Supporting Information

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SI Materials and Methods

Strains and Media. Strains and plasmids used in this work are listed in Tables S2 and S3.

Saccharomyces cerevisiae was grown in yeast extract peptone (YEP) medium (10 g/L yeast extract, 20 g/L bacteriological peptone), synthetic complete (SC) medium (1.7 g/L yeast nitrogen base without amino acids and ammonium sulfate, 5 g/L ammonium sulfate, amino acid supplements), or synthetic minimal (SM) medium (same as SC, but without amino acid supplements and with 20 mM monopotassium phosphate) at 30 °C. Synthetic media were adjusted to pH 6.3 with potassium hydroxide and were supplemented with uracil, L-leucine, L-tryptophan, and L-histidine as needed for selection of auxotrophic plasmid markers (1). G418 (200 µg/mL), hygromycin B (200 µg/mL), and clonNAT/nourseothricin (100 µg/mL) were added for selection of kanMX, hphNT1, and natNT2 markers, respectively. If not stated otherwise, carbon sources were added to concentrations of 20 g/L [glucose (D), ethanol (E), galactose (G)] or 10 g/L [xylose (X), maltose (M)]. EBY.VW4000 was cultured on maltose or ethanol, AFY10 and AFY10X on ethanol.

Plasmids were amplified in *Escherichia coli* strains DH5 α (Gibco BRL) and SURE (Stratagene). *E. coli* was grown in lysogeny broth (LB) at 37 °C or 30 °C (for *HXT7* plasmids) with 100 μ g/mL ampicillin or 50 μ g/mL kanamycin for plasmid selection.

Transformation and DNA Preparation. *E. coli* transformations were performed via electroporation according to the method of Dower et al. (2). Plasmids were isolated from *E. coli* using the GeneJET Plasmid Miniprep Kit (Fisher Scientific). *S. cerevisiae* was transformed according to protocols by Gietz et al. (3, 4). DNA from *S. cerevisiae* for plasmid recovery, PCR analysis of mutant transporter ORFs, or verification of genomic gene deletions/integrations was isolated as described in ref. 5.

Plasmid Construction. Plasmids were constructed in vivo by homologous recombination in *S. cerevisiae* as described in ref. 6. Suitable DNA fragments were generated by PCR using primers with a 5' sequence homologous to the integration region in the vector backbone (Table S4). Plasmids were linearized by restriction digestion. Assembled plasmids were recovered from yeast DNA preparations by *E. coli* transformation, clone selection, and plasmid isolation. Constructs were verified by restriction digestion analysis and DNA sequencing of the ORF. *HXT7* and *GAL2* were amplified from CEN.PK2-1c genomic DNA (sequences identical to CEN.PK113-7D).

For easy separation of transporter-coding plasmids and the xylose isomerase (XI)-coding plasmid, the ORF of the ampicillin resistance in YEp181_pHXT7-optXI_Clos (7) was replaced by the kanamycin resistance gene from pDONR222 (Invitrogen).

The integrative plasmid pAF-HD8.3 was generated from pHD8 (8) by successive excision of HXT7 and opt *xylA*, and the 2 μ origin by restriction digestion, religation of the vector, amplification in *E. coli*, and confirmation by restriction digestion analysis.

Strain Construction. Genes were targeted by the homologous regions flanking the deletion cassettes. For *HXK1* and *YLR446W*, *hphNT1* cassettes were amplified from plasmid pZC1 using primers containing the homologous sequence in their 5' part. *KanMX* cassettes for deletion of *GLK1* and *HXK2* were amplified from genomic DNA of preexisting deletion strains (7), resulting in cassettes with extended homologous regions (200–300 bp), which increases target efficiency. All genome integrations and deletions

were confirmed by PCR analysis. For cassette removal, we relied on the low basal expression of cre recombinase from plasmid pSH47 on maltose or ethanol medium because full induction by galactose is lethal to EBY.VW4000 due to multiple *loxP* sites across the genome. Removal of cassettes was confirmed by PCR. The *hxk*⁰-phenotype in the *hxt*⁰ background was verified on maltose media. Maltose is transported by specific transporters that are still present in the *hxt*⁰ strain (Mal21, Mal31) and is then cleaved into two glucose monomers. Disruption of the hexo- and glucokinase genes prevents maltose utilization. Codon optimization is described in ref. 9. Primers are listed in Table S4.

epPCR Mutagenesis. The GeneMorph II Random Mutagenesis Kit (Agilent Technologies) was used for error-prone PCR (epPCR) mutagenesis of *GAL2* and *HXT7* with low (0–4.5 mutations per kb), medium (4.5–9 mutations per kb), and high (9–16 mutations per kb) mutation frequencies. epPCR was performed with primer pairs Amp_GAL2_F/R and Amp_HXT7_F/R, with p426_GAL2 and p426_HXT7 as templates, respectively. The epPCR fragments were purified by agarose-gel electrophoresis and gel extraction (NucleoSpin Extract II; Macherey-Nagel) and used as templates for a PCR with primer pairs Clon_GAL2_F/R and Clon_HXT7_F/R to prepare fragments for recombinational cloning. AFY10X was transformed with linearized p426H7 and the PCR products and then screened as described in *Clone Selection and Analysis* in the *Materials and Methods* section of the main text.

Site-Directed Mutagenesis. Mutations were inserted into *HXT7* or *GAL2* by site-directed PCR mutagenesis in combination with in vivo recombinational cloning. Two fragments of the ORF, overlapping at the target site, were amplified from the wild-type-plasmids. Two mutagenesis primers covered the target codon and the neighboring 15–20 nucleotides and were directed in opposing directions (forward and reverse). They were combined with the respective reverse and forward cloning primers (as used for the wild type) in separate PCRs. Mutant constructs were assembled from the two fragments and a pRS62N vector backbone by homologous recombination directly in the screening strain AFY10X. The screening procedure is described in *Clone Selection and Analysis* in the *Materials and Methods* section of the main text. Single amino acid mutations were done accordingly, but in EBY.VW4000.

Primers for (semi)random mutagenesis were used as equal mixtures of oligonucleotides with variations at one or more positions of the target codon (expressed in ambiguity code in the primer sequence). The threonine 219/213 codon in both ORFs was altered in three separate sets (defined by three sets of mutagenesis primers), grouping amino acid changes from threonine to alanine/glycine/valine (codon gbt), serine/cysteine (codon tst), and asparagine/aspartic (codon rac). The asparagine 376/370 residue was randomly changed to the 20 proteinogenic amino acids (codon nnn). All primers are listed in Table S4.

Uptake Assays. EBY.VW4000 containing the respective plasmid was grown in selective yeast extract peptone ethanol (YEPE) to an OD₆₀₀ of 1.1–1.6, harvested by centrifugation, and washed twice in ice-cold uptake buffer (100 mM potassium phosphate, pH 6.5). Cell suspensions of 60 mg_{wetweight}/mL were prepared in uptake buffer, aliquotted, and stored on ice. For each measurement, 50 µL of 3× concentrated sugar solution and 100 µL of cell suspension were equilibrated to 30 °C for 4–5 min and then mixed. After 5 s (glucose) or 20 s (xylose), 100 µL of the mixture

were pipetted into 10 mL of ice-cold quenching buffer (100 mM potassium phosphate, 500 mM glucose, pH 6.5) to stop the reaction. Cells were filtered through a Durapore membrane filter with a 0.22-µm pore size (Millipore) and washed twice with 10 mL of ice-cold quenching buffer. The filter was transferred to a scintillation vial containing 4 mL of Rotiszint eco plus (Roth). Then, 10 µL of each reaction were transferred directly to a scintillation vial with 4 mL of scintillation mixture for determination of the total counts in each reaction. To determine a value for sugar that is unspecifically bound to the cell surface or the filter, samples of 33.3 µL of sugar solution and 66.6 µL of cell suspension were mixed in 10 mL of ice-cold quenching buffer and then treated as

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described for the other samples. Radioactivity was analyzed in a Wallac 1409 liquid scintillation counter.

Uptake was measured at sugar concentrations 0.2, 1, 5, 25, and 100 mM for glucose and 1, 5, 25, 66, 100, 200, and 500 mM for xylose. Inhibition of xylose uptake by glucose was measured at 25, 66, and 100 mM xylose with additional 25 and 100 mM unlabeled glucose. Sugar solutions contained 0.135–0.608 μ Ci of D-[U-¹⁴C]-glucose (290-300 mCi/mmol) or D-[1-¹⁴C]-xylose (55 mCi/mmol) (American Radiolabeled Chemicals). Calculation of $K_{\rm m}$ (Michaelis constant), $V_{\rm max}$ (maximal initial uptake velocity), and K_i (inhibitor constant for competitive inhibition) was done by nonlinear regression analysis and global curve fitting in Prism 5 (GraphPad Software) with values of three independent measurements.

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~	100 10-1 10-2 10-3 10-4	100 10-1 10-2 10-3 10-4	20 g/l glucose			
CEN.PK2-1c						
EBY.VW4000						
			10 g/l xylose			
AFY10X						
AFY10X-Gal2						
В	2 g/l xylose	2 g/l xylose + 10 a/l alucose	10 g/l xylose	10 g/l xylose + 50 g/l glucose	2 g/l glucose	
	10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	10° 10-1 10-2 10-3 10-4	10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	1
Hxt7 wt		0	🗩 🐥 🐉 🛷 🔗		· · · · · · · · · · · · · · · · · · ·	
T213G						
T213N						
N370S						
N370L						
IN 37 UF						
•						
С	2 g/l galactose	20 g/l galactose	2 g/l fructose	20 a/l fructose	2 g/l mannose	20 g/l mannose
С	2 g/l galactose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	20 g/l galactose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	2 g/l fructose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	20 g/l fructose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	2 g/l mannose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	20 g/l mannose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴
C Gal2 wt	2 g/l galactose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	20 g/l galactose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	2 g/l fructose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	20 g/l fructose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	2 g/l mannose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	20 g/l mannose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴
C Gal2 wt T219S	2 g/l galactose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	20 g/l galactose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	2 g/l fructose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	20 g/l fructose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	2 g/l mannose	20 g/l mannose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴
C Gal2 wt T219S T219N	2 g/l galactose 10° 10-1 10-2 10-3 10-4	20 g/l galactose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	2 g/l fructose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	20 g/l fructose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	2 g/l mannose	20 g/l mannose
C Gal2 wt T219S T219N N376Y	2 g/l galactose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	20 g/l galactose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	2 g/l fructose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	20 g/l fructose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	2 g/l mannose	20 g/l mannose
C Gal2 wt T219S T219N N376Y N376V	2 g/l galactose 10° 10-1 10-2 10-3 10-4	20 g/l galactose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	2 g/l fructose 10° 10-1 10-2 10-3 10-4	20 g/l fructose 10° 10' 10' 10' 10' 10' 10'	2 g/l mannose	20 g/l mannose
C Gal2 wt T219S T219N N376Y N376V N376F	2 g/l galactose 10° 10-1 10-2 10-3 10-4	20 g/l galactose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	2 g/l fructose 10° 10-1 10-2 10-3 10-4	20 g/l fructose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	2 g/l mannose	20 g/l mannose
C Gal2 wt T219S T219N N376Y N376V N376F Hxt7 wt	2 g/l galactose 10° 10-1 10-2 10-3 10-4	20 g/l galactose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	2 g/l fructose 10° 10-1 10-2 10-3 10-4	20 g/l fructose 10° 10' 10' 10' 10' 10' 0000000000000000000	2 g/l mannose	20 g/l mannose
C Gal2 wt T219S T219N N376Y N376V N376F Hxt7 wt T213G	2 g/l galactose 10° 10-1 10-2 10-3 10-4	20 g/l galactose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	2 g/l fructose 10° 10-1 10-2 10-3 10-4	20 g/l fructose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	2 g/l mannose	20 g/l mannose
C Gal2 wt T219S T219N N376Y N376V N376F Hxt7 wt T213G T213N	2 g/l galactose 10° 10-1 10-2 10-3 10-4	20 g/l galactose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	2 g/l fructose 10° 10-1 10-2 10-3 10-4	20 g/l fructose 10° 10' 10' 10' 10' 10' 000000	2 g/l mannose	20 g/l mannose
C Gal2 wt T219S T219N N376Y N376V N376F Hxt7 wt T213G T213N N370S	2 g/l galactose 10° 10° 10° 10° 10° 10° 10° 10° 00° 10° 10° 10° 10° 10° 10° 10° 10° 10°	20 g/l galactose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	2 g/l fructose 10° 10-1 10-2 10-3 10-4	20 g/l fructose 10° 10' 10' 10' 10' 10' 10'	2 g/l mannose	20 g/l mannose
C Gal2 wt T219S T219N N376Y N376V N376F Hxt7 wt T213G T213N N370S N370L	2 g/l galactose 10° 10-1 10° 10° 10° 10° 000 000 000 000 000 000 000 000 000 0	20 g/l galactose 10° 10 ⁻¹ 10 ⁻² 10 ⁻³ 10 ⁻⁴	2 g/l fructose 10° 10-1 10-2 10-3 10-4	20 g/l fructose 10° 10' 10' 10' 10' 10' 10'	2 g/l mannose	20 g/l mannose
C Gal2 wt T219S T219N N376V N376V N376F Hxt7 wt T213G T213N N370S N370L N370F	2 g/l galactose 10° 10-1 10-2 10-3 10-4	20 g/l galactose 10° 10-1 10-2 10-3 10-4	2 g/l fructose 10° 10-1 10-2 10-3 10-4	20 g/l fructose 10° 10' 10' 10' 10' 10' 10'	2 g/l mannose	20 g/l mannose
C Gal2 wt T219S T219N N376Y N376V N376F Hxt7 wt T213G T213N N370S N370L N370F CEN.PK2-1c	2 g/l galactose 10° 10-1 10° 10-3 10-4	20 g/l galactose 10° 10° 10° 10° 10° 10° 000 000 000 000 000 000 000 000 000 000 000 000000	2 g/l fructose 10° 10-1 10-2 10-3 10-4	20 g/l fructose 10° 10' 10' 10' 10' 10' 000 000 000 000 000 000 000 000 000 000 000 000 000 000 000 000 000 000	2 g/l mannose	20 g/l mannose

Fig. S1. (*A*) Growth analysis of the screening strain AFY10X and its ancestors. All strains were pregrown in liquid selective synthetic complete ethanol (SCE) medium. Serial dilutions of washed cells were dropped on solid SC media with the indicated carbon sources. Cells were grown at 30 °C for 3 d (glucose, maltose) or 6 d (ethanol, xylose). AFY10 was transformed with empty plasmid pRS62N. EBY.VW4000 and CEN.PK2-1c did not grow on xylose, AFY10 and AFY10X did not grow on glucose. (*B*) Functional characterization of Hxt7 wild type and mutants. Growth assay of AFY10X (xylose and xylose–glucose mixtures) and EBY. VW4000 (glucose) overexpressing the indicated transporters. Cells were pregrown in liquid selective SCE or YEPE medium, respectively. Serial dilutions of washed cells were dropped on solid SC media with the indicated carbon sources. Cells were grown at 30 °C for 3 d (xylose and mixtures). (C) Functional characterization of Gal2 and Hxt7 wild type and mutants regarding hexose transport. Growth assay of EBY.VW4000 overexpressing the indicated transporters. Cells were grown at 30 °C for 3 d (xylose and mixtures). (C) Functional characterization of Gal2 and Hxt7 wild type and mutants regarding hexose transport. Growth assay of EBY.VW4000 overexpressing the indicated transporters. Cells were pregrown in assay of SC media with the indicated carbon sources. Cells were dropped on solid SC media with the indicated transporters. Cells were pregrown at 30 °C for 3 d. CEN.PK2-1c and EBY.VW4000 contain empty plasmid pRS62N.



Fig. 52. (*A* and *B*) Glucose (*A*) and xylose (*B*) transport of wild-type and mutant transporters measured in zero-trans influx experiments. EBY.VW4000 overexpressing *GAL2* (solid lines and filled symbols) or *HXT7* (dashed lines and open symbols) variants were grown in selective YEPE. Shown are Gal2 wild type (filled triangle), Gal2-N376F (filled circle), Gal2-N376V (filled square), Hxt7 wild type (open triangle), and Hxt7-N376S (open circle). Curve fitting for Michaelis-Menten kinetics was applied to data of three independent measurements at each concentration. Error bars are given as SEM. Notice the different scales for glucose and xylose concentration. (*C* and *D*) Inhibitory effect of glucose on xylose transport of wild-type and mutant Hxt7. Zero-trans influx of xylose was measured in the absence of glucose (solid line, filled circle) and in the presence of 25 mM (dashed line, open square) or 100 mM glucose (dotted line, open triangle). EBY.VW4000 overexpressing Hxt7 wild type (*C*) or Hxt7-N370S (*D*) was grown in selective YEPE. Global curve fitting for Michaelis-Menten kinetics with competitive inhibition was applied to data of three independent measurements at each concentration. Error bars are given as SEM but are often smaller than the respective symbol. v, initial rate of uptake.



Fig. S3. Homology model of the Gal2 structure generated using the SWISS-MODEL software (1). The model is based on the outward-facing partly occluded structure of *E. coli* XyIE with bound glucose (PDB ID code 4GBZ). The N-terminal (amino acids 1–70) and C-terminal (amino acids 536–574) cytosolic tails could not be modeled due to the absence of corresponding sequences in XyIE. The side view, with the extracellular side at the top, shows the subdomains N (orange) and C (cyan) and all residues contributing to glucose binding in stick representation. Glucose is shown in space filling model (black and red), and the C6 is oriented to the back. The 3D images were created with PyMOL (2).

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	1 10	20	30			40	50	60	
ScGal2 ScHxt7 ScHxt5 EcXyIE EcXyIE SeGIcPse * HsGLUT1 * HsGLUT2 * GiGxs1 CiGxs1 CiGxf1 SsXut1 SsXut3 DhXyIHP	MAVEENNMPV MSQDAAIAEQ MS.ELENAHQ	VSQQPQAGED) TPVEHLSAVD GPLEGSATVS	/ISSLSKDSH. SASHSVLSTP. INSNSYNEKSG MSQDSHSSG.	NSTAPGTAGYN	LSAQSQK SI IDNLAQAKPVSS AMHO	YSNDELKAGI NKAERDEIK. YISHEGPPKI MI ATPVNGSILI GGGDGNDITI	ESGSEGSQSVP AYGEGEEHEPV DELEELQKEVD LKRFHKGIIIS GLEDNRMVKRF EKEKEDSPVLQ EIIAARRLQIA .MREVGILDVA	IEIPKKPMSE VEIPKRPASA KQLEKKSKSD LAIEKGMIRM MOTQYN LAIEKGMIRM MTEDKV VNVGEKKAGS VDAPQKGFKD GKSGVAGLVA HGNVVTIMMK MSK	67 61 82 6 22 8 6 22 40 32 20 3
	70 -0	80 TM1	90	100				110	
ScGal2 ScHxt7 ScHxt5 EcXyIE SeGlcPse * HsGLUT1 * CiGx51 CiGx51 CiGxf1 SsXut1 SsXut3 DhXyIHP	YVTVSLLCLC YVTVSIMCIM LLFVSVCCLM SSYIFSTLV KANKYLIFIL TGRLWLAVGG TGTLVFTVIT TAMAIIVGLF YIVISIFCFM DFVVFLVILF DPVVFLVILF	A F G F W F G W II A F G F V F G W IV A F G F V F G W A T L G L L F G Y G A L G L L F G Y G A L G L L F G Y A V L G S L O F G Y A S G G V L F G Y A S L G G L V F G F A S L G G L L F G Y A S L G G L L F G Y A S L G G L L F G Y A S L G G L L F G Y	TGTISGFVVQ TGTISGFVRQ TGTISGFVRS NGVISGAL TGVINAPQKV IGVINAPQKV IGVINAPQKV TGTISGFVRM TGTISGFVRM QGMFGGISGM QGVISGIVTM	.TDFLRRFGMM .TDFIRRFGMM .TDFIRRFGST .LNTVFVAP .LFIHKD IEEFYNQTWU. ISHYRHVLGV .DYVLARYP .SDFKDRFGQH YSFSKAIG ESFGAKFP	PLDDRKAINNY	VINSTDELP	TISYSMNPKPT	HKDGT RANGT QNLS HRYGESIL PWAEEETVAA SNK HADGT R	113 107 128 49 57 57 89 64 86 74 60 43
	120	130 TM2	140	150	160 TM3	170		-0	
ScGal2 ScHxt7 ScHxt5 EcXylE SeGlcPse * HsGLUT1 * HsGLUT2 * CiGxf1 SsXut1 SsXut3 DhXylHP	HYLSNVRTGL NYLSKVRTGL ESAANSLLGF IPLNSTTEGI PTTLTTLWSL AGLITMLWSL HSFTADESSL IQDNPTLGGL IFMDADYKGW IYMDPDYKGW	IVAIFNIGCAI VSIFNIGCA CVASALIGCI VVSSMLIGAI SVAIFSVGGM IVSILSVGTFI MISIFNVGCA LTSILELGAW (FVSTFLLCAWI	GGIILSKGGD GGIILSKLGD GGIVLSKLGD GGALGGYCSN GACSSGPLAD GSFSVGLFVN ASFFGGWLGD GGIFLCKVAD GVLMNGYIAD GSIINTPIVD GSLINSPIVD	MYGRKKGLSI. MYGRKIGLMT. RFGRRDSLKI. KLGRRRLVML. RFGRRNSMLM. TLGRIKAMLV. TLGRRWCLILS VWGRRIGLMF. RLGRKKSVVV. RFGRRDSITI.	VVSVYIVGIII VVVIYIGIII AVVLFIGALII MVVFIGALII AVLFFSQVG ALVFIGALI ALISLVGALL ANILSLVGALL GVFFFGVIV GVFFFFGVIV ACVVFVIGSAF ACVVFVIGSF	QIASIN.K. QIASIN.K. QIASIN.K. QIASIN.K. AWPELGFT LAASTN WGFSKLGKS QVIST.A. QISSST.K. QAVARGGN. QCAGIS.	SINPDNTVPVY	WYQYFI WYQYFI LAGYVPEFVI LALLII FEMLIL HILIA IPLLCA WYQFFI YDYILG TSMLFG VSMLFA	178 172 193 131 121 124 156 129 151 140 124 107
	180	190	200 2	10 22	230				
ScGal2 ScHxt7 ScHxt5 EcXylE SeGlcPse * HsGLUT1 * HsGLUT2 * CiGxf1 CiGxf1 SsXut1 SsXut3 DhXylHP	GR I I S G L G V G GR I I S G L G V G GR I I S G L G V G GR I I G G I G V G GR I I G U S V C GR S I S G L Y C G GR S I S G L Y C G GR L I A G L A V G GR A V A G L A V G GR A V A G L A V G GR A V A G L G V G	GIAVLCPMLI GIAVLSPMLI GITVLAPMLI LASMLSPMYIL LTGFVPMYU LISGLVPMYU LISGLVPMYU LISATIPLYQ TVSVVSPLFI ILSMVVPLYN QLTMVVPMYM QLTMVVPMYM	SE IAPKHLRGT EVSPKHLRGT EVSPKHLRGT ELAPAHIRGK SEVSPTALRGA EVSPTALRGA ETAPKWIRGA ETAPKWIRGA EVSPKQIRGT EVSPPEIRGS ELAPPSVRGG SELAPPSVRGG	LVSCYDLM TA LVSCYDLM TA LVSCYDLM TA LVSCYDLM TA LGSLNOLM T LGSLNOLM TU LGTLHDLGI VV LGTFHDLA VT IVSCYDWA TU LVCCFDLCTL LVALQOLA TT LVVLQOLS TT LVVLQQLS T	GIFLGYCTNYG GIFLGYCTNFG GIFLGYCTNFG GULLYYCVNYA GILIAQVFGLD GILISQIIGLI GIFLSCVNKG GIFLGYCTYG GIMISYWITYG GIMISYWLDYG GILISFWINYG	TKSY TKNY FADI FADI FIL TEHM TKTY TNYIGGTG THFIGGTRC, TGFIGGTKC,	APSHPYQGETF APGRNYQGDVF	NPNVDVPPGG DPYVDVPKQG	235 229 250 191 178 180 212 186 208 201 207 190
	240	250 TM6	260	270	280	290	300	310	
ScGal2 ScHxt7 ScHxt5 EcXyIE SeGIcPse * HsGLUT1 * CiGxs1 CiGxf1 SsXut1 SsXut3 DhXyIHP	SUSTICATION STATES SUSTICATION	VPLGLCFAWS VPLGLCFAWS WFASECIPA WMLGLAVVPS ILLSIIFIPA ILLGLSGVRA IPLAIQCLWG IPLGLCFAWA VPICIQLVPA IPFGVQIAPA IPFGLQIAPA	FMIGALTLVP FMIGGMTFVP FMIVGMTFVP FMIVGMTFVP FULFULTVP ILFUNGIYFMP LQSLUFFCP LQSLUFFCP LLVVGMLNMP LLVVGMLNMP LLGVGIFFMP LLGIGMIFFP LLGIGMIFFP	ESPRYLCEVN. ESPRYLVEVG ESPRWLWSRG ESPRWLLENR. ESPRFLLINRN ESPRFLVIKL ETPRFWISKG ESPRYLVEKH. ESPRWLLSKG. RSPRWLLSKG.	KVEDAKRSIAKS KIEEAKRSIAYS KIEEAKRSIAYS KIEEAKRSIAYS KIEEAKRSIA REEAARQVMKI EENRAKSVLKK NQEKAAESLAR RIDEAKRSIARS REDECLSVLSNI REEEAWSSLKYI REEEAWSSLKYI	SNKVSPEDP SNKVAVDDP ANKTTEDSP IMGNT TYDDS LRGYDI LRGYDI LRKLPIDHP SNKIPEDP LRSLSKEDT LRSLSKEDT LRRKS.HEDO LRRRN.NPDI	AVQA ELDLIMA SVLAEVEAVLA LVTLEMENYQS LATQAVOEIKH EIDKELKEMKE DVTHDLQEMKE DVTHDLNEMRK DSLEELRDITA FVYTEVQLIQA LVQMEFLEMKA QVEREFAEIKA MIEAEFNEIRS	GIEAEKLAGN GVEAEKLAGN SIEAERLAGS SLDHGRKTG. INAISE ESRQMMREKK EREEASSEQK AYEFETVYGK GIEREALAGQ QKLFERELSA EVVYEDKYKE DVIFEKKYNE	314 308 329 267 246 256 288 265 287 282 288 271
			320	330 +	340	350	360	370 TM8	
ScGal2 ScHxt7 ScHxt5 EcXyIE SeGicPse * HsGLUT1 * HsGLUT2 * CiGxs1 CiGxf1 SsXut1 SsXut1 SsXut3 DhXyIHP	KYFPHLODGS KRFPGKTG. KRFPGKEG.		WGELFSTKTK WGELFSSKTK WGELVTGKPQ GRLUMF WTVIKSPW ILELFRS.PA SIIQLFTN.SS WSQVFSHKNH WKELIGKPK YKSMITH.YP YWDLFST.KS	VFORLLMGVFV VLORLIMGAMI MFRRTLMGMMI GVGVIVIGVML LGRILIVGCIF YROPILVALMI YROPILVALMI JRRVINGIM FRKVMGIM FRKVMGIMGSAV	IAAI A IAAI A QREQUITENNY A QSLOQUITENNY A SIFQOFIGINY A ISIFQOFIGINY A ISIFQOFISINY A QSLOQUITENNY A QSLOQUITENNY A QSIFQOFISINY A Q	● ▲ ● ▲ ● ▲ ● ▲ ● ▲ ● ▲ ● ▲ ● ▲ ● ■ ■	KSVGL DDS KAVGL SDS DAVGL EDS KAGV DIA AKAGLGE . AAS EKAGV QQP DIAGI SKP KRAGV KDS SSLGLSGNTIS SOLGMDSNTTA	FETSIVIGVV FETSIVLGVV FETAIVLGVV LLQTIIVGVI ILGSVGIGTI VYATIGSGIV VYATIGVGAV FTISLATNIV LLSGVVGIV LLGTGVVGIV	375 369 390 324 307 316 348 325 348 364 364 364 347

Fig. S4. (Continued)

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	380	390	400	410	420	430	440	450	
ScGal2 ScHxt7 ScHxt5 EcXylE SeGlcPse * HsGLUT2 * CiGxs1 CiGxf1 SsXut1 SsXut3 DhXylHP	MA N F AST F F S LW N F AST F F S LY N F V S T F F S LY N T F T V LA I M N T AF T V V S LF N V C S T I P G I L N F AST F V G I Y N C L S T L P AV L N C L S T L P A V F	TVENLOHRKC VVERYGRRTC VVDKFGRRNC VVDKIDRKKL VVERAGRRSL LVEKAGRRSL LMEVLGRRNM VIERLGRRLC WYDRLGRKPV LIDRCGRKPV LIDRCGRKFL	TMB LLLGAATMMACM LLWGCAGMICCY QIIGALGMAIGU LVGGNIGGMIASL HLIGLAGMAGGA FLIGMSGMFVGA LMGGATGMSLSC LLTGSAAMFICF LISGAIMGICF LMAGAAGTFVSL	IV I YAS VGV IV VYAS VGV IFS LGTAFY I SLGTAFY I LIMAILIW I LMTIALA I FMS VGLV I I YSLIGT I FVXAAILG .VI VGAIVG .VI VGAIVG	TRLYPHOKSQPS TRLWPNGQDQPS T	CALL CALL CALL CALL CALL CALL CALL CALL	VFTCFYIFC VFACFYIFC LSMLFYVAA VCLSLFIVF VAIFGFVAF AFSCIFIAF FITCLYIFA AFSCIFIAF FITCLYIFAIG AFIFIYDVN AFIFIYDFN	MID A YA T TWAP VAW A FA TTWAP VAW FA TSWAP VAW FAMSWCP VAY FASSWCP VAW FE IGPCF I PW FE IGPCF I PW FE A TSWCP CAW FAA TSWCP CAW FA STWACG VY FAA TSWCP CAW FA STWACG VY FA STWACG VY FA STWACG VY FA STWACG VY FS SSWAP I GW FASSWAP I GW	455 449 470 392 392 392 420 392 431 440 440 440 42
ScGal2 ScHxt7 ScHxt5 EcXylE HsGLU71 * HsGLU72 * CiGxs1 CiGxf1 SsXut1 SsXut3 DhXylHP	460 VITAESFPLR VUSETFPLR VLISESYPLR VLISEIFPNA FIVAELFSQG FWVAEFSQG VVVGELFPLR CIISESYPLR VLVSEIFPIG VLVSEIFFIG VLPSEIFSIG	470 VKSKCMALAS VKSKAMSIAT VRGKAMSIAS IRGKALAIAV ARGAATGISA PRPAAIAVAG PRPAALAIAA TRAKSVSLCT IRSKAMSIAT IRSKAVSIGA IRSNAISITT	480 1411 A ANNUWG FLI A A A SNWVWG FLI SF A ANNUWG FLI SF A ADM LANYFYSW VLNI GTLI SS FSNWT CNFI VAL A SNWLWNWG I AY A ANNUWG FLI SF SSNWT NN FI I GL SSTWMNN FI I GL	490 FTPFITSA FTPFITSA TFPMMDKN FFPILSDA CFQYIADF ATPYMVDE FTPFITSA ISTPDFVAK VTPHMLET ITPRMLNT	500 1N	2 510 THI2 GYV FMGCLVAMF GYV FMGCLVFMF GYV FMGCMVFAY YWI YGCMGVLAA FLI FAFLGVLAMF FFL FAQVLAFT FFLWGGFNLACV GFV FTGCLAFSF YI FLGAGALIAF YI FLGAAFALIAF	520 FYVFFFVP FYVFFFVP LFMWKFVPE LFMWKFVPE LFTFKVPE FFAWYFIYE FFAWYFIYE FFAWYFIYE FFTWLIIPE VFTWYMIPE	530 TKGLS LEE IQ TKGLT LEE VN TKGKT LEE VN TRGRS LEE IE TRGRS LEE IE TKGKS FE IA TKGLS LEO VD TKGLS LEO VD TKGLS LEO VD TKGLS LEO D TKGVP LEEMD	531 528 548 458 464 498 478 500 510 510 498
ScGal2 ScHxt7 ScHxt5 EcXyIE SeGIcPse * HsGLUT2 * HsGLUT2 * CiGxs1 CiGxf1 SsXut1 SsXut3 DhXyIHP	540 ELWEEGVLPW MWEEGVLPW ALWEPETKKT YELRERT.GA SGFRQGGASQ AEFQKKSGSA ELYEHVSKKSSA ELYEHVSKSGSA ELYEHVSKSGSA ELFGDTSGTS AVFGDTAALG FVFGDTAALG	550 KSEGWIPSSR KSASWVPPSR KSTKWIPPSR RTE SDKTPEELFH HRPKAAVEMK KSKGFVPSKH KSASWVPNL KMEKEIHEQK EKQRFSDTNA	560 RGNNYDLEDLQH RGANYDAEEMTH RTTDYDLDATRN PLGADSQV SFREQVDQQMDS SFREQVDQQMDS EHMAHSAGYAGA LKEVGLLQLLGE SESDAKDRNSIE JSKIHINTGTKV	570 IDDKPWYKA IDDRPLYKR IDPRPFYKR SKTEAIMSE D.KATDEQ ENASESEN IMSE WSE	ML	73 70 92 91 97 92 92 92 92 94 95 95 95 95 95 95 95 95 95 95 95 95 95			

Fig. 54. Protein sequence alignment of different sugar transporters. Transporters mentioned in the main text and additionally further xylose transporters found to be functional in *S. cerevisiae* (1) were aligned using the PRALINE multiple sequence alignment server (2). Transporters aligned are *Saccharomyces cerevisiae* Gal2, Hxt7, and Hxt5, *Escherichia coli* XylE, *Staphylococcus epidermidis* GlcP_{SE}, *Homo sapiens* GLUT1 and GLUT2, *Candida intermedia* Gxs1 and Gxf1, *Scheffersomyces stipitis* Xut1 and Xut3, and *Debaryomyces hansenii* xylHP. Transporters not able to transport xylose are marked by asterisks. Transmembrane helices refer to XylE (as adapted from the supporting information of ref. 3). The numbering above refers to the Gal2 sequence. Important residues are boxed, and black symbols indicate their contribution to binding of xylose and glucose (triangle) or glucose only (square), referring to Sun et al. (3). Contribution of F350 (diamond) to glucose binding was inferred from the Gal2 homology model. Green squares indicate the two identified amino acid positions T219/213 and N370/376 in Gal2 and Hxt7, respectively. Similarities of residues are indicated in blue. The figure was created with ALINE software (4).

1. Young E, Poucher A, Comer A, Bailey A, Alper H (2011) Functional survey for heterologous sugar transport proteins, using Saccharomyces cerevisiae as a host. Appl Environ Microbiol 77(10):3311–3319.

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- 3. Sun L, et al. (2012) Crystal structure of a bacterial homologue of glucose transporters GLUT1-4. Nature 490(7420):361-366.
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Fig. S5. Sugar coordination of XylE and Gal2. (A) Schematic map of glucose coordination in XylE. The residues of the XylE crystal structure (PDB ID code 4GBZ) forming hydrogen bonds (blue) or hydrophobic contacts (red) with β -D-glucose are shown, and the corresponding amino acids of Gal2 are indicated below. The water molecules are omitted for clarity. Conserved residues are indicated in bold. The Gal2 positions that were subjected to site-directed mutagenesis are marked by asterisks. (*B*) A 3D model of the coordination of glucose in the XylE crystal structure (*Left*) and the Gal2 homology model (*Right*). Only TM2, which does not contribute to sugar binding, is omitted for better view of the binding pocket. Residues participating in xylose and glucose binding are indicated in cyan, and residues that exclusively bind to glucose are indicated in salmon. The two identified amino acid positions T219 and N376 in Gal2 and their homologs in XylE (1172 and N325, respectively) are shown in green. The Gal2 positions that were subjected to site-directed mutagenesis.

Positio	n in		Growth on				
XylE	Gal2	Changed to	Glucose	Xylose	Glucose + xylose		
1171	1218	G	_	+	_		
		А	+++	+++	_		
		D	++	++	_		
		F/K	_	_	_		
Q175	1222	G/A/D/F/K	_	_	_		
		Q	++	++	_		
L297	F350	G	+	_	_		
		A/Y	++	_	_		
		D/K/W	_	_	_		
		L	+++	++	_		
		М	+++	_	_		
F383	Y446	W	—	—	—		

Table S1. Growth of EBY.VW4000 (glucose) and AFY10X (xylose and xylose–glucose mixtures) overexpressing individual Gal2 mutants

Growth was compared with the Gal2 wild type. Growth comparable with the wild type is denoted as (+++), weaker growth with (++) or (+), and no growth as (-).

Table S2. S. cerevisiae strains used in this work

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Name	Relevant genotype	Source
CEN.PK2-1C	MATa leu2-3,112 ura3-52 trp1-289 his3∆1 MAL2-8 ^c SUC2	E.B. laboratory stocks
EBY.VW4000	CEN.PK2-1C ∆hxt1-17 gal2∆::loxP stl1∆::loxP agt1∆::loxP mph2∆::loxP mph3∆::loxP	(1)
AFY10	EBY.VW4000 glk1Δ::loxP hxk1Δ::loxP hxk2Δ::loxP ylr446wΔ::loxP pyk2Δ::pPGK1-opt.XKS1-tPGK1 pTPI1-TAL1-tTAL1 pTDH3-TKL1-tTKL1 pPFK1-RPE1-tRPE1 pFBA-RKI1-tRKI1 loxP	This work
AFY10X	AFY10, pHXT7-opt.xylA-tHXT7, LEU2 (plasmid YEp-kanR_optXI)	This work

1. Wieczorke R, et al. (1999) Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in Sacccharomyces cervisiae. FEBS Lett 464(3):123-128.

Table S3. Plasmids used in this work

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Name	Description	Source
YEp181_pHXT7-optXI_Clos	2µ-plasmid, LEU2 marker, codon-optimized xylose isomerase	(1)
	gene xyIA of C. phytofermentans (opt.XyIA) under control of shortened	
	HXT7-promoter and CYC1-terminator, E. coli ampicillin marker	
YEp181-kanR_optXI	2μ-plasmid, <i>LEU2</i> marker, codon-optimized xylose isomerase gene xylA of	This work
	C. phytofermentans (opt.XyIA) under control of shortened HXT7-promoter	
	and CYC1-terminator, E. coli kanamycin marker	
pDONR222	Gateway-system donor-vector, E. coli kanamycin-marker,	Life Technologies
	chloramphenicol-marker, pUC-origin	(2)
рнов	integrative 2µ-plasmid (PYK2-locus), kanMX marker, pHX17-opt.XyIA-tCYC1	(2)
	pPGK1-opt.XKS1-tPGK1, p1PI1-1AL1-t1AL1 p1DH3-1KL1-t1KL1, pPFK1-RPE1-tRPE1	
	pFBA1-KKI1-tRKI1 pPGM1-HX1/-tHX1/	This second
PAF-HD8.3	Integrative plasmid (PYK2-locus), kanivix marker, pPGK1-opt.XKS1-tPGK1,	I his work
-761	pipii-iali-tiali pidha-ikli-tikli pprki-kpei-tkpei prbai-kkii-tkkii	(2)
	CENCARCHARLES (10.10) of the nphilin determined of the nphilin determined of the	(3)
pSH47	CEN6/ARSH4-plasmid, URA3 marker, cre-recombinase under control of	(4)
	inducible GALI-promoter and CYCI-terminators	
	2µ-plasmid; URA3 marker, GAL2 under control of ADH1-promoter;	(5)
P426H7	2µ-plasmid; UKA3 marker, shortened HX17-promoter and CYC7-terminator	(0) This second
p426_GAL2	ORF of GALZ IN P426H7	inis work
P426_HX17	ORF of HX17 In p426H7	I NIS WORK
PRS62N	2µ-plasmid; hativiz marker; shortened HX17-promoter and CYC7-terminator	E.B. laboratory stock
PRS62N_GAL2	ORF of GAL2 in pRS62N	This work
PRS62N_GAL2-12195	ORF of GAL2-12195 IN pRS62N	inis work
PRS62N_GAL2-1219N	ORF of GAL2-1219N IN PRS62N	inis work
PRS62N_GAL2-N376Y	ORF OF GALZ-N376Y IN PR562N	Inis work
PRS62N_GAL2-N376V	ORF OF GAL2-N376V IN PRS62N	inis work
PRS62N_GAL2-N376F	ORF of GALZ-N3/6F IN PRS62N	inis work
		This work
PR562N_HX17-1213G	ORF OF HX17-1213G IN DRS62N	Inis work
	ORF OF HX17-1213N IN PRS62N	This work
PRS62N_HX17-N3705		This work
	ORF OF HX17-N370L IN PRS62N	This work
		Inis work
p426_GAL2-I218G	ORF of GAL2-1218G in p426H7	This work
P426_GAL2-I218A		This work
p426_GAL2-I218D	ORF of GAL2-1218D in p426H7	This work
P426_GAL2-I218K	ORF of GAL2-1218K IN P426H7	Inis work
P426_GAL2-I218F	ORF of GAL2-1218F in p426H7	This work
P426_GAL2-I222G		inis work
P426_GAL2-I222A	ORF of GAL2-I222A IN P426H7	Inis work
p426_GAL2-I222D	ORF of GAL2-I222D in p426H7	This work
P426_GAL2-I222K	ORF of GAL2-1222K in p426H7	This work
P426_GAL2-I222F	ORF of GAL2-1222F in p426H7	This work
p426_GAL2-I222Q	ORF of GAL2-I222Q IN P426H7	Inis work
P426_GAL2-Y446VV	ORF of GAL2-Y446W in p426H7	This work
P420_GAL2-F350A	OKE OF GALZ-F350A IN P426H/	I NIS WORK
P426_GAL2-F350D	OKE OT GALZ-F350D IN P426H7	This work
P426_GAL2-F350G	OKF OT GALZ-F350G IN P426H7	This work
P426_GAL2-F350K	OKF OT GAL2-F35UK IN P426H7	This work
P426_GAL2-F350L		This work
		I NIS WORK
P426_GAL2-F350W		This work
P420_GAL2-F350Y	ORF 01 0AL2-F35UT IN P420H7	I DIS WORK

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2. Demeke MM, et al. (2013) Development of a D-xylose fermenting and inhibitor tolerant industrial Saccharomyces cerevisiae strain with high performance in lignocellulose hydrolysates using metabolic and evolutionary engineering. Biotechnol Biofuels 6(1):89.

3. Carter Z, Delneri D (2010) New generation of loxP-mutated deletion cassettes for the genetic manipulation of yeast natural isolates. Yeast 27(9):765-775.

4. Güldener U, Heck S, Fielder T, Beinhauer J, Hegemann JH (1996) A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res 24(13):2519–2524.

5. Liang H, Gaber RF (1996) A novel signal transduction pathway in Saccharomyces cerevisiae defined by Snf3-regulated expression of HXT6. Mol Biol Cell 7(12):1953–1966.

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Table S4. Primers used in this work

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Name	Sequence*	Description
del_GLK1_F	CCGCCCGACAGGGTAACATA	Forward primer for amplification of a <i>kanMX</i> -deletion cassette for <i>GLK1</i>
del_GLK1_R	AGTACGCCCCTTGGAAGTG	Reverse primer for amplification of a <i>kanMX</i> -deletion cassette for <i>GLK1</i>
A1_GLK1	TAGACATGCTGCTTGCAAC	Forward primer for verification of a <i>GLK1</i> deletion (upstream)
A2_GLK1	TTCCTTTGGTGGAGCTAGAC	Reverse primer for verification of a <i>GLK1</i> deletion (ORF)
A4_GLK1	TTCGGACCTTAGCGGAGAG	Reverse primer for verification of a <i>GLK1</i> deletion (downstream)
del_HXK2_F	CTTTGATTGCGAGATCCACG	Forward primer for amplification of a <i>kanMX</i> -deletion cassette for <i>HXK2</i>
del_HXK2_R	CGTTCGTTCCAGAATTATCACG	Reverse primer for amplification of a <i>kanMX</i> -deletion cassette for <i>HXK2</i>
A1_HXK2	GCACCGGGCAATAAACCGG	Forward primer for verification of a <i>HXK2</i> deletion (upstream)
A2_HXK2	CAAAGTACCGGTAGTGTCGTTTATC	Reverse primer for verification of a <i>HXK2</i> deletion (ORF)
A4_HXK2	CGGGTATGAAGTGGTTGTGAGAATTAG	Reverse primer for verification of a <i>HXK2</i> deletion (downstream)
del_HXK1_F	ATGGTTCATTTAGGTCCAAAGAAACCACAGGCTAGAAAGGGT TCCATGGCTTCGTACGCTGCAGGTCGAC	Forward primer for amplification of a <i>hphNT1</i> -deletion cassette for <i>HXK1</i> from pZC1
del_HXK1_R	TTAAGCGCCAATGATACCAAGAGACCTTACCTTCGGCAATTCTTTTT CGGCCATAGGCCACTAGTGGATCTG	Reverse primer for amplification of a <i>hphNT1</i> -deletion cassette for <i>HXK1</i> from pZC1
A1_HXK1	CAAGGTCTCGCTGTCAACTG	Forward primer for verification of a <i>HXK1</i> deletion (upstream)
A2_HXK1	CTAGACCATGGGATGCAACT	Reverse primer for verification of a <i>HXK1</i> deletion (ORF)
A4_HXK1	CCTGGAAGTAGGTGCCCTTG	Reverse primer for verification of a <i>HXK1</i> deletion (downstream)
del_YLR446w_F	ATGACAATTGAAAGCACTCTAGCTCGGGAATTA	Forward primer for amplification of a hphNT1-deletion
	GAAAGCTTGATTTTACC TTCGTACGCTGCAGGTCGAC	cassette for YLR446w from pZC1
del_ YLR446w _R		Reverse primer for amplification of a hphNT1-deletion
		Cassette for YLR446W from p2C1
		(upstream)
A4_ YLR446w	GCACAAAACCAAAGAGAAAG	Reverse primer for verification of a YLR446w deletion (downstream)
K2_kanMX	TTGTCGCACCTGATTGCCCG	Reverse primer for detection of an genome-integrated <i>kanMX</i> deletion cassette
K3_kanMX	GATAATCCTGATATGAATAAATTGC	Forward primer for detection of an genome-integrated <i>kanMX</i> deletion cassette
K2_hphNT1	GAAAGCACGAGATTCTTC	Reverse primer for detection of an genome-integrated hphNT1 deletion cassette
K3_hphNT1	TACACAAATCGCCCGCAGAAG	Forward primer for detection of an genome-integrated <i>hphNT1</i> deletion cassette
A1_PYK2	CATCCTCTACGTCCATTGTAAG	Forward primer to confirm pAF-HD8.3 integration into <i>PYK2</i> (upstream)
A2_PYK2	ACCCACATCCGATACTTCAG	Reverse primer for verification of <i>PYK2</i> replacement by pAF-HD8.3 (ORF)
A3_PYK2	AGTATCGGATGTGGGTAACG	Forward primer for verification of <i>PYK2</i> replacement by pAF-HD8.3 (ORF)
A4_PYK2	CCAGACTGTGCGTAAACTTG	revers primer to confirm pAF-HD8.3 integration into <i>PYK2</i> (downstream)
K2_pAF-HD8.3	ATACTAACGCCGCCATCC	revers primer to confirm pAF-HD8.3 integration into PYK2 (cassette)
K3_pAF-HD8.3	CTAGGACCTTGTTGTGTG	Forward primer to confirm pAF-HD8.3 integration into <i>PYK2</i> (cassette)
Clon_kanR_F	ATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTA TGAGCCATATTCAACGG	Forward primer for cloning of the kanamycin resistance gene
Clon_kanR_R	AATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGT TAGAAAAACTCATCGAGCATC	Reverse primer for cloning of the kanamycin resistance gene

Table S4. Cont.

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Name	Sequence*	Description
Clon_HXT7_F	ΑΤΑΑΑCΑCΑΑΑΑΑCΑΑΑΑΑGTTTTTTTAATTTAATCAAAAA ΑΤΩΤΩΓΑΓΑΑGΑCCCTGCTATTC	Forward primer for cloning of HXT7
Clon_HXT7_R	GAGGGCGTGAATGTAAGCGTGGCTGAACATTACTACTACTACTACTACTACTACTACTACTACTA	Reverse primer for cloning of HXT7
Clon_GAL2_F	AACACAAAAACAAAAAGTTTTTTTTTTTAATTTAATCAAAAAA TGGCAGTTGAGGAGACAA	Forward primer for cloning of GAL2
Clon_GAL2_R	GAATGTAAGCGTGACATAACTAATTACATGACTCGA	Reverse primer for cloning of GAL2
Amp HXT7 F		Forward primer for amplification of the HXTZ ORF
Amp_HXT7_R	ттатттсстсстсаасаттс	Reverse primer for amplification of the HXT7 ORF
Amp GAL2 F		Forward primer for amplification of the GAL2 ORF
Amp GAL2 R	TTATTCTAGCATGGCCTTG	Reverse primer for amplification of the GAL2 ORE
HXT7 mut1-T213 F	CTTGCTACCAATTGATGATTGBTGCCGGTATTTTCTTGG	Forward primer for mutagenesis of T213 to A/G/V in HXT2
HXT7 mut1-T213 R	GTAACCCAAGAAAATACCGGCAVCAATCATCAATTGGTAG	Reverse primer for mutagenesis of T213 to A/G/V in HXT7
HXT7 mut2-T213 F	CTTGCTACCAATTGATGATTTSTGCCGGTATTTTCTTGG	Forward primer for mutagenesis of T213 to S/C in HXT7
HXT7 mut2-T213 R	GTAACCCAAGAAAATACCGGCASAAATCATCAATTGGTAG	Reverse primer for mutagenesis of T213 to S/C in HXT7
HXT7 mut3-T213 F	CTTGCTACCAATTGATGATTRACGCCGGTATTTTCTTGG	Forward primer for mutagenesis of T213 to D/N in HXT7
HXT7 mut3-T213 R	GTAACCCAAGAAAATACCGGCGTYAATCATCAATTGGTAG	Reverse primer for mutagenesis of T213 to D/N in HXT7
GAL2 mut1-T219 F	CTTGTTATCAGCTGATGATTGBTGCAGGTATCTTTTGG	Forward primer for mutagenesis of T219 to A/G/V in GAL2
GAL2 mut1-T219 R	TAGCCCAAAAAGATACCTGCAVCAATCATCAGCTGATAAC	Reverse primer for mutagenesis of T219 to A/G/V in GA/2
GAL2 mut2-T219 F	CTTGTTATCAGCTGATGATGATTTSTGCAGGTATCTTTTGG	Forward primer for mutagenesis of T219 to S/C in GAL2
GAL2 mut2-T219 R	TAGCCCAAAAAGATACCTGCASAAATCATCAGCTGATAAC	Reverse primer for mutagenesis of T219 to S/C in GAL2
GAL2 mut3-T219 F		Forward primer for mutagenesis of T219 to D/N in GAL2
GAL2 mut3-T219 R	TAGCCCAAAAAGATACCTGCGTYAATCATCAGCTGATAAC	Reverse primer for mutagenesis of T219 to D/N in GAL2
HXT7 mut-N370 F	CTATTGTCTTGGGTATTGTTNNNTTTGCTTCCACCTTTG	Forward primer for random mutagenesis of N370 in HXT7
HXT7 mut-N370 R		Reverse primer for random mutagenesis of N370 in HXT7
GAL2 mut-N376 F	CCATTGTCATTGGTGTGTAGTCNNNTTTGCCTCCACTTTC	Forward primer for random mutagenesis of N376 in GAL2
GAL2_mut-N376_		Reverse primer for random mutagenesis of N376 in GAL2
HXT7 N370F F		Forward primer for mutagenesis of N370 to F in HXT7
HXT7 N370F R		Reverse primer for mutagenesis of N370 to F in HXT7
GAL2 N376Y F		Forward primer for mutagenesis of N376 to Y in GAL2
GAL2 N376Y R		Reverse primer for mutagenesis of N376 to Y in $GAI2$
GAL2 1218G F	TGTTATCAGCTGATGGGTACTGCAGGTATCTTTTTGG	Forward primer for mutagenesis of 1218 to G in GAL2
GAL2 1218G R	AAAGATACCTGCAGTACCCATCAGCTGATAACAAG	Reverse primer for mutagenesis of 1218 to G in GAL2
GAL2 1218A F	TGTTATCAGCTGATGGCTACTGCAGGTATCTTTTGG	Forward primer for mutagenesis of I218 to A in GAL2
GAL2 1218A R	AAAGATACCTGCAGTAGCCATCAGCTGATAACAAG	Reverse primer for mutagenesis of I218 to A in GAL2
GAL2 1218D F	TGTTATCAGCTGATGGACACTGCAGGTATCTTTTGG	Forward primer for mutagenesis of I218 to D in GAL2
GAL2 1218D R	AAAGATACCTGCAGTGTCCATCAGCTGATAACAAG	Reverse primer for mutagenesis of I218 to D in GAL2
GAL2 1218K F	TGTTATCAGCTGATGAAGACTGCAGGTATCTTTTGG	Forward primer for mutagenesis of I218 to K in GAL2
GAL2 1218K R	AAAGATACCTGCAGTCTTCATCAGCTGATAACAAG	Reverse primer for mutagenesis of I218 to K in GAL2
GAL2 1218F F	TGTTATCAGCTGATGTTCACTGCAGGTATCTTTTTGG	Forward primer for mutagenesis of I218 to F in GAL2
GAL2 I218F R	AAAGATACCTGCAGTGAACATCAGCTGATAACAAG	Reverse primer for mutagenesis of I218 to F in GAL2
GAL2 1222G F	ATGATTACTGCAGGTGGTTTTTTGGGCTACTGTAC	Forward primer for mutagenesis of 1222 to G in GAL2
GAL2 1222G R	ACAGTAGCCCAAAAAACCACCTGCAGTAATCATCAGC	Reverse primer for mutagenesis of I222 to G in GAL2
GAL2 1222A F	ATGATTACTGCAGGTGCTTTTTTGGGCTACTGTAC	Forward primer for mutagenesis of 1222 to A in GAL2
GAL2 1222A R	ACAGTAGCCCAAAAAAGCACCTGCAGTAATCATCAGC	Reverse primer for mutagenesis of I222 to A in GAL2
GAL2 1222D F	ATGATTACTGCAGGTGACTTTTTGGGCTACTGTAC	Forward primer for mutagenesis of I222 to D in GAL2
GAL2 1222D R	ACAGTAGCCCAAAAAGTCACCTGCAGTAATCATCAGC	Reverse primer for mutagenesis of I222 to D in GAL2
GAL2 1222K F	ATGATTACTGCAGGTAAGTTTTTTGGGCTACTGTAC	Forward primer for mutagenesis of I222 to K in GAL2
GAL2 1222K R	ACAGTAGCCCAAAAACTTACCTGCAGTAATCATCAGC	Reverse primer for mutagenesis of I222 to K in GAL2
GAL2 1222F F	ATGATTACTGCAGGTTTCTTTTTGGGCTACTGTAC	Forward primer for mutagenesis of I222 to F in GAL2
GAL2 1222F R	ACAGTAGCCCAAAAAGAAACCTGCAGTAATCATCAGC	Reverse primer for mutagenesis of I222 to F in GAL2
GAL2 12220 F	ATGATTACTGCAGGTCAATTTTTGGGCTACTGTAC	Forward primer for mutagenesis of I222 to O in GAL2
GAL2_12220 R	ACAGTAGCCCAAAAATTGACCTGCAGTAATCATCAGC	Reverse primer for mutagenesis of 1222 to O in GAL2
GAL2 Y446W F	TTTTATATTTTCTGTTGGGCCACAACCTGGGCGCC	Forward primer for mutagenesis of Y446 to W in GAL2
GAL2 Y446W R	CGCCCAGGTTGTGGCCCAACAGAAAATATAAAAACAGG	Reverse primer for mutagenesis of Y446 to W in GAL2
GAL2 F350A F	GGTAACAATTATTTTGCTTACTACGGTACCGTTATTTTCAAGTC	Forward primer for mutagenesis of F350 to A in GAL2
GAL2 F350A R	TAACGGTACCGTAGTAAGCAAAATAATTGTTACCGG	Reverse primer for mutagenesis of F350 to A in GAI 2
GAL2 F350D F	GGTAACAATTATTTTGACTACCGGTACCGTTATTTTCAAGTC	Forward primer for mutagenesis of F350 to D in GAL2
GAL2 F350D R	TAACGGTACCGTAGTAGTCAAAATAATTCTTACCCC	Reverse primer for mutagenesis of F350 to D in $GA12$
GAL2 F350G F	GGTAACAATTATTTTGGTTACTACGGTACCGTTATTTTCAAGTC	Forward primer for mutagenesis of F350 to G in $GAI2$
GAL2 F350G R	TAACCGTACCGTAGTAACCAAAATAATTCTTACCCCC	Reverse primer for mutagenesis of F350 to G in GAL2
	THEODIACCOINGIANTANIALIGIIACCOG	Reverse primer for mutagenesis of 1550 to d in GALZ

Table S4. Cont.

PNAS PNAS

Name	Sequence*	Description
GAL2_F350K_F	GGTAACAATTATTTT <u>AAG</u> TACTACGGTACCGTTATTTTCAAGTC	Forward primer for mutagenesis of F350 to K in GAL2
GAL2_F350K_R	TAACGGTACCGTAGTACTTAAAATAATTGTTACCGG	Reverse primer for mutagenesis of F350 to K in GAL2
GAL2_F350L_F	GGTAACAATTATTTTTTTGTACTACGGTACCGTTATTTTCAAGTC	Forward primer for mutagenesis of F350 to L in GAL2
GAL2_F350L_R	TAACGGTACCGTAGTACAAAAAAATAATTGTTACCGG	Reverse primer for mutagenesis of F350 to L in GAL2
GAL2_F350M_F	GGTAACAATTATTTTA <u>TG</u> TACTACGGTACCGTTATTTTCAAGTC	Forward primer for mutagenesis of F350 to M in GAL2
GAL2_F350M_R	TAACGGTACCGTAGTACATAAAATAATTGTTACCGG	Reverse primer for mutagenesis of F350 to M in GAL2
GAL2_F350W_F	GGTAACAATTATTTT <u>TGG</u> TACTACGGTACCGTTATTTTCAAGTC	Forward primer for mutagenesis of F350 to W in GAL2
GAL2_F350W_R	TAACGGTACCGTAGTACCAAAAATAATTGTTACCGG	Reverse primer for mutagenesis of F350 to W in GAL2
GAL2_F350Y_F	GGTAACAATTATTTT <u>TAC</u> TACTACGGTACCGTTATTTTCAAGTC	Forward primer for mutagenesis of F350 to Y in GAL2
GAL2_F350Y_R	TAACGGTACCGTAGTAGTAAAAATAATTGTTACCGG	Reverse primer for mutagenesis of F350 to Y in GAL2

*Sequences that are homologous to target sequences (for recombinatorial cloning or genomic deletion/integration) are printed in bold; the mutated codon in primers for site-directed mutagenesis is underlined.