# Functional analysis of *CLPT1*, a Rab/GTPase required for protein secretion and pathogenesis in the plant fungal pathogen *Colletotrichum lindemuthianum*

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## Summary

**In eukaryotic cells, Rab/GTPases are major regulators of vesicular trafficking and are involved in essential processes including exocytosis, endocytosis and cellular differentiation. To investigate the role of these proteins in fungal pathogenicity, a dominant-negative mutant allele of *CLPT1*, a Rab/GTPase of the bean pathogen *Colletotrichum lindemuthianum*, was expressed in transgenic strains. This mutated gene encodes the amino-acid substitution N123I analogous to the N133I substitution in a known *trans*-dominant inhibitor of the Sec4 Rab/GTPase from *Saccharomyces cerevisiae*. A pectinase gene promoter was used to drive the *CLPT1(N123I)* allele in *C. lindemuthianum*, allowing the expression of the foreign gene on pectin medium and during pathogenesis, but not on glucose. The same strategy was used to overexpress the wild-type *CLPT1* allele. During growth on pectin medium, production of extracellular pectinases was strongly impaired only in *CLPT1(N123I)*-expressing strains. Cytological analysis revealed that *CLPT1(N123I)* strains accumulated intracellular aggregates only on pectin, resulting from the fusion of vesicles containing polysaccharides or glycoproteins. Moreover, these strains showed a severe reduction of pathogenesis and were unable to penetrate the host cells. These results indicated that the Rab/GTPase *CLPT1* is essential for fungal pathogenesis by regulating the intracellular transport of secretory vesicles involved in the delivery of proteins to the extracellular medium and differentiation of infectious structures.**

Key words: Fungal pathogenicity, *Colletotrichum lindemuthianum*, Secretion

## Introduction

During infection of their hosts, plant and animal pathogenic fungi secrete a variety of extracellular proteins that were shown in a number of cases to contribute to pathogenicity. These include hydrolytic enzymes produced during different stages of pathogenesis such as cutinases and pectinases by phytopathogens (Li et al., 2003; Oeser et al., 2002) and proteases by animal pathogens (Naglik et al., 2003). Extracellular proteins can also play a role in the molecular dialogue that establishes between the parasite and the host, contributing to the outcome of the host-microbe interaction (Esquerré-Tugayé et al., 2000). Thus, a precise regulation of the production of these proteins should occur during pathogenesis. While several studies have focused on the transcriptional regulation of extracellular proteins during pathogenesis (Herbert et al., 2002) little is known about the molecular mechanisms regulating the transports of these virulence factors to the extracellular medium. In eukaryotic cells, the intracellular vesicle trafficking is essential for many aspects of cellular processes including polarized growth and secretion of extracellular proteins. The Rab/Ypt family of small GTPases has a central role in the control of vesicular transport (Novick and Zerial, 1997). These GTPases cycle between donor and acceptor membranes and each step of the transport is governed by specific members of this protein family. In the budding yeast *Saccharomyces cerevisiae*, 11 such proteins have been identified (Lazar et al., 1997) and most of them are essential for yeast development. In animal and plant pathogenic fungi, the role of these proteins in regulating protein transport is largely unknown. Recently, two Rab/GTPases from the human pathogen *Candida albicans* were analyzed (Lee et al., 2001; Mao et al., 1999). To study the role of these essential proteins, dominant-negative mutations were introduced into the wild-type sequence of their genes which were expressed under the control of an inducible promoter. This strategy provided a means to show that the *C. albicans* orthologues of yeast *YPT1* and *SEC4* are essential for growth and protease secretion (Lee et al., 2001; Mao et al., 1999).

We have studied the regulation of the production of extracellular pathogenicity factors in the plant pathogen *Colletotrichum lindemuthianum*. The genus *Colletotrichum* comprises several hundred species, mostly plant pathogens. Although only rarely pathogenic to humans, *Colletotrichum* spp. have been reported as almost exclusively causing keratis (Fernandez et al., 2002) but cutaneous infection has also been described (Guarro et al., 1998; O'Quinn et al., 2001). *C. lindemuthianum* is a hemibiotrophic fungus that causes anthracnose disease on bean (Perfect et al., 1999). During

pathogenic development, the fungus produces a series of infection structures including germ tubes, appressoria, intracellular hyphae and secondary necrotrophic hyphae. Each stage of infection is characterized by the production of specific cell-wall-localised glycoproteins and extracellular enzymes (Centis et al., 1997; Herbert et al., 2004; Hutchinson et al., 2002) illustrating the importance of the secretory pathway in this system. Recently, we identified a Rab/GTPase gene, *CLPT1*, from *C. lindemuthianum. CLPT1* is able to complement the yeast thermosensitive mutation *sec4-8* (Dumas et al., 2001) suggesting that it is involved in the last step of exocytosis, in the transport of post-Golgi vesicles to the plasma membrane. Interestingly, expression of *CLPT1* was up-regulated on pectin medium, which also induces the production of extracellular pectinases. This suggests that activation of the secretory pathway can sustain the production of extracellular enzymes (Dumas et al., 2001). In the present study, we characterized *C. lindemuthianum* transgenic strains expressing the wild-type sequence of CLPT1 or a trans-dominant inhibitor of CLPT1, which was placed under the control of a pectinase gene promoter, induced during growth on pectin medium and pathogenesis, and repressed on glucose medium. This strategy allowed us to study precisely the function of CLPT1 in protein secretion and pathogenicity.

## Materials and Methods

### Strains, culture conditions and transformation

*Colletotrichum lindemuthianum* (Sacc. et Magn.) Briosi and Cav., race β was maintained on synthetic agar medium (Bannerot, 1965). Liquid cultures were obtained in a 250 ml Erlenmeyer flask containing 50 ml of synthetic medium (Barthe et al., 1981) supplemented with D+ glucose (10 g/l) or apple pectin (10 g/l). For microscopic examinations, the mycelium was grown on cellophane disks laid down onto the surface of solid synthetic medium supplemented with glucose or pectin. Transformation of *C. lindemuthianum* was done according to the method of Hargreaves and Turner (Hargreaves and Turner, 1992). Transformants were regenerated on a medium containing hygromycin at a final concentration of 50 μg/ml.

### Plasmid constructions and mutagenesis

The *CLPT1* coding sequence (Dumas et al., 2001) was amplified by PCR and the two restriction sites *Nco*I and *Pst*I were added at the 5′ and 3′ ends, respectively. The PCR fragment was introduced between the *Nco*I and *Pst*I site of the PG2-90 plasmid (Herbert et al., 2002) to replace the GFP nucleotide sequence. A mutated version of CLPT1, in which the ATC/N123 codon was replaced by AAC/I123, was produced by site-directed mutagenesis using the QuickChange site directed mutagenesis kit (Stratagene, La Jolla, CA) and following the supplier's instructions.

### Northern blotting

Total RNA was isolated from the mycelium of *C. lindemuthianum* using the Extract-all kit (Eurobio, Les Ullis, France) following the supplier's instructions. Samples containing 10 μg of total RNA were denatured with glyoxal and submitted to electrophoresis in a 1.2% (w/v) agarose gel in 10 mM phosphate buffer (pH 7). The gels were transferred to Hybond $N^+$ membranes (Amersham Pharmacia Biotech, France) and fixed by baking for 2 hours at 80°C. The membranes were then prehybridized for 2 hours at 42°C in 50% formamide, 0.1% SDS, 1× Denhardt's solution, 2× SSC and 50 ng/ml denatured salmon sperm DNA. Hybridization was carried out overnight under the same conditions, after addition of the $^{32}$P-labeled DNA probe.

### Production of antibodies directed against CLPT1 expressed in *Escherichia coli*

The *CLPT1* cDNA (Dumas et al., 2001) was cloned into the pMAL-cR1 vector (New England Biolabs, Beverly, MA) in the same translational reading frame as the *malE* gene, which encodes the maltose-binding protein (MBP). The resulting plasmid was introduced in BL21 cells by electroporation. The bacterial culture (1 l) was induced with IPTG (10 mM) and the MBP-CLPT1 fusion protein was purified by affinity chromatography on an amylose column (New England Biolabs) according to the manufacturer's instructions. A rabbit was given four intramuscular injections of the purified fusion protein (100 μg each). Ten days after the last boost, the serum was collected, clarified by centrifugation and stored at –20°C.

### Western blotting

The mycelium was ground in liquid nitrogen. The powder (200 mg fresh weight; FW) was suspended in 0.5 ml of 0.05 M acetate buffer, pH 5.2. After centrifugation at 4°C for 15 minutes at 10,000 ***g***, the soluble extract was recovered and the protein content was determined by the method of Bradford (1976). Culture filtrates (25 μl) and mycelium soluble extracts (20 μg protein) were subjected to SDS-PAGE in a 12% polyacrylamide gel (Laemmli, 1970). After migration, the proteins were transferred to a nitrocellulose membrane using a Biorad semi-dry apparatus at a constant current (1.8 $mA/cm^2$). The membrane was soaked for 30 minutes in a Tris-buffered saline solution (Tris-HCl pH 7.5, NaCl 150 mM) containing 3% non-fat milk before being incubated overnight in the same buffer containing the MBP-CLPT1 antiserum which was used at a 1000× dilution (Hugouvieux et al., 1995). The antigen-antibody complex was visualized by colorimetric detection using alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Sigma, France).

### Polygalacturonase activity

The extracellular medium was dialyzed against acetate buffer 50 mM, pH 5.2 overnight at 4°C. Three flasks were sampled for each assay. The enzymatic assay consisted of a sample (50 μl) of the dialysed medium and 1 ml of polygalacturonic acid 0.1% (w/v) in acetate buffer (50 mM pH 5.2). After incubation for 30 minutes at 30°C, total reducing sugars were assayed by the colourimetric method of Somogyi (Somogyi, 1952). Controls in which either the dialyzed growth medium, or the substrate were omitted were simultaneously performed. The enzymic activity was expressed in nanokatals (nkat), 1 nkat corresponding to the release of 1 nmol of reducing group equivalent per second. α-D-galacturonic acid (Sigma) was used as a standard.

### Fluorescence confocal microscopy and TEM

For confocal laser scanning microscopy, samples were stained with Congo Red (0.5% in water) for 5-10 minutes and washed in distilled water. They were mounted on microscope slides and observed using a Leica SP-2 confocal spectral microscope (Germany) with a 40× (1.25 NA) oil immersion objective. The 543 nm ray line of a helium laser was used for excitation and emitted light was collected between 560-630 nm. Pictures were computed by projection of 10-15 plan-confocal images acquired in z-dimension. There were finally treated by image analysis using Image Pro-Plus (Media Cybernetics, Silver Spring, MD) for best look-up-table adjustment.

For transmission electron microscopy, the samples were fixed for 2 hours at room temperature in 50 mM sodium cacodylate buffer (pH 7.0) containing 2% (w/v) glutaraldehyde (Oxford Agar, Oxford, UK)

Journal of Cell Science

Journal of Cell Science

and then washed in the same buffer without glutaraldehyde. They were post-fixed with osmium tetroxide (1%, w/v) in the same cacodylate buffer for 1 hour at room temperature. The samples were washed in water and dehydrated in a series of aqueous solutions of increasing ethanol concentration (20, 40, 60, 70, 80, 90, 100% v/v; 30 minutes each). Progressive infiltration with Spurr's epoxy resin (Oxford Agar, Oxford, UK) was carried out by serial incubation in ethanolic solutions of increasing concentration of Spurr's resin (30, 50, 80%, v/v; 12 hours each) and two incubations in undiluted resin. Infiltrated samples were then embedded in capsules and allowed to polymerize for 24 hours at 70°C. Ultrathin sections (80-90 nm thickness) were prepared using an UltraCut E ultramicrotome (Reichert-Leica, Germany) and collected on gold grids. They were either submitted to the periodic acid-thiocarbohydrazide-silver proteinate reaction (PATAg) according to the method of Thiéry (Thiéry, 1967) or uranyl acetate, lead citrate staining. For PATAg staining, sections were floated on a 1% (w/v) aqueous solution of periodic acid for 30 minutes at room temperature and rinsed twice in distilled water for 15 minutes. They were treated overnight at 4°C with a 20% aqueous solution of acetic acid containing 0.2% thiocarbohydrazide, washed in solutions of decreasing acetic acid concentration and finally in water. They were floated on a 1% (w/v) aqueous solution of silver proteinate for 30 minutes in the dark, washed in water and air dried before examination with a transmission electron microscope (Hitachi, H-600, Japan) operating at 75 kV. Photographs were taken using Kodak-Electron films (Kodak, France). For uranyl acetate and lead citrate staining, sections were floated for 5 minutes in the dark on an aqueous solution of 5% (w/v) uranyl acetate containing 50% (v/v) ethanol, washed in water and then treated with an aqueous solution of 0.4% (w/v) lead citrate for 2 minutes in the dark.

## Results

### Inducible expression of *CLPT1(N123I)* in transgenic *C. lindemuthianum* strains

A strategy based on the inducible expression of a dominant-negative mutant allele of *CLPT1* was used. Since constitutive expression of dominant-negative mutations can lead to a lethal phenotype, an inducible promoter fragment was used to express a mutated version of *CLPT1* in transgenic *C. lindemuthianum* strains. A truncated version of the pectin- and pathogenesis-responsive *CLPG2* promoter (PG2-90) (Herbert et al., 2002) was selected to develop an inducible expression system. *CLPG2* is a *C. lindemuthianum* polygalacturonase gene that is induced by pectin during the very first stages of pathogenesis (Centis et al., 1997; Dumas et al., 1999). By using the GFP coding sequence as a reporter gene, it was previously observed that *PG2-90* is induced by growing the mycelium on pectin and during appressorium formation (Herbert et al., 2002). As a prerequisite to using this promoter, the expression pattern of the GFP gene driven by PG2-90 was followed by northern blot analysis, after transfer of the mycelium onto pectin. As shown on Fig. 1, GFP expression was very similar to *CLPG2*, being transiently induced by pectin reaching maximum levels 24 hours after the transfer. A very weak expression was detected on glucose. Thus, this promoter fragment can be used to transiently express a dominant-negative allele of an essential gene.

The strategy that was used to construct the dominant-negative mutation, was based on the data reported for the yeast *SEC4* gene by Walworth et al. (Walworth et al., 1989). Thus, the Asn123 AAC codon present in the coding sequence of *CLPT1* was mutagenized to an ATC Ile codon, by site-directed

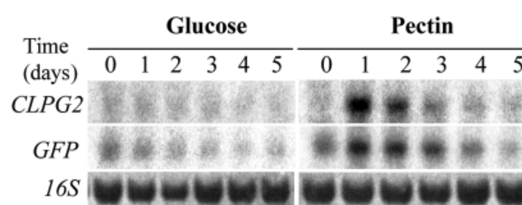


**Fig. 1.** Transcriptional regulation of the *GFP* gene fused to the *PG2-90* promoter fragment. Total RNA was extracted from a *PG2-90*::GFP transgenic strain (Herbert et al., 2002) grown on glucose or pectin medium for the time indicated. Northern blot analysis was performed using a *CLPG2* or a *GFP* probe. Equal loading of the RNAs (*16S*) on to the membrane was checked by staining with methylene blue.

mutagenesis. The resulting sequence was fused to the *PG2-90* promoter fragment as well as a control construct in which the wild-type *CLPT1* sequence was also fused to the *PG2-90* promoter fragment. The two constructs, named *PG2::CLPT1(N123I)* and *PG2::CLPT1*, were introduced into the *C. lindemuthianum* genome by protoplast transformation along with the selection plasmid pAN7, harbouring hygromycin resistance (Punt et al., 1987). Resistant strains were regenerated and analysed by PCR for the presence of the *PG2-90*::*CLPT1* or *PG2-90::CLPT1*(N123I) constructs (data not shown). For each construct, two transformants (CLPT1.4, CLPT1.9 and N123I.6, N123I.9) were selected for further studies.

Expression of *CLPT1* and *CLPT1(N123I)* in the selected transgenic strains was followed by northern blot analysis using RNA extracted from the mycelium grown on pectin. A signal corresponding to the wild-type *CLPT1* mRNA was visible in all tested samples. A second faster migrating band corresponding to the additional copy of *CLPT1* or *CLPT1(N123I)*, was detected only in the transgenic strains (Fig. 2A). Different levels of expression were obtained between the different strains, probably reflecting a position effect of the transgene insertion site.

In order to obtain specific antibodies directed against CLPT1, the protein was produced in *E. coli* as a fusion protein with the maltose binding protein (MBP). The fusion protein was purified on an amylose column and subsequently used to raise antibodies in rabbits. The antibodies were purified with the CLPT1-MBP fusion protein and used for western blots experiments. Protein extracts were prepared from the mycelium grown on glucose or pectin medium. A protein with a molecular mass of approximately 23 kDa was detected by the CLPT1 antibodies, and was shown to accumulate preferentially on pectin (Fig. 2B). However, we did not detect a stronger accumulation in the strains expressing additional copies of *CLPT1* compare to the wild type.

### Effect of *CLPT1(N123I)* on protein secretion

The ability of the *CLPT1* and *CLPT1(N123I)* transgenic strains to secrete extracellular polygalacturonases was examined. To use the same mycelium biomass for each strain, the mycelium was first grown on glucose for 3 days before transfer to pectin. Extracellular polygalacturonase (PG) activity was determined using polygalacturonic acid as substrate. In the case of the

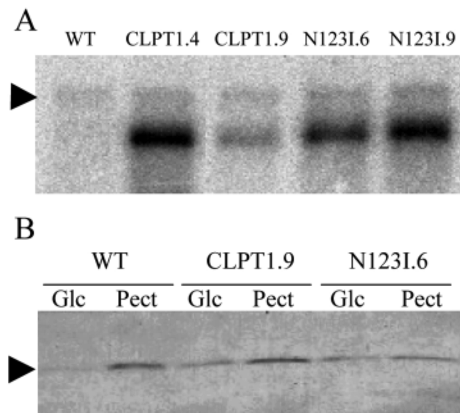


**Fig. 2.** Characterization of *C. lindemuthianum* strains transformed with *PG2-90::CLPT1* or *PG2-90::CLPT1(N123I)* constructs. (A) Northern blot analysis of total RNA extracted from the different strains grown on pectin medium and probed with *CLPT1* coding sequence. The band from the wild-type gene is indicated by the arrowhead. (B) Detection by western blot analysis of CLPT1 from wild-type and transgenic strains. Protein extracts were prepared from the mycelium grown for 4 days on glucose or pectin medium as indicated. Equal amounts of protein were loaded onto the gel. CLPT1 was revealed using purified anti-MBP-CLPT1 antibodies and anti-rabbit IgG labelled with alkaline phosphatase as secondary antibodies.

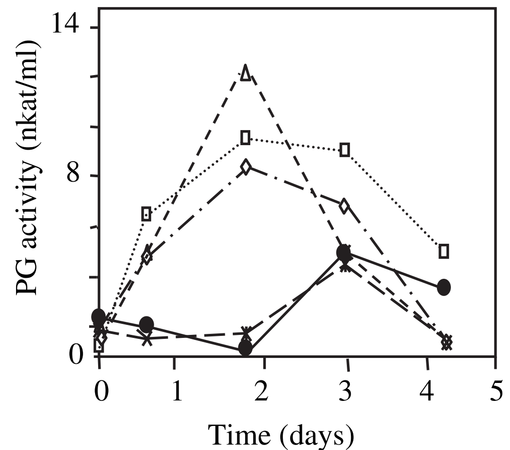


**Fig. 3.** Effect of *CLPT1* and *CLPT1(N123I)* on pectinase secretion. The mycelium was grown for 4 days on glucose medium before being transferred to pectin medium. Extracellular polygalacturonase activity produced by the different strains was measured using polygalacturonic acid as substrate. The data are the mean of three independent experiments. □, wild-type, △, transgenic control strain (empty vector); ◇, CLPT1.4; ×, N123I.6; ●, N123I. 9.

wild-type strain, the production of PG was transient, reaching a peak about 2 days after the transfer to pectin medium (Fig. 3). Expression of *CLPT1* under the control of the *PG2-90* promoter fragment had no significant effect on the production of extracellular PGs. However, induction of *CLPT1(N123I)* led to a dramatic reduction of PG activity. Interestingly, after 3 days in culture, by which time there is a decrease in the activity of the *CLPG2* promoter (Fig. 1), the level of PG increased. This showed that expression of *CLPT1(N123I)* is tightly correlated with an inhibition of PG production.

## Effect of *CLPT1(N123I)* on ultrastructural morphology

The cell wall of living hyphae from various strains were stained with Congo Red, a dye with high affinity for β-1,4 polysaccharides, and observed with a confocal laser scanning microscope. The hyphal walls of WT and *CLPT1* strains grown on glucose medium were labelled homogeneously along the germ tube. In contrast, strongly stained patches were detected along the hyphae of the *CLPT1(N123I)* strain grown on pectin, indicating abnormal accumulation and location of Congo Red stained material (Fig. 4).

Ultra-thin sections of hyphae were obtained from wild-type cultures and from the two transgenic *CLPT1* or *CLPT1(N123I)* strains. The three samples were indistinguishable if they had been grown on glucose (Fig. 5A-C). Each had some vesicle-like material (arrowheads) appressed along the cell wall. However, in *CLPT1(N123I)* strains grown on pectin a dramatic accumulation of vesicle-like material (arrowheads) within the hyphae was observed (Fig. 5F). Further investigations were performed on ultra-thin sections submitted to the PATAg reaction (Fig. 5G-J). This staining mainly revealed polysaccharidic components with high amounts of vicinal hydroxyl groups (Roland and Vian, 1991). Large accumulation

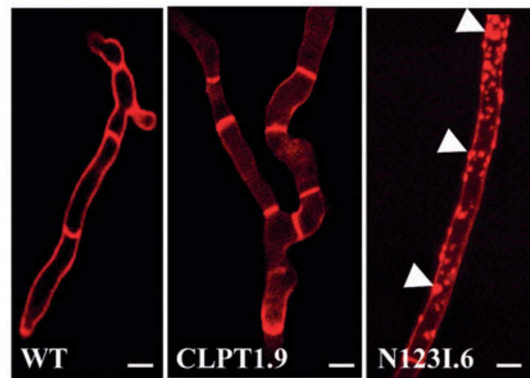


**Fig. 4.** Cell wall thickenings induced by *CLPT1(N123I)*. Confocal laser scanning microscopy of Congo Red-stained hyphae from wild type (WT), *CLPT1*- (CLPT1.9) or *CLPT1(N123I)* (N123I.6) expressing strains grown on a cellophane membrane placed on pectin medium. Each image is a projection of 10-15 confocal planes acquired in the *z* dimension. Note the accumulation of numerous Congo Red stained spots (white arrows) along the hyphal cell wall of N123I.6. Bars, 5 µm.

of PATAg-stained material was visible only within the *CLPT1(N123I)* hyphae grown on pectin (Fig. 5J). The PATAg-stained material appeared as vesicle-like structures (arrowheads) and also as large and highly reactive spots (white arrows).

## Effect of *CLPT1(N123I)* on appressoria development an pathogenesis

Production of appressoria was monitored for the wild-type and mutant strains by placing the conidia on a glass slide in water. It was shown previously that the PG2-90 promoter fragment allowed the expression of the GFP marker gene during appressorium formation (Herbert et al., 2002). The wild-type strain and the two strains expressing the *CLPT1(N123I)* gene

Journal of Cell Science

Journal of Cell Science

developed melanized appresoria. However, it was noted occasionally that the two *CLPT1(N123I)* strains produced abnormal appressorial cells with multiple germ tubes, which were never observed for the wild-type strain (Fig. 6). This suggested that expression of the negative-dominant mutation can impair appressorial differentiation.

To test the pathogenicity of the strains expressing *CLPT1(N123I)*, conidia suspensions were used to inoculate bean leaves and symptoms were recorded 7 days post-inoculation. The strain N123I.6 produced some necrotic spots with a very limited maceration of plant tissues, whereas the strain N123I.9 did not produce any visible symptoms (Fig. 6). This might reflect different levels of expression of the dominant-negative CLPT1 allele in the two transgenic strains. Microscopic examination of the infected tissues revealed that N123I mutants failed to penetrate the host cells. Whereas the wild-type strain formed an appressorium and differentiated an infection vesicle inside a host cell, N123I strains grew saprophytically on the plant surface (Fig. 6).

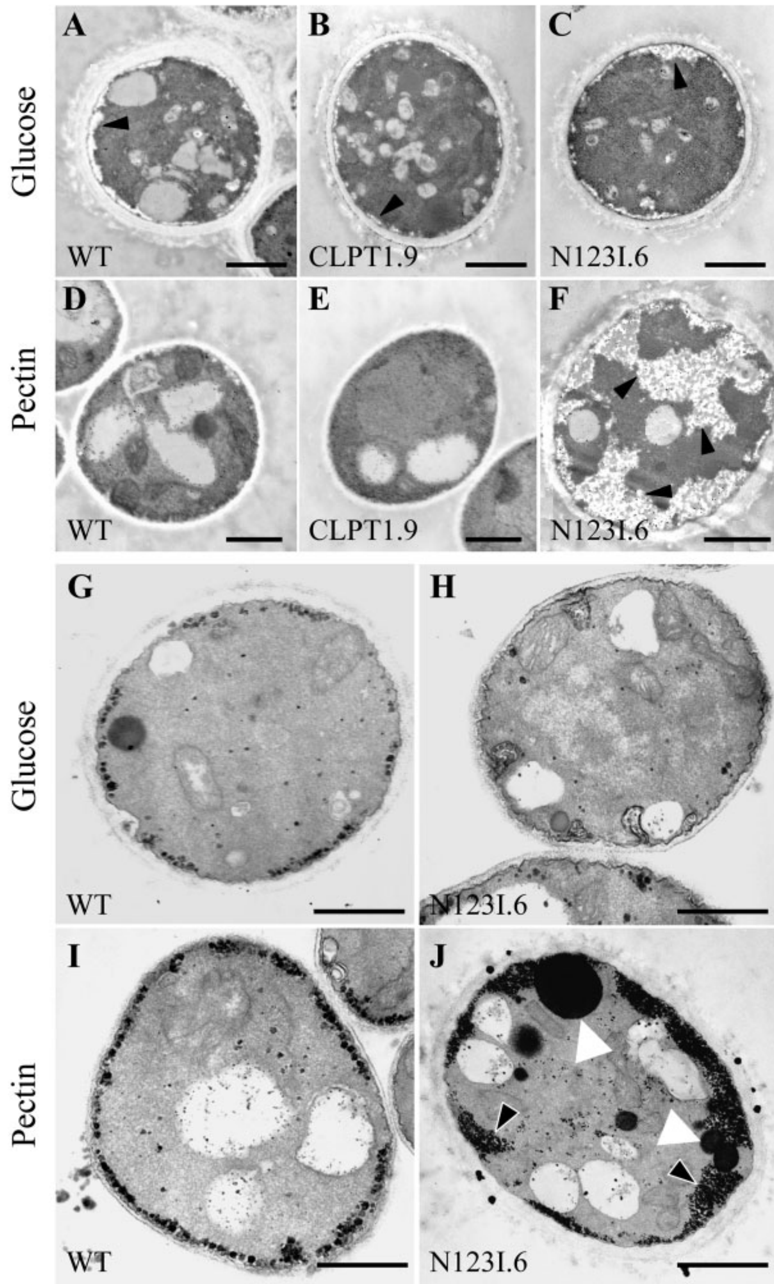


**Fig. 5.** Effect of *CLPT1(N123I)* on ultrastructural morphology. Conidia from the wild-type strain or the transgenic strains were allowed to germinate on a cellophane membrane placed on top of a solid medium containing glucose. After 24 hours of growth, the filters were transferred to a fresh medium containing either glucose (A-C,G,H) or pectin (D-F,I,J) as sole carbon sources. Micrographs of ultrathin sections of hyphae stained with (A-F) uranyl acetate and lead citrate or (G-J) PATAg. Arrowheads indicate the vesicle-like material; white arrows, the large spots accumulated within the hyphae of the *CLPT1 (N123I)*-overexpressing strains grown on pectin. Bars, 1 μm.

## Discussion

In plant and animal pathogenic fungi, it is assumed that the production of extracellular proteins occurs during all stages of fungal development in host tissues. However, little is known about the molecular mechanisms regulating this process. We focused our studies on Rab/GTPases because it is well-established that these proteins have a central role in the transport of secretory vesicles in eukaryotic cells. Here, the role of *CLPT1*, a *SEC4*-like gene from *C. lindemuthianum,* was investigated by expressing a dominant-negative allele of *CLPT1* in transgenic strains. This strategy was retained because Sec4p is an essential protein in yeast and an attempted deletion of a *Candida albicans SEC4* orthologue has been unsuccessful (Mao et al., 1999).

The regulated expression of a dominant-negative allele is particularly useful when one wants to study the function of genes that might play an essential role in development. In this case, gene disruption can lead to a lethal phenotype or pleiotropic phenotypes that could hide the primary function of the gene. Alternative approaches to study the functions of essential genes that are commonly used in *S. cerevisiae* and other model fungi such as *C. albicans* include the generation of conditional lethal mutants, expression of an antisense gene or overexpression of a dominant-negative allele under the control of a tightly regulated promoter. Proteins belonging to the small Ras-like GTPases family are well suited for this latter strategy since they contain conserved domains required for guanine nucleotide binding, GTP-GDP exchange and GTP hydrolysis, whose site-directed mutagenesis might lead to dominant-negative mutations. In CLPT1, the N123I substitution corresponds to the N133I substitution in Sec4p (Walworth et al., 1989), N121I substitution in YPT1 (Schmitt et al., 1986) and to the N124I substitution in the human Rab1 (Pind et al., 1994). This amino acid is located in the G4 region found in Ras (Lazar et al., 1997) and is involved in guanine nucleotide binding (Pind et al., 1994). In the case of Ras, mutations in the consensus guanine nucleotide binding sequence reduced guanine nucleotide binding affinity, leading to an enhancement of GDP/GTP exchange and to a dominant-negative phenotype probably through the

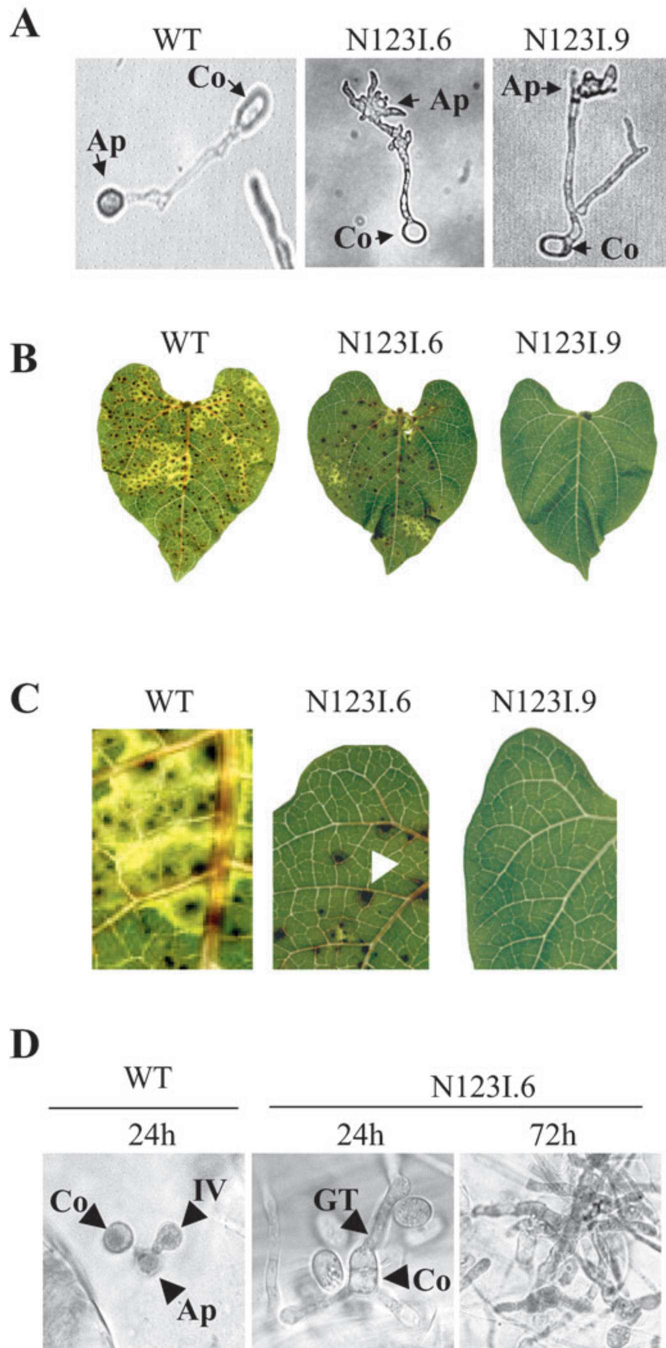


**Fig. 6.** Development of infection structures and pathogenicity of transgenic strains expressing *CLPT1(N123I)*. (A) Conidia (Co) of *C. lindemuthianum* wild-type (WT) and *CLPT1(N123I)* strains (N123I.6 and N123I.9) germinating on glass slides to form appressoria (Ap). Note the abnormal development of appressoria in the N123I strains. (B) Bean leaves were inoculated with a suspension of conidia from the wild-type strain (WT) or from the *CLPT1(N123I)* strains (N123I.6; N123I.9). (C) Details of anthracnose lesions produced by the different strains. (D) Microscopic examination of infected bean tissues. The wild-type strain (WT) develops an appressorium (Ap) that penetrates a hypocotyl epidermal cell and differentiates an infection vesicle (IV) 24 hours after inoculation. The N123I.6 conidia germinate, fail to penetrate host cells and grow saprophytically on the surface of the plant tissue.

sequestering of regulatory factors. A similar substitution in Sec4p resulted in a mutant protein that does not bind GTP and produces dominant lethal phenotypes and secretory defects when expressed in yeast (Walworth et al., 1989). Therefore, we reasoned that it might be possible to define the functions of CLPT1 by expressing, in a conditional manner, a N123I allele of CLPT1 in a wild-type strain of *C. lindemuthianum*.

To express the N123I allele, the *CLPG2* promoter was fused to the mutated *CLPT1* gene. This promoter is not active on glucose medium and is highly, but transiently, induced on pectin medium and during pathogenesis (Dumas et al., 1999; Herbert et al., 2002). This strategy allowed us to study the effect of the dominant-negative mutation on pectinase secretion and pathogenicity. Induction of the N123I allele on pectin medium led to a nearly complete inhibition of PG secretion. This showed that CLPT1 is essential for the intracellular vesicular transport of pectinases. Similarly, overexpression of a *SEC4* allele harbouring a dominant-negative mutation inhibited aspartyl protease secretion in the human pathogen *C. albicans* (Mao et al., 1999). Ultrastructural examination of the strains expressing the *CLPT1(N123I)* allele revealed the abnormal accumulation of vesicles that were heavily stained by the PATAg reagent and by Congo Red. Thus, it is clear that the presence of the N123I mutation blocks the transport of vesicles in a dominant fashion by preventing interaction of the wild-type CLPT1 with a transport component required for targeting and fusion of the vesicles with the plasma membrane. No morphological differences were noticed between the different strains during saprophytic growth, indicating that CLPT1 is not essential for hyphal elongation. This is in contrast to the results obtained on the role of *SRGA*, a putative *SEC4* homologue from the filamentous fungus *Aspergillus niger*. *SRGA* mutants displayed a twofold increase in their hyphal diameter and unusual apical branching (Punt et al., 2001). While comparison of SRGA and CLPT1 amino acid sequences revealed a high percentage of identity [82.6% (Dumas et al., 2001)] these two proteins could have different functional roles since CLPT1 is able to complement a yeast *sec4* mutant (Dumas et al., 2001) whereas SRGA cannot (Punt et al., 2001).

Pathogenicity was strongly reduced in the strains expressing *CLPT1(N123I)*. This suggests that the inhibition of protein secretion is an essential process at this stage of infection. It has been showed in a number of plant and animal systems that extracellular proteins play an essential role in pathogenic development. These include several plant cell wall degrading enzymes from phytopathogens (Rogers et al., 2000; Oeser et al., 2002; Isshiki et al., 2001), aspartyl proteases from the animal pathogen *C. albicans* and an extracellular phospholipase from *Cryptococcus neoformans* (Cox et al., 2001). While our data suggest that the reduced virulence of *CLPT1* dominant negative mutants could arise from a strong decrease of cell wall degrading enzyme secretion, other extracellular or cell surface proteins are known to also play a major role in pathogenesis. For example it has been reported that the synthesis of hydrophobin is necessary for efficient appressorial differentiation in the rice pathogen *Magnaporthe grisea* (Talbot et al., 1996). In *C. lindemuthianum*, pathogenic development is accompanied by a highly coordinated secretion of cell-surface proteins, which could have different roles in cell adhesion or biotrophic development (Hutchinson et al., 2002).

Alteration of the secretory pathway through the expression of the dominant negative CLPT1 allele could have a detrimental effect on all these processes leading to a non-pathogenic phenotype. Future work will aim at obtaining a more global view of the modification of the cell-surface proteome of the *CLPT1(N123I)*-expressing strains to identify essential secreted components of pathogenesis.

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