APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1997, p. 351–354 Vol. 63, No. 1
0099-2240/97/$04.00+0

Temporal Transcriptional Pattern of Three Melanin Biosynthesis Genes, *PKS1*, *SCD1*, and *THR1*, in Appressorium-Differentiating and Nondifferentiating Conidia of *Colletotrichum lagenarium*

YOSHITAKA TAKANO,[1] YASUYUKI KUBO,[2]* ITARU KURODA,[2] AND IWAO FURUSAWA[1]

Laboratory of Plant Pathology, Faculty of Agriculture, Kyoto University, Kyoto 606-01,[1] and Laboratory of Plant Pathology, Faculty of Agriculture, Kyoto Prefectural University, Kyoto 606,[2] Japan

Received 12 July 1996/Accepted 9 October 1996

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 incubated in water at 32°C germinated and produced germ tubes that elongated without appressorium differentiation. Conidia in 0.1% yeast extract solution at 32°C germinated and developed into vegetative hyphae. In this study, we investigated the temporal transcriptional pattern of cloned melanin biosynthesis genes, *PKS1*, *SCD1*, and *THR1*, in these differentiating and nondifferentiating conidia. During appressorium differentiation, de novo transcripts of the three melanin biosynthesis genes accumulated 1 to 2 h after the start of conidial incubation at 24°C, and the amount of transcripts began to decrease at 6 h. In conidia germinating in water at 32°C, the transcriptional pattern of the *PKS1*, *SCD1*, and *THR1* genes was similar to that of these genes in appressorium-forming conidia, although no appressoria were formed. However, in conidia in 0.1% yeast extract solution at 32°C, transcripts of the three melanin biosynthesis genes hardly accumulated.

Colletotrichum lagenarium (Pass.) Ellis et Halsted (syn. *Colletotrichum orbiculare* (Berk. et Mont.) von Arx) is the causal agent of anthracnose of cucumber. Infection by this fungus requires cellular differentiation, initiated by conidial attachment and proceeding to conidial germination, germ tube elongation, appressorium differentiation, infection peg formation, and cuticular penetration. Appressorium formation is essential for penetration by many phytopathogenic fungi of their host plants (3, 16). Appressorium formation is triggered by specific environmental cues. Several physical and chemical signals necessary for induction of appressorium formation have been reported (4, 13, 15).

In several fungi, including *Colletotrichum* and *Magnaporthe* species, appressoria are darkly melanized. Studies of melanin biosynthesis inhibitors in melanin-deficient mutants of *C. lagenarium* demonstrate that appressorial melanization is essential for penetration of the host plant (7–9, 22). Similarly, in *Magnaporthe grisea* (Hebert) Barr and *Colletotrichum lindemuthianum* (Saccardo et Magnus) Briosi et Cavara, appressorial melanization is essential for penetration of host plants (2, 20, 21). Recently we reported the isolation and structural analysis of three melanin biosynthesis genes, *PKS1*, *SCD1*, and *THR1*, of *C. lagenarium* (11, 12, 14, 18). Melanin biosynthesis starts with pentaketide synthesis to form scytalone. Polyketide synthase (encoded by *PKS1*) is involved in this early step (18). Subsequent steps consist of two dehydrations and one reduction reaction. The dehydrations of scytalone to 1,3,8-trihydroxynaphthalene (1,3,8-THN) and vermelone to 1,8-dihydroxynaphthalene are performed by scytalone dehydratase (encoded by *SCD1*) (12). Reduction of 1,3,8-THN to vermelone is performed by 1,3,8-THN reductase (encoded by *THR1*) (14). 1,8-Dihydroxynaphthalene is then polymerized and oxidized to yield melanin. Isolation of a gene cluster involved in melanin biosynthesis in *Alternaria alternata* and isolation and structural analysis of the 1,3,6,8-tetrahydroxynaphthalene (T_4HN) reductase gene of *M. grisea* have also been reported (5, 19). 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. In several fungi, appressorium formation is dependent on environmental conditions. Appressorium differentiation of *C. lagenarium* conidia is temperature sensitive. When *C. lagenarium* conidia suspended in water were exposed to a hard surface such as a petri dish and incubated at 24°C, they germinated and germ tubes differentiated into melanized appressoria (Fig. 1A). When conidia were incubated at 32°C, they germinated but no appressorium differentiation was observed (Fig. 1B and C). In this fungus, conditions for nearly synchronous cellular differentiation in vitro are established. This enabled us 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, we determined the temporal transcriptional patterns of three melanin biosynthesis genes, *PKS1*, *SCD1*, and *THR1*, in differentiating and nondifferentiating conidia.

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 met as follows. Conidia harvested from 7-day-old cultures were suspended in sterile, deionized water containing 0.01% (vol/vol) Tween 20 to obtain about 10^5 conidia per ml. A 4.5-ml portion of conidial suspension was placed in a 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

* Corresponding author. Mailing address: Laboratory of Plant Pathology, Faculty of Agriculture, Kyoto Prefectural University, Shimogamo, Kyoto 606, Japan. Phone: 81-75-702-0957. Fax: 81-75-702-0957. E-mail: y_kubo@kpu.ac.jp.

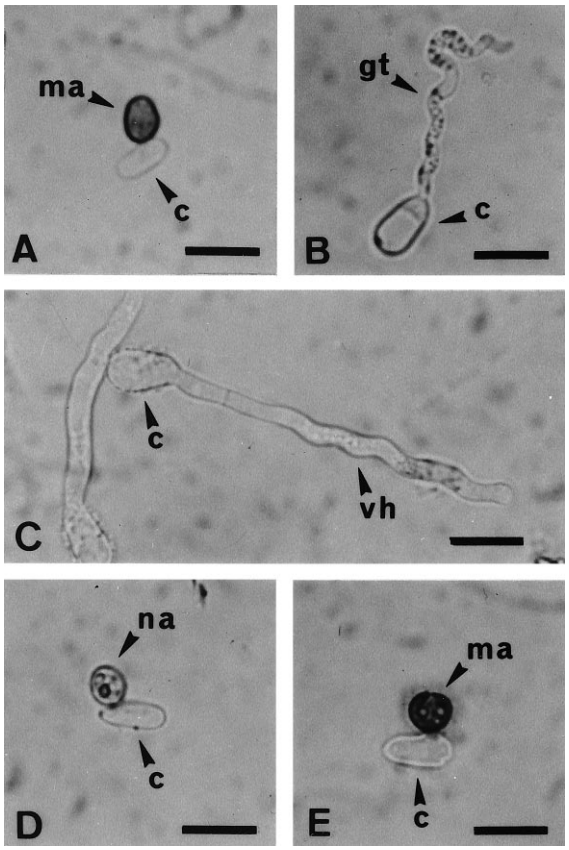

FIG. 1. Differentiation and nondifferentiation of conidia of *C. lagenarium*. Conidia were incubated for 12 h on petri dishes in water at 24°C (A), in water at 32°C (B), or in 0.1% yeast extract solution at 32°C (C). Conidia preincubated in water at 24°C for 4.5 h were subsequently incubated in water at 32°C in the absence of scytalone (D) or in the presence of 0.75 mM scytalone (E). Bar, 10 μm. Abbreviations: ma, melanized appressorium; na, nonmelanized appressorium; c, conidium; gt, germ tube; vh, vegetative hypha.

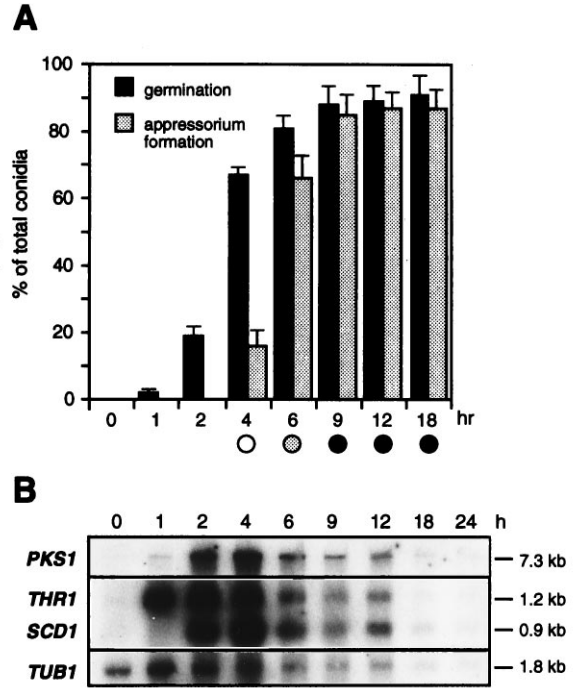

FIG. 2. Time course of expression of the *PKS1*, *SCD1*, and *THR1* genes during appressorium formation by *C. lagenarium* conidia. (A) Time course of germination and appressorium formation by *C. lagenarium* conidia. Approximately 100 conidia were observed per petri dish with a light microscope. Five replicates were examined at each time and the values were averaged. Standard deviations are indicated by vertical bars. The shading of the circles represents the degree of appressorial melanization. (B) RNA blot analysis showing time course of *PKS1*, *SCD1*, *THR1*, and *TUB1* gene expression during appressorium differentiation. Total RNA (2 μg) isolated from conidia was electrophoresed, blotted onto a nylon membrane, and hybridized with ^{32}P-labelled 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.

incubation under this condition, the proportions of nongerminating conidia, germinating conidia, and appressorium-forming conidia were determined. Figure 2A shows that 70% of conidia began to germinate within 2 to 4 h and that 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. 1A).

Next, we isolated total RNA from differentiating conidia for RNA blot analysis. Total RNA was prepared by the following procedure. Conidia were harvested by scraping them off petri dishes with a brush and were collected by centrifugation at 2,000 × *g* for 2 min. They were resuspended in a solution containing 600 μl of extraction buffer (100 mM glycine, 100 mM NaCl, 10 mM EDTA [pH 9.5]), 60 μl of 10% sodium dodecyl sulfate, and 12 μl of 6% (wt/vol) bentonite solution (6). 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 with 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. To prepare RNA from conidia before 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 (14). 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 ^{32}P-labelled in vitro transcripts of the respective genes as antisense RNA probes (12, 14, 18). In addition, expression of the β-tubulin gene, considered not linked to melanin biosynthesis, was investigated as a control. We isolated cDNA of the β-tubulin gene of *C. lagenarium*, designated *TUB1*, from a cDNA library using a genomic fragment containing the β-tubulin gene as a probe (11). The 0.4-kb β-tubulin 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 activities were used for detecting each transcript.

The results showed that no transcripts of the three melanin biosynthesis genes were detected at 0 h, though transcripts of the β-tubulin gene were detected (Fig. 2B). 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 to 2 h after the start of incubation, i.e., at an early developmental stage when germ tubes were not yet visible. The accumulation of transcripts of these genes increased

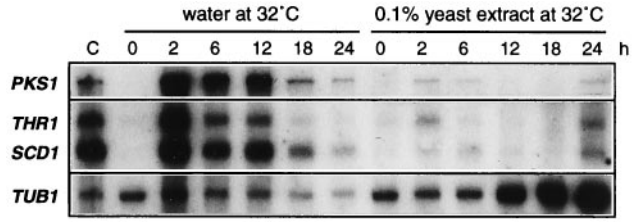

FIG. 3. RNA blot analysis showing the time course of expression of the *PKS1*, *SCD1*, *THR1*, and *TUB1* genes during development of nondifferentiating conidia. Conidia were incubated in water or 0.1% yeast extract solution. Total RNA (2 μg) isolated from conidia was analyzed as described in the legend to Fig. 2B. RNA isolated from conidia at 6 h after the start of incubation in water at 24°C was electrophoresed as a control.

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 BAS2000 bioimaging analyzer (FUJIX) showed that the ratios between the *PKS1*, *SCD1*, and *THR1* transcripts remained consistent at each time point from 2 to 12 h (data not shown).

The level of transcripts of the β-tubulin gene began to decrease at 6 h. In general, β-tubulins are known to be involved in various phases, including mitosis, morphogenesis, and cytoskeleton formation, as subunits of microtubules (1). Presumably, the decrease of *TUB1* transcripts was correlated with the finish of cellular morphogenesis at 6 to 9 h, i.e., appressorium formation.

Transcriptional pattern of the *PKS1*, *SCD1*, and *THR1* genes during development of nondifferentiating conidia. *C. lagenarium* conidia incubated in water at 32°C germinated and produced elongated germ tubes but no appressoria (Fig. 1B) (17). On the other hand, conidia incubated in 0.1% (wt/vol) yeast extract solution at 32°C developed into vegetative hyphae (Fig. 1C) 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. 3), even though these conidia produced no appressoria and no melanization was observed. *TUB1* transcripts 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 from our laboratory showed that appressorial melanization as well as appressorium formation was temperature sensitive (10). 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. 1D). However, when the colorless appressoria were instead incubated in 0.75 mM scytalone at 32°C, they became melanized (Fig. 1E). This suggested that melanin biosynthesis enzymes involved in steps after scytalone synthesis were active at 32°C, though melanin biosynthesis enzymes involved in earlier steps were temperature sensitive (10). On the other hand, conidia incubated continuously at 32°C, which produced germ tubes but no appressoria, were not melanized after the addition of scytalone (data not shown), although the three melanin biosynthesis genes were transcribed as highly in these conidia as they were in appressorium-forming conidia (Fig. 3). We conclude that some regulation system, perhaps involved in posttranscriptional regulation of the melanin biosynthesis genes, did not function properly in nondifferentiating conidia.

On the other hand, in conidia incubated in 0.1% yeast extract solution at 32°C, transcripts of the three melanin biosynthesis genes were hardly detected, although *TUB1* transcripts gradually accumulated (Fig. 3). Conidia incubated in 0.1% yeast extract solution at 32°C produced vegetative hyphae that continued to elongate, whereas conidia incubated in water at 24 and 32°C finished appressorium formation and germ tube elongation, respectively, at 6 to 9 h. We considered the possibility that *TUB1* transcripts did not decrease in conidia incubated in 0.1% yeast extract solution at 32°C because of continuous elongation of vegetative hyphae. We found that conidia formed vegetative hyphae when incubated in 0.1% tryptone solution at 32°C. This suggests that complex nutrients, such as yeast extract and tryptone, induce vegetative-hyphal elongation in conidia at 32°C. We 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 similar time courses 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, we assume that factors regulating transcription of the *PKS1*, *SCD1*, and *THR1* genes were not identical because *PKS1*, *SCD1*, and *THR1* transcripts did not appear synchronously during appressorium differentiation. The order of expression of the three melanin biosynthesis genes (*THR1*, *PKS1*, and *SCD1*) was not consistent with the order of the melanin biosynthesis pathway (*PKS1*, *SCD1*, and *THR1*). We suppose that the posttranscriptional regulation of the melanin biosynthesis genes, as suggested above, determines 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.

The results presented here suggest that both transcriptional regulation and posttranscriptional regulation of the three melanin biosynthesis genes are involved in appressorial melanization in *C. lagenarium*. We believe that determining the accumulation pattern and intracellular localization of the three enzymes during appressorium differentiation could help to unravel their posttranscriptional regulation. Also, analysis of the promoter regions and transcriptional factors of the three melanin biosynthesis genes is needed to elucidate the transcriptional controls which determine the order of the expression of the three genes.

We are grateful to Richard O'Connell (IACR-Long Ashton, University of Bristol) and Kazuyuki Mise (Kyoto University) for reviewing the manuscript.

This work was supported in part by research grant 07556080 from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. **Avila, J.** 1992. Microtubule functions. Life Sci. **50:**327–334.
2. **Chumley, F. G., and B. Valent.** 1990. Genetic analysis of melanin-deficient, non-pathogenic mutants of *Magnaporthe grisea*. Mol. Plant-Microbe Interact. **3:**135–143.
3. **Emmett, R. W., and D. G. Parbery.** 1975. Appressoria. Annu. Rev. Phytopathol. **13:**147–167.
4. **Hoch, H. C., R. C. Staples, B. Whitehead, J. Comeau, and E. D. Wolfe.** 1987. Signaling for growth orientation and cell differentiation by surface topography in *Uromyces*. Science **234:**1659–1662.
5. **Kimura, N., and T. Tsuge.** 1993. Gene cluster involved in melanin biosynthesis of the filamentous fungus *Alternaria alternata*. J. Bacteriol. **175:**4427–4435.
6. **Kroner, P., and P. Ahlquist.** 1992. RNA-based viruses, p. 23–34. *In* S. J. Gurr, M. J. McPherson, and D. J. Bowles (ed.), Molecular plant pathology, a practical approach. Oxford University Press, New York, N.Y.
7. **Kubo, Y., and I. Furusawa.** 1991. Melanin biosynthesis: prerequisite for

successful invasion of the plant host by appressoria of *Colletotrichum* and *Pyricularia*, p. 205–217. *In* G. T. Cole and H. C. Hoch (ed.), The fungal spore and disease initiation in plants and animals. Plenum Publishing Corp., New York, N.Y.

8. **Kubo, Y., K. Suzuki, I. Furusawa, N. Ishida, and M. Yamamoto.** 1982. Relation of appressorium pigmentation and penetration of nitrocellulose membranes by *Colletotrichum lagenarium*. Phytopathology **72:**498–501.
9. **Kubo, Y., K. Suzuki, I. Furusawa, and M. Yamamoto.** 1985. Melanin biosynthesis as a prerequisite for penetration by appressoria of *Colletotrichum lagenarium*: site inhibition by melanin-inhibiting fungicides and their action on appressoria. Pestic. Biochem. Physiol. **23:**47–55.
10. **Kubo, Y., I. Furusawa, and M. Yamamoto.** 1984. Regulation of melanin biosynthesis during appressorium formation in *Colletotrichum lagenarium*. Exp. Mycol. **8:**364–369.
11. **Kubo, Y., H. Nakamura, K. Kobayashi, T. Okuno, and I. Furusawa.** 1991. Cloning of a melanin biosynthetic gene essential for appressorial penetration of *Colletotrichum lagenarium*. Mol. Plant-Microbe Interact. **5:**440–445.
12. **Kubo, Y., Y. Takano, N. Endo, N. Yasuda, S. Tajima, and I. Furusawa.** 1996. Cloning and structural analysis of the melanin biosynthesis gene encoding scytalone dehydratase in *Colletotrichum lagenarium*. Appl. Environ. Microbiol. **62:**4340–4344.
13. **Lee, Y.-H., and R. A. Dean.** 1993. cAMP regulates infection structure formation in the plant pathogenic fungus *Magnaporthe grisea*. Plant Cell **5:**693–700.
14. **Perpetua, N. S., Y. Kubo, N. Yasuda, Y. Takano, and I. Furusawa.** 1996. Cloning and characterization of a melanin biosynthetic *THR1* reductase gene essential for appressorial penetration of *Colletotrichum lagenarium*. Mol. Plant-Microbe Interact. **9:**323–329.
15. **Podila, G. K., L. M. Rogers, and P. E. Kolattukudy.** 1993. Chemical signals from avocado surface wax trigger germination and appressorium formation in *C. gloeosporioides*. Plant Physiol. (Rockville) **103:**267–272.
16. **Staples, R. C., and H. C. Hoch.** 1987. Infection structure—form and function. Exp. Mycol. **11:**163–169.
17. **Suzuki, K., I. Furusawa, N. Ishida, and M. Yamamoto.** 1981. Protein synthesis during germination and appressorium formation of *Colletotrichum lagenarium* spores. J. Gen. Microbiol. **124:**61–69.
18. **Takano, Y., Y. Kubo, K. Shimizu, K. Mise, T. Okuno, and I. Furusawa.** 1995. Structural analysis of *PKS1*, a polyketide synthase gene involved in melanin biosynthesis of *Colletotrichum lagenarium*. Mol. Gen. Genet. **249:**162–167.
19. **Vidal-Cros, A., F. Viviani, G. Labesse, M. Bocara, and M. Gaudry.** 1994. Polyhydroxynaphthalene reductase involved in melanin biosynthesis in *Magnaporthe grisea*. Eur. J. Biochem. **219:**985–992.
20. **Wolkow, P. M., H. D. Sisler, and E. L. Vigil.** 1983. Effect of inhibitors of melanin biosynthesis on structure and function of appressoria of *Colletotrichum lindemuthianum*. Physiol. Plant Pathol. **22:**55–71.
21. **Woloshuk, C. P., H. D. Sisler, and E. L. Vigil.** 1983. Action of the antipenetrant, tricyclazole, on appressoria of *Pyricularia oryzae*. Physiol. Plant Pathol. **22:**245–259.
22. **Yamaguchi, I., and Y. Kubo.** 1992. Target sites of melanin biosynthesis inhibitors, p. 101–118. *In* W. Köller (ed.), Target sites of fungicide action. CRC Press, London, England.