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Molecular Genetic Evidence for the Involvement of a Specific Polygalacturonase, P2c, in the Invasion and Spread of *Aspergillus flavus* in Cotton Bolls†

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Isolates of *Aspergillus flavus* can be differentiated based on production of the polygalacturonase P2c. One group of isolates produces P2c, whereas the other group does not. In general, the group that produces P2c causes more damage and spreads to a greater extent in cotton bolls than those isolates that do not produce P2c. To determine whether P2c contributes to disease, the expression of *pecA*, the gene previously determined to encode P2c, was genetically altered. Adding the *pecA* gene to a strain previously lacking the gene resulted in the ability to cause significantly more damage to the intercarpellary membrane and the ability spread to a greater extent within the adjacent locule compared to the abilities of a control transformant. Conversely, eliminating the expression of *pecA* by targeted disruption caused a significant reduction in aggressiveness compared to that of a nondisrupted control transformant. These results provide direct evidence that P2c contributes to the invasion and spread of *A. flavus* during infection of cotton bolls. However, other factors not evaluated in this study also contribute to aggressiveness.

Many plant pathogenic and saprophytic organisms produce a battery of plant cell wall-degrading enzymes as the first step in gaining access to nutrients from plants (22). Attention has focused on enzymes which degrade pectin, a major constituent of plant cell wall, including the exo- and endo- forms of polygalacturonase (PG), the exo- and endo- forms of pectate lyase, and pectin methylesterase (PME). These enzymes, referred to as pectinases, are the first cell wall-degrading enzymes produced by bacterial or fungal pathogens when cultured on isolated plant cell walls or during infection, and it has been proposed that they are important in some plant-pathogen interactions (3, 6, 29). In addition, pectinases play a role in activating disease resistance mechanisms. Some pectinases are phytotoxic (27) and may induce the hypersensitive response in incompatible interactions (22). Endo-PG also releases oligogalacturonides from cell walls, and this can result in the elicitation of plant defense responses (13).

Detailed studies on the role of pectinases in pathogenesis have been undertaken with bacterial pathogens. For example, the role of pectinases in soft-rot erwinias has been examined extensively through physiological and genetic approaches (5, 16). On the other hand, the role of fungal pectinases in plant-pathogen interaction is not well understood and somewhat controversial. Molecular genetic approaches have been used to obtain direct evidence for the importance of pectinase enzymes in disease. Genes for several fungal endo-PGs have been cloned and characterized, including those from *Aspergillus niger*, *Aspergillus tubingensis*, *Aspergillus parasiticus*, *Cochliobolus carbonum*, *Colletotrichum lindemuthianum*, *Cryphonectria parasitica*, *Fusarium moniliforme*, and *Sclerotinia sclerotiorum* (7–12, 20, 28, 33). In several instances targeted deletion of representative PG genes has failed to find an important function in disease. Disruption of the endo-PG gene of *C. carbonum* had no effect on pathogenicity on maize (30). Similarly, disease caused by *Penicillium oilskin* on *Arabidopsis thaliana* was not affected by deletion of the PG gene (25). It was also reported recently that disruption of the endo-PG gene from *C. parasitica* resulted in no reduction in canker formation on dormant American chestnut stems (20). However, these studies were far from exhaustive, and further studies are required before general statements can be made concerning the role of PG and other pectinases in fungal infection.

Aspergillus flavus is a serious problem because it produces aflatoxins, the most carcinogenic naturally occurring compounds known, during infection of agronomically important crops, such as corn, peanuts, and cotton. A wide spectrum of *A. flavus* strains can be isolated from naturally infected cotton bolls. At one extreme, certain isolates can degrade the intercarpellary membrane that divides a cotton boll and are able to spread throughout the boll, as measured by the presence of bright greenish yellow fluorescence (BGYF) on the cotton lint (4, 17). Conversely, at the other end of the spectrum other isolates cannot degrade this membrane, and thus their growth in the cotton boll is limited. One consistent difference between these isolates is in their pectinase production. All produce three pectinase activities: two endo-PGs (P1 and P3) and a PME. However, expression of an additional endo-PG (termed P2c) has been correlated with increased damage to the intercarpellary membrane and greater spread within infected tissues (14, 15). P2c, the predominant pectinase activity, accumulates in medium containing glucose or pectin, whereas the

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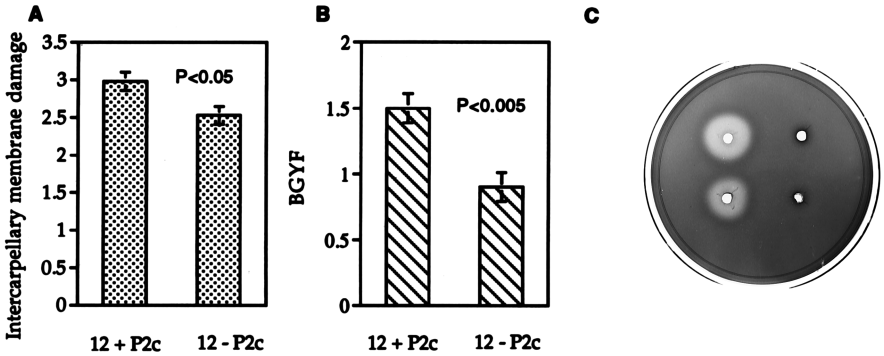

FIG. 1. Aggressiveness, spread, and pectinase activities of *A. flavus* transformants of a P2c$^-$ strain in cotton bolls. Transformant 12 + P2c contains an active *pecA* gene, and transformant 12 − P2c is the control. (A) Intercarpellary membrane damage caused by the transformants. Values represent the rating means and standard errors for 6 to 10 repetitions for three tests, calculated with an ANOVA test (SAS). Intercarpellary membrane damage was rated as described in Materials and Methods. (B) BGYF. Values represent the rating means and standard errors for 6 to 10 repetitions (two locules per repetition) for four tests, calculated with an ANOVA test (SAS). BGYF on the lint of each locule was rated as described in Materials and Methods. (C) Cup-plate assay of pectinase activities from the extracts of inoculated cotton bolls. The clear zone produced by extracts from two independent bolls inoculated with transformant 12 + P2c (left) indicates activity. No activity was detected in extracts from two independent bolls inoculated with the control, transformant 12 − P2c (right).

accumulation of the other enzymes is repressed by glucose and is produced only in pectin medium. The ability to produce endo-PG in the presence of glucose is rare in filamentous fungi (2). However, the ability of some organisms to produce enzyme activity in the absence of an inducer may be a key element in pathogenesis caused by those organisms.

Recently, we have isolated two pectinase genes, *pecA* and *pecB*, from *A. flavus* and have shown that one of these genes, *pecA*, encodes the PG activity P2c (34). In this study, we have genetically altered the expression of P2c, both by gene addition and targeted deletion. The results show that P2c does contribute to fungal aggressiveness, but it is not the sole factor responsible for pathogenicity.

MATERIALS AND METHODS

Strains and culture conditions. *A. flavus* strains 70 (P2c$^+$) and 12 (P2c$^-$) (17) and transformants were maintained on 4% V-8 agar medium, pH 6.5, at 30°C. *Escherichia coli* DH5α was used for all bacterial manipulations.

A. flavus was grown in the liquid medium according to the method described by Adye and Mateles (1) at 30°C with shaking at 200 rpm. The medium contained either 0.5% pectin (Sigma Chemical Company, St. Louis, Mo.) or potato dextrose broth.

DNA isolation and manipulation. Plasmid DNA was isolated by the alkaline lysis method. *A. flavus* genomic DNA was isolated according to the method of Horng et al. (24).

For Southern analysis, digested genomic DNA was fractionated on a 0.8% agarose gel before being transferred to a Hybond N nylon filter (Amersham Corp., Arlington Heights, Ill.), according to the method of Maniatis et al. (26). DNA labeling and Southern hybridizations were performed according to the procedures of Maniatis et al. (26). Southern hybridizations were performed in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's solution–0.1% sodium dodecyl sulfate (SDS)–50 mM PO_4 buffer, pH 6.6, at 65°C. Membranes were washed three times at 65°C in 0.1× SSC–0.1% SDS before being exposed to autoradiographic film.

Construction of plasmid pMS3. Plasmid pMS3 was constructed by first cloning a 1.4-kb *Bam*HI-*Xba*I fragment of pCFC12 (containing the 5′ end of *pecA*) and a 1.6-kb *Xba*I-*Bam*HI fragment of pCFC41 (containing the 3′ end of *pecA*) into pUC8 (34). A 6.7-kb *Xba*I fragment from pSL82 (containing the *niaD* gene, encoding nitrate reductase, from *A. parasiticus*) was then inserted into the *Xba*I site.

Transformation. *niaD* mutants from strain 70 (P2c$^+$) were isolated from chlorate medium and analyzed as described previously (31). Polyethylene glycol-mediated transformation was adapted from the method of Unkels et al. (32). Protoplasts of one *niaD* mutant were transformed with plasmid pMS3. Following transformation, protoplasts were plated on selective medium (minimal medium containing 1.2 M sorbitol and 10 mM nitrate as the sole nitrogen source). Transformants were streaked to obtain single colonies and further evaluated.

Pectinase activity assays. Transformants were grown in potato dextrose liquid medium for 48 h. Culture filtrates were then collected and examined for P2c activity by the cup-plate assay described by Cleveland and Cotty (14). Alternatively, culture filtrates were collected from selected transformants grown in liquid medium containing pectin, transferred to dialysis tubing, and concentrated by overlaying with sucrose followed by dialysis against 1% glycine (15). The 10-fold concentrated filtrates were applied to isoelectrofocusing (IEF) gels, pH 3.5 to 9.5. IEF gels were assayed for pectinase activity by a pectin-agarose overlay technique (15).

Inoculation and sampling of cotton bolls. Cotton plants (Acala SJ-2 in tests 1, 2, and 3; Delta Pine 90 in test 4) were grown in a greenhouse in 1-gal pots containing an equal mixture of sand, Pro-mix (Premier Brands Inc., New Rochelle, N.Y.), and top soil. Plants were then fertilized weekly with Peters fertilizer (Grace-Sierra Inc., Milpitas, Calif.) at 150 ppm. Flowers were dated at opening (18). Unopened cotton bolls were wounded once 25 to 28 days postanthesis with a cork borer (3-mm diameter) to a depth of 2 mm. The resulting plug from the carpel area was removed. The exposed lint was inoculated with a 10-μl spore suspension (4.0×10^6 spores per ml) of fungal strain. Wounded, noninoculated bolls served as controls (6). Bolls for pectinase extraction or for examination of fungal aggressiveness were collected after boll opening, usually 10 to 14 days postinoculation. Each treatment was replicated 6 to 10 times; each replicate consisted of one boll. Tests to assess pectinase production were performed twice, while tests to assess fungal aggressiveness were performed at least three times.

Extraction of pectinases from cotton bolls. Pectinases were extracted from bolls as previously described (15). To each locule, 10 ml of 0.1 M sodium acetate buffer (pH 4.5) was added. Air was then squeezed from the lint with a spatula, and then the tissue was soaked in the buffer with continuous agitation for 30 min. Buffer containing the pectinases was squeezed from the lint and seed and centrifuged for 10 min at 27,200 × *g* to remove insoluble material. The supernatant was dialyzed against the acetate buffer and concentrated 100-fold with Centricon concentrators and Centriprep microconcentrators (10,000-Da cutoff) (Amicon Co., Danvers, Mass.), and the concentrated solution was subsequently used in cup-plate assays (14).

Evaluation of fungal aggressiveness and spread. Fungal spread between locules was used as one measure of virulence (6). During growth on cotton lint, *A. flavus* produces kojic acid, which is converted by host peroxidase into a compound with BGYF. The presence of BGYF on cotton lint is a reliable indicator of the activity of *A. flavus* (3, 17).

Mature harvested bolls were dried in a forced air oven at 60°C for 2 days and kept at room temperature in sealed plastic bags containing silica gel dessicant until evaluated. Wound-inoculated locules adjacent to noninoculated locules were examined under UV light after drying, and BGYF on the lint of each locule was rated as follows: 0, no BGYF; 1, BGYF on less than 25% of the lint; 2, BGYF on 25 to 50% of the lint; and 3, BGYF on more than 50% of the lint.

Intercarpellary membranes adjacent to inoculated locules and membranes from noninoculated control bolls were examined to measure fungal aggression (6). Membranes were rated as follows: 0, no symptoms; 1, slightly discolored on surface next to inoculated locule; 2, same as a rating of 1 plus slightly discolored on surface away from inoculated locule; 3, highly discolored on surface next to inoculated locule and slightly discolored on surface away from inoculated locule; and 4, highly discolored on both surfaces.

Statistical analysis. Analyses of BGYF ratings and intercarpellary membrane damage ratings were performed with the Statistical Analysis Software System (SAS Institute, Inc., Cary, N.C.). Treatment replicates from each test were first subjected to analysis of variance (ANOVA) followed by mean comparisons of BGYF or membrane damage values. Differences among treatment means were determined by the least-significant-difference test. Pearson product moment correlations were calculated for relationships between intercarpellary membrane

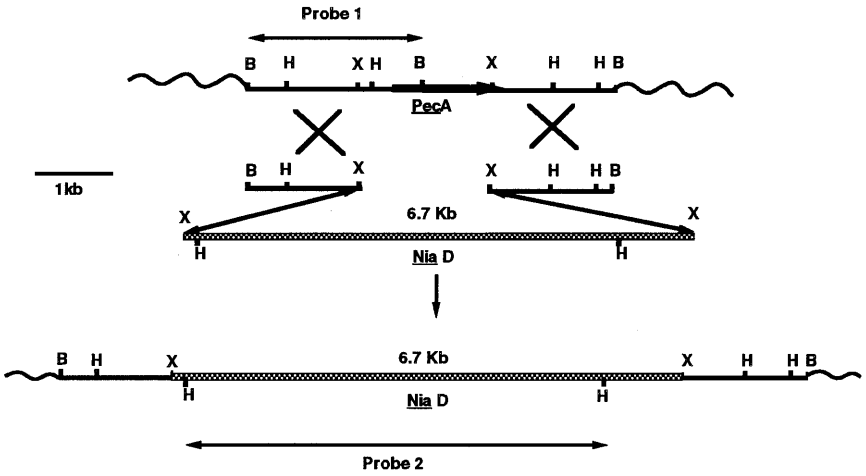

FIG. 2. Schematic diagram for the gene replacement strategy used for targeted disruption of *pecA*. B, *Bam*HI; H, *Hin*dIII; X, *Xba*I. Probes 1 and 2 were used for Southern hybridization analysis as described in the legend to Fig. 4.

damage and the amount of BGYF present on lint for tests 2, 3, and 4 with Statistica vol. 1 software (StatSoft Corp., Tulsa, Okla.).

RESULTS

Introduction of the P2c gene into P2c$^-$ strain 12 results in increases in aggressiveness. Previously, transformants from strain 12 (P2c$^-$) carrying active *pecA* genes were shown to produce P2c activity in vitro (34). To study the effect of an active *pecA* gene on *A. flavus* infection of cotton bolls, transformant 9-10-3 (strain 12 + P2c) and control transformant 6-10-19 (strain 12 − P2c), a transformant carrying the *niaD* gene only, were used to inoculate cotton bolls. Fungal aggressiveness and spread were examined by observing damage to intercarpellary membranes and by measuring the presence of BGYF on the cotton lint (4, 17). Four independent experiments with 66 cotton bolls were conducted. Tight locks (failure of mature locks to expand and fluff out) were induced in inoculated cotton bolls by both *A. flavus* transformants. For noninoculated control bolls, locules showed no tight lock formation, BGYF was not present on the lint, and intercarpellary membranes showed no symptoms of *A. flavus* infection. Statistical analysis on the combined data indicated that strain 12 + P2c caused significantly greater damage on intercarpellary membrane ($P < 0.05$) and spread to a greater extent within the locule (BGYF; $P < 0.005$) than strain 12 − P2c (Fig. 1A and B). To verify P2c expression by the transformants in planta, pectinase activities were measured with the extract of inoculated cotton bolls. P2c enzyme activity was detected only in cotton bolls infected with transformant 12 + P2c and not in bolls infected with the control strain 12 − P2c (Fig. 1C).

Targeted disruption of *pecA* in P2c$^+$ strain 70. Previously, we have shown that *pecA* is a single-copy gene; therefore, creating strains lacking P2c activity was readily possible (34). Plasmid pMS3, containing the *niaD* gene from *A. parasiticus* flanked by the 5′ and 3′ ends of *pecA*, was used to transform strain 70 by polyethylene glycol-mediated transformation (Fig. 2). Transformants grown in medium containing glucose were screened for their ability to produce P2c by the cup-plate assay (14). Of a total of 120 transformants, 7 were negative for P2c production. To confirm the loss of P2c activity, several transformants expressing P2c and several others not expressing P2c were further evaluated by using a pectin-agarose overlay of IEF gels (15). While putative *pecA* gene knockout transformants (strain 70 − P2c) showed only PE bands, both PE and P2c bands were detected in the recipient parental strain and putative ectopic transformants (strain 70 + P2c) (Fig. 3). P1 and P3 could be detected only with extended incubation of pectin overlay. To check the genotypes of these transformants, genomic DNA from the wild type and the 70 − P2c transformants was digested with *Xba*I and hybridized with *niaD* (Fig. 4A) or *pecA* (Fig. 4B). When probed with *niaD*, a 6.7-kb *A. parasiticus niaD* fragment was detected, as predicted from the restriction map (Fig. 2). An additional 8.5 kb was detected in the transformants and the parent, which may have resulted from cross-hybridization of *A. parasiticus niaD* with the endogenous *A. flavus niaD* gene. The *pecA* probe hybridized, as predicted, to two fragments (1.6 kb and 9 kb) in strain 70 and only to a 9-kb fragment in the 70 − P2c transformants. The lack of the 1.6-kb fragment resulted from double homologous recombination and replacement of the *pecA* coding region with the *niaD* gene from pMS3. For all the transformants tested, Southern hybridization data correlated with P2c activity in vitro (data not shown).

Disruption of P2c in the P2c$^+$ strain reduces aggressiveness. To obtain additional direct evidence for a role of P2c in disease caused by *A. flavus*, transformant 5-3-14 (strain 70 − P2c) and control ectopic transformant 5-3-35 (strain 70 + P2c) were used to inoculate cotton bolls. Results from four independent experiments, again with a total of 66 cotton bolls, are shown in Fig. 5. Deletion of *pecA* from strain 70 resulted in a significant

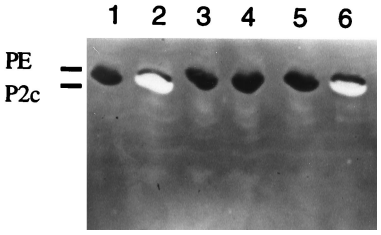

FIG. 3. Pectinase activities in culture filtrates of transformants grown in pectin medium. Activities were examined by pectin-agarose overlay of IEF gels. Lane 1, culture filtrates from strain 12; lane 2, culture filtrates from an ectopic transformant of strain 70 (strain 70 + P2c; transformant 5-3-35) expressing P2c; lanes 3 to 5, culture filtrate from transformants of strain 70 (strain 70 − P2c; transformants 5-3-1, 5-3-22, and 5-3-14) in which *pecA* had been deleted and P2c was not expressed. Culture filtrate of strain 70 is shown in lane 6.

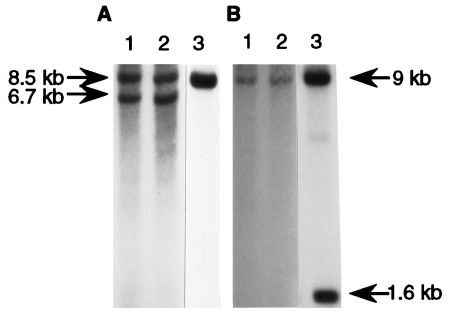

FIG. 4. Hybridization of *niaD* and *pecA* to strain 70 transformants. (A) A 5.0-kb *Hin*dIII fragment from pSL82 containing the *niaD* gene (probe 2, Fig. 2) was radiolabelled and hybridized to *Xba*I-digested genomic DNA from strain 70 (lane 3) and from the 70 − P2c transformants (5-3-14 and 5-3-22; lane 1 and 2, respectively). (B) Membrane used in panel A was stripped and probed with a 2.3-kb *Bam*HI fragment from pCFC12 (probe 1, Fig. 2) which contains the *pecA* gene.

decrease in the ability to damage intercarpellary membranes ($P < 0.05$) (Fig. 5A) and in spread within locules (BGYF; $P < 0.05$) (Fig. 5B) when compared with the results obtained with the control transformant. The phenotypes of transformants in planta were verified by determining pectinase activities in extracts from inoculated cotton bolls. As expected, P2c enzyme activity was detected in extracts from bolls infected with the control transformant (containing *pecA*) but not in extracts from bolls infected with the transformant with pecA deleted (Fig. 5C). The expression of other pectinase enzymes appeared to be unaffected.

Correlation of fungal spread and aggressiveness. Pearson product moment correlations were calculated to correlate the ability to damage intercarpellary membranes and the amount of BGYF present on lint (Fig. 6). A strong correlation was detected for all four *A. flavus* transformants tested ($r = 0.73$; $P < 0.01$), consistent with previous findings (6).

DISCUSSION

The role of pectinase in the pathogenesis of microbial infection, particularly for fungal pathogens, is not well established in spite of the number of studies on a variety of organisms. Molecular genetic studies have not been completed, in large part because of the presence of multiple pectinase genes that need to be evaluated.

Previous studies suggested that P2c plays a role in the aggressiveness of the infection of cotton bolls by *A. flavus* (14, 15). Our studies demonstrate that the addition of the *pecA* gene to a P2c null strain increases both fungal aggressiveness and spread. Furthermore, the removal of the *pecA* gene from a P2c$^+$ strain decreases the aggressiveness of fungal infection. It is always possible that other events related to the transformation procedure could account for the differences observed. However, this seems unlikely, since the addition of P2c resulted in increased aggressiveness and the disruption of P2c resulted in reduced aggressiveness. Also, these results are consistent with previous findings (6, 14, 15). Taken together, these data suggest that the PG P2c produced in *A. flavus* contributes to fungal invasion and colonization. To our knowledge, this is the first report of molecular genetic evidence for the involvement of a specific pectinase in fungal pathogenesis.

Although previous studies demonstrated a correlation between the aggressiveness of *A. flavus* and P2c enzyme activity, there are clearly other factors involved. As a group, P2c strains are more aggressive than P2c$^-$ strains; however, there is a continuum of measurable aggressiveness within each strain type. As shown in Fig. 1 and 5, there is little difference in the fungal aggressiveness between the ectopic controls, strain 12 (P2c$^-$) and strain 70 (P2c$^+$). Strain 12 and strain 70 were chosen for this study because of their similarity in growth rate and morphology. Also, the fact that the P2c null strain and the *pecA*-disrupted transformant are still capable of causing disease further indicates that P2c is not the only factor involved in infection and colonization. Multiple factors, including other cell wall-degrading enzymes, are likely to be involved. Even when considering only pectinases, usually more than one pectinase and more than one type of pectinase are produced. Two additional endo-PGs, P1 and P3, and PME are produced by *A. flavus*. Although the PG activities of P1 and P3 are relatively low when compared to P2c expression in vitro and in planta, it is possible that these or other pectinase activities are sufficient for the initial stages of infection. Both strain 12 and the *pecA*-disrupted transformant (strain 70 − P2c) produced small amounts of P1 and P3 activity, which were detected in the extract from infected cotton bolls (data not shown). Previously, we isolated another PG gene, *pecB*, which may encode either P1 or P3. Pathogenesis studies with a double disruptant (disruption of both *pecA* and *pecB*) should provide further information on the role of PGs in disease.

Most studies of pectinase enzymes have revealed that gene expression is substrate-stimulated and catabolite-repressed (19). In filamentous fungi, it is rare that pectinase gene expression is not subject to glucose repression. An exception has been

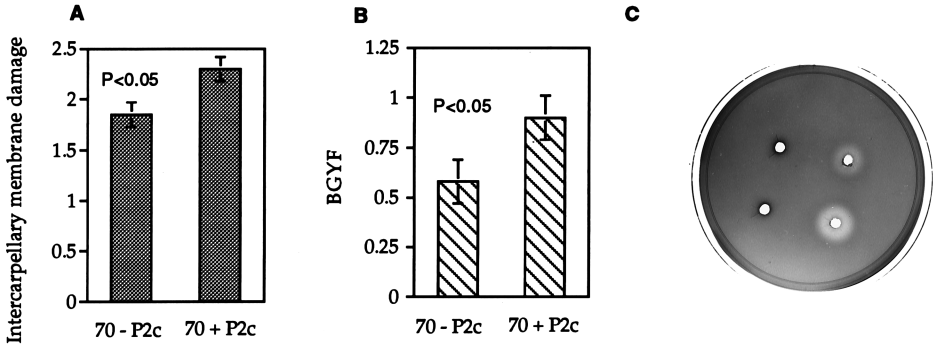

FIG. 5. Aggressiveness, spread, and pectinase activities of *A. flavus* transformants of a P2c$^+$ strain in cotton bolls. Transformant 70 − P2c lacks the *pecA* gene, and 70 + P2c is the control. (A) Intercarpellary membrane damage caused by the transformants. (B) BGYF produced by transformants. (C) Cup-plate assay of pectinase activity from the extract of inoculated cotton bolls. No P2c activity was detected in two independent boll extracts from transformant 70 − P2c (left), whereas clear zones were produced by two independent boll extracts from the control transformant 70 + P2c (right). See the legend to Fig. 1 for further explanation.

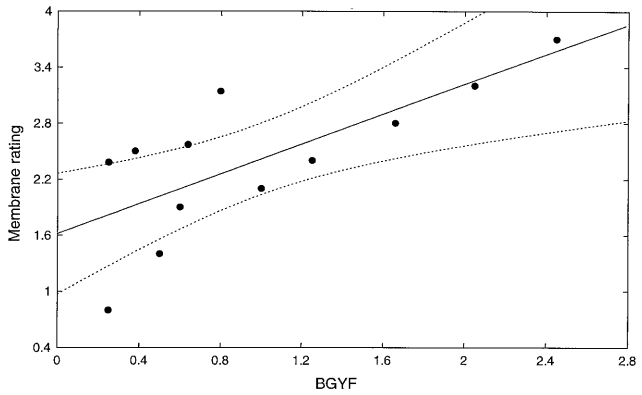

FIG. 6. Correlation of intercarpellary membrane damage with BGYF. Both ratings are measurements of fungal virulence (see text). The membranes and lint analyzed were from cotton boll locules inoculated with *A. flavus* transformant 12 − P2c, 12 + P2c, 70 + P2c, or 70 − P2c in three independent experiments. Dotted lines indicate 95% confidence bands.

recently reported for pectate lyase (*pelB*) from *Fusarium solani* f. sp. *pisi* (21). In our case, the fact that P2c plays a role in the invasion of the fungus and that *pecA* expression is not inhibited by glucose suggests that the regulation of *pecA* is important in pathogenesis. Previous studies on *Fusarium oxysporum* f. sp. *cepae* suggested that high sugar concentration in onion cells plays a role in resistance to the pathogen by imposing catabolite repression on the expression of the pectinase enzymes (23). Therefore, insensitivity of *pecA* to glucose repression allows expression of P2c production in planta, thus facilitating fungal infection. The cell wall degradation products from P2c activity may induce expression of other pectinases or other cell wall-degrading enzymes and result in further tissue damage. Experiments are under way to determine the control mechanisms for PG expression, especially for P2c expression.

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