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# Cloning and Characterization of a Melanin Biosynthetic *THR1* Reductase Gene Essential for Appressorial Penetration of *Colletotrichum lagenarium*

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**Melanin biosynthesis of *Colletotrichum lagenarium* is essential for appressorial penetration of the host plant. A melanin deficient mutant 9141 (Thr$^-$) has a defect in the conversion of 1,3,8-trihydroxynaphthalene to vermelone in the melanin biosynthetic pathway. The mutant formed nonmelanized appressoria and had little infectivity on cucumber leaves. A cosmid clone pCR1 was selected from a cosmid library of wild-type *C. lagenarium* by means of a heterologous probe *BRM2,* one of the clustered genes involved in melanin biosynthesis of *Alternaria alternata.* pCR1 transformed the Thr$^-$ mutant 9141 to wild-type phenotype. A DNA fragment (*THR1*) homologous to *BRM2* was subcloned from pCR1 and the nucleotide sequence determined. *THR1* contains one open reading frame that encodes a protein of 282 amino acids. A transformant resulting from gene disruption showed a light brown phenotype different from the dark brown phenotype of the wild-type 104-T. The transformant formed nonmelanized appressoria and had little infectivity. The *THR1* amino acid sequence contains a region highly similar to the *Ver1* gene involved in the conversion of versicolorin A to sterigmatocystin in aflatoxin biosynthesis by *Aspergillus parasiticus* and to the $T_4$HN reductase gene involved in the conversion of 1,3,6,8-tetrahydroxynaphthalene to scytalone and 1,3,8-trihydroxynaphthalene to vermelone in melanin biosynthesis by *Magnaporthe grisea.* Expression of the *THR1* gene during spore germination of *C. lagenarium* was detected by RNA blotting. We propose that the *C. lagenarium THR1* gene encodes a reductase involved in conversion of 1,3,8-trihydroxynaphthalene to vermelone.**

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Nucleotide sequence data to be found at GenBank as accession number D83988.

Most fungal pathogens produce a dark brown or black pigment known as melanin. Fungal melanins are produced by a polyketide biosynthetic pathway in which the final step is polymerization of 1,8-dihydroxynaphthalene (DHN) (Bell and Wheeler 1986).

*Colletotrichum lagenarium* (Pass.) Ellis & Halsted produces melanized appressoria during the infection process. Appressorial melanization has been implicated as an important factor for penetration and pathogenicity. It is envisaged that melanin mediates the build-up of pressure in the appressorium and that this high pressure provides the essential driving force for mechanical penetration (Howard and Ferrari 1989). Melanin also provides cell wall rigidity to the appressorium, which is necessary for focusing the turgor forces for vertical penetration (Kubo and Furusawa 1991). We have previously cloned a melanin biosynthetic gene, *PKS1,* which is involved in polyketide synthesis by *C. lagenarium* (Kubo et al. 1991; Takano et al. 1995).

Several chemical compounds have been shown to prevent direct penetration by inhibiting the formation of melanin in the appressorial cell wall of fungal pathogens. These commercially available fungicides include tricyclazole, which is used to control the rice blast pathogen *Magnaporthe grisea* (Hebert) Barr. Tricyclazole in *M. grisea* acts to interfere with DHN melanin biosynthesis (Woloshuk et al. 1980). In *C. lagenarium,* the inhibition site of tricyclazole was elucidated by comparing the shunt products produced by the wild-type 104-T treated with tricyclazole with the shunt products produced by other melanin-deficient mutants (Kubo et al. 1985).

Several key enzymes such as reductases, dehydratases, and oxidases are involved in melanin biosynthesis (Bell and Wheeler 1986). Mutants that are deficient in these enzymes have been isolated and their shunt products identified. The Buf$^-$ mutants of *M. grisea* (Chumley and Valent 1990) and Brm2$^-$ (brown) mutants of *Alternaria alternata* (Kimura and Tsuge 1993) are deficient in the reductase that converts 1,3,8-trihydroxynaphthalene (1,3,8-THN) to vermelone. The Buf$^-$ mutants are not pathogenic to rice.

In the experiments reported here, we also isolated a reductase-deficient mutant of *C. lagenarium* that does not infect cucumber. We are interested in defining the significance of the reductase gene in melanin biosynthesis and pathogenicity and its pattern of expression during spore germination. We report

here the cloning and sequence of the *THR1* gene of *C. lagenarium*, and provide evidence for its involvement in pathogenicity.

## RESULTS

### Characterization of Thr⁻ mutant 9141.

The Thr⁻ mutant 9141 of *C. lagenarium* had a defect in the conversion of 1,3,8-THN to vermelone in the melanin biosynthetic pathway (Fig. 1). A shunt metabolite, 3,4-dihydro-4,8-dihydroxy-1(2H) naphthalenone (DDN), the last shunt product derived from 1,3,8-THN through the inhibition of its reduction to vermelone (Kubo et al. 1985), was identified in the culture filtrate of the mutant 9141 (Fig. 2). The product was also obtained from the culture filtrate of the wild-type strain treated with tricyclazole, which inhibits the conversion of 1,3,8-THN to vermelone in *C. lagenarium* (Kubo et al. 1985) (Fig. 2). The mutant produced a light brown phenotype on potato sucrose agar medium and formed nonmelanized appressoria on glass slides (Fig. 3). It does not have the ability to infect cucumber leaves (Fig. 4).

### Cloning and sequencing of the *THR1* gene of *C. lagenarium*.

A genomic library of *C. lagenarium* wild-type strain 104-T was constructed in the cosmid vector pKVβ (Kubo et al. 1991). The genomic DNA containing the reductase sequences was isolated using a heterologous probe, *BRM2*, one of the clustered genes involved in melanin biosynthesis by *Alternaria alternata* (Kimura and Tsuge 1993). From about 10,000 bacterial colonies screened by colony blot hybridization, one positive clone was identified and designated pCR1. The Thr⁻ mutant 9141 of *C. lagenarium* was transformed with cosmid clone pCR1 and several dark melanin-restored transformants

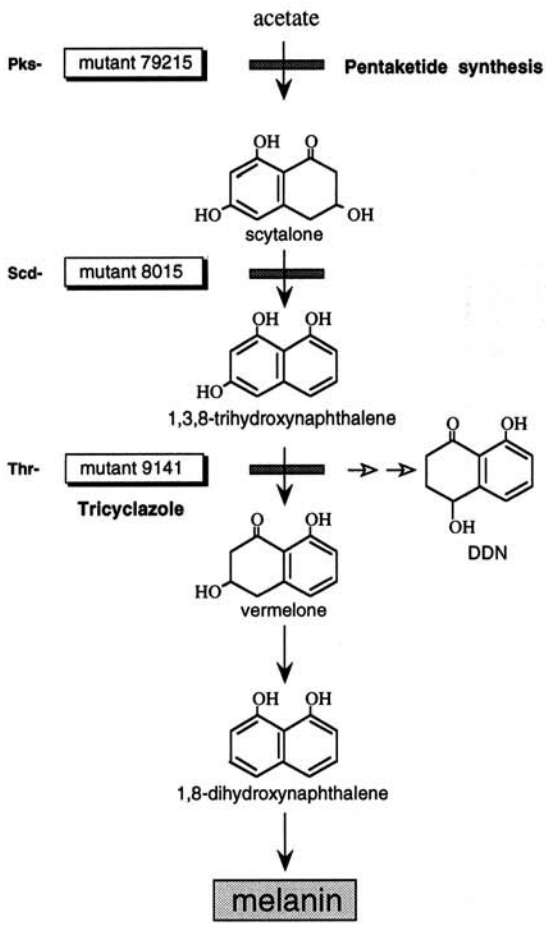


**Fig. 1.** The melanin biosynthetic pathway of *Colletotrichum lagenarium* showing sites that are blocked in mutants and inhibited by chemicals. 1,3,8-THN = 1,3,8-trihydroxynaphthalene; 1,8-DHN = 1,8-dihydroxynaphthalene.

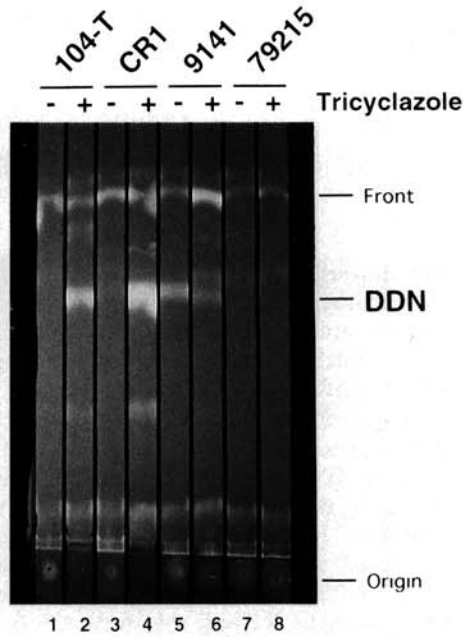


**Fig. 2.** Thin-layer chromatography of the wild-type strain 104-T, a melanin-restored pCR1 transformed strain of Thr⁻ mutant 9141, and the Thr⁻ mutant 9141 and Pks⁻ albino mutant 79215 of *Colletotrichum lagenarium* treated with or without 100 μM tricyclazole. Chromatograms were developed with benzene-ethyl acetate (1:1, vol/vol) solvent and observed by fluorescence in 365 nm light. 3,4-dihydro-4,8-dihydroxy-1(2H)naphthalenone (DDN) was recognized at $R_f$ value 0.66 in 104-T and CR1 treated with tricyclazole, and Thr⁻ mutant 9141 in the absence or presence of tricyclazole.

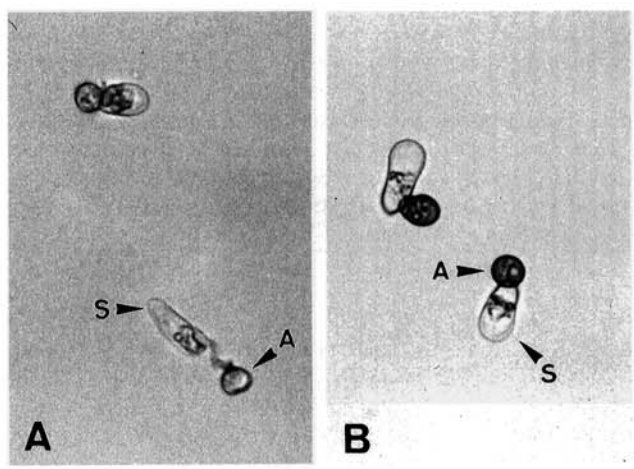


**Fig. 3.** Appressorium formation by (**A**) Thr⁻ mutant 9141and (**B**) wild-type 104-T of *Colletotrichum lagenarium* on a glass slide incubated at 24°C for 24 h. Wild-type 104-T formed melanized appressoria and Thr⁻ mutant 9141 formed nonmelanized appressoria. S, spore; A, appressorium.

were recovered. These transformants have a phenotypic appearance similar to that of the wild-type 104-T and accumulate the melanin shunt product DDN when treated with tricyclazole, as does the wild-type 104-T (Fig. 2). Cellulose membrane tests showed that the appressoria were melanized in a manner indistinguishable from that of the wild-type 104-T and formed penetration hyphae into cellulose membranes as effectively as wild type.

Restriction-map analysis of pCR1 revealed that the heterologous probe *BRM2* hybridized to the *Acc*I and *Sal*I internal region of pCR1 (Fig. 5). We designate the region of pCR1 that is homologous to *BRM2* as *THR1*, and the region was subcloned into pBluescriptII as a series of overlapping DNA fragments. Sequencing of the *THR1* gene was done with commercially available and synthetic primers (Fig. 5). The sequenced DNA contains one open reading frame that codes for 282 amino acids (Fig. 6). The first ATG occurs 113 nucleotides downstream of the major transcriptional start site, designated as +1. The transcription initiation sites of the *THR1* gene were determined by primer extension analysis of melanin induced–hyphal poly(A)$^+$ RNA (Fig. 7) and the polyadenylation sites at the 3′ end were deduced from three independent cDNA clones. The four intron sites were confirmed by comparing the sequence obtained from the cDNA and that of the genomic DNA clone. The signal sequences in the presumptive introns match the consensus sequences for fungal 5′ splice sites [GT(A/G/T)(A/C/T)G(TC)], the 3′ splice sites [(C/T)AG] and internal splicing signals [(G/A)CT(A/G)AC] of *Neurospora crassa* Shear & B. O. Dodge and the β-tubulin genes of *Colletotrichum graminicola* (Ces.) G. W. Wils. (Bruchez et al. 1993; Panaccione and Hanau 1990).

A search of GenBank for the deduced amino acid sequence of *THR1* revealed a strikingly high similarity to the *Ver1* gene involved in the conversion of versicolorin A to sterigmatocystin in aflatoxin biosynthesis by *Aspergillus parasiticus* Speare (Fig. 8) (Skory et al. 1992) and the $T_4$HN reductase gene involved in the conversion of 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) to scytalone and 1,3,8-THN to vermelone in melanin biosynthesis by *M. grisea* (Fig. 8) (Vidal-Cros et al. 1994).

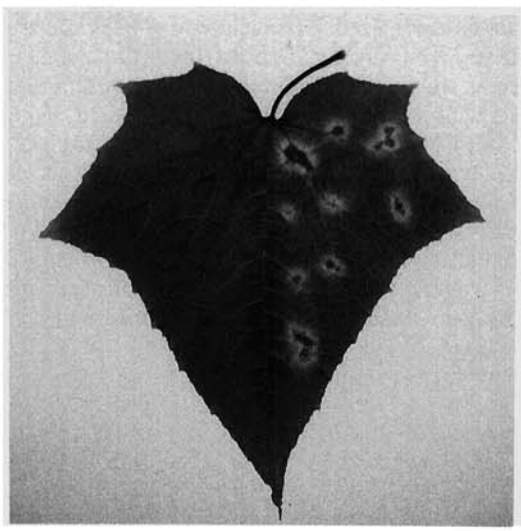

**Fig. 4.** Pathogenicity test of Thr$^-$ mutant 9141. A cucumber leaf was inoculated with Thr$^-$ mutant 9141 and the wild-type strain 104-T and incubated at 20°C for 7 days. Thr$^-$ mutant 9141 could not form lesions on the cucumber leaf. Left half of leaf, Thr- mutant 9141; right half of leaf, wild-type strain 104-T.

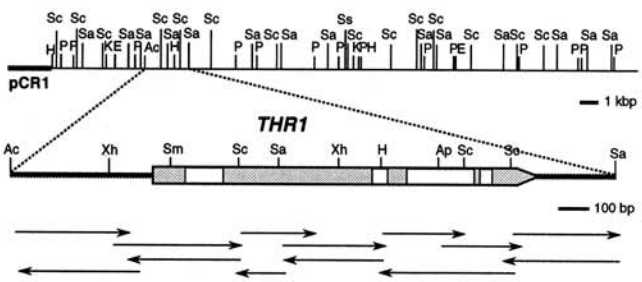


**Fig. 5.** Cloning and sequencing of the *THR1* gene of *Colletotrichum lagenarium*. A cosmid clone pCR1 was selected from a wild-type cosmid library of *Colletotrichum lagenarium* probed with the *BRM2* gene of *Alternaria alternata*. A hybridizing fragment and its flanking regions were subcloned into pBluescriptII and sequenced. The arrows indicate the extent and direction of sequencing with M13 universal primers or synthetic oligonucleotide primers. The shaded arrow indicates the open reading frame. Open boxes within the shaded arrow indicate introns. Ac, *Acc*I; Xh, *Xho*I; Sc, *Sac*I; H, *Hin*dIII; Ap, *Apa*I

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-343  GTCTACGTGTTGCTACCGACAACTCTCGGCTCTTCGCCCTTTGGTGCGGGAAGCGATGCT  -284
-283  CTGTACGGCTGGTCCCAGAGCTAGAGACGGAAATGGTGTCGTTTCACGGACGTCCGGCGC  -224
-223  GCACGTCCAAGGTCCGCCGGTCTCTCCACGGGCCTCCGTCGCGAGACGAACCGCCTCACC  -164
-163  TGAGGGACGATCCTTGGGACTATGGGGGCGGACGCGGGAAAGGAGGAGATCAACACAAAC  -104
-103  AACAAGCGACGAACTGACAAGCTCGGCGTGGAACCTGTGCTGAAACGTCGTATTTAAGAC  -44
                                                    +1
 -43  TCGAGATTCCGACCTCGAGATTGGGATTTTTTTTCGAAACACGACACAGCTCAACCAACC   16

  17  CAATCCAACCAACTGTTTCTTCCGGTCTTTTCTCGACGACTCTTCAACCTTCACTTAGAC   76

  77  ATCCAAGTCAACCCTTTTTAATCAGTCAAGATGCCTGGCGTTACTTCTCAGTCTGCTGGT  136
   1                                  M  P  G  V  T  S  Q  S  A  G    10
 137  TCCAAGTACGATGCCATCCCGGGTCCCCTGGGTTTGGCCTCTGCTTCTTTGATGGGCAAG  196
  11  S  K  Y  D  A  I  P  G  P  L  G  L  A  S  A  S  L  M  G  K    30
 197  GTTGCTCTCGTCACCGGTGCCGgtaagtcgatctgatcttccagtcacccttttcgtctca  256
  31  V  A  L  V  T  G  A  G                                        38
 257  gcacagaaaagagacagagagagaatcagggtctggttcgtctgaactcctgaatatccg  316

 317  ctgactctcacgcccccgcacagGCCGCGGTATCGGTCGTGAGATGGCCATGGAGCTCGG  376
  39                          R  G  I  G  R  E  M  A  M  E  L  G    50
 377  CCGCCGCGGCGCCAAGGTCATTGTCAACTACGCCAACAGCGCCGAGACGGCCGAGGAGGT  436
  51   R  R  G  A  K  V  I  V  N  Y  A  N  S  A  E  T  A  E  E  V    70
 437  CGTCCAGGCCATCAAGAAGTCCGGCTCCGACGCCGCCTCCATCAAGGCCAACGTCTCCGA  496
  71   V  Q  A  I  K  K  S  G  S  D  A  A  S  I  K  A  N  V  S  D    90
 497  CGTCGACCAGATCGTCAAGATGTTCGGCGAGGCCAAGCAGATCTGGGGCAGGCTCGACAT  556
  91   V  D  Q  I  V  K  M  F  G  E  A  K  Q  I  W  G  R  L  D  I   110
 557  CGTCTGCTCCAACAGCGGCGTCGTCTCCTTTGGCCACGTCAAGGACGTCACCCCCGAGGA  616
 111   V  C  S  N  S  G  V  V  S  F  G  H  V  K  D  V  T  P  E  E   130
 617  GTTTGACCGCGTCTTCGCCATCAACACCCGCGGCCAGTTCTTCGTCGCCCGCGAGGCCTA  676
 131   F  D  R  V  F  A  I  N  T  R  G  Q  F  F  V  A  R  E  A  Y   150
 677  CAAGCACCTCGAGGTCGGCGGCCGCCTGATCCTCATGGGCTCCATCACCGGCCAGGCCAA  736
 151   K  H  L  E  V  G  G  R  L  I  L  M  G  S  I  T  G  Q  A  K   170
 737  GGGCGTCCCCAAGCACGCCGTCTACTCTGGTTCCAAGGGCACCATCGAGACCTTTGTCCG  796
 171   G  V  P  K  H  A  V  Y  S  G  S  K  G  T  I  E  T  F  V  R   190
 797  CTGCATGGCCATCGgtaagcttacgcgatagctcacgagctgggagctggctgaccatcc  856
 191   C  M  A  I  D                                               195
 857  tcagACTTTGGTGACAAGAAGATCACCGTCAACGCTGTTGCCCCCGGTGGCATCAAGACC  916
 196        F  G  D  K  K  I  T  V  N  A  V  A  P  G  G  I  K  T    213
 917  Ggtaagtcacttccccgcatccacgcacccatggcccatcccaccagcctcgttccctc  976
 214  D                                                            214
 977  acggcattgccaactctgccgggacccactcctccgacgtggacccacctttcccgctcc 1036

1037  atacttcgatttcttccggggcccccgaatctctccatatcatcatccgatgagctcttt 1096

1097  tgataaaaacaccgctaacatactatgcttcaatcagACATGTACAGAGACGTTTGCAGg 1156
 215                                  M  Y  R  D  V  C  R           221
1157  tcagtacacccttattcctattccctgagtgaaagctgaccgtcaacagAGAGTACATCC 1216
 222                                              E  Y  I  P       225
1217  CCAACGGAGGTGAGCTCGACGACGAGGGTGTTGATGAGTTCGCCGCTGGCTGGTCCCCCA 1276
 226   N  G  G  E  L  D  D  E  G  V  D  E  F  A  A  G  W  S  P  M   245
1277  TGCACCGTGTCGGTCTCCCGATCGACATTGCCCGCGTCGTCTGCTTCCTGGCTTCCCAGG 1336
 246   H  R  V  G  L  P  I  D  I  A  R  V  V  C  F  L  A  S  Q  D   265
1337  ACGGCGAGTGGATCAACGGCAAGGTCCTCGGCATCGACGGTGCCGCCTGCATGTAAATAC 1396
 266   G  E  W  I  N  G  K  V  L  G  I  D  G  A  A  C  M  •        282
1397  TTGGCGAGAATCACATAGGAGCCGGTTGGAGTTGTGATTGATATTGATGGGTAATGGTAG 1456

1457  ACTGGAACCAATGTAAATCTTAGACATAATACCAAATAGTTGCTAAAAGCTAGTGTCTGT 1516
                                                    *
1517  GAATACAGTCTAATGTCAAGGTTGATGAGTATATTTTTGGCAAATCATTACTGGACACGT 1576

1577  CGAC
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**Fig. 6.** Nucleotide and deduced amino acid sequences of the *C. lagenarium THR1* gene. The transcription initiation sites were determined by primer extension mapping. Two major and three minor 5′ ends are indicated by arrows. The most intensive transcription site is indicated as +1. Four introns are indicated by lower case letters. A putative polyadenylation signal sequence in the 3′ untranslated region is underlined at positions 1484 and 1491. The poly(A) addition site deduced from the sequence of three independent cDNA clones is indicated with an asterisk.

**Disruption of *THR1* gene.**

The gene disruption vector pRH1211 was constructed by inserting the internal *SalI/Hin*dIII region of the *THR1* gene into the *Sal*I and *Hin*dIII sites of pBluescriptII SK$^+$ and *hph* hygromycin B resistance gene obtained from pSH75 (Kimura and Tsuge 1993) between the *Hin*dIII and *Eco*RI sites. Plasmid pRH1211 should disrupt the *THR1* gene in the wild-type strain through single-cross over homologous recombination. Transformants were screened and selected on potato dextrose agar containing 100 μg of hygromycin B per ml. From 300 hygromycin-resistant transformants, one transformant (Rho1) with a phenotype similar to that of the Thr$^-$ mutant 9141 was isolated. Rho1 and three hygromycin-resistant transformants (Rhe1, 2, and 3) with wild-type phenotypic appearance were analyzed by DNA blotting with pSH75 (5.5 kb) and the *Sal*I fragment of *THR1* used as probes (Fig. 9). Rho1 showed the predicted homologous recombination pattern between the pRH1211 and the *THR1* gene in the chromosomal DNA. When the genomic DNA of Rho1 was digested with *Eco*RI and probed with the *THR1* gene, the resulting fragments of 22.3 and 3.1 kb were observed (Fig. 9). Also, when the genomic DNA of Rho1 was digested with *Hin*dIII, the expected internal 6.3-kb *Hin*dIII fragment was detected with pSH75 used as a probe. On the other hand, in the case of hygromycin-resistant transformants (Rhe1, 2, and 3) digested with *Eco*RI and probed with the *THR1* gene, 19.1-kb bands were detected that corresponded to the intact band of wild-type 104-T, indicating that no disruption had occurred (Fig. 9). Rhe2 showed a relatively faint band due to loading variance; however, the 19.1-kb band still could be detected. Also, when the transformants were digested with *Hin*dIII, the internal 6.3-kb *Hin*dIII fragment expected from the disruption of the *THR1* gene was not detected with pSH75 used as a probe. Again, Rhe2 showed a faint band of very high molecular size.

Rho1 transformant accumulated DDN in the same way as Thr$^-$ mutant 9141 and wild-type 104-T treated with tricyclazole. The Rho1 transformant formed nonmelanized appressoria with little ability to penetrate host cucumber leaves (Fig. 10).

**Expression of *THR1* mRNA.**

To determine the expression of *THR1* mRNA during spore germination, an RNA blot was made with total RNA from spores obtained 0, 1, and 2 h after the start of spore incubation at 24°C. At 1 h, emergence of germination tips from spores could not be recognized. Germination tips could be seen 2 h after the start of incubation. Structurally mature nonmelanized appressoria were formed after 6 h of incubation and they started melanization, forming visually melanized appressoria at 12 h of incubation. The RNA blot indicated that no signal was detected at 0 time but was detected 1 h and 2 h after the start of incubation as two distinct narrow bands of approximately 1.0 kb RNA (Fig. 11). Therefore, the *THR1* gene is expressed at the onset of spore incubation. Currently, we do not know whether either or both of these bands correspond to the *THR1* gene transcript. Total rRNA stained with ethidium bromide was used as a loading control.

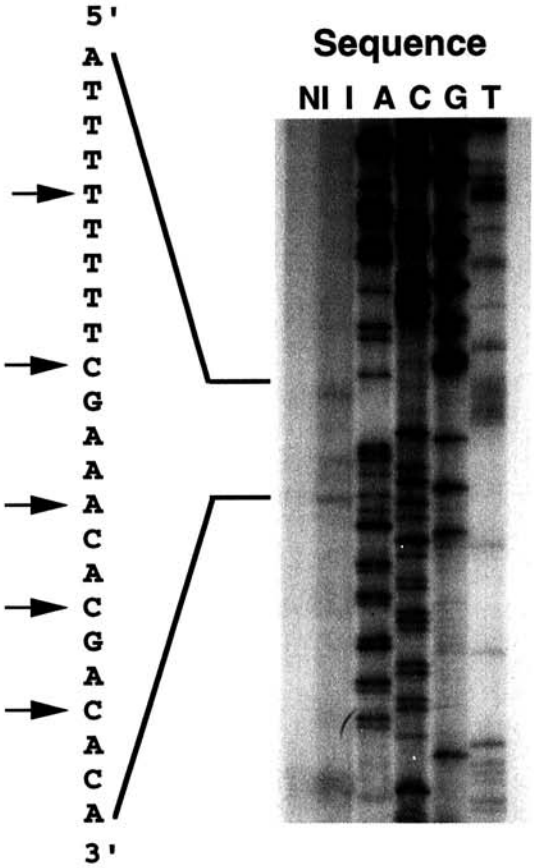


**Fig. 7.** Primer extension mapping of melanin-induced hyphal poly(A)$^+$ RNA. NI: noninduced hyphal poly(A)+ RNA; I: melanin-induced hyphal poly(A)$^+$ RNA. The sequence of the 5′ region of the *THR1* gene (see Figure 6) is given as a sequence ladder. The transcription initiation sites are indicated by arrows.

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Thr1:
ThnR:
Ver1:

  1 MPGVTSQSAGSKYDAIPGPLGLASASLMGKVALVTGAGRGIGREMAMELG
  1 ..A.. PRGE..........P ....E.....................
  1 -------------------MSDNHR.D.............AAI.VA..
                              * ***************   *  **

 51 RRGAKVIVNYANSAETAEEVVQAIKKSGSDAASIKANVSDVDQIVKMFGE
 51 .C..........T.S.....A....N.....CV....GV.ED..R..E.
 32 E.....V....HCRRPRRKWLNRSRPMVPMLSQSQ.D.G.PEATA.LMA.
      **** ****                          * *

101 AKQIWGRLDIVCSNSGVVSFGHVKDVTPEEFDRVFAINTRGQFFVAREAY
101 .VK.F.K.............................T.............
 82 TVRHF.Y....S..A.I.....L............RV............
         * **** ** * ***** ************  **************

151 KHLEVGGRLILMGSITGQAKGVPKHAVYSGSKGTIETFVRCMAIDFGDKK
151 ....I.............A.............A....A......MA...
132 R.MRE...I..TS.N.ACV..............A.D.........C....
     *   *** **  * *   * ************ * ** ******  ***

201 ITVNAVAPGGIKTDMYRDVCREYIPNGGELDDEGVDEFAA-GW-SPMHRV
201 ....V...........HA.........EN.SN.E...Y..SA.-..L...
182 .........A.....FLA.S.......ETFT..Q...C.--.L..LN...
    **** **** *****   * *******      * *** **  * **  **

249 GLPIDIARVVCFLASQDGEWINGKVLGIDGAACM
250 ..............N..G.VT...I....G...
230 ...V.V....S....DTA..VS..II.V..G.FR
    *** * **** ****    *  **  * ** *
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**Fig. 8.** Alignment of deduced amino acid sequences coded by the *THR1* gene of *C. lagenarium* (Thr1) with the tetrahydroxynaphthalene reductase gene of *Magnaporthe grisea* (ThnR) and the *Ver1* gene of *Aspergillus parasiticus* (Ver1). Amino acid residues conserved among the three fungi are marked with asterisks.

## DISCUSSION

The involvement of fungal melanin in pathogenicity has been well established in plant pathogenic fungi such as *M. grisea* (Woloshuk et al. 1980; Chumley and Valent 1990), *Colletotrichum lagenarium,* and *C. lindemuthianum* (for review, see Kubo and Furusawa 1991). The role of melanin in appressorial penetration has been envisaged as providing the physical rigidity necessary for focusing the turgor pressure of the protoplasm into the host cuticle to facilitate penetration. The cloning of a melanin biosynthetic gene from *C. lagenarium* by complementation provides strong evidence for its role in penetration and pathogenicity (Kubo et al. 1991; Takano et al. 1995). Recently, the $T_4HN$ reductase gene of *M. grisea* has been cloned and characterized (Vidal-Cros et al. 1994); however, its role in penetration and pathogenicity has not been documented. We report here the cloning of the *THR1* reductase gene involved in melanin biosynthesis of *C. lagenarium.* Several lines of evidence indicate that the cloned gene is the *THR1* reductase gene of *C. lagenarium*: (i) the isolated genomic DNA, pCR1, restored the wild-type phenotype of Thr⁻ mutant 9141 by DNA complementation; (ii) disruption of *THR1* gene brought about Thr⁻ phenotype; and (iii) the cloned gene shares a high deduced amino acid similarity to the sequence of the *Ver1* gene of *A. parasiticus* and the $T_4HN$ reductase gene of *M. grisea.*

pCR1 transformed the Thr⁻ mutant 9141 to the wild-type phenotype. Such complementation studies have conventionally been used as an important step in isolating genes of interest. We have identified pathogenicity-related genes such as the albino gene of *C. lagenarium* (Kubo et al. 1991; Takano et al. 1995) and a gene involved in penetration peg formation (Perpetua et al. 1994) by DNA complementation.

Gene disruption experiments through homologous recombination in melanin biosynthetic genes have been done in *Alternaria alternata* (Kimura and Tsuge 1993). The vector pRH1211, which carries an internal fragment of the *THR1* gene, disrupted the *THR1* gene in wild-type 104-T. The Thr⁻ disrupted transformant Rho1 showed a mutant phenotype similar to that of the Thr⁻ mutant 9141. Rho1 transformant accumulated melanin shunt product DDN in the same way as Thr⁻ mutant 9141. Appressoria of Rho1 were not melanized and had little ability to penetrate and infect cucumber leaves.

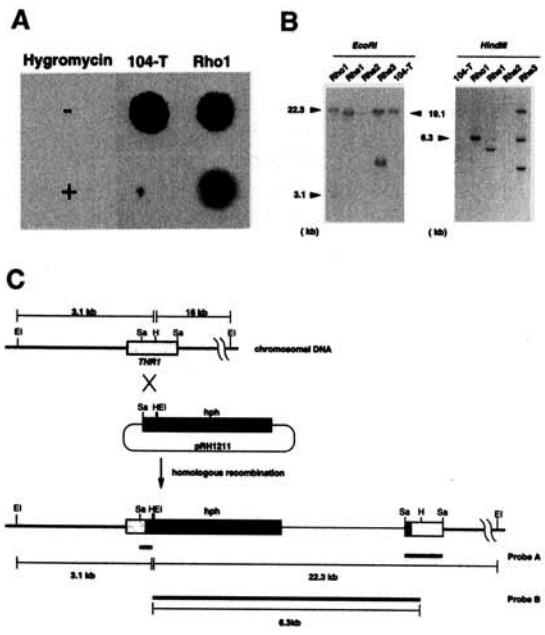


**Fig. 9.** Analysis of *THR1*-disruption mutant Rho1. **A,** Growth of disrupted Rho1 and wild-type 104-T in the absence and presence of hygromycin B (100 μg/ml). **B,** DNA blot analysis of transformants Rho1, Rhe1, Rhe2, and Rhe3, and wild-type strain 104-T. Genomic DNA was digested with *Eco*RI or *Hin*dIII and probed with the *Sal*I fragment of the *THR1* gene for *Eco*RI digestion and pSH75, vector DNA with the hygromycin resistance gene, for *Hin*dIII digestion. Homologous recombination is predicted to disrupt the 19.1-kb *Eco*RI fragment in wild-type 104-T to make *Eco*RI fragments of 3.1 and 22.3 kb. In the transformant Rho1 the expected 3.1- and 22.3-kb bands were detected when probed with the *Sal*I fragment of the *THR1* gene. Also, pSH75 is predicted to hybridize to a 6.3-kb *Hin*dIII fragment in the *THR1*-disrupted transformant. The band was detected for the transformant Rho1 with pSH75 used as a probe. In transformants Rhe1, Rhe2, and Rhe3, the bands predicted from homologous recombination were detected for neither blot. **C,** Predicted restriction map of *THR1* gene disruption by homologous recombination with pRH1211. EI, *Eco*RI; H, *Hin*dIII; Sa, *Sal*I.

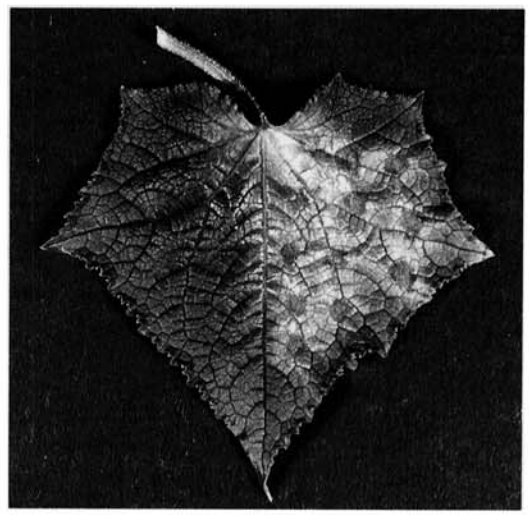

**Fig. 10.** Pathogenicity test of *THR1* gene disruptant Rho1. A cucumber leaf was inoculated with Rho1 and the wild-type strain 104-T and incubated at 20°C for 7 days. Rho1 could not form lesions on the cucumber leaf. Left half of leaf, Rho1; right half of leaf, wild-type strain 104-T.

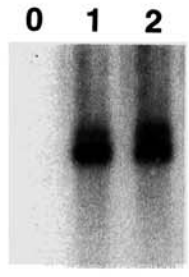


**Fig. 11.** RNA blot analysis of total RNA obtained from spores at 0, 1, and 2 h after the start of spore incubation. The blot was probed with $^{32}P$-labeled *THR1* gene transcripts made in vitro from a clone containing a *THR1* sequence from 1228 to 1577 (Fig. 6). Two narrow bands of approximately 1 kb indicate *THR1* transcripts were detected 1 h and 2 h after the start of incubation. Total rRNA stained with ethidium bromide was used as a loading control.

These results indicate that *THR1* of *C. lagenarium* is involved in conversion of 1,3,8-THN to vermelone in melanin biosynthesis and is essential for penetration.

The size of the transcript of *THR1* gene detected by RNA blotting was approximately 1.0 kb. This size is similar to that of other reported reductase genes of *A. alternata* (Kimura and Tsuge 1991) and *M. grisea* (Vidal-Cros et al. 1994). *THR1* mRNA could be detected 1 h after the start of spore incubation. Expression of the *THR1* gene at the onset of spore incubation agrees with earlier results that dehydratase and reductase, both of which are involved in melanin biosynthesis, are synthesized within 1 h of spore incubation (Kubo et al. 1984). In our RNA blot experiment, we detected two distinct narrow bands. We do not know whether both of them are transcripts from the *THR1* gene, differing only in the size of the transcript, or are derived from another gene that hybridizes with the *THR1* probe.

The deduced amino acid sequence of the *C. lagenarium* *THR1* reductase gene shares an especially high similarity to that of the $T_4$HN reductase gene of *M. grisea* (Vidal-Cros et al. 1994). Eighty-three percent of the amino acid residues was identical throughout the whole amino acid sequence. Since the two fungi have quite similar melanin biosynthetic pathways and the genes are involved in the same reduction process, it is not surprising that the two sequences are so similar. However, in our experiments, we could not get any affirmative data indicating that the *THR1* gene product was involved in the conversion of 1,3,6,8-THN to scytalone, in contrast to the result with the *M. grisea* $T_4$HN reductase (Vidal-Cros et al. 1994). Accumulation of the shunt product DDN in Thr$^-$ mutant 9141 and Rho1 indicated that at least 1,3,8-THN was synthesized in those mutants even though the reductase was not functional. In our previous experiments using *Cochliobolus miyabeanus* (Ito & Kuribayashi) Drechs. ex Dastur (Kubo et al. 1989) and *Cochliobolus heterostrophus* (Drechs.) Drechs. (Tanaka et al. 1991), we made double mutants defective in both reduction of 1,3,8-THN and dehydration of scytalone by crossing of the single mutant. The double mutants showed a dehydratase-defective phenotype, indicating that dehydration of scytalone is epistatic to reduction of 1,3,8-THN. This clearly indicates that in those *Cochliobolus* species the reductase is not involved in the reduction of 1,3,6,8-THN. Similar results were obtained in our experiments with *C. lagenarium*. Although we cannot exclude the possibility that another reductase gene may be involved in the putative reduction step between 1,3,6,8-THN and scytalone, currently we have not been able to isolate any mutants of *C. lagenarium* that indicate defectiveness in reduction of 1,3,6,8-THN to scytalone.

The amino acid sequence of the *THR1* reductase gene also shows similarity to the *Ver1* gene of *A. parasiticus* that is associated with the conversion of versicolorin A to sterigmatocystin (Skory et al. 1992). The similarity between the $T_4$HN reductase gene involved in melanin biosynthesis and the *Ver1* gene involved in aflatoxin biosynthesis has been described by Vidal-Cros et al. (1994). The similarity of the amino acid sequence of the *C. lagenarium* *THR1* reductase gene to *Ver1* was 56% over the whole amino acid sequence; however, more highly conserved subregions can be recognized. The active site residue involved in the common reduction process may be conserved.

## MATERIALS AND METHODS

### Fungal strains.

*Colletotrichum lagenarium* strain 104-T (stock culture of the Laboratory of Plant Pathology, Kyoto University) was used as the wild-type strain. Melanin-deficient Thr$^-$ mutant strain 9141 was isolated by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment (Kubo et al. 1982). Wild-type and mutant strains were cultured on potato sucrose agar medium at 24°C. Pathogenicity tests on cucumber leaves were made as previously described by Kubo et al. (1982).

### Analysis of melanin intermediates.

Analysis of melanin intermediates by thin-layer chromatography was done as described by Kubo et al. (1985).

### Fungal transformation.

The wild-type 104-T genomic DNA libraries were constructed with cosmid vector pKVβ, which contains the β-tubulin gene as a selective marker for transformation studies. Selections were made on regeneration medium (potato dextrose agar conditioned with 1 M glucose containing 1.2 μg of benomyl per ml) as earlier described (Kubo et al. 1991). Fungal transformation vector pSH75 (Kimura and Tsuge 1993) containing the hygromycin resistance *hph* gene was used to subclone the *THR1* genomic DNA fragment. Hygromycin-resistant transformants were selected on regeneration medium containing 100 μg of hygromycin B per ml.

### DNA sequencing.

Several overlapping restriction fragments containing the *THR1* gene were subcloned into pBSII(KS$^-$). DNA sequence information was obtained from both strands by means of the dideoxy chain termination method (Sanger et al. 1977) with *Bca*BEST DNA polymerase (Takara Corp., Kyoto, Japan) according to the manufacturer's instructions. Primers used were M13 universal primers and two synthetic oligonucleotide primers derived from established sequences.

### Induction of melanin biosynthesis and RNA isolation.

Melanin biosynthesis during mycelial growth was induced by transferring nonmelanized mycelia grown in potato-sucrose liquid media containing 0.2% yeast extract to 1.2 M sucrose water solution (Takano et al. 1995). RNA was isolated as described by Takano et al. (1995).

### Construction of induced-hyphal cDNA libraries.

The melanin-induced hyphal cDNA libraries were constructed in lambda GEM-4 (Promega, Madison, WI) by means of the Boehringer cDNA synthesis kit with slight modifications. Poly(A)$^+$ RNA (7 μg) was annealed to an *Xba*I oligo (dT) primer-adapter. The first-strand cDNA synthesis reaction mixtures contained the following: 5 mM dATP, dGTP, dTTP, 5-methyl dCTP, 25 units of RNasin, and 40 units of AMV reverse transcriptase. The mixtures were incubated for 1 h at 42°C. The DNA/RNA hybrids were collected by ethanol precipitation and second-strand DNA synthesis reaction mixtures contained the following: 5 mM dATP, dGTP, dTTP, 5-methyl dCTP, 20 μCi $^{32}$P-dCTP, 25 units of *Escherichia coli* DNA polymerase, 20 units of RNase, and 80 units of T4 DNA polymerase. The cDNA was phenol extracted and ethanol pre-

cipitated, and ligated to *Eco*RI linkers by means of 350 units of T4 DNA ligase for 16 h at 15°C. The DNA was digested with *Xba*I and *Eco*RI and inserts were gel eluted, phenol extracted, and ethanol precipitated. The cDNA inserts were ligated to lambda GEM-4 and packaged.

**Primer extension analysis.**

Poly(A)$^+$ RNA (10 μg) from melanin-induced hyphae was used to map the 5′ end of the *THR1* transcript. The oligonucleotide 5′-GGTGACGAGAGCAACCTT-3′ is complementary to the *THR1* mRNA for 18 nucleotides starting 100 nucleotides downstream of the ATG initiation codon. A 20-μl reaction mixture composed of 4.0 pmol oligonucleotide primer, 10 mCi $^{32}$P-dCTP (3,000 Ci/mmol), 5 mM dATP, dGTP, dTTP, 0.5 mM dCTP, 25 units of RNasin, and 20 units of AMV reverse transcriptase was prepared and incubated for 1 h at 42°C. The products were analyzed by electrophoresis on a 6% urea (wt/vol) polyacrylamide sequencing gel. A ladder sequence was used to determine the reverse transcribed products.

**RNA blot hybridization.**

RNA samples were denatured in formaldehyde/morpholinepropanesulfonic acid (MOPS)/EDTA buffer at 65°C for 10 min. Gel electrophoresis was done on 1.5% agarose gels containing 5% formaldehyde and 20 mM MOPS. The gel was blotted onto Hybond N$^+$ nylon membrane and hybridized by means of in vitro T7 RNA polymerase transcripts of a clone containing a *THR1* sequence from 1228 to 1577 (Fig. 6) as probe. The membrane was washed four times with 0.1% sodium dodecyl sulfate/0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 60°C for 20 min.

**Preparation of fungal genomic DNA.**

Genomic fungal DNA was isolated by the procedure described in Perpetua et al. (1994).

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