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Diversity of cutinases from plant pathogenic fungi: differential and sequential expression of cutinolytic esterases by *Alternaria brassicicola*

Chao-Yun Fan, Wolfram Köller *

Department of Plant Pathology, Cornell University, New York State Agricultural Experiment Station, Geneva, NY 14456, USA

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Abstract

Plant cuticles provide a protective layer that has to be penetrated by fungal pathogens. Evidence is provided for a differential and sequential induction of two classes of cutinolytic esterases by *Alternaria brassicicola*. Serine esterases with cutinolytic activities were expressed by conidia germinating on host surfaces. The enzymes were not induced by surface wax or cutin monomers. They were only expressed during initial (24 h) contact of conidia with cutin on host surfaces freed from wax, and with cutin in aqueous suspensions. In contrast, contact with cutin had no immediate effect on the expression of *CUTAB1*, a gene encoding two cutinase isozymes with crucial functions in the saprophytic utilization of cutin. Presence of a cutin monomer or prolonged exposure to cutin was required for the induction of *CUTAB1* expression. The differential induction of cutinolytic esterases indicates a sequential recognition of cutin as a barrier to be penetrated and to be utilized as a carbon source in saprophytic stages.

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Keywords: *Alternaria brassicicola*; Cutinase; Cutin; Cutin monomer

1. Introduction

Many fungal pathogens encounter the plant cuticle, a hydroxyfatty acid polyester impregnated with waxes, as the initial barrier to be breached during penetration into their hosts. The crucial involvement of cutinases during this early step in host invasion has been suggested for numerous pathogens, with *Fusarium solani* (*Nectria haematococca*) infecting pea hypocotyls as the most comprehensively studied example [1–4].

Multiple lines of evidence have indicated that cutinases expressed during saprophytic utilization of cutin by fungal pathogens were also crucially involved in the penetration of cuticles [1–4,9]. Recent results obtained with cutinase gene-disrupted mutants of *Magnaporte grisea* [5], *F. solani* [6–8], *Alternaria brassicicola* [9] and *Botrytis cinerea* [10,11] have not confirmed this concept of a dual cutinase function. Although virulence penalties reported for a gene-disrupted mutant of *F. solani* remain controversial [7,12], the disruption of cutinase genes strongly

* Corresponding author. Tel.: +1 (315) 7872375; Fax: +1 (315) 7872389; E-mail: wk11@cornell.edu

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expressed during saprophytic growth of the pathogens on cutin had no apparent effect on the pathogenicity of respective mutants. Instead, cutin was no longer accepted as a saprophytic carbon source by gene-disrupted mutants of *F. solani* [6] and *A. brassicicola* [9].

All cutinase genes disrupted thus far and including *CUTAB1* of *A. brassicicola* are induced by cutin monomers [1–4]. In full recognition of this induction mechanism, the original concept of cutinase involvement in pathogenicity implied that cutin monomers were generated by small amounts of a 'sensing' cutinase released from conidia after their attachment to host surfaces [1–4]. In our infection of cabbage leaves with *A. brassicicola*, a rapid generation of cutin monomers during invasion of the host was not apparent; the monomer-induced cutinases A_c and B_a encoded by *CUTAB1* and strongly expressed during saprophytic utilization of cutin were not expressed during host penetration [3,9]. Instead, two different esterases with cutinolytic activities when tested in mixture were recovered from host cuticles inoculated with both the wild-type strain and CUTAB1-minus mutants [3,9]. The induction of different cutinolytic enzymes expressed either in planta or during a saprophytic stage was investigated in this study.

2. Materials and methods

2.1. Fungal culture and conidial germination

Wax was eluted from 80 cabbage leaves by a 10-s dip into chloroform, the chloroform solution was concentrated and spread over the surfaces of 10 glass plates (20×20 cm) equivalent to the extracted leaf area, and the chloroform was evaporated at room temperature. In order to remove chloroform traces, the plates were heated at 45°C for 4 h. Cabbage leaves freed from wax were washed in running water for 5 h.

Preparation of conidia from the wild-type strain of *A. brassicicola* was described earlier [9,13,14]. Conidia (2×10^7 ml^{-1}) were suspended in water and misted onto glass plates, glass plates coated with cabbage leaf wax, and cabbage leaves freed from surface wax. After 24 h, the glass plates and leaves were rinsed with water as described before [9]. The rinsates were filtered and freeze dried. For enzyme expression in aqueous cultures, conidia (2×10^6 ml^{-1}) were suspended in water, in water amended with 5 mg ml^{-1} cutin, and in water containing 0.25 mg ml^{-1} of the cutin monomer 16-hydroxyhexadecanoic acid. Conidia were removed by centrifugation after 24 h or 96 h of incubation at 22°C, and the supernatants were freeze-dried.

2.2. Detection of serine esterases and enzyme assays

Freeze-dried samples were resuspended in 2 ml 10 mM HEPES pH 7.5 and centrifuged. Aliquots containing 2 nkat esterase activity were analyzed by active site-labeling with [^{3}H]diisopropylfluorophosphate (DFP), separation of proteins by SDS-PAGE and subsequent fluorography as described elsewhere [9,13]. Esterase activity was assayed with *p*-nitrophenyl butyrate as substrate and cutinase activity was assayed with [^{3}H]grapefruit cutin as described previously [9].

2.3. Reverse transcription-polymerase chain reaction

Total RNA was extracted as described previously [14] from conidia after 24 and 96 h germination in the presence of cutin, and after 24 h in the presence of 16-hydroxyhexadecanoic acid. RNA (4 μg) was reverse-transcribed with oligo-T as primer at 42°C for 1 h.

Primers used for PCR corresponded to nucleotides 106–127 and 746–766 of *CUTAB1* [14]. Amplifications of cDNA were performed with aliquots of the reverse-transcribed RNA fractions in 50 μl using 2.5 mM $MgCl_2$, 1 U Taq polymerase, 0.2 mM each of deoxyribonucleotide triphosphates, 50 mM KCl, 10 mM Tris-HCl (pH 8.3) and 20 ng of each primer. Water and the two primers alone and as primer pair were used as negative controls. A Perkin Elmer GeneAmp PCR System 9600 was programmed as follows: 94°C for 30 s, 50°C for 30 s, 72°C for 2 min; 35 cycles. Aliquots of PCR products were electrophoresed on agarose gel (1.2%), stained with ethidium bromide and visualized with a UV transilluminator.

3. Results

3.1. Inductive properties of cabbage wax

Germinating conidia of *A. brassicicola* form simple appressoria and penetrate host cuticles within 24 h; disease symptoms are expressed 72 h after inoculation [3,9]. In order to reflect this time frame of host infection, all experiments with relevance to cutinase induction during penetration were performed after 24 h of conidial germination. As before [13,14], the saprophytic stage of cutin utilization was investigated after 96 h. All experiments were done with the wild-type strain of *A. brassicicola* containing the intact cutinase gene *CUTAB1* [9,13,14] and were repeated several times with very similar results.

As shown in Fig. 1, serine esterases with molecular masses of 52 kDa and 26 kDa were released from conidia of *A. brassicicola* suspended in water. The enzymes were fully released after 4 h (data not shown) indicating a preformed nature of these esterases. Although the esterases were clearly different from the 31-kDa and 19-kDa cutinolytic esterases expressed on cabbage leaves [3,9] and the monomer-induced cutinase isozymes A_c (24 kDa) and B_a (21 kDa) encoded by *CUTAB1* [13,14], the mixture of enzymes was cutinolytically active (16 Bq h^{-1} mg^{-1}). The same two 52-kDa and 26-kDa esterases released by conidia suspended in water were also released from conidia germinating on glass surfaces (Fig. 1).

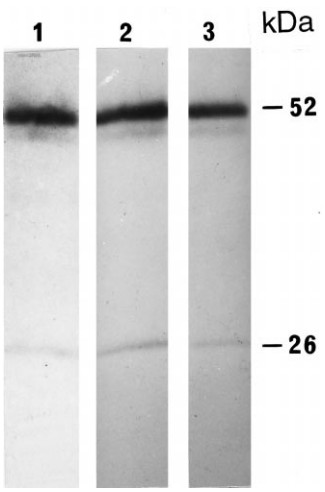

Fig. 1. SDS-PAGE and fluorography of DFP-labeled serine esterases under non-inductive conditions. Conidia were germinated in water (lane 1), on glass plates (lane 2) or on glass plates coated with wax isolated from cabbage leaves (lane 3). Samples were analyzed after 24 h.

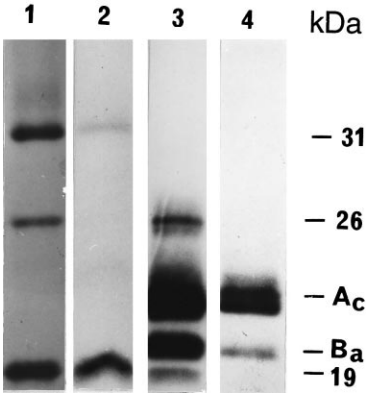

Fig. 2. SDS-PAGE and fluorography of DFP-labeled serine hydrolases under inductive conditions. Conidia germinated for 24 h on cabbage leaves freed from wax (lane 1), for 24 h (lane 2) or 96 h (lane 3) in aqueous suspensions of apple cutin, or for 24 h in the presence of 16-hydroxyhexadeconoic acid (lane 4). Cutinases A_c and B_a have molecular masses of 24 and 21 kDa, respectively.

The expression of the 31-kDa and 19-kDa cutinolytic esterases on host surfaces has been studied with intact leaves [3,9], and their induction by surface wax rather than cutin could not be excluded. However, the esterases released from germinating conidia exposed to a layer of wax removed from cabbage leaves and coated on glass plates were not different from conidia germinating in water (Fig. 1). The result indicated that cabbage wax was inactive in inducing the cutinolytic 31-kDa and 19-kDa esterases strongly expressed on host surfaces during infection by the pathogen [3,9].

3.2. Induction of cutinolytic esterases by cutin and cutin monomers

The lack of esterase induction by surface wax mandated a study on the inductive properties of the polymer cutin. The results are summarized in Fig. 3. In the labeling experiments, equal amounts of esterases determined with *p*-nitrophenyl butyrate as a substrate were subjected to labeling with [^{3}H]DFP [14], and the absence or presence of the 26-kDa esterase released under noninductive condi-

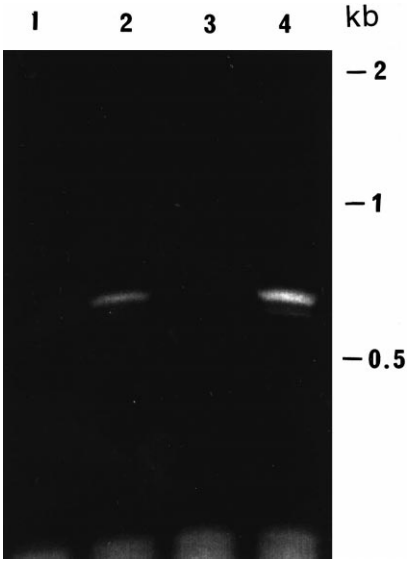

Fig. 3. RT-PCR analysis of *CUTAB1* expression. Conidia were germinated for 24 h in water (lane 1), in water containing 0.25 mg ml^{-1} 16-hydroxyhexadeconoic acid (lane 2), and for 24 h (lane 3) or 96 h (lane 4) in water containing 5 mg ml^{-1} apple cutin. The expected PCR product had a size of 0.6 kb.

tions (Fig. 1) reflects only the proportion of the enzyme relative to other esterases. As shown in Fig. 2, the surface of cabbage leaves freed from wax and, thus, with cutin in immediate contact with germinating conidia retained their activity of inducing the two esterases with molecular masses of 31 kDa and 19 kDa, which also had been recovered from wax-covered leaves [3,14]. Removal of surface wax had no impact on the induction of *CUTAB1* expression, as evidenced by the lack of cutinase A_c and B_a expression (Fig. 3).

The induction of the 31-kDa and 19-kDa esterases and lack of *CUTAB1* expression on both intact [9] and dewaxed surfaces (Fig. 2) prompted us to investigate the inductive properties of apple cutin suspended in aqueous medium and, thus, under the saprophytic conditions employed in the purification of cutinases A_c and B_a [13]. Similar to the results obtained with conidia of *A. brassicicola* in contact with host cuticles, only the 31-kDa and 19-kDa esterases were expressed during the initial 24-h exposure phase (Fig. 2). The result confirmed the fungal origin of these esterases and also indicated that their induction was dependent neither on germination on cuticular surfaces nor on the presence of host cutin. Very similar to host surfaces, cutinases A_c and B_a encoded by *CUTAB1* were not detected after this initial 24-h exposure of conidia to a cutin suspension. As expected from our previous results [13], however, the isozymes became the predominant esterases expressed after 96 h of exposure to cutin (Fig. 2).

In complete reversal, cutinases A_c and B_a were expressed by conidia germinating for 24 h in the presence of the cutin monomer 16-hydroxypalmitic acid (Fig. 2) as a known inducer of *CUTAB1* expression [13,14]. The cutinolytic 31-kDa and 19-kDa esterases were not induced by the monomer (Fig. 2).

3.3. Detection of CUTAB1 mRNA under inductive conditions

The delayed expression of *CUTAB1* in the presence of cutin as opposed to cutin monomers was fully confirmed in a RT-PCR analysis with specific primers derived from the *CUTAB1* sequence (Fig. 3). Conidia germinating for 24 h in water and in the presence of an aqueous cutin suspension contained no detectable *CUTAB1* mRNA. As expected from the expression pattern shown in Fig. 2, gene transcription was evident in conidia germinating for 24 h in the presence of a cutin monomer and after 96 h of saprophytic exposure of the fungus to cutin (Fig. 3).

4. Discussion

In summary, the results described in this study suggest a regulatory sequence more complex than suggested for *F. solani* [1,4]. With *A. brassicicola*, two serine esterases with cutinolytic activity and molecular masses of 52 kDa and 26 kDa were constitutively released shortly after conidia were suspended in water. Surface wax as the first layer encountered by conidia attaching to host surfaces was inactive in the induction of additional serine esterases. Exposure of germinating conidia to host cutin on dewaxed leaves or to nonhost cutin in aqueous suspension induced the expression of the 31-kDa and 19-kDa cutinolytic esterases described before [3,9], but cutin monomers were inactive as inducers. The situation for the well characterized cutinases A_c and B_a [13]

encoded by *CUTAB1* [14] was reversed. The gene was rapidly induced by a cutin monomer, but induction by cutin was delayed beyond the 24-h phase of cuticle penetration during host infection.

The sequential induction of different cutinolytic enzymes implies the existence of a regulatory 'switch'. The polymer appears to be first perceived as a barrier to be penetrated in order to gain access into the host. Once the pathogen reaches a subcuticular location, the availability of carbohydrate sources provided by the host would repress the subsequent induction of other cutinases [1–4,13]. If such repressive conditions are not met within the normal time frame of host surface penetration, cutin monomers as inducers of cutinases functional in a saprophytic stage might slowly be generated, and cutin would then be recognized as a saprophytic carbon source.

While the activity of cutin monomers as specific inducers of *CUTAB1* in *A. brassicicola* was confirmed in this study, the induction mechanism of the 31-kDa and 19-kDa cutinolytic esterases expressed in the presence of cutin remains elusive. The mixture of esterases constitutively released from conidia was cutinolytically active, and these esterases might generate cutin-derived inducers different from cutin monomers. Although highly speculative, cutin oligomers generated through *endo*-cutinase action could serve this function as initial inducers. Such oligomers might slowly be hydrolyzed to cutin monomers as inducers of *CUTAB1* crucial in saprophytic utilization of cutin rather than in cuticle penetration [9].

As summarized and discussed recently [15], it has not been demonstrated thus far that any extracellular hydrolase is crucially involved in fungal pathogenesis. One of the explanations offered was that many of these polymer-degrading enzymes are genetically redundant. This would imply that the disruption of one of the redundant genes is counteracted by related enzymes, which in concert would overcome any recognizable virulence penalty. Our results with *A. brassicicola* described above might point in a different direction. Cutinases might have evolved separately, and according to their specific roles they play in either pathogenic or saprophytic niches occupied by a fungal pathogen. This alternative explanation would imply that the evolution of pathogenic and saprophytic traits diverged more stringently than anticipated in the past.

In support of this hypothesis of cutinase adaptation to distinct ecological roles, the *Fusarium* cutinase has been described to be also active as a lipase [16,17]. Crucial involvement of the *Fusarium* cutinase in saprophytic degradation of the synthetic polyester polycaprolactone [18], and a close relationship between the *Fusarium* cutinase and a lipolytic esterase gene cloned from the nonpathogenic fungus *Aspergillus oryzae* [19] supports a diverse and flexible role of the enzyme in saprophytic stages of plant pathogenic fungi. The broad enzymatic activity of the *Fusarium* cutinase makes it appealing to speculate that the cutinases characterized in the past are 'multi-purpose esterases' hydrolyzing a variety of saprophytically relevant esters including cutin.

While cutinases functional in saprophytic stages of plant pathogenic fungi and induced by cutin monomers are exceptionally well understood [1–4,16–18], the functions and properties of cutinolytic esterases expressed by *A. brassicicola* during initial contact with cutin remain largely unexplored. The results described above demonstrate that such cutinase genes exist and that, in addition, the two classes of cutinases are induced by different mechanisms. The differential and sequential expression of different cutinolytic esterases described in this study will aid in the functional characterization of the cutin-induced esterases expressed at an early stage of host invasion and in the identification of mechanisms involved in a regulatory 'switch' between parasitic and saprophytic stages of a fungal pathogen.

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