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Comparison of Biologically Different Isolates of Odontoglossum Ringspot Virus from *Cymbidium* in Japan by Peptide Mapping

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Symptoms on *Cymbidium*, double-stranded (ds) RNA pattern and peptide mapping of coat protein (CP) of five isolates of odontoglossum ringspot virus from *Cymbidium* in Japan were compared. One of the isolates, Cy-1, that produced unique symptoms on *Cymbidium*, showed a distinct peptide mapping pattern from those of the other four isolates by partial digestion of CP with pepsin. All the isolates produced three major dsRNA species of Mr=4.3, 1.4 and 0.6×10^6 in the infected plants.

Key words : Odontoglossum ringspot virus, *Cymbidium*, Peptide mapping, Double-stranded RNA.

INTRODUCTION

Odontoglossum ringspot virus (ORSV), a member of *Tobamovirus*, was first described by Jensen and Gold (1951) as a pathogen of *Odontoglossum grande*. It occurs world-wide and infects commercially important cultivated orchids. Virions of ORSV contain a single molecule of, positive-sense ssRNA, approximately 6 kb in size and a single protein with a $Mr=17.6\times10^3$ (Paul 1975). Coat protein is synthesized by translation of subgenomic RNA, whose nucleotide sequence is identical to that of the 3' end of the viral genome (Dawson 1992, Isomura *et al.* 1991).

This virus causes diamond mottle and mosaic symptoms in *Cymbidium* (Inouye 1966, Paul 1975). Previously, we studied four ORSV isolates from *Cymbidium* for biological and physical properties (Kondo *et al.* 1991). The Cy-1 isolate was originally obtained from naturally infected *Cymbidium* showing clear mosaic and many dark brown spots on the older leaves

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(Inouye 1966), but symptoms of the other isolates in original host plants consisted of mosaic symptoms only (Kondo *et al.* 1991). Coat protein of Cy-1 isolate migrated slightly more slowly than that of the other isolates by SDS-PAGE. However, no serological differences could be detected between the four isolates of ORSV by immunodiffusion tests or DAS-ELISA.

Here we confirmed that the Cy-1 isolate was distinguishable from the other isolates by symptoms on *Cymbidium* and peptide mapping of coat protein.

MATERIALS AND METHODS

Virus isolates

ORSV isolates Cy-Kan, Cy-Kei (Kondo *et al.* 1991) and Cy-Ama (in the present study) were obtained from Oriental *Cymbidium* plants, *Cym. kanran*, *Cym. ensifolium* and *Cym. koran*, respectively. Other isolates of ORSV were Cy-1 (Inouye 1966) and Cy-46 (Inouye 1983), isolated from *Cymbidium* spp., came from our virus collection. All isolates were propagated in *Chenopodium quinoa* and purified from infected leaves as described previously (Kondo *et al.* 1991).

Inoculation to Cymbidium *plant*

Small seedlings of *Cymbidium* (cv. Kenny "Wine Color") were inoculated mechanically with sap from infected leaves of *Tetragonia expansa*. The inoculated plants were maintained in a greenhouse and symptoms were observed over one year.

Preparation of viral coat protein

ORSV coat proteins were prepared from purified viral preparations according to the method of Fraenkel-Conrat (1957).

Peptide mapping

One-dimensional peptide mapping by limited proteolysis of protein was carried out as described by Cleveland *et al.* (1977). *Staphylococcus aureus* V8 protease (EC 3. 4. 21. 19) (Sigma), pepsin (EC 3. 4. 23. 1) (Sigma), chymotrypsin (EC 3. 4. 21. 4) (Sigma) and papain (EC 3. 4. 22. 2) (Merck) were used at a 1 : 5, 1 : 5, 1 : 10 and 1 : 30 enzyme to substrate ratio, respectively. All coat protein samples ($OD_{280nm}^{0.1\%}=1.3$) in distilled water were partially digested for 0.25 hr (papain), 0.5 hr (chymotrypsin), 1.0 hr (V8 protease) or 16 hr (pepsin) at 37°C. The reaction was terminated by adding an equal volume of 0.01M Tris-HCl buffer, pH 6.8, containing 25% sucrose, 2.5% SDS, 5% 2-mercaptoethanol

and the mixture was boiled for 5min. Digested samples were electrophoresed in discontinuous SDS-PAGE (Laemmli 1970), using 15% acrylamide in the resolving gel. The gels were stained with Coomassie Brilliant Blue.

dsRNA extraction and electrophoresis

dsRNAs were extracted from ORSV infected leaves of *C. quinoa* as described by Valverde *et al.* (1990). The dsRNAs were analyzed by electrophoresis using a 6% polyacrylamide slab gel (70×80×1mm) in Tris-acetate-EDTA (TAE) buffer at 50V for 3-4hr. After electrophoresis, the gels were stained with a silver staining kit (Kanto Chemical Co., Tokyo). To estimate molecular weight, dsRNAs isolated from plants infected with tobacco mosaic virus-ordinary strain (TMV-OM, Mr=4.3×10^6) and cucumber mosaic virus-Y strain (CMV-Y, Mr=2.54, 2.26, 1.64, 0.7, 0.2×10^6) were used as standards.

RESULTS

Symptoms on Cymbidium

The primary symptoms of all five isolates were systemic chlorotic spots and streaks on newly developed leaves of *Cymbidium* several months after inoculation. Later these symptoms became mosaic (Fig. 1A) and eventually disappeared on most of the matured leaves of *Cymbidium* inoculated with Cy-Kei, Cy-Ama, Cy-Kan or Cy-46 isolates. On the other hand, the Cy-1 isolate produced many small necrotic spots on younger leaves showing

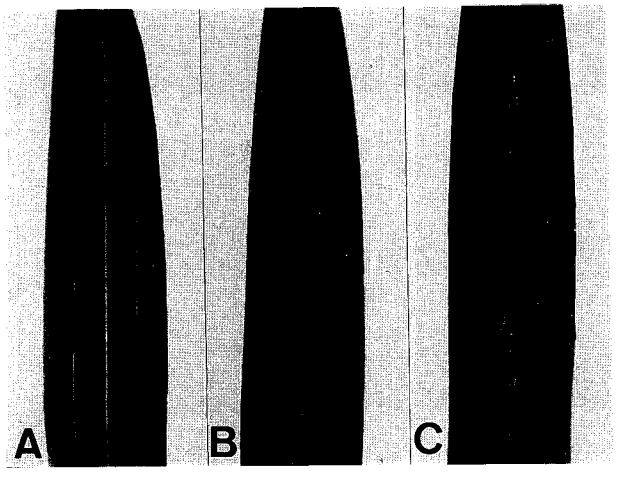

Fig. 1. Systemic symptoms of odontoglossum ringspot virus in *Cymbidium* plants. A. Mosaic on young leaf induced by Cy-46 isolate. B. Mosaic and small necrotic spots on young leaf induced by Cy-1 isolate. C. Mosaic and necrotic spots on older matured leaf induced by Cy-1 isolate.

mosaic symptoms (Fig. 1B) and these necrotic spot symptoms became progressively severe (Fig. 1C). Mosaic and necrotic spots produced by the Cy-1 isolate did not disappear on older matured leaves.

Peptide mapping

Coat proteins of five ORSV isolates formed a single band in SDS-PAGE. The coat protein of the Cy-1 isolate migrated slightly more slowly than that of Cy-Kei, Cy-Ama, Cy-Kan and Cy-46 isolates (data not shown).

Limited digestion of coat protein of five ORSV isolates with V8 protease, pepsin, chymotrypsin and papain produced the band patterns shown in Fig. 2. Partial digestion with pepsin gave patterns with four major bands for the Cy-1 isolate (Fig. 2B, lane 4), but very faint minor bands (Fig. 2B, lane 1, 5) or no visible digested bands (Fig. 2B, lane 2, 3) for the other isolates. Digestion of the Cy-1 and Cy-46 isolates with V8 protease produced five major bands (Fig. 2A, lane 4, 5), but, the smallest bands in the Cy-1 and Cy-46 coat protein digests were absent in the Cy-Kei, Cy-Ama and Cy-Kan digests (Fig. 2A, lane 1, 2, 3). In contrast, the band patterns of partial

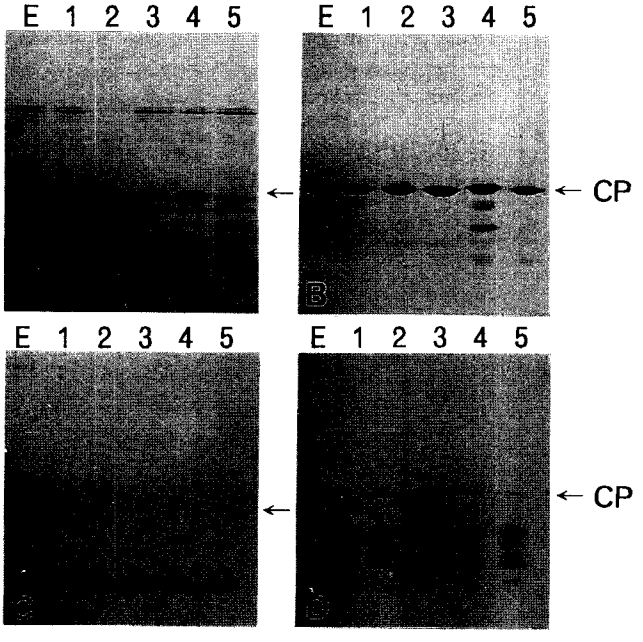

Fig. 2. Peptide maps obtained by discontinuous SDS-PAGE (15% acrylamide in resolving gel) of partial enzymatic digests of coat proteins of five ORSV isolates. The gels were stained with Coomassie Brilliant Blue. Lane 1-5: isolates Cy-Kei, Cy-Ama, Cy-Kan, Cy-1 and Cy-46, respectively. Lane E: protease alone. A-D: electrophoresis of partial digests with V8 protease, pepsin, chymotrypsin and papain, respectively. Arrows indicate the positions of coat proteins (CP).

digestion of all isolates with chymotrypsin (Fig. 2C) and pepsin (Fig. 2B) were indistinguishable except for the intensity of each band.

Patterns of dsRNA

dsRNA was isolated from the leaves of *C. quinoa* infected with five ORSV isolates and analyzed by PAGE. Three major dsRNA species of estimated Mr 4.3×10^6 (dsRNA 1), 1.4×10^6 (dsRNA 2) and 0.6×10^6 (dsRNA 3) were consistently detected with some faint subsidiary bands, in all extracts from plants infected with the five ORSV isolates (Fig. 3). No comparable

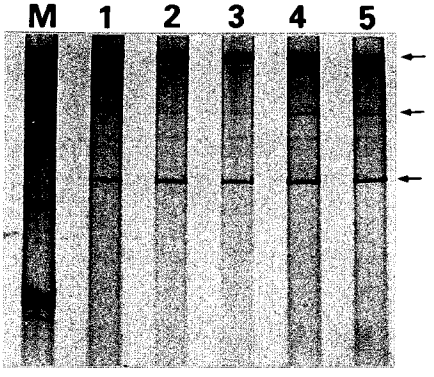

Fig. 3. Polyacrylamide gel electrophoresis (6%) of double-stranded (ds) RNAs extracted from *Chenopodium quinoa* infected with five ORSV isolates. Gel was run at 50V for 4 hr and stained with silver. Lane 1-5: isolates Cy-Kei, Cy-Ama, Cy-Kan, Cy-1 and Cy-46, respectively. Lane 6: extract from healthy *C. quinoa*. Lane M: mixture of dsRNAs extracted from *Nicotiana tabacum* infected with TMV-OM and *N. glutinosa* infected with CMV-Y. Arrows indicate three species of dsRNAs of ORSV isolates.

dsRNAs were detected in the healthy control (Fig. 3, lane 6). No differences could be seen in the mobility of dsRNA species of any isolate studied. The double-stranded nature of bands was confirmed by treatment with RNase A under low- and high-salt conditions (data not shown).

DISCUSSION

Previously, we studied the biological and physical properties of four ORSV isolates from *Cymbidium* (Kondo *et al.* 1991), and showed that the coat protein of the Cy-1 isolate migrated slightly more slowly than that of the other three isolates. In this study, we confirmed that the Cy-1 isolate was distinguishable from the other four isolates by typical symptoms on *Cymbidium*. We performed peptide mapping of these isolates to assess the heterogeneity of the coat protein sequence.

Peptide mapping by partial digestion with pepsin differentiated the Cy-1 isolate from the other four isolates. Since traditional immunological tests such as immunodiffusion and ELISA did not discriminate the Cy-1 isolate from the others, comparison of peptide maps of coat proteins may be a useful tool to differentiate ORSV isolates, as reported on strains of red clover necrotic mosaic virus (Rao *et al.* 1987), cucumber mosaic virus (Edwards and Gonsalves 1983) and citrus tristeza virus (Guerri *et al.* 1990).

The dsRNA analysis revealed that two subgenomic dsRNAs (Mr=1.4 and 0.6×10^6), in addition to a replicative form of genomic RNA (Mr=4.3×10^6) were detected in plants infected with all five ORSV isolates. No differences in the mobility of the fastest migrating dsRNAs of the five ORSV isolates studied here could be found. Three major dsRNAs were involved in plants infected with several tobamoviruses including ORSV (Valverde *et al.* 1986). Valverde *et al.* (1986) noted the size of the fastest migrating dsRNA coding coat protein was variable in size among TMV-type, TMV-U5 and ORSV. However, we found no differences in the mobility of the fastest migrating dsRNAs of the five ORSV isolates studied here.

These findings indicate that the Cy-1 isolate of ORSV which shows unique mosaic and necrotic spots on infected *Cymbidium* can be distinguished from other isolates by peptide mapping using pepsin. Dawson *et al.* (1988) noted that the TMV coat protein gene has biological functions such as symptom expression and induction of hypersensitive reactions in plants with N gene. The significance of our results in relation to differences in symptoms on *Cymbidium* can not yet be assessed. As the nucleotide sequence of Cy-1 coat protein gene has been determined (Isomura *et al.* 1991), analysis of the coat protein gene of other isolates is awaited.

REFERENCES

Cleveland, D. W., Fischer, S. G., Kirschner, M. W. and Laemmli, U. K. (1977). Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. 252 : 1102-1106.

Dawson, W. O., Bubrick, P. and Grantham, G. L. (1988). Modifications of the tobacco mosaic virus coat protein gene affecting replication, movement, and symptomatology. Phytopathology 78 : 783-789.

Dawson, W. O. (1992). Tobamovirus-plant interactions. Virology 186 : 359-367.

Edwards, M. C. and Gonsalves, D. (1983). Grouping of seven biologically defined isolates of cucumber mosaic virus by peptide mapping. Phytopathology 73 : 1117-1120.

Fraenkel-Conrat, H. (1957). Degradation of tobacco mosaic virus with acetic acid. Virology 4 : 1-4.

Guerri, J., Moreno, P. and Lee, R. F. (1990). Identification of citrus tristeza virus strains by peptide maps of virion coat protein. Phytopathology 80 : 692-698.

Inouye, N. (1966). A virus disease of *Cymbidium* caused by odontoglossum ringspot virus. Ber. Ohara Inst. landw. Biol. Okayama Univ. 13 : 149-159.

Inouye, N. (1983). Host range and properties of a strain of odontoglossum ringspot virus in Japan. Nogaku Kenkyu 60 : 53-67. (in Japanese with English abstract).

Isomura, Y., Matumoto, Y., Murayama, A., Chatani, M., Inouye, N. and Ikegami, M. (1991). Molecular cloning, sequencing and expression in *Escherichia coli* of the odontoglossum ringspot virus coat protein gene. J. Gen. Virol. 72 : 2247-2249.

Jensen, D. D. and Gold, H. A. (1951). A virus ringspot of *Odontoglossum* orchid : symptoms, transmission and electron microscopy. Phytopathology 41 : 648-653.

Kondo, H., Maeda, T. and Inouye, N. (1991). Host range and properties of odontoglossum ringspot tobamovirus (ORSV) isolated from miniature *Cymbidium* in Japan. Bull. Res. Inst. Bioresour. Okayama Univ. 1 : 21-34. (in Japanese with English abstract).

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 : 680-685.

Paul, H. L. (1975). Odontoglossum ringspot virus. CMI/AAB Descriptions of Plant Viruses No.155. 4pp.

Rao, A. L. N., Lakshman, D. K., Ohki, S. T. and Hiruki, C. (1987). Identification of a new serotype and antigenic relationships among six strains of red clover necrotic mosaic virus. Phytopathology 77 : 995-999.

Valverde, R. A., Dodds, J. A. and Heick, J. A. (1986). Double-stranded ribonucleic acid from plants infected with viruses having elongated particles and undivided genomes. Phytopathology 76 : 459-465.

Valverde, R. A., Nemeth, S. T. and Jordan, R. L. (1990). Analysis of double-stranded RNA for plant virus diagnosis. Plant Dis. 74 : 255-258.

日本において *Cymbidium* 属植物から分離された生物学的性質の異なる Odontoglossum Ringspot Virus 分離株のペプチドマッピングによる比較

近藤秀樹・前田孚憲・井上成信

日本において *Cymbidium* 属植物から分離された odontoglossum ringspot virus (ORSV) 5分離株間の比較を行った．接種試験の結果，Cy-1分離株（井上 1966）は *Cymbidium* での病徴が他の4分離株と異なっていた．外被タンパク質のペプチドマッピングでは，ペプシンを用いた場合 Cy-1分離株において特異的な部分分解バンドが認められ，そのバンドパターンが他の分離株と大きく異なった．また，V8プロテアーゼによる部分分解パターンも分離株間でわずかに異なったが，キモトリプシンとパパインでは差は認められなかった．ORSV 感染葉から2本鎖 RNA を抽出し PAGE を行ったところ，すべての分離株で分子量が4.3，1.4，0.6×10^6の3種のバンドが検出されたが，これらの2本鎖 RNA の分子量は分離株間で差がなかった．

キーワード：オドントグロッサムリングスポットウイルス，シンビジウム，ペプチドマッピング，二本鎖 RNA