The Presence of Double-Stranded RNAs in *Alternaria alternata* Japanese Pear Pathotype and Their Participation in AK-toxin Productivity*

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The intracellular occurrence of viruses has been reported in major fungal taxa¹⁾. Most of the characterized mycoviruses contain segmented double-stranded RNA (dsRNA) genomes¹⁾. The presence of mycoviruses has been usually indicated by the occurrence of dsRNA and confirmed by the visualization of particles. In many cases, virus infection causes the morphological abnormalities and the unusual biological activities of fungi¹⁾. The dsRNA has been shown to also associate with different levels of virulence in several phytopathogenic fungi, *e.g.* hypovirulence in *Endothia parasitica* causing blight of chestnut^{1,2)}.

Alternaria alternata Japanese pear pathotype, the causal organism of black spot disease of Japanese pear, produces AK-toxin as a host-specific toxin (HST)³⁾. The HST is now known to be a critical determinant of pathogenesis of HST-producing fungi. Thus, the infectivity and host specificity of such pathogens are most likely governed by the ability to produce HST³⁾. The AK-toxin productivity has been observed to be relatively unstable^{4,5)}. The field isolates frequently lose their ability to produce AK-toxin under laboratory and even field conditions^{4,5)}. Such toxin-less mutants simultaneously lose their pathogenicity to their host plants, *e.g.* Japanese pear cv. Nijisseiki, and become indistinguishable from saprophytic *A. alternata*.

In the present investigation, we have studied on the relationship between the occurrence of dsRNA and AK-toxin productivity by using AK-toxin-producing isolates and AK-toxin-less mutants.

The isolates of *A. alternata* examined in this study are listed in Table 1. The AK-toxin-less mutants are those which had expressed pathogenicity to the host plant just after isolation from naturally occurred lesions, but lost pathogenicity along with the loss of AK-toxin productivity during culture in laboratory.

The dsRNA was extracted from 10 g of frozen mycelia grown in a potato dextrose broth

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Table 1. List of isolates of *A. alternata* Japanese pear pathotype

Isolate	Geographic origin	Pathogenicity	AK-toxin production	Mycelial growth ^{a)}	dsRNA ^{b)}
No. 15A	Tottori	+	+	13.6	—
A85-4	Anjo	+	+	14.5	—
TAK-41	Tottori	+	+	11.4	+
TAK-43	Tottori	+	+	11.0	+
TAK-44	Tottori	+	+	13.3	+
TAK-47	Tottori	+	+	15.0	+
No. 15B	Tottori	—	—	14.3	—
TAK-2	Tottori	—	—	7.0	—
TAK-6	Tottori	—	—	9.5	+
TAK-7	Tottori	—	—	12.2	—
L-8192	Anjo	—	—	12.8	+
L-8205	Anjo	—	—	13.1	+

a) Mycelial growth was represented by the fresh weight (g) of 7 days old mycelia cultured in potato-dextrose broth in a 1-liter Roux's bottle.

b) Double-stranded RNA, observed in this work.

medium for 7 days at 25 C, and purified by CF-11 cellulose chromatography (Whatman) as described by Morris and Dodds⁶⁾. The dsRNA samples were suspended in 30 μ l of sterile distilled water. Each 10 μ l of sample was treated with RNase-free DNase (Promega Biotec) at 20 U/ml with 20 mM MgCl₂ for 20 min at 37 C and electrophoresed on 1.5% agarose slab gel with 89 mM Tris base, 89 mM boric acid, 2 mM disodium EDTA. The electrophoresed gel was soaked with DNase-free RNase A (Sigma) at 50 μ g/ml in 300 mM NaCl for 30 min at 37 C. Subsequently, the gel was stained with ethidium bromide (10 ng/ml). Some bands resistant to RNase at high concentration of salt were visualized on UV transilluminator after staining, and the gel was photographed (Fig. 1). To confirm that these bands would be dsRNAs, the gel was destained and again soaked with RNase A at 50 μ g/ml in sterile distilled water instead of the salt solution for 2 hr at 37 C. The gel was restained with ethidium bromide. After the second treatment with RNase A, all bands disappeared. These results showed that visualized bands after first treatment with RNase A must be dsRNAs.

Among the 12 tested isolates which consisted of 6 AK-toxin-producing isolates and 6 AK-toxin-less mutants, 7 isolates were proved to contain dsRNAs (Fig. 1). These dsRNA-possessing isolates were composed of 4 AK-toxin producers and 3 AK-toxin-less mutants. Consequently, there was no consistent association between the AK-toxin productivity and the presence of dsRNA in mycelia. No significant differences were found in the growth rate in an artificial medium (Table 1) and in the sporulation on mycelial mats among isolates bearing or lacking dsRNAs. In this study, no correlation was observed between the presence of dsRNAs and the morphological and biological changes of *A. alternata*.

Isolates L-8192 and L-8205, or isolates TAK-41, TAK-44 and TAK-47 were observed to have the same kinds of dsRNA, respectively. Each one of these 2 groups of isolates was taken from an individual orchard at the same time. Hunst *et al.*⁷⁾ compared the distribution patterns of dsRNA in mycelia of *Pyricularia oryzae* isolates from geographically separated locations and showed the possibility that the patterns can be useful as an effective molecular marker for ecological studies of fungi. In *A. alternata* pathogens, more extensive studies on the distribution of dsRNAs in the large population of isolates from geographically different places may also provide valuable information concerning the ecology of this fungus.

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Fig. 1. (A) Electrophoretic analysis of dsRNA extracted from mycelia of AK-toxin producing isolates (Tox⁺) and AK-toxin-less mutants (Tox⁻) of *A. alternata*. The dsRNAs were analyzed on a 1.5% agarose gel, soaked in DNase-free RNase A solution with 0.3 M NaCl, and stained with ethidium bromide. Lanes 1=No. 15A, 2=A85-4, 3=TAK-44, 4=TAK-41, 5=TAK-43, 6=TAK-47, 7=No. 15B, 8=TAK-2, 9=TAK-6, 10=TAK-7, 11=L-8192 and 12=L-8205. (B) Schematic representation of electrophoretic mobility and intensity of dsRNAs detected in Fig. 1(A). Molecular weight (Mol Wt.) was calculated from the mobility of *Hind*III-digested λ DNA.

和 文 摘 要

林 律器・柘植尚志・小林裕和・西村正暘：ナシ黒斑病菌の菌糸中に存在する二本鎖RNAとAK-毒素生成能の関係

AK-毒素生成6菌株とAK-毒素生成能失活6菌株の培養菌糸から抽出した核酸分画を、CF-11 cellulose クロマトグラフィーによって精製し、二本鎖RNAを得た。分離した二本鎖RNAを、アガロース電気泳動により比較検討した結果、AK-毒素生成4菌株と毒素生成能失活3菌株から、二本鎖RNAが検出された。なお、電気泳動により検出したバンドは、DNaseおよびRNaseに対する感受性の検定から、二本鎖RNAであることが確認された。しかし、毒素生成の有無と二本鎖RNAの存在との間には直接的な相関は見出されなかった。また、二本鎖RNAの存在は、菌糸生長や孢子形成にも影響していなかった。

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