

# Unraveling the Difference between Invertases and Fructan Exohydrolases: A Single Amino Acid (Asp-239) Substitution Transforms Arabidopsis Cell Wall Invertase1 into a Fructan 1-Exohydrolase<sup>1[C]</sup>

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Plant cell wall invertases and fructan exohydrolases (FEHs) are very closely related enzymes at the molecular and structural level (family 32 of glycoside hydrolases), but they are functionally different and are believed to fulfill distinct roles in plants. Invertases preferentially hydrolyze the glucose (Glc)-fructose (Fru) linkage in sucrose (Suc), whereas plant FEHs have no invertase activity and only split terminal Fru-Fru linkages in fructans. Recently, the three-dimensional structures of *Arabidopsis* (*Arabidopsis thaliana*) cell wall Invertase1 (AtcwINV1) and chicory (*Cichorium intybus*) 1-FEH IIa were resolved. Until now, it remained unknown which amino acid residues determine whether Suc or fructan is used as a donor substrate in the hydrolysis reaction of the glycosidic bond. In this article, we present site-directed mutagenesis-based data on AtcwINV1 showing that the aspartate (Asp)-239 residue fulfills an important role in both binding and hydrolysis of Suc. Moreover, it was found that the presence of a hydrophobic zone at the rim of the active site is important for optimal and stable binding of Suc. Surprisingly, a D239A mutant acted as a 1-FEH, preferentially degrading 1-kestose, indicating that plant FEHs lacking invertase activity could have evolved from a cell wall invertase-type ancestor by a few mutational changes. In general, family 32 and 68 enzymes containing an Asp-239 functional homolog have Suc as a preferential substrate, whereas enzymes lacking this homolog use fructans as a donor substrate. The presence or absence of such an Asp-239 homolog is proposed as a reliable determinant to discriminate between real invertases and defective invertases/FEHs.

The nonreducing disaccharide Suc ( $\alpha$ -D-Glc-(1  $\rightarrow$  2)- $\beta$ -D-Fru; Fig. 1A) is, besides starch, one of the most common reserve carbohydrates in plants, playing a key role in plant metabolism and development (Sturm and Tang, 1999). Suc is synthesized in source leaves during photosynthesis and is thereof transported to sink tissues. Upon arrival into sink cells, Suc can serve as a source of carbon for the synthesis of polysaccharides, such as starch and fructans, or as a source of energy through respiration. Metabolic use of Suc as a carbon-building block or energy source and its channeling into the sink metabolism require the cleavage of the  $\alpha$ 1- $\beta$ 2 glycosidic bond. In plants, this reaction can be catalyzed

by two distinct enzymes: Suc synthase (EC 2.4.1.13) and invertases (EC 3.2.1.26). Suc synthase is a glycosyl-transferase catalyzing the UDP-dependent reversible cleavage of Suc into UDP-Glc and Fru, whereas invertase irreversibly hydrolyzes Suc into Glc and Fru. Because the substrate and reaction products of invertases are not only important nutrients, but also regulators of different classes of genes, invertases are fundamental enzymes in the control of cell differentiation and plant development (Koch, 1996).

Based on their subcellular localization, pH optima, solubility, and isoelectric point, three different types of invertase isoenzymes could be distinguished: vacuolar, cell wall-bound, and cytoplasmic invertases (Tymowska-Lananne and Kreis, 1998). Cell wall invertases are key metabolic enzymes involved in the regulation of Suc partitioning (Eschrich, 1980; Sturm, 1999; Sherson et al., 2003; Roitsch and Gonzalez, 2004), developmental processes such as pollen and seed development (Cheng et al., 1996; Goetz et al., 2001), and the response to wounding and pathogen infection (Sturm and Crispeels, 1990; Benhamou et al., 1991; Fotopoulos et al., 2003; Ditt et al., 2006). In *Arabidopsis* (*Arabidopsis thaliana*), two vacuolar, six putative cell wall, and nine cytoplasmic invertase genes are present (Ji et al., 2005). However, two of the putative cell wall invertase

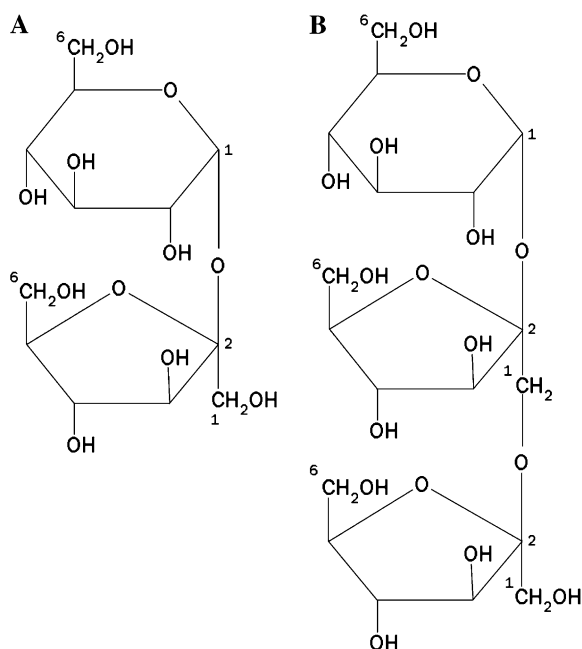
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**Figure 1.** Schematic structure of Suc (A) and 1-kestose (B).

genes turned out to encode fructan exohydrolases (FEHs), enzymes that hydrolyze terminal Fru moieties from fructans but not from Suc (De Coninck et al., 2005).

Both vacuolar and cell wall invertases are grouped together with fructan biosynthetic and degrading enzymes and microbial  $\beta$ -fructosidases in family 32 of glycoside hydrolases (GH32; www.cazy.org). Family GH32 can be combined in the clan GH-J together with the related family GH68 (Naumoff, 2001), harboring bacterial invertases, levansucrases, and inulosucrases. These enzymes use Suc as the preferential donor and transfer the fructosyl moiety to a variety of acceptors, including water (Suc hydrolysis), Suc, and fructan (levan or inulin). Within this GH-J clan, three-dimensional (3-D) structures of six enzymes have been determined, namely, the levansucrases from *Bacillus subtilis* (Meng and Fütterer, 2003; Protein Data Bank [PDB] code 1OYG) and *Gluconacetobacter diazotrophicus* (Martinez-Fleites et al., 2005; PDB code 1W18), an exoinulinase from *Aspergillus awamori* (Nagem et al., 2004; PDB code 1Y4W), a  $\beta$ -fructosidase from *Thermotoga maritima* (Alberto et al., 2004; PDB code 1UYP; Alberto et al., 2006; PDB code 1W2T), a fructan 1-exohydrolase (1-FEH) from chicory (*Cichorium intybus*; Verhaest et al., 2005b; PDB code 1ST8), and, most recently, a cell wall invertase from *Arabidopsis* (Verhaest et al., 2006; PDB code 2AC1). All these proteins show a common fold; they consist of an N-terminal five-bladed  $\beta$ -propeller domain (GH32 and GH68) followed by a C-terminal domain formed by two  $\beta$ -sheets (only in GH32). Superposition of these structures indicates that the active sites are located in the  $\beta$ -propeller domain. The active site is characterized

by the presence of three highly conserved acidic groups that play a crucial role in the catalytic mechanism for the hydrolysis of the glycosidic bond (Reddy and Maley, 1990, 1996; Batista et al., 1999; Yanase et al., 2002).

A phylogenetic tree containing deduced amino acid sequences from vacuolar and cell wall invertases and fructan biosynthetic and breakdown enzymes revealed that FEHs are more related to cell wall invertases than to fructan biosynthetic enzymes, which, in turn, are more related to vacuolar invertases. FEHs and cell wall invertases are believed to have evolved from a common ancestor (Van den Ende et al., 2000). FEHs and invertases are both hydrolases but use different preferential donor substrates. Suc is the preferential donor substrate for cell wall invertases, but other donor substrates, such as 1-kestose (Fig. 1B), raffinose, and stachyose, were also reported to be degraded to a certain extent (Tymowska-Lananne and Kreis, 1998; De Coninck et al., 2005). It was reported that the side activity against raffinose is determined by a single amino acid (Goetz and Roitsch, 1999). Plant FEHs seem to be unifunctional enzymes, only degrading fructans but not Suc (Van Laere and Van den Ende, 2002), in contrast with microbial FEHs that can degrade Suc as well. Depending on the linkage type that is hydrolyzed, 1-FEH or inulinase ( $\beta$ -2,1 linkages) and 6-FEH or levanase ( $\beta$ -2,6 linkages) can be distinguished.

To gain insight into (1) which amino acids determine the preference of Glu-Fru linkage (Suc, raffinose, stachyose) hydrolysis versus Fru-Fru linkage (1-kestose and higher degree of polymerization [DP] fructans) hydrolysis and, more particularly, (2) which amino acids are important for the binding and degradation of Suc, site-directed mutagenesis studies were performed focusing on the identification of putative important residues as determined by sequence and 3-D structure comparisons. To our knowledge, this is the first report specifically considering the difference between GH32 hydrolases only differing in donor substrate specificity (Suc versus fructan). All studies were performed on *Arabidopsis* cell wall Invertase1 (AtcwINV1; *Arabidopsis* gene code At3g13790), a genuine cell wall invertase (De Coninck et al., 2005).

## RESULTS AND DISCUSSION

### The Active Site of AtcwINV1 Consists of Three Highly Conserved Amino Acids

By multiple-sequence alignments of members of GH32, GH68, and GH43 (harboring  $\beta$ -xylanases,  $\beta$ -xylosidases,  $\alpha$ -L-arabinases, and  $\alpha$ -L-arabinofuranosidases), and GH62 (harboring some  $\alpha$ -L-arabinofuranosidases), three highly conserved amino acids, at an equivalent position in all the enzymes, can be identified (Pons et al., 2004). The general GH reaction mechanism requires at least two critical acidic residues, a proton donor, and a nucleophile. The reaction involves two major mechanisms resulting in either an overall retention (GH32 and GH68)

or inversion (GH43) of the anomeric configuration (Davies and Henrissat, 1995). Several mutation experiments on enzyme members of these families and on a plant invertase in particular (Goetz and Roitsch, 2000), revealed a crucial role for Asp and Glu residues in substrate hydrolysis (Reddy and Maley, 1990, 1996; Batista et al., 1999; Nurizzo et al., 2002; Yanase et al., 2002; Meng and Fütterer, 2003; Ozimek et al., 2004; Altenbach et al., 2005; Ritsema et al., 2006). Recently, the 3-D structures of chicory 1-FEH IIa/AtcwINV1 were resolved, and the acidic residues Asp-22/Asp-23, Asp-147/Asp-149, and Glu-201/Glu-203 were considered as nucleophile, transition-state stabilizer, and acid/base catalyst, respectively (Verhaest et al., 2005b, 2006). These residues belong to the conserved NDPNG, RDP, and EC regions within the GH32 family (Fig. 2; Naumoff, 2001). Because the Asp of the RDP motif is considered to be too distant to take part in the catalytic process (Meng and Fütterer, 2003), we focused on Asp-23 and Glu-203 from AtcwINV1 and mutated them into Ala residues. The total activity, measured as the release of Fru from Suc, of wild-type and mutant enzymes, were compared and their kinetic parameters determined (Table I). Compared to the wild-type enzyme, the  $k_{\text{cat}}$  for both mutant enzymes drastically decreased (600- to 900-fold), whereas the  $K_m$  was modified to a lesser extent (8-fold). The large reduction in  $k_{\text{cat}}$  values for both D23A and E203A mutants confirms that these residues are essential for catalysis in AtcwINV1. In accordance with previous studies on yeast (*Saccharomyces cerevisiae*) invertase (Reddy and Maley, 1996) in which the nucleophile and proton donor were identified, we conclude that the Asp-23 and Glu-203 residues in AtcwINV1 act as the nucleophile and proton donor in the hydrolysis reaction of Suc.

#### Presence of an Additional Asp/Asn Residue Is Important for Binding and Hydrolysis of Suc in AtcwINV1

A comparison of the AtcwINV1 and chicory 1-FEH IIa structures reveals some marked differences in their active site regions (Fig. 3). In AtcwINV1, an extra acidic residue (Asp-239) is observed in the vicinity of the conserved acid/base catalyst Glu-203 (Fig. 3A). An acidic amino acid is also found in 1-FEH IIa (Glu-234), but the acidic chain is twisted over 180° and oriented away from the active site (Fig. 3B). This different orientation is most probably due to the presence of a double deletion in the vicinity of this residue (Fig. 4). The presence of an Asp-239 homolog is highly conserved in cell wall invertases, a noticeable exception being Arabidopsis cell wall invertase 5 (Fig. 4). Besides cell wall

invertases, vacuolar invertases in general also share this feature (data not shown). It can be hypothesized that the presence of an Asp-239 homolog contributes to the specificity of Suc recognition of plant acid invertases. By contrast, analogous to chicory 1-FEH IIa, all other known FEHs are generally characterized by the absence of such a structural Asp-239 homolog. In some cases, an acidic residue is present, but, analogous to chicory 1-FEH IIa, always surrounded by deletions (Fig. 4). Taking into account the orientation of Glu-234 in chicory 1-FEH IIa away from the active site, most probably due to a double deletion, it can be assumed that the presence of deletions in this region might be a reason for the absence of a structural Asp-239 homolog in FEHs.

Because polar forces (i.e. the formation of H-bonds between the side chains of polar amino acids and the hydroxyl groups of the carbohydrates) can play an important role in the recognition and binding of carbohydrate substrates in the active site, the possible role of Asp-239 in the binding and hydrolysis of Suc was further investigated by site-directed mutagenesis studies on AtcwINV1. Several mutants were constructed in which Asp-239 was changed into an Ala (D239A), a Phe (D239F), or an Asn (D239N). A comparison of the invertase activities of wild-type and mutant enzymes is presented in Figure 5. The kinetic parameters of the purified mutant enzymes were determined and compared with those obtained for the wild-type invertase (Table II). In both D239A and D239F mutants,  $K_m$  increased 6- to 11-fold, respectively, inferring an important role for the Asp-239 residue in substrate binding. The lower substrate affinity of D239F compared to D239A can result from the more extended steric hindrance of the bulky Phe residue. A similar tendency is observed on  $k_{\text{cat}}$  values, which decrease 10- to 20-fold, suggesting that Asp-239 is important for efficient catalysis as well. In contrast,  $K_m$  and  $k_{\text{cat}}$  values of the D239N mutant differ only slightly from the wild type, convincingly demonstrating that an acidic group is not essential at this position and can be replaced by an Asn. This is not surprising because both Asp and Asn, containing a carbonyl on their side chain, can form H-bonds with the substrate in almost exactly the same way. Conclusively, these data show that the presence of an additional Asp or Asn residue, adjacent to the Glu-203 proton donor, is important for optimal binding and efficient catalysis of Suc.

Remarkably, Asp-239 interacts very strongly with the Lys-242 residue (Fig. 3A), which can be considered as the structural homolog of the Arg-360 residue in *B. subtilis*

**Figure 2.** Multiple sequence alignment (using EMBL-EBI ClustalW) of three conserved regions of some plant GH32 enzymes. The following sequences were used for the alignment: AtcwINV1, chicory vacuolar invertase, 1-FEH IIa, 1-SST, 1-FFT, and an invertase from yeast. The three crucial catalytic residues are shown in bold. Arrows indicate the residues subjected to mutagenesis. Numbering refers to AtcwINV1.

	↓ 23	149	↓ 203
AtcwINV1	WMNDP <b>NG</b> PM	FRDP	SGM <b>W</b> EC <b>PD</b>
1-FEH IIa <i>C. intybus</i>	WMNDP <b>NG</b> PM	FRDP	TGT <b>W</b> ED <b>PD</b>
Vacin <i>C. intybus</i>	WMNDP <b>NG</b> PV	FRDP	TGM <b>W</b> EC <b>VD</b>
1-SST <i>C. intybus</i>	FISDP <b>NG</b> PM	FRDP	TGM <b>W</b> EC <b>VD</b>
1-FFT <i>C. intybus</i>	FIYDP <b>NG</b> PL	FRDP	TDM <b>W</b> EC <b>VD</b>
Inv <i>S. cerevisiae</i>	WMNDP <b>NG</b> LW	FRDP	GYQ <b>Y</b> EC <b>PG</b>

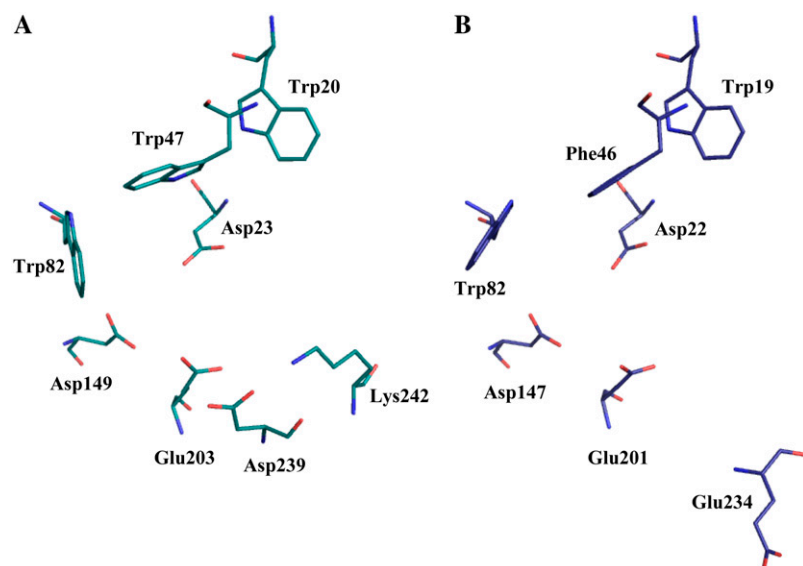
**Table I.** Kinetic parameters for the hydrolysis of Suc of purified AtcwINV1 wild-type, D23A mutant, and E203A mutant proteins

Enzyme	$K_m$ <i>mM</i>	$k_{cat}$ $s^{-1}$	$k_{cat}/K_m$ $mM^{-1} s^{-1}$
Wild type	$0.35 \pm 0.05$	$59 \pm 4$	168.6
D23A	$2.4 \pm 0.4$	$10 \times 10^{-2} \pm 0.1 \times 10^{-2}$	$42 \times 10^{-3}$
E203A	$2.7 \pm 0.3$	$7 \times 10^{-2} \pm 0.5 \times 10^{-2}$	$26 \times 10^{-3}$

levansucrase. It was demonstrated that Arg-360 fulfills a crucial role in the transfructosylation process and interacts via H-bonds with the bound substrate (Chambert and Petit-Glatron, 1991; Meng and Fütterer, 2003). Modeling studies (data not shown) indicate that the Asp-Lys or Asp-Arg interaction might occur in all cell wall invertases and is more probable than a Lys substrate interaction homologous to the Arg substrate interaction observed in levansucrase. Indeed, at the Lys-242 position, a Lys or Arg residue is invariably observed among all cell wall invertases, except for *Arabidopsis* cwINV5 (Fig. 4). To investigate whether the preceding results on the Asp-239 mutants were due to the removal of this Asp residue itself, or indirectly caused by the disturbance of the Asp-239-Lys-242 interaction, a K242L mutant was constructed and the kinetic properties of the purified enzyme were determined (Table II). The K242L mutant enzyme  $K_m$  increased with a comparable factor as those of the Asp-239 mutants. However,  $k_{cat}$  value only decreased 2-fold in comparison with the wild-type enzyme. According to these data, a crucial role for the Lys-242 residue itself is excluded. However, regarding the conserved interaction between Asp-239 and Lys-242, it is plausible that Lys-242 is necessary to keep Asp-239 in the right orientation toward the active site.

A superposition of the *B. subtilis* levansucrase and AtcwINV1 structures strongly suggests that Glu-340 in

levansucrase can be considered as the functional (but not structural) homolog of Asp-239 in AtcwINV1 (Fig. 6A). The presence of a Glu or Gln besides the proton donor Glu-342 is strictly conserved in levansucrases, implying a possible important role for this residue in binding and/or degradation of Suc in these enzymes. Indeed, it was shown that Glu-340 forms strong H-bridges with the Glc moiety of Suc (O3 and O4 groups) in the active site of *B. subtilis* levansucrase (Fig. 6B; Meng and Fütterer, 2003). As a consequence of these interactions, we can presume that Glu-342 is not hindered by these groups in its proton donation to the glycosidic oxygen. Regarding this superposition, it can be assumed that the Glu-340 residue is important for both Suc binding and degradation in *B. subtilis* levansucrase, although this was not checked by site-directed mutagenesis in this system. By analogy with these findings, our results on the Asp-239 AtcwINV1 mutants could be explained (Fig. 6C). Although the exact position of Suc in the active site of AtcwINV1 is unknown and the structure of the AtcwINV1-Suc complex remains to be determined, the orientation of Suc in AtcwINV1 can be predicted (Fig. 6C) based on the position of Suc in the active site of *B. subtilis* levansucrase because the position of the terminal fructosyl at the -1 subsite is equal for all GH32 and GH68 enzymes (Verhaest et al., 2007). An Asp-239/Glu-340 functional homolog can also be found in *G. diazotrophicus* levansucrase (Gln-399)



**Figure 3.** Overview of the 3-D structure of the active site of AtcwINV1 (PDB code 2AC1; A) and chicory 1-FEH IIa (PDB code 1ST8; B). Figures were prepared with PyMol (Delano, 2002). The sequence motifs containing the indicated residues are shown in Figures 2, 4, and 7: Asp-23/Asp-22 (DPN region), Asp-149/Asp-147 (RDP region), Glu-203/Glu-201 (EC region), Asp-239/Glu-234, Lys-242 (LDDTKH/FEG--H region), Trp-19/Trp-20 (WMN region), Trp-47/Phe-46 (WGN/FGD region), and Trp-82/Trp-82 (WSGSAT region). [See online article for color version of this figure.]

**Figure 4.** Multiple sequence alignment of the Asp-239/Lys-242 region (shown in bold) in cell wall invertases and all known plant FEHs. The following sequences were used for the alignment: Arabidopsis cell wall invertase 1, *Solanum tuberosum* cell wall invertase 1, *Nicotiana tabacum* cell wall invertase, *S. tuberosum* cell wall invertase 2, *S. tuberosum* cell wall invertase 3, tomato cell wall invertase Lin5, *Vicia faba* cell wall invertase, *Pisum sativum* cell wall invertase, *V. faba* cell wall invertase 2, *Chenopodium rubrum* cell wall invertase, Arabidopsis cell wall invertase 2, Arabidopsis cell wall invertase 4, *Daucus carota* cell wall invertase 1, *Oryza sativa* cell wall invertase, wheat cell wall invertase, *Zea mays* cell wall invertase 1, *Z. mays* cell wall invertase 2, *Z. mays* cell wall invertase 3, Arabidopsis cell wall invertase 5, Arabidopsis 6-FEH (ancient cell wall invertase 3), *Beta vulgaris* 6-FEH, chicory 1-FEH I, chicory 1-FEH IIa, chicory 1-FEH IIb, *Campanula rapunculoides* 1-FEH, Arabidopsis 6&1-FEH (ancient cell wall invertase 6), wheat 1-FEH w1, wheat 1-FEH w2, wheat 6&1-FEH, *Hordeum vulgare* 1-FEH, wheat 6-KEH w1, wheat 6-KEH w2, *Lolium perenne* 1-FEH, and wheat 6-FEH. Functionally characterized enzymes are marked with an asterisk.

		239 242
<b>CWINV1</b>	<b><i>A. thaliana</i>*</b>	HVLKISL <b>DDT</b> KHDYYTIGTYDRVKDKF
CWINV1	<i>S. tuberosum</i>	HVLKVS <b>FDV</b> TRFDHYTIGTYDTKKDKY
CWINV	<i>N. tabacum</i> *	HVLKVS <b>LDV</b> TRFEYTVGTGTYDTRKDRY
CWINV2	<i>S. tuberosum</i>	HVLKNS <b>MDL</b> TRFEYTLGKYDTKKDRY
CWINV3	<i>S. tuberosum</i>	HVLKNS <b>LDV</b> NRFEYTTIGMYDTKKDRY
CWINV	Lin5 <i>L. esculentum</i> *	IVLKNS <b>LDV</b> ARFDYTTIGMYHTKIDRY
CwINV	<i>V. faba</i>	HVLKNS <b>LDI</b> TRFEYTTIGTYLQNDKY
CwINV	<i>P. sativum</i>	HVLKVS <b>LD</b> DTKHDHYLIGSYDVVKDVF
CWINV2	<i>V. faba</i>	HVLKVS <b>LD</b> DKKHYYLIGSYDEEKDRF
CwINV	<i>C. rubrum</i> *	HVLKVS <b>LDV</b> SKHDVYTTIGGYDTKKDAY
CWINV2	<i>A. thaliana</i>	HVLKVS <b>LDL</b> TRFEYTLGTYDTRKDRY
CWINV4	<i>A. thaliana</i>	HVLKVS <b>LDI</b> TRFEYTLGKYDLKKDRY
CWINV1	<i>D. carota</i> *	HVLKVS <b>LDL</b> TRYEYTVGTGTYLTKDRY
CWINV	<i>O. sativa</i>	YVLKNS <b>LDL</b> TRYDYTTVGIYKVTERY
CWINV	<i>T. aestivum</i>	YVLKVS <b>LDV</b> TRYEYTTIGWYDHAKDRY
CWINV1	<i>Z. mays</i>	YVLKSS <b>LDL</b> TRYDYTTIGSYDGGKDRY
CWINV3	<i>Z. mays</i>	YVLKVS <b>LDL</b> TRYEYTVGTGTYDHATDRY
CWINV2	<i>Z. mays</i>	HVLKNS <b>LDL</b> RRYDYTTVGTGTYHPRARY
CWINV5	<i>A. thaliana</i>	HVLKVS <b>LD</b> TDTFNDYYTIGTYDEVDRVY
6-FEH	<i>A. thaliana</i> *	HVLKVS <b>LI</b> ETLHDYYTIGSYDREKDVY
6-FEH	<i>B. vulgaris</i> *	HVLKLS <b>LF</b> DFTQYEEYTTIGRYDIEKDIY
1-FEH I	<i>C. intybus</i> *	HVLKVS <b>FNS</b> --REYVYVIGTYDPIKDKF
1-FEH IIa	<i>C. intybus</i> *	HVMKAG <b>FEG</b> --HDWYTTIGTYS PDRENF
1-FEH IIb	<i>C. intybus</i> *	HVMKAG <b>FEG</b> --HDWYTTIGTYS PDRENF
1-FEH	<i>C. rapunculoides</i> *	HVVKVG <b>FNG</b> --IDWYTTIGTLS -ERDNY
6&1-FEH	<i>A. thaliana</i> *	HVLKAS <b>FGG</b> --NDCYVIGKYSSETEGF
1-FEHw1	<i>T. aestivum</i> *	HALKMS <b>VDS</b> --VDKYMIGVYDLQRDAF
1-FEHw2	<i>T. aestivum</i> *	HALKMS <b>VDS</b> --VDKYMIGVYDLQRDAF
6&1-FEH	<i>T. aestivum</i> *	HVLKMS <b>INS</b> --CDMYIVGVYDLKRDEF
1-FEH	<i>H. vulgare</i>	HALKMS <b>VDS</b> --VDKYMIGVYDLQRDAF
6-KEHw1	<i>T. aestivum</i> *	HVLKMG <b>MNF</b> -GEDVYVIGVYDLKRDAF
6-KEHw2	<i>T. aestivum</i> *	HVLKMG <b>LNF</b> -GEDVYVIGVYDLKRDAF
1-FEH	<i>L. perenne</i>	HVLKMD <b>LDS</b> --CDKYMIGVYDLKSDTF
6-FEH	<i>T. aestivum</i> *	HVLKLS <b>VM</b> NTTQDYAVAGRYDDVADTF

and *T. maritima* invertase (Glu-188). On the contrary, chicory 1-FEH IIa lacks this additional residue. Like all other known plant FEHs, 1-FEH IIa is unable to degrade Suc. Moreover, Suc is a strong inhibitor of most FEHs, including 1-FEH IIa (De Roover et al., 1999). Unlike plant FEHs, microbial FEHs are able to degrade Suc to a certain extent (Nagem et al., 2004). Although *A. awamori* exoinulinase lacks a structural Asp-239 homolog, the function of this residue could probably be partly taken over by Trp-335.

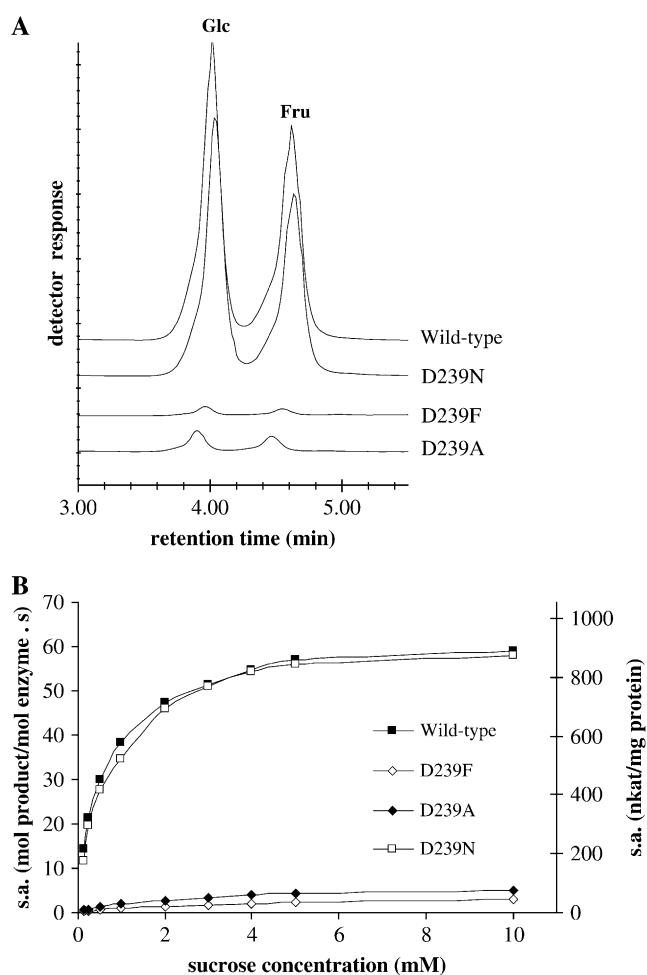
Taken together, our data strongly suggest that the presence of an extra Asp/Asn/Glu/Gln group in GH32 and GH68 Suc-metabolizing enzymes might be important for optimal binding and efficient catalysis of Suc. Overall, the presence or absence of a carbonyl-containing residue adjacent to the acid/base catalyst could be an important determinant of differences in substrate specificity between 1-FEH IIa and fungal exoinulinase (fructan as preferential donor), on one hand, and invertase and levansucrase (Suc as preferential donor) on the other hand. However, this general hypothesis should be further tested by additional site-directed mutagenesis work on a wider array of enzymes

also including microbial invertases, fructan hydrolases, and levansucrases.

#### Conserved Trp Residues Are Important for Stable Substrate Binding

Besides polar forces that are involved in the carbohydrate recognition of various enzymes, it has been reported that other interactions can play a distinctive role. Depending on the stereochemistry of the carbohydrate monomers, the presence of a number of apolar C-H groups (aromatic residues) can play an important role by stabilizing the sugar-enzyme complexes by means of interactions between the aromatic and sugar rings through van der Waals contacts and CH- $\pi$  stackings (Van der Veen et al., 2001; Muraki, 2002; Chavez et al., 2005).

At the rim of the AtcWINV1 active site, there is a prominent presence of three Trp residues, namely, Trp-20, Trp-47, and Trp-82, together forming an aromatic zone (Fig. 3A). These residues belong to the conserved (among invertases) WMNDPN, WGN, and WSGSAT regions (Fig. 7). Mutagenesis experiments were performed in



**Figure 5.** A, Chromatographic pattern of AtcwINV1 wild-type and Asp-239 mutant enzymatic activities. Reaction conditions were as follows: incubation of 50 ng purified enzyme with 10 mM Suc in 50 mM acetate buffer, pH 5.0, during 30 min at 30°C. B, Comparison of specific enzymatic activities [specific (enzymatic) activity in (mol Fru) × (mol enzyme)<sup>-1</sup> (s)<sup>-1</sup> and (nkat) × (mg protein)<sup>-1</sup>] of AtcwINV1 wild-type and Asp-239 mutant purified proteins in function of increasing substrate concentrations.

which these Trp residues were changed into a Leu. Determination of the kinetic parameters of the purified mutant proteins and comparison with the wild-type enzyme revealed an important role for these hydrophobic residues in substrate binding (Table III).  $K_m$

values increased 200, 600, and 120 times for the W20L, W47L, and W82L mutants, respectively, in comparison with the wild-type enzyme. The especially high value of the W47L mutant is not surprising because this residue is very close to the active site and the hydrophobic ring has a good orientation for interacting with the substrate. As a consequence of the very unstable substrate binding in all three mutants, the release of the Glc and Fru residues out of the active site probably takes place at a higher velocity, giving rise to an increase of the  $k_{cat}$  values in two of the three cases.

Taken together, these data strongly suggest that the presence of an aromatic zone in the active site is important for optimal and stable binding of Suc in invertases. Interestingly, most FEHs lack a Trp-47 homolog at this position, but often contain a Phe, which has a less hydrophobic character compared to a Trp residue. A structural Trp-47 homolog can also be detected in *T. maritima* invertase (Trp-41) and *A. awamori* exoinulinase (Trp-65). On the contrary, such a homolog is absent in all levansucrases. However, in *G. diazotrophicus* levansucrase, His-172 might possibly fulfill an equal function because it is present at a similar position in the active site (Martinez-Fleites et al., 2005). Although the active sites of both *B. subtilis* levansucrase and *G. diazotrophicus* levansucrase show quite similar architecture, a His-172 homolog is absent in *B. subtilis* levansucrase. It has been reported that the ratio between high DP fructan and oligosaccharide formation differs significantly between *B. subtilis* levansucrase, on one hand, and *G. diazotrophicus* levansucrase, on the other hand (Hernandez et al., 1995). In this context, further research on the absence or presence of a His-172 homolog could be interesting.

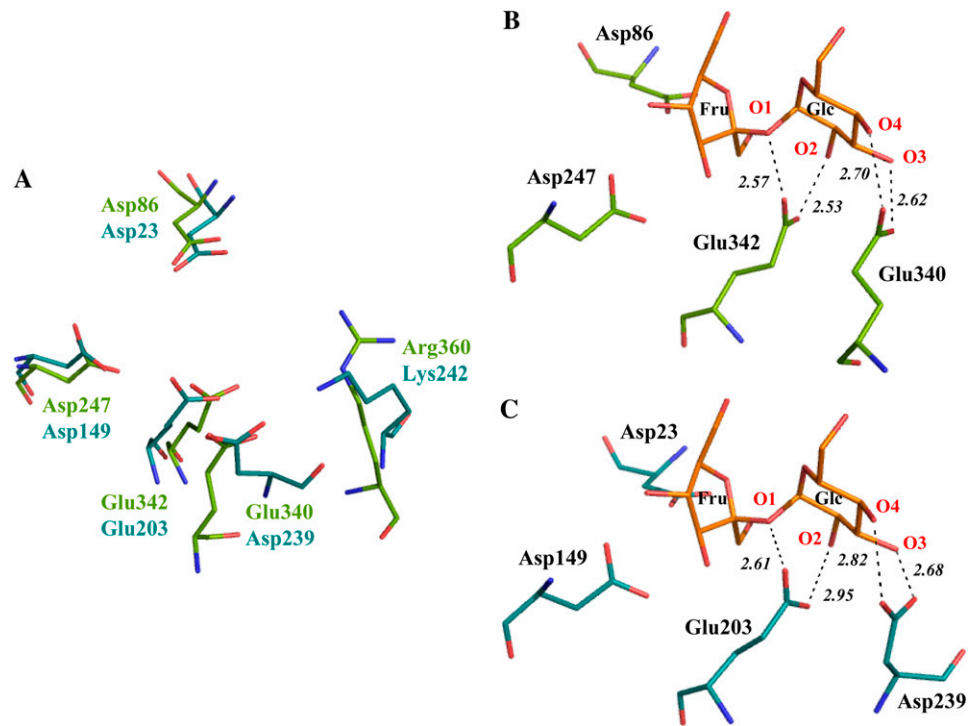
### 1-Kestose as a Substrate for AtcwINV1: The D239A Mutant Acts as a FEH

Cell wall invertases can, besides Suc, degrade other substrates like 1-kestose, raffinose, and stachyose, although to a much lesser extent. Because 1-kestose is the preferential substrate of chicory 1-FEH IIa (De Roover et al., 1999) and an Asp-239 homolog is missing in 1-FEH IIa (Fig. 3B), we investigated whether the breakdown of 1-kestose by AtcwINV1 was also affected by mutagenesis on the Asp-239 residue. The kinetic parameters for the degradation of 1-kestose were determined for the wild-type, D239A, and D239F mutant proteins

**Table II.** Kinetic parameters for the hydrolysis of Suc of purified AtcwINV1 wild-type, D239A mutant, D239F mutant, D239N mutant, and K242L mutant proteins

Enzyme	$K_m$ mM	$k_{cat}$ $s^{-1}$	$k_{cat}/K_m$ $mM^{-1} s^{-1}$
Wild type	$0.35 \pm 0.05$	$59 \pm 4$	168.6
D239A	$2.1 \pm 0.2$	$6 \pm 0.3$	2.9
D239F	$4.5 \pm 0.5$	$3 \pm 0.2$	0.7
D239N	$0.6 \pm 0.07$	$61 \pm 5$	101.7
K242L	$3.7 \pm 0.4$	$29 \pm 1$	7.8

**Figure 6.** A, Superposition of the active sites of AtcwINV1 (turquoise) and *B. subtilis* levansucrase (green; PDB code 1OYG) showing similarities in the catalytic region. B, Details of Suc (orange) in the active site of *B. subtilis* levansucrase Glu-342A mutant (PDB code 1PT2) in which Ala-342 was replaced in silico by Glu-342 (wild-type orientation; PDB code 1OYG). C, Illustration of the putative position of Suc in the active site of AtcwINV1 based on the known structural complex of *B. subtilis* levansucrase with Suc. Putative Suc-protein interactions are indicated with dashed lines. Figures were prepared with PyMol (Delano, 2002).



(Table IV). There was no marked difference in  $K_m$  and  $k_{cat}$  values between the wild-type and D239F mutant proteins. D239A was seriously affected in its ability to degrade Suc (Fig. 5; Table II), but retained its ability to degrade 1-kestose, supporting the idea that plant FEHs lacking invertase activity can evolve from cell wall invertases by a minimal number of mutations or deletions. Similarly, degradation of raffinose and stachyose was affected to the same extent as observed for Suc (data not shown). Higher DP inulin-type fructans were found to be very poor substrates for AtcwINV1 (De Coninck et al., 2005) and for the mutant D239A enzyme (data not shown), so the mutant D239A enzyme can in fact be considered as a special 1-FEH preferentially degrading 1-kestose, the smallest inulin-type fructan (a 1-kestose exohydrolase [1-KEH]). Enzymes that preferentially degrade fructan trisaccharides were

recently discovered in fructan plants. Specific 6-kestosidase (6-KEH) enzymes have been purified and cloned from wheat (*Triticum aestivum*; Van den Ende et al., 2005) and 1-KEH activities were reported in onion (*Allium cepa*; Benkeblia et al., 2005).

Strikingly, the D239A mutant showed superior affinity ( $K_m$  600  $\mu M$ ) for 1-kestose compared to the naturally occurring FEHs from chicory (1-FEH IIa: 58 mM; De Roover et al., 1999) and wheat (1-FEH w1 and w2: 7 mM; Van den Ende et al., 2003a). However, compared to chicory 1-FEH IIa, the D239A mutant has a similar  $k_{cat}$  (11  $s^{-1}$ ). The difference in  $K_m$  between invertases and FEHs is not surprising because cell wall invertases and FEHs fulfill different functions in plants. Cell wall invertases fulfill crucial roles in phloem unloading, carbohydrate partitioning, and sugar signaling, determining normal plant growth and development. It is reasonable

**Figure 7.** Multiple sequence alignment of the three conserved hydrophobic residues (shown in bold) of some cell wall invertases: Arabidopsis cell wall invertase 1, *S. tuberosum* cell wall invertase 1, *N. tabacum* cell wall invertase, *S. tuberosum* cell wall invertase 2, *S. tuberosum* cell wall invertase 3, *V. faba* cell wall invertase, *P. sativum* cell wall invertase, *V. faba* cell wall invertase 2, *O. sativa* cell wall invertase, wheat cell wall invertase, *Z. mays* cell wall invertase 3, *Z. mays* cell wall invertase 1, and *Z. mays* cell wall invertase 2.

		20	47	82
CWINV	<i>A. thaliana</i>	WMNDPNG	VWGNIV	WSGSAT
CWINV1	<i>S. tuberosum</i>	WINDPNG	WGNIV	WSGSAT
CWINV	<i>N. tabacum</i>	WINDPNG	VWGNIV	WSGSAT
CWINV2	<i>S. tuberosum</i>	WINDPNA	VWGNIV	WSGSAT
CWINV3	<i>S. tuberosum</i>	WINDPNA	VWGNIV	WSGSAT
CwINV	<i>V. faba</i>	WINDPNG	VWGNIV	WSGSAT
CwINV	<i>P. sativum</i>	WINDPNG	VWGNIV	WSGSAT
CwINV2	<i>V. faba</i>	WINDPNG	QWGNIV	WSGSAT
CWINV	<i>O. sativa</i>	WINDPNG	VWGNIV	WSGSAT
CWINV	<i>T. aestivum</i>	WINDPNG	VWGNII	WSGSAT
CWINV3	<i>Z. mays</i>	WINDPNG	VWGNIV	WSGSAT
CWINV1	<i>Z. mays</i>	WINDPNA	VWGNIV	WSGSAT
CWINV2	<i>Z. mays</i>	WINDPNA	VWGNIV	WSGSAT

**Table III.** Kinetic parameters for the hydrolysis of Suc of purified AtcwINV1 wild-type, W20L mutant, W47L mutant, and W82L mutant proteins

Enzyme	$K_m$	$k_{cat}$	$k_{cat}/K_m$
	mm	$s^{-1}$	$mm^{-1} s^{-1}$
Wild type	$0.35 \pm 0.05$	$59 \pm 4$	168.6
W20L	$72 \pm 8$	$89 \pm 6$	1.2
W47L	$212 \pm 20$	$175 \pm 15$	0.8
W82L	$43 \pm 4$	$39 \pm 4$	0.9

to assume that high affinity for Suc is absolutely essential for this purpose. FEHs play a role in remobilization of large amounts of fructans (accumulating up to 20% on a fresh weight basis) and very high affinity of FEHs for their substrates might be less critical for this function. The active site of AtcwINV1 is well designed to bind and hydrolyze Suc in a very efficient way. This appears intrinsically linked to efficient 1-kestose binding and degradation as well. The fact that the D239A mutant has superior affinity for 1-kestose (especially when compared to naturally occurring FEHs) shows that site-directed mutagenesis in the active region of GH32 family members can lead to the development of new enzymes with changed substrate specificities and better kinetics. This allows further exploration of the biotechnological potential of interfering with invertase function in plants. Indeed, it is not unthinkable that further manipulation of AtcwINV1 will result in the development of enzymes with even lower  $K_m$  values (higher affinities). Such enzymes might be used in the future to potentially increase sink strength and increase yield, enhance fruit quality, or secondary product formation in sink organs of crop plants (Sonnewald et al., 1997). As an example, it has recently been illustrated that a natural cell wall invertase isoform with superior kinetics determines fruit quality in a particular tomato (*Lycopersicon esculentum*) cultivar (Fridman et al., 2004).

#### Asp-239 as a Reliable Determinant for Prediction of Functionality within the Group of Plant Cell Wall Invertases/FEHs

Without exception, all functionally characterized FEHs lack an Asp-239 homolog and are unable to break down Suc (Van den Ende et al., 2000, 2001, 2003a, 2003b, 2005; De Coninck et al., 2005; Van Riet et al., 2006). On the contrary, the presence of such an Asp-239 homolog seems to be a general feature of cell wall invertases. Unfortunately, only four so-called cell wall invertases are functionally characterized as real invertases (Sturm

and Crispeels, 1990; Roitsch et al., 1995; Fridman et al., 2004; De Coninck et al., 2005). Functional characterization and reliable modeling studies (data not shown) now revealed that three of six of the previously termed cell wall invertase genes of Arabidopsis do not encode real invertases, but defective invertases/FEHs with different substrate specificities (De Coninck et al., 2005). Based on mutagenesis data, extensive functional characterization, and modeling studies on many FEHs, Asp-239 can be proposed as a reliable marker to predict whether a new, uncharacterized member within the cell wall invertase/FEH group encodes a real invertase or a defective invertase/FEH.

#### CONCLUSION

The GH32 family harbors  $\beta$ -fructosidases, invertases, FEHs, and various types of fructosyltransferases, differing only in their donor and acceptor substrate specificities. High levels of amino acid sequence similarities between these enzymes reveal evolutionary relationships. Fructan biosynthesizing enzymes are closely related to vacuolar invertases (Vijn and Smeekens, 1999; Francki et al., 2006; Ritsema et al., 2006), whereas FEHs are closely related to cell wall invertases, suggesting that they both evolved from a common  $\beta$ -fructosidase ancestor (Van den Ende et al., 2000). Site-directed mutagenesis-based data on AtcwINV1 demonstrate an important role for the Asp-239 residue in both binding and hydrolysis of Suc. A hydrophobic zone at the rim of the active site further stabilizes the optimal binding of Suc. Moreover, an D239A mutant acted as a 1-FEH, preferentially degrading 1-kestose. In general, GH32 family enzymes containing an Asp-239 functional homolog have Suc as a preferential donor, whereas enzymes lacking this homolog use fructans as their donor substrate. Asp-239 is proposed as a reliable determinant for the identification of noncharacterized members within the group of cell wall invertases/FEHs.

**Table IV.** Kinetic parameters for the hydrolysis of 1-kestose of purified AtcwINV1 wild-type, D239A mutant, and D239F mutant proteins

Enzyme	$K_m$	$k_{cat}$	$k_{cat}/K_m$
	mm	$s^{-1}$	$mm^{-1} s^{-1}$
Wild type	$1 \pm 0.1$	$21 \pm 2$	21.0
D239A	$0.6 \pm 0.03$	$21 \pm 3$	35.0
D239F	$1.2 \pm 0.1$	$20 \pm 2$	16.7



## MATERIALS AND METHODS

### Cloning and Site-Directed Mutagenesis

*Arabidopsis* (*Arabidopsis thaliana*) AtcwINV1 (gene accession no. At3g13790) was cloned into the pPICZαA vector as described by De Coninck et al. (2005). Single amino acid substitutions were generated following the Quick Change protocol (Stratagene), using the pPICZαA-AtcwINV1 construct as a template. For site-directed mutagenesis, the following forward oligonucleotide primers (and complementary reverse primers) were used: 5'-GGATGAACGCTCC-TAATGGG-3' (D23A), 5'-GGAAGTGGAAATGTGGGCATGTCTGATTTTTTC-3' (E203A), 5'-GAAAATAAGTTTGGCCGACACGAAACATG-3' (D239A), 5'-GTT-GAAAATAAGTCTGTTCGACACGAAACATG-3' (D239F), 5'-GTTGAAAAT-AAGTCTGAACGACACGAAACATG-3' (D239N), 5'-GTTTGGACGACACG-CTACATGATTATTAC-3' (K242L), 5'-CCCCCAAAAATTGATGAACGATCC-3' (W20L), 5'-GGAGCCGTGTGGGTAACATC-3' (W47L), and 5'-CAACGGA-TGCCTGTCGGTTCAG-3' (W82L; mutations are in bold). After *DpnI* digestion, an additional purification step was performed (QiaQuick PCR purification kit; Qiagen) and 3 μL of the purified plasmids were used for transformation of *Escherichia coli* TOP10 cells. Mutations were confirmed by sequence analysis.

### Expression and Purification

The methylotrophic yeast *Pichia pastoris* was used for extracellular gene expression as described in De Coninck et al. (2005). Purification of the protein from the culture supernatant was achieved by 80% ammonium sulfate precipitation. After centrifugation (8,500g, 30 min at 4°C), the pellet was redissolved in 50 mM sodium acetate buffer, pH 5.0. The solution was further centrifuged (10,000g, 10 min at 4°C) to spin down undissolved material. The supernatant was subsequently dialyzed in 50 mM sodium acetate buffer, pH 5.0, for 4 h at 4°C. As a final purification step, the dialyzed solution was loaded onto a Mono S column as described in Verhaest et al. (2005a). The purity of the protein was checked by SDS-PAGE and a single band could be observed after gel staining with Coomassie Blue. Concentration measurements of purified enzymes were performed using the Bradford method with bovine serum albumin as a standard (Sedmak and Grossberg, 1977). The amount of pure protein yielded from one *P. pastoris* expression culture varied depending on the *P. pastoris* clone and ranged between 50 and 200 μg.

### Enzyme Assays

For kinetic determination, appropriate aliquots of enzyme were mixed with Suc or 1-kestose (final concentration ranging from 250 μM to 1 M) in 50 mM sodium acetate buffer, pH 5.0. Reaction mixtures were incubated at 30°C for different time periods. Sodium azide (0.02% [v/v]) was added to prevent microbial growth. Total enzyme activity was determined by measuring the amount of released Fru by anion-exchange chromatography with pulsed amperometric detection (Van den Ende and Van Laere, 1996). For all reactions, different time points were analyzed and only data from the linear range were used. The experiments were repeated three times with consistent results. Kinetic parameters ( $K_m$  and  $k_{cat}$ ) were determined using the linear Hanes plot.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers: AY079422 (*Arabidopsis* cell wall invertase 1), AJ419971 (chicory [*Cichorium intybus*] vacuolar invertase), AJ295033 (chicory 1-FEH IIa), U81520 (chicory 1-SST), U84398 (chicory 1-FFT), P10594 (yeast [*Saccharomyces cerevisiae*] invertase), Q43171 (*Solanum tuberosum* cell wall invertase 1), X81834 (*Nicotiana tabacum* cell wall invertase), Q9M4K7 (*S. tuberosum* cell wall invertase 2), Q9M4K8 (*S. tuberosum* cell wall invertase 3), AJ272304 (tomato [*Lycopersicon esculentum*] cell wall invertase Lin5), Z35162 (*Vicia faba* cell wall invertase), AK118343 (*Arabidopsis* cell wall invertase 2), AB049617 (*Arabidopsis* cell wall invertase 4), X69321 (*Daucus carota* cell wall invertase 1), AF063246 (*Pisum sativum* cell wall invertase), Z35163 (*V. faba* cell wall invertase 2), X81792 (*Chenopodium rubrum* cell wall invertase), AB073749 (*Oryza sativa* cell wall invertase), AF030420 (wheat [*Triticum aestivum*] cell wall invertase), AF050129 (*Zea mays* cell wall invertase 1), AF050128 (*Z. mays* cell wall invertase 2), AF050631 (*Z. mays* cell wall invertase 3), AB029310 (*Arabidopsis* 6-FEH [ancient cell wall invertase 3]), BAB01929 (*Arabidopsis* cell wall invertase 5), AJ508534 (*Beta vulgaris* 6-FEH), AJ242538 (chicory 1-FEH), AJ295034 (chicory 1-FEH IIb), AJ509808 (*Campanula rapunculoides* 1-FEH), AY060533 (*Arabidopsis* 6&1-FEH [ancient cell wall invertase 6]), AJ516025 (wheat 1-FEH w1), AJ508387 (wheat 1-FEH w2), AB089269 (wheat 6&1-FEH), AJ605333 (*Hordeum vulgare* 1-FEH),

AB089271 (wheat 6-KEH w1), AB089270 (wheat 1-FEH w2), DQ073968 (*Lolium perenne* 1-FEH), and AM075205 (wheat 6-FEH).

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