Electronic Journal of Biotechnology 17 (2014) 150–155

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

The *ICY1* gene from *Saccharomyces cerevisiae* affects nitrogen consumption during alcoholic fermentation

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ARTICLE INFO

Article history:
Received 27 September 2013
Accepted 10 April 2014
Available online 3 May 2014

Keywords:
Enological traits
Gene expression
Wine fermentation
Wine yeast

ABSTRACT

Background: Saccharomyces cerevisiae is the main microorganism responsible for alcoholic fermentation. In this process, the consumption of nitrogen is of great importance since it is found in limiting quantities and its deficiency produces sluggish and/or stuck fermentations generating large economic losses in the wine-making industry. In a previous work we compared the transcriptional profiles between genetically related strains with differences in nitrogen consumption, detecting genes with differential expression that could be associated to the differences in the levels of nitrogen consumed. One of the genes identified was *ICY1*. With the aim of confirming this observation, in the present work we evaluated the consumption of ammonium during the fermentation of strains that have deleted or overexpressed this gene.
Results: Our results confirm the effect of *ICY1* on nitrogen uptake by evaluating its expression in wine yeasts during the first stages of fermentation under low (MS60) and normal (MS300) assimilable nitrogen. Our results show that the mRNA levels of *ICY1* diminish when the amount of assimilable nitrogen is low. Furthermore, we constructed strains derived from the industrial strain EC1118 as a null mutant in this gene as well as one that overexpressed it.
Conclusions: Our results suggest that the expression of *ICY1* is regulated by the amount of nitrogen available in the must and it is involved in the consumption of ammonium, given the increase in the consumption of this nitrogen source observed in the null mutant strain.

1. Introduction

Wine fermentation is a complex process involving numerous microorganisms, where the yeast *Saccharomyces cerevisiae* is one of the most important and responsible for alcoholic fermentation. During this process, the amount of yeast nitrogen assimilable is limiting [1], which is, in many cases, the cause of sluggish or stuck fermentations producing large economic losses in the wine-making industry [2,3,4]. The main nitrogen sources in the must are amino acids and the ammonium ion; both compounds are incorporated into the cell through active transport by specific permeases [5,6,7,8]. Once inside the cell, these compounds are incorporated into the main nitrogen metabolism where ammonium ions transported from the exterior of the cell or from amino acids are assimilated by their incorporation into an α-ketoglutarate molecule, carried out by the enzyme glutamate dehydrogenase [5]. On the other side, the nitrogen from amino acids could be incorporated to glutamate generating glutamine by the glutamine synthetase enzyme [5,9,10]. The different sources of nitrogen available in the must are consumed in the order of preference with respect to their capacity to be incorporated into the main nitrogen metabolism [6,11]. The main system of regulation of genes involved in nitrogen consumption is the nitrogen catabolic repression system [5], which allows the consumption of the different nitrogen sources available to the yeast to be regulated and is of great importance in nitrogen consumption during alcoholic fermentation [5,12]. All these processes explain the complexity of nitrogen metabolism in the yeast and therefore the participation of numerous genes in this trait of great enological interest [5,10].

Due to the importance of nitrogen metabolism in alcoholic fermentation, numerous studies have been carried out to identify the genes involved in this process [13,14,15]. Within this context, Jiménez-Martí et al. [16] described the expression of various genes

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.ejbt.2014.04.006

as a response of the yeast to the substitution of the must with different nitrogen sources observing that the gene response depends on the nitrogen source used. Furthermore, the effect of some specific genes in the consumption of nitrogen has been defined, for example *GAP1* [7], the genes encoding for the ammonium permeases *MEP1*, *2* and *3* [17], amongst others, which have allowed the identification of the main genes involved in nitrogen metabolism regulation during alcoholic fermentation. On the other hand, various genome-wide studies which analyze the transcriptional changes associated to the consumption of nitrogen have been carried out [13,14,17,18,19]. Thus, the addition of ammonium phosphate has been described to promote expression changes of 350 genes involved in the transport of small molecules, protein and purine syntheses, amongst others [14]. Moreover, Varela et al. [19] used SAGE to analyze the genic expression during fermentation noting variations in important genes involved in transport, *GAP1*, in the metabolism, *GDH2*, *GDH3* and *PUT1* or in the regulation, *GCN4* and *MET30*. In spite of the large amount of information available on this important enological trait, it is vital to remember that like other enological traits, the consumption of nitrogen is a polygenic phenotype where various genes contribute to the observed trait, and therefore many other genes could potentially participate in this process. In this regard, our laboratory implemented an experimental approach based on breeding to identify genes that have yet to be related to this trait [15]. This strategy consisted in obtaining a hybrid from the mating of wild wine strains and the subsequent generation, by sporulation and autodiploidization of 115 monosporic cultures from this hybrid, producing a population of genetically related individuals presenting a normal distribution for the nitrogen consumption trait. Strains AC19 and AC114, which differed in the consumption of nitrogen from ammonium during alcoholic fermentation, were selected from this population. These strains were subject to a transcriptional comparative analysis using microarray allowing the identification of differential expression of 121 genes, amongst them, gene *ICY1* [15]. This gene is associated to the "negative petit" phenotype suggesting it is essential in strains that lose the mitochondrial DNA [20]. Other mutations of genes have been described that show this phenotype, which are associated with: protein transport to the mitochondria [20,21], components of the F1-ATPase [22], components of the ATP/ADP antiport and proteins that form part of the i-AAA system [23,24]. All these processes are related to the capacity of the mitochondria to maintain the potential difference between the exterior and interior of this organelle. However, there is no information to indicate the specific function of *ICY1* in these processes or the relationship with the nitrogen consumption.

In the current work, we evaluated the effect of the gene *ICY1* in the consumption of nitrogen during alcoholic fermentation using transcriptional and mutational analysis.

2. Materials and methods

2.1. Strains and culture media

The yeasts used in this study were the monosporic cultures AC19 and AC114, isolated by micromanipulation of tetrads obtained from hybrid L3044 formed by the mating of the two wild strains, L3217 and L3218, collected from a wine area in Valle del Maipo, Santiago, Chile [15]. The commercial strain EC1118 (Lalvin) was also used.

The synthetic must (MS300) was prepared as described before in Rossignol et al. [18] with some modifications. This medium contains 125 g l^{-1} of glucose, 125 g l^{-1} of fructose, mineral salts (750 mg l^{-1} KH_2PO_4, 500 mg l^{-1} K_2SO_4, 250 mg l^{-1} $MgSO_4 \cdot 7H_2O$, mg l^{-1} $CaCl_2 \cdot 2H_2O$, 200 mg l^{-1} NaCl, 4 mg l^{-1} $MnSO_4 \cdot H_2O$, 4 mg l^{-1} $ZnSO_4$, 1 mg l^{-1} $CuSO_4 \cdot 5H_2O$, 1 mg l^{-1} KI, 0.4 mg l^{-1} $CoCl_2 \cdot 6H_2O$, 1 mg l^{-1} H_3BO_3, 1 mg l^{-1} $NaMoO_4 \cdot 2H_2O$), and vitamins (20 mg/l myo-inositol, 2 mg l^{-1} nicotinic acid, 1.5 mg l^{-1} calcium panthothenate, 0.25 mg l^{-1} thiamine HCl, 0.25 mg l^{-1} pyridoxine HCl, 0.003 mg l^{-1} biotin). The medium contained anaerobic factors (15 mg l^{-1} ergosterol; 5 mg l^{-1} sodium oleate), added to the medium in 1 ml Tween 80/ethanol (50/50 v/v). The pH was buffered at 3.3 with NaOH. The nitrogen sources were 300 mg l^{-1} of assimilable nitrogen where 120 mg l^{-1} comes from 460 mg l^{-1} of ammonium chloride and 180 mg l^{-1} from a mixture of 19 amino acids (612.6 mg l^{-1} L-proline, 505.3 mg l^{-1} L-glutamine, 374.4 mg l^{-1} L-arginine, 179.3 mg l^{-1} L-tryptophan, 145.3 mg l^{-1} L-alanine, 120.4 mg l^{-1} L-glutamic acid, 78.5 mg l^{-1} L-serine, 75.9 mg l^{-1} L-threonine, 48.4 mg l^{-1} L-leucine, 44.5 mg l^{-1} L-aspartic acid, 44.5 mg l^{-1} L-valine, 37.9 mg l^{-1} L-phenylalanine, 32.7 mg l^{-1} L-isoleucine, 32.7 mg l^{-1} L-histidine, 31.4 mg l^{-1} L-methionine, 18.3 mg l^{-1} L-tyrosine, 18.3 mg l^{-1} L-glycine, 17.0 mg l^{-1} L-lysine, and 13.1 mg l^{-1} L-cysteine). For the experiments carried out under low nitrogen content conditions (60 mg l^{-1} of assimilable nitrogen called MS60), the synthetic must contained only 20% of the nitrogen sources mentioned. The fermentations were done at 25°C for 20 d without shaking in 15 ml conic tubes containing 12 ml of synthetic must in six replicates. The inoculum consisted of 1×10^6 cells ml^{-1} obtained from a 16 h culture in the same must with shaking. CO_2 production was monitored by weighing the tubes to determine weight loss.

2.2. Gene sequences

Yeast DNA extractions were carried out with the Wizard Genomic DNA Purification Kit (Promega, Madison, USA) using 10 mg ml^{-1} of Zymolyase 20 T (Seikagaku Corp., Tokyo, Japan) and measured spectrophotometrically at 260 nm. The gene *ICY1* was amplificated using the primers VGM56 and VGM57 (Table 1) and the PCR product was purified using the E.Z.N.A. Cycle Pure kit (Omega, Biotek, Georgia, USA) following the protocol described by the manufacturer. The PCR product was subcloned in the vector pGEM-T easy (Stratagene, California, USA) following the protocols described by the manufacturer. This sample was automatically sequenced by Macrogen (Seoul, Korea). The analysis of the sequences was carried out with the program vector NTI 9.0 (Life Technology, California, USA).

2.3. RNA preparation

RNA extraction was performed from cultures grown in synthetic must without shaking in conic tubes of 15 ml with 12 ml of MS300 or MS60 at 25°C. The samples were collected by centrifugation during the early phase of the fermentation, because it has been described that in this step the genes associated with nitrogen metabolism reach higher expression levels [25]. RNA extraction was performed immediately using a commercial kit (RNeasy Mini Kit, Qiagen, USA) according to the protocol described by the manufacturer and was followed by DNAseI treatment (Promega, Madison, USA). The RNA quantity and quality were determined using UV spectrophotometry and determining the 260/280 nm ratio. The RNA extractions showed a 260/280 ratio of 1.9–2.0 confirming good RNA quality. The samples were stored at -80°C.

2.4. RT-qPCR assay

The reverse transcription reaction was carried out using one unit of M-MLV Reverse transcriptase, 1 μg of Oligo $(dT)_{15}$ Primer (Promega, Madison, USA) and 3 μg of digested RNA in a final volume of 25 μl according to the protocols described by manufacturer. cDNA samples obtained were quantified by UV spectroscopy and used as template in the qPCR reaction. The qPCR reaction was carried out in a final volume of 20 μl. The reaction mixture contained 10 μl of 2× Brilliant II SYBR Green qPCR Master mix (Stratagene, California, USA), 0.1 mg ml^{-1} of BSA (New England BioLabs, USA) and 2 μM of each primer. The qPCR reaction was carried out in LightCycler 1.5 equipment by triplicate (Roche, Germany). Results were analyzed using the LightCycler 4.0 software (Roche, Germany). The genes evaluated and primers used are listed in Table 1. The relative expression of each gene was quantified

Table 1
Primers used in this work.

Name	Sequence 5′ → 3′	Origin
VGM57	GTGTTATCAAACAGGTTTTC	This study
VGM58	AACCATGGCTTCAAACTATGCCACTCC	This study
VGM62	AAGTCGACAAATGAAGACAGCCAC	This study
VGM88	TTGAAGTACAATTTATACTGCTCAGAGGCTCATTCGATTGGTCTAAAGGAGTGGCATAG	This study
VGM89	CTTGAGAAGGTTTTGGGACGCTCGAAGGCTTTAATTTGCAAGCTGATTAAGGAATGATAATCA	This study
VGM85	TTGATTATCATTCCTTAATCGTTATTCTAAAATGAAGACAGCCACAATCTGTAGTTTTGTAGCTTGCAAATTAAAGC	This study
VGM84	CTCATATAACACATACAATAAAACCAAGAAATGTCTTCAAACTATGCCACTCCTTTAGACCAATCGAATGAGCCTCTGAG	This study
VGM83	ACTAGTGGCCTATGCGGCCGCGGATCTGCCGGTCTCCCTATAGTGAGTCGTATTAATTTCATTGACCGATCTTTATGCC	This study
VGM82	GAAGTTATATTAAGGGTTGTCGACCTGCAGCGTACGAAGCTTCAG CTGGCGGCCGCGTTCAATAATGCTCACCAAGTGAC	This study
VGM50	CTCATATAACACATACAATAAAACCAAGAAATGTCTTCAAACTATGCCACTCCTTTAGACCAGCTGAAGCTTCGTACGC	This study
VGM51	TTGATTATCATTCCTTAATCGTTATTCTAAAATGAAGACAGCCACAATCTGTAGTTTTGTGCATAGGCCACTAGTGGATCTG	This study
VGM56	AGCTCGATTTTCAGCACCTTG	This study
NCOITR	GAGTCTTTTCCTTACCCATAGTTGTTTATGTTCGG	This study
NCOITF	CCGAACATAAACAACTATGGGTAAGGAAAAGACTC	This study
SP6	ATTTAGGTGACACTATAG	Stratagene
T7	GTAATACGAACTCACTATAGGGC	Stratagene
ACT1F	TTG GCC GGT AGA GAT TTG AC	[15]
ACT1R	CCC AAA ACA GAA GGT GGA A	[15]
ICY1F	CGA GAG ACA CCG GGA AGT	[15]
ICY1R	TTG GCA TAA AGA TCG GTC AA	[15]
TUB1F	AGAAAATTCGATTTA	This study
TUB1R	TTAAAATTCCTCTTC	This study
PGK1F	CTTGCCAGTCGACTTCATCA	[42]
PGK1R	CCTTTGCAACAGTAGCAGCA	[42]

using the mathematical method described by Livak and Schmittgen [26], using *ACT1*, *TUB1* and *PGK1* as reference genes after a previous selection of reference genes according to the geNorm kit [27].

2.5. Enological parameters

The residual sugar, glycerol and ethanol production were determined by HPLC in a Shimadzu Prominence HPLC equipment (Shimadzu, USA) using an HPX-87H Aminex ion-exclusion column following the method of Nissen et al. [28]. The consumption of ammonia and the 19 amino acids present in the must (see Section 2.1) were determined by derivatization with DEEMM and separation by HPLC using column 5 C18-HL as was described by Gómez-Alonso [29].

2.6. Cloning and sequencing of gene ICY1

The *ICY1* gene was cloned in the YEpACT4MX vector obtained from the vector YepACT4 [30] incorporating the G418 (*KANMX*) resistance cassette in the *Eco*RV restriction site obtained from the vector pUG6. Previously, and to eliminate the *Nco*I site in the *KANMX* cassette, the pUG6 vector was subjected to a site-directed mutagenesis using the QuikChange® Site-Directed Mutagenesis system (Stratagene, California, USA) and the primers NCOITF and NCOITR (Table 1). As a result, the CCATGG site was replaced by the sequence CTATGG using the *Nco*I site in the cloning strategy in the vector YEpACT4MX. The gene *ICY1* was amplified from the genomic DNA of the strains EC1118 or S288c and subcloned in the vector pGEMT easy (Promega, Madison, USA) using the primers VGM56 and VGM57. The construct was verified using PCR, digestion and sequencing. Many of the clones obtained in this stage were analyzed by sequencing using the primers T7 and SP6 (Table 1) (Macrogen, Korea, Japan). Subsequently, the insert was cloned in the vector YEpACT4MX in the *Nco*I and *Sal*I sites. The construct was used to transform strain EC1118 according to the method described by Becker and Guarente [31]. The analysis of the sequences obtained was carried out with the program vector NTI 9.0 (Life Technology, California, USA).

2.7. Deletion of gen ICY1 in the commercial wine strain EC1118

The deletion mutant of the gene *ICY1* was carried out according to Shao et al. [32]. The primers VGM56, VGM82, VGM50, VGM51, VGM83 and VGM57 were used (Table 1). The transformation of strain EC1118 was done as previously described [31]. The second allele was mutated using the same strategy but using the resistance gene to hygromycin and the primers VGM84, VGM85, VGM88, VGM89 and VGM57 as markers (Table 1). The PCR products were purified with the E.Z.N.A. Cycle pure kit (Omega Biotek, Georgia, USA) and used to transform strain EC1118. The transformant clones were selected in G418 or G418 and hygromycin and verified by PCR using the primers VGM58 and VGM62 (Table 1).

2.8. Statistical analysis

The enological parameter data were subjected to analysis of variance (ANOVA) and the mean values of the experiments were statistically analyzed using the LSD test. Differences were considered significant when the probability was ≤ 0.05.

3. Results

3.1. Expression of the ICY1 gene in wine strains

To evaluate the participation of *ICY1* in the consumption of nitrogen, the expression levels of this gene in strains AC19 and AC114, the hybrid L3044, the parental strains L3217 and L3218, and the commercial wine strain EC1118 were quantified in must containing 300 mg l^{-1} and 60 mg l^{-1} of assimilable nitrogen (Table 2). The results indicate that gene *ICY1* shows a greater amount of transcripts in fermentations containing 300 mg l^{-1} than in low nitrogen conditions. This behavior is observed in all strains analyzed. On the other hand, the relative expression of

Table 2
Relative expression of the gene *ICY1* in fermentations containing 300 mg l^{-1} and 60 mg l^{-1} nitrogen.

Strain	Major expression condition	QPCR[a]
L3217	MS300	14.47 ± 0.50
L3218	MS300	13.77 ± 2.39
L3044	MS300	3.92 ± 1.60
AC19	MS300	33.05 ± 7.58
AC114	MS300	3.90 ± 2.01
EC1118	MS300	4.92 ± 0.40

[a] Relative expression calculated following the mathematical method described by Livak and Schmittgen [26].

Table 3
Nitrogen consumption of strains AC114, AC19 and EC1118 during the fermentation in MS300.

Strain	Nitrogen uptake from ammonium (mg l^{-1})	Nitrogen uptake from amino acids (mg l^{-1})	YAN consumption (mg l^{-1})
AC19	53.23 ± 0.64[b]	94.76 ± 0.99[c]	148.00 ± 1.55[b]
AC114	28.96 ± 2.41[a]	72.51 ± 0.89[a]	101.48 ± 3.02[a]
EC1118	89.04 ± 5.10[c]	87.48 ± 0.97[b]	176.52 ± 4.12[c]

Different letters in the same column indicate significant statistical differences according to the LSD test $P \leq 0.05$.

the gene in the strains analyzed showed differences in the magnitude of the response. Thus, strains EC1118, AC114 and L3044 show the smallest difference in transcripts between the two conditions analyzed, whilst the monosporic strain AC19 shows the greatest difference (Table 2).

3.2. Sequence of the gene ICY1 in the industrial strain EC1118

The differences in the relative transcript levels of the gene *ICY1* in response to the varying amounts of nitrogen in the must, in addition to the differences in expression of this gene in the monosporic strains AC114 and AC19 reported by Contreras et al. [15], could be a result of the differences in the promoter region sequence of this gene in the strains analyzed (*cis* regulation). To evaluate this proposal, the gene was sequenced in the monosporic strains and the commercial strain EC1118, however, the results show that the promoter region is identical in all these strains indicating that the differences in the levels of transcripts are not due to *cis* factors.

Moreover, the sequence comparison of the gene encoding region in strains AC19 and AC114 with the reference strain S288c sequence shows a base change from thymine to cytosine in nucleotide 267 in the monosporic strains implying a synonymous mutation which does not alter the sequence of the translated protein. Furthermore, when various subclones obtained in the vector pGEM-T easy by genomic DNA amplifications of strain EC1118 were sequenced, two alleles were observed in this strain. One of these shows 99% similarity with the gene *ICY1* of the reference strain S288c, with a difference in nucleotide 31 where a guanine is replaced by an adenine resulting in a change of an aspartic acid for an asparagine in amino acid residue 11. The other allele obtained, in addition to the previously mentioned polymorphism, has a polymorphism in position 267 resulting in a thymine instead of a cytosine, translating in a synonymous mutation that does not affect the protein sequence. The latter mutation was the same as that obtained for the monosporic strains AC114 and AC19. These results differ from those described by Novo et al. [33] and suggest that strain EC1118 is heterozygous for the gene *ICY1*, which both alleles encode for identical proteins of 127 amino acids and the asparagine amino acid at position 11 is replaced by an asparagine residue when compared to reference strain S288c.

3.3. Nitrogen consumption in the monosporic and industrial strain EC1118

To compare the final consumption of each available nitrogen source in the must, the monosporic strains AC19 and AC114 and the industrial strain EC1118 were fermented in MS300 and MS60 (Table 3). The results indicate that both monosporic strains consume less total nitrogen than the industrial strain and this difference is mainly given by a lower ammonium consumption. The detailed comparison of the consumption of the different amino acids (Table 4) shows that strain EC1118 consumes the greatest amount of aspartic acid (41.23 ± 1.53 mg l^{-1}) and glutamic acid (108.00 ± 4.48 mg l^{-1}) in comparison to both monosporic strains. However, strain AC19 consumes greater amounts of glutamine, serine, arginine and valine, with values of 428.43 ± 1.85, 51.71 ± 1.30, and 82.27 ± 3.96 and 40.63 ± 0.50 mg l^{-1} respectively. Strain AC114 shows lower consumptions of these amino acids reaching values of 26.70 ± 0.85 mg l^{-1} for aspartic acid, 47.50 ± 3.46 mg l^{-1} for glutamic acid, 373.67 ± 0.84 mg l^{-1} for glutamine, 29.20 ± 0.35 mg l^{-1} for serine, 3.93 ± 1.96 mg l^{-1} for valine and 26.07 ± 0.67 mg l^{-1} for phenylalanine. On the other hand, strain EC1118 shows the lowest consumption of arginine with a value of 23.40 ± 13.36 mg l^{-1}, whilst strain AC114 shows a value of 53.10 ± 6.29 mg l^{-1}.

Alternatively, in the fermentations carried out by strain EC1118 the amount of nitrogen consumed from amino acids is between the amounts consumed by the monosporic strains (Table 3). However, this strain consumes a large amount of nitrogen from ammonium (89.04 ± 5.10 mg l^{-1}) as opposed to that observed for monosporic strains. On the other hand, fermentations in MS60 did not show differences in the consumption of nitrogen from ammonium in the strains analyzed since this compound is completely consumed by all the strains during fermentation. With regard to the amino acids in MS60, there is a total consumption of the majority of amino acids present with a YAN from the amino acids close to 27 mg l^{-1} in all the strains.

3.4. Overexpression and mutation of the gene ICY1 in the industrial strain EC1118 and its effect on the consumption of nitrogen during the fermentation

An approach used to evaluate the effect of the gene *ICY1* in the consumption of nitrogen was to analyze the uptake of this compound in fermentations carried out by mutant strains or strains that overexpress the gene *ICY1*. The characterization of the fermentations of the strains that overexpress the allele of the strain EC1118 does not show a significant statistical difference in the enological parameters analyzed, such as sugar consumption or ethanol and glycerol production with respect to the strain EC1118YEPMX, including the consumption of nitrogen from ammonium. However, there is a tendency to consume less nitrogen from this source. Due to the sequence differences found in the gene *ICY1* in strains EC1118 and S288c, the effect in nitrogen consumption of the overexpression of each allele in the strain EC1118 was evaluated (Table 5). The results showed that the strain that overexpresses the allele from strain S288c consumes a lower amount of nitrogen from ammonium, which is different to what is observed with the

Table 4
Consumption of amino acids with statistical differences between the strains AC19, AC114 and EC1118 during alcoholic fermentation in MS300.

Strain	Amino acid consumption (mg l^{-1})						
	Aspartic acid	Glutamic acid	Serine	Glutamine	Arginine	Valine	Phenylalanine
AC19	37.60 ± 1.61[b]	73.93 ± 4.38[b]	51.70 ± 1.30[c]	428.43 ± 1.85[c]	82.27 ± 3.96[c]	40.63 ± 0.50[c]	37.90 ± 0.01[b]
AC114	26.70 ± 0.85[a]	47.50 ± 3.46[a]	29.20 ± 0.35[a]	373.67 ± 0.84[a]	53.10 ± 6.29[b]	3.93 ± 1.96[a]	26.07 ± 0.67[a]
EC1118	41.23 ± 1.53[c]	108, 00 ± 4.48[c]	33.13 ± 1.53[b]	396.57 ± 2.80[b]	23.40 ± 13.36[a]	34.40 ± 4.85[b]	36.33 ± 0.40[b]

Different letters in the same column indicate significant statistical differences according to the LSD test $P \leq 0.05$.

Table 5
Enological parameters of the fermentations.

Strain	Sugar consumption ($g\ l^{-1}$)	Ethanol production (%)	Glycerol production ($g\ l^{-1}$)	YAN consumption ($mg\ l^{-1}$)	Consumption of nitrogen from ammonium ($mg\ l^{-1}$)	Consumption of nitrogen from amino acids ($mg\ l^{-1}$)
EC1118	202.63 ± 30.33^{b}	11.94 ± 2.11^{bc}	13.05 ± 1.32^{b}	164.28 ± 20.09^{cd}	80.19 ± 15.19^{bc}	83.78 ± 4.67^{c}
EC1118 YEpACT4MX	177.32 ± 41.62^{ab}	11.11 ± 2.34^{abc}	11.04 ± 1.51^{a}	151.47 ± 17.55^{bc}	68.95 ± 16.27^{bc}	82.47 ± 4.55^{bc}
EC1118 YEP*ICY1*	145.84 ± 30.88^{a}	9.48 ± 1.91^{a}	10.68 ± 1.75^{a}	145.56 ± 25.32^{bc}	64.95 ± 17.63^{b}	80.61 ± 6.89^{abc}
EC1118 YEP ICY1 S288c	170.45 ± 11.78^{ab}	10.28 ± 1.53^{ab}	11.35 ± 0.86^{a}	120.49 ± 14.27^{a}	42.89 ± 13.07^{a}	77.60 ± 1.60^{ab}
EC1118Δ*ICY1*	202.50 ± 54.02^{b}	13.40 ± 3.03^{abc}	11.43 ± 1.39^{ab}	169.44 ± 36.47^{bcd}	88.80 ± 30.26^{cd}	80.64 ± 6.41^{abc}
EC1118ΔΔ*ICY1*	184.7 ± 36.20^{ab}	11.73 ± 2.65^{bc}	11.80 ± 0.62^{ab}	180.68 ± 14.04^{d}	105.72 ± 8.87^{d}	74.97 ± 6.18^{a}

Different letters in the same column indicate significant statistical differences according to the LSD test $P \leq 0.05$.

overexpression of the allele from strain EC1118. On the other hand, the fermentation carried out by the null mutant strain in gene *ICY1* showed a tendency toward greater total nitrogen consumption as a result from the greater consumption of nitrogen from ammonium. It is important to highlight that the difference in nitrogen consumption does not affect the other enological parameters, indicating that this gene only affects the consumption of this compound and not the fermentation itself (Table 5).

4. Discussion

To identify genes involved in the consumption of nitrogen during alcoholic fermentation, our laboratory compared the transcriptional profiles of the monosporic strains AC19 and AC114, which come from the same hybrid making them genetically related, but differing in their nitrogen consumption [15]. The results showed a correlation between the consumption of nitrogen with differences in the expression of some genes such as *ICY1*. This shows that strain AC19, which consumes a greater amount of nitrogen from ammonium, has a higher expression of the gene *ICY1* than strain AC114. In the current work, where the consumption of the diverse nitrogenated sources was evaluated in detail in the aforementioned strains, it was found that they differ not only in the consumption of ammonium but also in the consumption of some specific amino acids when the fermentations are carried out in must with 300 $mg\ l^{-1}$ of assimilable nitrogen. The detailed consumption of amino acids in the studied strains shows that strain AC114 consumes the lowest amount of the amino acids, aspartic acid, glutamic acid, glutamine, serine, valine and phenylalanine. Hence, the differences in the consumption of certain amino acids explain the differences described for YAN from amino acids. This observation was not been detected in the experiments described by Contreras et al. [15] perhaps because the must used in these experiments simulates industrial conditions and therefore contains a high concentration of assimilable nitrogen (518 $mg\ l^{-1}$) of which 339 mg of nitrogen comes from ammonia. Furthermore, it only contains 9 amino acids corresponding to those preferentially consumed by the yeast: arginine, serine, threonine, lysine, aspartic acid, glutamic acid, asparagine, glutamine and leucine. Finally, many of the amino acids consumed differentially by the strains AC19 and AC114 have been described as those of major consumption during alcoholic fermentation [11] which may have important implications in the development of alcoholic fermentation.

The prediction of regulation sites of the *ICY1* gene promoter using the server YEASTRACT (http://www.yeastract.com/findregulators.php) and Yeast Promoter Atlas (http://ypa.ee.ncku.edu.tw/) suggests that this gene is transcriptionally regulated by many factors, such as Gcn4p [34], Phd1p [35], Hap5p [36], and Nrg1p [37], for which the binding to the promoter zone of the gene *ICY1* has been described. However, the majority of these interactions were detected by mass techniques such as CHIP on chip [38], and therefore there is no experimental evidence that identifies the effect of these factors in the expression of the gene. As a result, and given that the sequences of the promoter region of the gene *ICY1* in strains AC19 and AC114 are identical, the differences in the amount of mRNA may be explained by the differential expression of a transcriptional regulator or as a result of polymorphisms in one of these regulators that affects the binding to the DNA or the interaction with the transcriptional machinery, which directly or indirectly produces changes in the amount of mRNA of this gene. In this context, the expression of the general control system pathway transcriptional factor (GCN), Gcn4p, is triggered by the lack of amino acid. This transcriptional factor activates the expression of about 500 genes. One of the regulation targets of this factor is the gene *ICY1*, which could explain the increase in its expression under nitrogen starvation conditions [39,40]. In our experiments, the expression of the gene *ICY1* is greater when the strains are in must containing 300 $mg\ l^{-1}$ of assimilable nitrogen. A possible explanation for this observation is although it is a high assimilable nitrogen condition, there may be another transcriptional factor involved, because the expression of GCN4 is overexpressed in MS60 (data not shown). However, it is necessary to carry out further work to determine the specific transcription factors which regulate the expression of this gene and how the differences in expression between strains AC114 and AC19 may be explained.

In the present work the participation of gene *ICY1* in the consumption of nitrogen is observed by an increase in the consumption of ammonia by the null mutant strain and is confirmed by the lower consumption of this compound by the strain that overexpress the allele from strain S288c. However, the strain that overexpresses the EC1118 allele does not show differences in nitrogen uptake in relation to control strain. These results could be explained for the differences in the protein sequences suggesting that the change in the amino acid 11 of an aspartic acid residue for asparagine changes the functions of the protein and therefore varies nitrogen consumption during fermentation. These together with the differential expression of the gene *ICY1* regarding the amount of available nitrogen in the must suggest that this gene plays an important role in the nitrogen consumed under fermentation conditions.

Regarding the effect of the gene *ICY1* in nitrogen metabolism, there is insufficient information to enable the definition of a mechanism of action. Nevertheless, the results from this study suggest that gene *ICY1* affects the consumption of ammonium in fermentation conditions and that the expression of this gene is regulated by the amount of available nitrogen in the must. Some studies, such as those by Dunn and Jensen [20] indicate that this gene is related to cell survival in the absence of mtDNA as previously mentioned. Furthermore, there is a direct relationship between the function of this organelle and the main nitrogen metabolism, since in fermentation conditions the α-ketoglutarate necessary for the assimilation of ammonium is produced by the retrograde pathway of the Krebs cycle, the pathway that affects the maintenance of mtDNA [41]. Therefore, gene *ICY1* could affect the retrograde pathway of the Krebs cycle and the normal functioning of the mitochondria and the consumption of ammonium by α-ketoglutarate.

Financial support

Agency/Institution: CONICYT; Program: FONDECYT Postdoctoral Fellowship and regular FONDECYT grant; Project number: grants 3120107, FONDECYT Postdoctoral Fellowship. Grant FONDECYT 1100509.

Author contributions

Proposed theoretical frame: VG; Conceived and designed the experiments: VG, AC; Contributed reagents/materials/analysis tools: VG, CM; Wrote the paper: VG, CM; Performed the experiments: VG, OA; Analyzed the data: VG, CM, AG.

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