Transforming a Fructan:Fructan 6G-Fructosyltransferase from Perennial Ryegrass into a Sucrose:Sucrose 1-Fructosyltransferase[1[C]]

Bertrand Lasseur[2], Lindsey Schroeven, Willem Lammens, Katrien Le Roy, German Spangenberg, Hélène Manduzio, Rudy Vergauwen, Jérémy Lothier[3], Marie-Pascale Prud'homme, and Wim Van den Ende*

UMR INRA UCBN 950 EVA, Ecophysiologie Végétale, Agronomie et Nutritions NCS, Université de Caen, 14032 Caen cedex, France (B.L., J.L., M.-P.P.); Katholieke Universiteit Leuven, Laboratorium voor Moleculaire Plantenfysiologie, B–3001 Leuven, Belgium (L.S., W.L., K.L.R., R.V., W.V.d.E.); Department of Primary Industries, Victorian AgriBiosciences Centre, Bundoora, Victoria 3083, Australia (G.S.); and CNRS UMR 6037 IFRMP 23, UFR des Sciences, Université de Rouen, 76821 Mont Saint Aignan, France (H.M.)

Fructosyltransferases (FTs) synthesize fructans, fructose polymers accumulating in economically important cool-season grasses and cereals. FTs might be crucial for plant survival under stress conditions in species in which fructans represent the major form of reserve carbohydrate, such as perennial ryegrass (*Lolium perenne*). Two FT types can be distinguished: those using sucrose (S-type enzymes: sucrose:sucrose 1-fructosyltransferase [1-SST], sucrose:fructan 6-fructosyltransferase) and those using fructans (F-type enzymes: fructan:fructan 1-fructosyltransferase [1-FFT], fructan:fructan 6G-fructosyltransferase [6G-FFT]) as preferential donor substrate. Here, we report, to our knowledge for the first time, the transformation of an F-type enzyme (6G-FFT/1-FFT) into an S-type enzyme (1-SST) using perennial ryegrass 6G-FFT/1-FFT (Lp6G-FFT/1-FFT) and 1-SST (Lp1-SST) as model enzymes. This transformation was accomplished by mutating three amino acids (N340D, W343R, and S415N) in the vicinity of the active site of Lp6G-FFT/1-FFT. In addition, effects of each amino acid mutation alone or in combination have been studied. Our results strongly suggest that the amino acid at position 343 (tryptophan or arginine) can greatly determine the donor substrate characteristics by influencing the position of the amino acid at position 340. Moreover, the presence of arginine-343 negatively affects the formation of neofructan-type linkages. The results are compared with recent findings on donor substrate selectivity within the group of plant cell wall invertases and fructan exohydrolases. Taken together, these insights contribute to our knowledge of structure/function relationships within plant family 32 glycosyl hydrolases and open the way to the production of tailor-made fructans on a larger scale.

Fructans are Fru polymers that can be considered as an extension of Suc, accumulating above a certain threshold Suc concentration (Cairns and Pollock, 1988; Guerrand et al., 1996; Maleux and Van den Ende, 2007). Fructans occur in many dicot and monocot plant species mainly belonging to Asteraceae, Liliaceae, and Poaceae (Hendry, 1993; Prud'homme et al., 2007; Shiomi et al., 2007; Van den Ende and Van Laere, 2007; Yoshida et al., 2007). Nowadays, research is mainly focused on economically important cereals (wheat [*Triticum aestivum*] and barley [*Hordeum vulgare*]) and forage grasses (mainly *Lolium* species). Depending on the linkage type between the fructosyl residues and the position of the Glc residue (Lewis, 1993), several fructan types can be distinguished. Fructans with a terminal Glc 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 cereals, respectively). Fructans with an internal Glc residue include the neoinulin and neolevan types (occurring in monocots such as *Allium*, *Asparagus*, and *Lolium*).

Apart from their function as a vacuolar storage carbohydrate, fructans may help to protect plants against abiotic stresses (Hisano et al., 2004, 2008; Li et al., 2007; Kawakami et al., 2008; Xue et al., 2008) by stabilizing cellular membranes (Hincha et al., 2007). Grass fructans, mainly stored in mature leaf sheaths and elongating leaf bases, support leaf regrowth after defoliation (Morvan-Bertrand et al., 2001).

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[2] Present address: Cornell University, 262 Plant Science Building, Tower Road, Ithaca, NY 14850.

[3] Present address: Laboratoire de Nutrition Azotée des Plantes, Unité de Recherche 511, Département de Biologie Végétale, Centre de Recherche de Versailles-Grignon, INRA, RD 10, Route de Saint-Cyr, 78026 Versailles cedex, France.

* Corresponding author; e-mail wim.vandenende@bio.kuleuven.be.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Wim Van den Ende (wim.vandenende@bio.kuleuven.be).

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The different fructan classes are biosynthesized by different types of fructosyltransferases (FTs), while fructan breakdown is catalyzed by different types of fructan exohydrolases (FEHs; for review, see De Coninck et al., 2007). In a first step, Suc:Suc 1-fructosyltransferase (1-SST) catalyzes the production of the trisaccharide 1-kestose (1K) from two Suc molecules. In dicots, 1K acts as a preferential donor substrate for the elongation enzyme fructan:fructan 1-fructosyltransferase (1-FFT), producing inulin-type fructans (Edelman and Jefford, 1968; Van den Ende and Van Laere, 2007) with higher degrees of polymerization. In cereals, the key enzyme Suc:fructan 6-fructosyltransferase (6-SFT) uses Suc as a donor substrate and 1K as an acceptor substrate to synthesize the tetrasaccharide bifurcose (1&6-kestotetraose; Duchateau et al., 1995; Sprenger et al., 1995). Further elongation is believed to occur by the concerted action of 6-SFT and 1-FFT (Yoshida et al., 2007). Recently, the dual-specificity enzyme fructan:fructan 6G-fructosyltransferase/fructan: fructan 1-fructosyltransferase (6G-FFT/1-FFT) was described in onion (*Allium cepa*; Ritsema et al., 2003) and in perennial ryegrass (*Lolium perenne*; Lasseur et al., 2006). This enzyme allows the biosynthesis of neoinulin series and small inulin-type fructans but cannot account for the synthesis of the neolevan type of fructans in perennial ryegrass, which is catalyzed by a 6-SFT type of enzyme (Lasseur et al., 2006; B. Lasseur, J. Lothier, A. Wiemken, A. Van Laere, A. Morvan-Bertrand, W. Van den Ende, and M.P. Prud'homme, unpublished data).

FTs might be crucial enzymes for plant survival under stress conditions, especially in plants that use fructans as a major reserve carbohydrate, such as perennial ryegrass (Hisano et al., 2008), explaining the extended research efforts on the key enzymes 1-SST and 6G-FFT/1-FFT from perennial ryegrass. In general, FTs are reported to be regulated by Suc, light, and cold (Gallagher et al., 2007; Van den Ende and Van Laere, 2007; Hisano et al., 2008). Besides regulation at the transcriptional level, posttranslational regulation has also been suggested (Amiard et al., 2003; Lasseur et al., 2006).

FTs are believed to have evolved from vacuole-type invertases (VIs), and this process occurred independently in monocots and dicots (Vijn and Smeekens, 1999; Wei and Chatterton, 2001). By contrast, FEHs are believed to have evolved from cell wall invertases (CWIs) or from an ancestral β-fructosidase type of enzyme (Le Roy et al., 2007, 2008) capable of degrading both Suc and fructans. VIs, CWIs, FEHs, and FTs all belong to family 32 of glycoside hydrolases (GH32; Lammens et al., 2008).

Among the FTs, two major groups can be further discerned: FTs that use Suc as a preferential donor substrate on the one hand (S-type enzymes: 1-SST and 6-SFT) and FTs that use fructans (and not Suc) as a preferential donor substrate on the other hand (F-type enzymes: 1-FFT and 6G-FFT). From a functional point of view, S-type enzymes are still more similar to the VIs from which they evolved, since both enzymes split Suc, a property that was lost or strongly reduced in F-type enzymes.

Perennial ryegrass is an economically important species and is an ideal system in which to perform detailed structure-function work on FTs and VIs. Indeed, many cDNAs became available from this species in recent years (Chalmers et al., 2005; Lasseur et al., 2006). Moreover, the overall identity between these cDNAs is very high, and recombinant enzymes are available through heterologous expression in *Pichia pastoris*. The very high identity at the sequence level between Lp6G-FFT/1-FFT and Lp1-SST inspired us to study in depth the molecular differences between these functionally different enzymes. Despite their high identity, these enzymes differ greatly in substrate specificity and product formation, the two most crucial differences being that (1) Lp1-SST uses Suc as a donor substrate, while Suc is a very bad donor substrate for the recombinant Lp6G-FFT/1-FFT and (2) Lp1-SST can only create a β(2-1) linkage between two fructosyl residues, while Lp6G-FFT/1-FFT can create both a β(2-1) linkage between two fructosyl residues and a β(2-6) linkage between one fructosyl residue and one glucosyl residue.

Although substantial progress has been made to transform a VI into a FT (Ritsema et al., 2006; Schroeven et al., 2008) and a CWI into a FEH (Le Roy et al., 2007), the transformation of one type of FT activity into another type of FT activity has not been reported before. Here, we describe, to our knowledge for the first time, the transformation of an F-type enzyme (6G-FFT/1-FFT from perennial ryegrass: Lp6G-FFT/1-FFT) into an S-type enzyme (1-SST) by site-directed mutagenesis and compare the kinetic properties of the best mutant with the wild-type 1-SST from perennial ryegrass (Lp1-SST).

RESULTS

Designing Mutants Based on Multiple Sequence Alignments and Modeling

As argued in the introduction, two major classes of FTs can be discerned: the S- and F-type FTs. It is widely admitted that all of these FTs are composed of two subunits, one large subunit and one small subunit (Sprenger et al., 1995; Altenbach et al., 2004). Interestingly, these functionally different types of enzymes can be very similar at the sequence level. For instance, the Lp6G-FFT/1-FFT and Lp1-SST enzymes share 83% identical amino acids in their mature proteins. As a first step to understanding the difference at the molecular level between these enzymes, a multiple sequence alignment (Fig. 1) was made containing Lp1-SST, Lp6G-FFT/1-FFT, the 6G-FFT/1-FFT from onion (Ritsema et al., 2003; Fujishima et al., 2005), and the 6G-FFT from *Asparagus officinalis* (Ueno et al., 2004). Since the substrate specificity of FTs is believed to be determined by their large subunit carrying the active

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Ac6G-FFT    MDAQDIESR-----------HPLIGARPRRRALRSLS------------ILLAAALLLGL 37
Ao6G-FFT    ---MATSLQ-----------APILGSRPPRRTLRFLS------------FALFSALVLVV 34
Lp6G-FFT    MESSAVVVQGTTAPLLPYAYAPLPSSADDARENQSSGGGVRWRACA---ASALVVLLVVV 57
Lp1-SST     MESPSAVVPGTTAPLLPYAYAPLPSSADDARQNRSGG---RWRACAAVLAASALAVVVVV 57
                                 *: .:    *  :  .                .::: :

Ac6G-FFT    VLFY--------------------------ANGTGSGTAVDPV---RVDNEFPWTNDML 67
Ao6G-FFT    ASFS--------------------------SRKSESGSGLRSG---SVEPEYAWTNQML 64
Lp6G-FFT    GFFAGGRVDLGQDGEVSATSSVPGS------SRGKDSGVSEKES---PADGGFPWSNAML 108
Lp1-SST     GLLAGGRVDRVPAGGDVASATVPAVPMEFPRSRGKDLGVSEKSSGAYSTDGGFPWSNAML 117
              :                          :.  .  * .         .:  :.*:* **

Ac6G-FFT    AWQRCGFHFRTVRNYMNDPSGPMYYKGWYHLFYQHNKDFAYWGNITWGHAVSRDLINWQH 127
Ao6G-FFT    TWQRAGFHFRTVKNYMNDPSGPMYYKGWYHLFYQHNPNYAYWGDISWGHAVSRDLLNWFH 124
Lp6G-FFT    QWQHTGFHFQPLKHYMNDPNGPVYYGGWYHLFYQHNPYGDSWGNVSWGHAVSKDLVNWRH 168
Lp1-SST     QWQRTGFHFQPEQHYMNDPNGPVYYGGWYHLFYQHNPKGDSWGNIAWAHAVSKDMVNWRH 177
             **: ****:. ::*****.**:** **********     **:::*.****:*::** *

Ac6G-FFT    LPVAVGPDHWYDISGVWTGSIIVVSED-RVVMLFTGGTKSFDQSINLAEAADPSDPLLLK 186
Ao6G-FFT    LPVAVKPDRWYDIYGVWTGSITVMPDDGRVVMLYTGGTKEKYQIMSVAMAADPSDPLLVE 184
Lp6G-FFT    LPVALVPDQWYDINGVLTGSITVLPDG-RVILLYTGNTDTFSQVQCLAVPADPSDPLLRS 227
Lp1-SST     LPLAMVPDQWYDSNGVLTGSITVLPDG-QVILLYTGNTDTLAQVQCLATPADPSDPLLRE 236
            **:*: **:***  ** **** *:.:. :*::*:**.*.    *    :* .******** .

Ac6G-FFT    WIKYDN-NPILWPPPGIVRDDFRDPNPIWYNASESTYHIVVGSKNDSLQHTGIALVYLTK 245
Ao6G-FFT    WVKYDEVNPVLRPPPGIGLTDFRDPNPIWYNTTDSTWQLVIGSKNDSLQHTGIAMVYTTK 244
Lp6G-FFT    WIKHPA-NPILFPPPGIGLKDFRDPLTAWFEHSDNTWRTIIGSKDDD-GHAGIVLSYKTT 285
Lp1-SST     WIKHPA-NPILFPPPGIGLKDFRDPLTAWFDHSDHTWRTVIGSKDDD-GHAGIILSYKTK 294
            *:*:   **:* *****    ***** . *:: :: *:: ::***:*.  *:** : * *.

Ac6G-FFT    DFKKFDLLPTVLHS-VDKVGMWECVEVYPVATTGPLLHKAIDNFDVDRVLDRSTVKHVLK 304
Ao6G-FFT    DFINLTLLPGVLHS-VDHVGMWECVDLFPVASSGPLIGRGLD----RSMMLADNVKHVLK 299
Lp6G-FFT    DFVNYELMPGNMHRGPDGTGMYECLDIYPVGGNSSEMLGGDS---------SPEVLFVLK 336
Lp1-SST     DFVNYELMPGNMHRGPDGTGMYECIDLYPVGGNSSEMLGGDD---------SPDVLFVLK 345
            ** :  *:*  :*   *  .**:**::::**. ... :  . .              * .***

Ac6G-FFT    ASMNDEWHDYYAIGTFDPIGNKWTPDDETVDVGIGLRYDWGKFYASRTFFDPLKQRRIIW 364
Ao6G-FFT    ASMNDEWHDYYAIGSYDVATHRWVPDDESVDVGIGMRIDWGKFYASRTFYDPVKERRVMW 359
Lp6G-FFT    ESANDEWHDYYALGWFDAAANTWTPQDPEADLGIGLRYDWGKYYASKSFYDPIKNRRVVW 396
Lp1-SST     ESSDDERHDYYALGRFDAVANVWTPIDRELDLGIGLRYDWGKYYASKSFYDQKKNRRIVW 405
             * :** *****:* :*   : *.* *   *:***:* ****:***::*:*  *:**::*

Ac6G-FFT    GYIGEVDSQKADIAKGWASLQGIPRSVLYDVKTGTNVLTWPIEEMEGLRMARKDFSGIKI 424
Ao6G-FFT    GYVGETDSGDADVAKGWASFQGIPRTVLFDVKTGTNVLTWPIEEVESLRMTRKDFSDIVV 419
Lp6G-FFT    AFVGETDSEQADKAKGWASLMSIPRTVELDKKTRTNLIQWPVEEIETLRRNVTDLGGITV 456
Lp1-SST     AYIGETDSEQADITKGWANLMTIPRTVELDRKTRTNLIQWPVEEVDTLRRNSTDLGRITV 465
            .::**.** .** :****.:  ***:*  * ** **::**:**:: **   .*:. * :

Ac6G-FFT    KKGSTVELSDFGDAFQIDIEAEFTISKEALEATIEADVGYNCSSSGGAAIRGTLGPFGLL 484
Ao6G-FFT    NKGSTVEL-HVGDANQLDIEAEFEMDKDALETAIEADIGYNCSSSGGAVSRGVLGPFGLF 478
Lp6G-FFT    EAGSVIHL-PLQQGGQLDIEASFRLNSSDIDALNEADVGFNCSSSDGAAVRGALGPFGLL 515
Lp1-SST     NAGSVIRL-PLHQGAQLDIEASFQLNSSDVDAINEADVGYNCSTS-GAAVRGALGPFGLL 523
            : **.:.*  . :. *:****.* :... :::  ***:*:***:* **. **.******:

Ac6G-FFT    VLANQDLTENTATYFYVSKGIDGSLITHFCQDETRSSKANDIVKRVVGGTVPVLDGETFA 544
Ao6G-FFT    VLANQDLTELTATYFYVSRATDGSLHTHLCHDEMRSSKANDIVKRVVGGTFTVLDGELLS 538
Lp6G-FFT    VFADG-RHEQTAAYFYVSKGLDGSLLTHYCHDESRSTRAKDVVSRVVGGTVPVLDGETFS 574
Lp1-SST     VLANG-RTEQTAVYFYVSKGVDGGLQTHFCHDESRSTRAKDVVNRMIGSIVPVLDGETFS 582
            *:*:    * **.*****:. **.* ** *:** **::*:*:*.*::*. ..***** ::

Ac6G-FFT    VRILVDHSVIESFAMGGRTSATSRAYPTEAINSAARVFLFNNATGVDVIAESVKIWQMNS 604
Ao6G-FFT    LRILVDHSIVESFAQGGRTSATSRVYPTEAIYERARVFLFNNATGATITAKAVKVWQMNS 598
Lp6G-FFT    VRVLVDHSIVQSFVMGGRTTVTSRAYPTEAIYAAAGVYLFNNATSATITAEGLVVYEMAS 634
Lp1-SST     VRVLVDHSIVQSFAMGGRITATSRAYPTEAIYAAAGVYLFNNATGATVTAERLVVHEMAS 642
            :*:*****:::**. *** :.***.******   * *:******.. : *: : : :* *

Ac6G-FFT    TYNDFYHF---- 612
Ao6G-FFT    TSNQYYPFTSSN 610
Lp6G-FFT    AESRAFLADDM- 645
Lp1-SST     ADNHIFTNDDL- 653
            : .  :
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Figure 1. Multiple alignment of the amino acid sequences of Lp1-SST (accession no. AY245431) and all plant 6G-FFT-type enzymes characterized so far (Ac, *Allium cepa* [AY07838]; Ao, *Asparagus officinalis* [AB084283]; Lp, *Lolium perenne* [AF492836]). Conserved regions are boxed. The three carboxylic acids that are crucial for enzyme catalysis (Verhaest et al., 2007) are marked in these regions. Arrows indicate the start of the large and small subunits. Amino acids that are identical in all 6G-FFTs but absent in Lp1-SST are boldface and underlined. Consensus line symbols are as follows: asterisks, identical residues (also marked with a gray background); colons, conserved substitutions; periods, semiconserved substitutions.

site (Altenbach et al., 2004), among the 189 amino acids common to the large subunits of the three 6G-FFTs, 10 amino acids were not found in Lp1-SST (Fig. 1). Therefore, these amino acids represent interesting targets for site-directed mutagenesis. However, most of these amino acids represent semiconserved substitutions (Fig. 1). Moreover, a molecular modeling approach realized in silico (using Arabidopsis [*Arabidopsis thaliana*] cell wall invertase 1 [AtcwINV1] as a template; Verhaest et al., 2006; Protein Data Bank identifier 2AC1) allowed us to further reduce the number of promising candidates. The active site of all GH32 enzymes is characterized by three highly conserved amino acids in the WMN**D**PNG, R**D**P, and **E**C motifs (Reddy and Maley, 1996; Schroeyen et al., 2008), and only the amino acids within a 15-Å distance from the catalytic triad center were taken into account (Fig. 2). In Lp6G-FFT/1-FFT, the catalytic triad consists of the nucleophile D126, the transition state stabilizer D250, and the proton donor E308 (Fig. 2A). The three most promising amino acids in the vicinity of the active site of Lp6G-FFT/1-FFT are N340, W343, and S415 (Fig. 2A), which are replaced by D349, R352, and N424 in Lp1-SST (Fig. 2C). Therefore, the single mu-

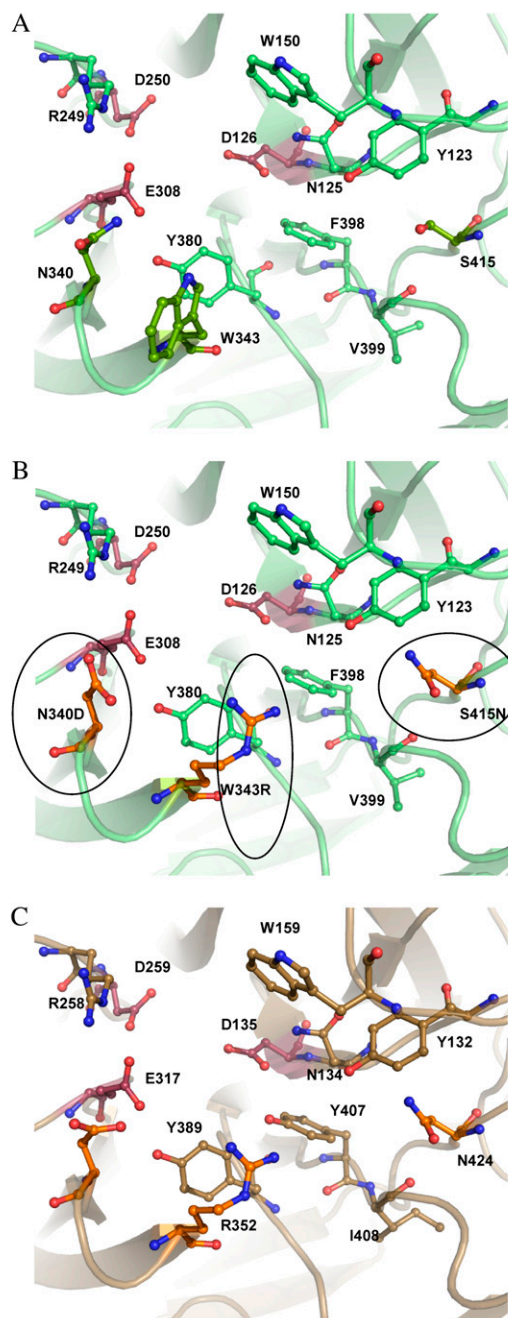

Figure 2. Localization of the considered amino acids in the active sites of wild-type Lp6G-FFT/1-FFT (A), the triple mutant N340D+W343R+S415N from Lp6G-FFT/1-FFT (B), and wild-type Lp1-SST (C). The mutated amino acids are circled; they are located in the large subunit as well as the conserved domains NDPNG, RDP, and EC (based on the sequence homology with barley 6-SFT, the large subunit of the mature Lp6G-FFT begins at amino acid 97 and ends at amino acid 489; Lasseur et al., 2006). AtcwINV1 was used as a template for the three-dimensional models. The images were drawn with Pymol (DeLano, 2002). [See online article for color version of this figure.]

tants N340D (Fig. 3C), W343R (Fig. 3D), and S415N (Fig. 3E), the double mutants N340D+W343R (Fig. 3F), N340D+S415N (Fig. 3G), and W343R+S415N (Fig. 3H), and finally the triple mutant N340D+W343R+S415N (Fig. 3I) were constructed and transformed to *P. pastoris* for functional characterization and comparison with the wild-type enzymes Lp6G-FFT/1-FFT (Fig. 3A) and Lp1-SST (Fig. 3B).

Comparison of FT Activities of Wild-Type and Mutant Enzymes

Lp1-SST uses Suc as a preferential donor and acceptor substrate (Chalmers et al., 2003), while the bifunctional Lp6G-FFT/1-FFT uses 1K as a preferential donor substrate (Lasseur et al., 2006). Previously, both recombinant enzymes were successfully produced in *P. pastoris* (Chalmers et al., 2003; Lasseur et al., 2006). The three main oligosaccharides produced by Lp6G-FFT/1-FFT from 1K (GFF) as a single substrate during short-term incubations are 1&6GK (FGFF), Nys (GFFF), and 6G-kestotriose or neokestose (nK; FGF). 1&6GK results from 6G-FFT activity, while Nys results from 1-FFT activity (Fig. 4). Both reactions produce Suc (GF), which is a good acceptor as well for the Lp6G-FFT/1-FFT, resulting in the formation of nK (Fig. 4). However, Suc can act as a competitive inhibitor at the donor site, especially at 1K:Suc ratios < 1 (see figure 4E in Lasseur et al., 2006). To allow Suc to fulfill its role as acceptor substrate but at the same time prevent its inhibitory role at the donor site, a 1K:Suc ratio of 2.5 was chosen in our experiments. Therefore, equal protein amounts of wild-type and mutant recombinant enzymes were incubated with 50 mM 1K, or 50 mM 1K + 20 mM Suc, or 200 mM Suc. A higher concentration with Suc as a single substrate was chosen because Suc is a very bad donor substrate for the Lp6G-FFT/1-FFT (Lasseur et al., 2006) and to provide enough substrate to the wild-type Lp1-SST, showing a high apparent K_m for Suc (see Fig. 6 below). Representative chromatograms of the 1K- and Suc-only reactions are presented (Fig. 5A). All quantitative data are presented in Tables I to III. It should be noted that the recombinant Lp6G-FFT/1-FFT enzyme also shows considerable 1K exohydrolase activity (Fig. 4) at lower 1K concentrations (Lasseur et al., 2006; Fig. 5). It cannot be excluded that this side activity is caused by the recombinant nature of this enzyme. Indeed, additional side activities have been reported before (Sprenger et al., 1995; Hochstrasser et al., 1998).

Compared with the wild-type Lp6G-FFT/1-FFT, the N340D mutant shows only very low overall activity levels (Fig. 5; Tables I–III). The S415N mutant shows slightly lower 6G-FFT/1-FFT activities, but the ratio between the three main reaction products is not significantly affected (Fig. 5A; Tables I and II). A lower 1-SST activity is also observed for this mutant (Fig. 5B; Table III). Compared with the wild-type Lp6G-FFT/1-FFT and the S415N mutant, the W343R mutant shows a similar 1-FFT activity but a largely reduced 6G-FFT

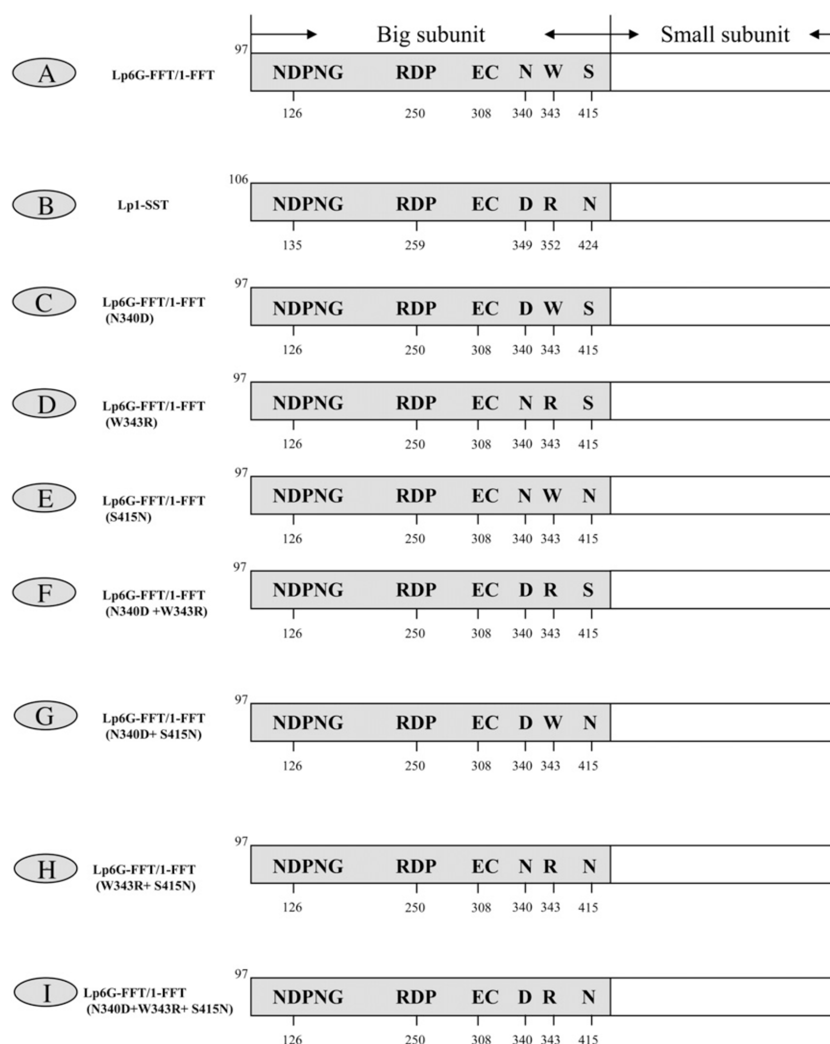

Figure 3. Schematic representation of the wild-type and mutant enzymes considered in this work. The conserved regions NDPNG, RDP, and EC and the three amino acid positions of the catalytic acids are indicated, as well as the positions of the three amino acids that were subjected to mutagenesis. A, Wild-type Lp6G-FFT/1-FFT. B, Wild-type Lp1-SST. C to E, Single mutants. F to H, Double mutants. I, Triple mutant.

activity (Fig. 5A; Tables I and II), resulting in strongly increased Nys-nK and Nys-1&6GK ratios (Tables I and II). Comparable to the N340D mutant, the W343R mutant shows only a very low 1-SST activity (Fig. 5B; Table III). Only a slightly increased activity is observed for the N340D+S415N mutant in comparison with the N340D mutant. The W343R+S415N mutant shows similar properties to the W343R mutant, but the Nys-nK ratio is decreased (Tables I and II). Most important, while the N340D+W343R mutant shows 6G-FFT and 1-FFT activities that hold the middle between those of the N340D and W343R single mutants, its 1-SST activity is increased drastically (Fig. 5B; Table III). The 1-SST characteristics are increased further in the triple N340D+W343R+S415N mutant (Fig. 5B; Table III), while its intrinsic 1-FFT activity is further decreased (Fig. 5A). The properties of the triple mutant resemble those of the wild-type Lp1-SST (Fig. 5B; Table III), although a slightly higher intrinsic 1-FFT activity remains in the mutant (Fig. 5A; Table I). With the exception of the W343R mutant, the hydrolase activity is decreased compared with the wild-type Lp6G-FFT/1-FFT (Fig. 5A; Tables I and II). Interestingly, the Fru-Nys ratio is increased strongly in all mutants containing the N340D mutation (Tables I and II).

The general fructan patterns from the 1K + Suc reaction mixtures did not differ greatly from that of the 1K-only reaction mixtures (data not shown). However, as expected, for all enzymes under study, the addition of 20 mM Suc to 50 mM 1K resulted in a decrease in total oligosaccharide formation and a lower Nys-nK ratio (Tables I and II) caused by the fact that Suc competes with 1K as an acceptor substrate.

Comparing the Kinetic Parameters of Lp1-SST and the Triple Mutant

FTs are very difficult enzymes with respect to kinetic studies, but an estimation of kinetic parameters is sometimes possible (Van den Ende et al., 1996a, 1996b; Vergauwen et al., 2003) for initial reactions catalyzed by "unifunctional" enzymes and provided that the products formed are not better substrates (compared with the original substrates) or strong inhibitors. The

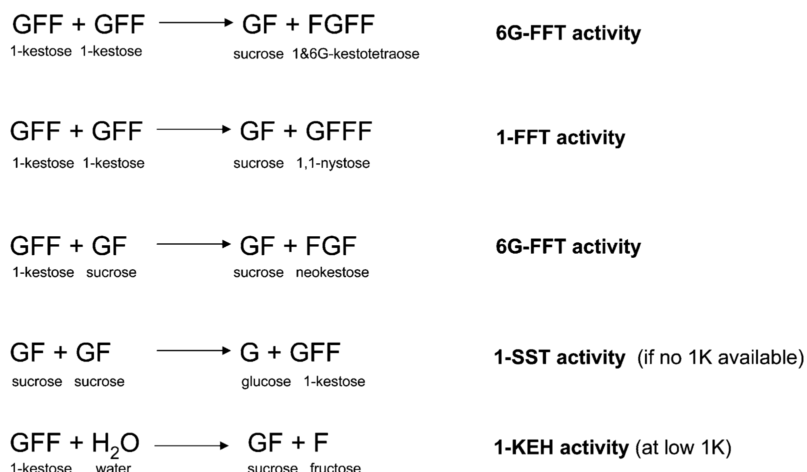

Figure 4. An overview of the initial reactions that are typically catalyzed by the recombinant Lp6G-FFT/1-FFT provided with 1K, Suc, or both. A minor 1-SST activity is only found with Suc as a single substrate, while hydrolytic activity becomes gradually more important at low 1K concentrations (Lasseur et al., 2006).

bifunctional Lp6G-FFT/1-FFT enzyme forms a very complex system, since it can catalyze multiple reactions (Fig. 4). Moreover, Lp6G-FFT/1-FFT produces Suc, which is a good acceptor substrate, rapidly competing with 1K as an acceptor substrate, especially at lower 1K concentrations. The situation is further complicated by the fact that Suc can also inhibit the binding of 1K as a donor substrate. Therefore, a kinetic approach to the Lp6G-FFT/1-FFT system is an extremely difficult task. On the contrary, Lp1-SST and the triple mutant N340D+W343R+S415N catalyze only one major type of reaction, allowing a reasonable estimation of the apparent K_m and V_{max} values. The remaining question is the following: Is there still some kinetic difference between the triple mutant N340D+W343R+S415N and the wild-type Lp1-SST, taking into account a whole range of Suc concentrations? Figure 6 compares the substrate/velocity curves of the triple mutant in comparison with both wild-type enzymes. Interestingly, although both enzymes can be considered as 1-SSTs, they differed in their saturation characteristics. While the wild-type Lp1-SST showed a tendency to saturate at the highest Suc concentrations, this saturation behavior was absent for the triple mutant (Fig. 6). At the lower Suc concentrations, the wild-type Lp1-SST showed a higher activity compared with the triple mutant. Linear Hanes plots were obtained and the kinetic parameters were estimated for both enzymes. An apparent K_m of 272 mM was obtained for the Lp1-SST, and the V_{max} was estimated at 78 nkat mg^{-1} protein. Considerably higher K_m (976 mM) and V_{max} (118 nkat mg^{-1} protein) values were obtained for the triple mutant.

Homologous Amino Acids in Other GH32 Plant Enzymes

The results show that N340, W343, and S415 are important determinants for explaining the particular donor substrate characteristics of the wild-type Lp6G-FFT/1-FFT. What are the homologous amino acids in other FTs and in the structurally well-characterized chicory (*Cichorium intybus*) 1-FEH IIa and AtcwINV1? Figure 7 shows a multiple alignment of a selection of FTs together with chicory 1-FEH IIa and AtcwINV1. With the help of modeling studies, it could be confirmed that the N340/W343 couple is equivalent to the D239/K242 couple in AtcwINV1. In AtcwINV1, the D239/K242 couple is believed to be essential for binding Suc (for more details, see "Discussion").

DISCUSSION

Marked Differences between S and F Enzyme Types

Using a combined multiple alignment and modeling approach, we could rationally limit the number of putative amino acids involved in donor substrate selectivity between Lp6G-FFT/1-FFT (preferred donor, 1K) and Lp1-SST (preferred donor, Suc). Only three amino acids, being different between Lp6G-FFT/1-FFT and Lp1-SST, were selected on this basis: N340, W343, and S415 (Figs. 1 and 2). N340 and W343 are unique amino acids among GH32 plant members (occurring only in 6G-FFTs; Fig. 7), which are very close in place (Fig. 2A). The N/W couple is part of a flexible loop structure, comparable to the D/K couple, as observed in AtcwINV1 (Fig. 7; Lammens et al., 2008). Intriguingly, all S-type enzymes (1-SSTs, 6-SFTs, and vacuolar invertases) contain a D and an R at these positions (Fig. 7), which form a couple (D/R couple; Fig. 2C). Strikingly, the D/R couple is absent in all F-type enzymes, including the FEHs in the CWI/FEH group (Fig. 7; Le Roy et al., 2008).

Understanding the Complex Role of W343

The S415N substitution did not greatly affect the activity of Lp6G-FFT/1-FFT (Tables I–III). When changing N340 alone (creating the D/W couple), low overall activity levels are generated (Fig. 5; Tables I–III). More important, no additional S-type characteristics are created. This result strongly suggests that

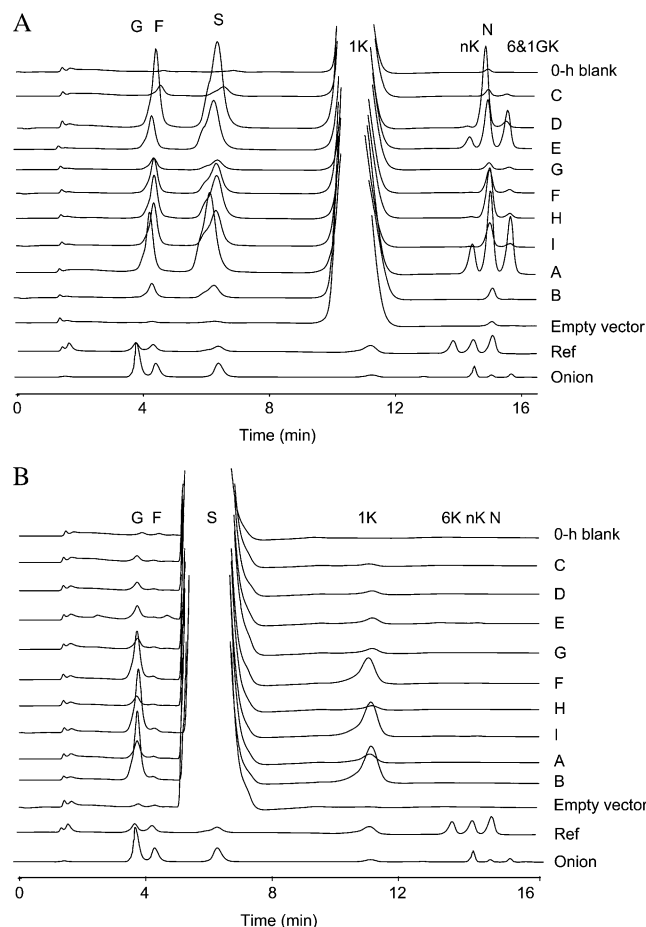

Figure 5. HPAEC-PAD chromatograms of reaction mixtures of the wild-type and mutant enzymes under study (compare with Fig. 3). Aliquots of the enzyme (final concentration, 40 μg mL^{-1}) were incubated with 50 mM 1K for 10 min (A) or 200 mM Suc for 25 min (B) at 30°C. A 0-h sample (blank) and an empty vector control are also presented. For comparison, references with standard compounds (1.25 nmol of each carbohydrate) and an authentic extract from an onion bulb are also indicated. G, Glc; F, Fru; S, Suc; N, 1,1-kestotetraose or nystose; 6&1GK, 6&1G-kestotetraose.

introduction of an Asp residue alone is not sufficient to introduce S-type activity. Most likely, the Asp should be positioned in a correct orientation to be able to interact with Suc as a donor. This orientation might be determined by the neighboring amino acid in the couple.

When changing W343 alone (creating the N/R couple), the 6G-FFT activity was greatly affected but the 1-FFT or 1-SST activity was not (Tables I–III). Therefore, the result strongly suggests that W343 as such is not involved in donor substrate selectivity but rather influences the binding orientation of the acceptor substrate, favoring the formation of a β(2-6) linkage between Fru and Glc. This is the first report, to our knowledge, demonstrating that amino acids outside the WMNDPNG motif are very important in this respect. Previously, Ritsema et al. (2005) showed for Ac6G-FFT/1-FFT that mutagenesis at N84 (into S, A, or Q) in the WMNDPNG (β-fructosidase) motif could change the 6G-FFT/1-FFT activity ratio. However, these mutations are artificial, since S, A, and Q never occur at this position in any F-type enzymes (Altenbach and Ritsema, 2007). Therefore, it seems likely that the linkage specificity in the wild-type Lp6G-FFT/1-FFT, and perhaps also in other naturally occurring 6G-FFT/1-FFTs, is determined by W343 and not by N125 (Fig. 2A). Indeed, N125 is invariably present in Lp6G-FFT/1-FFT (Fig. 2A), Lp1-SST (Fig. 2C), and all LpVIs (data not shown). Overall, Trps are known to interact with sugars, as observed for many other sugar-metabolizing enzymes (Törrönen and Rouvinen, 1997). The introduction of a bulky hydrophobic Trp in the active site often enhances the transglycosylation capacity (Fujita and Takegawa, 2002; Tang et al., 2006). The finding that only a few amino acids are essential to discriminate between transferases and hydrolases was also reported by Schroeven et al. (2008), by Chambert and Petit-Glatron (1991), and by Kelly et al. (2007).

The N340/W343 Couple Greatly Determines the Donor Substrate Selectivity

The most prominent result is obtained after changing the N/W couple into a D/R couple, causing a drastic shift from an F-type to an S-type activity (Tables I–III), as could be expected from comparing the whole range of naturally occurring F-type and S-type enzymes (Fig. 7). These results strongly suggest that R343 is able to bring D340 into a correct position for binding Suc as a donor substrate. Since no FT crystal structure-substrate complexes are available, only modeled results can be generated. These results are in favor of a putative hydrogen bond between D340 and R343 (Fig. 2C), although the estimated distance (4.5 Å) is higher than that observed between D239 and K242 in AtcwINV1 (Lammens et al., 2008), raising doubts about the effective presence of a hydrogen bond between these residues. To resolve the exact nature of this interaction, Lp6G-FFT/1-FFT crystallization and three-dimensional structural elucidation would be a path-breaking task, but so far this is hampered by technical difficulties in obtaining significant amounts of purified enzyme.

Taken together, these results strongly suggest that the amino acid at position 343 (W or R) in Lp6G-FFT/1-FFT can greatly determine the donor substrate characteristics by influencing the position of the amino acid at position 340. On the other hand, this amino acid can also influence the acceptor substrate characteristics, the presence of R343 negatively affecting the formation of neofructan-type linkages. It can be speculated that the N-W or D-R interaction can be destroyed after binding the donor substrate, allowing more freedom to these amino acid side chains, so that W or R can fulfill a fundamental role in acceptor substrate binding as well. In this respect, it is important to realize that W343 (Lp6G-FFT/1-FFT) and R352

Table I. *Quantification of nK, Nys, and 6&1GK formation in reaction mixtures (compare with Fig. 5) containing the enzymes under study (compare with Fig. 3) together with 50 mM 1K*

Nys formation equals 1-FFT activity. The sum of nK and 6&1GK productions represents the total 6G-FFT activity. Fru production reflects the overall hydrolytic activity.

Enzyme	Name	Fru	nK	Nys	6&1GK	Nys-nK Ratio	Nys-6&1GK Ratio	nK-6&1GK Ratio	Fru-Nys Ratio
			nkat mg^{-1} protein						
Lp6G-FFT	A	179.45 ± 10.57	7.75 ± 0.45	126.13 ± 13.49	156.75 ± 8.72	16.27	0.80	0.05	1.42
Lp1-SST	B	67.97 ± 1.15	0.02 ± 0.01	5.38 ± 0.21	0.31 ± 0.01	297.69	17.12	0.06	4.22
N340D	C	28.71 ± 0.23	0.16 ± 0.05	3.92 ± 0.18	2.48 ± 0.07	23.87	1.58	0.07	7.32
W343R	D	196.38 ± 7.56	0.90 ± 0.03	121.56 ± 4.25	11.26 ± 0.30	129.58	10.36	0.08	1.61
S415N	E	111.52 ± 6.25	7.15 ± 0.14	71.28 ± 0.65	81.68 ± 0.65	9.97	0.87	0.09	1.56
N340D+W343R	F	140.50 ± 2.80	0.27 ± 0.18	31.34 ± 2.21	6.80 ± 0.36	116.79	4.61	0.04	4.48
N340D+S415N	G	33.48 ± 0.20	0.28 ± 0.06	4.98 ± 0.29	3.07 ± 0.05	17.51	1.62	0.09	6.72
W343R+S415N	H	163.17 ± 1.86	1.10 ± 0.23	76.63 ± 2.27	8.25 ± 0.36	69.66	9.29	0.13	2.13
N340D+W343R+S415N	I	157.31 ± 6.82	0.92 ± 0.12	28.07 ± 0.59	4.71 ± 0.05	30.45	5.96	0.20	5.60

(Lp1-SST) are the functional (but not structural) homologs of R360 in *Bacillus subtilis* levansucrase (Lammens et al., 2008) and H296 in *Zymomonas mobilis* levansucrase (Yanase et al., 2002), both members of family GH68 of structurally related FTs. Mutagenesis of these amino acids revealed that they greatly determine the transfructosylation properties of these enzymes (Chambert and Petit-Glatron, 1991; Yanase et al., 2002; Li et al., 2008). Taken together, the available data in GH68 and GH32 now lead to the general hypothesis that the conformational flexibility of R360 (or its homologs W343/R352 in *Lolium* enzymes) is linked to the formation of a transient docking site for the fructosyl-acceptor substrate (Seibel et al., 2006; Meng and Fütterer, 2008; this work).

The Effect of the S415N Substitution

Lp6G-FFT has an S at position 415 (Fig. 2A), similar to VIs (Fig. 7). Many 1-SSTs show an N at this position, while dicot 1-FFTs have a T (Fig. 7). The ability to use Suc as a donor and acceptor substrate increased further in the triple N340D+W343R+S415N mutant (Fig. 5B; Table III) compared with the double N340D+W343R mutant (Fig. 5B; Table III), and its 1-FFT activity was further decreased (Fig. 5A), although the level remained higher than that observed for Lp1-SST. These results strongly suggest that the S-to-N transition is beneficial for further optimizing 1K production from Suc. The relatively higher nK production (N340D+W343R+S415N compared with N340D+W343R, N340D+S415N compared with N340D, and W343R+S415N compared with W343R; Table I) shows that the Suc formed from 1K can be transformed more efficiently to nK if N is present at position 415. Although the S415N mutant shows a slightly reduced overall activity, it shows the lowest Nys-nK ratio of all enzymes tested, indicating that the N residue plays a particular role for binding Suc in a configuration in which it can efficiently accept a fructosyl moiety at the primary hydroxyl group at the C6 position of Glc. Conclusively, the combined presence of W343 and N415 (not observed in any natural 6G-FFT/1-FFT; Fig. 1) seems most optimal for the formation of neofructan types. Rational enzyme design in this area might lead to further optimization and the production of tailor-made fructans by mutant enzymes, either in transgenic plants or in bioreactors.

Table II. *Quantification of nK, Nys, and 6&1GK formation in reaction mixtures (compare with Fig. 5) containing the enzymes under study (compare with Fig. 3) together with 50 mM 1K and 20 mM Suc*

Nys formation equals 1-FFT activity. The sum of nK and 6&1GK productions represents the total 6G-FFT activity. Fru production reflects the overall hydrolytic activity.

Enzyme	Name	Fru	nK	Nys	6&1GK	Nys-nK Ratio	Nys-6&1GK Ratio	nK-6&1GK Ratio	Fru-Nys Ratio
			nkat mg^{-1} protein						
Lp6G-FFT	A	74.86 ± 10.99	11.25 ± 1.13	49.63 ± 3.78	70.00 ± 1.32	4.41	0.71	0.16	1.51
Lp1-SST	B	18.21 ± 0.45	0.03 ± 0.01	1.45 ± 0.02	0.06 ± 0.01	41.68	23.68	0.57	4.19
N340D	C	10.20 ± 0.46	0.24 ± 0.03	1.51 ± 0.27	0.94 ± 0.10	6.27	1.60	0.26	6.76
W343R	D	69.98 ± 4.48	1.35 ± 0.13	42.08 ± 1.78	4.10 ± 0.22	31.27	10.27	0.33	1.66
S415N	E	43.45 ± 1.79	9.46 ± 0.04	28.67 ± 0.40	32.43 ± 0.38	3.03	0.88	0.29	1.52
N340D+W343R	F	36.14 ± 0.84	0.74 ± 0.16	9.65 ± 0.86	1.98 ± 0.19	13.07	4.87	0.37	3.75
N340D+S415N	G	11.76 ± 0.82	0.26 ± 0.01	1.44 ± 0.27	1.04 ± 0.06	5.52	1.38	0.25	8.18
W343R+S415N	H	62.38 ± 0.34	1.48 ± 0.05	29.08 ± 1.08	3.00 ± 0.08	19.66	9.69	0.49	2.14
N340D+W343R+S415N	I	58.10 ± 0.23	0.90 ± 0.03	10.65 ± 0.38	1.80 ± 0.07	11.86	5.91	0.50	5.46

Table III. *Quantification of 1K formation in reaction mixtures (compare with Fig. 5) containing the enzymes under study (compare with Fig. 3) together with 200 mM Suc*

1K formation equals 1-SST activity.

Enzyme	Name	1K
		nkat mg⁻¹ protein
Lp6G-FFT	A	5.38 ± 0.66
Lp1-SST	B	32.22 ± 0.69
N340D	C	1.71 ± 0.13
W343R	D	2.04 ± 0.67
S415N	E	3.32 ± 0.31
N340D+W343R	F	15.17 ± 0.23
N340D+S415N	G	2.69 ± 0.16
W343R+S415N	H	2.28 ± 0.04
N340D+W343R+S415N	I	21.17 ± 0.17

The Triple Mutant Resembles Lp1-SST

When tested at 1 M Suc, the triple mutant and wild-type Lp1-SST produced equal amounts of 1K (Fig. 6). When comparing their kinetic parameters (Lp1-SST, K_m ~ 272 mM; V_{max} ~ 78 nkat mg^{-1} protein; triple mutant, K_m ~ 976 mM; V_{max} ~ 118 nkat mg^{-1} protein) with those of the recombinant 1-SST from *Agave tequilana* (K_m ~ 633 mM; V_{max} ~ 3.85 nkat mg^{-1} protein; Avila-Fernandez et al., 2007) and the purified native 1-SST from chicory (K_m ~ 313 mM; V_{max} ~ 324 nkat mg^{-1} protein; Van den Ende et al., 1996a), it is striking that the *Agave* 1-SST has a much lower specific activity and a higher apparent K_m than the wild-type chicory and *Lolium* 1-SSTs.

It is clear that the triple mutant gained the characteristic of using Suc as a donor substrate. However, at the same time, this mutant can still use 1K as a donor substrate, but in a much less efficient way than the wild-type Lp6G-FFT/1-FFT. We propose that this residual 1-FFT activity can (at least partly) explain why the triple mutant differs in substrate concentration/velocity plots (Fig. 6). Indeed, next to the 1-SST reaction Suc + Suc → Glc + 1K, the competitive futile reaction 1K + Suc → Suc + 1K might become particularly important for the mutant enzyme, especially at the lower Suc concentrations. Obviously, this causes an underestimation of the velocity at the lower Suc concentrations and an overestimation of the apparent K_m values. For a partially purified enzyme fraction from *Lolium rigidum*, containing both 6G-FFT and 1-FFT activities (and thus probably representing the Lr6G-FFT/1-FFT), work with radioactively labeled Suc convincingly demonstrated that the major reaction was the transfer of a fructosyl residue from 1K to Suc, resulting in resynthesis of 1K (St. John et al., 1997). Therefore, most likely the futile reaction 1K + Suc → Suc + 1K might even be more important than the reaction 1K + Suc → Suc + nK using the same substrates (Lasseur et al., 2006). Overall, it is generally assumed that these futile reactions play a prominent role for all F-type enzymes (Van den Ende et al., 1996b), also explaining why a long lag phase is observed during the in vitro synthesis of longer polymerization fructans by combining S- and F-type enzymes with Suc as a single substrate (Van den Ende and Van Laere, 1996).

Another explanation for the reduced 1K synthesis by the triple mutant (compared with Lp1-SST) might be that Suc cannot optimally bind as an acceptor at the lower concentrations. A comparison of the active sites of Lp1-SST and the triple mutant revealed that Lp1-SST contains Y407 as a homolog of F398 (Fig. 2, A versus C). This residue was not retained for mutagenesis, since all other plant S- and F-type FTs contain a Y at this position (Fig. 7), strongly suggesting that this amino acid could not be important with respect to donor substrate selectivity, which was the major goal of this study. However, Altenbach and Ritsema (2007) reported that mutagenizing the homolog of Y407/F398 in onion VI, W440, into a Y resulted in an increased capacity to use Suc as an acceptor substrate and an increased production of 1K. Taken together with the fact that a Y is invariably present at this position in all natural 1-SSTs (Fig. 7), these findings strongly suggest that a Y is needed at this position to optimize the binding of Suc as an acceptor substrate.

Parallels with Donor Substrate Selectivity in the CWI/FEH Group

Several three-dimensional structures have been unraveled within GH32 (for review, see Lammens et al., 2008), including a 1-FEH from chicory (Verhaest et al., 2005) and, most recently, a CWI from Arabidopsis (Verhaest et al., 2006).

Within family GH32, differences in donor substrate preference occur in both the VI/FT and CWI/FEH subgroups. On the other hand, the most marked difference in acceptor substrate selectivity (water: hydrolases versus fructan:FTs or Suc:FTs) seems to be

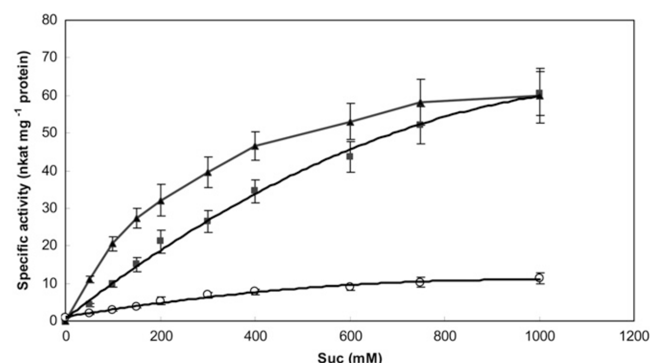

Figure 6. Suc concentration versus velocity (1K formation) plots for the recombinant wild-type Lp1-SST (black triangles), the recombinant 6G-FFT/1-FFT N340D+W343R+S415N triple mutant (black squares), and the recombinant wild-type Lp6G-FFT/1-FFT (white circles). Recombinant proteins were incubated with 0 to 1,000 mM Suc for 25 min at 30°C, and 1K production was quantified by HPAEC-PAD. The SE for n = 3 is indicated.

Figure 7. Multiple alignment of a selection of plant GH32 VI/FT members in the regions surrounding the amino acids under study (boldface). Both S-type and F-type enzymes can be discriminated. For comparison, structurally characterized representatives of S-type (AtcwINV1) and F-type (Ci1-FEH IIa) enzymes within the plant GH32 CWI/FEH subgroup are also presented (boldface and underlined). We refer to Le Roy et al. (2008) for more extended multiple alignments within the CWI/FEH subgroup.

Enzyme			
Lolium perenne 6G-FFT/1-FFT	340**N**DE**W**343	396WG**F**398	412GWA**S**415
Allium cepa 6G-FFT/1-FFT	**N**DE**W**	WG**Y**	GWA**S**
Asparagus officinalis 6G-FFT	**N**DE**W**	WG**Y**	GWA**S**
Cichorium intybus 1-FFT	**W**EG**H**	WG**Y**	GWA**T**
Helianthus tuberosus 1-FFT	**W**EG**H**	WG**Y**	GWA**T**
Cynara scolymus 1-FFT	**W**EG**H**	WG**Y**	GWA**T**
***Cichorium intybus* 1-FEH IIa**	233**F**EG**H**236	290WA**W**292	306GWA**G**309
Lolium perenne 1-SST	349**D**DE**R**352	405WA**Y**407	421GWA**N**424
Festuca arundinacea 1-SST	**D**DE**R**	WA**Y**	GWA**N**
Agava tequiliana 1-SST	**D**DE**R**	WG**Y**	GWA**S**
Allium cepa 1-SST	**D**DE**R**	WG**Y**	GWA**S**
Cichorium intybus 1-SST	**D**ED**R**	WG**Y**	GWA**N**
Helianthus tuberosus 1-SST	**D**ED**R**	WG**Y**	GWA**N**
Cynara scolymus 1-SST	**D**ED**R**	WG**Y**	GWA**N**
Hordeum vulgare 6-SFT	**D**DE**R**	MG**Y**	GWA**S**
Lolium perenne 6-SFT (putative)	**D**DE**R**	LG**Y**	GWA**S**
Lolium perenne VI	**D**DD**R**	WG**W**	GWA**S**
Allium cepa VI	**D**DD**R**	WS**W**	GWA**S**
Asparagus officinalis VI	**D**DD**R**	WG**W**	GWA**S**
Cichorium intybus VI	**D**DD**R**	WS**W**	GWA**S**
Orysa sativa VI1	**D**DD**R**	WG**W**	GWA**S**
Orysa sativa VI2	**D**DD**R**	WG**W**	GWA**S**
Arabidopsis thaliana VI1	**D**DT**R**	WG**W**	GWS**S**
Arabidopsis thaliana VI2	**D**DT**R**	WS**W**	GWS**S**
***Arabidopsis thaliana* cwINV1**	239**D**DT**K**242	295WG**W**297	311GWS**G**314

limited to the VI/FT subgroup, since no FT-type enzymes have been discovered in the CWI/FEH subgroup. According to Lammens et al. (2008), so far all family GH32 enzymes bind the terminal Fru (from Suc or a fructan as the donor substrate) in exactly the same way at the −1 position, consisting of the active site formed by the catalytic triad (Fig. 2). However, a considerable variation is observed in the binding position of the Fru (from 1K) or Glc (from Suc) residue at the +1 position in the donor site (Verhaest et al., 2007; Lammens et al., 2008; Matrai et al., 2008). The exact orientation of the sugar residue at this position is believed to be determined by the presence or absence of a D239 homolog (AtcwINV1 terminology) and/or by the orientation of a W82 homolog (1-FEH IIa terminology; Le Roy et al., 2007, 2008; Lammens et al., 2008). Indeed, site-directed mutagenesis on AtcwINV1 showed that the removal of a D239 homolog results in the destruction of invertase activity, so that the endogenous 1K exohydrolase activity became the main activity (Le Roy et al., 2007). So far, all functionally characterized FEHs lack a D239 homolog. It was found that introduction of a D239 homolog in sugar beet (*Beta vulgaris*) 6-FEH and chicory 1-FEH IIa resulted in mutant enzymes that were able, at least to a certain extent, to use Suc as a donor substrate (Le Roy et al., 2008). Therefore, it was proposed that the presence of a D239 is an essential requirement to bind and stabilize Suc (Le Roy et al., 2007), and this was supported by the generation of several AtcwINV1/Suc complexes in which D239 strongly interacts with the Glc part of the Suc molecule (Lammens et al., 2008). From all of this, it became clear that D239 (or its homologs) is a key role player for determining substrate selectivity in the CWI/FEH group. Since K242 forms a close H bond with D239 in AtcwINV1 (Lammens et al., 2008) and K242 mutations negatively affect the binding of Suc (Le Roy et al., 2007), it became evident that the D/K couple in AtcwINV1 is essential for optimal binding and catalysis of Suc. The D/K or D/R couple is invariably present in all CWIs but not in FEHs (Le Roy et al., 2008). Multiple alignments (Fig. 7) and modeling studies indeed confirmed that D349 and R352 are the functional homologs of D239 and K242 in AtcwINV1. By comparing our results with those generated within the CWI/FEH subgroup (Le Roy et al., 2007, 2008), it can now be concluded that very similar mechanisms are used to control the donor substrate selectivity in both subgroups. In support of this, after providing 1K as a single substrate, the Fru-Nys ratio strongly increased in all mutants containing the N340D mutation (Tables I and II). Very likely this can at least partly be explained by the fact that these enzymes, after splitting 1K to Suc and Fru, can further split Suc to Glc and Fru, due to the presence of a real D239 homolog.

Predicting the Major Functionality of Any Plant GH32 Member

By combining the results from the CWI/FEH group with the results obtained here, it can be concluded that the presence of a functional D239 homolog, forming a couple with an adjacent R or K residue (Fig. 7; Le Roy et al., 2008), can be considered a typical characteristic of all enzymes that are able to use Suc as a donor substrate (1-SSTs, 6-SFTs, VIs, and CWIs). On the other hand, the complete absence of such a D/R or D/K couple and/or the presence of deletions in this area are typical for all F-type enzymes (FEHs, 1-FFTs, and 6G-FFTs). These characteristics can easily be derived directly from the sequence level, paving the way to

predict the functionality of any new plant GH32 member. After aligning with GH32 members that have been fully studied in this respect (chicory 1-FEH IIa, sugar beet 6-FEH, AtcwINV1, Lp1-SST, and Lp6G-FFT/1-FFT), it can simply be predicted whether the enzyme belongs to the S or F donor type by checking for the absence or presence of a D/R or D/K couple (Fig. 7). Regarding the acceptor substrate specificity (hydrolase versus transferase), the W(A/S/G)W motif is typical for hydrolases, while an h(A/G)Y/F motif (where h is any hydrophobic amino acid) is typical for FTs (Fig. 7). Interestingly, this runs parallel to the presence (invertases) or absence (FTs) of an intact WMNDPNG motif (Ritsema et al., 2006). By combining all of these features, one can reasonably predict the major functionality (invertase, FEH, SST/SFT, or FFT) of any GH32 member. It should be noted, however, that a functional characterization by heterologous expression and/or purification of the native enzyme remains necessary to decipher the exact nature of the enzyme. In particular, it remains unknown which factors determine whether an enzyme is involved in the degradation or synthesis of β(2-1) versus β(2-6) fructosyl linkages, a challenging area for future research.

CONCLUSION

Most likely, FTs are crucial enzymes for fructan plant survival under stress conditions, especially in plants in which fructans represent the major form of reserve carbohydrate, such as in perennial ryegrass and other economically important fodder grasses and cereals. Here, we used a site-directed mutagenesis and modeling approach to understand the difference in substrate selectivity between Lp1-SST and Lp6G-FFT/1-FFT, two key players of fructan anabolism in perennial ryegrass, and succeeded, to our knowledge for the first time, to transform an F-type FT (Lp6G-FFT/1-FFT) into an S-type FT (1-SST). Together with recent findings within the CWI/FEH group, these insights allow us to propose a general hypothesis to predict the functionality of any GH32 plant member and open the way to rational enzyme design for use in biotechnological applications.

MATERIALS AND METHODS

Cloning and Mutagenesis of Perennial Ryegrass 6G-FFT

The wild-type enzymes (Lp6G-FFT and Lp1-SST) from perennial ryegrass (*Lolium perenne*) were cloned in the expression vector pPICZαA as described previously (Chalmers et al., 2003; Lasseur et al., 2006) in frame behind the yeast α-factor signal to allow entry into the secretory pathway (Hochstrasser et al., 1998).

To create single amino acid mutants, the mature part of Lp6G-FFT, cloned in pPICZαA and previously functionally characterized (Lasseur et al., 2006), was used as a template. Mutagenesis was performed by site-directed mutagenesis (Quickchange Site-Directed Mutagenesis Kit; Stratagene). Single mutants (constructs C, D, and E in Fig. 3) were obtained using the following primer pairs: N6GFFT1 (5′-GAGAGCGCC**G**ACGACGAGTGGCA-3′) and N6GFFT2 (5′-TGCCACTCGTCGT**C**GGCGCTCTC-3′), W6GFFT1 (5′-CCAACGACGAG**A**GGCACGACTAC-3′) and W6GFFT2 (5′-GTAGTCGTGCC**T**CTCGTCGTTGG-3′), and finally, S6GFFT1 (5′-GGGATGGGCG**AA**CCTCATGTCG-3′) and S6GFFT2 (5′-CGACATGAGG**TT**CGCCCATCCC-3′). Introduced mutations are shown in boldface.

To create double amino acid mutants (mutants F, G, and H in Fig. 3), single mutants obtained as above were used as templates, following the same method of site-directed mutagenesis. Using mutant C as a template and W6GFFT1 and W6GFFT2 as primers, double mutant F was generated. Using mutant C as a template and S6GFFT1 and S6GFFT2 as primers, double mutant G was created. Finally, mutant G was realized using mutant D as a template for mutagenesis PCR and S6GFFT1 and S6GFFT2 as primers.

The triple amino acid mutant (mutant I in Fig. 3) was obtained using the double mutant F as a template and S6GFFT1 and S6GFFT2 as primers. After *Dpn*I digestion to remove methylated DNA (template DNA), an additional purification was performed (Qiaquick PCR Kit; Qiagen).

For each mutant, 3 μL of plasmid DNA was transformed into *Escherichia coli* competent cells as described by Van den Ende et al. (2001). Cells were plated on 2xYT medium supplemented with zeocine as a selection marker. Positive clones were used for vector amplification. The *Pichia pastoris* wild-type strain X33 was transformed by electroporation with 20 μg of *Pme*I-linearized pPICZαA-desired construct. Transformants were selected on YPDS/zeocine plates.

Heterologous Expression in *P. pastoris*

In order to produce recombinant wild-type Lp6G-FFT and Lp1-SST enzymes (Chalmers et al., 2003; Lasseur et al., 2006) and all of the mutant proteins previously described for functional characterization, a 90-mL preculture medium (BMGY) was inoculated with a single colony carrying one of these constructs and incubated overnight at 30°C and 200 rpm. Cells were harvested by centrifugation, resuspended in 20 mL of induction medium (BMMY), and incubated for 4 d at 29°C. Methanol was replenished every day to a final concentration of 2% (v/v). Protein enrichment was done by following the protocol described below.

Protein Enrichment, Antibody Production, Electrophoresis, and Western Blotting

After 5 d, the yeast supernatant was centrifuged for 10 min at 1,500*g*. Ammonium sulfate was added until 80% saturation was reached, and 20 mM citrate-phosphate buffer, pH 5.0, was added to a final concentration of 10 mM. After 1 h of stirring on ice, enzymes were pelleted by a 30-min centrifugation at 8,000*g* and 4°C. The pellet was then washed three times with 20 mM citrate-phosphate buffer, pH 5.0, containing ammonium sulfate until 80% saturation was reached. Finally, this pellet was redissolved in 50 mM sodium acetate buffer, pH 5.0, and subsequently centrifuged for 3 min at 10,000*g* (De Coninck et al., 2005). Several attempts (concanavalin A, Mono Q, Mono S) to further purify Lp6G-FFT/1-FFT and Lp1-SST enzymes were unsuccessful, probably due to the instability of the enzyme and/or the rather low concentration in the yeast's expression medium. Therefore, we worked with concentrated protein solutions derived from redissolved ammonium sulfate pellets. In order to compare the mutants and wild-type recombinant enzymes at an equal protein concentration, both protein determination and western blotting with an antibody against the Lp6G-FFT/1-FFT C-terminal ESRAFLADDM were used (only suitable to detect Lp6G-FFT/1-FFT and derived mutants, not for Lp1-SST). Anti-peptide antibodies were raised in rabbits immunized with an ESRAFLADDM-BSA conjugate (Faye et al., 1986), and western blotting was performed essentially as described by Van den Ende et al. (1996a). Protein concentrations were also calculated as total protein minus total protein derived from a medium of an empty vector control. The Coomassie Brilliant Blue reagents method (Sedmak and Grossberg, 1977) was used for this purpose.

Enzyme Activity Measurements

Aliquots (final protein concentration, 40 $\mu g\ mL^{-1}$) were incubated at 30°C for different time periods with 50 mM 1K, or 50 mM 1K and 20 mM Suc, or 200 mM Suc. The different substrates were dissolved in 50 mM sodium acetate buffer, pH 5.0, also containing 0.02% (w/v) sodium azide. Carbohydrates of the assay mixture were separated and quantified by high-performance anion-exchange chromatography and pulsed amperometric detection (HPAEC-PAD; DX-300; Dionex) on an analytical CarboPac PA100 column (4 $\times$ 250 mm) using a sodium acetate gradient (1 mL min^{-1}) in 90 mM NaOH. Using solutions A (90

mM NaOH) and B (90 mM NaOH and 500 mM NaOAc), the following running profile was applied: time 0, 100% A; 4 min, 100% A; 10 min, 98% A, 2% B; 20.1 min, 100% B; 25 min, 100% B; 25.1 min, 100% A; 30 min, 100% A. The experiments were repeated three times with consistent results. Identities were assigned to peaks by comparison with commercial standards and identified onion (*Allium cepa*) carbohydrates (Lasseur et al., 2006).

Kinetics

For the kinetic analyses of wild-type Lp1-SST, wild-type Lp6G-FFT/1-FFT, and the triple mutant of Lp6G-FFT/1-FFT, great care was taken to select time points in the linear region, ensuring that less than 10% of the original substrate was consumed. Enzymes (final protein concentration, 40 μg mL^{-1}) were incubated at 30°C in 50 mM sodium acetate buffer, pH 5.0, containing 0.02% (w/v) sodium azide and different Suc concentrations (50, 100, 150, 200, 300, 400, 600, 750, and 1,000 mM) for 10 and 60 min. In contrast to invertases, most FTs cannot be fully saturated with Suc (Van den Ende et al., 1996a, 1996b). Hence, their "apparent" K_m, based on Michaelis-Menten kinetics, can only be estimated. Kinetic parameters (apparent K_m, V_{max}) were estimated using the linear Hanes plot.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AF492836 (*Lolium perenne* 6G-FFT/1-FFT), AY07838 (*Allium cepa* 6G-FFT/1-FFT), AB084283 (*Asparagus officinalis* 6G-FFT), U84398 (*Cichorium intybus* 1-FFT), AJ009756 (*Helianthus tuberosus* 1-FFT), AJ000481 (*Cynara scolymus* 1-FFT), AY323935 (*Cichorium intybus* 1-FEH IIa), AY245431 (*Lolium perenne* 1-SST), AJ297369 (*Festuca arundinacea* 1-SST), DQ535031 (*Agave tequiliana* 1-SST), AJ006066 (*Allium cepa* 1-SST), AJ567377 (*Hordeum vulgare* 1-SST), U85120 (*Cichorium intybus* 1-SST), AJ009757 (*Helianthus tuberosus* 1-SST), Y09662 (*Cynara scolymus* 1-SST), X83233 (*Hordeum vulgare* 6-SFT), AF494041 (putative *Lolium perenne* 6-SFT), AY082350 (*Lolium perenne* VI), AJ006067 (*Allium cepa* VI), AF002656 (*Asparagus officinalis* VI), AJ419971 (*Cichorium intybus* VI), AF276703 (*Oryza sativa* VI1), AF276704 (*Oryza sativa* VI2), AY039610 (*Arabidopsis thaliana* VI1), Y11559 (*Arabidopsis thaliana* VI2), and X74514 (*Arabidopsis thaliana* cwINV1).

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