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NOTE

Characterization of a Mutant Strain of a Filamentous Fungus *Cladosporium phlei* for the Mass Production of the Secondary Metabolite Phleichrome

Min-Hee Yi[1], Jung-Ae Kim[1], Jung-Mi Kim[1], Jin-Ah Park[1], Beom-Tae Kim[2], Seung-Moon Park[2], Moon-Sik Yang[2], Ki-Jun Hwang[3], and Dae-Hyuk Kim[1]*

[1]Institute for Molecular Biology and Genetics, Center for Fungal Pathogenesis, [2]Research Center of Bioactive Materials, [3]Department of Chemistry, Chonbuk National University, Chonbuk 561-756, Republic of Korea

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UV-mutagenesis was performed to obtain mutant strains that demonstrate altered production of phleichrome, a secondary metabolite of *Cladosporium phlei*. Among fifty mutants selected, based on the increased area and intensity of the purple pigment surrounding the colonies, the strain M0035 showed the highest production of phleichrome, more than seven fold over wild type. Plate cultures of the M0035 strain resulted in a total of 592 mg phleichrome consisting of 146 mg and 446 mg from the mycelia and agar media, respectively. The M0035 strain displayed a growth rate and a mycelial mass comparable to the parental strain but had significantly reduced asexual sporulation.

***Keywords*:** *Cladosporium phlei*, UV-mutagenesis, perylenequinone, phleichrome

Photodynamic therapy (PDT) is a treatment that uses a photosensitizing agent and a particular type of light. When photosensitizers are exposed to a specific wavelength of light, they produce a form of oxygen that kills nearby cancer cells. The most commonly used and studied PDT agent to date is Photofrin II, the only commercially available photosensitizer (Reynold *et al*., 1997); however, many new compounds have been studied in an attempt to create more efficient treatments. Certain compounds derived from 4,9-dihydroxy-3,10-perylenequinone are associated with photogenic diseases (e.g., hypericism, fagopyrism) and exhibit photodynamic properties (Diwu and Lown, 1995). Compounds belonging to the latter class include fagopyrin, elsinochrome, cercosporin, phleichrome, and hypocrellin. Among these, phleichrome has been intensively studied with regard to its photodynamic activity (Olivo and Chin, 2006) and potential as a pharmacophore to produce various derivatives.

Phleichrome, a derivative of 4,9-dihydroxyperylene-3,10-quinone, is a member of a group of fungal perylenequinones and is present as a deep red pigment in mycelia of the phytopathogenic fungus, *Cladosporium phlei* (C. T. Gregory) de Vries (Yoshihara *et al*., 1975). *C. phlei*, is a hypomycetous fungus that causes the purple eyespot disease in timothy (*Phleum pratense*). The disease is typified by circular purple (and later brown) spots with white to grayish-fawn centers on host leaves and has been implicated as the most common foliar disease affecting timothy (Shimanuki, 1987). Although it has been shown that phleichrome can be synthesized chemically and further transformed into more effective agents, this alternative chemical synthetic methodology for the preparation of a key precursor compound was itself a rate-limiting step. Thus, as a preparation methodology for the sustainable production of phleichrome, the establishment of biological methodologies, including strain improvement and the optimization of the fermentation process, is considered as the best, probably the only, alternative option. Recently we reported the culture characteristics of *C. phlei* and the extraction of phleichrome, which resulted in 43 mg/L and 2 mg/L of phleichrome in mycelia and the culture filtrate, respectively (Lee *et al*., 2007). In addition, a fungal transformation system was recently established for the genetic manipulation of *C. phlei* (Kim *et al*., 2009). However, no other studies on the recombinant or classical genetic manipulation of *C. phlei* strains have been conducted yet for the improvement of characteristics of this fungus. In the current study, we used UV-mutagenesis of *C. phlei* to breed a fungal strain for the stable overproduction of phleichrome.

The *C. phlei* wild-type strain (ATCC 36193) was used in mutagenesis experiments. Conidiospores of *C. phlei* used in the mutagenesis were harvested from cultures on V8 juice agar plates, which had been incubated for 4 weeks at 20°C. The conidia from each plate were thoroughly scraped with 10 ml sterile water containing 0.01% Tween 20 (Ichinomiya *et al*., 2005), filtered through Miracloth (Calbiochem, USA) and washed in sterile distilled water. The numbers of conidia were counted using a hemacytometer, and colony-forming units (CFU) per plate were counted by plating serially diluted spore suspensions on the V8 juice agar plates. For UV-mutagenesis, the suspension of conidia (usually 0.2 ml, 2×10^3 CFU mg/L) was spread on potato dextrose agar (PDA) plates and irradiated with UV light (Witteveen *et al*., 1990). The resulting mutants were maintained on V8 juice agar plates under con-

* For correspondence. E-mail: dhkim@chonbuk.ac.kr; Tel.: +82-63-270-3440; Fax: +82-63-270-3345

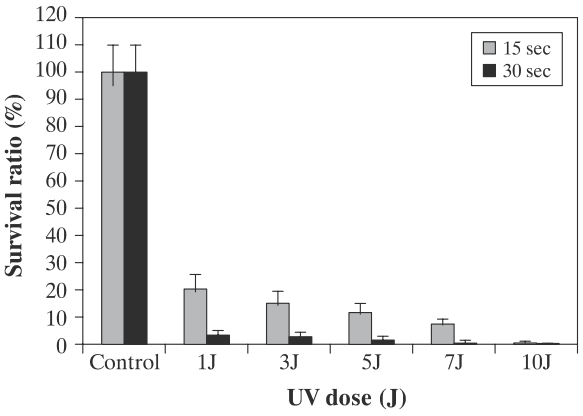

Fig. 1. Survival ratio of UV-irradiated spores.

stant low-level (<2,000 l x) light at 20°C and stored as frozen agar plugs harboring actively growing young hyphae in 5% DMSO solution at -70°C. As shown in Fig. 1, a UV dose of 5.0 J for 30 sec was sufficient to kill almost all of the spores. A UV dose of 5.0 J for 15 sec was selected for an approximately 10% survival rate, which was obtained under this intensity with little variation.

Although the V8 juice agar provided the optimal media for the growth of *C. phlei*, the cloudy nature of the media limited the visual inspection of pigment production. We therefore adopted PDA for screening the over-pigmented mutants. In addition to an enhanced color contrast, PDA screening is advantageous because a larger number of colonies can be screened on a single plate due to compact colonial growth on PDA. Mutant colonies that displayed increased purple pigmentation were selected from the PDA plate. Selected mutants were successively transferred at least three times on fresh PDA media to confirm the altered pigmentation and were preserved as described. Mutants showing increased purple pigmentation were found in high frequency (10^{-3}), while mutants lacking this pigmentation were sparse ($<10^{-4}$). A total of 50 overproducing mutants from 50,000 stable CFU were selected for further screening.

As pigmentation of the wild-type strain changed to a dark green color from the characteristic purple, we examined the phleichrome production according to the culture age using thin-layer chromatography (TLC) of the crude extract from the agar plugs, which contained actively growing mycelia (Lee *et al*., 2007). Briefly, 10 agar blocks of uniform size (0.5-cm-diameter) were excised from 0.5-cm behind margins of actively growing colonies and were gently agitated for 5 h in 2 ml of ethyl acetate (EtOAc) at 20°C (Smedsgaard, 1997). The samples were then centrifuged for 5 min at 5,000×g and the crude extract was analyzed using TLC with a resolving solution (CH_2Cl_2:MeOH=9:1, v/v) and a purified phleichrome as a control (Lee *et al*., 2007). As shown in Fig. 2, phleichrome production based on the TLC peaked at 12, 13, 14, 15, and 16 days after incubation and was followed by a decrease that corresponded to the change of pigment color from purple to dark green. Interestingly, although pigmentation on the PDA was evident, based on the characteristic purple pig-

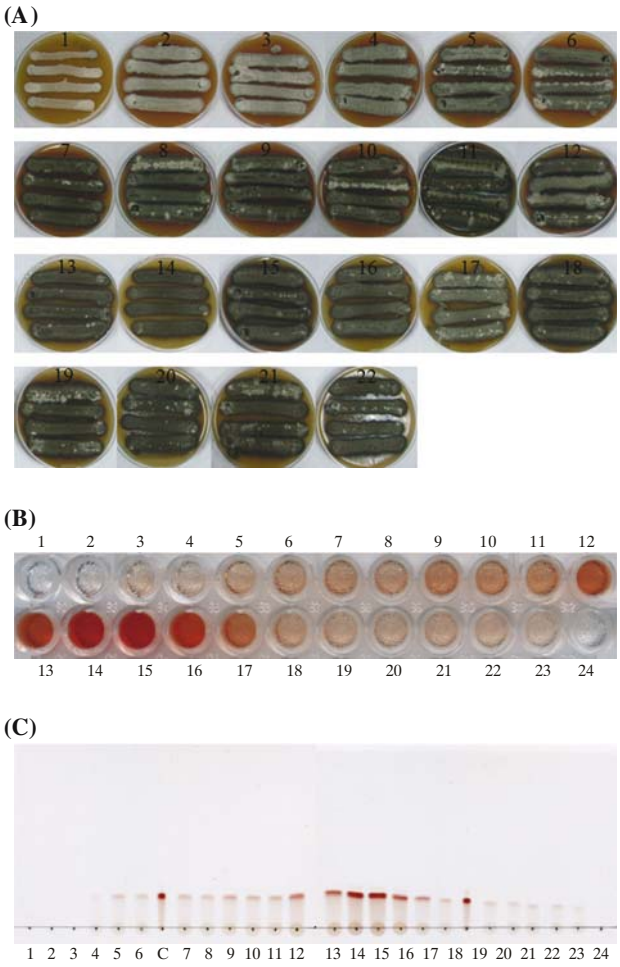

Fig. 2. Assessment of the colony morphology. (A) the color of ethyl acetate extract from the agar plugs containing actively growing mycelia (B) and the TLC analysis of corresponding ethyl acetate extract (C). Numbers indicate sample preparations from the corresponding days after the incubation. Lane C in the TLC plate contains purified phleichrome from previous studies (Lee *et al*., 2007).

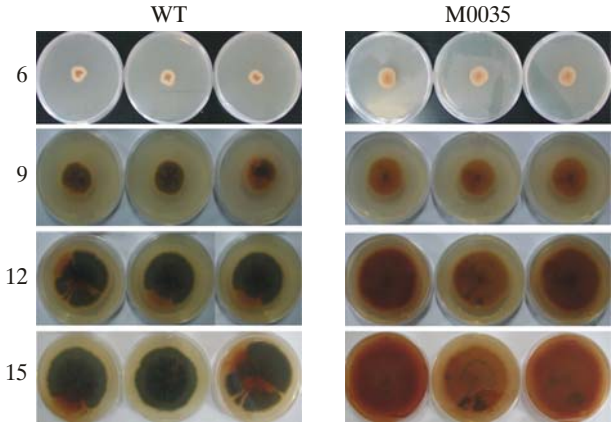

Fig. 3. Colony morphology of the M0035 and wild-type (WT) strains. Numbers indicate the days after cultivation on the PDA plate.

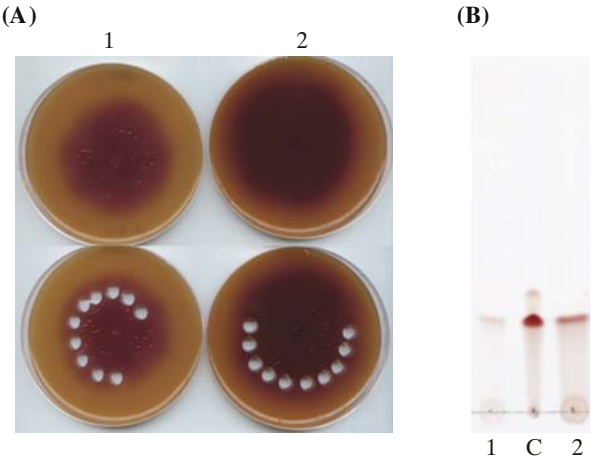

Fig. 4. Phleichrome extraction from the agar media. (A) Cellophane layer containing the actively growing mycelia on the V8 juice plate were removed and 10 agar plugs were obtained to extract the phleichrome. (B) TLC analysis of the ethyl acetate extract of agar plugs. Numbers 1 and 2 indicate the wild-type and mutant M0035 strains, respectively.

ment around the colony, phleichrome production on PDA was significantly reduced compared to that on the V8 juice agar. Approximately 10-fold lower amounts of phleichrome, estimated by TLC from serially diluted samples, were produced through the time course of *C. phlei* culturing on PDA. Therefore, TLC of the crude extract from mutants cultured for 15 days on the V8 juice agar plate or prior to the pigment change to dark green was performed for the rapid screening of a phleichrome overproducer.

Among the 50 screened mutants, mutant strain M0035 was selected. As shown in Fig. 3, the M0035 strain showed an increased purple pigment production compared to the wild-type strain. Interestingly, the characteristic purple pigment remained as the culture proceeded, suggesting that the increased levels of phleichrome in the M0035 mutant could be ascribed at least in part to the delayed metabolic conversion of phleichrome. In addition, as shown in Fig. 4, the presence of phleichrome in the agar media following the removal of the cellophane layer indicated that phleichrome was secreted from the cells into the media. Thus, the larger area and more intense color of the M0035 strain suggested that increased levels of secreted phleichrome were present, an observation confirmed by TLC analysis. The mycelial mass, harvested following prolonged incubation (>20 days) of liquid culture, showed the characteristic purple pigment compared to the dark green of the wild-type, suggesting that the delayed conversion of the fungal pigment also occurred in liquid media.

The growth rate measured from the radial growth of colonies on the plate indicated no differences between the wild-type and M0035 strains. In addition, the liquid culture of M0035 in V8 juice supplemented with 5% glucose and 2% malt extract yielded mycelial masses of 20.5 g/L after 15 days incubation, comparable to that of the wild-type strain, indicating no growth defects (Lee *et al*., 2007). However, when the conidial production of the M0035 on V8 juice agar plates was analyzed, a 1,000-fold reduction in the number of conidia per plate was observed regardless of the light exposure. Although the number of conidia was reduced, the morphological characteristics of the spores were unchanged and they sustained an oblong to cylindrical appearance with rounded edges, verruculose on the surface and pigmentation from pale brown to olive brown. This suggests that the M0035 strain has a defect in the frequency of conidiation, most likely in the process of conidiophore genesis; however, once the spores are produced, they appear comparable to the wild-type.

Table 1. The yield of phleichrome from mycelia and cultured media of the M0035 strain

Culture type	Location	Amount of phleichrome (mg/L)
Liquid	Mycelia	311
	Cultured media	45
Solid	Mycelia	146
	Cultured media	446

The yield of phleichrome from the M0035 strain was measured following purification as described previously (Lee *et al*., 2007). When compared to the wild-type, the phleichrome yields from the M0035 strain were increased more than seven fold; the phleichrome yields from the mycelia and culture filtrates were 311 mg/L and 45 mg/L, respectively. As the M0035 strain showed reduced conidiation, the solid culture of the mutant strain was adopted and the phleichrome production on the V8 juice agar plate was measured. The extraction of phleichrome from the agar medium was performed by applying the same ratios between the sample volume and EtOAc that were used in the extraction from agar blocks. We obtained a total of 592 mg of phleichrome from 25 plates produced by pouring 40 ml of melted V8 juice agar. The phleichrome production was around 146 mg and 446 mg from mycelia and agar medium, respectively, suggesting that solid instead of liquid fermentation is the preferred culture condition for the mass production of phleichrome (Pham *et al*., 2010) (Table 1). These results indicate that UV-mutagenesis followed by visual inspection and rapid TLC analysis using agar blocks is a successful method for selecting a mutant strain that overproduces phleichrome.

The genetic manipulation of fungi has been suggested as an efficient tool for improving the fungal strain of interest. We have recently established the transformation of *C. phlei* (Kim *et al*., 2009). Although fungal pigment belonging to the perylenequinones is generally known to be synthesized by polyketide synthase (PKS), the identity of the specific PKS responsible for phleichrome has been difficult to assess due to there being multiple genes for fungal PKS (Choquer *et al*., 2005). PCR amplification of the PKS gene using degenerate primers for the conserved region of the fungal PKS revealed the presence of multiple copies of the PKS gene in *C. pheli* (unpublished data). Thus, obtaining both over- and underproducing mutants may, in the future, aid the identification of the PKS responsible for the production of phleichrome.

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