Structural and Functional Complexity of the Genomic Region Controlling AK-Toxin Biosynthesis and Pathogenicity in the Japanese Pear Pathotype of *Alternaria alternata*

Aiko Tanaka and Takashi Tsuge

Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan

Accepted 8 May 2000.

The Japanese pear pathotype of *Alternaria alternata* produces host-specific AK-toxin and causes black spot of Japanese pear. Previously, a cosmid clone, pcAKT-1, was isolated that contains two genes, *AKT1* and *AKT2*, within a 5.0-kb region required for AK-toxin biosynthesis. The wild-type strain has multiple, nonfunctional copies of these genes. In the present study, two additional genes, *AKTR-1* and *AKT3-1*, downstream of *AKT2* were identified. Transformation of the wild type with *AKTR-1*- and *AKT3-1*-targeting vectors produced toxin-deficient (*Tox*[−]), nonpathogenic mutants. DNA gel blot analysis, however, demonstrated that the fragments targeted in *Tox*[−] mutants were different from those containing *AKTR-1* and *AKT3-1* on the transforming vectors. A cosmid clone, pcAKT-2, containing the targeted DNA was isolated and shown to carry two genes, *AKTR-2* and *AKT3-2*, with high similarity to *AKTR-1* and *AKT3-1*, respectively. Transcripts from not only *AKTR-2* and *AKT3-2* but also *AKTR-1* and *AKT3-1* were found in the wild type. DNA gel blot analysis with pulsed-field gel electrophoresis showed that *AKT1*, *AKT2*, *AKT3*, and *AKTR* and their homologues are on a single chromosome. These results indicate the structural and functional complexity of the genomic region controlling AK-toxin biosynthesis.

The imperfect fungus *Alternaria alternata* is one of the most cosmopolitan fungal species and is generally saprophytic (Rotem 1994). This species, however, contains seven pathogens that cause severe disease on different plants due to production of toxins effective only against the host plants (Nishimura and Kohmoto 1983; Kohmoto et al. 1995). These *A. alternata* host-specific forms have been designated as pathotypes (Nishimura 1980; Nishimura and Kohmoto 1983; Kohmoto et al. 1995). The toxins are low-molecular-weight metabolites of diverse structure, and their participation in es-

tablishment 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). *A. alternata* pathotypes are a fascinating case for studying intraspecific variation and evolution of pathogenicity in plant-pathogenic fungi.

The Japanese pear pathotype of *A. alternata* produces AK-toxin 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). This disease 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).

Recently, we isolated AK-toxin-deficient (*Tox*[−]) mutants of *A. alternata* by restriction enzyme-mediated integration (REMI) mutagenesis and cloned the genes for toxin biosynthesis from a tagged *Tox*[−] mutant by recovery of genomic DNA flanking the tag (Tanaka et al. 1999). Structural and functional analyses of this DNA revealed two genes, designated *AKT1* and *AKT2* (Tanaka et al. 1999). *AKT1* encodes a carboxyl-activating enzymes homologue, and *AKT2* encodes a protein of unknown function (Tanaka et al. 1999). Their essential roles in toxin biosynthesis and in pathogenicity were proven by targeted gene disruption, which caused complete loss of both abilities (Tanaka et al. 1999). DNA gel blot analysis of wild type and the *AKT1* or *AKT2* mutants demonstrated that the wild-type strain has additional copies of these genes, which are nonfunctional in toxin production (Tanaka et al. 1999).

DNA gel blot analysis revealed that *AKT1* and *AKT2* are unique to three *A. alternata* pathotypes: Japanese pear, strawberry, and tangerine (Tanaka et al. 1999; Masunaka et al. 2000). The strawberry pathotype produces AF-toxin and causes Alternaria black spot of strawberry (Maekawa et al. 1984; Nakatsuka et al. 1986). The tangerine pathotype produces ACT-toxin and causes brown spot of tangerines and mandarins, a disease that has not occurred in Japan so far (Pegg 1966; Kohmoto et al. 1993). Toxins of these three pathotypes have a common 9,10-epoxy-8-hydroxy-9-methyl-decatricenoic acid structural moiety (Nakashima et al. 1985; Nakatsuka et al. 1986; Kohmoto et al. 1993), a precursor of all these toxins (Feng et al. 1990; Nakatsuka et al. 1990). Thus, we hypothesized that the genes encode the enzymes for biosynthesis of this

Corresponding author: Takashi Tsuge
E-mail: ttsuge@agr.nagoya-u.ac.jp

Nucleotide and/or amino acid sequence data for *AKTR-1*, *AKT3-1*, *AKTR-2*, and *AKT3-2* are to be found in the DDBJ, GenBank, and EMBL data bases as accession numbers AB035491, AB035492, AB035493, and AB035494, respectively.

common precursor. The *AKT1*- or *AKT2*-targeted mutants fail to produce AK-toxin and this precursor (Tanaka et al. 1999).

There is increasing evidence that genes for secondary metabolites are clustered in fungi (Keller and Hohn 1997). We determined previously that *AKT1* and *AKT2* are located within a 5.0-kb region (Tanaka et al. 1999). In this study, we describe two additional open reading frames (ORFR and ORF3) downstream of *AKT2* that are present in multiple copies. When particular versions of these ORFs are disrupted, *Tox*⁻ mutants result. As with *AKT1* and *AKT2*, we determined the presence of additional, nonfunctional copies of these ORFs in wild type and identified the functional copies for toxin biosynthesis. Thus, it appears that the locus encoding ability to produce AK-toxin is structurally and functionally complex: multiple, non-identical copies of the genes are present; however, only one of each is functional.

RESULTS

Identification of two ORFs downstream of the *AKT2* gene.

The nucleotide sequence of a 7.0-kb region downstream of *AKT2* was analyzed, and two ORFs (ORFR and ORF3) identified (Fig. 1).

ORFR (1,332 bp) is a single exon encoding a 444 amino acid protein with two recognizable domains: a zinc binuclear cluster DNA-binding domain (CX₂CX₆CX₆CX₂CX₆C) in the amino terminal region and an internal leucine zipper domain (LX₆LX₆LX₆L) (Fig. 2). 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). The highest similarity is to the *afIR*-encoding proteins of *Aspergillus* species, which are positive regulators required for expression of other pathway genes for biosynthesis of aflatoxin and its derivatives (Woloshuk et al. 1994; Yu et al. 1996) (Fig. 2). The zinc binuclear cluster domain of the ORFR

product has similarity also to those of Pip2 (Rottensteiner et al. 1996) and Gal4 (Laughon and Gesteland 1984) of *Saccharomyces cerevisiae*, the type proteins for this family.

ORF3 (888 bp) encodes a protein of 296 amino acids after splicing of three putative introns (Fig. 1). Introns were deduced on the basis of presence of consensus sequences for

A

```

MLQCAPKKNERLRGS•CD•F•CTOSKLR•CNKNKPS•CRR•CTIOO 40
QP•C•VYSVARRTGRPPKHPRKANDCQEANGQHGEQDPVTST 80
PGGSCQQQSNHLLDVEGDGANFTLADASTTAQGRETAASS 120
ALDNALLVGETFGFSSLLDDPLIQSDDFLSFLCMPPEGEE 160
EGHMASPRALNGSTGPCSPTVLSSIDVPHLPARFGFLESS 200
VESGLHGRTGPHLVEQDPKIVPSSSFSEMEKIYDEGLTFSG 240
LDSAINAVTNNKGEPASGTMAAHPSKRCQFCSTSMK 280
LQMLISHPTLCQKNSRARFDMTLFLEEVVFNHRDVLQCL 320
VCQSKSLHSLASLCICTDWVIEA•LRDVAOD•LSGODN•LGG 360
FRAG•LCPPKDKFSCVGRFVLDDQLRESC•TRSLVKYRLRK 400
LVPIMDTMMKLNRYGAGGALSQAIRTMVEDVRHKIESALG 440
MMEL 444

```

B

```

ORFR  KKNE•RLRGSC•DF•CTOSKLR•CNKNKPS•CRR•CTIOOQPCVYSVARRTGR
AflR  QGTRKLR•ES•CIS•CSRSK•VK•CK•KE•KPT•CSRCVRRGLPC•Y•MSRRRTGR
Pip2  KKRNL•SFV•COAC•RRK•AK•TK•CD•QK•PRC•TRK•ONL•FC•YDVARQAAP
Gal4  KLLS•SEI•EQAC•DF•CTOSKLR•CNKNKPS•CRR•CTIOOQPCVYSVARRTGR

```

Fig. 2. Similarity of the open reading frame (ORF) ORFR-encoded protein to other proteins. **A**, Amino acid sequence encoded by ORFR. Zinc binuclear DNA-binding domain in amino terminal region and internal leucine zipper domain are underlined. Dots above sequences indicate highly conserved cysteines and leucines in these domains. **B**, Amino acid sequence alignment of zinc binuclear cluster domain of ORFR-encoded protein with those of AflR of *Aspergillus nidulans* (Yu et al. 1996) and Pip2 (Rottensteiner et al. 1996) and Gal4 (Laughon and Gesteland 1984) of *Saccharomyces cerevisiae*. Amino acids conserved between the ORFR product and any of the others are indicated as white letters on a black background. Dots above sequence indicate highly conserved cysteines in this domain.

Fig. 1. Partial structure of cosmid clones pcAKT-1 and pcAKT-2. These clones were isolated from a genomic cosmid library of the wild-type strain 15A. The *AKT1* and *AKT2* genes are essential for AK-toxin biosynthesis (Tanaka et al. 1999). Open reading frames (ORFs) ORFR (*AKTR-1*) and ORF3 (*AKT3-1*) in pcAKT-1 and ORFRh (*AKTR-2*) and ORF3h (*AKT3-2*) in pcAKT-2 were identified in this study. Arrows indicate protein coding regions; white segments indicate positions of introns. Arrowheads above ORFs denote orientation and location of oligonucleotide primers used in polymerase chain reaction (PCR) and reverse transcription-PCR experiments. p1EX, p2XS, pRPX, pRS, pRhS, p3HB, and p3hS were made by cloning indicated fragments in pBluescript KS⁺ or pGEM-T Easy, and used as probes for hybridization experiments. Abbreviations: B, *Bam*HI; H, *Hind*III; E, *Eco*RI; Ps, *Pst*I; Pv, *Pvu*II; S, *Sph*I; V, *Eco*RV; X, *Xho*I.

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). A BLAST data base search revealed that the putative ORF3-encoded protein has similarity to members of the hydratase/isomerase enzyme family, such as enoyl-CoA hydratases, crotonases, naphthoate synthases, and 4-chlorobenzoyl-CoA dehalogenases (Müller-Newen and Stoffel 1993) (Fig. 3). The ORF3 product reveals the highest similarity (28.6 and 24.8% identities, respectively) to enoyl-CoA hydratase of *Rhodobacter capsulatus*, which is involved in β -oxidation of fatty acids (Beckman and Kranz 1991), and crotonase of *Thermoanaerobacterium thermosaccharolyticum*, which is involved in butanoate metabolism (GenBank accession no. CAB07495) (Fig. 3). The ORF3-encoded protein terminates with a peroxisomal targeting signal type 1 (PTS1) tripeptide PKL that conforms to the PTS1 consensus motif established for *S. cerevisiae* (Elgersma et al. 1996).

Mutation of ORFR and ORF3 by gene targeting.

To determine the function of ORFR in AK-toxin biosynthesis, homologous recombination was employed to replace ORFR with plasmid pGDTR, which contains the hygromycin B phosphotransferase gene (*hph*) flanked by 5' and 3' sequences from ORFR (Fig. 4A). For ORF3, pGDT3, which was constructed by inserting a 1.0-kb ORF3 fragment in a transformation vector containing *hph*, was used for disruption (Fig. 5A). Transformation of the wild-type strain 15A with pGDTR and pGDT3 yielded eight out of 100 pGDTR transformants and six out of 100 pGDT3 transformants with complete loss of pathogenicity (Fig. 6). Culture filtrates of the wild type and all pathogenic transformants tested showed

ORF3	1:MLNRFSSYSSNAWHNLRVDPDADGIAVIVLARSQSRNA	
ECH	1:.....MSYHTIRYEIS..EGLAVITLDRPEVMNA	
Crt	1:.....MDFNNVLLNKD..DGIATLIINRPKALNA	
ORF3	39:LTLPMLTDMVOLLSAMDADDSVKCIVFTGEG.PFFCSG	
ECH	28:LNAAMRHETAAE..HRRGEARATVLTGSG.RAFCSG	
Crt	28:LNYEELKELDSVDIVENDKEIKVLIITCSGKETFVAG	
ORF3	76:VDLTGEGFGSIGKTRDTHRDAGGKIALALHNCRKPTIAA	
ECH	63:QDLGDGAAAGLNLSTVLRREYEPLQATYSCTPEVLAA	
Crt	66:ADIAEMSNMTPLEAKKFSLYGQKVFRRKLEMLSKENVIAA	
ORF3	114:INGTAVGVGITMTTPMSIRIAAKTAKISFFVRRGIVA	
ECH	101:VNGAAAGAGANLAAADVIAAQAFAAFMQAFTRIGLMP	
Crt	104:VNGFALGGCELSMACDRIASKNKFGQPEVGLGIIP	
ORF3	152:DAASSFYLPRLVGYGRALHFTTCALYPAESGLLHGLF	
ECH	139:DAGGTWWLPROVGMARAGMALFAEKIGAEAAARMGLI	
Crt	142:GFSGTQRLPRLIGTSKAKELIFTCGDMINSDEAKIGLI	
ORF3	190:SETVNPASSTLPRALLEVARDIYNASQVGVILTRDLVI	
ECH	177:WEAV.PDVDFEHWRARAAHLAR.GPSAAFAAVKKAFH	
Crt	180:SKVV.ELSDLIEEAKKLAKKMMS.KSQTATISLAKKAIN	
ORF3	228:RSPRSPEQAHLLSAAALYTRY.QSRDFEEGVKSFLEKR	
ECH	213:AGLSNPLPAAOLALEARLQELGQSADEFEGVQAFLEKR	
Crt	216:KGMETDLDTGNTIEAEKFSLCFTTDDQEGMIAFSEKR	
ORF3	265:KPRFQDTMREQSSEGVLERGDCVVGSLASKPKL	
ECH	251:PPHETGR..... (28.6%)	
Crt	254:APKFGK..... (24.8%)	

Fig. 3. Similarity of the open reading frame (ORF) ORF3-encoded protein to other proteins. Amino acid sequence of the ORF3-encoded protein was aligned with those of enoyl-CoA hydratase of *Rhodobacter capsulatus* (ECH) (Beckman and Kranz 1991) and crotonase of *Thermoanaerobacterium thermosaccharolyticum* (Crt) (GenBank accession no. CAB07495). Amino acids conserved between the ORF3 product and any of the others are indicated as white letters on a black background. Amino acids identical in all sequences are indicated by asterisks. The peroxisomal targeting signal type 1-like tripeptide PKL at the carboxy terminus of the ORF3 product is shown with dots.

Fig. 4. Transformation-mediated targeting of the open reading frame (ORF) ORFR. A, Structure of ORFR before and after homologous integration of the ORF3-targeting vector pGDTR. pGDTR contains the hygromycin B phosphotransferase gene (*hph*) flanked by the 5' sequence (0.8 kb) and 3' sequence (1.3 kb) from ORFR in pSH75 (Kimura and Tsuge 1993). Abbreviations: B, *Bam*HI; Ps, *Pst*I; Pv, *Pvu*II; V, *Eco*RV; Xb, *Xba*I; Xh, *Xho*I. B and C, DNA gel blot analysis of pGDTR transformants. Total DNA from the wild-type strain (W), Tox⁻ transformants or Tox⁺ transformants was digested with *Pvu*II and fractionated in a 0.8% agarose gel. Blots were hybridized with the pRPX insert, which was replaced with the *hph* gene in pGDTR (see Figure 1). C, Blots of B were stripped of probe and rehybridized with the pRhS insert (see Figure 1). Sizes (in kb) of marker DNA fragments (*Hind*III-digested λ DNA) are indicated on left.

toxicity to Nijisseiki pear leaves, but those of the nonpathogenic transformants had no toxicity (Fig. 6). High-performance liquid chromatography analysis also could not detect AK-toxin in culture filtrates of the nonpathogenic transformants, indicating loss of AK-toxin production.

The level of 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 of a subset of toxin-producing (Tox^+) and toxin-deficient (Tox^-) transformants was quantified by high-performance liquid chromatography. Culture filtrates of Tox^+ transformants contained the precursor molecule: 37 to 308 ng per ml in five pGDTR transformants and 208 to 834 ng per ml in five pGDT3 transformants. In contrast, 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid could not be detected in culture filtrates of each of five Tox^- transformants obtained by transformation with pGDTR and pGDT3.

All Tox^- transformants 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 loss of pathogenicity is attributable to loss of AK-toxin production.

The mode of integration of the vector in a subset of Tox^+ and Tox^- transformants was analyzed by DNA gel blot analysis (Figs. 4B and 5B). Total DNA of the wild type and pGDTR transformants was digested with *PvuII*, and the blot probed with the pRPX insert, which was replaced with the *hph* gene in pGDTR (Figs. 1 and 4A). The probe hybridized to a 2.0-kb fragment in not only wild type and Tox^+ transformants but also Tox^- transformants (Fig. 4B). The 2.0-kb fragment corresponds to the *PvuII* fragment containing ORFR in pcAKT-1 (Figs. 1 and 4A), suggesting that ORFR had not been disrupted in the Tox^- transformants.

Total DNA of pGDT3 transformants was digested with *SphI*, which has no site in pGDT3, and the blot was probed with the pGDT3 insert (Fig. 5A). The restriction map of pcAKT-1 predicts that the probe would hybridize to a 2.5-kb *SphI* fragment in the wild type (Fig. 5A); however, two bands (2.5 and 4.0 kb) hybridized (Fig. 5B). The 2.5-kb signal was more intense. Thus, the wild type has multiple copies of the ORF3 homologue. All Tox^+ transformants contained these two bands (Fig. 5B). All Tox^- transformants lacked the 4.0-kb band, implying that pGDT3 did not disrupt the genomic copy of ORF3, but did disrupt the 4.0-kb *SphI* copy (Fig. 5B). Tox^+ and Tox^- transformants both carried high-molecular-weight hybridizing bands (>23 kb), indicating integration of plasmids as tandem repeats (Fig. 5B). Such integration patterns are usual in transformation of *A. alternata* (Tsuge et al. 1990; Shiotani and Tsuge 1995; Tanaka et al. 1999).

Isolation and characterization of ORFRh and ORF3h.

DNA gel blot analysis of Tox^- transformants demonstrated that genomic copies of ORFR and ORF3 cloned in pcAKT-1 had not been disrupted. To isolate the targeted actual genes, a cosmid genomic library of the wild-type strain was screened with an ORF3 probe (p3HB insert) (Fig. 1), and 30 positive clones selected. DNA of positive clones was digested with *SphI* and hybridized with the same probe. Among 30 clones, 29 contained the 2.5-kb *SphI* fragment hybridizing to the probe; the remaining one, which we have named pcAKT-2, contained the 4.0-kb *SphI* fragment (Fig. 1). This result strongly suggests that the wild-type strain has multiple copies of ORF3, and indicates that pcAKT-2 contains the copy, which was disrupted in Tox^- pGDT3 transformants. Positive clones were also digested with *PvuII* and hybridized with the ORFR probe (pRPX insert) (Fig. 1). Unexpectedly, pcAKT-2 had a 3.6-kb *PvuII* fragment hybridizing to the probe, while the other clones had the 2.0-kb fragment. The 3.6-kb signal was weaker than the 2.0-kb signal, suggesting that pcAKT-2 contains an ORFR homologue.

Sequencing of the 6.5-kb region of pcAKT-2 containing the ORFR and ORF3 homologues detected two putative protein coding regions, designated ORFRh and ORF3h (Fig. 1), which are highly similar to ORFR and ORF3. Figure 7 is a comparison of the 6.5-kb regions of pcAKT-1 and pcAKT-2. ORFR (1,332 bp) and ORFRh (1,305 bp) are single exons, 84.8 and 74.1% identical in nucleotide and amino acid se-

Fig. 5. Transformation-mediated targeting of the open reading frame (ORF) ORF3. **A**, Structure of ORF3 before and after homologous integration of the ORF3-targeting vector pGDT3. pGDT3 contains the 1.0-kb *BamHI*-*HindIII* fragment of ORF3 in pSH75 (Kimura and Tsuge 1993). Abbreviations: B, *BamHI*; S, *SphI*. **B**, DNA gel blot analysis of pGDT3 transformants. Total DNA from the wild-type strain (W), Tox^- transformants or Tox^+ transformants was digested with *SphI* and fractionated in a 0.8% agarose gel. Blots were probed with the pGDT3 insert. Sizes (in kb) of marker DNA fragments (*HindIII*-digested λ DNA) are indicated on left.

quences, respectively (Fig. 7A and B). The amino terminal regions (59 amino acids) have 98.3% identity, including the zinc binuclear cluster domain with only one amino acid residue difference (Fig. 7B). The carboxy terminal regions (59

amino acids) are also highly conserved (96.6% identity) (Fig. 7B). However, the internal regions (326 and 317 amino acids in ORFR and ORFRh, respectively) containing the leucine zipper domain are only 65.6% identical (Fig. 7B). The vari-

Fig. 6. AK-toxin production and pathogenicity of wild-type strain (W) and a mutant (M) produced by transformation with (A) the open reading frame (ORF) ORFR-targeting vector pGDTR or (B) the ORF3-targeting vector pGDT3. Leaves of susceptible pear cv. Nijisseiki were wounded slightly, treated with culture filtrates, and incubated in a moist box at 25°C for 20 h (left). Leaves were spray inoculated with spore suspensions (about 5×10^5 spores per ml) and incubated in a moist box at 25°C for 20 h (right).

Fig. 7. Structure of open reading frame (ORF) ORFR/ORF3 and ORFRh/ORF3h regions. **A**, Identity of nucleotide sequences of regions containing ORFR and ORF3 in pcAKT-1 and ORFRh and ORF3h in pcAKT-2. **B**, Amino acid sequence alignment of proteins encoded by ORFR and ORFRh. Conserved amino acids are indicated as white letters on a black background. Zinc binuclear DNA-binding domain in amino terminal region and internal leucine zipper domain are underlined. Dots above sequences indicate highly conserved cysteines and leucines in these domains. Amino acid sequence identity is shown in parentheses. **C**, Amino acid sequence alignment of proteins encoded by ORF3 and ORF3h. Conserved amino acids are indicated as white letters on a black background. Amino acid sequence identity is shown in parentheses.

able regions of ORFR and ORFRh (78.1% identity in nucleotide sequence) were cloned to make pRS and pRhS (Fig. 1), and used to distinguish between the pcAKT-1 and pcAKT-2 genes in the following experiments.

The DNA gel blot of the wild type and pGDTR transformants used in Figure 4B was stripped of the pRPX probe and rehybridized with the pRhS insert. This probe hybridized to the 3.6-kb band containing ORFRh in wild type and all Tox⁺ transformants (Fig. 4C). In contrast, all Tox⁻ transformants lacked the 3.6-kb band, indicating that ORFRh had been targeted in the Tox⁻ pGDTR transformants. The genes encoding ORFR and ORFRh were designated *AKTR-1* and *AKTR-2*, respectively.

ORF3 and ORF3h both consist of putative four exons of the same sizes (Fig. 1). Of three introns, only the second intron is different in size (ORF3 = 56 bp; ORF3h = 60 bp). ORF3 and ORF3h are 91.5% identical in nucleotide sequence of the exon plus intron regions and 94.3% identical in deduced amino acid sequence (Figs. 7A and C). As with the ORF3-encoded protein, the ORF3h-encoded protein reveals the highest similarity (27.4 and 24.2% identities, respectively) to enoyl-CoA hydratase of *R. capsulatus* (Beckman and Kranz 1991) and crotonase of *T. thermosaccharolyticum* (GenBank accession no. CAB07495); 43 out of 44 amino acid residues that are identical in all the ORF3 product, enoyl-CoA hydratase, and crotonase are conserved in the ORF3h product (Fig. 3). The ORF3h product has the same PTS1 tripeptide (PKL) as the ORF3 product (Fig. 7C). The genes encoding ORF3 and ORF3h were designated *AKT3-1* and *AKT3-2*, respectively.

The homology between *AKTR-1* or *AKT3-1* and *AKTR-2* or *AKT3-2* extends beyond the ORFs in both directions (Fig. 7A). Between the *AKTR* and *AKT3* genes are pcAKT-1- and pcAKT-2-specific insertions of 110 and 282 bp, respectively (Fig. 7A). There is also a pcAKT-1-specific insertion of 221 bp at the 3' flanking region of *AKT3-1* (Fig. 7A).

Expression of the toxin biosynthesis genes.

Transcription of *AKTR-1* and *AKTR-2* in wild type and the pGDTR transformants was investigated by reverse transcription-polymerase chain reaction (RT-PCR). The *AKTR-1*-specific primer pair (RTR-f/RTR-r) (Figs. 1 and 8A) yielded a 0.92-kb fragment of DNA from total RNA of not only the wild type and Tox⁺ transformants but also Tox⁻ transformants (Fig. 8B). The *AKTR-2*-specific primers (RTRh-f/RTRh-r) (Figs. 1 and 8A) generated a 0.89-kb fragment of DNA from total RNA of the wild type and Tox⁺ transformants, but not from the Tox⁻ transformants (Fig. 8B). Transcription of *AKT3-1* and *AKT3-2* in wild type and pGDT3 transformants was also investigated by RT-PCR. The *AKT3-1*-specific primer pair (RT3-f/RT3-r) (Figs. 1 and 8C) produced a 0.63-kb fragment of DNA from total RNA of the wild type, Tox⁺ transformants and Tox⁻ transformants (Fig. 8D). The *AKT3-2*-specific primer pair (RT3h-f/RT3h-r) (Figs. 1 and 8C) generated a 0.63-kb fragment of DNA from total RNA of the wild type and Tox⁺ transformants, but not from the Tox⁻ transformants (Fig. 8D). RT-PCR analysis, together with DNA gel blot analysis (Figs. 4 and 5), clearly demonstrated that *AKTR-2* and *AKT3-2* had been targeted in Tox⁻ mutants produced by integration of pGDTR and pGDT3, respectively.

To confirm introns in *AKT3-1* and *AKT3-2*, primer pairs RT3-f/RT3c-r and RT3h-f/RT3c-r were used for RT-PCR amplification of *AKT3-1* and *AKT3-2* cDNAs, respectively, from total

RNA of wild type. RT3c-r (5'-TCAGAGCTTTG GCTTGGAA G-3') consists of sequences identical in the 3' ends of *AKT3-1* and *AKT3-2* ORFs (Fig. 1). RT-PCR amplification produced DNA fragments of the expected size (0.92 kb) by both primer pairs (data not shown). Sequencing of the RT-PCR products confirmed that the three introns in *AKT3-1* and *AKT3-2* are spliced.

Chromosomal distribution of the *AKT* gene clusters.

We showed previously that the wild-type strain has single copies of *AKT1* and *AKT2* and multiple copies of homologues (*AKT1h* and *AKT2h*) that are nonfunctional in toxin production (Tanaka et al. 1999). DNA gel blot analysis allowed the identification of polymorphisms between *AKT1*/*AKT2*-type and *AKT1h*/*AKT2h*-type restriction fragments (Tanaka et al. 1999). The 30 cosmid clones selected above were hybridized with p1EX and p2XS inserts containing the *AKT1* and *AKT2* internal fragments, respectively (Fig. 1). These probes did not hybridize to pcAKT-2, showing the lack of the *AKT1* and *AKT2* homologues on this cosmid. Among the remaining 29 clones, 19 and 26 clones hybridized to the *AKT1* and *AKT2*

probes, respectively. The diagnostic *AKT1*/*AKT2*-type restriction fragments were not present, however, showing that these clones contain homologues (*AKT1h* and *AKT2h*). Nucleotide sequences of the regions containing the *AKTR-1* and *AKT3-1* homologues in six clones, which had *AKT1h* and *AKT2h*, were analyzed. The analysis detected the same sequences as *AKTR-1* and *AKT3-1* in all the six clones, suggesting the presence of multiple copies of *AKTR-1* and *AKT3-1* in the genome of the wild type. One clone, which carries *AKT1h*, *AKT2h*, *AKTR-1*, and *AKT3-1*, was named pcAKT-3.

To investigate the genomic distribution of the *AKT* genes and their homologues, the chromosomal DNA of wild type was separated by pulsed-field gel electrophoresis and probed with p1EX, p2XS, pRS, pRhS, and p3HB containing internal fragments of *AKT1*, *AKT2*, *AKTR-1*, *AKTR-2*, and *AKT3-1*, respectively (Figs. 1 and 9). *AKT3-1* and *AKT3-2* could not be distinguished by DNA gel blot analysis, because their sequences are 91.5% identical. All probes hybridized to a single band of about 4.1 Mb, suggesting that these genes and their homologues all are located on a single chromosome (Fig. 9).

Distribution of the *AKTR* and *AKT3* homologues in *A. alternata* strains.

DNA from 12 strains of the Japanese pear pathotype (Table 1) was digested with restriction enzymes, and the blots were probed with pRS, pRhS and p3HB containing the *AKTR-1*, *AKTR-2*, and *AKT3-1* fragments, respectively (Fig. 1). All the probes detected bands in all strains tested, and their hybridization patterns were similar to those of 15A (Fig. 10). Figure 10 shows DNA blots of representative seven strains of the Japanese pear pathotype. The *AKTR-1* and *AKTR-2* probes hybridized to 2.0-kb and 3.6-kb bands, respectively, in *PvuII*-digested DNA of all strains (Fig. 10A and B). There were no

Fig. 8. Detection of transcripts from *AKTR* and *AKT3* in *Tox*⁻ mutants. Total RNA of the wild-type strain (W), *Tox*⁺ transformants or *Tox*⁻ transformants was used as a template for reverse transcription-polymerase chain reaction (RT-PCR) amplification. RT-PCR products were electrophoresed in a 1.2% agarose gel. **A**, Primers used for detection of *AKTR-1* and *AKTR-2* transcripts. Primer pairs specific for *AKTR-1* (RTR-f/RTR-r) and *AKTR-2* (RTRh-f/RTRh-r) were used in RT-PCR (see Figure 1). Nucleotides conserved between two primers are indicated as white letters on a black background. **B**, Detection of transcripts from *AKTR-1* and *AKTR-2* in the pGDTR transformants. **C**, Primers used for detection of the *AKT3-1* and *AKT3-2* transcripts. Primer pairs specific for *AKT3-1* (RT3-f/RT3-r) and *AKT3-2* (RT3h-f/RT3h-r) were used in RT-PCR (see Figure 1). Nucleotides conserved between two primers are indicated as white letters on a black background. **D**, Detection of transcripts from *AKT3-1* and *AKT3-2* in the pGDT3 transformants.

Fig. 9. Chromosomal distribution of genes involved in AK-toxin biosynthesis. Chromosome-sized DNA molecules were separated by contour-clamped homogeneous electric field (CHEF) electrophoresis, and the blots were hybridized with the *AKT1*, *AKT2*, *AKTR-1*, *AKTR-2*, and *AKT3-1* fragments from p1EX, p2XS, pRS, pRhS, and p3HB, respectively (see Figure 1). Sizes (in Mb) of marker DNAs (chromosome-sized DNAs of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*) are indicated on left.

apparent differences in signal intensity of the 2.0-kb or 3.6-kb fragment between 15A and each of others. The *AKT3-1* probe hybridized strongly to 2.5-kb bands and weakly to 4.0-kb bands in *SphI*-digested DNA of all strains (Fig. 10C). The *AKT3-2* probe (p3hS) (Fig. 1) also hybridized strongly to 2.5-kb bands and weakly to 4.0-kb bands (data not shown). This analysis, together with the analysis of the 30 cosmid clones of 15A, strongly suggests that all strains contain multiple copies of *AKTR-1* and *AKT3-1* and single copies of *AKTR-2* and *AKT3-2*.

Nineteen strains from an additional six pathotypes and five nonpathogenic strains of *A. alternata* (Table 1) were also analyzed for distribution of the gene homologues. Figure 10 shows DNA blots of a representative strain each of the six pathotypes and a nonpathogenic *A. alternata* strain. The *AKTR-1* and *AKT3-1* probes detected their homologues in all strains of the tangerine pathotype, which produces ACT-toxin (Pegg 1966; Kohmoto et al. 1993), and of the strawberry pathotype, which produces AF-toxin (Maekawa et al. 1984; Nakatsuka et al. 1986) (Fig. 10A and C). The *AKTR-2* probe, however, did not hybridize to DNA of these pathotype strains, suggesting *AKTR-2* is unique for the Japanese pear pathotype (Fig. 10B). Strains from other pathotypes and nonpathogenic *A. alternata* had no detectable homologues of these genes (Fig. 10).

DISCUSSION

Previously, we described a cosmid clone pcAKT-1, which contains the *AKT1* and *AKT2* genes essential for AK-toxin biosynthesis (Tanaka et al. 1999). Here we identified two additional genes, *AKTR-1* and *AKT3-1*, downstream of *AKT2* in pcAKT-1. Transformation of the wild-type strain 15A with the *AKTR-1*- and *AKT3-1*-targeting vectors produced *Tox⁻* transformants. However, DNAs targeted by the vectors in *Tox⁻* transformants were not the same as the transforming *AKTR-1* and *AKT3-1* DNA. A cosmid clone pcAKT-2 carrying the actual targeted genes, *AKTR-2* and *AKT3-2*, was isolated. Sequence analysis showed that *AKTR-2* and *AKT3-2* are highly similar, but not identical to *AKTR-1* and *AKT3-1*, respectively, indicating that the vectors were targeted to heterologous locations. We previously analyzed homologous recombination of transforming DNA in strain 15A with its *BRM1* gene, which is essential for melanin biosynthesis (Kimura and Tsuge 1993; Shiotani and Tsuge 1995). Targeting vectors were constructed

by inserting 0.6- to 3.1-kb internal *BRM1* fragments into a plasmid containing *hph* as a selectable marker. When circular plasmids were used, melanin-deficient transformants accounted for 30 to 80%, correlating closely with the size of the *BRM1* segment in the vectors. This result demonstrated that homologous DNA of 0.6 kb could be enough for targeted integration of exogenous DNA in this fungus (Shiotani and Tsuge 1995). Effect of the homology level on targeted integration of exogenous DNA has not been examined. The *AKT3-1*-targeting vector contained a 1.0-kb segment of *AKT3-1*, which has 91.5% identity in nucleotide sequence to the corresponding region of *AKT3-2*. The *AKTR-1*-targeting vector had *hph* flanked by the 5' sequence (0.8 kb) and 3' sequence (1.3 kb) from ORFR. The 5' and 3' sequences reveal 76.1 and 67.0% identities to the corresponding regions of *AKTR-2*, respectively. Thus, these identities and lengths could be enough for homologous integration of exogenous DNA in this fungus.

DNA gel blot analysis revealed restriction fragment length polymorphisms (RFLPs) between *AKTR-1*/*AKT3-1* and *AKTR-2*/*AKT3-2* types. Among 30 positive clones selected from a cosmid genomic library of 15A probed with *AKT3-1*, only pcAKT-2 had the *AKTR-2*/*AKT3-2* type of restriction fragments; the remaining 29 clones had the *AKTR-1*/*AKT3-1* type. Sequence analysis of six out of these 29 clones showed the presence of the same sequences as *AKTR-1* and *AKT3-1*. DNA gel blot analysis of genomic DNA of 15A showed that the *AKT3-1* and *AKT3-2* probes both hybridized strongly to the *AKT3-1*-type DNA and weakly to the *AKT3-2*-type DNA. These results indicate that the wild-type strain has multiple copies of *AKTR-1* and *AKT3-1*, and fewer copies of *AKTR-2* and *AKT3-2*.

We have previously demonstrated that the wild-type strain 15A has single copies of *AKT1* and *AKT2*, and multiple copies of their homologues, AKT1h and AKT2h (Tanaka et al. 1999). The gene targeting experiments showed that toxin biosynthesis requires *AKT1* and *AKT2*, but not their homologues (Tanaka et al. 1999). Although the *AKT1*- or *AKT2*-targeted transformants maintained the homologues in their genomes, RT-PCR analysis could not detect RNA transcription from these homologues (Tanaka et al. 1999). Cosmid pcAKT-1 carries *AKT1*, *AKT2*, *AKTR-1*, and *AKT3-1*, in that order, within a 12-kb region. Cosmid pcAKT-2 contains only *AKTR-2* and *AKT3-2*. Cosmid pcAKT-3 contains AKT1h, AKT2h, *AKTR-1*, and *AKT3-1*. DNA gel blot analysis with pulsed field gel

Fig. 10. Distribution of the *AKTR-1*, *AKTR-2*, and *AKT3* homologues in *Alternaria alternata* strains. Total DNA of each strain was digested with (A and B) *PvuII* or (C) *SphI*, and separated in a 0.8% agarose gel. Blots were probed with the (A) *AKTR-1*, (B) *AKTR-2*, and (C) *AKT3-1* fragments from pRS, pRhS, and p3hS, respectively (see Figure 1). Sizes (in kb) of marker DNA fragments (*HindIII*-digested λ DNA) are indicated on left. Lanes 1–7, 15A, T88-11, T88-107, Nu89-22, N18, G31, and G90-A2, respectively, of the Japanese pear pathotype; lanes 8–14, AM-1 of the apple pathotype, AT204 of the tobacco pathotype, ATCC38962 of the rough lemon pathotype, ATCC38963 of the tangerine pathotype, O-187 of the strawberry pathotype, AL-4 of the tomato pathotype, and IFO031188 of nonpathogenic *A. alternata*, respectively (see Table 1).

electrophoresis showed that these genes and their homologues are all on the same 4.1-Mb chromosome. The 4.1-Mb chromosome is one of 11 chromosomes also estimated previously in 15A (Adachi et al. 1996; Akamatsu et al. 1999). These results imply that the genomic region controlling AK-toxin biosynthesis is structurally and functionally complex. It is possible that there is a cluster consisting of a set of the genes essential for AK-toxin biosynthesis, and that pcAKT-1 and pcAKT-2 are versions that contain only parts of the cluster. Taga and Murata (1994) reported successful use of fluorescence in situ hybridization (FISH) for allocation of rDNA to a particular

chromosome in the *A. alternata* genome. We are further investigating the chromosomal distribution of the toxin biosynthesis genes and their homologues by the FISH method.

Complexity at loci controlling host-specific toxin biosynthesis has been reported for *Cochliobolus heterostrophus* race T (Tzeng et al. 1992; Chang and Bronson 1996; Yoder 1998; Kodama et al. 1999) and *C. carbonum* race 1 (Ahn and Walton 1996; Walton et al. 1998). In *C. heterostrophus*, early genetic studies indicated that a single Mendelian locus, *Tox1*, controls T-toxin biosynthesis (Leach et al. 1982). Molecular data, however, revealed that *Tox1* is two loci (*Tox1A* and *Tox1B*), one on each of two chromosomes in race T that are translocated with respect to their counterparts in race O, which does not produce T-toxin (Tzeng et al. 1992; Chang and Bronson 1996; Yoder 1998; Kodama et al. 1999). *Tox1A* carries the *PKS1* gene, which encodes a Type I polyketide synthase; *Tox1B* carries the *DEC1* gene, which encodes a decarboxylase (Lu et al. 1994; Yang et al. 1996; Yoder 1998; Kodama et al. 1999). In *C. carbonum*, a single locus, *Tox2*, controlling HC-toxin biosynthesis was defined by segregation analysis (Nelson and Ullstrup 1961; Scheffer et al. 1967). Molecular studies showed that *Tox2* carries two or three copies each of *HTS1*, *TOXA*, *TOXC*, *TOXE*, and *TOXF*, known to be essential for toxin biosynthesis (Panaccione et al. 1992; Scott-Craig et al. 1992; Ahn and Walton 1996, 1997, 1998; Pitkin et al. 1996; Cheng et al. 1999). All copies of these genes are located in a region of >540 kb and are functional (Ahn and Walton 1996; Walton et al. 1998). Thus, the genomic regions controlling biosynthesis of T-toxin, HC-toxin, and AK-toxin in three fungi all are not simple, genetic loci, but instead are large, complex regions of DNA.

AKTR-1 and *AKT3-1* sequences are highly similar to, but significantly different from, those of *AKTR-2* and *AKT3-2*, respectively (84.8 and 91.5%). Similarity between *AKTR-1* or *AKT3-1* and *AKTR-2* or *AKT3-2* extends beyond the coding sequence in both directions; however, the conserved regions are divided by the *AKTR-1/AKT3-1*- and *AKTR-2/AKT3-2*-specific insertions. Thus, it is unlikely that variability between the *AKTR-1/AKT3-1* and *AKTR-2/AKT3-2* regions originated by simple mutation. Although the gene targeting experiments showed that *AKTR-2* and *AKT3-2*, at least, are essential for toxin biosynthesis, RT-PCR experiments detected not only *AKTR-2* and *AKT3-2* mRNAs but also *AKTR-1* and *AKT3-1* mRNAs in the wild type. Thus, it is possible that *AKTR-1* and *AKT3-1* might encode proteins that have different functions from those encoded by *AKTR-2* and *AKT3-2* and might be essential for toxin biosynthesis. However, the functional characterization of *AKTR-1* and *AKT3-1* by gene targeting is very difficult or perhaps impossible, because of the presence of multiple copies of these genes.

The *AKTR-1*- and *AKTR-2*-encoded proteins (AktR-1 and AktR-2) contain the zinc binuclear cluster DNA-binding domain typical in the Zn(II)2Cys6 family proteins (Todd and Andrianopoulos 1997). Proteins with this domain are known to be typical regulatory factors in fungi and to be involved in a diverse array of cellular processes, including primary and secondary metabolism, drug resistance, and meiotic development in fungi (Todd and Andrianopoulos 1997). The presence of zinc binuclear cluster domain in *AKTR-1* and *AKTR-2* suggests that they bind to DNA to regulate expression of other pathway genes, but this has not yet been experimentally demonstrated.

Table 1. Strains of *Alternaria alternata* used in this study

Pathotype and strain ^a	Origin	
	Location	Source ^b
Japanese pear (AK-toxin)		
15A	Tottori	TU
T88-11	Tottori	THES
T88-52	Tottori	THES
T88-58	Tottori	THES
T88-107	Tottori	THES
91H-18	Aichi	NU
91H-27	Aichi	NU
Nu89-22	Aichi	NU
N18	Aichi	NU
G16	Gifu	NU
G31	Gifu	NU
G90-A2	Gifu	NU
Apple (AM-toxin)		
AM-1	Aichi	NU
IFO08984	Unknown ^c	IFO
Ku-16Ro	Aomori	FTRS
AM91-1	Nagano	NFTES
Tobacco (AT-toxin)		
AT-204	Aichi	NU
No. 122-2	Tochigi	JT
86-1	Kanagawa	JT
ATL-10	Tottori	JT
IFO6149	Unknown ^c	IFO
Rough lemon (ACR-toxin)		
ATCC38962	United States	ATCC
Tangerine (ACT-toxin)		
ATCC38963	United States	ATCC
Strawberry (AF-toxin)		
O-187	Iwate	TU
M-30	Tottori	TU
T-32	Tottori	TU
NAF-2	Aichi	NU
NAF-3	Aichi	NU
Tomato (AAL-toxin)		
AL-4	Mie	NU
AL-12	Mie	NU
No. 91080804	Mie	MARC
Nonpathogen		
IFO031188	Kagawa	IFO
IFO031189	Unknown ^c	IFO
ATCC66981	United States	ATCC
ATCC66982	United States	ATCC
O-94	Tottori	TU

^a Host-specific toxin produced by each pathotype is shown in parentheses.

^b TU, Tottori University, Tottori, Japan; THES, Tottori Horticultural Experimental Station, Tottori, Japan; NU, Nagoya University, Aichi, Japan; IFO, Institute for Fermentation, Osaka, Japan; FTRS, Fruit Tree Research Station, Ibaraki, Japan; NFTES, Nagano Fruit Tree Experiment Station, Nagano, Japan; JT, Japan Tobacco, Kanagawa, Japan; ATCC, American Type Culture Collection, MD; MARC, Mie Agricultural Research Center, Mie, Japan (Kusaba and Tsuge 1994; Adachi et al. 1996).

^c Unknown, but in Japan.

AktR-1 and AktR-2 have lower identity (65.6%) in the internal regions containing the leucine zipper domains, which are necessary for homodimerization or heterodimerization (Todd and Andrianopoulos 1997). DNA gel blot analysis with probes corresponding to this variable region distinguished between *AKTR-1* and *AKTR-2*. The *AKTR-1* homologues were found in the strawberry and tangerine pathotypes as well as the Japanese pear pathotype. Their toxins have a common moiety, 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid (Nakashima et al. 1985; Nakatsuka et al. 1986; Kohmoto et al. 1993). In contrast, the *AKTR-2* homologues were detected only in the Japanese pear pathotype. These results suggest that AktR-1 and AktR-2 are required for expression of pathway genes for biosynthesis of 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid and AK-toxin-specific moiety, respectively. The *AKTR-2*-targeted mutants, however, failed to produce 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid, indicating that AktR-2 is required for biosynthesis of this moiety. The zinc binuclear cluster domain has been shown to determine DNA binding specificity for numerous proteins in this family (Todd and Andrianopoulos 1997). AktR-1 and AktR-2 have 98.3% identity in the amino terminal regions (59 amino acids), including this domain, with only one amino acid residue difference. Detection of DNA-binding activity of AktR-1 and AktR-2 and identification of their binding sequences are required to assess the role of these proteins in regulation of toxin pathway genes and in toxin biosynthesis.

The *AKT3-1*- and *AKT3-2*-encoded proteins (Akt3-1 and Akt3-2) show homology to members of the hydratase/isomerase family, such as enoyl-CoA hydratases, crotonases, naphthoate synthases, and 4-chlorobenzoyl-CoA dehalogenases (Müller-Newen and Stoffel 1993). This family consists of enzymes that use CoA derivatives as substrates, and their amino acid sequences have low, but significant, homology one another (Müller-Newen and Stoffel 1993). This family contains enzymes involved in the fatty acid modification (Müller-Newen and Stoffel 1993). The *AKT3-2*-targeted, *Tox⁻* mutants failed to produce 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid. The *AKT3* homologues were also found in the strawberry and tangerine pathotypes. Thus, Akt3-2 is likely to be involved in 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid biosynthesis via modification of its earlier precursor.

Akt3-1 and Akt3-2 terminate with a peroxisomal targeting signal type 1 (PTS1)-like tripeptide PKL that conforms to the PTS1 consensus motif established for *S. cerevisiae* (Elgersma et al. 1996). These proteins show similarity to enoyl-CoA hydratases involved in β -oxidation pathway of fatty acids, which can take place in peroxisomes and/or mitochondria in eukaryotes (Kunau et al. 1988). The peroxisomal enoyl-CoA hydratase is part of the trifunctional enzyme, which consists of 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase, and 3,2-*trans*-enoyl-CoA isomerase domains (Osumi et al. 1985; Müller-Newen and Stoffel 1993). Although Akt3-1 and Akt3-2 have the PTS1, their amino acid sequences are more similar to those of the mitochondrion-type enzymes than to those of the peroxisome-type enzymes, indicating that Akt3-1 and Akt3-2 are novel proteins of the hydratase/isomerase family. The *AKT1*- and *AKT2*-encoded proteins (Akt1 and Akt2) also have the PTS1 tripeptides SKI and SKL, respectively, at the carboxy termini (Tanaka et al. 1999). The presence of the PTS1 in Akt1, Akt2, and Akt3 suggests that 9,10-

epoxy-8-hydroxy-9-methyl-decatrienoic acid is biosynthesized in peroxisomes. We are now investigating peroxisomal import of these proteins with green fluorescent protein fusions.

The Japanese pear, strawberry, and tangerine pathotypes are good models for studying intraspecific variation and evolution of pathogenicity in *A. alternata*. We previously measured genetic relatedness among the *A. alternata* pathotypes on the basis of three DNA markers: RFLPs of nuclear rDNA, nucleotide sequence variation in rDNA internal transcribed spacer regions, and RFLPs of mitochondrial DNA (Kusaba and Tsuge 1994, 1995, 1997). None of these markers could differentiate strains in correlation with pathotypes: single-pathotype populations carried multiple DNA variants, and different pathotype populations shared similar variation (Kusaba and Tsuge 1994, 1995, 1997). These data suggest that single-pathotype populations of *A. alternata* are not clonal. Comparison of the structure, function, and chromosomal distribution of the toxin biosynthesis genes from three pathotypes is of great importance in the study of the evolution of toxin biosynthesis and the origin of genes for toxin biosynthesis. Isolation of the *AKT* gene homologues from the strawberry and tangerine pathotypes is now in progress.

MATERIALS AND METHODS

Fungal strains.

A total of 36 strains from seven pathotypes and nonpathogenic *A. alternata* were used in this study (Table 1). Strain 15A of the Japanese pear pathotype was the source of the AK-toxin biosynthesis genes (Tanaka et al. 1999). The others 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.

Transformation vector pSH75 (Kimura and Tsuge 1993) was used for construction of gene targeting vectors in *A. alternata*. pSH75 carries the hygromycin B resistance gene (*hph*), which is fused to the *A. nidulans trpC* promoter and terminator (Cullen et al. 1987; Kimura and Tsuge 1993). p1EX contains the 1.1-kb fragment internal to *AKT1*, and p2XS contains the 0.9-kb fragment internal to *AKT2* (Fig. 1) (Tanaka et al. 1999). The 1.6-kb *PstI-XbaI* fragment internal to ORFR was cloned in the *PstI-XbaI* site of pBluescript KS+ (Stratagene, La Jolla, CA) to make pRPX (Fig. 1). The 0.9-kb fragments internal to ORFR and ORFRh were amplified from pcAKT-1 and pcAKT-2, respectively, with the following primer pairs: RTR-f/RTR-r for ORFR and RTRh-f/RTRh-r for ORFRh (Figs. 1 and 8A). The PCR products were cloned in pGEM-T Easy vector (Promega, Madison, WI) to produce pRS and pRhS containing the ORFR and ORFRh fragments, respectively (Fig. 1). The 1.3-kb *XbaI-XhoI* fragment containing the 3' region of ORFR was cloned in the *XbaI-XhoI* site of pSH75 to produce pRXX-SH (Fig. 4A). The 0.8-kb *PstI* fragment containing the 5' region of ORFR was cloned in pBluescript KS+ to make pPs0.9 (Fig. 4A). The pPs0.9 insert was cut out with *Bam*HI and *Eco*RV (restriction enzyme sites in pBluescript KS+) and cloned in the *Bg*III-*Eco*RV site of the pRXX-SH to make the ORFR targeting vector pGDTR (Fig. 4A). The 1.0-kb *Hind*III-*Bam*HI fragment internal to ORF3 was cloned in pBluescript KS+ to produce p3HB (Fig. 1). The

same fragment was cloned in the *Hind*III-*Bam*HI site of pSH75 to produce the ORF3 targeting vector pGDT3 (Fig. 5A). The 0.75-kb fragment of ORF3h was amplified from pcAKT-2 with the primer pair RT3h-f/RT-3h-r and cloned in pGEM-T easy to produce p3hS (Fig. 1).

A genomic cosmid library of strain 15A has been previously described (Kimura and Tsuge 1993). The cosmid clone pcAKT-1 was isolated from the library and characterized to contain the *AKT1* and *AKT2* genes (Tanaka et al. 1999).

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 to 10 days after plating on the selective regeneration media (Tsuge et al. 1990) were transferred to PDA containing hygromycin B at 100 µg per ml, and transformants were selected after incubation at 25°C for 5 days.

Assay for pathogenicity and AK-toxin production.

Pathogenicity of wild type and transformants was assayed with leaves of Japanese pear cultivar Nijisseiki as previously described (Hayashi et al. 1990; Tanaka et al. 1999).

AK-toxin production during growth in potato dextrose broth (PDB; Difco) was tested with a bioassay involving leaves of Japanese pear cultivar Nijisseiki, which is the most sensitive method for detection of toxin (Hayashi et al. 1990), as previously described (Tanaka et al. 1999). AK-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 (Tanaka et al. 1999).

DNA manipulations.

Isolation of total DNA and RNA from *A. alternata*, isolation of plasmid and cosmid DNAs, agarose gel electrophoresis, screening of cosmid libraries, and DNA gel blot hybridization were performed as previously described (Tanaka et al. 1999). Chromosome-sized DNA molecules were prepared, fractionated by pulsed-field gel electrophoresis with a contour-clamped homogeneous electric field (CHEF) apparatus (CHEF-DRII; Bio-Rad Laboratories, Hercules, CA) and subjected to hybridization as described by Adachi et al. (1996) and Akamatsu et al. (1999). All blots were washed twice in 2× SSPE (1× SSPE = 180 mM NaCl, 10 mM NaH₂PO₄ [pH 7.7] and 1 mM EDTA) plus 0.1% sodium dodecyl sulfate at 65°C for 10 min and once in 1× SSPE plus 0.1% sodium dodecyl sulfate at 65°C for 10 min.

RT-PCR was performed with the RNA PCR Kit version 2.1 (Takara, Ohtsu, Japan) according to the manufacturer's instructions. The following primer pairs were used: RTR-f/RTR-r for *AKTR-1*, RTRh-f/RTRh-r for *AKTR-2*, RT3-f/RT3-r and RT3-f/RT3c-r for *AKT3-1*, and RT3h-f/RT3h-r and RT3h-f/RT3c-r for *AKT3-2* (Figs. 1 and 8).

For analysis of nucleotide sequences, DNA was cloned in pBluescript KS+ or pGEM-T Easy. DNA sequences were determined 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). Alignment of nucleotide and amino acid se-

quences was made with the CLUSTAL W program (Thompson et al. 1994).

ACKNOWLEDGMENTS

We express our special thanks to Gillian Turgeon for critical suggestions and reading of the manuscript. We thank Mikihiro Yamamoto and Kazuya Akimitsu for critical suggestions and discussion; Hirofumi Yoshioka, Kazuhito Kawakita, and Noriyuki Doke for valuable suggestions; and the Radioisotope Research Center, Nagoya University, for technical assistance. This work was supported by grants-in-aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (T. T.), special coordination funds for promoting science and technology from the Science and Technology Agency of Japan (T. T.), Daiko Foundation (T. T.), and a Research Fellowship of Japan Society for the Promotion of Science for Young Scientists (A. T.).

LITERATURE CITED

- Adachi, Y., Watanabe, H., and Tsuge, T. 1996. Relationships between genetic polymorphisms and fungicide resistance within *Alternaria alternata*. *Phytopathology* 86:1248-1254.
- Ahn, J.-H., and Walton, J. D. 1996. Chromosomal organization of *TOX2*, the complex locus controlling host-selective toxin biosynthesis in *Cochliobolus carbonum*. *Plant Cell* 8:887-897.
- Ahn, J.-H., and Walton, J. D. 1997. A fatty acid synthase gene in *Cochliobolus carbonum* required for production of HC-toxin, cyclo(D-prolyl-L-alanyl-D-alanyl-L-2-amino-9,10-epoxy-8-oxodecanoyl). *Mol. Plant-Microbe Interact.* 10:207-214.
- Ahn, J.-H., and Walton, J. D. 1998. Regulation of cyclic peptide biosynthesis and pathogenicity in *Cochliobolus carbonum* by TOXEp, a novel protein with bZIP basic DNA-binding motif and four ankyrin repeats. *Mol. Gen. Genet.* 260:462-469.
- Akamatsu, H., Taga, M., Kodama, M., Johnson, R., Otani, H., and Kohmoto, K. 1999. Molecular karyotypes for *Alternaria* plant pathogens known to produce host-specific toxins. *Curr. Genet.* 35:647-656.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment sequence tool. *J. Mol. Biol.* 215:403-410.
- Beckman, D. L., and Kranz, R. G. 1991. A bacterial homolog to the mitochondrial enoyl-CoA hydratase. *Gene* 107:171-172.
- Bruchez, J. J. P., Eberle, J., and Russo, V. E. 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.
- Chang, H.-R., and Bronson, C. R. 1996. A reciprocal translocation and possible insertion(s) tightly associated with host-specific virulence in *Cochliobolus heterostrophus*. *Genome* 39:549-557.
- Cheng, Y.-Q., Ahn, J.-H., and Walton, J. D. 1999. A putative branched-chain-amino-acid transaminase gene required for HC-toxin biosynthesis and pathogenicity in *Cochliobolus carbonum*. *Microbiology* 145:3539-3546.
- Cullen, D., Leong, S. A., Wilson, L. J., and Henner, D. J. 1987. Transformation of *Aspergillus nidulans* with the hygromycin-resistance gene, *hph*. *Gene* 57:21-26.
- Elgersma, Y., Vos, A., van den Berg, M., van Roemund, C. W. T., van der Sluijs, P., Distel, B., and Tabak, H. F. 1996. Analysis of the carboxyl-terminal peroxisomal targeting signal 1 in a homologous context in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 271:26375-26382.
- Feng, B.-N., Nakatsuka, S., Goto, T., Tsuge, T., and Nishimura, S. 1990. Biosynthesis of host-selective toxins produced by *Alternaria alternata* pathogens. I. (8R,9S)-9,10-epoxy-8-hydroxy-9-methyl-deca-(2E,4Z,6E)-trienoic acid as a biological precursor of AK-toxins. *Agric. Biol. Chem.* 54:845-848.
- Hayashi, N., Tanabe, K., Tsuge, T., Nishimura, S., Kohmoto, K., and Otani, H. 1990. Determination of host-selective toxin production during spore germination of *Alternaria alternata* by high-performance liquid chromatography. *Phytopathology* 80:1088-1091.
- Keller, N. P., and Hohn, T. M. 1997. Metabolic pathway gene clusters in filamentous fungi. *Fungal Genet. Biol.* 21:17-29.
- Kimura, N., and Tsuge, T. 1993. Gene cluster involved in melanin biosynthesis of the filamentous fungus *Alternaria alternata*. *J. Bacteriol.* 175:4427-4435.
- Kodama, M., Rose, M. S., Yang, G., Yun, S. H., Yoder, O. C., and Turgeon, B. G. 1999. The translocation-associated *Tox1* locus of *Coch-*

- liobolus heterostrophus* is two genetic elements on two different chromosomes. *Genetics* 151:585-596.
- Kohmoto, K., Itoh, Y., Shimomura, N., Kondoh, Y., Otani, H., Kodama, M., Nishimura, S., and Nakatsuka, S. 1993. Isolation and biological activities of two host-specific toxins from the tangerine pathotype of *Alternaria alternata*. *Phytopathology* 83:495-502.
- Kohmoto, K., Otani, H., and Tsuge, T. 1995. *Alternaria alternata* pathogens. Pages 51-63 in: *Pathogenesis and Host Specificity in Plant Diseases: Histopathological Biochemical, Genetic and Molecular Bases*, Vol. 2. Eukaryotes. K. Kohmoto, U. S. Singh, and R. P. Singh, eds. Pergamon, Oxford.
- Kunau, W.-H., Böhne, S., de la Garza, M., Kionka, C., Mateblowski, M., Schultz-Borchard, U., and Thieringer, R. 1988. Comparative enzymology of β -oxidation. *Biochem. Soc. Trans.* 18:418-420.
- Kusaba, M., and Tsuge, T. 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 Tsuge, T. 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 Tsuge, T. 1997. Mitochondrial DNA variation in host-specific toxin-producing pathogens in the genus *Alternaria*. *Ann. Phytopathol. Soc. Jpn.* 63:463-469.
- Laughon, A., and Gesteland, R. F. 1984. Primary structure of the *Saccharomyces cerevisiae* GAL4 gene. *Mol. Cell. Biol.* 4:260-267.
- Leach, J., Tegtmeyer, K. J., Daly, J. M., and Yoder, O. C. 1982. Dominance at the *Tox1* locus controlling T-toxin production by *Cochliobolus heterostrophus*. *Physiol. Plant Pathol.* 21:327-333.
- Lu, S., Lyngholm, L., Yang, G., Bronson, C., Yoder, O. C., and Turgeon, B. G. 1994. Tagged mutations at the *Tox1* locus of *Cochliobolus heterostrophus* by restriction enzyme-mediated integration. *Proc. Natl. Acad. Sci. USA* 91:12649-12653.
- Maekawa, N., Yamamoto, M., Nishimura, S., Kohmoto, K., Kuwata, M., and Watanabe, Y. 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.
- Masunaka, A., Tanaka, A., Tsuge, T., Peever, L. W., Yamamoto, M., Yamamoto, H., and Akimitsu, K. 2000. Distribution and characterization of AKT homologs in the tangerine pathotype of *Alternaria alternata*. *Phytopathology* 90:762-768.
- Müller-Newen, G., and Stoffel, W. 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., Ueno, T., Fukami, H., Taga, T., Masuda, H., Osaki, K., Otani, H., Kohmoto, K., and Nishimura, S. 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., Feng, B.-N., Goto, T., Tsuge, T., and Nishimura, S. 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.
- Nakatsuka, S., Ueda, K., Goto, T., Yamamoto, M., Nishimura, S., and Kohmoto, K. 1986. Structure of AF-toxin II, one of the host-specific toxins produced by *Alternaria alternata* strawberry pathotype. *Tetrahedron Lett.* 27:2753-2756.
- Nelson, R. R., and Ullstrup, A. J. 1961. The inheritance of pathogenicity in *Cochliobolus carbonum*. *Phytopathology* 51:1-2.
- Nishimura, S. 1980. Host-specific toxins from *Alternaria alternata*: problems and prospects. *Proc. Japan Acad. (Ser. B)* 56:362-366.
- Nishimura, S., and Kohmoto, K. 1983. Host-specific toxins and chemical structures from *Alternaria* species. *Annu. Rev. Phytopathol.* 21:87-116.
- Osumi, T., Ishii, N., Hijikata, M., Kamijo, K., Ozasa, H., Furuta, S., Miyazawa, S., Kondo, K., Inoue, K., Kagamiyama, H., and Hashimoto, T. 1985. Molecular cloning and nucleotide sequence of the cDNA for rat peroxisomal enoyl-CoA: Hydratase-3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme. *J. Biol. Chem.* 260:8905-8910.
- Otani, H., Kohmoto, K., Nishimura, S., Nakashima, T., Ueno, T., and Fukami, H. 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.
- Panaccione, D. G., Scott-Craig, J. S., Pacard, J. A., and Walton, J. D. 1992. A cyclic peptide synthetase gene required for pathogenicity of the fungus *Cochliobolus carbonum* on maize. *Proc. Natl. Acad. Sci. USA* 89:6590-6594.
- 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.
- Pitkin, J. W., Panaccione, D. G., and Walton, J. D. 1996. A putative cyclic peptide efflux pump encoded by the *TOXA* gene of the plant-pathogenic fungus *Cochliobolus carbonum*. *Microbiology* 142:1557-1565.
- Rotem, J. 1994. The Genus *Alternaria*: Biology, Epidemiology, and Pathogenicity. The American Phytopathological Society, St. Paul, MN.
- Rottensteiner, H., Kal, A. J., Filipits, M., Binder, M., Hamilton, B., Tabak, H. F., and Ruis, H. 1996. Pip2p: A transcriptional regulator of peroxisome proliferation in the yeast *Saccharomyces cerevisiae*. *EMBO J.* 15:2924-2934.
- Scheffer, R. P., and Livingston, R. S. 1984. Host-selective toxins and their role in plant diseases. *Science* 223:17-21.
- Scheffer, R. P., Nelson, R. R., and Ullstrup, A. J. 1967. Inheritance of toxin production and pathogenicity in *Cochliobolus carbonum* and *Cochliobolus victoriae*. *Phytopathology* 57:1288-1291.
- Scott-Craig, J. S., Panaccione, D. G., Pocard, J. A., and Walton, J. D. 1992. The multifunctional cyclic peptide synthetase catalyzing HC-toxin production in the filamentous fungus *Cochliobolus carbonum* is encoded by a 15.7 kb open reading frame. *J. Biol. Chem.* 267:26044-26049.
- Shiotani, H., and Tsuge, T. 1995. Efficient gene targeting in the filamentous fungus *Alternaria alternata*. *Mol. Gen. Genet.* 248:142-150.
- Taga, M., and Murata, M. 1994. Visualization of mitotic chromosomes in filamentous fungi by fluorescence staining and fluorescence in situ hybridization. *Chromosoma* 103:408-413.
- Tanaka, S. 1933. Studies on black spot disease of Japanese pear (*Pyrus serotina* Rehd.). *Mem. Coll. Agric. Kyoto Imp. Univ.* 28:1-31.
- Tanaka, A., Shiotani, H., Yamamoto, M., and Tsuge, T. 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.
- Thompson, J. D., Higgins, D. G., and Gibson, T. J. 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 Andrianopoulos, A. 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., Nishimura, S., and Kobayashi, H. 1990. Efficient integrative transformation of the phytopathogenic fungus *Alternaria alternata* mediated by the repetitive rDNA sequences. *Gene* 90:207-214.
- Tzeng, T.-W., Lyngholm, L. K., Ford, C. F., and Bronson, C. R. 1992. A restriction fragment length polymorphism map and electrophoretic karyotype of the fungal maize pathogen *Cochliobolus heterostrophus*. *Genetics* 130:81-96.
- Walton, J. D. 1996. Host-selective toxins: agents of compatibility. *Plant Cell* 8:1723-1733.
- Walton, J. D., Ahn, J.-H., Pitkin, J. W., Cheng, Y.-Q., Nikolskaya, A. N., Ransom, R., and Wegener, S. 1998. Enzymology, molecular genetics, and regulation of biosynthesis of the host-selective toxin HC-toxin. Pages 25-34 in: *Molecular Genetics of Host-Specific Toxins in Plant Disease*. K. Kohmoto and O. C. Yoder, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Woloshuk, C. P., Fount, K. R., Brewer, J. F., Bhatnagar, D., Cleaveland, T. E., and Payne, G. A. 1994. Molecular characterization of *aflR*, a regulatory locus for aflatoxin biosynthesis. *Appl. Environ. Microbiol.* 60:2408-2414.
- Yang, G., Rose, M. S., Turgeon, B. G., and Yoder, O. C. 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. Pages 3-15 in: *Molecular Genetics of Host-Specific Toxins in Plant Disease*. K. Kohmoto and O. C. Yoder, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Yu, J.-H., Butchko, R. A. E., Fernandes, M., Keller, N. P., Leonard, T. J., Adams, T. H. 1996. Conservation of structure and function of the aflatoxin regulatory gene *aflR* from *Aspergillus nidulans* and *A. flavus*. *Curr. Genet.* 29:549-555.