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Masao Arakawa · Hitoshi Nakamura · Yukari Uetake
Naoyuki Matsumoto

Presence and distribution of double-stranded RNA elements in the white root rot fungus *Rosellinia necatrix*

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Abstract Double-stranded (ds)RNA of various types was detected in 65 (21.8%) of 298 isolates from vegetative hyphae of *Rosellinia necatrix* by electrophoresis, but dsRNA was not detected from 39 ascosporic isolates. There were 45 distinct dsRNA profiles in the 65 isolates: they varied in the number of electrophoretic bands from 1 to 12 and in size from less than 1000 bp to more than 10 kbp. Each dsRNA profile was unique to each locality. dsRNAs having the same profiles were restricted to isolates of the same mycelial compatibility groups (MCG) from the same trees, with an exception where different profiles were detected in different isolates of the same MCGs.

Key words double-stranded RNA (dsRNA) · Mycelial compatibility group (MCG) · *Rosellinia necatrix* · White root rot

Introduction

White root rot, caused by *Rosellinia necatrix* Prillieux, is one of the most serious diseases of fruit trees such as Japanese pear and apple. The causal fungus attacks a wide range of plants belonging to 197 species in 50 families (Ito and Nakamura 1984). Diseased plants show yellowing of leaves, white plumose mycelia on the surface of decayed roots, and fan-shaped mycelial strands under the bark. Plants are killed when the infected area girdles the whole trunk base. Consequently, a large amount of fungicides is soil-drenched to individual trees to control the disease (Arai et al. 1989; Fukushima 1998; Kanadani et al. 1998). Chemical control is effective; however, concerns about environmental pollution demand the development of alternative methods. Recently, a novel biocontrol attempt using the hypovirulence factor against root diseases of fruit trees has been proposed (Matsumoto 1998). Fungal double-stranded (ds)RNA elements, which are known as hypovirulence factors, have been the subject of considerable research interest because of their potential as biocontrol agents against plant pathogenic fungi (Elliston 1982; Zhou and Boland 1998).

Circumstantial evidence (H. Nakamura, unpublished data) suggests that the white root rot fungus spreads clonally through root contact between host plants and develops mycelial networks in patches. dsRNA is considered to spread within the mycelial network. In this article, isolates of the white root rot fungus were collected from many plants from a wide geographic range in Japan and Australia, and the diversity of dsRNA elements was investigated. We also compared distribution patterns of dsRNA elements in terms of population structure of the pathogenic fungus in the orchard.

M. Arakawa[1] · H. Nakamura · Y. Uetake[2]
Bio-oriented Technology Research Advancement Institution, National Institute for Agro-Environmental Sciences, Tsukuba, Japan

N. Matsumoto (✉)
National Institute for Agro-Environmental Sciences, 3-1-3 Kan-non dai, Tsukuba 305-8604, Japan
Tel. +81-298-38-8267; Fax +81-298-38-8267
e-mail: nowmat@niaes.affrc.go.jp

Present address:
[1] Faculty of Agriculture, Meijo University, Shiogamaguchi, Tenpaku, Nagoya 468-8502, Japan
[2] Bio-oriented Technology Research Advancement Institution, National Institute of Livestock and Grassland Science, Nishinasuno, Tochigi 329-2793, Japan

Materials and methods

Fungal isolates

Two hundred and ninety-eight isolates of *R. necatrix* were obtained from vegetative hyphae of fan-shaped mycelial strands, from infected sapwood of diverse plants belonging to 27 species and unidentified dead shrubs, and from soil by the baiting method with mulberry twigs (Table 1). Thirty-nine ascosporic isolates were also used. Pure cultures were obtained by using 1.5% water agar (WA) containing 200 μg/ml of streptomycin sulfate. Agar plates were incubated in

Table 1. Numbers of isolates of *Rosellinia necatrix* examined

Criteria	Detail of items (number of isolates)
Locality	26 prefectures in Japan (335), Australia (2)
Host plant	27 species in 24 genera (257), 24 unidentified[a] (45) and baited from soil[b] (35)
Land use	Cultivated land (269), uncultivated land (68)
Source	Mycelium (298), ascospore (39)
MCG	174 MCGs of 337 isolates
dsRNA	65 of 337 isolates

MCG, mycelial compatibility group
[a] Host species were not identified because specimens were badly decayed
[b] Baited with dried twigs of *Pyrus pyrifolia* or *Morus alba* in agricultural fields

the dark at 25°C for 1–7 days. Single hyphae were removed from the colony margin and transferred to Difco oatmeal agar (OA; Difco, Detroit, MI, USA) slants. Cultures were maintained at 25°C, and duplicate stock cultures were stored at 10°C.

Determination of mycelial compatibility group

Mycelial compatibility groups (MCGs) were distinguished by the presence of a demarcation line along the colony junction in paired cultures. Small agar blocks with mycelia were cut from the colony margin of OA plate cultures. Six blocks were inoculated at equal distance around the edge of 9-cm OA plates and one was inoculated in the center. Plates were incubated at 25°C for 2 weeks. When a dark demarcation line such as is usually associated with the production of pseudosclerotia was observed between colonies, the two isolates involved were regarded as different MCGs.

Detection of dsRNA elements

Each isolate was pregrown on OA plates for 1 week at 25°C in the dark, and small blocks with mycelia were cut from the colony and placed in the center of a cellulose membrane overlaid on 5-cm potato-dextrose agar plates. After incubation for 1 week, the cellulose membrane with mycelia was stripped from the plates and ground to a fine powder in liquid nitrogen using a mortar and pestle. About 100 mg of mycelial powder, containing the cellulose membrane, was suspended in 750 µl extraction buffer [0.1 M Tris-HCl, 0.1 M NaCl, 1% sodium dodecyl sulfate (SDS), 0.1% β-mercaptoethanol, pH 8.0] and centrifuged at 15,000*g* for 10 min, 20°C. The supernatant was collected, mixed with an equal volume of PCI solution (TE-saturated phenol:chloroform:isoamylalchol, 25: 24:1, v/v) in a 1.5-ml microtube, and centrifuged at 10,000*g* for 10 min at 20°C. The PCI treatment was repeated several times until the middle layer containing protein disappeared. The aqueous phase was mixed gently with 40 µl 5 M NaCl and 600 µl 2-propanol in a new microtube and stored at 4°C for at least 1 h. Total nucleic acids were collected by centrifugation at 20,000*g* for 15 min at 4°C and the pellet was dissolved in 150 µl nuclease reaction buffer [50 units of DNase (DNase I, Takara, Ohtsu, Japan), 20 units of S1 nuclease (Takara), 30 mM sodium acetate, 5 mM $MgCl_2$, 100 mM NaCl, 1 mM $ZnSO_4$]. DNA and single-stranded nucleic acids in the solution were completely digested after overnight incubation at 37°C. After subsequent PCI treatment, 0.1 volume of 3 M sodium acetate and 2.5 volume of ethanol were added to the microtube, and the sample was precipitated by centrifugation at 20,000*g* for 15 min at 4°C. The pellet was dissolved in 30 µl TE buffer [10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0] and stored at −20°C as the dsRNA fraction.

Samples were separated by electrophoresis on 1.5% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) or 5.0% polyacrylamide gel in TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.0) and stained with ethidium bromide; fragments of dsRNA were visualized under UV irradiation. In preliminary experiments, electrophoresis was conducted before and after the nuclease treatment to confirm that signals of DNA and rRNA in samples were digested completely. Isolates containing dsRNA were denoted as dsRNA positive. dsRNA genome segments of rice dwarf virus given by Dr. T. Ohmura, National Agriculture Research Center, were used as dsRNA markers in electrophoresis to quantify band size.

Results

dsRNA elements detected in *R. necatrix*

dsRNA elements were detected in 65 of 298 (21.8%) isolates from vegetative hyphae, consisting of 136 MCGs. In most cases, no clear difference was evident in colony morphology between dsRNA-positive and dsRNA-negative isolates. Various patterns of dsRNA were observed (Fig. 1), and consequently 45 distinct dsRNA profiles were found within the 65 isolates (Table 2). These profiles were divided into five types (Fig. 1); (a) one or two bands of about 10 kbp, (b) one or four bands in the size range of 3.5–4.5 kbp, (c) more than 10 bands of less than 4.5 kbp, (d) one or more bands in the size range of 1.5–2.5 kbp, and (e) other dsRNA patterns made up of combinations of the above four patterns.

Of our isolates, 269 originated from cultivated land and 68 from uncultivated lands; 56 (20.8%) dsRNA-positive isolates were obtained from the former and 9 (13.2%) from the latter. dsRNA was not detected in a total of 39 ascosporic isolates, originating from eight distinct stroma samples. A stroma sample whose mycelium produced a dsRNA-positive isolate had no dsRNA-positive ascosporic isolates (5 isolates were examined).

Distribution of dsRNA in field populations of *R. necatrix*

The distribution of dsRNA elements in four orchards of Japanese pear is summarized in Table 3 in terms of the population structure of the white root rot fungus. Each dsRNA profile was unique to each orchard, and dsRNAs

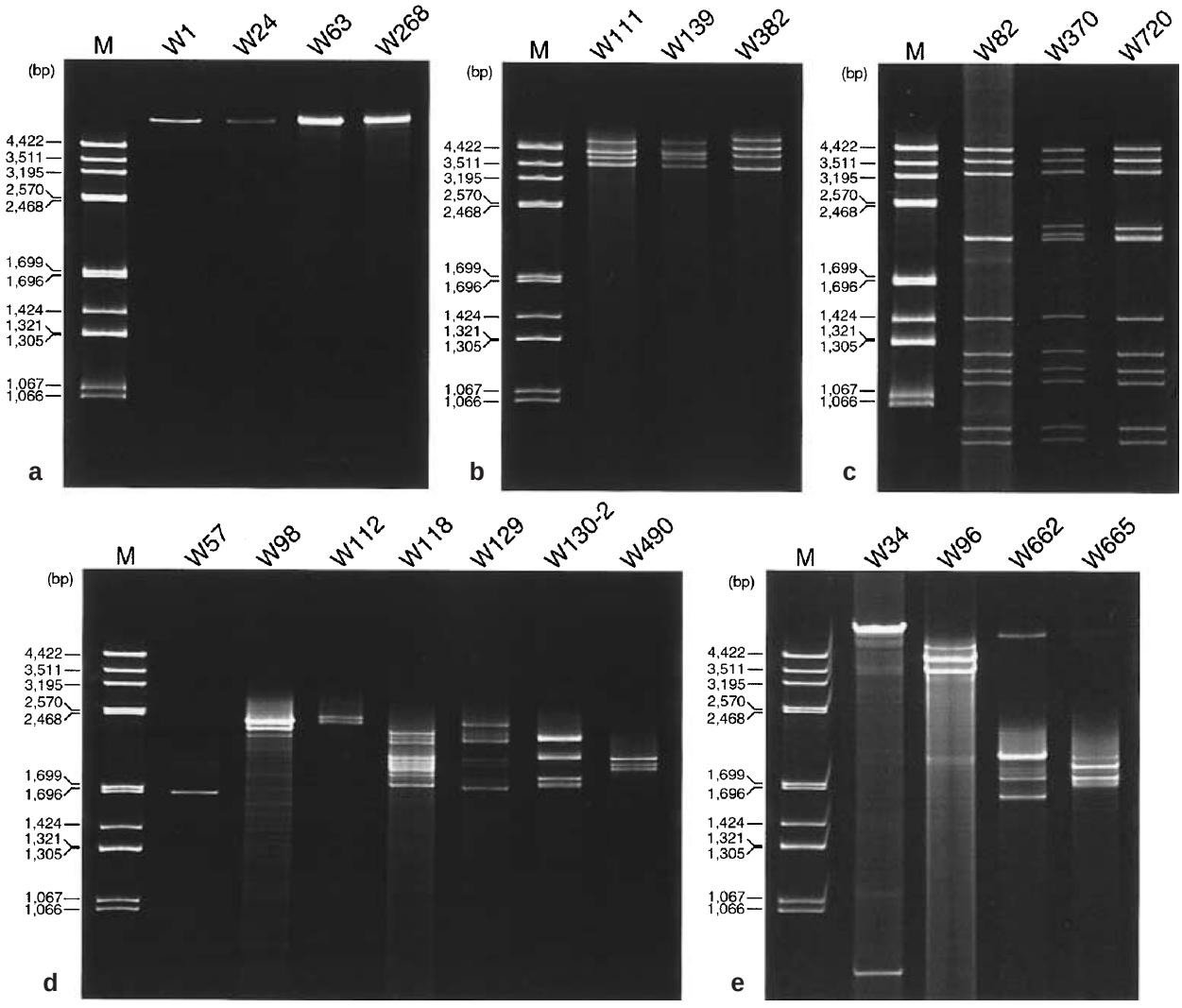

Fig. 1. Double-stranded RNA (dsRNA) types based on electrophoretic mobility found in isolates of *Rosellinia necatrix*. *Isolate number* is shown above each lane. *M*, genome segments of rice dwarf virus (RDV) as size marker. **a** One or two bands of about 10 kbp. **b** One or four bands in the size range of 3.5–4.5 kbp. **c** More than 10 bands of less than 4.5 kbp. **d** One or more bands in the size range of 1.5–2.5 kbp. **e** Combination of all four patterns

having the same profile were found exclusively in isolates from the same trees, i.e., A-I, A-VIII, B-II, and D-I. An exception was C-II, which produced three different dsRNA profiles in three isolates. The distribution of each dsRNA was mostly restricted within an MCG. For example, dsRNA profile B was detected from isolates W98, W99, W101, and W102 of MCG 80 but not from other MCGs. However, different dsRNA profiles were observed occasionally in the identical MCGs from the same tree (dsRNA profiles H, I, and J from MCG 88 on tree C-II) and from different trees (dsRNA profiles E and F from MCG 85 on trees B-I and II and dsRNA profiles K and L from MCG 90 on trees D-I and II). MCGs 139 and 302 also had different dsRNAs (see Table 2). The same dsRNA profiles were never found from isolates belonging to different MCGs.

Discussion

dsRNA showing various patterns of electrophoretic mobility was detected in isolates of the white root rot fungus. Genetic elements of dsRNA have been found in many fungi (Nuss and Koltin 1990; Hong et al. 1998). These elements are mostly encapsulated into virus-like particles (Buck 1986). We have not examined if dsRNAs detected in *R. necatrix* originated from mycovirus. Fungal viruses are characterized to the family level by their genome size and segment patterns of dsRNA (Buck 1998). Some patterns of dsRNA bands detected in *R. necatrix* isolates corresponded to those of dsRNA virus. The determination of nucleotide sequence and the observation of viral particles are further required for identification and to reveal diversity in dsRNA in *R. necatrix*.

Mycoviruses and related dsRNA genetic elements are frequently found associated with fungi and are often associ-

Table 2. List of 45 representative isolates[a] with dsRNA

Fungal isolate	Locality	Host plant	MCG	dsRNA type (number of fragments)
W1	Okayama	*Vitis vinifera*	1	I (1)
W8	Okayama	*Vitis vinifera*	5	V (3)
W24	Tottori	Unidentified[b]	16	I (1)
W27	Gunma	*Morus alba*	18	I (1)
W34	Ibaraki	*Pyrus pyrifolia*	31	V (3)
W57	Iwate	*Morus alba*	54	IV (1)
W63	Kagoshima	*Morus alba*	60	I (2)
W71	Kagawa	*Citrus* sp.	68	I (1)
W82	Saga	*Pyrus pyrifolia*	78	III (10)
W96	Saga	*Pyrus pyrifolia*	82	V (4)
W98	Saga	*Pyrus pyrifolia*	80	IV (3)
W111	Saga	*Pyrus pyrifolia*	85	II (4)
W112	Saga	*Pyrus pyrifolia*	85	IV (2)
W118	Saga	*Pyrus pyrifolia*	86	IV (9)
W124	Fukuoka	*Pyrus pyrifolia*	87	IV (1)
W129	Fukuoka	*Pyrus pyrifolia*	88	IV (5)
W130-1	Fukuoka	*Pyrus pyrifolia*	88	IV (3)
W130-2	Fukuoka	*Pyrus pyrifolia*	88	IV (4)
W133	Fukuoka	*Pyrus pyrifolia*	90	IV (1)
W139	Fukuoka	*Pyrus pyrifolia*	90	II (4)
W242	Chiba	*Eriobotrya joponica*	106	IV (2)
W262	Chiba	*Eriobotrya joponica*	115	I (1)
W267	Chiba	*Eriobotrya joponica*	117	I (1)
W268	Australia	*Malus domestica*	118	I (2)
W287	Tottori	*Pyrus pyrifolia*	130	I (1)
W302	Hiroshima	Soil[c]	133	I (1)
W333	Hiroshima	Soil[c]	139	IV (2)
W340	Hiroshima	Soil[c]	141	IV (1)
W343	Hiroshima	Soil[c]	139	IV (1)
W349	Hiroshima	Soil[c]	143	IV (2)
W369	Hiroshima	*Pyrus pyrifolia*	139	IV (1)
W370	Hiroshima	*Pyrus pyrifolia*	139	III (12)
W382	Hiroshima	*Pyrus pyrifolia*	139	II (4)
W392	Hiroshima	*Vitis vinifera*	136	IV (1)
W422	Mie	*Pyrus pyrifolia*	158	IV (4)
W490[d]	Chiba	*Quercus* sp.	212	IV (3)
W528[d]	Tochigi	*Lindera umbellata*	244	IV (2)
W530[d]	Chiba	Unidentified[b]	261	IV (2)
W567[d]	Chiba	Unidentified[b]	264	V (2)
W584[d]	Ibaraki	Unidentified[b]	275	II (1)
W659[d]	Tokyo	*Dendropanax trifidus*	299	IV (1)
W662	Gunma	*Malus domestica*	301	V (4)
W665	Gunma	*Malus domestica*	302	V (5)
W666	Gunma	*Malus domestica*	302	II (1)
W720	Saga	*Pyrus pyrifolia*	313	III (11)

MCG, mycelial compatibility group
[a] Isolates with the same dsRNA patterns based on electrophoresis were represented by one isolate
[b] Host species were not identified because specimens were badly decayed
[c] Baited with dried twigs of *P. pyrifolia* in a Japanese pear orchard
[d] Isolates obtained from nonagricultural lands

ated with fungal phenotypes (Nuss and Koltin 1990). However, not all dsRNAs found in a fungus alter the fungal phenotype. In *Cryphonectira parasitica*, a 12-kb segment of dsRNA present in 25% of the isolates recovered from virulent cankers was associated with neither hypovirulence nor altered morphology (Enebak et al. 1994). Similarly, no general correlation between the presence of dsRNA and virulence was reported in *Rhizoctonia solani* (Bharathan and Tavantzis 1990, 1991; Kousik et al. 1994), although specific segments of dsRNA were considered to be associated with hypovirulence (Bharathan and Tavantzis 1990). No relationship between the presence or absence of dsRNA and colony morphology was evident in *R. necatrix* (data not shown).

Rosellinia necatrix spreads mycelia and extends its domain through root contact between plants (H. Nakamura, unpublished data), and dsRNA translocates with mycelial growth (Buck 1998). These facts agree with the observations that the same dsRNAs existed in the same MCGs but not in different MCGs. dsRNAs in the white root rot fungus are, therefore, considered not to prevail beyond the barrier of MCG in nature.

Irregular distribution within isolates of the same MCGs seems to be unique to *R. necatrix*; there were both dsRNA-

Table 3. Distribution of dsRNA within populations of *R. necatrix* in orchards of Japanese pear[a]

Fungal isolate	MCG	Host tree[b]	dsRNA profile[c]	Fungal isolate	MCG	Host tree[b]	dsRNA profile[c]
Orchard A				Orchard B			
W81	78	A-I	–	W110	85	B-I	–
W82	78	A-I	A[d]	W111	85	B-I	E[d]
W83	78	A-I	–	W112	85	B-II	F[d]
W712	78	A-I	–	W113	85	B-II	F
W713	78	A-I	A	W114	85	B-II	–
W714	78	A-I	–	W115	85	B-II	–
W715	78	A-I	–	W116	86	B-IV	–
W716	78	A-I	–	W117	86	B-IV	–
W718	78	A-I	A	W118	86	B-V	G[d]
W719	78	A-I	–	Orchard C			
W95	78	A-II	–	W125	88	C-I	–
W84	79	A-III	–	W127	88	C-I	–
W85	79	A-III	–	W128	88	C-I	–
W86	79	A-III	–	W129	88	C-II	H[d]
W87	79	A-III	–	W130–1	88	C-II	I
W88	79	A-IV	–	W130–2	88	C-II	J[d]
W722	79	A-V	–	W131	89	C-III	–
W89	80	A-VI	–	W132	89	C-III	–
W90	80	A-VI	–	Orchard D			
W97	80	A-VII	–	W133	90	D-I	K
W98	80	A-VIII	B[d]	W134	90	D-I	K
W99	80	A-VIII	B	W135	90	D-I	K
W100	80	A-VIII	–	W136	90	D-I	–
W101	80	A-VIII	B	W137	90	D-II	–
W102	80	A-VIII	B	W138	90	D-II	–
W91	81	A-IX	–	W139	90	D-II	L[d]
W92	81	A-IX	–	W140	91	D-III	–
W94	81	A-X	–	W141	91	D-III	–
W723	81	A-XI	–	W142	91	D-III	–
W724	81	A-XI	–				
W96	82	A-XII	C[d]				
W725	82	A-XIII	C				
W726	82	A-XIII	C				
W720	313	A-XIV	D[d]				
W721	313	A-XIV	–				

[a] Orchards A and B were located in Saga and orchards C and D in Fukuoka
[b] Individual host trees were distinguished by the code numbers
[c] dsRNAs with the same character were indistinguishable by electrophoretic profile
[d] dsRNAs shown in Fig. 1

positive and -negative isolates in isolates from the same trees and, moreover, different dsRNAs were found in five cases, i.e., MCGs 85, 88, 90, 139, and 302. In the former, all or a part of the dsRNA segments could have been eliminated on isolation or through transfer. dsRNA is considered to have coexisted in the fungal cytoplasm through the process of speciation of hosts (Ghabrial 1998), but host–dsRNA interaction in *R. necatrix* is considered to be incompatible in most cases. For example, vertical transmission of dsRNA into ascospores is unlikely to occur, and consequently sexual reproduction is effective to remove dsRNA in *R. necatrix* (M. Arakawa, unpublished data). Also, the percentage of isolates with dsRNA in *R. necatrix* was much lower than that of *Helicobasidium mompa*, in which vertical transmission was found to occur, and about 70% of isolates had dsRNA (M. Arakawa, unpublished data). In conclusion, host–dsRNA interaction is not stable in *R. necatrix*, and mechanisms of dsRNA infection and removal may operate in nature.

dsRNA elements were detected more frequently from isolates in agricultural lands than from those from uncultivated lands. Fifty-six (20.8%) dsRNA-positive isolates were obtained from 269 isolates collected from agricultural lands and 9 of 68 (13.2%) isolates obtained from uncultivated lands; this may be attributed to the difference in the mode of fungal spread between cultivated and uncultivated lands. The fungus produces stromata on the trunk base more than 2 years after infection (Teixeira de Sousa and Whalley 1991; Nakamura et al. 2000), and the present study revealed that isolates originating from ascospores were all dsRNA negative. The teleomorph functions as a mechanism to eliminate dsRNA in *R. necatrix* as in other fungi (Chun and Lee 1997; Nuss 1993). Because killed plants are readily removed by the farmer, there is not enough time for the fungus to develop stromata. The fungus propagates exclusively through vegetative growth under agricultural conditions, allowing infection and persistence of dsRNA.

Several methods were used to isolate dsRNA from *R. necatrix*. A method using cellulose column (Morris and Dodds 1979) is widely used but needs a large amount of material: 1–10 g fresh weight of mycelia. Molecular analyses require a large quantity of dsRNA and consequently need a

large amount of mycelia, whereas 50–100 mg of fresh mycelia was sufficient to confirm the presence or absence of dsRNA using the present method. This method minimizes both dsRNA extraction and enzyme treatment and enables us to examine many samples at one time.

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