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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 *Alternaria alternata* pathogens.

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 *Alternaria 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].

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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].

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) (Table 1) [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) [20,22,24,27]. 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 (Table 1) [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].