JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 4145–4152 4145
DOI:10.1021/jf9044556

Effects of a Novel Pathogenesis-Related Class 10 (PR-10) Protein from *Crotalaria pallida* Roots with Papain Inhibitory Activity against Root-Knot Nematode *Meloidogyne incognita*

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A novel pathogenesis-related class 10 (PR-10) protein with papain inhibitory activity, named CpPRI, was purified from *Crotalaria pallida* roots by ammonium sulfate precipitation followed by three reverse-phase high-performance liquid chromatographies (HPLCs). CpPRI is made up of a single polypeptide chain with a M_r of 15 kDa, as determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). This protein exhibited a K_i value of 1.8×10^{-9} M and operates via a noncompetitive inhibition mechanism. The alignment of the N-terminal amino acid sequence of CpPRI with other proteins revealed its identity with PR-10 proteins. CpPRI acts against digestive proteinase from root-knot nematode *Meloidogyne incognita* and demonstrated nematostatic and nematicide effects on this parasite in bioassays. In a localization study, fluorescein-5-isothiocyanate (FITC)−CpPRI was observed to internalize and diffuse over the entire J2 body after 6 h of incubation. This fact could explain the natural tolerance of this plant species to nematodes.

KEYWORDS: Papain inhibitor; PR-10; nematode; *Crotalaria pallida*; *Meloidogyne incognita*

1. INTRODUCTION

The root-knot nematodes of the genus *Meloidogyne* are the most widespread pests, attacking almost every type of crop, including monocotyledonous and dicotyledonous herbaceous and woody plants. These nematodes can cause high yield losses and can strongly affect the quality of production. Approximately 2000 types of plants are susceptible to infection by root-knot nematodes, causing up to 12% of global crop loss annually (*1*) and reaching as high as 20% for certain crops (*2*). Nematode control is difficult because treatment options are often limited to chemical nematicides, which are highly toxic to the environment and humans (*3*).

Several plants have been screened for natural resistance or tolerance to nematodes. Among these, the species *Crotalaria pallida*, which belongs to the Fabaceae family, is found to have resistance. The members of this family are herbs or shrubs and are commonly found in tropical and subtropical areas. Suppression of parasitic nematodes, most of which are the sedentary endoparasitic nematodes, such as *Meloidogyne incognita*, by *Crotalaria* spp. has been known for decades (*4*). The mechanism of this nematode resistance is still not known, although it is thought to involve defense proteins, such as enzyme inhibitors, lectins, chitinases, and pathogenesis-related proteins (*5*).

Protease cysteine inhibitors are widely distributed in plants and have been purified from various sources including seeds, leaves, and fruits (*6*−*10*). Plant cysteine inhibitors are called phytocistatins as a class and consist of at least one distinct family, where most phytocistatins exhibit Mrs from 5 to 80 kDa (*11*). It seems that these inhibitors are able to defend plants against pest and pathogen attack. This presents pathogen and pest proteinases as promising targets to control these organisms, giving proteinaceous inhibitors high potential in defense strategies (*8*, *12*−*14*); for example, phytocystatin from *Oriza sativa* seeds, named oryzacystatin, expressed and regenerated in *Arabidopsis thaliana*, showed an effect on growth and development of nematodes, such as *Globera pallida* (*15*), *Heterodera schachtii*, and *M. incognita* (*16*).

Other defense plant proteins are the pathogenesis-related (PR) proteins, which represent a component of the active plant defense repertoire. PR proteins are defined as plant proteins that are induced in response to challenge by pathogens (*17*). On the basis of serological and molecular properties, 11 families of closely related PR proteins (PR-1−PR-11) have been found in most plants studied (*18*). Several of the PR proteins are hydrolytic enzymes, such as chitinases (*19*) and β-glucanases (*20*). Others

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Published on Web 03/03/2010

have structural sequence similarity to osmotin (*21*), proteinase-inhibitor-like properties (*22*), or thionin-like characteristics (*23*).

In this study, we report the purification and characterization of the first pathogenesis-related class 10 (PR-10) protein with papain inhibitory activity from *C. pallida* roots. This protein offers a potential strategy for nematode control.

2. MATERIALS AND METHODS

2.1. Purification of the Proteinaceous Papain Inhibitor (CpPRI). Mature *C. pallida* seeds were collected in Fortaleza, Brazil, and morphologically identified at the Department of Biology, University Federal of Ceará, Fortaleza, Brazil. Seeds were hydrated for 30 min in distilled water, germinated in soil, and maintained in a greenhouse. Roots were collected from the plants after 30 days, washed, and stored at −80 °C until use. For sample preparation, the roots were ground to a fine powder with a mortar and pestle and extracted for 75 min with 50 mM Tris-HCl buffer at pH 7.5 containing 0.15 M NaCl (1:5, w/v). The homogenate was filtered and centrifuged at 10000*g* for 30 min. The supernatant was subjected to 0−30% (F030), 30−60% (F3060), and 60−90% (F6090) ammonium sulfate fractionation. The precipitate of each fraction was collected by centrifugation, dissolved and dialyzed against water, and lyophilized. The fraction F6090, containing high inhibitory activity, was applied onto a reverse-phase column (Vydac C-18) using a high-performance liquid chromatography (HPLC) system equilibrated with a solution of 0.1% trifluoroacetic acid (TFA) at a flow rate of 1 mL min^{-1}. The sample was eluted with a gradient of solvent B (80% acetonitrile/0.1% TFA/water), and the chromatograph was monitored at 220 nm. Two peaks with papain inhibitory activities were detected. Peak 1 with high and specific papain inhibitory activity was applied on an additional HPLC column (2.2 × 25.0 cm) with a gradient of 0−5% solvent B for 3 min, 5−95% solvent B for 90 min, 95% solvent B for 10 min, 95−5% solvent B for 2 min, and 5% solvent B for 1 min. Two peaks were obtained (1a and 1b). Peak 1b with papain inhibitory activity was again purified on an analytical reverse-phase (0.46 × 25.0 cm) column at a flow rate of 1 mL min^{-1} as follows: 0−5% solvent B for 5 min, 5−45% solvent B for 5 min, 45−65% solvent B for 60 min, 65−95% solvent B for 2 min, 95% solvent B for 10 min, 95−5% solvent B for 2 min, and 5% solvent B for 3 min. The final purified protein, named CpPRI, was used in further analyses.

2.2. Protein Concentration. The protein concentration was determined by the dye-binding method of Bradford (*24*), using bovine serum albumin (BSA) as the standard.

2.3. Polyacrylamide Gel Electrophoresis (PAGE). Sodium dodecyl sulfate (SDS)−PAGE (12.5 and 15%) was performed at room temperature in the absence or presence of β-mercaptoethanol (0.1 M), as described by Laemmli (*25*). Gels were analyzed with silver stain or Coomassie Brilliant Blue. Protein molecular mass markers (BenchMark prestained protein ladder) were 190, 120, 85, 60, 50, 40, 25, 20, 15, and 10 kDa.

2.4. Characterization of the CpPRI. *2.4.1. Papain Activity Inhibitory Assay*. The papain inhibitory assay was performed as described by Abe et al. (*26*), using benzoyl-arginine-naphthylamide (BANA) as the substrate. To 20 μL of papain solution (0.1 mg mL^{-1} in 25 mM sodium phosphate buffer at pH 6.0) was added 40 μL of an activation solution [containing 2 mM ethylenediaminetetraacetic acid (EDTA) and 3 mM dithiothreitol (DTT) at pH 6.0], 100 μL of inhibitor solution, and 340 μL of 25 mM sodium phosphate buffer at pH 6.0. The mixture was incubated for 10 min at 37 °C. The reaction was initiated with 200 μL of 1 mM BANA solution, prepared in 1% (v/v) dimethylsulfoxide (DMSO) and 25 mM sodium phosphate buffer at pH 6.0. After 20 min at 37 °C, the reaction was stopped with the addition of 500 μL of 2% HCl in 95% ethanol. The color product was developed by adding 500 μL of 0.06% *p*-dimethylaminocinnamaldehyde in ethanol, and the absorbance was measured at 540 nm. To determine the K_i value of the reaction between CpPRI and papain, a similar assay was conducted at 37 °C with increasing concentrations of purified CpPRI (3.85, 7.7, and 15.4 μg) in 50 mM Tris-HCl at pH 7.5, followed by measurement of the residual activity using the synthetic substrate BANA (0.4, 0.8, 1, 1.5, 2.0, and 3.0 mM). The velocity rate of this reaction was expressed as $1/V$ $(OD_{540}/h/mL)^{-1}$, and the K_i value was determined using a double-reciprocal plot of the data.

2.4.2. N-Terminal Sequencing. The N-terminal sequence of CpPRI was determined using a Shimadzu PPSQ-23 automatic protein sequencer based on Edman degradation, followed by derivatization of the released amino acids to PTH derivatives, which were analyzed by a reverse-phase C18 column and identified through their characteristic retention time. The sequence and homology of the amino acids were analyzed by alignment using the BLASTP program, and sequences were deposited in the National Center for Biotechnology Information (NCBI) Genbank.

2.5. Nematodes. Populations of *M. incognita* race 3 were maintained on tomato plants (*Lycopersicon esculentum*) grown in potting compost in a contained greenhouse at a temperature of approximately 25−30 °C. Nematode eggs were extracted from the roots with 0.5% NaOCl (*27*, *28*). Eggs collected from a 600 mesh sieve were washed and placed in distilled water for hatching. Juvenile 2 (J2) was collected after the emersion was washed, centrifuged, and stored at −20 °C for posterior digestibility assays. Another part of J2 was concentrated to 100 nematodes per milliliter of distilled water by sedimentation in test tubes for posterior bioassays. Adult females of *M. incognita* were released from galled roots by incubation of the roots in Macerozyme (10 mg mL^{-1} solution in 0.1 M sodium acetate at pH 4.5) at 30 °C for 5 h with gentle agitation. Released nematodes were collected on a 600 mesh, rinsed, and handpicked from any root debris under a dissecting microscope.

2.5.1. Nematode Protein Extracts. A total of 50 adult females and 100 J2 were ground directly in Eppendorf tubes containing 50 μL of 50 mM sodium phosphate buffer at pH 6.0. The mixtures were extracted for 10 min and centrifuged at 10000*g*. The supernatants obtained were used in proteolytic activity assays.

2.5.2. M. incognita Proteolytic Activity Assays. Proteolytic and inhibitory assays were assessed by denaturing gelatin/PAGE 0.1% gelatin/SDS−PAGE (*29*). To 10 μL of extracts from *M. incognita* female adults, and J2, was added 10 μg of CpPRI in 10 μL of 50 mM Tris-HCl at pH 7.5. The mixture was incubated for 20 min at 37 °C. Next, the mixture was diluted 2-fold in sample buffer [62.5 mM Tris-HCl at pH 6.8, 2% (w/v) SDS, 2% (w/v) sucrose, and 0.001% (w/v) bromophenol blue]. The mixture was then centrifuged for 5 min at 10000*g*, and the supernatant was subjected to electrophoresis at 4 °C using the "Mighty Small II SE 250/SE260-Hoefer" system. After migration, the gels were transferred to a 2.5% (v/v) aqueous solution of Triton X-100 for 30 min at room temperature. The gel was then placed in proteolysis buffer (0.25 M sodium phosphate at pH 6.0, 1 mM EDTA, and 2 mM DTT) for 12 h at 37 °C. Proteolysis was stopped by transferring the gels into a staining solution (Phastgel blue R-350-Coomassie R). As a positive control, the enzyme total extract without inhibitor solution was made.

2.6. Nematicide or Nematostatic Assays. The bioassays were performed according to the methodology developed by Coimbra and Campos (*30*). At room temperature, 50 μL of sterilized H_2O containing 35 free-living nematodes of J2 stage and 50 μL of sterilized H_2O containing 10, 25, and 50 μg of CpPRI per well were added to 12 well Kline plates. Sterilized distilled water was used as a control. BSA in the same concentrations of the protein test was used as a control too. The first observation was made after 12 h, reaching a final time of 48 h. The CpPRI and control solutions were removed, and the contents of each well were resuspended in 100 μL of distilled water. In the bioassay, nematodes were considered dead when no movement was observed under a dissecting microscope and gentle tapping of nematodes by a stiletto did not result in movement. The mortality of nematodes was defined as the ratio of dead nematodes over the tested nematodes. The percentages of immobilized J2s were determined with an inverted microscope (40×). These assays were performed 3 times.

2.7. Localization of Fluorescein-5-isothiocyanate (FITC)−CpPRI Binding on J2 Body. FITC was covalently coupled to CpPRI using a modified protocol by Howard (*31*). Before use, a FITC solution (1 mg in 1 mL of anhydrous DMSO) was immediately added to 50 mM Tris-HCl buffer at pH 7.5, followed by CpPRI (2 mg mL^{-1}). The mixture was incubated in a dark glass tube and rotated for 1 h at room temperature. The FITC-labeled CpPRI was purified by ammonium precipitation, dialyzed against distilled water, and freeze-dried. A total of 100 J2s were added to 500 μL of a FITC−CpPRI (400 μg) solution. A control was made where 100 J2s were incubated with 500 μL of 50 mM Tris-HCl buffer at pH 7.5. After incubation in the dark for 24 h, the mixtures were washed

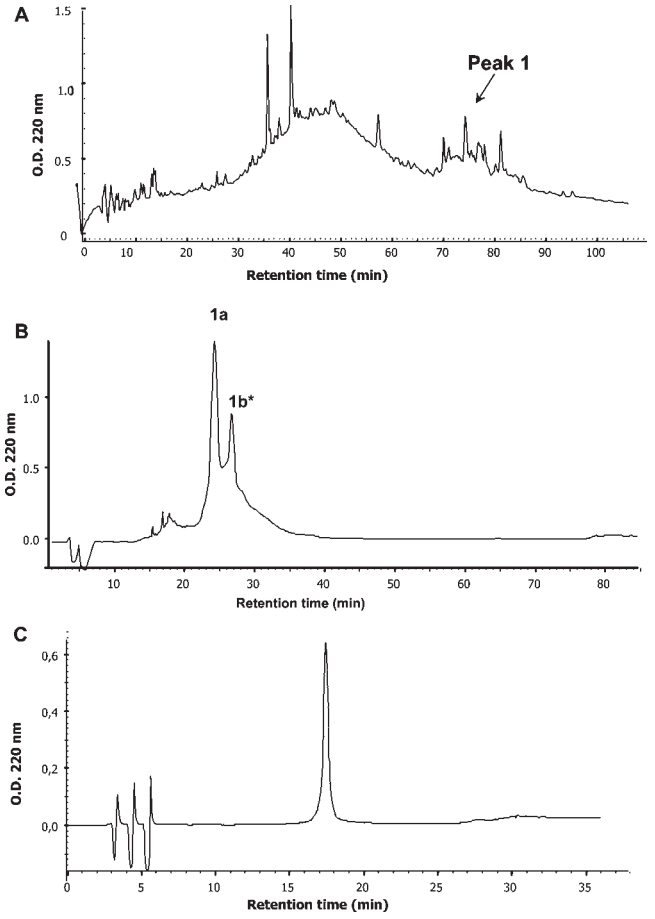

Figure 1. (A) Elution profile of the F6090 fraction on HPLC (Vydac C-18). F6090 was separated by a semi-preparative reverse-phase HPLC column at a flow rate of 9 mL/min. The sample was eluted with a gradient of solvent B (80% acetonitrile/0.1% TFA/water), and the chromatography was monitored at 220 nm. The proteins were separated in a semi-preparative (2.2 × 25.0 cm) column with a gradient of 0−5% solvent B for 3 min, 5−95% solvent B for 90 min, 95% solvent B for 10 min, 95−5% solvent B for 2 min, and 5% solvent B for 1 min. Elution profile of the peak 1 fraction on HPLC (Vydac C-18). (B) Peak I was then again subject to an analytical reverse-phase column at a flow rate of 1 mL/min, eluted using a gradient of solvent B (80% acetonitrile in 0.1% TFA) in solvent A (0.1% TFA/H_2O), and monitored at 220 nm. The used gradient was 0−5% solvent B for 5 min, 5−45% solvent B for 5 min, 45−65% solvent B for 60 min, 65−95% solvent B for 2 min, 95% solvent B for 10 min, 95−5% solvent B for 2 min, and 5% solvent B for 3 min. Two peaks were obtained (peaks 1a and 1b). (C) Peak 1b with antipapain activity was rechromatographed in the same conditions. The used gradient was 45% solvent B for 5 min, 45−60% solvent B for 15 min, 60−95% solvent B for 2 min, 95% solvent B for 10 min, 95−45% solvent B for 2 min, and 45% solvent B for 2 min. The purified papain inhibitor was designed as CpPRI.

10 times with distilled water and centrifuged. The pieces were examined on a fluorescence microscope (Nikon Eclipse E200).

3. RESULTS

3.1. Purification of PR-10 Protein with Papain Inhibitory Activity. Soluble protein obtained from *C. pallida* roots was precipitated at 30, 60, and 90% saturation with ammonium sulfate. Three protein fractions, named F030, F3060, and F6090, were obtained. The F6090 fractions showed the highest inhibitory activity against papain, a cysteine proteinase, and were applied to a Vydac C-18 column for HPLC purification (**Figure 1A**). This was followed by two additional HPLC purification steps using reverse-phase columns (panels **B** and **C** of **Figure 1**). The papain inhibitor obtained was named CpPRI and was shown by

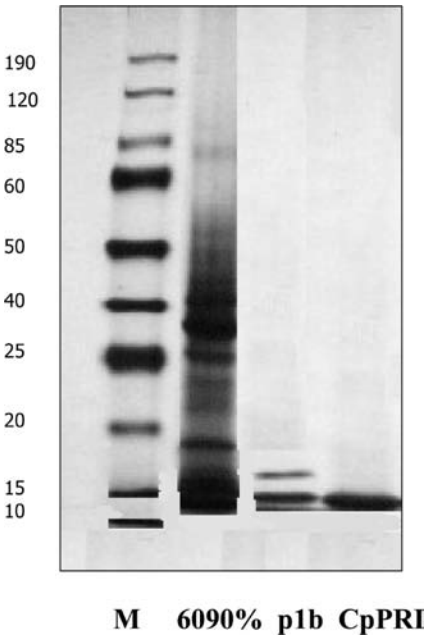

Figure 2. SDS−PAGE analysis at 12.5% of purified CpPRI stained with silver showed one protein band with a molecular mass of approximately 15 kDa. (M) Protein molecular mass markers (BenchMark prestained protein ladder) of 190, 120, 85, 60, 50, 40, 25, 20, 15, and 10 kDa. (6090%) Fraction 6090% precipitated with ammonium sulfate. (p1b) Peak 1b. (CpPRI) Purified inhibitor CpPRI.

Table 1. Purification Steps of the Papain Inhibitor from *C. pallida* Roots[a]

steps	total inhibitory units (IU)	total protein (mg)	specific activity (IU mg^{-1})	purification (fold)	yield (%)
EB	69.757	323.02	215.95	1	100.00
F6090	10.560	23.13	456.54	2.11	15.13
peak 1	2.320	0.5	4.640	10.16	3.32
peak 1b	1.118	0.23	4.860	10.64	1.60
CpPRI	1.954	0.02	9.770	210.40	0.280

[a] One papain protein inhibitor unit (1 IU) was defined as the inhibitor amount that decreased the absorbance at 540 nm by 0.01 arbitrary unit (UA) in the papain assay conditions.

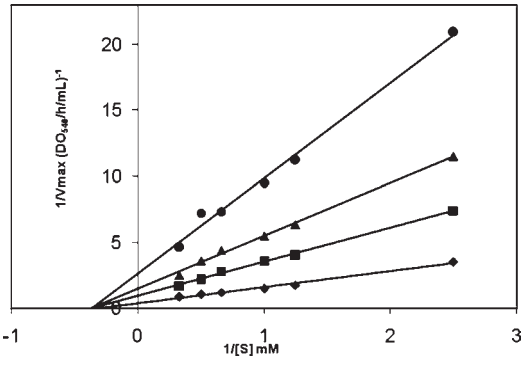

Figure 3. Inhibition of papain activity by CpPRI was noncompetitive. Inhibition kinetic data are illustrated with Lineweaver−Burk double-reciprocal plots. CpPRI activity was determined using various concentrations of BANA as the substrate. (◆) No inhibitor in the assay mixture, (■) 3.85 μg of CpPRI, (●) 7.7 μg of CpPRI, and (○) 15.4 μg of CpPRI.

Table 2. Alignment of the N-Terminal Amino Acid Sequence from CpPRI with Other PR-10 Proteins[a]

species	N-terminal sequence	identity (%)	reference
CpPRI (*C. pallida*)	**FA**F**ED**E**NT**S**PVAPA**KLF**KA**LT**KD**A**D**VII**PK**VI**E**PD**Q**	100	
L. lupus	**FA**F**EN**E**QS**S**TVAPA**KLY**KA**LT**KD**S**D**EIV**PK**VI**E**PI**Q**	75	*62*
R. raetam	**FT**F**KE**E**NV**S**PVAPA**KLF**KA**FV**KD**S**D**TII**PK**VV**E**PI**Q**	72	*63*
L. albus	**FT**F**ED**E**ST**S**TVAPA**RLY**KA**LV**KD**A**D**TII**PK**AV**E**AI**Q**	69	*64*

[a] CpPRI aligned with other proteins in the NCBI. *L. lupus* PR10.1A, gi: 2183275; *R. raetam* PR-10, gi: 17352485; and *L. albus* pr-10, gi: 30025158. Bold letters are indicative of 100% identity.

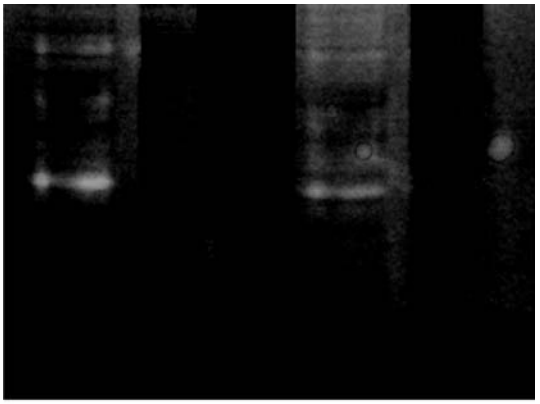

Figure 4. Gel electrophoresis using 10% gelatin/SDS−PAGE. *M. incognita* female (Mif) and *M. incognita* J2 (MiJ2) gelatinasic activities are indicated. Mif + I and MiJ2 + I are *M. incognita* female and J2 in the presence of 10 μg of purified inhibitor (CpPRI), respectively.

SDS−PAGE to have a molecular mass of 15 kDa (**Figure 2**). It consists of a single polypeptide chain (data not showed). The chromatographic procedures on CpPRI resulted in a 214.0-fold purification with a 0.28% yield (**Table 1**).

3.3. Kinetics of CpPRI. To determine the mechanism of CpPRI against papain, we analyzed the inhibition kinetic data by Lineweaver−Burk plots (**Figure 3**). This analysis showed that inhibition of papain follows noncompetitive-type kinetics. The K_i value of papain was 0.15×10^{-9} M.

3.4. N-Terminal Amino Acid Sequences. The alignment of the N-terminal amino acid sequence of CpPRI with other proteins suggested identity with pathogenesis-related class 10 proteins (**Table 2**). The alignment identities of CpPRI with PR-10 proteins from *Lupinus lupus* roots, *Retama raetam* roots, *Lupinus albus* leaves were 75, 72, and 69%, respectively. The alignment of the N-terminal amino acid sequence of CpPRI with other papain inhibitors showed low identity below 10%.

3.5. Inhibitory Effect of CpPRI to *M. incognita* Female and J2 Proteinases. Using PAGE containing 0.1% gelatin, *M. incognita* proteolytic activities were visualized as a clear zone in the gel against a dark blue background. As illustrated in **Figure 4**, one to two proteolytic activity bands were visualized for both female and J2 nematodes, respectively. When treated with CpPRI, these activities were completely abrogated.

3.7. Nematostic or Nematicide Assays. An effect on the mobility of J2 nematodes was observed with doses of 10, 25, and 50 μg of CpPRI at 48 h of incubation. After 48 h of incubation, the 10 μg dose of CpPRI was nematostic to J2s (**Figure 5A**), at the 25 μg dose, a nematostic effect was observed, with 48% mortality (**Figure 5B**), and at the 50 μg dose, CpPRI was lethal, causing 95% mortality (**Figure 5C**). Three controls were used: sterilized water (**Figure 5D**), 50 mM Tris-HCl at pH 7.5 (**Figure 5E**), and 50 μg BSA in 50 mM Tris-HCl at pH 7.5 (**Figure 5F**). After 48 h, the mortalities were around 20% compared to the maximum concentration. The effects on the mobility of J2 nematodes were observed with doses of 25 and 50 μg of CpPRI at 24 and 36 h of incubation.

3.8. Localization of FITC−CpPRI Binding on J2 Body. FITC−CpPRI was observed to internalize and diffuse over the entire J2 digestive tract after 6 h of incubation (panels **B** and **D** of **Figure 6**).

DISCUSSION

Crops can be attacked by a number of disease agents and pests, both in the field and during storage. Root-knot, caused by nematodes of various species of *Meloidogyne*, is one of the most destructive plant diseases that farmers must combat. Chemical and/or biological strategies have been used to control nematodes. In soil, for example, the roots of tolerant and/or resistant plants can act as a pitfall to pathogens. The most reported tolerant and/or resistant plants comprise the species *Tagetes* spp. (family Asteraceae), *Azadirachta indica* (family Meliaceae), *Crotalaria juncea*, *Mucuna* spp. (family Fabaceae), and several species of grasses from the Poaceae family (*32*, *33*). Among these plants, the *Crotalaria* genus has the ability to attract and capture these pathogens. This fact was demonstrated by Ochese and Brewton (*34*) when they analyzed 20 species of plants of the *Crotalaria* genus and observed that the plants were toxic to nematodes. The species *C. juncea* was indicated as a good antagonist candidate for breeding purposes. When tested, this plant suppressed *M. incognita* and *Rotylenchulus reniformis* population densities, as compared to controls (*35*). All juvenile stages of *M. incognita* and a few adults were present within the root system, indicating that there was penetration and reproduction; freeze-dried root exudates were able to kill both *M. incognita* and *R. reniformis* juveniles. The mechanism responsible for nematode mortality is still not clear, but it might be due to nematotoxic compounds released from the roots (*35*).

In this work, a papain inhibitor named CpPRI was purified from *C. pallida* roots, which could have evolved to confer resistance against nematodes. This inhibitor was purified 214.0-fold with a yield of 0.28%. CpPRI has a molecular mass of 15 kDa, as determined by SDS−PAGE, and is made up of a single polypeptide chain. Plant papain inhibitors from seeds and roots have been characterized in both monocotyledons and dicotyledons, including *O. sativa* (*36*), *Zea mays* (*37*), *A. thaliana* (*38*), *Hordeum vulgare* (*39*), *Vigna unguiculata* (*9*), *Castanea sativa* (*40*), *Brassica campestris* (*41*), *Glycine max* (*42*), *Solanum tuberosum* (*43*), and *Solanum lycopersicum* (*44*). They are involved in plant abiotic stress (*37*, *39*, *40*) and as modulators in programmed cell death (*38*). Also, plant papain inhibitors may play roles in defense against pathogens, such as viruses (*45*), fungi (*46*), insects (*47*), and nematodes (*48*). In the case of nematodes, the mechanism of action of inhibitors could be due to the interaction of this molecule with papain-like enzymes present in the digestive tube and in the body of the pathogens (especially in the cuticles). In J2 and mature females of *M. incognita*, intestines express the papain

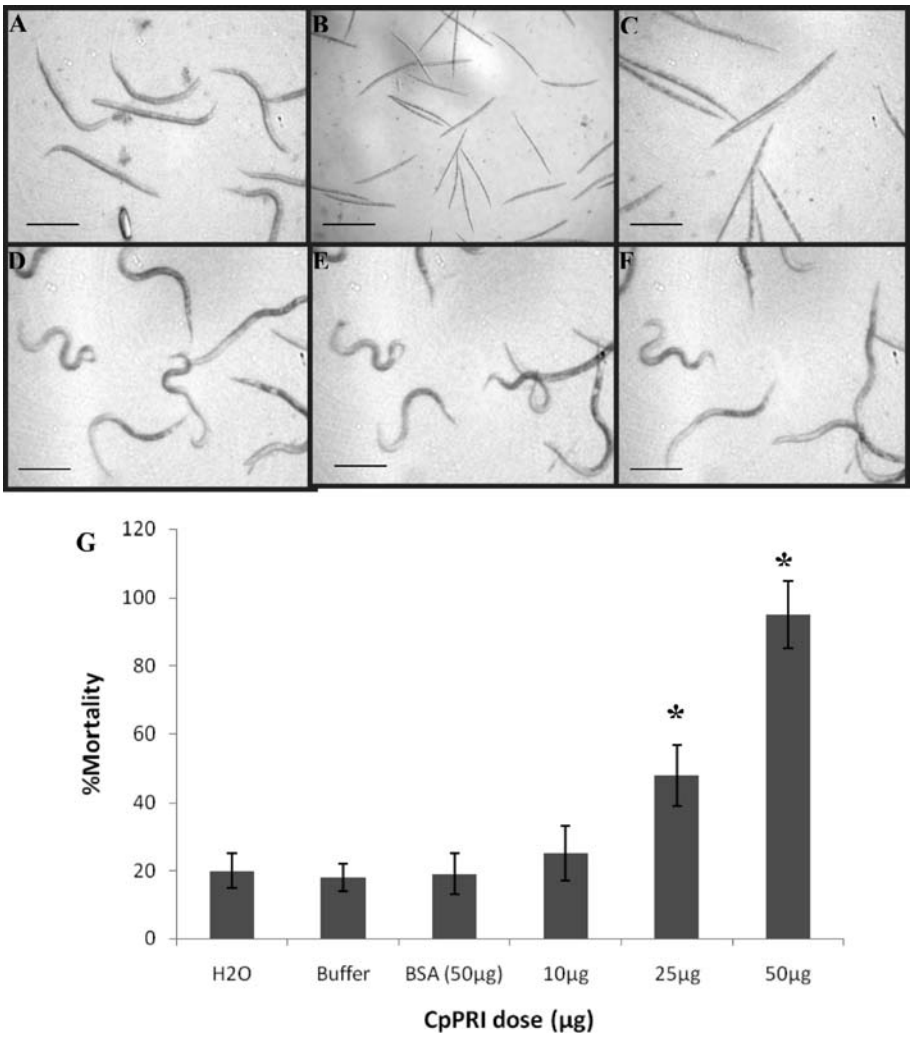

Figure 5. Nematicide effects of CpPRI. (A) Nematostatic effect of 10 μg of CpPRI after 48 h of contact. (B) Nematicide effects of 25 μg of CpPRI after 48 h. (C) Nematicide effects of 50 μg of CpPRI after 48 h. (D) J2 incubated with distilled water after 48 h. (E) J2 incubated with 50 mM Tris-HCl at pH 7.5 after 48 h. (F) J2 incubated with 50 μg of BSA in 50 mM Tris-HCl at pH 7.5 after 48 h. The scale in A and C−F is 10 μm. The scale in B is 5 μm. (G) Quantification of nematicide effects of CpPRI in the percentage of mortality after 48 h of incubation. Controls: J2 incubated with distilled water had 20%; J2 incubated with 50 mM Tris-HCl at pH 7.5 had 18%; J2 incubated with 50 μg of BSA had 19%. Tests: 10 μg of CpPRI had 25%; 25 μg of CpPRI had 48%; and 50 μg of CpPRI had 95%. Mean values followed by an asterisk were statistically different ($p < 0.05$) by Tukey's test. The error bars represent the standard deviation.

enzymes cathepsin L, B, or Z type (*49*, *50*). These enzymes have been characterized by different approaches, such as mildly denaturing gelatin PAGE (*29*). In this study, approximately 1−2 gelatinasic activities at pH 5.5 in J2 and female extracts of *M. incognita* were detected by zymogram and were completely inhibited by CpPRI. CpPRI is a noncompetitive type of inhibitor against papain, with a K_i value of 0.15×10^{-9} M. Papain inhibitors from plants were tested against nematodes and were found to be very effective against *Globodera* (*51*), *Meloidogyne* (*52*), and *Rotylenchulus* (*53*). CpPRI purified from *C. pallida* roots had nematostatic and nematicide effects on J2 *M. incognita*. The effects on the mobility (nematostatic effects) of J2 nematodes were observed with doses of 25 and 50 μg of CpPRI at 24 and 36 h of incubation. After 48 h of incubation, at the 25 μg dose of CpPRI, nematostatic and nematicide effects were observed, causing 48% mortality to J2, and at the 50 μg dose of CpPRI, nematostatic and nematicide effects were found to be lethal, with 95% mortality. These effects could be due to the association of CpPRI with intestinal cysteine enzymes. Performing a localization experiment, FITC−CpPRI was found to internalize and diffuse over the entire J2 body after 6 h of incubation. This inhibition of cysteine proteinases in the J2 intestinal tract could explain, together with other protein and non-protein factors, such as lectins, chitinases, other PR proteins, and secondary metabolites, the tolerance or resistance of the *Crotalaria* genus against *M. incognita* nematodes.

The alignment of the N-terminal amino acid sequence of the CpPRI with other proteins suggested high identity with PR-10 proteins from *L. lupus* roots, *R. raetam* roots, and *L. albus* leaves and low identity, around 10%, with papain inhibitors. PR-10 proteins are found in various species of the plant kingdom, including both dicots and monocots. Unlike many other PR proteins, members of the PR-10 family are constitutively expressed in some plant organs and tissue, making detection easy in uninoculated plants (*54*). The biological functions of PR-10 proteins are unknown, although there is evidence that some, such as cotton PR-10 and birch PR-10 proteins, act as ribonucleases (*55*, *56*). There is also evidence that PR-10 proteins have a role in general plant defense mechanisms, because their induction occurs after pathogen or elicitor treatment (*57*, *58*), wounding (*59*), and other environmental stresses (*60*, *61*).

CpPRI is a PR-10 protein purified from *C. pallida* roots that exhibits papain inhibitory activity, being the first PR-10 protein purified that was lethal against J2 *M. incognita* nematodes in

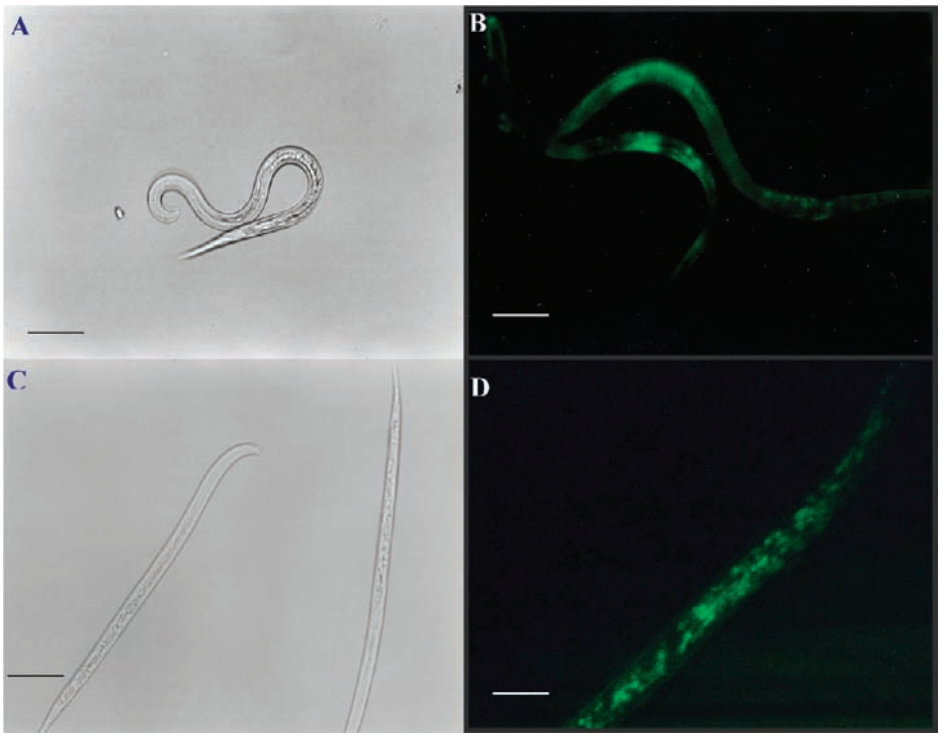

Figure 6. Fluorescence micrographs showing FITC−CpPRI binding in J2 *M. incognita* race 3. (A and C) Light microscopy and (B and D) fluorescence microscopy of the intestine and body of *M. incognita*. The scale in A−D is 20 μm.

bioassays. This activity could explain, together with others protein and non-protein factors, the tolerance and resistance of this species against nematodes. On the basis of these results, CpPRI is an excellent candidate to offer resistance to nematodes via DNA recombinant technology to plants of agronomic interests.

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Received for review December 17, 2009. Revised manuscript received February 10, 2010. Accepted February 18, 2010. This study was supported by the following Brazilian Agencies: RENORBIO-FINEP, CAPES, and CNPq.