Front. Agric. China 2011, 5(3): 361–365
DOI 10.1007/s11703-011-1109-z

RESEARCH ARTICLE

mRNA level of *PKA-c* gene in *Setosphaeria turcica* with different nutrition sources under metal ion or osmotic stress

Qian WANG *, Wei ZHAO *, Zhimin HAO *, Jingao DONG (✉)

Molecular Plant Pathology Laboratory, Agricultural University of Hebei, Baoding 071001, China

Abstract **Intracellular signal transduction pathways including MAPK, Ca^{2+}, and cAMP signal transduction pathways play important roles in regulating growth, development, and pathogenesis of phytopathogenic fungi. Protein kinase A (PKA) is a key enzyme in cAMP signaling pathway. The transcription level of the gene encoding catalytic subunit of PKA in *Setosphaeria turcica* under different culture conditions was analyzed by the semiquantitative RT-PCR method. The expression level of *PKA-c* gene was the lowest on the medium containing sucrose and starch as the carbon source, and it was distinctly inhibited by Cu^{2+}, but it was independent of nitrogen source. After the addition of different concentrations of sorbitol, it showed the positive correlation between the inhibition affection and concentrations. However, the highest expression was observed in response to NaCl (0.9 mol/L). This research enriched the biological information resource of filamentous fungi and laid a foundation for the functional analysis about signal transduction pathway in phytopathogenic fungi.**

Keywords *Setosphaeria turcica*, PKA, gene expression, semiquantitative RT-PCR

Introduction

Northern corn leaf blight is caused by the ascomycetous fungus *Setosphaeria turcica*. Its appearance and overspreading causes significant yield losses (Li et al., 2000; Zhou et al., 2003). *S. turcica* generates appressorium to penetrate the surface of plant tissue with mechanical pressure and creates typical brown lesions (Fan et al., 2004). Several studies of the signaling pathway in fungi have enhanced the intracellular signal transduction pathways including MAPK, Ca^{2+}, and cAMP, which play important roles in regulating growth, development, and pathogenesis of phytopathogenic fungi. PKA is the major downstream factor that affects the cAMP-dependent signaling pathway. PKA holoenzyme is an inactive tetrameric protein that consists of two regulatory subunits (PKA-R) and two catalytic subunits (PKA-C) and has close relationship with pathogen pathogenicity. In phytopathogenic fungi, PKA has been shown to regulate dimorphic transition and pathogenesis in *Ustilago maydis* (Dürrenberger et al., 1998). cAMP, as a mediator of infection structure formation, provided a clue to the regulation of this developmental process (Lee and Dean, 1993); *cpkA* gene regulated the appressorium formation and pathogenicity in *Magnaporthe grisea* (Mitchell and Dean, 1995). PKA regulated the germination of *Mucor rouxii* sporangiospores (Rossi and Moreno, 1994). Recent studies showed that cAMP signaling pathway is related to the adaptive capacity of fungi under nutritional stress (Kronstad et al., 1998; Liebmann et al., 2004; Yamauchi et al., 2004; Mehrabi and Kema, 2006), but the expression level of *PKA-c* gene in fungi under different stresses has not been reported currently.

In this study, we obtained the *PKA-c* gene and used the semiquantitative RT-PCR method to analyze the expression level of the *PKA-c* gene in *S. turcica*. This research laid a foundation for the functional analysis about signal-transduction pathway in phytopathogenic fungi.

Materials and methods

Fungal strains and culture conditions

S. turcica strain 01–23 (stock culture in the Laboratory of Molecular Plant Pathology Laboratory, Agricultural University of Hebei) was used as the wild-type strain. The fungi

Received March 29, 2011; accepted April 14, 2011

Correspondence: Jingao DONG

E-mail: dongjingao@126.com

*They contributed equally to this work.

grew on potato dextrose agar (PDA) medium at 25°C in the dark.

The first base medium was Richard's solution, including 10 g KNO_3, 10 g KH_2PO_4, 2.5 g $MgSO_4$, 0.02 g $FeCl_3$, 50 g sucrose, and 1000 mL distilled water. Sucrose, glucose, lactose, starch, maltose, sorbitol, and fructose were used as carbon sources, with KNO_3, $NaNO_3$, NH_4Cl, and $CO(NH_2)_2$ as nitrogen sources.

The second medium was potato dextrose (PD) medium. Briefly, exogenous stresses containing metal ion stress (Mg^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}) and penetration stress were added in the 7-day-old fungi in PD medium. Osmotic stress involved sorbitol (0, 0.5, 1.0, 2.0 mol/L) and NaCl (0.3, 0.5, 0.7, 0.9 mol/L). The mycelia were dried by Freeze Dryer after the treatment at 25°C for 72 h.

Verified gene copy number

Genomic DNA was extracted from *S. turcica* mycelia. For Southern blot analysis, 20 mg genomic DNA was digested with restriction endonuclease and separated by electrophoresis on a 1% agarose gel. The blots were hybridized using a digoxigenin (DIG)-labeled *PKA-c* fragment as a probe. The fragments were digested with the restriction enzymes (non in fragments) *Xba*I, *Sac*II, and *Bgl*II, respectively. DNA labeling, gel electrophoresis, transmembrane, hybridization, and detection were carried out using the DIG system according to the manufacturer's recommendations (DIG DNA Labeling and Detection Kit).

Total RNA extraction

Total RNA was extracted with an RNAiso reagent kit (Sangon, Shanghai, China). The concentration and purity of the nucleic acids were determined by electrophoresis on 1.2% agarose gels.

Synthesis of the first chain cDNA

Each mycelia sample of the Total RNA (equal) was used for first-strand cDNA synthesis. The reverse transcription system including 1 L total RNA, 1 L Oligo (dT), 1 L dNTP, and 7 L DEPC-H_2O, was kept at 65°C for 5 min and 2 min on ice immediately, and then, it was centrifuged for a few seconds and added with 4 L 5 × buffer, 0.5 L RNase inhibitor, 0.5 L PrimeScript Reverse Transcriptase (TaKaRa, Dalian, China), and 5 L DEPC-H_2O, for 30 min at 42°C and 15 min at 70°C. Products were preserved in refrigerator at 20°C.

Primer design

Primers used in this study were chemically synthesized by Sangon (Sangon, Shanghai, China) and were listed in Table 1.

Table 1 Primers used for semiquantitative RT-PCR

Primer name	Primer sequence
Tublin-R	5′-TAACAACTGGGCAAAGGGTCA-3′
Tublin-L	5′-GGGAACTCCTCACGGATGTTG-3′
B-L	5′-CGCATCCAAGGGCTACAACAAGTCTG-3′
B-R	5′-CTTGACCGTATGCCTCCGTCTCTTCC-3′

Semiquantitative RT-PCR and data analysis

PCR reactions were carried out as follows: predenature for 5 min at 95°C, followed by 35 cycles for 30 s at 94°C, 56°C, and 72°C; with the final 10 min extension at 72°C. RT-PCR was carried out using the PrimeScript RT-PCR kit (TaKaRa).

The bands on the Gel electrophoresis graph density were scanned by Quantitative Gel Analysis Software to receive the integrated optical density (IOD). The average and standard deviation was repeated three times.

Results

Gene homology analysis of *PKA-c*

The *PKA-c* nucleotide sequence was compared in GenBank with other known *PKA-c* gene sequences in fungi. The results showed that it shared 87%, 90%, 88%, 80%, 81%, 77%, 79%, 83%, and 72% identities with the corresponding genes of *Alternaria alternata*, *Phaeosphaeria nodorum*, *Pyrenophora tritici-repentis*, *Magnaporthe grisea*, *Neurospora crassa*, *Colletotrichum lagenarium*, *Colletotrichum gloeosporioides*, and *Colletotrichum trifolii* individually.

These sequences were analyzed using MEGA4 software, as shown in Fig. 1.

Copy number analysis of PKA-c

The genome DNA was digested by restriction enzymes *Xba*I, *Sac*II, and *Bgl*II completely. After the electrophoresis of DNA transferred to Nitrocellulose Filters, the filter was hybridized with *PKA-c* fragment labeled with DIG. The result showed that all genome DNA libraries were consistent with the presence of a single hybridization band (Fig. 2). It was suggested that *PKA-c* gene was present as a single copy in the genome of *S. turcica*.

Expression level of *PKA-c* in different cultivation conditions

Semiquantitative RT-PCR product was detected by 1.2% agarose gel electrophoresis, then, its brightness and specificity were identified, and finally, the cycling number for 32 was determined.

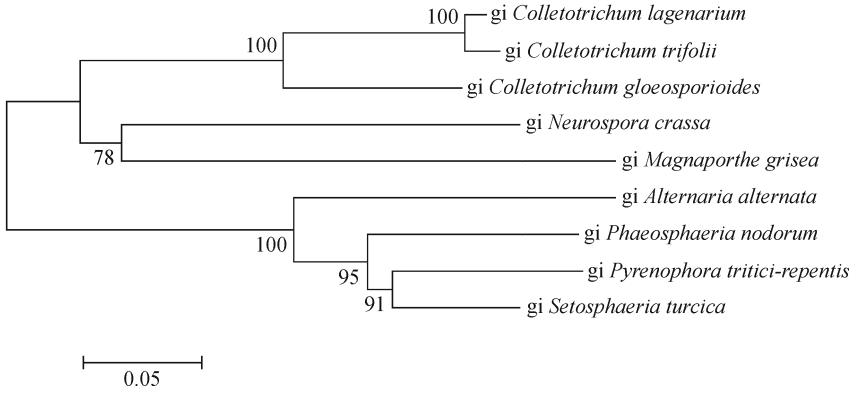

Figure 1 Homology tree of gene sequence of *PKA-c*.

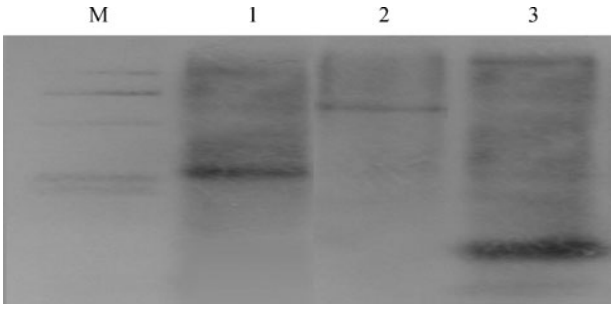

Figure 2 *PKA-c* gene copy number analysis in the genome of *S. turcica*. M is DNA molecular weight marker II, Digoxigenin-labeled; 1–3 are *Xba*I 2, *Sac*II 3, and *Bgl*II, respectively.

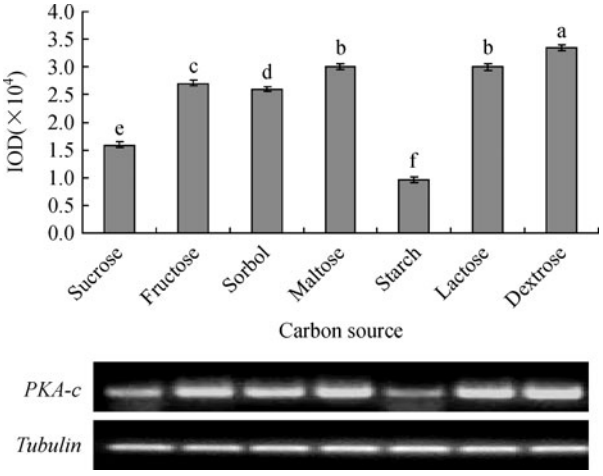

Figure 3 The expression of *PKA-c* gene in *S. turcica* on different carbon media. Average±standard error is shown; different lower case letters (a–f) at the top of the bars represent significantly different means ($P < 0.001$).

The affected expression level of PKA-c gene by different source of carbon and nitrogen source

Gene expression of *PKA-c* in different nutrient conditions including source of carbon and nitrogen was analyzed. The results of the different sources of carbon (Fig. 3) showed that *PKA-c* was highly expressed in glucose, maltose, and lactose. The expression level in fructose and sorbitol was lower than the former. *PKA-c* expression was downregulated obviously in sugar and starch as the carbon sources. It was suggested that sugar and starch could inhibit the expression of *PKA-c* gene obviously and glucose could induce its expression. The expression level of *PKA-c* gene was the highest in KNO_3 as nitrogen source on the medium; whereas, when $NaNO_3$, $CO(NH)_2$, and NH_4NO_3 were used as nitrogen source on the medium, the expression of the *PKA-c* reduced slightly, but the difference was not obvious (Fig. 4). We speculated that PKA-dependent cAMP signal pathway may be more sensitive to KNO_3 but somewhat less sensitive to other nitrogen sources.

The affected expression level of PKA-c gene by different metal ions

To analyze the expression level of *PKA-c* gene in the culture medium, different kinds of metal ions were added to it. The results (Fig. 5) showed that the expression level of *PKA-c* gene was the highest in the medium, with the presence of Mg^{2+} and Zn^{2+}, followed by the expression of *PKA-c* gene with $MnSO_4$ treatment. The expression of the *PKA-c* gene was downregulated significantly with $CuSO_4$ treatment. It was indicated that Mg^{2+} or Zn^{2+} could induce *PKA-c* gene expression, and Cu^{2+} could inhibit its expression.

The affected level expression of PKA-c gene by hypertonic stress

The analysis of the expression of *PKA-c* treated with different concentrations of sorbitol (Fig. 6) showed that it was significantly reduced under hypertonic stress by sorbitol, as compared with the control. When the concentration of the sorbitol was increased, the expression level of *PKA-c* was reduced. Particularly, 2.0 mol/L sorbitol treatment inhibited the gene expression significantly. These results showed that sorbitol could inhibit the expression of *PKA-c* gene, and the inhibition was positively correlated with the concentration of sorbitol.

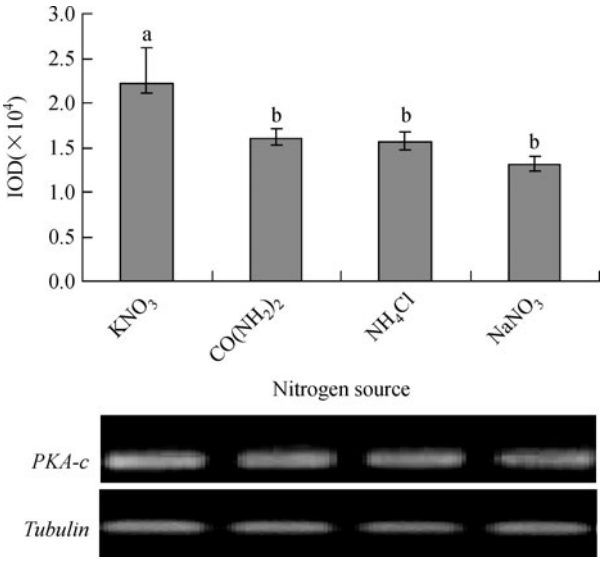

Figure 4 The expression of *PKA-c* gene in *S. turcica* on different nitrogen media. Average±standard error is shown; different lower case letters (a–b) at the top of the bars represent significantly different means ($P < 0.001$).

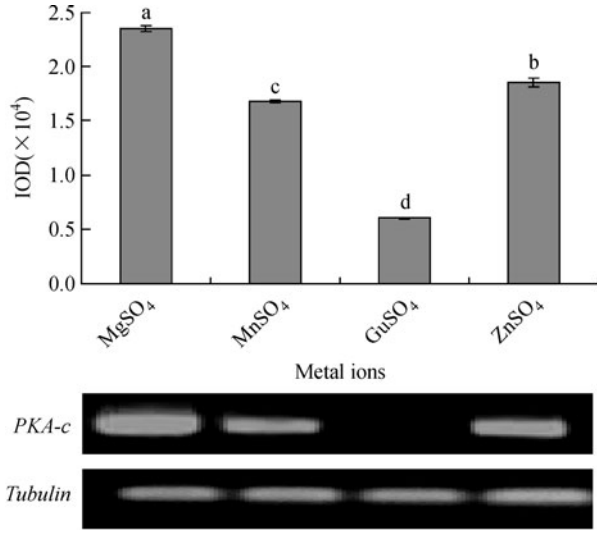

Figure 5 The expression of *PKA-c* gene in *S. turcica* under different metal ions. Average±standard error is shown; different lower case letters (a–d) at the top of the bars represent significantly different means ($P < 0.001$).

When *S. turcica* was treated with different concentrations of NaCl, it was revealed that the lower concentration, the expression level of *PKA-c* was higher (Fig. 7). This indicated that the lower concentration of NaCl would inhibit the expression level of *PKA-c*, while that with the higher concentration could stimulate its expression. These facts showed that the signal pathway mediated by *PKA-c* gene might be involved in the osmotic stress response under high concentration of NaCl in *S. turcica*.

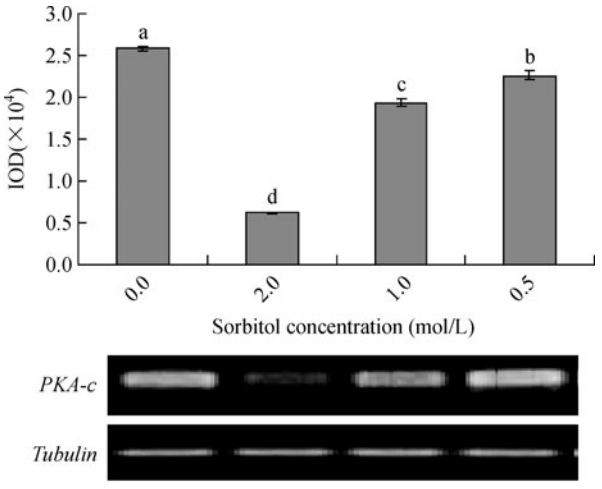

Figure 6 The expression of *PKA-c* gene in *S. turcica* under different concentrations of sorbitol. Average±standard error is shown; different lower-case letters (a–d) at the top of the bars represent significantly different means ($P < 0.001$).

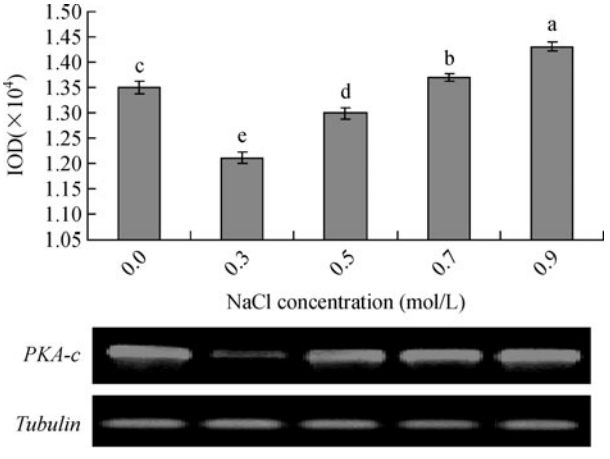

Figure 7 The expression of *PKA-c* gene in *S. turcica* under different concentrations of NaCl. Average±standard error is shown; different lower case letters (a–e) on top of the bars represent significantly different means ($P < 0.001$).

Discussion

The cAMP-PKA pathway was directly activated by sugars through the G-protein-coupled receptor Gpr1, and the affinity of Gpr1 for different sugar was dissimilar (Sam and Johan, 2008). PKA has been extensively studied in yeast; cAMP–PKA pathways were activated by sugar and induced pseudohyphae transition (Lemaire et al., 2004).

In addition, cAMP singling pathway could also accelerate the conidia germination process (Doehlemann et al., 2006). Our results obtained the upregulated expression of *PKA-c* gene when glucose, lactose, starch, and maltose were added as carbon sources, the downregulated expression in the presence of sucrose or starch. Different carbon sources might

activate the cAMP-PKA pathway in pathogenic fungi probably by influencing the expression level of *PKA-c* gene.

It was reported that *Inonotus obliquus* could not develop when the medium contained Cu^{2+} (Zhang and Chen, 2006). Our research found that metal ion stress, such as adding extra Cu^{2+} also inhibited the development of *S. turcica*. Meanwhile, RT-PCR results showed that the expression of *PKA-c* gene was inhibited significantly. While for the lesser influence of expression level of *PKA-c* gene in *S. turcica*, the strain could develop slowly in the medium containing Zn^{2+}. We inferred that Cu^{2+} inhibited *PKA-c* gene expression and then affected the growth of the strain. Under the osmotic stress, sorbitol could inhibit the expression of *PKA-c* gene, and the inhibition degree showed a positive correlation with its concentration. This suggested that cAMP-PKA signal transduction process might be influenced by sorbitol. In the meantime, low concentration of NaCl could inhibit the expression of *PKA-c* gene, with the concentration increasing the expression of *PKA-c* gene was higher. The results indicated that PKA might be involved in the stress reaction of plant pathogenic fungi under hypertonic environment by sodium salt.

As the key component of signal transduction pathways, the expression of *PKA-c* gene could be induced or inhibited by different environmental factors. It also suggested that the cAMP-PKA pathway probably responded in different levels of active state by environmental factors' stimulation. These laid a foundation for understanding this gene and cAMP-PKA signaling pathways.

References

Doehlemann G, Berndt P, Hahn M (2006). Trehalose metabolism is important for heat stress tolerance and spore germination of *Botrytis cinerea*. Microbiology, 152(9): 2625–2634

Dürrenberger F, Wong K, Kronstad J W (1998). Identification of a cAMP-dependent protein kinase catalytic subunit required for virulence and morphogenesis in *Ustilago maydis*. Proc Natl Acad Sci USA, 95(10): 5684–5689

Fan Y S, Cao Z Y, Gu S Q, Dong J G (2004). Effect of different induction factors on appressorium of *Setosphaeria turcica*. Scientia Agricultura Sinica, 37(5): 769–772

Kronstad J, De Maria A D, Funnell D, Laidlaw R D, Lee N, de Sá M M, Ramesh M (1998). Signaling via cAMP in fungi: interconnections with mitogen-activated protein kinase pathways. Arch Microbiol, 170(6): 395–404

Lee Y H, Dean R A (1993). cAMP regulates infection structure formation in the plant pathogenic fungus *Magnaporthe grisea*. Plant Cell, 5(6): 693–700

Lemaire K, Van de Velde S, Van Dijck P, Thevelein J M (2004). Glucose and sucrose act as agonist and mannose as antagonist ligands of the G protein-coupled receptor Gpr1 in the yeast *Saccharomyces cerevisiae*. Mol Cell, 16(2): 293–299

Li C X, Su J, Gong S C, Song X Z, Li G L, Hu G H (2000). Study on Physiological form of corn leaf blight in Heilongjiang Province. Journal of maize Sciences, 8(2): 89–91

Liebmann B, Müller M, Braun A, Brakhage A A (2004). The cyclic AMP-dependent protein kinase a network regulates development and virulence in *Aspergillus fumigatus*. Infect Immun, 72(9): 5193–5203

Mehrabi R, Kema G H J (2006). Protein kinase A subunits of the ascomycete pathogen *Mycosphaerella graminicola* regulate asexual fructification, filamentation, melanization and osmosensing. Mol Plant Pathol, 7(6): 565–577

Mitchell T K, Dean R A, 1995, The cAMP-dependent protein kinase catalytic subunit is required for appressorium formation and pathogenesis by the rice blast pathogen *Magnaporthe grisea*, Plant Cell, 7 (11): 1869–1878

Rossi S, Moreno S (1994). Regulation of protein kinase A subunits during germination of *Mucor rouxii* sporangiospores. Eur J Biochem, 222(2): 501–506

Sam V V, Johan M T, (2008). Cyclic AMP-protein kinase A and Snf1 signaling mechanisms underlie the superior potency of sucrose for induction of filamentation in *Saccharomyces cerevisiae*. Eukaryot Cell, 7(2): 286–293

Yamauchi J, Takayanagi N, Komeda K, Takano Y, Okuno T (2004). cAMP-pKA signaling regulates multiple steps of fungal infection cooperatively with Cmk1 MAP kinase in *Colletotrichum lagenarium*. Mol Plant Microbe Interact, 17(12): 1355–1365

Zhang L Q, Chen Y Q (2006). Effects of different carbon and nitrogen sources and inorganic salts on the culture characteristices of *Inontus Obliquus* mycelia. Journal of Agricultural Science Yanbian University, 28(1): 41–45 (in Chinese)

Zhou Y L, Tanaka C, Matsushim S, Tsuda M (2003). Protoplast isolation and transformation of *Exserohilum turcicum*. Chinese Journal of Biotechnology, 19(3): 364–367 (in Chinese)