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# The pH regulator PacC: a host-dependent virulence factor in *Botrytis cinerea*

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## Summary

The phytopathogenic fungus *Botrytis cinerea* is able to infect a wide variety of plants and plant tissues with differing chemical compositions. During its interaction with the host, this pathogen modulates its ambient pH by secreting acids or ammonia. In this work, we examined the Pal/Pac pathway, the fungal ambient pH-responsive signalling circuit, and investigated the role of the PacC transcription factor. Characterization of the *BcpacC* deletion mutant revealed an alteration of both fungal growth and virulence depending on the pH of the culture medium or of the host tissue. The pathogenicity of the mutant was altered on plants exhibiting a neutral pH and not on plants with acidic tissues. The capacity of the mutant to acidify its environment and, more particularly, to produce oxalic acid was affected, as was production of reactive oxygen species. Finally, proteomic profiling of the mutant secretome revealed significant changes in plant cell wall polysaccharides proteins and lipid degradation and oxidoreduction, highlighting the importance of BcPacC in the necrotrophic lifestyle of *B. cinerea*.

## Originality-significant statement

Our experimental data demonstrate the importance of the pH-responsive transcription factor PacC in a fungus which modifies the ambient pH during the infection process. The *BcpacC* encoding gene has been deleted in

the phytopathogenic fungus *Botrytis cinerea* and the deletion mutant exhibits a defect in virulence depending on the pH of the host tissues. Our study reveals that *pacC* controls also production of oxalic acid and several enzymes involved in hydrolysis of plant cell wall components or production of ROS.

## Introduction

Ambient pH is one of the most important physicochemical factors affecting cell growth and development. Faced with pH fluctuations, microorganisms must adapt rapidly, and they do so by monitoring external pH and accordingly adjusting metabolic gene regulation, protein synthesis and transport/secretion.

Fungi are known to thrive over a very large pH range from pH 2.0 to 9.0 (Caddick *et al.*, 1986), and adaptation to environmental pH is important to saprophytic as well as pathogenic species. In the case of pathogenic fungi, understanding pH adaptation could lead to better control over the associated animal or plant diseases. Numerous phytopathogenic fungi can attack various hosts and/or tissues with different pH(s), and several of them can also modify the pH of their environment by secreting organic acids or ammonia (Bateman and Beer, 1965; Prusky *et al.*, 2001; Alkan *et al.*, 2013a). They have evolved mechanisms to ensure that molecules directly exposed to the environment are produced only at pH values in which they can be active, including permeases, exported metabolites and secreted enzymes involved in fungal nutrition and/or virulence.

Fungi possess a pH-specific genetic regulatory system that acts through a single signalling pathway that was first described in the saprophytic fungus *Aspergillus nidulans* (Prusky and Yakobi, 2003). This pathway is composed of the products of six *pal* genes (*palA*, *palB*, *palC*, *palF*, *palH* and *palI*) and targets the zinc finger transcription factor PacC (Peñalva *et al.*, 2008). PacC activates genes expressed under alkaline conditions and represses genes expressed under acidic conditions. This pH-responsive pathway has been extensively studied, and in a revised model, Peñalva and his collaborators proposed that sensing the external pH involves the two transmembrane domain proteins PalH, PalI and the arrestin-like protein PalF, which form a membrane complex under neutral or alkaline

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pH. PalF bound to PalH is phosphorylated, ubiquitinated and interacts with Vps23, a component of the endosomal sorting complex I required for transport (ESCRT-I) for the recruitment of PalC and PalA, which interact with two components of ESCRT-III (Vps32/Vps20). PalA contains a 'V-shaped' domain that is involved in the interaction with two YPX(L/I) motifs present in PacC and flanks a signalling protease target region. PalB, the signalling cysteine protease, is then recruited by Vps24 to mediate cytoplasmic proteolysis of PacC. In *A. nidulans*, a second proteolysis event, independent of PalB, yields a final truncated PacC that translocates to the cell nucleus and modulates the expression of target genes (Peñalva *et al.*, 2014). Similar mechanisms involving the PacC orthologue Rim101 have been described in the yeasts *Saccharomyces cerevisiae*, *Candida albicans*, *Yarrowia lipolytica* (Comet and Gaillardin, 2014) and *Cryptococcus neoformans* (Ost *et al.*, 2015). During the last decade, numerous studies have confirmed that this pH signalling pathway is specific and conserved throughout the fungal kingdom. The construction and study of PacC/Rim101 deletion strains in multiple fungal species have revealed that this regulator is required for virulence in human, insect, fungus and plant pathogens (for review, see Alkan *et al.*, 2013a; Landraud *et al.*, 2013; Bertuzzi *et al.*, 2014; Comet and Gaillardin, 2014; Huang *et al.*, 2015; Ost *et al.*, 2015; Lou *et al.*, 2015; Luo *et al.*, 2017). In most cases, PacC plays a role in enzyme and metabolite production and/or fungal cell wall remodelling that are required in the infectious process. The role of this pH regulator has also been studied in a symbiotic interaction between the biotrophic fungus *Epichloë festucae* and the grass *Lolium perenne* (Lukito *et al.*, 2015).

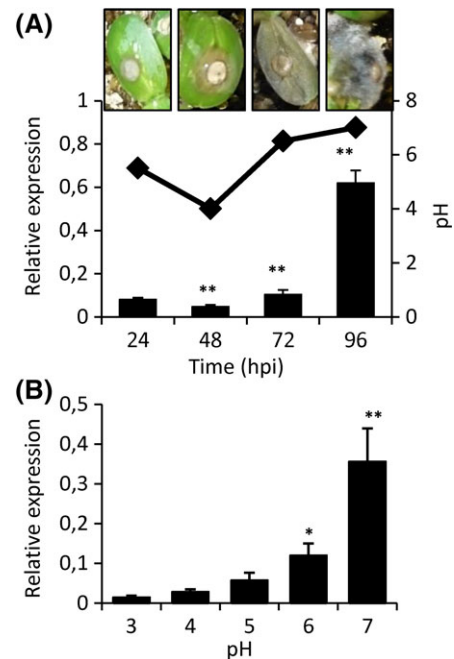
Our study focuses on the grey mould fungus *Botrytis cinerea*, an important plant pathogen that can infect more than 500 genera of plants, including high-value crops, such as grapevine, strawberry, tomato and ornamental flowers (Elad *et al.*, 2016). *B. cinerea* is considered to be a typical necrotrophic fungus. It secretes an arsenal of toxins, including organic acids, and cell wall degrading enzymes to kill the plant tissues and feed on the dead cells during colonization. This fungus is able to attack plant tissues with different pH values (for example, grape berries pH 3.5, cucumber pH 6.3) (Manteau *et al.*, 2003). It is known for its capacity to acidify some of these tissues during colonization by secreting large amounts of organic acids, including oxalic acid (OA). However, it can also alkalize plant tissues during infection probably due to the secretion of ammonia (Billon-Grand *et al.*, 2012). Here, we investigate the adaptive response of *B. cinerea* to pH and provide information about the role of the pH regulator BcPacC in the development and pathogenicity of this fungus. We show that the pathogenicity of the *BcpacC* deletion mutant is altered only in plants exhibiting tissues with neutral pH values and not in those with

acidic tissues. Our data reveal that PacC controls OA production and enzyme secretion, which are key virulence determinants influencing the infectious process.

## Results

### Expression of the pH-signalling pathway genes in *B. cinerea*

The protein sequence of the *pacC* gene and of the six *pal* genes (*palA*, *palB*, *palC*, *palF*, *palH*, *palI*) of *A. nidulans* was used to search for homologues in the genome of *B. cinerea*. Because of strong conservation of these seven genes across fungi, homologues were easily identified (see Supporting Information Table S1). *BcpacC* expression investigated during the course of sunflower infection revealed a low expression level during the first 2 days of the interaction and then an increase in expression up to more than 10-fold at 4 days post-inoculation (dpi). In parallel, the pH of the host tissues dropped from pH 6.0 to 4.0 during the first 48 h and then increased to reach pH 7.0 and 7.5 at 3 and 4 dpi respectively (Fig. 1A). This result suggested a pH regulation of



**Fig. 1.** Expression of the *B. cinerea pac* and *pal* genes. A. Expression levels of the *BcpacC* gene during the kinetics of sunflower cotyledon infection by *B. cinerea*. The stages of infection are shown for each time, and the pH values of the colonized plant tissues are indicated (♦). B. Expression levels of *BcpacC* gene in mycelia grown on solid non-buffered medium and transferred to liquid medium buffered at different pH values. The actin-encoding gene was used as a reference. Three independent biological replicates were assessed for each experiment. Standard deviations are indicated, and asterisks indicate a significant difference (Student's *t* test, \**p*-value < 0.05, \*\**p*-value < 0.005) in gene expression compared with the previous time point (A) or the previous pH value (B).

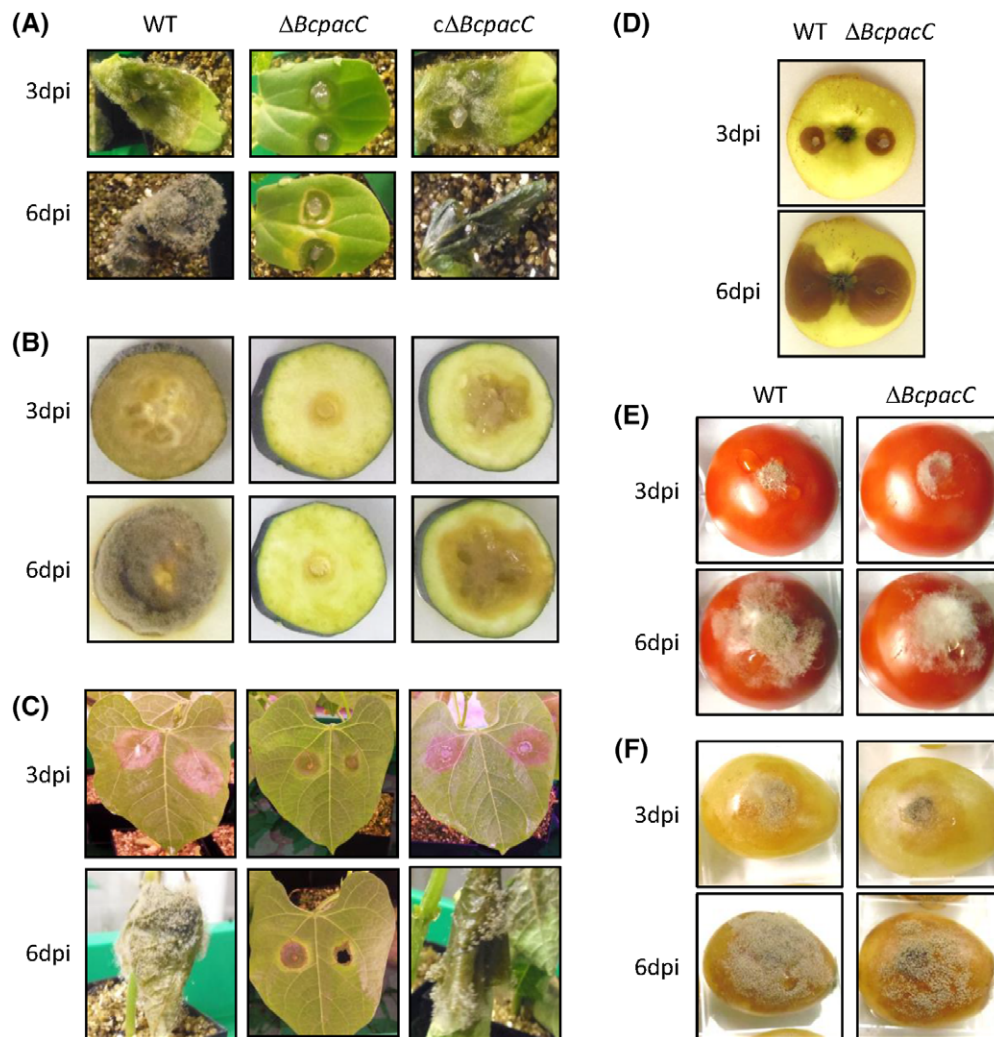
*BcpacC* expression. Transfer of the mycelium into medium buffered at different pH values confirmed this result, as transcription of *BcpacC* remained low under acidic conditions (pH 3.0–5.0) and increased when the fungus was exposed to pH value increases from pH 6.0 to 7.0 (Fig. 1B).

Expression of all *Bcpal* (*BcpalA-I*)-encoding genes was also measured during the course of sunflower infection, and the data showed that it remained low in comparison with *BcpacC* (Supporting Information Fig. S1).

#### *BcPacC* contributes to virulence in *B. cinerea*

To investigate the role of BcPacC in *B. cinerea* virulence, deletion mutants lacking the *pacC* coding region were constructed and three knock-out mutants were independently isolated (Supporting Information Fig. S2 and

Table S2) and tested in plant infection assays. One-week-old cucumber cotyledons (pH 6.7) showed complete colonization and tissue maceration by the wild type (WT) strain within 4 dpi, and sporulation of the pathogen was visible at 6 dpi. In comparison, only reduced primary lesions could be observed with the mutant strain at 6 dpi or at later time points (Fig. 2A). Zucchini fruits (pH 6.5) displayed colonization and complete maceration by the WT strain at 3 and 6 dpi respectively (Fig. 2B). The  $\Delta BcpacC$  mutant showed a delay in infection, as the first symptoms were observed around the inoculation point at 3 dpi and no evolution of the necrotic area could be observed over the following days. This defect in virulence was also noted on primary bean leaves (pH 6.3), as only reduced lesions could be observed with the mutant strain after 6 days of incubation. Moreover, a hypersensitive-like response was particularly observed around the



**Fig. 2.** Effect of *BcpacC* deletion on the virulence of *B. cinerea*. Time course of infection of cucumber cotyledons (A), zucchini fruits (B), French bean leaves (C), apple fruits (D), cherry tomatoes (E) and grape berries (F) inoculated with the WT strain, the deletion mutant strain ( $\Delta BcpacC$ ) or the complemented strain ( $c\Delta BcpacC$ ). Mycelial plugs served as inocula. All tests were assessed in three independent experiments with at least five technical replicates.

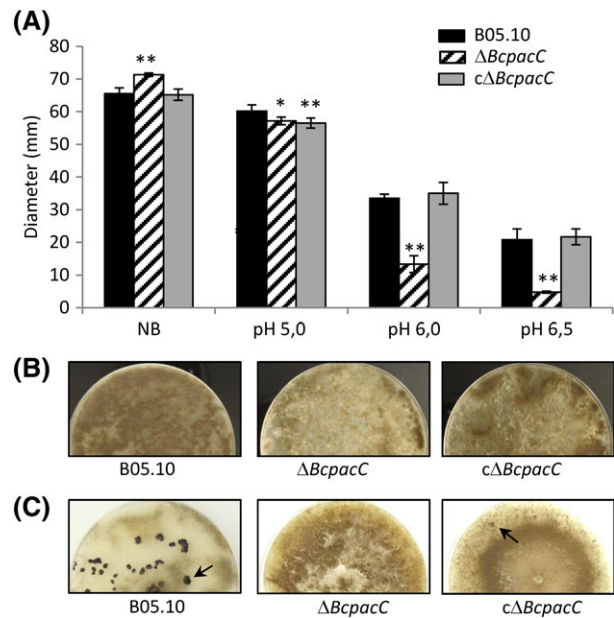


lesions produced by the mutant on bean leaves or cucumber cotyledons (Fig. 2A and C). By contrast, no difference between the WT and mutant strains could be observed on apple (pH 4.0), cherry tomato fruits (pH 4.7) or grape berries (pH 3.8) since the necrotic areas appeared around the inoculation point and the infection then progressed similarly for both strains (Fig. 2D–F). Overall, these results indicate that PacC is not required for infection of acidic tissues but is involved in the infection of non-acidic plant tissues. Under these conditions, PacC would not contribute to the establishment of primary lesions, but rather would be implicated later in the infectious process. Complementation of one deletion mutant was accomplished by reintroduction of the *BcpacC* gene into the deletion strain. Infection assays run in parallel with the complemented strain showed apparent restoration of virulence. Indeed, the strain was able to colonize and macerate the host tissues but conidiation observed on plant tissues was less important in comparison with the WT (Fig. 2A–C; Supporting Information Fig. S3).

#### Impact of the deletion of *BcpacC* on the physiology of *B. cinerea*

As plant colonization necessitates mycelial development, fungal growth of the WT and mutant strains was investigated under several pH conditions (Fig. 3A). Using medium buffered at different pH values, optimal growth was achieved under acidic conditions (pH 5.0) for the WT strain, while growth reduction was observed at pH 6.5 (more than 60% compared with growth at pH 5.0). At pH 5.0, the  $\Delta BcpacC$  mutant showed a 5% growth reduction in comparison with the WT strain, and this difference increased with the pH: 60% at pH 6.0 and 77% at pH 6.5. These differences were abolished in the complemented strain (Fig. 3A). These results indicate that the adaptation of  $\Delta BcpacC$  mutant to ambient pH is affected and it exhibits an acidic-mimicking phenotype.

Asexual reproduction was also investigated by cultivating the strains on complete medium under continuous near UV-light conditions. After 11 days of incubation at 21 °C, the mutant displayed significantly reduced (more than 70% in comparison with the WT strain, data not shown) formation of asexual spores (Fig. 3B). Longer incubation of the cultures confirmed that this defect in sporulation was quantitative and did not represent a delay in conidial differentiation. Sclerotium differentiation was also evaluated after 4 weeks of mycelial growth in continuous darkness. The mutant exhibited melanized hyphae with no conidia and no sclerotia differentiated compared with the WT strain (Fig. 3C). Although the three independent mutants we analysed revealed the same altered phenotypes, conidiation and sclerotium

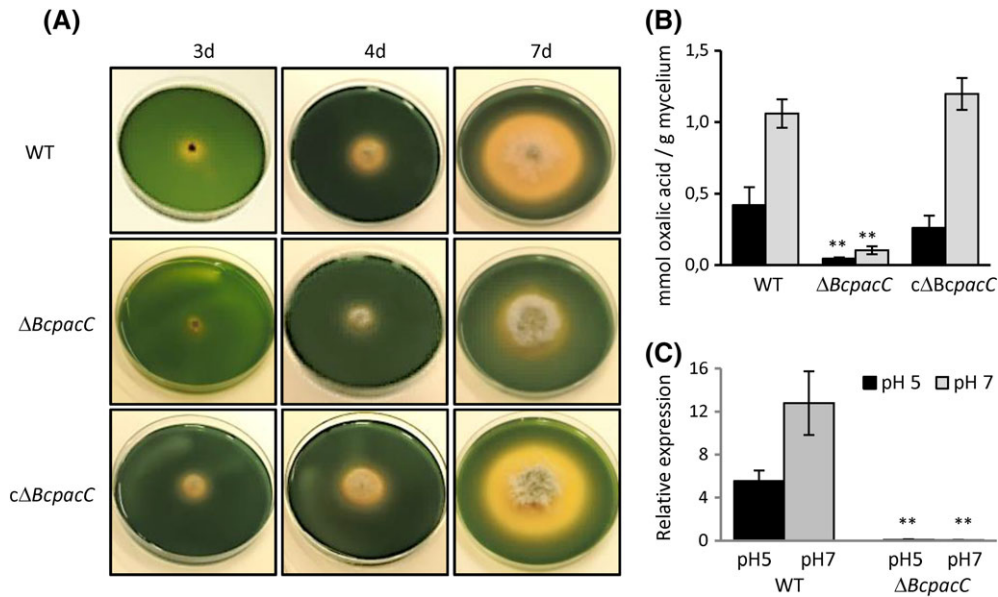


**Fig. 3.** Effect of *BcpacC* deletion on fungal growth, conidial production and sclerotium formation. A. Radial growth of the WT, deletion ( $\Delta BcpacC$ ) and complemented ( $c\Delta BcpacC$ ) strains was measured after 3 days on complete solid medium buffered at different pH values (NB: non-buffered medium) and supplemented with 0.5% glucose. B. Conidiation was observed after 11 days of culture on complete sporulation medium under continuous near-UV light. C. Sclerotium formation was observed after 3 weeks of culture on complete medium maintained in the dark. Data are the means of five technical replicates and three biological replicates. Standard deviations are indicated, and asterisks represent significant differences compared with WT for each condition (Student's *t* test, (\**p*-value < 0.05, \*\**p*-value < 0.005).

differentiation were not fully restored in the complemented strain suggesting that PacC is not the main regulator controlling these biological processes.

#### *BcPacC* is involved in acidification of the ambient environment and the synthesis of OA

*Botrytis cinerea* is known for its ability to acidify its ambient environment through the secretion of organic acids (Billon-Grand *et al.*, 2012). pH values of several host tissues were followed throughout the infection process of the WT and the *BcpacC* deletion mutant. On tissues exhibiting neutral values, a defect in acidification was observed during the first days of infection with the mutant (Supporting Information Fig. S3). Medium acidification by the WT, mutant and complemented strains were then examined using solid complete medium supplemented with the pH indicator bromothymol blue (Schumacher *et al.*, 2014). In comparison to the WT strain, the  $\Delta BcpacC$  strain showed reduced acidification of its environment, and this defect was not observed in the complemented strain (Fig. 4A). Following growth on the surface of liquid medium buffered at pH 5.0 or 7.0, OA production

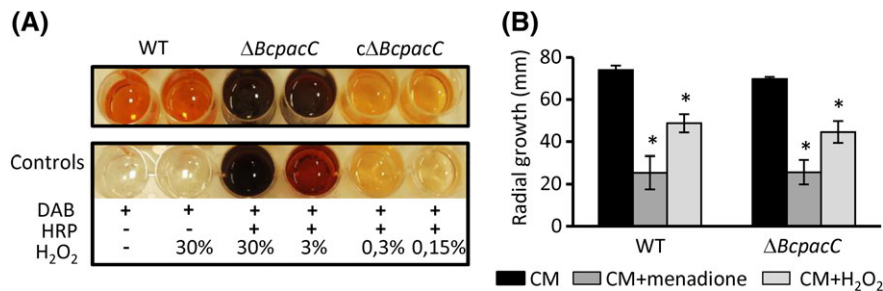


**Fig. 4.** Effect of *BcpacC* deletion on medium acidification and oxalic acid production. A. Medium acidification. The WT, deletion mutant and complemented strains were grown on solid complete medium supplemented with a pH indicator. The colour change from green to yellow indicates acidification (pH < 6.0). Photos are representatives of three independent experiments at 3, 4 and 7 days after inoculation with a mycelial plug. B. HPLC quantification of oxalic acid secreted into the liquid medium. The strains were cultivated for 72 h on the surface of cellophane placed on complete solid medium and then transferred for 4 h onto the surface of Gamborg liquid medium buffered at pH 5.0 or 7.0. C. Expression of the oxaloacetate hydrolase-encoding gene (*Bcoah*) in the WT and  $\Delta BcpacC$  strains. The mycelia grown for the experiment shown in B were collected for total RNA extraction, and the actin-encoding gene was used as a reference. The results shown in B and C are the means of three independent biological experiments and at least three technical replicates. Standard deviations are indicated, and asterisks represent significant differences compared with WT (Student's *t* test, \*\**p*-value < 0.005).

was quantified, and we observed that the production of this organic acid by *B. cinerea* was pH dependent (induced at pH 7.0) as previously described for *Sclerotinia sclerotiorum* (Rollins, 2003). Moreover, the mutant strain showed a 90% defect when compared with WT (Fig. 4B; Supporting Information Fig. S4). In parallel, expression of the oxaloacetate hydrolase-encoding gene [*BcoahA* (Bcin12g01020.1)] involved in OA biogenesis was measured. Higher expression was recorded at pH 7.0 than at pH 5.0 in the WT strain, while very low expression was measured in the mutant strain under both pH conditions (Fig. 4C).

#### *BcPacC* is involved in ROS production

During the early phase of the plant-fungus interaction, reactive oxygen species (ROS) are considered of key importance because they play a role in fungal colonization as well as in plant defence (for a review, see Siegmund and Viefhues, 2016). To investigate the capacity of the mutant to produce ROS, the WT, the *BcpacC* deletion and complemented mutant strains were grown on complete medium and equal amounts of biomass were incubated in diaminobenzidine (DAB) solution as described by Schumacher and colleagues (2014). The



**Fig. 5.** Effect of *BcpacC* deletion on ROS production and response to oxidative stress. A.  $H_2O_2$  production in mycelium grown for 3 days on complete medium buffered at pH 6.0. Control wells containing 1 ml of DAB solution and 1  $\mu$ l of the indicated dilutions of  $H_2O_2$  were incubated in the presence of horseradish peroxidase (HRP) under the same conditions used for the tested strains. B. Radial growth of the WT and  $\Delta BcpacC$  strain in the presence of 500  $\mu$ M menadione or 10 mM  $H_2O_2$ . All results are the means of three independent biological experiments and five technical replicates. Standard deviations are indicated, and asterisks represent significant differences compared with the results obtained on non-supplemented complete medium (CM) (Student's *t* test, \**p*-value < 0.05).

intensive brown colouration observed with the mutant indicated an increased accumulation of H<sub>2</sub>O<sub>2</sub> compared with the WT or complemented strains (Fig. 5A) and suggested a negative role of PacC in the ROS production. Considering the plant-fungus interaction, primary leaves of *Phaseolus vulgaris* were inoculated with *B. cinerea* and coloured with DAB (Schumacher *et al.*, 2014). A brown coloration typical of H<sub>2</sub>O<sub>2</sub> accumulation could be detected in plant tissues infected with the WT strain as early as 24 hpi and maintained during the infectious process. (Supporting Information Fig. S5). A more intense brown coloration typical of H<sub>2</sub>O<sub>2</sub> accumulation appeared around the small and arrested regions of necrosis caused by the mutant after 6 days of incubation. However, comparing different sized lesions was difficult and we cannot exclude a stronger HR response by the host in presence of the mutant. To complete this study, we placed the WT and mutant strains under oxidative stress *in vitro* (H<sub>2</sub>O<sub>2</sub> (10 mM) or menadione (500 µM)) and observed no significant difference in fungal growth (Fig. 5B). Altogether, these results suggest that BcPacC is not required to resist oxidative stress, but would play a negative role in the production of ROS by *B. cinerea*.

#### *Role of BcPacC in the production of the exoproteome of B. cinerea*

As secreted proteins are considered key determinants of the necrotrophic strategy of *B. cinerea*, the exoproteomes of the WT and the *BcpacC* deletion mutant were explored. The two strains were grown on complete solid medium on the surface of cellophane sheets and then transferred for 24 h onto minimal liquid medium buffered at pH 5.0 or 7.0. The proteins of three independent biological replicates were collected from the liquid phases and analysed by mass spectrometry. Altogether, 272 and 360 proteins were identified with a minimum of two unique peptides in the WT and mutant strains respectively. A total of 398 different proteins were detected. Using SignalP and SecretomeP software (Bendtsen *et al.*, 2004a,b), 263 of these proteins were classified as conventional secreted proteins (presence of a predicted signal peptide), and 84 as proteins putatively secreted through a non-classical secretory pathway (SecretomeP score > 0.5). According to their predicted function, the secreted proteins were classified into 13 functional categories with biological relevance to fungal development or infection (Supporting Information Table S3 and Fig. S6).

In the WT strain, 82 proteins were filtered (ANOVA test, *p*-value < 0.05; Supporting Information Table S3) to analyse the effect of pH on the fungal secretome. Twenty-nine of these proteins were up-accumulated at pH 5.0, while 52 were more abundant at pH 7.0. No significant change between pH 5.0 and 7.0 was observed

for one protein only, indicating that pH has a major influence on the secretome (pie chart in Fig. 6). The predicted function of these proteins (15 could not be associated with a known function) and their distribution indicated the occurrence of plant cell wall polysaccharide (PCWP) degradation as well as protein and lipid degradation and oxidoreduction (Fig. 6). Proteins involved in the degradation of cellulose, hemicellulose and other polysaccharides, oxidoreduction and cellular control were more abundant at pH 7.0 than at pH 5.0. Conversely, functional categories corresponding to pectin, lipid degradation and metabolism were characterized by more proteins at pH 5.0 than at pH 7.0.

In the mutant strain, 205 proteins were filtered (ANOVA test, *p*-value < 0.05; Supporting Information Table S3), of which 72 were up-accumulated at pH 5.0 and 125 were up-accumulated at pH 7.0 and 8 similarly to pH 5.0 and 7.0 (Fig. 6; pie chart). These proteins were distributed into the same functional categories presented above, but they exhibited a different pattern from that of WT (Fig. 6). Of the 30 proteins involved in PCWP degradation detected in WT, 9 (30%) were up-accumulated under acidic conditions and 21 (70%) up-accumulated at pH 7.0. These proportions varied in the mutant, as 27 proteins (55%) of the 49 detected proteins related to PCWP degradation were up-accumulated at pH 5.0 and 22 proteins (45%) at pH 7.0. Similarly, the categories 'protein degradation' and 'lipid degradation' were characterized by more proteins detected at acidic pH in the mutant. These results indicate that the mutant exhibits an acidic-mimicking phenotype. However, this observation could not be extended to all the functional categories analysed. For example, the abundance of proteins involved in oxidoreduction was particularly more elevated at pH 7.0 (18 proteins) than at pH 5.0 (six proteins) in the mutant, and the same was noted for proteins classified as 'cellular control or component' and 'metabolism'. Finally, 31 proteins detected in the mutant could not be associated with a known function, and most of them were also up-accumulated at pH 7.0 (Fig. 6). To estimate the fraction of proteins that accumulated in a manner dependent on pH and PacC, we considered the 39 proteins with a significant *p*-value (< 0.05) in both the WT and mutant strains (Fig. 7 and Supporting Information Table S3). Most of these proteins (22) accumulated differentially at pH 5.0 and 7.0 in the WT strain (fold change > 2) and revealed a different accumulation profile in the mutant strain, suggesting a PacC-dependence of the production of these proteins (Fig. 7). For example, the acid protease (Bcin15g02380.1) accumulated 46.3-fold more at pH 5.0 than at pH 7.0 in the WT strain and only 6.4-fold more in the mutant strain. Similarly, a metalloproteinase (Bcin11g00300.1) was 55.6-fold more abundant at pH 7.0 than at pH 5.0 in the WT strain and only 15.6-fold

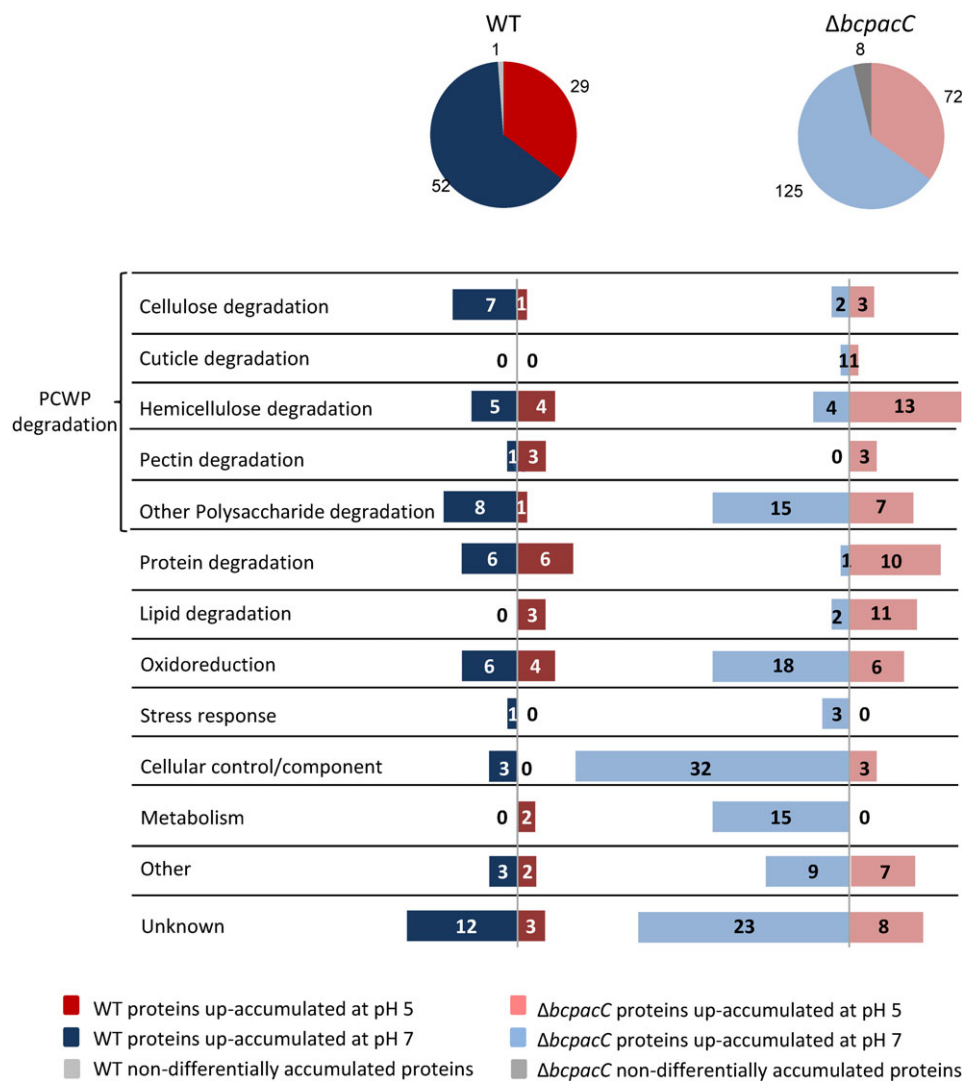
more abundant in the mutant strain. The peroxiredoxin-5 (Bcin10g01030.1) also constitutes a good example of a protein affected by the deletion of *BcpacC* as it showed 1587.3-fold greater accumulation at pH 7.0 than at pH 5.0 in the WT strain and only 37.0-fold greater accumulation in the mutant strain. A cupredoxin (Bcin13g02010.1) was over-accumulated at pH 5.0 in the WT strain, but showed no pH-dependence in the mutant strain. Finally, a hemicellulase (Bcin11g01370.1) exhibited an opposite accumulation profile between the WT and mutant strains as it over-accumulated at pH 7.0 in the WT strain and exhibited an excess accumulation under acidic conditions in the mutant strain (Fig.7). Taken together, these results indicate that BcPacC can positively or negatively influence the relative abundance of

several proteins that are secreted under acidic or neutral pH conditions. Moreover, we observed that proteins belonging to the same functional category could be regulated differentially by PacC (Fig. 7).

Finally, 16 secreted proteins exhibited a pH-dependent accumulation profile in the WT strain (4 up-accumulated at pH 5.0 and 12 up-accumulated at pH 7.0), and this profile was not affected by the deletion of *BcpacC* (Fig. 7; Supporting Information Table S3), suggesting the presence of a pH regulation that would be independent of PacC.

## Discussion

The ability to adapt to and thrive in a broad range of environmental pH conditions is a hallmark of fungal biology.



**Fig. 6.** Exoproteome analysis. Distribution of up-accumulated proteins of the WT and  $\Delta BcpacC$  strain according to ambient pH (pie charts), and distribution of proteins according to their functional classification (histograms). The two strains were grown on complete solid medium on the surface of cellophane sheets and then transferred for 24 h onto minimal liquid media buffered at pH 5.0 or pH 7.0. PCWP: plant cell wall polysaccharides. All results are the means of three independent biological replicates and two for WT at pH 7.0. Proteins were identified with at least two unique peptides ( $p$ -value < 0.05 and fold change > 2).



## PACC dependent secreted proteins

Functional Classification	Ratio pH5/pH7 WT	Ratio pH5/pH7 $\Delta bcpacC$	Gene ID	Description
Cellulose degradation	7,217	3,531	Bcin04g03510.1	barwin-like endoglucanase
Hemicellulose degradation	0,018	0,327	Bcin05g01660.1	glucan 1,3-beta-glucosidase precursor
	0,083	5,106	Bcin11g01370.1	beta-1,3-exoglucanase
	2,459	14,641	Bcin09g04440.1	alpha-L-arabinofuranosidase A precursor
	7,042	16,752	Bcin03g03840.1	glucan 1,3-beta-glucosidase precursor
	11,607	2,973	Bcin09g00190.1	mannan endo-1,6-alpha-mannosidase precursor
Other Polysaccharide degradation	0,310	0,091	Bcin07g02860.1	glycoside hydrolase
Pectin degradation	3,349	27,014	Bcin10g06130.1	polygalacturonase
Protein degradation	0,018	0,064	Bcin11g00300.1	metallopeptidase, catalytic domain
	2,266	6,468	Bcin01g06210.1	carboxypeptidase S1
	32,734	6,595	Bcin12g02040.1	rhizopuspepsin-2 precursor
	46,273	6,440	Bcin15g02380.1	acid protease
Lipid degradation	2,780	7,462	Bcin01g07230.1	neutral ceramidase precursor
	3,986	10,361	Bcin06g07020.1	acetylcholinesterase
Oxidoreduction	0,001	0,027	Bcin10g01030.1	peroxiredoxin-5, mitochondrial precursor
	0,010	0,135	Bcin03g07730.1	tyrosinase
	0,075	0,020	Bcin16g01700.1	riboflavin-aldehyde forming enzyme
	0,215	0,098	Bcin03g01540.1	choline dehydrogenase
	2,681	1,833	Bcin13g02010.1	cupredoxin
	0,002	0,017	Bcin02g01060.1	nucleoside diphosphate kinase
Cellular control/component	0,074	0,035	Bcin12g01360.1	peptidyl-prolyl cis-trans isomerase
	0,179	0,039	Bcin06g01000.1	spherulin 4-like cell surface protein
	0,522	0,307	Bcin02g07630.1	predicted protein

## PACC non-dependent secreted proteins

Functional Classification	Ratio pH5/pH7 WT	Ratio pH5/pH7 $\Delta bcpacC$	Gene ID	Description
Cellulose degradation	0,118	0,104	Bcin12g06630.1	endoglucanase-5, (1,4)-beta-D-glucosidic linkage
Other Polysaccharide degradation	0,018	0,021	Bcin07g03770.1	glycoside hydrolase
	0,045	0,026	Bcin16g01590.1	endochitinase precursor
	0,029	0,033	Bcin06g04830.1	beta-1,6-glucanase precursor
	0,052	0,037	Bcin02g02410.1	glycoside hydrolase
	0,125	0,195	Bcin01g05720.1	glycoside hydrolase/deacetylase, $\beta/\alpha$ -barrel
Protein degradation	5,343	9,12	Bcin10g01890.1	tripeptidyl-peptidase 1 precursor
Oxidoreduction	4,862	3,581	Bcin06g01930.1	glyoxal oxidase precursor
	8,025	8,941	Bcin12g01520.1	glyoxal oxidase precursor
Stress response	0,071	0,103	Bcin03g06600.1	heat shock protein
Other	0,025	0,037	Bcin01g06450.1	enolase allergen asp f 22
Unknown	0,01	0,014	Bcin08g01550.1	predicted protein
	0,031	0,028	Bcin12g04930.1	predicted protein
	0,019	0,036	Bcin02g04350.1	predicted protein
	0,1	0,1	Bcin08g03320.1	extracellular conserved serine-rich protein
	5,37	6,151	Bcin05g01510.1	predicted protein

<0,01	0,01-0,05	0,05-0,1	0,1-0,5	0,5-2	2-5	5-10	>10
Protein up-accumulated at pH 7				Protein non-differentially accumulated		Protein up-accumulated at pH 5	

**Fig. 7.** BcPacC-dependent and non-dependent secreted proteins. The WT and  $\Delta BcpacC$  strains were grown on complete solid medium on the surface of cellophane sheets and then transferred for 24 h onto minimal liquid media buffered at pH 5.0 or pH 7.0. A colour code was used to visualize the up-accumulation of proteins according to the ratios pH 5.0/pH 7.0. Comparing these ratios between the two strains, a fold change > 2 indicated PacC-dependence. Ratio pH 5/pH 7 WT: ratio between the average protein abundances for B05.10 strain at pH 5.0 and 7.0. Ratio pH 5/pH 7  $\Delta BcpacC$ : ratio between the average protein abundances for  $\Delta BcpacC$  strain at pH 5.0 and 7.0. All results are means of three independent biological experiments and two for WT at pH 7.0. Proteins were identified with a minimum of two unique peptides ( $p$ -value < 0.05 and fold change > 2).

This is particularly important in the case of pathogenic fungi, which can modify the pH of contaminated tissues as an attack strategy. The grey mould fungus *B. cinerea* appears to be an interesting model to study the relationship between ambient pH and infection because it is able to colonize plants with acidic or neutral tissues (Manteau *et al.*, 2003) and to modulate the ambient pH during the infection process by secreting either organic acids or ammonia (Billon-Grand *et al.*, 2012). This capacity to modify the ambient pH during host colonization suggests an efficient mechanism controlling the production and secretion of virulence factors regardless of the pH encountered by the pathogen. In the present study, we investigated the role of the pH regulator PacC in the biology of *B. cinerea*.

The signalling pathway for pH sensing and adaptation in fungi consists of the products of six *pal* genes and one *pac* gene. All these genes are present in the genome of *B. cinerea*, and expression analysis showed that all of them were functional and that *BcpacC* was over-expressed under neutral conditions, reinforcing the established strong conservation of the fungal pH signalling pathway and its transcriptional regulation (Peñalva *et al.*, 2014).

In the absence of PacC, *B. cinerea* exhibits a significant defect in radial growth on complex media at a pH equal or greater than 6.0. This phenotype has been previously reported in other pathogenic fungi, such as the white mould *S. sclerotiorum* (Rollins, 2003; Kim *et al.*, 2007), rice blast pathogen *Magnaporthe oryzae* (Landraud *et al.*, 2013), post-harvest pathogen *Penicillium digitatum* (Zhang *et al.*, 2013), and insect pathogen *Metarhizium robertsii* (Huang *et al.*, 2015). The absence of BcPacC also causes a defect in conidial production and sclerotium formation, and one or both of these traits has also been reported in the *pacC* deletion mutants of the aforementioned species, *S. sclerotiorum*, *M. oryzae*, *P. digitatum* and *M. robertsii*. The redundancy of *pacC* genes in fungi belonging to different genera and the common phenotypes characterizing several *pacC* deletion mutants could indicate the existence of a regulatory pathway implicating PacC and controlling genes involved in fundamental biological processes, such as fungal growth or asexual reproduction. The connection between pH, PacC and these processes awaits molecular characterization.

PacC has been shown to regulate fungal virulence towards mammalian, plant, insect and fungal hosts (Cornet and Gaillardin, 2014; Rollins, 2003; Kim *et al.*, 2007; Alkan *et al.*, 2013a, 2013b; Landraud *et al.*, 2013; Zhang *et al.*, 2013; Huang *et al.*, 2015; Lou *et al.*, 2015). In most cases, the absence or reduced production of PacC leads to a partial to total absence of pathogenicity, but increased virulence has also been reported in

*Fusarium* species (Caracuel *et al.*, 2003; Merhej *et al.*, 2011). In the present study, the *BcpacC* deletion mutant exhibited a defect in virulence depending on the pH of the host tissues. The mutant only produced reduced primary lesions on neutral (pH equal or greater than 6.0) plant tissues, such as leaves, cotyledons or zucchini fruits. In the closest relative, *S. sclerotiorum*, the decreased virulence of the *pacC* deletion mutant is associated with a defect in OA production (Godoy *et al.*, 1990; Kim *et al.*, 2007), likely due to impaired regulation of the oxaloacetate hydrolase-encoding gene (*Ssoah1*) by SsPacC (Liang *et al.*, 2015). A parallel can be drawn in *B. cinerea* since the expression of *Bcoah*, the *Ssoah1* orthologue, and the production of OA dropped when the *BcpacC* gene was deleted. However, we must consider the multifunctional role of OA during the interaction between pathogens and plants. It has been previously shown in *S. sclerotiorum* that this acid suppresses host defences by manipulating the host redox environment, including suppression of the oxidative burst (Williams *et al.*, 2011). The same phenomenon appears to occur in *B. cinerea*, as infection of bean leaves or cucumber cotyledons with the *pacC* deletion mutant revealed a hypersensitive-like response that was not observed on leaves or cotyledons inoculated with WT. These limited lesions were characterized by a marked accumulation of H<sub>2</sub>O<sub>2</sub> detected by DAB coloration. However, we have also to take in consideration that the fungus produces ROS that participate in fungal colonization by damaging plant tissues. The capacity of the mutant to secrete ROS was investigated *in vitro*, and the higher ROS production by the mutant compared with the WT strain suggested a negative role of PacC in the production of these molecules. Considering all these findings, we suppose that the H<sub>2</sub>O<sub>2</sub> accumulation observed in bean leaves several days after infection by the mutant resulted in part from an overproduction of ROS by the fungus and ROS accumulation by the plant due to the absence of OA. In addition, the proteomic analysis revealed that, under neutral pH conditions, the mutant accumulated more proteins that contribute to the oxidative burst or participate to the oxidative stress response to cope with ROS (see Supporting Information Table S3). We could imagine that the mutant adopts the same behaviour during colonization of neutral tissues.

*Botrytis cinerea* secretes a large set of enzymes to degrade plant tissues, and several proteomic approaches have highlighted the significant changes in the composition of its secretome in response to different environments and hosts (Li *et al.*, 2012; Blanco-Ulate *et al.*, 2014; González-Fernández *et al.*, 2015). The *BcpacC* deletion strain secreted a reduced number of proteins involved in cellulose, hemicellulose, pectin and protein degradation in comparison to the WT strain when grown

*in vitro* at pH 7.0. In hosts exhibiting neutral tissues, the mutant strain that is affected in its ability to produce OA and acidify its environment, might therefore secrete less of these enzymes necessary for plant tissue degradation. As a consequence, penetration and progression through the host might be impaired and fungal growth would be reduced, as observed *in vitro*.

The virulence defect described in hosts characterized by neutral tissues was not observed in acidic plant tissues, such as apple or tomato fruits, suggesting that PacC is rather a key regulator of pH adaptation than a key regulator of virulence in *B. cinerea*. Our results also showed that acidic ambient pH was more critical to virulence than OA production in this fungus. Similar conclusions have been drawn from the results obtained by Xu and colleagues (2015) in *S. sclerotiorum* since the *Ssoah* mutant, in which OA production is affected, could colonize hosts with a low pH or weak buffering capacity, but failed to colonize tissues characterized by a neutral pH. Xu and colleagues suggested that low pH and not OA itself establishes the necessary conditions for disease development. Indeed, acidification of the host tissue with citric acid-phosphate buffer significantly increased virulence, in contrast to supplementation of oxalate at the site of infection without buffering.

A previous transcriptomic analysis of grape berry (pH 3–4) infection by *B. cinerea* revealed weak expression of the *BcpacC* gene (Kelloniemi *et al.*, 2015), which we confirmed *in vitro*. This finding is consistent with the proposed role of PacC in fungal adaptation to neutral and alkaline pH and not to acidic pH, and it could explain, in part, the non-affected virulence phenotype of the *BcpacC* mutant on apple, tomato or grape berry tissues. In contrast to our results, Zhang and colleagues have shown that PacC can be considered as a key regulator of the virulence of *P. digitatum*, the most important post-harvest pathogen of citrus fruits (Zhang *et al.*, 2013). Although the authors have shown that *PdpacC* is an alkaline pH-induced gene under *in vitro* conditions, this gene is highly expressed during infection of citrus fruits (acidic tissues), suggesting that the composition of nutrients present in the medium affects the expression of this gene. In *Penicillium expansum*, the causal agent of blue mould rot, downregulation of *pacC* expression in *pacC*-RNAi transformants also leads to a reduction of disease development in *Golden delicious* apple fruits (Barad *et al.*, 2014). Further investigation revealed that ammonia accumulation by the pathogen under acidic conditions could be a cue for *pacC* activation towards the accumulation of pathogenicity factors such as patulin accumulation (Barad *et al.*, 2016). Fungal fruit pathogens, therefore, seem to vary in their infectious biology, and the role of PacC would have evolved accordingly.

Finally, the investigation of the secretome performed herein revealed the existence of several secreted pH-regulated proteins that were not dependent on PacC. Some of these proteins involved in cellulose or other PCWP degradation were upregulated at pH 7.0, whereas others, such as a tripeptidyl peptidase or two glyoxal oxidases (H<sub>2</sub>O<sub>2</sub> generating enzymes), were upregulated under acidic conditions. Several transcriptional analysis revealing the existence of genes pH-regulated independently of PacC have been previously described in several saprophytic (Kunitake *et al.*, 2016), mycotrophic (Trushina *et al.*, 2013) and phytopathogenic fungi (Franco-Frias *et al.*, 2014). For example, in *Trichoderma reesei*, several genes encoding cellulose and hemicellulose degrading enzymes respond to change of pH but are not dependent on PacC, suggesting the presence of other regulatory processes (Häkkinen *et al.*, 2015).

In conclusion, although this regulator seems to play a minor role in *B. cinerea* virulence towards host plants with an acidic pH, its presence seems to be required for infection of all other host plants. Taken together, our findings highlight the important regulatory position of PacC in the biology of *B. cinerea*, controlling not only fungal growth, but also production of OA and several enzymes involved in hydrolysis of plant cell wall components or production of ROS.

## Experimental procedures

### *Fungal strains, growth conditions and phytopathogenicity assays*

*Botrytis cinerea* (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) strain B05.10 was maintained on complete solid medium (Pontecorvo *et al.*, 1953) or on solid sporulation medium, as previously described (Antal *et al.*, 2012) and was used as a recipient strain for genetic modifications.

For DNA preparations, the mycelium was grown on solid sporulation medium for 3 days on the surface of cellophane membranes inoculated with 4-mm mycelial plugs. For quantification of pH signalling-related gene expression by real-time quantitative polymerase chain reaction (qRT-PCR), spores were spread on the surface of cellophane membranes deposited onto solid sporulation medium and grown for 3 days. The membranes were then transferred for 4 h onto the surface of 2 ml of Gamborg medium (Gamborg *et al.*, 1968) buffered at pH 3.0, 4.0, 5.0, 6.0 or 7.0. Infected plant tissues were collected after inoculation of 1-week-old sunflower (*Helianthus annuus*) cotyledons with 4-mm mycelial plugs. The infected plant material was incubated at 21 °C under 100% relative humidity and dark (10 h)-daylight (14 h) conditions. At different stages of symptom development,

cotyledons were harvested and frozen at  $-80\text{ }^{\circ}\text{C}$ . Infection assays were also performed with 1-week-old cucumber (*Cucumis sativus*) cotyledons, apple var 'Golden delicious', cherry tomatoes (*Solanum lycopersicum* var *cerasiforme*) zucchini (*Cucurbita pepo*) fruits and French bean (*P. vulgaris* var *Saxa*) leaves using 4-mm plugs collected from 72-h-old mycelial sporulation medium. For visualization of ROS in infected French bean leaves, 10  $\mu\text{l}$  droplets of conidia were used. The pH values of the plant tissues were measured with a flat contact electrode (Fisher-Bioblock Scientific, France) placed directly either on the surface of non-infected plant tissues or on the lesion centre for infected tissues. pH values were recorded after 2 min. For quantification of OA and *BcoaH* gene expression,  $10^3$  spores were inoculated on the surface of cellophane placed on solid sporulation medium. After 3 days at  $21\text{ }^{\circ}\text{C}$ , the membranes were transferred for 4 h onto the surface of 2 ml of Gamborg B5 liquid medium (Duchefa Biochemie) containing glucose (0.5%) and buffered at pH 5.0 or 7.0 (Tris/maleate 0.2 M). Three independent biological replicates were assessed. For the secretome analysis, the same protocol was used except that the membranes were transferred for 24 h onto the surface of 2 ml of Gamborg B5 liquid medium containing glucose (0.1%) and buffered at pH 5.0 or 7.0. Three independent biological replicates were assessed.

#### Construction of *BcpacC* mutants

Knock-out mutants were constructed using a gene replacement strategy (Supporting Information Fig. S2 and Appendix S1).

#### Expression profiling by quantitative PCR analysis

Experiments were performed as described by Billon-Grand and colleagues (2012). DNA-free total RNA (2.5  $\mu\text{g}$ ) was used for the synthesis of cDNA using the Thermoscript™ RT-PCR system kit (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. qRT-PCR experiments were performed in 96-well plates using ABI-7900 Applied Biosystems (Applied Biosystems, USA). Primer pairs (Supporting Information Table S1) were designed using Primer Express (Applied Biosystems, USA). After evaluating the primers efficiencies, the amplification reactions were carried out using SYBR Green PCR Master Mix (Applied Biosystems, USA) with the following protocol:  $95\text{ }^{\circ}\text{C}$  for 10 min and 40 cycles of  $95\text{ }^{\circ}\text{C}$  for 30 s and  $60\text{ }^{\circ}\text{C}$  for 1 min. Relative quantification was based on the  $2^{-\Delta\text{Ct}}$  method (Avrova *et al.*, 2003) using the actin-encoding gene (*Bcin16g02020.1*) as a normalization internal control. Three independent biological replicates were analysed.

#### Phenotypic tests

Radial growth was measured daily following inoculation of solid malt medium (Billon-Grand *et al.*, 2012) buffered at pH 5.0, 6.0 or 6.5 with 4-mm mycelial plugs and incubation at  $21\text{ }^{\circ}\text{C}$  in the dark. Conidial production was analysed by counting the conidia under a microscope after collecting them from 11-day-old cultures on solid malt medium incubated at  $21\text{ }^{\circ}\text{C}$  under continuous near-UV light. Sclerotium formation was observed after 3 weeks of culture on complete medium at  $21\text{ }^{\circ}\text{C}$  in the dark. Medium acidification was tested by growing the strains on solid complete medium adjusted to pH 8.0 and supplemented with bromothymol blue (yellow at  $\text{pH} < 6$ ).  $\text{H}_2\text{O}_2$  production was analysed according to Schumacher and colleagues (2014) using DAB. Following 3 days of culture on cellophane membranes overlaying complete medium buffered at pH 6.0, 25 mg of fresh mycelium was incubated for 2 h in DAB solution. Sensitivity to oxidative stress was determined by measuring radial growth after 72 h on complete medium plates buffered at pH 5.0 or pH 6.0 and supplemented with 10 mM  $\text{H}_2\text{O}_2$  or 500  $\mu\text{M}$  menadione (Schumacher *et al.*, 2014). For each test, three independent biological experiments were performed. To visualize ROS in infected French bean, leaves contaminated with conidia were detached from plants and incubated in DAB solution (0.05% in 100 mM citric acid, pH 3.7) for 2 h to detect  $\text{H}_2\text{O}_2$ . They were then discoloured after transfer for 5 min in boiling ethanol and observed under a stereomicroscope.

#### Quantification of secreted OA

Mycelial plugs were inoculated on the surface of cellophane overlaying complete solid medium. Following 72 h at  $21\text{ }^{\circ}\text{C}$  in the dark, the cellophane sheets were transferred for 4 h onto the surface of Gamborg liquid medium buffered at pH 5.0 or pH 7.0. The liquid medium was collected for oxalic quantification using high performance liquid chromatography (HPLC). A Hypersyl GOLD aQ (250 mm  $\times$  4.6 mm) was used with a particle size of 5  $\mu\text{m}$  (ThermoFisher, France). OA was separated using the following elution protocol with solvents A and B composed of 50 mM  $\text{KH}_2\text{PO}_4$  (in milliQ water) adjusted to pH 2.65 with  $\text{H}_3\text{PO}_4$  and acetonitrile (or methanol), respectively, all of which were HPLC grade. Separation was carried out at  $25\text{ }^{\circ}\text{C}$  at a flow rate of  $1\text{ ml}\cdot\text{min}^{-1}$  under the following conditions: pre-equilibration for 10 min in buffer A before injection, 0 min, 100% A; 8 min, 100% A, 10 min, 100% B; 12 min, 100% B; 13 min 100% A; 15 min, 100% A before a new run as described. The separation procedure was performed with an Alliance HT Waters 2795 separation module, and detection as carried out using a Waters 2996 photodiode array detector (Waters, France).



A wavelength of 210 nm and spectrum from 210 to 400 nm were utilized for detection. Under our conditions, OA was eluted at a retention time of 3.13 min. In the culture medium runs, the identity of OA was evaluated by co-injection with a solution of OA. Quantification was performed by measuring peak areas using the Mass-Link 4.1 software (Waters, France). Peak areas were then compared with a standard curve of OA to measure the amount in each sample in milligrams per millilitre. The linear standard curve was obtained by increasing the injected quantity of OA to determine the slope as a correction factor. The slope was  $9.E^{+7}$  ( $R^2$  of 0.98), and the final results are expressed as millimoles per gram dry mycelium.

#### Secretome analysis

To perform the secretome analysis, the fungal strains were grown for 3 days on complete solid medium on the surface of cellophane membranes inoculated with 4-mm mycelial plugs and then transferred for 24 h onto minimal liquid medium buffered at pH 5.0 or pH 7.0. Three independent biological replicates were assessed for each condition. Secreted proteins were recovered in the supernatant of the collected liquid medium centrifuged at 4 °C for 15 min at 5000g. Proteins were precipitated using trichloroacetic acid (TCA). Briefly, a 1/10 volume of 100% (w/v) TCA (Sigma, France) was added to the supernatant and stored at 4 °C for 12 h. Proteins were pelleted by centrifugation at 4 °C for 15 min at 14 000g and washed three times with acetone pre-cooled at -20 °C. The pellet was suspended into Laemmli sample buffer (Laemmli, 1970), and the proteins were separated by SDS-PAGE using the Laemmli buffer system. After migration, the proteins were visualized by staining with InstantBlue, Expedon (Sigma, France) and scanning with the ChemiDoc XRS camera (Bio-Rad, France). The proteins were quantified with Image Lab Software v5.0 (Bio-Rad, France) according to manufacturer's recommendations before shotgun analysis. For shotgun analysis, see Appendix S1.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Table S1. Accession numbers and homology of *Botrytis cinerea* pac/pal genes of different filamentous fungi (*Sclerotinia sclerotiorum*, *Aspergillus nidulans*, *Magnaporthe oryzae*, *Fusarium oxysporum*, *Colletotrichum graminicola*, *Trichoderma reesei*).**

**Table S2. Oligonucleotides used in this study.**

**Table S3. List of proteins (identified with at least two unique peptides) detected in the exoproteomes of the WT and  $\Delta BcpacC$  strains after transfer onto minimal media buffered at pH 5.0 and 7.0.** For quantification, all unique peptides of an identified protein were included, and the total cumulative abundance was calculated by summing the abundances of all peptides allocated to the respective protein. ANOVA test was applied at the protein level and filtered at  $p < 0.05$ . A greater than 2-fold decrease in abundance at pH 5.0 compared with pH 7.0 is indicated by blue shading. A greater than 2-fold increase in abundance at pH 5.0 compared with pH 7.0 is indicated by pink shading. Three independent biological replicates were conducted and analysed for each condition except for WT pH 7.0 (two replicates could be analysed). n.d. not determined.

**Fig. S1. Expression of the *B. cinerea* pal genes during sunflower cotyledon infection.** Total mRNA was extracted, and quantitative PCR analysis was carried out using specific primers (Table S2). The actin-encoding gene was used as a reference. Three independent biological replicates were analysed for each gene. Standard deviations are indicated. For each gene, asterisks indicate a significant difference (Student's *t* test, \*  $p$ -value  $< 0.05$ , \*\*  $p$ -value  $< 0.005$ ) in gene expression compared with the expression at pH 5.0.

**Fig. S2. Construction and identification of *B. cinerea* BcPacC null mutants and complemented strains.**

(A) Schematic representation of *BcpacC* gene replacement by the nourseothricin resistance gene (*nat*) flanked by 1,083 kb of 5' and 1,126 kb of 3' sequences from the *BcpacC* locus. Primers (black arrows; Table S1) used for PCR analysis and the probe used for Southern analysis are indicated. To confirm that the phenotypes observed in the mutants were caused by the deletion of *BcpacC*, the  $\Delta pacC$ -11 mutant was transformed with a complementation construct containing the full-length WT *BcpacC* gene and a selection cassette conferring resistance to hygromycin B. (B) Diagnostic PCR to identify the deletion mutants. Primers pairs are indicated. (C) Southern analysis of the wild type strain (WT: B05.10) and the  $\Delta pacC$ -11 transformant. Genomic DNA was digested with EcoRI (E). Hybridization with a specific probe confirmed the presence of a unique fragment exhibiting a different size from the WT strain signal. The absence of WT signal in the  $\Delta pacC$ -11 transformant confirmed the homokaryotic state. (D) Diagnostic PCR to identify  $\Delta pacC$ -11 complemented transformants. Primers pairs are indicated.

**Fig. S3.** (A) French bean leaves after 7 days of infection by WT B05.10,  $\Delta BcpacC$  and  $c\Delta BcpacC$  strains. Black arrows indicate control plant infected by WT. (B) pH evolution of the necrosis zone during infection of different hosts. From top to bottom: cucumber cotyledons, zucchini fruits, French bean leaves, apple, cherry tomatoes.

**Fig. S4. High-performance liquid chromatography profiles of culture filtrates of *B. cinerea* strains.** The strains were cultivated on the surface of cellophane membranes placed on solid sporulation medium for 3 days and then transferred for 4 h onto the surface of liquid Gamborg medium buffered at pH 5.0 (top) or 7.0 (bottom). Oxalic acid was used as a chemical standard and was eluted at a retention time of 3.28 min under the conditions used.

**Fig. S5. H<sub>2</sub>O<sub>2</sub> production during infection.** French bean leaves were infected with WT,  $\Delta BcpacC$  and  $c\Delta BcpacC$  conidia then detached after 3 and 6 days and incubated for 2 h in DAB solution. Leaves were then discoloured and observed under stereomicroscope. Six days after inoculation, leaves infected by WT and complemented strain were fully necrosed and could not be collected (X). Black bars represent 4 mm.

**Fig. S6. Global distribution in 13 functional classes of proteins secreted by *B. cinerea* and  $\Delta BcpacC$  strains in minimal liquid media buffered at pH 5.0 and 7.0.** The results are expressed as the percentage of total accumulated proteins identified with a minimum of two peptides.