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Characterization of a new, nonpathogenic mutant of *Botrytis cinerea* with impaired plant colonization capacity

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Summary

- *Botrytis cinerea* is a necrotrophic pathogen that attacks more than 200 plant species.
- Here, the nonpathogenic mutant A336, obtained via insertional mutagenesis, was characterized.
- Mutant A336 was nonpathogenic on leaves and fruits, on intact and wounded tissue, while still able to penetrate the host plant. It grew normally *in vitro* on rich media but its conidiation pattern was altered. The mutant did not produce oxalic acid and exhibited a modified regulation of the production of some secreted proteins (acid protease 1 and endopolygalacturonase 1). Culture filtrates of the mutant triggered an important oxidative burst in grapevine (*Vitis vinifera*) suspension cells, and the mutant–plant interaction resulted in the formation of hypersensitive response-like necrosis. Genetic segregation analyses revealed that the pathogenicity phenotype was linked to a single locus, but showed that the mutated gene was not tagged by the plasmid pAN7-1.
- Mutant A336 is the first oxalate-deficient mutant to be described in *B. cinerea* and it differs from all the nonpathogenic *B. cinerea* mutants described to date.

Key words: *Botrytis cinerea*, endopolygalacturonase BcPG1, hypersensitive response, nonaspartyl acid protease ACP1, nonpathogenic mutant, oxalate, oxidative burst.

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Introduction

The hyphomycete *Botrytis cinerea* is widely known as a fungus causing destructive and economically important plant diseases. It is concentrated in the temperate areas of the world where it can infect an extremely wide range of host plants (Jarvis, 1977; Elad *et al.*, 2004). *B. cinerea* is also noteworthy as a spoilage organism causing considerable losses of plants during storage and transit (Hammer *et al.*, 1990; De Kock & Holz, 1992; Berrie, 1994; Elad, 1998; Droby & Lichter, 2004). Disease control is extremely difficult because the fungus is capable of attacking crops at almost any stage in their growth or storage, and affects all plant parts, including cotyledons, leaves, stems, flowers, fruits and roots. Chemical control remains the main way to reduce *B. cinerea* disease (Leroux, 2004).

B. cinerea enters a plant via direct penetration or through natural openings or wounds (Verhoeff, 1980). As a necrotroph, it then derives nutrients from dead or dying cells (Agrios, 1997), and the colonization of the plant therefore depends upon the ability of the fungus to kill the host cells. Many factors potentially involved in the pathogenicity of the fungus have been studied (for a review, see Staples & Mayer, 1995). Several genes that code for these factors have been cloned and the corresponding mutants have been analysed (for reviews, see Prins *et al.*, 2000; Kars & van Kan, 2004). The tetraspanin BcPls1 (Gourgues *et al.*, 2004) and the Mitogen-Activated

Protein (MAP) kinase BMP1 (Zheng *et al.*, 2000) are two factors involved in penetration, and mutants of either gene are nonpathogenic. Recently, components of some signalling pathways have also been associated with virulence (Schulze Gronover *et al.*, 2001; Klimpel *et al.*, 2002; Viaud *et al.*, 2003) and a mutation in one of them (*bcg1*) leads to a nonpathogenic strain impaired in plant colonization (Schulze Gronover *et al.*, 2001). Several virulence factors involved in symptom development have also been described, but none of them is required for pathogenicity, probably because of gene redundancy. Among these factors are cell wall degrading enzymes (Ten Have *et al.*, 1998; Valette-Collet *et al.*, 2003), transporter proteins (Schoonbeek *et al.*, 2001; Hayashi *et al.*, 2002) and enzymes protecting the fungus from oxidative stress (Schouten *et al.*, 2002; Rolke *et al.*, 2004).

Production of phytotoxins by *B. cinerea* has been proposed to promote cell death in the host (Rebordinos *et al.*, 1996; Colmenares *et al.*, 2002). Further, Govrin & Levin (2000) suggested that induced cell death is related to the plant hypersensitive response (HR), and that its triggering by the fungus constitutes an important component of virulence. HR, however, is often associated with plant resistance to pathogens (for a review, see Lam *et al.*, 2001) and a delicate balance between the attack of the pathogen and the defence of the host could control the outcome of the interaction.

Here we report on a nonpathogenic mutant of *B. cinerea* that fails to overcome the defence system of the plant and whose interaction with its host results in HR-like necrosis. The phenotype of the mutant is different from any other described before. The mutant is blocked after penetration and does not colonize the plant tissue. It is deficient in oxalic acid production and does not produce the nonaspartyl acid protease 1 (ACP1). Moreover, it produces the *Botrytis cinerea* endopolygalacturonase 1 (BcPG1) elicitor in large amounts relative to the wild-type strain.

Materials and Methods

Fungal strains

The wild-type strain Bd90 of *Botrytis cinerea* pers. was collected from grapevine in Bordeaux, France (Reignault *et al.*, 1994). The monoascospore strain SAS405 was kindly provided by Dr F. Faretra (University of Bari, Italy). Mutant A336 had been obtained previously by transformation of the Bd90 strain, by applying a polyethylene glycol (PEG)-mediated method using the plasmid pAN7-1 (Hamada *et al.*, 1994). This plasmid carries the *Escherichia coli, hygromycin phosphotransferase (hph)* gene, conferring hygromycin B resistance.

Culture techniques

All *B. cinerea* strains and transformants were maintained on 2% [weight/volume (w/v)] malt-agar medium. In some tests, strains were grown on potato dextrose agar (PDA). *In vitro* growth tests (liquid or solid medium) used the minimal Czapeck medium containing (l^{-1}): $NaNO_3$ 2.5 g, KCl 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 10 mg, K_2HPO_4 1 g and Na molybdate 10 mg. The medium was supplemented with 2% glucose or polygalacturonic acid (PGA), (Sigma-Aldrich, Saint Quentin Fallavier, France), and with 15 g l^{-1} bacto agar for solid medium. Initial pH values in unbuffered media were adjusted using HCl or NaOH, while buffered media were prepared in appropriate 0.15 M citrate/phosphate buffer. All cultures were incubated at 21°C under constant light. Culture filtrates were collected from liquid cultures or cellophane-overlaid cultures after filtration on tissue with pore size 100 µm. If indicated, culture filtrates were concentrated 20-fold against PEG (6 M; Prolabo, Paris, France) in dialysis bags (molecular weight cut off (MWCO) 6–8,000 Dalton; Spectrum Laboratories, Carl Roth GmbH, Karlsruhe, Germany) and dialysed against 0.05 mM acetate buffer, pH 5. Filtrates used for acid protease and phosphatase assays were precipitated with 10% trichloroacetic acid (TCA) (Rolland *et al.*, 2003).

Sexual crosses

Microconidia from transformant A336, issued from wild-type strain Bd90 (mating type *mat1-1*), was crossed with sclerotia from monoascospore strain SAS405, mating type *mat1-2*. Matings were conducted as described by Faretra *et al.* (1988).

Plant material and inoculation

Pathogenicity tests on leaves of grapevine (*Vitis vinifera* L. cv. Chardonnay), bean (*Phaseolus vulgaris* L. cv. Victoire nain) and *Arabidopsis thaliana* ecotype Wassilewskija (Ws) were conducted as described by Reignault *et al.* (2000). Leaves, collected just before infection, were inoculated on their upper sides with malt-agar plugs from 4-d-old, nonsporulating cultures of wild-type strain Bd90 or mutant A336. The leaves were kept on wet filter paper in a Petri dish under high humidity conditions and daylight. The degree of colonization on plants was observed 4 d after inoculation.

Infection-related morphogenesis was observed on *Allium cepa* L. (onion) epidermis, as described in Gourgues *et al.* (2004). Malt-agar mycelium plugs were deposited onto the hydrophobic side of the onion epidermis. After 16 h of inoculation in a humid environment at 21°C and 16 h daylight, fungal cells were stained with methyl blue (cotton blue) and observed with light microscopy or epifluorescence microscopy.

Microscopy

Fragments of infected grapevine leaves or onion epidermis were observed using a Axiophot microscope (Carl Zeiss, Le Pecq, France) equipped for epifluorescence and bright field

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viewing. The light source was a HBO 50-W mercury lamp (Carl Zeiss, Le Pecq, France). For fluorescence, an excitation filter (395–440 nm) and a barrier filter that passes light above 470 nm were used.

Active oxygen species (AOS) production measurements

H_2O_2 production was determined using chemiluminescence of luminol. Aliquots (250 µl) of cell suspensions were added to 300 µl of buffer containing 50 mM HEPES, pH 8.5, 175 mM mannitol, 5 mM $CaCl_2$, 0.5 mM K_2SO_4, and 50 µl of 0.3 mM luminol. Chemiluminescence, measured within a 10-s period with a luminometer (Lumat LB 9507; EG & G Berthold Evry, France) was integrated and expressed in nmol of H_2O_2 per gram of fresh weight of cells (FWC), using a standard calibration curve obtained by addition of H_2O_2 in grapevine cell suspension.

Polygalacturonase activity

The polygalacturonase (PG) activity was determined spectrophotometrically using polygalacturonic acid as substrate and using the modified 2-cyanoacetamide method (Poinssot *et al*., 2003). One reducing group unit (RGU) corresponded to 1 µmol of reducing sugar released per min at 30°C.

BcPG1 and ACP1 immunodetection

Proteins from concentrated culture filtrates were submitted to 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) before transfer to nitrocellulose membrane. Detection of BcPG1 by western blot was performed with polyclonal rabbit anti-BcPG1 primary antibodies. Probing and detection were performed as described in the manufacturer's instructions for the ECL Western detection kit (Pharmacia Biosciences, Piscataway, NL, USA) with goat anti-rabbit horseradish peroxidase-coupled secondary antibodies. The acid protease ACP1 was detected using western blot analysis as described by Girard *et al.* (2004). The membrane was probed for 16 h with anti-ACP1 polyclonal antibodies, washed, and then incubated for 45 min in the presence of rabbit anti-immunoglobulin G (IgG) secondary antibodies. Chemiluminescent reaction was used to reveal the immunoreactive proteins. The western blot analysis was repeated three times.

Alkaline phosphatase assay

Enzyme activity was determined as described by Caddick & Arst (1986) in culture filtrate from cellophane-overlaid cultures buffered to pH 4 or 7. A volume of 50 µl of filtrate was made up to 1 ml using the appropriate buffer containing 1 mM p-nitrophenylphosphate (disodium salt) as substrate. Alkaline phosphatase was assayed in 100 mM diethanolamine (hydrochloride) buffer, at pH 9, containing 10 mM $MgCl_2$. All phosphatase reactions were terminated by addition of 2 ml of 100 mM NaOH and p-nitrophenol was estimated from the absorbance at 450 nm.

Oxalic acid quantification

Filtrated supernatants from cultures grown on PGA at different initial pHs were analysed for oxalic acid content with a commercial enzymatic assay kit (oxalate procedure no. 591; Trinity Biotech, St. Louis, MO, USA) according to the manufacturer's instructions. The oxalic acid concentration was calculated by extrapolation from a standard and adjusted for the dilution factors.

Results

Selection of the nonpathogenic mutant A336

Mutant A336 was obtained by PEG-mediated random insertional mutagenesis (Hamada *et al*., 1994) using the plasmid pAN7-1. It was selected among 900 hygromycin-resistant transformants for its lack of pathogenicity towards several plant hosts. When compared with the wild-type strain Bd90, mutant A336 indeed caused no infection of grapevine (*Vitis vinifera*), bean (*Phaseolus vulgaris*) or *Arabidopsis thaliana* (Fig. 1) and the same result was obtained using wounded plant tissues. On apple (Golden Delicious [*Malus domestica* cv.]), the mutant produced < 5% of the maceration in the tissue caused by the Bd90 strain (Reignault *et al*., 2000). The mutation carried by the mutant A336 therefore affects one or several genes whose products are essential to *B. cinerea* virulence.

Genetic characterization of mutant A336

We analysed the genetic segregation of the pathogenicity phenotype and the pAN7-1 plasmid insertion in the mutant strain. Mutant A336 (mating type *mat1-1*) was crossed with the strain SAS405 derived from a single ascospore (mating type *mat1-2*). Apothecia were obtained and five asci were dissected into 40 single-ascospore-derived colonies. A 1 : 1 segregation pattern was found for pathogenicity and for pAN7-1 insertion, indicating a monogenic inheritance (data not shown). However, no cosegregation was found between the pathogenicity phenotype and the insertion of pAN7-1 (Table 1); in two of the nonpathogenic progenies (a1.6 and a2.4), pAN7-1 was absent and, conversely, one pathogenic progeny (a2.7) carried the plasmid while another (a1.7) did not. The insertion of the plasmid was hence not responsible for the pathogenicity phenotype of mutant A336 and the locus carrying the mutation was named *BcPTH1* (for *B. cinerea* pathogenicity 1).

In vitro growth and reproduction of mutant A336

When grown on malt or potato dextrose agar at pH 5, hyphal growth of mutant A336 was identical to that of the wild

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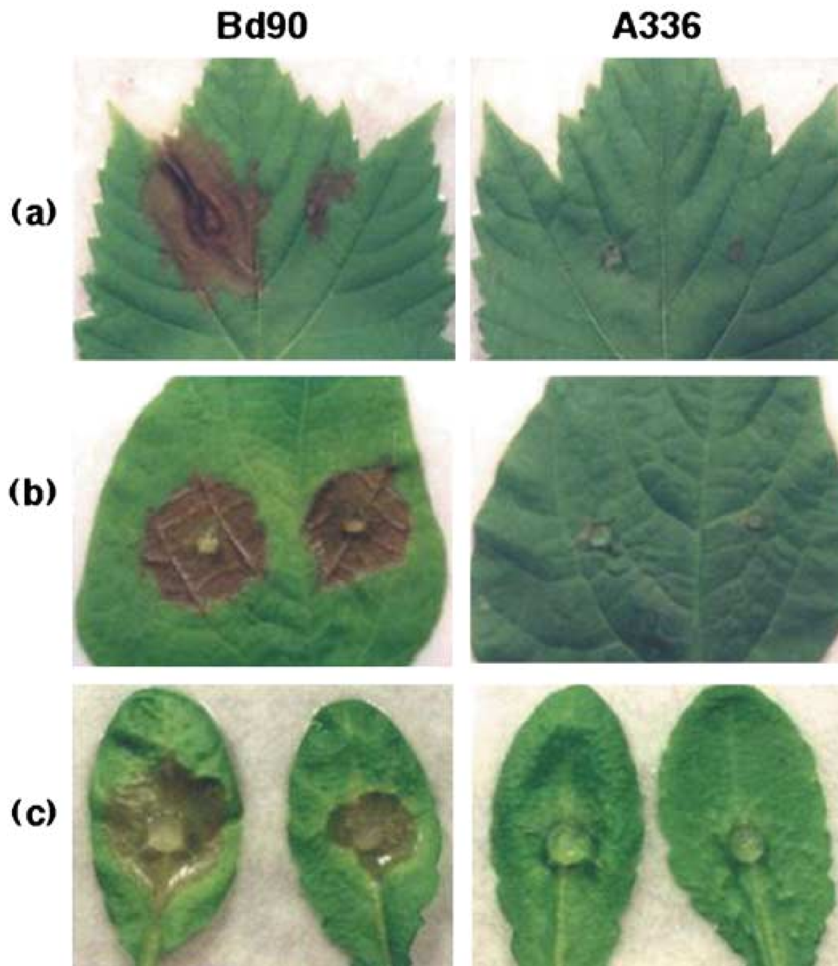

Fig. 1 Virulence assays on three different hosts. Mycelium plugs of *Botrytis cinerea* wild-type strain Bd90 and mutant A336 were applied to the upper face of detached (a) grapevine (*Vitis vinifera* cv. Chardonnay), (b) bean (*Phaseolus vulgaris* cv. Victoire nain), or (c) *Arabidopsis thaliana* ecotype Ws leaves. On each grapevine or bean leaf a mycelium plug was applied to a wounded site (left) and to an intact site (right). Two different *A. thaliana* leaves were used: a wounded leaf is shown on the left and an intact leaf on the right of each photograph. Observations were made 4 d post inoculation.

type. However, the mycelium of mutant A336 did not form conidiophores, conidia or sclerotia (Fig. 2a). Production of conidia and sclerotia was also analysed in the mutant progenies and a cosegregation was observed between their absence and the pathogenicity phenotype (Table 1). This indicates that the *BcPTH1* mutation does not affect fungal growth, but affects some of the developmental programmes of the fungus under the conditions tested.

When inoculated onto minimal unbuffered medium with 1% PGA as the carbon source, mutant A336 and the wild-type strain again grew at the same rate at pH 5, but the mutant strain exhibited a reduced growth rate at pH 6 and 7 (44% and 68% reductions, respectively) when compared with the wild-type strain. Surprisingly, however, at pH 7, mutant A336 produced viable conidia (Fig. 2b) which again produced conidia-free mycelium when plated on potato dextrose medium. Finally, mutant A336 was also able to form conidia when mycelium plugs were applied to grapevine leaves (Fig. 2c). These results indicate that the *BcPTH1* locus is involved in regulating conidiation but not morphogenesis or viability of the conidia.

Defective production of oxalic acid in mutant A336

Growth on PGA-containing medium requires the production of endopolygalacturonases by the fungus. The action of these enzymes needs an acidic environment (Magro *et al.*, 1984; Favaron *et al.*, 2004; Kars *et al.*, 2005) and such an environment is achieved through the secretion of oxalic acid by the fungus (Dutton & Evans, 1996). One possible reason for mutant A336 not performing very well on PGA at pH 6 or 7 could therefore be a defect in oxalic acid production. The mutant and the wild-type strains were grown for 3 d in liquid PGA unbuffered media (pH 5, 6 or 7) and both the production of oxalic acid and the final culture pH were measured (Table 2). The wild-type strain secreted millimolar amounts of acid when exposed to neutral pH and hence adjusted its surrounding pH to similar values in all cases. In contrast, the mutant produced negligible amounts of oxalic acid and the pH of the culture filtrate increased over time. These data were also collected for the mutant progenies and cosegregation was observed between the lack of acid production and the pathogenicity phenotype (Table 1).

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Table 1 Segregation analysis of the pleiotropic phenotype of *Botrytis cinerea* nonpathogenic mutant A336 in the progeny of the cross between the mutant A336 and monoascospore strain SAS405

	Phenotype analysed				
Strain	Virulence[1]	PAN7-1 insertion[2]	Final culture pH[3]	Oxalic acid production[3]	Conidia and sclerotia[4]
Wild-type strain Bd90	P	No	4.13	3.04	Yes
Parental strain SAS405	P	No	4.60	ND	Yes
Parental strain A336	NP	Yes	6.30	0.03	No
Progeny a1.6[5]	NP	No	5.69	0	No
Progeny a1.7	P	No	4.91	1.04	Yes
Progeny a2.4	NP	No	6.55	0.02	No
Progeny a2.7	P	Yes	4.39	1.05	Yes
Progeny a3.3	P	ND	4.42	4.74	Yes
Progeny a3.5	NP	ND	6.26	0.07	No
Progeny a4.4	P	ND	4.93	4.31	Yes
Progeny a4.8	NP	ND	5.57	0.07	No
Progeny a5.4	P	ND	4.23	1.65	Yes
Progeny a5.5	NP	ND	6.20	0.02	No

[1]P, pathogenic; NP, nonpathogenic.
[2]PAN7-1 insertion was tested using PCR for the detection of the *hygromycin phosphotransferase (hph)* gene; ND, not determined.
[3]pH and oxalic acid production were estimated in liquid cultures (Czapek glucose medium, initial pH 5.5) after 4 d of culture.
[4]Conidia and sclerotia production was tested on malt agar, pH 5.
[5]Progenies were obtained from cross A336 (microconidia) × SAS405 (sclerotia). For five asci dissected (a1 to a5), one nonpathogenic and one pathogenic progeny per ascus was chosen for the cosegregation analysis.

Table 2 Oxalic acid production and pH adjustment in Bd90 and A336 unbuffered polygalacturonic acid liquid culture filtrates

	Final pH		Oxalic acid (mM)	
Initial pH	Bd90[1]	A336[1]	Bd90[1]	A336[1]
5	5.70	6.92	0.15	0.06
6	5.20	7.18	2.51	0.13
7	5.63	7.42	2.74	0.08

Different initial pHs were tested. Each value for final pH is a mean with $n = 3$. Each value for oxalic acid concentration is a mean with $n = 2$. [1]Values are strain dependent ($P < 0.0001$ for the strain effect in the analysis of variance).

Altered regulation of secreted enzymes in mutant A336

As oxalic acid production by *B. cinerea* is higher under alkaline than acidic conditions, pH sensing by the fungus is likely to be involved in the regulation of at least part of that production. The effect of the *BcPTH1* mutation on oxalic acid production could therefore arise from a misperception of the extracellular pH. This possibility was investigated by monitoring the production of an acidic protease whose pH-dependent production has been shown in both *Sclerotinia sclerotiorum* (Lib.) de Bary and *B. cinerea* (Poussereau *et al.*, 2001; Cotton *et al.*, 2003; Rolland *et al.*, 2003). Mutant A336 and the wild-type strain were grown under acidic (pH 4) or neutral (pH 7) conditions and the secreted proteins were analysed by western blot using polyclonal antibodies raised against the *S. sclerotiorum* acid protease ACP1 (Fig. 3a). As expected, a single band corresponding to a protein of 29 kDa was detected in the wild-type culture broth buffered to pH 4 and was absent in the broth buffered to pH 7. This band could not be detected in the culture broths of the mutant, and the same result was obtained with the nonpathogenic progenies (data not shown). In parallel, we performed alkaline phosphatase assays on the culture broths from the wild-type and the mutant strain (Fig. 3b). More alkaline phosphatase was measured when both strains were grown at pH 7 than when they were grown at pH 4. Taken together, these results indicate that pH regulation is not abolished in the mutant A336 but that the regulation of ACP1 production is affected by the mutation. Interestingly, phosphatase activity was twice as high in the mutant culture broth as in that of the wild type, suggesting that the amount of phosphatase produced in the mutant is changed relative to the wild type. With ACP1 production also being modified, we clarified this particular issue by comparing the production of a third enzyme in the mutant and wild-type strains. Following growth in glucose medium at pH 5.5, the production of the endo-PG1 was analysed by western blotting using specific antibodies raised against the *B. cinerea* BcPG1 (Fig. 3c). The presence of the 40-kDa protein was clearly revealed in the mutant culture filtrate, whereas no signal could be observed under these conditions for the wild-type strain. Complementary experiments in fact revealed the presence of

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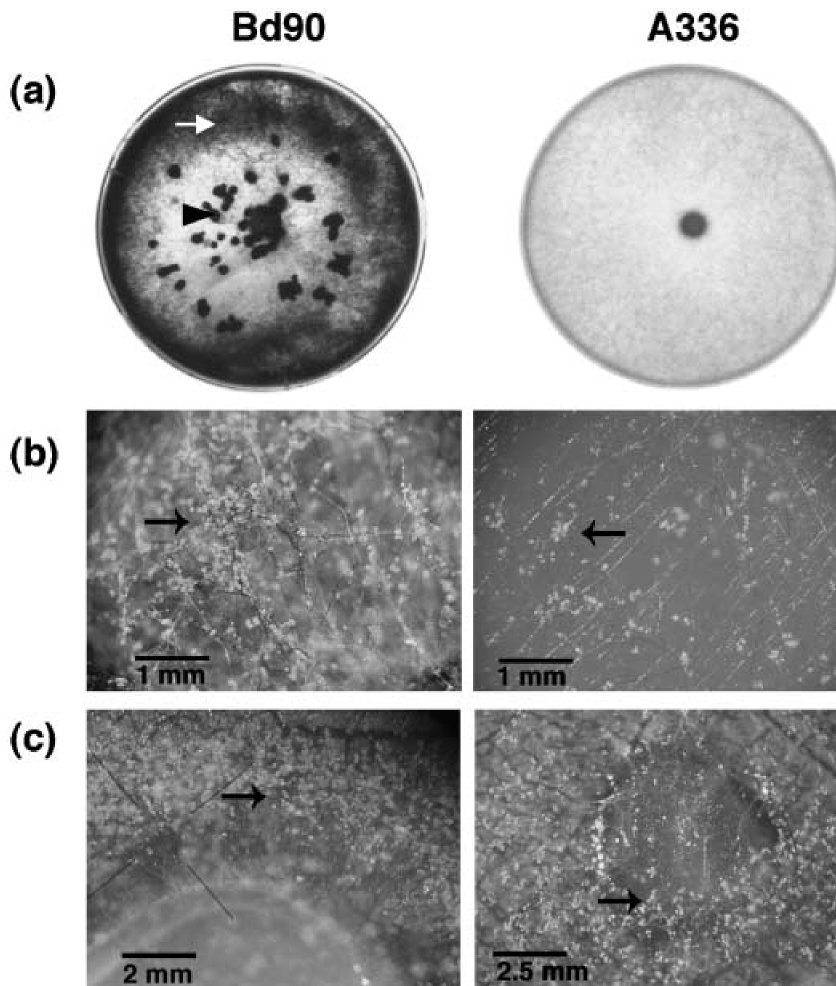

Fig. 2 *Botrytis cinerea* conidia and sclerotia production. (a) *In vitro* growth of Bd90 and A336 on potato dextrose agar (PDA) pH5 medium under constant light. The arrowhead indicates sclerotia. The dark coloration at the periphery of the Bd90 culture corresponds to conidia (white arrow). (b, c) Bd90 and A336 mycelium plugs inoculated on (b) polygalacturonic acid (PGA) medium, pH 7, and (c) the lower side of a grapevine (*Vitis vinifera* cv. Chardonnay) leaf. Conidia (arrows) were observed using binocular microscopy 7 d after inoculation.

BcPG1 in wild-type strain Bd90 in low amounts (data not shown). In parallel, an almost 20-fold higher polygalacturonase activity was found in the mutant culture filtrate when compared with the wild type (Fig. 3c). Taken together, these results show that the production of different secreted enzymes is affected by the *BcPTH1* mutation. Curiously, some enzymes (BcPG1 and alkaline phosphatase) were overproduced in the mutant A336 and one enzyme (ACP1) was less abundant in the mutant culture filtrate compared with the wild-type strain Bd90.

Mutant A336 is able to penetrate plant tissues

To test whether mutant A336 was able to penetrate plant tissues, we investigated fungal penetration of the onion epidermis. Onion is not a typical host for *B. cinerea* but its translucid cells combined with staining of the fungus with methyl blue allow clear visualization of hyphal penetration; internal hyphae are not stained and can be seen through the upper layers of plant cells. We applied mycelium plugs to onion epidermal strips and found that hyphae from both Bd90 and A336 had reached the epidermis surface at 16 hrs post inoculation (hpi). Noninoculated epidermal tissue was still alive at that time, as tested by staining with neutral red dye (data not shown). The wild-type hyphae had grown abundantly over the epidermal surface and had formed densely ramified 'claw-like' structures at many locations (Fig. 4a). Transparent hyphae were visible at these sites, indicating that penetration had occurred. At the same time-point, hyphae of mutant A336 had formed the same type of structures, albeit less frequently (Fig. 4b). In both the wild type and the mutant strain, penetration clearly occurred at the tip of slightly swollen hyphae (appressorium) within the claw-like structures (Fig. 4a,c). These results indicate that mutant A336 is not impaired in its capacity to penetrate the plant.

Mutant A336 is impaired in its capacity to invade plant tissues

We next explored how the mutant developed inside the plant. Grapevine leaves were infected with either the wild type or the mutant strain and microscopic observations were carried out (Fig. 5). At 4 d after inoculation, significant maceration of the host tissue was observed on the leaves infected by the

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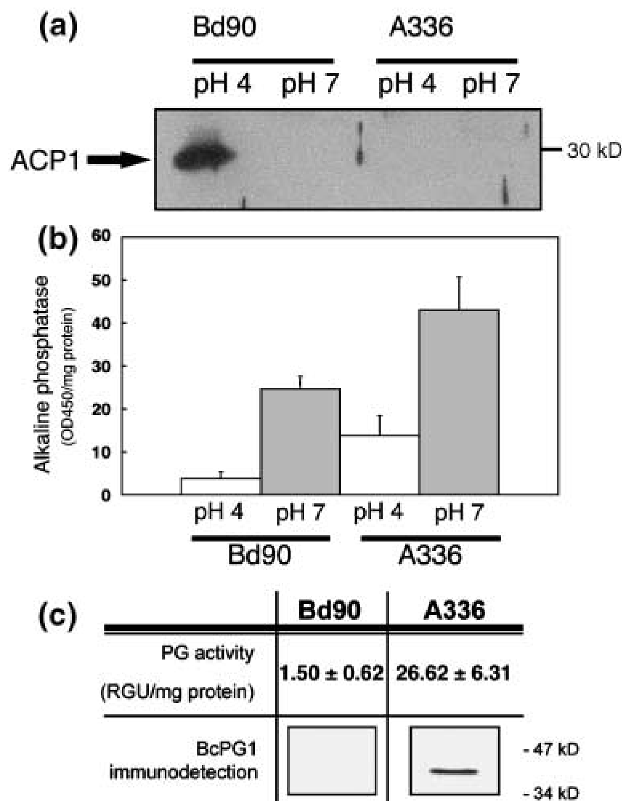

Fig. 3 Production of secreted enzymes nonaspartyl acid protease 1 (ACP1), alkaline phosphatase and *Botrytis cinerea* endopolygalacturonase 1 (BcPG1). (a, b) ACP1 and alkaline phosphatase were detected in culture filtrates of *B. cinerea* wild-type strain Bd90 and mutant A336 grown at pH 4 and 7. (a) ACP1 was detected using polyclonal antibodies raised against *Sclerotinia sclerotiorum* ACP1. (b) Alkaline phosphatase was determined by measuring p-nitrophenol absorbance at 450 nm. (c) BcPG1 was analysed in culture filtrates of wild-type strain Bd90 and mutant A336 grown at pH 5.5 on glucose medium. The polygalacturonase activity of 2 μg of total protein from culture filtrates was determined spectrophotometrically using polygalacturonic acid as substrate and the 2-cyanoacetamide method. One reducing group unit (RGU) corresponded to 1 μmol of reducing sugar released per min at 30°C. Data represent the mean ± standard deviation of duplicate assays from one representative experiment out of three. Immunodetection with a specific antibody raised against BcPG1 was performed on 2 μg of total protein from each culture filtrate.

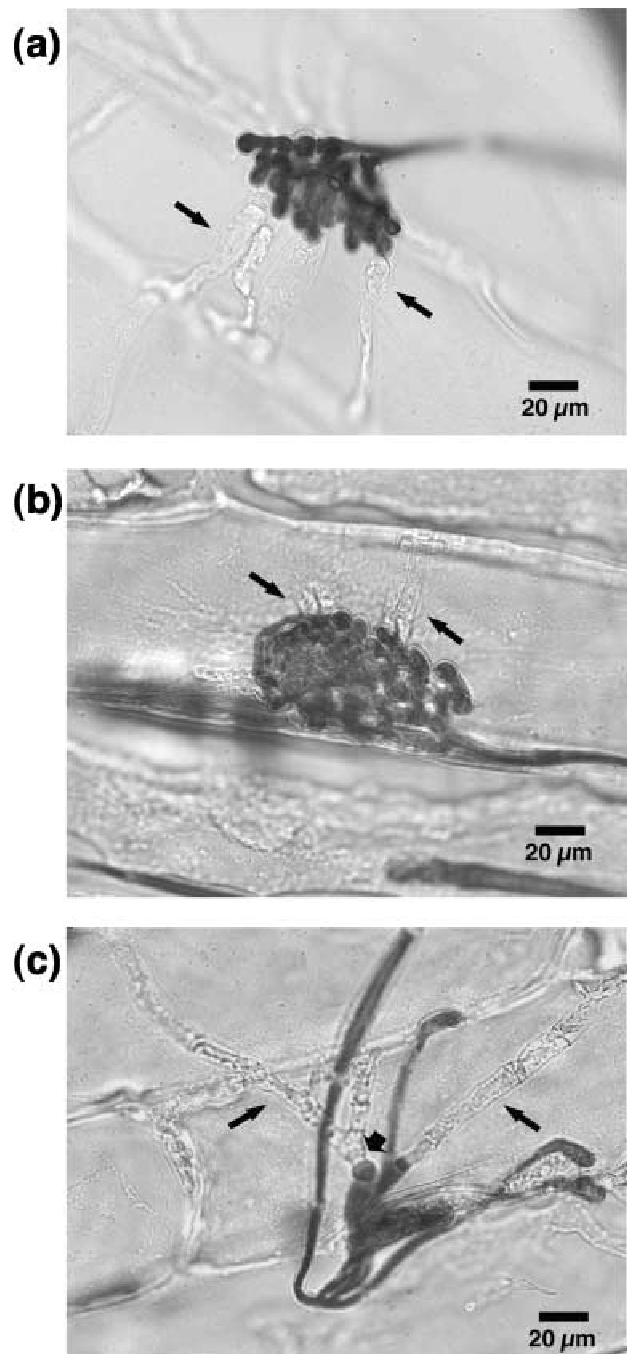

Fig. 4 Penetration assays on onion epidermal tissue. Mycelium plugs of *Botrytis cinerea* wild-type strain Bd90 (a) and mutant A336 (b, c) were applied to the upper side of onion epidermis and pictures were taken 16 hrs post inoculation (hpi). Hyphae were stained with methyl blue and observations made using light microscopy. Arrows indicate internal hyphae, and the arrowhead shows the penetration site in (c).

wild-type strain (Fig. 5a). In comparison, only small HR-like necrotic lesions near the inocula appeared on the mutant-infected leaves (Fig. 5b), and the same results were obtained when the experiments were performed with bean leaves (data not shown). As early as 24 hpi, multiple fluorescent spots could be observed in the mutant-infected leaves by epifluorescence microscopy (Fig. 5d). In exactly the same observation field, but using light microscopy, these fluorescent areas corresponded to healthy green tissue (Fig. 5f) and they disappeared with the formation of the dark-brown necrotic lesions 4 d after inoculation (Fig. 5b). It should be pointed out that, in Figs 5d and f, we focused on A336 hyphae, which are above the surface of the leaf, so the plant leaf cells are out of focus. In comparison, the Bd90 wild-type hyphae were observed within the healthy green plant tissues beyond the maceration front

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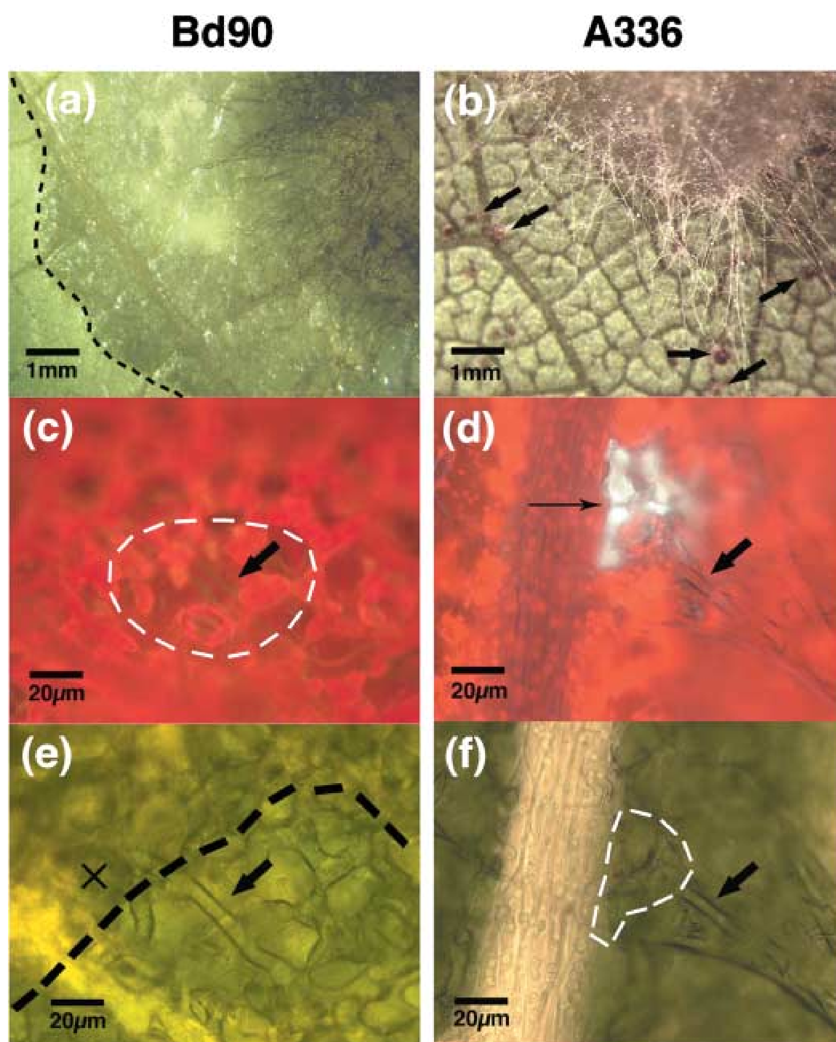

Fig. 5 Hypersensitive response (HR)-like necrosis and fluorescence on infected grapevine (*Vitis vinifera* cv. Chardonnay) leaves. Mycelium plugs of *Botrytis cinerea* wild-type strain Bd90 and mutant A336 were applied to the lower side of unwounded detached grapevine leaves. (a, b) Observations made with a binocular microscope. The frontier between macerated tissue and healthy tissue for strain Bd90 is indicated with a dotted line. The arrows indicate HR-like necrotic lesions for mutant A336. (c, d) Observations made using epifluorescence microscopy. The long thin arrow indicates the autofluorescent spot in the presence of mutant A336 hyphae. The area showing slightly enhanced fluorescence in the presence of Bd90 hyphae is surrounded by a dotted line. Thick arrows indicate fungal hyphae. (e, f) Observations made using light microscopy. The frontier between macerated tissue (marked with a cross) and healthy tissue for strain Bd90 is indicated with a dotted line. For mutant A336 the area that is strongly fluorescent in (d) is surrounded by a dotted line. Arrows indicate fungal hyphae. Images for wild-type strain Bd90 (c, e) and for mutant A336 (d, f) are for the same respective observation fields.

(Fig. 5e) and only faint and diffused fluorescence were seen in this area (Fig. 5c). A similar difference in fluorescence between the mutant and wild-type strains was also observed in bean leaves and onion epidermal strips (data not shown). These results show that mutant A336 stops its infection process soon after penetration of the plant. This is associated with the production of fluorescent material by the plant at the contact sites and the development of HR-like necrotic lesions.

Mutant A336 elicits a stronger host-plant defence response

The different outcomes of the fungal–plant interaction for the wild-type and mutant strains could be related to the differences in their production of extracellular enzymes (see 'Altered regulation of secreted enzymes in mutant A336'). The different outcomes could also be a result of a stronger plant defence response towards the mutant strain. To test this possibility, the mutant and wild-type strains were grown in glucose medium at pH 5.5 and the dialysed and concentrated culture filtrates were used to treat grapevine cell suspensions. AOS production by the plant cells was then measured. As shown in Fig. 6, a significant and transient H_2O_2 production by the plant cells was detected 20 min after treatment with the mutant filtrate, whereas its wild-type counterpart triggered a very small reaction. Interestingly, the treatment of the grapevine cells with increasing amounts of the PG inhibitor PG inhibitor protein 2 (PGIP2) from *P. vulgaris*, a specific inhibitor of BcPG1 (Poinssot *et al.*, 2003), caused up to 60% reduction in AOS production (Fig. 6b). These results indicate that the A336 mutant cells secrete an elicitor of the plant cell defence reaction in larger amounts than the cells of their wild-type counterparts. This elicitor activity seems mainly to derive from the BcPG1 protein.

Discussion

Total loss of pathogenicity in *B. cinerea* has been reported only rarely (Zheng *et al.*, 2000; Schulze Gronover *et al.*, 2001;

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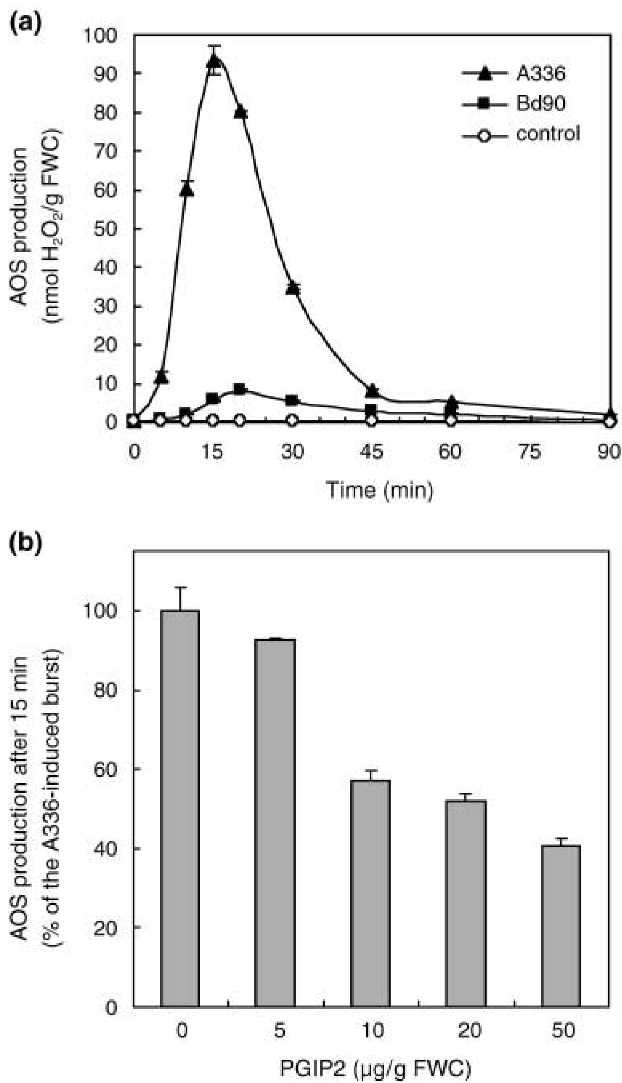

Fig. 6 Active oxygen species (AOS) production triggered by culture filtrates. (a) Kinetics of AOS production by grapevine (*Vitis vinifera* cv. Chardonnay) cells not treated (open circles), or treated with concentrated and dialysed culture filtrates from the *Botrytis cinerea* wild-type strain Bd90 (black squares) or the A336 mutant (black triangles) used at the same protein concentration [5 µg g^{-1} fresh weight of cells (FWC)]. Results are from triplicate assays and represent the mean ± standard deviation for one representative experiment out of three. (b) The oxidative burst triggered by the A336 culture filtrate is inhibited by increasing polygalacturonase inhibitor protein 2 (PGIP2) concentrations. The H_2O_2 production was measured in grapevine cells (1 ml) 15 min after treatment with the A336 culture filtrate (5 µg g^{-1} FWC). PGIP2 was added 30 s before culture filtrate treatment at the indicated concentration. Results are from triplicate assays and represent the mean ± standard deviation for one representative experiment out of two.

Gourgues *et al.*, 2004). Gene redundancy has frustrated attempts to produce nonpathogenic strains via disruption of a single gene or of small numbers of genes, and little is known today about the master regulations that govern the virulence process of this fungus.

In this paper, we describe the mutant A336 whose ability to infect plant tissues as diverse as apple fruit and grapevine, bean and *A. thaliana* leaves has been lost. When inoculated as mycelium onto onion epidermis, the mutant is able to penetrate the host but it forms penetration structures less frequently than the wild-type strain. Recent studies by Viaud *et al.* (2003) and Gourgues *et al.* (2004) showed that germinated conidia of *B. cinerea* form appressoria on onion epidermis, without previous ramification of germ tubes. In our study, using mycelium for inoculation, we have shown that the hyphae densely ramify in a claw-like structure, swell at their tips (appressoria) and then penetrate. This claw-like structure does not form when conidia of the same wild-type strain (Bd90) are used (Soulié *et al.*, 2006). To our knowledge, observation of this claw-like structure has not previously been reported, and suggests the existence of different methods of penetration for *B. cinerea* that have until now not been recognized.

The mutant has completely lost the ability to invade the host after penetration. Penetration and colonization of the plant constitute two distinct stages of the *B. cinerea* infection process (Tenberge, 2004), and mutant A336 hence appears only to be affected in the second stage of infection. So far, only one nonpathogenic mutant capable of penetration but impaired in tissue colonization has been described (Schulze Gronover *et al.*, 2001); this mutant lacks one subunit of a GTP-binding protein. Interestingly, mutant A336 differs from this mutant in its inability to produce conidia under normal *in vitro* growth conditions, and different components required for plant colonization are hence likely to be affected in the two strains.

HR-like lesions are visible on both bean and grapevine leaves at the penetration sites of the mutant, but are not visible in infection by the wild-type strain. Strong fluorescence can be observed in the plant cells neighbouring the penetrating mutant hyphae and significant production of hydrogen peroxide can be detected in culture of grapevine cells treated with dialysed fungal culture filtrates of the mutant. The accumulation of fluorescent phenolic compounds has often been observed in relation to efficient plant resistance to pathogens (Koga *et al.*, 1988), and the early production of AOS has been proposed to play a role in the onset of HR (Dixon *et al.*, 1994; Levine *et al.*, 1994; Jabs, 1999). On the one hand, Govrin & Levine (2000) proposed that *B. cinerea* triggers the HR to facilitate its spread within dead plant tissues. Dickman *et al.* (2001) also showed that inhibition of HR-related cell death in tobacco leads to enhanced resistance to *B. cinerea*. On the other hand, Derckel *et al.* (1999) showed that the aggressiveness of different *B. cinerea* wild-type strains correlated inversely with the strength of induction of plant defences. Similarly, Keller *et al.* (1999) showed that transgenic tobacco plants *Nicotiana Tabacum* L. responding to pathogen attack with localized HR-like necrosis were more resistant to *B. cinerea*. Finally, Unger *et al.* (2005) showed that the aggressiveness of *B. cinerea* is linked to an oxidative burst-suppressing agent that suppresses HR. The precise role of HR in the interaction between *B. cinerea* and its

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hosts thus remains to be elucidated. Our results are consistent with a difference in the mutant–plant interaction that leads to the development of a HR-like response that stops the progression of the mutant. This could arise from a stronger and faster reaction of the plant to the mutant than to the wild type, from a weakness of the mutant in overriding the plant defence system, or from both. Indeed, *B. cinerea* infection may require cell death in the plant to be able to proceed, but the timing and/or the relative strengths of the virulence and defence reactions may also affect the balance between the fungus and its host.

Mutant A336 is greatly affected in the production of oxalic acid, making it the first oxalate-deficient *B. cinerea* strain to be described. This deficiency explains the poor growth of the mutant on PGA medium as it prevents the acidification of the medium and, consequently, the action of the polygalacturonases needed to degrade the substrate (Favaron *et al.*, 2004; Kars *et al.*, 2005). Although the role of oxalic acid in the pathogenicity of *B. cinerea* is not yet well defined, it has been reported that oxalic acid chelates Ca^{2+} ions from pectin in the plant middle lamellae and in doing so facilitates the degradation of the plant cell wall by fungal enzymes (Bateman & Beer, 1965; Marciano *et al.*, 1983). In *S. sclerotiorum*, a necrotrophic fungus closely related to *B. cinerea*, a key role has been postulated for oxalic acid in pathogenesis and plant maceration (Godoy *et al.*, 1990, Guimaraes & Stotz, 2004) and its suppressor effect on the oxidative burst of the plant has also been shown (Cessna *et al.*, 2000). Finally, its role in plant pathogenicity has been shown in other fungi from various taxonomic classes (Dutton & Evans, 1996). The oxalate deficiency of mutant A336 therefore could contribute to its lack of virulence.

Mutant A336 is also affected in the production of extracellular enzymes; some of these enzymes, such as phosphatases and the endopolygalacturonase BcPG1, are produced in larger amounts than in the wild-type strain, while others, such as the acid protease ACP1, are not produced at all. This is also likely to contribute to the absence of virulence of the mutant strain. On the one hand, fungal secreted proteases participate in the degradation of the plant tissues during infection and could weaken the plant defence system by targeting the plant defence proteins. The total absence of ACP1 in the mutant could hence diminish its proteolytic potential. On the other hand, BcPG1 has been shown to activate defence reactions against *B. cinerea* in grapevine (Poinssot *et al.*, 2003) and therefore appears to act both as a lytic enzyme for the fungus (Ten Have *et al.*, 1998; Kars *et al.*, 2005) and as an elicitor for the plant (Poinssot *et al.*, 2003). In this study, BcPG1 was shown to be abundant in the mutant A336 culture filtrates, and these filtrates triggered a strong oxidative burst in grapevine cells that could be largely reduced by the specific BcPG1 inhibitor PGIP2. The overproduction of BcPG1 in the mutant therefore is likely to boost the plant defence reaction and to act against the fungus during the interaction with the plant.

As ACP1 is essentially produced under acidic conditions (Poussereau *et al.*, 2001), and as oxalate production is impaired in the mutant, we contemplated the possibility that a link existed between the modification of the production of this enzyme and the absence of acid secretion during pathogenesis of the mutant. We also investigated whether a perturbation of the pH sensing system resulted in reduced oxalate and altered enzyme production. However, pH regulation of phosphatase production is not disturbed in mutant A336 and addition of oxalic acid alongside the mutant fungus during pathogenicity tests did not restore virulence (Fig. 7). The absence of oxalate production alone therefore does not seem to be responsible for the nonpathogenic phenotype of the mutant, and pH sensing seems to be functional in this strain.

One feature of mutant A336 is an inability to produce sclerotia and to show a modified pattern of conidiation. Conidia do not form when the fungus grows on rich medium but do form when the mycelium is placed under conditions in which it cannot grow (on plant tissues or on PGA medium at pH 7). As deficiency in oxalate production is associated with the loss of sclerotia production in *S. sclerotiorum* (Godoy *et al.*, 1990), the absence of sclerotia in the mutant is also likely to result from its lack of acid production. This strongly supports the existence of a link between oxalate and sclerotia development whose molecular basis is completely unknown. Conidia formation has mainly been studied in *Aspeigillus nidulans* (for a review, see Adams *et al.*, 1998) and *Neurospace crassa* (for a review, see Springer, 1993). In the former, it requires the acquisition of developmental competence (Champe *et al.*, 1981), developmental induction (Morton, 1961; Mooney & Yager, 1990) and/or the signal for starvation-related stress (Skromne *et al.*, 1995). In *N. crassa*, it can be triggered by a drop in nutrients in the growth medium (Toledo & Hansberg, 1995). Two different pathways therefore seem to control this process: one involved in the setting up of a developmental programme, and one involved in the reaction to a stress. The involvement of G-protein signalling and regulators in the first pathway in *A. nidulans* has been recently demonstrated (for a review, see Yu & Keller, 2005) while components of the stress counterpart await identification. Our results on mutant A336 suggest that this strain is only impaired in the first conidiation-related signalling pathway. The mutant is thus affected in the unfolding of two developmental programmes that lead to either sclerotia or conidia formation. The possibility that oxalate could also play a role in conidiation is ruled out by the failure to restore the production of conidia in the mutant by adding oxalate to the culture medium.

Overall, our data describe a mutant deregulated in several aggressiveness factors, leading to total loss of pathogenicity accompanied by HR-like lesions. We cannot, at present, exclude the possibility that other aggressiveness factors not described in this work are also disturbed in mutant A336. The genetic analysis demonstrated that all the characteristics of the mutant cosegregated and only one locus (*BcPTH1*) is therefore responsible for the observed phenotype. As this phenotype is pleiotropic, we propose that a gene regulator might be mutated in this

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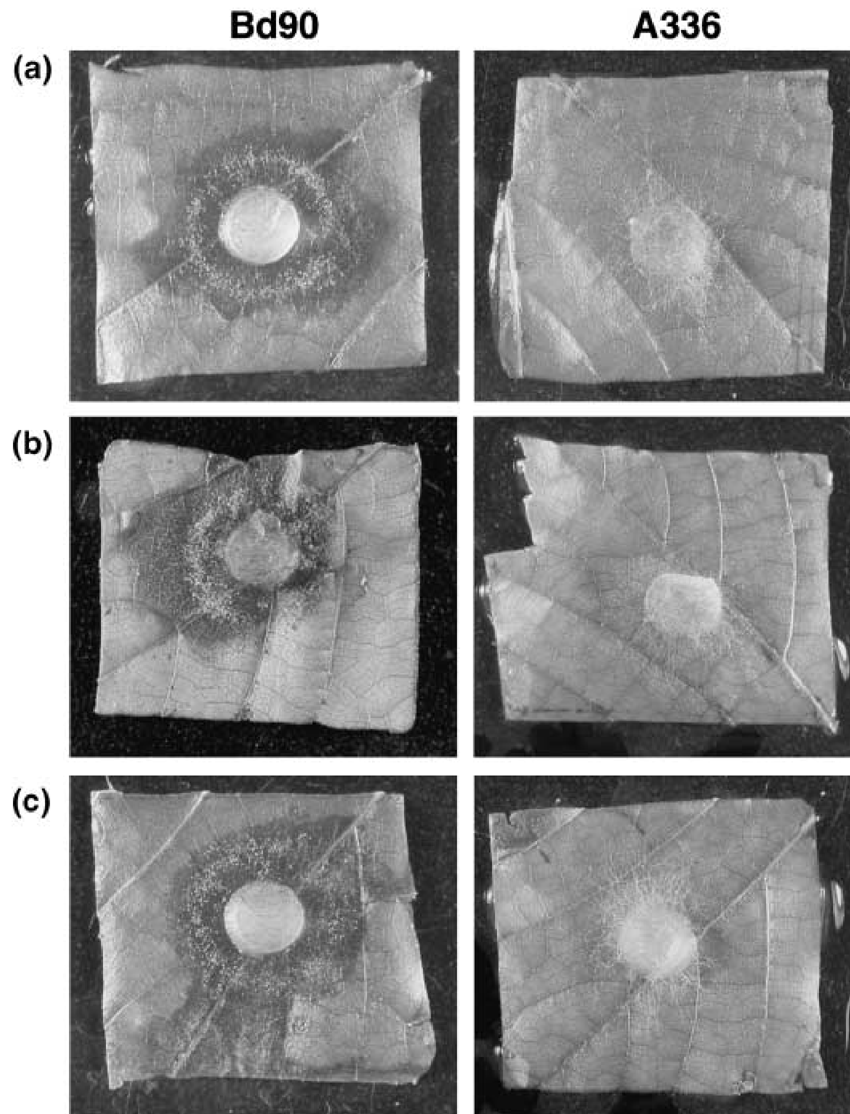

Fig. 7 Maceration symptoms on grapevine (*Vitis vinifera* cv. Chardonnay) leaf discs in the presence of oxalic acid. Mycelium plugs of *Botrytis cinerea* strain Bd90 and mutant A336 were applied to the upper side of unwounded leaf discs placed on oxalic acid solutions: (a) 0 mM, (b) 1 mM and (c) 10 mM. The oxalic acid solutions had been adjusted beforehand to pH 5. Maceration symptoms are visible as dark tissue around the mycelium plug for wild-type strain Bd90.

strain. We established that the mutation is not tagged by the plasmid used for the mutagenesis, a situation that has previously been reported by Tudzynski & Siewers (2004). Further, the lack of an established system for functional complementation studies in this fungus prevented us from identifying the mutated gene(s). However, the recent advances in *B. cinerea* complete genome sequencing (http://www.ncbi.nlm.nih.gov/genomes/leuks.cgi?p3=11:Fungi&taxgroup=11:Fungi|12: and http://www.genoscope.cns.fr/externe/English/Projets/Projet_LN/LN.html) will make the identification of the mutated gene in locus *BcPTH1* possible. Indeed, microsatellite markers are currently being developed to build a genetic map of the *B. cinerea* genome. This will allow a positional cloning strategy to identify the genomic region cosegregating with the pathogenicity phenotype. Furthermore, because these markers will be anchored on the *B. cinerea* complete genome sequence, the search for potential candidate genes for the mutated gene in mutant A336 will be facilitated. It is also possible that the overall effect of the mutation on metabolic and signalling pathways might be elucidated by transcriptome and metabolome analysis. Thus the fungal component(s) revealed by this study, which clearly plays an essential role in virulence, awaits further characterization.

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Supplementary material

The following supplementary material is available for this article online:

Table S1 *Botrytis cinerea* transformants with reduced virulence and simple plasmid (pAN7-1) insertion.

This material is available as part of the online article from http://www.blackwellpublishing-synergy.com

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