ACTTS3 Encoding a Polyketide Synthase Is Essential for the Biosynthesis of ACT-Toxin and Pathogenicity in the Tangerine Pathotype of *Alternaria alternata*

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The tangerine pathotype of *Alternaria alternata* produces host-selective ACT-toxin and causes Alternaria brown spot disease of tangerine and tangerine hybrids. Sequence analysis of a genomic BAC clone identified part of the ACT-toxin TOX (*ACTT*) gene cluster, and knockout experiments have implicated several open reading frames (ORF) contained within the cluster in the biosynthesis of ACT-toxin. One of the ORF, designated *ACTTS3*, encoding a putative polyketide synthase, was isolated by rapid amplification of cDNA ends and genomic/reverse transcription-polymerase chain reactions using the specific primers designed from the BAC sequences. The 7,374-bp ORF encodes a polyketide synthase with putative β -ketoacyl synthase, acyltransferase, methyltransferase, β -ketoacyl reductase, and phosphopantetheine attachment site domains. Genomic Southern blots demonstrated that *ACTTS3* is present on the smallest chromosome in the tangerine pathotype of *A. alternata*, and the presence of *ACTTS3* is highly correlated with ACT-toxin production and pathogenicity. Targeted gene disruption of two copies of *ACTTS3* led to a complete loss of ACT-toxin production and pathogenicity. These results indicate that *ACTTS3* is an essential gene for ACT-toxin biosynthesis in the tangerine pathotype of *A. alternata* and is required for pathogenicity of this fungus.

Seven strains of *Alternaria alternata* (Fr.) Keissl. are known to produce host-selective toxins (HST) that are selectively toxic to certain cultivars of plants. HST of *A. alternata* are low molecular weight, secondary metabolites with toxicity toward distinct plant genotypes, and have the same specificity as infection by the toxin-producing pathogens (Nishimura and Kohmoto 1983; Scheffer and Livingston 1984; Walton 1996; Wolpert et al. 2003; Yoder 1980). These HST-producing strains are defined as seven distinct pathotypes of *A. alternata* (Kohmoto

and Otani 1991; Nishimura and Kohmoto 1983) to emphasize the host specificity of each strain. Despite their close phylogenetic relationship (Kusaba and Tsuge 1994; 1995; Peever et al. 2004), each pathotype can be easily distinguished following inoculation on their respective host plants. Only a susceptible host can be damaged and reveal necrosis following inoculation of the pathotype as well as treatment with HST produced by the pathotype. No symptoms are visible on nonhosts following these treatments. The chemical structures of HST from six pathotypes of *A. alternata* have been elucidated (Kohmoto and Otani 1991), and HST biosynthetic genes were recently cloned from the Japanese pear pathotype, strawberry pathotype, tangerine pathotype, tomato pathotype, and apple pathotype of *A. alternata* (Akamatsu et al. 1997, 2003; Tanaka et al. 1999; Johnson et al. 2000; Masunaka et al. 2000, 2005; Tanaka and Tsuge 2000; Hatta et al. 2002; Ito et al. 2004; Ruswandi et al. 2005; Harimoto et al. 2007, 2008; Miyamoto et al. 2008, 2009).

The tangerine pathotype of *A. alternata* causes Alternaria brown spot disease, which affects many tangerine and mandarin cultivars and their hybrids, and the pathogenicity is dependent on the production of ACT-toxin (Kohmoto et al. 1979, 1991; Akimitsu et al. 2003). The structure of ACT-toxin is related to AK- and AF-toxins, which are the HST produced by the Japanese pear and strawberry pathotypes of *A. alternata*, and these toxins share a common 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid (decatrienoic acid) moiety in their structures (Nakashima et al. 1985, 1986; Kohmoto et al. 1993; Akimitsu et al. 2003) (Fig. 1). Homologues of the genes responsible for biosynthesis of the decatrienoic acid moiety were found in genomes from the tangerine, Japanese pear, and strawberry pathotypes (Tanaka et al. 1999; Masunaka et al. 2000, 2005; Tanaka and Tsuge 2000; Ruswandi et al. 2005; Miyamoto et al. 2008, 2009), and several genes in the tangerine pathotype identified and designated as ACT-toxin TOX (*ACTT1*), *ACTT2*, *ACTT3*, *ACTTR*, *ACTT5*, and *ACTT6* (Masunaka et al. 2000, 2005; Miyamoto et al. 2008, 2009).

RNA silencing of multiple copies of *ACTT2* encoding a putative hydrolase in the genome of tangerine pathotype led to a complete loss of *ACTT2* transcripts and ACT-toxin production as well as pathogenicity (Miyamoto et al. 2008). *ACTT5* encoding a putative acyl-CoA synthetase and *ACTT6* encoding a putative enoyl-CoA hydratase were also identified by sequencing of cosmid clone containing open reading frames (ORF) of these genes together with *ACTT2*, *ACTT3*, and *ACTTR* (Miyamoto et al. 2009). Targeted gene disruption of two copies of *ACTT5* significantly reduced ACT-toxin pro-

Nucleotide sequence data for *ACTTS3* is available in the DDBJ/GenBank database under accession number AB516321.

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duction and virulence, and targeted gene disruption of two copies of *ACTT6* led to a complete loss of ACT-toxin production and pathogenicity (Miyamoto et al. 2009).

Using partial regions of a chromosomal DNA sequence from a BAC clone selected by the *ACTTR* probe, we identified several ORF of the tangerine pathotype, isolate SH20, and we report here the distribution and role in ACT-toxin production and pathogenicity of one of these ORF encoding a putative polyketide synthase (PKS) designated as *ACTTS3*.

RESULTS

Sequence identification and characterization of *ACTTS3* in isolate SH20 of the tangerine pathotype of *A. alternata*.

BAC clone 2H2 was sequenced to 5× redundancy by shotgun sequencing using a capillary DNA sequencer. Sequence analysis identified several ORF of known ACTTS genes, including *ACTT1*, *ACTT2*, *ACTT3*, *ACTTR*, *ACTT5*, and *ACTT6*, and additional ORF, including *ACTTS1* encoding a putative nonribosomal peptide synthetase (NRPS), *ACTTS2* encoding a putative enoyl-reductase, *ACTTS4* encoding a putative NRPS, some additional ORF of unknown function, multiple transposon-like sequences, and a putative PKS.

Using the genomic sequence of the ORF encoding the putative PKS in this BAC clone, we designed several pairs of gene-specific primers for reverse-transcription polymerase chain reaction (RT-PCR) and 5' and 3' rapid amplification of cDNA ends (RACE) to identify the entire transcript (Table 1). The PKS gene designated *ACTTS3* (accession no. AB516321) is 7,374 bp in nucleotide length and predicted to encode a protein of 2,457 amino acids. The promoter region of *ACTTS3* has a GATA consensus motif (−50), a TATC motif (−339), potential binding sites for the nitrogen-induced AreA (Marzluf 1993), an ATTG motif (−324) of the light-regulated WC1/ WC2 (Linden and Macino 1997), transcriptional activators, and C/EBP (camp-activated protein) (Rangan et al. 1996) (data not shown). FASTA searches using the sequence of the β-ketoacyl synthase domain indicated that *ACTTS3* had sequence similarity to fungal PKS. *ACTTS3* had a significant match to the 6-methylsalicylic acid synthase gene (6MSAS; X55776) from *Penicillium patulum* (Beck et al. 1990) and pksL2 of *Aspergillus parasiticus* (APU52151) (Feng and Leonard 1995). This latter gene is involved in the biosynthesis of aflatoxin and is classified through phylogenetic analysis as a fungal type I PKS nested within a large clade comprising all bacterial type I PKS (Kroken et al. 2003; Moss et al. 2004) (Fig. 2; Supplementary Fig. 1). Functional domain searches with *ACTTS3* using the Pfam program (Finn et al. 2008) identified five catalytic domains arranged in order from the N terminal β-ketoacyl synthase (KS; 6 to 385), acyltransferase (AT; 549 to 889), methyltransferase (ME; 1,422 to 1,525), β-ketoacyl reductase (KR; 2,089 to 2,265), and phosphopantetheine attachment site (PP; 2,381 to 2,447) (Fig. 2; Supplementary Fig. 2). The organization of PKS functional domains in *ACTTS3* is similar to that of fungal reducing PKS, such as PKS4 and PKS5 of *Botryotinia fuckeliana* (Kroken et al. 2003), PKS16 of *Cochliobolus heterostrophus* (Kroken et al. 2003), and lvB of *A. terreus* (Kennedy et al. 1999) (Fig. 2).

The distribution of *ACTTS3* in the genome of seven pathotypes and nonpathogenic isolates of *Alternaria alternata* was examined by genomic Southern blotting. The *ACTTS3* probe hybridized only to genomes of tangerine pathotype isolates producing ACT-toxin (Fig. 3A). The *ACTTS3* probe hybridized the genomes of tangerine pathotype isolates from Israel, Australia, and Colombia (Fig. 3B), indicating that *ACTTS3* is distributed among all worldwide phylogenetic lineages of the pathogen. Southern blots of pulsed-field gel electrophoresis (PFGE) gels

demonstrated that *ACTTS3* located to the same small chromosome that was previously shown to carry other *ACTT* genes among Florida tangerine pathotype isolates (Masunaka et al. 2005; Miyamoto et al. 2008, 2009) (Fig. 3C).

The distribution of *ACTTS3* copies in the genome of tangerine pathotype isolate SH20 was further examined by genomic Southern blotting using multiple restriction enzymes. The *ACTTS3* probe detected one fragment when genomic DNA was digested with *EcoRV*, *PstI*, *HindIII*, and *BglII*, which have no predicted restriction sites within the region of sequence made for the probe (Fig. 4B). The expected two bands were observed upon digestion with *BanIII*, which has one restriction site predicted within the *ACTTS3* probe sequence. Expected sizes of bands matching with the sequence-based map (Fig. 4B) are shown with examples of a 3.0-kb fragment digested with *HindIII* (Fig. 4A and B) and a 1.2-kb fragment digested with *SacI*, which had one predicted restriction site within the probe sequence (Fig. 4A and B); however, only one band was observed upon digestion with the enzyme, as expected due to the location of the site within the probe region; it was placed at the edge of the sequence and the shorter fragment was not long enough to detect by the probe. *XbaI* also has one site at the edge within the *ACTTS3* probe sequence, similar to the case of *SacI* digestion, and only a single band was observed, as expected.

Targeted gene disruption of *ACTTS3* for functional analysis of ACT-toxin biosynthesis.

To examine the role of *ACTTS3* in ACT-toxin production and pathogenicity, targeting vector *ACTTS3hph* was constructed. The vector contained two partial regions of *ACTTS3*

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 (Nakashima et al. 1985, 1986; Kohmoto et al. 1993).

with an *hph* cassette (Fig. 5A) which was used for *ACTTS3* disruption mediated by homologous recombination. Screening for reduced ACT-toxin production and Southern hybridization identified transformant rep14-2, which showed a reduction in the toxicity in culture filtrates, and a shift in the 10-kb *ACTTS3* band present in the wild type to a 13.0-kb band in the transformant, indicating insertion of the vector construct at the target site (Fig. 5A and C). The *hph* probe hybridized only to the 13.0-kb-shifted band of transformant rep14-2 (data not shown) and the 10-kb band shown in the wild type still remained in rep14-2 (Fig. 5C), suggesting that *ACTTS3* exists in more than one copy in the genome of SH20 and the vector construct was inserted in only one of the multiple copies. Transcription analysis of *ACTTS3* by RT-PCR with the primer set of PKS1F3 and PKS1R3 generated an amplicon of predicted size (966 bp) from total RNAs of both the wild type and transformant rep14-2, and the product hybridized to the *ACTTS3* probe (Fig. 6A). Intensity of the band was reduced in the transformant rep14-2 compared with that of the wild type (Fig. 6A), and the PCR reaction without addition of reverse transcriptase did not amplify any product. The actin gene, which is known to be expressed constitutively, was amplified equally to that observed for the wild type (Fig. 6A). ACT-toxin production in culture filtrates seeded with either the wild type or transformant rep14-2 were compared by high-performance liquid chromatography (HPLC). A 61% reduction in ACT-toxin peak area was observed in transformant rep14-2 (Fig. 6B). When spores of transformant rep14-2 were spray inoculated on the lower leaf surfaces of susceptible cv. Iyokan, typical leaf spots appeared but disease severity was reduced significantly compared with inoculation with the wild type (Fig. 6C).

To disrupt the second copy of *ACTTS3*, the PCR fragment corresponding to a partial region of *ACTTS3* was amplified using the primer set PKS1F12NotI and PKS1R11BamHI and subcloned into the *NotI* and *BamHI* sites of transformation

Fig. 2. Organization of catalytic domains in *ACTTS3* and several fungal polyketide synthase (PKS) genes. Comparison of domain organization of *ACTTS3* with **A**, fungal 6MSA and **B**, fungal reducing PKS based on a genealogy of the β -ketoacyl synthase (KS) domain by Kroken and associates (2003). Catalytic domains: AT = acyltransferase, ME = methyl-transferase, KR = β -ketoreductase, PP = peptidyl carrier domain, C = condensation, and A = AMP-binding. Accession numbers for these PKS are X55776 for 6MSAS (*Penicillium patulum*), AY495609 for PKS4 (*Botryotinia fuckeliana*), AY495657 for PKS16 (*Cochliobolus heterostrophus*), AF151722 for lovB (*Aspergillus terreus*), and AY495610 for PKS5 (*Botryotinia fuckeliana*).

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

Primer name	Primer sequences (5'–3')	Purpose ^a	Reference
ACTTRF1	CAACAGCAGTCGAACC	ACTTR probe	Miyamoto et al. 2008
ACTTRR1	CGTCTAGAACGAATCG	ACTTR probe	Miyamoto et al. 2008
PKS1F3	GCTGACGAGACTGTTG	RT-PCR for <i>ACTTS3</i>	This study
PKS1R3	CTGTGTTTCCCCACAG	RT-PCR for <i>ACTTS3</i>	This study
actinFor	AGATGACACAGATTGTTTTCGAGACC	RT-PCR for actin gene	Miyamoto et al. 2009
actinRev	AGTTGAAAGTGGTGACGTGGATACC	RT-PCR for actin gene	Miyamoto et al. 2009
PKS1F1	TGGATCCTGCTCAACG	RT-PCR for <i>ACTTS3</i>	This study
PKS1R1	ATTGGCGGGTATGGTG	RT-PCR for <i>ACTTS3</i>	This study
2H2C27R1	GATGTGCAGGCCTAGCA	RT-PCR for <i>ACTTS3</i>	This study
2H2C27F1	AGATGCAGTCCGACCG	RT-PCR for <i>ACTTS3</i>	This study
PKS1F2	TGCGCCGAACCTTATC	RT-PCR for <i>ACTTS3</i>	This study
PKS1R2	TGGCGGCAACTTCACA	RT-PCR for <i>ACTTS3</i>	This study
C27Fe2	CAACGTGCGTGTA	RT-PCR for <i>ACTTS3</i>	This study
C27Rm2	TTCGAGCTCAACATCCAGGT	RT-PCR for <i>ACTTS3</i>	This study
C26Rm	GCTTTGTATCGTATGCC	RT-PCR for <i>ACTTS3</i>	This study
2H2C26F1	TTAGTCAGGCGGTCTGA	RT-PCR for <i>ACTTS3</i>	This study
2H2C26R1	TGCAGCAAGTCGGACT	RT-PCR for <i>ACTTS3</i>	This study
PKS1F4	GACACCGCAATCATG	RT-PCR for <i>ACTTS3</i>	This study
PKS1R4	CTGGCAACGTACCCAA	RT-PCR for <i>ACTTS3</i>	This study
PKS1R6	CGCGCATCTGCAGATCCCAATCGGA	5' RACE for <i>ACTTS3</i>	This study
PKS1R5	CAACAAACACAGACGTGTGGGTACCCTGC	5' RACE for <i>ACTTS3</i>	This study
PKS1F5	GGTGGTCAATCCAATTACCACG	3' RACE for <i>ACTTS3</i>	This study
PKS1F6	CGTGTCTCGAGGTCTTGCTGCATCGGTCA	3' RACE for <i>ACTTS3</i>	This study
PKS1F10HindIII	CCCAAGCTTGGGTGTGAAGTTGCCGCCA	<i>ACTTS3</i> hph construct	This study
PKS1R3EcoRV	GATATCCTGTGTTTCCCCACAG	<i>ACTTS3</i> hph construct	This study
PKS1F11BamHI	CGGGATCCCGTGTGGTTGACCAAGGG	<i>ACTTS3</i> hph construct	This study
PKS1R10KpnI	GGGGTACCCCGACATTTGCGGTGCGCA	<i>ACTTS3</i> hph construct	This study
PKS1F12NotI	GCGGCCGCCAGACTGTCTGGGA	<i>ACTTS3</i> NPTII construct	This study
PKS1R11BamHI	CGGGATCCCGCAAGTAGCCACCAGGT	<i>ACTTS3</i> NPTII construct	This study
NPTIIF1EcoRI	GGAATTCCATGATTGAACAAGATGGATTGC	<i>ACTTS3</i> NPTII construct	This study
NPTIIR1EcoRI	GGAATTCCTCAGAAGAACTCGTCAAGAA	<i>ACTTS3</i> NPTII construct	This study

^a RACE = rapid amplification of cDNA ends and RT-PCR = reverse-transcription polymerase chain reaction.

vector pAGY carrying the *NPTII* cassette to generate ACTTS3NPTII (Fig. 5B). The single-copy disruptant rep14-2 was retransformed with the ACTTS3NPTII vector, and the double mutants which showed resistance to both hygromycin and geneticin were selected. Culture filtrates from one of the transformants (D1-2) did not show any toxicity to *Iyokan*. Integration of ACTTS3NPTII at the target site was analyzed by Southern blotting and transformant D1-2 showed the expected bands of 13.0 and 20.0 kb, whereas the native 10.0-kb band was observed in the wild type (Fig. 5C). No *ACTTS3* transcript was detected by RT-PCR in total RNA from transformant D1-2 (Fig. 6A). HPLC analysis also did not detect any trace of ACT-toxin in the culture filtrates of transformant D1-2 (Fig. 6B), and inoculation of spores from transformant D1-2 on the *Iyokan* leaves were nonpathogenic (Fig. 6C). No apparent differences in growth (Fig. 6D), sporulation, and spore germination in vitro were detected among the wild type, single-

copy disruptant rep14-2, and double-copy disruptant D1-2 (data not shown).

DISCUSSION

Several ORF responsible for ACT-toxin biosynthesis have been identified in the tangerine pathotype of *A. alternata*, and these ORF are clustered on a 1.9-Mb chromosome in reference isolate SH20 (Masunaka et al. 2000, 2005; Miyamoto et al. 2008, 2009). In the current study, we have identified a 7,374-bp ORF encoding a putative PKS and named it *ACTTS3*. We determined the distribution of *ACTTS3* among different pathogenic and nonpathogenic isolates of *A. alternata* and provided biochemical and molecular genetic evidence for the involvement of *ACTTS3* in ACT-toxin production and pathogenicity.

FASTA searches using the KS domain sequence of *ACTTS3* revealed that *ACTTS3* belongs to the fungal type I PKS family

Fig. 3. Distribution of *ACTTS3* in the genomes of different pathogenic and nonpathogenic isolates of *Alternaria alternata*. **A**, Genomic Southern blot analysis of *ACTTS3* hybridizing to seven distinct host-selective toxin-producing pathotypes and non-pathogenic strains of *A. alternata*. Abbreviations used are: Tg, tangerine pathotype, isolates SH11 (lane 1) and SH20 (lane 2); RL, rough lemon pathotype, isolates HC1 (lane 1) and HC8 (lane 2); JP, Japanese pear pathotype, isolates G31(1) and G90-A2 (2); Sb, strawberry pathotype, isolates NAF-3 (lane 1) and NAF-8 (lane 2); Tm, tomato pathotype, isolates 91080804 (lane 1) and AL-4 (lane 2); Ap, apple pathotype, isolates AM-1 (1) and O-159 (2); Tb, Tobacco pathotype, isolates No. 122-2 (lane 1) and AT204 (lane 2); and No, nonpathogenic isolates O-94 (lane 1) and HC14 (lane 2). **B**, Genomic Southern blot analysis of the distribution of *ACTTS3* among a worldwide sample of tangerine pathotype isolates of *A. alternata*. Genomic DNA was digested with *EcoRV*. Isolates used were 45-001 (lane 1), 45-003 (lane 2), and 45-013 (lane 3) from Israel; Aus-1 (lane 4) and Aus-9 (lane 5) from Australia; and 45-014 (lane 6) and 11-CEN-S (lane 7) from Colombia. Sizes of marker DNA fragments (*HindIII*-digested λ DNA) are indicated at left in both panels. **C**, Chromosomal location of *ACTTS3* among tangerine pathotype isolates from citrus in Florida. Chromosomal DNAs of each isolate were separated using pulsed-field gel electrophoresis in 0.8% agarose gel (left panel). Chromosomes were transferred to nylon membrane and detected with digoxigenin-labeled *ACTTS3* probe (right panel). *Hansenula wingei* chromosome size markers are indicated at left. Isolates examined were from two *A. alternata* populations separated by approximately 20 km (SH = Shinn Grove and EV = Evenhouse Rd.) (Peever et al. 1999) in order from left to right: SH11, SH12, SH13, SH14, SH15, SH18, SH19, SH20, SH21, EV10, EV13, and EV14. Pathogenicity to *Iyokan* leaves (Patho) and ACT-toxin production (Toxin) are indicated at bottom of figure.

(Moss et al. 2004). This family includes the 6MSAS gene of *P. patulum* (Beck et al. 1990) which was nested within a large clade comprising all bacterial type I PKS genes (Kroken et al. 2003). Classifying PKS genes by domain organization in addition to sequence similarity within domains is a more effective way to categorize PKS genes because genes encoding fungal PKS generally consist of large, multifunctional ORF. Topologies of phylogenies estimated from different regions of these ORF can be incongruent (Kroken et al. 2003; Moss et al. 2004). Indeed, the organization of PKS functional domains in *ACTTS3* consisting of KS-AT-ME-KR-PP suggests that it may be more similar to the organization of fungal reducing PKS, which have lost the DH domain but gained the ME domain. An example of this is PKS16 of *C. heterostrophus* (Kroken et al. 2003), which synthesizes a precursor of T-toxin, one of the HST produced by *Cochliobolus* spp. (Yang et al. 1996). These reduced polyketides are frequently synthesized from CoA thioesterified carboxylic acids rather than acetyl and malonyl CoA, and the extent of keto-group processing varies from one condensation cycle to the next (Hopwood 1997). In addition, one or more sites may be methylated by a ME domain (Abe et al. 2002), although many of the predicted PKS in the PKS clade that produces reduced PKS have become highly divergent and some contain a presumably nonfunctional ME domain (Kroken et al. 2003).

Genes required for the synthesis of *Alternaria* spp. HST, such as the *AK-toxin TOX* (*AKT*) cluster controlling AK-toxin pro-

duction (Tanaka et al. 1999) and the *AF-toxin TOX* (*AFT*) cluster controlling AF-toxin production (Hatta et al. 2002), are tightly clustered. Genes responsible for ACT-toxin production (*ACTT1*, *ACTT2*, *ACTT3*, *ACTTR*, *ACTT5*, and *ACTT6* and *ACTTS1*, *ACTTS2*, *ACTTS3*, and *ACTTS4*) are also clustered, and this cluster has been designated as the *ACTT*. These genes are located on a single chromosome of 1.9 to 2.0 Mb in ACT-toxin-producing isolates from Florida (Masunaka et al. 2000, 2005; Miyamoto et al. 2008, 2009). Generally, only *A. alternata* strains producing HST are known to carry chromosomes of less than 2.0 Mb and these chromosomes appear to be completely absent from nonpathogenic strains (Akamatsu et al. 1999; Masunaka et al. 2000, 2005; Hatta et al. 2002). The small chromosomes carrying toxin genes do not appear to carry genes essential for normal growth and reproduction. Loss of these chromosomes has no obvious phenotypic effect on in vitro growth, resulting in these chromosomes being called conditionally dispensable (Covert 1998; VanEtten et al. 1998; Hatta et al. 2002). Interestingly, gene clusters responsible for the biosynthesis of HST, such as the *AKT*, *AFT*, *AMT*, *ALLT*, and *ACTT* clusters in the Japanese pear, strawberry, apple, tomato, and tangerine pathotypes, respectively, are all located on small, dispensable chromosomes (Akamatsu et al. 1997, 2003; Tanaka et al. 1999; Masunaka et al. 2000, 2005; Tanaka and Tsuge 2000; Harimoto et al. 2007, 2008; Miyamoto et al. 2008, 2009). The *AKT*, *AFT*, and *ACTT* clusters share homologous coding and noncoding regions, some of which are responsible for the production of the common decatienic acid moiety in the structures of AK-, AF-, and ACT-toxins (Tanaka et al. 1999; Masunaka et al. 2000; Tanaka and Tsuge 2000; Hatta et al. 2002; Ito et al. 2004; Ruswandi et al. 2005; Miyamoto et al. 2008, 2009).

Fig. 4. Estimation of copy number of *ACTTS3* in the genome of the tangerine pathotype of *Alternaria alternata*. **A**, Genomic Southern blot analysis of *ACTTS3* in isolate SH20 of the tangerine pathotype of *A. alternata* digested with several different restriction enzymes. DNA of isolate SH20 was digested with *EcoRV* (lane E), *PstI* (lane P), *SacI* (lane S), *HindIII* (lane H), *XbaI* (lane Xb), *BanIII* (lane Bn), and *BglII* (lane Bg), and the digested DNA was transferred to nylon membrane probed with a digoxigenin-labeled *ACTTS3* probe. Sizes of marker DNA fragments (*HindIII*-digested λ DNA) are indicated at left. **B**, Restriction and primer annealing site map of *ACTTS3* open reading frame and flanking regions. Abbreviations H and S indicate *HindIII* and *SacI* restriction sites, respectively. Arrows indicate location of oligonucleotide primers used in polymerase chain reaction. Scale bar indicates 1 kb.

Fig. 5. Targeted gene disruption of *ACTTS3* in the tangerine pathotype of *Alternaria alternata*. **A**, Disruption construct *ACTTS3hph* and predicted integration pattern in single-copy disruptant rep14-2 of tangerine pathotype isolate SH20. N, K, and B indicate *NotI*, *KpnI*, and *BglII* restriction sites, respectively. **B**, Disruption construct *ACTTS3NPTII* and predicted integration pattern in double-copy disruptant D1-2. B indicates a *BglII* restriction site. **C**, Genomic Southern blot of *ACTTS3* hybridizing to the wild type (lane W), single-copy disruptant rep14-2 (1st), and double-copy disruptant D1-2 (2nd). Genomic DNA was digested with *BglII* and fractionated in 0.8% agarose following hybridization with digoxigenin-labeled *ACTTS3* probe. Sizes of marker DNA fragments (*HindIII*-digested λ DNA) are indicated at left.

ACTTS3 only hybridized to tangerine pathotype genomes and not to the genomes of seven noncitrus pathotypes and non-pathogenic isolates of *A. alternata*, indicating that *ACTTS3* is unique to the tangerine pathotype. *ACTTS3* was found in tangerine pathotype isolates from Australia, Israel, and Colombia but did not hybridize to the genomes of the Japanese pear pathotype or strawberry pathotype isolates. This suggests that *ACTTS3* is not required for the biosynthesis of decatrienoic acid, a common moiety in ACT-, AK-, and AF-toxins, but, rather, is required for synthesis of some unique component of ACT-toxin. ACT-toxin consists of three parts, including decatrienoic acid, valine (amino acid), and a polyketide moiety (Fig. 1). Because the 2,457 amino acid sequence of *ACTTS3* shows a strong sequence similarity to a PKS, *ACTTS3* is predicted to be responsible for the biosynthesis of the polyketide moiety of ACT-toxin. *ACTTS3* was found only on the smallest chromosomes in the genomes (1.9 to 2.0 Mb) of all tangerine pathotype isolates obtained in Florida citrus fields, and the presence of this gene is correlated with ACT-toxin production and pathogenicity to Iyokan tangerine.

To definitively test the hypothesis that *ACTTS3* is required for biosynthesis of the polyketide moiety of ACT-toxin, we generated single- and double-copy disruptants of *ACTTS3* using gene replacement and targeted gene disruption mediated by homologous recombination. The single-copy mutant rep14-2 showed reductions in transcript accumulation of *ACTTS3*, ACT-toxin production, and pathogenicity, and the double-copy disruptant D1-2 showed a complete loss of *ACTTS3* transcription as well as ACT-toxin production and pathogenicity. Genomic Southern blots of the double-copy mutant probed with the *ACTTS3* probe revealed a band of equal size to that observed in the wild type, which might indicate additional copies of *ACTTS3* in the genome of D1-2. However, because there was no trace of *ACTTS3* transcript and ACT-toxin production detected in the double-copy disruptant D1-2, the band likely represents a pseudogene with defects in the promoter region, resulting in transcriptional dysfunction. Lack of ACT-toxin production was consistently associated with a loss of pathogenicity which has been consistently demonstrated in several independent studies of HST (Akamatsu et al. 1997, 2003; Tanaka et al. 1999; Johnson et al. 2000; Masunaka et al. 2000, 2005; Tanaka and Tsuge 2000; Hatta et al. 2002; Ito et al. 2004; Ruswandi et al. 2005; Harimoto et al. 2007, 2008; Miyamoto et al. 2008, 2009). We conclude that *ACTTS3* is critically required for ACT-toxin biosynthesis and is essential for pathogenicity of the tangerine pathotype of *A. alternata*. We speculate that the function of *ACTTS3* is to elongate the polyketide chain portion of ACT-toxin that is unique to this toxin.

MATERIALS AND METHODS

Fungal strains and plants.

Young leaves (midrib length: 2 to 4 cm) were obtained from the citrus cv. Iyokan tanger (*Citrus iyo* hort. Ex Tanaka) grown in the greenhouse and used for inoculation and toxin sensitivity assays as described previously (Masunaka et al. 2000; Miyamoto et al. 2008, 2009). Iyokan is susceptible to the tangerine pathotype of *A. alternata* and sensitive to ACT-toxin (Kohmoto et al. 1991). Isolates of *A. alternata* tangerine pathotype were previously sampled from leaf lesions of Minneola tangelo (*C. reticulata* Blanco × *C. paradisi* Macf.) in Florida for studies of the population structure and the mechanism of host specificity of the *Alternaria* brown spot pathogen (Peever et al. 1999; Masunaka et al. 2000, 2005; Miyamoto et al. 2008, 2009) and listed in Supplementary Table S1. Isolates SH11, SH12, SH13, SH14, SH15, SH18, SH19, SH20, and

SH21 and EV10, EV13, and EV14 of the tangerine pathotype and isolates HC1 and HC8 of the rough lemon pathotype were isolated from leaf lesions on both hosts in central Florida (Peever et al. 1999; Masunaka et al. 2000, 2005). Isolate SH20 has been used as the reference isolate for the brown spot pathogen in several previous studies (Masunaka et al. 2000, 2005; Miyamoto et al. 2008, 2009). Isolate BC3-5-1-OS2A, a dual pathotype isolate producing both ACT-toxin and ACR-toxin (Masunaka et al. 2005), was isolated from a rough lemon leaf lesion in central Florida. Isolates 45-001, 45-003, and 45-013 from Israel; Aus-1 and 9 from Australia; and 45-014 and 11-CEN-S from Colombia were also used (Peever et al. 1999). Laboratory stocks (Ito et al. 2004; Miyamoto et al. 2009) 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 122-2 and AT-204 were included for comparative purposes. Nonpathogenic isolates HC14 from rough lemon (Peever et al. 1999; Masunaka et al. 2000) and *A. alternata* isolate O-94, obtained from H. Otani, Tottori University, Japan, were included as nonpathogenic controls. All isolates, including transformants of isolate SH20, were stored in 25% (vol/vol) glycerol at -85°C and cultures

Fig. 6. ACT-toxin production and pathogenicity of *ACTTS3* disruptants. **A**, reverse-transcription polymerase chain reaction (RT-PCR) and Southern blot analysis of *ACTTS3* transcription in disruptants. Top panel: RT-PCR products of *ACTTS3* from total RNA of the wild type (lane W), single-copy disruptant rep14-2 (1st), and double-copy disruptant D1-2 (2nd) hybridized with digoxigenin-labeled *ACTTS3* probe. Middle panel: RT- indicates negative controls without addition of reverse transcriptase. Lower panel: Amplification of actin gene was used as positive control for RT-PCR system as well as to test RNA loading. **B**, High-performance liquid chromatography charts of each extract from culture filtrates of *ACTTS3* disruptants. Arrows indicate peaks for ACT-toxin for the wild type (lane W), single-copy disruptant rep14-2 (1st), and double-copy disruptant D1-2 (2nd). **C**, Symptoms caused by inoculation of Iyokan leaves with the wild type (lane W), single-copy disruptant rep14-2 (1st), or double-copy disruptant D1-2 (2nd) 48 h after inoculation. **D**, Morphology in culture on potato dextrose agar of the wild type (lane W), single-copy disruptant rep14-2 (1st), and double-copy disruptant D1-2 (2nd).

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 PCR, RT-PCR, RACE, and Southern blot hybridization using digoxigenin (DIG)-PCR-labeled probes (Roche, Basel, Switzerland) were carried out as previously described (Isshiki et al. 2001; Masunaka et al. 2000, 2005; Katoh et al. 2006, 2007; Miyamoto et al. 2008, 2009). Restriction enzyme digestion of genomic DNA (5 µg), agarose gel electrophoresis, and capillary transfer were conducted under standard conditions (Sambrook and Russell 2001). A BAC library was constructed using *A. alternata* isolate BC3-5-1-OS2A as described previously (Masunaka et al. 2005; Hatta et al. 2006). Genomic DNA fragments of approximately 100 kb from BC-3-5-1-OS2A were ligated into the *Bam*HI site of pcc1 BACTM vector (Epicentre, Madison, WI, U.S.A.) (Masunaka et al. 2005; Miyamoto et al. 2008). The ligated vector was introduced into *Escherichia coli* (DH10B) (Invitrogen, San Diego, CA, U.S.A.) by electroporation. After checking the titer of the transformation, colonies screened by blue-white selection were randomly picked into 384-well plates (total of 9 plates) as a glycerol stock of the library. The genomic BAC library was screened by colony hybridization with a DIG-PCR-labeled *ACTT*R probe using the primer set ACTTFR1 (5'-CAACAGCAGTCGAACC-3') and ACTTRR1 (5'-CGTCTAGAACGAATCG-3'), which is the same probe used previously for the identification of the cosmid clone pcCL4-1 (AB176941) (Miyamoto et al. 2008). After colony hybridization, BAC DNA was extracted from the positive clones as described previously (Sambrook and Russell 2001; Hatta et al. 2006). The insert of BAC clone 2H2 was sequenced to 5× redundancy by shotgun sequencing using a capillary DNA sequencer. Transcripts of *ACTT*S3 were amplified with or without addition of reverse transcriptase (Takara, Shiga, Japan) by RT-PCR using the primer sets PKS1F3 (5'-GCTGACGAGACTGTTG-3') and PKS1R3 (5'-CTGTGTTTCCCCACAG-3') for transcript amplification of *ACTT*S3, and actinFor (5'-AGATGACACAGATTGTTTTCGAGACC-3') and actinRev (5'-AGTTGAAAGTGGTGACGTGGATACC-3') for actin gene (*act1* of *A. alternata*) (AY748985). RT-PCR products were transferred to Hybond-N+ membrane using standard methods (Sambrook and Russell 2001) and detected with a DIG-PCR-labeled *ACTT*S3 probe prepared using the same primer sets used for RT-PCR with genomic DNA of SH20 as the template by the methods described previously (Isshiki et al. 2001; Masunaka et al. 2000, 2005; Katoh et al. 2006, 2007; Miyamoto et al. 2008, 2009). The region containing the entire ORF of *ACTT*S3 was amplified from DNA and cDNA of SH20 by PCR consisting of 30 cycles (denaturing for 1 min at 95°C, primer annealing for 1 min, and extension for 1 min per 1 kb of amplicon at 72°C) using multiple primer sets of PKS1F1 (5'-TGGATCCTGCTCAACG-3') and PKS1R1 (5'-ATTGGCGGGTATGGTG-3'), 2H2C27R1 (5'-GATGTGCAGGCCTAGCA-3') and 2H2C27F1 (5'-AGATGCAGTCCGACCG-3'), PKS1F2 (5'-TGCGCCGAACCTTATC-3') and PKS1R2 (5'-TGGCGGCAACTTCAACA-3'), PKS1F3 (5'-GCTGACGAGACTGTTG-3') and C27Fe2 (5'-CAACGTGCGTGTA-3'), C27Rm2 (5'-TTCGAGCTCAACATCCAGGT-3') and C26Rm (5'-GCTTTGTATC GTATGCC-3'), 2H2C26F1 (5'-TTAGTCAGGCGGTCTGA-3') and 2H2C26R1 (5'-TGCAGCAAGTCGGACT-3'), and PKS1F4 (5'-GACACCGCGAATCATG-3') and PKS1R4 (5'-CTGGCAACGTACCCAA-3'). The transcription initiation site was identified with PKS1R6 (5'-CGCGCATCTGCAGATCCCACC AATCGGA-3') and GeneRacerTM 5' Primer, and PKS1R5 (5'-CAACAAACACAGACGTGTGGGTACCCCTGC-3') and GeneRacerTM 5' Nested Primer for nested PCR by 5' RACE with

the GeneRacer kit (Invitrogen) according to the manufacturer's instructions. The transcription termination site was identified with PKS1F5 (5'-GGTGGTCAATCCAATTACCACG-3') and GeneRacerTM 3' Primer, and PKS1F6 (5'-CGTGCTCGAGGTCTTGCTGCATCGGTCA-3') and GeneRacerTM 3' Nested Primer for nested PCR by 3' RACE. Amplicons were subcloned into pT7Blue-2 vector (Novagen, Madison, WI, U.S.A.) following the manufacturer's instructions. 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). Sequence identities were estimated using FASTA searches at the DNA Databank of Japan, and domain organizations were estimated using Pfam program (Finn et al. 2008).

PFGE and hybridization.

Preparation of chromosomes from fungal protoplasts and PFGE was carried out according to methods described previously (Hatta et al. 2002; Masunaka et al. 2005; Miyamoto et al. 2008, 2009). Running conditions (duration, voltage, and linear gradient of switching time) in a PFGE apparatus (CHEF-DRII; Bio-Rad, Richmond, CA, U.S.A.) consisted of 0.5× Tris-borate-EDTA (Sambrook and Russell 2001) at 8°C in 0.8% agarose gel (Seakem Gold agarose; BioWhittaker Molecular Applications) for 25 h at 1.6 V/cm/500 s, 30 h at 1.6 V/cm/1,000 s, 50 h at 1.6 V/cm/1,500 s, and 32 h at 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) and 0.4 N NaOH plus 1.5 M NaCl for 1 h to transfer Hybond-N+ membrane (Amersham Biosciences, Piscataway, NJ, U.S.A.) by alkaline capillary transfer (Sambrook and Russell 2001) 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 *ACTT*S3 probes. Hybridization and washes were conducted under high-stringency conditions according to the manufacturer's instructions as described previously (Masunaka et al. 2005).

Plasmid vector constructs and fungal transformation.

The gene replacement vector *ACTT*S3hph was constructed using two partial regions of *ACTT*S3 sequences amplified by PCR with the primer set of PKS1F10HindIII (5'-CCCAAGCTTGGGTGTGAAGTTGCCGCA-3') and PKS1R3EcoRV (5'-GATATCCTGTGTTTCCCCACAG-3'), and PKS1F11BamHI (5'-CGGGATCCCGTGTGGTTGACCAAGGG-3') and PKS1R10KpnI (5'-GGGGTACCCCGACATTTGCGGTCGCA-3'), containing *Hind*III, *Eco*RV, *Bam*HI, and *Kpn*I sites at the ends of their respective PCR-amplified sequences, using genomic DNA of SH20 as the template. Partial *ACTT*S3 sequences amplified by PCR were subcloned into the subcloning vector pT7Blue-2 (Novagen). An *hph* cassette (Cullen et al. 1987) from pSH75 (Kimura and Tsuge 1993) was inserted into pT7Blue-2 vector at *Eco*RV and *Bam*HI sites and the constructed vector was named pAHY. The partial *ACTT*S3 sequence (PCR product using PKS1F11BamHI and PKS1R10KpnI) in pT7Blue-2 vector was subcloned into the pAHY at *Bam*HI and *Kpn*I. The pT7Blue-2 vectors containing the *hph* cassette and the partial *ACTT*S3 sequence were digested with *Hind*III and *Eco*RV, and were ligated with another partial *ACTT*S3 sequence (PCR product using PKS1F10HindIII and PKS1R3EcoRV) digested with the same restriction enzymes to produce the gene replacement vector named *ACTT*S3hph. The vector was digested with *Not*I and *Kpn*I and linearized before transformation to isolate SH20.

For the second disruption of *ACTTS3*, *ACTTS3NPTII* was constructed using a partial sequence of *ACTTS3* amplified by PCR with the primer set PKS1F12NotI (5'-GCGGCCGCCCA GACTGTCTGGGA-3') and PKS1R11BamHI (5'-CGGGATC CCGCAAGTAGCCACCAGGT-3') containing *NotI* and *BamHI* sites at the end of the PCR product using genomic DNA of SH20 as the template. Partial *ACTTS3* sequences amplified by PCR were first subcloned into the subcloning vector pT7Blue-2 (Novagen). The geneticin resistance gene *NPTII* was amplified with the primer set of NPTIIF1EcoRI (5'-GGAATTCCAT GATTGAACAAGATGGATTGC-3') and NPTIIR1EcoRI (5'-GGAATTCCTCAGAAGAACTCGTCAAGAA-3') containing *EcoRI* sites at the ends using pII99 vector (Namiki et al. 2001) as the template, and the product was subcloned in the *Alternaria* overexpression vector pAOEIK (Katoh et al. 2007) at the *EcoRI* site of pAOEIK, at the region between the promoter and terminator sequences of *Aspergillus nidulans trpC*, and the vector was named pAGY. The partial *ACTTS3* sequence subcloned in the pT7Blue-2 vector was then digested with *NotI* and *BamHI*, and subcloned into the pAGY at the same restriction sites to complete the construction of *ACTTS3NPTII* vector. These vector constructs of *ACTTS3hph* and *ACTTS3NPTII* were confirmed by sequencing.

Protoplast preparation and transformation of isolate SH20 were carried out using methods described previously (Issshiki et al. 2001, 2003; Katoh et al. 2006, 2007; Miyamoto et al. 2008, 2009). 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 (Issshiki et al. 2001, 2003; Katoh et al. 2006, 2007; Miyamoto et al. 2008, 2009).

Detection of plasmid integration, toxin production, and pathogenicity.

Integration patterns of transformation vectors in the genome of isolate SH20 were determined using genomic Southern blots and a DIG-labeled *ACTTS3* probe. Hybridization and detection were conducted as described previously (Masunaka et al. 2000, 2005; Issshiki et al. 2001, 2003; Katoh et al. 2006, 2007; Miyamoto et al. 2008, 2009). Production of ACT-toxin in culture filtrates was determined by HPLC and bioassay by the following methods. *Alternaria 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 AC-toxin medium (Kohmoto et al. 1979, 1993) for 25 to 30 days at 24°C for examination of ACT-toxin production in the culture filtrates. Culture filtrates were adsorbed to Amberlite XAD2, eluted with methanol, extracted twice with ethyl acetate, and suspended in methanol after passing through a Sep-Pak column plus C18 cartridge (Waters, Milford, MA, U.S.A.) as the protocol described previously (Masunaka et al. 2000, 2005; Miyamoto et al. 2008, 2009) for culture filtrate extract preparation for HPLC analysis. HPLC analysis of ACT-toxin was carried out on a Model PU-2080 HPLC system (Japan Spectroscopic, Tokyo) with a Develosil ODS-5 column (4.6 by 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) for 30 min at a flow rate of 1 ml/min, with peak detection with an absorbance at 290 nm (Kohmoto et al. 1993; Masunaka et al. 2000, 2005; Miyamoto et al. 2008, 2009). Ethyl-acetate-extracted samples (50 μ l) from culture filtrates (50 ml) were suspended in 1 ml of methanol, passed through a Sep-Pak column Plus C18 Cartridge (Waters), and injected for HPLC analysis. Retention time and quantification of ACT-toxin was determined for each HPLC analysis using previously purified ACT-toxin I (Kohmoto

et al. 1993; Masunaka et al. 2000, 2005; Miyamoto et al. 2008, 2009).

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 cv. Iyokan as described previously (Masunaka et al. 2000, 2005; Miyamoto et al. 2008, 2009). 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.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

DNA Data Bank of Japan (DDBJ): www.ddbj.nig.ac.jp
Wellcome Trust Sanger Institute Pfam program:
www.sanger.ac.uk/Software/Pfam