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Identification and Characterization of a Ringspot Isolate of *Odontoglossum ringspot virus* from *Cymbidium* var. 'Grace Kelly'

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An isolate of *Odontoglossum ringspot virus* (ORSV) was identified from *Cymbidium* var. 'Grace Kelly' showing ringspot symptom on the floral and leaf parts, and was denoted as cymbidium ringspot isolate (ORSV-CR). In ultrathin sections of leaf tissue from diseased *Cymbidium* plants, clusters of virus particles were observed in the vacuole and cytoplasm. In the Western blot hybridization, the virus strongly reacted with ORSV-specific antiserum indistinguishable from ORSV, suggesting that the virus is serologically identical with ORSV. ORSV-CR sap was inoculated onto 20 species belonging to 12 genera. Systemic infection occurred in *Cymbidium* sp., *Nicotiana benthamiana* and *N. clevelandii,* the host of which was found to be different from that of ORSV-Cy, the Korean strain of ORSV. The analysis of coat protein (CP) gene showed that ORSV-CR was highly homologous to the known isolates of ORSV, with over 95.6% identity in amino acid level. Phylogenetic tree analysis of CP showed that ORSV-CR was clustered with the known ORSV isolates, suggesting that ORSV is a very stable tobamovirus.

Keywords : coat protein, *Cymbidium*, identification, *Odontoglossum ringspot virus*, *Tobamovirus*.

Orchids are one of the most important ornamental plants in Korea, and are valued for their flowers, which produce diverse colors and shapes. Of the more than 50 orchid viruses, the genus *Tobamovirus Odontoglossum ringspot virus* (ORSV) and the *Potexvirus Cymbidium mosaic virus* (CymMV) cause severe damage to orchid plants. Both CymMV and ORSV are distributed throughout the world (Ryu and Park, 1995; Zettler et al., 1990). ORSV has elongated, rigid rod-shaped particles, which are non-enveloped, 300 nm long and 18 nm wide, similar with that of *Tobacco mosaic virus* (TMV). The virus is mechanically and wound transmissible, but has no efficient natural vectors, such as aphid (Gibbs, 1977; Namba and Ishii, 1971).

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Some studies have demonstrated that ORSV caused streak or stripped mosaic, diamond mottle, or ringspot symptoms on leaves (Corbett, 1967; Matthews, 1991). Sequences of full-length cDNAs for three isolates of ORSV RNA geonome have been reported (Chng et al., 1996; Ikegami et al., 1995; Ryu and Park, 1995). This study described the general characteristics and the CP gene analysis of ORSV strain isolated from *Cymbidium* var. 'Grace Kelly', including biological and physiochemical properties, and sequence differences with previously reported ORSV strains.

Materials and Methods

Sources of plant and virus. *Cymbidium* var. 'Grace Kelly' showing black ringspot symptom on leaves was used as source for isolation of the virus. Ringspot and necrosis were observed on flower parts (Fig. 1). To identify the casual agent, the existence of ORSV in the diseased *Cymbidium* var. 'Grace Kelly' was investigated by electron microscopy and serological method. Virus particles were extracted by the method of Park et al. (1990).

To identify the virus, Western blot hybridization was carried out using the antiserum against ORSV. Total proteins were extracted from the virus-infected leaf sample, as described previously (Ryu et al., 1994). The total proteins were separated on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with the Cy strain of ORSV (Ryu and Park, 1995) as the control, according to standard protocol (Sambrook et al., 1989), and were electroblotted to nitrocellulose membrane. The membrane was incubated with the antiserum specific to ORSV (1:1,000 dilutions) and was washed three times by TBST buffer (Choi et al., 2002). Subsequently, the membrane was incubated with the solution of anti-rabbit-goat IgG (1:7,500 dilutions, Promega, USA) antibody conjugated with alkaline phosphatase as the secondary antibody. Color reaction was developed with Western Blue Stabilizer Substrate solution (Promega).

Host range test. Biological properties of the virus were investigated by symptomatology on selected indicator plants and by

Fig. 1. Ringspot symptoms on leaves (**A**) and flowers (**B**) of *Cymbidium* var. 'Grace Kelly' induced by *Odontoglossum ringspot virus*.

comparing with those of ORSV-Cy (Park et al., 1990). Indicator plants were mechanically inoculated at the second or third foliage stage (Table 1). The local or systemic symptoms of tested hosts were analyzed 2-4 weeks after inoculation, while symptoms of orchid plants inoculated with the virus were observed after more than 6 months of inoculation.

Virus purification. The identified virus, designated as CR strain of ORSV, was serially passed onto tobacco plants (cv. Xanthi-nc) by mechanical inoculation. The local legion was used as the inoculum with 0.1 M phosphate buffer (pH 7.2), and inoculated onto tobacco cv. Samsun. The systemically latent infected leaves were harvested 14 days after inoculation, and grinded with 0.2 M sodium phosphate buffer (pH 7.4) containing 0.1% thioglycolic acid and 20 mM EDTA. Crude saps were passed through three layers of Miracloth, and were incubated with chloroform. The supernatant was subjected to differential ultracentrifugation and was further purified by sucrose density-gradient centrifugation (Ryu et al., 1994). The pellet containing purified virus particles was resuspended with 0.01 M phosphate buffer (pH 7.4).

RT-PCR and analysis of coat protein gene. To examine the genetic variability of ORSV-CR, RT-PCR was carried out by using the method previously described (Ryu et al., 1995). ORSV-specific primers (ORP1: 5'-AATGAACCTGGAGAAGGATT-3', AJ001098 and ORP2: 5'-AATTTGCCGGACAATTGCAA-3', AJ 001099) with RNAs of ORSV-Cy strain were used for amplification of target gene. TMV was obtained from the Plant Virus GenBank, Seoul, Korea and used as the control. Total

Table 1. Comparison of host reactions of two strains of *Odontoglossum ringspot virus*

Indicator plant	Symptom[a]	
	CR strain	Cy strain
Brassica perkinensis L. *rapifera*	–/–[b]	–/–
B. perkinensis L. *campestris*	–/–	–/–
Cattleys lababita	–/–	mM/Ch
Chenopodium amaranticolor	Cl/–	Cl/–
C. quinoa	Cl/–	Cl/–
Cucumis sativus	–/–	–/–
Cucurbita pepo	–/–	–/–
Cymbidium var. 'Grace Kelly'	N/NR	mM/M
Cymbidium goeringii	mM/M	mM/M
Datura stramonium	Nl/–	–/–
Gomphrena globosa	–/–	N/–
Lycopersicon esculentum	–/–	–/–
Nicotiana benthamiana	L/M	–/–
N. clevelandii	L/M	L/–
N. rustica	L/–	–/–
N. tabacum cv. Samsun	L/L	L/–
N. tabacum cv. Xanthi-nc	Cl/–	Cl/–
Phalaenopsis violacea	–/–	mM/mM
Phaseolus vulgaris	–/–	–/–

[a] Symbol for symptom: Ch=chlorosis; Cl=chlorotic local legion; Nl=necrotic local legion; N=necrosis; NR=necrotic ringspot; mM=mild mosaic; M=mosaic; L=latent infection; – =no infection.
[b] Inoculatead leaf/systemic leaf.

RNAs were extracted from purified virus particles as described previously (Ryu et al., 1995), and were used as templates for RT-PCR analysis.

RT reaction was performed at 42°C for 60 minutes in 20 μl volume containing 10 mM Tris-HCl buffer (pH 8.3), sample RNA (ca. 100 ng), 5 mM $MgCl_2$, 50 mM KCl, 1 mM dNTPs, primer ORP1 (50 pmol), 1 unit of RNase inhibitor, and 2.5 units Moloney murine leukemia virus reverse transcriptase. The cDNAs were amplified with 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer), ORP1, ORP2, and 0.2 mM dNTPs. The PCR condition was followed: 94°C for 5 minutes (1 cycle), 94°C for 1 minute, 46°C for 1 minute, 72°C for 1 minute (35 cycles), and 72°C for 5 minutes as post-elongation step. The synthesized PCR fragments were separated on 1.6% agarose gel (Sambrook et al., 1989).

Amplified DNA fragments from CR strain were completely digested by restriction endonucleases, *Hin*dIII, *Pst*I, *Xba*I, *Bsr*I, *Dpn*I, and *Rsa*I, and compared with those of Cy strain. Restriction-digested PCR products were fractionated on 1.6% agarose gel. The amplified product was cloned into pGEM-T Easy vector (Promega) and the clone containing CP gene was sequenced by dideoxy termination method. Sequence analysis was done by using the DNASTAR program (Lagergene, USA) for multiple alignment and phylogenetic tree analysis.

Results

Identification of ORSV from diseased orchid plant. *Cymbidium* var. 'Grace Kelly' showing ringspot symptom on both leaves and flowers (Fig. 1) was analyzed for identification of causal virus by using the electron microscopy and immunological methods. The morphology of the causal virus was typical rigid rod-shaped particles similar with that of other tobamoviruses, approximately 300 nm long and 18 nm wide, and the virus particles were dispersed through the cytoplasm (Fig. 2). The virus particles appeared as a bundle of irregular aggregates of various lengths called stacked plates or round plates in ultrathin section of the tested orchid (Edwardson and Christie, 1978; Milicic and Stefanac, 1971). Moreover, chloroplast and other cell organelles showed malformed shapes in the cells infected by the virus. Distinct inclusion body called X-body and paramural bodies were also observed on both the cellular membrane and cytoplasm containing vacuole-like cavities in the cells of 'Grace Kelly' orchid (data not shown). The

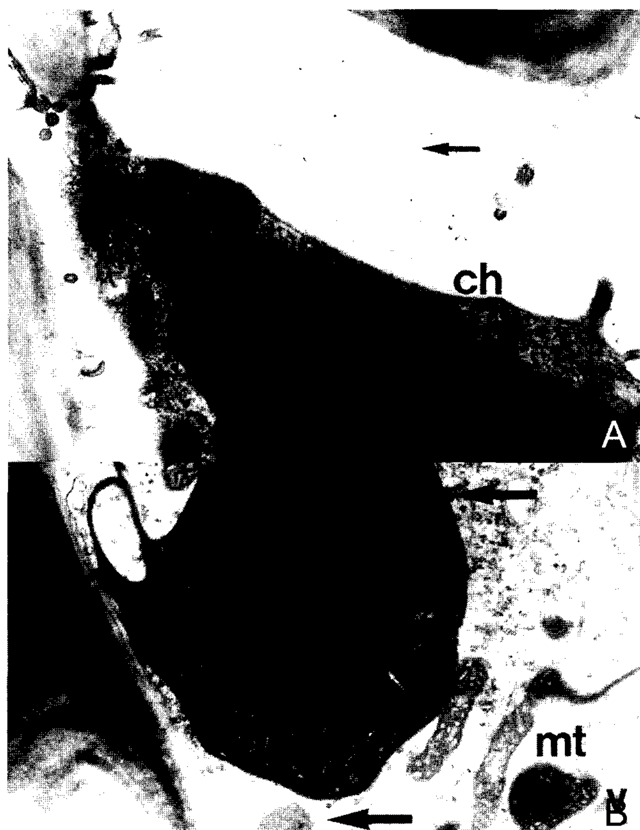

Fig. 2. Electron micrographs of leaves from *Cymbidium* var. 'Grace Kelly' infected by ORSV. (**A**) The rigid rod-shaped virus particles were observed in cytoplasm (indicated by arrow) arranged adjacent to chloroplast (**B**). The virus particles were stacked and rounded on plate indicated by arrow (ch: chloroplast, mt: mitochondria).

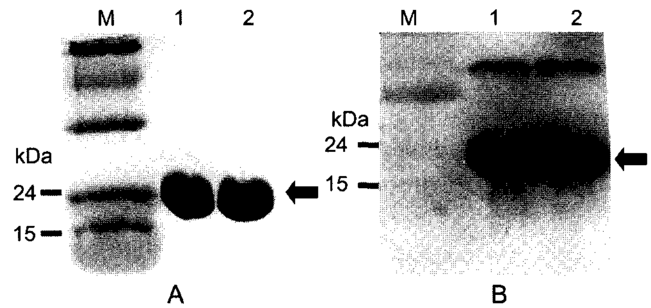

Fig. 3. Analysis of coat protein of ORSV by Coomassie-stained SDS-PAGE (**A**) and Western blot assay (**B**). Lane M, protein marker; lane 1, ORSV-CR; lane 2, ORSV-Cy. The arrows indicate the 18 kDa CP of ORSV.

analysis of coat proteins for ORSV-CR on SDS-PAGE revealed the electrophoretic homogeneity with ORSV-Cy strain (Fig. 3A). The molecular weight of the CP of CR isolate was estimated at about 18 KDa. In the Western blot assay, ORSV-CR particles reacted positively to the polyclonal antibody against ORSV (Fig. 3B). The results suggest that ORSV-CR is an isolate of ORSV.

Purification and host reaction of ORSV-CR. ORSV-CR particles were purified from tobacco plants showing systemic symptom by the combination methods of PEG precipitation, differential centrifugation, and sucrose gradient centrifugation (Ryu et al., 1995). Purified ORSV-CR particles were confirmed by electron microscopy. The purified virions showed rigid rod-shaped particles about

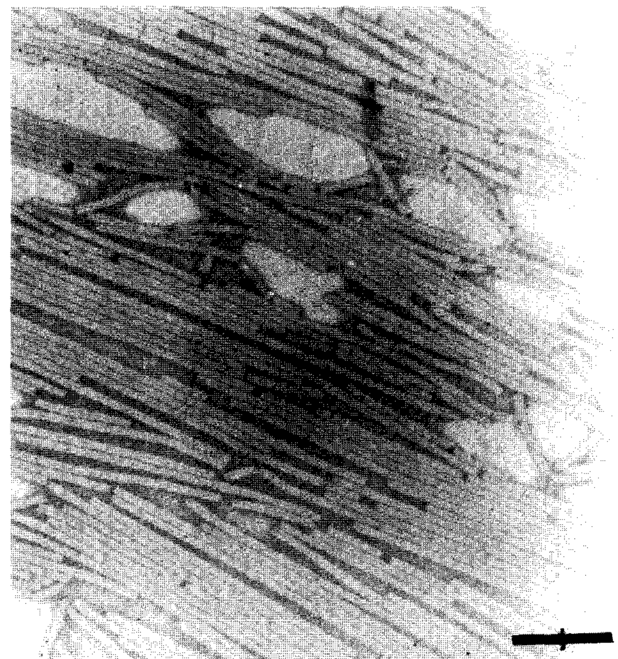

Fig. 4. Electron micrograph of purified ORSV-CR particles stained with 2% sodium phosphothungstic acid. The scale bar represents 100 nm in length.

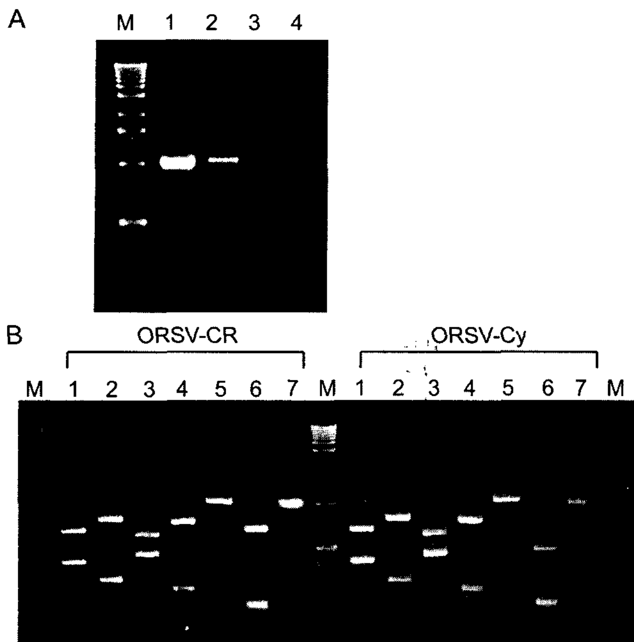

Fig. 5. Gel electrophoresis of RT-PCR products (**A**) and restriction-digested PCR fragments (**B**) by restriction endonucleases. Panel (A), lane M, 1 kb DNA marker; 1, ORSV-CR; 2, ORSV-Cy; 3, TMV; 4, CymMV. Panel (B), lane M, 1 kb DNA marker (BRL); 1, *Hind*III; 2, *Pst*I; 3, *Xba*I; 4, *Bsr*I; 5, *Dpn*I; 6, *Rsa*I; 7, undigested.

300 nm×18 nm, which is a typical morphology of the genus *Tobamovirus* (Fig. 4). Based on the common tobamovirus extinction coefficient that 3.0 A_{260} unit is equivalent to 1.0 mg/ml (Gibbs, 1977; Zatlin 1954), the virus yield of ORSV-CR was 40 mg per 100 g of fresh leaf weight and A_{260}/A_{280} was 1.29 (data not shown).

To know the difference in biological properties with previously reported strains of ORSV, 20 species of plants in 12 genera were used as host range of ORSV-CR. As shown in Table 1, host reactions to ORSV-CR were different from those of ORSV-Cy. ORSV-CR did not infect *Brassica* sp., *Cucumis sativus, Cucurbita pepo, Gomphrena globosa, Lycorpersicon esculentum, N. tabacum* cv. Xanthi, and *Phaseolus vulgaris*. ORSV-CR was not able to infect two orchids, *Cattleys lababita and Phalaenopsis violacea,* while ORSV-Cy did infect these two orchids.

Analysis of coat protein gene. To compare the genetic variability of ORSV-CR, RT-PCR was carried out using a pair of ORSV-specific primers, and PCR products were analyzed by RFLP and sequence determination. Single band of RT-PCR product generated from the sample containing RNA of ORSV-CR was detected (Fig. 5A). The same result was obtained with total RNA sample from diseased orchid plants (data not shown). The results suggest that the virus is a strain of ORSV.

The patterns of RT-PCR products of ORSV-CR and Cy strains digested by *Hind*III, *Pst*I, *Xba*I, *Bsr*I, *Dpn*I, and *Rsa*I were not identical (Fig. 5B). CP of CR isolate consisted of 158 amino acids as determined by sequence analysis of CP gene. The length was the same as that of previously published sequences of ORSV isolates. The N-terminal region of the CP was more conserved than the C-terminals and there were no distinct regions of variability in the CP. Sequence identity of the CP indicated 95.6-99.4% homology in amino acid level between ORSV-CR and other ORSV isolates. The phylogenetic tree analysis showed that the CR isolate could not be distinguished from other isolates of ORSV (Fig. 6). These results indicated that the genetic diversity in the CP gene sequences of ORSV infecting orchids was very low, and that ORSV isolates did not cluster according to origin of hosts or geographic origin.

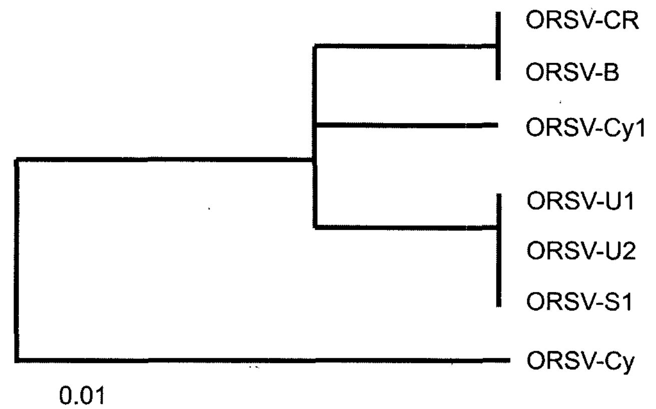

Fig. 6. Phylogenetic tree of the CP gene of ORSV at amino acid level. Bar represents a genetic distance of 0.01. The CP sequences of 6 isolates of ORSV were obtained from EMBL/GenBank databases (B, AF515606; CR, AF033848; Cy, X82130; Cy1, E04305; S1, U34586; U1, U89894; U2, AF141927).

Discussion

ORSV, a member of the genus *Tobamovirus*, has infected orchid species worldwide causing considerable economical losses (Edwardson and Zettler, 1986). In this study, ORSV-CR isolated from *Cymbidium* var. 'Grace Kelly' was characterized biologically and molecularly. The virus induced ringspot symptom on floral and leaf parts (Fig. 1). Initial diagnostic tests indicated that this virus was an isolate of ORSV and could be clearly distinguished from other ORSV strains by host reactions and PCR-RFLP analysis.

The symptoms of ORSV CR isolate, which is considered as a severe isolate of ORSV, have been causing serious damage in orchids although experimental host range is narrower than other known isolates of ORSV (Park et al., 1990). Symptom of the virus is very similar to the first reported ORSV by Jensen and Gold (1951). The observed

differences in host range test and symptom on orchids and tobacco plants appeared to be caused by the difference in the virus strain level. Interestingly, CR isolate could not infect two orchids (Table 1), which were known to be severely damaged by ORSV. However, the virus was able to infect *N. benthamiana* and *N. clevelandii*, while most ORSV isolates could not infect this species. Based on the results of the host range tests, the CR isolate that caused severe loss of quality of *Cymbidium* orchids might be an adaptive strain after specific host-interactions. These results demonstrated that the adaptation ability of TMV is correlated with host specificity and host range (Dawson, 1992), which might confer to fitness and evolution of viruses. In addition, host range and symptoms of some isolates of CMV from lily plants were different from those of representative CMV strains, in spite of the highly conserved sequences of genomic RNAs isolated from different geographic locations (Chen et al., 2001).

In many cases, the inclusion body of the virus is the key in identifying the specific viruses. Tobamoviruses mostly induce hexagonal, stacked, rounded plates, paracrystallines, and angled-layer aggregates in infected tissues (Christie and Edwardson, 1977; Edwardson and Christie, 1978; Toussaint et al., 1984). In ultrastructural investigations, ORSV-CR particles from infected leaf tissues of *Cymbidium* var. 'Grace Kelly' were concentrated and found in most parts of the cytoplasm. The distribution of the CR isolate was within the cytoplasm and intercellular space, and the stacking morphology of the viruses appeared as bundles of irregular aggregates that were stacked plates or round plates similar to that of other ORSV strains and tobamoviruses (Fig. 3B; Milicic and Stefanac, 1971). Especially, the distortion and /or disruption of chloroplasts in the virus-infected leaf may be responsible for the expression of symptom induced by the virus. In addition, the X-body of ORSV was observed to be quantitatively distinct from that of TMV (Granett and Shalla, 1970).

Analysis of the CP gene suggested that the sequences of CP gene for CR isolate was highly conserved, compared with that of the other strains, but not identical. As reported previously, members of the genus *Tobamovirus* have demonstrated to be a genetically stable population. Ajjikuttira et al. (2002) recently reported that isolates of ORSV collected from four different countries were very stable in the CP gene. Genetic variation is very limited in natural selection, resulting in maintenance of highly stable populations in both time and space (Fraile et al., 1996). This study did not determine the full nucleotide sequences of genomic RNAs. The difference in sequences within the CR strains might have affected the virus movement, which plays a role in host-specific determinant (Dawson, 1992), and which produces distinct symptom reactions or spread of virus in host plants. The relationship between sequences and host specificity of ORSV may be determined in the next study.

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