Disruption of *Botrytis cinerea* Pectin Methylesterase Gene *Bcpme1* Reduces Virulence on Several Host Plants

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The pectinolytic enzyme pectin methylesterase (PME) hydrolyses pectin in methanol and polygalacturonic acid. In the expressed sequence tag library of *Botrytis cinerea* T4, we identified a 1,041 bp *Bcpme1* cDNA potentially encoding a 346-amino acid protein of 37 kDa showing 46.8% identity with *Aspergillus* sp. PMEs. *Bcpme1* is a single copy gene and is similarly expressed in glucose and pectin containing media. To evaluate the role of *Bcpme1* in *Botrytis cinerea* virulence, a mutant in *Bcpme1* was generated by gene disruption. The *Bcpme1* mutant showed similar growth on rich medium but reduced growth on pectin medium. Two isozymes of pI 7.4 and 7.1 were detected in pectin liquid-culture supernatants of wild-type strain Bd90 analyzed by isoelectric focusing–polyacrylamide gel electrophoresis, while those of *Bcpme1* mutant possessed only the pI 7.1 isozyme. BCPME1, the pI 7.4 isozyme, is the major PME activity, as PME activity is 75% reduced in *Bcpme1* mutant. Moreover, the *Bcpme1* mutant was less virulent on apple fruits, grapevine, and *Arabidopsis thaliana* leaves. Those phenotypes were complemented by reintroducing a *Bcpme1* copy in the *Bcpme1* mutant. These results showed that *B. cinerea* possessed more than one PME-encoding gene and that BCPME1 is an important determinant of *B. cinerea* virulence.

While invading their hosts, many phytopathogenic bacteria and fungi synthesize extracellular enzymes that degrade pectin, the major component and the most complex polysaccharide in the plant cell wall, particularly abundant in the middle lamella. Pectin consists of “hairy” and “smooth” regions. The hairy region, known as rhamnogalacturonan, is characterized by stretches of α -1-2-L-rhamnose and α -1-4-D-galacturonic acid dimers. The smooth region, or homogalacturonan part, consists of a backbone of α -1-4-linked D-galacturonic acid residues, which are partly methylated and can be acetylated. Pathogens produce an arsenal of suitable smooth pectin depolymerizing enzymes: pectin methylesterases (PME, E.C. 1.1.11), which deesterify pectin into methanol and polygalacturonic acid (PGA), allowing subsequent action of polygalacturonases (PG, E.C. 3.2.1.15), which cleave glycosidic bonds by hydrolysis

and pectate lyases (PL, E.C. 4.2.2.2), which break PGA into oligogalacturonides by β -elimination. Pectin lyases (PNL, E.C. 4.2.2.10) also depolymerize native pectin by breaking bonds between methylated galacturonide residues via β -elimination.

The role of these different activities during bacterial or fungal infections has been addressed for several years. Approaches used in initial studies, such as analysis of enzyme activities in culture supernatant or decayed tissues, provided only correlations between the amount of pectinolytic enzymes produced and the extent of symptom development, especially in diseases characterized by soft-rotting symptoms (Alghisi and Favaron 1995). But in the last decade, gene disruption has allowed researchers to determine which attributes could be considered as factors of virulence. Concerning pectinolytic enzymes in filamentous fungi, the majority of studies focused on PGs. Their clear involvement in fungal pathogenicity is still debated. Indeed, several targeted mutants showed no reduction in pathogenicity (Bowen et al. 1995; Gao et al. 1996; Scott-Craig et al. 1998), while others demonstrated that the studied pectinolytic enzyme was essential for fungal attack (Isshiki et al. 2001; Oeser et al. 2002; Rogers et al. 2000; Shieh et al. 1997; Ten Have et al. 2002; Yakoby et al. 2000).

B. cinerea Pers.:F. (teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel), a broad-host-range necrotrophic pathogen, is responsible for many economic losses on fruits, vegetables, and flowers, causing soft-rotting symptoms (Coley-Smith et al. 1980). *B. cinerea* secretes numerous pectinolytic enzymes, especially PGs and PMEs. Ten Have and associates (1998) showed by partial gene replacement that mutants in *Bcpgl*, a gene coding for an endopolygalacturonase, were still pathogenic but that *Bcpgl* was required for full virulence of *B. cinerea*, i.e., for expanding lesions beyond the inoculation point. As endo-PGs are encoded by a multigenic family in *B. cinerea* (Wubben et al. 1999), the abolition of one gene of endo-PG can be compensated by the others.

Hardly anything is known about the contribution of PMEs in filamentous fungi pathogenicity, although evidence for PME activities has been found in many phytopathogenic fungi (Rexova-Benkova and Markovic 1976), including biotrophic fungi such as *Uromyces viciae-fabae* (Frittang et al. 1992). Nevertheless, PME activity may be of major importance for complete degradation of pectin by PGs and PLs, since these enzymes are not able to cleave highly methyl-esterified pectin. Evidence was provided by the disruption of the PMEA encoding gene in the soft-rotting bacterium *Erwinia chrysanthemi*, resulting in a strong reduction in virulence on the African violet *Saintpaulia ionantha* (Beaulieu et al. 1993; Boccara and Châtain 1989).

For all the reasons above, we chose to focus our work on the role of *B. cinerea* PME activity in virulence. In previous studies,

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The *Bcpme1* gene is expressed in glucose and pectin-containing media.

The nucleotidic sequence comparison between polymerase chain reaction (PCR) amplifications on Bd90 genomic DNA and cDNA *Bcpme1* sequence showed the existence of two introns of 133 and 48 bp, respectively (Fig. 1). Gene walking was performed to clone upstream and downstream genomic regions of *Bcpme1* coding sequence. The 5' untranslated region revealed a putative TATA box located at -72 nt and three putative CAAT boxes between -227 and -104 nt (Fig. 1).

The expression of *Bcpme1* gene was studied by semiquantitative reverse transcription (RT)-PCR experiments (discussed below) with RNA extracted from strain Bd90 grown for 48 h in glucose or pectin-containing media. Signal intensities of amplified fragments were very similar (in glucose, $92,764 \pm 5,552$ arbitrary unites (AU) and in pectin, $95,252 \pm 5,463$ AU).

The *Bcpme1* upstream sequence also contained several GATA motifs, which constituted the recognition sequence of major nitrogen regulatory proteins AREA in *A. nidulans* (Kudla et al. 1990) and NIT2 in *Neurospora crassa* (Fu and Mazluf 1990). We tested whether *Bcpme1* could be regulated by nitrogen level in the medium. No difference in signal intensity of amplified fragments was observed between media containing 0.3 mM and 30 mM NO_3^- (data not shown).

The *Bcpme1* gene is a single copy gene.

Southern blot analysis of genomic DNA, isolated from several strains of *B. cinerea* and digested with *Bam*HI, *Eco*RI, or *Hind*III and hybridized with *Bcpme1* cDNA as probe, showed only one hybridizing fragment (Fig. 2). The two hybridized fragments observed with *Bam*HI digestion could be explained by the presence of a *Bam*HI restriction site in the *Bcpme1* sequence. These results suggested that *Bcpme1* was present as a single copy gene in the haploid genome of *B. cinerea* and that a slight polymorphism existed between different strains at this locus.

Construction of a *Bcpme1* interrupted mutant.

A *Bcpme1* mutant was obtained by a transformation-mediated gene disruption in the wild-type strain Bd90. An 831-bp fragment was PCR-amplified with homologous primers (WOD and AFBR) on Bd90 genomic DNA and was cloned in plasmid pAN7-1. The derived plasmid pibcpmel contained the *hph* gene conferring resistance to hygromycin and was used to transform strain Bd90. From one transformation experiment, 30 independent transformants were obtained. Seventeen hygromycin-resistant colonies were analyzed by PCR, using AVWOD and FTTrpC primers to select transformants containing pibcpmel inserted at the *Bcpme1* locus (Fig. 3A). No PCR product was amplified with genomic DNA from strain Bd90, while a 1-kb

amplification product was expected with transformant genomic DNA where insertion of pibcpmel at the *Bcpme1* locus had occurred. Of 17 hygromycin-resistant transformants, 12 transformants for which no amplification was obtained exhibited ectopic insertions of pibcpmel, and four for which the 1-kb product was amplified with AVWOD and FTTrpC primers were disrupted at the *Bcpme1* locus. In one transformant, a fragment of more than 1 kb was amplified, suggesting rearranged insertion at the *Bcpme1* locus (data not shown). Southern analysis of the four disrupted transformants was performed, and mutant B21 was found to be the only homocaryotic transformant with multiple tandem insertions of pibcpmel at the *Bcpme1* locus (Fig. 3B). The three other disrupted transformants after four rounds of monospore isolation still possessed the wild-type copy of the *Bcpme1* gene. Mutant B21 was chosen for further characterization and pathogenicity studies.

Bcpme1 encodes the major PME activity of *B. cinerea*.

PME activity was measured in supernatants from Czapeck-pectin liquid medium. Mutant B21 (PME-specific activity, 0.32 ± 0.12 U per 100 mg mycelial dry weight) showed about 75% PME activity reduction compared with PME activity of wild-type strain Bd90. The same supernatants were subjected to a thin-layer polyacrylamide gel isoelectric focusing (IEF) and were analyzed for PME activities. The two expected activities corresponding to the isozymes of pI 7.1 and 7.4, respectively, were observed in the culture filtrate of wild-type strain Bd90. The supernatant of mutant B21 neither contained the pI 7.4 isozyme nor the pI 6.5 isozyme, indicating that the latter is also encoded by the *Bcpme1* gene (Fig. 4A). The activity of pI 6.5 resulted probably from partial modification of BCPME1.

Extracts from uninfected apple fruits showed a strong basic isozyme of pI 8.4 and a diffuse and faint band corresponding to an isozyme of pI 6.8. IEF-polyacrylamide gel electrophoresis (IEF-PAGE) with protein extracts from macerated regions of apple fruits infected with either wild-type strain Bd90 or mutant B21 showed that PME profile was as complex as already shown by Reignault and associates (2000). However, we noticed the absence of the isozyme corresponding to BCPME1 in extracts from apples infected by mutant B21. The intense band of pI 7.4 observed with extracts from apples infected with strain Bd90 demonstrated that BCPME1 was strongly secreted in planta (Fig. 4B).

The *Bcpme1* mutant B21 is reduced in its ability to grow on pectin and in its virulence on several host plants.

The radial growth on solid medium was measured on medium containing different carbon sources. On rich media, such as potato dextrose agar (PDA) and nutrient yeast (NY), and on Czapeck-PGA 1%, no difference was found between the mutant B21 and wild-type strain Bd90 (data not shown). Furthermore, mutant B21 sporulated and produced sclerotia equally as well as Bd90 (data not shown). However, on medium containing 66% esterified pectin as the sole carbon source, mutant B21 growth was reduced in about 20% at 96 h postinoculation (Fig. 5). The similar growth observed for mutant B21 and wild-type strain Bd90 after 24 h could be explained by the utilization by the fungus of carbon sources contained in the agar plug used as inoculum.

Mutant B21 pathogenicity was checked on three different host plants, *Arabidopsis thaliana* ecotype Ws leaves, *Vitis vinifera* cv. Chardonnay leaves, and *Malus domestica* cv. Granny Smith apple fruits. On apple fruits, mutant B21 produced brown, rotten lesions around the inoculation sites, as did wild-type strain Bd90. There was no significant difference in necrosis diameter between mutant B21 and wild-type strain Bd90 (data not shown). However, while measuring fresh

Fig. 2. Southern analysis of genomic DNA from several strains of *Botrytis cinerea* and one strain of *B. allii* digested by *Bam*HI, *Eco*RI, and *Hind*III and hybridized with *Bcpme1* cDNA as probe. Lane 1, Bd90; lane 2, B05.10; lane 3, Br94; lane 4, SAR2108; lane 5, SAR10993; and lane 6, *B. allii*.

weight of macerated tissues four days after inoculation, apples inoculated with mutant B21 showed a 30% reduction in tissue maceration compared with those inoculated with the wild-type strain Bd90 (Fig. 6A). Grapevine leaves inoculated with mutant B21 presented reduced lesions compared with those observed on leaves infected by strain Bd90. Globally, leaves infected by mutant B21 showed about 50% symptom reduction (Fig. 6B). The virulence of mutant B21 was even more reduced on *Arabidopsis thaliana* leaves. For most inoculated leaves, mutant B21 was not able to cause lesions and sporulated abundantly on the agar plug used as inoculum (Fig. 6C and Table 1). When infection was successful, lesion sizes were 77% reduced compared with lesions produced by wild-type strain Bd90 (Table 1). A Student's *t* test made for each experiment on the three tested host plants showed that lesions caused by the mutant B21 were significantly smaller than those caused by the wild-type strain Bd90.

Complementation of the mutant B21.

The mutant B21 was complemented using a plasmid containing the *Bcpme1* gene with part of its promoter (929 bp) and of its terminator (458 bp) and the *bar* gene conferring resistance to the herbicide Basta. Five complemented clones were analyzed. When grown on Czapeck-pectin medium, they all showed PME isozyme profiles identical to the profile of wild-type strain Bd90, including PME activity of pI 7.4 (Fig. 4A). The complemented clones possessed approximately the same total PME activity (1.26 ± 0.07 U per 100 mg mycelial dry weight) as wild-type strain Bd90. In addition, the complemented clones were as pathogenic as wild-type strain Bd90 on *A. thaliana* (Table 1) and grapevine leaves (Table 2).

DISCUSSION

In this paper, we described the cloning of the *Bcpme1* gene encoding for a pectin methylsterase from *Botrytis cinerea* and the construction of a mutant disrupted in the *Bcpme1* gene. We showed that *Bcpme1* encodes the major PME activity secreted by *B. cinerea*, that a mutation in this gene has a profound effect on fungal development in pectin-containing medium and in planta, and that complementation of the *Bcpme1* mutant restores wild type phenotype.

To our knowledge, the *Bcpme1* gene is the first *pme* gene cloned from a phytopathogenic fungus causing soft-rotting

symptoms. As expected, BCPME1-deduced protein sequence is closer to fungal PMEs (48% mean identity) than to bacterial or plant PMEs (21%). Molecular mass of BCPME1 is in the same range as other known fungal PMEs, such as those of *A. aculeatus* PME I (36.2 kDa) (Christgau et al. 1996), *A. niger* PME (43 kDa) (Khanh et al. 1991), and *A. oryzae* PME (38.5 kDa) (Kitamoto et al. 1999). Southern analysis shows that the *Bcpme1* gene occurs as a single copy gene in Bd90 as well as in all analyzed strains. Furthermore, the positions of the two introns are conserved in two other strains, T4 and K1 (data not shown).

It has been shown previously that PME isozymes were detected whatever the carbon source, glucose, pectin, or polygalacturonic acid (Reignault et al. 1994), suggesting that *B. cinerea* PME-encoding genes were not submitted to catabolite repression. Consistent with those observations, no CRE binding site (Kulmburg et al. 1993) is detected in *Bcpme1* promoter and *Bcpme1* is expressed in glucose-containing medium to the same extent as in pectin-containing medium. Putative GATA binding sequences of nitrogen regulatory proteins are present in the *Bcpme1* promoter region. The EST library from which *Bcpme1* was isolated was constructed with mRNA from *B. cinerea* grown in nitrogen starvation conditions. However, the expression of *Bcpme1* is not stimulated in glucose medium, in which nitrogen is limiting, suggesting that *Bcpme1* cloning from the EST library is more certainly due to its strong expression in glucose medium. The *Bcpme1* promoter contains the sequence 5'GCCARG3', a putative site of recognition for PacC transcription factor. PacC is known to regulate gene expression according to the ambient pH (Tilburn et al. 1995). Variations in *Bcpme1* expression when strain Bd90 is grown at different pH levels should confirm the validity of this box in *Bcpme1* promoter.

We constructed a mutant lacking BCPME1 activity by transformation-mediated gene disruption. The disruption of the *Bcpme1* gene leads to a nonfunctional protein, as the PME of pI 7.4 is not detected by IEF-PAGE analysis, neither in culture supernatants of the mutant B21 nor in apple fruit tissues infected by the mutant B21. Thus, the C terminal portion of the *Bcpme1* gene (86 amino acid residues) appears to be essential for BCPME1 activity. This portion of the protein contains one conserved box that could be either part of the catalytic site or could be necessary for the correct folding of the protein BCPME1. So far, the protein structures of only two PMEs

Fig. 3. A, Schematic representation of plasmid pibcpme1 used to transform strain Bd90 of *Botrytis cinerea* and primer positions for transformant screening by polymerase chain reaction. Restriction enzyme sites: B (*Bam*HI), E (*Eco*RI). **B**, Southern blot analysis of genomic DNA digested by *Eco*RI from wild-type strain Bd90 (lane 1), and the mutant B21 (lane 2) probed with *Bcpme1* complete cDNA. The intensity of the band at 5.5 kb suggests tandem insertions of pibcpme1 at the *Bcpme1* locus.

have been published, from *Erwinia chrysanthemi* (Jenkins et al. 2001) and from *Daucus carota* (Johansson et al. 2002). PEMA from *E. chrysanthemi* and *Daucus carota* PME have a right-handed parallel β -helix, as seen in all bacterial and fungal pectic enzymes. It should be interesting to address whether the BCPME1 enzyme adopts the same folding, since no fungal PME has been crystallized, and since the two known structures are from PMEs having highly basic pI (PEMA, 9.6 and *Daucus carota* PME, 9.8), whereas BCPME1 has a neutral pI (7.4).

PME activity is found to be 75% reduced in the mutant B21. This reduction could be correlated to the reduced growth of mutant B21 in a pectin medium. Indeed, reduced PME activity may result in less substrate for the PG activities and difficulty for mutant B21 to use pectin as sole carbon source. Mutant B21 growth is not reduced on medium containing PGA as the sole carbon source, suggesting that B21 is not affected in PG production. Moreover, IEF-PAGE analysis of supernatants from culture on pectin as the sole carbon source and with infected apple fruit tissues showed identical PG profiles for strains Bd90 and B21 (data not shown).

Since *B. cinerea* causes maceration on a wide range of plants, and since physiological properties of cell wall pectin polymers are controlled by the degree and distribution of methylation, it was legitimate to hypothesize that *B. cinerea* PME activity can be an important determinant of its pathogenicity. Indeed, the potential role of PME in *B. cinerea* pathogenicity has also been recently suggested by Kapat and associates (1998), who showed that *T. harzarium* isolate T39, a biocontrol agent of *B. cinerea*, may act directly on the fungus by reducing the level of hydrolytic enzymes, including PME, on bean leaves. We demonstrate here that BCPME1 is an essential determinant of *B. cinerea* virulence. Differences in virulence reduction of mutant B21 according to the tested host plant might be due to the diverse degrees of methylation of the pectic compounds of the three tested hosts (Voragen et al. 1986). Recently, the sequencing of the *Arabidopsis* genome allowed the identification of 67 PME-related genes (The Arabidopsis Genome Initiative 2000). These numerous genes may be involved in regulation of the pectin methylation level in different tissues during *A. thaliana* growth development. The strong decrease observed for virulence of the mutant B21 on *A. thaliana* leaves could be correlated with a high methylation level of corresponding pectins.

Finally, although the progression of mutant B21 in vine leaf is slowed down, immunocytological studies with JIM7 antibody (Knox et al. 1990), which recognizes highly methylated pectin, shows that mutant B21 is still able to degrade pectin (data not shown). This suggests that other activities can contribute to pectin degradation. On one hand, it has been shown that *B. cinerea* produces pectin lyase, which is able to depolymerize methylated pectin (Chilosi and Milagro 1997). On the other hand, disruption of the *Bcpme1* gene revealed the existence of other PME activity. Cloning of other *pme* genes and construction of mutants should confirm the importance of PME activity in *B. cinerea* virulence.

MATERIALS AND METHODS

Fungal strains and growth conditions.

The wild-type reference strain of *B. cinerea* was Bd90 isolated from grapevine. Other *B. cinerea* strains used were SAR2108, SAR10993 isolated from grapevine (Faretra et al. 1988), Br94 (isolated from an apple fruit), B05.10 (haploid laboratory strain) (Büttner et al. 1994), and *B. allii* (from *Al-lium* sp.). They were grown on PDA or NY (malt extract, 20 g per l; agar, 20 g per l; and yeast extract, 2 g per l) at 23°C. Czapeck synthetic medium contains NaNO₃, 30mM; KCl, 6.7 mM; MgSO₄, 7 H₂O, 0.2 mM; K₂HPO₄, 5.7 mM; FeSO₄, 7 H₂O, 36 μ M; and NaMo, 84 μ M supplemented with either 3% glucose, PGA (Sigma P3889), or 1% pectin (Sigma P9165) and adjusted to pH 6. For culture in solid medium, 1.5% agar was added. For liquid cultures, conidia (5×10^7 spores) were inoculated into a 250 ml Erlenmeyer flask containing 50 ml of medium.

Genomic Southern analysis and sequencing of Bcpme1.

Genomic DNA from *B. cinerea* strains was extracted after 48 h culture in liquid medium, as described by Sambrook and associates (1989). DNA was digested with *Bam*HI, *Eco*RI, and *Hind*III and was size-separated on 0.8% agarose Tris-borate-EDTA 1 $\times$ gel. After electrophoresis, DNA was blotted to Hybond N⁺ membranes (Amersham, Saclay, France) by transfer capillarity and hybridized in Church buffer (NaH₂PO₄; H₂O, 27.6 g per l; Na₂HPO₄, 42.6 g per l; SDS, 70 g per l; EDTA 0.5 M, pH 8, 2 ml per l; and bovine serum albumin [BSA], 10 g

Fig. 4. Revelation of pectin methylesterase activity after isoelectric focusing–polyacrylamide gel electrophoresis (pH range 3 to 10). **A**, Concentrated filtrates from 3-day-old liquid culture on 1% Czapeck-pectin, strain Bd90 (1), mutant B21 (2), and complemented B21 (3). Amount of protein loaded: (1) 9.6 μ g, (2) 9.5 μ g, (3) 19.2 μ g. **B**, Proteins extracted from healthy apple fruit (1), apple fruit infected by wild-type strain Bd90 (2), and by mutant B21 (3). Amount of sample loaded on each lane corresponds to 200 mg (fresh weight) of healthy or rotten apple tissues.

per l) at 65°C overnight. Full-length cDNA was used as a probe and labeled with [α -32 P] dATP using a random primer DNA labeling system from Boehringer (Roche Diagnostics, Meylan, France). Blots were washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 1% SDS for half an hour and once in 0.2× SSC and 1% SDS at 65°C and exposed to Kodak X-OMAT film for 48 h.

Gene walking to clone upstream and downstream regions of *Bcpme1* was performed using the Universal Genome Walker kit (Clontech Labs, Inc., Palo Alto, CA, U.S.A.) following the conditions recommended by the supplier. Primers used were *bcpme1pr1* (5' GCGGCAGAGACTGCTCCTAGAAAGAGTGG 3') and *bcpme1tr1* (5' GGCATCAGCAAAGCCATTCCTATC-ACC 3') for the 5' and 3' noncoding regions, respectively. DNA sequencing was performed by ESGS (European Sequence Gene Service, Groupe Cybergene, Evry, France).

Micro-sequencing of the BCPME1 protein.

Purified PME protein from *B. cinerea* Bd90 was subjected to SDS-PAGE (Reignault et al. 1994). Protein bands were stained with Coomassie brilliant blue and cut out. Then trypsin digestion was performed as follows: 200 μ l Tris HCl 0.1 M, pH 8.6, and 0.01% Tween buffer supplemented with 0.5 μ g of trypsin during 18 h at 30°C. Peptides were separated with high-pressure liquid chromatography on a DEAE-C18 column using an acetonitrile and trifluoroacetic acid 0.1% gradient (0 to 45%). N-terminus and internal amino acid microsequences were obtained by Edman degradation with Sequencer Applied Biosystem 494 (Institut Pasteur, Paris).

RNA extraction and RT-PCR analysis.

RNAs were extracted from frozen mycelium ground in liquid nitrogen, using the RNeasy plant MiniKit (Qiagen, Courtaboeuf, France). RT-PCR reactions were performed with a dilution series from 2 to 50 ng of total RNA, using the one-step RT-PCR kit (Qiagen) and following the manufacturer's instructions. Specific primers were designed (5'TCGA-TGCAACCGGCAAAACAG3' and 5'GCGGCAATGGTGCG-GATATCG3') to span an intron, as contaminating DNA would result in a larger size fragment. Steps of RT-PCR were: RT, 50°C 30 min; PCR, 15 min at 95°C; 25 cycles of 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C; followed by 10 min at 72°C. Amplification products were loaded on a 1.2% agarose gel and were quantified by densitometric analysis using the BIOPROFIL program (Vilber Lourmat, Marne la Vallée,

Fig. 5. Radial mycelium growth on 66% esterified pectin-containing medium at 24, 48, 72, and 96 h postinoculation of Bd90 wild-type strain and the mutant B21 strain. Bars indicate standard deviation of mean of three experiments.

France). Results were expressed in AU representing signal intensity. Proportionality between the fluorescence intensities and the amount of input total RNA was observed, allowing comparisons of fractions of specific RNAs between two growth conditions. Experiments have been repeated three times.

Construction of the gene disruption vector and fungal transformation.

Homologous primers WOD (5'CGCGAAGCTTGTCTCCA-GGACCTCTGCA3') and AFBR (5'CGCGAAGCTTTCTCC-ATGGACGTCCCAA3') containing *Hind*III sites were designed on the *Bcpme1* cDNA sequence and were used to amplify an 831-bp product on genomic DNA by PCR (3 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 62°C, and 1 min at 72°C, followed by 7 min at 72°C). This product was cloned into the *Hind*III site of pAN7-1, leading to the plasmid *pibcpme1* used for transformation. Strain Bd90 was transformed with undigested *pibcpme1* according to Hamada and associates (1994). Transformants were single spores isolated on hygromycin-containing medium (100 μ g per ml). In order to make a first screening, two primers, AVWOD (5'CTTCTA-GGAGCAGTCTCTG3') and FTtrpC (5'CACTCCACATCTC-CACTCGA3'), were designed so that only DNA of homologous interrupted transformants could be amplified as 1-kb product. Those transformants were further analyzed by Southern blotting.

Fig. 6. Pathogenicity assays with the wild-type Bd90 and mutant B21 strains. **A**, On Granny Smith apple fruits. Fresh weight of macerated tissues at 96 h postinoculation (hpi). **B**, On *Vitis vinifera* Chardonnay leaves. Evolution of necrosis diameter. **C**, On *Arabidopsis thaliana* ecotype Ws leaves. Symptoms caused by strains Bd90 and B21 at 96 hpi. Bars indicate standard deviation of mean of three experiments.

Table 1. Pathogenicity assay on *Arabidopsis thaliana* ecotype Ws leaves

Strain ^a	Necrosis diameter mean (cm) at 96 hpi	Successful infection ^b (%) at 96 hpi
Bd90	1.89 ± 0.31	100
B21	0.44 ± 0.21	33
B21C	1.60 ± 0.16	100

^a Bd90 (parental wild-type strain), B21 (*Bcpme1* disrupted mutant), and B21C (complemented *Bcpme1* transformant of B21).

^b Percentage of successful infections corresponds to the number of inoculations leading to infections divided by the number of total inoculations. An inoculation leading to an infection was considered successful when the lesion was more than 4 mm in diameter.

Complementation of mutant B21.

With primers *bcpme1*prCgata (5'GGAAAGCGTGATATTG-TTGAT3'), located in the promoter of the *Bcpme1* gene, and *bcpme1*trC (5'GGGGTTCCAAGATGGGATGTTAG3'), located at the end of the known portion of the *Bcpme1* terminator, a product of 2,610 bp was amplified (Expand High Fidelity System; Roche Diagnostics, Meylan, France) on genomic DNA of wild-type strain Bd90. This PCR product was cloned in pGEM-T (Promega, Charbonnières, France), was digested by *ApaI* and *SsrI*, and was introduced in vector pCB1265 (provided by M. H. Lebrun, Bayer Lyon), which contained a *bar* gene conferring resistance to the herbicide Basta, leading to plasmid p**bcpme1**C to transform mutant B21. For selection to Basta resistance, protoplast suspension was resuspended in MS1 medium (NaNO₃, 23.5 mM; glucose, 111 mM; sucrose, 584 mM; 1% mineral solution [KH₂PO₄, 735 mM; MgSO₄, 7 H₂O, 0.2 M; KCl, 0.67 M; FeSO₄, 7 H₂O, 3.6 mM, pH 3.5] and agar 0.7%) supplemented with 100 µg of Basta per ml (Ammonium Glufosinate, Ehrenstorfer-Schäfers GmbH Laboratory) and was spread over a petri dish with MS1 medium containing Basta. Transformants resistant to Basta appeared after 4 to 5 days.

Quantitative measurement of PME activity.

PME activity was measured by a colorimetric method with the pH indicator bromocresol green, according to Reignault and associates (1994). One unit of PME was the amount of enzyme that released 1 µmole of H⁺ ions per min at pH 5.5 and 25°C.

IEF-PAGE analysis.

Fungus was cultivated in Czapeck synthetic medium supplemented with either 3% glucose, 1% PGA, or 1% pectin during three days at 21 ± 1°C. The culture media filtrated on miracloth were concentrated against 20 M PEG carboxylate and then dialyzed in 50 mM citrate phosphate buffer, pH 6.4. IEF-PAGE was performed as described by Bertheau and associates (1984). Preparation of extracts from healthy and infected apple fruits was performed as described by Reignault and associates (2000).

Inoculation of plants and pathogenicity assays.

Apple fruits (*Malus domestica* cv. Granny Smith) were acquired commercially and wound-inoculated with a 5-mm-diameter plug of mycelium from the edge of 3-day-old culture on solid NY. Strain Bd90 and mutant B21 were inoculated on the same apple fruit, and five apple fruits were used in each of the three experiments. For grapevine (*Vitis vinifera* cv. Chardonnay) and *Arabidopsis thaliana* ecotype Ws, detached leaves were inoculated without wounding on the adaxial face (plug of 3- or 5-mm diameter for *A. thaliana* and grapevine leaves, respectively). The leaves were put in petri dishes containing water and small beads to maintain a high humidity level. The petri dishes were incubated at 21 ± 1°C and under daylight. After

Table 2. Pathogenicity assay on *Vitis vinifera* leaves

Strain ^a	Necrosis diameter mean (cm) at 96 hpi
Bd90	2.30 ± 0.40
B21	1.25 ± 0.32
B21C	2.28 ± 0.31

^a Bd90 (parental wild-type strain), B21 (*Bcpme1* disrupted mutant), and B21C (complemented *Bcpme1* transformant of B21).

24, 48, 72, and 96 h postinoculation, the necrosis diameters were noted. The experiments were repeated three times with five leaves for grapevine tests and ten leaves for *A. thaliana* tests. Wild-type strain Bd90 was applied on the left side and mutant B21 on the right side of grapevine leaves. Strain Bd90 and mutant B21 were applied on different *A. thaliana* leaves, as they were too small to put the two strains on the same leaf. For *Arabidopsis thaliana* pathogenicity tests, we calculated a percentage of successful infections, which corresponded to the number of inoculations leading to infections divided by the number of total inoculations. An inoculation leading to infection was considered successful when the lesion was more than 4 mm in diameter (including plug diameter). The data shown in Table 1 and in Figures 5, 6A, and 6B are accumulated data from three experiments.

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