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The Observation of PlcA Mutation and Localization in *Aspergillus nidulans*[§]

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To know the function of the *plcA* gene, which encodes a putative phosphoinositide-specific phospholipase C, in a model filamentous fungus *Aspergillus nidulans*, it was disrupted thorough homologous recombination and examined. The germination rate of *ΔplcA* was reduced by approximately 65% and germination of *ΔplcA* at a lower temperature (25°C) was much slower than germination under normal conditions (37°C), suggesting the *plcA* is responsible for cold-sensitivity. The hyphal growth of *ΔplcA* was slightly reduced at 37°C and conspicuously reduced at 25°C. While germinating *ΔplcA* formed giant swollen spores, and generated short and thick hyphae. The results of the nuclear examination of *ΔplcA* showed nuclear division with mis-segregation, and the rate of nuclear division was lower than that of wild type at both 25°C and 37°C. The results of this study showed that *plcA* is localized to the nucleus through intracellular calcium signaling in *A. nidulans*. The abnormal nuclear division, resulting from *plcA* gene deletion, affects conidiation in asexual development. Taken together, these results suggested that *plcA* is required for normal vegetative growth, morphogenesis, conidiation, and nuclear division in *A. nidulans*.

Keywords: *Aspergillus nidulans*, PlcA, germination, conidiation, nuclear division, nuclear translocation

Introduction

The mammalian phosphoinositide-specific phospholipase C (PLC) superfamily comprises of six major groups, including PLCβ, PLCγ, PLCδ, PLCε, PLCζ, and PLCη, and of at least 13 isoforms based on the enzymatic activity (Katan, 2005). These enzymes functions are known well in mammalian cells. PLCβ and –γ isoforms increase intracellular calcium level by G protein subunits and tyrosine protein kinase, respectively (Katan, 2005). In small GTPase (such as Ras-, Rho-, and Gαβγ) mediated signaling, PLCε carried out as an effector or a multifunctional nexus protein (Wing *et al*., 2003). PLCη is known that has putative neuronal functions by Ca^{2+} signal modulation in neuronal and neuroendocrine tissues (Stewart *et al*., 2007; Popovics and Stewart, 2012). PLCζ, called a sperm-specific PLC, is investigated that it's involved in fertilization through the calcium mobilization (Saunders *et al*., 2002). PLCδ is translocated into the nucleus by the Ca^{2+} dependent manner, but not clearly functions of this (Okada *et al*., 2005).

In yeasts and some filamentous fungi, the δ isoforms are considered as typical PLC proteins in their organisms (Flick and Thorner, 1993; Yoko-o *et al*., 1993; Andoh *et al*., 1995; Jung *et al*., 1997; Chung *et al*., 2006; Gavric *et al*., 2007; Vanzela *et al*., 2011). Yeasts, such as *Saccharomyces cerevisiae*, possess only one PLC isoform of the δ type, Plc1p, encoded by the *PLC1* gene (Flick and Thorner, 1993; Yoko-o *et al*., 1993). The mutations in the catalytic domains of Plc1p in *S. cerevisiae* lead to growth defects under hyper-osmotic conditions, and in synthetic media under high temperatures these yeast mutants display defective cytokinesis, unusual sensitivity to UV-irradiation and vacuole fragmentation (Flick and Thorner, 1993, 1998; Payne and Fitzgerald-Hayes, 1993; Yoko-o *et al*., 1993, 1995; Andoh *et al*., 1998; Seeley *et al*., 2002). Plc1p of *S. cerevisiae* was identified in the nucleus and is localized to centromeric loci at the G2/M check point, affecting kinetochore function potentially through modulating the structure of centromeric chromatin (DeLillo *et al*., 2003). In filamentous fungi, *N. crassa plc-1* is necessary for normal growth, as the mutant shows several defects, notably in growth rate, hyphal size and proportion, and branching (Gavric *et al*., 2007).

Recently, three PLC coding genes (AN0664, AN2947, and AN6382) have been identified in *A. nidulans*, a model organisms used for the study of filamentous fungi in eukaryotes (Vanzela *et al*., 2011). Of them, PlcA is contained with PH (pleckstrin homology), EF-hand (calcium binding motif), catalytic X-Y, and C2 (Ca^{2+}-dependent membrane-targeting) domain, which is typical δ type. Unlike PlcA, however, AN2947 containing catalytic domain and C2 domain except for PH and EF-hand domain is like seem to be another type PLC in filamentous fungi, and AN6382 has a similar structure of bacterial type PLC, which containing only catalytic X domain. *A. nidulans plcA* has been directly associated with high-molecular-weight carbon source sensing.

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Table 1. **Plasmid and *A. nidulans* and bacterial strains used in this study**

Strain or plasmids	Genotype	Reference
A. nidulans		
A4	Glasgow wild type (*veA*$^{+}$)	FGSC[a]
A26	*biA1 veA1*	FGSC
A773	*wA3 pyroA4 pyrG89 veA1*	FGSC
A850	*biA1 methG ΔargB::trpC trpC801 veA1*	FGSC
A851	*yA1 pabaA1 ΔargB::trpC trpC801 veA1*	FGSC
ACS-1	*biA1 methG veA1*	This study
PCD15	*biA1 methG ΔplcA::argB ΔargB::trpC trpC801 veA1*	This study
PAG-11	*yA1 pabaA1 plcA::sGFP veA1*	This study
Escherichia coli DH10B^{TM}	*F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ-rpsL(Str^{R}) nupG*	Invitrogen
Plasmids		
pMT-sGFP	Amp^{r} *argB*$^{+}$ *alcAp attR1 ccdB-box attR2 sGFP*	FGSC
pAT-sGFP	modified from pMT-sGFP	This study
pAT-*plcA::sGFP*	Amp^{r} *argB*$^{+}$ *alcAp plcA sGFP*	This study

[a] Fungal Genetic Stock Center

PlcA-deficient *A. nidulans* mutants showed a reduction in nuclear duplication, after 8 h of cultivation on glucose (Chellegatti *et al*., 2010; Vanzela *et al*., 2011).

Although some functions are considered previously, the regulation of cellular effects and other functions of *A. nidulans* PLCs are not fully elucidated. Therefore, in this study, we further examined the functions of PLCs in *A. nidulans*. To determine the role of PlcA, we observed morphological changes using *plcA*-deletion mutants and examined cellular localization using the GFP fusion proteins in *A. nidulans*. Moreover, we attempted to identify other *plcA*-related genes using quantitative real-time RT-PCR.

Materials and Methods

Strains and culture media

The *A. nidulans* strains used in this study were obtained from FGSC (Fungal Genetics Stock Center), as listed in Table 1. For the generation of the arginine auxotroph-complemented *A. nidulans* strain ACS-1, A850 was crossed with *A. nidulans* strain A773. *A. nidulans* strains were cultured as previously described (Harsanyi *et al*., 1977).

Construction of plasmids for *plcA* deletion and GFP tagging

Genomic DNA was extracted from wild type strain and transformants, respectively, according to the protocol of Irelan and Selker (1997). Genomic DNA of wild type was used for construction of library (data not shown) and mutant, and transformant genomic DNA was extracted for distinction with wild type strain through the colony hybridization. As shown in Supplementary data Fig. S1A, we constructed with *plcA*-disruption plasmid using fragment containing *plcA* gene from genomic library. The resulting circular form of the *plcA*-disruption plasmid was approximately 8.2 kb in length. For transformation, the circular form of the *plcA*-disruption plasmid was digested with *PvuII*, to generate a linear *plcA*-disruption plasmid approximately 5.8 kb in length.

Plasmid pMT-sGFP (FGSC) was used for the construction of the GFP-fusion vector, pAT-*plcA::sGFP*. Synthetic multiple cloning sites (MCS, Supplementary data Table S1) were manufactured to modify pMT-sGFP. For the construction of the *plcA::sGFP* vector, the *plcA* gene without a stop codon was PCR amplified and inserted into the MCS sites of pAT-sGFP using various restriction enzymes.

Fungal transformation

The transformation of the *plcA*-disruption plasmid and *plcA::sGFP* vector was performed as previously described (Szewczyk *et al*., 2006; Breakspear *et al*., 2007). After transformation of the *plcA*-disruption plasmid into a recipient strain, A850, *argB*+ transformants were purified and screened for substitution into the *plcA* locus using colony formation on MM agar plates. All putative mutants were identified and further characterized through Southern blot analysis.

Southern blot analysis

For the Southern blot analysis of *ΔplcA* transformants, the *plcA* gene was digested with *Eco*RI and *Kpn*I to obtain probes 1 and 2, respectively (Supplementary data Fig. S1A). The *plcA::sGFP* transformants were verified using Southern blot analysis, and a *sgfp* probe was amplified using specific primer set as described in Supplementary data Table S1. The probe 1, probe 2, and *sgfp* genes were labeled with ^{32}P-dCTP for the Southern blot analysis. The genomic DNA of the transformants of *ΔplcA* was digested with *Hin*dIII, and the genomic DNA of transformants of *plcA::sGFP* was digested with *Xho*I, *Pst*I, and *Bgl*II. Southern blot analysis was performed following standard procedures (Sambrook and Russell, 2001).

Fluorescent and confocal microscopic analysis

Morphological change in *A. nidulans* was observed using a BX51 fluorescent microscope (OLYMPUS, Japan). The conidia were incubated in 5 ml MMR liquid media at 37°C and

25°C, respectively, Wild type and mutant *A. nidulans* were cultured on MM or MMR agar plates for 3 days, to measure the hyphal growth. The hyphal growth of wild type and mutant *A. nidulans* were measured for colony diameter at 5 days after growth at 37°C and 25°C. When the radial extension of the wild type and mutant *A. nidulans* was approximately 6 cm in diameter, the 1-cm^2 agar blocks from middle region of colony were vortexed in 2 ml of 0.08% Triton X-100 solution of 2 min, and the resulting conidial suspension was counted using a hemocytometer (Chang *et al.*, 2004).

To examine nuclear division in *A. nidulans*, 1×10^5 of conidia were incubated in 20 ml MMR liquid media with coverslips at 37°C and 25°C. The coverslips were sunk in MMR liquid media every 2 h at 37°C and every 4 h at 25°C. The hyphae was fixed with 3 ml fixer solution (3.7% formaldehyde, 0.2% Triton X-100, and 50 mM phosphate buffer, pH 7.0), and incubated for 30 min at room temperature. Nuclear staining was performed as previously described (Harris *et al.*, 1994). For counting of nuclei number and localization of PlcA, the stained nuclei of the *plcA* deletion mutant, wild type, and GFP-fusion strains of *A. nidulans* were observed through a BX51 fluorescent microscope and a FluoView FV1000 confocal microscope (Olympus).

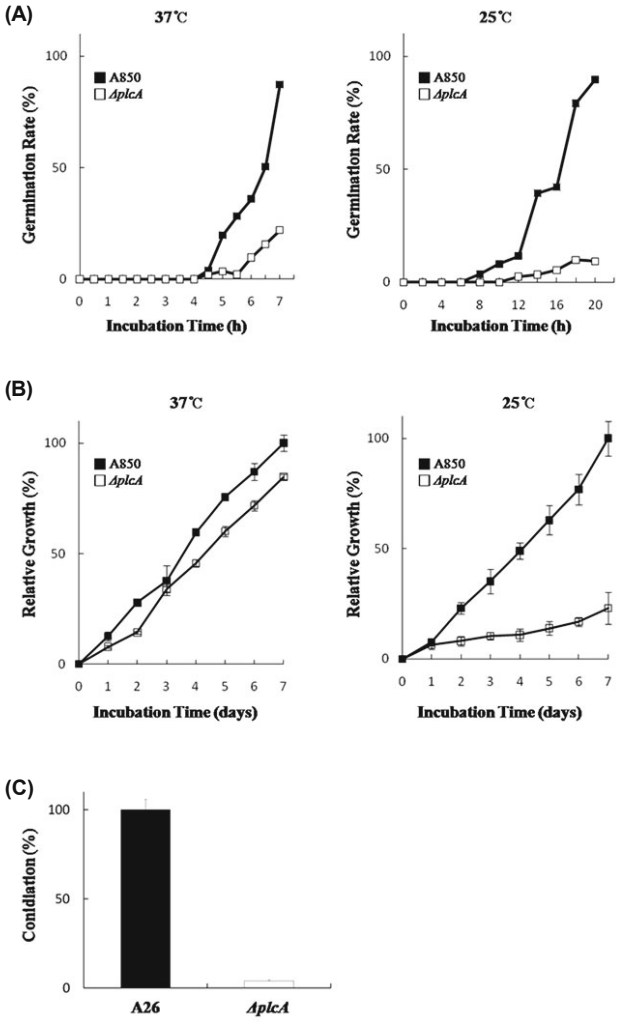

Fig. 1. Germination, hyphal growth and conidiation of *ΔplcA*. (A) Comparison of control (A850) and *ΔplcA* for germination. Germinated conidia were counted from total 100 conidia at every each time point. (B) Hyphal growth measurement of control (A850) and *ΔplcA*. Hyphal growth was measured colony diameter, and converted as a percentage compared with control. The error bars represent the standard deviation of triplicates in three independent experiments. (C) Conidiation of wild type (A26) and *ΔplcA* strains. The error bars represent the standard deviation of triplicates in three independent experiments (*P* value < 0.0001).

Quantitative Real-Time PCR

A. nidulans A26 and PCD15 were incubated with shaking (120 rpm) in MM for 18 h at 37°C. Total RNA was purified from the mycelium of A26 and PCD15, respectively, using an RNeasy Plant Mini Kit (QIAGEN, Germany) according to the manufacturer's protocol. Total RNA was reverse-transcribed into cDNA using the Power cDNA Synthesis Kit (iNtRON, Korea) according to the the manufacturer's instructions. The gene-specific primer sets, *nimA*, *nimX*, *nimE*, and *sonA*, are listed in Supplementary data Table S1. Each gene-specific primer was optimized for expression analysis through real-time PCR on a LightCycler®Nano (Roche, Germany) using FastStart Essential DNA Green Master (Roche). The PCR cycling parameters were 10 min at 95°C, followed by 45 cycles of 10 sec at 95°C, 10 sec at 55°C, and 15 sec at 72°C, and a melting curve analysis to confirm specificity. Gamma-actin (*gAct*) was used for normalization, and the relative expression was calculated using comparative Cq quantification (Hellemans *et al.*, 2007).

Results

Construction of the *plcA*-deletion mutant and PlcA-sGFP fusion strain in *A. nidulans*

To investigate the physiological effect of the *plcA* gene in *A. nidulans*, a disruption plasmid was constructed as shown in Supplementary data Fig. S1A. The Southern blot analysis was performed with probes 1 and 2 to select specific transformants (Supplementary data Fig. S1A). A 2.8 kb wild type *plcA* gene was detected using both probes 1 and 2. However, a 1.9 kb *plcA*-deletion gene was only detected using probe 1. In addition, 1.9 and 1.5 kb *plcA*-deletion genes were detected through Southern blotting using an *argB* gene probe. Three *plcA*-deletion mutant strains, PCD15, PCD16, and PCD31, were identified through Southern blot analysis (Supplementary data Fig. S1B, lanes 3, 4, and 7). The *plcA*-deletion gene of PCD15 was confirmed through PCR using specific primers (see Supplementary data Table S1, *plcA* forward and reverse primers). The PCR fragments for the wild type *plcA* genes FGSC A4 and A850 were 1.9 kb, and the PCR fragments for the *plcA*-deletion gene in PCD15 were 3.1 kb (Supplementary data Fig. S1C). A *ΔplcA* mutant showed colorless than wild type strains, and reduced colony diameter during growth for 5 days at 37°C compared with wild type strains (Supplementary data Fig. S1D).

The *plcA::sGFP* vector was inserted into genome of *A. nidulans* through homologous recombination, and we obtained with a PlcA-sGFP fusion strain, which was confirmed through Southern blot and PCR analyses (Supplementary data Fig. S2).

Phenotypic analysis of the *ΔplcA* mutant

To know the cellular function of the *plcA* gene, we examined various phenotypes of the *ΔplcA* mutant. The germination rate of *ΔplcA* was reduced 65% compared with that of *A. nidulans* A850, as shown in Fig. 1A. The germination rate of *ΔplcA* was much lower at 25°C than at 37°C. The hyphal growth of *ΔplcA* was similar with that of the normal *A. nidulans* A850 strain at 37°C, as shown in Fig. 1B. However, the growth rate was conspicuously reduced at 25°C. Compared with *A. nidulans* A850, the *ΔplcA* mutant was cold sensitive, suggesting that the *plcA* gene is necessary for the normal hyphal growth and germination of *A. nidulans* especially in lower temperature.

Since germination was largely affected by the *plcA* gene, we now speculated asexual sporulation or conidiation. The conidiation of *ΔplcA* was significantly reduced, producing less than 10% conidia compare to wild type control (Fig. 1C). These results suggest that *plcA* is also required for conidiation in *A. nidulans* and deletion of *plcA* causes pleiotropic effects.

In addition, the conidia swelling in the *ΔplcA* mutant was different from that in wild-type strains. The *ΔplcA* mutant forms giant swollen spores. After germ tube emergence, the radial growth of conidia was continuously increased to form giant swollen spores at both 25°C and 37°C. Furthermore, the morphology of the hyphae was abnormal under microscopic observation. The *ΔplcA* hyphae were shorter and thicker than that of the wild-type strains (Supplementary data Fig. S3), indicating that PlcA is required for normal morphogenesis.

Nuclear division in *ΔplcA* during the delayed germination

As *ΔplcA* showed reduce germination and conidiation, we examined whether nuclear division in spores and hyphae occurs normally in *ΔplcA* mutant through nuclear staining using DAPI. As shown in Fig. 2, *A. nidulans* A850 control strain showed normal nuclear division when cultured in MMR medium for 8 h at 37°C. In contrast, *ΔplcA* mutant did not show normal nuclear division (Fig. 2A, upper panel). To confirm the nuclear division, the number of stained nuclei was counted in control and mutant. When wild type A850 was cultured as a control, the number of nuclei increased as a function of time. During the early phase of growth, up to 2 h, only one nucleus was observed. At 4 h, the number of nuclei was increased, and more than five nuclei were observed after 8 h of culture at 37°C (Fig. 2A, lower panel). At 25°C, the number of nuclei in *A. nidulans* A850 increased slowly as a function of time. When *ΔplcA* was cultured for 8 h at 37°C, about 1.8 times more uninucleated spores were observed compared with the control. At 25°C, 60% of the spores were uni-nucleate compared with the control (Fig. 2B). These results suggest that the germination retardation of the *ΔplcA* might due to the nuclear division defect caused by the absence of functional PlcA and, therefore, this result lead us to conclude that PlcA is also involved in nuclear division in *A. nidulans*.

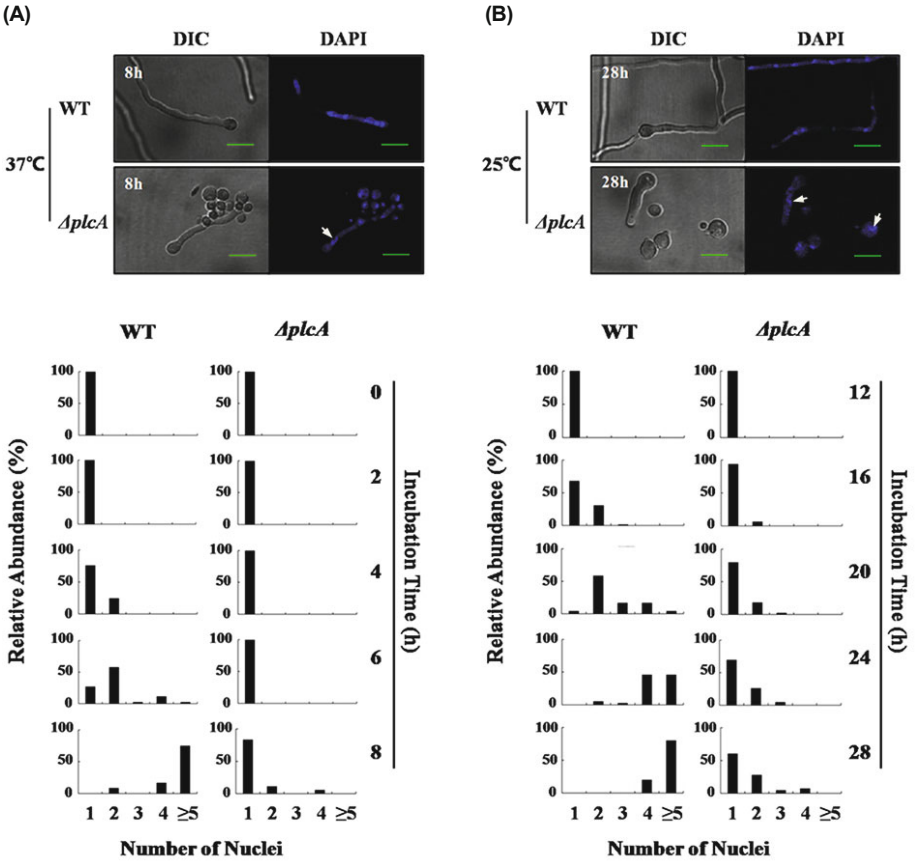

Fig. 2. Comparative analysis of nuclear division between wild-type and *ΔplcA*. The measurement of the number of nuclei was performed in 37°C (A) and 25°C (B), respectively. The upper layer shows the DAPI-stained image, and lower layer shows the graphical image of the counted nuclei. The nuclei in wild type and *ΔplcA* were counted in over one hundred conidia and hyphae. White arrow showed abnormal nuclear division in *ΔplcA*.

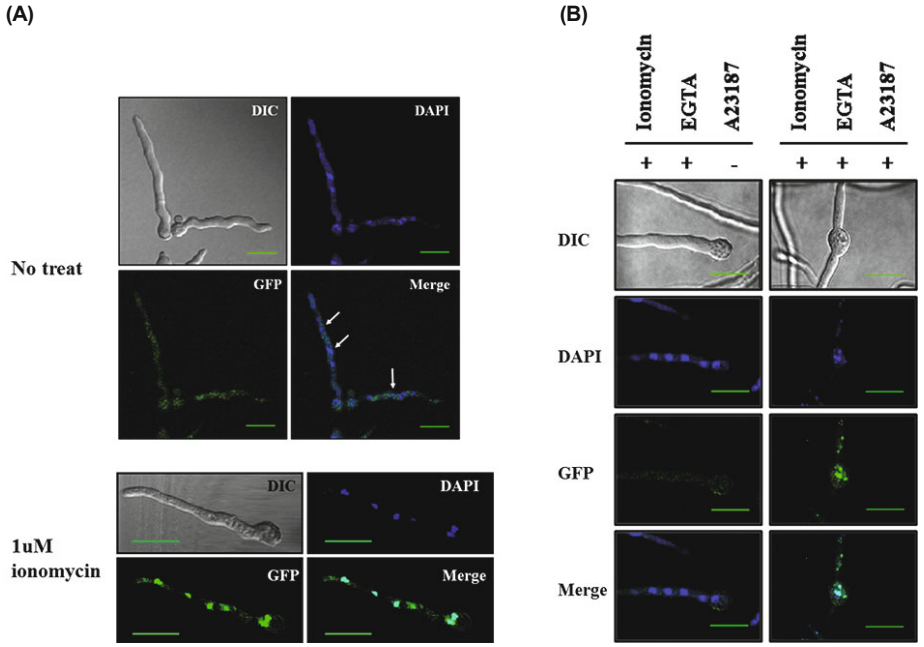

Fig. 3. The PlcA localization and relationship with intracellular calcium contents in *A. nidulans*. (A) The observation of the localization of GFP-tagged PlcA with or without 1 μM ionomycin. (B) Translocation of PlcA into the nucleus through the elevation of intracellular calcium level. The PAG-11 strain was cultivated in MM supplemented with ρ-aminobenzoic acid at 37°C, and treated with 1 μM ionomycin, 5 mM EGTA and 1 μM A23187. Scale bars are 10 μm.

Nuclear localization of PlcA through the intracellular calcium contents

We next examined whether PlcA was translocated to the nucleus. The vegetative hyphae were grown for 10 h at 37°C, and PlcA was detected using confocal microscopy. Under the same conditions, the localization of the PlcA-GFP fusion protein was coincident with those of the nuclei as well as cytosol, which were stained with DAPI (Fig. 3A, upper panel). These results showed that PlcA was translocated to the nucleus from the cytosol. To know the translocation of PlcA is affected by calcium signaling, as previously described (Okada *et al*., 2005; Yagisawa *et al*., 2006), 1 uM ionomycin, an ionophore raising intracellular calcium concentration, was added to the liquid medium, and then PlcA was clearly detected in nucleus (Fig. 3A, lower panel). Also, to examine the effect of extracellular Ca^{2+} in the translocation of PlcA to the nucleus, the hyphae of *A. nidulans* were permeabilized with 0.02% Triton X-100, and treated with an excess of EGTA (5 mM) to chelate the extracellular calcium (Fig. 3B). Although presence of ionomycin, PlcA could not be translocated into nucleus causes by EGTA. However, the obvious translocation of PlcA into the nucleus was observed in the presence of A23187 (1 μM), a calcium ionophore for Ca^{2+} release from intracellular calcium store, in the presence of excess EGTA, indicating that calcium release from intracellular stores might be important for the localization of PlcA.

Quantitative real-time PCR of mitosis-related genes in *ΔplcA*

To further confirm the involvement of nuclear division with PlcA in *A. nidulans*, we examined the expression of the mitosis-related genes *nimA*, *nimX*, and *nimE*, identified as β-casein kinase, histone H1 kinase and cylinB, respectively (Osmani *et al*., 1987; Wu *et al*., 1998; Ukil *et al*., 2008). Comparison of expression level of the *nimA*, *nimX*, and *nimE* genes between control and *ΔplcA* mutant strain by using the quantitative real-time PCR analysis revealed that *ΔplcA* was enriched for *nimA*, approximately 2.4 fold. In addition, *nimX*, and *nimE* were increased slightly about both 1.6-fold (Fig. 4), respectively. However, no significant difference in

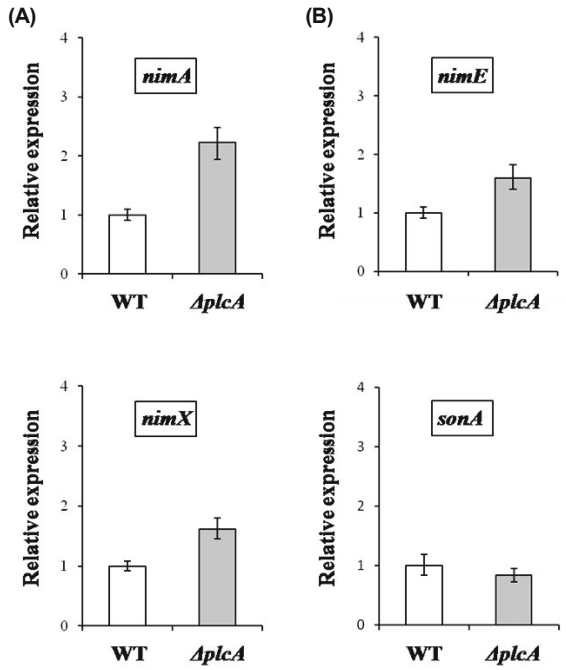

Fig. 4. Relative expression of *nimA*, *nimE*, *nimX*, and *sonA* in wild type and *ΔplcA* was examined by quantitative real-time PCR. Quantitative real-time PCR was performed with cDNA obtained from mycelia cultivated for 18 h at 37°C in MM supplemented with ρ-aminobensoic acid using optimized gene-specific primer sets to analyze the expression levels of *nimA*, *nimE*, *nimX*, and *sonA* normalized to *gAct* expression in the corresponding samples. The error bars represent the standard deviation of triplicates in three independent experiments.

sonA expression was observed. Therefore, we suggested that *plcA* might be related dependently or independently with *nimA*, and required for including the mitotic phase.

Discussion

A. nidulans possesses one homologous *Aras* gene, as previously described (Som and Kolaparthi, 1994). The activity of A-Ras influences the developmental decisions of *A. nidulans*. In constitutively acitived conditional *Aras* mutants, the cells fail to initiate germ tube emergence and germ tube formation is inhibited. However, conidia swelling and nuclear division persists in these mutants, producing giant swollen spores. In addition, the shape of the mycelia is wider and shorter than that of the wild type, with large vacuoles. In the present study, the deletion of *plcA* resulted in giant swollen spores and thicker and shorter hyphae, compared with WT (Supplementary data Fig. S3). However, *ΔplcA* and Aras mutants showed different phenomena in nuclear division. Constitutively active *ras* inhibited the initiation of germ tube formation and showed multinucleated, giant swollen spores, while *ΔplcA* revealed poor nuclear division (Fig. 2). Thus, the deletion of *plcA* critically influenced nuclear division in *A. nidulans*.

Rat PLCδ-1 was accumulated in the nucleus in a Ca^{2+}-dependent manner (Yagisawa *et al*., 2006). The accumulation of PLCδ-1 was observed after treating serum-starved MDCK cells with ionomycin (Okada *et al*., 2005). In the filamentous fungus *A. nidulans*, intracellular calcium is stored in vacuoles (Harris *et al*., 2009; Findon *et al*., 2010). In the present study, PlcA was translocated into the nucleus through the elevation of intracellular calcium ion level after ionomycin and A23187 treatment (Fig. 3). Therefore, these results suggested that PlcA needs to be translocated into nuclei and subsequently induces of mitosis.

NIMA is a β-casein kinase and serine/threonine protein kinases (Osmani *et al*., 1988; Lu *et al*., 1993; Pu and Osmani, 1995; Pu *et al*., 1995). $NIMX^{CDC2}$ is an essential histone H1 kinase that is structurally and functionally homologous to fission yeast $p34^{cdc2}$, containing an amino-terminal catalytic domain and a carboxyl-terminal regulatory domain (Osmani *et al*., 1994). The activation of $NIMX^{CDC2}$ during G2 in *Aspergillus* is mediated through binding to $NIME^{Cyclin\ B}$, the Cyclin B homolog, which is the principle B-type cyclin associated with activated $NIMX^{CDC2}$ during G2 (Bergen *et al*., 1984; Osmani *et al*., 1994; James *et al*., 1995). The localization of SONA to the nuclear periphery is consistent with a role for SONA in nucleocytoplasmic transport (Wu *et al*., 1998). The production of the $NIME^{Cyclin\ B}/NIMX^{CDC2}$ complex is increased by NIMA activity in the early G2 phase and reduced by SONA activity in the late G2 phase for the transition of mitosis. Therefore, we examined expression of the cell cycle-related genes, *nimA*, *nimE*, *nimX*, and *sonA*. As shown in Fig. 4, the transcript levels of *nimA* in *ΔplcA* mutants were higher than those in wild type, and *nimE*, and *nimX* showed a little change of transcript level in *ΔplcA* mutants, but *sonA* expression showed no difference. These results indicate a G2-M phase transition is delayed through the deletion of *plcA* because *nimA*, *nimE*, and *nimX* expression was not reduced during the late G2 phase. Therefore, we proposed that the deletion of *plcA* in *A. nidulans* prevents the regulation cell cycle-related gene expression which eventually resulted in germination delays and conidiation defect.

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