

FUNGAL DISEASES

Sheila Ruswandi · Keiji Kitani · Kazuya Akimitsu
Takashi Tsuge · Tomonori Shiraishi · Mikihiro Yamamoto

Structural analysis of cosmid clone pcAFT-2 carrying *AFT10-1* encoding an acyl-CoA dehydrogenase involved in AF-toxin production in the strawberry pathotype of *Alternaria alternata*

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Abstract The strawberry pathotype of *Alternaria alternata* produces the host-specific AF-toxin and causes black spot of strawberry. The genes in the toxin gene cluster are currently being identified and characterized. In this study, a genomic cosmid clone, pcAFT-2, was structurally characterized. This cosmid contains *AFT* homologs, which were found to be involved in AF-toxin biosynthesis. These homologs were designated *AFTR-2* and *AFT3-2*. Four new open reading frames (ORFs) (*AFT9-1*, *AFT10-1*, *AFT11-1*, *AFT12-1*) and two transposon-like sequences (TLS-S4, TLS-S5) were also identified. These ORFs were shown to encode for polyketide synthase, acyl-CoA dehydrogenase, P450 monooxygenase, and an oxidoreductase, respectively. Transcripts of all the ORFs were detected. DNA gel blot analysis detected homologs of these four ORFs only in the tangerine, strawberry, and Japanese pear pathotypes, which share a common 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid moiety in their toxin structure. Targeting of *AFT10-1*, which encodes an acyl-CoA dehydrogenase, produced single- and double-copy mutants with highly reduced numbers of lesions on host leaves concomitant with reduced toxin production, confirming its role in pathogenicity. Thus, *AFT10-1* exists in multiple copies in the genome of *Alternaria alternata*; and based on the presence of homologs in the tangerine and Japanese pear pathotypes, it is

involved in the formation of the 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid moiety of the toxin molecule.

Key words *Alternaria alternata* strawberry pathotype · Acyl-CoA dehydrogenase (ACD) · Host-specific toxin (HST)

Introduction

Host-specific toxins (HSTs) are agents of virulence produced by certain fungal pathogens such as the genera *Alternaria* and *Cochliobolus* (Kohmoto and Otani 1991; Walton and Panaccione 1993). *Alternaria alternata* is a saprophyte or a weak pathogen on a number of plants (Nishimura et al. 1979). Even during host–pathogen interactions, hyphal ingress can be halted in the cell wall or in the lumen of plants. Thus, the differentiation of specialized pathogens from opportunistic pathogens may depend on the production of host-specific or host-selective toxins (McRoberts and Lennard 1996; Nishimura and Kohmoto 1983); thus, the function and biosynthesis of HSTs has gained much attention (Thomma 2003).

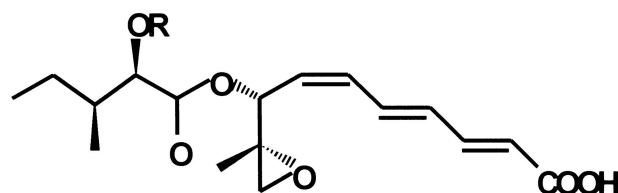
In *Alternaria*, host-specific toxins are involved in at least 10 plant diseases (Akamatsu et al. 1999). Generally, HSTs have been proposed to act directly on the plasmalemma or mitochondria of sensitive cells (Yoder 1980). In this species, HSTs are classified into groups in terms of the primary site of action; the first group consists of ACT-, AF-, and AK-toxins produced by tangerine, strawberry, and Japanese pear pathotypes of *A. alternata*, respectively. These toxins share a common 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid structure (Kohmoto et al. 1993; Nakashima et al. 1985; Nakatsuka et al. 1986), suggesting that these toxins share a common biochemical pathway. Genes controlling AK-toxin biosynthesis in the Japanese pear pathotype have been cloned, and their homologs were also found in the tangerine and strawberry pathotypes (Masunaka et al. 2000; Tanaka et al. 1999; Tanaka and Tsuge 2000).

S. Ruswandi · K. Kitani · T. Shiraishi · M. Yamamoto (✉)
Faculty of Agriculture, Okayama University, 1-1-1 Tsushimanaka,
Okayama 700-8530, Japan
Tel. +81-86-251-8301; Fax +81-86-251-8301
e-mail: myama@cc.okayama-u.ac.jp

K. Akimitsu
Faculty of Agriculture, Kagawa University, Kagawa, Japan

T. Tsuge
Graduate School of Bioagricultural Sciences, Nagoya University,
Nagoya, Japan

The nucleotide sequence data of pcAFT-2 is available in DDBJ/
EMBL/GenBank databases under accession number AB179766



AF-Toxin I : R= COCH(OH)C(CH₃)₂OH

AF-Toxin II : R= H

AF-Toxin III: R= COCH(OH)CH(CH₃)₂

Fig. 1. Structure of AF-toxin produced by *Alternaria alternata* strawberry pathotype

The strawberry pathotype, which causes black spot of strawberry, is pathogenic only on strawberry cultivar Morioka-16 (Maekawa et al. 1984). This pathogen produces AF-toxins I, II, and III (Fig. 1). AF-toxin I is thought to be the major toxin based on its role in pathogenesis (Yamamoto et al. 1984, 2000). AF-toxin I is toxic to the strawberry cultivar and to certain cultivars of the Japanese pear. AF-toxin II is active on Japanese pear only; and AF-toxin III is toxic to strawberry and less toxic to Japanese pear (Maekawa et al. 1984).

Previously, cosmid clones containing *AKT* homologs were screened in the NAF8 isolate of *Alternaria alternata* strawberry pathotype. One of the cosmid clones, pcAFT-1, was characterized and was found to contain three *AKT* homologs designated *AFT1-1*, *AFTR-1*, and *AFT3-1*. Targeting of *AFT1-1* and *AFT3-1* produced AF-toxin-minus, nonpathogenic mutants that lacked a 1.05-Mb chromosome encoding these *AFT* genes (Hatta et al. 2002). Another gene, *AFTS1*, which is also found in this cosmid clone and encodes an enzyme of the aldo-ketoreductase family, is involved specifically in the formation of AF-toxin I (Ito et al. 2004).

In this study, another cosmid clone, pcAFT-2, was characterized. This cosmid clone contains homologs of *AKTR* and *AKT3* designated *AFTR-2* and *AFT3-2* in the strawberry pathotype. Four new open reading frames (ORFs) were also identified that potentially encode for a polyketide synthase (PKS), an acyl-coenzyme A (CoA) dehydrogenase, cytochrome P450 monooxygenase, and an oxidoreductase. Southern blots using probes corresponding to these new ORFs suggest that they are found only in the strawberry, Japanese pear, and tangerine pathotypes. The results of this study suggest that these four genes may be involved in biosynthesis of the 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid structure of the AF-, AK-, and AC-toxins.

One of the genes in pcAFT-2, *AFT10-1*, encodes an acyl-CoA dehydrogenase. This FAD-containing enzyme is implicated in β -oxidation in fungi and higher plants (Masterson et al. 2000; Richmond and Blecker 1999; van Roermund et al. 1998). One important product of β -oxidation is acetyl-CoA, which is used for the biosynthesis of various products including polyketides, which are sec-

Table 1. Fungal isolates used in this study

No. ^a	Isolate	Pathotype	Source
1	G31	Japanese pear	Gifu, Japan
2	T88-52	Japanese pear	Tottori, Japan
3	Nu89-22	Japanese pear	Aichi, Japan
4	G90-A2	Japanese pear	Gifu, Japan
5	15A	Japanese pear	Tottori, Japan
6	O-187	Strawberry	Iwate, Japan
7	M-30	Strawberry	Tottori, Japan
8	NAF2	Strawberry	Aichi, Japan
9	T-32	Strawberry	Tottori, Japan
10	NAF8	Strawberry	Aichi, Japan
11	ATCC38963	Tangerine	United States
12	ATCC38962	Rough lemon	United States
13	O-159	Apple	Unknown
14	AM-1	Apple	Aichi, Japan
15	AT-204	Tobacco	Aichi, Japan
16	No.122-2	Tobacco	Tochigi, Japan
17	No. 91080804	Tomato	Mie, Japan
18	AL-4	Tomato	Mie, Japan
19	O-94	Nonpathogenic	Tottori, Japan
20	IFO0131189	Nonpathogenic	Unknown

^aThe numbers correspond to the lane numbers in Fig. 5

ondary fungal metabolites (Brown et al. 1996). To determine the function of *AFT10-1* in the pathogenesis and toxin production in *Alternaria alternata* strawberry pathotype, disruption vectors were constructed to disrupt two copies of the gene. Single- and double-copy mutants produced by double crossover homologous recombination events produced less toxin and fewer lesions on strawberry and Japanese pear leaves. This study shows that an acyl-CoA dehydrogenase is involved in the pathogenicity of *Alternaria alternata* strawberry pathotype.

Materials and methods

Fungal isolates and plants

The fungal isolates (Table 1) were maintained on potato sucrose agar (PSA) and kept at 25°C. The susceptible strawberry cultivar Morioka-16 and the Japanese pear cultivar Nijisseiki were used in the pathogenicity tests.

Pathogenicity test and determination of penetration behavior

Pathogenicity tests were performed as described previously (Maekawa et al. 1984; Tanaka et al. 1999). Lesions were counted, and the average number of lesions per square centimeter of leaf was determined. Penetration behavior was observed by placing spore suspensions (10³ spores/ml) on cellophane sheets mounted on glass slides. The slides were then incubated in a moist chamber for 24-48h and observed with a light microscope. Germinated and ungerminated conidia were then counted. Appressorial formation and formation of penetration hyphae were also noted.

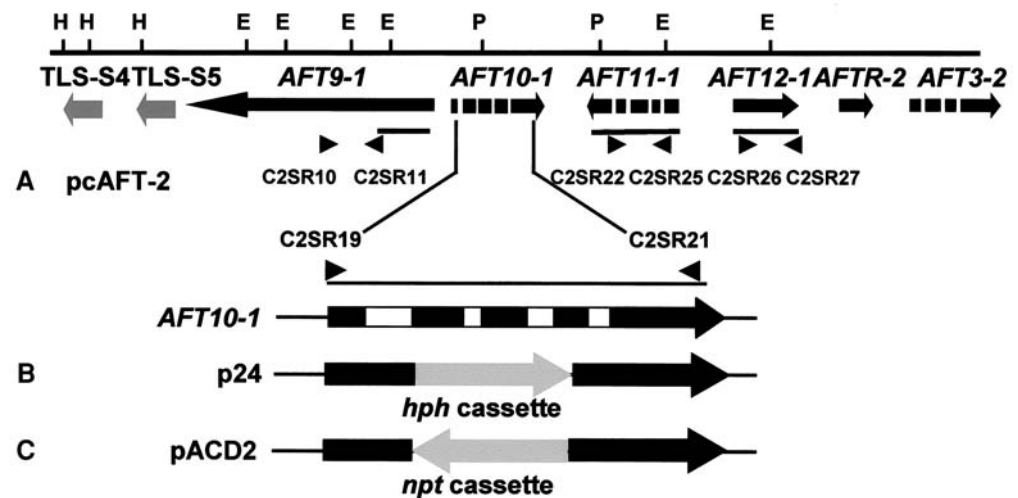


Fig. 2. Maps of pcAFT-2, p24, and pACD2. **A** Map of cosmid clone pcAFT-2 showing the different ORFs. pcAFT-2 was isolated from the genomic library of isolate NAF8 of *Alternaria alternata* strawberry pathotype. Arrows and boxes indicate the protein-coding regions. The blank segments indicate the intron positions. Lines indicate the probes used for the distribution analysis. Arrowheads indicate the primers used to amplify the various open reading frames (ORFs). H, *Hind*III; E, *Eco*RI; P, *Pvu*II. The sequence of pcAFT-2 can be accessed in the

DDBJ/GenBank/EMBL databases under accession no. AB179766. **B** Structure of disruption vector p24. Disruption vector p24 was constructed as described in the text. This vector, containing the hygromycin phosphotransferase (*hph*) gene cassette, was utilized to disrupt the first copy of *AFT10-1*. **C** Structure of disruption vector pACD2. Disruption vector pACD2 was constructed as described in the text. This vector, containing the neomycin phosphotransferase (*npt*) gene cassette, was utilized to disrupt the second copy of *AFT10-1*.

Protoplast preparation and fungal transformation

Protoplasts of isolate NAF8 were prepared and transformed as described previously (Shiotani and Tsuge 1995; Tsuge et al. 1990). Colonies that formed were picked up and transferred to potato dextrose agar (PDA) plates containing hygromycin B or geneticin at 100 µg/ml.

Cosmid and plasmids

The cosmid clone pcAFT-2 contains the genomic sequences of isolate NAF8 of *Alternaria alternata* and the *AKT* homologs from the Japanese pear pathotype with c. 31-kb region upstream of the *AKTR* homolog (Hatta et al. 2002). The pGEM-T Easy vector (Promega, Madison, WI, USA) was used to subclone *AFT10-1*, *AFT11-1*, *AFT12-1*, and *AFT9-1*.

The disruption vectors used were p24 (Fig. 2B) and pACD2 (Fig. 2C). The p24 was produced by first amplifying the *AFT10-1* ORF using the primers C2SR19 and C2SR21 and subcloning the polymerase chain reaction (PCR) fragment into a pGEM-T Easy vector to produce the plasmid p41ACD. The hygromycin phosphotransferase (*hph*) gene cassette from the plasmid pSH75 (Kimura and Tsuge 1993) was excised using *Eco*RV and *Pvu*II and ligated into the *Eco*RV-linearized p41ACD to form the disruption vector p24. Two sets of primers were used for constructing pACD2: primers C2SR21B and ACD2E, which add *Bgl*III and *Eco*RI restriction sites to the ACD fragment, and C2SR19H and ACD1X, which add *Hind*III and *Xba*I cutting sites. The fragments produced were 871bp and 932bp, respectively. To ensure that these restriction

sites were added to the fragments during amplification, the PCR products were subcloned in a pGEM-T Easy vector to form the plasmid pBE and pXH, respectively. The pBE was digested with *Bgl*III and *Eco*RI to release the insert. This fragment was then ligated into pII99 vector (Namiki et al. 2001) containing a neomycin phosphotransferase (*npt*) gene cassette digested with the same enzymes. The resulting plasmid was pBE8. The insert from pXH was reisolated using *Hind*III and *Xba*I and was ligated into *Hind*III- and *Xba*I-digested pBE8 to form the disruption vector pACD2. The primers used are listed in Table 2.

DNA manipulations

For DNA isolation, *Alternaria alternata* isolates were grown on PSA plates for 2–3 days at 25°C. DNA was then isolated as described previously (Tanaka et al. 1999). For Southern blotting, genomic DNA was digested with restriction enzymes and fractionated on agarose gels using standard procedures (Sambrook et al. 1989). Probes were labeled using an AlkPhos Direct Labelling kit (Amersham-Pharmacia, Piscataway, NJ, USA), and hybridizations were done according to the manufacturer's instructions. Detection was done using the CDP Star detection kit. The blots were then exposed in an imager (Fluor-S Max MultiImager; BioRad, Hercules, CA, USA) for 2h. Probes used in the distribution analysis were the purified PCR products of C2SR14/C2SR17, C2SR19/C2SR21, C2SR28/C2SR29, and C2SR26/C2SR27 for *AFT9-1*, *AFT10-1*, *AFT11-1*, and *AFT12-1*, respectively. Table 2 shows the list of primers used in this study.

Table 2. Primers

Primer	Sequence (5'–3')	ORF amplified
C2SR10	GAGGACGCATGTATTGTGCTGGC	<i>AFT9-1</i>
C2SR11	GATCAAGCATGGACATCATGTCG	<i>AFT9-1</i>
C2SR14	GTGTATCATGTTGGCTCGATATCC	<i>AFT9-1</i>
C2SR17	CGATGGATCCGCAGCAGCGCC	<i>AFT9-1</i>
C2SR19	CGGCTCCACGGTCCCTTTCGCGG	<i>AFT10-1</i>
C2SR21	CAGCTAGCTCGAAGGTTCTTGCC	<i>AFT10-1</i>
C2SR19H	AAGCTTCGGCTCCACGGTCCCTTTCGCGG	<i>AFT10-1</i>
ACD1X	TCTAGAGCAATATCAGAGCCCACTGCAC	<i>AFT10-1</i>
C2SR21B	AGATCTCAGCTAGCTCGAAGGTTCTTGCC	<i>AFT10-1</i>
ACD2E	GAATTCGTCAATTACGGAACCGAGGAGC	<i>AFT10-1</i>
C2SR22	CATCAGTTCAACTTCGTGTGCGCC	<i>AFT11-1</i>
C2SR25	CAAGTCGGATGTCGTGGCTAGGG	<i>AFT11-1</i>
C2SR28	ATTAATTCCCCGAAACACGT	<i>AFT11-1</i>
C2SR29	CCGAATGACTTATGAAAAGC	<i>AFT11-1</i>
C2SR26	GCTGCCATGTTTCATGGTTG	<i>AFT12-1</i>
C2SR27	GTAGATGTCTAAAGAATCACCAG	<i>AFT12-1</i>

ORF, open reading frame

For sequence analysis, PCR products were subcloned into pGEM-T Easy vector. Sequence data were obtained using a fluorescent DNA sequencer (DSQ1000L; Shimadzu, Kyoto, Japan). DNA sequences were analyzed with BLAST (Altschul et al. 1997) and Vector NTI (Infomax, Taiwan, ROC).

Reverse transcription-polymerase chain reaction

Total RNA was extracted from the NAF8 isolate of *Alternaria alternata* using the Qiagen RNeasy Mini kit (Qiagen, Toyko, Japan). mRNA was then extracted from the total RNA using the Oligotex–dT30 (Super) mRNA Purification kit (Takara Biochemicals, Shiga, Japan). Reverse transcription-polymerase chain reaction (RT-PCR) was then performed using the Qiagen RT-PCR kit (Qiagen). Primer pairs C2SR10/C2SR11, C2SR19/C2SR21, C2SR22/C2SR25, and C2SR26/C2SR27 were used for amplifying *AFT9-1*, *AFT10-1*, *AFT11-1*, and *AFT12-1*, respectively.

Toxin production

Wild-type and mutant strains were grown on 10ml potato sucrose broth for 7 days without agitation and incubated at 25°C. Mycelial mats and culture filtrates were then harvested. Culture filtrates were used to quantify AF-toxins I and II by high performance liquid chromatography (HPLC) analysis as previously described (Hayashi et al. 1990). AF-toxin III was not quantified because it is usually present only in trace amounts in culture filtrates, even in wild-type strains (Maekawa et al. 1984).

Results

Structure of pcAFT-2

Previously, four cosmid clones containing AKT homologs from the Japanese pear pathotype were isolated (Hatta

et al. 2002). One of the cosmid clones, pcAFT-1, has been characterized and four ORFs (*AFT1-1*, *AFTR-1*, *AFT3-1*, *AFTS1*) were revealed (Hatta et al. 2002; Ito et al. 2004). In this study, a second cosmid clone, pcAFT-2, which contains a 40-kb insert, was reported. This cosmid clone contains two AKT homologs designated *AFTR-2* and *AFT3-2*, which are found at the border of the insert. *AFTR-2* and *AFT3-2* have 99% and 100% homology to *AFTR-1* and *AFT3-1*, respectively, which are found in pcAFT-1. Upstream of *AFTR-2* and *AFT3-2* are four new ORFs that are presumed to encode polyketide synthase, acyl-CoA dehydrogenase, cytochrome P450 monooxygenase, and oxidoreductase. These ORFs were designated *AFT9-1*, *AFT10-1*, *AFT11-1*, and *AFT12-1*, respectively. Figure 2A shows the map of pcAFT-2 showing these ORFs.

At the other border of the insert are two transposon-like sequences designated TLS-S4 and TLS-S5. Transposon-like sequences were also found in pcAFT-1 (Hatta et al. 2002). TLS-S4 has high homology to restless transposase of *Nectria haematococca* (Han et al. 2001) and a transposase-like protein of the Ac-type transposon Tfo1 of *Fusarium oxysporum* (Okuda et al. 1998). TLS-S5, on the other hand, has high homology to that of the Fot2 transposase identified in *Aspergillus niger* var. *awamori*, which is a member of the Fot1 family (Nyyssonen et al. 1996).

The coding sequence of *AFT11-1* is around 1764bp. It encodes a protein consisting of more than 500 amino acids. The sequence of the cDNA compared with that of the genomic DNA had four introns separating the five exons (Fig. 2A). The intron sizes are 62, 42, 53, and 49 basepairs (bp). Two of the introns do not show the typical 5'[GT(A/G/T)(A/C/T)G(T/C)] and 3'[(C/T)AG] splice sites of fungi (Bruchez et al. 1993). BLAST search revealed that *AFT11-1* encodes a P450 monooxygenase similar to that of *Aspergillus parasiticus*, *Nostoc punctiforme*, *Arabidopsis thaliana*, and *Musa acuminata*. *AFT12-1* consists of a single exon of around 290 amino acids and encodes a protein belonging to the oxidoreductase family (Fig. 2A). It has high homology to the hypothetical proteins of *Gibberella zeae*, *Magnaporthe grisea*, *Aspergillus nidulans*, and *Neuro-*

orientation of the nucleotides. The *lines* indicate the forward and reverse primers used to amplify the ORF

(Fig. 4). Primer pair C2SR19 and C2SR21 was used to amplify *AFT10-I*. This produced a 1.1-kb fragment from the mRNA of NAF8, which corresponds to the size when the introns are spliced out. Control reactions without reverse transcriptase produced no amplification products, indicating that the fragments were amplified from the cDNA of NAF8. Primer pair C2SR22 and C2SR25 was used to amplify *AFT11-I* and produced a 969-bp fragment from the mRNA of NAF8 and a 1.0-kb fragment from the genomic DNA. Primer pair C2SR26 and C2SR27 was used to amplify *AFT12-I*, producing a 894-bp fragment from both genomic DNA and cDNA of NAF8, indicating an intronless gene. The expression of *AFT9-I* was also investigated using primer pair C2SR10 and C2SR11 where the intron site has been predicted. Several transcripts were produced with a major band the same size as that of the genomic DNA amplicon. Sequencing of the minor bands did not reveal any intron. Thus, the entire *AFT9-I* ORF needs to be further investigated to reveal the function of this gene.

Distribution of *AFT9-1*, *AFT10-1*, *AFT11-1*, and *AFT12-1* in *Alternaria alternata*

Southern hybridization was done to determine the distribution of *AFT9-1*, *AFT10-1*, *AFT11-1*, and *AFT12-1* in *Alternaria alternata* isolates. Genomic DNA was digested with *Eco*RI and probed with fragments synthesized from the internal regions of these ORFs. All probes hybridized only to the Japanese pear, strawberry, and tangerine pathotypes (Fig. 5A,C,E,G). These pathotypes are known to have the

Transcription products of *AFT9-1*, *AFT10-1*, *AFT11-1*, and *AFT12-1* in the NAF8 isolate were detected by RT-PCR

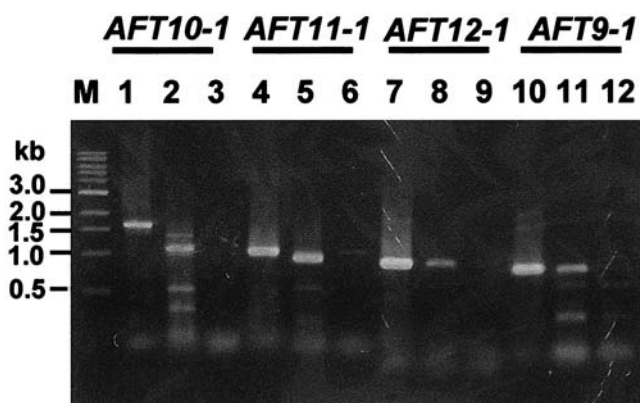


Fig. 4. Detection of transcripts. The mRNA of isolate NAF8 *Alternaria alternata* strawberry pathotype was used as a template for reverse transcription, and the synthesized cDNA was used as a template for polymerase chain reaction (PCR) (lanes 2, 5, 8, 11). Amplifications were done using primer pairs C2SR19/C2SR21, C2SR22/C2SR25, C2SR26/C2SR27, and C2SR10/C2SR11 to amplify *AFT10-1*, *AFT11-1*, *AFT12-1*, and *AFT9-1*, respectively. Controls using genomic DNA as templates were used for band comparisons (lanes 1, 4, 7, 10). Negative reverse transcription (RT) controls were conducted without reverse transcriptase (lanes 3, 6, 9, 12). The reaction products were electrophoresed in 1.2% agarose gel. The marker DNA fragments (M) are indicated on the left

9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid moiety in their toxin structures (Kohmoto et al. 1993; Nakashima et al. 1985; Nakatsuka et al. 1986). No hybridization was found in the rough lemon, apple, tobacco, tomato, or non-pathogenic isolates (Fig. 5B,D,F,H).

Functional analysis of *AFT10-1*

Among the ORFs in pcAFT-2, the function of *AFT10-1* was analyzed because in preliminary Southern blots using various restriction enzymes it appeared to exist as a single copy in the genome of NAF8 (data not shown). The first disruption vector, p24, was expected to disrupt *AFT10-1* by double-crossover homologous recombination. Five hygromycin-resistant mutants, including four double-crossover mutants (44B1, 24.1, 24.4, 24.5) were identified based on Southern hybridization (Fig. 6A). Three fragments (4.9kb, 845bp, 3kb) upon *Hind*III digestion were expected to hybridize to the ACD probe (Fig. 6B). The presence of the 3-kb native band in all the mutants suggested that *AFT10-1* exists in more than one copy in NAF8. The second copy of mutant *AFT10-1* was disrupted using the vector pACD2. This vector was also expected to disrupt the gene by double-crossover homologous recombination. Several geneticin-resistant mutants were obtained. These mutants were also resistant to hygromycin, indicating the integration of the vectors. Based on the Southern blot analysis of 24.1.8, derived from 24.1, the second copy was disrupted by a double-crossover homologous recombination event (Fig. 6C–E).

In pathogenicity tests, disruption of two copies of the gene greatly reduced the number of lesions but did not

completely abolish pathogenicity on pear and strawberry leaves (Figs. 7, 8A). The other mutants produced from the first- and second-round disruption events also had reduced pathogenicity. A greater reduction was produced by the second-round mutants compared with that of the first-round mutants (data not shown). Southern hybridization showed the presence of the native band, indicating that *AFT10-1* was not totally disrupted. Results, however, confirmed the role of *AFT10-1* in the pathogenicity of *Alternaria alternata* strawberry pathotype. The wild type and the mutants did not differ significantly in their penetration behavior (Fig. 8B), so the decrease in pathogenicity cannot be attributed to a fitness penalty. A decrease in toxin production is more likely to be responsible.

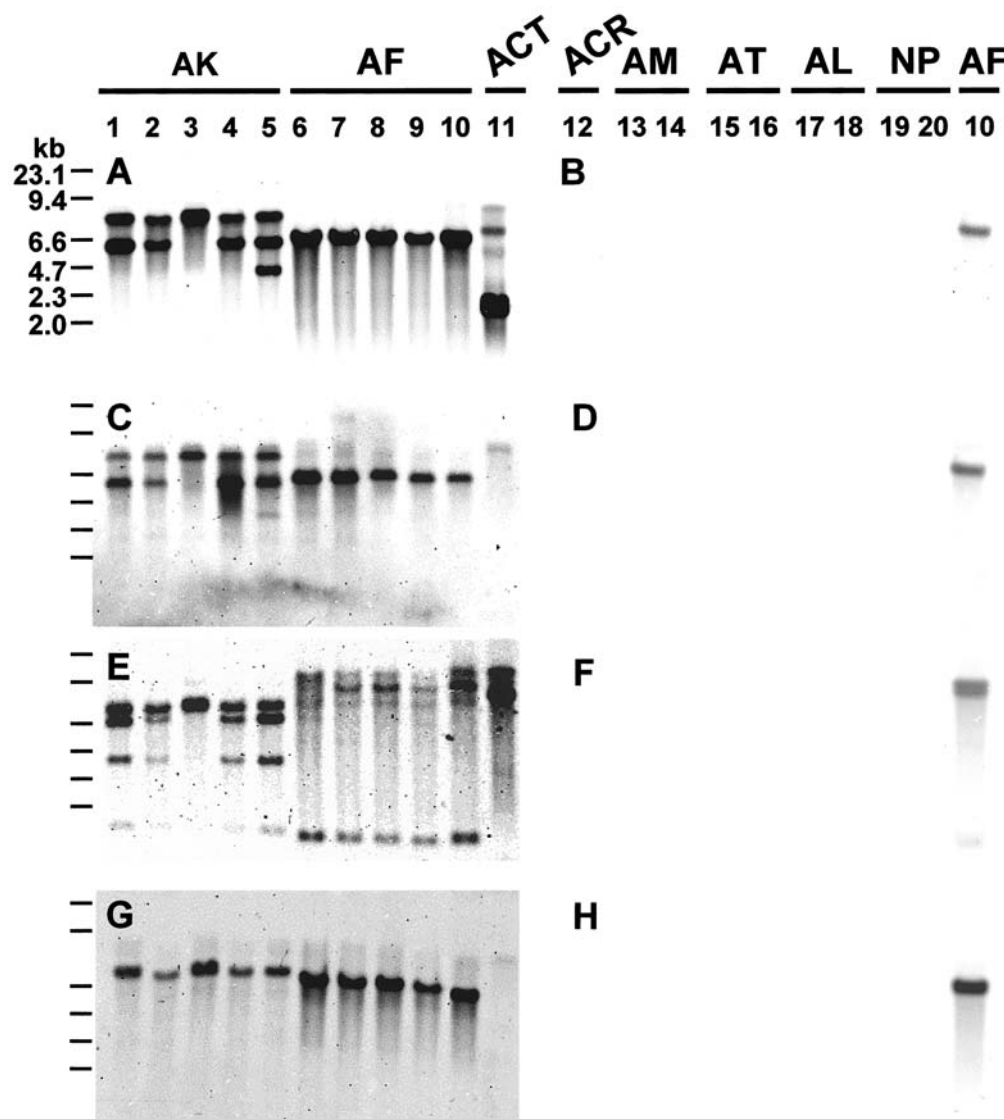
Toxin productivity was reduced in the mutants compared with that in the wild type (Fig. 8C). AF-toxin I was 55.5% and 97.9% lower in mutant strains 24.1 and 24.1.8, respectively, than in the wild type. AF-toxin II production decreased to almost 70% in 24.1 and up to 90% in 24.1.8. This shows that each copy of *AFT10-1* disrupted was functional and contributed to toxin production.

Discussion

Previously, a cosmid genomic library of NAF8 was screened and examined for the distribution of *AKT* homologs by DNA gel blot analysis. Four cosmid clones were obtained. Structural analysis of pcAFT-2 was reported in this article. The *AKT* homologs in pcAFT-2 are *AFTR-2* and *AFT3-2*. These genes have corresponding homologs in pcAFT-1, designated *AFTR-1* and *AFT3-1* (Hatta et al. 2002). *AFTR-2* is homologous to *AKTR-1* and *AKTR-2*, which are found in the Japanese pear pathotype. Both *AKTR-1* and *AKTR-2* have been studied previously and were suggested to be required for the expression of pathway genes of the 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid structure in the Japanese pear, strawberry, and tangerine pathotypes (Tanaka and Tsuge 2000). *AFT3-2*, on the other hand, is homologous to both *AKT3-1* and *AKT3-2*. Targeting of *AKT3-2* produced nonpathogenic, toxin-deficient mutants, indicating its role in toxin biosynthesis. Based on their homology to that of the *AKT* genes, it is likely that *AFTR-2* and *AFT3-2* may also be involved in toxin biosynthesis.

Four new ORFs that were identified upstream of the *AKT* homologs encode a polyketide synthase (*AFT9-1*), an acyl-CoA dehydrogenase (*AFT10-1*), a cytochrome P450 monooxygenase (*AFT11-1*), and an oxidoreductase (*AFT12-1*). The presence of RT-PCR products indicates that these ORFs are transcriptionally active. The function of the *AFT9-1* mentioned here could not yet be established. It is possible that in *Alternaria alternata* isolate NAF8 only part of *AFT9-1* is functional – thus the difficulty in establishing the intron position. Based on the BLAST search, the terminal portion of *AFT9-1* is also part of a carnitine acyltransferase, suggesting that it may indeed be a pseudogene. However, the functionality of the *AFT9-1* should be further investigated because AF-toxin is a polyketide mol-

Fig. 5. Distribution of the ORFs in *Alternaria alternata*. Distribution of *AFT11-1*, *AFT10-1*, *AFT12-1*, and *AFT9-1* in Japanese pear (AK), strawberry (AF), and tangerine (ACT) pathotypes (A, C, E, and G, respectively) and in rough lemon (ACR), apple (AM), tobacco (AT), tomato (AL), and nonpathogenic (NP) pathotypes of *A. alternata* (B, D, F, and H, respectively) were determined. Total DNA of each strain was digested with *Eco*RI and run in 0.8% agarose gel. The blots were probed with PCR fragments amplified for each ORF. Molecular size markers are indicated on the left side of the blots. Lane numbers found on top of the blots correspond to the isolate number found in Table 1. In B, D, F, and H, NAF8 isolate was included to serve as a positive control



ecule, and so a functional PKS should be part of the toxin gene cluster. Distribution analysis also showed that the four ORFs are found only in the Japanese pear, strawberry, and tangerine pathotypes, which share a common 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid structure (Kohmoto et al. 1993; Nakashima et al. 1985; Nakatsuka et al. 1986), suggesting their role in AF-toxin production. The *AFT* genes in pcAFT-2 appear to be part of the AF-toxin gene cluster. The physical clustering of genes suggests that these genes are acquired through gene transfer (Walton 2000). Because the total set of biosynthetic genes should be transmitted from one species to the other to provide the recipient with a complete biosynthetic pathway, the chance is higher if the genes are clustered (Walton 2000). The *A. alternata* HST-producing pathotypes have been suggested to evolve as highly specialized parasites with a distinct host via a common biosynthetic pathway (Masunaka et al. 2000). Another important point is that the *AFT* genes have been found on a 1.05-Mb supernumerary or conditionally dis-

pensable (CD) chromosome (Hatta et al. 2002). Thus it is hypothesized that the ability to produce HST in *Alternaria* may have been due to transfer of a CD chromosome (Ito et al. 2004). A CD chromosome is also associated with the toxin phenotype in *Alternaria alternata* apple pathotype (Johnson et al. 2001).

AFT10-1 encodes an acyl-CoA dehydrogenase. Generally, acyl-CoA dehydrogenase is involved in the first step in β -oxidation of fatty acids. This enzyme has an important role in the generation of acetyl-CoA, which is a substrate for the biosynthesis of natural products and a central metabolite in a variety of important physiological processes (Qiu et al. 1999). Disruption of two copies of *AFT10-1* resulted in highly reduced pathogenicity on both strawberry and pear leaves, indicating that this gene plays a role in pathogenicity. Because homologs of this gene are found only in the Japanese pear, strawberry, and tangerine pathotypes, this enzyme must be involved in the formation of 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid

Fig. 6. Southern hybridization patterns of isolate NAF8 of *Alternaria alternata* strawberry pathotype and the disruption mutants. **A** Total genomic DNA was digested with *Hind*III and run in 0.8% gel. The blots were probed with PCR fragment amplified using C2SR19 and C2SR21 with NAF8 as template. **B** Analysis of the integration pattern of the first round mutants using p24 as the disruption vector containing the *hph* cassette as marker. **C** Total genomic DNA was digested with *Hind*III and run in 0.8% gel. The blots were probed with the PCR fragment amplified using C2SR19 and C2SR21 with NAF8 as template. **D** Total genomic DNA was digested with *Hind*III and run in 0.8% gel. The blots were then probed with *npt* cassette excised from pACD2 using *Bam*HI and *Cla*I. **E** Analysis of the integration pattern of the second-round mutants using pACD2 containing the *npt* cassette as the disruption vector

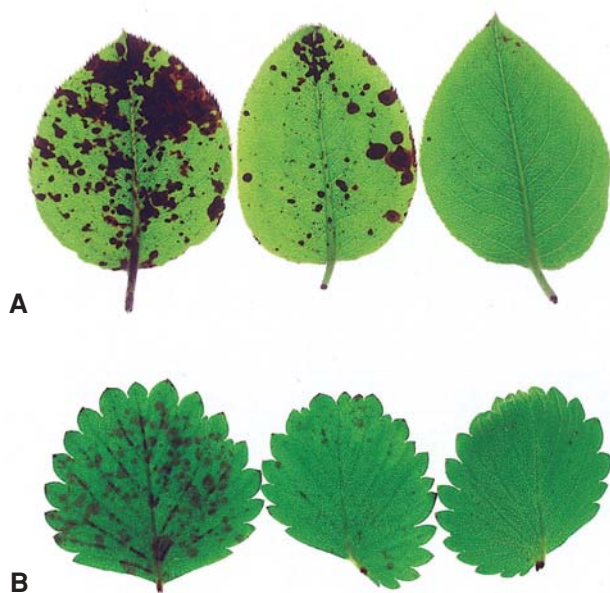
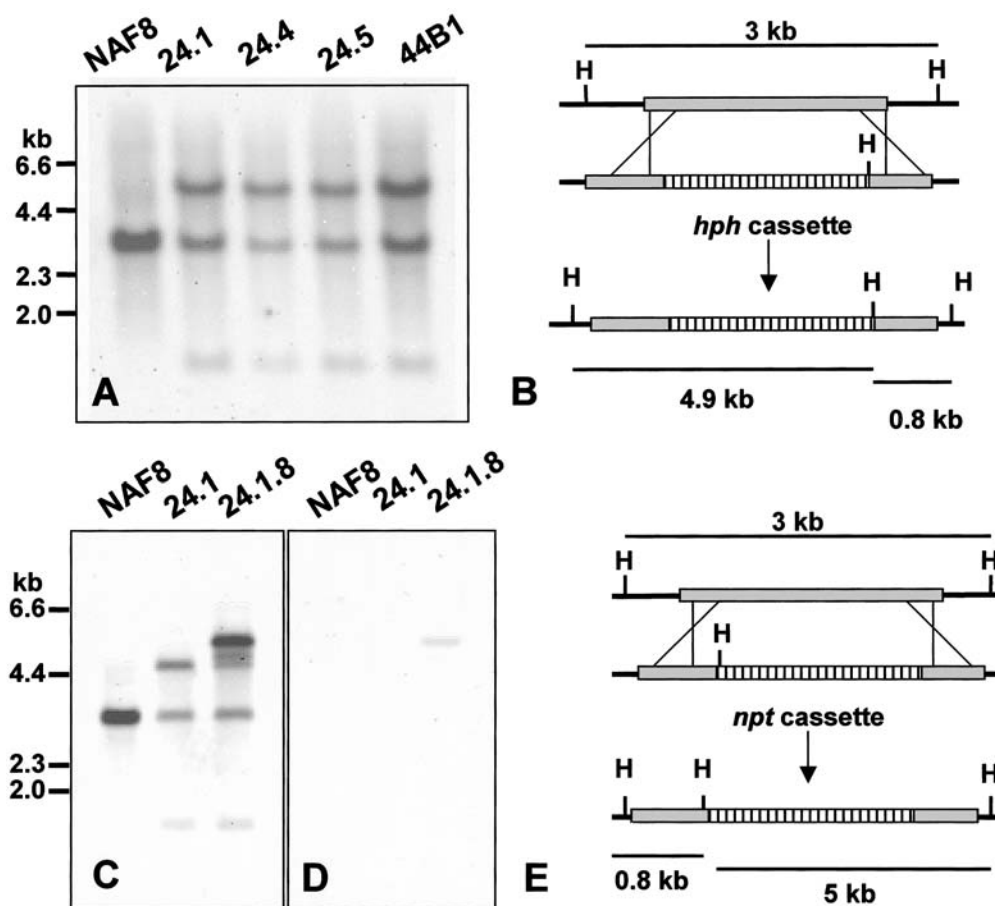


Fig. 7. Pathogenicity test in strawberry and pear leaves. **A** Spore suspensions were adjusted to 1×10^5 spores/ml and sprayed on the leaf surface of the Japanese pear cultivar Nijisseiki. Leaves were incubated in a moist chamber for 36 h at 25°C. The leaves were inoculated with isolate NAF8 of *Alternaria alternata* strawberry pathotype, 24.1, and 24.1.8 (left to right). **B** Spore suspensions were adjusted to 1×10^5 spores/ml and sprayed on the leaf surface of Morioka-16. Leaves were incubated in a moist chamber for 36 h at 25°C. The leaves were inoculated with NAF8, 24.1, and 24.1.8 (left to right)

structure (Kohmoto et al. 1993; Nakashima et al. 1985; Nakatsuka et al. 1986), which is shared by these pathotypes. Several other genes previously identified in the toxin cluster were also unique to the three pathotypes (Hatta et al. 2002; Masunaka et al. 2000; Tanaka and Tsuge 2000; Tanaka et al. 1999), although there are also other genes in the cluster that are pathotype-specific (Ito et al. 2004). The ability of the double copy mutant to cause disease symptoms after disruption of two copies of the gene indicates that there is still a functional copy of the gene. Based on preliminary Southern blot analysis, *AFT10-1* exists as a single-copy gene in the NAF8 isolate. However, the gene may exist as three copies; the adjacent regions of the gene may be conserved within the genome so the banding patterns were initially thought to be that of a single copy gene. Also, based on the results, all three copies may be functional. Thus, disruption of two copies is not enough for total loss in pathogenicity. Results, however, showed that each copy of the gene that was disrupted contributed significantly to toxin production and the pathogenicity of the organism, although it cannot be ascertained yet which copy contributes the most. Production of a null mutant is still ongoing. This article, therefore, is the first report on the disruption of a multicopy gene involved in the AF-toxin gene cluster. It is also interesting to note that although the previous gene-targeting experiments on *AFT1-1* and *AFT3-1* produced nonpathogenic and toxin-deficient mutants through loss of a 1.05-Mb chromosome

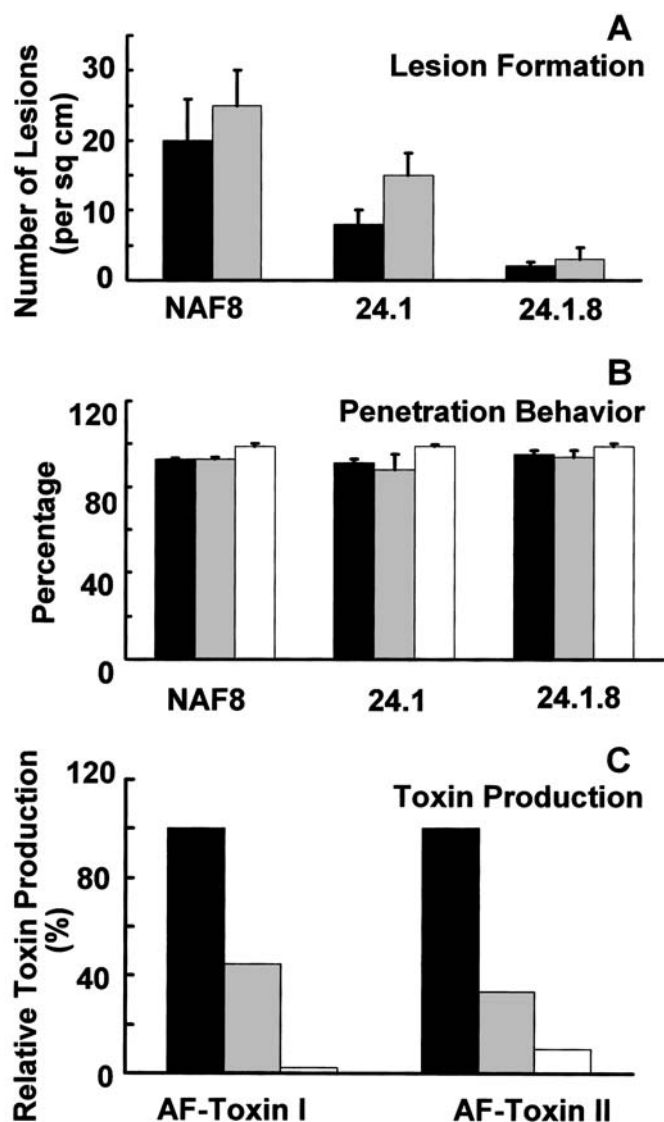


Fig. 8. Lesion formation, penetration behavior, and toxin production of isolate NAF8 of *Alternaria alternata* strawberry pathotype and mutant strains. **A** Strawberry and pear leaves were inoculated with 1×10^5 spores/ml and incubated for 36 h. The average number of lesions from six leaves was computed per square centimeter of leaf area. The vertical lines indicate the standard error of the mean. Black bars represent lesion counts in strawberry. Gray bars represent counts in Japanese pear leaves. **B** Germination rate, appressorial formation, and penetration hyphae formation of the mutants and the wild-type isolate NAF8 were observed. Conidia were allowed to germinate on cellophane sheets, and the number of germinated conidia in about 100 conidia was counted. Counts were done in duplicate. Appressoria and penetration hyphae formation were also noted. The values are given as a percentage. Black, gray, and white bars represent the germination rate, appressorium formation, and penetration hyphae formation, respectively. The vertical lines indicate the standard error of the means. **C** Culture filtrates from the wild-type and mutant strains were harvested and production of AF-toxins I and II was quantified using high-performance liquid chromatography (HPLC) analysis. Relative toxin production was determined by comparing with toxin production by the wild-type isolate NAF8, which was assigned a value of 100%. Black, gray, and white bars represent toxin production in the wild type, NAF8, and mutants 24.1 and 24.1.8, respectively.

and not by site-directed mutagenesis (Hatta et al. 2002), this did not occur here. The same gene-targeting strategy was used in this experiment. However, the difference may be in the position of the genes involved in the chromosome and on the number of copies of the genes. The mechanism underlying the loss of the 1.05-Mb chromosome, however, is still unknown; we therefore cannot yet explain why site-directed gene targeting was possible here without loss of the 1.05-Mb chromosome.

The presence of transcripts and their distribution in the 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid-producing isolates of *Alternaria alternata* suggests that *AFT9-1*, *AFT10-1*, *AFT11-1*, and *AFT12-1* are involved in AF-toxin production and the pathogenicity of the organism. The sequence similarity of *AFTR-2* and *AFT3-2* with that of the *AKT* genes suggests that they may be involved in the formation of the 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid structure (Kohmoto et al. 1993; Nakashima et al. 1985; Nakatsuka et al. 1986). The results of this study provide additional support for the clustering of genes involved in AF-toxin biosynthesis. It also confirms the involvement of *AFT10-1* in toxin production and pathogenicity of NAF8 isolate of *Alternaria alternata*. However, the other genes still need to be characterized to determine their respective roles in the toxin cluster.

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