FEMS Yeast Research, 16, 2016, fow021

doi: 10.1093/femsyr/fow021
Advance Access Publication Date: 4 March 2016
Research Article

RESEARCH ARTICLE

RIM15 antagonistic pleiotropy is responsible for differences in fermentation and stress response kinetics in budding yeast

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One sentence summary: The antagonist pleiotropic nature of a *RIM15* null variant positively affects a series of fermentation-related phenotypes.

Editor: Isak Pretorius

ABSTRACT

Different natural yeast populations have faced dissimilar selective pressures due to the heterogeneous fermentation substrates available around the world; this increases the genetic and phenotypic diversity in *Saccharomyces cerevisiae*. In this context, we expect prominent differences between isolates when exposed to a particular condition, such as wine or sake musts. To better comprehend the mechanisms underlying niche adaptation between two *S. cerevisiae* isolates obtained from wine and sake fermentation processes, we evaluated fermentative and fungicide resistance phenotypes and identify the molecular origin of such adaptive variation. Multiple regions were associated with fermentation rate under different nitrogen conditions and fungicide resistance, with a single QTL co-localizing in all traits. Analysis around this region identified *RIM15* as the causative locus driving fungicide sensitivity, together with efficient nitrogen utilization and glycerol production in the wine strain. A null *RIM15* variant confers a greater fermentation rate through the utilization of available glucose instead of its storage. However, this variant has a detrimental effect on fungicide resistance since complex sugars are not synthesized and transported into the membrane. Together, our results reveal the antagonist pleiotropic nature of a *RIM15* null variant, positively affecting a series of fermentation related phenotypes, but apparently detrimental in the wild.

Keywords: *Saccharomyces cerevisiae*; natural variation; *RIM15*; fermentation; fungicide; QTL

Received: 10 November 2015; **Accepted:** 29 February 2016

INTRODUCTION

Efforts in biology have long been focused on the phenotypic and molecular consequences of alternative allelic variants in nature and the genetic basis of adaptation (Mackay, Stone and Ayroles 2009). The budding yeast *Saccharomyces cerevisiae* represents an outstanding model for understanding trait adaptation history, since it is distributed worldwide in an extensive range of growth substrates, thus, encountering diverse ecological conditions (Legras *et al*. 2007; Liti *et al*. 2009; Parts 2014). The Saccharomyces Genome Resequencing Project (SGRP) released genome sequences of dozens of *S. cerevisiae* strains isolated from several geographic locations and sources, such as oak trees, vineyards, palm trees and human patients (Liti *et al*. 2009). These genome sequences were used to determine the phylogenetic relationship among the strains, with as many as half of them falling into five distinct clean lineages, according to the geographic origin or isolation source. Nowadays, the genome sequences of many more strains are publically available (Novo *et al*. 2009; Skelly *et al*. 2013; Bergstrom *et al*. 2014) together with recombinant families derived from directed crosses between representative strains (Ehrenreich, Gerke and Kruglyak 2009; Cubillos *et al*. 2011; Cubillos *et al*. 2013; Illingworth *et al*. 2013; Brice *et al*. 2014). Most important agronomical and industrial traits vary quantitatively in nature due to the extent of natural variation in multiple genes contributing to a trait and their interaction with the environment (Liti and Louis 2012). This information serves as a workhorse for the identification of ecologically and industrially relevant genes and can, then, support predictions for the evolutionary paths of yeast populations. In this context, the latest improvements in molecular technologies have allowed the identification of several genomic regions underlying adaptive traits in yeast related to the fermentation processes (Katou *et al*. 2008; Salinas *et al*. 2012; Steyer *et al*. 2012; Almeida *et al*. 2014; Brice *et al*. 2014).

The fermentation of grape must is a complex microbiological process with an economic and social impact upon human history across centuries (Legras *et al*. 2007; Sicard and Legras 2011). Among the many different microorganisms exploited, *S. cerevisiae* represents the main species responsible for the alcoholic fermentation of different beverages, able to efficiently withstand the unfavourable growth conditions (Querol *et al*. 2003). For example, during the fermentation process, yeast encounters severe osmotic stresses such as high sugar levels and low nitrogen with respect to carbon ratios (Pretorius, Curtin and Chambers 2012). Consequently, it is critical for the outcome of the process to maintain high fermentation rates under these conditions along with high ethanol levels to dominate the fermentation over other microorganisms. Moreover, besides tolerating hostile conditions during the process, yeast strains have to overcome several other stresses in their natural niches during their life cycle. Indeed, previously it has been shown that industrial practices can negatively affect yeast diversity in the wild, reducing the available biota for native or spontaneous fermentations (Ganga and Martinez 2004; Cubillos, Louis and Liti 2009). A notable example of this is the utilization of fungicides in the industry to control for fungus infections in vineyards. These fungicides can have an impact on the yeast diversity present in grapes (Milanovic, Comitini and Ciani 2013). Copper sulphate is widely used in vineyards as a fungicide to treat powdery mildew, and natural yeast strains have shown differences in their ability to proliferate in the presence of this fungicide (Fay *et al*. 2004). In this context, each genetic background uses alternative biochemical pathways to tolerate the stresses of vineyard conditions (Crepin *et al*. 2012).

The establishment of comparative genomics-based strategies between *Saccharomyces* strains has been successfully applied to the identification of chromosomal rearrangements as a signature of selection for optimal sugar utilization in brewing strains (Nakao *et al*. 2009). Likewise, assimilable nitrogen is a key nutrient during the fermentation process, where nitrogen starvation conditions can activate stress responsive genes through the TOR pathway (Tesniere, Brice and Blondin 2015). Grape must contains various potential nitrogen sources such as: proteins, peptides, amino acids and in smaller quantities urea and allantoin. Like most traits in nature, nitrogen assimilation varies quantitatively between strains (Gutierrez *et al*. 2013; Ibstedt *et al*. 2015). Previously, several genetic variants were shown to underlie different nitrogen preferences between isolates (Gutierrez *et al*. 2013; Brice *et al*. 2014; Jara *et al*. 2014). Nevertheless, most natural variants have been mapped utilising non-restrictive nitrogen conditions. Therefore, how genetic variants respond to different nitrogen concentrations, and the consequences for yeast metabolism in stress conditions is still unclear.

Similarly, little is known about the genomic regions responsible for variation to stressors in the wild, such as fungicide resistance. For example, fungicide resistance variation between strains has been linked to copy number variation of the metallothionein *CUP1*, where wine and sake isolates show higher resistance levels due to a greater number of tandem amplifications of this genomic region (Warringer *et al*. 2011). Nevertheless, still little is known about the effect of other fungicides (such as captan and maneb, two fungicides widely used in vineyards), besides copper sulphate, on natural yeast isolates. Up to now, no gene has been identified as responsible for captan resistance in *S. cerevisiae*, whereas for mancozeb (another fungicide of close structural similarity with maneb) a small network of transcription factors (Yap1p and Rpn4p) and plasma membrane multidrug transporters (Flr1p and Tpo1p) have been identified (Teixeira *et al*. 2008; Dias *et al*. 2010). Therefore, despite the efforts made to understand how yeast could tolerate fungicide utilization in the wild, the practical implications of adaptive trait variation is still unclear, stressing the need for a better identification of other genetic factors underlying fungicide resistance.

Thus far, only a minority of the heritable contributions to phenotypes of industrial interest have been explained, and therefore, understanding the genetic basis underlying natural variation in these traits is still a milestone in yeast genetics. Several recent studies have focused on finding causal intermediates utilizing recombinant populations, yielding many loci underlying complex traits in nature (Liti and Louis 2012; Salinas *et al*. 2012; Parts 2014). Some of these loci, like *IRA2*, were shown to underlie more than a single trait, exhibiting opposing effects upon fitness depending on the phenotype (Yadav *et al*. 2015). This genetic trade-off or antagonistic pleiotropy is based on the premise that no mutation can be advantageous for all traits and its role would be to balance fitness over the course of evolution (Fisher 1930). Therefore, in order to propose new alleles, and eventually new yeast strains for industry, it is imperative to assess the fitness of these alleles under several environmental conditions.

Here, we aim to identify genetic loci involved in several phenotypes such as: fermentation kinetics, nitrogen assimilation and fungicide resistance. For this purpose, we used a set of segregants derived from the cross of two widely utilized strains for genetic studies, a sake and a wine isolate obtained from different fermentation processes. We identified genetic variants influencing the fermentation kinetic profile under different nitrogen concentrations and underlying fungicide resistance differences between strains. The results obtained for the different

traits analysed converged towards *RIM15* as a single major locus explaining the phenotypic differences between the two backgrounds. Having the wine allele was a fitness advantage in phenotypes related to the fermentation process, but had a detrimental effect in response to fungicide exposure. The results here demonstrate the antagonistic nature of *RIM15* allelic variants and provide evidence of the importance of analysing each locus in a wide variety of environmental conditions.

MATERIALS AND METHODS

Yeast strains and media culture

Haploid parental strains Y12 (named as Sake, 'SA', *Mat alpha ho::HygMX, ura3::KanMX*), YPS128 (named as North American, 'NA', *Mat alpha ho::HygMX, ura3::KanMX*), DBVPG6044 (named as West African, 'WA', *Mat alpha ho::HygMX, ura3::KanMX*) and DBVPG6765 (named as Wine/European, 'WE', *Mat a, ho::HygMX, ura3::KanMX*), together with the F1 hybrid and 288 segregants (96 per F1 cross) utilised in this study (for the WE × SA, WE × NA and WE × WA crosses) were previously described (Cubillos, Louis and Liti 2009; Cubillos *et al.* 2011).

Fermentation in synthetic wine must

Fermentations were carried out in triplicates in synthetic wine must and MS300 prepared according to Rossignol *et al.* (2003) and Jara *et al.* (2014), where the nitrogen content was adjusted depending on the MS (Synthetic Must) tested. For MS60 and MS600, each nitrogen source was proportionally reduced from MS300 five times and duplicated two times, respectively. The amount of each amino acid and ammonium source is explained in detail in Table S1 (Supporting Information). For each experiment, the strains were initially grown under constant agitation in 10 mL of MS300 during 16 hours at 25°C. Next, 1×10^6 cells mL^{-1} were inoculated into 12 mL of each MS (in 15 mL conical tubes) and incubated at 25°C, with no agitation. Fermentations were weighed every day to calculate the CO_2 output until the daily CO_2 lost represented less than 10% of the accumulated CO_2 lost.

To estimate nitrogen consumption differences, 12 mL of synthetic grape must (MS300) was harvested at day 6, centrifuged at 9000 × *g* for 10 min, and the supernatant was collected. A total of 20 uL of MS300 was injected into Shimadzu Prominence HPLC equipment (SHIMADZU, Kyoto, Japan) using a Bio-Rad HPX–87H column according to Nissen *et al.* (1997). The concentration of each amino acid was estimated using the HPLC analysis as previously described (Gomez-Alonso, Hermosin-Gutierrez and Garcia-Romero 2007). The consumption of each nitrogen source was estimated as the difference between the initial and final amounts of each source before and after fermentation, respectively. Similarly, succinic acid and glycerol were estimated at the end of the fermentation for each case.

QTL mapping

The mapping of QTLs was performed as previously described (Cubillos *et al.* 2011) using the rQTL software (Broman *et al.* 2003). LOD scores were estimated using a non-parametric model and the significance of each region was determined from permutations. Briefly, for each trait and cross, we permuted the phenotype values 1000 times, recording the maximum LOD score each time. We called a QTL significant if its LOD score was greater than the 0.05 tail of the 1000 permuted LOD scores. The percentage of phenotypic variance explained for a QTL was calculated using the following formula, where n represents the sample size:

$$\text{Percentage of variance explained} = 100(1–10^{(-2\text{LOD}/n)}).$$

Fungicide resistance assay

The solid media phenotyping assay was carried out by spotting each segregant in YPDA medium and incubating for 48 and 96 h at 28°C for the control (YPDA) and fungicide treatment, respectively. For each experiment, fungicide was added to the sterilized medium immediately before solidifying in the Petri dish. Each experiment was performed in triplicate, and the SA parental strain was included as a normalizer between experiments. For colony growth estimation, plates were directly scanned and phenotyping was performed by obtaining the pixel intensity of each spot/strain using ImageJ software. For each case, the pixel intensity was normalized to that of the SA strain within every plate. The relative phenotypic value of the fungicide treatment was then estimated as the ratio of the colony growth in fungicide divided by that of the control condition.

For the microculture phenotyping assay, segregants were precultivated in 200 uL of YNB medium supplemented with uracil (0.67% yeast nitrogen base, 2% glucose, 0.2% uracil) during 48 h at 28°C. For the experimental run, segregants were inoculated to an optical density (OD) of 0.03–0.1 (wavelenght 630 nm) in 200 uL and incubated without agitation at 28°C for 24 h (YNB control) and 72 h (fungicide treatments) in a BioTek EL808TM (BioTek, Winooski, VT, USA) absorbance microplate reader. OD was measured every 20 min using a 630 nm filter. Each experiment was performed in duplicate, and the laboratory strain S288c was included to normalize between experiments. Relative fitness variables for each strain and trait were calculated as previously described (Warringer *et al.* 2011). Briefly, the mitotic proliferation rate (maximum first derivative), lag (inverse of the time when the second derivative is maximum) and efficiency (difference between the maximum and minimum OD) were extracted from high-density growth curves and $\log_2$ transformed (the measure of proliferation lag was inverted to maintain directionality between fitness components).

Reciprocal hemizygosity

In order to generate the reciprocal hemizygotes, initially *RIM15* knockouts were generated from each parental strain (Table S2, Supporting Information). For both strains, the *URA3* gene was used as a selectable marker. Transformations were performed as previously described (Jara *et al.* 2014). Briefly, haploid versions of the parental strains (WE *MAT a, ho::HygMX, ura3::KanMX* and SA *MAT alpha, ho::NatMX, ura3::KanMX*) were used to delete *RIM15* and construct all the possible combinations of single deletions in the hybrid. For this, mutated parental strains were crossed to generate the reciprocal hemizygote strains and selected on double drug plates (50 mg mL^{-1} Hygromycin B and 100 mg mL^{-1} Nourseothricin). Diploid hybrid strains were confirmed using *MAT* locus PCR (Huxley, Green and Dunham 1990) and the *RIM15* deletions were confirmed by PCR using the primer pairs A1/S8 or A4/S5. A1 and A4 primers are listed in Table S2 (Supporting Information), while S5 and S8 have been previously described elsewhere (Salinas *et al.* 2012). *GAT1* and *YFL040W* reciprocal hemizygotes were obtained in a previous study (Salinas *et al.* 2012).

RIM15 sequencing and analysis

Genomic yeast DNA from Chilean isolates was extracted using the MasterPure Yeast DNA Purification Kit (Epicentre). DNA sequence around the *rim15c459_460insCA* was directly obtained from PCR products by Macrogen (http://dna.macrogen.com/) (Goujon *et al.* 2010; Kearse *et al.* 2012). Primers used are listed in Table S2 (Supporting Information).

The Rim15 protein sequences were obtained from 38 strains in the SGD database, eight strains with full Rim15 protein sequences from the SGRP database (DBVPG1106, DBVPG1373, DBVPG6765, UWOPS03-461.4, UWOPS83-787.3, UWOPS87-2421, Y12 and YJM975) and the information from two Chilean strains (L-323 and L-348), where we fully sequenced *RIM15* ORF using Sanger sequencing. A total of 48 protein sequences were aligned using Clustal omega (Goujon *et al.* 2010). We used the alignment information to generate a complete list of amino acid changes in Rim15p across strains using Rim15p from S288c strain as a reference. The secondary structure modifications due to amino acid changes relative to the reference sequence were predicted using EMBOSS 6.5.7 and the GOR method (Garnier, Osguthorpe and Robson 1978). Based on the Rim15 alignment information, we built a phylogenetic tree using PHYML (Guindon *et al.* 2010).

RESULTS

Mapping Quantitative trait loci in different nitrogen fermentation conditions

In order to identify genomic intervals underlying fermentation kinetic differences in diverse nitrogen fermentation conditions, we selected two *S. cerevisiae* isolates belonging to clean linages and which are associated with human activities, a Sake (SA, Y12) and a Wine/European strain (WE, DBVPG6765). These isolates have been previously shown to display dissimilar patterns of nitrogen assimilation preferences and fermentation kinetic profiles (Jara *et al.* 2014), representing an interesting background of genetic diversity for quantitative trait studies. Initially, we characterized the fermentation kinetics profile of the parental strains by growing them under different nitrogen conditions in three synthetic wine musts (MS): (i) MS60, (ii) MS300 and (iii) MS600 (see Methods). We followed the CO_2 output for 21 days and compared the CO_2 fermentation rate between isolates. We estimated that the fermentation profile of the two strains significantly differed, with the WE isolate showing a greater maximal fermentation rate (V_{max}) compared to the SA isolate at all concentrations (Fig. 1A; *P*-value < 0.05). Moreover, in MS60 the WE strain reaches its V_{max} at an earlier time (37 hours, 0.57 g L^{-1}*h) than the SA isolate (53 hours, 0.41 g L^{-1}*h), suggesting a stronger capacity of adaptation and ability to assimilate nitrogen in low nitrogen fermentation musts only, in agreement with its niche adaptation to grape must.

Next, in order to map the genetic variants underlying the phenotypic differences previously observed between the two strains, we utilized a segregating population consisting of 96 segregants derived from a WE × SA F1 hybrid (Cubillos *et al.* 2011). For each segregant, we repeated the fermentation strategy performed in the parental strains for all nitrogen conditions and measured the CO_2 output across 21 days. The fermentations were carried out in triplicate, with three completely independent biological replicates for each segregant. We estimated the fermentation rate for the whole population (Table S3, Supporting Information) and the individual distributions are shown in Fig. 1B. Interestingly, we only observed negative transgressive segregants, where the WE parental strain exhibited the greatest fermentation rate in all conditions (Fig. 1B). To identify quantitative trait loci (QTLs) responsible for the phenotypic differences in the WE × SA population, we performed interval mapping utilizing the genotype database from our previous studies (Cubillos *et al.* 2011; Salinas *et al.* 2012; Jara *et al.* 2014). Overall, we identified four QTLs, one of them found at several intervals during the fermentation process (Table S4, Supporting Information). The greatest QTL effect in all three nitrogen conditions correspond(s) to QTL VI.65, explaining up to 20% of the phenotypic variance in the different fermentation musts, while the rest of the genomic regions were only found in MS600 (Fig. 1C). Segregants carrying the WE variant for QTL VI.65 evidenced a greater fermentation rate in the initial stages of the fermentation process compared to those carrying the SA allele; QTL VI.65 was therefore a good candidate region to identify alleles involved in high fermentation rates (Fig. 1D). Interestingly, this QTL has also been mapped in our two previous studies underlying glycerol, residual sugar, malic acid (Salinas *et al.* 2012) and nitrogen assimilation differences (Jara *et al.* 2014), suggesting a pleiotropic effect in several oenological phenotypes. Altogether, these results suggest a shared genetic regulator influencing the fermentation kinetics profiles and several wine-related phenotypes in the WE × SA population, independently of the nitrogen available in the must.

Identification of genomic regions underlying fungicide resistance in yeast

Yeasts face several stress conditions not only in the fermentation must, but also in nature. An important example is the need to resist fungicides widely used in vineyards. In order to characterize natural variation of fungicide resistance in yeasts, a fungicide-sensitivity plating assay was performed. This entailed spotting a 10-fold serial dilution of cells on YPDA media supplemented with two widely used fungicides in vineyards, captan and maneb. The two haploid versions of the industrial strains previously used for our QTL mapping strategy (WE and SA) as well as a wild North American (NA) isolate and a West African (WA) strain were evaluated, and their sensitivities to the fungicides scored (Liti *et al.* 2009). For both fungicides, we used the necessary concentration to better discriminate the phenotype of the four strains; this concentration was close to the previously reported IC_{50} (Fig. S1, Supporting Information) (Fai and Grant 2009). The SA and the WE strains showed the two most extreme phenotypes, with the SA isolate showing the greatest tolerance levels to captan and maneb, while the WE isolate grew poorly in the presence of these fungicides (Fig. S1, Supporting Information).

To investigate the genetic architecture underlying fungicide resistance variation between industrial isolates, we initially estimated levels of resistance in the WE × SA recombinant population in solid medium. For this purpose, we spotted each segregant on a YPDA plate containing fungicide and estimated colony growth after 96 h (see Methods). All phenotypes showed a continuous distribution, suggesting, once again, a polygenic contribution. We subsequently performed linkage analysis as in the previous section and mapped two QTLs for maneb tolerance (Fig. 2A) and a single QTL for captan resistance at a 5% FDR (Fig. 2A). Interestingly, the single QTL mapped for captan (VI.65; previously mapped as QTL3 in Jara *et al.* 2014) was also found for maneb and represents the strongest interval for both fungicides, explaining 30.5% and 49.9% of the phenotypic variation

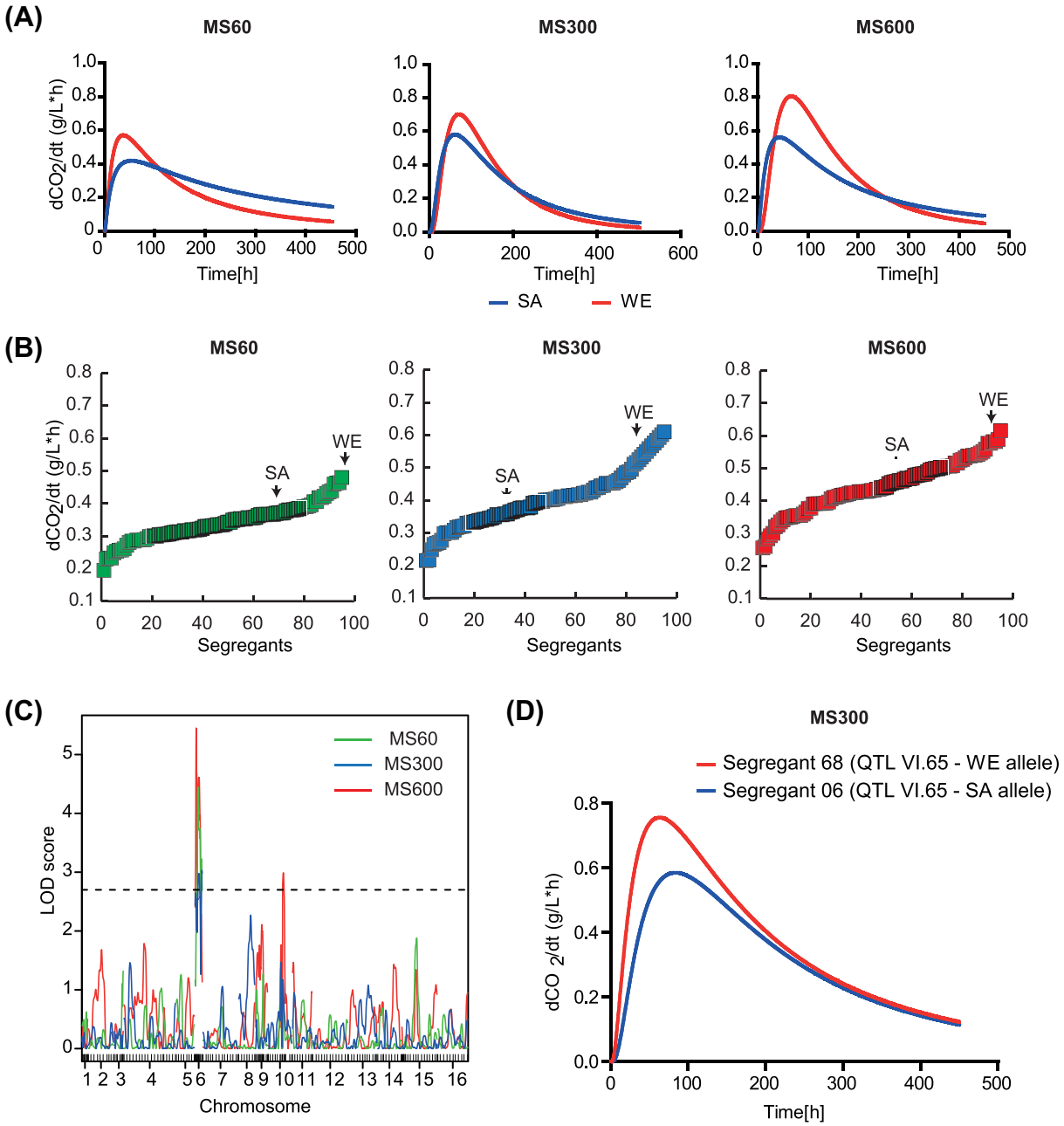

Figure 1. Fermentation kinetics in WE × SA individuals. (**A**) The fermentation rate (g L^{-1}*h) was estimated in MS60 (Left panel), MS300 (central panel) and MS600 (right panel) for the WE (red) and SA (blue) isolates. Plots represent a Gaussian (log) regression of three replicates (**B**) The WE × SA maximum fermentation rate profile for each segregant is shown in the different fermentation musts (MS60: green; MS300: blue; MS600: red). (**C**) LOD plot from linkage analysis for fermentation rate in MS60 (green), MS300 (blue) and MS600 (red) using a nonparametric model. (**D**) Fermentation rate for two segregants carrying alternative genotypes for QTL VI.65 (WE in red and SA in blue).

for maneb and captan, respectively (Table S5, Supporting Information). Furthermore, in order to investigate how pervasive this QTL was, we performed linkage mapping in two additional F1 populations, having WE as a common parent crossed against the NA (fungicide resistant) and WA (fungicide sensitive) isolates. In the WE × NA cross, we also found QTL VI.65 for the same two fungicides, but not in the cross between the two most sensitive strains (WE × WA), suggesting a context independent effect (Fig. S2, Supporting Information).

Next, in order to exhaustively assess the genetics underlying fungicide resistance differences between the WE and SA strains utilizing a quantitative approach, segregants were subjected to precise growth phenotyping while exposed to both fungicides. We quantified mitotic growth properties (rate, lag and efficiency) (Warringer *et al.* 2011) and performed linkage mapping as previously explained. A total of two and three QTLs were found for maneb and captan, respectively (Table S5, Supporting Information; Fig. 2B and C), expanding the number of QTLs from two in solid media to a total of three in microculture. The strongest QTL for maneb was mapped in chromosome VII (VII.331) for all three parameters and explaining over 20% of the phenotypic variance (Table S5, Supporting Information). Nevertheless, the

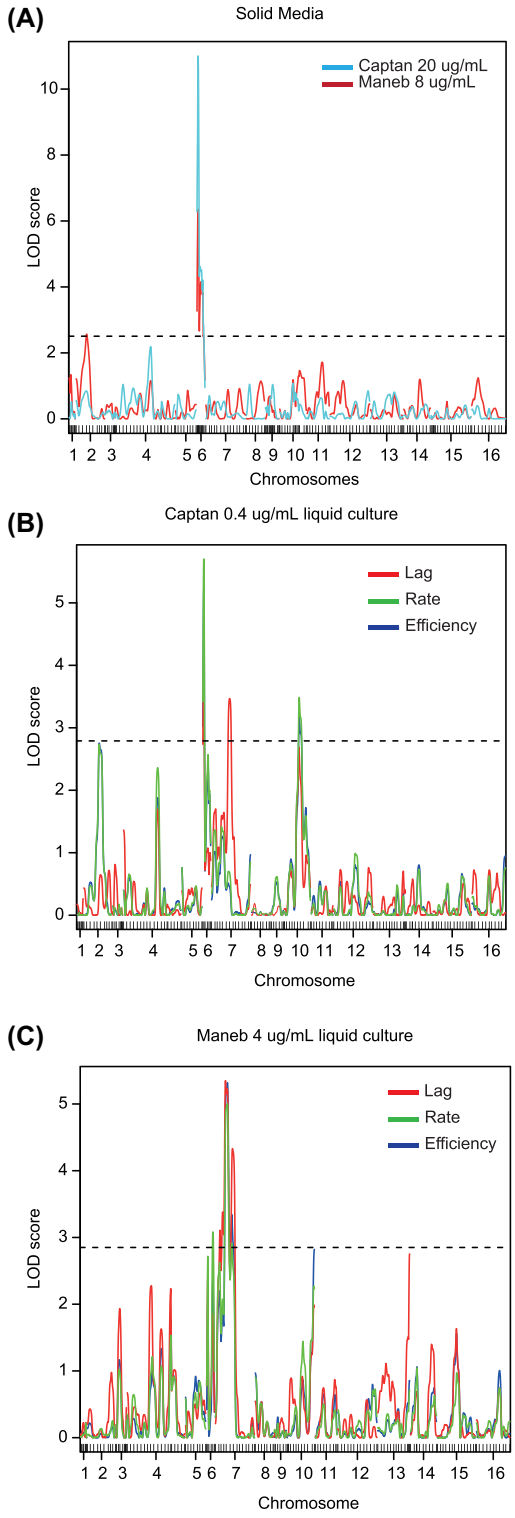

Figure 2. Fungicide Linkage Analysis. LOD plots from linkage analysis for the different traits analysed, (**A**) Captan 20 ug mL^{-1} and Maneb 8 ug mL^{-1} in solid media, (**B**) Captan 0.4 ug mL^{-1} in microcultures and (**C**) Maneb 4 ug mL^{-1} in microcultures. For (B) and (C) Lag (red), Rate (green) and Efficiency (blue) parameters are plotted.

QTL VI.65 mapped in solid media conditions was also identified in liquid cultures for both fungicides and for several growth parameters, also representing the strongest QTL for captan tolerance (Fig. 2B-C). Overall, our results suggest the presence of a major locus within chromosome VI underlying fungicide resistance, together with a series of other oenological phenotypes.

RIM15 exhibits antagonistic pleiotropy underlying natural variation between WE and SA isolates

In order to find candidate genes underlying the previously observed phenotypic differences between segregants, we further analysed the pleiotropic genomic interval surrounding QTL VI.65, which contains ~28 genes. Among this set of genes, one represented a good functional candidate, *RIM15*. This gene encodes for a protein kinase involved in cell proliferation in response to nutrients and has been previously shown to vary between strains, causing phenotypic differences for traits such as: fermentation rate (Watanabe *et al.* 2012), nitrogen utilization (Ibstedt *et al.* 2015) and sporulation efficiency (Bergstrom *et al.* 2014), the last two explained due to a loss-of-function mutation originating from a CA insertion in the *RIM15* WE allele (*rim15c459_460insCA*) (Bergstrom *et al.* 2014). Therefore, *RIM15* was chosen as an initial candidate for a reciprocal hemizygosity assay in order to estimate the phenotypic contribution of each allele towards fermentation kinetics and fungicide resistance.

Initially, we evaluated the fermentation kinetics for each *RIM15* reciprocal hemizygote in MS60, MS300 and MS600 and did not find significant differences in the total CO_2 output or fermentation rate in any of the nitrogen conditions tested (Fig. 3A). We further investigated the role of *RIM15* in fermentation kinetics and compared the CO_2 output profile in MS300 between the wild type and the corresponding *RIM15* knockout strain for each genetic background. In the WE background, we did not observe any differences between the wild-type strain and the WE *rim15::URA3* knockout strain, confirming null activity of the $RIM15^{WE}$ allele (Fig. S3, Supporting Information). In contrast, in the SA background, we observed a greater CO_2 output and a greater fermentation rate in the SA *rim15::URA3* knockout strain with respect to the wild-type strain, demonstrating the positive effect upon fermentation kinetics of a *RIM15* null variant. In parallel, and in order to evaluate whether other genes within the genomic interval of QTL VI.65 were implicated in fermentation kinetic differences between WE and SA strains, we evaluated reciprocal hemizygotes for two other candidate genes, *GAT1* and *YFL040W*. These genes were previously described to be involved in glycerol production differences between WE and SA isolates (Salinas *et al.* 2012). As observed for *RIM15*, the total CO_2 output between reciprocal hemizygotes for these two genes did not differ under any nitrogen condition (Fig. S4, Supporting Information), suggesting that alternative genetic factors within the same interval could underlie fermentation kinetic differences between WE and SA isolates.

Subsequently, based on the differences observed in musts with diverse nitrogen concentration and our previous findings of a QTL interval mapped for nitrogen assimilation differences (specifically tryptophan and lysine, previously defined as QTL3 in (Jara *et al.* 2014)) in the *RIM15* region (Jara *et al.* 2014), we evaluated the nitrogen consumption profile in MS300 at day 6 for the *RIM15* reciprocal hemizygotes. Overall, the total yeast assimilable nitrogen (YAN) for amino acids did not differ (paired student test, $P = 0.1$). However, we found individual differences for aspartic acid, histidine, glutamine, tryptophane and leucine (ANOVA, $P < 0.05$), where the $RIM15^{WE}$ allele showed greater nitrogen consumption levels, while the opposite was observed for lysine, with the $RIM15^{SA}$ allele exhibiting higher assimilation levels (Fig. 3B). Likewise, $RIM15^{WE}$ showed greater ammonium

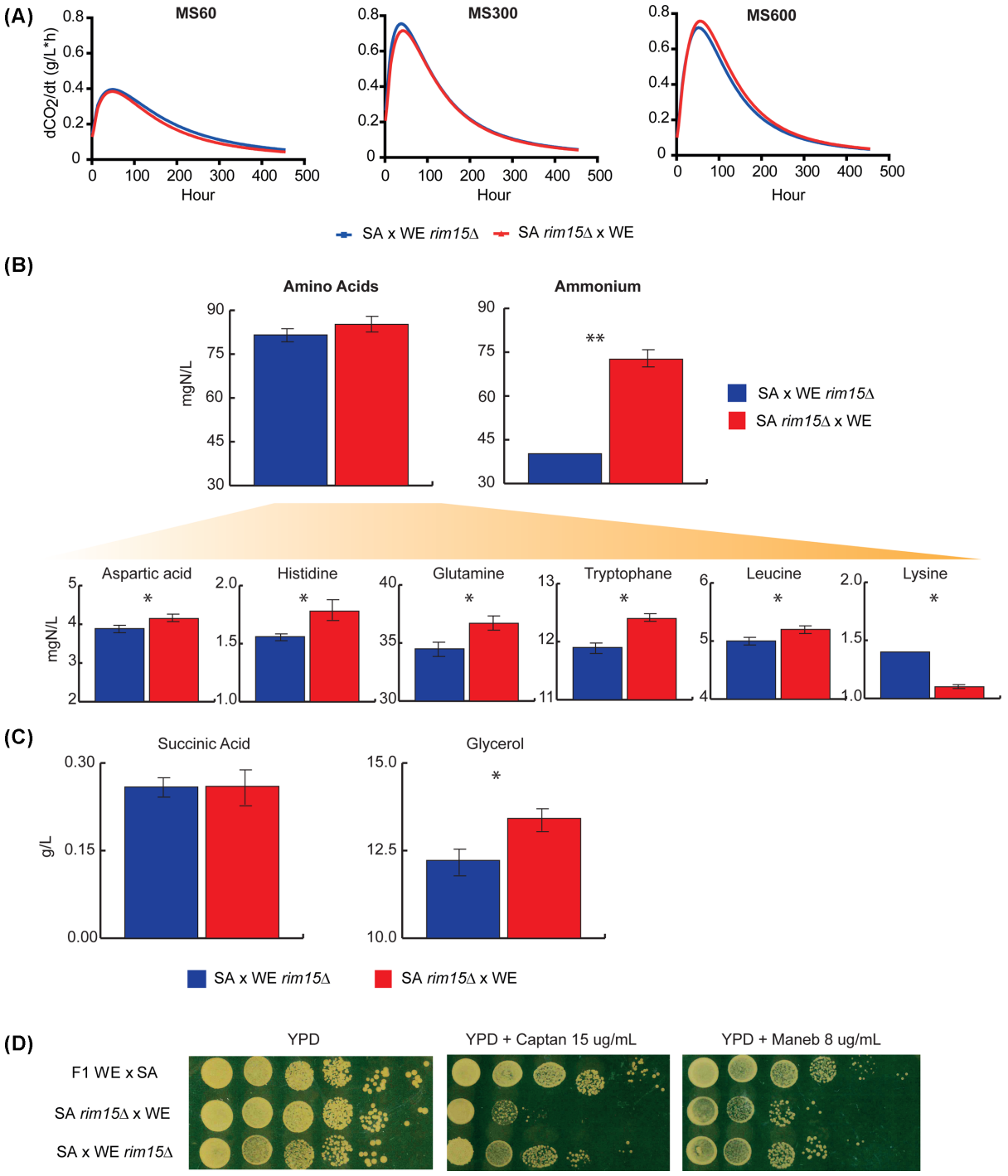

Figure 3. Validation of *RIM15* reciprocal hemizygotes phenotypes. Reciprocal hemizygotes SA × WE *rim15*Δ (blue) and SA *rim15*Δ × WE (red) were evaluated in triplicates for (**A**) Fermentation rate in MS60 (left panel), MS300 (central panel) and MS600 (right panel), (**B**) Nitrogen consumption as total Amino Acid YAN (left superior panel), ammonium YAN (right superior panel), Aspartic acid, Histidine, Glutamine, Tryptophane, Leucine and Lysine YAN (bottom panel), (**C**) Succinic acid and Glycerol and (**D**) Fungicide resistance in Captan (15 ug mL^{-1}) and Maneb (8 ug mL^{-1}). (*) < 0.05 and (**) < 0.01. In all cases, *P*-values were obtained using a one-way ANOVA.

consumption levels with respect to $RIM15^{SA}$, validating the role of *RIM15* in nitrogen consumption differences between WE and SA isolates. In addition, this genomic interval (VI.65) has also been implicated in succinic acid and glycerol production during grape must fermentation in previous studies (Salinas *et al.* 2012). Thus, we evaluated the production of these two compounds in the reciprocal hemizygotes after 21 days of fermentation and found significant differences for the total glycerol content in the final fermentation must (Fig. 3C). Interestingly, the $RIM15^{WE}$ variant showed greater glycerol production levels compared with the $RIM15^{SA}$ allele, $RIM15^{WE}$ therefore represents an interesting allele for the wine industry.

Finally, QTL VI.65 was also found for the two fungicides evaluated in this study, captan and maneb. We evaluated the response of each reciprocal hemizygote in the presence of both fungicides in solid media. Contrasting what was observed for the

previous oenological phenotypes, the *RIM15*^{WE} variant showed a detrimental effect for fungicide resistance. Particularly, in the case of captan as we observed up to 100× better growth of the hybrid when the SA allele was present (Fig. 3D). Similarly, for maneb we estimated a 10× growth difference with a superior performance of the SA allele over the WE allele. These results suggest a detrimental effect of the loss-of-function *RIM15*^{WE} variant in response to these strong stress conditions. Altogether our results suggest an antagonistic pleiotropic effect of the *RIM15*^{WE} variant, with detrimental or beneficial effects depending on the environmental condition.

The WE strain carries a rare insertion not found in other wine isolates

The *RIM15*^{WE} variant carries a CA insertion at position 549 (*rim15c459_460insCA*), causing an early stop codon. Initially, in order to determine the allele frequency of the *rim15c459_460insCA* insertion (Bergstrom *et al*. 2014) in other populations, we examined the *RIM15* sequence from genomes available in the SGD and SGRP databases (38 and six isolates, respectively). The chosen strains included wild, wine, clinical and domesticated isolates (Liti *et al*. 2009). For each strain, we extracted the region around the insertion and performed a sequence alignment. We found that the CA insertion was not present in any other strain (Fig. 4A), suggesting that this could be a rare variant in yeast. In order to further test this hypothesis, we analysed the region in other strains from the Wine/European cluster. For this, we re-sequenced by Sanger sequencing the region containing the CA insertion in 27 Chilean isolates obtained directly from vineyards and determined the sequence for each strain. Once again, the CA insertion was not found in any strain (Fig. 4B), suggesting that the *RIM15*^{WE} allele represents a rare variant event among wine strains and, subsequently, within the species.

Moreover, in order to determine if there were other *RIM15* null mutants, we re-sequenced by Sanger sequencing the full *RIM15* ORF of two Chilean isolates and we analysed these sequences together with the 46 isolates mentioned above for which full sequences of Rim15p are available. We observed several amino acid changes across strains, some of them affecting the secondary structure of the Rim15p. For example, we found that a group of wine strains (L-323, EC1118, L-1528 and L-348) have a turn disruption due to a T1378I amino acid change, which instead generates a new beta strand. These results strongly suggest a possible impaired function of Rim15p in these strains, which are closely related to the WE isolate (Table S6 and Fig. S5, Supporting Information).

DISCUSSION

Quantitative genetic studies have assisted the exploration of allelic variants between strains that can explain variation in performance, in terms of adaptation to local stressors and fermentation musts (Marullo *et al*. 2007; Katou *et al*. 2008; Salinas *et al*. 2012; Brice *et al*. 2014; Garcia-Rios, Lopez-Malo and Guillamon 2014). In this study, we have attempted to identify genetic variants underlying differences in several fermentative-related phenotypes between two domesticated yeast strains, a wine and sake isolate. For this, we exposed the strains to a series of synthetic wine musts with varying levels of available nitrogen and to two widely used fungicides in the industry. For all phenotypes, we found substantial differences between both strains (Fig. 1; Fig. S1, Supporting Information). The WE strain was hardly affected by low nitrogen levels in the must, exhibiting greater fermentation rates at all the concentrations tested in comparison to the SA strain. These results are not surprising, since the synthetic wine must utilized resembles the natural environment of the WE strain, and therefore, greater fitness compared to the SA strain is expected. In contrast, the opposite result was obtained for fungicide tolerance, where the WE strain exhibited a poor performance with low levels of resistance. As a result, the main question in this study is what genomic regions underly these differences and can they help us to understand the evolutionary processes occurring in these two backgrounds? To partially solve this question, we utilized an F1 recombinant population derived from these two strains and performed a linkage analysis strategy. In this way, we mapped multiple QTLs (Fig. 2; Table S4 and 5, Supporting Information), with QTL VI.65, common to all traits. Interestingly, in our previous studies in similar fermentation musts, the same region was also implicated in glycerol and malic acid production, together with glucose, fructose, tryptophan and lysine consumption (QTL3) (Salinas *et al*. 2012; Jara *et al*. 2014). Taken together, we decided to look for candidate genes within the VI.65 genomic interval and identified *RIM15* as a potential candidate that required validation. *RIM15* encodes a 1770 amino acid protein kinase involved in multiple cellular processes such as: stress response, diauxic shift, nutrient starvation, fungicide sensitivity and entry into stationary phase (Reinders *et al*. 1998; Cameroni *et al*. 2004; Wei *et al*. 2009; Costa *et al*. 2015; Ibstedt *et al*. 2015). Furthermore, in previous reports, *RIM15* has been shown to underlie differences between strains for nitrogen limiting conditions (Ibstedt *et al*. 2015), sporulation efficiency (Bergstrom *et al*. 2014) and fermentation kinetics in modern sake strains (Watanabe *et al*. 2012). *RIM15* is part of the TORC1 and Ras/PKA signalling pathways and is phosphorylated through Sch 9, establishing its subcellular localisation (either in the cytoplasm or the nucleus) (Pedruzzi *et al*. 2003; Swinnen *et al*. 2006; Mirisola *et al*. 2014). When present in the nucleus, Rim15p directly activates Msn2/4 through phosphorylation, which then activates stress response genes (Martinez-Pastor *et al*. 1996; Gasch *et al*. 2000). Moreover, Rim15p is also able to interact with the Gis1 transcriptional factor, which is involved in the diauxic shift response (Pedruzzi *et al*. 2000).

RIM15^{WE} contains a two base pair insertion, *rim15c459_460insCA*, causing an early stop codon (Bergstrom *et al*. 2014). This mutation is not present in the SA background, where the *RIM15* allele would be fully functional. In our study, the different stresses imposed converged toward *RIM15* as a putative causal locus underlying a series of traits. A reciprocal hemizygosity assay did not show any significant differences between allelic variants at any of the nitrogen concentrations evaluated, suggesting that *RIM15* would not be the responsible locus for QTL VI.65 (Fig. 3A). Similarly, evaluation of other candidate genes within the same interval did not show differences in the fermentation rate between WE and SA alleles (Fig. S4, Supporting Information), suggesting that either other genes within the same interval underlie the trait or more complex genetic interactions are involved. Nevertheless, further evaluation of *RIM15* knockouts confirmed in the SA background that a deletion of *RIM15* confers a more efficient fermentation rate and therefore null *RIM15* alleles could confer an advantage upon fermentation kinetics (Fig. S3, Supporting Information). Further, evaluation of other oenological phenotypes allowed us to validate differences between hemizygotes for ammonium and glycerol production. Strains containing the *RIM15*^{WE} allele showed greater ammonium consumption and glycerol production levels compared to the active *RIM15*^{SA} variant. These results

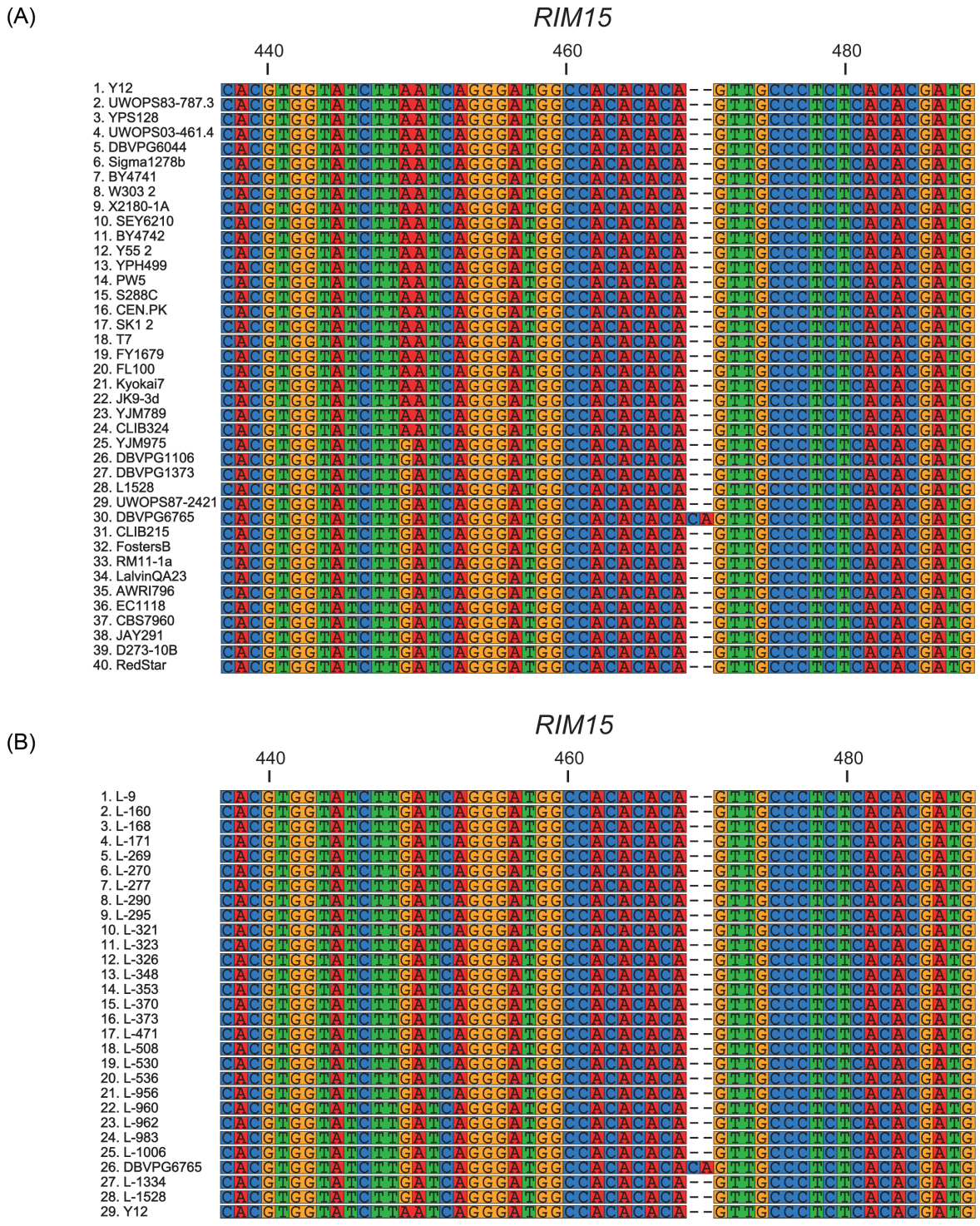

Figure 4. Sequences of natural isolates in the region around the *RIM15* insertion. The *RIM15* sequence alignment between nucleotides 437 and 489 within the ORF is shown for (**A**) 40 isolates of different geographical origins and (**B**) 27 Chilean isolates isolated from vineyards.

could suggest that a null *RIM15* variant would be selected for in wine fermentation conditions, given that ammonium is the predominant form of nitrogen in the must and the relative importance of glycerol in the final wine product. However, the *RIM15*^{WE} allele would not confer an advantage in the field given the low resistance levels to the two fungicides evaluated in this study, suggesting an antagonistic pleiotropic effect. Similar cases have been described for many other genes in yeast, for which in some conditions null alleles confer an adaptive advantage, while in others a detrimental effect (Qian *et al.* 2012; Yadav *et al.* 2015). *IRA2* and multiple other components of the Ras/PKA signalling pathway have also shown antagonistic pleiotropy in natural populations (Yadav *et al.* 2015). The *RIM15*^{WE} variant would represent another example exhibiting opposing effects

in different phenotypes; however, this variant has not been found in any other strain (Fig. 4) (Liti *et al*. 2009).

Our results agree well with previous observations on modern sake yeast strains, which also contain an early stop codon due to an insertion in nucleotide 5055 in the C-terminal region of the encoded protein (Watanabe *et al*. 2012). These strains displayed quiescence-related deficient phenotypes and increased fermentation rates compared to other sake strains. The Y12 sake strain used in our study does not belong to the group of strains containing an early stop codon and therefore contains an active *RIM15* allelic variant, contrasting with the WE strain which contains a null allele (Bergstrom *et al*. 2014). Moreover, it has recently been shown that the absence of *RIM15* would decrease the stores of sugars, such as trehalose and beta-glucans, since *Pgm2* (phosphoglucomutase which catalyses the conversion from glucose-1-phosphate to glucose-6-phosphate) and *Ugp1* [UDP-glucose pyrophosphorylase, which catalyses the reversible formation of UDP-Glucose from glucose 1-phosphate and UTP will not be expressed (normally activated through Msn2/4 and Hsf1; Watanabe *et al*. 2015). Trehalose and 1,3 beta-glucans are essential for survival under stress conditions, and particularly, given that beta-glucans are among the main components of the yeast cell wall, which would be affected in the absence of *RIM15*. These antecedents agree well with our findings and could explain the greater sensitivity of the *RIM15* null strain (WE) to fungicide exposure. Moreover, since glucose is not being stored, it is now available for the glycolysis and the fermentation process, explaining the greater fermentation rate of natural null mutants and *rim15*Δ strains (Qian *et al*. 2012; Tesniere, Brice and Blondin 2015). In parallel, ammonium uptake (which represents the main nitrogen source in the must) is also improved in *RIM15* null strains, since the TORC1 pathway activates, through Gcn4, the GAAC-amino acid biosynthesis activator (Tesniere, Brice and Blondin 2015).

In conclusion, we provide evidence of the antagonistic pleiotropic effect of a *RIM15* natural variant exposed to different environments related to wine and grape phenotypes. Particularly, we have shown how a null *RIM15* variant positively affects a series of phenotypes related to alcoholic fermentation through available glucose utilization, conferring an advantage. In contrast, the same variant would have a detrimental effect in the vineyard when exposed to fungicides, due to the absence of an effective stress response. This provides a plausible real example of an antagonistic pleiotropic effect in nature. Nevertheless, the lack of other strains containing the same mutation suggests that this variant has not been selected for in the wine fermentation process, likely due to its sensitivity to stress conditions, and instead represents a rare allele. This finding provides evidence pointing to selection against the *RIM15* null variant in natural environments and stresses the need for large-scales studies to decipher the role of this allele in other genetic backgrounds and in different environments.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

ACKNOWLEDGEMENTS

We thank Walter Tapia and Sindy Gutierrez for technical help.

FUNDING

This work was supported by grants from Comisión Nacional de Investigación Científica y Tecnológica CONICYT Programa de Atracción e Inserción/Concurso Nacional de Apoyo al retorno de investigadores/as desde el extranjero [grant no. 82130010], CONICYT FONDECYT [grant no. 11140097], CONICYT FONDECYT [grant no. 3150156], MN-FISB [grant no. 120043] and Proyectos Basales y Vicerrectoría de Investigación, Desarrollo e Innovación, Universidad de Santiago de Chile.

Conflict of interest. None declared.

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