

# A Nonpathogenic Mutant of *Colletotrichum magna* Is Deficient in Extracellular Secretion of Pectate Lyase

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A nonpathogenic mutant strain (path-1) of *Colletotrichum magna* (teleomorph *Glomerella magna*) was isolated and grew endophytically in cucurbit seedlings. Decay development on the pericarp of avocado fruits was significantly delayed in the wild-type (wt) isolate of *C. magna* compared with *C. gloeosporioides* isolate Cg-14, but both isolates colonized mesocarp tissue equally well. However, path-1 did not cause disease symptoms in either the pericarp or the mesocarp. Electrofocusing of culture filtrates of *Colletotrichum* strains revealed the presence of pectate lyase (PL) and polygalacturonase (PG) at pIs of 7.9 and 4.8, respectively, but no significant activity from path-1 was detected. Western blots detected PL in avocado fruit tissue infected with Cg-14 and wild-type *C. magna*. DNA of the three *Colletotrichum* isolates hybridized to a *pel* probe from *C. gloeosporioides* indicating that at the DNA level no deletion in the *pel* gene was evident in path-1. Similar levels of *pel* mRNA expression were also found for wild-type and path-1 of *C. magna*, and Cg-14 of *C. gloeosporioides*. However, when PL antibodies were cross-reacted with cell lysate of *Colletotrichum* strains, PL accumulated in the hyphae of path-1 of *C. magna*, but was not detected in Cg-14 cell lysate. This suggests that the malfunction in extracellular secretion of PL proteins results in differential pathogenicity of path-1 isolate on avocado.

*Additional keywords:* *Persea americana*, quiescent infection.

*Colletotrichum gloeosporioides* produces an endopolygalacturonase (PG) (Prusky et al. 1989) and pectate lyase (PL) (Wattad et al. 1994a). Both enzymes degrade avocado tissue but their role in pathogenicity of avocado fruit has not been determined. The correlation between the enzyme level and tissue maceration suggests the importance of pectolytic enzymes as basic compatibility factors (Wattad et al. 1994). This indicates that the enzyme is required by the pathogen but does not determine race or cultivar specificity (Walton 1994). The best tool to determine if a pectolytic enzyme affects pathogenicity is site-directed mutagenesis of the structural gene. Nevertheless, new questions may be raised in such

cases. When all the *pel* genes from *Erwinia chrysanthemi* were mutated by marker exchange-ejection, the bacteria could still macerate plant tissue (Reid and Collmer 1988). *E. chrysanthemi* produced an entire new set of pectinases only when grown in planta (Kelemu and Collmer 1993). Although few genes encoding pectolytic enzymes from fungi have been cloned, the available molecular data for polygalacturonase from *Cochliobolus carbonum*, a pathogen of maize (Scott-Craig et al. 1990) and *Penicillium olsonii* pathogenic on *Arabidopsis thaliana* (Kusserow and Schafer 1994) revealed that the enzyme is not required for pathogenicity and residual pectinase activity supported full growth on pectin.

*C. gloeosporioides* is the the most destructive fungal pathogen decaying tropical fruits after harvest. *C. magna* is a causal agent of cucurbit anthracnose and is not a specific pathogen on avocado fruit but can cause reduced symptoms of decay (Prusky et al. 1994). A nonpathogenic mutant of *C. magna* was obtained by UV mutagenesis and developed endophytically in cucurbit plants without causing symptoms of disease (Freeman and Rodriguez 1992, 1993). The nonpathogenic mutant could not colonize avocado tissue and lacks the ability to macerate avocado tissue (Prusky et al. 1994). The presence of three *Colletotrichum* isolates differing in pathogenicity on avocado was used as a tool for understanding the importance of pectolytic enzymes during symptom development. In the present study, we report that the path-1 strain of *C. magna* which is nonpathogenic on avocado is impaired in the export of pectate lyase and produces minor levels of PG. The *pel* gene of *C. gloeosporioides* recently cloned by Wattad et al. (1994 b) was used for the analysis.

## RESULTS

### Decay development of avocado pericarp and mesocarp by *Colletotrichum* isolates.

Initial symptoms of decay on the pericarp of avocado fruit inoculated with *C. gloeosporioides* isolate Cg-14 were observed at 4 to 5 days after inoculation, as reported previously (Prusky et al. 1994). A delay in decay development of the pericarp was observed with the wild-type *C. magna* isolate, which produced minor symptoms 7 days after inoculation. However, the nonpathogenic path-1 mutant strain of *C. magna* induced no significant symptoms on inoculated pericarp (Fig. 1A).

Decay development on avocado mesocarp was more rapid than that observed in pericarp inoculations. Cg-14 and wild-type *C. magna* isolates caused significant maceration of the mesocarp 2 to 3 days after inoculation, whereas no symptoms were observed following path-1 mesocarp inoculations (Fig. 1B).

#### Presence of PL and PG in culture filtrates of *Colletotrichum* isolates.

Culture filtrates of the *Colletotrichum* isolates were concentrated, dialyzed, and applied to a preparative isoelectric focusing column. PL activity had focused at pI 7.9 from the culture media of Cg-14 and wild-type *C. magna* but no PL was detected from path-1 culture fluid (Fig. 2). PG activity from Cg-14 and wild-type *C. magna* focused at pI values of 4.8 and 4.9, respectively. PG activity from path-1 was only about one sixth that of the wild-type isolate and peaked at pH 4.2 (Fig. 2).

Western blot analysis with antibodies specific to PL from Cg-14 (Wattad et al. 1994a) were used to determine the presence of the protein in cultures of the three *Colletotrichum* isolates grown on pectin and polygalacturonic acid as sole carbon sources. PL protein was detected as a 39-kDa protein from Cg-14 and the wild-type *C. magna* culture but no PL antigens were detected from the path-1 culture filtrate (Fig. 3).

#### Detection of PL in inoculated avocado pericarp and mesocarp.

Pericarp and mesocarp of avocado fruits were inoculated with the three *Colletotrichum* isolates and PL protein was determined by Western blot analysis. In Cg-14-inoculated fruit, the 39-kDa PL protein was detected in both pericarp and mesocarp. However, the enzyme was detected only in the mesocarp and not in the pericarp of avocado fruit inoculated with the wild-type *C. magna* isolate (Fig. 4). In path-1-inoculated fruit, PL was not detected and similar banding

patterns were observed with the corresponding noninoculated control fruits (Fig. 4).

#### Presence and function of the PL gene.

To determine whether any visible differences were distinguishable among genomic DNA of the three *Colletotrichum* isolates, total DNA was digested with *Bam*HI, *Hind*III, and *Pst*I, and hybridized to a *Pel* cDNA clone (Wattad et al. 1994b) isolated from Cg-14 (Fig. 5). DNA restriction digest patterns of path-1 and wild-type *C. magna* were alike for the three enzymes, suggesting that no deletion in the *Pel* gene was evident for path-1. *Bam*HI digestion of Cg-14 genomic DNA revealed a band of hybridization of approximately 9.0 kb in size.

Total RNA was extracted from the three *Colletotrichum* isolates which had been grown on pectin and polygalacturonic acid as sole carbon sources and probed with the cloned cDNA *Pel* gene. Northern blot analysis showed similar levels of mRNA expression for all three isolates of *Colletotrichum* (Fig. 6A).

To determine whether the PL gene product was produced but accumulated within PL-induced path-1 cells, Western blot analysis was performed on cell lysate. As shown in Figure 6B, a higher level of PL was detected in path-1 cell lysate, compared to a lower level in wild-type *C. magna*, and no visible accumulation for isolate Cg-14. In path-1 cell lysate, enhanced as well as lower levels of additional proteins could be observed, compared with the protein profile for wild-type *C. magna* (Fig. 7).

#### DISCUSSION

The role of cell wall-degrading enzymes as pathogenicity determinants in fungi is not well established, although these enzymes are considered to be examples of basic compatibility factors (Walton 1994). Most of the available evidence regarding the importance of pectate lyase during fungal attack is

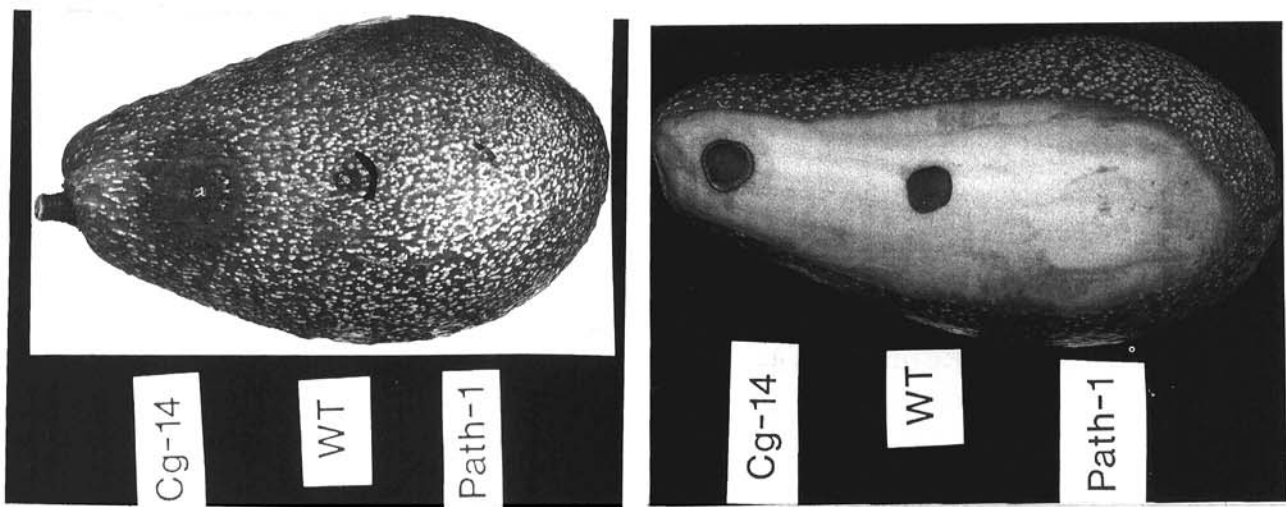


Fig. 1. Disease symptoms on avocado pericarp (A) and mesocarp (B) inoculated with *Colletotrichum gloeosporioides* (isolate Cg-14), the wild-type (WT) and nonpathogenic mutant (path-1) isolates of *C. magna* at concentrations of  $2 \times 10^6$  conidia/ml. Symptoms observed on avocado pericarp (A) and mesocarp (B) are shown 7 and 2 days after inoculation, respectively.

circumstantial and is based on correlations between the presence of pectolytic enzymes, disease symptoms, and relative virulence (Collmer and Keen 1986). In *Nectria haematococca*, specific antibodies raised against PL reduced virulence in pea seedlings (Crawford and Kolattukudy 1987) and similarly, antibodies against PL of *C. gloeosporioides* inhibited avocado mesocarp maceration (Wattad et al. 1994a). Similarly, *C. gloeosporioides* Cg-14 and *C. magna* wild-type strains produce decay symptoms on avocado fruits while the mutant path-1 failed to induce visible symptoms of decay. The mutant lacks the capability to produce and secrete significant amounts of PL and PG required to cause maceration in avocado fruit.

Studies in which pectinase-deficient mutants were produced with UV irradiation or with chemicals had contributed

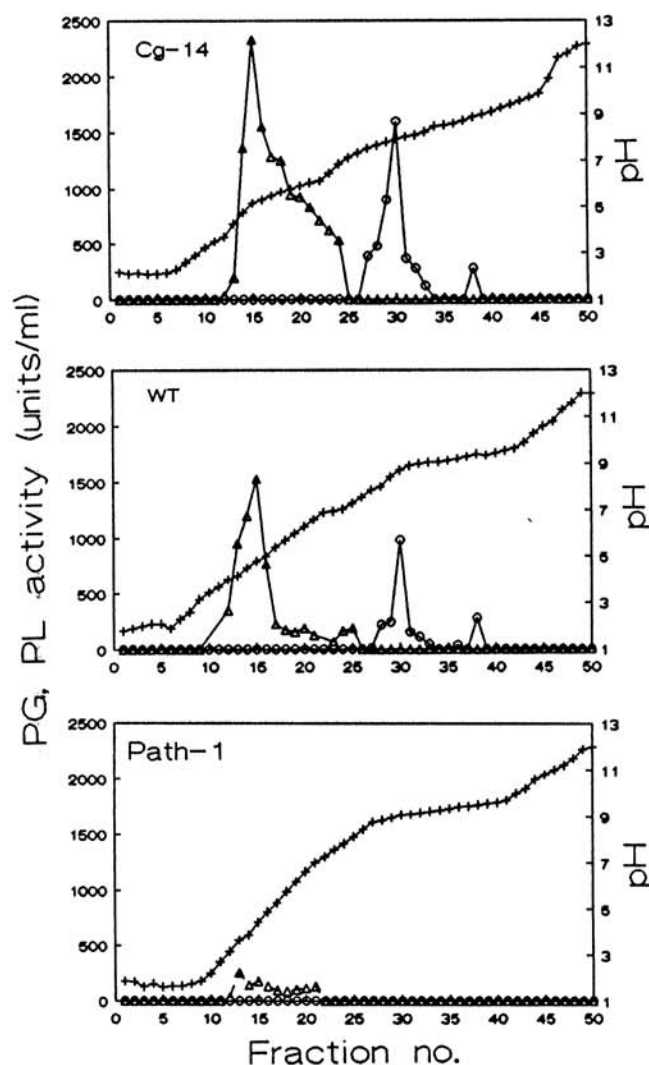


Fig. 2. Column electrofocusing of 10 $\times$  culture filtrates of *Colletotrichum gloeosporioides* (isolate Cg-14), the wild-type (WT) and nonpathogenic mutant (path-1) of *C. magna*. The dialyzed solution was subjected to preparative isoelectric focusing by the use of an LKB 81001-1 column with 1% carrier ampholytes (pH 3.0 to 10). Samples were applied to a linear sucrose gradient 50 to 5% (w/v) and electrophoresed at a constant power of 7 W for 48 h to a final 2,500 V. Fraction pH is indicated by (+), polygalacturonase (PG) activity by ( $\Delta$ ) and pectate lyase (PL) activity by (o).

little to the determination of virulence in filamentous fungi such as *Fusarium*, *Verticillium*, *Sclerotinia*, and *Alternaria* since they remained pathogenic (Walton 1994). In avocado, the nonpathogenic mutant of *C. magna*, path-1, did not induce visible symptoms of decay in the pericarp or mesocarp of harvested fruits (Prusky et al. 1994; Fig. 1). This is in contrast to wild-type *C. magna*, a nonspecific pathogen and *C. gloeosporioides* Cg-14 a specific pathogen of avocado, which caused significant maceration of the mesocarp. In avocado however, the degree of symptom development in the pericarp depends on the levels of PL and PG produced by the *Colletotrichum* strain in vitro. The nonpathogenic strain of *C. magna* path-1 is unable to secrete the enzyme in vivo and in vitro. Since similar *pel* transcription products were observed in all the *Colletotrichum* strains we propose that the lack of PL in vivo is the result of a deficient extracellular secretion of the enzyme. This is the first report, to the best of our knowledge, that a fungal pathogen impaired in extracellular secretion is lacking in pathogenicity.

Several bacterial mutants were reported to be impaired in export of extracellular proteins (EXPs) that affected pathogenicity (Dow et al. 1987; Andro et al. 1984; Thurn and Chatterjee 1985; Kang et al. 1994). Expert and Toussaint (1985), and Andro et al. (1984) isolated *E. chrysanthemi*

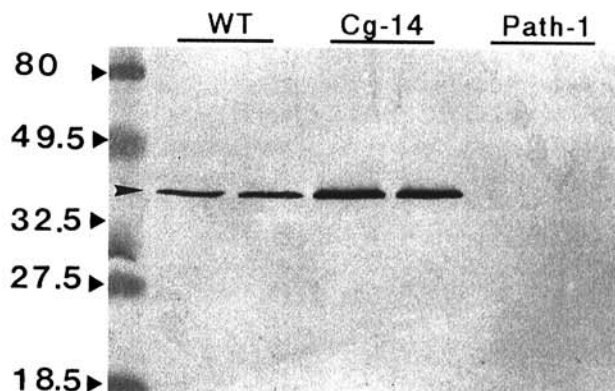


Fig. 3. Western blot analysis of 10 $\times$  culture filtrates of the wild-type (WT) isolate of *Colletotrichum magna*, *C. gloeosporioides* (isolate Cg-14), and the nonpathogenic mutant (path-1) of *C. magna*. Samples of 5 mg of protein were analyzed on 12.5% SDS-PAGE and transferred to a nitrocellulose membrane and probed with anti-PL of *C. gloeosporioides*. Results of two different preparations per isolate are shown, with molecular weight standards denoted in kilodaltons.

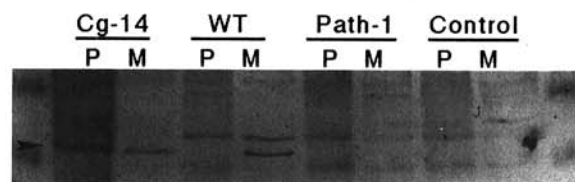


Fig. 4. Western blot analysis of avocado pericarp (P) and mesocarp (M) tissue inoculated with *Colletotrichum gloeosporioides* (isolate Cg-14), the wild-type (WT) and nonpathogenic mutant (path-1) isolates of *C. magna* were compared to noninoculated fruits (control). Proteins were extracted from 10 g of inoculated tissue and 5 mg of protein samples were analyzed on 12.5% SDS-PAGE and transferred to nitrocellulose membrane. The control treatment show the extract of a noninoculated fruit. Pectate lyase is indicated by an arrow.

mutants with outer membrane alterations that were nonpathogenic to *Saintpaulia ionantha* although they were shown to produce wild-type levels of PL. Mutants of *P. solanacearum* that accumulated EXPs inside the cell showed reduced virulence to tomato and potato (Kang et al. 1994). This suggests that specific regulatory mechanism(s) involved in the export of extracellular proteins are not functioning adequately and may be the cause of lack of disease symptoms produced by path-1 inoculations. Daniels et al. (1984) restored pathogenicity to chemically induced mutants of *X. campestris* pv. *campestris* by introduction of a 10-kb region of cloned wild-type DNA containing two linked regions involved in the export of PL and protease.

One obvious possibility is that *Colletotrichum* PL is involved in penetration and/or degradation of pericarp tissue and thus facilitates infection and fruit colonization. Dickman et al. (1989) and Dickman and Patil (1989) suggested that cutinase production is a key factor for penetration of the fruit cuticle. However, present results suggest that a *Colletotrichum* strain impaired in PL secretion could not cause symptoms of decay although conidia germinated and formed appressoria that breached the fruit cuticle (Prusky et al. 1994). It is clear that PL is crucial for efficient and rapid infection and further tissue colonization in avocado fruit. In mesocarp tissue, lower level of PL secretion by the wild-type *C. magna* is sufficient to cause symptoms similar to those produced by Cg-14 but not enough to produce symptoms on the pericarp of the fruit. Symptom development on the pericarp of the fruit appear to depend on the levels of PL produced and secreted in infected tissue. When protein profiles from cell lysate of path-1 and wild type were compared, other proteins accumulated in path-1, suggesting that additional proteins besides PL are not secreted. Those proteins might be

involved in symptom development of the wild-type in cucurbits. This indicates that regulation of protein secretion might be a general mechanism for selective attack by *Colletotrichum* sp.

The path-1 mutation mapped to a single genetic locus (Freeman and Rodriguez 1992); however, it was considered that besides a single gene or multiple-pathogenicity genes, regulatory genes as well as additional gene mutations may be responsible for the lack of pathogenicity (Tyler 1993). The lack of secretion of PL in path-1 clearly suggests the importance of PL for pathogenicity in avocado fruits, but its not clear whether this is the only limiting factor that enables the mutant to grow endophytically within cucurbit host tissue without causing necrotrophic reactions as observed with the wild-type isolate (Freeman and Rodriguez 1993).

Besides path-1 being nonpathogenic it also activated host defense responses and induced resistance in cucurbits against challenge inoculations of various *Colletotrichum* and other fungal pathogens, without visibly initiating a hypersensitive response (Freeman and Rodriguez 1993). Autoclaved extracts of cell walls of path-1 and Cg-14 have induced significantly higher levels of phenylammonia lyase in avocado cell suspension culture (Prusky et al. 1994). Challenge inoculation of both strains has also elicited the increase of epicatechin that inhibits the activity of avocado lipoxygenase and delayed the breakdown of the preformed antifungal compound in whole fruit (Karni et al. 1989; Prusky et al. 1994). In fruits inoculated with Cg-14 the decrease in epicatechin concentration during fruit ripening (Prusky et al. 1994) lead to the initiation of PL secretion and early fungal attack (Wattad, Dinoor, and Prusky, unpublished data). The high level of epicatechin that was still present in path-1-inoculated ripe fruit suggests that path-1 may continuously stimulate host defense in ripening avocado (Prusky et al. 1994). This could be explained by the ability of path-1 to remain viable and have the constitutive

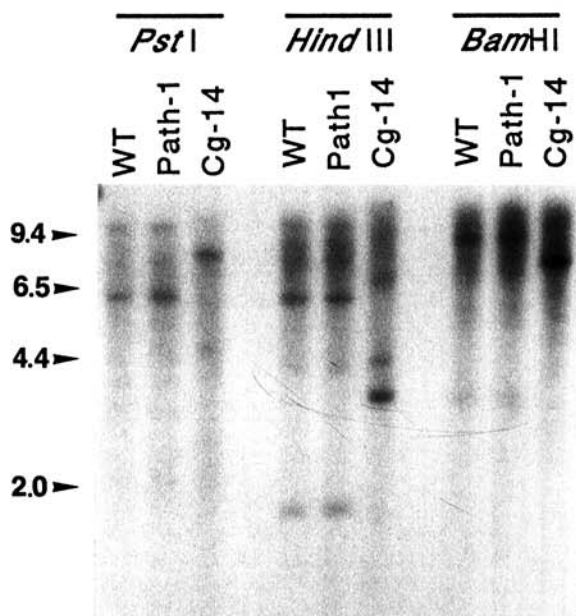


Fig. 5. Southern blot analysis of genomic DNA from *Colletotrichum gloeosporioides* (isolate Cg-14), the wild-type (WT) and nonpathogenic mutant (path-1) isolates of *C. magna*. A 5-mg DNA sample of each isolate was digested with various restriction enzymes and probed with a  $^{32}$ P random-primed clone of pectate lyase from *C. gloeosporioides*.

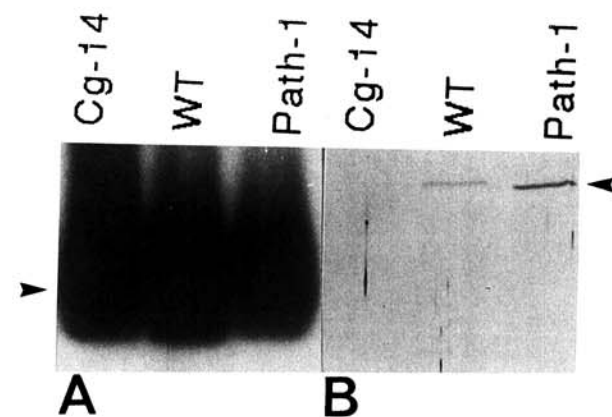


Fig. 6. Northern blot analysis (A) and Western blot (B) of pectate lyase-induced cell lysate from *Colletotrichum gloeosporioides* (isolate Cg-14), the wild-type (WT) and nonpathogenic mutant (path-1) isolates of *C. magna*. For Northern blot analysis 10 mg of RNA was fractionated on a 1.2% agarose formaldehyde gel and probed with a  $^{32}$ P random-primed clone of pectate lyase from *C. gloeosporioides*, transcription products are denoted by an arrow. For Western blot analysis proteins were extracted from 0.5 g of lyophilized hyphae and 5 mg of protein were analyzed on 12.5% SDS-PAGE, transferred to a nitrocellulose membrane and probed with anti-PL of *C. gloeosporioides* translation products are indicated by an arrow.

capability present in the cell walls, for the continuous activation of defense responses in spite of secreting insignificant levels of pectolytic enzymes.

## MATERIALS AND METHODS

### Fungal isolates, bacterial strains, growth conditions, and fruit inoculation.

*Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. pathogen on avocado fruit (*Persea americana* Miller var. *drymifolia* (Schldl. and Cham.) S. F. Blake 'Fuerte') was isolated from decayed fruit and maintained on potato-dextrose agar (PDA) at 95% RH, 26°C. *C. magna* S. F. Jenkins & Winstead (teleomorph: *Glomerella magna*) wild-type isolate (obtained from S. Brown and O. C. Yoder, Cornell University, Ithaca, NY) and a nonpathogenic mutant (path-1) derived from the wild-type isolate by ultraviolet and chemical treatments were maintained as described (Freeman and Rodriguez 1992).

For determining pectate lyase (PL) production and PL RNA extraction, isolates of *Colletotrichum* were grown in an induction culture medium as described by Wattad et al. (1994a). For DNA preparation the isolates were grown for 3 to 4 days in 500 ml of potato-dextrose broth (PDB) in the presence of 250 mg/liter of chloramphenicol.

Spot inoculation either on the pericarp or mesocarp of freshly harvested avocado fruits, were performed as described (Prusky et al. 1994). Total fruit inoculation, was performed by dipping the fruits in a water suspension of the various *Colletotrichum* isolates ( $2 \times 10^6$  conidia/ml) for a few seconds. Inoculated fruits were then incubated at 95% RH at 26°C. Ten fruits per treatment were inoculated and experiments repeated

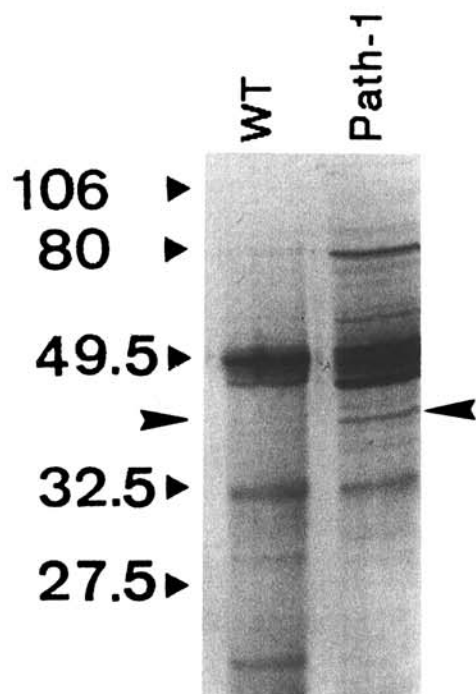


Fig. 7. SDS-PAGE analysis of cell lysate from the wild-type (WT) and nonpathogenic mutant (path-1) isolates of *Colletotrichum magna*. Molecular weight standards are denoted in kilodaltons with arrows indicating pectate lyase.

three times.

The *E. coli* strain, XL1Blue, harboring the pBLUESCRIPT SK+ plasmid containing the *pel* gene of *C. gloeosporioides*, as an 1,100-bp *EcoRI XhoI* fragment was isolated from a cDNA library by subsequent screening with *Aspergillus nidulans* PRD-091 clone of pectate lyase (kindly supplied by R. Dean). The isolated clones were confirmed as a *Pel* gene by the use of GCG software (Wattad et al. 1994b).

### Pectate lyase and polygalacturonase enzyme assays.

PL was assayed by monitoring the release of 4,5 unsaturated galacturonide from sodium polypectate at 232 nm (Wattad et al. 1994a). One unit of enzyme activity was defined as that liberating 1 mmole 4,5-unsaturated galacturonide/min under the assay conditions.

PG was assayed by monitoring the release of reducing groups from polygalacturonase as measured by the arsenomolybdate method (Prusky et al. 1989). One unit of enzyme activity was defined as that liberating 1 mmole of reducing equivalents per minute.

### Electrofocusing of culture filtrates.

Five hundred milliliters of culture filtrate were concentrated tenfold by rotary evaporation and dialyzed against two changes of 10 liters of ddH<sub>2</sub>O. The dialyzed solution was subjected to preparative isoelectric focusing using an LKB 8100-1 column. Samples were applied to a linear sucrose gradient 5 to 50% (v/w) in the presence of 1% carrier ampholyte (pH 3.0 to 10) and electrophoresed at a constant power of 7 W for 48 h (Wattad et al. 1994a). Fractions of 2.0 ml were collected and their pH determined. PL and PG activities were determined in a 50-ml aliquot.

### Detection of PL in decayed tissue.

Samples of 10 g of pericarp and mesocarp from 10 fruits per treatment were collected separately and placed in 50-ml tubes in liquid nitrogen. The frozen tissue was lyophilized until dry and ground in liquid nitrogen in the presence of 3 g of acid-purified sand. Proteins were extracted from the ground powder by resuspending 50 mg of tissue in 2.5 ml 4× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (0.25 mM Tris-HCl, 4% SDS, 10% β-mercaptoethanol, 40% glycerol), homogenized using a hand homogenizer (Thomas, 5 ml) and incubated at 75°C for 15 min. Samples were centrifuged at 14,000 × g for 10 min at room temperature and the interface was collected in duplicate. Fifty milliliters of the supernatant in 0.0012% bromophenol blue, 1× SDS-PAGE loading buffer were boiled for 5 min. A duplicate of each sample was dialyzed using Centrecon-10 units (Amicon Corp.) and proteins were quantified (Bradford 1976). Proteins were separated on 12.5% SDS-PAGE gels (Mini-Protein II, Bio-Rad) and Western blot analysis was performed as described (Wattad et al. 1994a).

### DNA, RNA, and protein isolation from fungi.

Total DNA was extracted from 1 g of lyophilized mycelium by grinding in liquid nitrogen in the presence of 3 g of acid-purified sand. The powder was transferred to 50 ml of extraction buffer (150 mM EDTA, 50 mM Tris-HCl pH 8, 2% sarkosyl) and 5 mg/ml proteinase K. The lysate was incubated at 55°C for 3 h and cell debris was removed by centrifugation for 10 min at 10,000 × g at room temperature. The super-

natant was collected and extracted three times with phenol chloroform and once more with chloroform. Nucleic acids were ethanol precipitated and dissolved in 5 ml of ddH<sub>2</sub>O. One hundred units of Rnase A Dnase free (Promega), were added and incubated at 37°C for 45 min. Polysaccharides were removed by addition of 1/10 volume of 5 M NaCl, 1/10 volume of 3M Na-acetate pH 3.8, 1% SDS, and incubated on ice for 1 h. The solution was centrifuged for 20 min at 15,000 × g at 4°C, the supernatant was collected and the DNA was precipitated in 95% ethanol.

Total RNA was isolated from induced cultures 5 days after seeding using the hot SDS-phenol chloroform method for plant RNA (Ausubel et al. 1991).

Total proteins were extracted from lyophilized mycelium by grinding 0.5 g in the presence of 2 g of acid-purified sand in 10 ml of 4× SDS-PAGE loading buffer. Cell debris was removed by centrifugation at 14,000 × g for 5 min. The supernatant was used without further treatment in Western blot analysis as described above.

Plasmid DNA was isolated from a 3-ml overnight culture of the cloned *pel* of *C. gloeosporioides* using standard protocols (Ausubel et al. 1991).

#### Western, Southern, and Northern blot analyses.

Five-milligram protein samples (Bradford 1976) were analyzed on denaturing SDS-PAGE gels (12.5%) run for 1 h at 150 V constant voltage (Laemmli 1970) and Western blot analysis was performed using antibodies raised against PL of *C. gloeosporioides*, essentially as described by Wattad et al. (1994a).

Genomic DNA was digested overnight with *Hind*III, *Pst*I, and *Bam*HI restriction enzymes, and fractionated in 1% agarose gel in TAE buffer. DNA was transferred to a nylon membrane (Nytran, Schleicher & Schuell) and hybridized overnight in an aqueous solution at 55°C to a <sup>32</sup>P random primed *Pel* gene of *C. gloeosporioides* (Wattad et al. 1994b). The membrane was washed twice in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS for 15 min at room temperature and a final wash of 0.25× SSC, 0.1% SDS at 55°C for 30 min.

Total RNA was fractionated in 1.2% formaldehyde gels as described by Ausubel et al. (1991). RNA was transferred to a Nytran nylon membrane and hybridized under similar conditions to those used for DNA analysis.

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