

Comparison of Chitinases from Dogwood Anthracnose Resistant and Susceptible *Cornus* Species

Nicole A. Cardwell¹ and Gary L. McDaniel²

¹Department of Ornamental Horticulture and Landscape Design, The University of Tennessee, Knoxville, TN 37996-4500

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Abstract. The pathogenesis-related protein, chitinase, is implicated in the resistance mechanisms involved in dogwood anthracnose, which is caused by *Discula destructiva*. Chitinase isozymes were isolated from *Discula*-inoculated *Cornus mas*, a highly resistant species, and from inoculated *C. florida*, a highly susceptible species. Chitinase activity was identified in *C. mas* on days 2–12 following inoculation, but was expressed only on day 8 following inoculation in *C. florida*. Both dogwood species expressed a constitutive chitinase level in noninoculated control leaves, but *Discula*-inoculated leaves of *C. mas* contained three chitinase isozymes, whereas *C. florida* leaves expressed only two. Molecular masses of isozymes were 21, 32, and 35 kDa for *C. mas* and 21 and 35 kDa for *C. florida*. Isoelectric focusing demonstrated three chitinase isozyme isoelectric points for *C. mas* (pI = 5.6, 6.8, and 8.9), but only two for *C. florida* (pI = 5.6 and 6.8). These differences in synthesized isozymes and rate of accumulation suggest that chitinase may have a role in the defense of dogwood against *D. destructiva* infection.

The economic status of ornamental flowering dogwood (*Cornus florida* L.) is threatened because of the widespread appearance within its natural range of the anthracnose disease caused by the fungus *Discula destructiva* Red. (Redlin, 1991). The disease has destroyed large numbers of trees in naturally forested areas from New England to the southern Appalachian mountain range in northern Georgia and Alabama. Nurseries in the southeastern states grow >20 named flowering dogwood cultivars and ship them to other regions of the United States (Brooker et al., 1995). While Tennessee is the largest producer of these cultivars, other southeastern states contribute significantly in their production. Incidence of the disease is spreading to urban landscapes as far away as Kansas and Michigan; this has been attributed to movement of nursery stock from infected areas (Daughtrey et al., 1996). Thus, there is considerable concern over the negative impact this disease may have on flowering dogwood sales.

Since the 1970s, research has emphasized epidemiological control strategies, identification, and characterization of the pathogen (Daughtrey and Hibben, 1994; Daughtrey et al., 1996; Redlin, 1991; Trigiano et al., 1995).

Plants often synthesize enzymes that inhibit the growth of pathogens. One group of proteins synthesized are termed "pathogenesis-related" proteins (PR-proteins), and includes the fungal cell wall degrading chitinases [poly [1,4-(N-acetyl-β-D-glucosaminide)] glycanohydrolase, EC 3.2.1.14] and β-1,3-glucanases (EC 3.2.1.39). These enzymes reportedly act directly on the invading pathogen (Cutt and Klessig, 1992; Graham and Sticklen, 1994; Mauch et al., 1988; Punja and Zhang, 1993; Simmons, 1994). The biochemical basis for resistance of the dogwood species to dogwood anthracnose disease has not been investigated.

Plant chitinases are categorized in three broad classes (Shinshi et al., 1990): 1) Class I chitinases are basic, generally located in the vacuole, and contain a cysteine-rich N-terminal domain with chitin-binding properties; 2) Class II chitinases are acidic, extracellular, and lack the N-terminal domain of Class I chitinases; and 3) Class III chitinases share sequence homology with the other classes, but lack serological relationship. These are extracellular and may be either acidic or basic and function as lysozymes with chitinase activity.

Many plant chitinases are produced at a low constitutive level in noninfected tissue. Chitinases may be induced by several unrelated factors, such as infection by pathogens, exposure to ethylene, and wounding of tissues (Graham and Sticklen, 1994). Mauch and Staehelin (1989) proposed the following model for plant defense against invading fungal pathogens. Early in the course of infection, β-1,3-glucanases release β-1,3-glucans from fungal hyphae, which serve as elicitors and induce plant defense genes, such as those for extracel-

lular chitinases. Cell lysis due to fungal invasion releases large quantities of invacuolated chitinase. The large outflux of chitinase results in the osmotic lysis of the hyphae. This results in formation of a hypersensitive reaction in resistant hosts that effectively limits infection damage.

While *D. destructiva* produces hyphae having glucan and chitin outer surfaces, whether anthracnose-resistant dogwood species actively produce either of these PR-proteins in response to infection has not been determined. A possible donor for the resistance gene could be the nonnative *C. kousa* (Buerger ex Hance), which is sexually compatible with *C. florida*, but is only moderately resistant to *D. destructiva* (Holmes and Hibben, 1989; Santamour et al., 1989; Sherald et al., 1994). Better potential for obtaining resistance genes is offered by other highly resistant native species, including *C. racemosa* (L.) and *C. canadensis* (L.) (Daughtrey and Hibben, 1994), as well as *C. amomum* (Mill.), *C. alternifolia* (L.), and *C. mas* (L.) (Brown et al., 1992; Sherald et al., 1994). This study was conducted to determine whether *Cornus mas* (highly resistant) and *C. florida* (highly susceptible) express chitinase activity in response to *Discula* infection, and to characterize this protein in both dogwood species.

Materials and Methods

Discula destructiva isolate GA-1 was maintained on potato dextrose agar (PDA) at 16 h light/8 h dark, 50 μmol·m⁻²·s⁻¹, and 17 °C for use in these experiments. Thirty 1-year-old plants each of *C. mas* and *C. florida* were planted in 100% pine bark in 7.6-L containers and placed in a greenhouse maintained at a constant 22 °C. Plants were fertilized with 20N–8.8P–16.6K soluble fertilizer with N at 300 mg·mL⁻¹ once per week. After 5 weeks, plants were transferred to a clear polyethylene plastic-enclosed bench with 75% shade, two humidifiers, and an air conditioner. Plants were maintained at 25 °C and 90% to 95% relative humidity.

Leaves from 10 plants each of *C. mas* and *C. florida* were wounded with a floral needle-point holder (20-mm diameter). Agar plugs containing mycelia and spores of GA-1 (6-mm diameter) were gently applied to wounded leaf surfaces. Leaves were then enclosed in plastic bags. Mock-inoculated leaves (10 plants of each species) were wounded as described and inoculated using ultrapure H₂O and PDA before enclosing in bags. Leaves on 10 control plants of each species were not wounded or treated before enclosing in bags. Following inoculation, plants were placed in a completely randomized design in the chamber. About 10 g of leaves (mean of 6×2.5 cm) were randomly collected from each dogwood species at 12 h, 24 h, and 2, 4, 6, 8, 10, and 12 d following inoculation and placed in liquid N. Samples were stored at –80 °C until used.

Leaves were placed in a prechilled mortar and ground to a fine powder in liquid N with polyvinylpyrrolidone (PVP). Extraction buffer (De Tapia et al., 1986) was added and the

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¹Current address: Dept. of Horticulture, Walters State Community College, Morristown, TN 37814. To whom reprint requests should be addressed.

sample was reground, allowed to thaw, and centrifuged at $23.5 \times 10^3 g_n$ for 30 min at 4 °C. The supernatant was saturated to 40% (w/v) with ammonium sulfate, placed on ice for 2 h, and centrifuged at $23.5 \times 10^3 g_n$ for 15 min at 4 °C. The resulting supernatant was then saturated to 60% (w/v) with ammonium sulfate on ice for 2 h and recentrifuged for 30 min, and the pellet dissolved in ultrapure H₂O. The solution was dialyzed against ultrapure H₂O overnight and protein concentrations were measured according to Bradford (Bradford, 1976), using the protein assay developed by Bio-Rad (Richmond, Calif.) with bovine albumin as the standard protein. Proteins to be used for electrophoresis were extracted as described with the exception that ammonium sulfate saturation was omitted.

Chitinase specific activity was determined by a modification of the Sigma chitinase assay (Sigma, St. Louis). Samples were incubated at 45 °C for 3 h on a rotary shaker, boiled for 15 min, and placed on ice to cool at room temperature. Chitinase was determined photometrically at 540 nm. Sample analysis was replicated three times and specific activities were subjected to analysis of variance (ANOVA) (SAS, 1988).

Proteins from both *Cornus* species were separated by electrophoresis according to the modified method of Pan et al. (1991) in a 15% native polyacrylamide resolving gel (PAGE) using 30 mA constant current at 8 °C. These gels were then overlaid with a 7.5% PAGE containing 0.04% (m/v) glycol chitin and incubated for 12 h at 45 °C under moist conditions (Trudel and Asselin, 1989). Overlay gels were then incubated at room temperature for 5 min with 0.01% (m/v) Calcofluor White M2R in 0.5 M tris-HCl (pH 8.9) and held at 15 °C for 8 h. Protein bands were visualized on overlay gels using a UV transilluminator at 312 nm and photographed with a ISO-1000 Digital Imaging System (Alpha Innotech Corp., San Leandro, Calif.).

For molecular mass determinations, chitinase bands were excised from the original PAGE using overlay positions, combined, and separated by electrophoresis in a 15% PAGE-0.1% sodium dodecyl sulfate (SDS) gel at 200 V for 6 h. Calibration of the PAGE-SDS gel was with Kaleidoscope prestained molecular weight marker kit (Bio-Rad Laboratories, Hercules, Calif.). Following electrophoresis, gels were fixed in 10% (v/v) glutaraldehyde, stained with silver nitrate, and developed according to the methods of Morrissey (1981).

Proteins precipitated without ammonium sulfate were dialyzed against ultrapure H₂O overnight at 4 °C for isoelectric focusing (IEF). Proteins were focused in PhastGel IEF ampholyte (pH 3–9) using a Phastsystem (Pharmacia Biotech, Piscataway, N.J.) and compared with standards from an IEF calibration kit. Focusing conditions were 200 V, 2.5 mA, 3.5 W, 15 °C, and 410 Vh for 30 min. Following electrophoresis, gels were overlaid with glycol chitin-containing PAGE, and incubated as previously described, and isoelectric points (pI) were determined.

Chitinase Activity

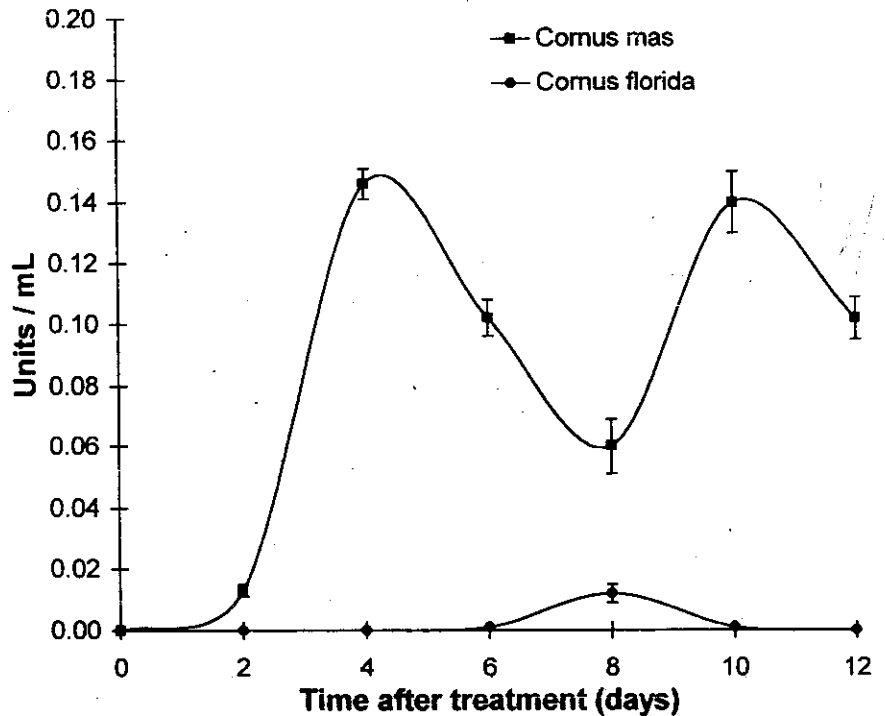


Fig. 1. Chitinase activity in *Cornus mas* and *Cornus florida* following inoculation with *Discula destructiva*. Vertical bars represent means ($n = 3$) \pm se. Where no error bars are shown, se was smaller than the mean indicator symbol.

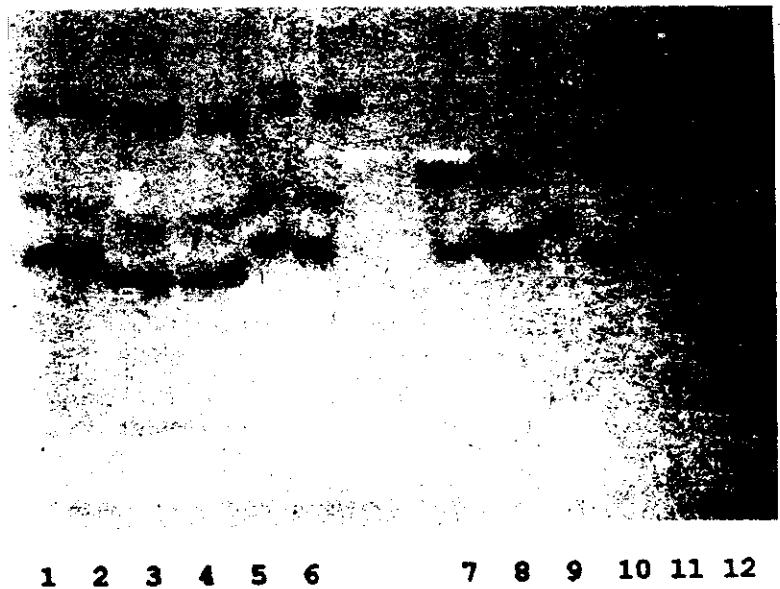


Fig. 2. Glycol chitin-PAGE overlay gel demonstrating chitinase bands from dogwood following inoculation with *Discula destructiva*. Lanes 1–6 = *Cornus mas* (lane 1 = 24 h, lane 2 = day 2, lane 3 = day 4, lane 4 = day 6, lane 5 = day 8, lane 6 = day 10). Lanes 7–12 = *Cornus florida* (lane 7 = 24 h, lane 8 = day 2, lane 9 = day 4, lane 10 = day 6, lane 11 = day 8, lane 12 = day 10).

Results and Discussion

Foliar reactions to wounding and inoculation with *D. destructiva* in *C. florida* were initial tan spots at puncture sites, followed by small purple-rimmed spots. These spots became larger brown patches that expanded progressively from the puncture sites; nearly the entire surface was covered at the end of the 12-d collection period. Inoculated *C. mas* leaves displayed tan spots within 24 h that became rimmed in red. These "hypersensitive" spots did not exceed 1 mm in diameter. Both mock-inoculated species produced tan spots that did not display additional color or increase in size around the puncture sites. No changes in leaf morphology were observed in control plants of either species.

Measurable levels of chitinase were detected in *C. mas* beginning on day 2 following inoculation, with peak activity ($0.14 \pm \text{SE}$ units·mL⁻¹ protein) at days 4 and 10 (Fig. 1). Measurable chitinase levels on inoculated *C. florida* were observed only on day 8 at $0.012 \pm \text{SE}$ units·mL⁻¹ protein. No chitinase was detectable from controls or mock-inoculated *C. florida* at any collection date. A rapid rise in chitinase activity was observed in mock-inoculated *C. mas* on day 4 (0.02 units·mL⁻¹ protein) that was not measurable thereafter (data not shown). This has been previously reported as a response to wounding (mock-inoculation), where single punctures to plant tissue result in a sharp but temporary rise in chitinase activity (Bradshaw et al., 1991; Brederode et al., 1991; Hedrick et al., 1988).

When *Discula*-inoculated *C. mas* and *C. florida* proteins were subjected to electrophoresis in a 15% PAGE and overlaid with glycol chitin-PAGE, chitinase activity was present on all sampling dates (Fig. 2). Under these electrophoretic conditions, three chitinase isozymes were found in *C. mas*, but only two in *C. florida*. Noninoculated control and mock-inoculated *C. mas* overlay gels were similar, but the bands were less dense (not shown). Bands on control and mock-inoculated *C. florida* were also present, but were extremely weak and difficult to visualize (not shown). Because this overlay technique utilizes enzymatic degradation of the glycol chitin substrate, chitinase activity below the limits of detection for the quantitative assay are visible. These results show that a low level of chitinase is expressed constitutively by both dogwood species, although it is not measurable by standard assay methods.

Molecular masses for the three chitinase isozymes from *C. mas* were determined as 35, 32, and 21 ± 5 kDa (Fig. 3). The two chitinase isozymes from *C. florida* had molecular masses of 35 and 21 ± 5 kDa (not shown). Plant chitinases generally range in molecular mass from 25–36 kDa (Graham and Stucklen, 1994), but also have been reported to have masses of from 21 kDa (Ishige et al., 1991) to as small as 12.5 kDa (Kurosaki et al., 1986). The chitinase isozyme isoelectric points were determined for *C. mas* at 5.6, 6.8, and 8.9 and for *C. florida* at 5.6 and 6.8 (Fig. 4). Since the overlay gel only records chitinase degradation of glycol chitin, the standard bands are not visible.

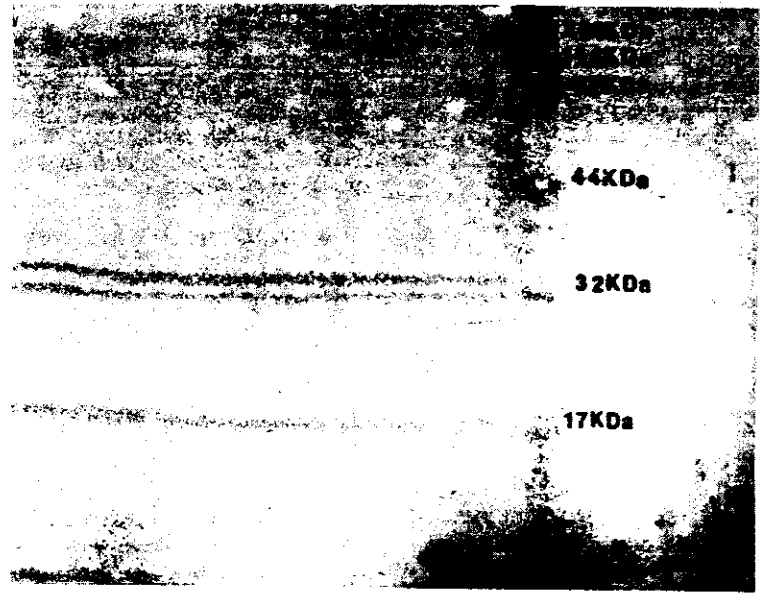


Fig. 3. Molecular mass determinations of chitinase isozymes from *Cornus mas* are estimated at 35, 32, and 21 kDa.

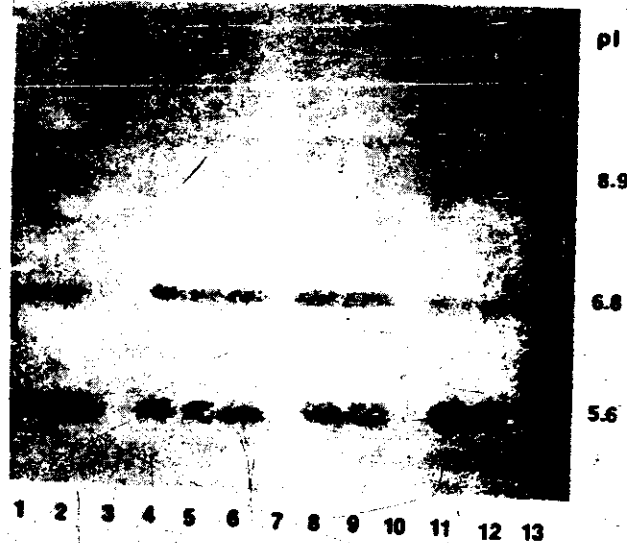


Fig. 4. Isoelectric focusing points for chitinases from *Cornus mas* (CM) and *C. florida* (CF) inoculated with *Discula destructiva*. Lane 1 = CM 24 h, lane 2 = CM day 4, lane 3 = standard, lane 4 = CF 24 h, lane 5 = CF day 4, lane 6 = day 6, lane 7 = not used, lane 8 = CF day 8, lane 9 = CF day 10, lane 10 = standard, lane 11 = CM day 6, lane 12 = CM day 8, and lane 13 = CM day 10. Standard bands do not appear on overlay gels.

Chitinase accumulation in *Discula*-inoculated leaves of *C. mas* differed from that in *C. florida*. The rapid and sustained synthesis of chitinase in *C. mas* apparently contributed to limiting infection to localized penetration sites. In contrast, *Discula* invasion of *C. florida* leaves proceeded unabated, presumably because PR-proteins, such as chitinase, were not present soon enough or in effective concentration to hydrolyze fungal mycelia. The differences in response between the two dogwood species indicates that chitinase may have a role in the resistance to infection by *D. destructiva*.

Further examination of chitinases derived from both dogwood species using electrophoretic separation revealed that an additional isozyme is present in *C. mas*. This chitinase isozyme ($m = 32$ kDa, $\text{pI} = 8.9$) is consistent with basic, invacuolated, class I chitinases previously reported. The absence of accumulated chitinases in vacuoles upon cell lysis by *D. destructiva* would allow rapid invasion of *C. florida* leaf tissue. This study demonstrates that chitinase activity is inducible with *D. destructiva* and that a resistant species and the highly susceptible *C. florida* differ in response.

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