Radish Yellow Edge Virus, a Seed-borne Virus with Double-stranded RNA, of a Possible New Group

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夏秋知英*・山下修一**・土居養二**・奥田誠一*・寺中理明*：ダイコン葉縁黄化
ウイルス—新グループの種子伝染性二本鎖 RNA ウィルス

Abstract

Radish yellow edge virus (RYEV) was purified from infected Japanese radish (*Raphanus sativus* L.) by CsCl equilibrium density gradient centrifugation. Virus particles banded with a buoyant density of about 1.37g/cm^3 in CsCl. Purified RYEV preparations showed UV absorption spectrum characteristic of a nucleoprotein with $A_{\text{max}}/A_{\text{min}}$ and A_{260}/A_{280} ratios of about 1.10 and 1.39, respectively. Proteins from purified RYEV preparations, electrophoresed in SDS-polyacrylamide gels, migrated as two species with mol. wt. of about 63,000 and 61,000. RYEV nucleic acid was judged to be double-stranded ribonucleic acid (dsRNA) from its resistance to RNase in high ionic strength conditions and from its reaction with an antiserum to poly (I): poly (C). In polyacrylamide gel electrophoresis, RYEV dsRNA was fractionated into three major classes with mol. wt. of approximately $1.30, 1.25, 1.21 \times 10^6$ and two minor ones of $1.14, 1.09 \times 10^6$. RYEV was not related serologically to turnip crinkle, turnip rosette, turnip mosaic and cucumber mosaic viruses which infect crucifers or to ten seed-borne viruses which resemble RYEV in particle morphology and biological aspects. It is highly likely that RYEV and several other seed-borne dsRNA viruses constitute a new group of plant viruses.

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Key Words: radish yellow edge virus, seed-borne virus, double-stranded RNA.

Introduction

Radish yellow edge virus (RYEV), detected in Japanese radish (*Raphanus sativus* L.) is a seed-borne virus with small isometric particles about 30 nm in diameter¹⁷⁾. RYEV-infected seedlings usually show no symptoms but sometimes cause mild yellow edge of leaves. RYEV was transmitted through seed in high frequency but not by sap. Virus particles were observed only in cells of vascular bundles in thin sections. Nine seed-borne "temperate" viruses, including spinach temperate virus, carrot temperate virus etc., have been recognized in nine plant species from six families^{14, 16, 17, 18)}. They closely resemble RYEV in particle morphology, transmissibility and some other characteristics. Beet cryptic virus⁵⁾ and other "cryptic" viruses^{6, 7, 10)} also share

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properties similar to those of RYEV.

This paper describes nucleic acid and some other properties of RYEV.

Materials and Methods

RYEV purification. RYEV was purified from infected Japanese radish cv. Minowase grown in sterilized soil. Frozen leaves were homogenized in 0.1 M phosphate buffer (2 ml/g tissue) containing 0.1% thioglycolic acid (pH 7.6). The homogenate was squeezed through two layers of cheese cloth, mixed with half volume of 1:1 chloroform and *n*-butanol and stirred. The emulsion was centrifuged for 10 min at *ca.* $8,000 \times g$ and the supernatant centrifuged for 1.5 hr at $123,400 \times g$. After resuspending the pellets in 0.1 M phosphate buffer and two further cycles of differential centrifugation, final preparation was subjected to 10–40% linear sucrose density gradient centrifugation for 2.5 hr at $68,900 \times g$. Then the partially purified preparation was subjected to 35% w/v CsCl equilibrium density gradient centrifugation for 20 hr at $117,800 \times g$.

Analysis of the protein. Purified RYEV preparation and marker proteins, boiled for 5 min in 0.05 M Tris-HCl (pH 9) containing 2% sodium dodecyl sulfate (SDS) and 1% 2-mercaptoethanol (2-MEC), were subjected to electrophoresis on 12.5% SDS-polyacrylamide slab gel at 40 mA for about 4.5 hr and stained with Coomassie brilliant blue as described by Laemmli⁸⁾.

Nucleic acid extraction and analysis. Purified or partially purified RYEV preparation were incubated with 1% SDS, 0.1% bentonite and 1% 2-MEC in 0.01 M sodium phosphate buffer (pH 7.0) containing 1 mM sodium ethylenediaminetetraacetic acid (Na-EDTA) for 5 min at 50°C and then extracted two times with 80% phenol containing 0.1% 8-hydroxyquinoline. The nucleic acid was precipitated from aqueous phase by adding 2 vol cold ethanol and stored at -20°C . The double-stranded ribonucleic acids (dsRNA) of *Penicillium chrysogenum* virus (PcV, a fungal virus from strain IAM 7326) and rice dwarf virus (RDV, a member of plant reovirus) were prepared as described by Wood and Bozarth²⁰⁾ and Reddy *et al.*¹⁹⁾ respectively.

Nucleic acids were analysed by electrophoresis in 5% polyacrylamide gel. The buffer used were Loening's (0.04 M Tris, 0.02 M sodium acetate, 1 mM Na-EDTA, pH 7.2)¹¹⁾. Gels were stained with 'Stains all'¹²⁾. For nucleic acid identification, after electrophoresis polyacrylamide gels were incubated with 50 $\mu\text{g}/\text{ml}$ of ribonuclease (RNase) in either distilled water or 0.3M NaCl as described by Morris and Dodds¹³⁾.

Poly (I): poly(C) antiserum. Antiserum to dsRNA was made against complex of synthetic polyinosinic: polycytidylic acid (poly (I): poly (C), Yamasa Co., Choshi, Japan) and methylated bovine serum albumin, and serological tests were done by two dimensional immunodiffusion as described by Francki and Jackson²⁾. The sensitivity of nucleic acids to RNase and deoxyribonuclease (DNase) were tested by incubation of nucleic acids with 0.1 $\mu\text{g}/\text{ml}$ of RNase in 2 or $0.1 \times \text{SSC}$ buffer (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and with 1 $\mu\text{g}/\text{ml}$ of DNase in 0.02 M phosphate buffer containing 3 mM MgCl_2 . The samples, after incubated for 20 min at 30°C, were added 2 vol cold ethanol and then subjected to immunodiffusion tests.

Serology. Antiserum to RYEV was obtained from a rabbit immunized with

four intramuscular injections of purified virus preparation emulsified with Freund's complete adjuvant. Antisera for comparative serological tests were kindly supplied by Dr. R. Hull (turnip crinkle and turnip rosette viruses) and Dr. R. F. White (beet cryptic virus).

Results

Properties of purified RYEV preparation

RYEV formed one band at a density of about 1.37 g/cm³ (Fig. 1). The purified preparation (Fig. 2) showed an UV absorption spectrum characteristic of a nucleoprotein, with $A_{\max}/A_{\min}$ and A_{260}/A_{280} ratios of about 1.10 and 1.39 respectively. The yield of virus was 1 mg per 3 kg tissues, assuming a value of 5.0 for A_{260} (1 mg/ml).

Protein

When electrophoresed in SDS-polyacrylamide gels, proteins from RYEV particles migrated as two species, with estimated mol. wt. of about 63,000 and 61,000 (Fig. 3 A and B).

Fig. 1. A band (arrow) of RYEV particles (shown in Fig. 2) formed by CsCl equilibrium density gradient centrifugation.

Fig. 2. Purified RYEV particles stained with 2 % phosphotungstate pH 6. Bar represents 100 nm.

Nucleic acid

The gels in which the preparation of RYEV nucleic acid was electrophoresed were incubated with RNase in either distilled water or 0.3 M NaCl. Bands of RYEV nucleic acid were readily digested in distilled water but not in 0.3 M NaCl. RYEV

Fig. 3. A: Electrophoresis of RYEV protein in 12.5% SDS-polyacrylamide slab gel, (a) RYEV protein, (b) cucumber mosaic virus protein, (c) tobacco mosaic virus (TMV) protein.

B: Molecular weights of RYEV and marker proteins estimated from mobility in 12.5% SDS-polyacrylamide slab gel. Marker proteins used are; bovine serum albumin (BSA, MW 66,000), aldolase (AI, MW 39,000), carbonic anhydrase (CA, MW 28,800), and TMV protein (TMV, MW 17,500).

Fig. 4. Gel immunodiffusion test. A: well (As) contained poly (I) : poly (C) antiserum, the rest contained PcV dsRNA (1), RYEV nucleic acid (2), total nucleic acid from healthy tobacco leaves (3), and cucumber mosaic virus single-stranded RNA (4), B: well (As) contained poly (I) : poly (C) antiserum, the rest contained RYEV nucleic acid treated with RNase in 0.1×SSC (1), treated with RNase in 2×SSC (2), treated with DNase (3), and untreated (4).

nucleic acid gave one band in two dimensional immunodiffusion tests against poly (I): poly (C) antiserum (Fig. 4), reacting with 1 mg/ml of poly (I): poly (C) to a titer of 1: 64. Precipitine lines were detected when RYEV nucleic acid was incubated with DNase or RNase in $2 \times \text{SSC}$ but not with RNase in $0.1 \times \text{SSC}$. The nucleic acid gave three major and two minor bands when electrophoresed. The apparent mol. wt. of RYEV nucleic acid segments, using RDV dsRNA as markers, were approximately $1.30, 1.25, 1.21 \times 10^6$ for majors and $1.14, 1.09 \times 10^6$ for minors (Fig. 5). These results indicated that the nucleic acid of RYEV consists of dsRNAs. The determination of whether RYEV segmented dsRNAs are encapsidated in one particle or separate ones must await further studies.

Serology

In double diffusion tests, purified RYEV preparation didn't react with any antisera to turnip crinkle, turnip rosette and beet cryptic viruses. Antiserum to RYEV reacted specifically with the purified preparation to dilutions of 1/128 but not with crude radish extract, turnip mosaic or cucumber mosaic viruses.

In immune electron microscopic observations, RYEV antiserum didn't react with seed-borne "temperate" viruses isolated from spinach (*Spinacia oleracea*), leaf beet (*Beta vulgaris* var. *cicla*), carrot (*Daucus carota*), garland chrysanthemum (*Chrysanthemum coronarium*), santosai (*Brassica rapa* var. *amplexicaulis* subvar. *dentata*), mibuna (*B. rapa* var. *laciniiifolia*), garden rhubarb (*Rheum rhabonticum*), alfalfa (*Medicago sativa* subsp. *sativa*) and white clover (*Trifolium repens*)^{14,16,17,18}.

Fig. 5. Electrophoresis of RYEV ds RNA in 5 % polyacrylamide slab gel: (a) RDV dsRNA as marker, MW 2.50×10^6 (2), 2.25×10^6 (3), 1.90×10^6 (4), 1.80×10^6 (5), 1.15×10^6 (6), 1.13×10^6 (7), 0.86×10^6 (8), 0.78×10^6 (9), 0.75×10^6 (10) and 0.52×10^6 (11,12), segment No. 1, MW 3.10×10^6 , is not shown here; (b) RDV dsRNA and RYEV dsRNA; (c) RYEV dsRNA, detected three major and two minor species (arrows).

Discussion

The data presented here and in the previous report¹⁷⁾ show that RYEV has properties as follows; (1) high frequency (80-100%) of seed transmission, (2) untransmissibility

by mechanical inoculation, (3) isometric particles about 30 nm in diameter, (4) low concentration in infected plants which usually show no symptoms, (5) virus particles confined to vascular bundle cells, (6) sedimentation coefficient of 118S and buoyant density of about 1.37 g/cm³, (7) containing two polypeptides, and (8) the nucleic acid consist of segmented dsRNA. These properties are evidence to distinguish RYEV from all plant virus groups described by Matthews¹²⁾. The only other viruses which have similar properties to those of RYEV are spinach temperate virus (STV), beet temperate virus (BTV), garland chrysanthemum temperate virus, rhubarb temperate virus, carrot temperate virus, alfalfa temperate virus (ATV) and white clover temperate virus (WCTV)^{14,16,17,18)} and "cryptic" viruses^{5,6,7,10)}. Because of the lack of suitable infectivity test, of the very low concentration of virus in sap and of the symptomless infection, it is difficult to study the biological and physicochemical properties of "temperate" viruses and the serological relationships among them. Serological experiments have shown that RYEV clearly differs from all "temperate" viruses and beet cryptic virus. Furthermore, STV¹⁵⁾, BTV¹⁵⁾, ATV¹⁴⁾ and WCTV¹⁴⁾ have been revealed to contain segmented dsRNA. There are differences in molecular weight distribution of dsRNA among STV, BTV, ATV, WCTV and RYEV. Carnation cryptic virus also contain dsRNA⁹⁾. Because these seed-borne dsRNA viruses differ from all plant virus groups established¹²⁾, they can be grouped as a new type of plant viruses.

As for nucleic acid, it is known that reoviruses, bacteriophage $\phi 6$ and fungal viruses contain dsRNA genome¹²⁾. Reoviruses and bacteriophage $\phi 6$ differ from RYEV in particle morphology and sizes of dsRNA. Although fungal viruses differ from RYEV in type of host, some of them are similar to RYEV in particle morphology, latent infection, sedimentation coefficient of virus particles, protein mol. wt. and sizes of dsRNA^{3,4)}. It may be interesting to study on the relationships between RYEV and fungal viruses.

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和文摘要

ダイコン葉縁黄化ウイルス—新グループの 種子伝染性二本鎖 RNA ウィルス

夏秋知英・山下修一・土居養二・奥田誠一・寺中理明

ダイコン葉縁黄化ウイルス (RYEV) を CsCl 平衡密度勾配遠心分離により純化した。ウイルス粒子は CsCl 中で浮遊密度が約 1.37 g/cm^3 のところに1本のバンドを形成した。RYEV 純化試料は典型的な核タンパクの紫外線吸収を示し、その $A_{\text{max}}/A_{\text{min}}$ と A_{260}/A_{280} の比はそれぞれ約1.10と1.39であった。RYEV はアブラナ科植物に感染する4種のウイルス (turnip crinkle virus, turnip rosette virus, カブモザイクウイルス, キュウリモザイクウイルス) および RYEV に似た粒子形態と生物学的性状を有する10種の種子伝染性ウイルスと血清反応を示さなかった。RYEV の純化試料から SDS-ポリアクリルアミド電気泳動により分子量約63,000と61,000の2種のタンパクのバンドが得られた。RYEV の核酸は高塩濃度下で RNase に耐性で、poly (I): poly (C) 抗血清と反応することから二本鎖 RNA であることが判明した。RYEV の二本鎖 RNA はポリアクリルアミド電気泳動で分子量が約 1.30, 1.25, 1.21×10^6 の3本のメジャーバンドと、同じく $1.14, 1.09 \times 10^6$ の2本のマイナーバンドに分かれた。RYEV とそれに類似する数種の種子伝染性二本鎖 RNA ウィルスは新しい植物ウイルスグループを形成するものと考えられた。