

Molecular Genetic Studies on Appressorial Melanization of *Colletotrichum lagenarium*

Yoshitaka Takano

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Anthrachnose are diseases of the foliage, stems or fruits that typically appear as small or large, dark colored-spots or slightly sunken lesions. Anthracnose is probably the most destructive disease on cucurbits, especially severe on watermelon, cantaloupe , and cucumber. Symptoms appear first on the leaves as small, water soaked, yellowish areas that enlarge from several mm to 1-2 cm and turn brown (Agrios 1988). The plant pathogenic fungus, *Colletotrichum lagenarium* causes anthracnose of cucumber. During the infection process, germ tubes from conidia differentiate into specific infection structure, appressoria prerequisite for penetration into its host plant (Fig. I-1). From appressoria, penetration pegs are formed which directly penetrates the epidermal cell walls of the host plant. In this infection process, appressoria are darkly pigmented by melanin. Melanins are dark, generally black, biological macromolecules composed of various types of phenolic or indolic monomers. In 1982, an albino mutant 79215 carrying a deficiency of melanin biosynthesis was isolated from *C. lagenarium* wild type (Kubo et al. 1982a). The albino mutant 79215 forms non-melanized orange colony (Fig. I-2), and its conidia form colorless non-melanized appressoria (Fig. I-3). The albino mutants are non-pathogenic on their host cucumber leaves (Fig. I-4). Studies with the albino mutants demonstrate that appressorial melanization of *C. lagenarium* is essential for penetration into the host plant (Kubo et al. 1982a; Kubo and Furusawa 1991).

Rice blast disease caused by *Magnaporthe grisea* (*Pyricularia oryzae*) has been controlled with compounds that act through mechanisms other than those relating to conventional fungicide activities. One of the most attractive groups of such compounds are inhibitors of melanin biosynthesis such as tricyclazole, pyroquilon, carpropamid (Woloshuk and Sisler 1982; Hattori et al. 1994; Kurahashi et al. 1997). These chemicals are highly effective in controlling rice blast

at concentrations well below those inhibit hyphal growth. The mechanism of action of these chemicals on pathogens is investigated in studies of infection by *M.grisea*. In *C. lagenarium*, the use of melanin-deficient mutants and melanin-inhibiting fungicides afford clear evaluation of the effect of these chemicals on melanin biosynthesis and penetration by appressoria (Kubo et al. 1982b). In *M.grisea*, melanin functions as semi-permeable membrane, mediating the osmotic generations of large turgor pressures which provide the driving force for mechanical penetration (Howard and Ferrari, 1989). Also, in *C. lagenarium*, it is envisaged that increase in structural rigidity of appressorial cell walls generated by melanin resists the high turgor pressures and directs the force vertically downwards through the penetration pore which is non-melanized region (Kubo and Furusawa 1986; Kubo and Furusawa 1991).

Melanin produced by fungi such as *C. lagenarium* and *M.grisea* is dihydroxynaphthalene (DHN) melanin, otherwise known as polyketide melanin (Bell and Wheeler 1986). The starting molecule of the DHN-melanin pathway, 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) is formed by the "head to tail " joining and cyclization of acetate molecules (Fig. I-5). This is also referred to as polyketide synthesis, and in the case of DHN melanin more specifically as pentaketide synthesis since the base naphthalene ring structure of the DHN melanin pathway presumably forms by the conjoining and cyclization of 5 ketide subunits obtained from 5 acetate molecules. Beginning with 1,3,6,8-THN, an alternating pair of reduction and dehydration reactions result in the formation of the immediate precursor, DHN (the monomer). In brief, 1,3,6,8-THN is reduced to scytalone, a dehydration reaction then forms 1,3,8-trihydroxynaphthalene (1,3,8-THN) from scytalone. A second reduction reaction forms **vermelone** from 1,3,8-THN. **Vermelone** is converted to DHN by a second dehydration reaction, and DHN is finally **polymerized** to DHN melanin. During cellular differentiation of *C. lagenarium* from conidial germination to penetration hyphae formation, melanization is confined to the appressoria; conidia, germ tubes, and penetration hyphae are never melanized, indicating that melanin

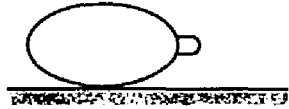
biosynthesis is developmentally regulated at appressorium differentiation process. However, mechanisms of appressorial melanization are unclear. A few genes involved in melanin biosynthesis have been identified, and almost nothing has been known about transcription of melanin biosynthesis genes and accumulation and localization of melanin biosynthesis enzymes at appressorium differentiation process.

The objectives of this study are to elucidate mechanisms of appressorial melanization of *C. lagenarium* by molecular genetic approaches. This thesis is composed of four chapters. In Chapter II, structural analysis of melanin biosynthesis gene *PKS1* of *C. lagenarium* was performed. The *PKS1* gene complemented the melanin biosynthesis and penetrating ability of the melanin-deficient albino mutant 79215. The nucleotide sequence of the *PKS1* gene demonstrated that the *PKS1* gene encodes type-I polyketide synthase involved in pentaketide synthesis of melanin biosynthesis. There are differences of localization and roles of melanin between melanin-producing fungi. The plant pathogenic fungus *Alternaria alternata* produce melanized conidia and colorless appressoria whereas *C. lagenarium* produce colorless conidia and melanized appressoria, and *A. alternata* albino mutant (*alm*-) retained pathogenicity whereas *C. lagenarium* albino mutant (*pks1*-) lost penetrating ability to the host plant. In Chapter III, I performed functional comparison of polyketide synthase genes involved in melanin biosynthesis between these two melanin-producing fungi by the complementation test of *C. lagenarium* albino mutant (*pks1*-) with the polyketide synthase gene *ALM* of *A. alternata*. In Chapter IV, a transcriptional pattern of the melanin biosynthesis genes of *C. lagenarium* was studied. In addition to characterization of the *PKS1* gene, two melanin biosynthesis genes, *SCD1* gene encoding scytalone dehydratase and *THR1* gene encoding 1,3,8-trihydroxynaphthalene reductase were isolated and characterized. The temporal transcriptional patterns of the three major melanin biosynthesis genes *PKS1*, *SCD1*, and *THR1* during appressorium differentiation were investigated. Also, the temporal transcriptional patterns of the three genes during non-appressori-

um differentiation of conidia were investigated. In Chapter V, accumulation and localization of scytalone dehydratase (SCD1) protein during appressorium differentiation of conidia were investigated by using SCD1 fusion protein with green fluorescent protein (GFP).



Attachment of conidia



Conidial germination



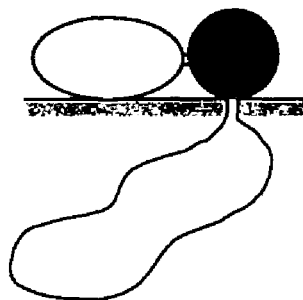
Appressorium formation



Appressorial melanization



Infection peg formation



Infectious hyphae formation

Fig. I-1. The plant infection process of *Colletotrichum lagenarium*. Conidia attach to the host leaf surface and produce germ tubes that differentiate into appressoria. After melanization of appressoria, appressoria produce the infection peg that penetrates the plant surface and produces infectious hyphae.

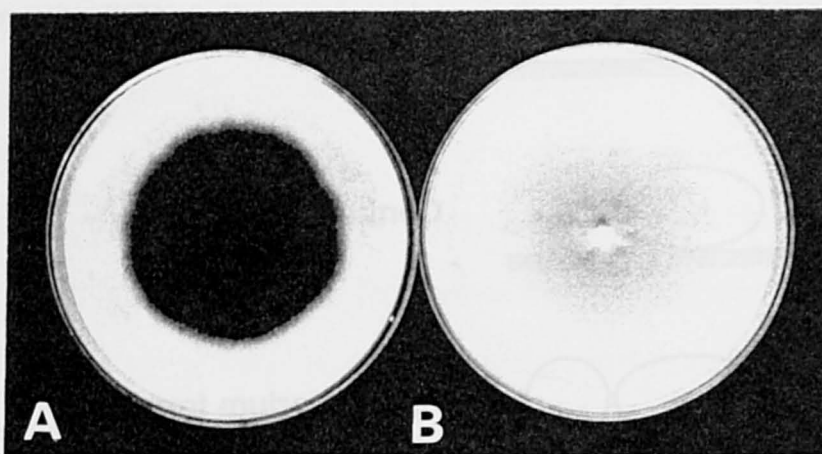


Fig. I-2. Colonies of a wild type 104-T (A) and an albino mutant 79215 (B) of *C. lagenarium* on PDA medium.

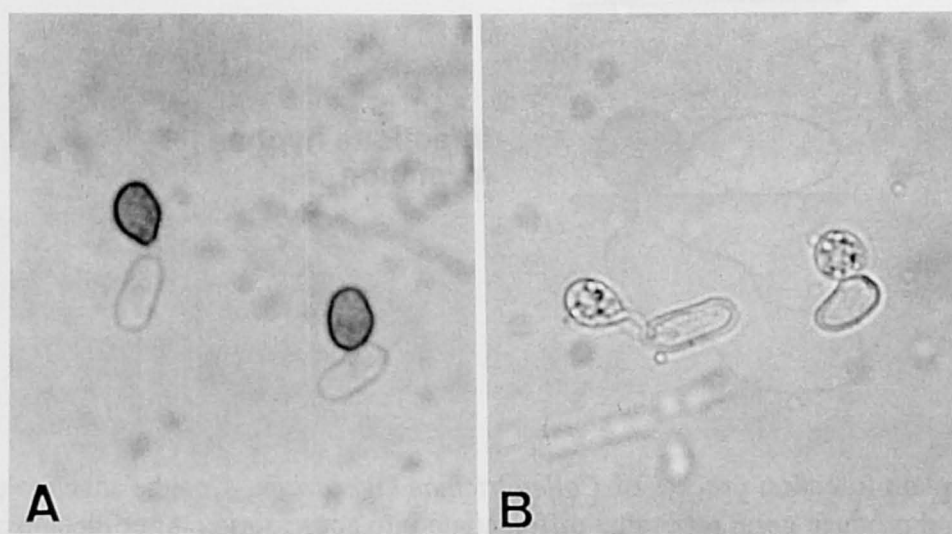


Fig. I-3. Appressoria of the wild type 104-T and the albino mutant 79215 of *C. lagenarium*. Conidia were incubated at 24°C on petri dishes: (A) melanized appressoria of the wild type 104-T; (B) colorless appressoria of the albino mutant 79215.



Fig. I-4. Pathogenicity of the albino mutant 79215 of *Colletotrichum lagenarium*. Drops of conidial suspension of the wild type 104-T (left) and mutant 79215 (right) were spotted on the cucumber leaves and incubated for 5 days at 24°C.

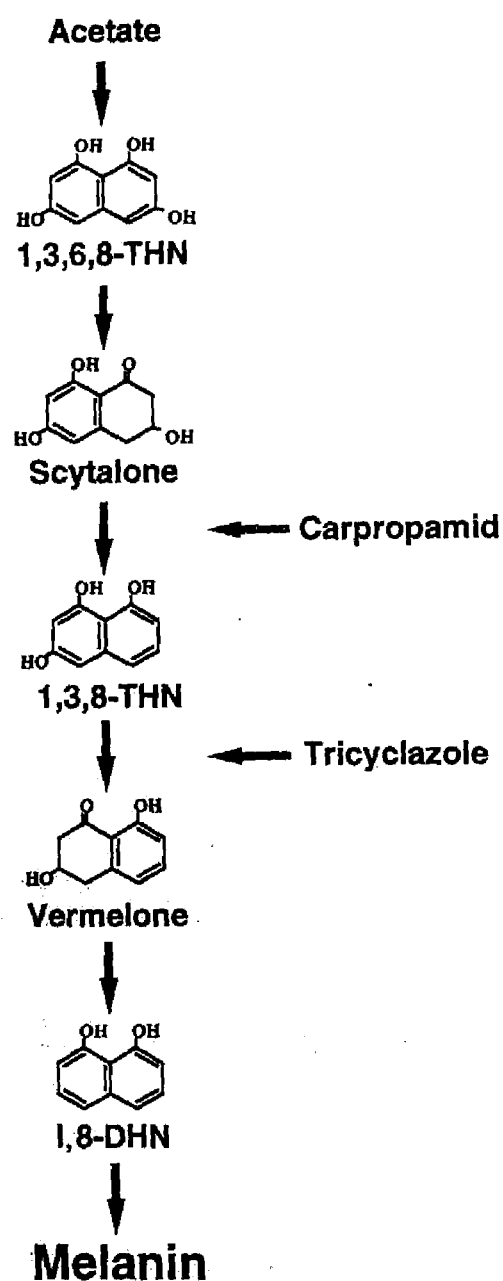


Fig. I-5. Biosynthetic pathway of melanin in *C. lagenarium* with inhibition sites by chemicals. Abbreviations: 1,3,6,8-THN, 1,3,6,8-tetrahydroxynaphthalene; 1,3,8-THN, 1,3,8-trihydroxynaphthalene; 1,8-DHN, 1,8-dihydroxynaphthalene.

Chapter II. Structural Analysis of *PKS1*, a Polyketide Synthase Gene Involved in Melanin Biosynthesis.

Introduction

The biosynthetic pathway of melanin in *C. lagenarium* starts from pentaketide synthesis and proceeds to form scytalone. Following steps consist of two dehydration and one reduction steps, i.e., dehydration of scytalone to 1,3,8-trihydroxynaphthalene (1,3,8-THN), reduction of 1,3,8-THN to vermelone and dehydration of vermelone to DHN. DHN is then polymerized and oxidized to yield melanin. The biochemical analysis of the two enzymes, dehydratase and reductase involved melanin biosynthetic pathway has been reported in *Verticillium dahliae*, *Cochliobolus miyabeanus* and *Magnaporthe grisea* (Wheeler et al. 1982; Tajima et al. 1989; Vidal-Cros et al 1994).

Conventional genetic analysis has been made with melanin-deficient mutants in several plant pathogenic fungi, such as *Cochliobolus miyabeanus*, *Cochliobolus heterostrophus* and *Magnaporthe grisea* (Kubo et al. 1989; Tanaka et al. 1990; Chumley and Valent 1990). There were three reports in cloning and structural analysis of genes coding for melanin biosynthesis enzymes before the study in this chapter.

In *C. lagenarium*, several melanin-deficient mutants have been isolated. The albino mutant 79215 (*pks1*-) of *C. lagenarium* forms colorless appressoria and has little penetrating ability to its host plant. The defective site of the albino mutant was considered to be at or before pentaketide cyclization, the earliest step of melanin biosynthesis (Kubo and Furusawa 1991). A melanin biosynthesis gene was first cloned from cosmid library of *C. lagenarium* by complementation of the albino mutant (*pks1*-) and partially characterized (Kubo et al. 1991). The

cloned melanin biosynthesis gene of *C. lagenarium* was assumed to be involved in pentaketide synthesis and/or pentaketide cyclization. Besides the cloning of the melanin biosynthesis gene of *C. lagenarium*, the isolation of the gene cluster involved in melanin biosynthesis of plant pathogenic fungus *Alternaria alternata* (Kimura and Tsuge 1993) and cloning and sequencing of T⁴HN reductase gene of *M. grisea* (Vidal-Cros et al. 1994) have been reported.

In this chapter, I made structural analysis of the melanin biosynthesis gene of *C. lagenarium*, designated as the *PKS1* gene, by sequencing the *PKS1* gene and its flanking regions and by determining its transcription architecture.

Materials and Methods

Fungal strains

Colletotrichum lagenarium (Pass.) Ellis and Halsted strain 104-T (culture stock of Laboratory of Plant Pathology, Kyoto University) was used as a parent stock. Albino mutant strain 79215 (*pks1*-) was isolated by ultraviolet light irradiation of conidia (Kubo et al. 1982a). These strains were routinely cultured in potato sucrose agar (PSA) medium at 24°C.

Plasmids

A cosmid pAC7 transforms the albino mutant 79215 (*pks1*-) to melanin restored phenotype. pAC71 was constructed by inserting a 8.0 kb *Bam*HI fragment of pAC7 into the *Bam*HI site of pBluescript (SK-) (Kubo et al. 1991). The 8.0 kb *Bam*HI fragment of pAC71 was subcloned into the *Bam*HI site of pSH75 containing the *hph* (hygromycin B phosphotransferase) gene as a selective marker for fungal transformation (Kimura and Tsuge 1993), which was kindly

provided by Dr. T. Tsuge. This plasmid was designated as pAH71 and used for complementation of albino mutant 79215 (*pks1*-) (Fig. II-1).

Fungal transformation

To obtain protoplasts, conidia were incubated for 3 days in 250 ml of potato sucrose broth supplemented with yeast extract (PSY : 200 ml extract from 200 g potato, 20 g sucrose, 2 g yeast extract per 1000 ml deionized water). Mycelia were harvested by filtration with a sterile cheezegauze, treated with 20 ml of enzyme solution containing 5 mg/ml of lysing enzyme from *Trichoderma harzianum* (Sigma) in 1.2 M MgSO_4 and 10 mM of Na_2HPO_4 to release the protoplasts (Rodriguez and Yoder 1987). Transformation experiments were made by the procedure of Vollmer and Yanofsky (1986) and Kubo et al. (1991).

Genomic DNA blot hybridization

The total DNAs of *C. lagenarium* were isolated from their mycelia by the following procedure. Approximately 5 g of mycelia was added to a mortar and crushed under liquid nitrogen using a pestle. The resulting homogenate powder was suspended in 5 ml of lysis buffer (150 mM EDTA, 50 mM Tris-HCl pH 7.4, 1% (w/v) sodium N-lauroylsarcosine, 500 $\mu\text{g/ml}$ pronase E) and incubated at 65°C for 15 min. The cell homogenate was centrifugated at 2,000 g and the supernatant was then extracted with equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) (P/C). Then the aqueous phase was extracted once with P/C and twice with chloroform/isoamyl alcohol (24:1). The DNA was precipitated by addition of 2 volumes of ethanol and pelleted by centrifugation. The pellet was resuspended in 2 ml of TE containing 100 $\mu\text{g/ml}$ RNase A and incubated at 37°C for 1 h. The DNA solution was extracted once with P/C, once with chloroform/isoamyl alcohol, and the DNA was precipitated with ethanol. Genomic DNA was digested with *Xba*I, subjected to agarose gel electrophoresis and transferred onto

Hybond N+ nylon membrane (Amersham) as described by Southern (1975). The DNA probe was labelled with [α - 32 P] dCTP using random oligonucleotide primers as described by Sambrook et al. (1989). Hybridization was carried out using hybridization buffer (50% (w/v) formamide, 5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl, 0.015M sodium citrate), 0.1% (w/v) SDS, 5% (w/v) sodium dextran sulfate) at 42°C for 16 h. The membrane was washed three times with 1 $\times$ SSC at 42°C for 20 min.

Sequencing of PKS1

The DNA sequence of the *PKS1* gene was determined by the dideoxy chain termination method (Sanger et al. 1977) with the *BcaBEST*TM DNA polymerase kit (Takara Shuzo, Kyoto) or cycle sequencing kit (Applied Biosystem) according to the manufacturers' instructions. The restriction DNA fragments of the *PKS1* gene were subcloned into the pBluescript (SK-). Nested deletions of these clones were made with ExoIII and mung bean nucleases as described previously (Henikoff 1984). Primers were M13 universal primers (Takara Shuzo, Kyoto) and 12 synthetic oligonucleotide primers derived from the established sequences.

RNA isolation

Mycelia of *C. lagenarium* were inoculated into 250 ml PSY and grown at 24°C in a reciprocal shaker for 3 days. At this time, no visual melanization of mycelia was recognized. For rapid induction of melanin biosynthesis, the grown mycelia were filtered with a sterile cheesegauze and transferred to 1.2 M sucrose solution and incubated at 24°C in a reciprocal shaker (110 rpm) for additional 16 h. Total RNA was isolated by modification of the method by Kroner and Ahlquist (1992). To 1 cm³ of collected mycelial mat, 800 μ l of extraction buffer (0.5 M glycine, 0.5 M NaCl, 5 mM EDTA, pH 9.5), 80 μ l of 10% SDS, 16 μ l of 6% bentonite solution and 2.8 ml

phenol/chloroform/isoamyl alcohol (24:24:1) were added. Mycelia were crushed and homogenized in liquid nitrogen, then the aqueous phase was separated from phenol/chloroform/isoamyl alcohol by centrifugation at 1,000 g for 5 min. The aqueous phase was extracted with phenol/chloroform/isoamyl alcohol three times. Total RNA was precipitated by addition of 2 volumes of ethanol. Poly(A)+ RNA was isolated using an oligo(dT) cellulose column (Collaborative Research) according to the manufacturer's instructions.

Primer extension analysis

Poly(A)+ RNA (7 µg) from melanin induced-hyphae was used to map the 5' end of the *PKS1* transcript. The oligonucleotide 5'-TCTTCGCAAGGATGCTCG-3' is complementary to the *PKS1* mRNA for 18 nucleotides starting 86 nucleotides downstream of the predicted ATG initiation codon. A 20 µl reaction mixtures of 4.0 pmol of oligonucleotide primer, 10 µCi [α -³²P] dCTP (3000Ci/mmol), 5 mM dATP, dGTP, dTTP, 0.5mM dCTP and 4µl Buffer I from cDNA synthesis kit (Boehringer Mannheim), 25 units RNasin (Promega) and 20 units AMV reverse transcriptase (Boehringer Mannheim) were prepared and were incubated for 1 h at 42°C. The products were analyzed by electrophoresis on a 6% urea polyacrylamide sequencing gel. Ladder sequence was used to determine the reverse transcribed products.

RT-PCR and cDNA Cloning

RT-PCR was performed essentially as described by Kawasaki (1990). First strand cDNAs were synthesized with poly(A)+ RNA prepared from melanin induced hyphae using AMV reverse transcriptase and either adapter linked oligo(dT), 5'-CGACGGCCAGTGCCAAGCTTTTTTTTTT-3', as the primer for inference of polyadenylation site or 5'- ACTGGATGACGACACAGC-3' as the primer for mapping of introns. PCR amplification for inference of polyadenylation site

was performed using 5'-CGACGGCCAGTGCCAAGC-3' and 5'-GGATTACATAGCGAGG-TTG3'. PCR amplification for mapping of introns was performed using 5'-CTGGATGACGACACAGC-3' and 5'-GCTCAACGAACGATACCA-3'. Products were fractionated electrophoretically in 1 % agarose gels in TAE buffer. cDNAs amplified by RT-PCR were cloned into pCRTMII by using TA cloning kit (Invitrogen) according to the manufacturer's instructions.

RNA blot hybridization

Total RNA samples were denatured in formamide/MOPS/EDTA buffer at 65°C for 10 min. Gel electrophoresis was made on 1.5% agarose gels containing 5% formaldehyde and 1% MOPS. Gel was blotted onto Hybond N+ nylon membrane and hybridization (buffer: 5 x SSPE, 50% formamide, 0.1% SDS, 5% dextran sulfate) was carried out using the in vitro T7 RNA polymerase transcript of the cloned *PKS1* gene as a probe. The membrane was washed four times with 0.1% SDS / 0.1 x SSC at 60°C for 20 min.

Results

Genomic DNA blot analysis of melanin restored transformants by pAH71

The 8.0 kb *Bam*HI fragment of pAC7 was considered to contain a gene involved in restoration of melanization to the albino mutant 79215 (Kubo et al. 1991). Since this gene was thought to be involved in pentaketide synthesis and/or pentaketide cyclization in melanin biosynthetic pathway, I designated this gene as *PKS1*. This fragment was subcloned into pSH75 containing hygromycin resistance gene *hph* and designated as pAH71 (Fig.II-1). pAH71 transformed the albino mutant (*pks1*-) effectively to a melanin restored wild phenotype; 40-50 % of hygromycin

resistant transformants were dark brown melanin restorants. These melanin restored (*PKS1*+) transformants formed darkly melanized appressoria indistinguishable from those formed by the wild type strain. To examine whether complementation of the albino mutant (*pks1*-) by pAH71 was the result of homologous integration or nonhomologous integration, genomic DNA blot analysis was made. Genomic DNAs of the wild type 104-T and eight *PKS1*+ transformants were isolated and digested with *Xba*I. The 8.0 kb *Bam*HI fragment from pAC71 was used as a probe. DNA blot analysis revealed that all *PKS1*+ transformants DNA showed band pattern indicating homologous integration of pAH71 (Fig. II-2A). In all *PKS1*+ transformants, the original 19.0 kb fragment of the wild type was disrupted to 17.0 and 10.0 kb fragments, which were expected to be generated by the homologous integration of pAH71 (Fig. II-2B). In one transformant (lane 6), nonhomologous integration was observed besides the homologous integration.

Expression of the PKS1 gene

To analyze the expression of the *PKS1* gene during melanization, I established the condition where melanin biosynthesis was induced. For induction of melanin biosynthesis, the grown mycelia in PSY were transferred to a high osmotic solution including 1.2 M sucrose. A little mycelial melanization occurred at 8 h after the induction of melanin biosynthesis and melanization increased gradually until 16 h (data not shown). Total RNAs of mycelia at 0, 4, 8, 12 and 16 h of melanin biosynthesis induction were isolated. The total rRNA was used as a loading control. RNA blot analysis using the *PKS1* gene as a hybridization probe showed that a 7.3 kb *PKS1* transcript was detected at 8, 12, 16h after induction of melanin biosynthesis (Fig. II-3). The amount of the *PKS1* transcript increased in parallel with the degree of melanization.

DNA sequencing and identification of the PKS1 gene

The 8.0 kb *Bam*HI fragment of pAC7 containing the *PKS1* gene and its flanking regions were sequenced on both strands. The sequence strategy for bidirectional sequencing of this region is shown in Fig. II-4. The transcription initiation site of the *PKS1* gene was determined by primer extension analysis of poly(A)+ RNA from the melanin induced-hyphae (Fig. II-5). The major transcription initiation site was preceded by a potential TATA box at position -50 and -45. The two closest ATG initiation codons were 37 and 56 nucleotides downstream of the major transcription initiation site. The latter, which was followed by a long open reading frame, was chosen as the most likely *PKS1* translation initiation codon. The context of the presumed *PKS1* translation initiation sequence (CTGACAAGATGGCG, the initiation codon is underlined) was consistent with the *Neurospora crassa* consensus Kozak sequence [CANNCA(A/C)~~T~~ATGGC] (Bruchez et al. 1993a). Two tentative intervening sequences were predicted on the basis of 5' splicing signals [GT(AGT)(ACT)G(T/C)], the 3' splicing signals [(C/T)AG]) and the internal splicing signals [(G/A)CT(A/G)AC] referred to consensus sequence in *N. crassa* and common sequence in *Colletotrichum graminicola* β -tubulin genes (Bruchez et al. 1993b; Panaccinone and Hanau 1990). The introns and polyadenylation site of the *PKS1* gene were determined from appropriate cDNA sequences. The cDNA clones were generated by reverse transcription-polymerase chain reaction (RT-PCR). PCR primer pairs were used to amplify small regions for analysis using poly(A)+ RNA as a template. The two introns were confirmed by comparing the nucleotide sequence obtained from one cDNA clone with that of genomic DNA clones. The polyadenylation site was deduced from two independent cDNA clones and preceded by two potential polyadenylation signals (AATA). The nucleotide sequence and the predicted amino acid sequence of the *PKS1* gene are presented in Fig. II-6. The *PKS1* sequence contains one open reading frame which codes for 2187 amino acids.

Comparison of PKS1 to polyketide synthases

The *PKS1* polypeptide is assumed to be involved in pentaketide synthesis and/or pentaketide cyclization. The eukaryotic polyketide synthases (PKSs) are generally thought to be large multifunctional proteins (type I PKSs) (Hopwood and Sherman 1990). Since the *PKS1* gene is considered to code for a polyketide synthase, I compared the *PKS1* sequence with sequences of type I PKSs. Dot plot comparisons of the predicted amino acid sequence of *PKS1* with that of two type I PKSs which showed significant homology are shown in Fig. II-7. *Penicillium patulum* MSAS gene encodes a PKS involved in the synthesis of 6-methylsalicylic acid (Beck et al. 1990). *Aspergillus nidulans* *wA* gene coding for a PKS involved in conidial pigmentation (Mayorga and Timberlake 1992), especially exhibits a most striking homology. The *PKS1* polypeptide conserved the β -ketoacyl synthase, acetyl/malonyl transferase, and two acyl carrier protein (ACP) domains. The *PKS1* polypeptide showed complete conservation of the active site cysteine residue of the β -ketoacyl synthase, the active site serine residue of the acetyl/malonyl transferase and the pantetheine-binding serine residue of the ACP (Fig. II-8).

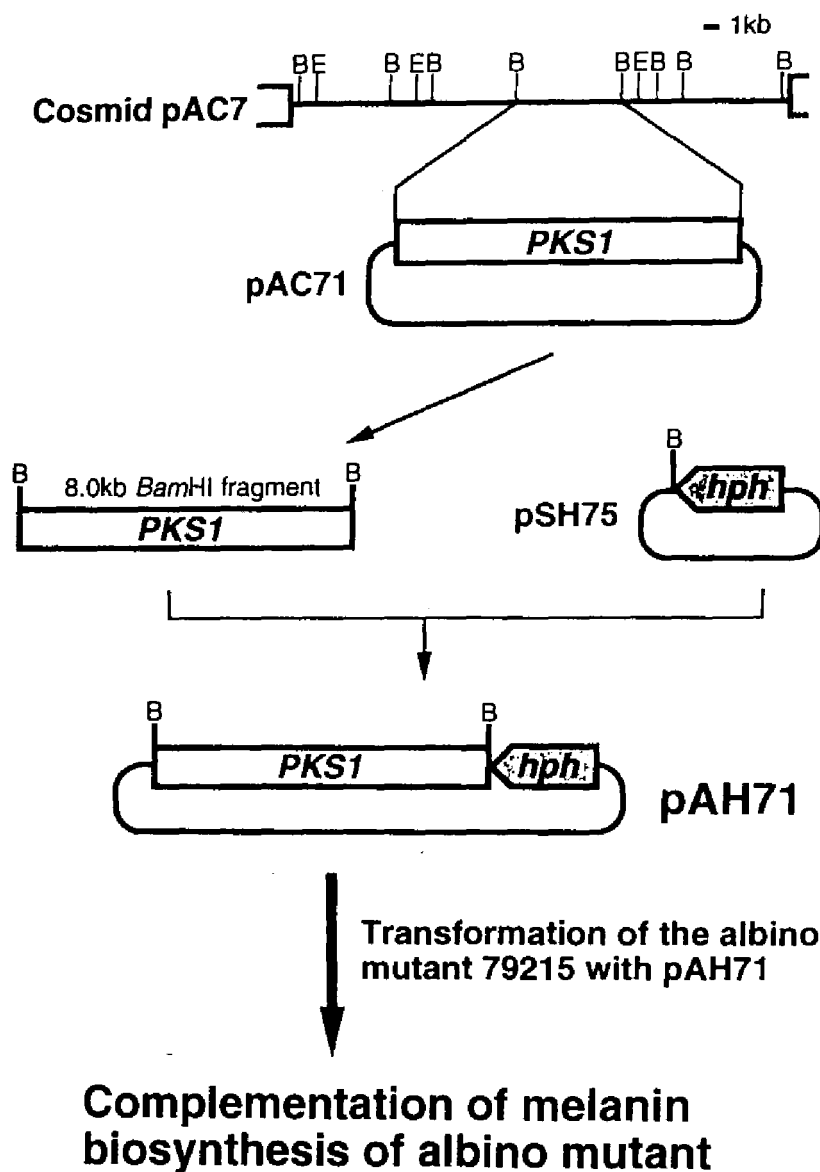


Fig. II-1. Construction of plasmid pAH71 used for transformation of the albino mutant 79215. The 8.0 kb *Bam*HI fragment of cosmid pAC7 was subcloned into *Bam*HI site of pBlueScript, designated pAH71. The 8.0 kb *Bam*HI fragment of pAH71 was reintroduced into *Bam*HI site of pSH75 containing hygromycin resistance gene *hph*.

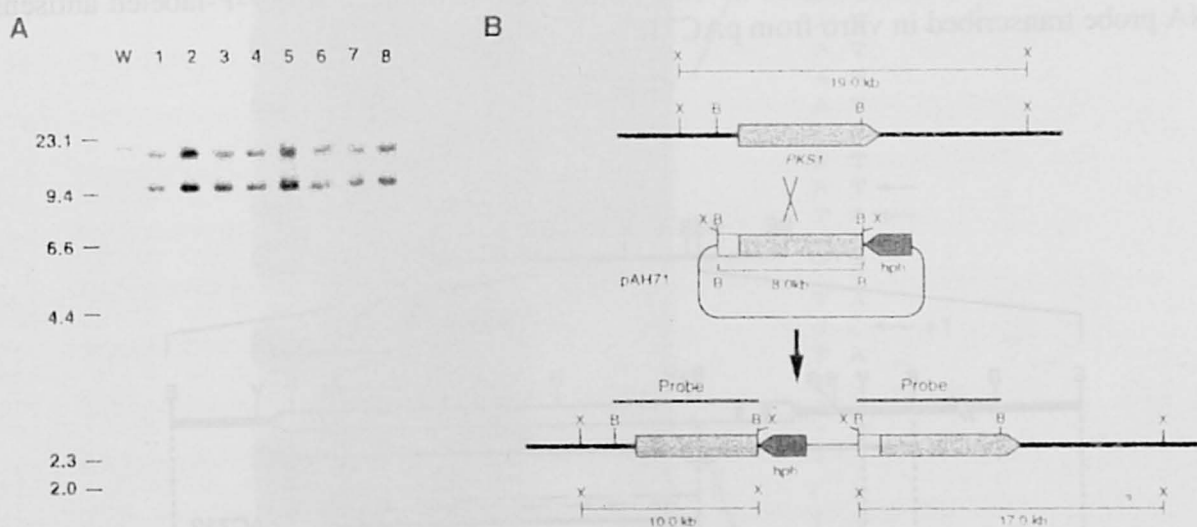


Fig. II-2. Genomic DNA blot analysis of wild type strain and melanin-restored transformants of the albino mutant 79215 (*pks1*-) by pAH71

(A) Genomic DNA blot of *C. lagenarium* wild type, melanin-restored transformants with pAH71 using the ^{32}P -labeled 8.0 kb *Bam*HI fragment from pAH71 as a probe. Genomic DNAs were digested with *Xba*I. 19.0 kb *Xba*I fragment observed in the wild type was disrupted to 17.0 and 10.0 kb *Xba*I fragments in all melanin-restored transformants (*PKS1*+). Lanes: W, wild type strain 104-T; 1-8, melanin-restored transformants.

(B) A scheme representing the complementation of the albino mutant by homologous integration with pAH71. Abbreviations: B, *Bam*HI; X, *Xba*I.

0 4 8 12 16 (hr)

7.3kb

Fig. II-3. RNA blot analysis of *PKS1* transcript during induction of mycelial melanization in *C. lagenarium*. Total RNA was isolated from grown mycelia incubated under induction of melanin biosynthesis for 0, 4, 8, 12, and 16 h. RNA blots were hybridized with 32 P-labeled antisense RNA probe transcribed in vitro from pAC712.

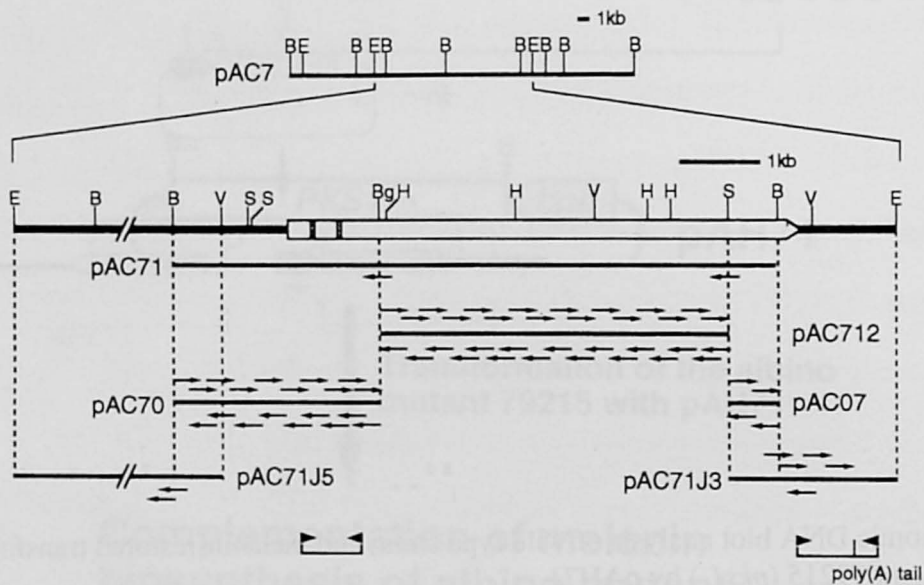


Fig. II-4. Sequencing strategy of the *C. lagenarium* *PKS1* gene. The restriction fragments including the *PKS1* gene of pAC7 were cloned into pBluescript. Unidirectional deletions of pAC712 and pAC70 were constructed with ExoIII and mung bean nucleases. The arrows indicate the extent and direction of sequencing with the use of either M13 universal primers or synthetic oligonucleotide primers. The open arrow indicates the open reading frame. Shaded boxes in the open arrow indicate introns. Arrowheads indicate RT-PCR primers for identification of introns and polyadenylation sites. RT-PCR primers were used to amplify poly(A)⁺ RNA. The PCR fragments were cloned and sequenced. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; S, *Sma*I; V, *Eco*RV.

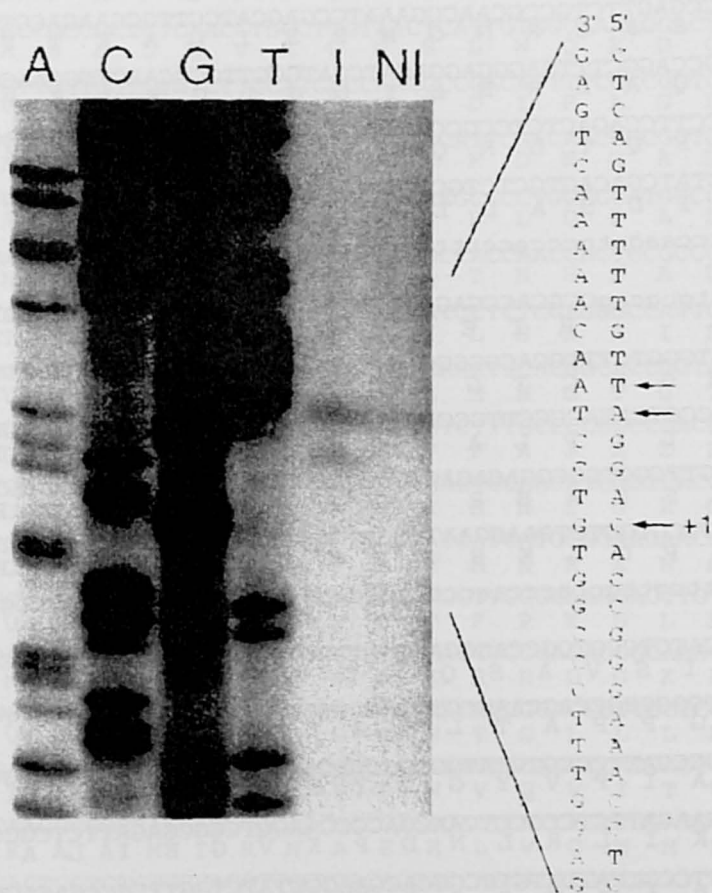


Fig. II-5. Primer extension mapping of melanin induced-hyphal poly (A)+ RNA. NI: poly(A)+ RNA from the non-induced hyphae; I: poly(A)+ RNA from the melanin induced-hyphae. The sequence of 5' region of the *PKS1* gene is given as a sequence ladder. The transcription initiation sites are indicated by the arrows. The major transcription initiation site is marked +1.

-304 GGAAAGGAAGGGGTGCAAGGGGGGCGTGGGATACATGGGACGTCTTCACCACACCCCCT -245
 -244 TCTCCTTCTCTCGTTCTCACCTCACTCCCTCGACCCAGACCACTTGGGTTGTGTGTTCCA -185
 -184 TGTGCAATTCCGCTATCCATCAGCCATGACTTTGACAGATGGGTTTGATTATTTTGGGG -125
 -124 AGACGCCAGCTCGTGGTGGTGTGACCTTGTCCATGGTGTGTACACCCCGTCTGTCCCTTT -65
 -64 GGCCCATCTCTTTCATATAAGTTCCAGACTCGCTCCCAGTATCCCAATCTCAGTTTTTGT -5
 -4 TAGGACACCGGGAAACCTCTCAAACACCTAGTAGGAAATTCATGTCGACAACCTGACAAG 55
 56 ATGGCCGACACGATGTCTTATCTCTCTTCGGAGATCAGAGCCTTGATACCCATGGATTT 115
 1 M A D T M S Y L L F G D Q S L D T H G F 20
 116 CTTGCCGAGTTCTGCCGCAACGGAAATCCGAGCATCCTTGCGAAGACGTTCTCGAGCAG 175
 21 L A E F C R N G N P S I L A K T F L E Q 40
 176 GCTGGCCAGGCTTTGAGGGAGGAGATCGATGGGTTGGGCAAGTTGGAACGGTCGAAACTG 235
 41 A G Q A L R E E I D G L G K L E R S K L 60
 236 CCCACCTTCCAGACTCTCCGCCAGCTCAACGAACGATACCACGCCAGAGCATCAAGCAC 295
 61 P T F Q T L R Q L N E R Y H A Q S I K H 80
 296 CCAGGTATCGACAGTGTCTGTCTTTGCACCACCCAGCTGGCTCACTACATCGAgtacgtc 355
 81 P G I D S A L L C T T Q L A H Y I D 98
 356 gccatccaagatgccacaccttagagggaatgccccacgaagcgttgaaccgctcacta 415
 416 acacttgcgagCCGCACCGAGAAGGAGCCTCAAGATGCTTGCCTTCACGACCACACTTT 475
 99 R T E K E P Q D A C L H D H T F 114
 476 CTTTCATGGGTCTTTGCACGGGGCTGTTCCGCCCGCTGCCATCGCCTCGACCCCTCAGT 535
 115 F M G L C T G L F A A A A I A S T P S V 134
 536 CTCTACCTCATACCCCTTGCCGTTCAAGTAGTCTCATGGCCTTCAGGACTGGCTCTCA 595
 135 S T L I P L A V Q V V L M A F R T G S H 154
 596 CGTCGGTTCGCTGGCGGAGAGACTCAGCCCGCCGGTCGGCCAATCTGAACCCTGGACCCA 655
 155 V G S L A E R L S P P V G Q S E P W T H 174
 656 TATCCTTCTGGTCTGAAGGAATCCGATGCCAAAGAGGCACTCGCTAATTTCCATGAATC 715
 175 I L P G L K E S D A K E A L A N F H E S 194
 716 TAATgttcgttgctacccaacctgcagctgtccagttatcgcaatggctgaccacgtcta 775
 195 N
 776 cagTACATCTCCGTCGCCAGCCAAGCATACGTTAGCGCTGTGTCTCATCCAGTCTGGCA 835
 196 Y I S V A S Q A Y V S A V S S S S L A 214
 836 ATCTCTGGGCGGCCAGCAACCCTGAAGGCTCTCGACGACCAGAACGTCTTTGGCGTCAAG 895
 215 I S G P P A T L K A L D D Q N V F G V K 234
 896 TCGACGGCGATTCCCGTCTATGGTCCCTACCATGCTGCCCATCTTCACGGCACCGCCGAT 955
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1616 ACTACGGCTTACGAGGCTCTGGAGATGTGCGGTACGTGCCCAACCGTACTCCCTCCACG 1675
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1796 CGTATCAACTACCACCTTTGGTTTCAGCGGTCCAGTCTCAACGTCGACACTGCCTGTTCC 1855
535 R I N Y H F G F S G P S L N V D T A C S 554
1856 TCCAGTGCCGCCGCTCTCAACGTGGCTTGTAACTCATTGTGGCAAAAGGACTGTGACACC 1915
555 S S A A A L N V A C N S L W Q K D C D T 574
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975 G L C C F E M A L A R L W A S W G I R P 994
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 1995 K A R Q S K G P Y S L A G W S A G G V I 2014
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7316 ATACCCCCTGTCCCGGCGCCCC

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Fig. II-6. Nucleotide and deduced amino acid sequences of the *C. lagenarium* *PKS1* gene. One major and two **minor** transcription sites are indicated by bent arrows. Major transcription initiation site is indicated as +1. Intron sequences are indicated as small letters. A potential TATA box and two putative polyadenylation signals are underlined. The polyadenylation site deduced from two independent cDNA clones is indicated with asterisk.

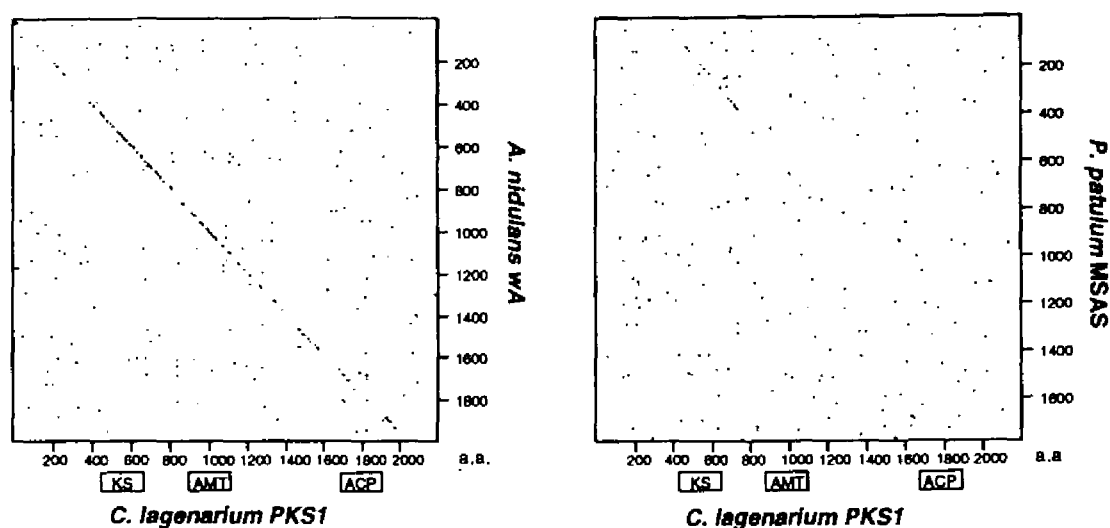


Fig. II-7. Dot matrix comparison of *PKS1* with polyketide synthases using a window of 5 and a stringency of 4. The *PKS1* polypeptide was compared with the *A. nidulans* wA polypeptide (Mayorga and Timberlake 1992) and the *P. patulum* MSAS polypeptide (Beck et al. 1990). Abbreviations: KS, β -ketoacyl synthase; AMT, acetyl/malonyl transferase; ACP, acyl carrier protein

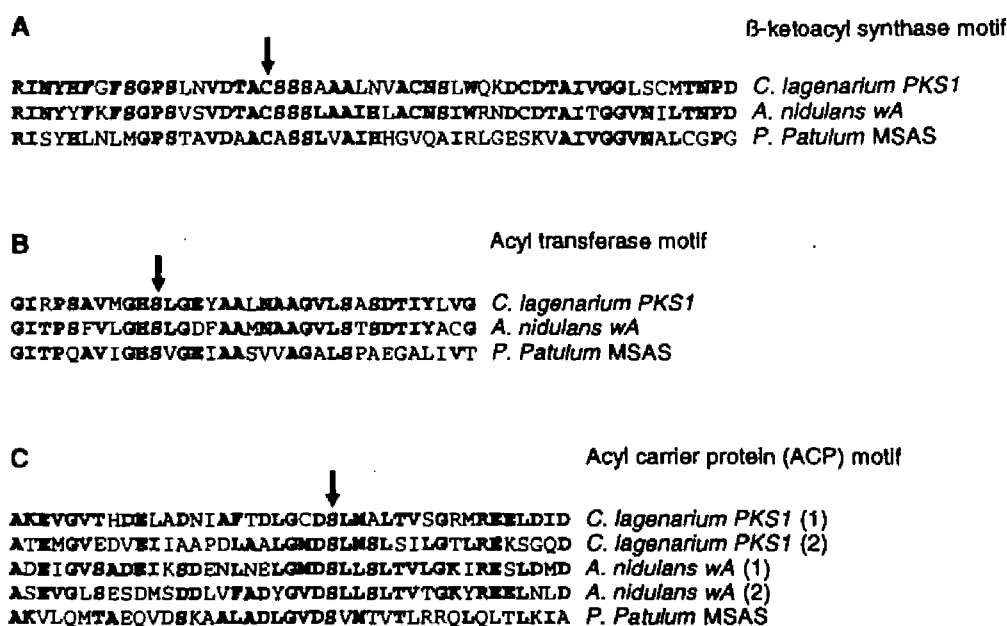


Fig. II-8A-C. Alignment of the *PKS1* amino acid sequence with active site regions of polyketide synthases.

A β -ketoacyl synthase motif. B Acyl transferase motif. C Acyl carrier protein motif (Beck et al. 1990; Mayorga and Timberlake 1992). Arrows indicate the active site cysteine residue (A), the active site serine residue (B) and the pantetheine-binding serine residue (C). Amino acids conserved between at least two of the aligned sequences are indicated in bold faces.

Discussion

In general, melanin plays a role in the survival and longevity of fungal propagules (Bell and Wheeler 1986). Particularly in *Colletotrichum* and *Magnaporthe* (*Pyricularia*) species, melanin biosynthesis during appressorial formation has been shown to be directly involved in penetration of host plants. Cell wall rigidity provided by melanin is considered to be needed for focusing the turgor forces in the vertical direction to facilitate penetration (Kubo and Furusawa 1991). The importance of appressorial melanization in penetration was first noted in *C. lagenarium* albino mutant (*pks1*-) (Kubo et al. 1982). The *PKS1* gene was cloned by complementation of an albino mutant (*pks1*-) to the wild phenotype (Kubo et al. 1991). In this chapter, I showed complementation of the albino mutant (*pks1*-) by pAH71 was due to the homologous integration of the 8.0 kb *Bam*HI fragment of pAH71, suggesting this fragment did not contain the *PKS1* gene completely and could not be functional itself. I sequenced the 8.0kb *Bam*HI fragment and its flanking regions. The predicted *PKS1* polypeptide of *C. lagenarium* shared highly significant homology with type I PKSs. This result strongly suggests that the *PKS1* gene encodes for a polyketide synthase involved in pentaketide synthesis of melanin biosynthesis of *C. lagenarium*. The *PKS1* polyketide synthase is considered to be a type I PKS containing β -ketoacyl synthase, acetyl/malonyl transferase and two ACP domains. A region similar to typical keto reductase domains was not found in the predicted *PKS1* polypeptide. While homology of *C. lagenarium* *PKS1* to *P. patulum* MSAS is restricted to active domains of PKSs, *A. nidulans* *wA* showed high homology with *PKS1* throughout the entire amino acid sequence (Fig. II-7, 8). Although the polyketide products of the *A. nidulans* *wA* gene have not been identified, high homology of the *wA* gene to the *PKS1* gene suggests the possibility that the polyketide products of the *wA* gene may be similar to pentaketides, the products of the *PKS1* gene. In *C. lagenarium*,

cyclization steps would be required to form scytalone via pentaketide synthesis. The *PKS1* polypeptide, therefore, might contain active domains of cyclase. However, these domains have not been identified in *PKS1*. Some other enzymes may be involved in cyclization steps via pentaketide synthesis to form scytalone.

Chapter III. Restoration of Appressorial Melanization and Penetration of Cellulose Membranes in the Melanin-Deficient Albino Mutant with the *Alternaria Alternata* Melanin Biosynthesis Gene.

Introduction

Among melanin-producing fungi, there is diversity of fungal cell types where melanization is observed during the penetration process. Fungi such as *Alternaria* and *Cochliobolus* species produce melanized conidia and colorless tiny appressoria (Fig. III-1). On the other hand, fungi such as *Colletotrichum* and *Magnaporthe* species produce non-melanized conidia and well-developed appressoria pigmented with melanin (Emmet and Parberry 1975). In general, melanin is important for survival and longevity of fungal propagules (Bell and Wheeler 1986). Conidial and mycelial melanization of *A. alternata* is shown to be not directly related to fungal pathogenicity (Tanabe et al. 1995). However, appressorial melanization of *C. lagenarium* (Kubo et al. 1982a; Kubo and Furusawa 1991), *C. lindemuthianum* (Wolkow et al. 1983), and *M. grisea* (Woloshuk et al., 1983; Yamaguchi and Kubo 1992), is essential for penetration of their host plants. These suggest that there is great diversity among fungi in roles of melanin.

In several fungi, cloning and structural analysis of melanin biosynthesis genes have been reported. In *C. lagenarium*, three melanin biosynthesis genes, *PKS1* (Kubo et al. 1991; Takano et al. 1995; Chapter II), *SCD1* (Kubo et al. 1996), and *THR1* (Perpetua et al. 1996) were isolated and their DNA sequences were determined just before the study of this chapter. In *A. alternata*, a gene cluster containing *ALM*, *BRM1*, and *BRM2* genes involved in melanin biosynthesis was isolated (Kimura and Tsuge, 1993). In *M. grisea*, isolation and structural analysis of T₄HN reductase gene and scytalone dehydratase gene have been also reported (Vidal-

Cros et al., 1994; Lundqvist et al., 1993; Lundqvist et al., 1994). The *C. lagenarium* *PKS1* gene complemented the melanin-deficient albino mutant (*pks1*⁻) which forms colorless appressoria and lacks pathogenicity to the host plant. The *PKS1* gene encodes a large multifunctional protein, a type I polyketide synthase (PKS), involved in synthesis of the pentaketide intermediate of melanin biosynthesis (Takano et al. 1995; Chapter II). Although the DNA sequence of *A. alternata* *ALM* gene has not been determined yet, the *ALM* gene probably encodes a type I PKS (Fig. III-2) because the *ALM* gene complemented *A. alternata* albino mutant (*Alm*⁻), and because the *ALM* gene produced a large transcript (ca. 7.2 kb), consistent with the typical size of type I PKS genes (Kimura and Tsuge 1993).

I consider that comparison of sequences and functions of melanin biosynthesis genes among fungi could help to elucidate the diversity in regulation systems for expression of melanin biosynthesis genes and roles of melanin. For comparison of polyketide synthase genes involved in melanin biosynthesis among fungi, I performed comparison between the *PKS1* gene and the *ALM* gene. In this chapter, I examined whether the *A. alternata* *ALM* gene could substitute for the *C. lagenarium* *PKS1* gene by transformation of the *C. lagenarium* albino mutant (*pks1*⁻) with the *ALM* gene.

Materials and Methods

Fungal strains and culture conditions

C. lagenarium (Pass.) Ellis and Halsted strain 104-T was used as a wild-type strain. Albino mutant 79215 (*pks1*⁻) was isolated from wild-type 104-T by ultraviolet light irradiation (Kubo et al. 1982) and was used for fungal transformation studies. Wild-type strain 15A of the Japanese pear pathotype of *A. alternata* was used for isolation of its genomic DNA. These strains were routinely cultured in potato dextrose agar (PDA) medium at 24°C in the dark.

Plasmid construction

The *A. alternata* *ALM* gene was cloned into pBR322 and designated pMBE1 (Kimura and Tsuge 1993). The 9.4 kb *Eco*RI fragment of pMBE1 was subcloned into the *Eco*RI site of pCB1004 containing the *hph* (hygromycin B phosphotransferase) gene as a selective marker for fungal transformation (Carroll et al. 1994). This plasmid was designated pCBALM and used for transformation of the albino mutant 79215 (*pks1*⁻) (Fig. III-3). The 1.4 kb *Bam*HI-*Hind*III fragment, and the 1.2 kb *Hind*III fragment of pCBALM were introduced into pBluescript (KS-), designated pBALMBH, and pBALMH respectively (Fig. III-3). The 0.45 kb *Bgl*II-*Sal*I in pAC712 containing 4.6 kb *Bgl*II-*Sma*I fragment of the *PKS1* gene (Takano et al. 1995) was introduced into pBluescript (SK-), designated pAC712S.

Fungal transformation

Preparation of protoplasts and transformation experiment of *C. lagenarium* were performed according to the method described previously (Kubo et al. 1991). Hygromycin resistant transformants were selected on regeneration medium containing 100 µg hygromycin B per ml. CAL transformants were obtained from transformation of the albino mutant 79215 (*pks1*⁻) with pCBALM. *PKS1*⁺ transformant was co-transformant of the albino mutant 79215 (*pks1*⁻) with pAC712 containing the *PKS1* fragment and pSH 75 containing the *hph* gene (Kimura and Tsuge 1993).

Genomic DNA blot analysis

The total DNAs of *C. lagenarium* and *A. alternata* were isolated by the procedure described in Chapter II. Genomic DNA was digested with *Eco*RI or *Sal*I, subjected to agarose gel electrophoresis and transferred onto Hybond N+ nylon membrane (Amersham) as described by

Southern (1975). The DNA probe was labelled with [α - 32 P] dCTP using random oligonucleotide primers as described by Sambrook et al. (1989). Hybridization was carried out by the procedure described in Chapter II.

Isolation of total RNA and RNA blot analysis

Total RNA was prepared by the following procedure. To prepare RNA from conidia before the start of incubation (at 0 h), conidia harvested from 7-day-old cultures were directly subjected to RNA isolation. To start conidial germination, conidial suspension (10^5 conidia/ml) were poured into 9-cm-diameter petri dishes at 24°C. Approximately 10^7 conidia were harvested at 1, 2, and 4 h by scraping them off from Petri dishes with a brush and collected by centrifugation at 2,000 g for 2 min. To obtain non-melanized mycelia, *C. lagenarium* was cultured in 250 ml of potato sucrose broth supplemented with 0.2% (w/v) yeast extract (PSY) at 24°C with shaking. The resulting mycelia were collected at 40, 60, and 80 h after inoculation in PSY. 10^7 conidia or 0.5 g of mycelia were suspended in 600 μ l of extraction buffer (100 mM glycine, 100 mM NaCl, 10 mM EDTA, pH 9.5), 60 μ l of 10% (w/v) SDS and 12 μ l of 6% (w/v) bentonite solution (Kroner and Alquist 1992). The suspension was transferred to a sterile mortar and 1.2 ml of phenol/chloroform/isoamyl alcohol (25:24:1) was added. Conidia were crushed and homogenized under liquid nitrogen using a pestle, and the homogenate was collected into polypropylene centrifuge tubes. The aqueous phase was then separated by centrifugation at 2,000 g for 5 min. The aqueous phase was extracted twice with phenol/chloroform/isoamyl alcohol. Total RNA was prepared by ethanol precipitation. RNA probes were made with T3 or T7 RNA polymerase by use of an in vitro transcription system in the presence of [α - 32 P] UTP. RNA blot hybridization was performed as previously described (Perpetua et al., 1996).

Thin-layer chromatography analysis

Carpropamid (KTU3616) is a novel blast fungicide inhibiting the conversion of scytalone to 1,3,8-trihydroxynaphthalene (Hattori et al. 1994). Fungi were cultured on 20 ml of PDA medium with or without 100 µg/ml Carpropamid at 24°C for 7 days. Scytalone was extracted according to the method described previously (Kubo et al. 1983). Chromatograms were developed with benzene-ethyl acetate (1:1, vol/vol) solvent and scanned by fluorescence in 254 nm light.

Penetration and pathogenicity test

The penetration ability to cellulose membranes was tested by the procedures previously described (Kubo et al. 1982a). Inoculation assay on cucumber was performed as described (Kubo et al. 1982b; Perpetua et al. 1994). 20 µl of conidial suspension (10^5 conidia /ml) were spotted on the surface of cucumber leaves. After inoculation, the leaves were placed in humid petri dishes at 24°C for 7 days. For microscopic observation, 20 µl of conidial suspension were spotted on the lower epidermis of cucumber cotyledons. Epidermal layers of cotyledons were peeled-off, stained with lactophenol aniline blue and observed by light microscopy 3 days after inoculation.

Electron microscopy

Appressoria formed on petri dishes were fixed at 0°C for 12 h with 1% (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. The appressoria were removed with a brush and collected, postfixed at 0°C for 12 h with 1% (w/v) osmium tetroxide, and dehydrated through an ethanol series. The ethanol was then replaced with propylene oxide and the materials were infiltrated and embedded in Lubeac 812 mixture (Nacalai Chemicals, Japan). Ultrathin sections were examined by a Hitachi H-7100FA electron microscope. About 50 appressoria were observed in each sample.

Results

Transformation of C. lagenarium albino mutant (pks1-) with A. alternata ALM gene

The 9.4 kb *Eco*RI fragment containing *A. alternata* ALM gene was introduced into pCB1004 carrying *hph* gene to produce pCBALM (Fig.III-3). pCBALM was used to transform the melanin-deficient albino mutant (*pks1*-) of *C. lagenarium*. The albino mutant (*pks1*-) forms an orange colony on PDA medium, and its conidia form colorless appressoria. Approximately 20% of hygromycin-resistant transformants formed dark-brown colonies. These *C. lagenarium* dark brown transformants were designated CAL transformants. CAL transformants could produce pigmented appressoria, although the pigment intensity of colonies and appressoria of CAL transformants was weaker than that of the wild-type 104-T (Fig. III-1).

To analyze the integration of pCBALM into the genomic DNA of CAL transformants, DNA gel blot analysis was performed. Total DNAs of *A. alternata* wild-type 15A, *C. lagenarium* wild-type 104-T and six of CAL transformants (CAL-1 to CAL-6) were isolated and digested with *Eco*RI or *Sal*I. The 1.4 kb *Bam*HI-*Hind*III ALM fragment of pCBALM was used as DNA probe (Fig. III-3). *Eco*RI digestion showed that the 9.4 kb *Eco*RI fragment of pCBALM was observed in all six CAL transformants (Fig. III-4). This suggested that pCBALM was integrated into the CAL transformants without the cleavage of the 9.4 kb *Eco*RI fragment. The analysis of DNA digested with *Sal*I suggested that pCBALM was introduced as 1 or 2 copies through random integration into the genome of the CAL transformants (Fig. III-4). Unless otherwise stated, I used CAL-1 in following experiments to characterize CAL transformants.

Detection of melanin intermediate, scytalone in CAL transformants

To confirm restoration of melanin biosynthesis of CAL transformants, I examined the accumulation of melanin intermediate in CAL transformants by using melanin biosynthesis inhibitor. Carpropamid was reported to interfere with the conversion of scytalone to 1,3,8-trihydroxynaphthalene in melanin biosynthesis pathway (Hattori et al. 1994; Kurahashi et al. 1997). *C. lagenarium* wild-type 104-T accumulated scytalone when grown on the culture medium containing 100 µg Carpropamid per ml (Fig. III-5). Accumulation of scytalone of CAL transformants incubated in the presence of Carpropamid was investigated by thin-layer chromatography (TLC) analysis. TLC analysis showed that the CAL transformant accumulated scytalone, whereas the albino mutant 79215 did not accumulate scytalone (Fig. III-5). This demonstrated that melanin biosynthesis was restored in CAL transformants. However, the quantity of scytalone in the CAL transformant was about 10% of that in the wild-type 104-T (Fig. III-5).

Localization of melanin in cell walls of appressoria of CAL transformants

To investigate localization of melanin in the cell walls of appressoria of CAL transformants, ultrastructural studies of appressoria were performed. In wild type, melanin was deposited in the inner thin layer, the middle layer, and the outer layer of appressorial cell walls (Fig. III-6A). The inner thin layer was observed just outside of the plasma membrane as high electron-dense melanized layer in the wild type (Fig. III-6A). By contrast, in appressorial cell walls of the albino mutant, the outer melanized layer was not observed, and the inner thin layer and middle layer were observed but less electron-dense compared with those of the wild type (Fig. III-6B). In appressorial cell walls of the CAL transformant, the outer melanized layer was formed same as wild type (Fig. III-6C). However, inner thin layer and middle layer of the CAL transformant

were less electron-dense compared with those of the wild type (Fig. III-6C).

Penetration ability to cellulose membranes and pathogenicity on cucumber in CAL transformants

When conidia of *C. lagenarium* wild-type 104-T were incubated at 24°C, melanized appressoria were formed within 12 h, and appressoria penetrated cellulose membranes during incubation for 48 h at 24°C. On the other hand, the albino mutant forms non-melanized appressoria, and have little penetrating ability to cellulose membranes (Table III-1). I investigated the penetrating ability of CAL transformants by using cellulose membranes. As a result, CAL transformants (CAL-1 to CAL-4) formed penetration hyphae into cellulose membranes as effectively as the wild-type 104-T (Table III-1).

I tested pathogenicity of the CAL transformants on host cucumber leaves. They could form lesion on cucumber leaves (Fig. III-7). However, the both number and size of lesions produced by the CAL transformants (CAL-1 to CAL-4) were obviously reduced compared to those of wild type (Fig. III-7 and data not shown). And also on cucumber cotyledons, the CAL transformants produced lesions of which number and size were smaller than wild type (data not shown). To evaluate pathogenicity of the CAL transformants on cucumber in more detail, we examined the penetration frequency of the CAL transformants on cucumber cotyledons. Fig. III-8A shows that appressoria of wild type produced penetration hyphae effectively. In wild type, 49% of appressoria examined (n=300) formed penetration hyphae. On the other hand, the CAL transformants could not penetrate cucumber cotyledons efficiently (Fig. III-8B). I found that only 1.5% of appressoria of CAL transformants formed visible penetration hyphae in epidermal layers of cotyledons.

To determine whether the reduction of penetration frequency of CAL transformants on cotyledons was due to *ALM* gene or other possible mutations of the albino mutant 79215 (*pks1-*

), I examined the penetrating ability of a melanin restored transformant, *PKS1*⁺ transformant, which was obtained from transformation of the albino mutant (*pks1*⁻) with the pAC712 containing the fragment of the *PKS1* gene. The *PKS1*⁺ transformant could produce melanized appressoria, of which pigment intensity was indistinguishable from wild type, and could penetrate cellulose membranes effectively same as wild type (data not shown). The size and number of lesions produced by the *PKS1*⁺ transformant were nearly equal to those of wild type on both cucumber leaves and cotyledons (Fig. III-7 and data not shown). The *PKS1*⁺ transformants produced penetration hyphae in cucumber cotyledon effectively (Fig. III-8C). 38% of appressoria of the *PKS1*⁺ transformant produced penetration hyphae in cotyledons.

The expression of the ALM gene in CAL transformants

In *C. lagenarium*, conidia germinated and germ tubes differentiated into melanized appressoria in petri dishes. Germ tubes became visible at 2 h, and began to swell and subsequently differentiate into appressoria by 4 h. To examine transcription of the *ALM* gene in CAL transformants during conidial germination and appressorium differentiation, RNA blot analysis was performed with total RNA from conidia at 0, 1, 2 and 4 h after the onset of conidial incubation. The RNA probe prepared from pBALMH detected the *ALM* transcript specifically (Fig. III-9A). During conidial germination and appressorium differentiation process, the accumulation pattern of the *ALM* transcript in the CAL transformant was quite different from that of the *PKS1* transcript in wild-type 104-T. The *PKS1* transcript was not detected in conidia of both the wild type and CAL transformants before the start of conidial incubation. The *PKS1* transcript appeared at 1 h after the start of conidial incubation, and then the amount of the *PKS1* transcript increased gradually (Fig. III-9A). On the other hand, the *ALM* transcript accumulated in conidia of the CAL transformant before the start of conidial incubation (at 0 h) (Fig. III-9A). At 1 h after the start of

conidial incubation, the amount of the *ALM* transcript decreased to about 15% of the amount of the *ALM* transcript present at 0 h. The accumulation of the *ALM* transcript in conidia of other 5 CAL transformants (CAL-2 to CAL-6) showed similar pattern (data not shown).

The expression of the *ALM* gene during mycelial growth of CAL transformants was also investigated. When *C. lagenarium* was cultured in liquid medium (PSY) with shaking at 24°C, the mycelia were non-melanized. In wild type, the *PKSI* transcript was hardly detected in these non-melanized mycelia at 40, 60, and 80 h after inoculation in PSY (Fig. III-9B). On the other hand, the *ALM* transcript was always detected in non-melanized mycelia of the CAL transformant at 40, 60, and 80 h (Fig. III-9B).

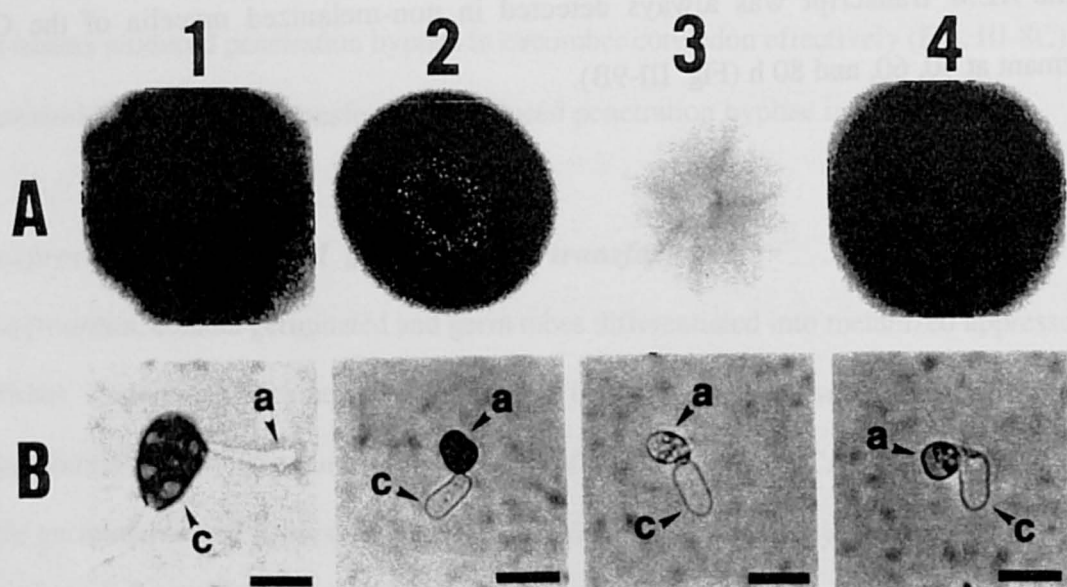


Figure III-1. Melanization of *A. alternata* wild type, *C. lagenarium* wild type, *C. lagenarium* albino mutant, and the CAL transformant. (A) Melanization of colony. Each strain was incubated on PDA medium at 24°C for 7 days. (B) Melanization of appressorium or conidium. Conidia of *A. alternata* and *C. lagenarium* harvested from 7-day-old cultures were suspended in water and incubated on petri dishes at 24°C for 6 h and 12 h respectively. (1) *A. alternata* wild type 15A. (2) *C. lagenarium* wild type 104-T. (3) *C. lagenarium* albino mutant 79215 (Pks1-). (4) CAL transformant (CAL-1). Bars = 10 μ m. Structures: a, appressorium; c, conidium.

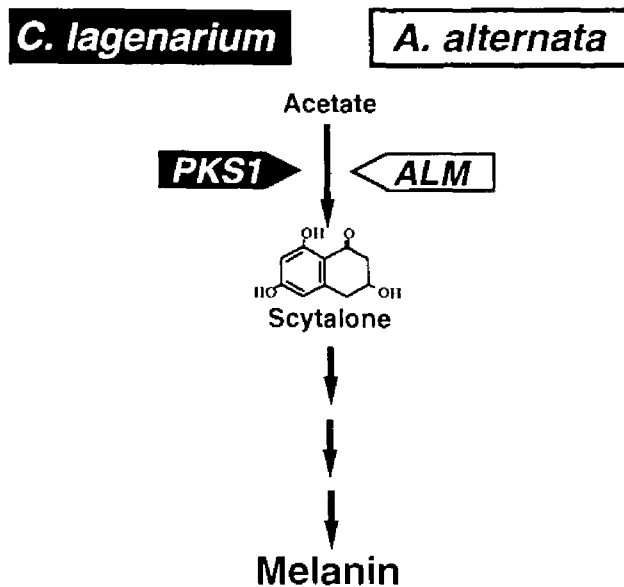


Figure III-2. Polyketide synthase genes involved in melanin biosynthesis of *C. lagenarium* and *A. alternata*. *PKS1* encodes type-I polyketide synthase involved in melanin biosynthesis of *C. lagenarium*. *ALM* is considered to encode type-I polyketide synthase involved in melanin biosynthesis of *A. alternata* because *ALM* complemented *A. alternata* albino mutant (*Alm*⁻), and the size of its transcript was 7.2kb.

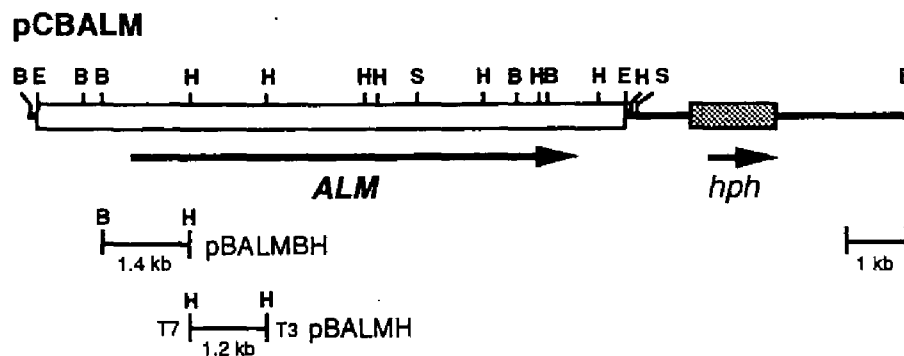


Figure III-3. Schematic representation of plasmid pCBALM. The 9.4 kb *EcoRI* fragment of pMBE1 (Kimura and Tsuge, 1993) was subcloned into pCB1004 to produce pCBALM. The 1.2 kb *HindIII* fragment of pCBALM insert was introduced into pBluescript (KS-) to prepare strand specific RNA probe for RNA blot analysis. The location of promoter sequence in resulting plasmid was shown. The 1.4 kb *BamHI*-*HindIII* fragment of pCBALM was introduced into pBluescript (KS-), and used to prepare the DNA probe for DNA blot analysis. Abbreviations: B, *BamHI*; E, *EcoRI*; H, *HindIII*; S, *Sall*.

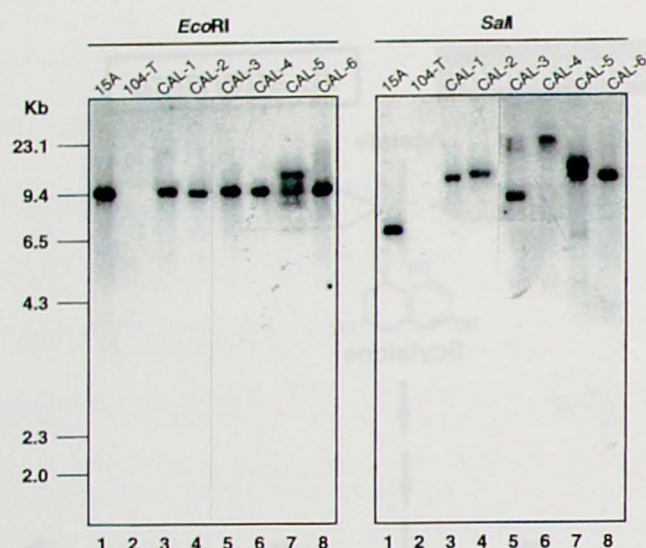


Figure III-4.

DNA gel blot analysis of the CAL transformants. Genomic DNAs were digested with *EcoRI* or *SalI* and probed with 1.4 kb *BamHI-HindIII* fragment of the *ALM* gene. Lane 1, *A. alternata* wild type 15A; lane 2, *C. lagenarium* wild type 104-T; lanes 3 to 8, the CAL transformants (CAL-1 to CAL-6) respectively.

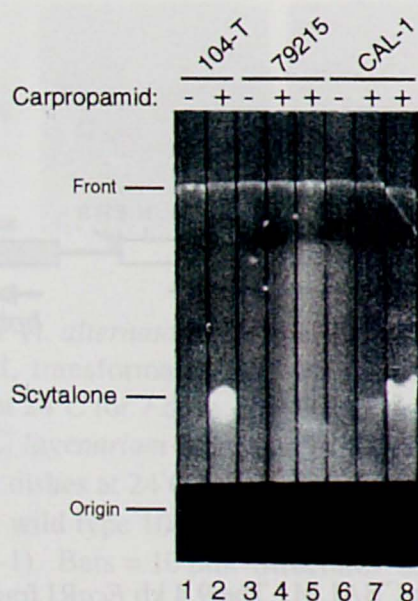


Figure III-5. Analysis of accumulation of scytalone in the CAL transformant by thin-layer chromatography. The wild-type 104-T, the albino mutant 79215(*pks1*⁻), and the CAL transformant (CAL-1) were cultured on 20 ml of PDA medium with or without 100 μ g/ml Carpropamid at 24°C for 7 days. Extraction and detection of scytalone were described in Materials and Methods. 10 volumes of chromatograms of lane 4 and 7 were developed in lane 5 and 8 respectively. Scytalone was recognized at R_f value 0.33 in lanes, 7 and 8.

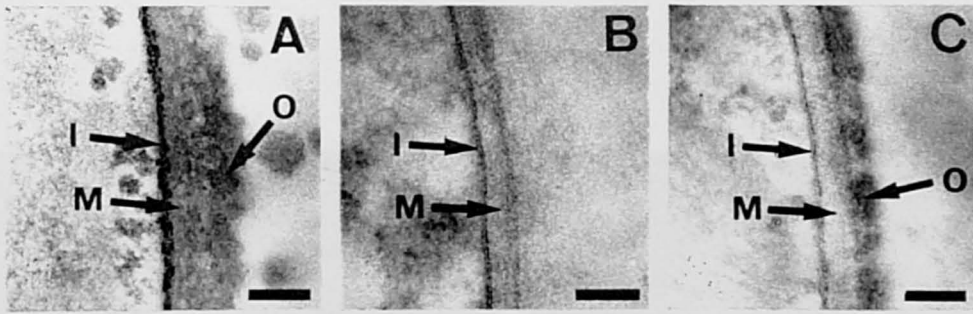


Figure III-6. Electron micrographs of appressorial cell walls of the wild type, the albino mutant, and the CAL transformant. (A) The wild-type 104-T. (B) The albino mutant 79215(*pks1*⁻). (C) The CAL transformant (CAL-1). Structures: I, inner thin layer; M, middle layer, O, outer layer. Bar markers represent 0.2 μ m.

Table III-1.

The penetration ability of the CAL transformants on cellulose membranes.

Strain	Appressorial pigmentation ^a	Appressorium formation	Penetration hyphae ^b
104-T	++	88.0	93.3
79215	-	75.5	2.0
CAL-1	+	85.1	90.2
CAL-2	+	88.2	92.3
CAL-3	+	81.3	90.3
CAL-4	+	80.4	92.7

Conidia of the wild type 104-T, the albino mutant 79215, or the CAL transformants (CAL-1 to CAL-4) were incubated at 24°C for 48 h on cellulose membranes. Approximately 300 conidia were counted. ^a Appressoria were darkly melanized (++), slightly melanized (+), or colorless (-). ^b Percentage of penetration hyphae was based on the total number of appressoria.

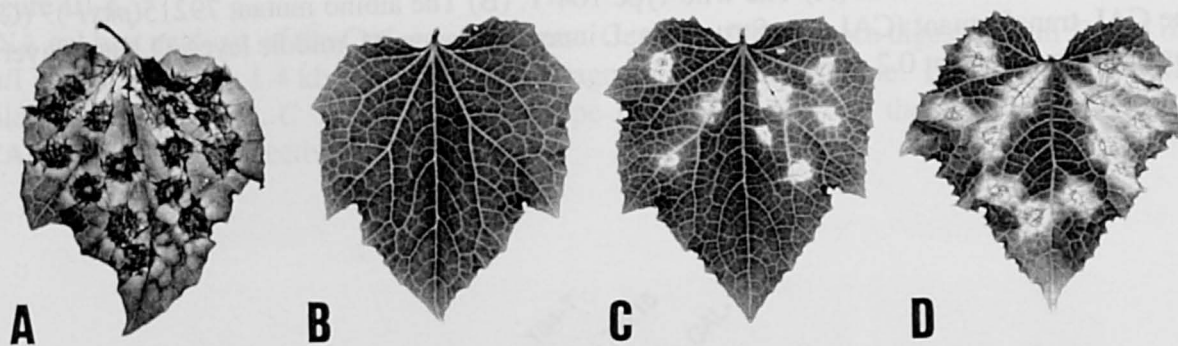


Figure III-7.

Pathogenicity test of the CAL transformant. Cucumber leaves were inoculated with the wild type, the albino mutant, the CAL transformant, or the *PKS1*⁺ transformant, and then incubated at 24°C for 7 days. (A) the wild type 104-T. (B) the albino mutant 79215 (*pks1*⁻). (C) the CAL transformant (CAL-1). (D) the *PKS1*⁺ transformant.

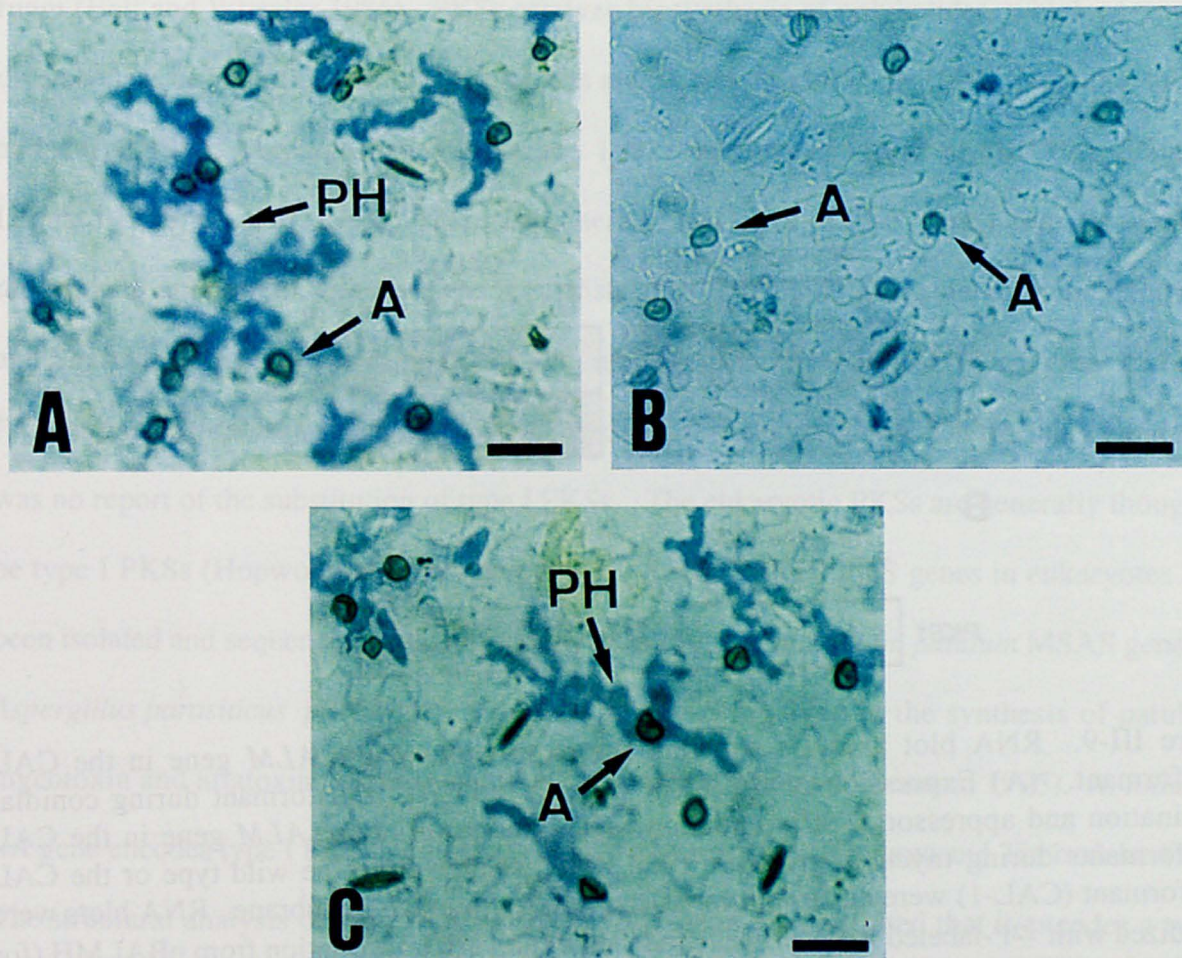


Figure III-8. The penetrating ability of *C. lagenarium* in the epidermal layer of the cucumber cotyledon. (A) Appressoria of wild-type 104-T forming penetration hyphae effectively. (B) Appressoria of the CAL transformant (CAL-1) with no penetration hyphae. (C) Appressoria of the *PKS1*+ transformant forming penetration hyphae effectively. A, appressorium; PH, penetration hyphae. Bar=20 μm.

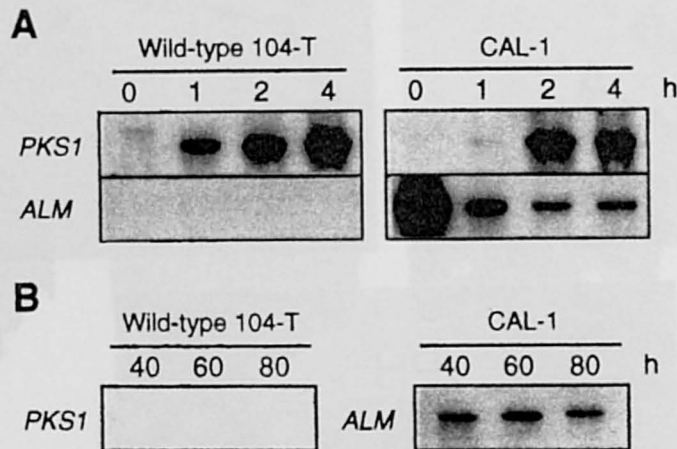


Figure III-9. RNA blot analysis showing the expression of the *ALM* gene in the CAL transformant. (A) Expression of the *ALM* gene in the CAL transformant during conidial germination and appressorial differentiation. (B) Expression of the *ALM* gene in the CAL transformants during mycelial growth. Total RNA isolated from the wild type or the CAL transformant (CAL-1) were electrophoresed, blotted onto a nylon membrane. RNA blots were hybridized with ^{32}P -labeled RNA probes produced by in vitro transcription from pBALMH (for *ALM*) or pAC712S (for *PKS1*) by use of T3 or T7 RNA polymerase (see materials and methods) respectively. Total rRNA stained with ethidium bromide was used as a loading control.

Discussion

Polyketide synthases (PKSs) are involved in the initial steps of melanin biosynthesis of most fungi (Bell and Wheeler 1986). PKSs catalyze biosynthesis of polyketides, which comprise a diverse class of natural products that includes antibiotics (Cortes et al. 1990), pigments (Brown and Salvo 1994; Mayorga and Timberlake 1992), and toxins (Beck et al. 1990; Feng and Leonard 1995; Lu et al. 1994). There are generally two types of PKS: type I consists of a large multifunctional protein, whereas type II consists of several monofunctional proteins (Hopwood and Sherman 1990). Previously, the substitution of several monofunctional enzymes of type II PKSs among *Streptomyces* species was reported (Sherman et al. 1992). On the other hand there was no report of the substitution of type I PKSs. The eukaryotic PKSs are generally thought to be type I PKSs (Hopwood and Sherman 1990). Several type I PKS genes in eukaryotes have been isolated and sequenced (Hutchinson and Fujii 1995). *Penicillium patulum* MSAS gene and *Aspergillus parasiticus pksLI* gene encode type I PKS involved in the synthesis of patulin, a mycotoxin and aflatoxin, respectively (Beck et al. 1990; Feng and Leonard 1995). *A. nidulans wA* gene encodes type I PKS involved in conidial pigmentation (Mayorga and Timberlake 1992). The structural analysis of the *C. lagenarium PKS1* gene demonstrated that it encodes a type I PKS involved in melanin biosynthesis (Takano et al. 1995). The *A. alternaria ALM* gene, which complements the albino mutant (*Alm⁻*), was considered to encode type I PKS (Kimura and Tsuge 1993).

In this chapter, I demonstrated that *A. alternata ALM* gene could restore melanization of *C. lagenarium* albino mutant (*pksI⁻*). The melanin-restored transformants (CAL transformants) could form melanized colonies and appressoria. However, the pigment intensity of both melanized appressoria and colonies of the CAL transformants was lower than that of the wild-

type 104-T. TLC analysis of products formed in the presence of the melanin biosynthesis inhibitor showed lower levels of scytalone in CAL transformants, suggesting that CAL transformants synthesized less melanin than wild type. I consider that the lower level of pigment intensity of CAL transformants depends on the level of synthesized melanin. Although the *ALM* transcript of the CAL transformant was continuously detected from 0 h to 4 h after the start of conidial incubation, there may be a possibility that the lower level of synthesized melanin was due to the amount of the *ALM* transcript in the CAL transformants. And also there is a possibility that it was due to a post-transcriptional process, i.e. efficiency of translation of the *ALM* transcript, and enzyme activity and cellular localization of ALM polyketide synthase.

Melanin is a component of appressorial cell walls of several fungi (Emmet and Parbery 1975; Kubo and Furusawa 1986; Howard and Ferrari 1989). In a previous report, melanin was interpreted as the inner thin layer of appressorial cell walls of *C. lagenarium* (Kubo and Furusawa 1986). In addition, I found that melanin was located in the middle and outer layers as well as in the inner thin layer of *C. lagenarium* wild type from comparison of appressorial cell walls among the wild type, the albino mutant, and the CAL transformants. In appressorial cell walls of the CAL transformant, the outer layer was formed as in the wild type. On the other hand, the inner thin layer and middle layer of the CAL transformant were less electron-dense compared to the wild type. This pattern may depend on lower level of synthesized melanin in CAL transformants, and/or on the difference of localization of melanin based on cellular localization of ALM polyketide synthase in CAL transformants.

CAL transformants could penetrate cellulose membranes as effectively as the wild type, even though pigment intensity of appressoria of the CAL transformants was weaker than that of the wild type. This was consistent to the previous report that *C. lagenarium* mutant 8004 forming slightly melanized appressoria could penetrate cellulose membranes as effectively as the wild type

(Kubo et al., 1987). The CAL transformants formed lesions on cucumber leaves. However, the pathogenicity of the CAL transformants was weaker than wild type. The penetration-frequency of the CAL transformants in cucumber cotyledons was remarkably reduced compared to the wild type. Thus, I conclude that the reduction of size and number of lesions produced by CAL transformants is due to the reduction of penetration-frequency of CAL transformants. It is considered that the reduction of penetration frequency of CAL transformants in cotyledons did not depend on the recipient strain, the albino mutant 79215 (*pks1*⁻) but on the *ALM* gene since the melanin-restorant (the *PKS1*⁺ transformant) of the albino mutant by the *PKS1* gene could penetrate cotyledons effectively. Thus, appressorial melanization of CAL transformants is sufficient for penetration of cellulose membranes, but is not sufficient for penetration of cucumber cotyledons. I consider that the pigment intensity reached wild type level, in the inner thin and middle layers as well as in the outer layer of appressorial cell walls, is required for appressorial penetration of cucumber cotyledons. In *M.grisea*, it is shown that melanin confers pathogenicity by facilitating the development of turgor pressure within appressoria (Howard and Ferrari, 1989; Money and Howard, 1996). There is a possibility that the turgor pressure within appressoria of CAL transformants is reduced compared to that of wild type, which causes the reduction of penetration-efficiency of CAL transformants in cucumber cotyledons.

The pattern of *ALM* transcript accumulation in the CAL transformants was quite different from that of the *PKS1* transcript. During conidial germination process, the *PKS1* transcript was not detected in conidia of the wild type before the start of conidial incubation, and the accumulation of the *PKS1* transcript increased gradually after the start of conidial incubation (Takano et al. 1997). On the other hand, the *ALM* transcript was detected in conidia of the CAL transformants before the start of conidial incubation. Analysis of 6 CAL transformants showed similar accumulation pattern of the *ALM* transcript, although the integration positions of the *ALM*

gene were not identical. Thus, the accumulation pattern of the *ALM* transcript depended not on the integration positions of pCBALM.

During vegetative growth, the *ALM* transcript accumulated in non-melanized vegetative mycelia of CAL transformants, whereas the *PKSI* transcript hardly detected. By contrast, in *A. alternata*, the *ALM* transcript was not detected during vegetative growth in liquid culture but detected specifically during mycelial melanization (Kimura and Tsuge 1993). Thus, the expression of the *ALM* gene was not normally regulated in *C. lagenarium*. This may be due to the following reasons: (i) pCBALM does not contain the entire promoter region which is essential for transcriptional regulation of the *ALM* gene in *A. alternata*, or (ii) transcriptional factors of *C. lagenarium* do not recognize the promoter region of the *ALM* gene properly. Structural and functional comparison of promoter regions of the *ALM* gene and the *PKSI* gene will result in a better understanding of the temporal and spatial control of melanin synthesis.

Chapter IV. Temporal transcriptional pattern of three melanin biosynthesis genes, *PKS1*, *SCD1*, and *THR1*, in appressorium-differentiating and nondifferentiating conidia.

Introduction

In several fungi including *Colletotrichum* and *Magnaporthe* species, appressoria are darkly melanized. In *C. lagenarium*, studies with melanin biosynthesis inhibitors and melanin-deficient mutants demonstrate that appressorial melanization is essential for penetration of the host plant (Kubo and Furusawa 1991; Kubo et al. 1982a; Kubo et al. 1985; Yamaguchi and Kubo 1992). Similarly, in *Magnaporthe grisea* (Hebert) Barr and *C. lindemuthianum* (Saccardo et Magnus) Briosi et Cavara, appressorial melanization is essential for penetration of host plants (Chumley and Valent 1990; Wolkow et al. 1983; Woloshuk et al. 1983). Isolation and structural analysis of three melanin biosynthesis genes, *PKS1*, *SCD1*, and *THR1* of *C. lagenarium* was reported just before the study of this chapter (Fig. IV-1; Kubo et al. 1991; Kubo et al. 1996; Perpetua et al. 1996; Takano et al. 1995). Melanin biosynthesis starts from pentaketide synthesis to form 1,3,6,8-THN. Polyketide synthase (*PKS1*) is involved in this early steps (Takano et al. 1995). Subsequent steps consist of two dehydrations and two reduction reaction. It is considered that 1,3,6,8-THN may be converted to scytalone by unidentified reductase. Both dehydrations of scytalone to 1,3,8-trihydroxynaphthalene (1,3,8-THN) and vermelone to 1,8-dihydroxynaphthalene (DHN) are performed by scytalone dehydratase (*SCD1*) (Kubo et al. 1996). Reduction of 1,3,8-THN to vermelone is performed by 1,3,8-THN reductase (*THR1*) (Perpetua et al. 1996). DHN is then polymerized and oxidized to yield melanin. In *C. lagenarium*, melanization is confined to appressoria; conidia and germ tubes are not melanized. There is little information concerning the mechanism(s) that confines melanin to appressoria.

Appressorium formation is triggered by specific environmental cues. Several physical and chemical signals necessary for induction of appressorium formation are reported (Hoch et al. 1987; Lee and Dean 1993; Podila et al. 1993). In several fungi, appressorium formation is dependent on environmental conditions. Appressorium differentiation of *C. lagenarium* conidia is temperature-sensitive. In *C. lagenarium*, when conidia suspended in water were exposed to a hard surface such as a Petri dish and incubated at 24°C, conidia germinated and germ tubes differentiated into melanized appressoria (Fig. IV-2A). When conidia were incubated at a higher temperature 32°C, conidia germinate but appressorium differentiation was not observed (Figs. IV-2B and IV-2C). In this fungus, conditions for nearly synchronous cellular differentiation in vitro are established. This enabled me to prepare abundant conidia at nearly synchronous developmental stages for RNA isolation. As a first step to elucidate the mechanism(s) confining melanin to appressoria, I describe here the temporal transcriptional patterns of three melanin biosynthesis genes, *PKS1*, *SCD1*, and *THR1* in differentiating and non-differentiating conidia.

Result and Discussion

Transcriptional pattern of the *PKS1*, *SCD1*, and *THR1* genes during appressorium differentiation

The time course of appressorium differentiation of conidia was examined. The condition that conidia proceed to cellular differentiation in vitro synchronously was as follows. Conidia harvested from 7-day-old cultures were suspended in sterile, deionized water containing 0.01 % (v/v) Tween 20 to give about 10⁵ conidia per ml. 4.5 ml of conidial suspension was placed in 9-cm-diameter Petri dish and incubated at 24°C.

At 0, 1, 2, 4, 6, 9, 12, and 18 h after the start of conidial incubation in this condition, the

proportions of non-germinating conidia, germinating conidia and appressorium-forming conidia were determined. Fig. IV-3A shows that 70% of conidia began to germinate within 2 to 4 h, and 70% of conidia produced appressoria at 6 h. Slight melanization of appressoria was observed at 6 h and melanization of appressoria was complete by 12 h (data not shown and Fig. IV-2A).

Next, I isolated total RNA from differentiating conidia for RNA blot analysis. Total RNA was prepared by the procedure described in Chapter III. To start conidial germination, conidial suspension (10^5 conidia/ml) were poured into 9-cm-diameter petri dishes at 24°C. Approximately 10^7 conidia were harvested at 1, 2, 4, 6, 9, 12, and 18 h after the start of conidial incubation and by scraping them off from Petri dishes with a brush and collected by centrifugation at 2,000 g for 2 min. To prepare RNA from conidia before the start of incubation (at 0 h), conidia harvested from 7-day-old cultures were directly suspended in extraction buffer for RNA isolation. RNA blot hybridization was performed as previously described (Perpetua et al. 1996). The 0.45 kb *Bgl*II-*Sal*I fragment of pAC712 containing the *PKS1* gene, the 0.65 kb *Eco*RI-*Xba*I fragment of pSD2SK containing the *SCD1* gene and the 0.55 kb *Sac*I-*Sal*I fragment of pCR1 containing the *THR1* gene were used to prepare [α - 32 P] UTP labelled in vitro transcripts of the respective genes as antisense RNA probes (Kubo et al. 1996; Perpetua et al. 1996; Takano et al. 1995). In addition, expression of the β -tubulin gene, considered to be not linked to melanin biosynthesis, was investigated as a control. cDNA of the β -tubulin gene of *C. lagenarium*, designated *TUB1* was isolated from a cDNA library using a genomic fragment containing the β -tubulin gene as a probe (Kubo et al. 1991). The 0.4 kb β -tubulin (*TUB1*) cDNA fragment was used as a template for preparing 32 P-labelled in vitro transcript. In RNA blot analyses, RNA probes with almost the same specific activity were used for detecting each transcript.

Results showed that transcripts of the three melanin biosynthesis genes were not detected at 0 h, though transcripts of the β -tubulin gene were detected (Fig. IV-3B). At 1 h *THR1* transcripts

accumulated to a higher level than *PKS1* transcripts, whereas *SCD1* transcripts had not accumulated. The *PKS1* transcript accumulated to a higher level and the *SCD1* transcript started to accumulate at 2 h. This result demonstrated that these melanin biosynthesis genes were not expressed in conidia before the start of incubation, and they were transcribed de novo 1-2 h after the start of incubation, i.e. at early developmental stage when germ tubes were not yet visible. The accumulation of transcripts of these genes increased until 4 h and began to decrease at 6 h. At 18 h, transcripts of the three melanin biosynthesis genes were hardly detected. Measurement of the radioactivities of each transcript with a Bio-Imaging Analyzer BAS2000 (FUJIX), showed that the ratios between the *PKS1*, *SCD1*, and *THRI* transcripts remained consistent at each time point from 2 h to 12 h (data not shown).

The amount of the transcript of the β -tubulin (*TUB1*) gene began to decrease at 6 h. In general, β -tubulins are known to be involved in various phases including mitosis, morphogenesis, and cytoskeleton as subunits of microtubules (Avila 1992). Presumably, the decrease of the *TUB1* transcript was correlated with the finish of cellular morphogenesis at 6-9 h, i.e. appressorium formation.

Transcriptional pattern of the *PKS1*, *SCD1*, and *THRI* genes during development of nondifferentiating conidia

C. lagenarium conidia incubated in water at 32°C germinated and produced elongated germ tubes but no appressoria (Suzuki et al. 1981; Fig. IV-2B). On the other hand, conidia incubated in 0.1% (w/v) yeast extract solution at 32°C developed into vegetative hyphae (Fig. IV-2C) that were distinguishable from germ tubes by their greater diameters. Furthermore, nuclear division and septum formation were observed during elongation of vegetative hyphae but not during elongation of germ tubes (data not shown).

All three melanin biosynthesis genes were expressed in conidia incubated in water at 32°C

(Fig. IV-4), even though conidia produced no appressoria and melanization was not observed. The *TUB1* transcript began to decrease gradually at 6 h, which seemed to be correlated with the finish of elongation of germ tubes. The transcriptional pattern in conidia incubated in water at 32°C was similar to that in conidia incubated at 24°C.

A previous report showed that appressorial melanization as well as appressorium formation was temperature-sensitive (Kubo et al. 1984). When conidia were preincubated in water for 4.5 h at 24°C so that fully expanded but colorless appressoria were formed, and then incubated in water at 32°C, appressoria did not become melanized (Fig. IV-5). However, when the colorless appressoria were instead incubated in 0.75 mM scytalone at 32°C, they became melanized (Fig. IV-5). It suggested that melanin biosynthesis enzymes involved in steps after scytalone synthesis, i.e. both *SCD1* and *THR1* were active at 32°C though melanin biosynthesis enzymes involved in earlier steps were temperature-sensitive (Kubo et al. 1984). On the other hand, conidia incubated continuously at 32°C, which elongate germ tubes and produce no appressoria, were not melanized after the addition of scytalone, suggesting biosynthesis steps after scytalone synthesis was inactive (Fig. IV-5), although the melanin biosynthesis genes *SCD1* and *THR1* were transcribed as highly as in appressorium-forming conidia. Thus, I conclude that some regulation system, perhaps involved in post-transcriptional regulation of the melanin biosynthesis genes *SCD1* and/or *THR1*, did not function properly in non-differentiating conidia.

On the other hand, in conidia incubated in 0.1% yeast extract solution at 32°C, the transcripts of the three melanin biosynthesis genes were hardly detected although the *TUB1* transcript gradually accumulated (Fig. IV-4). Conidia incubated in 0.1 % yeast extract solution at 32°C continued to elongate vegetative hyphae whereas conidia incubated in water at 24°C and 32°C finished appressorium formation and elongation of germ tubes respectively at 6-9 h. I considered that the *TUB1* transcript did not decrease in conidia incubated in 0.1 % yeast extract solution at 32°C because of continuous elongation of vegetative hyphae. I found that conidia

formed vegetative hyphae when conidia were incubated in 0.1% tryptone solution at 32°C. This suggests that complex nutrients such as yeast extract and tryptone induce vegetative-hyphal elongation of conidia at 32°C. I assume that the expression of the melanin biosynthesis genes is repressed by these complex nutrients.

Transcripts of the *PKS1*, *SCD1*, and *THR1* genes accumulated and diminished in a similar time course during appressorium differentiation, and none of the three transcripts accumulated in conidia incubated in 0.1% yeast extract solution at 32°C. These observations suggested that common mechanisms regulate transcription of the three melanin biosynthesis genes. However, I assume that factors regulating transcription of the *PKS1*, *SCD1* and *THR1* genes were not identical because the *PKS1*, *SCD1*, and *THR1* transcripts did not appear synchronously during appressorium differentiation. The order of expression of the three melanin biosynthesis genes (*THR1*-*PKS1*-*SCD1*) was not consistent with the order of the melanin biosynthesis pathway (*PKS1*-*SCD1*-*THR1*). I suppose that the post-transcriptional regulation of the melanin biosynthesis genes, as suggested above, determine the time when melanin biosynthesis enzymes should work, and that the order of expression of the three genes should not necessarily correlate with the order of the pathway.

Results presented here suggest that both transcriptional and post-transcriptional regulations of the three melanin biosynthesis genes are involved in appressorial melanization of *C. lagenarium*. I consider that the accumulation pattern and intracellular localization of the three enzymes during appressorium differentiation could help to unravel their post-transcriptional regulation. And also analyses of the promoter regions and transcriptional factors of the three melanin biosynthesis genes is needed to elucidate the transcriptional control which determine the order of the expression of the three genes.

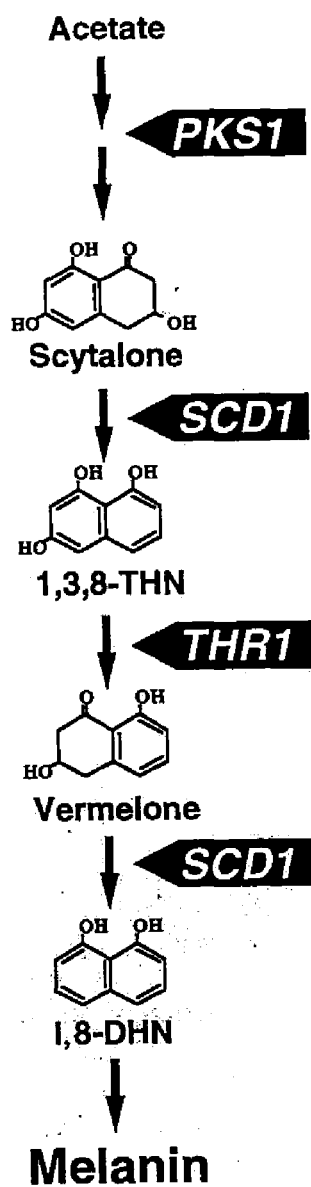


Figure IV-1. Melanin biosynthetic pathway and cloned melanin biosynthesis genes *PKS1*, *SCD1*, and *THR1* of *C. lagenarium*.

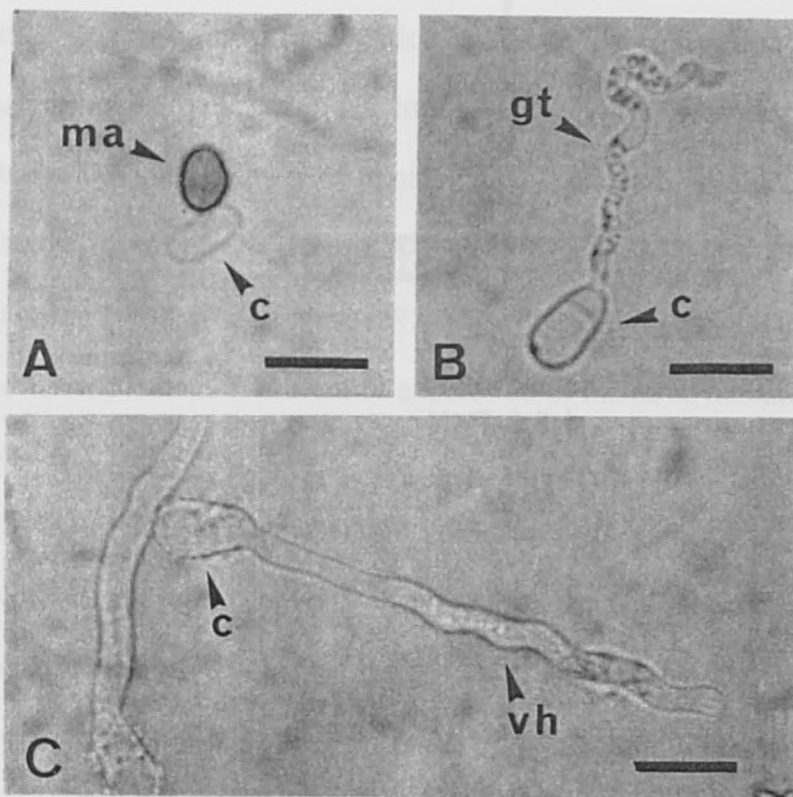


Figure IV-2. Differentiation and nondifferentiation of conidia of *C. lagenarium*. Conidia were incubated for 12 h on Petri dishes in water at 24°C (A), in water at 32°C (B) or in 0.1% yeast extract solution at 32°C (C). The bar markers represent 10 µm. Structures : ma, melanized appressorium; c, conidium; gt, germ tube; vh, vegetative hypha.

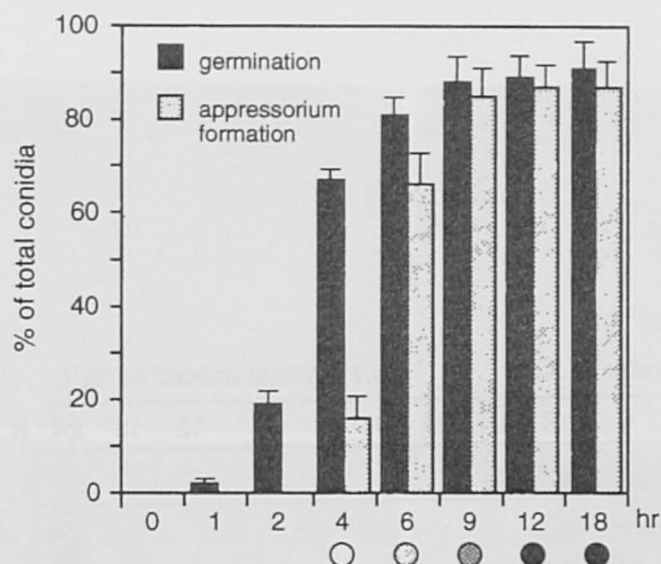
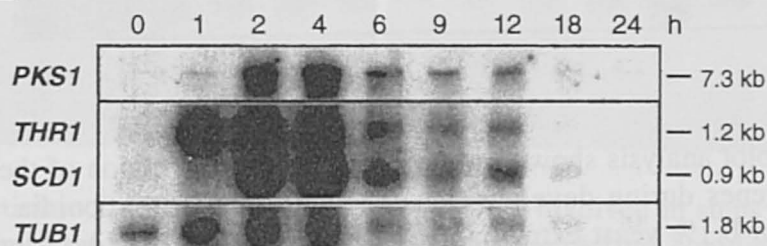
A**B**

Figure IV-3. Time course of the expression of *PKS1*, *SCD1*, and *THR1* genes during appressorium formation by *C. lagenarium*. (A) Time course of germination and appressorium formation of *C. lagenarium* conidia. Approximately 100 conidia were observed per Petri dish with a light microscope. Five replicates were examined at each time and the values were averaged. SD was indicated by vertical bars. The color of circles represents the degree of appressorial melanization. (B) RNA blot analysis showing time course of the *PKS1*, *SCD1*, *THR1* and *TUB1* gene expression during appressorium differentiation. Total RNA (2 μ g) isolated from conidia were electrophoresed, blotted onto a nylon membrane and hybridized with 32 P-labeled in vitro transcripts complementary to mRNA of the *PKS1*, *SCD1*, *THR1* and *TUB1* genes. Total rRNA stained with ethidium bromide was used as a loading control.

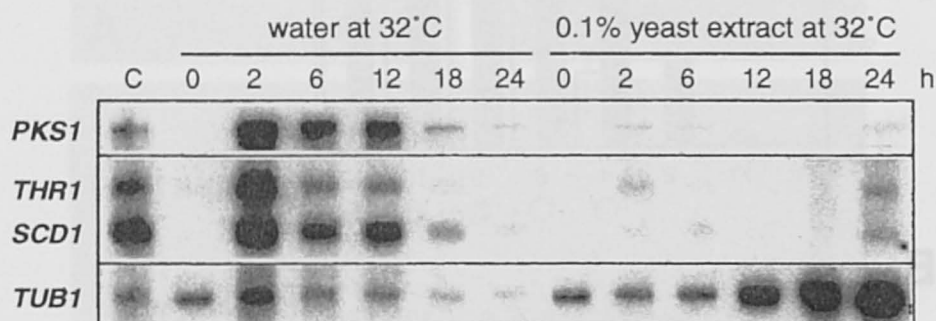


Figure IV-4. RNA blot analysis showing time course of the expression of the *PKS1*, *SCD1*, *THR1*, and *TUB1* genes during development of nondifferentiating conidia. Conidia were incubated in water or 0.1% yeast extract solution at 32°C. Total RNA (2 µg) isolated from conidia were analyzed as described in the legend to Fig. IV-2B. RNA isolated from conidia at 6 h after the start of incubation in water at 24°C was electrophoresed as a control (lane C).

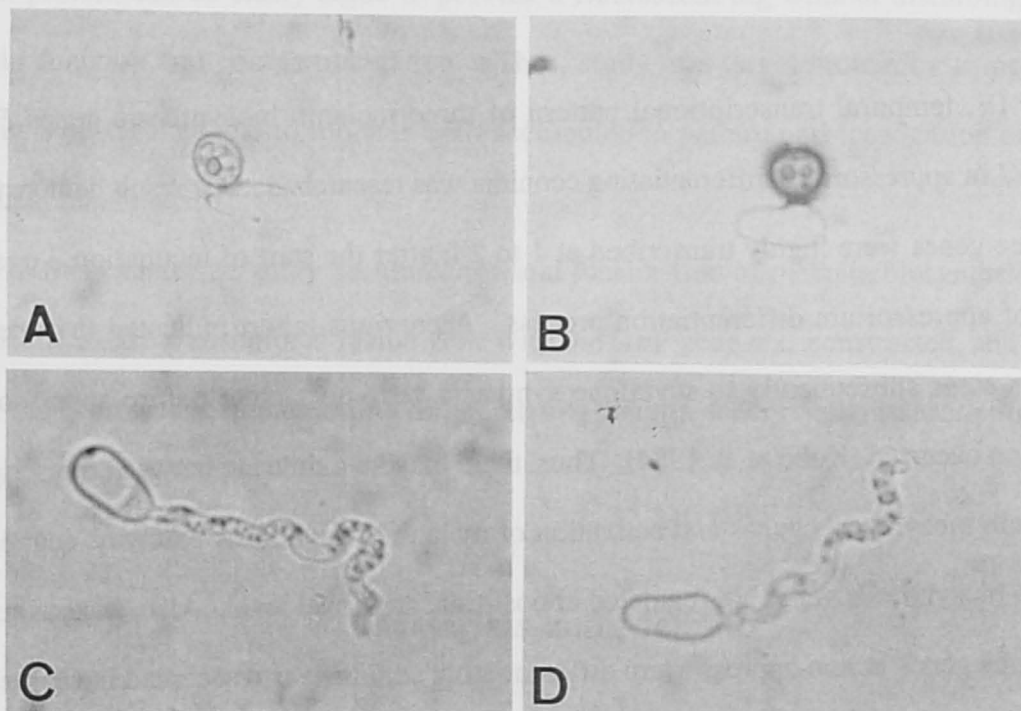


Figure IV-5. Activity of melanin biosynthesis enzymes involved in steps after the scytalone synthesis at appressorium-differentiating and nondifferentiating conidia at 32°C. Conidia preincubated in water at 24°C for 4.5 h were subsequently incubated in water at 32°C in the absence of scytalone (A) or the presence of 0.75mM scytalone (B). Conidia incubated in water at 32°C for 12 h in the absence of scytalone (C) or the presence of 0.75mM scytalone (D).

Chapter V. Studies on accumulation and localization of melanin biosynthesis enzyme SCD1 during appressorium differentiation by using SCD1 fusion protein with green fluorescent protein (GFP)

Introduction

In Chapter IV, temporal transcriptional pattern of three melanin biosynthesis genes *PKS1*, *SCD1*, *THR1* in appressorium-differentiating conidia was researched. The result demonstrated that the three genes were highly transcribed at 1 to 2 h after the start of incubation, i.e., at the early step of appressorium differentiation process. A previous report indicated that melanin biosynthesis steps subsequently to scytalone synthesis were not active before appressorium differentiation occurred (Kubo et al. 1984). Thus, there may be a time lag between transcription of the melanin biosynthesis genes and activation of melanin biosynthesis pathway, suggesting that melanin biosynthesis genes are regulated at post-transcriptional level. Also, transcriptional pattern of three genes in non-appressorium differentiating conidia was researched in chapter IV. The result suggested that post-transcriptional regulations of the *SCD1* and/or the *THR1* genes were involved in appressorium differentiation. Post-transcriptional regulations are considered to be translational and/or post-translational regulations. For elucidation of post transcriptional regulations of melanin biosynthesis genes, it is useful to study translational pattern and localization of melanin biosynthesis enzymes in appressorium-differentiating conidia. For this purpose, a new vital marker, green fluorescence protein (GFP) from the jellyfish *Aequorea victoria*, is powerful tool. GFP is a small protein of 238 amino acids responsible for green bioluminescence in Cnidaria. Since formation of the fluorescent chromophore is not species dependent, GFP has been successfully expressed in a variety of heterologous system, e.g.

Escherichia coli, worm, fly, yeast, mammals, and plants (Chalfie et al. 1994; Wang and Hazelrigg 1994; Haseloff and Amos 1995; Cubitt et al. 1995; Baulcombe et al. 1995; Sheen et al. 1995). Recently, GFP has been used in phytopathogenic fungi *Ustilago maydis* as a vital marker (Spellig et al. 1996). When GFP is expressed, blue or UV light and oxygen are the only requirements to induce green fluorescence; exogenous substrates are not needed. In addition, fusion protein can be easily made to provide a fluorescent tag without disturbing the native protein function and compartmentation. Thus, study of a target protein by using the fusion protein with GFP enable to monitor both accumulation pattern and localization of the target protein at real time.

In this chapter, to study accumulation and localization of melanin biosynthesis enzymes SCD1, the *SCD1* translational fusion gene with the GFP gene was constructed, and this fusion gene was expressed in appressorium differentiating conidia under regular transcriptional manner of the *SCD1* gene.

Materials and Methods

plasmids

SmaI-ClaI fragment containing the SGFP-TYG gene and NOS terminator in pOTEFSG, kindly provided by Dr. Regine Kahmann, was introduced into *SmaI-ClaI* sites in pBS (KS-), resultant plasmide was designated as pBSGFP. For an addition of glycine spacers to N-terminal region of SGFP-TYG, SGFP-TYG was amplified by using primer GLY-ATG-GFP (5'-CGGGATCCTC GGTGGTGGTGGCATGGTGAGCAAGGGCG-3'), and universal primer M13 RV. Amplified product was digested with *Bam*HI and *Sal*II, and introduced into pBS, designated pGLSGFP. In case of construction of EGFP (P64L, S65T), site directed mutagenesis of SGFP-TYG (S65T) in

pBSGFP was performed. First PCR was performed by using one primer set consisting GLY-ATG-GFP and MUTBSL1 (5'-CCCTCGAGGTAGACGGTATCGATAAG-3'), and the other primer set consisting M13 RV and P64LGFP (5'-CACCCCTCGTGACCACCCTGACCTACGGCGTGCA-3') respectively. Second PCR reaction was performed in mixture of both first PCR products as template by using primer GLY-ATG-GFP and M13RV. Amplified products by second PCR was digested with *Bam*HI and *Sall*, and introduced into pBS, designated pGLEGFP. A *Sall* fragment containing hygromycin-resistant *hph* gene in pSH75 was introduced into *Sall* site of pGLSGFP and pGLEGFP, designated as pHPGLSGFP and pHPGLEGFP respectively. The *SCD1* genome fragment was amplified from pCBSD by using M13RV and SDTAABM (5'-CGGGATCCAGTGAGCAGCGGCGAACTTG-3'). Amplified products was digested with *Bam*HI, and introduced into *Bam*HI site of pHPGLSGFP and pHPGLEGFP, and resultant plasmids were designated pHPGLSGFP-SD and pHPGLEGFP-SD respectively.

Conidia PCR

10⁴-10⁵ conidia were mixed in 100ul of lysing solution (2mg/ml Lysing Enzyme (Sigma), 10mM Tris-HCl (pH9.0), 50mM KCl, 2.5mM MgCl₂, 0.2% Triton X), and incubated at 24C for 1 h. First PCR was carried out by adding 4ul of conidia solution to each tube containing 26 ul of 10mM Tris-HCl (pH9.0), 50mM KCl, 2.5mM MgCl₂, 0.2% Triton X, 0.25mM each dNTP, 1.0mM each primer, 1.25 units of Taq polymerase. Samples are overlaid with 50ul mineral oil and subjected to the following PCR condition: 1 cycle of 95 C for 3 min / 55 C for 5 min / 72 C for 3 min; 30 cycles of 95 C for 40 sec / 55 C for 1 min / 72 C for 2 min; 1 cycle of 95 C for 40 sec / 55 C for 1 min / 72 C for 15 min. Primers used for first PCR were GFPAS1 (5'-CCGGTGGTGCAGATGAAC-3'), and M13 universal primer M4. First PCR products were subjected to nested PCR. Nested PCR was carried out by adding 4 ul of first PCR product

to 26 ul reaction solution. Reaction solution and condition of nest PCR were same as those of first PCR except primers used for PCR. Primers used for nest PCR were T7 primer and SDTAABM primer. Transformants which showed no amplified products were selected, and subjected to fluorescence microscopic observation and genomic DNA blot analysis.

RNA blot analysis

Procedure for isolation of total RNA was described in Chapter III. Conidia were harvested at 3 and 6 h after the start of incubation. To prepare RNA from conidia before the start of incubation (at 0 h), conidia harvested from cultures were directly subjected to RNA extraction. Probes of the GFP genes were produced from *Bam*HI digested pGLEGFP by use of T3 RNA polymerase. The DNA region used for in vitro transcription of *SCD1* was described in Chapter IV. RNA was blotted and hybridized following procedures described by Perpetua et al. 1966 with slight modification.

Genomic DNA blot analysis

Total DNA of *C. lagenarium* was isolated from mycelia as described in Chapter III. The DNA of wild type, transformant EGSD2, and transformant EGSD53 was digested with *Bam*HI, subjected to agarose gel electrophoresis, and transferred onto a Hybond N+ membrane (Amersham) as described by Sambrook et al. DNA probes were labelled with [α -³²P] dCTP using the BcaBEST TM labeling kit. 0.95 kb *Bam* HI fragment containing the *SCD1* gene in pHPGLEGFP-SD was used as a template of probe.

Microscopic observation

For light and fluorescence microscopy, a ZEISS Axioskop microscope was used. For

fluorescence microscopy, the ZEISS filter set for fluorescein isothiocyanate fluorescence was employed (BP 450-490 excitation filter, 510 nm dichroic and BP515-565 barrier filter).

Results

SCD1-EGFP fusion reporter system

In order to study accumulation and localization of melanin biosynthesis enzymes, scytalone dehydratase (SCD1), translational fusion gene of the *SCD1* gene with the green fluorescent protein (GFP) gene was constructed. Initially, an improved version of the GFP gene, SGFP-TYG was used. In SGFP-TYG, a Ser 65→Thr point mutation has been introduced in the chromophore domain, resulting in a GFP with a single excitation and emission peak, brighter fluorescence and more rapid chromophore generation. In addition the codon usage was optimized for higher plant. Recently, the SGFP-TYG gene was used as new vital marker in phytopathogenic fungus *Ustilago maydis*. Plasmid pHPGLSGFP-SD containing the *SCD1*-SGFP fusion gene where glycine spacer was inserted between both genes was constructed (Fig. V-1). Promoter region of the *SCD1* gene in pHPGLSGFP-SD was 185 bp. The pHPGLSGFP-SD was introduced into the wild type 104-T. As a result, most of hygromycin-resistant transformants showed green fluorescence, but intensity of their fluorescence was weak (data not shown), suggesting fluorescence intensity of the SCD1 fusion protein with SGFP-TYG was not enough to study accumulation and localization of the SCD1 protein in conidia. In an attempt to overcome the problem, a recently described improve version of GFP (Cormack et al. 1996), EGFP (Ser 65 Thr and Phe 64 Leu) was constructed from SGFP-TYG (Ser 65 Thr) by site directed mutagenesis. Next, SGFP-TYG in pHPGLSGFP-SD was replaced by EGFP, and resultant plasmid was designated pHPGLEGFP-SD (Fig. V-1). pHPGLEGFP-SD was

introduced into wild type by the transformation experiment. As a result, most of transformants containing pHPGLEGFP-SD exhibited strong green fluorescence in conidia and mycelia compared with that of transformants containing pGLSGFP-SD (Fig. V-2 and data not shown). Fluorescence intensity of EGFP was considered to be enough for study of accumulation and localization of SCD1 in conidia. To research whether the SCD1-EGFP fusion protein retain activity of scytalone dehydratase (SCD1), a complementation test of *SCD1* deleted mutant 9201Y by introduction of pHPGLEGFP-SD containing was performed. As a result of transformation of 9201Y with pHPGLEGFP-SD, transformants containing pHPGLEGFP-SD formed melanized mycelia (Fig. V-3). It suggested that the SCD1-EGFP fusion protein retained SCD1 activity.

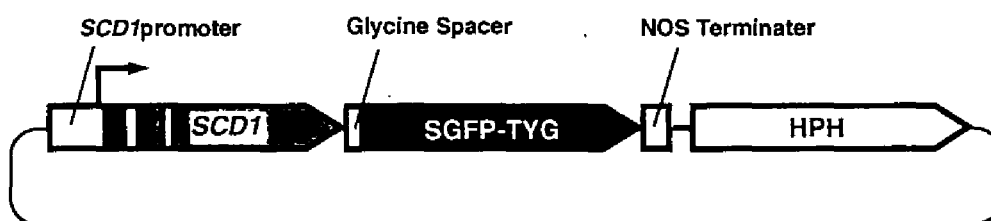
Accumulation and localization of SCD1-EGFP fusion protein during appressorium differentiation

In most of transformants containing pHPGLEGFP-SD, green fluorescence was observed in conidia before the start of incubation (Fig. V-2). This indicated that the *SCD1*-EGFP fusion gene was transcribed before the start of incubation of conidia, whereas the transcript of the *SCD1* gene was never detected at this time. Thus, it was estimated that promoter region of the *SCD1*-EGFP fusion gene contained in pHPGLEGFP-SD was not enough for regular transcription of the *SCD1* gene. In order to study accumulation and localization of the SCD1 protein under the regular transcriptional pattern of the *SCD1* gene, I screened a transformant where homologous recombination between the *SCD1*-EGFP fusion gene and the wild type *SCD1* gene occurred, resulting that the *SCD1*-EGFP fusion gene has full *SCD1* promoter (Fig. V-4). As a result of screening of 60 transformants by using conidia PCR, one candidate transformant EGSD53 was obtained. Recombination event between the *SCD1*-EGFP fusion gene and wild type *SCD1* gene in EGSD53 was researched by genomic DNA blot analysis (Fig. V-4). In wild type, 1.8kb

*Bam*HI fragment containing the *SCD1* gene was observed. In EGSD2, 1.8kb *Bam*HI fragment containing wildtype *SCD1* gene and 0.95 *Bam*HI fragment in pHPGLEGFP-SD were observed, suggesting that non-homologous integration occurred. On the other hand, 1.8kb *Bam*HI fragment was separated to 1.6 kb and 1.15 kb fragments in EGSD53, which were expected to be generated by homologous integration of pHPGLEGFP-SD (Fig. V-4). Thus, I considered that the *SCD1*-EGFP fusion gene in EGSD53 contained full *SCD1* promoter.

By RNA blot analysis, I researched whether the *SCD1*-EGFP fusion gene in EGSD53 was transcribed the same as the wild type *SCD1* gene (Fig. V-5). In EGSD53, the transcript of the fusion gene was not detected in conidia before the start of conidial incubation. Its transcript highly accumulated at 3 h, and it was hardly detected at 6 h. This suggested that transcriptional pattern of the fusion gene was almost same as that of the *SCD1* gene. I researched fluorescence pattern of the *SCD1*-EGFP fusion protein during appressorium differentiation in EGSD53. At 0 h, i.e., before the start of conidial incubation, no fluorescence was observed in EGSD53 whereas strong fluorescence was observed in EGSD2 (Fig. V-6). Until 4 h when conidia formed nonmelanized appressoria, no fluorescence was observed in EGSD53. At 5 h when appresorial melanization was started, fluorescence began to be observed. Intensity of fluorescence increased in parallel with progress of appresorial melanization (Fig. V-7). In appressorium-differentiating conidia of EGSD53, fluorescence was hardly detected in conidia, and localized in appressoria. On the other hand, in EGSD2, fluorescence was observed in both conidia and appressoria (Fig. V-8).

pHPGLSGFP-SD



pHPGLEGFP-SD

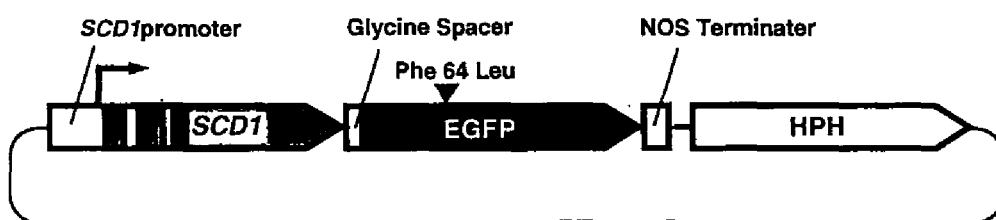


Figure V-1. Schematic representation of plasmid pHPGLSGFP-SD and pHPGLEGFP-SD.

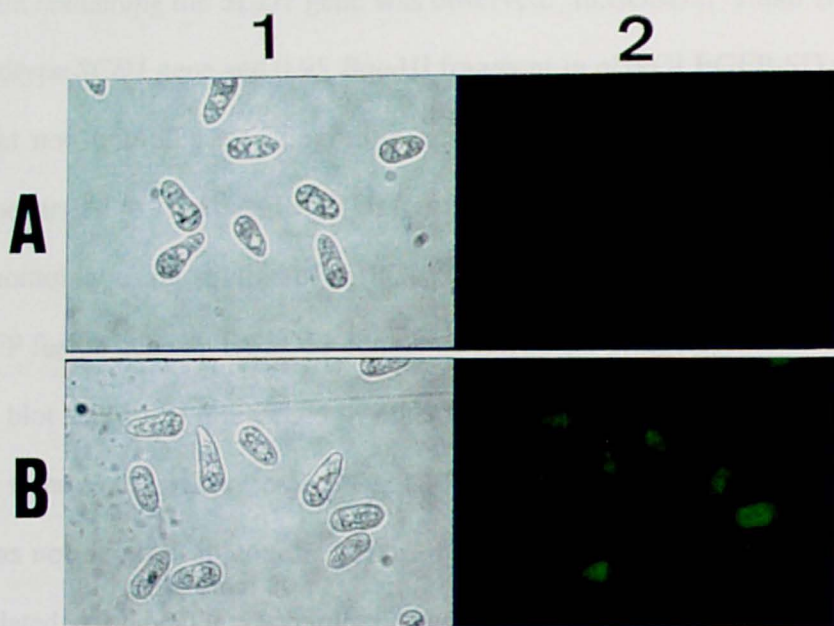


Figure V-2. Fluorescence of SCD1-EGFP in conidia of a transformant EGSD2. Conidia of wild type (A) and transformant EGSD2 (B) were observed before the start of incubation. Samples were observed before the start of incubation by light microscopy (panels 1) or epifluorescence microscopy (panels 2).

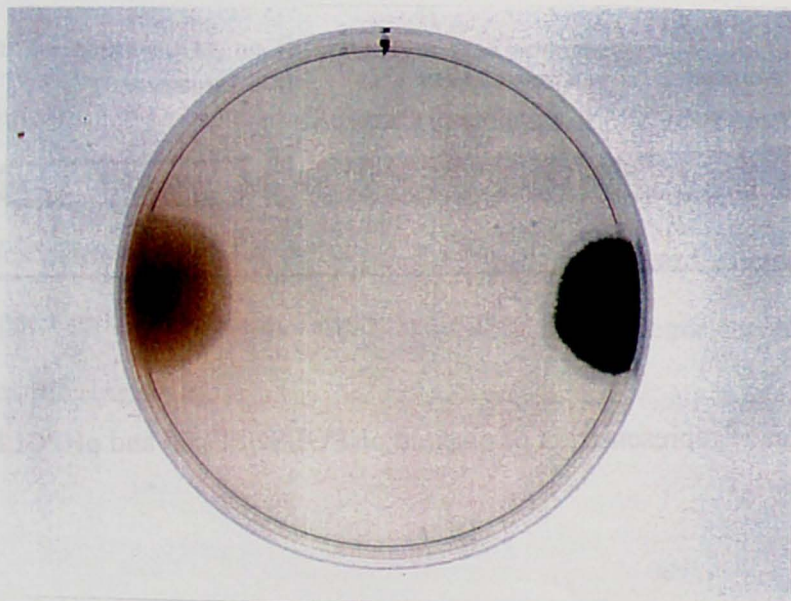


Figure V-3. Complementmentation of melanization of *SCD1* deleted mutant 9201Y by introduction of the *SCD1*-EGFP fusion gene. 9201Y (*scd1*-) was transformed with pHPGLEGFP-SD. 9201Y (left) and melanin-restored transformant of 9201Y with pHPGLEGFP-SD (right) were cultured on PDA medium for 4 days.

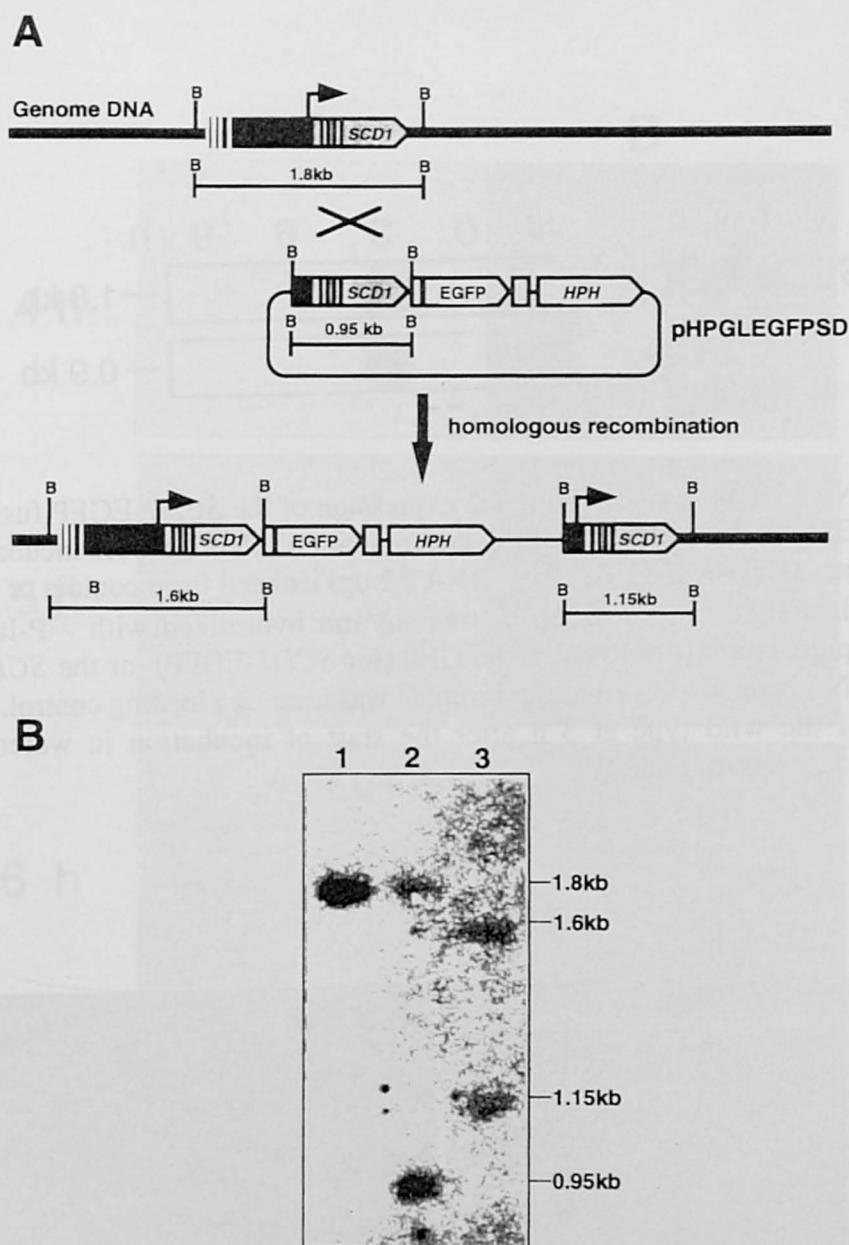


Figure V-4. Genomic DNA blot analysis of a transformant EGSD53.

(A) A scheme representing the exchange of the *SCD1* promoter in the wild type and the *SCD1*-EGFP promoter in pHPGLEGFPSD by homologous integration.

(B) Genomic DNA blot of *C. lagenarium* wild type, the transformant EGSD2, and the transformant EGSD53 using the ^{32}P -labeled 0.95 kb *Bam*HI fragment from pHPGLEGFPSD as a probe. Genomic DNAs were digested with *Bam*HI. 1.8 kb *Bam*HI fragment observed in the wild type was disrupted to 1.6 kb and 1.15 kb *Bam*HI fragments in EGSD53. Lanes: 1, the wild type 104-T; 2, the transformant EGSD2; 3, the transformant EGSD53. Abbreviations: B, *Bam*HI.



Figure V-5. RNA blot analysis showing the expression of the *SCD1*-EGFP fusion gene in the transformant EGSD53 during appressorium differentiation. Conidia were incubated in water or 0.1% yeast extract solution at 32°C. Total RNA (2 µg) isolated from conidia of EGSD53 were electrophoresed, blotted onto a nylon membrane and hybridized with ³²P-labeled in vitro transcripts complementary to mRNA of the GFP (for *SCD1*-EGFP) or the *SCD1* (for *SCD1*) genes. Total rRNA stained with ethidium bromide was used as a loading control. RNA isolated from conidia of the wild type at 3 h after the start of incubation in water at 24°C was electrophoresed as a control (lane W).

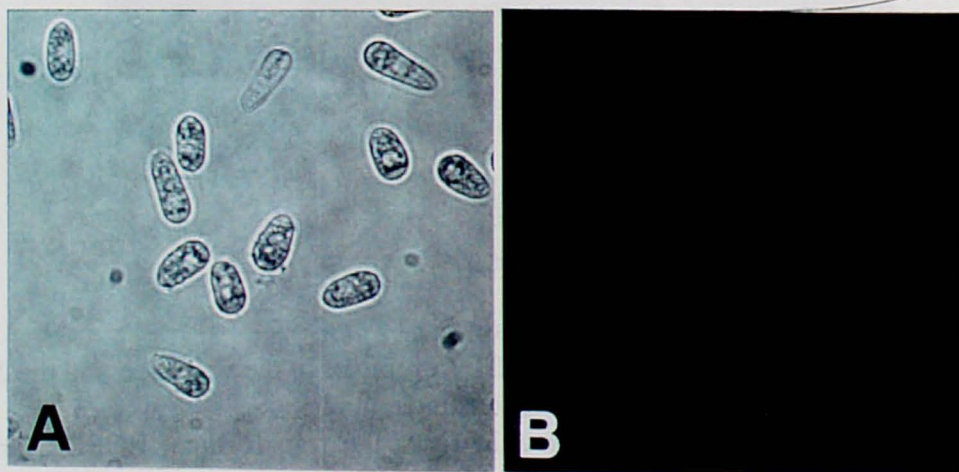


Figure V-6. Fluorescence of *SCD1*-EGFP in conidia of a transformant EGSD53. Conidia of transformant EGSD53 were observed before the start of incubation. Samples were observed before the start of incubation by light microscopy (A) or epifluorescence microscopy (B).

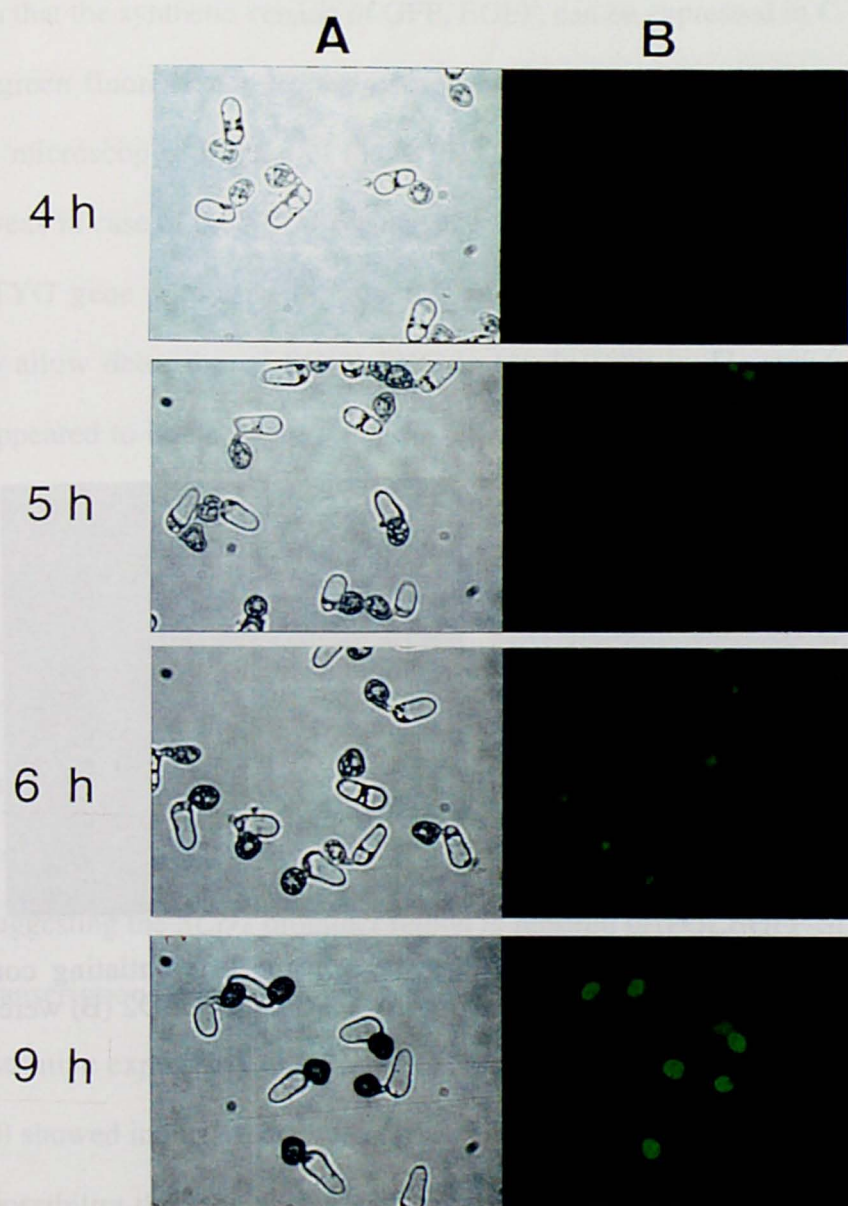


Figure V-7. Increase of fluorescence of SCD1-EGFP in conidia of a transformant EGSD53 during appressorium differentiation.. Conidia of transformant EGSD53 were observed at 4, 5, 6, and 9h after the start of incubation. Samples were observed by light microscopy (A) or epifluorescence microscopy (B).

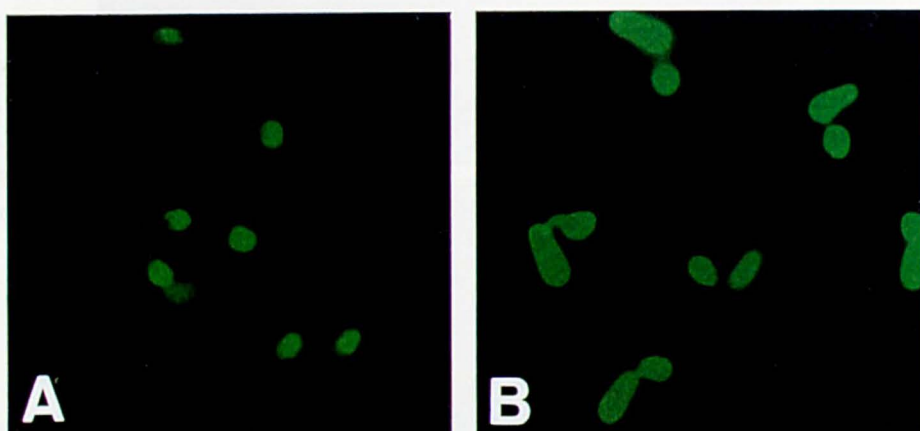


Figure V-8. Localization of fluorescence in appressorium-differentiating conidia of the transformant EGSD53. Conidia of transformant EGSD53 (A) or EGSD2 (B) were observed at 9h after the start of incubation by epifluorescence microscopy.

Discussion

I have shown that the synthetic version of GFP, EGFP, can be expressed in *C. lagenarium*, and its intrinsic green fluorescence is easily detected in the morphologically distinct forms by fluorescence microscopy. In case of the SGFP-TYG gene, fluorescence is detected but its intensity is weak in case of the *SCD1* fusion gene with the SGFP-TYG gene in *C. lagenarium*. The SGFP-TYG gene was used for reporter gene in phytopathogenic fungus *U. maydis*. However, to allow detection of SGFP-TYG in single cells in *U. maydis*, high levels of expression appeared to be necessary. On the other hand, EGFP showed strong fluorescence, enough to visualize accumulation and localization of target proteins in single cells in *C. lagenarium*. Thus, it is considered that the EGFP fusion protein system is useful to study accumulation and localization of target proteins in the level of single cells of filamentous fungi containing *C. lagenarium*. To study accumulation and localization of the *SCD1* protein, the *SCD1* fusion protein with EGFP was expressed in wild-type 104-T. However, in most of transformants, conidia showed fluorescence of *SCD1*-EGFP before the start of conidial incubation, suggesting the *SCD1* promoter region in plasmid pHPGLEGFP-SD was not enough for regular transcription of the *SCD1* gene. The *SCD1* promoter region in pHPGLEGFP-SD resulted constitutive expression whereas the *SCD1* gene containing full promoter region (not characterized) showed inductive expression after the start of conidial incubation. These results suggest the possibility that an upstream promoter region of the *SCD1* gene, not contained in pHPGLEGFP-SD, have a regulatory region of transcription of the *SCD1* gene. In transformant EGSD53, the transcriptional pattern of the *SCD1*-EGFP gene was almost identical to that of the *SCD1* gene during appressorium differentiation at 24°C. The fluorescence of the *SCD1*-EGFP fusion protein was observed just before the beginning of appressorial melanization (at 5 h). It

has been reported that formation of the fluorescent chromophore in S65T GFP protein takes 30 min. Thus, it is considered that the timing when the SCD1 protein started to accumulate is approximately at 4.5h, although the time of the fluorescent chromophore formation in SCD1-EGFP fusion protein is not investigated. In appressorium-differentiating conidia of the transformant EGSD53, fluorescence of the SCD1-EGFP fusion protein was localized in appressoria. On the other hand, in those of EGSD2, fluorescence was observed in both conidia and appressoria. This suggests that regular transcription of the *SCD1* gene is necessary for localization of SCD1 in appressoria. However, fluorescence in appressoria of EGSD53 was observed uniformly, thus, intracellular localization of fluorescence in appressoria was not found. Melanin layers are present outside plasma membrane. Perhaps, the SCD1-EGFP fusion protein might be localized outside plasma membrane. However, I could not omit the possibility that the fusion protein are abundant in cytoplasm. Confocal microscopical observation is necessary to evaluate intracellular localization of the SCD-EGFP fusion protein.

In *C. lagenarium*, several factors required for the penetration of its host plant by appressoria have been determined by using penetration-deficient mutants. Melanin biosynthesis (Kubo and Furusawa 1991), synthesis and secretion of cellulase (Suzuki et al. 1983) and penetration peg formation (Katoh et al. 1988; Perpetua et al. 1994) have been reported as these factors. Recently, several genes involved in infection process including appressorium differentiation and penetration have been identified in *Magnaporthe* and *Colletotrichum* species. The *PKS1*, *SCD1*, and *THR1* genes involved in appressorial melanization are isolated and characterized in *C. lagenarium* (Takano et al. 1995; Chapter II, Kubo et al. 1996; Perpetua et al. 1996). In *M. grisea*, cAMP signal transduction pathway and MAP kinase cascade are shown to be involved in pathogenicity (Dean et al. 1997). The *cpkA* gene encoding for cAMP dependent protein kinase is involved in appressorial penetration (Mitchell and Dean 1995; Xu et al. 1997). The *MAC1* gene encoding adenylate cyclase is involved in appressorium differentiation and other aspects of growth and development (Choi and Dean 1997). The *PMK1* gene encoding MAP kinase homologous to *Saccharomyces cerevisiae* *FUS3* is involved in appressorium differentiation and pathogenic growth (Xu and Hamer 1996).

The nucleotide sequence of the *PKS1* gene complementing the albino mutant of *C. lagenarium* was determined (Chapter II). The determination of the nucleotide sequences indicated that the *PKS1* gene encoded type-I polyketide synthase involved in pentaketide synthesis of melanin biosynthesis in *C. lagenarium*. The product synthesized by *PKS1* polyketide synthase has not been identified. Recently, *C. lagenarium* *PKS1* polyketide synthase was heterologously expressed by using *Aspergillus oryzae* expression system, and the product by *PKS1* in *A. oryzae* was identified as 1,3,6,8,-THN (Tsuji et al. 1998). It suggests that

PKS1 synthesize 1,3,6,8,-THN directly from acetate units. It has been estimated that conversion of 1,3,6,8-THN to scytalone is performed by another enzymes, 1,3,6,8-THN reductase (not identified) and 1, 3, 8-THN reductase (THR1) (Tsuji et al. 1998 and unpublished result).

A. alternata melanin biosynthesis gene *ALM* complemented the melanin biosynthesis and penetrating ability to cellulose membranes of the *PKS1* deficient albino mutant of *C. lagenarium* (Takano et al. 1997; Chapter III). A similar study was performed with *A. alternata* and *M. grisea*. The *ALM* gene complemented the melanin biosynthesis and pathogenicity of the albino mutant (*alb1*) of *M. grisea* (Kawamura et al. 1997). These results suggest that polyketide synthases involved in melanin biosynthesis are functionally conserved among melanin-producing fungi. However, the *ALM* gene could not enable the albino mutant of *C. lagenarium* to penetrate efficiently into the host plant. The electromicroscopic observation of appressoria indicated that the *ALM* transformants formed outer melanin layers but lacked inner and middle melanin layers. It is considered that inner and middle melanin layers have important roles for appressorial penetration into the host plant. In *M. grisea*, it is estimated that appressoria generate turgor pressures of up to 8.0 MPa because of accumulation of glycerol in appressoria (Howard et al. 1991). De Jong have now confirmed that melanized appressoria are impermeable to glycerol, and also that glycerol permeates the appressorial wall of two isogenic melanin-deficient mutant of *M. grisea* (De Jong et al. 1997). It is mentioned that glycerol molecules diffuse from cytoplasm through the plasma membrane, but are trapped close to the outer surface of the membrane by inner melanized layer of the cell wall (Money 1997). Thus, it is considered that glycerol permeates the appressorial walls in the *ALM* transformants because of lack of inner and/or middle melanin layers, resulting that the turgor pressure in the *ALM* transformants could not reach the level enough for penetration into the host plant. It is necessary to compare the turgor pressure between *C. lagenarium* wild type and the *ALM* transformants. Also, the *ALM*

transformants formed outer melanin layers and penetrated into cellulose membranes the same as the wild type whereas the albino mutant hardly penetrated. It suggests that formation of outer melanin layers is enough for penetration into cellulose membranes. It remains to elucidate the role of outer melanin layers for appressorial penetration.

Temporal transcriptional pattern of three melanin biosynthesis genes *PKS1*, *SCD1*, and *THR1* was investigated in appressorium-differentiating conidia, and nondifferentiating conidia (Takano et al. 1997; Chapter IV). During appressorium differentiation, melanin biosynthesis genes were transcribed at 1 to 2 h after the start of conidial incubation. This period is early stage of appressorium differentiation; some conidia germinated, and others did not form germ tubes yet. Analysis of accumulation of the *SCD1* fusion protein with green fluorescent protein (GFP) suggested that the *SCD1* transcript was translated at the beginning of appressorial melanization (at 4.5 h). It seems that there is a time lag between transcription (at 2 h) and translation (at 4.5 h) of the *SCD1* gene. The transcriptional pattern of the melanin biosynthesis genes in non-differentiating conidia incubated in water at 32°C was similar to that in appressorium-differentiating conidia although melanization was never observed in non-differentiating conidia. It suggested that post-transcriptional regulation i.e. translational and/or post-translational regulation was involved in appressorial melanization. Study of accumulation of the *SCD1* protein by using GFP and study of transcriptional pattern in nondifferentiating conidia strongly suggested that translational regulation of melanin biosynthesis gene *SCD1* should be involved in appressorial melanization. Study of the translational pattern of *SCD1* during appressorium differentiation by using antibody against *SCD1* protein is needed to evaluate translational regulation to the *SCD1* gene. It is also necessary to investigate whether another melanin biosynthesis genes, *PKS1* and *THR1* are regulated at post-transcriptional level by using GFP system and antibody against each enzyme.

In nondifferentiating conidia incubated in 0.1% yeast extract solution, the melanin

biosynthesis genes were hardly transcribed. This result suggested that transcription of melanin biosynthesis genes was induced by the start of the distinct developmental process. Comparison of accumulation of the SCD1-GFP fusion protein between the transformant EGSD2 showing constitutive expression and EGSD53 showing induced expression suggested that regular transcription of the *SCD1* gene enabled accumulation of SCD1 to be confined to appressoria. Thus, transcriptional regulation of melanin biosynthesis genes play pivotal roles in appressorial melanization. Recently, *CMR1* gene encoding a putative transcriptional factor for melanin biosynthesis genes has been isolated and characterized (Kenmochi et al. 1997). The *CMR1* deficient mutant accumulated scytalone in culture media; however, it produced normally melanized appressoria, suggesting that CMR1 is a transcriptional factor involved in mycelial melanization. Identification and characterization of transcriptional factors involved in appressorial melanization may be necessary to elucidate transcriptional regulation of melanin biosynthesis genes. Transcriptional and translational regulations of melanin biosynthesis genes in conidia are considered to be strongly related with morphological developmental process, i.e., appressorium differentiation. In *C. lagenarium*, it remains to elucidate signal transduction pathways involved in appressorium differentiation. To elucidate the relation between signal transduction pathway for appressorium differentiation and the transcriptional regulation of melanin biosynthesis genes should enhance total understanding of appressorial melanization and appressorium differentiation.

SUMMARY

I. Structural analysis of *PKS1*, polyketide synthase gene involved in melanin biosynthesis.

Albino mutants of *Colletotrichum lagenarium* form nonmelanized appressoria and possess little penetrating ability to the host plant. The defective site of the albino mutant 79215 is considered to be pentaketide biosynthesis and/or pentaketide cyclization in melanin biosynthesis. A cosmid pAC7 carrying the *PKS1* gene transforms the albino mutant to melanin restored phenotype. I resolved the DNA sequence and the transcriptional architecture of the *PKS1* gene. The *PKS1* gene contains one open reading frame, consisting of 3 exons separated by two short introns. The predicted *PKS1* polypeptide consists of 2187 amino acids and has significant similarities with polyketide synthases, particularly *Aspergillus nidulans* *wA* involved in conidial pigmentation. The *PKS1* gene contains highly conserved β -ketoacyl synthase, acetyl/malonyl transferase, and acyl carrier protein domains. The *C. lagenarium* *PKS1* gene encodes a polyketide synthase involved in melanin biosynthesis.

II. Restoration of appressorial melanization and penetration of cellulose membranes in the melanin-deficient albino mutant with the *Alternaria alternata* melanin biosynthesis gene.

Colletotrichum lagenarium and *Alternaria alternata* produce a dark pigment, melanin. The *C. lagenarium* *PKS1* and *A. alternata* *ALM* genes are involved in polyketide synthesis in the melanin biosynthesis pathway. *PKS1* encodes a type I polyketide synthase. For functional comparison of the *ALM* gene with the *PKS1* gene, I examined whether the *A. alternata* *ALM* gene could restore melanin synthesis in *C. lagenarium* albino mutant (*pks1*⁻). The *ALM* gene

transformed the albino mutant (*pks1*⁻) to melanin-producing phenotype, designated CAL transformants. Pigment intensity of both melanized colonies and appressoria of CAL transformants was weaker than that of the wild type. Ultrastructural studies of the cell walls of appressoria demonstrated that CAL transformants formed an outer melanized layer as the wild type. On the other hand, thin inner and middle melanin layers were less electron dense compared with those of wild type. CAL transformants were able to penetrate cellulose membranes as effectively as the wild type. By contrast, the penetration frequency of CAL transformants on the cucumber cotyledons was remarkably reduced compared to wild type. During conidial germination, the *PKS1* transcript accumulated de novo in both the wild type and CAL transformants after the start of conidial incubation. On the other hand, the *ALM* transcript accumulated in conidia of CAL transformants before the start of conidial incubation.

III. Temporal transcriptional pattern of three melanin biosynthesis genes, *PKS1*, *SCD1*, and *THR1*, in appressorium-differentiating and nondifferentiating conidia.

A phytopathogenic fungus, *Colletotrichum lagenarium* produces melanized appressoria that display temperature-sensitive differentiation. Conidia incubated in water at 24°C germinated and germ tubes differentiated into melanized appressoria. On the other hand, conidia in water at 32°C germinated and elongated germ tubes without appressorium differentiation. Conidia in 0.1% yeast extract solution at 32°C germinated and developed into vegetative hyphae. I investigated the temporal transcriptional pattern of cloned melanin biosynthesis genes, *PKS1*, *SCD1*, and *THR1* in these differentiating and non-differentiating conidia. During appressorium differentiation, de novo transcripts of the three melanin biosynthesis genes accumulated by 1-2 h after the start of conidial incubation at 24°C and began to decrease at 6 h. In conidia elongating germ tubes in water at 32°C, the transcriptional pattern of the *PKS1*, *SCD1*, and *THR1* genes

was similar to that in appressorium-differentiating conidia, although no melanization was observed. However, in conidia in 0.1% yeast extract solution at 32°C, transcripts of the three melanin biosynthesis genes hardly accumulated.

IV. Studies on accumulation and localization of melanin biosynthesis enzyme SCD1 during appressorium differentiation by using SCD1 fusion protein with green fluorescent protein (GFP)

In order to accumulation and localization of melanin biosynthesis enzymes during appressorium differentiation, fusion protein reporter system with green fluorescent protein (GFP) was developed. The SCD1 fusion gene with improved version of GFP, EGFP (Phe 64 Leu, Ser 65 Thr) was expressed in wild type, resulting that strong fluorescence was observed in transformants expressing SCD1-EGFP fusion protein. The *SCD1*-EGFP fusion gene complemented melanin biosynthesis of *SCD1* deficient mutant, suggesting the SCD1-EGFP fusion protein retained function of SCD1. The transcriptional pattern of the *SCD1*-EGFP gene was similar to that of the wild-type *SCD1* gene in the transformant EGSD53 where short *SCD1* promoter of *SCD1*-EGFP fusion gene was replaced with full *SCD1* promoter through homologous recombination. In transformant EGSD53, fluorescence of *SCD1*-EGFP was observed just before appressorial melanization. Fluorescence was confined to appressoria in appressorium-differentiating conidia of the transformant EGSD53 whereas fluorescence was observed in both conidia and appressoria in the transformant expressing fusion gene constitutively.

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