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Transforming wheat vacuolar invertase into a high affinity sucrose:sucrose 1-fructosyltransferase

Lindsey Schroeven[1], Willem Lammens[1,2], André Van Laere[1] and Wim Van den Ende[1]

[1]K. U. Leuven, Laboratorium voor Moleculaire Plantenfysiologie, Kasteelpark Arenberg 31, bus 2434, B-3001 Leuven, Belgium; [2]K. U. Leuven, Laboratorium voor Biokristallografie, Herestraat O&N II, bus 822, B-3000 Leuven, Belgium

Author for correspondence:
Wim Van den Ende
Tel: +32 16321952
Fax: +32 16321967
Email: wim.vandenende@bio.kuleuven.be

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Summary

• Vacuolar invertases (VIs) degrade sucrose to glucose and fructose. Additionally, the fructan plant wheat (*Triticum aestivum*) contains different fructosyltransferases (FTs), which have evolved from VIs by developing the capacity to bind sucrose or fructans as acceptor substrates. Modelling studies revealed a hydrogen bonding network in the conserved WMNDPNG motif of VIs, which is absent in FTs.

• In this study, the hydrogen bonding network of wheat VI was disrupted by site-directed mutagenesis in the 23WMNDPNG29 motif. While the single mutants (W23Y, N25S) showed a moderate increase in 1-kestose production, a synergistic effect was observed for the double mutant (W23Y+N25S), showing a 17-fold increase in transfructosylation capacity, and becoming a real sucrose:sucrose 1-fructosyltransferase.

• Vacuolar invertases are fully saturable enzymes, contrary to FTs. This is the first report on the development of a fully saturable FT with respect to 1-kestose formation. The superior kinetics (K_m ~ 43 mM) make the enzyme useful for biotechnological applications.

• The results indicate that changes in the WMNDPNG motif are necessary to develop transfructosylating capability. The shift towards smaller and/or more hydrophilic residues in this motif might contribute to the formation of a specific acceptor site for binding of sugar, instead of water.

Key words: 1-SST (sucrose:sucrose 1-fructosyltransferase), fructan, hydrolase, invertase, transferase.

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Introduction

Fructans are polymers of fructose and an extension of sucrose occurring in many plant species mainly belonging to Asteraceae, Liliaceae and Poaceae (Pollock & Cairns, 1991; Hendry, 1993). Different types of fructan molecules can be distinguished depending on the linkage type between the fructosyl residues and the position of the glucose residue (Lewis, 1993). Fructans with a terminal glucose residue include the β(2-1)-type fructans (inulin, principally occurring in dicots), and the linear β(2-6) (levan) or branched-type fructans (graminan) with both β(2-6) and β(2-1) linkages (as occurring in bacteria and monocots). Fructans with an internal glucose residue include the neo-inulin and neo-levan types (occurring in monocots such as *Allium*, *Asparagus* and *Lolium*; Prud'homme *et al.*, 2007).

Substantial work has been dedicated to fructan metabolism in economically important cereals (e.g. wheat and barley (*Hordeum vulgare*); Yoshida *et al.*, 2007) and forage grasses (e.g. *Lolium* species; Prud'homme *et al.*, 2007). Apart from their function as a vacuolar storage carbohydrate, fructans may protect plants from drought and cold stress by stabilizing cellular membranes (Hincha *et al.*, 2007). Grass fructans, mainly stored in leaf sheaths and bases, support regrowth after defoliation (Morvan-Bertrand *et al.*, 2001). In wheat, fructans temporarily accumulate in the upper parts of the stem (Gebbing,

2003), forming important carbon sources for grain weight, and yield under water-limited conditions (Schnyder *et al.*, 1993; Xue *et al.*, 2008). Furthermore, fructo-oligosaccharides (FOS) have become increasingly popular as low caloric sweeteners and have prebiotic qualities (Roberfroid, 2007).

Fructans are biosynthesized by fructosyltransferases (FTs). Sucrose:sucrose 1-fructosyltransferase (1-SST) and sucrose: fructan 6-fructosyltransferase (6-SFT) play key roles in graminan biosynthesis in cereals such as wheat and barley (Sprenger *et al.*, 1995; Nagaraj *et al.*, 2004). Starting from two sucrose molecules, 1-SST catalyzes the production of the trisaccharide 1-kestose. 6-SFT subsequently synthesizes the tetrasaccharide bifurcose (1&6-kestotetraose) by transferring a fructosyl unit from sucrose to 1-kestose (Duchateau *et al.*, 1995). Further β(2-6)- and β(2-1)-linked fructosyl chain elongation to graminans (up to DP 20) is established by 6-SFT and fructan:fructan 1-fructosyltransferase (1-FFT) (Kawakami & Yoshida, 2005). Fructan:fructan 6G-fructosyltransferase (6G-FFT) is a key enzyme involved in the biosynthesis of neoseries fructans in *Lolium perenne* (Lasseur *et al.*, 2006). Many specific fructan exohydrolases (FEHs; degrading fructans and not sucrose) have been cloned from wheat and perennial ryegrass as model plants (De Coninck *et al.*, 2007). The functionally characterized cDNAs include 1-FEH (Van den Ende *et al.*, 2003; Chalmers *et al.*, 2005), 6-FEH (Van Riet *et al.*, 2006), 6-kestose exohydrolase (6-KEH) (Van den Ende *et al.*, 2005) and 6&1-FEH (Kawakami *et al.*, 2005). Interestingly, the 1-FEHs might act as β(2-1) trimmers during the period of active fructan biosynthesis (Van den Ende *et al.*, 2003; Lothier *et al.*, 2007).

Plant invertases degrade sucrose into glucose and fructose. Cell wall invertases (CWIs) are key metabolic enzymes involved in the regulation of sucrose partitioning, while vacuolar invertases (VIs) control the sugar composition in fruits and storage organs and play a role in response to abiotic stress, osmoregulation and cell elongation (Roitsch & Gonzalez, 2004). Invertases can be regulated in many ways (Huang *et al.*, 2007). FTs produce fructans and are considered to be critical enzymes for the acquisition of freezing tolerance, especially in plants in which fructans are the main storage carbohydrates, such as perennial ryegrass (Chalmers *et al.*, 2005; Hisano *et al.*, 2004, 2008). FT genes are believed to be mainly regulated at the transcriptional level by sucrose, light (Gallagher *et al.*, 2007; Van den Ende & Van Laere, 2007) and cold (Hisano *et al.*, 2008). However, posttranslational regulation was also suggested (Amiard *et al.*, 2003; Lasseur *et al.*, 2006).

Different types of FTs, FEHs, CWIs and VIs are grouped together with microbial β-fructosidases (degrading both sucrose and fructans) in the family 32 of glycoside hydrolases (GH32) (www.cazy.org) (Henrissat, 1991). Family GH32 can be combined with family GH68 in the clan GH-J. GH68 harbours bacterial invertases, levansucrases and inulosucrases. Several 3-D structures have been unravelled within GH32 (Lammens *et al.*, 2008 and references therein). All these proteins consist of an N-terminal five-bladed β-propeller domain (GH32 and GH68) followed by a C-terminal domain formed by two β-sheets (only in GH32). The active site is characterized by the presence of three highly conserved acidic groups (in the WMN**D**PNG, R**D**P and **E**C motifs). The Asp from the first motif is the nucleophile, the Asp from the second motif is proposed to act as transition state stabilizer and the Glu residue from the **E**C motif acts as acid/base catalyst, playing a crucial role in the catalytic mechanism (Reddy & Maley, 1996; Alberto *et al.*, 2004; Le Roy *et al.*, 2007). Invertases usually show an intact WMNDPNG motif, while FTs are altered in this motif (Gallagher *et al.*, 2004; Ritsema *et al.*, 2006).

Based on multiple alignments of FTs and VIs, it was postulated that FTs evolved from VIs (Vijn & Smeekens, 1999), independently from each other in monocots and dicots (Wei & Chatterton, 2001). Also the fact that VIs can synthesize small amounts of 1-kestose or 6-kestose (Housley *et al.*, 1991; Vijn *et al.*, 1998) at high sucrose concentrations (> 100 mM) contributes to this theory. In addition, FTs that use sucrose as a donor substrate show invertase activity at low sucrose concentrations (Sprenger *et al.*, 1995; Van den Ende *et al.*, 1996). On the other hand, FEHs have evolved from CWIs. Thanks to the available 3-D structures of a FEH and a CWI, the crucial differences between FEHs and CWIs have been recently demonstrated by site-directed mutagenesis (Le Roy *et al.*, 2007, 2008).

Many VIs and FTs have been cloned but only a limited number of the encoded enzymes have been functionally characterized (see the references in Fig. 1). In severe contrast to VIs, which are saturable enzymes with K_m values in the low-millimolar range (Sturm, 1999), all FTs that use sucrose as donor substrate are essentially unsaturable enzymes showing apparent K_m values of several hundreds of millimolars (Van Laere & Van den Ende, 2002). The typical low-affinity character of these FTs is consistent with the point of view that fructans can be considered to be a surplus pool of sucrose, only synthesized by FTs when a threshold sucrose concentration is surpassed (Maleux & Van den Ende, 2007).

A rather limited increase in transfructosylation capability was realized by mutagenesis adjacent to the nucleophile in a VI from onion (Ritsema *et al.*, 2006), but the mutant enzyme mainly behaved as an invertase with fully saturable kinetics for hexose production (hydrolytic reaction). However, no full saturation was observed for 1-kestose production (transfer reaction).

Wheat as an economically important species is an ideal model plant to perform detailed structure-function work on VIs and FTs. Indeed, it is a unique fact that 1-SST, 6-SFT, 1-FFT and VI cDNAs are available from the same species. Moreover, the percentage of identity between wheat INV and the different FTs is high (ranging between 63 and 65%).

As a first and necessary step to understand the molecular basis for the observed substrate specificities among wheat FTs and to learn more about their peculiar kinetics, this study aims to further increase the transfructosylation capacity of a VI

1	23 25	321	Enzyme	Acc.no.	Ref.
FSWTNAMLAWQRTAFHFQPPKNWMNDPNGPLYHK	KGWASVQSIP	Zm INV	P49175	(Xu *et al.*, 1995)
YAWTNSMLSWQRTAFHFQPPNNWMNDPNGPLYYK	KGWASLQSIP	Os INV	AF276704	(Ji *et al.*, 2007)
FPWTDAMLQWQRTGFHFQPEKNWMNDPDGPMFYK	KGWASVLPIP	Tg INV	X97642	(Balk & de Boer, 1999)
FPWSNEMLQWQRSGYHFQTAKNYMSDPNGLMYYR	KGWASIQSVP	Hv 6-SFT	X83233	(Sprenger *et al.*, 1995)
FPWSNEMLQWQRSGYHFQTAKNYMSDPNGLMYYR	KGWASIQSVP	Ta 6-SFT	AB029887	(Kawakami & Yoshida, 2002)
FPWSNEMLQWQRSSYHFQPAKNYMSDPDGLLYYG	KGWASIQSVP	*Ac* 6-SFT?	AAK27319	(Wei & Chatterton, 2001)
FPWSNSMLQWQRAGFHFQTEKNFMSDPNGPVYYR	KGWASIQSIP	Pa 6-SFT	AF192394	(Wei *et al.*, 2002)
FPWSNAMLQWQRTGFHFQPEQHYMNDPNGPVYYG	KGWANLMTIP	Lp 1-SST	AY245431	(Chalmers *et al.*, 2003)
FPWSNAMLQWQRTGFHFQPDKYYQNDPNGPVYYG	KGWANLQSIP	Hv 1-SST	AJ567377	(Nagaraj *et al.*, 2004)
FPWSNAMLQWQRTGFHFQPEKHYMNDPNGPVYYG	KGWANLMTIP	Fa 1-SST	AJ297369	(Luscher *et al.*, 2000)
FPWSNAMLQWQRTGYHFQPDKYYQNDPNGPVYYG	KGWANLQSIP	Ta 1-SST	AB029888	(Kawakami & Yoshida, 2002)
FPWSNAMLQWQRTGFHFQPEKNWMNDPNGPVYYK	KGWASLQSIP	Lp INV	AY082350	(Johnson *et al.*, 2003)
FPWSNAMLQWQRTGFHFQPEKNWMNDPNGPVYYK	KGWASLQSTP	Hv INV	AJ623275	(Nagaraj *et al.*, 2005)
FPWSNAMLQWQRTGFHFQPEKNWMNDPNGPVYYK	KGWASLQSIP	Ta INV	AJ635225	(Ji *et al.*, 2007)
FPWSNAMLQWQRTGFHFQPEKNWMNDPNGPVYYK	**KGWASLQSTP**	Lt INV?	AJ532549	(Gallagher *et al.*, 2004)
FPWSNAMLQWQRTGFHFQPQRNWMNDPNGPVYYK	KGWASLQGIP	So INV?	AAP59436	unpublished
FPWSNAMLQWQRTGFHFQPQKNWMNDPNGPVYYK	KGWASLQSIP	Bo INV	DQ267825	(Hsieh *et al.*, 2006)
YPWTNKMLSWQRTGFHFQPEKNWMNDPNGPLYYK	KGWASLQGIP	Ao INV?	AAB71136	(Dwyer, 1997)
YPWTNQMLSWQRTGFHFQPVKNWMNDPNGPLYYK	KGWASLQGVP	Ac INV	AJ006067	(Ritsema *et al.*, 2006)
YPWTNEMLKWQRTGYHFQPPNHFMADPNAAMYYK	KGWASVQNIA	Ac 1-SST	AJ006066	(Vijn *et al.*, 1998)
YPWTNDMFEWQRPGYHFQPPYHFMGDPNAAMYYK	KGWANVLNIA	As 1-SST?	AAM21931	unpublished
YPWTNDMLRWQRTGFHFQPEKNFQADPNAAMFYK	KGWASLQNIP	At 1-SST	DQ535031	(Avila-Fernandez *et al.*, 2007)
KLESNAGVEWERSAYHFQPDKNFISDPDGPMYHM	KGWANMLNIP	To 1-SST	AJ250634	(Van den Ende *et al.*, 2000)
KLESNADVEWQRSAYHFQPDKNFISDPDGPMYHM	KGWANILNIP	Ci 1-SST?	AAB58909	(de Halleux & Van Cutsem, 1997)
KLDSSAEVEWQRSTYHFQPDKNFISDPDGPMYHM	KGWANILNIP	Ht 1-SST	AJ009757	(van der Meer *et al.*, 1998)
ELKTNAEVEWQRSAYHFQPDKNYISDPDGPMYHM	KGWANILNIP	Cs 1-SST	Y09662	(Hellwege *et al.*, 1997)
FAWSNLMLSWQRTSYHFQPVKNWMNDPNGPLYYK	KGWASLQAIP	Ib INV	AY037938	(Wang *et al.*, 2005)

Fig. 1 Multiple alignment of amino acid sequences of closely related vacuolar invertases (VIs), sucrose:sucrose 1-fructosyltransferases (1-SSTs) and sucrose:fructan 6-fructosyltransferases (6-SFTs) containing the conserved VI motifs WMNDPNG and GWAS. Prominent differences are highlighted (Ac, *Allium cepa*; *Ac*, *Agropyron crista*; Ao, *Asparagus officinalis*; As, *Allium sativum*; At, *Agave tequilana*; Bo, *Bambusa oldhamii*; Ci, *Cichorium intybus*; Cs, *Cynara scolymus*; Fa, *Festuca arundinacea*; Ht, *Helianthus tuberosus*; Hv, *Hordeum vulgare*; Ib; *Ipomoea batatus*; Lp, *Lolium perenne*; Lt, *Lolium temulentum*; Os, *Oryza sativa*; Pa, *Poa ampla*; So, *Saccharum officinarum*; Ta, *Triticum aestivum*; Tg, *Tulipa gesneriana*; To, *Taraxacum officinale*; Zm, *Zea mays*).

by site-directed mutagenesis and to develop a high-affinity FT with fully saturable kinetics for fructan trisaccharide production.

Materials and Methods

Cloning and site-directed mutagenesis on wheat VI

The cloning of the full-length cDNA of winter wheat (*Triticum aestivum* L.) VI (accession number AJ635225) has been described (Ji *et al.*, 2007). The cDNA was cloned into the pPICZαA expression vector (obtained from Invitrogen, Groningen, the Netherlands), in frame behind the α-factor secretion signal and using the EcoRI/XbaI restriction site. This expression plasmid encoding wheat VI was used as a template to introduce mutations.

Following the QuikChange TM site-directed mutagenesis protocol (Stratagene, La Jolla, CA, USA), single or double amino acid mutations were introduced in the WMNDPNG and GWAS motifs of wheat VI. The following oligonucleotide primers (and their reverse complement) were used to introduce the amino acid substitutions in the WMNDPNG region: N25Sf, GAACTGGATGA**G**CGACCCCAATG (Tm = 71.5°); W23Yf, GAGAAGAACT**AC**ATGAACGACC (Tm = 60°C); and W23Y+N25Sf, CGAGAAGAACT**AC**ATGA**G**CGAC-CCCAATG (Tm = 70°C). An additional mutation was introduced in the GWAS region with the primer S321Nf: GGGATGGGCC**AA**CCTGCAGTCG (Tm = 70°C). In this way, mutants W23Y, N25S, W23Y+N25S, W23Y+S321N and N25S+S321N were generated by PCR mutagenesis.

The nonmethylated strand was digested by 1 μl DpnI (37°C, 90 min.) and the samples were purified by QiaQuick PCR purification kit (Qiagen, Valencia, CA, USA). Four microlitres of the purified vector-DNA was used for transformation of *E. coli* TOP10 cells by heat shock. Positive single colonies were selected on low-salt YT agar plates supplemented with zeocine (30 μg ml^{-1}) and checked with PCR. All constructs were sequenced (BaseClear, Leiden, the Netherlands) to check for the desired mutation.

Wild-type (wt) 1-SST cDNA (accession number AB029888) and wt 6-SFT cDNA (accession number AB029887), both isolated from winter wheat and cloned into the pPICZαA expression vector, were kindly provided by Drs Midori Yoshida and Akira Kawakami (National Agricultural Research Centre for Hokkaido Region, Japan).

Heterologous expression in *Pichia pastoris* and enzyme purification

Wild-type and mutated cDNAs were expressed in the methylotrophic yeast *Pichia pastoris.* Transformation of the *Pichia* cells with the expression plasmids was carried out with

the EasySelect™ Pichia Expression Kit (Invitrogen). According to the supplier's instruction, *Pichia pastoris* strain X33 was cultured and transformed by electroporation with 10 μg of PmeI-linearized vector-DNA and with an empty vector as a control. Cells were selected on YPDS agar plates containing 100 μg ml^{-1} zeocine. Components of the different culture media are described in the manual.

Single positive colonies (checked with PCR) were grown overnight (30°C, 200 rpm) in 5 ml preculture of YPD and zeocine. Five hundred microlitres of this start culture was transferred to 80 ml BMGY (pH 6.0) for overnight growth (30°C, 200 rpm). Cells were harvested by centrifugation (1000 *g*, 5 min, 4°C) and subsequently dissolved in 20 ml induction medium BMMY (pH 6.0). During incubation (96 h, 30°C, 200 rpm), 400 μl methanol (2% v/v) was added to the cells every day.

After 4 d of induction, the yeast supernatant, containing the recombinant proteins, was collected (1000 *g*, 10 min, 4°C). Citrate phosphate buffer (20 ml, pH 5.0, 20 mM) and $(NH_4)_2SO_4$ (80% final saturation) were added to precipitate the proteins. Enzymes were pelleted (40 000 *g*, 15 min, 4°C) after incubation on ice for 45 min. The pellet was dissolved in 500 μl sodium acetate buffer (pH 5.0, 50 mM) supplemented with 0.02% (w/v) sodium azide and centrifuged for 5 min at 17 200 *g*.

Subsequently, wild-type and mutant enzymes were further purified on a Fast Desalting Column HR 10/10 (Amersham Biosciences, Uppsala, Sweden). Fractions of 0.5 ml were collected. Most active fractions with identical retention time were selected and used for subsequent enzyme incubations (see next section). Protein concentrations in these fractions were calculated as total protein minus total protein in Fast Desalting fractions derived from a medium of an empty vector control. Protein concentrations were determined by the Coomassie Brilliant Blue Reagens method (Sedmak & Grossberg, 1977) and were found to vary between 0.5 and 1.0 μg μl^{-1}.

Substrate specificity and kinetic parameters of wild-type and mutant enzymes

Specific activity determinations revealed that the enzymes greatly differed, specific activities ranging between 10 133 nkat product mg^{-1} protein (wt VI) and 0.64 nkat product mg^{-1} protein (wt 6-SFT). Therefore, it was practically impossible to compare the enzymes at an equal protein concentration. To allow a visual prospection of the hydrolase to transfructosylase ratio, the protein concentrations and reaction times were adapted to obtain an equal amount of fructosyl transfers (either to water or to sucrose). The following amounts of protein were used per 80 μl reaction mixture: wt VI, 0.03 μg; W23Y, 0.35 μg; N25S, 0.44 μg; W23Y+N25S, 0.85 μg; wt 1-SST, 5 μg; wt 6-SFT (fructose production), 2.5 μg; wt 6-SFT (1&6-kestotetraose production), 5 μg.

For the kinetical analyses, great care was taken to select time-points in the linear region, ensuring that < 10% of the original substrate was consumed. All enzymes were incubated at 30°C in a total reaction volume of 80 μl in 50 mM sodium acetate buffer (pH 5.0) containing 0.02% (w/v) sodium azide and different sucrose concentrations ranging between 5.0 mM and 1.0 M (see figures). After 10 and 30 min for mutants and wt invertase, after 2 h and 4 h for 1-SST, and after 30 min and 2 h for 6-SFT, the incubation reactions were stopped by heating at 90°C for 5 min. 6-SFT was also incubated with 0–1000 mM sucrose and 50 mM 1-kestose for 2 and 4 h at 30°C to check for 1&6-kestotetraose production. All experiments were done in triplicate. The reaction products were analysed by high-pressure anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD, Dionex, Sunnyvale, CA, USA) as previously described (Van den Ende & Van Laere, 1996). By comparing the peak areas with known amounts of standard compounds, the amounts of products were determined.

In contrast to invertases, most FTs cannot be fully saturated with sucrose (Van den Ende *et al.*, 1996). Hence their 'apparent' K_m, based on Michaelis–Menten kinetics, can only be estimated. Kinetic parameters (apparent K_m, specific activity) were determined using the linear Hanes plot. Considering the substrate velocity curves, samples with high sucrose concentrations (1.0 M) sometimes resulted in deviating measurements. This might be the result of pipetting errors, caused by the high viscosity of the samples. Consequently, those outliers were deleted in the Hanes plots (Supporting information, Fig. S1).

Results and Discussion

Designing mutants based on multiple sequence alignments

In an attempt to understand the evolutionary steps that were involved to develop FTs from an ancestral VI in wheat, a multiple-sequence alignment of VIs and sucrose-splitting FTs (1-SSTs and 6-SFTs) was performed to detect amino acid residues that are unique to VIs, on the one hand, and FTs on the other. Prominent differences were observed in two conserved VI regions (WMNDPNG and GWAS) as shown in Fig. 1. Since 1-SSTs of Poaceae always contain a tyrosine (Y) instead of a tryptophan (W) in the WMNDPNG region, the W23Y VI mutant was constructed. In addition, 1-SSTs of dicotyledonous plants and 6-SFTs from Poaceae contain a serine (S) instead of an asparagine (N) adjacent to the nucleophile. To test the contribution of this residue to the development of FT activity, the N25S and W23Y+N25S mutants were designed. The alignment also revealed that, with the exception of Ac1-SST and At1-SST, 1-SSTs always contain an N in the GWAS/N region, whereas all VIs and 6-SFTs have a S. Therefore, these residues were additionally mutated in the W23Y and the N25S mutants, yielding the double mutants W23Y+S321N and N25S+S321N.

The position of these mutated amino acids in the active site of wheat VI is estimated by modelling studies by using the

Arabidopsis thaliana cell wall invertase 1 (AtcwINV1, Protein Data Bank (PDB) ID 2AC1) 3-D structure (Verhaest *et al.*, 2006) as a template (Fig. 2). The active site residues include the nucleophile D26, the transition state stabilizer D151 and the proton donor E207. The mutated residues, W23 and N25, flank the negatively charged central pocket of the N-terminal domain. Interestingly, S321 is positioned close to W23. Therefore, it was also considered to be a good candidate for mutagenesis.

Comparison of the transfructosylation capacity (TFC) of wt and mutant enzymes

Vacuolar invertase, 1-SST and 6-SFT cDNAs were previously expressed in *Pichia pastoris* (Kawakami & Yoshida, 2002; Ji *et al.*, 2007). All these enzymes use sucrose as donor substrate. 1-SST also uses sucrose as acceptor substrate. The typical acceptor substrate of 6-SFT is 1-kestose (Sprenger *et al.*, 1995), but the enzyme also accepts sucrose as an acceptor, although to a limited extent (Kawakami *et al.*, 2008). Reaction mixtures containing the heterologously expressed wheat VI, 1-SST or 6-SFT wt and mutant VI enzymes were compared after incubation with 500 mM sucrose as a single substrate (Fig. 3a). The wt VI showed no significant production of 1-kestose. The W23Y mutant synthesized a small amount of 1-kestose, comparable to the amount produced by the 6-SFT. Additionally, the 6-SFT produced a small amount of 6-kestose as well. Compared with the W23Y mutant, the N25S mutant produced much more 1-kestose. Interestingly, a synergistic effect is observed when the two mutations are combined (W23Y+N25S), resulting in a strongly increased 1-kestose synthesis and a decreased fructose to glucose ratio. Although Ritsema *et al.* (2005) demonstrated that mutagenesis of an N25 homologue (into S, A, Q) influenced the 1,1 nystose/neokestose product ratio in another type of onion fructosyltransferase (6G-FFT), homologues of the N25S and W23Y+N25S mutants have not been studied in the onion invertase system. The wt 1-SST, showing nearly zero hydrolytic activity at 500 mM sucrose concentration, is also shown for comparison (Fig. 3a).

A typical characteristic of the wt 1-SST is the very limited hydrolytic capacity in the lower sucrose range (Fig. 3b,c). While the W23Y mutant showed no transfructosylase characteristics in this range, the N25S mutant produced 1-kestose at 125 mM but not at 25 mM. The W23Y+N25S produced 1-kestose at 25 mM sucrose as well. The double mutants W23Y+S321N and N25S+S321N did not show an extended TFC compared with the single mutants W23Y and N25S (data not shown).

Optimizing the TFC by breaking the typical hydrogen bond network in VI

The percentage of 1-kestose (transfer to sucrose) over the total of fructose (transfer to water: hydrolysis) and 1-kestose is a measure for the TFC. Typically, the TFC increases with increasing substrate concentration; this is the case for all enzymes tested (Fig. 4). There is a clear shift from hydrolysis to transfructosylation for the mutant invertases, especially at the higher sucrose concentrations. The mutant W23Y shows only a slight increase (twofold) in TFC compared with the wt VI; the main activity of this mutant remained invertase activity. However, the mutants N25S and W23Y+N25S show a 10- and 17-fold increase in TFC. At high sucrose concentrations, the W23Y+N25S mutant reaches a maximal TFC of more than 50%. For comparison, the TFC of the best-performing onion VI mutant only resulted in a 3.5-fold increase in TFC (Ritsema *et al.*, 2006). This prominent change in acceptor substrate convincingly demonstrates that amino acids W23 and N25 of the WMNDPN region greatly determine the difference between a hydrolase and a transferase in the wheat VI/FT system.

N25 forms a hydrogen bonding network with W23 (2.8 Å) and D26 (3.4 Å) (Fig. 2a). This configuration is typical for all GH32 plant invertases, suggesting that it promotes hydrolytic activity. The disturbance of this network seems to form a first and essential step to develop transfructosylation capability. Most likely, S25 forms one H-linkage with the nucleophile D26 in mutant N25S (Fig. 2e), relieving the fixed position of W23 as observed in VI. Besides the fact that S and Y are smaller than N and W, creating extra space to allow the development of a specific sugar acceptor site, it is tempting to speculate that the hydroxyl groups of the mobile S and Y side chains (Fig. 2f) can form H-linkages to specifically stabilize sucrose as an acceptor substrate in the double mutant. However, it is likely that some extra amino acids in the neighbourhood are needed to fully stabilize sucrose as an acceptor substrate. This needs to be further corroborated by crystallizing an FT in complex with sucrose as an acceptor substrate.

Analysis of the kinetic parameters

Typically, VIs and FTs show 'side' activities besides their main activity (Van den Ende *et al.*, 1996). Wheat VI produces small amounts of 1-kestose at higher sucrose concentrations, and wheat 1-SST produces low amounts of fructose (compared with 1-kestose) at the lowest sucrose concentrations. The concentration of these side-products was too low and the substrate concentration window too narrow to derive reliable kinetic parameters. Therefore, we focused only on the main reaction products (either fructose or 1-kestose) to determine the kinetic parameters of the different recombinant enzymes under study. The reaction velocity was plotted against increasing substrate concentrations and linear Hanes plots were generated to estimate the kinetic parameters (Fig. S1). The derived specific activities and estimated K_m values are summarized in Table 1 (fructose formation) and Table 2 (1-kestose or 1&6-kestotetraose formation).

For the wt wheat VI, a K_m-value of 15 mM was obtained. This is close to that of barley VI (K_m = 14 mM; Nagaraj *et al.*,

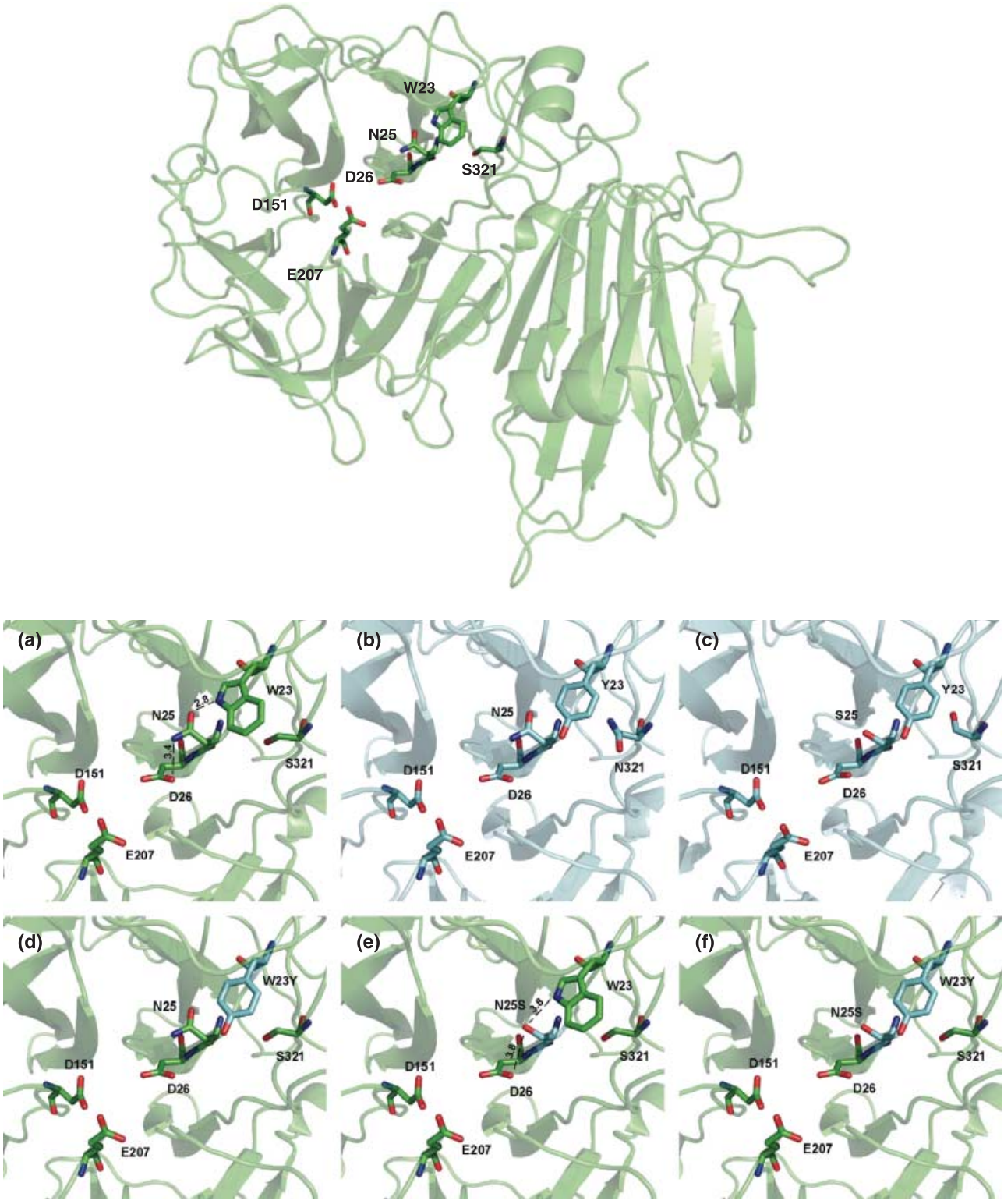

Fig. 2 Schematic of wheat (*Triticum aestivum*) vacuolar invertase (VI), showing the catalytic triad and the amino acids that were mutated and localization of the considered amino acids in the active sites of wild-type (wt) wheat VI (a), sucrose:sucrose 1-fructosyltransferase (1-SST) (b), and sucrose:fructan 6-fructosyltransferase (6-SFT) (c), and in the mutant VI enzymes W23Y (d), N25S (e) and W23+N25S (f). Labelling of 1-SST and 6-SFT according to equivalent residues in the wild-type VI. AtcwINV1 was used as template for the three-dimensional models. The figure was drawn with Pymol (DeLano, 2002).

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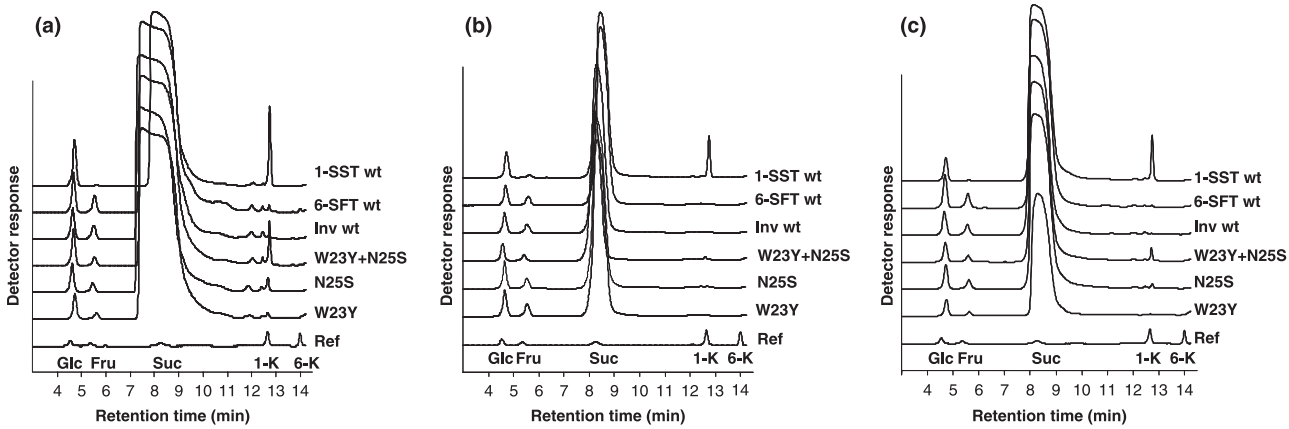

Fig. 3 Anion-exchange chromatography with pulsed-amperometric detection (AEC-PAD) chromatograms of reaction mixtures of wild-type (wt) vacuolar invertase (VI), sucrose:fructan 6-fructosyltransferase (6-SFT), sucrose:sucrose 1-fructosyltransferase (1-SST) and mutant VI W23Y, N25S and W23Y+N25S enzymes. All enzymes were incubated with 500 mM sucrose (a), 25 mM sucrose (b) and 125 mM sucrose (c) at 30°C for 10 min (VI and mutants derived thereof), 30 min (6-SFT) and 2 h (1-SST). See the Materials and Methods section for protein amounts used. 1-K, 1-kestose; 6-K, 6-kestose; Fru, fructose; Glc, glucose; Suc, sucrose.

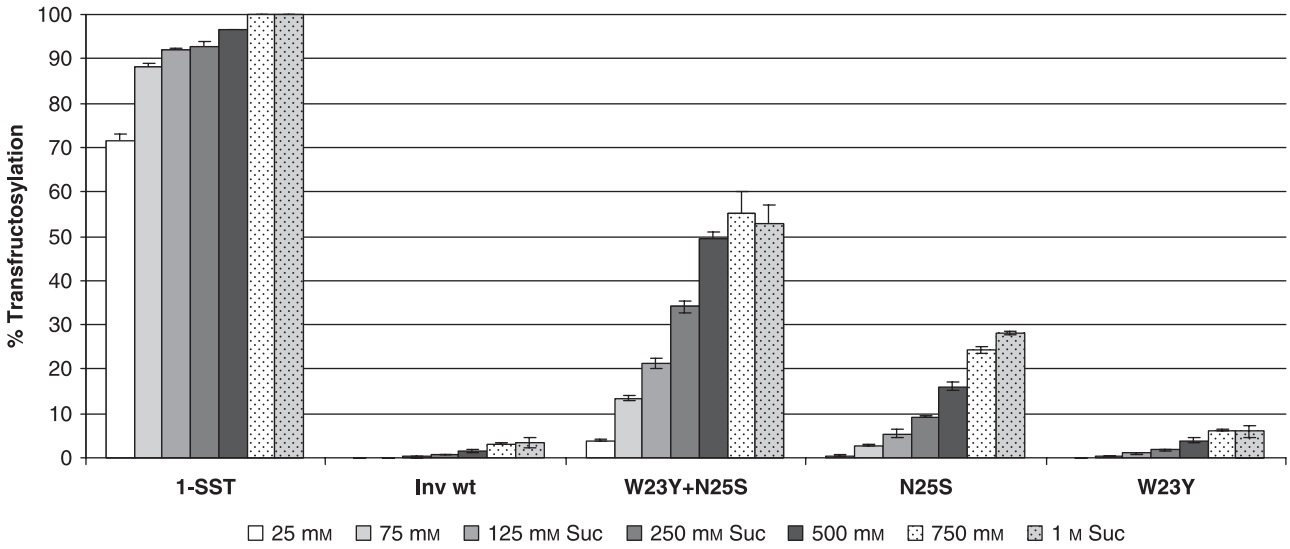

Fig. 4 Transfructosylation percentage at increasing sucrose concentrations (25–1000 mM). Results from three independent experiments are shown, with error bars representing the SEM. Reaction conditions: 30°C, 30 min (wild-type vacuolar invertase (Inv wt) and mutants derived), 4 h (sucrose:sucrose 1-fructosyltransferase (1-SST)). See the Materials and Methods section for protein amounts used.

2005). The specific activity was relatively high (10 133 nkat fructose mg^{-1} protein). The mutant W23Y K_m increased 10-fold while the specific activity only slightly decreased (7086 nkat fructose mg^{-1} protein). This strong increase in K_m-value demonstrates that W23 is an essential amino acid for efficiently binding sucrose as donor substrate in invertase. Recent site-directed mutagenesis work on AtcwINV1 showed that the K_m of a W20L mutant (W20 is the homologue of W23 in wheat VI) increased from 0.35 to 72 mM (Le Roy *et al.*, 2007). AtcwINV1 contains a hydrophobic rim consisting of three tryptophanes (W20, W47 and W82) near the active site, believed to assist in substrate stabilization (Le Roy *et al.*, 2007). Ritsema *et al.* (2006) mutated W161 (corresponding to W23 of wheat VI) of the WMNDPN region in onion VI. Comparable to the wheat VI mutant W23Y, the onion W161Y mutant showed a two- to threefold increase in transfructosylation capacity compared with the wt onion invertase. Considering the hydrolytic reaction, the K_m increased from 6 (wt onion invertase) to 29 mM (W161Y).

In contrast to mutant W23Y, 6-SFT showed a similar K_m (17 mM) as the wt VI, but a much lower specific activity (16 nkat fructose mg^{-1} protein). Also the specific activity (127 nkat product mg^{-1} protein) of the N25S mutant (1-kestose production) is much lower than the one of the W23Y mutant (fructose production) (Tables 1, 2), suggesting a much slower release of the elongated acceptor substrate.

Table 1 Kinetic parameters for the hydrolysis of sucrose by wild-type vacuolar invertase (VI) and sucrose:fructan 6-fructosyltransferase (6-SFT) and mutant enzyme W23Y. Fru, fructose

Enzyme	Product	Apparent K_m (mM)	Specific activity (nkat mg^{-1} protein)
Invertase	Fru	15 ± 2	10 133 ± 400
W23Y	Fru	152 ± 6	7 086 ± 560
6-SFT	Fru	17 ± 3	16 ± 1.6

Specific activity is calculated at V_{max}.

Table 2 Kinetic parameters for the production of 1-kestose (1-K) from sucrose by wild-type sucrose:sucrose 1-fructosyltransferase (1-SST) and mutant enzyme N25S and W23Y+N25S and for the production of bifurcose (Bif) by wild-type sucrose:fructan 6-fructosyltransferase (6-SFT)

Enzyme	Product	Apparent K_m (mM)	Specific activity (nkat mg^{-1} protein)
N25S	1-K	215 ± 11	127 ± 8.0
W23Y+N25S	1-K	43 ± 11	85 ± 5.6
1-SST	1-K	551 ± 66	13 ± 1.6
6-SFT	Bif	472 ± 73	0.64 ± 0.08

Specific activity is calculated at V_{max}.

Although FTs do not show a typical saturation behaviour, the kinetic parameters could be estimated since they did fit the linear Hanes plot (Figs S1e,g). The apparent K_m values of the wt 1-SST and 6-SFT were estimated at 551 and 472 mM, respectively. The estimated values for the specific activity of 1-SST (1-kestose production, Table 2) and 6-SFT (1&6-kestotetraose production, Table 2) were very low (13 and 0.64 nkat product mg^{-1} protein, respectively) compared with the wt VI (fructose production, Table 1). An apparent K_m (1-kestose production) of 215 mM was obtained for the N25S mutant and its specific activity was 10 times higher compared with the wt 1-SST.

Interestingly, superior kinetics were derived for the W23Y+N25S double mutant, showing an apparent K_m (43 mM) 13 times lower than that obtained for the wt 1-SST and five times lower than that for the N25S mutant (Table 2). Moreover, the derived value for the specific activity of the W23Y+N25S mutant was more than six times higher compared with the wt 1-SST (Table 2). In contrast to the wt 1-SST (Fig. S1e), the W23Y+N25S mutant is a fully saturable enzyme (Fig. S1f).

Point mutations affecting the overall transglycosylation capacity

The introduction of a bulky hydrophobic tryptophane in the active site often enhances the transglycosylation capacity (Fujita & Takegawa, 2002; Tang *et al.*, 2006). By contrast, the removal of a bulky, hydrophobic W23 seems a necessary (but not sufficient) step to develop a transfructosylase from a hydrolase in family GH32 VIs, suggesting that the creation of extra space is a necessity to bind sucrose as an acceptor substrate. The finding that only a few amino acids are essential to discriminate between transfructosylases and hydrolases was also reported by Kelly *et al.* (2007), by changing a cyclodexin glucano-transferase into an α-amylase. Moreover, it was shown that R360 is a critical amino acid to establish a fructan polymerase activity in *Bacillus subtilis* levansucrase (Chambert & Petit-Glatron, 1991).

The fact that only a few amino acid substitutions are needed to develop a transfructosylase from a hydrolase (Ritsema *et al.*, 2006; this work) and to develop a FEH from a cell wall invertase (Le Roy *et al.*, 2007) might explain the polyphyletic origin of the fructan accumulation (and degradation) trait.

Biotechnological applications

Here we prove for the first time that it is possible to develop a high-affinity 1-SST starting from a VI, but showing superior kinetic parameters compared with the natural 1-SST and 6-SFT enzymes (Table 2). It would be interesting to introduce similar mutations in rice VIs since rice and wheat are very closely related species, but wheat is a fructan accumulator and rice is not. Structure-function work will contribute to the introduction of the fructan trait in the edible parts of rice, ensuring incorporation of healthy fructans into the diet of millions of people, but also making rice more resistant to abiotic stresses. Indeed, recent investigations demonstrated that introduction of wheat 1-SST in the nonfructan plants tobacco (Li *et al.*, 2007) and rice (Kawakami *et al.*, 2008) effectively contributes to freezing or chilling tolerance.

Owing to its superior kinetics, the W23Y+N25S mutant could also be very useful for efficiently producing the prebiotic 1-kestose. In general, the development of high-affinity FTs could contribute to a rapid and cheaper production of tailor-made fructans or, when different sugar acceptors are used, to the production of new biomaterials for specific industrial, pharmaceutical and functional food applications (Lammens *et al.*, 2008; Zuccaro *et al.*, 2008).

Conclusions and perspectives

We succeeded in causing a drastic shift from water to sucrose as preferred acceptor molecule in wheat VI, by disturbing the typical hydrogen bonding network as occurring in VIs. The double mutant W23Y+N25S is saturable with regard to 1-kestose formation and shows superior kinetics compared with natural 1-SSTs. In addition, we compared, for the first time, the kinetic parameters of wt and mutant VI, 1-SST and 6-SFT enzymes derived from the same species. The double mutant W23Y+N25S contains the YMSDPNG motif, as observed in 6-SFT enzymes (Fig. 1). Interestingly, this mutant cannot produce 6-kestose from sucrose as a single substrate or

1&6-kestotetraose from sucrose as donor substrate and 1-kestose as acceptor substrate. Deciphering which factors determine whether an enzyme is involved in the degradation or synthesis of β(2-1) vs β(2-6) Fru-Fru linkages remains a challenging area for future research.

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References

Alberto F, Bignon C, Sulzenbacher G, Henrissat B, Czjzek M. 2004. The three-dimensional structure of invertase (beta-fructosidase) from *Thermotoga maritima* reveals a bimodular arrangement and an evolutionary relationship between retaining and inverting glycosidases. *Journal of Biological Chemistry* **279**: 18 903–18 910.

Amiard V, Morvan-Bertrand A, Billard JP, Huault C, Keller F, Prud'homme MP. 2003. Fructans, but not the sucrosyl-galactosides, raffinose and loliose, are affected by drought stress in perennial ryegrass. *Plant Physiology* **132**: 2218–2229.

Chalmers J, Lidgett A, Cummings N, Cao YY, Forster J, Spangenberg G. 2005. Molecular genetics of fructan metabolism in perennial ryegrass. *Plant Biotechnology Journal* **3**: 459–474.

Chambert R, Petit-Glatron MF. 1991. Polymerase and hydrolase activities of *Bacillus subtilis* levansucrase can be separately modulated by site-directed mutagenesis. *Biochemical Journal* **279**: 35–41.

De Coninck B, Van den Ende W, Le Roy K. 2007. Fructan ExoHydrolases (FEHs) in plants: properties, occurrence and 3-D structure. In: Shiomi N, Benkeblia N, Onodera S, eds. *Recent advances in fructooligosaccharides research*. Kerala, India: Research Signpost, 157–179.

DeLano WL. 2002. *The PyMOL molecular graphics system*. San Carlos, CA, USA: DeLano Scientific.

Duchateau N, Bortlik K, Simmen U, Wiemken A, Bancal P. 1995. Sucrose-fructan 6-fructosyltransferase, a key enzyme for diverting carbon from sucrose to fructan in barley leaves. *Plant Physiology* **107**: 1249–1255.

Fujita K, Takegawa K. 2002. Tryptophan-216 is essential for the transglycosylation activity of endo-β-N-acetylglucosaminidase A. *Biochemical and Biophysical Research Communications* **283**: 680–686.

Gallagher JA, Cairns AJ, Pollock CJ. 2004. Cloning and characterization of a putative fructosyltransferase and two putative invertase genes from the temperate grass *Lolium temulentum* L. *Journal of Experimental Botany* **55**: 557–569.

Gallagher JA, Cairns AJ, Turner LB 2007. Fructan in temperate forage grasses; agronomy, physiology. In: Shiomi N, Benkeblia N, Onodera S, eds. *Recent advances in fructooligosaccharides research*. Kerala, India: Research Signpost, 15–46.

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

Henrissat B. 1991. A classification of glycosyl hydrolases based on amino-acid-sequence similarities. *Biochemical Journal* **280**: 309–316.

Hincha DK, Livingston DP, Premakumar R, Zuther E, Obel N, Cacela C, Heyer AG. 2007. Fructans from oat and rye: composition and effects on membrane stability during drying. *Biochimica et Biophysica Acta-Biomembranes* **1768**: 1611–1619.

Hisano H, Kanazawa A, Kawakami A, Yoshida M, Shimamoto Y, Yamada T. 2004. Transgenic perennial ryegrass plants expressing wheat fructosyltransferase genes accumulate increased amounts of fructan and acquire increased tolerance on a cellular level to freezing. *Plant Science* **167**: 861–868.

Hisano H, Kanazawa A, Yoshida M, Humphreys MO, Iizuka M, Kitamura K, Yamada T. 2008. Coordinated expression of functionally diverse fructosyltransferase genes is associated with fructan accumulation in response to low temperature in perennial ryegrass. *New Phytologist* **178**: 766–780.

Housley TL, Gibeaut DM, Carpita NC, Sims I, Pollock CJ. 1991. Fructosyl transfer from sucrose and oligosaccharides during fructan synthesis in excised leaves of *Lolium temulentum* L. *New Phytologist* **119**: 491–497.

Huang LF, Bocock PN, Davis JM, Koch KE. 2007. Regulation of invertase: a suite of transcriptional and post-transcriptional mechanisms. *Functional Plant Biology* **34**: 499–507.

Ji XM, Van den Ende W, Schroeven L, Clerens S, Geuten K, Cheng SH, Bennett J. 2007. The rice genome encodes two vacuolar invertases with fructan exohydrolase activity but lacks the related fructan biosynthesis genes of the Pooideae. *New Phytologist* **173**: 50–62.

Kawakami A, Sato Y, Yoshida M. 2008. Genetic engineering of rice capable of synthesizing fructans and enhancing chilling tolerance. *Journal of Experimental Botany* **59**: 793–802.

Kawakami A, Yoshida M. 2002. Molecular characterization of sucrose:sucrose 1-fructosyltransferase and sucrose:fructan 6-fructosyltransferase associated with fructan accumulation in winter wheat during cold hardening. *Bioscience Biotechnology and Biochemistry* **66**: 2297–2305.

Kawakami A, Yoshida M. 2005. Fructan: fructan 1-fructosyltransferase, a key enzyme for biosynthesis of graminan oligomers in hardened wheat. *Planta* **223**: 90–104.

Kawakami A, Yoshida M, Van den Ende W. 2005. Molecular cloning and functional analysis of a novel 6&1-FEH from wheat (*Triticum aestivum* L.) preferentially degrading small graminans like bifurcose. *Gene* **358**: 93–101.

Kelly R, Leemhuis H, Dijkhuizen L. 2007. Conversion of a cyclodextrin glucanotransferase into an R-amylase: assessment of directed evolution strategies. *Biochemistry* **46**: 11216–11222.

Lammens W, Le Roy K, Van Laere A, Rabijns A, Van den Ende W. 2008. Crystal structures of *Arabidopsis thaliana* cell-wall invertase mutants in complex with sucrose. *Journal of Molecular Biology* **377**: 378–385.

Lasseur B, Lothier J, Djoumad A, De Coninck B, Smeekens S, Van Laere A, Morvan-Bertrand A, Van den Ende W, Prud'homme MP. 2006. 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.). *Journal of Experimental Botany* **57**: 2719–2734.

Le Roy K, Lammens W, Van Laere A, Van den Ende W. 2008. Influencing the binding configuration of sucrose in the active sites of chicory fructan 1-exohydrolase and sugar beet fructan 6-exohydrolase. *New Phytologist* **178**: 572–580.

Le Roy K, Lammens W, Verhaest M, De Coninck B, Rabijns A, Van Laere A, Van den Ende W. 2007. Unraveling the difference between invertases and fructan exohydrolases: a single amino acid (Asp239) substitution transforms *Arabidopsis* cell-wall invertase 1 into a fructan 1-exohydrolase. *Plant Physiology* **145**: 616–625.

Lewis DH. 1993. Nomenclature and diagrammatic representation of oligomeric fructans – a paper for discussion. *New Phytologist* **124**: 583–594.

Li HJ, Yang AF, Zhang XC, Gao F, Zhang JR. 2007. Improving freezing tolerance of transgenic tobacco expressing sucrose:sucrose 1-fructosyltransferase gene from *Lactuca sativa*. *Plant Cell, Tissue and Organ Culture* **89**: 37–48.

Lothier J, Lasseur B, Le Roy K, Van Laere A, Prud'homme MP, Barre P, Van den Ende W, Morvan-Bertrand A. 2007. Cloning, gene mapping,

and functional analysis of a fructan 1-exohydrolase (1-FEH) from *Lolium perenne* implicated in fructan synthesis rather than in fructan mobilization. *Journal of Experimental Botany* **58**: 1969–1983.

Maleux K, Van den Ende W. 2007. Levans in excised leaves of *Dactylis glomerata:* effects of temperature, light, sugars and senescence. *Journal of Plant Biology* **50**: 671–680.

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

Nagaraj VJ, Altenbach D, Galati V, Luscher M, Meyer AD, Boller T, Wiemken A. 2004. Distinct regulation of sucrose:sucrose-1-fructosyltransferase (1-SST) and sucrose:fructan-6-fructosyltransferase (6-SFT), the key enzymes of fructan synthesis in barley leaves: 1-SST as the pacemaker. *New Phytologist* **161**: 735–748.

Nagaraj VJ, Galati V, Luscher M, Boller T, Wiemken A. 2005. Cloning and functional characterization of a cDNA encoding barley soluble acid invertase (HvINV1). *Plant Science* **168**: 249–258.

Pollock CJ, Cairns AJ. 1991. Fructan metabolism in grasses and cereals. *Annual Review of Plant Physiology and Plant Molecular Biology* **42**: 77–101.

Prud'homme MP, Morvan-Bertrand A, Lasseur B, Lothier J, Meuriot F, Decau ML, Noiraud-Romy N. 2007. *Lolium perenne*, backbone of sustainable development, source of fructans for grazing animals and potential source of novel enzymes. In: Shiomi N, Benkeblia N, Onodera S, eds. *Recent advances in fructooligosaccharides research*. Kerala, India: Research Signpost, 231–258.

Reddy A, Maley F. 1996. Studies on identifying the catalytic role of Glu-204 in the active site of yeast invertase. *Journal of Biological Chemistry* **271**: 13953–13958.

Ritsema T, Hernandez L, Verhaar A, Altenbach D, Boller T, Wiemken A, Smeekens S. 2006. Developing fructan-synthesizing capability in a plant invertase via mutations in the sucrose-binding box. *Plant Journal* **48**: 228–237.

Ritsema T, Verhaar A, Vijn I, Smeekens S. 2005. Using natural variation to investigate the function of individual amino acids in the sucrose-binding box of fructan: fructan 6G-fructosyltransferase (6G-FFT) in product formation. *Plant Molecular Biology* **58**: 597–607.

Roberfroid MB. 2007. Prebiotics: the concept revisited. *Journal of Nutrition* **137**: 830S–837S.

Roitsch T, Gonzalez MC. 2004. Function and regulation of plant invertases: sweet sensations. *Trends in Plant Science* **9**: 606–613.

Schnyder H, Gillenberg C, Hinz J. 1993. Fructan contents and dry-matter deposition in different tissues of the wheat-grain during development. *Plant, Cell & Environment* **16**: 179–187.

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

Sprenger N, Bortlik K, Brandt A, Boller T, Wiemken A. 1995. Purification, cloning, and functional expression of sucrose: fructan 6-fructosyltransferase, a key enzyme of fructan synthesis in barley. *Proceedings of the National Academy of Sciences, USA* **92**: 11652–11656.

Sturm A. 1999. Invertases. Primary structures, functions, and roles in plant development and sucrose partitioning. *Plant Physiology* **121**: 1–7.

Tang S, Yang S, Cha H, Woo E, Park C, Park K. 2006. Contribution of W229 to the transglycosylation activity of 4-alpha-glucanotransferase from *Pyrococcus furiosus*. *Biochimica et Biophysica Acta-Proteins and Proteomics* **1764**: 1633–1638.

Van den Ende W, Clerens S, Vergauwen R, Van Riet L, Van Laere A, Yoshida M, Kawakami A. 2003. 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, Van Laere A. 1996. Fructan synthesizing and degrading activities in chicory roots (*Cichorium intybus* L.) during field-growth, storage and forcing. *Journal of Plant Physiology* **149**: 43–50.

Van den Ende W, Van Laere A. 2007. Fructans in dicotyledonous plants: occurrence and metabolism. In: Shiomi N, Benkeblia N, Onodera S, eds. *Recent advances in fructooligosaccharides research*. Kerala, India: Research Signpost, 1–14.

Van den Ende W, Van Wonterghem D, Dewil E, Verhaert P, De Loof A, Van Laere A. 1996. Purification and characterization of 1-SST, the key enzyme initiating fructan biosynthesis in young chicory roots (*Cichorium intybus*). *Physiologia Plantarum* **98**: 455–466.

Van den Ende W, Yoshida M, Clerens S, Vergauwen R, Kawakami A. 2005. Cloning, characterization and functional analysis of novel 6-kestose exohydrolases (6-KEHs) from wheat (*Triticum aestivum*). *New Phytologist* **166**: 917–932.

Van Laere A, Van den Ende W. 2002. Inulin metabolism in dicots: chicory as a model system. *Plant, Cell & Environment* **25**: 803–813.

Van Riet L, Nagaraj V, Van den Ende W, Clerens S, Wiemken A, Van Laere A. 2006. Purification, cloning and functional characterization of a fructan 6-exohydrolase from wheat (*Triticum aestivum* L.). *Journal of Experimental Botany* **57**: 213–223.

Verhaest M, Lammens W, Le Roy K, De Coninck B, De Ranter CJ, Van Laere A, Van den Ende W, Rabijns A. 2006. X-ray diffraction structure of a cell-wall invertase from *Arabidopsis thaliana*. *Acta Crystallographica Section D-Biological Crystallography* **62**: 1555–1563.

Vijn I, van Dijken A, Luscher M, Bos A, Smeets E, Weisbeek P, Wiemken A, Smeekens S. 1998. Cloning of sucrose:sucrose 1-fructosyltransferase from onion and synthesis of structurally defined fructan molecules from sucrose. *Plant Physiology* **117**: 1507–1513.

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

Wei JZ, Chatterton NJ. 2001. Fructan biosynthesis and fructosyltransferase evolution: expression of the 6-SFT (sucrose:fructan 6-fructosyltransferase) gene in crested wheatgrass (*Agropyron cristatum*). *Journal of Plant Physiology* **158**: 1203–1213.

Xue GP, McIntyre CL, Jenkins CLD, Glassop D, van Herwaarden AF, Shorter R. 2008. Molecular dissection of variation in carbohydrate metabolism related to water-soluble carbohydrate accumulation in stems of wheat. *Plant Physiology* **146**: 441–454.

Yoshida M, Kawakami A, Van den Ende W. 2007. Graminan metabolism in cereals: wheat as a model system. In: Shiomi N, Benkeblia N, Onodera S, eds. *Recent advances in fructooligosaccharides research*. Kerala, India: Research Signpost, 201–212.

Zuccaro A, Götze S, Kneip S, Dersch P, Seibel J. 2008. Tailor-made fructooligosaccharides by a combination of substrate and genetic engineering. *ChemBioChem* **9**: 143–149.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Substrate–velocity curves and linear Hanes plots for the mutant enzymes W23Y (a), N25S (b) and W23Y+N25S (c) and the wild-type VI (d), 1-SST (e) and 6-SFT (f, g).

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