

Induction and expression of cutinase activity during saprophytic growth of the fungal plant pathogen, *Glomerella cingulata*.

Farah Diba Abu Bakar^{1*}, Abdul Munir Abdul Murad¹, Aidil Abdul Hamid¹,
Zulkeflie Zamrod¹, Nor Muhammad Mahadi¹ and Patrick Sullivan².

¹*School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi 43600, Selangor, Malaysia.*

²*Institute of Molecular Biosciences, Massey University, Palmerston North, New Zealand.*

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Abstract. The fungus *Glomerella cingulata* damages a wide range of crops in the tropics and subtropics. This fungus produces an extracellular enzyme, cutinase, which has been implicated in the plant disease process. A cutin monomer, 16-hydroxyhexadecanoic acid was used to induce cutinolytic esterase activity during saprophytic growth of *G. cingulata*. Cutinolytic esterase was induced in cultures grown in cutin monomer but was repressed by glucose. The pattern of cutinolytic activity obtained from the induction and enzyme assays correlates with the results of the Northern blot analyses. The cutinolytic activity and expression from this study demonstrates that the enzyme is tightly regulated by the cutin monomer, 16-hydroxyhexadecanoic acid.

INTRODUCTION

The plant cuticle, a major component of which is cutin, covers and protects aerial surfaces of plants against physical, chemical and biological factors in the environment, including pathogens. The cuticle is thus the first layer that many pathogens must breach. Cutin is an insoluble polymer comprised of esterified hydroxyl and hydroxyepoxy fatty acids which is embedded in a complex mixture of wax (Kolattukudy, 2001). The breaching of this first barrier of plant defense has had considerable attention from fungal plant pathologists. While some reports suggested that physical force was sufficient for the penetration by pathogens, others presented evidence that a cutinolytic enzyme, cutinase, was essential for penetration in at least some plant-fungal interactions.

There has been emphasis in the study of the role of fungal cutinases as a pathogenicity factor and determinant in several pathogens. A critical role for fungal cutinases in the penetration of unwounded host tissues was demonstrated for some fungi by the use of antibodies, inhibitors and by using cutinase-deficient fungal mutants (Dickman and Patil, 1986; Dickman *et al.*, 1983; Maiti and Kolattukudy, 1979; Shaykh *et al.*, 1977). In another report, it was demonstrated that a fungal pathogen that normally infects only wounded papaya fruit was able to

penetrate the cuticle only when the cuticle was pretreated with purified cutinase enzyme (Dickman *et al.*, 1982). In addition, recent gene disruption studies on the cutinase gene of *Pyrenopeziza brassicae*, an ascomycete, showed molecular evidence that cutinase is required for pathogenicity (Li *et al.*, 2003). However, cutinase gene disruption studies with other fungi for example in *Fusarium solani* (Stahl and Schafer, 1992), *Magnaporthe grisea* (Sweigard *et al.*, 1992) and *Botrytis cinerea* (Van Kahn *et al.*, 1997), have produced conflicting evidence, suggesting that cutinase is not required for fungal pathogenicity.

Cutinases hydrolyse ester bonds linking the fatty acids which make-up the cutin polymer (Kolattukudy, 2001; Carvalho *et al.*, 1999). Cutinases are classified as serine hydrolases that hydrolyse fatty acid esters and emulsified triglycerides as efficiently as lipases (Carvalho *et al.*, 1999). Cutinases however, differ from lipases as they do not exhibit enhancement of activity in the presence of a lipid-water interface and are active on both soluble and emulsified triglycerols (Verger, 1976; Martinez *et al.*, 1992). These surface hydrophobicity characteristic and lipolytic activity of cutinases have been

*Author for Correspondence.

Mailing address: School of BioSciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Malaysia. Tel : 03-89215980; Fax : 03- 89252698; E-mail : fabyff@pkrisc.cc.ukm.my

exploited in their use in laundry detergent formulations (Carvalho *et al.*, 1999).

Besides fungal penetration of the cuticular layer during initial stages of infection, cutinases have been suggested to have a role in spore attachment (Deising *et al.*, 1992) and in carbon acquisition for saprophytic growth (Köller and Parker, 1989). Cutinases secreted by the fungus may partially break down the plant cuticle to produce cutin monomers, which have been shown not only to induce the expression of cutinase (Lin and Kolattukudy, 1978) but to trigger conidial germination and differentiation of appressoria (Gilbert *et al.*, 1996; Hegde and Kolattukudy, 1997). Thus, cutinases may have a role in surface signaling that is crucial for the differentiation of essential infection structure and expression of pathogenicity factors. In quiescent infections whereby spores remain latent on the plant host surface, host factors could signal the pathogen to remain in latency and prevent its differentiation into an infectious form. Cutinases may be representative of a series of cuticle and cell wall degrading enzymes produced by fungal pathogens. Understanding their regulation and role in quiescence may contribute to improved control strategies (Wang *et al.*, 2002).

Glomerella cingulata (Stoneman) Spaulding et Schrenk (anamorph *Colletotrichum gloeosporioides*), an ascomycete, is a pathogenic fungus of a wide variety of crops in the subtropical and tropical regions (Jeffries *et al.*, 1990). Post harvest crop loss significantly limits export quality of produce and severely reduces the quantity and quality of fruit crops (Waller, 1992; Prusky *et al.*, 2000). Previously we reported the cloning and characterisation of a *G. cingulata* cutinase (*cutA*) gene and its cDNA (Farah Diba *et al.*, 2001). This current work presents the induction and expression profile of the cutinase during saprophytic growth of *G. cingulata* and a proposed scheme of the way in which the plant cuticle induces cutinase in *G. cingulata*.

MATERIALS AND METHODS

Organisms and plasmids. *G. cingulata* ICMP 11061 was originally obtained from the International Collection of Microorganisms from Plants (ICMP), Maanaki Whenua Landcare, New Zealand. The *G. cingulata* culture was maintained on Potato Dextrose Agar (PDA). Plasmid pDMC1 carries a 5.85 kb *G. cingulata* genomic sequence that includes the full length cutinase gene. This plasmid was constructed using pGEM7f (-) [Promega, USA] (Farah Diba *et al.*, 2001).

Cutinase induction and cutinolytic esterase enzyme assay. The method of Van der Vlugt-Bergmans *et al.* (1997) was adopted. *G. cingulata* was first grown on PDA at 30°C for 7 days. Thirty agar plugs (6 mm in diameter) of this mycelial plate culture was inoculated into 200 ml of Wickerham's solution (Wickerham, 1946), 0.5% ammonium sulfate and 0.3 % glucose. This was incubated at 30°C for 48 hr. The

culture was stirred using a magnetic stirrer throughout the incubation in a one Litre flask, in order to produce a homogeneous suspension of mycelia. Aliquots of 5 ml of this start culture were transferred into media containing, 0.05% 16-hydroxyhexadecanoic acid (16-hha) [Medium CM], 16-hha (0.05 % 16-hha) + glucose (0.3 %) [Medium CMGLC] and 0.3 % glucose (Medium GLC). These were incubated at 30°C, 150 rpm and sampled each day for the following 8 days. The dry weight of the mycelia and *p*-nitrophenyl butyrate hydrolase activity of the filtrates were determined. Extracellular culture fluid collected after filtering the mycelia was diluted in 25 mM potassium phosphate buffer, pH 8 and 0.05% Triton X-100 in a final volume of 450 μ l. To this, 50 μ l of *p*-nitrophenyl butyrate (PNB) [Sigma] resuspended at a concentration of 10 mM in 0.25 mM phosphate buffer, pH7.0, and 0.5% (v/v) Triton X-100 was added. Aliquots of 80 μ l of this were transferred into the wells of a 96-well microtitre plate. After 1 hr of incubation at 30°C, PNB hydrolysis was measured spectrophotometrically at 405 nm using an ELISA plate reader. Background caused by yellowish culture filtrate and/or nonenzymatic substrate degradation was determined in a parallel assay containing protein fraction that had been treated for 6 min at 100°C, and subtracted from the values of non-treated enzyme fractions. Thus, the start culture of *G. cingulata* was inoculated (day 0) in medium CM, containing the cutin monomer, 16-hha, as the sole carbon source, medium CMGLC, containing 16-hha and glucose, or medium GLC, containing glucose as sole carbon source. The culture supernatants were sampled during each of the following 8 days. A 2 days post-induction (d.p.i.) culture grown in medium CMGLC was also harvested and transferred to a fresh growth medium (Medium MM: minimal medium and no carbon source) and assayed for cutinolytic activity for each of the following 6 days. These experiments were carried-out in triplicates and repeated at least three times.

Measurement of glucose concentration. The glucose concentration in the extracellular culture fluid of the culture grown in induction media was determined using the Glu GOD-PAP kit (Boehringer Mannheim/Roche Molecular Biochemicals). The culture fluid (10 μ l) was added to 1000 μ l of working reagent containing 200 mmol phosphate buffer, pH 7.2, 10 mmol 4-hydroxybenzoic acid, 0.75 mmol 4-amino anti pyrine (PAP), 250 kat glucose oxidase (GOD), 20 kat peroxidase (POD) and <0.1 % sodium azide. This was mixed and incubated for 10 min at 37°C and subsequently, the absorbance was read.

Cutinase probe construction. A cutinase gene probe was constructed by using Polymerase Chain Reaction (PCR). PCR of the pDMC1 plasmid was carried out using primers designed based on the +1 to +23 and the +705 to +726 regions of a cutinase gene from *C. gloeosporioides* (Accession No. M21443 ; Ettinger *et al.*, 1987). The parameters for the

PCR cycles used were 94°C, 5 min (1 cycle), 94°C, 1 min, 52°C, 30 sec, 72°C, 2 min (30 cycles) and 72°C, 5 min (1 cycle). The MgCl₂ concentration was 1.25 mM. The amplification yielded a ~730 bp product.

Total RNA extraction. *G. cingulata* mycelia were filtered and washed with sterile diethylpyrocarbonate-treated water. The washed mycelia were then immediately frozen in liquid nitrogen and grounded into powder. Total RNA extraction of the mycelia was then carried out using TriPure Isolation Reagent (Roche Molecular Biochemicals, Germany) as described by the manufacturer.

Northern transfer. Approximately 20 µg of total RNA from several samples were fractionated by electrophoresis using standard methods (Sambrook *et al.*, 1989). The fractionated samples were transferred to Hybond N+ (Amersham Biosciences) membrane using standard capillary transfer (Sambrook *et al.*, 1989).

Random primer labelling. The DNA probe was radiolabelled with α-³²P-dCTP (Amersham Biosciences) using Ready to Go™ DNA Labelling Beads (-dCTP) (Amersham Biosciences) kit prior to hybridisation.

Hybridisation to nylon membranes and post-hybridisation washes. Hybridisation was performed according to the manufacturer's instructions (Amersham Biosciences) using a hybridisation solution of 0.5 M phosphate buffer, pH 7.2, 7 % SDS and 0.01 M EDTA, pH 8.0 (Phosphate buffer, pH 7.2 [70.4 ml 0.5 M Na₂HPO₄, 29.6 ml 0.5 M NaH₂PO₄]). Prehybridisation and hybridisation were carried out at 65°C. Post-hybridisation membrane washes and autoradiography were carried out using standard procedures (Sambrook *et al.*, 1989).

RESULTS AND DISCUSSION

Cutinolytic esterase enzyme activity of *G. cingulata*. Induction studies were carried-out using glucose and a cutin monomer, 16-hydroxyhexadecanoic acid. Expression of the *cutA* gene was measured indirectly by esterase activity and by Northern hybridisations. Cutinase assays are generally performed on the extracellular fluids of cultures induced with cutin hydrolysate or the cutin monomer, 16-hydroxyhexadecanoic acid (Sweigard *et al.*, 1992; Van der Vlugt-Bergmans *et al.* 1997; Carlvaho *et al.*, 1999). Cutinase activity can be directly measured by the release of radioactivity from tritiated cutin. However, the assay is tedious and the substrate is difficult to obtain. Thus, cutinase activity is often measured indirectly as general esterase activity using PNB as substrate (p-nitrophenyl hydrolase activity).

In this study, cutinase gene induction was carried out in cultures of *G. cingulata* grown under different conditions and

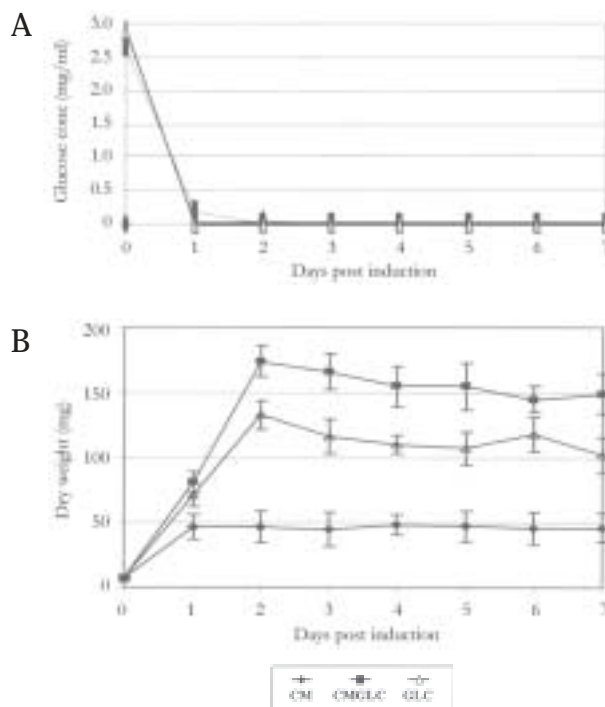


Figure 1. Glucose concentration (A) and mycelial dry weight (B) of *G. cingulata* grown under different conditions. A start culture of *G. cingulata* was inoculated at day 0 in medium CM (16-hydroxyhexadecanoic acid [hha]), CMGLC (16-hha+glucose) and GLC (glucose).

compared for PNB hydrolytic activity. Assays showed that glucose was depleted from the culture media CMGLC and GLC at day 2 (Figure 1 A). In the absence of glucose as seen for the CM culture sample, less fungal biomass developed (Figure 1 B). Production of PNB hydrolytic activities in the start and subcultures sampled during 8 days post-induction (d.p.i.) is shown in Figure 2. The culture grown in 16-hha (medium CM) was induced but induction was slow and the highest PNB hydrolase activity of 76.5 nmole/hr was at 7 d.p.i. In the absence of the cutin monomer (medium GLC), no PNB hydrolytic activity was detected suggesting that glucose acts as a repressor. In medium CMGLC however, no PNB hydrolase activity was detected throughout the 8-day incubation.

PNB hydrolase activity of the cultures grown in medium CM and medium MM was compared. The cultures grown in medium MM showed higher PNB hydrolytic activities (nmole/hr) than the culture filtrates of medium CM at days 4, 5, 6, 7 and 8 (Figures 2 A and B). However, when mycelial dry weights of these cultures were taken into consideration, culture medium CM grown in 16-hydroxyhexadecanoic gave the highest PNB hydrolase activity of 377 nmole/hr/mg dry weight on the 7th day as compared to 257 nmole/hr/mg dry weight for the medium MM culture.

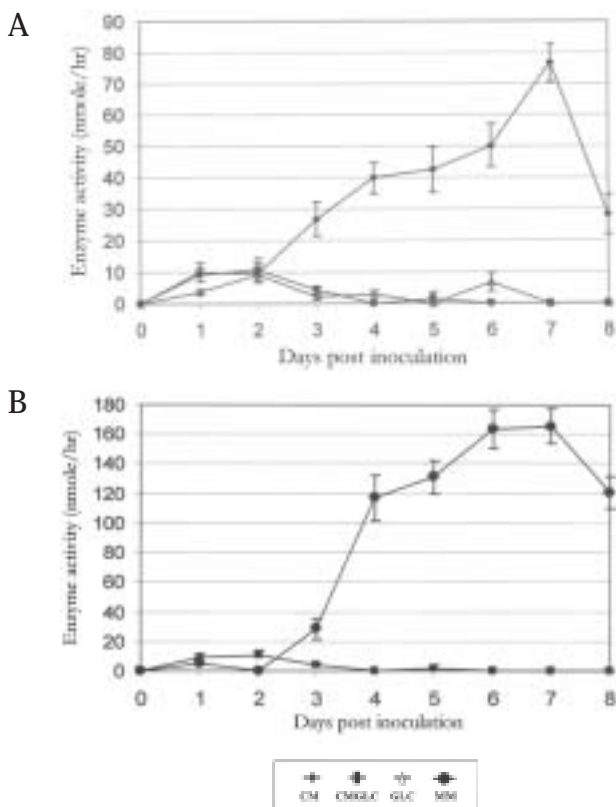


Figure 2. PNB hydrolytic activity in culture filtrates of mycelia (unwashed [A] and washed [B]) of *G. cingulata*, grown under different conditions. A start culture of *G. cingulata* was inoculated at day 0 in medium CM (16-hha), CMGLC (16-hha + glucose), GLC (glucose) and MM (minimal medium). The culture used to inoculate medium MM was harvested from medium CMGLC after 2 days, washed and transferred to minimal medium (MM).

The results of these assays seem to suggest that the cutinase/PNB hydrolase is tightly regulated by 16-hydroxyhexadecanoic. In the work of Van der Vlugt-Bergmans *et al.* (1997), the PNB hydrolase activity of *B. cinerea* was shown to be induced by the cutin monomer 16-hydroxyhexadecanoic and repressed by glucose. However, in a medium containing 16-hydroxyhexadecanoic and glucose, PNB hydrolase activity increased after depletion of glucose at 2 d.p.i.. A similar derepression trend was also observed in the work of Hawthorne *et al.* (2001) with the cutinolytic esterase of *F. solani* f. sp. *cucurbitae*. However, the analogue of the cutin fraction, hexadecanols (C16 alcohol) and cutin from fruits were used.

Northern blot analysis. RNA blot analyses of total RNA isolated from day 0 and days 2, 4, 6 and 8 of the cultures grown on media CM, CMGLC, GLC and MM were carried out. The RNA blots were hybridised using a 727 bp DNA probe that encodes the *cutA* gene. No hybridisation signals

were observed with the blots of the RNA extracted from the cultures grown on media supplemented with 16-hha and glucose (medium CMGLC), and glucose alone (medium GLC) (results not shown). This correlates with the PNB hydrolytic activity of the culture filtrates of these media (Figure 2 A). Figure 3 shows the Northern blots of the RNA samples from cultures grown on media CM (16-hha) and MM (washed culture pellet of medium CMGLC transferred to minimal medium). In medium CM, in the absence of glucose, the *cutA* mRNA was expressed at 2 d.p.i. and highest expression was detected at 6 d.p.i. At 8 d.p.i. the *cutA* mRNA expression appears to be reduced. Whereas in medium MM, the *cutA* mRNA was not expressed at 0 and 2 d.p.i. but expression was detected at 4 d.p.i. and was highest at 6 d.p.i. This indicated that the *cutA* gene is repressed by glucose. At 8 d.p.i. the hybridisation signal was low.

The pattern of expression seen in Figure 3 (A and B) correlated with the PNB hydrolytic activity of the culture filtrates (Figures 2 A and B). However, the low hybridisation signal detected in the RNA culture of medium MM (Figure 3 B) at 8 d.p.i. seems to indicate that the high level of PNB hydrolytic activity displayed at 8 d.p.i. is due to esterases other than cutinase (Figure 2 B). This suggestion is made because the PNB substrate is not a specific substrate for cutinase as it is also hydrolysed by other esterases. On the other hand, the mRNA level could have decreased between days 6 and 8 while the secreted enzyme was still active. The results obtained from the induction, enzyme activity and expression assays indicate that cutinase is induced by 16-hydroxyhexadecanoic and repressed by glucose. The absence of hybridisation signals by Northern blot analyses in the RNA samples of cultures grown in media containing glucose may also indicate that basal expression due to constitutive expression of cutinase was not detected.

Most cutinase of plant fungal pathogens are induced by cutin monomers, however, there are exceptions reported otherwise. For example, while *F. solani* f. sp. *pisi* showed induction of cutinase in cultures supplied with cutin monomers and their analogues such as C₁₆ alcohol (Lin and Kolattukudy, 1978), Hawthorne *et al.*, (2001) showed that the cutinase of *F. solani* f. sp. *cucurbitae* MPV strain was not induced by the C₁₆ alcohol, hexadecanol. In other fungi such as *Alternaria brassicicola* (Yao and Köller, 1995; Fan and Köller, 1998), two classes of cutinolytic esterases were found. One class was only expressed during short duration (24 hr) contact with intact cutin and this class of enzyme was not induced by surface wax or cutin monomers. In contrast, the second class of enzyme was induced by cutin monomers or by prolonged exposure to intact cutin.

Southern blot analysis showed that there is only one copy of the cutinase gene in *G. cingulata* ICMP 11061 (Farah Diba *et al.*, 2001). However, this is in contrast to the *F. solani* f. sp. *pisi* as this fungus showed multiple cutinase genes that can also be detected by Southern hybridisation with genomic DNA (Kolattukudy and Crawford 1987). To date, three

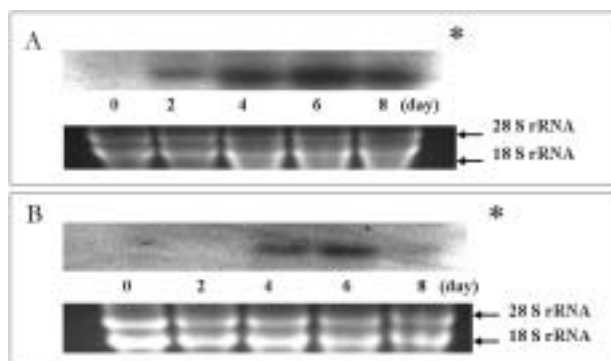


Figure 3. RNA Northern blot analysis of *cutA* expression from cultures grown in medium CM (A) and cultures grown in medium MM (B). Asterisks indicate the RNA Northern blot. The ethidium bromide stained total RNA samples below the blots were used for the Northern transfers. The arrows show the 28S and 18S rRNA bands that were used to standardise RNA loadings by band intensities. Each well was loaded with approximately 20 μ g total RNA.

cutinase genes (*cut1*, *cut2* and *cut3*) have been cloned from *F. solani* f. sp. *pisi* (Ettinger *et al.*, 1987; Li *et al.*, 2002). *Cut1* is suggested to be the inducible gene while *cut2* and *cut3* are constitutively expressed (Kolattukudy, 2001; Li *et al.*, 2002). Detailed studies on the regulation of the *F. solani* f. sp. *pisi* suggest that a low level of constitutively expressed cutinase, present on the conidia that land on the plant surface, generates small amounts of cutin monomers from the host cuticle. These monomers then trigger transcriptional activation of the inducible cutinase gene (Kolattukudy *et al.*, 1995). The transcription factors from *F. solani* f. sp. *pisi* have been cloned and studied (Kolattukudy *et al.*, 1995; Li and Kolattukudy, 1995; Li and Kolattukudy, 1997; Li *et al.*, 2002). However, similar studies to determine the mechanism of cutinase gene regulation in *G. cingulata* or any other *Colletotrichum* and fungal species have yet to be reported.

Many fungi do not possess multiple copies of cutinase as was observed from our previous study on *G. cingulata* (Farah Diba *et al.*, 2001) and other studies on *C. gloeosporioides*, *C. capsici*, *C. lindemuthianum*, *C. graminicola*, *M. grisea*, *Ascochyta rabiei* and *B. cinerea* (Ettinger *et al.*, 1987; Sweigard *et al.*, 1998, Tenhaken *et al.*, 1997; Van der Vlugt-Bergmans *et al.*, 1997). Thus, for these fungi, the mechanism by which the cutinase gene expression is triggered may be different from that proposed for *F. solani* f. sp. *pisi*.

Analyses of our previous study have shown that there are differences in the cutinase genes of *G. cingulata* and that of *F. solani* f. sp. *pisi* namely, in copy number and in the sequences of the 5' flanking regulatory regions (Farah Diba *et al.*, 2001). These differences may indicate that the mechanism of cutinase gene regulation in *Colletotrichum* is different from that of *F. solani* f. sp. *pisi*. The data gathered from this study and the experimental studies of others working on single copy fungal cutinases showed that cutin monomers such as 16-

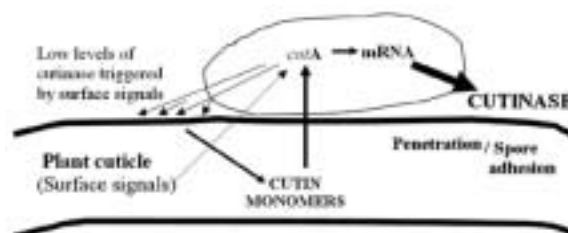


Figure 4. A hypothetical scheme depicting the way by which the plant cuticle induces cutinase in a *G. cingulata* spore.

hydroxyhexadecanoic acid, induce cutinase gene expression. Thus, a hypothetical scheme depicting the way by which the plant cuticle induces cutinase in a *G. cingulata* spore is proposed (Figure 4). In this postulation, in the absence of cutin monomers on an intact plant cuticle, a *G. cingulata* spore that lands on a plant surface produce low levels of cutinase triggered by specific plant surface signaling factors. The cutin monomers generated from the degradation of cutin in the plant cuticle can then act as inducers thereby allowing the fungus to produce sufficient quantities of cutinase to gain access into the host by penetration or to facilitate spore adhesion onto the plant cuticle. Thus, it is postulated that the cutinase gene regulation in *G. cingulata* involves interaction between activators and repressors that initially produce low-level expression of cutinase that degrade cutin and produce cutin monomers, followed by induced expression of cutinase by cutin monomers. This differs from the way by which cutinase is produced by *F. solani* whereby induction of cutinase by cutin monomers is followed by production of low levels of cutinase by constitutive expression.

The initial signals perceived by pathogenic fungi prior to penetration of the plant host are not well understood. Surface signals are crucial for the differentiation of vital infection structures such as appressoria and pathogenicity factors such as toxins and depolymerases. Thus, understanding the regulation of cutinase during pre-penetration and its involvement in quiescence could contribute to improved control strategies.

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