Identification of a Polyketide Synthase Gene in the Synthesis of Phleichrome of the Phytopathogenic Fungus Cladosporium phlei

Kum-Kang So\textsuperscript{1}, Yun-Jo Chung\textsuperscript{2}, Jung-Mi Kim\textsuperscript{2}, Beom-Tae Kim\textsuperscript{1}, Seung-Moon Park\textsuperscript{1}, and Dae-Hyuk Kim\textsuperscript{1,*}

Phleichrome, a pigment produced by the phytopathogenic fungus Cladosporium phlei, is a fungal perylenequinone whose photodynamic activity has been studied intensively. To determine the biological function of phleichrome and to engineer a strain with enhanced production of phleichrome, we identified the gene responsible for the synthesis of phleichrome. Structural comparison of phleichrome with other fungal perylenequinones suggested that phleichrome is synthesized via polyketide pathway. We recently identified four different polyketide synthase (PKS) genes encompassing three major clades of fungal PKSs that differ with respect to reducing conditions for the polyketide product. Based on in silico analysis of cloned genes, we hypothesized that the non-reducing PKS gene, Cppks1, is involved in phleichrome biosynthesis. Increased accumulation of Cppks1 transcript was observed in response to supplementation with the application of synthetic inducer cyclo-(L-Pro-L-Phe). In addition, heterologous expression of the Cppks1 gene in Cryptonectria parasitica resulted in the production of phleichrome. These results provide convincing evidence that the Cppks1 gene is responsible for the biosynthesis of phleichrome.

INTRODUCTION

Cladosporium phlei (George) de Vries belongs to a group of hypomycetous fungus that causes purple eyespot disease in timothy (Phleum pratense). This disease is one of the most common foliar diseases of timothy, which is easily distinguishable by eye-shaped spots of light greyish-fawn centers with pur-

Materials and Methods

Fungal strains, culture media, and growth conditions

Cladosporium phlei (ATCC 36193) was stored in the form of...
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frozen agar plugs containing actively growing young hyphae in 5% DMSO solution at -70°C and maintained on potato dextrose agar (PDA) as described previously (Lee et al., 2007). Culture conditions and methods for phleichrome induction using 150 μM cyclo-(-Phe-L-Pro) inducer are provided in our previous study (So et al., 2015). Mycelia on cellophane membranes overlaying agar plates were collected and lyophilized as described previously, and stored until use (So et al., 2015). All chemicals were obtained from Sigma-Aldrich Co. (USA), unless otherwise specified.

Analysis of Cppks1 transcript expression
RNA from mycelia grown on cellophane layered on top of PDA media supplemented with 150 μM cyclo-(-Pro-L-Phe) inducer was extracted as described previously (Park et al., 2004). Northern blot analysis was used to compare the level of Cppks1 (GenBank accession no. JX129223) transcript before and after induction. Semi-quantitative analysis of the accumulation of Cppks1 transcript was performed by RT-PCR using total RNA extracted from cultures at 6 and 18 days after inoculation with and without induction, as described previously (Park et al., 2012). Cppks1 transcript levels were compared with those of another PKS gene encoding a highly-reducing (HR)-PKS, namely Cppks3 (GenBank accession no. JX129225), and normalized to levels of the gene encoding beta-tubulin (β-tub) as an internal control (Choi and Nuss, 1990). Analyses were conducted at least twice, in triplicate for each transcript, from at least two independent RNA preparations of the same sample with primers specific for β-tub, Cppks3, and Cppks1 genes. Primer pairs for each gene are described in Table 1. To determine relative phleichrome content from the corresponding mycelia, phleichrome was extracted from mycelia using ethyl acetate (EtOAc) and compared using thin-layer chromatography (TLC) on a silica gel with a resolving solution (CH2Cl2/MeOH = 19:1, v/v), as described previously (Yi et al., 2011).

Construction of a Cppks1 expression cassette for heterologous expression
We selected a genomic fosmid clone containing full-length Cppks1 from our previous study. The deduced Cppks1 sequence contained no introns, and the protein product of CppKS1 consisted of 2,174 codons, with an estimated molecular weight of 245,316 Da. It contained no introns, and the protein product of Cppks1 contained no introns, and the protein product of Cppks1 sequence contained no introns, and the protein product of CppKS1 consisted of 2,174 codons, with an estimated molecular weight of 245,316 Da.

RESULTS AND DISCUSSION

Bioinformatic analyses of a gene involved in phleichrome biosynthesis
Structural comparison with other fungal perylenequinones revealed that phleichrome is a close analogue of cercosporin. The only gross structural differences between the two substances are the two methoxy groups in phleichrome instead of the methoxy groups in cercosporin (Arnone et al., 1985). Cercosporin is known to be synthesized via the polyketide metabolic pathway using acetate and malonate subunits (Choquer et al., 2005; Kurobane et al., 1981; Kusari et al., 2009; Liao and
Chung, 2008; Okubo et al., 1975). Structural similarity suggests that phleichrome is also synthesized via the polyketide pathway, which in fungi is orchestrated by polyketide synthases (PKS), which are multimeric enzymes that function analogously to fatty acid synthases joining carboxylic acid units in a stepwise fashion (Crawford and Townsend, 2010). All PKSs are currently divided into three general classes according to the organization of their active sites; fungal PKSs are multidomain systems (iterative type I) (Crawford and Townsend, 2010; Hutchinson and Fujii, 1995). In our previous study, we cloned four C. phlei PKS genes, which included three representative subclasses of PKS based on the level of reductive processing during chain assembly (So et al., 2012). In general, fungal aromatic polyketides, including phleichrome, are known to be synthesized by non-reducing (NR)-PKSs (Crawford and Townsend, 2010). Phylogenetic analysis of the four cloned C. phlei PKS genes indicated that the Cppks1 protein product appeared to be a NR-PKS (So et al., 2012). It was most closely related to other fungal PKS genes involved in the synthesis of red and orange perylenequinone pigments, including close analogues of phleichrome such as cercosporin (Choquer et al., 2005) and elsinochromes (Liao and Chung, 2008). In addition, encoded proteins of Cppks1 and two other genes for cercosporin and elsinochrome biosynthesis consisted of five catalytic domains, including keto synthase (KS), acyltransferase (AT), thioesterase/claisen cyclase (TE/CYC), and two consecutive acyl carrier proteins (ACP), that were distributed at comparable distances in the same order and at a similar length (Choquer et al., 2005; Liao and Chung, 2008; So et al., 2012). Altogether, our results suggested that Cppks1 is responsible for the biosynthesis of phleichrome via the fungal polyketide pathway.

**Regulation of the expression of Cppks1**

In our previous studies, we found that phleichrome production was significantly increased by addition of 150 μM cyclo-(L-Pro-L-Phe) synthetic inducer into the culture media (Fig. 1). Thus, we analyzed levels of the Cppks1 transcript in response to the inducer using semi-quantitative RT-PCR. Total RNA was extracted from cultures at 6 and 18 days postinoculation. These timepoints were chosen because discernable pigmentation was observed on the PDA plates by 6 days postinoculation and the maximum amount of phleichrome was produced by 18 days postinoculation. As shown in Fig. 2, supplementation of the synthetic inducer into culture significantly increased the accumulation of Cppks1 transcript. The level of Cppks1 transcript increased along with incubation time, similar to the temporal pattern of phleichrome production under induction conditions. However, the expression of Cppks3, which is an HR-PKS, did not change in response to the synthetic inducer supplementation. The coordinated response of Cppks1 transcript to the inducer suggests that Cppks1 gene is associated with phleichrome biosynthesis.

**Fig. 1.** Phleichrome production from the mycelia of wild-type C. phlei grown on PDA supplemented with 150 μM cyclo-(L-Pro-L-Phe). Phleichrome production without induction as a control is also shown. Left panels demonstrate the characteristics of each colony from above, while right panels display the characteristics from the bottom of the plate.

**Fig. 2.** Semi-quantitative RT-PCR analysis of Cppks1 transcript levels relative to levels of β-tubulin (β-tub). Total RNA was extracted 6- and 18-days after induction. Accumulation of Cppks1 transcript was compared with and without an induction. Experimental results were normalized to β-tub gene and the PKS gene Cppks3, a member of the HR-PKS subclass, was analyzed as an internal control. Note that equal amounts of RNA samples were loaded as shown in the bottom panel by the expression level of β-tub gene showing similar band intensities among samples and a representative ethidium bromide-stained rRNA bands from one of three independent experiments.
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**Fig. 3.** Pigmentation of Cppks1-transformed *C. parasitica*. The bottoms of plates containing colonies after 10 days of culture on PDA supplemented with 100 mg/L of L-methionine and 1 mg/L of biotin (PDAmb) are shown. Strains used, which are indicated above the panel, were wild-type *C. parasitica* EP155/2 strain and three single-spored transformants (TNF#44, #16, and #56). Note that TNF#16 and #56 had more pinkish pigments at the bottom compared to wild type.

**Analysis of *C. parasitica* transformants**

Heterologous expression is one of the most efficient methods for functional analysis of a given cloned gene. *C. parasitica* is a genetically tractable organism (Churchill et al., 1990) capable of producing highly complexed pigments such as skyrin, oxyskyrin, and rugulosin via the polyketide pathway (Shibata, 1973). Therefore, we expressed Cppks1 in *C. parasitica* to see if the resulting recombinant fungus would produce phleichrome. Thirty putative transformants were selected from the top agar plates supplemented with 150 μg/ml geneticin, passed three times on selective media supplemented with 50 μg/ml geneticin, and single-spore isolated prior to further analyses. Mitotic stability of each transformant was confirmed by successive transfers alternating on selective and non-selective media. In order to detect integration of the transforming vector into the chromosome of *C. parasitica*, PCR analysis using Cppks1-specific primers was conducted. All 30 transformants were positive for the 646-bp PCR amplicon, indicating stable integrative transformants.

Because phleichrome is responsible for the characteristic deep red pigmentation in the mycelia and culture medium, we looked for changes in the colour of colonies in single-spored transformants. As shown in Fig. 3, two colonies became pinkish over time, in contrast to the original orange colour, as culture aged. However, no discernible changes in any of the other characteristics, including growth rate and sporulation, were observed in these transformants. Therefore, we assessed the presence of phleichrome in these selected transformants. TLC analysis using an EtOAc extract of the mycelia revealed the presence of yellowish pigment at an Rf value of 0.24, the same Rf as for purified phleichrome (Fig. 4A). However, these pigments were also present in wild-type *C. parasitica*. These results indicated that TLC was not sensitive enough to differentiate phleichrome from other residual pigments, such as skyrin and oxyskyrin. The amount of heterologous phleichrome produced may also have been too low for detection by TLC.

Thus, pigment spots migrating at the same Rf value as purified phleichrome on TLC plates were scraped off and extracted with methanol, and the presence of phleichrome was determined by LC/MS/MS analysis with purified phleichrome from *C. phlei* as a control. As shown in Fig. 4B, methanol extract of TLC spots from recombinant *C. parasitica* had a new peak with the same retention time as the control phleichrome, and the molecular weight of the corresponding peak was the same as that of phleichrome, confirming the presence of phleichrome in extracts. These results clearly indicated that phleichrome is synthesized via the polyketide pathway and that Cppks1 encodes a protein responsible for the biosynthesis of phleichrome. It would be of great interest to carry out functional analysis of the Cppks1 gene product using gene replacement or silencing. In addition to the PKS gene, tailoring proteins that catalyze subsequent reactions including oxidation, hydration, methylation, and hydroxylation are also required for successful cercosporin biosynthesis (Chen and Nuss, 2007). *C. parasitica* does not produce phleichrome but does produce secondary metabolites such as skyrin, oxyskyrin, and rugulosin via the polyketide pathway (Shibata, 1973).

Genome survey of *C. parasitica* suggested the presence of at least 31 PKS genes and/or clusters (http://genome.jgi-psf.org/Crypa1/Crypa1.home.html). Thus, genes involved in the processing of the core component of *C. parasitica* may be involved in further processing of phleichrome. In addition, heterologous production of a fungal polyketide in the yeast *Saccharomyces cerevisiae* requires co-expression of the corresponding PKS gene and a heterologous 4′-phosphopantetheinyl transferase (PPTase) (Kealey et al., 1998; Wattanachaisaeree-kul et al., 2007). PPTases catalyze the post-translational modification of proteins by the covalent attachment of a 4′-phosphopantetheine moiety of coenzyme A to a conserved serine residue of an inactive form of ACP, resulting in conversion to the active form during the biosynthesis of metabolites (Walsh et al., 1997). Interestingly, no PPTase domain or genes were found in the gene cluster for cercosporin biosynthesis. Therefore, the successful heterologous production of phleichrome suggests that there was an appropriate PPTase that acted in trans in *C. parasitica*, that converted heterologously expressed inactive apo-CpPKS1 to active holo-CpPKS1 by modifying the ACP domain of CpPKS1 with a 4′-phosphopantetheine moiety. Almost all organisms that utilize more than one 4′-phosphopantetheine-dependent pathway also have more than one PPTase (Gehring et al., 1998; Quadri et al., 1998). A genome survey of *C. parasitica* suggested the presence of three PPTase genes (http://genome.jgi-psf.org/Crypa1/Crypa1.home.html). However, filamentous fungi such as *Neurospora crassa*, *A. fumigatus*, *A. nidulans*, and *Fusarium fujikuroi* harbor only a single PPTase (Kim et al., 2015).

Genes involved in the secondary metabolite pathways in filamentous fungi are often organized in clusters (Keller et al., 2005). Therefore, we are currently characterizing the loci surrounding Cppks1 to determine if genes involved in the biosyn-
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Thesis of phleichrome are clustered. Additionally, Cppks1 may not be the only gene for phleichrome synthesis. The survey of genomic library suggested that more NR-PKS genes remain to be identified in addition to the four PKS genes cloned in a previous study (So et al., 2012). Further studies are required to characterize all cloned PKS genes. Functional analysis of Cppks1 gene will allow determination of the redundancy of the phleichrome gene.

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