Reduction of Laccase Activity in dsRNA-Containing Hypovirulent Strains of *Cryphonectria (Endothia) parasitica*

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ABSTRACT

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Double-stranded RNA (dsRNA) was detected in hypovirulent but not in virulent strains of *Cryphonectria (Endothia) parasitica* isolated in Switzerland. To test for phenol oxidase activity we grew the strains on malt extract agar containing tannic acid (Bavendamm test). All virulent strains produced a strong color reaction, indicating phenol oxidase activity, whereas hypovirulent strains showed weak or no activity. Transfer of dsRNA into virulent strains via hyphal anastamosis resulted in transfer of hypovirulence as tested in the field and loss of phenol oxidase activity. Phenol oxidase is secreted into the medium at the advancing edge of the fungal colony. The enzyme was identified as phenol oxidase of the laccase type. The results suggest that laccase might play a role in pathogenicity of *C. parasitica* and is affected by dsRNA.

Cryphonectria (Endothia) parasitica (Murr.) Barr. is the fungus causing chestnut blight. Hypovirulent strains of the pathogen were isolated in nature and have been associated with the decline of the disease in Europe and in some areas of North America (5,16,19,21, 28,38). Hypovirulent strains differ from virulent strains by their abnormal culture morphology, reduced sporulation, and reduced virulence (14). The hypovirulent phenotype is cytoplasmically controlled and correlated with the presence of double-stranded RNA (dsRNA), presumably of viral origin (12,15,20,40). Hypovirulent strains can convert virulent strains to hypovirulence by transfer of dsRNA via hyphal anastamosis (2). This has been successfully applied to control actively growing cankers (18,27).

Various efforts have been made to correlate hypovirulence with specific biochemical and physiological parameters. Reduced production of oxalate has been associated with the hypovirulent genome (24). However, in another study no difference in oxalate production was found between virulent and hypovirulent strains (7). Recently it was reported that dsRNA reduces the accumulation of specific virulence-associated polypeptides, indicating that hypovirulence is not the result of general debilitation of the pathogen (35). Van Alfen (39) suggested that a locus controlling pigmentation, sporulation, and virulence is affected by dsRNA.

In the present study we report the detection of dsRNA in hypovirulent strains isolated in Switzerland and evidence that the dsRNA affects laccase activity in *C. parasitica*.

MATERIALS AND METHODS

Fungal strains. Virulent and hypovirulent strains of *C. parasitica* used in this study were isolated from natural cankers on European chestnut trees (*Castanea sativa* Mill.) in the southern part of Switzerland (5). The strains were stored lyophilized as described by Bazzigher and Kanzler (6) or maintained on slants of Difco potato-dextrose agar with L-methionine (100 mg/L) and biotin (1 mg/L) (PDAmb).

Culture methods. Mycelia used for dsRNA extraction and pathogenicity tests were grown in KYG medium, which consists of Knop's solution (37) amended with 2% glucose and 0.5% Difco yeast extract, final pH adjusted to pH 4.2. The media were inoculated with conidial suspensions prepared from sporulating cultures grown on slants of PDAmb under white fluorescent light with a 14-hr photoperiod. The cultures (100 ml/500-ml Erlenmeyer

flask) were incubated by shaking at 20 C in the dark for 7 days. The mycelial pellets produced were harvested by filtration in a Büchner funnel, washed with distilled water, and used for pathogenicity tests within 1 day as described below. For isolation of dsRNA the mycelia were frozen at −25 C.

Conversion of virulent strains. Conversions were made by placing virulent and hypovirulent isolates of *C. parasitica* approximately 1 cm apart at the edge of a petri dish containing PDAmb. The converted strains were easily distinguished from the original virulent strains, because they developed white mycelia characteristic of hypovirulent strains (2). Mycelia of the converted strains were removed and transferred to fresh PDAmb.

Pathogenicity test. Five-year-old trees grown from seeds of a single European chestnut tree were used to determine pathogenicity of strains of *C. parasitica*. Wounds were made by removing a plug (5 mm in diameter) of bark to the depth of the cambium. Mycelium was placed into the wound with a flamed spatula and covered with tape to prevent desiccation. Each strain was inoculated into five plants at the beginning of July. After 3 mo, canker length and width were measured, and the canker area was calculated by using the formula for an ellipse.

Isolation of dsRNA. DsRNA was extracted with a procedure modified from that of Day et al (12) and Dodds (13). Frozen mycelia (1.5–3.0 g wet weight) were ground with mortar and pestle in 5 ml of TSE buffer (0.05 M Tris-HCl, 0.1 M NaCl, 0.001 M EDTA, pH 7.0) containing 0.1% bentonite. After adding 0.5 ml of 10% sodium dodecyl sulfate (SDS), total nucleic acid was extracted once with 6 ml of a mixture of phenol (saturated with TSE buffer, and containing 0.1% hydroxyquinoline):chloroform:isoamyl alcohol (25:24:1, by volume) and once with 6 ml of chloroform:isoamyl alcohol (24:1). The aqueous phase was adjusted to 15% ethanol, and dsRNA was purified by chromatography on cellulose CF-11. The dsRNA was concentrated by ethanol precipitation and electrophoresed through a 0.8% agarose gel in TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.2) at 60 V for 2 hr. The gels were stained with ethidium bromide (1.5 μg/ml) and photographed in transmitted UV light on Polaroid type 665 film.

To confirm the nature of the dsRNA, its sensitivity to nucleases was tested before gel electrophoresis. Samples were incubated for 20 min at 30 C with 5 μg of DNase I (Boehringer Mannheim, West Germany) per milliliter in TSE buffer with 30 mM $MgCl_2$. RNase sensitivity of the dsRNA was tested by incubation for 30 min at 25 C with 0.5 μg of RNase (Boehringer Mannheim, West Germany) per milliliter at high (0.3 M NaCl) and low (0.015 M) salt concentrations. To destroy the RNase before electrophoresis, the samples were incubated for 2 hr at room temperature with 2 μg of Proteinase K (Boehringer Mannheim, West Germany) per milliliter in 0.03 M sodium citrate (pH 7.0) with 0.3 M NaCl and 0.05% SDS (42).

Phenol oxidase test (Bavendamm test). To test for phenol oxidase activity the strains were grown on Bavendamm's medium (4). It contains 0.5% tannic acid (Fluka AG, Switzerland, or Merck AG, West Germany), 1.5% Difco malt extract, 2% Difco bacto-agar, adjusted with NaOH to pH 4.5. The tannic acid solution and malt extract agar suspension were autoclaved separately and mixed before dispensing into petri dishes.

Fungal strains were inoculated as plugs (5 mm in diameter) cut with a cork borer from the growing edge of PDAmb cultures. Plates were incubated at 25 C in the dark. Coloring of the agar medium indicates phenol oxidase activity.

Crude enzyme preparation. Bavendamm agar media together with the mycelial mat were cut into small pieces (approximately 2 mm in width, 5 mm in length, and 5 mm in depth), extracted with distilled water (200 μl/150 mg of agar) at 4 C for 30 min, and centrifuged for 3 min at 10,000 *g*. The supernatant solutions were assayed for enzyme activity.

Laccase assay. The activity was determined with 2,6 dimethoxyphenol (DMOP) (Fluka AG, Switzerland) as substrate (8). Enzyme extract (0.2 ml) was added to 0.8 ml of 2.5 mM DMOP in 80 mM sodium tartrate buffer at pH 3.0. The enzyme activity showed a broad optimum between pH 2.5 and 4.5. The increase in absorbance at 468 nm was measured continuously at 25 C with a Perkin-Elmer Lambda 1 spectrophotometer equipped with a thermostatable cell holder. Enzyme activity is given as relative activity (absorbance change per 5 min per sample).

RESULTS

Strains were tested for pathogenicity and the presence of dsRNA (Fig. 1). The strains showing high virulence were found to contain no detectable dsRNA. These virulent strains had normal culture characteristics: Orange pigmentation and abundant orange pycnidia on PDAmb. In contrast, the dsRNA-containing strains were less virulent. These hypovirulent strains showed no orange pigmentation for at least the first 4 days after inoculation on PDAmb and reduced sporulation compared with the virulent strains.

All hypovirulent strains examined contained a high molecular weight dsRNA with identical mobility in agarose gels (Fig. 2). By

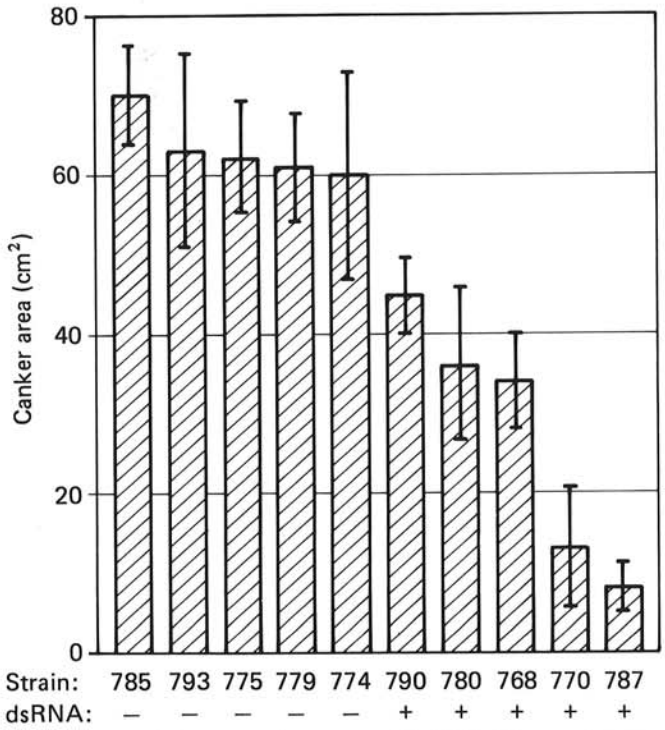

Fig. 1. Pathogenicity tests and presence of dsRNA. Mean canker area produced by dsRNA-free and dsRNA-containing strains of *Cryphonectria parasitica* isolated in Switzerland. Each strain was inoculated into five plants of *Castanea sativa*. Bars represent standard deviations. +, dsRNA detected; −, dsRNA not detected.

Fig. 2. The two types of dsRNA isolated from Swiss hypovirulent strains of *Cryphonectria parasitica*. Agarose gel electrophoresis of dsRNA extracted from H strains M 787 (lane 1) and M 770 (lane 2).

comparison with dsRNA of the French hypovirulent strain B 2025 (Ep 113) of *C. parasitica* (1,13) its molecular weight was estimated to be approximately 6.0×10^6. Additionally, a second smaller dsRNA was found in strain M 770 (Fig. 2). The nature of the dsRNA was confirmed by its resistance to DNase I, resistance to RNase at high salt concentration and susceptibility to RNase at low salt concentration (Fig. 3). Three strains (M 790, M 770, and M 787) representing different levels of hypovirulence were used to convert three virulent strains (M 779, M 775, and M 785) to hypovirulence. Each of the strains was then tested for phenol oxidase activity using the Bavendamm test (Fig. 4). The virulent strains produced a strong color reaction, whereas hypovirulent strains (original and converted)—with one exception—showed a weak or no reaction. Growth of the hypovirulent strains on Bavendamm medium is comparable to that of the virulent strains

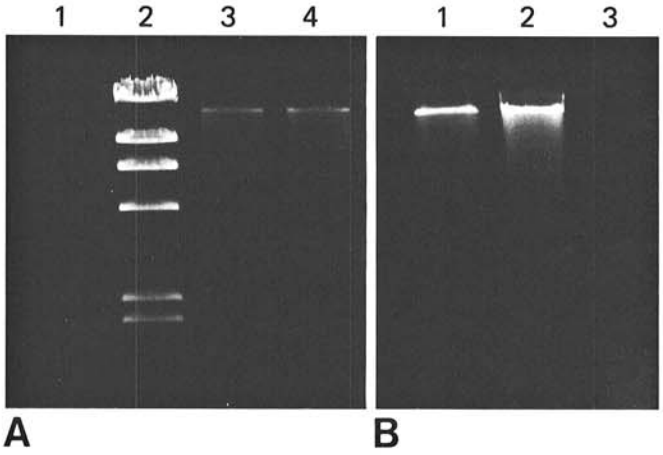

Fig. 3. Nuclease analysis of dsRNA from strain M 787 of *Cryphonectria parasitica*. **A,** Agarose gel electrophoresis of nucleic acids treated with DNaseI. Lane 1, lambda DNA (*Hin*dIII digest) + DNaseI; lane 2, lambda DNA (*Hin*dIII digest), untreated; lane 3, dsRNA + DNaseI; lane 4, dsRNA, untreated. **B,** Agarose gel electrophoresis of dsRNA treated with RNase. Lane 1, untreated dsRNA; lane 2; dsRNA + RNase in high salt (0.3 M NaCl); lane 3, dsRNA + RNase in low salt (0.015 M NaCl).

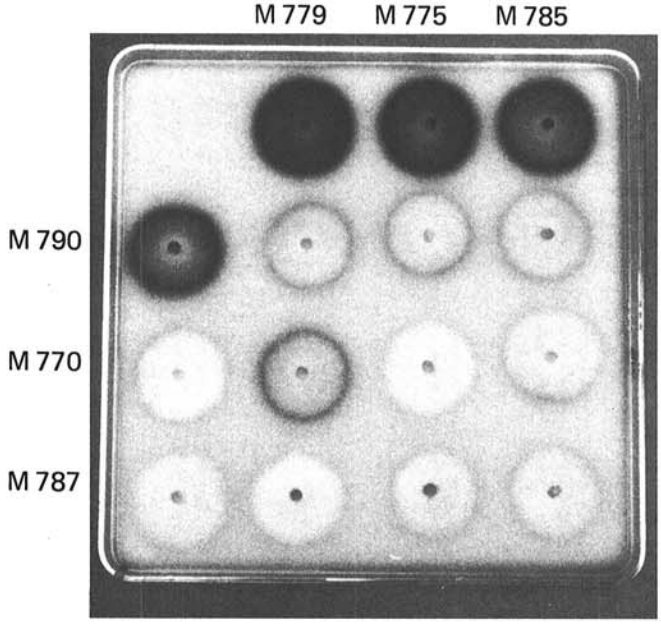

Fig. 4. Bavendamm reaction of three virulent strains (top), three hypovirulent strains (left), and corresponding converts of *Cryphonectria parasitica*. Strains were inoculated 5.5 cm apart from each other on a 23-× 23-cm bioassay plate containing Bavendamm medium, and incubated for 4 days at 25 C in the dark. The photograph was made with transmitted white fluorescent light.

(Fig. 4). Neither the virulent nor the hypovirulent strains gave a color reaction on malt extract agar containing 0.5% gallic acid.

Because laccase activity has been correlated with the Bavendamm reaction in other fungi (23,32), 2,6-dimethoxyphenol, a substrate for laccase and peroxidase but not for tyrosinase (8), was used for the quantitative assay. Laccase activity was found beyond and at the advancing edge of the colony, but not under the older mycelium. Because it was found in front of the colony and easily extractable from the agar, it was concluded that the laccase activity is extracellular. To test the possibility that peroxidases were responsible for the phenol oxidase activity, catalase (80 units/ml) was added in the DMOP assay to destroy the peroxides. No decrease in activity was observed. No activity was detected with tyrosine as substrate.

Characteristics of the 15 strains shown in Figure 4 are summarized in Table 1. Laccase activity was found in agar cultures of all three dsRNA-free virulent strains. The same strains converted to hypovirulence with M 790, M 770, and M 787, respectively, produced no detectable laccase activity. Among the dsRNA-containing strains, only M 790 showed a color reaction and laccase activity, which was approximately half that produced by the virulent strains. The mean canker area produced by this strain was 64–74% of that produced by the virulent strains. Upon subcultivation, the strains M 790 and M 779(770) showed variable Bavendamm reaction ranging from no reaction to that shown in Figure 4.

DISCUSSION

Strains of *C. parasitica* isolated in Switzerland were separable into two groups: dsRNA-free strains with high virulence and dsRNA-containing strains showing different levels of hypovirulence. Similar findings were reported for Italian, French, and American hypovirulent strains of *C. parasitica* (14,16). Electrophoretic analysis of dsRNA of Swiss hypovirulent strains revealed banding patterns also found in Italian hypovirulent strains (1,12,31). This is not surprising because the Swiss chestnut stands are close to the Italian border. Using the Bavendamm test, we found clear differences in phenol oxidase activity between virulent and hypovirulent strains of *C. parasitica*. Transfer of dsRNA into virulent strains via hyphal anastomosis resulted in

TABLE 1. Mean canker area, Bavendamm reaction, and laccase activity of dsRNA-free and dsRNA-containing strains of *Cryphonectria parasitica*

Strain	dsRNA[a]	Canker area[b] (cm^2)	Bavendamm-reaction[c]	Laccase-activity[d] (ΔA_{468}/5 min)
M 779	−	61 ± 7	+++	0.11
M 775	−	62 ± 6	+++	0.12
M 785	−	70 ± 6	+++	0.11
M 790	+	45 ± 5	(++)	0.05
M 779(790)[e]	+	32 ± 5	−	0.0
M 775(790)	+	36 ± 6	−	0.0
M 785(790)	+	37 ± 6	−	0.0
M 770	+	13 ± 7	−	0.0
M 779(770)	ND	13 ± 7	(+)	0.0
M 775(770)	+	10 ± 3	−	0.0
M 785(770)	ND	8 ± 2	−	0.0
M 787	+	8 ± 3	−	0.0
M 779(787)	+	8 ± 3	−	0.0
M 775(787)	+	7 ± 2	−	0.0
M 785(787)	+	8 ± 2	−	0.0

[a] +, dsRNA detected; −, dsRNA not detected; ND, not determined.
[b] Mean canker area (± standard deviation), 3 mo after inoculation into 5-yr-old European chestnut plants, $n = 5$.
[c] Color reaction on Bavendamm medium: + to +++, denotes increasing reaction; (+), (++), variable reactions; −, no reaction.
[d] Laccase activity at the edge of agar cultures grown on Bavendamm medium. Relative activity per sample is given.
[e] Designates a converted strain: Strain M 779 is converted by strain M 790, and so forth.

transfer of hypovirulence and loss of phenol oxidase activity. The evidence that growth of virulent and hypovirulent strains on Bavendamm medium is comparable suggests that dsRNA specifically affects phenol oxidase activity and not general viability of the strains. The enzyme responsible for the color reaction was identified as phenol oxidase of the laccase type. The laccase activity is found at the advancing edge of the fungal colony. This is an important requirement for an enzyme activity that is suggested to play a role in the infection process.

Most fungi that oxidize tannic acid also oxidize gallic acid (11). The negative color reaction with gallic acid suggests that specific inducers are necessary for laccase production in *C. parasitica*. The physiological function of laccase in fungi is still not clear, in spite of its widespread occurrence (34). Laccase activity has been suggested to be involved in degradation of lignin (3,29), pathogenesis (17,33), formation of fruiting bodies (30), and pigmentation (10). With regard to lignification as a defense reaction of chestnut bark attacked by *C. parasitica* (9,25), laccase perhaps interferes with this process or participates in the penetration of the mycelial fan through lignified zones. It also was suggested that laccase plays a role during the infection process by detoxifying host phenolics (34). An interesting feature of laccase is its ability to polymerize phenolic materials into powerful binding substances (22). It was suggested by some authors that a similar mechanism, i.e., strengthening of cell-to-cell adhesion, may be involved in fungal morphogenesis (26,30,41).

Several mutants of the ascomycete *Podospora anserina* deficient in laccase formation exhibited defects in growth rate and in mycelial and sexual morphology (36). Van Alfen (39) pointed out that mycelial fan formation, an important step in expression of virulence (25), and sporulation, were morphogenic events presumably affected by dsRNA in hypovirulent strains of *C. parasitica*. Thus, a deficiency in laccase activity in hypovirulent strains could be an explanation for both reduced sporulation and reduced mycelial fan formation. The use of mutants lacking laccase activity could help to clarify the physiological function of laccase in *C. parasitica*.

Our results corroborate the hypothesis that the cause of hypovirulence in *C. parasitica* is not general debilitation. The postulated locus controlling pigmentation, sporulation, and virulence (39) could be one that controls phenol oxidase activity and which is affected by dsRNA in hypovirulent strains. The fact, however, that the laccase negative strains show different levels of hypovirulence suggests that other factors might also contribute to this dsRNA induced phenomenon.

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