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Isolation and Characterization of Polygalacturonase Genes (*pecA* and *pecB*) from *Aspergillus flavus*†

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Two genes, *pecA* and *pecB*, encoding endopolyglacturonases were cloned from a highly aggressive strain of *Aspergillus flavus*. The *pecA* gene consisted of 1,228 bp encoding a protein of 363 amino acids with a predicted molecular mass of 37.6 kDa, interrupted by two introns of 58 and 81 bp in length. Accumulation of *pecA* mRNA in both pectin- or glucose-grown mycelia in the highly aggressive strain matched the activity profile of a pectinase previously identified as P2c. Transformants of a weakly aggressive strain containing a functional copy of the *pecA* gene produced P2c in vitro, confirming that *pecA* encodes P2c. The coding region of *pecB* was determined to be 1,217 bp in length interrupted by two introns of 65 and 54 bp in length. The predicted protein of 366 amino acids had an estimated molecular mass of 38 kDa. Transcripts of this gene accumulated in mycelia grown in medium containing pectin alone, never in mycelia grown in glucose-containing medium, for both highly and weakly aggressive strains. Thus, *pecB* encodes the activity previously identified as P1 or P3. *pecA* and *pecB* share a high degree of sequence identity with polygalacturonase genes from *Aspergillus parasiticus* and *Aspergillus oryzae*, further establishing the close relationships between members of the *A. flavus* group. Conservation of intron positions in these genes also indicates that they share a common ancestor with genes encoding endopolyglacturonases of *Aspergillus niger*.

The ability to degrade plant tissues is an important characteristic for many plant pathogenic and saprophytic organisms. Hence, microorganisms produce a battery of plant cell wall-degrading enzymes (19). Among these are endopolygalacturonases (EC 3.2.1.15), which degrade pectin, a major constituent of plant cell walls. Endopolygalacturonases have been proposed as being important in some plant pathogen interactions (4, 38). They are the first cell wall-degrading enzymes produced by fungal pathogens when cultured on isolated plant cell walls or during infection (3). Additionally, endopolygalacturonases are sometimes phytotoxic (31), which may induce the hypersensitive response in incompatible interactions (19). Endopolygalacturonases also release oligogalacturonides from cell walls which can result in the elicitation of plant defense responses (10).

Several fungal endopolygalacturonases have been cloned and characterized, including those from *Aspergillus niger*, *Aspergillus tubigensis*, *Cochliobolus carbonum*, *Fusarium moniliforme*, and *Sclerotinia sclerotiorum* (5–8, 46). Disruption of the endopolygalacturonase gene of *C. carbonum* had no effect on the pathogenicity to maize (41). Analyses of genes from *A. niger* have concentrated on their use in the food industry and for juice clarification. However, endopolygalacturonase genes have not been previously cloned from an opportunistic plant-pathogenic fungus, such as *Aspergillus flavus*, in which endopolygalacturonases are thought to be essential in aggressiveness towards infection of cotton bolls (4, 13).

A. flavus produces aflatoxins, the most carcinogenic naturally occurring compounds known, during infection of agronomically important crops such as corn, peanuts, and cotton. Two widely occurring strains of *A. flavus* can be isolated from naturally infected cotton bolls. Highly aggressive strains degrade the intercarpellary membrane that divides a cotton boll and are able to spread throughout the boll. Weakly aggressive strains cannot degrade this membrane, and thus their growth is restricted to individual locules. The only consistent difference detected between these two strain types is their pectinase production. Both produce three pectinase activities, two endopolygalacturonases (P1 and P3) and a pectin methyl esterase (PE). However, highly aggressive isolates produce an additional endopolygalacturonase (termed P2c) (13, 14). P2c activity accumulates in medium containing glucose or pectin, whereas the activity of the other enzymes is repressed by glucose and is only produced in pectin medium. The ability to produce endopolygalacturonases in the presence of glucose is rare in filamentous fungi (2). However, the ability to produce enzyme activity in the absence of an inducer may be a key element in the pathogenesis by some organisms.

The two strain types of *A. flavus* provide an excellent opportunity to analyze, at the molecular level, production of endopolygalacturonases in an opportunistic pathogen and to determine their role in pathogenesis. Further knowledge of the control and regulation of endopolygalacturonase also may prove useful to industrial production of this enzyme. We describe here the cloning and characterization of two endopolygalacturonase genes from a highly aggressive strain of *A. flavus*. We establish by DNA transformation that one of these genes, *pecA*, encodes the pectinase P2c.

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MATERIALS AND METHODS

Strains and culture conditions. *A. flavus* 70 and 12 (15) were maintained on 4% V-8 agar medium (pH 6.5) at 30°C. *Escherichia coli* DH5α and XL1-blue were used for all bacterial manipulations.

A. flavus was grown in the liquid medium as described by Adye and Mateles (1) at 30°C and 200 rpm. The medium contained either pectin at 0.5% or other carbon sources at 1%. Mycelia were grown in glucose medium for 15 h before being shifted to glucose, pectin, pectin and glucose, or mannitol for 5 h prior to harvest for RNA extraction (17). All carbon sources were purchased from Sigma Chemical Company (St. Louis, Mo.).

DNA and RNA isolation and manipulation. Plasmid DNA was isolated by the alkaline lysis method. Restriction enzyme site mapping was conducted according to standard methods (30). *A. flavus* genomic DNA was isolated as described by Horng et al. (20). To isolate RNA, approximately 0.5 g of press dried mycelia was ground to a fine powder in liquid N_2. The powder was placed in oak ridge tubes containing liquid N_2, 3 ml of 8 M guanidinium HCl, and 3 ml of phenol-chloroform. The powder was thawed on ice and centrifuged at 3,000 rpm for 10 min in an SS34 rotor. The supernatant was placed over a 1.5-ml 5.7 M $CsCl_2$ cushion and centrifuged for 20 h at 26,000 rpm in an SW41 swinging bucket rotor. The resulting RNA pellet was resuspended in deionized water.

Digested genomic DNA was fractionated on a 0.8% TAE (Tris-acetic acid-EDTA) agarose gel, while total RNA was separated on a 1% formaldehyde agarose gel, before being transferred to a Hybond N nylon filter (Amersham Corp., Arlington Heights, Ill.), as described by Maniatis et al. (30). DNA labelling and Southern and Northern (RNA) hybridizations were performed according to the method of Maniatis et al. (30) 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). The temperature of the hybridizations and washes varied between 56 and 68°C for different experiments. The membranes were washed in 3× to 0.1× SSC–0.1% SDS, before being exposed to autoradiographic film.

DNA sequencing. Restriction enzyme fragments from pCFC9, pCFC12, and pCFC41 were subcloned into pBluescript KS+ or SK+. Both strands of the subclones were sequenced by the dideoxynucleotide chain termination method (40). Either a Sequenase version 2.0 (United States Biochemical, Cleveland, Ohio) with [α-^{35}S]dCTP or a *Taq* Dye Deoxy Terminator Cycle Sequencing (Applied Biosystem, Foster City, Calif.) kit was used to label the DNA. Reactions were analyzed on a standard sequencing gel (30) or an Applied Biosystems autosequencer, respectively. DNA and protein sequence analyses were performed by using the software package GeneWorks 2.2 for Macintosh (Intelligentics, Inc., Mountain View, Calif.).

Genomic library screening and phage manipulations. A genomic library of *A. flavus* 70 was constructed by cloning partially digested *Sau*3A genomic DNA into EMBL3. Phage clones were maintained in *E. coli* LE392. Screening of the genomic library was performed according to the method of Sambrook et al. (39). Hybridization of the *C. carbonum pgn-1* gene or *Aspergillus parasiticus pec-1* gene was done as described above at 56 or 60°C, respectively. Individual purified plaques were amplified, and DNA was isolated by using Magic Lambda Preps (Promega, Madison, Wis.) according to the manufacturer's instructions.

Construction of plasmid pCFC80. Plasmid pCFC80 was constructed by first cloning a 1.7-kb *Bam*HI-*Hin*dIII fragment of pCFC41 into pBluescript SK+. A 2.3-kb *Bam*HI fragment from pCFC12 was then inserted into the *Bam*HI site to create plasmid pCFC80, which contains the entire *pecA* gene.

Transformation. *niaD* mutants from strain 12 (weakly aggressive) were isolated from chlorate medium as described previously (44). Several mutants that were unable to produce P2c were defective in *niaD*. Polyethylene glycol-mediated transformation was adapted from the method of Unkels et al. (44). Protoplasts of one *niaD* mutant were cotransformed with plasmids pCFC80 and pSTA10 (containing the *niaD* gene from *A. niger*) (43). 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 for single colonies and further evaluated.

Pectinase activity assays. Transformants were grown in liquid medium containing glucose as the sole carbon source for 48 h. Culture filtrates were then collected and examined for P2c activity by cup-plate assay as described previously (13). Alternatively, culture filtrates were collected from selected transformants grown in liquid medium containing pectin. These culture filtrates were then concentrated by sucrose overlay and dialyzed against 1% glycine (14). The 10-fold-concentrated filtrates were then applied to isoelectric focusing gels (pH 3.5 to 9.5). Isoelectric focusing gels were assayed for pectinase activity by pectin-agarose overlay (14).

Nucleotide sequence accession numbers. The GenBank accession numbers of the nucleotide sequences of *pecA* and *pecB* (see Fig. 2 and 3) are U05015 and U05020, respectively.

RESULTS

Isolation of genes encoding endopolygalacturonases. A strategy of cloning by heterologous hybridization was employed. A 541-bp *Pst*I-*Sac*I fragment of the *C. carbonum pgn-1* endopolygalacturonase gene (46) was used to probe genomic *A. flavus* DNA isolated from strains 70 (highly aggressive) and 12 (weakly aggressive). This fragment consistently hybridized to an approximately 3.8-kb *Bam*HI fragment in both strains (data not shown) and was therefore hybridized to a genomic library of *A. flavus* 70 constructed in EMBL3. Strongly hybridizing plaques were isolated and analyzed in detail. A 3.8-kb *Bam*HI fragment, which hybridized to the *C. carbonium pgn-1* gene, was isolated and cloned into pBluescript KS+ to create pCFC9. pCFC9, in addition to hybridizing to a 3.8-kb *Bam*HI fragment, produced a weak hybridization signal at 2.5 kb when hybridized to *Bam*HI-digested *A. flavus* DNA (data not shown). pCFC9 was restriction mapped, and the region hybridizing to the *C. carbonum Pst*I-*Sac*I fragment was determined by Southern analysis (Fig. 1A). This putative endopolygalacturonase was termed *pecB.*

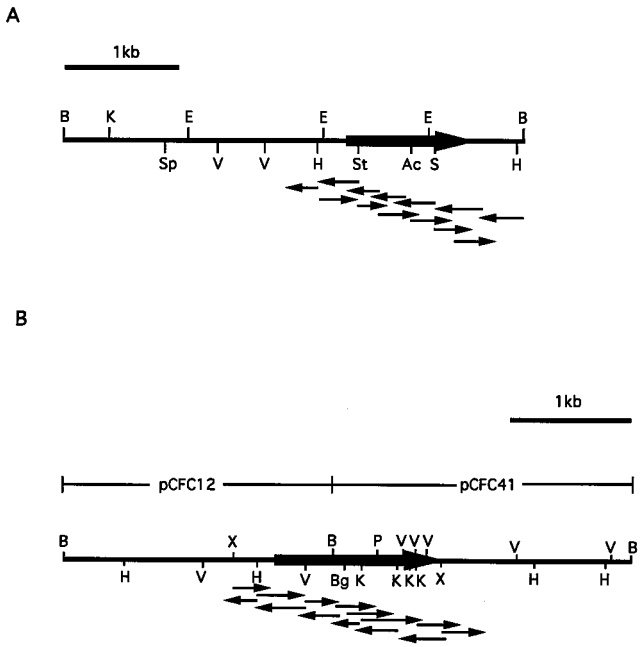

FIG. 1. Partial restriction map and localization of *pecB* in pCFC9 (A) and *pecA* in pCFC12 and pCFC41 (B). The large solid arrows indicate the positions of the genes and the direction of transcription. The sequencing strategy for each gene is outlined below the restriction map by small arrows. Ac, *Acc*I; Bg, BglI; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sal*I; St, *Stu*I; V, *Eco*RV; X, *Xba*I.

In order to isolate additional endopolygalacturonase genes, a cDNA clone of the endopolygalacturonase, *pec-1*, of *A. parasiticus* (9) was used to probe *A. flavus* 70 and 12 genomic DNA. The *pec-1* gene preferentially hybridized in 6× SSC at 60°C to 2.5- and 2.3-kb *Bam*HI fragments and only very slightly to the 3.8-kb *Bam*HI fragment which contains *pecB* (data not shown). The *pec-1* gene was therefore used to screen the genomic library of *A. flavus* 70. Strongly hybridizing plaques were isolated and analyzed further. *Bam*HI fragments, 2.5 and 2.3 kb, were isolated from one clone and subcloned into pBluescript KS+ to create pCFC41 and pCFC12, respectively. These were restriction mapped, and the regions of hybridization to *pec-1* were determined by Southern analysis (Fig. 1B). These two clones were assessed to contain one putative endopolygalacturonase gene, termed *pecA.*

Sequence analysis of *pecA* and *pecB*. The nucleotide sequence of *pecA* (Fig. 2) and *pecB* (Fig. 3) were determined by using the subcloning strategy outlined in Fig. 1. The coding

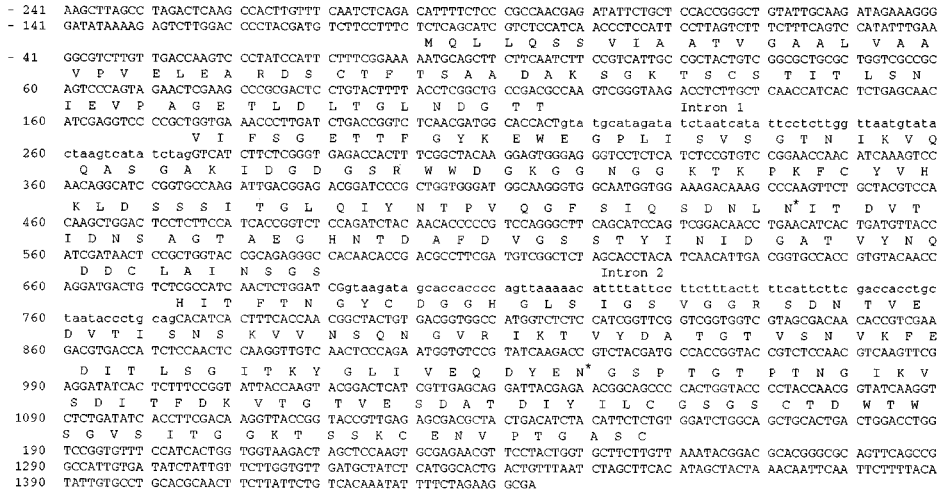

FIG. 2. Nucleotide sequence of region containing *A. flavus pecA* and deduced amino acid sequence of the encoded protein. The amino acid sequence is shown in single-letter code above the corresponding codons. Intron sequences are in lowercase letters. Potential N glycosylation sites are indicated by asterisks.

region of *pecA* consists of 1,228 bp (including introns) with the potential of encoding a protein of 363 amino acids with a molecular mass of 37,650 Da. Potential N glycosylation sites are found at Asn-162–Ile–Thr-164 and Asn-294–Gly–Ser-296. The optimum site for cleavage of the signal peptide is at Ala-16 according to the rules of von Heijne (45). The coding region of *pecA* is interrupted by two introns with lengths of 58 bp (intron 1) and 81 bp (intron 2) which are bounded by GTA--TAG and GTA--CAG, respectively. These conform to the consensus for the boundaries of fungal introns as determined by Gurr et al. (18). Additionally, the internal conserved sequence of filamentous fungal introns, PyGCTAAC, is present in both introns: TACTAAG (intron 1) and TGCTAAT (intron 2).

The coding region of the *pecB* gene is 1,217 bp (including introns) long interrupted by two introns with lengths of 65 bp (intron 1) and 54 bp (intron 2). Again, these contain the conserved splice sites of GTA--TAG and GTA--CAG characteristic of fungal introns. However, the internal intron recognition sequence is not so highly conserved, with sequences of AGTTAAT and ATATTAC being observed for intron 1 and intron 2, respectively. The *pecB* gene encodes in a protein of 366 amino acids with a predicted molecular mass of 38,146 Da. A signal sequence cleavage site is predicted at Ala-17 (45). One potential N glycosylation site is found at Asn-278–Ile–Thr-280.

No recognizable TATAA or CAAT motif was observed in the 5′ region of *pecA*. However, sequences of TATACA and CAAAT were observed at −57 and −108, respectively, for *pecB*. Both genes obeyed the translation start rule of having a purine, normally an A, at the −3 position, to direct efficient ribosomal binding (26).

Comparisons of endopolygalacturonase sequences. The nucleic acid of the coding region and predicted amino acid sequences of *pecA* and *pecB* were compared with other fungal endopolygalacturonase sequences, by using the alignment algorithms of the GeneWorks analysis program (IntelliGenetics) (Fig. 4). The degree of nucleic acid identity ranged from 55 to 65% for the majority of genes analyzed, with some notable exceptions. The nucleotide identity between the *A. flavus pecA* gene and the *A. oryzae* gene encoding polygalacturonase (PG) and *A. parasiticus pec-1* gene was very high, 99 and 96%, respectively. Also, the regions 5′ (240 bp) and 3′ (180 bp) to the coding sequence of these genes demonstrated exceptional levels of identity with the *pecA* gene of *A. flavus. pecA* was 99% identical to the *A. oryzae* PG gene and 92% identical to *A.*

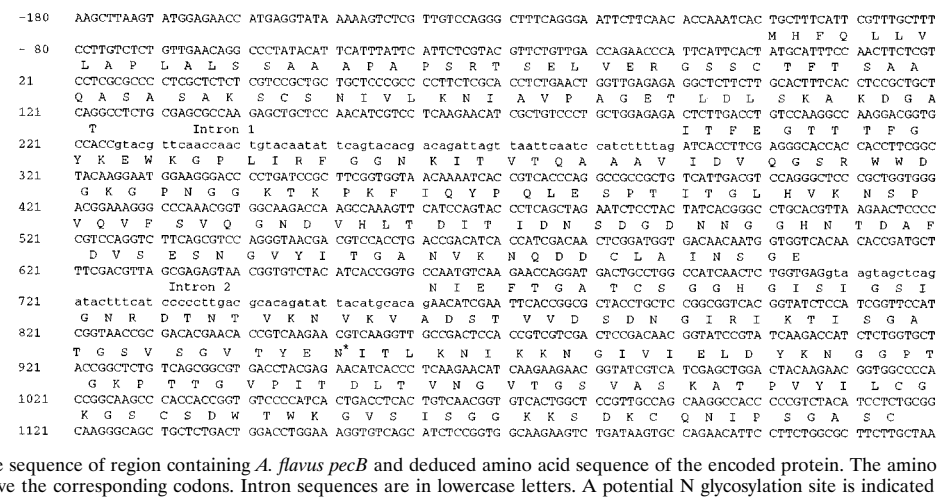

FIG. 3. Nucleotide sequence of region containing *A. flavus pecB* and deduced amino acid sequence of the encoded protein. The amino acid sequence is shown in single-letter code above the corresponding codons. Intron sequences are in lowercase letters. A potential N glycosylation site is indicated by an asterisk.

	A.flavus pec A	*A. oryzae*	*A. parasiticus*	*A. niger Pga II*	*A.tubigensis*	*A.niger pga I*	*A.flavus pec B*	*S.sclerotiorum*	*C.carbonum*	*A.niger Pga C*	*F. moniliforme*
A.flavus pec A		99	96	65	64	67	67	63	67	59	55
A. oryzae	98		96	65	64	67	67	63	67	59	55
A. parasiticus	97	97		65	64	66	65	62	67	60	56
A. niger Pga II	59	59	60		90	63	62	58	65	57	55
A.tubigensis	61	61	62	94		64	63	57	64	55	55
A.niger pga I	61	60	61	58	58		76	61	65	60	55
A.flavus pec B	58	57	58	57	58	67		60	67	59	51
S.sclerotiorum	58	58	59	56	59	54	54		64	59	52
C.carbonum	61	62	63	58	58	56	63	61		58	57
A.niger Pga C	54	54	56	50	51	56	53	55	53		46
F. moniliforme	46	46	45	41	41	39	42	43	47	38	

N.A. / A.A.

FIG. 4. Percentage identity of nucleotide and deduced amino acid sequences of 11 fungal endopolygalacturonase genes. The sequences were compared in all pairwise combinations from *A. oryzae* (9), *A. parasiticus* (9), *A. niger* (5–7), *S. sclerotiorum* (37), *C. carbonum* (46), *F. moniliforme* (8), and *A. flavus* (this study). N.A., nucleotides; A.A., amino acids.

parasiticus pec-1. High levels of nucleotide identity were also observed between the *A. flavus pecB* gene and the *A. niger pga-1* gene, levels much higher (76%) than that observed between the *A. niger* endopolygalacturonase genes (57 to 63%) or between the *pecA* and *pecB* genes of *A. flavus* (67%). The nucleic acid identity observed between the 5′ regions of *pga-1* and *pecB* (62%) was unusually high compared with the degree of identity in the same region between *pecA* and *pecB* (34%).

Gross structural similarities between the fungal endopolygalacturonase genes were analyzed. Certain introns of the *A. niger*, *A. flavus*, and *A. parasiticus* genes are conserved in the same position (Fig. 5). The introns for the *A. oryzae* PG gene were identical in sequence and position to those of the *A. flavus pecA* gene. Thus, according to the endopolygalacturonase sequence data available, all the genes possess introns 1 and 2, except for *A. niger pga-II*, which lacks intron 1. The *A. niger pgaC* gene has a third intron not found in other genes.

Expression analysis of *pecA* and *pecB*. The *pecB* gene transcript was found to accumulate in the presence of pectin only when glucose was absent. A 1.2-kb transcript accumulated in mycelia of strain 70 when grown in pectin medium but was completely absent when *A. flavus* was grown in either glucose or glucose and pectin (Fig. 6A). This pattern was observed for both strains 70 and 12.

The *pecA* gene product (1.3 kb) was found to accumulate in both pectin- and glucose-grown mycelia for *A. flavus* 70 (Fig. 6B). However, the level of transcript accumulation when cells were grown in pectin was increased compared with the glucose levels. *pecA* mRNA was not detected in *A. flavus* 12 grown in either pectin or glucose (data not shown).

Production of P2c by introducing active *pecA* into a weakly aggressive strain. To directly determine whether *pecA* encodes P2c, a plasmid containing the *pecA* gene (pCFC80) was introduced into strain 12 (weakly aggressive), and P2c production was examined. The *pecA* gene was introduced into *niaD* mutants of *A. flavus* 12 by cotransformation with a plasmid containing the *niaD* gene and a plasmid containing the *pecA* gene (pCFC80) through polyethylene glycol-mediated transformation. Transformants were then screened for their ability to produce P2c when grown in medium containing glucose. Pectinase activities were detected by the cup-plate assay (13). Several transformants potentially expressing P2c and several others not expressing P2c were further evaluated for pectinase activity by using a pectin agarose overlay of isoelectric focusing gels (15). While the recipient parental strain and negative (not expressing P2c) transformants showed only PE bands, both PE and P2c bands were detected in the positive (expressing P2c) transformants (Fig. 7). To check the genotypes of these transformants, genomic DNA from positive and negative transformants was digested with *Hin*dIII and hybridized with *pecA* (Fig. 8). As predicted from the restriction map of *pecA* (Fig. 1B), three fragments (1.1, 2.3, and 2.5 kb) were detected in strain 70 (Fig. 8A, lane 1). On the other hand, hybridization of *pecA* to *Hin*dIII-digested DNA from strain 12 detected two of the same three bands (1.1 and 2.5 kb), with the exception that the probe hybridized to a 2.9-kb band instead of a 2.3-kb band (Fig. 8A, lanes 2 and 3). This polymorphism was valuable in establishing the addition of the *pecA* gene from strain 70 to strain 12. All transformants tested expressing P2c contained both the 2.3- and 2.9-kb bands, while only the 2.9-kb band was detected in the negative transformants (Fig. 8B). The additional bands detected in two of the transformants expressing P2c suggest multiple integrations of the *pecA* gene (Fig. 8B, lanes 2 and 3).

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A. flavus pecA                GTCTCAACGA TGGCACCACT 216  Intron 1 275 GTCATCTTCT CGGGTGAGAC
A. parasiticus pec1           GTCTCAACGA TGGCACCACT 216  Intron 1 269 GTCATCTTCT CGGGTGAGAC
A. niger pgaII                GTCTCACCAG CGGTACCAAG 213----------214 GTCATCTTCG AGGGCACCAC
A. niger pgaI                 ATGCTGCTGA TGGCTCCACC 228  Intron 1 281 ATCACCTTCG AGGGCACCAC
A. flavus pecB                AGGCCAAGGA CGGTGCCACC 225  Intron 1 291 ATCACCTTCG AGGGCACCAC
A. niger pgaC                 ACCTGAATGA TGGAACCCAC 264  Intron 1 340 GTGATCTTCC AGGGAGAAAC

A. flavus pecA                TCGCCATCAA CTCTGGATCG 691  Intron 2 773 CACATCACTT TCACCAACGG
A. parasiticus pec1           TCGCCATCAA CTCTGGATCG 685  Intron 2 762 CATATCACTT TCACCAACGG
A. niger pgaII                TTGCGGTTAA CTCTGGCGAG 630  Intron 1 683 AACATCTGGT TCACCGGCGG
A. niger pgaI                 TTGCCATCAA CTCTGGCGAG 700  Intron 2 763 AGCATCTCTT TCACCGGCGG
A. flavus pecB                TGGCCATCAA CTCTGGTGAG 707  Intron 2 763 AACATCGAAT TCACCGGCGC
A. niger pgaC                 TTGCCATCAA TTCTGGAGAG 765  Intron 2 822 AACATTTATT TCAGTGCCAG

A. flavus pecA                GGTTGTCAAC TCCCAGAATG 902----------903 GTGTCCGTAT CAAGACCGTC
A. parasiticus pec1           GGTTGTCAAC TCTCAGAACG 891----------892 GTGTCCGTAT CAAGACCGTC
A. niger pgaII                CGTGAGCAAT TCCGAAAACG 812----------813 CCGTCCGAAT TAAGACCATC
A. niger pgaI                 TGTCAGCAAC TCCGCCAACG 892----------893 GTGTCCGCAT CAAGACCATC
A. flavus pecB                CGTCGTCGAC TCCGACAACG 892----------893 GTATCCGTAT CAAGACCATC
A. niger pgaC                 TGTTCTCAAG TCCCAGCAAG 951  Intron 3 1005 CAATCCGTAT CAAGACCATC
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FIG. 5. Alignment of intron positions for endopolygalacturonase genes from *Aspergillus* species. The sequences aligned are from *A. parasiticus* (9), *A. niger* (5–7), and *A. flavus* (this study). Where present, introns are located in the same position in the nucleotide sequence.

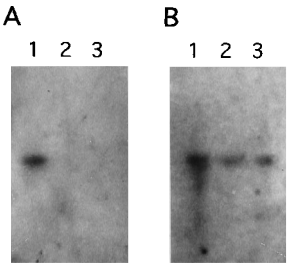

FIG. 6. Transcript accumulation profiles of *pecA* and *pecB* in *A. flavus* 70 grown on various carbon sources. A 3.8-kb *Bam*HI fragment containing the *pecB* gene (A) and a 2.5-kb *Bam*HI fragment containing part of the *pecA* gene (B) were radiolabelled and hybridized to total RNA (10 μg per lane) isolated from *A. flavus* 70 grown on pectin (lane 1), pectin and glucose (lane 2), or glucose (lane 3).

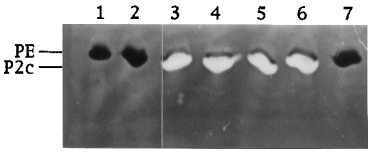

FIG. 7. Pectinase activities in culture filtrates of transformants grown in pectin medium. Activities were examined by pectin-agarose overlay of isoelectric focusing gels. Lanes: 1 and 2, culture filtrates from transformants (7-82-11 and 6-10-19) not expressing P2c; 3 to 5, culture filtrates from transformants (7-82-14, 9-10-14, and 9-10-3) expressing P2c; 6 and 7, culture filtrates of strain 70 and strain 12, respectively.

DISCUSSION

Two genes, *pecA* and *pecB*, encoding endopolygalacturonases were cloned from a highly aggressive strain of *A. flavus*. One gene, *pecB*, was found in both weakly and highly aggressive strains of *A. flavus* and was expressed at apparently similar levels in both organisms. This gene probably encodes either the P1 or P3 activity, because the transcript accumulates only in mycelia grown in medium containing pectin alone. The transcript of *pecA* was found to accumulate in mycelia grown in pectin- and glucose-containing medium, although the level of expression in pectin-grown mycelia was elevated compared with the level in glucose-grown cells. Expression of *pecA* was not detected in the weakly aggressive strain under any conditions. A *pecA* homolog was detected in the weakly aggressive strain by Southern hybridization; however, no transcript was detected. The *Hin*dIII polymorphism (2.3 versus 2.9 kb) between the two strains indicates differences in DNA composition and may account for the lack of *pecA* expression in the weakly aggressive strain. These data are consistent with *pecA* encoding the pectinase P2c. Further evidence that *pecA* encodes P2c is provided by transformation studies. A pectinase with the same activity profile as P2c was detected in the weakly aggressive strain when the *pecA* gene from an aggressive strain was introduced.

The expression of *pecA* was increased by the addition of pectin; hence, this gene retains a mechanism for pectin induction as observed for the expression of *pecB*. The *pecB* transcript did not accumulate in mycelia grown on neutral carbon sources, such as mannitol (results not shown), indicating that only pectin will induce the expression of *pecB*. However, *pecA* is expressed at similar levels in cells grown in glucose and in mannitol. Thus, its promoter contains elements for constitutive expression in the absence of pectin and also elements that enhance transcription in the presence of pectin.

There are a number of possible explanations for this finding. The low-level constitutive production of extracellular enzymes by plant-pathogenic organisms in the absence of an inducer has been postulated to be important for infection (25, 32) and may enable some plant pathogens to be primed to infect their hosts, quickly degrading and overcoming the mechanical barriers to infection. Breakdown products of the structural components of the plant cell wall are rapidly produced and further act to induce expression of extracellular enzymes, facilitating the infection process. The isolation of *pecA* provides an excellent opportunity to study this mechanism in a plant-pathogenic organism.

The elucidation of the mechanisms regulating the expression of *pecA* also may be useful to the industrial production of pectinases. *A. niger* strains are employed as the main industrial producer of pectinases for use in the food industry. Considerable effort has been placed on increasing the production of pectinases in culture. If the mechanism by which *pecA* produces pectinase in the presence of glucose is determined, it may be possible to genetically alter native *A. niger* pectinase genes or introduce the *A. flavus pecA* gene and increase the productivity of pectinase manufacturers.

The analysis of *pecA* and *pecB* revealed an extremely high degree of sequence identity between *A. flavus pecA*, the *A. oryzae* PG gene, and *A. parasiticus pec-1*. These organisms are all part of the *A. flavus* group of fungi, which exhibits a high degree of variation (42). The question of speciation within this group has been intensely debated and is unsettled (12, 16, 22–24, 27, 33–36).

An extremely high degree of nucleotide identity has been found in other genes in the *A. flavus* group of fungi. The *nor-1* genes of *A. flavus* and *A. parasiticus* (4a) as well as the *apa-2* gene of *A. parasiticus* and the *aflR* gene of *A. flavus* are approximately 95% identical (11). On the basis of DNA reassociation experiments, the degree of complementarity between *A. flavus* and *A. oryzae* is 100% (29). Perhaps it is not surprising that, as shown here, the *A. flavus pecA* and *A. oryzae* PG genes are essentially identical. These observations are consistent with arguments that species in the *A. flavus* group should be reduced to the subspecies level (29).

The conservation of intron positions between *A. niger*, *A. flavus*, and *A. parasiticus* endopolygalacturonase genes indicates that these organisms and endopolygalacturonase genes

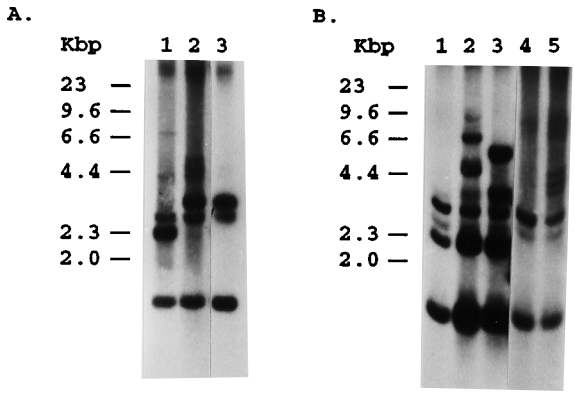

FIG. 8. Hybridization of *pecA* to strain 12 transformants. (A) A 2.3-kb *Bam*HI fragment from pCFC12 was radiolabelled and hybridized to *Hin*dIII-digested strain 70 (lane 1), strain 12 (lane 2), and a *niaD* mutant of strain 12 (lane 3). (B) The probe described for panel A was hybridized to three transformants expressing P2c, 7-82-14, 9-10-3, and 9-10-14 (lanes 1 to 3, respectively) and two transformants not expressing P2c, 6-10-19 and 7-82-11 (lanes 4 and 5, respectively).

share a common ancestor in their phylogeny. This finding and the high level of nucleotide identity seen between the *A. flavus pecB* gene and the *A. niger pgaI* gene suggest that these are analogous genes. Duplication events that caused a number of endopolygalacturonase genes to be present in the genome of a common ancestor may have occurred before the divergence of *A. niger* from *A. parasiticus* and *A. flavus.*

The cloning and characterization of *pecA* and *pecB* from *A. flavus* now makes it possible to determine the role that pectinases, P2c (encoded by *pecA*) in particular, play in the infection of cotton bolls. As shown here, we have isolated transformants from a weakly aggressive strain which contain the *pecA* gene and produce P2c activities in vitro. In addition, transformants are being constructed from highly aggressive strains which contain a disrupted and therefore inactive *pecA* gene. Studies of the abilities of those strains to infect cotton bolls should provide further information on the genetic basis for the role of P2c in fungal pathogenesis. Other experiments are under way to determine the control mechanisms for endopolygalacturonase expression. These results will enhance our understanding of the role of pectinases in plant-pathogen interactions and provide further information for the manipulation of pectinase production by industrial filamentous fungi.

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