Evolution of pathogenicity controlled by small, dispensable chromosomes in *Alternaria alternata* pathogens

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ABSTRACT

Alternaria alternata includes seven pathogenic variants, called pathotypes, which produce host-selective toxins (HSTs) as determinant factors for pathogenicity. The gene clusters for HST biosynthesis were identified from six pathotypes (Japanese pear, strawberry, tangerine, apple, tomato and rough lemon) and were found to reside on small chromosomes of < 2.0 Mb in most strains tested. We isolated mutants lacking the small chromosomes from the strawberry, apple and tomato pathotypes and showed that the small chromosomes are dispensable for growth. In this review, we summarize our current understanding of the evolution of pathogenicity controlled by small, dispensable chromosomes in *A. alternata* pathogens.

Keywords:
Alternaria alternata
Host-selective toxin
Evolution
Conditionally dispensable chromosome

1. Introduction

There are seven known host-parasite combinations in which host-selective toxins (HSTs) produced by the mitosporic fungus *Alternaria alternata* act as effectors for the establishment of plant diseases (Table 1) [1,2]. Chemical structures of six HSTs of *A. alternata*, excluding the AT-toxin from the tobacco pathotype, have been determined [1,2]. These HSTs are low-molecular weight substances with a diverse range of structures. The participation of HSTs in plant diseases is one of the most clearly understood mechanisms of host-specific pathogenesis [1-5].

Previously, different species names were adopted for *Alternaria* pathogens that produce different HSTs. However, Syoyo Nishimura and his colleagues at Tottori University found that measurements of the conidial size of these pathogens fall within the statistical range described for *A. alternata* [6]. *A. alternata* is fundamentally a ubiquitous, saprophytic fungus frequently found on decaying plant tissues [7,8]. Regardless of similarity in conidial morphology of the HST-producing pathogens, they cause diseases on different plants sensitive to a particular HST, and it is possible to distinguish one type of pathogen from another. All isolates belonging to *A. alternata* possess a general aggressiveness, recognizable as the ability to penetrate artificial membranes through appressoria of germinated conidia [1,2,6]. It is likely that the pathogenicity of *A. alternata* pathogens consists of potential aggressiveness, common to all isolates belonging to *A. alternata*, and HSTs, which are essential for host-specific infection and disease development. Based on these features, the HST-producing pathogens have been defined as pathotypes of *A. alternata* (Table 1) [6]. This classification was supported by analyses of genetic relatedness among the pathogens using molecular markers [9-11]. Thus, these pathogens are good models for studying intraspecific evolution of pathogenicity, from saprophyte to pathogen, in plant pathogenic fungi because their HSTs are well-characterized factors responsible for host-specific pathogenesis [1-5].

2. HST biosynthetic genes of *A. alternata* pathogens

To assess the molecular basis of the pathogenic specialization of *A. alternata* pathogens, we identified the HST biosynthetic genes, called *TOX* genes, from six pathotypes (Japanese pear, strawberry, tangerine, apple, tomato and rough lemon) (Table 1) [1,2]. The *TOX* genes of each pathotype are located in a single locus in the genome as a gene cluster.

2.1. ***AK-toxin, AF-toxin and ACT-toxin***

AK-toxins, AF-toxin and ACT-toxins produced by the Japanese pear, strawberry and tangerine pathotypes, respectively, are structurally analogous substances that are esters of 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid (EDA) (Table1) [12-14]. The *TOX* cluster was first isolated from the Japanese pear pathotype [15]. We used restriction enzyme-mediated integration transformation to isolate AK-toxin-minus mutants and identified the affected gene in a mutant, which is essential for AK-toxin biosynthesis. Structural and functional analysis of a cosmid clone containing the tagged site identified six AK-toxin biosynthetic genes, named *AKT* genes (Table 1). Of six genes, one (*AKTR*) encodes a putative transcription regulator containing a zinc binuclear cluster DNA-binding domain typical of the fungal Zn(II)2Cys6 family proteins, and the remaining five genes encode enzymes possibly involved in secondary metabolism [15-18]. In Southern blot analysis using *AKT* probes in *A. alternata* pathogens, five genes including *AKTR* were also present in the strawberry and tangerine pathotypes, and the remaining one (*AKTS1*) was unique to the Japanese pear pathotype [15, 16, 18-20]. The enzyme genes shared by three pathotypes were found to be involved in EDA biosynthesis. The Japanese pear pathotype strains appeared to have multiple copies of functional and nonfunctional homologs of the *AKT* genes [15-18].

Genomic cosmid libraries of the strawberry and tangerine pathotypes were screened with the *AKT* probes to isolate the AF-toxin and ACT-toxin biosynthetic genes, named *AFT* and *ACTT* genes, respectively (Table 1). By the molecular analysis of selected clones, we identified *AFT* and *ACTT* genes having strong similarity to *AKT* genes [18,20-23]. The corresponding gene pairs from the three pathotypes have > 90% nucleotide identity in pairwise comparisons. However, the arrangement of genes in the clusters differs among the three pathotypes. We also found new genes including *AFTS* and *ACTTS* genes, which are pathotype-specific [18,24-26]. Thus, the pathotype-specific genes as well as genes common to the three pathotypes coexist in the *TOX* clusters of respective pathotypes. Strains of the strawberry and tangerine pathotypes also have multiple copies of *TOX* genes in their genomes, as do the Japanese pear pathotype strains, indicating that the genomic regions controlling HST biosynthesis in these three pathotypes have complex structures [20-23,25,26].

We examined the chromosomal distribution of *TOX* genes in the three pathotypes and found that the genes are clustered on single small chromosomes of < 2.0 Mb in most strains tested (Table 1) [20,24,22,27]. Recently, we determined sequences of the 1.0-Mb chromosome of the strawberry pathotype strain NAF8 and identified the entire *AFT* region of about 390 kb in the chromosome [Unpublished results]. This region

includes two to seven copies of more than 20 putative *AFT* genes and many transposon-like sequences, most of which are inactive transposon fossils [28,Unpublished results].

2.2. ***AM-toxin***

The apple pathotype produces cyclic peptide AM-toxins (Table1) [29]. Cyclic peptides are generally synthesized via non-ribosomal pathways by large multifunctional enzymes called non-ribosomal peptide synthetases (NRPS) [30]. *AMT1* encoding a 479-kDa NRPS was isolated by PCR-based cloning with primers designed to amplify conserved domains of fungal NRPS genes known to be essential for AM-toxin biosynthesis [31]. We also identified three genes, *AMT2*, *AMT3* and *AMT4*, from a BAC clone that contains *AMT1* [32,33]. This clone contains the four *AMT* genes and other genes, which are synchronously up-regulated in AM-toxin producing cultures relative to non-producing cultures [32]. These genes encode proteins with similarity to enzymes involved in secondary metabolism and amino acid modification [32,33].

Chromosomal distribution analysis of the *AMT* genes in the apple pathotype strains demonstrated that the genes are encoded by single small chromosomes of < 1.8 Mb (Table 1) [32]. Structural analysis of the 1.3-Mb chromosome from strain IFO8984 identified multiple sets of putative *AMT* clusters [Unpublished results]. This chromosome includes a cluster containing more than 10 putative *AMT* genes and additional copies of parts of the cluster. The *AMT* region is enriched in transposon fossils, resembling the *AFT* region of the strawberry pathotype [Unpublished results].

2.3. ***AAL-toxin***

The tomato pathotype produces AAL-toxins, which are polyketide-derived compounds similar to the sphinganine-analog mycotoxins, fumonisins, produced by *Fusarium* species [34,35]. Polyketides are produced by the activity of polyketide synthases (PKSs) and then modified by other enzymes [30]. *ALT1*, which encodes a type I PKS required for AAL-toxin biosynthesis, was isolated by PCR-based cloning with primers designed to amplify conserved domains of fungal PKS genes [36]. *ALT1* has similarity to *FUM1*, encoding a PKS required for fumonisin biosynthesis in *F. verticillioides* [36-38]. A BAC clone of the tomato pathotype strain As-27 was isolated using the *ALT1* probe, and a 120-kb region of this clone was found to include at least 13 genes with significant similarity to the genes in the *FUM* cluster, suggesting that this region is responsible for AAL-toxin biosynthesis [39,Uunpublished results]. Disruption

of gene in this cluster confirmed their involvement in AAL-toxin biosynthesis by the tomato pathotype [36,40,Unpublished results). The *ALT* genes were located on single small 1.0-Mb chromosomes in the tomato pathotype strains (Table 1) [40]. Structural analysis of the 1.0-Mb chromosome identified two sets of putative *ALT* clusters.

2.4. ***ACR-toxin***

The rough lemon pathotype produces polyketide ACR-toxin [41]. Strains of this pathotype were found to have pathotype-specific small chromosomes of 1.2 to 1.5 Mb (Table 1) [27]. Partial sequencing of the 1.5-Mb chromosome of strain HC1 identified multiple genes, designated *ACRT* genes, which are possibly involved in ACR-toxin biosynthesis [42,43]. The functions of the candidate *ACRT* genes were examined by gene disruption and RNA silencing experiments. *ACRTS1* and *ACRTS2* encoding a putative hydroxylase and PKS, respectively, were found to be unique to the rough lemon pathotype and essential for ACR-toxin biosynthesis [42,43]. Both genes have multiple paralogs on the same chromosome.

2.5. ***Duplication of TOX clusters***

A. alternata pathotypes have multiple copies of *TOX* genes in their genomes. This is one of the molecular evolutionary characteristics of HST biosynthesis and has pathological significance. In experiments involving disruption of *TOX* genes in various pathotypes, it was frequently observed that mutants having a remaining functional copy of a *TOX* gene produced trace or undetectable amounts of toxin and showed markedly reduced pathogenicity, indicating that the pathotypes need multiple copies of the genes to produce enough toxin for expressing significant virulence [21-23,25,32,33,42,43]. The gene clusters for synthesis of secondary metabolites in fungi generally exist as a single set in their genomes [30]. This suggests that the duplication of the *TOX* clusters in the genomes of *A. alternata* pathotypes was the critical event for increasing virulence and thereby, fitness by enabling efficient infection of host plants.

3. Conditionally dispensable chromosomes encoding *TOX* clusters

Small chromosomes of several fungi have been identified as supernumerary chromosomes, which have been also called dispensable, B or accessory chromosomes [44,45]. Such chromosomes are generally dispensable for growth and inherited in a non-Mendelian manner [44,45]. The pea pathogen *Nectria haematococca* was the first

fungus found to carry a supernumerary chromosome, which encodes functional genes essential for virulence on host plants [46,47]. In this pathogen, genes for phytoalexin detoxification and other virulence determinants are on the supernumerary chromosomes. Loss of these chromosomes does not affect growth in culture, but does affect virulence on host plants [46,47]. Such supernumerary chromosomes have been termed conditionally dispensable (CD) chromosomes [44].

Before the *TOX* genes were isolated from *A. alternata* pathogens, electrophoretic karyotypes of *A. alternata* strains, including seven pathotypes and nonpathogenic strains, were analyzed by pulsed-field gel electrophoresis (PFGE) [48]. The PFGE analysis demonstrated that *A. alternata* strains have nine to eleven chromosomal bands of 0.4 to 5.7 Mb, with genome sizes of about 30 Mb. This analysis found an interesting difference in karyotypes between pathogenic and nonpathogenic strains: all strains from the pathotypes had small chromosomes of < 2.0 Mb, but nonpathogenic strains did not have such small chromosomes [48]. The presence of additional small chromosomes only in the pathotype strains suggested that the small chromosomes are involved in HST biosynthesis.

The chromosomal distribution of *TOX* genes in six pathotypes was analyzed by PFGE-Southern blot analysis using *TOX* probes. The analysis showed that most strains tested had *TOX* genes on small chromosomes of < 2 Mb in the six pathotypes (Table 1) [20,22-25,27,32,40,42]. For example, all strains of the strawberry pathotype had at least 10 chromosomal DNAs of about 0.9 to 5.7 Mb [20]. When the gel blots were probed with the *AFT* genes, all probes hybridized to chromosomal bands of about 1.0 Mb in all strains (Table 1) [20,24].

During the course of targeting the *AFT* genes in the strawberry pathotype strain NAF8, three mutants that lacked the 1.0-Mb chromosome encoding *AFT* genes were isolated [20]. The mutants lost the ability to produce AF-toxin and hence to cause disease on strawberry leaves. However, they grew and formed conidia on culture media, as did the wild type. Thus, this chromosome appeared to be a CD chromosome [20,44]. Mutants lacking the *TOX* chromosomes were also isolated from strains of the apple and tomato pathotypes, and their *TOX* chromosomes were also CD [40,49]. Since most strains of the Japanese pear, tangerine and rough lemon pathotypes also carry *TOX* clusters on small chromosomes, pathogenicity of all the *A. alternata* pathotypes seems to be controlled by small, CD chromosomes.

We observed the structural differences between the CD chromosome and the core chromosomes within the genomes of *A. alternata* pathogens. In PFGE-Southern blot analysis using the CD chromosome probe in the strawberry, apple and tangerine pathotypes, the probe strongly hybridized to only the CD chromosome in each

pathotype [20,32,40]. The CD chromosomes of the tomato pathotype strains collected worldwide had similar sizes of about 1.0 Mb and identical *Not*I-digestion patterns, and the sequences of some genes on the CD chromosomes were identical among the strains, suggesting that their structures are conserved [40]. In contrast, the sequences of other genes located on the core chromosomes, such as β-tubulin, melanin biosynthetic PKS and mating-type genes, had considerable polymorphisms among the strains [40]. These results indicated that the CD chromosomes might have an evolutionary history different from those of the core chromosomes in the tomato pathotype genome.

We are interested in where and how the CD chromosomes were acquired in the first place. To understand the evolution of pathogenicity mediated by HST biosynthesis and the origin of CD chromosomes, we attempted to determine complete structures of the CD chromosomes from the strawberry, apple and tomato pathotypes, which produce HSTs classified into different chemical groups (Table 1). The CD chromosome DNAs were recovered from PFGE gels and subjected to sequencing using Sanger or 454 pyrosequencing technology. To connect the contigs obtained, we isolated BAC clones containing CD chromosome DNAs and selected clones bridging contigs. By sequencing the bridge clones, we obtained almost complete sequences of the CD chromosomes and confirmed their structures by a physical mapping using the Optical Mapping technology [Unpublished results].

Although the origin of the CD chromosomes in *A. alternata* pathotypes is as yet unknown, we discovered evidence for a common origin of the CD chromosomes. Comparison of the entire lengths of CD chromosomes from the strawberry, apple and tomato pathotypes identified large syntenic regions among the three CD chromosomes [Unpublished results]. The chromosome regions including *TOX* clusters are unique to the respective pathotypes, but the remaining regions are conserved among the three chromosomes. On the basis of such structural similarity among the CD chromosomes from three pathotypes, we hypothesize that the syntenic regions are the core of the original, supernumerary chromosome, and that *TOX* clusters were integrated into the original chromosome, probably by horizontal gene transfer (HGT).

4. Intraspecies transfer of CD chromosomes

Analysis of genetic relatedness among *A. alternata* pathotypes using DNA markers could not resolve strains by correlation with pathotypes: a single pathotype population does not form a monophyletic group [9-11,50]. The fact that the *A. alternata* pathotypes have *TOX* clusters on small CD chromosomes, which nonpathogenic strains do not have, suggests that the pathotypes could be generated by intraspecies transfer of CD

chromosomes to nonpathogenic strains of *A. alternata*.

Intraspecies transfer of CD chromosomes in *A. alternata* was observed in protoplast fusion experiments. The protoplast fusion made hybrid strains between the tomato and apple pathotypes and between the tomato and strawberry pathotypes, which produced two toxins produced by the parental strains and were pathogenic to both plants affected by the parents [40,51,52]. The hybrid strains carried two CD chromosomes from both parental strains, suggesting that *A. alternata* is able to accept and maintain a small, exogenous chromosome in its genome.

Masunaka et al. [27] discovered an *A. alternata* isolate from a leaf spot on rough lemon in Florida that produces two distinct HSTs, ACT-toxin and ACR-toxin, and causes lesions on both ACT-toxin-sensitive and ACR-toxin-sensitive citrus cultivars. This isolate harbors two small chromosomes of 1.05 and 2.0 Mb, which encode the *ACTT* and *ACRT* genes, respectively, indicating that the acquisition of two small chromosomes in the same genome conferred the ability to produce two HSTs [27]. A possible mechanism of intraspecies horizontal chromosome transfer (HCT) in *A. alternata* is a parasexual cycle through hyphal anastomosis. Hyphal anastomosis has been observed between different pathotype strains and between pathogenic and nonpathogenic strains of *A. alternata* in laboratory experiments [53].

The occurrence of HCT between pathogenic and nonpathogenic strains has also been reported in *Fusarium oxysporum* [54]. In co-cultivation experiments using strains of the tomato wilt pathogen *F. oxysporum* f. sp. *lycopersici* and nonpathogenic *F. oxysporum*, the non-pathogenic strain received the 2.0-Mb supernumerary chromosome of the tomato pathogen and became pathogenic towards tomato [54]. Together with our finding of a double toxin producer within *A. alternata*, this finding may explain the occurrence of new pathogenic genotypes within a fungal species and suggest that HCT events have occurred in nature.

5. Concluding remarks

The pathogenic forms (pathotypes) of *A. alternata* are considered to have arisen through the acquisition of respective HSTs. The HST biosynthetic genes, *TOX* genes, have been isolated from six pathotypes of *A. alternata* and found to be clustered on a single chromosome, as are the genes responsible for synthesis of other secondary metabolites in fungi. The *TOX* clusters appeared to have characteristic features, such as the presence of multiple sets on a single chromosome, accumulation of inactive transposon-like sequences in the clusters and location of the clusters on small CD chromosomes. Extensive phylogenetic analysis of the genes in the CD chromosomes

and comparative genomics of CD chromosomes among multiple strains from different pathotypes are still needed to understand the origin of the CD chromosomes, the mechanisms that established these chromosomes, and the acquisition of the *TOX* clusters that created intraspecific pathogenic variations in the ubiquitous, saprophytic fungus *A. alternata.*

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Table 1
Alternaria alternata pathotypes known to produce host-selective toxins (HSTs)

Pathogen[a]	Disease name	Host plants[b]	HST	*TOX* genes[d]
Japanese pear pathotype (*A. kikuchiana*)	Black spot of Japanese pear	Nijisseiki, Shinsui	AF-toxin EDA ester[c]	*AKT* genes (< 2.0 Mb[e])
Strawberry pathotype	Black spot of strawberry	Morioka-16	AK-toxin EDA ester[c]	*AFT* genes (1.0 Mb)
Tangerine pathotype (*A. citri*)	Brown spot of tangerine	Dancy, Minneola, etc	ACT-toxin EDA ester[c]	*ACTT* genes (< 2.0 Mb)
Apple pathotype (*A. mali*)	Alternaria blotch of apple	Red Gold, Starking, etc	AM-toxin Cyclic depsipeptide	*AMT* genes (< 1.8 Mb)
Tomato pathotype (*A. alternata* f. sp. *lycopersici*)	Alternaria stem canker of tomato	Earlypak 7, First	AAL-toxin Polyketide ester	*ALT* genes (1.0 Mb)
Rough lemon pathotype (*A. citri*)	Leaf spot of rough lemon	Rough lemon (citrus rootstocks)	ACR-toxin Polyketide	*ACRT* genes (< 1.5 Mb)
Tobacco pathotype (*A. longipes*)	Brown spot of tobacco	*Nicotiana* plants	AT-toxin Unknown	Unknown

[a] Previous species name is shown in parentheses.
[b] Susceptible cultivars or plants.
[c] EDA, 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid.
[d] Sizes of chromosomes encoding *TOX* genes are shown in parentheses.
[e] Exceptional strain 15A was found to have a 4.1-Mb chromosome encoding *AKT* genes [16].