Hyphal anastomosis and complementary growth of fused cells in *Alternaria alternata*

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Hyphal anastomosis and complementary growth of fused cells in *Alternaria alternata* were investigated. Sixty-four experimental isolates were divided into anastomosis-positive (A^+) and anastomosis-negative (A^-) groups based on their self-anastomosing ability. Nonself-anastomoses (interisolate) were readily distinguished from self-anastomoses (intraisolate) by using a mixed culture of conidia and hyphal fragments prepared from the respective isolates. Nonself-anastomosis occurred only between the A^+ isolates irrespective of their pathogenicity and geographic origin. The breakdown of cell walls and the establishment of cytoplasmic continuity between fused cells were microscopically observed only in the self-anastomoses. The frequency of the nonself-anastomosis was, in general, lower than that of the self-anastomosis. For analysis of complementation between the fused cells, mutants doubly marked with auxotrophy and hygromycin B (Hyg) resistance were prepared from wild-type isolates. The identity of the mutants was confirmed by RAPD analysis using three arbitrary primers. Complementary growth occurred only between an A^+ isolate and its mutant(s) on a minimal medium containing Hyg, demonstrating that the self-anastomoses resulted in perfect cell fusions and the nonself-anastomoses were contact or imperfect fusions.

Key Words——*Alternaria alternata*; complementary growth; hyphal anastomosis.

Alternaria alternata (Fries) Keissler (formerly *Alternaria tenuis* Nees) is a ubiquitous and cosmopolitan fungus which can be found on many kinds of plants and other substrata including foodstuffs, soil and textiles (Ellis, 1970). Many strains of this fungus are known to be saprophytic, but some are notorious for causing severe diseases on various plants. Among such plant pathogenic *A. alternata*, at least seven pathogens are known to produce host-specific toxins (HSTs) which play a role as initiation factors in pathogenesis (Kohmoto and Otani, 1991). However, these HST-producing pathogens were previously classified as different species irrespective of the lack of significant difference in conidial morphology and size (Nishimura et al., 1978). Nishimura (1980) and Nishimura et al. (1982, 1983) proposed a pathotype system for designating these pathogens, which should be considered as biochemical mutants of saprophytic *A. alternata*, and their acquisition of the ability to produce a HST that generates pathogenicity to specific plant species or cultivars. Recently, this proposal has been supported by molecular genetic evidence. Unlike the case of *Fusarium* spp., in which formae speciales or vegetative compatibility groups are clearly distinguishable by DNA restriction fragment length polymorphism (RFLP) (Manicom et al., 1990), DNA fingerprinting, and random amplification of polymorphic DNA (RAPD) (Crowhurst et al., 1991; Kelly et al., 1994; Manulis et al., 1994), *A. alternata* shows considerable genetic diversity among isolates of its saprophyte and pathotypes, as indicated by isozyme analysis (Hwang et al., 1987; Petrunak and Christ, 1992), RFLP analysis of nuclear ribosomal RNA genes, and DNA fingerprinting (Adachi et al., 1993). Therefore, the saprophyte and pathotypes of *A. alternata* could not be discriminated from each other by these molecular methods. This variation in *A. alternata*, however, is acceptable at the species level: *A. alternata* is clearly distinguished, as a species, from other species of *Alternaria*, based on molecular data (Kusaba and Tsuge, 1994a, b).

Alternaria alternata belongs to Deuteromycotina (Fungi Imperfecti) as its sexual phase has been found neither in nature nor in vitro. Accordingly, asexual recombination becomes an important candidate for explaining this genetic variation in *A. alternata*, and hyphal anastomosis is the only way that two nuclei can come together to give birth to recombinant(s). Hyphal anastomosis has been reported in some pathogenic *A. alternata* (Nagai and Takahashi, 1966; Fukuda, 1968). Recently, Adachi and Tsuge (1994) reported the occurrence of coinfection in single black lesions of Japanese pear leaves by different isolates of *A. alternata*, and heterokaryosis was confirmed in *A. alternata* (Tsuge et al., 1987), indicating the possibility of genetic interaction between individuals of *A. alternata* by means of hyphal anastomosis. From the viewpoint of the prerequisite for subsequent heterokayosis, the objectives of the present research were to investigate the hyphal anastomosis of *A. alternata* in depth under the microscope, and to confirm whether it leads to complementary growth of the

fused cells.

Materials and Methods

Fungal isolates Sixty-four isolates of *A. alternata*, including 29 isolates of the Japanese pear pathotype, 17 of the apple pathotype, 5 of the tangerine pathotype, 3 of the strawberry pathotype, and 10 nonpathogenic isolates were used. They are listed in Table 1 with their origins and some biological characters examined. Of the nonpathogenic isolates, EGS35-193 and IMI89343 were obtained from the Simmons Culture Collection, U.S.A. and International Mycological Institute, U.K., respectively, and used as standard isolates of *A. alternata*.

Preparation of conidia Mycelial plugs were inoculated on V-8 juice agar plates and incubated at 21°C under continuous irradiation of near-UV light (FL20S, Matsushita, Osaka) for 6 to 10 d. Conidia formed on the plates were harvested with sterile deionized water containing 0.5% polyoxyethylene (20) sorbitan monolaurate by gently shaking the plates. This conidial suspension was centrifuged at 2000 rpm for 3 min, then the collected conidia were washed once with sterile deionized water by centrifugation (2000 rpm, 3 min).

Preparation of hyphal fragments The mycelial plugs were inoculated on gellan gum plates (3% glucose, 1.5% gellan gum, and 0.1% $MgSO_4 \cdot 7H_2O$, w/v) and incubated at 25°C for 2 to 4 d. Two or three mycelial blocks (3×3 mm) were cut out and transferred to 100 ml of potato-dextrose broth (PDB, pH 5.5) in an 100-ml Erlenmeyer flask. After incubation at 25°C for 5 to 10 d, cottony mycelia in the liquid medium were harvested and fragmented with a homogenizer (Precise®, Products Corporation Racine, Wisconsin) at a medium speed for 4 min. After removal of air from bubbles in vacuo, the hyphal fragments in PDB were filtered through a sterile nylon mesh (110 μm openings) and washed once with sterile deionized water by centrifugation (2000 rpm, 3 min).

Observation of hyphal anastomosis To examine the ability of self-anastomosis in *A. alternata*, conidia prepared from 55 isolates were suspended in PDB (3×10^5 conidia/ml), and the conidial suspensions were incubated at 25°C for 6 h on sterile glass slides. After removal of superfluous PDB, the germlings on the glass slides were fixed with 70% ethanol, stained with 0.1% aniline blue in lactophenol and anastomoses were observed under a light microscope (Nikon, AFX-II). The self-anastomosing ability of an isolate was evaluated by the number of anastomoses per 1000 germlings observed. Nonself-anastomosis (interisolate) was examined in mixed cultures of conidia and hyphal fragments. Conidia of one isolate of *A. alternata* were mixed with hyphal fragments of a different isolate and suspended in PDB (3×10^5 conidia and 7×10^4 hyphal fragments/ml). This mixed suspension (1.5 ml) was placed on a sterile glass slide and incubated at 25°C for 7.5 h. After fixation and staining, anastomoses between germlings and regenerating hyphal fragments were microscopically judged to be nonself-anastomosis and those between germlings or between regenerating hyphal fragments to be self-anastomosis (intraisolate). For a nonsporulating isolate, its self-anastomosing ability was determined by the presence or absence of anastomosis between its regenerating hyphal fragments.

Transformation Fungal protoplasts were prepared by the method of Tsuge et al. (1990) with the following modifications. Cottony mycelia were obtained by growing cultures in PDB as described above, then rinsed with 0.7 M NaCl and steeped in an enzyme solution. The components of the enzyme solution were the same as described by Tsuge et al. (1990). After incubation at 30°C for 4 h on an orbital shaker at 120 rpm, the resultant protoplasts were separated from cell debris by filtering through a nylon mesh (60 μm openings) and washed once with 0.7 M NaCl. Plasmid pAN7-1 constructed by Punt et al. (1987) was used for transforming the protoplasts to hygromycin B resistance (Hyg®). Transformation was performed by the method of Vollmer and Yanofsky (1986) with modifications as previously described by Itoh et al. (1994).

Mutagenesis and isolation of auxotrophic mutants Mutagenesis and isolation of auxotrophic mutants of *A. alternata* were carried out by the method of Tsuge et al. (1987) with the following modifications. For mutagenesis, approximately 0.01 ml of 0.3% (w/v) *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was added to 0.99 ml of conidial suspension (10^6 conidia/ml) in a 10-ml sterile centrifuge tube. After incubation for 4–5 h at 25°C with gentle vortexing two or three times, the MNNG-treated conidia were washed three times with sterile deionized water by centrifugation (2000 rpm, 3 min), and plated on a complementary medium (CM). Tips of germ tubes or hyphae were separately transferred to a minimal medium (MM) after 1 to 2 d of incubation at 25°C. The components of CM and MM were the same as described by Tsuge et al. (1987). Slow-growing colonies were separately transferred to CM and MM after incubation for 4 d at 25°C. Colonies showing growth on CM but not on MM were selected as auxotrophic mutants. Specific nutritional requirements of the isolated mutants were determined by Holliday's method (1956).

RAPD analysis Genomic DNA of *A. alternata* was isolated by the method of Yoder (1988) with modifications as previously described by Itoh et al. (1994). The polymerase chain reaction (PCR) amplification conditions used were based on those of Williams et al. (1990) with modifications as described by Crowhurst et al. (1991). Amplification reactions were carried out with GeneAmp PCR system 9600 (Perkin Elmer, U.S.A.) in a final reaction volume of 20 μl containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 0.001% (w/v) gelatin, 125 mM concentration of each dNTP, 200 nM primer, 1 unit of *Taq* DNA polymerase (Takara, Japan) and approximately 15 ng of genomic DNA. The temperature cycle used for amplification was: 3 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 35°C and 3 min at 72°C. Amplified products were analysed by agarose gel (1.2%) electrophoresis. Primers used were: GT-02 (5'-TGGTGGGTCC-3'), OPA-02 (5'-TGCCGAGCTG-

Table 1. *Alternaria alternata* isolates used in this study and their sporulating and self-anastomosing abilities.

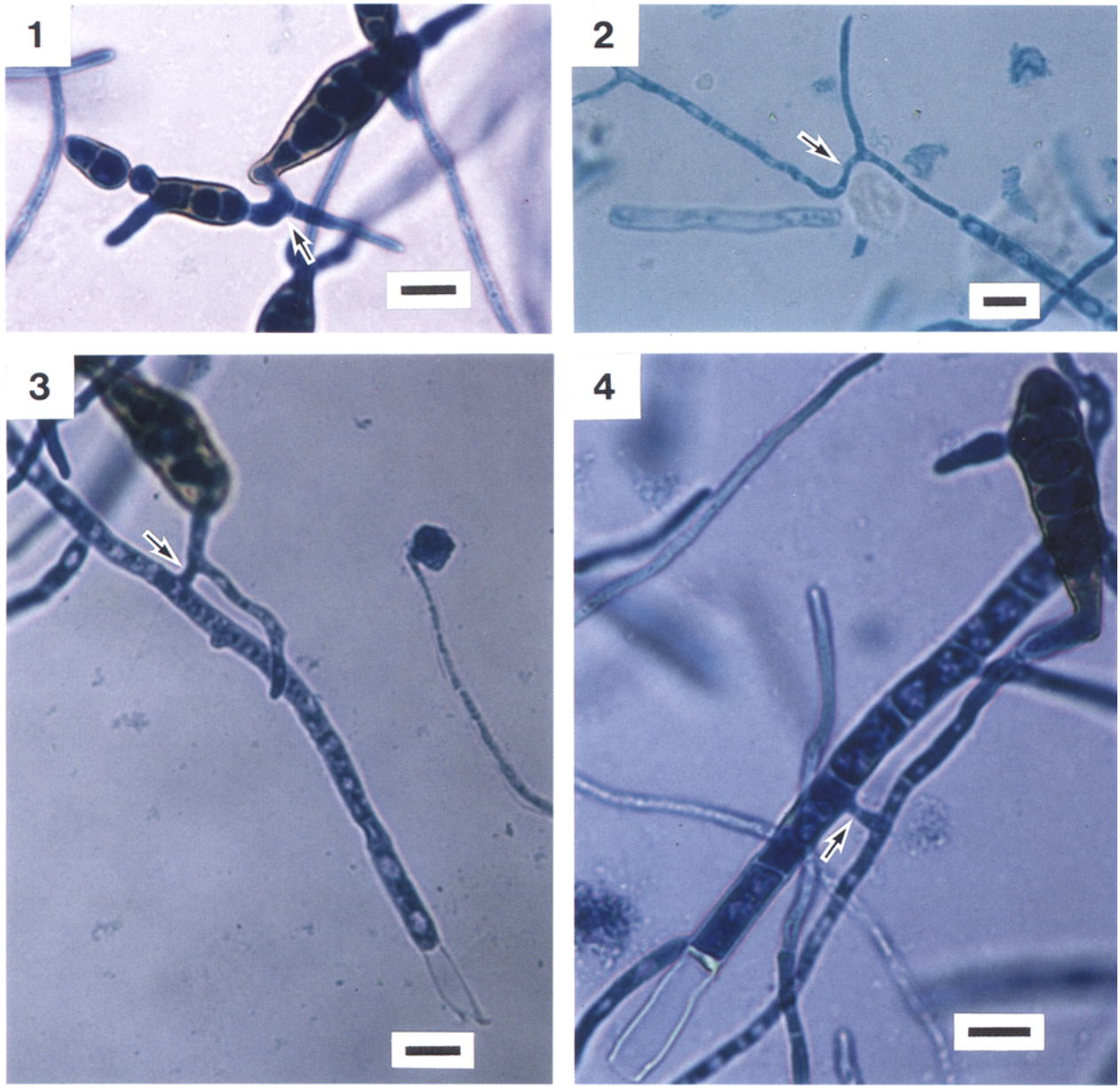
Isolate	Host	Geographic origin	Year isolated	Sporulation[a)]	Self-anastomosis[b)]
A-1		Tottori, Japan	1992	+	A^+
A-2		Tottori, Japan	1992	+	A^+
A-3		Tottori, Japan	1992	+	A^+
A-4		Tottori, Japan	1992	+	A^-
A-5		Tottori, Japan	1992	+	A^+
EGS35-193		U. S. A.		+	A^+
IMI89343		U.K.		+	A^+
No. 19		Tottori, Japan		+	A^-
T-5		Tottori, Japan	1991	+	A^-
O-94		Tottori, Japan		+	A^-
176	Japanese pear	Tottori, Japan	1993	+	A^+
203	Japanese pear	Tottori, Japan	1993	+	A^+
204	Japanese pear	Tottori, Japan	1993	+	A^+
210	Japanese pear	Tottori, Japan	1993	+	A^+
234	Japanese pear	Tottori, Japan	1993	+	A^+
239	Japanese pear	Tottori, Japan	1993	+	A^+
247	Japanese pear	Tottori, Japan	1993	+	A^+
GN-16	Japanese pear	Tottori, Japan	1992	+	A^+
ML-10-E	Japanese pear	Tottori, Japan	1989	+	A^-
No. 2	Japanese pear	Tottori, Japan		+	A^+
No. 3	Japanese pear	Tottori, Japan		+	A^-
No. 5	Japanese pear	Tottori, Japan		+	A^+
No. 6	Japanese pear	Tottori, Japan		+	A^+
No. 8	Japanese pear	Tottori, Japan		+	A^+
No. 9	Japanese pear	Tottori, Japan		+	A^+
No. 15A	Japanese pear	Tottori, Japan		+	A^-
O-274	Japanese pear	Tottori, Japan	1993	+	A^+
O-275	Japanese pear	Tottori, Japan	1993	+	A^+
O-276	Japanese pear	Tottori, Japan	1993	+	A^-
P-3	Japanese pear	Tottori, Japan	1992	+	A^-
P-7	Japanese pear	Tottori, Japan	1992	+	A^+
P-8	Japanese pear	Tottori, Japan	1992	+	A^-
P-9	Japanese pear	Tottori, Japan	1992	+	A^+
T-2	Japanese pear	Tottori, Japan	1991	+	A^+
T-3	Japanese pear	Tottori, Japan	1991	+	A^+
T-7	Japanese pear	Tottori, Japan	1991	+	A^+
T-8	Japanese pear	Tottori, Japan	1991	+	A^+
T-10	Japanese pear	Tottori, Japan	1991	+	A^+
Y-42	Japanese pear	Tottori, Japan	1968	+	A^+
K-1	Apple	Akita, Japan	1985	+	A^-
K-2	Apple	Akita, Japan	1985	+	A^+
K-3	Apple	Akita, Japan	1985	+	A^-
K-5	Apple	Akita, Japan	1985	+	A^+
KSA-01	Apple	Aomori, Japan	1992	+	A^+
KSA-02	Apple	Aomori, Japan	1992	+	A^-
KSA-03	Apple	Aomori, Japan	1992	+	A^-
KSA-05	Apple	Aomori, Japan	1992	+	A^-
KSA-07	Apple	Aomori, Japan	1992	+	A^-
KSA-08	Apple	Aomori, Japan	1992	+	A^+
KSA-09	Apple	Aomori, Japan	1992	+	A^-
KSA-10	Apple	Aomori, Japan	1992	+	A^+
KSA-11	Apple	Aomori, Japan	1992	+	A^-
M-71	Apple	Toyama, Japan	1974	−	A^-
O-159	Apple	Nagano, Japan	1971	−	A^-
O-210	Apple	Aomori, Japan	1981	−	A^-
O-211	Apple	Iwate, Japan	1981	−	A^-
10626-1	Tangerine	Australia	1993	−	A^-
10626-2	Tangerine	Australia	1993	−	A^+
10627-A4-1	Tangerine	Australia	1993	+	A^+
10627-A4-2	Tangerine	Australia	1993	+	A^-
10626-A4-2	Tangerine	Australia	1993	+	A^-
M-30	Strawberry	Tottori, Japan		−	A^-
O-165	Strawberry	Iwate, Japan	1977	−	A^-
T-32	Strawberry	Tottori, Japan		−	A^+

a) "+" and "−" denote sporulating and nonsporulating isolates, respectively.
b) Isolates with the ability to self-anastomose are denoted as A^+, and those without as A^-.

3'), and RC-07 (5'-TGCGACAATC-3'). These primers were purchased from Nippon Gene, Japan for this study.

DNA manipulations and Southern hybridization Restriction enzyme digestion of DNA and agarose gel electrophoresis conditions for separation of DNA fragments were the same as described by Sambrook et al. (1989). Genomic DNA digests were transferred from agarose gels to Hybond N^+ nylon membranes (Amersham, U.K.) by capillary transfer (Southern, 1975) under alkaline conditions, then probed with digoxigenin (DIG)-11-dUTP labelled pAN7-1. Probe labelling was done by primed synthesis (Feinberg and Vogelstein, 1984) using a DNA labelling kit (Boehringer Mannheim, Germany). Hybridizations were carried out at 65°C and a DIG-ELISA detection kit (Boehringer Mannheim, Germany) was used to identify DNA fragments that hybridized.

Complementation tests Auxotrophic Hyg® mutants (doubly marked mutants) were obtained by transforming the monosporic cultures of wild-type isolates to Hyg® with pAN7-1 plasmid, followed by mutating Hyg® transformants to auxotrophy with MNNG, or vice versa. Single-spore isolations of the doubly marked mutants were



Figs. 1–4. Hyphal anastomoses of *A. alternata* (arrows indicate the fused sites).
1. Self-anastomosis between two germlings of isolate EGS35-193. 2. Self-anastomosis between two regenerating hyphal fragments of isolate IMI89343. 3. Self-anastomosis between a germling and a regenerating hyphal fragment of isolate EGS35-193. 4. Interisolate (nonself) anastomosis between a germling of isolate EGS35-193 and a regenerating hyphal fragment of isolate IMI89343.
All scale bars = 10 μm.

carried out by the method of Huang and Kohmoto (1991). Monosporic cultures of the doubly marked mutants and their parent wild-type isolates were used for the complementation tests. Conidia of a doubly marked mutant were mixed with hyphal fragments of its parent or nonparent wild-type isolate in PDB (3×10^5 conidia and 7×10^4 hyphal fragments/ml). This mixed suspension (3 ml) was divided into two equal parts: one part was used for microscopic observation of hyphal anastomosis between the doubly marked mutant and its parent or nonparent isolate by the method described above, and the remaining 1.5 ml was used for complementation tests by the following method.

The mixed suspension was placed on a sterile plastic Petri dish (90×20 mm, Iwaki Glass, Japan) and incubated at 25°C for 8 h. It was then gently rinsed twice with 20 to 25 ml of sterile deionized water and overlaid with 20 ml of molten (50°C) MM containing 100 μg/ml of Hyg (MMH). After incubation at 25°C for 6 d, the appearance of a complementing colony on the plate was examined.

Results

Microscopic observation of hyphal anastomosis Following incubation of conidia or hyphal fragments prepared from an *A. alternata* isolate as described in Materials and Methods, hyphal anastomosis was examined under the microscope (Figs. 1 and 2, respectively). Self-anastomosis was observed in 35 out of 55 sporulating isolates of *A. alternata* examined (Table 1), and the number of anastomoses in these 35 isolates varied greatly from a minimum of 2 to a maximum of 110 in 1000 conidial germlings observed, with an average and a standard deviation of 20.7±25.5. Of 9 nonsporulating isolates, only 2 (10626-2 and T-32) showed the ability to self-anastomose (Table 1). The proportion (63.6%) of the sporulating isolates that were capable of self-anastomosis was clearly higher than that (22.2%) of the nonsporulating isolates. There was a marked difference in the proportion of isolates with self-anastomosing ability among the saprophyte (60%), the Japanese pear pathotype (79.3%), the apple pathotype (29.4%), the tangerine pathotype (40%), and the strawberry pathotype (33.3%) (Table 2).

Table 2. Frequencies of isolates capable (A^+) or incapable (A^-) of self-anastomosis in saprophytic or pathogenic *A. alternata*.

Group of *A. alternata*	No. of isolates	
	A^+	A^-
Saprophyte	6 (60.0)[a)]	4 (40.0)
Japanese pear pathotype	23 (79.3)	6 (20.7)
Apple pathotype	5 (29.4)	12 (70.6)
Tangerine pathotype	2 (40.0)	3 (60.0)
Strawberry pathotype	1 (33.3)	2 (66.7)

a) Percent of isolates.

Three isolates, EGS35-193, IMI89343 and 204, which showed high, moderate and low rates of self-anastomosis, respectively, and isolate T-5, which lacked the ability of self-anastomosis, were chosen for the following tests. The frequency of self-anastomosis was investigated under different pH, temperature and nutritional conditions (Figs. 5A, B and 6, respectively). Although the numbers of self-anastomoses in each of the three isolates (EGS35-193, IMI89343, and 204) varied significantly with pH, temperature and medium and were maximal under certain optimal conditions, the isolates

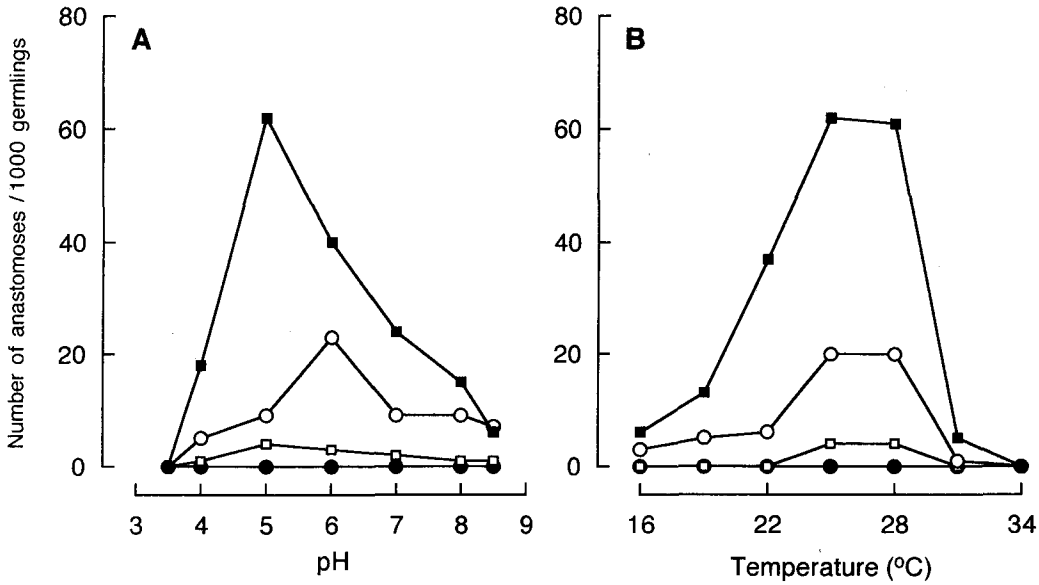


Fig. 5. Frequency of self-anastomosis in *A. alternata* isolates (■-■, EGS35-193; ○-○, IMI89343; □-□, 204; ●-●, T-5) incubated under different cultural conditions.
A. Conidia were incubated in PDB of different pHs at 25°C for 6 h. B. Conidia were incubated in PDB (pH 5.5) at different temperatures for 7 h.

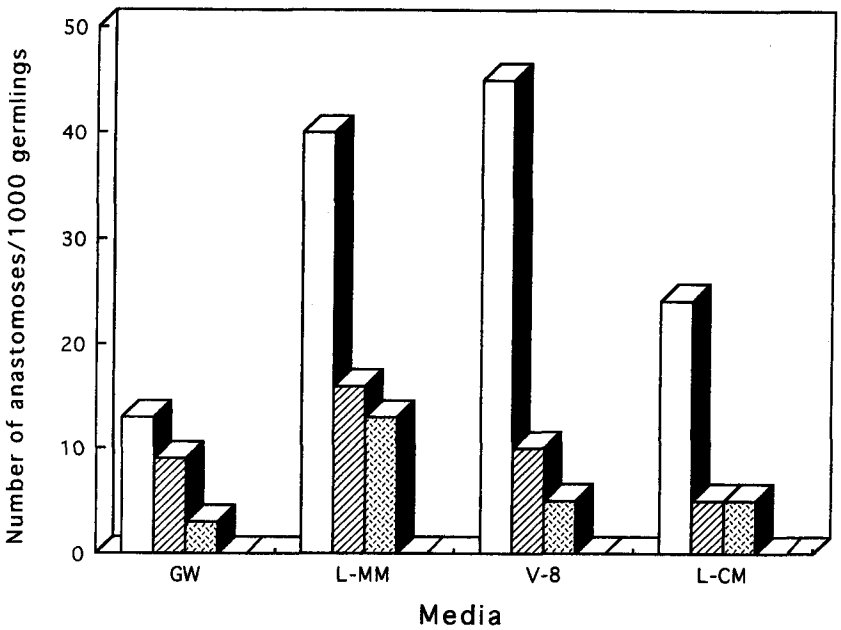


Fig. 6. Frequency of self-anastomosis in *A. alternata* isolates (□, EGS35-193; ▨, IMI89343; ▩, 204). Conidia were incubated in different liquid media (pH 7.0) at 25°C for 6 h. GW, 1% glucose in deionized water; L-MM, liquid MM; V-8, V-8 juice; and L-CM, liquid CM. No anastomosis was observed in strain T-3.

showed parallel patterns of variation. On the other hand, isolate T-5 showed no apparent self-anastomosis even under the optimal conditions for the other isolates, indicating that it is characterized by the lack of self-anastomosing ability. Although the possibility of observing an anastomosis event would be increased by increasing the sampling size, we have divided the *A. alternata* isolates into 2 groups: anastomosis-negative (A^-) isolates, in which no self-anastomosis was found in 1000 germlings at 25°C in PDB (pH 5.5); and anastomosis-positive (A^+) isolates, in which self-anastomoses were observed under the same conditions.

Hyphal anastomosis also occurred between different isolates (Fig. 4). To distinguish nonself-anastomosis from self-anastomosis, conidia and hyphal fragments were prepared from different isolates and anastomosis was observed in this mixed culture. Nonself-anastomosis (conidial germling and hyphal fragment type, CH-type) was easily recognized from self-anastomosis (conidial germling or hyphal fragment type). This nonself-anastomosis was, however, observed only when both the conidia and the hyphal fragments were A^+ (Table 3). The frequency of self-anastomosis was approximately 2 to 20 times higher than that of nonself-anastomosis (Table 3).

Two distinctive modes of fusion between vegetative cells of *A. alternata* were detected microscopically. One mode of the fusion was seen only in the self-anastomoses and involved the breakdown of cell walls and the establishment of cytoplasmic continuity between two fused cells (Figs. 1-3). The other was found mainly in the nonself-anastomoses, in which no cytoplasmic fusion occurred between different isolates (Fig. 4).

Pathogenicity and geographic origins of the isolates examined were apparently unrelated to the occurrence of nonself-anastomosis in *A. alternata*. Anastomosis was observed between saprophyte and pathogenic isolates, and between different pathotypes (Table 3).

Complementary growth of fused cells To confirm whether hyphal anastomosis leads to further growth of the fused cells, *A. alternata* mutants doubly marked with auxotrophy and drug resistance were employed for complementation tests.

Two A^+ isolates, 204 and EGS35-193, were first mutated to auxotrophy by MNNG treatment, then the resultant auxotrophs were transformed to Hyg® with pAN7-1. Other A^+ isolates, P-7 and KSA-10, and the A^- isolates T-5 and No. 15A were first transformed to Hyg® with pAN7-1, then mutated to auxotrophy by MNNG treatment. The nutritional requirements of the doubly marked mutants were as follows: 204-AH-26 required guanine, EGS-AH-1, KSA-AH-2, P-AH-5, T-AH-8 and T-AH-28 required adenine, KSA-AH-33 and P-AH-3 required L-methionine, and 15-AH-12 required L-histidine. Most of the adenine-requiring auxotrophs also grew on MM containing 500 μg/ml of hypoxanthine (Hyp).

Integration of the plasmid into the genomic sequences of mutants was confirmed by Southern blot analysis with pAN7-1 as probe. The *Eco*R I digests of all transformants showed signals at 2.5 and 4.1 kb, which were characteristic of a head-to-tail tandem repeat of pAN7-1 integration, and signals at various sizes unique to each transformant were also detected, while no signal was detected in wild-type isolates (data not shown). All the doubly marked mutants showed the same level of ability of self-anastomosis as their parent isolates.

The identity of the doubly marked mutants was

Table 3. Number of anastomoses between germlings and regenerating hyphal fragments of *A. alternata* in mixed cultures of conidia and hyphal fragments.

Isolate from which hyphal fragments derived[a]		No. of anastomoses/1000 germlings					
		Isolate from which conidia derived[a]					
		EGS35-193 (A^+)	IMI89343 (A^+)	P-7 (A^+)	No.15A (A^-)	O-94 (A^-)	T-5 (A^-)
Saprophyte							
EGS35-193	(A^+)	46[b]	10	22	0	0	0
IMI89343	(A^+)	10	41[b]	14	0	0	0
O-94	(A^-)	0	0	0	0	0[b]	0
T-5	(A^-)	0	0	0	0	0	0[b]
Japanese pear pathotype							
204	(A^+)	11	12	19	0	0	0
P-7	(A^+)	8	12	120[b]	0	0	0
Y-42	(A^+)	4	12	15	0	0	0
ML-10-E	(A^-)	0	0	0	0	0	0
No.15A	(A^-)	0	0	0	0[b]	0	0
Apple pathotype							
KSA-10	(A^+)	7	15	36	0	0	0
M-71	(A^-)	0	0	0	0	0	0
O-159	(A^-)	0	0	0	0	0	0
O-210	(A^-)	0	0	0	0	0	0
O-211	(A^-)	0	0	0	0	0	0
Tangerine pathotype							
10626-2	(A^+)	4	4	19	0	0	0
10627-A4-1	(A^+)	5	10	24	0	0	0
10626-1	(A^-)	0	0	0	0	0	0
10626-C-4	(A^-)	0	0	0	0	0	0
10627-A4-2	(A^-)	0	0	0	0	0	0
Strawberry pathotype							
T-32	(A^+)	14	5	6	0	0	0
M-30	(A^-)	0	0	0	0	0	0
O-165	(A^-)	0	0	0	0	0	0

a) A^+ and A^- in parentheses denote the ability and inability to self-anastomose, respectively.
b) Number of self-anastomoses.

confirmed by RAPD analysis using three arbitrary primers, GT-02, OPA-02 and RC-07. Amplification products were obtained with these primers for all the parent isolates and their mutants, and the patterns of amplified bands obtained with all primers for each mutant were identical to their parents, but were considerably different between isolates (Fig. 7, data of P-7 and its mutants not shown), indicating that these doubly marked mutants were indeed derived from their wild-type parents without contamination during the process of adding these genetic markers.

The growth of the doubly marked mutant EGS-AH-1 and its parent isolate EGS35-193 was compared after 10 d of incubation at 25°C on various media (Fig. 8). The parent isolate grew on MM, while the mutant did not. On the other hand, the mutant could grow on MM containing 500 μg/ml of Hyp and 100 μg/ml of Hyg (MMNH), but the parent isolate could not. Further, both the parent isolate and the mutant grew on MM containing only Hyp (MMN), but neither of them grew on MMH. The remaining combinations of the parent isolate and the doubly marked mutant(s) showed the same growth pattern except for the difference in nutritional requirements (data not shown).

When a doubly marked mutant and its parent A^+ wild-type isolate were precultured together at 25°C for 8 h, then overlayed with MMH, a complementing colony appeared on the MMH plate after 6 d of incubation (Fig. 9). In the combination of the doubly marked mutant EGS-AH-1 and its parent wild-type EGS35-193, numerous conidia were formed on the resultant complementing colony (data not shown). Single-spore analysis was carried out for this colony. Out of 124 germinating conidia isolated, 64 were found to be prototrophic and Hyg-sensitive as the wild-type (EGS35-193), the remaining 60 to be auxotrophic and Hyg® as the

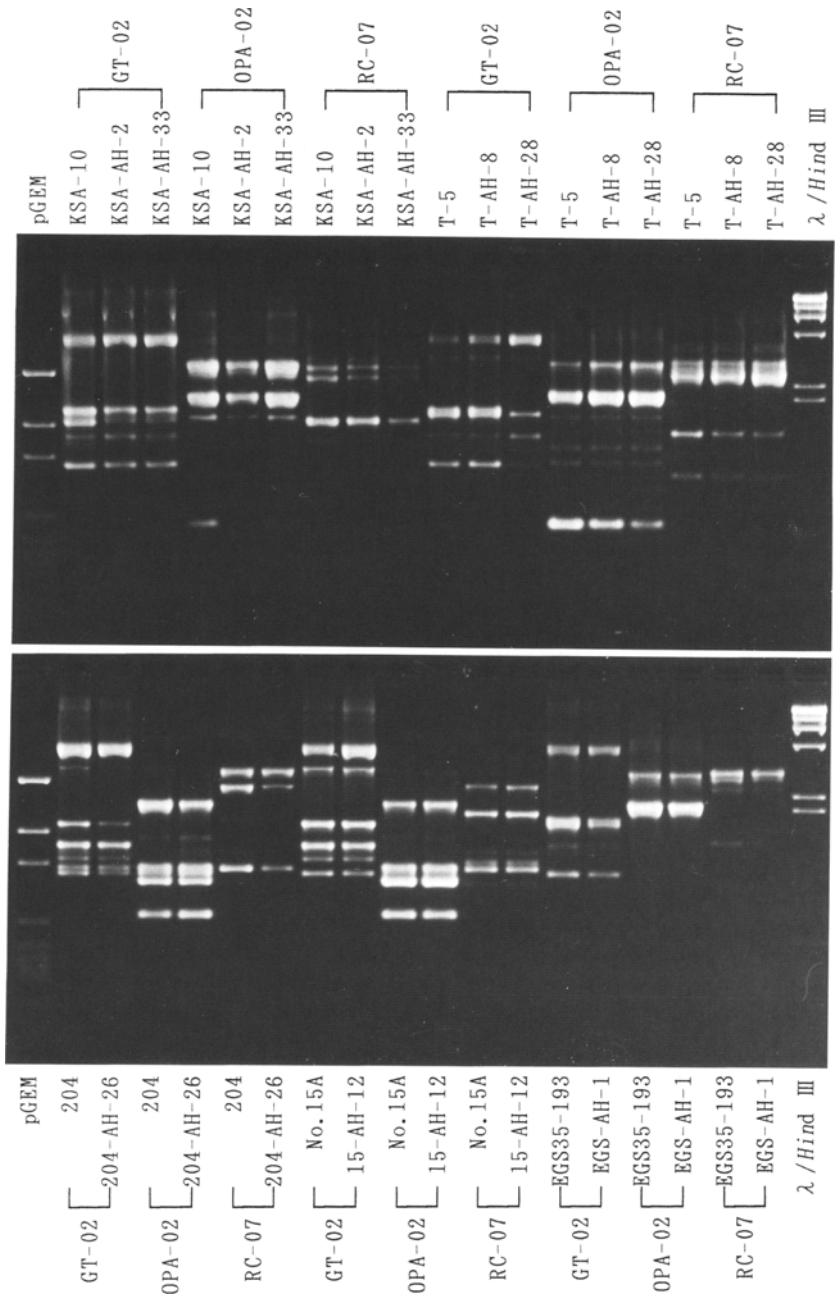
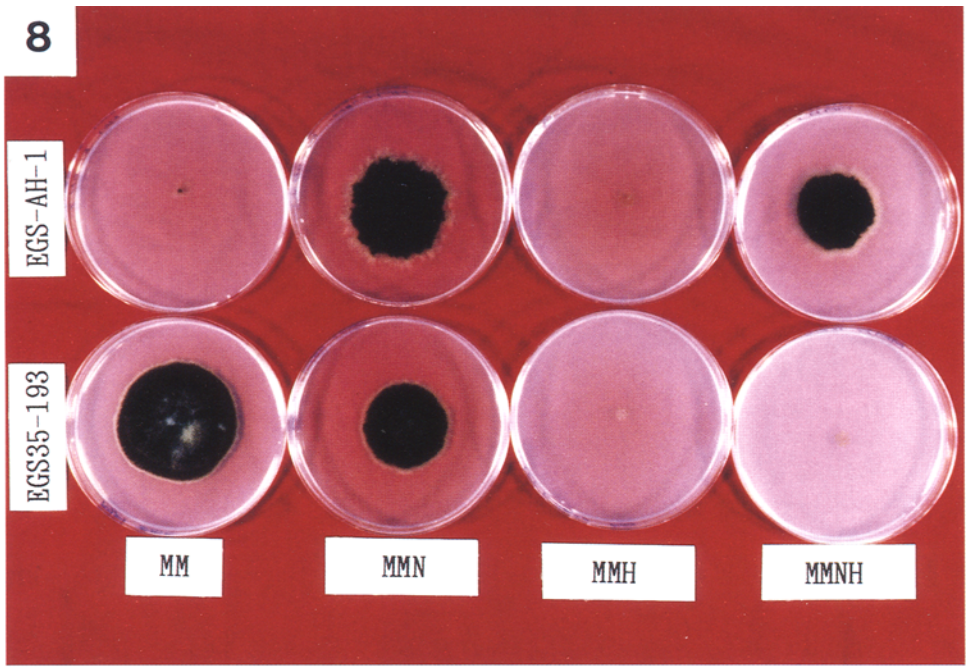


Fig. 7. Comparison of the random amplified polymorphic DNA patterns between the doubly marked mutants and their parent isolates of *A. alternata*. Polymerase chain reactions were carried out with three primers: GT-02, OPA-02 and RC-07.

mutant (EGS-AH-1). Although the CH-type anastomoses were microscopically confirmed for all the combinations of A^+ isolates and A^+ mutants, the complementary growth occurred only between the parent isolates and their own mutants (Table 4). The hyphal anastomoses between mutants and nonparent isolates did not result in further growth of fused cells on MMH (Table 4). As described above, anastomosis was not observed under the microscope in any combination of A^- and A^- or A^- and A^+ constituents, including A^- parent isolates and their mutants. Complementary growth was also not observed in such combinations.

Discussion

Hyphal anastomosis has been investigated in many filamentous fungi. Among them, *Rhizoctonia solani* Kühn has been most intensively studied, and 12 anastomosis groups were reported in this fungus (Rovira et al., 1986; Ogoshi, 1987; Carling et al., 1987, 1994). Since these groups were closely related to host ranges (Ogoshi, 1987) and genomic organizations of the fungus (Kuninaga and Yokosawa, 1980; Vilgalys, 1988), hyphal anastomosis appeared to be an effective means of grouping isolates of this phytopathogen. In contrast to the case of



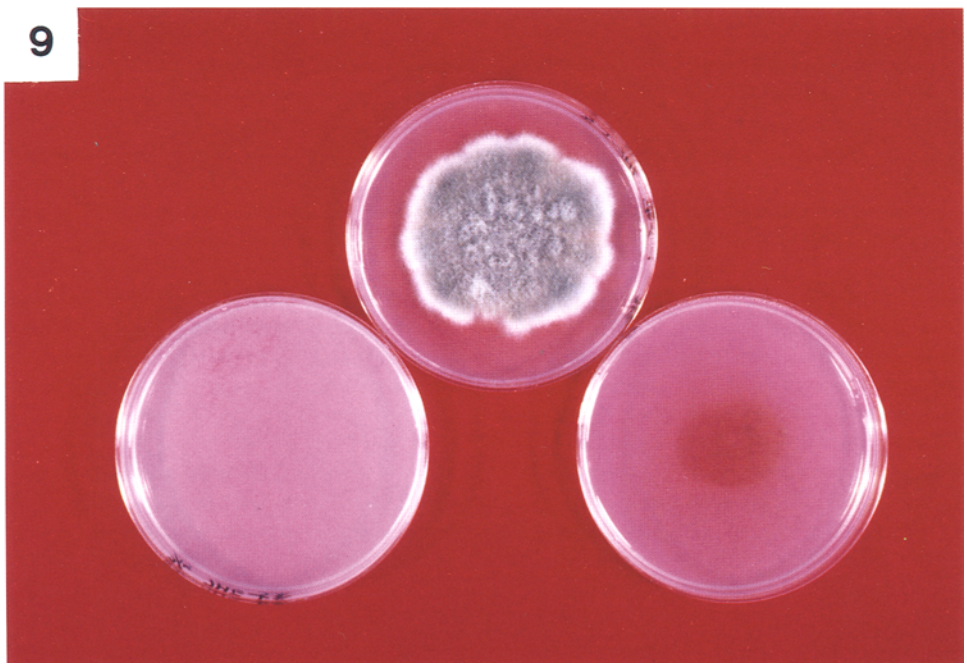


Fig. 8. Comparison of growth between the doubly marked mutant EGS-AH-1 and its parent isolate EGS35-193 of *A. alternata* on various media. MM, minimal medium; MMN, MM containing Hyp (500 μg/ml); MMH, MM containing Hyg (100 μg/ml); and MMNH, MM containing Hyp (500 μg/ml) and Hyg (100 μg/ml).

Fig. 9. Complementary growth in the mixed culture of conidia of the doubly marked mutant P-AH-5 and hyphal fragments of its parent wild-type isolate P-7 of *A. alternata* on MMH (the upper plate). Neither the germlings of the mutant (right) nor the regenerating hyphal fragments of the wild-type (left) grew on this medium alone.

R. solani, however, successful anastomosis of *A. alternata* occurred only between isolates with self-anastomosing ability, and was not related to their pathogenicity or geographic origin (Tables 3, 4). Recently, we have confirmed the occurrence of hyphal anastomosis between *A. alternata* and some small-spored *Alternaria* spp. including *A. brassicicola* (Schweinitz) Wiltshire, *A. japonica* Yoshii, and *A. radicina* Meier, Drechsler & Eddy (unpublished data). Therefore, it seems difficult to employ hyphal anastomosis as a criterion for grouping isolates of *A. alternata*.

Examination on 64 sporulating and nonsporulating

Table 4. Anastomosis and complementing reactions between doubly marked mutants and wild-type isolates of *A. alternata*.

Doubly marked mutant[a]	No. of anastomoses[b] (complementing reaction[c])					
	Wild-type isolate[a]					
	204(A^+)	EGS35-193(A^+)	KSA-10(A^+)	P-7(A^+)	T-5(A^-)	No.15A(A^-)
204-AH-26 (A^+)	65 (+)[d]	16 (−)	22 (−)	26 (−)	0 (−)	0 (−)
EGS-AH-1 (A^+)	10 (−)	36 (+)[d]	8 (−)	13 (−)	0 (−)	0 (−)
KSA-AH-2 (A^+)	42 (−)	7 (−)	23 (+)[d]	20 (−)	0 (−)	0 (−)
KSA-AH-33 (A^+)	28 (−)	17 (−)	57 (+)[d]	26 (−)	0 (−)	0 (−)
P-AH-3 (A^+)	18 (−)	12 (−)	16 (−)	41 (+)[d]	0 (−)	0 (−)
P-AH-5 (A^+)	8 (−)	5 (−)	6 (−)	24 (+)[d]	0 (−)	0 (−)
T-AH-8 (A^-)	0 (−)	0 (−)	0 (−)	0 (−)	0 (−)[d]	0 (−)
T-AH-28 (A^-)	0 (−)	0 (−)	0 (−)	0 (−)	0 (−)[d]	0 (−)
15-AH-12 (A^-)	0 (−)	0 (−)	0 (−)	0 (−)	0 (−)	0 (−)[d]

a) A^+ and A^- in parentheses denote the ability and inability to self-anastomose, respectively.
b) Number of anastomoses between a doubly marked mutant and a wild-type isolate per 1000 germlings.
c) Complementing colony occurred (+) or did not occur (−) between a doubly marked mutant and a wild-type isolate after 6 d of incubation at 25°C on MMH.
d) The combination of a doubly marked mutant and its parent wild-type isolate.

isolates of *A. alternata* showed that 37 isolates (57.8%) had the ability to self-anastomose and the remaining 27 isolates (42.2%) did not (Table 1). We designated the former as A^+ isolates and the latter as A^- isolates. The A^- isolates never anastomosed either with other A^- or A^+ isolates, even those belonging to the same pathotype (Tables 3, 4), though the nonself-anastomosing isolates of *R. solani* could anastomose with the self-anastomosing isolates belonging to the same anastomosis group (Kuninaga, 1980; Hyakumachi and Ui, 1987). Thus, the A^- isolates of *A. alternata* apparently differ in anastomosis reactions from the nonself-anastomosing isolates of *R. solani*, in spite of their inability to self-anastomose.

The numbers of self-anastomoses in each of three A^+ isolates (EGS35-193, IMI89343 and 204) varied significantly with cultural conditions (Figs. 5, 6), but the patterns of self-anastomosis were parallel in the isolates, indicating that each has own degree of ability to self-anastomoses. On the other hand, although there was a marked difference in the proportion of A^+ isolates among the various groups of *A. alternata* (Table 2), it is difficult to conclude whether the different rates reflect a distinctive feature of each group.

Since nonself-anastomoses could theoretically occur simultaneously with self-anastomoses in *A. alternata* as well as in *R. solani*, it is necessary to distinguish these two events in the study of hyphal anastomosis between individuals of the respective fungi. The method routinely used for such studies of *R. solani* is to oppose mycelial transfers of two isolates on water agar (Flentje and Stretton, 1964; Garza-Chapa and Anderson, 1966; Parmeter et al., 1969; Tu et al., 1969; Ogoshi, 1972; Kuninaga et al., 1978; Burpee et al., 1980; Kuninaga, 1980; Hyakumachi and Ui, 1987) or on cellophane resting on water agar (Whitney and Parmeter, 1963; Flentje and Stretton, 1964; Parmeter et al., 1969; Ogoshi, 1972). Because the regular branching habit of *R. solani* indicates the direction of growth, individual hypha can readily be traced back to insure that anastomoses are between paired isolates and not between branches of the same isolate (Parmeter et al., 1969). Anastomoses of *A. alternata* also occurred on water agar or on cellophane resting on water agar (unpublished data). However, the fungus has variable branching habit. Thus, it was time-consuming and laborious to use the routine method for such work in *A. alternata*. In contrast, the occurrence of anastomosis between a germling and a regenerating hyphal fragment (Figs. 3, 4) evidently shows that conidia and hyphal fragments can be used as reliable morphological markers for distinguishing self-anastomoses from nonself-anastomoses in *A. alternata*.

To ascertain whether anastomosis within the same isolate and between different isolates of *A. alternata* leads to further growth of the fused cells, mutants doubly marked with auxotrophy and Hyg® were employed in the present study. To confirm that the doubly marked mutants are derived from their original parents without contamination, RAPD analysis was carried out. This analysis was originally developed by Williams et al. (1990) and Welsh and McClelland (1990), and now is used for a variety of organisms to assess genomic variability and to construct genetic maps. In addition, this molecular method is also very useful for strain identification, has advantages over the traditional markers such as auxotrophy, drug resistance and morphological difference, and is rapid in performance. The information collected by the method can easily be increased by increasing the number of primers used. Therefore, more precise and definitive identification of mutants is possible. The isolated doubly marked mutants may be used as testers to check viability of growth of fused cells on MMH and to test efficiently the complementing ability of

a lot of field isolates.

Hyphal anastomoses microscopically observed in *Hypochnus sasakii* Shirai were classified into perfect, imperfect and contact fusions (Matsumoto et al., 1932). Perfect fusion had at least two features: 1) a breakdown of the contacting cell walls between fused cells, and 2) confluence of two masses of cytoplasm without causing the death of fused cells. Imperfect fusion means that the cell membranes of contacting hyphae were incompletely dissolved, or the contents of the two hyphal cells did not mix together, either case resulting in no intimate organic union (Matsumoto et al., 1932). Additionally, imperfect fusion resulting in the death of fused and adjacent cells was observed microscopically in *R. solani* (Ogoshi, 1972). The term "contact fusion" was used to describe another mode of cell fusion, in which two growing hyphae came in close contact but did not fuse, simply making contact, as in the case of appressoria of certain fungi (Matsumoto et al., 1932). In the present study, the disappearence of cell walls at the contact site and the cytoplasmic confluence between the two fused cells were observed only in the self-anastomoses of *A. alternata* (Figs. 1-3). Further, such cytological events were also found in the anastomoses between the A^+ doubly marked mutants and their original A^+ parents, but not in the anastomoses between the A^+ doubly marked mutants and A^+ nonparents (data not shown). The results based on microscopic observation are compatible with the complementing reactions in which further growth of fused cells occurred only in the combination of an A^+ doubly marked mutant and its original parent on MMH (Table 4), demonstrating that the self-anastomoses microscopically observed in *A. alternata* were fully perfect fusion and the nonself-anastomoses were imperfect or contact fusion. This is similar to the hyphal anastomosis of *R. solani*, which microscopically showed perfect fusion between the cells of the same isolate and imperfect or contact fusion between the cells of different isolates belonging to the same anastomosis group (Ogoshi, 1972).

The lack of complementation between the doubly marked mutants and A^+ or A^- nonparent wild-type isolates, or between the mutants and their A^- parent wild-type isolates clearly indicates that vegetative (somatic or heterokaryon) incompatibility is present in the population of *A. alternata*, although its ecological significance is not yet understood. Vegetative incompatibility is common in many fungal species. In some Basidiomycetes, anastomosis was not blocked between incompatible hyphae, but fusion cells were subsequently isolated and deteriorated (Rayner and Todd, 1979). Transmission of myco-viruses to a strain of *Penicillium stoloniferum* Thom occurred through heterokaryonsis (Lhoas, 1971). The adaptive significance of vegetative incompatibility may be to limit the spread of viruses (Caten, 1972) or maladapted nuclei (Hartl et al., 1975) through a fungal population, or more generally to reduce risks of genetical or physical take over or disharmony (Rayner, 1991) by maintaining the integrity of fungal individuals (Rayner et al., 1984).

Anastomosis of *R. solani* was suggested to be genetically controlled (Kuninaga, 1980). It is clear that anastomosis of *A. alternata* is also genetically controlled, based on the following facts: 1) the offspring (conidia) from an A^+ or A^- isolate generally kept the same self-anastomosing ability as the parent; 2) doubly marked mutants derived from the A^+ isolates had the ability to self-anastomose, while doubly marked mutants derived from the A^- isolates lacked the ability; and 3) cultural conditions did not affect the inability to self-anastomose in the A^- isolate (T-5).

Asexual recombination is an important mechanism of generating new genotypes (variants) for an imperfect fungus. Although the parasexulity in *A. alternata* remains to be confirmed, due to the existence of vegetative incompatibility in this fungus as described above, the potential production of heterokaryons and asexual recombinants between individuals of the fungus may be limited to certain combinants with the same A^+ original parents or the same genetic loci governing the process of perfect fusion. Perfect fusion should be studied in detail to gain a better understanding of the genetic diversity of *A. alternata*, because it is a prerequisite for establishment of heterokaryons between individuals of the fungus. Heterokaryons were formed between auxotrophic mutants of the Japanese pear pathotype and the apple pathotype of *A. alternata* (Tsuge et al., 1987). Although complementing reactions between each doubly marked mutant and its nonparent wild-type isolate could not be observed in the present work, this does not exclude the possibility that complementation might be found between an A^+ doubly marked mutant and a field isolate of *A. alternata* if the sampling numbers were increased.

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