Journal of Microbiology (2013) Vol. 51, No. 4, pp. 461–470
DOI 10.1007/s12275-013-3078-9

The Pectate Lyase Encoded by the *pecCl*1 Gene Is an Important Determinant for the Aggressiveness of *Colletotrichum lindemuthianum*

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(Received February 4, 2013 / Accepted March 10, 2013)

Colletotrichum lindemuthianum **is the causal agent of anthracnose in the common bean, and the genes that encode its cell-wall-degrading enzymes are crucial for the development of the disease. Pectinases are the most important group of cell wall-degrading enzymes produced by phytopathogenic fungi. The *pecCl*1 gene, which encodes a pectate lyase in *C. lindemuthianum*, was isolated and characterized. Possible cis-regulatory elements and transcription factor binding sites that may be involved in the regulation of genetic expression were detected in the promoter region of the gene. *pecCl*1 is represented by a single copy in the genome of *C. lindemuthianum*, though in silico analyses of the genomes of *Colletotrichum graminicola* and *Colletotrichum higginsianum* suggest that the genome of *C. lindemuthianum* includes other genes that encode pectate lyases. Phylogenetic analysis detected two groups that clustered based on different members of the pectate lyase family. Analysis of the differential expression of *pecCl*1 during different stages of infection showed a significant increase in *pecCl*1 expression five days after infection, at the onset of the necrotrophic phase. The split-maker technique proved to be an efficient method for inactivation of the *pecCl*1 gene, which allowed functional study of a mutant with a site-specific integration. Though gene inactivation did not result in complete loss of pectate lyase activity, the symptoms of anthracnose were reduced. Analysis of pectate lyases might not only contribute to the understanding of anthracnose in the common bean but might also lead to the discovery of an additional target for controlling anthracnose.**

Keywords: pectate lyase, *Colletotrichum lindemuthianum*, anthracnose, necrotrophic phase, aggressiveness

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Introduction

Colletotrichum ssp. is one of the most common and important genera of phytopathogenic fungi and includes several species that cause large economic losses (Bailey and Jeger, 1992). The hemibiotrophic fungus *Colletotrichum lindemuthianum* (Sacc. and Magn.) Scrib. is the causal agent of anthracnose in the common bean (*Phaseolus vulgaris* L.) (Geffroy *et al*., 1999, 2000; Perfect *et al*., 1999). Because anthracnose can cause significant damage to common bean crops, several strategies are used to control the disease. One particular strategy is the use of resistant cultivars. However, genetic variability is one of the most important traits exhibited by *C. lindemuthianum*, which complicates the long-term use of resistant cultivars (Sicard *et al*., 1997; Damasceno e Silva *et al*., 2007; Barcelos *et al*., 2011).

The cell wall is considered to be the first barrier established by the host to prevent infection, colonization, and access to nutrients. To degrade the cell wall, phytopathogenic fungi produce many extracellular enzymes, including pectinolytic enzymes. Pectinolytic polygalacturonases (Barthe *et al*., 1981), endopolygalacturonases (Centis *et al*., 1996, 1997; Herbert *et al*., 2004) and pectin lyases (Wijesundera *et al*., 1984) were identified in *C. lindemuthianum*. Necrotrophy is associated with increased expression of wall cell-degrading enzymes such as endopolygalacturonases and pectin lyases. The necrotrophic phase of the *Colletotrichum* species was primarily studied using the *Colletotrichum*-common bean interaction (Perfect *et al*., 1999; Münch *et al*., 2008).

After the YSST (*Yeast Signal Sequence Trap*) library was developed, several secreted proteins were detected in the hemibiotrophic fungus *Colletotrichum graminicola*, including the enzyme pectate lyase (Krijger *et al*., 2008). Several genes affecting virulence were identified in *C. graminicola* using *Agrobacterium tumefaciens*-mediated mutation in which the mutants exhibited a significant reduction in virulence (Münch *et al*., 2011). Comparative genomic analysis of the fungi *C. graminicola* and *Colletotrichum higginsianum* showed that both species have large sets of genes related to their pathogenicity. These include families of genes encoding cell wall-degrading enzymes (O'Connell *et al*., 2012).

Genes encoding cell wall-degrading enzymes are necessary for the development of anthracnose. Of the cell wall-degrading enzymes produced by phytopathogenic fungi, pectinases are characterized as the most important group. Among the pectinases, pectate lyase is one enzyme that is crucial for pectin depolymerization (Lebeda *et al*., 2001; Reignault *et al*., 2008). Consequently, the knowledge of the genes involved at each stage of the fungal lifecycle is important for control of anthracnose.

Recently, subtractive libraries of the *Colletotrichum*-common bean association were constructed and analyzed at the Laboratory of Molecular Genetics of Microorganisms at the Federal University of Viçosa. Among the differentially expressed genes, a gene encoding the pectate lyase *pecCl*1 was identified in leaves seven days after infection (Fontenelle, 2010). After obtaining a partial sequence of the *pecCl*1 gene, isolation and characterization of its complete sequence were possible. This facilitated the production of mutants and analysis of gene regulation during anthracnose development, allowing the determination of whether pectate lyase was important for *C. lindemuthianum* aggressiveness. Therefore, the aim of our study was to structurally and functionally characterize the gene encoding pectate lyase in *C. lindemuthianum*.

Materials and Methods

Microorganisms and culture conditions

The present study used the following eight isolates from several physiological races of *C. lindemuthianum* fungus: 65451, 72801, 73320, 73497, 75485, 81, 81538, and 89$A_2$2-3. *C. lindemuthianum* isolate $A_2$2-3 of race 89 was used as the control wild-type lineage and the acceptor lineage during the creation of mutants. Wild-type and mutant lineages were cultured and maintained in PDA (potato dextrose agar) culture medium at 22°C.

Extraction of total DNA

To obtain mycelia, approximately 10^6 conidia were inoculated in GPYECH liquid medium (20 g glucose, 5 g peptone, 1 g yeast extract, and 1 g casein hydrolysate per L) (Ansari *et al*., 2004) and incubated in a growth chamber at 22°C for five days. Total DNA extraction was performed following a modification of the protocol for fungi (Specht *et al*., 1982).

Inverse PCR

Total DNA from isolate 89$A_2$2-3 of *C. lindemuthianum* was digested using the restriction enzyme *Hin*dIII and the fragments were ligated using enzyme T4 ligase (Promega). The ligation product was used as a template for inverse PCR with two specific primers iCl7d124F and iCl7d124R, which are shown in Table 1. The amplified fragments were cloned into the *pGEM-T* Easy vector using the *pGEM®-T* Easy Vector System kit (Promega). The product from the ligation reaction was used to transform ultra competent *E. coli* DH5α cells, and the plasmid DNA was extracted and purified using the GeneJET™ Plasmid Miniprep Kit (Fermentas Life Sciences).

In silico analysis of the *pecCl*1 gene sequence

The complete nucleotide sequence of the *pecCl*1 gene was deposited in GenBank under the accession number JX270683. The complete sequence of the *pecCl*1 gene was compared to sequences of other fungi available from the NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/) protein database using the basic local alignment search tool (BLAST) algorithm (Altschul *et al*., 1997). The presence of the signal peptide was analyzed based on the deduced amino acid sequence using the online software SignalP version 3.0 (Emanuelsson *et al*., 2007). To find genes with sequence identity, the complete sequence of the *pecCl*1 gene was compared to the databases of *C. graminicola* and *C. higginsianum* (*Colletotrichum* database [http://www.broadinstitute.org/]) using the BLASTn algorithm.

Manual alignment of the pectate lyase amino acid sequences from *C. lindemuthianum* with reference sequences obtained from the GenBank database was performed using the software MEGA 5.0 (Tamura *et al*., 2011). A maximum parsimony (MP) tree was constructed and tests were performed using PAUP* 4.0b10 (Swo Vord, 2002). The heuristic search method was used to construct the tree with

Table 1. Primers used in the present study

Primers	Sequence (5′→3′)	Finality
iCl7d124F	AAAGGTCAGCTGCCAAATGT	Inverse PCR
iCl7d124R	GCGGTAGAGCTTGCCATAGT	
PLCol1F	GCAACGTTTGCTTCATCTTG	Probe 1
iCl7d124R	GCGGTAGAGCTTGCCATAGT	
pecCl1FP1	GGTTTGAGGATCCGGAGATT	Split-Marker; Probe 2
pecCl1RP1	TCCTGTGTGAAATTGTTATCCGCTCTGGTTCTTGCCGATGATG	
pecCl1FP2	GTCGTGACTGGGAAAACCCTGGCGCACCCTGGAGTTTGTCTGGT	Split-Marker
pecCl1RP2	TCGTCATCTGCTCAGTCGTC	
NLC37	GGATGCCTCCGCTCGAAGTA	Split-Marker
NLC38	CGTTGCAAGACCTGCCTGAA	
M13F	CGCCAGGGTTTTCCCAGTCACGAC	Split-Marker
M13R	AGCGGATAACAATTTCACACAGGA	
pecCl1ForaF	GCAACGTTTGCTTCATCTTG	Inactivation control
pecCl1ForaR	GGAAGCATTCCAGCAGAAAA	
pClRTF2	GCAGCTCTGCCTTCCACACT	qPCR
pClRTR2	CTCGATGACCTTGCTGTTGGT	
ClrRNA3	CCTGTTCGAGCGTCATTTCA	qPCR
ClrRNA4	CCGGTGCGAGGTGGTATG	

tree bisection and reconnection (TBR). Tree View was used to visualize the tree (Page, 1996).

Number of copies of the *pecCl*1 gene

The total DNA from eight isolates of several physiological races of *C. lindemuthianum* was extracted and cleaved and the fragments were detected by Southern blot (Sambrook and Russell, 2001). Hybridization was performed using the Dig High Prime DNA Labeling and Detection Starter Kit II (Roche). Probe 1 was labeled in an independent PCR reaction using the Dig Probe Synthesis Kit (Roche). Primers used were PLCol1F and iCl7d124R, shown in Table 1.

Analysis of gene expression

Conidia were used to infect the cotyledonary leaves of the common bean. Leaves were inoculated with 10^6 conidia using a brush (Dufresne *et al.*, 1998) and were collected three, five, and seven days after inoculation. Real-time PCR (qPCR)

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-494    GATTACGCCAGCTATTTAGGTGACACTATAGAATACTCAAGCTATGCATCCAACGCGTTG

-434    GGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGATTGCAACGTT

-374    TGCTTCATCTTGTTCATGTCGAAACAAACTTGATTTTTGAAGGATACAGGCATCTGATTG

-314    GACCGCATAAGCATCCTGCGGTCCTTGGTAGTATCGTGTTGGGTTTGAGGATCCGGAGAT

-254    TCATGATGCGTGAAAATGCTGGCTGTGGGCTTTTGCGATGGCAACAATGCTTCGATGTAA

-194    GTCCACCAATAGTGGCCATGTCGACCGCGTGAGCGGGAAGAGAGATTCAACCTGTCTCGT

-134    GGGCTCAACCCCCGAGATGAGTATCTATATAAACTAAATGTCTTGAGGCCTTGACTCTCG

-74     CCATCAACCCTCACTCACAGTCTTCAGCATCATCAGCAGCCCATCTCCCTAGCACAGTTT

-14     CGAGATCTTCCATCATGTACTTCTCCAAGAGCTCCATCGTAGCTTTTCTGGCAGCTCTGC
 1                  M  Y  F  S  K  S  S  I  V  A  F  L  A  A  L

46      CTTCCACACTTGCCTGTCTCGGATACGAGGGTGGTATTCCTGCTGCCACCTCGACCAAGA
16      P  S  T  L  A  C  L  G  Y  E  G  G  I  P  A  A  T  S  T  K

106     CCAACAGCAAGGTCATCGAGGTCAAGGCCGGAGAGGTTTACGACGGAGGATGGGCCCGCT
36      T  N  S  K  V  I  E  V  K  A  G  E  V  Y  D  G  G  W  A  R

166     TCGACCGCGAGTCGGGTGCCTGCAATGATCAAGCTGAAGGAGgtaagccattaaatggtt
56      F  D  R  E  S  G  A  C  N  D  Q  A  E  G

226     caagtcatgggaagaaaccgttgacctaacgaacgcagGCGACGCCGATGCTGTCTTTCT
70                                            G  D  A  D  A  V  F  L

286     GCTTCGCCGCGGCGCCACGCTGAAGAACGCCATCATCGGCAAGAACCAGGCCGAGGGAGT
78        L  R  R  G  A  T  L  K  N  A  I  I  G  K  N  Q  A  E  G  V

346     TCACTGCGACGGCCCTTGCACCCTGGAGTTTGTCTGGTTCGAGGACGTGTGCGAGGATGC
98        H  C  D  G  P  C  T  L  E  F  V  W  F  E  D  V  C  E  D  A

406     CATCTCTGTCgtgagtcattcaggctgaccagaagcaagactcataaacccctgacaccc
118       I  S  V

466     atacagaagAACGACAAGGCCGGCGACCAAACCTGGATCATCGGCGGTGGCGCCTACAAG
121               N  D  K  A  G  D  Q  T  W  I  I  G  G  G  A  Y  K

526     GCGTCGGACAAAATTGTCCAGCATAATGGTTGCGGTACCGTTAACgtaagtcatgaccga
138      A  S  D  K  I  V  Q  H  N  G  C  G  T  V  N

586     cgcctcctaagggatcatgctgagtcttattcagATCATCGACTTCTACGCCAACGACTA
153                                      I  I  D  F  Y  A  N  D  Y

646     TGGCAAGCTCTACCGCTCTTGCGGCAACgtgagtgaacatgacatttctgcgtcatcatg
162       G  K  L  Y  R  S  C  G  N

706     ggtctctgaccgtttcaatacagTGCAGCAGCCAGTGCAAGAGAAACGTATACgtagagg
171                           C  S  S  Q  C  K  R  N  V  Y

766     caagttcacatgtccgtatttatagatcacggttactaacgttcgggcagGGAACGACCG
181                                                     G  T  T

826     CTTACAACGGTGGTGAGATCGTCGGCATCAACTCCAACTACGGCGACACGGCGACCCTGA
184     A  Y  N  G  G  E  I  V  G  I  N  S  N  Y  G  D  T  A  T  L


886     AGAACGTCTGCACCGACGCAAAGGTCAGCTGCCAAATGTACAACGGCTGCGCGGGTGGCT
204     K  N  V  C  T  D  A  K  V  S  C  Q  M  Y  N  G  C  A  G  G

946     GCGAGCCCACCAAGTCCGGCGTTTGCTCTGGTTGAGGTCCGTTGTCGGATACACCTTGAC
224     C  E  P  T  K  S  G  V  C  S  G  *

1006    GACTGAGCAGATGACGAGATCCGGATGTGAGGCAGATGCCCCATCATCAGCCACAAACTC

1066    TGAACTGCCTGATATGAACCTGAACAAAGTTCTTTGTACATAGTTTTCTGCTGGAATGCT

1126    TCCAATCGAATTCCCGCGGCCGCCATGGCGGCCGGGAGCATGCGACGTCGGGCCCAATCG

1186    CC
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Fig. 1. Nucleotide sequence of the gene encoding the pectate lyase in *C. lindemuthianum* and the deduced amino acid sequence. The putative CAAT box, TATA box, transcription factor binding sites (CreA-CCGGAG, AreA-GATA, AceI-AGGCA), and the polyadenylation sites are underlined. The CT-rich regions are indicated by the dotted lines. The stop codon is indicated with an asterisk. The start codon, together with the consensus sequence around it, is underlined in bold. Introns are expressed in lowercase letters. The cleavage site of the signal peptide is highlighted in gray.

was performed to quantify the expression of the *pecCl*1 gene at each stage of infection. Mycelia and infected leaves were separately macerated and total RNA was extracted using TRIzol (Invitrogen) following the manufacturer's instructions. Samples were treated with DNAse RQI RNAse-free (Promega) and cDNA was synthesized using the ImProm-II Reverse Transcription System (Promega) kit. PCR was performed using the SYBR Green PCR-Master Mix (Applied Biosystems) kit and readings were performed using a CFX96 Touch™ Real-Time PCR Detection System device (Bio-Rad). Each experiment was performed in biological replicates using two different RNA samples. The ribosomal RNA gene (rRNA) was used as an endogenous control to normalize expression levels.

Inactivation of the *pecCl*1 gene

The Δ*pecCl*1 mutant of the *C. lindemuthianum* strain $A_2$2-3 race 89 isolate was obtained using the split-marker method described by Catlett *et al.* (2003). The split-marker technique was performed using two PCR rounds with four universal primers targeting the hygromycin-resistance selection marker (M13R, M13F, NLC37, and NLC38) and four primers specific for the *pecCl*1 gene (pecCl1FP1, pecCl1RP1, pecCl1FP2, and pecCl1RP2), which are shown in Table 1. The "HY" and "YG" DNA fragments of the hygromycin phosphotransferase cassette (HYG) were amplified from the plasmid pAN7.1 (Punt *et al.*, 1987) using the primers M13R/NLC37 and M13F/NLC38. The PCR reactions were performed using Platinum *Taq* DNA polymerase High Fidelity (Invitrogen).

Transformation of *Colletotrichum lindemuthianum*

C. lindemuthianum was transformed using a modification to published methods (Rodriguez and Yoder, 1987; Redman and Rodriguez, 1994). Approximately 10^6 conidia/ml were inoculated in GPYECH medium at 22°C for three days. Mycelia were collected, cleaned, and resuspended in 20 ml of phosphate buffer supplemented with 7 mg/ml of Lysing Enzyme (Sigma). Protoplastization was performed at 30°C under agitation of 80 rpm for 4 h. A solution containing the inactivation cassette constructs and 50 µl of polyethylene glycol (25% PEG 6000, 50 mM $CaCl_2$) was added to 200 µl of the suspension containing 10^7 protoplasts/ml. The mixture was incubated at 0°C for 20 min. An additional 500 µl of PEG solution was added, followed by incubation at room temperature for 20 min. Protoplasts were plated in PDA medium containing 0.56 M saccharose and incubated at 22°C for 48 h. After protoplast regeneration, 5 ml of semisolid PDA medium containing 60 µg/ml of hygromycin (Sigma-Aldrich) was added. Later, the hygromycin-resistant transformants were selected and purified using monosporic isolation.

Selection of transformants with site-specific integration

To select transformants with site-specific integration in the locus of the *pecCl*1 gene, PCR was performed using the specific primers pecCl1ForaF and pecCl1ForaR; primers are shown in Table 1. These primers amplify from the flanking regions of the *pecCl*1 gene. Amplification was performed using the Platinum *Taq* DNA polymerase High Fidelity (Invitrogen).

Molecular characterization of transformants was performed by extracting and cleaving their total DNA. The resulting DNA fragments were detected by Southern blotting (Sambrook and Russell, 2001). Hybridization was performed using the Dig High Prime DNA Labeling and Detection Starter Kit II (Roche). Probe 2 was labeled in an independent PCR reaction using the PCR Dig Probe Synthesis (Roche) kit following the manufacturer's instructions and the specific primers pecCl1FP1 and pecCl1RP1; primers are shown in Table 1.

Pathogenicity test

To test for pathogenicity, detached common bean leaves were inoculated with 10^6 conidia of the wild-type and mutant lineages, maintained in Petri dishes containing paper disks moistened with sterile distilled water, and incubated for seven days at 22°C under a photoperiod of 16 h of light (166 µE/sec/m^2) and 8 h of dark (Dufresne *et al.*, 1998). Over seven consecutive days, the leaves were observed and photographed every 24 h to document the stages of infection by *C. lindemuthianum*. To verify the reproducibility of the results, inoculation was performed in triplicate.

Pectate lyase activity assay

A suspension with approximately 10^6 conidia/ml of the wild-type and mutant lineages was inoculated into supplemented buffered mineral medium [6.98 g/L K_2HPO_4, 5.44 g/L KH_2PO_4, 1.0 g/L $(NH_4)_2SO_4$, 1.1 g/L $MgSO_4{\cdot}7H_2O$, 0.6 g/L yeast extract, and 3.0 g/L pectin] pH 6.8 at 22°C under agitation of 120 rpm for three and five days. The mycelia were filtered and the supernatant was used to detect extracellular pectinases. Pectate lyase activity was determined using spectrophotometry at 230 nm by incubating the culture filtrate with 1% (w/v) polygalacturonic acid in 0.1 M Tris/HCl buffer pH 8.5 supplemented with 1 mM $CaCl_2$ (Collmer *et al.*, 1988). The reaction was processed at 40°C for 40 min. One unit of pectate lyase activity is defined as the amount of enzyme needed to produce 1 µmol of unsaturated product per liter of culture per minute. For the calculation, the molar absorption coefficient of the unsaturated product was established as 5,200 L/cm/mol (Moran *et al.*, 1968).

Results

Isolation and structural characterization of the *pecCl*1 gene

Inverse polymerase chain reaction (PCR) was used to obtain the complete sequence of the *pecCl*1 gene. The inverse PCR product was approximately 1.7 kb. The *pecCl*1 gene sequence obtained in this assay contains 494 nucleotides in the promoter region, 981 nucleotides in the coding region, and 207 nucleotides in the terminator region. Analysis of the promoter region revealed some possible cis-regulatory elements that are involved in regulating pectate lyase genetic expression, a TATA box at position -109, CAAT boxes at positions -189 and -201, and CT-rich regions. In addition, possible transcription factor binding sites were detected: CreA at position -244; AreA at position -416; and AceI at position

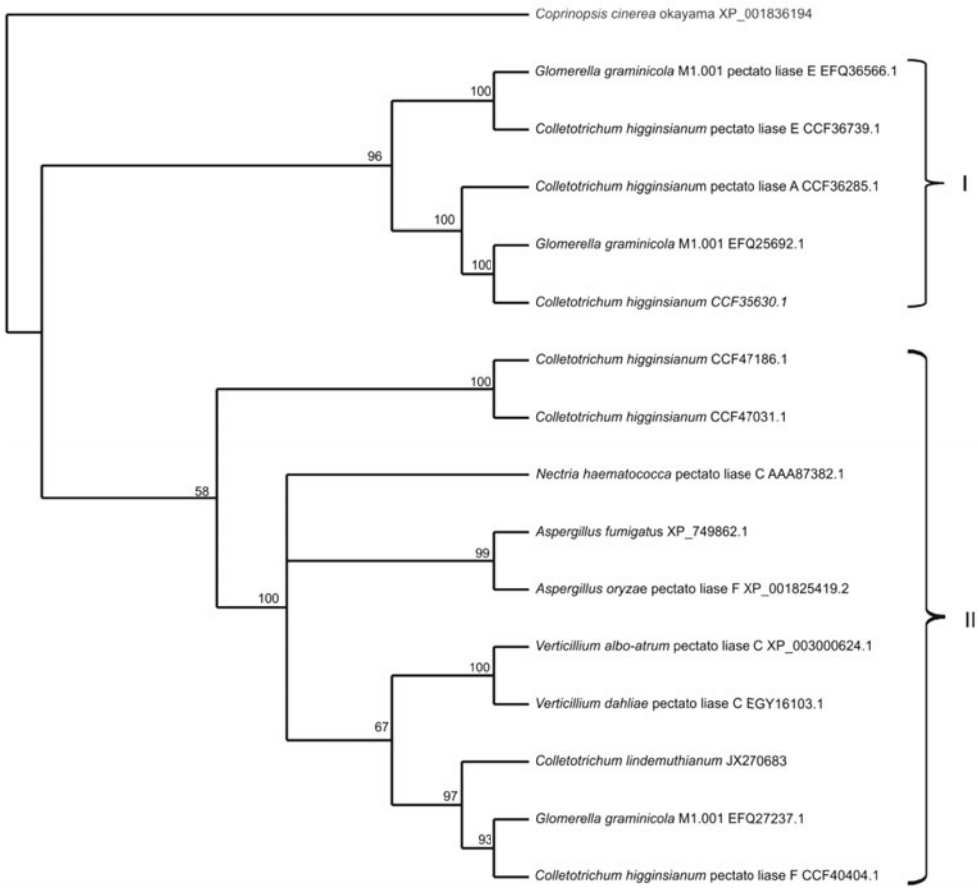

Fig. 2. Phylogeny of the amino acids sequences from *Colletotrichum* spp. pectate lyases. The tree rooted with Basidiomycota *Coprinopsis cinerea* fungus was obtained using the maximum parsimony method and PAUP * 4.0b10. The numbers close to the branches indicate the bootstrap values based on 1,000 replicates. Clade I grouped the members of the pectate lyases A and E, and clade II grouped the members of the pectate lyases C and F.

-421. Five putative introns were found interrupting the coding region and possible polyadenylation sites were found at the terminator region. Within the deduced amino acid sequence, a signal peptide was found with a probability of 0.994 and is cleaved at the TLA-CL site between amino acids 20 and 21 (Fig. 1). The complete nucleotide sequence of the *pecCl*1 gene was deposited in GenBank under the accession number JX270683.

The amino acid sequence of *pecCl*1 exhibited sequence identity with the pectate lyase of *C. higginsianum* (89% identity, accession number CCF40404.1) and the pectate lyase C of *Glomerella graminicola* strain M1.001 (83% identity, accession number EFQ27237.1). In addition, a possibly conserved domain, pfam03211, was detected in the pectate lyase superfamily between amino acids 20 and 228.

A single copy of the *pecCl*1 gene was detected in the genomes of isolates from the eight physiological races of *C. lindemuthianum* that were analyzed; a single specific band was observed from the various isolates when a restriction enzyme that does not cleave within the gene and under high stringency conditions was used (data not shown). However, three sequences in the genome of *C. graminicola* that encode pectate lyase exhibited sequence identity (68, 74, and 76%, respectively) with the *pecCl*1 gene of *C. lindemuthianum*, and seven sequences from the genome of *C. higginsianum* encoding pectate lyase exhibited sequence identity (70, 89, 81, 74, 75, 97, and 79%, respectively) with the *pecCl*1 gene of *C. lindemuthianum*. This indicates that the genome of *C. lindemuthianum* might also contain other genes encoding pectate lyases in addition to the *pecCl*1 gene.

Phylogenetic analysis

A phylogenetic tree was constructed based on the alignment of the amino acid sequences of pectate lyases. Figure 2 shows the occurrence of two different groups which clustered based on different members of the pectate lyase family, suggesting that such pectate lyases differ significantly in their amino acid composition. Clade I grouped the members of pectate lyase A and E, and clade II grouped the members of pectate lyase C and F. The pectate lyase of *C. lindemuthianum* clustered in clade II, more specifically with pectate lyase F of *C. higginsianum* (CCF40404.1) and the pectate lyase of *G. graminicola* strain M1.001 (EFQ27237.1).

Gene expression

A real-time PCR assay (qPCR) was performed to quantify *pecCl*1 gene expression at the following stages of infection: three days (biotrophic phase), five days (onset of the necrotrophic phase), and seven days (end of the necrotrophic phase). In Fig. 3A, the fungal cDNA sample was used as a control. In Fig. 3B, the sample from three days post-infection was used as the control. The efficiency of amplification of *pecCl*1 gene's rRNA was relatively similar, which permitted the use of the comparative Ct method for relative quantification as described by Livak and Schmittgen (2001). Although

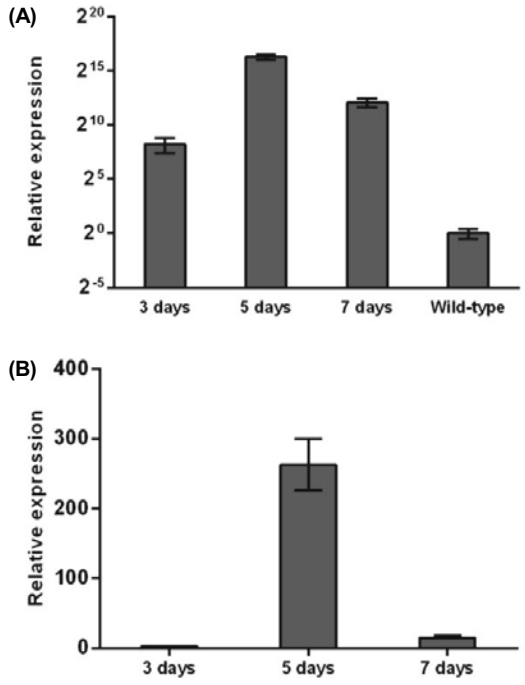

Fig. 3. Relative expression of *pecCl*1 during *C. lindemuthianum* infection of the common bean. (A) The fungus sample was used as the control, and samples that were collected at three, five, and seven days represent the different stages of infection. The Y-axis values are expressed on a logarithm scale to base 2. (B) The sample at three days post-infection was used as a control, and samples collected at five and seven days represent the different stages of infection. The Y-values are expressed on a linear scale.

*pecCl*1 transcripts were observed at all stages of fungal development in the plant, they exhibited a significant increase five days after infection, corresponding to the onset of the fungal necrotrophic phase.

Inactivation of the *pecCl*1 gene

The function of the *pecCl*1 gene was assessed using the genetic inactivation strategy known as split-marker. The first PCR round was performed using the plasmid pAN7.1 as template. The products obtained were DNA fragments of 3,101 bp and 1,584 bp that were amplified using M13R/NLC37 and M13F/NLC38 primers, respectively. Also in this first round, genomic DNA was used as a template and two DNA fragments of 608 bp and 659 bp were obtained; these fragments were amplified using the primers FP1/RP1 and FP2/RP2, respectively. The second PCR round yielded two DNA fragments with 3,709 bp and 2,243 bp. These fragments served as the inactivation cassettes used to transform *C. lindemuthianum* protoplasts.

After protoplast transformation, 16 transformants were selected and purified. Confirmation of gene disruption was performed using PCR with pecCIForaF and pecCl1ForaR primers. Among the analyzed transformants, the occurrence of site-specific integration was identified in only three transformants (T1, T2, and T11). Amplification was performed on the genomic DNA from T1, T2, and T11, resulting in a DNA fragment of 5,730 bp amplified from the junction of the *pecCl*1 flanking regions with the gene conferring resistance to hygromycin, *hyg* gene. A 1,511 bp DNA fragment corresponding to *pecCl*1 was amplified from wild-type and transformants with ectopic integration.

Southern blotting was performed to acquire a more detailed molecular characterization of integration of DNA fragments into the transformants' genomes. A DNA fragment of 608 bp corresponding to the initial part of *pecCl*1 was used as the probe. This analysis was performed on the three transformants with confirmed gene disruption. Figure 4 shows that when the enzyme *Eco*RV was used, which does not cleave within *pecCl*1 gene nor within the *hyg* gene, the size of the band relative to the gene *pecCl*1 is greater than that of wild-type (WT), indicating that site-specific integration occurred in the T1, T2, and T3 transformants. A DNA

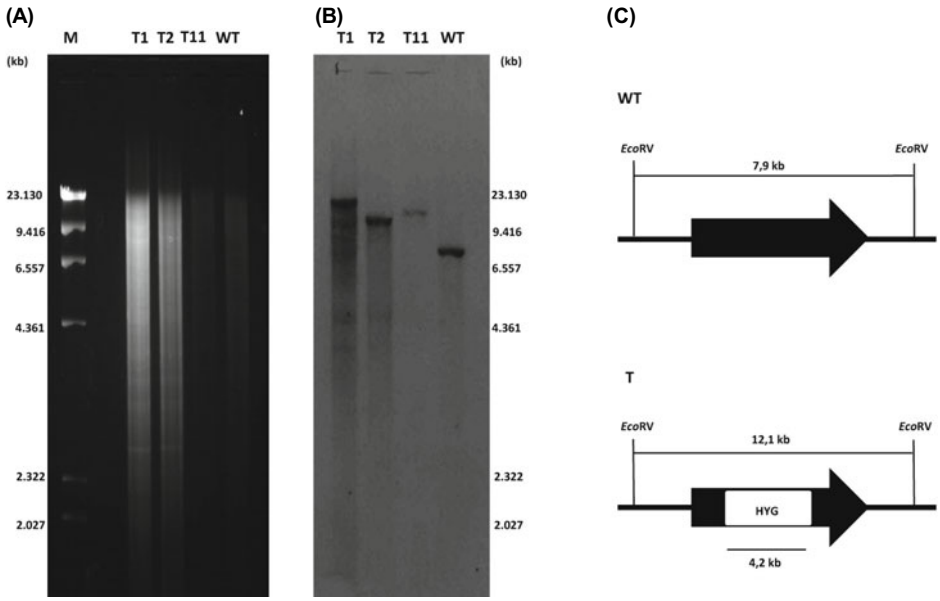

Fig. 4. Molecular characterization of transformants. (A) Electrophoresis in a 0.8% agarose gel of the cleavage of the total DNA from the wild-type (WT) and transformants (T1, T2, and T11) *C. lindemuthianum* with the enzyme *Eco*RV. (M) Phage lambda DNA marker digested with *Hin*dIII. (B) Autoradiograph of the total DNA hybridization of wild-type (WT) and transformants (T1, T2, and T11) *C. lindemuthianum* with the 608 bp probe. (C) Profile of the site-specific integration in the locus of the *pecCl*1.

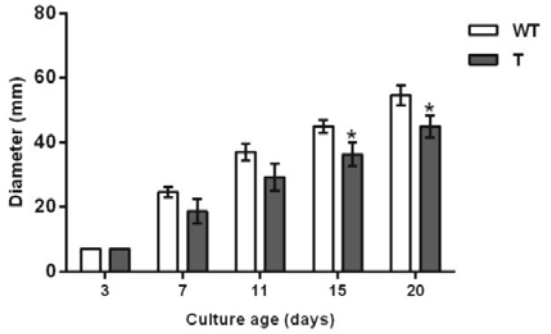

Fig. 5. Analysis of mycelial growth in the Δ*pecCl*1 mutant (T). The wild-type (WT) and Δ*pecCl*1 mutant (T) were re-inoculated in PDA medium. The average growth diameter of the three dishes was used to generate the graph. *Mean is significantly different (P<0.01) compared to the wild-type using Student's t-test.

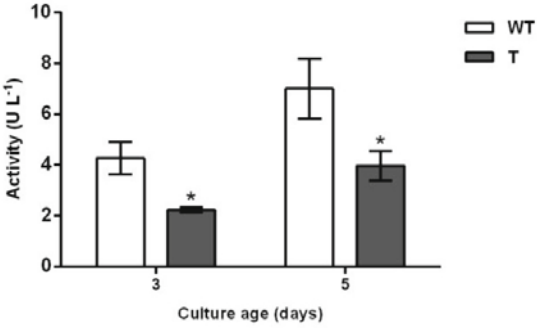

Fig. 7. Enzymatic activity of pectate lyase. Enzymatic assay was assessed using the supernatant obtained from wild-type (WT) and Δ*pecCl*1 mutant (T) lineages after three and five days of growth in liquid medium. The mean is significantly different (P<0.05) compared to the wild-type using Student's t-test.

fragment of approximately 7.9 kb was observed from the wild-type genome, whereas fragments of approximately 16.8, 12.1, and 14.8 kb were observed from the T1, T2, and T11 transformants, respectively. A fragment of exactly 4.2 kb larger than the gene present in the wild-type (WT) was observed from the transformant T2, corresponding to the size of the *hyg* gene. In transformants T1 and T11, the size difference between bands was greater than 4.2 kb, suggesting that rearrangements occurred during recombination.

The results confirm that transformant T2 had an inactivated *pecCl*1 gene and therefore it is designated as the Δ*pecCl*1 mutant. This mutant has a site-specific integration, and no additional copies of the inactivation cassette were detected. Thus, the Δ*pecCl*1 mutant could be used for functional inference gene analysis.

Functional analysis of the *pecCl*1 gene

Macroscopic observation of colonies did not reveal morphological differences between the Δ*pecCl*1 mutant and the *C. lindemuthianum* wild-type lineage. Conidiation was similar between the Δ*pecCl*1 mutant and the *C. lindemuthianum* wild-type lineage. However, growth rate was affected and the Δ*pecCl*1 mutant exhibited a significant reduction of the mycelial growth 15 days after subculture (Fig. 5).

The possible relationship between *pecCl*1 and the pathogenicity of the fungus *C. lindemuthianum* race 89A$_2$2-3 in a susceptible common bean was investigated by inoculating leaves of the common bean cultivar Rosinha, which is susceptible to anthracnose, with conidia from the Δ*pecCl*1 mutant or with the wild-type isolate as a control to observe the symptoms of disease. The symptoms of anthracnose appeared on the leaves five days after infection with the wild-type isolate; this corresponds to the onset of the necrotrophic phase. However, leaves inoculated with Δ*pecCl*1 mutant conidia exhibited a reduction in anthracnose symptoms primarily after seven days post-inoculation. Small lesions were observed starting on day five, but the extension of the affected area did not increase and the tissue was not macerated when compared to leaves inoculated with wild-type conidia (Fig. 6).

To detect pectate lyase secretion by the Δ*pecCl*1 mutant, an enzymatic assay was conducted with the supernatants collected from the wild-type and mutant lineages after three and five days of growth in liquid medium (Fig. 7). At both time points, the pectate lyase enzymatic activity was significantly reduced in the Δ*pecCl*1 mutant. Therefore, the enzymatic assay confirms that the decrease of the anthracnose symptoms observed for the Δ*pecCl*1 mutant conidia was due

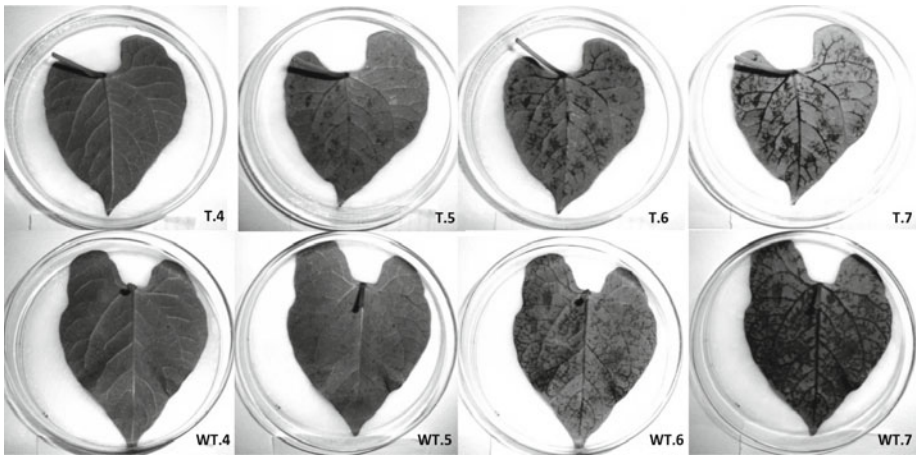

Fig. 6. Analysis of *C. lindemuthianum* pathogenicity. Letters and numbers represent the wild-type (WT) and Δ*pecCl*1 mutant (T) and the number of days after inoculation of the detached leaves of the susceptible common bean, respectively.

to reduced secretion of pectate lyase and not to a secondary effect (Figs. 6 and 7).

Discussion

Like the *Colletotrichum gloeosporioides* f. sp. *malvae* genes encoding pectate lyases *pel*-1 and *pel*-2 (Shih *et al*., 2000), *pecCl*1 is present as a single copy in the *C. lindemuthianum* genome. The production of cell wall-degrading enzymes by filamentous fungi is primarily regulated at the transcriptional level. These genes are induced in the presence of polymers or derived molecules and are repressed under growth conditions where the production of such enzymes is not necessary. Genetic expression of these genes is regulated by environmental and cellular factors and host components, some of which are common whereas others are unique for a given fungus or enzyme class (Aro *et al*., 2005; Lara-Márquez *et al*., 2011b). Possible cis-regulatory elements (TATA box, CAAT box) and transcription factor binding sites (CreA, AreA, and AceI) were found in the promoter region of *pecCl*1.

Though the transcription factor CreA has been demonstrated to be involved in the catabolic repression of several pectinases (Aro *et al*., 2005), not all pectinases are subject to glucose repression. For instance, the *pel*B gene encodes pectate lyase in *C. gloeosporioides* and is activated in the presence of glucose (Miyara *et al*., 2008). Four CreA-binding sites were found in the promoter region of *ccpel*A, which encodes the pectate lyase in *Colletotrichum coccodes*; however, pectate lyase was secreted in the presence of glucose, which indicates that the binding sites found might be nonfunctional (Ben-Daniel *et al*., 2011).

Several genes in phytopathogenic fungi are activated under conditions of nitrogen deprivation. The regulation of nitrogen metabolism in *Aspergillus nidulans* and *Neurospora crassa* involves the GATA family of transcription factors, including AreA and Nit2 (Basse and Farfsing, 2006). In *C. gloeosporioides*, nitrogen deprivation activates transcription of *are*A and *nit* mutants (which cannot use nitrate) do not secrete pectate lyase (Kramer-Haimovich *et al*., 2006). In *C. lindemuthianum, clnr*1 mutants (homologous of *are*A) are not pathogenic and are unable to progress to the necrotrophic phase of infection (Pellier *et al*., 2003). Similarly, *C. coccodes are*A and *nit* mutants have impaired ammonia secretion and their virulence in the host is reduced (Alkan *et al*., 2008). The promoter region of the gene encoding the pectate lyase *pel*A was compared between moderately and highly aggressive isolates of *C. coccodes* and the only difference found in the promoters was that the highly aggressive isolate contained an AreA-binding site (Ben-Daniel *et al*., 2011). Therefore, the transcription factor AreA might be involved in fungal aggressiveness and consequently, *pecCl*1 might be a virulence factor that is involved in *C. lindemuthianum* pathogenicity.

The transcription factor AceI might act as a repressor of the genetic expression of cell wall-degrading enzymes. A role for AceI as a general regulator was suggested because deletion of *ace*I caused reduction of *Trichoderma reesei* growth when sorbitol was used as the exclusive source of carbon (Aro *et al*., 2002, 2005).

Transcripts of *pecCl*1 were observed during all fungal developmental stages in the plant but they exhibited a significant increase five days after infection, at the onset of the necrotrophic phase. During this phase, the secondary hyphae cause extensive degradation of the plant cell wall through secretion of a wide variety of depolymerases, including pectate lyase (Münch *et al*., 2008). The expression of *pel*-2 was greater in *C. gloeosporioides* at the onset of the necrotrophic phase (Shih *et al*., 2000). Expression of *cgmpg*2, which encodes endopolygalacturonase in *C. gloeosporioides*, was similarly detected during all phases of infection; however, *cgmpg*2 had greater differential expression at the onset of the necrotrophic phase (Li and Goodwin, 2002).

The split-marker technique efficiently inactivated *pecCl*1 of *C. lindemuthianum,* allowing functional analysis of *pecCl*1 in a mutant with a site-specific integration and no ectopic integrations. This mutant exhibited a low rate of transformation and some transformants had high numbers of ectopic integrations, which poses a problem when disruption of only one gene is desired due to unintended interference with the mutant's phenotype. The efficiency of homologous integration might depend on the mechanism of homologous recombination that is used by the species of interest. The split-marker technique stands out as a simple, fast, and low-cost inactivation technique that has been applied successfully to other filamentous fungi (Colot *et al*., 2006; You *et al*., 2009; Gravelat *et al*., 2012).

Pectate lyase is secreted by fungi into the extracellular environment where the enzyme degrades the plant cell wall. Such secretion is orchestrated by a signal peptide that is present in the amino acid sequence of the *C. lindemuthianum* pectate lyase. Cell wall depolymerases supply nutrients and allow for the growth of fungi, which suggests a nutritional rather than an infection-specific function (Jia and Wheals, 2000; Münch *et al*., 2008).

The appearance of macroscopic symptoms of anthracnose on the common bean leaves starting on day five after *C. lindemuthianum* infection indicates the development of secondary hyphae, which secrete pectinolytic enzymes that degrade the cell wall as they progress. This phenomenon leads to the production of necrotrophic lesions which cause symptoms of anthracnose at more advanced stages (Perfect *et al*., 1999; Münch *et al*., 2008).

Phylogenetic analysis demonstrated that the pectate lyase family members differ significantly in their amino acid composition. Nevertheless, multiple forms of these enzymes catalyze similar reactions. The existence of multiple isoenzymes might be due to multiple genes encoding pectate lyases. Furthermore, these isoenzymes might originate from post-translational modifications of proteins (Annis and Goodwin, 1997).

Pectinolytic enzymes might be important in the aggressiveness of *C. lindemuthianum*, but this same role has not been confirmed in some fungi because some enzymes are frequently encoded by multigenic families. This means that the deletion of one gene might not affect pathogenicity because other genes might mask the loss of function (Lebeda *et al*., 2001; Reignault *et al*., 2008). In our study, inactivation of the *pecCl*1 gene did not result in complete loss of pectate lyase activity and only reduced the symptoms of anthracnose.

This finding is consistent with the presence of other genes encoding pectate lyases that confer greater flexibility to the pathogen's aggressiveness.

Studies conducted in *Fusarium solani* f. sp. *pisi* suggest that only two of the four known pectate lyases are essential for fungus pathogenicity (Rogers *et al*., 2000). In *C. gloeosporioides*, inactivation of the pectate lyase gene *pelB* resulted in lower enzymatic activity and an approximately 40% reduction in disease severity in avocado fruits (Yakoby *et al*., 2001). Similarly, inactivation of the pectate lyase gene *pelA* of *C. coccodes* resulted in reduced aggressiveness (20–25%) in tomato fruits and affected the secretion and extracellular activity of the pectate lyase (Ben-Daniel *et al*., 2011).

In addition to an important role in the infection of plants by pathogenic fungi, pectate lyase exhibits high industrial biotechnological potential in several areas: to reduce viscosity and increase yield in the production of fruit juice; to degum fibers in the textile and paper industries; during fermentation in the coffee and tea industries; to increase output and shorten the process of oil extraction; to remove pectin-containing materials during the treatment of industrial effluents; and during the preliminary treatment of grape juice in the wine industry (Lara-Márquez *et al*., 2011a).

This study was the first to identify and characterize the gene encoding pectate lyase in *C. lindemuthianum* during the necrotrophic phase. The pectate lyase encoded by the *pecCl*1 gene is an important factor in the aggressiveness of *C. lindemuthianum*. Analysis of pectate lyase might not only contribute to the understanding of anthracnose in the common bean but might also lead to the discovery of additional targets for the control of anthracnose.

Acknowledgements

This research was supported by the following Brazilian agencies: the Minas Gerais Science Foundation (FAPEMIG–Fundação de Amparo à Pesquisa do Estado de Minas Gerais), the Brazilian Federal Agency of Support and Evaluation of Postgraduate Education (CAPES–Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and the National Council of Scientific and Technological Development (CNPq–Conselho Nacional de Desenvolvimento Científico e Tecnológico).

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