A Conditionally Dispensable Chromosome Controls Host-Specific Pathogenicity in the Fungal Plant Pathogen *Alternaria alternata*

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ABSTRACT

The filamentous fungus *Alternaria alternata* contains seven pathogenic variants (pathotypes), which produce host-specific toxins and cause diseases on different plants. Previously, the gene cluster involved in host-specific AK-toxin biosynthesis of the Japanese pear pathotype was isolated, and four genes, named *AKT* genes, were identified. The *AKT* homologs were also found in the strawberry and tangerine pathotypes, which produce AF-toxin and ACT-toxin, respectively. This result is consistent with the fact that the toxins of these pathotypes share a common 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid structural moiety. In this study, three of the *AKT* homologs (*AFT1-1*, *AFTR-1*, and *AFT3-1*) were isolated on a single cosmid clone from strain NAF8 of the strawberry pathotype. In NAF8, all of the *AKT* homologs were present in multiple copies on a 1.05-Mb chromosome. Transformation-mediated targeting of *AFT1-1* and *AFT3-1* in NAF8 produced AF-toxin-minus, nonpathogenic mutants. All of the mutants lacked the 1.05-Mb chromosome encoding the *AFT* genes. This chromosome was not essential for saprophytic growth of this pathogen. Thus, we propose that a conditionally dispensable chromosome controls host-specific pathogenicity of this pathogen.

HOST-SPECIFIC toxins, which are produced by plant pathogenic fungi, are generally low-molecular-weight secondary metabolites (Yoder 1980; Nishimura and Kohmoto 1983; Scheffer and Livingston 1984; Walton 1996). They are critical determinants of host-specific pathogenicity or virulence in several plant-pathogen interactions (Yoder 1980; Nishimura and Kohmoto 1983; Scheffer and Livingston 1984; Walton 1996). The imperfect fungus *Alternaria alternata* contains seven variants, which produce host-specific toxins and cause necrotic diseases on different plants (Nishimura and Kohmoto 1983; Kohmoto *et al.* 1995). Since *A. alternata* is one of the most cosmopolitan fungal species and is generally saprophytic (Rotem 1994), these host-specific forms have been designated as pathotypes of *A. alternata* (Nishimura and Kohmoto 1983; Kohmoto *et al.* 1995). *A. alternata* pathotypes are a fascinating case for studying intraspecific variation and evolution of pathogenicity in plant pathogenic fungi.

Host-specific toxins from *A. alternata* are diverse in structure (Kohmoto *et al.* 1995). However, AF-toxin of the strawberry pathotype, AK-toxin of the Japanese pear pathotype, and ACT-toxin of the tangerine pathotype have a common 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid structural moiety (Figure 1; Nakashima *et al.* 1985; Nakatsuka *et al.* 1986, 1990; Feng *et al.* 1990; Kohmoto *et al.* 1993). Thus, these three pathotypes should share genes required for biosynthesis of this common moiety.

The strawberry pathotype causes Alternaria black spot of strawberry and affects only one Japanese strawberry cultivar, Morioka-16 (Maekawa *et al.* 1984). The Japanese pear pathotype causes black spot of Japanese pear on a narrow range of susceptible cultivars, including the commercially important cultivar Nijisseiki (Tanaka 1933; Otani *et al.* 1985). The tangerine pathotype causes citrus brown spot on tangerine, grapefruit, and grapefruit × tangerine hybrids, and this disease has not occurred in Japan so far (Pegg 1966; Kohmoto *et al.* 1991, 1993; Peever *et al.* 2000). Interestingly, the strawberry and tangerine pathotypes were also found pathogenic to Japanese pear cultivars susceptible to the Japanese pear pathotype (Maekawa *et al.* 1984; Kohmoto *et al.* 1993). The host ranges of these pathotypes can be explained by toxicity of their toxins. Each of these toxins consists of multiple related molecules (Figure 1). AK-toxins I and II of the Japanese pear pathotype are toxic only to susceptible cultivars of Japanese pear (Otani *et al.* 1985). AF-toxin I of the strawberry pathotype is toxic to susceptible cultivars of both strawberry and pear; toxin II is toxic only to pear; and toxin III is toxic to strawberry (Maekawa *et al.* 1984). ACT-toxin I of the tangerine pathotype is toxic to susceptible cultivars of both citrus and pear; toxin II is more toxic to pear than to citrus (Kohmoto *et al.* 1993).

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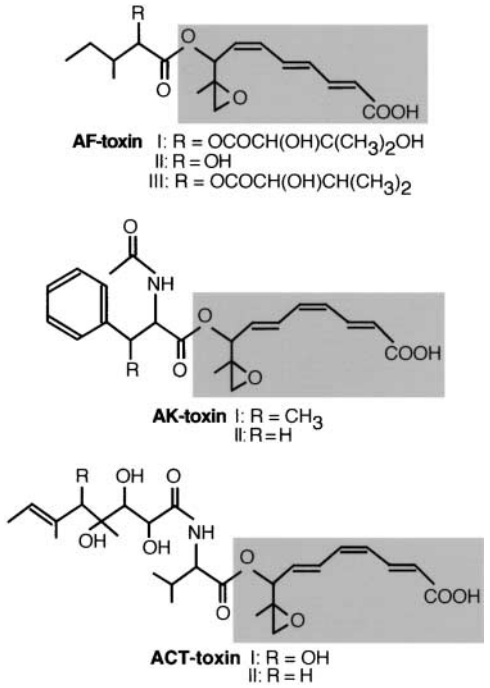

FIGURE 1.—Host-specific toxins produced by three pathotypes of *A. alternata*. AF-toxins of the strawberry pathotype (NAKATSUKA *et al.* 1986), AK-toxins of the Japanese pear pathotype (NAKASHIMA *et al.* 1985), and ACT-toxins of the tangerine pathotype (KOHMOTO *et al.* 1993) have a common moiety, 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid, indicated in shaded boxes.

We previously isolated AK-toxin-minus, nonpathogenic mutants of the Japanese pear pathotype by restriction enzyme-mediated integration (REMI) mutagenesis and selected a cosmid clone (pcAKT-1) of the wild-type strain 15A, which contains the tagged site in a mutant (TANAKA *et al.* 1999). Structural and expression analyses identified four genes (*AKT1*, *AKT2*, *AKTR-1*, and *AKT3-1*) within an ~15-kb region in pcAKT-1 (Figure 2; TANAKA *et al.* 1999; TANAKA and TSUGE 2000, 2001). Transformation of the wild type with the targeting vectors of these genes produced toxin-minus, nonpathogenic mutants. DNA gel blot analysis demonstrated that *AKT1* and *AKT2* were targeted in the mutants (TANAKA *et al.* 1999). *AKTR-1* and *AKT3-1*, however, were not disrupted in the mutants, and the targeting vectors were integrated into different regions (TANAKA and TSUGE 2000). Thus, we isolated a cosmid clone, pcAKT-2, containing the targeted DNA and found that pcAKT-2 carries two genes, *AKTR-2* and *AKT3-2*, with strong similarity to *AKTR-1* and *AKT3-1*, respectively (Figure 2; TANAKA and TSUGE 2000). DNA gel blot analysis demonstrated that the wild-type strain has multiple copies of nonfunctional homologs of all the *AKT* genes and that these genes and their homologs are on a 4.1-Mb chromosome (TANAKA *et al.* 1999; TANAKA and TSUGE 2000). These results imply the structural and functional complexity of the genomic region controlling AK-toxin biosynthesis.

In DNA gel blot analysis, these genes are unique to three pathotypes of *A. alternata*: Japanese pear, strawberry, and tangerine (TANAKA *et al.* 1999; TANAKA and TSUGE 2000). In the tangerine pathotype, polymerase chain reaction (PCR) amplification and sequence analysis of the *AKT1* and *AKT2* homologs showed that the sequences are ~90% identical to *AKT1* and *AKT2* (MASUNAKA *et al.* 2000). The *AKT* homologs in the strawberry and tangerine pathotypes are hypothesized to be the genes responsible for biosynthesis of 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid.

Our objective in this study was to characterize the structure and function of the *AKT* homologs of the strawberry pathotype. We found that all of the *AKT* homologs are located on a single chromosome of 1.05 Mb in strain NAF8 of the strawberry pathotype. We identified a genomic cosmid clone of NAF8, which contains the *AFT1-1*, *AFTR-1*, and *AFT3-1* genes with strong similarity to *AKT1*, *AKTR*, and *AKT3*, respectively. Transformation-mediated targeting of *AFT1-1* and *AFT3-1* produced AF-toxin-minus (Tox^-) mutants, which also lost pathogenicity completely. These mutants were found to lack the 1.05-Mb chromosome, which was dispensable for saprophytic growth. Thus, it appears that AF-toxin biosynthesis genes are clustered on a supernumerary chromosome.

MATERIALS AND METHODS

Fungal strains: Strains NAF1, NAF8, T-32, O-187, and M-30 of the strawberry pathotype of *A. alternata* (KUSABA and TSUGE 1994) were used in this study. NAF1 and NAF8 were collected from the same field in Aichi Prefecture, Japan (KUSABA and TSUGE 1994). T-32, O-187, and M-30 were isolated in Tottori, Iwate, and Tottori Prefectures, respectively (KUSABA and TSUGE 1994). NAF8 was used for isolation of AF-toxin biosynthesis genes. The others were used for analysis of chromosomal distribution of the *AKT* gene homologs. Strains were routinely maintained on potato dextrose agar (PDA; Difco, Detroit).

Plasmids and genomic libraries: The plasmid clones p1EX, p2XS, pRS, and p3HB, which contain the internal fragments of *AKT1*, *AKT2*, *AKTR-1*, and *AKT3-1*, respectively, in pBluescript KS+ (Stratagene, La Jolla, CA) or pGEMT Easy (Promega, Madison, WI) (Figure 2; TANAKA *et al.* 1999; TANAKA and TSUGE 2000), were used as probes in hybridization experiments. The targeting vectors pGDT1 and pGDT3 were used for transformation-mediated disruption of *AFT1-1* and *AFT3-1*, respectively, in NAF8. To make pGDT1 and pGDT3, the internal fragments of *AKT1* and *AKT3-1*, respectively, were cloned in a transformation vector pSH75 (Figure 2; TANAKA *et al.* 1999; TANAKA and TSUGE 2000), which carries the hygromycin B resistance gene (*hph*) fused to the *Aspergillus nidulans trpC* promoter and terminator (MULLANEY *et al.* 1985; KIMURA and TSUGE 1993). The plasmid clone pGDB2-2, which contains the *A. alternata BRM2* gene, was used as a probe for hybridization experiments. *BRM2* encodes 1,3,8-trihydroxy-naphthalene reductase involved in melanin biosynthesis (KAWAMURA *et al.* 1999).

A genomic cosmid library of strain NAF8 was constructed with a cosmid vector pMLF2 (AN *et al.* 1996) using half-site fill-in reactions (SAMBROOK *et al.* 1989). Total DNA of NAF8 was partially digested with *Sau*3AI to generate fragments of ~40 kb and partially filled with dATP and dGTP. The cosmid

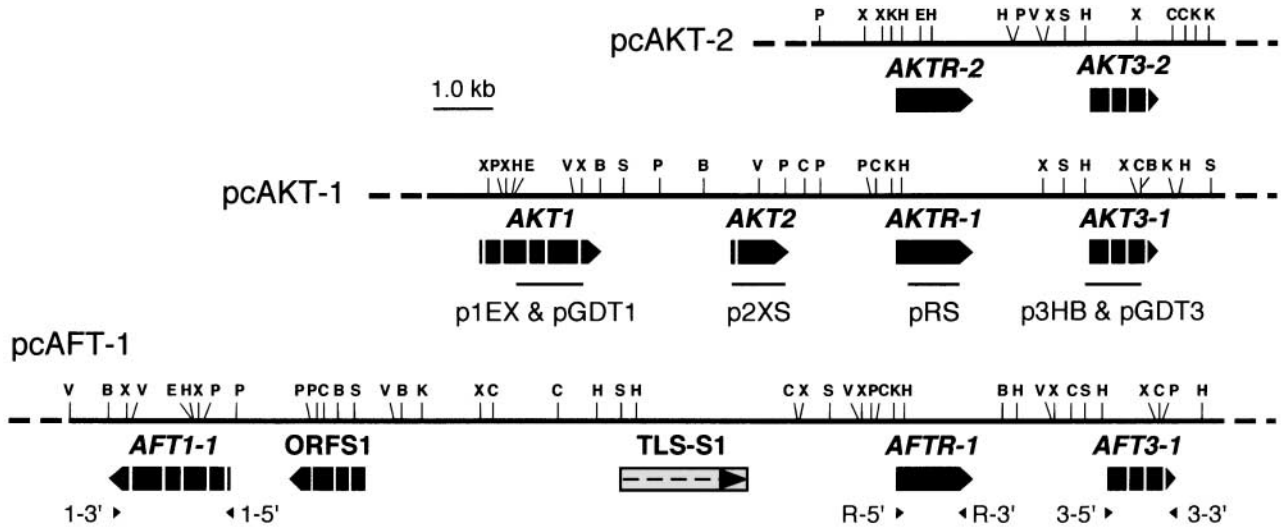

FIGURE 2.—Partial structure of cosmid clones pcAKT-1, pcAKT-2, and pcAFT-1. The cosmid clones pcAKT-1 and pcAKT-2 were isolated from a genomic library of strain 15A of the Japanese pear pathotype (TANAKA *et al.* 1999; TANAKA and TSUGE 2000). The cosmid clone pcAFT-1 was isolated from a genomic library of strain NAF8 of the strawberry pathotype in this study. Arrows indicate the protein coding regions, and white segments indicate positions of introns. *AFT1-1*, *AFTR-1*, and *AFT3-1* are the *AKT1*, *AKTR*, and *AKT3* homologs, respectively. The plasmid clones p1EX, p2XS, pRS, and p3HB were made by cloning the indicated fragments in pBluescript KS+ or pGEM-T Easy (TANAKA *et al.* 1999; TANAKA and TSUGE 2000) and used as probes for hybridization experiments. The fragments from p1EX and p3HB were cloned in pSH75 (KIMURA and TSUGE 1993) to make the targeting vectors pGDT1 and pGDT3, respectively (TANAKA *et al.* 1999; TANAKA and TSUGE 2000). Arrowheads denote the orientation and location of oligonucleotide primers used in RT-PCR experiments. B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sph*I; V, *Eco*RV; X, *Xho*I.

vector pMLF2 was completely digested with *Xho*I and partially filled with dCTP and dTTP. Partially filled *Sau*3AI genomic DNA fragments were cloned at the partially filled *Xho*I site of pMLF2 to construct a genomic library. Screening of the library by colony hybridization was conducted by the standard method (SAMBROOK *et al.* 1989).

Fungal transformation: Protoplast preparation and transformation of *A. alternata* were performed by the methods previously described (TSUGE *et al.* 1990; SHIOTANI and TSUGE 1995). Colonies that appeared 5–10 days after plating on the selective regeneration media (TSUGE *et al.* 1990) were transferred to PDA containing hygromycin B (Wako Pure Chemicals, Osaka, Japan) at 100 μg/ml, and transformants were selected after incubation at 25° for 5 days.

Assay for AF-toxin production, pathogenicity, and vegetative growth: The wild type and transformants were grown statically in 5 ml of potato dextrose broth (PDB, Difco) in test tubes at 25° for 7 days, and culture filtrates and mycelial mats were harvested. Culture filtrates were tested for toxicity to leaves of strawberry cultivar Morioke-16 and Japanese pear cultivar Nijisseiki as previously described (MAEKAWA *et al.* 1984; TANAKA *et al.* 1999). AF-toxin and 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid in culture filtrates were extracted and quantified by reverse-phase high-performance liquid chromatography as previously described (FENG *et al.* 1990; HAYASHI *et al.* 1990; TANAKA *et al.* 1999).

Mycelial mats were used for preparation of conidia as previously described (HAYASHI *et al.* 1990). Pathogenicity was assayed by spray inoculation of conidial suspension ($\sim 5 \times 10^5$ conidia/ml) to leaves of cultivar Morioka-16 and cultivar Nijisseiki as previously described (MAEKAWA *et al.* 1984; HAYASHI *et al.* 1990; TANAKA *et al.* 1999).

The wild type and transformants were grown on PDA at 25° for 4 days. Agar blocks (3 mm in diameter) carrying mycelia were prepared from the resulting colonies and inoculated on PDA. After incubation at 25° for 4 days, colony growth and morphology were observed. Prototrophy of transformants was tested on a minimal agar medium (10 g KNO_3, 5 g KH_2PO_4, 2.5 g $MgSO_4 \cdot 7H_2O$, 0.02 g $FeCl_3$, 10 g glucose, 20 g agar per liter).

DNA manipulations: Isolation of total DNA and RNA from *A. alternata*, isolation of plasmid and cosmid DNA, agarose gel electrophoresis, and DNA gel blot hybridization were performed as previously described (TANAKA *et al.* 1999). Hybridized blots were washed twice in 2× SSPE [1× SSPE: 180 mM NaCl, 10 mM NaH_2PO_4 (pH 7.7) and 1 mM EDTA] plus 0.1% sodium dodecyl sulfate at 65° for 10 min and once in 1× SSPE plus 0.1% sodium dodecyl sulfate at 65° for 10 min.

Reverse transcription-polymerase chain reaction (RT-PCR) was performed using the RNA PCR kit, version 2.1 (Takara, Shiga, Japan), according to the manufacturer's instructions. The following primers were used: 1-5′ (5′-CTACCGCCTGAG TACATGCGTC-3′) and 1-3′ (5′-AGCAACAGCACCCTGGGG TT-3′) for *AFT1-1*; R-5′ (5′-GCATGGGGACAAGATCCAG-3′) and R-3′ (5′-CCAACACAGATGCTGAACTT-3′) for *AFTR-1*; and 3-5′ (5′-CCTGCGAAACTCTACCTCTG-3′) and 3-3′ (5′-TCA GAGCTTTGGCTTGGAAG-3′) for *AFT3-1* (Figure 2).

For analysis of nucleotide sequences, DNA was cloned in pBluescript KS+. DNA sequences were determined using the PRISM dye termination cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) and an automated fluorescent DNA sequencer (Model 373A, Applied Biosystems) according to the manufacturer's instructions. DNA sequences were analyzed with BLAST (ALTSCHUL *et al.* 1997). Alignment of nucleotide and amino acid sequences was made using the CLUSTAL W program (THOMPSON *et al.* 1994).

Pulsed-field gel electrophoresis (PFGE): Chromosome-sized DNA molecules were prepared from fungal protoplasts as previously described (ADACHI *et al.* 1996). PFGE was carried out on a contour-clamped homogeneous electric field (CHU *et al.* 1986) apparatus (CHEF-DRII, Bio-Rad Laboratories, Hercules, CA) using 0.5× TBE (SAMBROOK *et al.* 1989) at 8° in 0.8% agarose gel (Seakem Gold agarose, BioWhittaker Molecular Applications, Rockland, ME). The following electrophoresis

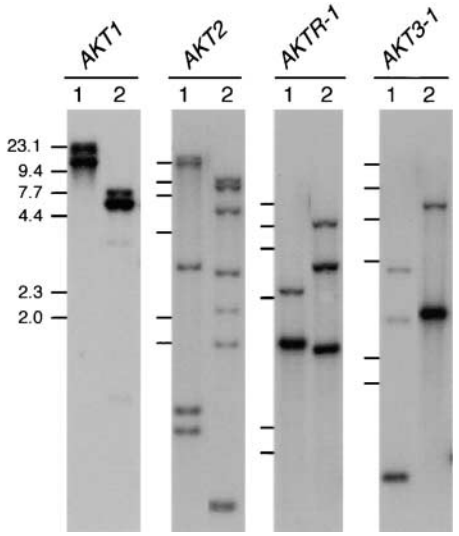

FIGURE 3.—Distribution of the *AKT* homologs in strain NAF8. Total DNA of NAF8 was digested with *Cla*I (lane 1) or *Eco*RV (lane 2) and separated in 0.8% agarose gel. The blots were probed with the *AKT1*, *AKT2*, *AKTR-1*, and *AKT3-1* fragments from p1EX, p2XS, pRS, and p3HB, respectively (Figure 2). Sizes (in kilobases) of marker DNA fragments (*Hin*dIII-digested λDNA) are indicated on the left.

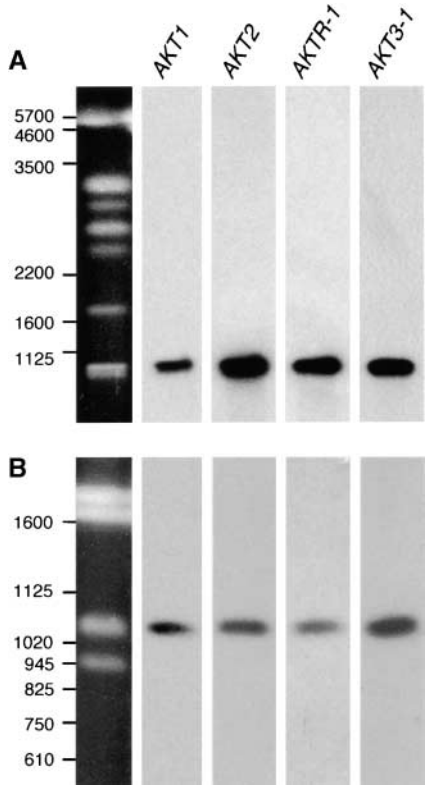

FIGURE 4.—Chromosomal distribution of the *AKT* homologs in strain NAF8. Chromosome-sized DNA molecules of strain NAF8 were separated by pulsed-field gel electrophoresis under the condition for 1.0–6.0 Mb DNA (A) and for <2.0 Mb DNA (B). The blots were probed with the *AKT1*, *AKT2*, *AKTR-1*, and *AKT3-1* fragments from p1EX, p2XS, pRS, and p3HB, respectively (Figure 2). Sizes (in kilobases) of chromosomes of *S. pombe* and *S. cerevisiae* (A) and of *S. cerevisiae* (B) are indicated on the left.

conditions (duration/voltage/linear gradient of switching time; MORALES *et al.* 1993) were used: for separating 1.0–6.0 Mb DNA, 115 hr/50 V/3600–1800 sec, 24 hr/50 V/1800–1300 sec, 28 hr/60 V/1300–800 sec, and 28 hr/80 V/800–600 sec, and, for separating <2.0 Mb DNA, 13 hr/180 V/120 sec and 13 hr/180 V/180 sec. The gels were stained with ethidium bromide for 30 min and destained in distilled water for 30 min. Size standards were chromosome preparations of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* (BioWhittaker Molecular Applications).

DNA gel blotting and hybridization were performed as previously described (ADACHI *et al.* 1996). Chromosomal DNA separated by PFGE was excised from the gel, nicked by UV irradiation, and purified using the GeneClean II kit (Bio 101, Vista, CA). Recovered DNA was used as a probe in hybridization experiments.

RESULTS

Distribution of the *AKT* homologs in the strawberry pathotype strains: We previously showed that the *AKT1*, *AKT2*, *AKTR*, and *AKT3* probes hybridized to DNA of all strains of the strawberry pathotype collected from different locations in Japan (TANAKA *et al.* 1999; TANAKA and TSUGE 2000). Hybridization patterns were similar in these strains and suggested that the homologs are present in multiple copies (TANAKA *et al.* 1999; TANAKA and TSUGE 2000). In the present study, strain NAF8 was selected, because it showed the high level of pathogenicity and of AF-toxin production.

The DNA of strain NAF8 was digested separately with 12 restriction enzymes and probed with the *AKT1*, *AKT2*, *AKTR-1*, and *AKT3-1* fragments from p1EX, p2XS, pRS, and p3HB, respectively (Figures 2 and 3). Figure 3 shows blots of *Cla*I- or *Eco*RV-digested DNA. All probes hybridized to multiple bands (Figure 3). To confirm complete digestion of DNA, the blots were subsequently stripped of the probes and rehybridized with the *BRM2* fragment from pGDB2-2 (KAWAMURA *et al.* 1999) as a single-copy gene probe. The *BRM1* fragment has no *Cla*I and *Eco*RV sites. The *BRM2* probe hybridized to a single band (6.6-kb *Cla*I fragment or 13.0-kb *Eco*RV fragment) in each lane (data not shown). These results suggest that NAF8 has multiple copies of each homolog of four *AKT* genes.

To investigate the genomic distribution of the *AKT* homologs, the chromosome-sized DNA of NAF8 was separated by PFGE, and the blot was probed with the *AKT1*, *AKT2*, *AKTR-1*, and *AKT3-1* fragments (Figures 2 and 4). Under the electrophoresis condition for separating 1.0–6.0 Mb DNA, at least 10 chromosomal DNAs of ~1.0–5.7 Mb were separated (Figure 4A). All probes hybridized to a single band of ~1.0 Mb. Chromosome-sized DNA was also separated by PFGE under the condition for providing good resolution of <2.0 Mb DNA. Under this condition, ~1.0 Mb DNA detected in Figure 4A was resolved into two chromosomal DNAs of ~0.95 and 1.05 Mb (Figure 4B). All probes hybridized to the

1.05 Mb DNA, indicating that the small chromosome encodes all of the *AKT* homologs (Figure 4B).

The chromosomal distribution of the *AKT* homologs in other strains of the strawberry pathotype was also examined. Strains NAF1 and NAF8 were collected from the same field in Aichi Prefecture, Japan, and the others were from different prefectures (Kusaba and Tsuge 1994). Chromosome-sized DNA of each strain was separated by PFGE under the condition for <2.0 Mb DNA. All strains had 1.05 Mb chromosomal DNA (Figure 5). NAF1, T-32, and M-30 had additional small DNAs of ~0.95, 1.13, and 0.37 Mb, respectively (Figure 5). The gel blot was hybridized with the *AKT1*, *AKT2*, *AKTR-1*, and *AKT3-1* probes. Figure 5 shows the blot hybridized with the *AKT1* probe. All probes hybridized to the 1.05 Mb DNA in all strains (Figure 5). The 1.13 Mb DNA of T-32 was also hybridized with all probes (Figure 5). All of the *AKT* homologs are located on small chromosomes in the strawberry pathotype strains.

Isolation of cosmid clones containing the *AKT* homologs: A cosmid genomic library of strain NAF8 was screened with the *AKT2* or *AKTR-1* probes (p2XS and pRS inserts, respectively; Figure 2), and 39 positive clones were isolated. These clones were examined for distribution of the *AKT* homologs by DNA gel blot analysis with the *AKT1*, *AKT2*, *AKTR-1*, and *AKT3-1* probes. This analysis divided these clones into at least four groups (Groups 1–4), which contain *AKT1-AKTR-AKT3*, *AKTR-AKT3*, *AKT1-AKT2*, and *AKT1-AKT2-AKTR* homologs, respectively. No clones had homologs for all four *AKT* genes.

Preliminary mapping of the *AKT* homologs in the representative clones of four groups suggested that these clones did not correspond to overlapping fragments of a single cluster of the *AKT* homologs. The cosmid clones pcAFT-1 (Group 1) and pcAFT-4 (Group 4) contain three homologs in the order *AKT1-AKTR-AKT3* homologs and *AKT1-AKT2-AKTR* homologs, respectively. The cosmid clone pcAFT-2 (Group 2) has the *AKTR* and *AKT3* homologs with an ~31-kb region upstream of the *AKTR* homolog. The cosmid clone pcAFT-3 (Group 3) contains the *AKT1* and *AKT2* homologs with an ~29-kb region downstream of the *AKT2* homolog. The 31-kb and 29-kb regions in pcAFT2 and pcAFT-3, respectively, are apparently longer in size than the intergenic region upstream of the *AKTR* homologs in pcAFT-1 and pcAFT-4 (data not shown). These results strongly suggest that NAF8 has multiple clusters of the *AKT* homologs, which are different in distribution patterns of the homologs. Here we analyzed detailed structure of pcAFT-1 (Figure 2).

Structure of pcAFT-1: The nucleotide sequence of a 32-kb region in pcAFT-1 was determined, and complete open reading frames (ORFs) with high similarity to *AKT1*, *AKTR-1*, and *AKT3-1* were identified within a 24-kb region (Figure 2). We designated the *AKT1*, *AKTR-1*, and *AKT3-1* homologs in pcAFT-1 as *AFT1-1*, *AFTR-1*, and *AFT3-1*, respectively (GenBank accession

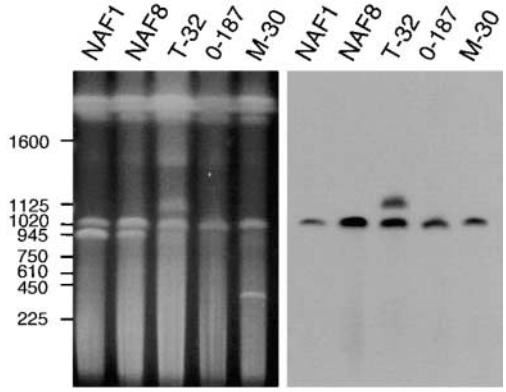

Figure 5.—Chromosomal distribution of the *AKT* homologs in the strawberry pathotype strains. Chromosome-sized DNA molecules of strains NAF1, NAF8, T-32, O-187, and M-30 were separated by pulsed-field gel electrophoresis under the condition for <2.0 Mb DNA (left). (Right) The blots were probed with the *AKT1* fragment from p1EX (Figure 2). Sizes (in kilobases) of chromosomes of *S. cerevisiae* are indicated on left.

nos. AB070711, AB070712, and AB070713; Figure 2). Figure 2 shows the map of the 25-kb region containing these genes. The remaining 7.0-kb region downstream of *AFT3-1* contained no putative ORFs with significant size, although short sequences homologous to fungal transposons were detected.

AFT1-1, *AFTR-1*, and *AFT3-1* were deduced to consist of six, one, and four exons, respectively, on the basis of alignments with *AKT1*, *AKTR-1*, and *AKT3-1* sequences. Introns of *AFT1-1* and *AFT3-1* contain consensus sequences for 5′ splice sites [GT(A/G/T)(A/C/T)G(T/C)] and 3′ splice sites [(C/T)AG], which is typical of fungal genes (Bruchez *et al.* 1993). The cosmid clone pcAKT-1 of the Japanese pear pathotype encodes *AKT1*, *AKT2*, *AKTR-1*, and *AKT3-1* on the same strand within a 15-kb region (Figure 2). However, pcAFT-1 lacks the *AKT2* homolog and encodes *AFT1-1* on the different strand from *AFTR-1* and *AFT3-1* (Figure 2).

The intergenic region between *AFT1-1* and *AFTR-1* was found to encode an additional ORF, ORFS1, and a transposon-like sequence, TLS-S1 (*t*ransposon-*l*ike *s*equence of the *s*trawberry pathotype 1; Figure 2). These sequences have not been detected in the *AKT* cluster of the Japanese pear pathotype. ORFS1 possibly encodes 366 amino acids after splicing three introns. TLS-S1 has significant similarity to transposase genes of *restless* of *Tolypocladium inflatum* (Kempken and Kück 1996) and *Tfo1* of *Fusarium oxysporum* (Okuda *et al.* 1998), which are members of the hAT transposon family. TLS-S1, however, contains several stop codons and is probably a pseudogene of transposase. Structural and functional characterization of ORFS1 and TLS-S1 will be reported elsewhere.

Structure of the *AFT* genes: *AFT1-1* and *AKT1* are almost identical in sequence (Figure 6). *AFT1-1* potentially encodes a 578-amino-acid protein, the same size

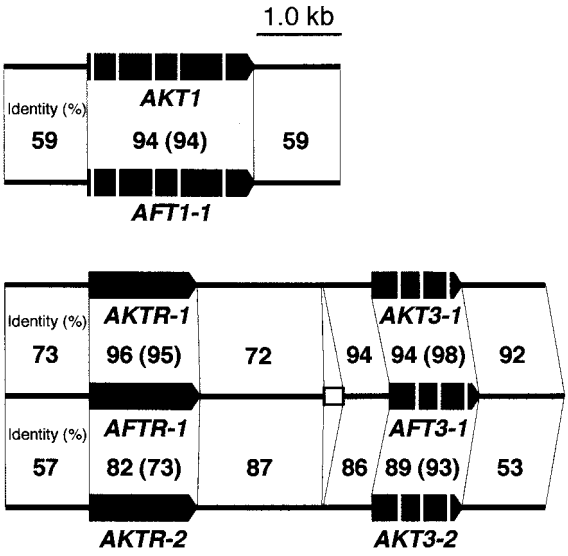

FIGURE 6.—Comparison of structure of the *AFT* and *AKT* genes. Identity of amino acid sequences of the gene products is shown in parentheses. The open box in the intergenic region between *AFTR-1* and *AFT3-1* indicates the specific insertion sequence of 185 bp.

as Akt1 (TANAKA *et al.* 1999). *AFT1-1* and *AKT1* both consist of six exons of the same size (Figure 6). Of five introns, only the fourth intron is different in size (*AFT1-1* = 60 bp; *AKT1* = 61 bp). As with Akt1, Aft1-1 reveals similarity to carboxyl-activating enzymes, such as 4-coumarate-CoA ligases of higher plants, long chain acyl-CoA synthetases of microorganisms, and luciferases of insects (PAVELA-VRANCIC *et al.* 1994; TANAKA *et al.* 1999). Aft1-1 and Akt1 terminate with Ser-Lys-Ile, a peroxisomal targeting signal type 1 (PTS1) tripeptide that conforms to the PTS1 consensus motif established for *S. cerevisiae* (ELGERSMA *et al.* 1996).

AFTR-1 is a single exon encoding a 445-amino-acid protein. It is more similar to *AKTR-1* than to *AKTR-2* (Figure 6). As with AktR-1 and AktR-2, AftR-1 contains two recognizable domains: a zinc binuclear cluster DNA-binding domain (Cys-X_2-Cys-X_6-Cys-X_6-Cys-X_2-Cys-X_6-Cys) in the amino terminal region and an internal leucine zipper domain (Leu-X_6-Leu-X_6-Leu-X_6-Leu) (TODD and ANDRIANOPOULOS 1997; TANAKA and TSUGE 2000). Proteins with the zinc binuclear cluster DNA-binding domain are known to be typical regulatory factors in fungi and have been placed in a single protein family, designated Zn(II)2Cys6 (TODD and ANDRIANOPOULOS 1997).

AFT3-1 encodes a protein of 296 amino acids. *AFT3-1*, *AKT3-1*, and *AKT3-2* all consist of four exons of the same size (Figure 6; TANAKA and TSUGE 2000). Of three introns, only the second intron is different in size (*AFT3-1* = 69 bp; *AKT3-1* = 56 bp; *AKT3-2* = 60 bp). *AFT3-1* is more similar to *AKT3-1* than to *AKT3-2* (Figure 6). As with Akt3-1 and Akt3-2, Aft3-1 has similarity to members of the hydratase/isomerase enzyme family, such as enoyl-CoA hydratases, crotonases, and naphthoate synthases of microorganisms (MÜLLER-NEWEN and STOFFEL 1993; TANAKA and TSUGE 2000). Aft3-1 terminates with the same PTS1 tripeptide, Pro-Lys-Leu, as Akt3-1 and Akt3-2 (ELGERSMA *et al.* 1996; TANAKA and TSUGE 2000).

The 5′ and 3′ flanking sequences of *AFT3-1* and *AKT3-1* ORFs are highly conserved (Figure 6). However, the flanking sequences of the *AFT1-1* and *AFTR-1* ORFs show lower homology with those of the *AKT1* and *AKTR* ORFs (Figure 6). The intergenic region between *AFTR-1* and *AFT3-1* is longer than that between *AKTR* and *AKT3* due to a specific insertion of 185 bp (Figure 6).

Expression of the *AFT* genes: Transcription of *AFT1-1*, *AFTR-1*, and *AFT3-1* in strain NAF8 was investigated by RT-PCR. The *AFT1-1*-, *AFTR-1*-, and *AFT3-1*-specific primer pairs (1-5′/1-3′, R-5′/R-3′, and 3-5′/3-3′, respectively; Figure 2) produced DNA fragments of 1.58, 0.92, and 0.92 kb, respectively, from total RNA of strain NAF8 (data not shown). Control reactions without reverse transcriptase produced no DNA fragments (data not shown), indicating that the RT-PCR products were amplified from cDNA templates. The sizes of RT-PCR products for *AFT1-1* and *AFT3-1* corresponded to those expected, when five and three introns, respectively, were spliced. Thus, *AFT1-1*, *AFTR-1*, and *AFT3-1* are transcribed in NAF8.

Mutation of *AFT1* and *AFT3* by gene targeting: To determine the function of *AFT1-1* and *AFT3-1* in AF-toxin biosynthesis, homologous recombination was employed to disrupt these genes in strain NAF8 with pGDT1 and pGDT3, which contain internal sequences of *AKT1* and *AKT3-1*, respectively, of the Japanese pear pathotype in a fungal transformation vector pSH75 (Figure 2; TANAKA *et al.* 1999; TANAKA and TSUGE 2000). The targeting vectors pGDT1 and pGDT3 were expected to be useful to disrupt *AFT1-1* and *AFT3-1*, respectively, in the strawberry pathotype, because the *AKT1* and *AKT3-1* sequences in these vectors are ~94% identical to the corresponding regions of *AFT1-1* and *AFT3-1*, respectively. Strain NAF8 was transformed with pGDT1 and pGDT3, and 162 and 92 transformants, respectively, were selected. AF-toxin production of transformants was evaluated on the basis of toxicity of culture filtrates to leaves of strawberry cultivar Morioka-16 and Japanese pear cultivar Nijisseiki. Two pGDT1 transformants (GD1-1 and GD1-2) and one pGDT3 transformant (GD3-1) were found to lose toxin production. When culture filtrates were applied to three leaves of each plant, culture filtrate of the wild type showed toxicity to all leaves of strawberry and pear, but those of GD1-1, GD1-2, and GD3-1 showed no toxicity to both plants (Figure 7A). When conidial suspensions were spray inoculated on three leaves of each plant, the wild type caused a number of spots on all leaves, but the transformants caused no spots on the leaves (Figure 7B). We used three sets of culture filtrates and conidia separately

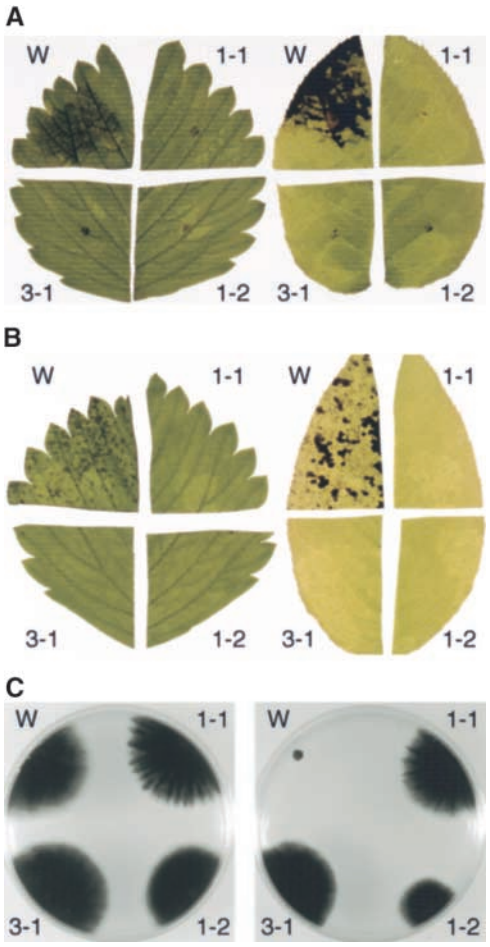

FIGURE 7.—AF-toxin production (A), pathogenicity (B), and colony growth (C) of the wild-type strain and Tox$^-$ transformants. Tox$^-$ transformants GD1-1 (1-1) and GD1-2 (1-2) were produced by transformation of the wild-type strain NAF8 (W) with the *AFT1*-targeting vector pGDT1; GD3-1 (3-1) was produced by transformation of NAF8 with the *AFT3*-targeting vector pGDT3 (Figure 2). (A) Leaves of strawberry cultivar Morioka-16 (left) and Japanese pear cultivar Nijisseiki (right) were wounded slightly, treated with culture filtrates, and incubated in a moist box at 25° for 20 hr. (B) Leaves were spray inoculated with conidial suspensions (~5×10^5 conidia/ml) and incubated in a moist box at 25° for 20 hr. (C) The wild type and transformants were grown on PDA (left) and PDA containing 100 μg/ml hygromycin B (right) at 25° for 4 days.

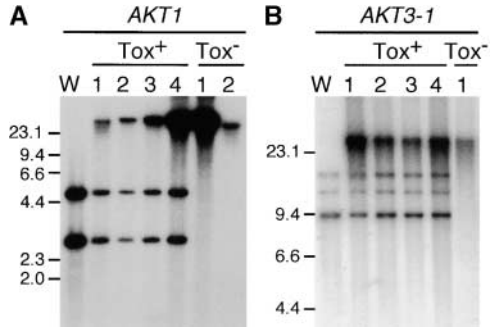

FIGURE 8.—DNA gel blot analysis of pGDT1 transformants (A) and pGDT3 transformants (B). Total DNA from the wild-type strain NAF8 (W), toxin-producing (Tox$^+$), or toxin-minus (Tox$^-$) transformants was digested with *Hin*dIII (A) or *Kpn*I (B) and fractionated in 0.8% agarose gel. The blots were probed with the *AKT1* (A) and *AKT3-1* (B) fragments from pGDT1 and pGDT3, respectively (Figure 2). Sizes (in kilobases) of marker DNA fragments (*Hin*dIII-digested λDNA) are indicated on the left.

prepared and confirmed loss of toxin production and pathogenicity in these transformants.

AF-toxin has been characterized as three related molecular species: AF-toxins I, II, and III (Figure 1), with toxin I being the predominant species with respect both to yield and to biological activity (MAEKAWA *et al.* 1984; NAKATSUKA *et al.* 1986; HAYASHI *et al.* 1990). Thus, toxins I, II, and III were quantified by reverse-phase high-performance liquid chromatography. Toxins I and II were detected in culture filtrates of the wild-type strain (1.81 and 0.11 μg/ml, respectively, in the averages of three cultures), but not in those of GD1-1, GD1-2, and GD3-1. Toxin III was not detectable in the wild type and transformants. The level of a precursor of AF-toxin, 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid (FENG *et al.* 1990; NAKATSUKA *et al.* 1990), in culture filtrates was also quantified by high-performance liquid chromatography. Culture filtrates of the wild type contained the precursor molecule (0.75 μg/ml in the average of three cultures). In contrast, the same analysis could not detect the precursor molecule in culture filtrates of the Tox$^-$ transformants.

All the Tox$^-$ transformants were prototrophic (data not shown). To compare vegetative growth between the wild type and Tox$^-$ transformants, they were grown together on three PDA plates at 25° for 4 days. On all plates, colony diameter of GD1-1 was similar to that of the wild type, but its colony morphology was different (Figure 7C). Colony growth of GD1-2 was slightly slower than that of the wild type (Figure 7C). Colony growth and morphology of GD3-1 were indistinguishable from those of the wild type (Figure 7C). The wild type and transformants were also grown on three PDA plates containing hygromycin B at 100 μg/ml. On all plates, colony diameter of GD1-2 was smaller than that of GD1-1 or GD3-1 (Figure 7C). This seemed to be also due to differences in copy number of the integrated *hph* cassette as shown below. When conidia of the Tox$^-$ transformants were suspended in water, placed on glass slides, and incubated at 25°, they normally germinated and formed appressoria within 20 hr. These results strongly suggest that mutations, which affect pathogenicity in these transformants, are attributable to loss of AF-toxin production.

DNA gel blot analysis of the *AFT1*- or *AFT3*-targeted mutants: The mode of integration of the vector in a subset of toxin-producing (Tox$^+$) and Tox$^-$ transformants was analyzed by DNA gel blot hybridization (Figure 8). Total DNA of the wild type and pGDT1 transformants was digested with *Hin*dIII, which has no

site in pGDT1 and a single site in *AFT1-1*, and the blot was probed with the *AKT1* fragment inserted in pGDT1 (Figures 2 and 8A). The *Hin*dIII site in *AFT1-1* is outside the region hybridizing to the *AKT1* probe (Figure 2). The probe hybridized to two bands in the wild type (Figure 8A), showing the presence of multiple copies of the *AFT1* homolog. Although Tox^+ transformants preserved these two bands, Tox^- transformants lost both bands (Figure 8A). Tox^+ and Tox^- transformants both carried high-molecular-weight hybridizing bands (>23 kb), suggesting integration of multiple copies of plasmids as tandem repeats (Figure 8A).

Total DNA of pGDT3 transformants was digested with *Kpn*I, which does not digest within pGDT3 and *AFT3-1*, and probed with the *AKT3-1* fragment inserted in pGDT3 (Figures 2 and 8B). The probe hybridized to three bands in the wild type (Figure 8B), showing the presence of multiple copies of the *AFT3* homolog. Tox^+ transformants preserved these three bands, and Tox^- transformants lost all of these bands (Figure 8B). The pGDT3 transformants also carried high-molecular-weight hybridizing bands (>23 kb) resulting from the integration of multiple copies as tandem repeats (Figure 8B).

DNA gel blot analysis suggested that all copies of the *AFT1* or *AFT3* gene were targeted in the Tox^- transformants. To determine whether the DNA flanking *AFT1* or *AFT3* was altered in the Tox^- transformants, the *Hin*dIII-digested DNA of pGDT1 transformants and the *Kpn*I-digested DNA of pGDT3 transformants were hybridized with other *AKT* gene probes. The *AKT2*, *AKTR-1*, and *AKT3-1* probes did not hybridize to DNA of the *AFT1*-targeted, Tox^- transformants (data not shown). The *AKT1*, *AKT2*, and *AKTR-1* probes did not hybridize to DNA of the *AFT3*-targeted, Tox^- transformant (data not shown). Thus, it appeared that these Tox^- transformants lost all of the *AKT* homologs through deletion of a large part of the region controlling AF-toxin biosynthesis.

Electrophoretic karyotypes of the *AFT1*- or *AFT3*-targeted mutants: To gain additional information on the extent of the deletion, which occurred in the Tox^- mutants, the chromosome-sized DNA of the wild type and mutants were separated by PFGE under the condition for <2.0 Mb DNA (Figure 9A). All mutants were found to lack the 1.05-Mb chromosome, which carries all of the *AKT* homologs in the wild type (Figures 4 and 9A). No deletion products that originated from the 1.05-Mb chromosome were found in the 0.2- to 0.9-Mb region on the gel (Figure 9A). The pGDT1 transformant GD1-1 also lacked the 0.95-Mb chromosome (Figure 9A). The chromosome-sized DNA of the wild type and mutants were also separated by PFGE under the condition for 1.0–6.0 Mb DNA (Figure 9B). No other alterations were observed in the karyotype of GD1-2 (Figure 9B). However, GD1-1 also lacked a chromosomal DNA of ~2.9 Mb, and the ethidium bromide-stained signal of ~3.3 Mb DNA increased (Figure 9B). This suggested that the 2.9-Mb chromosome was increased in size to 3.3 Mb in GD1-1. In the pGDT3 transformant GD3-1, the signal of 3.3 Mb DNA decreased, and a larger-sized DNA of ~3.4 Mb newly appeared (Figure 9B). The wild type probably has two chromosomes of ~3.3 Mb, one of which was increased in size in GD3-1. These results indicated the possibility that the translocation of part of the 1.05-Mb chromosome to other chromosomes occurred in GD1-1 and GD3-1.

To detect such translocation in the mutants, the 1.05 Mb DNA of the wild type was recovered and used as a probe for hybridization of the gel blots. The probe hybridized to the 1.05 Mb DNA in the wild type, but apparently not to any chromosomes in the mutants (Figure 9). Thus, it seemed that the translocation of the 1.05-Mb chromosome did not occur in the mutants. Longer exposure of the hybridized blot detected the 3.3 Mb DNA in GD1-1 with very weak signal. Such weak signal was probably due to hybridization of the probe to multiple copies of the *AKT1* fragment in pGDT1 introduced into the chromosome as shown below.

Examination of the chromosomal distribution of targeting vectors in the mutants showed that integration of multiple copies of targeting vectors into the 2.9- and 3.3-Mb chromosomes of the wild type produced the 3.3- and 3.4-Mb chromosomes of GD1-1 and GD3-1, respectively. The gel blot shown in Figure 9B was stripped of the 1.05-Mb DNA probe and rehybridized with the backbone plasmid pSH75 of pGDT1 and pGDT3. This probe hybridized to 3.3, 2.6, and 3.4 Mb DNA of GD1-1, GD1-2, and GD3-1, respectively (Figure 9B). The hybridization signals in GD1-1 and GD3-1 were markedly intense compared with that in GD1-2 (Figure 9B). This result revealed that the 2.6 Mb DNA of GD1-2 contains single or few copies of targeting vector and that the 3.3 and 3.4 Mb DNA of GD1-1 and GD3-1, respectively, contain many copies of targeting vectors. The integration of pGDT1 or pGDT3 in these chromosomes of transformants was confirmed by rehybridizing the blot with the *AKT1* and *AKT3-1* fragments from pGDT1 and pGDT3, respectively (Figure 9B). The 3.3 Mb DNA of GD1-1 possibly resulted from the integration of ~60 copies of pGDT1 (6.6 kb) into the original 2.9-Mb chromosome. The 3.4 Mb DNA of GD3-1 could be originated through the integration of ~15 copies of pGDT3 (6.5 kb) into the 3.3-Mb chromosome. Such multiple-copy integration of transformation vectors is usual in transformation of *A. alternata* (Tsuge *et al.* 1990; Shiotani and Tsuge 1995; Tanaka and Tsuge 2000). It appeared that these mutants originated through loss of the 1.05-Mb chromosome simultaneously with ectopic integration of pGDT1 or pGDT3 into other chromosomes.

DISCUSSION

Here we found that the *AKT* homologs are present in multiple copies on a single chromosome of 1.05 Mb in strain NAF8 of the strawberry pathotype. We identified

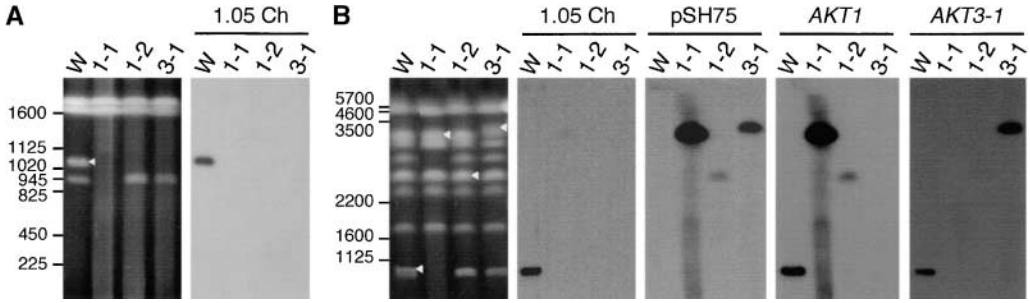

FIGURE 9.—Electrophoretic karyotypes of Tox⁻ transformants. Chromosome-sized DNA molecules of the wild-type strain NAF8 (W) and Tox⁻ transformants GD1-1 (1-1), GD1-2 (1-2), and GD3-1 (3-1) were separated by pulsed-field gel electrophoresis under the condition for <2.0 Mb DNA (A) and for 1.0–6.0 Mb DNA (B). The blots were probed with the 1.05 Mb chromosomal DNA of the wild-type strain (1.05 Ch), the backbone vector pSH75 of pGDT1 and pGDT3, the *AKT1* fragment from pGDT1, and the *AKT3-1* fragment from pGDT3. Arrowheads indicate chromosomal DNA hybridizing to any of the probes. Sizes (in kilobases) of chromosomes of *S. cerevisiae* (A) and chromosomes of *S. pombe* and *S. cerevisiae* (B) are indicated on the left.

three *AKT* homologs (*AFT1-1*, *AFTR-1*, and *AFT3-1*) on a cosmid clone pcAFT-1 of NAF8 and also isolated three other types of cosmid clones, which are different in the distribution pattern of the *AKT* homologs. These results imply the structural complexity of the genomic region controlling AF-toxin biosynthesis.

In strain 15A of the Japanese pear pathotype, multiple copies of the *AKT* genes are also present on a single chromosome of 4.1 Mb; however, only one of each is functional (TANAKA *et al.* 1999; TANAKA and TSUGE 2000). DNA gel blot analysis of AK-toxin-minus mutants produced by transformation-mediated gene disruption allowed us to identify functional copies: toxin-minus mutants resulted when particular versions of the *AKT* genes were disrupted (TANAKA *et al.* 1999; TANAKA and TSUGE 2000). Transformation-mediated targeting of *AFT1* and *AFT3* in strain NAF8 of the strawberry pathotype also produced Tox⁻, nonpathogenic mutants. However, these mutants resulted from loss of the 1.05-Mb chromosome encoding the *AFT* genes and not from site-directed integration of the disruption vectors into certain copies. Thus, we could not address whether all or particular copies of each of the *AFT* genes were functional. If multiple copies of the genes are functional, it is probably impossible to disrupt all of the copies by site-directed integration of targeting vectors. In this case, Tox⁻ mutants could result only when a large deletion of the region containing the *AFT* genes or loss of the *AFT* chromosome would result.

All strains of the strawberry pathotype tested had 1.05-Mb chromosomes encoding the *AFT* genes. Small chromosomes of several fungi have been identified as supernumerary (dispensable) chromosomes (COVERT 1998). Such chromosomes are similar to B chromosomes in other eukaryotic organisms, which are described as small, dispensable for growth, and inherited in a non-Mendelian manner (JONES 1991). The function of supernumerary chromosomes in most species is still cryptic. However, in the pea pathogen *Nectria haematococca*, the supernumerary chromosomes have been characterized to encode functional genes, which have roles in virulence to host plants (MIAO *et al.* 1991a,b; COVERT *et al.* 1996; KISTLER *et al.* 1996; WASMANN and VANETTEN 1996; VANETTEN *et al.* 1998; HAN *et al.* 2001). In this pathogen, genes for phytoalexin detoxification as well as other virulence determinants are on the 1.6-Mb supernumerary chromosomes. Inherent instability of the supernumerary chromosomes does not affect growth, but does affect the disease-causing capacity on host plants (MIAO *et al.* 1991a,b; KISTLER *et al.* 1996; WASMANN and VANETTEN 1996; VANETTEN *et al.* 1998; HAN *et al.* 2001). The apparent loss of a supernumerary chromosome following transformation was also reported in this pathogen (WASMANN and VANETTEN 1996). The chromosome-deletion transformants showed markedly reduced virulence on pea. Fungal supernumerary chromosomes, which are not required for growth but confer an advantage for colonizing certain ecological niches, have been termed conditionally dispensable (CD) chromosomes (COVERT 1998). The 1.05-Mb chromosome controlling AF-toxin biosynthesis and pathogenicity was found dispensable for saprophytic growth of strain NAF8. Thus, we propose that the 1.05-Mb *AFT* chromosome is a CD chromosome.

AKAMATSU *et al.* (1999) observed that all strains of *A. alternata* pathotypes have small chromosomes of <1.7 Mb, but that nonpathogenic isolates do not have such small chromosomes. In the apple pathotype, the *AMT* gene encoding a cyclic peptide synthetase was identified to be required for biosynthesis of cyclic peptide AM-toxin (JOHNSON *et al.* 2000a). This gene was found to be located on a small chromosome of 1.1–1.7 Mb, depending on the strains (AKAMATSU *et al.* 1999; JOHNSON *et al.* 2000a). JOHNSON *et al.* (2001) found an AM-toxin-minus, nonpathogenic isolate from laboratory stocks of the apple pathotype isolates and showed that it had no small chromosomes. This suggests that *AMT* is also located on CD chromosomes in the apple pathotype (JOHNSON *et al.* 2001). We observed previously that the *AKT* genes are located on the 4.1-Mb chromosome of strain 15A of the Japanese pear pathotype (TANAKA and TSUGE 2000). However, other strains of the Japanese pear pathotype tested were found to have the genes on small chromosomes of <1.8 Mb (A. TANAKA and T. TSUGE, unpublished results). Thus, most of the strains from the strawberry, apple, and Japanese pear patho-

types carry the genes for host-specific toxin biosynthesis on small chromosomes. It is possible that the 4.1-Mb *AKT* chromosome of 15A might result from translocation of an original smaller chromosome.

The patterns of repeated DNA sequences on certain supernumerary chromosomes of fungi suggest that they have a different evolutionary history from the essential chromosomes in the same genome and that they may have been introduced into the genome by horizontal transfer from another species (Enkerli *et al.* 1997; Covert 1998; Rosewich and Kistler 2000). Supernumerary chromosomes have been shown to have the capacity for transfer between otherwise genetically isolated strains of *Colletotrichum gloeosporioides* (Masel *et al.* 1996; He *et al.* 1998). He *et al.* (1998) provided experimental evidence that the 2.0-Mb supernumerary chromosome can be selectively transferred from the A-biotype strain to a vegetatively incompatible B-biotype strain in this fungus. Two biotypes both cause anthracnose diseases on the tropical legumes of *Stylosanthes* spp., but are different in host range and symptoms. Transfer of the 2.0-Mb chromosome did not affect pathogenicity of the recipient B-biotype strain, indicating that the chromosome might not carry genes determining pathogenicity (Masel *et al.* 1996; He *et al.* 1998). Although the pathological role of the 2.0-Mb chromosome is unknown, this observation indicates that the horizontal transfer of supernumerary chromosomes across incompatibility barriers can occur in fungi.

We previously measured genetic relatedness among *A. alternata* pathotypes on the basis of three DNA markers: restriction fragment length polymorphisms (RFLPs) of nuclear rDNA, nucleotide sequence variation in rDNA internal transcribed spacer regions, and RFLPs of mitochondrial DNA (Kusaba and Tsuge 1994, 1995, 1997). These analyses showed that a single pathotype population does not form a monophyletic group. The *AKT* homologs are unique in the Japanese pear, strawberry, and tangerine pathotypes (Tanaka *et al.* 1999; Masunaka *et al.* 2000; Tanaka and Tsuge 2000); the *AMT* gene is only in the apple pathotype (Johnson *et al.* 2000b). These data indicate that the evolution of toxin production in *A. alternata* may involve horizontal transfer of DNA. All strains of the strawberry pathotype tested share the 1.05-Mb chromosome encoding the *AFT* genes. Thus, the ability to produce AF-toxin in the strawberry pathotype could be potentially imparted by transfer of the 1.05-Mb chromosome, rather than the DNA segment, containing the *AFT* genes to a strain of *A. alternata*. Horizontal transfer has also been proposed for the acquisition of genes for host-specific toxin biosynthesis in *Cochliobolus carbonum* (Nikolskaya *et al.* 1995; Ahn and Walton 1996; Walton *et al.* 1998; Walton 2000) and *C. heterostrophus* (Yang *et al.* 1996; Yoder 1998; Kodama *et al.* 1999).

AFT1-1, *AFTR-1*, and *AFT3-1* are 94, 96, and 94% identical in nucleotide sequence to *AKT1*, *AKTR-1*, and *AKT3-1*, respectively. The organization of the genes, however, is different between the *AFT* and *AKT* clusters. Furthermore, strains of both pathotypes have multiple sets of different clusters in the gene organization. Thus, it is unlikely that variability between the *AFT* and *AKT* clusters was originated by simple mutation. We found a new ORF, designated ORFS1, in the *AFT* cluster. DNA gel blot analysis revealed that ORFS1 was present in the strawberry pathotype strains, but not in strains of the Japanese pear and tangerine pathotypes (K. Ito, R. Hatta, A. Tanaka, M. Yamamoto, K. Akimitsu and T. Tsuge, unpublished results). The *AKT* cluster in pcAKT-1 of the Japanese pear pathotype was found to contain two additional genes, *AKT4* and *AKTS1*, upstream of *AKT1* (Tsuge *et al.* 2001). *AKTS1* is present only in the Japanese pear pathotype (Tsuge *et al.* 2001). Thus, the *AFT* and *AKT* clusters contain the pathotype-specific genes as well as the genes common in the strawberry, Japanese pear, and tangerine pathotypes. Comparison of the structure, function, and chromosomal distribution of the gene clusters from these pathotypes is of great importance in the study of the evolution of toxin biosynthesis and the origin of genes for toxin biosynthesis.

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LITERATURE CITED

Adachi, Y., H. Watanabe and T. Tsuge, 1996 Relationships between genetic polymorphisms and fungicide resistance within *Alternaria alternata*. Phytopathology **86:** 1248–1254.

Ahn, J.-H., and J. D. Walton, 1996 Chromosomal organization of *TOX2*, the complex locus controlling host-selective toxin biosynthesis in *Cochliobolus carbonum*. Plant Cell **8:** 887–897.

Akamatsu, H., M. Taga, M. Kodama, R. D. Johnson, H. Otani *et al.*, 1999 Molecular karyotypes for *Alternaria* plant pathogens known to produce host-specific toxins. Curr. Genet. **35:** 647–656.

Altschul, S. F., T. L. Madden, A. A. Schäfer, J. Zhang, Z. Zhang *et al.*, 1997 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:** 3389–3402.

An, Z., M. L. Farman, A. Budde, S. Taura and S. A. Leong, 1996 New cosmid vectors for library construction, chromosome walking and restriction mapping in filamentous fungi. Gene **176:** 93–96.

Bruchez, J. J. P., J. Eberle and V. E. Russo, 1993 Regulatory sequences in the transcription of *Neurospora crassa* gene: CAAT box, TATA box, intron, poly(A) tail formation sequences. Fungal Genet. Newsl. **40:** 89–96.

Chu, G., D. Vollrath and R. W. Davis, 1986 Separation of large DNA molecules by contour clamped homogeneous electric fields. Science **234:** 1582–1585.

Covert, S. F., 1998 Supernumerary chromosomes in filamentous fungi. Curr. Genet. **33:** 311–319.

Covert, S. F., J. Enkerli, V. P. W. Miao and H. D. VanEtten, 1996 A gene for macckiain detoxification from a dispensable chromosome of *Nectria haematococca*. Mol. Gen. Genet. **251:** 397–406.

Elgersma, Y., A. Vos, M. van den Berg, C. W. T. van Roemund, P. van der Sluijs *et al.*, 1996 Analysis of the carboxyl-terminal peroxisomal targeting signal 1 in a homologous context in *Saccharomyces cerevisiae*. J. Biol. Chem. **271:** 26375–26382.

Enkerli, J., G. Bhatt and S. F. Covert, 1997 *Nht1*, a transposable element cloned from a dispensable chromosome in *Nectria haematococca*. Mol. Plant-Microbe Interact. **10:** 742–749.

Feng, B.-N., S. Nakatsuka, T. Goto, T. Tsuge and S. Nishimura, 1990 Biosynthesis of host-selective toxins produced by *Alternaria alternata* pathogens. I. (*8R,9S*)-9,10-epoxy-8-hydroxy-9-methyldeca-(*2E,4Z,6E*)-trienoic acid as a biological precursor of AK-toxins. Agric. Biol. Chem. **54:** 845–848.

Han, Y., X. Liu, U. Benny, H. C. Kistler and H. D. VanEtten, 2001 Genes determining pathogenicity to pea are clustered on a supernumerary chromosome in the fungal plant pathogen *Nectria haematococca*. Plant J. **25:** 305–314.

Hayashi, N., K. Tanabe, T. Tsuge, S. Nishimura, K. Kohmoto *et al.*, 1990 Determination of host-selective toxin production during spore germination of *Alternaria alternata* by high-performance liquid chromatography. Phytopathology **80:** 1088–1091.

He, C. Z., A. G. Rusa, A. M. Polawski, J. A. G. Irwin and J. M. Manners, 1998 Transfer of a supernumerary chromosome between vegetatively incompatible biotypes of the fungus *Colletotrichum gloeosporioides*. Genetics **150:** 1459–1466.

Johnson, R. D., L. Johnson, Y. Itoh, M. Kodama, H. Otani *et al.*, 2000a Cloning and characterization of a cyclic peptide synthetase gene from *Alternaria alternata* apple pathotype whose product is involved in AM-toxin synthesis and pathogenicity. Mol. Plant-Microbe Interact. **13:** 742–753.

Johnson, R. D., L. Johnson, K. Kohmoto, H. Otani, C. R. Lane *et al.*, 2000b A polymerase chain reaction-based method to specifically detect *Alternaria alternata* apple pathotype (*A. mali*), the causal agent of Alternaria blotch of apple. Phytopathology **90:** 973–976.

Johnson, L., R. D. Johnson, H. Akamatsu, A. Salamiah, H. Otani *et al.*, 2001 Spontaneous loss of a conditionally dispensable chromosome from *Alternaria alternata* apple pathotype leads to loss of toxin production and pathogenicity. Curr. Genet. **40:** 65–72.

Jones, R. N., 1991 B-chromosome drive. Am. Nat. **137:** 430–442.

Kawamura, C., T. Tsujimoto and T. Tsuge, 1999 Targeted disruption of a melanin biosynthesis gene affects conidial development and UV tolerance in the Japanese pear pathotype of *Alternaria alternata*. Mol. Plant-Microbe Interact. **12:** 59–63.

Kempken, F., and U. Kück, 1996 *restless*, an active *Ac*-like transposon from the fungus *Tolypocladium inflatum*: structure, expression, and alternative RNA splicing. Mol. Cell. Biol. **16:** 6563–6572.

Kimura, N., and T. Tsuge, 1993 Gene cluster involved in melanin biosynthesis of the filamentous fungus *Alternaria alternata*. J. Bacteriol. **175:** 4427–4435.

Kistler, H. C., L. W. Meinhardt and U. Benny, 1996 Mutants of *Nectria haematococca* created by a site-directed chromosome breakage are greatly reduced in virulence toward pea. Mol. Plant-Microbe Interact. **9:** 804–809.

Kodama, M., M. S. Rose, G. Yang, S. H. Yun, O. C. Yoder *et al.*, 1999 The translocation-associated *Tox1* locus of *Cochliobolus heterostrophus* is two genetic elements on two different chromosomes. Genetics **151:** 585–596.

Kohmoto, K., K. Akimitsu and H. Otani, 1991 Correlation of resistance and susceptibility of citrus to *Alternaria alternata* with sensitivity to host-specific toxins. Phytopathology **81:** 719–722.

Kohmoto, K., Y. Itoh, N. Shimomura, Y. Kondoh, H. Otani *et al.*, 1993 Isolation and biological activities of two host-specific toxins from the tangerine pathotype of *Alternaria alternata*. Phytopathology **83:** 495–502.

Kohmoto, K., H. Otani and T. Tsuge, 1995 *Alternaria alternata* pathogens, pp. 3–22 in *Pathogenesis and Host Specificity in Plant Diseases: Histopathological, Biochemical, Genetic and Molecular Bases, Vol II, Eukaryotes*, edited by K. Kohmoto, U. S. Singh and R. P. Singh. Pergamon, Oxford.

Kusaba, M., and T. Tsuge, 1994 Nuclear ribosomal DNA variation and pathogenic specialization in *Alternaria* fungi known to produce host-specific toxins. Appl. Environ. Microbiol. **60:** 3055–3062.

Kusaba, M., and T. Tsuge, 1995 Phylogeny of *Alternaria* fungi known to produce host-specific toxins on the basis of variation in internal transcribed spacers of ribosomal DNA. Curr. Genet. **28:** 491–498.

Kusaba, M., and T. Tsuge, 1997 Mitochondrial DNA variation in host-specific toxin-producing pathogens in the genus *Alternaria*. Ann. Phytopathol. Soc. Jpn. **63:** 463–469.

Maekawa, N., M. Yamamoto, S. Nishimura, K. Kohmoto, M. Kuwata *et al.*, 1984 Studies on host-specific AF-toxins produced by *Alternaria alternata* strawberry pathotype causing Alternaria black spot of strawberry. I. Production of host-specific toxins and their biological activity. Ann. Phytopathol. Soc. Jpn. **50:** 600–609.

Masel, A. M., C. Z. He, A. M. Polawski, J. A. G. Irwin and J. M. Manners, 1996 Molecular evidence for chromosome transfer between biotypes of the fungus *Colletotrichum gloeosporioides*. Mol. Plant-Microbe Interact. **9:** 339–348.

Masunaka, A., A. Tanaka, T. Tsuge, T. L. Peever, L. W. Timmer *et al.*, 2000 Distribution and characterization of *AKT* homologs in the tangerine pathotype of *Alternaria alternata*. Phytopathology **90:** 762–768.

Miao, V. P. W., S. F. Covert and H. D. VanEtten, 1991a A fungal gene for antibiotics resistance on a dispensable (B) chromosome. Science **254:** 1773–1776.

Miao, V. P. W., D. E. Mathews and H. D. VanEtten, 1991b Identification and chromosomal locations of a family of cytochrome P-450 genes for pisatin detoxification in the fungus *Nectria haematococca*. Mol. Gen. Genet. **226:** 214–223.

Morales, V. M., G. Seguin-Swartz and J. L. Tayler, 1993 Chromosome size polymorphism in *Leptosphaeria maculans*. Phytopathology **83:** 503–509.

Mullaney, E. J., J. E. Hamer, K. A. Roberti, M. M. Yelton and W. E. Timberlake, 1985 Primary structure of the *trpC* gene from *Aspergillus nidulans*. Mol. Gen. Genet. **199:** 37–45.

Müller-Newen, G., and W. Stoffel, 1993 Site-directed mutagenesis of putative active-site amino acid residues of 3,2-*trans*-enoyl-CoA isomerase, conserved within the low-homology isomerase/hydratase enzyme family. Biochemistry **32:** 11405–11412.

Nakashima, T., T. Ueno, H. Fukami, T. Taga, H. Masuda *et al.*, 1985 Isolation and structures of AK-toxin I and II, host-specific phytotoxic metabolites produced by *Alternaria alternata* Japanese pear pathotype. Agric. Biol. Chem. **49:** 807–815.

Nakatsuka, S., K. Ueda, T. Goto, M. Yamamoto, S. Nishimura *et al.*, 1986 Structure of AF-toxin II, one of the host-specific toxins produced by *Alternaria alternata* strawberry pathotype. Tetrahedron Lett. **27:** 2753–2756.

Nakatsuka, S., B.-N. Feng, T. Goto, T. Tsuge and S. Nishimura, 1990 Biosynthetic origin of (*8R,9S*)-9,10-epoxy-8-hydroxy-9-methyl-deca-(*2E,4Z,6E*)-trienoic acid, a precursor of AK-toxins produced by *Alternaria alternata* Japanese pear pathotype. Phytochemistry **29:** 1529–1531.

Nikolskaya, A. N., D. G. Panaccione and J. D. Walton, 1995 Identification of peptide synthetase-encoding genes from filamentous fungi producing host-selective phytotoxins or analogs. Gene **165:** 207–211.

Nishimura, S., and K. Kohmoto, 1983 Host-specific toxins and chemical structures from *Alternaria* species. Annu. Rev. Phytopathol. **21:** 87–116.

Okuda, M., K. Ikeda, F. Namiki, K. Nishi and T. Tsuge, 1998 *Tfo1*: an *Ac*-like transposon from the plant pathogenic fungus *Fusarium oxysporum*. Mol. Gen. Genet. **258:** 599–607.

Otani, H., K. Kohmoto, S. Nishimura, T. Nakashima, T. Ueno *et al.*, 1985 Biological activities of AK-toxins I and II, host-specific toxins from *Alternaria alternata* Japanese pear pathotype. Ann. Phytopathol. Soc. Jpn. **51:** 285–293.

Pavela-Vrancic, M., E. Pfeifer, H. van Liempt, H. J. Schäfer, H. von Döhren *et al.*, 1994 ATP binding in peptide synthetases: determination of contact sites of the adenine moiety by photoaffinity labeling of tyrocidine synthetase 1 with 2-azidoadenosine triphosphate. Biochemistry **33:** 6276–6283.

Peever, T. L., L. Olsen, A. Ibañez and L. W. Timmer, 2000 Genetic differentiation and host-specificity among population of *Alternaria* spp. causing brown spot of grapefruit and tangerine × grapefruit hybrids in Florida. Phytopathology **90:** 407–414.

Pegg, K. G., 1966 Studies of a strain of *Alternaria citri* Pierce, the causal agent of brown spot of emperor mandarin (*Citrus reticulata*). Queensland J. Agric. Anim. Sci. **23:** 15–28.

Rosewich, U. L., and H. C. Kistler, 2000 Role of horizontal gene transfer in the evolution of fungi. Annu. Rev. Phytopathol. **38:** 325–363.

Rotem, J., 1994 *The Genus Alternaria: Biology, Epidemiology, and Pathogenicity*. The American Phytopathological Society, St. Paul.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual*, Ed. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Scheffer, R. P., and R. S. Livingston, 1984 Host-selective toxins and their role in plant diseases. Science **223:** 17–21.

Shiotani, H., and T. Tsuge, 1995 Efficient gene targeting in the filamentous fungus *Alternaria alternata*. Mol. Gen. Genet. **248:** 142–150.

Tanaka, A., and T. Tsuge, 2000 Structural and functional complexity of the genomic region controlling AK-toxin biosynthesis and pathogenicity in the Japanese pear pathotype of *Alternaria alternata*. Mol. Plant-Microbe Interact. **13:** 975–986.

Tanaka, A., and T. Tsuge, 2001 Reporter gene analysis of *AKT3-1* and *AKT3-2* expression during conidial germination of the Japanese pear pathotype of *Alternaria alternata*. J. Gen. Plant Pathol. **67:** 15–22.

Tanaka, A., H. Shiotani, M. Yamamoto and T. Tsuge, 1999 Insertional mutagenesis and cloning of the genes required for biosynthesis of the host-specific AK-toxin in the Japanese pear pathotype of *Alternaria alternata*. Mol. Plant-Microbe Interact. **12:** 691–702.

Tanaka, S., 1933 Studies on black spot disease of Japanese pear (*Pyrus serotina* Rehd.). Mem. Coll. Agric. Kyoto Imp. Univ. **28:** 1–31.

Thompson, J. D., D. G. Higgins and T. J. Gibson, 1994 CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:** 4673–4680.

Todd, R. B., and A. Andrianopoulos, 1997 Evolution of a fungal regulatory gene family: the Zn(II)2Cys6 binuclear cluster DNA binding motif. Fungal Genet. Biol. **21:** 388–405.

Tsuge, T., S. Nishimura and H. Kobayashi, 1990 Efficient integrative transformation of the phytopathogenic fungus *Alternaria alternata* mediated by the repetitive *rDNA* sequences. Gene **90:** 207–214.

Tsuge, T., A. Tanaka, R. Nishikawa, R. Hatta, K. Ito *et al.*, 2001 Molecular genetics of host-specific toxin biosynthesis in *Alternaria alternata*, pp. 87–96 in *Delivery and Perception of Pathogen Signals in Plants*, edited by N. Keen, S. Mayama, J. L. Leach and S. Tsuyumu. The American Phytopathological Society Press, St. Paul.

VanEtten, H. D., S. Jorgensen, J. Enkerli and S. F. Covert, 1998 Inducing the loss of conditionally dispensable chromosomes in *Nectria haematococca* during vegetative growth. Curr. Genet. **33:** 299–303.

Walton, J. D., 1996 Host-selective toxins: agents of compatibility. Plant Cell **8:** 1723–1733.

Walton, J. D., 2000 Horizontal gene transfer and the evolution of secondary metabolite gene clusters in fungi: an hypothesis. Fungal Genet. Biol. **30:** 167–171.

Walton, J. D., J.-H. Ahn, J. W. Pitkin, Y.-Q. Cheng, A. N. Nikolskaya *et al.*, 1998 Enzymology, molecular genetics, and regulation of biosynthesis of the host-selective toxin HC-toxin, pp. 25–34 in *Molecular Genetics of Host-Specific Toxins in Plant Disease*, edited by K. Kohmoto and O. C. Yoder. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Wasmann, C. C., and H. D. VanEtten, 1996 Transformation-mediated chromosome loss and disruption of a gene for pisatin demethylase decrease the virulence of *Nectria haematococca*. Mol. Plant-Microbe Interact. **9:** 793–803.

Yang, G., M. S. Rose, B. G. Turgeon and O. C. Yoder, 1996 A polyketide synthase is required for fungal virulence and production of the polyketide T-toxin. Plant Cell **8:** 2139–2150.

Yoder, O. C., 1980 Toxins in pathogenesis. Annu. Rev. Phytopathol. **18:** 103–129.

Yoder, O. C., 1998 A mechanistic view of the fungal/plant interaction based on host-specific toxin studies, pp. 3–15 in *Molecular Genetics of Host-Specific Toxins in Plant Disease*, edited by K. Kohmoto and O. C. Yoder. Kluwer Academic Publishers, Dordrecht, The Netherlands.

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