An Isolate of *Alternaria alternata* That Is Pathogenic to Both Tangerines and Rough Lemon and Produces Two Host-Selective Toxins, ACT- and ACR-Toxins

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ABSTRACT

Masunaka, A., Ohtani, K., Peever, T. L., Timmer, L. W., Tsuge, T., Yamamoto, M., Yamamoto, H., and Akimitsu, K. 2005. An isolate of *Alternaria alternata* that is pathogenic to both tangerines and rough lemon and produces two host-selective toxins, ACT- and ACR-toxins. *Phytopathology* 95:241-247.

Two different pathotypes of *Alternaria alternata* cause *Alternaria* brown spot of tangerines and *Alternaria* leaf spot of rough lemon. The former produces the host-selective ACT-toxin and the latter produces ACR-toxin. Both pathogens induce similar symptoms on leaves or young fruits of their respective hosts, but the host ranges of these pathogens are distinct and one pathogen can be easily distinguished from another by comparing host ranges. We isolated strain BC3-5-1-OS2A from a leaf spot on rough lemon in Florida, and this isolate is pathogenic on both cv. Iyokan tangor and rough lemon and also produces both ACT-toxin and

ACR-toxin. Isolate BC3-5-1-OS2A carries both genomic regions, one of which was known only to be present in ACT-toxin producers and the other was known to exist only in ACR-toxin producers. Each of the genomic regions is present on distinct small chromosomes, one of 1.05 Mb and the other of 2.0 Mb. *Alternaria* species have no known sexual or parasexual cycle in nature and populations of *A. alternata* on citrus are clonal. Therefore, the ability to produce both toxins was not likely acquired through meiotic or mitotic recombination. We hypothesize that a dispensable chromosome carrying the gene cluster controlling biosynthesis of one of the host-selective toxins was transferred horizontally and rearranged by duplication or translocation in another isolate of the fungus carrying genes for biosynthesis of the other host-selective toxin.

Additional keyword: anastomosis.

Alternaria species cause four diseases of citrus including *Alternaria* brown spot of tangerines (*Citrus reticulata* Blanco), *Alternaria* leaf spot of rough lemon (*C. jambhiri* Lush.) and rangpur lime (*C. limonia* Osbeck), *Alternaria* black rot of many citrus cultivars, and Mancha foliar of Mexican lime (*C. aurantifolia* Swingle) (3,23,26,33). *Alternaria* brown spot affects many tangerines, grapefruit (*C. paradisi* Macf.), and their hybrids resulting in lesions on, and abscission of, immature fruit and leaves. *Alternaria* leaf spot of rough lemon produces similar symptoms to those described above on leaves and fruits. The fungi causing brown spot and leaf spot are morphologically similar, have small catenulate spores, and are considered to be distinct pathotypes of the same species, *A. alternata* (26). Both brown spot and leaf spot pathogens produce host-selective toxins (HSTs) and the host range of each is very narrow (3,14–16).

Alternaria and *Cochliobolus* species are well-known producers of HSTs (21,31,34,35,37). HSTs are low molecular weight, secondary metabolites that are produced during germination of spores on plant surfaces. These toxins have high specificity and toxicity and induce cell death at 10^{-9} to 10^{-8} M when applied to susceptible plants. The selective toxicity of HSTs is correlated with the pathogenicity reactions of the fungi that produce them (14–16), and HSTs are demonstrated pathogenicity factors (13,17,21,

32,34,35,37). Nishimura and Kohmoto (21) proposed that these *Alternaria* strains should be designated as pathotypes of *A. alternata*. Despite the evolutionary relatedness of these host-specific *Alternaria* strains, each produces a chemically distinct HST and they can be easily distinguished by comparing host range. The chemical structures of HSTs from six pathotypes of *A. alternata* including ACT- and ACR-toxins (Fig. 1) have been elucidated (4,15,19), and we have identified *ACTT* genes that are thought to be involved in the biosynthesis of ACT-toxin (18).

Three major evolutionary lineages of *A. alternata* cause brown spot and leaf spot of tangerines and rough lemon in Florida (24,26). Two of these lineages are associated with brown spot of tangerines (the SH1 and SH4 lineages) and the other with leaf spot of rough lemon (HC1 lineage). All three can be recovered from healthy rough lemon tissue in Florida, but most isolates are nonpathogenic on either tangerine or rough lemon (24; T. L. Peever, *unpublished data*). Abandoned citrus groves on rough lemon rootstocks in Florida may be located adjacent to tangerine groves. This proximity of both hosts provides an opportunity for each pathotype to interact in a common host (rough lemon). Since all lineages can occur in a common host, we predict that an isolate of *A. alternata* may evolve that would possess genomic regions carrying genes responsible for the biosynthesis of both ACT- and ACR-toxins. One genomic region carrying either *ACTT* or *ACR* biosynthetic genes could have been transferred to a cell carrying the other genomic region during cell fusion through hyphal anastomosis. To test this hypothesis, we screened isolates of *A. alternata* from citrus groves in Florida to identify strains pro-

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ducing both ACT- and ACR-toxins and examined the pathogenicity and presence of predicted genes responsible for the biosynthesis of each HST.

MATERIALS AND METHODS

Fungal isolates and host materials. Thirteen isolates of the tangerine pathotype (isolate numbers SH11, 12, 13, 14, 15, 18, 19, 20, 21, EV10, 13, 14, and 24) were isolated from brown spot lesions on Minneola tangelo (*C. paradisi* × *C. reticulata*), and six isolates of the rough lemon pathotype (HC1, 7, 8, BC19, 27, and 35) were isolated from leaf lesions on rough lemon in central Florida (Table 1). Three isolates of nonpathogenic *A. alternata* (HC14, BC17, and 36) were isolated from leaf lesions on rough lemon from the same locations (Table 1). Isolate BC3-5-1-OS2A was isolated from a leaf lesion on rough lemon at the Baseball City location (Table 1). Tangerine pathotype isolate SH20 and rough lemon pathotype isolate HC1 were selected as reference strains for each pathotype (18). In addition, laboratory stocks (12) of nonpathogenic *A. alternata* isolate O-94, Japanese pear pathotype isolates 15A and Nu88-22, strawberry pathotype isolates NAF-3 and NAF-8, tomato pathotype isolates 91080804 and AL-4, apple pathotype isolates O-159 and AM-1, tobacco pathotype isolates 122-2 and AT-204, rough lemon pathotype isolate AC325 (2), and ATCC58171 for *A. citri* (11) were used in this study (Table 1). All isolates were stored in 25% glycerol at -85°C and cultures were initiated from the stocks for each experiment. Leaves for inoculation studies were obtained from cv. Iyokan tangor (*C. iyo* hort. Ex Tanaka) and rough lemon grown in the greenhouse as described previously (18,22). Iyokan tangor is sensitive to ACT-toxin and susceptible to the tangerine pathotype of *A. alternata* (14,18). Rough lemon is sensitive to ACR-toxin and susceptible to the rough lemon pathotype of *A. alternata* (2,14,18,22). The two hosts were insensitive to the toxin produced by the other pathotype.

Toxin production and pathogenicity. The pathogenicity of each isolate was tested using the spray inoculation assay of Kohmoto et al. (15). Isolates were grown in potato dextrose broth (PDB), and mycelial mats were washed three times with sterile water to prepare conidia as described previously (15,18). The pathogenicity of each isolate was determined by spray inoculation of a conidial suspension (1 × 10⁵ conidia/ml) on detached leaves of citrus cv. Iyokan tangor and rough lemon (15,18). Inoculated leaves were incubated in a moist chamber for 48 h at 24°C in the

dark, and the development of necrotic spots on the leaves was monitored.

Toxin production by each isolate was analyzed by high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) as previously described (2,4,15,18,19) with some modifications. Stationary cultures of each isolate were initiated in 50 ml of modified Richards' medium (15,16,18) at 24°C for 25 days, and each culture filtrate was collected through four layers of gauze. ACT-toxin and ACR-toxin were extracted from the culture filtrates using ethyl acetate as described by Kohmoto et al. (15). Ethyl acetate extracts of culture filtrates were analyzed using HPLC (Model 880 PU, Japan Spectroscopic, Tokyo) to detect ACT-toxin. After passing through Sep-Pak Plus C18 Cartridge (Waters, Milford, MA), the extracts were analyzed by HPLC on a Develosil ODS-5 column (4.6 by 250 mm, Nomura Chemical, Nagoya, Japan) using a gradient solvent system composed of acetonitrile/deionized water/acetic acid (from 30:70:1 to 60:40:1, vol/vol) for 30 min at a flow rate of 1 ml/min (15). Peaks were detected by monitoring absorbance at 290 nm. An authentic standard of ACT-toxin (Fig. 1) was prepared from culture filtrates of isolate SH20 as described previously (15).

The presence of ACR-toxin in the extracts was determined by the TLC system using silica gel 60 F254 TLC plates (Merck, Darmstadt, Germany). Ethyl acetate extracts were developed using a solvent system of benzene/acetone/acetic acid (60:40:1, vol/vol), and ACR-toxin was detected as a purple spot under 254 nm UV. An authentic preparation of ACR-toxin (Fig. 1) was prepared from culture filtrates of isolate HC1 as described previously (2,4,19).

Elution of the corresponding peak for ACT-toxin and the TLC spot for ACR-toxin were tested for their selective toxicities on different hosts by the leaf necrosis assay described previously

TABLE 1. Isolates of *Alternaria* spp. used in this study

Isolate	Pathotype	References
BC3-5-1-OS2A	Dual pathotypes	This study
SH11	Tangerine	(18)
SH12	Tangerine	(18)
SH13	Tangerine	(18)
SH14	Tangerine	(18)
SH15	Tangerine	(18)
SH18	Tangerine	(18)
SH19	Tangerine	(18)
SH20	Tangerine	(18)
SH21	Tangerine	(18)
EV10	Tangerine	(18)
EV13	Tangerine	(18)
EV14	Tangerine	(18)
EV24	Tangerine	(18)
HC1	Rough lemon	(18)
HC7	Rough lemon	(18)
HC8	Rough lemon	(18)
BC19	Rough lemon	(18)
BC27	Rough lemon	(18)
BC35	Rough lemon	(18)
AC325	Rough lemon	(2)
HC14	Nonpathogen	(18)
BC17	Nonpathogen	(18)
BC36	Nonpathogen	(18)
O-94	Nonpathogen	(2)
ATCC58171	Black rot pathogen	(11)
15A	Japanese pear	(12)
Nu88-22	Japanese pear	(12)
NAF-3	Strawberry	(12)
NAF-8	Strawberry	(12)
91080804	Tomato	(12)
AL-4	Tomato	(12)
O-159	Apple	(12)
AM-1	Apple	(12)
122-2	Tobacco	(12)
AT-204	Tobacco	(12)

ACT-toxin

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

ACR-toxin I

Fig. 1. Chemical structures of ACT-toxin and ACR-toxin-I produced by the tangerine pathotype and rough lemon pathotypes of *Alternaria alternata* (4,15,19).

(15,18). Toxicity of culture filtrates was also determined by the leaf necrosis assay (15,18). The supernatant of culture filtrates harvested by centrifugation at $1,500 \times g$ for 5 min, or purified toxin samples, was diluted in distilled water (1:100, vol/vol) and 30 μ l was placed on a puncture-wounded site with a needle on the lower surface of leaves of Iyokan tangor and rough lemon. Leaves were incubated in a moist chamber at 24°C in the dark, and necrosis development on leaves was observed after 48 h.

DNA manipulations. Isolation of total genomic DNA of *A. alternata* for genomic polymerase chain reaction (PCR) and genomic Southern blot hybridization was carried out as described previously (11,18), and nucleic acid manipulations followed standard procedures (30). Genomic DNA (5 μ g) was digested with 10 units of restriction enzyme *EcoRV* (Takara, Shiga, Japan) and fractionated in 0.8% agarose gels. Fractionated DNA was transferred to Hybond N⁺ membrane (Amersham Biosciences, Piscataway, NJ) by capillary transfer (30).

The ACTT-specific genes *ACTT1* and *ACTT2*, present only in ACT-toxin-producing isolates, were prepared as described previously (18) using ACTT1F2 (5'-CACAGGCTATCTTCACATGC-3') and ACTT1R2 (5'-CGTAGCTCCATAGCATTGCT-3') for amplification of *ACTT1* and ACTT2F1 (5'-AGATGCAGCAGCCC-ATCATT-3') and ACTT2R1 (5'-CGACTGCAGATATTTTCATCC-3') for amplification of *ACTT2*. The specific DNA fragment, present only in ACR-toxin-producing isolates, was designated RP10-2. This fragment was amplified from isolated 1.5 Mb dispensable chromosome from the isolate HC1 of the rough lemon pathotype of *A. alternata* by PCR with a random primer set (RP-A30 primer, 5'-GACCTGCGATCT-3', and RP-A68 primer, 5'-GCG-GAGGAACCA-3'). The 403-bp PCR product was subcloned into pT7Blue-2 (Novagen, Madison, WI). Digoxigenin (DIG)-labeling of DNA was performed with a DIG PCR-labeling kit (Roche, Mannheim, Germany) with the same primer sets, and hybridization and washing conditions for genomic Southern blotting were conducted under high stringency according to the manufacturer's instructions. Hybridization of the probes was visualized by anti-DIG antibody-alkaline phosphatase and CSPD or SD-star (Roche) and by exposing the membranes to X-ray film (Fujifilm RX-U, Tokyo).

Preparation of protoplasts for pulse field gel electrophoresis. Fungal protoplasts were prepared by methods described previously (11) with modifications. Fungi grown on V8-juice agar plates were collected in 7 ml of sterile distilled water, and all mycelia and spores collected were added to 50 ml of PDB in 200-ml flasks. Mycelia were incubated at 28°C for 24 h with reciprocating shaking (125 rpm). Mycelia were harvested by centrifugation at 3,000 rpm for 5 min. Pelleted mycelia were washed twice with 30 ml of sterile distilled water and once with 30 ml of osmotic (OM) buffer (1.2 M MgSO₄ and 10 mM Na₂HPO₄, pH 5.8) by centrifugation at 4,000 rpm for 5 min. Then mycelia were incubated in 5 ml of sterile enzyme solution (20 mg of Lysing enzymes per ml [Sigma, St. Louis] and 3 mg of kitalase per ml [Wako, Tokyo] in OM buffer) at 28°C for 6 h with shaking at 200 rpm. After incubation, the digested culture was transferred into 15-ml centrifuge tubes (Corning, Acton, MA) and overlain with 2 ml of ST buffer (0.6 M sorbitol and 100 mM Tris-HCl, pH 8.0) and then centrifuged at 3,000 rpm for 5 min. Protoplasts banded at the interface of OM buffer and ST buffer were transferred to a new centrifuge tube (Corning). The protoplast suspension was mixed with 5 ml of STC buffer (1 M sorbitol, 50 mM CaCl₂, and 50 mM Tris-HCl, pH 8.0) and pelleted at 3,000 rpm for 5 min. Protoplasts were washed twice with 5 ml of SE buffer (1 M sorbitol and 50 mM EDTA) at 3,000 rpm for 5 min. Protoplasts were resuspended in SE buffer and adjusted to a final concentration of 5.0×10^8 protoplasts per ml. The protoplast solution was mixed with an equal volume of 1% low melting point agarose (Invitrogen, Carlsbad, CA) in SE buffer, and 100 μ l was pipetted into plug molds (Bio-Rad, Hercules, CA). Plugs were

incubated in ET buffer (0.5 M EDTA and 10 mM Tris-HCl, pH 8.0) containing protease K (0.5 mg/ml, Wako) at 50°C for 48 h. Plugs were washed three times for 1 h in 50 mM EDTA and stored at 4°C until use.

Pulse field gel electrophoresis and Southern blot hybridization. Chromosomal DNA in the plugs was separated by pulse field gel electrophoresis (PFGE) using CHEF-DRII unit (Bio-Rad). Agarose gels (0.8%, 0.5 $\times$ Tris-borate-EDTA [TBE]) were prepared with Seakem Gold agarose (BMA, Rockland, ME) and run in 0.5 $\times$ TBE buffer at 8°C. Running conditions used to separate chromosomes of less than 6 Mb were as follows: 1.5 V/cm with pulse intervals of 3,600 – 1,800 s for 115 h, 1.5 V/cm with pulse intervals of 1,800 – 1,300 s for 24 h, 1.8 V/cm with pulse intervals of 1,300 – 800 s for 28 h, and 2.4 V/cm with pulse intervals of 800 – 600 s for 28 h. Chromosomal size standards of *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Hansenula wingei* were obtained from Bio-Rad.

PFGE was used to isolate the 1.5 Mb chromosome from isolate HC1 of the rough lemon pathotype. Running conditions used to separate chromosomes of less than 2 Mb were as follows: 5.4 V/cm with pulse intervals of 120 s for 13 h and 5.4 V/cm with pulse intervals of 180 s for 13 h. *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* chromosomes were used as size standards. DNA bands were visualized by ethidium bromide staining, and the 1.5 Mb chromosome was excised from the gel and purified using NucleoTrap spin columns (Macherey-Nagel, Easton, PA) according to the manufacturer's instructions. The purified 1.5 Mb chromosome from the rough lemon pathotype HC1 was used as the template for PCR with random primers.

For transfer of PFGE-separated chromosomes to nylon membranes for Southern hybridization, chromosomal DNA in PFGE gels was nicked with 0.2 N HCl for 20 min and then soaked in 0.4 N NaOH plus 1.5 M NaCl for 30 min with shaking at 50 rpm. DNA was transferred to Hybond N⁺ membranes (Amersham Biosciences) for 48 h by alkaline capillary transfer (30) with 0.4 N NaOH plus 1.5 M NaCl. Membranes were baked at 120°C for 30 min to fix the DNA. Membranes were washed once with sterile water at room temperature for 5 min and then twice with 0.2 N NaOH plus 1% (vol/vol) sodium dodecyl sulfate at 40°C for 20 min. Prehybridization was performed at 68°C for 30 min. Probe labeling with the DIG PCR-labeling kit (Roche), hybridization, and washing conditions were conducted under high stringency according to the manufacturer's instructions. Hybridization of the probes was visualized as described previously.

RESULTS

Pathogenicity of isolate BC3-5-1-OS2A. The standard tangerine pathotype isolate SH20 induced necrotic symptoms only on the ACT-toxin-sensitive cv. Iyokan and produced no symptoms on leaves of rough lemon (Fig. 2A), whereas rough lemon pathotype isolate HC1 was pathogenic only on rough lemon and not on Iyokan (Fig. 2A). BC3-5-1-OS2A was isolated from a leaf spot lesion on rough lemon, which is sensitive to ACR-toxin and susceptible to rough lemon pathotype isolate HC1. Isolate BC3-5-1-OS2A was pathogenic on both Iyokan tangor and rough lemon (Fig. 2A). BC3-5-1-OS2A was also pathogenic to Minneola and Seminole tangelos, which are sensitive to ACT-toxin and susceptible to the tangerine pathotype (14,24).

Production of two HSTs by BC3-5-1-OS2A. The pathogenicity phenotype of isolate BC3-5-1-OS2A on both Iyokan tangor and rough lemon indicated the possibility that two distinct HSTs, ACT-toxin and ACR-toxin, are produced by this strain (Fig. 1). Since the culture filtrate of BC3-5-1-OS2A showed toxicity to both Iyokan tangor and rough lemon leaves by the leaf necrosis assay (Fig. 2B), the production of both ACT- and ACR-toxins was examined in further detail from ethyl acetate extracts of the culture filtrate by HPLC analysis for ACT-toxin and TLC analysis

for ACR-toxin (Fig. 3). HPLC analysis detected a peak from the extract of BC3-5-1-OS2A that was equivalent to authentic ACT-toxin from the tangerine pathotype SH20 with a retention time of 19.5 min (Fig. 3A). TLC analysis also detected an equivalent spot for ACR-toxin from the extract of BC3-5-1-OS2A which was the same as authentic ACR-toxin from the rough lemon pathotype HC1 with an R_f value of 0.24 (Fig. 3B). The recovered HPLC peak and TLC spot also showed identical host-selective toxicities to that of the authentic ACT- and ACR-toxins.

Presence of ACT-toxin-specific genes (*ACTTs*) in BC3-5-1-OS2A. Southern hybridization with probes made from portions of the specific genes *ACTT1* and *ACTT2*, present only in ACT-toxin-producing isolates (18), to genomic DNA of isolate BC3-5-1-OS2A demonstrated that the *ACTT* probes hybridized to genomic DNA of both SH20 and BC3-5-1-OS2A, but not to genomic DNA of rough lemon isolate HC1 (Fig. 4). Hybridization patterns of the *ACTT* probes to SH20 and BC3-5-1-OS2A were different. The *ACTT1* probe hybridized to two restriction fragments of 4.0 and 4.5 kb in SH20, but only to a single band of 4.3 kb in BC3-5-1-OS2A. The *ACTT2* probe hybridized to 1.5-, 2.0-, 3.5-, and 6.0-kb

bands in SH20 and to 1.5-, 2.0-, and 3.5-kb bands in BC3-5-1-OS2A (Fig. 4).

To determine the chromosomal location of the ACT-toxin-specific genes, Southern hybridizations of transferred PFGE gels were performed (Fig. 5). The tangerine pathotype isolate SH20 had nine chromosomes ranging from 1.9 to 5.7 Mb and gave an estimated total genome size of approximately 30 Mb (Fig. 5). Isolate BC3-5-1-OS2A had 12 chromosomes ranging from 0.6 to 5.7 Mb with a similar total genome size of about 30 Mb, but the

Fig. 2. Spray inoculation of isolate BC3-5-1-OS2A on leaves of Iyokan tanger and rough lemon. Leaves of rough lemon are sensitive to ACR-toxin and susceptible to the rough lemon pathotype of *Alternaria alternata*, while leaves of Iyokan tanger are sensitive to ACT-toxin and susceptible to the tangerine pathotype. **A**, Spray inoculation with conidia of the tangerine pathotype (isolate SH20), the rough lemon pathotype (HC1), or isolate BC3-5-1-OS2A (OS2A), or **B**, application of culture filtrates of each isolate to leaves. Water was used as a control and the leaves were incubated under the same conditions as respective treatments.

Fig. 3. High-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) determination of host-selective toxin (HST) production by *Alternaria alternata* isolate BC3-5-3-OS2A. HSTs produced by isolate BC3-5-3-OS2A (OS2A) were analyzed by **A**, HPLC for ACT-toxin and **B**, TLC for ACR-toxin. Ethyl acetate extracts of culture filtrates from isolates of BC3-5-3-OS2A in modified Richards' medium (15,16,18) were further analyzed by **A**, HPLC to detect ACT-toxin with a Develosil ODS-5 column (15). An authentic preparation of ACT-toxin (Fig. 1) was purified from culture filtrates of isolate SH20 (tangerine pathotype) by the method of Kohmoto et al. (15). The presence of ACR-toxin in the extracts was determined by TLC, and ACR-toxin was detected as a purple spot under 254 nm UV. The authentic preparation of ACR-toxin (Fig. 1) was purified from culture filtrates of isolate HC1 (rough lemon pathotype) by the method described previously (2,4,19). Arrows indicate the corresponding peak for ACT-toxin in the HPLC chart in **A** and the corresponding spot of ACR-toxin on the TLC plate in **B**.

Fig. 4. Genomic Southern analysis of isolate BC3-5-3-OS2A. Total genomic DNA of *Alternaria alternata* isolate BC3-5-1-OS2A (OS2A), standard rough lemon pathotype isolate HC1 (HC1), and standard tangerine pathotype strain SH20 (SH20) digested with *EcoRV* were fractionated in 0.8% agarose gels and transferred to nylon membranes. Blots were hybridized with probes made from portions of the ACT-toxin producer-specific genes *ACTT1* and *ACTT2* or the RP10-2 region specific to ACR-toxin producers. Sizes of marker DNA fragments (*HindIII*-digested λ DNA) are indicated at left.

To determine which chromosome carried the ACR-toxin producer-specific gene in the rough lemon pathotype, PFGE and Southern blot hybridizations were carried out (Fig. 5). The RP10-2 probe specific to the 1.5 Mb chromosome of the rough lemon pathotype HC1 hybridized to two chromosomes of 1.05 and 2.0 Mb in BC3-5-1-OS2A (Fig. 5).

Fig. 5. Chromosomal location of *ACTT* genes and the RP10-2 region in isolate BC3-5-1-OS2A. Isolate BC3-5-1-OS2A (OS2A), standard rough lemon pathotype isolate HC1 (HC1), and standard tangerine pathotype strain SH20 (SH20) were determined by Southern blots of pulse field gel electrophoresis (PFGE). Sizes of *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Hansenula wingei* marker chromosomes are indicated at left. E.B.S. indicates the ethidium bromide-stained PFGE gel, and each panel shows hybridization with each probe.

The expansion of host range may increase the fitness of pathogens because it increases their capacity for infection of and reproduction on a larger number of plant species. However, there may be a fitness cost to this expanded host range that limits the frequency of broad host range isolates like BC3-5-1-OS2A. One hypothesis is that one of the HSTs produced by BC3-5-1-OS2A could act as an elicitor of plant defense responses in toxin-insensitive plants. For example, when BC3-5-1-OS2A infects rough lemon leaves, ACR-toxin functions as a pathogenicity factor while at the same time, ACT-toxin might act as an elicitor of defense responses in rough lemon. We have recently identified a possible role of ACT-toxin as an elicitor in the toxin-insensitive

Fig. 6. Distribution of the RP10-2 region among field-collected pathogenic and nonpathogenic isolates of the rough lemon and tangerine pathotypes of *Alternaria alternata* as determined by pulse field gel electrophoresis and Southern blot. Rough lemon pathotypes are coded with HC, BC, and AC, and tangerine pathotypes are coded with SH and EV. Sizes of *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Hansenula wingei* marker chromosomes are indicated at left. Isolates producing ACR-toxin are indicated with a "+" and isolates pathogenic on rough lemon are also indicated with a "+" for pathogenicity at the bottom of the figures.

cultivar of rough lemon (5–9,20). Transcriptional analysis of several defense-related genes in rough lemon including lipoxygenase (6), hydroperoxide lyase (8), allene oxide synthase (8), epoxide hydrolase (7), polygalacturonase-inhibiting protein (9,20), chalcone synthase (9), and chitinases (5) following inoculation with isolate SH20 induced higher transcript accumulation than nonpathogenic isolate O-94. Because there appear to be no major genetic differences, either morphologically or biochemically, between SH20 and O-94, except that SH20 produces ACT-toxin (15) that is toxic to tangerines but not to rough lemon (2,3,14,15,18), ACT-toxin could be functioning as an elicitor in toxin-insensitive rough lemon. Although there is no significant difference found in necrosis caused by spray inoculation of BC3-5-1-OS2A and HC1 in the experiment described in this paper, a large number of conidia (1×10^5 conidia per ml) were inoculated in this assay, and BC3-5-1-OS2A might have reduced fitness compared with that of the pathotype producing a single HST under natural conditions where infection by pathogen is likely initiated by infection of a single conidium.

We have demonstrated that a single isolate of *A. alternata* on citrus is able to cause disease on two distinct hosts and produce two distinct HSTs. These abilities appear to be conferred through the acquisition of two small chromosomes in the same nucleus. Phylogenetic analysis based on sequence data from three regions of the genome (26) has 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 (T. L. Peever, unpublished data). BC3-5-1-OS2A is found in clade 3 of the combined phylogeny (26) containing several reference isolates of the rough lemon pathotype (T. L. Peever, unpublished data). However, BC3-5-1-OS2A has specific genes *ACTT1* and *ACTT2*, present only in the tangerine pathotype producing ACT-toxin, as well as the genomic region, which is correlated with ACR-toxin producers of the rough lemon pathotype, on two small chromosomes of 1.05 and 2.0 Mb. The sizes of these small chromosomes appear similar to other small, conditionally dispensable chromo-

somes (<2 Mb) in *Alternaria* isolates that are related to production of HSTs and pathogenicity (1,10,12,13). It is not known if the small chromosomes studied here are conditionally dispensable chromosomes but the lack of these chromosomes in nonpathogenic *A. alternata* isolates suggests that they are. The minimum chromosome size that carries all *ACTT* genes required for ACT-toxin biosynthesis is 1.1 Mb for isolates from Turkey, South Africa, and Israel (A. Masunaka and K. Akimitsu, unpublished data), and the 1.05 Mb of BC3-5-1-OS2A is similar to this size. The pathotype-specific HST genes studied here will be useful for population genetic studies of these fungi as well as for molecular evolution studies of toxin genes.

All tested isolates causing leaf spot of rough lemon that produce ACR-toxin carry the RP10-2 genomic region. This region occurs on the 1.5 Mb chromosome of isolate HC1, whereas all non-ACR-toxin-producing strain including all other HST-producing *Alternaria* pathotypes (rough lemon, tangerine, Japanese pear, strawberry, tomato, apple, and tobacco pathotypes) and nonpathogenic *A. alternata* do not have this sequence in their genomes. ACR-toxin is a polyketide and we hypothesize that the production of this toxin is under the control of a polyketide synthase that may be part of the toxin biosynthesis cluster controlling ACR-toxin biosynthesis. A BLAST search revealed that RP10-2 likely encodes a glycosyl transferase, a gene that is often tightly linked to the polyketide synthase gene in the genomes of filamentous fungi (27,28,36). It may be possible to exploit this linkage to clone and characterize the genes responsible for ACR-toxin biosynthesis. We have isolated several bacterial artificial chromosome clones from a genomic library of isolate HC1 using RP10-2 as a probe and are currently sequencing and performing functional analysis of the region surrounding RP10-2 on the 1.5 Mb chromosome (A. Masunaka and K. Akimitsu, unpublished data).

The 1.05 Mb chromosome of BC3-5-1-OS2A carries portions of at least two distinct gene clusters responsible for the production of both ACR- and ACT-toxins because *ACTT* probes as well as the RP10-2 probe hybridized to this same chromosome. The acquisition of both toxin gene clusters by isolate BC3-5-1-OS2A may have resulted in rearrangement of the genes in the clusters such as duplication or dispersal through unequal crossing over or translocation. Comparisons of chromosomal organization and toxin sequences between the 1.9 Mb chromosome of isolate SH20, 1.5 Mb chromosome of isolate HC1, and 1.05 Mb chromosome of isolate BC3-5-1-OS2A may give some insight into the evolution of toxin production and pathogenicity as well as the evolution of eukaryotic genomes.

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Fig. 7. Distribution of RP10-2 region among various host-selective toxin (HST)-producing isolates of *Alternaria alternata*. Total genomic DNA from HST-producing *A. alternata* pathotypes were digested with *EcoRV* and fractionated in 0.8% agarose gels. Blots were hybridized with the RP10-2 probe from the 1.5 Mb chromosome specific to ACR-toxin producers. Sizes of marker DNA fragments (*HindIII*-digested λ DNA) in kilobases are indicated at left. Abbreviations used in this figure are as follows: RL (rough lemon pathotype, isolates HC1 [lane 1] and AC325 [lane 2]), Ta (tangerine pathotype isolates SH14 [lane 1] and SH20 [lane 2]), JP (Japanese pear pathotype isolates 15A [lane 1] and Nu88-22 [lane 2]), St (strawberry pathotype isolates NAF-3 [lane 1] and NAF-8 [lane 2]), To (tomato pathotype isolates 91080804 [lane 1] and AL-4 [lane 2]), Ap (apple pathotype isolates O-159 [lane 1] and AM-1 [lane 2]), Tb (tobacco pathotype isolates 122-2 [lane 1] and AT-204 [lane 2]), and Non (nonpathogenic isolate O-94).

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