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Genes involved in thiol release from cysteine and glutathione conjugates by Saccharomyces cerevisiae during wine fermentation

Margarita Paz Santiago Calderón

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Biological Sciences,
The University of Auckland, 30th May 2014
Abstract

The aroma of Sauvignon blanc is highly influenced by three volatile thiols: 4MMP, 3MH and 3MHA. Considerable effort has been made during the last decade in order to understand how these desirable thiols are released by *Saccharomyces cerevisiae* during wine fermentation. So far, three types of thiol precursors have been found in grape: cysteinylated-conjugates (C-4MMP and C-3MH), glutathionylated-conjugates (G-4MMP and G-3MH) and C6 compounds ((E)-2-hexen-1-ol and (E)-2-hexenal), providing a huge thiol potential. All these precursors have been shown to release their respective thiol by the action of yeast. However, their rate of conversion is low (less than 10%) and their relative contribution is unclear. Little is known about yeast genes that participate in thiol release. So far, the most important gene is *IRC7F*, which is necessary for 4MMP release. Additionally, the deletion of the transporters, *GAP1* and *OPT1*, partially reduced 3MH release.

The aim of this thesis is to identify genes involved in thiol release from cysteinylated and glutathionylated precursors. A full-length allele, *IRC7F*, was shown to be necessary and sufficient to release 4MMP from C-4MMP and 3MH from C-3MH and G-3MH in synthetic grape media. A wine yeast strain overexpressing *IRC7F* cleaved around 50% of C-4MMP and 10% of G-3MH into 4MMP and 3MH, respectively, in synthetic media. In addition, it was demonstrated that Irc7F cleaves L-cysteine *in vitro* (enzymatic assay with purified Irc7Fp) and *in vivo* (growth in L-cysteine in fermentative-like conditions), suggesting that it plays a biological role in yeast sulfur metabolism. Moreover, yeast selected for growth on L-cysteine showed improved 4MMP production. However, the uptake of C-4MMP does not occur via the known cysteine permeases, but it is controlled by the nitrogen catabolism repression.

The uptake of G-3MH occurs through Opt1p and its cleavage needs the action of the γ-glutamyltransferase, Cis2p, to release 3MH. It was demonstrated that yeast can use G-3MH as both a sulfur and nitrogen source, pathways that may account for losses in thiol precursor. Interestingly, glutathione seems to play a role in 3MH release from precursors different than C-3MH or G-3MH.

The results from this thesis contribute to the understanding and improvement of thiol production from cysteinylated and glutathionylated precursors. It also provides tools to search for novel 3MH precursors that may account for most of 3MH production.
To Simon for taking this trip with me.
This thesis would not have been finished without the support and help of many of people. I want to say a big THANK YOU to:

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To my dear parents: Emilio, Maggie for the being supportive, for their visits, laughs, skype talks and love.

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<tr>
<td>°C</td>
<td>Celsius degrees</td>
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<tr>
<td>3MH</td>
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<td>IRC7 short or deleted allele</td>
</tr>
<tr>
<td>kan</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>kb</td>
<td>Kilo base</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>NAT</td>
<td>Nourseothricin</td>
</tr>
<tr>
<td>NCR</td>
<td>Nitrogen catabolism repression</td>
</tr>
<tr>
<td>ND</td>
<td>Not detected</td>
</tr>
<tr>
<td>NG</td>
<td>No growth</td>
</tr>
<tr>
<td>NR</td>
<td>Not reached</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>O/N</td>
<td>Overnight</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SB</td>
<td>Sauvignon blanc</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SEC</td>
<td>S-ethyl-cysteine</td>
</tr>
<tr>
<td>SGD</td>
<td>Saccharomyces Genome Database</td>
</tr>
<tr>
<td>SGM</td>
<td>Synthetic Grape Medium</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris-hydroxymethylaminomethane</td>
</tr>
<tr>
<td>Vₚmax</td>
<td>Maximum velocity</td>
</tr>
<tr>
<td>wt</td>
<td>Wild-type</td>
</tr>
<tr>
<td>YAN</td>
<td>Yeast assimilable nitrogen (N mg/L)</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Overview of New Zealand winemaking: emphasis in Sauvignon blanc

New Zealand has a short but intense winemaking history. The first record of planted vines, brought from Australia, was at Kerikeri (Bay of Islands) by Samuel Marsden during the early 19th century. Years later, in 1836, D’Urville, the French Commander of the Astrolabe, tasted and made notes about a wine produced by James Busby, but it might not be the first wine made in New Zealand.

In 1838 Bishop Pompallier brought French vines to Hokianga and vineyards spread to supply priest requirements. The first capitalization on winemaking potential came with Charles Levet who planted 2.8 ha in 1863 around the Kaipara Harbour, beginning four decades of wine production at that site. During the late 19th century, vineyard planting was stimulated by the Victorian government assessor Romeo Bragato, who recognized and publicized the potential of New Zealand for winemaking.

After prohibition and vineyard infections during the early 20th century, the New Zealand wine industry started in earnest and New Zealanders attitude towards wine matured. Production jumped from 4.1 million litres in 1960 to 45 million litres by 1989.

The 1980 Grape Survey showed that the leading red grape planted was Cabernet Sauvignon followed by Pinotage; while the white varieties were Muller-Thurgau and Palomino. New regions were included for grape growing, including Gisborne in the late 1960s, Marlborough and Nelson in the early 1970s, and during late 1970s and 1980s the promising new regions of Canterbury, Wairarapa and Central Otago. A growing range of French grape varieties filled New Zealand vineyards. After a depression at the beginning of the 80s, there was large-scale replanting and the industry emerged onto the international stage and began a period of very rapid growth that has continued until now (Cooper (1990) Thorpy (1983)).

In a short period, Sauvignon blanc became a success. Matua Winery planted the first Sauvignon Blanc (SB) in 1969, which were imported from the University of California vineyard. These clones became the ones that developed the industry in New Zealand. In 1972, appointed by Montana Wines, Wayne Thomas of the Department of Scientific and Industrial Research (DSIR) recommended Marlborough as a suitable region for SB. In the mid 80’s Ernie Hunter, took his SB to the London International Wine Show, wining the three top trophies
with a fresh and fruity quality wine, so different from the French wine. After his success, SB grapes were planted in large quantities (Martech (2004)).

In 2013, New Zealand harvested 345,000 tonnes of wine grapes, of which 68% were SB (Winegrowers (2013)). SB also provided over 80% of the $1b in total export revenues earned by wine. It has consolidated as the dominant and most renowned variety from New Zealand. This consolidation has been due to the enhanced varietal aroma profile of SB wines, especially those from Marlborough that has resulted in a worldwide recognition. Marlborough SB wines have distinctive sensory properties with higher intensity levels when compared with international SB wines (Lund et al. (2009)).
1.2 Volatile compounds in wine

A small fraction of wine, approximately 1%, correspond to volatile compounds (Swiegers et al. (2005)). These volatiles are characterized by their low perception threshold, and consequently have a great impact in the resulting wine, despite their low concentrations.

Volatile compounds are classified depending on the timing of their appearance during the winemaking process (Rapp et al. (1986)).

- The aroma compounds derived from grape berries that are not changed during fermentation.
- The aroma compounds formed by yeast during fermentation of grape juice. The principal volatile compounds within this group are: esters, higher alcohols, carbonyl compounds, volatile acids, volatile phenols and sulfur compounds.
- The aroma compounds produced during wine aging and storage in wood barrels and bottles, where some volatile compounds are modified and some new compounds are released.

Wines present a large diversity of volatile compounds, whose perception depends on their concentration and the human perception threshold (Ferreira et al. (2009)). Interactions between volatiles and wine composition also influence wine aroma (Sáenz-Navajas et al. (2010)).
1.3 Sauvignon blanc aroma profile

SB wines have a characteristic aroma that is distinct from wines made from other grape varieties. Most of the volatile compounds that contribute to its distinctive aroma are undetected in grape juice and develop during alcoholic fermentation (Dubourdieu et al. (2006)). SB aroma has been described as having capsicum, tomato leaf, asparagus, grapefruit, gooseberry and passion fruit nuances. These descriptors will fall into two more general categories: ‘green’ i.e. capsicum, tomato leaf and asparagus, or ‘tropical’ i.e. grapefruit, gooseberry and passion fruit (Swiegers et al. (2006a)).

The green aroma characters results from methoxypyrazines. These are secondary metabolites from grape berries (Augustyn et al. (1982)), so are present in grape must and are not changed significantly by fermentation. Lacey (1991) concluded that there are at least three different methoxypyrazines in SB wines: 2-sec-butyl-3-methoxypyrazine, 2-isopropyl-3-methoxypyrazine and 2-isobutyl-3-methoxypyrazine. The latter, usually referred to as IBMP, is the most abundant methoxypyrazine in New Zealand wines, and contributes the capsicum character (Allen et al. (1991), Lund et al. (2009)).

The tropical characters come from sulfur compounds or thiols and form during yeast fermentation. The most important volatile thiols are 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA). They will be deeply described in the following Section 1.4, because they are the main subject of this thesis.

In addition, New Zealand SB had important differences from SB made in other countries. For example, New Zealand SB from Marlborough has been described by a sensory panel as tropical and sweet, sweaty, passion fruit, while French and South African were flinty/mineral and bourbon-like (Lund et al. (2009)). A significant role of volatile thiols in the unique character of Marlborough SB has been demonstrated (Mateo-Vivaracho et al. (2010), Benkwitz et al. (2012)).
1.4 Thiol aroma compounds in Sauvignon blanc

The varietal characters in SB wines come primarily from three volatile thiols in the wine. These volatile thiols are extremely potent, having low perception thresholds (Dubourdieu et al. (2006)).

Unlike methoxypyrazines, volatile thiols are almost non-existent in grape juice and are developed only during fermentation.

Table 1.1 describes the three main volatile thiols for SB varietal aroma. These are 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptahexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA). They were first identified in SB wines, but later found in other varieties like Scheruebe (Guth (1997a), Guth (1997b)), Gewürztraminer, Pinot gris, Riesling, Muscat, Sylvaner, Pinot blanc, Petit Manseng, Semillon (Tominaga et al. (2000)), Cabernet Sauvignon, Merlot (Murat et al. (2001b)), Rioja (Aznar et al. (2001)) and Koshu (Kobayashi et al. (2010)), as well as Syrah (Gerard Logan, personal communication) and Pinot noir (Tanya Rutan, personal communication).

Table 1.1: Volatile thiols in SB.

<table>
<thead>
<tr>
<th>Thiol</th>
<th>Structure</th>
<th>Perception threshold [ng/L]</th>
<th>Concentration in SB [ng/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>4MMP</td>
<td><img src="image" alt="Structure" /></td>
<td>$3^2$</td>
<td>$0 - 88^{2,4,5,7}$</td>
</tr>
<tr>
<td>3MH</td>
<td><img src="image" alt="Structure" /></td>
<td>$^{*}50 - 60^8$</td>
<td>$157 - 12800^{1,3,4,6,7}$</td>
</tr>
<tr>
<td>3MHA</td>
<td><img src="image" alt="Structure" /></td>
<td>$^{*}9 - 2.5^8$</td>
<td>$0 - 2,500^{1,3,4,6,7}$</td>
</tr>
</tbody>
</table>

* Perception threshold of R and S enantiomer, respectively.

1.4.1 4MMP

4MMP was first detected by Darriet (1993) in SB. The perception threshold was determined in wine (close to 3 ng/L). 4MMP is found in SB in a range of concentration (see Table 1.1) and has a low perception threshold. The compound was described as imparting box tree and broom aromas (Darriet et al. (1995)).

1.4.2 3MH and 3MHA

3MH was identified for the first time by Engel et al. (1991) in passion fruit. Later, Tominaga et al. (1998a) identified 3MH in SB. Its perception threshold in a 12% aqueous alcohol solution is 50-60 ng/L. This compound contributes to wine aroma with grapefruit and passion fruit notes (see Table 1.1).

3MHA is the acetylated form of 3MH. 3MHA smells like box tree and passion fruit and it can be perceived at a concentration of 2-4 ng/L in a hydro-alcoholic model solution (Tominaga et al. (1996)). It is described as ‘extremely fruity, suggestive of passion fruit, with a Riesling type note’.

3MH and 3MHA are chiral molecules with two enantiomers: R and S (Tominaga et al. (2006)). It has been shown that both enantiomers are distinguishable from each other by their olfactory descriptors in a hydroalcoholic model solution. R-3MH was described as grapefruit and S-3MH as passion fruit, with perception thresholds of 50 and 60 ng/L respectively. On the other hand, the S-form of 3MHA has nuances of passion fruit, while the R-form was more like box tree. 3MHA enantiomers are perceived at much lower concentrations of about 9 and 2.5 ng/L for the R and S-form.

Stereoisomers of 3MH and 3MHA have been reported in SB wines (Tominaga et al. (2006)). These authors found the two enantiomers (R:S) of 3MH and 3MHA at a ratio of approximately 50:50 and 30:70, respectively, at the end of fermentation. It was also shown that at early stages of fermentation, 3MH-S was more abundant (60%) than 3MH-R (40%). However, as the fermentation continues it stabilizes at 50:50 ratios. On the other hand, the ratios of 3MHA enantiomers stay constant during fermentation under the same conditions.

In contrast, when fermentation was carried out on synthetic juice spiked with 15 µM of C-3MH (Thibon et al. (2008a)), R and S enantiomers of 3MH were produced in 50:50 ratios throughout the fermentation. In the same study, the authors showed that a ure2 mutant of yeast produced 72:28 ratios of R and S forms of 3MH. The absence of Ure2p favored R-3MH production, suggesting that it may be a negative regulator of the cleavage pathway of the R-enantiomer.
1.5 Thiol Precursors

Different molecules have been proposed as precursors of thiols. 3MH and 4MMP are generated from odorless precursors in grapes, first identified as cysteinylated conjugates (Tominaga et al. (1998c)). Later glutathionylated conjugates (Peyrot des Gachons et al. (2002b), Fedrizzi et al. (2009)) were also detected and proposed as precursors.

Schneider (2006) proposed a third way of thiol release from non-amino acid precursors. The importance of each precursor and their ability to be converted into thiols will be reviewed in the following sections.

1.5.1 Cysteine conjugates

Cysteine-conjugates (cys-conjugates) were proposed to be thiol precursors when Tominaga (1998c) was able to detect 4MMP and 3MH after incubating a non-volatile crude extract of SB with a cell-free extract of *Eubacterium limosum*, which has known carbon-sulfur β-lyase activity (see Section 1.6.1). By measuring simultaneous 3MH release and the degradation of C-3MH *in vitro* in this experiment and in fermentations of experimental juice spiked with synthesized C-3MH) using VL3c yeast strain, these authors strongly suggested that there was conversion of cysteine precursors into thiols.

Cysteinyalted precursors have been quantified in several studies, with general agreement for the range of concentrations present in SB must. C-4MMP range from 5 to 29 µg/L (Roland et al. (2010c), Roland et al. (2010d)). For C-3MH, Subileau et al. (2008a) found between 11 and 55 µg/L, Thibon and colleagues (2008b) determined values of 48 to 78 µg/L, Capone et al. (2010) found 10 - 55 µg/L and Pinu et al. (2012) reported between 1 and 126 µg/L. Both C-4MMP and C-3MH are found at similar concentrations in SB must (Roland et al. (2010d)). The exception was found on botrytis-affected SB grapes, in which C-3MH was present at 1,100 to 2,000 µg/L (Thibon et al. (2008b)). In addition, higher proportions of (S)-C-3MH over the (R)-form have been reported in SB wines (Capone et al. (2010)) which is accentuated especially in botrytis-affected wines (Thibon et al. (2008b)).

Cysteinyalted precursors are required for production of thiols. Synthesized precursors added to synthetic juice resulted in 4MMP and 3MH release (see Tables 1.2 and 1.3).

More specifically, after fermenting synthetic juice spiked with a racemic mixture of C-3MH (3 mg/L), using VIN13 (overexpressing *tnaA*) the concentrations of R-3MH and S-3M were 53 µg/L and 141 µg/L (1:3 ratio),
respectively. This corresponds to approximately 14% conversion rate. No thiols were detected (limit detection 5 µg/L) when unmodified VIN13 was used (Grant-Preece et al. (2010)).

In addition, when different SB musts were supplemented with deuterated C-3MH and subjected to fermentation, deuterated 3MH was released. However, less than 1% molar conversion occurred (Subileau et al. (2008a)). Together these results have demonstrated the conversion of 4MMP and 3MH from synthetic cysteine conjugates by yeast. The proportion of thiols that originates from cysteinylated precursors in natural conditions is yet to be determined.

1.5.2 Glutathione conjugates

Glutathione-conjugates (Glut-conjugates) were discovered later than cysteine precursors. The original identification of G-3MH in must was performed by Peyrot des Gachons (2002b), but the first quantification of G-3MH precursors came from Capone et al. (2010). Concentrations ranged between 140 and 640 µg/L, up to 35 times higher than C-3MH in the same SB juices. In the same way as C-3MH, the S-form was more abundant than R-G-3MH. Simultaneously, another group (Roland et al. (2010a)) published completely contrasting amounts of G-3MH (from 1.3 to 7.5 µg/L) in SB juices, less than the cysteinylated precursors in the same juice. This 100x lower concentration of glutathione conjugates found in French musts could be due to differences in the must origin, differences in the harvesting and pressing conditions used to obtain the musts, or differences in the quantification methods used.

Direct evidence of conversion from G-3MH into the free thiol using synthesized precursors was recently obtained. For example, when VIN13 and VIN13 (tnaA) were allowed to ferment synthetic grape juice spiked with R-G-3MH (3 mg/L), only the latter was able to produce R-3MH (over the detection limit 5 µg/L) with a conversion rate of ~ 3% (Grant-Preece et al. (2010)). Additionally, Winter et al. (2011) has shown that 3MH formation from G-3MH is significantly less efficient than that from C-3MH.

Quite recently, the presence of G-4MMP in grape juice was confirmed by Fedrizzi (2009), but at approximately 5 µg/L in SB juice, a much lower concentration than its cysteine counterpart. Conversion of labeled G-4MMP (synthetic media and must spiked with 4.5 nmol/L of deuterated precursor) into the deuterated thiol through the action of VIN13 during fermentation was monitored. The conversion rate was near 0.3 % (Roland et al. (2010b)).
The publications cited above confirm the conversion of glut-conjugate precursors into thiols. However, it remains to be determined how much of 3MH and 4MMP come from the various types of precursor in natural fermentations.

### 1.5.3 Conversion of glutathionylated precursors into cysteinylated precursors

The first evidence that Cys-conjugates could be derived from glut-conjugates during fermentation arose from a study that showed an increase of C-3MH after percolating grape must through a $\gamma$-glutamyltranspeptidase column (Peyrot des Gachons et al. (2002b)). However C-4MMP did not show any difference after the treatment. These results suggest that there are important amounts of G-3MH, at least in this particular SB must, but not G-4MMP (Peyrot des Gachons et al. (2002b)).

The idea of conversion of glut-conjugates into Cys-conjugates was supported in 2009 (Wunschmann et al.) when the degradation pathway of glutathione-conjugates was unraveled in yeast. It was shown (Figure 1.1) that glut-conjugates can follow two different pathways to produce Cys-conjugates, and that a high proportion of the input glut-conjugate ends up as Cys-conjugates. Important amounts of the Cys-conjugate and the CysGly-conjugate were found in the medium. Also, direct demonstration of this conversion was provided by Grant Preece et al. (2010), who showed that when fermenting deuterated R-G-3MH with VIN13 ($tnaA$), deuterated R-C-3MH was present in the media at the end of the fermentation.

![Figure 1.1: Glutathione-conjugates degradation pathway.](image)

The degradation of glutathione conjugates can proceed through two pathways both requiring CIS2 and they are likely to occur in the vacuole (blue compartment). Contrary, glutathione turnover occurs in the cytoplasm via different enzymes. CIS2: $\gamma$-glutamyltranspeptidase, CPC: peptidase (YBR139w), CPY1: peptidase (YMR297w), GSH1: $\gamma$-glutamylcysteine synthetase, GHS2: glutathione synthetase, GST: glutathione-S-transferases, YCF1: Vacuolar glutathione S-conjugate transporter. Adapted from (Wunschmann et al. (2009) and Baudouin-Cornu et al. (2012)).
1.5.4 Other possible precursors: mesityl oxide and \(E\)-2-hexenal

Based on chemical similarity, Schneider (2006) proposed two additional molecules as possible precursors for 4MMP and 3MH, mesityl oxide and \(E\)-2-hexenal respectively. Both molecules must be subjected to a sulfur addition (from \(\text{H}_2\text{S}\) or cysteine) on the double bond to be transformed into the free thiol. In addition, \(E\)-2-hexenal should suffer a reduction of the carbonyl group to be transformed into 3MH. \(E\)-2-hexenal is a common and relative abundant component of grape juice, but the presence of mesityl oxide has never been described in must (Schneider et al. (2006)).

In order to test the ability of these two molecules to release thiols, fermentations were carried out using Melon B. must with added deuterated precursors, mesityl oxide and \(E\)-2-hexenal. The corresponding deuterated 4MMP and 3MH were detected, but comprised only about 10% of the total. Subileau et al. (2008a) have repeated the same experiment using deuterated \(E\)-2-hexenal and obtained a 3MH conversion rate of 0.05%. They also note that natural concentration of \(E\)-2-hexenal in the must used was 100 times less than the added precursor. For this reason the amount of 3MH produced through this pathway was considered not important. Harsch et al. (2013a) have shown that \(E\)-2-hexenal is rapidly catabolized by yeast early during fermentation without having the possibility to interact with the \(\text{H}_2\text{S}\) produced later during fermentation. However, adding \(\text{H}_2\text{S}\) at early stages of fermentation hugely increased 3MH.

Based on the results of these two research groups, it seems likely that 2-hexenal is able to be converted to 3MH, but in most juices is not a major contributor to the total 3MH made.
1.6 Yeast genes involved in thiol aroma release

1.6.1 Carbon-sulfur β-lyases

Carbon-sulfur (C-S) β-lyases cleave the carbon-sulfur bonds (β-elimination) of cysteine-S-conjugates, using pyridoxal phosphate (a vitamin B6 derivative) as cofactor. The reaction produces pyruvate, ammonium and a free thiol.

Howell et al. (2005) used a gene deletion approach in yeast to test the importance of possible C-S yeast β-lyase genes on 4MMP production. Based on sequence similarity with known C-S β-lyases, four candidate genes were selected from S. cerevisiae genome: YJL060w (BNA3), YAL012w (CYS3), YFR055w (IRC7) and YML004c (GLO1). Deletions were conducted in laboratory (BY4742) and wine yeast strains (VL3 derivative). Fermentation of the deleted laboratory strains in defined media with added C-4MMP (at concentrations 10,000x higher than those found naturally in grape juice) showed a 40 to 50% reduction in 4MMP release. When the deletions were tested in a wine strain, reductions of 30% for bna3Δ, 40% for irc7Δ and about 50% for cys3Δ were detected. The authors concluded that all four of these gene products are involved in 4MMP production.

Thibon (2008a) repeated these experiments for bna3Δ, cys3Δ and irc7Δ mutants using natural amounts of precursor added to ferments of synthetic medium. Under these conditions, IRC7 was the main β-lyase involved in thiol production as the deletion mutant showed a 96% reduction on 4MMP and 42% reduction on 3MH release. The other two mutants had no effect on thiol release under more normal physiological conditions.

These results suggest that the release of 4MMP from C-4MMP is mainly performed by one yeast enzyme, Irc7p, and that 3MH is released by more than one.

Results from our laboratory support the idea that 4MMP is produced in yeast by the IRC7 full-length gene product (Roncoroni et al. (2011)). A kanamycin-resistant cassette was used to delete a copy of IRC7 in a clinical yeast strain, YJM450 (a strain that produces good levels of 4MMP). Once sporulated, the progeny from one tetrad (segregating with a 2:2 ratio of wt: kanamycin-resistant phenotypes) was use to ferment grape juice. Both wt progeny produced normal amounts of 4MMP, but the two kanamycin-resistant progeny were unable to release 4MMP. No differences were found in 3MH production between the four progeny.

These results suggested that Irc7p is the major β-lyase involved in 4MMP production but is not fundamental for 3MH production in grape juice. Carbon-sulfur β-lyase activity was confirmed for Irc7p using YJM450 protein
extracts that were incubated with C-4MMP as substrate, resulting in an estimated Km of ~ 5 mM. Irc7p showed substrate preference for C-4MMP over C-3MH.

The IRC7 gene from YJM450 was highly similar to its homologue in Saccharomyces paradoxus strain, including upstream sequences, suggesting that there has been an introgression event from S. paradoxus to YJM450 of a fragment between 5 to 30 kb in the telomeric region of chromosome VI.

Further comparison of IRC7 sequence within different strains, has shown that the YJM450 gene (and a few more from the SGRP sequenced strains, which includes 37 S. cerevisiae and 27 S. paradoxus strains) have a 38-bp insertion (near 3’ end of the gene) that eliminates a stop codon in the laboratory strain (and most other natural strains), generating a larger gene product. This long version of IRC7 correlates with strains that produce 4MMP.

IRC7 is located in the subtelomeric region of chromosome IV and it has an unknown biological role. Nevertheless various phenotypes has been associated to its deletion. IRC7 (Increased Recombination Centre) was originally named by (Alvaro et al. (2007)), since its deletion showed increased levels of spontaneous Rad52 foci (Rad52 is a homologous recombination marker that localizes into sub-nuclear foci in response to DNA damage). In addition, IRC7 deletion also suppressed the temperature sensitive mutant cdc13-1 (Addinall et al. (2008)), so that the double mutant was able to grow at the non-permissive temperature (CDC13 is involved in telomere capping and telomerase recruitment).

The IRC7 transcript has been reported to be 6-fold induced after yeast was treated with an alkylating agent, methylmethanesulfonate (Jelinsky et al. (1999)), implicating this gene in DNA repair. IRC7 has been also characterized as an NCR-regulated gene (Bertram et al. (2000), Usaite et al. (2006), Scherens et al. (2006)), although it was not regulated by nitrogen or in NCR mutants of a wine yeast during fermentation (Deed et al. (2011)). IRC7 was both activated under copper deficiency, mediated by MAC1 (Gross et al. (2000), van Bakel et al. (2005)) and down-regulated by copper (Yasokawa et al. (2008)). It was downregulated in the cold during fermentation of M2 wine yeast (Deed (2003)).

Most of the IRC7 studies described above were carried out on laboratory strains, i.e., yeasts that possess the short alleles of the gene, except for the studies analyzing thiol release (Thibon et al. (2008a), Roncoroni et al. (2011)).
1.6.2 Cysteine amino acid transporters

Only small non polar molecules can diffuse across the membrane therefore it is believed that thiol precursors need to be transported inside the cell. It seems likely that membrane transporters involved in the uptake of cysteine could also be responsible for uptake of cysteine-conjugated thiol precursors.

Cysteine uptake is conducted non-specifically by several amino acid permeases in the plasma membrane belonging to the amino acid permease (AAP) family, which also take up other amino acids. The first AAP shown to be involved in cysteine uptake was GAP1, the General Amino acid Permease (Greasham et al. (1973)).

During-Olsen (1999) used an overexpression and deletion strategy, to show that several members of AAP family could transport cysteine under different nitrogen conditions. Under non-repressive nitrogen conditions, GAP1 and AGP1 (high Affinity Glutamine Permease) were the main transporters involved in cysteine uptake. In repressive nitrogen media, GNP1 (Glutamine Permease), BAP2 and BAP3 (Branched-chain Amino acid Permeases) and TAT1 and TAT2 (Tyrosine and tryptophan Amino acid Transporters) all contribute to cysteine uptake.

Later, Kosugi (2001) used a gap1Δ background to isolate a chemical mutant resistant to DL-allylglycine. This toxic cysteine analogue was used as a sole sulfur source in minimal media during the screening. After complementing the resistant mutant with a genomic library, MUP1 (Methionine UPtake) was found to be responsible of restoring DL-allylglycine sensitivity and also showed an increased cysteine uptake activity. This result suggested that MUP1 mediates methionine and cysteine transport in S. cerevisiae under these conditions.

All the above-mentioned AAPs (except GAP1) involved in cysteine uptake are upregulated by extracellular amino acids, sensed by the SPS complex (Ljungdahl (2009)). Figure 1.2 illustrates how this sensor works. A mutant deleted for SSY1 had an affected the uptake of different amino acids, especially Leu, Ile, Val, Phe, Tyr, Glu, Trp, Ser, Met and Gln (Didion et al. (1998)). However, cysteine uptake has not been studied in this strain.

Finally, Kaur (2007) described the unique high affinity cysteine-specific transporter YCT1 (Yeast Cysteine Transporter), a member of the Dal5 family of transporters. The yct1Δ met15Δ mutant showed a growth defective phenotype at low cysteine concentrations compared to the met15Δ mutant. At higher concentrations of cysteine, no differences in growth were observed, probably due to the transport activity of other permeases. Cysteine uptake was also shown to be disrupted in yct1Δ met15Δ mutant.
Figure 1.2: SPS sensor. The SPS sensor consists in three components: SSY1, PTR3 and SSY5. The Ssy1p component (membrane protein) senses extracellular aa and together with Ptr3, transduces amino acid-initiated signals resulting in the release of Ssy5p inhibitory pro-domain. The free active catalytic form of Ssy5p (serine protease) cleaves the cytoplasmic retention motif from Stp1 and Stp2 (transcription factors). The processed forms of Stp1 and Stp2, enter the nucleus, bind relevant promoters, and activate gene expression of several aa permeases. Adapted from Ljungdahl (2009).

In summary, the current evidence suggests that cysteine uptake is mediated by at least eight transporters at high concentrations, but that YCT1 plays the major role at low cysteine concentrations (less than ~ 200µM).

Genes shown to transport cysteine belong to two families: eight permeases from the AAP transporter family and one transporter belonging to the Dal5 family. However, not much research has been undertaken on cysteine transport under fermentative conditions.

Subileau (2008b) investigated the role of GAP1 in the production of volatile thiols. A gap1 deletion mutant in a laboratory yeast strain background was used to ferment synthetic media spiked with 250 µg/L of C-3MH. The resulting wine showed lower levels of 3MH and 3MHA than that fermented by the wildtype strain. Similarly, a wine yeast strain mutant, in NCR i.e. with GAP1 expression not subjected to repression by nitrogen availability, produced double the amount of 3MH compare to the wildtype. However, when both mutants were fermented using grape must no differences were detected.

No cysteine transporters have been involved in thiol precursor uptake leaving a lot to do in this area of research.
1.6.3 Glutathione and oligo-peptide transporters

As glutathione is a tri-peptide, glutathione-conjugated thiol precursors are likely to be taken up using peptide transporters in *S. cerevisiae*.

Different systems of proton-driven peptide transporters have been reported in *Saccharomyces cerevisiae*: the Peptide TRansport system (PTR), the OligoPeptide Transporters (OPT) and the Fungal Oligopeptide Transporter (FOT).

Ptr2p is the only member of the PTR family that takes up di/tri peptides, with a wide range of substrates (Perry et al. (1994)). However, it has been shown that glutathione is not a substrate for Ptr2p in YHP499 strain (Bourbouloux et al. (2000)). *PTR2* is down-regulated by NCR (Magasanik et al. (2002)) and up-regulated through the SPS complex by micromolar amounts of several amino acids, especially leucine and tryptophan (Island et al. (1987), Perry et al. (1994)). In addition, dipeptides induce *PTR2* transcription through degradation of the repressor Cup9p (SPS-independent pathway Hauser et al. (2001)).

Two transporters, *OPT1* and *OPT2*, belong to the OPT family. *OPT1* has been described as a high affinity glutathione transporter Bourbouloux et al. (2000) and as a tetra/pentapeptide transporter (Hauser et al. (2000)). Up-regulation of *OPT1* was observed when glutathione was deficient, but not sulfur (Bourbouloux et al. (2000)). Using another yeast background, Wiles (2006) showed exactly the opposite results. In addition, when yeasts were starved for sulfur, all amino acids (except sulfur-containing amino acids) induced *OPT1*, in a SPS-dependent manner (Wiles et al. (2006)).

On the other hand, *OPT2* did not participate in glutathione transport (Bourbouloux et al. (2000)). However, Lubkowitz (1998) placed *OPT2* under a constituitive promoter and concluded that it does encode an tetra and pentapeptide transporter, but the overexpression strain had no increased sensitivity to toxic di or tripeptides. *OPT2* has a very low expression levels during normal growth. Additionally, an opt2 mutant was sensitive to a wide variety of toxic compounds that are normally detoxified by vacuoles Aouida et al. (2009). In the same study, it was proposed that *OPT2* is involved in the formation of mature vacuoles because the deletion mutant contains several small vesicles instead of a large vacuole. Almost no information is known about how *OPT2* is regulated, only that its expression is 12-fold higher in a cup9 deletion mutant, suggesting that Cup9p may be acting as a repressor just as it does for *PTR2*.

Of these three known peptide transporters, the only one so far shown to be involved in volatile thiol release by yeast is *OPT1*. Subileau (2008a) fermented 100% SB juice with the lab strain BY4743 and an Δopt1 mutant. Fermentation was stopped at 8% of residual sugars and thiols were measured. Half of 3MH and 3MHA were
detected in the absence of OPT1, suggesting that this transporter is responsible for uptake of ~50% of 3MH precursor or that glutathione transport is important for 3MH release.

No information of whether PTR2 or OPT2 could be involved in volatile thiol release can be found in the literature.

The FOT transporters have a broad di and tripeptide specificity (Damon et al. (2011)). Heterologous expression of a FOT in Xenopus has shown its capacity to transport cysteine. They have a patchy distribution in S.cerevisiae, being present in the EC1118 genome but not in S288c. So far they have not been associated to thiol release in yeast strains.

1.6.4 Acetyltransferases and esterases

No specific precursor has been described for 3MHA. A likely route for 3MHA synthesis is through 3MH acetylation. Swiegers (2006b) probed this theory through overexpression in a wine yeast background (VIN13) of known enzymes involved in ester formation and degradation: two acetyltransferases (ATF1 and ATF2), an ethanol hexanoyl transferase (EHT1) and an esterase (IAH1). Levels of 3MHA were quantified after 2 d fermentation of different strains on model medium supplemented with 1 mg/L of 3MH (approximately 1,000 higher than the natural occurring amount). Overexpression of ATF1, but not of ATF2 or EHT1, increased 3MHA formation six fold. In the other hand, IAH1 overexpression reduced 3MHA by half compared to the wildtype. In the same way, ATF1 deletion and overexpression were tested on a lab strain, showing ~30% reduction and 1,000-fold increase, respectively. Thus, there are more genes other than ATF1 involved in 3MHA formation, but its overexpression is sufficient to increase it.

Work in our laboratory (Keith Richards and Soon Lee, unpublished) has confirmed a role for ATF1 in 3MHA production; overexpression of ATF1 in wine yeast F15 resulted in a doubling of 3MHA after fermentation in SB grape juice.

For simplification purposes, in this thesis 3MH and 3MHA will be quantified and expressed together as 3MH levels.
1.7 Factors influencing thiol release

Every wine is different from each other, because it is made from grapes coming from different regions and made by different winemakers. Many parameters can influence the final wine aroma and several factors have been studied that change thiols composition in wine.

1.7.1 Grape juice composition

The different composition of grape musts of the same variety is a result of differences in grape geographical origin, which involves climate, soils and vineyard management. All these variables contribute to the aromatic profile of wines.

Berna (2009) described a method based on chemical analysis of aroma profiles to group SB wine from six different geographical origins over three countries, with a very low error prediction (6.5%). In addition, other authors have associated wine composition to geographic regions (Benkwitz (2009)).

Thiol precursors have been found in grape musts from different varieties, but SB wines have the highest values (Capone et al. (2010)). This suggests that SB grapes produce more precursors as part of their metabolism. In addition, a huge range of thiol concentrations can be found between different SB wines. Murat (2001a) performed an experiment that clearly reflects the importance of must composition on thiol release. After fermenting four different SB musts with four different yeast strains, no statistical variation in 3MH levels was found when the same juice was fermented with different yeast strains. However, there were important differences among the four wines coming from different musts. This could be explained by environmental and soil conditions, among other factors, that create a specific grape must which varies from vineyard to vineyard even in the same geographical region.

1.7.2 Yeast strains

In addition to grape must, yeast strain is also very important for the aroma profile of the final product. This is true for thiols in general, but critically for 4MMP. Murat (2001a) was the first to demonstrate that 4MMP production was dependent on the chosen yeast strain. From the four strains studied, only two (EG8 and VL3c) produced high amounts of 4MMP in model and real fermentations, conditions in which no differences were found in 3MH levels (see preceding paragraph).
Howell (2004) also found a large variation in 4MMP levels between eight S. cerevisiae strains that are commercially available, and are usually used for the production of white wines. These experiments were performed in model medium fermented with added C-4MMP as precursor. Only three strains produced high 4MMP levels, including VL3c. Similarly, Swiegers (2009) tested seven S. cerevisiae commonly used in vinification and analyzed 4MMP, 3MH and 3MHA. Again 4MMP showed a high variability within strains, with the best producer VIN7 followed by VIN13 and VL3. Less variation was found in 3MH, but 3MHA levels were also unpredictable and showed high variation. QA23, NT116, VL3 and VIN7 were good 3MHA producers according to the coefficient between 3MHA:3MH, which is an important parameter for defining strains with high conversion rate.

Some non-cerevisiae strains have been found to produce high amount of thiols. Dubourdieu (2006) showed that S. bayanus var. uvarum was better at 4MMP and 3MH production when compared with S. cerevisiae strains. Seven out of nine inter-specific hybrids between VL3c and a strain of S. bayanus var. uvarum showed an increased ability to produce 4MMP compare to VL3c (parent with the lowest production).

Different yeast strains can act synergistically to improve wine aroma profile and to affect thiol production. This has been proved by using co-inoculation of S. cerevisiae strains or even strains from different species. When fermenting grape must using VIN7 in combination with QA23 or VIN13, both co-inoculations improve 3MH levels compare to single inoculations or blend between wines from same strains. Similarly, co-inoculation of Vin7 with QA23 improves 3MHA levels (King et al. (2008)).

In a later report, King (2010) showed that all the double or triple co-inoculation of selected S. cerevisiae strains improved 3MH and 3MHA production compared to single inoculations. Specially the triple co-inoculation was highly rated in good attributes (estery, floral, fresh citrus, passion fruit and fresh green) and also had high concentrations of many ester compounds, 3MH and 3MHA. However, the majority of consumers did not prefer the wine derived from triple-inoculation, highlighting the importance of including consumer tastings to evaluate the real impact of the final wine.

Co-inoculation with non-Saccharomyces species has also been shown to alter aroma profiles in SB wines. Anfang (2009) showed how a New Zealand isolate of Pichia kluveri increased 3MH and 3MHA when co-inoculated with VL3 (9:1 ratio), but this was a specific interaction and was not generalized to all Saccharomyces strains.
In summary, yeast strain selection is important for thiol release, especially 4MMP. Co-inoculation can be an interesting tool for improving the aromatic profile, including volatile thiols, but this is not necessarily linked to customer preference.

### 1.7.3 Nitrogen Catabolism Repression (NCR)

NCR is a metabolic response by which yeast preferentially uses good nitrogen sources (which include asparagine, glutamine or ammonia for some strains). Under these conditions, genes encoding permeases and enzymes required for the uptake and degradation of poor nitrogen sources (such as proline or allantoin) are expressed at low levels (Cooper et al. (1983)).

This repression involves the inactivation of two transcription factors Gln3p and Gat1p by Ure2p in the presence of nitrogen-rich sources (Ter Schure et al. (2000)).

When only poor nitrogen sources are available, these transcription factors are expressed at much higher levels, inducing the expression of genes for taking up and metabolizing these non-preferred nitrogen sources. For example, various permeases that transport nitrogen compounds are activated by the release of NCR: GAP1, AGP1, CAN1, DAL4, DAL5, DUR3, PTR2 and PUT4 (Ter Schure et al. (2000), Rossignol et al. (2003), Deed et al. (2011)).

Grape juice has two main sources of nitrogen that can be used by yeast: ammonia and amino acids. In SB juice, the major nitrogen sources are ammonia (~40%) followed by arginine, proline, glutamic acid and alanine (Soon Lee, data from nitrogen composition of Marlborough SB must).

Beltran (2004) studied NCR in fermentation conditions. Most of the assimilable nitrogen was consumed in the first 24 h of fermentation. Measuring GAP1 expression as a marker of NCR it was showed that yeast evolved from a nitrogen-repressed condition (low expression of GAP1) at the beginning of the ferment to a nitrogen-derepressed condition as the ammonia was consumed, allowing amino acid to be used as nitrogen source. In a global expression experiment throughout fermentation, Rossignol et al. (2003) showed major changes in genes expression triggered by nitrogen depletion. Induction of genes encoding nitrogen permeases (GAP1, CAN1, DUR3, MEP2, PTR2) are good examples.

Thiol release is affected by NCR. This was demonstrated most clearly by Thibon (2008a) when assessing both ure2Δ mutant (constitutively de-repressed for NCR) and a gln3Δ mutant (constitutively NCR) on synthetic grape must with added cys-precursors. The ure2Δ mutant showed three times more volatile thiols than the wt and the
gln3 mutant produced severely decreased amounts of thiols. It was demonstrated that ure2 mutant has a higher β-lyase cleavage activity resulting in higher thiol production. It was confirmed that ure2-mediated enhancement of thiol production was through high expression of IRC7 gene. In addition, Subileau et al. (2008b) showed that yeast release more 3MH from its cysteinylated precursor when grown on synthetic media with urea as a nitrogen source, compared to DAP. Moreover, after yeast fermentation no precursor was left in the media for the urea condition contrary to the media with DAP. The result was confirmed in grape juice supplemented with 2.5 mM DAP resulted in a significant decrease in 3MH production that was unrelated to GAP1 repression.

However, there appears to be important differences in yeast strains in their response to nitrogen generally (Ambroset et al. (2011), Treu et al. (2014)) and also in the effect of NCR on thiol production. Harsch et al. (2013b) showed that deletion mutants of three NCR genes, URE2, GLN3 and NPR1, all affected thiol production in a wine strain F15, as well as in the laboratory strain BY4743. However, the direction of the change often varied between the two strains, with some increasing and some decreasing thiols. In contrast, in the M2 commercial wine strain, Deed et al. (2011) showed no difference in thiol production in two NCR mutant strains (ure2Δ or a dal80Δ gzf3Δ double mutant), and no change in IRC7 transcript levels in the double mutant strain.

Summarizing, NCR on wine yeast strains is typically lifted during progress of the fermentation, which may also affect the release of thiols. In the VL3 strain, constitutive de-repression as seen in ure2 mutant increased thiols over wt quantities, suggesting that normal NCR repression is not completely abolished under fermentative conditions. Thiol regulation through NCR appears to be yeast strain-dependent, like most of the variables presented in this section.

1.7.4 Fermentation temperature

Under winemaking conditions, SB musts are fermented at low temperatures to increase some desirable characteristics, especially the perceived ‘fruitness’ of the wine. Studies have revealed that 4MMP production is sensitive to low temperatures. Fermentation of synthetic media with added C-4MMP using the strain AWRI130 at 18°C produce four-fold less 4MMP than at 28°C (Howell et al. (2004)).

Masneuf-Pomarede (2006) compared thiol production of VL3c, VIN13 and X5 at 13° and 20°C after fermenting two different grape juices. The amount of 4MMP was higher at 20°C for the three strains and it was undetectable when fermenting any of the two juices using VL3 and VIN13 at 13°C. For 3MH the three strains showed significantly less 3MH only in one juice (this juice showed 10-fold more production at 20°C). Low temperature also affected 3MHA release by the three strains.
The evidence reviewed above shows that cold ferments can often produce lower amounts of volatile thiols, but that the magnitude of the reduction is dependent on yeast strain and juice composition.

### 1.7.5 Wine making practices

Several parameters of wine-making also affect the yields of volatile thiols in wine.

- **Skin contact**
  
  Skin contact during wine making is very important for the final amount of precursor available in the must. It has been demonstrated that C-3MH content is 11-times higher in skin than the juice fraction of Merlot grape berries (Murat et al. (2001b)), and that the skin contains ~60% of total C-3MH. Increasing skin contact times increase the extraction of C-3MH precursor in the must. Higher temperatures during skin contact (up to 25°C) also increased precursor in Merlot and Cabernet Sauvignon must that correlates with amounts of 3MH released.

  In SB berries at maturity the skin possess ~20% of C-4MMP and 50% of C-3MH. After skin contact (19 h) C-4MMP and C-3MH increased in the juice by 30% and 50% respectively. Skin contact at higher temperatures (18°C) favoured the increase of C-3MH only (Peyrot des Gachons et al. (2002a)). Additionally, Patel et al. (2010) and Allen et al. (2011) showed that precursor was higher in heavy pressed fractions, but the level of 3MH and 3MHA were lower. In contrast, Roland et al. (2011) found more precursor in press fractions that led to slightly higher 3MH levels.

- **Nitrogen addition**
  
  Musts have different amount of yeast assimilable nitrogen (YAN) depending on the nitrogen status of the grape at harvesting time. YAN is the sum of ammonia nitrogen and primary amino acid nitrogen (excluding proline). An optimum YAN is approximately 200 to 300 mg/L of total nitrogen, and it is needed to avoid stuck fermentations and excessive hydrogen sulfide production (Jiranek et al. (1995)). If grape juice has a lower YAN, nitrogen supplementation is used, commonly by the addition of di-ammonium phosphate (DAP).

  Nitrogen availability and quality affects the sensory quality of wine (Hernández-Orte et al. (2005), Vilanova et al. (2007)). Subileau (2008b) investigated the role of nitrogen sources in thiol release. An industrial yeast strain was used to ferment synthetic media containing C-3MH and supplemented with 5 and 10 mM of urea or DAP as nitrogen source. The highest DAP concentration showed left-over
precursor in the media and reduced 3MH production, suggesting that an excess of DAP induces NCR and reduces C-3MH conversion to thiols.

- **Copper fining**
  Small quantities of copper added to SB wine very quickly decrease the characteristic Sauvignon Blanc varietal aroma. This happens because the copper readily reacts with a family of complex sulfur-containing compounds.
  Because copper sulfate is used to remove H$_2$S and undesirable thiols from wines, this practice can also potentially remove positive thiols (4MMP, 3MH and 3MHA) from SB wines. This was demonstrated by Ugliano et al. (2009) when thiols were reduced in copper fined wines.
1.8 Low conversion from precursors into volatile thiols

During all the experiments on thiol release, only a limited proportion of the precursors present in the initial fermentation media are released as volatile thiols in the wine by yeast fermentation. Estimates of the proportion of precursors actually converted range from less than 1% up to 10% (see Sections 1.5.1 and 1.5.2). Table 1.2 – 1.4 summarizes the conversion yield on synthetic media with added precursor performed by different authors until the completion of this thesis.

| Table 1.2: 4MMP conversion yield from C-4MMP by different authors in synthetic medium.* |
|----------------------------------|---|---|---|---|---|
| C-4MMP [µg/L] | 100,000 | 83 | 2 | 16,000 | 2 |
| 4MMP\textsuperscript{pot} [µg/L] | 60,000 | 50 | 1.2 | 9,600 | 1.2 |
| 4MMP [µg/L] | 950 | 0.033 – 0.32 | 0.01 – 0.15 | 0.015 | 0.003 |
| Yeast | VL3c, VL3, NW3, VIN13 | X5, F10 |

\* Concentration of 4MMP assuming total conversion of precursor

**References:** (1) Howell et al. (2005), (2) Murat et al. (2001a), (3) Masneuf-Pomarède et al. (2006), (4) Swiegers et al. (2007), (5) Zott et al. (2011).

| Table 1.3: 3MH conversion yield from C-3MH by different authors in synthetic medium.* |
|----------------------------------|---|---|---|---|---|---|---|---|---|
| C-3MH [µg/L] | 217 | 544 | 438 | 500 | 12.5 | 293 | 438 | 500 | 438 |
| 3MH\textsuperscript{pot} [µg/L] | 143 | 359 | 289 | 300 | 7.5 | 176 | 289 | 300 | 289 |
| 3MH [µg/L] | 0.336 | 0.310 | 0.3 – 0.5 | 0.03- 0.06 | 0.18- 1.23 | 9.4 – 11.4 | 1.2 | 1 |
| Yield | 0.24 | 0.086 | 0.1 – 0.17 | 0 | 0.4 – 0.8 | 0.1- 0.7 | 3.25 – 3.9 | 0.4 | 0.35 |
| Yeast | VL3c, Siha8, NW3, VIN13 | IS1, VIN13 | IS1 | VL3, VIN13 | X5, F10 |

\* Concentration of 3MH assuming total conversion of precursor

Some authors have shown low yields despite all precursor being consumed by yeast from the media. For example, Dubourdieu (2006) showed that although C-3MH degradation totaled 200 nM after 5 d of SB fermentation, the free thiol released amounted only to 20 nM. This low conversion rate may mean that a large part of the precursors is not converted to volatile thiols, but is otherwise metabolized by yeast. Alternatively it may be that the volatile thiols produced are not stable (but may be oxidized or metabolized) (Murat et al. (2001b)).

Some evidence reveals that enzyme (activity and/or expression) in yeast could be responsible for limiting thiol production. For example, a wine yeast (VIN13) overexpressing the *Escherichia coli* *tnaA* gene (encoding a tryptophanase with strong cysteine-β-lyase activity) produced more than a 10-fold increase of 4MMP and released substantially higher amounts of 3MH compared to the control strain (Swiegers et al. (2007)). Another example of improvement was shown by Thibon (2008a) when a deletion in *URE2*, that results in a more highly expressed *IRC7*, had an enhanced precursor bioconversion rate from 3–5% to 7–10%. These results suggest that expression of the lyase activity in yeast limits thiol production, at least in some circumstances.

However it is also possible that the regulation of precursor uptake by yeast might be limiting thiol production in some circumstances. Precursor uptake is expected to be affected by the activity of membrane transporters and regulation of their expression. Capone (2010) noted that there were significant amounts of C-3MH (9-50 µg/L) and G-3MH (90-480 µg/L) in some bottled wine samples from Riesling, Chardonnay and SB (highest values), suggesting that not all the available precursor is taken up by yeast.

Finally, one study has suggested that the quantity of 3MH released into wine is proportional to the initial precursor content in must, which means that higher precursor concentrations in grapes result in higher thiol concentrations in the wine (Murat et al. (2001b)). In contrast, results from New Zealand SB samples does not

<table>
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<td>G-3MH [µg/L]</td>
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<td>806</td>
<td>925</td>
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<td>49.5</td>
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<td>0.5 – 0.8</td>
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<td>VIN13</td>
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† Concentration of 3MH assuming total conversion of precursor

* References: (1) Roland et al. (2010a), (2) Kobayashi et al. (2010), (3) Winter et al. (2011)
show such correlation between initial precursor amount and final thiols released (Pinu et al. (2012)). The reason for these different findings is unclear.

A better understanding of how precursors are taken up by yeast, how thiols are produced during fermentation, and what alternative pathway of precursor utilization are present in yeast, will help us to develop new yeast strains with increased volatile release that will finally improve wine characteristics.
1.9 Aims of this research

In spite of the fact that a lot of knowledge in thiol contribution to wine aroma has been accumulated during the last few years, there is still lot of controversy about the real importance of cysteinylated and glutathionylated precursors. Also there is a lack of information on the yeast genes involved in volatile thiol release from these precursors.

The main objective of this thesis is to understand how yeast produces 4MMP and 3MH during fermentation from C-4MMP and G-3MH, respectively. In particular, the goal is to identify the yeast genes involved in the uptake of these thiol precursors from grape juice and their cleavage to generate volatile thiols inside the cell. To accomplish these goals, the following specific objectives will be addressed during the thesis research:

- Overexpress the \textit{IRC7} gene in non-thiol producing yeast.
- Identify yeast mutants in membrane transporters with impaired thiol production.
- Overexpress membrane transporters involved in uptake of cysteine and glutathione precursors.
- Assess how much 4MMP and 3MH is released from glutathionylated and cysteinylated precursors.
- Test the ability of yeast to use cysteine and glutathione precursors as nitrogen and/or sulfur sources.
- Study the biological function of \textit{IRC7}.
2 Materials and Methods

All solutions and media were prepared with distilled water and autoclaved at 121 °C for 20 min or filter sterilized using a 0.2-µm syringe filter (Minisart, Sartorius). All percentages are weight/volume (w/v) unless indicated.

For primer dilution and PCR reactions, sterile water was used.

Culture of microorganisms was performed under sterile conditions using a laminar flow hood.

Disposal of liquid microbial cultures were done into the sink after a 60-min treatment with bleach solution (final concentration 1%). Agar plate cultures were placed in appropriate bags, autoclaved and disposed.
2.1 Organisms

2.1.1 Bacterial strains

*Escherichia coli* strain DH10β (Grant et al. (1990)) was used for transformation of purified plasmids or ligations.

The strain BL21 (Studier et al. (1986)) was utilized for bacterial overexpression of *IRC7* and further protein purification.

2.1.2 Yeast strains

*Saccharomyces cerevisiae* strains used in this thesis are listed in Table 2.1.

2.1.3 Long-term storage of microorganisms

Microorganisms were stored at -80°C in growth media with glycerol, added to a final concentration of 15% (v/v).
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype and strain information</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>F15 Zymaflore</td>
<td>Commercial wine yeast, diploid</td>
<td>Laffort Oenologie</td>
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<tr>
<td>F15 h(a)</td>
<td><em>MATα</em>, <em>ho::hphMX</em>, HB41</td>
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S288C

MATα, SUC2, mal, mel, gal2, CUP1, flo1, flo8-1; laboratory strain; rotting fig; California

S288c IRC7^ox

P<sub>PGK</sub>-IRC7^ recombined into HO locus of S288c

This thesis

X5 Zymaflore

Wine yeast

Laffort Oenologie

YJM450

Clinical isolate

John McCusker

CP-A-A3

Strain selected, from a cross between WP11 (Soon Lee, this lab) and EC1118, after several rounds of breeding under cysteine as nitrogen source,

BY4743

MATα, his3-Δ1/his3-Δ1, leu2-Δ0/leu2-Δ0, LYS2/lys2-Δ0, met15-Δ0/met15, ura3-Δ0/ura3-Δ0, derived from S288c

BY4743 IRC7^ox

P<sub>PGK</sub>-IRC7^ recombined into HO locus of BY4743

This thesis

BY4743 IRC7^ox

Cross between BY4741 IRC7^ox and BY4742 IRC7^ox

This thesis

BY4743 opt1Δ

opt1::kanMX

Euroscarf

BY4743 opt1Δ IRC7^ox

P<sub>PGK</sub>-IRC7^ recombined into HO locus of BY4743 opt1Δ

This thesis

BY4743 dug1Δ

dug1::kanMX

Euroscarf

BY4743 dug1Δ IRC7^ox

P<sub>PGK</sub>-IRC7^ recombined into HO locus of BY4743 dug1Δ

This thesis

BY4743 agp1Δ

agp1::kanMX

Euroscarf

BY4743 agp1Δ IRC7^ox

P<sub>PGK</sub>-IRC7^ recombined into HO locus of BY4743 agp1Δ

This thesis

BY4743 bap2Δ

bap2::kanMX

Euroscarf

BY4743 bap2Δ IRC7^ox

P<sub>PGK</sub>-IRC7^ recombined into HO locus of BY4743 bap2Δ

This thesis

BY4743 bap3Δ

bap3::kanMX

Euroscarf

BY4743 bap1Δ IRC7^ox

P<sub>PGK</sub>-IRC7^ recombined into HO locus of BY4743 bap3Δ

This thesis

BY4743 gnp1Δ

gnp1::kanMX

Euroscarf

BY4743 gnp1Δ IRC7^ox

P<sub>PGK</sub>-IRC7^ recombined into HO locus of BY4743 gnp1Δ

This thesis

BY4743 mup1Δ

mup1::kanMX

Euroscarf

BY4743 mup1Δ IRC7^ox

P<sub>PGK</sub>-IRC7^ recombined into HO locus of BY4743 mup1Δ

This thesis
BY4743 tat1Δ
BY4743 tat1Δ IRC7ox
BY4743 tat2Δ
BY4743 tat2Δ IRC7ox
BY4743 yct1Δ
BY4743 yct1Δ IRC7ox
BY4741 IRC7ox
BY4741 cis2Δ
BY4741 cis2Δ IRC7ox
BY4741 cis2Δ cpcΔ
BY4741 cis2Δ cpcΔ IRC7ox
BY4741 cis2Δ cpcΔ cpyΔ
BY4741 cis2Δ cpcΔ cpyΔ IRC7ox
BY4742 IRC7ox
BY4742 IRC7ox

BY4743 tat1Δ tat1Δ::kanMX Eurosscarf
BY4743 tat1Δ IRC7ox PPGK-IRC7ox recombined into HO locus of BY4743 tat1Δ
BY4743 tat2Δ tat2Δ::kanMX Euroscarf
BY4743 tat2Δ IRC7ox PPGK-IRC7ox recombined into HO locus of BY4743 tat2Δ
BY4743 yct1Δ yct1::kanMX Euroscarf
BY4743 yct1Δ IRC7ox PPGK-IRC7ox recombined into HO locus of BY4743 yct1Δ
BY4741 IRC7ox PPGK-IRC7ox recombined into HO locus of BY4741
BY4741 cis2Δ cis2::kanMX Euroscarf
BY4741 cis2Δ IRC7ox PPGK-IRC7ox recombined into HO locus of BY4741 Δcis2
BY4741 cis2Δ cpcΔ cis2::kanMX, Δybr139w::LEU2 Erwin Grill (Wunschmann et al. (2009))
BY4741 cis2Δ cpcΔ IRC7ox PPGK-IRC7ox recombined into HO locus of BY4741 cis2Δ ybr139wΔ
BY4741 cis2Δ cpcΔ cpyΔ cis2::kanMX, ybr139w::LEU2, cpy::HIS3 Erwin Grill (Wunschmann et al. (2009))
BY4741 cis2Δ cpcΔ cpyΔ IRC7ox PPGK-IRC7ox recombined into HO locus of BY4741 cis2Δ ybr139wΔ cpyΔ
BY4742 IRC7ox MATα, his3-Δ1, leu2-Δ0, lys2-Δ0, ura3-Δ0 Euroscarf
BY4742 IRC7ox PPGK-IRC7ox recombined into HO locus of BY4742
BY4742 IRC7ox PPGK-IRC7ox recombined into HO locus of BY4742
X5-2 IRC7ox/irc7Δ::kanMX in X5 Keith Richards, this lab homosporic background
EC1118 Lalvin Prise de Mousse; wine yeast Lallemard isolated in Champagne
M4054 MATa gal2 ura3 gap1-Δ101, Birgitte Regenberg isogenic to S288c Grauslund et al. (1995))
M4054 IRC7ox PPGK-IRC7ox recombined into HO locus of M4054
M4246  
\textit{MATa ura3 gap1}\Delta agp1\Delta \hspace{1cm} \text{Birgitte Regenberg (Regenberg et al. (1998))}

M4246 \textit{IRC}^\text{ox} \hspace{1cm} P_{\text{PGK}}^{-1}\textit{IRC}^\text{ox} \text{ recombined into HO locus of M4246} 
\text{This thesis}

M4578  
\textit{MATa ura3 gap1}\Delta agp1\Delta gnp1\Delta \hspace{1cm} \text{Birgitte Regenberg (During-Olsen et al. (1999))}

M4578 \textit{IRC}^\text{ox} \hspace{1cm} P_{\text{PGK}}^{-1}\textit{IRC}^\text{ox} \text{ recombined into HO locus of M4578} 
\text{This thesis}

M4581  
\textit{MATa ura3 gap1}\Delta agp1\Delta gnp1\Delta \Delta (\text{bap2-tat1}) \hspace{1cm} \text{Jürgen Wendland (During-Olsen et al. (1999))}

M4581 \textit{IRC}^\text{ox} \hspace{1cm} P_{\text{PGK}}^{-1}\textit{IRC}^\text{ox} \text{ recombined into HO locus of M4581} 
\text{This thesis}

M4582  
\textit{MATa ura3 gap1}\Delta \Delta (\text{bap2-tat1}) \textit{bap3}\Delta \textit{tat2}\Delta \hspace{1cm} \text{Jürgen Wendland (During-Olsen et al. (1999))}

M4582 \textit{IRC}^\text{ox} \hspace{1cm} P_{\text{PGK}}^{-1}\textit{IRC}^\text{ox} \text{ recombined into HO locus of M4582} 
\text{This thesis}

M4583  
\textit{MATa ura3 gap1}\Delta agp1\Delta gnp1\Delta \Delta (\text{bap2-tat1}) \textit{tat2}\Delta \hspace{1cm} \text{Jürgen Wendland (During-Olsen et al. (1999))}

M4583 \textit{IRC}^\text{ox} \hspace{1cm} P_{\text{PGK}}^{-1}\textit{IRC}^\text{ox} \text{ recombined into HO locus of M4583} 
\text{This thesis}

M4584  
\textit{MATa ura3 gap1}\Delta agp1\Delta gnp1\Delta \Delta (\text{bap2-tat1}) \textit{bap3}\Delta \textit{tat2}\Delta \hspace{1cm} \text{Birgitte Regenberg (During-Olsen et al. (1999))}

M4584 \textit{IRC}^\text{ox} \hspace{1cm} P_{\text{PGK}}^{-1}\textit{IRC}^\text{ox} \text{ recombined into HO locus of M4584} 
\text{This thesis}

MS1 \hspace{1cm} yct1::\textit{URA3} \text{ in M4584 }\textit{IRC}^\text{ox} \hspace{1cm} \text{This thesis}

MS2 \hspace{1cm} mup1::\textit{hphMX} \text{ in M4585} \hspace{1cm} \text{This thesis}

M4238  
\textit{MATa gal2 ura3 gap1-}\textit{\Delta 101 ssy1-}\textit{\Delta 1} \hspace{1cm} \text{Birgitte Regenberg (Didion et al. (1998))}

M4238 \textit{IRC}^\text{ox} \hspace{1cm} P_{\text{PGK}}^{-1}\textit{IRC}^\text{ox} \text{ recombined into HO locus of M4238} 
\text{This thesis}

M4238 \textit{IRC}^\text{ox AGP1ox} \hspace{1cm} \text{kanMX:} \textit{URA3:}\textit{P}_{\text{Tef2}} \text{ cassette recombined into AGP1 promoter region in M4238 }\textit{IRC}^\text{ox} 
\text{This thesis}

M4238 \textit{IRC}^\text{ox BAP2ox} \hspace{1cm} \text{kanMX:} \textit{URA3:}\textit{P}_{\text{Tef2}} \text{ cassette recombined into BAP2 promoter region in M4238 }\textit{IRC}^\text{ox} 
\text{This thesis}

M4238 \textit{IRC}^\text{ox BAP3ox} \hspace{1cm} \text{kanMX:} \textit{URA3:}\textit{P}_{\text{Tef2}} \text{ cassette recombined into BAP3 promoter region in M4238 }\textit{IRC}^\text{ox} 
\text{This thesis}

M4238 \textit{IRC}^\text{ox DIP5ox} \hspace{1cm} \text{kanMX:} \textit{URA3:}\textit{P}_{\text{Tef2}} \text{ cassette} 
\text{This thesis}
recombined into *DIP5* promoter region in M4238 *IRC7*ox

M4238 *IRC7*ox *GNP1*ox kanMX: *URA3:P_{TEF2}* cassette recombined into *GNP1* promoter region in M4238 *IRC7*ox

M4238 *IRC7*ox *MUP1*ox kanMX: *URA3:P_{TEF2}* cassette recombined into *MUP1* promoter region in M4238 *IRC7*ox

M4238 *IRC7*ox *MUP3*ox kanMX: *URA3:P_{TEF2}* cassette recombined into *MUP3* promoter region in M4238 *IRC7*ox

M4238 *IRC7*ox *TAT1*ox kanMX: *URA3:P_{TEF2}* cassette recombined into *TAT1* promoter region in M4238 *IRC7*ox

M4238 *IRC7*ox *TAT2*ox kanMX: *URA3:P_{TEF2}* cassette recombined into *TAT2* promoter region in M4238 *IRC7*ox

M4238 *IRC7*ox *YCT1*ox kanMX: *URA3:P_{TEF2}* cassette recombined into *YCT1* promoter region in M4238 *IRC7*ox

M4238 *IRC7*ox *CIS2*ox kanMX: *URA3:P_{TEF2}* cassette recombined into *CIS2* promoter region in M4238 *IRC7*ox

M4238 *IRC7*ox *OPT1*ox kanMX: *URA3:P_{TEF2}* cassette recombined into *OPT1* promoter region in M4238 *IRC7*ox

This thesis
2.2 Growth media

2.2.1 Bacterial growth media

- Luria Bertani (LB)

This medium was used to grow *Escherichia coli* (Miller (1987)). It was prepared as follows: 1% peptone, 1% NaCl, 0.5% yeast extract and 1.5% agar (for solid media). Solid components were added to distilled water and autoclaved. If antibiotic selection was required (Table 2.6), it was incorporated after autoclaving.

- SOC

SOC medium was used for the recovery of *Escherichia coli* after electrotransformation (Hanahan (1983)). It consisted of 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose. All components, except glucose, were added into distilled water and autoclaved. After the medium cooled to ~65°C, filter-sterilized glucose was added.

- ZYP-5052 autoinduction media (Al)

Al media (Studier (2005)) was used for protein expression by *E. coli* BL21. It was prepared four times concentrated (4x) and diluted with sterile water before use. Table 2.2 shows the composition for one liter of ZYP-5052.

ZY, NPS and 5052 solutions were autoclaved separately and MgSO₄ was filter sterilized. The solutions were then combined as needed. The solution was stored in 250-mL sterile Schott bottles.
Table 2.2 AI media components.

<table>
<thead>
<tr>
<th>Components</th>
<th>1x</th>
<th>4x</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZY solution (720 mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tryptone</td>
<td>1%</td>
<td>4%</td>
</tr>
<tr>
<td>yeast extract</td>
<td>0.5%</td>
<td>2%</td>
</tr>
<tr>
<td>NPS solution (200 mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>50 mM</td>
<td>200 mM</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>50 mM</td>
<td>200 mM</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>25 mM</td>
<td>100 mM</td>
</tr>
<tr>
<td>5052 solution (80 mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.5%</td>
<td>2%</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.05%</td>
<td>0.2%</td>
</tr>
<tr>
<td>α-lactose</td>
<td>0.2%</td>
<td>0.8%</td>
</tr>
<tr>
<td>MgSO₄ solution (4 mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgSO₄</td>
<td>2 mM</td>
<td>8 mM</td>
</tr>
</tbody>
</table>

2.2.2 Yeast growth and testing media

- **YPD**

This rich media was used to culture yeast strains (Rose et al. (1990)). It was made with 1% yeast extract, 2% peptone, 2% dextrose, 1.5% agar (for solid medium).

- **Modified synthetic dextrose (MSD)**

MSD is a modified version of SD (Cherest et al. (1992)), used in this thesis to test yeast growth on various toxic compounds. NFM (Nitrogen-free MSD) is a MSD variant used to sulfur-starve yeast. Table 2.3 lists components used to prepare MSD and its variant.

Concentrated solutions of mineral salts (10X), magnesium chloride (10X), calcium chloride (10X) and trace elements (1000X) were mixed together and autoclaved. Vitamins and growth factors (100X), were filter sterilized and added after autoclaving. Auxotrophic supplementations were performed according to Table 2.5, and also added after autoclaving.

- **BiGGY Agar**

BiGGY Agar (Becton, Dickinson and Company, NJ, USA) was prepared according to manufacturer’s instruction, and used to assess H₂S production by yeast (Jiranek et al. (1995)).
<table>
<thead>
<tr>
<th><strong>Table 2.3 MSD components</strong></th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th><strong>Carbon Source</strong></th>
<th><strong>g L^−1</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>D- glucose</td>
<td>20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Vitamins and growth factors</strong></th>
<th><strong>mg L^−1</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>myo-inositol</td>
<td>20</td>
</tr>
<tr>
<td>calcium pentothenate</td>
<td>2</td>
</tr>
<tr>
<td>niacin (nicotinic acid)</td>
<td>2</td>
</tr>
<tr>
<td>pyridoxine hydrochloride</td>
<td>2</td>
</tr>
<tr>
<td>thiamine hydrochloride</td>
<td>2</td>
</tr>
<tr>
<td>folic acid</td>
<td>0.2</td>
</tr>
<tr>
<td>para-aminobenzoic acid</td>
<td>0.2</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.2</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.02</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Mineral Salts</strong></th>
<th><strong>mM</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>6.6</td>
</tr>
<tr>
<td>NaCl</td>
<td>1.7</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.5</td>
</tr>
<tr>
<td>MgCl₂ * 6 H₂O</td>
<td>2</td>
</tr>
<tr>
<td>CaCl₂ * H₂O</td>
<td>0.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Trace elements</strong></th>
<th><strong>µg L^−1</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>H₃BO₃</td>
<td>500</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>190</td>
</tr>
<tr>
<td>KI</td>
<td>100</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>50</td>
</tr>
<tr>
<td>CuCl₂</td>
<td>40</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Nitrogen source</strong></th>
<th><strong>mM</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄Cl₂</td>
<td>15</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Sulfur (omitted in SFM)</strong></th>
<th><strong>mM</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaSO₄</td>
<td>0.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Gelling agent</strong></th>
<th><strong>g L^−1</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar or phytagel</td>
<td>20 or 8</td>
</tr>
</tbody>
</table>
2.2.3 Yeast fermenting media

- Synthetic grape media (SGM)

SGM is a chemically defined medium that resembles grape juice based on the recipe described in Table 2.4 (Harsch et al. (2010)).

Most SGM components are derived from the medium described by Henschke et al. (1993), but DAP and amino acid composition was calculated from the concentrations found in Marlborough Sauvignon blanc grape juices.

<table>
<thead>
<tr>
<th>Carbon sources</th>
<th>g L⁻¹</th>
<th>Major components</th>
<th>g L⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>105</td>
<td>potassium tartrate</td>
<td>5</td>
</tr>
<tr>
<td>Fructose</td>
<td>105</td>
<td>Malic acid</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Amino acids and DAP</th>
<th>mg L⁻¹</th>
<th>Trace minerals</th>
<th>µg L⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-alanine</td>
<td>100</td>
<td>MgSO₄·7H₂O</td>
<td>1.23</td>
</tr>
<tr>
<td>L-arginine</td>
<td>400</td>
<td>CaCl₂·2H₂O</td>
<td>0.44</td>
</tr>
<tr>
<td>L-aspartic acid</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-cysteine</td>
<td>5</td>
<td>MnCl₂·4H₂O</td>
<td>198.2</td>
</tr>
<tr>
<td>L-glutamic acid</td>
<td>100</td>
<td>ZnSO₄·7H₂O</td>
<td>287.5</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>125</td>
<td>FeSO₄·7H₂O</td>
<td>70.1</td>
</tr>
<tr>
<td>L-glycine</td>
<td>5</td>
<td>CuSO₄</td>
<td>25.3</td>
</tr>
<tr>
<td>L-histidine</td>
<td>20</td>
<td>H₃BO₃</td>
<td>5.7</td>
</tr>
<tr>
<td>L-isoleucine</td>
<td>25</td>
<td>CoCl₂·6H₂O</td>
<td>23.8</td>
</tr>
<tr>
<td>L-leucine</td>
<td>25</td>
<td>NaMoO₄·2H₂O</td>
<td>24.2</td>
</tr>
<tr>
<td>L-lysine</td>
<td>5</td>
<td>KIO₃</td>
<td>10.8</td>
</tr>
<tr>
<td>L-methionine</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-phenylalanine</td>
<td>40</td>
<td>myo-Inositol</td>
<td>100</td>
</tr>
<tr>
<td>L-proline</td>
<td>300</td>
<td>pyridoxine hydrochloride</td>
<td>2</td>
</tr>
<tr>
<td>L-serine</td>
<td>60</td>
<td>nicotinic acid</td>
<td>2</td>
</tr>
<tr>
<td>L-threonine</td>
<td>75</td>
<td>Ca-pantothenate</td>
<td>1</td>
</tr>
<tr>
<td>L-tryptophan</td>
<td>10</td>
<td>thiamine hydrochloride</td>
<td>0.5</td>
</tr>
<tr>
<td>L-tyrosine</td>
<td>10</td>
<td>p-amino benzoic acid</td>
<td>0.2</td>
</tr>
<tr>
<td>L-valine</td>
<td>30</td>
<td>riboflavin</td>
<td>0.2</td>
</tr>
<tr>
<td>(NH₄)₂HPO₄</td>
<td>363</td>
<td>biotin</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td></td>
<td>folic Acid</td>
<td>0.2</td>
</tr>
</tbody>
</table>
Sugars and major components were mixed together in water (equivalent to 80% of final SGM volume). The pH was adjusted with concentrated HCl to 3.2 and autoclaved. Concentrated solutions of vitamins (100X, filter sterilized), trace elements (1000X, autoclaved) and amino acid and DAP (10X, autoclaved) were added to the solution. If necessary, auxotrophic supplementations were also added according to Table 2.5.

SGM was used as a fermenting media for wine yeast strains. However when laboratory yeast strains were used, only 75% of sugars were added. In addition, SGM (with no added sugars) was used for diluting grape juice, in a 1:3 ratio, when laboratory yeast strains were fermented (Harsch et al. (2010)). If thiols precursors were added, it was at a concentration of 50 µg/L C-4MMP (0.23 µM), C-3MH (0.23 µM), and 500 µg/L [3H2] G-3MH (1.24 µM).

Fermentation performed in high nitrogen consisted in SGM (YAN = 327) with supplemented DAP up to 1,200 YAN.

Two variations of SGM were used for growth rate measurements (Section 2.5): SGM without nitrogen and without sulfur. The former did not contain “10x Amino acids and DAP” solution. For the latter medium, cysteine and methionine were excluded from “10x amino acid and DAP” solution and the major component MgSO₄·7H₂O was replaced by MgCl₂·6H₂O.

- **Grape Juice**

Malborough Sauvignon blanc juices (M1016, Blanw, SB2007, Squire) from different vintages were used during the development of this thesis. These were supplied by Andy Frost (Pernod Ricard NZ Ltda) from their Rapaura winery and stored frozen at -20°C in 2L polyethylene terephthalate (PET) bottles.

Sterilization was performed by adding 1 mL of a 20% v/v dimethyl dicarbonate (DMDC) in ethanol per liter of juice. This was incubated at 25°C with shaking (100 rpm) over night before fermentation.

### 2.2.4 Yeast sporulation media

Pre-sporulation media was prepared using 0.8% yeast extract, 0.3% peptone and 10% glucose and autoclaved.

Sporulation plates were prepared by mixing 1% potassium acetate, 0.1% yeast extract, 0.05% glucose, 2% agar. If necessary auxotrophy supplementations were added after autoclaving (Rose et al. (1990)).
2.2.5 Auxotrophy supplementation

Amino acid and nitrogen base solutions were used to supplement different medium when yeast strains with auxotrophies were cultured. Stock solutions and final concentration of all amino acid used are listed in Table 2.5. All stock solutions were stored at room temperature.

Table 2.5 Auxotrophy supplementations

<table>
<thead>
<tr>
<th>Nitrogen base or amino acid</th>
<th>Stock solution [mg/mL]</th>
<th>Final concentration [mg/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>Histidine</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Leucine</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>Lysine</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>Methionine</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Uracil</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>10</td>
<td>20</td>
</tr>
</tbody>
</table>

2.2.6 Antibiotics selection

Antibiotics were used to select yeast and bacteria strains containing a selection cassette or plasmid as shown in Table 2.6.

Table 2.6 Antibiotic supplementations for selection

<table>
<thead>
<tr>
<th>Organism</th>
<th>Antibiotic</th>
<th>Selection cassette or gene</th>
<th>Final concentration [µg/mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast</td>
<td>ClonNat or nourseothricin</td>
<td>natMX</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Hygromycin B</td>
<td>hphMX</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>Geneticin or G418</td>
<td>kanMX</td>
<td>200</td>
</tr>
<tr>
<td>Bacteria</td>
<td>Ampicillin</td>
<td>β-lactamase</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Kanamycin</td>
<td>Neomycin</td>
<td>50</td>
</tr>
</tbody>
</table>
\[phosphotransferase II\]
2.3 Microvinification

Microvinifications were carried out on 200 mL of grape juice or SGM or a mix of both in 250 flasks, sealed with airlocks. Wine strains were fermented in 100% grape juice and laboratory strains in 75% grape juice and 25% SGM (no added sugars and supplemented with auxotrophies requirements if necessary).

Daily weight measurements were performed until no weight loss is observed (end of fermentation). Wine was collected into three 50 mL containers after 5 min centrifugation at 6,000 x g to discard yeast cells. If genetically modified yeast were used, the wine was treated with 12 µL DMDC during 4 hours at 25°C with shaking. Samples were stored at -20°C.
2.4 Thiol analysis

2.4.1 Thiol extraction and quantification by $p$-hydroxymercuribenzoate (PHMB) method

This method is a modification from (Tominaga et al. (1998b)) that uses only 50 mL of wine sample, instead of 500mL. It was written by Robert Winz based on notes from Takatoshi Tominaga’s visit in 2004, and subsequently modified (Herbst-Johnstone et al. (2013)).

More in detail, Dowex resin (2x1, Sigma-Aldrich #44290) was activated by adding 2 or 3 mL of 0.1M HCl and washed out with MQdH2O water until pH 5-6, leaving it in water suspension. A volume of 13.5 mL of this suspension was pipetted into a glass column with a silanised wool plug on the bottom end. Bubbles were removed by gently stirring the resin with a Pasteur pipette and a filter paper disc (12mm diameter) was carefully floated onto the top of the resin. The column was washed with 100 mL MQdH2O using a flow rate of one drop every 3 sec.

In the meantime, wine sample was mixed with 5mL of 2mM pHMB, 0.5 mL of 2mM BHA, 25µL of 1 nmol 4M2M2MB and 50µL of a mixed of 0.3 nmol of [1-2H2]3-MHA and 1.5 nmol of [1-2H2]3-MH. The pH was adjusted to 7 using 10M NaOH and the sample was flush through the column (1 drop every 5 sec).

The column was washed with 50mL of Wash Buffer (0.1M NaAc pH 6.0, 0.5 mL 2mM BHA) adjusting the flux to 1 drop every 5 seconds. Thiols were eluted into a 100mL volumetric flask by adding 50mL of Elution Buffer (0.1M NaAc pH 6, 50 mM L-cysteine, and 0.5 mL 2mM BHA) at a flow rate of 1 drop every 7 sec.

Thiols extraction was performed twice using 4 and 2 mL of dichloromethane, adding 0.5mL ethyl acetate to the first extraction. After 5 min of magnetic stirring, the organic phase was collected, using a separating funnel, into a container with anhydrous sodium sulfate. The sample was filtered into a 10 mL graduated tube through a pipette Pasteur with silanised wool. Under a nitrogen flux, the sample was concentrated to 100-200 µL, transferred to a vial and finally concentrated to 25 µL.

The gas chromatographic analysis of thiols is carried out using an Agilent 6890N gas chromatograph (Santa Clara, CA) equipped with a 7683B automatic liquid sampler, a G2614A autosampler, and a 5973 mass selective detector. Samples are placed into a tray cooled to 9°C for automated injection. A volume of 1 µL of the sample was injected. Some GC/MS important parameters are listed below:
• Inlet temperature: 240°C
• Column: Agilent HP-INNOWax capillary column (60 m x 0.252 mm ID, 0.25 μm film thickness)
• Carrier gas: helium (112 kPa) at an initial flow rate of 1 mL/min (for 43.60 minutes), raised to 2.4 mL/min for 7 minutes after the separation of compounds of interest, dropping back to 1 mL/min for 2 minutes.
• Oven temperature: initially 50°C for 5 minutes, then ramped to 162°C at a rate of 3°C/min, then raised to 250°C at 70°C/min (held for 10 min) before dropping down to 50°C.
• Temperature of the interface line: 250°C.
• Ion source: electron impact mode at 70 eV, 250°C.
• Quadrupole temperature: 150°C.

After the run was finished, thiols quantification was carried out using ChemStation or MassHunter Workstation Software Version B.04.00 software (Agilent Technologies). Table 2.7 show the ions used for identification and quantification of each thiol and internal standard peaks. Ratios between a thiol and its respectively internal standard were calculated and introduced into the linear equation (see Section 2.4.3) to obtain the concentration in ng/L.

<table>
<thead>
<tr>
<th>Thiols</th>
<th>retention time* [min]</th>
<th>ions [m/z]</th>
</tr>
</thead>
<tbody>
<tr>
<td>4M2M2MB</td>
<td>18.88</td>
<td>134, 75</td>
</tr>
<tr>
<td>4MMP</td>
<td>26.80</td>
<td>132, 75, 99</td>
</tr>
<tr>
<td>[\textsuperscript{3}H\textsubscript{2}]3MHA</td>
<td>40.50</td>
<td>119, 104</td>
</tr>
<tr>
<td>[1-\textsuperscript{2}H\textsubscript{2}]3MHA</td>
<td>40.52</td>
<td>118, 103</td>
</tr>
<tr>
<td>3MHA</td>
<td>40.59</td>
<td>116, 101</td>
</tr>
<tr>
<td>[\textsuperscript{3}H\textsubscript{2}]3MH</td>
<td>43.60</td>
<td>137, 103</td>
</tr>
<tr>
<td>[1-\textsuperscript{2}H\textsubscript{2}]3MH</td>
<td>43.67</td>
<td>136, 102</td>
</tr>
<tr>
<td>3MH</td>
<td>43.72</td>
<td>134, 100</td>
</tr>
</tbody>
</table>

*retention times change, but are around these values.
2.4.2 Thiol extraction and quantification by ethyl propiolate derivatisation (ETP) method

This method was developed by (Herbst-Johnstone et al. (2013)). Briefly, to 50 mL of wine was added 500 µL of 2 mM BHA, 50 µL of the internal standard mix (0.3 mmol d2-3MH and 1.5 nmol d2-3MH), and 500 µL of 100 mM ETP ethanolic solution. The mixture was stirred for 2 min at 500 rpm and pH adjusted to 10.0 ± 0.1. The solution was mixed for 10 min at 500 rpm, followed by centrifugation of the sample for 10 min at 6000 rpm.

In the meantime, the SPE cartridge was activated with methanol (10 mL) and washed with ultrapure water (10 mL).

Then the sample was passed through the Supelclean ENVI-18 SPE cartridge attached to a Biotage VacMaster-10 sample processing manifolds. The cartridge was rinsed with water (10 mL) and then the thiols were recovered by passing 10 mL of dichloromethane into a container with Na2SO4. The sample was filtered into a 10 mL graduated tube through a pipette Pasteur with silanised wool and concentrated to about 20 µL under a gentle stream of nitrogen.

The gas chromatographic analysis of thiols is carried out using an Agilent 6890N gas chromatograph (Santa Clara, CA) equipped with a 7683B automatic liquid sampler, a G2614A autosampler, and a 5973 mass selective detector. Samples are placed into a tray cooled to 9°C for automated injection. A volume of 2 µL of the sample was injected. Some GC/MS important parameters are listed below:

- Inlet temperature: 250°C
- Column: Agilent HP-INNOWax capillary column (60 m x 0.250 mm ID, 0.25 µm film thickness).
- Carrier gas: helium at an initial flow rate of 1.2 mL/min.
- Oven temperature: initially 150°C for 2 minutes, then ramped to 250°C at a rate of 10°C/min, and held for 20 min.
- Temperature of the interface line: 250°C.
- Ion source: electron impact mode at 70 eV, 250°C.
- Quadrupole temperature: 150°C.
After the run was finished, thiols quantification was carried out using ChemStation or MassHunter Workstation Software Version B.04.00 software (Agilent Technologies). Table 2.8 show the ions used for identification and quantification of each thiol and internal standard peaks. Ratios between a thiol and its respectively internal standard were calculated and introduced into the linear equation (see Section 2.4.3) to obtain the concentration in ng/L.

Table 2.8: Ions and retention time used in thiols quantification using the ETP method.

<table>
<thead>
<tr>
<th>Thiols</th>
<th>retention time* [min]</th>
<th>ions [m/z]</th>
</tr>
</thead>
<tbody>
<tr>
<td>d10-4MMP-ETP</td>
<td>13.2, 15.2</td>
<td>240, 142, 109</td>
</tr>
<tr>
<td>4MMP-ETP</td>
<td>13.3, 15.3</td>
<td>230, 132, 157</td>
</tr>
<tr>
<td>[3H2]3MHA</td>
<td>16, 18.5</td>
<td>277, 232, 86</td>
</tr>
<tr>
<td>[1-2H2]3MHA-ETP</td>
<td>16.1, 18.6</td>
<td>276, 231, 85</td>
</tr>
<tr>
<td>3MHA-ETP</td>
<td>16.2, 18.7</td>
<td>274, 229, 83</td>
</tr>
<tr>
<td>[3H2]3MH</td>
<td>19.3; 22.6</td>
<td>235, 190, 135</td>
</tr>
<tr>
<td>[1-2H2]3MH-ETP</td>
<td>19.4, 22.7</td>
<td>234, 189, 134</td>
</tr>
<tr>
<td>3MH-ETP</td>
<td>19.5, 22.8</td>
<td>232, 187, 132</td>
</tr>
</tbody>
</table>

*Retention times change, but are around these values.

2.4.3 Calibration curves

A calibration curve was done on 50 mL of a model white wine (12% ethanol, 5g/L tartaric acid, pH 3.2) by adding increasing amounts of each compound in a range of occurrence (4-MMP 10-15000 ng/L, 3-MHA 20-3000 ng/L, and 3-MH 500-20,000 ng/L) plus the same amount of internal standards used in Section 2.4.1 or Section 2.4.2.

For each sample, thiols were extracted from the model white wine according to one of the methods described above. After the run was finished, thiols quantification was carried out using ChemStation or MassHunter Workstation Software Version B.04.00 software (Agilent Technologies). The area of each peak corresponding to the selected ion was calculated and the ratios of the peak area of thiol to peak area of internal standard were plotted against the corresponding thiol concentration (linear regression) obtaining a linear equation used to calculated thiol concentrations.
2.4.4 Thiol molar conversion from added precursors

Molar conversion yields were calculated as the total concentration of a particular thiol (nM) quantified at the end of fermentation divided by the concentration of “potential thiol” added to the media.

\[
\% \text{ 4MMP yield} = \left( \frac{[\text{4MMP}^{\text{quantified}}]}{[\text{4MMP}^{\text{potential}}]} \right) \times 100
\]

\[
\% \text{ 3MH yield} = \left( \frac{([\text{3MH}^{\text{quantified}}] + [\text{3MHA}^{\text{quantified}}])}{[\text{3MH}^{\text{potential}}]} \right) \times 100
\]
2.5 Growth curves using Bioscreen

Growth curves were done using the Bioscreen C Analyzer, measuring O.D. every 15 min during three d. The initial cell number was $1 \times 10^3$ cell/mL. Temperatures used were 25°C (for fermentative conditions) and 28°C (for normal growing conditions).

2.5.1 Growth curves in SGM without nitrogen or sulfur

Yeast strains were growth overnight on YPD. Next day, yeast were pelleted by centrifugation and resuspended in water at a concentration of $3 \times 10^4$ cells/mL. A volume 150 µL of SGM per well (without nitrogen or sulfur, see Section 2.6.1), supplemented with the appropriated nitrogen (15 mM) or sulfur source (0.5mM), was display in a honey comb plate and inoculated with 5 µL of the yeast suspension.

2.5.2 Growth curve parameters

Bioscreen data was plotted and three growth parameters (lag phase, doubling time and growth efficiency) were calculated as described by Gutiérrez et al. (2013). Briefly, Lag phase is the mean of the two highest intercepts between a slope (calculated every eight OD measurement points) and a straight horizontal line (corresponding to the initial OD). The doubling time correspond to the mean of 5 of the highest slopes calculated every three consecutive OD measurement points through the whole growth curve. Growth efficiency was calculated as the difference between end OD (mean of the six last OD measurements) and initial OD.

![Growth curves and parameters](image)

**Figure 2.1: Growth curves and parameters.** Plotted growth curves were used to obtained growth parameters: lag phase (time to initiate proliferation), rate or generation time and efficiency (total change in population density during growth). Diagram extracted from Gutiérrez et al. (2013).
2.6 Statistical test applied to results

The thiol quantities were subjected to one-way analysis of variance (ANOVA) using the SPSS Software (IBM). The significance of the differences was studied by a Tukey's HSD (honest significant test) using the SPSS Software (IBM).
2.7 Plasmids

2.7.1 E. coli plasmids

pAG32 (Goldstein et al. (1999)) was used to amplify hphMX cassette, for gene deletion purposes.

pKT127 (Sheff et al. (2004)) was used to amplify yEGFP cassette for Irc7p localization in yeast cells.

pET28a(+) (Novagen, Darmstadt, Germany) and pET28MBP (modification of pET28 made by Kien Ly that contains the Maltose Binding Protein) were used for overexpressed IRC7 gene from X5 in E. coli.

2.7.2 Yeast plasmids

pDH1 (David Hooks, this laboratory) and ptnaA (Keith Richards, this laboratory) were used to overexpress IRC7F (from X5) and tnaA (from E. coli) genes in S. cerevisiae by homologous recombination of the overexpression cassette (pGK1promoter-gene), into HO gene.

pDH1 plasmid was further used to overexpress STR3 (from X5) and IRC7 (from S288c). This was done by replacing the original gene in pDH1, using homologous recombination.

pCHUKTEF (Hester Sheehan, this laboratory) was used to amplify the URA3-Kan-TEF3prom cassette.
# 2.8 Oligonucleotides

All oligonucleotides used in this thesis are listed in Table 2.8.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Application (source, if other than author)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRC7 Inside F</td>
<td>ACAGGATATTCCAGCTTTGGTCTCTGT</td>
<td>Amplifies 3' half of IRC7 gene (David Hook).</td>
</tr>
<tr>
<td>IRC7 Inside R</td>
<td>TCGATTTCCGAGCCAAGCAGCCA</td>
<td>Amplifies 5' half of IRC7 gene (David Hook)</td>
</tr>
<tr>
<td>IRC7delFor</td>
<td>AGCTGGTCTGGAGAAAATGG</td>
<td>Amplifies from the upstream region of the IRC7 deleted fragment. Used with IRC7delRev to test for full length IRC7 locus (Miguel Roncoroni)</td>
</tr>
<tr>
<td>IRC7delRev</td>
<td>TCTTCTGCGAGACGTCTCAAA</td>
<td>See above (Miguel Roncoroni)</td>
</tr>
<tr>
<td>pGK F</td>
<td>ACTTGCATAAATTGGTCAATGC</td>
<td>Amplifies from PGK promoter (Keith Richards)</td>
</tr>
<tr>
<td>pGK R</td>
<td>TAGCGTAAAGGATGGGAAAA</td>
<td>Amplifies from PGK terminator (Keith Richards)</td>
</tr>
<tr>
<td>TNAtest1 F</td>
<td>CCCTGACTTGGCAACCTAA</td>
<td>Amplifies from the upstream HO 5' region that is the genomic flanking region of insertion cassettes derived from pDH1 (Keith Richards). Used with TNAtest1 R to check genomic insertion of PGK:tnaA or IRC7 or STR3 cassette.</td>
</tr>
<tr>
<td>TNAtest1 R</td>
<td>CCGTGTGCTCAAGAGTGGTA</td>
<td>Amplifies from NAT gene in cassettes derived from pDH1 plasmid (Keith Richards). See above.</td>
</tr>
<tr>
<td>TNAtest3 F</td>
<td>AATCTCGACTGGCTGGCTTA</td>
<td>Amplifies from TNA 3’region (Keith Richards). Used with TNAtest3 R to check genomic insertion of PGK:tnaA cassette.</td>
</tr>
<tr>
<td>TNAtest3 R</td>
<td>CATGTGCTGACAACCAAACC</td>
<td>Amplifies from downstream HO 3’region that is the genomic flanking region of insertion cassettes derived from pDH1 (Keith Richards). See above. Also used with IRC7 inside F to check genomic insertion of PGK:IRC7 cassette.</td>
</tr>
<tr>
<td>Primer</td>
<td>Sequence</td>
<td>Function</td>
</tr>
<tr>
<td>--------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td>PGK-STR3-F</td>
<td>ACAGATCATCAAGGAAGTAATTATCTACTTTTACAACAAATATAAAAACCGGACGCCGC</td>
<td>Used with PGK-STR3-R to place STR3 downstream PGKprom in pDH1 plasmid</td>
</tr>
<tr>
<td>PGK-STR3-R</td>
<td>AATAAAGCTTTATTTTAGCGTAAAGGATGGGGAAAGAGAAAAAGACCCAGCCGCG</td>
<td>See above</td>
</tr>
<tr>
<td>STR3test-R</td>
<td>AAGAATCCATTGGGATAAT</td>
<td>Used with PGK-F to test cloning of STR3</td>
</tr>
<tr>
<td>STR3del-R</td>
<td>GAATGCCTTGGAAATTAAGTG</td>
<td>Used with pGK-STR3-F to delete STR3</td>
</tr>
<tr>
<td>STR3deltest-R</td>
<td>GGAAATGACTCTTGTTTCGAT</td>
<td>Used with 3’ Kan I to check STR3 deletion</td>
</tr>
<tr>
<td>STR2del-F</td>
<td>TTAACCCACATTTTGTCTCAC</td>
<td>Used with STR2del-R to delete STR2</td>
</tr>
<tr>
<td>STR2del-R</td>
<td>TAGCTCGATTTTTCTGCTTT</td>
<td>See above</td>
</tr>
<tr>
<td>GSH1del-F</td>
<td>AGCCGACTCTACTACAACTGC</td>
<td>Used with GSH1-R to delete GSH1</td>
</tr>
<tr>
<td>GSH1del-R</td>
<td>CGTGGGCTATAAGTGTGTGTA</td>
<td>See above</td>
</tr>
<tr>
<td>3’Kan I F</td>
<td>GGTCCTATATCGTCTGTC</td>
<td>Amplifies from 3’ region of kanMX marker (Heather Brown)</td>
</tr>
<tr>
<td>5’Kan I R</td>
<td>GCACGCTAAGAGCTGTAAGGG</td>
<td>Amplifies from 5’ region of kanMX marker (Heather Brown)</td>
</tr>
<tr>
<td>MAT-sextype</td>
<td>AGTCACATCAAGATCGTTTATGG</td>
<td>Used with either a or α-sextype primer to detect mating type (Heather Brown)</td>
</tr>
<tr>
<td>a-sextype</td>
<td>ACTCCACCTTCAAGTAAGAGTTT</td>
<td>See above (Heather Brown)</td>
</tr>
<tr>
<td>α-sextype</td>
<td>GCACGGAATATGGGACTACTTCG</td>
<td>See MAT-sextype (Heather Brown)</td>
</tr>
<tr>
<td>cis2test-R</td>
<td>TGCGGCTATGTCGGCGTAGTTA</td>
<td>Used with 3’Kan I F to confirm CIS2 deletion</td>
</tr>
<tr>
<td>cpctest-R</td>
<td>AGAGAGGCTTGTACAGTTGG</td>
<td>Used with 3’Kan I F to confirm CPC deletion</td>
</tr>
<tr>
<td>opt1test-R</td>
<td>ATTGTATGCTCGTGCCCGTT</td>
<td>Used with 3’Kan I F to confirm OPT1 deletion</td>
</tr>
<tr>
<td>Mup1del-F</td>
<td>CGGTGCTACCTTCGCAGTTA</td>
<td>Used with Mup1del-R to delete MUP1 with kanMX cassette</td>
</tr>
<tr>
<td>Mup1del-R</td>
<td>TGCTATTTTGATTTTCGCGCA</td>
<td>See above</td>
</tr>
<tr>
<td>Opt1del-F</td>
<td>GACTGTGGGAGAAATAACCGCAACAAATATATCGCACAGAAACAGATATCAAGCTTCCTCGTC</td>
<td>Used with Opt1del-R to delete OPT1 with hphMX cassette</td>
</tr>
<tr>
<td>Opt1del-R</td>
<td>AATTTAAACTTGATATGAACTCTGCAGGTTTATATTATGTTTTTCATGAGTGCGACACTG</td>
<td>See above</td>
</tr>
<tr>
<td>GATGGCGGGCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primer Name</td>
<td>Sequences</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------</td>
<td>-------------</td>
</tr>
<tr>
<td><strong>Mup1-For</strong></td>
<td>TCACTTCGGCTCCGTAAGCGGGCGCTGCA TCCGCAACACCAGTTTTTTTTGTCGGGGCTTGGCTAACTAT</td>
<td>Used with Mup1-Rev to place URA3-kanMX-TEF2 prom cassette upstream of MUP1</td>
</tr>
<tr>
<td><strong>Mup1-Rev</strong></td>
<td>TCCTTGGTGAAGACATTCAACTGTGACAG AAACGTTTCTCTCCTCCGACATGTTTAGTTA ATTATAGTTTCG</td>
<td>See above</td>
</tr>
<tr>
<td><strong>Mup1Oxtest-Rev</strong></td>
<td>GACGGGCCACCAACAAATCCTC</td>
<td>Used with TEFtest-F to confirm the insertion of URA3-kanMX-TEF2 prom cassette</td>
</tr>
<tr>
<td><strong>Gnp1-For</strong></td>
<td>TTTTTTCATATTTGTTGATTTAAAACATAT TCCCTTTGTGCTTCTTCAAGTCGGGGCTTGGCTTAACTAT</td>
<td>Used with Gnp1-Rev to place URA3-kanMX-TEF2 prom cassette upstream of GNP1</td>
</tr>
<tr>
<td><strong>Gnp1-Rev</strong></td>
<td>TTGAGCTTTCCCTCATTTCCGCCCATGG CGTCTATTACAAAGCGTACTGTTTAGTTAATTTAGTTTCG</td>
<td>See above</td>
</tr>
<tr>
<td><strong>Gnp1Oxtest-Rev</strong></td>
<td>CCCGGACCTGCATTGCTCAA</td>
<td>Used with TEFtest-F to confirm the insertion of URA3-kanMX-TEF2 prom cassette</td>
</tr>
<tr>
<td><strong>Agp1-For</strong></td>
<td>TTTGTTATTATTTCCTCCTGAATACTCATATTGT TTTACATACTATATAAGTGTCGGGGCTTGCTTAACTAT</td>
<td>Used with Agp1-Rev to place URA3-kanMX-TEF2 prom cassette upstream of AGP1</td>
</tr>
<tr>
<td><strong>Agp1-Rev</strong></td>
<td>GAGCTATTTTTTCAAGTCTTTTCATGGTGTTTAGTTAAATTATAGTTTCG</td>
<td>See above</td>
</tr>
<tr>
<td><strong>Agp1Oxtest-Rev</strong></td>
<td>GAACCAACCGCGGTACCGTTA</td>
<td>Used with TEFtest-F to confirm the insertion of URA3-kanMX-TEF2 prom cassette</td>
</tr>
<tr>
<td><strong>Tat2-For</strong></td>
<td>TTTCATATTTTTGTTGATATACATCTGAGCATTGCGGATCTAAATAGTGTG</td>
<td>Used with Tat2-Rev to place URA3-kanMX-TEF2 prom cassette upstream of TAT2</td>
</tr>
<tr>
<td><strong>Tat2-Rev</strong></td>
<td>TTTAGCTTCCTCATTTTGAAGCGTGGCTGACAGAGAAATAAAGTCTCTCGGTCATGTTTAGTTAATTATAGTTTCG</td>
<td>See above</td>
</tr>
<tr>
<td><strong>Tat2Oxtest-Rev</strong></td>
<td>TGCCGCGGCAATGATTTC</td>
<td>Used with TEFtest-F to confirm the insertion of URA3-kanMX-TEF2 prom cassette</td>
</tr>
<tr>
<td>Primer</td>
<td>Sequence</td>
<td>Function</td>
</tr>
<tr>
<td>----------------</td>
<td>----------------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Tat1-For</td>
<td>TTTTGTTGACATCTATTCTCTTTTCTCTGA CGGTTAAAAAAAAGCAGACC GCTGTTAACTAT</td>
<td>Used with Tat1-Rev to place URA3-kanMX-TEF2prom cassette upstream of TAT1</td>
</tr>
<tr>
<td>Tat1-Rev</td>
<td>TATTTGTGGCTGGACTGCTTCTTTGGAAT GAAACTGACACTATCGTCCAT ATTAGTTGACTGCTGTTA</td>
<td>See above</td>
</tr>
<tr>
<td>Tat1Oxtest-Rev</td>
<td>TGCAAAAACCAACGAAGGGT</td>
<td>Used with TEFFtest-F to confirm the insertion of URA3-kanMX-TEF2prom cassette</td>
</tr>
<tr>
<td>Bap2-For</td>
<td>TTTGGGGTGCCGCAAAAAGAAAGGACCCCTT TTTTCGATCTAGGAATTTAAG GGCTTAAACTAT</td>
<td>Used with Bap2-Rev to place URA3-kanMX-TEF2prom cassette upstream of BAP2</td>
</tr>
<tr>
<td>Bap2-Rev</td>
<td>GGAGAAAAGTTCCTTTTTTCCAGAAGATCC AAAACTTCTGAGATAGCAT ATTATAGTTGACTGCTGTTA</td>
<td>See above</td>
</tr>
<tr>
<td>Bap2Ox-Rev</td>
<td>CCATCTCACCTGCAGCTTG</td>
<td>Used with TEFFtest-F to confirm the insertion of URA3-kanMX-TEF2prom cassette</td>
</tr>
<tr>
<td>Bap3-For</td>
<td>TTCAGTGGCCAGTAGTGTAGTTTCTTGC GGCTTAAACTAT</td>
<td>Used with Bap3-Rev to place URA3-kanMX-TEF2prom cassette upstream of BAP3</td>
</tr>
<tr>
<td>Bap3-Rev</td>
<td>AACTCTGCACCTTTTTTCATTTTTGGAGGAC GGTTACTAGAGATAGCAT ATTATAGTTGACTGCTGTTA</td>
<td>See above</td>
</tr>
<tr>
<td>Bap3Oxtest-Rev</td>
<td>TCGCGAACCACCAAGCAGCTTTC</td>
<td>Used with TEFFtest-F to confirm the insertion of URA3-kanMX-TEF2prom cassette</td>
</tr>
<tr>
<td>Yct1-For</td>
<td>AAAAATGCTACTGTGTTAATGGAGGAG TCGGAAAATCAAAAAAGTCAATAGTCTGGGCTGGCTTAAACTAT</td>
<td>Used with Yct1-Rev to place URA3-kanMX-TEF2prom cassette upstream of YCT1</td>
</tr>
<tr>
<td>Yct1-Rev</td>
<td>TCAAGAAGAGGAGATCGAGTCTGCTCCTAAAT TTTACGTCAACTTTTTGGACATTTTAGTAAATTAGGTTCG</td>
<td>See above</td>
</tr>
<tr>
<td>Yct1Oxtest-Rev</td>
<td>ATCTCAACGCAACAGACACCG</td>
<td>Used with TEFFtest-F to confirm the insertion of URA3-kanMX-TEF2prom cassette</td>
</tr>
<tr>
<td>Primer Name</td>
<td>Sequence</td>
<td>Function</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Opt1-For</td>
<td>TCAATTTCGTTTTTTTTTTTCAATTGGGTTCATTCAATGAAATTCGAGTGCAGTCGTTTACTCATGTTTAGTT</td>
<td>Used with Opt1-Rev to place URA3-kanMX-TEF2prom cassette upstream of OPT1</td>
</tr>
<tr>
<td>Opt1-Rev</td>
<td>GGGCAGGCTCCGACTCCACGAGTGCAGTCGTTTACTCATGTTTAGTT AATTATAGTTCG</td>
<td>See above</td>
</tr>
<tr>
<td>Opt1Oxtest-Rev</td>
<td>TTTTGTTAAATGGGCCCCGGG</td>
<td>Used with TEFtest-F to confirm the insertion of URA3-kanMX-TEF2prom cassette</td>
</tr>
<tr>
<td>Cis2-For</td>
<td>AATATTATAATTCTTCCGTAAGTTTCGATCTGACTTTCTCGGTGACAGTGTGGGGGTACGTTAAGTTAGTTA</td>
<td>Used with Cis2-Rev to place URA3-kanMX-TEF2prom cassette upstream of CIS2</td>
</tr>
<tr>
<td>Cis2-Rev</td>
<td>ATAAAAACAGGTATTTAAAGTCTTGGGACATTTCTATTACACAACAGCATGTTTAGTTA ATTATAGTTCG</td>
<td>See above</td>
</tr>
<tr>
<td>Cis2Oxtest-Rev</td>
<td>ACTACGATGCCCACCACCTCC</td>
<td>Used with TEFtest-F to confirm the insertion of URA3-kanMX-TEF2prom cassette</td>
</tr>
<tr>
<td>TEFtest-F</td>
<td>CGAGTTGCTGACAGAAGCCT</td>
<td>Amplifies from TEF2 promoter</td>
</tr>
<tr>
<td>pGK-IRC7ox</td>
<td>ACAGATCATCAAGGAAAGTTAAATTATCTACTCTT</td>
<td>Used with pGK-IRC7oxshort-R to place IRC7S downstream PGKprom in pDH1 plasmid</td>
</tr>
<tr>
<td>short-F</td>
<td>TTTACAACAAATATAAAACCAGGCCGTCGTTAAGTTAGTTA ATGATGATCGTACCAGGT</td>
<td></td>
</tr>
<tr>
<td>pGK-IRC7ox</td>
<td>AATAAAACTATTATTTAGCTAAAGGATGGA</td>
<td></td>
</tr>
<tr>
<td>short-R</td>
<td>GGAAAGAGAAAGAAGGTAAAGGATGGA</td>
<td></td>
</tr>
<tr>
<td>MBP-IRC-HIS-F</td>
<td>AATATAACATATGTGATTGATCGTACCAGGT</td>
<td>Used with IRC7-HIS-R to clone IRC7 into pET28MBP</td>
</tr>
<tr>
<td>IRC7-HIS-F</td>
<td>ATATAAAACTATGATTGATCGTACCAGGT</td>
<td>Used with IRC7-HIS-R to clone IRC7 into pET28a(+)</td>
</tr>
<tr>
<td>IRC7-HIS-R</td>
<td>GGCACAATCGGATTGTAAAGGATTTTCCAAGGAG</td>
<td>See MBP-IRC-HIS-F and IRC7-HIS-F</td>
</tr>
<tr>
<td>IRC7-GFP-F</td>
<td>GCTTTGACGCTTCGCAAGGAAGAAATTTCATTTAATGACTTCTACCTCTTGCGTACGGGTGCTGTTTAA</td>
<td>Used with IRC7-GFP-R to place yEGFP-ADH1term-kanMX cassette downstream IRC7ox (X5)</td>
</tr>
<tr>
<td>IRC7-GFP-R</td>
<td>TACATACAACATATTTAATTACACAATTACCAATTTACGCTGACAGGACACATTCGATGAATTCGAGCTCG</td>
<td>See above</td>
</tr>
</tbody>
</table>
2.9 DNA isolation and quantification

2.9.1 Isolation of plasmids from bacteria

Plasmid isolation from \textit{E. coli} was performed using the High pure plasmid isolation kit (Roche, Darmstadt, Germany), following the manufacturer’s instructions.

2.9.2 Genomic DNA extraction from yeast

Chelex DNA extraction was used for routine genomic DNA isolation to be used as PCR template. Using toothpick, a piece of yeast colony was resuspended into 150 µL of 5% chelex solution (in H$_2$O). Heated at 100°C during 10 min, vortex 10 sec and spined at 12,000 rpm for 3 min. DNA solution was stored at -20°C for up to one month.

2.9.3 Plasmid DNA isolation from yeast

An aliquot of 1.5 mL of an overnight yeast culture was centrifuged (2,000 x g, 10 min). After discarding the supernatant, 200 µL of breaking buffer (2% Triton-X 100, 1% SDS, 100 mM NaCl, 10 mM Tris, 1 mM EDTA), 0.3 g of glass beads, 100 µL of phenol, 100 µL of chloroform and 200 µL of TE were added. The mix was vortexed for 2 min and re-pelleted by centrifugation. The supernatant was transferred to a new tube and 1/10 volume of 3 M NaOAc and 2.5 volumes of ethanol were added. After centrifugation, the supernatant was discarded and the pellet was washed with 70% ethanol and finally resuspended in 20 µL of water. DNA was stored at -20°C. One to five µL were used to transform \textit{E. coli}.

2.9.4 DNA quantification and quality

DNA concentration in solution was determined using the Nanodrop spectrophotometer (ND-1000). DNA concentration was given in ng/µL. In addition, quality parameters like the ratio of absorbance at 260/280 nm (~1.8) and the ratio of absorbance at 260/230 (2 – 2.2) were checked for DNA purity.
2.9.5 DNA sequencing

All sequencing work was done by Kristine Boxen at the DNA Sequencing Facility, SBS, University of Auckland. Reactions were carried out using Applied Biosystems BigDye version 3.1 terminator chemistry on Applied Biosystems 9700 Gold Block thermal cyclers. Unincorporated dye terminators were cleaned up using CleanSeq magnetic bead technology.

Sequence analysis was performed using Geneious Pro software (Biomatters, Auckland, New Zealand).
2.10 DNA manipulation and visualization

2.10.1 PCR

Standard PCR reactions were set up in 200 µL tubes using any Taq polymerase available in the laboratory, e.g. Fast Start DNA polymerase (Roche, Darmstadt, Germany), Phire Hot Start II DNA polymerase (Thermo Scientific).

For cloning and sequencing, high fidelity polymerase was used like Fast Start High Fidelity PCR system (Roche, Darmstadt, Germany) or Phusion Hot Start II High-fidelity polymerase (Thermo Scientific, MA, USA).

PCR reactions were run in a Gene Amp PCR System 2400 (Perkin Elmer, MA, USA) or 9700 termocycler (Applied Biosystems, MA, USA).

PCR amplifying conditions were followed as recommended by the polymerase manufacturer.

2.10.2 DNA Electrophoresis

Agarose was dissolved in 1x TBE buffer through microwave heating. In most cases, 1% gel was run in 1x TBE buffer for 30-40 min at 100V. For small fragments (100-200 bp) 2% gels were used. DNA samples were mixed with 10x Blue Juice gel loading buffer (Invitrogen, MA, USA), into a final concentration of 1x. For estimating the molecular weight of DNA fragments, 500 ng of 1 Kb plus DNA ladder solution (Invitrogen) was loaded in every gel. Ethidium bromide staining (0.5mg/L) was carried out for 20 min after run. Gel visualization was done under UV light (302 nm) through Gel Doc system (Bio-Rad, CA, USA) attached to a computer with Quantity One software (Bio-Rad, CA, USA) that allowed image acquisition.

2.10.3 DNA purification

- Agarose gel band

Band excision was done over a transilluminator using a clean razor blade. Gel slide was dissolved and purified using PureLink Quick Gel Extraction Kit (Invitrogen, MA, USA).

- PCR product
PCR product was cleaned up from a PCR reaction using the High Pure PCR Product Purification Kit (Roche, Darmstadt, Germany) following the product instructions.

### 2.10.4 DNA digestion

DNA digestion using restriction endonucleases was performed following the manufacturer’s instructions. In most of the cases digestion were incubated over night at the enzyme optimal temperature.

### 2.10.5 Ligation

PCR product and plasmid were digested and purified before ligation. A ratio of 1:3 of plasmid: insert was used, using 100 ng of plasmid. Ligations were carried out following T4 DNA ligase (Fermentas, MA, USA) instructions. Ligations were dialyzed prior to *E. coli* electrotransformation (Section 2.11).
2.11 Bacterial transformation

2.11.1 Electrocompetent cell

One litre of LB media was inoculated with 100 mL of an overnight DH10β culture. Cells were grown at 37°C until an O.D$^{600}$ of 0.5 to 1. The flask was chilled for 30 min on ice, and centrifuged in a pre-chilled rotor at 4,000 x g for 15 min. Cells were gently resuspended in one litre of cold water and pelleted. Half litre was added to resuspend cells and harvested again by centrifugation. Cells were resuspended in 20 mL of 10% glycerol and centrifuged. Finally, cells were resuspended to a final volume of 2 or 3 mL using 10% glycerol. Aliquots of 40 µL were frozen in liquid nitrogen and stored at -80°C.

2.11.2 Electrotransformation

Electrocompetent cells were thawed in ice. DNA was added and the cell suspension was transferred into a pre-chilled 1mm electroporation cuvette. Cuvette was place in the Gene Pulser (Bio-Rad, CA, USA) with the following settings: 25 uF, 1.8 kV and 200 Ω. Quickly after the pulse, one mL of SOC media was added into the cells and mixed gently by pipetting up and down. Cells were transferred to a culture tube and incubate at 37°C during two hours, with shaking. Cells were placed on a plate with selection media, if necessary dilutions were plated.
2.12 Yeast competent cells and transformation

The protocol is based on Gietz et al. (2007). Basically, a single yeast colony was inoculated into 10 mL of liquid YPD in 50mL flask and incubate overnight on a rotary shaker at 200 r.p.m. and 28 C. Part of the overnight culture was added to 50 mL of YPD in 250 mL flask (until O.D 0.2) and incubated in the shaking incubator at 28 C and 200 rpm until the O.D is 0.6 (three hours approximately).

Cells were harvested by centrifugation at 3,000g for 5 min and the pellet resuspended in 25 mL of sterile water and then collected at 3,000g for 5 min at 20 C. This wash was repeated with another 25 mL of sterile water. Cells were resuspended in 1.0 mL of sterile water.

The cell suspension was transferred to a 1.5 mL microcentrifuge tube, centrifuged for 30 s at 13,000g and supernatant was discarded. Cells were resuspended in 1.0 mL of sterile water and 100 µL aliquots were transferred into 1.5 mL microcentrifuge tubes, one for each transformation, and centrifuged to remove the supernatant.

A volume of 360 mL of transformation mix (240 µL PEG 50%, 36 µL LiAc 1M, 50 µL denaturated carrier DNA 2 mg mL⁻¹, 34 µL of DNA solution in water) was added to each tube and cells were resuspended by vortexing vigorously. Tubes were incubated in a water bath at 42 C for 40 min.

Tubes were centrifuged at 13,000g for 30 s in a microcentrifuge and supernatant removed with a micropipettor. Cells were resuspended with one mL of YPD liquid medium and incubated for 2–3 h at 28 C.

Cell suspension was plated onto the appropriate selection medium and incubated at 28 C for 3–4 d.
2.13 Gene modification

2.13.1 Gene deletion in *S. cerevisiae*

In general, genes were deleted by replacing them with the kanMX cassette through homologous recombination. This was achieved by amplifying the kanMX cassette, using genomic DNA, from the BY4743 deleted strain in the selected gene. Primers must anneal 200-300 bp outside the cassette to amplify a fragment with homologous regions to the target gene. The PCR product was transformed into the strain to be modified and selected on YDP kanamycin plates.

Similarly, hphMX cassette was amplified from pAG32 plasmid (Goldstein et al. (1999)) with primers with at least 50 bp homologous sequences to the gene to be replaced. The PCR product was transformed into the strain to be modified and selected on YPD hygromycin plates.

2.13.2 Gene overexpression in *S. cerevisiae*

Overexpression was done by two different ways:

- *IRC7* (from X5), *IRC7* (from S288c) and *STR3* (from F15) overexpression was performed by recombining the gene in pDHI plasmid (David Hook). After this, the cassette containing TEFprom, NAT R gene, TEF2term, PGK1prom, gene of interest, PGK1term, was excised by Pael cleavage. Digestion was used to transform yeast. Overexpression cassette edges recombines into HO gene, transformants were selected in YPD plates with NAT (Figure 2.2).

- Cysteine permeases, *OPT1* and *CIS2* overexpression was done placing a cassette, containing the kanamycin resistance gene, *URA2* selection marker and *TEF2* promoter, upstream of the start codon of the gene to be overexpressed. The cassette was amplified from pCHUK-TEF2 plasmid (Hester Sheehan) using primers with at least 50 bp of homologous sequences to the target gene promoter. The PCR product was transformed into the strain to be modified and selected in SD or YPD kanamycin plates (Figure 2.3).
Figure 2.2: IRC7 overexpression strategy of IRC7 in yeast. The gene of interest (IRC7) was amplified with primers with overhang ends, complementary to the HO locus, for following chromosome integration. After transformation transformants were selected in YPD plates supplemented with NAT.

Figure 2.3: Overexpression of AAP in S. cerevisiae. The overexpression cassette URA3:kanMX.TEF2prom was amplified with primers with overhang ends, complementary to the target gene (AAP, aa permease), for following chromosome integration. After transformation transformants were selected in SD or YPD plates supplemented with kanamycin.
2.13.3 Gene overexpression in *E. coli*

*IRC7* gene was cloned into pET28(a) and pETMBP. For this purpose, primers were designed (see table 2.8) to amplify *IRC7* from pDHI plasmid with some specific features: maintain the translation frame, erase the stop codon and include restriction enzyme cleavage sequences. After PCR amplification, the PCR product and the plasmid were digested with the corresponding restriction enzymes and ligated. Ligations were transformed into DH10β and transformants were selected on LB supplemented with kanamycin. Plasmid purification was carried out and *IRC7* cloning was confirmed by PCR and used to transform BL21.

2.13.4 GFP fusion

The yEGFP-ADH1term-kanMX cassette from pKT127 was amplified using IRC7-GFP-F and IRC7-GFP-R primers (see Table 2.8). The PCR product was transformed into F15 *IRC7*ox strain to introduce yEGFP in the carboxyl-terminal end of *IRC7*ox.
2.14 Protein extraction and purification from *E. coli*

2.14.1 Protein induction

Irc7p induction in BL21 was tested under several conditions. Two inductive media were tested:

1. LB with 0.5 mM IPTG incubated at 37°C for 1, 2 and 3 hrs and 18°C O/N
2. AI media (see Section 2.2.1) incubated 18°C O/N

2.14.2 Small scale protein extraction

Ten mL cultures were grown under the conditions described on 2.13.1. Five mL of culture were harvested by centrifugation. The pellet was resuspended in suspension buffer (20 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol, 0.025% NaN₃) to give a final O.D. of 0.1 per µL. Aliquots of 25 µL/tube were stored in the freezer until used.

After thawing, 25 µL of buffer was added to each tube (0.5% DDM, 0.005M EDTA in suspension buffer) and incubated 20 min at RT, dissolving clumps.

A volume of 50 µL of (0.2 mg/mL of zymoliase, DNAse and RNAase in suspension buffer) was added to each sample and incubated for 20 min, dissolving clumps. Samples were sonicated 10 sec at 4°C, until they cleared.

Tubes were spun for 30 min at 4°C, max speed (top bench centrifuge) and supernatant transferred to a new tube. Pellet was resuspended in 100 µL suspension buffer. After running SDS-PAGE (see Section 2.13.6), the best induction treatment was selected and scaled up for purifying the protein (see Section 2.13.3 to 2.13.5).

2.14.3 Large scale protein extraction

BL21 containing overexpression plasmid was pre-culture on 10 mL of the selective medium overnight. Next day, one litre of Autoinduction media was inoculated with O/N culture and incubated for 24 hrs at 18°C (this was the induction condition that gave the best result in small scale trial). The culture was centrifuged for 15 min at 4,000g and pellet was resuspended using 5 mL of buffer (20 mM Tris pH 8, 150 mM NaCl, 10% glycerol, 0.025% NaN₃ and 5 mM TCEP, added at the moment the buffer was used) per 1 g of pellet. One tablet of protease inhibitor cocktail tablet (Roche, 11 873 580 001) was added per 10 g of pellet. The bacterial suspension was freeze and kept at -20°C until used. Bacterial suspension was defrosted at RT for 20 min. Then, enzyme mix (zymoliase,
DNAse, RNase 10 mg/mL each) was added in a proportion of 10 µL of mix per 1 g bacterial pellet and incubated at RT for 20 min. The sample was sonicated for 20 min, using intervals of 5 min to avoid heating (sample was on ice). Volume was adjusted to 10 mL per 1 g pellet, leaving the following concentrations: 25% glycerol, 0.5 M NaCl, 30 mM imidazol and water until final volume. The solution was centrifuged for 1 hr at 15,000 g at 4°C. The supernatant was filtered using a 0.45 µm filter.

2.14.4 His-tag purification (Affinity chromatography)

Protein extract was purified using Hi Trap™ 5 mL Chelating HP (GE Healthcare, Buckinghamshire, United Kingdom) following the manufacturer’s instructions detailed below.

Two column volumes (CV, 5 mL in this case) of 1% SDS were passed through the column at a flow rate of 5 mL/min. Then 5 CV of H₂O, followed by ½ CV of 0.1 M CoSO₄ and washed with 5 CV of water.

Column was then equilibrated with 3 CV of a 25% glycerol, 0.5 M NaCl, 30 mM imidazole solution. Then protein extract was passed through the column and collected in 2 tubes (1st and 2nd flow through).

Column was washed with 25 mL of 25% glycerol, 0.5 M NaCl, 30 mM imidazole and flow through was collected. Then, 25 mL of 25% glycerol, 0.5 M NaCl, 50 mM imidazole were passed and collected. This last step was repeated.

Elution was performed passing 10 mL of 10% glycerol, 0.5 M NaCl, 0.2 M imidazole and collecting 5mL fractions. A second elution was done by passing 15 mL of 10% glycerol, 0.5 M NaCl, 0.5 M imidazole and collecting 5 mL fractions.

The column was stripped with 1 CV of 50 mM EDTA, 500 mM NaCl, flow through was collected. Then, 2 CV of 25 mM EDTA, 250 mM NaCl, and 0.5% SDS was passed through the column and collected. Finally, water was passed and 20% EtOH. The column was stored in 20% EtOH at 4°C.

Elution fractions were run in a SDS-PAGE and those with high amount of protein were kept in 50% glycerol until further use.
2.14.5 Size exclusion gel filtration

Fractions with high protein concentration were mixed together (a backup of 500 µL was kept at -20 °C) and diluted with buffer (10 mM HEPES pH 7.0, 150 mM NaCl, 100 µM PLP, 0.025% NaN₃) until glycerol concentration was 20%. Sample was concentrated until 1 mL using a Vivaspin 20 (30 KDa, GE Healthcare, Buckinghamshire, United Kingdom).

Part of the sample (500 µL in first injection and 250 µL in second injection) was subjected to high performance gel filtration using a Superdex™200 10/300GL (GE Healthcare, Buckinghamshire, United Kingdom) column connected to an ÄKTA™ purifier system, following manufacturer recommendations, and using the following eluent buffer: 10 mM HEPES pH 7.0, 150 mM NaCl, 10% glycerol, 100 µM PLP.

Collected fractions were subjected to OD measurements and SDS-PAGE to double check the fraction that contained the protein. Selected fraction was concentrated using an Amicon Ultra-4 device (10,000 MWCO, Millipore, MA, USA) until the protein concentration was 10 mg/mL.

2.14.6 SDS-PAGE

Protein extracts were run in SDS-PAGE. Gels (4% acrylamide stacking gel and 10% acrylamide resolving gel) prepared using Mini Protean II Cell system (Bio-Rad, CA, USA). The protein sample was mixed with 5x loading buffer (10% SDS, 10 mM β-mercaptoethanol, 20% v/v glycerol, 0.2 M Tris-HCl pH 6.8, 0.05% bromophenol blue) at a final concentration of 1x. Gel was run for 1h at 200V in 1x running buffer (25 mM Tris-HCl, 200 mM glycine, 0.1% SDS). Finally, gel was stained using Coomassie staining (0.1% Coomassie R250, 10% glacial acetic acid, 40% methanol) for at least 20 min. Gel was de-stained (20% methanol, 10% acetic acid) overnight.

2.14.7 Protein sequencing

Irc7p solution was sequenced by the Center for Genomics and Proteomics at SBS in The University of Auckland. The sample was digested using trypsin and the peptides were sequenced (MALDI-MS/MS) and compared against the NCBI non-redundant *Saccharomyces cerevisiae* database using Mascot tool (Perkins et al. (1999)).
2.15 Enzymatic assays

Irc7p activity was tested by two enzymatic assays, Ellman’s reaction that detects a thiol group (useful for all substrate, except cysteine) and pyruvate assay. The substrates used were: L-cysteine (in water), L-methionine (in water), L-cystine (in 0.5 M HCl), L-cystathionine (in 0.5 M HCl), S-ethyl-L-cysteine (in water), cysteine-4MMP (in water) and cysteine-3MH (in water).

2.15.1 Thiol assay

Ellman’s reagent or DTNB (3,3’-dithio-bis(6-nitrobenzoic acid)) was used to determined Irc7p enzymatic activity. After Irc7p cleavage of C-S bond, the thiol produced will react with DTNB to produce a disulfide compound and TNB\(^2\) which gives an intense yellow colour at 412 nm (Figure 2.4). Substrates with a thiol group are not compatible with this assay (e.g: L-cysteine).

![Figure 2.4: Ellman’s reaction for detecting thiols during Irc7 enzymatic reaction.](image)

The reaction between Ellman’s reagent and the thiol produce after the enzymatic cleavage of a substrate by IRC7 leads to a compound that could be determine by absorbance measurement at 412nm.

The reaction mix was set up as follows:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume [µL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lyase reaction buffer</td>
<td>18</td>
</tr>
<tr>
<td>(0.1 mM EDTA, 0.1 mM PLP)</td>
<td></td>
</tr>
<tr>
<td>Irc7p solution (0.4 µg/µL)</td>
<td>1</td>
</tr>
<tr>
<td>DTNB</td>
<td>37</td>
</tr>
<tr>
<td>(1mM in 0.1 M NaPO(_4))</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>39</td>
</tr>
<tr>
<td><strong>Total volume [µL]</strong></td>
<td><strong>95</strong></td>
</tr>
</tbody>
</table>
The reactions were organized in a 96-well plate. Substrates (5 µL) were directly added into the well and the reaction was started by adding 95 µL of the reaction mix into the well. Absorbance was measured every minute at 412 nm for two hours using the Enspire plate reader (Perkin Elmer, MA, USA).

2.15.2 Pyruvate assay

After Irc7p cleaves a C-S bond, from cysteine or a cysteine-conjugate, it produces a thiol, pyruvate and ammonia. Pyruvate was measured using an assay kit from Biovision (Milpitas, CA, USA). The enzymatic reactions that occurred when using the kit are described in Figure 2.5. This assay did not work properly with substrates dissolved in HCl solution (e.g: L-cystine and L-cystathionine). To overcome that problem an equal amount of NaOH was added. Reactions that release H₂S also interfere with this assay (e.g: L-cysteine).

![Figure 2.5: Reaction involved in the detection of pyruvate during Irc7 enzymatic reaction.](image)

The assay involves two enzymes that act in a sequential way. First, pyruvate oxidase catalize the conversion of pyruvate (formed by Irc7p enzymatic cleavage of a substrate), phosphate and oxygen into acetyl phosphate, CO₂ and H₂O₂. Second, the horseradish peroxidase oxidizes Oxired™ in the presence of H₂O₂ to form Resorufin a coloured compound that can be detected by absorbance measurement at 570 nm.

Each reaction mix was set up as follows:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume [µL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lyase reaction buffer</td>
<td>18</td>
</tr>
<tr>
<td>Irc7p solution (0.4 µg/µL)</td>
<td>1</td>
</tr>
<tr>
<td>Pyruvate assay buffer</td>
<td>72</td>
</tr>
<tr>
<td>Pyruvate probe</td>
<td>2</td>
</tr>
<tr>
<td>Enzyme mix</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total volume [µL]</strong></td>
<td><strong>95</strong></td>
</tr>
</tbody>
</table>
The reactions were organized in a 96-well plate. Substrates (5µL) were directly added into the well and the reaction was started by adding 95 µL of the reaction mix into the well. Absorbance was measured every minute at 412 nm for two hours using the Enspire plate reader (Perkin Elmer, MA, USA).

### 2.15.3 Lead acetate assay

To determine H₂S formation from L-cysteine by Irc7p enzymatic activity an assay described by Chiku et al. (2009) was performed. Briefly, lead acetate reacts with H₂S to produce lead sulfide (coloured) and acetic acid [\(\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)\_2 + \text{H}_2\text{S} = \text{PbS} + 2 \text{C}_2\text{H}_4\text{O}_2\)].

Each enzymatic reaction was performed as follows:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume [µL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lyase reaction buffer</td>
<td>18</td>
</tr>
<tr>
<td>IRC7p solution (0.4 µg/µL)</td>
<td>1</td>
</tr>
<tr>
<td>HEPES 1M pH 7.4</td>
<td>10</td>
</tr>
<tr>
<td>PbAc 4 mM</td>
<td>10</td>
</tr>
<tr>
<td>Water</td>
<td>56</td>
</tr>
<tr>
<td><strong>Total volume [µL]</strong></td>
<td><strong>95</strong></td>
</tr>
</tbody>
</table>

The reactions were organized in a 96-well plate. Substrates (5µL) were directly added into the well and the reaction was started by adding 95 µL of the reaction mix into the well. Absorbance was measured every minute at 390nm for 15 min using the Enspire plate reader (Perkin Elmer, MA, USA).

### 2.15.4 \(V_{\text{max}}\) and \(K_m\) determination

Irc7p initial rates \((V_0)\) for different substrates concentrations were calculated from the plots (Absorbance versus Time) obtained using the enzymatic assays described in section 2.14. The parameter \(V_{\text{max}}\) and \(K_m\) were obtained by fitting a hyperbolic curve to the \(V_0\) values at different substrates concentrations using Table curve 2D software (Systat Software, CA, USA).
2.16 Protein crystallization

Irc7p purified solution (10 mg/mL) was subjected to a crystallization screening using a high throughput approach of 576 different conditions. The screening contains in-house and commercially available buffer screens, described in (Moreland et al. (2005)).

A MultiPROBE II HT/EX liquid-handling robot was used to prepare and dispense the 576 screening solutions in a temperature-controlled environment (291 K). A volume of 50 µL of each crystallization buffer was transferred from the deep-well plates, where they were prepared, into six 96-well Intelli-Plates (Hampton Research, CA, USA).

The Intelli-Plate was manually transferred to the Cartesian nanolitre-dispensing robot, where 100 nl of protein (10 mg/mL) and crystallization buffers were dispensed for sitting-drop vapour-diffusion crystallization, under controlled temperature conditions (291 K) and relative humidity (85%).

After the appearance of crystals, the successful condition was scaled-up to obtain crystals suitable, in size and quality, for X-ray diffraction.
2.17 Yeast sporulation and mating

2.17.1 Sporulation and tetrad dissection

The method used was a modification from the one in Rose et al. (1990). Diploid strains were grown overnight in 2 mL of pre-sporulation media (Section 2.2.4). Next morning, yeast were harvested and washed twice with 4 mL sterile distilled water and finally resuspended in 500 µL of water. The suspension was spread onto a sporulation plate and incubated for 7 d at 18°C.

Tetrads were scraped from a sporulation plate using a toothpick and dissolved in 10 µL of water. Then, 2.5 µL zymoliase (5mg/mL) were added, followed by 5 min incubation at 28°C. Digested tetrad solution was placed in the form of a line on the top of a YPD plate and allowed to dry. Spores were dissected using a micromanipulator.

2.17.2 Yeast mating

Haploid strains of each MAT type were used for mating. A solution of one of the strains (10 µL) was spotted onto a YPD plate. After the spot was dried, the solution of the other strain were pipette on top of the spot and the YPD plate incubated for 36 h at 28°C. Yeast colonies were streaked onto hybrids selective medium plates.
3 The role of \textit{IRC7} in thiol release and cysteine metabolism

3.1 Introduction

The biological function of \textit{IRC7} is unclear, but it has been linked to different possible roles according to deletion phenotypes and transcript regulation (see Section 1.6.1). So far three different \textit{IRC7} alleles have been described:

\textit{IRC7}^S – a short version found in S288C and in most other strains of \textit{S. cerevisiae}, which contains a 38-bp deletion compared to wild-type strains (eg X5) and is predicted to express a 340-amino acid protein;

\textit{IRC7}^F - the full-length version of the \textit{S. cerevisiae} allele with the 38-bp present and predicted to encode a 400-aa protein; this allele is found in the wine yeast strains X5 and VL3;

\textit{IRC7}^P – a full-length version of an allele from \textit{S. paradoxus} that has been introgressed into a clinical strain of \textit{S. cerevisiae}, YJM450; it encodes a 400-aa protein that differs from that of \textit{IRC7}^F at 34 residues, but the \textit{K_m}'s of \textit{Irc7p}^F and \textit{Irc7p}^P are similar (Roncoroni et al. (2011)).

The full length allele, \textit{IRC7}^F, has an important enological role: its deletion suppresses 4MMP release and has a partial impact on 3MH (Thibon et al. (2008a), Roncoroni et al. (2011)). Results from this thesis (published in Roncoroni et al. (2011)) showed that \textit{IRC7}^F overexpression is sufficient to restore 4MMP production in an \textit{IRC7}-deleted strain, and also has some impact on 3MH.

The \textit{IRC7} full-length allele is rare in \textit{S. cerevisiae}. It is present in some wine yeasts but in none of the laboratory yeast strains (Roncoroni et al. (2011)). Despite efforts to understand thiol release during alcoholic fermentation of wine, there is still a lack of clarity of the capacity of the short allele, \textit{IRC7}^S, to produce 4MMP. This chapter explores the role of both \textit{IRC7} alleles in thiol release. It also proposes explanations for low conversion of thiols from cysteine and glutathione precursors. Finally the chapter outlines evidence for a proposed new role for \textit{IRC7} in sulfur metabolism.
3.2 Only strains with \( \text{IRC7}^F \) are able to release 4MMP from grape juice

To examine the performance of two different full-length \( \text{IRC7} \) alleles, initial lab-scale fermentation was performed using three SB grape juices and three \( S. \text{cerevisiae} \) strains: X5, YJM450 and F15. A detailed difference between \( \text{IRC7} \) coding proteins is showed in Figure A.1. The X5 strain has an \( S. \text{cerevisiae} \) full-length \( \text{IRC7} \) allele (\( \text{IRC7}^F \)) and the YJM450 has an introgressed \( S. \text{paradoxus} \) full-length allele (\( \text{IRC7}^P \)) that possesses 34 amino acid substitutions compared to the protein sequence of X5. The winemaking yeast strain F15 was included as it has a short version of \( \text{IRC7} \) (\( \text{IRC7}^S \)) that codes for a truncated 340-aa protein, like the laboratory strain S288c.

After fermentation, the concentrations of varietal thiols in the resulting wines were quantified by GC-MS and the results are shown in Table 3.1.

<table>
<thead>
<tr>
<th>Juice</th>
<th>Strain</th>
<th>4MMP [ng/L]</th>
<th>3MH+3MHA [ng/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blanw</td>
<td>F15</td>
<td>ND</td>
<td>4205</td>
</tr>
<tr>
<td></td>
<td>X5</td>
<td>63</td>
<td>5239</td>
</tr>
<tr>
<td></td>
<td>YJM450</td>
<td>279</td>
<td>6128</td>
</tr>
<tr>
<td>M1016</td>
<td>F15</td>
<td>ND</td>
<td>7110</td>
</tr>
<tr>
<td></td>
<td>X5</td>
<td>151</td>
<td>7240</td>
</tr>
<tr>
<td></td>
<td>YJM450</td>
<td>424</td>
<td>8720</td>
</tr>
<tr>
<td>SB2007</td>
<td>F15</td>
<td>ND</td>
<td>4175</td>
</tr>
<tr>
<td></td>
<td>X5</td>
<td>180</td>
<td>3879</td>
</tr>
<tr>
<td></td>
<td>YJM450</td>
<td>389</td>
<td>4313</td>
</tr>
</tbody>
</table>

Fermentations were carried out at 25°C using three full-strength different S. blanc juices. Thiols were extracted by the PHMB method. \( n = 1 \). ND: not detected.

Both strains carrying the full-length \( \text{IRC7} \) allele released 4MMP in all juices compared to F15, which did not produce detectable peaks corresponding to 4MMP in the GC-MS analysis. In all juices YJM450 seemed to produce higher amounts of 4MMP than X5, but this experiment was performed as a preliminary test without replicates, so no statistical test confirmed this result.

All strains including F15 produced 3MH and 3MHA from all three juices, confirming that a full-length \( \text{IRC7} \) is not necessary for production of these volatiles.
3.3 \(IRC7^F\) overexpression leads to 4MMP production in \(IRC7\)-deleted backgrounds

In order to assess whether the expression of \(IRC7\) is sufficient for 4MMP release, \(IRC7^F\) from X5 was overexpressed under the control of the \(PGK1\) promoter in wine (F15) and laboratory (S288c and BY4743) yeast strains that carry the short allele of \(IRC7\) (\(IRC7^S\)). Two additional genes that code for enzymes with known \(\beta\)-lyase activity, \(STR3\) from \(S.\ cerevisiae\) and \(tnaA\) from \(E.\ coli\), were also overexpressed in F15 to compare their effect on thiol release (refer to Section 2.12.2).

The wine and the laboratory strains overexpressing \(IRC7^F\) were used to ferment full or three-quarters- strength M1016 juice respectively, and thiols were quantified in the wine, as shown in Figure 3.1 and Figure 3.2. Overexpression of \(IRC7^F\) had a significant impact on 4MMP, giving a yield of around 1,000 ng/L and 200 ng/L of 4MMP in wine and laboratory yeast, respectively. \(IRC7^F\) overexpression also significantly increased the amount of 3MH and 3MHA produced by all strains compared to the respective wt strain.

When comparing the F15 \(IRC7^F\) ox strain (Figure 3.1) with the endogenous performance of \(IRC7^F\) in X5 (Table 3.1, M1016 juice) the increase was \(\sim 7\)-fold for 4MMP (from \(\sim 150\) to \(\sim 1000\) ng/L) and \(\sim 2\)-fold for 3MH/3MHA (from \(\sim 7000\) to \(\sim 14000\) ng/L). On the other hand, laboratory strains overexpressing \(IRC7^F\) (Figure 3.2) reached similar levels than X5 for both thiols (Table 3.1, M1016 juice), releasing 4MMP up to \(\sim 200\) ng/L and increasing 3MH up to \(\sim 7000\) ng/L. These comparisons assume that thiol production by laboratory strains is not reduced by the use of three-quarters-strength juice for its fermentation compared to full-strength juice fermentation for X5 (experiments demonstrating this result are shown in Appendix A2.1).

Overexpression of the yeast cystathionine beta-lyase \(STR3\) did not have any effect on final thiols (Figure 3.1). Overexpression of the \(E.\ coli\) \(\beta\)-lyase \(tnaA\), previously shown to affect thiols (Swiegers et al. (2007)), produced a small amount of 4MMP (\(\sim 90\) ng/L) but was significantly the most effective 3MH/3MHA producer (Figure 3.1).

The production of 4MMP on a deleted-\(IRC7\) background by overexpressing \(IRC7^F\) provides evidence that \(IRC7^F\) is sufficient for its release, either in a wine or a laboratory strain. The high concentrations obtained from the overexpression strains, especially for the wine yeast, strongly suggested that \(IRC7^F\) expression levels are limiting thiol release during fermentation. The differences in thiol release between the wine and laboratory strains may be due to additional genetic factors that affect thiol release.
However, the 4MMP released by overexpressing *IRC7* in lab strains was sufficiently high to be detected. This result opened up the possibility to use the existing BY4743 deletion strains for analyzing the genetics of thiol production, provided that *IRC7* was overexpressed in each mutant.

**Figure 3.1: Overexpression of *IRC7* produced a 7-fold increase in 4MMP and 2-fold increase in 3MH and 3MHA.** Full-strength M1016 juice was fermented at 25°C using F15 overexpressing three β-lyase genes. Thiols were extracted by the PHMB method. Note the different scales for the two thiols here and in subsequent figures. 

*n = 2; error bars = SE; different letter labels above bars mean that samples are significantly different (p < 0.001, ANOVA, Tukey’s HSD).*

**Figure 3.2: Laboratory strains overexpressing *IRC7* release 4MMP during fermentation.** Three-quarter-strength M1016 juice (diluted with SGM with no added sugars) was fermented at 25°C using S288c and BY4743 and its *IRC7* overexpression counterparts. Juice was supplemented with histidine [20 mg/L], leucine [30 mg/L] and uracil [20mg/L] for BY4743. Thiols were extracted by the PHMB method. 

*n = 2; error bars = SE; asterisks above bars represent significant differences compared to the respective wt strain (* p< 0.05, ** p < 0.01).*
3.4 The deleted form of *IRC7* makes no thiols

The performance of the truncated allele of *IRC7* (*IRC7\*) on thiol precursors was studied by overexpressing *IRC7* from S288c in F15 (haploid *MATa*, *ura-*) and BY4742. To determine the conversion rate of thiols from its precursors, fermentation was performed in a defined media resembling grape juice (SGM, see Section 2.2.3) to which 50 µg/L C-4MMP and 500 µg/L G-3MH was added. Table 3.2 shows the calculated final thiol concentration that would be reached if 100% of the precursor was converted into thiol, referred as thiol potential. The thiol potential was used to calculate thiol yield in subsequent figures (see Section 2.4.4 for details). The experiments described below used isotopically labelled G-3MH containing three deuterium ions, and produced similarly labeled thiol products, as an additional check on the origin of the thiol products.

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Precursor concentration [µg/L]</th>
<th>Thiol potential [ng/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-4MMP</td>
<td>50</td>
<td>30,150 (4MMP)</td>
</tr>
<tr>
<td>C-3MH</td>
<td>50</td>
<td>30,300 (3MH)</td>
</tr>
<tr>
<td>G-3MH</td>
<td>500</td>
<td>164,000 (3MH)</td>
</tr>
</tbody>
</table>

Apart from testing *IRC7*\*ox strains, the strains overexpressing F15 *IRC7*\*ox (haploid *MATa*) and BY4741 *IRC7*\*ox were included as a positive control. In addition, a diploid hybrid strain between the two haploid overexpression strains (*IRC7*\*o/*IRC7*\*) was constructed and used to test if the truncated allele acts antagonistically to the full-length allele (dominant negative mutation). Such interaction may be expected because enzymes in this class are typically active as homotetramers (Clausen et al. (1996), Breitinger et al. (2001), Ku et al. (2006)). Figure 3.3 shows that overexpression of *IRC7*\* allele did not produce thiols from the added precursor and the hybrid strain overexpressing both alleles (*IRC7*\*o/*IRC7*\*) showed no difference in 4MMP released to the *IRC7*\*ox strain. However, the hybrid had a reduced 3MH production compared to the strain overexpressing *IRC7*\*, both in F15 (upper graphs Fig. 3.3) and BY4743 (bottom graphs Fig. 3.3). This result suggests that the truncated form of Irc7p is affecting the function of the full-length protein. One possibility is that both alleles form a multimeric enzyme with a tridimensional structure the affects 3MH release from G-3MH.
3.4.1 \(IRC^F\) is necessary for converting G-3MH, C-3MH and C-4MMP into the corresponding thiol

The results shown in Figure 3.3 suggest that \(IRC^F\) is necessary for the conversion of G-3MH into 3MH and of C-4MMP into 4MMP. To confirm this result, F15 and F15 \(IRC^F\) ox were used to ferment SGM to which all three putative thiol precursors were added, alone or in combination, in a concentration similar to natural grape juice (Table 3.2).

Figure 3.4 shows that SGM samples fermented using F15 \(IRC^F\) gave approximately 34% conversion of the added C-4MMP precursor to thiol. The 3MH and 3MHA released corresponded to just 2% yield when C-3MH
was added, and G-3MH resulted in ~8% conversion. When all the precursors were added this turned into a 10% overall 3MH release, coming from C-3MH and D3-3MH-glut. The addition of the other precursors did not affect 4MMP or 3MH release.

Samples fermented in parallel with the control F15 strain did not produce 4MMP, as expected, but they also did not release any 3MH/3MHA (data not shown). This lack of 3MH/3MHA production by F15 in this experiment, using SGM with added precursors, contrasts with the results from grape juice fermentation (Table 3.1 and Figure 3.1) in which F15 produced 3MH and 3MHA. This result, combined with that in Figure 3.3, strongly suggests that strains carrying \( IRC7^F \) are not able to cleave cysteine - or glutathione - conjugated precursors, and that \( IRC7^F \) is the main enzyme involved in thiol release from these conjugates, at least under these conditions.

![Figure 3.4](image)

**Figure 3.4:** \( IRC7^F \) overexpression gives a high conversion yield of 4MMP from C-4MMP. SGM with added precursors (single or multiple additions as indicated, at the concentrations listed in Table 3.1) was fermented at 25°C using the F15 overexpression strain. Thiols were extracted by the ETP method. The labels above the bars show the percentage of thiol conversion from the added precursor. \( n = 2 \); error bars = SE.

### 3.4.2 A wine yeast strain overexpressing \( IRC7^F \) produces higher thiols levels than a laboratory strain overexpressing \( IRC7^F \)

Figure 3.3 shows that F15 \( IRC7^F \)ox gave a higher thiol yield than BY4743 \( IRC7^F \)ox in SGM with added precursors. Figure 3.5 shows a repeat fermentation of these two strains, with the wine strain F15 again yielding higher thiol concentrations than the lab strain. This comparison was repeated in different experimental batches during this thesis with some experimental variation, but BY4743 always yielded lower thiols. For example, BY4743 \( IRC7^F \)ox showed a conversion rate of 5-11% from C-4MMP precursor and 5% from G-3MH precursor.
(Figures 3.3 and 3.5). These rates were lower that F15 IIRC7ox, which in these experiments converted 44-67% of C-4MMP into 4MMP and 6-10% of the added G-3MH into 3MH.

Early in this thesis the hypothesis that low fermentation rates in laboratory strains were responsible for this low thiol yield was tested. Harsch et al. (2010) showed that three-quarters-strength grape juice plus 10x auxotrophy supplementation improved the lab strain fermentation to levels comparable with those of wine strains. In Appendix A2.1 it is shown that despite fermentation improvement with grape juice dilution and elevated auxotrophy supplementation (Figure A4), 3MH levels remain stable and were significantly and consistently lower (around 4,000 ng/L) in the BY4743 strain compared to F15. This result suggests that BY4743 despite its fermentation performance had a lower capacity to produce thiols than F15. Therefore, inferior fermentation performance was discarded as the cause for low thiol yields in the laboratory strain.

From the experiments described above, it is concluded that F15 and F15 IIRC7ox had a greater capacity to convert C-4MMP and G-3MH into thiols than the BY4743 strains, due to unknown genetic differences that may exist between both strains.

Figure 3.5: IIRC7ox overexpression in BY4743 gave less yield than in F15. Three-quarters-strength or full-strength SGM with added precursors (50 µg/L C-4MMP and 500 µg/L D3-3MH-glut) was fermented at 25°C using F15 and BY4743 overexpression strains, respectively. SGM was supplemented with histidine [20 mg/L], leucine [30 mg/L] and uracil [20mg/L] for BY4743. Thiols were extracted by the PHMB method. The labels above the bars show the percentage of thiol conversion from the added precursor. n = 2; error bars = SE; different letter labels above bars mean that samples are significantly different (p < 0.01, ANOVA).
3.4.3 Wine strains with a full-length *IRC7* allele release very low levels of thiols from C-4MMP and G-3MH

Strains with natural alleles of *IRC7*\(^F\), X5 (homozygous for *IRC7*\(^F\)) and EC1118 (heterozygous for *IRC7*\(^F\)) were fermented using SGM with added C-4MMP and D3-3MH-glut. Both strains produced 4MMP and 3MH from the added precursors (Figure 3.6). A significant difference was found for 4MMP production with a yield of ~1% for X5 compared to ~0.2% for EC1118. The release of 3MH was similar for both strains (around 0.2% conversion). Note that these fermentations were performed as part of the same experiment shown in Figure 3.3 so that F15 *IRC7*\(^F\)ox acted as a negative control and F15 *IRC7*\(^F\)ox as a positive control. Overall, thiol release from cysteinylated and glutathionylated precursors by two wine strains carrying at least one copy of *IRC7*\(^F\) allele was very low, especially when compared to F15 *IRC7*\(^F\)ox strain (summarized below in Table 3.3).

![Figure 3.6: Conversion yield of natural *IRC7* alleles. SGM with added precursors (50 µg/L C-4MMP and 500 µg/L G-3MH) was fermented at 25°C using X5 and EC1118 strains. Thiols were extracted by the PHMB method. The labels above bars show the percentage of thiol conversion from the added precursor. \(n = 2\); error bars = SE; different letter labels above bars mean that samples are significantly different (\(p < 0.05\), ANOVA).](image)

3.4.4 Summary

Results in this section have shown that overexpression of the naturally deleted form of *IRC7* does not influence thiol release, only strains carrying a full-length *IRC7* gene are able to release 4MMP during grape juice fermentation. When *IRC7*\(^F\) was overexpressed in F15 (wine strain, *IRC7*\(^S\)) high concentrations of 4MMP were produced and 3MH doubled after grape juice fermentation. This result suggests that *IRC7*\(^F\) expression is limiting
varietal thiol release. STR3 overexpression did not affect 4MMP or 3MH production. The laboratory strains, S288c and BY4743, were able to release 4MMP and give improved 3MH production after IRC7F overexpression.

Table 3.3 provides a summary of conversion from added precursors into thiols in different yeast backgrounds with different IRC7 genotypes. Overexpression of the full-length IRC7 was most effective in thiol release, with natural alleles showing low thiol yields, and the short allele completely ineffective. However, differences were observed between yeast strains, with the F15 background giving better yield than the BY4743.

The fact that IRC7-deleted strain are not able to release 4MMP from grape juice (Figure 3.1) or from SGM with added C-4MMP (Figure 3.3) is consistent with the hypothesis that C-4MMP is an important contributor to 4MMP in wine. In contrast, the large amount of 3MH release by IRC7-deleted strains in grape juice (Figure 3.1), matched with the low conversion yield of G-3MH into 3MH by wine strains, suggests that G-3MH may be a minor precursor in most juices.

Table 3.3: Summary of thiol yields by different yeast backgrounds.

<table>
<thead>
<tr>
<th>Strain</th>
<th>4MMP yield (%)</th>
<th>3MH/3MHA yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F15 IRC7F ox</td>
<td>34-67</td>
<td>6-10</td>
</tr>
<tr>
<td>BY4743 IRC7F ox</td>
<td>6-11</td>
<td>5-6</td>
</tr>
<tr>
<td>X5 (IRC7F/IRC7F)</td>
<td>1</td>
<td>0.3</td>
</tr>
<tr>
<td>EC1118 (IRC7F/IRC7S)</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>F15 IRC7S ox</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Added precursors: 50 µg/L C-4MMP and 500 µg/L G-3MH. ND: not detected.
3.5 **IRC7\textsuperscript{F} allows yeast to utilize cysteine**

Previous studies performed by Watson (1976) showed that cysteine was not able to support yeast growth when used as a nitrogen source (2 mM). In addition, (Kumar, 2006 #551) demonstrated that cysteine and homocysteine, but not glutathione, inhibit yeast growth in a concentration-dependent way (0.25 mM – 5 mM). Kumar (2006) did not find increased ROS production after cysteine or homocysteine treatment, suggesting that their induced growth defect is not due to the oxidative stress. They proposed that yeast growth inhibition by cysteine could be attributed to ER stress. Other authors suggested that toxicity may come from respiration inhibition ((Bhuvaneswaran, 1964 #692)).

### 3.5.1 Full length IRC7 is necessary for yeast to grow on L-cysteine

To test if IRC7\textsuperscript{F} has any implications on utilization of sulfur compounds, due to its known β-lyase activity (Roncoroni et al. (2011)), F15 and F15 IRC7\textsuperscript{Ox} strains were grown on four different sulfur compounds: L-cysteine, L-methionine, L-glutathione and S-ethyl-L-cysteine (SEC) (illustrated in Figure 3.7). Each was used as a sole source of either sulfur (at 0.5 mM) or nitrogen (at 15 mM).

![Structure of sulfur compounds](image)

**Figure 3.7: Structure of the sulfur compound tested as sulfur or nitrogen sources for yeast growth.**

When the different sulfur sources (0.5 mM) were tested, F15 was able to grow well on Na\textsubscript{2}SO\textsubscript{4} (positive control), L-methionine, L-glutathione and L-cysteine, but poorly on SEC where it started to grow only after 2 d (see Figure 3.8A, left side). The same strain overexpressing IRC7\textsuperscript{F} also showed growth on Na\textsubscript{2}SO\textsubscript{4} (positive control), L-methionine, L-glutathione and L-cysteine, but no growth at all in SEC (see Figure 3.8A, right side).
When the compounds were supplied as a sole nitrogen source (15 mM), F15 showed growth on NH₄Cl (positive control), L-methionine and SEC, but did not grow in L-cysteine and L-glutathione (see Figure 3.9B, left side). On the other hand, the F15 \( \text{IRC7}^{\text{F}} \) ox strain had the same performance as F15 on some compounds, growing on NH₄Cl (positive control) and L-methionine, but not growing on glutathione. The main differences were its ability to grow on L-cysteine and the lack of growth on SEC (see Figure 3.9A, right side) compared to F15, which showed the opposite phenotypes. In summary, the overexpression of \( \text{IRC7}^{\text{F}} \) allowed yeast growth on L-cysteine as a nitrogen source and inhibited growth on SEC as both sulfur or nitrogen sources.

![Figure 3.8: Growth of F15 and F15 \( \text{IRC7}^{\text{F}} \) strains in different sources of sulfur.](image)

Strains were grown in 150 µL SGM at 25°C for 3 d using different sulfur compounds as the sole sulfur source (0.5 mM). Absorbance was measured every 15 min and plotted versus time. Average curves were plotted. \( n = 2 \).

![Figure 3.9: Growth of F15 and F15 \( \text{IRC7}^{\text{F}} \) strains in different sources of nitrogen.](image)

Strains were grown in 150 µL SGM at 25°C for 3 d using different sulfur compounds as the sole nitrogen source (15 mM). Absorbance was measured every 15 min and plotted versus time. Average curves were plotted. \( n = 2 \).
The ability of \( IRC^F \) to allow growth on L-cysteine as a nitrogen source was studied further using BY4743 and F15 wt and \( IRC^F_{\text{ox}} \) strains, comparing growth on sodium-L-glutamate (positive control) and L-cysteine. All four strains grew in 15 mM sodium-L-glutamate (Figure 3.10 and Table 3.4), although F15 backgrounds had significantly faster doubling time and higher growth efficiency in SGM than BY4347 backgrounds. In L-cysteine, only F15 \( IRC^F_{\text{ox}} \) and BY4743 \( IRC^F_{\text{ox}} \) strains were able to use cysteine as a nitrogen source (Fig. 3.10 and Table 3.4). However, both strains had longer lag phases in L-cysteine compared to L-glutamate (Table 3.4), with F15 \( IRC^F_{\text{ox}} \) growing significantly better and faster than BY4743 \( IRC^F_{\text{ox}} \). This result supports the conclusion that \( IRC^F \) overexpression can sustain growth on L-cysteine as a nitrogen source despite the yeast background used.

**Figure 3.10:** Growth on cysteine by overexpressing \( IRC^F \) in a wine (F15) and a laboratory (BY4743) yeast strain backgrounds. Strains were grown in 150 µL SGM at 25°C for 3 d using 15 mM of sodium-L-glutamate or L-cysteine as nitrogen sources. SGM was supplemented with histidine [20 mg/L], leucine [30 mg/L] and uracil [20mg/L] for BY4743. Absorbance was measured every 15 min and plotted versus time. Average curves were plotted. \( n = 2 \).
Table 3.4: Growth parameters of F15 \(IRC7^{\text{ox}}\) and BY4743 \(IRC7^{\text{ox}}\) yeast strains in sodium-L-glutamate and L-cysteine as nitrogen sources.

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>7.02 ± 0.27(^a)</td>
<td>3.27 ± 0.02(^a)</td>
<td>1.60 ± 0.02(^a)</td>
<td>F15</td>
<td>7.87 ± 0.03(^a)</td>
<td>5.04 ± 0.14(^b)</td>
<td>0.58 ± 0.03(^b)</td>
</tr>
<tr>
<td>9.80 ± 0.24(^a)</td>
<td>2.93 ± 0.02(^a)</td>
<td>1.62 ± 0(^a)</td>
<td>F15 IRC7ox</td>
<td>23.60 ± 0.2(^a)</td>
<td>10.57 ± 0.9(^a)</td>
<td>0.72 ± 0.01(^a)</td>
</tr>
<tr>
<td>8.64 ± 0.21(^b)</td>
<td>4.95 ± 0.09(^b)</td>
<td>0.59 ± 0(^b)</td>
<td>BY4743</td>
<td>29.60 ± 0.34(^b)</td>
<td>24.34 ± 1.34(^b)</td>
<td>NR(^b)</td>
</tr>
</tbody>
</table>

Values are the mean of two values ± SEM. Samples in each column that are not connected by the same letter are significantly different (\(p < 0.001\), ANOVA, Tukey’s HSD). Growth parameters are defined in Section 2.5.3. NG: no growth; NR: not reached.

To confirm that growth on L-cysteine and SEC as nitrogen sources is also affected in strains possessing a natural full-length \(IRC7\) allele, growth curves on L-cysteine and SEC were generated for four progeny coming from single tetrads derived from either diploid X5 or diploid YJM450 strains that had been made heterozygous for \(IRC7 (IRC7^{\text{F}/IRC7^{\text{F}}}:\text{KAN})\). Figure 3.11 demonstrates that the two progeny carrying \(IRC7^{\text{F}}\), either from X5 or YJM450, were able to grow on L-cysteine, but that the two progeny with deleted \(IRC7\) could not (Figure 3.11, left side graphs). YJM450 grew faster than X5 and reached stationary phase within 3 d. When SEC was used as a nitrogen source (Figure 3.11, right side graphs), only the progeny with deleted \(IRC7 (IRC7::\text{KAN})\) were able to grow, and again the YJM450 strain grew faster and reached stationary phase. The two YJM450 progeny with the \(IRC7^{\text{F}}\) allele were finally able to grow after a long lag phase (aprox.1 d), but X5 progeny with the \(IRC7^{\text{F}}\) allele did not show any growth on SEC during 3 d. This result confirms that \(IRC7^{\text{F}}\) is necessary for the utilization of L-cysteine as a nitrogen source, and that \(IRC7^{\text{F}}\) is detrimental for growth on SEC as a nitrogen source.
Figure 3.11: Full-length IRC7 strains allow yeast to grow on cysteine as a nitrogen source. Strains were grown in 150 µL SGM at 25°C for 3 d using 15 mM L-cysteine or SEC as nitrogen sources. Absorbance was measured every 15 min and plotted versus time. Average curves were plotted. n = 2.

3.5.2 IRC7 helps yeast to overcome cysteine growth inhibition

To understand the effect of L-cysteine on yeast growth and the role of IRC7-fl in allowing yeast to grow on L-cysteine, F15 and F15 IRC7-fl ox were grown in 15 mM NH₄Cl or L-cysteine as controls and in 15 mM NH₄Cl with two concentrations of L-cysteine (2 mM and 15 mM). Growth curves (Figure 3.12 and Table 3.5) showed that increasing amounts of L-cysteine, added to a media containing NH₄Cl, prolonged lag phase in a dose-dependent way for both strains. L-cysteine at 15 mM, but not 2 mM, significantly increased doubling time and reduced efficiency in both strains, but the negative effect was softer when IRC7-fl was overexpressed. Using 15 mM of L-cysteine as a sole nitrogen source did not support F15 growth as shown in the previous section, but the presence of IRC7-fl eventually allowed growth in 15 mM, comparable to the F15 growth in 15 mM NH₄Cl with 15 mM L-cysteine.

It was concluded that IRC7-fl not only confers growth on L-cysteine as a sole nitrogen source, but also reduces the inhibitory effect of L-cysteine on yeast growth.
Figure 3.12: *IRC7* is needed to partially overcome cysteine toxicity in F15. F15 (solid lines) and F15 *IRC7*ox (dotted lines) were grown in 150 µL SGM at 25°C for 3 d using NH₄Cl alone or with L-cysteine (2 mM and 15 mM) as nitrogen sources. Absorbance was measured every 15 min and plotted versus time. Average curves were plotted. *n* = 2.

Table 3.5: Growth parameters of F15 and F15 *IRC7*ox yeast strains on NH₄Cl alone or with increasing amounts of L-cysteine.

<table>
<thead>
<tr>
<th>Nitrogen additions</th>
<th>Strains</th>
<th>Lag [h]</th>
<th>Doubling [h]</th>
<th>Efficiency [OD]</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 mM NH₄Cl</td>
<td>F15</td>
<td>10.99 ± 0.04a</td>
<td>3.10 ± 0.01a</td>
<td>1.63 ± 0.04a</td>
</tr>
<tr>
<td></td>
<td>F15 <em>IRC7</em>ox</td>
<td>11.77 ± 0.15a</td>
<td>3.09 ± 0a</td>
<td>1.61 ± 0.03a</td>
</tr>
<tr>
<td>15 mM NH₄Cl + 2 mM cysteine</td>
<td>F15</td>
<td>21.39 ± 0.42b</td>
<td>4.05 ± 0.02a</td>
<td>1.44 ± 0.15a</td>
</tr>
<tr>
<td></td>
<td>F15 <em>IRC7</em>ox</td>
<td>17.67 ± 0.2b</td>
<td>3.81 ± 0.03a</td>
<td>1.48 ± 0.05b</td>
</tr>
<tr>
<td>15 mM NH₄Cl + 15 mM cysteine</td>
<td>F15</td>
<td>38.51 ± 0.29c</td>
<td>14.90 ± 0.04b</td>
<td>0.49 ± 1.27b</td>
</tr>
<tr>
<td></td>
<td>F15 <em>IRC7</em>ox</td>
<td>34.82 ± 1.16c</td>
<td>8.46 ± 0.04b</td>
<td>1.06 ± 0.16c</td>
</tr>
<tr>
<td>15 mM cysteine</td>
<td>F15</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td></td>
<td>F15 <em>IRC7</em>ox</td>
<td>37.25 ± 0.82c</td>
<td>14.86 ± 0.01b</td>
<td>0.57 ± 0.45b</td>
</tr>
</tbody>
</table>

Values are the mean of two values ± SEM. Samples in the same column that are not connected by the same letter are significantly different (*p* < 0.001, ANOVA, Tukey’s HSD). Growth parameters are defined in Section 2.5.3. NG: no growth.
3.5.3 Enzymes involved in cysteine metabolism are not needed for growth on L-cysteine as a nitrogen source

Irc7p has been described as a ‘β-lyase’ (Roncoroni et al. (2011), SGD) with activity on cysteine-conjugates with importance in winemaking. Growth on cysteine as a nitrogen source provides a simple method to identify the biological substrate for Irc7F, which may be cystathionine, or some other metabolite derived from the transulfuration or glutathione conversion pathways. To test these alternatives, BY4743 deletion mutants in key genes (see Figure 3.13) coding for enzymes involved in L-cysteine conversion into L-methionine (STR2 and STR3) or L-glutathione (GSH1) were transformed with the IRC7F overexpression construct and grown in L-cysteine as a nitrogen source.

Figure 3.13: Cysteine conversion pathway. Highlighted are key enzymes involved in cysteine conversion into methionine and glutathione.

Figure 3.14 shows that BY4743 IRC7F ox and the deletion mutants str2Δ, str3Δ and gsh1Δ overexpressing IRC7F grew in a similar way in the control media (15 mM NH₄Cl), with the exception of Δgsh1 that needed to be supplemented with L-glutathione to show a normal phenotype. When grown on L-cysteine, all the deletion mutants grew similarly to BY4743 IRC7F ox. Interestingly, this included the gsh1Δ strain when grown without glutathione supplementation. This result supports findings by Grant et al. (1996) who showed that cysteine and other reducing agents containing a thiol group can restore gsh1Δ growth defect.

It was concluded that the products of STR2, STR3 or GSH1 are not required for either L-cysteine toxicity or growth on L-cysteine conferred by IRC7F ox. These results suggest that IRC7F may be acting on L-cysteine directly to release a compound suitable to be used as a nitrogen source by yeast.
Figure 3.14: Cysteine use by the F15 *IRC7*ox strain is independent of cysteine conversion. The BY4743 deletion mutant strains indicated were each transformed with an *IRC7*ox construct and grown in 150 µL SGM at 25°C for 3 d using NH₄Cl or L-cysteine (15 mM) as nitrogen sources. Absorbance was measured every 15 min and plotted versus time. Average curves were plotted. \( n = 2 \).

Furthermore, it was observed that *IRC7*ox overexpression restored the growth defect in both the str3Δ mutant (Figure 3.15) and the str2Δ mutant (not shown) growing in L-cysteine or L-glutathione as a sulfur source. This result shows that possession of *IRC7*ox allows yeast to bypass the known transulfuration pathway to provide assimilable sulfur from cysteine and glutathione through STR2 and STR3.

Figure 3.15: *IRC7*ox suppressed STR3 deletion in F15. The F15 and F15 str3Δ strains with and without *IRC7*ox overexpression were grown in 150 µL SGM at 25°C for 3 d using L-cysteine or L-glutathione (0.5 mM) as sulfur sources. Absorbance was measured every 15 min and plotted versus time. Average curves were plotted. \( n = 2 \).
3.5.4 Overexpression of $IRC7^F$ results in increased $H_2S$ production

If $IRC7^F$ acts on cysteine directly, the predicted thiol product from the reaction is $H_2S$ (see Fig. 3.22C in Section 3.8.2). To further test the idea that cysteine is a substrate of Irc7p, the overexpressing strain was assayed on BiGGY plates which monitor $H_2S$ production via a colony color reaction. In Figure 3.16, the wt F15 strain showed a $H_2S$–producer phenotype as indicated by the brown-colored colonies on BiGGY plates (in agreement with Huang et al. (2014)); however, both F15 $IRC7^Fox$ and F15 $IRC7^Pox$ had darker colonies on the same plate. Strains overexpressing either the $IRC7$ short allele or $tnaA$ were not darker than the wt.

![Figure 3.16: Strains overexpressing full-length $IRC7$ showed darker-colored colonies on BiGGY plates. F15 and variants overexpressing $IRC7^F$, $IRC7^S$, $IRC7^P$ and $tnaA$ were grown on BiGGY plates to assess colorimetric differences between them that reflect $H_2S$ production.](image)

3.5.5 Summary

These results demonstrate that $IRC7^F$ is both necessary and sufficient for yeast to grow on L-cysteine as a sole nitrogen source. L-cysteine has a dose-dependent negative effect on yeast growth, which $IRC7^F$ also helps to overcome in the presence of another nitrogen source. L-cysteine seems to be the direct substrate of Irc7p, because enzymes involved in cysteine metabolism are not required for its utilization by an $IRC7^Fox$ strain, and because $IRC7^F$ overexpression results in $H_2S$ production by the strain. The results were also consistent with the idea that $IRC7^F$ acts on the substrate SEC to make it toxic to yeast cells.
3.6 Yeast selected for growth on L-cysteine increased 4MMP yield

The fact that \( IRC7^F \) confers both 4MMP production and the ability to grow on L-cysteine as a nitrogen source, combined with the finding that \( IRC7^F \) expression likely limits 4MMP production, suggested an interesting potential application for breeding: high 4MMP producer strains might be obtained by selecting for growth on L-cysteine. Hester Sheehan (this laboratory) performed an experiment in which 10 hybrid parents (possessing a range of full-length alleles, both \( IRC7^F \) and \( IRC7^P \)) were subjected to three rounds of breeding and selection. Each round consisted of sporulation, crossing and growth on L-cysteine as a sole nitrogen source at 15°C; the low temperature was used because SB fermentation is usually performed at low temperatures, and because \( IRC7^P \) transcript expression is lower in the cold (Deed (2003)). After this recurrent selection process, a pool of genetically diverse progeny was screened for growth on SGM plates containing L-cysteine as a nitrogen source. Eleven individual strains that gave large colonies when grown on L-cysteine were used to ferment grape juice at 15°C using as a control the strain HHA7 (\( IRC7^P \)), one of the best parental strains for thiol production. The author then quantified thiols (in these non-replicated ferments) as shown in Figure 3.17. In this initial screen, all the strains selected showed elevated 4MMP levels, but no improvement for 3MH.

![Figure 3.17: Increased thiol levels of yeast selected to use cysteine as a nitrogen source at low temperatures. Blanw juice was fermented at 15°C using HHA7 and 11 strains selected for growth on cysteine. Thiols were extracted by the PHMB method. \( n = 1 \).](image-url)
The best strain for 4MMP release (CP-A-A3) plus F15 IRC7ox and X5 were used in duplicate ferments of SGM with added C-4MMP and D3-3MH-glut to calculate conversion rates. Figure 3.18 shows that, CP-A-A3 exhibited a 20% conversion yield of C-4MMP into 4MMP, almost half of the yield of the IRC7 overexpression strain and around 20-fold higher than the best commercial wine yeast X5. The conversion from D3-3MH-glut to 3MH was also significantly improved to 2%. This result indicates that cysteine selection successfully resulted in improved 4MMP and 3MH release from cysteinylated and glutathionylated precursors.

Figure 3.18: Cysteine selection improves conversion yields from C-4MMP into 4MMP. SGM with added precursors (50 µg/L C-4MMP and 500 µg/L D3-3MH-glut) was fermented at 25°C using F15 IRC7ox, X5 and CP-A-A3 strains. The labels above bars show the percentage of thiol conversion from the added precursor. n = 2; error bars = SE; different letter labels above bars mean that samples are significantly different (p < 0.05, ANOVA, Tukey’s HSD).
3.7 Irc7p biochemical characterization

Experiments carried out in this laboratory (Roncoroni et al. (2011)) have shown that crude protein extracts from yeast overexpressing IRC7\(^F\) or IRC7\(^P\), in the 2\(\mu\) plasmid, possess \(\beta\)-lyase activity. The extracts were active against S-ethyl-cysteine (SEC), C-4MMP and C-3MH, in that order of preference. The calculated Km for C-4MMP for protein extracts containing Irc7\(^F\)p and Irc7\(^P\)p was 5 ± 1 mM and 1.7 ± 0.4 mM, respectively (David Hook, BSc hons thesis, 2009). The results suggest that the two enzymes are very similar in substrate affinity.

Irc7\(^P\)p was overexpressed and purified from \(E.\ coli\) in order to characterize the biochemical properties of this protein.

3.7.1 Successful Irc7p purification in \(E.\ coli\)

IRC7\(^F\) was cloned with a C-terminal 6xHIS extension (for purification) into two bacterial plasmid vectors: pET28 and pETMBP, which has an N-terminal fusion to the maltose binding protein (MBP) that has been reported to aid in both protein yield and solubility. The expressed fusion proteins were purified from \(E.\ coli\) following the pipeline shown in Figure 3.19 (for details refer to Section 2.14).

The results of induction trials showed that overnight induction at 18°C either with IPTG or AutoInductive (AI) media (see Section 2.13.3) produced better results than fast IPTG induction (1-3 hours) at 37°C for both the Irc7\(^F\)-6xHIS (45 kDa) and MBP-Irc7\(^F\)-6xHIS (88 kDa) protein fusions. This difference is shown in Figure 3.20; after running a denaturing protein gel, most of the protein is in the soluble fraction when overnight induction at 18°C was used (Figure 3.20A, lanes 10 to 14 marked by arrow heads). In contrast, most of the protein was in the pellet fraction (Figure 3.20B, lanes 5 to 10 marked by arrows) when fast IPTG induction was performed at 37°C.
The treatment chosen to scale up the culture and protein purification was an overnight induction treatment at 18°C in AI media (Figure 3.20A lane 13) using *E. coli* transformed with pET28-IRC². The use of AI media has been shown to be a more convenient, efficient and economical alternative to IPTG induction (Studier (2005)) and was successfully applied to Irc7p induction in this study.

**Figure 3.20: Irc7²p induction trials.** Protein extract from *E. coli* cultures subjected to different induction treatments were centrifuged to obtain a soluble fraction (A) and a pellet fraction (B); see Section 2.13.2 for details. The fractions were electrophoresed in denaturing PAGE and stained with Coomassie blue. Arrow heads show candidate bands for Irc²-6xHIS (black arrow heads, 45 kDa) and for MBP-Irc²-6xHIS (white arrow heads, 88 kDa). Lane 1: Molecular weight size marker; Lane 2: untransformed *E. coli* protein extract; Lane 3: protein extract from uninduced *E. coli* transformed with pET28-IRC²; Lane 4: protein extract from uninduced *E. coli* transformed with pETMBP-IRC²; Lanes 5-7: protein extract from *E. coli* transformed with pET28-IRC² induced with IPTG at 37°C for 1, 2 or 3 h; Lanes 8-10: protein extract from *E. coli* transformed with pETMBP-IRC² induced with IPTG at 37°C for 1, 2 or 3 h; Lane 11: protein extract from *E. coli* transformed with pET28-IRC² induced with IPTG at 18°C O/N; Lane 12: protein extract from *E. coli* transformed with pETMBP-IRC² induced with IPTG at 18°C O/N; Lane 13: protein extract from *E. coli* transformed with pET28-IRC² induced with AI media at 18°C overnight; Lane 14: protein extract from *E. coli* transformed with pETMBP-IRC² induced with AI media at 18°C overnight.
After preparing the protein extract from one liter of BL21 culture, two purification steps were performed to obtain Irc7p. First, the protein extract was subjected to affinity chromatography purification in which the 6xHIS tag was bound to immobilized metal ions (cobalt, in this case) under strict buffer conditions. After three washes the protein was eluted and a band of the expected size was clearly visible in several fractions (Figure 3.21A, lanes 9 to 13), with most of the protein eluted within the first three fractions (Figure 3.21A, lanes 9 to 11). The second purification step was size exclusion chromatography; Irc7p eluted mainly in fractions 6 to 8, as detected by absorbance measurements at 280 nm (not shown) and PAGE (Figure 3.21B, lanes 7 to 9). A final concentration step using a centrifugal filter unit resulted in a highly pure protein preparation at a concentration of 10 mg/mL (Figure 3.21B, lane 11). The sample identity was confirmed by sequencing peptides fractionated after trypsin digestion that were aligned against the NCBI non-redundant S. cerevisiae data base. The top hit was Irc7p from S. cerevisiae FostersO (see Appendix A1.3), which is 100% identical to X5 Irc7p.

**Figure 3.21: Irc7p purification steps.** Protein extract from an *E. coli* culture incubated overnight in Al media at 18°C was centrifuged at high speed to obtain a soluble fraction, and subsequently purified (see Section 2.14). Protein samples from each purification step of affinity chromatography (A) and size-exclusion chromatography (B) were run in denaturing PAGE and stained with Coomassie blue. (A) Lane 1: Molecular weight size marker; Lane 2: soluble fraction of *E. coli* protein extract; Lane 3: pellet fraction of *E. coli* protein extract; Lanes 4-5: flow-through after passing protein through column; Lanes 6-8: washes; Lanes 9-13: elution steps; Lanes 14-15: column stripping washes. (B) Lane 1: Molecular weight size marker; Lanes 2-10: elution fractions from size-exclusion chromatography; Lane 11: purified and concentrated Irc7p; Lane 12: filtrate from concentration step.
3.7.2 Irc7p acts on L-cysteine

Irc7p function as a β-lyase was tested by three different enzymatic assays illustrated in Figure 3.22 (details in Section 2.15), which utilized spectrophotometric detection. Known substrates like SEC, C-4MMP and C-3MH were retested to compare the activity of the purified enzyme to the activity of the crude extract studied previously in this laboratory (Roncoroni et al. (2011)). Some new putative substrates like cystathionine, methionine and L-cysteine were also tested.

Figure 3.22: Enzymatic assays used to test different compounds as Irc7p substrates.
Purified Irc7p cleaved C-4MMP, SEC and C-3MH to give a free thiol and pyruvate detectable by thiol assay (Figure 3.23 A) or pyruvate assay (Figure 3.24 A) respectively. L-methionine and L-cystathionine did not react. Substrates alone gave no or very low reaction background (Figure 3.23 B and 3.24 B).

The putative substrate L-cysteine could not be tested using the thiol assay - because it contains a free thiol - and gave very unusual kinetics on the pyruvate assay (see Appendix A3). The PbAc assay was used to measure Irc7Fp activity on L-cysteine. The results confirmed that Irc7p cleaves L-cysteine to produce H₂S (dark color, Figure 3.25A). The reaction was followed during 15 min to give the kinetics shown in Figure 3.25B.

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**Figure 3.23: Irc7Fp activity using the thiol assay.** Reactions were carried out in 96-well plates using the DTNB reagent for thiol detection. Each well contained either 1 mM substrate and 0.4 µg/µL Irc7Fp (A) or only 1 mM substrate (B) in a final volume of 100 µL. Absorbance (412 nm) was measured every min for 2 hours. Average curves were plotted. n = 3.

---

**Figure 3.24: Irc7Fp activity using the pyruvate assay.** Reactions (100 µl) were carried out in 96-well plates using the pyruvate assay kit. Each well contained either 1 mM substrate and 0.4 µg Irc7Fp (A) or 1 mM substrate (B). Absorbance (570 nm) was measured every min for 2 hours. Average curves were plotted. n = 3.
Figure 3.25: Irc7Fp activity on L-cysteine using PbAc assay. Reactions were carried out in 96-well plates using the PbAc assay. Each well contained 1 mM substrate and 0.4 µg Irc7p in 100 µL final volume. (A) Picture of the dark coloured product obtained after H2S, produced when Irc7p cleaved L-cysteine, which reacted with PbAc to form PbSH. (B). Kinetics of PbSH formation using L-cysteine as a substrate. Absorbance (390 nm) was measured every min for 15 min. Average curves were plotted. n = 2.

Biochemical parameters for Irc7p on C-4MMP, SEC and L-cysteine were calculated using the thiol assay and on L-cysteine using the PbAc assay (Table 3.6). The Km values were similar for C-4MMP, SEC and L-cysteine (around 3 mM). The Vmax was similar for C-4MMP and SEC, but much higher for L-cysteine. The calculation of Km and Vmax for C-3MH was not achieved because Irc7p had a very low activity on this substrate under the experimental conditions of the thiol assay.

**Table 3.6: Irc7p biochemical parameters for different substrates.**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Km [mM]</th>
<th>Vmax [µM/min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-4MMP</td>
<td>3.3</td>
<td>3.8</td>
</tr>
<tr>
<td>SEC</td>
<td>2.7</td>
<td>4.2</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>3.8</td>
<td>45.5</td>
</tr>
</tbody>
</table>

* 0.4 ug of Irc7p were added to a 200-µL reaction volume.

In summary, these results have shown that purified Irc7Fp has β-lyase activity against SEC, C-4MMP and C-3MH, confirming previous whole cell extract experiments (Roncoroni et al. (2011)). However, L-methionine and L-cystathionine were not substrates of the enzyme. In addition, Irc7Fp has been shown to cleave L-cysteine to release H2S.
3.7.3 Irc7Fp formed small crystals when subjected to a crystallization screening

Purified Irc7Fp was put through a crystallization screening (see Section 2.16 for details). After concentration, Irc7Fp (10 mg/mL) was kept at 4°C and an aliquot diluted to 5 mg/mL with glycerol was stored at -20°C. The screening was performed in six 96-well Intelli-Plates, where 100 nL of protein were dispensed (5 mg/mL in 50% glycerol in the first two plates and 10 mg/mL in the following plates) followed by crystallization buffer addition.

One of the 576 conditions tested produced a small crystal (not shown) by two weeks of incubation. The solution in this condition was 20% ethanol, 0.1 M Tris pH 8.5. The crystallization process was scaled up using the successful conditions, but after more than 6 months no crystals appeared.

3.7.4 Irc7Fp localizes to the cytoplasm in yeast

Because the laboratory strains all have the shortened version of IRC7, most of the results published to date pertain solely to this allelic form of the protein. To determine the subcellular localization of Irc7Fp, the coding sequence of yEGFP was fused to the 3´ end of the open reading frame of IRC7Fp in the F15 IRC7Fpox background (see Section 2.13.4). Cells carrying the Irc7p-GFP fusion were able to grow on L-cysteine as a nitrogen source, indicating that Irc7Fp functionality was preserved (data not shown). For fluorescent microscopy, SGM and SGM with NH4Cl cultures were grown until exponential growth and then transferred and diluted into fresh media containing NH4Cl or L-cysteine as nitrogen source (15 mM). Samples were grown and observed under a fluorescence microscope after both 3 h and 24 h. Under all conditions Irc7p-GFP fusion was localized in the cytoplasm (Figure 3.26). These results confirm conclusions drawn with the shorter IRC7 form of the protein and suggest that the C-terminal region does not contain signals that alter its localization within the yeast cell.
Figure 3.26: Irc7\textsuperscript{p}p-GFP localization. Yeast F15 \textit{IRC7}\textsuperscript{ox-GFP} grown in SGM with NH\textsubscript{4}Cl (upper panels) or L-cysteine (lower panels) as a nitrogen source for 3 and 24 h (right and left images, respectively) were observed under fluorescent microscope (x100) to visualize Irc7p-GFP. The clumping of the haploid cells occurs because F15 has an active form of the \textit{AMN1} gene.

3.7.5 Summary

\textit{IRC7}\textsuperscript{F} codes for a \(\beta\)-lyase able to cleave cysteine and cysteine conjugates such as C-4MMP and SEC. In yeast, \textit{IRC7}\textsuperscript{F} allows yeast to use L-cysteine as a nitrogen source, helping to overcome cysteine toxicity. In addition, \textit{IRC7}\textsuperscript{F} allows the use of L-cysteine as a sulfur source independently from \textit{STR2} and \textit{STR3}, by providing a H\textsubscript{2}S input into the sulfur metabolism pathway. Moreover, it was demonstrated that \textit{IRC7}\textsuperscript{S} does not help yeast to grow on L-cysteine and that it also does not participate in the conversion of C-4MMP and G-3MG into their respective volatile thiols, which was demonstrated to be \textit{IRC7}\textsuperscript{F}-dependent.
3.8 Discussion

In this chapter the role of \textit{IRC7}\textsuperscript{F} in thiol release during fermentative conditions was examined in detail and compared to \textit{IRC7}\textsuperscript{S}. It was concluded that \textit{IRC7}\textsuperscript{S} is not capable of thiol release by yeast. In addition, \textit{Irc7}\textsuperscript{F}p was characterized to have activity on L-cysteine, which has been described in yeast before (Tokuyama et al. (1973)) but with no gene assignment for the function. The major conclusions of Chapter 3 will be discussed below.

3.8.1 \textit{IRC7}\textsuperscript{F}, but not \textit{IRC7}\textsuperscript{S}, is sufficient for 4MMP release

Testing strains with full-length \textit{IRC7} alleles versus a strain with a deleted version of the gene gave an initial indication that \textit{IRC7}\textsuperscript{F} is sufficient for 4MMP release. This was evidenced by the fact that X5 and YJM450 carrying \textit{IRC7}\textsuperscript{F} and \textit{IRC7}\textsuperscript{F}p respectively, released 4MMP in the three different SB juices, while F15 (\textit{IRC7}\textsuperscript{S}) did not. The YJM450 (\textit{IRC7}\textsuperscript{F}) strain produced more 4MMP than X5 strain. This difference could be due to differences in aa sequence between the \textit{IRC7} sequences (detailed in Figure A1) or because of other differences between the two genetic backgrounds tested, one being a wine strain bred by hybridization (X5) and the other a clinical isolate (YJM450). In synthetic media, X5 homozygous for \textit{IRC7}\textsuperscript{F} released 4MMP from C-4MMP. Furthermore, EC1118, a wine strain widely used for winemaking that is heterozygous for \textit{IRC7} (Roncoroni et al. (2011)), was also able to produce 4MMP in synthetic media from the precursor C-4MMP (Figure 3.6).

The preliminary results discussed above are in agreement with Murat et al. (2001a) who showed that VL3c and VL1 produce 4MMP when fermenting grape juice and model fermentation medium with added C-4MMP (~80 µg/L). VL3c is a homozygous diploid strain derived from VL3, which is known to be homozygous for the full-length \textit{IRC7} (Roncoroni et al. (2011)). VL1 showed the lowest 4MMP level in this work, which is probably explained by the heterozygosity for \textit{IRC7}\textsuperscript{F} in this strain (this thesis, data not shown). Contrary to the results described above, Howell et al. (2005) using synthetic medium (0.67% yeast nitrogen base, an amino acid mix and 8% D-glucose) with very high levels of C-4MMP (~100,000 µg/L), showed that deleting \textit{IRC7}\textsuperscript{F} in VL3c reduced but did not stop 4MMP formation (27% of the wt) and, more controversially, that deleting \textit{IRC7}\textsuperscript{S} from BY4742 also reduced 4MMP (to 54% of the control). However, these results were refuted by Thibon et al. (2008a), who used the \textit{irc7}\textsuperscript{Δ} mutant in VL3-1D strain (derived from VL3c) to ferment synthetic grape juice supplemented with C-4MMP at more natural amounts (4 µg/L) and obtained 96% 4MMP reduction compared to wt.

A second important conclusion was to determine that \textit{IRC7}\textsuperscript{F} is necessary to release thiols from cysteinylated and glutathionylated precursors. Figure 3.4 showed that \textit{IRC7}\textsuperscript{F} overexpression is required to produce 4MMP from C-
4MMP and 3MH from C-3MH and G-3MH. The conversion yield was higher for 4MMP release from the C-4MMP precursor (~34 %) than for 3MH from C-3MH (2%) and from G-3MH (8%). This difference can be partially explained by the enzymatic properties of IRC7F upon the cysteinylated substrates; Irc7Fp had very low activity on C-3MH compared to C-4MMP (Figures 3.23 and 3.24). The more complex result came from the higher 3MH yield obtained from G-3MH compared to C-3MH (8% versus 2%), because G-3MH is supposed to be metabolized into C-3MH by yeast. One possible explanation is that the C-3MH conjugate is taken up by yeast in a more inefficient way than G-3MH, so there is less substrate availability for Irc7Fp. Another option is that Irc7Fp is acting on the dipeptide conjugate intermediate (Cys-Gly-conjugate or Glu-Cys conjugate), produced during the turnover of G-3MH into C-3MH, with more affinity than C-3MH. The relative 3MH production from C-3MH (2% conversion) and G-3MH (8% conversion) in Figure 3.4 were opposite to the 3MH levels obtained by Winter et al. (2011) when fermenting VL3 with added C-3MH (1% conversion) and G-3MH (0.5% conversion). It is possible those experimental conditions, e.g: precursor amount, media composition, and/or genetic differences between strains, e.g: transport of precursor, conversion rates of G-3MH into C-3MH, might be having an effect on 3MH release from cysteinylated and glutathionylated precursors.

One of the most important conclusions is that IRC7S has no function in thiol release by yeast during fermentation. Overexpression of IRC7S did not result in the conversion of either C-4MMP or G-3MH. The overexpression of IRC7S was performed in F15 and BY4742 strains and no thiol release was seen in either case. The hybrid strains overexpressing IRC7S and IRC7F showed a significantly lower conversion of 3MH from G-3MH, supporting the idea of an Irc7Sp interacting with Irc7Fp to give an “altered enzyme”. The fact that 4MMP release was not altered may indicate that the dominant negative effect only alters the binding of C-3MH. Another possibility is that dominant effect alters the binding of the dipeptide Gly-Cys-3MH, which has not been discarded as a substrate for Irc7Fp. The negative effect on the truncated Irc7p could be perfectly possible if the enzyme behaves as a multimer, similar to other β-lyase enzymes belonging to PLP-dependent group do (Clausen et al. (1996), Breitinger et al. (2001), Ku et al. (2006)). Nevertheless, IRC7 has not been crystallized and has not been proved to be PLP-dependent although PLP was included in the purification and reaction buffer for enzymatic assays. In addition, Irc7p has shown a putative binding site for PLP (Figures A1 and A2). The attempt to crystallize IRC7F during this thesis was unsuccessful because of difficulties in scaling up the crystal-producing trial conditions.
3.8.2 **IRC7^F**, but not IRC7^S, is necessary to release 3MH from C-3MH and G-3MH

All strains tested in this thesis released 3MH when used to ferment grape juice, including strains with the short allele of IRC7 (F15 in Figure 3.1 and BY4743 in Figure 3.2). Therefore, 3MH production is independent of IRC7^F. Overexpression of IRC7^F in F15 and BY4743 resulted in more 3MH release when fermenting grape juice, compared to the wt strains (Figures 3.1 and 3.2). This result means that there is 3MH precursor in grape juice suitable to be cleaved by Irc7^Fp. These precursors used by Irc7^Fp are probably G-3MH and C-3MH, because both released 3MH using F15 IRC7^F in synthetic media (Figure 3.4) but were not cleaved by F15 wt (not shown). Thus, in direct contrast to the results with 4MMP, it is clear that 3MH can be produced from precursors in grape juice using an alternative, IRC7-independent pathway (or pathways). Given that most wine yeasts do not have a full-length thiol-active IRC7 allele, it appears that the great majority of 3MH/3MHA in wine is produced by such pathway(s). This has been supported by other authors showing lack of correlation between 3MH and its precursors C-3MH and G-3MH (Pini et al. (2012), Allen et al. (2011)) and by the discovery of other precursors: (E)-2-hexenal and (E)-2-hexen-1-ol (Schneider et al. (2006), Harsch et al. (2013a)). The contribution of the cysteinylated and glutathionylated precursors for 3MH/3MHA has also been shown to be low when using synthetic media with added precursors or when adding labeled precursor to wine (Winter et al. (2011), Subileau et al. (2008a), Roland et al. (2010a)), even when using IRC7^F strains like X5. This low conversion might be explained by low expression of IRC7^F or by the poor inherent activity of purified Irc7p on C-3MH in vitro (Figures 3.23 and 3.24). Other possible explanation might be that the cysteinylated and glutathionylated precursors are not efficiently taken up by yeast, which is supported by studies reporting that high amounts of these precursors remain in some wines (Capone et al. (2010), Capone et al. (2011)).

3.8.3 **STR3 has no effect on thiols**

Str3p from S. cerevisiae was a promising enzyme for cleaving cysteinylated precursors as it has β-lyase activity against cystathionine (Holt et al. (2011)). However, the results shown in this thesis demonstrate that STR3 is not necessary or sufficient for thiol production.

STR3 along with IRC7^F and tnaA were independently overexpressed in F15 to confirm previous results from other authors and especially to clarify the role of STR3 in thiol release. Overexpressing STR3 had no effect on either 4MMP or 3MH when the strain was used to ferment grape juice (Figure 3.1). Deleting STR3 in F15 also had no effect on 3MH (not shown). The null effect of F15 STR3ox in thiol production found in this thesis is directly opposed to the increase in 3MH reported by Holt et al. (2011) when fermenting the VIN13 STR3ox strain.
(1,362 ± 84 ng/L) compared to the wt (1,084 ± 66 ng/L). One possible explanation for the null effect of STR3 on thiol release during fermentation in this thesis (Figure 3.1), despite it having some substrate specificity for thiol precursors (Holt et al. (2011)), might be that STR3 is localized in the peroxisome within the cell (Schafer et al. (2001), Yi et al. (2002)). Evidence that Irc7Fp is located to the cytoplasm was presented in Figure 3.27, where it may perform its activity on thiol precursors in yeast cells.

On the other hand, overexpression of tnaA from E. coli in F15 resulted in a large increase in thiol production (Figure 3.1), supporting earlier findings by Swiegers et al. (2007). F15 tnaAox produced 4MMP (90 ng/L) and increased 3MH almost 3-fold when fermenting grape juice. This 3MH increase was much higher than that observed by IRC7F overexpression in the same experiment. From these results it could be suggested that tnaA, which has a strong β-lyase activity, might have more affinity than Irc7Fp for the 3MH precursor.

3.8.4 Cysteinylated and glutathionylated precursors give low thiol yield

Low conversion from cysteine and glutathione precursors into thiols by strains carrying natural alleles of IRC7F, like X5 and EC118, has been shown in this thesis (Figure 3.6) and reported by other authors (Subileau et al. (2008a), Roland et al. (2010a), Winter et al. (2011)). Overexpression of IRC7F in a wine strain improved yield, especially of 4MMP, which increased from 1% in X5 to 34-67% conversion in F15 IRC7F ox, an increase of over 30-fold. For 3MH there was an improvement of over 25-fold, from 0.3% conversion in X5 to 8-10% conversion in F15 IRC7F ox. It was also noted that IRC7F ox in laboratory strains had reduced yield of 3MH compared to overexpressing IRC7F in a wine yeast (Table 3.3). This difference might be explained by genetic differences between strains. Perhaps these differences result from human selection for wine yeast strains that efficiently uptake/metabolize compounds present in grape juice.

The low overall conversion of precursors to varietal thiols by Irc7Fp might have multiple causes, including:

- IRC7F low expression levels.
  Low expression levels can be inferred by comparing 4MMP yields between X5 and the F15 IRC7F ox strain in synthetic media with added C-4MMP (Table 3.2). The same gene under a high-level constitutive promoter was able to yield about 25 to 50-fold more 4MMP in F15 compared to the endogenous promoter in X5. However, it cannot be discounted that F15 offers a better genetic background for thiol release than X5. Other authors have shown that in some yeast backgrounds a ure2 mutant, which has a release of NCR and increased IRC7 expression, showed elevated thiol levels (Thibon et al. (2008a), Dufour et al. (2013)).
• Inefficient precursor uptake by yeast.

Thibon et al. (2008a) showed that C-4MMP and C-3MH are completely assimilated by VL3-1D yeast strain from synthetic grape juice when 30% of CO\textsubscript{2} has been released. However, significant amounts of C-3MH (up to \textasciitilde 100 µg/L) and G-3MH (up to \textasciitilde 500 µg/L) have been found in SB wines (Capone et al. (2010), Capone et al. (2011)) suggesting that there is an inefficient uptake of the precursors in some circumstances.

Some evidence on transport impairment was given by Subileau et al. Subileau et al. (2008b) which demonstrated that high DAP (10 mM) in synthetic media with added C-3MH was able to block precursor transport, probably through GAP1 repression by NCR; however, a gap1\textsuperscript{Δ} strain did not show any effect on 3MH when fermenting grape juice. These results suggest that available nitrogen in grape juice is not blocking precursor uptake by yeast, but that nitrogen additions may have an effect. In addition, deletion of the glutathione transporter. OPT1, reduced 3MH by 2-fold in grape juice (Subileau et al. (2008a)), which could be the result of either a transport defect of the glutathionylated precursor or the need for functional glutathione transport to achieve 3MH levels.

• Precursor or thiol metabolism by yeast.

It is possible that yeast may use cysteine and glutathione precursors and/or thiols as a sulfur source. So far there is no direct evidence for this, but this hypothesis is examined in Chapter 4 of this thesis.

• Thiol precursor or thiol modification by yeast.

It is also possible that cysteine and glutathione precursors and/or thiols are modified by yeast into other compounds to be stored, compartmentalized or secreted. For example, Wunschmann et al. (2009) showed that yeast catabolized a glutathione-S-bimane conjugate into the intermediates γ-GluCys-bimane or CysGly-bimane, by two different pathways, and they were then converted into a cysteine-bimane conjugate. In addition, some of the CysGly-bimane (15%) and the cysteine-bimane (40%) were found to be secreted by yeast into the media. Knowing this, it cannot be ruled out that yeast take up thiol precursors then later secrete them or their derivatives out of the cell. Supporting this idea, Grant-Preece et al. (2010) found C-3MH was present in the ferments when G-3MH had been added to the juice. In addition, plants have developed different mechanisms to avoid the formation of free thiols derived from cysteine conjugates (Dixon et al. (2010)). For example, N-malonylation, or N-glucosylation of the amino group on cysteine conjugates prevent the action of C-S lyases. Also in plants, free thiols can be subjected to S-methylation before C-S cleavage. Moreover, Howell et al. (2005) identified the formation of 2-methyl-tetrathiophen-3-one (MTHT) by BY4742 yeast; this formation was highly enhanced by addition of a high amount of C-4MMP to the fermenting medium, especially when BY4742 cys3\textsuperscript{Δ} was
used. However, the compound has also been proposed to be formed from methionine in cheese-ripening yeast (López Del Castillo-Lozano et al. (2007)). These findings open new possibilities to be studied in precursor or thiol metabolism by yeast that results in thiol low yields.

### 3.8.5 \( IRC^F \) cleaves L-cysteine

The inability of yeast strains to grow on cysteine as a nitrogen source was overcome by overexpressing \( IRC^F \). It was demonstrated that cysteine additions (2 and 15 mM) in the presence of a preferred nitrogen source, extended lag phase in a dose-dependent way and also slowed yeast growth (only 15 mM); however both of these effects were reduced when \( IRC^F \) was overexpressed. Moreover, progeny sporulated from a heterozygous parent (\( IRC^F/IRC^F::KAN \)) carrying \( irc7 \Delta \) did not grow on L-cysteine (15 mM) compared to the wt progeny. These results demonstrate that \( IRC^F \) is both sufficient and necessary for cysteine utilization as a nitrogen source.

This thesis has further proposed a new biochemical function for Irc7p, which is an L-cysteine desulphhydrase activity (CD), which is the cleavage of L-cysteine to release three products: \( \text{H}_2\text{S} \), ammonia and pyruvate.

The release of \( \text{H}_2\text{S} \) was initially detected by the darker color of \( IRC^F \) ox and \( IRC^F \) ox colonies grown in BiGGY agar and later confirmed by directly monitoring \( \text{H}_2\text{S} \) release from L-cysteine in vitro using purified Irc7p (Figure 3.25).

The production of \( \text{H}_2\text{S} \) was not measured. Growth on L-cysteine as a nitrogen source by yeast having a full-length \( IRC7 \), without the necessity for enzymes involved in cysteine transformation, provided good evidence that L-cysteine is transformed into a useful nitrogen source. However, it is interesting to point out the long lag phase observed when yeast grew on L-cysteine, as opposed to the fast growth provided by ammonia (Figure 3.12). One explanation could be that there is not enough L-cysteine available in the cytoplasm to be cleaved by Irc7p during approximately the first 24 h of culture, which needs a mM level (\( \text{K}_\text{m} = 3.8 \times 10^{-3} \)). Perhaps transport is not fast enough to reach mM concentration in the cell. Cysteine has been reported to enter the cell very slowly at low concentrations but increase substantially at increasing cysteine concentration (Maw (1963), Ono et al. (1991)). In addition, due to the high cysteine concentrations used (15 mM) the toxic effect remains even in the presence of a good nitrogen source (ammonia) and despite the help of Irc7p activity to overcome this toxicity (see Figure 3.12).

The production of pyruvate by Irc7p action on cysteine, gave very unusual kinetics using the pyruvate assay (see appendix A2). The addition of 0.4 mM CuSO4 to the reaction corrected the defect, suggesting that \( \text{H}_2\text{S} \) was
interfering with the reaction. The mechanism by which H₂S is causing this blockage is unknown but it could involve any of the two enzymes of the pyruvate assay or even by inducing the production of 2-aminoacrylate under these experimental conditions, which is proposed to be an intermediate in the degradation of cysteine by CD enzymes (Kredich et al. (1973), Morra et al. (1991)). This unstable intermediate can rapidly be hydrolyzed non-enzymatically to ammonia and pyruvate.

The data from the Irc7Fp in vitro assays discussed above confirm that Irc7Fp has a CD activity on L-cysteine. This is the first time CD activity has been linked to a gene product (Irc7Fp) in S. cerevisiae. Previously Wainwright (1970) and Tokuyama et al. (1973) showed increased H₂S production using cell cultures and crude extracts of yeast growth in pantothenic acid-deficient media. In addition, Tokuyama et al. (1973) characterized and purified two homogeneous enzyme preparations from Baker’s yeast showing CD activity in vitro: one was a proper cysteine desulfhydrase and the other was a cysteine synthetase. Both enzyme preparations were PLP-dependent.

From this previous evidence, it seems that in normal conditions the CD activity is low or null in yeast, but in situations where intracellular cysteine accumulation occurs (e.g. pantothenic acid deficiency) CD activity is observed. In this thesis, the amount of L-cysteine added to the growth experiments (15 mM) is large compared to the “standard amounts” in yeast culture media (e.g. ~40 µM in SGM). This might increase intracellular cysteine to a level where Irc7Fp can act on it. Irc7Fp might correspond to one of the enzymes described by Tokuyama et al. (1973), but more experiments will need to confirm this hypothesis. The Irc7Fp Km value (Km = 3.0 x 10⁻³ M) for cysteine does not match with either of the two enzyme Km described by Tokuyama et al. (1973), but this could be due to differences in experimental conditions.

The CD activity observed in Irc7Fp seems to be rare in S. cerevisiae. However, the available sequences of other sensu stricto species reveal predominance of the full-length gene. In addition, cysteine desulfhydrases are widely found in bacteria (Awano et al. (2005), Yano et al. (2009), Oguri et al. (2012)), plants (Papenbrock et al. (2007), Álvarez et al. (2010), Romero et al. (2013)) and mammals (Singh et al. (2011)). Irc7Fp has been proposed to be horizontally transferred to S. cerevisiae from bacteria (Hall et al. (2005)), because it has homologues in bacterial genomes but not in other yeast genomes (like E. gossypii). The bacterial homologue most similar to Irc7Fp is metC from Yersinia pestis. The biological role of metC in Y. pestis is unknown; however, in E. coli, the metC gene encodes a CBL with reported CD activity in vitro (Dwivedi et al. (1982)) and in vivo (Awano et al. (2003)). The biological role of metC in E. coli is to hydrolyzed cystathionine into homocysteine. In addition, disruption of the gene caused L-cysteine overproduction in E. coli (Awano et al. (2003)).
3.8.6 Irc7^Fp gave sensitivity to SEC

Yeast cells with an IRC7^S allele (like F15) are able to use SEC as both a nitrogen and sulfur source, but with a prolonged lag phase of 24 h and 48 h, respectively (Figures 3.8 and 3.9). This suggests that SEC is transformed into different compound(s) that are used either as a nitrogen or sulfur source. Methionine is also used by yeast as a nitrogen or sulfur source, but is has the opposite effect of SEC, being better as a sulfur source rather than as a nitrogen source.

When IRC7^F was overexpressed in F15, there was no growth at all in SEC as either a sulfur or nitrogen source in the time course of the experiment (Figures 3.8 and 3.9). The same no-growth phenotype was seen for a strain with an IRC7^F allele (X5), but when IRC7^F was deleted the growth was restored (Figure 3.11). Although YJM450 (having the IRC7^P allele) showed some growth on SEC as a nitrogen source, the growth was also highly improved if IRC7^P was deleted (Figure 3.11). These results showed that IRC7^F confers sensitivity to SEC, which can vary depending on the yeast genetic background.

It has been shown that Irc7^Fp acts on SEC as a substrate (Figures 3.23 and 3.24), so there is a possibility that the thiol released after Irc^F7p cleavage (presumably ethanethiol or derivatives obtained from it) is causing toxicity to yeast cells.

3.8.7 Growth on L-cysteine used as a selection tool to breed good 4MMP yeast producers

Selection on L-cysteine was shown to improve 4MMP production by wine yeast. In the breeding pool used here, full-length IRC7 was present in all of the parental strains, since each hybrid contained one allele of full-length IRC7 (IRC7^F or IRC7^P) as well as a second variable IRC7 allele (IRC7^S, IRC7^F, or unknown). After three rounds of selection for growth on cysteine, all the selected strains produced high 4MMP levels from grape juice (Figure 3.17), and all of them had only the IRC7^P allele (H. Sheehan, personal communication). For one strain, CP-A-A3, the 4MMP improvement was shown to be associated with an elevated conversion of C-4MMP in synthetic medium (Figure 3.18). The genetic origin of this improvement was not studied in this thesis, but it might be caused by mutations affecting the IRC7^F promoter or a transcription/repressor factor that enhanced its expression. Alternatively it might be that selection on L-cysteine improved C-4MMP transport or reduced losses of the precursor or thiol. Direct sequencing of IRC7 from the CP-A-A3 strain (Cecile Nieto, this laboratory) ruled out the possibility that the increase is due to a mutation in the coding sequence that gives better enzymatic properties, like improved substrate affinity or processing.
Cysteine growth is a simple way to test, select and improve strains with the \( IRC7^F \) allele. Based on the frequency of the full-length allele among strains of \( S. \) \textit{cerevisiae}, one can hypothesize that \( IRC7^F \) is being removed from the \( S. \) \textit{cerevisiae} population compared to other strains from the \textit{sensu stricto} group. Genome sequences from \( S. \) \textit{paradoxus}, \( S. \) \textit{mikatae} and \( S. \) \textit{bayanus} (three genomes available in SGD data and 28 genomes of \( S. \) \textit{paradoxus} available in Sanger Institute) have all shown to have a full-length \( IRC7 \) gene (data not shown), suggesting that they might be good 4MMP producers. For example, \( S. \) \textit{bayanus} has been shown to release higher amounts of 4MMP than \( S. \) \textit{cerevisiae} (Dubourdieu et al. (2006)). Also, hybrids between \( S. \) \textit{cerevisiae} and \( S. \) \textit{bayanus var. uvarum} have been described to have improved 4MMP production compared to their \( S. \) \textit{cerevisiae} parent VL3c (Dubourdieu et al. (2006)). In addition, YJM450 carrying an introgressed full-length \( IRC7 \) from \( S. \) \textit{paradoxus} released high amounts of 4MMP (Roncoroni et al. (2011)).

It is highly probable that any improvement in \( IRC7 \) expression or enzymatic performance in CP-A-A3 may have simultaneously raised \( \text{H}_2\text{S} \) production levels, but this was not confirmed in this thesis. The potential new source of \( \text{H}_2\text{S} \) could be detrimental for wine as an off-flavor, but it might be beneficial if the timing of \( \text{H}_2\text{S} \) release occurs early during fermentation. As shown by Harsch et al. (2013a), providing a good source of \( \text{H}_2\text{S} \) on the onset of fermentation increased 3MH potential drastically.
3.9 Concluding summary and remarks

Chapter 3 studied in detail the role of different \( IRC7 \) alleles, determining their involvement in thiol release during wine fermentation and their biological function. The main experimental findings were:

- \( IRC7^B \) is not necessary or sufficient for thiol release.
- \( IRC7^F \) is necessary and sufficient for 4MMP and 3MH release from C-4MMP and G-3MH.
- \( IRC7^F \) is necessary and sufficient for growth on L-cysteine as a nitrogen source.
- \( IRC7^F \) codes for an enzyme with \( \beta \)-lyase and L-cysteine desulphhydrase activities.

Based on these results, it may be that yeast acquired \( IRC7 \) by horizontal transfer from bacteria in order to provide yeast cells with the capability to cleave cysteine directly. This capability may have provided yeast cells with a sulfur source (\( \text{H}_2\text{S} \)) that they can use independently from the Str2p-Str3p transsulfuration pathway, and a nitrogen source (\( \text{NH}_4^+ \)) that yeast can use directly without the need for the Erhlich pathway of amino acid degradation.
4 Transporters and other genes involved in thiol release

4.1 Introduction

The thiol precursors studied in this thesis are conjugated to the amino acid L-cysteine or to the tripeptide L-glutathione. In this chapter the major focus will be to establish the involvement of L-cysteine and L-glutathione transporters in thiol precursor uptake. In addition, genes involved in the transformation of glutathione conjugates into 3MH will also be studied.

There are nine L-cysteine transporters described so far in *S. cerevisiae* (see Section 1.6.2 for details). In contrast, L-glutathione is mainly transported by *OPT1*, but some authors suggest that it can also be taken up by *OPT2* (see Section 1.6.3).

The degradation of glutathione conjugates has been shown to occur in yeast through two different pathways, both requiring the *CIS2*-encoded γ-glutamyltranspeptidase (Wunschmann et al. (2009), see Section 1.6.3).
4.2 Screening tools to search for genes transporting thiol precursors

In order to identify genes that encode transporters of C-4MMP and G-3MH, the available tool for screening mutants is thiol quantification in wine after yeast fermentation, a process which is time consuming and expensive. In this section some tools were tested that potentially will allow faster and cheaper screening of candidate genes involved in cysteinylated and glutathionylated thiol precursors.

4.2.1 Growth on C-4MMP and G-3MH as a selection tool for genes involved in their uptake

One approach was to measure yeast growth on thiol precursors as a sole source of nitrogen, with the idea of utilizing transporter deletion mutants to screen for lack of growth. In addition, testing for cell growth on thiol precursors as a sulfur source will also help to establish possible losses that account for low conversion of these precursors into thiols.

Figure 4.1 shows that the cysteine-conjugates (C-3MH and C-4MMP) did not support growth in F15 and F15 \textit{IRC7} \textit{Fox} when used as either a nitrogen or sulfur source. However, G-3MH allowed moderate growth of both strains as a nitrogen source, and very slight growth as a sulfur source. This was a very interesting result, given the inability of the same yeast strain to grow on L-glutathione as a nitrogen source (Figure 3.9). The fact that \textit{IRC7} \textit{Fox} overexpression did not give an advantage for yeast growth suggests that \textit{IRC7} \textit{Fox} is not involved in the utilization of G-3MH as either a nitrogen or sulfur source.

This result showed that C-4MMP or C-3MH cannot be used for screening growth of the deletion mutant on genes coding for cysteine transporters, at least in the F15 and BY4743 strain backgrounds. However, growth on G-3MH (especially as a nitrogen source) can be used as a rapid screen to test mutants in some candidate genes for glutathione transporters and for glutathione degradation enzymes. The results of these screens would need to be further confirmed by fermenting and analyzing thiols using pre-selected mutants.
Figure 4.1: F15 and F15 \( IRC7^{\text{Fox}} \) growth on thiol conjugates as sulfur and nitrogen sources. The F15 (continuous lines) and F15 \( IRC7^{\text{Fox}} \) (dashed lines) strains were grown in 150 µL SGM at 25°C for 3 d using Na\(_2\)SO\(_4\) (sulfur positive control) or NH\(_4\)Cl (nitrogen positive control), C-4MMP, C-3MH or D3-3MH-G as sulfur sources (0.5 mM) and nitrogen sources (15 mM). Absorbance was measured every 15 min and plotted versus time. Average curves were plotted. \( n = 2 \).

To test if the lack of growth was caused by an inhibitory or toxic effect of the cysteinylated conjugates, yeasts were grown in SGM with 0.5 mM Na\(_2\)SO\(_4\) as a sulfur source with and without 0.5 mM C-4MMP supplementation. No positive or negative effect on yeast growth was observed in strains F15 and F15 \( IRC7^{\text{Fox}} \) when adding C-4MMP. It was concluded that C-4MMP does not interfere with yeast growth at a concentration of 0.5 mM. Thus, absence of growth when C-4MMP is used as a sulfur source in Figure 4.1 was attributed to the inability of yeast to metabolize the compound into assimilable sulfur or nitrogen under these experimental conditions.

Figure 4.2: C-4MMP does not act as a growth inhibitor. The strains were grown in 150 µL SGM at 25°C for 3 d using only 0.5 mM Na\(_2\)SO\(_4\) as a sulfur source with and without supplementation by 0.5 mM C-4MMP. Absorbance was measured every 15 min and plotted versus time. Average curves were plotted. \( n = 2 \).
4.2.2 Growth on toxic analogues as a selection tool for genes involved in C-4MMP uptake

Two known yeast growth inhibitors (Maw (1961)), that are similar in structure to C-4MMP, SEC and ethionine (illustrated in Figure 4.3) were used to screen several BY4743 single mutants deleted for different permeases. These compounds were chosen because of their solubility in water. Other cysteine-like compounds such as S-t-butyl-L-cysteine, S-carboxymethyl-L-cysteine and S-benzyl-L-cysteine were discarded as they could not easily be dissolved (Harsch (2009), PhD thesis).

![C-4MMP, Ethionine, SEC](image)

**Figure 4.3:** Toxic compounds used to screen for resistance in several permease deletion mutants. The cysteine backbone is conserved between the three compounds, with the main differences observed in the sulfur side chain.

All single permease mutants in both the AAP and DAL families (listed in Table 4.1) in the BY4743 deletion collection were used to screen for resistance to ethionine and SEC. Cells were starved for sulfur in SFM plates for 2 d and then grown in MSD plates supplemented with 0.025 mM ethionine or 2 mM SEC, concentrations at which the wt BY4743 was not able to grow. Adding the sulfur starvation step before growing yeast on the toxic compounds gave a much cleaner result. Figure 4.3 shows that the Δmup1 and Δgnp1 strains were able to grow in 0.025 mM ethionine and 2 mM SEC, respectively.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Regulation</th>
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<tbody>
<tr>
<td>AAP</td>
<td>Intermediate to low-affinity AAP with broad substrate range (Asn, Gln, and other amino acids like Leu, Ile, Met, Phe, Ser, Thr, Trp, Tyr, Val, Ala, Gly, Cys) ({})</td>
<td>NCR-regulated, aa induction through SSY1. GAAC regulated ({}).</td>
</tr>
<tr>
<td>AGP1</td>
<td>Arg transporter ({}).</td>
<td>()</td>
</tr>
<tr>
<td>ALP1</td>
<td>High-affinity branched-chain amino acid permease (Leu, Ile and Val) and other amino acids like Phe, Tyr, Trp, Met, Cys, Ala ({}).</td>
<td>Aa induction (especially Leu) through SSY1. GAAC-regulated ({}).</td>
</tr>
<tr>
<td>BAP2</td>
<td>Amino acid permease involved in the uptake of branched amino acids (Ile, Leu, Val) and Cys, Phe, Tyr, Trp, Met, Thr, Ala ({}).</td>
<td>Aa induced through SSY1 ({}).</td>
</tr>
<tr>
<td>BAP3</td>
<td>Arg permease ({}).</td>
<td>()</td>
</tr>
<tr>
<td>CAN1</td>
<td>Dicarboxylic amino acid permease, mediates high-affinity transport of Glu and Asp; also a transporter for Gln, Asn, Ser, Ala, and Gly ({}).</td>
<td>NCR ({}) and SSY1-regulated ({}).</td>
</tr>
<tr>
<td>DIP5</td>
<td>Diacidic permease, transports all common L-amino acids, also several D-amino acids, aa related compounds and aa toxic analogues ({}).</td>
<td>NCR and SSY1-regulated ({}).</td>
</tr>
<tr>
<td>GAP1</td>
<td>General aa permease, transports all common L-amino acids, also several D-amino acids, aa related compounds and aa toxic analogues ({}).</td>
<td>NCR and GAAC-regulated ({}).</td>
</tr>
<tr>
<td>GNP1</td>
<td>High-affinity glutamine permease, also transports Leu, Ser, Thr, Cys, Met and Asn ({}).</td>
<td>Expressed on both rich and poor nitrogen sources. Induced by aa, through SSY1 ({}). Higher transcription under sulfur-limitation ({}).</td>
</tr>
<tr>
<td>LYP1</td>
<td>Lysine permease, also transports methionine. ({}).</td>
<td>GAAC-regulated expression ({}).</td>
</tr>
<tr>
<td>MMP1</td>
<td>High-affinity S-methylmethionine permease ({}).</td>
<td>Higher transcription under sulfur-limitation ({}). Downregulated by methionine as nitrogen source ({}).</td>
</tr>
<tr>
<td>MUP1</td>
<td>High affinity methionine permease, also involved in cysteine uptake ({}).</td>
<td>SSY1-regulated ({}). Higher transcription under sulfur-limitation ({}).</td>
</tr>
<tr>
<td>MUP3</td>
<td>Low affinity methionine permease ({}).</td>
<td>GAAC-regulated ({}). Higher transcription under sulfur-limitation ({}).</td>
</tr>
<tr>
<td>PUT4</td>
<td>Proline permease, also Ala, Gly and GABA ({}).</td>
<td>NCR-regulated ({}).</td>
</tr>
<tr>
<td>SAM3</td>
<td>High-affinity S-adenosylmethionine permease ({}).</td>
<td>()</td>
</tr>
<tr>
<td>TAT1</td>
<td>Tyr high-affinity transporter and low-affinity uptake of Trp and His. Also transport Val, Thr, Cys, Leu and Ile ({}).</td>
<td>Aa induced through SSY1 ({}).</td>
</tr>
<tr>
<td>TAT2</td>
<td>High-affinity Trp. Also transports Tyr, Phe, Gly, ()</td>
<td>Aa induced through SSY1 ({}).</td>
</tr>
</tbody>
</table>
**UGA4**

Gamma-aminobutyrate (GABA) permease. NCR-regulated\(^{12}\).  
Vacuolar localization\(^1\).

**DAL PERMEASES**

<table>
<thead>
<tr>
<th><strong>DAL4</strong></th>
<th>Allantoin permease(^{28}). NCR-regulated(^{13}).</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DAL5</strong></td>
<td>Allantoate and ureidosuccinate permease; also transports dipeptides(^7,8). NCR-regulated, allantoate feedback inhibition and trans-inhibition by L and D-aa(^{7,26}).</td>
</tr>
<tr>
<td><strong>FEN2</strong></td>
<td>High-affinity H(^+)-pantothenate symporter(^{34}). Regulated by pantothenate extracellular concentrations, being maximal at low levels of the compound.</td>
</tr>
<tr>
<td><strong>SEO1</strong></td>
<td>Putative permease; mutation confers resistance to ethionine sulfoxide(^{16}).</td>
</tr>
<tr>
<td><strong>THI73</strong></td>
<td>Putative plasma membrane permease proposed to be involved in carboxylic acid uptake(^{23,22}). Repressed by thiamine(^{22}).</td>
</tr>
<tr>
<td><strong>TNA1</strong></td>
<td>High affinity nicotinic acid plasma membrane permease(^{22}). Expression increases in the absence of extracellular nicotinic acid(^{22}).</td>
</tr>
<tr>
<td><strong>YCT1</strong></td>
<td>High-affinity cysteine-specific transporter(^{18}). Derepressed in sulfur-limiting conditions and repressed in the presence of organic sulfur(^{18}).</td>
</tr>
<tr>
<td><strong>YIL166C</strong></td>
<td>Putative protein with similarity to Dal5p(^{29}). Expression is elevated by sulfur limitation(^7).</td>
</tr>
</tbody>
</table>

* Permeases that have been described as transporters of cysteine are shown in red.  
NCR: nitrogen catabolism repression is a mechanism triggered by the presence of preferable nitrogen compounds to repress genes involved in the usage of poor nitrogen sources.  
SSY1: one of three components of the Ssy1p–Ptr3p–Ssy5 (SPS) sensor of extracellular amino acids, which induce the expression of different aa and peptide transporter genes when sensing extracellular aa.  
GAAC: General Amino Acid Control, activated by aa starvation inducing the expression of genes involved in aa biosynthesis through Gcn4p.


The growth observed by the *mup1Δ* and *gnp1Δ* strains in the presence of toxic levels of the inhibitors suggested that under these experimental conditions, *MUP1* is the major transporter involved in the uptake of SEC and *GNP1* is the major transporter for ethionine. The transporters encoded by *MUP1* and *GNP1* have both been related to cysteine transport (Kosugi et al. (2001), During-Olsen et al. (1999)), which made them good candidates for putative C-4MMP transporters.
Figure 4.3: MUP1 and GNP1 deletion mutants showing resistant phenotype to ethionine and SEC on MSD plates. After sulfur starvation different BY4743 permease mutant were incubated on MSD with 0.025 mM ethionine or 2 mM SEC, supplemented with histidine [20 mg/L], leucine [30 mg/L] and uracil [20 mg/L]. The picture showed two different plates in which one mutant grew on each of the toxic compounds.

4.2.3 Summary

Cysteinylated thiol precursors were not used by yeast as nitrogen or sulfur sources, making them not suitable to be used in phenotype screening for growth defects. The lack of yeast growth on C-4MMP and C-3MH as sulfur sources in fermentation medium also showed that low thiol yields are not due to losses by sulfur assimilation. However, G-3MH was well used as a nitrogen source and gave some growth as a sulfur source. Growth on G-3MH as a nitrogen source allows this loss-of-growth phenotype screening in the search of permease(s) involved in its uptake or genes involved in its processing inside the cell, while the growth on G-3MH as a sulfur source can help to explain losses in thiol yield.

The use of toxic compounds (SEC and ethionine) with similar structures to cysteinylated precursors suggested two candidate permeases for C-4MMP uptake that will be tested in the next section: Gnp1p and Mup1p.
4.3 C-4MMP is not transported by known L-cysteine transporters

To identify putative candidates for a C-4MMP transporter, the two cysteine transporters identified in Section 4.2.2, \textit{MUP1} and \textit{GNP1}, along with other seven known cysteine transporters, were included in the search.

Overexpression and deletion of the cysteine transporters was the strategy followed to test for their involvement in C-4MMP uptake. The strain M4238 \textit{gap1\Delta ssy1\Delta} (Didion et al. (1998)), which has a downregulation of multiple permeases, was selected to construct overexpression strains. In addition, different single and multiple cysteine deletion mutants were tested. The mutants used were all constructed in laboratory yeast genetic backgrounds and all needed to be firstly transformed with \textit{IRC7}\textsuperscript{F} to allow detection of 4MMP and/or growth on L-cysteine as nitrogen source. Untransformed deletion mutants were only useful for screening growth on L-cysteine as a sulfur source.

4.3.1 Overexpression of cysteine amino acid transporters does not improve 4MMP production

Eight of the nine genes known to code for cysteine permeases (except \textit{GAP1}) were overexpressed in a \textit{gap1\Delta ssy1\Delta} mutant strain, which is known to have decreased transport of certain aa, especially branched aa (Didion et al. (1998)). \textit{GAP1} is the major transporter of aa in low nitrogen conditions while \textit{SSY1} regulates several aa permeases (see Table 4.1), including most of those that transport L-cysteine (all except \textit{GAP1} and \textit{YCT1}). The overexpression strategy consisted of introducing a \textit{TEF2} promoter cassette into the yeast chromosomes just in front of the start codon of each permease gene (see Section 2.13.2).

The growth defect of \textit{gap1\Delta ssy1\Delta} on SGM was studied to confirm that it showed impaired aa uptake under the experimental conditions used in this thesis. Growth on SGM with 19 different aa as nitrogen sources was compared for the starting \textit{gap1\Delta} and \textit{gap1\Delta ssy1\Delta} strains as an indirect measure of aa transport impairment. Table 4.2 summarizes the results and Appendix A4.1 contains detailed information on strain growth rates. The main differences between the strains were found on six aas: Ile, Leu, Met, Phe, Val and Thr, where the \textit{gap1\Delta ssy1\Delta} did not grow, but the \textit{gap1\Delta} did. Slight differences were found on Ala, Asn, Asp, Glu, Ser where the \textit{gap1\Delta ssy1\Delta} had longer lag phase than the \textit{gap1\Delta} mutant, but only Glu and Asp also increased generation time.

No reduction in growth in the \textit{gap1\Delta ssy1\Delta} strain was seen on Arg, Gln and Pro. Cysteine showed a more complex phenotype: the \textit{gap1\Delta ssy1\Delta} mutant had a significantly shorter lag phase and higher efficiency (final cell titre) than \textit{gap1\Delta}, which could be interpreted as lower levels of Cys entering the \textit{gap1\Delta ssy1\Delta} cells that will have
a reduced negative impact on growth. However, the fact that Cys supported growth of the \( \text{gap1}\Delta\text{ssy1}\Delta \) mutant indicated that transport is not completely blocked in this mutant. Despite this the overexpression of different aa permeases was performed in this background.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Same phenotype for ( \text{gap1}\Delta ) and ( \text{gap1}\Delta \text{ssy1}\Delta )</td>
<td>Arg, Gln, Pro (growth) Gly, His, Lys, Trp (no growth)</td>
</tr>
<tr>
<td>Growth for both strains, but altered growth for ( \text{gap1}\Delta\text{ssy1}\Delta )</td>
<td>Ala, Asn, Asp, Cys, Glu, Ser</td>
</tr>
<tr>
<td>Growth for ( \text{gap1}\Delta ), but no growth for ( \text{gap1}\Delta \text{ssy1}\Delta )</td>
<td>Ile, Leu, Met, Phe, Thr, Val</td>
</tr>
</tbody>
</table>

After overexpression of eight individual permeases (\( \text{AGP1}, \text{BAP2}, \text{BAP3}, \text{GNP1}, \text{MUP1}, \text{TAT1}, \text{TAT2} \) and \( \text{YCT1} \)) in the \( \text{gap1}\Delta\text{ssy1}\Delta \) background, growth on 12 aa as nitrogen sources (aa that gave altered growth compared to \( \text{gap1}\Delta \), Table 4.2) were retested to check for growth differences due to permease overexpression. Only four of the eight overexpression strains gave a clear phenotype as listed below and detailed in Appendix A4.2:

- Overexpression of \( \text{AGP1} \) allowed the strain \( \text{gap1}\Delta\text{ssy1}\Delta \) to grow on Ile, Leu, Met, Phe, Thr and Val.
- Overexpression of \( \text{BAP3} \) allowed the strain \( \text{gap1}\Delta\text{ssy1}\Delta \) to grow on Ile, Leu, Met, Phe, Thr and Val.
- Overexpression of \( \text{MUP1} \) allowed the strain \( \text{gap1}\Delta\text{ssy1}\Delta \) to grow on Ile, Leu, Met, Phe, Thr and Val, but greatly slowed growth on Cys.
- Overexpression of \( \text{TAT1} \) slowed growth of the strain \( \text{gap1}\Delta\text{ssy1}\Delta \) on Glu and greatly slowed growth on Cys.

These changes in phenotype provide good evidence that overexpression of these four permeases was successful. The fact that overexpressing \( \text{BAP2}, \text{GNP1}, \text{TAT2} \) and \( \text{YCT1} \) did not change \( \text{gap1}\Delta\text{ssy1}\Delta \) growth on 12 tested aa could have different explanations. First, it may be that the constructs were not functional. Second, it may be that transcriptional overexpression does not affect gene activity for some of the permeases (for example, their activity may be translationally regulated). Finally, there is the possibility that they are functionally active but their effect was not seen when using 15 mM of the putative substrates, because they may be acting as high-affinity permeases. Nevertheless, the eight permease overexpression strains were used in the following experiments.
An initial fermentation using grape juice M1016 (widely used in Chapter 3) was completed using gap1Δ and gap1Δssy1Δ, both overexpressing IRC7F. The double mutant did not show any 4MMP when compared to the gap1Δ strain (Figure 4.4). In contrast, 3MH levels were not significantly different between strains.

![Graph showing 4MMP and 3MH levels](image)

**Figure 4.4: The strain gap1Δssy1Δ IRC7Fox did not produce 4MMP in M1016 juice.** M1016 juice was fermented at 25°C using gap1Δ IRC7F and gap1Δ ssy1Δ IRC7F. Juice was three-quarters-strength by diluting with SGM with no added sugars and supplemented with uracil [20mg/L]. Thiols were extracted by the PHMB method. n = 2; error bars = SE; asterisks above bars represent significant differences compared to gap1Δ strain (**p < 0.01, ANOVA).

Unfortunately juice M1016 was finished and replaced with different SB juice for testing the permease overexpression strains. The first attempt was performed by fermenting gap1Δssy1Δ IRC7fox and gap1Δssy1Δ IRC7fox MUP1ox using three SB juices and SGM with added precursors. Results were completely different this time, because gap1Δssy1Δ IRC7fox produced 4MMP in all juices (Table 4.3). The overexpression of MUP1 significantly increased 4MMP in one juice and 3MH release in two of the three grape juices tested. However, MUP1 overexpression significantly reduced 4MMP production in SGM with added C-4MMP and G-3MH.
Table 4.3: Overexpression of MUP1 gave more 4MMP in grape juice but less in SGM.

<table>
<thead>
<tr>
<th>Juice†</th>
<th>Strain</th>
<th>4MMP [ng/L]</th>
<th>3MH/3MHA [ng/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blanw</td>
<td>gap1Δssy1Δ IRC7ox</td>
<td>121 ± 6</td>
<td>2,468 ± 26</td>
</tr>
<tr>
<td></td>
<td>gap1Δssy1Δ IRC7ox MUP1ox</td>
<td>284 ± 23*</td>
<td>3,212 ± 167*</td>
</tr>
<tr>
<td>Squire</td>
<td>gap1Δssy1Δ IRC7ox</td>
<td>427 ± 13</td>
<td>3,198 ± 22</td>
</tr>
<tr>
<td></td>
<td>gap1Δssy1Δ IRC7ox MUP1ox</td>
<td>505 ±34</td>
<td>4,212 ± 160*</td>
</tr>
<tr>
<td>M1008</td>
<td>gap1Δssy1Δ IRC7ox</td>
<td>25 ± 25</td>
<td>1,752 ± 281</td>
</tr>
<tr>
<td></td>
<td>gap1Δssy1Δ IRC7ox MUP1ox</td>
<td>37 ± 20</td>
<td>1,480 ± 115</td>
</tr>
<tr>
<td>SGM</td>
<td>gap1Δssy1Δ IRC7ox</td>
<td>3,788 ± 13</td>
<td>7,741 ± 210</td>
</tr>
<tr>
<td></td>
<td>gap1Δssy1Δ IRC7ox MUP1ox</td>
<td>2,962 ±10*</td>
<td>7,068 ± 170</td>
</tr>
</tbody>
</table>

† Grape juices was three-quarters-strength by diluting with SGM with no added sugars. Fermentation in SGM was supplemented with 50 µg/L C-4MMP and 500 µg/L G-3MH. All juices for gap1Δssy1Δ IRC7ox fermentation were supplemented with uracil [20mg/L].
* Significant differences compared to gap1Δ ssy1Δ IRC7ox strain (p < 0.05, ANOVA).

The inconsistent increase of thiol when using different grape juices to ferment the gap1Δssy1Δ IRC7ox MUP1 overexpression strain suggested that there must be interactions between yeast strain and the grape juice composition that are determining the changes. Moreover, the decrease in 4MMP production observed in synthetic medium with added precursors indicates that some complex interactions or regulation may be also occurring in this situation. One possible explanation is that increased uptake of a Mup1p substrate (methionine, cysteine or other) may be causing a decrease in uptake of C-4MMP or a reduction in its processing into 4MMP. Also, it may be that Mup1p suffered degradation or recycling into another cell compartment.

Next, the eight strains overexpressing the different permeases were used to ferment SGM with added C-4MMP and G-3MH. Figure 4.5 shows that none of them increased thiol release under this conditions but five of them again decreased production of either 4MMP, 3MH or both. This time gap1Δssy1Δ MUP1ox was not significantly lower than gap1Δ, indicating that there is an experimental variation between batches. The decrease in thiol production was not studied further.
Figure 4.5: 4MMP release is not improved by yeast overexpressing different permeases in SGM with added C-4MMP. SGM with added precursors (50 µg/L C-4MMP and 500 µg/L D3-3MH-glut) was fermented at 25°C using gap1Δssy1Δ IRC7F ox and the AAP overexpression strains. SGM was three-quarters-strength in sugars for all strains and supplemented with uracil [20mg/L] for the gap1Δssy1Δ strain. Thiols were extracted by the PHMB method. The labels on bars are percentages of conversion yields. Note the difference scale in the two graphs. n = 2; error bars = SE; asterisks above bars represent significant differences compared to gap1Δ strain (* p < 0.05, ** p < 0.001, ANOVA).

I next attempted to define fermenting conditions that would reduce uptake of precursors and therefore allow a better resolution for testing the permease overexpression strains. SGM with high nitrogen was used, which might give a constant NCR during fermentation, thus maintaining repression of the genes involved in the utilization of less preferred nitrogen sources (Beltran et al. (2004)). An experiment using high nitrogen addition was performed by fermenting SGM with added precursors using the gap1Δssy1Δ IRC7F ox strain (Appendix A2.2). It was very interesting to observe that the high nitrogen condition only reduced 4MMP release from C-4MMP (~3-fold reduction), suggesting that NCR might be controlling either uptake of the cysteinylated precursor or affecting Irc7F p activity. However, 3MH production was unaffected in high nitrogen, suggesting that Irc7F p activity was not affected, and that the added nitrogen was affecting uptake of C-4MMP precursor.

Then a subset of six permease overexpression strains was used to ferment SGM (high DAP) with added C-4MMP and G-3MH. Figure 4.6 confirmed that only 4MMP release was affected by high nitrogen in the gap1Δssy1Δ strain (~400 ng/L, compared to ~4,000 ng/L in the normal conditions in Figures 4.5 and A5), while 3MH remained similar (around 11,000 ng/L). Under these conditions, MUP1 overexpression gave significantly higher 4MMP and 3MH levels. In addition, TAT1 overexpression increased 4MMP, but TAT2 overexpression still decreased 4MMP production.
Overall the results from the overexpression strains were inconclusive and disappointing. The largest phenotypic changes were seen with overexpression of \textit{MUP1}, but these changes were not consistent between media. Moreover, conversion of both cysteinylated and glutathionylated precursors was affected, and growth on cysteine was greatly reduced. This range of results suggests that the phenotypes seen are indirect and may not be the result of increases in the uptake of these different precursors by the \textit{MUP1} transporter.

\subsection{4.3.2 Single deletion of cysteine transporters does not affect 4MMP release}

To complement results from overexpressing cysteine transporters and to have a clearer perspective about the involvement of cysteine transporters in C-4MMP uptake, BY4743 single deletion mutants in each of the nine cysteine transporters were tested using three assays.

Firstly, growth on SGM with L-cysteine as a sulfur source was performed with the aim of identifying the main cysteine transporter in conditions similar to wine fermentation. In Figure 4.7, \textit{YCT1} deletion seems to result in a complete block of L-cysteine transport at this concentration (0.5 mM) in these conditions, making it a good candidate for C-4MMP transport. Although the \textit{BAP2} deletion mutant also showed no growth in L-cysteine, it had
poor growth in N₂SO₄, suggesting that the strain has a growth defect rather than a specific cysteine-related phenotype.

![Graph showing growth in Na₂SO₄ and L-cysteine](image)

**Figure 4.7: BY4743 single deletion mutants growth in L-cysteine as sulfur source.** The strain was grown in 150 µL SGM at 25°C for 3 d using 0.5 mM Na₂SO₄ (sulfur positive control) or L-cysteine as a sulfur source (0.5 mM). SGM was supplemented with histidine [20 mg/L], leucine [30 mg/L] and uracil [20mg/L]. Absorbance was measured every 15 min and plotted versus time. Average curves were plotted. *n = 2.*

Secondly, IRC7 was overexpressed in all eight single deletion mutants to allow testing of growth on L-cysteine as a nitrogen source in fermentation medium (gap1Δ was excluded because it was tested before, see Table 4.2 and Figure 4.4). Again bap2Δ was the only single deletion mutant showing altered growth on L-cysteine as a nitrogen source (15 mM). This result suggests that more than one of the known cysteine transporters might be involved in its uptake at this higher concentration (not shown).

Finally, each of the eight single deletion permease mutants overexpressing IRC7 was used to ferment SGM with added C-4MMP and G-3MH and thiols were quantified. Figure 4.8 shows that no significant differences were seen in 4MMP, but mup1Δ and gnp1Δ gave significant reduction on 3MH production under these conditions (*p < 0.05*, not shown). BAP2 deletion gave the lowest 4MMP release (*p = 0.068*, ANOVA) compared to the BY4743 wt strain, but it showed a slower fermentation rate compared to the rest of the strains (not shown).
4.3.3 Deletion of all nine cysteine transporters does not block 4MMP release

The effect on thiol production was tested using mutants deleted for multiple cysteine permeases. Eight of the strains used were kindly provided by Birgitte Regenberg and Jurgen Wendland (During-Olsen et al. (1999)) and are detailed in Table 4.4. Two additional strains, MS1 and MS2 were constructed from M4584 IRC7\textsuperscript{ox}. In MS1 the permease \textit{YCT1} was replaced by the \textit{URA3} gene (\textit{yct1\textDelta}::\textit{URA3}) and MS2 corresponds to MS1 but with \textit{MUP1} replaced by the \textit{hphMX} cassette (\textit{mup1\textDelta}::\textit{hphMX}). All ten mutant strains were transformed with the \textit{IRC7}\textsuperscript{ox} overexpression cassette to allow growth on L-cysteine as a nitrogen source and 4MMP detection during fermentation.
Table 4.4: Cysteine transporter deletion strains.*

<table>
<thead>
<tr>
<th>Strain \ deletion</th>
<th>∆gap1</th>
<th>∆agp1</th>
<th>∆gnp1</th>
<th>∆bap2</th>
<th>∆tat1</th>
<th>∆tat2</th>
<th>∆bap3</th>
<th>∆yct1</th>
<th>∆mup1</th>
</tr>
</thead>
<tbody>
<tr>
<td>M3750</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>M4054</td>
<td>X</td>
<td></td>
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<td></td>
</tr>
<tr>
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<td>X</td>
<td>X</td>
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<td></td>
</tr>
<tr>
<td>M4578</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td>X</td>
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</tr>
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<td>M4584</td>
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<td>X</td>
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<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS1</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS2</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Deleted genes are shown by an X symbol

Prior to fermentation, a growth experiment on L-cysteine as a nitrogen source was performed to test functionality of IRC7 overexpression and to assess cysteine transport indirectly. All strains grew well in the ammonia control medium (Figure 4.9 left side graph). Surprisingly, no mutants stopped yeast growth completely on L-cysteine, although growth on other aa was abolished in some strains (e.g. Ile, Leu, Met, Phe, Thr, Val, see Appendix A4.3). This result showed that cysteine transport is still occurring despite all the nine known cysteine transporters having been deleted. However, the seven mutants carrying the AGP1 deletion showed lower growth rate than the wt (Figure 4.9, right side graph), suggesting that these strains are taking up cysteine to a greater extent and being inhibited. It was concluded that AGP1 deletion affects cysteine uptake in fermentative-like conditions (SGM media).
Figure 4.9: Deletion of all nine known cysteine transporters did not completely block yeast growth on cysteine as a nitrogen source. The strains were grown in 150 µL SGM at 25°C for 6 d using either 15 mM of NH₄Cl or L-cysteine as a nitrogen source. SGM was supplemented with uracil [20mg/L]. Absorbance was measured every 15 min and plotted versus time. Average curves were plotted. n = 2.

The set of multiple deletion mutants was then used to ferment SGM with added C-4MMP and G-3MH to look for decreased 4MMP concentrations in wine. All the mutants produced 4MMP (Figure 4.10) with no significant differences compared to the wt. The release of 3MH from G-3MH was significantly lower in five strains, which seems to be an experimental variation more than a real difference because some strains with deletions that overlap the ones that gave a difference (e.g. MS1 and MS2) showed no significant change.

These results suggest that permease(s) different from the nine known cysteine transporters are required to take up C-4MMP into the yeast cell. As cysteine uptake is also not completely blocked in the mutants tested, it cannot be ruled out that a different transporter is taking up both cysteine and C-4MMP.
Figure 4.10: Deletion of all nine cysteine transporters does not affect 4MMP release. Nine mutants with increasing numbers of deleted cysteine transporters (see Table 4.4) and transformed with the ho::P$_{PGK}$-IRC7 overexpression cassette were used to ferment SGM with added 50 µg/L of C-4MMP and 500 µg/L of G-3MH at 25°C. SGM was three-quarters-strength in sugars and supplemented with uracil [20mg/L]. Thiols were extracted by the ETP method. The labels above the bars show the percentage of thiol conversion from the added precursor. n = 2; error bars = SE; asterisks above bars represent significant differences compared to gap1Δ strain (* p < 0.05, ** p < 0.001, ANOVA).

4.3.4 Summary

The nine known cysteine transporters seemed to be good candidates for C-4MMP. However, strains with multiple deletions of all transporters grew on L-cysteine as a nitrogen source and produced 4MMP from C-4MMP in fermenting conditions with no significant difference from the wt strain. Moreover, overexpression of individual cysteine permeases in strains with impaired aa uptake had no positive effect on 4MMP in SGM with added C-4MMP. However, when high nitrogen fermentations were carried out, which hugely reduced total 4MMP levels, MUP1 and TAT1 overexpression significantly increased 4MMP. The former strain also showed a significant increase in one grape juice, but was lower in SGM. These differences suggest that media composition modulates the effect of MUP1 in 4MMP release, and that this effect may be indirect.

Taken together, the results suggest that none of the nine known cysteine transporters seem to be key in C-4MMP uptake.
4.4 G-3MH is transported by OPT1 and cleaved by the γ-glutamyltranspeptidase CIS2

G-3MH has been shown to be converted to 3MH by yeast in an IRC7∗-dependent way in Chapter 3 (see Figures 3.3 and 3.4). Because IRC7-deleted strains produce 3MH abundantly from grape juice (Figures 3.1 and 3.2), it was suggested that G-3MH is not the major precursor in grape juice. This conclusion is consistent with other findings that G-3MH concentration in grape juice has no correlation with 3MH release (Pinu et al. (2012)) and with low conversion efficiencies measured for this precursors (Winter et al. (2011), Grant-Preece et al. (2010)). However, G-3MH has clearly been shown to be a precursor for 3MH by different authors, and can contribute to the final 3MH production in wine (Figure 3.4). For this reason genes involved in G-3MH uptake and its degradation into 3MH were investigated in this section (for more information see Sections 1.6.3 and 1.7.3).

4.4.1 OPT1 deletion blocked 3MH release from G-3MH in several strains, but only partially in BY4743

To confirm the role of the major glutathione transporter OPT1 in G-3MH transport, growth on L-glutathione (control) and G-3MH as sulfur sources were tested for different genetic background strains of yeast (BY4743 and F15) and their corresponding opt1Δ mutants. G-3MH had previously supported only slightly growth as a sulfur source compared to its utilization as a nitrogen source (Figure 4.1).

Figure 4.11 shows that deletion of OPT1 stopped F15 growth on L-glutathione, but only slowed down BY4743 growth. However, the opt1Δ mutants all showed reduced growth on G-3MH as a sulfur source. These results suggested that only OPT1 was taking up G-3MH in the two strains, but glutathione was also taken up by another transporter in BY4743.
Figure 4.11: *OPT1* deletion mutants showed reduced growth on glutathione and G-3MH as a sulfur source. The strains were grown in 150 µL SGM at 25°C for 3 d using glutathione and G-3MH as sulfur source (0.5 mM). For BY4743 strains SGM was supplemented with histidine [20 mg/L], leucine [30 mg/L] and uracil [20 mg/L]. Absorbance was measured every 15 min and plotted versus time. Average curves were plotted. *n* = 2.

The spiked growth curve of F15 in G-3MH is caused by the “clumping” phenotype showed by haploid F15 due to its active form of the *AMN1* gene, enhanced at low OD readings.

Subsequently, synthetic juice with added precursors was fermented using the same three strains (BY4743 and F15) and their corresponding *opt1*Δ mutants. The deletion of *OPT1* did not affect 4MMP yields, but in F15 and M4238 (not shown) gave no detectable 3MH, and significantly reduced its production in BY4743. These results exactly parallel the findings above for growth on L-glutathione as a sulfur source, and confirm that *OPT1* is the transporter for G-3MH in F15 and M4238. Surprisingly, BY4743 seems to have another transporter for G-3MH, as observed for glutathione uptake.
Figure 4.12: **OPT1** deletion block 3MH release in F15, but not in BY4743. Mutants with a deleted **OPT1** gene were transformed with the ho::P<sub>PGK</sub>::IRC<sub>F</sub> overexpression cassette and used to ferment SGM with added 50 µg/L of C-4MMP and 500 µg/L of G-3MH at 25°C. For BY4743 strains fermentations SGM was three-quarters-strength in sugars and supplemented with histidine [20 mg/L], leucine [30 mg/L] and uracil [20 mg/L]. Thiols were extracted by the PHMB method. The labels above the bars show the percentage of thiol conversion from the added precursor. n = 2; error bars = SE; asterisks above bars represent significant differences compared to gap1Δ strain (** p < 0.001, ANOVA).

### 4.4.2 **CIS2** deletion strongly reduced 3MH release from G-3MH

To get some knowledge on the pathway by which G-3MH is converted into 3MH, deletion mutants in genes that code for glutathione degradation enzymes were used to grow on G-3MH and also to ferment SGM with added thiol precursors.

Figure 4.13 shows that **DUG1** seems to be important for the use of glutathione as a sulfur source in BY4743, but **CIS2** is not required, as expected from the results of Baudouin-Cornu et al. (2012). Growth in G-3MH as a sulfur source by BY4743 was also reduced by **DUG1** deletion but only slightly affected in the **CIS2** deletion strain. These results suggest that G-3MH undergoes different breakdown pathways inside the cell when it is used as sulfur source compared to those when it is released as 3MH.

Figure 4.14 shows that **DUG1** deletion did not cause any effect in 3MH release from G-3MH. In contrast, **CIS2** deletion hugely decreased 3MH in BY4743. Additional, deletion strains used to study the degradation pathway of glutathione conjugates in yeast (schemed in Figure 1.1, Wunschmann et al. (2009)) were also tested and confirmed that **CIS2** is causing the main impact in 3MH release from G-3MH (Appendix 5).
Figure 4.13: DUG1 deletion mutants had no growth in G-3MH as sulfur source. The strains were grown in 150 µL SGM at 25°C for 3 d using glutathione and G-3MH as a sulfur source (0.5 mM). SGM was supplemented with histidine [20 mg/L], leucine [30 mg/L] and uracil [20 mg/L]. Absorbance was measured every 15 min and plotted versus time. Average curves were plotted. n = 2.

Figure 4.14: CIS2 deletion reduced 3MH release from its precursor G-3MH in BY4743. Mutants in glutathione degradation enzymes (CIS2 and DUG1) were transformed with the ho::P<sub>PGK</sub>-IRC7<sup>F</sup> overexpression cassette and used to ferment SGM with added 50 µg/L of C-4MMP and 500 µg/L of G-3MH at 25°C. SGM was three-quarters-strength in sugars and supplemented with histidine [20 mg/L], leucine [30 mg/L] and uracil [20 mg/L]. Thiols were extracted by the PHMB method. The labels above the bars show the percentage of thiol conversion from the added precursor. n = 2; error bars = SE; asterisks above bars represent significant differences compared to gap1<sup>Δ</sup> strain (** p < 0.001, ANOVA).
To see if CIS2 and OPT1 could improve 3MH release from G-3MH, both genes were overexpressed independently in the F15 IRC7ox strain by inserting a TEF2 promoter cassette just in front of the start codon of the genes (see Section 2.13.2). Figure 4.15 shows that 3MH yield was not improved by overexpressing these two genes, despite their deletion affecting its release from G-3MH.

To check if the overexpressed genes were functional, F15 IRC7ox CIS2ox and F15 IRC7ox OPT1ox were grown on glutathione and G-3MH as sulfur sources. Figure 4.16 shows that CIS2 overexpression allowed yeast to better use glutathione as a sulfur source, comparable to the control strain deleted for GSH1. This contrasts with results in Figure 4.13 and from Baudouin-Cornu et al. (2012) who showed that CIS2 is not required for L-glutathione degradation in the W303-1A strain. No improvement was observed for G-3MH usage by F15 IRC7ox CIS2ox, again suggesting that CIS2 is not involved in the use of this compound as a sulfur source. OPT1 overexpression gave reduced growth in L-glutathione and G-3MH, suggesting degradation, negative regulation or an imbalance in sulfur utilization caused by an excess of Opt1p.

It was concluded from the lack of an effect of overexpressing OPT1 and CIS2 that the limited 3MH yield from G-3MH cannot be improved by enhancing G-3MH transport or degradation. It is possible that low yield of thiols from G-3MH might result from production of unknown intermediates and/or secretion of G-3MH and its intermediates back to the media. Additionally, vacuolar transportation can also be a limiting step.

Figure 4.15: CIS2 and OPT1 overexpression do not improve 3MH release from G-3MH. F15 IRC7ox strains overexpressing CIS2 or OPT1 were used to ferment SGM with added 50 µg/L of C-4MMP and 500 µg/L of G-3MH at 25°C. Thiols were extracted by the PHMB method. The labels above the bars show the percentage of thiol conversion from the added precursor. n = 2; error bars = SE. No significant differences compared to F15 IRC7ox (p < 0.05, ANOVA).
Figure 4.16: *CIS2* overexpression in *F15 IRC7*ox improved growth on L-glutathione as a sulfur source, but not on G-3MH. The strains were grown in 150 µL SGM at 25°C for 3 d using glutathione and G-3MH as sulfur source (0.5 mM). Absorbance was measured every 15 min and plotted versus time. Average curves were plotted. *n* = 2.

### 4.4.3 *OPT1* deletion reduced 3MH release from grape juice in *IRC7*<sup>s</sup> yeast strains

Subileau et al. (2008a) showed that a *BY4743 opt1Δ* strain produced half the 3MH/3MHA of the wt when fermenting grape juice. Two hypotheses were proposed from this result: (1) half of the 3MH precursor entered the cell through *OPT1* or (2) glutathione could be an activator of 3MH from its precursor.

To confirm that the deletion of *OPT1* in a strain with a deleted *IRC7* allele produces low levels of 3MH, fermentations were carried out in two different grape juices using *BY4743 opt1Δ*. Table 4.5 shows that the *opt1Δ* mutation reduced 3MH in wine made by fermentation with *BY4743*.

<table>
<thead>
<tr>
<th>Juice†</th>
<th>Strain</th>
<th>3MH/3MHA [ng/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>75 % Blanw</td>
<td><em>BY4743</em></td>
<td>1326 ± 25</td>
</tr>
<tr>
<td></td>
<td><em>BY4743 opt1Δ</em></td>
<td>830 ± 55*</td>
</tr>
<tr>
<td>75% M1016</td>
<td><em>BY4743</em></td>
<td>3728 ± 11</td>
</tr>
<tr>
<td></td>
<td><em>BY4743 opt1Δ</em></td>
<td>1240 ± 55**</td>
</tr>
</tbody>
</table>

† Grape juices were three-quarters-strength by diluting with SGM with no added sugars. All juices were supplemented with histidine [20 mg/L], leucine [30 mg/L] and uracil [20mg/L]. Thiols were extracted by the PHMB method.

* Significant differences compared to *BY4743* strain (*p* < 0.05, **p** < 0.001, ANOVA).
The reduced release of 3MH in strains with the short \textit{IRC7} allele suggests that glutathione transport is important for the production of this thiol from precursors that are different to G-3MH, or that an \textit{IRC7}\textsuperscript{F}-independent pathway may dominate thiol production during grape juice fermentation.

4.4.4 \textit{OPT1} deletion does not affect 4MMP release in grape juice fermentation

Strains carrying a deleted \textit{OPT1} in an \textit{IRC7}\textsuperscript{F}ox background showed no effect on 4MMP release from grape juice compared to their respective wt (Table 4.6). This was shown for two different genetic backgrounds (BY4742 and M4238). Assuming that \textit{OPT1} is the only transporter for the glutathionylated precursor of this thiol, as it is for G-3MH, the result suggests that the G-4MMP found in juice (Fedrizzi et al. (2009), Roland et al. (2010c)) is not a major contributor to 4MMP. However, the existence of another transporter able to take up G-4MMP cannot be ruled out.

\begin{table}[h]
\centering
\begin{tabular}{lrr}
\hline
Strain & 4MMP [ng/L] & 3MH+3MH [ng/L] \\
\hline
BY4743 \textit{IRC7} ox & 74 \pm 1.3 & 2,104 \pm 13 \\
BY4743 \textit{IRC7} ox opt1\Delta & 63 \pm 0.6 & 1,114 \pm 37* \\
M4238 \textit{IRC7} ox & 121 \pm 6 & 2,468 \pm 26 \\
M4238 \textit{IRC7} ox opt1\Delta & 110 \pm 10 & 983 \pm 39* \\
\hline
\end{tabular}
\caption{\textit{OPT1} deletion does not affect 4MMP release}
\end{table}

Blanw juice was made three-quarters-strength by diluting with SGM with no added sugars. BY4743 fermentations were supplemented with histidine [20 mg/L], leucine [30 mg/L] and uracil [20mg/L] and M4238 with uracil [20mg/L]. Thiols were extracted by the PHMB method.

* Significant differences compared to BY4743 strain (*\textit{p} < 0.01, ANOVA).

4.4.5 Summary

The \textit{OPT1} transporter is absolutely necessary for the release of 3MH from G-3MH in some strains, such as F15. Assaying growth on G-3MH as a sulfur source confirmed the transporter (\textit{OPT1}) as the uptake gene for this precursor. However, in the BY4743 background, the opt1\Delta mutation still produced some 3MH, and moreover was still able to utilize glutathione as a sulfur source, suggesting the involvement of another transporter in this strain (different from \textit{OPT1}) that is able to transport both glutathione and G-3MH.
In addition, the deletion of the γ-glutamyltranspeptidase \textit{CIS2} significantly reduced 3MH release from G-3MH, suggesting that this protein has a role in the cleavage of the glutathionylated precursor into the 3MH-Cys-Gly intermediary and the subsequent 3MH production. Dug1p was not required for this process. The Irc7\textsuperscript{F}p lyase is then responsible for production of 3MH in this pathway.

The assay for growth on G-3MH as a sulfur source was used to identify potential genes for losses in thiol production. This assay produced different results from thiol quantification, with the \textit{DUG1} gene essential and \textit{CIS2} playing a minor role. The results were similar to those for growth on glutathione, consistent with the idea that some G-3MH is converted to glutathione in yeast cells and therefore lost to thiol production.
4.5 Discussion

In this chapter the role of cysteine permeases in C-4MMP transport during fermentative conditions was studied. The overexpression of the nine cysteine permeases did not increase 4MMP release from C-4MMP in synthetic media. However, in high nitrogen conditions that caused impaired 4MMP release, MUP1 and TAT1 overexpression significantly increased 4MMP from C-4MMP. In grape juice, only a MUP1 overexpressing strain was tested and it occasionally gave a significant increase in 4MMP. In addition after deleting all nine known cysteine permeases, it was concluded that none of them were absolutely needed for taking up C-4MMP in synthetic media.

G-3MH transport and degradation was also studied in this chapter. The glutathione transporter, OPT1 was shown to be essential for 3MH release from G-3MH in F15, but only partially affected 3MH release in BY4743. In addition, CIS2 and IRC7 are necessary for the cleavage of 3MH from G-3MH. The major conclusions of Chapter 4 will be discussed below.

4.5.1 Using thiol precursors and related compounds to screen for genes involved in its uptake and degradation

Identifying the transporters for C-4MMP and G-3MH was one of the major goals of this thesis, because this knowledge will help to modulate 4MMP and 3MH release at the precursor transport level. Initially, it was thought that growing yeast in C-4MMP or G-3MH as a sulfur or nitrogen source would be an easy and quick way to look for lack of growth in different permease deletion mutants.

This did not prove possible because C-4MMP did not support yeast growth at all in F15 and F15 IRC7 ox (Figure 4.1). This suggested that C-4MMP is not being converted into cysteine or other metabolizable sulfur compounds in yeast cells. In addition, it was shown that C-4MMP was not toxic to yeast cells at a concentration of 0.5 mM. It remains possible that C-4MMP is subjected to other types of modifications, maybe as has been shown to occur in plant cells (Brazier-Hicks et al. (2008), Dixon et al. (2010)). Secretion back to the medium is another possible way of loss that might be causing lack of growth in C-4MMP. One option is that C-4MMP could be detoxified from yeast cell by the PDR5 pleiotropic drug resistant ABC transporter (Balzi et al. (1994)) or other mechanism. For example, in yeast it has been shown that radiolabelled sulfur from SEC was rapidly taken up for a short period, but that 75% of the sulfur of this compound was then released back to the media as SEC and an unknown sulfur compound (Maw (1963)). However, it does not seem like C-4MMP is always subject to major
losses, because if IRC7 is overexpressed then 4MMP will be released to a high yield (34 – 67%) showing that at least an important part of C-4MMP remains inside the cell. Nevertheless, the C-4MMP concentration in a ferment is very low (0.23 µM) compared to the concentrations used as a sulfur (0.5 mM) or nitrogen source (15 mM), so it may be secreted when present in higher amounts.

Two permease deletions, GNP1 and MUP1, gave resistance to toxic cysteine-like compounds SEC and ethionine, respectively. However, a direct demonstration that Mup1p transports ethionine and that Gnp1p transports SEC are still required. Growth on these two compounds provides a simple and novel negative selection to obtain null mutants in these two genes, which can be useful for a range of genetic purposes. Both permeases have been shown to have a broad spectrum of substrates. Gnp1 is involved in the uptake of several aa: Leu, Ser, Thr, Cys, Met, Gln, Asn and a toxic analogue of glutamine: L-glutamic acid γ-monohydroxamate (Zhu et al. (1996), Regenberg et al. (1999)). On the other hand, Mup1 has been shown to transport methionine, oxidized analogues of methionine (methionine sulphoxide and ethionine sulphoxide) and cysteine (Isnard et al. (1996), Kosugi et al. (2001)). After further testing, Gnp1 and Mup1 were shown to not be involved in C-4MMP uptake, results that will be discussed in the following section. There must be important structural differences between SEC and ethionine compared to C-4MMP, which can be distinguished by different transporters; it is clear that, despite having a similar backbone, each aa has one or more specific transporters.

The story was different for G-3MH, because it allowed moderate growth of F15 and F15 IRC7 Fox as a nitrogen source, and slight growth as a sulfur source (Figure 4.1). The use of G-3MH as a sulfur source was preferred to screen deletion mutants, because this screen uses less of the precursor. This screen identified Opt1p as the main transporter for G-3MH (discussed in Section 4.5.3). The fact that IRC7 Fox gave no improvement in growth on G-3MH suggests that the intracellular pathway followed to use this precursor as a sulfur or nitrogen source is different to that of thiol formation. G-3MH might be used as a sulfur source by being converted into glutathione and then into cysteine, because the utilization of both G-3MH and glutathione was affected by both dug1∆ (Figure 4.14) and str2∆ mutants (not shown). The limited growth provided by this degradation pathway suggests that the rate of G-3MH conversion into glutathione is very low. On the contrary, 3-GMH provides better growth as a nitrogen source, in complete contrast to glutathione, which was not able to be used a nitrogen source in the same experiment. This has also been observed by Baudouin-Cornu et al. (2012), who suggested that glutathione is rapidly degraded via the DUG pathway into Cys to be used as a sulfur source, but not at a sufficient rate to provide nitrogen (Glu) for growth. The use of G-3MH as a nitrogen source may use degradation via Cis2p to provide enough Glu to allow yeast growth, or may use an unknown pathway.
Because overexpression of IRC7 did not affect growth on G-3MH as a nitrogen source (Figure 4.1), cysteine breakdown to $\text{NH}_4^+$ by this enzyme does not seem to be important for this pathway. Indeed, the two cysteine precursors tested were unable to be used as nitrogen sources (Figure 4.1), suggesting that part of the glutathione moiety is central for this pathway.

4.5.2 None of the known cysteine transporters is taking up C-4MMP

Overexpression of all nine known cysteine permease did not have any improvement on 4MMP from C-4MMP on synthetic media. On the contrary, some overexpressing strains showed reduced 4MMP and 3MH (Figure 4.5). The MUP1 overexpression strain was independently tested twice and some experimental differences were observed, with a significant decrease in 4MMP concentration compared to the wt only in one of them (Table 4.3 and Figure 4.5). When the MUP1 overexpression strain was used to ferment different grape juices, the response was again variable, with a significant increase in 4MMP release in some juices (Table 4.3). These different responses shown by the MUP1 overexpression strain suggest that important interactions between juice composition and cellular component might be acting to modulate 4MMP release. Similar interactions involving both nitrogen and sulfur compounds and pathways were noted by Harsch et al. (2013b). Small thiol decreases in SGM were also observed in other permease overexpression strains, suggesting that SGM composition might be regulating permease degradation, stability or other factors that affect thiol release in a negative way when using permease overexpression strains.

The observation that some cysteine permease overexpression (Figures 4.5 and 4.6) and deletion lines changed 3MH yields from G-3MH was not studied further. One possible explanation is that somehow the absence or excess of an aa permease in the membrane, or some down-stream effect of these modifications, may trigger modified intracellular levels of its substrate that might modulate other permeases that affect G-3MH uptake. However, in some cases, the differences were attributed to experimental variation more than a real effect, as mentioned in Section 4.3.3 for strains in Figure 4.10.

High nitrogen additions affected 4MMP release from C-4MMP, but had no effect on 3MH coming from G-3MH. Since both did not change, this result suggests that Irc7p activity and stability were not affected. One possibility is that C-4MMP transport is down-regulated by high nitrogen additions, leading to the idea that nitrogen catabolism repression has an important role in C-4MMP uptake. There are several permeases shown to be regulated by NCR, different from GAP1, which could make putative candidates for C-4MMP transport: CAN1 (Ter Schure et al. (2000)), DAL4 (Godard et al. (2007)), DAL5 (Rai et al. (1987)), DIP5 (Deed et al. (2011)),...
DUR3 (Deed et al. (2011)), PUT4 (Jauniaux et al. (1987)), PTR2 (Deed et al. (2011)) and UGA4 (Godard et al. (2007)). Another possibility is that NCR modifies C-4MMP to be metabolized differently, producing less 4MMP.

Other authors have shown that NCR affects thiol release. Subileau et al. (2008b) published that 3MH increased when urea is used, instead of DAP, as a sole nitrogen source on synthetic medium. On grape juice, DAP additions decrease 3MH release. In addition, Thibon et al. (2008a) showed that 4MMP release was affected by IRC7 repression through URE2 in VL3 (full-length allele). On the contrary, Deed et al. (2011) found no difference in IRC7 expression in M2 (truncated allele) strain via NCR, suggesting strain differences or more specific, regulatory differences between IRC7 alleles.

Individual deletion of all nine cysteine permeases did not give a reduction of 4MMP from C-4MMP in synthetic media. BAP2 deletion in BY4743 was the only strain that showed a significant reduction of 4MMP (Figure 4.8), but also had a reduced growth and fermentation rate in SGM. Multiple cysteine permease deletion that included BAP2 deletion, in a genetic background different from BY4743, did not showed any effect (Figure 4.10).

Even when all the previously known cysteine transporters have been deleted, yeast can still grow on L-cysteine as a nitrogen source and moreover 4MMP was still released from C-4MMP (Figures 4.9 and 4.10). AGP1 deletion was the only mutation that gave delayed yeast growth on L-cysteine as a nitrogen source, hypothesized to be caused by increased cysteine uptake that provokes more toxicity. AGP1 and GAP1 deletion have been shown to induce expression of AGP2 and AGP3, which are low-affinity and low-specific permeases (Schreve et al. (2004)) that could be participating in L-cysteine and in C-4MMP transport. This behavior could apply for other permeases with low expression levels that are induced by the absence of one or more permeases.

The results presented here have shown the complexity of the genes involved in amino acid uptake in yeast. There is not a one-to-one relationship between genes and transport of individual amino acids. Rather there is a large family of transporters with a high degree of overlap in their specificities for different substrates (see also Appendix A4.2 and Regenberg et al. (1999)). In addition, different genes are used for high and low affinity transport of different amino acids, there is relaxation in specificity at higher amino acid concentrations for some of the transporters, and their regulation is complex and overlapping. All of these features make it very difficult to unravel the process for any one amino acid, and unfortunately it seems that the situation is particularly complex for cysteine.
4.5.3 OPT1 and CIS2 are necessary for 3MH release from G-3MH

The main glutathione transporter OPT1 is also the major G-3MH transporter in yeast cells. This was first confirmed by the absence of growth in the F15 IRC7Fox opt1Δ strain when using G-3MH as a sulfur source, and later confirmed by the undetectable levels of 3MH when fermenting the F15 IRC7Fox opt1Δ strain using SGM with added G-3MH. The deletion of OPT1 in BY4743 IRC7Fox reduced growth when using G-3MH as a sulfur source, but it seems to grow slightly more than F15 IRC7Fox strain; however, this small difference could be an experimental bias and needs to be confirmed. On the other hand, 3MH is only partially reduced from G-3MH. This result suggested that OPT1 is important for G-3MH in BY4743, but also an additional transporter for this precursor (Figure 4.12).

The γ-glutamyltranspeptidase CIS2 was shown to be necessary for 3MH release from G-3MH. The BY4743 cis2Δ IRC7Fox strain strongly reduced 3MH (Figure 4.13). Nevertheless, low levels of 3MH were still detected suggesting a minor role of another degradation pathway. According to Wunschmann et al. (2009) glutathione conjugates can be degraded in two different ways. Briefly, one requires the removal of the glutamyl residue through Cis2p, followed by the removal of glycine residue by unknown enzyme(s). The other pathway requires Cpcp (YBR139w) or Cpy1p (YMR297w) to remove the glycine residue and then Cis2p to remove the glutamyl residue. Both pathways lead to a cysteine-conjugate (see Figure 1.1). The Wunschmann mutants (BY4741 cis2Δ, BY4741 cis2Δ cpcΔ and BY4741 cis2Δ cpcΔ cpy1Δ, all overexpressing IRC7Fox) showed that deletion of CPC and CPY1, in a BY4741 cis2Δ strain gave no significant extra reduction of 3MH release from G-3MH (Figure A13), suggesting that these two enzymes are not participating in the degradation. Moreover, both cis2Δ cpcΔ and cis2Δ cpcΔ cpy1Δ showed reduced 4MMP release from C-4MMP compared to the BY4741 IRC7Fox strain. This phenotype was observed in two independent IRC7F ox transformants for each strain, suggesting a real effect on 4MMP. The Wunschmann strains are in a BY4741 background (met17Δ), which is affecting 4MMP via an unknown mechanism. Nevertheless, the main reduction in 3MH is given by CIS2 deletion in BY4741 (which has normal levels of 4MMP), and is very similar to the reduction shown by BY4743 cis2Δ. The residual 3MH formation in the cis2Δ mutants (Figures 4.13 and A11) might come from a different and unknown pathway.

Despite the critical roles of CIS2 and OPT1 in 3MH release from G-3MH, the overexpression of either gene did not cause an increased release of 3MH in synthetic media. The CIS2 overexpression result suggests that G-3MH degradation is not limiting 3MH release, supported by the fact that the overexpressed Cis2p was functional and able to improve glutathione usage. However, a transport limitation cannot be discounted, because the OPT1 overexpression strain showed reduced growth on L-glutathione and G-3MH suggesting a down-regulation of uptake or some other downstream reaction in this strain. The role of OPT1 and CIS2 in 3MH release in this
thesis was observed in yeast background overexpressing $IRC7^F$ and they need to be confirmed in a yeast strain with a natural allele for $IRC7^F$, like X5.

4.5.4 Glutathione transport is necessary for 3MH release from sources different from G-3MH

BY4743 needs $IRC7^F$ to release 3MH from G-3MH (Figure 3.3). In addition, BY4743 has more than one L-glutathione and G-3MH transporter, but $OPT1$ is very important (Figures 4.11 and 4.12). However, BY4743 opt1Δ produces reduced levels of 3MH from grape juices compared to BY4743 (Table 4.5 and Subileau et al. (2008a)). This evidence strongly suggests that $OPT1$, and glutathione uptake, has an effect on 3MH coming from a source different than G-3MH. One explanation could be that a reduced glutathione uptake leads to elevated glutathione synthesis, by consuming cysteine and other sulfur sources that eventually interfere with other ways of producing 3MH. For example, less H$_2$S to react with (E)-2-hexenal may cause reduced 3MH (Schneider et al. (2006), Harsch et al. (2013a)).
4.6 Concluding summary and remarks

Chapter 4 assessed the involvement of different transporters in the uptake of C-4MMP and G-3MH and addressed the importance of the glutathione degradation pathways in G-3MH metabolization.

The main conclusions from Chapter 4 are:

- None of the nine known cysteine transporters are needed for 4MMP release from C-4MMP. The results also suggest the existence of other, as yet unidentified, low-affinity cysteine transporter(s) in yeast that operate in fermentation conditions.
- OPT1 is the only G-3MH transporter in F15 and M4238, but there is an additional transport system in BY4743.
- CIS2 is the main enzyme involved in G-3MH breakdown to give thiols.
- Impaired glutathione transport affects 3MH release from sources different than G-3MH.

These results have established OPT1 and CIS2 as genes that are required for the conversion of G-3MH to thiols. In combination with the identification of IRC7 as being essential for the conversion of both G-3MH and C-4MMP in the previous chapter, the findings may allow the use of yeast strains mutant in these genes to distinguish the contributions made by these precursors to thiols during grape juice fermentation.

In addition, the results collectively suggest that there may be two “loss pathways” for G-3MH that prevent it being fully converted into 3MH and contribute to its low conversion rate. One pathway may involve a ‘reverse glutathione-S-transferase’ reaction that cleaves the hexenal moiety from G-3MH to give glutathione that is then broken down via the DUG pathway to cysteine, which can be used as a sulfur source via the transsulfuration pathway (including Str2p). The second involves degradation of G-3MH via an unidentified but IRC7-independent pathway that allows it to be used as a nitrogen source.
5 Concluding discussion

5.1 Main findings of this thesis

4MMP, 3MH and 3MHA are important volatile thiols released by *S. cerevisiae* during wine fermentation. So far three types of thiol precursors have been found in grape must: cysteinylation-conjugates (C-4MMP and C-3MH), glutathionylation-conjugates (G-4MMP and G-3MH) and C6 compounds ((E)-2-hexen-1-ol and (E)-2-hexenal). All known precursors require the action of yeast to produce their respective thiol when they are added to synthetic media or must. However, a major problem is the low conversion yield (less than 10%) from precursor to thiols. In addition, the contribution that each precursor makes to total thiol is unclear.

The major findings of this thesis are that:

- *IRC7* is not needed for 4MMP and 3MH release from C-4MMP and G-3MH, respectively.
- *IRC7F* is sufficient and necessary for 4MMP and 3MH release from C-4MMP and C-3MH or G-3MH, respectively.
- *OPT1* and *CIS2* are also necessary for 3MH release from G-3MH.
- Known cysteine transporters are not needed for C-4MMP uptake.
- *Irc7* has cysteine sulfhydrylase activity that allows yeast to grow on L-cysteine as a nitrogen source.
5.2 Remarks on the study of thiols from cysteinylated and glutathionylated precursors

5.2.1 Introduction

Thiol extraction and quantification was performed extensively during this thesis research. Studying thiols produced by yeast during wine fermentation is a long and time consuming procedure. Grape juice or synthetic media is fermented at a specific temperature, for several days, then the wine is collected and thiols extracted and quantified. Thiol extraction was improved recently by the ETP method (Herbst-Johnstone et al. (2013)), which only takes ~ 5 h compared to the ~8 h spent with the PHMB method. Both methods were compared during this thesis and gave similar results. Comparison of strains, treatments and conditions is difficult due to the time, material and equipment involved. Alternative methods to measure thiol release would allow a more high-throughput approach to thiols analysis.

5.2.2 Fermentation and thiol analysis

Defining fermentation conditions is very important and will depend on the objectives of the research. In this case, for the study of thiol release by yeast, a controlled and invariable medium was preferred. During the initial stages of this thesis, grape juice was fermented to analyze thiols. Grape juice is variable, and contains different amounts of the known thiol precursors. It also might contain some unknown precursors or components that affect thiol release. We and others have observed huge variability from different SB juices origins and also important differences from one vintage to the next one (Benkwitz et al. (2012), Jouanneau et al. (2012), Pinu et al. (2012)). It was not possible to perform all the experiments required for this thesis using exclusively one batch of grape must. For these reasons, a synthetic defined medium (SGM) was used with known amounts of added precursors to give a controlled media to work with. It allowed more flexibility, for example making three-quarters sugar strength SGM without altering the rest of the components, in order to ferment laboratory yeast strains more efficiently (Harsch et al. (2010)). SGM used in this thesis resembles Marlborough SB must composition (Harsch et al. (2010)).

The amount of precursors added to the fermentation media, should resemble their natural occurrence. It was observed that thiol analysis gave wrong results when adding precursors in excess (Howell et al. (2005), Thibon et al. (2008a)). Researching the available data at the beginning of this thesis, it was found that C-4MMP occurs
between 2.3 and 6.3 µg/L, C-3MH from 4.7 to 78 µg/L and G-3MH from 1.2 to 640 µg/L. For this thesis, 50 µg/L of C-4MMP or C-3MH and 500 µg/L of G-3MH were used. Once the media was established all the experiments were carried out the same way and at the same temperature. This approach made it possible to identify yeast genes required for the conversion of individual precursors into thiols.

### 5.2.3 Yeast strains

One of the major conclusions from this work is that Irc7Fp is necessary for thiol release from C-3MH, C-4MMP and G-3MH. For studying thiol release from these precursors, all the strains used in this thesis were transformed with the overexpression cassette \( \text{ho::PGK-IRC7} \), which recombines into the HO locus. This provided high Irc7Fp levels that gave 4MMP yields from C-4MMP of about ~50% or ~10% for F15 and BY4743, respectively. On the other hand, the conversion from G-3MH was around ~8% or ~ 6% for F15 and BY4743, respectively. Variation between the wine strain and the laboratory strain in 4MMP release may be useful in future to study genetic differences that result in the lower release from the wine strain. This knowledge that \( IRC7 \) is required for thiol release from cysteinylated and glutathionylated precursors will allow the study of thiol precursors that are different from these, especially those involved in 3MH production, by using \( IRC7 \) strains.

### 5.2.4 Future directions

Having a fast and accurate way to detect thiol release using small volume fermentations that allow screening of multiple strains, would be one important improvement. In particular, it would be interesting to try methods for thiol detection, more sensitive than DTNB (Peng et al. (2012)), that utilize rapid fluorescent or colorimetric assays. By using \( IRC7 \) strains in small scale ferments using SGM with added precursors (avoiding other thiols like cysteine or glutathione), thiol levels in a range from 0.02 to 0.1 µM can be obtained. Finding a simple and rapid assay that would work at this concentration would be enormously helpful in the study of thiols.

The mutant strains and overexpression lines developed in this thesis should be useful for determining the source of thiols made from grape juice. For example, future experiments to confirm the low conversion yield from known thiol precursors could use F15 and F15 \( IRC7 \) to ferment grape must with additions of C-3MH, C-4MMP, G-3MH and (E)-2-hexenal precursors that are differentially labeled. This experimental design will allow accurate predictions of how much of these labeled precursors are converted into thiols and comparison with the unlabeled thiols produced from grape juice. From all the data available (Subileau et al. (2008a), Roland et al. (2010a)),

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these precursors should account for only a small proportion of the total 3MH thiol. After confirming this, an experiment that allows detection of any unknown 3MH precursor could be performed. For example, I have shown that glutathione uptake is important for thiol release from sources different that C-3MH and G-3MH. One potentially interesting experiment could be to remove all sulfur compounds from grape must (PHMB extraction or other) and check if thiols are produced using F15 (which does not convert either the cysteinylated or glutathionylated precursors). If not, glutathione and other sulfur compounds can be added one at a time and fermentation with F15 used to see if thiols are recovered. This experiment may clarify whether other sulfur compounds found in grape juice are precursors. Juice fractionation and fermentation might be another way to find novel 3MH precursors. Each fraction and groups of mixed fractions should be fermented with F15 and analyzed for 3MH. Such experiments are currently underway in this laboratory using the yeast strains developed in this thesis to distinguish production from different precursors, and to try to identify novel precursors.
5.3 Remarks on the study of low yields and losses

5.3.1 Introduction

Transport of precursors was measured indirectly during this thesis. By quantifying thiols after fermenting SGM with added precursor it was concluded that the precursor was taken up by yeast. This method was only informative if thiols conversion took place, but did not show how much of the precursor was transported. Both precursors, C-4MMP and G-3MH, produced thiols after fermenting with an IRC7fox strain. Amounts of precursors left in the medium were also not quantified in this thesis.

Another approach taken to study transport and losses indirectly was by growing yeast on the precursors as sole nitrogen and sulfur sources. For these experiments, very high concentrations of precursors were added to the media (0.5 mM or 15 mM), compared to the amounts used during fermentation (0.23 µM or 1.24 µM). For unknown reasons, neither C-4MMP nor C-3MH were able to be used as sulfur or nitrogen sources by yeast under these conditions. However G-3MH did support growth, allowing use of this method for the study of mutants with deletions of candidate genes.

5.3.2 Thiol precursor transport

My results have shown that at least half of C-4MMP is taken up by some yeast strains, because ~50% was obtained yield when overexpressing IRC7 in F15. Based on the data presented in Chapter 3, it seems clear that C-4MMP transport is not limiting 4MMP yields, but that IRC7 expression is the main restriction. Modification of the fermenting media (SGM) allowed me to show that C-4MMP transport could be significantly reduced by high nitrogen. The used of a strain carrying multiple deletions of known cysteine transporters was very useful to conclude that none of them was required for C-4MMP uptake (Chapter 4). Growth experiments on L-cysteine as a nitrogen source using the same multiple deletion mutants clearly showed that cysteine uptake was occurring under fermentative conditions. Thus other transporters must be involved in the uptake of both cysteine and cysteinylated thiol precursors.

Results in Chapter 4 have shown that Opt1p is the membrane transporter for G-3MH in F15, but a second gene may be involved in BY4743 strain. IRC7ox greatly increased thiol release from G-3MH but not to the same high yields as it did for C-4MMP. The lower final yields could result because of the several transportation steps and enzymatic degradations involved in 3MH release from G-3MH. Results from my research were not conclusive as
whether or not G-3MH transport from the media is limiting 3MH release. An OPT1 overexpression strain did not show any 3MH increase, but the strain had impaired glutathione transport, as shown by its growth defect on L-glutathione as nitrogen source. This is another example where a growth experiment provided complementary data that was associated with thiol release information to provide a better understanding of yeast metabolism. Overexpression of CIS2 did not have any positive effect on 3MH release from G-3MH, suggesting that vacuolar transportation might be limiting 3MH release from G3MH. Assuming that G-3MH is converted into C-3MH in the vacuole it may need another transporter to return to the cytoplasm and allow IrC7p cleavage.

5.3.3 Precursor losses

A possible source of precursor losses is that yeast is utilizing the precursors as sulfur sources. Sulfur assimilation was studied by growing yeast on C-4MMP, C-3MH or G-3MH as sole sulfur sources. Only 3-MH slightly supported yeast growth. The results with dug1Δ and str2Δ deletion strains suggested that this yeast growth was occurring by the precursor being converted back to glutathione and degraded to cysteine, which was then used as a sulfur source. Toxicity was ruled out at least for C-4MMP. In addition, the precursors were tested as nitrogen sources and again only G-3MH supported growth. The fate of the sulfur after yeast utilization as nitrogen source is unknown. Other ways of losses need to be studied, such as precursor secretion back to the media and modification of the precursor or the thiol.

5.3.4 Future directions

Despite that the fact that C-4MMP transport seems not to be limiting 4MMP, knowing its transporter would allow alternative approaches to modulating thiol release. Results in this thesis suggest that the candidate genes have been reduced to permeases that are controlled by NCR. A more detailed studied on 4MMP release from C-4MMP could be performed to narrow the putative candidates. For example, 4MMP release could be measured under sulfur limitation or using sulfur compound additions (methionine, SEC, glutathione, homocysteine), as well as using different nitrogen sources. Then, deletion mutants in each putative permease can be transformed with IRC7pox cassette and thiols quantified after fermentation.

The gene for transport of G-3MH in yeast cell has been identified, but its transportation within the cell should be resolved. Deletion mutants in vacuolar transporters for the import of G-3MH and the export of C-3MH back to the cytoplasm can be transformed with IRC7pox cassette and thiols quantified after fermentation. Good candidates
are YCF1 and BPT1, which transports glutathione-conjugates into the vacuole (Li et al. (1996), Klein et al. (2002)). Other transporters from the AVT family (Russnak et al. (2001)) and the PQ-loop family (Jézégou et al. (2012)) are also possible candidates. Similarly, G-3MH degradation needs to be resolved further, since only CIS2 was identified as a critical step in this research. The used of a cis2Δ strain will help to look for other enzymes involved in the glutamyl residue removal that explains the residual 3MH formation from G-3MH. Mutants in different peptidases can be used to unravel the cleavage of the glycine residue to produce C-3MH.

Information on precursor conversion into other compounds would be useful information to understand losses and will help improve thiol release from cystenylated and glutathionylated precursors. This issue could be addressed by using S34 labelled precursor and analyzing the metabolite profile from the cells and wine after fermentation. F15 could be used to avoid thiol formation, so the results will reflect the fate of the sulfur from precursors after fermentation. In addition, fermentation with F15 overexpressing IRC7 might help identify any modification of thiols that cause them to be retained in the cell (see Chapter 4.5.1)

Understanding how yeast use G-3MH as a nitrogen source will be an interesting experiment, because glutathione cannot be used. To address this question, deletion mutants in putative degradation genes and transporter genes can be used to define if it is happening in the vacuole and if the pathway goes through utilization of the glutamyl residue cleaved by Cis2p. The slight growth of yeast on G-3MH as a sulfur source would be useful to identify an enzyme capable to performed the reverse reaction of a glutathione transferase for the conversion of a glutathione-conjugate into glutathione that can be then cleave by the DUG pathway.
5.4 Remarks on the biological role of \textit{IRC7F}

5.4.1 Introduction

The study of the biological function of \textit{IRC7F} came after the work on understanding its role in wine making. Results from Chapter 3 demonstrated that Irc7Fp acts as a $\beta$-lyase on C-4MMP, but not on cystathionine, and that \textit{IRC7F} is both necessary and sufficient to allow yeast to growth on L-cysteine. These data strongly suggested that cysteine is its biological substrate.

5.4.2 Irc7Fp biochemical characterization

Two enzymatic assays were used for assessing the $\beta$-lyase activity of Irc7Fp: one for measuring production of free thiols (thiol assay) and the other for measuring pyruvate (pyruvate assay) that is released after cleavage of a cysteine-conjugate. The thiol and the pyruvate enzymatic assays were used previously in the lab and performed well in detecting substrates coming from the cleavage of Irc7Fp on C-4MMP, C-3MH and SEC. However, cysteine was not suitable for the thiol assay and the pyruvate assay gave unusual kinetics. Added copper sulfate mitigated these unusual kinetics but itself disrupted the assay (Appendix 3). I next tested the methylene blue formation method to assess H$_2$S production in this assay (not shown); it showed H$_2$S release from L-cysteine after cleavage with Irc7Fp, but is a destructive assay that only measures final points (Yoshida et al. (2010)). To follow the kinetics of the reaction the lead acetate assay gave good results and was used to determine biochemical parameter of Irc7Fp on L-cysteine.

5.4.3 Growth experiments in different sulfur compounds

Biochemical characterization was complemented with \textit{in vivo} experiments that mainly involved the use of F15 and F15 \textit{IRC}7F ox strains grown on different sulfur compounds. The main discovery was that possession or overexpression of \textit{IRC7F} allowed yeast to grow on cysteine and gave increased sensitivity to SEC. Using growth on L-cysteine was an important tool to screen different mutants on enzymes that participate in cysteine conversion into methionine or glutathione. This simple method allowed me to confirm that \textit{in vivo} Irc7Fp was acting on L-cysteine. Indicator plates also confirmed H$_2$S production in vivo from strains overexpressing \textit{IRC7F}.
5.4.4 Using other techniques to understand Irc7^Fp role

Other methods were used to understand Irc7^Fp role on yeast metabolism. GFP fusion to \textit{IRC7^F} showed that the protein is cytoplasmic. Growth on L-cysteine showed that Irc7^Fp was functional after the fusion. Additionally, crystallization trials were successful, but scaling up the conditions did not give any result.

5.4.5 Future directions

A major focus of work should be to examine the activity of the truncated allele of \textit{IRC7}. To understand its function will give an important complement to my work. If the truncated allele has a different biological role, it may be an interesting example of gene functional diversion of an allele variant. Regarding \textit{IRC7^F} I would suggest some expression analysis using different media to understand how \textit{IRC7^F} transcript is regulated. This work will help to understand the impact that \textit{IRC7^F} can have on H$_2$S production and the sulfur metabolic pathway. Also, Irc7^Fp might play a key role in modulating the intracellular cysteine pool under certain conditions, and thereby could affect intracellular redox control. Experiments to test the effect of Irc7^Fp on H$_2$O$_2$ or other ROS would provide useful information, for example via growth analyses of \textit{IRC7^F}ox strain subjected to stress conditions. It would also be interesting to find out how Irc7^Fp is helping yeast to overcome cysteine toxicity, respiration measurements could give a clue according to Bhuvaneswaran et al. (1964).

Resuming crystallization attempts to reveal its structure might also be a good thing to pursue, because possession of a cysteine desulphhydrase activity is rare in \textit{S. cerevisiae}. Unraveling the structure of Irc7Fp will be advantageous to understand its function further and in particular to understand the effect of the truncated protein over the full-length protein on heterozygous strains. A good understanding of its structure might also allow site direct mutagenesis to improve its affinity for C-3MH, which may be limiting its activity on this substrate.
5.5 Closing thoughts

The work in this thesis has contributed to our understanding of IRC7 role in thiol release during wine making, by clarifying that the activity of this gene is completely necessary for the thiol production from C-4MMP, C-3MH and G-3MH. The research has also provided evidence for the involvement of other yeast genes in thiol release, like OPT1 and CIS2, and provided evidence of some possible pathways of losses for G-3MH. Outcomes of this work include the suggestion of some new ideas to improve thiol release from cysteinylated and glutationylated precursors, and the provision of new impetus to identify additional pathways of thiol formation. One of the most important findings was the identification of a direct role of Irc7p on cysteine metabolism; further work to understand the cellular role of IRC7 might have far-reaching impacts on our understanding of the sulfur metabolism of yeast.
Appendices

Appendix 1  IRC7 alleles

A1.1 Comparing three different IRC7 alleles from S. cerevisiae

Previous work from this laboratory has shown that within S. cerevisiae there are at least three different IRC7 alleles: IRC7^S, IRC7^F and IRC7^P. Table A1 classified different S. cerevisiae strains according to its IRC7 allele. It is interesting to notice that some strains are heterozygous for IRC7 and that good thiol producer strains (e.g. X5, VL3, VIN13) all have an IRC7^F allele.

<table>
<thead>
<tr>
<th>IRC7 allele</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRC7^S</td>
<td>S288c, AWRI1631, AWRI796, BY4741, BY4742, CBS7960, CEN.PK113-7D, Collection Cepage Chardonnay*, CLIB215, CLIB324, CLIB382, F15*, FL100, LalvinBA11*, LalvinD254*, LalvinSVG*, Merit*, M22, RM11*, Sigma1278b, Siha7, T73, VL2*, W303</td>
</tr>
<tr>
<td>IRC7^S/IRC7^F</td>
<td>Cepage Sauvignon**, EC1118*, VIN13**, VL1**</td>
</tr>
<tr>
<td>IRC7^F</td>
<td>EC9-8, FostersO, Kyokai7, LalvinQA23, PW5, T7, UC5, VL3*, X5**, YJM269, YJM789, YPS163, ZTW1</td>
</tr>
<tr>
<td>IRC7^P</td>
<td>YJM450*</td>
</tr>
</tbody>
</table>

IRC7 sequences were obtained from SGD database for most of strains, except for strains marked with * which were tested by PCR or sequenced by Roncoroni et al. (2011) and strains marked by ** which were subjected to PCR during this thesis.

In this thesis, strains carrying the three alleles of IRC7 have been used. Figure A1 shows a comparison of the aa sequences coded by IRC7 alleles from F15, X5 and YJM450 (sequenced by David Hooks and Miguel Roncoroni). Irc7^Pp from YJM450 shows 34 substitutions compared to X5 protein. Irc7^Sp from F15 is missing 60 aa from the carboxyl-terminal end and has one substitution compared to X5 (P146A).
Figure A1: *IRC7* protein variants in *S. cerevisiae*. Clustal alignment showing Irc7p amino acid residues differences between strains used in this thesis. The red box shows the PS00868 PROSITE consensus pattern for PLP-binding site, in which K is the pyridoxal-P attachment site.
A1.2 IRC7\textsuperscript{F} shows high homology to \textit{metC} from \textit{Y. pestis}

The IRC7 gene has been proposed to be acquired from \textit{Yersinia pestis} (Hall et al. (2005)), a facultative anaerobic bacterium that causes plague in humans and other animals. The \textit{metC} gene product from \textit{Y. pestis}, annotated as a cystathionine beta-lyase (CBL), is the gene most similar to Irc7p\textsuperscript{F} of \textit{S. cerevisiae}. The bacterial sequence looks like a full-length gene, coding a 393-aa protein, compared to the 400-aa \textit{S. cerevisiae} Irc7\textsuperscript{F}p protein, with 62% aa identity between them (Figure A2).

| IRC7S  | 1 | MIRTEL6SFCTITQLSIVORPDEGSQGFMVVPVTLYKSTIIILASDLRQKGRF- | YTA |
| IRCTF  | 1 | MIRTEL6SFCTITQLSIVORPDEGSQGFMVVPVTLYKSTIIILASDLRQKGRF- | YTA |
| \textit{metC} | 1 | -MNKADEPSDGPHVEQKGRFEQTVTPYKSTIIILASDLRQKGRF- | YTA |
| IRC7S  | 60 | CISPPTDLENANWTHLTQAGTVL5ALGSIGLALLAISKGDHILMTDSVYPTRNLCC | |
| IRCTF  | 60 | CISPPTDLENANWTHLTQAGTVL5ALGSIGLALLAISKGDHILMTDSVYPTRNLCC | |
| \textit{metC} | 60 | CISPPTDLENANWTHLTQAGTVL5ALGSIGLALLAISKGDHILMTDSVYPTRNLCC | |
| IRC7S  | 120 | CLAFECVTDYDPICKDRKLTVKVPETTVIPLRDSCTWQVDIALSVAFLKICL | |
| IRCTF  | 120 | CLAFECVTDYDPICKDRKLTVKVPETTVIPLRDSCTWQVDIALSVAFLKICL | |
| \textit{metC} | 120 | CLAFECVTDYDPICKDRKLTVKVPETTVIPLRDSCTWQVDIALSVAFLKICL | |
| IRC7S  | 180 | TIIDNTWAPPLPPDHRACDISVEACTYKLGHSDDLIGLASHANRCPWPLRRSTYDMA | |
| IRCTF  | 180 | TIIDNTWAPPLPPDHRACDISVEACTYKLGHSDDLIGLASHANRCPWPLRRSTYDMA | |
| \textit{metC} | 180 | TIIDNTWAPPLPPDHRACDISVEACTYKLGHSDDLIGLASHANRCPWPLRRSTYDMA | |
| IRC7S  | 240 | MLPCEDCQLAGHRLTTLIKSVERKLDLLAALWGRNDEVSVILLHPEDCPGEHYW | |
| IRCTF  | 240 | MLPCEDCQLAGHRLTTLIKSVERKLDLLAALWGRNDEVSVILLHPEDCPGEHYW | |
| \textit{metC} | 240 | MLPCEDCQLAGHRLTTLIKSVERKLDLLAALWGRNDEVSVILLHPEDCPGEHYW | |
| IRC7S  | 300 | RDYKSGSFIPvLNLGFTRAGLSINVMEIVLQIGF8WGG | |
| IRCTF  | 300 | RDYKSGSFIPvLNLGFTRAGLSINVMEIVLQIGF8WGG | |
| \textit{metC} | 300 | RDYKSGSFIPvLNLGFTRAGLSINVMEIVLQIGF8WGG | |
| IRC7S  | 360 | PTKCEPSTRCLEKGLCDRKLIEVSLPL | |
| IRCTF  | 360 | PTKCEPSTRCLEKGLCDRKLIEVSLPL | |
| \textit{metC} | 360 | PTKCEPSTRCLEKGLCDRKLIEVSLPL | |

Figure A2: Irc7 protein variants in \textit{S. cerevisiae} compared to the \textit{metC} gene from \textit{Y. pestis}. Clustal alignment showing Irc7p amino acid residues differences between \textit{S. cerevisiae} strains and \textit{Y. pestis}. The red box shows the PS00868 PROSITE consensus pattern for PLP-binding site.
A1.3 Confirming the identity of the purified Irc7\(^F\)p

Irc7\(^F\)p was purified from *E. coli* and used in several in vitro enzymatic assays. Its identity was confirmed by protein sequencing and alignment of the resulting peptide to a protein database. The best match was to FostersO Irc7\(^F\)p (Figure A4).

![Figure A3: The purified Irc7\(^F\)p is homologous to other Irc7-full length protein in database. In red are sequenced peptides from the purified Irc7\(^F\)p with homology to Irc7p from *S. cerevisiae* FostersO (gi|323309282).]
Appendix 2  Effect of additions to SGM on the production of thiols

A2.1 Using 10x auxotrophy supplementation improved fermentation but not 3MH release in BY4743

In previous studies performed in this laboratory, Harsch et al (2010) showed that three-quarters strength juice with 10x auxotrophies supplementation improved laboratory strains fermentation capacity. These results were confirmed as shown in Figure A4 (left side graph). BY4742 was faster to ferment the 10x supplemented juice (green squares) and failed to finish the fermentation of full-strength juice (black squares). F15 (black circles) consumed all sugars by Day 8. Thiol data for these ferments (Figure A4, right graph) demonstrates that the juice modifications did not significantly affect 3MH release.

![Graph showing fermentation and thiol release](image_url)

**Figure A4: Improved fermentation conditions for BY4743 do not result in improved thiol release.** M1016 juice was fermented at 25°C using F15 and BY4743. To optimize fermentation by the laboratory strain, juice was diluted with SGM with no added sugars in a 3:1 proportion (see Section 2.2.3) and auxotrophic supplementations were added to the ferments (1x and 10x concentrated, Section 2.2.5). Thiols were extracted using the PHMB method. \( n = 2; \) error bars = SE; different letter labels above bars mean that samples are significantly different (\( p < 0.05, \) ANOVA, Tukey’s HSD).
A2.2 High nitrogen additions affects 4MMP release from C-4MMP

NCR has been shown to affect thiol release by repressing IRC7 expression (Thibon et al. (2008a)). However, in high nitrogen conditions (Table A1) a strain overexpressing IRC7 showed reduced 4MMP levels (Figure A5, left side), but 3MH remained constant compared to a strain fermented in standard nitrogen concentration (Figure A5, right side). This difference supports the idea that the strains have an altered C-4MMP transport rather than a reduced IRC7 expression or Irc7p degradation, which is expected to affect both thiols.

Table A1: Nitrogen additions to SGM.

<table>
<thead>
<tr>
<th>Juice</th>
<th>DAP YAN [mg/L of N]</th>
<th>AA YAN [mg/L of N]</th>
<th>Total YAN [mg/L] of N</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGM</td>
<td>77</td>
<td>250</td>
<td>327</td>
</tr>
<tr>
<td>SGM (High DAP)</td>
<td>950</td>
<td>250</td>
<td>1,200</td>
</tr>
</tbody>
</table>

Figure A5: High nitrogen strongly reduced 4MMP in synthetic media fermented by gap1 ssy1Δ IRC7ox strain. The strain was used to ferment SGM with added 50 µg/L of C-4MMP and 500 µg/L of G-3MH at 25°C. SGM was 3/4 strength in sugars and supplemented with uracil [20mg/L]. Nitrogen was added as shown in Table A1. Thiols were extracted by the ETP method. The labels above the bars show the percentage of thiol conversion from the added precursor. n = 2; error bars = SE; asterisks above bars represent significant differences compared to gap1Δ strain in SGM (*p < 0.01, ANOVA).
Appendix 3  Cysteine kinetics when using the pyruvate assay

Irc7fp in vitro enzymatic reaction using the pyruvate assay gave unusual kinetics when using L-cysteine as a substrate, as shown in Figure A6 (left side graph, solid black line). Copper sulfate was added, to test if H2S was interfering with the assay, and normal kinetics were obtained (left side graph, dotted black line). However, the addition of 0.4 mM CuSO4 had a negative impact on SEC kinetics (blue solid line compared to blue dotted line), suggesting that copper addition is affecting the assay somehow. Because of this, L-cysteine was further assayed by measuring H2S via a lead acetate assay (Figure A6, right side graph).

**Figure A6:** CuSO4 helps fixing L-cysteine kinetic, but had a negative impact in pyruvate assay. Reactions were carried out in 96-well plates using the pyruvate assay kit or lead acetate assay. Each well contained both a defined concentration of substrate (2 mM in the pyruvate assay) and 0.4 µg Irc7fp in a final volume of 200 µL. Absorbance 570 nm or 390 nm was measured every min for 2 hours or 10 minutes for the pyruvate and the lead acetate assay, respectively. n = 2.
A4 Overexpression or deletion of L-cysteine transporters affects growth on several aa

A4.1 The strain gap1Δ ssy1Δ IRC7ox grew on SGM with L-cysteine as nitrogen source but not with Ile, Leu, Met, Phe, Thr and Val

The strain M4238 IRC7ox (gap1Δ ssy1Δ, Didion et al. (1996)) was used to overexpress cysteine permease, because it has impaired aa uptake. The strain was tested to growth on different aa as nitrogen sources, to indirectly study aa transport in this mutant compared to M4054 IRC7ox strain (gap1Δ, Grauslund et al. (1995)). The double mutant showed a significantly altered growth on 12 of the 19 aa tested (summarized in Table A2 and detailed in Figure A7). The most extreme phenotype was lack of growth of the gap1Δ ssy1Δ strain, which occurred in six aa (black cells in Table A2): Leu, Ile, Met, Phe, Thr and Val. In addition, the double mutant had a significant growth impairment in other six aa (Grey cells in Table A2): Ala, Asn, Asp, Cys, Glu and Ser. These results showed that aa uptake in gap1Δ ssy1Δ IRC7ox strain is highly affected in fermentative conditions. Nevertheless, cysteine transport was not completely blocked. Despite this, the gap1Δ ssy1Δ IRC7ox strain was chosen to overexpress all known cysteine permeases.

Table A2: Summary of growth phenotype of gap1Δ ssy1Δ compared to gap1Δ in 12 aa.

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<th>Asn</th>
<th>Glu</th>
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<th>Thr</th>
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Black cells: gap1Δ ssy1Δ IRC7ox has a significant and extreme change compared to gap1Δ IRC7ox.
Grey cells: gap1Δ ssy1Δ IRC7ox has a significant and intermediate change compared to gap1Δ IRC7ox.
White cell: gap1Δ ssy1Δ IRC7ox has no significant change compared to gap1Δ IRC7ox.
Figure A7: Deletion of SSY1 changed growth parameters on several aa when used as nitrogen sources.

The gap1Δ and gap1Δ ssy1Δ strains were grown in 150 µL SGM at 25°C for 3 d using different aa as sole nitrogen source (15 mM). SGM was supplemented with uracil [20mg/L]. Absorbance was measured every 15 min. Growth parameters were calculated as described in Section 2.5.2. Data is ordered left to right from lowest lag phase in gap1Δ strain. n = 2; error bars = SE; asterisks above bars represent significant differences compared to the gap1Δ strain (* p < 0.01, ANOVA).
A4.2 Overexpression of L-cysteine permeases modified growth of \( \text{gap1}\Delta \text{ ssy1}\Delta \text{ IRC7}\text{ox} \) on cysteine and several aa as nitrogen sources

Eight known cysteine permeases were overexpressed in the \( \text{gap1}\Delta \text{ ssy1}\Delta \text{ IRC7}\text{ox} \) strain and the strains were grown on the 12 aa that gave impaired growth, as nitrogen sources. Only four of the overexpressed genes (\( \text{AGP1}, \text{BAP3}, \text{MUP1} \) and \( \text{TAT1} \)) gave a distinguishable phenotype. As shown in Figure A8, the inability of \( \text{gap1}\Delta \text{ ssy1}\Delta \text{ IRC7}\text{ox} \) to grow on Ile, Leu, Met, Phe, Thr and Val were all overcome by the overexpression of these three permeases: \( \text{AGP1}, \text{BAP3} \) and \( \text{MUP1} \). In addition, the use of Asp and Glu was improved by the overexpression of these three permeases (Figure A9). \( \text{MUP1ox} \) also improved growth on Ala and Asn (Figure A10). Growth on Ser and Gln showed no difference between \( \text{gap1}\Delta \text{ ssy1}\Delta \text{ IRC7}\text{ox} \) and the overexpression strains (not shown). It needs to be pointed out that effect of high affinity permease is probably missed because of the high aa concentration used.

None of the eight overexpression lines showed any sign of improved growth on cysteine as a nitrogen source; in contrast, growth on L-cysteine was actually blocked by the overexpression of \( \text{MUP1} \) and \( \text{TAT1} \) (Figure A11). This might suggest that excess of Mup1p and Tat1p in the membrane increased L-cysteine, therefore more toxicity.
Figure A8: Growth was restored on Ile, Leu, Met, Phe, Thr, Val by overexpressing AGP1, BAP3 or MUP1 in gap1Δ ssy1Δ IRC7 ox. The gap1Δ ssy1Δ IRC7 ox and the different permease overexpression strains were grown in 150 μL SGM at 25°C for 3 d using different aa as sole nitrogen source (15 mM). SGM was supplemented with uracil [20mg/L] for gap1Δ ssy1Δ IRC7 ox. Absorbance was measured every 15 min. Average curves were plotted. n = 2.
Figure A9: Growth was improved on Asp and Glu by overexpressing AGP1, BAP3 or MUP1 in gap1Δ ssy1Δ IRC7ox. The gap1Δ ssy1Δ IRC7ox and the different permease overexpression strains were grown in 150 µL SGM at 25°C for 3 d using different aa as sole nitrogen source (15 mM). SGM was supplemented with uracil [20mg/L] for gap1Δ ssy1Δ IRC7ox. Absorbance was measured every 15 min. Average curves were plotted. n = 2.

Figure A10: MUP1 overexpression improved the use of Ala and Asn. The gap1Δ ssy1Δ IRC7ox and the different permease overexpression strains were grown in 150 µL SGM at 25°C for 3 d using different aa as sole nitrogen source (15 mM). SGM was supplemented with uracil [20mg/L] for gap1Δ ssy1Δ IRC7ox. Absorbance was measured every 15 min. Average curves were plotted. n = 2.
Figure A11: Growth on cysteine was blocked by overexpression of MUP1 and TAT1. The gap1Δ ssy1Δ IRC7 Fox and the different permease overexpression strains were grown in 150 µL SGM at 25°C for 3 d using different aa as sole nitrogen source (15 mM). SGM was supplemented with uracil [20mg/L] for gap1Δ ssy1Δ IRC7 Fox. Absorbance was measured every 15 min. Average curves were plotted. n = 2.
A4.3 Mutants in multiple L-cysteine transporters stopped yeast growth on SGM with several aa as nitrogen sources but not on cysteine

Deletion mutants in multiple cysteine transporter coding genes (see Table 4.4; all deletion strains were transformed to overexpress the \(IRC7^F\) allele) were used to test 4MMP production from C-4MMP. Firstly, they were grown on \(\text{NH}_4\text{Cl}\) (positive control) and L-cysteine as nitrogen sources (Figure 4.9) to check for growth impairment. However, deletion of all 9 cysteine transporters did not stop growth on cysteine. To check if the deletion strains were correct, growth on eight different aa as nitrogen sources was performed. The aa chosen were those that were substrates for the deleted permeases (Regenberg et al. (1999)). The results from different combinations of deleted permeases (Table 4.4), suggested that in fermentative-like conditions only \(AGP1\) and \(GNP1\) were important in transporting seven of the eight aa tested:

- \(GNP1\) deletion caused a slightly extended lag phase in Asn and Ser (Figure A12).
- \(AGP1\) deletion stopped growth on Ile and Val (Figure A13).
- \(AGP1\) deletion strongly reduced growth on Leu, Thr and Phe, and in combination with an additional \(GNP1\) deletion, blocked growth on all three aa. (Figure A13).

These results confirmed that deletion strains had impaired uptake of some aa under fermentative-like conditions. However, clear changes were mainly seen for two permeases, \(AGP1\) and \(GNP1\). Deletion of \(AGP1\) also positively affected cysteine uptake (Figure 4.9, reduced growth probably due to increased uptake) by an unknown mechanism. It needs to be pointed out that effect of high affinity permease is probably missed because of the high aa concentration used.

![Figure A12: Deletion of multiple cysteine extended lag phase on Asn and Ser.](image)

The strains were grown in 150 µL SGM at 25°C for 3 d using different aa as sole nitrogen source (15 mM). SGM was supplemented with uracil [20mg/L]. Absorbance was measured every 15 min. Average curves were plotted. \(n = 2\).
Figure A13: Deletion of multiple cysteine permease reduce growth on Ile, Leu, Met, Phe, Thr and Val. The strains were grown in 150 µL SGM at 25°C for 3 d using different aa as sole nitrogen source (15 mM). SGM was supplemented with uracil [20mg/L]. Absorbance was measured every 15 min. Average curves were plotted. n = 2.
Previously, Wunschmann et al. (2009) has unraveled two degradation pathways of a glutathione conjugate (glutathione-S-bimane) in *S. cerevisiae*. As pictured in Figure 1.1, one pathway started with the removal of the glutamyl residue by Cis2p followed by the removal of the glycine residue by unknown enzyme(s), and the other pathway started by the removal of the glycine residue by Cpcp or Cpy1p peptidases and followed removal of the glutamyl residue by Cis2p. The *cis2Δ, cis2Δ cpy1Δ* and *cis2Δ cpy1Δ cpcΔ* mutants (obtained from Wunschmann et al. (2009)) were transformed with the construct overexpressing *IRC7F* and used to ferment SGM with added C-4MMP and G-3MH. Figure A13 shows that *CIS2* deletion greatly reduced 3MH production. The *cis2Δ cpcΔ* and *cis2Δ cpy1Δ cpcΔ* strains were not significantly different from *cis2Δ* strain, suggesting that Cpcp and Cpy1p are not participating in the cleavage of G-3MH release. However, the *cis2Δ cpcΔ* and *cis2Δ cpy1Δ cpcΔ* showed an unexpected reduction in 4MMP release. Currently there is no obvious reason why CPC gene – a serine protease located in the vacuolar compartment of yeast cells – might impact on conversion of C-4MMP to thiols.

**Figure A13:** *CIS2* deletion reduced 3MH conversion from G-3MH in BY4741. The strains shown were used to ferment SGM with added 50 µg/L of C-4MMP and 500 µg/L of G-3MH at 25°C. SGM was 3/4 strength in sugars and supplemented with histidine [20 mg/L], leucine [30 mg/L], uracil [20 mg/L] and methionine [20 mg/L]. Thiols were extracted by the PHMB method. The vertical axis represents [ng/L] of thiols and the labels above the bars shows the percentage of thiol conversion from the added precursor. n = 2; error bars = SE; different letter labels above bars mean that samples are significantly different (p < 0.01, ANOVA, Tukey’s HSD).


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