Functional Analysis of a Multicopy Host-Selective ACT-Toxin Biosynthesis Gene in the Tangerine Pathotype of *Alternaria alternata* Using RNA Silencing

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Alternaria brown spot, caused by the tangerine pathotype of *Alternaria alternata*, is a serious disease of commercially important tangerines and their hybrids. The pathogen produces host-selective ACT toxin, and several genes (named *ACTT*) responsible for ACT-toxin biosynthesis have been identified. These genes have many paralogs, which are clustered on a small, conditionally dispensable chromosome, making it difficult to disrupt entire functional copies of *ACTT* genes using homologous recombination-mediated gene disruption. To overcome this problem, we attempted to use RNA silencing, which has never been employed in *Alternaria* spp., to knock down the functional copies of one *ACTT* gene with a single silencing event. *ACTT2*, which encodes a putative hydrolase and is present in multiple copies in the genome, was silenced by transforming the fungus with a plasmid construct expressing hairpin *ACTT2* RNAs. The *ACTT2* RNA-silenced transformant (S-7-24-2) completely lost *ACTT2* transcripts and ACT-toxin production as well as pathogenicity. These results indicated that RNA silencing may be a useful technique for studying the role of *ACTT* genes responsible for host-selective toxin biosynthesis in *A. alternata*. Further, this technique may be broadly applicable to the analysis of many genes present in multiple copies in fungal genomes that are difficult to analyze using recombination-mediated knockdowns.

The plant-pathogenic fungus *Alternaria alternata* has several biological variants that produce distinct host-selective toxins (HST) (Kohmoto and Otani 1991; Nishimura and Kohmoto 1983). HST are low-molecular weight secondary metabolites with toxicity toward distinct plant genotypes and have the same specificity as infection by the toxin-producing pathogen (Nishimura and Kohmoto 1983; Scheffer and Livingston 1984; Walton 1996; Wolpert et al. 2003; Yoder 1980).

The tangerine pathotype of *A. alternata* causes *Alternaria* brown spot disease, which affects many tangerines and their hybrids. Pathogenesis is thought to be due to the ability of the fungus to produce the host-selective ACT toxin (Akimitsu et al. 2003; Kohmoto and Otani 1991; Kohmoto et al. 1979;

1991). Toxin at a concentration of 2×10^{-8} M causes veinal necrosis on leaves, with a rapid loss of electrolytes from host cells (Kohmoto and Otani 1991; Kohmoto et al. 1991, 1993). The mode of action of ACT toxin appears to be complex, but ultrastructural changes in cells treated with the toxin indicated that the primary site of action is likely the plasma membrane (Kohmoto et al. 1993).

The major form of HST produced by the tangerine pathotype was designated ACT-toxin I (Fig. 1) (Kohmoto et al. 1993). ACT-toxin I and its derivatives are the only major products detected in fluids purified from germinating conidia (Kohmoto et al. 1993). The structure of ACT toxin is related to that of AK- and AF-toxin, which are the HST produced by the Japanese pear and strawberry pathotypes of *A. alternata*, respectively (Fig. 1) (Kohmoto et al. 1993; Nakashima et al. 1985; Nakatsuka et al. 1986). These toxins share a common 9,10-epoxy-8-hydroxy-9-methyl-decatricenoic acid moiety (Fig. 1) (Kohmoto et al. 1993; Nakashima et al. 1985; Nakatsuka et al. 1986).

We previously identified *ACTT1* and *ACTT2* from the tangerine pathotype, which were expected to participate in the biosynthesis of the common moiety in the structures of ACT-, AK-, and AF-toxins (Kohmoto et al. 1993; Masunaka et al. 2000, 2005; Nakashima et al. 1985; Nakatsuka et al. 1986; Tanaka et al. 1999). Both *ACTT1* and *ACTT2* locate to a single small chromosome of the tangerine pathotype, with a size of 1.1 to 1.9 Mb that varied in different field isolates (Masunaka et al. 2005). Disruption of *AKT1* in the Japanese pear pathotype, similar to *ACTT1* at the nucleotide level (90.6%), led to a loss of production of AK toxin as well as loss of decatrienoic acid (Masunaka et al. 2000; Tanaka et al. 1999). Southern blot analysis indicated that *ACTT2* exists as multiple copies in the genome of the tangerine pathotype (Masunaka et al. 2000; 2005). It has been difficult to disrupt entire functional copies of *ACTT* genes using homologous recombination-mediated gene disruption, due to redundancy, lack of selectable markers for multiple transformations with different vector constructs, as well as low efficiency of homologous recombination.

In this study, we attempted to use RNA silencing technology to knock down the function of *ACTT* genes, thus overcoming some of the limitations of gene knockouts using homologous recombination-mediated gene disruption. RNA silencing, initially reported in *Caenorhabditis elegans* (Fire et al. 1998), is a posttranscriptional gene silencing phenomenon induced by double-stranded RNA (dsRNA). In many organisms, dsRNA is cleaved by Dicer, a nuclease of the RNase III family, into

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small interfering RNAs (siRNA) that are then incorporated into a ribonucleoprotein complex (RNA-induced silencing complex [RISC]) (Bernstein et al. 2001; Hammond et al. 2001). RISC recognizes and degrades homologous mRNAs by complementary base pairing with the incorporated siRNA (Elbashir et al. 2001; Zamore et al. 2000). This gene-silencing system has also provided powerful reverse genetic tools for functional genomics in various filamentous fungi, including *Neurospora crassa* (Cogoni et al. 1996; Romano and Macino 1992), *Magnaporthe oryzae* (Kadotani et al. 2003), *Colletotrichum lagenarium* (Nakayashiki et al. 2005), *Venturia inaequalis* (Fitzgerald et al. 2004), *Phytophthora infestans* (van West et al. 1999), *Bipolaris oryzae* (Moriwaki et al. 2007), *Fusarium graminearum* (McDonald et al. 2005), and some of the *Aspergillus* spp. (Table 1) (Hammond and Keller 2005; Mouyna et al. 2004; Yamada et al. 2007). Although the potential use of this technique for analysis of fungal gene function in molecular plant-microbe interactions has been suggested, we are aware of only one instance in which it has been used to analyze fungal-plant interactions. The *Fusarium graminearum* silencing mutant *tri6* with reduced mycotoxin (deoxynivalenol

[DON]) production showed a reduction in virulence in a head blight assay (McDonald et al. 2005), providing evidence for DON as a virulence factor in this pathosystem.

RNA silencing has never been demonstrated in *Alternaria* spp., so we first designed a vector construct for generating hairpin RNA of the green fluorescent protein (*GFP*) gene in *A. alternata*. We then introduced the vector into a mutant strain of *A. alternata* causing citrus black rot (Isshiki et al. 2003), which overexpressed GFP as the silencing target, to determine if gene silencing could function in *A. alternata*. After this preliminary demonstration of RNA-silencing efficiency, we analyzed the role of *ACTT2* in ACT-toxin biosynthesis. *ACTT2* has multiple functional copies with high sequence identity in the genome of the *A. alternata* tangerine pathotype, which were silenced by introduction of a vector that generated *ACTT2* hairpin RNA.

RESULTS

Presence of multiple *ACTT2* copies in the genome of the tangerine pathotype of *A. alternata*.

One of the previously identified *ACTT* genes, *ACTT2* (accession AB432914), encodes a putative hydrolase (Masunaka et al. 2000) that is considered to play a role in the biosynthesis of the 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid moiety of ACT toxin (Fig. 1) (Masunaka et al. 2000, 2005; Tanaka et al. 1999). Pulsed-field gel electrophoresis (PFGE) and Southern blotting demonstrated that *ACTT2* locates to a single chromosome of 1.9 to 2.0 Mb, varying in different field isolates of ACT-toxin producers, but not to chromosomes from any other pathotype or nonpathogenic species of genus *Alternaria* (Fig. 2).

The distribution of *ACTT2* in the genome of strain SH20 was further examined by genomic Southern blotting (Fig. 3). The hybridization pattern of the *ACTT2* probe was complicated, and three to five fragments were detected with the probe following digestion by several restriction enzymes (Fig. 3). Expected sizes of bands have been confirmed, such as a 1.4-kb fragment digested with *Bam*HI, that recognize a single restriction site within the sequenced region of *ACTT2* (Masunaka et al. 2000), as well as fragments of 3.7 kb digested with *Bgl*II, 9.8 kb with *Eco*RI, and 10.1 kb with *Sal*I, which recognize no restriction site within the sequence (Fig. 3B). However, detection of many unexpected sizes of bands based on the restriction map of pcCL4-1 (Fig. 3) indicate the presence of additional *ACTT2* copies with different restriction sites in the flanking or open reading frame (ORF) region.

Examination of RNA-silencing efficiency in *Alternaria* spp. using the *GFP* gene as the target.

To establish a model system for elucidating RNA silencing in *Alternaria* spp., we attempted to silence *GFP* gene expression in the citrus black rot strain G1 of *A. alternata*, which constitutively expresses *GFP* (Isshiki et al. 2003). We constructed a plasmid vector pASY-GFP to make hairpin *GFP*

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, Japanese pear, and strawberry pathotypes of *Alternaria alternata* (Kohmoto et al. 1993; Nakashima et al. 1985; Nakatsuka et al. 1986).

Table 1. Phytopathogenic fungi used for RNA-silencing studies

Fungi	Target gene of RNA silencing	Role ^a	Reference
<i>Phytophthora infestans</i>	<i>infI</i> (elicitor protein)	ND	van West et al. 1999
<i>Magnaporthe oryzae</i>	GFP ^b , <i>MPG1</i> (hydrophobin, polyketide synthase-like gene)	ND	Nakayashiki et al. 2005
<i>Venturia inaequalis</i>	GFP, <i>THN</i> (trihydroxynaphthalene reductase)	ND	Fitzgerald et al. 2004
<i>Colletotrichum lagenarium</i>	GFP	ND	Nakayashiki et al. 2005
<i>Aspergillus flavus</i>	<i>aflR</i> (transcription factor for mycotoxin biosynthesis)	ND	McDonald et al. 2005
<i>Aspergillus parasiticus</i>	<i>aflR</i> (transcription factor for mycotoxin biosynthesis)	ND	McDonald et al. 2005
<i>Fusarium graminearum</i>	<i>tri6</i> (DNA-binding protein for mycotoxin biosynthesis)	Virulence reduced	McDonald et al. 2005
<i>Bipolaris oryzae</i>	<i>PKS1</i> (polyketide synthase)	ND	Moriwaki et al. 2007

^a Role of the target gene in pathogenesis. ND means not determined.

^b GFP = green fluorescent protein.

RNAs with sense and antisense sequences of *GFP* (720 bp) that formed a double-stranded structure at the stem (Fig. 4), and pASY-GFP was cotransformed into the GFP-expressing transformant of G1 with pII99 (Namiki et al. 2001) carrying *NPTII* as the selectable marker. Geneticin-resistant transformants ($n = 100$) were randomly selected, and the intensity of GFP fluorescence was measured microscopically with an image analyzer. Transformants were classified into three categories based on relative fluorescent intensity compared with G1. Ninety-five transformants had equal intensity to those of transformants 8-1 and 13-2 (Fig. 5), representing an approximately 85 to 90% reduction in fluorescence intensity relative to that of wild type. Transformants 9-1, 9-2, and 11 showed greater reduction (more than 95%) in intensity of fluorescence, and two transformants 2-1 and 2-2 lost GFP expression completely (Fig. 5).

For transformants 2-1 and 2-2 with no visible fluorescence, the integration of pASY-GFP was analyzed by polymerase chain reaction (PCR). The primer set of PacCITF1multisite and GFPRI was used for detection of PCR products corresponding to the hairpin head and sense part of the dsRNA region from genomic DNA of the transformants (Fig. 6A). PCR products with the expected size of 841 bp were amplified from all transformants but not from the original strain used for GFP transformation (Fig. 6A). Accumulation of *GFP* mRNA was reduced in the transformants, and no visible transcript was observed in transformants 2-1 and 2-2 (Fig. 6B). The presence of siRNA was also confirmed by Northern blots using both sense and antisense probes using one (2-2) of the transformants showing no visible GFP fluorescence and transcripts (Fig. 6C). Statistical analysis by a Tukey-Kramer test showed no differences in growth rate of cultures, sporulation, and spore germination found between the wild type, GFP-expressing transformant (G1) and the *GFP* RNA-silenced transformants.

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

Once the efficiency of RNA silencing had been established using *GFP* in *Alternaria* spp., we applied this method to knock down the function of *ACTT2* in the tangerine pathotype of *A. alternata*. The RNA silencing vector pASYII-*ACTT2* express-

Fig. 2. Distribution of *ACTT2* among chromosomes in the tangerine and rough lemon pathotypes and saprophytic strains of *Alternaria alternata*. Intact chromosomal DNAs of each isolate were separated with pulsed-field gel electrophoresis (0.8% agarose gel) under conditions optimized for separation of chromosomes <2.0 Mb (left panel). Chromosomes were transferred onto nylon membrane, and *ACTT2* sequences were detected with digoxigenin-labeled *ACTT2* probes (right panel). Sizes (in Mb) of *Hansenula wingei* chromosomes are indicated at the left.

Fig. 3. Genomic Southern blot analysis of *ACTT2* sequences in the tangerine pathotype strain SH20, using several restriction enzymes. DNA of strain SH20 digested with *Pst*I (lane P), *Sac*I (Sc), *Hind*III (H), *Xba*I (Xb), *Xho*I (Xh), *Sal*I (Sl), *Ban*III (Bn), *Bgl*II (Bg), *Eco*RI (E), and *Bam*HI (Bm). Sizes of marker DNA fragments (*Hind*III-digested λ DNA) are indicated at left.

Fig. 4. Construction and plasmid maps of RNA-silencing vectors pASY, pASY-II, pASY-GFP, and pASYII-*ACTT2*.

ing hairpin *ACTT2* RNA, constructed by sense and antisense sequences of *ACTT2* (780 bp) in pASY-II carrying *NPTII* as the selection marker (Fig. 4), was transformed into the strain SH20 *A. alternata* tangerine pathotype. A total of 75 transformants were examined for production of ACT toxin in the culture filtrates using a leaf necrosis assay, and 21 transformants showing less than 50% necrosis development compared with that caused by the wild-type SH20 in toxin-sensitive citrus leaves were selected. These transformants were further examined for loss of pathogenicity in a spray-inoculation assay on tangerine leaves and ability to produce ACT toxin detected by high-pressure liquid chromatography (HPLC). Two transformants, S-7-3 and S-7-24-2, were selected. Transformants carrying pASYII-*ACTT2* were selected by resistance to geneticin and PCR amplification of the vector-specific region (Fig. 7A). Transcript accumulation of *ACTT2* was detectable by reverse transcription (RT)-PCR but was significantly reduced in S-7-3 compared with that of wild-type SH20 (Fig. 7B). HPLC analysis of extracts from culture filtrates of S-7-3 indicated that the peak area of S-7-3 showed 84.6% reduction compared with that of the wild type (Fig. 7C). An additional two transformants showed similar reduction to that of S-7-3, and another 17 transformants also showed 20 to 30% reduction in ACT-toxin production compared with wild type. RT-PCR of transformant S-7-24-2 showed no detectable *ACTT2* transcript, and no peak of ACT toxin was detected by HPLC analysis (Fig. 7B and C). Leaves sprayed with conidia of the wild type were covered by necrotic brown lesions 48 h postinoculation, while those sprayed with conidia of S-7-3 showed significant reduction in the numbers of lesions, and no lesions were observed

on leaves sprayed with conidia of S-7-24-2 (Fig. 7D). Statistic analysis by Tukey-Kramer test showed no differences in growth rate of cultures, sporulation, and spore germination found between wild type and the *ACTT2* RNA silenced transformants.

DISCUSSION

Most of the HST biosynthesis genes in *A. alternata* are present in multiple functional copies with high sequence identity among paralogs. This creates challenges for gene manipulations that are required to demonstrate a causal connection between each of the genes and a phenotype. Repeated rounds of disruption are required to knock out all copies of a particular target gene. Each round of disruption needs an independent vector construct with a different antibiotic resistance marker; however, only a few such resistance genes are available for fungi, and low efficiency of homologous recombination in *Alternaria* spp. also limits the effectiveness of the approach. To solve these limitations, RNA silencing was applied to *ACTT2*, which has the highest number of copies among the putative ACT-toxin biosynthetic *ACTT* genes identified in the genome of the tangerine pathotype.

In this study, the occurrence of RNA silencing was initially demonstrated using GFP as the monitoring marker. The degree of suppression of GFP fluorescence varied significantly among transformants using the vector construct designed for generating a hairpin *GFP* RNA. All transformants examined (100) showed more than 85% reduction in fluorescence, including three (9-1, 9-2, 11) transformants reduced more than 95% and

Fig. 5. Images of green fluorescent protein (GFP) fluorescence in mycelia of silenced and nonsilenced transformants of the G1 mutant overexpressing GFP. Green fluorescence of the G1 mutant and transformants were detected in germinating spores on agar plates. Green fluorescence was detected with an Olympus BX51 microscope with a B excitation. Upper panel of each transformant shows the view under light microscope, and the lower panel shows the same view under fluorescence. All experiments were repeated at least three times with similar results. Scale bars represent 50 μ m.

two (2-1 and 2-2) transformants that lost GFP fluorescence altogether. Compared with that observed for GFP RNAi, approximately the same percentage of transformants lost or had strongly reduced transcript accumulation of *ACTT2*, ACT-toxin production, and pathogenicity when the *A. alternata* tangerine pathotype was transformed with the vector construct designed to generate a hairpin *ACTT2* RNA. Transformation constructs giving hairpin structure of RNA using sense and antisense sequence of the target gene with an intron sequence-based hairpin head spacer are known as an effective and relatively stable way for RNA silencing in fungi as well as many other organisms (Cogoni and Macino 1999; Hannon 2003; Kadotani et al. 2003; Liu et al. 2002). This is likely due to recognition of such dsRNA as aberrant RNA by RNA-dependent RNA polymerase-mediated process (Baulcombe 1996). In this study, RNA-silenced *A. alternata* transformants showed similar or greater expression reduction compared with other reports for filamentous fungi (Cogoni et al. 1996; Fitzgerald et al. 2004; Hammond and Keller 2005; Kadotani et al. 2003; Moriwaki et al. 2007; Mouyna et al. 2004; Nakayashiki et al. 2005; Romano and Macino 1992; Yamada et al. 2007). The low frequency (2.5%) of completely silenced strains recovered in this study might be related to copy number of the target genes. The frequency of completely silenced strains of other HST biosynthesis genes with lower copy number in other pathotypes of *A. alternata* was greater than the frequency recovered for silencing of *ACTT2* in this study (*unpublished data*).

ACTT2 is thought to play a role in the biosynthesis of the common decatrienoic acid backbone of ACT, AK, and AF toxins, and sequence similarities of *ACTT2* orthologs among these three pathotypes are high. *ACTT2* (accession AB432914) and *AKT2* (accession AB015352) are 90.6% similar and *ACTT2* and *AFT2* (AB434468) are also 90.6% similar. A complication

for comparative analysis of these genes is that they are duplicated in the genome. There are multiple copies of this gene carried on the 1.9-Mb chromosome of *A. alternata* tangerine pathotype strain SH20, and sequence identity among copies is high. For example, the sequence identity of *ACTT2* and *ACTT2-1* (accession AB432915) is 98.8%. Homologs of *ACTT2* identified from a dual toxin-producing strain of *A. alternata* (BC3-5-1-OS2A) (Masunaka et al. 2005) also showed similarities of 99 and 98.9% for *ACTT2-2* (accession AB432916) and *ACTT2-3* (accession AB432917), respectively. Although conventional disruption of *ACTT2* could not be accomplished due to the high number of the copies identified in the genome, the high sequence identity among these copies of *ACTT2* is likely an advantage for RNA silencing, because it allows knockdown of all copies of this gene simultaneously. Target sequences of RISC among respective *ACTT2* copies are likely to have high identities, and the RISC recognizes them as the target for silencing without selecting the source of original DNA copies. Since the target recognition process is highly sequence-specific but does not always require 100% identity (Elbashir et al. 2001), sequence identity among functional copies of *ACTT2* is likely high enough to be recognized by *ACTT2* siRNA-ori-

Fig. 6. RNA-silencing transformants for green fluorescent protein (GFP) expression in the GFP-overexpressing strain. **A**, Confirmation of insertion of GFP-silencing vector to the silenced transformants using polymerase chain reaction. Lane G1 = G1 mutant, lane 1 = strain 2-1, lane 2 = strain 2-2, lane 3 = strain 8-1, lane 4 = strain 9-1, lane 5 = strain 9-2, lane 6 = strain 13-2, and lane 7 = strain 11. **B**, Northern blot analysis of the accumulation of GFP mRNA in the silenced transformants. The rRNA, stained with methylene blue and photographed before blotting, is shown below the blot. **C**, Small interfering RNAs of GFP were also detected by Northern blot. Total RNA was separated on a 15% polyacrylamide gel and was hybridized with digoxigenin-labeled sense and antisense GFP RNA probes.

Fig. 7. Transformation and expression of a plasmid construct expressing hairpin *ACTT2* RNAs, reduced *ACTT2* transcripts, ACT-toxin production, as well as pathogenicity. **A**, Confirmation of insertion of *ACTT2*-silencing vector into silenced transformants using polymerase chain reaction (PCR). Lane W = wild-type strain SH20, lane 1 = strain S-7-3, and lane 2 = strain S-7-24-2. **B**, Reverse transcription-PCR detection of *ACTT2* transcripts in wild-type SH20 (WT) and the silenced transformants S-7-3 (1), and S-7-24-2 (2). **C**, High-pressure liquid chromatography charts of each extract from culture filtrates from the wild type SH20 (WT) and silenced transformants S-7-3 (1) and S-7-24-2 (2). **D**, Citrus leaves were spray-inoculated with conidia of wild type SH20 (WT) and silenced transformants S-7-3 (1) and S-7-24-2 (2).

ented RISC. Indeed, we obtained a transformant (S-7-24-2) that did not produce ACT toxin because of lack of *ACTT2* transcript by this method, thus demonstrating the role of *ACTT2* in the biosynthesis of ACT toxin and importance of the toxin in pathogenicity. To our knowledge, this is the first documented case of RNA silencing used to disrupt production of a host-specific toxin in a filamentous fungus. RNA silencing was used to suppress mycotoxin production and virulence of *F. graminearum* (McDonald et al. 2005). Our results indicate that RNA silencing using vectors that generate hairpin RNA of the target gene will be useful for functional analysis of HST genes in other pathotypes of *A. alternata* as well as HST produced by other filamentous fungi.

MATERIALS AND METHODS

Plants and fungal strains.

Young citrus leaves (midrib length 2 to 4 cm) of cultivar Iyokan tangor (*Citrus iyo* hort. Ex Tanaka) were produced in the greenhouse as described previously and used for inoculation and toxin sensitivity assays (Masunaka et al. 2000). Iyokan is susceptible to the tangerine pathotype of *A. alternata* and sensitive to ACT toxin (Kohmoto et al. 1991).

Strains of the tangerine pathotype of *A. alternata* were obtained from leaf lesions of *Minneola tangelo* (*Citrus reticulata* Blanco × *Citrus paradisi* Macf.) and rough lemon in Florida for a study of the population structure and host specificity of the *Alternaria* brown spot pathogen (Peever et al. 1999; Masunaka et al. 2000, 2005). Isolates SH11, SH12, SH14, SH15, SH18, SH19, SH20, SH21 and EV10, EV13, and EV14 are tangerine pathotype isolates, whereas isolates HC1, HC7, HC8, BC19, and BC27 are rough lemon pathotype isolates (Peever et al. 1999; Masunaka et al. 2000, 2005). Tangerine pathotype SH20 has been used previously as a reference strain for the brown spot pathogen (Masunaka et al. 2000). Isolates HC14 and BC17 are nonpathogenic isolates of *A. alternata* recovered from rough lemon leaves (Peever et al. 1999; Masunaka et al. 2000, 2005). In addition, laboratory stock strains of saprophytic *A. alternata* (isolates O-94 and IFO031189) (Tanaka et al. 1999) and a citrus black rot strain (G1) (ATCC58171) (Isshiki et al. 2001, 2003) were used in this study. A GFP-expressing mutant of G1 (Isshiki et al. 2003) was previously generated by introducing the *GFP* gene into wild-type black rot isolate ATCC58171. All isolates including transformants for RNA silencing of *GFP* and *ACTT2* were stored in 25% (vol/vol) glycerol at -85°C, and cultures were initiated from the stocks for each experiment by growth on V8 juice agar or potato dextrose agar (PDA) plates.

Nucleic acid manipulations.

Genomic DNA and total RNA were isolated from mycelium of wild type or mutants grown at 24°C for 21 to 25 days in potato dextrose broth (PDB) (for GFP-related studies) or AC medium (for analyzing *ACTT2*-silencing mutants), as described previously (Masunaka et al. 2000; Isshiki et al. 2001). Restriction enzyme digestion of genomic DNA (5 µg), agarose gel electrophoresis and transfer to Hybond N⁺ membrane (Amersham Biosciences, Piscataway, NJ, U.S.A.) by capillary transfer were conducted under standard conditions (Sambrook et al. 1989). PCR probes for Southern blots were prepared using the PCR DIG probe synthesis kit (Roche, Mannheim, Germany), with respective primer sets, following the manufacturer's instructions. Genomic Southern hybridization following various restriction-enzyme digestions was performed at 68°C overnight with digoxigenin (DIG)-labeled *ACTT2* probe prepared by the primer set of *ACTT2F1* (5'-ATGCAGCAGCCCA

TCATT-3') and *ACTT2R1* (5'-ACATATCGACAGGGCCTGT-3') for *ACTT2* detection by the method described previously (Masunaka et al. 2000) and with DIG-labeled GFP probe prepared by the primer set of *GFPF1* (5'-ATGGTGAGCAAGGGCGA-3') and *GFPR1* (5'-TTACTTGTACAGCTCGT-3') for *GFP* detection by the method described previously (Isshiki et al. 2003).

For Northern blots, total RNA (8 µg per lane) was loaded on agarose-formaldehyde gels and was transferred to Hybond N⁺ membrane by capillary transfer (Sambrook et al. 1989). Ribosomal RNAs on the membrane were stained with 0.02% (wt/vol) methylene blue to confirm equal RNA loading (Sambrook et al. 1989). Hybridization for *GFP* mRNA detection was performed at 50°C overnight with DIG-labeled GFP PCR probe.

Small RNAs were extracted and detected using methods described by Dalmay and associates (2000), with minor modifications. The DIG-labeled *GFP* RNA probe was prepared by in vitro transcription and labeling, using the DIG RNA labeling kit (Roche), from PCR products amplified using *GFPF1* (5'-ATGGTGAGCAAGGGCGA-3') and *GFPR1* (5'-TTACTTGTACAGCTCGT-3') (Isshiki et al. 2003) in the pSPT18 vector (included in the kit), following the manufacturer's instructions. Hybridization was performed at 68°C overnight with each probe (100 ng/ml). Detection was performed under the same conditions as the Southern and Northern blot analyses described above.

For RT-PCR, isolation of total RNA from mycelia and cycling conditions were carried out as previously described (Masunaka et al. 2000), and *ACTT2* transcripts were amplified using the same primer sets as used for synthesis of DIG-labeled *ACTT2* probe prepared with primers of *ACTT2F1* and *ACTT2R1*. RT-PCR products were run on agarose gels and were transferred to Hybond N⁺ membrane using standard methods (Sambrook et al. 1989), and were detected with the DIG-labeled *ACTT2* probe as described above.

Insertions of RNA-silencing vector constructs were confirmed by genomic PCR using the primer set of *PacCITF1* multisite (5'-GAATTCTGCAGATATCAGATCTGTAAGTTGACC GCCAAG-3') designed from the spacer region in the internal vector construct and *ACTT2R1* for *pASYII-ACCT2* vector construct and *PacCITF1* multisite and *GFPR1* for *pASY-GFP* vector construct.

PFGE and hybridization.

Preparation of chromosomes from fungal protoplasts and PFGE were carried out according to the methods described previously (Hatta et al. 2002; Masunaka et al. 2005). Running conditions (duration, voltage, linear gradient of switching time) in the PFGE apparatus (CHEF-DRII, BioRad, Hercules, CA, U.S.A.) consisted of 0.5× tris-borate-EDTA (Sambrook et al. 1989) at 8°C in 0.8% (wt/vol) agarose gel (Seakem Gold agarose; Cambrex BioScience Rockland, Inc., Rockland, ME, U.S.A.), 13:5.4:120 h/V cm⁻¹/s, 13:5.4: 180 h/V cm⁻¹/s. Chromosomal DNA bands were visualized by ethidium bromide staining, and the gel was nicked with 0.2 N HCl for 20 min and was soaked in 0.4 N NaOH plus 1.5 M NaCl for 30 min to transfer Hybond N⁺ membranes by alkaline capillary transfer (Sambrook et al. 1989) 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, was washed twice with sterile water at room temperature for 5 min with 0.2 N NaOH plus 1% (vol/vol) sodium dodecyl sulfate at 50°C for 20 min, and was hybridized at 68°C for 30 min with the same *ACTT2* probes used for the Southern blot analysis described above. Hybridization and washing were conducted under high stringency conditions, according to methods described previously (Hatta et al. 2002; Masunaka et al. 2005).

Plasmid constructs.

Plasmid vectors pASY and pASY-II (Fig. 4) were constructed from *Alternaria* overexpression vector pAOEIK (Katoh et al. 2007) for use as RNA silencing vectors in *Alternaria* spp. For construction of pASY, the intron region (97 bp) of AcPacC (accession AB433595), a pH-dependent regulatory gene of *A. alternata*, was amplified and added as the spacer sequence in the vector, for construction of a part of the hairpin head of dsRNA. The spacer region forming a hairpin head was amplified from genomic DNA of *A. alternata* tangerine pathotype strain SH20 by PCR consisting of 30 cycles of denaturing for 30 s at 95°C, primer annealing for 30 s at 50°C, and extension for 15 s at 72°C. The forward primer of PacCITF1multisite (5'-GAATTCTGCAGATATCAGATCTGTAAGTTGACCGCC AAG-3') contained *EcoRI*, *PstI*, *EcoRV*, and *BglIII* sites in the primer sequence, and the reverse primer PacCITR1multisite (5'-GAATTCAAGCTTTAAATTCGAACTGCAATCGAGTC AGC-3') contained *EcoRI*, *HindIII*, *DraI*, and *NspV* sites in the sequence. Both the PCR product (AB433596) and pAOEIK (Katoh et al. 2007) were digested with *EcoRI*, and the *EcoRI*-digested pAOEIK, following dephosphorylation with alkaline phosphatase (Takara, Shiga, Japan), was ligated with the PCR product at the region between the promoter and terminator sequences of *Aspergillus nidulans trpC* (Fig. 4). For construction of pASY-II, a cassette containing the *NPTII* gene, which confers geneticin resistance, under the *trpC* promoter and terminator regulation was digested by *SacI* and *NotI* from the plasmid vector pII99 (Namiki et al. 2001) and was ligated into the predigested pASY with the same restriction enzymes (Fig. 4). Insertions and direction of the PCR products were confirmed by sequencing with 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).

The entire ORF of *GFP* was amplified from pTEFEGFP (Van den Wymelenberg et al. 1997) by PCR consisting of 30 cycles of denaturing for 1 min at 95°C, primer annealing for 1 min at 48°C, and extension for 1 min at 72°C, using a primer set of either GFPF1NspV (5'-TTCGAAATGGTGAGCAAGGGCGA-3'), containing a *NspV* site, and GFPR1HindIII (5'-CCCAAG CTTGGGTACTTGTACAGCTCGT-3'), containing a *HindIII* site, or GFP F1BgIII (5'-GAAGATCTTCATGGTGAGCAAGG GCGA-3'), containing a *BglIII* site, and GFPR1EcoRV (5'-GA TATCTTACTTGTACAGCTCGT-3'), containing a *EcoRV* site. Amplicons were subcloned into pASY to construct the RNA-silencing vector pASY-GFP with the following procedures. The pASY vector was first digested with *BglIII* and *EcoRV*, and then, the PCR product was amplified with GFPF1BgIII, and GFPR1EcoRV was subcloned after digestion with *BglIII* and *EcoRV* (Fig. 4). The vector (Fig. 4) was redigested with *NspV* and *HindIII*, and the PCR product was amplified with GFPF1-*NspV*, and GFPR1HindIII was subcloned into the vector after digestion with *NspV* and *HindIII*. Insertions of the first PCR product between the *trpC* promoter and spacer region as well as the second PCR product between the spacer region and *trpC* terminator (Fig. 4) were confirmed by sequencing as described above.

For construction of the *ACTT2* RNA-silencing vector, inserts of *ACTT2* were amplified from subcloned *ACTT2* cDNA by PCR consisting of 30 cycles (denaturing for 1 min at 95°C, primer annealing for 1 min at 50°C, and extension for 1 min at 72°C), using a primer set of either ACTT2F1BgIII (5'-GAAG ATCTTCATGCAGCAGCCCATCATT-3'), containing a *BglIII* site, and ACTT2R1BgIII (5'-GAAGATCTTCCGACTGCAGA TATTCATCC-3'), containing a *BglIII* site, or ACTT2F1-*NspV* (5'-TTCGAAATGCAGCAGCCCATCATT-3'), contain-

ing a *NspV* site, and ACTT2R1HindIII (5'-AAGCTTCGAC TGCAGATATTCATCC-3'), containing a *HindIII* site. Both the PCR product, amplified with ACTT2F1BgIII and ACTT2-R1BgIII, and pASY-II were digested with *BglIII*, and the *BglIII*-digested pASY-II, following dephosphorylation with alkaline phosphatase (Takara), was ligated with the PCR product at the region between the *trpC* promoter and the spacer region (Fig. 4). The vector was redigested with *NspV* and *HindIII*, and the PCR product was amplified with ACTT2F1NspV, and ACTT2-R1HindIII was subcloned into the vector after digestion with *NspV* and *HindIII* at the region between the spacer region and *trpC* terminator (Fig. 4). Insertions and direction of respective PCR products were confirmed by sequencing as described above.

Fungal transformation.

Transformation to *Alternaria* spp. was carried out using methods described previously (Isshiki et al. 2001, 2003). Protoplasts (1×10^7) of mutant G1 (Isshiki et al. 2003) were transformed with 3 µg each of the vector pASY-GFP and pII99 carrying *NPTII* cassette as the selection marker (Namiki et al. 2001). Protoplasts (1×10^7) of strain SH20 were transformed with 3 µg of the vector pASYII-ACTT2 carrying a *NPTII* cassette as the selectable marker. Transformants were selected on PDA plates containing geneticin (200 µg/ml) by growth at 24°C for 5 to 7 days, and single-spore cultures were recovered as previously described (Isshiki et al. 2001; 2003).

Determination of GFP fluorescence, ACT-toxin production, and pathogenicity of RNA-silencing transformants.

GFP expressed in transformants was detected using an Olympus BX51 microscope (Olympus, Tokyo) with a B excitation filter (460 to 480 nm). Relative intensity of green fluorescence from germinating spores of transformants in PDB after 24 h of incubation at 24°C with 120 rpm shaking was estimated against that of the G1 mutant (Isshiki et al. 2003), using the Image Gauge program version 4.0 (Fuji Photo Film, Tokyo) following the manufacturer's instructions. Same microscopic view was also observed under light microscope by differential interference contrast with U-DICT in the Olympus BX51 microscope. Three different fields were observed independently for each treatment, and an average of the relative present rate of intensity was obtained.

ACT-toxin production was estimated by placing diluted culture filtrates (10^1 to 10^3 in water) onto wounded sites of young leaves of Iyokan and Nijisseiki, as previously described (Masunaka et al. 2000). Seventy-five *ACTT2*-silenced transformants were tested. Leaves were incubated in a moist chamber at 24°C in the dark, and necrosis development on the leaves was observed after 48 h. Transformants causing less than 50% necrosis compared with the wild type (strain SH20) were further examined for pathogenicity in a plant inoculation assay, and ACT-toxin production was assessed by HPLC.

Production of ACT toxin in culture filtrates was determined by HPLC. *A. alternata* SH20 and its mutants were grown in 200-ml flasks containing 50 ml of AC-toxin medium (Kohmoto et al. 1979, 1993) for 25 to 30 days at 24°C. Culture filtrates were adsorbed to Amberlite XAD2, extracted with methanol, extracted twice with ethyl acetate, and suspended in methanol (Kohmoto et al. 1993). The methanol phase was passed through a Sep-Pak Column Plus C18 cartridge (Waters, Milford, MA, U.S.A.) and were further analyzed by bioassay and HPLC (Model PU-2080; Japan Spectroscopic, Tokyo), with an absorbance at 290 nm, using a Develosil ODS-5 column (4.6 × 250 mm, Nomura Chemical, Nagoya, Japan) with a gradient solvent system composed of acetonitrile, water, and 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 (Kohmoto et al. 1993). Relative comparison of ACT toxin peak areas was carried out using a chromatographic analysis program JASCO-Borwin (version 1.50, Japan Spectroscopic) according to the manufacturer's instructions.

Pathogenicity was determined by spots and necrosis development on leaves spray-inoculated with conidia of *A. alternata* SH20 and its RNA-silenced mutants as previously described (Kohmoto et al. 1993). Conidia of each isolate were collected and suspended in water at a concentration of 1×10^5 spores per milliliter and were spray-inoculated onto detached, young leaves (midrib length 3 to 4 cm) of ACT toxin-sensitive citrus cultivar Iyokan as previously described (Masunaka et al. 2000, 2005). Inoculated 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 observed.

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