The CBEL glycoprotein of *Phytophthora parasitica* var. *nicotianae* is involved in cell wall deposition and adhesion to cellulosic substrates

Elodie Gaulin, Alain Jauneau, François Villalba, Martina Rickauer, Marie-Thérèse Esquerré-Tugayé and Arnaud Bottin*

UMR 5546 UPS-CNRS, Pôle de Biotechnologie Végétale, 24 Chemin de Borde-Rouge, BP17, Auzeville, F-31326 Castanet-Tolosan, France
*Author for correspondence (e-mail: bottin@smcv.ups-tlse.fr)

Accepted 3 September 2002
Journal of Cell Science 115, 4565-4575 © 2002 The Company of Biologists Ltd
doi:10.1242/jcs.00138

Summary

The cell wall of the oomycete plant pathogen *Phytophthora parasitica* var. *nicotianae* contains a protein called CBEL that shows cellulose-binding (CB), elicitor (E) of defense in plants and lectin-like (L) activities. The biological role of this molecule in *Phytophthora* was investigated by generating transgenic strains suppressed in *CBEL* expression. Phenotypic characterization of these strains showed that they were severely impaired in adhesion to a cellophane membrane, differentiation of lobed structures in contact with cellophane, and formation of branched aggregating hyphae on cellophane and on flax cellulose fibres. Infection assays revealed that the strains suppressed in *CBEL* expression were not greatly affected in pathogenicity and formed branched aggregating hyphae in contact with the roots of the host plant, thereby indicating that CBEL is involved in the perception of cellulose rather than in the morphogenesis of hyphal aggregates. Interestingly, the absence of CBEL was correlated with abnormal formation of papillae-like cell wall thickenings in vitro, suggesting that CBEL is involved in cell wall deposition in *Phytophthora*. Reverse genetics in oomycetes has long been hampered by their diploid nature and difficulties in transformation and regeneration. The gene inactivation approach reported in this work provides the first direct evidence for intrinsic functions of an elicitor and cell wall protein in oomycetes.

Key words: Oomycete, Transformation, Silencing, Branching, Lobed structures, Cellulose, CBD

Introduction

Oomycetes are a group of filamentous eukaryotic microorganisms comprising several of the most devastating plant pathogens. They were long classified in the kingdom *Fungi*, although they differ from true fungi in many aspects, such as the production of wall-less motile zoospores and the non-chitinous nature of the cell wall (Judelson, 1997). According to recent phylogenic analyses, they are now included in the kingdom *Chromista* (Cavalier-Smith, 1998) or in the Stramenopile phylum (Sogin and Silberman, 1998) together with brown algae and diatoms. Among oomycetes, the genus *Phytophthora* is composed mainly of phytopathogenic species that are destructive on a wide range of crop plants and woody species worldwide (Erwin and Ribeiro, 1996). The unique features of oomycetes and notably of *Phytophthora* make them insensitive to most fungicides, and necessitate studies specifically devoted to their biology in order to control them (Govers, 2001; Tyler, 2001).

Owing to the potential involvement of the cell wall in many aspects of *Phytophthora* biology, a better knowledge of this compartment is needed. In particular it is a reservoir of elicitors, a class of molecules whose identification is based on their ability to induce defense and/or hypersensitive response (HR) necrosis when externally supplied to plant tissues (Keen et al., 1972). This definition does not imply an intrinsic function of elicitors for the microorganism itself. Whether elicitor molecules do fulfil basic functions in the biology of the microorganisms which produce them is not known yet. Various molecules endowed with elicitor activity have been characterized in *Phytophthora*, notably glucan fragments (Keen and Yoshikawa, 1983; Sharp et al., 1984; Waldmüller et al., 1992), small polypeptides from the elicitin family (Ricci et al., 1989; Ponchet et al., 1999), glycoproteins (Farmer and Helgeson, 1987; Parker et al., 1991; Séjalon-Delmas et al., 1997) and fatty acids (Bostock et al., 1981). Among possible roles, one might conceive their involvement in cell wall structure, adhesion, signal perception, development or nutrition.

Working on *Phytophthora parasitica* var. *nicotianae* (*P.p.n.*), a root pathogen which is the causal agent of the black shank disease of tobacco, led us to the purification of a glycoprotein which elicits HR-like necrosis and defense gene expression in tobacco (Séjalon-Delmas et al., 1997). This glycoprotein was localized by immunogold-labelling to the external and internal layers of the hyphal cell wall. Early deposition from secretory vesicles was recorded at the onset of cell wall synthesis during encystement of zoospores. Labelling was associated with the living microorganism in vitro and in planta, and declined with mycelium cell death (Séjalon-Delmas et al., 1997). Cloning of the corresponding gene indicated that the protein portion is

composed of two cysteine-rich domains separated by a threonine/proline-rich linker region (Villalba Mateos et al., 1997). Interestingly, each repeated domain contains one sub-domain homologous to cellulose binding domains (CBDs) of glucan hydrolases (Tormo et al., 1996). CBDs are well characterized in microbial cellulolytic enzymes, and are supposed to enhance the efficiency of hydrolysis notably by docking the enzymes to their substrate (Gilkes et al., 1991). Further studies demonstrated that CBEL i) binds crystalline cellulose without showing enzyme activity on cellulose and various glycans, and ii) has lectin activity. Accordingly, the elicitor was named CBEL for Cellulose-Binding Elicitor Lectin (Villalba Mateos et al., 1997). With respect to its cell wall localization, expression pattern and multiple activities, it was proposed that CBEL is involved in cell surface properties during the life cycle of *P.p.n*. In order to check this hypothesis, it is necessary to generate *P.p.n.* strains with altered levels of CBEL production. So far, only a few *Phytophthora* (*P.*) species, comprising *P. infestans*, *P. sojae*, *P. capsici* and *P. palmivora*, have been transformed (Judelson et al., 1991; Judelson et al., 1993a; Ersek et al., 1994; van West et al., 1999b). We formerly established that *P.p.n.* is amenable to transformation with a reporter gene (Bottin et al., 1999). In the present study, this method was used to generate transgenic strains containing antisense and sense copies of *CBEL* cDNA. The obtained transformants were characterized with respect to *CBEL* gene expression and to their phenotype during saprophytic growth and upon inoculation of the tobacco host plant.

Materials and Methods

Strains and culture conditions

Phytophthora parasitica Dastur var. *nicotianae* (Breda de Haan) Tucker race 0 untransformed and transformed strains were grown at 25°C and maintained at 15°C in the dark on V-8 agar medium (50 ml of V-8 juice and 20 g agar per litre, pH 5.0). Culture media for the maintenance of transformant strains contained 70 μg ml^{-1} hygromycin B (Cayla SA, France). For growth kinetics on liquid medium or molecular analysis of mycelium, the strains were grown on liquid synthetic medium at 25°C in the dark and harvested on a sintered glass filter as described (Villalba Mateos et al., 1997). The mycelium was then either lyophilized for dry weight determination, or stored frozen before nucleic acid or protein extraction.

Phytophthora parasitica var. *nicotianae* (P.p.n.) transformation

DNA vectors used for *P.p.n.* transformation were pTH210 (Judelson et al., 1991), and plasmid constructs derived from it. In pTH210, an oomycete hygromycin B resistance cassette has been inserted into the pUC19 vector. This cassette is composed of the coding sequences from the hygromycin phosphotransferase gene *HPH* from *Escherichia coli* under the control of a *HSP70* gene promoter and a *HAM34* gene terminator from the oomycete *Bremia lactucae* (Fig. 1A). Plasmids derived from pTH210 were constructed according to standard procedures (Sambrook et al., 1989) using Life Technologies (France) restriction and modification enzymes. The *CBEL* cDNA was isolated as a 918 bp *Eco*RI-*Xho*I fragment (Villalba Mateos et al., 1997), and DNA ends were filled in by the Klenow fragment enzyme. The 1.2 kb *HPH* gene insert in pTH210 was released by *Sma*I digestion, and the remaining vector fragment was ligated with the *Eco*RI-*Xho*I *CBEL* cDNA blunt fragment. This resulted in either plasmid pTHEX3 or plasmid pTHEX11, both of 4.7 kb size, where the *CBEL* cDNA is inserted in place of the *HPH* gene in sense or antisense orientation respectively (Fig. 1A).

Genetic transformation was carried out as described (Bottin et al., 1999). Briefly, *P.p.n.* protoplasts were prepared by enzymatic digestion of mycelium and incubated with a mixture of *Bam*HI-linearized pTH210 (15 μg) and either pTHEX3 or pTHEX11 (15 μg) in the presence of $CaCl_2$ and polyethylene glycol. Regeneration was carried out on V-8 agar medium containing 70 μg ml^{-1} hygromycine B.

Molecular characterization of *P.p.n.* transformants

Genomic DNA and total RNA were extracted from the mycelium according to Dellaporta et al. and Villalba Mateos et al., respectively (Dellaporta et al., 1983; Villalba Mateos et al., 1997). Nucleic acid concentrations were measured spectrophotometrically at 260 nm. For Southern blot analysis, 10 μg of *Eco*RI-digested genomic DNA were electrophoresed on a 1% (w/v) agarose gel. After alkaline denaturation, DNA fragments were transferred onto a positive nylon membrane (Hybond N+, Amersham, France) and subjected to Southern hybridization (Sambrook et al., 1989). Northern blots were performed with 10 μg of total RNA as described previously (Rickauer et al., 1997). The 918 bp *Eco*RI-*Xho*I *CBEL* cDNA fragment (Villalba Mateos et al., 1997) was labelled with α-[^{32}P]dCTP by random priming using the RadPrime DNA Labelling System (Life Technologies, France) and used as the probe in Southern and northern blot analyses. A radiolabelled PCR fragment corresponding to 18S ribosomal DNA was used as a control of RNA amount in northern blot analysis. After hybridization and washing, the membranes were exposed to Hyperfilm MP films (Amersham, France) at –80°C.

For western blot analysis, protein extracts were prepared following a procedure adapted from Séjalon-Delmas et al. (Séjalon-Delmas et al., 1997). Briefly, 1 g of ground frozen mycelium was suspended in 1.5 ml of acidic ethanol, the extract was clarified by centrifugation and proteins were precipitated by cold acetone and air-dried. Protein samples (100 μg dry weight) were analyzed by sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (Laemmli, 1970) on a 12% (w/v) resolving gel. After transfer onto a nitrocellulose membrane (Protran, Schleicher & Schuell, Germany), proteins were probed with a polyclonal antiserum directed against CBEL as described (Séjalon-Delmas et al., 1997).

Characterization of *P.p.n.* transformant phenotypes

For adhesion assay and microscopy analysis of mycelium, a washed, autoclaved cellophane (Cellophane octaframe, MERCK-Eurolab, France) or polycarbonate (Polycarbonate cyclopore, MERCK-Eurolab, France) membrane was put down onto V-8 agar medium and subsequently inoculated on its center with a mycelium plug. In some experiments, a bed of depectinised flax cellulose fibres (a gift from C. Morvan, Rouen, France), boiled for 2 hours in 0.1 M NaOH to remove hemicellulose contaminants, was spread onto the cellophane sheet prior to inoculation. After incubation for 7 days at 25°C in the dark, the membrane together with the mycelium was gently removed from the underneath medium and cut into 8 sectors of similar sizes. These samples were either directly processed for microscopy observation, or incubated under gentle horizontal shaking at room temperature in a 50 ml polypropylene tube containing 30 ml of distilled water in the case of adhesion assay. After 2 hours, the presence of remaining adherent mycelium was checked with bright field microscopy.

For microscopy analysis of germlings, *P.p.n.* zoospores were obtained by starvation of mycelial cultures followed by cold shock (Gooding and Lucas, 1959), adjusted to 5×10^4 cell μl^{-1} in water, and vortexed at maximum speed for 1 minute in order to provoke encystment. Fifteen microliters aliquots were then dropped either directly onto the surface of a polystyrene Petri dish or onto cellophane sheets placed on top of a 4% (w/v) water agar. The samples were incubated at 24°C for 16 to 48 hours in the dark and observed under differential interference contrast microscopy.

Pathogenicity was assessed on tobacco 46-8 and 49-10 isolines, susceptible and resistant to *P.p.n.* race 0 respectively. Inoculation assays were performed on stem sections of 10 week-old 46-8 plants (Rancé et al., 1998), and on the root system of 3 week-old 49-10 seedlings. For this assay, 12 surface-sterilized seeds were sowed on water agar (1.2% w/v, pH 6.5) in square 12 cm-Petri dishes set up vertically in order for the roots to grow on the surface of the medium. After 3 weeks of culture at 25°C with 16 hours daily illumination at 30 $\mu E/m^2s$, mycelium plugs taken up from the periphery of *P.p.n.* colonies grown on V-8 agar medium were placed in contact with the roots beneath the collar. The dishes were further incubated in the same conditions and symptoms were scored during 12 days, according to the following notation: 0, no symptom; 1, leaf discoloration and black shank; 2, brown dead seedling with visible mycelium. For microscopy studies, infected seedlings were observed with an inverted light microscope either directly in the assay Petri dish, or after removal of the seedling and staining.

Microscopy techniques

Inoculated seedlings, as well as mycelium grown on cellophane or polycarbonate membranes, were observed without prior fixation with an inverted light microscope (Leitz DMIRBE, Leica, Germany). Microscopy views were obtained with a Color Cool View CCD camera (Photonic Science, UK) linked to an Image Acquisition Photolite software (Photonic Science, UK). In some cases, inoculated seedlings were bleached for 24 hours at 30°C in 10% KOH (w/v), washed in water, stained in rosazurin (3% w/v in water) and mounted in water for microscopy observation.

For transmission electron microscopy, the samples were fixed for 2 hours at room temperature in 0.1 M sodium cacodylate buffer (pH 7.2) containing 1.5% (w/v) glutaraldehyde (Oxford Agar, UK), then washed in the same buffer without glutaraldehyde. They were dehydrated in a series of aqueous solutions of increasing ethanol concentration (10, 20, 40, 50, 60, 70, 80, 90, 100% v/v, 20 minutes each). Progressive infiltration with LR White resin (Oxford, UK) was carried out by serial incubation in ethanolic solutions of increasing LR White resin concentration (10, 30, 50, 70, 90% v/v, at least 1 hour each), followed by several incubations in undiluted LR White resin. The infiltrated samples were then embedded in gelatine capsules and allowed to polymerize for 24 hours at 60°C. Ultrathin sections (90 nm thickness) were prepared using an UltraCut E ultramicrotome (Reichert-Leica, Germany) and collected on gold grids. They were submitted to the periodic acid, thiocarbohydrazide silver-proteinate reaction (PATAg) according to Thiéry (Thiéry, 1967). Sections were floated on a 1% (w/v) periodic acid aqueous solution for 30 minutes and rinsed twice in distilled water for 15 minutes. They were then maintained overnight at 4°C on a 20% (v/v) acetic acid aqueous solution containing 2 g l^{-1} thiocarbohydrazide, washed in solutions of acetic acid of decreasing concentrations, and finally in pure water. Sections were then treated with 1% (w/v) silver proteinate in water for 30 minutes in the dark. Grids were rinsed and air dried before examination with a transmission electron microscope operating at 75 kV (Hitachi, H-600, Japan) and photographs were taken on Kodak-Electron films (Kodak, France).

For scanning electron microscopy, the samples were fixed and dehydrated as described above and critical-point dried with CO_2 as transitional fluid. The dried samples were sputter-coated with gold-palladium using a Jeol JFC1100 apparatus (JEOL, Japan). Observations were made with a Hitachi C-450 (Japan) scanning electron microscope operating at 15 kV, and photographs were taken on Illford 125 ISO film.

For confocal laser scanning microscopy, samples were stained with Congo Red (1% w/v in water) for 15 minutes, then briefly washed in distilled water. Preparations were viewed with a Leica SP-2 (Germany) microscope equipped with a 40× (1.25 NA) oil immersion objective. Excitation was brought about by the 543 nm emission line of the He-Ne laser, and light emitted between 560 and 630 nm was collected. Pictures were computed by projection of 15 plan-confocal images acquired in z dimension with 0.5 μm increment between two focal planes.

Results

Inactivation of *CBEL* gene expression

In order to investigate the biological role of CBEL, attempts were made to modify *CBEL* gene expression by transformation. The *CBEL* cDNA in antisense or sense orientation was introduced in place of the *HPH* coding sequences in the oomycete transformation vector pTH210 (Judelson et al., 1991), resulting in pTHEX11 or pTHEX3 respectively, in which the *CBEL* sequences are under control of the *HSP70* gene promoter and *HAM34* gene terminator from *Bremia lactucae* (Fig. 1A). Transformation of *P.p.n.* race 0 was achieved by co-incubation of protoplasts with pTH210, conferring hygromycin resistance, and either pTHEX11 or pTHEX3. The presence of *CBEL* sense or antisense constructs in the transgenic strains was checked by Southern blot analysis of genomic DNA digested by *Eco*RI, an enzyme which recognizes a single restriction site in the two constructs. Hybridization with a *CBEL* probe showed a strong band at 6.8 kb in each transformant, corresponding to the *CBEL* endogenous gene (Fig. 1Ba). An additional *Eco*RI band of the size of the transforming plasmids pTHEX11 or pTHEX3 (4.7 kb) was detected in most transformants, suggesting tandem integration events. Other bands of various sizes were also detected in several transformants, for example in EX 11-4, representing either recombined plasmid molecules or border fragments.

CBEL gene expression in the transformants was assessed by northern blot analyses (Fig. 1Bb). The 1.3 kb hybridising band corresponding to the *CBEL* transcript was observed with RNA from the untransformed strain and some antisense transformants, but it was undetectable in the EX11-4 antisense transformant. Interestingly, the level of *CBEL* mRNA was not enhanced in any of the sense transformants, but was reduced in most of them, being undetectable in the EX3-3 transformant.

To determine the effect of *CBEL* mRNA suppression on the level of the CBEL protein, we conducted western blot analyses of protein extracts prepared from the untransformed and from four transformant strains (Fig. 1Bc). A major band of M_r of 34×10^3, corresponding to the CBEL glycoprotein, was detected in the untransformed, EX11-1 and EX3-5 strains, whereas the protein was not detected in EX11-4 and EX3-3 strains.

Altogether, northern and western blot analyses indicate that expression of the *CBEL* gene, and synthesis of the CBEL protein, were suppressed in transformants EX11-4 and EX3-3, whereas transformants EX11-1 and EX3-5 exhibited similar levels of *CBEL* expression as the untransformed control strain. These two expressing ($CBEL^+$) and the two non-expressing ($CBEL^-$) transformants were selected for further analyses. By analogy to suppression of gene expression in sense and antisense transformants of *P. infestans* (van West et al., 1999a), it can be supposed that the $CBEL^-$ strains resulted from homology-dependent gene silencing. It is worth noting that the transformants were highly impaired in zoospore production independently of *CBEL* gene expression. For this reason, all

experiments aimed at comparing transgenic CBEL$^+$ and CBEL$^-$ strains were performed with mycelium.

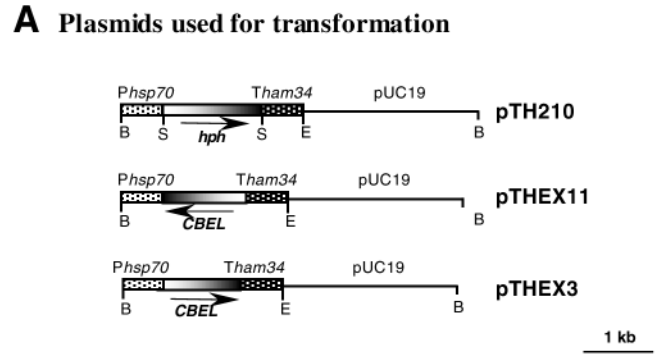

Fig. 1. Characterization of *P.p.n.* strains transformed with sense or antisense constructs of *CBEL* cDNA. (A) Plasmids used for transformation. *HPH*, coding sequences of the hygromycin phosphotransferase *HPH* gene; P*hsp70*, promoter of a *HSP70* gene from *Bremia lactucae*; *Tham34*, terminator of the *HAM34* gene from *B. lactucae*; B, *Bam*HI; E, *Eco*RI; S, *Sma*I. (B) Molecular characterization of the transformants. (a) Southern blot analysis. In each lane, *Eco*R1-fragmented genomic DNA from the untransformed control strain (c), and from twelve strains transformed either with plasmid pTHEX11 (EX11, 1-7) or plasmid pTHEX3 (EX3, 1-5), were analyzed and hybridized with a radiolabeled *CBEL* cDNA probe. (b) Northern blot analysis. Total RNA from the same strains was electrophoresed, blotted and probed with a *CBEL* cDNA probe. The size of *CBEL* transcripts is 1.3 kb. Relative amounts of blotted RNA were determined by hybridization with an 18S rRNA probe. (c) Immunodetection of CBEL protein. Protein extracts from the untransformed control strain (c) and from four strains transformed either with plasmid pTHEX11 (EX11, 1 and 4) or plasmid pTHEX3 (EX3, 5 and 3) were subjected to western blot analysis using an anti-CBEL polyclonal antiserum. The M_r of CBEL is 34×10^3.

CBEL silencing has no effect on mycelium growth

Cultures of the EX11-1 CBEL$^+$ and EX11-4 CBEL$^-$ strains were grown on liquid medium and the mycelium dry weight was recorded as a function of time. Fig. 2A shows that the two strains grew equally well under these conditions. As it has been demonstrated that CBEL has affinity for cellulose (Villalba Mateos et al., 1997), the growth of CBEL$^+$ and CBEL$^-$ strains was studied on a cellophane membrane placed on top of an agar medium. Mycelium growth, assessed by measuring the diameter of the colonies, was not affected under these conditions (Fig. 2B). The untransformed strain and all transformants colonized the whole surface of the cellophane sheet after 7 days of culture. Thus, *CBEL* silencing did not affect mycelium growth in vitro.

CBEL silencing reduces attachment to cellulosic surfaces

It has been shown previously that CBEL is localized at the surface of cysts and hyphae both in vitro and in planta (Séjalon-Delmas et al., 1997), exhibits lectin activity and binds to cellulose and tobacco root cell walls in vitro (Villaba Mateos et al., 1997). To gain insight into the intrinsic role for *P.p.n.* of the carbohydrate-binding capacity of CBEL, we investigated attachment of CBEL$^+$ and CBEL$^-$ strains to cellulosic or polycarbonate (PC) membranes. These strains were grown on membrane disks placed on top of V-8 agar medium for 7 days; the disks were then removed from the medium, cut in sectors of similar sizes (Fig. 3A-E), and the sectors were incubated with moderate shaking for 2 hours in water (Fig. 3F-J). Microscopic observation showed that the mycelium of the untransformed and CBEL$^+$ EX11-1 and EX3-5 strains remained attached to the cellophane membrane after this treatment (Fig. 3F,G,I). In contrast, most hyphae of the EX11-4 and EX3-3 CBEL$^-$ strains were detached from the membrane (Fig. 3H,J). None of the five strains remained attached to a PC membrane under the same conditions (data not shown). These results demonstrate that the ability of *P.p.n.* to stick to a cellulosic substrate is highly dependent on the presence of CBEL.

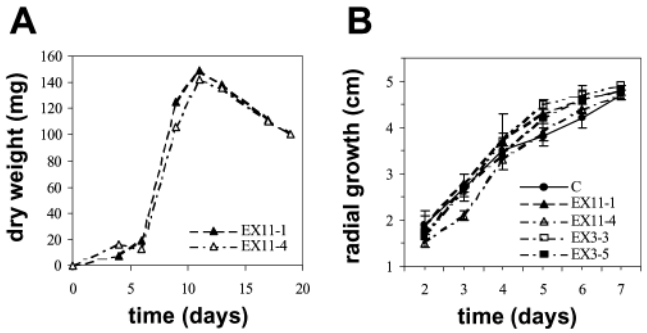

Fig. 2. In vitro growth of CBEL$^+$ and CBEL$^-$ strains. (A) EX11-1 (CBEL$^+$) and EX11-4 (CBEL$^-$) strains were grown at 25°C on synthetic liquid medium and the mycelium dry weight was determined. The means of two independent experiments are shown. (B) The untransformed (C) and transgenic (EX11-1, EX11-4, EX3-3, EX3-5) strains were grown at 25°C on a cellophane membrane placed on top of V-8 agar medium. Growth was determined by measuring the diameter of the colony. The data is the mean of three independent experiments±s.d.

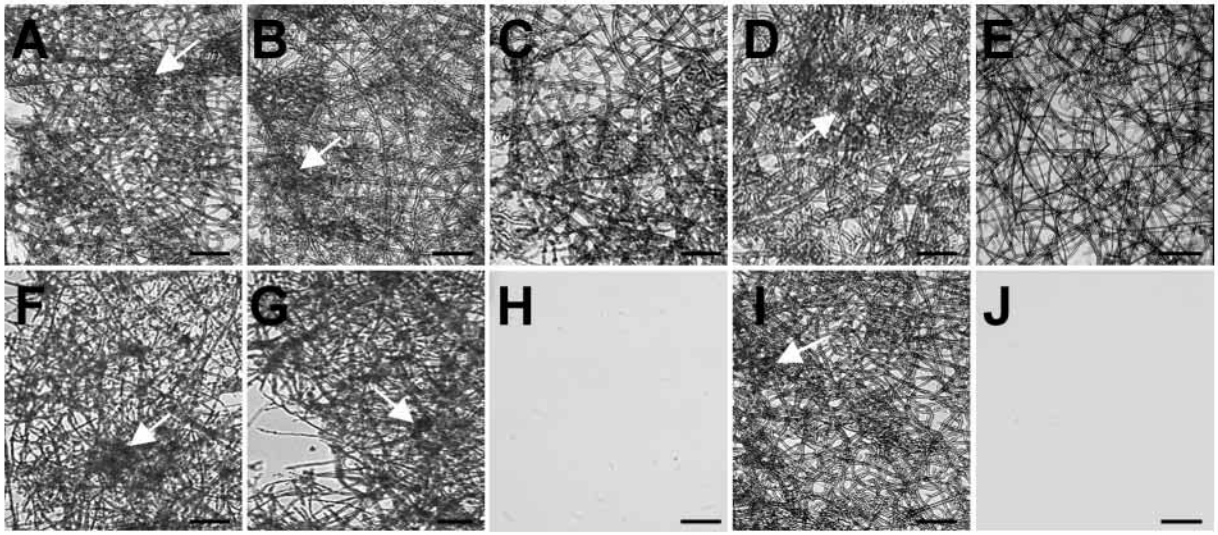

Fig. 3. Adhesion properties of mycelium from CBEL$^+$ and CBEL$^-$ *P.p.n.* strains growing on a cellophane membrane. The mycelium was grown on a cellophane membrane placed on top of V-8 agar medium. The membrane together with the mycelium was removed from the medium underneath (A-E), cut in sectors of similar sizes, which were then incubated for 2 hours under shaking in water. The presence of remaining adherent mycelium was checked by light microscopy (F-J). (A,F) Untransformed control strain; (B,G) EX11-1 CBEL$^+$ strain; (C,H) EX11-4 CBEL$^-$ strain; (D,I) EX3-5 CBEL$^+$ strain; (E,J) EX3-3 CBEL$^-$ strain. Results representative of three independent experiments are shown. The mycelial aggregates are indicated by arrows. Note the absence of residual mycelium in H and J. Bars, 70 μm.

CBEL silencing hampers differentiation in contact with cellulosic surfaces

In addition to a difference in adhesion, we observed that the untransformed strain and transgenic *CBEL*-expressing strains EX11-1 and EX3-5 differentiated two types of structures when grown on a cellophane membrane. One type appeared as small regions of densely aggregated hyphae (Fig. 3A,B,D,F,G,I, arrows). The thickness of these mycelial aggregates prevented clear visualisation with the light transmission microscope (Fig. 4A). The second type appeared in contact with cellophane under the form of lobed granular structures (Fig. 4A,B). These structures were not in the same focus plane as most hyphae (Fig. 4B) but seemed to develop within the cellophane sheet. They were clearly distinguishable from protoplasm leaking out of damaged hyphae (Fig. 4C). These differentiations were substrate-specific since they did not occur when the strains were grown on a PC membrane (data not shown). The *CBEL*-silenced strains EX11-4 and EX3-3 never presented either mycelial aggregates or lobed structures (Fig. 4D).

In order to get insight into the biological significance of the lobed structures, we looked for their occurrence during *P.p.n.* germling development on various substrates. Since the transgenic strains were impaired in zoospore production, experiments were conducted with the untransformed strain. Encysted zoospores germinated and produced globular appressoria

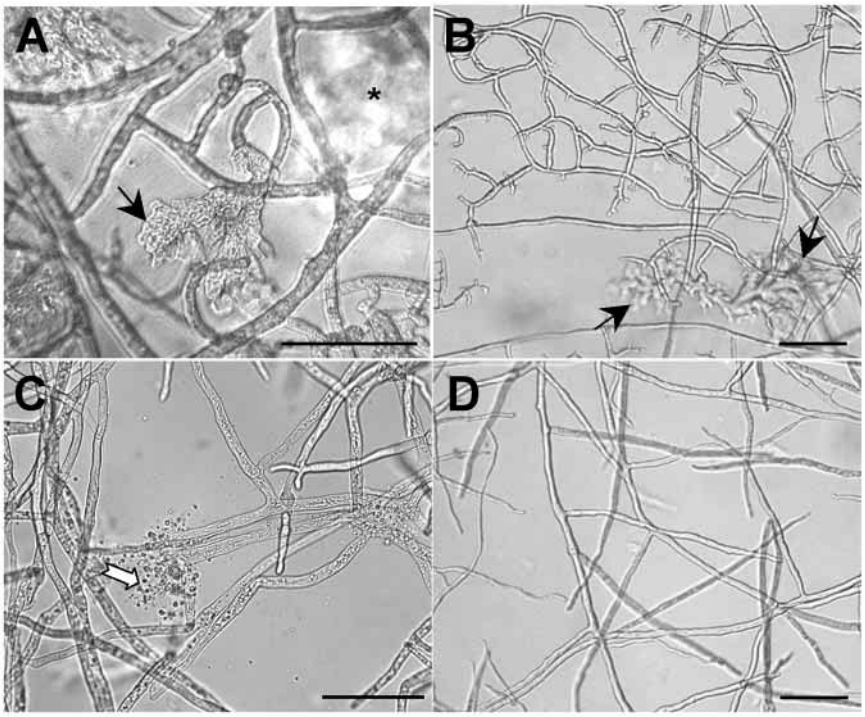

Fig. 4. The differentiation of lobed structures in mycelium from CBEL$^+$ and CBEL$^-$ *P.p.n.* strains in contact with a cellophane membrane. The mycelium was grown on a cellophane sheet placed on top of V-8 agar medium. The membrane with the mycelium was removed from the medium underneath, cut in sectors and observed under bright field microscopy. The whole membrane sector from each sample was screened, and typical data from triplicate samples are shown. (A,B) Lobed granular structures (arrows) from the EX11-1 (A) and EX3-5 (B) CBEL$^+$ strains. The asterisk indicates an aggregate of intricate hyphae on a different focus plane. (C) Leakage of protoplasm (open arrow) from a damaged hyphae in the untransformed strain. (D) Hyphae from the EX11-4 CBEL$^-$ strain. Note the absence of lobed structures. Bars, 40 μm.

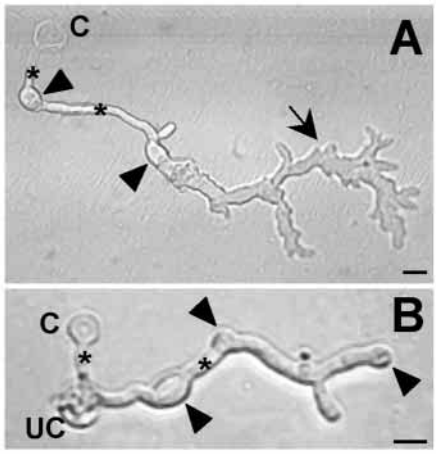

Fig. 5. Development of germlings from the untransformed *P.p.n.* strain in contact with solid surfaces. Freshly prepared zoospores were induced to encyst by vortexing and spread onto various surfaces. (A) Development on a cellophane membrane placed on top of water agar medium. After 40 hours, the spherical encysted zoospore (c) has germinated to form a germ-tube (asterisks) and two appressoria (arrowheads). A lobed structure (arrow) has emerged from the distal appressorium and has developed in a plane slightly out of focus. Note that the cytoplasm has migrated out of the cyst. (B) Development on the polystyrene surface of a Petri dish. After 20 hours, the encysted zoospore (c) produced a series of appressoria (arrowheads). No further development of different structures was observed after longer incubation. UC, ungerminated cyst. Bars, 10 μm.

within a few hours on a solid surface (Fig. 5). When the substrate was a cellophane membrane, lobed structures emerged from some appressoria (Fig. 5A). In contrast, only repeated appressorium formation was observed along the germ tube growing on a polystyrene surface (Fig. 5B) or on a glass slide (data not shown). The data indicate that lobed structures develop subsequently to appressorium formation and confirm that their formation is substrate-specific.

Additional experiments using scanning electron microscopy were undertaken in order to better characterize the interaction between *P.p.n.* and cellulosic substrates. In addition to cellophane, flax cellulose fibres were used as a more natural cellulose substrate. When grown on either substrate, the EX11-1 CBEL$^+$ strain formed highly intricate and branched hyphae that likely corresponded to the above-mentioned mycelial aggregates (Fig. 6A,C). These aggregates were localised in close vicinity of the cellulose fibres (Fig. 6C). No lobed structures could be detected in these conditions, a result which is in agreement with the hypothesis that they do not develop at the surface of a substrate, but within it. In accordance with light microscopy observations, the EX11-4 CBEL$^-$ transformant showed only loosely branched hyphae and no mycelial aggregates on either substrate (Fig. 6B,D).

Taken together, the data show that CBEL is required for differentiation of lobed structures and hyphal aggregates in contact with cellulosic surfaces.

CBEL silencing results in cell wall thickenings

Because CBEL is localised in the cell wall of *P.p.n.*, we hypothesized that it might also be involved in the cell wall architecture. Hyphae from the various strains were stained with Congo Red, a dye with high affinity for β-1,4 polysaccharides (Wood, 1980), and observed with a confocal laser scanning electron microscope. Strongly stained cell wall patches were detected along the hyphae of the CBEL$^-$ strains grown either on a cellophane or a polycarbonate membrane, as illustrated with the EX11-4 strain in Fig. 7B,D. In contrast, the hyphal walls of CBEL$^+$ strains did not exhibit any thickening and were labelled homogeneously regardless of the chemical nature of the substrate (Fig. 7A,C).

Further investigations were performed on ultra-thin sections of hyphae which were submitted to the PATAg reaction. This staining reveals the polysaccharides of the cell wall which contain vicinal hydroxyl groups (Roland and Vian, 1991). In transformants expressing *CBEL*, the hyphae were surrounded by a PATAg-stained cell wall of regular thickness, as illustrated with the EX11-1 CBEL$^+$ strain (Fig. 7E). However, in the two silenced strains, numerous hyphae exhibited paramural deposits (Fig. 7F,G), a pattern which was never observed with the untransformed and CBEL$^+$ transgenic strains. These appositions were stained with

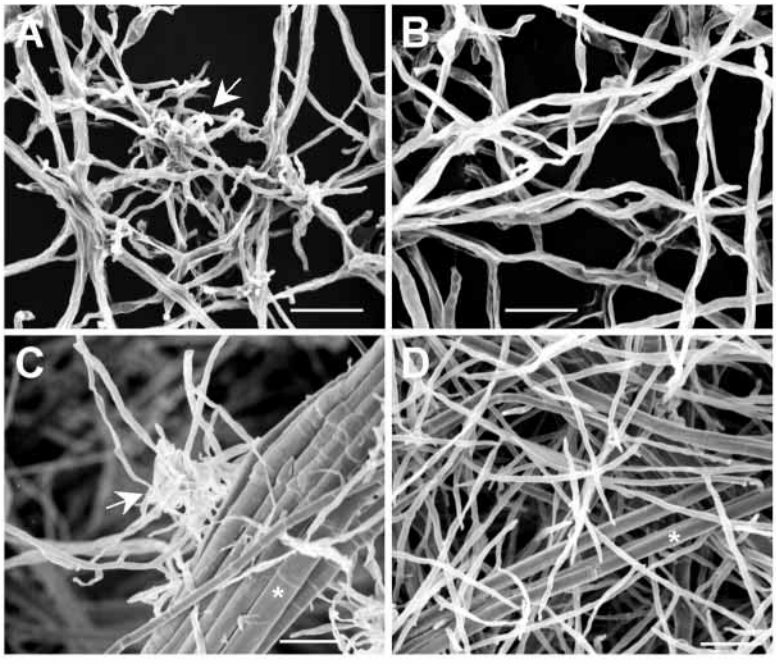

Fig. 6. The differentiation of lobed structures in mycelium from CBEL$^+$ and CBEL$^-$ *P.p.n.* strains in contact with cellulosic substrates. The mycelium was grown on a cellophane membrane (A,B) or on flax cellulose fibres dispersed onto a cellophane membrane (C,D) placed on top of V-8 agar medium. After 7 days of culture, the cellophane sheet was removed from the medium underneath and processed for scanning electron microscopy. (A,C) Aggregates of intricate ramified hyphae (arrows) were observed in the EX11-1 CBEL$^+$ strain grown on cellophane or flax fibres. (B,D) The EX11-4 CBEL$^-$ strain formed only smooth hyphae that rarely branched on either substrate. Asterisks indicate the flax cellulose fibres. Bars, 40 μm.

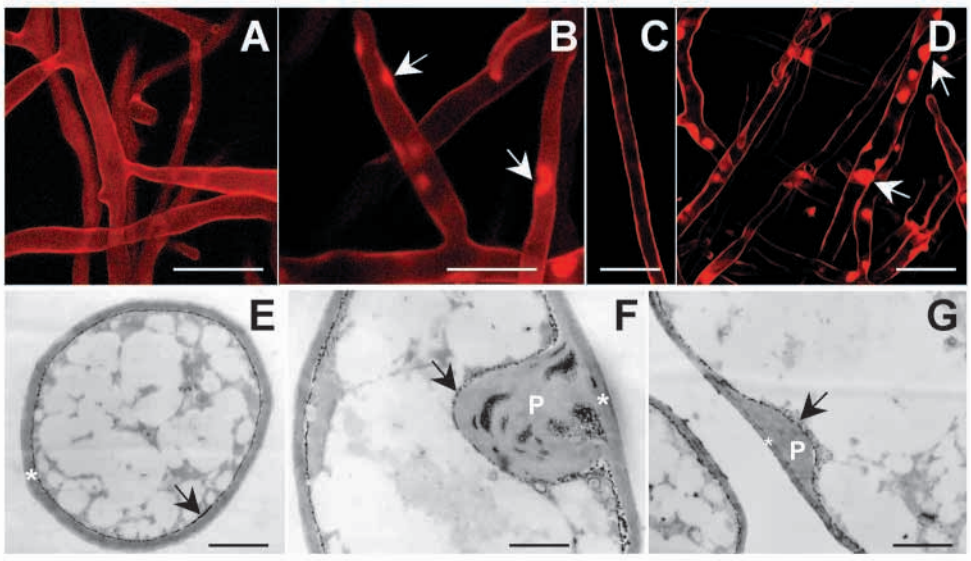

Fig. 7. Cell wall thickenings in mycelium from *P.p.n.* strains. (A-D) Confocal laser scanning microscopy of Congo Red-stained EX11-1 CBEL$^+$ (A,C) or EX11-4 CBEL$^-$ (B,D) hyphae grown either on a cellophane (A,B) or a polycarbonate membrane (C,D) placed on top of V-8 agar medium. Note the presence of patches of Congo Red-stained material (arrows) along the CBEL$^-$ EX11-4 hyphae grown on either substrate. Image acquisition parameters were adjusted to optimize contrast in images C and D. Bars, 40 μm. (E-G) Transmission electron microscopy of PATAg-stained hyphae grown on a cellophane membrane placed on top of V-8 agar medium. (E) Transverse section of a hypha from the EX11-1 CBEL$^+$ strain. The PATAg-stained cell wall appears as an electron dense layer (asterisk) and the plasmalemma-cell wall interface is strongly labelled (arrow). (F,G) Oblique sections of EX11-4 (F) or EX3-3 (G) CBEL$^-$ hyphae. Note the PATAg-stained papillae-like paramural deposits (P) between the cell wall (asterisk) and the plasma membrane (arrow). Bars, 1.5 μm.

PATAg and likely corresponded to the above-described Congo Red-stained material. In conclusion, the data indicate that the absence of CBEL results in abnormal cell wall deposition.

CBEL silencing has no major effect on the tobacco-*P.p.n.* race-cultivar specific interactions

The effect of *CBEL* silencing on pathogenicity was assessed on susceptible and resistant plants from two near-isogenic tobacco lines using mycelial explants as inoculum. When CBEL$^+$ or CBEL$^-$ transgenic strains were inoculated onto the stems of plants from the resistant line, a dark-brown HR-like necrosis was observed and disease symptoms did not develop further (data not shown), indicating that avirulence on the resistant tobacco line was retained in the strains silenced for *CBEL* expression. When the susceptible line was inoculated with the same strains, the *CBEL*-silenced strains were still pathogenic. A root inoculation assay with the susceptible line confirmed these data; the *CBEL*-silenced strains EX11-4 and EX3-3 were either more or less aggressive than the EX11-1 and EX3-5 CBEL$^+$ control strains in two independent experiments (Table 1). Light microscopy observation of inoculated roots did not allow detection of the presence of lobed structures. However, it showed that the untransformed and EX11-4 CBEL$^-$ strains differentiate appressoria and hyphal aggregates in contact with the root surface (Fig. 8). Taken together, the data show that *CBEL* silencing does not greatly alter the outcome of the race-cultivar specific interaction between tobacco and *P.p.n.* when the source of inoculum is a mycelial explant.

Table 1. Virulence of CBEL$^+$ and CBEL$^-$ strains on susceptible tobacco plants*

Strain	Experiment 1	Experiment 2
EX11-1 (CBEL$^+$)	0.46	0.41
EX11-4 (CBEL$^-$)	0.39	0.47
EX3-5 (CBEL$^+$)	0.74	0.30
EX3-3 (CBEL$^-$)	0.43	0.40

*In each experiment, 18 to 23 tobacco seedlings were inoculated with mycelium plugs from each strain, and symptoms were scored up to 12 days after inoculation. Representative data of the disease index (see Materials and Methods) scored 7 days post-inoculation are reported.

Discussion

The elicitor concept, which was first introduced by N. T. Keen (Keen et al., 1972) to designate molecules that induce defense responses in plants, does not provide any insight into the intrinsic biological function of these molecules for the organisms which produce them. In previous work, we purified a cell wall elicitor from the mycelium of *Phytophthora parasitica* var. *nicotianae* grown in vitro (Villalba Mateos et al., 1997), and reported its cellular localization in infected plants at the host-parasite interface (Séjalon-Delmas et al., 1997). The present work was undertaken in order to assess the biological significance of CBEL by a reverse genetic approach. Towards this end, we generated transgenic strains of *Phytophthora parasitica* var. *nicotianae* suppressed in *CBEL* gene expression, and studied their phenotype under saprophytic and parasitic growth conditions. The data indicated that strains lacking CBEL were deficient in adhesion and differentiation in contact with cellulosic substrates, and showed altered cell wall deposition. It was also found that CBEL$^-$ strains were not greatly altered in their specific interaction with tobacco cultivars. The contribution of this work to the knowledge of *Phytophthora* biology is severalfold, in that it illustrates in this organism (1) the use of reverse genetics for functional studies, (2) the involvement of cell wall proteins in adhesion and signal perception, and (3) biological significance of an elicitor.

Reverse genetics to address CBEL function in *Phytophthora parasitica* var. *nicotianae*

Attempts to inactivate or overexpress genes in *Phytophthora*

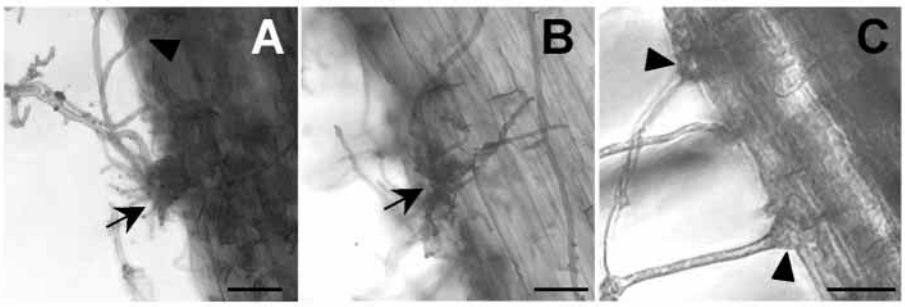

Fig. 8. Hyphal aggregates differentiation of mycelium from CBEL$^+$ and CBEL$^-$ *P.p.n.* strains in contact with susceptible tobacco roots. The untransformed (A) and EX11-4 CBEL$^-$ strain (B,C) were observed under bright-field microscopy either after rosazurin staining (A,B) or directly in the inoculation assay Petri dish (C). Arrows, hyphal aggregates; arrowheads, appressoria. Bars, 40 μm.

have been limited by the difficulty to transform most species. In addition, unlike the situation in fungi, gene inactivation in this organism cannot be performed by a single round of gene disruption because of the diploid nature of oomycetes and the low frequency of homologous DNA recombination (Judelson, 1997). The feasibility to suppress gene expression was first illustrated in *P. infestans* where a transgenic strain expressing the β-glucuronidase (*gus*) reporter gene was further transformed with *gus* antisense constructs resulting in suppression of *gus* expression (Judelson et al., 1993b). So far, this technology has been applied only once to inactivate an endogenous gene: the *inf*1 gene of *P. infestans*, which encodes a peptide elicitor from the elicitin protein family (Kamoun et al., 1998; van West et al., 1999a). However, the presence of multiple *inf* genes in the pathogen did not allow complete suppression of elicitin production. Since CBEL is encoded by a single gene (Villalba Mateos et al., 1997), it should be easier to significantly suppress its production. We therefore transformed *P.p.n.* with an antisense construct of *CBEL*. In parallel, we attempted to obtain overexpressing strains by introducing a *CBEL* sense construct. Both strategies only allowed recovery of strains in which *CBEL* expression was suppressed. This is in accordance with data obtained in *P. infestans*, where silencing resulted from both sense and antisense constructs (van West et al., 1999a).

At first sight, the phenotype of the transformants was normal when they were grown on standard media. Further analysis showed however that the transformants, expressing *CBEL* or not, were impaired in zoospore production. A similar sporulation defect was also reported by Ersek et al. in *P. capsici* transformants containing the pTH210 plasmid used in this study (Ersek et al., 1994). Our unpublished data, obtained with *P.p.n.* strains resistant to either hygromycin or G418 antibiotic, indicate that suppression of sporulation is due to conjugated effects of the presence of the antibiotic in culture medium and of the antibiotic resistance cassette in the genome of the transformants (E.G. and A.B., unpublished).

CBEL is necessary for adhesion and differentiation in contact with cellulosic surfaces

The presence of two cellulose binding domains in CBEL and the ability of the protein to bind to cellulose in vitro suggested that CBEL participates in adhesion of *P.p.n.* to cellulosic substrates. Adhesion to solid surfaces is a common feature of both saprophytic and parasitic microorganisms. In fungi and oomycetes, it is mediated by secreted adhesins that are part of the cell wall or physically associated with it (Nicholson, 1996; Carzaniga et al., 2001; Tucker and Talbot, 2001). Since the first report over 20 years ago (Sing and Bartnicki-Garcia, 1975a; Sing and Bartnicki-Garcia, 1975b), various proteins and glycoproteins have been putatively associated with adhesion in *Phytophthora*, notably during zoospore encystment and cyst germination. Among them, the Vsv1 protein of *P. cinnamomi* (Hardham and Mitchell, 1998), and mucin-like proteins from cyst germination fluids of *P. infestans* (Gornhardt et al., 2000) have been best characterized. The availability of *P.p.n.* strains suppressed in *CBEL* expression provides here the first genetic evidence that a cell wall glycoprotein of *Phytophthora* is required for adhesion to a cellulosic substrate in vitro.

In addition to their ability to attach to a cellophane membrane, the untransformed and transgenic CBEL$^+$ strains differentiated mycelial aggregates and flattened lobed structures when grown on this substrate. The aggregates were composed of intricate and branched hyphae best visualised by scanning electron microscopy. When flax cellulose fibres were used as a substrate, aggregates developed in close vicinity of the fibres, suggesting that they are produced in response to the contact of exogenous cellulose. The formation of lobed structures was clearly distinguishable from the formation of appressoria, from which they emerged in the untransformed *P.p.n.* strain. We assume that the lobed structures grew within the cellophane membrane because light microscopy observations indicated that they were localized beneath the focus plane of appressoria and of most hyphae; in addition, they were not detected by scanning electron microscopy, which visualises only aerial or surface structures. Interestingly, similar lobed structures, referred to as ‘fronds’, were described for dermatophytic fungi growing within keratin or cellophane sheets (English, 1965). It was proposed that they represent an adaptation to the layered structure and physical resistance of these substrates. The formation of polymorphic multilobed hyphae was also observed in *P.p.n.*-infected tobacco roots when the parasite was growing intramurally (Benhamou and Côté, 1992). Since the plant cell wall represents a multilayered and physically resistant environment, it is hypothesized that the lobed structures that we observed in vitro are related to the morphological changes that occur in planta when the parasite colonizes the host cell wall. Lobed structures were not convincingly identified when infected tobacco rootlets were observed with a light microscope, possibly as a result of difficulties in vizualising internal polymorphic flattened structures whose shape might depend on root tissue topography which is much more complex than a cellophane sheet.

Neither lobed structures nor mycelial aggregates could be observed in *CBEL*-silenced strains grown in vitro on a cellophane membrane or on flax cellulose fibres, indicating that CBEL is necessary for the morphological differentiation that

occurs in response to these substrates. As lobed structures and mycelial aggregates were shown to be physically associated with cellophane or flax cellulose fibres respectively, they might be directly involved in attachment of *P.p.n.* to cellulosic substrates.

Adhesion and differentiation are important processes for fungal and oomycete pathogenicity. In a number of systems, it has been reported that hyphal branching, aggregate formation, and appressorium differentiation are induced in response to the presence of host plants (Garrett, 1970; Kolattukudy et al., 1995; Nicholson, 1996; Morris et al., 1998; Hardham and Mitchell, 1998; Buée et al., 2000). Although *CBEL*-silenced strains are impaired in their ability to respond to cellulose in vitro, the EX11-4 CBEL$^-$ strain was still able to form hyphal aggregates in vivo, in contact with the host plant. This indicates that, besides cellulose, other plant components are able to induce mycelium differentiation, and that CBEL is not directly involved in the hyphal aggregate morphogenesis per se. It is worth mentioning that cellulose is embedded in the cell wall of the root epidermis, and not exposed to the rhizosphere. Interestingly, a similar difference between in vitro and in vivo behaviour has been recently reported in *Magnaporthe grisea*, where null mutants of a putative chitin-binding protein failed to differentiate appressoria normally on an artificial surface, but succeeded in differentiating them on the plant leaf surface (Kamakura et al., 2002).

CBEL is required for proper deposition of cell wall polymers

Knowledge about identity and role of cell wall proteins in oomycetes is still very limited, and no structural protein has been identified to date. CBEL-defective strains, though growing normally on standard media, formed frequent thickenings on the inner side of the cell wall. This suggests that CBEL is involved in the proper deposition of cell wall polymers, a property that might be related to the presence of cellulose-binding domains in the protein. In contrast to the cell wall of fungi, the oomycete cell wall is non-chitinous in nature but contains important amounts of cellulose that may participate in its scaffolding (Bartnicki-Garcia and Wang, 1983). CBEL, which has no enzyme activity but binds cellulose probably via its two CBDs, might be able to crosslink two cellulose chains and thus be involved in the organisation of the cell wall network. Such a role has also been suggested for a gametophytic cell wall protein of the red alga *Porphyrea purpurea* that contains four CBDs of the fungal type (Liu et al., 1996). Alternatively, CBEL might serve as a shuttle protein as has been suggested for AGPs (Arabino-Galactan-Protein) in plants (Gibeaut and Carpita, 1991). It is interesting to note that AGPs and CBEL share common features, notably their cell wall localization, the presence of hydroxyproline in their protein moiety, and the fact that their suppression (in the case of *CBEL*; this study) or inhibition [in the case of AGPs (Lord et al., 2000)] result in abnormal wall appositions. The reactivity to Congo Red and PATAg stainings of the cell wall thickenings in CBEL$^-$ strains suggests the presence of amorphous β-1,4-glucans such as non-crystalline cellulose. These thickenings are reminiscent of cellulosic cell wall appositions formed by *P. parasitica* in response to parasitism by *Pythium oligandrum* (Picard et al., 2000), and of papillae formed in plant cells in response to pathogen attack (Benhamou, 1995). Thus, absence of CBEL could either lead to deregulation of cellulose synthesis or packaging in the cell wall, or it might locally alter the cell wall in a way that mimicks pathogen attack and induces *P.p.n.* to respond by the production of papillae. In the oomycete *Saprolegnia ferax*, it has been reported that drug-induced disorganization of the cytoskeleton results in patterns of abnormal cell wall deposition (Bachewich and Heath, 1998) which resemble the cell wall thickenings of CBEL$^-$ strains. With regard to these observations, it will be of interest to compare the organization of the cytoskeleton in the *P.p.n.* CBEL$^+$ and CBEL$^-$ transgenic strains.

Biological significance of CBEL

Although CBEL was initially isolated and characterized in race 0 from *P. parasitica* var. *nicotianae*, Southern and western blot analyses indicated that CBEL homologues are also present in race 1 of *P.p.n.*, in a tomato isolate of *P. parasitica*, in strains of *P. citricola* and *P. sojae* and in *Pythium irregulare* (F.V. and M.R., unpublished). In addition to these species, homologues of CBEL have been detected in Expressed Sequence Tag libraries of *P. infestans* and *P. sojae* (available at https://xgi.ncgr.org/pgc). This distribution is consistent with a general role of CBEL in the biology of *Phytophthora* and related *Pythiaceae*.

According to its properties and localization in the innermost and outermost layers of the cell wall, CBEL might fulfil several functions. One of them relates to its elicitor activity. A few microbial elicitors have been shown to participate in virulence on susceptible hosts, or to avirulence on resistant plants (Rohe et al., 1995; Kamoun et al., 1998; Laugé and De Wit, 1998; White et al., 2000). The data reported in this work indicate that CBEL is not a primary determinant of the tobacco-*P.p.n.* race-cultivar specific interactions, since CBEL$^-$ strains remained virulent and avirulent on susceptible and resistant cultivars respectively. This is in accordance with unpublished results showing that infiltration of purified CBEL induces defence reactions in the two tobacco isolines (F.V. and M.R., unpublished). The question as to whether CBEL acts as an elicitor upon colonization of the host plant cannot be readily answered, mainly because CBEL suppression may have adverse effects on pathogenicity. Thus, lowering the elicitor effect might increase pathogenicity, whereas abnormal cell wall appositions might weaken the mycelium, hence the agressiveness of CBEL$^-$ strains. Opposite effects on elicitation and agressivity might then account for apparent unchanged pathogenicity.

Specific assay conditions and plant species or cultivars are often required in order to detect modifications in plant-parasite interactions, particularly when multiple virulence or avirulence factors are involved in the interaction (Kamoun et al., 1998; Isshiki et al., 2001). For example, the *Ecp2* gene of *Cladosporium fulvum* was first claimed not to be essential for pathogenicity on tomato (Marmeisse et al., 1994), whereas improvement of the infection assay in order to better mimick natural conditions allowed to demonstrate subsequently that it was a virulence gene (Laugé et al., 1997). Since the *P.p.n.* transformants used in this study do not release zoospores, the production of standardized inocula for fine quantitation of virulence is difficult, and the natural infection mode cannot be mimicked.

Another major characteristic of CBEL relates to adhesion to cellulose and associated differentiation of $CBEL^+$ strains as compared to *CBEL*-suppressed strains. This suggests that CBEL, which contains two cellulose-binding domains, acts as a sensor of exogenous cellulose either in the soil during the saprophytic life of *P.p.n*., and/or in the host plant after penetration has occurred. Besides the presence of two CBDs, a striking feature of CBEL is its high cysteine content, a structural property it shares with proteins of the hydrophobin family identified in filamentous fungi (Templeton et al., 1994). The fact that hydrophobins are cell wall proteins involved in development, attachment, and cell wall structure (Wösten, 2001) extends the parallel between CBEL and hydrophobins to functional properties.

In conclusion, the finding that cell surface properties are altered in $CBEL^-$ strains and that abnormal cell wall appositions occur in these strains, illustrates the concept that elicitor molecules have other functions than those related to their effect when externally supplied to host plants. Microbial cell walls are the subject of increasing interest as potential targets in the search for new antimicrobial compounds (Goldman and Branstrom, 1999). CBEL is the first cell wall protein of an oomycete microorganism which has been molecularly characterized. The demonstration that it is involved in the proper deposition of cell wall polymers represents a major step towards the understanding of cell wall biogenesis in this organism.

We thank H. S. Judelson (University of California, Riverside) and C. Morvan (UMR6037 CNRS-Université de Rouen, Mont-Saint-Aignan, France) for the generous gift of plasmid pTH210 and flax cellulose fibres respectively, L. Larché, L. Y. Martinez and P. Sanchez (UMR5546 CNRS-Université Paul Sabatier, Castanet-Tolosan, France) for technical help, and B. Dumas and P. Rech (UMR5546 CNRS-Université Paul Sabatier, Castanet-Tolosan, France) for critical reading of the manuscript.

References

Bachewich, C. and Heath, I. B. (1998). Radial F-actin arrays precede new hypha formation in *Saprolegnia*: implications for establishing polar growth and regulating tip morphogenesis. *J. Cell Sci.* **111**, 2005-2016.

Bartnicki-Garcia, S. and Wang, M. C. (1983). Biochemical aspects of morphogenesis in *Phytophthora*. In *Phytophthora, its Biology, Taxonomy, Ecology and Pathology* (ed. D. C. Erwin, S. Bartnicki-Garcia and P. H. Tsao), pp. 121-137. St Paul: APS Press.

Benhamou, N. (1995). Immunocytochemistry of plant defense mechanisms induced upon microbial attack. *Microsc. Res. Tech.* **31**, 63-78.

Benhamou, N. and Côté, F. (1992). Ultrastructure and cytochemistry of pectin and cellulose degradation in tobacco roots infected by *Phytophthora parasitica* var. *nicotianae. Phytopathology* **82**, 468-478.

Bostock, R. M., Kuc, J. A. and Laine, R. A. (1981). Eicosapentaenoic and arachidonic acids from *Phytophthora infestans* elicit fungitoxic sesquiternes in the potato. *Science* **212**, 67-69.

Bottin, A., Larché, L., Villalba, F., Gaulin, E., Esquerré-Tugayé, M. T. and Rickauer, M. (1999). Green fluorescent protein (GFP) as gene expression reporter and vital marker for studying development and microbe-plant interaction in the tobacco pathogen *Phytophthora parasitica* var. *nicotianae*. *FEMS Microbiol. Lett.* **176**, 51-56.

Buée, M., Rossignol, M., Jauneau, A., Ranjeva, R. and Becard, G. (2000). The pre-symbiotic growth of arbuscular mycorrhizal fungi is induced by a branching factor partially purified from plant root exudates. *Mol. Plant Microbe Interact.* **13**, 693-698.

Carzaniga, R., Bowyer, P. and O'Connell, R. J. (2001). Production of extracellular matrices during development of infection structures by the downy mildew *Peronospora parasitica*. *New Phytologist* **149**, 83-93.

Cavalier-Smith, T. (1998). A revised six-kingdom system of life. *Biol. Rev. Camb. Philos. Soc.* **73**, 203-266.

Dellaporta, S. L., Wood, J. and Hicks, J. B. (1983). A plant DNA minipreparation. In *Plant Molecular Biology Reporter*, pp. 19-21. New York: Cold Spring Harbor Laboratory Press.

English, M. P. (1965). The saprophytic growth of non-keratinophilic fungi on keratinized substrata, and a comparison with keratinophilic fungi. *Trans. Br. Mycol. Soc.* **48**, 219-235.

Ersek, T., Schoelz, J. E. and English, J. T. (1994). Characterization of selected drug-resistant mutants of *Phytophthora capsici*, *Phytophthora parasitica* and *Phytophthora citrophthora*. *Acta Phytopathologica et Entomologica Hungarica* **29**, 215-229.

Erwin, D. C. and Ribeiro, O. K. (1996). *Phytophthora* diseases worldwide. St Paul: APS Press.

Farmer, E. E. and Helgeson, J. P. (1987). An extracellular protein from *Phytophthora parasitica* var. *nicotianae* is associated with stress metabolite accumulation in tobacco callus. *Plant Physiol.* **85**, 733-740.

Garett, S. D. (1970). Unspecialized parasites. In *Pathogenic root-infecting fungi*, pp. 31-46. Cambridge: Cambridge University Press.

Gibeaut, D. M. and Carpita, N. C. (1991). Tracing cell wall biogenesis in intact cells and plants: selective turnover and alteration of soluble and cell wall polysaccharides in grasses. *Plant Physiol.* **97**, 551-561.

Gilkes, N. R., Henrissat, B., Kilburn, D. G., Miller, R. C. and Warren, R. A. (1991). Domains in microbial β1,4-glycanases: sequence, conservation, function, and enzyme families. *Microbiol Rev.* **55**, 303-315.

Goldman, R. C. and Branstrom, A. (1999). Targeting cell wall synthesis and assembly in microbes: similarities and contrasts between bacteria and fungi. *Curr. Pharm. Des.* **5**, 473-501.

Gooding, G. V. and Lucas, G. B. (1959). Factors influencing sporangial formation and zoospore activity in *Phytophthora parasitica* var. *nicotianae*. *Phytopathology* **49**, 277-281.

Gornhardt, B., Rouhara, I. and Schmelzer, E. (2000). Cyst germination proteins of the potato pathogen *Phytophthora infestans* share homology with human mucins. *Mol. Plant Microbe Interact.* **13**, 32-42.

Govers, F. (2001). Misclassification of pest as 'fungus' puts vital research on wrong track. *Nature* **411**, 633.

Hardham, A. R. and Mitchell, H. J. (1998). Use of molecular cytology to study the structure and biology of phytopathogenic and mycorrhizal fungi. *Fungal Genet. Biol.* **24**, 252-284.

Isshiki, A., Akimitsu, K., Yamamoto, M. and Yamamoto, H. (2001). Endopolygalacturonase is essential for citrus black rot caused by *Alternaria citri* but not brown spot caused by *Alternaria alternata*. *Mol. Plant Microbe Interact.* **14**, 749-757.

Judelson, H. S. (1997). The genetics and biology of *Phytophthora infestans:* modern approaches to a historical challenge. *Fungal Genet. Biol.* **22**, 65-76.

Judelson, H. S., Tyler, B. M. and Michelmore, R. W. (1991). Transformation of the oomycete pathogen, *Phytophthora infestans*. *Mol. Plant Microbe Interact.* **4**, 602-607.

Judelson, H. S., Coffey, M. D., Arredondo, F. R. and Tyler, B. M. (1993a). Transformation of the oomycete pathogen *Phytophthora megasperma* f. sp. *glycinea* occurs by DNA integration into single or multiple chromosomes. *Curr. Genet.* **23**, 211-218.

Judelson, H. S., Dudler, R., Pieterse, C. M., Unkles, S. E. and Michelmore, R. W. (1993b). Expression and antisense inhibition of transgenes in *Phytophthora infestans* is modulated by choice of promoter and position effects. *Gene* **133**, 63-69.

Kamakura, T., Yamaguchi, S., Saitoh, K., Teraoka, T. and Yamaguchi, I. (2002). A novel gene, CBP1, encoding a putative extracellular chitin-binding protein, may play an important role in the hydrophobic surface sensing of *Magnaporthe grisea* during appressorium differentiation. *Mol. Plant Microbe Interact.* **15**, 437-444.

Kamoun, S., van West, P., Vleeshouwers, V. G., de Groot, K. E. and Govers, F. (1998). Resistance of *Nicotiana benthamiana* to *Phytophthora infestans* is mediated by the recognition of the elicitor protein INF1. *Plant Cell* **10**, 1413-1426.

Keen, N. T. and Yoshikawa, M. (1983). β1,3-endoglucanase from soybean releases elicitor active carbohydrates from fungus cell wall. *Plant Physiol* **71**, 460-465.

Keen, N. T., Partridge, T. E. and Ai Zaki, T. (1972). Pathogen-produced elicitor of a chemical defense mechanism in soybeans monogenically resistant to *Phytophthora megasperma* f.sp. g*lycinea*. *Phytopathology* **62**, 768.

Kolattukudy, P. E., Rogers, L. M., Li, D., Hwang, C. S. and Flaishman,

M. A. (1995). Surface signaling in pathogenesis. *Proc. Natl. Acad. Sci. USA* **92**, 4080-4087.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Laugé, R. and de Wit, P. J. (1998). Fungal avirulence genes: structure and possible functions. *Fungal Genet. Biol.* **24**, 285-297.

Laugé, R., Joosten, M. H. A., van den Ackerveken, G. F. J., van den Broek, H. W. J. and de Wit, P. J. (1997). The in planta-produced extracellular proteins ECP1 and ECP2 of Cladosporium fulvum are virulence factors. *Mol. Plant Microbe Interact.* **10**, 725-734.

Liu, Q. J., Ross, N., Lanthier, P. and Reith, M. (1996). A gametophyte cell wall protein of the red alga *Porphyra purpurea* (Rhodophyta) contains four apparent polysaccharide-binding-domain. *J. Phycol.* **32**, 995-1003.

Lord, E. M., Holdaway-Clarke, S. J., Roy, S. J., Jauth, G. Y. and Helper, P. K. (2000). Arabinogalactan-protein in pollen tube growth. *In Cell and Developmental Biology of Arabinogalactan-protein* (ed. E. A. Nothnagel, A. Bacic and A. E. Clarke), pp. 153-167. New York: Kluwer Academic/Plenum Publishers.

Marmeisse, R., van den Ackerveken, G. F., Goosen, T., de Wit, P. J. and van den Broek, H. W. (1994). The in-planta induced ecp2 gene of the tomato pathogen *Cladosporium fulvum* is not essential for pathogenicity. *Curr. Genet.* **26**, 245-250.

Morris, P. F., Bone, E. and Tyler, B. M. (1998). Chemotropic and contact responses of *Phytophthora sojae* hyphae to soybean isoflavonoids and artificial substrates. *Plant Physiol.* **117**, 1171-1178.

Nicholson, R. (1996). Adhesion of fungal propagules. In *Histology, Ultrastructure and Molecular Cytology of Plant-Microorganism Interactions* (ed. M. Nicole and V. Gianinazzi-Pearson), pp. 117-134. Dordrecht, Netherlands: Kluwer Academic.

Parker, J. E., Schulte, W., Hahlbrock, K. and Scheel, D. (1991). An extracellular glycoprotein from *Phytophthora megasperma* f. sp. *glycinea* elicits phytoalexin synthesis in cultured parsley cell and protoplasts. *Mol. Plant Microbe Interact.* **4**, 19-27.

Picard, K., Tirilly, Y. and Benhamou, N. (2000). Cytological effects of cellulases in the parasitism of *Phytophthora parasitica* by *Pythium oligandrum*. *Appl. Environ. Microbiol.* **66**, 4305-4314.

Ponchet, M., Panabières, F., Milat, M. L., Mikes, V., Montillet, J. L., Suty, L., Triantaphylides, C., Tirilly, Y. and Blein, J. P. (1999). Are elicitins cryptograms in plant-oomycete communications? *Cell. Mol. Life Sci.* **56**, 1020-1047.

Rancé, I., Fournier, J. and Esquerré-Tugayé, M. T. (1998). The incompatible interaction between *Phytophthora parasitica* var. *nicotianae* race 0 and tobacco is suppressed in transgenic plant expressing antisense lipoxygenase sequences. *Proc. Natl. Acad. Sci. USA* **95**, 6554-6559.

Ricci, P., Bonnet, P., Huet, J. C., Sallantin, M., Beauvais-Cante, F., Bruneteau, M., Billard, V., Michel, G. and Pernollet, J. C. (1989). Structure and activity of proteins from pathogenic fungi *Phytophthora* eliciting necrosis and acquired resistance in tobacco. *Eur. J. Biochem.* **183**, 555-563.

Rickauer, M., Brodschelm, W., Bottin, A., Véronési, C., Grimal, H. and Esquerré-Tugayé, M. T. (1997). The jasmonate pathway is involved differentially in the regulation of different defence responses in tobacco cells. *Planta* **202**, 155-162.

Rohe, M., Gierlich, A., Hermann, H., Hahn, M., Schmidt, B., Rosahl, S. and Knogge, W. (1995). The race-specific elicitor, NIP1, from the barley pathogen, *Rhynchosporium secalis*, determines avirulence on host plants of the Rrs1 resistance genotype. *EMBO J.* **14**, 4168-4177.

Roland, J. C. and Vian, B. (1991). Use of purified endopolygalacturonase for a topochemical study of elongating cell walls at the ultrastructural level. *J. Cell Sci.* **48**, 333-343.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular cloning. In *A Laboratory Manual* (ed. J. Sambrook, E. F. Fritsch and T. Maniatis). New York: Cold Spring Harbor Laboratory Press.

Séjalon-Delmas, N., Villalba, F., Bottin, A., Rickauer, M., Dargent, R. and Esquerré-Tugayé, M. T. (1997). Purification, elicitor activity, and cell wall localisation of a glycoprotein from *Phytophthora parasitica* var. *nicotianae*, a fungal pathogen of tobacco. *Phytopathology* **87**, 899-909.

Sharp, J. K., McNeil, M. and Albersheim, P. (1984). The primary structure of one elicitor-active and seven elicitor-inactive hexa(β-D-glucopyranosyl)-D-glycitols isolated from the mycelia walls of *Phytophthora megasperma* f. sp. *glycinea*. *J. Biol. Chem.* **259**, 11321-11326.

Sing, V. O. and Bartnicki-Garcia, S. (1975a). Adhesion of *Phytophthora palmivora* zoospores: detection and ultrastructural visualization of concanavalin A-receptor sites appearing during encystment. *J. Cell Sci.* **19**, 11-20.

Sing, V. O. and Bartnicki-Garcia, S. (1975b). Adhesion of *Phytophthora palmivora* zoospores: electron microscopy of cell attachment and cyst wall fibril formation. *J. Cell Sci.* **18**, 123-132.

Sogin, M. L. and Silberman, J. D. (1998). Evolution of the protists and protistan parasites from the perspective of molecular systematics. *Int. J. Parasitol.* **28**, 11-20.

Templeton, M. D., Rikkerink, E. H. A. and Beever, R. E. (1994). Small, cysteine-rich proteins and recognition in fungal-plant interactions. *Mol. Plant Microbe Interact.* **7**, 320-325.

Thiéry, J. P. (1967). Mise en évidence des polysaccharides sur coupes fines en microscopies électroniques. *J. Microsc.* **6**, 987-1018.

Tormo, J., Lamed, R., Chirino, A. J., Morag, E., Bayer, E. A., Shoham, Y. and Steitz, T. A. (1996). Crystal structure of a bacterial family-III cellulose-binding domain: a general mechanism for attachment to cellulose. *EMBO J.* **15**, 5739-5751.

Tucker, S. L. and Talbot, N. J. (2001). Surface attachment and pre-penetration stage development by plant pathogenic fungi. *Annu. Rev. Phytopathol.* **39**, 385-417.

Tyler, B. M. (2001). Genetics and genomics of the oomycete-host interface. *Trends Genet.* **17**, 611-614.

van West, P., Kamoun, S., van't Klooster, J. W. and Govers, F. (1999a). Internuclear gene silencing in *Phytophthora infestans*. *Mol. Cell* **3**, 339-348.

van West, P., Reid, B., Campbell, T. A., Sandrock, R. W., Fry, W. E., Kamoun, S. and Gow, N. A. (1999b). Green fluorescent protein (GFP) as a reporter gene for the plant pathogenic oomycete *Phytophthora palmivora*. *FEMS Microbiol. Lett.* **178**, 71-80.

Villalba Mateos, F., Rickauer, M. and Esquerré-Tugayé, M. T. (1997) Cloning and characterization of a cDNA encoding an elicitor of *Phytophthora parasitica* var. *nicotianae*, that shows cellulose-binding and lectin-like activities. *Mol. Plant Microbe Interact.* **10**, 1045-1053.

Waldmüller, T., Cosio, E. G., Grisebach, H. and Ebel, J. (1992). Release of highly elicitor-active glucans by germinating zoospores of *Phytophthora megasperma* var. *glycinea*. *Planta* **188**, 498-505.

White, F. F., Yang, B. and Johnson, L. B. (2000). Prospects for understanding avirulence gene function. *Curr. Opin. Plant Biol.* **3**, 291-298.

Wood, P. J. (1980). Specificity in the interaction of direct dyes with polysaccharides. *Carbohydr Res.* **85**, 271-287.

Wösten, H. A. (2001). Hydrophobins: multipurpose proteins. *Annu. Rev. Microbiol.* **55**, 625-646.