A Polyketide Synthase Gene, *ACRTS2*, Is Responsible for Biosynthesis of Host-Selective ACR-Toxin in the Rough Lemon Pathotype of *Alternaria alternata*

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The rough lemon pathotype of *Alternaria alternata* produces host-selective ACR-toxin and causes *Alternaria* leaf spot disease of rough lemon (*Citrus jambhiri*). The structure of ACR-toxin I (MW = 496) consists of a polyketide with an α -dihydropyrone ring in a 19-carbon polyalcohol. Genes responsible for toxin production were localized to a 1.5-Mb chromosome in the genome of the rough lemon pathotype. Sequence analysis of this chromosome revealed an 8,338-bp open reading frame, *ACRTS2*, that was present only in the genomes of ACR-toxin-producing isolates. *ACRTS2* is predicted to encode a putative polyketide synthase of 2,513 amino acids and belongs to the fungal reducing type I polyketide synthases. Typical polyketide functional domains were identified in the predicted amino acid sequence, including β -ketoacyl synthase, acyl transferase, methyl transferase, dehydratase, β -ketoreductase, and phosphopantetheine attachment site domains. Combined use of homologous recombination-mediated gene disruption and RNA silencing allowed examination of the functional role of multiple paralogs in ACR-toxin production. *ACRTS2* was found to be essential for ACR-toxin production and pathogenicity of the rough lemon pathotype of *A. alternata*.

Seven strains of *Alternaria alternata* (Fr.) Keissl. are known to produce host-selective toxins (HST), and each strain is selectively toxic to a particular cultivar of each host species (Kohmoto and Otani 1991; Nishimura and Kohmoto 1983; Otani et al. 1995). HST of *A. alternata* are secondary metabolites of low molecular weight that induce necrotic cell death and have the same specificity as the toxin-producing pathogen during infection (Nishimura and Kohmoto 1983; Scheffer and Livingston 1984; Walton 1996; Wolpert et al. 2002; Yoder 1980). Morphological characteristics of the seven strains are highly similar, and this close evolutionary relationship has been verified by molecular phylogenetic studies (Kusaba and Tsuge 1995; Peever et al. 1999). Each pathotype can be distinguished only by its specificity on a particular host plant, which can be noted by the necrotic symptoms that develop after inoculation with the pathotype or after treatment with the HST produced

by the pathotype. Chemical structures of six of the seven HST have been elucidated (Kohmoto and Otani 1991). Further, HST-biosynthetic genes of *A. alternata* have been cloned from Japanese pear, strawberry, tangerine, tomato, apple, and rough lemon pathotypes. In a series of studies using homologous recombination-mediated gene disruption and RNA silencing, these genes were found to be essential for the production of a particular HST and required for pathogenicity (Ajiro et al. 2010; Akamatsu et al. 1997, 2003; Harimoto et al. 2007, 2008; Hata et al. 2002; Ito et al. 2004; Izumi et al. 2012; Johnson et al. 2000, 2001; Masunaka et al. 2000, 2005; Miyamoto et al. 2008, 2009, 2010; Ruswandi et al. 2005; Tanaka et al. 1999; Tanaka and Tsuge 2000).

The rough lemon pathotype of *A. alternata* causes *Alternaria* leaf spot disease of the common citrus root stock species rough lemon (*Citrus jambhiri* Lush.) and a hybrid of rough lemon and acid mandarin, rangpur lime (*C. limonia* Osbeck) (Akimitsu et al. 2003; Kohmoto et al. 1979, 1984, 1991). The pathogen produces host-selective ACR-toxin (Gardner et al. 1985a and b; Kohmoto et al. 1979; Nishimura and Kohmoto 1983). The structure of the major form of ACR-toxin (ACR-toxin-I, MW = 496) consists of an α -dihydropyrone ring in a 19-carbon polyalcohol, a typical polyketide structure (Gardner et al. 1985a and b; Nakatsuka et al. 1986) (Fig. 1). The molecular mechanisms conferring sensitivity to ACR-toxin in rough lemon also have been identified (Akimitsu et al. 1989, 2003; Kohmoto et al. 1984; Ohtani et al. 2002). The target site of ACR-toxin, the mitochondrion, becomes dysfunctional in rough lemon treated with the toxin (Akimitsu et al. 1989; Kohmoto et al. 1984). ACR-toxin uncouples oxidative phosphorylation and also causes leakage of cofactor NAD⁺ from the TCA cycle (Akimitsu et al. 1989). The *ACR-toxin sensitivity* (*ACRS*) gene that confers sensitivity to ACR-toxin was identified from the mitochondrial genome of rough lemon (Ohtani et al. 2002). Sequence analysis demonstrated that *ACRS* is located within the group II intron of the mitochondrial tRNA-Ala and encodes an amphiphilic helix type of tetrameric transmembrane receptor-like protein (Ohtani et al. 2002). This gene confers ACR-toxin sensitivity to *Escherichia coli*, and the addition of the toxin at a minimum concentration of 50 nM abolished oxygen uptake by *ACRS*-expressing *E. coli*. *ACRS* transcript analysis demonstrated that toxin sensitivity was not controlled by the presence or absence of *ACRS* but, rather, by post-transcriptional modification of the *ACRS* transcript (Ohtani et al. 2002).

The HST-biosynthetic genes of *A. alternata* pathotypes are clustered on small chromosomes that are generally less than

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2 Mb (Ajiro et al. 2010; Akagi et al. 2009; Akamatsu et al. 1999; Harimoto et al. 2007; Hatta et al. 2002; Ito et al. 2004; Izumi et al. 2012; Johnson et al. 2001; Masunaka et al. 2005; Miyamoto et al. 2008, 2009, 2010). Masunaka and associates (2005) found that strains of the rough lemon pathotype also carried a small chromosome, approximately 1.5 Mb, and the presence of this chromosome was correlated with production of ACR-toxin and pathogenicity to rough lemon.

We recently identified *ACRTS1*, which encodes a putative hydroxylase located in the 1.5 Mb-chromosome (Izumi et al. 2012; Masunaka et al. 2005). There are more than three copies of *ACRTS1* in the genome of the rough lemon pathotype but, by combining homologous recombination-mediated gene disruption and RNA silencing, we were able to functionally suppress all paralogs and demonstrate that this gene is required for biosynthesis of ACR-toxin and pathogenicity (Izumi et al. 2012).

Most of the genes responsible for HST production in *A. alternata* have been duplicated and multiple paralogs are present in all genomes studied to date (Ajiro et al. 2010; Akagi et al. 2009; Akamatsu et al. 1999; Harimoto et al. 2007; Hatta et al. 2002; Ito et al. 2004; Izumi et al. 2012; Johnson et al. 2001; Masunaka et al. 2005; Miyamoto et al. 2008, 2009, 2010). These duplications make it difficult to examine the role of these genes in toxin production when attempting to disrupt all functional copies using homologous recombination-mediated gene targeting. To overcome this problem, RNA silencing has been introduced to knock down all transcripts from all functional copies in HST-producing *A. alternata* (Miyamoto et al. 2008). The first example of this approach to examine HST biosynthesis genes with multiple paralogs was the use of RNA silencing of the tangerine pathotype HST biosynthesis gene *ACTT2*, which encodes a putative hydrolase and has the highest number of copies of the ACT-toxin biosynthesis genes identified in the genome of this pathotype (Miyamoto et al. 2008). The methodology followed protocols used previously (Ajiro et al. 2010; Izumi et al. 2012; Miyamoto et al. 2009, 2010).

In the present study, we identified an 8,338-bp gene with multiple paralogs encoding a putative fungal reducing type I polyketide synthase (PKS), named *ACRTS2*, located on the 1.5-Mb chromosome of the rough lemon pathotype of *A. alternata*. Functional suppression of *ACRTS2* through gene disruption

and RNA silencing prevented the construction of the polyketide structure of the toxin, with a subsequent loss in the biosynthesis of ACR-toxin (Fig. 1) and pathogenicity to rough lemon.

RESULTS

Isolation of a PKS gene from 1.5-Mb chromosome of *A. alternata* rough lemon pathotype.

Approximately 56 Mb of sequence data was obtained from a 1.5-Mb chromosome from the rough lemon pathotype of *A. alternata* (Izumi et al. 2012; Masunaka et al. 2005). The assembly consisted of 235 contigs greater than 4 kb, with the largest contig at approximately 41 kb. One 17-kb contig (C14014) contained three open reading frames (ORF) that encoded two unknown proteins and a putative PKS, identified via SoftBerry and BLAST/FASTA searches. The putative PKS-encoding ORF, named *ACRTS2* (accession number AB725683), was 8,338 bp in length and contained seven putative introns and coded for a predicted protein of 2,513 amino acids (Fig. 2) (Supplementary Fig. S1). *ACRTS2* contained six PKS catalytic domains identified by Pfam and SMART searches including β -ketoacyl synthase domain (KS; 13-1312), acyl transferase domain (AT; 1859-2961), methyl transferase domain (ME; 4578-4899), alcohol-dehydrogenase domain (DH; 5799-6561), β -ketoacyl reductase domain (KR; 6753-7404), and phosphopantetheine attachment site domain (PP; 7740-7925) (equal to acyl carrier protein), respectively (Fig. 2).

We recently cloned another PKS gene, *ACTTS3*, which is responsible for biosynthesis of ACT-toxin in the tangerine pathotype of *A. alternata* (Miyamoto et al. 2010). Although both genes are PKS, the chemical structures (Fig. 1) and host specificities for the toxicities of ACR-toxin produced by the rough lemon pathotype and the ACT-toxin produced by the tangerine pathotype of *A. alternata* are completely different (Gardner et al. 1985a and b; Kohmoto et al. 1991, 1993; Nakatsuka et al. 1986). Because both PKS genes were cloned from HST-producing *A. alternata* pathogenic to citrus, the evolutionary relationship between these PKS and among other fungal PKS genes was further examined. Comparison of the KS domains of the two genes, *ACRTS2* and *ACTTS3*, previously used in phylogenetic analysis of PKS sequences, revealed that they clearly belong to different clades (Supplementary Fig. S2). Comparison of the genome sequences of *ACRTS2* and *ACTTS3* using a Harr plot analysis and the Artemis Comparison Tool (Carver et al. 2005) also indicated minimal similarity between *ACRTS2* and *ACTTS3* (Supplementary Fig. S3). Alignment of nucleotide and putative amino acid sequences of *ACRTS2* and *ACTTS3* similarly revealed low levels of similarity. Similarity of the nucleotide sequences for each domain was 44.9% for β -ketoacyl synthase (KS), 49.5% for acyl transferase (AT), 55.4% for methyl transferase (ME), 47.7% for β -ketoacyl reductase (KR), 50.6% for the phosphopantetheine attachment site (PP), and 49.1% for the entire sequence homology. A homology search using the nucleotide sequence of the KS domain also indicated that *ACRTS2* was most similar to fungal PKS such as *PKS8* from *Botryotinia fuckeliana* (Amselem et al. 2011) with 49.7%, *PKS1* from *Cochliobolus heterostrophus* (Yang et al. 1996) with 48%, and *mlcB* from *Penicillium citrinum* (Abe et al. 2002) with 44.5% (Fig. 2).

Classifying PKS genes by domain organization in addition to sequence similarity of particular domains has proven a more effective way to categorize PKS genes because genes encoding fungal PKS generally consist of multifunctional domains within a large ORF, and phylogenetic analyses of different domains within the ORF can be incongruent (Kroken et al. 2003; Miyamoto et al. 2010; Moss et al. 2004). There are three

ACR-toxin

ACT-toxin

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

Fig. 1. Chemical structures of ACR- and ACT-toxins produced by the rough lemon pathotype and the tangerine pathotype of *Alternaria alternata* (Gardner et al. 1985a and b; Kohmoto et al. 1993; Nakatsuka et al. 1986).

major groups of fungal reducing PKS (PKS with no DH domain, with no ME domain, and with both domains) (Kroken et al. 2003; Miyamoto et al. 2010; Moss et al. 2004). *ACRTS2* belongs to the group with both domains, while *ACTTS3* from the tangerine pathotype belongs to the group with no DH domain (Fig. 2). Although these results indicated that *ACRTS2* and *ACTTS3* are both typical fungal reducing PKS genes, their sequences are highly dissimilar, and the domain organization of *ACRTS2* (KS-AT-ME-DH-KR-PP) is also different from that of *ACRTS2* (KS-AT-ME-KR-PP) (Fig. 2).

Distribution and copy number of *ACRTS2*.

The distribution of *ACRTS2* in the genomes of 14 isolates representing seven different *A. alternata* pathotypes and a dual pathotype strain (Masunaka et al. 2005) was examined by genomic Southern blotting. The *ACRTS2* probe hybridized only to the genome of ACR-toxin-producing isolates of the rough lemon pathotype and the dual pathotype (Fig. 3A). Pulsed-field gel electrophoresis (PFGE) and Southern blotting demonstrated that *ACRTS2* localized to the small, 1.5-Mb chromosome in the rough lemon pathotype isolates HC1 and HC8 and to the 2.0-Mb chromosome in dual-pathotype isolate BC3-5-1-OS2A (Fig. 3B). The distribution of *ACRTS2* in the genomes of several rough lemon pathotype ($n = 5$), tangerine pathotype ($n = 11$), and nonpathogenic *A. alternata* ($n = 5$) isolates was further examined (Fig. 3C). The *ACRTS2* probe only hybridized to the genome of ACR-toxin producers, indicating that the presence of *ACRTS2* is completely correlated with ACR-toxin production (Fig. 3).

The *ACRTS2* probe detected only one fragment when genomic DNA was digested with *Eco*RI or *Hind*III, which has no predicted restriction site within the sequence region targeted by the probe, and two expected bands were observed after digestion with *Alu*I, which has one predicted restriction site within the *ACRTS2* probe sequence (Fig. 4A). However, multiple bands were observed after digestion with *Bgl*II and *Sac*I, which also have no predicted restriction site within the target sequence of the *ACRTS2* probe, indicating the presence of multiple *ACRTS2* paralogs with different restriction sites in the flanking DNA or within the ORF region in the genome of isolate HC1 (Fig. 4A).

Targeted gene disruption of *ACRTS2* for functional analysis of ACR-toxin production.

To examine the role of *ACRTS2* in ACR-toxin production and pathogenicity, we constructed the targeting vector pTeasy-*ACRTS2* containing a partial region of *ACRTS2* in pGEM-T easy vector (Promega Corp., Madison, WI, U.S.A.) to disrupt *ACRTS2* by homologous recombination-mediated mutation (Fig. 4B). This vector was used to transform the wild-type isolate HC1 of the rough lemon pathotype, and 34 hygromycin B-resistant putative transformants (50 μ g/ml) were selected. In additional screening of these 34 hygromycin B-resistant isolates for reduced production of ACR-toxin using a leaf necrosis assay and for insertion of the vector construct using Southern hybridization, we identified four transformants (D-A5, D-A8, D-F1, and D-H15). Transformant D-A5 produced reduced toxin in culture filtrates and had a shift from the wild-type 1.1-kb *ACRTS2* band to an 8.5-kb band (Fig. 4C), which was the predicted shift based on the map of the *ACRTS2* region (Fig. 4B), indicating a tandem insertion of the vector construct at the target site. The remaining 1.1-kb band was not disrupted by the targeting vector pTeasy-*ACRTS2* (Fig. 4C), indicating the presence of an intact *ACRTS2* region among multiple copies of *ACRTS2*. D-H15 had the same integration pattern as that of D-A5, demonstrating reduced toxin production (data not shown).

Fig. 2. Catalytic domain organization in *ACRTS2* and comparison with those in other fungal polyketide synthases (PKS) among a fungal reducing PKS group. Catalytic domain organization of *ACRTS2* is shown and classified based on their types of organization in several fungal reducing PKS. PKS with no dehydratase (DH) domain are grouped and surrounded by a blue line; with no methyl-transferase (ME) domain by a green line, and with both domains by an orange line. Other functional domains used in the panel are KS = β-ketoacyl synthase, AT = acyltransferase, ER = enoyl reductase, KR = β-ketoreductase, and PP = phosphopantetheine attachment site (equal to acyl carrier protein). Accession numbers for these PKS are listed in Supplementary Table S1.

Transcript analysis of *ACRTS2* by reverse-transcriptase polymerase chain reaction (RT-PCR) with primer set C14014F10 and C14014R8 generated an amplicon of the predicted size from total RNA of the wild type and transformant D-A5, and the products hybridized to the *ACRTS2* probe (Fig. 5A). Intensity of the hybridization signal was reduced in transformant D-A5 compared with that of the wild type but not eliminated completely (Fig. 5A). The actin gene, which is expressed constitutively in this fungus, was amplified approximately equally in D-A5 and the wild type (Fig. 5A). The PCR reaction without RT did not amplify any product (Fig. 5A). Thin-layer chromatography (TLC) analysis of ACR-toxin production also detected ACR-toxin from the extract of disruptant D-A5 at the authentic ACR-toxin Rf value of 0.24 (Fig. 5B) (Izumi et al. 2012). Inoculation of rough lemon leaves with D-A5 resulted in a very limited number of necrotic spots (Fig. 5C). Trans-

formant D-H15 also had a pathogenicity phenotype that was comparable with D-A5 (data not shown).

RNA silencing of *ACRTS2* for functional analysis of ACR-toxin production.

RNA silencing of the green fluorescent protein (GFP) gene and *ACTS2* toxin gene has been previously demonstrated in *A. alternata* and this methodology has been successfully used for the functional analysis of HST biosynthesis genes with multiple paralogs (Ajiro et al. 2010; Izumi et al. 2012; Miyamoto et al. 2008). Plasmid vector pASY-*ACRTS2* was constructed to make double-stranded hairpin *ACRTS2* RNA with sense and antisense sequences of *ACRTS2* (Fig. 4D) and used to transform into wild-type isolate HC1. ACR-toxin production in culture filtrates by 67 transformants was measured with a leaf necrosis assay, and four transformants did not cause any necrosis. These transformants were further examined for loss of ACR-toxin production using TLC and a pathogenicity assay on rough lemon leaves. Thus, two transformants (S-B4 and S-X4) were selected for further analysis. Insertion of pASY-*ACRTS2* in transformant S-B4 was confirmed by genomic PCR amplification with primer sets C14014R8PstI and PacCITR1multisite (antisense region) and PacCITF1multisite and C14014R8HindIII (sense region) (Fig. 4E). The *ACRTS2* transcript was undetectable by RT-PCR followed by Southern blot with the *ACRTS2* probe (Fig. 5A), and no ACR-toxin was detected by TLC (Fig. 5B). Leaves inoculated with wild-type isolate HC1 were covered by necrotic spots, while those inoculated with S-B4 had no symptoms (Fig. 5C). Phenotypes of transformants of S-X4 were also identical to that of S-B4 (data

Fig. 3. Distribution of *ACRTS2* among different pathotypes and nonpathogenic isolates of *Alternaria alternata*. **A**, Southern blot analysis for *ACRTS2* in seven pathotypes, two nonpathogenic isolates, and a dual pathotype of *A. alternata*. Genomic DNA of each fungus was digested with *EcoRV*. Abbreviations used are Tg = tangerine pathotype isolates SH11 and SH20 (lanes 1 and 2), RL = rough lemon pathotype isolates HC1 and HC8 (lanes 3 and 4), JP = Japanese pear pathotype isolates G31 and G90-A2 (lanes 5 and 6), Sb = strawberry pathotype isolates NAF-3 and NAF-8 (lanes 7 and 8), Tm = tomato pathotype isolates 91080804 and AL-4 (lanes 9 and 10), Ap = apple pathotype, isolates AM-1 and O-159 (lanes 11 and 12), Tb = tobacco pathotype, isolates No. 122-2 and AT204 (lanes 13 and 14), No = nonpathogenic isolates O-94 and HC14 (lanes 15 and 16), and OS2A = dual pathotype, isolate BC3-5-1-OS2A (lane 17). Sizes of DNA marker fragments (*HindIII*-digested λ DNA) are indicated at left. **B**, Chromosomal location of *ACRTS2* among various isolates of *A. alternata* pathotypes. Chromosomes of each isolate were separated with pulsed-field gel electrophoresis in a 0.8% agarose gel (left panel). Chromosomes were transferred to a nylon membrane and hybridized with digoxigenin-labeled *ACRTS2* probe (right panel). Abbreviations and isolates used in respective lanes are Tg = tangerine pathotype isolates SH11 and SH20 (lanes 1 and 2), RL = rough lemon pathotype isolates HC1 and HC8 (lanes 3 and 4), JP = Japanese pear pathotype isolates G31 and G90-A2 (lanes 5 and 6), Sb = strawberry pathotype isolates NAF-3 and NAF-8 (lanes 7 and 8), Tm = tomato pathotype isolates 91080804 and AL-4 (lanes 9 and 10), Ap = apple pathotype, isolates AM-1 and O-159 (lanes 11 and 12), OS2A = dual pathotype, isolate BC3-5-1-OS2A (lane 13), and Tb = tobacco pathotype, isolates No. 122-2 and AT204 (lanes 14 and 15), respectively. Chromosome size markers in Mb for *Saccharomyces cerevisiae* are indicated at left. **C**, Southern blot analysis for *ACRTS2* in rough lemon pathotype (Rough Lemon) and nonpathogenic (Nonpatho) strains (left panel), and tangerine pathotype (Tangerine) strains (right panel). Genomic DNA of each strain was digested with *EcoRV*. Isolates in each lane of the left panel from lanes 1 to 10 are HC1, HC7, HC8, BC19, BC27, HC14, BC17, O-94, BC36, and *A. citri*, respectively; and, in right panel, lanes 1 to 11 are SH12, SH13, SH14, SH15, SH18, SH19, SH20, SH21, EV10, EV13, and EV14, respectively. Pathogenicity of each strain on rough lemon young leaves and ACR-toxin production are indicated as (+) or (-) at the bottom of both panels. Sizes of DNA marker fragments (*HindIII*-digested λ DNA) are indicated at left.

not shown). No significant differences in growth rate of cultures (Fig. 5D), sporulation, and spore germination were found between the wild type and the target gene disruptant D-A5 or *ACRTS2* RNA-silenced transformants S-B4.

Fig. 4. Disruption of *ACRTS2* using homologous recombination-mediated gene targeting or RNA silencing of *ACRTS2* in *Alternaria alternata* rough lemon pathotype. **A**, Southern blot analysis of *ACRTS2* hybridized to genomic DNA from *A. alternata* rough lemon pathotype digested with different restriction enzymes. Genomic DNA of wild-type isolate HC1 was digested with *EcoRI* (lane E), *AluI* (lane A), *HindIII* (lane H), *BglII* (lane Bg), or *SacI* (lane S). Digested DNA was transferred to a nylon membrane and probed with a digoxigenin-labeled *ACRTS2* probe. Sizes (kb) of DNA marker fragments (*HindIII*-digested λ DNA) are indicated at left. **B**, Disruption construct pTeasy-*ACRTS2* and tandem integration pattern of the vector in the single-copy disruptant D-A5. **C**, Genomic southern blot of *ACRTS2* in the wild type (WT, isolate HC1) and the single-copy disruptant (D-A5). Genomic DNA was digested with *PstI* and fractionated in agarose gel followed by hybridization with *ACRTS2* probe. Sizes of DNA marker fragments (*HindIII*-digested λ DNA) are indicated at left. **D**, Map of RNA silencing vector construct pASY-*ACRTS2*. **E**, Insertion of vector pASY-*ACRTS2* was confirmed by genomic polymerase chain reaction of *ACRTS2*-silenced transformant S-B4 using primer sets PacCITF1multisite/C14014R8HindIII (amplification of sense region) or PacCITR1multisite/C14014R8PstI (amplification of antisense region) followed by sequencing of both amplicons for confirmation.

DISCUSSION

The long-chain fatty-acid-like structure of ACR-toxin is structurally typical of a polyketide (Fig. 1) (Gardner et al. 1985a

Fig. 5. Detection of *ACRTS2* transcripts, ACR-toxin production, and pathogenicity of *ACRTS2*-disruptant and -silenced transformant of *Alternaria alternata* rough lemon pathotype. **A**, Reverse-transcriptase polymerase chain reaction (RT-PCR) detection of *ACRTS2* transcript abundance in wild-type HC1 (lane WT), single-copy *ACRTS2* disruptant D-A5 (lane D), and RNA-silenced transformant S-B4 (lane S). PCR using the same primer set with genomic DNA as the template (lane G) was also done. RT-PCR or genomic PCR products were hybridized with *ACRTS2* probe after agarose gel fractionation and transfer to membrane. RT+ and RT- indicate RT-PCR with or without addition of reverse transcriptase, respectively. Amplification of the *actin* gene was used as a positive control for RT-PCR and to indicate the use of an equal amount of RNA as the template as described previously (Ajiro et al. 2010; Izumi et al. 2012; Miyamoto et al. 2008, 2009, 2010). Sizes of DNA marker fragments (*HindIII*-digested λ DNA) are indicated at left. **B**, Thin-layer chromatography analysis for detection of ACR-toxin production in extracts from culture filtrates of wild-type HC1 (WT), single-copy *ACRTS2* disruptant D-A5 (D), and RNA-silenced transformant S-B4 (S). **C**, Disease symptoms in rough lemon leaves after inoculation of wild-type HC1 (WT), single-copy *ACRTS2* disruptant D-A5 (D), and RNA-silenced transformant S-B4 (S). **D**, Morphology and growth on PDA plate of wild type (WT), single-copy disruptant D-A5 (D), and RNA-silenced transformant S-B4 (S).

and b; Nakatsuka et al. 1986), and the role in ACR-toxin production and pathogenicity of *ACRTS2* encoding a PKS identified by a draft sequence of the 1.5-Mb chromosome from the rough lemon pathotype of *A. alternata* was examined. Among 33 HST-producing or toxin-nonproducing *A. alternata* isolates examined, only ACR-toxin-producing isolates carried *ACRTS2* in their genome, and the presence of this gene is perfectly correlated with toxin production. Our study of the function of *ACRTS2* in ACR-toxin production using a single-copy disruption of the *ACRTS2* gene by homologous recombination and *ACRTS2* transcript-knockdown by RNA silencing revealed that the PKS encoded by *ACRTS2* is essential for the biosynthesis of the polyketide ACR-toxin. We speculate that *ACRTS2* synthesizes the polyketide backbone of the toxin structure (Fig. 1) and a hydroxylase encoded by *ACRTS1*, which is also required for biosynthesis of ACR-toxin (Izumi et al. 2012), is used to close the ring at the end of the polyketide chain to complete synthesis of the toxin (Fig. 6). Based on the functions of these enzymes encoded by two essential genes *ACRTS1* and *ACRTS2* in ACR-toxin production, we propose a hypothetical biosynthetic pathway for ACR-toxin (Fig. 6). Because *ACRTS2* can be classified as a type I enzyme, which is a single, large, multifunctional polypeptide containing all the necessary enzymatic domains for multiple cycles of condensation and β -keto processing (Hopwood and Khosla 1992; Hopwood and Sherman 1990; Hutchinson and Fujii 1995), a few critical functional domains such as enoyl reductase domain, cyclase domain, and thioesterase domain, which may be required to synthesize the polyketide part of ACR-toxin structure, are likely missing from *ACRTS2*. However, the enoyl reductase domain as well as the β -ketoacyl reductase and dehydratase domains are often not utilized or are simply missing in many PKS, leaving any given β -keto group uncharged or reduced to a alkyl, enoyl, or hy-

droxyl function for the extender unit that follows (Hopwood and Khosla 1992; Hopwood and Sherman 1990; Hutchinson and Fujii 1995). Thus, the additional enzymes that complement the functions of the missing cyclase and thioesterase domains in *ACRTS2* need to be confirmed to completely elucidate the ACR-toxin biosynthetic pathway. Thioesterase function is essential for the release of the carbon chain from the acyl carrier protein, and PKS cannot terminate its enzymatic reaction without this function (Hopwood and Khosla 1992; Hopwood and Sherman 1990; Hutchinson and Fujii 1995), indicating that a gene encoding a thioesterase that works with *ACRTS2* is present elsewhere in the genome of the rough lemon pathotype. The cyclase domain has not yet been extensively studied in any fungal PKS; however, the claisen cyclase domain of the PKS responsible for naphthopyrone synthesis in *Aspergillus nidulans* has yielded interesting observations (Fujii et al. 2001). The C-terminus region of this PKS has a thioesterase domain, as identified in an analysis by the homology of the PKS with the fatty acid synthase of bacterial PKS. When the region consisting of the thioesterase domain was deleted, the mutated enzyme produced isocoumarins that were missing the cyclization of the second ring of naphthopyrone (Fujii et al. 2001). Fujii and associates (2001) discovered that the region initially found to be a thioesterase domain works not only as a thioesterase but also as a claisen cyclase. Based on this information, we searched the draft sequence of the 1.5-Mb chromosome and found no ORF encoding a cyclase but we did find one ORF encoding a putative thioesterase (designated as C14129ORF1) (S. Nikaido, Y. Izumi, and K. Akimitsu, unpublished data). Functional analyses of this ORF are in progress. If the gene product also possesses a dual function, as in the case of the thioesterase domain of naphthopyrone synthase, then the protein encoded by this ORF might be working at once to complement the missing thioesterase as well as the cyclase functions of *ACRTS2* (Fujii et al. 2001).

Two PKS genes, *ACTTS3* from the tangerine pathotype (Miyamoto et al. 2010) and *ACRTS2* from the rough lemon pathotype, have been identified as having functional roles in HST production in citrus-infecting strains of *Alternaria alternata*. Another *A. alternata* PKS, *ALT1* from the tomato pathotype, functionally complemented the biosynthesis of the mycotoxin fumonisin in FUM1-disrupted *Fusarium verticillioides* (Zhu et al. 2008), and it also plays a role in AAL-toxin production (M. Kodama, Tottori University, personal communication). We previously isolated strain BC3-5-1-OS2A, which is pathogenic on both tangerine and rough lemon and produces both ACT-toxin and ACR-toxin (Masunaka et al. 2005). This isolate also carries both small chromosomes with biosynthetic gene clusters for ACT-toxin and ACR-toxin. Because both *ACTTS3* and *ACRTS2* were isolated from specific chromosomes carrying the respective HST biosynthesis gene clusters in the genomes of two citrus pathotypes of *A. alternata*, these genes were compared to examine their evolutionary relationship. Although the domain organization of *ACTTS3* (KS-AT-ME-KR-PP) and *ACRTS2* (KS-AT-ME-DH-KR-PP) are similar, and both belong to type 1 fungal-reducing PKS, sequence similarity over the entire sequence and through the KS domain sequence is low enough to speculate that they have independent evolutionary origin. Phylogenetic analysis of three regions of the *A. alternata* genome (Peever et al. 2004) indicated that BC3-5-1-OS2A has a rough lemon pathotype genetic background, and there is no evidence that this isolate represents an ancestral form from which the rough lemon and tangerine pathotypes have evolved (Masunaka et al. 2005). Therefore, the origin of *ACRTS2* remains to be discovered. *ACRTS2* showed some similarities to other fungal PKS such as *PKS8*

Fig. 6. Proposed biosynthetic pathway for ACR-toxin synthesis using multiple polyketide synthesis functions of *ACRTS2* and a putative hydroxylase function of *ACRTS1*. Abbreviations used in this pathway are: ACP = acyl carrier protein, KS = ketosynthase, AT = acyl transferase, KR = β -ketoacyl reductase, ER = enoyl reductase, MeT = methyl transferase, DH = dehydratase, CYC = cyclase, and TE = thioesterase. These functions are catalyzed by the respective functional domain of *ACRTS2* (solid line) or possibly by separate enzymes (dotted line).

from *B. fuckeliana* (Amselem et al. 2011), *PKS1* from *C. heterostrophus* (Yang et al. 1996), and *mlcB* from *P. citrinum* (Abe et al. 2002); however, the similarities were low (49.7 to 44.5%). *ACRTS2* is unique to the genome of ACR-toxin producers, indicating that it may have an alien origin. *ACTTS3* is also located only in the genome of ACT-toxin producers and, unlike *ACRTS2*, *ACTTS3* does not have an intron with characteristics that are consistent with a possible prokaryotic source and with the phylogenetic tree that nests the KS domain from *ACTTS3* within a clade of bacterial type I PKS genes (Miyamoto et al. 2010). HST-producing *A. alternata* are known to carry dispensable chromosomes of less than 2 Mb, which appear to be missing from nonpathogenic strains, and all HST biosynthetic gene clusters are located in such dispensable small chromosomes (Ajiro et al. 2010; Akamatsu et al. 1997, 2003; Harimoto et al. 2007, 2008; Hatta et al. 2002; Izumi et al. 2012; Masunaka et al. 2000, 2005; Miyamoto et al. 2008, 2009, 2010; Tanaka and Tsuge 2000; Tanaka et al. 1999). Thus, *ACRTS2* and *ACTTS3* are likely pathogenesis-related genes that have been acquired by horizontal transfer like many other pathogenic fungi that acquired genes from distant relatives (Mehrabani et al. 2011; Yang et al. 1996; Walton 1996). Interestingly, both *ACTTS3* and *ACRTS2* are present in the dual pathotype BC3-5-1-OS2A (Miyamoto et al. 2010; this study). However, we hypothesize that one of these PKS genes, a gene in the HST biosynthetic gene cluster on the dispensable chromosome, was transferred horizontally, then rearranged in another isolate of the fungus that carried a different PKS gene for the biosynthesis of its respective HST (Masunaka et al. 2005).

Using a single-copy disruptant produced by targeted gene disruption mediated by homologous recombination and RNA silencing, we proved definitively that *ACRTS2* is required for the biosynthesis of ACR-toxin. The single-copy disruptant D-A5 had reductions in transcript accumulation of *ACRTS2*, ACR-toxin production, and pathogenicity, and the RNA-silencing transformant S-B4 showed no detectable transcription, ACR-toxin production, or pathogenicity. Lack of HST production was consistently associated with a loss of pathogenicity in the toxin producers, including the case of the rough lemon pathotype and *ACRTS1* (Izumi et al. 2012). We conclude that *ACRTS2* is also critical for ACR-toxin biosynthesis and is essential for pathogenicity of the rough lemon pathotype of *A. alternata* and the function of *ACRTS2* because it is responsible for elongating the unique polyketide backbone structure of ACR-toxin, which is the major structure of this toxin.

MATERIALS AND METHODS

Plants and fungal strains.

Detached young rough lemon leaves (midrib length 2 to 4 cm) were grown in the greenhouse and used for toxin sensitivity and pathogenicity assays as described previously (Ajiro et al. 2010; Akimitsu et al. 1989; Masunaka et al. 2000, 2005; Miyamoto et al. 2008, 2009, 2010; Ohtani et al. 2002). Rough lemon is susceptible to the rough lemon pathotype of *A. alternata* and sensitive to ACR-toxin (Kohmoto et al. 1979, 1991). Isolates HC1, HC7, and HC8 and BC19 and BC27 (Peever et al. 1999) are isolates of the rough lemon pathotype, and HC1 was used as the reference isolate for this pathotype (Izumi et al. 2012; Masunaka et al. 2005). Isolate BC3-5-1-OS2A, a dual-pathotype isolate that produces both ACT-toxin and ACR-toxin (Masunaka et al. 2005), was also used as a representative ACR-toxin-producing isolate. Isolates SH11, SH12, SH13, SH14, SH15, SH18, SH19, SH20, and SH21 and EV10, EV13, and EV14 (Peever et al. 1999) are ACT-toxin-producing isolates of the tangerine pathotype (Ajiro et al. 2010; Izumi et al.

2012; Masunaka et al. 2000, 2005; Miyamoto et al. 2008, 2009, 2010; Peever et al. 1999, 2004). Isolates HC14, BC17, BC36, and O-94 were used as nonpathogenic, citrus-associated isolates of *A. alternata* (Ajiro et al. 2010; Izumi et al. 2012; Masunaka et al. 2000, 2005; Miyamoto et al. 2008, 2009, 2010; Peever et al. 1999, 2004), and *Alternaria* black rot pathogen *A. citri* (ATCC58171) was also used as a non-HST-producing and representative nonpathogenic isolate for citrus leaves (Isshiki et al. 2001). Non-citrus-associated isolates of *A. alternata* included 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 (Ito et al. 2004; Miyamoto et al. 2009), all provided by T. Tsuge, Nagoya University, Japan; and O-94, provided by H. Otani, Tottori University. All isolates were stored in 25% (vol/vol) glycerol at -80°C , and cultures for each experiment were grown on V8 juice agar or potato dextrose agar (PDA) plates from stock cultures.

Nucleic acid manipulations.

Genomic DNA and total RNA extractions, genomic PCR and RT-PCR, and Southern blot hybridizations using digoxigenin (DIG)-PCR-labeled probes (Roche, Basel, Switzerland) were carried out as previously described (Ajiro et al. 2010; Isshiki et al. 2001; Izumi et al. 2012; Masunaka et al. 2000, 2005; Miyamoto et al. 2008, 2009, 2010). Restriction enzyme digestion of genomic DNA (5 μg), agarose gel electrophoresis, and capillary transfer were conducted under standard conditions (Sambrook et al. 1989). Transcripts of *ACRTS2* were amplified for detection and examination of transcript abundance, with or without RT (Invitrogen, San Diego, CA, U.S.A.), using RT-PCR and primer sets C14014F10 (5'-TTCTGGAGT CGACGTTGT-3')/C14014R8 (5'-GCTGCTTGGATTGTCA TC-3'), or actinFor (5'-AGATGACACAGATTGTTTTCGAG ACC-3')/actinRev (5'-AGTTGAAAGTGTTGACGTGGATAC C-3') for the actin gene as a control (*act1* of *A. alternata*) (AY748985) (Table 1). RT-PCR using the Thermoscript RT-PCR system (Invitrogen) was performed with 30 cycles of denaturing for 30 s at 94°C , primer annealing for 30 s at 52°C , and extension for 1 min at 72°C using total RNA (5 ng) as the template. RT-PCR products were either subcloned into pGEM-T easy vector (Promega Corp.) and sequenced or transferred to Hybond-N+ membranes (Amersham Biosciences, Piscataway, NJ, U.S.A.) after separation in agarose gels using standard methods (Sambrook et al. 1989) and detected with DIG-labeled PCR amplicons of *ACRTS2* probe using primer pair C14014F10/C14014R8 as described previously (Ajiro et al. 2010; Isshiki et al. 2001; Izumi et al. 2012; Masunaka et al. 2000, 2005; Miyamoto et al. 2008, 2009, 2010). For sequencing of the entire *ACRTS2* transcript, the entire cDNA was amplified separately by RT-PCR with various combinations of primer sets (Table 1), followed by sequencing. Sequencing was performed by the dideoxy chain termination method (Sanger et al. 1977) using an ABI PRISM Dye Termination Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, U.S.A.) and an automated fluorescent DNA sequencer (model 310; Applied Biosystems). For draft sequencing of the 1.5-Mb chromosome from *A. alternata* rough lemon pathotype isolate HC1, the chromosome was separated using PFGE by the methods described below and sequenced with 454 FLX systems (Roche). Approximately 5.8 μg of the 1.5-Mb chromosomal DNA was sonicated, and a 750-bp fragment-rich library was prepared using GS FLX Titanium General Library Preparation Kit (Roche). The 454 sequencing was performed using the GS Titanium Sequencing Kit XLR70 (Roche) and Genome Sequencer FLX system (GS FLX; Roche) and the

manufacturer's standard method. Total leads of 144,832 from sequence data of 56,283,076 bp were assembled with GS de novo Assembler version 2.0 (Roche). The 454 sequencing and assembling were performed at the Dragon Genomic Center (Takara, Mie, Japan). Sequences of respective contigs were analyzed for the presence of ORF by the SoftBerry program and BLAST and FASTA searches at the DNA Data Bank of Japan. PKS domain and motif analyses of were performed using a Pfam search and SMART search, and a phylogenetic tree search using the KS domain sequence was performed with the program Clustal W (Thompson et al. 1994) at the DNA Data Bank of Japan using 1,000 bootstraps and the consensus tree was visualized in TREEVIEW (Page 1996), respectively. *ACRTS2* and *ACTTS3* sequences were compared using Harr plot analysis (Harr et al. 1982) and the Artermis Comparison Tool (Carver et al. 2005).

Plasmid vector constructs and fungal transformation.

The gene-targeting vector pTeasy-*ACRTS2* (Fig. 4B) was constructed using a partial *ACRTS2* sequence amplified by PCR consisting of 30 cycles (denaturing for 30 s at 94°C, primer annealing for 30 s at 52°C, and extension for 1 min at 72°C) with primer set C14014F10/C14014R8 (Table 1) using HC1 genomic DNA as the template. The partial *ACRTS2* region amplified by PCR was subcloned into pGEM-T easy vector (Promega Corp.) and used to construct the targeting vector pTeasy-*ACRTS2* for disruption of *ACRTS2* using homologous recombination. The targeting vector was co-transformed with pSH75 carrying the *hph* cassette as a selection marker (Ajiro et

al. 2010; Isshiki et al. 2001; Izumi et al. 2012; Miyamoto et al. 2008, 2009, 2010). Plasmid vector pASY containing the intron region of AcPacC (accession AB433595) as the head portion of the hairpin structure of double-stranded (ds)RNA was prepared previously (Ajiro et al. 2010; Izumi et al. 2012; Miyamoto et al. 2009) and used as the RNA silencing vector for *ACRTS2* transcripts in *A. alternata* rough lemon pathotype. For construction of pASY-*ACRTS2* RNA-silencing vector (Fig. 4D), sense and antisense inserts of the partial region of *ACRTS2* were amplified by PCR consisting of 30 cycles (denaturing for 30 s at 94°C, primer annealing for 30 s at 56°C, and extension for 1 min at 72°C) using primer set C14014F10EcoRI (5'-GA ATTCTTCTGGAGTCGACGTTGT-3'), containing an *EcoRI* site, and C14014R8HinsIII (5'-AAGCTTGCTGCTTGATTG TCATC-3'), containing a *HindIII* site, or C14014F10EcoRV (5'-GATATCTTCTGGAGTCGACGTTGT-3'), containing an *EcoRV* site, and C14014R8PstI (5'-CTGCAGGCTGCTTGGG TTGTCATC-3'), containing a *PstI* site (Table 1). The PCR product for antisense *ACRTS2* amplified with C14014F10EcoRV and C14014R8PstI (Table 1) digested with *EcoRV* and *PstI* was ligated at the region between the *trpC* promoter and the inserted AcPacC intron region as the head portion of the hairpin structure of dsRNA. The PCR product of sense *ACRTS2* amplified with C14014F10EcoRI and C14014R8HindIII (Table 1) was ligated at the region between the inserted AcPacC region and *trpC* terminator of the pASY containing antisense *ACRTS2* digested with *EcoRI* and *HindIII*. The constructed RNA silencing vector pASY-*ACRTS2* was confirmed by PCR and subsequent sequencing using primer set PacCITF1multi-

Table 1. Sequences of gene-specific primers used in this study

Primer name	Primer sequence (5'-3')	Purpose ^a	Reference ^b
C14014F10	TTCTGGAGTCGACGTTGT	ACRTS2 probe, pTeasy-ACRTS2 construct	This study
C1014R8	GCTGCTTGGATTGTCATC	ACRTS2 probe, pTeasy-ACRTS2 construct	This study
C14014F10EcoRI	GAATTCTTCTGGAGTCGACGTTGT	Sense ACRTS2	This study
C14014R8HindIII	AAGCTTGCTGCTTGGATTGTCATC	Sense ACRTS2	This study
C14014F10EcoRV	GATATCTTCTGGAGTCGACGTTGT	Antisense ACRTS2	This study
C14014R8PstI	CTGCAGGCTGCTTGGATTGTCATC	Antisense ACRTS2	This study
PacCITF1multisite	GAATTCTGCAGATATCAGATCTGTAAGTTG ACCGCAAG	Silencing vector check	Ajiro-Miyamoto
PacCITR1multisite	GAATTCAAGCTTTAAATTCGAAGTCAATC GAGTCAGC	Silencing vector check	Ajiro-Miyamoto
actinFor	AGATGACACAGATTGTTTTCGAGACC	RT-PCR for actin gene	Ajiro-Masunaka-Miyamoto
actinRev	AGTTGAAAGTGGTGACGTGGATACC	RT-PCR for actin gene	Ajiro-Masunaka-Miyamoto
C14014L1-4it	CAGCGCGTATGCGGTTGATATGG	RT-PCR for TS and SP	This study
C14014R1-4it/ C14014R1-1	GATATCTGCGAGGTTCTTCGTACCCG	RT-PCR for TS and SP	This study
C14014L5it	CGAACCTGAAGCCAGCAAGATAAG	RT-PCR for TS and SP and intron check	This study
C14014R5it	CAGTTACCATGTTGGGAACAACGCC	RT-PCR for TS and SP and intron check	This study
C14014L6-7it	CATGCAATGTCGGCGATCGCGA	RT-PCR for TS and SP and intron check	This study
C14014R6-7it	CATATCCACGTCAGAACAACGGG	RT-PCR for TS and SP and intron check	This study
C14014F1-5'	CAGCAGTCTTTGCGTTGCGCT	RT-PCR for TS and SP and 5' end check	This study
C14014R1-5'	CCATATCAACCGCATACGCGC	RT-PCR for TS and SP and 5' end check	This study
C14014F1-3'	CTTCTTGGGCCATCGTCACC	RT-PCR for TS and SP and 3' end check	This study
C14014R1-3'	CTCGCTGCCGCTCACGTTA	RT-PCR for TS and SP and 3' end check	This study
C14014F1-1	CAGCGCGTATGCGGTTGATATGG	RT-PCR for TS and SP	This study
C14014F1-2	CGCCGGCTTCGAGAGAATCAAT	RT-PCR for TS and SP	This study
C14014R1-2	CATGTTGGGAACAACGCCCC	RT-PCR for TS and SP	This study
C14014F1-3	GATGCTGAGATCAGCCAGCC	RT-PCR for TS and SP	This study
C14014R1-3	GCTTTCTCCCTATGAGGGC	RT-PCR for TS and SP	This study
C14014F1-4	CCGCGCGTCACGCATGACAA	RT-PCR for TS and SP	This study
C14014R1-4	CCAGATCTCCGACAGACTCG	RT-PCR for TS and SP	This study
C14014F1-5	CAGCGTCCGTTCTTGACAAT	RT-PCR for TS and SP	This study
C14014R1-5	GAACGAGCAACTGATTGGGC	RT-PCR for TS and SP	This study
C14014F1-6	GGCAAGCAGGACGACAGCGA	RT-PCR for TS and SP	This study
C14014R1-6	CACAACGACGTCTACGCCTC	RT-PCR for TS and SP	This study
C14014F1-7	GGGACAGCTGTGTGTAAG	RT-PCR for TS and SP	This study
C14014R1-7	TGAAGCTCTGCCATTGCGGA	RT-PCR for TS and SP	This study

^a RT-PCR = reverse-transcriptase polymerase chain reaction; TS and SP = transcript check and sequence primer.

^b Ajiro-Miyamoto = Ajiro et al. 2010; Miyamoto et al. 2008, 2009, 2010; and Ajiro-Masunaka-Miyamoto = Ajiro et al. 2010; Masunaka et al. 2000, 2005; Miyamoto et al. 2008, 2009, 2010.

site (5'-GAATTCTGCAGATATCAGATCTGTAAGTTGACC GCCAAG-3') designed from the inserted AcPacC intron region and C14014R8HindIII for sense insertion or PacCITR1multisite (5'-GAATTCAAGCTTTAAATTCGAATCGAG TCAGC-3') and C14014R8PstI for antisense insertion (Table 1). The silencing vector was co-transformed with pSH75 carrying the *hph* cassette as a selection marker, as described previously (Ajiro et al. 2010; Izumi et al. 2012).

Protoplasts of isolate HC1 or its mutants (1×10^7) were transformed with 3 μ g of each vector using methods described previously (Ajiro et al. 2010; Isshiki et al. 2001; Izumi et al. 2012; Masunaka et al. 2000, 2005; Miyamoto et al. 2008, 2009, 2010). Colonies were transferred to PDA plates containing hygromycin B (50 μ g/ml) and grown at 24°C for 5 to 7 days, and single-spore cultures were recovered as described previously (Ajiro et al. 2010; Isshiki et al. 2001; Izumi et al. 2012; Masunaka et al. 2000, 2005; Miyamoto et al. 2008, 2009, 2010).

Detection of plasmid integration, ACR-toxin production, and pathogenicity.

Integration patterns of the transformation vectors for targeted gene disruption were examined by genomic Southern blots with an *ACRTS2* probe and *Pst*I-digested genomic DNA from each of the respective transformants. Hybridization and detection conditions for Southern blots were described previously (Ajiro et al. 2010; Isshiki et al. 2001; Izumi et al. 2012; Masunaka et al. 2000, 2005; Miyamoto et al. 2008, 2009, 2010). Integration of the RNA-silencing vector was determined by genomic PCR using primer set PacCITF1multisite/C14014R8HindIII (amplification of sense region) or PacCITR1multisite/C14014R8PstI (amplification of antisense region) (Table 1). The PCR products using these two primer sets (Table 1) were sequenced to confirm the presence of the inserted vector construct.

ACR-toxin production of 34 independent *ACRTS2* disruption transformants and 67 independent *ACRTS2*-silencing transformants were selected by growing on PDA containing hygromycin B (50 μ g/ml). Toxin initially quantified by placing dilute culture filtrates (10^1 to 10^3 in water) onto wound sites of detached young leaves of rough lemon as previously described (Akimitsu et al. 1989; Izumi et al. 2012; Kohmoto et al. 1979, 1991; Masunaka et al. 2005; Ohtani et al. 2002). Leaves were incubated in a moist chamber at 24°C in the dark and observed for necrosis development after 48 h. Transformants causing less than 50% necrosis compared with wild-type isolate HC1 were scored as mutants with reduced ability to cause necrosis.

Mutants that caused no or significantly reduced necrosis in this assay were further examined by TLC analysis for ACR-toxin production and pathogenicity assay. For TLC analysis of ACR-toxin production in culture filtrates, *A. alternata* strain HC1 and its mutants were grown in 200-ml flasks containing 50 ml of AC-toxin medium (Akimitsu et al. 1989; Izumi et al. 2012; Kohmoto et al. 1979, 1991; Masunaka et al. 2005; Ohtani et al. 2002) for 20 to 25 days at 24°C. The presence of ACR-toxin in the extracts was confirmed by a TLC system employing silica gel 60 F254 TLC plates (Merck, Darmstadt, Germany). Ethyl acetate extracts were obtained using a benzene/acetone/acetic acid solvent system (60:40:1, vol/vol), and ACR-toxin was detected as a purple spot on plates under UV illumination at 254 nm. An authentic preparation of ACR-toxin was prepared from culture filtrates of isolate HC1 as previously described (Akimitsu et al. 1989; Izumi et al. 2012; Kohmoto et al. 1979, 1991; Masunaka et al. 2005; Ohtani et al. 2002).

For the pathogenicity assay, conidia of each isolate were collected from mycelial mats grown in potato dextrose broth for 3 weeks. Conidia were suspended in water at 1×10^5 conidia/ml and used to inoculate the lower surface of detached young leaves (midrib length 3.0 to 4.0 cm) of ACR-toxin-sensitive

citrus cultivar rough lemon as described previously (Izumi et al. 2012; Masunaka et al. 2000, 2005). Leaves were incubated in a moist chamber at 24°C in the dark, and the development of leaf spots and necrosis was monitored after 48 h.

PFGE and hybridization.

Preparation of fungal protoplasts for separation of chromosomes by PFGE and running conditions for PFGE were as described previously (Ajiro et al. 2010; Izumi et al. 2012; Masunaka et al. 2000, 2005; Miyamoto et al. 2008, 2009, 2010). Running conditions (duration, voltage, and linear gradient of switching time) in the PFGE apparatus (CHEF-DRII; BioRad, Richmond, CA, U.S.A.) consisted of 0.5 $\times$ Tris-borate-EDTA at 8°C in 0.8% (wt/vol) agarose gel (Seakem Gold agarose; Cambrex BioScience Rockland, Rockland, ME, U.S.A.) as follows: 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. For transfer to membranes, the gel was soaked in 0.2 N HCl for 20 min to nick chromosomal DNA, then in 0.4 N NaOH plus 1.5 M NaCl for 1 h to transfer to Hybond-N⁺ membranes (Amersham Biosciences) by alkaline capillary transfer 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 *ACRTS2* probes. Hybridization and washes were conducted under high-stringency conditions according to the manufacturer's instructions, as described previously (Ajiro et al. 2010; Izumi et al. 2012; Masunaka et al. 2000, 2005; Miyamoto et al. 2008, 2009, 2010).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Artermis comparison tool WebACT: www.webact.org/WebACT/generate
 DNA Data Bank of Japan (DDBJ) database: www.ddbj.nig.ac.jp
 SMART database: smart.embl-heidelberg.de
 SoftBerry database: linux1.softberry.com/berry.phtml
 Wellcome Trust Sanger Institute Pfam database: pfam.sanger.ac.uk