

Cutinase A of *Botrytis cinerea* is Expressed, but not Essential, During Penetration of Gerbera and Tomato

J. A. L. van Kan, J. W. van't Klooster, C. A. M. Wagemakers, D. C. T. Dees, C. J. B. van der Vlugt-Bergmans

Department of Phytopathology, Wageningen Agricultural University, P.O. Box 8025, 6700 EE Wageningen, The Netherlands

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The plant pathogen *Botrytis cinerea* can infect undamaged plant tissue directly by penetration of the cuticle. This penetration has been suggested to be enzyme-mediated, and an important role for cutinase in the infection process has been proposed. In this study the expression of the cutinase encoding gene *cutA* of *B. cinerea* was analyzed using a *cutA* promoter-GUS reporter gene fusion. Transformants containing the fusion construct were examined for GUS expression on gerbera flowers and tomato fruits. High GUS activity was detected from the onset of conidial germination and during penetration into epidermal cells, indicating that *cutA* is expressed during the early stages of infection. To determine the biological relevance of cutinase A for successful penetration, cutinase A-deficient mutants were constructed by gene disruption. Pathogenicity of two transformants lacking a functional *cutA* gene was studied on gerbera flowers and tomato fruits. Their ability to penetrate and cause symptoms was unaltered compared to the wild-type strain. These results exclude an important role for cutinase A during direct penetration of host tissue by *B. cinerea*.

Additional keywords: gene disruption, grey mold, homologous recombination, pathogenicity gene, reporter gene.

The ubiquitous plant pathogen *Botrytis cinerea* Pers.:Fr. infects fruits, flowers, or green tissue of more than 200 plant species (Jarvis 1977). Direct penetration by germ tubes into undamaged plant tissue occurs via natural openings or through the cuticle (Verhoeff 1980). To breach the cutin layer, mechanical or enzymatic mechanisms have been suggested. Clark and Lorbeer (1976) reported the formation of appressorium-like structures that mediated penetration of onion leaves by mechanical force. McKeen (1974) and Rijkenberg et al. (1980) studied penetration of the cuticle of bean leaves and tomato fruits by electron microscopy. They observed a sharp pore without curled edges and no indentation of the cuticle or

epidermal wall during penetration by the infection peg. Moreover, the absence of appressorium-like structures indicated that penetration is an enzymatic process. Esterase activity at the tip of germ tubes (McKeen 1974) and the production of cutinase in liquid culture (Shishiyama et al. 1970; Baker and Bateman 1978) is consistent with a role for cutinase in penetration of the cuticle by *B. cinerea*. Involvement of cutinase in penetration by *B. cinerea* was indicated by the observation that monoclonal antibodies, raised against cutinase, reduced lesion formation on gerbera flowers by 80% when flowers were treated with this antibody after inoculation with conidia (Salinas 1992).

The cloning of the cutinase-encoding gene *cutA* of *B. cinerea* (Van der Vlugt-Bergmans et al. 1997) enables the use of a promoter-reporter gene fusion to investigate *cutA* gene expression during penetration of host tissue. The *uidA* reporter gene encoding β -glucuronidase (GUS, Jefferson et al. 1987) has been successfully used to study gene expression in fungal pathogens during colonization of host tissue (van den Ackerveken et al. 1994; Wubben et al. 1994). In this study, the GUS reporter system was employed to determine whether cutinase A is indeed produced by *B. cinerea* during penetration of host tissue, visualized as blue staining of fungal structures when GUS is expressed under control of the *cutA* promoter. To unequivocally determine the biological relevance of cutinase A for *B. cinerea* during penetration of its hosts, cutinase A-deficient mutants are required. The establishment of a transformation system for *B. cinerea* (Hilber et al. 1994; Hamada et al. 1994) and the availability of haploid *B. cinerea* strains (Büttner et al. 1994) allowed disruption of the single copy gene *cutA*, to obtain such mutants. Transformants lacking a functional *cutA* gene were evaluated for their ability to infect gerbera and tomato tissue.

RESULTS

Characterization of reporter gene transformants.

A fusion was made between the *cutA* promoter and the *uidA* reporter gene encoding β -glucuronidase (GUS, Jefferson et al. 1987). The terminator sequence of the *tubA* gene of *B. cinerea* (*TtubA*) was cloned upstream of the *cutA* promoter (*PcutA*) to minimize interference by regulatory sequences near the site of integration (Fig. 1A). The *trpC* terminator sequence from *Aspergillus nidulans* (*TtrpC*) was placed downstream of *uidA*. The resulting construct (pCutGUS) was introduced into *B.*

Corresponding author: J.A.L. van Kan;
E-mail: jan.vankan@medew.fyto.wau.nl

Present address of C. J. B. van der Vlugt-Bergmans: Wageningen Agricultural University, Department of Food Science, Division of Industrial Microbiology, P.O. Box 8129, 6700 EV Wageningen, The Netherlands.

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cinerea strain SAS56 by cotransformation with the plasmid pOHT. Twelve hygromycin B resistant transformants were analyzed for the presence of pCutGUS insertions by PCR using primers complementary to the *tubA* terminator sequence and the *uidA* coding sequence (Fig. 1A). In 10 of the 12 transformants a fragment of 2.0 kb was amplified, indicating that the *cutA* promoter-*uidA* reporter gene fusion was correctly cotransformed into the genome of *B. cinerea*. The number of pCutGUS insertions was determined by Southern blot analysis (Fig. 2). A blot containing genomic DNA of five transformants (T1, T2, T3, T5, T7) and recipient strain SAS56 digested with *EcoRV* was probed with an *EcoRV/HindIII* fragment of pCutGUS (Fig. 1A) containing parts of the *uidA* coding region and *trpC* terminator. T2 and T5 contained a single hybridizing fragment whereas T1, T3, and T7 contained a large number of hybridizing fragments.

To assess whether these transformants exhibit GUS activity upon induction with the cutin monomer 16-hha (van der Vlugt-Bergmans et al. 1997), transformants and the wild-type strain SAS56 were grown in liquid B5 medium containing 0.3% glucose. After 2 days of growth 16-hha was added (day 0) and incubation continued for 3 days. Samples were taken for intracellular GUS activity measurements. Low GUS activity

(<1 unit) was detected in transformants T2 and T7 at day 0. In both transformants GUS activity increased on days 1 and 2 after addition of 16-hha (Table 1) and it decreased again on day 3. In the recipient strain SAS56 lacking the pCutGUS construct, no significant GUS activity was detected (Table 1).

To investigate in closer detail whether *uidA* mRNA and *cutA* mRNA are expressed similarly from the *cutA* promoter under different cutinase inducing and repressing conditions (van der Vlugt-Bergmans et al. 1997), expression of the genes was also studied by RNA blot analysis. Transformants T2 and T7 were grown in a liquid culture for 2 days and subsequently diluted (day 0) in three different media: medium A contained 16-hha, medium B contained 16-hha and glucose and in medium C only glucose was present. The glucose concentration in media B and C decreased to undetectable levels between day 1 and day 2 after transfer of the mycelium.

Mycelia from the start culture and media A to C were sampled at day 0 and day 1 to 3, respectively, for isolation of RNA. Results of the RNA blot analysis are shown in Figure 3. Both *cutA* and *uidA* transcripts of 900 and 2,000 nt, respectively, were detected from day 1 onwards when mycelium was grown in the presence of 16-hha (medium A). In medium B, transformant T2 expressed *cutA* and *uidA* transcripts at days 2 and 3 when glucose had been depleted from the medium, indicating that glucose repression of the *cutA* promoter affects the expression of the *uidA* gene as well. In the absence of the inducer (T2, medium C), only weak hybridization signals of *cutA* and *uidA* were present. Hybridization of the blot with a

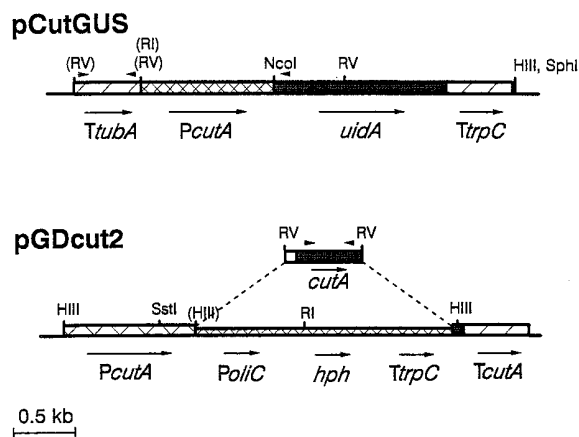


Fig. 1. Schematic representation of plasmids pCutGUS and pGDcut2 used for transformation of *Botrytis cinerea*. *TtubA* represents a terminator sequence of the *B. cinerea* β -tubulin gene. *PcutA* represents the promoter region of the cutinase A gene from *B. cinerea*. *uidA* (β -glucuronidase) and *hph* (hygromycin phosphotransferase) are *Escherichia coli* genes, *TtrpC* and *PoliC* are terminator and promoter sequences, respectively, from *Aspergillus nidulans*. Arrows indicate the orientation of the fragment. Small arrowheads above the boxes represent primers used for PCR. RI = *EcoRI*, RV = *EcoRV*, HIII = *HindIII*. Restriction sites between brackets were deleted during subcloning.

Table 1. GUS activity in mycelia of transformants T2 and T7 and the wild-type strain SAS56a

Day	T2	T7	SAS56
0	0.95	0.39	0.04
1	11.89	5.45	0.07
2	12.85	6.38	0.06
3	9.60	1.56	0.02

^a At day 0 the cutin monomer 16-hha was added. Units of GUS activity are defined in nmol 4-methyl umbelliferone produced/h/10 μ g protein. Data of one experiment are shown and were reproducible in a second independent experiment.

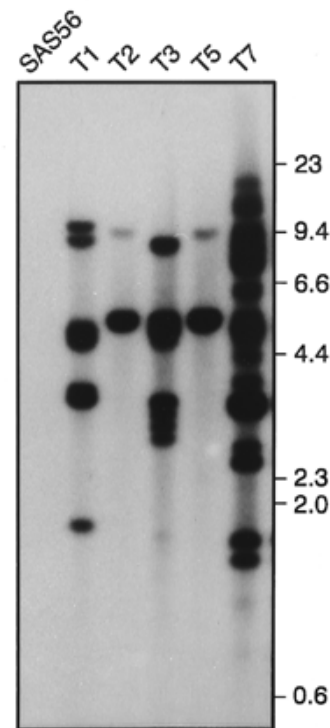


Fig. 2. Autoradiograph of a Southern blot containing genomic DNA of *Botrytis cinerea* wild-type strain SAS56 and the transformants T1, T2, T3, T5 and T7, digested with *EcoRV*. The blot was hybridized with the *EcoRV/HindIII* fragment of pCutGUS (Fig. 1A), containing parts of the *uidA* coding region and *trpC* terminator. Molecular size markers are indicated in kbp in the right margin.

probe derived from the constitutively expressed β -tubulin gene of *B. cinerea* (*tubA*, accession number Z69263 of the GenEMBL database) showed similar amounts of fungal RNA in each lane on the blot. Co-expression of *cutA* and *uidA* was also observed in transformant T7, except for the samples harvested at day 1 (medium B) and days 2 and 3 (medium C). Moreover, hybridization of the blot containing T7 RNA with the *uidA* probe shows an additional larger transcript, which is coregulated with the 2,000 nt *uidA* transcript. Since the pCut-GUS construct contained a terminator sequence upstream of the *cutA* promoter, this transcript probably results from termination downstream of the *trpC* terminator. Smears are observed below the *uidA* transcript and the hybridization signal decreases compared to that of *cutA* at day 3 (media A and B), suggesting that *uidA* mRNA is more rapidly turned over than *cutA* mRNA. Nevertheless it can be concluded from these analyses that the *cutA* gene and the *uidA* gene under control of the *cutA* promoter are synchronously induced in transformants T2 and T7. The induction by 16-hha and repression by glucose are consistent with the results described in the accompanying paper (Van der Vlugt-Bergmans et al. 1996).

Histochemical staining of GUS activity.

Conidia of both transformants T2 and T7, harvested from plates, did not contain any detectable GUS activity. When conidia were germinated on water agar containing X-Gluc, a pale blue colony was obtained. Addition of a suspension of 16-hha to the agar yielded colonies with a more intense blue color. When the conidia were germinated on X-Gluc plates

containing sucrose the resulting colonies were colorless (results not shown).

The activity of the *cutA* promoter during penetration of gerbera flowers and tomato fruits was examined in transformants T2 and T7 by monitoring GUS activity microscopically in a histochemical assay using X-Gluc as substrate. Both T2 and T7 were able to infect the hosts with the same timing and caused similar symptoms as the recipient strain SAS56. On gerbera flowers dry-inoculated with conidia of *B. cinerea*, necrotic lesions were clearly visible 24 h postinoculation (h.p.i.). Penetration of the cutin layer had occurred before that timepoint and, therefore, flower petals were harvested and examined for GUS activity at 6, 16 and 24 h.p.i.. At 6 h.p.i., several conidia showed a dark blue color, whereas other conidia were pale blue or colorless (Fig. 4A). At 16 h.p.i., nearly all conidia had germinated and were stained blue. Infection pegs were also stained dark blue (Fig. 4B). Penetration of host cells was observed and at 24 h.p.i. cell death was visible by yellow-brownish cell contents of penetrated epidermal cells (Fig. 4C). In SAS56, germination and penetration were observed with equal efficiency and timing. However, no blue staining was observed at any time during infection with SAS56 (not shown).

On tomato fruits *B. cinerea* developed more slowly than on gerbera flowers. Two days postinoculation (d.p.i.) 60% of the conidia had germinated and 40% of the total number of conidia had penetrated into epidermal cells. All fungal structures, except for some ungerminated conidia, were stained dark blue and penetration of host tissue was visible (Fig. 4D). At 3 d.p.i., up to 90% of the conidia had germinated and 50% had penetrated. Death of single epidermal cells was visible in the microscope, although no necrotic lesions were seen by eye.

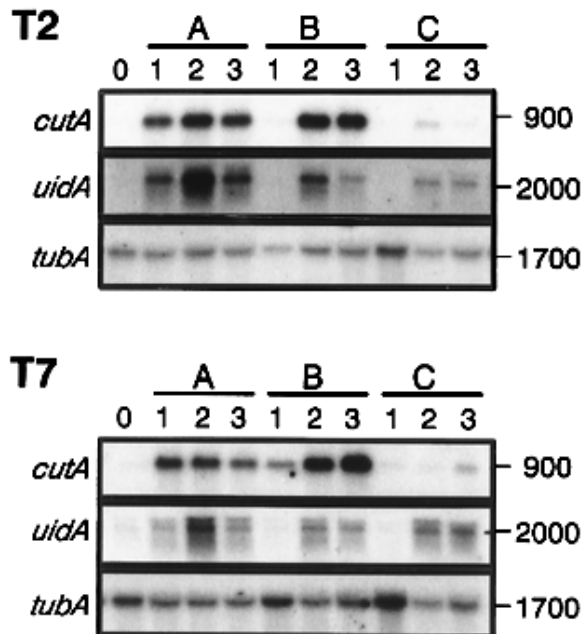


Fig. 3. Autoradiograph of an RNA blot containing total RNA of *Botrytis cinerea* transformants T2 and T7, grown under different conditions. Mycelium from a liquid start culture (lane 0) was diluted into fresh medium containing 16-hydroxyhexadecanoic acid (A), 16-hydroxyhexadecanoic acid and glucose (B), or only glucose (C), and harvested after 1, 2 and 3 days, as indicated above the lanes. The blot was hybridized with probes containing part of the coding regions of the *cutA*, *uidA* and *tubA* genes. Sizes of the transcripts are indicated in nt in the right margin.

Characterization of cutinase A-deficient transformants.

To determine whether cutinase A is essential for successful penetration of host tissue by *B. cinerea*, the single copy *cutA* gene was mutated by gene disruption. Transformation vector pGDcut2 containing a hygromycin B selection marker (*hph*) flanked by promoter and terminator sequences of *cutA* (*PcutA* and *TcutA*; Fig. 1B), was used to transform the haploid *B. cinerea* strain B05.10. Pathogenicity of B05.10 was indistinguishable from strain SAS56 from which it was derived (P. Büttner and P. Tudzinsky, unpublished). Homologous recombination between the *cutA* locus and *cutA* sequences present in pGDcut2 would allow replacement of the wild-type gene by the hygromycin cassette.

Eighty hygromycin B resistant colonies obtained after transformation of strain B05.10 with pGDcut2 were screened by PCR using primers complementary to *cutA* sequences which were deleted in pGDcut2 (Fig. 1B). A 400-bp PCR fragment was predicted from the wild-type *cutA* locus, whereas no amplification product was expected in transformants in which gene disruption had occurred. In 23 of the 80 transformants the 400-bp fragment was absent or only weakly amplified, as compared to the intensity of the amplification product in other transformants. Six of the 23 transformants were selected for further analysis by Southern blotting (Fig. 5A). Genomic DNA digested with *HindIII* was predicted to release a 2.1-kb fragment from the wild-type *cutA* gene or a 4.0-kb fragment from the mutated gene (Fig. 1B). Using a *HindIII/SstI* fragment of the *cutA* promoter (see Fig. 1B) as probe, a 4.0-kb

fragment was detected in all six transformants indicating that homologous recombination had occurred at the *cutA* locus. However, in three transformants (T124, T125, and T137) the wild-type 2.1-kb fragment also weakly hybridized, indicating that the wild-type *cutA* gene is still present in these transformants. Nonpredicted hybridization signals in T137 (Fig. 5A) demonstrate that ectopic vector integration had also occurred. Hybridization of the same blot with a *HindIII/EcoRI* fragment of the hygromycin cassette (Fig. 1B) showed only hybridization of the 4.0-kb fragment in T124, T125, T130, T132, and T136, and the same additional fragments of T137 as detected in Fig. 5A (results not shown). Since no ectopic integrations of the selection marker were detected in T124 and T125, a small proportion of untransformed nuclei harboring the wild-type *cutA* gene is apparently still present in these transformants.

To monitor *cutA* transcript levels in the recipient strain and transformants, RNA blot analysis was performed on total RNA isolated from transformants grown in cutinase inducing medium (Fig. 5B). A single band of 900 nt was detected in B05.10 and, to a lesser extent, in transformants T124, T125 and T137, in which the wild-type *cutA* gene was also detected by Southern blotting (see Fig. 5A). No *cutA* mRNA was detected in transformants T130, T132, and T136. Cohybridization with a probe derived from the constitutively expressed β -tubulin gene *tubA* of *B. cinerea* demonstrated the presence of approximately equal amounts of RNA on the blot.

Southern and RNA blot data demonstrate that the *cutA* gene is not expressed in transformants T130, T132, and T136. Viability of transformants T130, T132 and the recipient strain B05.10 was tested by plating conidia on solid medium. After 2 days of incubation 90% of T130 or T132 conidia had germi-

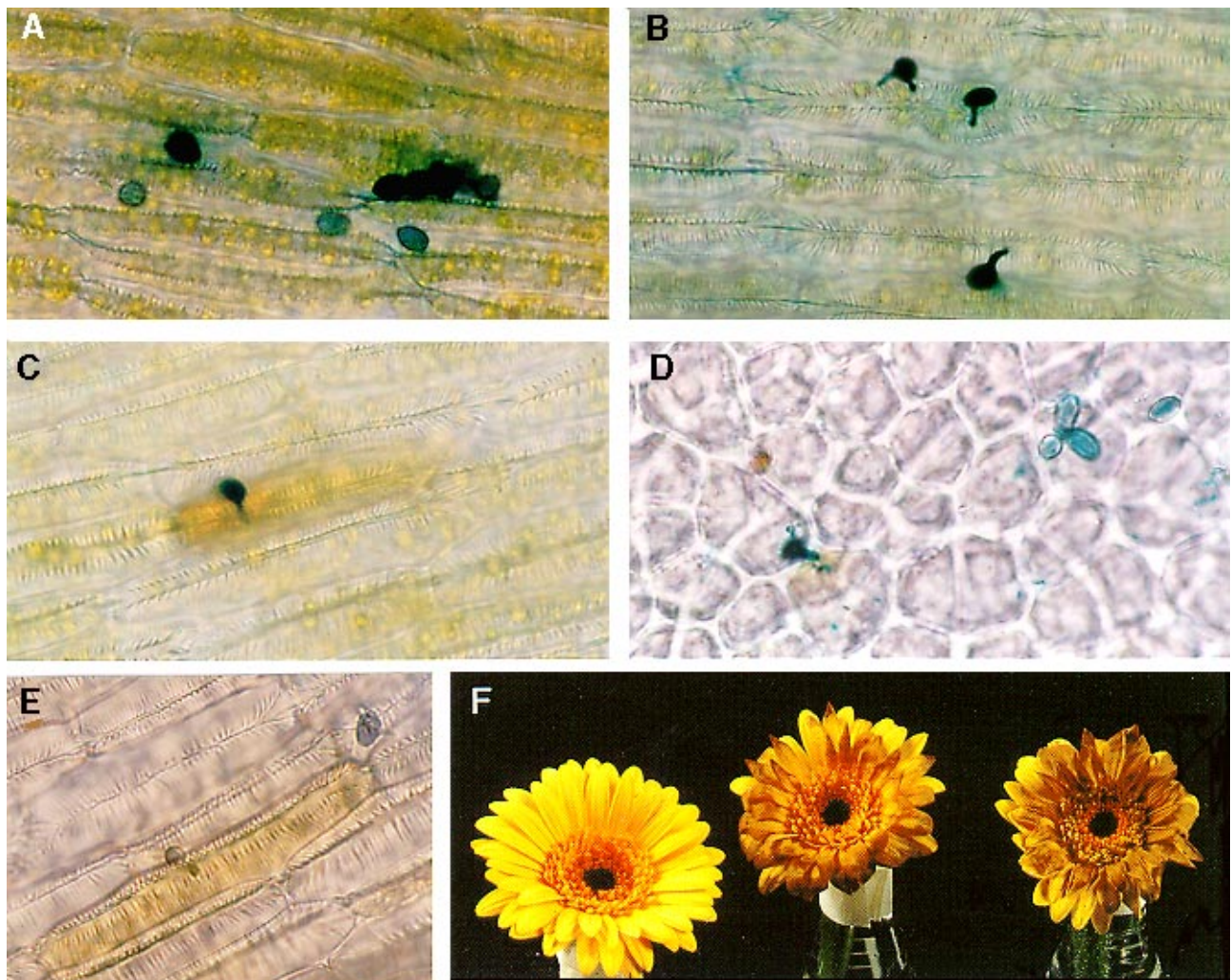


Figure 4. Histochemical localization of GUS activity on gerbera petals (A–C) and tomato fruit (D) inoculated with transformant T2 of *Botrytis cinerea* containing the *cutA* promoter–GUS fusion. **A**, Conidia on a gerbera petal showing different levels of GUS activity at 6 h.p.i. **B**, At 16 h.p.i. most conidia have germinated and show high GUS activity in conidia and infection pegs. **C**, At 24 h.p.i. entry of an infection peg into an epidermal cell has led to cell death visualized by yellowish-brown cell content. **D**, Three d.p.i. on tomato fruit, conidia show different levels of GUS activity and one germinated conidium has penetrated an epidermal cell. **E**, Conidia of transformant T130 lacking a functional cutinase A gene, germinate and penetrate epidermal cells of gerbera petals as well as the wild-type SAS56. Fungal structures are stained with lactophenol-cotton blue dye. Note the yellowish-brown cell content of the penetrated epidermal cell. **F**, Gerbera flowers noninoculated (left) and inoculated with B05.10 (middle) and T130 (right).

nated compared to 100% germination in B05.10. All germinated conidia developed into normal colonies. One of the mutants was further characterized biochemically. Recipient strain B05.10 and the mutant T132 were grown in liquid culture containing tomato cutin for 4 weeks. The culture filtrates were partially purified by acetone precipitation and gel filtration chromatography. Fractions containing PNB hydrolytic activity were pooled and analysed on a chromatofocusing column with a pH gradient of 7.4 to 5 (Fig. 6). The recipient strain B05.10 contained PNB hydrolytic activity in two peaks, eluting at pH 6.3 and 5.75. The latter peak corresponds to the cutinase A (van der Vlugt-Bergmans et al. 1997). Mutant T132 contained PNB hydrolytic activity, but none of this activity eluted at pH 5.75 (Fig. 6).

Pathogenicity assay of cutinase A-deficient mutants.

Conidia of recipient strain B05.10 and transformants T130 and T132 (lacking the *cutA* gene) were dry-inoculated on

flowers of gerbera cv. Sirtaki and on tomato fruits. Symptoms on gerbera flowers were first visible 24 h.p.i. as necrotic lesions occurring everywhere on the petals. At 48 h.p.i. flowers were completely rotten (Fig. 4F). Penetration of epidermal gerbera cells was examined microscopically by cotton blue staining of fungal hyphae in inoculated tissue. No morphological differences were observed among the fungal penetration structures of B05.10, T130 and T132. Penetration by all strains occurred via epidermal cells and all strains caused death of host cells, as indicated by yellow-brownish stained cell contents (Fig. 4E). Since the structure and thickness of the cuticle may vary with plant age or cultivar (Martin and Juniper 1970), gerbera flowers of different age and of a different cultivar ('Romana') were tested. Also on these flowers, no differences in pathogenicity of T130 and T132 as compared to B05.10 were observed.

On tomato fruits no necrotic lesions were visible on the fruit surface. At 3 d.p.i. the interior of the fruit was colonized resulting in complete maceration of the tissue 5 d.p.i. In two independent tests on two gerbera flowers and on tomato fruits, no significant differences in lesion size, lesion number, or rate of disease development were observed between B05.10 and the cutinase A-deficient transformants T130 and T132. *B. cinerea* was reisolated from tomato fruits inoculated with B05.10, T130, and T132 and analyzed by PCR using *cutA*-specific primers (Fig. 1B). A 0.4-kb fragment was amplified from the strain, reisolated from tomato fruits inoculated with B05.10. This fragment was not amplified in PCR assays using strains reisolated from tomato fruits inoculated with T130 and T132 (result not shown). This demonstrates that the strains obtained from the tomato fruit inoculated with mutant strains T130 and T132 had retained the *cutA* deletion.

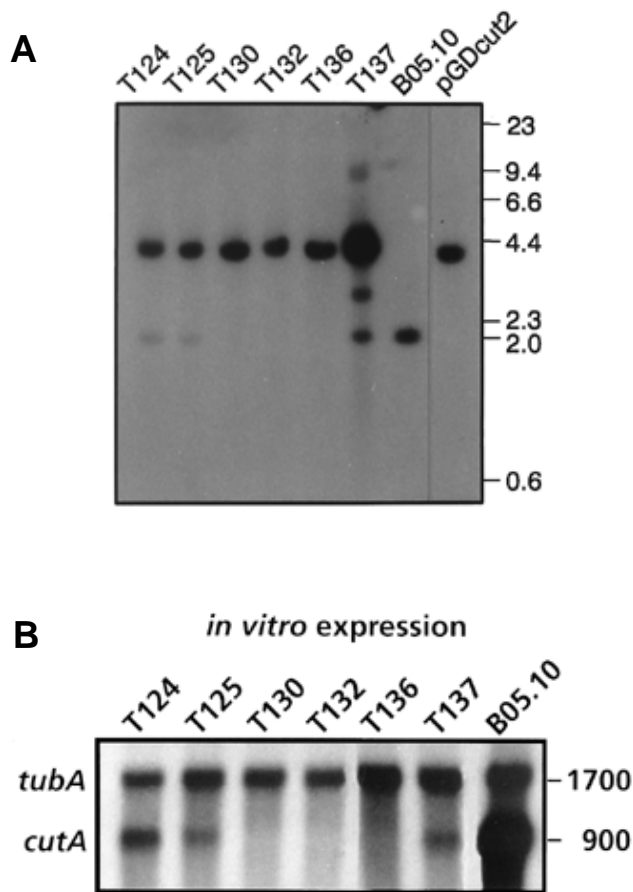


Fig. 5. A, Autoradiograph of a Southern blot containing genomic DNA of *Botrytis cinerea* wild-type strain B05.10 and the transformants T124, T125, T130, T132, T136, T137, and the gene disruption plasmid pGDcut2 digested with *Hind*III. The blot was hybridized with the *Hind*III/*Sst*I fragment of pGDcut2 (Fig. 1A), containing a part of the *cutA* promoter sequence. Molecular-size markers are indicated in kbp in the right margin. **B,** Autoradiograph of a Northern blot containing total RNA of *B. cinerea* wildtype strain B05.10 and the transformants T124, T125, T130, T132, T136, T137, isolated from mycelium induced for *cutA* expression. As probe parts of the *cutA* and *tubA* encoding regions of these *B. cinerea* genes have been used. Sizes of the transcripts are shown in nt at the right margin.

DISCUSSION

Cutinase was suggested to play an important role in penetrating host tissue by *B. cinerea*. This was based on electron microscopy studies (McKeen 1974; Rijkenberg et al. 1980) and on the observation that monoclonal antibodies raised against cutinase A reduced lesion formation by *B. cinerea* on

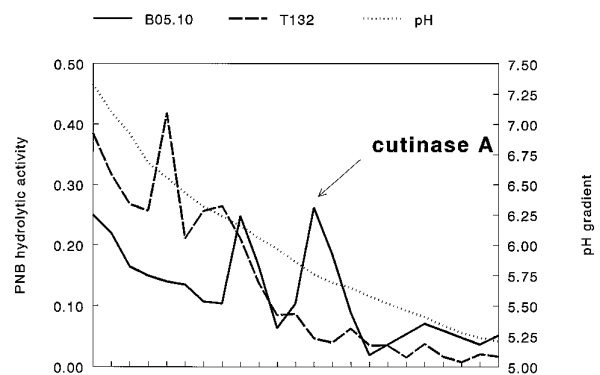


Fig. 6. Biochemical characterization of *cutA* mutant T132. Culture filtrates of strain B05.10 and transformant T132, grown in liquid culture containing tomato cutin, were precipitated with acetone, fractionated by gel filtration and subjected to chromatofocusing. In each fraction, the pH and the PNB hydrolytic activity ($\Delta A_{405}/h$) were determined. Solid line: strain B05.10. Dashed line: transformant T132. Dotted line: profile of the pH gradient.

gerbera flowers (Salinas 1992). However, proof for production of cutinase A at the site of penetration was lacking and, therefore, its role remained unclear. In this study the expression of cutinase A during penetration of host tissue by *B. cinerea* was analyzed by using transformants with the GUS reporter gene *uidA* under control of the *cutA* promoter. The requirement for cutinase A in the penetration process was investigated by using cutinase A-deficient mutants obtained by gene disruption in pathogenicity assays.

Transformants of *B. cinerea* containing the *cutA* promoter-GUS fusion construct were first tested in vitro by GUS activity assays and RNA blot analysis, in order to select transformants in which *uidA* and *cutA* mRNAs are similarly expressed from the *cutA* promoter. X-Gluc plate assays with transformants T2 and T7 provided further evidence for the activation of the *cutA* promoter by the cutin monomer 16-hha and for catabolite repression by glucose (van der Vlugt-Bergmans et al. 1997). These two transformants were used in histochemical analysis of GUS activity during penetration of host tissue. On gerbera petals and epidermal strips of tomato fruits inoculated with T2 and T7, intense blue staining was observed from the onset of conidial germination and in penetration pegs. Without exception, conidia that had germinated stained blue. Thus cutinase A expression starts during germination, just prior to the penetration event, in which the enzyme was proposed to play a role. GUS activity could not be followed throughout the infection of the gerbera flowers or the tomato fruits because the plant tissues were severely macerated at later stages of the infection, thus hampering histochemical staining techniques. However, the observation that *cutA* mRNA is expressed in *B. cinerea*-infected tomato leaves until the stage of sporulation (van der Vlugt-Bergmans et al. 1997) suggests that cutinase A is not only produced in early stages of the infection process. The pCutGUS transformants were not tested on tomato leaves. In this bioassay the addition of glucose in the inoculum is essential for the initiation of infection and glucose was shown to repress the *cutA* gene (Van der Vlugt-Bergmans et al. 1997).

The requirement of cutinase A for enzyme-mediated penetration was investigated by using cutinase A-deficient mutants, constructed by replacement of *cutA* coding sequences with a hygromycin marker. Southern blot analysis of six transformants showed three transformants lacking the wild-type *cutA* gene and three transformants containing both the disrupted and wild-type *cutA* gene. The latter gene was present in much lower abundance as shown by weaker hybridization signals. Since the selection marker was not ectopically integrated in transformants T124 and T125, the wild-type *cutA* gene is probably present in nuclei which are hygromycin B sensitive and thus untransformed. Due to the multinucleate, heterokaryotic nature of *B. cinerea* (van der Vlugt-Bergmans et al. 1993), transformed and untransformed nuclei can coexist in one thallus. Apparently, untransformed nuclei survived hygromycin B selection due to the presence of resistant nuclei in transformants T124 and T125. The presence of both types of nuclei hampered the initial detection of gene disruption by PCR, since the PCR assay was based on absence or presence of an amplification product of the *cutA* gene. In 23 out of 80 transformants the PCR fragment was weakly amplified or completely absent. Southern blot analysis of 6 out of these 23 showed gene disruption in all six transformants. This suggests that homologous recombination had occurred with a frequency

of approximately 25% (23:80). However, the actual frequency of cutinase A-deficient mutants is lower due to the presence of untransformed nuclei. In future gene disruption experiments, single spore cultures of *B. cinerea* transformants will be analyzed in order to reduce the number of heterokaryons containing wild-type nuclei. However, this will not guarantee that such heterokaryons are not obtained, because conidia of *B. cinerea* can contain up to 10 nuclei (Shirane et al. 1988).

Transformants T130, T132, and T136 lacked a functional *cutA* gene, since no *cutA* mRNA was detected by RNA blot analysis. Pathogenicity tests were conducted with the cutinase A-deficient transformants T130 and T132 and the recipient strain B05.10 by inoculation on gerbera flowers and tomato fruits. Both T130 and T132 induced disease symptoms indistinguishable from B05.10 which carries a functional *cutA* gene. The use of flowers of different age or a different gerbera cultivar, of which the cuticle might be of a different structure or thickness (Martin and Juniper 1970), did not result in altered symptoms. Microscopic examination at early stages of the infection showed that penetration of single germinating conidia of T130 into epidermal cells was similar to the wild-type strain. Thus, cutinase A-deficient mutants of *B. cinerea* show no altered penetration ability and cutinase A is not essential for successful penetration of undamaged host tissue. In contrast, Salinas (1992) observed that treatment of gerbera flowers with monoclonal antibodies raised against cutinase A of *B. cinerea* strain Bc7 reduced lesion formation by 80%. The discrepancy between these observations cannot easily be explained, but the results with the cutinase A-deficient mutants exclude a requirement of cutinase A for penetration.

This is the first report of successful gene disruption in *B. cinerea* as a means of specifically mutating a single defined locus, the *cutA* gene. Cutinase gene disruption in *Magnaporthe grisea* (Sweigard et al. 1992) and *Alternaria brassicicola* (Yao and Köller 1995) also did not alter the penetration ability of the fungus. In *M. grisea*, significant remnant cutinase activity was detected in the disruption mutant (Sweigard et al. 1992). In *A. brassicicola* the cutinase gene that had been disrupted was not expressed in planta to detectable level, and a low level of additional cutinase activity was produced specifically during the penetration of host tissue (Köller et al. 1995; Yao and Köller 1995). There is still controversy over the requirement of a cutinase gene of *F. solani* f.sp. *pisi* for pathogenicity on pea (Rogers et al. 1994; Stahl et al. 1994).

To our knowledge, the analysis of cutinase gene expression in a plant pathogenic fungus in single penetrating germ tubes by using reporter gene fusions has not been described before. In other pathosystems droplet inoculations with conidial suspensions were performed (Sweigard et al. 1992; Rogers et al. 1994; Stahl et al. 1994; Köller et al. 1995; Yao and Köller 1995). This makes it difficult to exclude side effects of clusters of conidia on plant surfaces. In our gerbera inoculation assays, cutinase A expression was observed in individual conidia that germinated on and penetrated into the host cuticle. In spite of the fact that single conidia were able to penetrate and cause disease, the deletion of the *cutA* gene did not affect pathogenicity of *B. cinerea* to any detectable extent. Thus, *B. cinerea* apparently possesses other means of penetrating the host surface. In this respect, the production of unrelated cutinases or other enzyme activities during the infection process will have to be investigated.

MATERIALS AND METHODS

Fungal growth.

Strain SAS56 of *B. cinerea* (van der Vlugt-Bergmans et al. 1993), haploid strain B05.10 (derived from SAS56, a gift from P. Büttner and P. Tudzynski, Institut für Botanik, Westfälische Wilhelms-Universität, Münster, Germany), and resulting transformants were grown on malt extract agar (Oxoid, Basingstoke, England) at 18°C in the dark. Prior to plant inoculations strains were grown on tPDA (potato dextrose agar containing 300 g of homogenized tomato leaves per litre). After 3 days the cultures were exposed for 16 h to near UV-light. One week later conidia were collected from sporulating cultures and inoculated (10^8 conidia per liter) in Gamborg's B5 medium (Duchefa Biochemie BV, Haarlem, The Netherlands) supplemented with 0.3% glucose. After 2 days of growth in a rotary shaker at 160 rpm, 20°C, mycelium was harvested for isolation of genomic DNA. For analysis of gene expression, 2-day-old cultures (start cultures) were diluted tenfold in the following media: B5 medium + 0.05% 16-hydroxyhexadecanoic acid (16-hha; A), B5 medium + 0.05% 16-hha + 0.3% glucose (B), B5 medium + 0.3% glucose (C). Remaining mycelium of the start cultures was harvested. All three subcultures were incubated at 20°C, 160 rpm and sampled during the following days. The glucose concentration in liquid culture media was determined according to the method of Nelson (1944).

Construction of transformation vectors.

Vector pCutGUS containing a fusion between the *cutA* promoter of *B. cinerea* and the GUS reporter gene *uidA* was constructed in the following way. A *NcoI* site (CCATGG) was introduced at the ATG start codon of the *cutA* gene by PCR using a primer complementary to a region upstream of the ATG (5'-CTCCATGGTGAAATTGATGTATTGATATTTG-3') in combination with an M13 primer at the 5'-end of the promoter on a subclone containing part of the *cutA* gene as template. The resulting 1.5-kb PCR fragment was ligated in pBluescript SK⁻ (Stratagene) digested with *EcoRV*. Plasmid pCF20 (Van den Ackerveken et al. 1994) contained the *gpd* promoter of *Aspergillus nidulans*, the *Escherichia coli uidA* gene encoding β -glucuronidase (GUS, Jefferson et al. 1987) and the terminator of the *A. nidulans trpC* gene. The *gpd* promoter was excised from pCF20 by digestion with *EcoRI* and *NcoI* and replaced by the *cutA* promoter present on a 1.5-kb *EcoRI/NcoI* fragment excised from the Bluescript construct. In this construct, the *EcoRI* site (upstream of the *cutA* promoter) was made blunt with the Klenow fragment of DNA polymerase and a 0.7-kb *EcoRV* fragment containing the terminator sequence of *tubA* gene of *B. cinerea* (*TubA*) was inserted (Fig. 1A). The correct orientation of the *tubA* terminator in vector pCutGUS was checked by PCR using one of the primers complementary to 5'- and 3'-sequences of the *tubA* terminator in combination with a primer in the *uidA* gene (Fig. 1A).

Vector pGDcut2 was derived from plasmid pCut1 (van der Vlugt-Bergmans et al. 1997). The main part of the coding sequence of *cutA* was deleted by digestion with *EcoRV* and replaced by a selection marker digested from plasmid pOHT (Hilber et al. 1994; provided by M. Ward, Genencor International, San Francisco, CA). The selection marker was present on a 2.6-kb *SmaI/HindIII* fragment of pOHT and consisted of

the bacterial hygromycin phosphotransferase gene (*hph*, conferring resistance to hygromycin B) fused to the *oliC* promoter and *trpC* terminator of *A. nidulans*. This fragment was incubated with the Klenow fragment of DNA polymerase to fill in the ends and used to replace the *cutA EcoRV* fragment from plasmid pCut1 (Fig. 1B). The orientation of the hygromycin B selection marker in the resulting pGDcut2 construct was determined by restriction analysis.

Transformation of *B. cinerea*.

Conidia from sporulating cultures of *B. cinerea* strain SAS56 or B05.10 were collected and inoculated in bacto malt extract (Difco, 10^5 conidia/ml). Cultures were incubated in a rotary shaker at 150 rpm at 22°C for 2 days. The following transformation protocol was adapted from Hamada et al. 1994. Mycelium was harvested by filtration and washed twice in sterile water and once in 0.6 M KCl, 50 mM CaCl₂ (KC-buffer). Protoplasts were generated by adding 10 ml of a filter-sterilized solution containing 5 mg of Novozyme 234 (Novolabs) in KC-buffer per gram of fresh weight mycelium. This suspension was incubated for 2 h at 23°C with shaking at 80 rpm. The digested mycelium was filtered using filters with 25 and 10 μ m mesh size, respectively. Protoplasts were pelleted by centrifugation at $2,000 \times g$ for 10 min, washed once and resuspended in KC-buffer to a final concentration of 10^8 protoplasts per ml. Portions of 100 μ l were maintained on ice for 5 min and, subsequently, plasmid DNA in 40 μ l of water, 5 μ l of 50 mM spermidine and 100 μ l of 25% PEG 3350 (Sigma) in 50 mM CaCl₂, 10 mM Tris-HCl, pH 7.5, were, one by one, gently added to the protoplasts. The mixture was kept on ice for 20 min. Five-hundred microliters of PEG solution were added, gently mixed and incubated for 10 min at room temperature. Finally, the volume was adjusted to 1 ml with KC-buffer. For selection, 100 μ l of protoplasts were spread on 10 ml of SH-agar (0.6 M sucrose, 5 mM HEPES, pH 5.3, 1 mM (NH₄)₂HPO₄, 1.2% agar) and incubated for 3 h at room temperature. Protoplasts were then overlaid with 3 ml of SH-top agar (0.6% agar) containing 100 μ g/ml of hygromycin B (Duchefa). After 3 to 7 days of incubation at 22°C, hygromycin B-resistant colonies of *B. cinerea* were individually transferred to malt extract agar and grown for 2 days at 22°C. Agar plugs with mycelium from the edge of each colony were subsequently transferred to malt extract agar amended with 100 μ g/ml hygromycin B.

Prior to transformation of *B. cinerea* strain SAS56, vector pCutGUS was linearized with *SphI* (Fig. 1A) and pOHT with *HindIII*. Ten micrograms of linearized vector and 1 μ g of linearized pOHT as selection marker were used for cotransformation. The haploid strain B05.10 of *B. cinerea* was transformed using 2 μ g of circular pGDcut2.

Southern blot analysis.

Genomic DNA was isolated according to Drenth et al. (1993), digested with restriction enzymes, size separated on a 0.7% agarose gel and blotted onto Hybond-N⁺ (Amersham) according to Sambrook et al. (1989). The blot was hybridized in modified Church buffer (0.5 M sodium phosphate buffer, pH 7.2 and 7% SDS; Church and Gilbert 1984) at 65°C for 18 h in the presence of a random-primed [α -³²P]dATP labeled probe. Blots were washed in 0.2 \times SSC and 0.5% SDS at 65°C and exposed to Kodak X-OMAT AR film.

RNA blot analysis.

Total RNA was isolated from mycelial samples using the Extract-A-Plant RNA kit (Clontech). Samples of 10 µg total RNA were denatured with formamide and formaldehyde and subjected to electrophoresis on a 1.0% agarose gel containing formaldehyde (Sambrook et al. 1989). After blotting to Hybond-N⁺ membranes, blots were hybridized as described above in the presence of a random-primed [α -³²P]dATP labelled probe and washed in 0.2× SSC and 0.5% SDS at 65°C before exposure to Kodak X-OMAT AR film.

Biochemical analysis.

Recipient strain B05.10 and *cutA* mutant T132 were grown in 125 ml of liquid medium as described above. After 3 days, 500 mg of tomato cutin was added and the culture was incubated under continuous shaking for 30 days. The culture filtrates were harvested, acetone was added to a final concentration of 20% and the sample was centrifuged at 2,000 × *g* at 4°C for 20 min. The supernatant was adjusted to 80% acetone and precipitated proteins were pelleted by centrifugation at 3,000 × *g* at 4°C for 30 min. The pellet was dried, dissolved in 40 mM Tris-HCl, 50 mM NaCl, pH 7.5 and loaded on a Sephadex G75 column (1.5 × 25 cm) equilibrated with the same buffer. Fractions of 4 ml were collected and measured for PNB hydrolytic activity. Fractions containing enzyme activity were pooled, dialyzed, and applied to a PBE 94 column as described in the accompanying paper (van der Vlugt-Bergmans et al. 1997). The pH and the PNB hydrolytic activity of each fraction were determined.

Fluorometric GUS assay.

One hundred milligrams of fresh mycelium was resuspended in GUS extraction buffer (GEB: 50 mM sodium phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% N-laurylsarcosine, 0.1% Triton X-100, 10 mM β-mercaptoethanol; Jefferson et al. 1987), ground with a pestle, vortexed and after centrifugation the supernatant was stored at 4°C. The protein concentration was determined using the Bio-Rad protein assay (Bradford 1976) and bovine IgG as a standard. Ten micrograms of protein of each sample was diluted to 100 µl in GEB and added to 500 µl of 1 mM MUG (4-methyl umbelliferyl glucuronide, Research Organics) dissolved in GEB. After mixing, enzyme assays were incubated at 37°C in the dark. After 1 and 2 h of incubation, 100 µl of each enzyme assay was mixed with 900-µl stop buffer (0.2 M Na₂CO₃). Fluorescence was measured in a spectrofluorimeter (excitation 365 nm, emission 450 nm), calibrated with MU (4-methyl umbelliferone, Sigma) in stop buffer.

Bioassays.

Gerbera flowers cv. Sirtaki and Romana and tomato fruits cv. Moneymaker (green fruits of 3 to 4 cm in diameter) were dry-inoculated with transformants or wild-type strains of *B. cinerea*. Conidia were transferred from sporulating cultures by a brush, or they were dusted on the host tissue by tapping the bottom of an inverted petri dish with a sporulating culture.

Flowers and fruits were incubated in closed plastic boxes with a transparent lid to obtain a humidity of 100%, at 20°C with a dark-light cycle of 16 h light and 8 h dark. Development of disease symptoms was followed by eye and fungal penetration was examined microscopically in whole-mount

preparations of inoculated petals or in epidermal strips of tomato fruits. Flower petals harvested at different time-points after inoculation were fixed in absolute alcohol-glacial acetic acid (1:1 v/v), cleared in 75% lactic acid for 48 h at 37°C, and mounted on a microscope slide in lactophenol-cotton blue dye. Epidermal strips of tomato fruits were similarly treated with omission of the clearing step.

For histochemical localization of GUS activity, gerbera petals or epidermal strips of tomato fruits were incubated with 0.5 mg/ml X-Gluc (5-bromo-4-chloro-3-indolyl β-D glucuronide, Biosynth AG) in 50 mM phosphate buffer, pH 7.0, 1 mM KFeCN and 0.05% (v/v) Triton-X100, overnight at 37°C. The tissue was mounted on microscope slides in 50% (v/v) glycerol.

The samples were examined with a Zeiss Axioscope microscope and photographs were taken using a Zeiss MC-100 camera unit. Bioassays were performed two times.

Reisolation of mycelium from infected tomato fruit was performed by incubating a piece of infected fruit tissue on water agar at 18°C. After 3 days of incubation mycelium bearing agar plugs were excised from the edge of the developing colony and incubated on malt extract agar as described above.

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