Glycoproteins from *Colletotrichum graminicola* that Bind Phenols: Implications for Survival and Virulence of Phytopathogenic Fungi

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ABSTRACT

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The anthracnose fungus *Colletotrichum graminicola* produces spores in a water-soluble mucilage. A glycoprotein fraction of the mucilage had an exceptionally high affinity for binding to purified condensed tannins (phenolic polymers of flavan-3-ols) and protected spores from inhibition of germination by a variety of phenolic compounds. We hypothesize that the ability of fungal glycoproteins to bind phenols is a mechanism by which fungi protect themselves from toxic phenols produced by plants in response to infection.

Plants produce phenolic compounds which may be toxic to microbes, animals, and other plants. This toxicity, and in particular that of oxidized phenols (quinones), generally is attributed to the compounds' ability to bind to and inactivate proteins (2,15,35). Phenol synthesis increases in plants in response to infection and the eventual accumulation of toxic levels of these compounds is considered an important mechanism through which tissue limits the spread of pathogens (7,23,38).

Knowledge of how fungi survive potentially inhibitory levels of phenols that accumulate in plants during infection or are present in the cuticle and cell wall before infection is fundamental to understanding the development of numerous plant diseases and wood rotting phenomena (4,8,14,18,22). With the exception of enzymatic modification of some phytoalexins (24,25,34), mechanisms of fungal detoxification of plant stress metabolites, of which phenols are prominent examples, have not been described. Our discovery of fungal glycoproteins with high affinity for phenols may represent one mechanism through which fungi inactivate toxic phenolic compounds in their environment.

Anthracnose, caused by the fungus *Colletotrichum graminicola* (Ces.) Wils., is a major disease of cereals throughout the world. The survival and dispersal of this fungus is aided by the presence of a water-soluble mucilaginous material (Fig. 1) which protects spores from desiccation (29). Studies of the composition of the spore mucilage revealed the presence of glycoproteins with carbohydrate and amino acid composition similar to those of animal mucins (31). A relatively high proline content of the glycoproteins (11 mol %) was of particular interest because selective binding to polyphenols is a property of proline-rich proteins (11). The importance of such binding is emphasized by the demonstration that rats and mice fed rations high in tannins (condensed flavan-3-ol polyphenols) produced specific salivary proline-rich glycoproteins. These glycoproteins bound dietary tannin and largely overcame its antinutritional effects (26,27).

The similarity of the *C. graminicola* glycoproteins to animal mucins, and the characteristic production of phenols by plants in response to infection, prompted us to investigate whether protection of spores involves the binding of polyphenols by the spore mucilage. Because proteins can be expected to form complexes with phenols, we asked not whether mucilage proteins bind polyphenols but whether components of the mucilage would exhibit a greater affinity for phenols and whether this affinity is important to survival of the fungus.

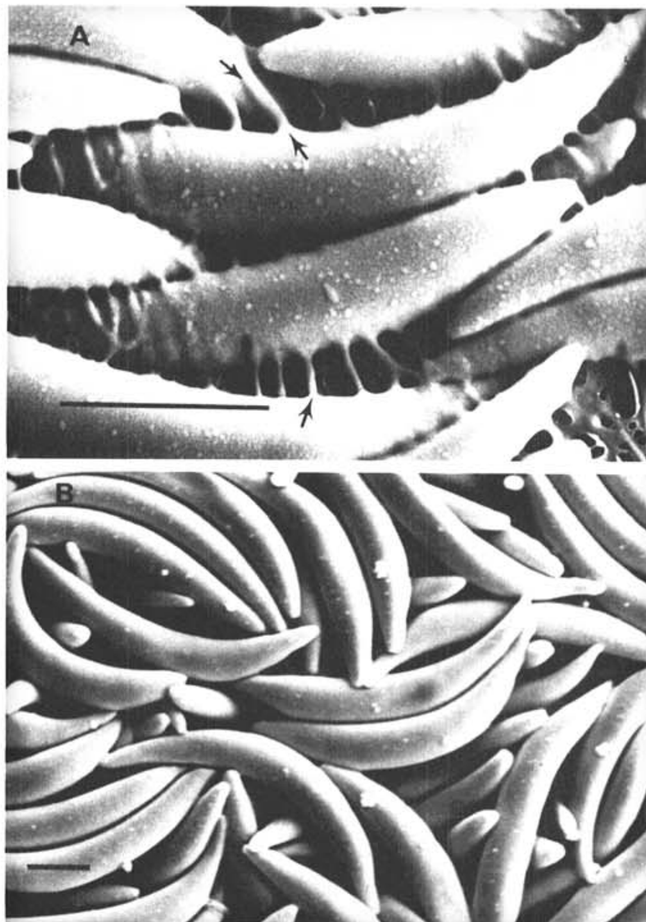

Fig. 1. Scanning electron micrographs of spores of *Colletotrichum graminicola*. **A,** Unwashed spores with associated mucilage (arrows). **B,** Spores washed free of the water soluble mucilage. Bars = 10 μm. Fresh samples were frozen in a nitrogen slush, transferred to the Joel 840-SEM cold stage (−185 C), sublimed at −85 C, sputter coated with gold, and returned to the cold stage for observation and photography.

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MATERIALS AND METHODS

C. graminicola was cultured on oatmeal agar under fluorescent light ($60 \mu E \cdot m^{-2} \cdot s^{-1}$) to induce sporulation and production of the spore mucilage (29). Spore masses with their associated mucilage were scraped from the surface of 10-day-old cultures, and the viscous mucilage was separated from spores by centrifugation (20,000 *g* for 15 min). Thus the viscous spore mucilage was never exposed to any solvent during the collection process. The resulting clear, yellow-brown supernatant (crude spore mucilage) was separated by gel chromatography into various components as described by Ramadoss et al (31). This involved separation of the high molecular weight Peak I glycoprotein complex (referred to as PI) by Sephacryl S-200 gel chromatography. The PI complex of glycoproteins was separated by DEAE cellulose chromatography into the components previously designated A, B, and C (31). Finally PI, A, B, and C glycoprotein components were dialyzed overnight at 4 C against sterile, glass distilled water and lyophilized for use in various experiments.

Competitive binding assay. The ability of glycoprotein fractions of the mucilage to selectively bind polyphenolic materials was determined in a competitive binding assay. The assay measures the relative affinity for polyphenols of proteins by assessing the degree to which they compete for purified tannin with ^{14}C-labeled bovine serum albumin (BSA) (1,11). The tannin-binding efficiency of the mucilage was compared with that of unlabeled BSA, calf skin gelatin, and the purified mouse salivary protein GP-66sm (26).

For competition assays, the competitors (lyophilized mucilage components PI and the PI fractions A, B, and C) were dissolved in 0.2 M acetate buffer (pH 4.8). Different amounts of a competitor were first mixed with 100 μg of ^{14}C-BSA (1 mg/ml in the same buffer) to give a total volume of 640 μl. Then 20 μg of purified condensed tannin in 120 μl of absolute methanol was added to the test protein mixtures. This amount of tannin precipitates $75 \pm 2\%$ of the ^{14}C-BSA in the absence of any competitor. Samples were mixed, incubated for 5 min at 25 C and centrifuged for 5 min at 1,000 *g*. Supernatants were discarded and the pellets representing the tannin-protein complexes were dissolved in 200 μl of 1% (w/v) sodium dodecyl sulfate (SDS). A low level of radioactivity in the SDS-protein solution indicated that the test ligand bound tannin more efficiently than the ^{14}C-BSA (1,11). Condensed tannins (phenolic polymers of flavan-3-ols) were extracted from high tannin sorghum (*Sorghum bicolor* (L.) Moench) grain and purified as described previously (10). BSA was covalently labeled with ^{14}C-formaldehyde by the method of Jentoft and Dearborn (17). Protein was determined by the method of Lowry et al (21).

Gel electrophoresis. Crude spore mucilage (4 ml) was mixed with 2.5 mg of tannin (1 mg/ml of H_2O). After 5 min, the insoluble protein-tannin complexes were removed by centrifugation (1,000 *g*). The pellet was washed by resuspension and centrifugation in distilled water and lyophilized. Native mucilage (100 μl), fraction A of the PI component of the mucilage (1 mg), and proteins precipitated by tannin (0.4 mg) were individually boiled for 1 min in 400 μl of a solution of 1% SDS, 400 mM glycine, 50 mM Trizma, 300 mM 2-mercaptoethanol, 0.004% bromphenol blue, and 20% (v/v) glycerol. Samples (50 μl) were analyzed on 7.5% SDS-polyacrylamide gels (33) and stained with silver stain (39).

Spore germination bioassay. The ability of mucilage components to protect spores from potentially toxic phenols was determined by spore germination bioassay. Spores washed free of mucilage by repetitive centrifugation with fresh, sterile, distilled water (seven washes at 12,000 *g* for 4 sec, Eppendorf Microfuge) were germinated on 0.5% sucrose agar at 28 C in the presence of various phenols and oxidized phenols. Phenols were oxidized with peroxidase and hydrogen peroxide as previously described (19). Counts of germination were made 10 hr after the beginning of the assay procedure. Six replicates of no fewer than 50 spores per replicate were counted for each of nine spore treatments.

Determination of phenols in leaf leachates. Corn (*Zea mays* L.) leaves of the hybrid $Mo17_{Ht} \times B73_{Ht}$ were inoculated with *C. graminicola* (5×10^5 spores per milliter) as previously described (12). When anthracnose lesions ceased to enlarge (10 days post-inoculation) leaves were cut and placed flat in petri dishes. Sterile distilled water (100 μl) was pipetted onto the surface of individual lesions and after 1 hr incubation the liquid (leachate) was collected and filtered (0.45μm fluoropolymer membrane, Gelman Sciences, Inc., Ann Arbor, MI) to remove particulate material. Samples were then assayed for total phenol content (expressed as milligrams of chlorogenic acid equivalents per milliliter) with the Folin-Ciocalteu reagent (3). Leachates collected from healthy leaves served as controls.

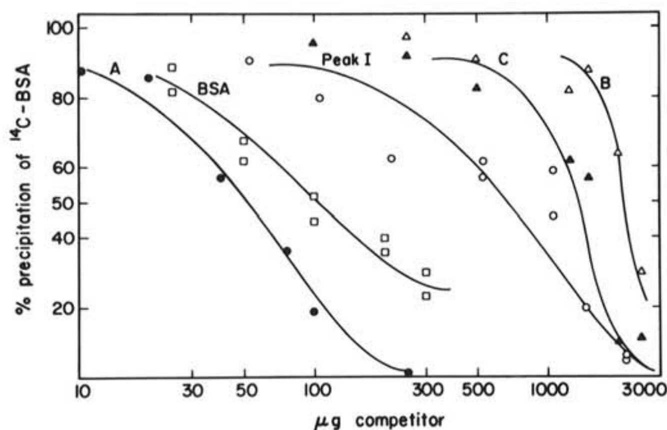

Fig. 2. Competitive binding curves of ^{14}C-bovine serum albumin with purified condensed tannin (phenolic polymers of flavan-3-ols). Competitors were unlabeled bovine serum albumin (BSA) and glycoprotein components (Peak I, A, B, and C) of the spore mucilage from *Colletotrichum graminicola*. Mucilage components obtained as previously described (31).

RESULTS AND DISCUSSION

Based on weight, fraction A of the mucilage glycoproteins was a significantly better competitor for tannin binding than BSA and other mucilage fractions (Fig. 2). The specificity of fraction A for tannin binding became more evident when the data were expressed as relative affinity based on protein content (Table 1). On this basis, fraction A was found to have 26-fold greater affinity for tannin than did BSA and approximately fourfold greater affinity than did the highly specific mouse salivary protein.

As fraction A is a mixture of glycoproteins (31), the association of specific components with tannin binding was assessed by separating tannin precipitable glycoproteins of the mucilage by electrophoresis. Three components of the native mucilage were precipitated by the tannin, and these exhibited migration in the polyacrylamide gel identical to glycoproteins present in fraction A and the native mucilage (Fig. 3). These proteins were estimated to be 66, 165, and 240 kdaltons. Dark areas below the 66-kdalton band in Figure 3 are artefacts.

The ability of the mucilage components PI and fraction A of PI

TABLE 1. Relative affinities[a] of *Colletotrichum gramnicola* spore mucilage fractions and various proteins for purified condensed tannin

Protein competitor	Relative affinity based on	
	Weight	Protein content
PI mucilage component	0.2	1.7
Fraction A	1.4	26.0
Fraction B	0.1	3.8
Fraction C	0.1	7.7
Unlabeled Bovine Serum Albumin	1.0	1.0
Gelatin	5.0	5.0
GP-66sm[b]	3.3	6.7

[a] Relative affinity = amount of ^{14}C-labeled bovine serum albumin in the assay divided by the amount of competitor required to inhibit precipitation of the ^{14}C-labeled protein by 50% (10).

[b] GP-66sm is a proline-rich glycoprotein from the mouse submandibular gland (21).

TABLE 2. Protection of *Colletotrichum graminicola* spores from inhibition of germination caused by various phenols

Spore treatments[a]	% Germination in the presence of[b]			
	Water	Peak I	Fraction A	GP-66sm
Water	90.3 ± 4.6[c]	90.9 ± 2.5	89.6 ± 2.3	89.4 ± 1.9
Ferulic	8.5 ± 3.6	92.2 ± 1.4	91.5 ± 3.2	87.4 ± 2.0
p-Coumaric	17.1 ± 1.6	91.1 ± 2.1	90.7 ± 3.7	79.8 ± 4.0
Vanillic	35.2 ± 5.8	90.8 ± 2.1	89.1 ± 4.8	84.0 ± 5.4
Protocatechuic	44.4 ± 16.7	91.9 ± 1.2	92.2 ± 3.9	89.0 ± 1.6
Chlorogenic	60.3 ± 13.7	90.8 ± 1.8	87.9 ± 3.8	82.2 ± 3.8
Catechin	71.9 ± 3.5	87.7 ± 1.6	88.5 ± 5.4	85.8 ± 2.5
Syringic	76.1 ± 4.9	91.2 ± 4.7	86.7 ± 2.8	78.9 ± 6.2
Tannic	77.8 ± 9.6	89.1 ± 1.6	88.9 ±1.0	83.7 ± 2.8

[a] Phenols administered at a rate of 6mM, except tannic acid (6 mg/ml).
[b] The mucilage component PI and the PI fraction A and mouse salivary protein GP-66sm applied as protectants at a rate of 1 mg/ml of final spore suspension (10^5 spores per milliliter).
[c] Values are means ± S.D. and are for unoxidized phenols.

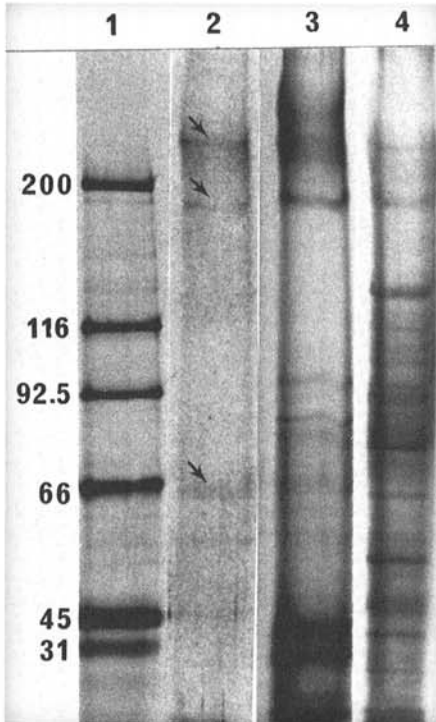

Fig. 3. SDS-polyacrylamide gel electrophoresis of tannin precipitable proteins from the crude spore mucilage of the fungus *Colletotrichum graminicola*. Lane 1 = molecular weight standards, carbonic anhydrase, ovalbumin, BSA, phosphorylase B, β-galactosidase, myosin (31, 45, 66, 92.5, 116.3, and 200 kdaltons, respectively); lane 2 = proteins (arrows) precipitated from crude spore mucilage by purified condensed tannin; lane 3 = mucilage fraction A, lane 4 = crude spore mucilage.

to protect spores from potentially toxic phenols was tested by spore germination bioassay. Spores were equally sensitive to both oxidized and unoxidized phenols and addition of the mucilage components as well as the mouse salivary protein GP-66sm protected spores from the inhibitory effects of the phenolic preparations (Table 2). Data presented in Table 2 are for unoxidized phenols only because results with oxidized phenols were not significantly different.

These results demonstrate that glycoprotein components of the *C. graminicola* spore mucilage have an exceptionally high affinity for binding phenolic materials and that spores are protected from the inhibitory effects of phenols when germinated in the presence of mucilage components.

We hypothesize that the phenol-binding capacity of the mucilage is an important aid to the secondary spread of this pathogen. Spores of the fungus are extruded in mucilage from the acervulus (29) and are dispersed across the leaf in an aqueous mixture of mucilage components. Potentially toxic phenols, including phenylpropanoids, flavonoids, and isoflavonoids, are known to be present as natural components of the leaf cuticle (13,32). However, a more significant source of potentially inhibitory compounds is the lesion margin where phenolic oxidation is assumed to restrict the continued growth of the fungus in the suscept tissue (9,12,19,30,37). Like many fungi, *C. graminicola* sporulates in the lesion center but not in the lesion margin. Phenols that leach from the lesion margin (either into dew or rain water) would be present in the phylloplane. Spores of *C. graminicola,* which must be dispersed by water across the leaf surface, could encounter potentially inhibitory levels of these toxicants. As a test of this hypothesis, we leached materials into water from the surface of uninfected leaves and from the surface of restricted anthracnose lesions on infected leaves. Within 1 hr a substantial amount of phenolic material (0.031–0.048 mg of chlorogenic acid equivalents per milliliter) had leached from leaf lesions. Over the same time no detectable phenols leached from uninfected leaves. In an additional test of our hypothesis, we suspended spores of *C. graminicola* (10^5 spores per milliliter) in the liquid that contained materials leached from infected leaves. Spore germination was compared between spores washed free of mucilage (Fig. 1B) and spores from which mucilage had not been removed (Fig. 1A). When mucilage was present spore germination was 91 ± 4%, whereas when mucilage was absent germination was only 56 ± 12%. In the absence of the leachate spore germination was the same (85%) regardless of the presence or absence of the mucilage.

As numerous fungi produce their spores in mucilage (5,20,36), it is reasonable to expect that the ability to sequester phenols may be common among plant pathogens. The mucilaginous sheaths that surround germ tubes and growing hyphae (6,16) may also contain proline-rich glycoproteins with high affinity for phenols. This passive binding could serve to reduce the accessible concentration of toxic phenols to levels that are not inhibitory. We propose that the ability to produce ligands that bind phenols should be considered as a virulence factor (28) for pathogenicity.

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