

Fusarium oxysporum 860926aを抗原としたモノクローナル抗体の作製とその性状

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Production and Partial Characterization of Monoclonal Antibodies against *Fusarium oxysporum* 860926a*

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Abstract

Stable hybridoma cell lines secreting monoclonal antibodies (MoAbs) to *Fusarium oxysporum* (860926a) were produced by fusing spleen cells from immunized BALB/c mice with SP2 myeloma cells. Antibody production by the cell lines, API9-2 and API9-3, was assayed by dot immunobinding assay (DIBA). These MoAbs showed positive reactions with 11 formae speciales (ff. sp.) of *F. oxysporum*, *Gibberella fujikuroi* (*F. moniliforme*), *G. zeae* (*F. roseum*), as well as with *Nectria haematococca* (*F. solani*). Whereas the MoAbs did not react with 35 plant pathogenic fungi, not classified in *Fusarium* spp. except *N. solani* (*Thielaviopsis basicola*). Thus these hybridoma-derived antibodies are considered to be specific to the genus *Fusarium*.

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Key words: monoclonal antibody (MoAb), *Fusarium oxysporum*, *Fusarium*-specific antibody.

INTRODUCTION

An integrated pathogen management strategy for crop protection and production seeks to achieve maximum yields while minimizing crop damage, cost of grower, and environmental hazards¹⁴⁾. An effective pathogen management strategy must include the accurate, timely and specific detection of pests and pathogens¹⁴⁾.

Soilborne disease brings about severe damage to agricultural production because the pathogen is difficult to control with fungicides or any other methods. In order to protect crop plants from soilborne disease, it is a first requisite to diagnose as early as possible if the nursery seedlings or field soils are infested with the pathogens and, next, to establish effective measures to control¹⁵⁾.

For the detection of some soilborne plant pathogens, several methods have been reported with selective medium¹⁰⁾ or indicator plant¹⁰⁾. But they are not efficient enough because of the difficulty or the consuming long period for the test¹⁰⁾.

Recent advances in immunology are being applied to the development of rapid, specific, and sensitive tools for the detection of plant pathogens^{12,14,15)}, especially for plant viruses. In soilborne plant pathogenic fungi, works on *Ophiobolus graminis*⁴⁾, *Verticillium dahliae*⁸⁾ and species in *Phytophthora*¹³⁾ showed that immunofluorescent assay (IFA) could be used for these

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fungi recognition. El-Nashaar *et al.*⁶⁾ quantified *Gaeumannomyces graminis* associated with wheat roots by enzyme linked immunosorbent assay (ELISA). Arie *et al.*¹⁾ and Lange *et al.*¹¹⁾ reported that resting spores of *Plasmodiophora brassicae* (pathogen of clubroot disease of cruciferous plants) were detectable with polyclonal antibodies against the pathogen, by IFA in soil and plant tissues, and by dot immunobinding assay (DIBA) in plant tissues, respectively.

Fusarium oxysporum is one of the most important soilborne pathogens and brings about severe damage to many kinds of vegetables, flowers and trees. In order to control *Fusarium* diseases, for example by biocontrol with *Pseudomonas gladioli* M-2196³⁾, effective detection method to diagnose soil and nursery seedlings has been demanded. Iannelli *et al.*⁹⁾ produced monoclonal antibody (MoAb) against *F. oxysporum* and showed its specificity.

This paper deals with preparation of genus specific monoclonal antibodies to establish the immunochemical method for the detection of *Fusarium* spp., and their characteristics.

MATERIALS AND METHODS

Preparation of antigen. *Fusarium oxysporum* 860926a¹⁶⁾ was incubated in potato dextrose broth (PDB) medium at 27°C for about 10 days. The mycelia formed were collected by centrifugation (3,000 rpm, 15 min, 20RPR rotor: Hitachi Co.), washed with distilled water, homogenized in liquid nitrogen with Waring blender (11,000 rpm, 5 min), and suspended in phosphate buffered saline, pH 7.0 (PBS). This fungal preparation (0.1 mg protein/ml) was used as antigen⁵⁾.

Hybridoma production. Female BALB/c mice were immunized intraperitoneally on day 0 with 50 μ l of antigen emulsified in Freund's complete adjuvant, followed by 10 additional immunizations with 50 μ l of antigen in Freund's incomplete adjuvant on days 31, 46, 61, 74, 87, 102, 116, 130, 152 and 190. Finally, 100 μ l of antigen was administered intravenously 3 days before cell fusion. Immune spleen cells from immunized mice were fused with SP2/O-Ag14 (SP2) myeloma cells at a ratio of 5:1, respectively, in the presence of 50% polyethylene glycol 4,000 (Merck) in serum-free RPMI1640 medium (Nissui Pharmaceutical Co.)^{3,7,17,18)}. The cells were then seeded in HAT medium³⁾ and incubated at 37°C in air containing 5% CO₂ in a humidified incubator.

Production of monoclonal antibodies. The culture supernatants were assayed by DIBA^{3,7)}. The selected hybridomas were cloned by limiting dilution method for two times. The hybridoma clones selected were cultured in RPMI1640 medium containing 10% (v/v) foetal bovine serum (FCS: Flow Lab. Co. or IS Japan Co.), and the supernatant fluids were obtained as solutions containing the monoclonal antibody (MoAb).

Assay for the immunological properties of the MoAbs. The Ig class and subclass of the MoAbs were determined with Monoclonal mouse immunoglobulin isotyping kit (PharMingen Co.) by ELISA³⁾. The reaction specificity of the MoAbs against some species of plant pathogenic fungi was also investigated by DIBA.

RESULTS AND DISCUSSION

Production of MoAbs by hybridoma cells

BALB/c mice were immunized by mycelial preparation of the isolate and their spleen cells were fused with myeloma cells. By DIBA, 2 stable hybridoma clones, API9-2 and API9-3, were obtained as positive ones which produce MoAbs against *F. oxysporum* 860926a. These hybridoma clones were incubated in 10% FCS-RPMI1640 under 37°C and 5% CO₂ condition, and the supernatant fluids containing MoAb were obtained. The titer of the supernatant fluids was determined by DIBA, and the reactions with the antigen were positive up to 10 times dilution.

Isotype and reaction specificity of the MoAbs

The isotype of the MoAbs was assayed with monoclonal mouse immunoglobulin isotyping kit by ELISA, and the result was shown in Table 1. As the MoAbs reacted with anti IgM antibody and anti kappa light chain antibody (IgL(κ)), the immunoglobulin produced by these clones was determined to be IgM(κ). API9-2 and API9-3 reacted with every 11 formae speciales of *F. oxysporum* tested (Table 2). They also showed positive reaction with *Gibberella fujikuroi* (*F. moniliforme*), *G. zeae* (*F. roseum*), *Nectria haematococca* (*F. solani*) and *N. solani* (*Thiel-*

Table 1. Reaction of the MoAbs to tested mouse immunoglobulin isotype-specific antibodies ^{a)}

Isotype-specific antibodies	Reaction ($A_{405/600}$)		
	API9-2	API9-3	Control
Anti IgG ₁	0.098	0.057	0.034
Anti IgG _{2a}	0.077	0.049	0.034
Anti IgG _{2b}	0.065	0.041	0.031
Anti IgG ₃	0.075	0.041	0.035
Anti IgM	2.589	2.076	0.034
Anti IgA	0.067	0.042	0.036
Anti IgL(κ)	1.002	1.157	0.034
Anti IgL(λ)	0.077	0.042	0.035

a) Monoclonal mouse immunoglobulin isotyping kit (PharMingen Co.) was used (ELISA).

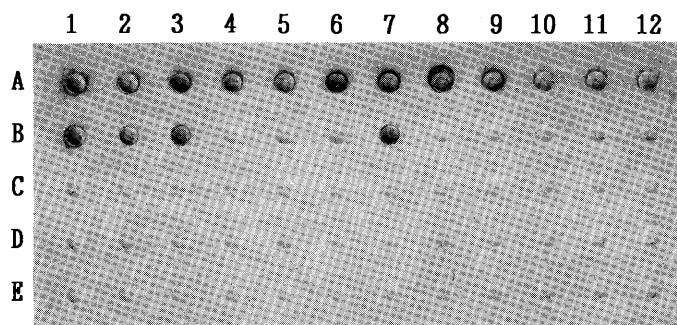


Fig. 1. DIBA of tested fungi with MoAb API9-2. A1: *F. oxysporum* 860926a, A2: *f. sp. lycopersici* race J2, A3: *f. sp. radicle-lycopersici*, A4: *f. sp. lagenariae*, A5: *f. sp. conglutinans*, A6: *f. sp. batatas*, A7: *f. sp. fragariae*, A8: *f. sp. raphani*, A9: *f. sp. spinaciae*, A10: *F. oxysporum* 891003a-1, A11: *f. sp. cucumerinum*, A12: *f. sp. lini*, B1: *Gibberella zeae* (*F. roseum*), B2: *G. fujikuroi* (*F. moniliforme*), B3: *Nectria haematococca* (*F. solani*), B4: *N. gliocladioides* (*Gliocladium roseum*), B5: *Rhizoctonia solani*, B6: *R. solani*, B7: *N. solani* (*Thielaviopsis basicola*), B8: *Verticillium dahliae*, B9: *V. dahliae*, B10: *Corticium rolfsii*, B11: *C. rolfsii*, B12: *C. rolfsii*, C1: *Cochliobolus heterostrophus*, C2: *C. heterostrophus*, C3: *Colletotrichum lagenarium*, C4: *Colletotrichum fragariae*, C5: *Pythium aphanidermatum*, C6: *Monosporascus cannonbollus*, C7: *Lepista sordida*, C8: *Botrytis cinerea*, C9: *Sclerotinia* sp., C10: *S. sclerotiorum*, C11: *Sclerotium cepivorum*, C12: *Phytophthora* sp., D1: *Pyricularia oryzae*, D2: *Diaporthe citri*, D3: *Alternaria mali*, D4: *A. kikuchiana*, D5: *A. bataticola*, D6: *Aspergillus niger*, D7: *Cladosporium fulvum*, D8: *Penicillium simplicissimum*, D9: *Penicillium* sp., D10: *Colletotrichum lindemuthianum*, D11: *Glomerella cingulata*, D12: *Gloeosporium laeticolor*, E1: *Gloeosporium kaki*, E2: *Ceratocystis fimbriata*, E3: *Colletotrichum lagenarium*, E4: *Plasmodiophora brassicae*, E5: *Cochliobolus miyabeanus*, E6: *Valsa ceratosperma*, E7-12: TBST (for negative control); A1-12, B1-3 (*Fusarium* spp.) and B7 showed positive reaction (blue), but others were negative (not blue).

Table 2. Reactivity of the MoAbs to *Fusarium oxysporum* by DIBA

Tested f. sp.	Isolate No.	Reaction by DIBA	
		API9-2	API9-3
<i>lycopersici</i> J1	NIAES5121	+	+
<i>lycopersici</i> J2	880621a-1	+	+
<i>radicis-lycopersici</i>	860320a-1	+	+
	860926a b)	+	+
<i>apii</i>	ATCC15636 c)	+	+
<i>apii</i>	ATCC18142 c)	+	+
<i>lagenariae</i>	0091	+	+
<i>cucumerinum</i>		+	+
<i>conglutinans</i>	851108a-1	+	+
<i>conglutinans</i>	880120h	+	+
<i>batatas</i>	860809c	+	+
<i>fragariae</i>	880531c	+	+
<i>raphani</i>	860807b	+	+
<i>spinaciae</i>	860926b	+	+
	891003a-1 d)	+	+
<i>lini</i>		+	+

a) Positive.

b) Isolated from Japanese honeysuckle¹⁶⁾, but the pathogenic form (f. sp.) has not been determined; Antigen of MoAbs, API9-2 and API9-3.

c) Fungal preparations were stocked under -80°C for about 2 years.

d) Isolated from chrysanthemum (*Chrysanthemum morifolium* Ramat.), but the pathogenic form has not been determined.

viopsis basicola), but did not react with other 35 species of fungi such as *N. gliocladioides* (*Gliocladium roseum*), *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Sclerotinia* sp., *Sclerotium cepivorum*, *Verticillium dahliae*, *Phytophthora* spp., *Pythium* sp., *Rhizoctonia solani* and *Plasmodiophora brassicae* (Table 3 and Fig. 1).

From the above results, the MoAbs, API9-2 and API9-3, appear to be *Fusarium*-specific, except *N. solani*, although they did not have enough forma-specificity in the species, *F. oxysporum*. Iannelli *et al.*⁹⁾ reported that a forma-specific MoAb was obtained when intact spores were used as antigen. In this study, in order to screen antibody against every epitope of antigen, we used a mixture of the spores and homogenized mycelia of *F. oxysporum* as a source of antigen. The result suggested that *Fusarium* spp. (and also *N. solani*) share common antigenic substances. Such common antigenic substances appear interesting to be analyzed immunologically, chemically and pathogenically.

Since the MoAbs obtained in this study are mostly genus specific for *Fusarium* spp., they may be usable for detection of *Fusarium* spp. in infected plant tissues or soil by IFA, ELISA or DIBA. Further studies are in progress.

For the more detailed detection of pathogenic form (f. sp.) of *F. oxysporum* in soil or plant tissue, we must prepare a specific MoAb for the each form. As mentioned above, forma-specific MoAb may be obtained with intact spores as antigen. Further research will be required to apply any fractions of *F. oxysporum* as antigen.

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Table 3. Reactivity of the MoAbs to fungi other than *Fusarium oxysporum* by DIBA

Tested fungi	Isolate No.	Reaction by DIBA	
		API9-2	API9-3
<i>Gibberella fujikuroi</i> (<i>Fusarium moniliforme</i>)	GF-1	+	+
<i>G. zeae</i> (<i>F. roseum</i>)		+	+
<i>Nectria haematococca</i> (<i>F. solani</i>)	880701c-1	+	+
<i>N. solani</i> (<i>Thielaviopsis basicola</i>)	NIAES 5215	+	+
<i>N. gliocladioides</i> (<i>Gliocladium roseum</i>)	850511	—	—
<i>Botrytis cinerea</i>	BC-A	—	—
<i>Sclerotinia sclerotiorum</i>	880323c-1	—	—
<i>S. sclerotiorum</i>	901220a-1	—	—
<i>S. sclerotiorum</i>	CHSS1	—	—
<i>Sclerotinia</i> sp.	880120a	—	—
<i>Sclerotium cepivorum</i>	303	—	—
<i>Pyricularia oryzae</i>	NIAES 5001	—	—
<i>Verticillium dahliae</i>	CH01	—	—
<i>V. dahliae</i>	880324a-1	—	—
<i>Colletotrichum lagenarium</i>	Cu101113	—	—
<i>Col. fragariae</i>	Nirayama1	—	—
<i>Col. fragariae</i>	Nirayama2	—	—
<i>Col. lindemuthianum</i>		—	—
<i>Gloeosporium laeticolor</i>		—	—
<i>Gloe. kaki</i>		—	—
<i>Glomerella cingulata</i>		—	—
<i>Cochliobolus heterostrophus</i>	Kagome86	—	—
<i>Coc. heterostrophus</i>	BMZM	—	—
<i>Coc. miyabeanus</i>		—	—
<i>Nakataea sigmoidea</i>		—	—
<i>Valsa ceratosperma</i>		—	—
<i>Diaporthe citri</i>		—	—
<i>Ceratocystis fimbriata</i>		—	—
<i>Cladosporium fulvum</i>		—	—
<i>Penicillium simplicissimum</i>	8	—	—
<i>Penicillium</i> sp.		—	—
<i>Aspergillus niger</i>		—	—
<i>Alternaria mali</i>		—	—
<i>Alt. kikuchiana</i>		—	—
<i>Alt. bataticola</i>		—	—
<i>Rhizoctonia solani</i>	880607	—	—
<i>Corticium rolfii</i>	860809a	—	—
<i>Cor. rolfii</i>	861107a	—	—
<i>Cor. rolfii</i>	860811a	—	—
<i>Lepista sordida</i>	880323d	—	—
<i>Phytophthora cryptogea</i>	VE-G-1	—	—
<i>Phytophthora infestans</i>	TK-II	—	—
<i>Phytophthora</i> sp.		—	—
<i>Pythium aphanidermatum</i>	K9001	—	—
<i>Pythium</i> sp.	K9002	—	—
<i>Plasmodiophora brassicae</i> ^{c)}		—	—

a) Positive.

b) Negative.

c) Solution of resting spores of *P. brassicae* from Chinese cabbage (*Brassica campestris* L. *pekinensis* group) in Oyama-shi, Tochigi Prefecture, Japan.

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和 文 摘 要

有江 力・林 義雄・長谷あきら・古谷雅樹・山口 勇: *Fusarium oxysporum* 860926a を抗原としたモノクローナル抗体の作製とその性状

ミツバ株枯病菌 (*Fusarium oxysporum* 860926a) を抗原として、モノクローナル抗体 API9-2 および API9-3 を得た。これらのモノクローナル抗体は、抗原である *F. oxysporum* 860926a をはじめとして *F. oxysporum* の試験した 11 のすべての分化型、*Gibberella fujikuroi* (*F. moniliforme*), *G. zeae* (*F. roseum*), *Nectria haematococca* (*F. solani*) および *N. solani* (*Thielaviopsis basicola*) と強い陽性反応を示した。供試したこれ以外の 35 種の植物病原菌とは、反応がみられなかった。このように、本モノクローナル抗体はほぼ *Fusarium* 属菌に特異的に反応した。