Distribution and Characterization of *AKT* Homologs in the Tangerine Pathotype of *Alternaria alternata*

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ABSTRACT

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The tangerine pathotype of *Alternaria alternata* produces a host-selective toxin (HST), known as ACT-toxin, and causes Alternaria brown spot disease of citrus. The structure of ACT-toxin is closely related to AK- and AF-toxins, which are HSTs produced by the Japanese pear and strawberry pathotypes of *A. alternata*, respectively. AC-, AK-, and AF-toxins are chemically similar and share a 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid moiety. Two genes controlling AK-toxin biosynthesis (*AKT1* and *AKT2*) were recently cloned from the Japanese pear pathotype of *A. alternata*. Portions of these genes were used as heterologous probes in Southern blots, that detected homologs in 13 isolates of *A. alternata* tangerine pathotype from Minneola tangelo in Florida. Partial sequencing of the homologs in one of these isolates demonstrated high sequence similarity to *AKT1* (89.8%) and to *AKT2* (90.7%). *AKT* homologs were not detected in nine isolates of *A. alternata* from rough lemon, six isolates of nonpathogenic *A. alternata*, and one isolate of *A. citri* that causes citrus black rot. The presence of homologs in the Minneola isolates and not in the rough lemon isolates, nonpathogens or black rot isolates, correlates perfectly to pathogenicity on Iyo tangerine and ACT-toxin production. Functionality of the homologs was demonstrated by detection of transcripts using reverse transcription-polymerase chain reaction (RT-PCR) in total RNA of the tangerine pathotype of *A. alternata.* The high sequence similarity of *AKT* and *AKT* homologs in the tangerine pathotype, combined with the structural similarity of AK-toxin and ACT-toxin, may indicate that these homologs are involved in the biosynthesis of the decatrienoic acid moiety of ACT-toxin.

Additional keywords: fungi, host-specific toxin, phytopathogen.

Some strains of *Alternaria alternata* (Fr.) Keissl. are known to produce host-selective toxins (HST) that are selectively toxic to certain plants. This selectivity correlates to the pathogenicity reactions of the fungi that produce them (9,18). Nishimura and Kohmoto (18) proposed that these *Alternaria* strains should be defined as distinct pathotypes of *A. alternata*. Despite the morphological similarity of these host-specific strains, one pathotype can be easily distinguished from another by comparing host range. Chemical structures of HSTs from six pathotypes of *A. alternata* have been elucidated (8), and two HST biosynthetic genes were recently cloned from the Japanese pear pathotype of *A. alternata* (29).

Citrus brown spot, caused by the tangerine pathotype of *A. alternata*, is a serious foliage and fruit disease of tangerines (*Citrus reticulata* Blanco), grapefruit (*C. paradisi* Macf.), and grapefruit × tangerine hybrids, including such cultivars as Minneola, Orlando, Nova, Murcott, and Sunburst. The pathogen causes abscission of immature fruit and blemishes on mature fruit that can lead to extensive fruit losses. The pathogen also affects young leaves, and infected leaves develop brown spots surrounded by a chlorotic halo due to production of the HST. Abscission of infected leaves is also commonly observed, and these leaves fall to the grove floor and serve as a source of primary inoculum for the disease (31). Alternaria brown spot was first reported in Australia on Emperor mandarin in 1903 (3), but the causal agent was not identified until 1959 (5,21). Brown spot appeared in Florida in 1974 (35) and now occurs in many parts of the world where susceptible cultivars are grown (31). The pathogen was initially identified as *A. citri* Ellis & Pierce (21,35) due to its morphological similarity to the fungus that causes storage rot of citrus known as citrus black rot (2). However, the pathogen that causes brown spot is now categorized as the tangerine pathotype of *A. alternata* and is distinguished from the black rot pathogen based on pathogenicity reaction and the production of an ACT-toxin (7,10). The major form of HST produced by the tangerine pathotype was designated as ACT-toxin I (Fig. 1) (7). Another selective toxin, ACTG-toxin, was identified by Kono et al. (11); however, ACT-toxin is at least 10-times more toxic to citrus, and ACT-toxin I and its derivatives are the only major products detected in germinating conidium fluids (7). The structure of ACT-toxin is closely related to AK- and AF-toxins, which are the HSTs produced by the Japanese pear and strawberry pathotypes of *A. alternata* (7,14,16). These toxins share a common 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid moiety (Fig. 1) (7,14,16).

Two genes (*AKT1* and *AKT2*) involved in AK-toxin biosynthesis were recently cloned using a strategy based on restriction enzyme-mediated integration (REMI) (29). Both genes are required for AK-toxin synthesis and are organized in a gene cluster designated the *AKT* locus. This study also reported on the distribution of *AKT* homologs in seven HST-producing strains and other non-

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Nucleotide and/or amino acid sequence have been deposited with GenBank as Accession no. AB034586 for the *AKT1* homolog sequence and Accession no. AB034587 for the *AKT2* homolog sequence.

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pathogenic strains of *A. alternata* using Southern blot analysis (29). *AKT* probes hybridized to all strains of the Japanese pear pathotype that produce AK-toxin. Probes also hybridized to all tested strains of the strawberry pathotype and the tangerine pathotype that produce AF-toxin and ACT-toxin, respectively (29). The *AKT1* and *AKT2* homologs in the strawberry and tangerine pathotypes are hypothesized to be the genes responsible for biosynthesis of 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid (29), which is the common structural moiety shared by these three toxins (7,14,16). Determination of the role of these genes in the biosynthesis of AK-, ACT-, and AF-toxins will be an important component of evolutionary studies of pathogenicity and host specificity in this fungus.

Our objective in this study was to characterize the distribution of *AKT* homologs in 25 isolates of *A. alternata* isolated from two *Citrus* species in Florida. Homologs were detected using Southern blot analysis with partial *AKT1* and *AKT2* sequences as probes. To confirm that these homologous sequences are transcribed, transcripts of the *AKT1* and *AKT2* homologs were identified in total RNA using reverse transcription-polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

Plants and fungal strains. The plants used in this study included Iyo (*C. iyo* hort. ex Tanaka), rough lemon (*C. jambhiri* Lush.), and Japanese pear (*Pyrus pyrifolia* Nakai var. *culta* Nakai) cv. Nijisseki. Iyo is susceptible to the tangerine pathotype of *A. alternata* and sensitive to ACT-toxin but resistant to the rough lemon pathotype of *A. alternata* and ACR-toxin (6). In contrast, rough lemon is susceptible to the rough lemon pathotype and sensitive to ACR-toxin but is resistant to the tangerine pathotype and ACT-toxin (6). Plants were maintained in a greenhouse and young leaves (midrib length 2 to 4 cm) were used for all experiments.

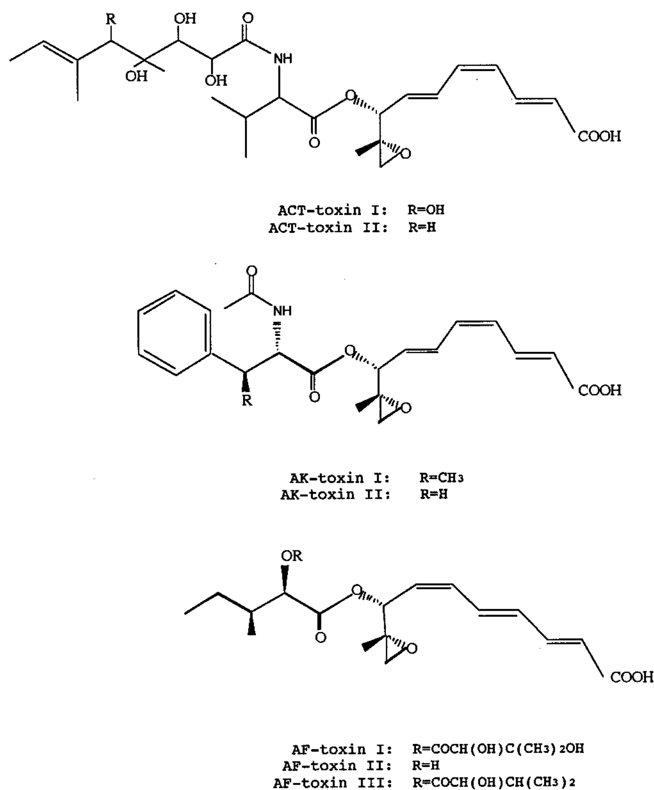

Fig. 1. Chemical structures of ACT-, AK-, and AF-toxins.

Twenty-five isolates of *A. alternata* were obtained: thirteen from leaf lesions of Minneola tangelo (*C. reticulata* × *C. paradisi*) and twelve from rough lemon in Florida as part of a study of the population structure and host specificity of Alternaria brown spot isolates (20). In addition, laboratory stock strains of the rough lemon pathotype of *A. alternata* (AC325), a nonpathogenic *A. alternata* (0-94) and a strain of the citrus black rot pathogen (*A. citri* strain ATCC58171) were used in this study. Codes for the field-collected Florida isolates were as follows: (i) codes beginning with SH were isolated from Minneola tangelo at Shinn Grove; (ii) codes beginning with EV were from Minneola tangelo at Evenhouse; (iii) codes beginning with HC were from rough lemon root sprouts at an abandoned grove in Haines City; and (iv) codes beginning with BC were from rough lemon root sprouts at an abandoned grove in Baseball City. All isolates were started from filter papers stored at –20°C (20) and grown on V8-juice agar plates (4). Mycelia and conidia formed on each plate and were collected with 7 ml of sterile distilled water and mixed with an equal volume of sterile 50% (vol/vol) glycerol and stored at –80°C.

Pathogenicity and toxin production. HST production and pathogenicity of field-collected isolates were determined by the methods of Kohmoto et al. (6,7,10). Briefly, the toxin production of each isolate was determined using a leaf necrosis assay with crude culture filtrates (6,7,10). Small pieces of mycelium of isolates grown on V8-juice agar plates (4) were inoculated in modified Richards' solution (10). Following incubation for 24 days at 24°C under cool-white fluorescent light, the mycelial mat was harvested by centrifugation at 1,500 × *g* for 5 min. The supernatant was diluted with distilled water (1:100, vol/vol), and 30 µl was placed on a puncture-wound site with a needle on the lower surface of

TABLE 1. Pathogenicity and host-selective toxin productivity of *Alternaria* spp. isolated in Florida

Alternaria spp. isolate	Pathogenicity[a]		Response to culture filtrate[b]	
	Iyo	RL	Iyo	RL
SH11	+	–	+	–
SH12	+	–	+	–
SH13	+	–	+	–
SH14	+	–	+	–
SH15	+	–	+	–
SH18	+	–	+	–
SH19	+	–	+	–
SH20	+	–	+	–
SH21	+	–	+	–
EV10	+	–	+	–
EV13	+	–	+	–
EV14	+	–	+	–
EV24	+	–	+	–
HC1	–	+	–	+
HC7	–	+	–	+
HC8	–	+	–	+
HC14	–	–	–	–
HC15	–	+	–	+
HC21	–	+	–	+
BC6	–	–	–	–
BC17	–	–	–	–
BC19	–	+	–	+
BC27	–	+	–	+
BC35	–	+	–	+
BC36	–	–	–	–

[a] Conidial solution (10^5 spores per ml) was sprayed on the lower surface of Iyo and rough lemon (RL) leaves by the method described by Kohmoto et al. (6,7,10). The leaves were incubated for 24 h at 24°C under dark, and leaves showing necrotic spots were determined as susceptible (+).

[b] Culture filtrate of each isolate was diluted 100 times with distilled water, and 30 µl of each diluted filtrate was placed on wounded site of lower surface of Iyo and rough lemon (RL) leaves by the method described by Kohmoto et al. (6,7,10). The leaves were incubated for 48 h at 24ºC under dark. Necrosis developed on entire leaves was counted as sensitive (+). All experiments described above were repeated three times and the same results were obtained.

leaves of Iyo, rough lemon, and Japanese pear cv. Nijisseki. Leaves were incubated in a moist, dark chamber at 24°C, and necrosis development on leaves was determined after 48 h.

The pathogenicity of each isolate was determined by spraying conidia on detached young leaves of Iyo, rough lemon, and Japanese pear cv. Nijisseki (6,7,10). The mycelial mat produced in the modified Richards' solution (10) was washed with water and dried briefly on paper towels. The mycelial mat was incubated for 24 h at 24ºC in the dark to induce sporulation. Conidia formed on the surface of the mat were removed with a paint brush dipped in water and were washed twice by centrifugation at $800 \times g$ for 15 min. The conidial solution was adjusted to a concentration of 10^5 spores per ml with a hemacytometer and sprayed on the lower surface of detached leaves of Iyo, rough lemon, and Japanese pear cv. Nijisseki. Leaves were incubated in a moist chamber for 24 h at 24ºC in the dark, and the development of necrotic spots on the leaves was monitored.

DNA extraction and Southern blot analysis. Genomic DNA was extracted using the method of Scott-Crag et al. (27) with some modifications. Ten microliters of freeze stocks of *A. alternata* produced on V8-juice agar plates was inoculated into 50 ml of potato dextrose broth (4) in 200-ml flasks and incubated for 20 h at 24ºC on an orbital shaker (120 rpm) in the dark. The resulting mycelium was harvested by passing through two layers of no. 4 filter paper (Whatman International, Maidstone, England) under vacuum. The mycelium was then ground to a fine powder in liquid nitrogen with a mortar and pestle and resuspended in 5 ml of TE extraction buffer (3.75 ml of 0.2 M EDTA, 0.25 ml of 1.0 M Tris [pH 8.0], 1.0 ml of sterile water, 0.05 g of sarkosyl, and 1.5 mg of proteinase K) and incubated for 15 min at 65ºC. After centrifugation for 5 min at 5,000 rpm, the supernatant was treated with RNase A at a concentration of 10 µg/ml for 30 min at 37ºC. Following RNase treatment, the supernatant was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol/vol) and once with chloroform. DNA was precipitated in EtOH-sodium acetate (23) and resuspended in 800 µl of TE buffer (pH 8.0) (23). DNA was reprecipitated at 4ºC overnight following addition of 2.5 M NaCl and 20% (wt/vol) PEG 8000 (550 µl). Pellets were resuspended in TE buffer (pH 8.0) (23) or water following centrifugation. DNA concentrations were estimated visually in ethidium bromide-stained agarose gels by comparing band intensity with known quantities of phage lambda DNA (Takara, Shiga, Japan).

Southern blot analysis was performed with the internal regions of the *AKT1* and *AKT2* genes as probes (29). Probes were labeled using the digoxigenin (DIG) High Prime DNA labeling and detection kit (Boehringer, Mannheim, Germany) following the manufacturer's directions with minor modifications. The subcloned 1.1-kb internal region of *AKT1* from cosmid clone pcAKT-1 (29) was digested with *Eco*RI and *Xho*I, and the 1.8-kb fragment containing *AKT2* was digested with *Bgl*II and *Apa*I. Each of these fragments was cloned into the Bluescript SK(+) vector (Stratagene, La Jolla, CA) and digested with the respective combinations of restriction enzymes. Digested inserts were labeled with the DIG system following the manufacturer's directions. Total genomic DNA (10 µg) of *A. alternata* was digested with several different restriction enzymes, including *Bam*HI, *Cla*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Not*I, *Sal*I, and *Xho*I (Takara). Digested DNA products were separated by electrophoresis in 1% (wt/vol) agarose gels with Tris-acetate-EDTA (TAE) buffer (23) and transferred to Hybond-N+ membrane (Amersham, Buckingham, UK) by capillary transfer following denaturation in 0.5 M NaOH and 1.5 M NaCl and neutralization in 1 M Tris-HCl, pH 7.4, plus 1.5 M NaCl (23). Five microliters of DIG-labeled probe was used for hybridization at 68°C in a RPN2510 hybridization oven (Amersham) for 16 h in hybridization buffer (10 ml) supplied by the kit. Following hybridization, the membrane was washed five times: twice with washing solution 1 (0.3 M NaCl plus 0.03 M sodium citrate [2X SSC] and 0.1% sodium dodecyl sulfate [SDS]) at room temperature for 5 min each, twice with washing solution 2 (0.1X SSC and 0.1% SDS) at 68°C for 15 min each, and once with washing solution 3 (0.15 M NaCl, 0.1 M maleic acid, 0.3% Tween 20) at room temperature for 5 min. Hybridization of the probes was visualized by anti-DIG antibody-alkaline phosphatase and CSPD or SD-star (Boehringer) and exposure of the membranes to X-ray films (Fujifilm RX-U, Tokyo).

PCR for *AKT* homolog amplification. PCR primers were designed from regions of the *AKT1* and *AKT2* genes to amplify *AKT* homologs from genomic DNA of isolate SH20. Primers included ACTT1F2 (5′-CACAGGCTATCTTCACATGC-3′) and ACTT1R2 (5′-CGTAGCTCCATAGCATTGCT-3′) for amplification of the *AKT1* homolog and ACTT2F1 (5′-AGATGCAGCAGCCCATCATT-3′) and ACTT2R1 (5′-CGACTGCAGATATTTCATCC-3′) for amplification of the *AKT2* homolog. Reaction mixtures (100 µl) contained 100 ng of genomic DNA, 100 pM each of forward and reverse primers, 2.5 units of *Taq* polymerase with supplied A501-3 reaction buffer and dNTP mixture (Takara). Reactions were carried out in a Perkin-Elmer DNA thermal cycler (PE Biosciences, Norwalk, CT). PCR consisted of 30 cycles (denaturing for 1 min at 95°C, primer annealing for 1 min at 60°C for *AKT1* homolog or at 50°C for *AKT2* homolog, and extension for 1 min at 72°C). PCR products were separated on 1% agarose gel, transferred to Hybond-N+ by capillary transfer (23), and the membrane was hybridized with *AKT1* or *AKT2* probes as described above.

RT-PCR. Transcripts of *AKT* homologs were detected using RT-PCR. Mycelium was produced in modified Richards' solution

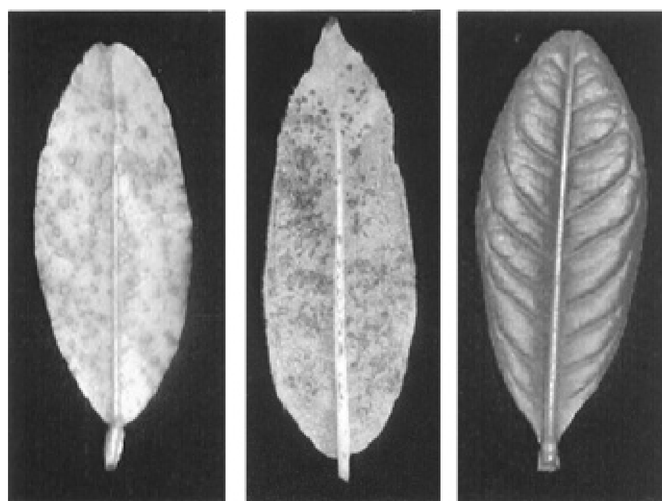

Fig. 2. Citrus and Japanese pear leaves inoculated with conidia of Minneola isolate SH20. Conidial suspension (10^5 spores per ml) was sprayed on the lower surface of detached young leaves of citrus cvs. Iyo (left), rough lemon (right), and Japanese pear cv. Nijisseki (center) with the method described by Kohmoto et al. (5,8). Inoculated leaves were incubated in a moist, dark chamber at 24ºC for 24 h.

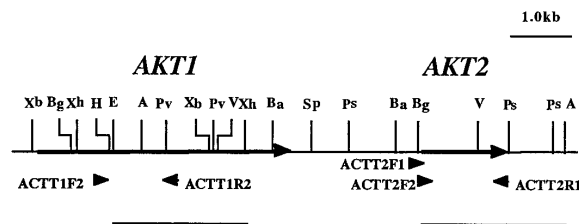

Fig. 3. Restriction enzyme map of *AKT1*, *AKT2,* and flanking regions. Open reading frames of *AKT1* and *AKT2* are indicated by arrows. Restriction sites are abbreviated as follows: Xb, *Xba*I; Bg, *Bgl*II; Xh, *Xho*I; H, *Hin*dIII; E, *Eco*RI; A, *Apa*I; Pv, *Pvu*II; V, *Eco*RV; Sp, *Sph*I; Ps, *Pst*I; Sa, *Sal*I. Regions of each gene used for probes are indicated as bars below the map. Arrowheads indicate the priming sites for amplification of the partial regions of the *AKT1* and *AKT2* homologs.

(10) for 30 days at 24°C under cool-white fluorescent light and ground to a fine powder with liquid nitrogen in a mortar with a pestle. Total RNA was isolated using the RNeasy plant mini kit (Qiagen, Hilden, Germany) following the manufacturer's directions.

One microgram of total RNA from isolates SH20, AC325, and O-94 was employed as the template for RT-PCR. RT-PCR was carried out in a two-step process using the ThermoScript RT-PCR system (GibcoBRL, Bethesda, MD) according to the manufacturer's directions. cDNA was synthesized in the first step with total RNA and oligo(dT)$_{20}$ at 50°C for 60 min. In the second step, PCR was performed with the cDNA as the template and the following primer sets: the same primers as described above for amplification of the partial region of the *AKT1* homolog and ACTT2F2 (5′-GCATGGGAGGCTGTCA-3′) and ACTT2R1 (5′-CGACTGCA-GATATTTCATCC-3′) for amplification of the partial region of the *AKT2* homolog. Reaction conditions for PCR were the same as described above for genomic PCR. RT-PCR products were separated on 1% agarose gel, transferred to Hybond-N+ membranes using capillary transfer (23), and probed with *AKT1* or *AKT2* probes.

Sequencing and sequence alignment. PCR products were subcloned into pT7Blue-2 T-vector (Novagen, Madison, WI) according to the manufacturer's directions. Sequence data were obtained from both strands by the dideoxy chain termination method (24) using an ABI PRISM dye termination cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) and an automated fluorescent DNA sequencer (Model 310, Applied Biosystems). DNA sequences were aligned with CLUSTAL W (30).

RESULTS

Pathogenicity and toxin production. Isolate samples from Minneola and rough lemon were pathogenic only on their respective hosts, which is consistent with previous reports (6,7,10,20). All 13 isolates obtained from Minneola tangelo at the SH and EV Florida locations were pathogenic on Iyo, while none of the rough lemon isolates were pathogenic on Iyo (Table 1). All Minneola isolates were nonpathogenic on rough lemon, and 4 of 12 rough lemon isolates were nonpathogenic on both hosts (Table 1). Toxin assays revealed a perfect correlation between pathogenicity reaction and response to toxin (Table 1), which is also consistent with previous studies (6,7). Typical symptoms of the disease caused by Minneola isolate SH20 when inoculated on Iyo, rough lemon, and Japanese pear cv. Nijisseki are shown in Figure 2. The symptoms produced by Minneola isolate SH20 on Japanese pear (Fig. 2) were identical to symptoms produced by the Japanese pear pathotype of *A. alternata* (29). Both the rough lemon pathotype and the nonpathogenic *A. alternata* isolates failed to show any effect on Japanese pear cv. Nijisseki (results not shown).

Distribution of *AKT* homologs among pathogenic and nonpathogenic *A. alternata*. Distribution of *AKT* homologs among field-collected Florida isolates was examined by Southern blotting with internal regions of the *AKT1* and *AKT2* genes as probes (Fig. 3). The *AKT1* probe hybridized to a 4-kb fragment from *Eco*RV-digested genomic DNA of all Minneola isolates. No hybridization of the *AKT1* probe was detected for DNA from the rough lemon isolates, nonpathogenic isolates, or the citrus black rot pathogen (Fig. 4A). The *AKT*2 probe produced a similar hybridization pattern hybridizing to all the Minneola isolates but not to rough lemon isolates, nonpathogenic isolates, or the black rot pathogen (Fig. 4B). The *AKT1* probe hybridized to a single restriction fragment in each isolate, while the *AKT2* probe hybridized from one to four fragments per isolate (Fig. 4). Hybridization of *AKT1* and *AKT2* was examined further in isolate SH20 with additional restriction enzymes. The *AKT1* probe hybridized to one restriction fragment for all enzymes, except *Sal*I and *Pst*I, which both had two fragments that hybridized (Fig. 5A). The *AKT2* probe hybridized to more than one fragment for all enzymes tested (Fig. 5B).

Amplification of a partial region of *AKT* homologs by PCR. PCR primers designed for *AKT1* and *AKT2* (Fig. 3) were used to amplify the internal region of the homologs from Minneola isolate SH20 (Fig. 6). A 785-bp PCR fragment amplified with ACTT1F2 and ACTT1R2 hybridized to the *AKT1* probe used in Southern blot analysis (Fig. 6A). The PCR product was subcloned, and the nucleotide sequence was determined (GenBank Accession no. AB034586) (Fig. 7). The sequence was 89.8% identical to *AKT1*. PCR primers ACTT2F1 and ACTT2R1, designed from the internal sequence of *AKT2,* amplified a 840-bp PCR product that hybridized to the *AKT2* probe (Fig. 6B). The sequence of the subcloned product (GenBank Accession no. AB034587) was 90.7% similar to *AKT2* at the DNA sequence level (Fig. 8). Neither primer set amplified products from genomic DNA of the rough lemon pathotype (AC325) or the nonpathogenic *A. alternata* isolate O-94 (Fig. 6).

Detection of *AKT* homolog expression by RT-PCR amplification. Expression of the *AKT1* and *AKT2* homologs from Minneola isolate SH20 was also examined by RT-PCR (Fig. 6).

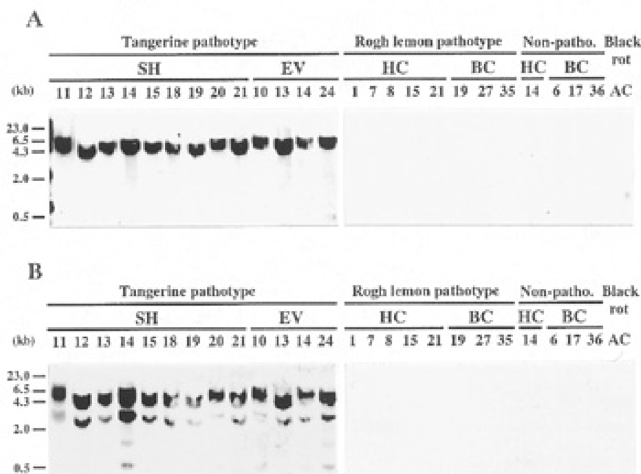

Fig. 4. Distribution of the *AKT1* and *AKT2* homologs in isolates of *A. alternata* isolated from Minneola tangelo and rough lemon in Florida. Total DNA of each strain was digested with *Eco*RV and separated in 1% agarose gel. The blot was probed with (A) *AKT1* and (B) *AKT2* (see Fig. 3). Molecular size markers in kb are indicated at the left side of the blot. SH = isolates from Minneola tangelo leaf lesions at Shinn Grove; EV = isolates from Minneola tangelo leaf lesions at Evenhouse; HC = isolates from rough lemon root sprouts in an abandoned grove in Haines City; BC = isolates from rough lemon root sprouts in an abandoned grove in Baseball City. A strain of the citrus black rot pathogen (*A. citri*, ATCC58171) is indicated as Black rot/Ac.

Fig. 5. Southern blot analysis of the *AKT1* and *AKT2* homologs in the tangerine pathotype of *A. alternata* (SH20) using several restriction enzymes. Total DNA of the Minneola isolate SH20 digested with *Eco*RI (E), *Xho*I (X), *Eco*RV (V), *Hin*dIII (H), *Bam*HI (B), *Cla*I (C), *Kpn*I (K), *Sal*I (S), and *Pst*I (P), and separated in 1% agarose gel. The blot was probed with (A) *AKT1* and (B) *AKT2* (see Fig. 3). Molecular size markers in kb are indicated at the left side of the blot.

Primers for the *AKT1* homolog (ACTT1F2 and ACTT1R2) amplified a 700-bp product (Fig. 6A), and primers for the *AKT2* homolog (ACTT2F2 and ACTT2R1) amplified a 750-bp product (Fig. 6B). RT-PCR products were amplified from the ACT-toxin producer (SH20) but not from the rough lemon pathotype (AC325) nor from nonpathogenic O-94 (Fig. 6). The 700-bp amplification product hybridized to the *AKT1* probe (Fig. 6A) and was smaller than the PCR product (785 bp) amplified from genomic DNA of the same isolate (Fig. 6A). The 750-bp RT-PCR product hybridized to the *AKT2* probe (Fig. 6B), and the size of the RT-PCR product was smaller than the PCR product (810 bp) amplified with the same primer set (ACTT2F2 and ACTT2R2) from genomic DNA (data not shown). Comparison of the sizes of the amplified products from genomic PCR and from RT-PCR indicated that both *AKT1* and *AKT2* homologs have introns, which have also been found in *AKT1* and *AKT2* (29).

DISCUSSION

Our present knowledge of HST in plant-pathogenic fungi is based mainly on studies of two genera, *Alternaria* and *Cochliobolus* (18,25,33,37). Genes involved in the production of HST in *C. carbonum* race 1 (1,19,22,28,34) and *C. heterostrophus* race T (12,36,38) have been cloned. These findings have facilitated investigations into the evolution of pathogenesis and host specificity in these systems. More recently, the *AKT* locus responsible for the biosynthesis of host-specific AK-toxin in *A. alternata* was tagged using REMI (29). Sequencing of the flanking regions of the tagged site identified two genes, that have been designated *AKT1* and *AKT2*. In the present study, we have demonstrated that *AKT1* and *AKT2* homologs are present in *A. alternata* isolates from Minneola tangelo in Florida. All Minneola isolates were pathogenic on Iyo tangerine but not on rough lemon, and the pathogenicity reactions and HST production patterns were identical to the laboratory strain of the tangerine pathotype of *A. alternata* (AC320) (6,7,10). *AKT* homologs were present only in the Minneola isolates and not in the eight rough lemon isolates or the five nonpathogenic *A. alternata* isolates. *AKT* homologs were also absent from laboratory stock strains of the rough lemon pathotype (AC325), the non-pathogenic *A. alternata* isolate O-94, and *A. citri.* Transcripts of the *AKT1* and *AKT2* homologs were only found in the Minneola isolates, indicating that functional *AKT* homologs were only present in the isolates that produced ACT-toxin and not in other *Alternaria* species isolated from citrus.

AKT homologs have also been identified in the strawberry pathotype of *A. alternata* (29), indicating that homologous genetic loci are present in AK-, ACT-, and AF-toxin producing pathotypes of *A. alternata.* Disruption of *AKT1* and *AKT2* in the Japanese pear pathotype led to a loss of production of AK-toxin as well as production of 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid (29). The decatrienoic acid moiety is a precursor of AK-toxin (15). Therefore, the *AKT* homologs identified in the Minneola isolates in this study and the strawberry pathotype in previous studies are likely to be involved in the biosynthesis of ACT- and AF-toxins. We speculate that these loci are involved in the biosynthesis of 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid, which is a common component of these HSTs.

Minneola isolates were able to infect Japanese pear cv. Nijisseki in laboratory inoculations, and the symptoms were identical to those caused by the Japanese pear pathotype of *A. alternata.* A previous study demonstrated that spraying leaves with isolated ACT-toxin mixed with conidia of nonpathogenic *A. alternata* (O-94) allowed penetration and colonization of Japanese pear leaves by nonpathogenic *A. alternata* (7). However, this is the first time that the pathogenicity of a tangerine pathotype on Japanese pear cv. Nijisseki has been demonstrated. ACT-toxin is selectively toxic to tangerines and tangerine hybrids is also toxic to Japanese pear cv. Nijisseki (7). AF-toxin and its derivatives contain the same decatrienoic acid moiety (Fig. 1) and are also toxic to Japanese pear (13,16).

Examination of the restriction pattern of Minneola isolate SH20, using *AKT* probes and nine restriction enzymes, revealed multiple

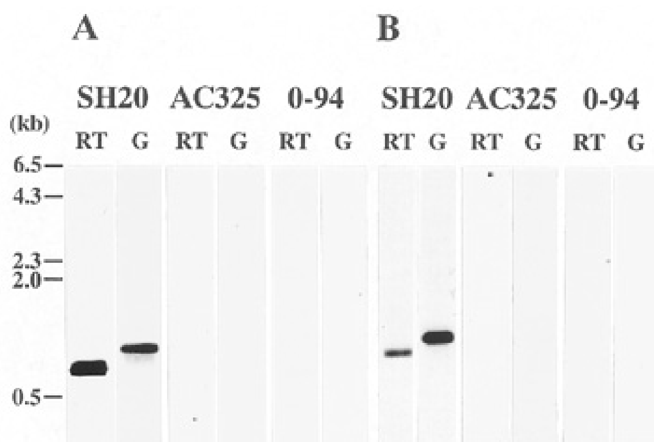

Fig. 6. PCR amplification of partial regions of the *AKT1* and *AKT2* homologs from Minneola isolate SH20, rough lemon isolate AC325, and nonpathogenic isolate O-94, and detection of transcripts. PCR with genomic DNA as the template (indicated as G) with primers ACTT1F2 and ACTT1R2 for the *AKT1* homolog (A), and primers ACTT2F1 and ACTT2R2 for the *AKT2* homolog (B). RT-PCR with total RNA as the template (indicated as RT) with the same primers as described above for the *AKT1* homolog (A) and primers ACTT2F2 and ACTT2R2 for the *AKT2* homolog (B). Southern hybridization of PCR (G) and RT-PCR (RT) products with (A) *AKT1* probe or (B) *AKT2* probe was described. Molecular size markers in kb are indicated at the left side of the blot.

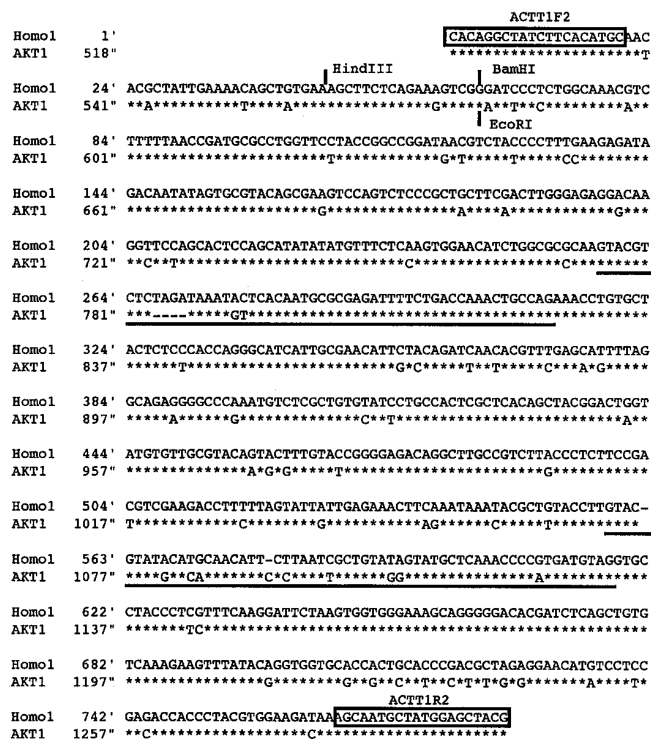

Fig. 7. Alignment of nucleotide sequences of the *AKT1* homolog (upper sequence) and *AKT1* (lower sequence). Asterisks represent residues that are identical between the two sequences. Underlined residues represent two introns. Boxes indicate priming sites for ACTT1F2 and ACTT1R2. Restriction sites of *Hin*dIII and *Bam*HI within the *AKT1* homolog, and *Eco*RI within *AKT1* are indicated. Genbank Accession no. for the *AKT1* homolog is AB034586.

copies of *AKT* homologs. The *AKT1* probe hybridized to a single restriction fragment for all enzymes, except *Pst*I and *Sac*I, which showed two bands hybridizing to the *AKT1* probe. Our sequence data identified *Bam*HI and *Hin*dIII restriction sites at the 5′ end region of the *AKT1* homolog (Fig. 7) however, these sites did not result in multiple hybridizing bands. The *Bam*HI and *Hin*dIII sites were near the 5′ end (*Eco*RI) of the *AKT1* probe, which was obtained from an *Eco*RI-*Xho*I fragment of *AKT1* (Fig. 7). Therefore, the *AKT1* probe could not detect these fragments as internal restriction sites of the homolog sequence. There were no *Pst*I and *Sac*I restriction sites in the *AKT1* homolog sequence, but two bands hybridized to the *AKT1* probe, indicating that there may be multiple copies of this gene. Hybridization of the *AKT2* probe was more complicated. More than one fragment was detected with the *AKT2* probe for all restriction enzymes tested, and multiple bands with similar molecular sizes were often observed after digestion with a single enzyme, such as *Hin*dIII, *Kpn*I, *Pst*I, *Sal*I, and *Xho*I. *Bam*HI, *Eco*RV, and *Pst*I recognize a single restriction site within the region of the *AKT2* homolog sequenced (Fig. 8), but none of the other enzymes tested had a restriction site within the sequence. These results also indicate that there may be multiple copies of these genes. Multiple copies of genes responsible for HST production have been reported in other systems. *TOX2*, controlling HC-toxin production in *C. carbonum* race 1, segregates as a single Mendelian locus in genetic crosses (17,26). However, the locus carries two copies of *HTS1,* which encodes a cycle peptide synthetase, and two or three copies each of *TOXA*, *TOXC*, *TOXD,* and *TOXE*, which are all essential for toxin production (1,19,22,28,34). Interestingly, all these genes are located within the *TOX2* locus, which is a large region (≈ 540 kb) of DNA that is present only in HC-toxin producing isolates (34). The *AKT* locus also appears to have a complex organization containing multiple copies of genes responsible for AK-toxin production (29,32). The data in this study indicate that gene duplication may be a common feature of *Tox* loci.

Peever et al. (20) recently reported high levels of inter-population genetic diversity and genetically diverse collections of clonal genotypes within populations of the citrus brown spot pathogen in Florida. They found no correlation between RAPD genotype and pathogenicity reaction on Minneola and rough lemon. If toxins are essential pathogenicity factors in this pathosystem and the toxin genes are similar among these RAPD groups, this may indicate that toxin genes have been transferred readily among genetically isolated groups of *A. alternata* on citrus. To date, *AKT* homologs have never been found in non ACT-toxin producing isolates of the *Alternaria* species. This may indicate that the evolution of genes responsible for toxin production is not due to point mutation or internal genetic rearrangement, but rather to larger-scale mutations, such as an insertion or deletion events or possibly horizontal transfer as hypothesized in other HST systems (32,34,38).

PCR amplification and sequence analysis of *AKT1* and *AKT2* homologs showed that the sequences of the partial regions were 89.8% similar to *AKT1* and 90.7% similar to *AKT2* at the nucleotide level. The sequence similarity of *AKT1* and *AKT2* and their homologs and the similarity of the chemical structures of ACT-, AK-, and AF-toxins indicates that a more detailed molecular study of these sequences, in the three distinct *Alternaria* pathotypes, may shed some light on the evolution of these host-specific forms and on their respective hosts. The HST-producing, *A. alternata* pathotypes appear to have evolved as highly specialized parasites with distinct host specificities through the elaboration of slightly different HST molecules via a common biosynthetic pathway. Further study of these genes will allow a more detailed understanding of the evolution of toxin production and its relationship to pathogenicity in these *Alternaria* species. Characterization of a cosmid clone containing the *AKT* homologs and targeted disruption of the sequences is currently underway in our laboratory. These experiments should allow us to determine the genetic organization of this locus and to definitively test the role of these genes in pathogenicity.

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                ACTT2F1                          ACTT2F2
Homo2    1' ATGCAGCAGCCCATCATTGGTGTGGACACAGCATGGGAGGCTGTCAGATGTAAGGGAAA
AKT2     1" ***********************T**************************C*TA***

Homo2   61' AATCGCGCCCCTGACCCGCACATACAACTCTTAGTAACAGGGATTGAAAGTGCAACGTTA
AKT2    61" ******AA********T*****G****G**C**C*C***CC****TC***A*********

Homo2  121' TCCGTTACTTCCCGCAGAATATTCAGTACTATGATACTGTTGGACCCTGCCATTGGACCA
AKT2   121" ********************G**T********************************T***

Homo2  181' CCTGAAATGGGCCTTGCAACCCTTGGCTTGGGTCAATTGACATTACGACGTAGGACGCAG
AKT2   181" *****C*******************A**********************************
                                                                 BamHI
Homo2  241' TGGCTGACAAGAGAAGATGCTGAGAAGGCGCTGAGAACTTCTTTCAGTACCTGGGATCCG
AKT2   241" ****CA**G**************A**************************G******T**

Homo2  301' CAAGTACTGGATCTCTTGATCAAACACTCTATCCATAGTGACAAACAGTCGGTTGAGATG
AKT2   301" *****C***A*****C***********************************A********
                                              EcoRV
Homo2  361' GAGGATGGCCCGTTCAGTCTAGTCACAGGACGATATCAGGAGCTGGTCAACTATATCAAA
AKT2   361" ************G**********************************************G

Homo2  421' CCCTCCTTCATTCGCTCTGGAAAGGTGGTTGGCCAAGAGTTAGTCCATCAGACAGGCCCT
AKT2   421" ***************************AA************GA*****************

Homo2  481' GTCGATATGTACCACATGCTTGGCCTCGTAACTTGTAGCACTCTATACTTGTGTGGCGGA
AKT2   481" ***************************************G********************

Homo2  541' GAGAGTACCTTGAGCACTCCTAGGGCCAGGGAGCTTTGGCTTAGCCGAACGGCAGAGCTT
AKT2   541" ***************GT********T******T**********A**********A****C

Homo2  601' TCGTACCCAAAGGACCCGGGAGAAATGAGGAAGGTGGATGAAAGAATTGTGCCAGACACA
AKT2   601" ******T*******G********G*C******A***********G***************

Homo2  661' GGACACTTCTTGCCTATGGAGGAGCCTAAGGAATGTGCAGACATTATAGCAGATTGGATC
AKT2   661" *****T********C***********C***A**********T******************

Homo2  721' GACAAGGACGAGTGTATGATATGGAATTGTCATATTGGTAAACAAGGAAAAATATGGCGT
AKT2   721" **A******A*******AGC**********TG*G*******G*G******G*C****T**
                                                         ACTT2R1  PstI
Homo2  781' GACCTAAGCAACACCAACAGGAAGATGAACGCGGAGGTTTGGATGAAATATCTGCAGTCG
AKT2   781" **GT********G***G**A*G******G********C**********************
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Fig. 8. Alignment of nucleotide sequences of the *AKT2* homolog (upper sequence) and *AKT2* (lower sequence). Asterisks represent residues that are identical between the two sequences. Underlined residues represent an intron. Boxes indicate priming sites for ACTT2F1, ACTT2F2, and ACTT2R1. Restriction sites of *Bam*HI, *Eco*RV, and *Pst*I within the *AKT2* homolog are indicated. Genbank Accession no. for the *AKT2* homolog is AB034587.

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