Insertional mutagenesis and characterization of a polyketide synthase gene (PKS1) required for melanin biosynthesis in Bipolaris oryzae

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Abstract

A polyketide synthase gene named PKS1, involved in the melanin biosynthesis pathway of the phytopathogenic fungus Bipolaris oryzae, was isolated using restriction enzyme-mediated integration. Sequence analysis showed that the PKS1 encodes a putative protein that has 2155 amino acids and significant similarity to other fungal polyketide synthases. Targeted disruption of the PKS1 gene showed that it is necessary for melanin biosynthesis in B. oryzae. Northern blot analysis showed that PKS1 transcripts were specifically enhanced by near-ultraviolet radiation (300–400 nm) and that its temporal transcriptional patterns were similar to those of THR1 and SCD1 genes involved in the melanin biosynthesis pathway of B. oryzae.

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1. Introduction

Melanin is a dark-pigmented polymer that protects organisms against environmental stress, and its production is also widespread in the fungal kingdom [1–3]. In general, melanin accumulates in fungal cell walls and has been believed to confer tolerance to environmental stresses such as UV radiation [4–6].

Bipolaris oryzae (Breda de Haan) Shoem., the causal agent of brown leaf spot disease in rice, produces 1,8-dihydroxynaphthalene (DHN)-melanin [7], as do Colletotrichum lagenarium, Magnaporthe grisea, and Alternaria alternata. DHN-melanin biosynthesis starts with a polyketide synthase (PKS) using acetate as a precursor. An hydroxynaphthalene reductase converts 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) to scytalone. Dehydration of scytalone forms 1,3,8-trihydroxynaphthalene (1,3,8-THN), which is converted to 1,8-DHN after an additional reduction and dehydration step. Finally, oxidative polymerization of 1,8-DHN gives the DHN-melanin. We have recently observed that the expression of two melanin biosynthesis genes, the scytalone dehydrogenase gene (SCD1) and 1,3,8-THN reductase gene (THR1), were specifically up-regulated by near-ultraviolet (NUV) radiation in B. oryzae [8,9]. However, the PKS gene involved in melanin biosynthesis has not yet been identified and characterized in B. oryzae.

In this study, we report restriction enzyme-mediated integration (REMI) tagging, cloning, and characterization of the PKS1 required for melanin biosynthesis of B. oryzae.
the rice brown spot fungus \textit{B. oryzae}. Furthermore, we compare the up-regulated gene expression of the \textit{PKS1} gene by NUV irradiation with those of two other melanin biosynthesis genes.

2. Materials and methods

2.1. Fungal strains, culture condition, and fungal transformation

\textit{Bipolaris oryzae} strain D9/F6-69 (a stock culture at the Laboratory of Plant Pathology, Shimane University) was used as the wild-type strain. Potato-dextrose agar (PDA) plates were inoculated with a small mycelial plug at the center, followed by incubation at 25 ± 1 °C in darkness. Fungal transformation using REMI methods were performed according to the methods previously described by [9] and fungal transformation vector pSH75 [10], containing a hygromycin-resistant \textit{hph} gene as a selectable marker between the \textit{Aspergillus nidulans} PtrpC promoter and TrpC terminator, was used. For REMI methods, approximately 0.5 × 10^7 protoplasts in 80 μl of STC buffer were gently mixed with 5 μg of linearized plasmid pSH75, 20 μl of PEG solution (40% PEG 4000 in STC buffer). About 20 μl of the same restriction enzyme was used to digest plasmid pSH75. The solution was incubated on ice for 30 min. A further 900 μl of PEG solution was added, and the solution was mixed and incubated at room temperature for 10 min. Approximately 1 ml of this reaction mixture was mixed with 7.5 ml of regeneration medium (RM; 0.1% yeast extract, 0.1% casein, 1 M sucrose, 0.8% agar) and overlaid onto a plate containing regeneration agar (1.5%). After incubation at 25 ± 1 °C overnight, 7.5 ml of regeneration agar (1.5%) containing 350 μg/ml of hygromycin B was poured onto the plate. Plates were incubated at 25 ± 1 °C. After 4–6 days of incubation, regenerated protoplasts on RM medium were transferred to PDA medium containing 50 μg/ml of hygromycin B.

2.2. Manipulation of DNA and plasmids

Southern blot analysis was performed as described [8]. The blots were hybridized with the DIG-labeled 3.0-kb fragment internal to the \textit{PKS1} gene amplified by PCR using the primers KS1 (5'-CCCGGCGCCGCTCAAATCA-3') and 5E/RI (5'-CTTACCCGGCAATCTTGATAGAT-3') and \textit{B. oryzae} genomic DNA as a template. DNA labeling, hybridization, and detection were carried out using the DIG system (Roche Diagnostics, Mannheim, Germany).

To recover flanking fungal genomic sequences from the integrated site, genomic DNA (50 μg) from REMI-transformants were digested with EcoRI or EcoRV and purified by phenol extraction and ethanol precipitation. Digested DNA was self-ligated using DNA Ligation High (TOYOBO, Osaka, Japan) followed by transformation into \textit{Escherichia coli} competent cells HB101 (TaKaRa, Shiga, Japan). The rescued plasmid was isolated from ampicillin-resistant \textit{E. coli} and analyzed further.

A cosmid library constructed from \textit{B. oryzae} genomic DNA using the pWEB Cosmid Cloning Kit (Epigenetics Technology, Madison, WI, USA) was screened by colony hybridization using DIG-labeled \textit{PKS1} fragment from \textit{B. oryzae}. DNA labeling, hybridization, and detection were carried out using a non-radioactive system (Roche Diagnostics GmbH, Mannheim, Germany). Sequencing reactions were performed using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA, USA) on an ABI PRISM 310 genetic analyzer (Applied Biosystems). Computer analysis of the DNA sequence data was performed using GENETYX (Software Development Co. Ltd., Tokyo, Japan). The GenBank, EMBL, and DDBJ nucleotide sequence databases were searched using the FASTA and BLAST programs at http://www.ddbj.nig.ac.jp/.

2.3. Disruption of \textit{PKS1}

Fungal transformation vector pSH75 [10], containing a hygromycin-resistant \textit{hph} gene as a selectable marker, was used to construct the \textit{PKS1} disruption vector. A 3.0-kb fragment internal to the \textit{PKS1} gene amplified by PCR using primers KS1 (5'-CCCGGCGCCGCTCAAATCA-3') and 5E/RI (5'-CTTACCCGGCAATCTTGATAGAT-3'), and \textit{B. oryzae} genomic DNA as a template was cloned into the transformation vector pSH75 to produce pSHGDPKS1. The strain D9/F6-69 was transformed with pSHGDPKS1 as described elsewhere [8,9].

2.4. Pathogenicity test

Seedlings of the rice (\textit{Oryza sativa} L., cv. Koshihikari) were planted in a seedling case containing Sun Soil (Nagata KK, Japan), and then grown in a greenhouse. A spore suspension (5 × 10^4 spores/ml) of \textit{B. oryzae} was sprayed onto intact rice plants at 5–6 leaf-stage. Inoculated rice plants were kept in a moist chamber for 24 h in darkness, and then in a greenhouse. Lesion formation was investigated at 7 days after inoculation.

2.5. Northern blot analysis

Irradiation and Northern blot analysis was performed as described elsewhere [9]. For temporal transcription pattern analysis, DIG-labeled cDNA of \textit{THRI} [8] and \textit{SCDI} [9] were also used as probes. In all Northern blot analyses, 18S rDNA from \textit{B. oryzae} was used as an internal control. DNA labeling, hybridization,
ization, and detection were carried out using the DIG system (Roche).

3. Results and discussion

Among 1200 REMI-transformants, we isolated 10 mycelial pigmentation-deficient transformants based on colony color on PDA medium. By Southern blot analysis, we selected two mutants that showed a single integration event. Mutants B5w and HDDB28 generated by REMI transformation using the HindIII restriction enzyme had completely lost their pigmentation ability, whereas mutant BT4 generated by REMI transformation using the BamHI restriction enzyme accumulated a darkish pigment (Fig. 1).

Wild-type genes corresponding to two mutant phenotypes mentioned above were cloned by obtaining the genomic DNA flanking the insertion point by plasmid rescue. The rescued plasmids containing the flanking genomic DNA sequences were designated as pB5E, pHDB28, and pSHBT4, corresponding to the mutants B5w, HDDB28, and BT4, respectively. These plasmids were then sequenced. A BLAST search of the DDBJ homology search system with the putative translation products in the flanking genomic DNA fragments of pB5E, pHDB28, and pSHBT4 showed the highest similarity to polyketide synthase (PKSs) from C. lagenarium [11], Nodulisporium sp. pks1 [12], A. nidulans wA [13], A. fumigatus alb1 (pksP) [14,15], and Glarea lozoyensis [16] with four potential catalytic modules: beta-ketoacyl synthase (KS), an acyltransferase (AT), two acyl carrier sites (AC1 and AC2), and a thioesterase (TE)/Claisen cyclase (CYC) (Figs. 2 and 3). The flanking genomic DNA fragment of pB5E and pHDB28 corresponded to the 3’ region of the PKS1 gene, and both integration events occurred at the same HindIII site (Fig. 2). These results suggested that the mutants B5w and HDDB28 could be due to a defective gene encoding PKS involved in the initial step of fungal DHN melanin pathway. It was noted that the flanking genomic DNA fragment of pSHBT4 was located 666-bp upstream from the deduced initiation codon of PKS1, and the integration event occurred at the BamHI site in the upstream region of the PKS1 gene. Thus, several regulatory cis-elements of the PKS1 gene could be located in the upstream region of the PKS1 gene.

Disruption of fungal melanin PKS genes has been previously demonstrated to generate fungal strains lacking pigmentation in C. lagenarium [11], Nodulisporium species [12], A. fumigatus [14,15], G. lozoyensis [16], Wangiella dermatitidis [17], and Ceratocystis resinifera [18]. In order to confirm the function of the PKS1 gene

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Fig. 1. Growth of melanin-deficient mutants and wild-type on PDA medium. The wild-type (WT) and mutants B5w, HDDB28 and BT4 were inoculated on PDA medium and incubated in darkness at 25 ± 1 °C for 3 days: (a) upper side of the plate; (b) lower side of the plate.
of B. oryzae, we next investigated the effects of disrupting the PKS1 gene. A PCR-amplified fragment (3.0-kp) internal to the PKS1 gene was cloned into the pSH75, producing the disruption vector pSHGDPKS1. The plasmid was predicted to disrupt the PKS1 gene in the wild type through single-crossover homologous recombination. As a result of transformation, 118 of 126 hygromycin-resistant transformants showed a melanin-deficient appearance, similar to that of the mutants B5w and HDDB28 (Fig. 4(a)). The occurrence of insertional transformation of PKS1 in six PKS1 disruptants (T1-T6) was confirmed by Southern hybridization (Fig. 4(b)). When the genomic DNA of the wild-type was digested with BamHI and probed using the PKS1 gene, a fragment of 10 kb resulted. On the other hand, when the genomic DNA of six PKS1 disruptants was digested with BamHI and probed with the PKS1 gene, fragments of 11 and 7.8 kb were observed instead of the 10-kb fragment of the wild-type. Thus, all six PKS1 disruptants showed the predicted homologous recombination pattern between pSHGDPKS1 and PKS1 in the chromosomal DNA of B. oryzae (Fig. 4(c)). These results indicated that the PKS1 gene is essential for melanin biosynthesis in B. oryzae.

Melanin production is known to be an essential factor in the phytopathogenic fungus M. grisea [19–21] and C. lagenarium [22]. To determine whether the PKS1 is essential for pathogenicity on rice, we inoculated rice plants with conidial suspensions of wild-type and the PKS1 disruptants. After seven days from the inoculation, both the wild-type and the PKS1 disruptants caused typical necrotic lesions with same size on the rice leaves (data not shown). These results indicated that the PKS1 gene is probably not relevant to the pathogenicity of B. oryzae, since the PKS1 gene of B. oryzae is not essential for its pathogenicity.

Previously, we reported that two melanin biosynthesis genes, the SCD1 and 1,3,8-THN reductase gene (THRI), were up-regulated by NUV radiation [8,9]. Thus, we investigated the PKS1 gene expression under several light conditions by Northern blot analysis. We first examined the relationship between PKS1 gene expression and sunlight by Northern blot analysis. The PKS1 gene was expressed weakly in darkness, whereas its expression was enhanced by sunlight irradiation for 5 min followed by darkness for 30 or 60 min (Fig. 5(a)). This suggested that light up-regulated PKS1 gene expression. In order to clarify the most effective wavelength range to enhance PKS1 gene expression, we next examined PKS1 gene expression in relation to several types of artificial light at two irradiance conditions (250 and 1000 J/m²) by Northern blot analysis (Fig. 5(b)). As a result, the expression of PKS1 was significantly enhanced in mycelia exposed to NUV radiation under low (250 J/m²) and high (1000 J/m²) irradiance conditions, but not in mycelia exposed to blue, green, yellow, and red radiation (Fig. 5(b)). In this experiment, control hybridizations with an 18S rDNA probe showed similar amounts of RNA in each RNA sample (Fig. 5(b)). These results indicated that the most effective wavelength range for enhancing PKS1 gene expression is the NUV region (300–400 nm). We also studied a time course in the expression of PKS1 transcripts compared with those of SCD1 and THRI transcripts in darkness after NUV radiation. The cultures for RNA isolation were irradiated with NUV for 5 min, and total RNA was extracted at different times thereafter. As shown in Fig. 5(c), the PKS1 transcripts started to accumulate at 30–45 min, increased until 1 h after NUV radiation, and began to gradually decrease until 12 h in darkness. Likewise, the transcripts of SCD1 and THRI increased until 1 h after NUV radiation, and began to gradually...
Fig. 3. Alignments of the polyketide motifs presented in the deduced amino acid sequence of B. oryzae PKS1 and those of various fungal polyketide synthase genes. The putative catalytic modules found in the deduced sequence of PKS1 from B. oryzae (Fig. 2) are compared with the equivalent motifs in PKSs involved in the synthesis of melanin or spore pigments: (a) N-terminus of the protein; (b) KS motif with the active site cysteine; (c) Acyl transferase (AT) motif with the active site serine; (d) Acyl carrier (AC) protein motif 1 with the active site serine; (e) TE or CYC motif 1 with the active site serine; (f) TE or CYC motif 2 and C-terminus of the protein with the active site histidine. Database accession numbers for the corresponding genes are as follows: Bo, B. oryzae PKS1 (in this study, AB176546); Cl, C. lagenarium PKS1 (D83643); Nd, Nodulisporium sp. pks1 (AF151533); An, A. nidulans wA (X65866); and Af, A. fumigatus alb1 (AF025541); and Gl, G. lozoyensis pks1 (AF549411). Amino acid residues conserved among the six fungi are marked with asterisks.
decrease until 12 h in darkness. The transcriptional patterns of PKS1, SCD1, and THR1 were thus quite similar to each other, suggesting that common mechanisms regulate transcription of the three melanin biosynthesis genes, although the PKS1 showed a slightly more rapid increase compared to SCD1 and THR1. These results indicated that the expression of PKS1 gene involved in melanin biosynthesis is specifically regulated by NUV radiation. Melanin is a major and ubiquitous pigment that plays a major role in photoprotection from UV radiation, whose many effects on fungi include death, inhibition of spore germination, growth delay, and mutation [23–27]. In general, melanin is not essential for fungal growth but, as it accumulates in cell walls, can influence survival and longevity of fungal hyphae and spores [28,29]. Melanin has also been considered to confer tolerance to environmental stresses such as UV radiation [4–6]. The results presented here suggest that three melanin biosynthesis genes are transcriptionally regulated by NUV radiation. This up-regulated expression of three melanin biosynthesis genes could promote the production of melanin, resulting in enhancement of the UV tolerance of B. oryzae. Furthermore, our previous experiment revealed that a melanin-deficient mutant of B. oryzae was highly UV-sensitive and lost UV tolerance (data not shown). Thus, up-regulated gene expression of the PKS1 gene as well as that of other melanin biosynthesis genes would be an important mechanism for B. oryzae to protect itself against the harmful effects of UV radiation.

It was reported that each transcriptional activator CMR1 and PIG1 containing two distinct DNA-binding motifs, a Cys2His2 zinc finger motif and Zn(II)2Cys6 binuclear cluster motif, regulate transcription of melanin biosynthesis genes in the phytopathogenic fungi C. lagenarium and M. grisea, respectively [30]. Furthermore, a dsg gene encoding a putative Zn(II)2Cys6 binuclear cluster DNA-binding protein was clustered with a polyketide synthase gene (pks1) of the fungus G. lozoyensis [16]. We are now cloning the gene that
encodes the fungal transcriptional activator for melanin biosynthesis genes, and we will analyze the relationship between the three melanin biosynthesis genes and the transcriptional activator in B. oryzae. Moreover, analysis of the promoter regions of the three melanin biosynthesis genes is necessary to elucidate the mechanism of the transcriptional regulation of these genes by NUV radiation.

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References


