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Accumulation of a Protein Associated with Plant Defense in Powdery Mildew Resistant Dogwood (*Cornus florida*)[1]

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Abstract

This study was conducted to better understand the nature of powdery mildew resistance in flowering dogwood (*Cornus florida* L.) and in particular to determine if inducible plant defense proteins are associated with powdery mildew resistance. Results from this study showed an accumulation of a new protein in resistant plants, but not in susceptible plants that were challenged with powdery mildew pathogen (*Erysiphe* (Sect. *Microsphaera*) *pulchra*). The protein accumulated in a high level in the resistant selections at 48 hr after inoculation with the pathogen and that was consistent with the production of pathogenesis related (PR) proteins. The protein was characterized as having an isoelectric point of 7.5 ± 0.5 and molecular weight of 18 ± 2 KD. Partial sequence analysis of this protein revealed homology with PR-10 protein associated with drought resistance in potato and was analogous to other proteins related to resistance in other crops. Repeated analysis showed similar results and suggested that a biochemical mode of resistance involving plant defense proteins may be associated with powdery mildew resistance in flowering dogwood.

Index words: powdery mildew, host resistance, biochemical resistance, plant proteins, PR proteins, *Erysiphe* (Sect. *Microsphaera) pulchra*.

Species used in this study: flowering dogwood (*Cornus florida* L.).

Significance to the Nursery Industry

The nature of powdery mildew resistance in the newly identified sources of powdery mildew resistance is not known and it is difficult and time consuming to use conventional breeding methods. However, understanding the basis of host resistance to powdery mildew in flowering dogwood will help further efforts to introduce genes for disease resistance in commercial cultivars. The objective of this research was to determine whether the resistance to powdery mildew in *Cornus florida* was associated with defense related PR proteins.

Introduction

Powdery mildew is an important constraint in dogwood production throughout the southeastern United States (10, 18, 19, 21, 23). Powdery mildew stunts growth and reduces flower bud set; severe infections have also been associated with death of young seedlings (5). Efforts to breed for powdery mildew resistance are underway with plant selections from natural crossing providing disease resistance genes (20, 30). However, the nature of powdery mildew resistance in the newly identified sources of powdery mildew resistance is not known (20, 21, 30).

The basis of host resistance to plant pathogens may be mechanical or biochemical, and in some cases, more than one mechanism may be associated with disease resistance (1). Structural characteristics of leaf surfaces that induce appresoria formation may also decrease receptivity to infection (2). Waxes and leaf pubescence may provide physical barriers to plant pathogens (13, 22). In addition, cuticle thickness may increase resistance to pathogens that do not depend upon epidermal openings to penetrate the host (6, 24).

Host resistance that is based on biochemical processes involves metabolic reactions in plant cells that result in the production of chemical compounds harmful to the pathogen (1). The chemical compounds include secondary metabolic compounds such as phytoalexins, lipid-derived metabolites, and a broad range of defense-related proteins and peptides or signaling compounds, such as salicylic acid and ethylene (3, 8, 9, 11, 12). Among the defense related proteins are pathogenesis-related-proteins (PR proteins) that are inducible and accumulate systemically in resistant plants during the early stages of host-parasite interaction (3, 26, 27, 28).

In the last 25 years, a wide spectrum of PR proteins have been characterized in diverse plant species and grouped into families according to their mode of action and chemical characteristics (7, 8). In a review published in 1999, 14 families were recognized and named PR-1 to PR-14 (26). PR proteins have been identified as chitinases, gluconases, proteinase inhibitors, endoproteases, peroxidases, ribonuclease-like proteins, and as human allergens (27). Many of the PR proteins are not unique to a given plant species, but are found in a wide range of organisms (25). Some PR protein genes have been sequenced and the regulation of these genes has been determined, but the activities of many of the PR-proteins have not been characterized (3). Chitinase PR-proteins were implicated in dogwood resistance to *Discula destrutiva* in *Cornus mas* (4), but the involvement of PR proteins in the interaction with powdery mildew fungi has not been reported. The objective of this research was to determine whether the resistance to powdery mildew in *Cornus florida* was associated with defense related PR proteins.

Materials and Methods

Plant material and inoculation with powdery mildew. Previously identified resistant (R) and susceptible (S) plants were selected for this study. *C. florida* selections RN14 (R), WR16 (R), WR20 (R), 'Cherokee Princess' (S), and Selection HL26

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(S), had exhibited field resistance (R) or susceptibility(S) to powdery mildew over a 4-year period. The selections were derived from natural out-crossing and evaluated for powdery mildew reaction at the Tennessee State University Nursery Crop Research Station evaluation plots in McMinnville, TN. Leaves from the selected plants were collected in early spring, before powdery mildew was observed in the local area. Leaves were transferred on ice to the laboratory, surface disinfected using 1% commercial bleach (Clorox®) for 2.5 min, rinsed twice with sterile-distilled water, blot-dried with sterile paper towels, and transferred to Petri plates containing 1% water agar with leaf petioles pushed into the agar for moisture.

Powdery mildew inoculum consisting of conidiospores of *E. pulchra* was collected from previously infected *C. florida* maintained in the greenhouse. A soft camel-hair paintbrush was used to release conidiospores from infected plants and inoculate the detached leaves. Inoculated and non-inoculated (control) leaves were incubated at room temperature (24 ± 2C (75 ± 4F)) for 12, 24, 48 and 72 hr. Replications of 16 inoculated and 16 non-inoculated leaves were used; at the end of each incubation period, leaf samples were removed from the plates in two sets of eight inoculated or non-inoculated leaves. Tissue from the leaf lamina of each set of eight leaves was combined for protein extraction. Leaves that were not processed immediately after incubation were stored at –80C (–112F) until protein extraction.

Protein extraction and protein electrophoresis. Total protein was extracted from the leaves using a modification of the phenol extraction method reported by Hurkman and Tanaka (15). Proteins were separated by two-dimensional isoelectric focusing (IEF)/SDS-polyacrylamide gel electrophoresis. The IEF was performed in capillary gels using a tube electrophoresis system (24-plate unit, CBS Scientific, Del Mar, CA) at 4C. Capillary gels, which were of 10-cm length and 1.5-mm diameter, were inserted in capillary glass. The gels contained 7.5 M urea, 4.5% acrylamide/bis-acrylamide, 1.5% CHAPS, 9% glycerol, 1.26% ampholyte pH 3–7, 0.54% ampholyte pH 3–10, 0.2% ammonium persulfate, and 0.15% TEMED. The capillary gels were pre-run at 200 V for 15 min, 300 V for 15 min and 400 V for 15 min loaded with 10 µl of sample buffer overlaid with 10 µl of a solution containing 8 M urea, 0.28% ampholyte pH 3–7, 0.12% ampholyte pH 3–10, 5% CHAPS, 0.1 M DTT (overlay solution). The cathode buffer was 10 mM phosphoric acid and the anode buffer was 20 mM NaOH. Protein concentration was estimated in the protein extract using Micro Protein Determination Phenol Reagent Method for biological fluids (Sigma, Diagnostics, St. Louis, MO), then aliquots corresponding to 100 µg of total protein were loaded in the capillary, overlaid with 10 µl of overlay solution and subjected to electrophoresis. The IEF electrophoresis was performed at 400 V for 15 hr and 800 V for 2 hr using the same anode and the cathode buffer solutions described for the pre-run. After the electrophoresis was completed, gels were removed from the capillary glass by air pressure and treated for 5 min with a buffer containing 0.06 M Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 100 mM DTT, and 10% glycerol. Capillary gels were stored at –20C (–4F) if the second electrophoresis was not performed immediately after IEF.

The second dimension electrophoresis was done using a Dual-Gel Electrophoresis system (Owl Separation Systems, Portsmouth, NH) according to the manufacturer's instructions. Capillary gels from the IEF were placed on top of a 16 × 14 (6.3 × 5.5 in) cm gel (10% polyacrylamide/bis-acrylamyde, 0.5 M Tris pH 8.8, 1% SDS, 1% ammonium persulfate, and 0.04% TEMED) and run at 140 V for 1 hr and at 220 V for 1 hr 40 min using TGS buffer (Tris-Glycine SDS buffer, Bio-Rad Laboratories, Hercules, CA). Gels were separated from the glass plates, silver stained and scanned using a Bio-Rad Flor-f multi-imaging scanner with Quality One, version 4.1.1 software (Bio Rad Laboratories). Protein profiles were examined for similarities and differences between inoculated and non-inoculated resistant and susceptible genotypes, and different incubation times.

Protein sequencing. A protein spot that appeared with high intensity after inoculation in the resistant genotypes was isolated and sequenced at the Protein Chemistry Laboratory, Biochemistry Department, Vanderbilt University, Nashville, TN. To improve resolution of the protein spot, the second dimension gel electrophoresis was performed in 15% polyacrylamide gels. For N-terminal sequencing, proteins were electro-transferred from the polyacrylamide gels to a polyvinylidene fluoride (PVDF) membrane (Bio Rad Laboratories) following the manufacturer's instructions. The membrane was stained with Coomassie blue R-20, and the protein spot was dissected and subjected to sequencing on a Procise 492 protein sequencer (Applied Biosystems, Foster City, CA). For internal sequencing of the protein, the spot was isolated from a Coomassie-stained gel and digested (in-gel) with trypsin using the method of Williams et al (29). Polypeptides were separated from the digestion solution by high performance liquid chromatography (HPLC). Twenty eluates were subjected to mass analysis using a matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The information on the molecular mass of the peptides were obtained utilizing the MS-Fit program Protein Prospector from University of California San Francisco, after calibrating the software with the trypsin autolytic peptides. Five randomly selected fractions were sequenced using Procise 492 protein sequencer (Applied Biosystems, Foster City, CA).

The protein sequences were analyzed to find analogous sequences using BLAST and information from the national Center of Biotechnology Information (NCBI) for Protein multi-alignments FASTAS3 from European Bioinformatics Institute**.**

Results and Discussion

There were no changes in protein profiles detectable at 12 hr and 24 hr post- inoculation, but at 48 hr post- inoculation, the inoculated leaves of the susceptible 'Cherokee Princess' and selection HL26 displayed fewer protein spots than the non-inoculated controls. The number and intensity of protein spots increased at 72 hr post-inoculation. Most of the new spots were of low molecular weight. These observations suggest that the susceptible plant response to pathogen challenge involved protein synthesis that occurred 72 hr post-inoculation. The results also suggest an initial decrease in protein synthesis between 24 to 48 hr after pathogen challenge and reactivation between 48 hr to 72 hr post-inoculation. Because the focus of this study was on proteins associated with disease resistance, further analysis of the proteins associated with susceptible responses was not pursued.

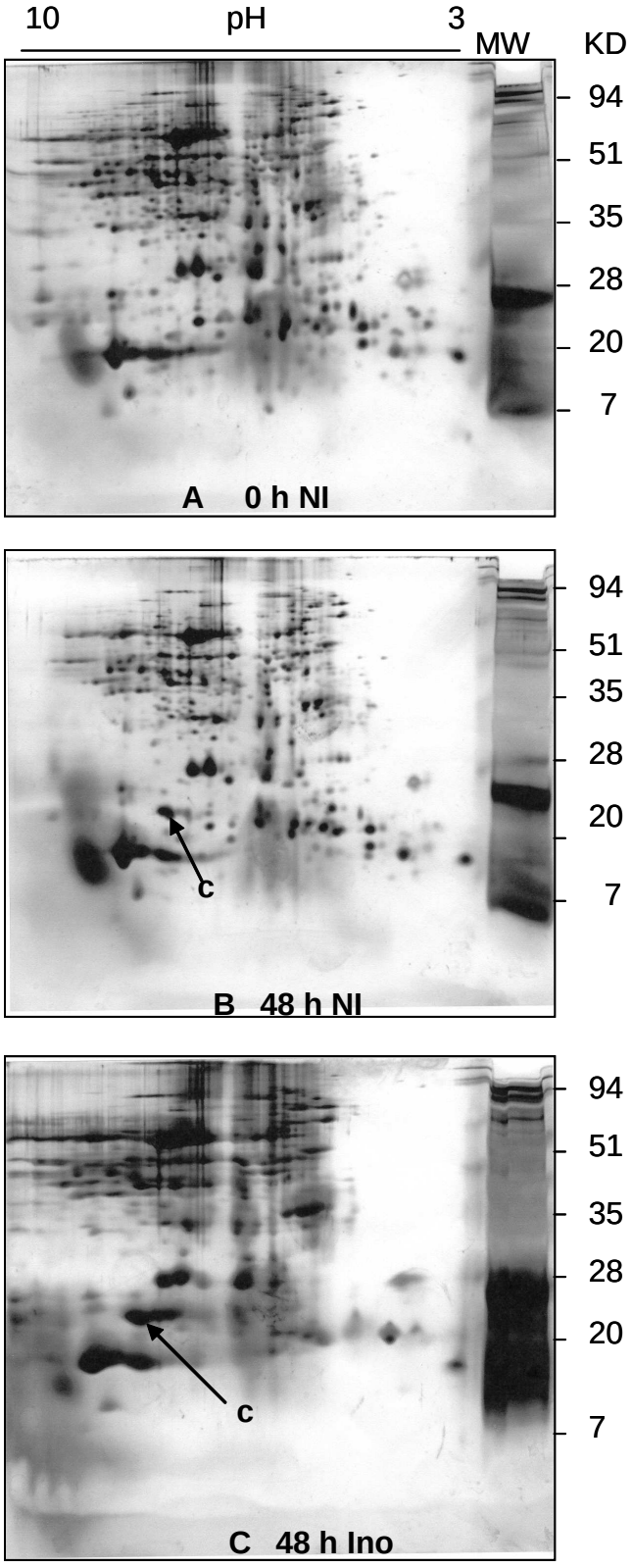

Fig. 1. Two-dimensional gel electrophoresis protein profiles from leaves of the resistant *Cornus florida.* IEF was in range pH 3–10 and second-dimension electrophoresis was performed in 15% polyacrylamide. (A) Non-inoculated control at the beginning of the experiment (0 h NI). (B) non-inoculated control at 48 hr (48 hr NI), and (C) Inoculated leaves 48 hr after inoculation (48 hr In) Molecular weight (MW) standards are indicated in the right of the gels. The PR protein 'c' is indicated by the arrow.

Differences in protein profiles in the resistant plants were detected at 48 hr post-inoculation. New protein spots in the resistant selections were detected at 48 hr and 72 hr post-inoculation and some proteins spots were more intense among the new proteins than in the non-inoculated controls. The majority of *de novo* proteins were observed at 48 hr after inoculation, and these proteins ranged in size from 7 to 25 kD, with a pI of 7.5 to 8.5. Among new protein spots observed in the inoculated resistant selections, a protein spot indicated by 'c' in Fig. 1 appeared at high intensity and was resolved further on a higher percentage polyacrylimide gel (Fig. 1). The molecular weight of the 'c' protein was 18 KD ± 2 and the pI was 7.5 ± 0.5. Analysis of molecular mass of the twenty eluates from trypsin digestion of the 'c' protein indicated that a protein with a similar digestion pattern has not been previously reported in plants.

Analysis of the sequences of the five polypeptides of the 'c' protein (HRIDALDTANFSS, QTNFHEASPFKS, VTYYQQEDAYSDATT, SIEFVHGDGGVGS and GVTQYTQEDASIVAP) using the multiple Edman sequence Search Visual Fasta of the European Bioinformatics Institute indicated that three of these sequences (HRIDALDTANFSS, SIEFVHGDGGVGS and GVTQYTQEDASIVAP) recognized simultaneously analog sequences in a drought-induced protein that belong to PR class 10 reported in *Retama raetam* (Accession number AAL32031; Pnueli and Mittler, 2002). Analysis of the partial sequences of the 'c' protein using BLAST from NCBI indicated that the 'c' protein sequences were also analogous to other proteins related to resistance, including the PR proteins STH-2 and STH-21 reported in potato (16, 17). These analogous proteins were of similar length (between 158 and 160 amino acids); 90% of these belong to the PR proteins in Class 10. Independent polypeptide analysis indicated that the 'c' protein polypeptides also share some homology with other PR-proteins (16, 17), allergen proteins (14) and ribonucleases (28).

Although the 'c' protein spot was also detected in the non-inoculated control at 48 h (Fig. 1-B), the intensity was much lower than that in the inoculated leaves (Fig. 1-C). Inducible proteins related to resistance may also be induced by abiotic stress including wounding (28). It is possible that the wound caused by detaching the leaves from the plants may have induced the low-level synthesis of the 'c' protein in the non-inoculated, control leaves. However, the high accumulation level of the 'c' protein in the inoculated leaves suggests that the powdery mildew challenge induced a higher level of the protein in the resistant genotypes. The 'c' protein sequences were consistently analogous to the PR10 proteins, thus suggesting that the 'c' protein is closely related to PR protein class 10. The activity of the 'c' protein and its mode of action have not been determined to confirm its role in resistance, but the increased accumulation induced by powdery mildew pathogen suggests that it may play a role in powdery mildew resistance. The partial sequences presented could be used to determine the presence of the 'c' messenger RNA in other *C. florida* genotypes that have powdery mildew resistance.

Comparison of protein profiles between the resistant and the susceptible genotypes indicated that the synthesis of new proteins occurred at an earlier time in the resistant genotype than in the susceptible genotypes. Similar results have been reported in other host-parasite interaction systems (3, 4). In studies conducted on PR-proteins in *C. mas* and *C. florida*

plants infected with *D. destrutiva*, chitinase activity occurred 2 days post-inoculation in resistant *C. mas*, whereas chitinase activity was detected 8 days post-inoculation in susceptible *C. florida* (4). The detection of protein 'c' in only the powdery mildew resistant selections was confirmed in repeated evaluations and suggests a need for further studies. In addition to PR proteins, small peptides with antimicrobial properties have also been associated with plant defense against fungal infections in plant systems (8). It is well known that resistance to infections by plant pathogens is the result of a combination of factors including physical and chemical barriers (6). The accumulation of a PR protein was induced by the powdery mildew pathogen in powdery mildew resistant plants and not in susceptible plants. Since the protein was analogous to other proteins related to resistance in other crops, including the PR proteins STH-2 and STH-21 reported in potato (16, 17), the proteins may be involved in powdery mildew resistance in *C. florida.*

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