1   **Molecular and biochemical characterization of a beta-fructofuranosidase from**

2   ***Xanthophyllomyces dendrorhous***

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16 **RUNNING TITLE:** A NOVEL FRUCTOFURANOSIDASE FROM *X. dendrorhous*

1 **ABSTRACT**

2 An extracellular β-fructofuranosidase from the yeast *Xanthophyllomyces dendrorhous*

3 was characterized biochemically, molecularly and phylogenetically. This enzyme is a

4 glycoprotein with an estimated molecular mass of 160 kDa, of which the N-linked

5 carbohydrate accounts for 60 % of the total mass. It displays optimum activity at pH

6 5.0-6.5, and its thermophilicity (with maximum activity at 65-70 ºC) and thermostability

7 (with a T_{50} in the range 66-71ºC) is higher than that exhibited by most yeast invertases.

8 The enzyme was able to hydrolyze fructosyl-β-(2→1)-linked carbohydrates such as

9 sucrose, 1-kestose or nystose, although its catalytic efficiency, defined by the k_{cat}/K_m

10 ratio, indicates that it hydrolyzes sucrose approximately 4.2 times more efficiently than

11 1-kestose. Unlike other microbial β-fructofuranosidases, the enzyme from *X.*

12 *dendrorhous* produces neokestose as the main transglycosylation product, a potentially

13 novel bifidogenic trisaccharide. Using a 41% (w/v) sucrose solution, the maximum FOS

14 concentration reached was 65.9 g l^{-1}. In addition, we isolated and sequenced the *X.*

15 *dendrorhous* β-fructofuranosidase gene (*Xd-INV*), showing that it encodes a putative

16 mature polypeptide of 595 amino acids and that it shares significant identity with other

17 fungal, yeast, and plant β-fructofuranosidases, all members of family 32 of the glycosyl-

18 hydrolases. We demonstrate that the *Xd-INV* could functionally complement the *suc2*

19 mutation of *Saccharomyces cerevisiae* and finally, a structural model of the new

20 enzyme based on the homologous invertase from *Arabidopsis thaliana* has also been

21 obtained.

1   **INTRODUCTION**

2   The basidiomycetous yeast *Xanthophyllomyces dendrorhous* (formerly *Phaffia*

3   *rhodozyma*) produces astaxanthin (3-3'-dihydroxy-β, β carotene-4,4 dione; 17, 25).

4   Different industries have displayed great interest in this carotenoid pigment due to its

5   attractive red-orange colour and antioxidant properties, which has intensified the

6   molecular and genetic study of this yeast. As a result, several genes involved in the

7   astaxanthin biosynthetic pathway have been cloned and/or characterized, as well as

8   some other genes such as those encoding actin (60), glyceraldehyde-3-phosphate

9   dehydrogenase (56), endo-β-1,3 glucanase and aspartic protease (4). In terms of the use

10   of carbon sources, a β-amylase (9) and an α-glucosidase (33) with glucosyltransferase

11   activity (12), as well as a yeast cell-associated invertase (41) have also been reported.

12   Invertases or β-fructofuranosidases (EC 3.2.1.26) catalyse the release of β-fructose from

13   the non-reducing termini of various β-D-fructofuranoside substrates. Yeast β-

14   fructofuranosidases have been widely studied, including that of *Saccharomyces*

15   *cerevisiae* (11, 14, 45, 46), *Schizosaccharomyces pombe* (36), *Pichia anomala* (40, 49),

16   *Candida utilis* (5, 8) or *Schwanniomyces occidentalis* (2). They generally exhibit strong

17   similarities where sequences are available, and they have been classified within family

18   32 of the glycosyl-hydrolases (GH) on the basis of their amino acid sequences. The

19   catalytic mechanism proposed for the *Saccharomyces cerevisiae* enzyme implies that an

20   aspartate close to the N terminus (Asp-23) acts as a nucleophile, and a glutamate (Glu-

21   204) acts as the acid/base catalyst (46). In addition, the three-dimensional structure of

22   some enzymes in this family have been resolved, such as that of an exoinulinase from

23   *Aspergillus niger* (*var. awamori*; 37) and the invertase from *Arabidopsis thaliana* (55).

24   As well as hydrolysing of sucrose, β-fructofuranosidases from microorganisms may

25   also catalyze the synthesis of short-chain fructooligosaccharides (FOS), in which one to

1   three fructosyl moieties are linked to the sucrose skeleton by different glycosidic bonds

2   depending on the source of the enzyme (3, 52). FOS are one of the most promising

3   ingredients for functional foods since they act as prebiotics (44), and they exert a

4   beneficial effect on human health, participating in the prevention of cardiovascular

5   diseases, colon cancer or osteoporosis (28). Currently, *Aspergillus* fructosyltransferase

6   is the main industrial producer of FOS (15, 52), producing a mixture of FOS with an

7   inulin-type structure, containing β-(2→1)-linked fructose-oligomers (^{1}F-FOS: 1-

8   kestose, nystose or ^{1}F- fructofuranosylnystose). However, there is certain interest in the

9   development of novel molecules that may have better prebiotic and physiological

10   properties. In this context, β-(2→6)-linked FOS, where this link exits between two

11   fructose units (^{6}F-FOS: 6-kestose) or between fructose and the glucosyl moiety (^{6}G-

12   FOS: neokestose, neonystose, neofructofuranosylnystose), may have enhanced prebiotic

13   properties when compared with commercial FOS (29, 34, 54). The enzymatic synthesis

14   of 6-kestose and other related β-(2→6)-linked fructosyl oligomers has already been

15   reported in yeasts such as *S. cerevisiae* (11) or *Schwanniomyces occidentalis* (2), and in

16   fungi such as *Thermoascus aurantiacus* (26) or *Sporotrichum thermophile* (27).

17   However, the production of FOS included in the ^{6}G-FOS series has not been widely

18   reported in microorganisms, probably as they are not generally produced (2, 15) or

19   because they represent only a minor biosynthetic product (e.g. with baker's yeast

20   invertase*;* 11). Most research into neo-FOS production has been carried out with

21   *Penicillium citrinum* cells (19, 31, 32, 39). In this context, neokestose is the main

22   transglycosylation product accumulated by whole *X. dendrorhous* cells from sucrose

23   (30), although the enzyme responsible for this reaction remained uncharacterized.

24   Here, we describe the molecular, phylogenetic and biochemical characterization of

25   an extracellular β-fructofuranosidase from *X. dendrorhous*. Kinetic studies of its

1   hydrolytic activity were performed using different substrates, and investigated its

2   fructosyltransferase capacity. The functionality of the gene analysed was verified

3   through its heterologous expression and a structural model of this enzyme based on the

4   homologous invertase from *A. thaliana* has also been obtained.

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6   **MATERIALS AND METHODS**

7   **Organisms, transformations and culture conditions.** The *Xanthophyllomyces*

8   *dendrorhous* strains ATCC MYA-131, ATCC24202 and ATCC24230 were grown at

9   23ºC on MM medium (0.7% yeast nitrogen base; Difco) supplemented with 2% (w/v)

10   maltose (MMM), glucose (MMG) or sucrose (MMS). Growth was monitored

11   spectrophotometrically at a wavelength of 660 nm (A_{660nm}). *Escherichia coli* DH5α

12   competent cells were prepared, stored and transformed by standard techniques (51). *E.*

13   *coli* XL10-Gold ultracompetent cells[®] (Stratagene) were used to obtain the cDNA

14   library. Invertase-deficient *Saccharomyces cerevisiae* SEY 2101 (MATα *ura3-52 leu2-*

15   3 *leu2*-112 *ade2*-101 *suc2*-Δ9) was transformed by the lithium acetate method (24).

16   **Protein purification and quantification.** The invertase activity secreted (2.5 U ml-

17   [1]) by *X. dendrorhous* ATCC MYA-131 (1 L of MMM during 60 h, A_{660}=4) was

18   concentrated through 30000MWCO PES using a VivaFlow 50 system

19   (VIVASCIENCE). The active fraction (150 ml) was dialyzed in 20 mM sodium

20   phosphate pH 7 (buffer A) and it was applied to DEAE-Sephacel chromatography

21   column (20 ml) equilibrated with buffer A. The protein was eluted with a 0 to 0.2 M

22   NaCl gradient at a flow rate of 1 ml min^{-1}. The fractions showing invertase activity

23   were eluted with 0.05 M and 0.1 M NaCl. The 0.1 M fractions (3 ml) were pooled,

24   dialyzed in 20 mM sodium acetate pH 5 (buffer B) and they were applied to a DEAE-

25   Sephacel column equilibrated with buffer B. The proteins were eluted as above and the

1   fractions (2 ml) showing invertase activity were pooled, dialyzed and stored at -70ºC

2   (70 U ml^{-1}; 12 µg ml^{-1}). All procedures were carried out at 4ºC. The protein profiles

3   were determined by column chromatography, measuring the absorbance of the eluates

4   at 280 nm. Silver stained (PlusOne^{TM}, Amersham Biosciences) SDS-PAGE (8%

5   polyacrylamide) gels of the samples confirmed the purity of the invertase. Broad range

6   protein markers (prestained-BioLabs or Bio-Rad) were used as a control. When

7   required, the samples were concentrated to the desired volume using the Microcon YM-

8   10 (Amicon) system. Peptide-N-glycosidase F (PNGase F; New England Biolabs)

9   treatment was performed according to the manufacturer's protocol. Invertase activity

10   was detected from native preparations by electrophoresis on 7% polyacrylamide gels

11   that were subsequently stained with 1% (w/v) 2,3,5-triphenyltetrazolium chloride in

12   0.25 M NaOH as described previously (47). Invertase activity from *S. cerevisiae*

13   (Novozymes) was used as a control in this test. The protein concentration was

14   determined using the Bio-Rad microprotein determination assay according to the

15   manufacturer's specifications and with bovine serum albumin as a standard.

16   For the N-terminal amino acid sequencing, the purified protein (500 ng) was

17   subjected to SDS-PAGE (8 %), and blotted onto polyvinylidene difluoride membranes

18   (Millipore). The membranes were stained with Coomassie Brilliant Blue R250 and the

19   band obtained was excised and processed for N-terminal amino acid sequencing (HZY,

20   Germany).

21   **Enzyme and kinetic analysis.** Unless otherwise indicated, β-fructofuranosidase

22   activity was determined by measuring the amount of glucose liberated from different

23   substrates (0.5% (w/v) in 50 mM sodium phosphate buffer, pH 5.5) over 10-20 min at

24   42ºC. The mixture was boiled for 5 min and the glucose was measured using a glucose

25   oxidase-peroxidase assay (Sigma Technical Bulletin nº 510). A calibration curve was

1 established with a 2 mg ml^{-1} glucose solution. One unit of activity (U) was defined as

2 that corresponding to the release of 1μmol of glucose per min under the conditions

3 described above. The enzyme associated to the cellular fractions was assayed after

4 addition of pearl glass and following five cycles of agitation in a Vortex for one-minute

5 as indicated previously (41).

6   The Michaelis-Menten kinetic constants were determined using sucrose (MERCK; 0-

7 60 mM) or 1-kestose (TCI Europe; 0-12 mM) and 0.5 U of pure enzyme (about 10 μl

8 conveniently diluted to fit the calibration curve). The plotting and analysis of the curves

9 was carried out using SigmaPlot software (version 7.101) and the kinetic parameters

10 were calculated by fitting the initial rate values to the Michaelis-Menten equation.

11   The estimation of hydrolase activity at different pH and temperatures was carried out

12 under the aforementioned conditions using sucrose as the substrate. The buffers used

13 were citric acid/sodium citrate (pH 3-4), Na_2HPO_4/NaH_2PO_4 (pH 4-7) and Tris/HCl (pH

14 7-8), all at 100 mM. Thermostability was determined by incubating 0.3 U of the pure

15 enzyme at different temperatures, removing the samples at regular intervals and

16 estimating the residual activity as described previously.

17   **Production of fructooligosaccharides.** The invertase was added to a sucrose

18 solution (410 g l^{-1}) in 0.2 M sodium acetate buffer (pH 5.6) and in a total reaction

19 volume of 2 ml. The activity in the mixture was adjusted to 0.5 U ml^{-1} and the mixture

20 was incubated at 50 °C in an orbital shaker (Vortemp) at 200 rpm. At different times, 40

21 µl aliquots were withdrawn, diluted with 160 µl water and incubated for 10 min at 90

22 °C to inactivate the enzyme. The samples were then centrifuged for 5 min at 6000 rpm

23 in an eppendorf with a 0.45 µm Durapore$^®$ membrane (Millipore), and they were

24 analysed by HPLC with a quaternary pump (Delta 600, Waters) coupled to a 5 μm

25 Lichrosorb-NH2 column (4.6 x 250 mm; Merck) as indicated previously (2).

1   **DNA techniques, cDNA library construction and cloning of the *X. dendrorhous***

2   **invertase.** Routine recombinant DNA techniques were used throughout (51). The *X.*

3   *dendrorhous* ATCC MYA-131 strain was grown in 100 ml MMM at 24ºC in an orbital

4   shaker (A_{660}=1.8). The cells were frozen in liquid nitrogen, total RNA was isolated

5   using TRIREAGENT (Molecular Research Centre), and polyadenylated mRNA was

6   enriched using Oligo-dT cellulose chromatography (Amersham Biosciences) in

7   accordance with the manufacturer's instructions. The *X. dendrorhous* cDNA library was

8   generated with the pBluescript® II XR cDNA Library Construction Kit (Stratagene) and

9   it contained 3.3×10^{5} clones with an average insert size of 1.3 kb and it was stored as

10   individual pools (1500-6000 colony-forming units/pool) in 43% glycerol at -80ºC.

11   The coding sequence of the extracellular invertase from *X. dendrorhous* was

12   amplified by PCR using the cDNA library as the template and the universal T7

13   (Stratagene) and INV-Nter primers (Table 1), the latter directed against part of the N-

14   terminal amino acid sequence (EGWMNDPMG) of the protein. The fragments were

15   amplified with the Pwo DNA polymerase (Roche Diagnostics, Germany) under the

16   following conditions of amplification: i) 94ºC for 120 s; ii) 10 cycles of 94ºC for 10 s,

17   50ºC for 30 s, and 68ºC for 300 s; iii) 30 cycles of 94ºC for 10 s, 50ºC for 30 s, and

18   finally 68ºC for 300 s in the first cycle, which gradually increased by 12 s per cycle up

19   to 660 s in the last one. The PCR fragment amplified (1.8 kb) was purified by agarose

20   gel electrophoresis, it was then recovered with the QiaExII gel extraction kit (Qiagen),

21   cloned into the pST-Blue1 vector (Invitrogene) as recommended by the supplier, and

22   sequenced (SIDI, Universidad Autónoma de Madrid, Spain). The GWMN, RDP and

23   FIN primers (Table 1) were used to complete the sequencing of the 1.8 kb fragment.

24   The cDNA library and the T3 (Stratagene) and GWMN(-) primers (Table 1) were used

1   to amplify and analyse the region that putatively lies upstream of the GWMN amino

2   acid sequence.

3   To characterize the genomic DNA encoding the invertase from *X. dendrorhous*, total

4   DNA from this yeast was isolated as described previously (7), and it was used as the

5   template in PCR amplifications. The GWMN and FVK primers (Table 1) were used to

6   amplify a 1.9 kb fragment that included most of this gene. Inverse PCR was used to

7   analyse the flanking sequences of this DNA fragment (38). Briefly, genomic DNA from

8   the yeast was digested with X*ho*I (an enzyme that has no restriction sites in the 1.9 kb

9   amplified fragment), it was incubated with T4 DNA ligase (Roche Diagnostics,

10   Germany), and then treated with B*am*HI (which has a single cleavage site within the 1.9

11   kb fragment at nucleotide 291). The digested product was used as a template in PCR

12   reactions with the GWMN(-) and FIN primers (Table 1). All the PCR products amplified

13   were introduced into the pST-Blue1 vector and sequenced.

14   To express the hypothetical invertase from *X. dendrorhous* in a heterologous system,

15   the 1788 nucleotide fragment was amplified from the cDNA library using the INVHindIII

16   and INVXhoI primers (Table 1). This fragment started at the TTC codon and it

17   terminated at the TAA stop codon, and it was introduced into the Bluescript Sk(+)

18   plasmid (Stratagene). The resulting INV-BS construct was then used as a template to fuse

19   an ATG codon followed by the MFα1 spacer region (KREAEA) using the INVBamHI

20   and INVXbaI(MF1α) primers (Table 1), thereby generating a sequence encoding the

21   putative extracellular protein. The INVBS-MF construct generated was verified by

22   sequencing, digested with *Xba*I and *Xho*I, and the 1809 nucleotide fragment obtained was

23   introduced into the pVT103-L plasmid (57) under the control of the *ADH1* promoter. The

24   resulting pINV-PVT plasmid was used to transform *S. cerevisiae* SEY 2101.

1   **Phylogenetic analysis and molecular modelling.** The amino acid sequence of the β-

2   fructofuranosidase gene from *X. dendrorhous* (the Gene Bank accession number will be

3   available on acceotance of the manuscript for publication) was blasted against the

4   protein database at SwissProt (http://www.expasy.org/tools/blast/), and the sequences

5   were aligned with the CLUSTALW interface in MEGA4.0

6   [http://www.megasoftware.net/] (pair wise alignment gap opening penalty, 10; gap

7   extension penalty, 0.1; multiple alignment gap opening penalty, 10; gap extension

8   penalty 0.2). The bootstrap test of phylogeny was used with the tree obtained.

9   A structure-based alignment of *X. dendrorhous* invertase and *Arabidopsis thaliana*

10   invertase (PDB identifier 2ac1) was performed with MUSCLE (10) and the resulting

11   alignment was further refined manually. This alignment was used to build a structural

12   model with MODELLER9v4 (50).

13   **Nucleotide sequence accession numbers**. The sequences encoding the invertase

14   from *X. dendrorhous* have been assigned the EMBL accession nº (they will be

15   submitted on acceptance of the manuscript).

16

17   **RESULTS**

18   **Biochemical characterization of a β-fructofuranosidase activity from**

19   ***Xanthophyllomyces dendrorhous.*** The yeast *X. dendrorhous* is able to consume sucrose

20   (17), and a cell-associated invertase activity has already been reported in this organism

21   (41). In an attempt to characterize this enzyme, the yeast was grown in liquid media

22   (33) and the invertase activity was determined from samples taken at different growth

23   times. In these conditions, maximum levels of activity (approximately 2-4 U ml^{-1}) were

24   detected in the culture filtrates at the beginning of the stationary phase ($A_{660nm} = 4$) and

25   they were maintained for at least 80 h of growth (Figure 1). However, and contrary to

1   previous reports (41), only low levels of activity (≤0.8 U ml^{-1}) were found in the cell-

2   associated fraction during this period. In addition, no activity was detected when

3   glucose was used as a carbon source (data not shown), pointing to the catabolic

4   repression of the enzyme analyzed. It was notable that similar activity levels and

5   profiles were obtained using three strains of this particular yeast (ATCC MYA-131,

6   ATCC24202 and ATCC24230; data not shown).

7   To purify the invertase activity from *X. dendrorhous*, the culture was collected and

8   processed as described in the Materials and Methods. The overall yield of the

9   purification was 30% (data not shown) and an only one band of about 160 kDa was

10   evident when assayed by SDS-PAGE (Figure 2A). Treatment with PNGase F resulted in

11   a shift in the apparent molecular mass of this protein to about 66 kDa (Figure 2B). Thus,

12   presuming that the glycosylated and unglycosylated forms behave similarly in the gel,

13   N-linked oligosaccharides appear to represent about 60% (94 kDa) of the total protein

14   mass.

15   The purified enzyme yielded a smeared band above 200 kDa in activity-staining gels

16   (Figure 2C). In this assay the invertase from *S. cerevisiae* was used as a control, and it

17   also produced a smeared band with a molecular mass above 200 kDa (Figure 2C),

18   which probably corresponded to the 270 kDa glycosylated, functionally-active

19   homodimer described previously (14). This correlation suggested that the active enzyme

20   from *X. dendrorhous* was also likely to function as a dimmer.

21   The biochemical properties of the enzyme purified from *X. dendrorhous* were

22   characterized, including the active pH range, optimal temperatures as well as its

23   thermostability and substrate specificity. This enzyme displayed maximum activity at

24   pH 5.0-6.5 (Figure 3A) and temperature of 65-70ºC, with almost 90% of its activity

25   maintained in the range of 60-75ºC (Figure 3B). In general, two properties should be

1   considered in association with high temperature adaptation: the thermophilicity, the

2   ability of an enzyme to exhibit activity at high temperatures; and the thermostability, the

3   ability to remain stable /active after storage at high temperature. To determine the

4   thermostability of the *X. dendrorhous* invertase, the purified enzyme was pre-incubated

5   for different periods of time prior to substrate addition at temperatures in the range of

6   40-85ºC. Only minor inactivation of the enzyme (<10%) was detected after 4 days at

7   40-50ºC, whereas incubation for 24 hours at 60ºC decreased its activity by 50% and it

8   was completely inactivated within 10 min at 85ºC (data not shown). Then, the enzyme

9   was pre-incubated at temperatures in the range of 60-85ºC and for 10-120 min. Under

10   these conditions, a 50% loss of activity (T_{50}) was produced in the 66-71ºC range (Figure

11   3C).

12   **Substrate specificity of the enzyme and kinetic properties.** The enzyme purified

13   from *X. dendrorhous* was able to liberate glucose from fructosyl-$\beta-(2\rightarrow1)$-linked non-

14   reducing carbohydrates such as sucrose [α-D-glucopyranosyl-$(1\rightarrow2)$-β-D-

15   fructofuranose], 1-kestose [α-D-glucopyranosyl-$(1\rightarrow2)$-β-D-fructofuranosyl-$(1\rightarrow2)$-β-

16   D-fructofuranose] or nystose [α-D-glucopyranosyl-$(1\rightarrow2)$-β-D-fructofuranosyl-$(1\rightarrow2)$-

17   β-D-fructofuranosyl-$(1\rightarrow2)$-β-D-fructofuranose], as well as from palatinose [α-D-

18   glucopyranosyl-$(1\rightarrow6)$-D-fructofuranose]. However, while a specific activity of about

19   5200 mU μg^{-1} was quantified for sucrose, only 1200 mU μg^{-1} was measured for 1-

20   kestose, and very weak activity was observed for nystose (220 mU μg^{-1}) and palatinose

21   (90 mU μg^{-1}). The enzyme was not active on maltose [α-D-glucopyranosyl-$(1\rightarrow4)$-D-

22   glucopyranose], lactose [β-D-galactopyranosyl-$(1\rightarrow4)$-D-glucopyranose], or leucrose

23   [α-D-glucopyranosyl-$(1\rightarrow5)$-D-fructofuranose], indicating that it only recognizes

24   carbohydrates containing fructosyl-β-(2-1) or -(6-1)-linked bonds. This enzyme

25   displayed Michaelis kinetics towards sucrose and 1-kestose (results not shown) and the

1   kinetic parameters obtained are presented in Table 2. The K_m value of 4 mM sucrose

2   was similar to that obtained with the *Sw. occidentalis* enzyme (4.9 mM; 2), about two-

3   fold that found for the *C. utilis* enzyme (1-2 mM; 5) and less than that measured for the

4   invertase from *P. anomala* (16 mM; 49) or *S. cerevisiae* (26.1 mM; 45). In addition, the

5   catalytic efficiency defined by the k_{cat}/K_m ratio showed that the *X. dendrorhous*

6   fructofuranosidase hydrolyzes sucrose approximately 4.2 times more efficiently than 1-

7   kestose.

8   **Transfructosylating activity.** The transfructosylating activity of the *X. dendrorhous*

9   enzyme was assayed with sucrose under the conditions indicated in the Materials and

10   Methods, and the analysis of the reaction products showed that the enzyme possessed

11   this activity (Figure 4A). The amount of fructose detected was slightly less than that

12   detected for glucose, indicative of the fructosyltransferase activity of the enzyme at high

13   sucrose concentrations. A blank reaction in the absence of enzyme was also assessed

14   and peaks 4 and 5 were not evident (data not shown).

15   Based on their chromatographic mobility, the compounds corresponding to peak

16   number 4 and 5 were identified as neokestose ([6]G-FOS series) and 1-kestose ([1]F-FOS

17   series), respectively. Figure 5 shows the reaction profile using a 410 g l^{-1} (41% w/v)

18   sucrose solution. The neokestose/1-kestose ratio varied during the reaction, with values

19   between 2/1 and 3/1. At the point of maximum FOS production (48 h), the reaction

20   mixture contained 132 g l^{-1} fructose, 179 g l^{-1} glucose, 49 g l^{-1} sucrose, 40 g l^{-1}

21   neokestose, 18 g l^{-1} 1-kestose and 8 g l^{-1} tetrasaccharides (mainly nystose). The total

22   FOS production is shown in Figure 5B. Maximum FOS concentration achieved was

23   65.9 g l^{-1}, which corresponded to 15.8% FOS percentage referred to the total amount of

24   sugars in the mixture.

1   **Molecular characterization of the fructofuranosidase from *X. dendrorhous*.** To

2   isolate the gene encoding the β-fructofuranosidase from *X. dendrorhous*, the enzyme

3   was initially processed for amino acid sequencing as indicated in the Materials and

4   Methods. The N-terminus of the extracellular mature protein was determined to be

5   FIAPEGWMNDPMGL, which already included part of the β-fructosidase NDPN motif

6   and it aligned with part of the amino acid sequences from yeast and fungal invertases in

7   BLAST searches. A cDNA library of this yeast was constructed and used as the

8   template for PCR reactions including an oligonucleotide targeted to the N-terminal

9   amino acid sequence of this protein (for details see Materials and Methods). In this way,

10   an open reading frame (ORF) of 1788 bp was identified (*Xd-INV*), corresponding to a

11   595 amino acid polypeptide. A molecular mass of 64.62 kDa was calculated for the

12   polypeptide derived from this sequence, with no signal peptide, and this was in

13   accordance with the apparent molecular mass of the 66 kDa for the purified

14   unglycosylated enzyme. The analysis of the sequences flanking the 1788 bp ORF

15   showed that the first in-frame ATG codon was located at position 210 upstream of the

16   TTC codon that encodes the initial Phe of the mature protein. This suggests the presence

17   of a putative signal peptide of 70 amino acids that will not be present in the mature

18   protein. In silico analysis of this putative peptide provided evidence of a potential

19   secretion signal (6) and a predicted cleavage site between position 17 and 18 (AYA-

20   AEL). In addition, the sequence of the mature protein had a predicted pI of 4.4 and it

21   contained 17 putative N-linked glycosylation sites (N-X-S/T), as well as another 4 such

22   sites located between the amino acids 24 and 57 of the presumptive signal peptide.

23   Comparing the cDNA sequence with that of the genomic DNA identified three introns

24   of 92, 95 and 117 bp, all located in the first third of the sequence encoding the protein

25   analysed.

1   The deduced protein sequence of *Xd-INV* was very similar to that of the β-

2   fructofuranosidases from basidiomyceta yeasts and *Aspergillus* spp. Indeed, it was most

3   similar to invertases from *Uromyces fabae* (41% identity over a 295 amino acids

4   overlap), *A. niger* 20611 (later reclassified as *A. japonicus* ATCC 20611; fopA: 33 %

5   over a 223 amino acids overlap), *A. niger* (SucA: 33 % over a 211 amino acids overlap)

6   and *A. sydowii* (31 % over a 175 amino acids overlap). Lower similarities were

7   observed for proteins from the yeasts *S. cerevisiae* or *C. utilis,* as well as for other

8   bacteria and plant proteins (Figure 6A). Xd-INV contained most of the elements

9   characteristic of β-fructofuranosidases and indeed, the six domains that are well

10   conserved among the microbial β-fructofuranosidases (A-F; 8) were all essentially

11   present in the *X. dendrorhous* protein (data not shown). Multiple-sequence alignment of

12   the glycosyl-hydrolase (GH) families 32, 43, 62, and 68 revealed the presence of three

13   conserved domains (A, D and E), each containing a key acidic residue that is implicated

14   in substrate binding and hydrolysis (43), and these residues were also present in the

15   enzyme isolated from *X. dendrorhous* (Figure 6B).

16   To prove the functionality of the *Xd-INV* gene isolated, we fused the 1788 bp ORF to

17   the ATG codon and a *S. cerevisiae* MFα1 spacer region in the pVT103-L expression

18   vector. The pINV-PVT plasmid generated was used to transform a *S. cerevisiae* strain

19   unable to ferment sucrose as a sole carbon source. The growth of transformants (leu+)

20   carrying either the pVT103-L vector or the pINV-PVT plasmid was no different on a

21   glucose-based medium (MMG). However, only the pINV-PVT was able to complement

22   the invertase negative phenotype of the *S. cerevisiae* strain on a sucrose-based medium

23   (MMS). Invertase activity was detected in positive transformants, but only weak activity

24   (10 mU ml^{-1}) was quantified from the cellular fraction. Together, theses data provide

25   direct evidence that *Xd-INV* gene truly acts as an invertase.

1

DISCUSSION

2

3   In contrast to previous studies that indicated the presence of an invertase exclusively

4   associated to the cell fraction of *X. dendrorhous* (41), we have purified an extracellular

5   activity from this yeast that is able to liberate glucose from sucrose. The enzyme was

6   glycosylated, and it presented a molecular mass of 160 kDa that was derived from a 66

7   kDa unglycosylated monomer. Its active form probably represents a homooligomeric

8   protein with an apparent molecular mass above 200 kDa as judged from its mobility in

9   seminative acrylamide gels. Similarly, invertases described in a number of yeasts are

10   also dimeric or multimeric enzymes generated from unglycosylated-monomeric

11   peptides with an average molecular mass of 60-65 kDa, including that of *S. cerevisiae*,

12   *S. pombe*, *P. anomala* or *C. utilis* (Table 3). The maximum activity of the enzyme from

13   *X. dendrorhous* reached at pH 5.0-6.5, which is also in accordance with the data for

14   other yeast invertases such as that of *P. anomala*, and it is only a slightly higher range

15   than that found for *S. cerevisiae* or *C. utilis* (Table 3). However, the thermophilicity

16   (maximum activity at 65-70 °C) and thermostability (T_{50} in the range 66-71°C) of this

17   enzyme were above that exhibited by most yeast invertases, the optimal temperatures of

18   which are generally around 40-50 °C and that are rather unstable at higher values (Table

19   3). Nevertheless, an invertase from *Rhodotorula* sp. that is very stable at temperatures

20   just below 66 °C was recently described (20), and another from *C. utilis* has an optimum

21   temperature of 70 °C, but its thermostability remains to be defined (5).

22   The enzyme purified from *X. dendrorhous* is a β-fructofuranosidase that can

23   hydrolyze fructosyl-β(2→1)-linked carbohydrates (sucrose, 1-kestose and nystose) and

24   palatinose [α-D-Glc-(1→6)-D-Fru]. The enzyme hydrolyzes sucrose very efficiently

25   and it has transfructosylating activity. In contrast to other microbial β-

1   fructofuranosidases that produce mainly [1]F-FOS and little or no [6]G-FOS (Table 3, and

2   19, 52), the main transglycosylation product of the *X. dendrorhous* enzyme is

3   neokestose ([6]G-FOS) followed by 1-kestose. The maximum FOS concentration of 65.9

4   g l^{-1} from a concentration of 410 g l^{-1} sucrose corresponded to 15.8% (w/w) of the total

5   sugar composition in the mixture. This yield could be improved by increasing the initial

6   sucrose concentration favouring the transglycosylation activity (42). In this context,

7   49.4 g l^{-1} neo-FOS (8.2% w/w in the sugar composition) was obtained with intact

8   immobilized *P. citrinum* cells and a 600 g l^{-1} sucrose solution (31) and the neo-FOS

9   production increased to 108.4 g l^{-1} (18% w/w of the total sugar composition) by co-

10   immobilization of *P. citrinum* cells and their neofructosyltransferase (32). Neokestose is

11   a bifidogenic substance with prebiotic effects that may surpass those of commercial

12   FOS (29, 34, 54). In addition, the branched structure of the neo-FOS confers enhanced

13   chemical stability in comparison to conventional FOS (32), and for this reason the new

14   enzyme characterized here could be of considerable biotechnological value.

15   Plants contain different forms of invertases that can be distinguished by their

16   subcellular localization as well as through their biochemical characteristics. Formation

17   of neokestose by plant fructosyltransferases (6G-FFT) that catalyze the transfer of a

18   fructose residue from 1-kestose to the C6 of the glucose moiety of sucrose has been well

19   studied in Liliaceous species such as onion and asparagus (13, 53, 58). In this context, a

20   comparative amino acid sequence analysis of theses proteins might help to clarify their

21   different properties (thermostabilities, specificities, regioselectivities, etc) and to

22   understand the unusual behaviour of the *X. dendrorhous* enzyme.

23   We have isolated and characterized the *Xd-INV* gene that encodes the invertase from

24   *X. dendrorhous* after determining the 14 N terminal amino acids of the purified protein.

25   This sequence was also found in the deduced amino acid sequence of the cloned gene

1   and furthermore, this *Xd-INV* encoded an invertase when it was expressed in an

2   invertase-deficient yeast strain. This provides convincing evidence that the gene

3   analyzed encodes the enzyme characterized from *X. dendrorhous.* The amino acid

4   sequence of the protein encoded by *Xd-INV* revealed close similarity to other β-

5   fructofuranosidases within the GH32 enzyme family, which includes invertases,

6   inulinases, levanases and fructosyltransferases. Indeed, a structural model of this

7   enzyme based on the homologous invertase from *A. thaliana* (55) has been obtained

8   (Figure 7). These two proteins only share 19% sequence identity but nevertheless, the

9   enzyme from *X. dendrorhous* has the five-fold propeller and β-sandwich motifs

10 characteristic of many family 32 glycoside hydrolases. The complete β-fructosidase

11 motif, also known as NDPN box (16), was reduced to NDP in this new protein. A

12 similar change was also found in the enzyme from *U. fabae* (59), as well as in the

13 putative enzyme from *U. maydis* and in other related fungal β-fructofuranosidases.

14 Similarly, the entire ECP/V box (16) could not be identified in any of these proteins,

15 including Xd-INV. Nevertheless, all the proteins analysed share a common acidic

16 residue in these two boxes, which has been previously identified experimentally in *S.*

17 *cerevisiae* invertase (46), *A. awamori* exoinulinase (37) or *T. maritima* β-fructosidase

18 (1). This residue appears to form part of the catalytic machinery responsible for the

19 cleavage of glycosidic bonds. On the basis of our multiple sequence alignment and our

20 structural model, we propose Asp80 and Glu303 (Asp10 and Glu233 in the mature

21 protein) to be the two presumptive catalytic residues in the *X. dendrorhous* enzyme. The

22 predicted Xd-INV sequence also contains the conserved RDP motif and we speculate

23 that as in the *A. awamori* enzyme (37), Arg220 and Asp221 (Arg150 and Asp151 in the

24 mature protein) within this motif could also participate in substrate recognition.

1   The main industrial FOS producers are currently enzymes from Aspergillus, which

2   generally provide a mixture of molecules of the inulin-type β (2→1) structure [1]F-FOS.

3   Despite the fact that catalytic specificity may be dependent on experimental

4   conditions, β-fructofuranosidase SucB of *A. niger* and FopA of *A. japonicus*

5   ATCC20611 (previously *A. niger* ATCC20611) produce 1-kestose and nystose, whereas

6   that of *A. sydowii* IAM 2544 produces some high molecular weight polymers (21 and

7   Table 3). In addition, the fructosyltransferase from *A. foetidus*, which clusters in a

8   separate branch of the phylogenetic tree (Figure 5A) to the other fungal proteins,

9   produces 1-kestose (Table 3). None of these enzymes produces [6]G-FOS (neokestose),

10   such as the *X. dendrorhous* enzyme (Table 3). Furthermore, and as far as we know, no

11   transferase activity has been reported for the β-fructofuranosidase SucA of *A. niger*, *A.*

12   *oryzae*, *U. fabae*, *U. maydis*, *P. anomala*, *C. utilis* and *S. pombe* (Table 3). Remarkably,

13   although the enzyme from *X. dendrorhous* and those of *A. niger* and *A. sydowii* cluster

14   in the same branch of the phylogenetic tree, their enzymatic activities appear to be fairly

15   different. However, the invertase from *S. cerevisiae* clusters in a different branch even

16   though it produces FOS with a levan-type β (2→6) structure, mainly 6-kestose ([6]F-

17   FOS), with neokestose ([6]G-FOS) being a side product of the reaction (11). *Penicillium*

18   *citrinum* cells also produced some neokestose was also from sucrose (19) but

19   unfortunately, no protein responsible for this biosynthetic reaction has yet been

20   identified and characterized.

21   The overall amino acid sequence similarity between the enzyme from *X.*

22   *dendrorhous* and that of the plant fructosyltransferases that produce neokestose or

23   fructans with a higher degree of polymerization (13, 53, 58) was low (<26% over an

24   approximate 100 amino acids overlap). Indeed, all of these proteins cluster as a distinct

25   group in the phylogenetic tree. Based on the sequence comparisons and enzymatic

1   properties, fructosyltransferases from plants are thought to evolve from vacuolar

2   invertases that lack transferase activity. In this context, replacing 33 amino acids that

3   correspond to the N-terminus of the mature onion vacuolar invertase with the

4   corresponding region of onion 6G-FFT led to a shift in activity from the hydrolysis of

5   sucrose towards a transferase reaction (48). In addition, site-directed mutagenesis

6   studies have revealed that positions relatively far from the N-terminus are involved in

7   fructosyl transfer reactions of levansucrases (GH68) from *Zymomonas mobilis* (61) or

8   *Bacillus subtilis* (35). In general, the structural motives required for transferase activity

9   of the β-fructofuranosidases are poorly defined. Further research into these structure-

10   specificity relationships should shed additional light on the determinants responsible for

11   fructosyltransferase activity within the GH32 enzyme family.

12

13   **ACKNOWLEDGMENTS**

23

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16   **Okamoto.** 2002. Identification of functionally important amino acid residues in

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FIGURE LEGENDS

1

FIG 1. **Extracellular invertase activity.** Inocula from *X. dendrorhous* ATCC MYA-
131 were grown (empty squares) in 250 ml flaks containing 50 ml of MMM. The
invertase activity was measured in 0.01 ml of culture filtrates at the times indicated
using sucrose (triangles) as the substrate. Each point represents the average of three
independent measurements with a standard deviation of ± 5%. Similar results were
obtained for two other different cultures (data not shown).

FIG 2. **SDS/PAGE analysis of the purified invertase and PNGase F treatment.** (A)
Purification: the concentrated culture filtrate from *X. dendrorhous* ATCC MYA-131
expressing the invertase activity was subjected to SDS/PAGE before (lane 2) or after
DEAE-Sephacel column chromatography pH 7 (lane 3) and pH 5 (lane 4). Lane 1,
protein standards. (B) Purified invertase digested (5 μg lane +) with 0.2 unit of PNGase
F for 90 min at 37ºC or undigested (10 μg, lane -). (C) Purified invertase activity was
revealed *in situ* (lane 2) and the *S. cerevisiae* enzyme was used as a control (lane 3).
Lane 1, protein standards. The positions of the molecular mass markers are indicated (in
kDa) at the left of (A) and (C).

FIG 3. Temperature, pH dependence and thermostability profiles. The effect of pH
(A) and temperature (B) on the *X. dendrorhous* invertase activity was evaluated at 42ºC
and at pH 5.5, respectively. (C) The purified invertase was incubated for 10 (circles), 20
(rhombus), 60 (squares) and 120 min (triangles) at temperatures in the range of 60-85ºC
in 50 mM sodium phosphate buffer, pH 5.5 prior to the addition of the substrate. The
remaining activity was determined at 42ºC as described in the Materials and Methods.
Each point represents the mean of four independent measurements with a standard
deviation of ±4%.

FIG 4. (A) HPLC chromatogram corresponding to the reaction of sucrose with the β-
fructofuranosidase from *X. dendrorhous*. (1) Fructose; (2) Glucose; (3) Sucrose; (4)
Neokestose; (5) 1-kestose; (6-8) Tetrasaccharides. (B) Schematic view of the
transfructosylation reactions.

1   **FIG 5.** (A) Time-course of neo-FOS and FOS production catalyzed by the β-

2   fructofuranosidase from *X. dendrorhous.* Experimental conditions: 410 g/l sucrose, 0.5

3   U ml^{-1}, 0.2 M sodium acetate buffer (pH 5.6), 50 °C (B) Formation of total FOS.

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5   **FIG 6. Phylogenetic analysis of the fructofuranosidase from *X. dendrorhous* and a**

6   **comparison of the catalytic residues**. A. The radical tree was constructed from the

7   alignment of the amino acid sequences using the CLUSTALW programme. The

8   GeneBank accession numbers are indicated. B. Alignment of conserved sequences

9   including the A, D, and E domains of fructosylhydrolases. The residues underlined

10   indicate the positions of the acidic residues implicated in substrate binding and

11   hydrolysis. The sequence identifiers are in accordance with the nomenclature in

12   SwissProt.

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14   **FIG 7. Molecular model of *X. dendrorhous* fructofuranosidase**. Ribbon

15   representation of the overall structure showing the catalytic residues: D10 (nucleophile)

16   and E233 (catalytic acid/base) catalytic residues. The profile-profile derived alignment

17   of *X. dendrorhous* fructofuranosidase (INV-Xd) and *A. thaliana* invertase (2ac1) is

18   presented in the supplemental data.

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TABLE 1. Primers used

Primer	Sequence
INV-Nter	GA(R)GG(N)TGGATGAA(Y)GA(Y)CC(N)ATGGG
GWMN	GCTGGATGAACGACCCTATGGGGTTGT
RDP	GAGCCCAACTTGATCGGTTTTCGAGAT
FIN	GCTGGCTTCCGAGTGCTTGCGTCCGA
FVK	GGGCGAGACGTTCTCGACGACCTT
GWMN(-)	CGCTGGTACAACCCCATAGGGTCGTTC
INVXhoI	GGGAA**CTCGAG**AGAAACACAAACAGATGGACA
INVHindIII	GCGTACGCA**AAGCTT**CTCGACCTTCCTAATT
INVBamHI	GGCGTGGAT**GGATCC**A
INVXbaI(MF1α)	CCCTA**TCTAGA**TGAAGAGAGAAGCTGAAGCTTTC ATTGCACCTGAAGGCTGGATGAACGACCCTATG

The MFα1 spacer region coding sequence is underlined and the
restriction sites are indicated in bold. The restriction site in INVBamHI
was not included artificially.

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TABLE 2. Kinetic analysis of the β-fructofuranosidase
from *X. dendrorhous*.

Substrate	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1} mM^{-1})
Sucrose	341 ± 14	4.0 ± 0.5	85.3 ± 6.8
1-Kestose	90.8 ± 7.3	4.5 ± 0.7	20.2 ± 2.3

Reaction rate measurements were performed in triplicate. Values of k_{cat} were
calculated from the V_{max} considering a protein molecular mass of 64.63 kDa.
The k_{cat}/K_m standard errors were obtained by fitting the normalized Michaelis-
Menten equation as: $v = (k_{cat} / K_m)[S]/(1 + [S]/Km)$

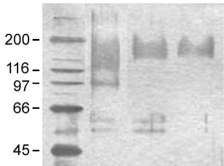**Table 3.** Properties of β-fructofuranosidases from different sources

Source	Glycosylated monomer MW (kDa)[a]	Native MW (kDa)	Optimum pH	Optimum temperature	Transglycosylation main product	Ref.
Aspergillus aculeatus	65	134	5.0-7.0	60℃	1-kestose	(15)
Aspergillus foetidus	90	180	n.r.	n.r	1-kestose	(47)
Aspergillus niger (SucB)	75	n.r.	5.0	40℃	1-kestose, nystose	(18)
Aspergillus japonicus ATCC 20611	100	340	5.0	50	1-kestose, nystose	(22, 23)
Candida utilis	150	300	4.4	70 ℃	n.r.	(5)
Pichia anomala	86	254	4.0-6.5	38 ℃	n.r.	(49)
Saccharomyces cerevisiae	135	270	3.5-5.5	50℃	6-kestose, 6β-fructofuranosylglucose	(11,14, 36)
Schizosaccharomyces pombe	205	1070	n.r.	n.r.	n.r.	(36)
Schwanniomyces occidentalis	85	85, 175	5.5	45-55 ℃	6-kestose	(2)
Uromyces fabae	118	n.r.	4.6	40℃	n.r.	(59)
Xanthophyllomyces dendrorhous	160	>200	5.0-6.5	65-70 ℃	neokestose	This work

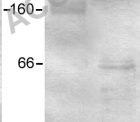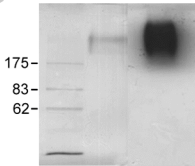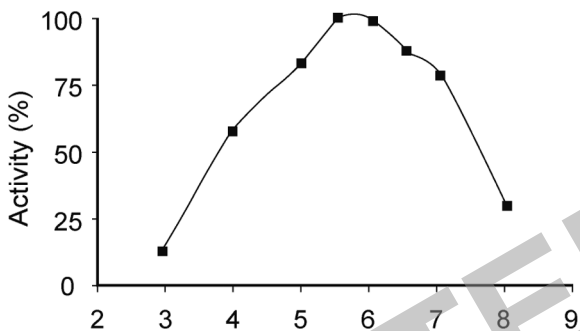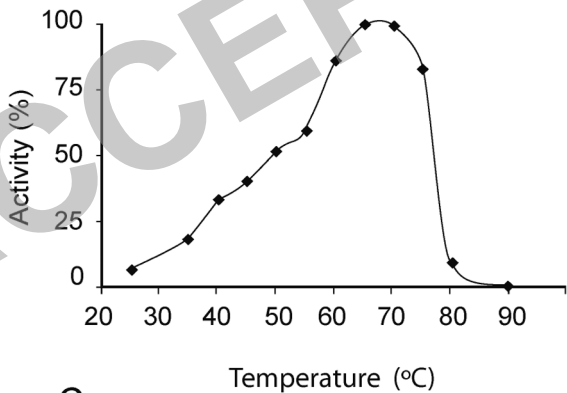[a] Approx. molecular weight obtained from SDS-PAGE. n.r., not reported.

A

1 2 3 4

200—
116—
97—

66—

45—

B

PNGaseF

— +

160—

66—

C

1 2 3

175—

83—
62—

A

B

A

A phylogenetic tree with bootstrap values:

- A.cepa (O81083)
- A.officinalis (Q5FC15) — 100
- O.sativa (Q0JBF1) — 100
- L.perenne (Q8S337) — 100
- A.thaliana (Q38801)
- S.lycopersicum (O82119) — 100
- N.tabacum (Q43799) — 100
- 61
- A.foetidus (O42801) — 100
- A.awamori (Q96TU3) — 100
- S.pombe (O59852)
- S.cerevisiae (Suc2) (P00724) — 87
- P.anomala (P40912) — 99
- C.utilis (O94224)
- T.maritima (O33833)
- A.niger (Suc B) (Q0ZR36) — 100
- A.oryzae (Q2U9G6) — 93
- U.maydis (Q4P8F8)
- X.dendrorhous — 100
- U.fabae (Q2PCS4) — 100
- A.sydowii (Q9P853)
- A.niger (Suc A) (Q0ZR37) — 91
- A.niger (fopA) (Q96VC5) — 100

0.1

B

Source		A		D		E
X.dendrorhous	76	GWMNDPMG	218	GFRDPYVFQ	301	NFETAGV
U.fabae Q2PCS4	88	GWMNDPMA	232	GFRDPFVFE	331	NFECGAI
A.niger (Suc A) Q0ZR37	60	CQIGDPCL	191	AFRDPYVFQ	331	NFETGNV
A.niger (fopA) Q6VC5	56	CQIGDPCA	188	AFRDPYVFQ	290	NFETGNV
A.sydowii Q9P853	53	CQIGDPCA	185	AFRDPYVFQ	263	NFETGNV
A.niger (Suc B) Q0ZR36	25	GWMNDPCG	164	GWRDPFVGA	241	NWEVVNW
A.oryzae Q2U9G6	58	GWLNDPCG	197	AWRDPYIGV	276	NWETTNW
U.maydis Q4P8F8	129	GWMNDPCG	272	SWRDPYIGE	248	NWEVANF
A.foetidus Q42801	37	NWMNDPNG	186	NFRDPVFVW	239	VWECPGLF
S.cerevisiae (Suc2) P00724	38	GWMNDPNG	186	QFRDPKVFW	221	QYECPGLI
A.awamori Q96TU3	37	NWMNDPNG	186	NFRDPVFVW	239	VWECPGLF
P.anomala P40912	38	GWMNDPNG	168	QQRDPKVLW	221	QYECPGLF
C.utilis O94224	40	GWMNDPNG	170	QFRDPKVIW	223	QYECPGLF
S.pombe O59852	93	GFMNDPNG	225	QFRDPKVIW	278	QYECPGMA
O.sativa Q0JBF1	131	NWMNDPNG	256	DFRDPTTAW	313	MWECVDLY
A.cepa O81083	160	NWMNDPNG	285	DFRDPTTAW	342	MWECVDFY
A.officinalis Q5FC15	127	NWMNDPNG	254	DFRDPTTAW	311	MWECIDFY
L.perenne Q8S337	122	HIMNDPNG	247	DFRDPTTAW	306	MYECLDIF
S.lycopersicum O82119	62	NWINDPNG	188	QFRDPTTAW	244	NWECPDFF
N.tabacum Q43799	61	NWINDPNG	187	QFRDPTTAW	242	NWECPDFF
A.thaliana Q38801	58	HWINDPNA	183	AFRDPTTAW	239	NWECPDFF
T.maritima O33833	13	GWMNDPNG	135	AFRDPKVNR	188	EIECPDLV