1 **Effect of deletion and overexpression of tryptophan metabolism genes on growth**

2 **and fermentation capacity at low temperature in wine yeast**

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Abstract

Low-temperature fermentations produce wines with greater aromatic complexity, but the success of these fermentations greatly depends on the adaptation of yeast cells to cold. Tryptophan has been previously reported to be a limiting amino acid during *S. cerevisiae* growth at low temperature. The objective of this study was to determine the influence of the tryptophan metabolism on growth and fermentation performance during low-temperature wine fermentation. To this end, we constructed the deletion mutants of the *TRP1* and *TAT2* genes in a derivative haploid of a commercial wine strain, and the *TAT2* gene was overexpressed in the prototroph and auxotroph (*Δtrp1*) backgrounds. Then we characterized growth and fermentation activity during wine fermentation at low and optimum temperatures. Our results partially support the role of this amino acid in cold yeast growth. Although deletion of *TRP1* impaired amino acid uptake and the growth rate at low temperature in synthetic must, this growth impairment did not affect the fermentation rate. Deletion of *TAT2* endorsed this strain with the highest nitrogen consumption capacity and the greatest fermentation activity at low temperature. Our results also evidenced reduced ammonium consumption in all the strains at low temperature.

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Introduction

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31   Many winemakers prefer low-temperature fermentation (10-15°C) for white and "rosé"

32   wine production because it improves the characteristics of taste and aroma. This

33   improved aroma profile can be attributed not only to the prevention of volatilization of

34   primary aromas, but also to the increased synthesis of secondary aromas. Thus the final

35   wine product has greater terpenes retention, a reduction in higher alcohols, and an

36   increased proportion of ethyl and acetate esters in the total volatile compounds. [1-3]

37   Another positive aspect is that low temperatures diminish the growth of acetic and lactic

38   bacteria, which makes alcoholic fermentation control easier. [4] However, low

39   temperatures lower the growth rate and prolong the lag phase, which lead to longer

40   fermentations and increase the risk of producing sluggish, stuck fermentations. [5]

41   Therefore, the quality of wines produced at low temperature depends on the yeast's

42   ability to adapt to cold.

43   The nitrogen fraction in grape must consists of organic (amino acids) and inorganic

44   (ammonium) nitrogen. *Saccharomyces cerevisiae* is able to regulate nitrogen uptake by

45   the so-called nitrogen catabolite repression (NCR) mechanism. [6] Recently, Crepin *et al.*[7]

46   determined the consumption kinetics of the amino acids and ammonium present in a

47   synthetic grape must. These authors found that the assimilation sequence of the nitrogen

48   compounds during fermentation is largely determined by the kinetic characteristics and

49   regulation of the transporters of amino acids and ammonium. Most late consumed

50   amino acids (i.e., arginine and alanine) are transported by permeases under NCR, except

51   valine, tryptophan and tyrosine, which are transported by SPS-regulated permeases. [7]

52   Quiros *et al.*[8] mostly confirmed the order of consumption of the different nitrogen

53   sources reported previously by Crepin *et al.*[7] These authors also observed that the

54   consumption of amino acids is also affected by both sugar concentration and

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55 temperature. Beltran *et al.*[9] showed that cells growing at low temperature had a less

56 effective NCR, which resulted in a higher uptake of two of these late consumed amino

57 acids, arginine and tryptophan, and that the consumption of glutamine, one of the earlier

58 consumed and more preferred amino acids, was lower. These authors[9] also reported that

59 the consumption of ammonium in cells growing at low temperature significantly

60 diminished.

61 It has been reported that tryptophan uptake at low temperature is a rate-limiting step for

62 *S. cerevisiae* growth.[10] Broad-range permease (Gap1p), or specific amino acid

63 permeases Tat1p and Tat2p, are able to transport tryptophan into the cell. Tat2p

64 mediates the high-affinity uptake of tryptophan, and Tat1p mediates the low-affinity

65 uptake of this amino acid.[11] Low temperature increases the rigidity of the plasma

66 membrane[12], which results in the slower lateral diffusion of membrane proteins, less

67 active membrane-associated enzymes, and a major reduction in membrane transport.[13]

68 Membrane permeases are highly temperature-dependent because changes in temperature

69 can cause conformational changes in their structure[14]. Sensitivity of tryptophan uptake

70 at low temperature has been related to a dramatic conformational change in Tat2p. Abe

71 and Horikoshi[15] demonstrated that the overexpression of *TAT2* in a laboratory *S.*

72 *cerevisiae* strain confers yeast cells good capacity to grow at low temperature.

73 Moreover, enhancement of the gene expression for tryptophan biosynthesis or uptake

74 has also been related to ethanol stress tolerance.[16] Recently in a metabolic comparison

75 between a wine strain of *S. cerevisiae* and a cryotolerant strain of *S. bayanus,*[17] we

76 detected more active tryptophan biosynthesis in the cryotolerant strain via the shikimate

77 pathway, which partially explains its improved growth at low temperature.

78 The aim of this study was to analyze the importance of tryptophan biosynthesis and

79 uptake in a context which mimics wine fermentation conditions. First we deleted the

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80   *TRP1* gene, which is involved in tryptophan biosynthesis, and the *TAT2* gene, which is

81   related to tryptophan uptake, in the genetic background of a derivative haploid of the

82   commercial wine strain QA23. We also constructed strains which overexpressed the

83   *TAT2* gene in the background of the wine strain and in its auxotroph for tryptophan

84   (Δ*trp1*). Then we analyzed the effect of deletions and overexpression on growth and

85   fermentation activity during wine fermentations at low and optimum temperatures.

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Materials and Methods

Construction of the mutant and overexpressing strains

89   The *TAT2* and *TRP1* genes were deleted on the derivative haploid of the commercial

90   wine strain QA23 (Lallemand S.A., Canada) *ho*QA23.[18] The primers used to construct

91   the mutant and overexpressing strains are listed in Table 1. The *TAT2* and *TRP1* genes

92   were deleted using the short flanking homology (SFH) method[19] based on the *KanMX4*

93   and the *NatMX4* deletion cassette, respectively, which provided geneticin (G418) and

94   nourseothricin resistance. Given the high GC content of the *NAT1* gene encoding

95   nourseothricin-resistance, the PCR reaction mixture contained 5% of DMSO, as

96   previously described.[20] The deletion cassettes were used to transform the haploid

97   *ho*QA23 strain by the lithium acetate procedure.[21] After selecting the transformants in

98   YPD with geneticin (G418) or noursethricin (clonNAT), the correct insertion of the

99   deletion cassettes was also verified by PCR using the primers upstream and downstream

100   of the deleted region, combined with the primers of the *KanMX4* or *NatMX4* genes

101   (Table 1).

102   The *TAT2* gene was overexpressed by cloning it into the centromeric plasmid

103   pGREG505 in strains *ho*QA23 and *ho*QA23 (Δ*trp1)*, as described in Jansen *et al.*[22]

104   *TAT2* was amplified from approximately 600 nucleotides upstream of the start codon to

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105 400 nucleotides downstream of the stop codon in order to ensure that the promoter and

106 terminator regions were included. The PCR protocol involved an initial denaturation at

107 94ºC (2 min), followed by 30 cycles of 10 s at 94ºC, 30 s at 52ºC and 3 min at 72ºC.

108 The last cycle was followed by a final extension step of 10 min at 72ºC. PCR fragments

109 were generated with oligonucleotides containing short sequences rec5 (forward) and

110 rec2 (reverse), which are homologous to the sequences in the plasmid (about 35 bp).

111 The plasmid was linearized by *Sal*I digestion and digested with *Asc*I to avoid sticky

112 ends and to make the recombination process easier.[22] Derivative wine yeasts *ho*QA23

113 and *ho*QA23 (Δ*trp1)* were co-transformed with the digested pGREG505 plasmid,

114 together with the PCR-amplified target gene that was flanked by recombination

115 sequences homologous to the plasmid ends. This co-transformation promotes an *in vivo*

116 homologous recombination between both fragments. This recombination process also

117 deleted the *GAL1* promoter of the plasmid (genes were cloned with their own

118 promoters). Transformants were selected by geneticin resistance, which is encoded by

119 the *KanMX4* gene in the plasmid. Correct integration of the gene into the vector was

120 verified by plasmid DNA isolation using a modified version of the protocol described

121 by Robzyk and Kassir[23], and further PCR amplification was performed with the primers

122 specified for sequences rec5 and rec2 (Table 1).

123

124 *Growth media*

125 Strains were cultured in the synthetic grape must (SM) (pH 3.3) described by Riou *et*

126 *al.*[24], but with 200 g/L of reducing sugars (100 g/L glucose + 100 g/L fructose) and

127 without anaerobic factors.[25] The following were utilized: organic acids, malic acid 5

128 g/L, citric acid 0.5 g/L and tartaric acid 3 g/L; mineral salts KH_2PO_4 750 mg/L, K_2SO_4

129 500 mg/L, $MgSO_4$ 250 mg/L, $CaCl_2$ 155 mg/L, NaCl 200 mg/L, $MnSO_4$ 4 mg/L, $ZnSO_4$

130 4 mg/L, $CuSO_4$ 1 mg/L, KI 1 mg/L, $CoCl_2$ 0.4 mg/L, H_3BO_3 1 mg/L and $(NH4)_6Mo_7O_{24}$

131 1 mg/L; vitamins myo-inositol 20 mg/L, calcium pantothenate 1.5 mg/L, nicotinic acid

132 2 mg/L, chlorohydrate thiamine 0.25 mg/L, chlorohydrate pyridoxine 0.25 mg/L and

133 biotin 0.003 mg/L. The assimilable nitrogen source used was 300 mg N/L (120 mg N/L

134 as ammonium and 180 mg N/L in the amino acid form). Tryptophan represented

135 approximately 6.5% of the total ==assimilable organic nitrogen== (12 mg N/L).

136 The population inoculated in the synthetic grape must came from an overnight culture in

137 YPD (1% yeast extract, 2% peptone, 2% glucose) at 30ºC. After counting

138 microscopically, the appropriate dilution of the overnight culture was transferred to SM

139 to achieve an initial cell concentration of 2×10^6 cells/mL.

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141 *Gene expression analysis by real-time quantitative PCR*

142 The total RNA of 10^8 cell/mL was isolated from the different samples, as described by

143 Sierkstra et al.[26], and was resuspended in 50 μL of DEPC-treated water. Total RNA

144 suspensions were purified using the High Pure Isolation kit (Roche Applied Science,

145 Germany), following the manufacturer's instructions. RNA concentrations were

146 determined in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies,

147 USA), and RNA quality was verified electrophoretically in 0.8% agarose gel. Solutions

148 and equipment were treated to leave them RNase-free, as outlined in Sambrook et al.[27]

149 Total RNA was reverse-transcribed with Superscript[TM] II RNase H[-] Reverse

150 Transcriptase (Invitrogen, USA) in a GenAmp PCR System 2700 (Applied Biosystems,

151 USA). The reaction contained 0.5 μg of Oligo $(dT)_{12-18}$ primer (Invitrogen, USA) and

152 0.8 μg of total RNA as a template in a total reaction volume of 20 μL. Following the

153 manufacturer's guidelines, cDNA was synthesized at 42ºC for 50 min after denaturation

154 at 70ºC for 10 min. Then the reaction was inactivated at 70ºC for 15 min.

The primers of the *TAT2* gene were designed according to the *Saccharomyces* Genome

Database. The primers of housekeeping gene *ACT1* have been previously described by

Beltran *et al.*[25] (Table 1). Amplicons were shorter than 100 bp, which ensured maximal

PCR efficiency and the most precise quantification. Real-time quantitative PCR was

performed using the LightCycler® 480 SYBR Green I Master (Roche, Germany). The

SYBR PCR reactions contained 2.5µM of each PCR primer, 5 µL of cDNA and 10 µL

of SYBR Green I Master (Roche, Germany) in a 20-µL reaction.

All the PCR reactions were mixed in a LightCycler® 480 Multiwell Plate 96 (Roche,

Germany) and cycled in a LightCycler® 480 Instrument II, 96-well thermal cycler

(Roche, Germany) under the following conditions: 95ºC for 5 min, and 45 cycles at

95ºC for 10 sec, at 60ºC for 10 sec and 72ºC 10 sec. Each sample had two controls that

were run in the same PCR: no amplification control (sample without reverse

transcriptase reaction) to avoid interference by contaminant genomic DNA, and no

template control (sample without RNA template) to avoid interference by primer-dimer

formation. All the samples were analyzed in triplicate with the LightCycler® 480

Software, version 1.5 (Roche, Germany), and the expression values were averaged. The

gene expression levels of *TAT2* in the overexpressing strains are shown as a relative

value in comparison to their control strains, *ho*QA23-pGREG and pGREG (Δ*trp1)*.

Housekeeping gene *ACT1* was used as an endogenous reference gene to normalize input

amounts. This endogenous *ACT1* gene showed excellent uniformity in the expression

levels of all the study samples.

Generation time (GT)

Growth was monitored at 600 nm in a SPECTROstar Omega instrument (BMG

Labtech, Germany) at 12ºC and 28ºC. Measurements were taken after pre-shaking the

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180   microplate for 20 sec every 30 min for 3 days. At 12ºC, the microplate had to be

181   incubated outside the SPECTROstar spectrophotometer and was then moved inside to

182   take measurements every 8 hours in the lag phase and every 3 hours in the exponential

183   phase. Microplate wells were filled with 0.25 mL of SM medium to reach an initial OD

184   of approximately 0.2 (inoculum level of 2 x 10^6 CFU/mL). Uninoculated wells for each

185   experimental series were also included in the microplate to determine, and to

186   consequently subtract, the noise signal. All the experiments were carried out in

187   triplicate.

188   Growth parameters were calculated from each treatment by directly fitting OD

189   measurements versus time to the reparameterized Gompertz equation proposed by

190   Zwietering *et al.*[28]:

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

192   where y=ln(OD_t/OD_0), OD_0 is the initial OD, OD_t is the OD at time t, D=ln(OD_t/OD_0) is

193   the asymptotic maximum, μ_{max} is the maximum specific growth rate (h^{-1}), and λ is the

194   lag phase period (h).[29] Generation time (GT) was calculated using the GT=ln2/μ_{max}

195   equation.

196   *Fermentations*

197   Fermentations were performed at 28ºC and 12ºC with continuous orbital shaking at 100

198   rpm, using 100-mL bottles filled with 60 mL of SM fitted with caps that enabled carbon

199   dioxide to escape and samples to be removed. The population inoculated in each flask

200   was 2 x 10^6 cells/mL from an overnight culture in YPD. Fermentation was monitored by

201   measuring media specific density with a Densito 30 PX densitometer (Mettler Toledo,

202   Switzerland). Fermentation was considered complete when density was below 0.998.

203   Yeast cell growth was also determined by absorbance at 600 nm and by plating samples

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204   at the end of fermentation on YPD medium in serial decimal dilutions, and they were

205   incubated at 28ºC for 2 days.

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207   *Nitrogen content analysis*

208   Samples were taken at 74 h and 240 h at 28ºC and 12ºC, respectively (around 1.020 of

209   specific density), during fermentation. The individual amino acids present in the media

210   were analyzed by OPA and FMOC derivatizations with the Agilent 1100 Series HPLC,

211   as described in Beltran *et al.*[25] Each amino acid concentration was calculated using

212   external and internal standards, and was expressed as mg N/L. The Agilent ChemSation

213   Plus software was used (Agilent Technologies, Germany).

214

215   *Statistical data processing*

216   All the experiments were repeated at least 3 times and data were reported as the mean

217   value ± SD. Significant differences among the control strain, the mutant and the

218   overexpressing strains were determined by *t*-tests (the SPSS 13 software package). The

219   statistical level of significance was set at $P \leq 0.05$.

220   A principal component analysis (PCA) was done using the *vegan* package (the *rda*

221   function) of the R v.2.15 statistical software (R Development Core Team, 2010).[30]

222

223   **Results**

224   *Gene expression analysis of overexpressing strains by real-time quantitative*

225   *PCR*

226   After constructing the overexpressing strains, we aimed to validate and quantify the

227   gene expression in these strains. Samples were taken at time 0 h (before inoculation), 6

228   and 18 h after inoculation in the synthetic grape must (SM) at low temperature (12ºC).

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229   The relative expression values of *TAT2* are shown in Figure 1. The two overexpressing

230   strains showed a significant overexpression of *TAT2* at time 0 h, which was 4-fold

231   greater than the control for the pGREG *TAT2* strain and 27-fold greater than the control

232   for the pGREG *TAT2* (Δ*trp1)* strain. Moreover, the pGREG *TAT2* (Δ*trp1)* strain also

233   showed overexpression at 6 h and 18 h after inoculation (20- and 5.5-fold more than the

234   control, respectively).

235

236   *Phenotype effect of the mutant and overexpressing strains*

237   *Determination of generation time (GT)*

238   In order to determine the effect of the *TRP1* and *TAT2* deletions on growth, and the

239   overexpression of *TAT2,* in two different genetic backgrounds (tryptophan prototroph

240   and auxotroph), the GT of the mutant and overexpressing strains at 12ºC and 28ºC in

241   the SM were calculated (Table 2). No significant differences in the growth rate of the

242   *TAT2* mutant and the overexpressing strains were found, and only the Δ*trp1* strain

243   showed lower growth rate than its control strain, *ho*QA23, at both temperatures.

244   *Fermentation activity*

245   The fermentation activity of the mutant and overexpressing strains was estimated by

246   calculating the time required to ferment 5% (T5), 50% (T50) and 100% (T100) of the

247   sugars in the SM (Fig. 2). T5, T50 and T100 approximately matched the beginning (lag

248   phase), middle (end of exponential phase) and end of fermentation, respectively. At the

249   beginning of fermentation (T5), no differences were observed at low temperature. The

250   Δ*tat2* strain displayed greater fermentation activity at low temperature, and required a

251   considerably shorter time to consume 50% and 100% of the sugars (17 h and 60 h less,

252   respectively) if compared to the fermentation of the control *ho*QA23 strain at 12ºC.

253   Conversely this strain finished later than the control for the fermentation at 28ºC.

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254 pGREG *TAT2* also finished earlier at 12ºC if compared to its control (*ho*QA23-

255 pGREG), but took longer to finish the fermentation at 28ºC. pGREG *TAT2* (Δ*trp1)* was

256 able to finish fermentation at 28ºC before its control pGREG (Δ*trp1),* but no differences

257 were found at 12ºC. These differences in fermentation activity cannot be ascribed to

258 biomass production because non significant differences were observed in the viable

259 population at the end of the fermentations among the different strains and temperatures,

260 except for the Δ*trp1* strain, which showed a larger population size at 28ºC (Table 3).

261

262 *Nitrogen consumption of mutant and overexpressing strains*

263 Different nitrogen requirements in yeast fermenting were observed at both temperatures.

264 At the same fermentation point (around 1.020 of specific density, which matches the

265 end of the exponential phase), nitrogen consumption by yeast was generally lower at

266 12ºC than at 28ºC (Table 4). These differences between both temperatures were greater

267 for ammonium consumption. Major differences in the amino acids present in the SM

268 between the temperatures were observed in the tryptophan auxotroph strains (Δ*trp1* and

269 pGREG (Δ*trp1*)). Conversely mutations and overexpressions did not significantly

270 modify the uptake of ammonium and amino acids at the same temperature. Only

271 pGREG *TAT2* (Δ*trp1*) at 28ºC consumed more amino acids and ammonium than its

272 control pGREG (Δ*trp1).*

273 In order to explore the effect of deletion/overexpression and temperature fermentation

274 on ammonium and amino acid preferences, a PCA was carried out (Fig. 3). This PCA

275 was performed on the seven strains using the residual concentration (mg N/L) of 20

276 amino acids and ammonium measured in the SM at the analyzed fermentation time

277 point (1.020 of specific density) (Table S1). The first two components were retained and

278 explained 86.7% of total variance. The first component (PC1) explained 68.8% of

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279   variation, and was marked by a high positive component loading for histidine (+0.366)

280   and high negative loadings for glutamine (-0.755) and ammonium (-0.509). The second

281   component explained 17.9% of variation, and was marked by a high positive component

282   loading for glutamine (+0.495) and a high negative loading for ammonium (-0.841).

283   This result revealed the different nitrogen preferences at both temperatures. The most

284   evident result was that control strain *ho*QA23 and its mutants consumed more histidine

285   at the low temperature, and more glutamine and ammonium at the optimum

286   temperature.

287

288   **Discussion**

289   Low temperature produces several effects on biochemical and physiological properties

290   in yeast cells. Recently in their metabolomic study on wine fermentations at low

291   temperature, López-Malo *et al*.[17] reported that the main differences in the metabolic

292   profiling of the *S. cerevisiae* industrial strain QA23 growing at 12ºC and 28ºC were

293   observed in the lipid and amino acid metabolisms. These differences in metabolites

294   might be related to the previously reported different nitrogen consumption pattern at

295   low temperature,[8,9] or with the greater demand of some specific amino acids at low

296   temperature, such as tryptophan.[8,10] In this study, most strains also showed higher

297   nitrogen consumption at the optimum temperature than at 12ºC. As already reported by

298   Beltran *et al*.[9], the main differences in nitrogen use at both temperatures are accounted

299   for by ammonium consumption. This result supports the idea that there is a problem

300   with the uptake or metabolization of one of the main nitrogen sources at low

301   temperature.[9] As previously reported[31], low temperature exerts strong nutritional stress,

302   similarly to growth under nitrogen-limiting conditions. Regarding amino acid

303   consumption, only the tryptophan auxotroph strains (Δ*trp1* and pGREG (Δ*trp1*))

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304   exhibited impaired amino acids uptake at low temperature, which was not affected at the

305   optimum temperature. This lower amino acid consumption resulted in a growth defect

306   in the Δ*trp1* strain, which showed a longer GT at 12ºC in comparison with the wild

307   type, as previously reported.[32] Nevertheless, we also observed impaired growth at the

308   optimal temperature. It is important to consider that our experimental approach differs

309   substantially from those previously reported using laboratory strains and growth

310   conditions in standard laboratory media. We used an industrial wine strain to mimic

311   wine fermentation conditions. Similar non fitting results were also obtained from the

312   fermentation activity of this Δ*trp1* strain. Some authors have revealed that the deletion

313   mutants of tryptophan biosynthesis show ethanol-sensitive growth at the optimal

314   temperature.[16] In comparison with the control strain however, no significant differences

315   were found with the Δ*trp1* strain during the time required to complete fermentation.

316   The cold-sensitive phenotype of the Δ*trp1* strain has been related with impaired

317   tryptophan uptake. Abe and Horikoshi[15] postulated that the increased rigidity of the

318   plasma membrane at low temperature induces conformational changes in permease

319   Tat2p and impairs tryptophan transport. In the present study, no differences were found

320   in the growth rate of Δ*tat2* at both temperatures if compared to *ho*QA23. Thus in this

321   industrial strain, tryptophan uptake must be compensated by the action of other

322   permeases, such as low-affinity tryptophan permease, Tat1p or the general amino acid

323   permease Gap1p. Although it was not statistically significant, this strain consumed more

324   amino acids and ammonium than its control strain *ho*QA23 at low temperature.

325   However one unexpected result revealed that this strain also displayed better

326   fermentation performance at low temperature. Although this direct correlation between

327   *TAT2* deletion and fermentation improvement at low temperature should be confirmed

328   with other industrial strains, our working hypothesis is that the greater nitrogen uptake

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329   of this strain produces a fermentation activity stimulus. Regulation of glycolytic

330   enzymes by ammonium and amino acids uptake has been widely reported.[33,34]

331   Abe and Horikoshi[15] demonstrated that the cells expressing Tat2p at high levels are

332   endowed with the ability to grow under low-temperature conditions in a tryptophan

333   auxotroph laboratory strain. However, the present study evidences no growth

334   improvement with the overexpression of *TAT2* in either the prototroph or the auxotroph

335   background, and this result highlights the importance of the genetic background. As

336   with the mutant strain, the overexpression of *TAT2* also significantly reduced

337   fermentation time.

338   Unexpectedly tryptophan uptake showed few differences between the different strains

339   and temperature. This amino acid was practically consumed completely under all the

340   fermentation conditions (Table S1). The PCA only revealed lower ammonium and

341   glutamine consumption, as previously reported,[9] and higher histidine consumption at

342   low temperature, which has not been reported to date.

343

344   **Conclusions**

345   One conclusion drawn from this study is the need to test the phenotypes observed in the

346   mutant laboratory strain in the genetic background of industrial strains under conditions

347   that mimic the industrial process. Our experience has revealed that many of these

348   phenotypes are not reproduced when transferred to more complex, robust and polyploid

349   industrial strains.[35,36] Tryptophan has been previously reported to be a limiting amino

350   acid during *S. cerevisiae* growth at low temperature, and the results of the present study

351   partially support this assessment. Deletion of *TRP1*, a key gene of tryptophan

352   biosynthesis, impaired the growth rate at low temperature in synthetic must. However, it

353   was also affected at 28ºC. Thus the cold sensitivity of this strain in this genetic

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354   background was not absolutely conclusive. Moreover this growth impairment did not

355   affect the fermentation rate. A more evident phenotype in Δ*trp1* was the lowest amino

356   acid consumption noted at low temperature, which might explain its lower growth rate.

357   Strangely enough, the overexpression of *TAT2* in the Δ*trp1* strain did not reveal that

358   nitrogen consumption recovered sufficiently, whereas deletion of *TAT2* (Δ*tat2*)

359   endorsed this strain with the highest nitrogen consumption capacity at low temperature.

360   This strain underwent significant reduction during the time required to complete

361   fermentation at low temperature. Although this result was unexpected, it may prove

362   promising to meet the objective of obtaining industrial strains with improved

363   fermentation capacity at low temperature through the selection of proper strains or the

364   genetic modification of current industrial yeast.

365

366   **Acknowledgments**

370

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470

471 **Figure legends**

472 **Figure 1.** Relative expression of *TAT2* during SM fermentation at 12ºC. Changes in

473 gene expression in the overexpressing strains are shown as compared to their controls,

474 *ho*QA23-pGREG and pGREG (Δ*trp1)*, at the same fermentation point (0 h, 6 h and 18

475 h), set as value 1. Values higher than 1 indicate a higher gene expression than the

476 control, whereas those lower than 1 denote a lower gene expression than the control.

477 *Statistically significant differences (P-value ≤ 0.05) versus their control strains.

478 **Figure 2.** Determination of the time required by the mutant and overexpressing strains

479 to ferment 5% (T5), 50% (T50) and 100% (T100) of the initial sugar content in a

480 synthetic must at 12ºC (gray bars) and 28ºC (black bars). Positive and negative values

481 represent the increases and decreases in time (h) of the mutant and overexpressing

482 strains if compared to the control strains (normalized as value 0). The fermentation

483 times of the control strains were: *ho*QA23 at 12ºC T5 = 62.75 h ± 2.29 h, T50 = 176.75

484 h ± 3.77 h T100 = 371.75 h ± 8.66 h; at 28ºC T5 = 17.94 h ± 2.26 h, T50 = 50.12 h ±

485 3.99 h, T100 = 133.84 h ± 8.66 h and *ho*QA23-pGREG at 12ºC T5 = 81.75 h ± 5.20 h,

486 T50 = 176.75 h ± 16.75 h, T100 = 361.75 h ± 1.73 h; at 28ºC T5 = 21.85 h ± 1.13 h,

487 T50 = 53.82 h ± 2.10 h, T100 = 136.34 h ± 13.56 h. **pGREG (Δ*trp1)*** at 12ºC T5 =

488 47.75 h ± 1.73 h, T50 = 151.75 h ± 7.40 h, T100 = 341.75 h ± 4.82 h; at 28ºC T5 =

489 20.12 h ± 1.36 h, T50 = 53.38 h ± 1.36 h, T100 = 148.41 h ± 1.85 h. *Statistically

490 significant differences (P-value ≤ 0.05) as compared with their control strains.

491 **Figure 3.** Biplot of the first two PCA components according to the residual amino acids

492 present in synthetic must at a specific density of 1.020 in the 28ºC and 12ºC

493 fermentations. Variables are represented in gray and samples are denoted in black.

Table 1. List of the DNA sequences of the oligonucleotides used in this study.

Primer	SEQUENCE (5´→ 3´)	Use
TRP1-K1[a]	ATGTCTGTTATTAATTTCACAGGTAGTTCTGGTCCATTGGCGTACGCTGCAGGTCGAC	Deletion *TRP1*
TRP1-K2[a]	ATTGTTTTATCGTTTAAAGCAGTTTTTACGATTCTTTATCATCGATGAATTCGAGCTCG	Deletion *TRP1*
cmTRP1F	AAAGACATGGAGGGCGTTAT	Verification deletion *TRP1*
cmTRP1R	GCACTCCTGATTCCGCTAATA	Verification deletion *TRP1*
TAT2- S1[b]	GCAAACAATTACCCGGAAATAGGGCTTAAATCAAGAATAAACCCCTAATTCGTACGCTGC	Deletion *TAT2*
TAT2-C2[b]	GAAGTCCTCATCCACGTATTCAAATGATAATTTTTCAAAATGTCAGGTTTACTAGTGGAT	Deletion *TAT2*
TAT2-ovF	CATGTCCGATAGCGGGAACTGA	Verification deletion *TAT2*
TAT2-ovR	ATTATCGCATGCAAACCAACTTGGCTACCG	Verification deletion *TAT2*
TAT2rec5F[c]	AACAAAAGCTGGAGCTCGTTTAAACGGCGCGCCCATGTCCGATAGCGGGAACTGA	Overexpression *TAT2*
TAT2rec2R[c]	GCGTGACATAACTAATTACATGACTCGAGGTCGACATTATCGCATGCAAACCAACTTGGCTACCG	Overexpression *TAT2*
rec5F[c]	AACAAAAGCTGGAGCTCGTTTAAACGGCGCGCC	Verification overexpression *TAT2*
rec2R[c]	GCGTGACATAACTAATTACATGACTCGAGGTCGAC	Verification overexpression TAT2
TAT2F	CTGGCCACGTGCATTGTCT	Real-time quantitative PCR *TAT2*
TAT2R	GCCTTCATCGCCAGTCTAAATC	Real-time quantitative PCR *TAT2*
ACT1F	TGGATTCCGGTGATGGTGTT	Real-time quantitative PCR *ACT1*
ACT1R	CGGCCAAATCGATTCTCAA	Real-time quantitative PCR *ACT1*

[a] The sequence with homology to the plasmid pAG25 is underlined. The remainder of the primer sequences is homologous to the flanking region of the deleted ORF.

[b] The sequence with homology to the plasmid pUG6 is underlined. The remainder of the primer sequences is homologous to the flanking region of the deleted ORF.

[c] The recombination sequences homologous to the plasmid pGREG 505 ends are underlined.

23

Table 2. Generation time (GT) of the strains.

Strain	12ºC	28ºC
*ho*QA23	22.73 ± 0.48	4.82 ± 0.15
Δtat2	24.20 ± 1.35	4.76 ± 0.10
Δtrp1	25.56± 1.26 *	6.00 ± 0.12 *
*ho*QA23 pGREG	25.55 ± 1.16	4.78 ± 0.14
pGREG *TAT2*	24.47 ± 0.72	4.99 ± 0.11
pGREG (*Δtrp1)*	26.00 ± 2.62	4.71 ± 0.13
pGREG *TAT2* (*Δtrp1)*	24.11 ± 0.18	4.81 ± 0.09

* Statistically significant differences (P-value ≤ 0.05) as compared with their control strain at the same temperature.

Table 3. Viable cells (CFU/mL (10^7) at the end of fermentation at 12ºC and 28ºC.

Strain	Viable cells (CFU/mL (10^7))	
	12ºC	28ºC
*ho*QA23	4.40 ± 1.04	7.53 ± 1.72
Δtat2	5.20 ± 1.76	5.37 ± 0.42
Δtrp1	2.80 ± 0.14	13.83 ± 1.20*
*ho*QA23 pGREG	7.97 ± 2.76	5.20 ± 2.43
pGREG *TAT2*	5.70 ± 2.91	6.57 ± 2.37
pGREG (*Δtrp1)*	4.60 ± 2.34	4.43 ± 1.82
pGREG *TAT2* (*Δtrp1)*	4.37 ± 1.80	4.50 ± 0.44

24

Table 4. Residual Yeast Assimilable Nitrogen (YAN) in the synthetic must (SM) at 1.020 density of fermentation at 28ºC and 12ºC, expressed as amino acid (YANaa) and ammonia (YAN NH₄) (mg N /L)

Strain	*ho*QA23	*Δtat2*	*Δtrp1*	*ho*QA23 pGREG	pGREG *TAT2*	pGREG (*Δtrp1*)	pGREG *TAT2* (*Δtrp1*)
28ºC							
YANaa	85.67 ± 11.34	90.12 ± 2.16	68.18 ± 9.67	102.32 ± 4.50	102.85 ± 13.20	87.57 ± 0.73	$78.75 \pm 3.90_a$
YAN NH₄	2.01 ± 0.43	3.58 ± 2.72	1.56 ± 0.18	1.73 ± 0.07	10.03 ± 7.61	1.62 ± 0.01	$1.52 \pm 0.03_a$
Total YAN	90.80 ± 16.77	93.71 ± 4.88	69.73 ± 9.80	104.11 ± 3.94	112.87 ± 19.43	89.20 ± 0.50	80.27 ± 3.93
12ºC							
YANaa	102.24 ± 7.92	76.27 ± 15.84	$114.33 \pm 8.19_b$	109.64 ± 0.88	118.96 ± 6.90	$120.47 \pm 11.10_b$	105.62 ± 18.93
YAN NH₄	$38.24 \pm 5.73_b$	11.29 ± 9.83	$20.62 \pm 3.05_b$	$22.88 \pm 2.98_b$	17.72 ± 4.77	$14.28 \pm 10.19_b$	14.41 ± 13.79
Total YAN	$140.48 \pm 13.65_b$	87.55 ± 25.67	$134.96 \pm 11.25_b$	$132.52 \pm 2.09_b$	136.68 ± 5.33	134.75 ± 21.28	120.04 ± 32.00

a Significant differences (P-value ≤ 0.05) as compared to their control at the same temperature.
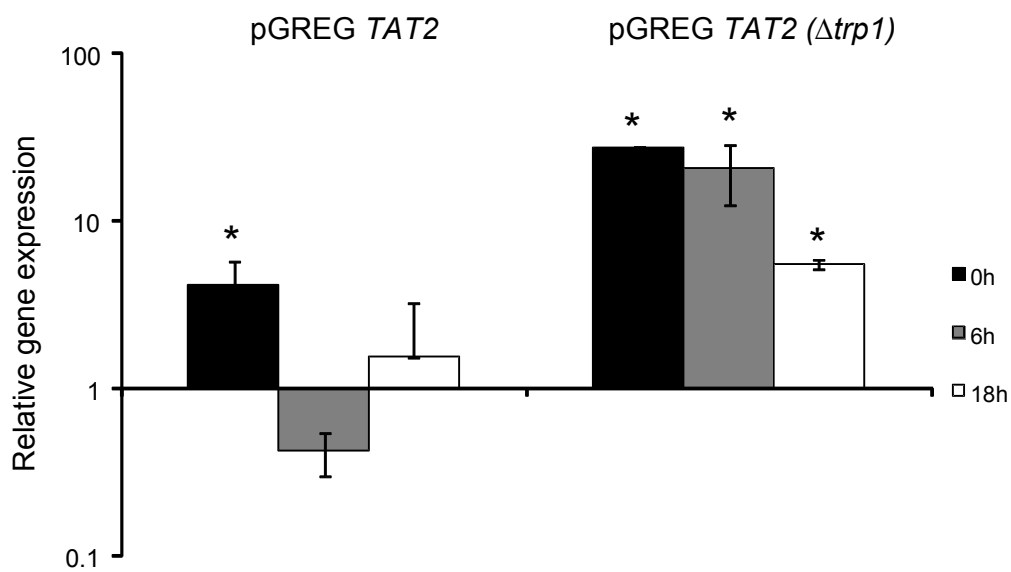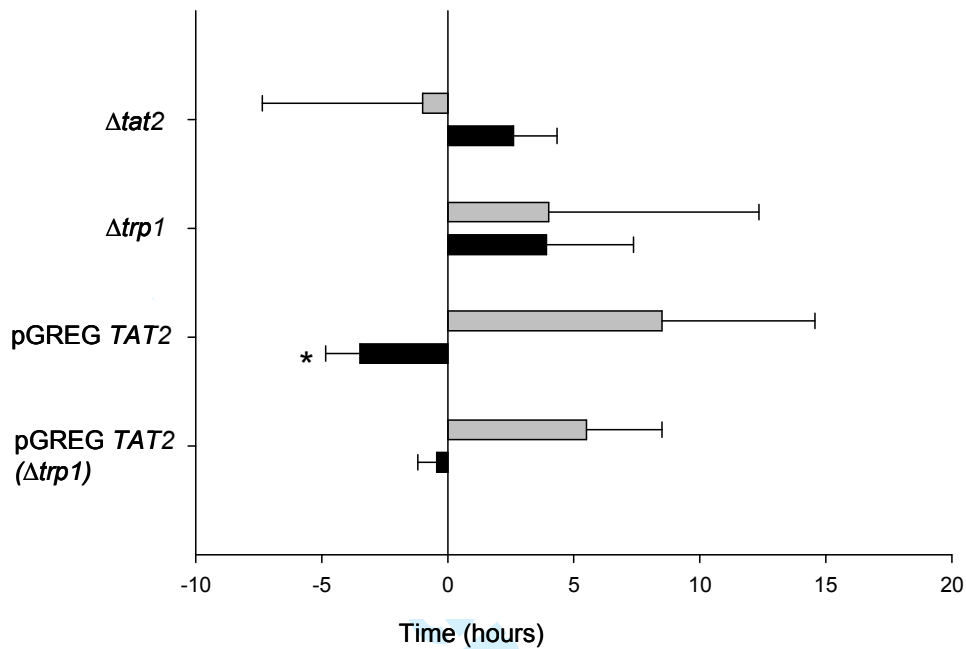b Significant differences (P-value ≤ 0.05) due to temperature.
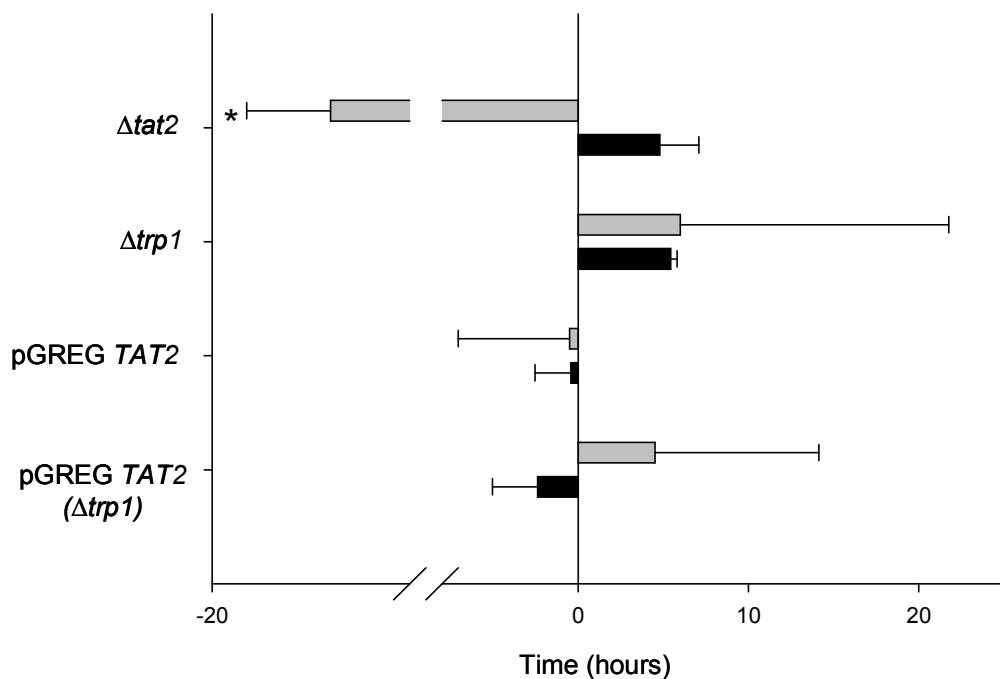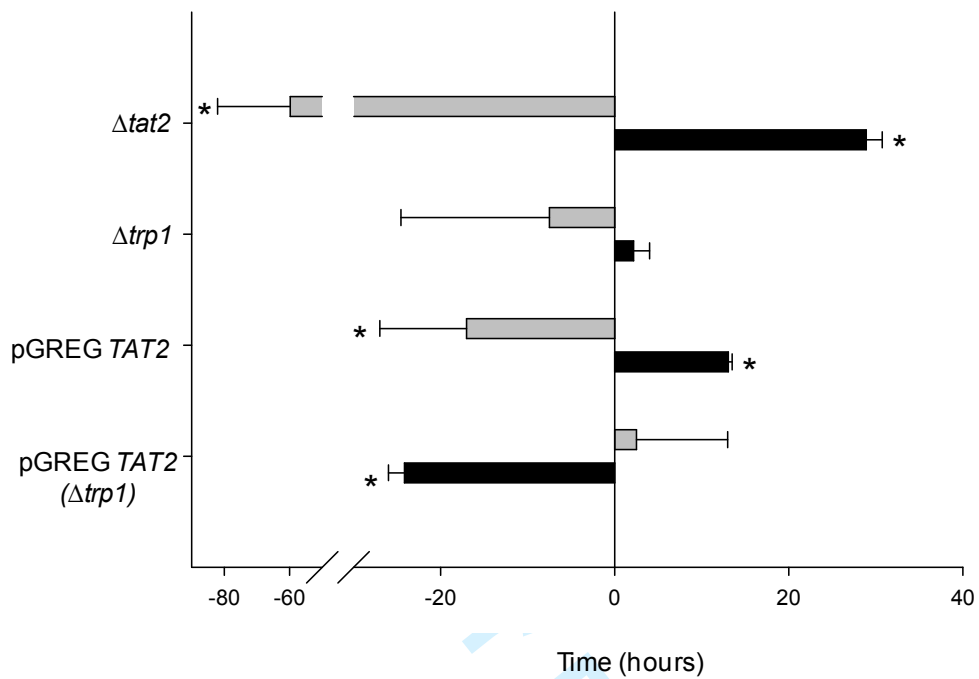
25

Figure 1

Figure 2

T5

T50

T100

Figure 3

Table S1: Residual amino acids present in synthetic must at a specific density of 1.020 in the fermentations at 28ºC and 12ºC, expressed as the mean ± SEM (standard error of the mean) of the amino acid concentration (mg N /L).

	Aspartic	Glutamic	Asparagine	Serine	Glutamine	Histidine	Glycine	Threonine	Arginine	Alanine
28 ºC										
*ho*QA23	0.57 ± 0.09	1.37 ± 0.48	0.1 ± 0.18	0.84 ± 1.00	4.27 ± 3.81	3 ± 0.51	3.53 ± 0.18	1.56 ± 0.44	24.92 ± 2.39	10.57 ± 2.04
Δ*tat2*	0.68 ± 0.07	1.82 ± 0.28	-	0.27 ± 0.01	6.11 ± 0.46	3 ± 0.15$_b$	3.29 ± 0.16	1.8 ± 0.11	25.43 ± 0.33	12.78 ± 0.67
Δ*trp1*	0.31 ± 0.15	0.95 ± 0.35	-	0.21 ± 0.04	1.94 ± 1.10	2.04 ± 0.50$_b$	2.8 ± 0.17$_a$	0.7 ± 1.32	23.28 ± 2.07	6.12 ± 2.00
*ho*QA23 pGREG	-	1.63 ± 0.11	-	2.7 ± 0.20	28.74 ± 3.26	-	3.33 ± 0.53	1.83 ± 0.17	24.21 ± 3.13	11.18 ± 0.75
pGREG *TAT2*	-	2.08 ± 0.33	-	3 ± 0.52	28.57 ± 3.51	-	3.59 ± 0.46	2.03 ± 0.38	26.13 ± 4.35	11.55 ± 1.46
pGREG (Δ*trp1*)	-	1.62 ± 0.06	-	2.23 ± 0.50	25.44 ± 0.53	-	3.85 ± 0.98	1.5 ± 0.31	21.98 ± 1.20	9.26 ± 1.19
pGREG *TAT2* (Δ*trp1*)	-	1.07 ± 0.12$_a$	-	1.46 ± 0.27	22.51 ± 2.14	-	3.29 ± 0.07	1.04 ± 0.17	21.52 ± 0.38	7.11 ± 0.61
12 ºC										
*ho*QA23	1.31 ± 0.18$_b$	2.85 ± 0.24$_b$	0.87 ± 0.01$_b$	2.23 ± 0.48	27.31 ± 4.07$_b$	-	3.14 ± 0.01	1.54 ± 0.38	24.39 ± 0.89	14.43 ± 0.92
Δ*tat2*	0.56 ± 0.34	1.33 ± 0.78	0.77 ± 0.29	0.75 ± 0.63	15.79 ± 7.71	-	3.33 ± 0.01$_a$	0.53 ± 0.41	22.68 ± 0.43$_b$	8.68 ± 4.61
Δ*trp1*	1.49 ± 0.11$_b$	2.88 ± 0.10$_b$	1 ± 0.08$_b$	2.71 ± 0.40$_b$	32.98 ± 3.41$_b$	-	3.4 ± 0.18$_b$	1.77 ± 0.25$_b$	26.79 ± 1.47	15.37 ± 1.05$_b$
*ho*QA23 pGREG	1.28 ± 0.04$_b$	2.9 ± 0.02$_b$	0.82 ± 0.20$_b$	1.98 ± 0.05$_b$	31.5 ± 1.56	-	3.3 ± 0.26	1.49 ± 0.16	25.89 ± 1.62	15.15 ± 0.71$_b$
pGREG *TAT2*	1.06 ± 0.15$_b$	2.37 ± 0.20$_a$	0.83 ± 0.08	1.57 ± 0.31$_b$	27.6 ± 3.18	-	2.65 ± 1.17	1.22 ± 0.23$_b$	23.75 ± 2.15	14.26 ± 0.59$_b$
pGREG (Δ*trp1*)	1.3 ± 0.38$_b$	2.4 ± 0.58	0.63 ± 0.56	2.39 ± 0.96	30.6 ± 6.35	-	3.84 ± 0.30	1.58 ± 0.63	29 ± 1.73$_b$	14.93 ± 2.22$_b$
pGREG *TAT2* (Δ*trp1*)	0.88 ± 0.78	2.25 ± 0.47$_b$	0.89 ± 0.13$_b$	1.76 ± 0.95	28.18 ± 6.04	-	3.97 ± 0.42	1.17 ± 0.55	26.90 ± 3.20	12.73 ± 3.25

a Significant differences (P-value ≤ 0.05) as compared to their control at the same temperature.
b Significant differences (P-value ≤ 0.05) due to temperature.

30

	Tyrosine	Valine	Methionine	Cysteine	Isoleucine	Tryptophan	Leucine	Phenylalanine	Ornithine	Lysine
28ºC										
*ho*QA23	0.03 ± 0.01	-	0.67 ± 0.09	1.06 ± 0.1	1.19 ± 0.10$_b$	-	0.97 ± 0.15	-	0.18 ± 0.07	0.15 ± 0.02
Δ*tat2*	0.04 ± 0	-	0.75 ± 0.09	1.17 ± 0.02	-	0.25 ± 0.02$_a$	1.24 ± 0.11	-	0.23 ± 0.12	0.14 ± 0.01
Δ*trp1*	0.03 ± 0.1	-	0.68 ± 0.11	1.07 ± 0.05	-	0.29 ± 0.01$_a$	1.18 ± 0.13	-	0.26 ± 0.06	0.16 ± 0.05
*ho*QA23 pGREG	-	-	0.65 ± 0.04	0.86 ± 0.14$_b$	-	0.27 ± 0.05$_b$	1.27 ± 0.05$_b$	-	0.28 ± 0.05$_b$	-
pGREG *TAT2*	-	-	1.08 ± 0.55	1.14 ± 0.20$_b$	-	0.28 ± 0.02	1.46 ± 0.25	-	0.23 ± 0.02	-
pGREG (Δ*trp1*)	-	-	0.63 ± 0.04	0.81 ± 0.09$_b$	-	0.27 ± 0.05	1.14 ± 0.16$_b$	-	0.22 ± 0.01	-
pGREG *TAT2 (Δtrp1)*	-	-	0.58 ± 0.02	0.9 ± 0.04	-	0.32 ± 0	0.86 ± 0.04$_b$	-	0.19 ± 0.08	-
12ºC										
*ho*QA23	0.04 ± 0	-	0.66 ± 0.03	1.07 ± 0	-	0.1 ± 0$_b$	1.73 ± 0.06$_b$	-	0.17 ± 0.06	0.11 ± 0.01
Δ*tat2*	0.03 ± 0.01	-	0.52 ± 0.08	0.98 ± 0.16	-	0.15 ± 0$_{ab}$	1.14 ± 0.41	-	0.12 ± 0.01	0.05 ± 0.07
Δ*trp1*	0.05 ± 0	-	0.75 ± 0.08	1 ± 0.06	0.27 ± 0.38	0.49 ± 0.54	0.81 ± 1.14	-	0.2 ± 0.01	0.1 ± 0.11
*ho*QA23 pGREG	0.05 ± 0.01$_b$	0.83 ± 0.03$_b$	0.5 ± 0.02$_b$	-	-	-	-	0.14 ± 0.20	-	2.28 ± 0.19$_b$
pGREG *TAT2*	-	0.82 ± 0.19$_b$	0.71 ± 0.11	-	2.4 ± 1.29$_b$	0.19 ± 0.17	1.11 ± 1.93	11.17 ± 17.20	7.51 ± 11.36	-
pGREG (Δ*trp1*)	0.03 ± 0.03	0.79 ± 0.23$_b$	0.38 ± 0.33	-	3.36 ± 0.45$_b$	0.16 ± 0.27	-	1.08 ± 1.43	1.44 ± 0.03$_b$	2.46 ± 0.19$_b$
pGREG *TAT2 (Δtrp1)*	0.02 ± 0.02	0.31 ± 0.53	0.69 ± 0.13	0.61 ± 0.53	1.28 ± 1.28	0.16 ± 0.28	-	0.12 ± 0.21	0.12 ± 0.13$_a$	1.23 ± 1.00

a Significant differences (P-value ≤ 0.05) as compared to their control at the same temperature.

b Significant differences (P-value ≤ 0.05) due to temperature.

(-) not detected

31