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Research article

PHYLOGENETIC ORIGIN AND TRANSCRIPTIONAL REGULATION AT THE POST-DIAUXIC PHASE OF *SPI1* IN *Saccharomyces cerevisiae*

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Abstract: The gene *SPI1* of *Saccharomyces cerevisiae* encodes a cell wall protein that is induced in several stress conditions, particularly in the post-diauxic and stationary phases of growth. It has a paralogue, *SED1*, which shows some common features in expression regulation and in the null mutant phenotype. In this work we have identified homologues in other species of yeasts and filamentous fungi, and we have also elucidated some aspects of the origin of *SPI1* by duplication and diversification of *SED1*. In terms of regulation, we have found that the expression in the post-diauxic phase is regulated by genes related to the PKA pathway and stress response (*MSN2*/*4*, *YAK1*, *POP2*, *SOK2*, *PHD1* and *PHO84*) and by genes involved in the PKC pathway (*WSC2*, *PKC1* and *MPK1*).

Key words: *SPI1*, Phylogenetic origin, Transcriptional regulation, Post-diauxic, Nutrient starvation, PKA, PKC

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Abbreviations used: AP – adaptor protein; C – control; GPI – glycophosphatidylinositol; mRNA – messenger RNA; OD – optical density; ONPG – ortho-nitrophenyl-β-galactoside; PD – post-diauxic; PKA – protein kinase A; PKC – protein kinase C; S – SD without glucose; SD – synthetic defined medium; YPD – yeast extract peptone dextrose medium

INTRODUCTION

There is a clear relationship between the nutritional state of the cell and its ability to resist stress conditions. When nutrients are available, a high activity of the Ras/adenylate cyclase/protein kinase A (PKA) pathway determines growth stimulation and cell division, as well as repression of stress, respiration, the protein kinase C (PKC) pathway and autophagy related genes [1]. Depletion of any essential nutrient stops the cell cycle, activates the stress response, and cells enter the stationary phase; when all the essential nutrients are available again, cells exit this phase [2]. These changes are regulated, at least partially, by PKA pathway and cyclin-dependent kinases Pho80/85p, which control the entry into the stationary phase, activating the transcription of genes characteristic of this phase via several transcription factors, such as Msn2/4p [3].

S. cerevisiae gene *SPI1* is a good example of a stress-response gene. It encodes a serine/threonine-rich protein anchored to the cell wall by glycophosphatidylinositol (GPI) [4]. It has been shown that it is important in resistance to herbicides, wall lytic enzymes, food preservatives and weak lipophilic acids [5, 6]; besides, its overexpression causes pseudohyphal growth [7]. Its expression is induced in several adverse conditions, particularly under oxidative, heat, ethanol, acetaldehyde and hyperosmotic stresses, lack of nitrogen and amino acids, and acidic or basic pH [8, 9], being particularly high during diauxic change, the stationary phase and nutrient starvation in laboratory and wine-making conditions [10, 11]. Its strong (and almost exclusive) expression under stress conditions has been used successfully to express stress response genes in the later stages of wine-making [12].

Some data on the transcriptional regulation of this gene are known. The induction by glucose starvation is partially dependent on Msn2p/Msn4p [10]. Expression in the post-diauxic phase is regulated by Sok2p and the ubiquitin ligase Rsp5p [11], as well as the phosphatidylinositol-4-phosphate kinase Mss4p and the flavodoxin-like proteins Rfs1p and Ycp4p [13].

The Spi1p homologous protein with the highest degree of similarity in *S. cerevisiae* is Sed1p. It is also a serine/threonine-rich protein anchored by GPI to the cell wall, and has important functions in cell wall structure and biogenesis [14]. It is also induced in nutrient starvation and the stationary phase, being the major protein of the cell wall in these conditions. The *sed1*Δ mutant is sensitive to lytic enzymes and oxidative stress and the gene is induced under stress conditions in a PKC-dependent manner [14].

In this work we have studied the phylogenetic origin of Spi1p and the role of some transduction pathways in its expression, concluding that *SPI1* was originated by duplication and diversification of *SED1*. In terms of regulation, expression in the post-diauxic phase of *SPI1* is controlled by genes related to the PKA pathway and stress response and by genes involved in the PKC pathway.

MATERIALS AND METHODS

Yeast strains, plasmids and growth conditions

The yeast strains and plasmids used in this work are listed in Supplementary Tables 1A and B respectively in Supplementary material at http://dx.doi.org/10.2478/s11658-012-0017-4. For yeast growth the following media were used: yeast extract peptone dextrose (YPD) medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose); synthetic defined (SD) medium (0.17% (w/v) yeast nitrogen base without amino acids and ammonium sulphate, 0.5% (w/v) ammonium sulphate, 2% (w/v) glucose) supplemented with the required amino acids; S (SD without glucose). Cultures were incubated at 30ºC with shaking, unless a different temperature is indicated. Solid plates contained in addition 2% (w/v) agar.

Gene expression analysis

The *SPI1p/lacZ* fusion expression was determined as β-galactosidase activity in liquid medium via the method of permeabilized cells, using ortho-nitrophenyl-β-galactoside (ONPG) as substrate, as described [11]. RNA isolation, quantification and analysis by Northern blot analysis were performed as previously described [11].

Phylogenetic studies

To search for homologous sequences the protein-protein BLAST tool of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with default settings was employed. The sequences obtained were aligned using Clustal 2.0 [15] and Probcons [16]. The alignments obtained are shown in Supplementary Figs 1 and 2 (http://dx.doi.org/10.2478/s11658-012-0017-4). For the phylogenetic analysis, we used the alignment obtained with Clustal 2.0 (Suppl. Fig. 1) edited with Gblocks 0.91b [17]. Preliminary phylogenetic trees were obtained using MEGA 4 [18]. The final tree was obtained using MrBayes 3.1.2 [19], using the parameters detailed in the supplementary material.

Identification of domains, motifs and repetitions

For these analyses we used the resources available at ExPASy (http://expasy.org/tools/) and Conserved Domains (CDD) of NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The search for structural homologues was made using Phyre 0.2 (http://www.sbg.bio.ic.ac.uk/~phyre/) and 3D-BLAST (http://3d-blast.life.nctu.edu.tw/dbsas.php).

Bioinformatics analysis

For structural modelling we used the resources available at ExPASy. We obtained similar results with several tools for secondary structure (only results obtained from PSIPRED are shown). For homology modelling, no adequate structural templates were found.

Threading structural modelling with acceptable results was performed using the LOMETS meta-server (http://zhanglab.ccmb.med.umich.edu/LOMETS/). The

structures generated were evaluated using the tools available in SWISS-MODEL. The models and the quality assessment data were deposited in the Protein Model Database (http://mi.caspur.it/PMDB/) to make them publicly available (PMDB IDs: Spi1p PM0077324; Sed1p PM0077325).

Statistical analysis of the results

The statistical significance of the numerical results was performed using a one-tailed Student's *t* test with 2 degrees of freedom (3 samples) available in Excel (Microsoft Office package). Data were considered significant if the p-value was 0.05 or lower, and the statistic *t* does not exceed the limits for considering the test as correct.

RESULTS

Phylogenetic study

To perform the phylogenetic study, we searched for homologous proteins by means of the BLAST tool of NCBI using the reference sequence database. To root the tree we used species belonging to the genera *Pichia* and *Yarrowia* as outgroups; they are frequently considered in this way because they are the most distant from *S. cerevisiae* among the yeast species commonly studied [20].

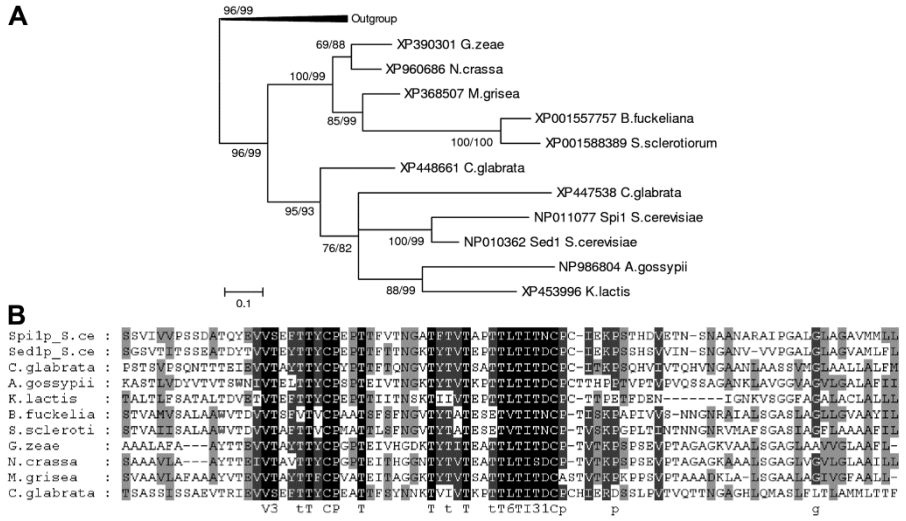

Fig. 1. Phylogenetic relationships between Spi1p and homologues in fungi. A – Phylogenetic tree of Sed1p and Spi1p obtained using MrBayes. In the tree are shown the posterior probabilities obtained by Bayesian statistics (MrBayes) and the bootstrap values (500 replicates) obtained using the Neighbour Joining method with a JTT matrix (Mega 4.0) for each branch. Also protein ID (NCBI) and the abbreviated name of the species are shown (G: *Gibberella*, N: *Neurospora*, Ss: *Sclerotinia*, M: *Magnaporthe*, B: *Botryotinia*, C: *Candida*, Sc: *Saccharomyces*, A: *Ashbya* and K: *Kluyveromyces*). B – Edited alignment used for the performance of the phylogenetic tree shown in A.

According to the phylogenetic tree obtained (Fig. 1A) by means of the sequence alignment performed (Fig. 1B and Suppl. Fig. 1), there are Spi1p orthologues in at least three species of yeast: *Kluyveromyces lactis*, *Candida glabrata* and *Ashbya gossypii*; and five filamentous fungi: *Sclerotinia sclerotiorum*, *Botryotinia fuckeliana*, *Magnaporthe grisea*, *Neurospora crassa* and *Gibberella zeae*, among the sequenced organisms. Moreover, as expected [4], Spi1p has one paralogue in *S. cerevisiae*, Sed1p. Also *C. glabrata* presents two paralogues of the protein.

Bioinformatics analysis of Spi1p and Sed1p domains and structure

Although we did not find conserved functional domains with acceptable probabilities, small similarity (e-value ≈ 3) was found in the conserved sequence (amino acids 17 to 52 in Fig. 1B) with PRK08383 (E subunit of H^+/monovalent cation antiporter), PRK11512 (putrescine-2-oxoglutarate aminotransferase) and DM13 (protein of unknown function present in *Drosophila melanogaster* and *Caenorhabditis elegans*). The prediction of potential sites of post-translational modifications is shown on the alignment of Sed1p and Spi1p (Suppl. Fig. 5). The N-terminal glycosylation has already been tested [14], as well as the phosphorylation [29], and there are several motifs that can potentially be a substrate of these post-translational modifications. There are also some

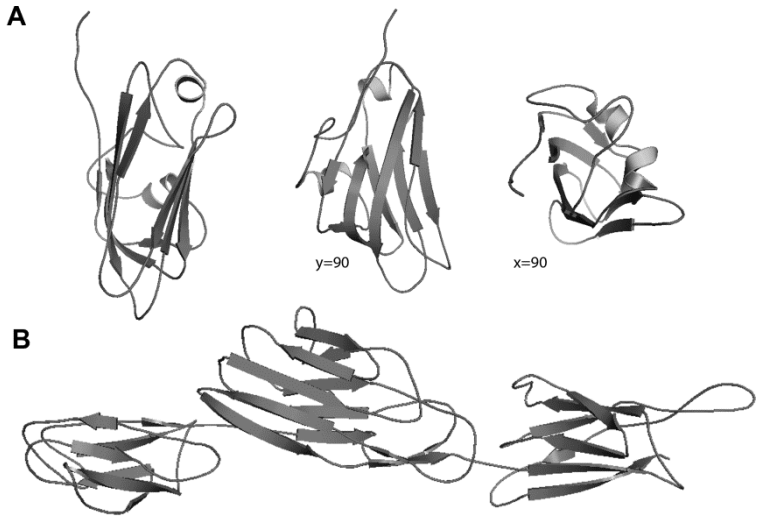

Fig. 2. Tertiary structure prediction obtained by threading using LOMETS meta-server. A – Three-dimensional model obtained for Spi1p. The structure shows the β-sheet barrels forming Ig-like domains. β-sheets and coiled-coil regions are dark-coloured, whereas α-loops are light-coloured. Three views of the protein are shown, referring the turn of the axis to the first view. B – Three-dimensional model obtained for Sed1p. The structure shows a three-lobular structure formed by Ig-like domains. Helices are shown in yellow and coiled-coil regions in green. The results show that it is also composed of three lobes of β-sheet barrels like those that form Spi1p.

interesting protein-binding motifs. There is one for AP (adaptor protein), typical of the endocytic route, very common in membrane or cell wall proteins, especially those involved in transport. The structural models obtained for Spi1p (Fig. 2A and Suppl. Fig. 6) show that the conserved sequences shown in Fig. 1B (TTFVT and TTLTITNCP) form β-sheets that are part of a domain similar to the eight-stranded anti-parallel β-sheet barrel (Ig-like domain), typical of porins, adhesins and other cell wall or outer-membrane proteins that bind hydrophobic ligands. Sed1p (Fig. 2B) is composed of three lobes of β-sheet barrels like those that form Spi1p, showing that these proteins also have related three-dimensional structure. The search for structural homologues shows that there are similar β-sheet barrels in solved structures, such as 2nnc (sulphur carrier protein from sulphur bacterium), 1st8 (fructan 1-exohydrolase from plants) and 3por (calcium porin from non-sulphur photosynthetic bacterium). These results point to a channel and/or enzymatic function of this protein in the yeast cell wall. The search for structural homologues for Sed1p also points to bacterial adhesins and surface antigens as the closest structures. It is also remarkable that the alignment of Spi1p and Sed1p (Suppl. Fig. 5) shows that cysteines forming disulfide bonds that maintain the Ig-like domain (see models deposited in PMDB) are often conserved in both proteins.

Study of the expression of *SPI1* during growth and its involvement in the response to nutrient starvation

To gain a global view on *SPI1* transcriptional regulation we performed a search for transcription factors for which a direct or indirect role in the regulation of *SPI1* has been described (Suppl. Table 2A). Notably, using transcription factors listed in this table, over-represented gene ontology terms were found for stimulus and nutrient response. Suppl. Table 2B shows the transcription factors which have binding consensus sequences in the promoter of *SPI1*. Although for some of them there is evidence indicating that they participate in the regulation of *SPI1* in other conditions (underlined; Crz1p, Gis1p, Hsf1p, Mcm1p, Msn2/4p, Sko1p, Ste12p and Yap1p), no studies have been carried out in post-diauxic conditions until now. Besides, in most cases there are no data about its involvement in the transcriptional regulation of this gene, so it remains to be demonstrated that the putative binding sequence is functional. In this case, many of the transcription factors included in this table are involved in filamentous growth and the stress response. It is remarkable that in both cases (Suppl. Tables 1 and 2), the most over-represented classes (adjusted p-value < 0.001) were the same (nitrogen compound metabolic process, ion-binding and nucleic acid metabolism), indicating the importance of these transcription factors in these processes.

The expression of *SPI1* during growth was studied by Northern blot in the strain BY4742 (Fig. 3A). The expression is increased up to 3 days, its maximum level being found between 1 and 1.5 days (coinciding with the diauxic shift), then decreased and maintained for 10 days. No expression was detected with the method used after 10 days.

The *spi1*Δ mutant presents a sensitive phenotype in starvation conditions. As shown in Fig. 3B, it has significantly lower viability than the wild type strain at 20 days in nutrient deficient medium (transferring the cells from YPD medium in exponential growth to S medium with no glucose), although these differences could also be explained by previously described variations in viability in rich medium after the diauxic shift [11].

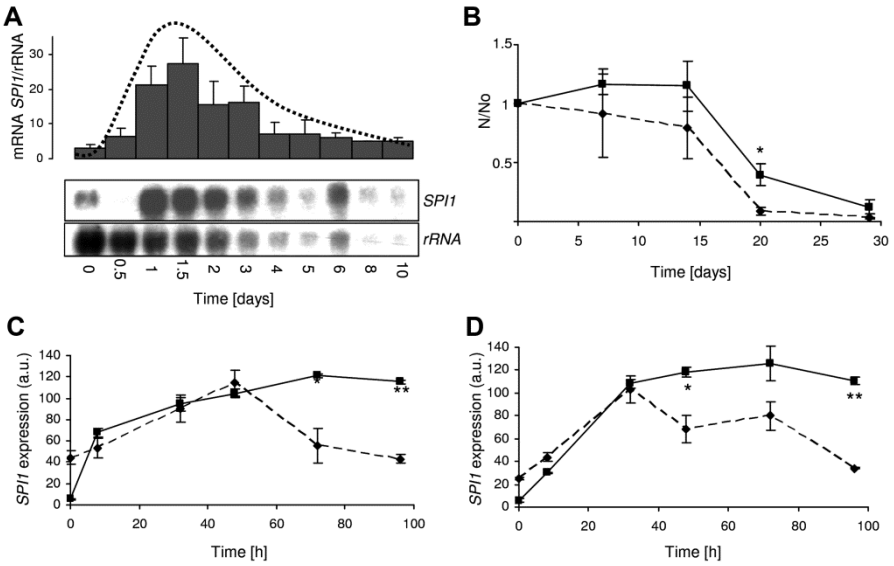

Fig. 3. Study of *SPI1* expression during the growth and its role in the response to nutrient starvation. A – *SPI1* expression in BY4742 was analysed by Northern blot. We present the quantification and the standard deviation of the data obtained with three different cultures. B – Loss of viability in starvation medium (S medium) in wild type strain (represented by squares and continuous line) and *spi1*Δ (diamonds and discontinuous line). Counts were made by plate count in YPD plates and are presented as N/No [(cfu/ml)/ initial (cfu/ml)] to cancel the effects due to different initial cfu/mL number. C – Comparison of β-galactosidase activity of *SPI1p/lacZ* fusion (squares) and Northern blot of *SPI1* mRNA (diamonds, discontinuous line) during growth in the strain BY4742. D – Same as C but for YPH499 strain. The data were multiplied by the factor needed to be represented in the same scale. For Northern blot analyses shown in this figure, data were obtained by normalization to rRNA. Statistically significant differences are marked with asterisks (* p-value < 0.05, ** p-value < 0.005).

Verification of the appropriateness of *SPI1*p/*lacZ* fusion and β-galactosidase activity as a simple method to analyse expression of *SPI1*

In order to use the β-galactosidase activity generated by the *SPI1p/lacZ* fusion as a reporter of *SPI1* expression, YPH499 and BY4742 strains were transformed with the fusion and the expression was measured in parallel by β-galactosidase activity and Northern blot during growth (Figs. 3C and D). It is noted that in early stages, until 32-48 hours depending on the strain, the results obtained using

the *SPI1*p/*lacZ* fusion are comparable to those obtained by Northern blot. In contrast, for longer times, the expression detected by Northern blot decays, while β-galactosidase activity remains. However, no significant differences were found between mRNA levels of the *lacZ* gene expressed under the control of *SPI1* promoter and mRNA levels of *SPI1* (Suppl. Fig. 3A). For both strains, the maximum β-galactosidase activity is approximately at 80 hours, while the highest mRNA level occurs at about 40 hours. This could be explained by enzyme accumulation due to its higher stability compared to *SPI1* mRNA, while differences in *SPI1* mRNA between strains could be due to strain-specific differences in transcriptional regulation. These differences among the strains could explain different vitality, so BY4742, which shows higher expression of *SPI1* at late phases, also grows to reach higher OD and during a longer time than YPH499 (Suppl. Fig. 3B).

Searching for transduction pathways that regulate *SPI1* transcription in post-diauxic phase

We studied the role of different transduction pathways and transcription factors in the regulation of *SPI1* transcription during the post-diauxic phase. These analyses were made using some mutant strains, including some genes that were described as regulators of *SPI1* by direct or indirect evidence in other conditions, as well as genes that encode transcription factors with binding consensus sequences in the *SPI1* promoter, according to the data shown in Suppl. Table 2. The results indicate that some of the selected transcription factors, such as Pho4p, Crz1p, Mig1p, Yap1p, Msn1p and Sko1p, have no relevant effect on the expression of *SPI1* at the post-diauxic phase (Suppl. Fig. 4), although they have been related to the regulation of *SPI1* expression in other conditions (Suppl. Table 2A). We also studied the effect of mutants in some genes that encode proteins involved in signal transduction and other functions related to gene expression, but no effect was detected in the deletion strains of *PHO85*, *CNB1*, *SST2*, *MSN5*, *PUF5* and *HOG1* (Suppl. Fig. 4). However, an effect was found in some deletion or thermosensitive mutants in genes related to PKA/stress and PKC pathways, as is shown below.

The PKA/stress pathway has a role in the regulation of SPI1 transcription at the post-diauxic phase

The expression of *SPI1* at the post-diauxic phase decreases in *msn2*Δ*msn4*Δ strain compared to the wild type (Fig. 4A). According to this, the transcription factors Msn2/4p are involved in the transcriptional regulation of this gene, as expected according to previous studies [10].

A regulatory effect on transcription of *SPI1* was found for Yak1p (Fig. 4A) and the related protein Pop2p (Fig. 4B), involved in the PKA pathway and response to glucose depletion [20]. Also *pho84*Δ (Fig. 4C) mutation has an effect on this expression, suggesting a role of phosphate starvation as a sensor for the induction of the gene by this pathway.

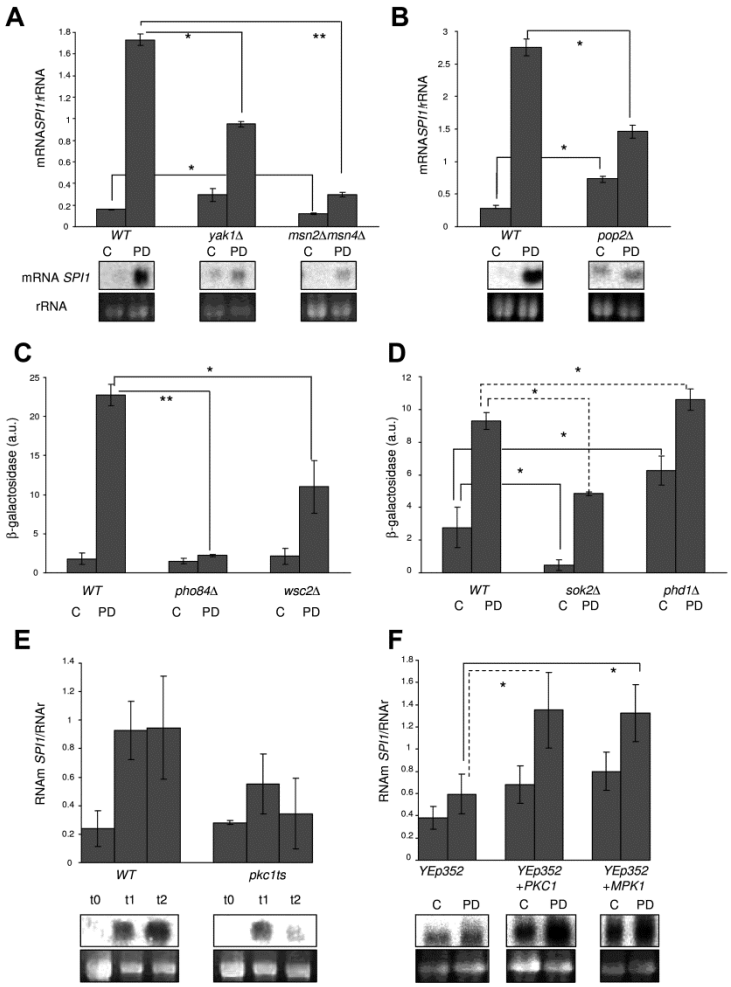

Fig. 4. Effect of several mutants in the expression of *SPI1* during exponential [C] and post-diauxic phase [PD]. A – Study by Northern blot in deletion strains in the *MSN2/MSN4* and *YAK1* genes, in *W303-1a* strain grown in YPD. B – Study by Northern blot of the effect of *POP2* gene deletion in BY4742 strain in YPD. C – Study of the effect of deletions in *WSC2* and *PHO84* in *BY4741* strain in SC-URA using *SPI1p/lacZ* fusion. D – Study of the effect of deletions in *SOK2* and *PHD1* in *MLY40* strain in SC-URA using *SPI1p/lacZ* fusion. E – Effect of mutations in PKC pathway genes in *SPI1* expression. Northern blot after 0, 6 and 9 hours at 37ºC in *W303-1a* and the thermosensitive mutant *pkc1ts* and its quantification are shown. F – Effect of the overexpression of PKC pathway genes in *SPI1* expression. Quantification in *W303-1a* containing multicopy plasmids with indicated genes and Northern blot spots are shown. Statistically significant differences are marked with asterisks (* p-value < 0.05, ** p-value < 0.005).

The transcriptional factor Sok2p and its antagonist Phd1p, also related to the PKA pathway, regulate the expression of *SPI1* in these conditions (Fig. 4D). In

the mutant *sok2*Δ, induced (post-diauxic) and basal (exponential phase) expression is lower, as we previously described [11], whereas in the mutant *phd1*Δ the mRNA levels are higher than in the wild type strain, under both control and post-diauxic conditions (Fig. 4D).

The PKC pathway has a role in the regulation of SPI1 transcription at the post-diauxic phase

Since Spi1p is a cell wall protein, we also studied the involvement of the PKC pathway in the expression of *SPI1*. In the thermosensitive mutant *pkc1ts*, *SPI1* expression decreases in post-diauxic phase cultures changed from 30 to 37°C in rich medium (Fig. 4E), although the differences are not statistically significant. Also *wsc2*Δ shows significantly lower induction of *SPI1* in post-diauxic conditions (Fig. 4C), pointing to activation via the canonical transduction pathway of PKC, which starts in this sensor protein. However, in *mpk1*Δ the induction was not affected in the post-diauxic phase (Suppl. Fig. 4), supporting the results of other authors who proposed that there is a bypass on the pathway at this level [22]. We also studied the expression in yeast transformed with multicopy plasmid containing *MPK1* and *PKC1*. The overexpression of both genes increases the *SPI1* expression in a statistically significant manner, both under control and post-diauxic conditions (Fig. 4D).

DISCUSSION

The results obtained from the phylogenetic analysis suggest that Sed1p duplication and subsequent differentiation of one copy could be the origin of Spi1p. Homologues of Sed1p and Spi1p are present in yeasts only in the subphylum *Saccharomycotina*/class *Saccharomycetes*/order *Saccharomycetales*, whereas in filamentous fungi they are present in the subphylum *Pezizomycotina*, classes *Leotiomycetes* (*B. fuckeliana* and *S. sclerotiorum*) and *Sordariomycetes* (*G. zeae*, *N. crassa* and *M. grisea*), but they are not found in *Eurotiomycetes* class (including other sequenced model organisms belonging to *Aspergillus* genus) or throughout the subphylum *Taphrinomycotina* (which includes other sequenced model organisms such as *Schizosaccharomyces pombe* and *Pneumocystis carini*). Based on the phylogenetic relationship of *Ascomycota* proposed by Scannell *et al.* [23], these results suggest that Sed1p and orthologous genes appear in the evolution of the subphylum *Pezizomycotina* when *Eurotiomycetes* diverge from *Sordariomycetes* and *Leotiomycetes*, and are maintained later on (*Leotiomycetes*, *Sordariomycetes* and *Saccharomycetales*) or duplicate (*Saccharomyces sensu stricto*), explaining the lack of these genes in *Eurotiomycetes* and *Taphrinomycotina*. It is interesting to note the presence of a conserved sequence of amino acids (TLTITDCPC, amino acids in positions 235-244 in the alignment) in these proteins (Fig. 1B). The protein of *A. gossypii* and Sed1p have two repetitions of this sequence (Suppl. Fig. 1, amino acids in positions 332-341 and 483-492 in the alignment), which could indicate that these

proteins have a common ancestor with Spi1p that would have originated by duplication and specialization in *S. cerevisiae*, after losing one copy of this sequence. The conservation of these proteins (even duplicated) in the mentioned species points to an important function, for instance in stress resistance, as has been demonstrated [11, 14]. In *S. cerevisiae*, gene expression changes at the diauxic shift and the stationary phase have been studied by genome-wide analyses [24, 25]. These works clearly showed the importance of protein biosynthesis, as well as carbohydrate and amino acid metabolism, in these conditions. In this sense, our group has reported the relation of Spi1p regulation with these pathways [11, 13]. In other yeasts, the homologue proteins have also been related to nutrient limitation. In *N. crassa*, Spi1p/Sed1p homologue (XP960686, named ccg6) is light-activated and development-related [26], and the expression of the gene is induced in glucose-limitation conditions, in medium with acetate as a carbon source [27]. In *K. lactis* it would be interesting to analyse the expression of the hypothetical protein XP453996 in post-diauxic conditions, to investigate the relationship of this protein with the changes that occur in this shift.

Sed1p duplication could have happened during the total genome duplication proposed by Wolfe in 1997 [28], so it would be present only in *S. cerevisiae* and *C. glabrata*, and the subsequent gene loss or specialization would have not been equal in both species, so the divergence may be different. Subsequent specialization of one copy probably originated Spi1p in *S. cerevisiae*, which may explain the partially coincident phenotypes of null mutants and regulation pathways in Sed1p and Spi1p, even more important considering that the conserved domain described in this work is present in both proteins, Sed1p (repeated) and Spi1p (single) (Suppl. Fig. 5).

There is controversy over what yeast genus (*Candida* or *Kluyveromyces*) is phylogenetically closer to *Saccharomyces* [20, 23, 30]. The tree obtained for Spi1p supports the theory proposed by Wolfe in 1997 [24], given that *C. glabrata* presents two sequences homologous to Spi1p and Sed1p, while *C. albicans* and *K. lactis* have only one. Thus, the results of this phylogenetic study point to a Sed1p gene duplication and diversification as the origin of Spi1p. This diversification would imply the loss of part of the sequence of Sed1p, including one repetition of the conserved motif TLTITDCPC, which is not lost in *A. gossypii*.

In this work we have analysed the involvement of several transcription factors in the regulation of the *SPI1* gene at the post-diauxic phase. Sok2p and Pdh1p, two transcription factors involved in pseudohyphal growth regulation, are involved in *SPI1* transcriptional regulation. In the mutant *sok2*Δ, β-galactosidase activity is decreased in the post-diauxic phase, whereas in *phd1*Δ it is increased (Fig. 4D). This result fits with the model proposed by Pan and Heitman [31] that suggests antagonist effects of these factors; Sok2p inhibits *PHD1* transcription, which induces *FLO11* (a glycoprotein anchored by GPI) and triggers pseudohyphal invasive growth. In the case of *SPI1*, Phd1p would act as a transcriptional

repressor to fit this model. In addition, *SOK2* is strongly induced under heat shock, osmotic stress and the stationary phase, and *PHD1* is activated under osmotic stress and nitrogen starvation [32], pointing to the involvement of these transcription factors in the stress response. Although *pho85*Δ mutation has no effect in *SPI1* induction in these conditions (Suppl. Fig. 4), *pho84*Δ has an evident effect (Fig. 4C), suggesting a role of inorganic phosphate signalling in the induction of the gene. This phosphate transporter has also been related to PKA pathway activation [33], suggesting an explanation for its role in *SPI1* induction.

Bioinformatics analysis of transcription factors which regulate *SPI1* transcription by gene ontology (Suppl. Table 2) shows the following as enriched terms: nitrogen compound metabolic process, ion binding, nucleic acid metabolism, stimulus response, glucose metabolism regulation, nutrient response, filamentous growth and stress transcriptional response. These results are also consistent with the phenotype observed (stress and nutrient starvation sensitivity) and together with the putative function proposed by the bioinformatics analysis open new fields that could be explored with these proteins, as transport or signal transduction.

In this work, a connection has been established between the expression of *SPI1* in the post-diauxic phase and the PKC pathway, confirming the relationship between this gene and the cell integrity pathway. Plasmids containing *MPK1* and *PKC1* show greater *SPI1* induction (Fig. 4F), and the *WSC2* (sensor where the pathway begins) deletion has significantly smaller induction (Fig. 4C). However, in the *mpk1*Δ strain (last kinase in the pathway) no altered expression was detected (Suppl. Fig. 4), so other proteins at the end of the pathway may be responsible for this transcriptional activation. Some proteins that have been described with a partially redundant function with Mpk1p are Rpi1p [34] and Mlp1p [35].

According to these results, *SPI1* expression is regulated by two major cell pathways: PKA, which regulates the stress response and cell growth, and PKC, which regulates cell integrity and cell wall metabolism and biogenesis. These pathways are the key to control of global cellular physiology, regulating the expression of a large number of genes in different conditions, and are probably controlled together by TOR kinase, also involved in the response to nutrient and environmental conditions. Thus, it seems clear that the transcription of this gene is regulated in a manner dependent on the availability of nutrients and cell growth, consistent with its expression pattern and the phenotype of the null mutant in starvation conditions (Fig. 3B).

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