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Cloning and Partial Characterization of Endopolygalacturonase Genes from *Botrytis cinerea*

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***Botrytis cinerea* is a plant-pathogenic fungus infecting over 200 different plant species. We use a molecular genetic approach to study the process of pectin degradation by the fungus. Recently, we described the cloning and characterization of an endopolygalacturonase (endoPG) gene from *B. cinerea* (*Bcpg*1) which is required for full virulence. Here we describe the cloning and characterization of five additional endoPG-encoding genes from *B. cinerea* SAS56. The identity at the amino acid level between the six endoPGs of *B. cinerea* varied from 34 to 73%. Phylogenetic analysis, by using a group of 35 related fungal endoPGs and as an outgroup one plant PG, resulted in the identification of five monophyletic groups of closely related proteins. The endoPG proteins from *B. cinerea* SAS56 could be assigned to three different monophyletic groups. DNA blot analysis revealed the presence of the complete endoPG gene family in other strains of *B. cinerea*, as well as in other *Botrytis* species. Differential gene expression of the gene family members was found in mycelium grown in liquid culture with either glucose or polygalacturonic acid as the carbon source.**

Botrytis cinerea Pers.:Fr. *Botryotinia fuckeliana* (de Bary) Wetz., also known as the gray mold fungus, is a plant pathogen infecting more than 200 different plant species, including many economically important crops (18). Primary infection often involves invasion of weak, damaged, or senescent tissues. After the initial establishment in the host, the fungus spreads throughout the plant causing severe damage by tissue maceration. During all stages of infection the fungus produces a spectrum of cell-wall-degrading enzymes (CWDEs), among which are several pectin-degrading enzymes such as pectin methyl esterase, pectin lyase, and a number of different polygalacturonases (PGs) (23, 26, 30). Although much biochemical research has been performed, the importance of these enzymes for pathogenesis of *B. cinerea* was not well understood until recently (13, 22, 34). We set out a molecular genetic approach to study this process.

Recently we described the cloning and characterization of an endopolygalacturonase (endoPG) from *B. cinerea* (*Bcpg*1) (38). Elimination of this gene resulted in a mutant with reduced virulence on different hosts, indicating that CWDEs can be involved in pathogenesis. Here we describe the cloning of five additional endoPG genes from *B. cinerea* and the partial characterization of the gene family.

MATERIALS AND METHODS

Fungal strains and culturing methods. *B. cinerea* strains used in this study are indicated in Table 1. Isolates of four other *Botrytis* species were also used as indicated in Table 1. Fungal strains were grown on malt extract agar (Oxoid, Basingstoke, United Kingdom) at 20°C. For liquid cultures conidia were harvested from 10-day-old plates and used to inoculate Gamborg's B5 medium (Duchefa Biochemie BV, Haarlem, The Netherlands) supplemented with 1% (wt/vol) glucose and 10 mM $(NH_4)H_2PO_4$. Cultures were incubated in a rotary shaker at 180 rpm and 20°C. Depending on the growth rate of the different strains and isolates used, cultures were grown for between 16 and 48 h postinoculation prior to harvesting the mycelium.

DNA recombinant techniques. Standard DNA recombinant protocols were used as described before (32). Host strains used were *Escherichia coli* LE392 for λEMBL3 phages and *E. coli* DH5α for plasmid propagation. The plasmid vectors pBluescript II SK/KS (Stratagene, La Jolla, Calif.) and pGEM-T-Easy (Promega, Madison, Wis.) were used for DNA fragment cloning.

DNA blot analysis. DNA was isolated as described previously (25), digested with *Eco*RI or *Hin*dIII (1 μg), separated on a 0.7% (wt/vol) agarose gel, and subsequently alkali blotted onto Hybond N^+ membranes according to the manufacturer's instructions (Amersham). Membranes were hybridized as described earlier (41) at 65 and 55°C for high and low stringency, respectively. High-stringency hybridizations were followed by washing in 0.3 M NaCl plus 0.03 M sodium citrate (pH 7.0) (2× SSC)–0.1% (wt/vol) sodium dodecyl sulfate (SDS), 0.5× SSC–0.1% (wt/vol) SDS, and 0.2× SSC–0.1% (wt/vol) SDS at 65°C for 30 min each. Low-stringency hybridizations were followed by washing in 2× SSC–0.1% (wt/vol) SDS and then 0.5× SSC–0.1% (wt/vol) SDS at 55°C for 15 min each. Autoradiographs were made by 96-h exposure of Kodak-LS/Kodak-AR films at −70°C with one intensifying screen. The following fragments were used for probe preparation for the different genes (numbers indicate the distances of restriction sites from the translation start site): *Bcpg*1, *Pst*I-*Bam*HI (+161, +862); *Bcpg*2, *Nco*I-*Eco*RI (+858, +1413); *Bcpg*3, *Kpn*I-*Kpn*I (+766, +1313); *Bcpg*4, *Bam*HI-*Bam*HI (+384, +891); *Bcpg*5, *Bgl*II-*Hin*dIII (+92, +1072); and *Bcpg*6, *Cla*I-*Cla*I (+308, +980).

Medium shift and RNA blot analysis. The expression of the endoPG gene family was analyzed on two different carbon sources. Gamborg's B5 medium supplemented with 1% (wt/vol) glucose, 0.05% yeast extract, and 10 mM NaH_2PO_4-Na_2HPO_4 (pH 6.0) was inoculated with 10^6 conidia ml^{-1} as described above. After 16 h of growth in a rotary shaker at 180 rpm and 20°C, the mycelium was harvested by using Miracloth (Calbiochem, La Jolla, Calif.) and washed thoroughly with Gamborg's B5 medium supplemented with 10 mM NaH_2PO_4-Na_2HPO_4 (pH 6.0). Wet mycelium was transferred to fresh Gamborg's B5 medium with 10 mM NaH_2PO_4-Na_2HPO_4 (pH 6.0) and supplemented with either 1.0% (wt/vol) glucose or 1.0% polygalacturonic acid (U.S. Biochemical Corp., Cleveland, Ohio). After transfer, the fungus was grown for 6, 12, 24, and 30 h prior to harvest of the mycelium by using Miracloth. The harvested mycelium was blotted dry on filter paper, quickly frozen in liquid nitrogen, and stored at −80°C prior to extraction of the RNA. RNA was extracted from frozen mycelium by using the Trizol reagent (Life Technologies, Inc., Gaithersburg, Md.). Then, 10 μg of total RNA was denatured by using glyoxal as described before (32) separated on 1.2% (wt/vol) agarose gel and blotted onto Hybond N membranes with 10× SSC according to the manufacturer's instructions (Amersham). Membranes were hybridized as described previously (41) at 65°C and washed with 2× SSC–0.1% (wt/vol) SDS (two times for 30 min) and 0.5× SSC–0.1% (wt/vol) SDS (30 min). Autoradiographs were made by exposure of Kodak-LS/Kodak-AR films at −70°C with two intensifying screens. DNA fragments used for probe preparation were similar to those described above for DNA blot analysis. A *B. cinerea* 27S ribosomal fragment (kindly provided by Theo

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TABLE 1. Strains of *B. cinerea* and other *Botrytis* species used

Strain or isolate	Type	Mating type	Origin
Strains			
SAS56	Monoascospore isolate	*MAT1-1*	Italy (of parental strains)
SAS405	Monoascospore isolate	*MAT1-2*	Italy (of parental strains)
B05.10	Haploid derivative SAS56	ND[c]	Italy (of parental strains)
Isolates[a]			
Bc7 (tomato, 1970)			The Netherlands
Bc12 (gerbera, 1986)			The Netherlands
Bc21 (rose, 1990)			The Netherlands
Bc29 (gerbera, 1991)			The Netherlands
J13 (*Vitis vinifera*, before 1985)			Jena, Germany
M15 (*Vitis vinifera*, 1993)			Freiburg, Germany
Other species[b]			
B. aclada (onion)			
B. gladiorum (gladiolus)			
B. paeoniae (peony)			
B. squamosa (onion)			

[a] The host from which the isolate was obtained and the year of isolation are indicated in parentheses.
[b] The known host is indicated in parentheses.
[c] ND, not determined.

Prins, Laboratory of Phytopathology, Wageningen Agricultural University, The Netherlands) was used to demonstrate equal loading of the gels.

Screening of genomic library. A genomic library (λEMBL3) of *B. cinerea* SAS56 (kindly provided by Theo Prins) was screened (10^5 phages) with an internal *Pst*I/*Bam*HI fragment (0.7 kb) of *Bcpg*1 as a probe. Hybridizations and washings were performed as described earlier (41) at 60°C and resulted in the isolation of positive phages. Hybridizing fragments of these phages were subcloned into pBluescript II SK/KS plasmids and further characterized by restriction analysis and Southern hybridizations. This resulted in the identification of different classes of hybridizing clones. Within each class, DNA fragments were further characterized by sequence analysis.

Nucleotide sequence analyses. Sequencing reactions were performed by using the ThermoSequenase fluorescent-labelled primer cycle sequencing kit (Amersham) with universal sequencing primers and the Cy5 Autoread Sequencing kit (Pharmacia Biotech, Uppsala, Sweden) with gene-specific oligonucleotides. The sequencing reactions were analyzed on an ALF Express sequencer (Pharmacia Biotech). Nucleotide sequence data were analyzed by using the Lasergene Biocomputing Software for Windows (DNASTAR, Inc., Madison, Wis.). BLAST database searches were performed by using the National Center for Biotechnology Information BLAST WWW server. Phylogenetic analyses were performed with PAUP 3.1 (35).

Nucleotide sequence accession numbers. The nucleotide sequences for the endoPG-encoding genes of *B. cinerea* are in the GenBank database under accession numbers U68715 (*Bcpg*1), U68716 (*Bcpg*2), U68717 (*Bcpg*3), U68719 (*Bcpg*4), U68721 (*Bcpg*5), and U68722 (*Bcpg*6).

RESULTS

Cloning of the *Bcpg* gene family members encoding endoPGs. The isolation and characterization of the *Bcpg*1 gene has been described (38). Southern analysis of genomic DNA of *B. cinerea* SAS56, with the *Bcpg*1 gene as a probe at low-stringency conditions, revealed the presence of at least three additional genes that are homologous to the *Bcpg*1 gene (38). Screening of the genomic library of *B. cinerea* (SAS56) with the *Bcpg*1 gene as a probe resulted in the identification of numerous positive phages, of which 100 hybridizing phages were chosen randomly. A total of 21 clones appeared to contain the *Bcpg*1 gene as determined by PCR analysis. Eighteen hybridizing phage clones (not containing *Bcpg*1) were further characterized by using restriction, Southern, and nucleotide sequence analyses. The phages were assigned to five groups of (overlapping) clones, each covering a separate region of the *B. cinerea* genome. This resulted in the identification of five additional *Bcpg* genes (*Bcpg*2 to -6). The complete nucleotide sequences of these genes have been determined and deposited in GenBank.

Genomic organization of the endoPG gene family. Intron positions in the nucleotide sequences were predicted based on codon usage by using the program Testcode (14) and amino acid sequence alignment with homologous fungal endoPGs from other species. Between 1 and 4 introns are present in the different *Bcpg* genes, exception for *Bcpg*1, which is intron-less (Fig. 1). The position of intron A of *Bcpg*3 was confirmed by sequencing of the reverse transcriptase PCR (RT-PCR) products (37). Indirect evidence for the presence of introns was provided for *Bcpg*2 (intron C), *Bcpg*4 (introns A and B), *Bcpg*5 (introns B and C), and *Bcpg*6 (intron B) by RT-PCR with primers specifically annealing to regions flanking either side of the intron (37). The border sequences of the introns in the *Bcpg* genes and the internal consensus for lariat formation corresponded with previously reported 5′ and 3′ splice sites in the fungal genes (39). The introns in the *Bcpg* genes varied in size between 46 and 60 nucleotides. Conservation of intron positions was only observed for *Bcpg*2 (intron C), *Bcpg*4 (intron B), and *Bcpg*5 (intron B) (Fig. 1).

Analysis of the deduced amino acid sequences. The amino acid sequences were deduced from the predicted open reading frames present in the genomic sequences of each of the five endoPG genes. The predicted endoPG proteins ranged in length from 371 to 515 amino acids (Fig. 1). All protein sequences contain a predicted signal sequence as determined according to the method of Nielsen et al. (27). Analogous to *Aspergillus niger* endoPGs, monobasic (Arg) and dibasic (Lys-Arg) cleavage sites (3) were present in most of the *Botrytis* endoPGs (Arg for BcPG1 and BcPG2; Lys-Arg for BcPG4 and BcPG5). The functionality of the cleavage sites remains to be confirmed by sequencing of the N terminus of the processed proteins. For BcPG6 no apparent propeptide cleavage site could be predicted. The BcPG3 structure is different from the other five genes. The protein is enlarged by the predicted presence of an N-terminal extension of approximately 150 amino acids. BcPG3 contains a predicted signal peptide of 16 amino acids (27) but no putative mono- or dibasic cleavage sites. Sequence identity between the unprocessed endoPGs

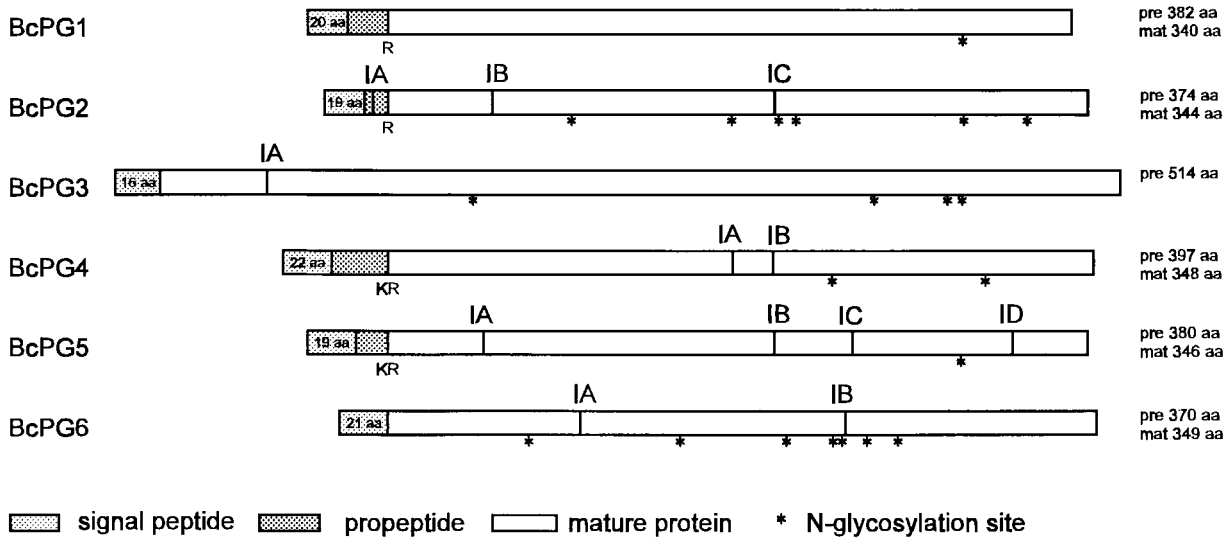

FIG. 1. Genomic organization of the endopolygalacturonase gene family of *B. cinerea*. Indicated are the positions of the introns in the original DNA sequence (IA, IB, IC, or ID), the presence of a putative monobasic (R) or dibasic (KR) cleavage sites, and the presence of *N*-glycosylation signals (*). Also depicted in the figure are the derived lengths of unprocessed proteins (pre) and mature processed proteins (mat). The lengths of predicted signal peptides for each of the proteins are indicated in the respective boxes.

varied between 34 and 73% (Table 2). Nine amino acid residues which are strictly conserved in all PGs (3) are present in each of the *Botrytis* endoPGs (also mentioned in reference 38). The presence of N-linked glycosylation signals in all of the endoPGs of *B. cinerea* (Fig. 1) indicates that they might be excreted as glycosylated enzymes, as is the case for *A. niger* (43).

Protein sequence alignment of fungal endoPGs. BLAST protein sequence similarity searches were performed by using deduced amino acid sequences of the six endoPG-encoding genes of *B. cinerea*. The striking homology between amino acid sequences of the *Botrytis* endoPGs and those from other filamentous fungi prompted us to perform a detailed phylogenetic analysis with a large number of fungal endoPGs. Related fungal endoPGs (Table 3) were used to generate a protein sequence alignment which was used for phylogenetic analysis. A general heuristic search was performed by using PAUP 3.1, and clade stability was assessed by bootstrap replications. Gaps in the alignment were treated as missing values. The *Arabidopsis thaliana* PG (GBGATHAL) was used as an outgroup (Fig. 2). Figure 2A shows the consensus tree generated from the three most parsimonious trees found in the analysis. Figure 2B shows one of the three most parsimonious trees. The phylogenetic analysis indicated the presence of several groups of related PGs as predicted from the BLAST protein sequence similarity searches. Five different monophyletic groups were distinguished, each containing a minimum of three endoPGs originating from more than one fungal species. Among the fungal species represented in the tree, several possessed PGs belonging to more than one group: for example, *A. niger* (groups I, II, IV, and V), *Sclerotinia sclerotiorum* (groups III and IV), *Aspergillus flavus* (groups I and II) and *B. cinerea* (groups III, IV, and V). With respect to the endoPGs of *B. cinerea*, BcPG1 belongs to group III together with three endoPGs of *S. sclerotiorum*, with sequence identities of around 90%. BcPG3 and BcPG6 cluster with PGD (*A. niger*) and several endoPGs isolated from different *Fusarium* species (group V). PGD (28) and BcPG3 are distinct from most endoPGs because of the presence of an N-terminal extension of approximately 150 amino acids. BcPG4 and BcPG5 were assigned to group IV together with PG5 of *S. sclerotiorum* and PGC and PGE of *A. niger*. BcPG5 and PG5 of *S. sclerotiorum* are 89.5% identical at the amino acid level. BcPG2 was related to BcPG1 and BcPG5; however, it was assigned to neither group III nor group IV but was assigned to a separate branch of the tree.

TABLE 2. Sequence pair distance of the endoPG family of *B. cinerea* as determined by the CLUSTAL method

endoPG	Sequence pair distance (% identity) of endoPG:				
	BcPG2	BcPG3	BcPG4	BcPG5	BcPG6
BcPG1	72.0	38.7	65.8	72.7	55.0
BcPG2		34.8	59.2	63.7	54.3
BcPG3			33.6	36.7	48.3
BcPG4				67.7	48.5
BcPG5					55.2

Presence of the endoPG gene family in different *B. cinerea* strains and *Botrytis* species. All genes have been cloned from a genomic library of strain SAS56 and might thus be present in only this particular strain. In order to examine the presence of the genes throughout the species *B. cinerea*, a high-stringency DNA blot analysis was performed with DNA obtained from nine different strains (Table 1). All strains tested showed a hybridizing fragment; however, some restriction fragment length polymorphisms were observed (Fig. 3). In addition, we performed a low-stringency DNA blot analysis with DNA isolated from *Botrytis aclada*, *Botrytis gladiorum*, *Botrytis paeoniae*, and *Botrytis squamosa*. All *Botrytis* species tested displayed at least one hybridizing fragment specific for each of the probes used (Fig. 4). For some probes (for example, *Bcpg*2) the signal was not strong but the observed hybridization pattern was distinct for each probe used. This excludes the occurrence of possible cross-hybridization between the different genes in this experiment, since blots used for the different hybridizations were identical. Apparently, homologues of the entire endoPG

TABLE 3. EndoPGs used for the construction of the protein sequence alignments

Organism	Protein	Accession number	Length (aa)[a]	Source or reference
Arabidopsis thaliana	GBGATHAL	S34266	445	Unpublished
Aspergillus flavus	PGAASPFL	P41749	363	42
	PGBASPFL	P41750	366	42
Aspergillus niger	PG1ASPNG	P26213	368	4
	PG2ASPNG	P26214	362	7
	PGAASPNG	Y18804	370	28
	PGBASPNG	Y18805	362	28
	PGCASPNG	X64356	383	5
	PGDASPNG	Y18806	495	28
	PGEASPNG	Y14386	378	29
Aspergillus oryzae	PGRASPOR	P35335	363	21
Aspergillus parasiticus	PGRASPPA	P49575	363	9
Aspergillus tubingensis	PG2ASPTU	P19805	362	6
Botrytis cinerea	BcPG1	U68715	382	38
	BcPG2	U68716	374	This study
	BcPG3	U68717	514	This study
	BcPG4	U68719	397	This study
	BcPG5	U68721	380	This study
	BcPG6	U68722	370	This study
Claviceps purpurea	CPPG1	Y10165	369	36
	CPPG2	Y10165	369	36
Cochliobolus carbonum	PGRCOCCA	P26215	364	33
Colletotrichum lindemuthianum	CLPG1END	X89370	363	10
	CLPG2END	X95475	366	11
Cryphonectria parasitica	CP47910	U49710	369	16
Fusarium moniliforme	PGRFUSMO	Q07181	373	8
Fusarium oxysporum	FOAB124	AB000124	371	Unpublished
	FOU96456	U96456	370	12
Kluyveromyces marxianus	KMAJ76	AJ000076	361	Unpublished
Saccharomyces cerevisiae	PGXSACSE	P47180	361	Unpublished
Sclerotinia sclerotiorum	PG1SCSCL	L12023	380	31
	PG2SCSCL	S62742	380	15
	PG3SCSCL	S63743	380	15
	PG5SCSCL	Y13669	387	Unpublished
Stereum purpureum	SPD072	D45072	404	24
Trichosporon penicillatum	TPD650	D89650	367	17

[a] aa, amino acids.

gene family found in *B. cinerea* are also present in other *Botrytis* species.

Expression of the *Bcpg* gene family on glucose and polygalacturonic acid. The expression of the *Bcpg* gene family members in *B. cinerea* was analyzed in a medium shift experiment in which the fungus was precultured on glucose-containing medium and transferred to medium supplemented with glucose or polygalacturonic acid as sole carbon sources (Fig. 5). RNA blot analysis revealed that each member of the gene family was expressed in liquid culture. High expression of the *Bcpg*1 gene was observed both on glucose and polygalacturonic acid; however, expression on glucose decreased after a longer incubation period (24 and 30 h posttransfer). The pattern of expression of the *Bcpg*2 gene was comparable to the *Bcpg*1 gene expression. Expression of the *Bcpg*3 gene was low at the time of mycelium transfer (lane S) but increased after prolonged periods of growth on glucose. On polygalacturonic acid hardly any expression of the *Bcpg*3 gene was observed. For *Bcpg*4, the highest expression was observed early after transfer to polygalacturonic acid. Expression of the *Bcpg*4 gene was low or not detectable on glucose. Expression of the *Bcpg*5 gene was observed on glucose and only at later time points on polygalacturonic acid. *Bcpg*6 expression was relatively low on glucose and an increased expression was observed after transfer to polygalacturonic acid. Three independent medium shift experiments resulted in the same expression pattern for each of the genes as shown in Fig. 5.

DISCUSSION

Over the last three decades, plant pathology research has focused on the identification of extracellular enzymes involved in fungal pathogenicity (1, 34). Among them were CWDEs, which include enzymes involved in pectin degradation. It was shown before that the broad-host-range pathogen *B. cinerea* produces a range of pectinolytic enzymes (19, 23, 26, 30), including up to 13 different PG isoforms (40). The complex pectinolytic system of *B. cinerea* prompted us to initiate a molecular genetic analysis to unravel the functional role of individual enzymes.

Six different endoPG genes were isolated from *B. cinerea* SAS56. The sequence identity at the amino acid level within the endoPG family of *B. cinerea* varied between 34 and 73%. Highly homologous proteins were found in other fungal species by using BLAST protein sequence similarity searches. The phylogenetic analysis performed with a group of 35 related endoPGs clearly resulted in the identification of distinct monophyletic groups of endoPGs originating from different species. This suggests that ancestor genes for these clusters existed prior to the divergence of these fungal species. It is also interesting to note that several species have endoPGs belonging to different monophyletic groups, while others produce only a single known PG. It is possible that other endoPG genes are present in some of these species but that they have not been found yet. The presence of three very closely related PGs

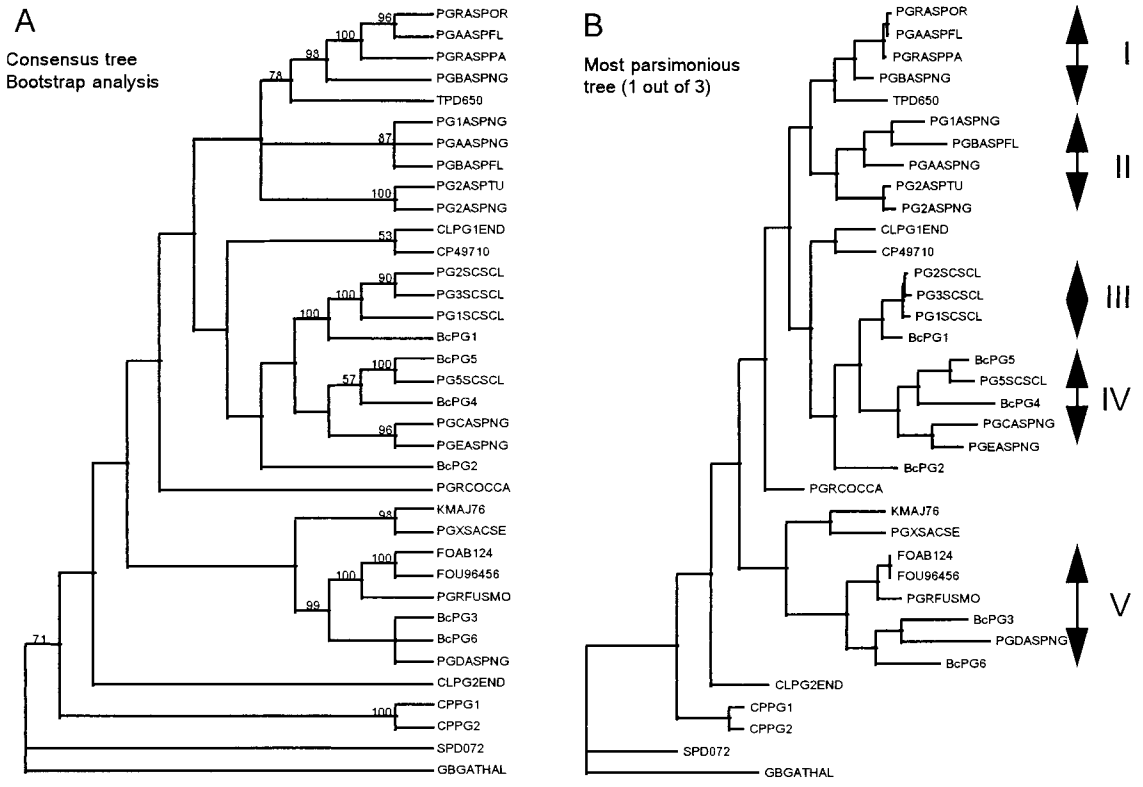

FIG. 2. Phylogenetic analysis of fungal endoPGs. The analysis was performed by using an optimal alignment generated from the PGs depicted in Table 3. Panel A shows the consensus tree derived from three most parsimonious trees calculated by using PAUP 3.1. The different values represent the percentage of occurrence obtained after bootstrap analysis (1,000 iterations) of the phylogenetic analysis. Panel B shows the one most parsimonious tree and identifies the different monophyletic groups that we defined as a result of the analysis. The abbreviations of protein names are indicated in Table 3.

(>98%) originating from a single species (*S. sclerotiorum*) in one group was explained as a recent gene duplication (15). The biological significance for the presence of more than one endoPG in *B. cinerea* is not known. Enzymes produced by the saprophytic fungus *A. niger* display considerable differences with respect to substrate specificity, cleavage rate, and optimal pH for activity (2, 20, 29). Information on endoPGs of other fungal species is required to show whether enzymes belonging to the same monophyletic group share biochemical properties which could also be related with a biological function.

We analyzed different *B. cinerea* strains and other *Botrytis* species for the presence of DNA homologous to the endoPG-encoding genes of *B. cinerea* SAS56. Without exception, each member of the gene family was present in all of the strains and species tested, although for some of the genes the hybridizing signal was not strong. Therefore, the presence of the endoPG gene family in *B. cinerea* is presumably not the sole explanation for its broad host range. The other *Botrytis* species tested can only infect a single host plant species, yet they contain the homologues of the complete gene family. Whether these genes

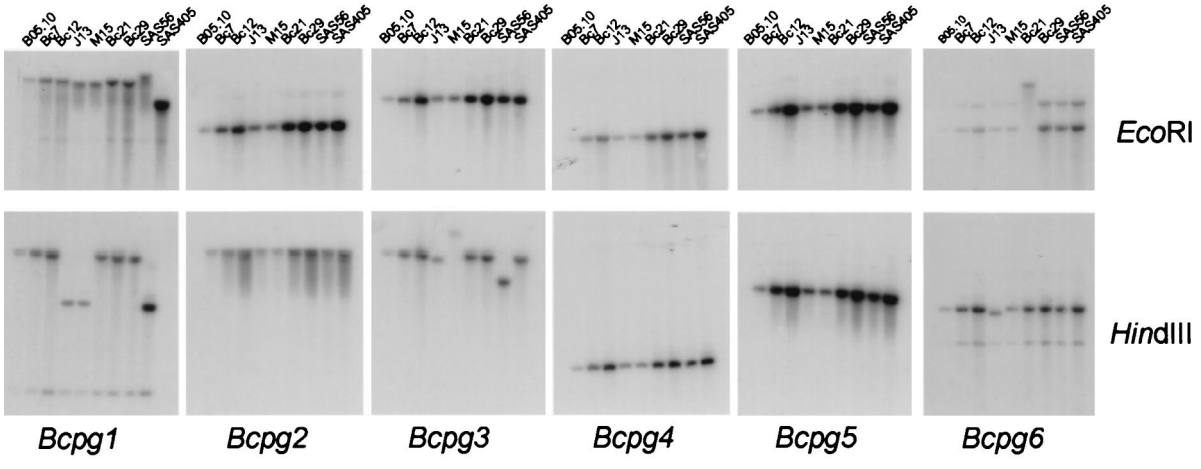

FIG. 3. Southern blot analysis of different strains of *B. cinerea* with the *Bcpg* genes as a probe. Fungal DNA isolated from the different strains of *B. cinerea* was digested with *Eco*RI (top panel) or *Hin*dIII (lower panel) and subjected to Southern hybridization with gene-specific probes (*Bcpg*1 to -6) under high-stringency conditions as described in Materials and Methods.

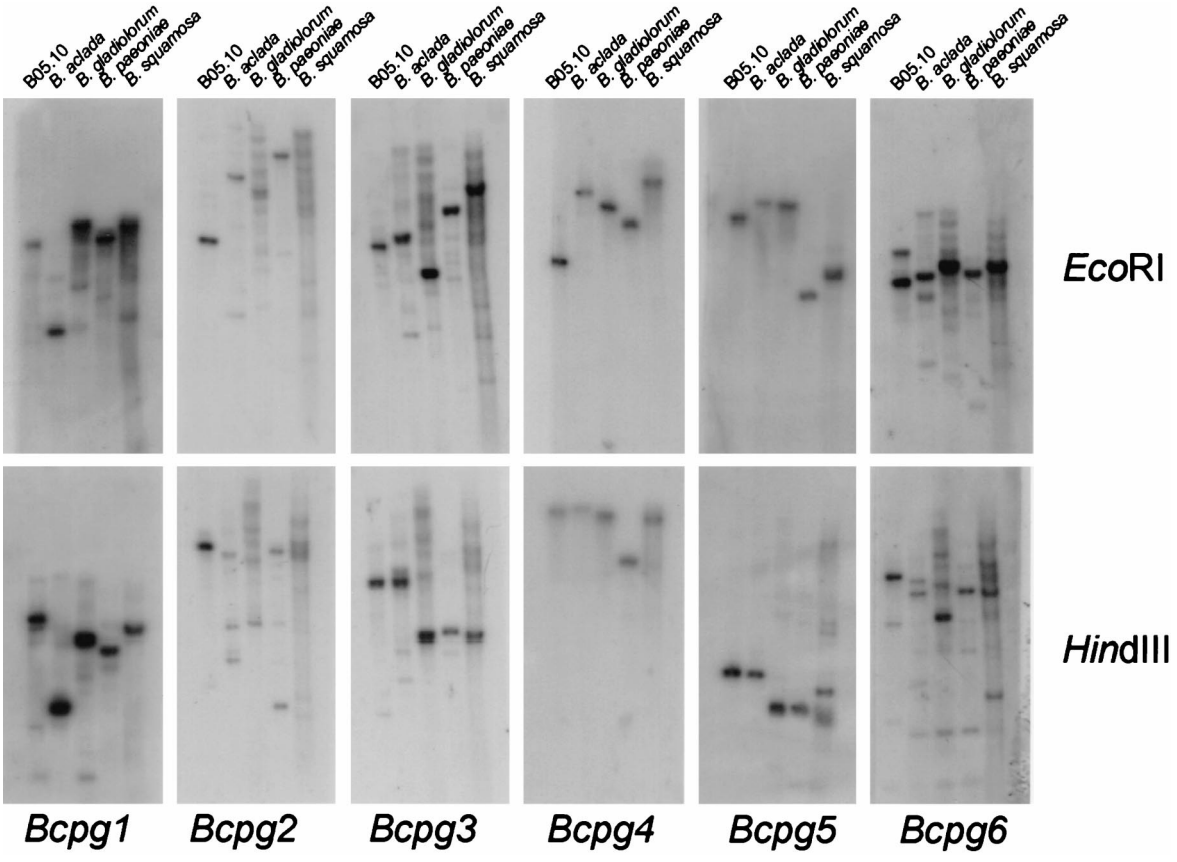

FIG. 4. Southern blot analysis of different species of *Botrytis* with the *Bcpg* genes as a probe. Fungal DNA was isolated from the different *Botrytis* species, digested with *Eco*RI (top panel) or *Hin*dIII (lower panel), and subjected to Southern hybridization with gene-specific probes (*Bcpg*1 to -6) under low-stringency conditions as described in Materials and Methods.

are functional in these species remains to be determined. Moreover, the saprophytic fungus *A. niger* produces a spectrum of endoPGs which, nevertheless, do not enable the fungus to infect living plant tissue.

EndoPG genes of *B. cinerea* appear to be differentially regulated in liquid culture since most of the isoforms produced are only found when the fungus is cultivated on pectin-related carbon sources (19, 40). Those factors affecting the expression pattern in liquid culture might also influence the expression of the endoPG-encoding genes during infection of plants. Previously, we reported that the *Bcpg*1 gene is expressed during the infection of tomato leaves (38). We analyzed here the expression of the *Bcpg* gene family when the fungus is grown on two different carbon sources: glucose and polygalacturonic acid. Clear differences in gene expression levels could be observed between members of the *Bcpg* gene family. Expression of some of the gene family members was observed on both carbon sources (*Bcpg*1, -2, and -6), while others were predominantly expressed on either glucose (*Bcpg*3 and -5) or polygalacturonic acid (*Bcpg*4). We are currently studying the expression of the *Bcpg* gene family on a range of other carbon sources to identify inducers that may affect gene expression during growth on different plant species. It can be envisaged that a coordinated regulation of gene expression occurs during infection of plants. EndoPGs from constitutively expressed genes might release pectin degradation products which can induce the expression of other endoPG-encoding genes.

Our aim is to unravel the role of the induced and concerted action of endoPGs of *B. cinerea* during infection of plants. We

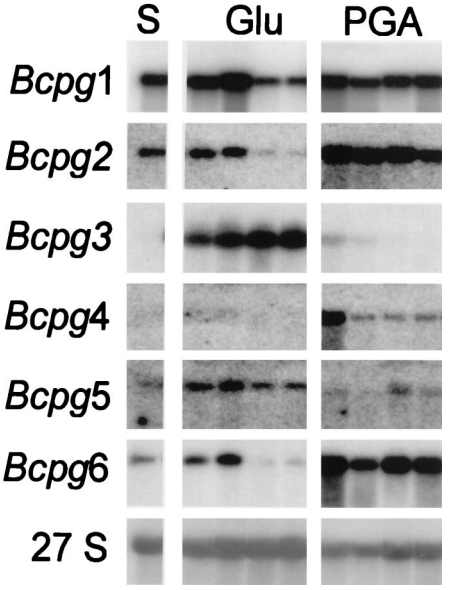

FIG. 5. Northern blot analysis of *B. cinerea* B05.10 in a medium shift experiment with the *Bcpg* genes as a probe. Fungal RNA was isolated from mycelium grown in liquid culture on glucose (Glu) and polygalacturonic acid (PGA) harvested 6, 12, 24, and 30 h after transfer from glucose (S). RNA was subjected to Northern blot hybridization with gene-specific probes (*Bcpg*1 to -6) under high-stringency conditions as described in Materials and Methods. As loading control, RNA was hybridized with a ribosomal probe (27S) from *B. cinerea*. Incubation times for autoradiography after hybridization with the different probes were adjusted to obtain equally exposed films.

have already reported that gene replacement of *Bcpg*1 yielded a fungal mutant with reduced virulence (38). Similar experiments are in progress with other *Bcpg* gene family members. It will be interesting to investigate whether each individual endoPG of *B. cinerea* has a specific function during the course of infection. Differential gene expression in combination with specific enzymatic properties of the endoPGs would justify the need for several enzymes in order to optimally degrade pectin polymers under different environmental conditions.

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