Role of the Host-Selective ACT-Toxin Synthesis Gene *ACTTS2* Encoding an Enoyl-Reductase in Pathogenicity of the Tangerine Pathotype of *Alternaria alternata*

Naoya Ajiro, Yoko Miyamoto, Akira Masunaka, Takashi Tsuge, Mikihiro Yamamoto, Kouhei Ohtani, Takeshi Fukumoto, Kenji Gomi, Tobin L. Peever, Yuriko Izumi, Yasuomi Tada, and Kazuya Akimitsu

First, second, third, sixth, seventh, eighth, tenth, eleventh, and twelfth authors: Faculty of Agriculture and Gene Research Center, Kagawa University, Miki, Kagawa 761-0795 Japan; fourth author: Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601 Japan; fifth author: Faculty of Agriculture, Okayama University, Okayama 700-8530 Japan; and ninth author: Department of Plant Pathology, Washington State University, Pullman 99164-6430.

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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 of tangerines and tangerine hybrids. Sequence analysis of a genomic BAC clone identified a previously uncharacterized portion of the ACT-toxin biosynthesis gene cluster (*ACTT*). A 1,034-bp gene encoding a putative enoyl-reductase was identified by using rapid amplification of cDNA ends and

polymerase chain reaction and designated *ACTTS2*. Genomic Southern blots demonstrated that *ACTTS2* is present only in ACT-toxin producers and is carried on a 1.9 Mb conditionally dispensable chromosome by the tangerine pathotype. Targeted gene disruption of *ACTTS2* led to a reduction in ACT-toxin production and pathogenicity, and transcriptional knockdown of *ACTTS2* using RNA silencing resulted in complete loss of ACT-toxin production and pathogenicity. These results indicate that *ACTTS2* is an essential gene for ACT-toxin biosynthesis in the tangerine pathotype of *A. alternata* and is required for pathogenicity 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). HSTs produced by *A. alternata* are secondary metabolites with toxicity to specific plant genotypes and have the same specificity as infection by the toxin-producing pathogen (28,33,38–40). These HST-producing strains are defined as distinct pathotypes of *A. alternata* (18,28). The chemical structures of HSTs from six pathotypes have been elucidated (18), and several HST biosynthetic genes were recently cloned from the Japanese pear, strawberry, tangerine, rough lemon, tomato, and apple pathotypes (1,2,6,7,10,11,20–23,30,35,36).

The tangerine pathotype of *A. alternata* causes *Alternaria* brown spot disease of many tangerines, mandarins, and their hybrids, and pathogenicity is dependent upon the production of ACT-toxin (4,16,19). ACT-toxin is structurally related to AK- and AF-toxins, which are the HSTs produced by the Japanese pear and strawberry pathotypes of *A. alternata*, respectively (4,24,25). These toxins share a common 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid moiety (4,18,24,25). Three homologues (*ACTT1*, *ACTT2*, and *ACTTR*) of the *AKT1*, *AKT2*, and *AKTTR* genes carried by the Japanese pear pathotype are responsible for bio-

synthesis of the decatrienoic acid moiety in the tangerine pathotype (20–23,30,35,36). RNA silencing of the multicopy *ACTT2* encoding a hydrolase in the tangerine pathotype led to complete losses of the *ACTT2* transcript, ACT-toxin production, and pathogenicity (23). *ACTT5*, encoding an acyl-CoA synthetase, and *ACTT6*, encoding an enoyl-CoA hydratase, were also identified by sequencing cosmid clone pcCL4-1 containing *ACTT1*, *ACTT2*, and *ACTTR* open reading frames (ORFs) (23). Targeted gene disruptions of two copies of *ACTT5* significantly reduced ACT-toxin production and virulence, and targeted gene disruption of two copies of *ACTT6* led to complete losses of *ACTT6* transcription, ACT-toxin production, and pathogenicity (23).

In this study, we identified a previously uncharacterized region of genomic DNA from a BAC clone of *A. alternata* dual-pathotype isolate BC3-5-1-OS2A producing both ACT- and ACR-toxins (20) using *ACTTR* as a heterologous probe (20,21). The genomic region contained several ORFs, and we report here the distribution and role in ACT-toxin production and the pathogenicity of one of these ORFs encoding a putative enoyl-reductase (1,034 bp) designated as *ACTTS2*.

MATERIALS AND METHODS

Plants and fungal strains. Young citrus leaves (midrib length 2 to 4 cm) of cultivar Iyokan tangor (*Citrus iyo* hort. Ex Tanaka) grown in the greenhouse were used for inoculation and toxin sensitivity assays as described previously (20–23). Iyokan is susceptible to the tangerine pathotype of *A. alternata* and sensitive to ACT-toxin (16). Tangerine pathotype isolates SH11, 12, 13, 14, 15, 18, 19, 20, 21, and EV 10, 13, 14, 24, and rough lemon

Corresponding author: K. Akimitsu; E-mail address: kazuya@ag.kagawa-u.ac.jp

Accession number for *ACTTS2* sequence in DDBJ/GenBank is AB516322.

*The e-Xtra logo stands for “electronic extra” and indicates that the online version contains a figure showing the sequence of *ACTTS2* from the isolate SH20 of the tangerine pathotype of *Alternaria alternata* and a table showing the sequence of gene-specific primers used in this study. Figure 5 appears in color online.

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pathotype isolates HC1, HC7, HC8, BC19, BC27, and BC35 were obtained from leaf lesions of Minneola tangelo (*Citrus reticulata* Blanco × *Citrus paradisi* Macf.) and rough lemon in Florida for previous studies of the population structure and pathogenicity of *Alternaria* brown spot pathogen (20–23,29). Tangerine pathotype isolate SH20 has been used as the reference strain of *Alternaria* brown spot pathogen in previous studies (20–23). Isolates HC14, BC17, and BC36 are nonpathogenic strains of *A. alternata* sampled from rough lemon leaves (21,29) and isolate BC3-5-1-OS2A, a dual-pathotype strain producing both ACT-toxin and ACR-toxin (20), was also used in this study. In addition, laboratory stocks (22,23) 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. Another nonpathogenic *A. alternata* isolate O-94 was obtained from H. Otani, Tottori University, Japan. 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) and reverse transcription-PCR (RT-PCR) and Southern blot hybridizations using digoxigenin (DIG)-PCR labeled probes (Roche, Basel, Switzerland) were carried out as previously described (9,12,13, 20–23). Restriction enzyme digestion of genomic DNA (5 µg), agarose gel electrophoresis, and capillary transfer were conducted under standard conditions (31). A BAC library was constructed from *A. alternata* dual-pathotype isolate BC3-5-1-OS2A (20). Genomic DNA fragments of approximately 100 kb from BC-3-5-1-OS2A separated by pulse field gel electrophoresis (PFGE) (20,22,23) were ligated into the *Bam*HI site of a pcc1 BAC vector (Epicentre, Madison, WI). Ligated vector was introduced into *E. coli* (DH10B) (Invitrogen, San Diego, CA) by electroporation. After verifying the titer and length of insert, colonies screened by blue-white selection were randomly picked onto 384 wells plates (total of nine plates) and maintained as glycerol stocks. The genomic BAC library was screened by colony hybridization with a DIG-labeled *ACTT*R probe generated using primers ACTTFR1 (5'-CAACAGCAGTCGAACC-3') and ACTTRR1 (5'-CGTCTA-GAACGAATCG-3') (e-Xtra), which were designed previously based on sequences from cosmid clone pcCL4-1 (AB176941) (22,23). BAC DNA was extracted as described previously (20,31), and the entire insert of BAC clone 2H2 was sequenced to 5× redundancy by shotgun sequencing using a capillary DNA sequencer. Transcripts of *ACTTS2* were amplified with or without addition of reverse transcriptase (Takara) by RT-PCR using the following primer sets: 2H2C7F1 (5'-TGTTGACTCGTCGTCG-3') and 2H2C7R2 (5'-TCCAGATAGCAGGCGA-3') for *ACTTS2*, and actinFor (5'-AGATGACACAGATTGTTTTTCGAGACC-3') and actinRev (5'-AGTTGAAAGTGGTGACGTGGATACC-3') for the actin gene *act1* of *A. alternata* (AY748985), respectively (e-Xtra). RT-PCR products were transferred to Hybond-N⁺ membranes using standard methods (31) and detected with DIG-labeled PCR amplicons of *ACTTS2* probe by the method described previously (9,12,13,20–23). Transcription initiation sites were identified with 2H2C7RACER1 (5'-CTTTGCTGTAACATAGGAGGCAAATCC-3') and GeneRacer 5' Primer, and 2H2C7RACER2 (5'-GATTTAGGGCTACAGCTTTGACTTTGAC-3') (e-Xtra) and GeneRacer 5' Nested Primer for nested PCR by 5' rapid amplification of cDNA ends (RACE) with the GeneRacer Kit (Invitrogen) according to the manufacturer's instructions. Transcription stop site was identified with 2H2C7RACEF1 (5'-CGACAGGAAGTCTAGCTGTACAATATGC-3') and GeneRacer 3' Primer, and 2H2C7RACEF2 (5'-GCTTTCTCTCGTCTTCG-ACACTATCTCT-3') (e-Xtra) and GeneRacer 3' Nested Primer for nested PCR by 3' RACE. Amplicons were subcloned into

pT7Blue-2 vector (Novagen, Madison, WI) following the manufacturer's instructions. Sequencing was performed by the dideoxy chain termination method (32) 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 (<http://www.ddbj.nig.ac.jp>), and domain and motif analysis were estimated using software at the Pfam (<http://www.sanger.ac.uk/Software/Pfam/>) (5).

Plasmid vector constructs and fungal transformation. Targeting vector pT7-ACTTS2 was constructed using a partial *ACTTS2* sequence amplified by PCR consisting of 30 cycles (denaturing for 2 min at 95°C, primer annealing for 1 min at 55°C, and extension for 1 min at 72°C) with the primer set 2H2C7F1 and 2H2C7R2 using genomic DNA of SH20 as the template. The partial *ACTTS2* sequence amplified by PCR was subcloned into the *Eco*RV site of subcloning vector pT7Blue-2 (Novagen) and used as the targeting vector pT7-ACTTS2 for disruption of *ACTTS2* using homologous recombination. The targeting vector was cotransformed with pSH75 (15) carrying *hph* cassette as a selection marker as described previously (9,12, 13,22,23).

For construction of the *ACTTS2* RNA-silencing vector, sense- and antisense-inserts of *ACTTS2* were amplified from genomic DNA of SH20 by PCR consisting of 30 cycles (denaturing for 1 min at 95°C, primer annealing for 1 min at 55°C, and extension for 1 min at 72°C) using a primer set of either 2H2C7RiF3-2BgIII (5'-GAAGATCTTCATCGATCTTGACACCAAC-3') containing a *Bg*III site and 2H2C7RiR3BgIII (5'-GAAGATCTTCATG-TTGACTCGTCGTGCTC-3') containing a *Bg*III site, or 2H2C7RiF4NspV (5'-TTCGAAATGTTGACTCGTGCTC-3') containing an *Nsp*V site and 2H2C7RiR4-3NspV (5'-TTC-GAAATCGATCTTGACACCAAC-3') containing an *Nsp*V site (e-Xtra). PCR products (anti-sense *ACTTS2*) amplified with 2H2C7RiF3-2BgIII and 2H2C7RiR3BgIII digested with *Bg*III and the RNA-silencing vector for *Alternaria* spp. (pASY) (23) digested with *Bg*III following dephosphorylation with alkaline phosphatase (Takara, Shiga, Japan), were ligated at the region between the *trpC* promoter and the spacer region of pASY. PCR products (sense *ACTTS2*) amplified with 2H2C7RiF4NspV and 2H2C7RiR4-3NspV digested with *Nsp*V, and the pASY containing antisense *ACTTS2* digested with *Nsp*V following dephosphorylation with alkaline phosphatase, were ligated at the region between the spacer region and *trpC* terminator. The vector construct of pASY-ACTTS2 for RNA-silencing of *ACTTS2* was confirmed by PCR and sequencing using a primer set of PacCITF1multisite (5'-GAATTCTGCAGATATCAGATCTGTA-AGTTGACCGCCAAG-3') designed from the spacer region in the internal vector construct and 2H2C7RiR4-3NspV for sense insertion, or of PacCITR1multisite (5'-GAATTCAAGCTTTAA-ATTCGAAGTGAATCGAGTCAGC-3') and 2H2C7RiR3BgIII for anti-sense insertion (e-Xtra).

Plasmid vector pAGY used for cotransformation with pASY-ACTTS2 was constructed in *Alternaria* overexpression vector pAOEIK (13) by insertion of *NPTII* gene amplified by PCR consisting of 30 cycles (denaturing for 1 min at 95°C, primer annealing for 1 min at 55°C, and extension for 1 min at 72°C) with the primer set NPTIIF1EcoRI (5'-GGAATTCATGATT-GAACAAGATGGATTG-3') and NPTIIR1EcoRI (5'-GGAATTC-CTCAGAAGAAGTCAAGAA-3') (e-Xtra) using the plasmid vector pII99 (26) as the template. The PCR amplicon and pAOEIK were digested with *Eco*RI, and the digested vector was dephosphorylation with alkaline phosphatase (Takara) following the ligation with the PCR product at the region between *trpC* promoter and *trpC* terminator to construct the *NPTII* cassette.

Protoplast preparation and transformation of isolate SH20 or its mutants were carried out using methods described previously

(9,12,13,22,23). Protoplasts (1×10^7) were transformed with 3 μ g of each vector. Colonies were transferred to PDA plates containing hygromycin B (50 μ g/ml) or geneticin (200 μ g/ml) and grown at 24°C for 5 to 7 days and single spore cultures were recovered as described previously (9,12,13,22,23).

Detection of plasmid integration, ACT-toxin production, and pathogenicity. Integration patterns of transformation vectors for targeted gene disruption were determined using genomic Southern blots probed with the *ACTTS2* probe. Integration of the RNA-silencing vector was determined using genomic Southern blots with DIG-labeled NTPII probe prepared by the primer set of NTPIIF1 (5'-ATGATTGAACAAGATGGATTG-3') and NTPIIR1 (5'-TCAGAAAGAACTCGTCAAGAA-3'), and genomic PCR using a primer set of PacCITF1multisite and 2H2C7RiR4-3NspV (e-Xtra). Hybridization and detection were conducted as described previously (9,12,13,20–23).

ACT-toxin production was quantified by placing dilute culture filtrates (10^1 to 10^3 in water) onto wounded sites of young leaves of Iyokan and Nijisseiki, as previously described (21–23). Sixty-one independent *ACTTS2*-silenced transformants were tested. Leaves were incubated in a moist chamber at 24°C in the dark, and necrosis development on the leaves was observed after 48 h. Transformants causing less than 50% necrosis compared with that caused by the wild type (isolate SH20) were further examined for the pathogenicity assay by inoculation tests and a high-pressure liquid chromatography (HPLC) analysis of ACT-toxin production. For HPLC examination of ACT-toxin production in culture filtrates, *A. alternata* strain SH20 and its mutants were grown in 200-ml flasks containing 50 ml of AC-toxin medium for 25 to 30 days at 24°C. Extracts of culture filtrates (50 ml) were prepared as described previously (17,18,20–23), and resuspended in 1 ml of methanol. Equal volume (50 μ l from the extract solution in 1 ml of methanol) of each sample was used for a further HPLC analysis and run for HPLC using a Model PU-2080 HPLC system (Japan Spectroscopic, Tokyo, Japan) with a Develosil ODS-5 column (4.6 $\times$ 250 mm, NOMURA 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 an absorbance at 290 nm was used as in the methods described previously (17,18,20–23).

For pathogenicity assays, spores of each isolate were collected from mycelial mats grown in potato dextrose broth 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 (20–23). Leaves were incubated in a moist chamber for 48 h at 24°C in the dark, and the development of leaf spots and necrosis was monitored.

PFGE and hybridization. Preparation of fungal protoplasts for separation of chromosomes by PFGE and running conditions for PFGE were described previously (3,7,21–23). Running conditions (duration, voltage, linear gradient of switching time) in the PFGE apparatus (CHEF-DRII, Bio-Rad, Richmond, CA) consisted of 0.5 $\times$ TBE (31) at 8°C in 0.8% (wt/vol) agarose gel (Seakem Gold agarose; Cambrex BioScience Rockland, Inc., Rockland, ME), 25 h/1.6 V/cm/500 s, 30 h/1.6 V/cm/1,000 s, and 50 h/1.6 V/cm/1,500 s, 32 h/1.6 V/cm/2,000 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), 0.4 N NaOH plus 1.5 M NaCl for 1 h to transfer Hybond-N⁺ membrane (Amersham Biosciences, Piscataway, NJ) by alkaline capillary transfer (31) with 0.4 N NaOH and 1.5 M NaCl for 48 h. The membrane was baked at 80°C for 2 h to fix the DNA and hybridized at 68°C overnight with *ACTTS2* probes. Hybridization and wash were conducted under high stringency conditions according to the manufacturer's instructions as described previously (20–23).

RESULTS

Identification and genomic analysis of *ACTTS2* from BAC clone 2H2. BAC clone 2H2 was selected for further analysis from approximately 1,000 BAC clones from isolate BC-3-5-1-OS2A based on hybridization to the *ACTTR* PCR probe. The presence of *ACTT* genes *ACTT1*, 2, 5, and 6 (20–23) in the inserted genomic region in the BAC clone 2H2 was verified with PCR. The entire insert of BAC clone 2H2 (100 kb) was sequenced to greater than 5 $\times$ redundancy by shotgun sequencing using a capillary sequencer. A total of 574,860 bp of sequence data was obtained. Annotation analysis identified 25 contigs of more than 4 kb with an average redundancy of 4.99 and containing putative ORFs including *ACTT1*, 2, 5, 6 (20–23), *AKT3*, and *AKTR* homologs (35,36), and other putative ORFs encoding a nonribosomal peptide synthetase (NRPS) of 1,218 bp; a HMG-CoA synthase of 1,179 bp; an enoyl-reductase of 1,034 bp; a monooxygenase of 1,560 bp; a polyketide synthase of 7,374 bp; an NRPS of 4,833 bp; and transposon-like sequences, and several ORFs of unknown function.

Sequence data from the ORF encoding a putative enoyl-reductase of 1,034 bp were used to design gene-specific primers and four primers for 5' and 3' RACEs in order to clone the gene from reference isolate SH20 of the tangerine pathotype of *A. alternata*. The gene designated as *ACTTS2* (accession no. AB516322) is 1,034 bp in length with a predicted 343 amino acid protein (e-Xtra). BLAST search indicates that *ACTTS2* likely encodes an enoyl-reductase and possesses domains similar to the alcohol dehydrogenase GroES-like domain and zinc-binding dehydrogenase domain, and showed high similarity to enoyl-reductases, especially those involved in fungal polyketide biosynthesis, such as *lovC* (14).

Presence of multiple *ACTTS2* copies in the genome of the tangerine pathotype of *A. alternata*. The distribution of *ACTTS2* in the genomes of seven pathotypes producing different HSTs or nonpathogenic strains of *A. alternata* and a dual pathotype strain BC3-5-1-OS2A was examined by genomic and PFGE Southern blotting (Fig. 1A and B). The distribution of *ACTTS2* in the genomes of several different isolates of tangerine pathotype, rough lemon pathotype, and nonpathogenic *A. alternata* was also examined (Fig. 1C). The *ACTTS2* probe only hybridized to the genome of ACT-toxin producers (tangerine pathotype and dual-pathotype isolates) (Fig. 1A and C), indicating that the presence of *ACTTS2* is highly correlated to ACT-toxin production. PFGE and Southern blots demonstrated that *ACTTS2* located to a single small chromosome of 1.9 Mb in the tangerine pathotype (SH20) but not to chromosomes from any other *A. alternata* pathotype (Fig. 1B).

The distribution of *ACTTS2* in the genome of isolate SH20 of the tangerine pathotype was further examined (Fig. 2). The *ACTTS2* probe detected one fragment when genomic DNA was digested with *EcoRI*, and *BglII*, which have no predicted restriction site within the probe sequence, and two bands were observed upon digestion with *EcoRV* which has one predicted restriction site within the *ACTTS2* probe sequence (Fig. 2). However, multiple bands were observed upon digestion with *BamHI*, *KpnI*, *XbaI*, and *SacI*, which have no predicted restriction site within the sequence of the *ACTTS2* probe (Fig. 2). These results indicated the presence of multiple *ACTTS2* copies with different restriction sites in the flanking DNA or within the ORF region in the genome of isolate SH20 (Fig. 2).

Targeted gene disruption of *ACTTS2* for functional analysis in ACT-toxin biosynthesis. To examine the role of *ACTTS2* in pathogenicity and ACT-toxin production, targeting vector pT7-ACTTS2 containing a partial region of *ACTTS2* (707 bp) in pT7-blue2 (Novagen) was constructed for disruption of *ACTTS2* by homologous recombination (Fig. 3A). This vector was cotransformed into the isolate SH20 of the tangerine pathotype with

Fig. 1. Distribution of *ACTTS2* among different pathotypes and nonpathogenic strains of *Alternaria alternata*. **A**, Southern blot analysis of *ACTTS2* with seven pathotypes, a nonpathogen, and the dual pathotype isolate BC3-5-1-OS2A (OS2A) (20) of *A. alternata*. Genomic DNA of each fungus was digested with *EcoRV*. Abbreviations used in panel **A** and **B** of this figure are as follows: Tg, tangerine pathotype isolates SH11 (lane 1) and SH20 (2); RL, rough lemon pathotype isolates HC1 (1) and HC8 (2); JP, Japanese pear pathotype isolates G31(1) and G90-A2 (2); Sb, strawberry pathotype isolates NAF-3 (1) and NAF-8 (2); Tm, tomato pathotype isolates 91080804 (1) and AL-4 (2); Ap, apple pathotype isolates AM-1 (1) and O-159 (2); Tb, tobacco pathotype isolates No. 122-2 (1) and AT204 (2); and No, nonpathogenic isolates O-94 (1) and HC14 (2). Sizes of marker DNA fragments (*HindIII*-digested λ DNA) are indicated at left. **B**, Chromosomal location of *ACTTS2* among isolates of seven pathotypes. Intact chromosomes of each isolate were separated with pulse field gel electrophoresis condition in 0.8% agarose gel (left). Chromosomes were transferred to nylon membrane and hybridized with DIG-labeled *ACTTS2* probe (right). Sizes of *Saccharomyces cerevisiae* chromosome size markers are indicated at left. Abbreviations and isolates used in respective lanes were as follows: Tg, isolates SH20 (lane 1); JP, isolates G31(1) and G90-A2 (2); Sb, isolates NAF-3 (1) and NAF-8 (2); Tm, isolates 91080804 (1) and AL-4 (2); Ap, isolates AM-1 (1) and O-159 (2); Tb, isolates No. 122-2 (1) and AT204 (2); and RL, isolates HC1 (1) and HC8 (2). **C**, Southern blot analysis of the distribution of *ACTTS2* among Florida rough lemon pathotype and nonpathogenic strains (left), and tangerine pathotype strains (right). Genomic DNA of each strain was digested with *EcoRV*. Isolates used in each lane were as follows: 1, HC1; 2, HC7; 3, HC8; 4, BC19; 5, BC27; 6, BC35; 7, HC14; 8, BC17; 9, O-94; 10, BC36; and 11, *A. citri* (left panel); and 1, SH12; 2, SH13; 3, SH14; 4, SH15; 5, SH18; 6, SH19; 7, SH20; 8, SH21; 9, EV10; 10, EV13; 11, EV14; and 12, EV24 (right panel). Pathogenicity of each strain on Iyokan young leaves and ACT-toxin production are indicated as $\pm$ at bottom of figure. Sizes of marker DNA fragments (*HindIII*-digested λ DNA) are indicated at left.

pSH75 (15) carrying *hph* cassette as a selection marker. Screening for reduction in ACT-toxin production by leaf symptom bioassay and for insertion of the vector construct by Southern hybridization identified transformant D6-2, which showed a reduction of the toxin production in culture filtrates and a shift of 7.7-kb *ACTTS2* band present in the wild type to 9.4, 3.9, and 2.2-kb bands in the transformant (Fig. 3B), which were the predicted shifts of bands based on the map of *ACTTS2* region, indicating an insertion of the vector construct at the target site (Fig. 3A). The remaining of the 7.7-kb band indicated the presence of multiple copies of *ACTTS2*. Transcript analysis of *ACTTS2* by RT-PCR with primer set 2H2C7F1 and 2H2C7R2 generated an amplicon of the predicted size (707 bp) from total RNA of wild-type and transformant D6-2, and the products hybridized to the *ACTTS2* probe (Fig. 4A). Intensity of the band was reduced but not eliminated in transformant D6-2 compared with that of wild type (Fig. 4A). The PCR reaction without addition of reverse transcriptase did not amplify any product (Fig. 4A). The actin gene, which is known to express constitutively in this fungus (22), was amplified approximately equally to that observed for wild type (Fig. 4A). An approximately 70% reduction in the peak area of ACT-toxin in HPLC analysis was observed in transformant D6-2 (Fig. 5A). When spores of transformant D6-2 were spray-inoculated on the lower leaf surfaces of susceptible cv. Iyokan, typical leaf spots appeared but disease severity was reduced relative to that caused by the wild-type strain (Fig. 5B).

Use of RNA silencing for functional disruption of *ACTT2*.

In a previous study, the efficiency of RNA silencing in *A. alternata* was established by knocking down *GFP* fluorescence in a GFP-overexpressing strain and silencing the multicopy *ACTT2* gene in the tangerine pathotype of *A. alternata* (23). We applied RNA silencing in the current study to knockdown the function of *ACTT2* in the tangerine pathotype of *A. alternata*. The RNA-silencing vector pASY-ACTT2S2 expressing hairpin *ACTT2* RNA, constructed by sense and antisense sequences of *ACTT2* (1,029 bp) in pASY, was cotransformed into the single-copy disruptant D6-2 with pAGY carrying *NTPII* as the selection marker. A total of 61 transformants were examined for their production of ACT-toxin in the culture filtrates using a leaf

Fig. 2. Southern blot analysis of *ACTTS2* in *Alternaria alternata* tangerine pathotype isolate SH20 using different restriction enzymes. Genomic DNA of isolate SH20 digested with *EcoRI* (lane EI), *ecoRV* (EV), *BamHI* (Bm), *BglIII* (Bg), *KpnI* (K), *XbaI* (X), and *SacI* (S), respectively. Digested DNA was transferred to nylon membrane probed with a DIG-labeled *ACTTS2*. Sizes of marker DNA fragments (*HindIII*-digested 1 DNA) are indicated at left.

necrosis assay. Twelve transformants showing less than 50% necrosis development than the wild-type SH20 when inoculated on toxin-sensitive citrus leaves were selected for further study. These transformants were examined for their loss of pathogenicity in a spray-inoculation assay on tangerine leaves and ability to produce ACT-toxin detected by HPLC, and the transformant S6-2-1 were selected. The transformant S6-2-1

Fig. 3. Targeted gene disruption of *ACTTS2* in *Alternaria alternata* tangerine pathotype isolate SH20. **A**, Disruption construct pT7-ACTTS2 and integration pattern in single-copy disruptant D6-2 (W, wild-type; D, D6-2). X signifies *XhoI* restriction sites. **B**, Genomic Southern blot of *ACTTS2* in wild-type (SH20), single-copy disruptant D6-2. Genomic DNA was digested with *XhoI* and fractionated in a 0.8% agarose gel following hybridization with *ACTTS2* probe. Sizes of marker DNA fragments (*HindIII*-digested λ DNA) are indicated at left.

Fig. 4. Reverse transcription-polymerase chain reaction (RT-PCR) and genomic PCR analysis of *ACTTS2* disruptant and *ACTTS2* RNA-silenced mutant. **A**, RT-PCR detection of *ACTTS2* transcripts in wild-type SH20 (lane W), single-copy *ACTTS2* disruptant D6-2 (D) and *ACTTS2* RNA-silenced mutant (S), respectively. RT⁻ indicates the PCR without addition of reverse transcriptase. Amplification of actin gene was used as positive control for RT-PCR as well as for loading of RNA. **B**, Insertion analysis of pASY-ACTTS2 by Southern blot of *NPTII* in wild-type (SH20), single-copy disruptant D6-2, *ACTTS2*-silencing mutant S6-2-1. Genomic DNA was digested with *XhoI* and fractionated in a 0.8% agarose gel following hybridization with *NPTII* probe. Insertion of the vector was confirmed by genomic PCR of wild-type (SH20), single-copy disruptant D6-2, *ACTTS2*-silencing mutant S6-2-1 using primer set of 2H2C7RiR4-3NspV and PacCITF1multisite (*ACTTS2*-PacCIT). **C**, Growth rate of *ACTTS2* in wild-type SH20, single-copy disruptant D6-2, *ACTTS2*-silencing mutant S6-2-1.

carrying pASY-ACTTS2 was resistant to geneticin, NTPII probe hybridized in genomic Southern blot, and PCR using the primer set 2H2C7RiR4-3NspV and PacCITF1multisite indicated insertion of silencing vector pASY-ACTTS2 (Fig. 4B). No *ACTTS2* transcript was detected in transformant S6-2-1 by RT-PCR (Fig. 4A). HPLC analysis also did not detect any trace of ACT-toxin in the culture filtrates of S6-2-1 (Fig. 5C). Leaves sprayed with spores of the wild type were covered by necrotic brown lesions 48 h after inoculation, while those sprayed with spores of S6-2-1 showed no lesions (Fig. 5D). No apparent differences were noticed in growth rate (Fig. 4C), spore formation, and spore germination rates were found among wild-type, single-copy disruptant D6-2, and RNA-silenced transformant S6-2-1.

DISCUSSION

Genes responsible for ACT-toxin production in the tangerine pathotype of *Alternaria alternata* identified to date include *ACTT1*, 2, 3, R, 5, and 6 (20–23). These genes are clustered and located to the smallest chromosomes in the *A. alternata* genome, generally less than 2 Mb in size. This group of genes has been designated as the ACT-toxin TOX (*ACTT*) cluster (4,20–23). Only plant-pathogenic *A. alternata* strains producing HSTs possess these small, dispensable chromosomes responsible for pathogenicity (3,7,20–23). Nonpathogenic strains of *Alternaria* spp. do not carry these small chromosomes (3,7,20–23). Since these small chromosomes do not appear to carry genes essential for normal growth and reproduction of fungi, their loss has no obvious phenotypic effect on in vitro growth or reproduction (7,35,36). Interestingly, gene clusters responsible for the biosynthesis of HSTs, such as the *AKT*, *AFT*, *ACTT*, *ACRT*, *ALT*, and *AMT* clusters in the Japanese pear, strawberry, tangerine, rough lemon,

Fig. 5. Effects of single copy disruption and RNA silencing of *ACTTS2* on ACT-toxin production and pathogenicity in *Alternaria alternata* tangerine pathotype isolate SH20. **A** and **C**, HPLC traces of extracts from culture filtrates of *ACTTS2* disruptant D6-2 (**A**) and *ACTTS2* RNA silenced mutant S6-2-1 (**C**). **B** and **D**, Pathogenicity tests of mutants using spray-inoculation with conidia of *ACTTS2* disruptant D6-2 (**B**) and *ACTTS2* RNA silencing mutant (**D**) on Iyokan leaves. W represents wild-type isolate SH20, D the single-copy *ACTTS2* disruptant D6-2, and S the *ACTTS2*-silencing mutant, respectively.

tomato, and apple pathotypes, respectively (1–4,7,10,11,20–23, 35,36), are all located to small, dispensable chromosomes. In addition, several genes in the *AKT*, *AFT*, and *ACTT* clusters are homologous and several genes responsible for production of the common decatrienoic acid moiety of AK-, AF-, and ACT-toxins (7,20–23,30,35,36) are shared among pathotypes. There are only a few examples where genes carried on conditionally dispensable chromosomes in plant-pathogenic fungi have been shown to play a role in pathogenicity. These include *Nectria haematococca* (37) and several pathotypes of HST-producing *A. alternata* (1–4,7, 10,11,20–23,35,36). The only apparent difference between HST-producing strains of *A. alternata* and saprophytic strains is the presence or absence of small, dispensable chromosomes. Therefore, more extensive study of these chromosomes including the ORFs encoded in biosynthesis clusters on the chromosomes as well as genomic comparisons of entire chromosomes and ORFs will be valuable for understanding the evolution of microbial pathogenicity.

In this study, we identified several putative ORFs in a 100-kb clone from the 1.9 Mb chromosome carried by reference tangerine pathotype isolate SH20. We are currently characterizing the roles of these additional ORFs in ACT-toxin production and pathogenicity, and the data presented here demonstrates the involvement of *ACTTS2* encoding an enoyl-reductase in HST production and pathogenicity. *ACTTS2* is only carried by the tangerine pathotype among seven pathotypes of HST-producing *A. alternata* tested and was not present in any other pathotypes or nonpathogenic strains. Multiple copies of *ACTTS2* were found on the 1.9 Mb chromosome, and presence of this gene is correlated with ACT-toxin production and pathogenicity among *A. alternata*. *ACTTS2* is likely an enoyl-reductase because it (i) displays sequence similarity to known enoyl-reductases especially those involved in fungal polyketide biosynthesis such as *lovC* (14), and (ii) possesses domains similar to the alcohol dehydrogenase GroES-like domain and zinc-binding dehydrogenase. *lovC* is one of the biosynthesis genes involved in synthesis of the polyketide lovastatin in *Aspergillus terreus* (14). The major biosynthetic enzyme for lovastatin is a polyketide synthase (PKS) named as lovastatin nonateride synthase encoded by *lovB* (8,14,34). PKSs usually consist of multiple functional domains of acyltransferase domain, β -ketoacyl synthase domain, β -ketoacyl reductase domain, dehydratase domain, enoyl-reductase domain, and acyl carrier protein domain (14,27). However, the enoyl-reductase domain is missing in *lovB*, and the function of *lovC* complements the insufficiency to synthesize the full structure of lovastatin together with *lovB* (8,14,34). ACT-toxin contains a polyketide component (17,18) and the involvement of a PKS in ACT-toxin biosynthesis is expected. Indeed, a gene, designated as *ACTTS3*, encoding a PKS of 7,374 bp located in the *ACTT* cluster was identified by sequence analysis of partial region of *ACTTs* in the BAC clone 2H2, and its role in ACT-toxin biosynthesis was confirmed by targeted disruption (Y. Miyamoto and K. Akimitsu, unpublished data). Interestingly, an enoyl-reductase domain is also missing in *ACTTS3*. We speculate that the function of *ACTTS2* might be to complement this insufficiency in *ACTTS3* in the synthesis of the polyketide portion of ACT-toxin, similar to the cooperation of *lovB* and *lovC* in lovastatin synthesis.

We generated a single-copy disruptant of *ACTTS2* using gene targeted disruption and also a functional disruptant of *ACTTS2* using RNA silencing. The *ACTTS2*-silenced mutant S6-2-1 had no detectable *ACTTS2* transcript or ACT-toxin production and was nonpathogenic. Although there are multiple copies of *ACTTS2* present in the genome of isolate SH20 of the tangerine pathotype, RNA silencing appeared to be effective in silencing all copies. RNA silencing was used previously to evaluate the role of *ACTT2* which is present in at least five copies in the genome of isolate SH20 (23). The data presented here demonstrate a reduction in toxin production and pathogenicity through both single-copy

disruption and RNA silencing, and confirm that *ACTTS2* plays an important role in ACT-toxin production as well as pathogenicity in the tangerine pathotype of *A. alternata*.

The tangerine, Japanese pear, and strawberry pathotypes are interesting models for understanding intraspecific variation in host specificity and the evolution of pathogenicity and host specificity in plant-pathogenic fungi. Although these three pathotypes have markedly different host specificities, their HSTs share a common structural moiety of decatrienoic acid in AK-, AF-, and ACT-toxins and homologous genes responsible for the biosynthesis of this moiety exist in genomes of these three distinct pathotypes (7,20–23,30,35,36). *ACTTS2* present only in the genome of the tangerine pathotype because the gene is responsible for the biosynthesis of polyketide moiety which is the unique structure in ACT-toxin and not in the structures of AK- and AF-toxins (4,18,24,25). Sequence comparisons of the toxin biosynthesis clusters in these pathotypes as well as comparisons of entire genomes of these fungi should prove fruitful in determining the evolutionary history and trajectories of these different *A. alternata* pathotypes.

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