ORIGINAL RESEARCH

PacCl, a pH-responsive transcriptional regulator, is essential in the pathogenicity of *Colletotrichum lindemuthianum*, a causal agent of anthracnose in bean plants

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Abstract In fungi, the expression of genes encoding proteins related to parasitism is regulated by several factors, including pH. This study reports the structural and functional characterization of the *pacCl* gene, which encodes the transcription factor PacC of *C. lindemuthianum*. The *pacCl* gene showed reduced expression in acidic pH, and its transcription was activated by elevated extracellular pH. The importance of this gene was demonstrated by the development of a pacC1 disruption mutant line of *C. lindemuthianum*. The mutant line was able to penetrate the host tissue through differentiation of primary hyphae. However, it was not able to cause maceration on the infected plant tissue. The results suggest that PacCl is a regulator of gene activation, and its expression is required for fungal growth in alkaline conditions, as well as for the transcription of genes necessary for the passage from the biotrophic to the necrotrophic phase.

Keywords *Colletotrichum lindemuthianum* · Anthracnose · pH · PacC

Introduction

The genus *Colletotrichum* represents one of the most important groups of phytopathogenic fungi; it is widely distributed worldwide and occurs mainly in tropical and sub—tropical regions of Latin America. Many of these species have been used as models to study differentiation and plant–pathogen interaction (Bailey and Jeger 1992; Perfect et al. 1999).

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The species *Colletotrichum lindemuthianum* (Sacc. & Magnus) is a phytopathogen with a hemibiotrophic life cycle and is responsible for anthracnose of common bean (*Phaseolus vulgaris* L.), a widespread disease in Brazil, especially in the south and southeast and in mountainous areas such as the southern region of Minas Gerais (Ansari et al. 2004; Damasceno e Silva et al. 2007; Bonett et al. 2008). The use of phytopathogen-resistant cultivars is the main strategy to contain this disease in Brazil (Damasceno e Silva et al. 2007). However, due to the large genetic variability of *C. lindemuthianum*, the breakdown of resistance prevents the use of cultivars for long periods of time, thus hindering the establishment of a permanent solution to the disease (Rodriguez-Guera et al. 2003; Ansari et al. 2004; Mahuku and Riascos 2004; Barcellos et al. 2011). The large genetic and pathogenic variability found in *Colletotrichum* species has been extensively studied, and it can be partially explained by recombinant events and mutations caused by the presence of transposons (Casela and Frederiksen 1994; Santos et al. 2012), and by the presence of anastomoses among conidia (Roca et al. 2003; Castro-Prado et al. 2007; Rosada et al. 2010; Franco et al. 2011).

Along with trying to understand the genetic variability in *C. lindemuthianum*, many researchers have focused on the molecular and regulatory mechanisms involved in the pathogen–host interaction, from the initial contact until disease establishment. In this context, the ambient pH is the most important factor because to survive and multiply, micro-organisms must have mechanisms to adapt to different ambient conditions (Peñalva et al. 2008). In particular, in fungi, the ambient pH plays an important role in determining the transcriptional levels of many genes, influencing growth, physiology, and differentiation processes (Lamb et al. 2001). Moreover, in plant–pathogen interactions, the ambient pH can be dynamically changed by the fungi. While some fungi change the extracellular pH through the production of organic acids, such as oxalic, citric, and glucuronic acids, other fungi, as in the case of *Colletotrichum*, alkalize the medium by producing and releasing ammonium (Caracuel et al. 2003a; Kramer-Haimovich et al. 2006).

Considering the ambient pH, a regulatory system controlling gene expression was identified and characterized for the first time in the filamentous fungus *Aspergillus nidulans* (Caddick et al. 1986). Since then, this mechanism has been studied and characterized in a wide variety of filamentous fungi and yeasts, including *Saccharomyces cerevisiae* (Su and Mitchell 1993), *Yarrowia lipolytica* (Blanchin-Roland et al. 1994), *Penicillium chrysogenum* (Suárez and Peñalva 1996), *Candida albicans* (Brown and Gow 1999), *Sclerotinia sclerotiorum* (Rollins and Dickman 2001), *Fusarium oxysporum* (Caracuel et al. 2003a), *Ustilago maydis* (Elva et al. 2005), *Colletotrichum acutatum* (You et al. 2007), *Wangiella* (*Exophiala*) *dermatitidis* (Wang and Szaniszlo 2009), *Clonostachys rosea* (Zou et al. 2010), and *Fusarium graminearum* (Merhej et al. 2011).

The general function of this regulatory system is well characterized in *A. nidulans* and requires the expression and integrity of seven dedicated genes, designated *pacC*, *palA*, *palB*, *palC*, *palF*, *palH*, and *palI*. In this gene group, *pacC* plays a central role, as it encodes a transcriptional regulator directly involved with the activation and repression of genes controlled by the ambient pH. (Denison 2000; Peñalva and Arst 2002, 2004; Arst and Peñalva 2003; Peñalva et al. 2008). In this work, we characterized the nucleotide sequence that encodes the protein PacC from *C. lindemuthianum* (denoted PacCl) and functionally demonstrated its essential role as a pathogenic determinant in the interaction with its host.

Methods

Organisms and culture conditions

The experiments were conducted with the isolates UPS9, LV 49 (pathotype 81), and LF (pathotype 89 A_2 2–3) of the fungus *C. lindemuthianum*. These isolates were kept at −80 °C in 25 % glycerol, using a concentrated suspension of conidia (approximately 10^8 conidia/ml). When necessary, the isolates were activated in potato dextrose agar (PDA) culture medium, pH 6.8, or malt-agar medium (Parisot et al. 2002), for growth at a temperature of 22 °C for 7 days. The susceptible bean (*P. vulgaris* L.) cultivar La Victoire was used in the pathogenicity experiments, and the resistant cultivar Jalo was used together with La Victoire to measure the apoplastic pH in bean leaves.

Isolation and structural characterization of the pacCl gene

For the isolation of the *pacCl* gene, the methods described by Benton and Davis (1977) were used for DNA

hybridization to single plaques, from the genomic library of *C. lindemuthianum*, constructed in the Lambda EMBL3 vector using the Lambda EMBL3/BamHI Vector Kit (Stratagene®). For the screening, the probe was a plasmid containing the cDNA corresponding to the *pacCl* gene, obtained from a cDNA library constructed by the LLPM Laboratory of the Institute of Plant Biology, University of Paris XI, France. The primer pairs pacCl1/pacCl9 and pacCl10/pacCl11 (Table 1) were designed based on the cDNA sequences of the *pacCl* gene and used for complete sequencing by PCR and inverse PCR (Ochman et al. 1988), respectively. The developed clones were propagated in ultracompetent *E. coli* DH5α (Inoue et al. 1990). From the deduced amino acid sequence, the programs WoLF PSORT (Horton et al. 2007) and ngLOC (King and Guda 2007) were used for prediction of subcellular localization. For the phylogenetic inferences, the neighbour-joining reconstruction method in MEGA 6.0 software (Tamura et al. 2007) was applied, using homologous sequences deposited in GenBank. The sequences of 31 organisms, including filamentous fungi and yeasts, were used for the analysis. GenBank accession numbers: *A. nidulans* (CAA87390); *A. niger* (Q00203); *A. fumigatus* (XP_754424); *A. oryzae* (BAB20756); *A. parasiticus* (Q96UW0); *A. giganteus* (Q5XL24); *P. chrysogenum* (Q01864); *S. cerevisiae* (NP_011836); *C. albicans* (Q9UW14); *S. Sclerotiorum* (Q9P413); *A. chrysogenum* (Q96X49); *F. oxysporum* (Q870A3); *F. verticillioides* (Q873X0); *N. crassa* (Q7RVQ8); *C. glabrata* (Q6FV94); *D. hansenii* (Q6BSZ4); *K. Lactis* (XP-453982); *C. neoformans* (XP_572292); *M. grisea* (Q52B93); *Y. lipolytica* (CAA67927); *C. acutatum* (ABL96218); *T. harzianum* (ABK60115); *B. fuckeliana* (AAV54519); *C. dubliniensis* (Q873Y3); *G. fujikuroi* (Q8J1U9); *T. rubrum* (Q9C1A4); *U. maydis* (CAG34353); *C. gloeosporioides* (ACD56154); *P. brasiliensis* (AAN06812); *C. lindemuthianum* (JX679084). *C. graminicola* and *C. higginsianum*: *Colletotrichum* Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/).

pacCl gene expression analysis

pacCl gene expression was analyzed by northern blot. Total RNA was extracted from mycelia that had grown for 5 days in buffered liquid medium with an initial pH ranging from 3 to 9. Samples containing 20 µg of RNA

Table 1 Oligonucleotides used for sequencing and RT-PCR

Oligonucleotide	Sequence (5' → 3')
R	GCAGGCATGCAAGCTTGGCACTGGC
F	CGTGCACGAAGACGGGGGTTCCC
N	ACTTTATTGTCATAGTTTAGATCTATTTTG
S	ATTAATCCTTAAAAACTCCATTTCCACC CCT
Opac1	AAGCGCCCCCAGGACTTGAAGAAGC
Opac2	CTCAAAGATGATGGGCGACCTGGGC
Opac3	CGACGCTTAGCGTGGGCGAAGAAGT
Clgpdq	CCGCACTGCTGCTCAGAAC
Clgpdq2	CTTCGTCTCCACCGACATGA
RTpacCl3F	CAGGACGACGACGGTGAA G
RTpacCl4R	AGGACTTGCCGCAGGAACT
Ohph3	GAGATGCAATAGGTCAGGCTCTCGCTG
Ohph4	AAGCGCGGCCGTCTGGACCGATGGC
pacCl1	CAACTCCCCGAAGAGCAGTA
pacCl9	ATACGCAGGATGGGGTACAG
pacCl10	GCATTGACCGTAGGGACTTC
pacCl11	TACTGCTCTTCGGGGAGT TG

were separated by electrophoresis in agarose gel containing formaldehyde and transferred to nylon N^+ membranes (Amersham France SA, Les Ulis, France) by capillarity (Sambrook et al. 1989). Hybridizations were performed as previously described (Dufresne et al. 1998). Fragments of the *pacCl* gene obtained by PCR with the primers Opac1/Opac2 (Table 1) were used as probes. To measure the transcript of the constitutively expressed *clgpd* gene, the PCR product obtained with the primers Ogpd1/Ogpd2 was labelled and used as probe. The probes were ^{32}P-labelled using the Ready-To-Go DNA labeling kit (Pharmacia Biotech S.A., Saint Quentin-en-Yvelines, France).

qRT-PCR was performed to study the transcription of the *pacCl* during the course of infection of bean plants. Total RNA was extracted with TRIzol reagent (TRI Reagent, Sigma®). The cDNA synthesis was performed from 1.0 µg total RNA using the Im Prom-II^{TM} Reverse Transcription System kit (Promega). The *gpd* gene from *C. lindemuthianum* was used as an endogenous control. The expression analysis was performed according to the standard curve based method for relative real time PCR data processing, proposed by Larionov et al. (2005). The experiments were repeated two times using preparations of total RNA obtained from independent biological

samples. The sequences of the oligonucleotides used in this study are listed in Table 1.

Construction of pacCI gene-inactivation vector

Amplification products obtained by the PCR of genomic DNA and the RT-PCR of total RNA from *C. lindemuthianum* with the primers Opac1/Opac3 (Table 1) were cloned into the TOPO® TA vector (Invitrogen®), resulting in the plasmids pPacgen and pPacrt, respectively. A DNA fragment from pPacrt cleavaged with EcoRI was subcloned into the pBluescript II KS+vector (Stratagene®), producing pBSKpac. This plasmid was used in the construction of the inactivation vector, through the mutagenesis system GPS™-M (New England Biolabs®). The plasmid pGPS3. Hygro-Amp (kindly provided by Marc-Henri Lebrun, CNRS Bayer Cropscience) was used as a donor vector. For the transposition reaction, 40 ng of donor vector pGPS3. Hygro-Amp and 60 ng of the recipient pBSKpac were mixed with the transposase TnsABC. After the transposition reaction, the restriction enzyme PI-SceI was used for the destruction of the donor vector. The transposition reaction products were used to transform ultra-competent *E. coli* DH5α (Invitrogen®) cells in kanamycin selective medium. Clones of interest were selected by PCR with the primer pairs Opac1/N and Opac1/S (Table 1) and confirmed using the primer pair Opac1/Opac2 (Table 1), according to the conditions suggested by the manufacturer of the GPS™-M kit. From this selection, the plasmid pBKSpac::hph2 was developed and used to disrupt the *pacCl* gene in *C. lindemuthianum*.

Development of the pacCl mutant

The pBKSpac::hph2 vector was transformed into *C. lindemuthianum* line USP9. The mycelium, grown in GPYECH liquid media (Ansari et al. 2004) for 48 h, was washed and placed in 100-ml Erlenmeyer flasks, containing 20 ml of 10 mg/ml glucanex in 10 mM phosphate buffer containing 0.8 M KCl as osmotic stabilizer. The obtained protoplasts were washed 3x in STC solution (1 M sorbitol, 50 mM CaCl$_2$ in 10 mM Tris–HCl buffer). At the end of the washes, the protoplasts were re-suspended in STC at a concentration of 10^8 protoplasts/ml. Next, 200 ml of protoplast suspension, 5 μg of pBKSpac::hph2, and 50 μl of 60 % polyethylene glycol (PEG) 6000 in STC were added to a

tube, and the tube was kept on ice. After 20 min, 500 ml of PEG 6000 was added, and the solution was incubated at room temperature for 20 min. At the end of this period, the protoplasts were plated on PDA medium containing 0.56 M sucrose as osmotic stabilizer. After 48 h of protoplast regeneration, a new layer of PDA medium containing 100 μg/ml hygromycin was added to the Petri dishes. The hygromycin-resistant transformants were transferred to new culture medium containing 50 μg/ml hygromycin and subsequently purified by monosporic isolation.

Molecular characterization of the functional deletion of the pacCl locus

The possible *pacCl* mutants were detected by PCR using the primer set Opac1/Opac2 (Table 1).

DNA hybridization was used to confirm *pacCl* interruption. First, DNA hybridization was performed with the wild-type isolate to determine the number of *pacCl* copies in the genome of *C. lindemuthianum*. For this purpose, 5 μg of total DNA was digested with the restriction enzymes *Xba*I, *Eco*RV, and *Hin*dIII (Promega®). The probe used was a 1.8-kb DNA fragment corresponding to the *pacCl* gene, obtained with the primer set pacCl1/pacCl9. To characterize the *pacCl⁻* mutant, total DNA from wild-type and mutant isolates were cleaved with *Hin*dIII. In this case, two probes were used: the aforementioned 1.8-kb DNA fragment and the empty pBluescript II KS+vector (Stratagene®). To label and detect the DNA fragments, the Gene Images AlkPhos Direct Labeling and Detection System (Amersham Biosciences®) was used. Total DNA from isolates was extracted by the method of Specht et al. (1982).

Phenotypic characterization of transformants in alkaline pH

To verify the influence of the *pacCl* gene on the growth of *C. lindemuthianum*, the conidium lines USP9 and Mutpac2 and the transformants Tr1 and Tr3 were inoculated in buffered solid media at pH 3, 6, and 9. In addition, growth curves were performed by inoculating 7-mm mycelial discs from the lines UPS9 and Mutpac2 in the center of Petri dishes containing culture media buffered at different pH values. The radial growth was evaluated at 2-day intervals.

Pathogenicity test

The common bean plant (*P. vulgaris* cv. La Victoire) was used as the susceptible cultivar in the pathogenicity tests. The seeds were germinated, and the bean plantlets were maintained in growth chambers for 7 days. The surface of the excised cotyledonary leaves were inoculated with a suspension of 10^7 conidia/ml. These leaves were kept in Petri dishes containing filter-paper discs soaked in water, then incubated at 19 °C in a growth chamber with 8-h dark and 16-h light periods (166 μE/s/m). Six leaves were used from each transformant line tested. The symptoms were observed 5–7 days after inoculation. The isolates showing a reduction or loss of pathogenicity were subsequently inoculated on plants kept in a greenhouse, to confirm their phenotypes. For microscopic observation of infection kinetics, excised bean hypocotyls were inoculated with 10 μl of the same conidial suspension and maintained under the same light, humidity, and temperature conditions.

Observation of infection process by fluorescence microscopy

The hypocotyl epidermal tissues were marked with a stylus, cleared twice for 10 min in 1 M KOH at 70 °C, and mounted in 67 mM K_2HPO_4 pH 9.0, containing 0.1 % aniline blue. The observations were obtained by epifluorescence microscopy in an Axioskop microscope (using filters BP365, FT395, and LP397 with UV light at 340 to 380 nm wavelength) (Zeiss, Oberkochen, Germany), and digital images were recorded with the aid of a video camera (Sony, Kolen, Germany) coupled to the computer.

Influence of the pacCl gene on the production and secretion of lipase

Tests were performed on solid medium containing 1.5 % agar or in liquid medium (10 g peptone, 5 g NaCl, 0.1 g $CaCl_2 \cdot 2H_2O$, 10 ml Tween 20, 100 ml H_2O). The dye bromocresol purple (25 mg/l) was used only on solid medium. Plaques were inoculated in the centre with 7-mm discs taken from the edge of the colony, and then the plaques were grown in M3S medium and maintained at 21 °C. After 7 days, the plaques were placed in a refrigerator to allow halos to form, which contained precipitated Ca^{+2} salts. Enzymatic activity was estimated using an enzymatic index (I), which expresses the ratio between the medium diameter of the halo and the medium diameter of the colony.

Liquid culture media were used to measure the amount of lipase produced by the different lines of *C. lindemuthianum*. Bottles containing 50 ml of culture medium were inoculated with 1 ml of a conidial suspension containing 10^7 conidia/ml and shaken at 150 rpm at 21 °C. After 8 days, the culture supernatant was removed, and the amount of lipase was estimated using the lipase dosage kit from Bioclin Laboratories.

Apoplastic pH measurement in the leaves of the susceptible common bean cultivar La Victoire and the resistant cultivar Jalo

Ten leaves were used for each experiment. These leaves were completely immersed in 100 ml of Milli-Q water in a beaker. The beaker was placed under a vacuum for 15 min. The vacuum was broken slowly to allow infiltration of the apoplast. Then, the excess surface water was removed from the leaves. These leaves were placed in syringe into Falcon tubes. The liquid obtained after 5 min of centrifugation at $4000 \times g$ was collected, and the pH was measured with a pH meter.

Results

Structural characterization of pacCl gene

Sequence analysis identified a protein containing 582 amino acids and a stop codon. This ORF was flanked by a 204-bp 5' UTR and by a 403-bp 3' UTR. The start codon (ATG) was present in a 5'-CACCATGTC-3' sequence, similar to that found in other filamentous fungi (Gurr et al. 1987). This sequence included the important A nucleotide of the Kozak consensus sequence (CAMM<u>ATG</u>NC) (Kozak 1984) at position −3 relative to the ATG. The comparison between the nucleotide sequence of the *pacCl* gene and the cDNA library sequence identified three putative introns. The first intron was 60 bp long and located at position 172–232. The second intron was 58 bp long and located at 446–504. The third intron was 74 bp long and located at 582–656. The *cis* element 5'-GT…AG-3' was found in all of the identified introns (Ballance 1986). The *C. lindemuthianum pacCl* gene nucleotide sequence was deposited in GenBank with accession number JX679084.

The alignment of the obtained protein sequence with sequences available in databases showed that its amino acid sequence had high identity and similarity values with the pH-response transcriptional regulator PacC from filamentous fungi. The PacC1 subcellular location prediction by WoLF PSORT and ngLOC showed nuclear localization in both programs with indices 26.5 and 23.51 %, respectively. Using the PacC/Him101 protein sequences in the NCBI database, we performed an alignment and constructed a phylogenetic tree. To construct the phylogenetic tree, sequences from 32 organisms were used, including filamentous fungi and yeast belonging to the Basidiomycota and Ascomycota phyla (data not shown). Interestingly, most clades grouped taxonomically related organisms at the class level. A monophyletic group was formed by the *Eurotiomycetes* class, including all species of the genus *Aspergillus* and the species *P. chrysogenum*, *T. rubrum*, and *P. brasiliensis*, with the group being supported by a bootstrap value of 99. The species *S. sclerotiorum* and *B. fuckeliana* were grouped in the Leotiomycetes class, with a consistency value of 100. The monophyly of the Sordariomycetes class, which included the three *Colletotrichum* species examined here (which were also grouped into a single clade, supported by a bootstrap value of 100), the genus *Fusarium*, and the species *M. grisea*, *N. crassa*, *T. harzianum*, *A. chrysogenum*, and *G. fujikuroi* (the species *C. lindemuthianum* is highlighted in the tree). This clade was supported by a consistency value of 99. Finally, more basal branches included the Saccharomycetes class, represented here by three species of the genus *Candida* and species *D. hansenii*, *K. lactis*, and *S. cerevisiae*.

pacCl gene expression is pH-dependent

To evaluate the influence of ambient pH on *pacCl* gene expression, its transcription was analyzed by northern blot. Total RNA was extracted from mycelia grown for 5 days in buffered liquid medium, with an initial pH between 3 and 9. Similarly to other works (Dufresne et al. 1998; Parisot et al. 2002), the constitutively transcribed *gpd* gene was used as the normalization control. The results are expressed as the ratio of *pacCl* to *gpd* band intensity (Fig. 1a). The densitometry values of the amplified products were obtained using ImageJ v1.37 software (http://rsb.info.nih.gov/ij). *pacCl* transcript was detected at all pH values tested, but the quantitative determination (Fig. 1a) clearly shows that

the expression level is pH-dependent, increasing at more alkaline pH values.

qRT-PCR was used to analyze *pacCl* expression in *C. lindemuthianum* during the course of infection of bean plants. The *pacCl* gene was transcribed in the cells of *C. lindemuthianum* during all the infection process, but the highest levels were found at the end of the necrotrophic phase. (Fig. 1b).

pacCl gene inactivation

The *pacCl* gene in *C. lindemuthianum* was interrupted by homologous recombination. The plasmid pBKSpac::hph2 (Electronic Supplementary Material A1), containing the hygromycin resistance cassette interrupting the cDNA sequence of *pacCl*, was transformed into the wild-type isolate UPS9.

Mutants of the *pacCl* gene were detected by PCR using the primer set Opac1/Opac2 (Table 1), as the interrupted *pacCl* sequence was inserted between these two oligonucleotides in the pBKSpac::hph2 vector. After PCR amplification using the transformant DNA and the Opac1/Opac2 primer set, the expected 1,221-bp fragment from DNA genomic was not amplified in one of the four lines tested (Electronic Supplementary Material A1). It was probable that in this transformant, denominated Mutpac2, the *pacCl* gene was interrupted. PCR experiments with the primer set Ohph3/Ohph4 (Table 1), which amplified a 712-bp DNA fragment of *hph*, were performed to verify the transformation event. In all transformant lines, the *hph* gene was inserted in the genome. The transformants Tr1, Tr3, and Tr4, were uncharacterised hygromycin resistant transformants having heterologous integration. In these lines, *pacCl* was likely not interrupted. The Tr2 line had a rearrangement in the *pacCl* locus because the amplified DNA fragment was smaller (861 bp) than expected when using the Opac1/Opac2 oligonucleotides (Electronic Supplementary Material A1).

To confirm *pacCl* interruption in Mutpac2, DNA hybridization was performed. First, this technique was used to determine *pacCl* copy number in the genome of the wild-type *C. lindemuthianum*. The result obtained from total DNA digested with the restriction enzymes *Apa*I, *Eco*RV, and *Hind*III, which did not cleave *pacCl*, allowed the visualization of a single positive band, confirming that the *pacCl* gene, similar to *pacC/HIM101* of other filamentous fungi and yeasts, was present in only one copy in the genome (Electronic
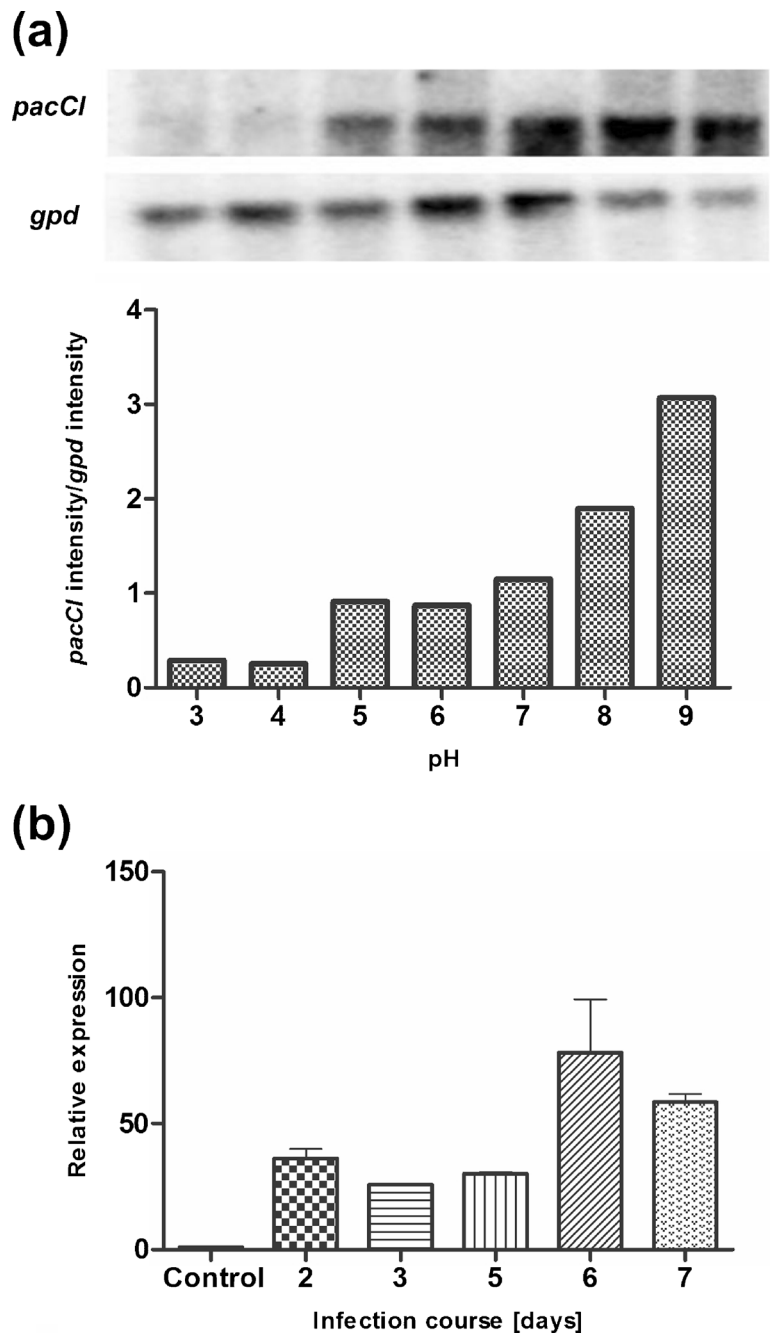
Supplementary Material A1). Then, to analyze and confirm the pattern of integration of the pBKSpac::hph2 vector in Mutpac2, DNA hybridization was again used. As shown in Electronic Supplementary Material A1, with the wild-type DNA and the *pacCl* probe, a single band was observed because the probe used was a fragment of the gene of interest, which exists as a single copy in the genome. In the case of Mutpac2, two positive bands were observed. When the pBluescriptSK[+] cloning vector was used as a probe, no positive bands were observed in the mutant or in the wild-type line. This could be explained by the strategy used in the transformation and by the type of inactivation vector used. Because site-directed mutagenesis was used, we

expected that homologous recombination would take place; in other words, a recombination between the *pacCl* gene contained in the inactivating vector and the wild-type gene contained in the *C. lindemuthianum* genome. Moreover, because the inactivation vector was designed to carry the *pacCl* gene interrupted by a central region containing a transposon and the *E. coli hph* gene, we also expected homologous recombination with gene exchange to occur, leaving no traces of the vector in the fungus genome. Finally, we expected that the hybridization with the 1.8-kb *pacCl* probe would show two bands in the mutant line because the new construct contained a *Hind*III restriction site. Fig. 2a and d show that these objectives were successfully achieved.

PacCl is necessary for the vegetative growth
of C. lindemuthianum in alkaline pH

Six days after inoculation, all tested lines had similar growth patterns in culture medium at pH 6 (Fig. 2a). The radial growth at pH 3 was much smaller compared to neutral pH, demonstrating that *C. lindemuthianum* grows preferably at a pH close to neutrality compared to more acidic values. The lines UPS9, Tr1, and Tr3 were capable of growing at pH 9, with a radial growth larger than at pH 3. Clearly, high pH values inhibit *C. lindemuthianum* growth. Interestingly, the growth of the Mutpac2 mutant was strongly inhibited at pH 9 (Fig. 2a).

The growth of the lines UPS9 and Mutpac2 was estimated measuring the radial growth every 2 days after inoculation in buffered media at different pH values (Fig. 2b). The wild-type line grew at pH values from 3 to 9. pH values of 5, 6, and 7 provided for the best growth of UPS9, followed by pH 8 and 9, at which the growth rates were the same. More acidic environments (pH 3 and 4) did not favour growth, which was more noticeable at pH 3, as observed above. The isolate UPS9 is able to change the pH of the culture medium, even if the medium is buffered.

In contrast, the growth of the Mutpac2 line suffered greater inhibition in more alkaline conditions. This line did not show any growth until day 5 after its inoculation in the culture medium. (Fig. 2b). This result clearly demonstrates that PacCl is necessary for the normal growth of *C. lindemuthianum* in alkaline pH, and it supports the hypothesis that in the transformant Mutpac2, the *pacCl* gene was interrupted, causing its loss of function.

The pacCl gene is essential for the pathogenicity
of C. lindemuthianum

Infection tests in excised leaves and bean plants were performed to determine the effect of *pacCl* loss of function on *C. lindemuthianum* pathogenicity.

When inoculated in excised leaves from the susceptible cultivar La Victoire, the *C. lindemuthianum* wild-type isolate UPS9 induced symptoms typical of anthracnose, with the formation of dark lesions in the primary veins 5 days after inoculation and subsequently extending to the secondary veins, resulting in complete maceration of the tissue 7 days after inoculation (Fig. 3a). The line Mutpac2, which carried an interrupted *pacCl* gene, did not cause anthracnose symptoms (Fig. 3a), even after longer incubation times. Rare, small, light-brown lesions could be observed on the leaf principal veins; however, the area of the lesions did not expand, showing a nonpathogenic phenotype.

Plants of the cultivar La Victoire were inoculated to confirm the phenotype shown by the mutant line. Symptoms appeared 4–5 days after inoculation, beginning with typical brown lesions on leaves, petioles, and hypocotyls. The lesions expanded with time, resulting in tissue maceration, followed by brownish exudates and eventually the death of these plants (Fig. 3b). The inoculation of conidia from this mutant line resulted in light lesions in the primary veins 6 days after inoculation. In contrast to what was observed in excised leaves, some lesions extended to the secondary veins, but no liquid exudates were observed from these regions. Small and rare lesions were also observed in the secondary veins, petioles, and hypocotyls. Because these lesions did not cause plant tissue maceration, the plants remained alive even 9 days after conidium inoculation.

To determine the Mutpac2 phenotype during the process of infection, the infection kinetics of this line were followed microscopically. Two days after conidium inoculation, the Mutpac2 mutant differentiated melanized appressoria, as did the wild-type line (Fig. 4a). Primary and secondary hyphae were observed in wild-type UPS9 7 days after inoculation, coinciding with the macroscopic visualization of plant tissue maceration (Fig. 4b). Mutpac2 differentiated conidia were functional and capable of epithelial tissue penetration, as primary hyphae were seen 7 days after inoculation of conidia (Fig. 4b). Contrary to the wild-type line, Mutpac2 did not differentiate secondary hyphae. In no field of observation were hyphae characteristic of the second phase of
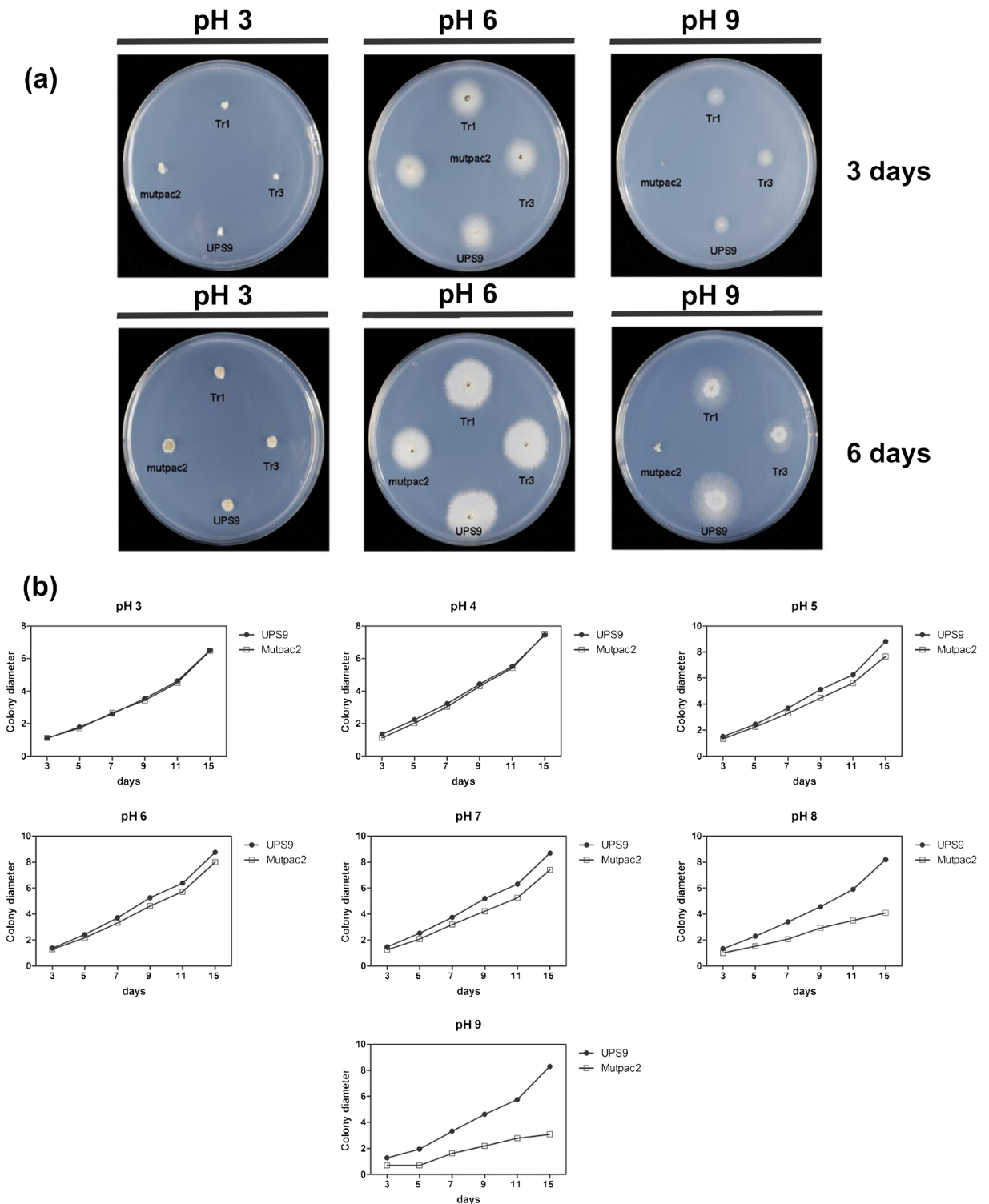
Fig. 2 Effect of ambient pH on the growth of transformants obtained with pBKSpac::hph2 transformation. (a) Conidia from the wild-type isolate (UPS9), the two transformants Tr1, Tr3, and Mutpac2 (mutant with loss of *pacCl* function) were inoculated in media with pH values of 3, 6, and 9, three days after conidium inoculation and six days after. (b) Growth curve of the wild-type isolate UPS9 and of the null mutant Mutpac2 at different pH values in buffered media

(a)

(b)
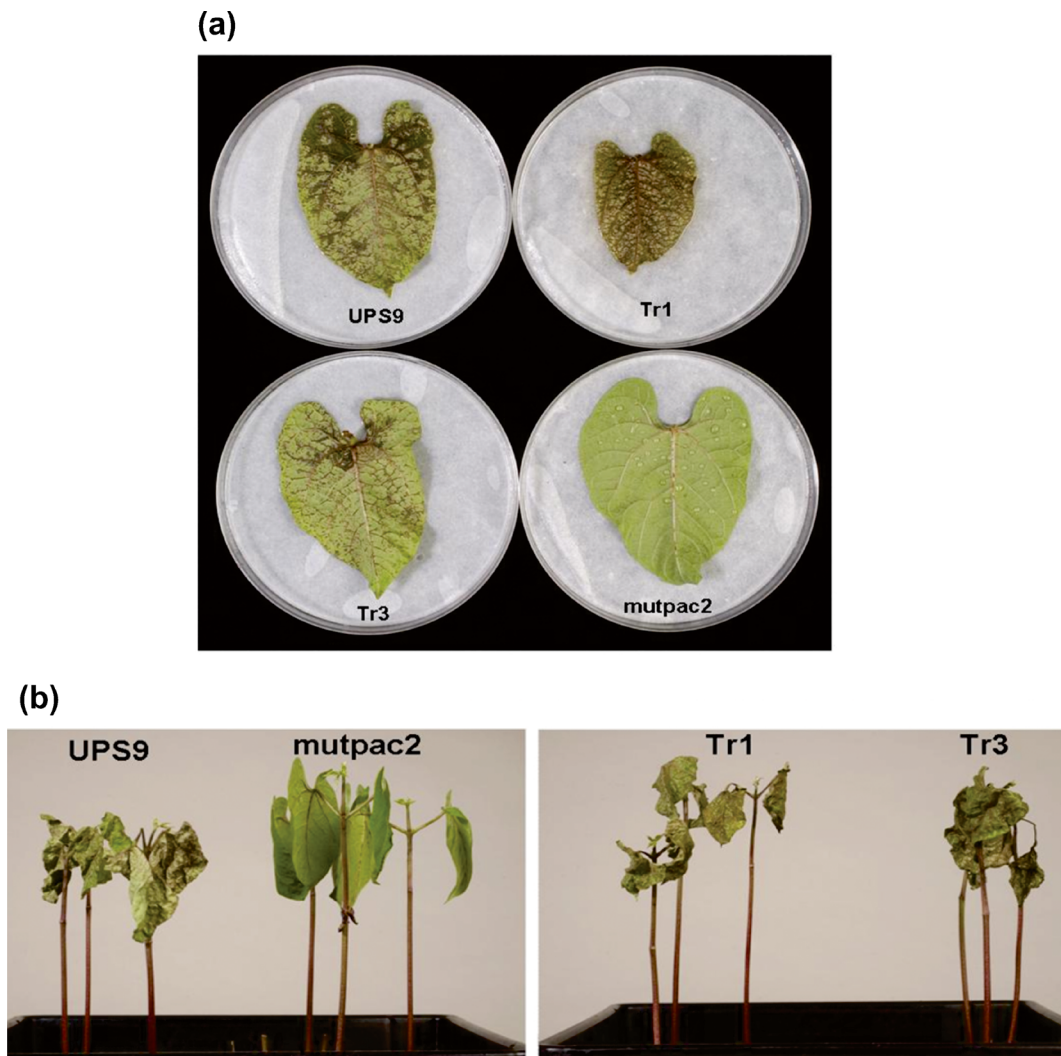
Fig. 3 Effect of *pacCl* gene inactivation on the pathogenicity of *C. lindemuthianum* inoculated on the susceptible cultivar La Victoire. (**a**) Excised cotyledonary leaves were inoculated with conidia from the wild-type isolate UPS9, the transformants Tr1 and Tr3, and the mutant line Mutpac2. (**b**) Plants from the anthracnose-susceptible cultivar La Victoire were pulverized with a conidial suspension, which was obtained from the same lines as in part **A**. Photographs were taken 9 days after inoculation

nutrition of this fungus observed, which suggests that PacCl is required for the transition from the biotrophic to necrotrophic phase in *C. lindemuthianum*. Because an organism's pathogenicity is characterized by its capacity to efficiently colonize the host and complete its life cycle, we propose that PacCl is involved in *C. lindemuthianum* pathogenicity.

The apoplastic pH from the leaves of the susceptible bean cultivar La Victoire was approximately 6.35. At this pH, the PacCl protein might be processed or modified in a way that converts it to its active form and confers the ability to activate the transcription of its own

gene. Therefore, PacCl was functional in the *C. lindemuthianum* UPS9 cells, while its hyphae grew in the interior of the La Victoire host tissue. Interestingly, the apoplastic pH of the resistant cultivar Jalo was approximately 5.6. Felle et al. (2004) concluded that changes in the apoplastic pH are important indicators of the plant–pathogen interaction, such that the different stages of infection of *Blumeria graminis* f. sp. *hordei* correlate with the different types of defence response of the host plant.

To evaluate the influence of *pacCl* inactivation on the expression of the lipase-encoding gene, lipase activity

(a)

(b)
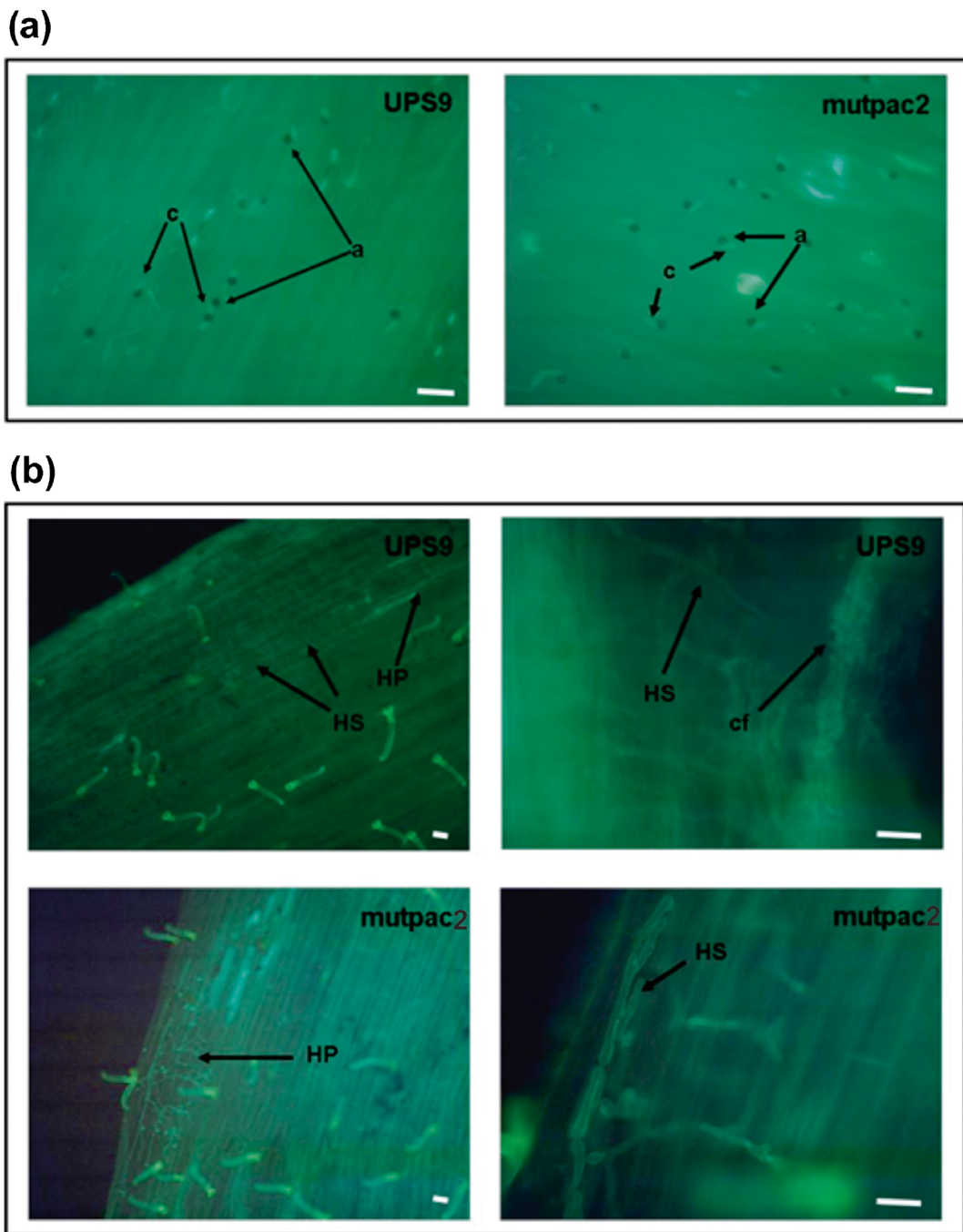
Fig. 4 Fluorescence microscopy analysis of the effect of *pacCl* inactivation on the infection cycle of *C. lindemuthianum* on the susceptible cultivar La Victoire. Excised hypocotyls were inoculated with conidia from wild-type isolate UPS9 and from Mutpac2. (**a**) Two days after inoculation, conidia from both lines developed melanized appressoria. (**b**) The wild-type line infected the vegetative tissue, developing primary and secondary hyphae and thereby completing its infection cycle. The Mutpac2 infected the vegetative tissue developing only primary hyphae (a): appressorium; (c): conidium; (*PH*): primary hyphae; (*SH*): secondary hyphae; (*cd*): conidiophore. Scale bar represents 25 μm

was detected in the culture media in the wild-type isolate UPS9 as well as the mutant Mutpac2. The lipase enzymatic activity index was lower in the wild-type, indicating that the *pacCl* gene might influence the

production capacity of this enzyme (Fig. 5a). To confirm this result, both lines were cultured in liquid medium and the amount of lipase in the supernatant determined. Lipase activity was detected in the wild-type culture, but not in the Mutpac2 culture supernatant (Fig. 5b).

Discussion

In this work, a 2,549-bp sequence of the *C. lindemuthianum* genome was isolated, containing a 1,749-bp ORF. The identified sequence was translated *in silico* into a putative polypeptide of 582 amino acids, which showed high similarity to the proteins from the PacC/Rim101 family of transcription factors identified in *A. nidulans* (Tilburn et al. 1995), *A. niger* (MacCabe et al. 1996), *F. oxysporum* (Caracuel et al. 2003a), *S. scleotiorum* (Rollins and Dickman 2001), *Y. lipolytica* (Lambert et al. 1997), and *C. albicans* (Ramon et al. 1999). The comparative analysis between the *pacCl* nucleotide sequence and the cDNA sequence identified three introns. These *pacCl* sequence characteristics are similar to those obtained in other *pacC* genes. In *F. oxysporum* (Caracuel et al. 2003a), *T. harzianum* (Moreno-Mateos et al. 2007), *C. acutatum* (You et al. 2007), and *C. gloeosporioides* (GenBank EU671075), for example, the putative ORFs have nucleotide sequences varying from 1,770 bp (*C. acutatum*) to 2,107 bp (*T. harzianum*). Moreover,

the *pacC* sequences of all these fungi show three introns, with sizes varying from 50 to 127 bp.

Similarly to the PacC/Rim101 protein from many fungi, the polypeptide analysis of PacCl identified the major elements that characterize this protein family. In the amino-terminal region, there were three zinc finger motifs, which are common in proteins that interact with DNA, such as transcription factors. In most cases, the zinc finger motif presents the $C(X_{2-4})C(X_{12})H(X_{3-5})H$ consensus sequence, i.e., the cysteine amino acid is separated from the other cysteine by 2–4 other amino acid residues, and these amino acids are separated from the two histidines by another 12 amino acid residues. The first and the third zinc finger discovered here exactly fit these characteristics; however, in the second zinc finger, 8 instead of 12 amino acid residues separated the extremities of the motif. The three motifs are located at the positions 44–67, 80–90 and 110–130 of the putative protein. Similar to what was identified in the PacC protein of *C. acutatum* (You et al. 2007) and *C. gloeosporioides*, at position 219–249 a nuclear localization signal was found, whose consensus sequence is $(K/R)_2–X_{10-12}–(K/R)_3$ (Nigg 1997). In the central portion of the protein, glycine- and glutamine-rich regions were found, as observed in other organisms, such as *A. nidulans* and *C. acutatum*. In the carboxy-terminal region at positions 429 and 575, two consensus sequences were found, YPNL and IPIL, which participate in the recognition and binding of the PalA protein and in
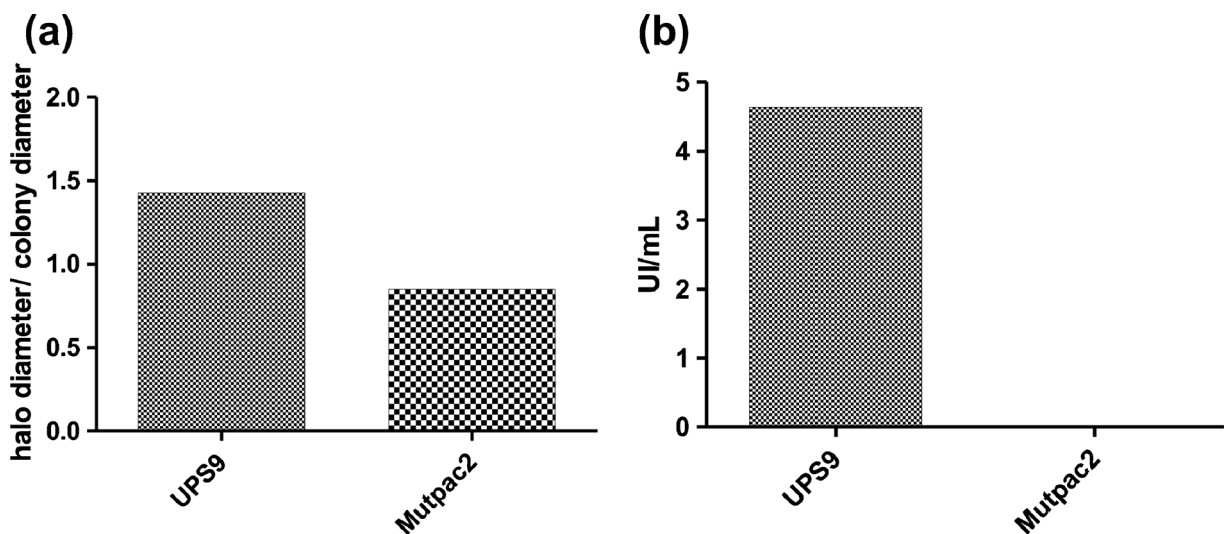
Fig. 5 Lipase activity in solid and liquid culture media from the isolates UPS9 and Mutpac2. (**a**) Index of lipase activity in solid medium. (**b**) Lipase units (UI) in the supernatant of liquid cultures.

One international lipase unit is defined as the enzymatic activity capable of releasing 1 μmol of -SH from the substrate dimercaprol tributyrate per minute per milliliter

the resultant signaling cascade, respectively. These two sequences flank another well-conserved region, the protease signaling box, which is located at position 446–467. This is the position where the first proteolytic cleavage takes place during the activation of the PacC protein, which is cleaved by PalB. The presence of all of these conserved domains in the deduced PacCl protein strongly indicates that it is homologous to the PacC/Rim101 proteins and that *C. lindemuthianum* is therefore able to regulate pH-mediated gene expression the same way as other fungi.

The *pacCl* transcript was detected at all pH values tested, from acidic to alkaline conditions. The quantitative analysis showed that *pacCl* expression is pH-dependent, increasing at more alkaline pH levels. The same transcription profile is observed for the *pacC* gene of *A. nidulans*, in which PacC binds to *cis* elements in its own promoter region, self-activating its transcription in more alkaline pH (Tilburn et al. 1995). The increase in the expression of *pacC/RIM101* genes at elevated extracellular pH appears to be common to many organisms, such as in *S. sclerotiorum* (Rollins 2003), *F. oxysporum* (Caracuel et al. 2003a), *F. verticillioides* (Flaherty et al. 2003), *C. albicans* (Ramon et al. 1999), *Y. lipolytica* (Lambert et al. 1997), and *U. maydis* (Aréchiga-Carvajal and Ruiz-Herrera 2005). Moreover, qRT-PCR demonstrated the expression of *pacCl* during the infection cycle of *C. lindemuthianum* in the biotrophic and, especially, in the necrotrophic phase.

The first physiological evidence that the *pacCl* gene was inactivated came from the growth evaluation of the wild-type and mutant lines cultured under different pH conditions. The Mutpac2 mutant showed a strong growth reduction in more alkaline pH in comparison with UPS9 isolates (Fig. 2). Decreased growth in mutants with *pacC/RIM101* loss of function is frequently observed when they are inoculated in environments with elevated pH (Peñalva and Arst. 2002). In an alkaline pH, these mutants show phenotypes that mimic growth in an acidic pH, resulting in the expression of genes that, in the wild-type, are preferentially expressed in acidic environments, as well as in the reduction of the expression of genes that, in the wild-type, are expressed preferentially in alkaline environments. Most likely, the genes required for *C. lindemuthianum* adaptation in alkaline environments and whose expression is activated by the PacCl transcription factor at these pH values are not expressed in Mutpac2. These results clearly show that PacCl is necessary for the normal growth of

C. lindemuthianum in alkaline pH, and they support the hypothesis that in the Mutpac2 transformant the *pacC1* gene is inactivated, leading to loss of its function. The *pacC/RIM101*-null mutants show other phenotypes in addition to the inhibition of growth at elevated pH values. In *S. sclerotiorum*, PacC is needed for the development and normal maturation of sclerotia (Rollins 2003). The mutant line grows normally at lower pH but is completely inhibited at higher pH levels. Moreover, the accumulation of oxalic acid is drastically reduced and the *pg1* gene derepressed at higher pH values (which repress this gene in wild-type lines). Morphological changes, increased sensitivity to lytic enzymes, and polysaccharide secretion are characteristics that had previously only been reported in the *pacC* mutant phytopathogen *U. maydis* (Aréchiga-Carvajal and Ruiz-Herrera 2005). This mutant is also sensitive to osmotic stress, caused by the increased Li^+ and Na^+ in the culture medium, as observed with $pacC^{+/-}$ mutants of *F. oxysporum* (Caracuel et al. 2003b). Altered conidiogenesis is reported for the fungi *A. nidulans* (Tilburn et al. 1995) and *A. niger* (MacCabe et al. 1996) when *pacC* is disrupted.

As shown in Fig. 3, the Mutpac2 line can be considered non-pathogenic due to its inability to macerate the plant tissue. Although it creates light-brown lesions, the infectious process leading to the development of anthracnose does not progress, and this line cannot complete its cycle of asexual reproduction in plants lacking lesions with exudates. *C. lindemuthianum* conidia can be found in bean tissue lesions, in which exudation occurs during the maceration process (Pellier et al. 2003).

During the colonization of the host tissue, the phytopathogen must maintain precise control over the timing of expression of its genes, efficiently adapting to the tissue ambient conditions to complete its life cycle. The limited availability of nutrients in plant tissue is one of the main signals that control the expression of genes involved in the pathogenicity of several pathogenic microorganisms (Divon and Fluhr 2007). Genes encoding general regulators of nitrogen and/or carbon absorption and utilization are important for the pathogenicity of phytopathogens, as they allow gene expression to adjust depending on nitrogen and carbon limitations in the host tissue. The *clnr1* gene from *C. lindemuthianum* encodes a transcription factor that enables this phytopathogen to use different nitrogen sources, and its transcription is necessary for

pathogenicity. This was demonstrated by the inability of the *clnr1*⁻ mutant to develop into the necrotrophic phase during the infection of susceptible cultivars of common bean (Pellier et al. 2003), as well as by the phenotype of the Mutpac2 line obtained in this work. A second gene, *clta1*, encodes another transcriptional activator in *C. lindemuthianum* that is necessary for biotrophic-to-necrotrophic phase transition (Dufresne et al. 2000). Clta1 shows homology to the GAL4 family of proteins. Thon et al. (2002) demonstrated that disruption of the CPR1 gene from C. graminicola results in a significant reduction in virulence. According to these authors, this gene encodes a component of a Signal Peptidase Complex (SPC), responsible for the signal peptide cleavage of proteins targeted to the endoplasmic reticulum. The authors believe, therefore, that the phenotype can be due the reduction in the transport of secretory proteins during the transition from biotrophy to a necrotrophic lifestyle, especially enzymes involved in degradation of plant cell wall polysaccharides. More recent work (Bhadauria et al. 2013), there is a novel effector gene, CtNUDIX, from C. truncatum that is exclusively expressed during the late biotrophic phase. It encodes a protein containing a signal peptide and a Nudix hydrolase domain, able to elicit the hypersensitive response (HR) pathway of defence against plant pathogens in tobacco. The ambient pH modulates the pathogenicity of many plant and insect pathogens (Prusky and Yakoby 2003; Akimitsu et al. 2004) and human pathogens, such as *C. albicans* (De Bernardis et al. 1998). Ambient pH controls the expression of virulence factors, such as cell wall hydrolytic enzymes and proteases, and even yeast filamentation. The phytopathogens can encounter both temporal and spatial pH variations during the process of host colonization. In this case, the transcription factor PacC/Rim101 could play a fundamental role in the regulation of genes necessary for efficient infection, thereby determining the virulence and/or pathogenicity of these organisms. In *S. sclerotiorum*, PacC functions as a virulence factor (Rollins 2003), similar to its orthologs in *A. nidulans* (Bignell et al. 2005) and *C. albicans* (Davis et al. 2000). Clearly, the role of PacC in virulence/pathogenicity varies according to the species in question. In the phytopathogen *F. oxysporum*, *pacC* mutants are more virulent than the wild-type, suggesting that PacC behaves as a negative virulence regulator (Caracuel et al. 2003a). Interestingly, the *pacC* mutant lines of *U. maydis* were as virulent as the wild-type when inoculated in maize

(Aréchiga-Carvajal and Ruiz-Herrera 2005). Thus, an organism's pathogenicity is characterized by its capacity to efficiently colonize the host and complete its life cycle. The results shown in Fig. 4 allow us to propose that *pacCl* is, in fact, involved in *C. lindemuthianum* pathogenicity.

PacC is a transcription factor involved in the activation or repression of a number of genes important in pathogenicity in filamentous fungi. To determine the effect of inactivating the *pacCl* gene on the expression of the gene encoding the *C. lindemuthianum* lipase enzyme, lipase activity was evaluated in the wild-type and Mutpac2 isolates. Lipase activity was detected in the wild-type culture, but not in the supernatant of the Mutpac2 culture (Fig. 5). Detecting lipase activity by the halo degradation method in different species of *Colletotrichum* has shown that lipase activity depends on pH. In general, lipase activity is absent at acidic pH and is prominent in the extracellular environment at alkaline pH (Maccheroni et al. 2004). In *F. graminearum*, as well as in other phytopathogenic fungi, the extracellular lipase is a necessary virulence factor for the infection of cereals (Voigt et al. 2005). The results in *C. lindemuthianum* indicate that Mutpac2 is deficient in the production or secretion of extracellular lipases. It is probable that PacCl is involved in the regulation of lipase synthesis in *C. lindemuthianum*. Taken together, our results indicate that the *pacCl* gene, which encodes a pH-responsive transcriptional regulator, is an important determinant of the pathogenicity of *C. lindemuthianum* towards its host, the common bean (*P. vulgaris*).

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