Biochimica et Biophysica Acta 1840 (2014) 344–349

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

A putative phospholipase C is involved in *Pichia fermentans* dimorphic transition

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ARTICLE INFO

Article history:
Received 21 April 2013
Received in revised form 17 September 2013
Accepted 19 September 2013
Available online 27 September 2013

Keywords:
Dimorphic shift
Phospholipase C
Pichia fermentans
Rapid subtraction hybridization (RaSH)
3′-5′-cyclic adenosine monophosphate (cAMP)
Pseudohypha

ABSTRACT

Background: Pichia fermentans DiSAABA 726 is a dimorphic yeast that reversibly shifts from yeast-like to pseudohyphal morphology. This yeast behaves as a promising antagonist of *Monilia* spp. in the yeast-like form, but becomes a destructive plant pathogen in the pseudohyphal form thus raising the problem of the biological risk associated with the use of dimorphic yeasts as microbial antagonists in the biocontrol of phytopathogenic fungi.
Methods: Pichia fermentans DiSAABA 726 was grown in urea- and methionine-containing media in order to induce and separate yeast-like and pseudohyphal morphologies. Total RNA was extracted from yeast-like cells and pseudohyphae and retro-transcribed into cDNA. A rapid subtraction hybridization approach was utilized to obtain the cDNA sequences putatively over-expressed during growth on methionine-containing medium and involved in pseudohyphal transition.
Results: Five genes that are over-expressed during yeast-like/pseudohyphal dimorphic transition were isolated. One of these, encoding a putative phospholipase C, is involved in *P. fermentans* filamentation. In fact, while the inhibition of phospholipase C, by means of 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphorylcholine (Et-18), is accompanied by a significant reduction of pseudohyphae formation in *P. fermentans*, the addition of exogenous cAMP fully restores pseudohyphal growth also in the presence of Et-18.
Conclusion: Phospholipase C is part of a putative "methionine sensing machinery" that activates cAMP-PKA signal transduction pathway and controls *P. fermentans* yeast-like/pseudohyphal dimorphic transition.
General significance: Phospholipase C is a promising molecular target for further investigations into the link between pseudohyphae formation and pathogenicity in *P. fermentans*.

1. Introduction

Several yeasts switch reversibly between yeast-like and pseudohyphal and/or hyphal morphologies in response to nutritional and environmental stimuli [1,2]. The production of pseudohyphae and hyphae, besides providing a selective advantage to non-motile yeast cells, is often, although not always, associated with pathogenic behavior [3–5]. For this reason the nutritional and environmental factors involved in hyphal/pseudohyphal growth were extensively studied in many different fungal models, including *Saccharomyces cerevisiae* and *Candida albicans*. Both of these microorganisms switch from yeast-like to pseudohyphal morphologies in response to nitrogen starvation [6–8]. Carbon starvation, pH and temperature changes, and the production of 'quorum sensing' molecules, which include higher alcohols, can also trigger this dimorphic transition [9–12]. Interestingly, despite the many different signals that can induce filamentous development, the signaling pathways involved in connecting external stimuli and cell differentiation are well conserved among distantly related fungi [1,13]. In particular, the mitogen-activating protein kinase (MAPK) and 3′-5′-cyclic adenosine monophosphate (cAMP)–protein kinase A (PKA) pathways are involved in the induction of cell filamentation in both *C. albicans* and *S. cerevisiae*.

Giobbe et al. [14] reported that while the dimorphic yeast *Pichia fermentans* can control brown rot caused by *Monilia* spp. on apples, it causes rapid fruit decay on peaches, where it shows pathogenic behavior. Interestingly, in both cases, *P. fermentans* forms a biofilm on the fruit surfaces, but this is made of yeast-like cells on apples and of pseudohyphae on peaches. Although a clear relationship between *P. fermentans* pseudohyphal growth and its pathogenic behavior on peaches has not yet been demonstrated, the formation of pseudohyphae is often considered a virulence factor. Moreover, this ambiguous performance of the dimorphic yeast *P. fermentans* raises the problem of the biological risk associated with the use of dimorphic yeasts as microbial antagonists in the biocontrol of phytopathogenic fungi.

It has been shown recently that by growing *P. fermentans* on urea-containing or methionine-containing media, it is possible to induce and separate its yeast-like and pseudohyphal morphologies under laboratory

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http://dx.doi.org/10.1016/j.bbagen.2013.09.030

conditions [15]. Here, with the aim of isolating the *P. fermentans* genes that are involved in this yeast-like to pseudohyphal transition, a rapid subtraction hybridization (RaSH) approach was used with *P. fermentans* DiSAABA 726 during growth in urea and methionine media.

2. Materials and methods

2.1. Strains and culture conditions

Pichia fermentans Lodder [anamorph: *Candida lambica* (Lindner & Genoud) Uden & H.R. Buckley ex S.A. Mey. & Ahearn (1983)] DISAABA 726 (=DBVPG 3627) is maintained in the culture collection of the Dipartimento di Agraria (Università degli Studi di Sassari) in YEPD (2% glucose, 2% peptone, 1% yeast extract, and 2% agar) at 4 °C for short-term storage, and in YEPD plus 20% glycerol at −80 °C for long-term storage. Other media utilized were YCBU (1.17% Yeast Carbon Base; 2% urea) and YCBM (1.17% Yeast Carbon Base, 0.2% methionine). Suspensions containing 1×10^6 cell/ml from overnight pre-cultures incubated in YEPD in an orbital shaker (180 rpm) were used as inocula. The cells were grown statically for 24 h in liquid YCBU or YCBM at 25 °C, and only the biofilm-forming cells were sampled for the further experiments.

Escherichia coli competent cells (One Shot TOP10 Chemically Competent *E. coli*, cat. No. C4040-03, Invitrogen, USA) were maintained in LBGLY (1% tryptone, 0.5% yeast extract, 1% sodium chloride, and 20% glycerol) at −80 °C, and cultured at 37 °C on LB with ampicillin (100 μg/ml) (LB-AMP) and X-GAL (40 μg/ml) when needed.

2.2. Construction of a cDNA library by the rapid subtraction hybridization approach

Total RNA was extracted from yeast-like cells grown for 24 h on YCBU ('driver') and pseudohyphae grown on YCBM ('tester') using Pure Link^{tm} RNA mini-kits (cat. No. 12183-018, Invitrogen, USA), and transcribed into cDNAs using Super SMART PCR cDNA Synthesis kits (cat. No. 635000, Clontech, USA), according to the manufacturer's instructions. The resulting driver and tester cDNAs were digested into small fragments with *EcoR*II, and 2 μg of each cDNA was ligated to adapters (see Table 1: XE-14, XEA-13 and XET-13) using DNA Ligation Ver 2.1 (cat. No 6022, Takara, Japan). Briefly, the ligation mixtures containing the cDNAs and adapters were incubated at 55 °C for 3 min, cooled at 8 °C, and left at 4 °C overnight for ligation. The cDNAs were amplified by PCR with the XEA-18 and XET-18 primers (see Table 1), in 25 μl reaction mix containing 1 μl cDNA, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 1 μM XEA-18, 1 μM XET-18, and 0.1 U TopTaq DNA polymerase (cat. No 200201, Qiagen, Italy) in 1 × TopTaq DNA polymerase buffer. The amplification protocol was as follows: 1 cycle at 72 °C for 5 min, followed by 25 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, and then 10 min 72 °C final extension. The amplicons were purified using QIAquick^R PCR Purification kits (cat. No. 28104, Qiagen, Italy), and the tester cDNA was digested with *Xho*I and hybridized to the driver cDNA. In particular, 100 ng tester cDNA was mixed with 3 μg driver cDNA (1:30 ratio) in 10 μl hybridization solution (0.5 M NaCl, 50 mM Tris–HCl, and pH 7.5, 0.2% [w/v] SDS, 40% [v/v] formamide), taken to 100 °C for 5 min, and then incubated at 42 °C for 48 h. The hybridization mixture was diluted to 100 μl, purified using QIAquick^R PCR Purification kit (cat. No 28104, Qiagen, Italy), cloned into pBlueScript SK (±), *Xho*I digested, and transformed into *E. coli*. The recombinant strains were subjected to blue/white selection.

2.3. cDNA library screening

White ampicillin-resistant bacterial colonies were cultured for 2 h at 37 °C in LB-AMP, and 1 μl of the culture was used as the template in the PCR reactions, with primers RaSHf and RaSHr, which align to pBluescript SK (see Table 1). In particular, the reaction mix contained 1 μl bacterial suspension, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 2 μM RaSHf, 2 μM RaSHr, and 0.1 U TopTaq DNA polymerase (cat. No 200201, Qiagen, Italy) in 1× TopTaq DNA polymerase buffer. The amplification protocol was 1 cycle at 94 °C for 3 min, followed by 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and then 10 min at 72 °C for the final extension. The PCR products were analyzed by gel electrophoresis, to determine the presence and length of the fragments. The amplicons were blotted in duplicate onto the same Hybond N^+ membrane (Amersham Biosciences, Freiburg, Germany). Three micrograms cDNAs that were PCR amplified from the tester or driver and labeled with DIG-High prime DNA labeling and detection starter kit II (Roche Applied Science, Mannheim, Germany) were used as probes for hybridization. Densitometric analysis of blot images was carried out using the ChemiDoc Quantity One 4.6.7 software. The clones of interest were sequenced (BMR-Genomics, Università di Padova, Italy).

2.4. BLAST analyses

The nucleotide sequences of individual clones were subjected to BLAST analyses [16] against the sequences available at http://blast.ncbi.nlm.nih.gov/Blast.cgi and http://www.candidagenome.org/.

2.5. Quantitative real-time PCR

Total RNA was extracted from yeast-like cells and the pseudohyphae obtained after 24 h growth on YCBU and YCBM, respectively. Primers to be used in the quantitative real-time PCR (qRT-PCR) were designed using the Primer3 software (http://frodo.wi.mit.edu/primer3/). The

Table 1
List of primer used in this study.

Sequence		Sequence	
Adapters			
XE-14	5′-CTGATCACTCGAGA-3′	XET-13	5′-CCTGGTCTCGAG-3′
XEA-13	5′-CCAGGTCTCGAG-3′		
cDNA amplification			
XE-18	5′-TGATCACTCGAGACCAGG-3′	XET-18	5′-TGATCACTCGAGACCTGG-3′
RaSH			
RaSHf	5′-ACTCACTATAGGGCGATTG-3′	RaSHr	5′-GGAATTCGATATCAAGCTTATC-3′
qPCR			
actFOR	5′-CCATGTTCCCAGGTATTGCT-3′	eloFOR	5′-GACAAGCAACTGGTGGTCAA-3′
actREV	5′-AGAAGATGGAGCCAAAGCAG-3′	eloREV	5′-GCTTGGTGGAAACGTCAGTT-3′
aldFOR	5′-TCGGAAGGCGCTAAACTAGA-3′	citFOR	5′-GTGGGAGAGCACCTTGGTTA-3′
aldREV	5′-CTTCTTCCGACTTGCCAAAG-3′	citREV	5′-CGACCTCGAGACCAGGTAA-3′
cellFOR	5′-AACCGTCGATGAGGATGACT-3′	plcFOR	5′-CATTGCAGCTTGAAAATGGA-3′
cellREV	5′-AGACACCTGCATTGTTGACG-3′	plcREV	5′-TGGGTATCATATGCAGCATCAC-3′

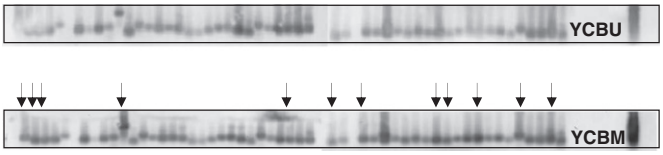

Fig. 1. Reverse Northern hybridization. Clones of the cDNA library were transferred onto Hybond N^+ membrane and probed with cDNA of the tester (YCMB) and of the driver (YCBU). Arrows indicate sequences that are more expressed during pseudohyphal growth (YCBM), as indicated by densitometric analyses of blot images.

primers specific for the actin gene, used as the housekeeping gene, were designed on conserved regions of the gene in the following microrganisms: *C. albicans*, *Candida dubliniensis*, *Candida glabrata*, *Candida famata*, *S. cerevisiae* and *Pichia manshurica* (Table 1). A total of 100 ng RNA extracted from the yeast-like cells grown on YCBU (driver) and the pseudohyphae grown on YCBM (tester) were transcribed into cDNAs using iScript® cDNA Sythesis kit (cat. No. 170-8890, Bio-Rad), according to the manufacturer's instructions. The qRT-PCR was performed using the SYBR® Green qPCR Supermix for iCycler® (cat. No. 11761-100, Invitrogen). The optimal PCR conditions in the 25 μl reaction volume were: 1 μl cDNA template, 12.5 μl 2× iQ SYBR® Green Supermix (cat. No 170 8882 Bio-Rad Laboratories Inc., Hercules, CA, USA), 400 nM forward primer, and 400 nM reverse primer. PCR was performed in quadruplicate on three independent biological replicates in 96-well optical plates on an iCycler iQ RT-PCR detection instrument (Bio-Rad Laboratories Inc., Hercules, CA, USA). The PCR program was as follows: 50 °C for 2 min, 95 °C for 8 min and 30 s, 40 cycles of 95 °C for 15 s, 54 °C for 60 s and 72 °C for 15 s, followed by measurement of fluorescence during a melting curve in which the temperature was raised from 55 °C to 95 °C in steps of 0.5 °C every 10 s.

2.6. Growth kinetics

The growth kinetics of *P. fermentans* DiSAABA 726 was monitored on YCBM without and with 100 and 150 μM 1-O-octadecyl-2-O-methyl-rac glycero-3-phosphorylcholine (Et-18; cat. No. O9262 Sigma-Aldrich, Italia) and 5 mM cAMP. Briefly, 5×10^6 cell/ml of *P. fermentans* DiSAABA 726 was inoculated into 200 μl medium in 96-well microtiter plates (cat. No. 5530100, Orange Scientific, Belgium). The optical density of the culture at 600 nm (OD_{600}) was evaluated every 15 min for 48 h, using a SPECTROstar Nano spectrometer (BMG LABTECH, Germany). The data were analyzed with the Statgraphics Centurion software (Statistical Graphics Corp. 1994–2001), using ANOVA and Multiple Range Duncan tests ($P < 0.05$) and expressed as means ± standard deviation of 4 independent replicates.

2.7. Image analysis

The cells were photographed at 100× magnification using an XM10 camera mounted on an Olympus BX61 microscope. At least 500 cells were sampled and analyzed from three independent cultures for each growth condition. Images of the representative cells are shown.

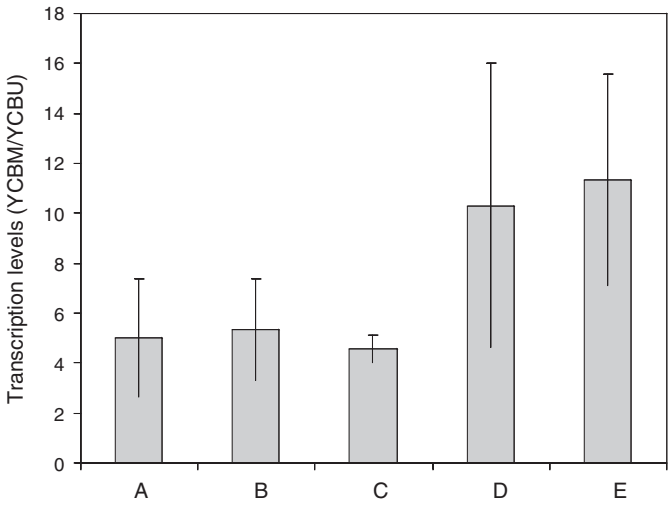

Fig. 2. Transcription levels of the five pseudohypae-related genes. The primers were designed based on the sequences of: prg1, putative phospholipase C (A); prg6, putative cell-wall glycosidase (B); prg7, putative aldose 1-epimerase (C); prg9, putative cytochrome C1 (D); prg10, putative GTPase cytoplasmic translation elongation factor 1-alpha t (E). The transcription levels were evaluated in pseudohyphae (YCBM) and yeast-like cells (YCBU), normalized with respect to the housekeeping gene (*ACT*1) and expressed as the ratio (YCBM/YCBU). The results refer to 4 technical replicates of 3 independent biological replicates.

2.8. Nucleotide sequences accession number

The sequences of DiSAABA 726 prg clones were deposited in GeneBank under the following accession numbers: JZ078735 (prg1 and prg5), JZ078736 (prg2, prg3, and prg4), JZ078737 (prg6), JZ078738 (prg7), JZ078739 (prg8), JZ078740 (prg9), JZ078741 (prg10, prg11, prg12).

3. Results and discussion

3.1. Cloning of genes differentially expressed during pseudohyphal growth

The dimorphic yeast *P. fermentans* DiSAABA 726 shows pathogenic behavior under pseudohyphal morphology [14]. To contribute to the elucidation of the molecular mechanisms involved in pseudohyphal transition and its associated pathogenicity, once the nutritional conditions that induce pseudohyphal morphology were individuated [15], an investigation on the genes over-expressed at the yeast-like *versus* pseudohyphal dimorphic transition was carried out. Based on the results obtained in other studies [17–19], a RaSH approach was used in the present study. The cDNA sequences putatively over-expressed on YCBM (pseudohyphal morphology) were cloned into pBlueScript SK (+/−) plasmid and transformed in *E. coli*. Among the resulting transformants, 349 carried recombinant plasmids containing cDNA fragments ranging from 100 bp and 600 bp. These were selected and subjected to reverse Northern hybridization against cDNAs of the tester (pseudohyphae; YCBM) and driver (yeast-like cells; YCBU). Densitometric analyses of reverse Northern blots highlighted 12 clones

Table 2
Characteristics and predicted functions of the sequences cloned by RaSH.

Clone	Predicted function	Identity (%)	*e* value	GeneBank accession n.
prg1, prg5	Phospholipase C of *C. orthopsilosis*	82.4	2.2e−10	JZ078735
prg2, prg3, prg4	Phospholipase C of *C. orthopsilosis*	75.6	1e−14	JZ078736
prg6	Cell-wall glycosidase of *C. albicans*	73	4e−11	JZ078737
prg7	Aldose 1−epimerase of *C. glabrata*	70.4	7e−06	JZ078738
prg8,	GTPase cytoplasmic elongation factor 1-alpha *P. fermentans*	98	2e−18	JZ078739
prg9	Cytochrome c1 of *C. tropicalis*	85	2e-60	JZ078740
prg10, prg11, prg12	GTPase cytoplasmic elongation factor 1-alpha *P. fermentans*	100	1e−19	JZ078741

Table 3
Pichia fermentans DiSAABA 726 growth rate, cell density, and cell morphology in YCBM without and with increasing concentrations of Et-18 and 5 mM cAMP.

Culture medium	Growth rate (h^{-1})	Cell density($0D_{600}$)	Pseudohyphal cell (%)¤	Cell length (μm)¤
YCBM	0.15 ± 0.00[a]	1.05 ± 0.04[a]	94.68	10.17 ± 1.69[bc]
YCBM + cAMP	0.14 ± 0.00[a]	1.19 ± 0.08[a]	94.87	10.04 ± 2.02[bc]
YCBM + 100 μM Et-18	0.13 ± 0.02[b]	1.21 ± 0.01[b]	11.19	7.66 ± 2.55[a]
YCBM + 150 μM Et-18	0.12 ± 0.01[b]	1.23 ± 0.03[b]	6.15	7.16 ± 2.09[a]
YCBM + 100 μM Et-18 + cAMP	0.13 ± 0.00[b]	1.19 ± 0.02[b]	94.84	9.12 ± 3.27[ab]
YCBM + 150 μM Et-18 + cAMP	0.12 ± 0.01[b]	1.24 ± 0.02[b]	93.72	11.05 ± 3.90[c]

Cell density is expressed as OD_{600} measured after 48 h growth. Data are means ± SD of 4 independent cultures. ¤ evaluated on at least 500 cells in four independent experiments. Different letters indicate statistically different values within columns ($P < 0.05$).

that showed more intense hybridization signals with the cDNA of pseudohyphae (YCBM) (Fig. 1) and that represent 3.5% of the total population of clones. This result is similar to that obtained by other authors [18] who, by applying the same technique to the identification of genes associated with changes in the morphology of *P. fermentans* on apple and peach fruit, highlighted a number of clones corresponding to 3.1% of the whole population. These 12 clones were potentially related to pseudohyphal development, and were arbitrarily indicated with the acronym prg (pseudohyphae-related genes) followed by their progressive number.

3.2. Identification of the sequences cloned

The 12 prg clones were sequenced and subjected to BLAST analyses. The results reported in Table 2 indicate that four of the 12 clones showed 98 to 100% identity with GTPase cytoplasmic elongation factor 1-alpha from *P. fermentans* (prg8, prg10, prg11 and prg12). The remaining 8 showed orthologs in the *Candida* spp. genome. Three of these showed identity with the cytochrome c1 gene of *C. tropicalis* (prg9), with SUN41 gene of *C. albicans* coding for a cell-wall glycosidase (prg6), and with an aldose 1-epimerase (prg7) of *C. glabrata*. The

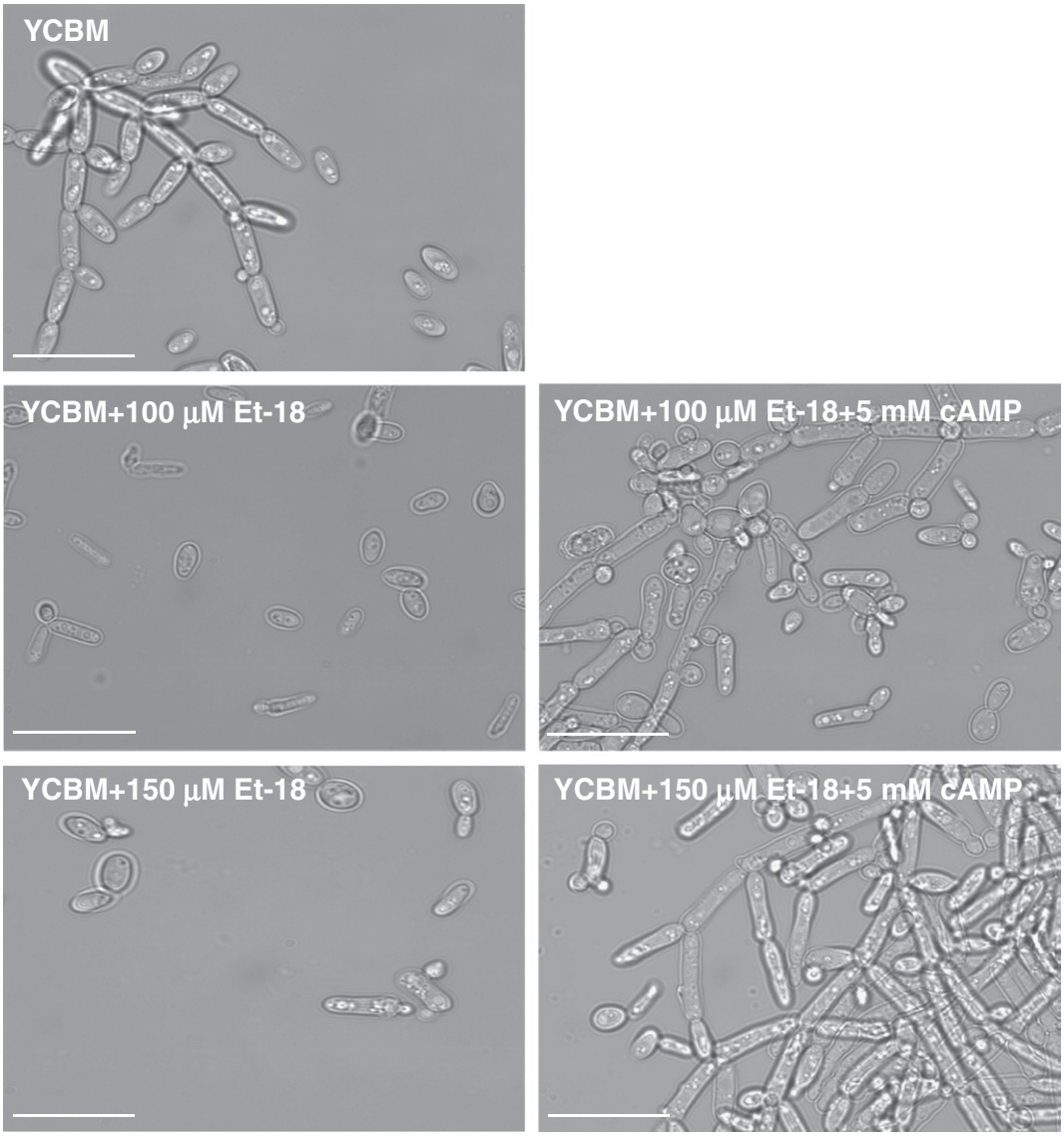

Fig. 3. Representative *P. fermentans* DiSAABA 726 morphology on YCBM without and with Et-18 and cAMP. Magnification, 100×. Data are representative of at least three independent experiments. Scale bars, 20 μm.

remaining five clones (prg1, prg2, prg3, prg4, and prg5) showed 75.6 to 82.4% identity to phospholipase C from *C. orthopsilosis*. Thus, BLAST analyses of these 12 sequences led to the identification of five genes whose higher level of transcription during pseudohyphal growth was confirmed by qRT-PCR (Fig. 2). Interestingly, the five genes code for proteins with possible roles in pseudohyphal growth. The overexpression of the gene coding for the GTPase cytoplasmic elongation factor 1-alpha from *P. fermentans* appears compatible with an increase in protein synthesis that might be needed for cell reorganization during the dimorphic transition from yeast-like to pseudohyphal [18]. The involvement of cytochrome C encoding gene can be envisaged taking into account that the mitochondrial electron transfer system is involved in hyphal growth in *C. albicans* [20]. For the possible involvement of the putative aldose 1-epimerase, it has been shown that the overexpression of glycolytic enzymes is related to hyphal growth in *C. albicans* [21] and to pseudohyphal growth in *P. fermentans* [18]. Interestingly, the transcription of some glycolytic enzymes is mediated by elongation factor 1 [22–24] that is up-regulated during pseudohyphal growth in *P. fermentans*, as shown in this work and by Fiori et al. [18]. Moreover, a gene showing an aldose 1-epimerase domain is a candidate virulence factor in the secretome of *Phytophtora infestans* [25]. Also the involvement of sequence prg6 that shows 73% identity with SUN41 gene of *C. albicans* might be related to pseudohyphal growth. Indeed, a *C. albicans sun41Δ* mutant cannot form hyphae on solid medium [26],

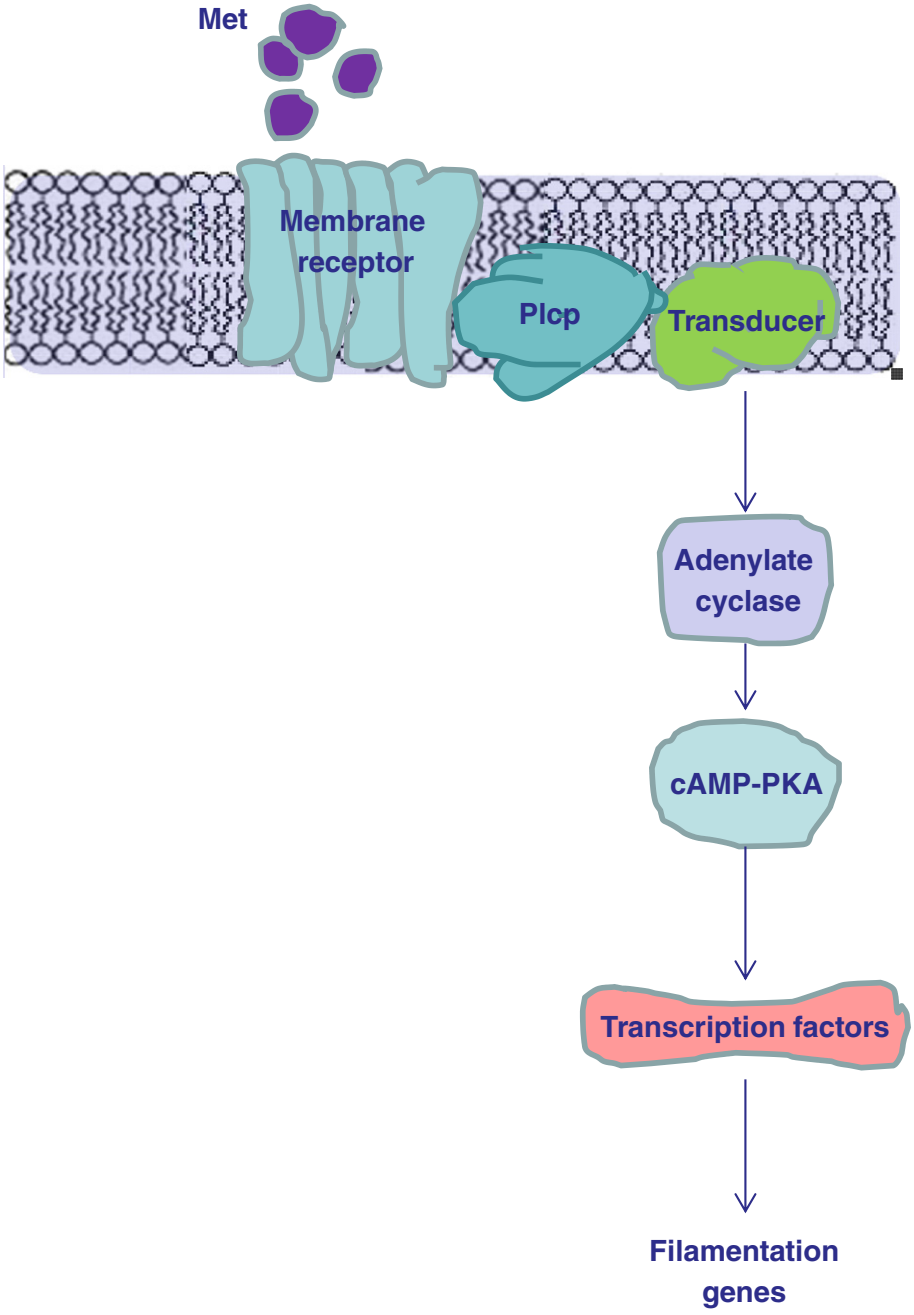

Fig. 4. Roles hypothesized for methionine and phospholipase C in *P. fermentans* DiSAABA 726 dimorphic transition. The presence of methionine is sensed by a "putative methionine sensing machinery" that involves cAMP-PKA signal transduction pathway through the participation of phospholipase C (Plcp) and leads to activation of the filamentation genes that favor pseudohyphal growth.

and this gene codes for a glycosidase involved in cytokinesis, cell-wall biogenesis, adhesion to host tissue, and biofilm formation, which have important roles in host–pathogen interactions [26–28].

Indeed, of the five genes that are more expressed under the pseudohyphal morphology, the one coding for a putative phospholipase C of *P. fermentans* was the most intriguing, both for its frequency of retrieval within the population of clones, and the role of phospholipase C in yeast filamentation. In *S. cerevisiae* phospholipase C (Plc1p) mediates the interactions between the membrane receptor Gpr1p and the G protein Gpa2p that activates the cAMP-PKA pathway. This, in turn, activates the transcription of genes involved in pseudohyphal growth [29]. In *C. albicans* reduced expression levels of phospholipase C are accompanied by defects in filamentous growth [30].

3.3. Role of putative *P. fermentans* phospholipase C in dimorphic transition

To study the role of phospholipase C in *P. fermentans* DiSAABA 726 dimorphic transition, this strain was grown in YCBM in the absence and in the presence of Et-18. Et-18 was used as it is a known inhibitor of phospholipase C [30] and both growth kinetics and cell morphology were analyzed. ET-18 addition resulted in an increase in biomass production that was accompanied by a decrease of growth rate (Table 3). Moreover, the morphology of *P. fermentans* DiSAABA 726 on YCBM with Et-18 saw dramatic changes with significant decreases in the percentages of pseudohyphae and in cell length (Fig. 3; Table 3) thus supporting a possible involvement of phospholipase C in filamentation also in *P. fermentans*.

Since the impairment of phospholipase C may inhibit *P. fermentans* filamentation possibly by affecting the initial step of the cAMP-PKA signal transduction pathway, it was hypothesized that the addition of exogenous cAMP, by circumventing the phospholipase C impairment, could restore filamentation. To evaluate this hypothesis 5 mM exogenous cAMP was added to YCBM in the absence and presence of Et-18. While not influencing cell morphology and growth kinetics in YCBM, the addition of cAMP fully restored pseudohyphal morphology in YCBM added with 100 and 150 μM Et-18 (Fig. 3).

These results are compatible with the hypothesis that *P. fermentans* harbors a putative "methionine sensing machinery" that senses the presence of methionine and activates cAMP-PKA signal transduction pathway through the involvement of phospholipase C (Fig. 4) thus leading to the transcription of filamentation genes. While the structure of this "methionine-sensing machinery" needs to be elucidated, phospholipase C is indeed a promising molecular target for further investigations into the formation of pseudohyphae in *P. fermentans* DiSAABA 726, and the link that this has with its associated pathogenicity. Should this link be confirmed, the dimorphic transition from yeast-like to pseudohyphal growth observed in *P. fermentans* DiSAABA 726 will need to be considered among the risk factors associated with the use of this microbial antagonist.

Acknowledgements

This work was financially supported by MURST PRIN Anno 2007-Prot. N 2007FRBK9N.

References

[1] H.D. Madhani, G.R. Fink, The control of filamentous differentiation and virulence in fungi, Trends Cell Biol. 8 (1998) 348–353.
[2] C. Sánchez-Martínez, J. Pérez-Martin, Dimorphism in fungal pathogens: *Candida albicans* and *Ustilago maydis*-similar inputs, different outputs, Curr. Opin. Microbiol. 4 (2001) 214–221.
[3] N.A.R. Gow, A.J.P. Brown, F.C. Odds, Fungal morphogenesis and host invasion, Curr. Opin. Microbiol. 5 (2002) 366–371.
[4] S.P. Saville, A.L. Lazzell, C. Monteagudo, J.L. Lopez-Ribot, Engineered control of cell morphology *in vivo* reveals distinct roles for yeast and filamentous forms of *Candida albicans* during infection, Eukaryot. Cell 2 (2003) 1053–1060.
[5] M. Whiteway, U. Oberholzer, *Candida* morphogenesis and host–pathogen interactions, Curr. Opin. Microbiol. 7 (2004) 350–357.
[6] C.J. Gimeno, P.O. Ljungdahl, C.A. Styles, G.R. Fink, Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: regulation by starvation and RAS, Cell 68 (1992) 1077–1090.
[7] B.L. Wickes, M.E. Mayorga, U. Edman, J.C. Edman, Dimorphism and haploid fruiting in *Cryptococcus neoformans*: association with the α-mating type, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 7327–7331.
[8] K. Biswas, J. Morschhäuser, The Mep2p ammonium permease controls nitrogen starvation-induced filamentous growth in *Candida albicans*, Mol. Microbiol. 56 (2005) 649–669.
[9] J.R. Dickinson, Fusel alcohols induce hyphal-like extensions and pseudohyphal formation in yeast, Microbiology 142 (1996) 1391–1397.
[10] M.C. Lorenz, N.S. Cutler, J. Heitman, Characterization of alcohol-induced filamentous growth in *Saccharomyces cerevisiae*, Mol. Biol. Cell 11 (2000) 183–199.
[11] M.M. Maidan, J.M. Thevelein, P. Van Dijck, Carbon source induced yeast-to-hypha transition in *Candida albicans* is dependent on the presence of amino acids and on the G-protein-coupled receptor Gpr1, Biochem. Soc. Trans. 33 (2005) 291–293.
[12] H.C. Chen, G.R. Fink, Feedback control of morphogenesis in fungi by aromatic alcohols, Genes Dev. 20 (2006) 1150–1161.
[13] P. Sudbery, N. Gow, J. Berman, The distinct morphogenic states of *Candida albicans*, Trends Microbiol. 12 (2004) 317–324.
[14] S. Giobbe, S. Marceddu, B. Scherm, G. Zara, V. Mazzarello, M. Budroni, Q. Migheli, The strange case of a biofilm-forming strain of *Pichia fermentans* which controls *Monilinia* brown rot on apple but is pathogenic on peach fruit, FEMS Yeast Res. 7 (2007) 1389–1398.
[15] M.L. Sanna, S. Zara, G. Zara, Q. Migheli, M. Budroni, I. Mannazzu, *Pichia fermentans* dimorphic changes depend on the nitrogen source, Fungal Biol. 116 (2012) 769–777.
[16] S.F. Altschul, T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 251 (1997) 3389–3402.
[17] B. Scherm, M. Schmoll, V. Balmas, C.P. Kubicek, Q. Migheli, Identification of potential marker genes for *Trichoderma harzianum* strains with high antagonistic potential against *Rhizoctonia solani* by a rapid subtraction hybridisation (RaSH) approach, Curr. Genet. 55 (2008) 81–91.
[18] S. Fiori, B. Scherm, J. Liu, R. Farrell, I. Mannazzu, M. Budroni, B.E. Maserti, M.E. Wisniewski, Q. Migheli, Identification of differentially expressed genes associated with changes in the morphology of *Pichia fermentans* on apple and peach fruit, FEMS Yeast Res. 12 (2012) 785–795.
[19] H. Jiang, D.C. Kang, D. Alexandre, P.B. Fisher, RaSH, a rapid subtraction hybridization approach for identifying and cloning differentially expressed genes, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 12684–12689.
[20] T. Watanabe, A. Ogasawara, T. Mikami, T. Matsumoto, Hyphal formation of *Candida albicans* is controlled by electron transfer system, Biochem. Biophys. Res. Commun. 348 (2006) 206–211.
[21] T.L. Han, R.D. Cannon, S.G. Villas-Bôas, The metabolic basis of *Candida albicans* morphogenesis and quorum sensing, Fungal Genet. Biol. 48 (2011) 747–763.
[22] T. Doedt, S. Krishnamurti, D.P. Bockmühl, B. Tebarth, C. Stempel, C.L. Russel, A.J.P. Brown, J.F. Ernst, APSES proteins regulate morphogenesis and metabolism in *Candida albicans*, Mol. Biol. Cell 15 (2004) 3167–3180.
[23] S. García-Sánchez, A.L. Mavor, C.L. Russel, S. Argimon, P. Dennison, B. Enjalbert, A.J.P. Brown, Global roles of Ssn6 in tup1- and nrg1-dependent gene regulation in the fungal pathogen *Candida albicans*, Mol. Biol. Cell 16 (2005) 2913–2925.
[24] J.A. Sexton, V. Brown, M. Johnston, Regulation of sugar transport and metabolism by the *Candida albicans* Rgt1 transcriptional repressor, Yeast 24 (2007) 847–860.
[25] S. Raffaele, J. Win, L.M. Cano, S. Kamoun, Analyses of genome architecture and gene expression reveal novel candidate virulence factors in the secretome of *Phytophthora infestans*, BMC Genomics 11 (2010) 637 (http://www.biomedcentralcom/1471-2164/11/637).
[26] E. Hiller, S. Heine, H. Brunner, S. Rupp, *Candida albicans* Sun41p, a putative glycosidase, is involved in morphogenesis, cell wall biogenesis, and biofilm formation, Eukaryot. Cell 6 (2007) 2056–2065.
[27] M. Mouassite, N. Camougrand, E. Schwob, G. Demaison, M. Laclau, M. Guerin, The "SUN" family: yeast *SUN4/SCW3* is involved in cell septation, Yeast 16 (2000) 905–919.
[28] C.T. Norice, F.J. Smith Jr., N. Solis, S.G. Filler, A.P. Mitchell, Requirement for *Candida albicans* Sun41 in biofilm formation and virulence, Eukaryot. Cell 6 (2007) 2046–2055.
[29] K. Ansari, S. Martin, M. Farkasovsky, I.M. Ehbrecht, H. Kuntzel, Phospholipase C binds to the receptor-like *GPR1* protein and controls pseudohyphal differentiation in *Saccharomyces cerevisiae*, J. Biol. Chem. 274 (1999) 30052–30058.
[30] D. Kunze, I. Melzer, D. Bennet, D. Sanglard, D. MacCallum, J. Norskau, D.C. Coleman, F.C. Odds, W. Schafer, B. Hube, Functional analysis of the phospholipase C gene *CaPLC1* and two unusual phospholipase C genes, CaPLC2 and CaPLC3, of *Candida albicans*, Microbiology 151 (2005) 3381–3394.