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Citrus as a molecular contact point for co-evolution of Alternaria pathogens☆

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ABSTRACT

Alternaria black rot, Alternaria leaf spot of rough lemon, and Alternaria brown spot of tangerines are three major citrus Alternaria pathogens. Citrus could be considered as a molecular contact point for host-selective toxin (HST)-mediated co-evolution of these Alternaria pathogens and susceptibility in the field. ACR-toxin is an HST produced by the rough lemon pathotype, and the target site of the toxin was identified as rough lemon mitochondria. The biosynthetic gene cluster for ACR-toxin production is on the 1.5 Mb-chromosome of the rough lemon pathotype. Another gene cluster for ACT-toxin production is located on the 1.9 Mb-chromosome of the tangerine pathotype. These TOX genes shown to have a role in ACR- or ACT-toxin biosynthesis by using gene disruption and silencing.

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1. Introduction

Alternaria species cause three major diseases of citrus including Alternaria black rot of many citrus fruits, Alternaria leaf spot of rough lemon, and Alternaria brown spot of tangerines [1]. Alternaria black rot causes internal rotting in fruits of all citrus species and is one of the major citrus post-harvest diseases worldwide. The Alternaria black rot pathogen is hardly distinguishable in morphological characteristics from the Alternaria brown spot or Alternaria leaf spot pathogens, and they are considered to be the same species, *Alternaria alternata* [2]. Unlike the black rot pathogen, the brown spot and leaf spot pathogens produce host-selective toxins (HST); ACT-toxin for the brown spot pathogen and ACR-toxin for the leaf spot pathogen, respectively [3,4]. The host ranges of these two pathogens are exactly the same as the sensitivity of citrus cultivars to these toxins [3,4]. The ACT-toxin producing brown spot pathogen affects many ACT-toxin-sensitive tangerines and mandarins causing necrotic spots on leaves, stems, and fruits, while the ACR-toxin producing leaf spot pathogen causes similar symptoms on ACR-toxin-sensitive rough lemon and rangpur lime [1,3].

The Alternaria black rot pathogen causes black-colored rotting at the central columella usually without showing any external symptoms in fruits [5]. Endopolygalacturonase (endoPG) appears to be an essential virulence factor for this pathogen [6]. A series of studies demonstrated that EndoPG is essential for the penetration of citrus peel and development of black rot symptoms [6,7]. The gene encoding endoPG is regulated by carbon catabolite repression mediated by the catabolite repressive element A (CreA) regulation factor, and endoPG does not have a central role for hyphal growth and colonization in the juice sac area in fruits, which contains sugars such as D-fructose and D-glucose [6–9]. The endoPG gene is conserved among *Alternaria* species [2], and endoPG sequences from black rot and leaf spot pathogens are highly similar [6]. However, necrotic symptoms induced by an endoPG-minus mutant of the rough lemon pathotype were unchanged, while and endoPG-minus mutant of the black rot pathogen was significantly reduced

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in its ability to cause symptoms [6]. It is probably a reasonable result for the Alternaria black rot pathogen may require endoPG because pectin degradation is likely essential to cause the typical maceration symptom as well as for successful infection by this pathogen, which penetrates fruit tissues that are very rich in pectin. Contrary to this case, host-selective ACR-toxin production by necrotrophic Alternaria leaf spot pathogen induces necrosis without extensive penetration and colonization as well as maceration of the cell wall, and the role of endoPG might be masked by the strong toxicity of HST which may allow necrotic symptoms to appear without endoPG functions [6]. If this hypothesis is correct, HST is a pathogenicity factor controlling host specificity with a strong virulence effect, which masks the effects of other virulence factors, e.g. endoPG.

2. The *ACRT* cluster for ACR-toxin biosynthesis in the *A. alternata* rough lemon pathotype

The genus *Alternaria* belongs to the Hyphomycetes in the Deuteromycotina [10]. Phylogenetic studies using multiple genes among hundreds of *Alternaria* isolates from citrus fields indicated that all citrus-associated *Alternaria* species are *A. alternata* [2]. All isolates of *A. alternata* possess a potential general aggressiveness, which is the fundamental ability to penetrate plant tissues [11,12]. Nishimura [11] proposed that HST-producing pathogens should be considered as a distinct pathotype of *A. alternata*, and one biotype among two citrus-associated *A. alternata* producing HST was identified as the rough lemon pathotype of *A. alternata*. The major toxin, ACR-toxin I, of the rough lemon pathotype has structural features typical of polyketides consisting of a C19 polyalcohol with an α-dihydropyrone ring [13–15]. Masunaka et al. [16] found that the rough lemon pathotype has pathotype-specific chromosomes with sizes of 1.2–1.5 Mb. Draft sequence analysis of the 1.5-Mb chromosome of the standard isolate HC-1 identified multiple *ACRT* genes, responsible for ACR-toxin biosynthesis. *ACRTS1*, encoding a hydroxylase, is essential for ACR-toxin biosynthesis in the rough lemon pathotype and is required for pathogenicity [17]. *ACRTS2* encodes a polyketide synthase of 2513 amino acids and is also essential for ACR-toxin biosynthesis in the rough lemon pathotype [18]. A third gene *ACRTS3* encoding a putative cyclase, was recently found to be essential for ACR-toxin biosynthesis (Nikaido et al., unpublished data). All three of these genes have multiple paralogs and were studied using targeted gene disruption and RNA silencing to knock-out and/or -down the functional copies for examination of their roles in ACR-toxin biosynthesis. Disruption of a few copies of the respective genes reduced toxin production, and the transformants in which these genes were silenced did not produce ACR-toxin and lost pathogenicity [17,18]. These genes are unique to ACR-toxin producers of the rough lemon pathotype, and they are clustered in the 1.5 Mb-chromosome. This chromosome exists only in the rough lemon pathotype and is known to be dispensable. Gain of these clustered genes on the 1.5 Mb-chromosome during evolution may have led to ACR-toxin production and hence the pathogenicity to rough lemon and rangpur lime. However, the origin of these genes, the gene cluster, or the chromosome has not been identified yet.

3. Target site of ACR-toxin in rough lemon

ACR-toxin has high specificity and toxicity and thus is generally recognized as a necrosis-inducing agent. However, ACR-toxin also causes delay or suppression of defense-related gene expression in rough lemon leaves inoculated with the rough lemon pathotype producing ACR-toxin [19–21].

The target site of ACR-toxin was found to be the mitochondria by electron microscopic examination [22] and monitoring of the toxin effects on oxidative phosphorylation using isolated physiologically active mitochondria [23]. ACR-toxin causes uncoupling of mitochondrial oxidative-phosphorylation with a loss of membrane potential and leakage of the co-factor NAD+ from the TCA cycle [23]. The *ACRS* gene that confers sensitivity to ACR-toxin has been identified from the mitochondrial genome of rough lemon [24]. The gene confers ACR-toxin sensitivity to *E. coli*, and the mechanism of specificity in plants is alternative processing of transcripts of the gene conferring ACR-toxin sensitivity [24]. *ACRS* is 171 bp and encodes a putative 7 kDa oligomeric pore-forming transmembrane protein controlling ACR-toxin sensitivity [24]. ACRS antibodies detected three proteins with molecular weights of 14, 21 and 28 kDa in extracts from rough lemon mitochondria, which could be the dimer, trimer and tetramer that are typical features of pore-forming receptors and not fully dissociated during SDS-PAGE [24]. Identification and evaluation of a protein complex which regulates alternative processing of the *ACRS* transcript is currently underway (Shimagami et al., unpublished data).

4. *ACTT* cluster for ACT-toxin biosynthesis in *A. alternata* tangerine pathotype

Alternaria brown spot disease on Emperor mandarin was first reported in Australia in 1903 [25], and the pathogen was initially identified as *Alternaria citri* Ellis & Pierce due to the morphological similarity to the black rot pathogen [26,27]. Later the pathogen was classified as *A. alternata* [4], as one of two biotypes causing leaf spot of rough lemon [13,14,28] and brown spot of tangerine [26,29].

The tangerine pathotype affects many species of tangerines and mandarins worldwide but there is no occurrence in Japan, because a major citrus cultivar grown in Japanese citrus fields Unshu is resistant to this pathogen [1]. In laboratory tests, this pathotype is also pathogenic to Japanese pear cultivars susceptible to the Japanese pear pathotype. However, the pathogen has never encountered susceptible pear cultivars, which are grown only in Japanese fields, and thus there is no field occurrence of this disease in Japan either [29]. The tangerine pathotype produces ACT-toxins I and II; ACT-toxin I is toxic to both citrus and pear, and toxin II is highly toxic to pear and slightly toxic to citrus [29]. ACT-toxin I causes necrosis on leaves of susceptible cultivars at concentrations as low as 10^{-8} to 10^{-9} M but no necrosis on leaves of resistant cultivars even at higher concentrations. ACT-toxins consist of three moieties, 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid (EDA), valine and a polyketide [29]. Toxins of the tangerine, Japanese pear and strawberry pathotypes are likely structurally analogous metabolites each containing a EDA moiety of (2E,4Z,6E)EDA for AK- and ACT-toxins or (2E,4E,6Z)EDA for AF-toxin [29–31].

ACTT genes responsible for the biosynthesis of the EDA moiety include pathotype-specific genes as well as genes common to the three pathotypes, which have multiple copies of functional or nonfunctional homologs sharing >90% nucleotide identity in the genomes from the tangerine, Japanese pear, and strawberry pathotypes [16,33–38]. These biosynthetic genes appear to be clustered on a chromosome with a size of 2.0-Mb in the standard isolate SH-20 of the tangerine pathotype [16,32,34–37]. Functional analyses of *ACTT1, ACTT2, ACTT3, ACTTR, ACTT5*, and *ACTT6* in ACT-toxin biosynthesis have been carried out using a combination of target gene disruption and gene silencing [16,32,34–37] (Tanaka et al., unpublished data). Four additional genes, *ACTTS1* to *ACTTS4*, were also identified as the tangerine pathotype-specific genes responsible for ACT-toxin biosynthesis; *ACTTS1* and *ACTTS4* encodes a putative non-ribosomal peptide synthase, *ACTTS2* encodes an enoyl-reductase, and *ACTTS3* encodes a polyketide synthase [16,37] (Katsumoto et al. unpublished data). Targeted gene disruption or

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