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NOTE

The Activity of Phosphoinositide-Specific Phospholipase C Is Required for Vegetative Growth and Cell Wall Regeneration in *Coprinopsis cinerea*

Young Taek Oh†, Chun-Seob Ahn†, Kyung-Jin Lee, Jeong-Geun Kim, Hyeon-Su Ro, Jae Won Kim*, and Chang-Won Lee*

Department of Microbiology, College of Natural Science/ Research Institute of Life Science, Gyeongsang National University, Jinju 660-701, Republic of Korea

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Three isotypes of phosphoinositide-specific phospholipase C designated *CcPLC1*, *CcPLC2*, and *CcPLC3* were identified in *Coprinopsis cinerea*, through a search of the genome sequence database. The functional role of the PI-PLCs were studied by using U73122, which specifically inhibits the activity of PI-PLC. The specificity of the inhibitor effect was confirmed by using an inactive structural analog U73433. The inhibition of PI-PLCs activity resulted in severely retarded germination of basidiospores and oidia, reduced hyphal growth, knobbly hyphal tips with many irregular side branches, and aberrant (branch-like structure) clamp cells. Furthermore, U73122 definitely inhibited cell wall formation. Here we report that PI-PLCs play important roles in various aspects of *C. cinerea* biology.

Keywords: phosphoinositide-specific phospholipase C (PI-PLC), *Coprinopsis cinerea*, U73122

The phosphoinositide-specific phospholipases C (PI-PLC) are relevant to various cellular metabolic and effector molecules in the signal transduction process. PI-PLCs hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP2) to generate two second messengers: diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). DAG and IP3 initiate further signal transduction pathways through activation of protein kinase C (PKC) and intracellular calcium release. The fungal PI-PLCs are most similar to the *PLC-δ* isoform of mammals. The model yeast *Saccharomyces cerevisiae* has only one δ type *PLC*, which is essential for cell wall integrity and involved in normal cell morphology, nuclear division and tolerance to osmotic stress (Yoko-o *et al.*, 1993). The pathogenic fungus *Candida albicans* has three isotypes: *CaPLC1* is a δ type and two additional *PLC* genes, *CaPLC2* and *CaPLC3*, encode two almost identical polypeptides similar to bacterial *PLCs.* The role of *CaPLC1* was demonstrated in conditional mutants. It is essential for viability, normal hyphal formation (a provirulence property), and high-temperature growth (Kunze *et al.*, 2005). In *Cryphonectria parasitica*, the *cplc1* gene is required for a normal mycelial growth rate and colony morphology (Chung *et al.*, 2006), and *Neurospora crassa plc-1* mutants show slow, aberrant growth and branching (Gavric *et al.*, 2006). To date, although it has been reported that PI-PLC was involved in survival and virulence in *Cryptococcus neoformans*, which is an opportunistic pathogenic basidiomycete (Chayakulkeeree *et al.*, 2008), the role of PI-PLC in mushroom-forming basidiomycota has never been examined.

Due to the rapid morphogenesis of its multicellular structure, the ink-cap mushroom *C. cinerea* is an attractive model for mushroom-forming homobasidiomycetes, and is used in a wide variety of genetic studies. From the ongoing genome annotation effort it is already apparent that there are large numbers of predicted genes with unknown function. There is now an urgent need for functional analysis of the *C. cinerea* genome. *C. cinerea* has been analyzed using genetic approaches by the transformation of an asexual spore of the haploid mycelium known as an oidium. Functional analysis of *C. cinerea* genes, however, is hampered by the lack of reliable tools for gene targeting. Although homologous recombination seems to occur in *C. cinerea*, targeted gene knockouts appear difficult to achieve, possibly due to very efficient non-homologous DNA end joining, as has been shown for the filamentous ascomycete *N. crassa* (Ninomiya *et al.*, 2004). Recently, the Sakaguchi and Künzler groups have developed gene repression by dsRNA-mediated gene silencing as an alternative reverse-genetics technique in *C. cinerea* (Namekawa *et al.*, 2005; Wälti *et al.*, 2006). To characterize *C. cinerea* PLCs, we tried gene disruption by homologous recombination as well as gene repression by dsRNA-mediated gene silencing. Unfortunately, we did not obtain any transformants with repressed *C. cinerea PLC* genes. So, we applied a specific inhibitor of the target protein or specific signal transduction pathway as an alternative method. We used U73122, a commonly employed specific inhibitor of receptor-mediated PLC, and its negative control, U73343 (Fig. 2A). U73122 inhibits the hydrolysis of PI (phosphatidylinositol) to IP3 (inositol trisphosphate) leading to a decrease in free cytosolic Ca^{2+}. Therefore, U73122 is now widely employed as a tool in the characterization of PI-PLC

† These authors contributed equally to this work.
*For correspondence. (C.W. Lee) E-mail: cwlee@gnu.ac.kr; Tel.: +82-55-772-1322; Fax: +82-55-772-1329 / (J.W. Kim) E-mail: jwkim@gnu.ac.kr; Tel.: +82-55-772-1325; Fax: +82-55-772-1329

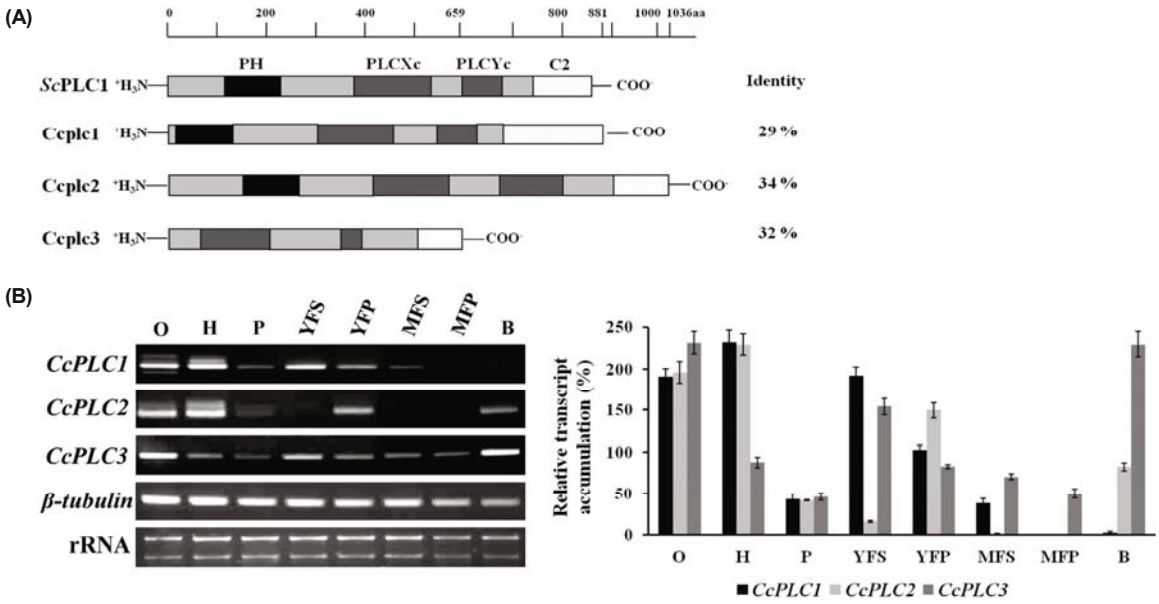


Fig. 1. Characteristics of *C. cinerea PLC* genes. (A) The schematic diagram of the predicted *CcPLC* gene products. Domain structures of putative phospholipase C proteins *of C. cinerea* in comparison of *S. cerevisiae* (Accession No. AAB27349). Phospholipase C (PLC) pleckstrin homology (PH) domains are indicated by black boxes, the catalytic domains of phospholipase C (domains X and Y) by black hatched boxes, and a calcium-binding (C2) motif by white boxes. (B) Expression of *CcPLC* mRNAs during the life cycle of *C. cinerea* by semi-quantitative PCR: O, oidia; H, hyphae (18 h); P, primordia; YFS, young fruiting body stipe; YFP, young fruiting body pileus; MFS, mature fruiting body stipe; MFP, mature fruiting body pileus; B, basidiospores. Beta-tubulin was used as the control. Values shown are the means of at least three independent determinations. Equal loading of total RNA was confirmed by ethidium bromide staining of rRNA.

and inositol phosphate-mediated Ca^{2+} signaling (Yule and Williams, 1992; Jun *et al*., 2004; Suzuki *et al*., 2005; Chayakulkeeree *et al*., 2008). In the current study, we determined the phenotypic effect of PI-PLC inhibition by U73122 on spore germination, hyphal growth and cell regeneration.

The *C. cinerea* wild-type strain Okayama-7 (FGSC #9003) and AmutBmut (*A43mut B43mut*, *pab-1*) (Swamy *et al*., 1984), a homothallic (self-compatible) strain, were used in the experiment. Strains were cultured on YMG (containing 0.4% glucose, yeast extract and malt extract) medium. Okayama-7 was used to harvest oidia from three-day cultured plates at 37°C under a controlled light regime. Basidiospores were harvested from mature fruiting bodies cultured at 25°C under a 12 h light/12 h dark regime for the AmutBmut strain. The culture method for fruiting-body formation used here has been described previously (Nara *et al*., 1999). To determine the effect of PI-PLC inhibitor (U73122) and the inactive substrate analog (U73343), strains were grown in MYG medium containing either U73122 or U73343 (each dissolved in DMSO) at concentrations of 5, 10, and 15 μM. An equivalent amount of DMSO was added to the control cultures.

We identified three putative *PLC* genes (designated *CcPLC1*, *CcPLC2*, and *CcPLC3*) by a genome database search (GenBank accession numbers EAU81219, EAU81283, and EAU83602, respectively) of the *C. cinerea* Okayama-7 strain, and a protein database search for the PLC1 sequence of *S. cerevisiae* (GenBank accession no.; AAB27349). The *CcPLC1* and *CcPLC2* coding regions are 2,577 and 3,111 bp and encode putative 881 and 1036 amino acid proteins, respectively. Deduced protein sequences (Ccplc1, Ccplc2) contain a pleckstrin homology (PH) domain, X / Y catalytic domains and a C2 (calcium-binding) domain. The *CcPLC3* coding region is 1,989 bp and encodes a putative 659 amino acid protein. Unlike Ccplc1 and Ccplc2, the deduced protein (Ccplc3) contains the X / Y catalytic domain and a C2 domain, but no PH domain. Ccplc1, Ccplc2, and Ccplc3 share 29%, 34%, and 32% amino acid sequence identity to corresponding *S. cerevisiae* proteins (Fig. 1A). Through an analysis of domain organization, Ccplc1 and Ccplc2 appear similar to the δ-isoform of eukaryotic PI-PLC. To predict the potential role of the three Ccplcs, we investigated transcript levels through the life cycle of *C. cinerea*. Total RNA was isolated from 1×10^9 oidia, 1×10^7 basidiospores and 150 mg of multi-cellular structures using the RNeasy kit (QIAGEN Sciences, USA) following the protocol provided by the manufacturer. Total RNA from each sample was used as a template for reverse transcriptase (RT)-PCR. The PCR reaction was performed using specific primers [(*CcPLC1*) forward: 5′-gcccatctccgaatacttca-3′/ reverse: 5′-gccttctcttctgccctctt-3′; (*CcPLC2*) forward: 5′-gtgtcag ccaagaagcacaa-3′/ reverse: 5′-ctggctcgagttgatgttga; (*CcPLC3*) forward: 5′-cggatgacttccctgtgttt-3′/ reverse: 5′-gtccgaaatctt gtcgcatt-3′; (*β-tubulin*) forward: 5′-ctcgtctccacttcttcatg-3′/ reverse: 5′-cgtcctggtattgctggtactcagc-3′). As shown in Fig. 1B, transcripts of *CcPLC1* and *CcPLC2* were highly expressed in oidia and hyphae. *CcPLC1* showed relatively high expression in the stipes of young fruiting bodies; whereas, *CcPLC2* showed relatively high expression in the pileus of the developing young fruiting body. In asexual spores (oidia), all three *CcPLCs* were highly expressed. In basidiospores, *CcPLC3* was

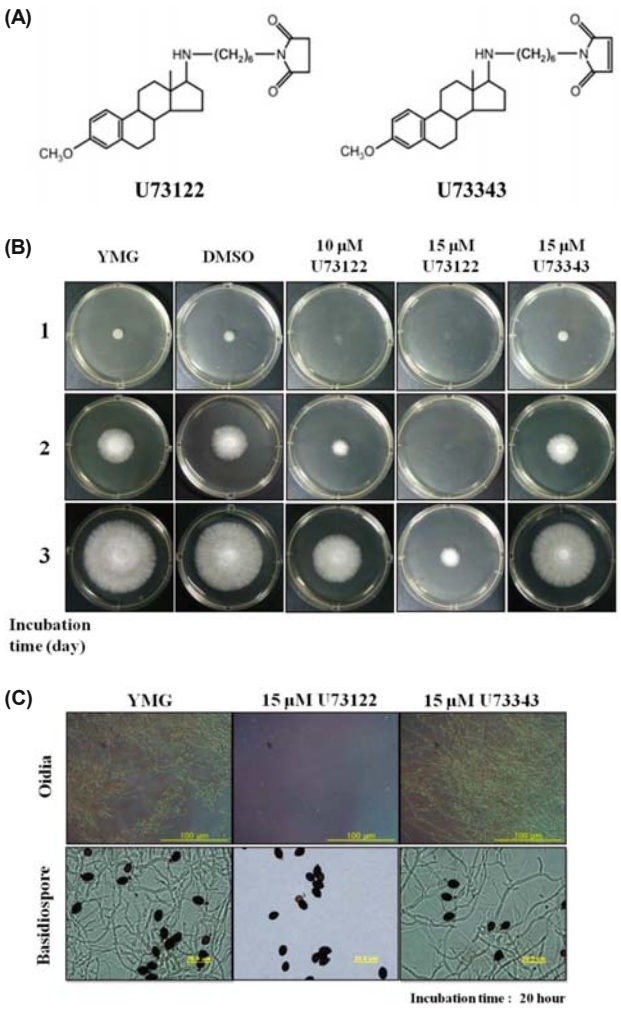
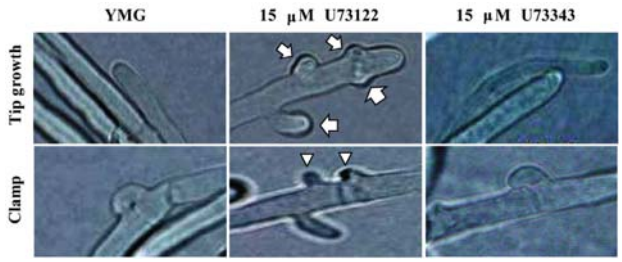


Fig. 2. Inhibition of Ccplc causes reduced hyphal growth and delayed germination. (A) Mycelial plug cut from the growing edge of 3-day colony of strain Okayama-7 was transferred to YMG plates containing DMSO, U73122 or U73343, at the indicated concentration, and incubated at 37°C for indicated times. (B) 1×10^7 oidia from strain Okyama-7 and 1×10^5 basidiospores from strain AmutBmut were inoculated on YMG medium containing 15 μM U73122 or 15 μM U73433, and incubated at 37°C for 20 h.

the most highly expressed.

To identify the function of the Ccplcs, we had to use U73122, a commonly employed specific inhibitor of receptor-mediated PLC, and its negative control, U73343. Because there are many previous studies indicating that fungal plc was required for normal vegetative growth and hyphal branching (Chung *et al*., 2006; Schumacher *et al*., 2008), we examined whether a PI-PLC inhibitor, U73122, could inhibit the hyphal growth of *C. cinerea*. Mycelial plugs were inoculated onto a YMG agar plate containing either U73122 or U73343 and incubated at 37°C for three days. To assess the hyphal growth rate, colony diameter was measured. U73122 (15 μM) inhibited the growth rate of 3-day cultured mycelia by more than 50%. In contrast, the inactive analog, U73343, showed less than 5% inhibition and DMSO had no effect (Fig. 2B).



Condition	Length between branches (μm)[a]
YMG	152.8 ± 14.8
DMSO	134.0 ± 29.6
5 μM U73122	109.0 ± 22.1
10 μM U73122	46.0 ± 21.9
15 μM U73122	28.0 ± 4.5
15 μM U73343	166.0 ± 50.9

[a] Distance between branches of mycelia

Fig. 3. Inhibition of Ccplc causes irregular polar growth and unfused clamp cells. Oidia from strain AmutBmut were inoculated on YMG medium containing 15 μM U73122 or 15 μM U73433, and incubated at 37°C. Arrows indicate side branches and aberrant clamp cells are marked by arrowheads.

We also checked spore germination. 1×10^7 oidia and 1×10^5 basidiospores were inoculated in 100 ml YMG media and incubated at 37°C with shaking (120 rpm) for 20 h. Spore germination was completely blocked by 15 μM U73122 until 20 h (Fig. 2C), while over 95% of the spores germinated with 15 μM U73343, which was no different than the control (YMG). After 36 h in the U73122 treatment, about 40% of the oidia and basidiospores germinated. Through these data, inhibition of Ccplc by U73122 resulted not only in inhibition of spore germination but also in inhibition of hyphal growth. These results indicate that the function of Ccplc is important for spore germination, and is also involved in normal hyphal growth. Interestingly, we observed irregular hyphal tip growth and open clamp connections with aberrant (branch-like structure) clamp cells in the presence of U73122. Unlike the normal sleek tip observed for growth with YMG or U73343, knobbly tips with many irregular side branches were observed for hyphae grown with U73122. The hyphae grown with U73122 had hyperbranching with narrow spaces

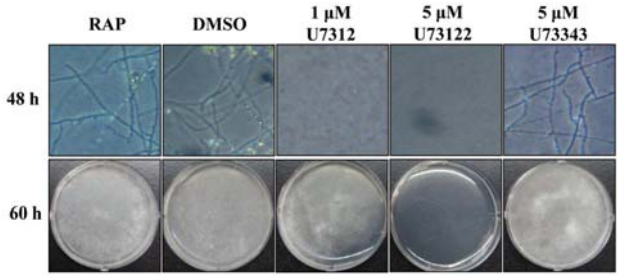


Fig. 4. Inhibition of Ccplc disturbs cell wall regeneration. 1×10^4 protoplasts from strain Okayama-7 were inoculated on RAP medium containing DMSO, U73122 or U73343, at the indicated concentration, and incubated at 37°C for indicated times.

between branches and aberrant clamp cells (Fig. 3). These data indicate that Ccplc has a role in establishing cell polarity and normal clamp cell connections. Our data is correlated with the function of *N. crassa plc-1*, which was shown to be involved in normal hyphal growth, branching and tip growth (Gavric *et al*., 2007).

Many previous studies indicate that fungal PI-PLCs have a critical role in maintenance of cell wall integrity and restoration following cell wall injury to retain normal growth and virulence (Chung *et al*., 2006; Chayakulkeeree *et al*., 2008). To determine the role in cell wall restoration, we investigated protoplast regeneration. The protoplasting and regeneration procedures were carried out as described by Granado *et al* (1997). Protoplasts were prepared from mycelia that were grown in YMG medium for 48 h with shaking, and digestion was with lysing enzymes (Sigma-Aldrich®, L1412, USA). After incubation for 4 h, the suspension was filtered through a disposable syringe (pore size: 0.2 μm) to remove mycelial debris and washed with washing buffer (0.5 M mannitol, 0.05 M disodium maleate, 25 mM $CaCl_2$). 1×10^4 protoplasts were spread on regeneration medium (RAP) (Granado *et al*., 1997) containing either U73122 or U73343 at concentrations of 1 and 5 μM. Incubation was carried out at 25°C for 48–60 h. As shown in Fig. 4, protoplast regeneration was inhibited according to the concentration of U73122 but regeneration in the presence of U73343 or DMSO was not different than with RAP. These results indicate that Ccplc has a role in cell wall regeneration.

In conclusion, this study using a specific inhibitor helps elucidate the role of PI-PLC, which is involved not only in spore germination and normal hyphal growth but also in cell wall generation in *C. cinerea*. Elucidation of the specific roles of each PLC isozyme would require the development of more reliable gene targeting tools. While waiting for such development, this study, by employing a biochemical approach using a specific inhibitor and an inactive analog, revealed functions of PI-PLCs in the basidiomycete *C. cinerea.*

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