Molecular characterization of dsRNA segments 2 and 5 and electron microscopy of a novel reovirus from a hypovirulent isolate, W370, of the plant pathogen \textit{Rosellinia necatrix}

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A hypovirulent isolate, W370, of the white root rot fungus \textit{Rosellinia necatrix} has previously been shown to harbour 12 dsRNA segments. In this study, complete nucleotide sequences of segments 2 and 5 of W370 dsRNAs were determined. The nucleotide sequence of genome segment 2 was 3773 bases long with a single long open reading frame (ORF) encoding 1226 amino acid residues with a predicted molecular mass of approximately 138 \textasciitilde 5 kDa. The nucleotide sequence of segment 5 was 2089 bases long with a single long ORF, whose deduced polypeptide contained 646 amino acid residues with a predicted molecular mass of about 72 kDa. Comparative analysis showed that the deduced protein sequence of segment 2 had significant homology with the putative VP2 of Colorado tick fever virus (CTFV) and European Eyach virus (EYAV) in the genus \textit{Coltivirus}, but the deduced protein sequence of segment 5 had no similarity with other virus proteins. Double-shelled spherical particles of approximately 80 nm in diameter associated with W370 dsRNAs were observed in a preparation from the mycelial tissue of isolate W370. The results demonstrated that the virus associated with W370 dsRNAs is a novel reovirus of the family \textit{Reoviridae}. The virus was named \textit{Rosellinia} anti-rot virus (RArV).

**INTRODUCTION**

White root rot, also known as \textit{Rosellinia} (\textit{Dematophora}) root rot, is a destructive disease of about 170 species of plants in 63 genera (Sztejnberg, 1994). The disease has a wide geographical distribution in all temperate regions of the world (Sztejnberg, 1994). In Japan, it is one of the most serious diseases of fruit trees and large amounts of fungicide have been used for its control in Japanese pear and apple orchards. During a search for hypovirulent isolates of the white root rot fungus \textit{Rosellinia necatrix} Prillieux to exploit for biocontrol of the disease, a hypovirulent isolate, W370, was found (Arakawa \textit{et al}., 2001, 2002). Isolate W370 was found to contain 12 dsRNA species and its hypovirulence was ascribed to the presence of these dsRNAs (Arakawa \textit{et al}., 2001, 2002).

The 12 dsRNA species were designated segments (S) 1–12, on the basis of their electrophoretic mobility (Osaki \textit{et al}., 2002). Previous sequence analysis of the eight smaller segments has suggested that W370 dsRNA segments might have originated from a member of the virus family \textit{Reoviridae} (Osaki \textit{et al}., 2002). In this paper we report the complete nucleotide sequences of W370 dsRNA genome segments 2 and 5. Double-shelled spherical particles of approximately 80 nm in diameter associated with W370 dsRNAs were observed in a preparation from the mycelial tissue of isolate W370. These results show that isolate W370 of \textit{R. necatrix} harbours a novel reovirus member of the family \textit{Reoviridae}. Since it has potential for use as a biocontrol agent of white root rot disease, we named it \textit{Rosellinia} anti-rot virus (RArV).

**METHODS**

\textbf{Fungal isolates and culture conditions.} Isolate W370 of \textit{R. necatrix} was a dsRNA-containing hypovirulent strain. An isogenic dsRNA-free isolate derived from isolate W370 and a dsRNA-free isolate W380 of \textit{R. necatrix} were used as control materials for electron microscopy (Arakawa \textit{et al}., 2001, 2002). They were cultured and maintained on oatmeal agar. For purification of dsRNAs and virus particles, the isolates were grown in 50 ml of oatmeal extract broth (15 g Quaker oatmeal in 1000 ml H\textsubscript{2}O) at 25 °C in the dark for 7 days, then transferred into 1000 ml of fresh oatmeal extract broth and incubated for 7 days at 25 °C in the dark.

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The GenBank accession numbers of the sequences reported in this paper are AB098022 (segment 2) and AB098023 (segment 5).
Extraction and purification of dsRNAs. Total dsRNA was prepared as described by Morris & Dodds (1979), with minor modifications (Osaki et al., 2002). The dsRNA was concentrated by ethanol precipitation and further purified using NReasy Plant Mini Kits (Qiagen). The RNaid kit (BIO101) was used to isolate and purify dsRNA segments from polyacrylamide gels.

cDNA synthesis and cloning. One cDNA library was synthesized, starting with 3 µg of purified total dsRNA. First-strand synthesis for the library was primed with 0-037 µg pd(N)₆, random hexamer. A total volume of 20 µl containing primers and dsRNA mixture in water was denatured by boiling for 10 min. The TimeSaver cDNA synthesis kit (Amerham Pharmacia) was used for cDNA synthesis. Double-stranded cDNA was passed through a Sизese 400 spin column to remove cDNA products with a length under 400 bp. The purified cDNA was ligated into the EcoRI site of pUC118, after addition of an EcoRRI/NolI adaptor and transformed into Escherichia coli DH5α (Toyobo).

General recombinant DNA methods were as described in Sambrook et al. (1989). Recombinant plasmids were screened from ampicillin-resistant white colonies using the QiAprep Spin Miniprep Kit (Qiagen). The RNaid kit (BIO101) was used to isolate and purify dsRNA segments from polyacrylamide gels.

Northern blotting. Northern blot hybridization analysis was performed as described by Valverde et al. (1990), with minor modifications. Prior to electrophoresing, dsRNA in polyacrylamide gels was denatured with 50 % (v/v) DMSO and 1 M glyoxal in 1 × TBE at 50 °C for 1 h. Gels were electrophoresed on to nylon membranes in the presence of 1 × TBE at 100 mA for 2 h. Membranes were baked at 120 °C for 2 h and used for hybridization. Digestoxigenin (Dig)-labelling and detection were carried out following the protocol of the DIG DNA labelling and detection kit (Boehringer Mannheim).

RT-PCR. Plasmids containing cDNA inserts of 1-5 kb and longer were sequenced. PCR primers were designed from the terminal sequences of inserts and used for RT-PCR on isolated segments to confirm the identity of the template. RT-PCR was carried out using the RNA LA PCR kit (AMV) (Version 1.1) (TaKaRa) and 20 µM of each primer was used. Reverse transcription was carried out at 42 °C for 60 min. PCR amplification was carried out using 35 cycles of denaturation at 94 °C for 0-5 min, annealing at 45 °C for 0-5 min and extension at 72 °C for 3 min, with a final extension step at 72 °C for 7 min.

The distal ends of S2 and S5 were amplified by the 5′ RACE (rapid amplification of cDNA ends) approach (Frohman, 1990) using a 5′ RACE kit (Invitrogen). Segments 2 and 5 isolated from polyacrylamide gel were used as template dsRNA. The RT-PCR products and 5′ RACE products were cloned into the pCR2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen).

Sequence analysis. DNA sequences were analysed using GENETYXWIN software (Software Development Co.). RNA secondary structures in both termini of the dsRNA segments were predicted using the program DNASIS Version 2.1 (Hitachi Software Engineering). Comparison of sequences with those available from nucleic acid and protein databases was performed using the NCBI BLAST 2.0 program (http://www3.ncbi.nlm.nih.gov/BLAST). Multiple sequence alignment was performed using CLUSTAL W (Thompson et al., 1994).

The Pfam program (http://www.sanger.ac.uk/Pfam/search.shtml) was used to search for previously described protein family domains. The Motif program (http://www.motif.genome.ad.jp) was used to analyse the theoretical protein sequences for the presence of known functional amino acid motifs.

Viruses particle purification and electron microscopy. Extraction of particle-containing fractions from the mycelia was performed with phosphate buffer (Kimura & Black, 1971; Omura et al., 1982), with minor modifications. W370 mycelia (100 g) were frozen in liquid nitrogen and ground to a fine powder. Potassium phosphate buffer (PB; 0-1 M, pH 7-2, 400 ml) was added to the ground mycelia. The mixture was stirred for 1 h, then passed through four layers of finely woven cotton cloth. Triton X-100 was added to a final concentration of 2 %. The mixture was stirred for 60 min, then centrifuged for 15 min at 3000 g. Polyethylene glycol 6000 and NaCl were added to the supernatant fluid to final concentrations of 6 % (w/v) and 0-3 M, respectively. The mixture was stirred for 40 min, then centrifuged for 15 min at 6000 g.

The resulting pellets were resuspended in 9 ml 0-1 M PB, pH 7-0. Carbon tetrachloride was added to a final concentration of 10 % and vortexed for 1 min. After centrifugation at 3000 g for 15 min, the supernatants were layered on to 1 ml sucrose cushions (20 % sucrose, w/v, in 0-1 M PB, pH 7-0) and centrifuged at 96 000 g for 2 h. The pellets were resuspended in 0-01 M PB, pH 7-0, containing 0-01 M MgCl₂ (PB-Mg). (MgCl₂ was added just before use) for 1 h and centrifuged for 15 min at 3000 g. The supernatant was layered on to 10-40 % (w/v) linear sucrose gradients in PB-Mg and centrifuged for 80 min at 62 800 g in a Hitachi SP-3000 rotor. Two bands, which corresponded to inner capsids and intact particles, respectively, were observed and collected separately. Appropriate fractions were centrifuged for 2 h at 96 000 g in a Hitachi SP-2500 rotor and the final pellets were resuspended in PB-Mg.

Particle preparations were stained with either 2 % phosphotungstic acid (PTA), pH 7-0, or 2 % aqueous uranyl acetate and then examined with a JEM-1200 EX electron microscope.

Isolate W380 and the isogenic dsRNA-free isolate from W370 were also treated in the same way.

Results

Sequence determination of S2 and S5

dsRNA isolated from the hypovirulent isolate of the white root rot fungus R. necatrix W370 was separated by electrophoresis into 12 discrete sizes (S1-S12) (Fig. 1). In our previous report (Osaki et al., 2002) we were unable to separate segments 4 and 5.

The S2 and S5 specificity of five cDNA clones with inserts longer than 1-5 kb were characterized by Northern blot analysis and preliminary sequence analysis. Primers (18- to 20-mers) were designed from the terminal sequences of the inserts and used for RT-PCR on isolated segments 2 and 5 to confirm the identity of the template. In all experiments, eight specific RT-PCR products were amplified by five sets of primers. On comparison of these sequences, all PCR products were divided into just two groups. Northern blot hybridization analysis confirmed that one corresponded to S2 and the other to S5.

Using the sequences of these RT-PCR products, the cDNA sequences of the entire dsRNA segments 2 and 5 could be completed, with the exception of the terminal sequences. The remainder of the S2 and S5 sequences was then determined by sequencing the 5′ RACE products, using specific primers derived from the RT-PCR products or nested primers from the truncated 5′ RACE products. The distal 3′ end of S5 was determined according to the protocol.
The extreme 5’ and 3’ ends of the sense strand of both segments had the sequence 5’-ACAAUUU...UGCAGAC-3’.

The complete nucleotide sequences of S2 and S5 were 3773 and 2089 bases long, with GC contents of 50% and 49-5%, respectively. Sequence comparisons of the two segments with those available from nucleic acid databases showed no significant homology with reported virus sequences at the nucleotide level.

Electron microscopy

The virus particles appeared to be tightly bound to cellular material, since only a few virus particles of approximately 80 nm in diameter were observed in the crude preparation of hypovirulent isolate W370 after cellular debris had been removed by low speed centrifugation (10,000 g, 10 min) (Fig. 3A). On the other hand, no such particles were observed in the preparation from mycelial tissue from the dsRNA-free strain that originated from the hypovirulent isolate W370 or from the healthy isolate W380 of R. necatrix. Double-shelled spherical particles approximately 80 nm in diameter were observed in the partially purified preparations from the mycelial tissue of hypovirulent strain W370 containing dsRNAs (Fig. 3B). Round particles 60–65 nm in diameter were also observed in the preparation from the mycelial tissue of strain W370 containing dsRNA that had been partially purified by centrifugation through sucrose cushions (Fig. 3C). From observations of partially degraded virus particles and virus particles variably penetrated by stain, it was concluded that the 60–65 nm particles represented the virion inner capsid core with B spikes (Milne & Lovisolo, 1977) and that the 80 nm particles represented intact virions with the typical double shells (Fig. 3B, C). This conclusion was confirmed by dsRNA extraction from different fractions of a sucrose density gradient containing the two particles of 80 nm and 60–65 nm in diameter (Fig. 3D, E); the two different particles contained the same 12 genome segments of dsRNA with the same electrophoretic profile as the dsRNA segments extracted from the mycelial tissue of R. necatrix W370 (data not shown). (These particles were disrupted with 2% PTA, pH 7-0; data not shown.)

The BLAST 2.0 program was used to search for protein sequence homologies. The deduced VP2 protein sequence showed homology with the putative VP2 of Colorado tick fever virus (CTFV; 23% identity, 39% similarity) and the putative VP2 of European Eyach virus (EYAV; 24% identity, 39% similarity) (Attoui et al., 2000, 2002). Analysis of amino acid sequences using the NCBI BLAST program showed that the deduced VP5 protein sequence had no similarity to other viral proteins.

A search for functional motifs in the deduced protein sequences encoded by the ORFs of S2 and S5 was conducted. The VP2 sequence was found to contain a methyltransferase family motif (LATKIAFLMNPLAYERNHVKAALFLDFIR) at aa 510–539, which is also conserved in VP2 of CTFV and EYAV (Fig. 2). Therefore, these putative proteins are proposed to act as methyltransferases (Attoui et al., 2000, 2002). No known functional amino acid motifs or previously described protein domains were found in the sequence of S5 using either the Motif or the Pfam programs.

Fig. 1. Electrophoresis of dsRNAs extracted from R. necatrix strain W370 on a 5% polyacrylamide gel. Lane 1, 12 dsRNA genome segments of rice dwarf virus as a dsRNA marker; lane 2, dsRNA preparation from mycelia of W370. The gels were stained with ethidium bromide and the nucleic acids visualized by UV.
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CTF-VP2
MSRTKEKTKDKTNDEQVTSTEAADFARNDREK----SSSSEPENAKETTLKDDSS- 54

EYA-VP2
MSTRKEKTKKE-KNEELTLENTQAQDTKVSSLVLSSRDRPQKEdwardQEGTD 59

Rar-VP2
MADSTPLDPLSTPNDQGTSSHPEK-------------------------- 26

*: *: .: .: .:

CTF-VP2
---SSKSVDDLAAMIEQOKVQMSGAGLGFRRPMPEITDAAALEREADVSKVVPSSL 111

EYA-VP2
KPGSRSIALDAAAIIEEKVAMSMTAGLDFRRPMPEITDAALLERKEDVSKVVPSSL 119

Rar-VP2
---SAAEESFRFLRKLDDILANT----SIAVFNQNLVALTRELSHIANSPLTTP-AF 77

*: *: .: .: .: .: .: .: .: .: .: .: .: .: .: .: .: .:

CTF-VP2
SKLDDGVVULLSVGEERTLSAQLFQLFLETVSIGYALTAKGIALGAQGGYLAKEHGE 171

EYA-VP2
SKLDDGVVULLSVGEERTLSAQLFQLFLETVSIGYALTAKGIALGAQGGYLAKEHGE 179

Rar-VP2
SVRDAFSAVLASKVVEHVAIATPEHPIHAYLARPDLMPDGVDGLFSLNP-GPAVDSP 136


CTF-VP2
QVYPFDIPAVANQGRGFRDFAVRYINTSTGGGVEIFIPDFDSDKGKVGGESLTGGLYNA 231

EYA-VP2
QVYPFDIPAVANFGRFLDFAVRYINTSTGGGIEIFIPDFDSDKGKVGGASLTLGGLYNA 239

Rar-VP2
MMFPFEQPTIKTKCLNMLGLGVVVIrishPVGMIRHPYDLSIDTVTMRDGIEEIDMGPSG 196


CTF-VP2
VGDEITYTDVKTEGMMEFVVLLIALAAGAKMGRFSSGVDARRRKLFTWVRRPVPNSDF 291

EYA-VP2
VGDEITYTDVKTEGMMEFVVLLIALAAGAKMGRFSSGVDARRRKLFTWVRRPVPNSDF 299

Rar-VP2
GLVTENDNMGFGLFLSGFLLKLDIDIGVSFRGFRMLMKSKIDDK-HCLTGLRNMARDPEY 255


CTF-VP2
RHVQWULLEDYENYPHVPEALTVPSEYDWFARERVDNMTDGLGTSQVMDPNNIESFF 351

EYA-VP2
RNIGWULLEDYENYPHVPEALTVPSEYDWFARERVDNMTDGLGTSQVMDPNNIESFF 359

Rar-VP2
ARISGMQKDDYERYHPYMEIARNHDLKDWRAERVDHEPRKLGTVESEG------VNLAH 310


CTF-VP2
NRKREAAICNLDIFTAEEAHNMVEEQLNEKMNVSAYVDLLQSCGMIFDIEGDTDTTILK 411

EYA-VP2
NRKREAAICNLDIFTAEEAHNMVEEQLNEKMNVSAYVDLLQSCGMIFDIEGDTTTLIK 419

Rar-VP2
NRKLQICYTRSDTLTKRANFELESMRGRFSLPDAYRDLLVNLVRQIDGRRMEMLRF 370


CTF-VP2
KVYMREVEDYAEELTRMLMFPGDOLLYVCDCRLAALYSPNRQGFLILSVLIAATSRGVGIE 471

EYA-VP2
KVYMREVEDYAEELTRMLMFPGDOLLYVCDCRLAALYSPNRQGFLILSVLIAATSRGVGIE 479

Rar-VP2
RKYDVDDLTFGRERAEFSAFIMYNQAQRLSCFCTLYLASYFRDFRFRSEVEGLAASLVRAT 430


CTF-VP2
LLPVAEMANSTLRNSVSHMALMTLAIGWEKLERRLTNPQIGKIVCDIMAPFVFDG- 530

EYA-VP2
LLPVAEMANSTLRNSVSHMALMTLAIGWEKLERRLTNPQIGKIVCDIMAPFVFDG- 538

Rar-VP2
VGTTPALNAMTRKDRKhVNSHINTTYGQAVFELIDLTINTSIEVRAWCLAEMLPMYDIS 490


CTF-VP2
LRMDSSGLQPUPTSLVSLLGGAKVALILTPNLNYDNLHLKAKHLVSTLSCFFPDQYGLAI 590

EYA-VP2
LRMDSSGLQPUPTSLVSLLGGAKVALILTPNLNYDNLHLKAKHLVSTLSCFFPDQYGLAI 598

Rar-VP2
TRPSADYGTDSRSKFAHVLLATKIAFLMNPLAYERNRHKKAALVDFIRFPLDFKNLEF 550


CTF-VP2
ASRGYHGVDVTGNSRDSIRGETKKNCRFATYTVLDPINPNPPDTRRRKWLMMKVQEWL 650

EYA-VP2
AARGYHGVDVTGNSRDSIRGETKKNCRFATYTVLDPINPNPPDTRRRKWLMMKVQEWL 658

Rar-VP2
RMPWUGYRSNGTPMVLEERGVTGRTISSNERFTTELEIPIPRANIPDAG-WNLMTRVLAFL 609


CTF-VP2
LRNLDVRREREIRDIYIRPYANVLRPLPGYTLPRQAGAPLPCEVLLNDNDAIAILEEYRTV 710

EYA-VP2
LRNLDVRREREIRDIYIRPYANVLRPLPGYTLPRQAGAPLPCEVLLNDNDAIAILEEYRTV 718

Rar-VP2
NAPGLS-FDQVRQARTRYSQFTMRIPGQAATPLAESQEYLVVRHLT-PAFIMSMFDKY 667


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DISCUSSION

This study reports the first observation of viral particles containing W370 dsRNAs in preparations from the mycelial tissue of isolate W370 of R. necatrix. RArV shares many properties with members of the family Reoviridae, such as double-shelled particles (Mertens et al., 2000). Intact particles of RArV were found to be similar in size to those of CTFV and EYAV (80 nm in diameter) (Attoui et al., 2000, 2002), but the inner capsids of RArV (60–65 nm) were slightly larger than those of CTFV and EYAV (50 nm). The RArV particles had a B-spiked core and showed a resemblance to Fijivirus particles (Milne & Lovisolo, 1977); however, similar surface projections have not been observed with CTFV or EYAV particles, in the genus Coltivirus. Whereas reoviruses such as Coltivirus and Fijivirus have B-spikes on the outer capsid, RArV appears to lack these surface projections.

Fig. 2. Alignment of the putative VP2 protein sequence from the ORF of segment 2 of R. necatrix W370 dsRNA virus (RAR-VP2) with VP2 of Colorado tick fever virus (CTF-VP2) and European Eyach virus (EYA-VP2) (Attoui et al., 2000, 2002). Amino acids identical among the three viruses are indicated with an asterisk; chemically similar amino acids are indicated with one or two dots.
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Fig. 3. Rosellinia anti-rot virus (RArV) particles from mycelia of W370 of R. necatrix. (A) Intact virus particles from the crude preparation. (B) An intact virus particle from the partially purified preparation. (C) The spiked core particles from the partially purified preparation (arrows indicate B spikes). (D) A purified intact virus particle. (E) A purified spiked core particle (arrow indicates B spike). Viral preparations were stained with uranyl acetate and are shown at the same magnification. Bar, 100 nm.


