Insertional Mutagenesis and Cloning of the Genes Required for Biosynthesis of the Host-Specific AK-Toxin in the Japanese Pear Pathotype of *Alternaria alternata*

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The Japanese pear pathotype of *Alternaria alternata* causes black spot of Japanese pear by producing a host-specific toxin known as AK-toxin. Restriction enzyme-mediated integration (REMI) mutagenesis was used to tag genes required for toxin biosynthesis. Protoplasts of a wild-type strain were treated with a linearized plasmid along with the restriction enzyme used to linearize the plasmid. Of 984 REMI transformants recovered, three produced no detectable AK-toxin and lost pathogenicity on pear leaves. Genomic DNA flanking the integrated plasmid was recovered from one of the mutants. With the recovered DNA used as a probe, a cosmid clone of the wild-type strain was isolated. Structural and functional analyses of an 8.0-kb region corresponding to the tagged site indicated the presence of two genes. One, designated *AKT1*, encodes a member of the class of carboxyl-activating enzymes. The other, *AKT2*, encodes a protein of unknown function. The essential roles of these two genes in both AK-toxin production and pathogenicity were confirmed by transformation-mediated gene disruption experiments. DNA gel blot analysis detected *AKT1* and *AKT2* homologues not only in the Japanese pear pathotype strains but also in strains from the tangerine and strawberry pathotypes. The host-specific toxins of these two pathotypes are similar in structure to AK-toxin. Homologues were not detected in other pathotypes or in non-pathogenic strains of *A. alternata*, suggesting acquisition of *AKT1* and *AKT2* by horizontal transfer.

There are seven known host-parasite combinations in which host-specific toxins produced by *Alternaria alternata* (Fr.:Fr.) Keissl. are responsible for disease (Nishimura and Kohmoto 1983; Kohmoto et al. 1995). Host-specific toxins are low-molecular-weight metabolites of diverse structure (Nishimura and Kohmoto 1983; Kohmoto et al. 1995). The participation of host-specific toxins in establishment of plant disease is one of the most clearly understood mechanisms of host-selective pathogenesis (Yoder 1980; Nishimura and Kohmoto 1983; Scheffer and Livingston 1984; Walton 1996).

Previously, species names were adopted for *Alternaria* pathogens that produce particular host-specific toxins (Nishimura and Kohmoto 1983). However, measurements of conidial size of these pathogens fall statistically within the range described for *A. alternata*, a ubiquitous, usually saprophytic, fungus (Nishimura et al. 1978; Nishimura 1980). Each pathogen has a distinct host range due to the production of a particular host-specific toxin, and thus it is possible to distinguish one type of pathogen from another (Nishimura et al. 1978; Nishimura 1980). Based on these features, Nishimura et al. (1978; Nishimura 1980) proposed that the *Alternaria* pathogens be defined as distinct pathotypes of *A. alternata*. Support for this classification was provided by molecular analyses of nuclear ribosomal DNA and mitochondrial DNA (Kusaba and Tsuge 1994, 1995, 1997). Thus, these pathogens provide an interesting example of intraspecific variation and evolution of pathogenicity in plant-pathogenic fungi.

To understand the molecular basis of pathogenic specialization, our efforts have focused on cloning the genes required for host-specific toxin biosynthesis. We have concentrated on the Japanese pear pathotype that produces a host-specific toxin known as AK-toxin (Fig. 1) and causes black spot of certain cultivars of Japanese pear, including the commercially important cultivar Nijisseiki (Tanaka 1933; Nakashima et al. 1985; Otani et al. 1985). Black spot disease of Japanese pear first became prominent in the early 1900s, shortly after cultivation of Nijisseiki pear was initiated, and has been the most critical problem in pear cultivation since (Tanaka 1933; Nishimura and Kohmoto 1983). Host-specific toxins from *A. alternata* pathogens are diverse in structure (Nishimura and Kohmoto 1983; Kohmoto et al. 1995). However, AK-toxin of the Japanese pear pathotype, AF-toxin of the strawberry pathotype, and ACT-toxin of the tangerine pathotype have a common moiety, 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic

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Nucleotide and/or amino acid sequence data for *AKT1* and *AKT2* are to be found in the DDBJ, GenBank, and EMBL data bases as accession numbers AB015351 and AB015352, respectively.

This paper is dedicated to the late Syoyo Nishimura, who provided us the opportunity to carry out this study and to learn his eminent way of thinking about host-specific toxins.

acid, in their structures (Fig. 1) (Nakashima et al. 1985; Nakatsuka et al. 1986; Kohmoto et al. 1993). This common moiety has been identified as a precursor of the toxins (Feng et al. 1990; Nakatsuka et al. 1990; Kohmoto et al. 1993). Thus, we hypothesize that these three pathotypes share genes encoding the enzymes for biosynthesis of 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid.

Although the chemical structures of the *Alternaria* host-specific toxins have been elucidated, knowledge of toxin biosynthesis is limited (Feng et al. 1990; Nakatsuka et al. 1990). Conventional genetic analysis is not possible with *A. alternata*, which lacks a known sexual cycle. Recently, the restriction enzyme-mediated integration (REMI) technique was developed for efficient transformation and tagging mutagenesis in fungi. This method was first described for *Saccharomyces cerevisiae* (Schiestl and Petes 1991) and then applied to *Dicystoselium discoideum* (Kuspa and Loomis 1992) and other fungi (Lu et al. 1994; Bölker et al. 1995; Shi et al. 1995; Sweigard et al. 1998). In the maize pathogen *Cochliobolus heterostrophus*, the REMI technique was successfully used to tag and clone a gene, *PKS1*, that encodes a polyketide synthase required for biosynthesis of a host-specific toxin known as T-toxin (Lu et al. 1994; Yang et al. 1996). Enhanced transformation efficiencies and mutagenesis by REMI have been recently reported in the tomato pathotype of *A. alternata* (Akamatsu et al. 1997).

We report here REMI tagging, cloning, and characterization of genes required for AK-toxin biosynthesis of the Japanese pear pathotype. We identified two genes, *AKT1* and *AKT2*, essential for both toxin production and pathogenicity.

RESULTS

REMI transformation.

Plasmid pSH75, which carries the hygromycin B resistance gene (*hph*) as a selectable marker with no homology to *A. alternata* DNA (Tsuge et al. 1990; Kimura and Tsuge 1993), was used for transformation. When protoplasts (about 10^7) of wild-type strain 15A were treated with circular or linearized pSH75 DNA (5 μ g) in 200 μ l of transformation mixture, transformants arose at low frequency (usually less than 10 transformants). Addition of restriction enzyme markedly increased the transformation rate: the best results (103 to 156 transformants) were obtained when 50 U of *Bam*HI or 100 U of *Hind*III was used.

Isolation of *Tox*⁻ mutants.

A total of 984 REMI transformants were tested for pathogenicity to Nijisseiki pear leaves. Three transformants, designated ATA95-1, ATA95-2, and ATA95-3, resulting from *Hind*III-mediated integration appeared to completely lose pathogenicity (Fig. 2A). AK-toxin production was tested with a bioassay involving Nijisseiki leaves, which is the most sensitive method for detection of toxin (Hayashi et al. 1990). Culture filtrates, mycelial extracts, and spore germination fluids of the wild-type strain showed toxicity to Nijisseiki pear leaves, but those of the nonpathogenic mutants had no toxicity (Fig. 2B–D), indicating loss of AK-toxin production. AK-toxin has been characterized as two related molecular species, AK-toxins I and II (Fig. 1), with toxin I being the predominant species with respect both to yield and to biological activity

(Nakashima et al. 1985; Otani et al. 1985). Toxins I and II were quantified by reverse-phase high-performance liquid chromatography. Toxin I was detected in culture filtrates (94.2 ng $\cdot$ ml⁻¹), mycelia (400 ng $\cdot$ g⁻¹), and spore germination fluid (12.5 ng $\cdot$ ml⁻¹) of the wild-type strain, but not in those of the mutants. Toxin II was not detectable in wild type and mutants. A precursor of AK-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. 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid was present in culture filtrate of the wild-type strain (686 ng $\cdot$ ml⁻¹) but not in those of three mutants, suggesting that these mutants lost the ability to produce this precursor. All mutants were prototrophic, and grew and sporulated as well as the wild-type strain (data not shown). There were no significant differences in spore germination, appressorium formation, or hyphal penetration on cellulose membranes between the wild type and each of these mutants (data not shown). These results suggest that all mutations that affect pathogenicity are attributable to loss of AK-toxin production.

Characterization of the plasmid insertion sites in *Tox*⁻ mutants.

To analyze plasmid integration events in three *Tox*⁻ mutants, total DNA was digested to completion with restriction

Fig. 1. Structure of host-specific toxins produced by three pathotypes of *Alternaria alternata*. AK-toxins of the Japanese pear pathotype (Nakashima et al. 1985), ACT-toxins of the tangerine pathotype (Kohmoto et al. 1993), and AF-toxins of the strawberry pathotype (Nakatsuka et al. 1986) have a common moiety, 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid, indicated in shadowed boxes.

enzymes, electrophoresed, blotted, and probed with ^{32}P -labeled pSH75. The pSH75 probe did not hybridize to total genomic DNA of the wild-type strain. The analysis showed that the plasmid integration events in these mutants were complex. *Apa*I, which has no site in pSH75, produced only high-molecular-weight hybridizing bands (>23 kb) in all mutants, suggesting multiple-copy integration of plasmids as tandem repeats (Fig. 3). *Hind*III, which was used in REMI transformation and has one site on pSH75, also produced only high-molecular-weight hybridizing bands (>23 kb), and no 5.5-kb band (the size of pSH75), demonstrating that the *Hind*III site on the plasmid had been lost, or that pSH75 did not insert at a *Hind*III site (Fig. 3). *Eco*RV, which has a single site in pSH75, was also used in the analysis. DNA of all the mutants shared a 5.5-kb *Eco*RV fragment, the size of pSH75, derived from tandem repeats of integrated pSH75 (Fig. 3). ATA95-3 had two faint additional bands, which probably correspond to the junctions between the recipient genomic DNA and integrated pSH75 (Fig. 3). ATA95-1 and ATA95-2 had several additional bands, which seem to have resulted from rearrangements of integrated plasmids (Fig. 3). These results demonstrate multiple-copy integration of plasmids in all mutants and rearrangements of integrated plasmids in ATA95-1 and ATA95-2.

Isolation of the DNA at the tagged locus.

Complex integration patterns of the transforming plasmid made it difficult to recover genomic DNA flanking the tag by plasmid rescue. Thus, genomic libraries of *Tox*⁻ mutants were screened with ^{32}P -labeled pSH75 and subsequently with ^{32}P -labeled total DNA of the wild-type strain. Two positive clones, which hybridized to both probes, were isolated each from the library of ATA95-1 or ATA95-3. We first analyzed two clones of ATA95-3, because the plasmid integration pat-

tern in this mutant was not as complex as in the other mutants (Fig. 3). Nucleotide sequencing of DNA flanking the plasmid insertion site in these clones detected the same sequences, which are highly homologous to long terminal repeat retrotransposons of *Magnaporthe grisea* (Dobinson et al. 1993; Farman et al. 1996). We did not think that the retrotransposon-like sequence has direct involvement in AK-toxin biosynthesis. Thus, we focused on the two clones of ATA95-1, named λ ATA1-1 and λ ATA1-2.

Fig. 3. DNA gel blot analysis of the *Tox*⁻ mutants of *Alternaria alternata*. Total DNA was digested with (lane 1) *Apa*I, (lane 2) *Hind*III, or (lane 3) *Eco*RV and separated in an 0.8% agarose gel. Blots were probed with pSH75. Sizes (in kilobases) of marker DNA fragments (*Hind*III-digested λ DNA) are indicated on left.

Fig. 2. Pathogenicity and AK-toxin production by *Alternaria alternata* wild-type strain 15A (W) and mutants ATA95-1 (1), ATA95-2 (2), and ATA95-3 (3). **A**, Detached leaves of susceptible pear cultivar Nijisseiki were spray inoculated with spore suspensions and incubated in a moist box at 25°C for 20 h. **B–D**, Detached leaves were wounded slightly: **(B)** culture filtrates, **(C)** mycelial extracts, or **(D)** spore-germination fluids were placed on the wounded portion and incubated in a moist box at 25°C for 20 h.

Restriction mapping of these clones with *EcoRI* and *EcoRV* showed that they contained the same side of the plasmid integration site (Fig. 4). λ ATA1-1 confirmed that multiple copies of the vector were present (Fig. 4). The 6.2-kb *EcoRI* fragment, which consists of the *Escherichia coli* plasmid pSP72 and flanking fungal DNA, was rescued in *E. coli* as pATA1-1 (Fig. 4). Restriction mapping of pATA1-1 showed that there were no *HindIII* sites at the junction of genomic DNA and integrated plasmid as predicted (Fig. 4).

To confirm that the recovered DNA corresponds to the plasmid integration site in mutant ATA95-1, the *SphI-XhoI* fragment (Fr. S-X) in the pATA1-1 insert (Fig. 4) was used to probe a DNA gel blot. Total DNA of wild type and mutant was digested with *EcoRV*, which has no site in the probe fragment (Fig. 4). Unexpectedly, the probe hybridized to two bands of 2.8 and 3.2 kb in the wild-type strain; the 2.8-kb band was more intense than the 3.2-kb band, indicating that the wild-type strain has multiple copies of genomic DNA homologous to the probe (Fig. 5). The mutant had the intense 2.8-kb band found in the wild type, but not the weak 3.2-kb band, and carried a new band of 5.1 kb resulting from plasmid integration (Figs. 4 and 5). These results imply that the 3.2-kb band represents a gene essential for toxin production, and that

Fig. 5. DNA gel blot analysis of (lane 1) wild-type strain 15A and (lane 2) mutant ATA95-1. Total DNA was digested with *EcoRV* and separated in an 0.8% agarose gel. Blot was probed with the *SphI-XhoI* fragment (Fr. S-X) in the pATA1-1 insert (see Figure 4). Sizes (in kilobases) of marker DNA fragments (*HindIII*-digested λ DNA) are indicated on left.

Fig. 4. Structure of the genomic locus tagged with pSH75 in *Tox⁻* mutant ATA95-1. λ ATA1-1 and λ ATA1-2 were isolated from a genomic phage library of ATA95-1. The 6.2-kb *EcoRI* fragment consisting of the *Escherichia coli* plasmid pSP72 and its flanking fungal DNA was recovered from λ ATA1-1 by plasmid rescue as pATA1-1. The *SphI-XhoI* fragment (Fr. S-X) in pATA1-1 was used as a probe for hybridization experiments. pcAKT-1 was isolated from a genomic cosmid library of wild-type strain 15A by screening with Fr. S-X probe, and the 8.0-kb region was sequenced. Arrows indicate the protein coding regions, designated ORF1 and ORF2. Positions of introns are indicated by "V" shapes. Arrowheads above ORF1 and ORF2 denote orientation and location of oligonucleotide primers used in polymerase chain reaction (PCR) and reverse transcription-PCR experiments. p1EX and p2XS were made by cloning the indicated fragments in pBluescript KS⁺ and pGEM-T Easy, respectively, and used as probes for hybridization experiments. Fragments were also cloned in pSH75 (Kimura and Tsuge 1993) to produce the targeted disruption vectors pGDT1 and pGDT2. Abbreviations: A, *ApaI*; Ba, *BamHI*; Bg, *BglII*; E, *EcoRI*; H, *HindIII*; Ps, *PstI*; Pv, *PvuII*; Sa, *Sall*; Sp, *SphI*; V, *EcoRV*; Xb, *XbaI*; Xh, *XhoI*.

the intense band might carry multiple copies of homologues nonfunctional in toxin production.

A cosmid genomic library of the wild-type strain was screened with ^{32}P -labeled Fr. S-X (Fig. 4). Positive clones were digested with *EcoRV* and hybridized with the same probe. A clone containing the 3.2-kb *EcoRV* fragment hybridizing to the probe was isolated and named pcAKT-1 (Fig. 4).

Identification of two open reading frames in the tagged locus.

The region within pcAKT-1 corresponding to the tagged site in mutant ATA95-1 was identified, and the nucleotide sequence of an 8.0-kb region determined. No *HindIII* (the enzyme used in the original REMI transformation) site was detected at the plasmid integration site in ATA95-1 (Fig. 4). Sequencing of the junction of plasmid and genomic DNA in pATA1-1 demonstrated that eight nucleotides at the end of the plasmid including the *HindIII* site had been deleted during the integration process. The DNA sequence of the 8.0-kb genomic region was analyzed for putative protein coding regions. The analysis identified two open reading frames, designated ORF1 and ORF2 (Fig. 4).

ORF1 (1,734 bp) potentially encodes a 578 amino acid protein after splicing of five putative introns (Fig. 4). Introns were deduced on the basis of presence of consensus sequences for 5' splice sites [GT(A/G/T)(A/C/T)G(T/C)] and 3' splice sites [(C/T)AG] typical of fungal genes (Bruchez et al. 1993). The ORF1 product has high similarity in amino acid sequence to 4-coumarate-CoA ligases of higher plants, acetyl-CoA ligases of microorganisms, long chain acyl-CoA synthetases of microorganisms, and luciferases of insects (Fig. 6). These enzymes have been identified as members of a superfamily, designated the carboxyl-activating enzymes or the AMP-binding proteins (Fulda et al. 1994; Pavela-Vrancic et al. 1994). The ORF1 product contains the highly conserved stretch of amino acids (amino acid positions 205 to 224) found in the carboxyl-activating enzyme family (Fulda et al. 1994; Pavela-Vrancic et al. 1994) (Fig. 6). The conserved lysine residue (position 218) within this sequence has been proposed to be essential for catalysis (Pavela-Vrancic et al. 1994). The ORF1 product also contains a consensus sequence TGD (amino acid positions 436 to 438) for the putative ATP binding region (Pavela-Vrancic et al. 1994) (Fig. 6).

Transcription of ORF1 and confirmation of introns were tested by reverse transcription-polymerase chain reaction (RT-PCR) experiments. RT-PCR amplification produced DNA fragments of the expected size from total RNA of the wild type (Fig. 7A-C). RT-PCR products were cloned in pGEM-T Easy and sequenced. Comparison of the genomic sequence with those of the RT-PCR products confirmed that the five introns are spliced.

ORF2 (786 bp) potentially encodes a 262 amino acid protein after splicing of one intron (Fig. 4). The ORF2 product has no similarity to known proteins in the data bases. RT-PCR amplification produced DNA fragments of the expected size from total RNA of the wild-type strain (Fig. 7D). Nucleotide sequencing of the products confirmed the presence of the intron in this region.

Mutation of ORF1 and ORF2 in the wild type by targeted gene disruption.

To assess the function of ORF1 and ORF2 in AK-toxin biosynthesis, fragments of the reading frames were cloned in pSH75 to produce targeting vectors pGDT1 and pGDT2 (Fig. 4). Transformation of the wild type with pGDT1 and pGDT2 yielded 12 out of 100 pGDT1 transformants and three out of 100 pGDT2 transformants that had completely lost AK-toxin production and pathogenicity. AK-toxin I and 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid in culture filtrates of a subset of toxin-producing (Tox^+) and toxin-deficient (Tox^-) transformants were quantified by high-performance liquid chromatography. Toxin I was detected in culture filtrates of all Tox^+ transformants tested: 60 to 393 $\text{ng} \cdot \text{ml}^{-1}$ in five pGDT1 transformants and 180 to 857 $\text{ng} \cdot \text{ml}^{-1}$ in five pGDT2 transformants. Culture filtrates of Tox^+ transformants also contained 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid: 274 to 514 $\text{ng} \cdot \text{ml}^{-1}$ in pGDT1 transformants and 331 to 1,166 $\text{ng} \cdot \text{ml}^{-1}$ in pGDT2 transformants. In contrast, the same analyses could detect neither toxin I nor 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid in culture filtrates of five and three of Tox^- transformants produced by pGDT1 and pGDT2, respectively.

The integration mode of the targeting vectors in these Tox^+ and Tox^- transformants was analyzed by DNA gel blot analysis (Fig. 8). Total DNA of the wild-type strain and pGDT1 transformants was digested with both *HindIII* and *SphI*, and the blot was probed with the ORF1 fragment in pGDT1. These enzymes have no site in pGDT1. On the basis of the restriction map of pcAKT-1, a 1.9-kb fragment was expected in the wild type (Fig. 4); however, the probe hybridized to two bands (1.9 and 2.3 kb) (Fig. 8A). The 2.3-kb signal was more intense than that of the 1.9-kb band, demonstrating that the wild-type strain contains multiple copies of the ORF1 homologue. All five Tox^+ transformants carried these two hybridizing bands (Fig. 8A). In contrast, the 1.9-kb hybridizing band was not detected in the Tox^- transformants (Fig. 8A). One of the Tox^- transformants also lost the 2.3-kb band. The other Tox^- transformants had the 2.3-kb bands, although their hybridizing signal was weaker than those of the wild type (Fig. 8A). These results suggest that the 1.9-kb fragment represents the functional ORF1. Tox^+ and Tox^- transformants both carried high-molecular-weight hybridizing bands (>23 kb) with markedly intense signals, suggesting high-copy-number integration of plasmids as tandem repeats (Fig. 8A). Such integration has been observed in transformation of *A. alternata* with plasmids that contain or lack the fungal DNA (Tsuge et al. 1990; Shiotani and Tsuge 1995). To control for the relative amounts of DNA loaded in each lane, the same blot was subsequently stripped of the ORF1 probe and rehybridized with the 1.4-kb *BamHI* fragment in pBBR2 (Kawamura et al. 1999) as a single-copy gene probe (Fig. 8C). pBBR2 contains the *A. alternata* *BRM2* gene involved in melanin biosynthesis (Kawamura et al. 1999). The *BRM2* probe visualized single bands of the expected size in all lanes, indicating complete digestion of DNA (Fig. 8C). Although intensity of hybridization signal was not equal in all lanes, there were no lanes in which DNA was considerably overloaded or underloaded (Fig. 8C).

To confirm ORF1 disruption in Tox^- transformants, the presence of ORF1 transcript was tested by RT-PCR with

primers RT1-5 and RT1-6 (Figs. 4 and 8E). RT-PCR produced a DNA fragment of 0.69 kb from total RNA of the wild-type and Tox⁺ transformants, but not from those of Tox⁻ transformants (Fig. 8E).

Total DNA of pGDT2 transformants was digested with *Pvu*II, which has no site in pGDT2, and the blot was probed with the ORF2 fragment in pGDT2. The restriction map of pcAKT-1 predicts that the probe would hybridize to one 5.0-kb *Pvu*II fragment in the wild type (Fig. 4); however, two bands (5.0 and 6.7 kb) hybridized (Fig. 8B). The 6.7-kb signal was more intense than the other. Thus, there are also multiple copies of the ORF2 homologue in the wild type. All Tox⁺ transformants contained these two bands (Fig. 8B). All Tox⁻

transformants lacked the 5.0-kb bands (Fig. 8B). Rehybridization of the blot with the *BRM2* probe showed that the relative amounts of DNA were not considerably different among lanes (Fig. 8D). An RT-PCR experiment with primers RT2-1 and RT2-2 (Fig. 4) confirmed ORF2 disruption in Tox⁻ transformants: the expected 0.78-kb DNA fragment was detected in wild-type and Tox⁺ transformants, but not in Tox⁻ transformants (Fig. 8F). These results imply that ORF2 on the 5.0-kb *Pvu*II fragment encodes the functional gene.

Since these gene disruption experiments demonstrated the essential roles of ORF1 and ORF2 in AK-toxin biosynthesis and pathogenicity, the genes encoding ORF1 and ORF2 were designated *AKT1* and *AKT2*, respectively.

ORF1	1	-----MVFAAP-----CWVPLP-SDLPDSTLEEF-----IFCQVKDSQTRSELD--RSILICGTQ
4CL	1	MAPQEQAVSQVMEKQSNNSNDVIFRSKLPDIYIPNHLSDHY-----IFQNISEFATKP-----CLINGPT
ACUA	1	---MNLKALPAIEGDHNLKNYEETIRHFDW-AEAKEHFSWHETGKLNAAEYEDRHAESFRKNKVALYKDAKR
LCFA	1	-----MRVMEVPEFVKN--RYWLERAWIEGVADVEIPEK-----PLYEVIDEVCQKY-----ADRTAIFY
LUCI	1	-----ME-----DKN-----ILYGPEP-FHPADGTAEGQ-----MEYALSRYADISG-----CIALTNAHT
ORF1	50	GKEYTVQESMERTGRLLAQLSAWLDWPKPSEEDWKVAIFENINCVFFESISHAIHRLGGTVSAINASSTADEL
4CL	63	GHVYTVSDVHVVISRQIAANFHKLG-VNQN-----DVMLLLPNCPEFVLSFLAASFRGATATAANPFFTPAEI
ACUA	71	DEKYTFKEMKEESNRAGNVLRRYGNVEKG-----DRVFIEMRSPPELYFIMLGAIKIGAIAGPLFEAFMEGAV
LCFA	56	GAELIKYGOLKEYTDRFATSLAKMG-IKKG-----DVAIYSPNCPQFVIAYYGAMKAGATVLTALSPLFAPREV
LUCI	47	KENVLYEEFLKLKSLCLAESFKKYG-LKQN-----DTIAVCSENGLOQFELPLIASLYLGIIAAPVSDKYIEREL
ORF1	124	EAOLRLSNAQAIFTCNTLLKIAMKASQKVGIPLANIFLTDAPGSYRFDVYPPQFIDNIVRTARSSSLPLLQLGR
4CL	130	AKQAKASNTKLIITEARYVDKIKPLQN--DDGVVIVCIDDNESVPIPEGCLRFTELQSTTEASEVIDSVEISP
ACUA	139	KDRLENSEAKVVVTTPELLERIPVDKLP-HLQHVVFVVGGEAESGTNIINYDEAAKQ-ESTRLDIEWMD--K-K
LCFA	123	EYQINDSGAKVLVTVEQLYPNFAAVRENTGVEEVLVANIAGGEAKVEGKFRDFREMLASPPPEPVS--WNVK
LUCI	114	IHSLGIVKPRIIFCSKNTFQKVLNVKSRLKYVETIITLDLNEDLGGYQCLNNEISQNSDINLDVKKFKPNSFNR
ORF1	198	GQGSSTPAYICFSSGTSGAQKPVLLSHQGIAN-----IVQINT-----FE
4CL	202	DD-V---VALPYSSGTTGLPKGVMLTHKGLVT-----SVAQQVDG-----ENP
ACUA	207	DG-----FLLHYTSGSTGTTPKGVLVHVEAMIQ-----QYQTG-----KW
LCFA	194	DD-V---AVLQYTGTTGLPKAAMLTHTNVVANMYELIPFTDLIRGWLKSHKVIREEGDYVIVDLGKEYRTFR
LUCI	188	DDQV---ALVMFSSGTTGVSKGVMLTHKNIVA-----RFSHCKDP-----TFG
ORF1	239	KFRQKGPNVSLCILPLAHSYGLVC---VAYNALYRGDRLAYLSSSDVEDLLSIVEKLRINTLYLVPTLLSRILS
4CL	241	NLYFHSDDVILCVLPMPFHIIVALNS---IMLCGLRVGAAILMPKFEINLLELIQRCKVTVAPMVPIVLAIK
ACUA	241	VLDLKEEDIYWCADPGWVTGTIVYG---IFAPWLNAGATNVIYGRFSPEISWYGTIEQLGVNVWVSAPTAFRMLMG
LCFA	264	DYYNDYSPRVAIILPWYHIYQGTV---DLNTGLATGDIIVFAQFEPEKILEAIERYRVATFMGAPAIFFVLAN
LUCI	228	N-AINPTTAILLTVIPFHHGFGMTTTLGYFTCGFRV---ALMHTFEKLFQLQSLQDYKVESTLLVPTLMAFFPK
ORF1	310	G--GKAGGHDLSCVKEVYTGAPLHPMLGEHILRHHPTW---KFKQCYGATEAGTAVSVTS-----DCDLW-PG
4CL	312	S--SETEKYDLSIRRVKSGAAPLGKELEDVAVNAKFPNA---KLGQGYGTEAGPVLAMSLGFAKEPFPVK-SG
ACUA	313	AGDEMAAKYDLTSLRHVLSVGEPLNPEVIRWGHVKFNKR---IHDTWMTETGSLICNY---PCMDEIK-PG
LCFA	335	NP-DLLKRYDLTSLLYVNNAGAPVPEIEIKWDELKDAGRGILVEGYLSBASPVTHTVG---PPFRKRKIG
LUCI	297	S--ALVEKYDLSHLKEIASGGAPLSKEIGEMVKRRFKLN---FVRQGYGLTETTSAVLITP-----DTDVR-PG
ORF1	373	SVGCLLPGVQAKIIRS DGSETTKHDES GELWVSS--PSLAI GYLSNPLATEATFTVDNTGRTWLRTGDEAKICL
4CL	380	ACGTVVRNAEMKIVDPDTGDSLSRNQPGGEICIRG--HOIMKGYLNPPAATAETIDKDQ---WLHTGDIIGLIDD
ACUA	378	SMGKPIPGVEAAIVD-NQGNELPPYRMGNLAIKKGWPSMMHTIWNNEKYESYFMPGG---WYVSGDSAYMDE
LCFA	405	SVGPPPIPTYAALINPETMEFLPIGEE GELVIAG--POVMKGYWNRERETEDVFEAGG-MKWLRGTGDIKMDDE
LUCI	360	STGKIVPFHAVKVVDPTTGKILGPNETGELYFKG--DMIMKSYNNEEATKAIINKDQ---WLRSGDIAIYDYN
ORF1	445	SPNGNEHLFIVDRIKDIIVKGFQVAPVELEQLLSNDFVEVAITSRQDKRGEERPOAFVVRTHEGLKEPQDA
4CL	447	--DD--ELFIVDRLKELIKYKGFQVAPAELEALLIGHPDITDVAVVAMKEEAAAGEVPVAFVVK-SKDSE---L
ACUA	446	--EG--YFWFQGRVDDVIMTSGERVGPFEVESKLVEHPAIAEAGVIGKPDVVRGEIIKAFIAL-REGFEPS-DK
LCFA	475	--DG--YFYIVDRLKDIIVKGFQVAPVELEQLLSNDFVEVAITSRQDKRGEERPOAFVVRTHEGLKEPQDA
LUCI	427	--DG--HFFYIVDRLKSLIKYKGFQVAPAEIEGILLQHPYIVDAGVTGIPDEAAGELPAAGVUVV-QTGKY---L
ORF1	519	VSESLQALVKKARKARVKKLHPHVI FVDSLPKTTS GSKIMRRALRNMC PANSEVNGRLSSKI
4CL	513	SEDDVKQFVSKQGVVFYKRIN-KVFFTESIPKAPSGKILRKDLRAKLANGL----- (28.4%)
ACUA	515	LKEEIRLFVKQGLAHAAPR-EIEFKDKLKPTRSGKIMRRVLKAWELNLPAGDLSTMED- (21.0%)
LCFA	543	TEQDIINWCKERMALVKKYPR-IVEFRDELPKSAAGKYLRRIEELKMKR----- (27.8%)
LUCI	493	NEQIVQNFVSSQVSTAKWLRGGVKFLDEIPKSGTGKIDRKVLRQMFEEKHKS KL----- (27.6%)

Fig. 6. Similarity of the ORF1-encoded protein with other proteins. Amino acid sequence of the ORF1-encoded protein was aligned with those of 4-coumarate-CoA ligase of *Arabidopsis thaliana* (4CL) (Lee et al. 1995), acetyl-CoA ligase of *Bacillus subtilis* (ACUA) (Grundy et al. 1993), long chain acyl-CoA synthetase of *Archaeoglobus fulgidus* (LCFA) (Klenk et al. 1997), and luciferase of *Photuris pennsylvanica* (LUCI) (Ye et al. 1997) by the CLUSTAL W program (Thompson et al. 1994). Amino acids that are conserved between the ORF1 product and any of the others are indicated as white letters on a black background. Amino acid identity between the ORF1 product and each of the others is shown in parentheses. The highly conserved stretch of 20 amino acids in the carboxyl-activating enzyme family (Fulda et al. 1994) and a consensus sequence TGD for the putative ATP binding region (Pavela-Vrancic et al. 1994) are underlined.

Distribution of the *AKT1* and *AKT2* homologues in *A. alternata* strains.

DNA from 12 strains of the Japanese pear pathotype was digested with *EcoRI*, and the blot was hybridized with the *AKT1*- and *AKT2*-specific probes p1EX and p2XS, respectively (Fig. 4). Each probe detected bands in all strains (Fig. 9). Use of other restriction enzymes showed that all strains carry multiple copies of the gene homologues (data not shown). Nineteen strains from an additional six pathotypes and five nonpathogenic strains of *A. alternata* were also analyzed for distribution of these gene homologues. Strains from the tangerine pathotype, which produces ACT-toxin (Fig. 1) (Pegg 1966; Kohmoto et al. 1993), and the strawberry pathotype, which produces AF-toxin (Fig. 1) (Maekawa et al. 1984; Nakatsuka et al. 1986), were found to have the *AKT1* and *AKT2* homologues (Fig. 9). However, strains from other pathotypes and nonpathogenic *A. alternata* had no detectable homologues of these genes (Fig. 9).

DISCUSSION

The REMI technique was used to mutagenize and simultaneously tag the genes required for AK-toxin biosynthesis of the Japanese pear pathotype of *A. alternata*. The presence of the restriction enzyme during transformation increased transformation frequency in *A. alternata* as reported by Akamatsu et al. (1997). Three *Tox*⁻, nonpathogenic mutants were isolated from 984 REMI transformants. This frequency (about 0.3%) is considerably higher than would be expected with random integrations (Kang and Metzenberg 1993). This result

suggests that REMI-based insertions are not random in *A. alternata*, or that a large number of genes participate in AK-toxin biosynthesis.

The REMI technique has been reported to generate single-copy integration events with high frequency, allowing efficient isolation of tagged genes (Kuspa and Loomis 1992; Lu et al. 1994; Bölker et al. 1995; Yang et al. 1996; Sweigard et al. 1998). However, in this study, DNA gel blot analysis of the three *Tox*⁻ mutants showed more complex plasmid integration events. All three mutants contained multiple copies of plasmid DNA. In other fungi, digestion of DNA of REMI transformants with the restriction enzyme used in REMI frequently produces a fragment equal to the size of the inserted plasmid (Schiestl and Petes 1991; Lu et al. 1994; Bölker et al. 1995; Shi et al. 1995). However, *HindIII*, which was used in REMI transformation of the Japanese pear pathotype, produced bands larger than 5.5 kb (the size of pSH75) in every mutant. Nucleotide sequencing of the junction of plasmid and genomic DNA recovered from mutant ATA95-1 demonstrated the deletion of eight terminal nucleotides of integrated plasmid. These results indicate that the *HindIII* sites at the junctions of plasmid and genomic DNA were lost during plasmid integration in these mutants. The addition of restriction enzyme to transformation mixture markedly enhanced transformation efficiency, probably by promotion of plasmid integration into host chromosomes. We assume that plasmid DNA is predominantly integrated into digested restriction enzyme sites. However, the REMI plasmid integration process is more complicated in *A. alternata* than in other fungi. A complex REMI process was also reported in *Mycosphaerella zeae-maydis*, which produces a host-specific toxin known as PM-toxin (Yun et al. 1998). Yun et al. (1998) isolated toxin-deficient mutants of this pathogen by REMI transformation. Analysis of the plasmid insertion sites of mutants showed that the REMI procedure can cause deletions (Yun et al. 1998).

We recovered DNA flanking the plasmid insertion point of mutant ATA95-1. With the recovered DNA used as a probe, a cosmid clone, pcAKT-1, was isolated from a genomic library of the wild type. We identified two genes, *AKT1* and *AKT2*, in an 8.0-kb region corresponding to the plasmid integration site in the mutant. Their essential roles in AK-toxin biosynthesis were proven by targeted disruption, which caused complete loss of toxin production and pathogenicity. Loss of pathogenicity in the gene-targeted strains confirms that AK-toxin is an essential factor in pathogenicity of the Japanese pear pathotype.

We observed that REMI mutant ATA95-1 and *AKT1*- or *AKT2*-targeted mutants failed to produce not only AK-toxin but also 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid, which has been identified as a precursor of AK-toxin (Feng et al. 1990; Nakatsuka et al. 1990). This result indicates that *AKT1* and *AKT2* are involved in 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid biosynthesis. The putative *AKT2*-encoded protein shows no homology to any known proteins. The amino acid sequence of the *AKT1* product, however, has significant similarity to those of 4-coumarate-CoA ligases, acetyl-CoA ligases, long chain acyl-CoA synthetases, and luciferases. The first three play crucial roles in different pathways and subcellular compartments involved in lipid metabolism by a similar reaction mechanism: formation of an acyl adenylate from ATP and a fatty acid or acetate, and then trans-

Fig. 7. Detection of ORF1 and ORF2 transcripts. Total RNA of wild-type strain 15A was used as a template for reverse transcription (RT), and the synthesized cDNA was subjected to polymerase chain reaction (PCR) amplification (lane 1). Total DNA was also used as a template for PCR (lane 2). The PCR products were electrophoresed in a 1.2% agarose gel. Sizes (in kilobases) of marker DNA fragments (100-bp DNA ladder, Takara) are indicated on left. Primers for ORF1 (RT primer and PCR primer pair): RT1-2 and RT1-1/RT1-2 (A); RT1-4 and RT1-3/RT1-4 (B); RT1-6 and RT1-5/RT1-6 (C) (see Figure 4). Primers for ORF2 (RT primer and PCR primer pair): RT2-2 and RT2-1/RT2-2 (D) (see Figure 4).

fer of the acyl group to the sulfhydryl group of CoA, with simultaneous release of AMP (Fulda et al. 1994). These enzymes have been identified as members of a superfamily of carboxyl-activating enzymes or AMP-binding proteins (Fulda et al. 1994; Pavela-Vrancic et al. 1994). Based on sequence similarity, luciferase has been also identified as a member of this family (Fulda et al. 1994). Common to all enzymes belonging to this family is the activation of the carboxyl group at the expense of ATP. Thus, the *AKT1* product is likely to activate an earlier precursor of 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid for further modification by other enzymes.

The *AKT1* and *AKT2* homologues are found not only in the Japanese pear pathotype strains but also in strains of the tangerine and strawberry pathotypes. The presence of the homologous genomic DNA in these three pathotypes is consistent with the similarity of their host-specific toxins and their host specificity (Fig. 1). Their toxins have a common moiety, 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid (Fig. 1) (Nakashima et al. 1985; Nakatsuka et al. 1986; Kohmoto et al.

1993). These pathotypes should share the genes encoding the enzymes for biosynthesis of the common moiety.

The Japanese pear pathotype is pathogenic only to susceptible pear cultivars (Tanaka 1933; Otani et al. 1985). The tangerine pathotype has been identified as a causal agent of brown spot of tangerines and mandarins, and has not occurred in Japan so far (Pegg 1966; Kohmoto et al. 1993). The strawberry pathotype has been identified as a causal agent of *Alternaria* black spot of strawberry (Maekawa et al. 1984). Interestingly, these two pathotypes were found pathogenic also 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 host-specific toxins. Each of these toxins consists of multiple, related molecules (Fig. 1). AK-toxins I and II are toxic only to Japanese pear (Nakashima et al. 1985; Otani et al. 1985). ACT-toxin I is toxic to both tangerine and pear; toxin II is more toxic to pear than to tangerine (Kohmoto et al. 1993). AF-toxin I is toxic to both strawberry and pear; toxin II is toxic only to pear; toxin III is more toxic to strawberry than

Fig. 8. DNA gel blot and reverse transcription–polymerase chain reaction (RT-PCR) analyses of (A, C, and E) pGDT1 and (B, D, and F) pGDT2 transformants. A and B, Total DNA from wild-type strain 15A (W), toxin-producing (Tox⁺), or toxin-deficient (Tox⁻) transformants was digested with (A) *Hind*III-*Sph*I or (B) *Pvu*II and fractionated in an 0.8% agarose gel. Blots were probed with (A) the ORF1 fragment in pGDT1 or (B) the ORF2 fragment in pGDT2 (see Figure 4). Sizes (in kilobases) of marker DNA fragments (*Hind*III-digested λ DNA) are indicated on left. C and D, Blots of A and B were stripped of the probes and rehybridized with the 1.4-kb *Bam*HI fragment in pBBR2, which contains the *Alternaria alternata* *BRM2* gene involved in melanin biosynthesis (Kawamura et al. 1999). E and F, Total RNA was used as a template for RT, and the synthesized cDNA was subjected to PCR amplification. PCR products were electrophoresed in a 1.2% agarose gel. RT primer and PCR primer pair: RT1-6 and RT1-5/RT1-6 (E); RT2-2 and RT2-1/RT2-2 (F) (see Figure 4).

to pear (Maekawa et al. 1984; Nakatsuka et al. 1986). The crucial roles of *AKT1* and *AKT2* in AK-toxin biosynthesis might be characterized by chemical and biochemical analyses of production and accumulation of toxin-related metabolites in the *Tox*⁻ mutants.

Our results show that these three pathotypes are good models for studying intraspecific variation and evolution of pathogenicity in *A. alternata*. Strains from other pathotypes and nonpathogenic *A. alternata* have no detectable homologue of *AKT1* and *AKT2*, suggesting that the evolution of toxin production in these three pathotypes may involve horizontal transfer of DNA. Horizontal transfer of the genes required for host-specific toxin biosynthesis has been proposed for HC-toxin of *Cochliobolus carbonum* race 1 (Nikolskaya et al. 1995; Ahn and Walton 1996) and T-toxin of *C. heterostrophus* race T (Yang et al. 1996; Yoder 1998). Isolation and characterization of the *AKT1* and *AKT2* homologues from the tangerine and strawberry pathotypes are now in progress.

DNA gel blot analysis indicates that the Japanese pear pathotype strain 15A has additional gene homologues of *AKT1* and *AKT2*. DNA gel blot analysis showed that other strains of the pathotype also contain multiple copies of these gene homologues. The complexity of the genetic loci controlling

host-specific toxin biosynthesis has been reported for *C. carbonum* (Panaccione et al. 1992; Ahn and Walton 1996, 1997; Pitkin et al. 1996; Walton et al. 1998) and *C. heterostrophus* (Tzeng et al. 1992; Chang and Bronson 1996; Yoder 1998; Kodama et al. 1999). Characterization of the structure and chromosomal distribution of the *AKT1* and *AKT2* homologues in the Japanese pear pathotype strains is of great importance in the study of evolution of AK-toxin biosynthesis and origin of genes for toxin biosynthesis. Recently, it has been discovered that genes for certain types of metabolic pathways are clustered in filamentous fungi (Keller and Hohn 1997). There is a possibility that *AKT1* and *AKT2* are clustered with additional genes required for AK-toxin biosynthesis. Structural and functional analyses of the regions flanking these genes are worthy of further study.

MATERIALS AND METHODS

Fungal strains.

A total of 36 strains, consisting of 31 strains from seven pathotypes of *A. alternata* and five strains from nonpathogenic *A. alternata*, were used in this study. Strain 15A of the Japanese pear pathotype was used for REMI transformation and

Fig. 9. Distribution of the *AKT1* and *AKT2* homologues in strains of *Alternaria alternata*. Total DNA of each strain was digested with *EcoRI* and separated in an 0.8% agarose gel. Blots were probed with (A) p1EX or (B) p2XS (see Figure 4). Sizes (in kilobases) of marker DNA fragments (*HindIII*-digested λ DNA) are indicated on left. Lanes 1 to 12, strains 15A, T88-11, T88-52, T88-58, T88-107, 91H-18, 91H-27, Nu89-22, N18, G16, G31, and G90-A2 of the Japanese pear pathotype; lanes 13 to 16, strains AM-1, IFO08984, Ku-16Ro, and AM91-1 of the apple pathotype (AM-toxin producer); lanes 17 to 21, strains AT-204, IFO6149, no. 122-2, 86-1, and ATL-10 of the tobacco pathotype (AT-toxin producer); lane 22, strain ATCC38962 of the rough lemon pathotype (ACR-toxin producer); lane 23, strain ATCC38963 of the tangerine pathotype (ACT-toxin producer); lanes 24 to 28, strains O-187, M-30, T-32, NAF-2, and NAF-3 of the strawberry pathotype (AF-toxin producer); lanes 29 to 31, strains AL-4, AL-12, and no. 91080804 of the tomato pathotype (AAL-toxin producer); lanes 32 to 36, strains IFO031188, IFO031189, ATCC66981, ATCC66982, and O-94 of nonpathogenic *A. alternata* (Kusaba and Tsuge 1995).

isolation of the *AKT1* and *AKT2* genes. The other strains were used for analysis of distribution of the gene homologues. Strains were routinely maintained on potato dextrose agar (PDA; Difco, Detroit, MI).

Plasmids and genomic libraries.

Integrative transformation vector pSH75 was used for transformation of *A. alternata* (Kimura and Tsuge 1993). pSH75 carries the *hph* gene, which is regulated by the *Aspergillus nidulans trpC* promoter and terminator sequences, in pSP72 (Promega, Madison, WI) (Cullen et al. 1987; Kimura and Tsuge 1993). The 1.1-kb *EcoRI-XhoI* fragment internal to *AKT1* was cloned in pBluescript KS⁺ (Stratagene, La Jolla, CA) to produce p1EX (Fig. 4). The 1.1-kb insert was cut out from p1EX by digestion with *Bam*HI and *Xho*I and cloned in the *Bam*HI-*Xho*I site of pSH75 to produce the targeted disruption vector pGDT1 (Fig. 4). The 0.9-kb fragment internal to *AKT2* was amplified from pcAKT-1 by PCR with primers ORF2-X (5'-TTCTCGAGATCATGCGGACGAT-3') and ORF2-S (5'-TTGCATGCTCATCTCCTTGCT-3') (Fig. 4). ORF2-X and ORF2-S included *Xho*I and *Sph*I sites (underlined), respectively, flanking the *AKT2* sequences. The PCR mixture (50 µl) contained 1 ng of pcAKT-1, 1 µM concentrations of each primer, 200 µM deoxyribonucleotide triphosphate and 1.25 U of *Taq* DNA polymerase (Takara, Ohtsu, Japan) with buffer from the manufacturer. PCR was performed for 25 cycles with an initial 3 min at 94°C for denaturation. Each cycle consisted of 1 min at 94°C, 30 s at 60°C, and 1 min at 72°C. The PCR product was cloned in pGEM-T Easy vector (Promega) to produce p2XS (Fig. 4). The PCR product was digested with *Xho*I and *Sph*I, and then cloned in the *Xho*I-*Sph*I site of pSH75 to produce the targeted disruption vector pGDT2 (Fig. 4). Plasmid pBBR2 contains the *A. alternata BRM2* gene, which encodes 1,3,8-trihydroxynaphthalene reductase involved in melanin biosynthesis (Kawamura et al. 1999).

Genomic DNA of each Tox⁻ mutant was partially digested with *Sau*3AI to generate fragments of 15 to 20 kb and cloned in the λDASHII vector (Stratagene) to construct a genomic library. A genomic cosmid library of wild-type strain 15A has been previously described (Kimura and Tsuge 1993). Screening of the libraries by plaque or colony hybridization was conducted by the standard methods (Sambrook et al. 1989).

Fungal transformation.

Protoplast preparation and transformation of *A. alternata* were performed by the methods previously described (Tsuge et al. 1990). For REMI, pSH75 was completely digested with *Bam*HI or *Hind*III, and enzymes were removed by phenol extraction and ethanol precipitation (Sambrook et al. 1989). The digested DNA (5 µg) was resuspended in 100 µl of restriction enzyme buffer containing the enzyme used to linearize the plasmid and mixed with about 1×10^7 protoplasts in 100 µl of STC (1.2 M sorbitol, 10 mM Tris-HCl [pH 7.5], and 10 mM CaCl₂). The mixture was kept on ice for 10 min before addition of polyethylene glycol and completion of the usual transformation and plating protocols (Tsuge et al. 1990). As controls, circular plasmid DNA (5 µg) or enzyme-digested DNA (5 µg) was suspended in 100 µl of restriction enzyme buffer without enzyme and subjected to transformation protocols. Colonies that appeared 5 to 10 days after plating on the selec-

tive regeneration media were transferred to PDA containing hygromycin B (Wako Pure Chemicals, Osaka, Japan) at 100 µg per ml, and transformants were selected 5 days after incubation at 25°C.

Screening and characterization of Tox⁻ mutants.

Transformants were grown on PDA containing hygromycin B at 100 µg per ml for 5 days, and then agar blocks (about 20 mm³) bearing cultured mycelia were cut from the media. Detached young leaves of the susceptible cultivar Nijisseiki of Japanese pear were slightly wounded at the center of lamina with needle. An agar block was placed on the wounded portion of each leaf, and one drop of distilled water was added. After incubation at 25°C for 20 h, pathogenicity was evaluated: a characteristic veinal necrosis was caused on leaves by pathogenic transformants, but not by nonpathogenic candidates. Nonpathogenic candidates were further tested for pathogenicity by spray inoculation of spore suspensions (about 5×10^5 spores per ml) on Nijisseiki pear leaves as previously described (Hayashi et al. 1990).

AK-toxin production during growth in potato dextrose broth (PDB) (Difco) and during spore germination was tested by bioassay with Nijisseiki leaves and by reverse-phase high-performance liquid chromatography analysis as previously described (Hayashi et al. 1990). For high-performance liquid chromatography analysis, toxin in culture filtrates and spore germination fluids was extracted with ethyl acetate by the method of Katsuya et al. (1997), and toxin in mycelia was extracted by immersing mycelia in methanol for 24 h at 20°C. The methanol extracts were concentrated under reduced pressure and subjected to the analysis. For bioassay, culture filtrates and spore germination fluids were dropped on the wounded portion of Nijisseiki leaves. Methanol extracts of mycelia were evaporated to dryness under reduced pressure. The residue was dissolved in a small amount of acetone, diluted with water to give an acetone concentration at less than 5%, and applied to Nijisseiki leaves. Ethyl acetate extracts of culture filtrates were also used for analysis of production of 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid by reverse-phase high-performance liquid chromatography as previously described (Feng et al. 1990).

Prototrophy was tested on a minimal medium (10 g of KNO₃, 5 g of KH₂PO₄, 2.5 g of MgSO₄ · 7H₂O, 0.02 g of FeCl₃, 10 g of glucose, 20 g of agar per liter). Vegetative growth and sporulation was assayed by the method of Kawamura et al. (1999).

Nucleic acid extraction.

For extraction of DNA, fungi were grown in 50 ml of PDB in 100-ml Erlenmeyer flasks at 25°C for 3 days on an orbital shaker (120 rpm; Bio-Shaker BR-30LF; Taitec, Saitama, Japan). The resulting mycelia were ground in liquid nitrogen in a mortar with a pestle. Total cellular DNA was prepared from the powdered mycelia as previously described (Adachi et al. 1993). For extraction of RNA, fungi were grown on PDA at 25°C for 4 days. Mycelia were ground in liquid nitrogen in a mortar with a pestle. Total cellular RNA was extracted from the powdered mycelia as described by Timberlake (1986). Plasmid DNA, cosmid DNA, and recombinant λ phage DNA were isolated by the standard methods (Sambrook et al. 1989).

Nucleic acid hybridization.

Restriction enzyme digestion of DNA and fractionation in agarose gels were performed by the standard methods (Sambrook et al. 1989). Fractionated DNA was transferred to Hybond N⁺ nylon membranes (Amersham, Arlington Heights, IL) and subjected to hybridization according to the manufacturer's recommendations. Hybridized blots were washed at 65°C; the final wash was done in 1× SSPE (1× SSPE is 180 mM NaCl, 10 mM NaH₂PO₄ [pH 7.7], and 1 mM EDTA) plus 0.1% sodium dodecyl sulfate. DNA probes were labeled with [α -³²P]dCTP (ICN Biochemicals, Costa Mesa, CA) by the random-primer method (Feinberg and Vogelstein 1983).

RT-PCR analysis.

RT-PCR was performed with the RNA PCR Kit Ver. 2 (Takara). The RT reaction mixture (20 μ l) contained 1 μ g of total RNA, 2.5 μ M primer, 1 mM deoxyribonucleotide triphosphate and 5 U of AMV reverse transcriptase XL with buffer from the manufacturer. The mixture was incubated at 55°C for 30 min, and the enzyme was inactivated at 99°C for 5 min. For subsequent PCR, the mixture was added with 20 pmol of each primer, 2.5 U of *Taq* DNA polymerase, buffer from the manufacturer, and water to a final volume of 100 μ l. The PCR was performed for 25 cycles with an initial 3 min at 94°C for denaturation. Each cycle consisted of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C. The following primers were used: RT1-1 (5'-TATCGCCTGAGTACATGCGT-3'), RT1-2 (5'-GCATGTGAAGATAGCCTGTG-3'), RT1-3 (5'-CACAGGCTATCTTCACATGC-3'), RT1-4 (5'-CGTAGCTCCATAGCATTGCT-3'), RT1-5 (5'-AGCAATGCTATGGAGCTACG-3') and RT1-6 (5'-CTATATCTTGCTCGACAGCC-3') for ORF1 (Fig. 4); RT2-1 (5'-AGATGCAGCAGCCCATCATT-3') and RT2-2 (5'-CGACTGCAGATATTTTCATCC-3') for ORF2 (Fig. 4). Primer combinations used in RT-PCRs are indicated in Figures 7 and 8. For analysis of nucleotide sequences, RT-PCR products were cloned in pGEM-T Easy.

Nucleotide sequence analysis.

DNA was cloned in pBluescript KS⁺ or pGEM-T Easy. DNA sequence information was obtained from both strands with the PRISM Dye Termination Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, UK) 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. 1990). Amino acid alignments were made with the CLUSTAL W program (Thompson et al. 1994).

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