European Journal of Plant Pathology **105:** 273–283, 1999.

Evidence for three different specific saponin-detoxifying activities in *Botrytis cinerea* and cloning and functional analysis of a gene coding for a putative avenacinase

Thomas Quidde, Peter Büttner and Paul Tudzynski*
*Westf. Wilhelms-Universität, Institut für Botanik, Schlossgarten 3, D-48149 Münster, Germany; *Author for correspondence (Phone: +49-251-83 24998; Fax: +49-251-83 23823; E-mail: tudzyns@uni-muenster.de)*

Accepted 4 February 1999

Key words: saponin degradation, tomatine, avenacin, avenacoside

Abstract

Saponins are typical phytoanticipins, i.e. preformed fungitoxic compounds suspected to play an important role in plant defense. Several phytopathogenic fungi are able to detoxify these compounds by deglycosylation, and this ability could be a factor of general importance for the successful colonization of saponin-containing plants. *Botrytis cinerea*, causing 'grey mould' of many (especially greenhouse) crops and vegetables, among them several plants containing saponins, has been reported to deglycosylate and detoxify α-tomatine. Here we show that it can also degrade avenacin, avenacosides and digitonin, and that it possesses more than one saponin-specific enzyme. Using the tomatinase gene (*tom1*) from *Septoria lycopersici* as probe, we have cloned and characterized a gene (*sap1*) coding for a 83 kDa polypeptide which shows significant homology to *tom1* and to the avenacinase gene of *Gaeumannomyces graminis.* Disruption of *sap1* leads to loss of the ability to deglycosylate avenacin, whereas *sap1* deletion mutants still can detoxify tomatine, digitonin and avenacosides, i.e. *sap1* encodes an 'avenacinase'. The putative product of this gene has been purified and characterized: it has a MW of 90,000, an isoelectric point (IP) of 5.2, and it has no activity against avenacosides. Since a tomatinase-deficient field isolate of *B. cinerea*, M3, also lacks the ability to deglycosylate digitonin, but can degrade avenacin and avenacosides, these data confirm the existence of at least three distinct activities of saponin-specific glycosidases in *B. cinerea*: a xylosidase (α-tomatinase, digitoninase), and two different glucosidases (avenacinase and avenacosidase).

Introduction

Botrytis cinerea Pers.: Fr is a ubiquitous broad host range pathogen, causing considerable damage on many economic important crops. Efficiency of chemical control of the fungus is limited due to its genetic variability, and reliable resistance genes are not available. Considerable efforts have been undertaken to identify pathogenicity or virulence factors for *B. cinerea*, as a basis for the development of alternative control systems. In addition to biochemical and physiological studies on the role of toxins (Collado et al., 1996; Rebordinos et al., 1996), cell wall degrading enzymes (Verhoeff, 1978; Urbanek and Zalewska-Sobczak, 1984; Movahedi and Heale, 1990), and active oxygen species (Edlich et al., 1989; Lyr et al., 1995; von Tiedemann, 1997), in the last years molecular genetic studies also have been applied which allow unequivocal proof for or against the essential importance of a single factor. For example, a cutinase gene that was deleted by transformation-mediated gene disruption was found to be not essential for pathogenicity (van der Vlugt-Bergmans et al., 1997; van Kan et al., 1997); on the other hand inactivation of one of at least five polygalacturonase genes seems to reduce agressiveness of the fungus (ten Have et al., 1998).

The ability of *B. cinerea* to detoxify plant chemical defense agents has also been implicated as a potential pathogenicity determinant (Verhoeff and Liem, 1975; Pezet et al., 1991; Sandrock and VanEtten, 1998; Sbaghi et al., 1996). Recently we confirmed earlier reports that most *B. cinerea* strains can deglycosylate the tomato phytoanticipin α-tomatine; the only strain of the sample of field isolates tested which could not degrade α-tomatine turned out to be highly sensitive to the drug and was non-pathogenic on tomato, implicating that the ability to detoxify this defense agent plays an important role in this interaction (Quidde et al., 1998). Here we report that *B. cinerea* can degrade/detoxify other plant saponins and that this ability is conferred by at least two additional independent enzymatic activities, one of which is encoded by *sap1*, a β-glucosidase gene from *B. cinerea*.

Materials and methods

Strains and culture conditions

B. cinerea strains used in this study were previously described in Quidde et al. (1998). Strains were grown on potato dextrose agar (Oxoid) at 22 °C under near UV light for seven to 14 days to induce sporulation. Conidia were harvested by rinsing the surface of a sporulating culture with sterile distilled water. Spores were separated from mycelial debris by filtering through sterile glass wool. Shake cultures were performed by inoculating 10^5 spores/ml in a medium containing B5 salts (Duchefa) supplemented with 2% glucose for 48 h at 22 °C and 150 rpm. *Escherichia coli* TOP 10 F′ (Invitrogen) was used routinely for cloning and propagation of plasmids. Lambda clones were propagated in *E. coli* K803.

Protein extracts and purification scheme of avenacinase activity

Preparation of crude extracts was performed with shake cultures of 500 ml culture volume. Incubation of 48 h pre-grown mycelium was for 96 h at 22 °C, 150 rpm on a rotary shaker in citrate-phosphate buffer (200 mM, pH 5.4) supplemented with a concentrated methanolic extract of 1 g (fresh weight) four days old *Avena* roots. Culture filtrate was separated by filtration through nylon meshes (Nytal). $(NH_4)_2SO_4$ was added to 50% saturation, the solution was stirred at 0 °C for 60 min and subsequently centrifuged for 30 min at 20,000 × *g* $(NH_4)_2SO_4$ was added to the cleared supernatant to 96% saturation, stirred and centrifuged as above. The pellet was resuspended in distilled water and dialysed against the buffer for ConA-Sepharose chromatography (50 mM NaAcetate, 200 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, pH 6.0). ConA-Sepharose (Sigma) was suspended in buffer and added to the protein extract at 4 °C, suspension was gently shaken for 1 h. The slurry was filled into an empty PD10 column and washed with buffer until no protein was detectable in the flowthrough; bound glycoprotein was eluted by adding 30 ml of 0.5 M methyl-mannose. The protein solution was dialysed against 25 mM Bis-Tris, pH 6.0 and loaded onto a MonoP column (Pharmacia). Protein was eluted with 50 ml of 10% polybuffer74 adjusted to pH 4.0 (HCl) at a flow of 1 ml/min β-glucosidase containing fractions were identified by measuring para-nitrophenylglucopyranosid (pNPG) hydrolysing activity according to Margolles-Clark et al. (1996) and avenacinase activity was identified by rechecking pNPGase containing fractions by incubation with 1 µg avenacin for 2 h at 30 ° in citrate–phosphate buffer followed by thin layer chromatography.

Fractions containing avenacinase activity were dialysed against 20 mM Tris, pH 8.0 (HCl) and loaded onto a Fractogel EMD-TMAE 650(S) (Merck) anion exchange column. The column was washed with the same buffer until the absorbance at 280 nm reached the baseline. Bound protein was eluted with a 0–300 mM NaCl linear gradient at a flow rate of 1 ml/min (total volume 50 ml). Avenacinase activity containing fractions were pooled and dialysed against 50 mM phosphate, 200 mM NaCl (pH 6.5) buffer, concentrated by Centricon (Amicon, 30 kDa cut off) and loaded onto a Superose 12, HR10/300 (Pharmacia) size exclusion column at a flow rate of 0.25 ml/min. Fractions with avenacinase activity were subjected to SDS-PAGE.

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Total protein from active fractions was precipitated by adding 3 vol. cold acetone and centrifuged at 20,000 × *g*, 4 °C for 30 min; protein pellets were dried under vacuum. SDS-PAGE was performed according to Laemmli (1970) in a vertical mini gel system (BIO-RAD) at 100 V, proteins were visualized by Coomassie staining according to Merril (1990).

Gas chromatography and thin layer chromatography

For gas chromatographic analyses, dialysed enzyme preparations were incubated with 50 µmol digitonin in citrate/phosphate buffer (200 mM, pH 5.3) for 24 h at 30 °C followed by lyophilisation and resolving in pyridine. Derivatisation and GC analyses were performed as decribed by Quidde et al. (1998). Thin layer chromatographic analyses of avenacins and desgluco-avenacosides were performed on silica gel 60 plates (Schleicher and Schüll) in a solvent system consisting of chloroform, methanol and water (26 : 13 : 1 v/v). Avenacins were visualized by their autofluorescence under UV light (365 nm). Desgluco-avenacosides were visualized by their fluorescence under UV light after spraying with 0.5% para-anisaldehyde in methanol, sulphuric acid (49 : 1 v/v) and heating at 120 °C for 10 min. Saponin degradation products of fungal transformants were analysed by incubating pregrown mycelia with different saponins (50 µmol each) for 24 h in citrate/phosphate buffer at 22 °C. Culture filtrate was separated from mycelia by filtering over sterile glass wool, followed by lyophilisation and resolving saponins in chloroform/methanol (1 : 1, v/v).

Standard molecular techniques

Standard molecular techniques such as cloning, gel electrophoresis, DNA blotting, labelling of DNA fragments, PCR, RT-PCR and DNA preparation from *E. coli* were performed according to Sambrook et al. (1989) and Ausubel et al. (1987). Isolation of genomic DNA from *B. cinerea* was performed according to Cenis (1992) from 30 h grown shake cultures. mRNA for RT-PCR analysis was extracted from lyophylized and pulverized mycelium using Dynabeads (Dynal) according to the manufacturer's protocol. Sequences of PCR primers used in this study: tC: 5′CCCAGAATGCACAGGTACAC 3′, P2: 5′ATAGATCTCGGTTTCCGCCT 3′

Screening of the genomic EMBL3 library was performed by plaque filter hybridization according to Sambrook et al. (1989). Nucleotide sequences of DNA fragments cloned in pUC19 (Yanish-Perron et al., 1985), pBluescript II (Short et al., 1988), or pCR2.1 (Invitrogen) were determined in an automated sequencer (LiCor 4000) using the Thermo sequenase fluorescent labelled cycle sequencing kit (Amersham). Sequence analysis and computing of phylogenetic distances were performed using the GCG software package (GCG, Wisconsin).

Genomic library

A genomic library from *B. cinerea* SAS 56 was constructed by partial digestion of DNA with *Sau*3A followed by size fractionation of DNA in a sucrose gradient centrifugation step. Fragments of about 18–20 kb were ligated into Lambda EMBL3 arms and packaged using the packaging extract from Stratagene according to the manufacturer's protocol.

Transformation of B. cinerea

Transformation of *B. cinerea* B05.10 was performed according to Hamada et al. (1994) with the modifications described by van Kan et al. (1997) using a vector containing the phleomycin resistance cassette from pAN8-1 (Mattern et al., 1988). Protoplasts were regenerated on osmotically stabilized medium containing 6 µg/ml phleomycin (Cayla) at pH 8 (5 mM HEPES/NaOH). Primary transformants were cultivated on selection medium (B5 salts, 2% glucose, 40 µg/ml phleomycin, 1.5% agar, pH 8). Transformants were genetically purified by three rounds of plating spores on potato dextrose agar supplemented with 0.05% Triton X-100 to restrict colony diameter and subsequent growth on selection medium for sporulation.

Growth inhibition tests

Growth inhibition tests were performed by applying a mycelial plug of sporulating *B. cinerea* cultures (5 mm diameter) to the center of a Petri dish containing 20 ml 1.5% water agar. Whatman filter papers (5 mm diameter) were loaded with avenacin and the solvent was evaporated under a hood. Filter papers were subsequently placed onto the water agar at a distance of 25 mm from the mycelial plug. To avoid photodestruction of avenacins, plates were incubated in the dark until the colony edge reached the control filter paper (loaded with solvent). Growth restriction was documented photographically at the time point when the colony edge reached the control filter paper.

Pathogenicity tests

Biotests were performed according to Benito et al. (1996) on detached bean leaves (*Phaseolus vulgaris* cv. Doublette). Inoculated leaves were incubated at high humidity at 22 °C for 4 days (bean) in transparent plastic boxes under near UV light, and photographically documented.

Results

Degradation of saponins by B. cinerea

Recently we could prove that most strains of *B. cinerea* can detoxify α-tomatine by removing the terminal xylose to yield β1-tomatine (Quidde et al., 1998). Strain M3, which lacks this tomatinase activity, is non-pathogenic on tomato, indicating that specific deglycosylation of the major tomato saponin could be of relevance for the pathogenicity of *B. cinerea* on tomato. Since *B. cinerea* successfully infects a wide variety of plants, several of which are known to have saponins as defense agents, we wanted to test the ability of *B. cinerea* to degrade saponins other than α-tomatine. The range of saponins we could include in our test was limited by their availability: we tested the potato saponins α-solanine and α-chaconine, the *Avena* saponins avenacin and avenacosides A and B (see Osbourn, 1996), and digitonin from *Digitalis*. As shown in Figure 1A, the standard strain B05.10 is able to metabolize α-tomatine (as reported), avenacin and the two 26-desgluco-avenacosides, but not α-chaconine and α-solanine. Since the two 26-desgluco-avenacosides are metabolized to the same end product (the substrates

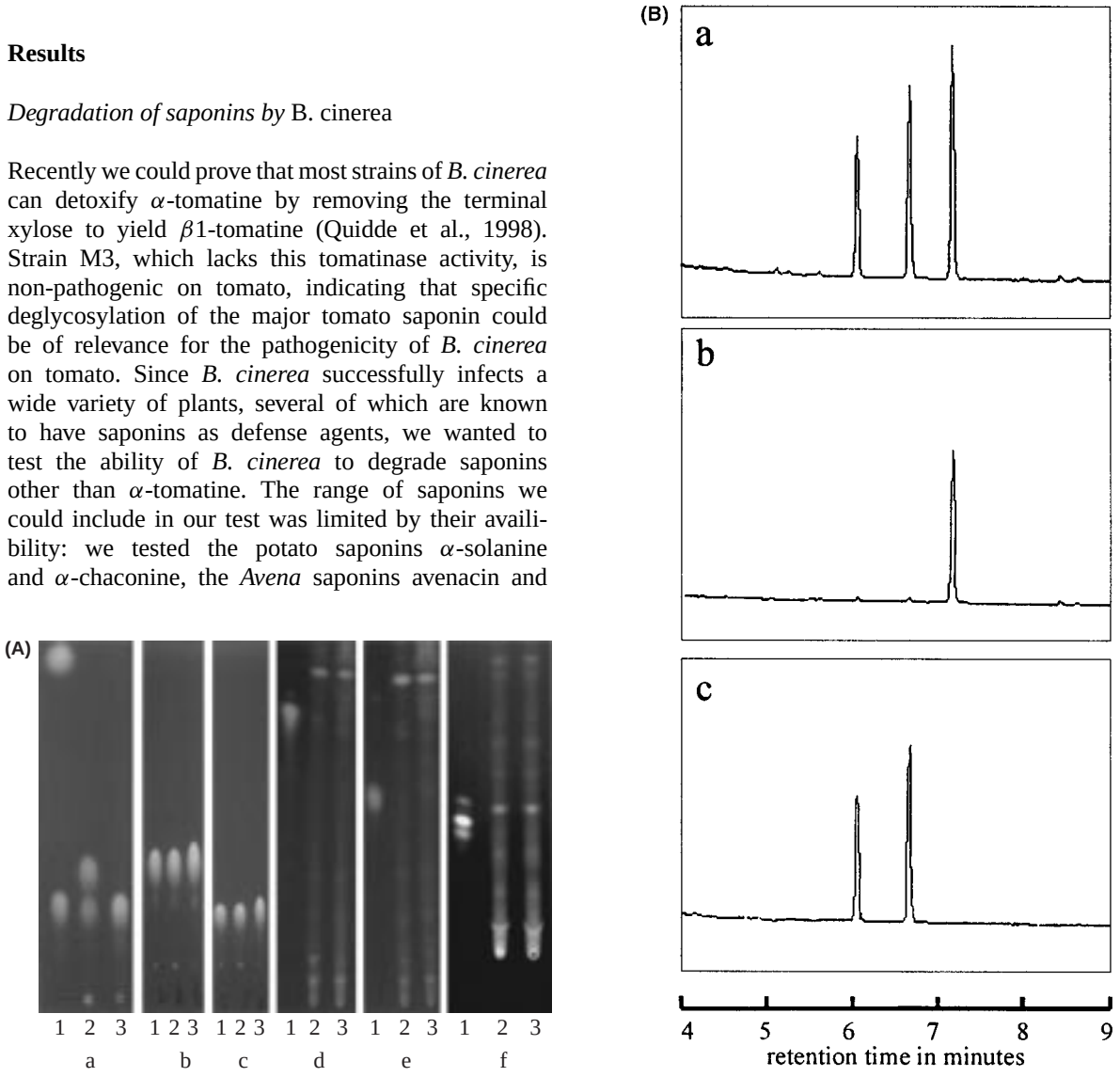

Figure 1. Saponin deglycosylation by *Botrytis cinerea.* A: TLC analysis. 1: substrates, 2,3: reaction products 24 h after inoculation for strain B05.10 and M3, respectively. (a) α-tomatin, (b) α-chaconin, (c) α-solanin, (d) 26-desgluco-avenacoside A, (e) 26-desgluco-avenacoside B, (f) avenacin. In sample A1 tomatidine was added for comparison. B: GC analyses of digitonin degradation by *B. cinerea*. (a) strain B05.10, (b) strain M3, (c) D-xylose standard. Digitonin standard: identical to b (not shown).

differ by the number of terminal glucose molecules), probably the glucose moieties are hydrolized. The same may be true for the deglycosylation of the avenacin, and therefore the enzymes involved are probably glucosidases. In addition, *B. cinerea* strain B05.10 is able to remove a xylose from digitonin (Figure 1B), as has been reported for the degradation of α-tomatine (Quidde et al., 1998). This indicates that there are at least two different enzymatic activities involved in deglycosylation of saponins by *B. cinerea*. We tested this assumption by analysing the ability of strain M3 (lacking tomatinase activity) to degrade the other saponins. As shown in Figure 1, strain M3 is able to deglycosylate all tested saponins except α-tomatine and digitonin, confirming that tomatinase/digitoninase activity is distinct from avenacinase/26-desgluco-avenacosidase activity.

Cloning and characterization of a saponinase gene

In order to be able to perform functional analyses of the role of the saponin-detoxifying enzymes in the pathogenicity of *B. cinerea*, we decided to clone the corresponding gene(s). We tested whether the available saponinase genes, the avenacinase gene from *Gaeumannomyces graminis* var. *avenae* and the tomatinase gene from *Septoria lycopersici* (*tom1*) could be used as heterologous probes. *Tom1* showed sufficient homology to genomic DNA of *B. cinerea*, as shown by Southern hybridization at moderately stringent conditions (60 °C hybridization temperature). It was used as probe to screen a genomic EMBL3 library of *B. cinerea* strain SAS56. Positive clones were analysed by restriction analysis and Southern hybridization. Restriction enzyme fragments that hybridized to the *tom1* probe were subcloned and sequenced and shown to contain a DNA region with significant homology to *tom1*. Sequence analysis of the whole region revealed the presence of a long ORF of 2414 bp, interrupted by an intron of 69 bp (the presence of which was confirmed by cDNA sequencing). Since the first 14 amino acids probably represent a signal peptide, the mature product of the gene (which we have called *sap1*) has a calculated molecular weight of 83 kDa, an IP of 6.0, and it contains nine potential N-glycosylation sites (data not shown). The derived amino acid sequence shows the highest homology to major parts of both, the avenacinase gene of *G. graminis* and *tom1* of *S. lycopersici* (66.8% and 69.5%, resp.), as well as homology, albeit lower, to other fungal family 3 glycosidases (e.g. *bgl1* of *Trichoderma reesei* 57.3%; see also Figure 8). A sequence alignment of the two saponinases to the *sap1* putative gene product is given in Figure 2.

Southern hybridization of genomic DNA of strain SAS56 using the internal 1.65 kb *Eco*RI fragment of the *sap1* region as probe, indicate that *sap1* is a single copy gene (Figure 4). Since even at moderate stringency (60 °C) no additional bands were observed, there seem to be no closely related genes in the *B. cinerea* genome.

Targeted inactivation of sap1

In order to elucidate the function of the *sap1* gene product we decided to inactivate the gene using a gene replacement approach. An internal *Eco*RV fragment (478 bp) within the 1650 bp *Eco*RI fragment of the *sap1* coding region was replaced by the phleomycin-resistance cassette (3451 bp) of vector pAN8-1 (Mattern et al., 1988) to yield the replacement vector pΔsap1phleo^R (Figure 3). The 4.6 kbp *Eco*RI fragment of this vector was used to transform *B. cinerea* strain B05.10, a haploid derivative of strain SAS56 (Büttner et al., 1994, and unpubl. data). Fifteen primary transformants were selected from 3 experiments and were checked by PCR for the presence of the replaced copy using a primer binding to sequences within the phleo^R cassette and a second one binding within the *sap1* coding region but outside the region contained in pΔsap1phleo^R (Figure 3). A PCR-amplified fragment of the expected size (750 bp) was obtained from three of the transformants (Bc135, Bc139, Bc146). The transformants were genetically purified by three rounds of single spore isolation and analysed by Southern hybridization. All three transformants lack the wild type *sap1* fragment, but have a larger fragment which corresponds to the expected size of the replaced fragment (see Figure 4A). Transformant Bc135 has an additional ectopic integration, whereas the other two transformants have not, as was also confirmed by hybridization with the phleo^R cassette (Figure 4B). These data show that the replacement approach was successful, and that 3 of 15 transformants contained the desired replacement at the *sap1* locus.

Characterization of sap1 *deletion mutants*

The *sap1* deletion mutants Bc135, Bc139 and Bc146 were checked for their capability to deglycosylate saponins. All three can still metabolize α-tomatine,

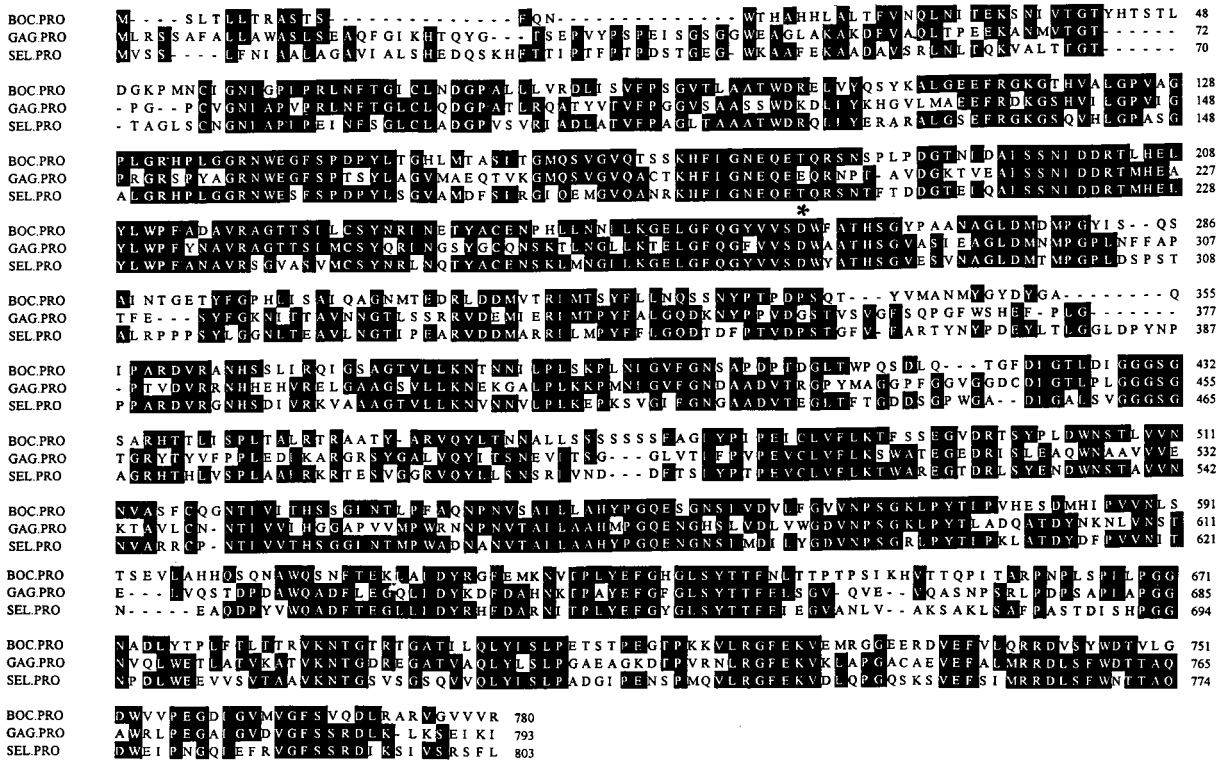

Figure 2. Sequence alignment between the *Botrytis cinerea sap1* gene product, *Septoria lycopersici* tomatinase and *Gaeumannomyces graminis* var. *avenae* avenacinase. Sequences were aligned by using the CLUSTAL method. Homologous residues are highlighted in black. The aspartic acid residue (position 260) which has been directly implicated as the catalytic residue (Bause and Legler, 1980) is marked with an asterisk. Abbreviations: Boc: *Botrytis cinerea sap1* gene product (accession number AJ130890), Gag: *Gaeumannomyces graminis* var. *avenae* avenacinase (accession number U35463), Sel: *Septoria lycopersici* tomatinase (accession number U35462).

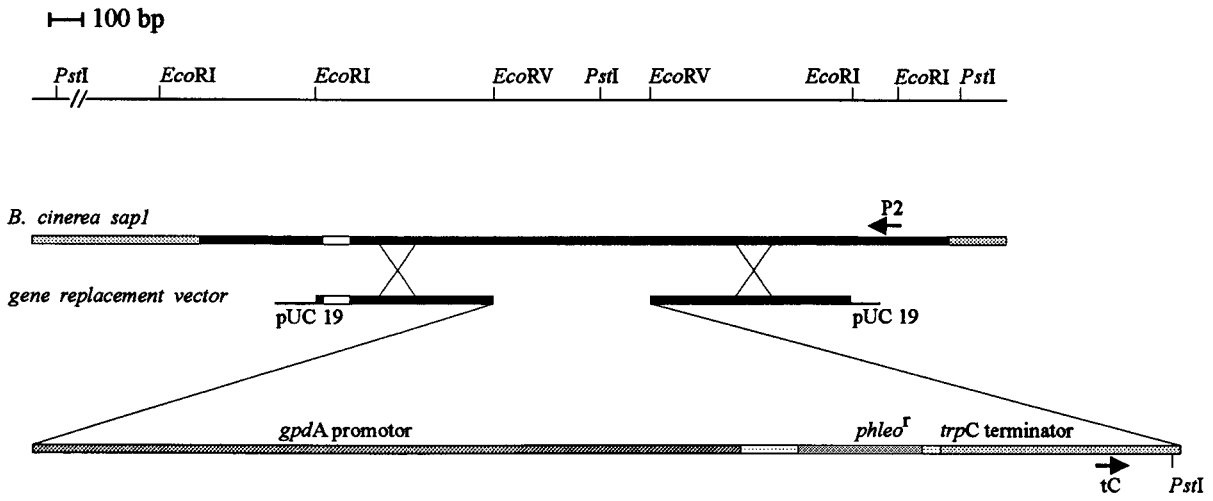

Figure 3. Cloning strategy of the gene replacement vector pΔ*sap*1*phleo*^{R}. A 478 bp *Eco*RV internal fragment of the *sap1* coding region was replaced by a 3451 bp fragment of vector p*AN8-1*, carrying the phleomycin-resistance cassette. Binding sites for PCR primers P2 and tC are indicated.

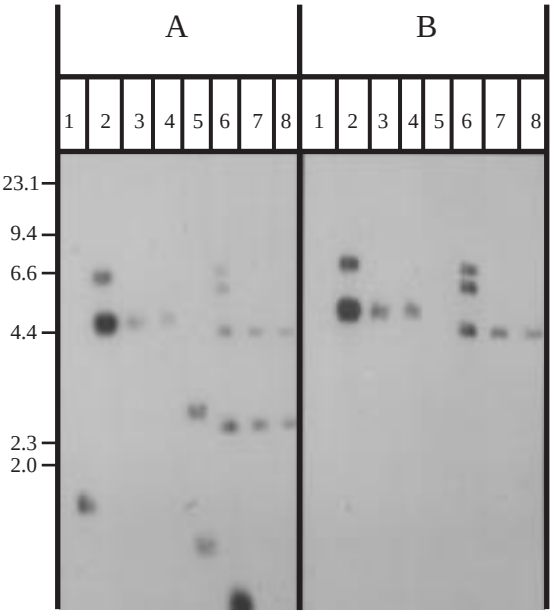

Figure 4. Southern analysis of *B. cinerea* transformants obtained with the pΔ*sap*1*phleo*^{R} construct. Genomic DNA of the recipient strain (1,5) and three transformants (2,6: Bc 135; 3,7: Bc 139; 4,8: Bc 146) was digested with *Eco*RI (1–4) and *Pst*I (5–8), separated on agarose gels, blotted on nylon membrane, and hybridized with the 1650 bp *Eco*R1 fragment of the *sap1* gene (A) and the phleo^R cassette from *pAN8-1* (B).

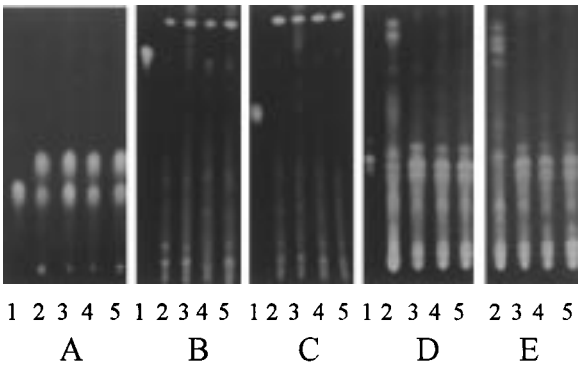

Figure 5. TLC analysis of saponin deglycosylation by *B. cinerea* strains. (1) substrate, (2) B05.10, (3) Bc135, (4) Bc139, (5) Bc146. (A) α-tomatin, (B) 26-desgluco-avenacoside A, (C) 26-desgluco-avenacoside B, (D) avenacin (24 h p.i.), (E) avenacin (48 h p.i).

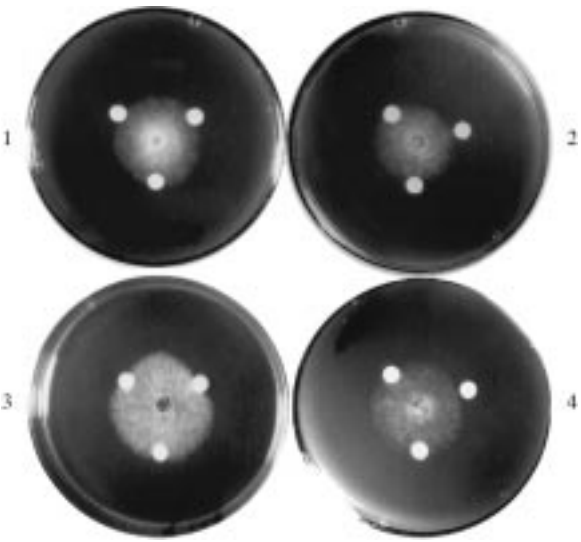

Figure 6. Sensitivity of *B. cinerea* strains to avenacin. The filters contain different amounts of avenacin: bottom: 0 μg; left top: 2 μg; right top: 10 μg. Plate 1: B05.10; 2: Bc135; 3: Bc139; 4: Bc 146.

26-desgluco-avenacoside A and B, but no longer avenacin (Figure 5). Even after 48 h of incubation avenacin was not affected; obviously the *sap1* gene product is specific for the deglycosylation of avenacin, but is not required for the detoxification of the avenacosides. In order to test if inactivation of *sap1* has a physiological effect with respect to tolerance against avenacin, a growth inhibition test was performed (Figure 6). It is obvious that the three deletion mutants are more sensitive to the highest concentration of avenacin than is strain B05.10. An *in planta* test showed no significant difference in pathogenicity on tomato and bean leaves between the *sap1*-inactivated transformants and the recipient strain (data not shown).

Purification and characterization of the putative sap1 *gene product*

To allow a characterization of the 'avenacinase' probably encoded by *sap1*, the enzyme was purified following the scheme optimized for the purification of the tomatinase of *B. cinerea* (Quidde et al., 1998). After induction with avenacin (see material and methods) avenacinase activity from axenic culture of *B. cinerea* strain SAS56 was quantitatively bound to concanavalinA-sepharose and further purified through a chromatofocusing column. Avenacinase activity eluted at pH 5.2–5.3. After anion-exchange chromatography and size exclusion chromatography the active fractions contained a single band in SPS-PAGE, with a molecular weight of about 90,000 (Figure 7), which is slightly larger than the calculated MW of the *sap1* gene product (83 kDa). The difference

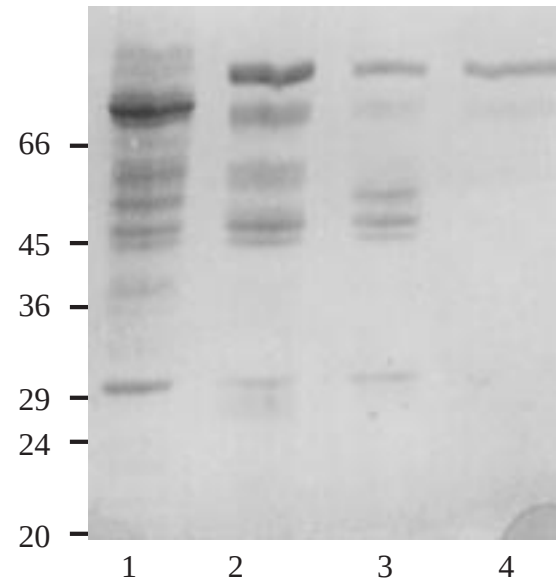

Figure 7. SDS-PAGE showing fractions containing avenacin hydrolyzing activity from *B. cinerea* isolate B05.10 at different stages of purification. Molecular weight markers (in kDa) are indicated. Proteins were visualized by Coomassie staining. (1) after conA sepharose purification, (2) after Mono P chromatofocusing, (3) after anion exchange chromatography, (4) after size exclusion chromatography.

is most likely due to glycosylation. This band showed in a Western blot analysis no cross reaction with a polyclonal antiserum raised against the *G. graminis* var. *avenae* avenacinase (Osbourn, pers. comm.).

Discussion

We have cloned the first gene from *B. cinerea* encoding a saponin-detoxifying enzyme. The derived gene product of *sap1* shows significant homology to family 3 β-glycosidases (Henrissat, 1991; Henrissat and Bairoch, 1996) in general, but the degree of amino acid homology is highest when compared to the two so-far characterized fungal saponinases, avenacinase from *G. graminis* (Bowyer et al., 1995) and tomatinase from *S. lycopersici* (Osbourn et al. 1995; Sandrock et al., 1995). As shown in Figure 8, a tree analysis groups these three enzymes together, as a distinct subgroup of the fungal family 3 β-glucosidases, which are clearly separated from the corresponding bacterial enzymes. Interestingly, these enzymes are further distinct from the other fungal enzymes as their

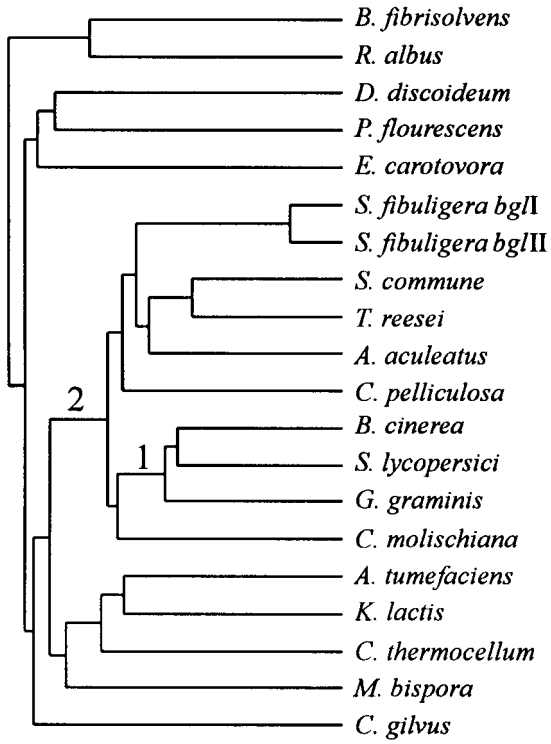

Figure 8. Tree analysis of family 3 β-glycosidases using the program 'Pileup' (GCG). Accession numbers of sequences: *Aspergillus aculeatus* P48825, *Agrobacterium tumefaciens* P27034, *Butyrivibrio fibrisolvens* M31120, *Cellvibrio gilvus* D14068, *Candida molischiana* JC4376, *Clostridium thermocellum* P14002, *Dictyostelium discoideum* L21014, *Erwinia carotovora* M32399, *Gaeumannomyces graminis* U35463, *Kluyveromyces lactis* X05918, *Microspora bispora* JC4825, *Pseudomonas flourescens* X65527, *Ruminococcus albus* X15885, *Saccharomycopsis fibuligera bgl*I M22475, *Saccharomycopsis fibuligera bgl*II M22476, *Septoria lycopersici* U24701, *Trichoderma reesei* U09580. 1: saponinases; 2: fungal enzymes.

corresponding genes contain introns; the intron of *sap1* is even localized at the same postition as the third intron of *tom1* (data not shown), indicating a potentially close evolutionary relationship of these two genes. Nevertheless, the substrate specificity of these enzymes is different: it has been shown that the *G. graminis* var. *avenae* avenacinase and TOM1 have only minor cross reactivity (Osbourn et al., 1995), and correspondingly SAP1 shows no activity on α-tomatine nor on avenacosides, indicating that a major part of the specificity may be based on the aglycon moiety. Nevertheless, the comparison of the three so-far sequenced saponinase encoding genes does not allow to discriminate saponinase-specific domains that

could separate these enzymes specifically from other family 3 β-glycosidases. It will be necessary to obtain sequence data from 'non-glucosidase' saponinases, e.g. the chaconinase of *Gibberella pulicaris* (Weltring et al., 1997), a rhamnosidase, or the tomatinase of *B. cinerea*, a xylosidase (Quidde et al., 1998). According to the genomic Southern analysis reported here the gene coding for the latter enzyme is not expected to have major sequence homology to *sap1*. The situation becomes even more complex if the side-activity of the *B. cinerea* tomatinase against digitonin is considered: here obviously a terminal β-1,3-linked-xylose is hydrolized from two saponins differing in their backbone and their carbohydrate components. Therefore far more sequence information (including 3D structure analyses) is needed to obtain a better understanding of the substrate specificity of this highly interesting type of detoxifying enzymes.

The product of the *sap1* gene has been identified as an avenacinase-like enzyme by creating a deletion in the ORF. A corresponding enzyme was partially purified from the wild type strain. To prove that this enzyme is indeed the product of the *sap1* gene (SAP1) requires partial sequencing of the protein or Western analyses demonstrating lack of the protein in the deletion mutant. However, the data presented here strongly suggest that the purified avenacinase corresponds to *sap1*: (1) no second avenacinase activity could be detected in the protein extracts; (2) the *sap1* deletion mutants showed no residual avenacinase activity, and (3) the molecular parameters of SAP1 as derived from the *sap1* sequence fit very well to the data obtained for the purified enzyme. The apparent molecular mass determined by SDS-PAGE (90 kDa) and the IP (5.2–5.3) differ from the calculated values based on the *sap1* sequence (83 kDa and 6.0, respectively). However, this difference could very well be due to glycosylation: the derived putative SAP1 sequence shows nine potential N-glycosylation sites (Bairoch, 1993). In addition, the purified avenacinase efficiently binds to concanavalin A. Also, the corresponding enzymes from *G. graminis* var. *avenae* and *S. lycopersici* are glycosylated (Bowyer et al., 1995; Sandrock et al., 1995), even more than SAP1: their corresponding 'calculated' MW is comparable to that of the putative *sap1* product (80–85 kDA), whereas the purified proteins are even larger than the purified avenacinase (about 110 kDa). This large size differentiates these saponin-specific glucosidases from other saponinases like the tomatinases from *B. cinerea* and *F. oxysporium* f. sp. *lycopersici* (70 and 50 kDa, resp. [Quidde et al., 1998; Lairini et al., 1996]), supporting the assumption that the saponinases having different glycosidic-bond specificities differ considerably.

The data presented here and in a previous paper (Quidde et al., 1998) show that *B. cinerea* is able to detoxify a wide range of saponins, probably far more than tested here. This is in contrast to the situation in *G. graminis* var. *avenae* and *S. lycopersici*, where sofar only one saponinase activity has been described, but well in accordance with analyses of other necrotrophic, broad host range pathogens like *Fusarium sambuccinum* (Weltring et al., 1997; Weltring et al., 1998) and could reflect the difference between highly specialized and broad-host-range pathogens. *B. cinerea* contains at least three different enzymatic activities (tomatinase/digitoninase; avenacinase; avenacosidase) and probably more, each of them obviously highly specific. The question arises why pathogens like *B. cinerea* have developed so many specific detoxification enzymes, which seems to be uneconomic at first glance. It will be interesting to learn more about the regulation of the different genes involved, especially with respect to the specificity of their induction and the receptors/signal chains involved in the substrate recognition.

The role of saponinases in the pathogenicity of *B. cinerea* remains to be evaluated. The significantly reduced virulence on tomato of a *B. cinerea* strain lacking tomatinase activity (Quidde et al., 1998) indicates that this enzyme might influence host specificity in *B. cinerea*. The *sap1* deletion mutants show no reduction of virulence on tomato and bean. However, to our knowledge, these species do not contain avenacin-like saponins and therefore this result was expected. The question remains for which of its numerous hosts this specific saponinase activity could be important; most probably not for *Avena*, since *B. cinerea* never has been reported to attack *Avena*, and – even more important – *B. cinerea* is no root pathogen, whereas avenacin is localized in *Avena* roots.

To elucidate the importance of saponin detoxification by *B. cinerea*, the genes coding for the tomatinase and the avenacosidase activity need to be identified, cloned and inactivated. Pathogenicity tests using the various saponinase deficient mutants on all major host plants of *B. cinerea* have to be performed. This approach might lead to the definition of a new target for specific fungicides or the development of new resistance breeding strategies for the major host plants.

Acknowledgements

We would like to thank Dr. Anne Osbourn (Norwich) for the generous gift of avenacosides and the *tom1* gene probe of *S. lycopersici*, and helpful advice, Dr. Jan van Kan and co-workers (Wageningen) for intensive discussions, A. Jülich for avenacin and A. Kammerahl for typing the manuscript, the Deutsche Forschungsgemeinschaft and the EC (HCM network 'Molecular phytopathology') for financial support.

References

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA and Struhl K (1987) Current Protocols in Molecular Biology, John Wiley and Sons, New York

Bairoch A (1993) The PROSITE dictionary of sites and patterns in proteins, its current status. Nucl Acids Research 21: 3097–3103

Bause E and Legler G (1980) Isolation and structure of a tryptic glycopeptide from the active site of β-glucosidase A3 from *Aspergillus wentii*. Biochim Biophys Acta 626: 459–465

Benito EP, Prins T and van Kan JAL (1996) Application of differential display RT-PCR to the analysis of gene expression in a plant–fungus interaction. Plant Molecular Biology 32: 947–957

Bowyer P, Clarke BR, Lunness P, Daniels MJ and Osbourn AE (1995) Host range of a plant pathogenic fungus determined by a saponin detoxifying enzyme. Science 267: 371–374

Büttner P, Koch F, Voigt K, Quidde T, Risch S, Blaich R, Brückner B and Tudzynski P (1994) Variations in ploidy among isolates of *Botrytis cinerea*: implications for genetic and molecular analysis. Curr Genet 25: 445–450

Cenis JL (1992) Rapid extraction of fungal DNA for PCR amplification. Nucl Acids Res 20: 2380

Collado IG, Hernandezgalan R, Prieto V, Hanson J and Rebordinos L (1996) Biologically active sequiterpenoid metabolites from the fungus *Botrytis cinerea*. Phytochem 41: 513–517

Edlich W, Lorenz G, Lyr H, Nega E and Pommer EH. (1989) New aspects in the infection mechanism of *Botrytis cinerea.* Neth J Plant Pathol 95: 53–62

Hamada W, Reignault P, Bompeix G, Boccara M (1994) Transformation of *Botrytis cinerea* with the hygromycin B resistance gene, hph. Curr Genet 26: 251–255

Henrissat B (1991) A classification of glycosylhydrolases based on amino acid sequence similarities. Biochem J 280: 309–316

Henrissat B and Bairoch A (1996) Updating the sequence-based classification of glycosyl hydrolases. Biochem J 316: 695–696

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriopahge T4. Nature 277: 680–685

Lairini K, Perez-Espinosa A, Pineda M and Ruiz-Rubio M (1996) Purification and characterization of tomatinase from *F. oxysporum* f. sp. *lycopersici*. Appl Env Microbiol 62: 1604–1609

Lyr H, Weigend M and Wegener A (1995) The role of various enzymes in the infection mechanism of *Botrytis cinerea.* In: Lyr H, Russell PE, Sisler HD (eds.) Modern funigcides and antifungal compounds. (pp 207–215) Intercept Ltd., Andover

Margolles-Clark E, Tenkanen M, Nakari-Setälä T and Pentillä M (1996) Cloning of genes encoding α-L-arabinofuranosidase and β-xylosidase from *Trichoderma reesei* by expression in *Saccharomyces cerevisiae*. Appl Env Microbiol 62: 3840–3846

Mattern IE, Punt PJ and van den Hondel CAMJJ (1988) A vector for *Aspergillus* transformation conferring phleomycin resistance. Fungal Genet Newslett 35: 25

Merril CR (1990) Gel-Staining methods. In: Deutscher MP (ed.) Methods in Enzymology. Vol. 182 (pp 477–488) Academic Press, San Francisco

Movahedi S and Heale JB (1990) The roles of aspartic proteinase and endo-pectin lyase enzymes in the primary stages of infection and pathogenesis of various host tissues by different isolates of *Botrytis cinerea* ex. Pers. Physiol Mol Plant Pathol 36: 303–324

Osbourn AE (1996) Preformed antimicrobial compounds and plant defense against fungal attack. The Plant Cell 8: 1821–1831

Osbourn AE, Bowyer P, Lunness P, Clarke B and Daniels MJ (1995) Fungal pathogens of oat roots and tomato leaves employ closely related enzymes to detoxify host plant saponins. Mol Plant-Microbe Interactions 8: 971–978

Pezet R, Pont V and Hoang-Van K (1991) Evidence for oxidative detoxication of pterostilbene and resveratrol by a laccase-like stilbene oxidase produced by *Botrytis cinerea*. Physiol Mol Plant Pathol 39: 441–450

Quidde T, Osbourn AE and Tudzynski P (1998) Detoxification of α-tomatine by *Botrytis cinerea*. Physiol Mol Plant Pathol 52: 11–165

Rebordinos L, Cantoral JM, Prieto V, Hanson JR and Collado IG (1996) The phytotoxic activity of some metabolites of *Botrytis cinerea*. Phytochem 42: 383–387

Sambrook J, Fritsch EF and Maniatis T (1989) Molecular cloning: A Laboratory Manual. 2nd Ed. Cold Spring Harbor Laboratory Press, New York

Sandrock RW and VanEtten HD (1998) Fungal sensitivity to and enzymatic degradation of the phytoanticipin α-tomatine. Phytopathology 88: 137–143

Sandrock RW, DellaPenna D and VanEtten HD (1995) Purification and characterization of $\beta 2$-tomatinase, an enzyme involved in the degradation of a-tomatine and isolation of the gene encoding $\beta 2$-tomatinase from *Septoria lycopersici*. Mol Plant-Microbe Interactions 8: 960–970

Sbaghi M, Jeandet P, Bessis R and Leroux P (1996) Degradation of stilbene type phytoalexins in relation to the pathogenicity of *Botrytis cinerea* to grapevines. Plant Pathology 45: 139–144

Short JM, Fernandez JM, Sorge JA and Huse WD (1988) Lambda ZAP: a bacteriophage lambda expression system with *in vivo* excision properties. Nucl Acids Res 16: 7583–7600

ten Have A, Mulder W, Visser J and van Kan JAL (1998) The endopolygalacturonase gene *Bcpg1* is required for full virulence of *Botrytis cinerea*. Mol Plant-Microbe Interactions 11: 1009–1016

Urbanek H and Zalewska-Sobczak J (1984) Multiplicity of cell wall degrading glycosidic hydrolases produced by apple infecting *Botrytis cinerea*. Phytopathol Zeitschrift 110: 261–271

van der Vlugt-Bergmans CJB, Wagemakers CAM and van Kan JAL (1997) Cloning and expression of the Cutinase A gene of *Botrytis cinerea.* Mol Plant-Microbe Interactions 10: 21–29

van Kan JAL, van't Klooster JW, Wagemakers CAM, Dees DCT and van der Vlugt-Bergmans (1997) Cutinase A of *Botrytis cinerea* is expressed, but not essential, during penetration by gerbera and tomato. Mol Plant-Microbe Interactions 10: 30–38

Verhoeff K (1978) The role of cell wall degrading enzymes in the pathogenesis of *Botrytis cinerea* in tomato plants. Annales Phytopathol 10: 137–144

Verhoeff K and Liem JI (1975) Toxicity of tomatine to *Botrytis cinerea*, in relation to latency. Phytopathol Zeitschrift 82: 333–338

von Tiedemann A (1997) Evidence for a primary role of active oxygen species in induction of host cell death during infection of bean leaves with *Botrytis cinerea*. Physiol Mol Plant Pathol 50: 151–166

Weltring KM, Wessels J and Geyer R (1997) Metabolism of the potato saponins α-chaconine and α-solanine by *Gibberella pulicaris*. Phytochem 46: 1005–1009

Weltring KM, Wessels J and Pauli GF (1998) Metabolism of the tomato saponin α-tomatine by *Gibberella pulicaris*. Phytochem 46: 1321–1328

Yanish-Perron C, Vieira J and Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene 33: 103–119