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# The histidine kinase *slnCl1* of *Colletotrichum lindemuthianum* as a pathogenicity factor against *Phaseolus vulgaris* L

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ABSTRACT

*Colletotrichum lindemuthianum*, the causal agent of anthracnose, is responsible for significant damage in the common bean (*Phaseolus vulgaris* L.). Unraveling the genetic mechanisms involved in the plant/pathogen interaction is a powerful approach for devising efficient methods to control this disease. In the present study, we employed the Restriction Enzyme-Mediated Integration (REMI) methodology to identify the gene *slnCl1*, encoding a histidine kinase protein, as involved in pathogenicity. The mutant strain, MutCl1, generated by REMI, showed an insertion in the *slnCl1* gene, deficiency of the production and melanization of appressoria, as well as the absence of pathogenicity on bean leaves when compared with the wild-type strain. The *slnCl1* gene encodes a histidine kinase class IV called SlnCl1 showing identity of 97% and 83% with histidine kinases from *Colletotrichum orbiculare* and *Colletotrichum gloesporioides*, respectively. RNA interference was used for silencing the histidine kinase gene and confirm *slnCl1* as a pathogenicity factor. Furthermore, we identified four major genes involved in the RNA interference-mediated gene silencing in *Colletotrichum* spp. and demonstrated the functionality of this process in *C. lindemuthianum*. Silencing of the *EGFP* reporter gene and *slnCl1* were demonstrated using qPCR. This work reports for the first time the isolation and characterization of a HK in *C. lindemuthianum* and the occurrence of gene silencing mediated by RNA interference in this organism, demonstrating its potential use in the functional characterization of pathogenicity genes.

## 1. Introduction

The genus *Colletotrichum* represents one of the most important groups of phytopathogenic fungi. *Colletotrichum* belongs to the phylum Ascomycota, which encompasses species capable of causing a disease known as anthracnose in a large variety of crops and ornamental plants. Many of these species have been used as models to study differentiation and plant-pathogen interactions (Perfect et al., 1999; Hyde et al., 2009), and recently, the genomes of some of these species have been sequenced (O'Connell et al., 2012; Gan et al., 2013; Zampounis et al., 2016; Gan et al., 2016). In particular, the species *Colletotrichum lindemuthianum* (Sacc & Magnus) Briosi & Cav. is the causal agent of anthracnose in the common bean (*Phaseolus vulgaris* L.). Anthracnose is a widely distributed disease in Brazil, with a greater incidence in the southern and southeastern regions. The use of infected seeds together with the occurrence of moderate temperatures and high humidity may lead to losses of up to 100% of crops (Ansari et al., 2004; Silva et al., 2007; Bonett et al., 2008). In this context, some groups have directed their efforts towards understanding the molecular mechanisms involved in plant-pathogen interactions from the initial contact until the emergence of symptoms and the disease establishment.

The Restriction Enzyme-Mediated Integration (REMI) method favors the random non-homologous integration of exogenous DNA on chromosomes of eukaryotic cells and have been widely employed to obtain mutants of fungi strains. High mutation frequencies are commonly observed by the employment of this technique, what makes it a powerful tool to understand the role of target genes, mainly when it is related to pathogenicity and development (Mullins and Kang, 2001; Riggle and Kumamoto, 1998; Wang et al., 2013). RNA interference-mediated gene silencing (RNAi) has become another prominent tool to

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study phytopathogenic fungi, and often replace the more traditional techniques of insertional inactivation (Panwar et al., 2013; Takahara et al., 2016). Commonly known as RNA interference (RNAi), may be understood as a mechanism of post-transcriptional gene silencing in which a double-stranded RNA (dsRNA) triggers the sequence-specific degradation of a target mRNA. This process appears to be primarily mediated by a group of the following proteins: QDE-1 (which encodes a RNA-dependent RNA polymerase - RdRP) (Cogoni and Macino, 1999), QDE-2 (argonaut protein, the catalytic component of RNA-induced silencing complex - RISC) (Cogoni and Macino, 2000), and DCL-1 and DCL-2 (Dicer-like enzymes, which cleaves long dsRNA into short double-stranded fragments) (Catalanotto et al., 2004).

The two-component system is a widely distributed signal transduction system that enables sensing, response and adaptation to different stimulus in the environment. Several data are found in literature regarding the strong importance of the two-component systems in response to osmotic and oxidative stress, sensitivity to fungicides, dimorphism, production of secondary metabolites, sporulation, virulence, melanin synthesis, cell wall integrity, hyphal morphogenesis and sexual/asexual development in filamentous fungi (Defosse et al., 2015; Fassler and West, 2013). The two-component system, extensively described in bacteria, archaea, yeasts, filamentous fungus, slime molds and plants (Dhiman et al., 2014; McCormick et al., 2012; Motoyama et al., 2005; Stock et al., 2000), includes a histidine kinase protein (HK); a sensor protein (SP), that receives an external signal and transmits to a response regulator protein (RR), which directs the received signal to the target (West and Stock, 2001; White, 2000). Few genes encoding HKs were described and characterized but their role on plant pathogenesis remains elusive. The two-component system was first described in *Saccharomyces cerevisiae* and is essential for its growth and sensor for environmental changes (Román et al., 2007). A histidine residue (His) in the active site of Sln1 HK is autophosphorylated, followed by the transference of the phosphoryl group (PG) to an aspartate residue (Asp) located in the acceptor domain and, subsequently, the PG is transferred to another His residue located in a phosphotransfer unit (Ypd1). Finally, the PG in Ypd1 is transferred to an Asp residue of the receptor domain of Ssk1, a response regulator protein, finishing the PG transference His-Asp-His-dependent (Fassler and West, 2013; Santos and Shiozaki, 2001).

To the best of our knowledge, this is the first work to identify and functionally characterized the gene HK in the pathogenic fungi *C. lindemuthianum* and its role in anthracnose, suggesting a potential target to the development of antimicrobial drugs. Furthermore, this also is the first work to describe the occurrence of RNAi mechanism in this organism, suggesting a powerful tool to study plant-pathogen interaction models.

## 2. Results

### 2.1. Isolation and characterization of histidine kinase slnCl1

Twenty-four, 21 and 21 transformant strains were obtained by REMI methodology employing 0 U, 10 U and 20 U of *Hind*III, respectively. One of the transformants strains obtained following the treatment with 10 U of *Hind*III showed deficiency on production of melanin when compared with *C. lindemuthianum* wild-type (Fig. 1a). This strain, called MutCl1, showed integration of pSM1 at one site of its genome (data not shown). The pathogenicity test showed the incidence of macroscopic brown spots on bean leaves surface after five days of infection with spores from wild-type *C. lindemuthianum.* In contrast, only a few leaves inoculated with MutCl1 spores showed little or rare injuries (Fig. 1b). Appressoria were observed in broth obtained from boiled leaves of cultivar Rosinha after 16 h of inoculation with spores of *C. lindemuthianum* wild-type and MutCl1. Melanization of appresoria was only observed for *C. lindemuthianum* wild-type after 19 h of inoculation (Fig. 1c).

The gene mutated through REMI was assessed by screening the *C. lindemuthianum* genomic bank using the plasmid pRescue24 as probe. pRescue24 contains the sequence of the gene interrupted by pSM1 obtained by PCR with the random primer OPG-17 and M13R, that anneal on pSM1. By this way, the positive phages clA, clD, clH and clI were identified by plaque hybridization. Subsequently, the DNA of positive phages were cleaved and submitted to a new round of *Southern-blot*. The hybridized fragments of DNA were isolated and cloned in pBluescript KS$^-$ vector given rise to the plasmids pDK1, pDS1, pHS1, pIS2, pAN1, pDN1 e pHC1. These plasmids were sequenced with the primers described in Table 1 and the full gene sequence was assembled.

The sequence obtained showed that the integration of pSM1 vector occurred on a promoter region, precisely at 83 base pairs (bp) upstream the Open Reading Frame (ORF) of a gene called *slnCl1* (NCBI Data Bank: GU907087.1) that encodes a HK. *slnCl1* sequence is interrupted by three putative introns containing 51, 87 and 89 base pairs (bp). The putative protein SlnCl1, deduced from *slnCl1*, presents 1183 amino acids and identity of 97%, 83%, 82%, 81%, 80% e 79% with HKs from *Colletotrichum orbiculare* (ENH80934.1), *Colletotrichum gloesporioides* (XP_007273712.1), *Colletotrichum fioriniae* (XP_007599157), *Colletotrichum sublineola* (KDN59768), *Colletotrichum graminicola* (EFQ28297), and *Colletotrichum higginsianum* (CCF39444), respectively. Moreover, the tridimensional structure of SlnCl1 (Figure S1) presents identity of 30.2% with an *E. coli* HK (PDB code: 1bxd:A).

Typical domains of HKs were found in SlnCl1: H-box, N-box, G1-box, F-box and G2-box (Wolanin et al., 2002) (Fig. 2a). The H-box domain, highlighted in Fig. 2a, contains the conserved His residue, the phosphorylation and dimerization site of HKs. The other domains form a packed α-helix on a big antiparallel β-sheet with a handle that closes the ATP binding site. Moreover, prediction of SlnCl1 transmembrane regions showed two sites between amino acids 11–35 and 398–422, as highlighted in Fig. 2b.

Regarding the phylogenetic analysis, the HK proteins were grouped in several classes as proposed by Cattlet et al., 2003. Using the phylogenetics analysis combined with the alignment results of HKs from *G. moniliformis*, *B. fuckeliana*, *C. heterostrophus* and *A. nidulans*, SlnCl1 was grouped in class VI that covers the SLN1/HHK5 (Fig. 3).

### 2.2. RNA interference-mediated gene silencing in Colletotrichum spp

To confirm the phenotype identified by inactivation of HK by REMI, the silencing mechanism of *C. lindemuthianum* was evaluated. Four genes potentially related with silence mechanism were detected on *C. lindemuthianum* when compared with *Neurospora crassa*, which strongly supports the hypothesis that the silencing mechanism also operates in *C. lindemuthianum*. The *qde-1* gene has 4200 nt and its ORF encodes a protein of 1356 amino acids interrupted by two introns. The gene *dcl-1* encodes the Dicer-like 1 protein and contains 4808 nt. Its ORF encodes a protein of 1535 amino acids and is interrupted by four introns. The *dcl-2* gene, which encodes the Dicer-like 2 proteins, contains 4563 nt, and its ORF encodes a protein containing 1452 amino acids that interrupted by four introns. And the *qde-2* gene that encodes an Argonaut protein has 2992 nt, and its ORF encodes a protein containing 980 amino acids that is interrupted by a single intron.

The amino acid sequence of these proteins revealed their typical domains (Figure S2). The QDE-1 protein (RdRP) has a long domain known as RdRP, a typical RNA-dependent RNA polymerase in eukaryotes that starts at position 512 and ends at position 1157 (Figure S2a). DCL-1 and DCL-2 (Dicer-like), which have function redundancy, contain the following four main domains: the helicase ATP-binding domain; the helicase C-Terminal domain; the dsRNA-binding domain and the ribonuclease III domain activity. Although DCL-1 and DCL-2 are considered distinct proteins, they have the same domains in equivalent positions (Figure S2b and S2c). Regarding the QDE-2 protein (Argonaut), the following two main conserved domains were identified: the PAZ domain located in the 351–436 position and the PIWI domain

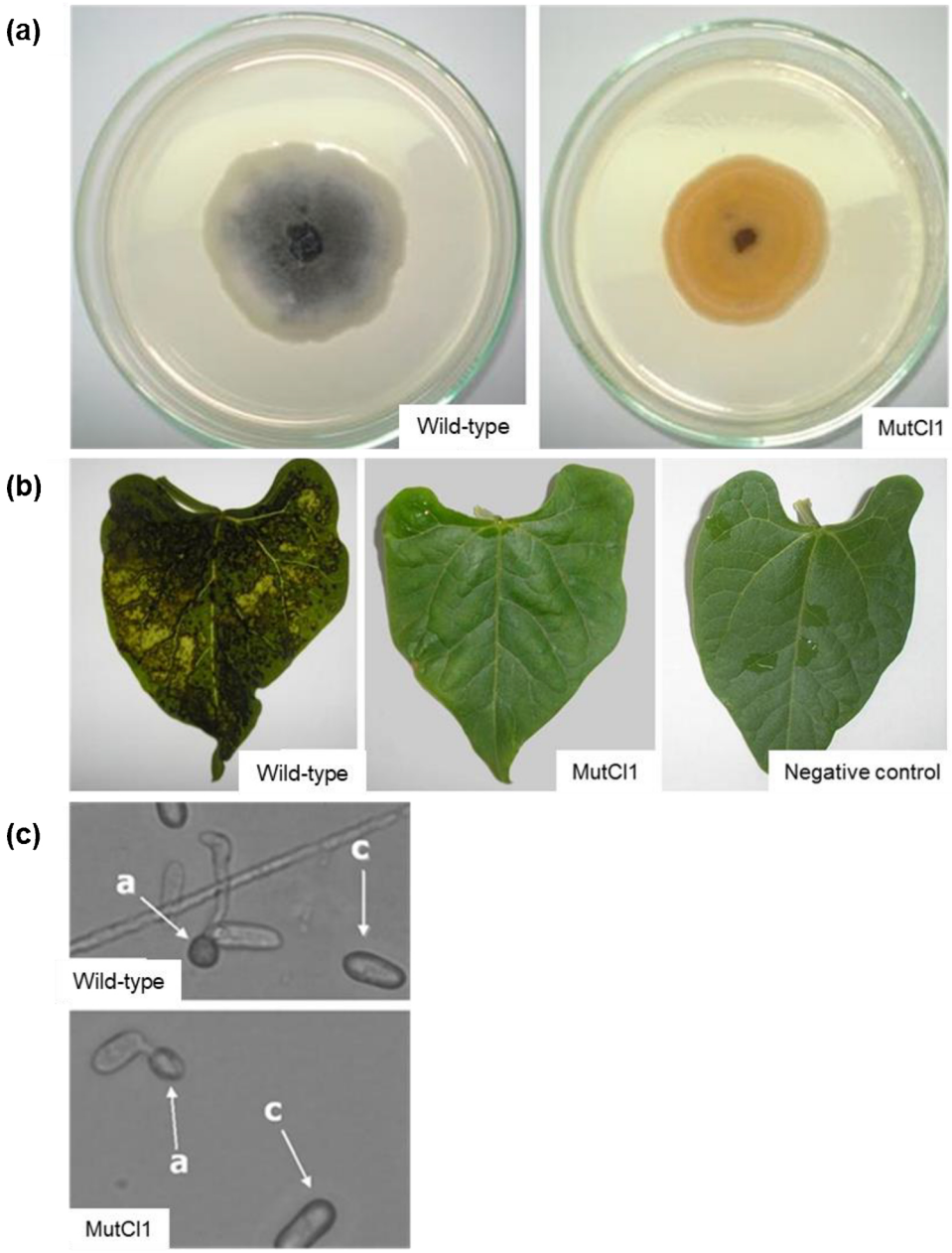


**Fig. 1.** Morphologies of *C. lindemuthianum* (Wild-type) and mutant (MutCl1) strains (A); pathogenicity test (B) where water was employed as the negative control; appressorium (a) and conidia germination (c) (C).

located at position 609–916 (Figure S2d).

For phylogenetic analysis of the proteins that constitute the central machinery of the silencing mechanism, representative amino acid sequences of 12 species of filamentous fungi were aligned to obtain a consensus phylogenetic tree for each protein (Figure S3). The results for the four proteins were very similar, consistently grouping the more taxonomically related organisms on the same branch of the tree. *C. lindemuthianum* are highlighted in each tree.

### 2.3. Silencing of the egfp reporter gene in Colletotrichum Lindemuthianum

To demonstrate the occurrence of RNA interference-mediated gene silencing in *C. lindemuthianum*, strains expressing the *egfp* reporter gene were obtained via genetic transformation of protoplasts with the pSM1 vector. Ten transformants were obtained, which were genetically stable and capable of emit fluorescence (Fig. 4a). In addition, PCR reactions with the oligonucleotide pairs *hph*F/*hph*R and *egfp*F/*egfp*R confirmed the presence of the genes *hph* and *egfp*, respectively, in the genome of transformant *C. lindemuthianum* (Fig. 4b). Hybridization analysis enabled the identification of a profile that varied in the pattern and number of integrations, with isolates containing from one to multiple copies of the vector in the genome, as presented in Fig. 4c. The strain identified as *Cl*pSM1-5 was selected for transformation with the silencing vector pSilentDual1-*egfp* because it had a single integration site in its genome, thus facilitating subsequent analyses.

Transformation of the *Cl*pSM1-5 strain with the pSilentDual1-*egfp* vector (Fig. 5a) yielded a recombinant strain called *Cl*pSD1-*egfp*. The *Cl*pSD1-*egfp* strain was able to grow in medium containing hygromycin due to the presence of the pSM1 vector and in the presence of geneticin due to the pSD1-*egfp* vector (Fig. 5b). PCR analysis using the oligonucleotides *nptII*F/*nptII*R confirmed the presence of pSilentDual1-*egfp* vector in the *Cl*pSD1-*egfp* strain (Fig. 5c). Furthermore, DNA hybridization revealed the occurrence of a single integration event because the *Eco*RI enzyme did not cleave the *nptII* gene, which was used as

**Table 1**
Oligonucleotides employed in this work.

| Primer | 5'-3' sequence | Application |
|---|---|---|
| T3 | AATTAACCCTCACTAAAGGG | Sequencing |
| T7 | GTAATACGACTCACTATAGGGC | Sequencing and probe |
| M13F | GTTTTCCCAGTCACGAC | Sequencing |
| M13R | CAGGAAACAGCTATGAC | Sequencing and plasmid recovery |
| SP6 | ATTTAGGTGACACTATAG | Sequencing |
| OPG-17 | ACGACCGACA | Plasmid recovery |
| pIS2T3 | GGACTTGAGCACACCGTTTT | Sequencing |
| pIS2T7 | ATGGGCTGAGAAGACGAAGA | Sequencing |
| pHSAT3 | GGAGCGAATGAGGGTATTCA | Sequencing |
| pHSAT7 | CCAGCGCAACCTTGAAAGTA | Sequencing |
| histsp6 | CCCTATGGTCCTCAACGAAA | Sequencing |
| histT7 | GCCTTTTTGCTGATTTCGAG | Sequencing |
| alb1F | CTGGCGATGGTGTCTGACT | PCR |
| alb1R | CTTTCTTGCACGCCAGTTTT | PCR and probe |
| CLGpDq1 | CCGCACTGCTGCTCAGAAC | RT-qPCR |
| CLGpDq2 | GGACATGCCGGTGAGCTT | RT-qPCR |
| histTRF | AGGTCGCAGATCCTGACCAT | RT-qPCR |
| histTRR | TGCTGCTCAGCTCGATGACT | RT-qPCR |
| si*sln*ClF | GAGAAGCTTCATCACCGTCCCGATCCGCG | Vector construction* |
| si*sln*ClR | GAGAAGCTTGACCGGGAGACCCTGCTCGT | Vector construction* |
| *hph*F | TTCGATGTAGGAGGGCGTGGAT | Confirmation of Integration (pSM1) |
| *hph*R | CGCGTCTGCTGCTCCATACAAG | Confirmation of Integration (pSM1) |
| *egfp*F | GAGAAGCTTAGCCGCTACCCCGACCACAT | Confirmation of Integration (pSM1) |
| *egfp*R | GAGAAGCTTTTGCTCAGGGCGGACTGGGT | Confirmation of Integration (pSM1) |
| *npt*IIF | AGATCCTCGCCGTCGGGCAT | Confirmation of Integration (pSD1) |
| *npt*IIF | CGGCTATCGTGGCTGGCCAC | Confirmation of Integration (pSD1) |
| qRT 18SF | TGAGTCGCACAAGCAAATAGTCA | qRT- PCR endogenous control (*rDNA*18S) |
| qRT 18SR | TGCAATTCACATTACTTATCGCATT | qRT- PCR endogenous control (*rDNA*18S) |
| qRT*egfp*F | GATCACTCTCGGCATGGA | qRT-PCR target gene (*egfp*) |
| qRT*egfp*R | GGTACCGTCGACTGCAGAA | qRT-PCR target gene (*egfp*) |
| qRT*sln*ClF1 | GGCCGACGAATGCCTCCAA | qRT-PCR target gene (*slnCl1*) |
| qRT*sln*ClR1 | ACTTCACGGGCACCTCGCTC | qRT-PCR target gene (*slnCl1*) |
| qRT*sln*ClF2 | CAGAATCTATTCCCGCAACA | qRT-PCR target gene (*slnCl1*) |
| qRT*sln*ClR2 | CGTAGGGCAGCTCAATGTC | qRT-PCR target gene (*slnCl1*) |

* The underlined sequence of the oligonucleotide pair si*sln*ClF/R represents the cleavage site for the *Hind*III enzyme.

a probe (Fig. 5d). Fluorescent microscopy analysis showed the silencing of *egfp* gene expression given their considerable reduction in fluorescence emission compared with the control strain *Cl*pSM1-5 (Fig. 5e). The confirmation of *egfp* gene silencing at the post-transcriptional level was achieved by qRT-PCR. The pSilentDual1-*egfp* vector led to a 53% reduction of *egfp* gene expression in the *Cl*pSD1-*egfp* compared with the *Cl*pSM1-5 (Fig. 5f). These results show for the first time the existence of a post-transcriptional RNA-mediated silencing mechanism (RNAi) in *C. lindemuthianum*.

### 2.4. *slnCl1* silencing in *C. lindemuthianum*

The pSilentDual1-*sln*Cl vector was constructed to silence the *slnCl1* gene and confirm its effects in the pathogenicity of *C. lindemuthianum* (Fig. 6a). Six *C. lindemuthianum* transformant strains were obtained and these strains remained genetically stable throughout successive cultivations in PDA culture media or PDA supplemented with 250 µg/mL geneticin (Fig. 6b). These strains were identified as *Cl*pSD1-*sln*(1–6) and were molecularly characterized by PCR (Fig. 6c) and *Southern blot* (Fig. 6d). Amplification of a 350 bp fragment corresponding to the *npt*II gene in all transformants evidenced the presence of the silencing vector in these selected strains. *Southern blot* analysis revealed a diversified pattern regarding the number and location of the integration sites, with some strains possessing a single one (*Cl*pSD1-*sln*1; *Cl*pSD1-*sln*4 and *Cl*pSD1-*sln*5), two (*Cl*pSD1-*sln*2 e *Cl*pSD1-*sln*6), or even three integration sites (*Cl*pSD1-*sln*3).

To demonstrate silencing of *slnCl1* at the molecular level, the qRT-PCR method was used, with the following strategy (Fig. 7): first, qRT-PCR was performed with the oligonucleotides qRT*sln*F1/R1, which amplified a 100-bp fragment of the *slnCl1* gene. This gene was contained within the 450-bp fragment that was cloned into the pSilentDual1-*sln*Cl vector. Thus, the relative quantification values of these transformant strains represented not only expression of the endogenous gene but also dsRNA expression. In five transformant strains, the relative expression values were significantly higher when compared with the wild-type strain, showing the correct integration of the vector and efficient expression of the dsRNA by these double constitutive promoters. To accurately assess the silencing of the *slnCl1* gene by the dsRNAs that were produced as described above, a second pair of oligonucleotides was used, namely, qRT*sln*F2/R2. Unlike the previous oligonucleotide pair, the qRT*sln*F2/R2 pair amplifies a 100-bp segment of the *slnCl1* gene that is present only in the endogenous copy (Fig. 7a). In this case, we observed decreased relative expression in six transformants compared with the wild-type strain (Fig. 7c). The reduction values ranged from 10% in strain *Cl*pSD1-*sln*3 to 36% in strain *Cl*pSD1-*sln*2 and reached a maximal value (46%) in strain *Cl*pSD1-*sln*6. Taken together, these results demonstrate *slnCl1* gene silencing through RNA interference in *C. lindemuthianum*.

### 2.5. The *slnCl1* gene encodes a pathogenicity determinant

A pathogenicity assay was performed to characterize the effects of silencing the *slnCl1* gene in strains transformed with the pSilentDual1-*sln*Cl vector. All six transformant strains displayed a decreased capacity to cause visible symptoms in detached common bean leaves compared with the pathogenic strain 89 $A_2$ 2–3 (results not shown). These findings demonstrate that the *slnCl1* gene may be an important pathogenicity determinant in the interaction of *C. lindemuthianum* with its host. To illustrate these results, Fig. 8a and b depicts the assay performed with the *Cl*pSD1-*sln*6 strain, which displayed the greatest reduction in the *slnCl1* gene expression levels as quantified by qRT-PCR. In the wild-type strain 89 $A_2$ 2–3, discrete symptoms of the disease were

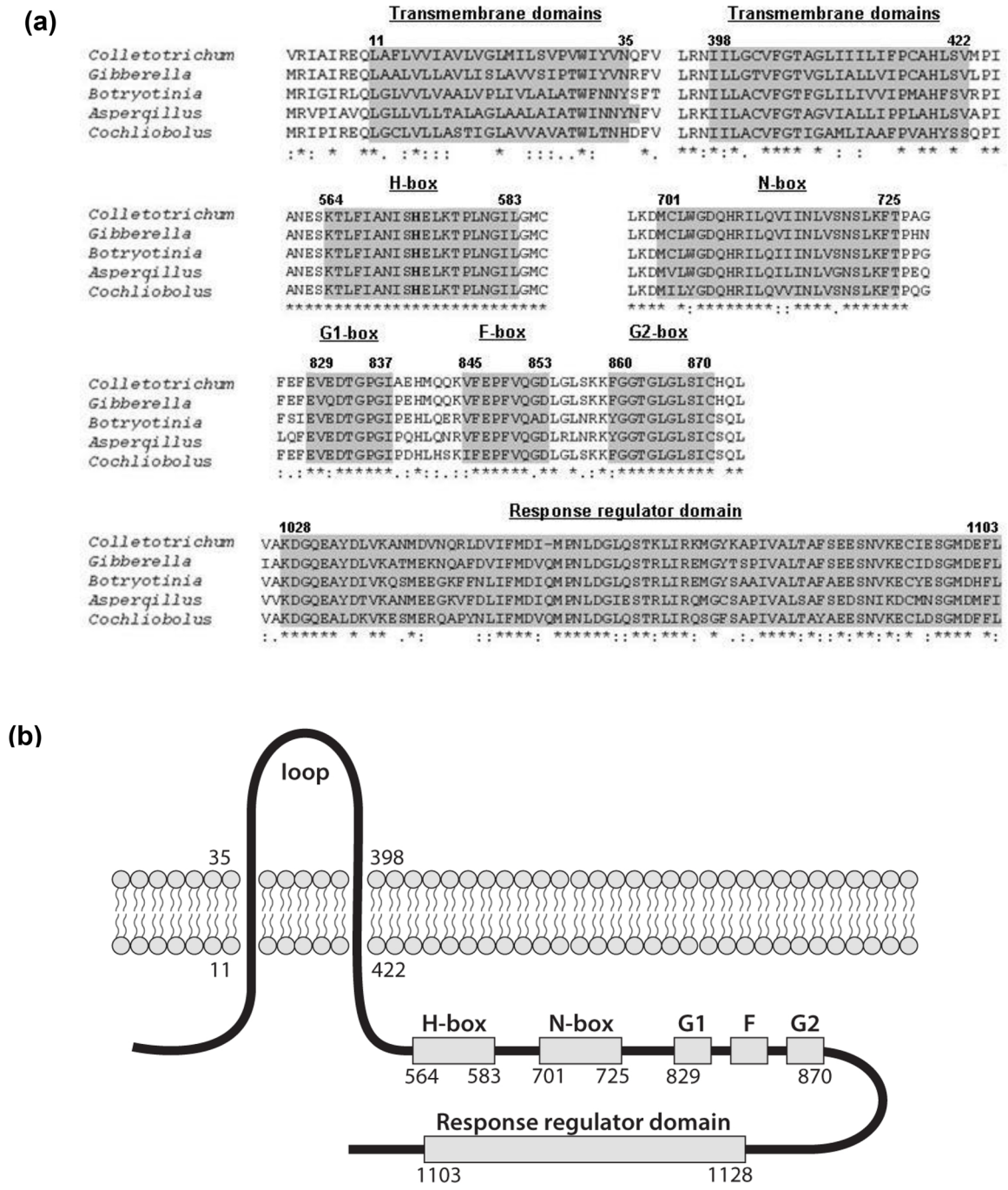


**Fig. 2.** The histidine kinase *slnCl1*. (A) Multiple sequence alignment of SnCl1 with HKs from other fungi species. Conserved sequences are highlighted in gray. The His residue, responsible for protein autophosphorilation, is showed in bold. Asterisks represents identical amino acids. (B) Schematic representation of *C. lindemuthianum* SlnCl1. The numbers are relative to amino acids position. Adapted from Hohmann, (2002).

only observed 5 days after and progressed significantly by day 6, with an affected leaf area of 24.93%. At day seven, this value increased abruptly to 52.49% and reached 57.95% at day 8. In the transformant strain *Clp*SD1-*sln*6, no symptoms were observed until day 5. At 6 days of infection, the first symptoms began to appear (4.14%) but were minimally visible. At day 7, the affected leaf area reached 8.72% and increased to 13.01% at day 8, all values considerably smaller than pathogenic strain 89 $A_2$ 2–3. These results demonstrated that silencing of the gene *slnCl1* delayed the ability of the fungus to cause the typically visible symptoms of the disease.

Bean leaves were inoculated with spores of *C. lindemuthianum* wild-type to evaluate the relation of the HK encoded by *slnCl1* with the infection process. The samples were collected at the 1st, 3th, 5th and 7th days and evaluated regarding *slnCl1* expression in comparison with *C. lindemuthianum* inoculated in culture medium. At the 3th day of infection, the expression of *slnCl1* was 17.35-fold higher than that presented by *C. lindemuthianum* inoculated in culture medium. Moreover, *slnCl1* expression at 1st, 5th and 7th days were low, being only 2.0-fold, 2.1-fold and 0.9-fold higher than *C. lindemuthianum* grown in culture media, respectively. These data, presented in Fig. 8C as the expression level, was related to the calibrator gene and was normalized against the gene expressed constitutively. A biological replicate was done obeying the same procedures developed in the first assay.

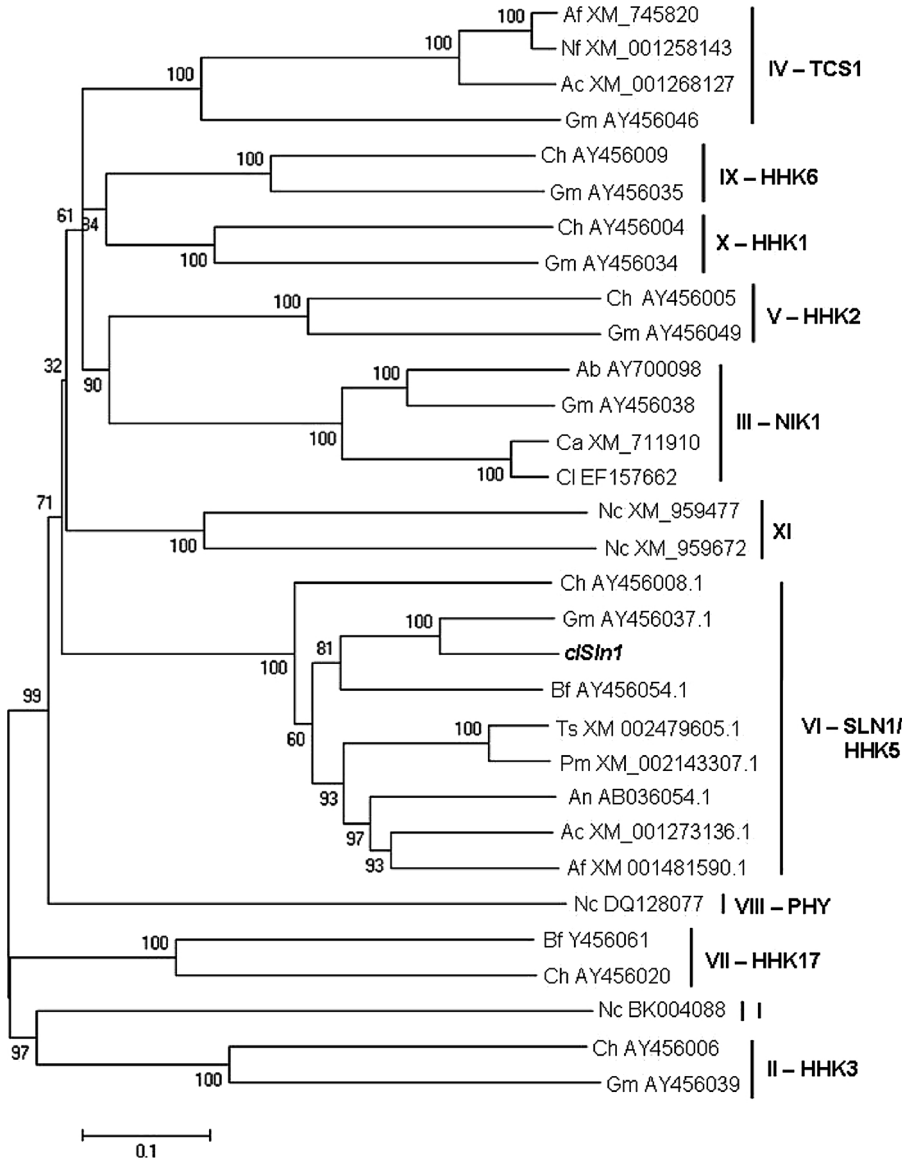


**Fig. 3.** Phylogenetic analysis of SlnCl1. The NCBI data bank numbers and the corresponding classes (I, II, III, IV, V, VI, VII and VIII) are showed. (Ab) *Alternaria brassicicola*, (Ac) *Aspergillus clavatus*, (Af) *Aspergillus fumigatus*, (An) *Aspergillus nidulans*, (Bf) *Botryotinia fuckeliana*, (Ca) *Candida albicans*, (Ch) *Cochliobolus heterostrophus*, (Cl) *Clavispora lusitaniae*, (Gm) *Gibberella moniliformis*, (Nc) *Neurospora crassa*, (Nf) *Neosartorya fischeri*, (Pm) *Penicillium marneffei*, (Ts) *Talaromyces stipitatus.*

### *2.6. slnCl1 knockout and pathogenicity assay*

To verify the pathogenicity of the selected mutant obtained by direct gene targeting using plasmid pSMIS2, spores from slnCl1$^{-}$ transformant were inoculated in leaves of Rosinha bean cultivar, susceptible to anthracnose. The wild strain was used as control for the observation of the disease symptoms. After five days of inoculation, the leaves with the wild strain as control began to show necrosis symptoms. The macroscopic appearance of these lesions suggests the development of secondary hyphae in this period, which secrete enzymes that degrade

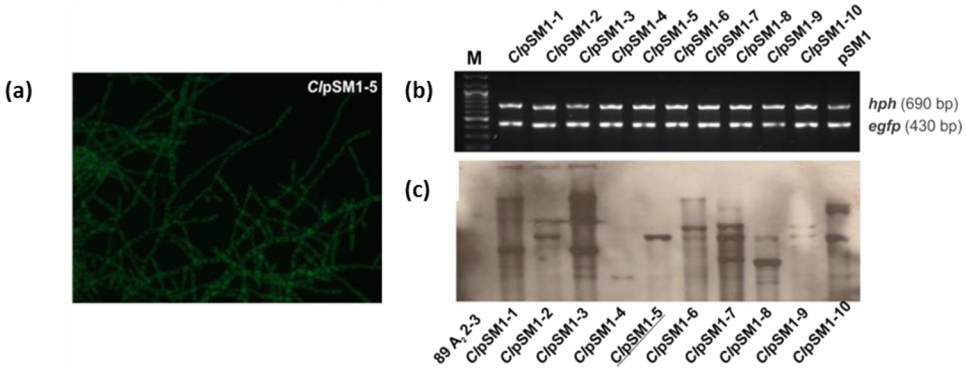


**Fig. 4.** Obtaining *C. lindemuthianum* strains expressing the *egfp* reporter gene. (A) Fluorescence microscopy displaying expression of the EGFP protein in *C. lindemuthianum*. (B) Amplification of the *hph* and *egfp* genes, demonstrating integration of the pSM1 vector into the *C. lindemtuhianum* genome. (C) DNA hybridization showing the pattern and number of integration sites of the pSM1 vector in the genome of the various transformant strains. M – Molecular size marker GeneRuler™ DNA ladder Mix (Fermentas Life Sciences).

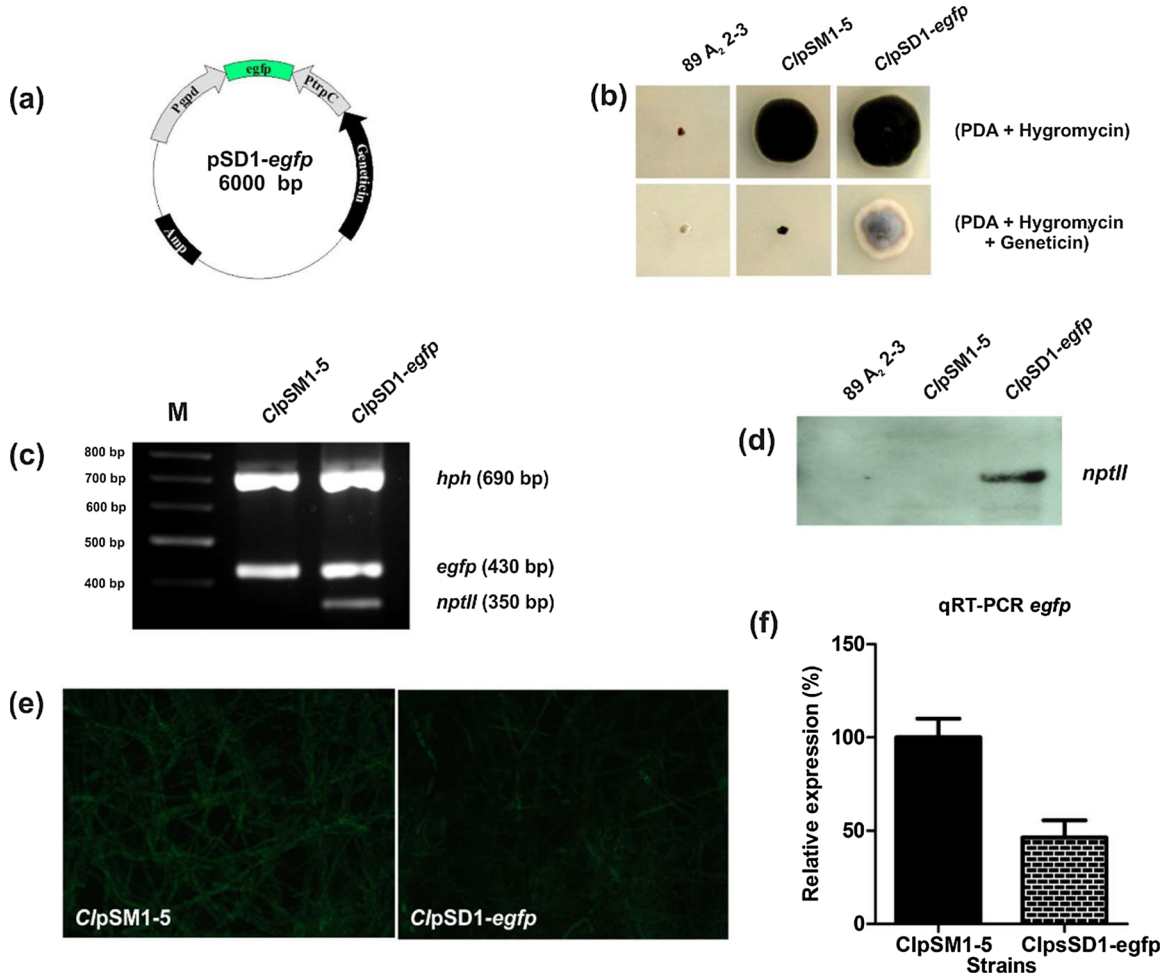


**Fig. 5.** Silencing of the *egfp* reporter gene in *C. lindemuthianum*. (A) Simplified physical map of the pSilentDual1-*egfp* vector used for silencing the *egfp* gene in *C. lindemuthianum*. (B) Growth of the wild-type and transformant strains of *C. lindemuthianum* in PDA culture media supplemented with hygromycin or hygromycin + geneticin. (C) Molecular characterization of the transformant strains by PCR amplification of the *hph*, *egfp*, and *nptII* genes. (D) DNA hybridization displaying the pattern and number of integration sites of the pSilentDual1-*egfp* vector in the *Cl*SD1-*egfp* strain. (E) Fluorescence microscopy showing evidence of the *egfp* gene silencing through reduction of the level of fluorescence emitted by the *Cl*SD1-*egfp* strain. (F) Real-time quantitative PCR demonstrating the reduction in expression of the *egfp* gene in the *Cl*SD1-*egfp* strain.

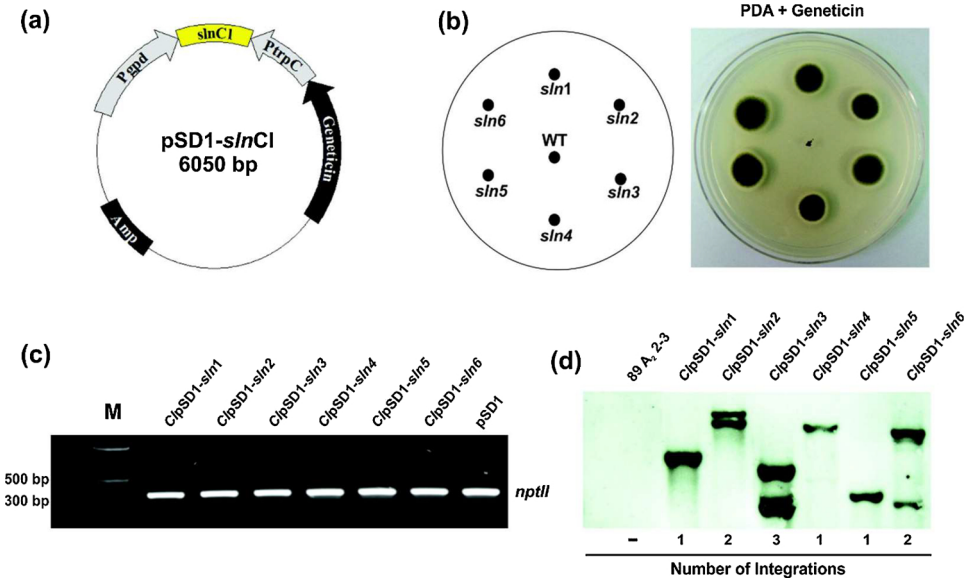


**Fig. 6.** Genetic transformation of *C. lindemuthianum* with the silencing vector pSilentDual1-*sln*Cl. (A) Simplified physical map of the pSilentDual1-*sln*Cl vector used for silencing the *slnCl1* gene in *C. lindemuthianum*. (B) Growth of the wild-type and transformant strains of *C. lindemuthianum* in PDA culture media supplemented with Geneticin. (C) Molecular characterization of the transformant strains by PCR amplification of the *nptII* gene. (D) DNA hybridization revealing the pattern and number of integration sites of the pSilentDual1-*sln*Cl vector in the various transformant strains.

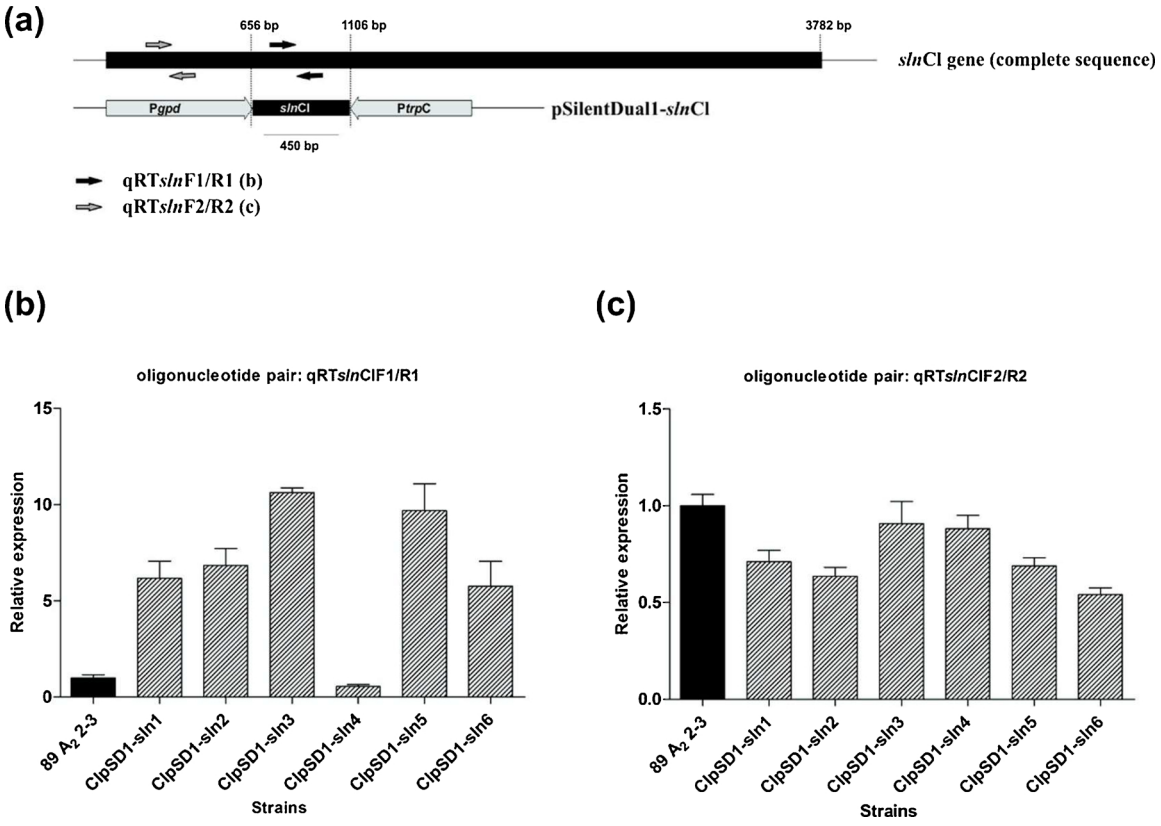


**Fig. 7.** Molecular characterization of the silenced strains using real-time quantitative PCR. (a) Strategy adopted to analyze the silenced strains using the transformation vector pSilentDual1-*sln*Cl. (b) Relative expression of the *slnCl1* gene using the oligonucleotide pair qRTslnClF1/R1. (c) Relative expression of the *slnCl1* gene using the oligonucleotide pair qRTslnClF2/R2.

the plant cell wall. This results in necrotrophic lesions formation. As expected, the leaves inoculated with the spores from slnCl1$^-$ mutant presented a significant reduction of anthracnose symptoms. Small lesions were observed only at day 5, but they did not evolve in the extension of the affected part, when compared to leaves inoculated with conidia of the wild isolate (Figure S4).

## 3. Discussion

By using REMI mutagenesis to mediate integration of pSM1 plasmid, we identified the gene *slnCl1* as a pathogenicity determinant in *C. lindemuthianum*. This work was the first to relate the isolation and characterization of a HK in *C. lindemuthianum* potentially involved with pathogenicity. To validate our results, we have chosen the methodology of *slnCl1* silencing. We identified the four major genes involved in RNA silencing in *Colletotrichum* spp. genomes, and we demonstrated the occurrence of this mechanism in *C. lindemuthianum* inducing silencing of the *egfp* reporter gene and the endogenous gene *slnCl1*.

Similar results (24, 21 and 24 transformant strains) were obtained when REMI methodology was carried out employing 0 U, 10 U or 20 U of *Hind*III, respectively. Therefore, it was concluded that the restriction enzyme did not improve significantly the number of transformant strains in *C. lindemuthianum*, the opposite pointed out for *Magnaporthe grisea* (Imazaki et al., 2007; Ruyi et al., 2007). However, in our work, REMI helped on isolation of sequences adjacent to the interrupted gene, making easier their identification and characterization. The mutant MutCl1 was not able to infect bean leaves of Rosinha cultivar. Also, this strain is deficient on melanin production and shows a delayed production of non-melanized apressoria compared to wild-type *C. lindemuthianum*. According to Langfelder et al. (2003) and Veneault-Fourrey et al. (2005), apressorium melanization occurs during their maturation. Melanin is strongly related with infection process in several phytopathogenic fungi due to the turgor pressure, resulting from glycerol accumulation on host cell wall (Mohammed, 2013). In conclusion, the absence of melanin could be the reason for the low infectivity showed by MutCl1 when inoculated in bean leaves (Fig. 1b).

The recovery of part of promoter region on pRescue24 favored the isolation of *slnCl1* full sequence. *slnCl1* encodes a 1183-amino acids protein classified as a HK VI as it displays an extracellular domain responsible for picking up extracellular signals (Catlett et al., 2003) and the domains PAS/PAC found in proteins related with signaling and transcription, phosphorous activation, ATP binding and Response Regulator protein (RR) (Taylor and Zhulin, 1999). Moreover, SlnCl1 is a hybrid protein as it presents both the portion HK that covers the autophosphorylation His residue, and the RR portion in the same polypeptide. As showed by alignments with HK protein sequences from other fungi species, the portions H-box, N-box, G1-box, F-box and G2-box are extremely conserved, suggesting the relevance of these consensus sequences to functionality and maintenance of HKs along the evolutive process. This two-component system is considered one of the main mechanisms by which certain organisms perceive and adapt to environmental conditions. This system is present in various organisms (including microorganisms such as bacteria, filamentous fungi, and yeasts) that participate in the regulation of processes, such as differentiation, chemotaxis, production of secondary metabolites, and even pathogenicity (West and Stock, 2001; Catlett et al., 2003; Defosse et al., 2015; Hérivaux et al., 2016).

We identified a silencing mechanism prospecting a sequence homologous to the QDE-1 protein of *N. crassa* in the genome of *C. lindemuthianum*. The QDE-1 gene, which encodes a RNA-dependent RNA polymerase (RdRP), was the first component of the eukaryotic silencing pathway discovered (Cogoni and Macino, 1999). Homologous sequences to DCL-1/DCL-2 protein of *N. crassa* also were identified in the genome of *C. lindemuthianum*. Although the DCL-1 and DCL-2 proteins

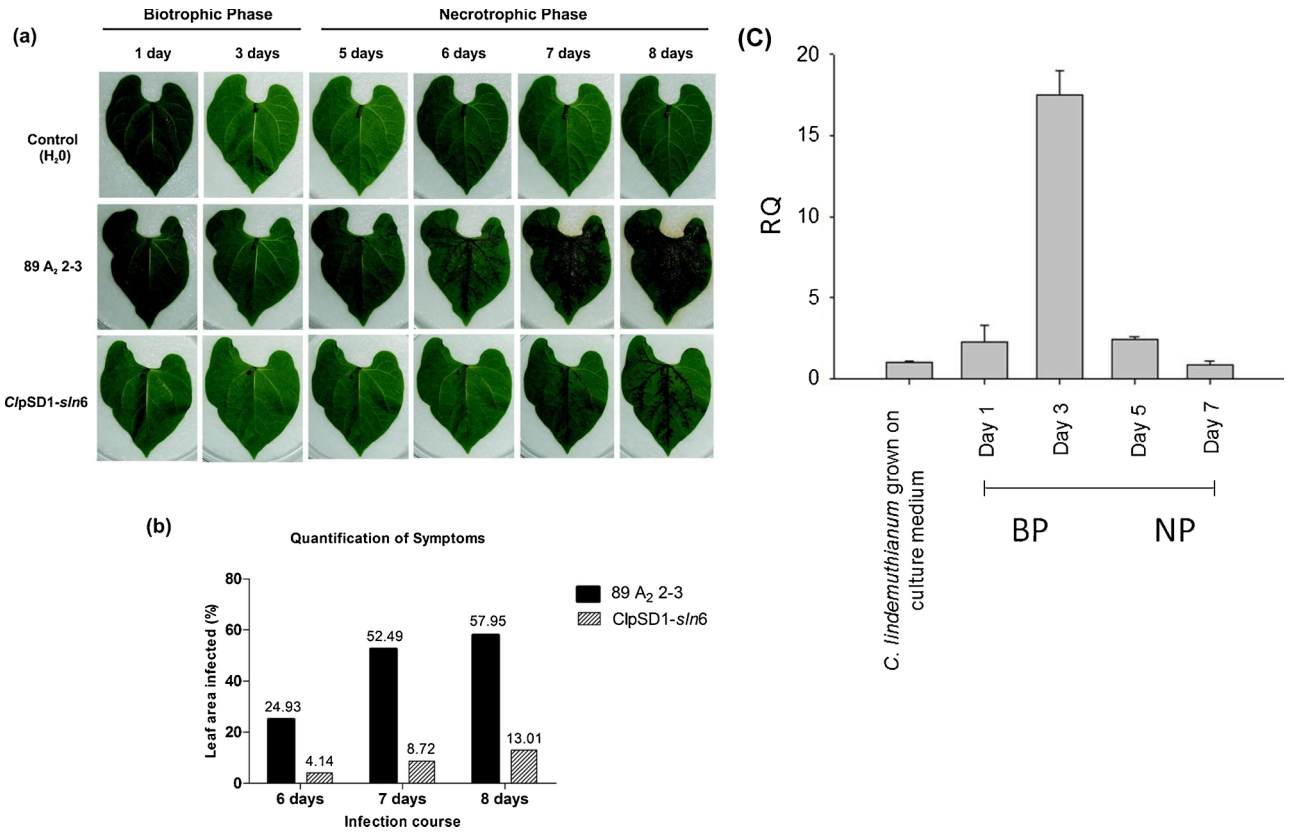


**Fig. 8.** Pathogenicity assay. (a) Pathogenicity tests in detached common bean leaves displaying the results of 1 and 3 days of inoculation (biotrophic phase), as well as 5, 6, 7, and 8 days of inoculation (necrotrophic phase). (b) Quantification of the visible symptoms in the common bean leaves at 6, 7, and 8 days after inoculation. The figure shows the results from the comparison between the 89 $A_2$ 2–3 wild-type strain and the *Clp*SD1-*sln*6 transformant strain. As a control, 100 µL of previously autoclaved, ultrapure deionized $H_2O$, was used to coat the healthy leaves. (C) Evaluation of *slnCl1* expression by Real Time quantitative PCR during the biotrophic and necrotrophic phases of infection. RQ, relative quantity; BP, biotrophic phase; NP, necrotrophic phase.

are considered distinct proteins, they have the same domains in equivalent positions. The QDE-2 protein is the central component of RISC (RNA-induced silencing complex) and has the following two main conserved domains: PAZ domain and the PIWI domain (Liu et al., 2004). Both domains have been identified in the sequences of *C. lindemuthianum*. A phylogenetic analysis of the proteins that constitute the central machinery of the silencing mechanism demonstrates that these proteins are relatively conserved among fungal species and may represent good evolutional phylogenetic markers for filamentous fungi. In fact, the RdRP, Dicer-like and Argonaut proteins occur in members of all groups of eukaryotic organisms. This wide taxonomic distribution and the direct demonstration of the occurrence of the phenomenon suggests that the central components of the RNAi machinery were present in the last common ancestor of eukaryotes.

For the silencing of genes *egfp* and *slnCl1* in *C. lindemuthianum*, we selected a vector based on a dual-promoter system, the pSilentDual1 (PSD1) (Nguyen et al., 2008). The transformation of *C. lindemuthianum* with the pSilentDual1-*egfp* vector resulted in a transformant strain (ClpSD1-*egfp*) that displayed a significant reduction in fluorescence emission (Fig. 5e) and a 53.6% reduction in the relative expression of the gene (Fig. 5f). The confirmation of *egfp* gene silencing in *C. lindemuthianum* demonstrates that this fungus, along with the vast majority of filamentous fungi, has at its disposal a functionally required protein arsenal to trigger the silencing of a target gene from a specific dsRNA. Thus, we built a vector called pSilentDual1-*sln*Cl to silence the *slnCl1* gene and confirm our previous results obtained by REMI regarding its effects on pathogenicity.

Six transformant strains were obtained with this vector, and the qRT-PCR analysis revealed a significant reduction in the endogenous expression level of the *slnCl1* gene. The strains *Clp*SD1-*sln*3 and *Clp*SD1-*sln*4 exhibited reductions of 12% and 10%, respectively, whereas strains *Clp*SD1-*sln*2 and *Clp*SD1-*sln*6 displayed reductions of 37% and 46%, respectively. It is possible to observe that two of the three strains with a lower reduction in the expression level (i.e., *Clp*SD1-*sln*4 and *Clp*SD1-*sln*5) presented a single integration site of the vector in the genome, whereas the two strains with greater reductions in the expression level (i.e., *Clp*SD1-*sln*2 and *Clp*SD1-*sln*6) possessed two integration sites. Regardless of the degree of silencing, the pathogenicity test in detached common bean leaves revealed a significant reduction of visible symptoms caused by all six transformant strains compared with the wild-type strain (data not shown), confirming that the *slnCl1* gene encodes a determinant of pathogenicity in the interaction of *C. lindemuthianum* and its host. Interestingly, the six transformants had no deficiency in melanin production, such as the initial mutant MutCl1, probably due to the degree of silencing. Fig. 8 illustrates the results obtained for the *Clp*SD1-*sln*6 strain, which presented the greatest degree of reduction in gene expression. The gene silencing did not prevent the *Clp*SD1-*sln*6 strain from causing tissue necrosis typical of anthracnose. However, these symptoms were delayed and only began to appear 6 days after the infection. It is therefore likely that the *slnCl1* gene plays an essential role in regulating the initial steps of the infection process, especially during the transition from the biotrophic phase to the necrotrophic phase. The evaluation of *slnCl1* expression during the infective process by *C. lindemuthianum* wild type was monitored from the 1$^{st}$ to the 7$^{th}$ day, counted from the inoculation (Fig. 8c). In fact, high expression was detected on the 3$^{th}$ day of infection, which corresponds to the final of biotrophic phase and transition to necrotrophic phase, indicating a high requirement of HK for infection. This result reinforces the hypothesis of a possible role of *slnCl1* gene on pathogenicity showed by *C. lindemuthianum*. Indeed, mutants unable to overcome the biotrophic phase

have been identified in other *Colletotrichum* species. For example, the *C. lindemuthianum* H433 strain grows biotrophically but fails to form necrotrophic secondary hyphae. In this case, the *CLTA1* (*GAL4*-like) gene, encoding for a zinc-finger transcriptional regulator, is responsible for preventing a successful biotrophic-necrotrophic transition (Dufresne et al., 2000). In a study with *C. graminicola*, disruption of the *CPR1* gene, encoding a microsomal signal peptidase, also affected the formation of secondary hyphae and therefore prevented host infection. The authors suggested that the mutant is unable to secrete sufficient amounts of proteins that are essential for necrotrophic development (Thon et al., 2002).

The present study is the first report that characterize and describe the role of the histidine kinase *slnCl1* in pathogenicity in *C. lindemuthianum*. Also, this is the first occurrence of an RNAi-mediated gene silencing mechanism in *C. lindemuthianum*. We demonstrate that all the four major proteins that constitute the central machinery of the silencing mechanism are present in the genome of *C. lindemuthianum* and that each of these proteins has conserved domains that are critical and properly function. Furthermore, this study also indicated the possibility of using this mechanism as a molecular tool for the functional characterization of the pathogenicity genes that are involved in the pathogen-host interaction. Silencing of the *slnCl1* endogenous gene suggests its role on pathogenicity reinforcing the elucidation of the regulatory mechanisms that are involved in the establishment of the infection, thereby facilitating the development of novel strategies to combat anthracnose in the common bean.

## 4. Material and methods

### 4.1. Microorganism, growth conditions and plant cultivar

Experiments were carried out with *C. lindemuthianum* 89 isolate $A_2$ 2–3. *C. lindemuthianum* was maintained in Potato Agar Dextrose (PDA) enriched with yeast extract (2 g/L), peptone (2 g/L) and hydrolyzed casein (1.5 g/L) at 22 °C. The mutant strains were grown on hygromycin-added (80 μg/mL) enriched PDA. The Rosinha cultivar, susceptible to anthracnose, was employed for pathogenicity assay. The propagation of plasmids was done in ultra-competent *Escherichia coli* DH5α cells (Inoue et al., 1990; Sambrook et al., 1989).

### 4.2. Genetic transformation of C. Lindemuthianum

*C. lindemuthianum* was grown during five days at 22 °C. The protoplasts were obtained from mycelial treatment with KCl 0.8 M in phosphate buffer 0.01 M (pH 5.8) containing 15 mg/mL Lysing Enzymes (Sigma Aldrich) and 10 mg.$mL^{-1}$ BSA at 30 °C for 3–4 hours/60 rpm. The protoplasts were washed by centrifugation in ST Buffer (sorbitol 1 M; Tris HCl, 0.01 M) and STC Buffer (sorbitol, 1 M; Tris HCl, 0.01 M e $CaCl_2$, 0.05 M) and ressuspended to a final concentration of $10^8$ protoplasts. $mL^{-1}$. For REMI, 200 μL of protoplasts, 10 μg pSM1 vector (Pöggeler et al., 2003), 0 U, 10 U or 20 U of *Hind*III and 50 μL of polyethylene glycol (PEG) 6000 25% were mixed. This mixture was then incubated on ice for 30 min following the addition of 500 μL of PEG 6000 25%. After 30 min at room temperature, the protoplasts were plated in enriched PDA with the osmotic stabilizer sucrose (0.56 M) and hygromycin (100 μg/mL) to select the transformants.

In the other transformation experiments 10 μg of the vector was used and the transformants strains were grown on enriched PDA containing hygromycin B (for the pSM1 vector) or geneticin to a final concentration of 250 μg/mL (for vectors pSilentDual1-egfp and pSilentDual1-slnCl).

### 4.3. Plant pathogenicity assay

The pathogenicity assay was carried out as proposed by Dufresne et al. (1998) employing the cultivar Rosinha, susceptible to antracnosis, as host. To obtain a large number of spores, *C. lindemuthianum* strains were inoculated in green beans (previously autoclaved two times with an interval of 24 h between them), following incubation for 10 days at 22 °C. Subsequently, the primary leaves of cultivar Rosinha, grown for 10 days at 23 °C, were excised, inoculated in abaxial surface with $10^6$ spores/mL and incubated at 22 ºC in a chamber with 16 h of light (166 $\mu E/s^{-1}/m^{-2}$) per day. The leaf symptoms were evaluated for 10 days. All the assays were performed in triplicate.

### 4.4. Apressoria analysis

Nearly 100 *g* of cultivar Rosinha leaves were excised, stored at −20 °C for 12 h and boiled in distilled water 1 L. The solution was then filtered in filter paper and autoclaved for 20 min at 121 °C. The filtrate portion was transferred to polypropylene tubes and centrifuged at 1000 *g* for 10 min. Twenty mL of supernatant was inoculated with $10^5$ spores. $mL^{-1}$ of *C. lindemuthianum* wild-type and MutCl strain separatelly. The supernatants were maintained for 12 h at 19 °C until observation with the inverted microscope Olympus IX70.

### 4.5. Hybridization analysis for copy-number determination

Total DNA from wild-type and mutant strains of *C. lindemuthianum* was extracted (Specht et al., 1982). In order to evaluate the number of integrations of the plasmid pSM1 in *C. lindemuthianum* genome, nearly 5 μg DNA were cleaved using the enzymes *Bgl*II and *Pst*I, which exhibit no cleavage site and one cleavage site, respectively, in pSM1 sequence. The products of the digestion were separated by electrophoresis in 0.8% agarose gel and transferred to a Duralon-UV membrane (Stratagene) and the hybridization was performed (Southern, 1975) employing the AlkPhos Direct Labeling and Detection Systems with CPD-Star (GE Healthcare) and the plasmid pSM1 as a probe.

### 4.6. Plasmid rescue

To determine the exact site of pSM1 integration, a Polymerase Chain Reaction (PCR) was employed with the primer M13R, homologous to region adjacent to the insertion of pSM1, and several arbitrary primers to amplify the sequence adjacent to the interrupted gene. Only the random primer OPG-17 in combination with M13R (Operon Tech) (Table 1) led a positive result. As a control, reactions were performed with only the M13R or only the arbitrary primer. The PCR reactions were carried out in a 25 μL reaction, consisted of 1X GoTaq Reaction Buffer with $MgCl_2$ 1.5 mM, dNTP 0.2 mM, primers 1 μM, GoTaq DNA polymerase 1.25 U and DNA 30 ng. The PCR conditions were as follows: initial denaturation at 90 °C for 1 min followed by 35 cycles of denaturation at 90 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min.

PCR product of the combination of primers M13R/OPG-17 were purified using the Wizard SV PCR kit and cloned into the pGEM-T Easy vector (Promega) given rise to the plasmid pRescue24 employed for gene screening in *C. lindemuthianum* genome bank.

### 4.7. Screening of slnCl1 from C. lindemuthianum genomic bank

The screening of the target gene was carried out employing *C. lindemuthianum* genome bank constructed with the Lambda EMBL3/BamHI Vetor Kit (Agilent Technologies). The hybridization was carried out following Benton and Davis (1977) recommendations using pRescue24 plasmid as probe. The DNA extraction of positive recombinant phages was performed (Sambrook et al., 1989) and the target gene was confirmed by PCR with the primers alb1F e alb1R (Table 1). The primers alb1F and alb1R were designed from pRescue24 sequence. The PCR conditions were the same as highlighted above. The DNA of positive phages (containing the target gene called *slnCl1*) were cleaved with the restriction enzymes *Cla*I, *Hind*III, *Kpn*I, *Not*I and *Sal*I, separated

by electrophoresis in agarose gel and hybridized by Southern blot (Southern, 1975) with the probe labeled by AlkPhos Direct Labeling and Detection Systems with CPD-Star Kit (GE Healthcare). Subsequently, the membranes were exposed for four hours to Hyperfilm (Amersham Pharmacia Biotech). The hybridized DNA fragments were recovered and cloned in pBluescript KS$^-$ vector (Agilent Technologies) giving rise to the plasmids pDK1, pDS1, pHS1, pIS2, pAN1, pDN1 e pHC1. The plasmids were sequenced by Macrogen Inc. (Seoul, South Korea) in order to obtain the gene sequence.

### 4.8. Sequence analysis and homology modeling

The deduced amino acid sequence was obtained using MEGA 6 (Tamura et al., 2013). The conserved domains of HKs were obtained from http://prosite.expasy.org/. Multiple alignments with HK sequences from *Gibberella moniliformis* (AY456037.1), *Botryotinia fuckeliana* (AY456054.1), *Cochliobolus heterostrophus* (AY456008.1) and *Aspergillus nidulans* (AB036054) were performed using ClustalW2 at http://www.ebi.ac.uk/Tools/msa/clustalw2/ (Thompson et al., 1994). The three-dimensional protein model was obtained at Phyre server (Kelley and Sternberg, 2009) and was compared with structures available at the Protein Data Bank (PDB) ProFunc server (Laskowski et al., 2005). The transmembrane regions were predicted by the TMHMM model of Markov (Krogh et al., 2001).

### 4.9. Phylogenetic analysis

Accession numbers of the sequences of HK used are provided in Table 2. The sequences were aligned on Clustal W (Thompson et al., 1994). Corrections were made manually following the recommendations of Tamura et al. (2013), employing MEGA 6. The Maximal Parsimony method was chosen to evaluate to which group of HK the *SlnCl1* belongs. The trees were obtained from PAUP 4.0b10 (Swofford, 2002) employing a heuristic search with the algorithm Subtree Pruning and Regrafting (SPR) and bootstrap of 1000 replicates.

For genes *qde-1*, *qde-2*, *dcl-1* and *dcl-2*, 12 species of filamentous fungi, including two strains of *C. lindemuthianum*, were selected. All of the sequences were aligned using the MEGA 6 program (Tamura et al., 2013). The phylogenetic method chosen was the maximum likelihood, and the robustness of the internal branches was determined by the bootstrap with 1000 replicates. Accession numbers of the sequences used are provided in Table 2.

### 4.10. Identification of RNA interference pathway in C. lindemuthianum genome

The sequences of genes encoding central components of the RNAi pathway were taken from the genome of the *C. lindemuthianum* strains 83.501 and $A_2$ 2–3. The sequences of the genes were subjected to the Augustus program (Stanke et al., 2004) to identify potential introns. The sequences of the protein were noted by the UniProtKB/SwissProt database, and possible conserved domains characteristic of each protein were identified using the CDD and PROSITE databases. All the sequences were deposited in the GenBank. The access numbers are provided in Table 2.

### 4.11. Assembly of silencing vectors

A pSM1 vector (Pöggeler et al., 2003) containing the *hph* gene was used to obtain a strain of *C. lindemuthianum* expressing the enhanced green fluorescent protein (EGFP). For silencing the *egfp* gene, we used the vector pSilentDual1-*egfp* (pSD1G) (Nguyen et al., 2008), which was acquired from the Fungal Genetics Stock Center (FGSC; Kansas City, Missouri USA) and contained the *nptII* gene, which confers resistance to the aminoglycoside Geneticin (G-418). For silencing the *slnCl1* gene, the pSilentDual1-*sln*Cl (pSD1-*sln*Cl) vector was constructed. For the construction of this vector, a 450-bp fragment of the *slnCl1* gene was amplified with the oligonucleotides si*sln*ClF/si*sln*ClR (Table 1) and cloned into the *Hind*III site of the pSilentDual1 (pSD1) vector, which was also acquired from FGSC. Both silencing vectors contained a target

**Table 2**
Microorganisms and accession of genes analyzed for construction of phylogenetic trees.

| Microorganisms | GenBank accession number | | | |
|---|---|---|---|---|
| | HK | | | |
| *Alternaria brassicicola* | AY700098 | | | |
| *Aspergillus clavatus* | XM_001268127 | XM_001273136.1 | | |
| *Aspergillus fumigatus* | XM_745820 | XM 001481590.1 | | |
| *Aspergillus nidulans* | AB036054.1 | | | |
| *Botryotinia fuckeliana* | AY456054.1 | Y456061 | | |
| *Candida albicans* | XM_711910 | | | |
| *Cochliobulus heterostrophus* | AY456009 | AY456004 | AY456005 | AY456008.1 |
| | AY456020 | AY456006 | | |
| *Clavispora lusitaniae* | EF157662 | | | |
| *Giberella moniliformis* | AY456046 | AY456035 | AY456034 | AY456049 |
| | AY456038 | AY456039 | AY456037.1 | |
| *Neurospora crassa* | XM_959477 | XM_959672 | DQ128077 | BK004088 |
| *Neosartorya fischeri* | XM_001258143 | | | |
| *Penicillium marneffei* | XM_002143307.1 | | | |
| *Talaromyces stipitatus* | XM 002479605.1 | | | |
| | QDE-1 | QDE-2 | DCL-1 | DCL-2 |
| *C. lindemuthianum strain 83* | ALJ83735.1 | ALJ83738.1 | ALJ83736.1 | ALJ83737.1 |
| *C. lindemuthianum strain 89* | ALJ83735.1 | ALJ83742.1 | ALJ83740.1 | ALJ83737.1 |
| *C. orbiculare* | ENH88131.1 | ENH85753.1 | ENH80776.1 | ENH87778.1 |
| *C. gloeosporioides* | XP_007279182.1 | XP_007275754.1 | XP_007286789.1 | EQB53060.1 |
| *C. fioriniae* | XP_007599611.1 | XP_007600899.1 | XP_007595264.1 | XP_007592384.1 |
| *C. graminicola* | EFQ34383.1 | EFQ31708.1 | EFQ33220.1 | EFQ30102.1 |
| *C. sublineola* | KDN68933.1 | KDN63192.1 | KDN70556.1 | KDN66022.1 |
| *C. higginsianum* | CCF36884.1 | CCF38692.1 | CCF41547.1 | CCF34628.1 |
| *M. oryzae* | XP_003711624.1 | XP_003716704.1 | ELQ37466.1 | XP_003715365.1 |
| *M. phaseolina* | EKG12628.1 | EKG10810.1 | EKG16741.1 | EKG11039.1 |
| *N. crassa* | CAB42634.1 | CAE85552.1 | Q7S8J7.1 | Q7SCC1.4 |
| *A. fumigatus* | XP_753696.1 | XP_747330.1 | Q4WVE3.3 | XP_746479.1 |
| *A.oryzae* | XP_001824860.2 | BAE55663.1 | XP_001824024.2 | XP_001818742.1 |

gene flanked by two promoters for RNA polymerase II of *Aspergillus nidulans*, namely, P*trpC* and P*gpd*, thereby leading to the production of dsRNA and causing the degradation of the target mRNA.

### 4.12. Molecular characterization of the transformant strains

The DNA of the transformant strains and the wild-type isolate was extracted using the UltraClean™ Microbial DNA isolation kit (MO BIO Laboratories). To confirm the presence of the pSM1 vector, a PCR reaction was performed using two pairs of oligonucleotides: *hph*F/*hph*R, and *egfp*F/*egfp*R (Table 1). To confirm the presence of the pSilentDual1-*egfp* or pSilentDual1-*sln*Cl vectors, a PCR reaction was performed with the oligonucleotide pair *npt*IIF/ *npt*IIR (Table 1). Southern blots were used to characterize the transformants regarding the pattern and number of integrations, as previously described. DNA fragments corresponding to the genes *hph* (for vector pSM1) or *npt*II (for vectors pSilentDual1-*egfp* and pSilentDual1-*sln*Cl) were used as probes, amplified using the oligonucleotides shown in Table 1, and labeled using the PCR DIG Probe Synthesis Kit (Roche).

### 4.13. Phenotypic characterization of the egfp strains

To assess the expression of the EGFP protein in strains that were transformed with only the pSM1 vector and in strains containing pSM1/pSilentDual1-*egfp*, slides containing mycelia from each transformant were prepared and photographed using the image capture system OLYMPUS Q-Color3™, which was coupled to the OLYMPUS BX50 fluorescence microscope with a BP 470/440-nm filter.

### 4.14. Gene targeting and phenotype confirmation

The inactivation of the *C. lindemuthianum slnCl1* gene was performed by integrating and disrupting the gene by plasmid pSMIS2. The pSMIS2 was assembled from plasmid pIS2, which carries part of the promoter region and 256 nucleotides of the initial region of the *slnCl1* gene cloned into the pSM1 vector. The wild strain 89 of *C. lindemuthianum* was used for transformation, which followed the procedures described previously. The plates were incubated and the hygromycin resistant transformants were further purified by monosporic isolation. The interruption of the histidine kinase gene in the mutant slnCl1$^-$ was checked by PCR.

### 4.15. Quantitative PCR

Rosinha cultivar leaves were inoculated with $10^6$ spores/mL of *C. lindemuthianum* wild-type and freezed in liquid nitrogen at intervals of 1, 3, 5 and 7 days of incubation. Leaves inoculated with distilled water were employed as negative control. The total RNA was extracted with Trizol (Invitrogen). The samples were treated with DNAse RQI RNAse-free (Promega) and the cDNA was obtained by the Kit ImProm-II Reverse Transcription System (Promega), following the manufacturer instructions.

The relative quantification of *slnCl1* expression was done in ABI 7500 Real Time PCR Systems (Applied Biosystems). The expression levels were normalized by the constitutively expressed gene *gpdA*, which expresses the enzyme glyceraldehyde dehydrogenase in *C. lindemuthianum*. The total RNA extracted from fungi grown on liquid culture medium was employed as a calibrator. The PCR reactions were done employing: cDNA 10 ng, primers 0.2 µM, and SYBR Green PCR Master Mix (Applied Biosystems) to the final concentration of 1X in 25 µL reaction. The standard curves were done with diluted cDNA (1:10). The experiments were done in triplicate employing leaves with the same age.

## Conflict of interests

All the authors declare no conflict of interests.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2018.10.005.

## References

Ansari, K.I., Palacios, N., Araya, C., Langin, T., Egan, D., Doohan, F.M., 2004. Pathogenic and genetic variability among *Colletotrichum lindemuthianum* isolates of different geographic origins. Plant Pathol. 53, 635–642.
Benton, W.D., Davis, R.W., 1977. Screening of Xgt recombinant clones by hybridization to single plaques in situ. Science 196, 180–183.
Bonett, L.P., Schewe, I., Silva, L.I., 2008. Variability of *Colletotrichum lindemuthianum* in common bean in western Paraná. Sci. Agric. 9, 207–210.
Catalanotto, C., Pallotta, M., ReFalo, P., Sachs, M.S., Vayssie, L., Macino, G., Cogoni, C., 2004. Redundancy of the two Dicer genes in transgene–induced posttrascriptional gene silencing in Neurospora crassa. Mol. Cell. Biol. 24, 2536–2545.
Catlett, N.L., Yoder, O.C., Turgeon, B.G., 2003. Whole-genome analysis of two-component signal transduction genes in fungal pathogens. Eukaryot. Cell 2, 1151–1161.
Cogoni, C., Macino, G., 1999. Gene silencing in *Neurospora crassa* requires a protein homologous to RNA–dependent RNA polymerase. Nature 399, 166–169.
Cogoni, C., Macino, G., 2000. Post–transcriptional gene silencing across kingdoms. Curr. Opin. Genet. Dev. 10, 638–643.
Defosse, T.A., Sharma, A., Mondal, A.K., de Bernonville, T.D., Latgé, J.-P., Calderone, R., Giglioli-Guivarc'h, N., Courdavault, V., Clastre, M., Papon, N., 2015. Hybrid histidine kinases in pathogenic fungi. Mol. Microbiol. 95, 914–924.
Dhiman, A., Bhatnagar, S., Kulshreshtha, P., Bhatnagar, R., 2014. Functional characterization of WalRK: a two-component signal transduction system from Bacillus anthracis. FEBS Open Biol. 4, 65–76.
Dufresne, M., Bailey, J.A., Dron, M., Langin, T., 1998. *clk*1, a serine/threonine protein kinase-encoding gene, is involved in pathogenicity of *Colletotrichum lindemuthianum* on common bean. Mol. Plant Microbe Interact. 11, 99–108.
Dufresne, M., Perfect, S., Pellier, A.L., Bailey, J.A., Langin, T., 2000. A GAL4–like protein is involved in the switch between biotrophic and necrotrophic phases of the infection process of *Colletotrichum lindemuthianum* on common bean. Plant Cell 12, 1579–1590.
Fassler, J.S., West, A.H., 2013. Histidine phosphotransfer proteins in fungal two-component signal transduction pathways. Eukaryot. Cell 12, 1058–1060.
Gan, P., Ikeda, K., Irieda, H., Narusaka, M., O'Connell, R., Narusaka, Y., Takano, Y., Kubo, Y., Shirasu, K., 2013. Comparative genomic and transcriptomic analyses reveal the hemibiotrophic stage shift of *Colletotrichum* fungi. New Phytol. 197, 1236–1249.
Gan, P., Narusaka, M., Kumakura, N., Tsushima, A., Takano, Y., Narusaka, Y., Shirasu, K., 2016. Genus-wide comparative genome analyses of *colletotrichum* species reveal specific gene family losses and gains during adaptation to specific infection lifestyles. Genome Biol. Evol. 8 (5), 1467–1481.
Hérivaux, A., So, Y.-S., Gastebois, A., Latgé, J.-P., Bouchara, J.-P., Bahn, Y.-S., Papon, N., 2016. Major sensing proteins in pathogenic Fungi: the hybrid histidine kinase family. PLoS Pathog. 12 (7) e1005683.
Hohmann, S., 2002. Osmotic stress signaling and osmoadaptation in yeasts. Microbiol. Mol. Biol. Rev. 66, 300–372.
Hyde, K., Cai, L., Cannon, P., et al., 2009. *Colletotrichum*–names in current use. Fungal Divers. 39, 147–182.
Imazaki, I., Kurahashi, M., Iida, Y., Tsuge, T., 2007. Fow2, a Zn(II)2Cys6-type transcription regulator, controls plant infection of the vascular wilt fungus Fusarium oxysporum. Mol. Microbiol. 63, 737–753.
Inoue, H., Nojima, H., Okayama, H., 1990. High efficiency transformation of *Escherichia coli* with plasmids. Gene 96, 23–28.
Kelley, L.A., Sternberg, M.J.E., 2009. Protein structure prediction on the web: a case study using the Phyre server. Nat. Protoc. 4, 363–371.
Krogh, A., Larsson, B., Heijne, G.V., Sonnhammer, E.L.L., 2001. Predicting transmembrane protein topology with a hidden Markov Model: application to complete genomes. J. Mol. Biol. 305, 567–580.

Langfelder, K., Streibel, M., Jahn, B., Haase, G., Brakhage, A.A., 2003. Biosynthesis of fungal melanins and their importance for human pathogenic fungi. Fungal Genet. Biol. 38, 143–158.
Laskowski, R.A., Watson, J.D., Thornton, J.M., 2005. ProFunc: a server for predicting protein function from 3D structure. Nucleic Acids Res. 33, W89–W93.
Liu, J., Carmell, M.A., Rivas, F.V., Marsden, C.G., Thomson, J.M., Song, J.J., Hammond, S.M., Joshua-Tor, L., Hannon, G.J., 2004. Argonaute2 is the catalytic engine of mammalian RNAi. Science 305, 1437–1441.
McCormick, A., Jacobsen, I.D., Broniszewska, M., Beck, J., Heesemann, J., Ebel, F., 2012. The two-component sensor kinase TcsC and its role in stress resistance of the human-pathogenic mold Aspergillus fumigatus. PLoS One 7, 1–11.
Mohammed, A., 2013. An overview of distribution, biology and the management of common bean anthracnose. J. Plant Pathol. Microbiol. 4, 1–6.
Motoyama, T., Kadokura, K., Ohira, T., Ichiishi, A., Fujimura, M., Yamaguchi, I., Kudo, T., 2005. A two-component histidine kinase of the rice blast fungus is involved in osmotic stress response and fungicide action. Fungal Genet. Biol. 42, 200–212.
Mullins, E.D., Kang, S., 2001. Transformation: a tool for studying fungal pathogens of plants. Cell. Mol. Life Sci. 58, 2043–2052.
Nguyen, Q.B., Kadotani, N., Kasahara, S., Tosa, Y., Mayuma, S., Nakayashiki, H., 2008. Systematic functional analysis of calcium–signalling proteins in the genome of the rice blast fungus, *Magnaporthe oryzae*, using a high–throughput RNA–silencing system. Mol. Microbiol. 68, 1348–1365.
O'Connell, R.J., Thon, M.R., Hacquard, S., et al., 2012. Lifestyle transitions in plant pathogenic *Colletotrichum* fungi deciphered by genome and transcriptome analyses. Nat. Genet. 44, 1060–1065.
Panwar, V., McCallum, B., Bakkeren, G., 2013. Endogenous silencing of *Puccinia triticina* pathogenicity genes through in plant–expressed sequences leads to the suppression of rust disease on wheat. Plant J. 73, 521–532.
Perfect, S.E., Hughes, H.B., O'Connell, R.J., Green, J., 1999. *Colletotrichum*: a model genus for studies on pathology and fungal–plant interaction. Fungal Genet. Biol. 27, 186–198.
Pöggeler, S., Masloff, S., Hoff, B., Mayrhofer, S., Kück, U., 2003. Versatile EGFP reporter plasmids for cellular localization of recombinant gene products in filamentous fungi. Curr. Genet. 43, 54–61.
Riggle, P.J., Kumamoto, C.A., 1998. Genetic analysis in fungi integration using restriction-enzyme mediated integration. Curr. Opin. Microbiol. 1, 395–399.
Román, E., Arana, D.M., Nombela, C., Alonso-Monge, R., Pla, J., 2007. MAP kinase pathways as regulators of fungal virulence. Trends Microbiol. 15, 181–190.
Ruyi, X., Juan, L., Yijun, Z., Yongjian, F., Xiaobo, Z., 2007. Screening and identification of mutants of *Magnaporthe grisea* by REMI. Front. Agric. China 1, 179–182.
Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.
Santos, J.L., Shiozaki, K., 2001. Fungal histidine kinases. Science 98, 1–14.
Silva, K.J.D., Souza, E.A., Ishikawa, F.H., 2007. Characterization of *Colletotrichum lindemuthianum* isolates from the State of Minas Gerais, Brasil. J. Phytopathol. 155, 241–247.
Southern, E., 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503–517.
Specht, C.A., Dirrusso, C.C., Novotny, C.P., Ullrich, R.C., 1982. A method for extracting high-molecular-weight deoxyribonucleic-acid from fungi. Anal. Biochem. 119, 158–163.
Stanke, M., Steinkamp, R., Waack, S., Morgenstern, B., 2004. AUGUSTUS: a web server for gene finding in eukaryotes. Nucleic Acids Res. 32, W309–W312.
Stock, A.M., Robinson, V.L., Goudreau, P.N., 2000. Two-component signal transduction. Annu. Rev. Biochem. 69, 183–215.
Swofford, D.L., 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.0b10. Available at. http://paup.csit.fsu.edu/index.html.
Takahara, H., Hacquard, S., Kombrink, A., Hughes, H.B., Halder, V., Robin, G.P., Hiruma, K., Neumann, U., Shinya, T., Kombrink, E., Shibuya, N., Thomma, B.P., O'Connell, R.J., 2016. *Colletotrichum higginsianum* extracellular LysM proteins play dual roles in appressorial function and suppression of chitin–triggered plant immunity. New Phytol. 211, 1323–1337.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.
Taylor, B.L., Zhulin, I.B., 1999. PAS domains: internal sensors of oxygen, redox potential, and light. Microbiol. Mol. Biol. Rev. 63, 479–506.
Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matriz choice. Nucleic Acids Res. 22, 4673–4680.
Thon, M.R., Nuckles, E.M., Takach, J.E., Vaillancourt, L.J., 2002. CPR1: a gene encoding a putative signal peptidase that functions in pathogenicity of *Colletotrichum graminicola* to maize. Mol. Plant Microbe Interact. 15, 120–128.
Veneault-Fourrey, C., Laugé, R., Langin, T., 2005. Nonpathogenic strains of *Colletotrichum lindemuthianum* trigger progressive bean defense responses during appressorium-mediated penetration. Appl. Environ. Microbiol. 71, 4761–4770.
Zampounis, A., Pigné, S., Dallery, J.-F., Wittenberg, A.H.J., Zhou, S., Schwartz, D.C., Thon, M.R., O'Connell, R.J., 2016. Genome sequence and annotation of *Colletotrichum higginsianum*, a causal agent of crucifer anthracnose disease. Genome Announc. 4, e00821–16.
Wang, Y.J., Liu, T., Zuo, Y.H., 2013. Isolation and identification of pathogenicity mutant of *Curvularia lunata* via restriction enzyme-mediated integration. Indian J. Microbiol. 53, 503–507.
West, A.H., Stock, A.M., 2001. Histidine kinases and response regulator proteins in two-component signaling systems. Trends Biochem. Sci. 26, 369–376.
White, D., 2000. The Physiology and Biochemistry of Prokaryotes. Oxford University Press, New York.
Wolanin, P.M., Thomason, P.A., Stock, G.B., 2002. Histidine protein kinases: key signal transducers outside the animal kingdom. Genome Biol. 10 (3013) 1-3013.8.