***clk1*, a Serine/Threonine Protein Kinase-Encoding Gene, Is Involved in Pathogenicity of *Colletotrichum lindemuthianum* on Common Bean**

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A random insertional mutagenesis in *Colletotrichum lindemuthianum*, the causal agent of common bean anthracnose, generated four mutants that showed altered pathogenicity when tested on intact seedlings, excised leaves, and/or excised hypocotyls. One of these mutants, H290, produced very few lesions on bean leaves and appeared affected in its ability to penetrate the leaf cuticle. Molecular analyses showed that the border sequences of the unique integration site of the disrupting pAN7-1 plasmid in the mutant exhibited homology with conserved domains of serine/threonine protein kinases. The corresponding wild-type sequences were cloned and a gene replacement vector with a mutated copy harboring a selection marker constructed. Transformation of the wild-type pathogen produced a strain with a phenotype identical to the original mutant. Genomic and cDNA sequences indicated that the disrupted gene is a member of the serine/threonine protein kinase family. The gene, called *clk1* (*Colletotrichum lindemuthianum* kinase 1), was weakly expressed in the mycelium of the wild-type strain grown on rich and minimal synthetic media but was undetectable during the infection even when a sensitive reverse transcriptase–polymerase chain reaction methodology was used. This study represents the first characterization of altered pathogenicity mutants in *C. lindemuthianum* produced by random mutagenesis and demonstrates the involvement of a member of the serine/threonine kinase gene family in the early steps of the infection process.

Many fungi are economically important plant pathogens. The need to understand the mechanisms leading to pathogenesis has driven a considerable amount of research on plant disease for many years. Knowledge of pathogenicity determinants will allow the identification of essential steps in the

infection processes, so that they may be targeted for the development of efficient and durable disease control.

In order to identify pathogenicity genes, different molecular approaches have been developed, as reviewed in Oliver and Osbourn (1995) and Hensel and Holden (1996). The first strategy concerns the targeted disruption of a gene whose function is postulated, on the basis of biochemical, cytological, and classical genetic data, to play a role in pathogenesis. To investigate its importance, a gene necessary for its expression is cloned and a mutant strain lacking the function constructed. The pathogenicity of the mutant strain is then compared with that of the corresponding wild-type strain. This approach has been adopted to investigate the role of cell wall–degrading enzymes such as cutinases (reviewed by Schäfer 1993).

The second approach is based on the assumption that expression of a pathogenicity gene is induced at a defined stage during infection. Differential expression studies have led to the cloning of several putative pathogenicity genes in fungi: for example, the *mpg1* gene in *M. grisea* (Talbot et al. 1993), *ipi* genes in *Phytophthora infestans* (Pieterse et al. 1993), and the *cap* genes in *Colletotrichum gloeosporioides* (Hwang and Kolattukudy 1995; Hwang et al. 1995). Major problems arise with this approach because the environment encountered in the host plant may also induce housekeeping genes, for example in response to nitrogen starvation, which could result in a high background in the differential screening. On the other hand, not all pathogenicity genes may be induced during disease development.

A third strategy, the candidate approach, consists of supposing that a gene is involved in the infection process by analogy with other organisms. Results in this emerging field essentially concern protein kinase-encoding genes. Several studies have been carried out on budding and fission yeasts and have allowed the identification of entire signal transduction pathways, in particular the MAP kinase pathways (reviewed by Herskowitz 1995). In the basidiomycete *Ustilago maydis*, the pheromone response, similar to that observed in *Saccharomyces* spp., is an important step in fungal development and therefore in pathogenesis. Based on homology with the *ste7* gene of *S. cerevisiae* encoding a MAPK kinase, the *U. maydis* *fuz7* gene was isolated and demonstrated to be

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required for several developmental processes such as tumor induction (Banuett and Herskowitz 1994). By means of a similar approach, the *M. grisea pmk1* gene, homologous to the *S. cerevisiae fus3* gene encoding a MAP kinase, was identified (Xu and Hamer 1996). *pmk1* mutants are unable to develop appressoria.

The last approach consists of mutants obtained by random insertional mutagenesis. The major asset of this strategy is that there are no a priori assumptions about the functions and expression of genes during infection. The second advantage is that mutant phenotypes are obtained before molecular analyses. However, the mutagenized organism must be at a haploid stage, at least during its development in the plant. Random integration events are carried out in the recipient genome with either transforming plasmids or transposable elements. Such a strategy has already been used to study development in *Dicystotium discoidum* (Kuspa and Loomis 1992). It has also proved highly effective in identifying pathogenicity genes, as exemplified by the results obtained in *M. grisea* (Sweigard et al. 1996) and *U. maydis* (Bölker et al. 1995).

Colletotrichum lindemuthianum (Sacc. & Magnus) Lams.-Scrib. is the causal agent of common bean anthracnose. Previous cytological studies provided a good knowledge of the infection process on *Phaseolus vulgaris* L. (O'Connell et al. 1985; Bailey et al. 1992). Conidia adhere to aerial parts of the plants such as leaves or stems and then germinate and differentiate an infection structure devoted to penetration, the appressorium. After penetration of an epidermal cell, the fungus develops intracellular infection vesicles that act as haustoria. Intracellular primary hyphae are formed from the vesicles and colonize several host cells. After a period of biotrophic growth lasting 3 to 4 days, the cytoplasm of infected epidermal cells gradually degenerates. Secondary hyphae form, growing both intracellularly and intramurally and causing extensive degradation of cell walls and cell death. At this stage, which coincides with the appearance of visible symptoms, the fungus behaves as a typical necrotroph. *C. lindemuthianum* is thus referred to as an hemibiotrophic pathogen. More recently, cytological studies have led to the identification of glycoproteins specific to particular infection structures formed during the interaction (Pain et al. 1994).

Despite these data, our knowledge of pathogenicity determinants is insufficient. We thus chose to develop an insertional mutagenesis strategy to better understand the molecular mechanisms that govern *C. lindemuthianum* pathogenesis. In this fungus, conventional transformation with supercoiled vectors had been shown to result in the integration of DNA vector molecules (Rodriguez and Yoder 1987; Daboussi et al. 1989) but not to give rise to complex insertion patterns. With the fungal transformation vector pAN7-1 (Punt et al. 1987), hygromycin-resistant transformants of *C. lindemuthianum* were generated and tested for pathogenicity on a susceptible cultivar of common bean. We report here the characterization of one of the pathogenicity-deficient strains identified in this screening.

RESULTS

Screening for pathogenicity mutants.

Among 600 transformants screened on the susceptible cultivar La Victoire, four strains showed reproducibly altered

pathogenicity. The mutant strain H290 was first assessed in axenic culture for radial growth, sporulation, and spore morphology. No obvious differences were observed when compared with the wild-type strain UPS9. When inoculated on excised primary leaves of the susceptible cultivar, the wild-type strain UPS9 induced reproducible macroscopic anthracnose symptoms as follows: brown lesions first appeared on main veins 4 to 5 days after inoculation, then on secondary veins, and within 24 to 36 h the whole leaf was macerated (Fig. 1A). When tested under the same conditions, H290 produced only a few lesions on the main veins 4 to 5 days after inoculation and these did not extend farther: the inoculated leaves were not macerated (Fig. 1A). Inoculation of whole plantlets resulted in similar observations. The wild-type strain UPS9 led to the death of all plantlets within 7 days after inoculation, but the mutant strain H290 only induced a few lesions on veins, leaving the plants alive (Fig. 1B and C). On excised hypocotyls, the wild-type strain UPS9 induced the appearance of spreading water-soaking lesions at all infection sites 6 to 7 days after inoculation; the mutant strain H290 also generated anthracnose symptoms, but always delayed by 2 to 3 days (data not shown).

H290 mutant phenotype.

H290 was tested for its ability to form appressoria on glass surfaces and excised hypocotyls. In both the wild-type strain UPS9 and the mutant strain, the majority of conidia germinated and produced appressoria (data not shown).

In order to test appressorium functionality, the lower surface of excised primary leaves was wounded by abrading with sand before inoculation. Seven days after inoculation, a marked increase in the symptoms produced by the mutant strain was clearly visible when inoculated on wounded leaves, compared with intact leaves (Fig. 2). Nevertheless, even if the number of lesions on veins was much higher on wounded leaves, their extension was still limited and no maceration was observed.

Gene replacement experiments.

Southern analysis was performed to establish the integration pattern of vector pAN7-1. H290 genomic DNA was digested by three restriction endonucleases: *KpnI*, which does not cut the plasmid, and *HindIII* and *EcoRI*, which cut once and twice, respectively, in the vector. As shown in Figure 3, the number and the respective sizes of the hybridizing bands reflect the insertion of a unique copy of the fungal vector at one locus in the *C. lindemuthianum* genome.

The restriction enzyme *KpnI* was used to recover the sequences flanking the pAN7-1 insertion in the mutant genome by a marker rescue strategy. Adjacent *C. lindemuthianum*-specific borders were isolated from plasmid sequences. The use of a *C. lindemuthianum*-specific, 360-bp *SacI-EcoRI* fragment as a probe on restriction digests of UPS9 genomic DNA displayed a simple hybridizing pattern coherent with the presence of a unique copy of these sequences in the *C. lindemuthianum* genome (Fig. 4). This probe was then used to screen a UPS9 genomic library to recover the entire corresponding wild-type region. A 5.5-kbp *XhoI* fragment present in all positive lambda clones was subcloned.

In order to check whether the integration event is responsible for the mutant phenotype, a gene replacement strategy was

set up. The gene replacement vector p290::hyg (Fig. 5A) was constructed by using the whole 5.5-kbp *Xho*I fragment as homologous sequences except that a 0.6-kbp *Sac*I-*Hind*III internal fragment extending on both sides of the insertion site was deleted and replaced by a 3.0-kbp *Sac*I-*Hind*III fragment from the pAN7-1 that had previously been demonstrated to be sufficient to govern the expression of the hygromycin resistance *hph* gene (Punt et al. 1990). UPS9 protoplasts were transformed with the resulting p290::hyg construct. One hundred and fifty transformants were isolated, purified, and tested for their mitotic stability. In a first step, the ectopic transformants were screened by polymerase chain reaction (PCR) with oligonucleotides located on both sides of the deletion. If the vector integrated at an ectopic locus, a 0.65-kbp amplification product corresponding to the wild-type region should be observed. No amplification product was expected under the same PCR conditions in case of homologous integration. A 0.65-kbp amplification product was obtained from 141 out of the 150 transformants tested. The remaining nine transformants were submitted to Southern blot analysis. Genomic

Fig. 2. Pathogenicity tests of the mutant strain H290 on intact (A) or wounded (B) cotyledonary leaves of the susceptible cultivar La Victoire. Wounded leaves were produced by abrading the lower face with Fontainebleau sand (Prolabo, Fontenay-sous-bois, France). Photographs were taken 7 days after inoculation.

Fig. 1. Pathogenicity tests of the mutant strain H290 on the *Phaseolus vulgaris* susceptible cultivar La Victoire. **A**, Excised cotyledonary leaves were inoculated with the wild-type strain UPS9 (left) and the mutant strain H290 (right) as described in Material and Methods. Eight-day-old plantlets were spray inoculated with either the wild-type strain (**B**) or the mutant strain (**C**). Photographs were taken 7 days after inoculation.

DNA of these transformants, of the wild-type strain UPS9, and of two ectopic transformants was digested by *KpnI* and probed with the 360-bp *SacI-EcoRI* probe. Gene replacement in the transformants was confirmed by the switch of the expected 1.6-kbp hybridizing fragment, present in the genomic DNA of the strain UPS9 and of the ectopic transformants, to a 14-kbp fragment in the replaced strains (Fig. 5B).

In order to establish whether the wild-type sequence was required for pathogenicity, excised primary leaves of the susceptible cultivar La Victoire were inoculated with spore suspensions of the replaced strains (kin5, 32, 75, 83, 89, 113, 129, 148, and 154), the two ectopic transformants (kin61 and 104), the mutant strain H290, and the wild-type strain UPS9. Re-

sults are presented in Figure 6. All the strains exhibiting a perfect replacement pattern exhibited the same mutant phenotype as the initial transformant H290, whereas the ectopic transformants retained their ability to produce normal anthracnose symptoms. These observations demonstrated that the H290 mutation was the basis for the altered pathogenicity phenotype.

Fig. 3. Analysis of pAN7-1 integration pattern in H290 genomic DNA. Total genomic DNA of the mutant strain H290 was digested with *KpnI* (lane 1), which does not cut the plasmid, and *HindIII* (lane 3) and *EcoRI* (lane 5) which cut once and twice, respectively, in the pAN7-1 vector. *HindIII* and *EcoRI* digests of the plasmid (lanes 2 and 4, respectively) were included as controls. The whole pAN7-1 vector was used as the probe. Sizes are indicated in kilobases.

Fig. 4. Occurrence of the genomic region surrounding the H290 insertion point in the genome of the strain UPS9 of *Colletotrichum lindemuthianum*. Total genomic DNA was digested with different restriction endonucleases and probed with the 360-bp *SacI-EcoRI* fragment. Sizes corresponding to the 1-kb DNA ladder (Gibco BRL, CITY, France) are indicated in kilobases. E: *EcoRI*, X: *XhoI*, S: *SacI*, H: *HindIII*, Xb: *XbaI*, P: *PstI*, K: *KpnI*.

B

Fig. 5. Targeted gene disruption of the H290 mutated region. **A**, p290::hyg contains the hygromycin resistance gene cassette (a 3-kbp *SacI-HindIII* fragment from the pAN7-1 plasmid) flanked with border sequences of the wild-type genomic locus. Left and right flanking regions are 3.0 kb (*XhoI-SacI* fragment) and 1.7 kb (*HindIII-XhoI* fragment), respectively. Restriction endonucleases sites are as follows: X, *XhoI*; K, *KpnI*; E, *EcoRI*; S, *SacI*; H: *HindIII*. Homologous recombination through a double crossing-over event results in replacement of part of the wild-type region by the hygromycin resistance gene. Curved lines represent the genome. Large arrow indicates location and direction of the *clk1* open reading frame. Asterisk indicates location of the pAN7-1 insertion site. **B**, Southern blot analysis of the replaced transformants. Total genomic DNA of the wild-type strain (lane 1), two ectopic transformants (lanes 2 and 3), and five out of the nine replaced transformants obtained (lanes 4 to 8) was digested with *KpnI* and probed with the 360-bp *SacI-EcoRI* fragment, indicated as probe in (A). As expected, the native 1.6-kbp band of strain UPS9 remains in the ectopic transformants but is no longer present in the replaced strains.

Mutant H290 is affected in a serine/threonine protein kinase-encoding gene.

The 5.5-kbp *Xho*I fragment previously mentioned was sequenced on both strands. Nucleotide sequence analysis revealed the existence of an open reading frame of 2,285 bp (including introns) potentially encoding for a 675-amino-acid polypeptide showing a high homology with serine/threonine protein kinases; the gene was thus called *clk1* (for *Colletotrichum lindemuthianum* protein kinase 1). The complete nucleotide and deduced amino acid sequences are presented in Figure 7. Three intervening sequences exhibiting expected splicing sites (Ballance 1986) were detected at nucleotide positions 2671 to 2791, 3720 to 3776, and 4365 to 4443. Within this open reading frame, the kinase catalytic core was identified between amino acids 32 and 316. The CLK1 polypeptide contains motifs and residues commonly found in other serine/threonine protein kinases (Hanks et al. 1988). These include the ATP binding motif, Gly-Lys-Gly-Ser-Phe-Ala, starting at position 32, the invariant Lys residue involved in phospho-transfer reaction at position 54, the consensus triplet Asp-Phe-Gly at position 204, and residues that are specifically conserved and that play a role in recognition of the correct hydroxyamino acid, Asp-Val-Lys-Pro-Gln-Asn at position 158 and Gly-Ser-Pro-Leu-Tyr-Met-Ala-Pro-Glu at position 222. Sequence comparison with the BLAST algorithm revealed that the CLK1 protein was closely related to the potential protein kinase KGTO of *Saccharomyces cerevisiae* (Fig. 8). These two proteins share 69.3% similarity and 53.7% identity within their catalytic domains.

clk1 expression analysis.

clk1 expression during the infection process was first studied with a Northern (RNA) blot analysis with total cellular and poly(A⁺) RNA from La Victoire leaves infected with the wild-type strain UPS9 at different points of the infection process and from cultures of the same strain grown either in complete or minimal medium. No hybridization signal was detected with the 360-bp *Sac*I-*Eco*RI fragment as a probe whatever the origin of the RNA. Nevertheless, clear signals corresponding to the appropriate sizes were observed when the plant-specific EF1- α or the fungal-specific actin probes were used (data not shown). It was thus concluded that *clk1* expression was either too weak or too transient to be detected by RNA gel blots.

A more sensitive reverse transcriptase (RT)-PCR approach was developed to detect *clk1* transcripts. In order to evaluate the occurrence of fungal transcripts in *P. vulgaris*-infected leaves, two fungal-specific genes were used. The first one corresponded to the *C. lindemuthianum* glyceraldehyde 3-phosphate dehydrogenase structural gene *gpd*, a constitutively and strongly expressed gene (Punt et al. 1990). Fungal-specific oligonucleotides were designed to avoid cross-amplification with plant materials. The second gene was the *C. lindemuthianum* structural nitrate-reductase gene *nia*, selected because of its inducibility under nitrogen starvation. Several pathogenicity genes have been shown to be induced by nitrogen starvation, for example the *M. grisea* *mpg1* gene (Talbot et al. 1993) and the *C. fulvum* *avr9* avirulence gene (Van den Ackerveken et al. 1994). *clk1*-specific oligonucleotides were synthesized for the detection of the kinase transcripts. Experiments for each gene were conducted three times to ensure reproducibility.

The *gpd* transcript was detected throughout the infection kinetics (Fig. 9A). The corresponding 345-bp amplification product was also observed in mycelium of strain UPS9. No amplification product was obtained in the (polyA⁺) RNA sample of noninfected leaves, proving the fungal specificity of the oligonucleotides. The very early detection of the amplification products specific for the *gpd* and *nia* genes of *C. lindemuthianum* (Fig. 9A and B) demonstrated the sensitivity of the method. Oligonucleotides specific for *clk1* discriminated between fungal and plant transcripts. The 340-bp amplification product of *clk1* was obtained in pure mycelium cultures, grown either in complete or minimal medium (Fig. 9C). It was also detected in the infection time-course from the second sample (12 h after inoculation; Fig. 9C). These results demonstrate that the *clk1* gene is expressed at a basal level.

DISCUSSION

Following a random insertional mutagenesis strategy, four *C. lindemuthianum* pathogenicity mutants were recovered out of 600 transformants, providing a frequency of 0.65%. Such a high frequency was also observed with similar approaches in *Ustilago maydis* and *Magnaporthe grisea* in which mutants more or less altered in their ability to infect their host plants were reported with a frequency of 1.4% (Bölker et al. 1995) and 0.65% (Sweigard et al. 1996), respectively. These results can be compared with those previously reported for tagging of specific genes *preg* and *pgov* in *Neurospora crassa*, which encode negative regulatory factors of the transcriptional activator NUC-1 (Kang and Metzenberg 1993). In that case, only two mutants out of 2×10^5 transformants were recovered. If the present study can be considered as representative of what obtained in filamentous fungi, it would provide a tagging frequency of 0.001% for a specific gene in the genome. These data demonstrate that a large number of genes may be involved in the pathogenesis of phytopathogenic fungi.

The mutant strain H290 appears to be affected at the penetration step, possibly because of the differentiation of defective appressoria. Additional experiments must be set up to identify which step is altered.

Molecular analyses of the mutant strain H290 led to the identification of the *clk1* gene. It encodes a putative serine/ threonine protein kinase. Gene replacement experiments showed that directed *clk1* mutants mimicked the phenotype of the original H290 mutant strain, thus demonstrating the requirement of *clk1* for *C. lindemuthianum* pathogenicity. The *clk1* transcripts are present in pure cultures of the fungus and in the infection time-course, suggesting that the *clk1* gene may be constitutively expressed. This may indicate that regulatory mechanisms occur at a posttranscriptional level, as widely described for protein kinase activities (Hunter 1995). On the other hand, the signal transduction pathway involving the CLK1 protein kinase may rather be regulated through its substrate(s).

Recent studies provide further evidence for a role of protein kinases in pathogenicity in phytopathogenic fungi. Pharmacological studies demonstrated that cyclic AMP (cAMP) was a regulatory element of appressorium differentiation in *M. grisea* (Lee and Dean 1993). Similar approaches showed that the Ca²⁺/calmodulin signal transduction pathway is important in the early development of *Colletotrichum trifolii* on the plant surface, i.e., germination of conidia and appressorium forma-

tion (Warwar and Dickman 1996). Subsequent work has led to the identification of elements of signal transduction pathways. In the rice blast fungus *M. grisea*, both insertional mutagenesis and the candidate approach allowed the identification of the *cpkA* gene, encoding the catalytic subunit of a cAMP-dependent protein kinase (Sweigard et al. 1996; Mitchell and Dean 1995). *cpkA* mutants showed delayed appressorium formation (Xu et al. 1997). By means of a candidate approach based on homology with the pheromone response pathway of *Saccharomyces cerevisiae*, members of MAP kinase pathways have also been identified in phytopathogenic fungi. In the maize smut fungus *U. maydis*, the *fuz7* gene, a homolog of the *ste7* gene from *S. cerevisiae* and of the *byr1* gene from *S. pombe*, was shown to be involved in several processes such as tumor induction and teliospore germination, two of the essential stages for pathogenicity (Banuett and Herskowitz 1994). In *M. grisea*, a similar approach led to the cloning of the *pmk1* gene, a *fus3* homolog involved in infection structure formation and fungal invasive growth in the host plant (Xu and Hamer 1996). Together with the present work, these results suggest that phosphorylation is an essential process in fungal differentiation and, consequently, plays a major role in the successful infection of host plants.

The identification of the *clk1* pathogenicity gene offers prospects for understanding a signal transduction pathway in-

involved in *C. lindemuthianum* infection process. In particular, heterologous expression experiments could be used to explore the functionality of the *CLK1* kinase and to facilitate the isolation of *CLK1*-interacting members by a two-hybrid strategy.

MATERIALS AND METHODS

Fungal cultures.

Colletotrichum lindemuthianum strain UPS9 (Fabre et al. 1995) was used as the recipient wild-type strain to generate hygromycin-resistant transformants. Cultures were grown in petri dishes on malt agar medium (N1) supplemented with spectinomycin sulfate (1 g/liter) and penicillin G (5×10^6 units/ml) to prevent bacterial contamination (N⁺). Conidia were obtained by gently scraping 10-day-old cultures flooded with 5 ml of sterilized, distilled water. Mycelia were grown in Roux's flasks (Fabre et al. 1995) containing either 50 ml of liquid potato dextrose medium or 50 ml of minimal medium (Daboussi et al. 1989). For genomic DNA extraction, the mycelium was collected after incubation for 48 h at 23°C, whereas for total RNA isolation it was harvested 36 h after inoculation. Mycelium was frozen in liquid nitrogen and conserved at -80°C until extraction.

Fungal transformation.

The fungal transformation vector pAN7-1 (Punt et al. 1987), containing the hygromycin resistance *hph* gene under the control of *Aspergillus nidulans* *gpdA* promoter and *trpC* terminator sequences, was used to generate a collection of hygromycin-resistant transformants and to construct the gene replacement vector. Preparation and transformation of *C. lindemuthianum* protoplasts were carried out as described by Rodriguez and Yoder (1987) with a slight modification: to select hygromycin-resistant transformants, hygromycin B was added after incubation at 23°C for 16 h in an overlay of 4 ml of molten complete medium at a final concentration of 400 µg/ml. Transformation frequency was approximately 30 to 35 transformants per µg of DNA. Hygromycin-resistant transformants were subcultured on N⁺ plates containing the antibiotic at a final concentration of 200 µg/ml, then purified by isolation of single spores. The mitotic stability of the hygromycin-resistance phenotype was tested after repeated subculturing on a nonselective medium. In total, 800 stable hygromycin-resistant *C. lindemuthianum* transformants were generated.

Pathogenicity tests.

Conidia of the wild-type strain UPS9 as well as the hygromycin-resistant transformants were obtained from N⁺ agar medium as previously described. Plantlets of *Phaseolus vulgaris* cv. La Victoire, which is highly susceptible to anthracnose, were grown for 8 days at 23°C, 75% relative humidity (RH), with 8-h dark and 16-h light photoperiods under fluorescent tubes ($166 \mu\text{E} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$), in trays filled with moist vermiculite. For the primary screening, primary leaves were excised and placed in petri dishes on a water-moistened filter paper. The lower surface of the leaf was inoculated by brushing the spore suspension at a concentration of 10^7 spores per ml. Petri dishes were closed to maintain high humidity and incubated at 19°C under the same light conditions as described above. Six leaves were used for each *C. lindemuthianum* strain tested. Symptoms were observed 5 and 7 days after

Fig. 6. Infected cotyledonary leaves of the susceptible cultivar La Victoire of *Phaseolus vulgaris*. Leaves were inoculated by either the wild-type strain UPS9, the mutant strain H290, an ectopic transformant (kin61), or a replacement strain (kin5).

inoculation. Uninoculated leaves survive at least 20 days on moist filter paper. When a mutant strain displaying reduced pathogenicity was apparent, leaves were maintained for 10 additional days under the same conditions.

When a hygromycin-resistant transformant exhibited a mutant phenotype, it was tested on excised primary leaves (10 replicates), on excised hypocotyls (5 replicates), and on whole plantlets (10 replicates). Excised hypocotyl segments were

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1      GTGGTCGAGTCAACATGACAGGTTGTCCCACTGAGAGCCAGCGTCAGATGCGTTGCCGAGAGATGCGTTTCCCGATGTG
79     CTCTCCCAACACGGAAGTTTGTATAACGGGGTACTCGGAATAGTCACTCTCTTCCGAGCGCTACCTTACTGCCACTGA
157    ATCTCATCTCGGCGCGCTGTAAACAGCGACTGAGGTGTCAAGGAGAAATGGAAGTCTGTGGGTACCTAGGTACAGGCA
235    AGCCATGGGCTTATCTGATACATGAGCCTTATCGGCCATGATGGCAATTGAGTGTGCGCAGCAGCAATACCCCTAC
313    TTCCCTTGTCTGATGACCGACACGAGGTGTACCAACCCAGGTATCCCTCTCCCACTGTATCCCTGCTCACCACCA
391    ACAGACCCCTATCTAATGACACGAGAAATACCGAGGGGATGCGACCATGTTCTTGCACTGGCTGCTACGGGCGGA
469    GTACGAGTGCCTGCACTGCGAAGCGCTCAAGTTAAGGTAGGAACCTACCTAAGATAGCGTCCCTTCTCTATAGTA
547    CTACATGAGACACGCGCAAGCAGGCACTACCTACCGACGAGCTTCTCCGCGCACTTGTTCTGCCCAATCAAGCT
625    AACCCAAACAGCTCAGCGCTGCGATGCGCAGCGCATCAGAGCCCAACTCCCAAGGAGCTGTTTGGAGTGGTCAI
703    TGGATTCACTTCSAAGACGCTGCTCTTTTATCCCGTTCTGTATATCCCGCTCTCTCTCTCTCTCTCTCTCTCTCT
781    TATCGCTTGTCTGCACTTGGTGCAGCATATAGGCCACTGGCTGACAGCTTCTGTGTATATGTGTTTCTCTCTCTCT
859    TACTTGATCGAGGTCCAAGTGCACAGTTTCTCCACGGGATCTTTACACCAAAATGACCTCAGCGGCTGGCTCTGCT
937    GCTGTCCCAACAGCGCGATGATCGCATATAGTGTCTACGTGCAACCTCAACTTCTGCTTTGGAAGATTAACACTCCC
1015   GTGGCTTAGGTTCTTCTGCTCAATTTCTGCTCACTTGTCTACTTCCGTACCGCAATTTCAACAGAACCTGAGCCAAAG
1093   CAACCAAGTGCACATCACATCGCATCTGCTGTACTCTCCCGAAGGACACGAGCGCGCTGGGAATATCTCCG
1171   GCTACTGCTACTGCTGCGCTATGCTGCGGTAGCAACTTGGAGTGTCTACCTTTATGCGATCGCATCAATCTCTCC
1249   ACATTTCCAGGAGCTGCAACCACTCATTTGATTCAGTGGTGGTGGCTGTCTGCACTGCAACCTTCAACCTCCTCC
1327   OCTATCTAATCTGCTGCGCGAATCCACTCCACACTTATCCACACCCATGCGCGGACCAAGACACACGCTCGCGA
1405   CCTAACCTTGGCGAGTACCTTGTCTAGCGCCAGTCACTGTCGCCAGTCTCTGTCACCGAGTGCACGCTGTCACTC
1483   GTTGCTCAGCGCTGCTTCTCTATCACTCACTCCCGACCGACGCGGCTTCTCTCTCTGCTCACTTCCACTC
1561   TGACCTCGATCTTGGCTTCAACCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT
1639   CTCACGACATCCCTGCACTTCTCTGCGCTTGTGCTGTTCTGCGACACACGACGACCACTTACGCGCTCGGCTCT
1717   TCGTTTCTATCGCGCTACCTTCACTTTAAACACATAGGCAGATACCGCAAGCGTCTGCTCACTGAGGTACATATCA
1795   AACACGACATCACCGAATGTATGCTCGGAGTCAATTAATTGACCTTGGATTTCACCTGACTCTGGAACAAGAAAGG
1873   TACCTTACCTCATCTCTCCACTGCTCCCAACGACCTTCAAACTTTTCAACGCTTACTTCTGCGAGCTCAACCGCT
1951   GGGTATGCTTCCATACAGGAGTACTTCTTGTTCACAGCTCACGCTTCACTGTATTAGTACAAATCTCGAGCTTTC
2029   CGCGCTCGCATTGTCTTCCCAATCGCGCTCTTCAAACTTCTGCTGCGCTCTTGTATCTCTCGCGAGTCTGCTCT
2107   TTCCACACTTCTGCTTGTGCTGAGACTACCGCGCTTCTGCGAGCGCTGACGCGCACAGGACGCTTCCGAGG
2185   GCGCACGCTTCTACCTTTCGACCGCATCTCGACACTGCTGCACTTGGTGGCGTGGCTCTTTCGACCTATCGGAAAG
2263   CTGCGGCTCTTCCACCGCTCTTACCAAGAAATACACAGGCTTCTGCTGCGAGTCTGTAGCTCTCTCAACCTGCG
2341   TTTGTTTTCTCCACGACATCCAGCAGCCATCAATACCTCGGAATCCATCGCAATCTATCTTTGCGAGCGTCACTGA
2419   ACGTGTACATGACGCGACCGCGCTTTCGCGAGTCTTTCGCGATGATTAGCGCTCGGCGCTGAACCACTCCCATGA
2497   CGCTTGTCTCCGACGAGTCTAACAGGGAAGAGGACAATA TGGCGGACGACTACCAACTCGACTCTCTCTGCGCGC

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M A D R L P T S T S S G R

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2575   AGGCGCGTGAAGCGATGCTCCATCGGAGAATTCTGATTGGCGGTGAATTTGGAAGGAGCTTCTGCTCAGGTC
R R R D G D A S I G E F V I G G E I G K G S F A Q V
2653   TACAGCGCGCATATAGGTATGTGACCTACTCCAAACCGTGGCGCTGGGCGAGCTTTCATCTGTAATCTCTCTGGA
Y S G H H K (
2731   CGCTAGAGTTATCAAGCGAGTCCGAATATGACAACTAGCAAGCTGACCAATCGCTTCCAGAACTCCAAAGCGCGT
) H S K A A V
2809   CGCATCAAGTCCGTTGAGATGGGCGCTCTGAACAACAACTTAGGAGAAATTTGATCGCGAGATCCAGATCACTCAA
A I K S V E M G R L N N K L R E N L Y G E I T K A V
2887   ACCCTACGTCATCCACACATCTGCTGCTGCAAGTTCGCTGCAATCGGCAAGCAGCAGCATCAACTCTGCTAGGATAC
T L R H P H I V A L H D C V E S A T H I N L V M E Y
2965   TCGGAGCTCGGGATCTTCTCTCTCAATTAAGAAGAGATCGCATGGCACTAATGCTGCCACCGAAGACATGGCA
C E L G D L S F F I K K R D R H G T N A A T E D M A
3043   AGGAATATCTGTCACCTCGGGCTCGGGTCTGCAAGTCTGTCACGCGCAATTTCTGCGAGCACTTGGCGCGCC
R K Y P V T P G S G L H E V V T R H F L Q Q L A S A
3121   CTAAAGTTTCTCAGAGAGAAGAACTACGTGCAAGAGACGTGAAGCGCCAAATCTGCTACTTCTGCGATCGCGCGG
L K F L R E K N Y V H R D V K P Q N L L L L P S P G
3199   TTCAGAAAGAAACTACGCGCCCATCTGACCGCGAGCAAGCTTCCCTGATTCCCAACGCGCGCTTGGCTTCTCTC
F R K E N S R P I L T A S N D S L I P N A G L A S L
3277   CCCATGCTAAAACTCGCGGATTTCTGCGCTTCTGCTGCGCTTCTGCGCATCAACATCGCTTCCGCGACGCTCTGCTG
P M L K L A D F G F A R V L P S T S L A D T L C G S
3355   CCATTTGATGATGCGCGCCGAAATTTCTCGCTACGAAAGATACGACGCGCAAGCGGACTTGTGTCGCTAGTACGTT
P L Y M A P E I L R Y E R Y D A K A D L W S V G T V
3433   TTGTACGAGATGATCAGCGAGCGCGCTCTTTCTGCTAGGAATCAGTCGAGTGTCTCGCGAAGATCGAAGCGCACA
L Y E M I T G R P P F R A R N H V E L L R K I E A T
3511   GAGGACAAGGTTAAGTACCGAAGGATGCTGTTGTGAGCAAGATCTGGTCAAGCTTATCGGCAAGCTACTACCGCG
E D K V K Y P K D A V V S K D L V K L I G K L L T R
3589   AACCCGCGGAGCGATCGCGTTGAGGATTTCTTCAACGACCGCGTCTGCTGTTGGTCCGATTCCAGGTGTAGTTGAG
N P V E R M R F E D F F N D P V V V G P I P G V V E
3667   GACGACATCCAAAGGTGAGCAGAAAGCATCAGAGACCTTCTGCTCTCTGCTAGTCCGATTTCGACATCTGCTACTG
D D I P K V E Q K P S R D L R S L E (
3745   TCTGACGCTTGTCTCTGCGCAGGACAGGCGGATCTCAACGGGAGCAAGGAACTGACCAATCGCGCGT
) A D P Q R E Q S E L A K S P R
3823   GAGGCTCTTTGAGATCCCCACAATTGCCAGCGCGGATGAGTACGAGTGGCGCAAGCAAGCTCTCAGCTCGCAC
E R P L R S P Q L P S P D E V R V P Q A N V S A R T
3901   GGGCAGTCTGCTGTCAGAGATCGGAGGGTCTTGGCACTTGCAGCACTCCATCCCGCAGCATCCACATCAGCG
G Q S P G R I G I G I G I R P P M P Q P S T S A
3979   CCGACGACCATAGGCTCTTCCAAAGCTCTGCTTATGCACTCCATCCGAGCATCTGCTTCAACGCGCACTCG
P S R P H R L S N A S L N R P P I R A S A P P T S
4057   TATCTGAATGAACGAAGCTCTGCTCCGTCACGAGCGAAGCATGCTGAGCAAGATAAGCGGCCGAGATGTGCA
Y L N E R K L R P V T E R S M T E Q D K A A Q D V A K
4135   TTTGAGAGATATGTTGCTGTGAAAGAACGCTAGAGGTCAATGCGCTTTGCGATGAATGGCCGCCAACCCG
F E R D Y V V V E K K H V E V N A F A D E M A A N P
4213   CGGTGACTCTGTTGTCACCAAGAACGCTCAGATGGTTCGACGGGCGACACAGCAAGGCGCTCCGACTTCTACCAG
R L T S L S P K N G Q M V R R A T Q Q G P P T S T T
4291   GGAGCAGGTGCTGATCAACCTTCCAGTGCAGTCCAAATGCTCAGGTTAAAGGACGGCCCGGCTGACCATCGTAG
G A G R M Q P S S A V Q I A Q G K G R P G H D H P (
4369   TCCTTTGACGCGCAAAAGCTATTCGGCGAGTCCACGACGCAAGGCTTCTATGCCAAGGCTATTTCCGACGCGAGG
) C
4447   CGTCTCTTGGCTTCAAGGTCCTTCAACGCTCTCGCGCGCAAGGCGGCTCTCGCGCCCTGTACAACCGCTTCCCC
V S L A S R S L N T S S A A R A P L R P C T T R S P
4525   GCGTATCCGACACCGACACCTCTGCTCTGTTGAGTCACTCGGTGACGCGCAGACAGGCGCACTCTGACGAGAT
R I R H R H P L L L D S S V T A D R A H H L T K M
4603   GCACGGTCTGCAAGCTCATCAAGACTTCGCACATCTGATGATTGTGTATATGGCTTTGCGGAAGTCAAGTTTAAAG
H G S S S S S K T S H I V V I V Y M A L P K S S L S
4681   CAACCTTTCCCTTTGGCACTTCCATGGATCAGCCCTGGGTGGCACCCCTGCCGCAAGTCTGATGAGGATGGCTT
N F S L W H L P W I T P W V A P L P T K S M R M V L
4759   ACTGTGAGGCGAGGTTGGCTCTGCTGAGGAGGCTCTGCTGCTTACGTCAGGCTCTGACACTGCTGGCGCGGCG
L S R Q R W L C L R R L S C F T S R L *
4837   ATGATGTGCGCAAGTCTGTGGTGACTCAGAAGACCGGGCGGATCTACCGGCGTACTGCGGCTGAGGTTTCCAAAG
4915   GATAGCTCGCGCGGAGATCACTCTACCGTTCAATGGGTGCGAGGGCGCTTAAACGAAGTCTTGGAGGAAGCGAA
4993   GCGGTACGATGAAGCTTCTCAAGTGCAGAGCTTCTGCTGAGGATCATCTGACCCACCGCAGCAACACGCGTGA
5071   GAGCGGCACTTTCGGCTCATCAAGCACAAAGACGTTCTCTCACCTCGGCTGACCTGAGCGCGAAAGCTCATGTAC
5149   GACCGGCTTGGAAATGAGCAAGACTGCTGCTATTGACGAAATACGAACGAGAAC

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Fig. 7. Nucleotide and predicted amino acid sequences of the *Colletotrichum lindemuthianum clk1* gene. Nucleotides are numbered with reference to the initiation codon. Introns are presented in lowercase letters. Nucleotide sequence of the *C. lindemuthianum clk1* gene has been deposited in the GenBank data base as accession number AF000309.

prepared as described by O'Connell et al. (1985). Five 10- μ l drops of each spore suspension (10^3 spores per ml) were placed on each of five hypocotyl replicas of the cultivar La Victoire. Hypocotyls were incubated in separate plastic boxes at 17°C with a 12-h dark and 12-h light photoperiod under moist conditions. Hypocotyls were examined daily for symptoms. Eight-day-old plantlets were spray inoculated with a spore suspension at a concentration of 10^6 spores per ml and then covered with a plastic cap to maintain high humidity. Symptoms were scored 7 days after inoculation.

Induction of germination and appressorium formation.

Spore suspensions of the wild-type strain UPS9 and the mutant strain H290 were diluted to a concentration of 10^3

spores per ml. Three 10- μ l drops of each suspension were deposited on the top surface of excised hypocotyls and incubated in separate plastic boxes at 17°C for 24 h. The suspension droplets were removed and replaced by 10- μ l droplets of 0.01% Calcofluor. After 30 min, the Calcofluor was removed and the area washed three times with sterile, distilled water. Thin layers of the inoculated surface were stripped off and mounted, inoculum side up, on slides and observed under a fluorescence microscope. The total number of appressoria in five microscope fields were counted in each of the three replicas for each isolate.

DNA isolation and manipulation.

Plasmid DNA was isolated by the alkaline lysis procedure (Sambrook et al. 1989). For the purpose of fungal transformation, it was purified by CsCl density gradient ultracentrifugation (Sambrook et al. 1989). *C. lindemuthianum* total genomic DNA was isolated following the methodology described in Fabre et al. (1995). DNA restriction, agarose gel fractionation, and transfer to Nylon membranes Hybond N (Amersham LIFE SCIENCE, Courtaboeuf, France) were performed according to manufacturer's instructions and standard procedures (Sambrook et al. 1989). Hybridizations for both Southern analysis and DNA library screening were carried out at 65°C according to standard procedures (Sambrook et al. 1989). Probes were 32 P-labeled with the Ready-To-Go DNA labeling kit (Pharmacia Biotech S.A., Saint Quentin-en-Yvelines, France). For the purpose of screening of the ectopic transformants in gene replacement experiments, PCR amplifications were conducted with 50 ng of total genomic DNA. Primers located on both sites of the deletion created in the gene replacement vector p290::hyg were designed as follows: 5'-GCGTCAATCGGCAACGC-3' and 5'-CCTGGAATGG GACCAACCACG-3'. Thirty cycles of denaturation, annealing, and polymerization were conducted at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, respectively.

CLK1	1	MADRLLPTSTS	SGRRRRDGDMA	SIGFVIGGR	IGKGSFAQVY	SGHKNKAA
KGTO	1	MGD-IKNDH	TTSVNNHLM	SAGNYTAEKE	IGKGSFAATVY	RGHLTSDKESQ
CLK1	51	-VAIKSVEMG	RENK-LREN	EYGEIOILAT	LRNHNVALH	DCVESATHIN
KGTO	51	HVAIKSVSRA	KLKNNKLEEM	LEISIAILKK	IKHFNHGLI	DCERTSDFY
CLK1	101	LVNEYCELDG	LSGFIRKR-D	RHGTNAATED	MARKYFVTPG	S--GLHEVVT
KGTO	101	LIMEYCALGD	LTFLLKRKE	LMENHPLLRT	VFEKYPPSE	NHNGHRAV
CLK1	151	RHFLQQLASA	LKFLREKNYV	HRDVKPQNL	L-LPSPGFRK	ENSRPILAS
KGTO	151	LSYLQQLASA	LKFLRSKNLV	HRDVKPQNL	LSTPLIGYHD	SKSFHEL---
CLK1	201	NDSLIPNAGL	ASLEPMKLAD	FGFARVLEST	SLADTLCGSE	LYMAPEILRY
KGTO	201	--GFV--GI	YNLFILKIAD	FGFARVLEST	SLAETLCGSE	LYMAPEILRY
CLK1	251	ERYDAKADLW	SVGTVLYEMI	TGRPPFRARN	HVELLRKIEA	TEQVKYKPD
KGTO	251	QKYNKADLW	SVGTVVFEMC	CGTTPFRASN	HLELFKKIKR	ANDVITFESY
CLK1	301	AVVSKDLVKL	IGKLLTRNEV	ERMRFEDFFN	DPVVVGPIPG	V-VEDDIEKV
KGTO	301	CNIEPEL-EL	ICSLITFDFA	QRIGFEEFFA	NKVVNEDLSS	YELEDDLEEL
CLK1	351	SKPSPDRDLS	LEADPQREQS	ELAKSPRER		
KGTO	351	ESK-SKGIVE	SNMFVSEYLS	KQPKSENSN		

Fig. 8. Sequence alignment between deduced amino acid sequences of *Saccharomyces cerevisiae* KGTO and *Colletotrichum lindemuthianum* CLK1 protein kinases. Roman numerals indicate conserved subdomains among the serine/threonine protein kinases family. Shaded letters indicate identical residues.

Fig. 9. Ethidium bromide-stained reverse transcriptase-polymerase chain reaction (RT-PCR) products corresponding to fungal-specific transcripts. Numbers refer to time of collection of the infected tissues (in hours after inoculation); CM and MM correspond to pure cultures of the UPS9 strain grown in complete and minimal medium, respectively; LV represents noninfected leaves of the cultivar La Victoire. Fungal genes tested are *gpd* (A), *nia* (B) as controls and *clk1* (C). Sizes of RT-PCR products are in base pairs: 345, 260, and 340 bp for the *gpd*, *nia*, and *clk1* genes, respectively.

Construction of the genomic library.

A *C. lindemuthianum* lambda (Lambda DASH II/BamHI Cloning Kit, Stratagene, Montigny-le-Bretonneux, France) genomic library was constructed from UPS9 genomic DNA. Total genomic DNA was partially digested with restriction endonuclease *Sau3A* (0.005 U per µg of DNA) and size fractionated on a 10 to 40% sucrose gradient. DNA fragments ranging between 10 and 15 kbp were selected and ligated with a vector:insert ratio of 1:3 into lambda DASH arms. After in vitro packaging with the GigapackII gold Packaging Extract Kit (Stratagene, Montigny-le-Bretonneux, France), recombinant phages were transfected in *Escherichia coli* strain P2392. The genomic library was found to represent two to three *C. lindemuthianum* genome equivalents as calculated from the equation of Clark and Carbon (1976). The *E. coli* strain Q358 (Sambrook et al. 1989) was used for amplification of recombinant phages.

RNA manipulation.

For the isolation of total RNA, an infection kinetics of the cultivar La Victoire was achieved. Plantlets were inoculated with strain UPS9. Four leaves were collected at the inoculation time (0 h) and then every 8 h from 12 to 108 h after inoculation (Mahé et al. 1992). The leaves were immediately frozen in liquid nitrogen and conserved at -80°C until RNA extraction. Total RNA was isolated following a cetyltrimethylammonium bromide (CTAB) procedure (Rogers and Bendich 1988). Total RNA was differentially precipitated from genomic DNA by two consecutive precipitations with lithium chloride (3.5 and 3 M, respectively). The final pellet was then resuspended in 500 µl of Tris-EDTA and precipitated with sodium acetate (final concentration 0.3 M) and 100% ethanol. The RNA concentration was measured spectrophotometrically. The messenger RNAs were isolated from total RNA with magnetic Dynabeads oligo(dT)₂₅ (Dynal, Compiègne, France) following the manufacturer's instructions. Northern blot experiments were conducted as described in Mahé et al. (1992) with a slight modification: hybridizations were carried out in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate), 5× Denhardt's solution (0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin) and 0.1% sodium dodecyl sulfate at 65°C. Membranes were washed at high stringency (Sambrook et al. 1989).

RT-PCR experiments.

RT-PCR experiments were realized following the manufacturer's instructions, except for annealing temperatures. Two microliters of total RNA was used for each reaction. The two fungal structural genes of the glyceraldehyde 3-phosphate dehydrogenase and nitrate-reductase, *gpd* and *nia*, respectively, were selected from the genomic library, subcloned, and at least partially sequenced to design the specific oligonucleotides described before. Two primers were designed for fungal-specific amplification of *gpd* transcripts: forward, 5'-CACATTACATATTCAATCAGACATAATGGC-3'; reverse, 5'-GCCTTGGCCTTGTCGATGGTGG-3'. Annealing was conducted at 56°C. For the amplification of fungal *nia* transcripts, the forward primer was 5'-GGTACTCGGGCA TCATCGCC-3' and the reverse was 5'-GTCAGTGAAGA GCCGTTTCAGG-3'. Annealing was conducted at 58°C. Two primers deduced from the *clk1* sequence were synthesized as

follows: forward, 5'-GGAAAAGGCAGCTTCGCTCAGG TCTACAGC-3'; reverse, 5'-CGTGACGACTTCGTGCA GACCCGAGCCCGG-3'. Annealing was conducted at 65°C.

DNA sequencing.

Sequencing of the *clk1* gene was performed by Genome Express (Grenoble, France). The complete sequence of *clk1* cDNA was obtained from both strands by means of the dideoxy chain termination method (Sanger et al. 1977) with the ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Roissy, France).

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