A Polymerase Chain Reaction-Based Method to Specifically Detect *Alternaria alternata* Apple Pathotype (*A. mali*), the Causal Agent of Alternaria Blotch of Apple

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ABSTRACT

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Alternaria alternata apple pathotype (previously *A. mali*) causes Alternaria blotch on susceptible apple cultivars through the production of a host-specific toxin, AM-toxin. Identification of some *Alternaria* species, especially those that produce host-specific toxins, has been extremely

difficult due to a high level of variability which extends even to nonpathogenic isolates. We have recently cloned and characterized a gene (*AMT*) that plays a crucial role in AM-toxin biosynthesis and demonstrated that it is only present in isolates of *A. alternata* apple pathotype. Using primers designed for the *AMT* gene, we developed a polymerase chain-reaction-based method to specifically detect AM-toxin producing isolates of *A. alternata* apple pathotype.

Additional keywords: host-selective toxin, molecular diagnostics.

The genus *Alternaria* encompasses both nonpathogenic and pathogenic species. Many of the species comprise different pathotypes of *A. alternata* (Fries) Keissler, each of which has a distinct and limited host range characterized by the production of host-specific toxins (HSTs) essential for pathogenesis (11,20,24). The mechanisms of host-selective pathogenesis through the participation of HSTs is well understood (20,32,39) and about 20 HSTs have been documented (38), of which at least nine are from *A. alternata* pathotypes (24). *A. alternata* apple pathotype (previously described as a distinct virulent form of *A. mali* Roberts) causes Alternaria blotch of apple on a narrow range of susceptible apple cultivars through the production of a host-specific AM-toxin (12). This disease is of worldwide importance (7,31) and is one of the most serious diseases of apple in Japan (10,30). AM-toxin consists of a four-membered cyclic depsipeptide (20,23,37). Physiological and ultrastructural studies suggest that susceptible cultivars of apple have two primary sites for the toxin; one on the plasma membrane and one on the chloroplast (11,20,24,25).

Identification of some *Alternaria* species, especially those that produce HSTs, has been extremely difficult due to a high level of variability in the formation and morphology of conidia, which extends even to nonpathogenic isolates (28,34,40). Recent work has indicated that *Alternaria* pathogens which produce HSTs are pathogenic variants within a single variable species, *A. alternata* (14,15).

Because apple blotch is widespread and has the potential to increase in incidence on susceptible apple cultivars, many of which are still grown commercially (7), it is essential to develop a fast and reliable method to differentiate this pathogen from other *A. alternata* pathotypes and nonpathogenic isolates. The polymerase chain reaction (PCR) has been shown to be useful as a diagnostic

tool for numerous fungal pathogens which are difficult to differentiate using conventional techniques (4,6,8,16,18,27,36). We have recently cloned a gene, *AMT* (GenBank accession no. AF184074), which has a crucial role in AM-toxin biosynthesis (9) and demonstrated that it is specific to the apple pathotype. *AMT* has an open reading frame of 13.1 kilobases (kb) in length which contains no introns. The derived amino acid sequence has a predicted molecular mass of 474 kDa and shows high homology to other cyclic peptide synthetase enzymes. Four catalytic domains, responsible for the activation of each residue in AM-toxin, were identified in *AMT*. This paper describes the use of a PCR-based technique, using primers designed for the cloned *AMT* gene, to specifically detect *A. alternata* apple pathotypes in field isolates of *Alternaria* pathogens.

MATERIALS AND METHODS

Fungal strains and culture conditions. Fungal strains used in this study are listed in Table 1. In addition, some of these isolates were described previously (2). All isolates were maintained on potato dextrose agar (Difco, Detroit, MI) slopes or as 15% glycerol spore suspensions at -80°C.

Leaf Necrosis Bioassay. For a leaf necrosis bioassay, cultures of representative *A. alternata* pathotypes and nonpathogenic *A. alternata* were grown at 26°C in 50 ml of Czapek-Dox medium (Oxoid, Hampshire, UK) supplemented with a 0.5% yeast extract (Difco) for 6 days with no shaking. The culture filtrate was collected and 20 µl was placed on the underside of a young leaf of the highly susceptible cv. Indo which had been slightly wounded by scratching the epidermis. Leaves were incubated overnight in the dark at 26°C and were assessed for necrosis after 24 h.

DNA manipulations. Genomic DNA was extracted as described previously (1), and nucleic acid manipulations followed standard procedures (29). Restriction digestions were carried out using 5 µg of DNA according to the manufacturer's instructions (Toyobo, Tokyo) and fractionated in 0.8% agarose gels. Frac-

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tionated DNA was transferred to Hybond-N membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK) by capillary transfer under alkaline conditions (26). A 0.25-kb DIG-labeled probe (300-1), corresponding to positions 2656–2914 in *AMT*, was generated by PCR using a DIG-DNA labeling and detection kit (Boehringer Mannheim GmbH, Mannheim, Germany). *Hind*III genomic digests of representative *A. alternata* pathotypes and nonpathogenic *A. alternata* were hybridized and washed under high stringency following the manufacturer's instructions.

Polymerase chain reaction. A pair of *AMT*-specific primers (LinF1, 5'-TAT CGC CTG GCC ACC TAC GC-3'; LinR, 5'-TGG CCA CGA CAA CCC ACA TA-3') whose target sequence corresponds to positions 8017–8036 (LinF1) and 8493–8512 (LinR) within the *AMT* gene (Genbank accession no. AF184074) or β -tubulin-specific primers (β -tub1, 5'-TCC GTC GTG CCT TCC CCC AAG GTC TCC GAC-3'; β -tub2, 5'-GGA GCG AAT CCG ACC ATG AAG AAG TGG AGA-3') for positive controls, were used in PCR experiments with genomic DNA of the appropriate *Alternaria* isolate under the following conditions: Twenty nanograms of genomic DNA was added to a 20- μ l reaction containing: dNTPs at a final concentration of 250 μ M (Takara, Tokyo), 1 $\times$ PCR reaction buffer (Takara), primers at a final concentration of 1 μ M (Pharmacia Biotech) and 1.0 unit of *Taq* DNA polymerase (Takara). PCR was performed in a GeneAmp PCR system 9600 (Perkin-Elmer, Norwalk, CT) with an initial step of 5 min at 95°C followed by 30 cycles of 30 s at 94°C, 30 s at 65°C, 45 s at 72°C, and a final step of 7 min at 72°C.

RESULTS

Leaf necrosis bioassay. Culture filtrates of *A. alternata* pathotypes (apple, Japanese pear, tomato, and strawberry) and a nonpathogenic *A. alternata* were tested for the presence of AM-toxin using a leaf necrosis bioassay (Fig. 1 and Table 1), as described in the methods. Only *A. alternata* apple pathotype gave

a positive result using the susceptible apple cv. Indo, demonstrating the host-specific nature of this toxin, which has been previously described (12).

Southern analysis. Southern analysis was carried out using a 0.25-kb fragment (300-1) of the cloned *AMT* gene as a probe to *Hind* III genomic digests of different *A. alternata* pathotypes and nonpathogenic isolates. The *AMT* probe hybridized only to the apple pathotype (Fig. 2), and together with the bioassay data provides strong evidence that strains unable to produce AM-toxin contain no homologs to *AMT*. Therefore, running a PCR with primers designed for the *AMT* gene should be a good strategy to specifically identify the *A. alternata* apple pathotype.

Polymerase chain reaction. Primers designed for the cloned *AMT* gene (see methods) were used in a PCR reaction with 20 ng of genomic DNA from various *A. alternata* pathogens (including five pathotypes), nonpathogenic *A. alternata*, and *A. brassicae* using conditions described in the methods. A PCR product of the expected size (0.5-kb) was only amplified from *A. alternata* apple pathotype. No other products were identified from different *A. alternata* pathotypes, nonpathogenic *A. alternata*, and other *Alternaria* species pathogenic on a range of different hosts (Fig. 3 and Table 1). *A. brassicae*, which produces the HST destruxin B (3), that is similar in structure to AM-toxin and is probably synthesized via a similar biosynthetic pathway, also gave no PCR product. All isolates amplified a 0.35-kb product using primers to the β -tubulin gene (Fig. 3) proving that negative amplification with the *AMT* primers was not due to poor template DNA. These results clearly demonstrate that PCR with *AMT* gene-specific primers is a reliable technique to specifically detect *A. alternata* apple pathotype, the causal agent of Alternaria blotch of apple.

DISCUSSION

The genus *Alternaria* encompasses both nonpathogenic and pathogenic species. Seven of these pathogens cannot be distin-

TABLE 1. *Alternaria* strains, AM-toxin bioassay and polymerase chain reaction

Strain	Disease	HST	Source	Pathogenicity ^a	PCR ^b	Reference
<i>A. alternata</i>						
Apple pathotype	Alternaria blotch of apple	AM-toxin				
M-71			Japan	+	+	Akamatsu et al. 1999
FIV-Cont45			Japan	+	+	Akamatsu et al. 1999
O-159			Japan	+	+	Akamatsu et al. 1999
O-210			Japan	+	+	Akamatsu et al. 1999
CSL-635			USA	+	+	This study
CSL-1166			USA	+	+	This study
CSL-1175			USA	+	+	This study
CSL-1177			USA	+	+	This study
Japanese pear pathotype	Black spot of Japanese pear	AK-toxin				
No.15A			Japan	–	–	Akamatsu et al. 1999
Strawberry pathotype (f. sp. <i>fragariae</i>)	Alternaria black spot of strawberry	AF-toxin				
M-30			Japan	–	–	Akamatsu et al. 1999
Tomato pathotype (f. sp. <i>lycopersici</i>)	Alternaria stem canker of tomato	A(A)L-toxin				
As-27			USA	–	–	
Tobacco pathotype	Brown spot of tobacco	AT-toxin				
ATL-5			Japan	–	–	Akamatsu et al. 1999
Non-pathogenic						
O-94			Japan	–	–	Akamatsu et al. 1999
EGS35-193			USA	–	–	Akamatsu et al. 1999
European pear isolate						
CSL648	UC ^c	UC	Portugal	–	–	This study
Chinese pear isolate						
CSL704	UC	UC	China	ND	–	This study
Wheat Isolate						
CSL325	UC	UC	Unknown	–	–	This study
<i>A. brassicae</i>						
MAFF712079	Gray spot of <i>Brassica</i>	Destruxin B	Japan	–	–	Bains & Tewart 1987

^a Isolates with a CSL designation were tested for pathogenicity by the Central Science Laboratory (Sand Hutton, York, YO4 1LZ, UK). All other isolates were tested for pathogenicity using a leaf necrosis bioassay as described. + designates pathogenic; – designates nonpathogenic; ND designates not done.

^b Genomic DNA (20 ng) was used in a polymerase chain reaction with *AMT* specific primers. + designates amplification; – designates no amplification.

^c Uncharacterized.

guished from ubiquitous saprophytic *A. alternata* (Fries) Keissler (5,17,33) on the basis of conidial morphology (19,21) but have been designated as distinct pathotypes based on their unique pathogenicity (19,21,22). These pathotypes have a distinct and limited host range characterized by the production of HSTs essential for pathogenesis (11,20,24). *A. alternata* apple pathotype produces the host-specific AM-toxin, and causes Alternaria blotch, a disease of

Fig. 1. Leaf necrosis bioassay of *Alternaria alternata* pathotypes and non-pathogenic *A. alternata*. Twenty microliters of culture filtrate was placed on the underside of a young leaf of the highly susceptible apple cv. Indo. A, apple pathotype (M-71); JP, Japanese pear pathotype (No.15A); S, strawberry pathotype (M-30); T, tomato pathotype (As-27); NP, non-pathogenic *A. alternata* (O-94); H₂O, water.

Fig. 2. Southern blot analysis of *Alternaria alternata* pathotypes using *AMT* probe 300-1. Total DNA was digested with *Hind*III, separated in a 0.8% agarose gel and hybridized to DIG-dUTP labeled 300-1 from *A. alternata* apple pathotype. Lane 1, apple pathotype (M-71); Lane 2, Japanese pear pathotype (No.15A); Lane 3, strawberry pathotype (M-30); Lane 4, tomato pathotype (As-27); Lane 5, non-pathogenic isolate (O-94). Size is in kilobases.

Fig. 3. Polymerase chain reaction using *Alternaria alternata* pathotypes, non-pathogenic *A. alternata* and other *Alternaria* pathogens. *AMT* specific primers (LinF1, 5'-TAT CGC CTG GCC ACC TAC GC-3'; LinR, 5'-TGG CCA CGA CAA CCC ACA TA-3') amplified a 0.5-kb product. Beta-tubulin specific primers (β -tub1, 5'-TCC GTC GTG CCT TCC CCC AAG GTC TCC GAC-3'; β -tub2, 5'-GGA GCG AAT CCG ACC ATG AAG TGG AGA-3') were used as a positive control and amplified a 0.35-kb product from all isolates. H₂O contains all reagents except DNA and acts as a negative control. Sizes are in kilobases.

worldwide importance (7,31) and one of the most serious diseases of apple in Japan (10,30), on a narrow range of susceptible apple cultivars (12).

The proposal that plant pathogenic *A. alternata* should be classified by pathotype (19,21,22) has not been widely accepted, partly because conventional classification of the imperfect fungus *Alternaria* depends on variation in formation and morphology of conidia (28,33,40). Molecular approaches to elucidate the taxonomy of *Alternaria* have also been used. DNA-DNA reassociation analysis between HST-producing *A. alternata* and nonpathogenic *A. alternata* suggested a close relationship (13). Restriction fragment length polymorphism (RFLP) analysis revealed that HST producing *A. alternata* could not be separated from nonpathogenic *A. alternata* (14). Comparison of the internal transcribed spacer regions (ITS1 and ITS2) of rDNA supported these results (15).

Identification of these important pathogens is therefore a lengthy and time-consuming process involving a combination of pathogenicity testing and conidial morphology analysis. The PCR has been shown to be useful as a diagnostic tool for numerous fungal pathogens which are difficult to differentiate using conventional techniques (4,6,8,16,18,27,36). Because the disease causing ability of the different *A. alternata* pathotypes is related to the production of HSTs, cloning of the genes required for their biosynthesis will not only further our understanding of the molecular basis of host specificity but will also provide molecular probes for diagnostic techniques. Recently we have cloned a gene, *AMT* (Genbank accession no. AF184074), from *A. alternata* apple pathotype involved in AM-toxin biosynthesis and shown it to be highly specific to the apple pathotype (9). Using primers designed for the *AMT* gene we have developed a PCR-based method to specifically identify *A. alternata* apple pathotype from other *A. alternata* pathotypes, nonpathogenic *A. alternata*, and other disease causing *Alternaria* species (Fig. 3). As other genes, from other *A. alternata* pathotypes, involved in HST production become characterized at the molecular level, similar diagnostic techniques will become available to identify these pathogens. Already, the genes for *AKT1* and *AKT2* involved in AK-toxin production from *A. alternata* Japanese pear pathotype have been cloned and shown to be specific to this pathotype (35).

Although the work here uses total DNA as a template for PCR, direct amplification from diseased plant material should be possible and we are currently refining the PCR procedure described here for this purpose.

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