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Inactivating Mutations in Irc7p Are Common in Wine Yeasts, Attenuating Carbon-Sulfur β-Lyase Activity and Volatile Sulfur Compound Production

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ABSTRACT During alcoholic fermentation of grape sugars, wine yeasts produce a range of secondary metabolites that play an important role in the aroma profile of wines. In this study, we have explored the ability of a large number of wine yeast strains to modulate wine aroma composition, focusing on the release of the "fruity" thiols 3-mercaptohexan-1-ol (3-MH) and 4-mercapto-4-methylpentan-2-one (4-MMP) from their respective cysteinylated nonvolatile precursors. The role of the yeast gene *IRC7* in thiol release has been well established, and it has been shown that a 38-bp deletion found in many wine strains cause them to express a truncated version of Irc7p that does not possess cysteine-*S*-conjugate β-lyase activity. In our data, we find that *IRC7* allele length alone does not fully explain the capacity of a strain to release thiols. Screening of a large number of strains coupled with analysis of genomic sequence data allowed us to identify several previously undescribed single-nucleotide polymorphisms (SNPs) in *IRC7* that, when coupled with allele length, more robustly explain the ability of a particular yeast strain to release thiols from their cysteinylated precursors. We also demonstrate that allelic variation of *IRC7* not only affects the release of thiols but modulates the formation of negative volatile sulfur compounds from the amino acid cysteine. The results of this study provide winemakers with an improved understanding of the genetic determinants that affect wine aroma and flavor, which can be used to guide the choice of yeast strains that are fit for purpose.

IMPORTANCE Volatile sulfur compounds contribute to wine aromas that may be considered pleasant, such as "tropical," "passionfruit," and "guava," as well as aromas that are considered undesirable, such as "rotten eggs," "onions," and "sewer." During fermentation, wine yeasts release some of these compounds from odorless precursor molecules, a process that is most efficient when performed by yeasts that express active forms of the protein Irc7p. We show that most wine yeasts carry mutations that reduce activity of this protein, affecting the formation of volatile sulfur compounds that impart both pleasant and unpleasant aromas. The results provide winemakers with guidance on the choice of yeasts that can emphasize or deemphasize this particular contribution to wine quality.

KEYWORDS aroma, lyase, sulfur, thiols, wine, yeasts

Volatile sulfur compounds (VSCs) are among the most impactful classes of aroma-active molecules in wine and can be considered a "double-edged sword" due to their potential to have positive and negative impacts on perceived wine quality (1). The production of negative VSCs, such as hydrogen sulfide (H_2S), is a significant problem for the wine industry, since it imparts an undesirable "rotten egg"-like flavor, even at low concentrations. On the other hand, some sulfur-containing flavor compounds,

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such as the polyfunctional thiols 4-mercapto-4-methyl-pentan-2-one (4-MMP), 3-mercaptohexan-1-ol (3-MH), and 3-mercaptohexyl acetate (3-MHA), impart "passionfruit," "grapefruit," "guava," and "box tree" aromas (2–4). These compounds have very low perception thresholds and are important contributors to the flavors of Sauvignon blanc and Chardonnay wines (5).

The formation and fate of VSCs in wine is mediated by several factors (6), although a prominent role is played by the *Saccharomyces cerevisiae* yeasts that are used to perform wine fermentation (1). The likelihood of a wine being negatively impacted by H_2S, for example, can be greatly reduced through choice of yeast strain. Propensity to produce H_2S has been linked to mutations in the sulfite reductase-encoding genes *MET10* and *MET5* (7, 8), and also to mutations in *SKP2* and *MET2* (9). Choice of yeast strain also modulates the concentration of 4-MMP, 3-MH, and 3-MHA in wine (10–13). Further opportunities to enhance polyfunctional thiol concentrations include sequential inoculation or cofermentation with non-*Saccharomyces* yeasts, such as *Torulaspora delbrueckii* and *Pichia kluyveri* (14–16), as well as the use of *S. cerevisiae* × *Saccharomyces kudriavzevii* hybrids (17).

It has been shown that 4-MMP and 3-MH exist in the grape berry as nonvolatile precursors, mainly as conjugates of glutathione (Glut-4-MMP and Glut-3-MH) and cysteine (Cys-4-MMP and Cys-3-MH) (18, 19). Dipeptide conjugates of 3-MH, *S*-3-cysteinyl glycinylhexan-1-ol (Cys-Gly-3-MH), and *S*-3-γ-glutamyl cysteinylhexan-1-ol (γ-Glu-Cys-3-MH) have also been identified (20–22). The acetate ester of 3-MH (3-MHA) is not formed from a nonvolatile precursor but results from acetylation of 3-MH by the yeast alcohol acetyltransferase *ATF1* (23). Biotransformation of conjugated precursors into free thiols by yeast involves their uptake through amino acid and glutathione (GSH) transporters in the plasma membrane (21, 24, 25). Once inside the cytoplasm, Glut-3-MH is cleaved to Cys-Gly-3-MH in the yeast vacuole by action of the γ-glutamate transpeptidase *ECM38*, which might also catalyze the formation of Cys-3-MH from the precursor dipeptide γ-Glu-Cys-3-MH (21, 26). The carboxypeptidase responsible for the cleavage of the dipeptide Cys-Gly-3-MH into Cys-3-MH remains to be identified. On the other hand, the degradation pathway of Glut-4-MMP to yield 4-MMP remains uncharacterized, as the two main GSH degradation pathways appear to be ineffective in metabolizing Glut-4-MMP (21).

Once the cysteinylated precursors are formed through degradation of their glutathionylated or dipeptide precursors, or are assimilated from grape must, these are cleaved by yeast enzymes with carbon–sulfur β-lyase activity to release the free thiols. In *S. cerevisiae*, there are at least three genes, *IRC7*, *STR3* and *CYS3*, encoding cysteine-*S*-conjugate β-lyase enzymes. Of these enzymes, Irc7p and Str3p have been directly shown to release polyfunctional thiols from cysteine conjugates (27, 28), while Cys3p and Str3p were recently shown to release 2-furfurylthiol from its cysteine conjugate (29). Interestingly, while Irc7p was found to be the key determinant of 4-MMP release from Cys-4-MMP and is a significant contributor to 3-MH release from Cys-3-MH (27), most strains of *S. cerevisiae* have a 38-bp deletion in the *IRC7* coding sequence that renders the protein inactive (27, 30). It has been hypothesized that the presence or absence of this deletion might account for the strain variation observed in 4-MMP release, and a positive correlation between the expression level of the full-length and active version of *IRC7* and 4-MMP production within industrial strains has been reported (31). Irc7p also shows cysteine desulfhydrase activity, thereby releasing ammonium and H_2S (32), and this deletion has the potential to affect formation of negative VSCs during winemaking.

The biological role of *IRC7* is not well established, as many functional studies have been conducted in strain backgrounds containing the inactive version of *IRC7* (33–35). While *IRC7* was originally suggested to be involved in DNA metabolism since the null mutant causes increased rates of recombination between homologous chromosomes (33), its role as cysteine desulfhydrase also points out to an involvement in the regulation of cysteine homeostasis (32).

In this study, across a large number of wine yeast strains, we show that while *IRC7*

TABLE 1 Distribution of *IRC7* genotypes across 179 *Saccharomyces cerevisiae* wine yeast strains

Genotype[a]	Total no. of isolates (%)	No. of commercial strains (% [$n = 94$])	No. of wine isolates (% [$n = 85$])
$IRC7^S$ $IRC7^S$	100 (56)	54 (57)	46 (54)
$IRC7^S$ $IRC7^L$	48 (27)	27 (29)	21 (25)
$IRC7^L$ $IRC7^L$	31 (17)	13 (14)	18 (21)

[a]$IRC7^S$, short allele of *IRC7*; $IRC7^L$, long or full-length allele of *IRC7*.

allele length does modulate formation of 3-MH and 4-MMP from their cysteinylated precursors, this alone does not fully explain the variation observed. We identify several single-nucleotide polymorphisms (SNPs) in *IRC7* that reduce activity of Irc7p against cysteine and cysteine conjugates, and, through allele replacement, demonstrate their impact upon the release of polyfunctional thiols and the formation of negative VSCs during wine fermentation.

RESULTS

It has been shown previously that a 38-bp deletion in *IRC7* (between nucleotides 1023 and 1060) results in a truncated protein of 340 amino acids (short Irc7p or $Irc7p^S$), that displays limited β-lyase activity compared with that of the 400-amino acid full-length and active protein (long Irc7p or $Irc7p^L$) (27). We took advantage of the recently published whole-genome sequencing data from 212 strains of *S. cerevisiae* (36) to determine the presence of the short or long versions of *IRC7* in a subset of 179 wine strains, comprising 94 commercial strains and 85 wine isolates from around the world. There was no difference between commercial strains and wine isolates with regard to the proportion that harbored copies of this short allele (Table 1). Thus, 56% of the sequenced strains were found to be homozygous for the short allele ($IRC7^S$ $IRC7^S$), 27% were heterozygous ($IRC7^S$ $IRC7^L$), and only 17% were homozygous for the long allele ($IRC7^L$ $IRC7^L$).

To assess variation among different strains in their ability to release the polyfunctional thiols 3-MH and 4-MMP, a series of small-scale fermentations were carried out in synthetic grape juice supplemented with grape-like concentrations of the cysteine precursors of 3-MH and 4-MMP (SGJ-thiol). The release of these polyfunctional thiols was analyzed in a subset of 80 strains, representative of each of the three *IRC7* genotypes observed in the larger population of sequenced strains (Table S1). Results showed a 20-fold difference across the yeasts studied in their ability to release 3-MH (from 131 to 2,508 ng·liter^{-1}), and an even greater difference, 30-fold, in the case of 4-MMP (from 171 to 5,298 ng·liter^{-1}). The release of both polyfunctional thiols was highly correlated ($R^2 = 0.78$, $P < 0.001$). Overall there was no difference between commercial strains and wine isolates in their ability to release either thiol from their cysteinylated precursors (Table 2). However, for the subset of 39 commercial strains, those recommended by yeast manufacturers for white winemaking released signifi-

TABLE 2 Release of 3-MH and 4-MMP by 80 wine yeast strains in SGJ-thiol medium according to their origin

Origin or recommended usage	No. of strains	3-MH (ng·liter^{-1})[b]	4-MMP (ng·liter^{-1})[b]
Origin			
Wine isolates	41	727 (181–2,508)	902 (150–5,298)
Commercial	39	696 (131–2,481)	844 (170–4,690)
Recommended usage[a]			
White	14	1,016 (412–2,481) A	1749 (170–4,690) A
Red	13	590 (131–839) B	618 (201–1,904) B
White/red	12	547 (170–1,290) B	512 (189–3,579) B

[a]Based upon commercial yeast manufacturers' published materials.
[b]Results are expressed as the mean for each of the groups, and the range of production within each group is shown in parentheses. Means with the same letter are not significantly different from each other (Tukey's HSD test, $P < 0.05$).

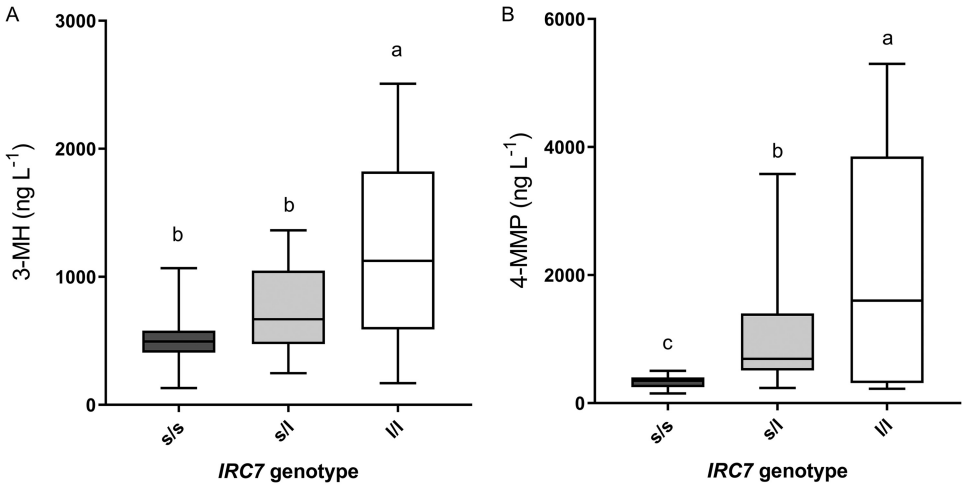

FIG 1 Release of 3-MH (A) and 4-MMP (B) by 80 wine strains in SGJ-thiol medium, grouped according to their *IRC7* genotype. s/s refers to the homozygous short allele (*IRC7*^{S} *IRC7*^{S}) and l/l to the homozygous long or full-length allele (*IRC7*^{L} *IRC7*^{L}), while s/l refers to heterozygous strains (*IRC7*^{S} *IRC7*^{L}). Means with the same letter are not significantly different from each other (Tukey's honestly significant difference [HSD] test, $P < 0.05$). Totals of 43, 20, and 17 strains from the s/s, s/l, and l/l *IRC7* genotypes, respectively, were included.

cantly more 3-MH and 4-MMP than did strains recommended either for red winemaking or for both red and white winemaking (Table 2). When the isolates were grouped according to genome sequencing-derived clades described by Borneman et al. (36), we observed that strains from the three major wine yeast clades released significantly less 4-MMP than did wine isolates from outside these clades (designated "Other"; Table S2).

When thiol release data were grouped according to *IRC7* genotype, strains that were homozygous for the *IRC7*^{L} allele produced, on average, 6 and 2.5 times more 4-MMP and 3-MH, respectively, than did strains homozygous for the *IRC7*^{S} allele (Fig. 1A and B). Within each genotype, particularly in the *IRC7*^{L} *IRC7*^{L} and *IRC7*^{S} *IRC7*^{L} groups, there was substantial variation in the release of these compounds between individual strains, as recently described (30). Of note, 5 of the 6 strains belonging to the Vin7 clade are homozygous for the *IRC7*^{L} allele yet produced levels of 4-MMP equivalent to those of the *IRC7*^{S} *IRC7*^{S} group (Table S2). Our results suggest that a substantial portion of variation between yeasts for capacity to release thiols is not explained solely by *IRC7* allele length.

Identification of *IRC7* sequence variants that affect polyfunctional thiol release. Upon closer examination of the *IRC7* open reading frame (ORF) across the 179 sequenced strains (36), we identified a series of single-nucleotide polymorphisms (SNPs). The most common of these SNPs leads to substitution of threonine in position 185 to alanine, which is linked with the long allele of *IRC7* (IRC7^{L} T185A). We found that 80% of the strains in the *IRC7*^{S} *IRC7*^{L} and *IRC7*^{L} *IRC7*^{L} groups exhibited this allele (data not shown). Other mutations were found in a smaller proportion of strains, and, in most cases, these were linked with the IRC7^{L} T185A allele (Table S1). A structural model of *S. cerevisiae* Irc7p^{L} was constructed, and all observed mutated amino acids were mapped against the model to facilitate prediction of impact on protein function. Only the T185A mutation was proximal to the active site of Irc7p^{L} (Fig. 2). The presence of this SNP appeared to have a deleterious effect on the release of both thiols, particularly that of 4-MMP (Table 3). Strains in the *IRC7*^{L} *IRC7*^{L} group that were homozygous for T185A produced 80% less 4-MMP (715 ng·liter^{-1}) than did strains without this mutation (4,618 ng·liter^{-1}).

Functional *in vitro* characterization of variant *IRC7*^{L} alleles. While none of the additional observed SNPs induced changes to protein sequences near functional sites, they may explain why some of the strains in the *IRC7*^{L} *IRC7*^{L} group produced levels of both thiols similar to *IRC7*^{S} *IRC7*^{S} strains. To investigate whether this was the case, each

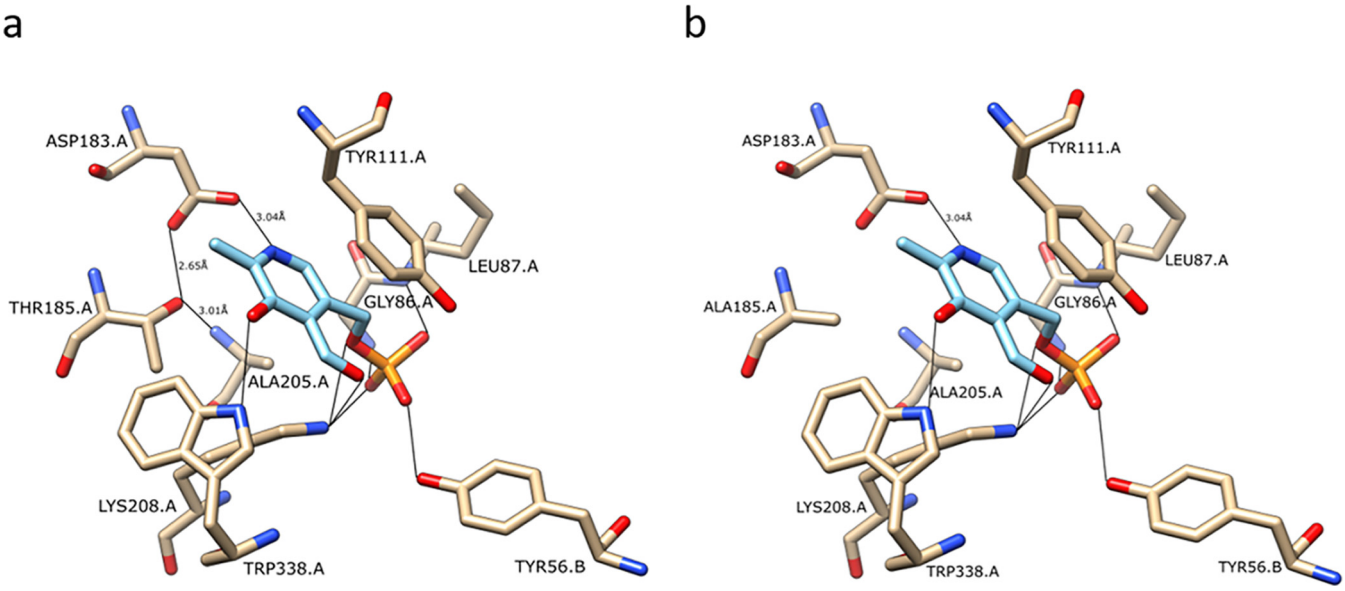

FIG 2 Model of the Irc7p^{L} active site in the presence of PLP cofactor (light blue). The model shows amino acid residues from two subunits (A and B) of Irc7p^{L} that form the catalytic dimer. In the model on the left (a), the hydroxyl group of Thr185 forms a hydrogen bond with the carboxylate group of Asp183, fixing it for optimal interaction with the nitrogen of the pyridine ring of PLP. In the model on the right (b), mutation on the Thr185 to Ala results in the loss of the hydrogen bond between this amino acid and Asp183.

of the most common SNPs was introduced individually in a multicopy plasmid containing *IRC7^{L}* (pHVX2-IRC7^{L}), and these were transformed into BY4742 Δ*IRC7*. Then, activity of protein extracts with the physiological substrate cysteine and with Cys-4-MMP was assayed (Fig. 3). Activity against Cys-3-MH was too small to be quantified by enzymatic methods. For both assayed substrates, the T185A mutation (pHVX2-IRC7^{L} T185A) resulted in a 50% decrease in the activity of the protein. The presence of additional mutations in the IRC7^{L} T185A construct resulted in further decreases in activity. In particular, the G253R and G321D mutations completely abolished the activity of Irc7p, while some residual activity could still be observed for the K43R, P146R, and E323G mutations. Enzymatic activity of the constructed *IRC7* alleles against Cys-4-MMP (Fig. 4) was highly correlated with end-of-ferment thiol concentrations for the corresponding diploid wine strains that exhibited the same alleles ($R^2 = 0.95$, $P < 0.0001$).

Impact of variant *IRC7^{L}* alleles on VSC and thiol formation during wine fermentation. To confirm the role of *IRC7* variant alleles in modulating formation of VSCs

TABLE 3 Release of 3-MH and 4-MMP by 80 wine yeast strains in SGJ-thiol medium according to their *IRC7* genotype

Genotype[a]	Amino acid in position 185[b]	No. of strains	3-MH (ng·liter^{-1})[c]	4-MMP (ng·liter^{-1})[c]
IRC7^{S} IRC7^{S}	T185	43	495 ± 169 C	324 ± 87 C
IRC7^{S} IRC7^{L}		20	755 ± 330	1,107 ± 973
	T185	5	1,074 ± 229 B	2,505 ± 960 B
	T185/A185	14	635 ± 297 C	666 ± 295 C
	A185	1	840 BC	275 C
IRC7^{L} IRC7^{L}		17	1,236 ± 764	2,027 ± 1,925
	T185	5	2,158 ± 388 A	4,618 ± 930 A
	T185/A185	2	1,326 ± 448 B	2,109 ± 719 B
	A185	10	757 ± 468 BC	715 ± 708 C

[a]*IRC7^{S}* indicates the short allele of *IRC7*, while *IRC7^{L}* indicates the long or full-length allele of *IRC7*.
[b]In the *IRC7^{S} IRC7^{L}* group, the presence of an alanine in position 185 (A185) is associated with *IRC7^{L}*.
[c]Means with the same letter are not significantly different from each other (Tukey's HSD test, $P < 0.05$).

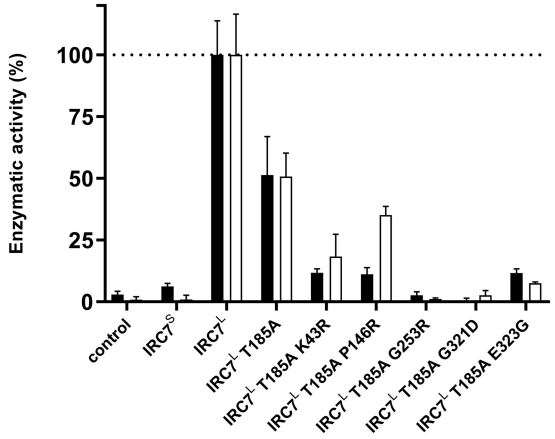

FIG 3 Enzymatic activity toward Cys-4-MMP (black) and cysteine (white) in protein extracts from BY4742 Δ*IRC7* yeast cells overexpressing the multicopy plasmid pHVX2 with different alleles of *IRC7*. Each result is expressed as the mean of three independent replicates. For both substrates, activity was expressed as a percentage relative to cells harboring the pHVX2-IRC7^{L} construct (100%) overexpressing the long or full-length version of *IRC7*. Enzymatic activity for this allele was found to be 214 ± 36 and 89.6 ± 12.4 nmol·min^{-1} μg·prot^{-1} for cysteine and Cys-4-MMP respectively.

during fermentation, each was integrated into the haploid wine strain AWRI1631 at the *IRC7* chromosomal location, under the control of the native promoter. Replacing the native *IRC7^{S}* allele of AWRI1631 with *IRC7^{L}* resulted in a dramatic increase in the levels of 4-MMP (22-fold) after fermentation of SGJ-thiol, while more modest increases were observed for both 3-MH and 3-MHA (3- and 6-fold, respectively) (Table 4). The conversion yields for the AWRI1631 IRC7^{L} strain were found to be 12.1% and 0.78%, for 4-MMP and 3-MH, respectively. The restoration of the fully active Irc7p^{L} also caused an increase in the levels of H_2S released during fermentation. In this background, the T185A mutation significantly decreased the amounts of 4-MMP and 3-MH (62% and 42% less) released relative to those released by the AWRI1631 IRC7^{L} strain. In combination with T185A, the G253R mutation had the greatest impact upon formation of polyfunctional thiols, which resulted in an *IRC7^{L}* allele that functioned equivalently to the *IRC7^{S}* allele.

Since we observed a significant increase in the levels of H_2S released throughout fermentation by restoration of Irc7p activity, we investigated the effect of the three main alleles of *IRC7* on the formation of other VSCs. We used increasing concentrations of cysteine to enhance the possible impact of Irc7p on the formation of sulfur

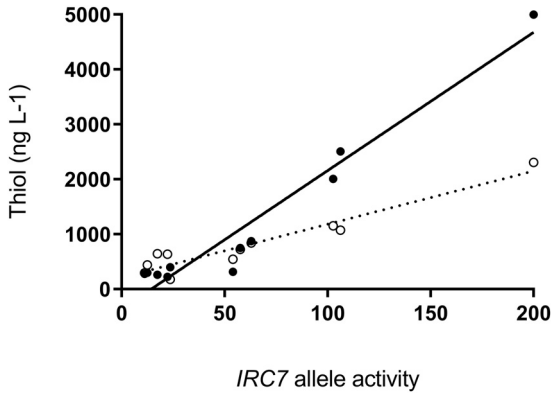

FIG 4 Correlation between the activity of combinations of different alleles of *IRC7* and the release of 4-MMP (filled circles) and 3-MH (empty circles) in SGJ-thiol medium by diploid strains exhibiting these alleles. Activity was evaluated in protein extracts in triplicate from cells overexpressing different alleles of *IRC7* using Cys-4-MMP as the substrate. The *IRC7^{L}* allele was given an arbitrary value of 100, and diploid strains with the *IRC7^{L} IRC7^{L}* genotype were given a value of 200. The correlation coefficient (R^2) for 4-MMP and 3-MH was found to be 0.95 and 0.90, respectively.

TABLE 4 Effect of chromosomal integration of different *IRC7* alleles in the haploid strain AWRI1631 in sulfur compound formation during fermentation of 90 ml of SGJ-thiol medium

AWRI1631 construct	Sulfur compound[a]			
	H_2S tube (μg)	4-MMP (ng·liter^{-1})	3-MH (ng·liter^{-1})	3-MHA (ng·liter^{-1})
No yeast	ND	107	ND	ND
IRC7^S	122 BC	662 G	324 F	9.6 E
IRC7^L	178 A	14,672 A	961 A	61.4 A
IRC7^L T185A	135 B	5,469 B	564 C	28.2 C
IRC7^L T185A K43R	121 BC	4,549 D	563 C	25.1 CD
IRC7^L T185A P146R	121 BC	3,559 E	531 CD	20.6 DE
IRC7^L T185A G253R	94 C	652 G	367 EF	7.6 F
IRC7^L T185A G321D	116 BC	2,303 F	453 DE	16.5 E
IRC7^L T185A E323G	123 BC	4,849 C	740 B	40.8 B

[a]Each results is expressed as the mean of three independent replicates; standard deviations were typically 10% and never exceeded 20%. The conversion yields of the AWRI1631 IRC7^L strain for Cys-3-MH and Cys-4-MMP were 0.78% and 12.1%, respectively. H_2S released during fermentation (H_2S tube) was measured with silver nitrate selective gas detector tubes. Means with the same letter are not significantly different from each other (Tukey's HSD test, $P < 0.05$). ND, not detected.

compounds, since it has a relatively high K_m for cysteine (3.8 mM, 460 mg·liter^{-1}) (32). We observed a general increase in the formation of the different VSCs analyzed with increasing concentrations of cysteine, particularly for H_2S released during fermentation (H_2S tube) and ethyl thioacetate (EtSAc) (Table 5). The magnitude of these changes was also dependent upon the allele of *IRC7* tested. Thus, the presence of the *IRC7*^{L} allele was responsible for significant increases in the formation of the different sulfur compounds, while fermentation with the *IRC7*^{L} T185A allele displayed an intermediate phenotype between the short and full-length versions of *IRC7*. Differences between the different alleles were more noticeable for H_2S tube, H_2S, and EtSAc for the whole range of concentration of cysteine, and in the case of ethanethiol (EtSH), methyl thioacetate (MeSAc), and carbon disulfide (CS_2), only for the highest concentration of cysteine. On the other hand, the formation of methanethiol (MeSH) was less responsive to the levels of cysteine or the allele of *IRC7* used.

DISCUSSION

Previous research identified Irc7p as being primarily responsible for release of the polyfunctional thiol 4-MMP from its cysteinylated precursor during fermentation (27), and subsequent work linked variation between strains in this capacity to the presence or absence of the full-length version of this protein (26, 30, 31). Overexpression of *IRC7*

TABLE 5 Effect of chromosomal integration of three *IRC7* alleles in the haploid strain AWRI1631 in VSC formation during fermentation of 90 ml of a synthetic grape juice (SGJ) supplemented with different concentrations of cysteine

Medium	AWRI1631 construct	VSC (μg·liter^{-1})[a]						
		H_2S	MeSH	EtSH	CS_2[c]	MeSAc	EtSAc	H_2S tube (μg)[b]
SGJ-Cys 17 mg·liter^{-1}	IRC7^S	2.6 ± 0.3 B	4.6 ± 0.5 A	<LOD	<LOD	12.3 ± 0.7 A	1.6 ± 0.1 B	118 ± 8 B
	IRC7^L T185A	2.1 ± 0.4 B	4.0 ± 0.5 A	<LOD	<LOD	16.8 ± 2.6 A	2.3 ± 0.6 AB	132 ± 9 B
	IRC7^L	4.0 ± 1.0 A	4.7 ± 0.6 A	<LOD	<LOD	16.9 ± 1.7 A	2.6 ± 0.3 A	175 ± 28 A
SGJ-Cys 50 mg·liter^{-1}	IRC7^S	3.7 ± 0.6 B	4.7 ± 0.7 A	<LOD	<LOD	17.2 ± 3.6 A	3.7 ± 1.1 B	140 ± 11 C
	IRC7^L T185A	7.6 ± 2.6 AB	4.1 ± 0.4 A	<LOD	<LOD	20.1 ± 1.2 A	6.8 ± 1.8 AB	228 ± 19 B
	IRC7^L	7.5 ± 1.7 A	4.3 ± 0.8 A	<LOD	<LOD	21.0 ± 2.8 A	9.4 ± 2.6 A	326 ± 58 A
SGJ-Cys 200 mg·liter^{-1}	IRC7^S	11.6 ± 1.2 C	4.5 ± 0.4 B	0.5 ± 0.1 C	<LOD	14.7 ± 1.5 B	7.3 ± 0.7 C	280 ± 22 C
	IRC7^L T185A	20.9 ± 3.3 B	5.5 ± 1.0 AB	1.9 ± 0.3 B	<LOD	23.2 ± 1.2 B	24.8 ± 2.7 B	630 ± 90 B
	IRC7^L	51.8 ± 3.0 A	8.6 ± 2.3 A	8.4 ± 2.9 A	1.2 ± 0.3	45.5 ± 8.3 A	61.8 ± 13.5 A	1,315 ± 221 A

[a]Results are expressed as the mean of three independent replicates. Means with the same letter are not significantly different from each other (Tukey's HSD test, $P < 0.05$).
[b]H_2S released during fermentation (H_2S tube) was measured with silver nitrate selective gas detector tubes.
[c]LOD, limit of detection.

was also recently implicated in production of H_2S in a solid medium containing cysteine (32). In this study, we have expanded upon this knowledge by providing evidence that many wine strains harbor a variety of mutations in *IRC7* that can have a profound effect on the activity of the encoded protein. This not only alters the capacity of these strains to release polyfunctional thiols from their cysteinylated-conjugated precursors but also their ability to produce other VSCs during wine fermentation.

The most common *IRC7* SNP observed across the 179 wine yeasts studied was the substitution of a threonine in position 185 with alanine, which was present in around 80% of the strains with at least one copy of *IRC7*^{L}. Thr185 is a highly conserved amino acid in pyridoxal-5′-phosphate (PLP)-dependent carbon-sulfur lyases, not only in fungi but also in prokaryotes and mammals (37). The hydroxyl group of Thr185 forms a strong hydrogen bond with the carboxylate group of Asp183, thereby tethering Asp183 in an optimal position to interact with the pyridine nitrogen of the PLP cofactor (37). As a result of the mutation to Ala, the hydrogen bond is then lost. This mutation results in a 50% loss of enzymatic activity, which corresponds to a similar decrease in the formation of the positive and negative VSCs during fermentation of a synthetic grape juice. In our model of the catalytic Irc7p^L dimer, the other common and deleterious mutations linked to the IRC7^L T185A allele (K43R, P146R, G253R, G321D, and E323G), were not located near the active site of the protein. Irc7p^L forms a homotetramer (32), as do other enzymes of the family of cysteine/methionine metabolism PLP-dependent enzymes (37, 38). Gly253 is a highly conserved amino acid in this family, and in the sequence is located next to an Arg252, which is involved in tetramer contact (37). Therefore, the mutation of the small and neutral Gly253 to Arg might create a disruption in tetramer formation due to the nearby presence of two positively charged amino acids.

It is striking that, in addition to the previously described prevalence of the inactive *IRC7*^{S} allele among wine strains, a significant percentage of those with one or two copies of the *IRC7*^{L} allele harbor other inactivating mutations. This indicates that *S. cerevisiae* has a strong selective pressure toward the inactivation of Irc7p, at least among wine strains, whereas other members of the *Saccharomyces sensu stricto* group have the full-length version of the gene (32). *IRC7* shows evidence of being horizontally transferred into an ancestral yeast from an unknown bacterium related to *Yersinia pestis* (39) prior to the whole-genome duplication event. Only a few other yeast species from the *Zygosaccharomyces*, *Lachancea*, and *Torulaspora* genera harbor *IRC7* orthologs. This suggests that among descendants of the ancestral yeast that acquired *IRC7,* there appear to be many lineages where this gene has been lost altogether. Overall, it seems reasonable to speculate that Irc7p carbon-sulfur β-lyase activity confers benefit to some species/strains, but not others.

It is important to note that most functional studies have been conducted by deletion of *IRC7*^{S} in different *S. cerevisiae* laboratory strain backgrounds (33–35). Even though we, and others, have shown that the Irc7p^S has no activity toward substrates such as cysteine or Cys-4-MMP, the truncated protein and/or the genomic locus itself may be functionally important in yeast. Indeed, deletion of the short *IRC7* allele in the diploid laboratory strain W303 increased the levels of mitotic recombination (33). Reduced risk of deleterious recombination events may explain both the common occurrence of inactivating truncations/mutations among wine strains and the absence of naturally occurring structural variants, based upon the assumption that Irc7p^L activity has some fitness cost under environmental conditions these yeasts encounter.

What might these conditions be? *IRC7* is under nitrogen catabolite repression (NCR) control, and increased transcription of *IRC7* is observed in poor nitrogen sources, such as methionine or proline (40). For this reason, complementation of grape must with an optimal nitrogen source, such as ammonium, decreases 3-MH production (25). Yeasts existing in environments lacking preferred nitrogen sources would benefit from cysteine desulfhydrase activity of Irc7p^L as a means to recover ammonium, so this seems an unlikely driver of the observed allelic variation in *S. cerevisiae* wine yeasts.

IRC7 is not only regulated by NCR but also by the copper-sensing transcription factor

Mac1p, which activates the expression of various genes, including *IRC7,* under copper-deficient conditions. Conversely, *IRC7* expression is inhibited under high copper conditions (41). The physiological explanation for *IRC7* being regulated by copper concentrations has not been elucidated but might be related to its putative biological role in cysteine homeostasis (32). In response to high concentrations of copper, transcription of the cysteine-rich metallothionein *CUP1* is heavily induced (41). Cup1p acts to sequester excess copper, which is toxic (42). Under these conditions, it might be necessary to repress the cysteine desulfhydrase activity of Irc7p to ensure that sufficient intracellular cysteine is available for synthesis of Cup1p in high quantities. One of the well-described domestication signatures of wine yeasts is tolerance to copper (43), which is a component of common vineyard sprays and is used during winemaking to sequester negative VSCs (1). It is possible that through long-term exposure to copper, wine strains of *S. cerevisiae* have accumulated *IRC7* mutations as a means to ensure cysteine availability in order to respond to acute copper exposure.

Regardless of the evolutionary drivers for Irc7p inactivation, allelic variation among *S. cerevisiae* strains at the *IRC7* locus has profound impact upon winemaking outcomes. In our experimental fermentation conditions, in which we spiked a synthetic grape juice with grape-like concentrations of both cysteinylated precursors of 4-MMP and 3-MH, the *IRC7* genotype seemed to explain much of the variation observed between wine strains for the release of these fruity thiols. Both our allele replacement experiments in the haploid AWRI1631 background and our survey of 80 wine yeast strains found that Irc7p^{L} was responsible for the release of at least 95% of 4-MMP from the Cys-4-MMP precursor during fermentation, confirming previous results (27). In the case of 3-MH, our results show that Irc7p^{L} is responsible for a significant part of the 3-MH released from Cys-3-MH, ranging from 66% in the haploid background to approximately 75% in the screening of strains. When combined with enzymatic assays, our results agree with previous studies (27, 28) that Irc7p^{L} is more active with Cys-4-MMP than with Cys-3-MH. All this evidence points toward other yeast β-lyases playing a small part in the release of 3-MH from its cysteinylated precursor. In this sense, Str3p has been shown to catalyze the release of 3-MH from Cys-3-MH (28).

The potential for contribution of cysteine as a precursor to VSCs in wine is generally disregarded, as other more abundant sources of sulfur exist. Nevertheless, we observed differences in H_2S and EtSAc concentrations according to *IRC7* allele during fermentation of model grape juice with cysteine at levels observed in natural musts (44). These observations were consistent with those of Kinzurik et al. (45), who demonstrated the role of H_2S made by yeast during fermentation in the formation of EtSH and EtSAc. Differences in VSC concentration according to the *IRC7* allele were exacerbated when the concentration of cysteine was increased to 50 and 200 mg liter^{-1}, which led to a general increase of most of the VSCs analyzed.

The cysteine-containing tripeptide GSH is naturally present in grape juice in concentrations ranging from 1 up to 102 mg liter^{-1} (46), and the International Organization of Vine and Wine (OIV) has recently allowed the addition of GSH to must or wine (20 mg liter^{-1}). In this context, research has shown that supplementation of must with GSH enhances the production of H_2S and other negative VSCs, which increases the intensity of sulfidic off flavors in wine (47, 48). Another source of GSH is nutrients that are commonly added to active dry yeast rehydration medium to improve yeast fermentation performance, which might act as a source for H_2S production during fermentation (49). The mechanisms of H_2S release due to GSH addition are not yet understood, but it has been suggested that this might occur via degradation of GSH to the individual amino acids and by subsequent degradation of cysteine to H_2S by cysteine desulfhydrase activity (49, 50). With this in mind, the relative impact of the wine yeast *IRC7* genotype in modulating VSC formation might be most important when the concentrations of cysteine and/or GSH are elevated, in combination with low-nitrogen or low-phenolic grape musts (47, 48).

Different wine yeast strains produce divergent profiles of aroma compounds, and the choice of a yeast strain can have a significant impact on the final flavor and aroma

TABLE 6 Primers used in this study

Name	Comments[b]	Sequence (5′–3′)[a]
TC1	Cloning *IRC7*^{S} or *IRC7*^{L} using *Eco*RI (underlined), forward	GACTGAATTCATGATTGATCGTACCGAGTTATCG
TC2	Cloning *IRC7*^{S} using *Xho*I (underlined), reverse	TAGCCTCGAGCTAGCCACCCCATGAAAATCCCA
TC3	Cloning *IRC7*^{L} using *Xho*I (underlined), reverse	GACTCTCGAGTCAGATTTGTAAAGGATTCAAAG
TC4	Sequencing primer (pHVX2 plasmid), forward	TCAAACAGAATTGTCCGAATCG
TC5	Sequencing primer (pHVX2 plasmid), reverse	CCGAACATAGAAATATCGAATGG
TC6	Site-directed mutagenesis (IRC7^L K43R), forward	GGGGTCAACCATCATTCTTAgAAAACTTAGTGATTTAGAAC
TC7	Site-directed mutagenesis (IRC7^L M106I), forward	GCTGGTGATCATATCTTGATtACTGATAGTGTCTACGTGC
TC8	Site-directed mutagenesis (IRC7^L P146R), forward	GATATAGAAAAACTAGTTAAGCgAAATACAACCGTCATTTTCCTC
TC9	Site-directed mutagenesis (IRC7^L G253R), forward	GTCAATTAGCATTGCGAaGAATGCGTACATTGCAC
TC10	Site-directed mutagenesis (IRC7^L W274R), forward	CCTGGATTTGGCTGCTcGGCTCGGAAATCGAGAT
TC11	Site-directed mutagenesis (IRC7^L G321D), forward	GAGGAAAATGACGGTTGTATTTcGCTTAACTAGTTTTTCTATATC
TC12	Site-directed mutagenesis (IRC7^L E323G), forward	CACAAGAGCTGGTCTGGgGAAAATGGTAGAAGGGA
TC13	Deletion of *IRC7* using CORE cassette, forward	ATGATTGATCGTACCGAGTTATCGAAGTTTGGTATTACTACGCAACT GTCgagcccccgatttagagctt
TC14	Deletion of *IRC7* using CORE cassette, reverse	TCAGATTTGTAAAGGATTCAAAGAAATTTCTTCTGCGAGACGTTCAAA GCCctcgaggtcgacggtatcgat
TC15	Confirmation deletion *IRC7* (5′-UTR IRC7), forward	ATTGTCGCCTCTTGTTTTGAG
TC16	Confirmation deletion *IRC7* (5′), reverse	AAGACTCTCCTCCGTGCGTC
TC17	Confirmation deletion *IRC7* (3′-UTR IRC7), reverse	GGAGTGAGATCACATGTCATATTG
TC18	Confirmation deletion *IRC7* (3′), forward	TGAGCATGCCCTGCCCCTA

[a]For primers TC6 through TC12, the reverse primers used for site-directed mutagenesis are the reverse complement of the corresponding forward primers; the nucleotide to be changed is indicated in lowercase in the sequence. For primers TC13 through TC14, the sequence annealing the CORE cassette of the pCORE5 plasmid is indicated in lowercase.
[b]UTR, untranscribed region.

of wine. In this study, we have comprehensively demonstrated the vast range of thiol-releasing capacities across a large number of wine yeasts. Furthermore, we have identified new molecular markers that can be used to predict this capacity. We also provide further evidence of the intrinsic link between polyfunctional thiol release and the potential for production of negative VSCs from organic sulfur precursors. This knowledge will guide strain development and choice of yeast to balance these flavor impacts.

MATERIALS AND METHODS

Chemicals. Analytical reagents were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) unless otherwise indicated. 4-Mercapto-4-methylpentan-2-one (4-MMP) and *S*-4-(4-methylpentan-2-one)-L-cysteine (Cys-4-MMP) were synthesized as described previously (10), and [$^2H_{10}$]4-MMP and 3-mercaptohexan-1-ol (3-MH), 3-mercaptohexyl acetate (3-MHA), and [$^2H_5$]-3-MHA were also synthesized as described previously (51). The synthesis of [$^2H_{10}$]3-MH was carried out according to a previously described method (52). *S*-3-(Hexan-1-ol)-L-cysteine (Cys-3-MH), as a mixture of diastereomers, was prepared as described previously (53).

Microorganisms and culture conditions. All yeast strains were obtained from The Australian Wine Research Institute (AWRI) culture collection. Yeast cultures were maintained on solid yeast extract-peptone-dextrose (YPD) agar plates (2% glucose, 2% peptone, 1% yeast extract, and 2% agar).

DNA manipulation, genome analysis and construction of plasmids. Standard procedures for the isolation and manipulation of DNA were used (54). Primers used in this study as summarized in Table 6. Genomic DNA from yeast strains was isolated using the Gentra Puregene Yeast/Bact. kit B (Qiagen, Australia).

Detection of *IRC7* short and long alleles, as well as of single nucleotide polymorphisms (SNPs) in its open reading frame (ORF), was performed by visualizing read alignments in a subset of 179 sequenced wine yeast strains (BioProject accession number PRJNA303109), data aligned to the S288c strain genome, and using the Integrated Genomics Viewer (IGV) (63). The presence of the long or short allele of *IRC7* was also confirmed by PCR as described previously (27).

The *IRC7* ORF was amplified by PCR using Phusion High-Fidelity DNA polymerase (New England Biolabs), with genomic DNA from the commercial wine strains AWRI838 and AWRI1688 and laboratory strain BY4742 (MATα *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0) as the templates. Primers TC1 and TC2 were used to amplify *IRC7* short version (*IRC7*^{S}) from BY4742 (1,023 bp), while primers TC1 and TC3 were used to amplify *IRC7* long or full-length version (*IRC7*^{L}) from both wine strains (1,203 bp); the restriction sites EcoRI (5′) and XhoI (3′) were introduced in the PCR. The fragments were purified and digested, and then cloned into the EcoRI and XhoI sites of the *S. cerevisiae* expression plasmid pHVX2 (55) to yield the following constructs: pHVX2-IRC7^S, pHVX2-IRC7^L, and pHVX2-IRC7^L T185A (Table 7).

In order to introduce some of the most common SNPs found in *IRC7* alleles from different wine yeast strains, the QuikChange site-directed mutagenesis kit (Agilent, Mulgrave, Australia) was used with the pHVX2-IRC7^L T185A plasmid as the template (Table 7). For each of the *IRC7* SNPs, both mutagenesis

TABLE 7 Strains and plasmids generated in this study

IRC7 allele (donor strain)	Mutation(s)	Strain[b]	Plasmid[c]
IRC7^S (BY4742)	1023_1060del[a]	AWRI1631 IRC7^S	pHVX2-IRC7^S
IRC7^L (AWRI838)		AWRI1631 IRC7^L	pHVX2-IRC7^L
IRC7^L T185A (AWRI1688)	553A→G	AWRI1631 IRC7^L T185A	pHVX2-IRC7^L T185A
IRC7^L T185A K43R (AWRI1493)	128A→G, 553A→G	AWRI1631 IRC7^L T185A K43R	pHVX2-IRC7^L T185A K43R
IRC7^L T185A P146R (AWRI722)	437C→G, 553A→G	AWRI1631 IRC7^L T185A P146R	pHVX2-IRC7^L T185A P146R
IRC7^L T185A G253R (AWRI1899)	553A→G, 757G→A	AWRI1631 IRC7^L T185A G253R	pHVX2-IRC7^L T185A G253R
IRC7^L T185A G321D (AWRI723)	553A→G, 962G→A	AWRI1631 IRC7^L T185A G321D	pHVX2-IRC7^L T185A G321D
IRC7^L T185A E323G (AWRI935)	553A→G, 968A→G	AWRI1631 IRC7^L T185A E323G	pHVX2-IRC7^L T185A E323G

[a]The deletion of 38 bp in the *IRC7*^{S} allele results in a protein of 340 amino acids (Gly340*), truncated compared to the full-length allele (*IRC7*^{L}), which encodes a protein of 400 amino acids.

[b]*IRC7* alleles from different donor strains were integrated at the *IRC7* chromosomal location of the haploid wine strain AWRI1631.

[c]The multicopy episomal plasmid pHVX2 (55) was used to overexpress *IRC7* alleles from different donor strains, under the control of the constitutive *PGK1* promoter.

primers were designed so they contained the desired mutation and were annealed to the same sequence on opposite strands of the plasmid (primers TC6 to TC12). The appropriate substitutions, as well as the absence of unwanted mutations, were confirmed by Sanger sequencing using primers TC4 and TC5.

Chromosomal integration of *IRC7* alleles in AWRI1631. Different *IRC7* alleles were inserted into the haploid wine yeast strain AWRI1631 via homologous recombination (54) to replace the corresponding *IRC7* wild-type locus (*IRC7*^{S}) so that no extraneous DNA was left behind (Table 7). First, the CORE-ΔIRC7 cassette (3 kb) was amplified from the pCORE5 plasmid (56) by PCR using Phusion DNA polymerase and primers TC13 and TC14. These primers were designed so they have at their 3′ end 20 bp complementary to flanking sequences of the CORE cassette and, at their 5′ end, 50 bp complementary to either the start (TC13) or the end (TC14) of the *IRC7* coding regions. The amplified CORE-ΔIRC7 cassette was then purified and transformed into AWRI1631, and positive transformants were selected in solid medium containing 100 mg·liter^{-1} of nourseothricin (clonNAT; Werner BioAgents, Germany). Confirmation of the replacement of the AWRI1631 *IRC7* wild-type locus by the CORE-ΔIRC7 cassette was confirmed by PCR using primers TC15 and TC16 (5′ integration), and TC17 and TC18 (3′ integration), yielding the AWRI1631-ΔIRC7-CORE strain.

Then, a fragment of 1.9 kb, encompassing the *IRC7* open reading frame plus a fragment of 280 bp before the start codon and one of 300 bp after the stop codon was amplified from genomic DNA of several strains of interest (Table 7) by PCR using Phusion DNA polymerase and primers TC15 and TC17. Positive transformants were selected in galactose plates, and integration of the each of the *IRC7* alleles was confirmed by PCR and Sanger sequencing.

Laboratory-scale fermentation in a synthetic grape juice. Laboratory-scale fermentations were performed in triplicate in a synthetic grape juice (SGJ) medium (21) with the following variations: sugar content was 100 g·liter^{-1} glucose and 100 g·liter^{-1} fructose, and 250 mg·liter^{-1} yeast-assimilable nitrogen was added. The concentrations of the sulfur-containing amino acids methionine and cysteine were set to 6 and 17 mg·liter^{-1}, respectively. Depending on the experiment, SGJ was supplemented with additional amounts of cysteine, so the total concentration of cysteine was 50 or 200 mg·liter^{-1}; or with 100 μg liter^{-1} of the cysteinylated precursors Cys-4-MMP and Cys-3-MH (SGJ-thiol). SGJ was filtered through 0.22 μm Stericup filters (Millipore). Yeast starter cultures were prepared by growing cells aerobically in YPD medium for 24 h to the stationary phase at 28°C. Then 1×10^6 cells ml^{-1} were inoculated into 50% diluted SGJ medium and grown for another 24 h at 22°C. The acclimatized cells were inoculated into 90 ml of SGJ at a density of 1×10^6 cells ml^{-1}. Fermentations were conducted at 22° in 100-ml Schott bottles fitted with stir bars and stirred at 200 rpm using a magnetic stirrer. The lids of the Schott bottles were fitted with H_2S detector tubes (Air-Met Scientific, South Australia) to measure the release of H_2S during fermentation (H_2S tube). Fermentation progress was followed by CO_2 weight loss, measured every 24 h. After fermentation, the ferments were cold settled at 4°C for 5 days and then sampled for VSC and nonvolatile compound analysis.

Assay of carbon–sulfur β-lyase activity. Strains were grown overnight in YPD medium at 28°C, and a volume of 8 ml of the culture was centrifuged for 2 min at 4,000 × *g*. Cells were washed twice with water, and the pellet was resuspended in 400 μl of cold buffer containing 100 mM HEPES (pH 7.5), 20 μM pyridoxal-5′-phosphate (PLP), 200 μM EDTA, 10% glycerol, and the protease inhibitors leupeptin (2 μg ml^{-1}) and PMSF (1 mM). Glass beads were added to the suspension, and cells were repeatedly vortexed for 30 s, then allowed to rest for another 30 s, for a total of 10 min at 4°C. The suspension was centrifuged for 30 min at 16,000 × *g* at 4°C, and the supernatant was used for enzyme assays. Protein concentrations were measured using the Bio-Rad protein assay (catalog number 5000006), with bovine serum albumin as a standard.

Reactions were carried out at 30°C in a total volume of 200 μl in 96-well microplates (UV-Star UV-transparent microplates; Greiner Bio-One) containing 100 mM HEPES buffer (pH 7.5), 20 μM PLP, 25 μM EDTA, and 2 mM concentration of the substrates L-cysteine or Cys-4-MMP. The reaction was started by adding 10 μg of the protein extracts. The release of pyruvate as a result of β-lyase activity (57) was analyzed via linked enzymatic conversion to lactate by L-lactic dehydrogenase enzyme (5 U μl^{-1}). Concomitant conversion of NADH (400 μM) to NAD^+ by this enzyme was monitored by measurement of absorbance at 340 nm (molar absorptivity = 6,220 liter mol^{-1}) every 5 min for 60 min.

Analysis of principal nonvolatile compounds. The concentrations of sugars, ethanol, glycerol, and organic acids were measured by high-performance liquid chromatography using a Bio-Rad HPX-87H column (7).

Analysis of the thiols 3-MH, 3-MHA, and 4-MMP. Analysis of 3-MH and 4-MMP occurred once the ferments achieved dryness and were clarified by centrifugation (2,500 × *g* for 5 min) to remove yeast cells. An aliquot (20 ml) of the clarified ferment samples was derivatized with 4,4′-dithiodipyridine, followed by solid-phase extraction (SPE) and analysis by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) as described previously (58). Extracts were stored at −20°C until analysis.

Analysis of volatile sulfur compounds. The volatile sulfur compounds (VSCs) H_2S, methanethiol (MeSH), dimethyl sulfide, diethyl sulfide, dimethyl disulfide, diethyl disulfide, ethanethiol (EtSH), carbon disulfide (CS_2), methyl thioacetate (MeSAc), ethyl thioacetate (EtSAc), were determined using an Agilent 355 sulfur chemiluminescence detector coupled to an Agilent 6890A gas chromatograph (Forest Hill, VIC, Australia). The analysis was carried out as described previously (59). H_2S released during fermentation was detected in the headspace using silver nitrate selective gas detector tubes (Komyo, Kitagawa, Japan) (49).

Statistical analysis. Minitab 17 statistical software (Minitab Inc., PA) was used for statistical analysis. Data were analyzed by one-way analysis of variance (ANOVA) using Tukey's honestly significant difference (HSD) test ($P < 0.05$), and *P* values were determined by a two-tailed Student's *t* test.

Irc7p protein modeling. Irc7p7 was modelled using the structures for *Escherichia coli* cystathionine β-lyase (60) (PDB accession number 1CL2) and *Trichomonas vaginalis* methionine γ-lyase (PDB accession number 1E5F) as comparative references. The A and B chains of 1CL2 were superposed to 1E5F. The sequences for Irc7p, 1CL2, and 1E5F were aligned with Clustal X (http://www.clustal.org/clustal2). Ten models were generated with UCSF Modeller v9.15 (61) and evaluated with PROCHECK (62). UCSF Chimera v1.10.2 (http://www.cgl.ucsf.edu/chimera) was used for visualizing models and modeling mutations.

Data availability. The best model of Irc7p7 was uploaded to the Protein Model DataBase under accession number PM0081794.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02684-18.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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