

The High-Capacity Specific Fructose Facilitator ZrFfz1 Is Essential for the Fructophilic Behavior of *Zygosaccharomyces rouxii* CBS 732^T

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Zygosaccharomyces rouxii is a fructophilic yeast that consumes fructose preferably to glucose. This behavior seems to be related to sugar uptake. In this study, we constructed *Z. rouxii* single-, double-, and triple-deletion mutants in the UL4 strain background (a *ura3* strain derived from CBS 732^T) by deleting the genes encoding the specific fructose facilitator *Z. rouxii* Ffz1 (ZrFfz1), the fructose/glucose facilitator ZrFfz2, and/or the fructose symporter ZrFsy1. We analyzed the effects on the growth phenotype, on kinetic parameters of fructose and glucose uptake, and on sugar consumption profiles. No growth phenotype was observed on fructose or glucose upon deletion of *FFZ* genes. Deletion of *ZrFFZ1* drastically reduced fructose transport capacity, increased glucose transport capacity, and eliminated the fructophilic character, while deletion of *ZrFFZ2* had almost no effect. The strain in which both *FFZ* genes were deleted presented even higher consumption of glucose than strain *Zrffz1Δ*, probably due to a reduced repressing effect of fructose. This study confirms the molecular basis of the *Z. rouxii* fructophilic character, demonstrating that ZrFfz1 is essential for *Z. rouxii* fructophilic behavior. The gene is a good candidate to improve the fructose fermentation performance of industrial *Saccharomyces cerevisiae* strains.

The most problematic spoilage yeasts in the food and beverage industry belong to the genus *Zygosaccharomyces*. *Zygosaccharomyces bailii* and *Zygosaccharomyces rouxii* are the main spoilage yeasts of sugar syrups, fruit juices, honey, and sweet sauces, due to their unique properties, such as high resistance to weak-acid preservatives, extreme osmotolerance (some *Z. rouxii* strains are able to grow on 90% [wt/vol] glucose), ability to vigorously ferment hexoses (which can cause swelling or bursting of packaging, due to gas accumulation), ability to adapt to high temperatures, and growth at low pH (1, 2). *Z. rouxii* is also industrially used in the fermentation of some salted condiments, such as soy sauce and miso paste, and in the production of balsamic vinegar (3, 4).

Z. rouxii and *Z. bailii* are fructophilic yeasts, meaning that they consume fructose faster than glucose, a behavior opposite to that of the glucophilic *Saccharomyces cerevisiae*, which prefers glucose over fructose (5). In 1956, Sols (6) showed that phosphorylation of sugars was not involved in fructophily of a “sauternes yeast” (probably *Z. bailii* [7]), suggesting that another step preceding phosphorylation was the main regulator of the fructophilic behavior. This was further supported by later studies that indicated that hexokinase activities were similar in *Z. bailii* and *S. cerevisiae* and that sugar transport should be involved in *Z. bailii* fructophily (5).

The transport across the plasma membrane is the first step at which the utilization of nutrients by cells can be controlled and regulated in response to extracellular conditions and intracellular requirements. The majority of sugar transporters belong to the sugar porter (SP) family, the largest member of the major facilitator superfamily (MFS) (8). Most yeasts use sugar facilitators and sugar-proton symporters, the latter being energy-consuming mechanisms that operate only when relatively low concentrations of sugars are available in the extracellular environment (9).

Fructose uptake in *Z. bailii* was shown to be mediated by a specific facilitator system with high capacity and low affinity and by a low-capacity and high-affinity facilitator system that also transports glucose (10). Fructose transport is thus privileged, mainly at high sugar concentrations. Three mechanisms seem to

be responsible for this behavior: (i) fructose is transported by a specific transport system with high capacity, (ii) fructose competes with glucose for the hexose transport system, and (iii) high fructose concentrations inactivate the glucose facilitator system (10). Similar results were also found for *Z. rouxii* (11).

A peculiar type of sugar transporter was identified and characterized in *Z. bailii* ISA 1307. The *Z. bailii* Ffz1 (ZbFfz1) protein, when expressed in a *hxt*-null mutant of *S. cerevisiae*, behaved as a high-capacity and low-affinity (K_m , ~80 mM; V_{max} , ~3 mmol h⁻¹ g⁻¹) specific fructose facilitator with low protein sequence identity to the sugar porter family (12). Two similar proteins, *Z. rouxii* Ffz1 (ZrFfz1) (a fructose-specific facilitator; K_m , ~400 mM; V_{max} , ~13 mmol h⁻¹ g⁻¹) and ZrFfz2 (a fructose/glucose facilitator; K_m glucose; fructose, ~200 mM; V_{max} glucose; fructose, ~4 mmol h⁻¹ g⁻¹), were subsequently characterized in *Z. rouxii* CBS 732^T, revealing the existence of a new family of sugar transporters (13). The Ffz proteins have highly conserved sequence motifs distinct from those of the sugar porter family (9) and are phylogenetically closer to the drug/H⁺ antiporter 1 (DHA1) family. This new family also includes several putative proteins from *Aspergillus* species and from several fungal plant pathogens (13). Another Ffz protein, CmFfz1, was recently characterized as a specific fructose transporter (K_m , ~100 mM; V_{max} , ~9 mmol h⁻¹ g⁻¹) in the osmotolerant fructophilic yeast *Candida magnoliae* (14). Besides the ZbFfz1 gene, three other putative *FFZ*-like genes were annotated in the recently sequenced genome of *Z. bailii* ISA 1307 (15). In the

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TABLE 1 Strains used or constructed in this study

Strain	Genotype	Reference
<i>S. cerevisiae</i> BW31a	<i>MATa leu2-3/122 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2 mal10 ena1-4Δ::HIS3 nha1::LEU2</i>	26
<i>Z. rouxii</i>		
CBS 732 ^T	Wild type	CBS strain database
UL4	<i>ura3</i>	19
F1	<i>ura3 Zrffz1Δ::loxP</i>	This study
F2	<i>ura3 Zrffz2Δ::loxP</i>	This study
F1F2	<i>ura3 Zrffz1Δ::loxP Zrffz2Δ::loxP</i>	This study
S1	<i>ura3 Zrfsy1Δ::loxP</i>	17
F1F2S1	<i>ura3 Zrffz1Δ::loxP Zrffz2Δ::loxP Zrfsy1Δ::loxP</i>	This study
F1F2x	<i>ura3 Zrffz1Δ::loxP Zrffz2Δ::loxP</i>	This study
F1F2(pZEU)	F1F2 + pZEU; <i>Zrffz1Δ::loxP Zrffz2Δ::loxP</i>	This study
F1F2(pZEU_ZrFFZ1)	F1F2 + pZEU_ZrFFZ1; <i>Zrffz2Δ::loxP</i>	This study
F1F2(pZCA_ZrFFZ1)	F1F2 + pZCA_ZrFFZ1; <i>Zrffz2Δ::loxP</i>	This study
F1F2(pZEU_ZrFFZ2)	F1F2 + pZEU_ZrFFZ2; <i>Zrffz1Δ::loxP</i>	This study
F1F2(pZGFP_ZrFFZ2)	F1F2 + pZGFP_ZrFFZ2; <i>Zrffz1Δ::loxP</i>	This study
F1F2(pZCA_ZrFFZ2)	F1F2 + pZCA_ZrFFZ2; <i>Zrffz1Δ::loxP</i>	This study

genome of the type strain *Z. bailii* CLIB13 (CBS 680) (16), we also detected two putative FFZ-like genes.

A high-affinity fructose/H⁺ symporter (K_m , ~0.4 mM; V_{max} , ~0.6 mmol h⁻¹ g⁻¹), Fsy1, was also recently described in *Z. rouxii* CBS 732^T. The symporter was tightly regulated, being expressed in *Z. rouxii* only when the cells were grown in media with extremely low fructose concentrations (<0.2% [wt/vol]) or with nonfermentable carbon sources, such as mannitol and xylitol (17).

The fructophilic behavior of *Z. rouxii* has not yet been unequivocally associated with the expression of a specific gene(s). The detection of the unusual Ffz transporters in at least three fructophilic yeasts points to a possible correlation between their presence and the fructophilic behavior of these yeasts.

In this paper, we analyze the effect of deletion of the genes encoding the atypical Ffz fructose transporters (*ZrFFZ1* and *ZrFFZ2*) on the fructophilic character of *Z. rouxii* CBS 732^T and demonstrate the importance of the expression of the specific fructose facilitator *ZrFfz1* in this behavior.

MATERIALS AND METHODS

Strains and growth media. The strains used or constructed in this study are listed in Table 1. *S. cerevisiae* strain BW31a was used for the construction of plasmids by homologous recombination. Yeast strains were grown in minimal YNB (yeast nitrogen base without amino acids containing the indicated carbon source and the required supplements) or rich YPD (1% yeast extract, 2% peptone, 2% dextrose) medium. Cultures were incubated at 30°C with orbital shaking. Sugar concentrations are given as percentages (weight/volume). *Escherichia coli* XL1-Blue (Stratagene) was used as the host for plasmid amplification. *E. coli* transformants were grown in standard Luria-Bertani medium supplemented with ampicillin (100 μg ml⁻¹).

Yeast transformations and construction of *Z. rouxii* deletion strains. *Z. rouxii* and *S. cerevisiae* cells were transformed by electroporation, as described in reference 18. The deletion of the *Z. rouxii* *ZrFFZ1* (ZYRO0E10054g; GenBank accession no. XM_002499318, GeneID 8204919) and *ZrFFZ2* (ZYRO0F02090g; GenBank accession no. XM_002497242, GeneID 8205043) genes in strain UL4 (a *ura3* auxotrophic strain derived from CBS 732^T) (19) was performed with PCR-amplified *loxP-kanMX-loxP* deletion cassettes (20). The primers (obtained from Sigma-Aldrich) used for the cassette amplifications are listed in Table S1 in the supplemental material (*ZrFFZ1*-Kan-F and *ZrFFZ1*-Kan-R for

ZrFFZ1; *ZrFFZ2*-Kan-F2 and *ZrFFZ2*-Kan-R2 for *ZrFFZ2*), and the pUG6 plasmid (21) was used as a template. The pZCRE plasmid expressing the Cre recombinase was used to remove the integrated *kanMX* marker (20).

The replacement of the original genes with the *loxP* sequence was confirmed by diagnostic PCR with the following combinations of primers (see Table S1 in the supplemental material): (i) for deletion of *ZrFFZ1*, *ZrFFZ1*-361up-F/KANX-R1, KANX-F1/*ZrFFZ1*-456d-R2, and *ZrFFZ1*-361up-F/*ZrFFZ1*-456d-R2; (ii) for deletion of *ZrFFZ2*, *ZrFFZ2*-521up-F2/KANX-R1, KANX-F1/*ZrFFZ2*-492d-R2, and *ZrFFZ2*-521up-F2/*ZrFFZ2*-492d-R2. The generated *Z. rouxii* deletion strains are listed in Table 1. Strain F1F2 was obtained by deletion of the *ZrFFZ1* gene in strain F2 (*Zrffz2Δ*). Strain F1F2x was obtained by deletion of the *ZrFFZ2* gene in strain F1 (*Zrffz1Δ*). Deletion of the *ZrFSY1* gene, as described in reference 17, was performed in strain F1F2, producing the triple-deletion mutant F1F2S1.

Growth assays. Yeast cells were grown at 30°C, and growth was monitored either in drop tests in solid media or in liquid media using an ELx808 Absorbance Microplate Reader (BioTek Instruments, Winooski, VT, USA) as described in reference 22 or by measuring optical densities at 640 nm (OD₆₄₀) in an Ultrospec 2100 pro (Amersham Biosciences) spectrophotometer.

DNA manipulations. DNA manipulations were performed according to standard protocols (23). Genomic DNA and plasmid DNA from yeast cells were isolated as described in reference 24. The high-fidelity DNA polymerase Phusion F-530 (Finnzymes) was used to avoid mismatch base pairing during the synthesis of PCR products. Plasmid DNA from *E. coli* was isolated using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich). Restriction enzymes were purchased from Roche. Sequencing was performed at Stab Vida (Caparica, Portugal).

Plasmid and *Z. rouxii* strain construction. The plasmids used for cloning were pZEU, pZGFP (20), and pZCA (18).

Plasmids containing the *ZrFFZ1* and *ZrFFZ2* genes with the respective promoters (828 and 869 bp long, respectively) were constructed by homologous recombination (25) in *S. cerevisiae* BW31a (26). *ZrFFZ2* (amplified by PCR with primers *ZrFFZ2*-ZEU-F and *ZrFFZ2*-ZEU-R) was inserted into the plasmid pZEU (previously digested with XbaI), resulting in the plasmid pZEU_ZrFFZ2. The same gene was also inserted into the plasmids pZGFP (previously digested with XbaI) and pZCA (previously digested with XbaI) (see Table S2 in the supplemental material). The same procedure was used to clone *ZrFFZ1* genes into plasmids pZEU and pZCA (see Table S2 in the supplemental material). The primers used are listed in Table S1 in the supplemental material. All the constructed plasmids and

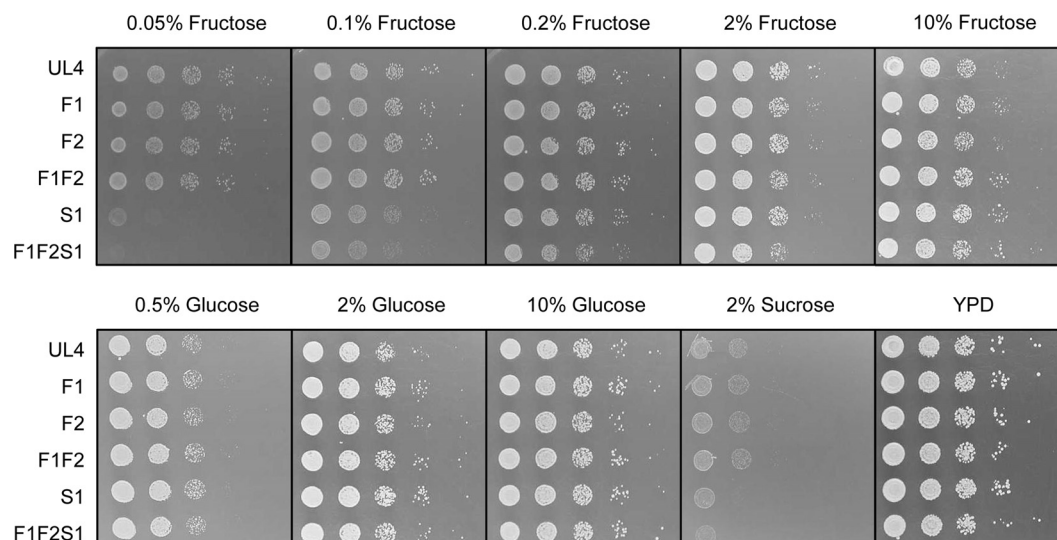


FIG 1 Growth of *Z. rouxii* strains UL4, F1 (*Zrffz1*Δ), F2 (*Zrffz2*Δ), F1F2 (*Zrffz1*Δ *Zrffz2*Δ), S1 (*Zrfsy1*Δ), and F1F2S1 (*Zrffz1*Δ *Zrffz2*Δ *Zrfsy1*Δ) on YPD and YNB media with different carbon sources after 3 days (fructose and glucose media) or 8 days (sucrose medium) of incubation at 30°C. The results from one of two independent experiments are shown.

the control plasmid pZEU were used to transform the *Z. rouxii* deletion strain F1F2.

Fluorescence microscopy. Cells grown to exponential phase were spotted onto microscope slides and observed with an Olympus AX70 fluorescence microscope. To visualize green fluorescent protein (GFP), a U-MWB fluorescence cube (Olympus) was used with a 450- to 480-nm excitation filter and a 515-nm barrier filter.

Sugar transport assays. Initial [U - ^{14}C]fructose and [U - ^{14}C]glucose (GE Healthcare [formerly Amersham Biosciences]) uptake rates were performed as previously described (13). Kinetic parameters were estimated using GraphPad Prism version 5.00 (Graphpad Software) for Michaelis-Menten regression analysis. The existence of H^+ movements associated with initial fructose uptake was assessed with a pH meter upon addition of fructose pulses to unbuffered cell suspensions, as described in reference 17. The dry weight was determined (in triplicate) by placing 100 μ l of cell suspensions into preweighed aluminum foil cups and drying in a 70°C oven for 24 h.

Sugar consumption assays. Strains were pregrown overnight in YNB plus 10 g liter $^{-1}$ fructose plus 10 g liter $^{-1}$ glucose (with supplements when necessary) or in YP (1% yeast extract, 2% peptone) plus 100 g liter $^{-1}$ fructose plus 100 g liter $^{-1}$ glucose, transferred to the same medium to an initial OD_{640} of 0.1 (in Erlenmeyer flasks with a medium/flask volume ratio of 1:5), and incubated at 25°C and 180 rpm. Growth was followed by measuring optical densities at 640 nm in an Ultrospec 2100 pro spectrophotometer (Amersham Biosciences). To monitor the evolution of sugar consumption, after removing the cells by filtration with a 0.2- μ m cellulose acetate filter (Advantec), glucose and fructose concentrations in supernatants, collected at several time points, were measured by enzymatic assays (kit no. 10 139 106 035; Boehringer Mannheim/Roche/r-biopharm).

Reproducibility of results. All experiments were performed at least three times. Mean values and corresponding standard deviations or results of typical experiments are presented, as indicated.

RESULTS

Construction and characterization of *Z. rouxii* FFZ deletion mutants. We recently characterized the fructose-specific facilitator ZrFfz1, the fructose/glucose facilitator ZrFfz2 (13), and the fructose/ H^+ symporter ZrFsy1 (17) from *Z. rouxii* CBS 732 T by heterologous expression in an *S. cerevisiae* strain lacking its own

hexose transporters (*hxt* null). *Z. rouxii* strain UL4 is a *ura3* auxotrophic strain derived from CBS 732 T (19). Strain UL4 was used as background for deletion of the genes encoding these three *Z. rouxii* fructose transporters. The constructed deletion mutants, F1 (*Zrffz1*Δ), F2 (*Zrffz2*Δ), F1F2 (*Zrffz1*Δ *Zrffz2*Δ), F1F2x (*Zrffz1*Δ *Zrffz2*Δ), and F1F2S1 (*Zrffz1*Δ *Zrffz2*Δ *Zrfsy1*Δ), were confirmed by PCR. Strain F1F2 was obtained by deletion of the *ZrFFZ1* gene in strain F2 (*Zrffz2*Δ). We also constructed strain F1F2x by deleting the *ZrFFZ2* gene in strain F1 (*Zrffz1*Δ). An extremely low targeting efficiency (sometimes as low as 0.5%) was observed, as previously reported for *Z. rouxii* UL4 (20).

The growth phenotype of single-, double-, and triple-deletion mutants was tested on solid (Fig. 1) and in liquid (Fig. 2) YNB media supplemented with fructose (0.05% [wt/vol] to 20% [wt/vol]), glucose (0.1% [wt/vol] to 20% [wt/vol]), or sucrose (0.5% [wt/vol] and 2% [wt/vol]).

Strains F1F2 (Fig. 1 and 2) and F1F2x (data not shown), both lacking the facilitators ZrFfz1 and ZrFfz2, displayed the same growth phenotype. For the single-, the double-, or even the triple-deletion mutants, there was not a visible difference in growth on solid (Fig. 1 and data not shown) or liquid (Fig. 2 and data not shown) medium for fructose concentrations above 0.2% or for any of the tested glucose concentrations. This means that under these growth conditions, other hexose transporters must be efficiently operating in *Z. rouxii*, compensating for the deletion of the three fructose transporters characterized so far. At fructose concentrations below 0.2%, the effect observed is probably due to the deletion of the fructose/ H^+ symporter ZrFsy1, which appears to be the main active fructose transporter at these extremely low concentrations, as previously observed for strain S1 (*Zrfsy1*Δ) (17).

Nevertheless, kinetic characterization of fructose and glucose transport in the deletion mutants presented some differences from strains CBS 732 T and UL4 (Table 2). *Z. rouxii* CBS 732 T presented a higher transport capacity for fructose than for glucose, in agreement with its fructophilic behavior. The *ura3* auxotrophic

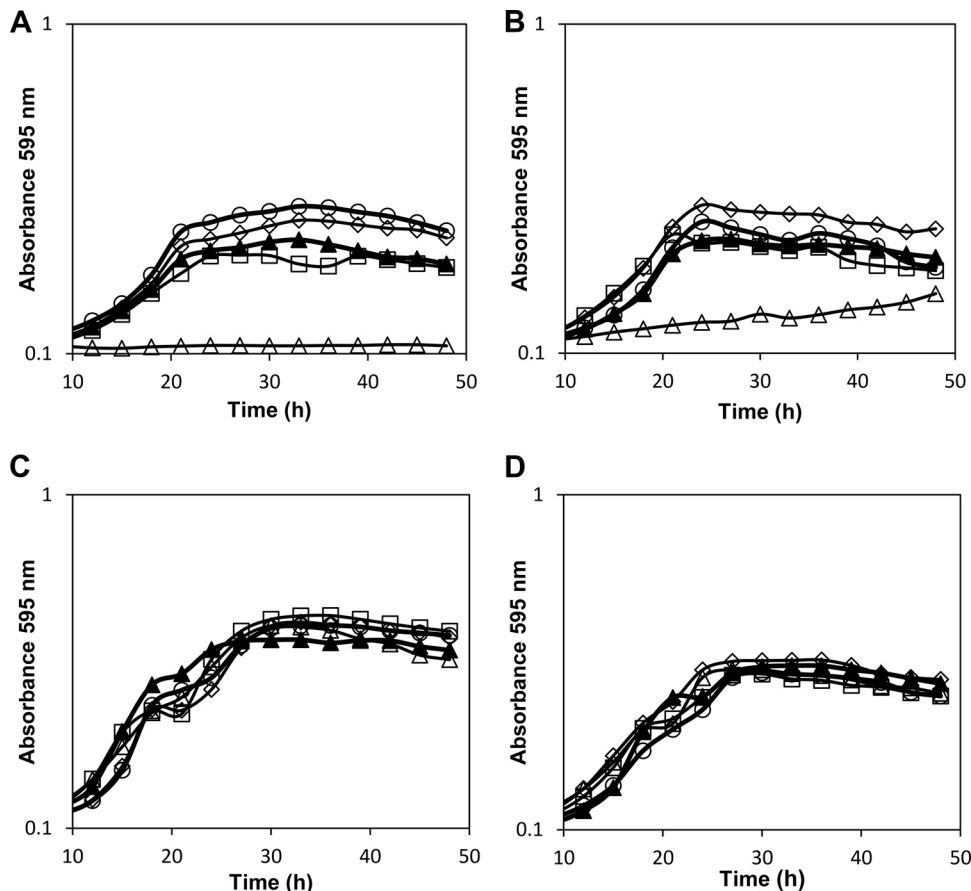


FIG 2 Growth curves (in microplates) of *Z. rouxii* strains UL4 (▲), F1 (*Zrffz1*Δ) (◇), F2 (*Zrffz2*Δ) (□), F1F2 (*Zrffz1*Δ *Zrffz2*Δ) (○), and F1F2S1 (*Zrffz1*Δ *Zrffz2*Δ *Zrfsy1*Δ) (△) in YNB medium supplemented with 0.05% (A), 0.1% (B), and 2% (C) fructose or 2% glucose (D). The data are representative of at least two independent experiments.

strain UL4 (derived from CBS 732^T) showed lower transport activities (a reduction of 36% for fructose and 48% for glucose V_{\max} values) than the *Z. rouxii* CBS 732^T strain (Table 2; see Fig. S1 in the supplemental material). The differences between the two strains were also visible in the respective growth patterns on fructose or glucose, as *Z. rouxii* CBS 732^T presented a shorter lag phase than UL4, slightly higher growth rates ($0.114 \pm 0.002 \text{ h}^{-1}$ versus $0.096 \pm 0.004 \text{ h}^{-1}$ in 2% fructose and $0.093 \pm 0.001 \text{ h}^{-1}$ versus $0.085 \pm 0.003 \text{ h}^{-1}$ in 2% glucose media) and higher final biomass (see Fig. S2 in the supplemental material).

When *ZrFFZ1* was deleted in *Z. rouxii* strain UL4, there was a strong reduction in fructose transport capacity ($V_{\max \text{ fructose}}$ was

reduced 73%), whereas deletion of *ZrFFZ2* had almost no effect ($V_{\max \text{ fructose}}$ was reduced only 3%). The double-deletion strain F1F2 exhibited fructose transport kinetic parameters very similar to those of strain F1 (*Zrffz1*Δ) (Table 2; see Fig. S1 in the supplemental material), which further supports the fact that *ZrFfz1* seems to be the main transporter system responsible for the high fructose transport capacity of *Z. rouxii*, as previously suggested by the kinetic parameters of the individual transporters *ZrFfz1* and *ZrFfz2* when expressed in a *hxt*-null *S. cerevisiae* strain ($V_{\max \text{ fructose } ZrFfz1}$, $12.7 \pm 3.3 \text{ mmol h}^{-1} \text{ g}^{-1}$ versus $V_{\max \text{ fructose } ZrFfz2}$, $4.51 \pm 0.56 \text{ mmol h}^{-1} \text{ g}^{-1}$) (13).

Deletion of the specific fructose facilitator *ZrFFZ1* also reduced K_m values (Table 2), increasing the overall affinity for

TABLE 2 Kinetic parameters of fructose and glucose transport in *Z. rouxii* strains grown in 2% glucose

Strain	Fructose transport ^b		Glucose transport ^b	
	K_m (mM)	V_{\max} (mmol g ⁻¹ h ⁻¹)	K_m (mM)	V_{\max} (mmol g ⁻¹ h ⁻¹)
CBS 732 ^{Ta}	284.5 ± 62.8	17.48 ± 2.45	14.28 ± 1.94	1.72 ± 0.04
UL4	239.2 ± 97.0	11.11 ± 2.45	19.76 ± 1.28	0.89 ± 0.02
F1 (<i>Zrffz1</i> Δ)	138.5 ± 31.7	2.95 ± 0.30	25.88 ± 2.72	1.34 ± 0.06
F2 (<i>Zrffz2</i> Δ)	339.6 ± 101.9	10.79 ± 1.94	13.28 ± 1.90	0.96 ± 0.04
F1F2 (<i>Zrffz1</i> Δ <i>Zrffz2</i> Δ)	97.30 ± 34.71	2.71 ± 0.45	12.10 ± 6.54	1.27 ± 0.16

^a Reference 11.

^b The values result from GraphPad Prism Michaelis-Menten regression analysis of data from at least two independent experiments. V_{\max} values were calculated for biomass expressed as dry weight.

fructose, in agreement with the very low affinity of the transporter ($K_{m\text{fructose ZrFfz1}}$, 424.2 ± 163.1 mM, when expressed in a *hxt*-null *S. cerevisiae* strain).

Regarding glucose transport, when the specific fructose facilitator *ZrFFZ1* was deleted, there was an increase (about 51%) in the glucose transport capacity (Table 2; see Fig. S1 in the supplemental material, strains F1 and F1F2), reaching a glucose transport capacity closer to that of the CBS 732^T strain. Deletion of the high-capacity, low-affinity fructose/glucose transporter *ZrFFZ2* did not significantly affect the glucose transport capacity (V_{max} increased only 8%) and slightly increased the overall affinity for glucose.

Fructophilic behavior of constructed strains. The *Z. rouxii* genes *ZrFFZ1* and *ZrFFZ2* were cloned into the *Z. rouxii* plasmids pZEU (multicopy), pZGFP (multicopy with C-terminal GFP tagging; only for *ZrFFZ2*), and pZCA (centromeric) by homologous recombination in *S. cerevisiae* BW31a and by plasmid rescue in *E. coli* XL-Blue. The constructed plasmids were confirmed by restriction analysis, PCR, and sequencing. Cloning of the constructed plasmids into the *Z. rouxii* deletion strain F1F2 produced the strains listed in Table 1.

Z. rouxii fructophilic behavior is characterized by a preference for consumption of fructose over glucose, more pronounced at high sugar concentrations. To correlate this behavior with the presence of the peculiar Ffz transporters, we analyzed sugar consumption in minimal and in rich media containing equimolar concentrations of glucose and fructose. Initially, we used minimal synthetic medium in order to define and control its sugar content (10 g liter⁻¹ fructose plus 10 g liter⁻¹ glucose) and then repeated the experiments in YP medium with high sugar concentrations (100 g liter⁻¹ fructose plus 100 g liter⁻¹ glucose). For these high sugar concentrations, we used YP instead of YNB medium to prevent the lack of some other nutrient possibly affecting sugar utilization. The results obtained for strains CBS 732^T and UL4 (*ura3*) (Fig. 3; see Fig. S3 in the supplemental material) show that in both media, fructose was consumed faster than glucose, with glucose consumption being much slower in strain UL4, especially in medium with a low sugar concentration. The phenotypic differences between strains CBS 732^T and UL4 (high transport capacity and higher growth rates of the former) are further evidenced by sugar consumption profiles (Fig. 3; see Fig. S3 in the supplemental material), as UL4 left about 80% residual glucose, even after 140 h of incubation, in medium with 10 g liter⁻¹ fructose plus 10 g liter⁻¹ glucose and about 30% residual glucose in medium with 100 g liter⁻¹ fructose plus 100 g liter⁻¹ glucose, whereas strain CBS 732^T was able to consume almost all the glucose available in both media (Fig. 3; see Fig. S3 in the supplemental material).

When the *ZrFFZ1* gene (strain F1) was deleted, both sugars started to be consumed at the same rate, and the characteristic *Z. rouxii* fructophilic character was lost. Deletion of *ZrFFZ2* (a fructose/glucose facilitator) did not affect the fructophilic behavior of *Z. rouxii* (Fig. 3; see Fig. S3 in the supplemental material). The double-deletion mutant F1F2 (as well as F1F2x [data not shown]) and the triple-deletion mutant F1F2S1 presented clear glucophilic behavior, consuming glucose faster than fructose (Fig. 3; see Fig. S3 in the supplemental material). These results reinforce the hypothesis that *ZrFFZ1* is the main factor responsible for *Z. rouxii* fructophilic behavior.

The *ZrFFZ1* and *ZrFFZ2* genes were individually cloned back into the double mutant F1F2 (*Zrffz1*Δ *Zrffz2*Δ). The resulting

strains, expressing *ZrFFZ1* from a multicopy (pZEU) or centromeric (pZCA) plasmid, are equivalent to strain F2 (*Zrffz2*Δ), lacking only the fructose/glucose facilitator *ZrFfz2*, and both strains recovered the original *Z. rouxii* fructophilic behavior at both high (Fig. 4) and low (see Fig. S4 in the supplemental material) sugar concentrations.

On the other hand, when the gene *ZrFFZ2* expressed from a multicopy (with or without GFP) or centromeric plasmid was cloned back into the double mutant F1F2, generating strains equivalent to strain F1 (*Zrffz1*Δ), lacking only the high-capacity fructose facilitator *ZrFfz1*, both sugars were consumed simultaneously (Fig. 4; see Fig. S4 in the supplemental material) and faster than in the F1F2(pZEU) strain.

These results strengthen the direct involvement of *ZrFFZ1* in *Z. rouxii* CBS 732^T fructophilic behavior.

DISCUSSION

In wine fermentations, grape must usually contains similar amounts of glucose and fructose that are cofermented to ethanol and carbon dioxide. Due to climate changes, sugar levels in grapes have been increasing, which leads to wines with high alcohol content, lower acidity, and compromised flavor profiles (27). Due to the predilection of *S. cerevisiae* Hxt transporters for glucose, this sugar is consumed faster, producing a discrepancy between the amounts of glucose and fructose consumed during fermentation, and consequently, the concentration of residual fructose is higher than that of glucose at the end of fermentation. As fructose is almost twice as sweet as glucose, this has a strong effect on the final sweetness of wine. The discrepancy between the utilization of glucose and that of fructose depends on the yeast strain used and on the external conditions, such as nitrogen and ethanol levels (28). During fermentation of grape must, arrest of yeast growth generally occurs when the medium still contains high sugar concentrations, especially the nonpreferred sugar fructose, and may lead to sluggish or stuck (i.e., very delayed or incomplete) fermentations. The fermentation rate decreases due to loss of hexose transport capacity, and the factors related to stuck fermentations also regulate transporter expression, turnover, and function (29). The inhibition of fermentative metabolism that occurs under these conditions could be due to several mechanisms, such as sugar transport inefficiency, intracellular accumulation of ethanol, and/or toxicity of some yeast fermentation by-products. In nitrogen-limited stuck fermentations, glucose transport inactivation seems to play an important role (30).

In *S. cerevisiae*, ethanol seems to increase the glucophilic character, since addition of ethanol has a stronger inhibitory effect on fructose utilization than on glucose utilization, whereas addition of assimilable nitrogen has the opposite effect, stimulating fructose utilization more than glucose utilization (28). In the glucophilic yeast *S. cerevisiae*, glucose not only is an energy and carbon source, it also has an important role as a global regulator of metabolism and growth. In the fructophilic yeasts *Z. bailii* and *Z. rouxii*, this critical regulatory role seems to be played by fructose, as fructose regulates and inactivates the glucose transporter system, especially at high fructose concentrations, since glucose transport is completely abolished subsequent to addition of 10% fructose to 2% glucose-grown cells after 4 h of incubation in *Z. bailii* (10) and in *Z. rouxii* (11).

The growth phenotype in fructose (concentrations above 0.2% [wt/vol]) or in glucose medium of the constructed *Z. rouxii* triple-

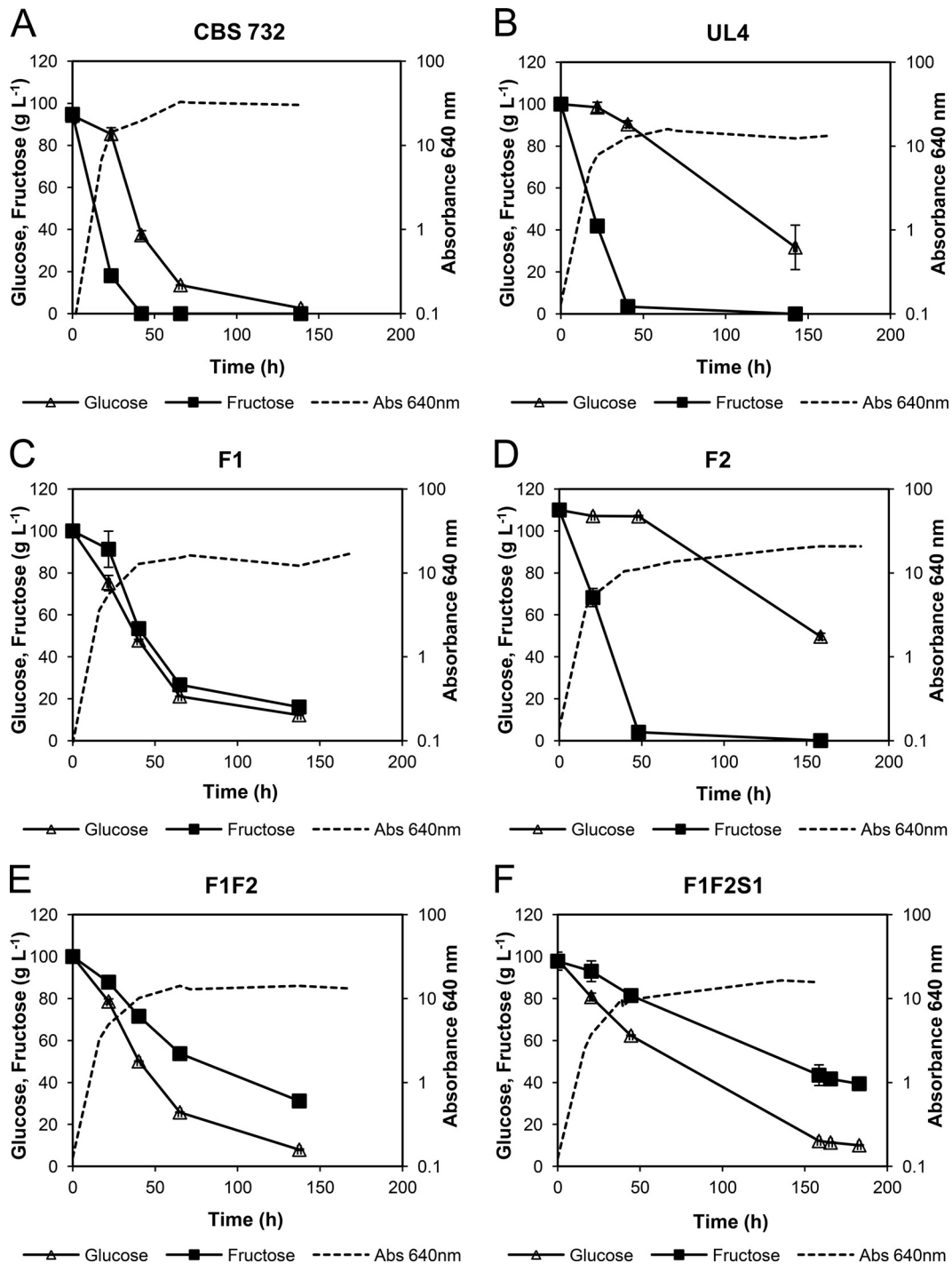


FIG 3 Fructose and glucose consumption profiles of *Z. rouxii* strains CBS 732^T (A), UL4 (B), F1 (*Zrffz1*Δ) (C), F2 (*Zrffz2*Δ) (D), F1F2 (*Zrffz1*Δ *Zrffz2*Δ) (E), and F1F2S1 (*Zrffz1*Δ *Zrffz2*Δ *Zrfsy1*Δ) (F) grown in YP plus 100 g liter⁻¹ fructose plus 100 g liter⁻¹ glucose. The data are representative of at least two independent experiments, and the error bars indicate standard deviations (SD).

deletion mutant (lacking the facilitators ZrFfz1 and ZrFfz2 and the symporter ZrFsy1) was not affected, indicating that other hexose transporters were still efficiently working in this strain, enabling growth on those carbon sources. A BLASTP analysis of the *Z. rouxii* CBS 732^T genome against known sugar transporters identified four putative hexose transporters similar to *S. cerevisiae*

Hxt proteins and one putative transporter similar to the *Kluyveromyces lactis* glucose/fructose/galactose transporter Hgt1 (17, 31).

When the gene encoding the specific fructose facilitator ZrFfz1 was deleted in *Z. rouxii*, fructose transport capacity was drastically reduced, in agreement with the very high fructose transport capacity of the transporter, while an increase in glucose transport

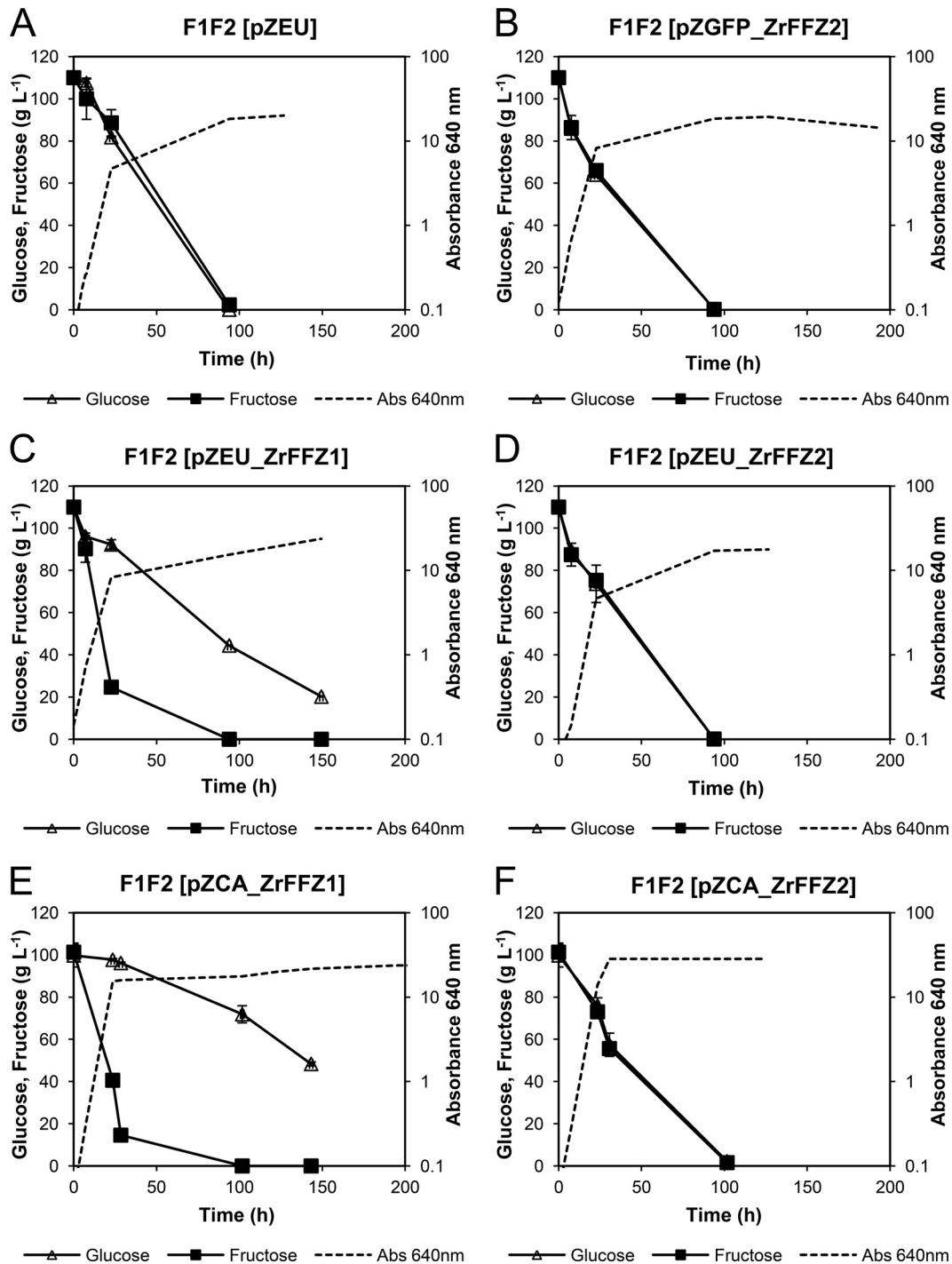


FIG 4 Fructose and glucose consumption profiles of *Z. rouxii* strains F1F2(pZEU) (A), F1F2(pZGFP_ZrFFZ2) (B), F1F2(pZEU_ZrFFZ1) (C), F1F2(pZEU_ZrFFZ2) (D), F1F2(pZCA_ZrFFZ1) (E), and F1F2(pZCA_ZrFFZ2) (F) grown in YP plus 100 g liter⁻¹ fructose plus 100 g liter⁻¹ glucose. The data are representative of at least two independent experiments, and the error bars represent SD.

capacity was observed ($V_{\max \text{ glucose}}$ increased about 51% in strain F1 compared to strain UL4 [Table 2]). This increase may result from derepression of glucose transport, since fructose influx is drastically reduced when *ZrFFZ1* is deleted. This effect is even more obvious in sugar consumption profiles of strain F1F2, lacking both Ffz facilitators, which has a lower fructose influx than

strain F1 and, consequently, a reduced putative repressing effect by fructose, displaying higher glucose consumption (Fig. 3; see Fig. S3 in the supplemental material). These results are indicative of a regulatory role of fructose in *Z. rouxii*.

Since it was reported that the high capacity of fructose transport is directly linked to the fructophilic character of *Z. bailii* (10)

and of *Z. rouxii* (11) and our results demonstrate that ZrFfz1 is responsible for the *Z. rouxii* fructophilic behavior, we advance the hypothesis that the presence of Ffz-like transporters can be an indicator of fructophily in other yeast species.

In *C. magnoliae*, also a fructophilic yeast, glucose is not consumed in the presence of fructose (32), and a comparative proteomic analysis of fructose-grown cells with glucose-grown cells revealed overexpression of proteins involved in stress response, in carbon metabolism, and in sugar uptake in fructose-grown cells, which further supports the crucial role of fructose uptake in the fructophilic behavior (33). This yeast also has a functional Ffz1 fructose transporter (CmFfz1) (14).

The fact that a wine strain with an altered Hxt3 hexose transporter has an enhanced fructose fermentation capacity, due to changes in fructose uptake (34), also supports the fundamental role of fructose uptake in fructophilic behavior.

To our knowledge, this is the first report that clearly demonstrates for a *Zygosaccharomyces* strain a striking change from an unambiguous fructophilic behavior to a glucophilic behavior due only to the deletion of one high-capacity specific fructose transporter gene, with complete reversal to the initial behavior when the gene is cloned back. This study confirms the molecular basis of the *Z. rouxii* fructophilic character, demonstrating the key role of ZrFfz1 in determining the fructose/glucose utilization preference during *Z. rouxii* fermentations.

Expression of this fructose transporter in industrial *S. cerevisiae* strains might improve the fructose utilization and fermentation performance of wine strains, accelerating the consumption of all sugars available and consequently reducing the risks of occurrence of stuck fermentations.

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