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Cloning of a Melanin Biosynthetic Gene Essential for Appressorial Penetration of *Colletotrichum lagenarium*

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We constructed a cosmid vector pKVβ for isolating genes by complementation of mutations in *Colletotrichum lagenarium*. pKVβ contains the bacteriophage λ *cos* site and the benomyl-resistant *C. lagenarium* β-tubulin gene as a selective marker for *Colletotrichum* transformation. A genomic DNA library of wild-type *C. lagenarium* was constructed in pKVβ. An albino mutant strain 79215 was transformed with DNA from this library and benomyl-resistant transformants were obtained at frequencies of approximately 20 transformants per microgram of DNA. Seven melanin-restored transformants were obtained from approximately 10,000 benomyl-resistant transformants. Albino mutants of *C. lagenarium* form nonmelanized appressoria and possess little penetrating ability. However, the transformants formed melanized appressoria with the ability to penetrate as efficiently as in the wild-type strain. From genomic DNA of a melanin-restored transformant integrated cosmid sequences (pAC7) were recovered by transduction of *Escherichia coli* to ampicillin resistance following treatment *in vitro* with λ packaging extract. pAC7 transformed albino mutant 79215 to a melanin-restored wild phenotype with high frequency. From structural analysis of pAC7, an 8.4-kb *Bam*HI fragment of pAC7 contains a wild-type copy of the gene involved in albino phenotype.

Colletotrichum lagenarium (Pass.) Ellis & Halsted is the causal agent of anthracnose of cucumber. Factors required for the penetration of host plant cell walls by appressoria of *C. lagenarium* have been determined using penetration-deficient mutants. These factors include melanin biosynthesis (Kubo and Furusawa 1986; Kubo *et al.* 1982a, 1982b, 1983, 1985, 1986, 1987; Suzuki *et al.* 1982b), synthesis and secretion of cellulase (Suzuki *et al.* 1981, 1982a, 1983; Katoh *et al.* 1988) and penetration peg formation (Katoh *et al.* 1988).

To demonstrate from the molecular genetic approach those metabolisms essential for penetration by appressoria requires construction of the transformation system and cloning vectors. In fungi, several chemicals have been used as selective agents for transformation. The antimicrotubule compound benomyl is one of those selective agents and β-tubulin genes conferring benomyl resistance have been cloned from several fungi including *Saccharomyces cerevisiae* Hansen (Thomas *et al.* 1985), *Neurospora crassa* Shear *et* Dodge (Orbach *et al.* 1986), *Aspergillus nidulans* (Eidam) G. Wint. (Sheir-Neiss *et al.* 1978), and *Colletotrichum graminicola* (Ces.) G. W. Wils. (Panaccione *et al.* 1988). In this experiment, we cloned a β-tubulin gene from a benomyl-resistant mutant of *C. lagenarium* using the *tub-2* gene of *N. crassa* as a probe and constructed cosmid vector pKVβ, which contains the cloned β-tubulin gene as a selective marker.

Complementation of mutations in *A. nidulans* (Yelton *et al.* 1985) and *N. crassa* (Vollmer and Yanofsky 1986) by transformation with recombinant DNA libraries constructed in a cosmid vector provided a convenient and valuable system for the isolation of specific genes of interest. The melanin biosynthetic genes are a good subject to study, because melanin biosynthesis is a metabolism essential for pathogenicity in *Colletotrichum* and *Pyricularia* species, melanin biosynthesis is a developmentally regulated system, the biosynthetic pathway is known, and several melanin-deficient mutants isolated are available (Kubo and Furusawa 1991). We have constructed a genomic DNA library of *C. lagenarium* and transformed an albino mutant, with the library DNA for complementation of the defect in melanin biosynthesis. As in the general case of filamentous fungi (Yelton *et al.* 1985; Vollmer and Yanofsky 1986), the *C. lagenarium* transformation system is an integration of transforming DNA sequences in the genomic DNA. In the *A. nidulans* system, integrated cosmid sequences were isolated by transduction of *Escherichia coli* to ampicillin resistance with the genomic DNA following treatment *in vitro* with λ packaging extract (Yelton *et al.* 1985).

In the experiment reported here, a wild-type copy of the albino gene was cloned from the genomic DNA of a melanin-restored transformant; the penetrating ability of the transformants was also investigated.

MATERIALS AND METHODS

Fungal strains. *C. lagenarium* strain 104-T (stock culture of the Laboratory of Plant Pathology, Kyoto University) was used as a wild-type strain. Albino mutant strain 79215 was isolated by ultraviolet light irradiation of spores (Kubo *et al.* 1982a). Benomyl-resistant mutants were selected on potato-dextrose agar medium containing 10 μg of benomyl following ultraviolet light irradiation of the spores of 104-T as described (Kubo *et al.* 1982a). Benomyl was made up fresh as a 1 mg/ml stock solution in 100% ethanol and was added to media after autoclaving. A benomyl-resistant mutant designated Bmr-1 was used as the source of benomyl-resistant β-tubulin gene to be cloned.

Preparation of DNA. Genomic DNA was isolated from

protoplasts by scaling up the method of Holm *et al.* (1986). Protoplasts prepared from mycelia as described below were used for DNA isolation. More than 500 μg of DNA was obtained from protoplasts prepared from 250 ml of liquid culture by this procedure. *E. coli* strain DH5α was used for routine propagation of plasmids and cosmids. For preparation of cosmid library DNA for fungal transformation, approximately 12,000 ampicillin-resistant transductants were grown in petri dishes and the bacterial cells were inoculated to 1 L of L broth containing ampicillin at 100 μg/ml. After 8 hr of incubation at 37° C, cosmid DNA was isolated by using standard procedures (Sambrook *et al.* 1989).

Cloning of β-tubulin DNA from *C. lagenarium* Bmr-1. A genomic DNA library of *C. lagenarium* strain Bmr-1 was constructed in the bacteriophage vector λ Fix following the conventional methods (Stratagene Cloning Systems, La Jolla, CA) (Sambrook *et al.* 1989). Packaged DNA libraries were plated on strain *E. coli* P2392 and recombinant phage that contained sequences with homology to the *N. crassa tub-2* gene were identified by plaque hybridization experiments according to the condition described by Orbach *et al.* (1986). pSV50 DNA contains the *N. crassa tub-2* gene and was used as a probe (Vollmer and Yanofsky 1986). Nitrocellulose filters were washed in 2× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate), 1% sodium dodecyl sulfate at 55° C for 30 min three times.

Fungal transformation. Mycelium for protoplasts was generated by incubating spores for 3 days at 25° C in 250 ml of potato-sucrose liquid media supplemented with 0.2% yeast extract by the method of Rodriguez and Yoder (1987). The mycelium was harvested by filtration through sterile gauze and treated with 20 ml of enzyme solution containing 10 mg/ml of Novozyme 234 (Novo Laboratories, Wilton, CT) in 1 M $MgSO_4$ and 10 mM Na_2HPO_4. Transformation experiments were performed following the procedure described by Vollmer and Yanofsky (1986). Plasmid or cosmid library DNA (10 μg) was mixed with 2 μl of 0.5 M spermidine 3HCl, 1 μl of 2 M spermine and 3 μl of heparin (50 mg/ml), which were made in STC (1 M sorbitol/50 mM Tris-HCl, pH 8.0/50 mM $CaCl_2$) solution. The DNA solution was added to 100 μl of protoplast suspension (5×10^7 protoplasts in STC) and the resulting suspension was incubated for 10 min at room temperature. This was followed by the addition of 1 ml of 60% polyethylene glycol (PEG) 4,000/50 mM Tris-HCl, pH 8.0/50 mM $CaCl_2$ and incubated for 20 min at room temperature. After removal of PEG solution by centrifugation, protoplasts were suspended in 1 ml of STC solution and mixed with 3 ml of regeneration agar (potato-dextrose agar osmotically conditioned with 1 M glucose). The mixture was poured onto 25 ml of regeneration agar plate containing 1.2 μg/ml of benomyl. The plate was incubated at 24° C and benomyl-resistant colonies appeared in 5 days and individual conidial isolates were obtained by plating dilutions of conidial suspensions on potato-sucrose agar medium.

Genomic Southern blot hybridization. Genomic DNA was digested with a restriction enzyme and transferred to Hybond N+ (Amersham Corp., Arlington Heights, IL) nylon membrane filters from agarose gel by the method of Southern (1975). After alkali fixation of the Southern blot, hybridization with nonradioactive chemi-probe and detection of specific sequences were made by the ECL gene detection system according to protocol given by the Amersham Corp.

Penetration and pathogenicity tests. The penetration ability by appressoria was tested on cellulose membranes, and the pathogenicity test to the host cucumber leaves was carried out by the procedures as described previously (Kubo *et al.* 1982b).

RESULTS

Cloning of β-tubulin DNA from benomyl-resistant *C. lagenarium*. Seven phage clones containing β-tubulin DNA fragments were selected from 20,000 recombinant phage clones from genomic DNA library of *C. lagenarium* Bmr-1 constructed in λ Fix by using pSV50, a cosmid containing the *N. crassa tub-2* gene, as a probe. Three of the seven phage clones (λBm-1, λBm-3, and λBm-4) were digested with *Xba*I and analyzed by Southern blot hybridization with pSV50. *Xba*I fragments (5.4, 6.4, and 6.4 kb) that hybridized to pSV50 were identified from λBm-1, λBm-3, and λBm-4 phage clones, respectively (Fig. 1). A 6.4-kb *Xba*I fragment from Bm-4 was subcloned in pUC19 and designated as pCTUB29 (Fig. 1). The 6.4-kb *Xba*I fragment contains 3.0-kb *Hin*dIII and 0.9- and 2.5-kb *Hin*dIII/*Xba*I fragments, pSV50 hybridized to the 2.5-kb *Hin*dIII/*Xba*I fragment only. The 3.0-kb *Hin*dIII and 0.9-kb *Hin*dIII/*Xba*I fragments in the 6.4-kb *Xba*I fragment of pCTUB29 were deleted and the resultant plasmid was designated as pCTUB1 (Fig. 1).

Transformation of *C. lagenarium* to benomyl resistance. *C. lagenarium* 104-T was transformed with pCTUB29 and pCTUB1. pCTUB29 transformed 104-T protoplast to benomyl resistance effectively. The transformation efficiency with pCTUB29 was about 20 transformants per microgram of DNA. This result indicated that the 6.4-kb *Xba*I fragment in pCTUB29 contains the complete β-tubulin gene that confers benomyl resistance. On the other hand, although pCTUB1 contains the 2.5-kb *Hin*dIII fragment that hybridized specifically with pSV50, this plasmid did not transform 104-T to benomyl resistance. Because the *N. crassa tub-2* gene was not completely homologous to *C. lagenarium* β-tubulin gene, a small overlap with the neighboring 3.0-kb *Hin*dIII fragment could not be detected by Southern blot hybridization analysis with pSV50.

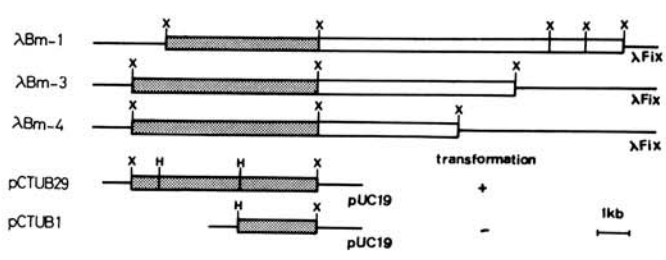

Fig. 1. Restriction map of *Colletotrichum lagenarium* β-tubulin DNA. The bar indicates the *C. lagenarium* DNA clones in each recombinant phage. The shaded region hybridized with the *Neurospura crassa tub-2* probe. In each recombinant phage cone, *Xba*I sites flanking inserted *C. lagenarium* DNA are restriction sites from λ Fix vector. X, *Xba*I; H, *Hin*dIII.

Hybridization analysis of benomyl-resistant transformants. Genomic DNA of *C. lagenarium* 104-T and the benomyl-resistant transformants was isolated and digested with *Xba*I. After electrophoresis, Southern blots were probed with the 6.4-kb *Xba*I fragment from pCTUB29. In nontransformed 104-T, a 7.4-kb DNA fragment was recognized (Fig. 2), and this fragment was indicative of the resident β-tubulin DNA in the genome. In transformants, in addition to the 7.4-kb *Xba*I fragment, several novel bands were recognized (Fig. 2). When pUC19, the vector used for cloning of β-tubulin DNA, was used as a probe, several bands were also identified in the transformants. In nontransformed control, no bands were detected. These results indicate that multiple copies of the plasmid were integrated in the fungal genomic DNA. The degree of resistance among transformants was different (data not shown). This may be correlated to the copy number of the integrated β-tubulin DNA in addition to its position effects.

Construction of cosmid vector and genomic DNA library. The cosmid vector pKVβ (Fig. 3) was constructed from pCTUB29. pCTUB29 was digested with *Bam*HI and a 1.6-kb *Bgl*II fragment that contains bacteriophage λ *cos* site was introduced from a cosmid pLAFR3 (Staskawicz *et al.* 1987). The resulting plasmid pKVβ was digested with *Sma*I and *Bam*HI linker was introduced to produce a cosmid pKVβ that possesses a unique *Bam*HI cloning site. The genomic DNA library of *C. lagenarium* 104-T was constructed using the cosmid pKVβ following conventional methods. Genomic DNA was partially digested with *Sau*3A1 and 30- to 40-kb DNA was sized-fractionated with 10–40% sucrose density gradient centrifugation, The DNA was ligated to pKVβ and introduced to DH5α following treatment with λ packaging extract.

Complementation of an albino gene by cosmid DNA. *C. lagenarium* albino mutant 79215, which is considered to be defective in the process of pentaketide cyclization in the melanin biosynthetic pathway (Kubo *et al.* 1983) (Fig. 4) was transformed by using DNA from the wild-type 104-T cosmic library. From approximately 10,000 benomyl-resistant transformants, seven dark brown melanin-restored transformants were identified. These transformants formed dark brown colonies similar in appearance to that of wild-type strain 104-T (Fig. 5). From one of the transformants designated AC-7, an integrated cosmid sequence was recovered by transducing *E. coli* to ampicillin resistance with the genomic DNA following immediate treatment *in vitro* with λ packaging extract. From a 5-μg genomic DNA treatment, 1,800 ampicillin-resistant *E. coli* transformants were obtained. Cosmid DNA was isolated

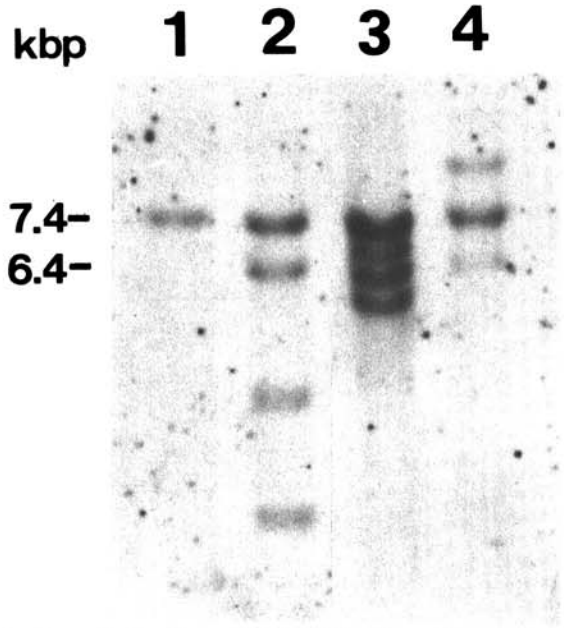

Fig. 2. Genomic Southern blot hybridization of benomyl-resistant transformants of *Colletotrichum lagenarium*. Genomic DNA digested with *Xba*I was electrophoresed with 0.7% agarose gel and transferred to Hybond N+. The DNA was probed with chemically labeled 6.4-kb *Xba*I β-tubulin fragment of pCTUB29 and specific sequences were detected by the ECL detection system of the Amersham Corp. Lane 1, wild-type strain 104-T; lanes 2–4, Benomyl-resistant transformants of strain 104-T.

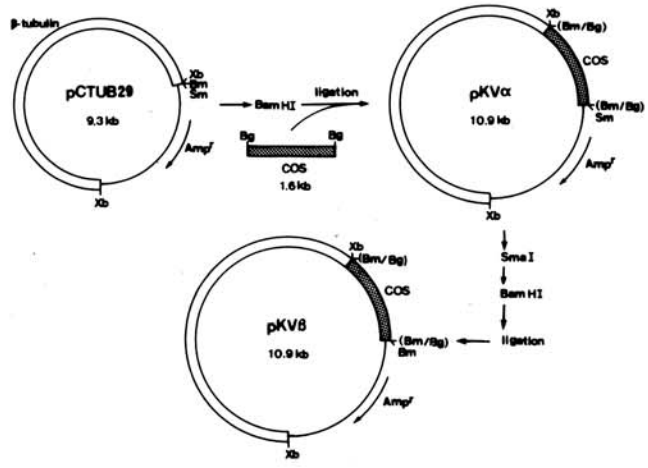

Fig. 3. Construction of a cosmid vector pKVβ. pCTUB29 was digested with *Bam*HI, and a 1.6-kb *Bgl*II fragment that contains bacteriophage λ *cos* site was introduced from a cosmid pLAFR3 (Staskawicz *et al.* 1987). The resulting plasmid pKVβ was digested with *Sma*I, and *Bam*HI linker was introduced to produce a cosmid pKVβ, which possesses a unique *Bam*HI cloning site. Xb, *Xba*I; Bm, *Bam*HI; Sm, *Sma*I; Bg, *Bgl*II.

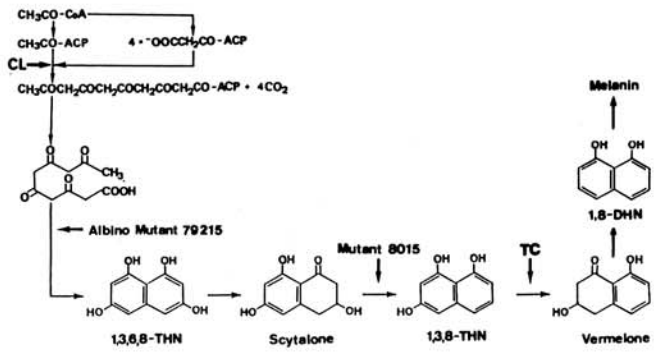

Fig. 4. Biosynthetic pathway of melanin in *Colletotrichum lagenarium* with deficient site of mutants and inhibition sites by chemicals (Kubo *et al.* 1983, 1985, 1986). 1,3,6,8-THN, 1.3.6.8- tetrahydroxynaphthalene; 1.3.8-THN, 1.3.8-trihydroxynaphthalene; 1, 8-DHN, 1, 8-dihydroxynaphthalene; CL, cerulenin; TC, tricyclazole.

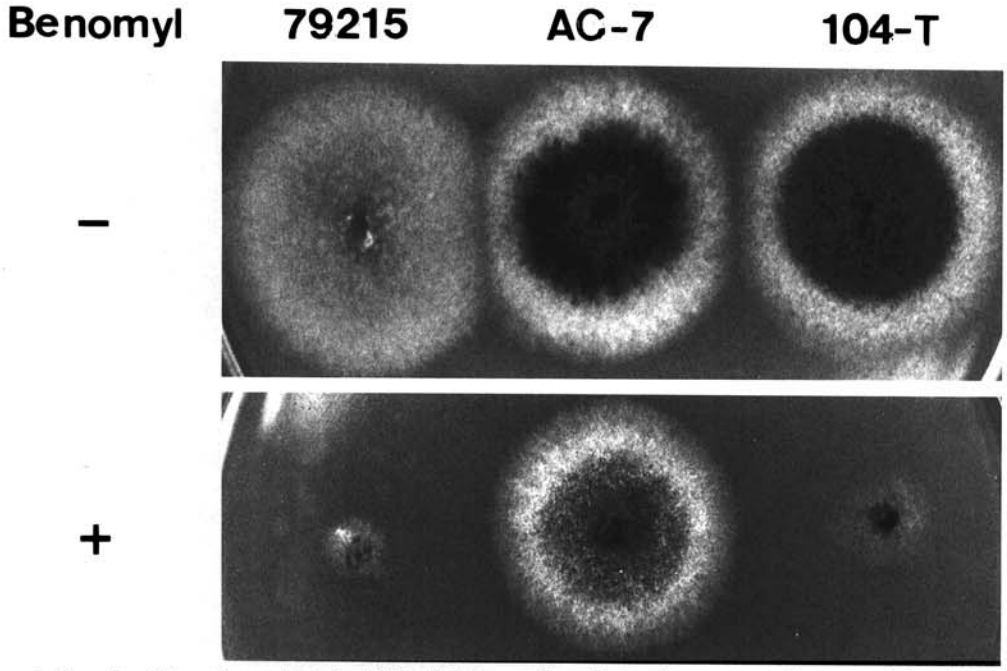

Fig. 5. Growth of a melanin-restored transformant strain AC-7 of *Colletotrichum lagenarium* on a medium containing 0.5 μg/ml benomyl. 104-T is a wild-type strain and 79215 is an albino strain derived from 104-T.

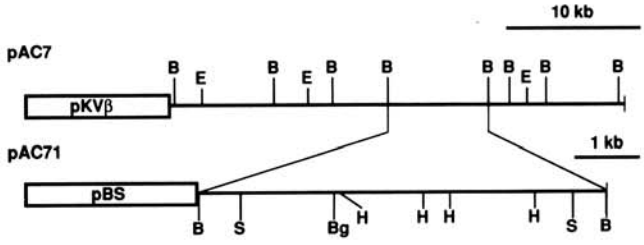

Fig. 6. Restriction maps of pAC7 and pAC71. pKVβ is a cosmid vector in which the genomic library of *Colletotrichum lagenarium* was constructed. An 8.4-kb *Bam*HI fragment which was involved in restoration of melanization to albino mutant strain 79215 was subcloned into pBS vector. B, *Bam*HI; E, *Eco*RI; S, *Sma*I; H, *Hin*dIII; Bg, *Bgl*II.

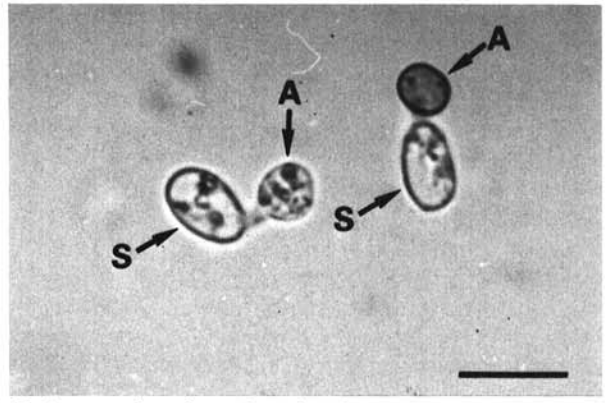

Fig. 7. Appressorium formation of albino mutant strain 79215 and its melanin-restored transformant strain AC-7 of *Colletotrichum lagenarium*. Left, strain 79215; right strain AC-7. Spores were incubated on a glass slide at 24° C for 12 hr. S, spores; A, appressoria.

from 12 of those transductants and subjected to restriction analysis. All but one plasmid showed identical electrophoretic patterns following digestion with *Bam*HI. One cosmid designated pAC7 was used to transform albino mutant 79215. pAC7 transformed the mutant effectively to a melanin-restored wild phenotype; 20–30% of benomyl-resistant transformants were melanin restorants. No reversion of albino to wild-type in regenerated protoplast that have been treated with pKVβ only was observed. From this result, it is indicated that the cosmid pAC7 contains a wild-type copy of albino gene. The *Bam*HI fragments of pAC7 were ligated into pBS. Albino mutant 79215 was cotransformed with each subcloned plasmid and pCTUB29, which contains benomyl-resistant β-tubulin gene as a selective marker. The combination of pAC71 (Fig. 6) that contains 8.4-kb *Bam*HI fragment with pCTUB29 transformed the albino mutant 79215 to a wild phenotype at the frequency of approximately 0.1 cotransformants per microgram of DNA. This indicated that the 8.4-kb *Bam*HI fragment (Fig. 6) contains a gene involved in restoration of melanization to the albino mutant.

Appressorium formation and penetration ability of transformants. Albino mutants of *C. lagenarium* form colorless appressoria and have little penetrating ability by germinating laterally from appressoria (Kubo *et al.* 1982a, 1983). The melanin-restored transformants formed darkly melanized appressoria indistinguishable from those formed by the wild-type strain 104-T (Fig. 7). These appressoria penetrated as effectively as those formed by the strain 104-T (Table 1). These transformants also have pathogenicity to the host cucumber plants (Fig. 8). The extent of the pathogenicity was the same as in the wild-type strain. The results clearly show that a functional wild-type copy of albino gene product is essential for pathogenicity.

DISCUSSION

We constructed a cosmid vector pKVβ that contains a benomyl-resistant β-tubulin gene of *C. lagenarium* as a selective marker for transformation. Benomyl resistance has been used as a selective marker for transformation for some fungi including *N. crassa* (Vollmer and Yanofsky 1986) and *C. graminicola* (Panaccione *et al.* 1988). Because the benomyl-resistant β-tubulin gene is reported to be functional in heterologous fungi (Vollmer and Yanofsky 1986; Panaccione *et al.* 1988), it seems natural that pSV50 containing benomyl-resistant β-tubulin gene of *N. crassa* should be functional also in *C. lagenarium*. However, there is a possibility that heterologous structural protein may not be fully functional in fungal cells so that the transformation frequency may be affected. This was avoided in our experiments by using β-tubulin gene cloned from a resistant strain of *C. lagenarium*. Cloning of a gene by complementation of a mutation with wild-type genomic library DNA, transformation efficiency is a critical point for effective cloning. The transformation efficiency of *C. lagenarium* using the benomyl-resistant β-tubulin gene was around 20 transformants per microgram of DNA. This value is quite sufficient for cloning of a gene by complementation of mutations. Melanin biosynthesis of *C. lagenarium* offers several interesting elements and advantages for the molecular genetic approach of plant pathogenic fungi; first, melanin biosynthesis is a metabolism essential for pathogenicity in *Colletotrichum* and *Pyricularia* species (Kubo and Furusawa 1990); second, melanin biosynthesis is a developmentally regulated system (Kubo *et al.* 1984); third, the biosynthetic pathway is known (Kubo *et al.* 1986); fourth, several melanin-deficient mutants isolated are available (Kubo *et al.* 1983).

By transformation of albino mutant strain 79215 with genomic library DNA of the wild-type strain, we got seven melanin-restored transformants out of approximately 10,000 benomyl-resistant transformants. This is quite a reasonable value assuming that the insert size in the cosmids is 40 kb and also that the genome size of *C. lagenarium* is around 40,000 kb (estimated from the genome size of *N. crassa* and other filamentous Ascomycetes).

The cloned DNA pAC7 and pAC71 from the genome of a melanin restorant, effectively transformed albino mutant strain 79215 to wild phenotype. In principle, most of the benomyl-resistant transformants of albino mutant strain 79215 transformed with pAC7 or pAC71 should become melanized, but the frequency was unexpectedly rather low. The reason is unclear, but the position effect of the transforming gene may be concerned. The integrated gene may not be expressed depending on the integrated position of the genome. This is quite conceivable considering that melanin biosynthesis is a developmentally regulated metabolism in *C. lagenarium*. Another possible reason is that essential genomic sequences for melanin restoration in the cosmid may be cleaved at the time of heterologous recombination.

Because albino mutants are not conditional mutants and the defective site is considered to be pentaketide cyclization (Kubo *et al.* 1983), the cloned gene is assumed to be a structural gene involved in pentaketide cyclization. However, since *in vitro* analysis for enzyme activity of pentaketide cyclization is currently impossible, we can not fully exclude the possibility that the cloned gene is a regulator gene rather than a structural gene. Anyway, our results clearly indicate that genomic sequences cloned in pAC71 are an essential gene for expressing pathogenicity. We consider the gene can be included the category of pathogenic gene.

Conventional genetics of melanin biosynthesis was carried out in several plant pathogenic fungi including *Cochliobolus miyabeanus* (Ito & Kunibayashi in Ito) (Kubo *et al.* 1989), *Cochliobolus heterostrophus* (Drechs.) Drechs. (Tanaka *et al.* 1990), *Magnaporthe grisea* (T. T. Hebert) Yaegashi & Udagawa (*Pyricularia oryzae* (Cooke) Sacc.) (Chumley and Valent 1990). The linkage relationship and arrangement of genes involved in melanin biosynthesis is quite different between *Cochliobolus* and *Magnaporthe* (*Pyricularia*). In the former fungi, albino gene and reductase gene are closely linked and dehydratase gene are independent of the two genes. In the latter fungus, no evident linkage was observed among the three genes. Recently in *Alternaria alternata* Holcomb *et* Antonpoulos, it is reported that melanin biosynthetic genes are are clustered from analysis of cloned gene (Kimura *et al.* 1990). It is conceivable that metabolic process of melanin biosynthesis is almost the same among different fungi; however, genes involved in the process have been arranged uniquely in

Table 1. Penetrating ability of melanin restored transformants of *Colletotrichum lagenarium* albino mutant 79215

Strain	Appressorial pigmentation	Percentage of appressoria with	
		Penetration hyphae	Lateral germination
104-T	+	89.3	2.1
79215	−	3.0	97.0
AC-1	+	83.1	2.7
AC-2	+	70.0	1.1
AC-3	+	70.7	1.2
AC-4	+	81.0	2.5
AC-5	+	71.7	2.8
AC-7	+	70.4	3.0

[a] Spores were incubated at 24° C for 48 hr on cellulose membranes. Approximately 300 spores were counted.

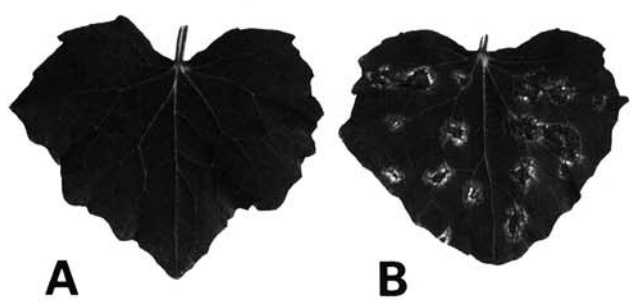

Fig. 8. Pathogenicity test of a melanin restorant AC-7. Albino mutant 79215 and wild-type strain 104-T were also inoculated as controls. Cucumber leaves inoculated with spores were incubated at 25° C for 7 days. **A,** Left half leaf, water control; right half leaf 79215 B: left half leaf, 104-T; right half leaf AC7.

different fungi. In the case of imperfect fungi, including *C. lagenarium*, molecular genetic approach is a sole means to demonstrate the linkage relationship and arrangement order of the genes. In our preliminary experiment, pAC7 did not transform mutant strain 8015, a mutant defective in scytalone dehydratase activity (Kubo *et al.* 1983), to wild phenotype. This suggests a possibility that albino gene and dehydratase gene are not linked. But more extensive experiments must be done to conclude the linkage relationship between an albino gene and scytalone dehydratase gene in *C. lagenarium*. The mutant strain 8015 is a conditional mutant; the mutant produce melanized appressoria indistinguishable from those of a wild-type strain, but during the vegetative growth on the medium the mutant express no scytalone dehydratase activity different from the wild-type strain. Mutant 8015 may be defective in regulatory region other than a structural gene itself.

Melanin biosynthesis during appressorium formation is developmentally regulated. We have shown that reductase and dehydratase involved in melanin biosynthesis are pre-existing enzymes in spores or synthesized during 1 hr of incubation, and that the enzymes are activated after 6 hr of incubation, that is the start of appressorium swelling (Kubo *et al.* 1984). Thus, the melanin biosynthesis of appressoria in *C. lagenarium* is quite a dextrously regulated system. To demonstrate the mechanisms of regulation at the molecular level is our next subject.

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