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

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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 $0.41 \times 10^6$ to $2.95 \times 10^6$. 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′-ACAUUU-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, Nilapavata 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
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 Na₂EDTA, 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.

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,