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SHORT COMMUNICATION

Transcription of the Hsp30, Hsp70, and Hsp90 heat shock protein genes is modulated by the PalA protein in response to acid pH-sensing in the fungus *Aspergillus nidulans*

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Abstract Heat shock proteins are molecular chaperones linked to a myriad of physiological functions in both prokaryotes and eukaryotes. In this study, we show that the *Aspergillus nidulans hsp30* (ANID_03555.1), *hsp70* (ANID_05129.1), and *hsp90* (ANID_08269.1) genes are preferentially expressed in an acidic milieu, whose expression is dependent on the *palA*$^{+}$ background under optimal temperature for fungal growth. Heat shock induction of these three *hsp* genes showed different patterns in response to extracellular pH changes in the *pal*A^{+} background. However, their accumulation upon heating for 2 h was almost unaffected by ambient pH changes in the *palA*$^{-}$ background. The PalA protein is a member of a conserved signaling cascade that is involved in the pH-mediated regulation of gene expression. Moreover, we identified several genes whose expression at pH 5.0 is also dependent on the *palA*$^{+}$ background. These results reveal novel aspects of the heat- and pH-sensing networks of *A. nidulans*.

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Introduction

The filamentous fungus, *Aspergillus nidulans*, has since long been an important tool for studying a number of cellular processes such as regulation of gene expression by the pH. Ambient pH signaling ensures that nutritional enzymes will be secreted at pH values at which they can function effectively; for instance, active acid and alkaline Pi-repressible phosphatases are secreted at acid and alkaline pH, respectively. This adaptive response involves the highly conserved PacC signal transduction pathway that mediates many metabolic events in model and pathogenic fungi, including the fungal virulence (Caddick et al. 1986; Ferreira-Nozawa et al. 2006; Leal et al. 2010; Silveira et al. 2010; Squina et al. 2010; Tilburn et al. 1995). The *pacC* gene encodes a Zn-finger transcription factor that is activated by a conserved signaling cascade composed of six *pal* genes (*palA*,*B*,*C*,*F*,*H*,*I*), and it has been reported that this activation would occur under exclusively alkaline growth conditions (Tilburn et al. 1995). The PalA protein interacts with the full-length version of the transcription regulator PacC, thereby mediating a protein–protein interaction that is necessary for the proteolytic activation of PacC (Vincent et al. 2003). Thus, irrespective of the ambient pH and assuming that PacC is inactive at acidic pH in the absence of *pal* signaling, loss-of-function mutations in any of the *pal* genes should lead to a wild-type acidic growth phenotype (Tilburn et al. 1995). However, there is increasing evidence that *pacC* and *pal* genes are functional when cultivation is carried out at both

acidic and alkaline pH (Leal et al. 2010; Silva et al. 2008; Silveira et al. 2010; Squina et al. 2010).

The aim of this study was to identify genes whose transcription is dependent on the *palA*$^{+}$ background at acidic pH. Functional characterization of these genes led to the identification of putative proteins involved in a number of cellular processes such as heat shock response, cell cycle, and development. In this study, we also show that the *A. nidulans hsp30*, *hsp70*, and *hsp90* genes are preferentially expressed in mycelia cultured at pH 5.0 and 37°C, which is the optimal culture temperature for fungal growth. These results improve our understanding of the metabolic functions of the *palA* gene and reveal novel aspects of the heat-sensing network of *A. nidulans*.

Materials and methods

A. nidulans strains

The *A. nidulans biA1* (biotin requiring, FGSC No. A26) and the strains carrying loss-of-function mutations in the *palA* (*biA1 palA1*, FGSC No. A243) and *palC* (*biA1 palC4*, FGSC No. 250) genes were obtained from the Fungal Genetic Stock Center (www.fgsc.net).

Culture and heat stress conditions

The conidia from each strain (approximately 10^7 cells/ml) were cultivated at 37°C with continuous shaking at 200 rpm for 7 h in low-Pi (200 μmol/l Pi) or high-Pi (10 mmol/l Pi) minimal liquid medium at pH 5.0 (buffered with 50 mmol/l sodium citrate) or pH 8.0 (buffered with 50 mmol/l Tris–HCl). The culture medium was supplemented with 1% D-glucose, 70 mmol/l sodium nitrate, and 10 mmol/l KCl (Cove 1966).

The *biA1* strain is the control strain used to study pH responses, Pi acquisition, and sensing. This strain responds positively to colony staining for Pi-repressible acid phosphatase and secretes this enzyme in liquid medium when cultured under limited Pi conditions at pH 5.0. The *biA1 palA1* and *biA1 palC4* strains enhanced the colony staining for acid phosphatase at pH 6.5. These strains grow very poorly on solid medium but reasonably well in liquid medium, both media adjusted to pH 8.0. The *palA1* and *palC4* mutations were induced in the *biA1* strain by exposure to UV light, and the mutant strains were selected for showing reduced alkaline phosphatase and increased acid phosphatase activities at pH 6.5. This was visualized by growing the colonies on solid medium lacking Pi and subsequent staining for Pi-repressible phosphatases (Dorn 1965a, b; Freitas et al. 2007). These isogenic strains were recently re-isolated by backcrosses, were maintained on silica at 4°C, and revived on the solid complete medium before use.

To determine the effect of heat stress, conidia germinated for 7 h at 37°C in low- or high-Pi medium buffered at pH 5.0 or pH 8.0 with shaking (200 rpm) were incubated for 0.5, 1, or 2 h at 50°C (Squina et al. 2010). The strains carrying the *biA1* mutation were cultured in medium supplemented with 2 μg biotin/ml.

Suppression subtractive hybridization (SSH) and screening of subtracted cDNA clones

Total RNA (1 μg) extracted from the mycelia with the NucleoSpin®RNA Plant Kit (BD Biosciences Clontech) was used to synthesize double-stranded cDNAs with the BD SMART™ PCR cDNA Synthesis Kit (BD Biosciences Clontech). The manufacturer's recommendations were followed throughout the cDNA synthesis procedure.

SSH was performed on *biA1* and *biA1 palA1* strains cultured for 7 h in low-Pi minimal medium buffered at pH 5.0. The PCR-Select™ cDNA Subtraction Kit (Clontech Laboratories) was used. For screening down-regulated genes in the mutant strain, forward subtractions were performed using the *biA1 palA1* strain as the driver and the *biA1* strain as the tester. The PCR products of the subtracted library were cloned into the pGEM-T-Easy Vector System (Promega) and transformed into *Escherichia coli* Mos-Blue-competent cells. Subtraction was also performed with the *biA1 palA1* strain as the tester to prepare reverse-subtracted cDNA probes for differential screening. The cDNAs corresponding to differentially expressed sequences in the *palA1* strain were amplified by PCR, and the products were screened by reverse northern hybridization, as described earlier (Gras et al. 2007; Silva et al. 2008).

DNA sequencing and validation of differentially expressed genes

The plasmids from arrayed clones that visually exhibited positive differential expression were purified and sequenced using the M13 forward primer, and the cDNA sequences were subjected to computational searches against the GenBank database. Expressed sequence tag (EST) sequences showing high nucleotide quality were processed with the CAP3 software for contig assembly, and the corresponding ORFs were identified in the *A. nidulans* genome database (http://www.broad.mit.edu/annotation/genome/aspergillusnidulans/Home.html) and subjected to BLASTX search against the GenBank database. The sequences were grouped into functional categories according to their putative BLASTX identification (Munich Information Center for Protein Sequences) (http:/mips.gsf.de). Mutation in the

Table 1 Genes downregulated in the *Aspergillus nidulans palA1* mutant strain identified by SSH and reverse northern hybridization

Predicted ORF[a]	Putative gene products	Number of clones[b]	FunCat MIPS[c]	PacC consensus[d] 5′-GCCARG-3′
ANID_03706.1	40S ribosomal protein S10b	32 (25.6%)	12	3
ANID_05996.1	60S acidic ribosomal protein P2 family	21 (16.8%)	12	0
ANID_01964.1	40S ribosomal protein S6	18 (14.4%)	12	0
ANID_08269.1[e]	hsp90-like protein	5 (4.0%)	32	1
ANID_00646.1	ATP-dependent helicase NAM7	3 (2.4%)	11, 12, 16	0
ANID_08704.1	60S ribosomal protein L24a	3 (2.4%)	12	0
ANID_07657.1[e]	1, 3-beta-glucanosyltransferase GelA	2 (1.6%)	43	4
ANID_04190.1	GPI-anchored serine-rich protein	2 (1.6%)	14	2
ANID_01624.1[e]	Mitochondrial ATP synthase (atp9)	2 (1.6%)	01, 20	2
ANID_07169.1	Flavohemoprotein	2 (1.6%)	32	2
ANID_08690.1	DUF1690 domain-containing protein	2 (1.6%)	99	1
ANID_04113.1[e]	Sensor histidine kinase/response regulator, putative	1 (0.8%)	14, 30	1
ANID_04508.1[e]	Bax Inhibitor family protein	1(0.8%)	16, 30	0
ANID_02282.1[e]	MFS transporter	1 (0.8%)	20	2
ANID_09168.1	MFS sugar transporter STL1	1 (0.8%)	20	4
ANID_05129.1[e]	hsp70-like protein	1 (0.8%)	32	1
ANID_03555.1[e]	30 kDa heat shock protein	1 (0.8%)	32	1
ANID_05781.1	Heat shock protein 30	1 (0.8%)	32	0
ANID_06048.1	Aspartate transaminase	1(0.8%)	01	0
ANID_01049.1	Zinc metalloproteinase	1 (0.8%)	14, 16	1
ANID_09488.1	Thiamine pyrophosphokinase Thi80	1 (0.8%)	01, 16	2
ANID_02367.1	Putative Zn(II)2Cys6 transcription factor	1 (0.8%)	01, 11	2
ANID_04908.1	Eukaryotic translation initiation factor subunit, putative	1 (0.8%)	12, 16	0
ANID_00465.1	40S ribosomal protein S8e	1 (0.8%)	12	1
ANID_02734.1	60S acidic ribosomal protein P0	1 (0.8%)	12	0
ANID_10416.1	60S ribosomal protein L27a	1 (0.8%)	12	2
ANID_04787.1	60S ribosomal protein L37	1 (0.8%)	12	2
ANID_02998.1	C1 tetrahydrofolate synthase	1 (0.8%)	01, 16	1
ANID_03873.1	Zinc-containing alcohol dehydrogenase	1 (0.8%)	01, 02	1
ANID_04064.1	ADP,ATP carrier protein	1 (0.8%)	01, 20	1
ANID_08793.1	Succinate dehydrogenase cytochrome b560 subunit	1 (0.8%)	01	0
ANID_02875.1	Fructose-bisphosphate aldolase	1 (0.8%)	01, 02	0
ANID_04163.1	Guanine nucleotide-binding protein subunit beta-like protein	1 (0.8%)	40, 43	0
ANID_08722.1	ATP-dependent RNA helicase sub2	1 (0.8%)	11, 16	2
ANID_03431.1	Nicotinate-nucleotide pyrophosphorylase	1 (0.8%)	01	2
ANID_12271.1	RNA annealing protein Yra1	1 (0.8%)	16, 20	0
ANID_00493.1	Surface protein 1	1 (0.8%)	99	2
ANID_07625.1	Myo-inositol-1-phosphate synthase	1 (0.8%)	01, 16	1
ANID_00752.1	RNA polymerase subunit	1 (0.8%)	11	0
ANID_01851.1	t-complex protein 1	1 (0.8%)	16, 42	2
ANID_05482.1	GTP-binding nuclear protein GSP1/Ran	1 (0.8%)	11, 20, 30	2
ANID_03802.1	Conserved hypothetical protein	1 (0.8%)	99	0
ANID_11096.1	Conserved hypothetical protein	1 (0.8%)	99	3

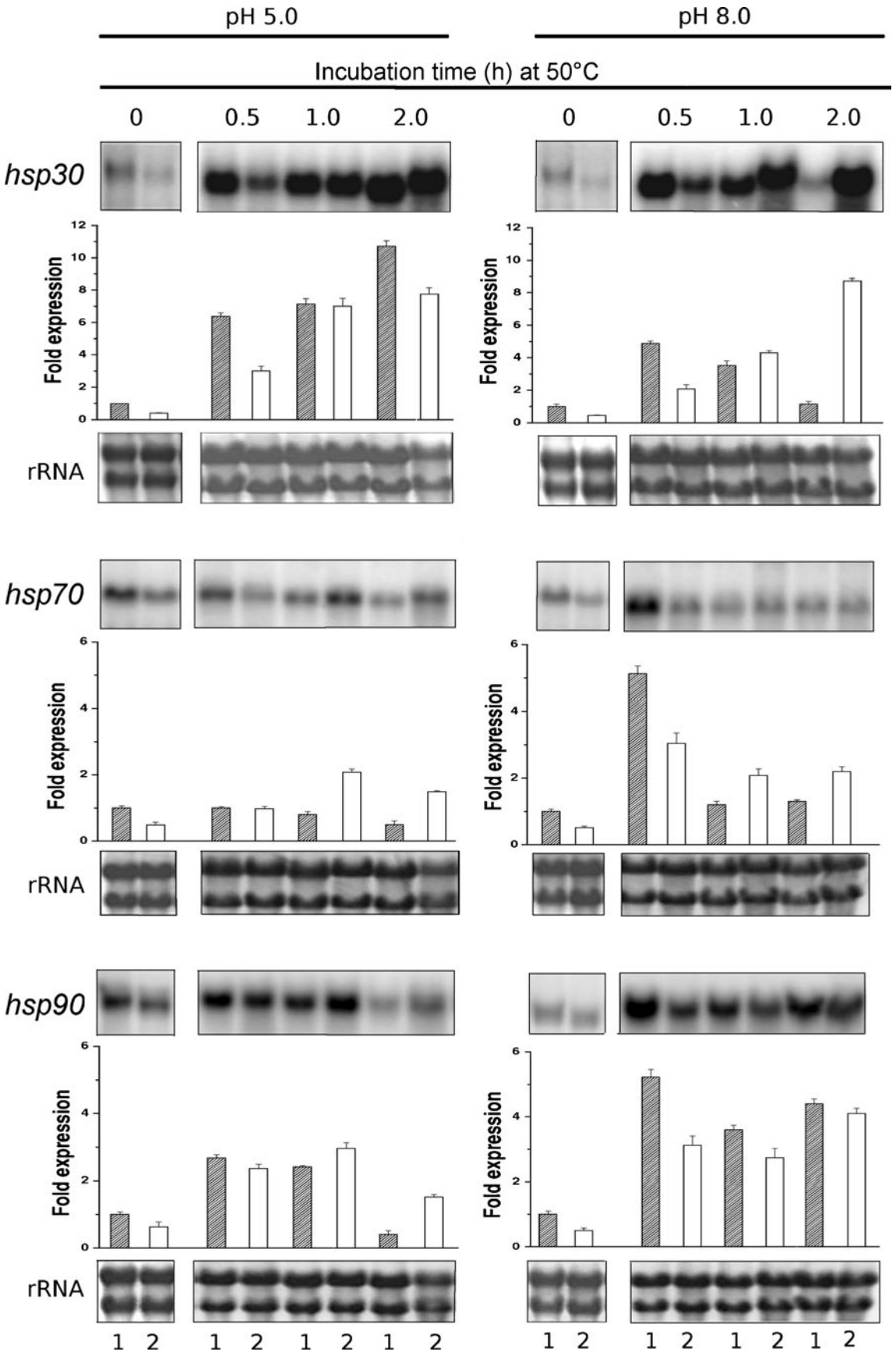
[a] Gene ID in *A. nidulans* Genome database (Broad Institute)

[b] Percentage in parentheses was calculated based on 124 cDNA clones analyzed

[c] MIPS: 01, metabolism; 02, energy; 11, transcription; 12, protein synthesis; 14, protein fate; 16, protein with binding function or cofactor requirement; 20, cellular transport; 30, cellular communication; 32 cell rescue, defense and virulence; 40, cell fate; 42, biogenesis of cellular components; 43, cell-type differentiation; 99, unclassified protein

[d] The consensus sequences 5′-GCCARG-3′ were screened over 1,000 nucleotides upstream the predicted ORFs in the Broad Institute database

[e] Transcripts downregulated in the *palA1* strain validated by Northern blot (Figs. 1, 2, and 3)


pH 5.0
pH 8.0
Incubation time (h) at 50°C
0
0.5
1.0
2.0
hsp30
hsp70
hsp90
Fold expression
rRNA
1 2 1 2 1 2 1 2

◀ **Fig. 1** Northern blot analysis of *hsp* transcripts using total RNA from the control and *palA1* strains of *A. nidulans*. The conidia from the control (*lane 1*) and *palA1* (*lane 2*) strains were grown for 7 h at 37°C in low-Pi medium buffered at pH 5.0 or pH 8.0. The mycelia were incubated at 50°C for the time indicated. The ethidium bromide-stained rRNA band is shown as the loading control. The *bars* indicate the relative fold expression determined from the densitometric analyses. The data represent the average values±standard deviation (SD) from three independent blot exposures

palA gene was identified by DNA sequencing of the *palA*$^{+}$ and *palA1* alleles.

For validating differential gene expression by northern blotting, the subtracted cDNA clones were amplified by PCR, radioactively labeled with [α-^{32}P]dCTP, purified, and used as probes (Gras et al. 2007; Silva et al. 2008).

Results and discussion

Following differential screening of the cDNA clones generated by both the forward and reverse-subtracted probes, 124 candidate clones were identified as being downregulated in the *palA1* strain, and these were isolated and sequenced. The results of similarity searches against the *A. nidulans* database revealed 43 non-redundant unigenes. Functional categorization of these genes led to the identification of putative proteins involved in diverse cellular processes (Table 1). We confirmed the differential expression of eight of these genes by northern blot analysis (Figs. 1, 2, and 3). Thus, the transcription of these genes and probably those listed in the Table 1 is positively modulated by the putative protein PalA. In order to extend the analysis of gene *palA*, the transcription profiling of some of these eight genes were also evaluated under different culture conditions such as heat stress, alkaline pH, or sufficient Pi media (Figs. 1, 2, and 3).

DNA sequencing of the *palA*$^{+}$ and *palA1* alleles showed a single T-to-A nucleotide exchange at nucleotide position 1802 in the *palA1* allele, which results in a stop codon in the *palA1* transcript. Thus, the predicted PalA1 protein is 247 amino acids shorter than the full-length PalA protein.

Transcripts of the *A. nidulans hsp30* (ANID_3555.1), *hsp70* (ANID_5129.1), and *hsp90* (ANID_8269.1) heat shock genes accumulated in the germinating conidia of the *palA*$^{+}$ strain incubated in an acidic milieu at 37°C, which is the optimal culture temperature for fungal growth (Fig. 1). This *hsp30* gene of *A. nidulans* codes for a putative alpha-crystallin-related small heat shock protein, which is homologous to the *HSP26* gene of *Saccharomyces cerevisiae*. Interestingly, this *HSP26* gene is strongly induced by a number of stressing conditions, including low ambient pH (Amoros and Estruch 2001; Burnie et al. 2006; Carmelo and Sa-Correia 1997; Haslbeck et al. 1999; Kusakabe et al. 1994). The 70-kDa class of heat shock protein genes is associated with the folding and translocation of proteins across membranes and also with cell division and developmental stage progression, among various other functions (Squina et al. 2010). Cytosolic Hsp90, an essential and highly conserved molecular chaperone that promote cancer cell growth, differentiation, and apoptosis in humans, is also involved in the activation of many key signal transducers in fungi (Cid et al. 2009; Cowen 2009; LaFayette et al. 2010; Mittelman et al. 2010; Shapiro et al. 2009; Solit and Chiosis 2008). The amount of the *hsp30* transcript induced upon heating for 2 h was greatly reduced in the *palA*$^{+}$ background at alkaline pH, an effect not

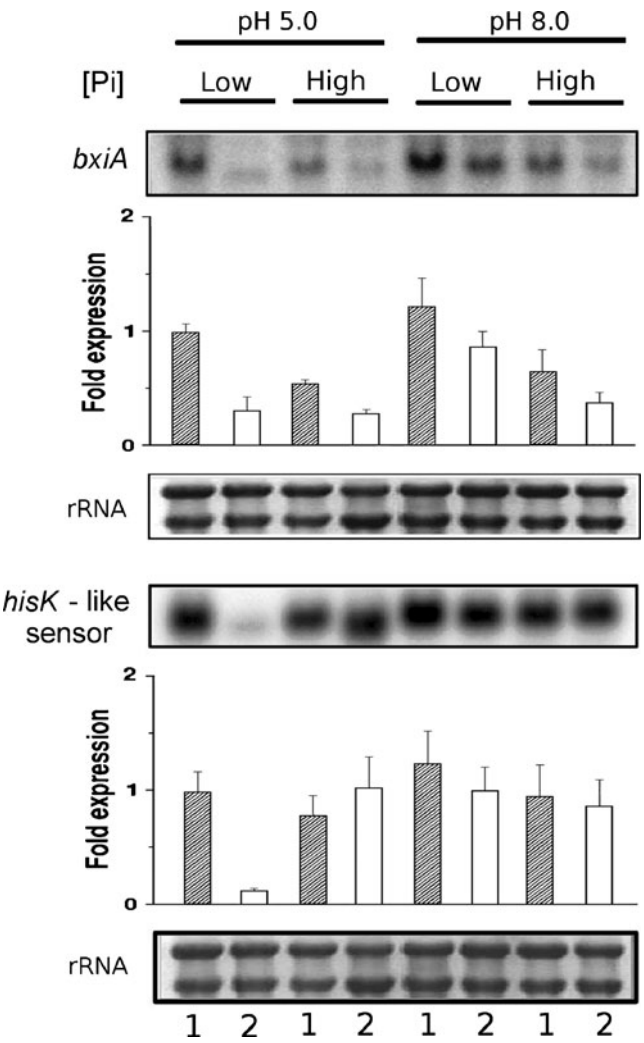


Fig. 2 Northern blot analysis of *bxiA* and sensor histidine kinase (*hisK*-like sensor) transcripts using total RNA from the control and *palA1* strains of *A. nidulans*. The conidia from the control (*lane 1*) and *palA1* (*lane 2*) strains were grown for 7 h at 37°C in low- or high-Pi liquid medium buffered at pH 5.0 or pH 8.0. The ethidium bromide-stained rRNA band is shown as the loading control. The *bars* indicate the relative fold expression determined from the densitometric analyses. The data represent the average values±standard deviation (SD) from three independent blot exposures

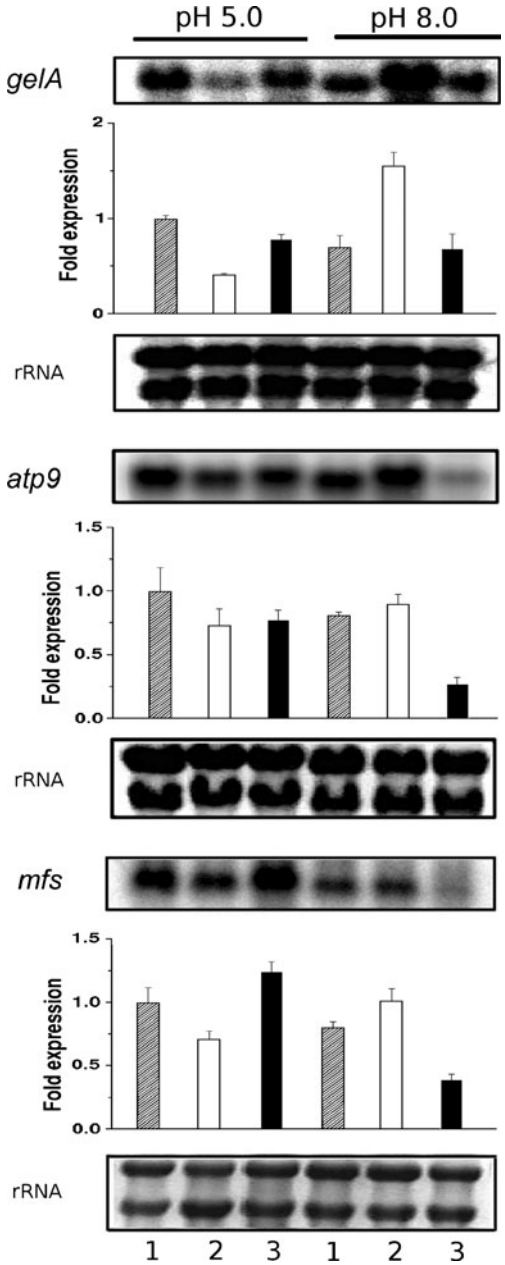


Fig. 3 Northern blot analysis of the *gelA*, *atp9*, and *mfs* transcripts using total RNA from the control, *palA1*, and *palC4* strains of *A. nidulans*. The conidia from the control (*lane 1*), *palA1* (*lane 2*), and *palC4* (*lane 3*) strains were grown for 7 h at 37°C in a low-Pi liquid medium buffered at pH 5.0 or pH 8.0. The ethidium bromide-stained rRNA band is shown as the loading control. The *bars* indicate the relative fold expression determined from the densitometric analyses. The data represent the average values±standard deviation (SD) from three independent blot exposures

observed at acidic pH (Fig. 1). However, the *hsp30* transcript accumulated in the *palA*$^-$ background upon heat shock under both acidic and alkaline culture conditions (Fig. 1). Expression of the *hsp70* gene was highly stimulated in the *palA*$^+$ background upon heating for 0.5 h at pH 8.0, and this transcript accumulated in the *palA*$^-$ background upon heating for 1 or 2 h in both acidic and alkaline cultures (Fig. 1). Extracellular pH changes had a limited effect on the levels of the *hsp90* gene induced by heat shock in both the *palA*$^+$ and *palA*$^-$ backgrounds; however, this transcript is probably unstable upon heating for 2 h at pH 5.0 (Fig. 1). Thus, in spite of the different patterns of induction, accumulation of these three *hsp* transcripts upon heating for 2 h was almost unaffected by ambient pH changes in the *palA*$^-$ background. The PalA protein mediates the proteolytic activation of PacC, a regulator required for the activation/repression of acid or alkaline genes in *A. nidulans* and other filamentous fungi (Leal et al. 2010; Silva et al. 2008; Silveira et al. 2010; Squina et al. 2010; Tilburn et al. 1995). PacC binds to 5′-GCCARG-3′ sequences upstream of pH-conditioned genes, and the presence of this hexanucleotide in the sequences upstream of the *hsp30*, *hsp70*, and *hsp90* genes (Table 1) suggests that their expression in an acidic milieu and at an optimal temperature for fungal growth might be directly controlled by PacC, i.e., the transcription of these heat shock genes may be PalA-dependent. Moreover, their accumulation upon heat shock for 2 h may be PalA-independent in both acidic and alkaline cultures. Thus, the *hsp30*, *hsp70*, and *hsp90* genes are preferentially expressed in the *palA*$^+$ strain incubated at an optimal temperature for fungal growth at acidic pH. Eukaryotic heat shock and stress-related (Sfl1) transcription factors regulate the constitutive and stress-inducible transcription of several genes (Galeote et al. 2007; Hjorth-Sorensen et al. 2001; Truman et al. 2007), including the *hsp* genes, and thereby play a central role in the regulation of numerous cellular reprogramming events (Sakurai and Takemori 2007; Squina et al. 2010). Therefore, interactions between the PacC/Pal conserved signaling cascade and these transcription factors, which include their competition for target genes, are highly complex.

Transcripts of the *A. nidulans* gene ANID_04508.1 (here named *bxiA* gene), which codes for a BI-1 homologous protein, accumulated in germinating conidia of the *palA*$^+$ strain incubated in either low- or high-Pi media at both acidic and alkaline pH values. Moreover, expression of this gene was reduced in the *palA*$^-$ background under both acidic and alkaline pH conditions, and this effect persisted even when the ambient Pi changed (Table 1, Fig. 2). Mitochondrial permeabilization associated with release of cytochrome *c* to the cytosol is a key step in the programmed cell death machinery, which includes both pro-apoptotic and anti-apoptotic members. Interestingly, the cell-death inhibitor BI-1 (Bax inhibitor 1), which is conserved in fungi but Bax itself is not, can suppress apoptosis induced by heterologous Bax in yeast (Chae et al. 2003; Eisenberg et al. 2007; Reimers et al. 2008; Xu and Reed 1998). In contrast, expression of the histidine kinase

sensor gene (ANID_04113.1) was strongly reduced in the *palA*$^{-}$ background in a low-Pi medium at pH 5.0, an effect that was almost undetected at alkaline pH (Fig. 2). Transcription of the 1, 3-beta-glucanosyltransferase (ANID_07657.1; *gelA* gene), mitochondrial ATP synthase (ANID_01624.1; homologous to *ATP9* gene of *S. cerevisiae*), and MFS transporter (ANID_02282.1) genes (Calderon et al. 2010; Jia et al. 2007; Tenreiro et al. 2000) was reduced in the *palA1* mutant grown in low-Pi cultures at acidic pH (Fig. 3). However, *gelA* transcripts accumulated to high levels in the *palA*$^{-}$ background at alkaline pH, but such an effect was not observed for the *atp9* and *mfs* transcripts (Fig. 3). Interestingly, the expression levels of these three genes were almost the same, being like wild type in the *palC*$^{-}$ background at acidic pH, whereas the expression of both the *atp9* and *mfs* transcripts was strongly reduced, relative to both wild type and *palA1*, in the *palC*$^{-}$ background at alkaline pH (Fig. 3). Gene *palC* was identified as a putative member of the conserved signaling cascade involved in ambient pH sensing whose function is to promote the activation of PacC (Freitas et al. 2007; Galindo et al. 2007). It has been recently demonstrated that PalF acts upstream of or at the level of PalC (Hervas-Aguilar et al. 2010), thus transmitting the ambient pH signal to the downstream elements of this regulatory pathway (PacC, PalA, and PalB). However, our results indicate that the gene *palA* is required for the induction of the genes *gelA*, *atp9*, and *mfs* at acidic pH, whereas *palC* is required for the induction of genes *atp9* and *mfs* under alkaline pH, i.e., genes *palA* and *palC* may act independently in the induction of these three genes and possibly many others. The PHR1 gene, a homologue of *gelA*, which codes for a pH-regulated protein in *Candida albicans*, plays a critical role in fungal cell wall biogenesis, apical growth, morphogenesis, adhesion, and virulence in this human opportunistic pathogen (Calderon et al. 2010). In *S. cerevisiae* and *Schizosaccharomyces pombe*, plasma membrane H^{+}-ATPases are strongly regulated by glucose or acidic pH, and the MFS transporters are carriers involved in various functions such as the transport of small solutes against chemiosmotic gradients or in multidrug resistance (Jia et al. 2007; Tenreiro et al. 2000).

In conclusion, SSH was successfully used to identify genes that were downregulated in the *palA1* mutant of *A. nidulans* grown in limiting Pi at pH 5.0 and 37°C. Functional grouping helped to identify genes known to play important roles in various cellular processes such as stress response and cell growth. Another interesting finding was that different patterns of heat shock induction were followed by the genes encoding the three Hsp proteins that were downregulated in a *palA*$^{-}$ background in mycelia cultured under optimal temperature conditions for growth. These results reveal novel aspects of the metabolic functions of the *palA* gene in response to pH-sensing in *A. nidulans*.

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