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Use of *Agrobacterium tumefaciens*-mediated transformation for the inactivation of pectinase genes in *Colletotrichum lindemuthianum*

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Abstract The fungus *Colletotrichum lindemuthianum* has genes that encode different enzymes involved in plant cell wall degradation such as pectinases, which are important for the pathogenicity of various fungi. To analyze the function of pectinase-encoding genes in *C. lindemuthianum*, the expression of these genes during the interaction with the common bean was evaluated by qPCR. Using these results, the transformation technique mediated by *Agrobacterium tumefaciens* was performed for the isolation of mutants (ATMT). The expression of 12 genes that code for pectinases in *C. lindemuthianum* was analyzed. Nine genes displayed differential expression only in the necrotrophic phase, and two only in the biotrophic phase. From the results obtained, four genes were selected for the isolation of mutants. From the 180 transformants analyzed, two mutants for the *pecCl6* gene were obtained. The Δ*pecCl6* mutants showed no difference in morphology, growth, sporulation, or pathogenicity compared to the wild type. No mutants were isolated from the other analyzed genes. The expression of pectinase-encoding genes indicates the importance of these enzymes during the interaction of *C. lindemuthianum* with the common bean. The advantages of using ATMT in *C. lindemuthianum* were its ability to present a single copy of the cassette in the genome of the transformants. A disadvantage was the low number of mutants in relation to the number of transformants obtained. Thus, the ATMT system was not efficient for the deletion of genes encoding pectinases in *C. lindemuthianum*. The results revealed that the *pecCl6* gene is not essential for the pathogenicity of *C. lindemuthianum*.

Keywords Pectinases · Gene expression · Anthracnose · Phytopathogenicity

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Introduction

The common bean (*Phaseolus vulgaris* L.) originated in the Americas, and its grains are mainly used as a source of protein in human food in several countries worldwide (Myers & Kmiecik, 2017). The productivity of this crop is reduced by the presence of anthracnose disease in cultivated fields when the meteorologic conditions are favorable for the development of the pathogen (Kumar et al., 1999). This disease is

caused by the fungus *Colletotrichum lindemuthianum* (Sacc. & Magnus), which is known to have multiple physiological races, making it difficult to control (dos Santos et al., 2012).

Like most fungi of the *Colletotrichum* genus, *C. lindemuthianum* presents a hemibiotrophic infection strategy. During the initial phase (the biotrophic phase) the pathogen penetrates the host tissue and develops primary colonization structures without causing damage to the host (Perfect et al., 1999). After a certain period, the pathogen hyphae are modified and colonization of other host cells occurs, which is preceded by tissue maceration by enzymatic action and cell death (the necrotrophic phase) (O'Connell et al., 1985). The symptoms of the disease observed in plants result from the action of enzymes that act in the maceration of plant tissue, such as pectinases, produced by the phytopathogen (Villa-Rivera et al., 2016). As pectin is one of the main components of the plant cell wall, fungi require a large complement of enzymes for its degradation (Glass et al., 2013). Due to the involvement of pectinases during the pathogenesis process, these enzymes are considered phytopathogen virulence factors (Schmitz et al., 2019).

The role of pectinases has been studied in many phytopathogenic fungi, as they are involved in the various stages of pathogen development (Yarullina et al., 2016). In particular, fungi of the genus *Colletotrichum* have an abundance of pectinolytic enzymes and a high expression of genes that encode these enzymes during host infection (Gan et al., 2012), and these enzymes have been studied for their involvement in the pathogenicity of several other fungi, along with *Colletotrichum* (Schmitz et al., 2019). The high number of pectinolytic enzymes produced by fungi causes challenges in determining their relative importance for the development of the disease; therefore, the use of techniques such as site-directed mutagenesis and analysis and differential gene expression helps in to identify the function of genes related to pathogenicity (Ruiz et al., 2015). Allowing the analysis of gene expression enables the monitoring of the transcription of genes related to pathogenicity at different stages of the pathogen-host interaction (Fontenelle et al., 2017). Through site-directed mutagenesis, it is possible to inactivate one or more genes of interest and analyze the behavior of the fungus in its interaction with its host (Ruiz et al., 2015).

A gene encoding a pectate lyase (*pecCl*1) was differentially expressed in the interaction of *C. lindemuthianum* with common bean using suppressive subtractive hybridization, (Fontenelle et al., 2017). The same gene was functionally evaluated through site-directed mutation (Cnossen-Fassoni et al., 2013) and proved to be important in the necrotrophic phase of the pathogen, since the typical symptoms of the disease were reduced in its absence (Cnossen-Fassoni et al., 2013).

For functional analysis by site-directed mutagenesis, the use of inactivation cassette construction methods and fungal transformation is required. Some construction methods are based on successive cloning (Rogers et al., 2000) or different rounds of PCR to construct a deletion cassette, as in the split-marker method (Catlett et al., 2003). Cassettes constructed by these methods were used for protoplast-mediated transformation (PMT). The one-step construction of agrobacterium-recombination-ready plasmids (OSCAR) was developed for the rapid construction of deletion cassettes used for *Agrobacterium tumefaciens*-mediated transformation (ATMT) (Paz et al., 2011). ATMT has been used in different fungi to inactivate known genes through site-directed mutagenesis or to discover new genes involved in pathogenicity through random mutagenesis (Korn et al., 2015).

Thus, the main objectives of this study were to analyze the expression of genes encoding pectinases in *C. lindemuthianum* during interaction with the common bean by qPCR and to functionally analyze candidate genes by site-directed mutagenesis using *A. tumefaciens*-mediated transformation.

Material and methods

Microorganisms and growing conditions

Colletotrichum lindemuthianum isolate A_2 2–3 belonging to the physiological race 89 and the strain C58C1 of *A. tumefaciens* from the collection of the Laboratório de Genética Molecular de Microrganismos/Bioagro of the Universidade Federal de Viçosa were used. To maintain the culture and induce sporulation of wild type and mutant strains, the fungi were cultivated in YMC medium (10 g L^{-1} malt extract; 2 g L^{-1} yeast extract, and 15 g L^{-1} agar) at 22 °C for 6 days. *Agrobacterium tumefaciens* was maintained

in LB medium with selection antibiotics (50 µg/mL rifampicin and 100 µg/mL spectinomycin). *Escherichia coli* Top10 (Invitrogen) was used for plasmid multiplication.

For RNA extraction, approximately 10^6 wild-type conidia/mL were inoculated in buffered mineral medium (MMT) pH 6.8 (K_2HPO_4 6.98 g L^{-1}, KH_2PO_4 5.44 g L^{-1}, and $(NH_4)_2SO_4$ 1.0 g L^{-1}), supplemented with $MgSO_4.7H_2O$ 1.1 g L^{-1} and glucose 10 g L^{-1}, and cultured without agitation for 5 days at 22 °C. For DNA extraction for Southern blotting, approximately 10^6 conidia/mL were inoculated in GPYECH medium (glucose 20 g L^{-1}, peptone 5 g L^{-1}, yeast extract 1.0 g L^{-1}, and hydrolyzed casein 1.0 g L^{-1}) and cultivated with agitation at 150 rpm for 4 days at 22 °C.

The co-cultivation of *C. lindemuthianum* with *A. tumefaciens* was performed in solid GI medium, composed of AB salts 1* (NH_4Cl 1 gL^{-1}, $MgSO_4.7H_2O$ 0.3 g L^{-1}, and KCl 0.15 g L^{-1}), AB salts 2* ($CaCl_2.2H_2O$ 10 mg L^{-1} and $FeSO_4$ 2.5 mg L^{-1}), GI phosphate ($NaH_2PO_4.H_2O$ 0.69 g L^{-1}), GI carbon (glucose 1.8 g L^{-1} and glycerol 5 mL L^{-1}), GI MES* pH 5.2 (4-Morpholineethanesulfonic acid 9.75 g L^{-1}), and 1.5% agar. GI phosphate was not added in the liquid GI medium. The star notation (*) indicates filter sterilization.

Interaction between *C. lindemuthianum* and *P. vulgaris*

To observe the infection and colonization processes established by the fungus *C. lindemuthianum*, detached leaves of Pérola commom bean, susceptible to anthracnose, were inoculated with 10^6 conidia/mL. The leaves were kept in Petri dishes for up to five days at 22 °C. Water was used as the negative control. At the times of 24, 48, 72, 96 and 120 h after inoculation, microscope slides were prepared with free-hand cut of the leaf veins. The microscope slides were prepared with a lactoglycerol solution (25% lactic acid; 50% glycerol) or cotton blue-lactoglycerol (25% lactic acid; 50% glycerol; 0.05% cotton blue). The microscope slides were observed in an Olympus BX53 optical microscope and the micrographs were captured by the coupled camera using the cellSens software.

RNA extraction and cDNA synthesis

The cotyledon leaves of Pérola common bean plants were inoculated 10 days post-germination with a suspension of 10^7 conidia/mL using a brush. The plants were kept in a mist chamber, and the leaves were collected every 24 h for 5 days. As a negative control, the fungus was grown on MMT for 5 days. Three biological repetitions were performed.

Total RNA from bean leaves inoculated with the phytopathogen at different times and from the fungus grown in culture medium was extracted using the Trizol® reagent (Invitrogen) following the procedures described by Teixeira et al., (2014). RNA samples were treated with RQ1 DNase (Promega) and cDNA synthesis was performed using 1 µg of treated RNA with the ImProm-II Reverse Transcription System kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's recommendations.

Gene selection and evaluation of differential expression by qPCR

The pectinase-encoding genes in *C. lindemuthianum* (unpublished data) were used for mapping of the transcriptome under construction of the interaction of *C. lindemuthianum* with common bean at different times of infection (unpublished data): 24 h, 72 h, and 120 h after inoculation. Genes that showed high expression levels during an infection time were selected for validation by quantitative PCR (Supplementary Table 1; Supplementary file 1). Specific primers for these genes were designed using the online tool PRIMER-3Plus (Untergasser et al., 2007).

Quantitative PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), and the CFX96 Touch TM Real-Time PCR Detection System (BIO-RAD) was used for the readings. The primers used for each gene are listed in Supplementary Table 2. The efficiency and coefficient of determination (R^2) of each primer pair were calculated. Relative quantification was performed using the standard curve obtained for each gene using the linear equation that relates the mean Ct and log10 of the cDNA concentration. The actin gene was used to normalize expression levels. The analysis of the results was performed using ANOVA

and Tukey's test using the GraphPad Prism 5.0 software (GraphPad Inc.).

Construction of deletion cassettes

The one-step construction of agrobacterium-recombination-ready plasmid (OSCAR) method (Paz et al., 2011) was used to construct the binary vector deletion cassettes. Approximately 1 Kb from the flanking regions of the target genes were separately amplified by PCR using primers with attB recombination sequences in the 5' region (Supplementary Table 3). PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega).

A 5 µl BP Clonase (Invitrogen) reaction was constructed using 1 µL of each of the following: 15–20 ng of a mixture of the purified PCR products, 1 µL with 60 ng of plasmid pA-Hyg-OSCAR, 1 µL with 60 ng of plasmid pOSCAR, 1 µL of BP Clonase II enzyme mix (Invitrogen), and 1 µL TE (10 mM Tris–HCl; 5 mM EDTA). The reaction mixture was incubated for 16 h at 25 °C. The reaction product was used to transform competent *E. coli* TOP10 cells according to the method described by Sambrook et al. (1989). Selection was performed in LB medium containing 100 µg/mL spectinomycin. The constructs were checked by simultaneous digestion with the restriction enzymes *Kpn*I and *Hind*III and by PCR.

Competent cells of *A. tumefaciens* C58C1 were transformed with vectors by heat shock (Wise et al., 2006). Transformants were selected in LB containing 100 µg/mL spectinomycin and 50 µg/mL rifampicin.

Transformation of C. lindemuthianum

The *A. tumefaciens* C58C1 strains carrying the different deletion cassettes were cultured in 50 mL of LB medium containing the selection antibiotics (100 µg/mL spectinomycin and 50 µg/mL rifampicin) for 16 h at 28 °C with stirring at 150 rpm until 0.3 – 0.4 (OD 600). An aliquot was removed, centrifuged, and the pellet was resuspended in GI medium and adjusted to 0.4 (O.D.600).

The bacterial cell suspension (150 µL) was mixed with 150 µL of a solution containing 10^7 conidia/mL of *C. lindemuthianum*, and 100 µL of the mixture was inoculated onto cellophane discs placed in three Petri dishes with GI agar medium containing 200 µM acetosyringone (Sigma-Aldrich). The plates were incubated at 22 °C for 48 h in the dark. After co-cultivation, the cellophane paper containing the inoculum was inverted and transferred to PDA plates supplemented with 60 µg/mL hygromycin and 100 µg/mL cefotaxime and incubated for 48 h at 22 °C. The cellophane paper was removed, and a thin layer of water agar containing 60 µg/mL hygromycin and 100 µg/mL cefotaxime was added to the culture medium. The plates were incubated at 22 °C until the growth of the transforming colonies.

DNA extraction

To rapidly extract DNA for PCR testing, a mycelium disk of transformants in culture medium was macerated in 500 µL of extraction buffer (0.2% SDS and 50 mM EDTA) and incubated at 65 °C for 30 min. The macerate was centrifuged for 15 min at 12,000 × g. The supernatant was transferred to a new tube and 0.5 volume of 7.5 M ammonium acetate and 1.5 volume of isopropanol were added. The mixture was shaken and centrifuged for 1 min at 12,000 × g. The pellet was washed with 70% ethanol, dried, and suspended in 50 µL of water.

For the Southern blot, the total DNA of the transformants was extracted following the methodology described by Specht et al. (1982), with modifications (Teixeira et al., 2011).

Transformant analysis: PCR and Southern blot

Initial selection was performed using PCR. Primers were used to amplify part of the coding and flanking regions of the target gene, the coding region of the *hph* gene (providing resistance to hygromycin), and the external regions of the target gene. To analyze the integration profile of the deletion cassette, the total DNA of the transformants was digested with restriction enzymes. The generated fragments were separated by electrophoresis on a 0.8% agarose gel. DNA was transferred to a Hybond-N + membrane (Amersham Biosciences) according to the methodology described by Sambrook et al. (1989). One of the flanking regions was used as a probe, and probe labeling was performed using the DIG Probe Synthesis PCR kit (Roche Applied Science), following the manufacturer's instructions. Signal hybridization and detection were performed using the DIG High Prime DNA Labeling and Detection Starter

Kit (Roche Applied Science), following the manufacturer's recommendations.

Pathogenicity testing and symptom assessment

The pathogenicity test was conducted using plants and detached leaves of the Pérola variety of the common bean. A suspension of 10^6 conidia/mL of the mutant and wild fungi was inoculated on the abaxial and adaxial faces of the cotyledonary leaves using a brush. Water was used as the negative control. The inoculated plants were placed in mist chambers, and the detached leaves were incubated in BOD at 22 °C. The development of symptoms (necrotic lesions on leaf veins) was followed for 7 days.

Results

Interaction between *C. lindemuthianum* and *P. vulgaris*

The process of infection and colonization of *P. vulgaris* by *C. lindemuthianum* was followed through microscopic observations (Fig. 1). Up to 72 h after inoculation, it is not possible to observe typical symptoms of the disease (Fig. 1a). From 96 h, it is possible to observe the characteristics symptoms of the disease, as necrosis in the leaf veins (Fig. 1b). Twenty-four hours after inoculation, it is possible to observe the formation of a melanized appressorium of *C. lindemuthianum* (Fig. 1d,1e). Forty-eight hours after inoculation, it is observed the primary hyphae formed from the appressorium inside the plant cell, starting the biotrophic phase of the fungus (Fig. 1f,1g). At 72 h, the initial development of thicker primary hyphae is observed, colonizing one or more plant cells (Fig. 1h). At 96 h, it is observed the colonization of adjacent cells of the host tissue by the secondary hypha, which is thinner, starting the necrotrophic phase with the collapse of plant cells together with the appearance of the characteristic symptoms (Fig. 1i). At 120 h, the plant tissue is largely colonized by the secondary hyphae of the phytopathogen, and during this period it is already observed the presence of acervulus (Fig. 1j-l).

Expression of pectinase-encoding genes in *C. lindemuthianum* in the interaction with common bean

Gene expression analysis was performed for 12 genes encoding different putative pectinases (Fig. 2). Of these, six coded for pectate lyase (*pecCl*2, *pecCl*3, *pecCl*4, *pecCl*5, *pecCl*6, and *pecCl*7), three for polygalacturonase (*pgaCl*2, *pgaCl*3, and *pgaCl*4), two for pectin methylesterases (*pmeCl*1 and *pmeCl*2), and one for pectin lyase (*pnlCl*3). The analyzed evaluation times refer to the phases of pathogen infection, which are the appressorial (24 h), biotrophic (48 h and 72 h), and necrotrophic (96 h and 120 h). With the exception of the *pgaCl*4 gene, transcripts were detected for all other genes at all times analyzed. Differential expression was observed in most of the genes analyzed (*pecCl*2, *pecCl*3, *pecCl*4, *pecCl*5, *pecCl*7, *pmeCl*1, *pnlCl*3, *pgaCl*2, and *pgaCl*4) only in the necrotrophic phase, and none in the appressorial phase. Two genes (*pecCl*6 and *pmeCl*2) showed differential expression in the biotrophic phase, and one (*pgaCl*3) in the biotrophic and necrotrophic phases. Three genes (*pecCl*7, *pgaCl*2, and *pnlCl*3) showed differential expression when the fungus was grown in buffered mineral medium, with glucose as the sole carbon source.

Transformation of *C. lindemuthianum* and analysis of transformants

Four genes (*pecCl*6, *pecCl*7, *pmeCl*2, and *pgaCl*3) with different expression profiles were chosen for functional analysis through deletion. The use of ATMT of *C. lindemuthianum* with the aim of gene deletion was reported for the first time in this study. After the transformation of *C. lindemuthianum*, 35–56 transformants were obtained for each target gene (Table 1), and initial analyses were performed by PCR with specific primers for the partial amplification of the coding region of each gene. The presence of an amplicon indicated that the target gene was not deleted in the analyzed transformant. In the absence of a band, a second PCR was performed using primers to amplify part of the *hph* gene, confirming the presence of the cassette in the correct locus. None of the transformants analyzed by PCR for the four target genes showed gene deletions; therefore, it is possible that all transformants revealed ectopic integration of the deletion cassette. Five transformants for the

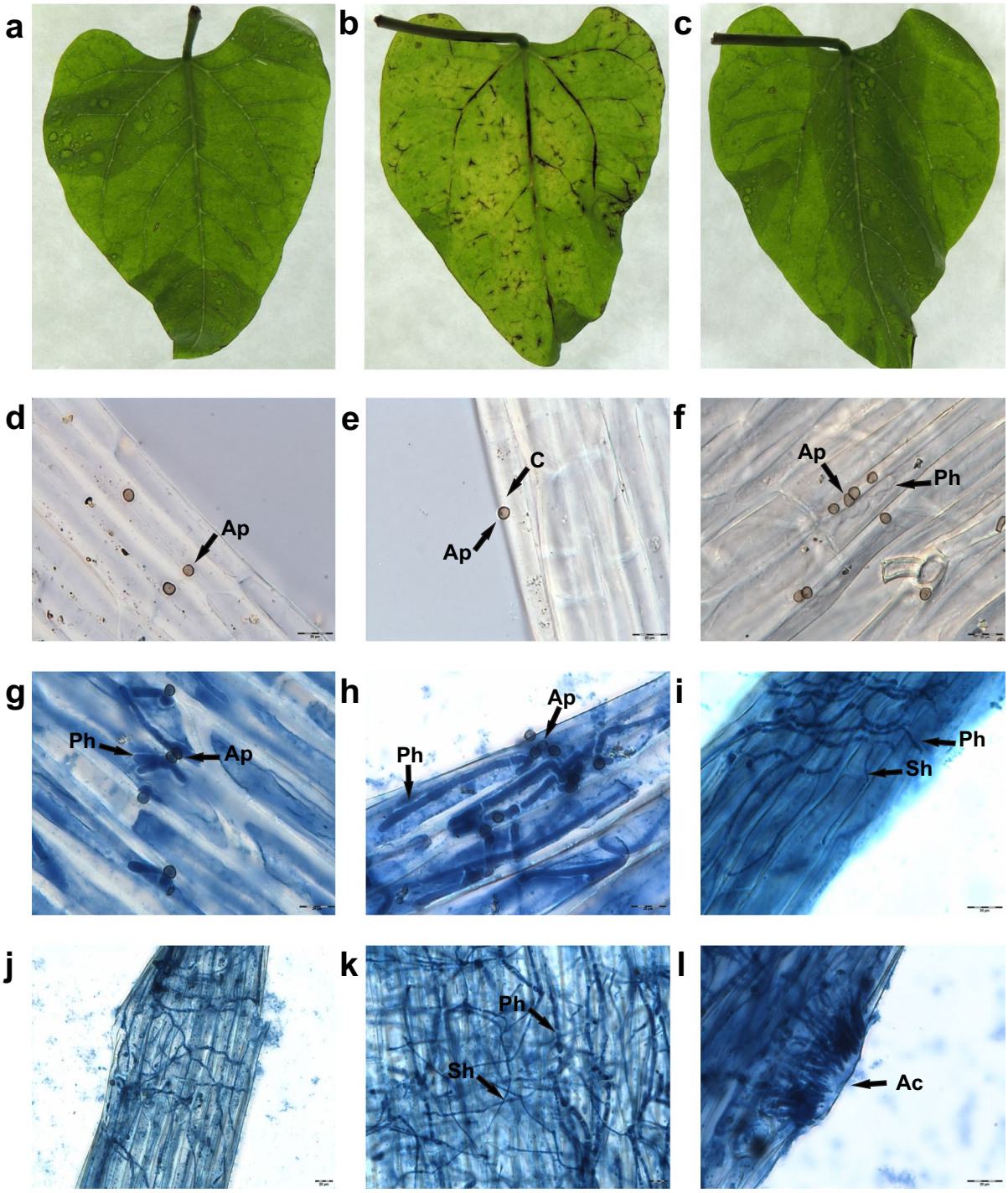

Fig. 1 Infection and colonization of common bean by *Colletotrichum lindemuthianum*. Common bean leaves 72 h (**a**) and 96 h (**b**) after inoculation with *C. lindemuthianum* and control (**c**). Micrographs of the structures formed by *C. lindemuthianum* at different times after inoculation: 24 h (d,e); 48 h (f,g); 72 h (h); 96 h (i); 120 h (j-m). Ap: appressorium; C: conidium; Ph: primary hypha; Sh: secondary hypha; Ac: acervulus

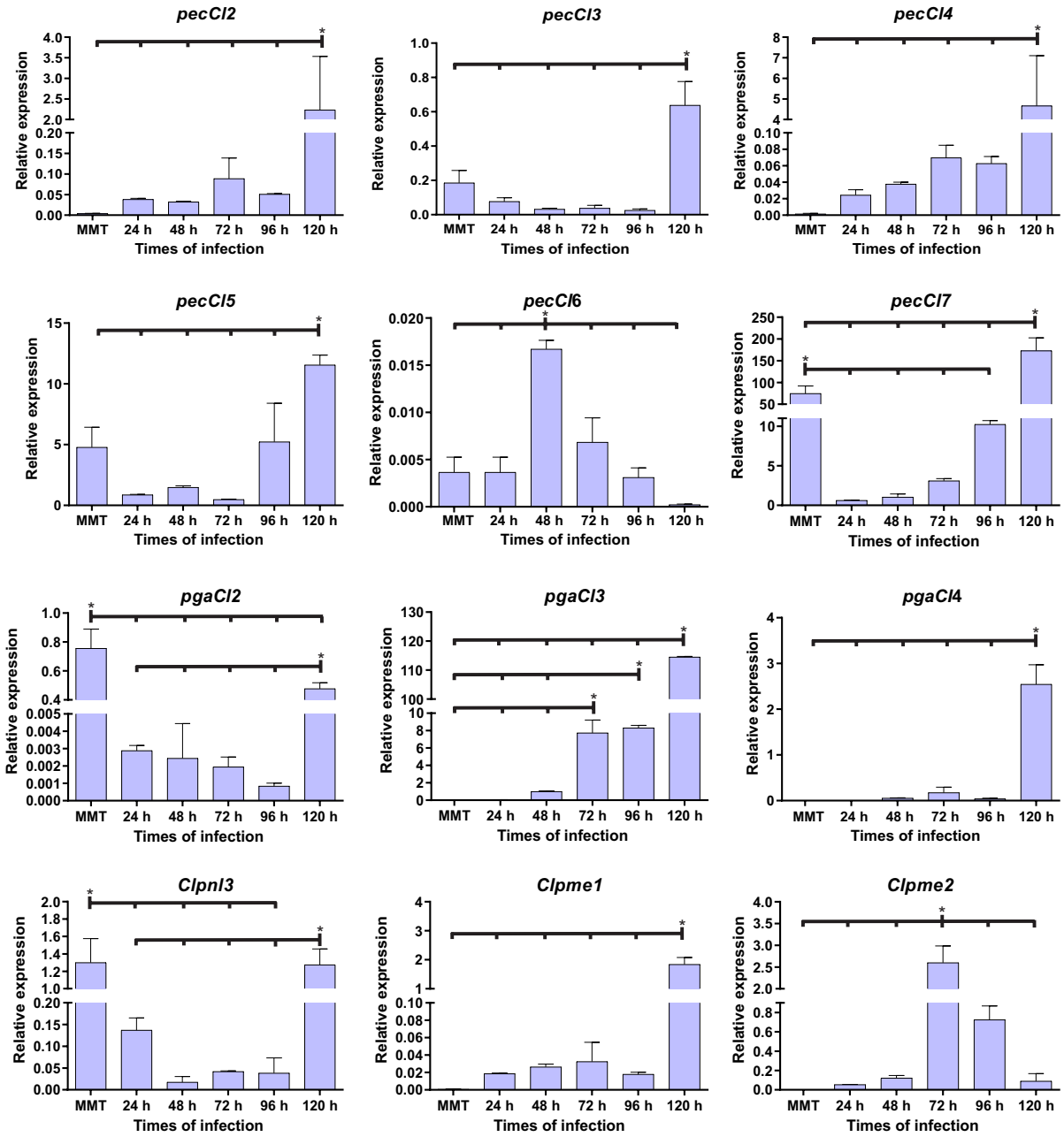

Fig. 2 Expression of genes from *C. lindemuthianum* encoding putative pectinases during the interaction of the fungus with the common bean host at different times of infection. (*) indicates the treatments in which the expression was considered differential by means of ANOVA and Tukey's test at 5% significance. MMT: buffered mineral medium

pgaCl3 gene were randomly chosen to check for the presence of the deletion cassette by Southern blotting (Fig. 3). *Sal*I was used as the restriction enzyme. The 5' flanking region of the gene was used as the probe, and the same region was used for the construction of the deletion cassette. In the wild-type strain, a single band of 2.9 kb in size was expected, and if the *pgaCl3* gene was deleted, a single 4.3 kb band should be observed (Fig. 3a). All five transformants analyzed showed a band corresponding to the *pgaCl3* gene

Table 1 Number of transformants obtained in each transformation experiment

Transformation	Gene	Number of transformants obtained and analyzed	Number of mutants
1	*pecCl6*	38	0
	pecCl7	35	0
	pmeCl2	56	0
	pgaCl3	45	0
2	*pecCl6*	154	2

and a second band correlating to the deletion cassette (Fig. 3b). A single integration of the deletion cassette was observed; however, all integrations were ectopic.

A new ATMT was performed using the *pecCl6* gene deletion cassette only. To obtain a greater number of transformants, the transformation protocol was repeated using 1.2 mL of the conidia and bacteria mixture equally distributed in 12 plates with GI agar medium for co-culture. After the second transformation of the fungus *C. lindemuthianum*, 154 transformants of the *pecCl6* gene were obtained (Table 1). Analysis of the presence of *pecCl6* for all transformants was performed by PCR (data not shown). Only two transformants did not exhibit target gene amplification. A second PCR confirmation was performed to check for the presence of the deletion cassette at the target gene locus. The PCR results indicated the replacement of the *pecCl6* gene with the deletion cassette (data not shown). The copy number of the deletion cassette was checked in the two mutants obtained by Southern blotting (Fig. 3). *BamH*I was used as the restriction enzyme with the 3' flanking region of the gene as a probe. In the wild-type strain, a single band with 5.5 kb in size was expected, or a 3.5 kb band if the *pecCl6* gene was replaced (Fig. 3c). The two analyzed transformants presented only one copy of the deletion cassette in the genome, which was in the *pecCl6* gene locus, confirming gene deletion (Fig. 3d).

Functional analysis of the *pecCl6* gene from *C. lindemuthianum*

The Δ*pecCl6* mutants did not show morphological differences from the wild-type strain of *C. lindemuthianum*. The growth rate and sporulation of Δ*pecCl6* mutants were not affected (data not shown). To assess the involvement of the *pecCl6* gene in the

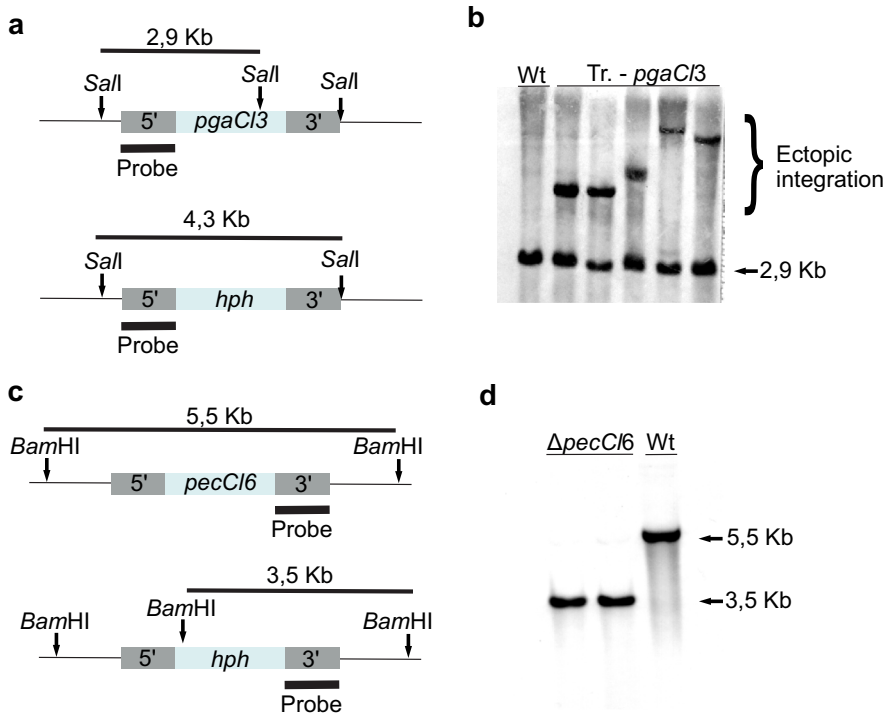

Fig. 3 Evaluation of the copy number of the deletion cassette in the transformant genomes. (**a** and **c**) Representation of the restriction enzyme recognition sites in the genome of the strains used for hybridization, and expected fragment size after enzyme digestion. (**b** and **d**) Integration profile of the deletion cassette in the genome of *C. lindemuthianum*. (**b**) ectopic integration. (**c**) homologous integration. Tr: transformants for the target gene *pgaCl3*

pathogenicity of *C. lindemuthianum*, the Δ*pecCl*6 mutants and the wild-type strain were tested for pathogenicity in bean plants of the Pérola variety that are susceptible to anthracnose, to assess the development of disease symptoms (Fig. 4). The typical symptoms of the disease began on the fourth day of inoculation, the beginning of the necrotrophic phase, in both the plant experiment and that with detached leaves for the Δ*pecCl*6 mutants and the wild type. On the fifth day after inoculation, the plants and detached leaves inoculated with the Δ*pecCl*6 mutants presented disease symptoms similar to those that received the wild-type isolate. The disease symptoms caused by Δ*pecCl*6 mutants did not differ from those caused by the wild-type strain, even after seven days of evaluation. These results indicate that the pecCl6 gene is not essential for the pathogenicity of *C. lindemuthianum*.

Discussion

All genes analyzed by qPCR that encode putative pectinases in *C. lindemuthianum* were expressed, and 75% of them were differentially expressed during the necrotrophic phase, when the host tissues were macerated by secreted enzymes. Increased expression of certain genes in the necrotrophic phase was also observed in other studies on *Colletotrichum* spp. Two genes encoding polygalacturonases in *C. lindemuthianum*, *Clpg*1 and *Clpg*2 (*pgaCl*2), were analyzed for expression during the necrotrophic phase, with only *Clpg*1 expression detected (Centis et al., 1997). Two genes encoding pectate lyase in *Colletotrichum gloeosporioides* (*pel*-1 and *pel*-2) were analyzed with only *pel*-2 showing expression, which occurred at the end of the biotrophic phase and continued to increase until the necrotrophic phase (Shih et al., 2000).

Of the 12 genes analyzed, 11 showed expression in the initial phase of infection; however, two genes showed a differential expression profile only in the biotrophic phase. The products of these genes may be involved in the initial process of interaction with the host. It is not evident whether only the turgor pressure exerted by the appressorium is sufficient to penetrate the plant tissue and start the infection process, if lytic enzymes are involved, or whether both are necessary (Chen et al., 2004; Walton, 1994). Some enzymes may participate in this process, such as pectinases and cutinases, but their roles have not been clearly demonstrated (Dickman et al., 1982; Dumas et al., 1999; Kleemann et al., 2008). The *Clpg*2 (*pgaCl*2) gene, which encodes a polygalacturonase, is detected only in the appressorial phase and not in the late phases of colonization of the common bean by *C. lindemuthianum* (Dumas et al., 1999; Herbert et al., 2004). Using immunofluorescence microscopy, the presence of CLPG2 (PgaCl2) was detected in the cell

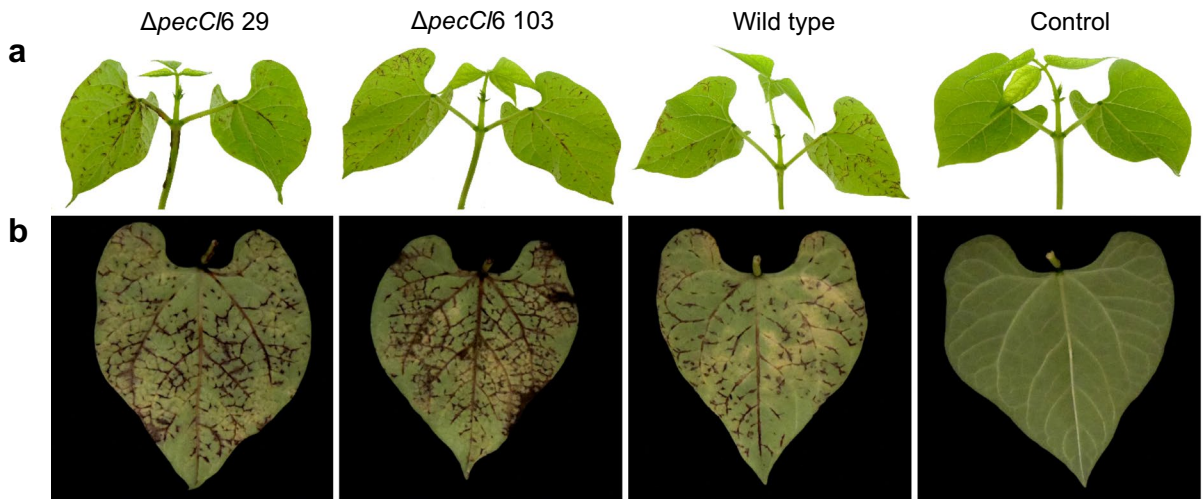

Fig. 4 Pathogenicity of mutant and wild-type strains of *C. lindemuthianum* in the common bean. **a** Plants kept in a mist chamber. **b** Detached leaves kept in BOD at 22 °C

wall of *C. lindemuthianum* during the formation of the appressorium in its interaction with its host (Herbert et al., 2004). The *Clpg*2 gene promoter (*pgaCl*2) fused to the GFP reporter gene allowing reporter gene expression in the initial phase of appressorium development (Dumas et al., 1999). When the expression of the *Clpg*2 gene (*pgaCl*2) was analyzed in this study, it occurred mainly in the necrotrophic phase. This variation may be due to the strain of the fungus used or the host genotype, as apparently pathogenic and non-pathogenic races of *C. lindemuthianum* presented different expression profiles for the *Clpn*2 gene in variable carbon sources (Lara-Márquez et al., 2011).

The expression of some genes (*pecCl*7, *pgaCl*2, and *pnlCl*3) that encode putative pectinases in *C. lindemuthianum* in a glucose-only medium indicates that the regulatory system is not the same for all genes. The promoter regions of pectinase-encoding genes in *C. lindemuthianum* were analyzed and variation in the presence and amount of putative binding sites for different transcriptional factors, including CreA, was observed (unpublished data). The transcription factor CreA is responsible for regulating the repression of genes involved in the metabolism of other carbon sources in the presence of glucose (Cubero & Scazzocchio, 1994). Of the 12 genes in which expression was analyzed, two (*pgaCl*2 and *pnlCl*3) did not present putative binding sites for the CreA protein in 500 base pairs (bp) before the initiation codon in their promoter region, and one (*pecCl*7) did not reveal putative sites for CreA in the 1200 bp analyzed. This suggests that these three genes are not regulated by CreA.

The OSCAR cassette construction method used is simpler and less laborious than other methods and allows for the use of a vector containing the deletion cassette by both ATMT and PMT. The transformation of *C. lindemuthianum* for the functional study of genes by ATMT was performed for the first time in this study, and was used to generate mutants of the chosen genes. Using this protocol, it was possible to obtain transformants; however, the number of transformants obtained in this study was low in relation to the number obtained by the amount of fungus spores used compared to other *Colletotrichum* species (Haghighi et al., 2013; Maruthachalam et al., 2008). A greater number of transformants can be obtained by modifying the protocol used for ATMT transformation of *C. lindemuthianum*, by changing the co-cultivation time, the proportion of bacteria and conidia in the fungus, or the concentration of acetosyringone, which has been performed with different fungi of the *Colletotrichum* genus to increase the amount of transformants obtained by the ATMT method (Auyong et al., 2012; Haghighi et al., 2013; Maruthachalam et al., 2008; Talhinhas et al., 2008).

Considering the number of mutants obtained in relation to the number of analyzed transformants, the ATMT method for deletion in *C. lindemuthianum* proved inefficient. For the three target genes, no mutants were detected for the average number of 40 transformants analyzed, and only the *pecCl*6 gene obtained mutants (two), after the analysis of more than 180 transformants, which indicates an efficiency lower than 2%. In *C. lindemuthianum*, the split-marker method for obtaining deletion cassettes and PMT has previously been used for the functional study of genes. To inactivate the *pecCl*1 gene of *C. lindemuthianum*, 16 transformants were obtained, three of which showed a single homologous recombination (Cnossen-Fassoni et al., 2013). In a study of a MFS transporter (*mfsCl*1) in *C. lindemuthianum*, one mutant with a single homologous integration between 10 analyzed transformants were obtained (Pereira et al., 2013). The split-marker method with PMT appears to be more efficient for *C. lindemuthianum* than the ATMT method. For certain fungi, ATMT can be advantageous owing to the high efficiency of obtaining mutants. Paz et al. (2011) described the OSCAR method for construction of the deletion cassette and showed that among 22 and 12 transformants analyzed for deletion of the VDAG_02161 and VDAG_09930 genes from *Verticillium dahliae*, seven and three transformants showed homologous integration, respectively. In *Pestalotiopsis microspora*, 80% of the transformants obtained by ATMT and OSCAR showed homologous integration (Yu et al., 2015), with less than 7% in *Metarhizium robertsii* (Xu et al., 2014). In *Colletotrichum higginsianum*, homologous integration by ATMT occurred in 15% of the cases (Korn et al., 2015). This demonstrates that the percentage of mutants among the transformants varies among fungal species, even for those of the same genus. All of the transformants analyzed by Southern blot in this study presented only one copy of the deletion cassette in the genome. One of the advantages of ATMT is the number of integrations of the deletion cassette in the fungal genome, since the integration of

a single copy of the cassette occurs more frequently (Michielse et al., 2005).

One of the main reasons for the low number of mutants obtained in *C. lindemuthianum* may be the greater efficiency of the non-homologous end joining (NHEJ) DNA repair mechanism compared to homologous recombination (HR), as already reported for other fungi (Krappmann, 2007). For gene inactivation in filamentous fungi, increasing the size of flanking regions increases the number of transformants with homologous recombination (Krappmann et al., 2006; Ninomiya et al., 2004) and it has been reported that at least 1 Kb of flanking regions favors HR (Korn et al., 2015; Lu et al., 2014; Paz et al., 2011). In this study, the flanking regions used are close to 1 Kb, which would be sufficient for this type of event as previously reported (Cnossen-Fassoni et al., 2013). Based on this, it is likely that in *C. lindemuthianum* the NHEJ is more efficient than the HR, which resulted in a low number of mutants in relation to the number of transformants obtained.

An alternative to increase the number of mutants with homologous integration in *C. lindemuthianum* is the use of mutants with deletion of the genes encoding Ku70 or Ku80 proteins as the recipient strain. The *ku70/80* genes are involved in the repair mechanism by non-homologous recombination, and when one of these genes is inactivated, this repair system is compromised, making ectopic DNA integration difficult. The number of homologous integrations in *C. higginsianum* ΔChku80 increased from 60 to 90% (Korn et al., 2015), and in *M. robertsii* ΔMrKu70, it was greater than 90% (Xu et al., 2014). Obtaining a smaller number of transformants will likely not be a limiting factor for gene deletion in *C. lindemuthianum* if using mutants for the ku70/80 genes, given the increase in the efficiency of homologous integration.

No change was observed in the symptoms of the host infected by the different Δ*pecCl*6 mutants in relation to the wild-type strain, indicating that this gene alone may not be essential for the pathogenicity of the fungus *C. lindemuthianum*. In other fungi, the inactivation of certain pectinases did not change or reduce the disease symptoms. Cnossen-Fassoni et al. (2013) demonstrated that the inactivation of the *pecCl*1 gene caused a reduction in symptoms caused by the fungus *C. lindemuthianum* in the common bean, although they were not completely eradicated. In addition, the deletion of two polygalacturonases in distinct mutants did not interfere with pathogenicity in *Fusarium oxysporum* (Ruiz et al., 2015).

In *C. lindemuthianum*, more than one gene codes for enzymes with the same activity, and several of these genes are expressed during the same phase. Thus, the maceration of plant tissues may occur from the sum of the activities of different enzymes during the same phase. Enzyme synergism is indicated for complete virulence, as observed in *Fusarium graminearum* mutants for *pg1* and *xyr1* genes, which encode polygalacturonase and xylanase, respectively. Strains with a single mutation for each gene showed no reduction in aggressiveness, whereas a significant reduction was observed with a double mutant in the plants evaluated (Paccanaro et al., 2017). Similarly, in *F. oxysporum*, only the double mutant for two polygalacturonases, Δ*pg1* and Δ*pgx6*, showed a reduction in disease symptoms in tomato (Ruiz et al., 2015).

Differential gene expression analysis in *C. lindemuthianum* can serve as a guide for selecting candidate genes for functional studies, although efficient transformation conditions for gene inactivation in *C. lindemuthianum* are yet to be determined. The results presented here show that analysis of a larger number of transformants is necessary to isolate mutants with homologous integration in the strain of *C. lindemuthianum* used in this study using ATMT under the conditions tested.

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Data availability The datasets generated during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Conflicts of interest/competing interests The authors declare that they have no conflict of interest.

Ethics approval Not applicable.

Consent to participate All authors contributed to the study conception and design. All authors read and approved the final manuscript.

Consent for publication All authors gave consent to submit for publication.

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