

Coinfection by Different Isolates of *Alternaria alternata* in Single Black Spot Lesions of Japanese Pear Leaves

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ABSTRACT

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Coinfection by different isolates of *Alternaria alternata* in single black spot lesions of Japanese pear leaves was detected by restriction fragment length polymorphism (RFLP) analysis of the nuclear ribosomal DNA (rDNA) for differentiation of individual fungal isolates. Mixed-rDNA isolates, whose total DNA contained two types of rDNA units, have been isolated from single black spot lesions of pear leaves in susceptible cultivar Nijisseiki plants. To examine whether mixed-rDNA isolates resulted from coinfection by different rDNA variants, single-spore isolates were prepared from nine mixed-rDNA isolates and subjected to the rDNA RFLP analysis. Among the nine mixed-rDNA isolates, five were identified as true mixed-rDNA types: All single-spore isolates carried two types

of rDNA units corresponding to the respective parent isolate. However, the remaining four isolates originated from lesions coinfecting by two variant rDNA isolates. All the single-spore isolates from the four mixed-rDNA isolates contained only one type of rDNA unit. Their rDNA types were identical to one or the other of the two types carried by the respective parent isolate, and two rDNA variants were detected in single-spore isolates from each parent. This result indicated that coinfection by different isolates of *A. alternata* might occur frequently in single black spot lesions of pear leaves in the field. Pathogenicity tests of rDNA variants obtained from coinfecting lesions showed that nonpathogenic isolates in conjunction with pathogenic isolates occasionally infected lesions.

Additional keywords: AK-toxin, black spot of Japanese pear, host-specific toxin, Japanese pear pathotype of *Alternaria alternata*.

At least seven plant diseases caused by *Alternaria alternata* (Fr.) Keissl. involve host-specific toxins as pathogenicity factors of the pathogens (15). The fact that host-specific toxins participate in the establishment of plant diseases in a host-selective manner is one of the most clearly understood mechanisms of fungal pathogenesis (15,20,29). *A. alternata* is a ubiquitous saprophytic fungus, and Nishimura and Kohmoto (15) now believe that the pathotypes appear in agroecosystems after acquiring the ability to produce host-specific toxins against certain susceptible host genotypes. Thus, the *A. alternata* pathogen seems to be a good model for studying the ecology and evolution of fungal pathogens through population genetics.

As an extension of population genetic studies of *A. alternata*, we used restriction fragment length polymorphism (RFLP) analysis of nuclear ribosomal RNA genes (rDNA) to detect genetic variability in the Japanese pear pathotype of *A. alternata* (1,23). The Japanese pear pathotype, which produces host-specific AK-toxins, is the causal agent of black spot of certain Japanese pear cultivars, including the commercially important cultivar Nijisseiki (14,16,24).

The rDNA RFLPs in the Japanese pear pathotype population from 322 isolates from 13 locations in Japan showed that the pathogen population contained at least eight rDNA variants (1). The eight variant types of rDNA differed in the length and the presence of the restriction sites in spacer DNA outside the coding regions for rRNAs (1). Although about 80% of isolates were classified in the eight rDNA types, the remaining 20% were identified as mixed-rDNA types (1). The mixed-rDNA isolates carried two types of rDNA units in their total DNA. Because the isolates used were obtained from single lesions on the Nijisseiki pear leaves,

but not from single spores on lesions, we suspected that some of the mixed-rDNA isolates originated from lesions coinfecting by two isolates with different rDNA types. Incidence of coinfection in single lesions by different isolates could have a significant effect on the population genetics of the pathogen, because coinfecting lesions could provide an opportunity for genetic interaction between different genotypes, such as heterokaryosis or parasexual reproduction.

We prepared single-spore isolates from some of the mixed-rDNA isolates and identified their rDNA types by RFLP analysis. If the mixed-rDNA isolates originated from lesions coinfecting by different rDNA variants, their single-spore isolates should carry either of the two rDNA units that were detected in the mixed-rDNA parents. This study provided the evidence for the frequent occurrence of coinfection in single black spot lesions by different isolates of *A. alternata*. Furthermore, pathogenicity tests of the single-spore rDNA variant isolates from coinfecting lesions suggested that nonpathogenic *A. alternata* in conjunction with pathogenic isolates occasionally infected the lesions.

MATERIALS AND METHODS

Fungal isolates. The *A. alternata* isolates used are listed in Table 1 (1). These isolates contained two types of rDNA units in their total DNA, as determined by RFLP analysis (Table 1) (1). They were obtained from naturally occurring lesions on leaves of Japanese pear cultivar Nijisseiki. Lesions on the leaves were cut into about 4-mm² pieces with a sterile blade, wetted by immersion in 70% ethanol for 15 s and surface-sterilized by immersion in 1% sodium hypochlorite for 3 min. After immersion in sterile distilled water, the leaf pieces were placed on potato-sucrose agar (PSA) in petri dishes and incubated at 25 C for 2 days. The growing fungal mycelia were transferred to fresh

PSA dishes. After incubation at 25 C for 5 days, the growing fungi were identified as the Japanese pear pathotype by light-microscopic observation and pathogenicity testing of the Nijisseiki pear leaves (1).

Preparation of spores and single-spore isolates. The fungal isolates were cultured statically in 30 ml of potato-sucrose broth (PSB) in 100-ml Erlenmeyer flasks at 25 C for 10 days. Spores were prepared from the resulting mycelial mats by the method previously described (6). The spores were suspended in sterilized water to give a concentration of 300 spores per milliliter, and 0.1–0.3 ml of the suspension was plated on PSA dishes (9 cm diameter). After incubation at 25 C for 2 days, the growing mycelia from single spores were transferred to fresh PSA dishes and were used as single-spore isolates.

Assay for pathogenicity. Pathogenicity of the fungal isolates to Japanese pear leaves was tested by spraying spore suspension (5×10^5 spores per milliliter) on detached young leaves of the susceptible pear cultivar Nijisseiki with a glass atomizer. After the leaves were incubated in a moist chamber at 25 C for 24 h, the necrotic spots that appeared on the leaves were counted. Pathogenicity was represented as the number of spots per square centimeter of leaf.

DNA probes. Plasmid pABM3 was used as a hybridization probe to determine the rDNA type of *A. alternata* isolates. Plasmid pABM3 was obtained by subcloning a 1.87-kb *Xba*I fragment of lambda DNA phage clone Alt1 that was isolated from a genomic library of the Japanese pear pathotype strain 15A (Fig. 1A) (26). We previously detected eight variant types of the nuclear rDNA units in the Japanese pear pathotype population (1): A1–A5 and B1–B3. The eight rDNA variants possessed single type-specific *Xba*I fragments in addition to their common fragments (Fig. 1B) (1). Because the pABM3 probe hybridized only to the type-specific *Xba*I fragments, the eight rDNA variants could be identified by Southern blot analysis of their total DNA with the pABM3 probe (Fig. 1B).

DNA extraction. Fungal isolates were grown in 50-ml of PSB in 100-ml Erlenmeyer flasks at 25 C for 3 days on an orbital shaker (120 rpm). Total DNA of each isolate was prepared from the resulting mycelia by the method described previously (1). Plasmid DNA was extracted by the alkaline lysis method (19).

Hybridization. Fungal DNA was digested to completion with *Xba*I and separated by electrophoresis in a 0.8% agarose gel by standard methods (19). The fractionated DNA was transferred to nylon membranes (Hybond N+, Amersham, Arlington Heights, IL) by the alkaline transfer method (18).

Hybridization with probe pABM3 and detection of specific sequences were performed with nonradioactive DNA labeling and detection kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's recommendations. A nonradioactive pABM3 probe was prepared by randomly-primed incorporation of digoxigenin-labeled dUTP (3).

TABLE 1. Mixed ribosomal DNA (rDNA) isolates of the Japanese pear pathotype of *Alternaria alternata* used in this study^a

Isolate ^b	rDNA type ^c
T88-11	A4 + B2
T88-58	B1 + B2
T88-60	A2 + B1
T88-67	A4 + B1
T88-95	B1 + B2
T88-120	A2 + A4
T88-148	B1 + B2
T88-223	B1 + B2
N89-65	A2 + B2

^a All isolates were obtained by mass hyphal transfer in single black spot lesions.

^b T88-series isolates were sampled from Tottori Prefecture, Japan, during 1988, and isolate N89-65 was collected from Aichi Prefecture, Japan, during 1989 (1).

^c Shown in Figures 2 and 3.

RESULTS

rDNA types of single-spore isolates obtained from mixed-rDNA isolates. Total DNA of the mixed-rDNA isolates used was digested with *Xba*I and hybridized with digoxigenin-labeled pABM3 DNA. The hybridization visualized two fragments in all isolates, indicating the coexistence of two types of rDNA units in their total DNA. (Figs. 2 and 3).

The rDNA types of single-spore isolates obtained from the mixed-rDNA parents were identified by Southern analysis of total DNA with probe pABM3. All single-spore isolates from the four mixed-rDNA isolates (T88-11, T88-60, T88-120, and N89-65) con-

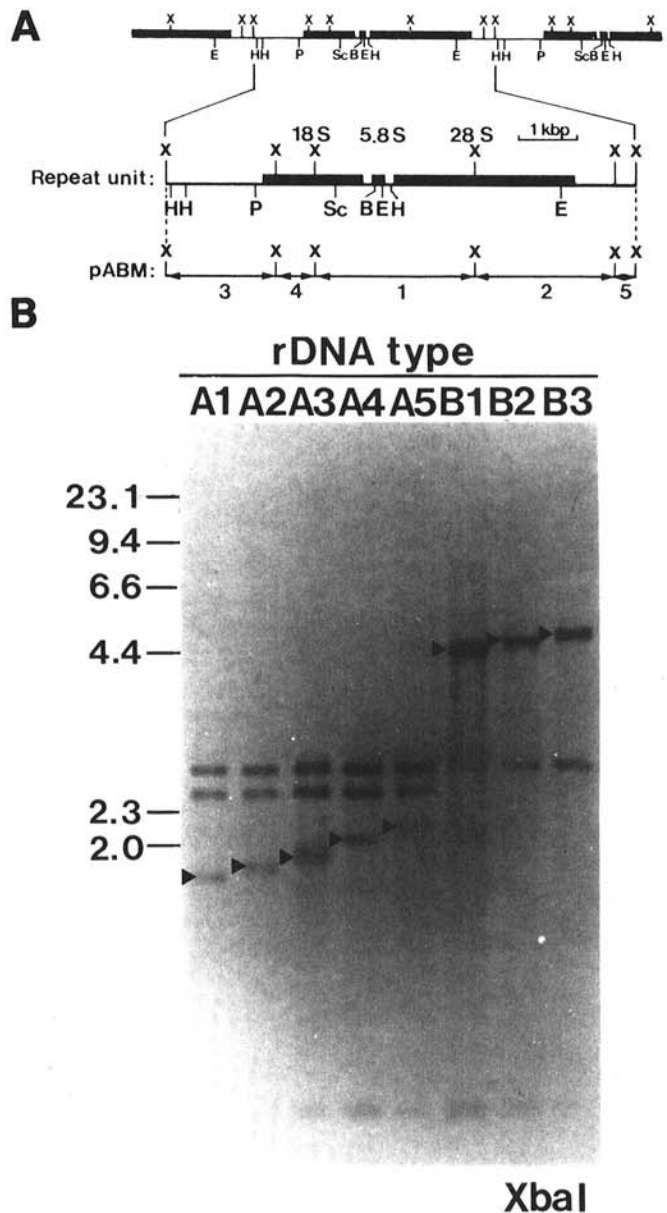


Fig. 1. A, Gene arrangement and restriction maps of a nuclear ribosomal DNA (rDNA) unit deduced for rDNA clone Alt1 of the Japanese pear pathotype and B, restriction fragment length polymorphisms of representative isolates of the eight rDNA variants of the pathogen population. A, Alt1 was cloned from a genomic library of the Japanese pear pathotype strain 15A (26). The five fragments produced by digestion of Alt1 DNA with *Xba*I were subcloned and designated the pABM series of plasmids (26). B = *Ban*III; E = *Eco*RI; H = *Hind*III; P = *Pst*I; Sc = *Sac*I; and X = *Xba*I. C, Total DNA was digested with *Xba*I and fractionated in 0.8% agarose gel. The Southern blot was hybridized with digoxigenin-labeled Alt1 probe. Arrow heads show the type-specific rDNA fragments detected by a subsequent rehybridization with the pABM3 probe. The sizes of marker DNA fragments (*Hind*III-digested lambda DNA) are given in kilobases to the left.

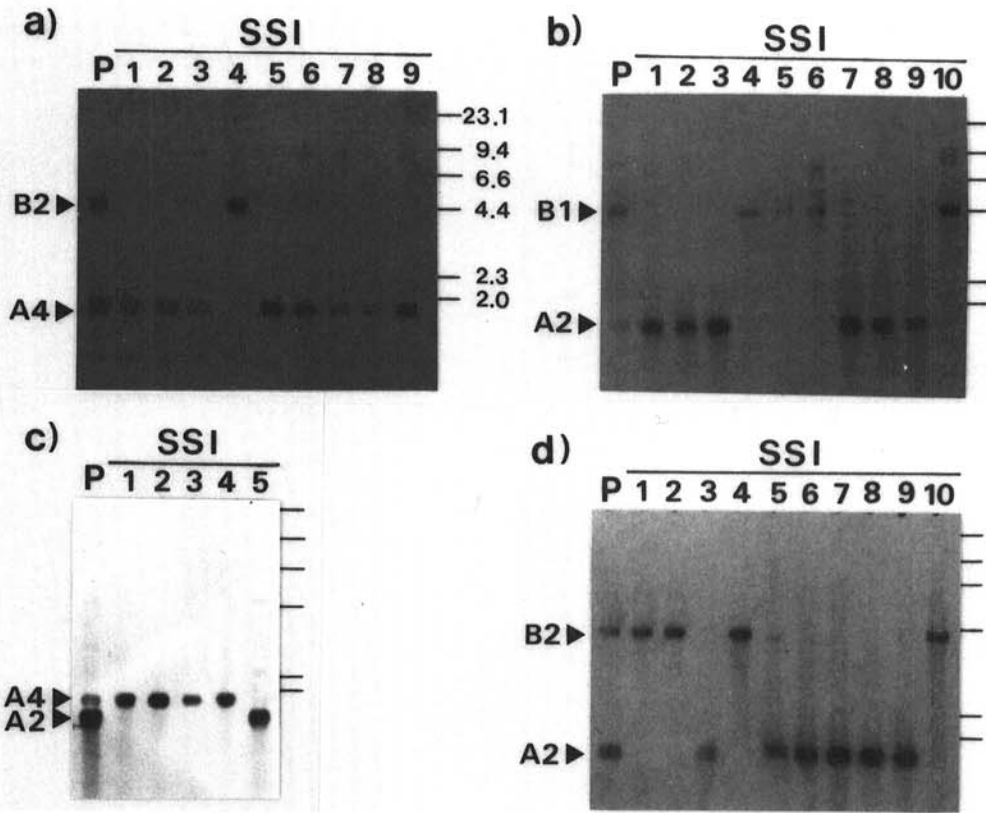


Fig. 2. Hybridization of total DNA of single-spore isolates (SSI) obtained from mass transfer isolates A, T88-11; B, T88-60; C, T88-120; and D, N89-65 with digoxigenin-labeled pABM3 probe. The *Xba*I-digested DNA was fractionated in 0.8% agarose gel, and the Southern blot was hybridized with the pABM3 probe. Arrow heads show the type-specific ribosomal DNA (rDNA) fragments (rDNA types A2, A4, B1, and B2). Lane P contains total DNA of the parent isolates. The sizes of marker DNA fragments (*Hind*III-digested lambda DNA) are given in kilobases to the right of A. The hash marks to the right of B-D indicate the same marker sizes as in A.

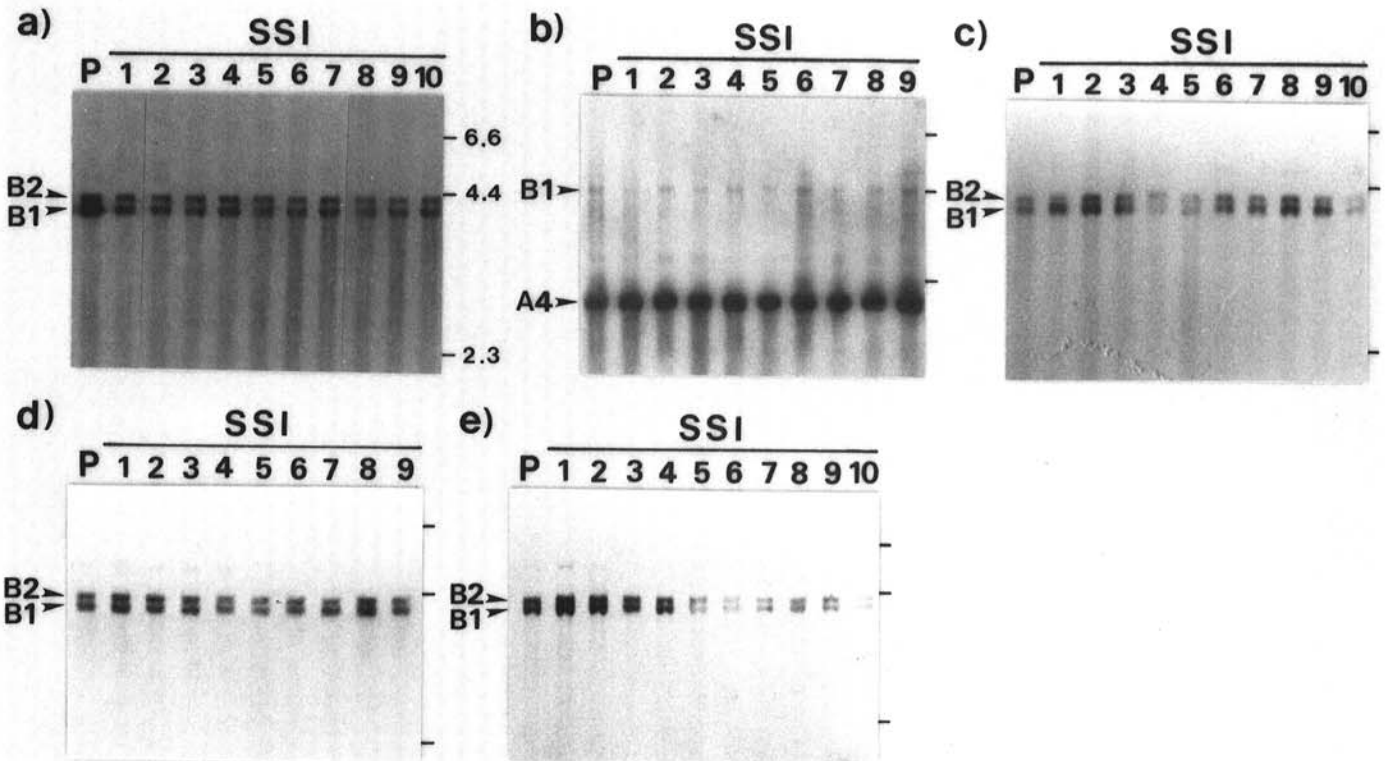


Fig. 3. Hybridization of total DNA of single-spore isolates (SSI) obtained from mass transfer isolates A, T88-58; B, T88-67; C, T88-95; D, T88-148; and E, T88-223 with digoxigenin-labeled pABM3 probe. The *Xba*I-digested DNA was fractionated in 0.8% agarose gel, and the Southern blot was hybridized with the pABM3 probe. Arrow heads show the type-specific ribosomal DNA (rDNA) fragments (rDNA types A4, B1, and B2). Lane P contains total DNA of the parent isolates. The sizes of marker DNA fragments (*Hind*III-digested lambda DNA) are given in kilobases to the right of A. The hash marks to the right of B-E indicate the same marker sizes as in A.

tained only one *Xba*I fragment that hybridized to the probe (Fig. 2). The detected fragments were identical in size to either of the two fragments carried by the respective parent isolates, and single-spore isolates were divided into two rDNA types based on the RFLPs (Fig. 2). For example, among nine single-spore isolates from isolate T88-11 (carrying both A4 and B2 rDNA units), one isolate was identified as B2 type and the others as A4 type (Fig. 2A). This indicated that these mixed-rDNA isolates were obtained from coinfecting lesions by two rDNA variants.

On the other hand, the pABM3 probe hybridized to two *Xba*I fragments in all single-spore isolates from the remaining five mixed-rDNA isolates (T88-58, T88-67, T88-95, T88-148, and T88-223) (Fig. 3). The hybridization profiles of single-spore isolates were the same as those of the parents, suggesting that two types of rDNA units coexisted in the genome of these isolates. To examine the mitotic stability of mixed-rDNA type, isolate T88-58 was passaged through three rounds of single-spore isolation, and the rDNA types of the resulting isolates were determined. The resulting isolates preserved the two types of rDNA units similarly to the parent (data not shown). This confirmed that this isolate was a true mixed-rDNA isolate carrying two types of rDNA units in the genome.

Pathogenicity of rDNA variants isolated from coinfecting lesions. Pathogenicity of two rDNA variants, which were obtained from four mixed-rDNA isolates (T88-11, T88-60, T88-120, and N89-65), was examined by spore inoculation of pear leaves of the susceptible cultivar Nijisseiki (Table 2). We selected one to three single-spore isolates as representatives of each rDNA variant. Both rDNA variants from isolates T88-11 and T88-120 were highly pathogenic to the pear leaves (Table 2). However, there were significant differences in pathogenicity between two rDNA variants from isolates T88-60 and N89-65. A2 variants from isolate T88-60 and B2 variants from N89-65 were weakly pathogenic or nonpathogenic to the pear leaves, although B1 and A2 variants from isolates T88-60 and N89-65, respectively, were highly pathogenic (Table 2).

DISCUSSION

This study provided evidence of the frequent occurrence of coinfection in single black spot lesions of Japanese pear leaves by two isolates of *A. alternata* in the field. Our previous study showed that about 20% of isolates obtained from single lesions

TABLE 2. Pathogenicity of ribosomal DNA (rDNA) variants obtained by single-spore isolation, from mass transfer isolates T88-11, T88-60, T88-120, and N89-65 of *Alternaria alternata*

Single-spore isolate	rDNA type ^a	Pathogenicity ^b
T88-11-1	A4	37.1 ± 5.6
T88-11-2	A4	70.0 ± 18.1
T88-11-9	A4	97.3 ± 5.0
T88-11-4	B2	79.1 ± 10.2
T88-60-1	A2	0.0 ± 0.0
T88-60-2	A2	0.3 ± 0.5
T88-60-3	A2	0.8 ± 1.4
T88-60-5	B1	41.2 ± 9.1
T88-60-6	B1	51.2 ± 13.7
T88-60-10	B1	51.2 ± 6.9
T88-120-1	A4	64.9 ± 22.8
T88-120-2	A4	78.7 ± 27.0
T88-120-4	A4	77.8 ± 14.3
T88-120-5	A2	116.4 ± 7.9
N89-65-1	B2	6.9 ± 3.9
N89-65-2	B2	0.4 ± 0.5
N89-65-3	A2	116.1 ± 10.6
N89-65-5	A2	100.5 ± 10.1

^a Shown in Figure 2.

^b Pathogenicity is represented as the number of necrotic spots per square centimeter of pear leaf inoculated with fungal spores. Each value represents the mean of four experiments ± SE.

contained two types of nuclear rDNA units in their total DNA (1). From among those, we randomly selected nine mixed-rDNA isolates and analyzed the rDNA types of their single-spore isolates. The results indicated that four of nine mixed-rDNA isolates, according to the rDNA types of their single-spore isolates, originated from coinfecting lesions. Experimental evidence for the occurrence of coinfection by phytopathogenic fungi has been very limited because of the lack of markers to distinguish individual isolates of fungi reliably. Kato (7) reported coinfection events in single ragi seeds by the ragi blast fungus *Pyricularia* sp., based on a mating-type marker; two mating-type isolates were detected in single ragi seeds. McDonald and Martinez (11) employed RFLP markers to analyze genetic variability of single-pycnidial isolates of *Mycosphaerella graminicola*, which were obtained from single lesions of wheat leaves. Their data suggested the occurrence of coinfection in wheat lesions by different isolates (11). We believe that evidence of coinfection events in phytopathogenic fungi will accumulate through studies of population genetics using DNA markers.

The incidence of coinfection by different isolates could be significant in the population genetics of the *A. alternata* pathotype, because coinfecting lesions enable different isolates to coexist and interact in a very small space. We previously detected a large variation within the Japanese pear pathotype population of *A. alternata*, based on the DNA fingerprints, using hybridization with a moderately repetitive DNA sequence of the fungus (1). Petrunak and Christ (17) also found considerable genetic variation in an *A. alternata* population on the basis of isozyme analysis. They believe that such variation occurs through recombination, whether asexual or sexual.

Asexual recombination (mitotic crossing-over and haploidization) can occur through the parasexual cycle, which may be a source of variation. Although it is unknown whether parasexual recombination occurs commonly in *A. alternata* in the field, heterokaryon formation has been reported in this fungus in laboratory experiments (22,25). A second possible route for recombination is through a sexual cycle. Although no sexual cycle is known to date in *A. alternata*, teleomorphs for several other *Alternaria* spp. have been discovered (21). If asexual or sexual recombination could occur in the field, coinfecting lesions would be an important place for genetic interaction between different genotypes. Layton and Kuhn (9) reported that in planta heterokaryon formation is significant in pathogenic specialization of *Phytophthora megasperma* f. sp. *glycinea*.

Pathogenicity tests of rDNA variants obtained from coinfecting lesions showed no correlation between pathogenicity and any of the variant rDNA types. We observed in two cases that both nonpathogenic and pathogenic isolates of *A. alternata* coinfecting single lesions. It is unknown whether the nonpathogenic *A. alternata* isolate establishes in the pear leaf tissues simultaneously with or after the infection event of the pathogenic isolate. Because *A. alternata* is a ubiquitous saprophytic fungus, a great number of spores are present in the air (15). Thus, the presence of a nonpathogenic isolate in a black spot lesion might indicate that an airborne saprophytic spore takes advantage of a preexisting area of necrotic leaf tissue. Although the coinfection process of different genotypes is unclear, coinfection events may play an important role in the development of new pathogenic variants and fungicide-resistant strains in phytopathogenic fungi. To examine in planta genetic recombination through heterokaryosis, we have applied genetic transformation to produce strains that can be identified easily by resistance to different drugs (27). We are now investigating heterokaryon formation in single lesions of the pear leaves inoculated with two strains.

True mixed-rDNA isolates that carried two types of rDNA units in their genome also were detected. Garber et al (5) reported that the rDNA cluster in fungi usually consists of tandem repeats of 100- to 300-copies of the identical units in a haploid genome. Heterogeneity in the rDNA units within individual genomes is rather unusual in fungi (2,8,10,28), although Bruns et al (2) reported that higher eukaryotes usually contain variation of rDNA units within individual genomes. A reason for the heterogeneity

within the rDNA cluster could be genetic recombination through a sexual cycle. Thus, the true mixed-rDNA isolates detected in the Japanese pear pathotype might result from recombination through either the asexual or sexual cycle.

DNA markers, such as RFLPs and DNA fingerprinting, have been used for population genetics of phytopathogenic fungi (2,4,12,13). An important application of such methods is the identification of individual isolates and the assessment of genetic relatedness within and between fungal pathogen populations (2,4). This study provides a good example of the effectiveness of DNA markers in differentiating individual isolates. We are currently trying to compare the genetic structure of the pathogen populations in different sampling years and geographic origins in micro- and macroscale using DNA fingerprinting with moderately repetitive DNA sequences of the fungus (1). Such analysis will provide a firmer basis for estimating the rate of gene flow between pathogen populations over time and space and might be useful for understanding the genetic potential of pathogenic specialization in this fungus.

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