Virus Genes 25:1, 101–107, 2002
© 2002 Kluwer Academic Publishers. Manufactured in The Netherlands.

Nucleotide Sequences of Double-Stranded RNA Segments from a Hypovirulent Strain of the White Root Rot Fungus *Rosellinia necatrix*: Possibility of the First Member of the *Reoviridae* from Fungus

HIDEKI OSAKI,[1*] CHUAN ZHAO WEI,[1] MASAO ARAKAWA,[2] TORU IWANAMI,[1] KINYA NOMURA,[1] NAOYUKI MATSUMOTO[2] & YOSHIHIRO OHTSU[1]

[1]*National Institute of Fruit Tree Science, Fujimoto, Tsukuba, 305-8605, Japan*
[2]*National Institute for Agro-Environmental Sciences, Kan-nondai, Tsukuba 305-8604, Japan*

Abstract. Twelve double-stranded (ds) RNA segments were detected from a hypovirulent strain W370 of the white root rot fungus *Rosellinia necatrix*. The estimated molecular weights ranged from 0.41×10^6 to 2.95×10^6. Full length cDNA clones for eight segments were obtained. Northern blot analysis suggested that each segment was genetically unique. The nucleotide sequences of eight full length dsRNA segments were determined. One long open reading frame was found in each segment. Conserved sequences at the 5′-end (5′-ACAAUUU-3′) and at the 3′-end (5′-UGCAGAC-3′) were identified in all eight segments. Segment-specific panhandle structures, formed by inverted terminal repeats, were also found in all segments. Comparative analyses of the predicted translational products of eight dsRNA segments showed that the deduced amino acid sequence partially matched those of the *Reoviridae* family members: Colorado tick fever virus, Nilaparvata lugens reovirus, and rice black streaked dwarf virus. The results suggested that W370 dsRNA is derived from a new member of the family *Reoviridae* detected in fungus.

Key words: dsRNA, hypovirulence, nucleotide sequence, *Rosellinia necatrix*, *Reoviridae*

Introduction

Rosellinia necatrix Prillieux is an ascomycetous fungus, which causes white root rot on a wide range of plants, including about 170 species in 63 genera and 30 families [1,2]. In spite of the serious damage to agricultural production, especially of fruit trees, there are few effective methods to control white root rot chemically or culturally.

Mycoviruses and double-stranded RNA (dsRNA) molecules have been observed in fungal isolates representing all major classes of fungi [3,4]. Although a large number of the viruses existing in plant pathogenic fungi are avirulent to the host fungi, it is becoming increasingly clear that some mycoviruses or certain dsRNA molecules are severely debilitating, and induce hypovirulence or hypervirulence of host fungi [4,5]. dsRNA is known as a hypovirulence factor of the chestnut blight fungus, *Cryphonectria parasitica* [6–8], and has been used as an effective biocontrol agent against the disease.

Similarly, we are trying to control white root rot by using dsRNAs or mycoviruses. About 20% of *R. necatrix* strains had dsRNA species [9]. One strain, W370, was found to be hypovirulent [10]. In this paper, we report twelve dsRNA segments detected from the hypovirulent strain W370 of *R. necatrix* and the eight nucleotide sequences out of the twelve dsRNA segments. The sequences showed similarities to those viruses in the family *Reoviridae*, which mainly infect invertebrate, vertebrate, and plant hosts, but not in fungal host. These data suggested that the dsRNA segments might have originated from a

*Author for all correspondence:
E-mail: hideki@affrc.go.jp

Reoviridae member. This is the first report describing possible mycovirus or dsRNA molecules found in *R. necatrix*.

Materials and Methods

R. necatrix *Strain*

Strain W370 of *R. necatrix*, obtained from Japanese pear tree, was shown to be hypovirulent by Arakawa et al. [10] and maintained on potato dextrose agar. For purification of dsRNAs, the fungus was grown in potato dextrose broth at 25°C. Mycelia were grown in petri dishes.

Purification of dsRNAs

One gram of mycelial tissue suspended in 1.8 ml of 2 × STE buffer (0.1 M NaCl, 50 mM Tris, 1 mM Na_2EDTA, pH 7.0), 0.2 ml of 10% SDS, 2 μl of mercaptoethanol, 1 ml of chloroform and 1 ml of water-saturated phenol containing 0.1% 8-hydroxyquinoline was homogenized for 5 min using a polytron, and the homogenate was centrifuged at 8,000 rpm. The aqueous phase was collected, and ethanol was added to make a final concentration of 15%. For each 10 ml of the 15% ethanol solution, 0.5 g CF-11 (Whatman) cellulose powder [11] was added. The mixture was contained in small columns, washed with a solution of 15% ethanol–STE buffer. The dsRNA was then eluted from the CF-11 cellulose with 100% STE buffer and was precipitated by adding 2.5 volumes of cold ethanol. The precipitate was collected by centrifugation, and was dissolved in a suitable buffer for electrophoretic analysis.

Purification of dsRNAs and Electrophoresis

dsRNAs were analyzed by electrophoresis in 5% polyacrylamide gel in TAE buffer (0.04 M Tris, 0.02 M acetic acid, 1 mM Na_2EDTA, pH 7.4). Electrophoresis was performed at 75 V for 17 h. The gels were stained with ethidium bromide. The nucleic acids were visualized on a UV transilluminator and were photographed using Polaroid 667 film. Rice dwarf virus (RDV) dsRNAs, kindly supplied by Dr. T. Omura (National Agriculture Research Center, Tsukuba, Japan), were used as molecular weight markers.

Cloning and Sequencing of dsRNAs

Full length cDNA clones of W370 dsRNAs with unknown sequences were obtained following the method of Isogai et al. [12]. Briefly, the 3′-ends of the plus and minus strands of the dsRNAs were polyadenylated and then used as templates for an initial reverse transcription using an oligo-dT-containing adapter primer (AP). The first-strand cDNAs of both polarities were annealed, filled in and amplified by the polymerase chain reaction using one primer containing an adapter region sequence identical to that in the AP. The amplified cDNA products were cloned using the TA Cloning Kit (Invitrogen). The DNA sequence was analyzed using an automated DNA sequencer (model 377, Applied Biosystems). Deduced proteins and RNA secondary structures in both termini of dsRNA segments were predicted by using the program DNASIS Version 2.1 (Hitachi Software Engineering). Terminal nucleotide sequences were also aligned for analysis of conserved sequences by DNASIS. Comparison of sequences with those available from nucleic acid and protein databases were performed using the BLAST program [13]. The Motif and Pfam programs (http://www.motif.genome.ad.jp) were used to analyze the theoretical protein sequences for the presence of known functional amino acid motifs, and to search for previously described protein family-domains.

Northern Blot Hybridization

For northern blot hybridization analysis, electrophoresis was carried out in 5% polyacrylamide gel. Northern blot hybridization analysis was performed as described [14]. Digoxigenin (DIG)-labeling and detection were done following the protocol of DIG DNA Labeling and Detection Kit (Roche).

Results and Discussion

dsRNAs from Strain W370 of R. necatrix

Twelve dsRNA species, designated as segment (S) 1–12, on the basis of electrophoretic mobility, were detected in 5% polyacrylamide gel electrophoresed with extract purified by CF-11 cellulose from strain W370 of *R. necatrix* (Fig. 1). The estimated molecular weights of the dsRNAs were about 2.95, 2.60, 2.40,

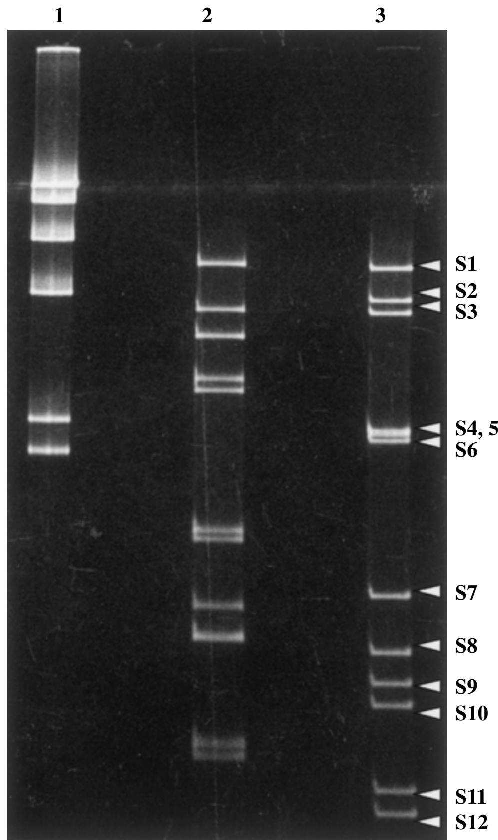


Fig. 1. Electrophoresis of dsRNAs extracted from strain W370 of *R. necatrix* in 5% polyacrylamide gel. Lane 1, λ/*Hind*III; lane 2, twelve dsRNA genome segments of rice dwarf virus; lane 3, dsRNA preparation from mycelia of W370.

1.55, 1.54, 1.50, 0.91, 0.74, 0.65, 0.61, 0.45, and 0.41×10^6, respectively.

Nucleotide Sequence of dsRNAs and Deduced Amino Acid Sequence

Full length cDNA clones for eight dsRNA segments were obtained. Northern blot analysis revealed that none of the clones hybridized to more than one of the 12 segments (Fig. 2), suggesting that each segment was genetically unique. Since it was unclear whether the largest clone hybridized to S4 or S5, whose electrophoretic mobilities were similar. We named this segment as segment X (SX).

Sequences of dsRNA segments (Fig. 3) were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under the following Accession

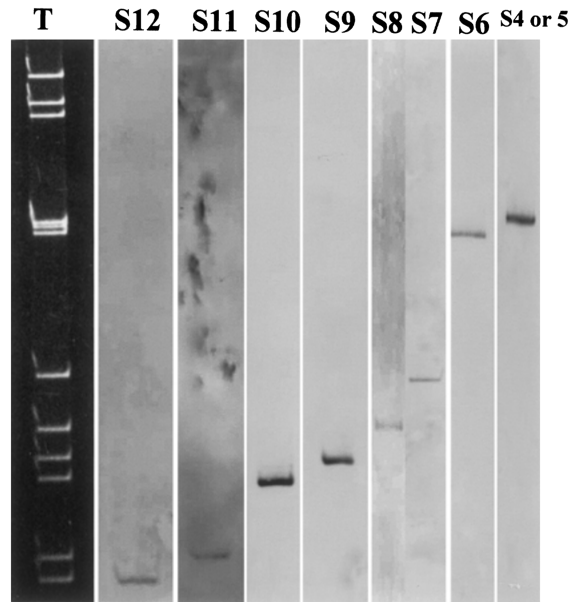


Fig. 2. Northern hybridization for assignment of cDNA clones to dsRNA segments. Total dsRNA of *R. necatrix* W370 was separated through a 5% polyacrylamide gel (T), blotted to nylon membranes and hybridized with DIG-labeled cDNA clones Nos. 13 (S12), 6 (S11), 15 (S10), 12 (S9), 53 (S8), 18 (S7), 28 (S6), and 20 (S4 or 5).

Table 1. The length and putative protein encoded of each dsRNA segment from *R. necatrix* W370

Segment No.	Nucleotides (bp)	Coding Region	Molecular Mass (kDa)
SX (4 or 5)	2259	29–2206	78.7
S6	2030	96–2000	71.5
S7	1509	20–1468	55.1
S8	1299	113–1090	36.5
S9	1226	46–1188	41.6
S10	1171	166–1098	33.7
S11	1003	82–930	31.1
S12	943	86–883	29.2

Numbers: AB073276 (SX), AB073277 (S6), AB073278 (S7), AB073279 (S8), AB073280 (S9), AB073281 (S10), AB073282 (S11), and AB073283 (S12), respectively. The lengths of the dsRNA segments are reported in Table 1. A unique long ORF was identified on the positive strand of each segment and proteins less than 96 amino acids (10.3 kDa) are encoded by the negative RNA strands.

As shown for members of the family *Reoviridae*, all eight segments of W370 dsRNAs had conserved sequences at the termini (5′- and 3′-ends). In all positive strands of the eight segments, the motifs

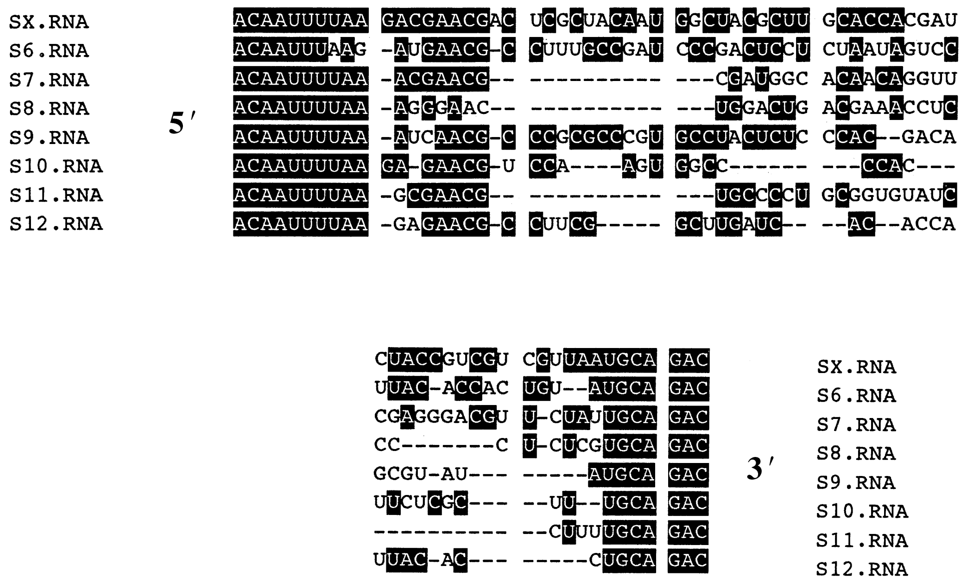


Fig. 3. Alignment of 5′- and 3′-terminal sequences of eight dsRNA segments of *R. necatrix* W370.

(5′-ACAAUUU-3′) and (5′-UGCAGAC-3′) were found in the 5′- and 3′-terminal non-coding regions, respectively (Fig. 3). The motifs were similar to those of Colorado tick fever virus (CTFV; 5′-$^{G}/_{C}$ACAUU-UUGU…UGCAGU$^{G}/_{C}$-3′) [15] in the *Reoviridae*. Segment-specific panhandle structures, formed by inverted terminal repeats, were also found in all eight segments (Fig. 4). A number of segment-specific inverted terminal repeats could interact by homologous base pairing and hold the RNA transcripts in a circular form. This circular form has been described as a panhandle structure that might function as a guiding site for an RNA-dependent RNA polymerase (RDRP). Alternatively, it has been suggested that such conserved motifs may act as sorting signals bringing a single copy of each genome segment into the nascent capsid [16,17].

The proteins encoded by the different dsRNA segments were designated P_N, where N refers to the number of the RNA segment based on its electrophoretic mobility. We have searched for protein sequence homologies using BLAST. Analyses of the predicted translational products of 8 dsRNA segments showed as follows. Amino acid (AA) residues 205 and 283 of PX showed partial similarity to VP4 of CTFV in the genus *Coltivirus*, one genus of the family *Reoviridae* (43% similarity, AA 495–573, Accession No. AF139760). VP4 is considered to be a cell-binding protein. AA 345–609, AA 415–615, AA 84–213, and AA 416–489 of P6 partially matched VP10 (possible nucleotide-binding function) of CTFV (43% similarity, AA 315–584, Accession No. AF139765), Segment 7 protein (core protein; possible NTP-binding function) of *Nilaparvata lugens* reovirus in the genus *Fijivirus*, another genus of the *Reoviridae* (40% similarity, AA 398–602, Accession No. D49699), tegument protein of murid herpesvirus in the family *Herpesviridae* (41% similarity, AA 79–214, Accession No. AF105037), and Segment 8 protein (core protein; possible NTP-binding function) of rice black streaked dwarf virus in the genus *Fijivirus* (52% similarity, AA 353–424, Accession No. AF399826), respectively. The BLAST analysis also revealed that the region between AA residues 146 and 233 of P12 exhibited resemblance to a capsid protein precursor of turkey astrovirus (41% similarity, AA 224–312, Accession No. Y15936). The deduced amino acids from the other segments revealed no clear similarity to other viruses. Moreover, the known functional amino acid motifs or previously described protein family-domains were not found in the sequences of W370 dsRNA segments by the Motif and Pfam programs.

dsRNA viruses are classified into six families: *Cystoviridae*, *Reoviridae*, *Birnaviridae*, *Totiviridae* [18], *Partitiviridae* [19] and *Hypoviridae* [20] on the

basis of physicochemical properties. The dsRNA genetic elements are commonly found in filamentous fungi, either within true virus particles or pleomorphic lipid vesicles. The dsRNA mycoviruses were classified into *Partitiviridae, Totiviridae* and *Hypoviridae*. Isometric mycoviruses containing dsRNA with divided genomes, which are generally two monocistronic dsRNA segments, usually of similar size (1.4–3.0 kb), are included in the *Partitiviridae*, and those with a monopartite genome, which ranges from 4.6 to 6.7 kb in size, are included in the *Totiviridae*. Members of the *Hypoviridae* has a larger genome (12–13 kbp) encapsulated in pleomorphic lipid vesicles, which are associated with hypovirulence in the host.

The properties of W370 dsRNAs suggest a close relationship with the viruses in the family *Reoviridae* [21]. Reoviruses have been found in invertebrate, vertebrate, and plant hosts, but not in fungal hosts. The W370 dsRNA consisted of 12 segments, ranging from ca. 0.41 to 2.95×10^6 in size. All sequenced segments of W370 dsRNA had conserved terminal sequences at both 5′- and 3′-ends. Segment-specific panhandle structures were also found in all eight

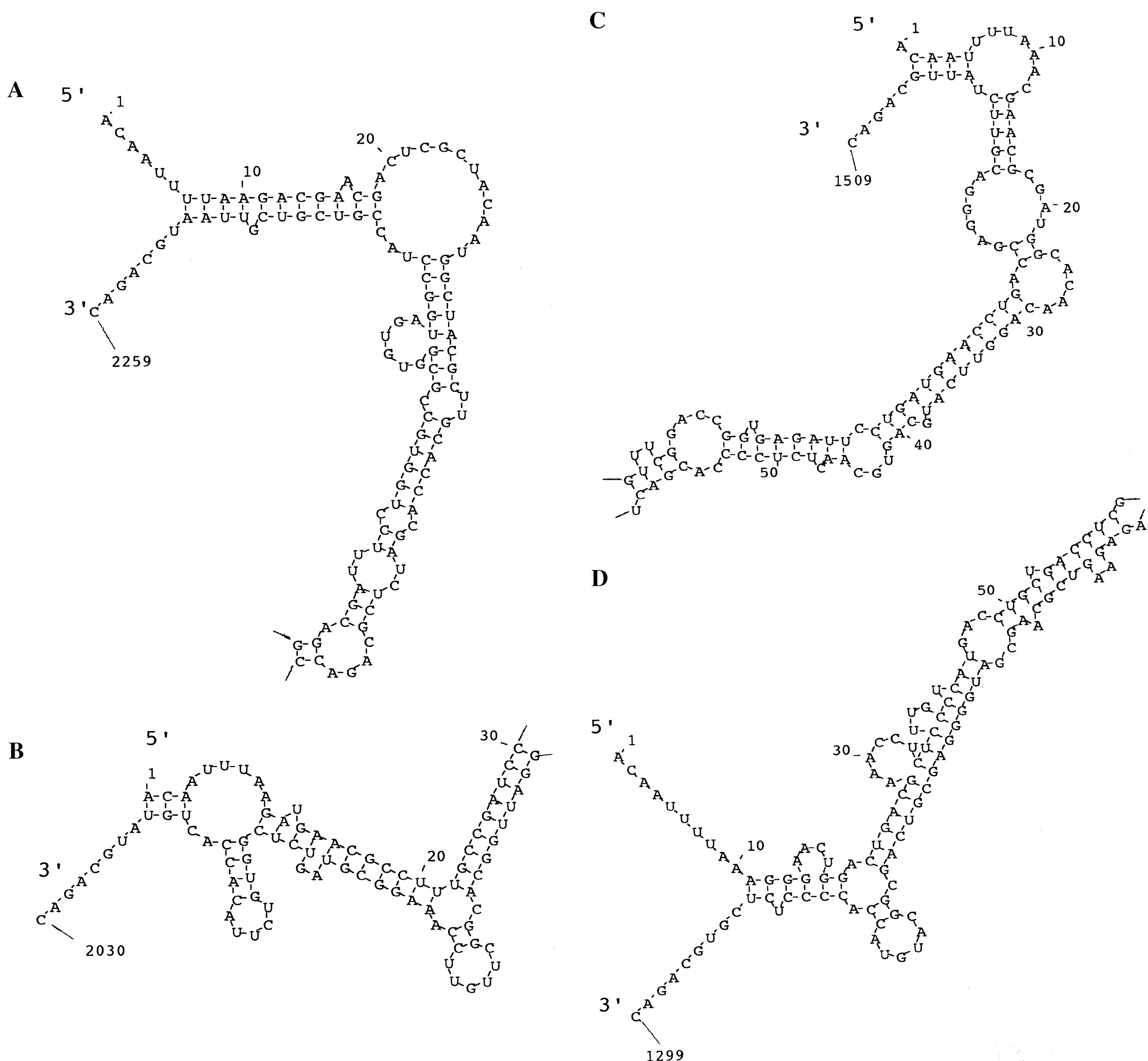
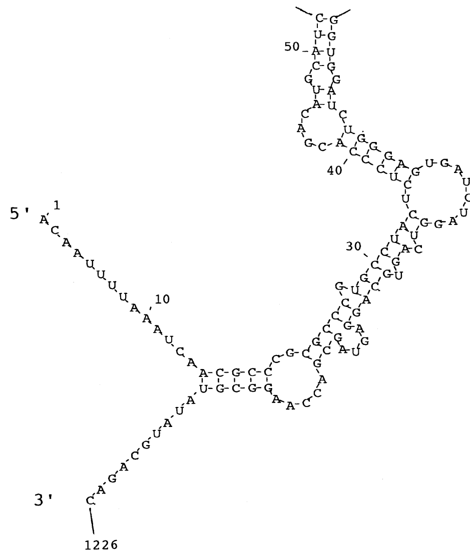
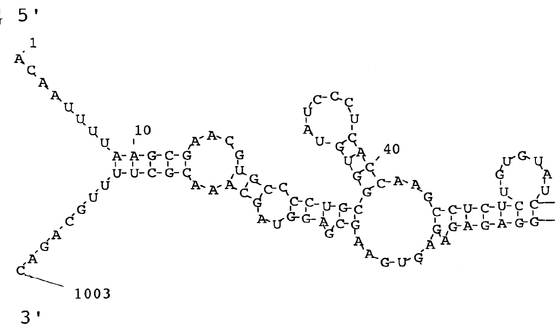
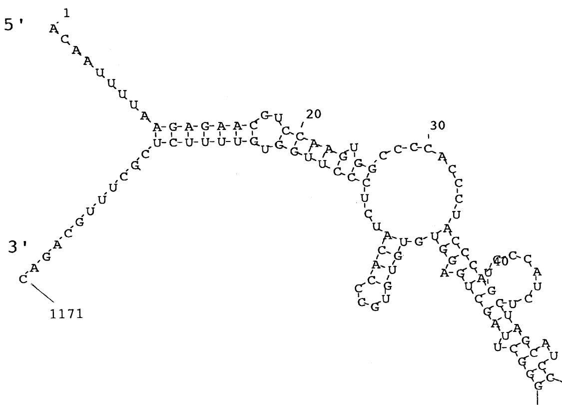


Fig. 4. The secondary structures of 5′- and 3′-terminal sequences of eight dsRNA segments of *R. necatrix* W370, segments X (A), 6 (B), 7 (C), 8 (D), 9 (E), 10 (F), 11 (G), and 12 (H).







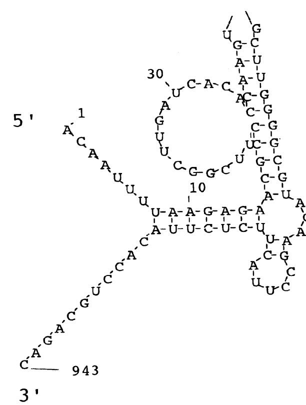


Fig. 4. Continued.

segments. These features are characteristic of the viruses in the family *Reoviridae*. Moreover, the deduced amino acid sequences of W370 dsRNAs showed partial similarities to several proteins of viruses in the *Reoviridae*. These results suggest that W370 contains dsRNA virus, although the viral particles have not yet been found, which might be the first member of the *Reoviridae* from fungus.

Similar dsRNA molecules, in size and with 11 segments, were also detected in a hypovirulent isolate of *Cryphonectria parasitica* [22]. Isometric particles (about 60 nm) were purified from the isolate. The relationship between dsRNAs from W370 and from *C. parasitica* is unclear because the latter has not been sequenced. We are now determining the nucleotide sequences of four other segments and searching for viral particles associated with these dsRNAs.

Isogenic dsRNA-free isolates from W370, which were bred by eliminating 12 dsRNA segments from W370 using hyphal tip isolation, recovered virulence [10]. This result suggests that W370 dsRNAs act as a hypovirulent factor to the host, *R. necatrix*. By further analyzing the mechanism of hypovirulence in W370, and identifying other mycoviruses or dsRNA molecules, we may be able to use dsRNAs that attenuate fungal virulence to control white root rot.

Acknowledgements

This work was funded through the Bio-oriented Technology Research Advancement Institution (BRAIN).

References

1. Khan A.H., Calif Dep Agric Bull *44*, 167–170, 1955.
2. Sztejnbrtg A. and Madar Z., Plant Dis *64*, 662–664, 1980.
3. Buck K.W. (ed.), *Fungal Virology*, CRC Press Boca Raton, 1985, pp. 2–84.
4. Nuss D.L. and Koltin Y., Annu Rev Phytopathol *28*, 37–58, 1990.
5. Ghabrial S.A., Adv Virus Res *43*, 303–388, 1994.
6. Biraghi A., Rep Congr Int Union For Res Org Rome *11*, 643–645, 1953.
7. Day P.R., Dodds J.A., Elliston J.E., Jaynes R.A., and Anagnostakis S.L., Phytopathology *67*, 1393–1396, 1977.
8. Elliston J.E., Jaynes R.A., Day P.R., and Anagnostakis S.L., Proc Am Phytopathol Soc *4*, 83–84, 1977.
9. Arakawa M., Nakamura H., Uetake Y., and Matsumoto N., Mycoscience *43*, 21–26, 2002.
10. Arakawa M., Nakamura H., Uetake Y., Nitta H., Naganawa T., Kakishima M., Okabe I., and Matsumoto N., Jpn J Phytopathol *67*, 192, 2001.
11. Dodds J.A. and Bar-Joseph M., Phytopathology *73*, 419–423, 1983.
12. Isogai M., Uyeda I., and Hataya T., Ann Phytopathol Soc Jpn *64*, 244–248, 1998.
13. Altschul S.F., Gish W., Miller W., Myers E.W., and Lipman D.J., J Mol Biol *215*, 403–410, 1990.
14. Valverde R.A., Nameth S., Abdallha O., Al-Musa O., Desjardins P., and Dodds A., Plant Sci *67*, 195–201, 1990.
15. Attoui H., Billoir F., Biagini P., Cantaloube J.F., de Chesse R., de Micco P., and de Lamballerie X., Biochem Biophys Res Commun *273*, 1121–1125, 2000.
16. Anzola J.V., Xu Z., Asamizu T., and Nuss D.L., Proc Natl Acad Sci USA *84*, 8301–8305, 1987.
17. Xu Z., Anzola J.V., Nalin C.M., and Nuss D.L., Virology, *170*, 511–522, 1989.
18. Wickner R.B., Ghabrial S.A., Bruenn J.A., Buck K.W., Patterson J.L., Stuart K.D., and Wang C.C., in van Regenmortel M.H.V., Fauquet C.M., Bishop D.H.L., Carstens E.B., Estes M.K., Lemon S.M., Maniloff J., Mayo M.A., McGeoch D.J., Pringle C.R., and Wickner R.B. (eds), *Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses*, Academic Press, San Diego, California, 2000, pp. 491–501.
19. Ghabrial S.A., Bozarth R.F., Buck K.W., Martelli G.P., and Milne R.G., in van Regenmortel M.H.V., Fauquet C.M., Bishop D.H.L., Carstens E.B., Estes M.K., Lemon S.M., Maniloff J., Mayo M.A., McGeoch D.J., Pringle C.R., and Wickner R.B. (eds), *Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses*, Academic Press, San Diego, California, pp. 503–513, 2000.
20. Hillman B.I., Fulbright D.W., Nuss D.L., and van Alfen N.K., in van Regenmortel M.H.V., Fauquet C.M., Bishop D.H.L., Carstens E.B., Estes M.K., Lemon S.M., Maniloff J., Mayo M.A., McGeoch D.J., Pringle C.R., and Wickner R.B. (eds), *Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses.* Academic Press, San Diego, California, 2000, pp. 515–520.
21. Mertens P.P.C., Arella M., Attoui H., Belloncik S., Bergoin M., Boccardo G., Booth T.F., Chiu W., Diprose J.M., Duncan R., ESTES M.K., Gorziglia M., Gouet P., Gould A.R., Grimes J.M., Hewat E., Holmes I.H., Hoshino Y., Hill C., Joklik W.K., Knowles N., Ferber M.L., Malby R., Marzachi C., McCrae M.A., Milne R.G., Nibert M., Nunn M., Omura T., Prasad B.V.V., Pritchard I., Roy P., Samal S.K., Schoehn G., Shikata E., Stoltz D.B., Stuart D.I., Suzuki N., Upadhyaya N., Uyeda I.,Waterhouse P., Williams C.F., Winton J.R., and Zhou H.Z., in van Regenmortel M.H.V., Fauquet C.M., Bishop D.H.L., Carstens E.B., Estes M.K., Lemon S.M., Maniloff J., Mayo M.A., McGeoch D.J., Pringle C.R., and Wickner R.B. (eds), *Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses.* Academic Press, San Diego, California, 2000, pp. 395–480.
22. Enebak S.A., Hillman B.I., and MacDonald W.L., MPMI *7*, 590–595, 1994.