A new chromosomal rearrangement improves the adaptation of wine yeasts to sulfite

Estéfani García-Ríos[1], Marcos Nuévalos[1], Eladio Barrio[1,2], Sergi Puig[1*] and José M. Guillamón[1]

[1]Departamento de Biotecnología de los Alimentos, Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC), Agustín Escardino 7, E-46980 Paterna, Valencia, Spain.

[2]Departament de Genètica, Universitat de València, Doctor Moliner 50, E-46100 Burjassot, Valencia, Spain.

***Corresponding author**: Sergi Puig

Email address: spuig@iata.csic.es

Running title: New sulfite-resistant yeast genome reorganization

ORIGINALITY-SIGNIFICANCE STATEMENT

Microorganisms utilize a great variety of genetic strategies to adapt to natural and human-made environments. Among them, genome-wide rearrangements constitute a mechanism to rapidly evolve by generating phenotypic diversity. Sulfite is widely used during winemaking because of its antimicrobial and antioxidant properties. Therefore, wine yeast strains have developed specific genetic changes to increase its resistance to sulfite. In this study, we have identified and characterized a novel chromosomal rearrangement that increases the sulfite resistance of *Saccharomyces cerevisiae* wine strains. Specifically, an inversion in chromosome XVI that involves the promoter of the cell surface sulfite efflux pump *SSU1* increases its expression and the strain sulfite tolerance. These data uncover a new genomic event that confers an evolutive advantage to wine yeast strains.

ABSTRACT

Sulfite-generating compounds are widely used during winemaking as preservatives because of its antimicrobial and antioxidant properties. Thus, wine yeast strains have developed different genetic strategies to increase its sulfite resistance. The most efficient sulfite detoxification mechanism in *Saccharomyces cerevisiae* uses a plasma membrane protein called Ssu1 to efflux sulfite. In wine yeast strains, two chromosomal translocations (VIIItXVI and XVtXVI) involving the *SSU1* promoter region have been shown to up-regulate *SSU1* expression and, as a result, increase sulfite tolerance. In this study, we have identified a novel chromosomal rearrangement that triggers wine yeast sulfite adaptation. An inversion in chromosome XVI (inv-XVI) probably due to sequence microhomology, which involves *SSU1* and *GCR1* regulatory regions, increases the expression of *SSU1* and the sulfite resistance of a commercial wine yeast strain. A detailed dissection of this chimeric *SSU1* promoter indicates that both the removed *SSU1* promoter sequence and the relocated *GCR1* sequence contribute to *SSU1* up-regulation and sulfite tolerance. However, no relevant function has been attributed to the *SSU1*-promoter binding transcription factor Fzf1. These results unveil a new genomic event that confers an evolutive advantage to wine yeast strains.

Keywords: Chromosomal rearrangement, inversion, *Saccharomyces cerevisiae*, *SSU1*, sulfite resistance, wine yeast.

INTRODUCTION

Wine fermentation is a complex ecological and biochemical process that involves the sequential growth of different microorganisms, mainly yeasts, but also filamentous fungi, lactic and acetic acid bacteria (Bauer and Pretorius 2000; Beltrán et al. 2002). However, the budding yeast *Saccharomyces cerevisiae* replaces other species found in grape musts because of its higher tolerance to ethanol and the production of heat via fermentation (Fleet, 2003; Goddard, 2008; Salvadó *et al.*, 2011). The use of this yeast in winemaking dates back to about 7400 years in the Cradle of Civilization, extending nowadays to all the temperate regions of the world (Bauer and Pretorius, 2000). Therefore, the wine strains of *S. cerevisiae* are highly specialized microorganisms that have evolved to use the different ecological niches provided by human activity. The specific genetic characteristics of the wine yeast strains are a consequence of the process of domestication (Liti *et al.*, 2009; Almeida *et al.*, 2015; Borneman *et al.*, 2016; Gayevskiy *et al.*, 2016; Gonçalves *et al.*, 2016; Legras *et al.*, 2018).

The study of patterns of genetic variation, population structure, and phenotypic diversity among strains of *S. cerevisiae* from different fermentative environments shows that the ecological specialization associated with human activity is accompanied by the accumulation of a great variety of potentially adaptive genetic changes (Legras *et al.*, 2018; Peter *et al.*, 2018). This rapid evolution is due to the ability of yeast to reshape its genome through expansion and contraction of gene families (Casadevall, 2008; Brown *et al.*, 2010; Lin and Li, 2011), duplication of entire genomes (Marcet-Houben *et al.*, 2015), acquisition of new genes through introgression (Neafsey *et al.*, 2010; Strope *et al.*, 2015; Barbosa *et al.*, 2016) or horizontal gene transfer (Novo *et al.*, 2009; Cheeseman *et al.*, 2014; Wisecaver and Rokas, 2015; Alexander *et al.*, 2016), and

directional selection (Hu *et al.*, 2014). Chromosomal rearrangements also underlie adaptation by affecting the expression of genes located in the proximity of the translocation breakpoints (Pérez-Ortín *et al.*, 2002; Zimmer *et al.*, 2014). All these genetic events promote a quicker adaptation to environmental changes than spontaneous mutations, which occur at comparatively lower rates (Doniger *et al.*, 2008).

Sulfite (SO_3^{2-}), which is produced by dissolution of sulfur dioxide (SO_2) in water, is used during winemaking as a microbial inhibitor and antioxidant (Bauer and Pretorius, 2000). Therefore, sulfite resistance is a desired trait for wine yeast strains (Divol *et al.*, 2012). The most common mechanisms to cope with the stress produced by sulfites include the increase in the production of acetaldehyde, which binds to SO_3^{2-}, the regulation of the sulfite uptake pathway, and sulfite efflux through a plasma membrane pump encoded by the *SSU1* gene (Casalone *et al.*, 1992). The latter is one of the most effective mechanisms of sulfite resistance in *S. cerevisiae* (Avram and Bakalinsky, 1997; Avram *et al.*, 1999). Strains lacking *SSU1* gene are more sensitive to sulfite than their wild-type counterparts, since *SSU1* deletion increases the accumulation of intracellular sulfite (Avram and Bakalinsky, 1997; Nadai *et al.*, 2016). Another gene involved in yeast sulfite resistance is the transcription factor *FZF1* (Casalone *et al.*, 1992, 1994; Avram *et al.*, 1999; Engle and Fay, 2012). Fzf1 interacts with two regions of DNA (CS1 and CS2) immediately upstream of *SSU1* and activates its transcription (Avram et al. 1999; Saver et al., 2005).

Among wine yeast strains, two chromosomal translocations (VIIItXVI and XVtXVI) have been shown to provide up-regulation of *SSU1* expression and increased sulfite tolerance (Pérez-Ortín *et al.*, 2002; Yuasa *et al.*, 2005; Zimmer *et al.*, 2014). In both cases, the translocation involves the *SSU1* promoter and leads to its transcriptional activation. A recent study showed that the *SSU1* translocations provide both ecological

divergence between wine and non-wine yeasts, due to sulfite resistance differences, and reproductive isolation by reducing spore viability in hybrids (Hyma and Fay, 2012; Hou et al. 2014; Clowers et al. 2015). In this work, we have identified an inversion in chromosome XVI that increases the expression of *SSU1* and the sulfite resistance of an industrial wine yeast strain. The aim of this work consists in the molecular and phenotypical characterization of this novel chromosomal rearrangement involving the yeast *SSU1* locus.

RESULTS

Identification of a sulfite-resistant wine yeast strain lacking VIIItXVI and XVtXVI chromosomal rearrangements

During the process of phenotypic characterization of the commercial wine yeast strain P5 (García-Ríos *et al.*, 2014, 2017), we decided to determine its sulfite resistance. For this purpose, we tested growth and viability by performing yeast spot assays in solid media containing increasing concentrations of $K_2S_2O_5$. For a better comparison, we included two sulfite resistant strains, T73 and VLI, which harbor genomic translocations affecting the *SSU1* locus that have been previously characterized (VIIItXVI and XVtXVI, respectively), and the control wine yeast strain P24 with no *SSU1* rearrangements (Goto-Yamamoto *et al.*, 1998; Pérez-Ortín *et al.*, 2002; Zimmer *et al.*, 2014; García-Ríos *et al.*, 2017). No growth differences in a medium without sulfites were observed among the wine yeast strains (Figure 1A). Upon addition of $K_2S_2O_5$, the P24 control strain displayed a slight sensitivity at 3 mM and no growth at 4 mM $K_2S_2O_5$, whereas the P5 strain was as resistant to sulfite as the T73 and VL1 reference strains (Figure 1A). When assayed in liquid medium, we observed a very slight growth defect for the T73 and VL1 strains as compared to the P5 strain under normal conditions, which was enhanced in the presence of 4 mM $K_2S_2O_5$ (Figure 1B). Again, T73, VL1 and P5 strains were all more resistant to sulfite than the P24 control strain (Figure 1B).

Previous studies have demonstrated that the resistance of yeast cells to sulfite is mainly determined by the expression of its *SSU1* gene (Avram and Bakalinsky, 1997). Therefore, to explore the potential mechanisms responsible for these phenotypic

differences, we first determined the expression levels of *SSU1* mRNA by RT-qPCR in the absence of $K_2S_2O_5$. As Figure 1C shows, the P5 yeast strain displayed the highest *SSU1* mRNA levels, followed by VLI and T73 strains respectively. The control P24 strain showed the lowest *SSU1* expression, which is consistent with its high sensitivity to sulfite (Figure 1A). No induction of *SSU1* expression by sulfite addition was observed for any of the studied yeast strains (data not shown).

To characterize the molecular bases of the elevated *SSU1* expression observed in the P5 wine yeast strain, we designed PCR primers to distinguish between a wild-type *SSU1* locus (nt-XVI), and the VIIItXVI and XVtXVI translocations (Figure 2). We observed specific bands that corresponded to the VIIItXVI and XVtXVI translocations only in T73 and VLI strains, respectively (Figure 2). Moreover, a PCR product indicative of a wild-type *SSU1* allele was also present in T73, VLI and P24 strains. Remarkably, the P5 sulfite-resistant strain neither presented the chromosomal rearrangements previously described nor the wild-type *SSU1* allele. Taken together, these results suggest that a novel genetic mechanism triggers the elevated expression of *SSU1* in the P5 wine strain, which results in greater resistance to sulfite.

The P5 wine yeast strain displays an inversion on chromosome XVI that involves the *SSU1* promoter region

The greater *SSU1* expression of the P5 strain prompted us to sequence its promoter region by using a reverse oligonucleotide within *SSU1* coding sequence (CDS). No abnormal sequences were observed up to 488 bp downstream of the *SSU1* CDS. However, the sequence identified beyond that point was identical to the promoter region 814 bp upstream of the *GCR1* gene CDS, which is normally located 38.5 Kb

172 away from the *SSU1* gene in chromosome XVI. An additional sequencing with a
173 reverse oligonucleotide within *GCR1* CDS supported that a chromosome XVI
174 reorganization, with breakpoints located at *SSU1* and *GCR1* promoters, had occurred in
175 the wine yeast strain P5 (see Figure 3A for a schematic representation of the
176 chromosome XVI inversion present in the P5 strain). To investigate the potential
177 molecular mechanism responsible for such an inversion, we aligned the nucleotide
178 sequence within both junctions of the inversion (Figure 3B; inv-XVI C and inv-XVI D
179 rev) and from the natural chromosome XVI (Figure 3B; nt-XVI A and nt-XVI B).
180 Approximately half of this sequence (18 out of 37 nucleotides) was identical among the
181 four fragments, which suggests that this illegitimate recombination has occurred
182 probably due to sequence microhomology.

183 To further characterize the chromosome XVI inversion (inv-XVI) as compared
184 to other chromosomal organizations, we designed specific primer pairs to amplify the
185 native *SSU1* (F1 and R1) and *GCR1* (F2 and R2) promoter regions (Figure 3A). In a
186 PCR contain all four primers, the T73, VL1 and P24 strains showed two bands
187 corresponding to the amplification of wild-type *SSU1* (F1-R1: 1256 bp) and *GCR1* (F2-
188 R2: 2088 bp) (Figure 3B). Importantly, the P5 strain did not exhibit any of these bands,
189 instead, P5 displayed two bands corresponding to the amplification with the F1-F2 and
190 R1-R2 primer pairs (1866 and 1634 bp, respectively) (Figure 3B). These results
191 demonstrate that the wine yeast strain P5 contains a large chromosome XVI inversion
192 with breakpoints located 488 bp upstream of the *SSU1* CDS and 814 bp upstream of the
193 *GCR1* CDS. Then, we decided to explore the presence of additional yeast strains with a
194 similar inversion at the *SSU1* locus of chromosome XVI. For this purpose, we extracted
195 the genomic DNA from 132 *S. cerevisiae* strains from different geographical origins
196 and isolation sources (Table S1), and used them as templates to perform specific PCRs

with the four primers described above (Figure 3C; F1, R1, F2 and R2). Most strains (106 out of 132) displayed a PCR pattern (2088 bp + 1256 bp) consistent with a wild-type *SSU1* locus (Table S1). A significant proportion of the strains (25 out of 132) exhibited only a 2088 bp band, but lacked the 1256 bp band corresponding to the native *SSU1* promoter (Table S1). This result was consistent with these 25 yeast strains containing a chromosomal rearrangement at the *SSU1* locus different from the chromosome XVI inversion described in the P5 strain, and probably due to either a VIIItXVI or a XVtXVI translocation. These results suggest that the inv-XVI present in the P5 yeast strain could be the consequence of a recent genetic rearrangement that did not spread yet among wine strains.

The Fzf1 transcription factor is not responsible for the increased *SSU1* expression and sulfite resistance of the P5 wine strain

The 488 bp fragment of *SSU1* promoter conserved in the P5 inv-XVI contains the binding sites for the Fzf1 transcription factor, which contributes to the basal expression of the *SSU1* gene (Figure 3A; Fzf1 binding sites located at -442 (CS1) and -387 bp (CS2) of *SSU1* CDS; Sarver and DeRisi, 2005; Avram et al., 1999). First, we determined the expression levels of *FZF1* in all wine strains included in this study. In this case, the strain P5 exhibited the lowest levels of *FZF1* mRNA (Figure S1). Then, we analyzed the implication of the transcriptional factor Fzf1 in the expression of *SSU1* observed in the P5 strain. For this purpose, the *FZF1* gene was deleted from a haploid version of P5 strain, and its effect on sulfite resistance and *SSU1* expression studied. Neither the growth in sulfite-containing media nor the expression of the *SSU1* gene of the P5 strain was altered when its *FZF1* gene was deleted (Figure 4). These data suggest

221 that the Fzf1 transcription factor does not account for the increased *SSU1* expression
222 and sulfite resistance displayed by the P5 wine strain.

223

224 **The ectopic expression of the P5 chimeric *SSU1* allele confers resistance to sulfite-**
225 **sensitive strains.**

226 To further ascertain the contribution of the P5 *SSU1* allele to sulfite resistance,
227 we amplified by PCR the natural wild-type *SSU1* allele (*WT-SSU1*) and the chimeric P5
228 *SSU1* allele (*P5-SSU1*) (containing either 810 or 1148 bp from their respective promoter
229 regions, *SSU1* CDS and terminator), and cloned them into a centromeric yeast
230 expression vector (pRS416; Sikorski and Hieter, 1989) (see Figure 5A for a schematic
231 representation). Both plasmids and empty vector were transformed into the wine yeast
232 strain P24 and the laboratory BY4743 strain, lacking either *SSU1* or *FZF1* genes (*ssu1Δ*
233 and *fzf1Δ* strains), all of them sensitive to sulfite. The plasmid expressing the wild-type
234 *SSU1* allele (*WT-SSU1*) increased *SSU1* mRNA levels except in the case of the BY4743
235 *fzf1Δ* strain, whereas the construct containing the chimeric *P5-SSU1* allele conferred the
236 highest expression independently of Fzf1 (Figure 5). To test the effect of the *P5-SSU1*
237 allele on sulfite resistance, we performed a yeast growth assay in media with increasing
238 concentrations of $K_2S_2O_5$. Notably, as compared to empty vector, the *P5-SSU1* allele
239 was able to confer resistance to all the sulfite-sensitive strains assayed, whereas the *WT-*
240 *SSU1* allele did not endow the sensitive strains with a higher sulfite tolerance (Figure 6).
241 These results indicate that the expression of *SSU1* gene under the control of the
242 chimeric *SSU1* promoter present in the wine yeast strain P5 is able by itself to increase
243 the sulfite resistance of different yeast strains.

244

The partial deletion of *SSU1* promoter is sufficient to increase expression and to confer sulfite resistance.

To determine the contribution of the *GCR1* promoter sequence acquired by the *P5-SSU1* recombinant allele to its expression and sulfite resistance, we cloned a truncated *SSU1* allele (*trunc-SSU1*), which only contained the 488 bp from the *SSU1* natural promoter region, but no *GCR1* sequence, into the pRS416 vector (as depicted in Figure 5A). The plasmid containing the *trunc-SSU1* allele was transformed into the sulfite-sensitive P24, BY4743 *ssu1Δ* and BY4743 *fzf1Δ* yeast strains. Then the *SSU1* mRNA levels and the sulfite resistance were assayed in comparison to the same strains expressing the *WT-SSU1* and *P5-SSU1* alleles. Interestingly, yeast strains with the *trunc-SSU1* plasmid displayed *SSU1* mRNA levels higher than those expressing the *WT-SSU1* allele, but slightly lower than the *P5-SSU1* transformed cells (Figure 5). Regarding growth in $K_2S_2O_5$-containing media, the *trunc-SSU1* allele increased the resistance to sulfite of all the strains in a similar manner to the *P5-SSU1* chimeric allele (Figure 6).

To discard a potential contribution of the pRS416 sequence to *SSU1* expression and sulfite resistance conferred by the cloned *trunc-SSU1* allele, we integrated the nourseothricin resistance gene (clonNAT) at -488 bp from the *SSU1* CDS of the wild-type and *fzf1Δ* laboratory BY4741 yeast strain. This genomic integration mimics the *trunc-SSU1* allele because it lacks the original sequence 488 bp downstream of the *WT-SSU1* and *P5-SSU1* alleles. Again, *SSU1* expression and sulfite growth assays demonstrated that the removal of the *SSU1* promoter region beyond 488 increased *SSU1* mRNA levels and conferred sulfite resistance in an Fzf1-independent way (Figure 7). Taken together, these results strongly suggest that the inv-XVI present in the P5 wine yeast strain eliminates a portion of the *SSU1* promoter region that was limiting *SSU1*

expression and sulfite resistance, and introduces a new *GCR1* sequence that further increases *SSU1* mRNA levels.

DISCUSSION

The stresses that yeast cells encounter during wine fermentations include the elevated content of sugar in grape musts, the high ethanol concentrations achieved, the temperature of the different fermentation types, and the sulfite added as an antimicrobial and antioxidant agent (Bauer and Pretorius, 2000; Divol *et al.*, 2012). These environmental stresses exerted for hundreds of years and thousands of generations have caused wine yeasts to evolve rapidly, shaping their genome through different genetic mechanisms (Marsit and Dequin, 2015; Guillamón and Barrio, 2017; Legras *et al.*, 2018).

Previous studies have shown that different chromosomal rearrangements of the wine yeast *SSU1* gene have led to an increase in their resistance against the stress generated by the sulfites added during the must fermentation process. In an initial study, we described that a translocation between chromosomes XVI and VIII (VIIItXVI) located the *SSU1* gene under the promoter region of the *EMC34* regulatory region, leading to a new *SSU1* promoter, which increased *SSU1* expression and tolerance to sulfites (Pérez-Ortín et al., 2002). A later study, showed that a translocation between chromosomes XVI and XV (XVtXVI) positioned the *SSU1* gene under the control of the promoter region of *ADH1*, a constitutively expressed gene encoding for the enzyme alcohol dehydrogenase *(*Zimmer et al., 2014). Again, this rearrangement enhanced the basal expression of *SSU1* and improved sulfite resistance. In the present work, we have identified and characterized a novel reorganization on chromosome XVI that involves

294 the *SSU1* gene. Specifically, we have identified an inversion in chromosome XVI (inv-
295 XVI) that increases the sulfite resistance capacity of the P5 wine yeast strain to a similar
296 strength of that of the VIIItXVI and XVtXVI translocations (Figure 1). We observed
297 that the yeast strain with the highest *SSU1* expression (P5) was the most resistant to
298 sulfite, whereas the most sensitive strain (P24) exhibited the lowest *SSU1* expression
299 (Figure 1). However, in the case of the T73 and VL1 yeast strains, *SSU1* mRNA levels
300 did not fit with their relative sulfite resistance probably due to the contribution of other
301 factors to yeast sulfite tolerance (Figure 1; Zimmer et al., 2014). No correlation was
302 observed between *FZF1* mRNA levels and sulfite resistance (Figure 1 and S1).

303 The molecular basis responsible for the increased expression of the different
304 *SSU1* alleles is not fully understood. The VIIItXVI reorganization present in the T73
305 and other wine strains conserved the two Fzf1-binding sites (CS1 and CS2) contained in
306 the original *SSU1* promoter region (Pérez-Ortín *et al.*, 2002). Moreover, cells acquired a
307 piece of *ECM34* promoter containing 76-bp repeats, whose number influences *SSU1*
308 expression and sulfite resistance (Pérez-Ortín et al., 2002; Yuasa et al, 2005). The
309 XVtXVI event occurred at an AT-rich region within the *ADH1* promoter on
310 chromosome XV (Zimmer et al., 2014). This translocation eliminated the Fzf1-binding
311 sites present in the native *SSU1* promoter, but added an Fzf1 and an Adr1 consensus-
312 binding site (Zimmer et al., 2014). The contribution of these regulatory elements to
313 *SSU1* expression has not been elucidated yet. To gain insight into the reasons
314 responsible for the increased *SSU1* expression conferred by the *GCR1-SSU1* regulatory
315 region present in the P5 wine strain, we compared how a battery of promoter constructs
316 (*WT-SSU1*, *P5-SSU1* and *trunc-SSU1*) influenced the expression of *SSU1* and the sulfite
317 adaptation of various yeast strains (P24, BY4743 *ssu1Δ* and BY4743 *fzf1Δ*). Our results
318 indicate that both the *GCR1* sequence positioned upstream of *SSU1* and the removal of

319 the *SSU1* sequence downstream of -488 bp contribute to the elevated *SSU1* mRNA
320 levels and sulfite resistance provided by the chimeric *GCR1-SSU1* promoter exhibited
321 by the P5 wine yeast strain. Despite the chimeric *P5-SSU1* promoter conserves both
322 original Fzf1-binding sites, the deletion of *FZF1* did not alter *SSU1* expression and
323 sulfite resistance (Figures 5-7). In fact, although *SSU1* is influenced by different stress
324 responses, including low oxygen and nitric oxide, its level of expression is not regulated
325 by sulfite present in the external milieu or into the cell (Sarver and DeRisi, 2005; Yuasa
326 *et al.*, 2005; Aranda *et al.*, 2006). There is only a previously described example of an
327 industrial strain (71B) that harbors a sulfur-inducible *SSU1* gene that may have gained a
328 new regulatory system (Nardi *et al.*, 2010). However, we did not observe any sulfite-
329 dependent regulation for the *P5-SSU1* allele. Further studies would be necessary to
330 decipher which transcription factors and *cis* elements enhance *SSU1* expression in the
331 different sulfite-resistant wine yeast strains.

332 Although we performed a screening of 132 different *S. cerevisiae* yeast strains,
333 we were unable to identify an inv-XVI event similar to the chromosomal reorganization
334 exhibited by the P5 wine strain (Table S1). We suggest that the inv-XVI could be a
335 recent genetic event in the evolution of wine yeasts that did not disseminate yet. We
336 consider that, from a genetic point of view, the inversion affecting the P5 strain could be
337 more beneficial than the VIIItXVI and XVtXVI translocations because the supergene
338 architecture generated by major chromosomal inversions, due to the localized reduction
339 in recombination within, offers a mechanism to the maintenance of balanced
340 polymorphism at multiple coadapted and tightly linked elements, without compromising
341 the viability of the heterozygous spores. In fact, the spore viability of a P5/BY4743
342 crossed strain resulted to be 100% as for the original P5 and BY4743 strains.
343 Contrastingly, *SSU1* translocations not only could generate an ecological differentiation

but also a reproductive barrier between wine and non-wine *S. cerevisiae* populations by reducing spore viability in hybrids (Hyma and Fay, 2012; Hou *et al.*, 2014; Clowers *et al.*, 2015). The inv-XVI identified here has a great potential to be used in breeding programs for sulfite resistance improvement in wine yeasts since it would not affect the viability of heterozygous spores, unlike previously described *SSU1* translocations.

EXPERIMENTAL PROCEDURES

Yeast strains and plasmids. The yeast strains genotyped for the *SSU1* locus are listed in Table S1, whereas the yeast strains and plasmids used in the main figures of this study are described in Table S2. We used the pAG25 plasmid as a template and specific oligonucleotides to generate integrative cassettes to truncate the genomic copy of *SSU1* promoter and to delete *FZF1* gene in the haploid version of the P5 strain. To construct the pRS416-based plasmids expressing chimeric *SSU1*, wild-type *SSU1* and truncated *SSU1*, 1148, 810 and 488 bp were respectively amplified from the promoter region of the *SSU1* gene of either P5 or BY4743 strains. The oligonucleotides used were SSU1_P5XhoI-F, SSU1_XhoI-F, promSSU1native_XhoI-F and SSU1_BamHI-R (Table S3). The PCR products and the plasmid were digested with the restriction enzymes *BamH*I (FastDigest, Thermo) and *Xho*I (FastDigest, Thermo) and ligation was performed. PCR amplifications were performed with the Phusion DNA polymerase (Finnzymes), and the cloned insert was sequenced. One Shot TOP10 chemically competent *Escherichia coli* cells (Invitrogen) were used to isolate and amplify plasmids.

Culture conditions. Yeast cultures were incubated at 28ºC. Sulfite tolerance was determined on YPD medium [1% (w/v) yeast extract, 2% (w/v) bacteriological peptone, 2% (w/v) glucose] containing 75 mM L-tartaric acid (TA) at pH 3.5 as previously described (Park *et al.*, 1999). The addition of sulfite to the agar medium after autoclaving, but just before pouring, was not effective. Therefore, sulfite-containing plates were prepared by spreading an appropriate amount of freshly filter-sterilized 1M $K_2O_5S_2$ stock solution on the top of YPD + TA plates. The strains transformed with the

plasmid pRS416 were selected on SC-Ura plates [0.17% (w/v) yeast nitrogen base without amino acids and without ammonium sulfate (Difco), 0.5% (w/v) ammonium sulfate (Panreac), 2% (w/v) glucose (Panreac), 2 g L^{-1} Kaiser drop-out (Formedium) and 2% (w/v) agar (Pronadisa)]. Likewise, liquid SC-Ura medium was used to keep the pRS416 plasmid during overnight growth. All other yeast precultures were carried out in YPD + TA under the same conditions. To assay solid growth, yeast cells were cultivated to exponential phase, and then spotted on 10-fold serial dilutions starting at an OD_{600} of 0.1, and incubated at 28°C for 3 days. The assays were performed on YPD + TA plates with concentrations of $K_2O_5S_2$ up to 4 mM. Growth rate in liquid medium was monitored by determining optical density at 600 nm in a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany). Measurements were taken every 30 min for 4 days after 20 seconds pre-shaking. Microplate wells were filled with the required volume of inoculum and 0.25 mL of medium to always ensure an initial OD of approximately 0.1 (inoculum level of about 10^6 cells mL^{-1}). Growth parameters were calculated from each treatment by directly fitting OD measurements versus time to the reparametrized Gompertz equation proposed by Zwietering *et al.*, (1990):

$$y = D*\exp\{-\exp[((\mu_{max} * e)/D) * (\lambda - t)) + 1]\}$$

where $y = \ln(OD_t/OD_0)$, OD_0 is the initial OD and OD_t is the OD at time t; $D = \ln(OD_t/OD_0)$ is the asymptotic maximum, μ_{max} is the maximum specific growth rate (h^{-1}), and λ is the lag phase period (h) (Aguilera *et al.*, 2007). To test the spore viability, sporulation was induced by incubating cells on acetate medium (1% potassium acetate and 2% agar) for 5 days at 30°C. Following the preliminary digestion of the asci walls with 2 mg mL^{-1} glucuronidase (Sigma), spores were dissected using micromanipulator (Singer instruments, United Kingdom). Viability was calculated as the percentage of

spores (from a total of 40 analyzed spores per strain) able to form a colony on YPD agar after 48-72 h at 28°C.

RNA analyses. Total RNA extraction and cellular mRNA levels were determined by RT-qPCR as previously described (Sanvisens *et al.*, 2014). The SSU1q_F/SSU1q_R, FZF1q_F/FZF1q_R and ACT1q_F/ACT1q_R primer pairs were used to determine the levels of *SSU1*, *FZF1* and *ACT1* mRNAs, respectively (Table S3). The data and error bars represent the average and the standard deviation of three independent biological samples.

Detection of the inversion within yeast population. The DNA extraction of the different strains was carried as previously described (Querol et al., 1992). To rapidly screen the genetic rearrangements involving the *SSU1* gene (nt-XVI, VIIItXVI, XVtXVI or inv-XVI), PCR tests were performed with appropriate primers (Table S3).

Statistical analyses. The results are expressed as mean and standard deviation of at least 3 independent biological replicates. To evaluate statistical significance, tailed t-student tests were applied. The asterisk (*) indicates statistically significant differences with p-value ≤ 0.05. Phenotypic data were fitted to the reparametrized Gompertz model by non-linear least-squares fitting using the Gauss-Newton algorithm as implemented in the nls function in the R statistical software, v.3.0.

ACKNOWLEDGMENTS

This work was supported by the Spanish Government through “Ministerio de Ciencia, Innovación y Universidades” (MICINN) and “Fondo Europeo de Desarrollo Regional” (FEDER) funds grant number PCIN-2015-143 and AGL2016-77503-C3-1-R to JMG, AGL2015-67504-C3-3-R to EB, and BIO2017-87828-C2-1-P to SP. This study has been carried out in the context of the European Project ERA-IB “YeastTempTation". The authors thank Dr. Philippe Marullo for kindly providing VL1 strain.

Disclosure Declaration

The authors declare no conflict of interest associated with this manuscript.

REFERENCES

Aguilera, J., Randez-Gil, F., and Prieto, J.A. (2007) Cold response in *Saccharomyces cerevisiae*: new functions for old mechanisms. *FEMS Microbiol. Rev.* **31**: 327–41.

Alexander, W.G., Wisecaver, J.H., Rokas, A., and Hittinger, C.T. (2016) Horizontally acquired genes in early-diverging pathogenic fungi enable the use of host nucleosides and nucleotides. *Proc. Natl. Acad. Sci. USA* **113**: 4116–4121.

Almeida, P., Barbosa, R., Zalar, P., Imanishi, Y., Shimizu, K., Turchetti, B., et al. (2015) A population genomics insight into the Mediterranean origins of wine yeast domestication. *Mol. Ecol.* **24**: 5412–5427.

Aranda, A., Jiménez-Martí, E., Orozco, H., Matallana, E., and Del Olmo, M. (2006) Sulfur and adenine metabolisms are linked, and both modulate sulfite resistance in wine yeast. *J. Agric. Food Chem.* **54**: 2839–5846.

Avram, D. and Bakalinsky, A.T. (1997) *SSU1* encodes a plasma membrane protein with a central role in a network of proteins conferring sulfite tolerance in *Saccharomyces cerevisiae*. *J. Bacteriol.* **179**: 5971–5974.

Avram, D., Leid, M., and Bakalinsky, A.T. (1999) Fzf1p of *Saccharomyces cerevisiae* is a positive regulator of *SSU1* transcription and its first zinc finger region is required for DNA binding. *Yeast* **15**: 473–480.

Barbosa, R., Almeida, P., Safar, S.V.B., Santos, R.O., Morais, P.B., Nielly-Thibault, L., et al. (2016) Evidence of natural hybridization in Brazilian wild lineages of *Saccharomyces cerevisiae*. *Genome Biol. Evol.* **8**: 317–329.

Bauer, F.F. and Pretorius, I.S. (2000) Yeast Stress Response and Fermentation Efficiency : How to Survive the Making of Wine - A Review. *South African J. Enol. Vitic.* **21**: 27–51.

Beltran, G., Torija, M.J., Novo, M., Ferrer, N.N., Poblet, M., Guillamón, J.M., et al. (2002) Analysis of yeast populations during alcoholic fermentation: a six year follow-up study. *Syst Appl Microbiol* **25**: 287–93.

Borneman, A.R., Forgan, A.H., Kolouchova, R., Fraser, J.A., and Schmidt, S.A. (2016) Whole Genome Comparison Reveals High Levels of Inbreeding and Strain Redundancy Across the Spectrum of Commercial Wine Strains of *Saccharomyces cerevisiae*. *G3 Genes|Genomes|Genetics* **6**: 957–971.

Brown, C.A., Murray, A.W., and Verstrepen, K.J. (2010) Rapid Expansion and Functional Divergence of Subtelomeric Gene Families in Yeasts. *Curr. Biol.* **20**: 895–903.

Casadevall, A. (2008) Evolution of Intracellular Pathogens. *Annu. Rev. Microbiol* **62**: 19–33.

Casalone, E., Colella, C.M., Daly, S., Fontana, S., Torricelli, I., and Polsinelli, M. (1994) Cloning and characterization of a sulphite-resistance gene of *Saccharomyces cerevisiae*. *Yeast* **10**: 1101–1110.

Casalone, E., Colella, C.M., Daly, S., Gallori, E., Moriani, L., and Polsinelli, M. (1992)

Mechanism of resistance to sulphite in *Saccharomyces cerevisiae*. *Curr. Genet.* **22**: 435–440.

Cheeseman, K., Ropars, J., Renault, P., Dupont, J., Gouzy, J., Branca, A., et al. (2014) Multiple recent horizontal transfers of a large genomic region in cheese making fungi. *Nat. Commun.* **5**: 2876.

Clowers, K.J., Heilberger, J., Piotrowski, J.S., Will, J.L., and Gasch, A.P. (2015) Ecological and genetic barriers differentiate natural populations of *Saccharomyces cerevisiae*. *Mol. Biol. Evol.* **32**: 2317–2327.

Divol, B., Du Toit, M., and Duckitt, E. (2012) Surviving in the presence of sulphur dioxide: Strategies developed by wine yeasts. *Appl. Microbiol. Biotechnol.* **95**: 601–613.

Doniger, S.W., Kim, H.S., Swain, D., Corcuera, D., Williams, M., Yang, S.P., and Fay, J.C. (2008) A catalog of neutral and deleterious polymorphism in yeast. *PLoS Genet.* **4**: e1000183.

Engle, E.K. and Fay, J.C. (2012) Divergence of the yeast transcription factor *FZF1* affects sulfite resistance. *PLoS Genet.* **8**: e1002763.

Fleet, G.H. (2003) Yeast interactions and wine flavour. *Int. J. Food Microbiol.* **86**: 11–22.

García-Ríos, E., López-Malo, M., and Guillamón, J.M. (2014) Global phenotypic and genomic comparison of two *Saccharomyces cerevisiae* wine strains reveals a novel role of the sulfur assimilation pathway in adaptation at low temperature fermentations. *BMC Genomics* **15**: 1059.

García-Ríos, E., Morard, M., Parts, L., Liti, G., and Guillamón, J.M. (2017) The genetic architecture of low-temperature adaptation in the wine yeast *Saccharomyces cerevisiae*. *BMC Genomics* **18**: 159.

Gayevskiy, V., Lee, S., and Goddard, M.R. (2016) European derived *Saccharomyces cerevisiae* colonisation of New Zealand vineyards aided by humans. *FEMS Yeast Res.* **16**: 1–12.

Goddard, M.R. (2008) Quantifying the complexities of *Saccharomyces cerevisiae*'s ecosystem engineering via fermentation. *Ecology* **89**: 2077–82.

Goldstein, A. and McCusker, J. (1999) Three New Dominant Drug Resistance Cassettes for Gene Disruption in *Saccharomyces cerevisiae*. *Yeast* **15**: 1541–1553.

Gonçalves, M., Pontes, A., Almeida, P., Barbosa, R., Serra, M., Libkind, D., et al. (2016) Distinct Domestication Trajectories in Top-Fermenting Beer Yeasts and Wine Yeasts. *Curr. Biol.* **26**: 2750–2761.

Goto-Yamamoto, N., Kitano, K., Shiki, K., Yoshida, Y., Suzuki, T., Iwata, T., et al. (1998) *SSU1*-R, a sulfite resistance gene of wine yeast, is an allele of *SSU1* with a different upstream sequence. *J. Ferment. Bioeng.* **86**: 427–433.

Guillamón, J.M. and Barrio, E. (2017) Genetic polymorphism in wine yeasts: Mechanisms and methods for its detection. *Front. Microbiol.* **8**: 1–20.

Hou, J., Friedrich, A., De Montigny, J., and Schacherer, J. (2014) Chromosomal rearrangements as a major mechanism in the onset of reproductive isolation in *Saccharomyces cerevisiae*. *Curr. Biol.* **24**: 1153–1159.

Hu, X., Xiao, G., Zheng, P., Shang, Y., Su, Y., Zhang, X., et al. (2014) Trajectory and genomic determinants of fungal-pathogen speciation and host adaptation. *Proc. Natl. Acad. Sci. USA* **111**: 16796–16801.

Hyma, K. and Fay, J. (2012) Mixing of vineyard and oak-tree ecotypes of *Saccharomyces cerevisiae* in North American vineyards. *Mol. Ecol.* **40**: 1301–1315.

Legras, J., Galeote, V., Bigey, F., Camarasa, C., Marsit, S., Nidelet, T., et al. (2018) Adaptation of *S. cerevisiae* to fermented food environments reveals remarkable genome plasticity and the footprints of domestication. *Mol. Biol. Evol.* **35**: 1712–1727.

Lin, Z. and Li, W.H. (2011) Expansion of hexose transporter genes was associated with the evolution of aerobic fermentation in yeasts. *Mol. Biol. Evol.* **28**: 131–142.

Liti, G., Carter, D.M., Moses, A.M., Warringer, J., Parts, L., James, S.A., et al. (2009) Population genomics of domestic and wild yeasts. *Nature* **458**: 337–341.

Marcet-Houben, M., Gabaldón, T., Jaillon, O., Aury, J.-M., Wincker, P., Coghlan, A., et al. (2015) Beyond the Whole-Genome Duplication: Phylogenetic Evidence for an Ancient Interspecies Hybridization in the Baker's Yeast Lineage. *PLoS Biol.* **13**: e1002220.

Marsit, S. and Dequin, S. (2015) Diversity and adaptive evolution of *Saccharomyces* wine yeast: a review. *FEMS Yeast Res.* **15**: fov067.

Nadai, C., Treu, L., Campanaro, S., Giacomini, A., and Corich, V. (2016) Different mechanisms of resistance modulate sulfite tolerance in wine yeasts. *Appl. Microbiol. Biotechnol.* **100**: 797–813.

Nardi, T., Corich, V., Giacomini, A., and Blondin, B. (2010) A sulphite-inducible form of the sulphite efflux gene *SSU1* in a *Saccharomyces cerevisiae* wine yeast. *Microbiology* **156**: 1686–1696.

Neafsey, D.E., Barker, B.M., Sharpton, T.J., Stajich, J.E., Park, D.J., Whiston, E., et al. (2010) Population genomic sequencing of *Coccidioides* fungi reveals recent hybridization and transposon control. *Genome Res.* **20**: 938–946.

Novo, M., Dé, F., Bigey, R., Beyne, E., Galeote, V., Gavory, R., et al. (2009) Eukaryote-to-eukaryote gene transfer events revealed by the genome sequence of the wine yeast *Saccharomyces cerevisiae* EC1118. *Proc. Natl. Acad. Sci. USA* **106**: 16333–16338.

Park, H., Lopez, I., and Bakalinsky, A.T. (1999) Use of sulfite resistance in *Saccharomyces cerevisiae* as a dominant selectable marker. *Curr. Genet.* **36**: 339–344.

Pérez-Ortín, J.E., Querol, A., Puig, S., and Barrio, E. (2002) Molecular characterization of a chromosomal rearrangement involved in the adaptive evolution of yeast

552 strains. *Genome Res.* **12**: 1533–9.

553 Peter, J., De Chiara, M., Friedrich, A., Yue, J.-X., Pflieger, D., Bergstrom, A., et al.
554 (2018) Genome evolution across 1,011 *Saccharomyces cerevisiae* isolates. *Nature*
555 **556**: 339–344.

556 Querol, A., Barrio, E., and Ramón, D. (1992) A Comparative Study of Different
557 Methods of Yeast Strain Characterization. *Syst. Appl. Microbiol.* **15**: 439–446.

558 Salvadó, Z., Arroyo-López, F.N., Barrio, E., Querol, A., and Guillamón, J.M. (2011)
559 Quantifying the individual effects of ethanol and temperature on the fitness
560 advantage of *Saccharomyces cerevisiae*. *Food Microbiol.* **28**: 1155–1161.

561 Sanvisens, N., Romero, A.M., An, X., Zhang, C., de Llanos, R., Martinez-Pastor, M.T.,
562 et al. (2014) Yeast Dun1 Kinase Regulates Ribonucleotide Reductase Inhibitor
563 Sml1 in Response to Iron Deficiency. *Mol. Cell. Biol.* **34**: 3259–3271.

564 Sarver, A. and DeRisi, J. (2005) Fzf1p Regulates an Inducible Response to Nitrosative
565 Stress in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **16**: 4781–4791.

566 Sikorski, R.S. and Hieter, P. (1989) A system of shuttle vectors and yeast host strains
567 designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*
568 **122**: 19–27.

569 Strope, P.K., Skelly, D.A., Kozmin, S.G., Mahadevan, G., Stone, E.A., Magwene, P.M.,
570 et al. (2015) The 100-genomes strains, an *S. cerevisiae* resource that illuminates its
571 natural phenotypic and genotypic variation and emergence as an opportunistic
572 pathogen. *Genome Res.* **125**: 762–774.

573 Wisecaver, J.H. and Rokas, A. (2015) Fungal metabolic gene clusters-caravans
574 traveling across genomes and environments. *Front. Microbiol.* **6**: 1–11.

575 Yuasa, N., Nakagawa, Y., Hayakawa, M., and Iimura, Y. (2005) Two alleles of the
576 sulfite resistance genes are differentially regulated in *Saccharomyces cerevisiae*.
577 *Biosci. Biotechnol. Biochem.* **69**: 1584–1588.

578 Zimmer, A., Durand, C., Loira, N., Durrens, P., Sherman, D.J., and Marullo, P. (2014)
579 QTL dissection of lag phase in wine fermentation reveals a new translocation
580 responsible for *Saccharomyces cerevisiae* adaptation to sulfite. *PLoS One* **9**:
581 e86298.

582 Zwietering, M.H., Jongenburger, I., Rombouts, F.M., and Van, K. (1990) Modeling of
583 the bacterial growth curve. *Appl. Environ. Microbiol.* **56**: 1875–1881.

584

585 **Figure Legends**

586

587 **Figure 1. Comparison of yeast strains holding different chromosomal**
588 **rearrangements in *SSU1* gene.** T73, VLI, P5 and P24 wine yeast strains were assayed
589 for growth on YPD + TA media with the different concentrations of $K_2S_2O_5$ in solid (A)
590 and liquid (B) media. C: Expression levels of *SSU1* mRNA after 6 h of growth in YPD
591 + TA obtained from RT-qPCR normalized with the constitutive gene *ACT1*. The
592 asterisk (*) indicates significant differences (p-value≤0.05) compared to P5.

593

594 **Figure 2. Detection of *SSU1*-mediated chromosomal translocations VIIItXVI and**
595 **XVtXVI.** Detection of the different chromosome XVI translocations by PCR. T73
596 showed a band corresponding to VIIItXVI (ECM34D and *SSU1*_R oligonucleotides).
597 VL1 showed an XVtXVI specific band (*ADH1*_F and R1 oligonucleotides). T73, VL1
598 and P24 conserved a wild-type *SSU1* allele (F1 and R1 oligonucleotides). P5 only
599 displayed a band for the amplification of *GCR1* used as DNA control (*NOG*_F and
600 *NOG*_R oligonucleotides).

601

602 **Figure 3. Genomic characterization of the chromosome XVI inversion present in**
603 **the P5 wine yeast strain**. A: Schematic representation of the region of chromosome
604 XVI comprising the *SSU1* gene and the 38.5 Kb inversion present in P5 strain. One of
605 the breakpoints is located 488 bp upstream of the *SSU1* CDS, whereas the other one
606 maps to 814 bp upstream of the *GCR1* CDS. CS1 and CS2 represent the Fzf1-binding
607 sites. B: Microhomology regions involved in the crossing-over that generated the

608 inversion in chromosome XVI. Black and white boxes highlight *GCR1* and *SSU1*
609 promoter sequences, respectively. Perfect sequence matches are shown in capitals, and
610 middle script corresponds to base pairs that are lost during the process. C: Detection of
611 the inv-XVI by PCR. T73, VL1 and P24 showed two bands corresponding to the
612 amplification of F1-R1 and F2-R2 (1256 and 2088 bp, respectively), while P5 exhibited
613 two bands corresponding to the amplification of F1-F2 and R1-R2 (1866 and 1634 bp,
614 respectively).

615

616 **Figure 4. The transcriptional factor Fzf1 is not required for sulfite resistance and**
617 ***SSU1* expression in the P5 strain**. A: Wild-type and *fzf1Δ* haploid P5 strains were
618 assayed for growth on YPD + TA media with the different concentrations of $K_2S_2O_5$. B:
619 Expression levels of *SSU1* mRNA after 6 h of growth in YPD + TA obtained from RT-
620 qPCR normalized with the constitutive gene *ACT1*. The asterisk (*) indicates significant
621 differences (p-value≤0.05) compared to P5.

622

623 **Figure 5. Effect of different promoters on *SSU1* expression.** A: Schematic
624 representation of the different *SSU1* promoter regions contained in the pRS416-*SSU1*
625 plasmids. Wild-type *SSU1* (810 bp promoter), chimeric P5 *SSU1* (1148 bp promoter),
626 truncated *SSU1* (488 bp promoter). B-D: Haploid P24 (B), BY4743 *ssu1Δ* (C) and
627 BY4743 *fzf1Δ* (D) yeast strains transformed with pRS416 (vector), pRS416-chimeric-
628 P5-*SSU1* (*P5-SSU1*) pRS416-truncated-*SSU1* (*trunc-SSU1*), and pRS416-wild-type-
629 SSU1 (*WT-SSU1*) were growth and *SSU1* mRNA levels determined by RT-qPCR with
630 specific oligonucleotides. *ACT1* mRNA levels were used to normalize. The asterisk (*)
631 indicates significant differences (p-value≤0.05) compared to cells with empty vector.

Figure 6. Effect of different *SSU1* promoters on sulfite resistance. The yeast strains obtained in Figure 5 were growth on YPD + TA plates containing different concentrations of $K_2S_2O_5$.

Figure 7. Deletion of a portion of *SSU1* promoter increases expression and sulfite resistance. A cassette conferring resistance to nourseothricin (clonNAT) was integrated at -488 bp from *SSU1* CDS of the laboratory BY4741 and BY4741 *fzf1Δ* haploid yeast strains. *SSU1* mRNA levels (A) and sulfite resistance (B) was determined as described in Figure 1. The asterisk (*) indicates significant differences (p-value≤0.05) compared to BY4741 and BY4741 *fzf1*Δ.

Supplementary figure legend

Figure S1. Expression levels of *FZF1* in various wine yeast strains. T73, VLI, P5 and P24 yeast strains were cultivated for 6 h in YPD + TA, total RNA was extracted, and *FZF1* mRNA levels determined by RT-qPCR with specific oligonucleotides. *ACT1* mRNA levels were used to normalize. The asterisk (*) indicates significant differences (p-value≤0.05) compared to P5.

Figure 1

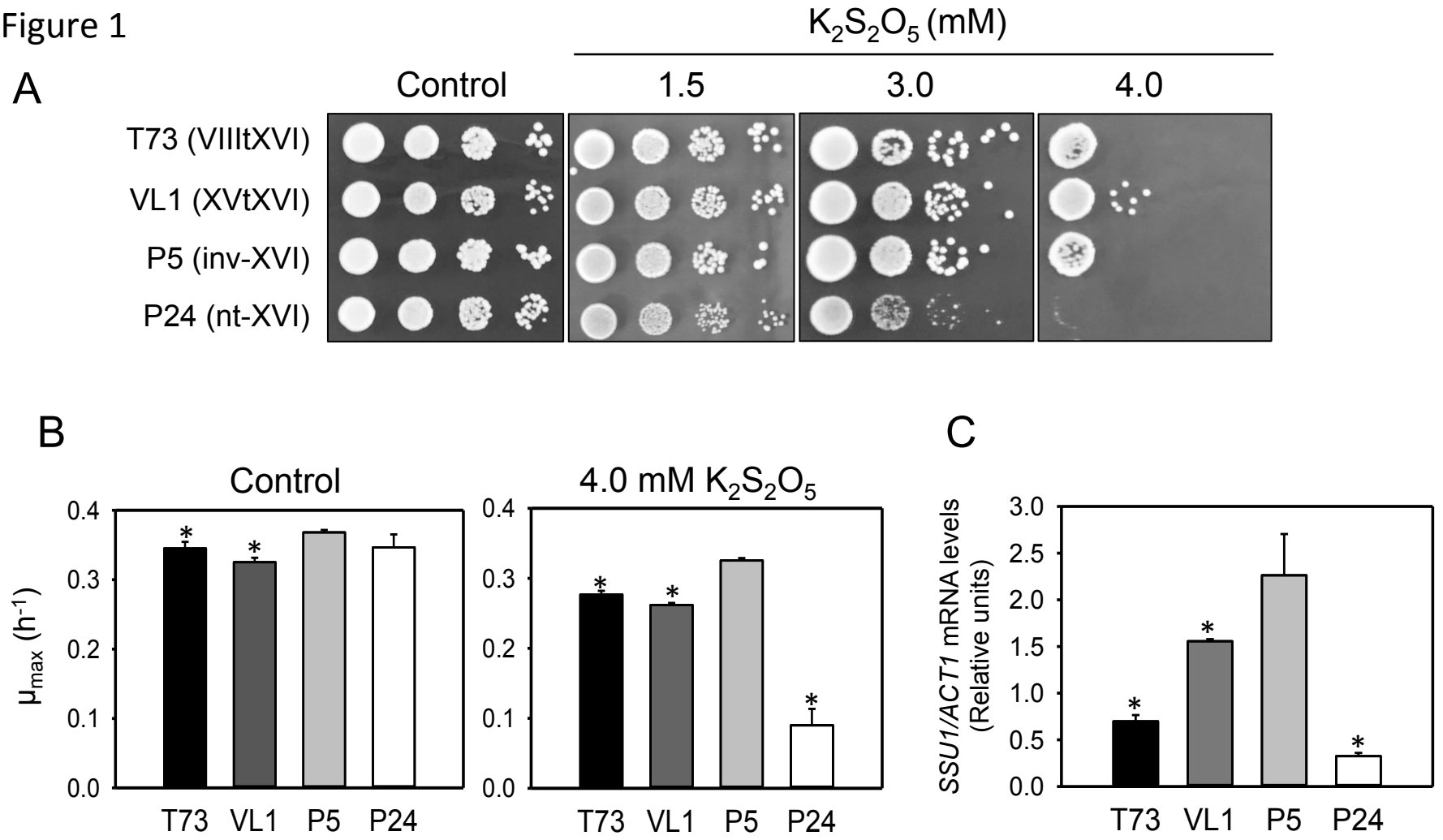

A
$K_2S_2O_5$ (mM)
Control
1.5
3.0
4.0
T73 (VIIItXVI)
VL1 (XVtXVI)
P5 (inv-XVI)
P24 (nt-XVI)
B
Control
4.0 mM $K_2S_2O_5$
μ_{max} (h^{-1})
0.0
0.1
0.2
0.3
0.4
T73
VL1
P5
P24
C
SSU1/ACT1 mRNA levels
(Relative units)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
T73
VL1
P5
P24

Figure 2

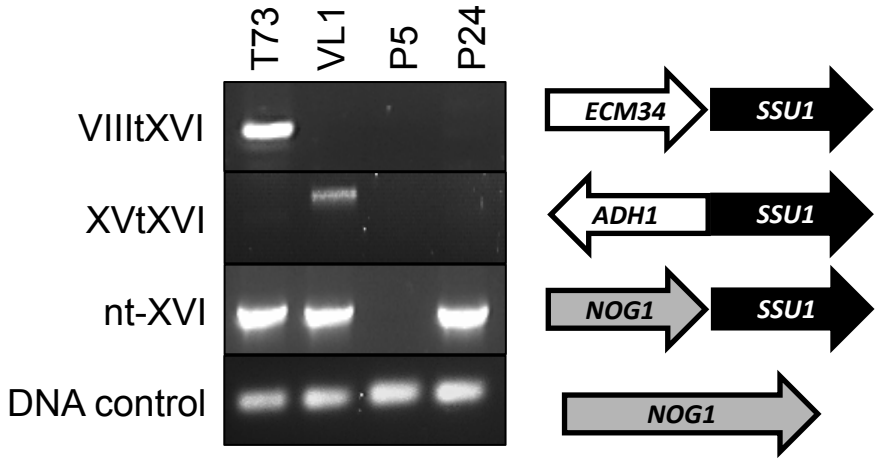
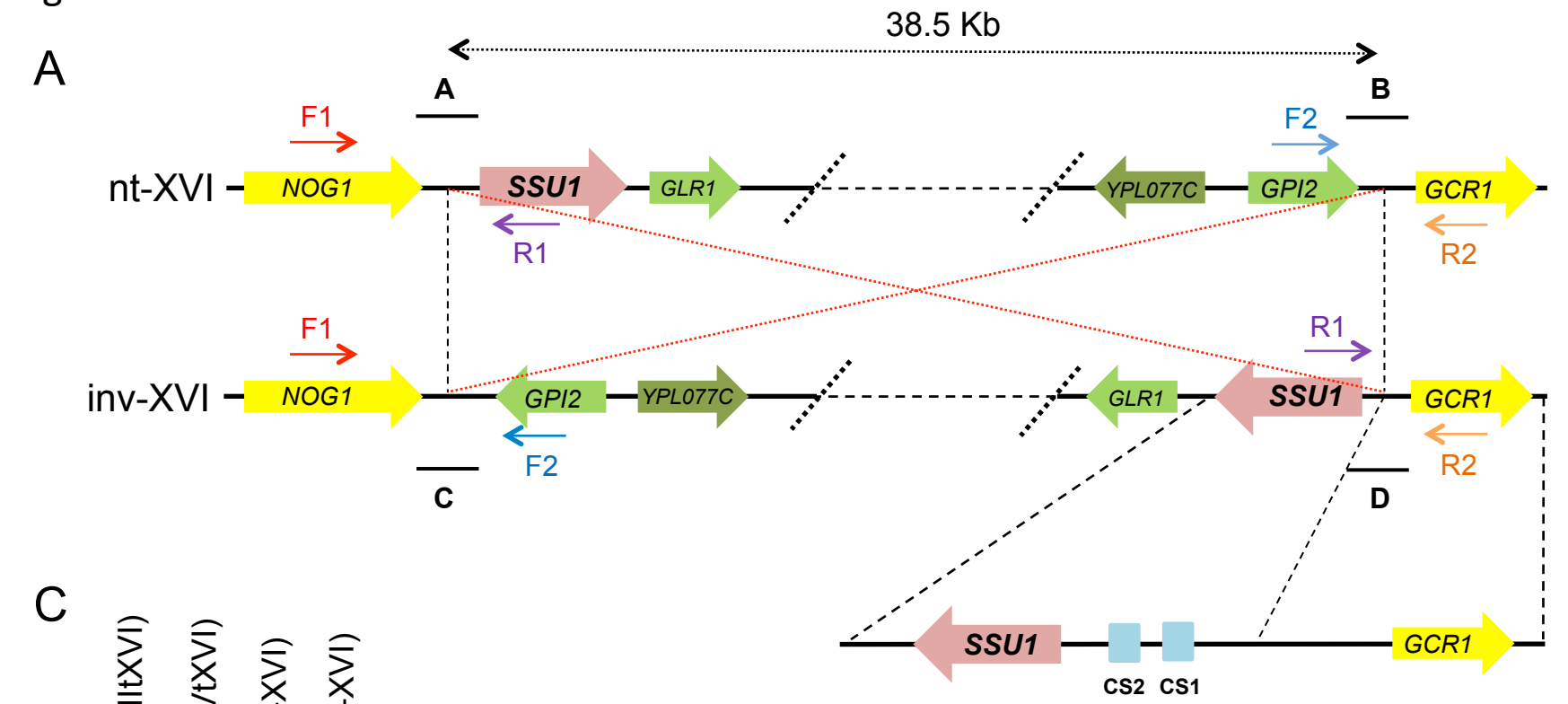
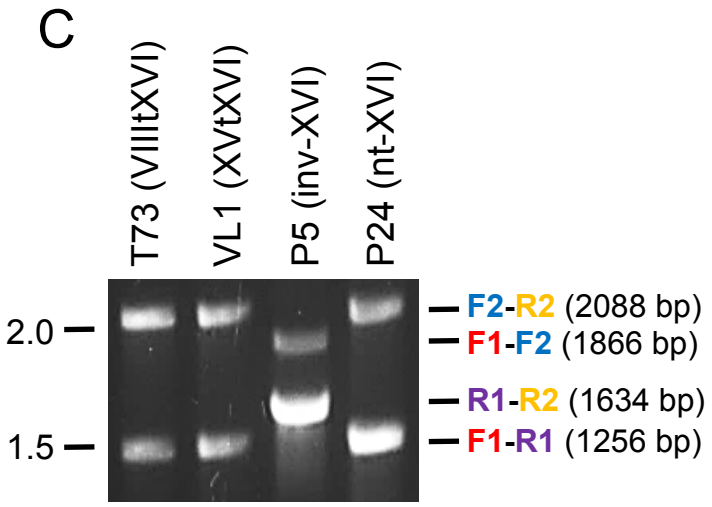

T73
VL1
P5
P24
VIIItXVI
XVtXVI
nt-XVI
DNA control
ECM34
SSU1
ADH1
SSU1
NOG1
SSU1
NOG1

Figure 3





B

```
nt-XVI A          aaTAaGaAaTTgttATCgTttTTgCagcgtAtcgtATAAgg
nt-XVI B rev      tgTAcGgAgTTtaaATCtTaaTTaCgtaaaAaatgATAAat
inv-XVI C         aaTAaGaAaTTgttATCgT--TTaCgtaaaAaatgATAAat
inv-XVI D rev     tgTAcGgAgTTtaaATCtTaaTTgCagcgtAtcgtATAAgg
```

Figure 4

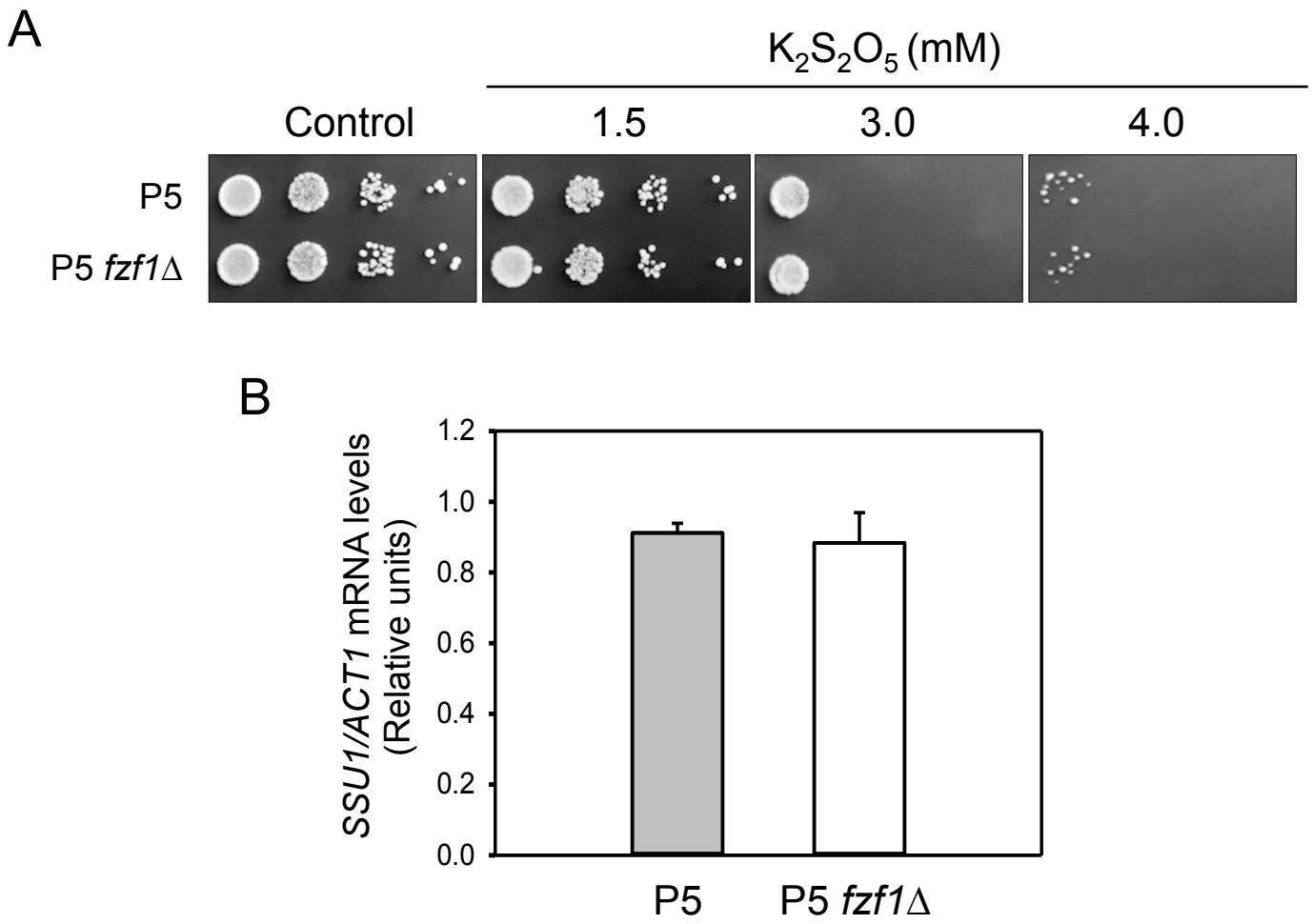

A
$K_2S_2O_5$ (mM)
Control
1.5
3.0
4.0
P5
P5 *fzf1*Δ
B
SSU1/ACT1 mRNA levels
(Relative units)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
P5
P5 *fzf1*Δ

Figure 5

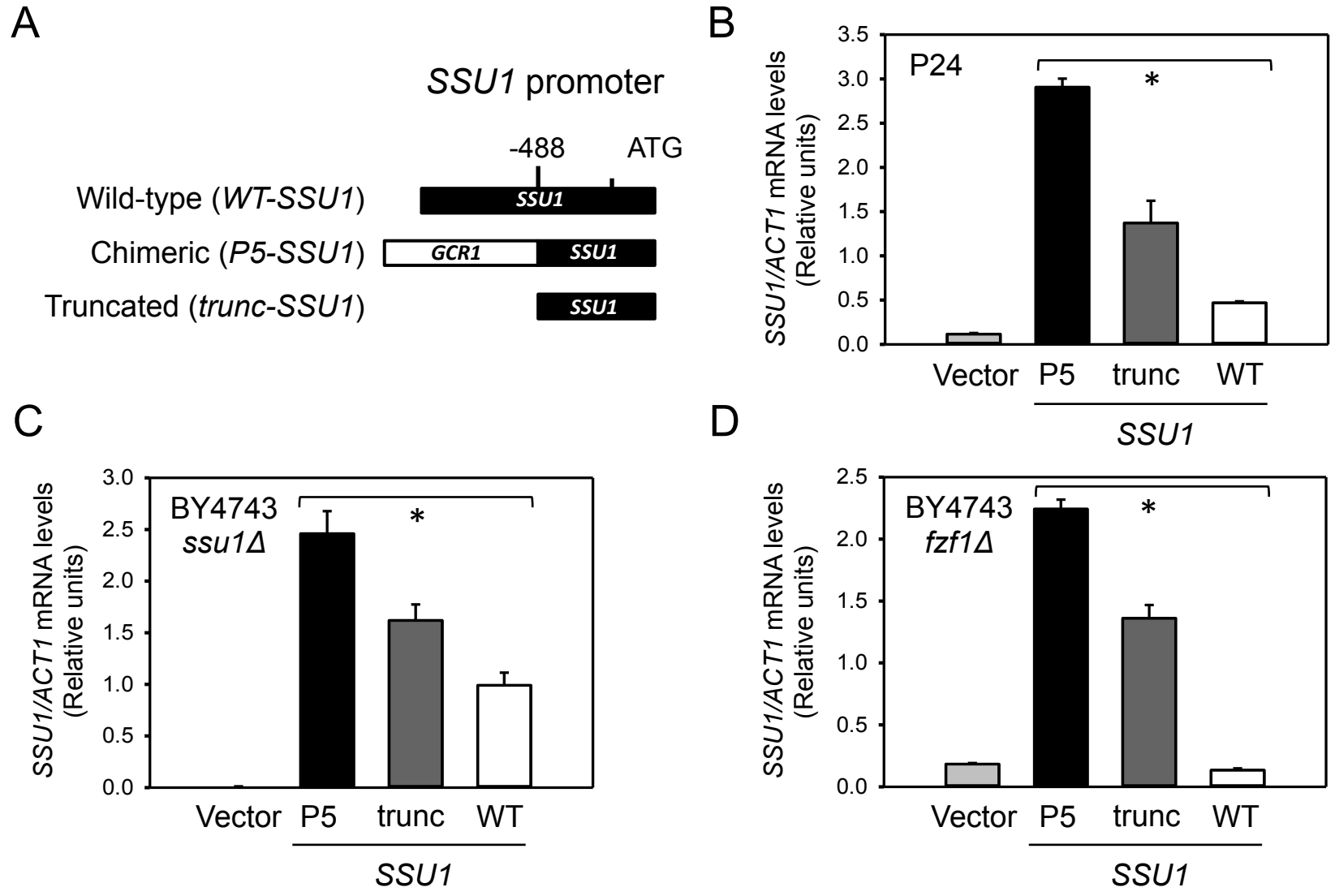

A
SSU1 promoter
-488
ATG
Wild-type (WT-SSU1)
SSU1
Chimeric (P5-SSU1)
GCR1
SSU1
Truncated (trunc-SSU1)
SSU1
B
P24
*
SSU1/ACT1 mRNA levels (Relative units)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Vector
P5
trunc
WT
SSU1
C
BY4743
ssu1Δ
*
SSU1/ACT1 mRNA levels (Relative units)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Vector
P5
trunc
WT
SSU1
D
BY4743
fzf1Δ
*
SSU1/ACT1 mRNA levels (Relative units)
2.5
2.0
1.5
1.0
0.5
0.0
Vector
P5
trunc
WT
SSU1

Figure 6

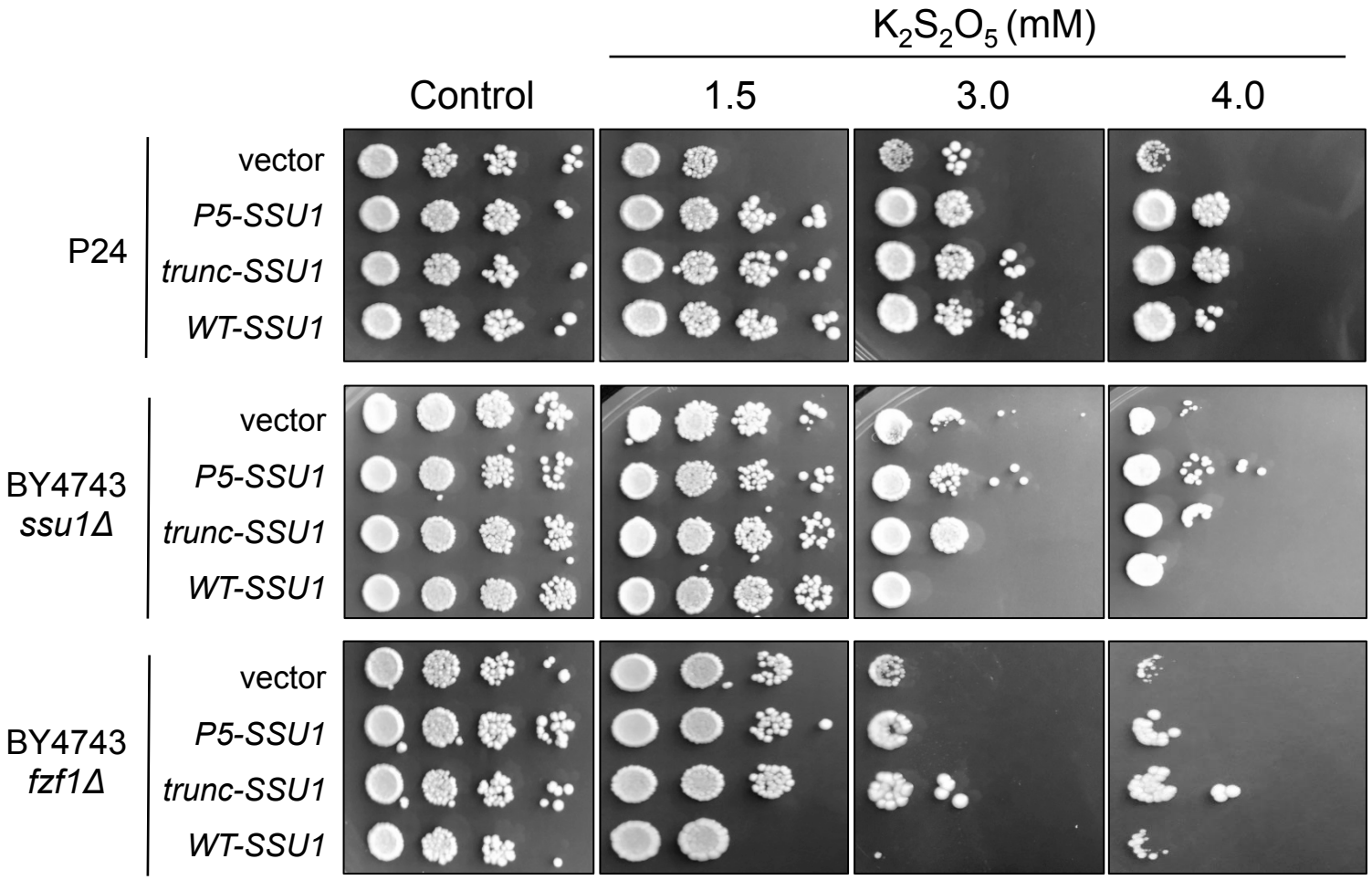

$K_2S_2O_5$ (mM)
Control
1.5
3.0
4.0
P24
vector
P5-SSU1
trunc-SSU1
WT-SSU1
BY4743
ssu1Δ
vector
P5-SSU1
trunc-SSU1
WT-SSU1
BY4743
fzf1Δ
vector
P5-SSU1
trunc-SSU1
WT-SSU1

Figure 7

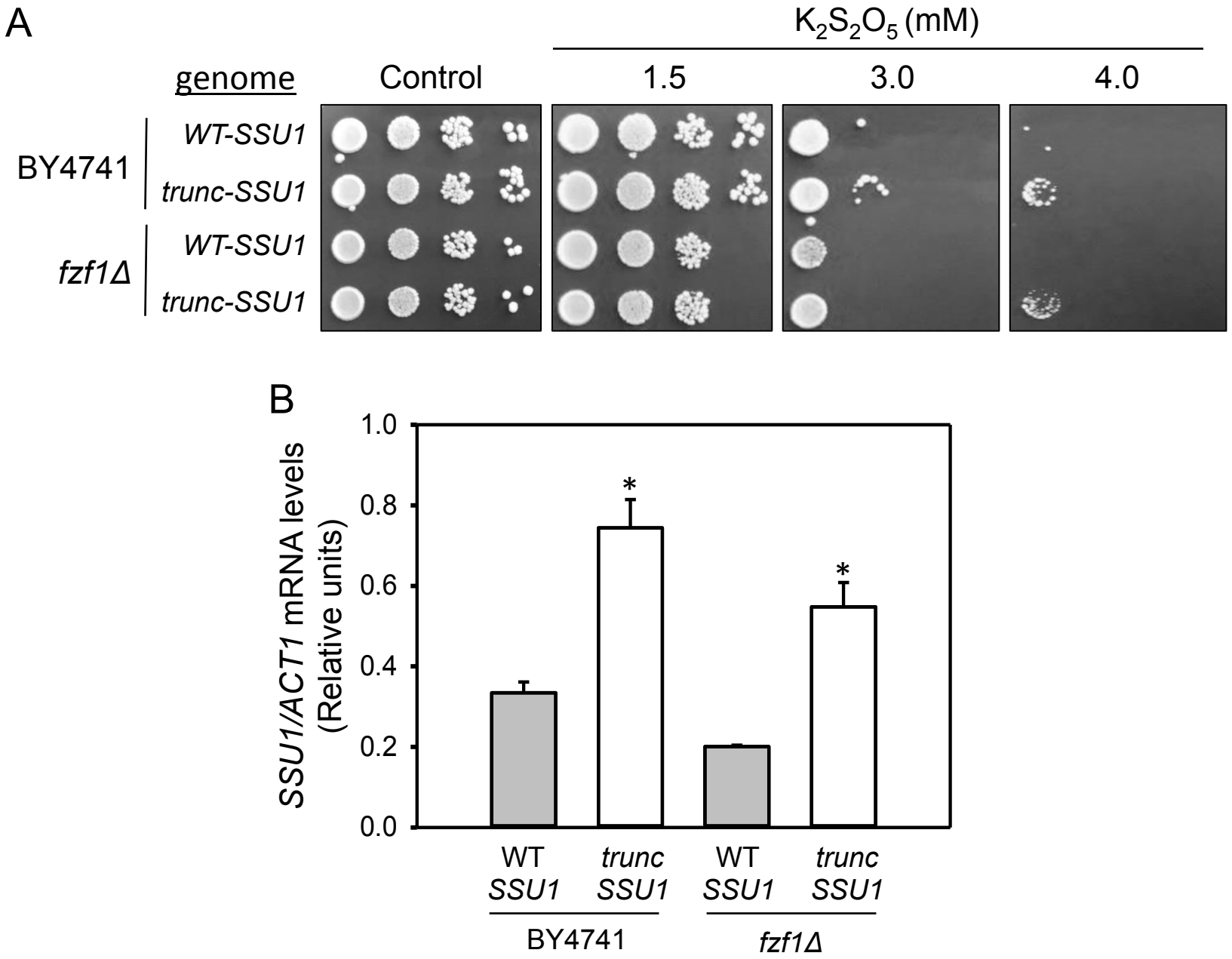
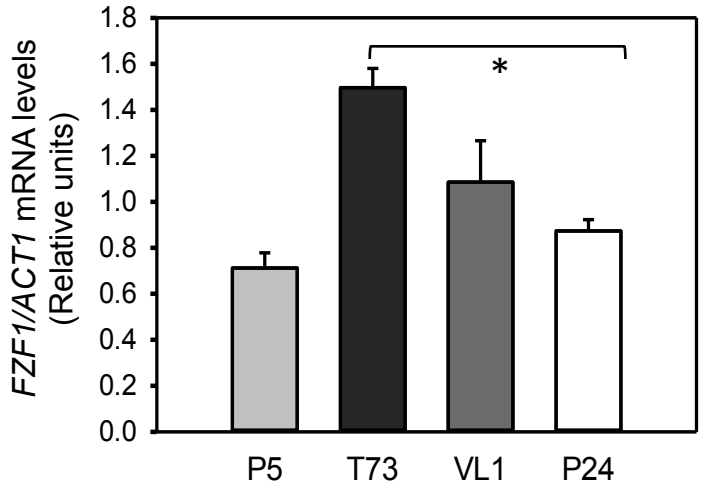

A
$K_2S_2O_5$ (mM)
genome
Control
1.5
3.0
4.0
BY4741
WT-SSU1
trunc-SSU1
fzf1Δ
WT-SSU1
trunc-SSU1
B
SSU1/ACT1 mRNA levels
(Relative units)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
WT
SSU1
trunc
SSU1
WT
SSU1
trunc
SSU1
BY4741
fzf1Δ

Figure S1


1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
FZF1/ACT1 mRNA levels
(Relative units)
P5
T73
VL1
P24