Function of Genes Encoding Acyl-CoA Synthetase and Enoyl-CoA Hydratase for Host-Selective ACT-Toxin Biosynthesis in the Tangerine Pathotype of *Alternaria alternata*

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ABSTRACT

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The tangerine pathotype of *Alternaria alternata* produces host-selective ACT-toxin and causes *Alternaria* brown spot disease. Sequence analysis of a genomic cosmid clone identified a part of the *ACTT* gene cluster and implicated two genes, *ACTT5* encoding an acyl-CoA synthetase and *ACTT6* encoding an enoyl-CoA hydratase, in the biosynthesis of ACT-toxin. Genomic Southern blots demonstrated that both genes were present in tangerine pathotype isolates producing ACT-toxin and also in Japanese pear pathotype isolates producing AK-toxin and strawberry pathotype iso-

lates producing AF-toxin. ACT-, AK-, and AF-toxins from these three pathotypes share a common 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid moiety. Targeted gene disruption of two copies of *ACTT5* significantly reduced ACT-toxin production and virulence. Targeted gene disruption of two copies of *ACTT6* led to complete loss of ACT-toxin production and pathogenicity and a putative decatrienoic acid intermediate in ACT-toxin biosynthesis accumulated in mycelial mats. These results indicate that *ACTT5* and *ACTT6* are essential genes in ACT-toxin biosynthesis in the tangerine pathotype of *A. alternata* and both are required for full virulence of this fungus.

Additional keywords: citrus, host-specific toxin, toxin biosynthesis gene cluster.

Seven strains of *Alternaria alternata* (Fr.) Keissl. are known to produce host-selective toxins (HST) that are selectively toxic to certain cultivars of host plants. *Alternaria* HSTs are low-molecular-weight, secondary metabolites with toxicity toward distinct plant genotypes, and have the same specificity as infection by the toxin-producing pathogen (24,30,34–36). These HST-producing strains are defined as seven distinct pathotypes (16,24) to reflect their morphological similarity. Pathotypes can be easily distinguished based on host range. The chemical structures of HSTs from six pathotypes of *A. alternata* have been elucidated (16), and HST biosynthetic genes were recently cloned from the Japanese pear pathotype, strawberry pathotype, tangerine pathotype, and apple pathotype of *A. alternata* (4–6,9,10,18–20,27,31,32).

The tangerine pathotype of *A. alternata* causes *Alternaria* brown spot disease, which affects many tangerines, mandarins, and their hybrids, and pathogenicity is dependent on the production of ACT-toxin (2,16,17). The structure of ACT-toxin is related to AK- and AF-toxins, which are the HSTs produced by the Japanese pear and strawberry pathotypes of *A. alternata* (15,21,22). These toxins share a common 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid moiety (Fig. 1) (15,21,22). Homologues

of three genes responsible for biosynthesis of the decatrienoic acid moiety in the Japanese pear pathotype (*AKT1*, *AKT2*, and *AKTTR*) are also found in the tangerine and strawberry pathotypes (18–20,31,32). Using *AKTR* (32) as a heterologous probe, we have identified a contiguous 33,974-bp region of chromosomal DNA (accession number AB176941) from a cosmid clone of the tangerine pathotype SH20. The 33,974-bp region contained five genes, and we report here the distributions and role in pathogenicity and virulence of two of these genes encoding a putative acyl-CoA synthetase (1,883 bp) designated *ACTT5* and a putative enoyl-CoA hydratase (897 bp) designated *ACTT6*.

MATERIALS AND METHODS

Fungal strains and plants. Young leaves (midrib length 2 to 4 cm) were obtained from citrus cv. Iyokan tanger (Citrus iyo hort. Ex Tanaka), grown in the greenhouse, and used for inoculation and toxin sensitivity assays as described previously (19,20). Iyokan is susceptible to the tangerine pathotype of *A. alternata* and sensitive to ACT-toxin (14). Strains of *A. alternata* tangerine pathotype were sampled from leaf lesions of Minneola tangelo (*C. reticulata* Blanco × *C. paradisi* Macf.) in Florida for a study of the population structure and host specificity of the *Alternaria* brown spot pathogen (18–20,25). Tangerine pathotype isolates SH11, 12, 14, 15, 18, 19, 20, 21, and EV10, 13, 14, and 17 and rough lemon pathotype isolates HC1 and 8 were isolated from leaf lesions on both hosts in central Florida (18,19,25). Tangerine pathotype strain SH20 has been used previously as a reference strain for the brown spot pathogen (18–20). Isolate BC3-5-1-

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First three authors contributed equally to this work. Accession numbers from DDBJ/GenBank of pcCL4-1, *ACTT5*, and *ACTT6* are AB176941, AB444613, and AB444614, respectively.

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OS2A, which is a dual pathotype strain producing both ACT-toxin and ACR-toxin (18), was isolated from a rough lemon leaf lesion in central Florida. In addition, laboratory stocks (9) of Japanese pear pathotype isolates G31 and G90-A2, strawberry pathotype isolates NAF-3 and NAF-8, tomato pathotype isolates 91080804 and AL-4, apple pathotype isolates AM-1 and O-159, and tobacco pathotype isolates No. 122-2 and AT-204 were used in this study. Isolate HC14 was a nonpathogenic strain of *A. alternata* sampled from rough lemon leaves (19,25), and another nonpathogenic *A. alternata* isolate O-94 was obtained from H. Otani, Tottori University, Japan. An additional 16 tangerine pathotype and non-pathotype strains were sampled worldwide from tangerines and mandarins (*C. reticulata* Blanco) or tangerine × grapefruit hybrids (*C. reticulata* × *C. paradisi* Macf.) in Israel, South Africa, Turkey, Australia, and Colombia (26). All isolates including transformants of isolate SH20 were stored in 25% (vol/vol) glycerol at -85°C , and cultures were initiated from the stocks for each experiment by growth on V8 juice agar or potato dextrose agar (PDA) plates.

Nucleic acid manipulations. Extraction of genomic DNA and total RNA, genomic polymerase chain reaction (PCR), reverse transcription (RT)-PCR and Southern blot hybridization using DIG-PCR-labeled probes were carried out as previously described (7,11,12,18–20). Restriction enzyme digestion of genomic DNA (5 μg), agarose gel electrophoresis, and capillary transfer were conducted under standard conditions (28). Transcripts of *ACTT5* or *ACTT6* were amplified with or without addition of reverse transcriptase (Takara, Shiga, Japan) by RT-PCR using the following primer sets: ACTT5S1F1 (5'-GTGTAGCAGCGGGACG-3') and ACTT5S1R1 (5'-CTACCGTTGGACCGCG-3') for *ACTT5*, ACTT6F1 (5'-TTTGATGTGCGATTCG-3') and ACTT6R1 (5'-CAATATGCCGCGCACTC-3') for *ACTT6*, and actinFor (5'-AGATGACACAGATTGTTTCGAGACC-3') and actinRev (5'-AGTTGAAAGTGGTGACGTGGATACC-3') for actin gene (*act1* of *A. alternata*) (AY748985), respectively. RT-PCR products for *ACTT5* were transferred to Hybond-N⁺ membrane using standard

methods (28) and detected with DIG-labeled PCR amplicons of *ACTT5* (*ACTT5* probe) or *ACTT6* (*ACTT6* probe) prepared with same primer sets used for RT-PCR and cosmid DNA of pcCL4-1 (AB176941) as the template by the method described previously (7,11,12,18–20). A probe for the hygromycin resistance gene *Hph* was prepared using a primer set of *hphF1* (5'-CTGAATC-ACCGCGACGT-3') and *hphR1* (5'-CCTTTGCCCTCGGACG-3') (3) in the pSH75 vector. This vector contains the *hph* cassette for hygromycin resistance as the selective marker and was labeled with same procedures as described for the *ACTT5* and *ACTT6* probes.

Sequencing was performed by the dideoxy chain termination method (29) 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). Sequence identities were estimated using FASTA searches at the DNA Data Bank of Japan.

Plasmid vector constructs and fungal transformation. The gene replacement vector pPCR-script::ACTT5::hph was constructed with partial *ACTT5* sequences amplified by PCR with the primer set ACTT5F1 (5'-GACTCAGAGCACCAGG-3') and ACTT5R1 (5'-ACTGCTCTGTCGACG-3') using cosmid DNA of pcCL4-1 (AB176941) as the template. The partial *ACTT5* sequence amplified by PCR was first subcloned into the *EcoRV* site of subcloning vector pT7Blue-2 (Novagen, Madison, WI) following digestion with *EcoRI* and *Sall*, and was resubcloned at the same enzyme sites (*EcoRI* and *Sall*) of pPCR-script Amp SK(+) (Stratagene, La Jolla, CA). The vector was then digested with *HindIII* and *EcoRV*, which cut in the middle of the *ACTT5* ORF, and ligated with a *hph* cassette from the fungal transformation vector pSH75 (3,7,13) excised with *HindIII* and *EcoRV*, to construct pPCR-script::ACTT5::hph for the first disruption of *ACTT5*. The vector was digested with *NotI* and *KpnI*, and linearized before transformation of isolate SH20. For the second disruption of *ACTT5*, a blunt-ended PCR fragment amplified from *ACTT5* using the primer set ACTT5S1F1 and ACTT5R2EcoRI (5'-CGGAATTCCGATTGGATGCCCCGTAC-3') with cosmid DNA of pcCL4-1 (AB176941) as the template, was subcloned at the *EcoRV* site of fungal transformation vector pII99 carrying an *NPTII* cassette (23) for geneticin resistance to produce pII99::ACTT5.

The gene replacement vector pSH75::ACTT6 was constructed using two partial sequences of *ACTT6* amplified by PCR using cosmid DNA of pcCL4-1 (AB176941) as the template with respective primer sets ACTT6F3BamHI (5'-CGGGATCCCGTC-TGCTGGGAGCAGGT-3') and ACTT6R3XhoI (5'-CCGCTCGA-GCGGGGTGGTTGGCCAAGAG-3'), and ACTT6F4BgIII (5'-G-AAGATCTTCAGCAATGCGGTGTGCC-3') and ACTT6R4SmaI (5'-TCCCCCGGGGAGTCTGGGCTCATTGCC-3'). One of the *ACTT6* PCR fragments amplified with the primer set ACTT6F3BamHI and ACTT6R3XhoI was digested with *BamHI* and *XhoI* and subcloned into the *BamHI* and *XhoI* sites of transformation vector pSH75. Another *ACTT6* PCR fragment amplified with the primer set ACTT6F4BgIII and ACTT6R4SmaI was digested with *BgIII* and *SmaI* and subcloned into the *BgIII* and *SmaI* sites of the vector pSH75 containing the first partial *ACTT6* fragment to generate pSH75::ACTT6. This vector was linearized by digestion with *HindIII* and *XhoI* before transformation of isolate SH20. For the second disruption of *ACTT6*, a blunt-ended *ACTT6* PCR fragment amplified using the primer set ACTT6F1 (5'-TTTGATGTGCGATTCG-3') and ACTT6R1 (5'-CAATATGCCGCGCACTC-3') was subcloned into the *EcoRV* site of transformation vector pII99 to generate pII99::ACTT6. Vector constructs for disruption of *ACTT5* and *ACTT6* were confirmed by sequencing.

Protoplast preparation and transformation of isolate SH20 were carried out using methods described previously (7,8,11,12,20). Protoplasts (1×10^7) were transformed with 3 μg of each vector.

ACT-toxin

ACT-toxin I: R=OH
ACT-toxin II: R=H

AK-toxin

AK-toxin I: R=CH₃
AK-toxin II: R=H

AF-toxin

AF-toxin I: R=COCH(OH)C(CH₃)₂OH
AF-toxin II: R=H
AF-toxin III: R=COCH(OH)CH(CH₃)₂

Fig. 1. Chemical structures of ACT-, AK-, and AF-toxins produced by the tangerine pathotype, Japanese pear pathotype, and strawberry pathotype of *Alternaria alternata*, respectively (15,21,22).

Colonies were transferred to PDA plates containing hygromycin B (50 µg/ml) or geneticin (200 µg/ml) and grown at 24°C for 5 to 7 days and single spore cultures were recovered as described previously (7,8,11,12,20).

Detection of plasmid integration, toxin production, and pathogenicity. Integration patterns of transformation vectors were determined using genomic Southern blots and DIG-labeled *ACTT5*, *ACTT6*, or *hph* probes. Hybridization and detection were conducted as described previously (7,8,11,12,18–20). Production of ACT-toxin and its putative intermediate compound in culture filtrates, spore germination fluid, and mycelial extracts was determined by high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC), and bioassay. *A. alternata* isolate SH20 and its mutants were grown on PDA for examination of fungal growth and also in 200-ml flasks containing 50 ml of ACT-toxin medium (15,17) for 25 to 30 days at 24°C for examination of ACT-toxin production in culture filtrates. Culture filtrates were adsorbed to Amberlite XAD2, extracted with methanol, extracted twice with ethyl acetate, and suspended in methanol following purification through a Sep-Pak Column Plus C18 Cartridge (Waters, Milford, MA) following the protocol described previously (18–20) for culture filtrate extract preparation for HPLC analysis. Mycelial mats were lyophilized using the LABCONCO Freeze dry System/Freezone 2.5 (Kansas City, MO) at –40°C (133×10^{-3} Mb) overnight. The mycelial mats (0.5 g dry weight) were ground under liquid nitrogen with a mortar and pestle until the mycelia became a fine gray powder. The powder was stirred in 200 ml of methanol for 1 h at 4°C and passed through paper filter (Whatman #4 filter; Maidstone, England) to remove mycelial residue. Extracts were dried and resuspended in 10 ml each of ethyl acetate and 10% (wt/vol) NaH_2PO_4 , and the extracted ethyl acetate layer was dried and resuspended in 5 ml of methanol. Methanol extracts were passed through a Sep-Pak Column Plus C18 Cartridge (Waters) and further analyzed by bioassay and HPLC. The method for spore germination fluid preparation was partially modified from the method of Kohmoto et al. (15). Conidia of isolate SH20 and its mutants were collected from mycelial mats grown in potato dextrose broth (PDB) for 3 weeks, and suspended in water at 1×10^7 spores/ml. Three milliliters of the spore suspension was placed as droplets (20 µl/drop) in the bottom of plastic 9 cm petri dishes and incubated covered at 24°C for 24 h. After incubation, droplets were pooled in 1.5-ml microcentrifuge tubes and centrifuged at 14,000 rpm for 10 min to pellet germinating spores. The supernatant was collected and evaporated with 10 times volume of methanol at 40°C under reduced pressure, and the residue was dissolved in 150 µl of methanol following purification through a Sep-Pak column Plus C18 Cartridge (Waters) for HPLC analysis.

HPLC analysis of ACT-toxin and a putative intermediate compound in ACT-toxin biosynthesis was carried out on a Model 880-PU HPLC system (Japan Spectroscopic, Tokyo, Japan) with a Develosil ODS-5 column (4.6 × 250 mm, NORUMA Chemical, Aichi, Japan) and a gradient solvent system composed of acetonitrile/water/acetic acid (linear gradient from 30:70:1 to 60:40:1, vol/vol/vol) in 30 min at a flow rate of 1 ml/min with peak detection with an absorbance at 290 nm (15,18–20). Fifty microliters of ethyl acetate extracted samples from either culture filtrates (50 ml) or mycelial mats (0.5 g dry weight) was suspended in 1 ml of methanol, passed through a Sep-Pak column Plus C18 Cartridge (Waters), and injected for HPLC analysis. Samples extracted from spore germination fluids (3 ml) were suspended in 150 µl of methanol, passed through Sep-Pak column Plus C18 Cartridge (Waters), and 50 µl of the sample was injected for HPLC analysis. For large-scale preparation of the putative ACT-toxin intermediate compound, HPLC of mycelia extracts was carried out using a Megapak SIL C₁₈ T preparative column (10 × 250 mm, Japan Spectroscopic) with acetonitrile/deionized water (50:50, vol/vol) solvent system under a flow rate of 3 ml per min

with 290 nm detection. A large peak with a retention time of 25 to 30 min was collected. The retention time of the collected material was confirmed using a Develosil ODS-5 column (4.6 × 250 mm) with acetonitrile/deionized water/acetic acid (30:70:1 to 60:40:1, vol/vol, gradient in 30 min) solvent system under a flow rate of 1 ml per min with 290 nm detection (15,18,19) and TLC analysis. The collected peak from the large-scale preparation was dried under reduced pressure, resuspended in 1 ml methanol, and 50 µl was used for further HPLC analysis for confirmation as well as TLC analysis. Retention time and quantification of ACT-toxin was determined for each HPLC analysis using previously purified ACT-toxin I (15,18–20).

For TLC determination of the putative intermediate compound in ACT-toxin biosynthesis, samples (10 µg per lane) were prepared from culture filtrates, mycelial extracts, and HPLC-purified mycelial extracts. Extracts were prepared from wild-type isolate SH20, single copy *ACTT6* disruptant 53, or double copy *ACTT6* disruptant 53-30 and run on TLC (Merck Silica gel 60F₂₅₄, Darmstadt, Germany) using a solvent system of benzene/acetic acid (90:10, vol/vol). Compounds were detected by irradiating plates with UV light at 315 nm or spraying plates with sulfuric acid/ethanol (5:95, vol/vol) following by heating at 180°C until spots appeared.

To determine the toxicity to plants of ACT-toxin and the putative intermediate compound in culture filtrates, HPLC fractions, and extracts from culture filtrate, spore germination fluid or mycelial mats, extracts were diluted 10 to 1,000 times in distilled water and placed onto the lower surface of detached leaves of *Citrus iyo* cv. Iyokan as described previously (18–20). Leaves were incubated in a moist chamber at 24°C in the dark, and necrosis development on the leaves was monitored for 48 h. For pathogenicity assays, spores of each isolate were collected from mycelial mats grown in PDB for 3 weeks and suspended in water at 1×10^5 spores/ml and spray-inoculated on the lower surface of detached young leaves (midrib length 3.0 to 4.0 cm) of ACT-toxin-sensitive citrus cultivar Iyokan as described previously (18–20). Leaves were incubated in a moist chamber for 24 to 48 h at 24°C in the dark, and the development of leaf spots and necrosis was monitored.

Pulse-field gel electrophoresis (PFGE) and hybridization. Preparation of chromosomes from fungal protoplasts and PFGE was carried out according to methods described previously (6, 18,20). Running conditions (duration, voltage, linear gradient of switching time) in a PFGE apparatus (CHEF-DRII, Bio-Rad, Richmond, CA) consisted of 0.5× TBE (28) at 8°C in a 0.8% agarose gel (Seakem Gold agarose, BioWhittaker Molecular Applications, Rockland, ME), 13 h/5.4 V/cm/120 s, 13 h/5.4 V/cm/180 s. Chromosomal DNA bands were visualized by ethidium bromide staining, and the gel was serially treated by soaking in 0.2 N HCl for 20 min (to nick chromosomal DNA), and then 0.4 N NaOH plus 1.5 M NaCl for 30 min and transferred to Hybond-N⁺ membrane (Amersham Biosciences, Piscataway, NJ) by alkaline capillary transfer (28) with 0.4 N NaOH and 1.5 M NaCl for 48 h. The membrane was baked at 120°C for 30 min to fix the DNA and hybridized at 68°C overnight with *ACTT5* or *ACTT6* probes labeled with the DIG-PCR labeling kit (Roche, Basel, Switzerland). Primer sets used for the preparation of *ACTT5* and *ACTT6* probes were as described for the RT-PCR analysis above. Hybridization and washes were conducted under highly stringent conditions according to the manufacturer's instructions as described previously (18,20).

RESULTS

Identification and genomic analysis of ACT-toxin biosynthesis genes *ACTT5* and *ACTT6* from cosmid clone pcCL4-1. Five genes including *ACTT5* (accession AB444613) encoding a putative acyl-CoA synthetase, and *ACTT6* (accession AB444614)

encoding a putative enoyl-CoA hydratase, were identified from the cosmid clone pcCL4-1 (accession AB176941) of the tangerine pathotype (Fig. 2A). *ACTT5* (accession AB444613) was 1,883 bp in length with a predicted 578 amino acid protein and three introns of 46 bp (base pairs 123 to 168), 47 bp (1,216 to 1,262), and 53 bp (1,468 to 1,520) confirmed by comparison with the cDNA sequence. *ACTT6* (accession AB444614) was 897 bp and encoded a deduced 298 amino acid protein with no introns. BLAST searches indicated that *ACTT5* likely encodes an acyl-CoA synthetase and *ACTT6* likely encodes an enoyl-CoA hydratase. PFGE and Southern blotting demonstrated that *ACTT5* and *ACTT6* are located on the same small chromosome (1.9 to 2.0 Mb) among several isolates of the tangerine pathotype of *A. alternata* obtained from citrus groves in Florida (Fig. 2B).

The distribution of *ACTT5* and *ACTT6* in the genome of strain SH20 of the tangerine pathotype was further examined by genomic Southern blotting (Fig. 3). The *ACTT5* probe detected two to four fragments when genomic DNA was digested with the restriction enzymes *Bam*HI, *Eco*RI, *Pst*I, *Sal*I, and *Xho*I, which have no predicted restriction sites within the gene sequence (Fig. 3A). Expected bands were observed, such as the 18.8-kb fragment in the *Bam*HI-digested DNA as well as 9.3-kb fragment in the *Xho*I digest (Fig. 3A and B). However, additional unexpected bands were observed based on the restriction map of pcCL4-1, such as the 9.0-kb band in the *Bam*HI digest and 7.5-, 13.5-, and 19.0-kb bands in the *Xho*I digest (Fig. 3A and B), indicating that there are multiple copies of *ACTT5* in the genome. The *ACTT6* probe detected one to three fragments when genomic DNA was digested with *Bam*HI, *Eco*RI, *Eco*RV, *Pst*I, *Sal*I, and *Xho*I, which have no predicted restriction sites within the gene sequence (Fig. 3A). Expected bands, such as the 18.8-kb fragment after digestion with *Bam*HI as well as 9.3-kb fragment in the *Xho*I digest (Fig. 3A and B), were observed using the *ACTT6* probe. However, similar to the results for *ACTT5*, detection of unexpected bands based on the restriction map of pcCL4-1, such as a 9.5-kb band after *Bam*HI digestion and 7.5- and 18.0-kb bands in the *Xho*I digest (Fig. 3A and B), indicated the presence of additional *ACTT6* copies.

The distribution of *ACTT5* and *ACTT6* among various *A. alternata* pathotypes (tangerine, rough lemon, Japanese pear, strawberry, tomato, apple, and tobacco), nonpathogenic strains of *A. alternata*, and a dual pathotype isolate producing both ACT- and ACR-toxins (18) was determined using genomic Southern blot analysis (Fig. 4). *ACTT5* and *ACTT6* homologues were detected in the genomes of Japanese pear and strawberry pathotype isolates and the dual pathotype isolate, but not in any other pathotype (Fig. 4). The distribution of *ACTT5* and *ACTT6* among 14 tangerine pathotype isolates sampled from different geographical regions was also determined using genomic Southern hybridization (Fig. 4). *ACTT5* and *ACTT6* probes hybridized to only those worldwide isolates that produced ACT-toxin and were pathogenic to citrus hosts susceptible to the tangerine pathotype. These probes did not hybridize to any nonpathogenic isolates in this worldwide sample, indicating that presence of *ACTT5* and *ACTT6* was highly correlated to ACT-toxin production and pathogenicity (Fig. 4).

Function of *ACTT5* in ACT-toxin biosynthesis. To examine the role of *ACTT5* in pathogenicity and ACT-toxin biosynthesis, the gene replacement vector pPCR-Script::ACTT5::hph was constructed. The vector contained partial *ACTT5* sequences with an *hph* cassette for replacement disruption of *ACTT5* mediated by homologous recombination (Fig. 5A). Reference tangerine pathotype isolate SH20 (18–20) was transformed with pPCR-Script::ACTT5::hph, and 10 transformants were selected. Screening the toxicity of culture filtrates and genomic Southern hybridization analysis of the transformants identified transformant 1-8, which showed a reduction in toxicity as well as a shift in the 3.0-kb *ACTT5* band present in the wild type to a 5.5-kb band in the transformant. This indicated insertion of the vector construct at the target site (Fig. 5A and C). Another native band of 2.1 kb remained intact (Fig. 5C). The *hph* probe hybridized only to the 5.5-kb band of transformant 1-8 (data not shown) suggesting that *ACTT5* exists in more than one copy in the genome of SH20 and the vector construct was inserted in only one of the copies. Transcript analysis of *ACTT5* by RT-PCR with primer set ACTT5S1F1 and ACTT5S1R1 generated an amplicon of predicted size

Fig. 2. Map showing location of *ACTT5* and *ACTT6* and distribution among chromosomes of Florida isolates of the tangerine pathotype of *Alternaria alternata*. **A**, Location and orientation of *ACTT5* and *ACTT6* genes on cosmid clone pcCL4-1. Scale indicates 1 kb. **B**, Chromosomal location of *ACTT5* and *ACTT6* among Florida tangerine pathotypes. Abbreviations of SH (Shinn Grove) and EV (Evenhouse Road) used in upper panels are codes of each isolate (18,20,25). Intact chromosomes were separated by pulsed-field gel electrophoresis with conditions adjusted for separation of chromosomes with sizes of <2.0 Mb in 0.8% agarose gels (left panel). Chromosomes were transferred to a nylon membrane and detected with digoxigenin-labeled *ACTT5* (middle panel) or *ACTT6* probes (right panel). *Hansenula wingei* chromosome size markers are indicated at left.

To disrupt the remaining copy of *ACTT5*, an *ACTT5* PCR fragment was amplified using primer set ACTT5S1F1 and ACTT5R2EcoRI and subcloned into the *EcoRV* site of transfor-

A

ACTT5

Agarose gel electrophoresis image for *ACTT5*. The gel shows DNA bands for seven lanes: EI, EV, Xh, Sl, P, Bm, and Bn. Molecular weight markers (kb) are indicated on the left: 23.0, 9.4, 6.6, 4.3, 2.3, and 2.0. The bands are as follows:

Lane	Band(s) (kb)
EI	23.0, 9.4, 6.6
EV	2.3
Xh	23.0, 9.4, 6.6
Sl	23.0, 9.4, 6.6
P	9.4, 6.6, 4.3
Bm	23.0, 9.4, 6.6
Bn	4.3, 2.3, 2.0

ACTT6

Agarose gel electrophoresis image for *ACTT6*. The gel shows DNA bands for seven lanes: EI, EV, P, Xh, Sl, Bm, and Bn. Molecular weight markers (kb) are indicated on the left: 23.0, 9.4, 6.6, 4.3, 2.3, and 2.0. The bands are as follows:

Lane	Band(s) (kb)
EI	9.4
EV	2.3
P	9.4, 6.6
Xh	9.4, 6.6
Sl	9.4, 6.6
Bm	9.4, 6.6
Bn	4.3, 2.3, 2.0

mation vector pII99 carrying the *NPTII* cassette to generate pII99::ACTT5. The single copy disruptant 1-8 was retransformed with pII99::ACTT5, and 24 transformants were selected. Bioassay of toxicity in the culture filtrates and genomic Southern hybridization analysis of the transformants identified transformant 8-32, which showed a reduction in toxicity as well as a shift of a 2.1-kb *ACTT5* band in the wild type to a 7.9-kb band in the transformant. This indicated that the vector construct inserted in another copy of the target gene (Fig. 5B and C). Although two distinct copies of *ACTT5* were disrupted with the respective vectors, the native 2.1-kb band remained (Fig. 5C), suggesting that *ACTT5* exists as more than two copies in the genome of isolate SH20 (Fig. 3). Transcript analysis of *ACTT5* by RT-PCR generated an amplicon of predicted size (959 bp) from total RNA of the transformant 8-32, and the product hybridized to the *ACTT5* probe (Fig. 6A). The intensity of the band was reduced in transformant 8-32 compared with that of the single copy disruptant 1-8 or wild type (Fig. 6A). Production of ACT-toxin in culture filtrates was greatly reduced (97.4% reduction in peak area) in transformant 8-32 (Fig. 6C), and inoculation of transformant 8-32 on detached leaves showed a great reduction in disease (Fig. 6D) compared to those of the single copy disruptant 1-8 or wild type. No differences in growth (Fig. 6B), sporulation, and spore germination rate were detected among wild type, single copy disruptant 1-8, or double copy disruptant 8-32.

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pathogenicity and ACT-toxin production, the disruption vector pSH75::ACTT6 was constructed. The vector contained two partial *ACTT6* sequences with an *hph* cassette (Fig. 5D) for *ACTT6* disruption mediated by homologous recombination. A screen for reduced ACT-toxin production and Southern hybridization identified transformant 53, which showed a reduction in the toxicity in culture filtrates and loss of a 9.5-kb *ACTT6* band. This indicated insertion of the vector at the target site due to replacement of the genomic region with the *hph* cassette carried on pSH75::ACTT6. The *hph* probe identified an expected 11.3-kb band, which has an insertion of pSH75::ACTT6 into the 9.5-kb band of the wild type (Fig. 5D and F). Transcript analysis of *ACTT6* by RT-PCR with primer set ACTT6F1 and ACTT6R1 generated an amplicon of predicted size (812 bp) from total RNA of the wild type and transformant 53, and the product hybridized to the *ACTT6* probe (Fig. 6E). Intensity of the band was greatly reduced in transformant 53 compared with that of wild type (Fig. 6E). The PCR reaction without addition of reverse transcriptase did not amplify a product, and the actin gene was amplified equally in both disruptant and wild type (Fig. 6E). Production of ACT-toxin by transformant 53 was reduced approximately 70% compared with wild type (Fig. 6G). When spores of transformant 53 were spray-

inoculated onto the lower surface of Iyokan leaves, typical leaf spots appeared but disease severity was reduced relative to wild type (Fig. 6H).

To disrupt the second copy of *ACTT6*, an *ACTT6* PCR fragment was amplified using the primer set ACTT6F1 and ACTT6R1 and subcloned into the *EcoRV* site of transformation vector pII99 carrying the *NPTII* cassette to generate pII99::ACTT6 (Fig. 5E). The single copy disruptant 53 was retransformed with pII99::ACTT6, and 36 transformants were tested for ACT-toxin production. Culture filtrates from seven transformants (53-6, -9, -30, -31, -35, -39, and -42) were not toxic to Iyokan. Integration of pII99::ACTT6 at the target site in one (53-30) of the transformants was

Fig. 5. Targeted gene disruption of *ACTT5* and *ACTT6* in the tangerine pathotype of *Alternaria alternata*. **A**, Disruption construct pPCR-script::ACTT5::hph and integration pattern in single copy disruptant 1-8. N, K, EV, and H are abbreviations used for *NotI*, *KpnI*, *EcoRV*, and *HindIII* restriction sites, respectively. **B**, Disruption construct pII99::ACTT5 and integration pattern in double copy disruptant 8-32. **C**, Genomic Southern blot of *ACTT5* in wild type (W), single copy disruptant 1-8, and double copy disruptant 8-32. Genomic DNA was digested with *EcoRV* and fractionated in a 0.8% agarose gel followed by hybridization with *ACTT5* probe. **D**, Disruption construct pSH75::ACTT6 and integration pattern in single copy disruptant 53. B and X are abbreviations used for *BamHI* and *XhoI* restriction sites, respectively. **E**, Disruption construct pII99::ACTT6 and integration pattern in double copy disruptant 53-30. **F**, Genomic Southern blot of *ACTT6* in wild type (W), single copy disruptant 53, and double copy disruptant 53-30. Genomic DNA was digested with *BamHI* and fractionated in a 0.8% agarose gel followed by hybridization with an *ACTT6* probe or *hph* probe. Sizes of marker DNA fragments (*HindIII*-digested λ DNA) are indicated at left.

Fig. 6. Reverse transcription-polymerase chain reaction (RT-PCR) detection, ACT-toxin production, and pathogenicity of *ACTT5*- or *ACTT6*-disruptants. **A** and **E**, RT-PCR detection of *ACTT5* (**A**) or *ACTT6* (**E**) transcripts in wild-type SH20 (lane W), single copy *ACTT5* disruptant 1-8, single copy *ACTT6* disruptant 53, double copy *ACTT5* disruptant 8-32, or double copy *ACTT6* disruptant 53-30, respectively. RT- indicates controls without addition of reverse transcriptase. Amplification of an actin gene was used as a positive control of the RT-PCR system as well as for equal addition of RNA. **B** and **F**, Growth of wild-type isolate SH20 (W) and disruptants on potato dextrose agar plates. **C** and **G**, High-performance liquid chromatography traces of extracts from culture filtrates of *ACTT5* disruptants (**C**) and *ACTT6* disruptants (**G**). **D** and **H**, Pathogenicity tests of disruptants using spray-inoculation with conidia of *ACTT5* disruptants (**D**) and *ACTT6* disruptants (**H**) on Iyokan leaves.

analyzed by Southern blotting, and transformant 53-30 showed the expected bands of 10.2 and 17.5 kb instead of the single 18.8-kb band in transformant 53 and the wild type (Fig. 5E and F). No *ACTT6* transcript was detected in transformant 53-30 by RT-PCR (Fig. 6E). HPLC analysis also did not detect any trace of ACT-toxin in the culture filtrates of transformant 53-30 (Fig. 6G), and inoculation of plants with transformant 53-30 did not induce any necrotic spots on Iyokan leaves (Fig. 6H). No differences in growth rate (Fig. 6F), sporulation and spore germination rate were detected among the wild type, single copy disruptant 53, and double-copy disruptant 53-30.

A putative precursor of ACT-toxin in *ACTT6* disruptants. Accumulation of a putative precursor of ACT-toxin was detected using HPLC of culture filtrates and mycelium of *ACTT6* disruptant 53-30. Disruptant 53-30 produced no detectable ACT-toxin nor any other major product in culture filtrates extracted with ethyl acetate (Figs. 6G and 7A), while a new peak appeared on an HPLC trace from methanol extracts of mycelium (Fig. 7A). The peak was not observed in the extracts of wild type mycelium, but appeared in the mycelium of single copy disruptant 53 and accumulated to a greater extent in the double copy disruptant 53-30 (Fig. 7A). The peak was collected following large-scale preparation, and run on a TLC plate after confirmation of retention time (28 min) as a single peak by HPLC under the same conditions (Fig. 7B). A single spot was observed with an *R_f* value of 0.28 under 315 nm UV irradiation and no other spot was observed even following H₂SO₄ spray detection (Fig. 7C). Although no peak was detected in the wild type by HPLC (Fig. 7A), a trace of a spot at *R_f* 0.28 was detected in the wild type when the corresponding HPLC fractions were collected and concentrated for TLC detection (Fig. 7C). Neither the extract from mycelial mats, the collected HPLC peak (Fig. 7B), nor the TLC spot at *R_f* 0.28 (Fig. 7C) from 53-30 showed toxicity to Iyokan leaves.

DISCUSSION

Five genes were identified on a 34-kb genomic sequence of the 1.9 Mb chromosome from the tangerine pathotype of *A. alternata*. We characterized two of these and present biochemical and molecular genetic evidence for the involvement of a putative acyl-CoA synthetase (*ACTT5*) and a putative enoyl-CoA hydratase (*ACTT6*) in ACT-toxin production and pathogenicity. The tangerine pathotype isolate SH20 makes ACT-toxin and has a 1.9 Mb chromosome that carries the biosynthetic genes controlling toxin production (18–20). Genes required for the synthesis of *Alternaria* HSTs are tightly clustered, such as the AK-toxin TOX cluster (*AKT*) controlling AK-toxin production (31), and the AF-toxin TOX cluster (*AFT*) controlling AF-toxin production (6). Genes responsible for ACT-toxin production (*ACTT1*, 2, 3, R, 5, and 6) are also clustered, and the cluster has been designated as ACT-toxin TOX cluster (*ACTT*) (A. Masunaka, Y. Miyamoto, K. Gomi, and K. Akimitsu, *unpublished data*). These genes are located on a single 1.9 to 2.0 Mb chromosome in ACT-toxin producing isolates from Florida (18). Generally only *A. alternata* strains producing HSTs are known to possess chromosomes of less than 2.0 Mb (1,19,20) and nonpathogenic strains do not have any small chromosomes. Since these small chromosomes do not carry genes essential for normal growth and reproduction and loss of these chromosomes has no obvious phenotypic effect, these chromosomes have been called conditionally dispensable chromosomes (6,33). Interestingly, gene clusters responsible for the biosynthesis of HST, such as the *AKT*, *AFT*, and *ACTT* clusters in the Japanese pear, strawberry, and tangerine pathotypes (4,18–20), are all located in small dispensable chromosomes. Genomic regions in these *AKT*, *AFT*, and *ACTT* clusters are similar and several genes responsible for production of the common 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic (decatrienoic) acid moiety of AK-, AF-, and ACT-toxins (6,9,18–20,27,31,32) were identi-

Fig. 7. High-performance liquid chromatography (HPLC) and thin-layer chromatographic (TLC) identification of a putative decatrienoic intermediate in ACT-toxin biosynthesis produced by double copy *ACTT6* disruptant 53-30. **A**, HPLC traces of extracts from spore germination fluid (left panel) and mycelial mats (right panel) of wild-type SH20 (W), *ACTT6* single copy disruptant 53, and double copy disruptant 53-30. Solid arrows indicate the ACT-toxin peak and open arrows indicate the peak of the putative intermediate compound. HPLC was carried out using a Develosil ODS-5 analytical column (4.6 × 250 mm) and 290 nm detection (15). **B**, HPLC traces of partial purification of mycelial extracts from *ACTT6* disruptant 53-30. HPLC was carried out for a large preparation (upper chart) using a Megapak SIL C₁₈ T preparative column (10×250 mm) with an acetonitrile/deionized water (50:50, vol/vol) solvent system, a flow rate of 3 ml per minute and 290 nm detection. The peak collected from retention time 25 to 30 min was run on a Develosil ODS-5 analytical column (lower panel) with same conditions as described for panel A for confirmation of the collected peak. **C**, The purified compounds were separated by TLC using a solvent system of benzene/acetic acid (90:10, vol/vol), and detected as a purple spot under 315 nm UV (upper panel) or detected by spraying with sulfuric acid/ethanol. Samples were prepared from culture filtrates or mycelia, and further purified using HPLC, from either wild-type isolate SH20 (W), single copy disruptant 53, or double copy disruptant 53-30 of *ACTT6*.

fied as homologues of each other. Distribution of *ACTT5* and *ACTT6* in seven pathotypes, a nonpathogenic strain, and worldwide strains of *A. alternata* corresponds well with these previous findings. *ACTT5* and *ACTT6* were found on the genomes of tangerine, Japanese pear, and strawberry pathotypes among seven pathotypes of *A. alternata*, and presence of these genes is correlated with ACT-toxin production and pathogenicity among *A. alternata* isolated from citrus fields around the world.

The nucleotide sequence of *ACTT5* is 1,883 bp in length with three introns and encodes a putative 578 amino acid protein which shows a sequence similarity to acyl-CoA synthetase. Our reference strain SH20 of the tangerine pathotype (18–20) has multiple copies of *ACTT5* as determined by genomic Southern blot analysis. We generated single and two copy disruptants of *ACTT5* using gene replacement mediated by homologous recombination. The two copy disruptant 8-32 showed markedly reduced ACT-toxin production and pathogenicity. A complete disruptant of *ACTT5* function has not been generated due to the remaining copies of *ACTT5*. Genes involved in HST production in *Alternaria* are often present as multiple copies (4–6,9,10,18–20,27,31,32), and this multiplication makes it difficult to disrupt all functional copies of the target genes due to lack of selectable markers for multiple transformations with different vector constructs as well as low the efficiency of homologous recombination in *Alternaria* spp. Since RNA silencing was successfully introduced for dysfunction of all copies of *ACTT2*, which has multiple copies in the genome of isolate SH20 (20), this method may be applicable for further functional analysis of *ACTT5*. However, we think the data presented here confirm that *ACTT5* is involved in ACT-toxin production, because each gene disruption correlated with a reduction in ACT-toxin production as well as reduced pathogenicity, as has been shown for other HST biosynthesis genes (4–6,9,10,18–20,27,31,32).

ACTT6 is comprised of 897 bp with no introns, and encodes a putative 298 amino acid protein. A BLAST search revealed that *ACTT6* shows sequence similarity to enoyl-CoA hydratase. The complete loss of *ACTT6* transcript accumulation by disruption of two functional copies of *ACTT6* led to a complete loss of ACT-toxin production and pathogenicity. Furthermore, we detected a putative intermediate compound due to the disruption of the step catalyzed by *ACTT6* in the ACT-toxin biosynthesis pathway, and accumulation of this compound was inversely proportional to reduction of ACT-toxin production by *ACTT6* disruption. Since *ACTT6* homologues are present in the genomes of the Japanese pear, strawberry and tangerine pathotypes, *ACTT6* is likely a part of the biosynthesis pathway of the 9,10-epoxy-8-hydroxy-9-methyl-decatricenoic acid moiety shared by AK-, AF-, and ACT-toxins (4–6,9,18–20,27,31,32). Thus, the accumulated putative compound may be an intermediate structure of decatrienoic acid with fatty acid properties, including high hydrophobicity. This property may be the reason why this compound is not secreted into the culture filtrate and accumulated in mycelial mats. Although the purified compound appears to be a single peak by HPLC and also a single spot by TLC, nuclear magnetic resonance analysis revealed some contamination in the fraction (data not shown) and further purification will be necessary to identify the chemical structure of the compound.

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