MOLECULAR AND CELLULAR BIOLOGY, Oct. 1992, p. 4539–4544 Vol. 12, No. 10
0270-7306/92/104539-06$02.00/0
Copyright © 1992, American Society for Microbiology

Cutinase in *Cryphonectria parasitica*, the Chestnut Blight Fungus: Suppression of Cutinase Gene Expression in Isogenic Hypovirulent Strains Containing Double-Stranded RNAs

D. A. VARLEY,[1] G. K. PODILA,[1] AND S. T. HIREMATH[2]*

Department of Biological Sciences, Michigan Technological University, Houghton, Michigan 49931,[1] and Northeastern Forest Experiment Station, Delaware, Ohio 43015[2]

Received 26 June 1992/Accepted 20 July 1992

Plant-pathogenic fungi produce cutinase, an enzyme required to degrade plant cuticles and facilitate penetration into the host. The absence of cutinase or a decrease in its production has been associated with a decrease in pathogenicity of the fungus. A set of isogenic strains of *Cryphonectria parasitica*, the chestnut blight fungus, was tested for the presence and amounts of cutinase activity. The virulent strain of *C. parasitica* produced and secreted significantly higher amounts of cutinase than the hypovirulent strains. Use of both nucleic acid and polyclonal antibody probes for cutinase from *Fusarium solani* f. sp. *pisi* showed that cutinase in *C. parasitica* is 25 kDa in size and is coded by a 1.1-kb mRNA. Both mRNA and protein were inducible by cutin hydrolysate, while hypovirulence agents suppressed the level of mRNA and the enzyme. Since all the strains had the cutinase gene, the suppression of expression was due to the hypovirulence agents. The data presented are the first report indicating that hypovirulence agents in *C. parasitica* regulate a gene associated with pathogenicity in other plant-pathogenic fungi.

Several strains of *Cryphonectria parasitica*, the causal agent of chestnut blight, have been found in nature which exhibit varying degrees of virulence in the development of the blight disease (2, 13). The most virulent (V) strains do not have any double-stranded RNAs (dsRNAs) in their cytoplasm. Strains which contain one or more dsRNAs display decreased virulence and therefore are called hypovirulent (H) strains (8, 17). There is a great variation among H strains in the number, size, concentration, and sequence similarities of the dsRNAs, and in the degree of hypovirulence (2, 8, 12, 14, 17, 30, 35). The dsRNAs, which have organizational features characteristic of the genomes of fungal viruses (18, 21, 22, 40, 50), are packaged in lipid vesicles (12) along with RNA-dependent RNA polymerase (18). Evidence suggests that the dsRNAs are the main, if not sole, H agents. The dsRNAs can move from H strains to a V strain through hyphal anastomosis, after which the recipient (V) strain will exhibit the phenotypic traits of the donor H strain (3). This phenomenon of transmissible hypovirulence has been the focus of recent efforts to use H strains for the biological control of this devastating disease (2, 34).

Although H strains have been successful in controlling the blight in Europe, H strains from Europe as well as the North American continent have had limited success in the United States. This has necessitated further study to understand the mechanism of hypovirulence. The major thrust of research to date has been in examining the structural organization of the dsRNAs and the genes coded by them. The European H strain EP713 has been shown to contain dsRNA coding for at least two proteins, both of which have autocatalytic protease activity similar to that found in potyviruses (6, 40, 46). Expression of one of these proteins in a virulent strain resulted in the strain exhibiting phenotypic traits characteristic of hypovirulent strains (5). However, there was no reduction in the virulence of the fungus. The role of the dsRNA and/or dsRNA-coded proteins in suppressing the virulence is yet to be determined.

Although considerable efforts have been undertaken to characterize the dsRNAs, very little effort has been made so far to identify the factors that determine the virulence of the blight fungus. Using differential hybridization techniques, genes expressed specifically in V strains have been isolated (37). Analyses of proteins from V and H strains have showed that accumulation of certain proteins in V strains is suppressed by the H agents (38). However, with the exception of the phenyl oxidase of the laccase type (19, 41, 42), these proteins and genes have not been identified and their function in hypovirulence is yet to be determined.

Recent studies have shown that the H strains produce reduced amounts of the enzyme laccase compared with the V strain (19, 41, 42). Transformation of a V strain with a cDNA copy of a gene coded by the dsRNA also resulted in a reduction of laccase production (5). The enzyme laccase is widely distributed in plants and fungi, and its function in fungi is largely unknown. It has been suggested to have a role in the formation of fruiting bodies and in the degradation of lignin. The presence of laccase in both pathogenic and nonpathogenic fungi suggests that the enzyme is not directly linked to pathogenesis.

To infect and cause the blight, it is likely that *C. parasitica* possesses genes for cutinases and pectinases to breach the outer barriers of the epidermal cells and other genes such as cellulases to colonize the host. There is considerable evidence indicating that cutinases play a crucial role in the penetration and establishment of disease by plant-pathogenic fungi (11, 24, 25, 26). Recent studies have shown that cutinase production by germinating spores is induced by cutin monomers (36). Furthermore, the de novo synthesis of cutinase is essential to produce enough cutinase for the dissolution of the plant cuticle during penetration by the fungus (26).

In this report, we provide evidence for the suppression of

* Corresponding author.

cutinase gene expression in *C. parasitica* by the cytoplasmic H agents.

MATERIALS AND METHODS

Fungal strains and growth conditions. A set of *C. parasitica* strains constructed by Anagnostakis (1) were used for this study. These strains were produced by converting the American V strain, EP155, with H isolates from Europe and North America. Since nuclear genetic elements in all these strains are identical, any changes in gene expression are the result of a change in the cytoplasm, including the H agents.

The fungal cultures were grown in EP basal medium (39) containing 2% glucose. For the induction of cutinase, conditions described for *Fusarium solani* f. sp. *pisi* (31) were used. The cultures were grown in EP basal medium containing 0.2% glucose as the carbon source. After 3 to 4 days when glucose was depleted (measured by the glucose oxidase assay [Sigma Chemical Co., St. Louis, Mo.]), cutin hydrolysate was added to the culture medium at a concentration of 50 μg/ml. The cultures were grown for ~2 days and harvested.

In all cases, the cultures were grown for a total of 6 days. The mycelia were harvested by filtration, the filtrate was centrifuged at 10,000 × *g* for 20 min, and the clear supernatants were used for activity measurements and protein analysis.

Cutinase assay. A cutinase assay with equal volumes of culture supernatants (100 μl) was performed as described by Lin and Kolattukudy (31) for *F. solani* f. sp. *pisi*.

Nucleic acids. Mycelia were lyophilized and ground to a powder in liquid nitrogen. The DNA was isolated by the method described by Davis et al. (7). The RNA was isolated essentially as described by Chirgwin et al. (4). The ground mycelial powder was homogenized in 4 M guanidinium thiocyanate solution and centrifuged at 10,000 × *g* for 10 min. Cesium chloride was added to give a concentration of 0.4 g/ml, and the solution was layered on a cushion of 5.7 M cesium chloride solution in an autoclaved polyallomer tube. The solution was subjected to centrifugation at 113,000 × *g* (Beckman Ultracentrifuge L8-80, SW41 rotor) at 22°C for 20 h to pellet the RNA. The RNA was further purified by extraction with a phenol-chloroform mixture and precipitation with ethanol (44).

Southern and Northern analyses. Genomic DNA (10 μg) from the fungal tissues was digested with *Pst*I, electrophoresed on a 0.8% agarose gel, and electrophoretically transferred to Magnagraph (Micro Separations Inc.) nylon membranes as described elsewhere (44). Total RNA (15 μg) from each strain was resolved by denaturing (formaldehyde) gel electrophoresis on a 1.5% agarose gel. The RNAs were visualized after staining with ethidium bromide. The profiles of RNA in all the lanes were identical (data not shown). The resolved RNAs were transferred to a nylon membrane (16).

A cDNA clone for cutinase from *F. solani* f. sp. *pisi* (48) was used as a probe. The cDNA insert was isolated by gel electrophoresis and electroelution and labeling with [α-^{32}P]dCTP by the random primer method (44). Hybridization and other analytical procedures were essentially as described by Sambrook et al. (44). Prehybridization was for 2 h at 42°C in a solution containing 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), 0.1% sodium dodecyl sulfate (SDS), 50% formamide, 5× Denhardt's solution, 100 μg of yeast RNA (Sigma) per ml, and 100 μg of sheared and denatured salmon sperm DNA per ml. Hybridization was at 45°C for 16 to 24 h in the same solution containing ~10^6 cpm of labeled probe per ml. The blots were washed to a final stringency of 0.1× SSPE–0.1% SDS at 65°C and exposed to Kodak XAR-5 film.

Protein analysis. Culture supernatants were either dialyzed against 10 mM Tris (pH 7.0) and lyophilized or subjected to precipitation with 80% acetone (20) to obtain proteins. The protein precipitate was suspended in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (29), heated at 100°C for 5 min, and subjected to SDS-PAGE on 4 to 20% polyacrylamide gradient gels (Bio-Rad Laboratories, Richmond, Calif.). Cellular proteins were extracted from lyophilized and powdered mycelia (38), and equal amounts of protein (20 μg) were analyzed by SDS-PAGE on 7.5% polyacrylamide gels.

Western blotting, (immunoblotting). The culture supernatants and the cell extracts resolved by SDS-PAGE as described above were transferred to Immobilon-P membranes (Amersham) essentially as described by Matsudaira (33). The transfer was performed at 4°C, using CAPS [3(cyclohexylamino)-1-propane sulfonic acid] buffer (pH 11.0) containing 10% methanol at 25 V for 16 to 20 h. The membranes were subjected to Western blotting with the Protoblot Western Blotting Kit (Promega Corp., Madison, Wis.). Antiserum raised against cutinase from *F. solani* f. sp. *pisi* was used (at a dilution of 10^{-4}) as a primary antibody.

RESULTS

Cutinase activity. The isogenic strains of *C. parasitica* were selected for this study since they differed only in their cytoplasmic contents. The parent strain was the American V strain, EP155. This was converted by the hypovirulent isolates from France, Italy, Michigan, Tennessee, and Virginia to produce EP713, EP779, EP868, EP905, and EP915, respectively (1).

Cutinase is synthesized by plant-pathogenic fungi and is secreted into the medium. The synthesis is known to be inhibited by the presence of glucose in the growth medium (26). Also, to synthesize cutinase, the fungi have to be induced by providing cutin monomers as the main carbon source in the growth medium. Therefore, the *C. parasitica* strains described above were grown in EP basal medium containing 2% glucose as well as under the inducible conditions.

The growth medium and the culture supernatants of strains grown in the presence of 2% glucose did not have detectable cutinase activity (data not shown).

Cultures grown in the presence of cutin hydrolysate indicated the presence of cutinase in their culture supernatants. The wet mycelial weight of all the strains grown under these conditions and recovered after 6 days was roughly the same. Therefore, an aliquot of 0.1 ml from each culture supernatant was used for the assays. The results of the assays and calculated values for activity are presented in Fig. 1. The highest cutinase activity was in the V strain, EP155. The H strains, on the other hand, had significantly lower cutinase activity in their culture supernatants. The strains EP713, EP779, and EP868 had the lowest activity (10% or less compared with EP155) among all the H strains. Two H strains, EP905 and EP915, showed slightly higher (~18 and 32%, respectively) activity but significantly less than that of EP155.

Southern analysis. Since cutinase genes of several fungi have been shown to contain conserved sequences (15), we decided to test the feasibility of using a probe from heterologous source. The DNA from *C. parasitica* strains was

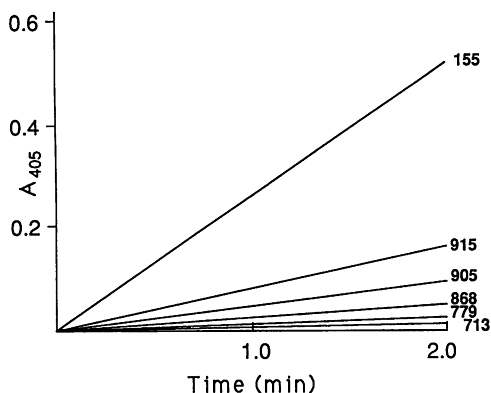

FIG. 1. Cutinase activity in the culture supernatants of *C. parasitica* strains. The virulent strain, EP155, and isogenic hypovirulent strains were grown in EP basal medium to which cutin hydrolysate was added (see Materials and Methods). The culture supernatants were separated by filtration and then centrifugation. Aliquots of 100 μl were used in the cutinase activity assay in which *p*-nitrophenyl butyrate was the substrate, and changes in the A_{450} were recorded. Activity units (1 U is nanomoles of substrate hydrolyzed per minute per milliliter of culture supernatant) for various strains were EP155, 189.0; EP713, 10.8; EP779, 17.8; EP868, 21.6; EP905, 35.6; EP915, 60.5.

digested with *Pst*I, and fragments were transferred to a nylon membrane and hybridized to a probe corresponding to cDNA of cutinase mRNA from *F. solani* f. sp. *pisi*. The hybridization analysis, performed under moderately stringent conditions (50% formamide, 45°C), suggested that the probe could be used to detect the homologous cutinase gene in *C. parasitica* (Fig. 2). The probe hybridized strongly to a single fragment of ~3.1 kbp. The hybridization profile for all the strains was almost identical.

Northern (RNA) analysis. The results obtained with RNA from strains grown under the inducible conditions are presented in Fig. 3. Equal amounts of total RNA from each strain (15 μg) were subjected to the analysis. A single RNA species, ~1.1 kb in size, hybridized strongly to the probe. This RNA species was not detectable in EP713, EP779, and EP868 under these conditions. However, samples from EP905 and EP915 showed the presence of the 1.1-kb RNA

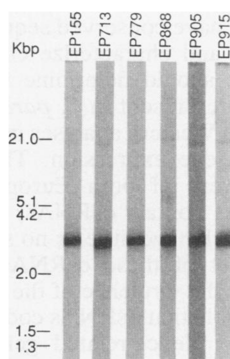

FIG. 2. Southern hybridization. Genomic DNA (10 μg) from *C. parasitica* strains was digested with *Pst*I. The fragments, resolved on 0.8% agarose gels, were transferred to a nylon membrane and hybridized to the probe. A cDNA clone corresponding to cutinase from *F. solani* f. sp. *pisi* was labeled with ^{32}P and used as a probe. Numbers on the left indicate mobilities and sizes of DNA markers.

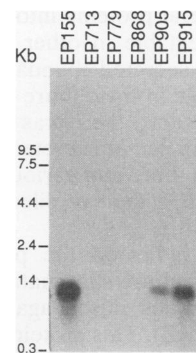

FIG. 3. Northern hybridization. Total RNA (15 μg) was isolated from *C. parasitica* strains grown under inducible conditions (see Materials and Methods) and resolved by denaturing (formamide) gel electrophoresis on 1.5% agarose gels. The separated RNAs were transferred to nylon membranes, and the blot was hybridized to the cutinase probe as described in the legend to Fig. 2. Numbers indicate the sizes of RNA markers (Bethesda Research Laboratories, Gaithersburg, Md.).

species, although the amounts were significantly lower than that in EP155. Analysis with 30 μg of total RNA indicated that strain EP779 contained trace amounts of this RNA species (data not shown). It was not detectable in EP868 and EP713 even under these conditions.

Similar analysis of RNA derived from cultures grown in the presence of 2% glucose indicated that the 1.1-kb RNA (hybridizable to the probe) was below detectable levels in all the strains (data not shown).

Protein analysis and Western blotting. Proteins were isolated from the culture supernatants of strains grown under inducible conditions as well as in the presence of 2% glucose. Aliquots corresponding to 150 μl of culture supernatant were subjected to SDS-PAGE on 4 to 20% polyacrylamide gradient gels. The Coomassie blue-stained proteins present in supernatants of strains grown under the inducible conditions are shown in Fig. 4A. The protein profiles of the V and various H strains were not identical. The V strain,

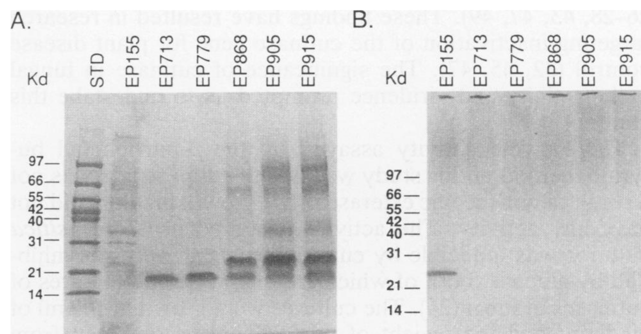

FIG. 4. Analysis of proteins present in the culture supernatants. Proteins were isolated from culture supernatants of strains grown in the presence of cutin hydrolysate. Aliquots corresponding to 150 μl of supernatant were analyzed by SDS-PAGE on 4 to 20% polyacrylamide gradient gels (Bio-Rad). (A) Gel was stained with Coomassie brilliant blue. (B) Resolved proteins were transferred to an Immobilon-P membrane and subjected to Western blotting. An antibody raised against cutinase from *F. solani* f. sp. *pisi* was used as the primary antibody in the Western blot. The positions of marker proteins (Promega Corp.) and their sizes are indicated.

EP155, secreted several proteins into the medium, some of which were not detectable in other samples. Conversely, some of the H strains secreted abundant quantities of certain proteins not detectable in the culture supernatant of EP155. Even among the H strains, there was considerable variation in the type and abundance of secreted proteins. Analysis of cellular proteins from the V and various H strains showed no noticeable differences in their profiles under the conditions used.

Western analysis indicated the presence of a 25-kDa protein in the EP155 culture supernatant which reacted with the polyclonal antibodies raised against cutinase from *F. solani* f. sp. *pisi* (Fig. 4B). This protein could not be detected in samples from EP713, EP779, and EP868 under these conditions. However, it was detected in the samples from EP905 and EP915. The relative levels of the 25-kDa protein among the strains were consistent with the relative levels of the 1.1-kb RNA observed in the Northern analysis and the levels of cutinase activity in the culture supernatants.

Western analyses of culture supernatants from strains grown in the presence of 2% glucose failed to detect the 25-kDa protein in any sample (data not shown).

DISCUSSION

The data presented here indicate that cutinase gene expression in *C. parasitica* is affected by the H agents present in the cytoplasm. This is the first report in *C. parasitica* demonstrating the H-agent regulation of a gene required by other plant-pathogenic fungi for pathogenicity. Earlier works of Powell and Van Alfen (37, 38) had shown that the H agents in the cytoplasm suppressed the expression of several genes. Two genes, Vir 1 and Vir 2, were cloned from the V strain. However, the proteins coded by these genes were not identified, and the potential role of these genes in pathogenicity was not investigated. The presence of H agents also leads to a reduction in production and accumulation of laccase (19, 41, 42). However, reduction in laccase levels did not affect the virulence of the fungus (5). The biological function of laccase in fungi is still unclear. In contrast, cutinase is one of the most important genes in plant-pathogenic fungi, and its involvement in the penetration of the barrier cuticles and in subsequent disease development has been demonstrated in several fungi (9, 10, 23, 26–28, 43, 47, 49). These findings have resulted in research targeting inactivation of the cutinase gene for plant disease control (32, 45, 47). The significance of cutinase in fungal pathogenicity and virulence prompted us to undertake this study.

The enzyme activity assayed by the *p*-nitrophenyl butyrate method in this study was due to cutinase and was not a result of nonspecific esterases. The growth medium did not have any activity. The activity observed in *C. parasitica* cultures was inducible by cutin hydrolysate and was inhibited by glucose, both of which are characteristic features of cutinases in fungi (26). The cultures were grown in 100 ml of medium, and the weight of mycelial tissue obtained from each strain was roughly the same. Therefore, the differences in the amount of cutinase activity per milliliter of the culture medium reflected differences in the level of synthesis and secretion of the enzyme into the medium. The V strain, EP155, secreted significantly higher amounts of the enzyme than the H strains. It has been shown for *F. solani* f. sp. *pisi* and other fungi that there is a direct relationship between pathogenicity and the amount of cutinase released from spores (10, 27, 32). Using mutants of *F. solani* f. sp. *pisi* which produced reduced amount of cutinase, they demonstrated that the efficiency of infection was correlated to the level of cutinase production. In light of this, our results suggest that a similar relationship between virulence and cutinase gene expression exists in *C. parasitica*.

Almost identical patterns were observed in the Southern analysis with the V and H strains. This was expected since the strains used were isogenic. The differences in RNA and proteins synthesized and secreted by the H strains thus were due to differential expression resulting from the cytoplasmic environment.

The analysis of RNA provided evidence that at least one step in the regulation of cutinase gene expression was at the transcriptional level. The hybridization probe detected a single RNA species under moderately stringent conditions. The size of the RNA, 1.1 kb, was similar to the sizes of cutinase mRNAs from other fungi (in *F. solani* f. sp. *pisi*, cutinase mRNA has a size of ~1.0 kb). The EP155 strain, which had the highest enzyme activity, had the highest amounts of this RNA compared with the H strains. As with the enzyme activity, this RNA species was inducible by cutin hydrolysate, while the presence of 2% glucose inhibited synthesis. The data suggest that the 1.1-kb RNA represents mRNA for cutinase in *C. parasitica*. The size of this RNA differs significantly from the 0.65- and 0.85-kb RNAs corresponding, respectively, to the Vir 1 or Vir 2 genes reported by Powell and Van Alfen (37).

The Western analysis further confirmed that cutinase gene expression was suppressed by the H agents. The size of the protein, 25 kDa, was consistent with the 1.1-kb RNA observed in the Northern analysis. Cutinases isolated from other fungi have been shown to have a molecular size of 22 to 32 kDa. The antibody cross-reaction in the Western analysis was very specific. As with activity and Northern analyses, the intensity of this band was consistent with the levels of the enzyme activity and levels of the 1.1-kb RNA species. This correlation, as well as the antigenic relatedness to cutinase from *F. solani* f. sp. *pisi*, suggested that the 25-kDa protein is cutinase from *C. parasitica*. A 24-kDa protein was reported to be synthesized specifically in the V strain of *C. parasitica* (38). This protein was detected in the cell as well as in culture supernatants. Since it was not characterized further, especially by cutin hydrolysate induction and glucose inhibition, it is difficult to infer whether the 24-kDa protein is related to the cutinase.

The data indicate that the *C. parasitica* cutinase is closely related to the cutinase from *F. solani* f. sp. *pisi*. Cutinases from different fungi have conserved sequences (15). Work in progress to isolate and characterize cutinase and its gene from *C. parasitica* should determine whether those conserved sequences are present in *C. parasitica*.

The Northern and Western analyses indicate that H agents suppress cutinase gene expression. The H agents in the strains analyzed were of both European and American origin. These strains contain dsRNAs, presumably the primary H agents, which have little or no sequence homology. However, the effect of these dsRNAs on cutinase gene expression, as with the virulence of the fungus, is relatively the same. It is possible that dsRNAs code for proteins which in turn suppress virulence-related genes. Two dsRNAs-coded proteins have been isolated from EP713 and were shown to have autocatalytic protease activity similar to potyviral proteinases (6, 46), but their effect on the virulence of *C. parasitica* has not been determined. Because of dissimilarities among dsRNA sequences from European and American strains, it is unlikely that they code for a specific

protein(s). Experiments to investigate the effect of these proteins on cutinase gene expression will be very useful in understanding the role of dsRNA in *C. parasitica*.

The degree of suppression of cutinase gene expression varied among the H strains. EP905 and EP915 showed significantly higher levels of expression than the rest of the H strains. Yet all the H strains used in this study showed a roughly equal degree of hypovirulence in the field tests (1, 3). This is not surprising since fungal virulence, presumably, is a combined effect of several gene functions and not an effect of a single gene. A pathogenic fungus uses cutinases, pectinases, ligninases, and cellulases among other enzymes to penetrate and colonize the host. Decrease in the expression of a single enzyme may not be enough to nullify the virulence. Hypovirulence, similarly, might result from the suppression of one or all pathogenic genes in *C. parasitica*. Preliminary work in our laboratories suggests that other genes are also regulated by the H agents. Although EP905 and EP915 showed higher cutinase levels relative to the other H strains tested, a higher degree of suppression of other pathogenicity genes in EP905 and EP915 may be responsible for the overall hypovirulence. Identification and regulatory analysis of other pathogenicity genes is necessary to understand the mechanism of hypovirulence in *C. parasitica*.

ACKNOWLEDGMENTS

We thank P. E. Kolattukudy, Ohio State University, Columbus, for the generous gifts of the cDNA clone and antiserum for cutinase from *F. solani* f. sp. *pisi*. We are grateful to Steven Eshita, Larry Schreiber, S. A. Ghabrial, and L. Shain for helpful discussions and in the preparation of the manuscript. We thank Mahalaxmi Nagarajan and Kirsten Lehtoma for technical help and Rhonda Cobourn for typing the manuscript.

REFERENCES

1. **Anagnostakis, S. L.** 1981. Stability of double-stranded RNA components of *Endothia parasitica* through transfer and subculture. Exp. Mycol. **5:**236–242.
2. **Anagnostakis, S. L.** 1982. Biological control of chestnut blight. Science **215:**466–471.
3. **Anagnostakis, S. L., and P. R. Day.** 1979. Hypovirulence conversion in *Endothia parasitica*. Phytopathology **69:**1226–1229.
4. **Chirgwin, J. M., A. E. Przybyla, R. J. McDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18:**5294–5299.
5. **Choi, G. H., and D. L. Nuss.** 1991. A viral gene confers hypovirulence-associated traits to the chestnut blight fungus. EMBO J. **11:**473–477.
6. **Choi, G. H., R. Shapira, and D. L. Nuss.** 1991. Co-translational autoproteolysis involved in gene expression from a double-stranded RNA genetic element associated with hypovirulence of the chestnut blight fungus. Proc. Natl. Acad. Sci. USA **88:**1167–1171.
7. **Davis, R. W., M. Thomas, J. Cameron, T. P. St. John, S. Scherer, and R. Padget.** 1980. Rapid DNA isolations for enzymatic and hybridization analysis. Methods Enzymol. **65:**404–411.
8. **Day, P. R., J. A. Dodds, J. E. Elliston, R. A. Jaynes, and S. L. Anagnostakis.** 1977. Double-stranded RNA in *Endothia parasitica*. Phytopathology **67:**1393–1396.
9. **Dickman, M. B., S. S. Patil, and P. E. Kolattukudy.** 1982. Purification, characterization, and role in infection of an extracellular cutinolytic enzyme from *Colletotrichum gloeosporioides* nz. on *Carica papaya* L. Physiol. Plant Pathol. **20:**333–347.
10. **Dickman, M. B., S. S. Patil, and P. E. Kolattukudy.** 1983. Effects of organophosphorous pesticides on cutinase activity and infection of papayas by *Colletotrichum gloeosporioides*. Phytopathology **73:**1209–1214.
11. **Dickman, M. B., G. K. Podila, and P. E. Kolattukudy.** 1989. Insertion of a cutinase gene into a wound pathogen enables it to infect intact host. Nature (London) **342:**486–488.
12. **Dodds, J. A.** 1980. Revised estimates of the molecular weights of dsRNA segments in hypovirulent strains of *Endothia parasitica*. Phytopathology **70:**151–158.
13. **Elliston, J. E.** 1982. Hypovirulence, p. 1–33. *In* D. S. Ingram and P. H. Williams (ed.), Advances in plant pathology, vol. 1. Academic Press, Inc., New York.
14. **Elliston, J. E.** 1985. Preliminary evidence for two debilitating cytoplasmic agents in a strain of *Endothia parasitica* from western Michigan. Phytopathology **75:**170–173.
15. **Ettinger, W. F., S. K. Thukral, and P. E. Kolattukudy.** 1987. Structure of cutinase gene, cDNA, and the derived amino acid sequence from phytopathogenic fungi. Biochemistry **26:**7883–7892.
16. **Fourney, R. M., J. Miyakoshi, R. S. Day, and M. C. Peterson.** 1988. Northern blotting: efficient RNA staining and transfer. Focus **10:**5–7.
17. **Fullbright, D. W.** 1984. Effect of eliminating dsRNA in hypovirulent *Endothia parasitica*. Phytopathology **74:**722–724.
18. **Hansen, D. R., N. K. Van Alfen, K. Gillies, and W. A. Powell.** 1985. Naked dsRNA associated with hypovirulence of *Endothia parasitica* is packaged in fungal vesicles. J. Gen. Virol. **66:**2605–2614.
19. **Hillman, B. I., R. Shapira, and D. L. Nuss.** 1990. Hypovirulence-associated suppression of host functions in *Cryphonectria parasitica* can be partially relieved by high light intensity. Phytopathology **80:**950–956.
20. **Hiremath, L. S., N. R. Webb, and R. E. Rhoads.** 1985. Immunological detection of the messenger RNA-binding protein. J. Biol. Chem. **260:**7843–7849.
21. **Hiremath, S. T., B. L'Hostis, S. A. Ghabrial, and R. E. Rhoads.** 1986. Terminal structure of hypovirulence-associated dsRNAs in the chestnut blight fungus *Endothia parasitica*. Nucleic Acids Res. **14:**9877–9896.
22. **Hiremath, S. T., D. L. Nuss, S. A. Ghabrial, and R. E. Rhoads.** 1988. Molecular cloning of hypovirulence-associated double-stranded RNA in *Endothia parasitica* and detection of sequence-related single-stranded RNA. J. Gen. Virol. **69:**2441–2453.
23. **Kolattukudy, P. E.** 1984. Fungal penetration of defensive barriers of plants, p. 31–37. *In* W. M. Dugger and S. Batnicki-Garcia (ed.), Structure, function and biosynthesis of plant cell walls. American Society of Plant Physiologists, Rockville, Md.
24. **Kolattukudy, P. E.** 1985. Enzymatic penetration of the plant cuticle by fungal pathogens. Annu. Rev. Phytopathol. **23:**223–250.
25. **Kölattukudy, P. E., J. Sebastian, W. F. Ettinger, and M. S. Crawford.** 1987. Cutinase and pectinase in host-pathogen and plant-bacterial interaction, p. 43–50. *In* D. P. S. Verma and N. Brisson (ed.), Molecular genetics of plant microbe interaction. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
26. **Köller, W.** 1990. Plant cuticles: the first barriers to be overcome by fungal plant pathogens, p. 219–246. *In* G. T. Cole and H. C. Hoch (ed.), The fungal spore and disease initiation in plants and animals. Plenum Press, New York.
27. **Köller, W., C. R. Allan, and P. E. Kolattukudy.** 1982. Role of cutinase and cell wall degrading enzymes in infection of *Pisum sativum* by *Fusarium solani* f. sp. *pisi*. Physiol. Plant Pathol. **20:**47–60.
28. **Köller, W., and D. M. Parker.** 1989. Purification and characterization of cutinase from *Venturia inaequalis*. Phytopathology **79:**278–283.
29. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
30. **L'Hostis, B., S. T. Hiremath, R. E. Rhoads, and S. A. Ghabrial.** 1985. Lack of sequence homology between double-stranded RNA from European and American hypovirulent strains of *Endothia parasitica*. J. Gen. Virol. **6:**351–355.

31. **Lin, T. S., and P. E. Kolattukudy.** 1978. Induction of a polyester hydrolase (cutinase) by low levels of cutin monomers in *Fusarium solani* f. sp. *pisi*. J. Bacteriol. **133:**942–951.
32. **Maiti, I. B., and P. E. Kolattukudy.** 1979. Prevention of fungal infection of plants by specific inhibitors of cutinase. Science **205:**507–508.
33. **Matsudaira, P.** 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. **262:**10035–10038.
34. **McDonald, W. L., and D. W. Fullbright.** 1991. Biological control of chestnut blight: use and limitations of transmissible hypovirulence. Plant Dis. **75:**656–661.
35. **Paul, C. P., and D. W. Fullbright.** 1988. Double-stranded RNA molecules from Michigan hypovirulent isolates of *Endothia parasitica* vary in size and sequence homology. Phytopathology **78:**751–755.
36. **Podila, G. K., M. B. Dickman, and P. E. Kolattukudy.** 1988. Transcriptional activation of a cutinase gene in isolated fungal nuclei by plant cutin monomers. Science **242:**922–925.
37. **Powell, W. A., and N. K. Van Alfen.** 1987. Differential accumulation of poly(A)$^+$ RNA between virulent and double-stranded RNA-induced hypovirulent strains of *Cryphonectria* (*Endothia*) *parasitica*. Mol. Cell. Biol. **7:**3688–3693.
38. **Powell, W. A., and N. K. Van Alfen.** 1987. Two nonhomologous viruses of *Cryphonectria* (*Endothia*) *parasitica* reduce accumulation of specific virulence-associated polypeptides. J. Bacteriol. **169:**5324–5326.
39. **Puhalla, J. E., and S. L. Anagnostakis.** 1971. Genetics and nutritional requirements of *Endothia parasitica*. Phytopathology **61:**169–173.
40. **Rae, B. P., B. I. Hillman, J. Tartaglia, and D. L. Nuss.** 1989. Characterization of double-stranded RNA genetic elements associated with biological control of chestnut blight: organization of terminal domains and identification of gene products. EMBO J. **8:**657–663.
41. **Rigling, D., U. Heiniger, and H. R. Hohl.** 1989. Reduction of laccase activity in dsRNA-containing hypovirulent strains of *Cryphonectria* (*Endothia*) *parasitica*. Phytopathology **79:**219–223.
42. **Rigling, D., and N. K. Van Alfen.** 1991. Regulation of laccase biosynthesis in the plant-pathogenic fungus *Cryphonectria parasitica* by double-stranded RNA. J. Bacteriol. **173:**8000–8003.
43. **Salinas, J., F. Warnaar, and K. Verhoeff.** 1986. Production of cutin hydrolyzing enzymes by *Botrytis cinerea* in vitro. J. Phytopathol. **116:**299–307.
44. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
45. **Schwinn, F., and H. Geissbuhler.** 1986. Towards a more rational approach to fungicide design. Crop Prot. **5:**33–40.
46. **Shapira, R., G. H. Choi, and D. L. Nuss.** 1991. Virus-like genetic organization and expression strategy for a double-stranded RNA genetic element associated with biological control of chestnut blight. EMBO J. **10:**31–739.
47. **Shaykh, M., C. Soliday, and P. E. Kolattukudy.** 1987. Proof for the production of cutinase by *Fusarium solani* f. *pisi* during the penetration into its host, *Pisum sativum*. Plant Physiol. **60:**170–172.
48. **Soliday, C. L., W. H. Flurkey, T. W. Okita, and P. E. Kolattukudy.** 1984. Cloning and structure determination of cDNA for cutinase, an enzyme involved in fungal penetration of plants. Proc. Natl. Acad. Sci. USA **81:**3939–3943.
49. **Tanabe, K., S. Nishimura, and K. Kohmoto.** 1988. Pathogenicity of cutinase- and pectic enzyme-deficient mutants of *Alternaria alternata* Japanese pear pathotype. Ann. Phytopathol. Soc. Jpn. **54:**552–555.
50. **Tartaglia, J., C. P. Paul, D. W. Fullbright, and D. L. Nuss.** 1986. Structural properties of double-stranded RNAs associated with biological control of chestnut blight fungus. Proc. Natl. Acad. Sci. USA **83:**9109–9113.