1 **Identification of novel transcriptional regulators of PKA subunits in *Saccharomyces***

2 ***cerevisiae* by Quantitative Promoter–Reporter Screening**

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24   **PKA transcriptional regulation**

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48   **ABSTRACT**

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50   The cAMP dependent protein kinase (PKA) signaling is a broad specificity pathway

51   that plays important roles in the transduction of environmental signals triggering precise

52   physiological responses. cAMP-signal transduction specificity is achieved and controlled at

53   several levels. The *Saccharomyces cereviciae* PKA holoenzyme consists of two catalytic

54   subunits encoded by *TPK1*, *TPK2* and *TPK3* genes, and two regulatory subunits encoded

55   by *BCY1* gene. In this work we studied the activity of these gene promoters using a

56   reporter-synthetic genetic array screen, with the goal of identifying novel regulators of

57   PKA subunits expression. Gene ontology (GO) analysis of the regulators identified showed

58   that these regulators were enriched for annotations associated with roles in several GO

59   biological process, as lipid and phosphate metabolism or transcription regulation and

60   regulate all or some of the four promoters. Further characterization of the effect of these

61   pathways on promoter activity and mRNA levels pointed to inositol, inositol

62   polyphosphates, choline and phosphate as novel upstream signals that regulate transcription

63   of PKA subunit genes. In addition, within each category there are genes that regulate only

64   one of the promoters and genes that regulate more than one of them at the same time. These

65   results support the role of transcription regulation of each PKA subunit in cAMP specificity

66   signaling. Interestingly, many of the known targets of PKA phosphorylation are associated

67   with the identified pathways, opening the possibility of a reciprocal regulation in which

68   PKA would be coordinating different metabolic pathways and these processes would in

69   turn, regulate expression of the kinase subunits.

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INTRODUCTION

The great variety of cellular processes regulated by the cAMP-protein kinase A (PKA)-pathway must be strictly controlled to maintain specificity in the response. Different regulatory mechanisms must exist to ensure the phosphorylation of the correct substrate in response to the proper stimulus. Several combined factors determine the differential effects of the signaling cascade initiated by cAMP. In mammals, different biochemical properties and substrate specificity displayed by PKA isoenzymes and their localization through the association with anchoring proteins (AKAPs) have been shown to contribute to specificity in the cAMP pathway (Skålhegg and Taskén 1997). Transcriptional regulation of the PKA subunits and their level of expression should also have a critical impact on signal specificity, but little is known about how these processes are regulated. The regulatory (R) and catalytic (C) subunits of mammalian PKAs are transcriptionally regulated by hormones and mitogens acting through different receptors like G-protein coupled receptors (Jahnsensg *et al.* 1985; Pariset *et al.* 1989; Landmark *et al.* 1993a, 1993b) or tyrosine kinases-associated receptors (Skålhegg and Taskén 1997). PKA subunits have shown differential expression patterns at different developmental and differentiation stages as well as in different tissues (Cadd and Mcknight 1989; Beebe *et al.* 1990; Hougel 1992; Landmark *et al.* 1993; Reinton *et al.* 1998; Cumming *et al.* 2007). cAMP positively modulates transcription of PKA subunits involving stabilization of their mRNAs as well as on both R and C protein stability after dissociation of the holoenzyme (Houge *et al.* 1990; Taskén *et al.* 1991; Knutsen *et al.* 1991; Hougel 1992).

In the model organism *Saccharomyces cerevisiae*, PKA controls a variety of essential cellular processes associated with fermentative growth, entrance into stationary phase,

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95   stress responses and development (Thevelein and Winde 1999; Thevelein *et al.* 2008;

96   Smets *et al.* 2010). This pleiotropic role of PKA also needs a tight regulation. The structure

97   of the PKA holoenzyme is conserved from mammals to yeast, and consists of a

98   heterotetramer composed of a regulatory subunit homodimer and two associated catalytic

99   subunits. The yeast catalytic subunits are encoded by three genes, *TPK1*, *TPK2* and *TPK3*,

100   and the regulatory subunit, by the *BCY1* gene. Among the factors contributing to the

101   cAMP-PKA pathway specificity in yeast are the synthesis, breakdown and spatial

102   localization of cAMP, Tpk isoform-dependent phosphorylation of substrates and

103   subcellular localization of the holoenzyme (Griffioen and Thevelein 2002; Vandamme *et*

104   *al.* 2012; Engelberg *et al.* 2014). We are interested in understanding how transcriptional

105   regulation of the PKA subunits contributes to the specificity of the cAMP-PKA signaling.

106   We have previously conducted investigations aimed at characterizing the promoter activity

107   of the *BCY1* and *TPK* genes and have demonstrated that the promoter of each isoform of

108   *TPK* and of *BCY1* is differentially regulated during growth phase and stress conditions

109   (Pautasso and Rossi 2014). *TPK1* promoter activity is positively regulated during heat

110   shock and saline stress but *TPK2, TPK3*, and *BCY1* promoters, unlike *TPK1*, are not

111   activated under these stress conditions. Therefore the expression of each PKA subunit

112   involves different mechanisms in response to heat shock or saline stress. However, the four

113   promoters of PKA subunits share an inhibitory autoregulatory mechanism since all of them

114   are downregulated by PKA activity (Pautasso and Rossi 2014). Taking into account these

115   antecedents, our aim in this work was to identify novel transcriptional regulators of PKA

116   subunits. Taking advantage of the unique tractability of yeast, we used an unbiased high-

117   throughput approach to uncover regulators of the promoters of the *BCY1* and *TPK* genes.

118   We performed a reporter-synthetic genetic array (R-SGA) screen previously described to

119   assess the effect of viable deletion mutants on the transcription of *TPK1*, *TPK2*, *TPK3* and

120   *BCY1* promoters. The R-SGA screen makes use of a two-colour promoter–reporter system

121   that is delivered to the array of viable haploid deletion mutants using high-throughput

122   genetics (Kainth *et al.* 2009). This reporter-based screen has shown to be a powerful

123   strategy for identification of regulatory proteins and upstream signals involved in promoter

124   regulation (Kainth and Andrews 2010). Using this approach, we were able to identify

125   unique pathways that differentially regulate the activity of the *BCY1*, *TPK1*, *TPK2* and

126   *TPK3* promoters. Clustering analysis of the genes identified revealed enrichment in genes

127   with roles in several GO biological process. From these GO, lipid and phosphate

128   metabolism, and regulation of transcription categories were further characterized and

129   validated using β-galactosidase reporter assays and qRT-PCR. The results of our genetic

130   screen pointed to inositol, choline and phosphate as novel upstream signals that regulate

131   transcription of PKA subunit genes.

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133   **EXPERIMENTAL PROCEDURES**

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135   ***Strains, plasmids and culture conditions***-Table 1 lists the genotype of the strains used in

136   this study. For the β-galactosidase reporter assays and RNA purification the strains were

137   cultivated at 30° to early log phase (OD_{600}=1) or late log phase (OD_{600}=3.5) in synthetic SD

138   media containing 0.67% yeast nitrogen base without amino acids, 2% glucose plus the

139   necessary additions to fulfill auxotrophic requirements . In the assays to measure inositol

140   and choline effects, yeast cultures were grown at 30° in a complete synthetic medium

141   lacking inositol, choline (Klig and Henry 1984) and uracil (in the case of reporter

142   plasmids). Where indicated, 75 µM inositol (I) and/or 1 mM choline (C) was added. The

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143   assays to analyze the phosphate effect were performed in low-Pi medium containing 0.15

144   mM KH$_2$PO4, and high-Pi medium containing 7.35 mM KH$_2$PO$_4$. High-throughput

145   functional assays were performed with plasmids containing the 5' regulatory region and

146   nucleotides of the coding region of *TPK1*, *TPK2*, *TPK3* and *BCY1* genes (positions -800 to

147   +10 with respect to the ATG initiation codon in each case), cloned into pBA1926

148   controlling GFP expression. The plasmids used in β-galactosidase reporter assays to

149   measure the promoter activities were derived from the YEp357 plasmid (Myers *et al.*

150   1986). The *TPK1-lacZ*, *TPK2-lacZ*, *TPK3-lacZ*, and *BCY1-lacZ* fusion genes contain the

151   same 810 bp regulatory fragments included in the pBA1926 constructs.

152   ***High-throughput functional assay***-The *Saccharomyces cerevisiae* array of 4500 viable

153   strains, each missing a non-essential gene (BY4741 background, Euroscarf) was used in the

154   R-SGA screen (Kainth *et al.* 2009) to detect genes that affect the expression of a GFP

155   reporter system under the control of *TPK1*, *TPK2*, *TPK3* and *BCY1* promoters The -800 to

156   +10 sequences from each promoter were cloned upstream the GFP reporter gene. The

157   constructs were used to transform the BY4256 strain carrying the RPL39pr–tdTomato

158   reporter (RFP). After mating the collection strain with the BY4256 MATα strain carrying

159   each of the *pr*GFP constructs, diploids were selected and sporulated followed by the

160   selection of MATa xxxΔ haploids containing the *pr*GFP plasmid. Robotic manipulation of

161   the collection was performed using a Biomatrix robot and plate imaging system (S&P

162   Robotics Inc.)..Colony size for each arrayed mutant was analyzed and positions on the

163   array with no or slow colony growth were eliminated from further analysis. Colony

164   fluorescence was assayed following 1 and 4 days of incubation on minimal glucose

165   medium using PharosFX Molecular Imager (Bio-Rad). The data was analyzed with Array

166   Gauge V1.2 software. The log2 GFP/RFP ratio from each colony on the array was

167 calculated as described (Kainth *et al.* 2009). A large number of deletion mutants were

168 represented twice in the array, and each screen was performed in duplicate, resulting in

169 quadruplicate measurements for some deletion mutants. These log2 ratios were transformed

170 to robust Z scores using median and median absolute deviation, and p-values were assigned

171 to these Z scores based on a normal distribution. A p-value cut-off of 0.01 and 0.05 was set.

172 A list of 260 deletion mutants that usually appear as hits in these screens (Henny Goettert

173 and Brenda Andrews, personal communication), was removed from further analysis of

174 specific *TPK*s and *BCY1* promoter regulators.

175 ***GO analysis-***The distribution of regulators into functional groups was assessed by

176 measuring the enrichment for genes in the same functional category according to the gene

177 annotation database of FunSpec (http://funspec.med.utoronto.ca/). The enrichment of the

178 genes belonging to the GO Biological Process, GO Molecular Function and MIPS

179 functional classification categories was calculated respect to the total gene number and the

180 total number of genes belonging to the GO category, with a p-value of 0.05 or 0.01. The

181 hypergeometric distribution was used to calculate a p-value for this fraction, and took p-

182 value <0.05 or p<0.01 to be significant. Only the results for stationary growth phase are

183 shown.

184 ***β-galactosidase assays-*** Cells were grown on SD medium at 30° up to an OD_{600} of 3.5.

185 Aliquots (10 ml) of each culture were collected by centrifugation and resuspended in 1 ml

186 buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$). β-

187 galactosidase activity was measured and expressed as Miller units (Miller 1972). Results

188 shown correspond to a representative experiment which was repeated at least three times,

189 each one performed in duplicates or triplicates.

190   ***qRT-PCR***-Total RNA was prepared from different yeast strains, grown at 30° to the same

191   OD_{600} as for β-galactosidase assays, using standard procedures. To determine the relative

192   levels of specific *TPK1*, *TPK2*, *TPK3* and *BCY1* mRNAs, a quantitative RT-PCR

193   experiment was carried out. Aliquots (~10 µg) of RNA were reverse-transcribed into

194   single-stranded complementary cDNA using an oligo-dT primer and Superscript II reverse

195   transcriptase (Life Technologies). The single-stranded cDNA products were amplified by

196   real time PCR using gene-specific sense and antisense primers (mRNA *TPK1*: Fw: 5'

197   CCGAAGCAGCCACATGTCAC 3', Rv: 5' GTACTAACGACCTCGGGTGC 3'; mRNA

198   *TPK2*: Fw: 5' GCTTGTGGAGCATCCGTTTC 3', Rv: 5' CACTAAACCATGGG

199   TGAGC 3'; mRNA *TPK3*: Fw: 5' CGTTGGACAAGACATTCCTG 3', Rv: 5' GTCGGT

200   TATCTTGATATGGCC 3'; mRNA *BCY1*: Fw: 5' CGAACAGGACACTCACCAGC 3',

201   Rv: 5' GGTATCCAGTGCATCGGCAAG 3'; mRNA *TUB1*(α-Tubulin gene): Fw: 5'

202   CAAGGGTTCTTGT TTACCCATTC 3', Rv: 5' GGATAAGACTGGAGAATATGAAAC

203   3'). The PCR products were visualized using SYBR® Green (Life Technologies). The

204   relative mRNA levels of *TPK1*, *TPK2*, *TPK3* and *BCY1* were first normalized to those of

205   *TUB1*and then compared to each other. Quantitative data were obtained from three

206   independent experiments and averaged.

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RESULTS and DISCUSSION

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Validation of the R-SGA genetic screen

We used the Dual-Reporter Functional Genomic Screen, R-SGA approach (Kainth *et al.* 2009) to survey the viable deletion collection for mutants that affect the transcription of promoters of the PKA subunits, (*prTPK1*-GFP, *prTPK2*-GFP, *prTPK3*-GFP and *prBCY1*-GFP). Differential GFP expression was assessed by scanning fluorescence intensities (GFP and control RFP) directly from colonies arrayed on agar plates obtaining in this way a genome-wide result of the effect of viable deletion mutants on the activity of the promoters of PKA subunits. The R-SGA assay was performed from colonies arrayed in defined media grown for four days with glucose as carbon source. The GFP:RFP ratios were normalized and the log2 values calculated as described (Figure S1). Decreased GFP:RFP ratios correspond to deletion of activators while increased ratios reveal deletion of putative repressors (Kainth *et al.* 2009). We considered the possibility that the genome-wide screen gave false positives using a p<0.05, however our control was the cAMP-signaling pathway as regulator. Although not much is known about transcriptional regulation of these genes, we have previously demonstrated that *TPK*s promoters are inhibited by PKA activity, and that Tpk2 had a stronger inhibitory effect on *TPK1* and *TPK3* promoters when cells were grown in liquid cultures (Pautasso and Rossi, 2014). Thus, we expected the deletion of *TPK2* to result in increased *prTPK1*-GFP and *prTPK3*-GFP expression compared to its effect on the control RPL39pr-RFP gene. We found that *TPK2* deletion did in fact cause a defect in *TPK1* transcription (Table S1). Reducing the cutoff to 1%, Tpk2 was not detected; thus, even though the false positives perhaps could be reduced using a lower cut off, this

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237   lower p-value could discard true regulators. The putative transcriptional regulators

238   identified when the cut off was 0.05 are summarized in Table S1.

239   The R-SGA analysis was also performed from colonies arrayed on defined media

240   with glucose as carbon source grown only one night instead of four days (data not shown).

241   The results in this case showed a higher number of negative regulators and a lower number

242   of positive regulators than those identified from plates grown four days (Figure 1). This is

243   in agreement with previous results from our laboratory indicating that all the promoter

244   subunits are upregulated during stationary growth phase (Pautasso and Rossi 2014). Here,

245   although strains were grown in solid media plates instead of liquid cultures, the results

246   showed the same tendency when comparing plates incubated for different periods of time.

247   Altogether, the results of our screen were consistent with previous findings, and

248   successful identifying some of the expected modulators, therefore validating the use of this

249   approach for the unbiased identification of novel regulators of the expression of PKA

250   subunits.

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252   *Novel transcriptional regulators of PKA subunits*

253   We focused our analysis on the results obtained from R-SGA screens performed from

254   colonies arrayed on defined medium with glucose as carbon source grown four days at 30°.

255   The log2 ratios were transformed to Z score and p-values assigned on the basis of a normal

256   distribution (see Materials and Methods). This analysis identified 469 putative

257   transcriptional regulators for *TPK1*, 437 for *TPK2*, 307 for *TPK3* and 299 for *BCY1* (Table

258   S1).

259   Clustering analysis of the genes identified revealed a discrete number of gene

260   ontology (GO) categories significantly enriched in each screen. The categories that were

261   found to be enriched for *TPK1, TPK2, TPK3* and *BCY1* promoters with a cutoff of a p-

262   value < 0.05 are listed in Figure 2 and Table S1. The results showed that several GO

263   categories differentially affected the expression of *TPKs* and *BCY1*, while many others

264   were common to all (Table 2).

265   Using a more stringent cutoff of 0.01 the list of categories regulating PKA promoter

266   activities was reduced from 42 to 9 (*TPK1*, 21,4%) 43 to 18 (*TPK2*, 41,8%) 86 to 19

267   (*TPK3*, 22%) and 57 to 7 (*BCY1*, 12,2%) (Table S1).

268   Overall, the results of the R-SGA screens have unveiled novel regulators of the PKA

269   subunits promoter activities. Different biological process affect different each PKA subunit

270   (twenty six categories), although some of them are shared for two (twelve categories), three

271   (eight categories) or all the subunits (seven categories) (Figure S1). Even more making a

272   detail analysis of all the genes included in the GO categories that were identified affecting

273   all the subunits or at least the catalytic subunits we could observe that not all the same

274   genes included in the GO category are identified as regulators. As is shown in the Figure 3,

275   the Venn diagrams clearly show this result. Taking as a whole these results strengthen our

276   hypothesis about the role of transcription regulation to collaborate with cAMP-PKA

277   specificity signaling.

278   Interestingly two interrelated categories, lipid metabolism and phosphate metabolism

279   were differentially enriched in the four screens (Figure 2). Lipid metabolism appeared as

280   modulator of only *TPKs* promoter activities (not *BCY1* promoter), while Pi metabolism as

281   regulator of *TPK2, TPK3* and *BCY1* promoters (Figure 3).

282   Lipids are major constituents of structural assembly of the cell and are integral to the

283   growth, development and maintenance of the organism. In *S.cerevisiae*, lipid metabolism is

284   altered by growth phase, by supplementation with inositol or choline and this regulation is

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285   controlled by genetic and biochemical mechanisms (Carman and Henry 2007). The

286   regulation of genes related to phospholipid biosynthesis in *S. cerevisiae* through cis-acting

287   upstream activating sequence (UAS$_{INO}$) by inositol supplementation is thoroughly known.

288   In addition, a role for inositol-polyphosphates, product of the reaction catalysed by the

289   phosphatidylinositol-specific phospholipase C (Plc1) in the direct regulation of lipid

290   biosynthesis has been proposed (Rupwate *et al.* 2012).

291   Lipids were only considered for years as building blocks for biological membranes,

292   without any other specific functions. However, this idea has changed during the last two

293   decades since certain classes of lipids are demonstrated to be involved in intracellular

294   signaling processes or work as sensors of the membrane status of a cell. Among the lipids

295   with important functions, phosphatidylinositol (PI) and derivatives are known to be

296   involved in transduction of intracellular signals (Lemmon 2003) besides their role in vesicle

297   trafficking (Odorizzi *et al.* 2000). Degradation products of phospholipid hydrolysis,

298   phosphatidic acid (PA), diacylglycerol (DAG) and fatty acids, as well as sphingolipids,

299   have also been shown to serve as messenger molecules. The intracellular amount of PA

300   triggers the transcriptional regulation of genes containing the UAS$_{ino}$. These genes are

301   regulated by the transcription factors Ino2, Ino4, and the repressor Opi1. The transcriptional

302   regulation of UAS$_{ino}$ genes is generally controlled by the rate of phospholipid synthesis.

303   (Henry *et al.* 2012). Under certain growth conditions (exponential phase, inositol

304   depletion), the levels of PA are relatively high (by the action of several enzymes, among

305   them Dgk1), and the Opi1 repressor is tethered to the nuclear/ER membrane through

306   interaction with the integral membrane protein Scs2, an association is stabilized by

307   interaction with PA. In this condition, UAS$_{INO}$-containing genes are maximally expressed

308   by the Ino2-Ino4 activator complex. In stationary phase, or with inositol supplementation,

309 the levels of PA are reduced, Opi1 dissociates from the nuclear/ER membrane, and enters

310 into the nucleus where it binds to Ino2 and attenuates transcriptional activation by the Ino2–

311 Ino4 complex.

312 Among the genes identified in the GO category of lipid metabolism (summarized in

313 Table S1), were many of the genes mentioned previously. these included genes known to be

314 regulated by inositol and choline, such as *INO1*, *CHO2*, *SCS2*, *EKI1, FEN1,*and the

315 transcription factors *INO2* and *INO4*, and genes involved in inositol polyphosphate

316 metabolism including *PLC1* and those encoding the inositol kinases *ARG82, IPK1* and

317 *KCS1*. The enrichment of this category survived was also detected with a more stringent

318 cutoff of $p<0.01$,. In addition, genes coding for the phosphoinositide phosphatases *SAC1*

319 and *INP51* as well as genes involved in sphingolipid metabolism like *ISC1* coding for the

320 inositol phosphosphingolipid phospholipase C and *ELO2* and *ELO3* coding for fatty acid

321 elongases involved in sphingolipid synthesis were identified. It is worth noting that

322 phosphatidiylinositol (PI) metabolism in yeast is directly linked to the synthesis of

323 sphingolipids as PI donates the phosphoinositol head group that is combined with ceramide

324 for the production of complex sphingolipids.

325 Altogether these results pointed to a possible novel role of inositol as an upstream

326 regulator of PKA subunits expression. To test this *TPK1*, *TPK2*, and *TPK3* promoter

327 activities were assessed using promoter-lacZ-based reporter assays, in wild type cells

328 (strain BY4741) grown to late log phase (OD_{600} 3.5) in minimal medium containing

329 glucose but lacking or supplemented with 75 μM inositol and 1mM- choline (Figure 4A,

330 upper panel). In fact, the activity of all three promoters were regulated by the presence of

331 inositol. Interestingly, addition of choline to inositol depleted medium also activated these

332 promoters and no major differences were found when inositol and choline were added

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333   together. This is somehow surprising as the effect of exogenous choline is usually much

334   less dramatic than the effects of exogenous inositol in UAS$_{INO}$ containing genes (Carman

335   and Henry 2007). In addition, no classic UAS$_{INO}$ sequences can be detected in any of the

336   promoters analyzed. Addition of choline is known to significantly alter phosphatidylcholine

337   turnover and the generation of the signalling lipids phosphatidic acid and diacylglycerol

338   (Dowd *et al.* 2001)Altogether this may suggest that the effect of inositol and choline on the

339   *TPKs* promoter activities is indirect by modulating the levels of signaling lipids and

340   signaling soluble products of phospholipid turnover like inositol polyphosphates. (Lee *et al.*

341   2008)

342   PKA subunit promoters were activated and not repressed by inositol. In fact, many

343   genes involved in phospholipid metabolism in yeast are repressed in response to inositol.

344   For example, INM1 gene, encoding inositol 3-phosphate phosphatase, DPP1 and PAH1

345   genes, encoding lipid phosphate phosphatases and PIS1 gene, encoding phosphatidyl-

346   inositol synthase are derepressed in the presence of inositol and in stationary phase

347   ((Murray and Greenberg 1997; Oshiro *et al.* 2000; Jani and Lopes 2008). In addition to

348   these, microarray analysis has also revealed many additional genes, that are regulated in

349   response to the presence or absece of inositol and choline which are not involved in lipid

350   metabolism (Santiago and Mamoun 2003; Jesch *et al.* 2005). In order to understand the

351   mechanism involved in the regulation of PKA subunits expression we further tested the

352   effect that deletion of various genes forming the inositol/PA sensing core had on the

353   promoter activities of the PKA subunits. Reporter assays using β-galactosidase activity for

354   each PKA subunit promoters were measured in mutant strains Δ*ino2*, Δ*ino4*, Δ*scs2*, Δ*opi1*

355   and Δ*dgk1* (Figure 5A). The results indicated that the lack of *INO2*, *INO4* or *DGK1*

356   upregulated *TPKs* promoters. On the contrary, in the Δ*opi1* strain *TPK1*, *TPK2* and *TPK3*

357   promoters were downregulated. Taking into account these results we suggest that the

358   regulation by inositol on *TPKs* promoters is indirect, and that Ino2/4 may regulate another

359   gene that has repressor activity on *TPKs* promoters. To validate the results with another

360   approach, the same mutant strains lacking the genes encoding regulatory proteins of the

361   phospholipid biosynthetic pathway were used to measure the endogenous mRNA levels of

362   each PKA subunit by RT-qPCR. The results showed that the mRNA levels of each subunit

363   were not completely in agreement with promoter activities (Figure 5B), In the the Δ*ino4*

364   strain for *TPK1* and *TPK2* promoters, and Δ*ino2* strain for the three *TPKs* promoters, the

365   mRNA levels were opposed to the promoter activity. Converse trends were also found for

366   Δ*opi1*.

367   These results, although unexpected, were not surprising since it was reported some

368   genes involved in lipid metabolism that are regulated at the level of mRNA stability *INO4,*

369   *INO1* and *CHO2* genes, which are also involved in the phospholipid biosynthetic pathway,

370   are regulated at the level of mRNA stability by inositol and choline (Robinson and Lopes

371   2000). The inverse relation with reporter assays in Opi1 mutant strain suggest that Opi1

372   could regulate the PKA subunits expression at the transcription level and mRNA stability

373   as it was already proposed for the regulation of *INO4* expression (Robinson and Lopes

374   2000).

375   This type of regulation has been further supported by the discover of synthegradase

376   factors that modulate not only the transcription but also the decay of mRNA as a two-arm

377   mechanism to be more responsive to regulatory signals (Henry *et al.* 2012). Recent studies

378   have demonstrated that environmentally induced genes are subject to transcriptional

379   upregulation along with an increase in decay rate of the transcripts (Bregman *et al.* 2011).

380   This coupling almost always involves enhancement of both mRNA synthesis and decay or,

381 conversely, repression of both mRNA synthesis and decay (Shalem *et al.*; Molin *et al.*

382 2009; Elkon *et al.* 2010; Rabani *et al.* 2011). Finally it has been proposed that an important

383 proportion of the yeast genes examined is likely to be regulated by synthegradases during

384 optimal proliferation conditions and the number of genes is likely to increase in stress

385 conditions (Dori-Bachash *et al.* 2011). All these antecedents would indicate an important

386 role of promoters, transcription factors and synthegradases in the regulation of both

387 synthesis and decay of the PKA subunits mRNAs.

388 Another GO category which showed significant gene enrichment was phosphate

389 metabolism including PHO80, PHO81, PHO84, PHO85 and PHO87 among other genes.

390 The complex formed by the regulatory proteins Pho80, Pho81 and the cyclin kinase Pho85

391 are known to regulate the response to phosphate limitation (Huang et al. 2007) Similar to

392 inositol and choline the presence of high phosphate (7.35 mM KH2PO4) increased the

393 activity of all promoters compared to low phosphate (0.15 mM KH2PO4) (Figure 4B).

394 Therefore promoter activity was lower in conditions of phosphate limitation. The effect of

395 phosphate may be linked to its regulatory role on inositol metabolism and the generation of

396 inositol polyphosphates, which are known regulators of gene expression in the phosphate

397 sensing pathway (Lee *et al.* 2008).In addition, it was reported that phospholipid

398 biosynthesis is coordinated with phosphate utilization via the bHLH proteins, Ino2/4 and

399 Pho2/4 (He *et al.* 2012). We have observed that the PKA subunit promoters responded to

400 the presence of inositol, choline and also Pi, suggesting a possible cross-regulation between

401 both pathways.

402 The repression of genes containing UAS_{INO} is considered to be a mechanism for

403 preferentially synthesizing large amounts of PI by inhibiting phosphatidylserine (PS)

404 synthase activity, thereby making CDP-DAG available for PI synthase (Kelleys *et al.*

17

405   1988). This in turn, would lead to PI phosphorylation and formation of phosphoinositides

406   Breakdown of PI(4,5)P$_2$ gives rise to a pool of inositol-polyphosphates with important roles

407   in the control of gene expression (Rupwate (Banfic $et\ al.$ 2013; Galdieri $et\ al.$ 2013). We

408   have found that several genes included in the pathway of biosynthesis of inositol-

409   polyphosphates were functionally enriched (p<0.01), indicating the possible importance of

410   these phosphoinositides in the regulation of the activity of PKA subunit promoters. The

411   hydrolysis of PI(4,5)P$_2$ by Plc1 yields two prominent eukaryotic second messengers: 1,2-

412   diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) (Divecha and Irvine 1995;

413   Carman and Han 2011). Plc1 and four inositol polyphosphate kinases (Ipk2p/Arg82p,

414   Ipk1p, Kcs1p, and Vip1p) constitute a nuclear signaling pathway that affects transcriptional

415   control (Odom $et\ al.$ 2000), export of mRNA from the nucleus (Monserrate and York

416   2010), homologous DNA recombination, cell death, and telomere length (Banfic $et\ al.$

417   2013). All of these genes have been identified in our screen as regulators of PKA subunit

418   promoters. We are further investigating the role of this important lipid signaling pathway in

419   the regulation of PKA subunits expression.

420   , The synthesis of phospholipids is regulated by controlling the expression of

421   enzymes and/or by modulating its catalytic activities. The protein kinases known to

422   regulate the function of catalytic and regulatory proteins in the phospholipid synthesis

423   include protein kinase A, protein kinase C, casein kinase II, and cyclin-dependent kinase.

424   Phospholipid synthesis enzymes, which are regulated by phosphorylation, include PS

425   synthase, CTP synthetase, choline kinase, and PA phosphatase. The transcriptional

426   repressor Opi1 is also regulated by phosphorylation by PKA. Is interesting to notice that

427   our results indicate the possibility of a regulatory loop in which the expression of PKA

428   subunits is controlled by lipid metabolism pathway and PKA activity regulates in turn,

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429   enzymes involved in phospholipids synthesis by phosphorylation. The same possible effect

430   was proposed above in the case of a reciprocal regulation among PKA coordinating

431   respiratory function and the respiratory metabolism upregulating PKA subunit promoter

432   activities. And in the same line of thought is the autoregulatory loop by which PKA activity

433   regulates the promoter activities of the enzyme subunits.

434   Finally we analyzed a group of transcriptional regulators that arose from the screen,

435   including transcription factors as Swi4, Cbf1, Rtg3, Mot3 and Flo8, and chromatin

436   remodelers Snf2 and Snf5, employing the reporter gene methodology in mutant strains

437   (Figure 6). The results demonstrate the different effect that the absence of each

438   transcription regulator caused on each PKA subunit promoter. The case of Flo8

439   transcription factor is noteworthy since it was identified as regulator of *TPK3* and *BCY1*

440   transcription in our R-SGA screens even though the colonies in the array have a BY4741

441   background. It is well known that yeast strains derived from S288c, like BY4741, are

442   incompetent for filamentous growth due to a mutation in the *FLO8* gene (*flo8-1*) that

443   produces a truncated transcription factor altering expression of *FLO11* (Liu *et al.* 1996).

444   The initial results from our screens suggested a role for this truncated protein and were

445   further confirmed by measuring the levels of mRNA in the *flo8Δ* strain (Figure-6). This

446   deletion mutant displayed robust changes in all *TPK* and *BCY1* mRNAs, with the greatest

447   effects on *TPK3* and *BCY1*. Our findings are in agreement with previous work reporting

448   mRNA changes in the *flo8Δ* mutant for all TPKs and BCY1, analyzed using microarray

449   technology (Hu *et al.* 2007). Interestingly, it has been recently reported that the C-terminal

450   region of Flo8 has a novel transcriptional activation domain with a crucial role in activating

451   transcription (Kim *et al.* 2014).

452　　　　In addition other ategories related to mitochondria, vacuole, amino acids metabolism,

453　　　transport and sporulation also affected the expression of all PKA subunits. While it is well

454　　　documented for example that the cAMP pathway regulates mitochondria function as

455　　　unregulated PKA activity can lead to the production of mitochondria that are prone to the

456　　　production of ROS, and to an apoptotic form of cell death (Feliciello *et al.* 2005; Aun *et al.*

457　　　2013; Galello *et al.* 2014). Other processes as the progression of sporulation are known to

458　　　be regulated from an interconnected signaling network that includes Ras/cAMP pathways

459　　　altered even on rich media (Honigberg and Purnapatre 2003). Taking into account the

460　　　reported antecedents mentioned above is clear how PKA activity regulates sporulation and

461　　　mitochondria functioning, however it is not clear how the dysfunction of these processes

462　　　would deregulate the expression of PKA subunits. Recently published results from our

463　　　laboratory (Pautasso and Rossi 2014) demonstrated that the *TPK1*, *TPK2*, *TPK3* and *BCY1*

464　　　promoter activities are upregulated during the stationary phase of growth in comparison

465　　　with logarithmic phase. Additionally we have also observed an upregulation of the

466　　　promoters of PKA subunits during growth in the presence of glycerol as carbon source,

467　　　(results to be published elsewhere). Thus, during a respiratory metabolism, when a proper

468　　　mitochondrial functioning is necessary, there is an activation of the PKA subunit

469　　　promoters. Here, our SGA results are suggesting that defective mitochondria deregulate

470　　　PKA subunits promoter activity. Therefore a reciprocal regulation could be proposed by

471　　　which PKA regulation plays a physiological role in coordinating respiratory function with

472　　　nutritional status in budding yeast and the respiratory metabolism would upregulate PKA

473　　　subunit promoter activities.

474　　　　Another interesting example is the regulation of vacuolar and proton acidification.

475　　　Mutants lacking several subunits of the vacuolar-ATPase (*vma2, vma4, vma5, vma7,*

476   *vma13, vma21 and vma22*) have been identified in our screen. The V-ATPase is a proton

477   pump that regulates cytosolic pH by pumping protons into the vacuole. Recent studies

478   suggest that the V-ATPase is required for the glucose-mediated stimulation of PKA and

479   that cytosolic pH serves as a second messenger in this regulatory pathway (Dechant and

480   Peter 2010). Activation of V-ATPase is required for full activation of PKA upon glucose

481   stimulation, thereby transducing, at least in part, the pH signal to PKA. As PKA activity

482   autoregulates the activity of its own subunit promoters, malfunction of V-ATPase could

483   indirectly impact the activity of the promoters by affecting the kinase activity.

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486   **CONCLUSIONS**

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488   Regulation of eukaryotic gene expression is controlled at multiple levels, each

489   distinguished by different spatial and temporal arrangements. The combination and

490   orchestration between regulatory mechanisms at various levels are central to a precise gene

491   expression pattern, which is essential to many critical biological processes. Kinases are

492   sensitive to environmental perturbations and have different functions under different

493   growth conditions. A kinase has many substrates and the signal specificity is key to achieve

494   the exact and appropriate response to a given stimulus. In the cAMP-PKA pathway, the

495   regulation of signal specificity is attained at several levels, one of them is the regulation of

496   PKA subunits transcription. Our results showed an integrate network of common and

497   singular regulators pathways of the transcription of each PKA subunit supporting the key

498   role of the expression regulation of each subunit to achieve the final specificity in the

499   cAMP-PKA signaling to sense and respond to environmental stimuli. Altogether, the

500 results of our screen were consistent with previous findings and successful in identifying

501 some of the expected modulators, while pointing to inositol and inositol-polyphosphates,

502 choline and phosphate as novel upstream signals that regulate transcription of PKA subunit

503 genes. Our results also open the possibility of a regulatory loop where pathways regulated

504 by PKA phosphorylation control in turn, the level of expression of different PKA isoforms.

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698   **FIGURE LEGENDS**

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700   **Figure 1-** Regulators, activators or inhibitors, identified in the high-throughput

701 functional assay from plates grown 1 (early log phase) or 4 (late log phase) days of

702 incubation.

703   **Figure 2-** Functional categorization of genes regulators identified in the screening for

704 *TPK1* promoter and of all genes in *S.cerevisiae*. Classifications were performed based on

705 same functional category according to the gene annotation database of FunSpec

706 (http://funspec.med.utoronto.ca/). Black and white bars represent the genome gene fraction

707 in this category and the regulators fraction identified respectively.

708

709   **Figure 3-** Venn diagrams including the regulators genes identified and indicating the

710 common and differential regulators for each PKA subunit. Each diagram corresponds to

711 one of the GO process analyzed: A) Lipids metabolism, B) Phosphate metabolism and C)

712 Transcription regulation.

713

714   **Figure 4 - A)** Upper panel, β-galactosidase activity was determined in WT cells

715 (strain BY4741) carrying the *TPKs*-lacZ or *BCY1*-lacZ fusion gene. Cells were grown up to

716 OD_{600}=3.5 in minimal medium glucose lacking or supplemented with I and C. β-

717 galactosidase activity is expressed in Miller Units. Results are expressed as the mean ± SD

718 from triplicates within a representative assay and normalized to the *TPK1* values. Lower

719 panel, *TPKs* and *BCY1* endogenous mRNA levels were determined in WT strain (BY4741)

720 grown in the same conditions described above. The values were normalized to *TUB1*

721 mRNA. The mRNA level for each subunit in the WT strain was defined as 1. **B)** β-

722   galactosidase activity was measured as in A but using minimal medium with high

723   phosphate (Pi) or low Pi.

724

725   **Figure 5- A)** β-galactosidase activity was determined in WT (BY4741) *opi1Δ*, *ino2Δ*,

726   *ino4Δ, scs2Δ* or *dgk1Δ* strains carrying the *TPKs*-lacZ or *BCY1*-lacZ fusion gene. The

727   results are expressed in Miller Units and as the mean ± SD for replicate samples (n=4) from

728   independent experiments. **B)** *TPKs* and *BCY1* endogenous mRNA levels were determined

729   in WT (BY4741), *opi1Δ*, *ino2Δ*, *ino4Δ, scs2Δ* or *dgk1Δ* strains by qRT-PCR and

730   normalized to *TUB1* mRNA. The mRNA level for each subunit in the WT strain was

731   defined as 1.

732

733   **Figure 6-** *TPKs* and *BCY1* endogenous mRNA levels were determined in WT

734   (BY4741), *cbf1Δ*, *mot3Δ*, *flo8Δ, swi4Δ*, *snf2Δ*, *snf5Δ* and *rtg2Δ* strains by qRT-PCR and

735   normalized to *TUB1* mRNA. The mRNA level for each subunit in the WT strain was

736   defined as 1.

737

738   **Supplementary Figure 1-** Distribution of log2 GFP:RFP ratios from genome-wide

739   analysis of the *TPK1*, *TPK2 TPK3* and *BCY1* promoters grown in SD medium. The y-axis

740   represents log2 GFP:RFP ratios measured from each deletion mutant displayed on the x-

741   axis. The promoters-GFP fluorescence intensities are standardized to the control RPL39pr-

742   RFP intensities.

743

744   **Supplementary Table 1-** Functional categorization of genes regulators identified in

745   the screening for *TPK1*, *TPK2, TPK3* and *BCY1* promoters and of all genes in *S.cerevisiae*.

33

746   Classifications were performed based on the categories defined in the GO biological

747   process, GO molecular function and MIPS databases using FunSpec.

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TABLE 1

YEAST STRAINS

Strain	Genotype	Reference or source
BY4256	*MATα can1Δ::STE2pr-his5 lyp1Δ HO::RPL39pr-tdTomato::hphMX*	Kainth *et al.*, 2009 [1]
BY4741 (WT)	*Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
ino2Δ	(BY4741) *ino2::KanMX4*	EUROSCARF
ino4Δ	(BY4741) *ino4::KanMX4*	EUROSCARF
dgk1Δ	(BY4741) *dgk1::KanMX4*	EUROSCARF
opi1Δ	(BY4741) *opi1::KanMX4*	EUROSCARF
rtg2Δ	(BY4741) *rtg2::KanMX4*	EUROSCARF
flo8Δ	(BY4741) *flo8::KanMX4*	EUROSCARF
swi4Δ	(BY4741) *swi4::KanMX4*	EUROSCARF
snf2Δ	(BY4741) *snf2::KanMX4*	EUROSCARF
snf5Δ	(BY4741) *snf5::KanMX4*	EUROSCARF
mot3Δ	(BY4741) *mot3::KanMX4*	EUROSCARF
cbf1Δ	(BY4741) *cbf1::KanMX4*	EUROSCARF

[1] Kainth, P., and B. Andrews, 2010. Illuminating transcription pathways using fluorescent reporter genes and yeast functional genomics. Transcription 1: 76–80.

TABLE 2 CLUSTERING

| CLUSTER	TPK1	TPK2	TPK3	BCY1
actin cortical patch localization				▪
activation of MAPK cascade				▪
aminoacid metabolism	▪	▪	▪	▪
cAMP-mediated signaling		▪		
carbohydrate metabolism	▪			
cell redox homeostasis	▪			
cellular bud site selection	▪			
cellular copper ion homeostasis	▪			
cellular ion homeostasis		▪		
cellular response to anoxia	▪			
cleavage of tricistronic rRNA transcript				▪
COPI coating of Golgi vesicle			▪	
defense response			▪	
DNA process			▪	
endocytosis		▪		
endoplasmic reticulum inheritance	▪			
filamentous growth			▪	
histone modification	▪		▪	
intracellular signal transduction			▪	
intralumenal vesicle formation	▪			
iron assimilation by reduction and transport				▪
karyogamy			▪	
lipid metabolic process	▪			
meiosis			▪	
metabolic process		▪		
methylation				▪
microautophagy		▪		
mitochondria process	▪			
mitotic recombination	▪			
mRNA processing	▪			
nucleoside metabolism	▪			
pectin catabolic process			▪	
plasma membrane fusion				▪
polyphosphate metabolic process	▪	▪	▪	▪
propionate metabolic process	▪			
protein metabolism	▪	▪	▪	▪
pyridoxine metabolic process			▪	▪
rDNA accumulation involved in cell aging			▪	
reactive oxygen species metabolic process	▪			
regulation of calcium ion-dependent exocytosis			▪	
regulation of cell budding			▪	
regulation of cell polarity	▪			
regulation of nitrogen utilization	▪			
response to DNA damage stimulus			▪	
response to drug	▪			
Rho protein signal transduction				▪
rRNA (guanine-N7)-methylation			▪	
sporulation	▪	▪	▪	
sulfate assimilation	▪	▪		
transcription	▪	▪	▪	▪
translation		▪	▪	▪
transport	▪	▪	▪	▪
vacuole process	▪	▪	▪	▪

Figure 1
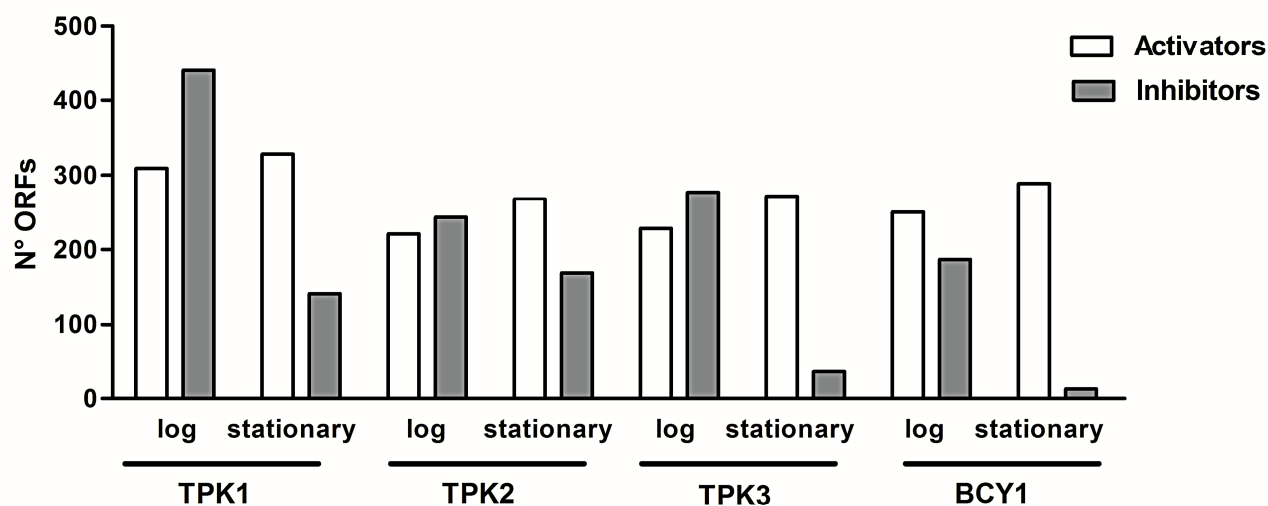
Figure 1- Regulators, activators or inhibitors, identified in the high -throughput functional assay from plates grown 1 (early log phase) or 4 (late log phase) days of incubation.
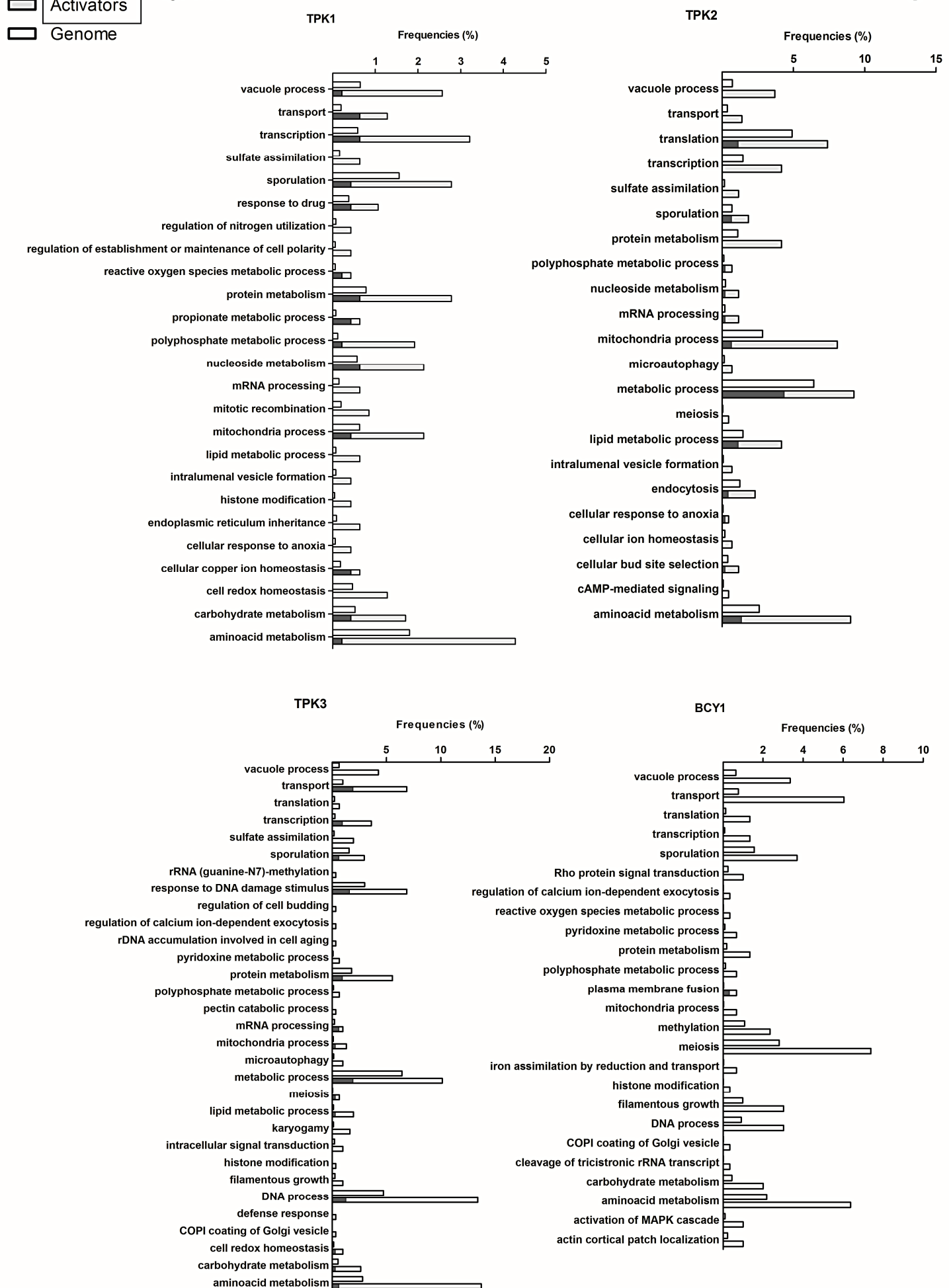
Figure 2

Figure 2- Functional categorization of genes regulators identified in the screening for *TPK1* promoter and of in *S.cerevisiae*. Classifications were performed based on same functional category according to the gene a database of FunSpec (http://funspec.med.utoronto.ca/). Black and white bars represent the genome gene fracti category and the regulators fraction identified respectively

Figure 3
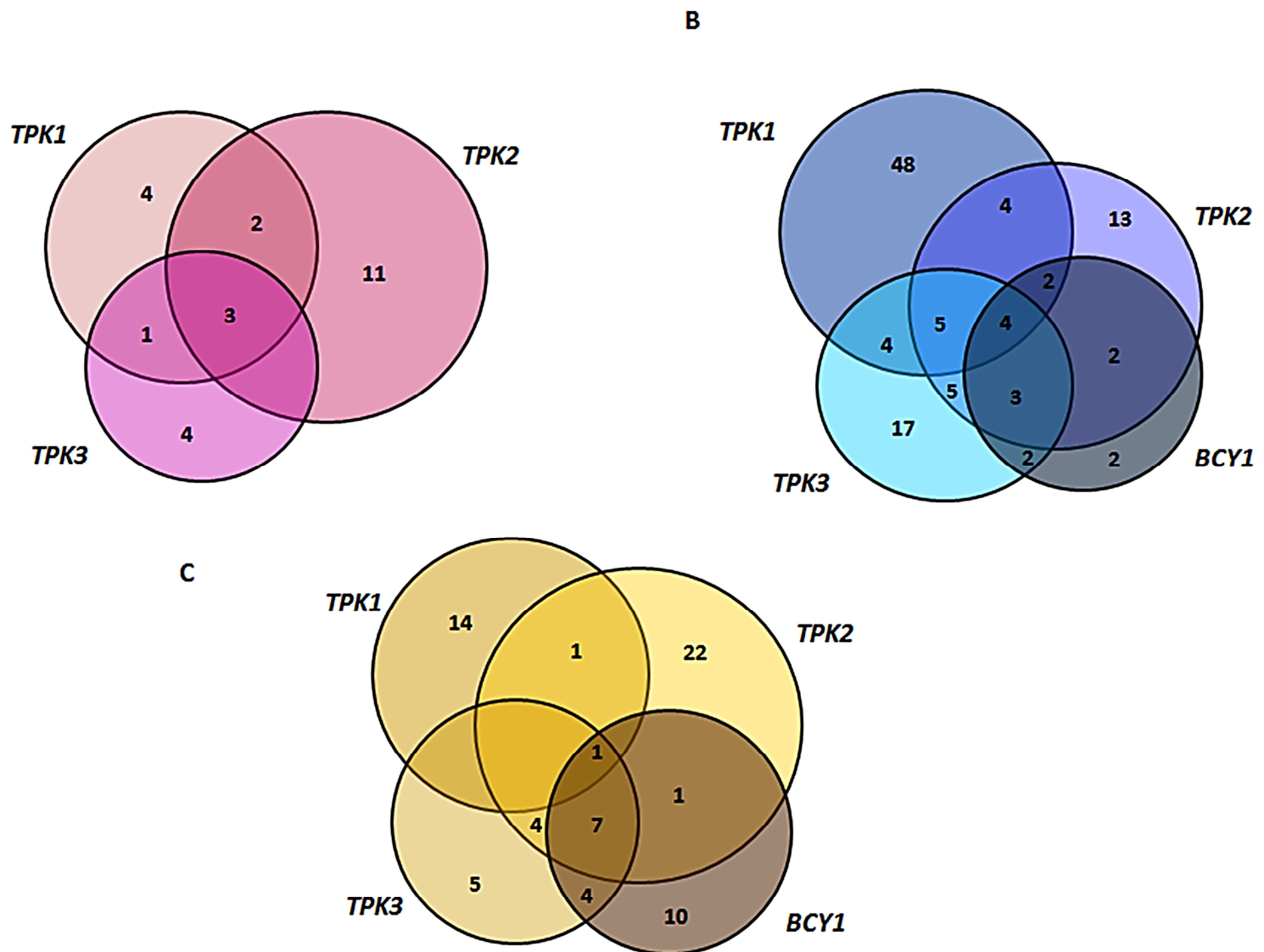
Figure 3- Venn diagrams including the regulators genes identified and indicating the common and differential regulators for each PKA subunit. Each diagram corresponds to one of the GO process analyzed: A) Lipids metabolism, B) Phosphate metabolism and C) Transcription regulation
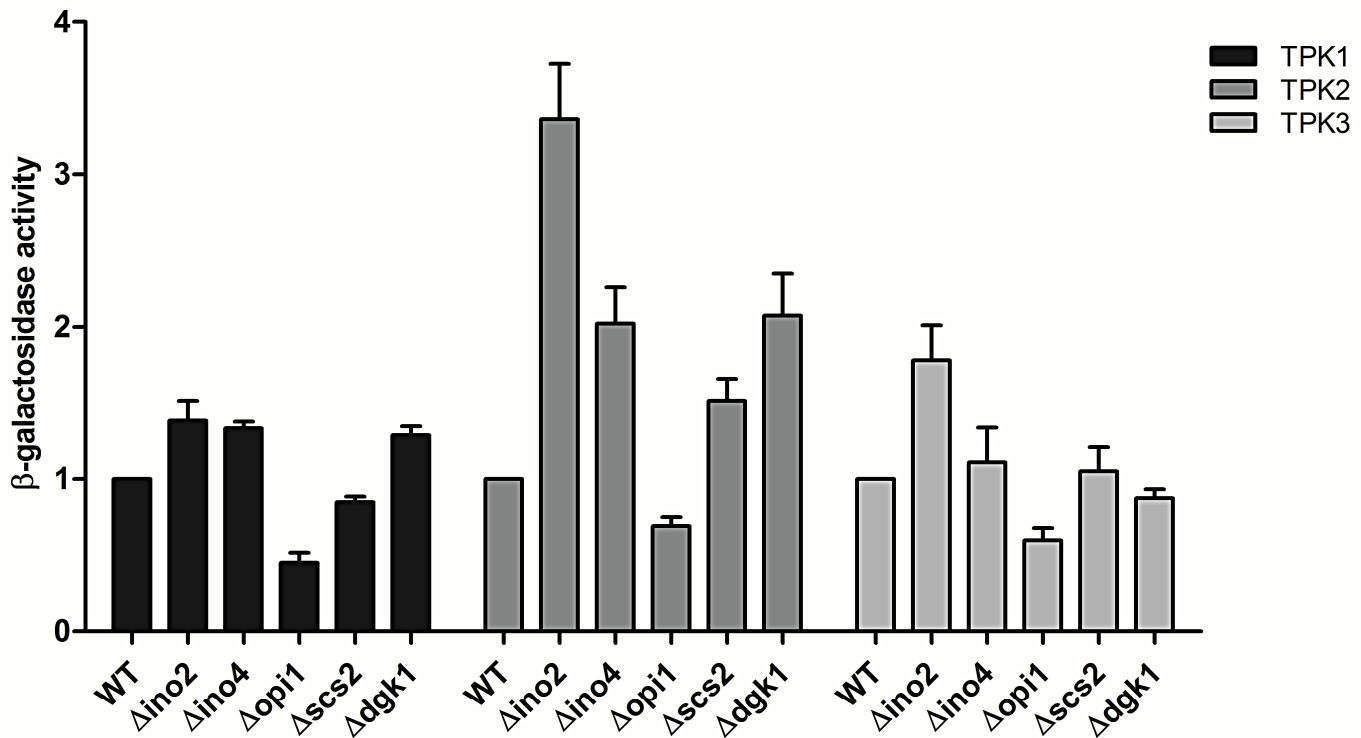
Figure 4
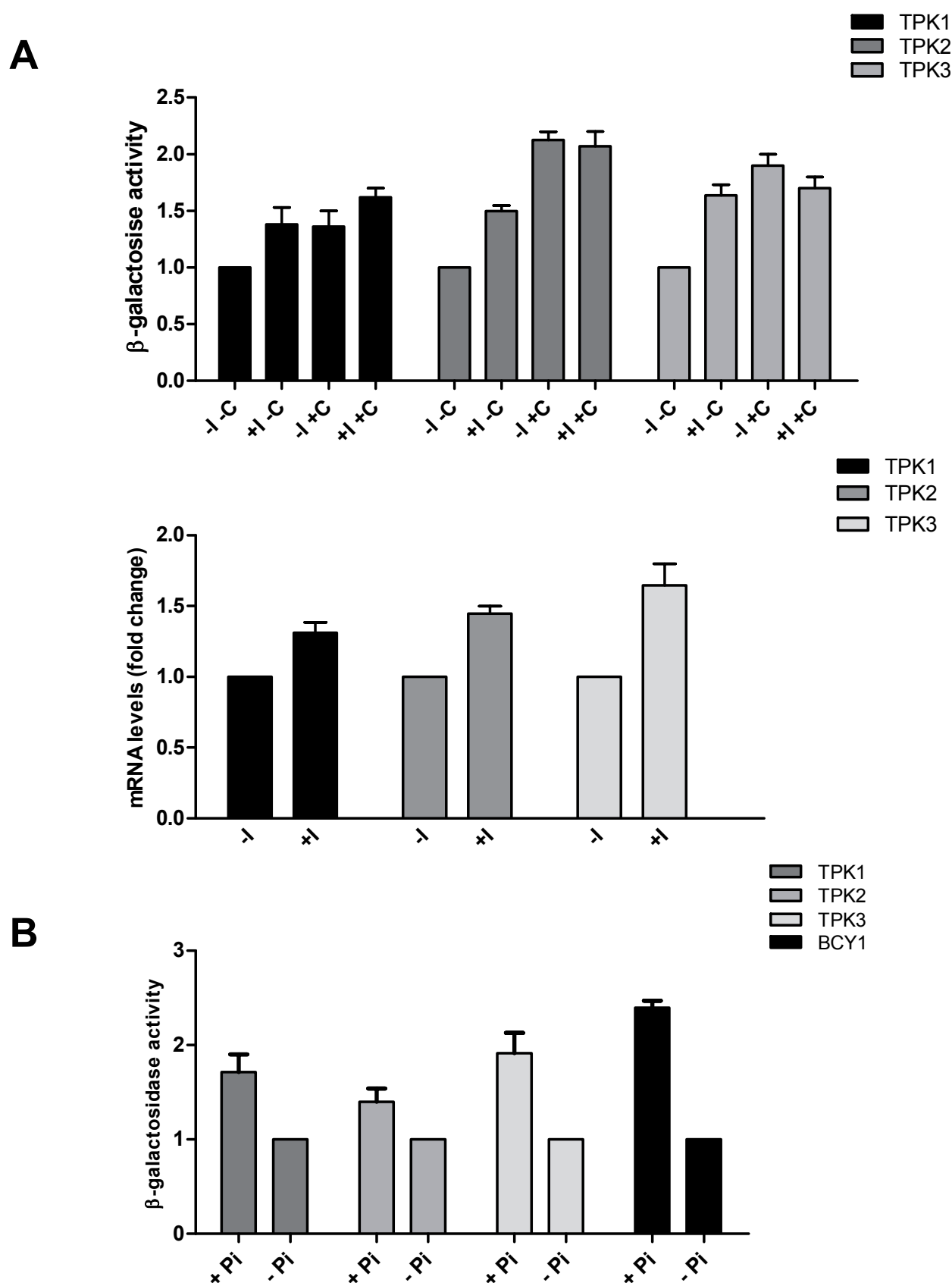
Figure 4 - A) Upper panel, β-galactosidase activity was determined in WT cells (strain BY4741) carrying the *TPKs*-lacZ or *BCY1*-lacZ fusion gene. Cells were grown up to OD_{600}=3.5 in minimal medium glucose lacking or supplemented with I and C. β-galactosidase activity is expressed in Miller Units. Results are expressed as the mean ± SD from triplicates within a representative assay and normalized to the *TPK1* values. Lower panel, *TPKs* and *BCY1* endogenous mRNA levels were determined in WT strain (B Y4741) grown in the same conditions described above. The values were normalized to *TUB1* mRNA. The mRNA level for each subunit in the WT strain was defined as 1. **B)** β-galactosidase activity was measured as in A but using minimal medium with high phosphate (Pi) or low Pi .
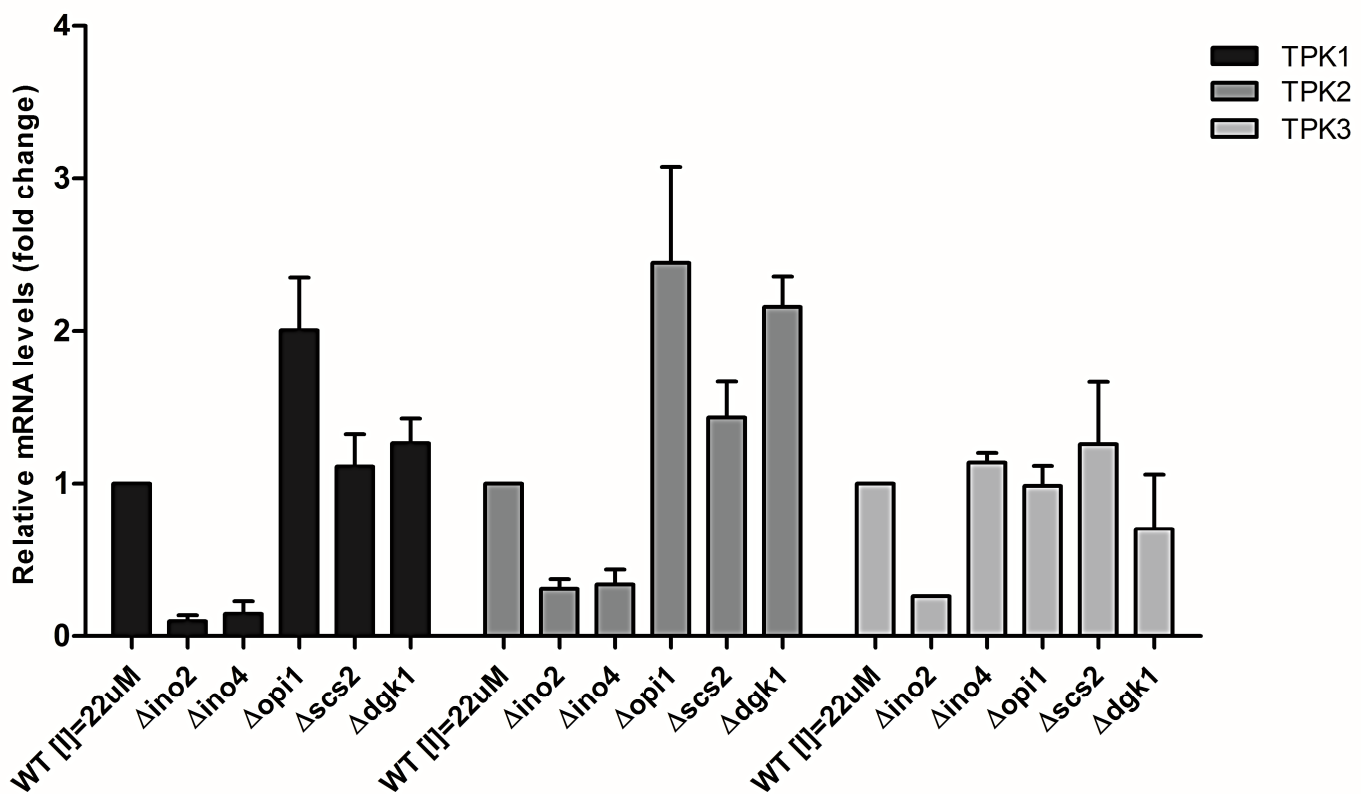
Figure 5

A

B

Figure 5- A) β-galactosidase activity was determined in WT (BY4741) *opi1* Δ *ino2* Δ *ino4* Δ *scs2* Δ or *dgk1* Δ strains carrying the *TPKs*-lacZ or *BCY1*-lacZ fusion gene. The results are expressed in Miller Units and as the mean ± SD for replicate samples (n=4) from independent experiments. **B)** *TPKs* and *BCY1* endogenous mRNA levels were determined in WT (BY4741), *opi1* Δ *ino2* Δ *ino4* Δ *scs2* Δ or *dgk1* Δ strains by qRT-PCR and normalized to *TUB1* mRNA. The mRNA level for each subunit in the WT strain was defined as 1.
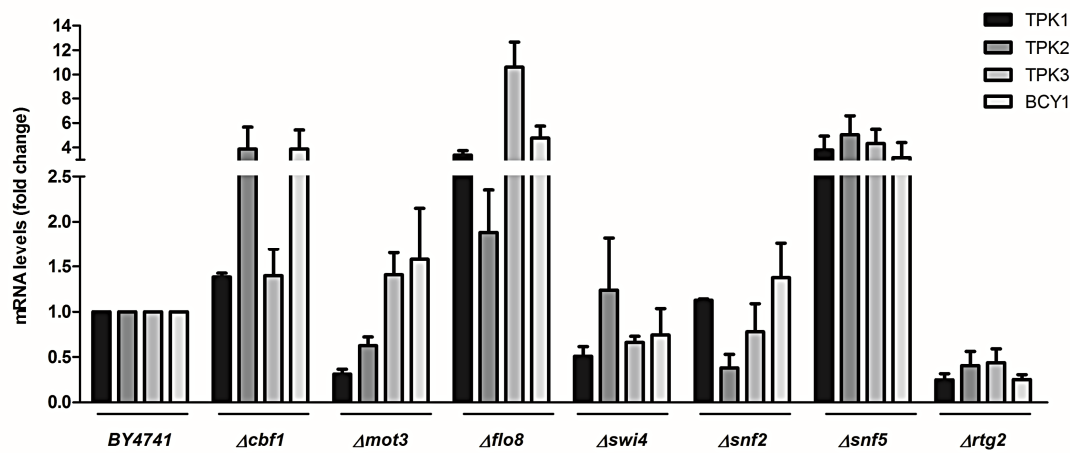
Figure 6

Figure 6- *TPKs* and *BCY1* endogenous mRNA levels were determined in WT (BY4741), *cbf1*Δ *mot3*Δ *flo8*Δ *swi4*Δ *snf2*Δ *snf5*Δ and *rtg2*Δ strains by qRT-PCR and normalized to *TUB1* mRNA. The mRNA level for each subunit in the WT strain was defined as 1