Supplementary material

Enhanced FOS synthesis by rational design of a novel β-fructofuranosidase from *Trichoderma atroviride*

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Table S1. Oligonucleotides employed in this study

Primer	Orientation	Sequence
pET28b(+) open	F	GCAGCCATCATCATCATCAT
	R	TGCCCATGGTATATCTCCTTC
TAINV	F	TTAAGAAGGAGATATACC**ATGTCTCTCCTAGTGG**
	R	GTGGTGGTGGTGGTGGTG**CCTCGATATTTTATGAGACA**
Int	F	AAAATTGGTCTCATATTGTATCACTCTGATGATCTTGTAAGCAAGACAAC
	R	TATCCAACGTCTCTTGGTC
W60Y	F	GTTAC**TAT**GTCAATGATCCGTGCGGTC
	R	TTGAC**ATA**GTAACGAGGCGGCATCAAG
N62S	F	GGTC**AGC**GATCCGTGCGGTCCAGGT
	R	GATC**GCT**GACCCAGTAACGAGGCGGC
D63A	F	CAAT**GCG**CCGTGCGGTCCAGGTTACAGC
	R	ACGG**CGC**ATTGACCCAGTAACGAGGCG
F125W	F	CGTA**TGG**ACAGGTTGTACTTGGCCAACT
	R	CTGT**CCA**TACGCCGCAAGGATCTTCAGT
T126D	F	ATTC**GAT**GGTTGTACTTGGCCAACTAACCC
	R	AACC**ATC**GAATACGCCGCAAGGATCT
H151Y	F	CCAA**TAT**TCACCAATACATTGGACATTACC
	R	GTGA**ATA**TTGGGCAGAAGTATAAAAGGTTG
E300R	F	TATA**CGC**GGCGCCCATGAAACGAAGACG
	R	CGCC**GCG**TATACTCATCAACAGGATGTCG
K328H	F	CAAAC**CAT**ATACAGAACTGGCTTTGCGG
	R	GTAT**ATG**GTTTGACCTTTGTGCTTTGCCA
K328V	F	CAAAC**GTG**ATACAGAACTGGCTTTGCGG
	R	GTAT**CAC**GTTTGACCTTTGTGCTTTGCCA
D201A	F	GCGC**GCG**CCCTTCATCGGAACATGGGA
	R	AGGG**CGC**GCCACCCTATCACGTCCA
E277A	F	ATTTT**GCG**GTGACCAATTTTATGACACT
	R	TCAC**CGC**AAAATTAGTGCCAAAATCTG

Substitutions and the beginning/ending of the gene *TaINV* are marked in bold.

Table S2. Specific activity of the TaINV variants including the referred substitutions

Variant	Specific activity (U/mg)
WT	416.4 ± 0.6
W60Y	25.1 ± 0.5
N62S	8.4 ± 0.02
F125W	225.8 ± 1
T126D	6 ± 0.1
H151Y	73.7 ± 0.9
E300R	0.11 ± 0.004
K328H	0.05 ± 0.001
K328V	0.04 ± 0.0009

Sucrose was used as substrate. Data are the average of three independent measures, and standard errors are indicated. WT, wild-type variant.

Table S3. Composition of the reaction mixture at the maximum FOS production point of the referred TaINV variants

TaINV variant	Sugars (g/L)									
	Glucose	Fructose	Sucrose	1-kestose	Nystose	Fructosylnystose	6-kestose	Neokestose	Blastose	Total FOS
WT	150±3	43±2	56.3±3	214.4±4	17.3±0.3	2.3±0.1	10.7±0.5	5.6±0.03	1.3±0.001	251.6±4
W60Y	134.6±5	19±0.7	33.5±1	257.5±5	46±1	3.4±0.2	2.4±0.07	1.2±0.02	2.8±0.01	313.3±5
N62S	148.6±3	43.2±1	20.5±0.8	231.8±4	38.9±2	-	3.5±0.1	5.3±0.2	8.2±0.7	287.7±5
F125W	184.9±6	146.2±3	48.2±2	103.7±4	-	-	6.4±0.09	10.5±0.2	4.6±0.1	125.2±4
T126D	132.2±6	53±2	58.8±2	220.3±8	13.7±0.4	-	10.8±0.3	5.2±0.07	6.8±0.4	256.8±8
H151Y	144±5	76.2±1	54.4±2	195.5±6	11.2±0.3	-	7.4±0.2	4.4±0.1	7±0.2	225.5±6

Data shown correspond to the following reaction times: WT and W60Y, 5 and 3 h, respectively; N62S and F125W, 2 h; T126D, 4 h; and H151Y, 24 h.

Data are expressed as mean ± standard errors of three independent experiments.

Table S4. Average degree of polymerization of FOS produced by the referred TaINV variants

Sucrose conversion (%)	FOS Degree of Polymerization					
	WT	W60Y	N62S	H151Y	F125W	T126D
85	3.06±0.09	3.07 ± 0.08	3.06 ±0.005	3.05 ± 0.07	3.06 ± 0.05	3.05 ± 0.09
90	3.09±0.08	3.17 ± 0.06	3.08 ± 0.01	3.07 ± 0.1	3.05 ± 0.08	3.07 ± 0.07
92	3.1±0.09	3.16 ± 0.05	3.1 ± 0.01	3.1 ± 0.08	3.06 ± 0.07	3.12 ± 0.01

Values of DP were calculated from the relative molar proportions of the FOS species produced in each reaction.

Data are expressed as mean ± standard errors of three independent experiments.

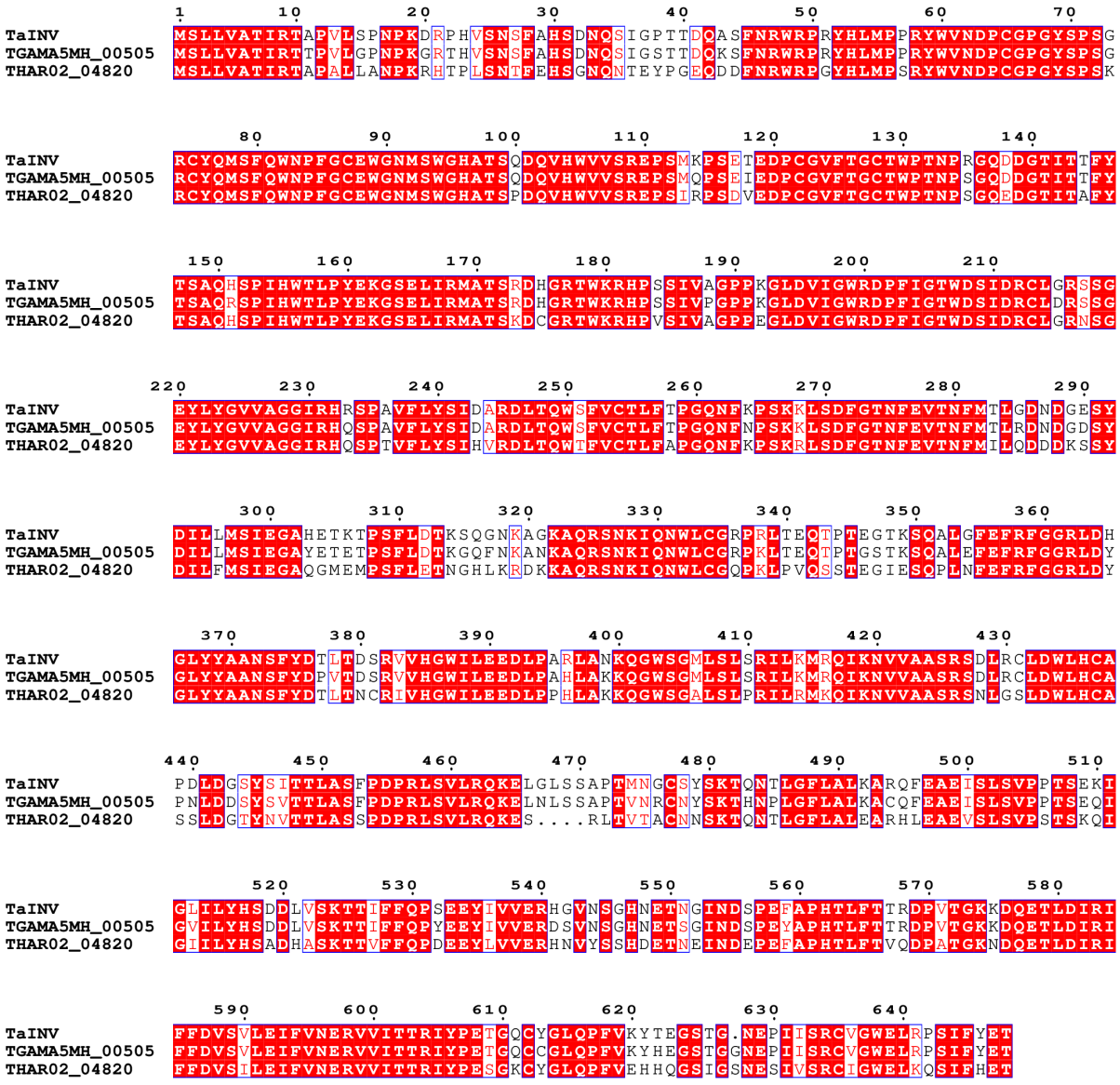

Figure S1. TaINV alignment with protein sequences from *Trichoderma* spp. Only sequences showing the highest homology are presented. TGAMA5MH_00505, hypothetical protein from *Trichoderma gamsii*; THAR02_04820, hypothetical protein from *Trichoderma harzianum.*

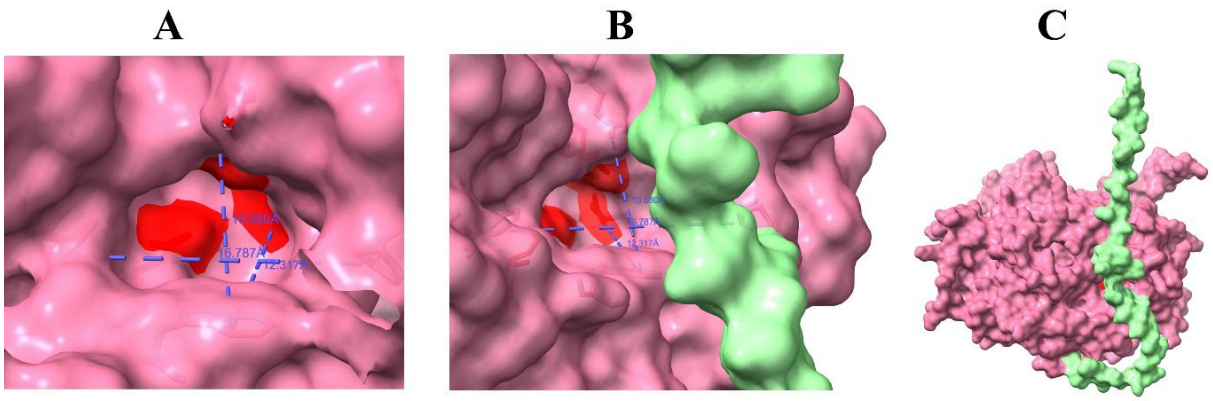

Figure S2. Catalytic cleft architecture of the β-fructofuranosidase TaINV. The structural organization of the TaINV active site is shown. Active site dimensions (width, length, and depth) are indicated with dashed blue lines and labelled in Å. The catalytic triad residues are highlighted in red, and the amino acids used for cleft measurements are displayed. (A) Close-up view of the TaINV catalytic pocket. (B) Catalytic site with the N-terminal extension (first 40 amino acids) shown in green, folded around the entrance of the cleft; the remainder of the protein is depicted in pink. (C) Overall structure of TaINV, illustrating the spatial position of the N-terminal extension (green) relative to the rest of the protein (pink).