Ribgrass Mosaic Tobamovirus Occurred on Chinese Cabbage in Korea

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A tobamovirus, Ribgrass mosaic virus (RMV), was identified newly from chinese cabbage (Brassica campestris L. pekinensis) in Korea. Virus disease incidence of RMV on chinese cabbage was 37.9% in alpine area on August in 1993. RMV induced the symptoms of necrotic ring spots, necrotic streak on midrib and malformation. RMV, Ca1 and Ca3 isolate, could infect 35 species out of 45 plants including Chenopodium amaranticolor. Physical properties of RMV Ca1 isolate were very stable as 10⁸ over for dilution end point, 95°C for temperature inactivation point and 18 weeks for longevity in vitro. RMV had the soil transmission rate of 75.0% for the chinese cabbages, ‘Chunhawang’ and ‘Seoul’ cultivars. The purified virions of RMV had the typical ultraviolet absorption spectrum of maximum at 260 nm and minimum at 247 nm. RMV of Ca1 isolate was related serologically with antisera of Tobacco mosaic virus (TMV)-Cym, TMV-O and Pepper mottle virus, but not related with antisera of Odontoglossum ring spot virus, coat protein gene of RMV-Ca1, sized 473 nucleotides, encoded 158 amino acid residues. Nucleotide identity of RMV-Ca1 CP gene was 96.4% with RMV-Shanghai (GenBank accession No. of AF185272) from China and 96.0% with RMV-Impatiens (GenBank accession No. of AM049074) from Germany. Identity of amino acids between RMV-Ca1 and the two RMV isolates was 96.8%. Specific three primers were selected for rapid and easy genetic detection of RMV using Virion Captured (VC)/RT-PCR method.

Keywords: identification, chinese cabbage, Ribgrass mosaic virus

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is needed only 3–4 hours to get results from virus infected plant sap in extraction buffer. VC/RT-PCR is a simple, accurate and economical genetic detection method without any works or commercial kits for the extraction of the nucleic acid from the infected plants, which has been developed for fast detection of plant viruses like TSWV, TYLCV etc (Cho, 2006; 2007; 2008).

Genomic studies of Tobamovirus isolates from cruciferous plants have been reported that they form a distinct group from other tobamoviruses (Aguilar et al., 1996). The group has no overlap between the viral replicase and movement protein genes and a long overlap between the movement protein and coat protein genes.

Materials and Methods

Biological test. Indicator plants of 45 species at the 3–4 leaf seedling stage were used. Inoculum was made by the maceration with mortar in a 4 Vol. (W/V) of 0.01M sodium phosphate buffer, pH 7.0. Virions in the inoculum were rubbed mechanically with a piece of wooden towel to the leaves of healthy plants scattered carborundum 600 mesh. Symptoms expressed were read for 2–3 weeks after inoculation.

Purification. Nicotiana debney showing necrotic spots systemically was used for material of virus purification. Purification method was modified as that of TMV (Zaitlin, 1975). Leaves 40 g were ground with Waring® blender for 60 sec in 4 Vol. of 0.1 M sodium phosphate buffer, pH 7.0, containing 0.01 M Na-DIECA and 0.01% thiglycolic acid. The homogenate was stirred for 30 min adding 8% chloroform and n-butanol, 1:1 mixture, followed by centrifugation at 8,000 g for 25 min. The supernatant obtained was stirred for 60 min and left in refrigerator. Pellets were suspended in 0.01 M sodium phosphate buffer, pH 7.0. After low centrifugation, virus into supernatant was pelletted by ultracentrifugation at 130,000 g for 2 hrs. After 2 repeat low- and ultra-centrifugation, the partially purified virus was purified further by 10–40% sucrose density gradient centrifugation at 24,000 rpm for 2 hrs (SRP28SA). Milkish virus band formed on medium of sucrose gradients was removed with hypodermic syringe, and the virions were concentrated by ultracentrifugation.

Serological test. Antisera of RMV-Ca1 was produced using purified virus proteins. Purified virions of 1.0 mg/ml were injected to rabbit by intravenous and muscular combinations for 4 times. Total bleeding was done at one week after final injection. Serological relationships of RMV and Tobamoviruses of TMV-Cym, TMV-0 and Pepper mottle virus were tested with Agar gel double diffusion. Antiserum of RMV- Ca1 was used in the isolation of r-globulin for Enzyme Linked Immuno-Sorbent Assay (ELISA). Gamma-globulin was purified column chromatography of DEAE washing in 0.1 M HCl and NaOH. Collection of r-globulin was done manually in 5 drops after UV absorption test. For ELISA test, the coating immunoglobulin and the enzyme conjugate were used as 1:1000 dilutions. Leaves of chinese cabbage from fields were homogenized in phosphate buffer, pH7.4, containing 0.05% Tween-20 and 2% polyvinylpyrrolidone with a mortar. Two hundred micro liters of the homogenate were added to micro plate wells after coating with gamma globulin. The plate was incubated overnight in the refrigerator. After washing, 200 μl of enzyme labeled gamma globulin was added. P-nitrophenyl phosphate was used as the substrate. Color intensity was read visually.

Electron microscopy. Quick dip preparation was used for the observation of virus particle morphology in samples from fields and purified materials. For ultrastructural studies of virus-infected cells, diseased plants were made by mechanical inoculation with RMV purified biologically. Systemically infected tissues past 2 weeks after inoculation were fixed with 2.5% glutaraldehyde in Millonig’s phosphate buffer, pH 7.3. After treatment of 2% osmium tetroxide, dehydration was done with 50-100% ethyl alcohol with six steps. The dehydrated tissues were embedded in Epon 812 and hardened at 60°C for overnight and then at 90°C for 2 hours. Ultrathin sectioning of 80-100 nm thickness was done with a diamond knife with Sorvall MT 7000, and the ribbons were stained with uranyl acetate and lead citrate for 5 minutes and 7 minutes, respectively. Electron microscope used was Zeiss EM902 at 80 kV.

Soil transmission test. The surface soils were collected from the fields in which chinese cabbage cultivated continuously at Pyungchang Alpine area in 1993, and transferred to the block field in the net house covered with vinyl at Suwon, a middle plane area in Korea. The seedlings at the 3-4 leaf stage of two commercial cultivars of chinese cabbage ‘Chunhawang’ and ‘Seoul’ were transplanted at the blocked field in 1993 and 1994. The viral inspection on the chinese cabbages was done at 80 days after transplanting with visual symptom, ELISA and electron microscopy.

Nucleic acid composition. The purified virus of RMV, Ca1 isolate, was used. Total RNA of Nicotiana tabacum Samsun was extracted from using a QIAGEN RNEasy Plant mini kit by following the manufacturer’s protocol. The nucleotide sequence of coat protein gene of RMV-Ca1 was determined by cloning and sequencing of reverse transcription polymerase chain reaction (RT-PCR). Reverse transcription
reaction was performed using 200 units of M-MLV Reverse Transcriptase (Promega) according to the manufacturer’s instructions. Five micro liter of the mixture were subject to PCR amplification using 2.5 units of Ex Taq DNA Polymerase (TAKARA) with primers R5 (5-ATCCACTGCG-ATGCCAAGCG AAA-3) and R3 (5-TGGGGCCCTAC-CGGGCTAGG-3). Amplifications were 30 cycles each of 30 sec at 95°C, 30 sec at 55°C and 3 min at 72°C with a final extension 5 min at 72°C. PCR fragment was cloned using pGEM-T easy vector system (Promega), and then it was sequenced by MACROGEN. Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems). Analysis of nucleotide and determine of amino acid sequences for RMV were performed through DNASTAR (Madison, WI, USA). The nucleotide and amino acid sequences of RMV-Sh, -Imp and those of other viruses of tobamovirus group were cited from GenBank in NCBI website. The phylogenetic relationship analysis and the genetic distance calculation were conducted through the software MEGA 4.0 with neighboring method. Bootstrap values were calculated out of 1000 replicates.

**Virion captured (VC)/RT-PCR.** Plants used for VC/RT-PCR were chinese cabbage of *Brassica campestris ‘Jeongsang’*, whole radish of *Raphanus acanthiformis ‘Jinjoo’*, and tobacco plants of *Nicotiana occidentalis*. Nine of primers for the RMV isolate were designed through DNASTAR Lasergene 7 with sequences encoding coat protein informed from GenBank accession No. NC-002792 (Table 6). Specificity of primers was tested with VC/RT-PCR using plant sap extracted with the extraction buffer. Condition of amplifications conducted with AccessQuick™ RT-PCR system (Promega®) for VC/RT-PCR was as follows; 48°C, 45m → 94°C, 2m [94°C, 30s → 55°C, 30s → 72°C, 1m30s] 35 cycles → 72°C, 7m. Extraction buffer for VC/RT-PCR (EB) was 0.01 M potassium phosphate buffer, pH 7.0 containing 0.5% sodium sulfite. Plant leaf for VC/RT-PCR was prepared by macerating in EB with 1:3 (weight of plant: volume of buffer) volume rate and centrifuged at 14,000 rpm for 1 minute if it needs to down of plant debris. The procedure of VC/RT-PCR was used reported by Cho et al., 2006. Add 30-50 ul of the macerated sap into PCR tube and incubation for 10 min at 37°C. Wash the PCR tubes with PBST (1x PBS containing 0.05% Tween-20) twice. Wash the tube with nuclease-free water after transfer the empty PCR tubes on the ice. Add master mix and primers for amplification (Promega® AccessQuick). And then the amplified cDNA was electrophoresis.

**Results and Discussion**

**Biological characteristics.** The virus causing the unusual symptom of midrib necrotic streak (MRNSt) on the chinese cabbage at Pyungchang alpine area (Fig. 1A) and Suwon plane area was identified as a *Ribgrass mosaic virus (RMV)* by the biological test, electron microscopy, serological test and so forth. At the fields of Pyungchang in 1993, the necrotic symptoms of MRNSt and necrotic locals could be seen easily on the inner leaves of almost all plants of chinese cabbage at harvesting stage (Fig. 1; B and C). Field samples of chinese cabbage were inoculated mechanically to the general indicator plants of *Chenopodium amaranticolor* and *Nicotiana debney* for obtaining of pure isolate of RMV (Table 1). In Table 2, the biologically purified isolates from the plants showing MRNS could infect systemically on *C. amaranticolor* and *N. debney*, however, those from the plants showing mild mosaic, mosaic or malformation could not. Five isolates of Ac1, AC3, Ac7, Ca1 and Ca4 out of 20 biologically purified isolates having the virulence of systemic infection on *C. amaranticolor* have the virus particles of rigid rod shape having 300 nm length. The biological characteristics for the above five isolates were studied further. The five RMV isolates could infect systemically on 21 plants out of 45 plants, and 12 plants were infected locally. The virus produced the symptoms of vein chlorosis (Fig. 1D), malformation and stunt (Fig. 1E) on chinese cabbage of ‘Chunhawnag’. The typical symptom of NRS was induced at one month later after mechanical inoculation on chinese cabbage ‘Chunhawang’ (Fig. 1F). On cabbage, systemic infection was occurred on the cultivars of ‘Red cabbage’ and ‘White cabbage’, but non infection was showed on ‘Savoy cabbage’. On radish, vein necrosis was produced on the inoculated leaves of the commercial cultivars of ‘Housealtari’ (Fig. 1G) and ‘Chunbaeg’, and then vein chlorosis was produced on the upper leaves (Fig. 1H). Systemic chlorotic spots were produced on *C. amaranticolor* (Fig. 1I). On *C. quinoa*, leaf necrosis of the inoculated leaves was occurred and followed by plant death with stem necrosis (Fig. 1J). In *Nicotiana* plant species, 7 species including *N. debney* were infected systemically with the symptoms of necrotic spots, ring spots, or stem necrosis (Fig. 1K) except *N. tabacum ‘Bright yellow’*. The systemic necrosis on *N. debney* could be used as a typical diagnostic indicator of RMV. The local infection of the isolates of Ac1, Ac3, Ac7, Ca1 and Ca4 on *N. debney* in Table 1 might be caused by the unrecovered virulence from field specimens, because they could infect systemically on the next experiment in Table 2. Plant death was occurred on *N. clevelnadii* (Fig. 2A). On *N. tabacum ‘Ky-57’*, stem necrosis and plant death were occurred (Fig. 2B) after
Chinese cabbages in a field at Pyungchang alpine area were infected severely with virus (A) which produced necrotic spots and mid rib necrotic streaks (B), and whole leaf necrosis (C) on the inner leaves in 1993. The causal agent identified as *Ribgrass mosaic virus* (RMV)-Ca1 produced vein clearing and mosaic (D), and severe stunt with malformation (E) on the upper leaves of Chinese cabbage by mechanical inoculation. The typical symptom of mid rib necrotic streaks of the Chinese cabbage ‘Seoul’ was induced by RMV-Ca1 at 4 weeks after mechanical inoculation (F). On radish of ‘Housealtari’, vein necrosis was produced on the inoculated leaves (G) and vein clearing was induced on the upper leaves (H). RMV-Ca1 could infect systemically after producing necrotic spots on the inoculated leaves of *Chenopodium amaranticolor* (I). On *C. quinoa*, the inoculated leaves were defoliated after showing necrotic spots and then stem necrosis was produced at the point of inoculated leaves, followed by plant death (J). *Nicotiana debney* was produced necrosis on whole plant after showing necrotic spots on the inoculated leaves (K).
showing large amount of necrotic locals on the inoculated leaves (Fig. 2C). RMV could produce large necrotic spots on the inoculated leaves, but could not infect systemically on *N. tabacum* ‘Bright yellow’ (Fig. 2D). RMV-Ca1 had severe virulence on *N. glutinosa* with stem necrosis (Fig. 2E), but no systemic infection was known on *N. glutinosa* (Oshima and Harrison, 1975). Necrotic locals were produced on *Petunia hybrida*, *Physalis floridana*, *Datura stramonium*, *Vigna sesquipedalis*, *Vicia faba*, and *Tetragonia expansa* (Fig. 2F). Malformation with chlorotic spots was produced on *Gomphrena globosa* (Fig. 2I). The RMV had no virulence on *Capsicum annuum*, *Lactuca sativa*, *Lycopersicon esculentum* and *Phaseolus vulgaris* (Oshima and Harrison, 1975). Therefore, the RMV from chinese cabbage in Korea could be an important virus especially on the chinese cabbage and *Nicotiana* species because of its wide virulence.

**Stability in sap.** Three properties of Dilution end point (DEP), Temperature inactivation point (TIP) and Longevity in vitro (LIV) were studied for RMV-Ca1 isolate from chinese cabbage (Table 3). DEP was $10^{-8}$. TIP was 95°C. LIV was 19 weeks. The physical properties of RMV in sap may be studied further with treatments of more dilutions, more longevity and long term keeping at room. RMV in Korea is important relatively in chinese cabbages because it had much more stable in sap than those of Holmes’ ribgrass isolate as $10^{-7}$ for DEP, 93°C for TIP and 18 weeks for LIV (Oshima, 1975), and those of other tobamoviruses including Tobacco mosaic virus (Zaitlin, 1975) or Cucumber green mottle mosaic virus (Hollings, 1975).

**Morphology of virus particles.** RMV was propagated using *N. debney* and purified further through 10-40% sucrose density gradient centrifugation. A milkish virus band was obtained (Fig. 3A). Virus particles were rigid rod shape having 300 nm in average length (Fig. 3B). Ultraviolet spectrum of the purified virus particles of RMV from chinese cabbage was a typical RNA virus curve as 260 nm for maximum absorbance and 247 nm for minimum. A$_{260}$/A$_{280}$ was 1.23. Virus was obtained 0.25 mg per gram tissue.

**Serological relationship.** Three isolates of RMV were
examined with 3 tobamovirus antisera of TMV-Cym from cymbidium, TMV-O from tobacco, Pepper mild mottle virus (PMMoV) from red pepper and Odontoglossum ringspot virus (ORSV) from cymbidium (Table 4, Fig. 3C). RMV-Ca1 was related serologically with three viruses of TMV-Cym, TMV-O and PMMoV, but not related with ORSV. RMV-Ac1 was related with TMV-Cym and TMV-O, however, RMV-Ac3 was related with TMV-Cym and PMMoV. RMV had the serological diversity within its strains of Holmes’s origin, Digitalis and Plantago (Cornelia Ernwein and Carl Wetter. 1987). RMV occurred on chinese cabbage might be related closely those from Solanaceae crops (Oshima, 1975) and their serological variation among RMV isolates may be studied further in the serological diagnosis.

**Ultrastructural characteristics.** The virus particles of RMV were presented in all plant parts including cytoplasm of parenchyma cells (Fig. 3D) and xylem vessel (Fig. 3E). The localization of RMV in conductive tissues might be related strongly with the typical symptoms of vein chlorosis, vein necrosis and mid rib necrotic streaks. The internal symptoms for mass of virus particles in cytoplasm and vacuole were almost same with those of RMV isolates (Oshima and Harrison, 1975), however, those of band structure and angled layer aggregates which were specific ultrastructure of TMV, TMV-U5, Tomato mosaic virus (ToMV) could not observed. Non formation of the tubular specific ultrastructure induced by RMV might be derived by the different virus isolate of ribgrass and chinese cabbage (Franci et al., 1985). An ultrastructure of spiral aggregate (SA) was specific induced by RMV-Digitalis strain (Cornelia Ernwein and Carl Wetter, 1987), but SA is induced easily in the susceptible cultivars or virus combinations, so it would be a specific ultrastructure in synergistic symptom expression by mixed infection with two different virions of Turnip mosaic virus (TuMV) and RMV (Cho et al., 2002, 2003).

**ELISA.** Three factors of ELISA test for RMV were determined as 0.01 μg/ml for r-globulin, 800 dilutions for the conjugated IgG and 1,000 dilutions for the crude sap.

**Nucleic acid composition.** Sequence analysis of the cloned cDNA fragment of RMV-Ca1 genome encoded a coat protein gene was conducted. It composed 473 nucleotides and 158 amino acid residues. The amino acid sequence inferred from the nucleotides which was aligned with other tobamoviruses, especially it had the closer relationship with RMV-Sh and RMV-Imp (Fig. 4) among

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**Table 2. Reactions on the indicator plants of RMV* purified biologically from chinese cabbage by mechanical inoculation**

| Plant name                          | Reactions on the leaves | Inoculated | upper |
|-------------------------------------|-------------------------|------------|-------|
| Brassica campestris pekinensis ‘Chun-hawang’ | NS                      | Vch, Mal, St |
| B. campestris pekinensis ‘Seoul’      | NS                      | Vch, Mal, St |
| B. oleracea ‘Savoy cabbage’          | -                       | -          |
| B. oleracea ‘Red cabbage’            | (-)                     | (-)        |
| B. oleracea ‘White cabbage’          | (-)                     | (-)        |
| Raphanus sativus ‘Housealturi’       | VN                      | -          |
| R. sativus ‘Chunbaeg’                | VN                      | -          |
| Chenopodium amaranticolor             | CS                      | CS, SN, Mal |
| C. quinoa                            | CS, D                   | CS, SN, D  |
| Cucurbita moschata ‘Aihobag-Jungang’ | CL                      | -          |
| C. moschata ‘Aihobag-Hungnong’       | CL                      | -          |
| C. pepo ‘Zucchini’                   | CL                      | -          |
| Cucumis sativus ‘Bagadagi’           | CL                      | -          |
| C. sativus ‘Pungnong’                | CL                      | -          |
| C. melo ‘Kumdoryung’                 | CL                      | -          |
| C. melo ‘Kumnari’                    | CL                      | -          |
| Capsicum annum ‘Hanhyul’             | -                       | -          |
| C. annum ‘Dabog’                     | -                       | -          |
| C. annum ‘Hanganghouse’              | -                       | -          |
| Celosia argentea                     | NS, D                   | M          |
| Datura stramonium                    | NL, SN, D               | -          |
| Gophrena globosa                     | CS, NS                  | CS, NS, Mal|
| Lactuca sativa ‘Cheonghima’          | -                       | -          |
| L. sativa ‘Jeogeohugmyeong’          | -                       | -          |
| Lycopersion esculentum               | -                       | -          |
| Nicotina clevelandii                 | NS, RS, D               | NS, RN     |
| N. debney                           | NS, RS, D               | NS, RS, SN, D |
| N. glutinosa                        | NS, D                   | SN         |
| N. rustica                          | LNS                     | NS, SN     |
| N. tabacum ‘Bright yellow’           | LNS                     | -          |
| N. tabacum ‘Ky-57’                   | LNS, D                  | NS, SN     |
| N. tabacum ‘Samsun NN’               | LNS, D                  | NS, SN     |
| N. tabacum ‘Xanthi NC’               | LNS, D                  | NS, SN     |
| Phaseolus vulgaris ‘Top crop’        | -                       | -          |
| Phaseolus vulgaris ‘Punghyubinnog’   | -                       | -          |
| Phaseolus vulgaris ‘Suwonjaerae’     | -                       | -          |
| Pettenia hybrida                     | NL                      | -          |
| Physalis floridana                   | NL                      | -          |
| Spinach oleracea                     | –                       | M, Mal     |
| Tetragonis expansa                   | NL                      | -          |
| Vigna sesquipedalis                 | NL                      | -          |
| V. unguiculata                      | NL                      | -          |
| Vicia faba                          | NL, Y, D                | (+)        |
| Vinca rosea                         | NS, RNS                 | NS, RNS    |
| Zina eleganum                       | –                       | M          |

*RMV-Ca1 isolates was used from Table 1.
* CS: Chlorotic spot, CL: Chlorotic local, Mal: Malformation, NL: Necrotic local, NS: Necrotic spot, LNS: Large necrotic spot, D: Leaf defoliation, RS: Ring spot, Vch: Vein chlorosis, SN: Stem necrosis, M: Malformation, D: Leaf defoliation, RS: Ring spot, Vch: Vein chlorosis, SN: Stem necrosis.
* (+): Infection without symptoms.
Fig. 2. RMV-Ca1 induced severe symptom of plant death in *N. clevelandii* (A) and stem necrosis and malformation in *N. tabacum* ‘Ky-57’ (B) after producing necrotic spots on the inoculated leaves (C). A large number of local lesions were produced on the inoculated leaves of *N. tabacum* ‘Bright yellow’ (D). The leaf necrosis was produced on the inoculated leaves of *N. glutinosa* and the necrosis was developed to the stem (E). Necrotic spots were produced on the inoculated leaves of *Petunia hybrid* (F), and leaf necrosis was occurred on the inoculated leaves of *Zinia elegance* (G). Whitish locals were produced on the inoculated leaves of *Datura stramonium* (H). Systemic infection with malformation was occurred on *Gomphrena globosa* after showing leaf necrosis of inoculated leaves (I). Necrotic locals on the inoculated leaves were produced on *Vigna sesquipedalis* (J) and *Vicia faba* (K), but large necrotic locals were induced on the inoculated leaves of *Tetragonia expansa* (L).
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**Table 3. Physical properties of RMV-Ca1 occurred on Chinese cabbage**

| Dilution end point | Temperature inactivation point | Longevity in vitro |
|--------------------|--------------------------------|-------------------|
| No. of local*      | Temp. (°C) | No. of local | week | No. of local |
| $10^{-4}$           | 19       | 75          | 61   | 15   | 60   |
| $10^{-5}$           | 18       | 80          | 186  | 16   | 33   |
| $10^{-6}$           | 4        | 85          | 200  | 17   | 13   |
| $10^{-7}$           | 4        | 90          | 7    | 18   | 20   |
| $10^{-8}$           | 3        | 95          | 2    | 19   | 11   |

*Average number of local lesions on the 3 leaves of Chenopodium amaranticolor by mechanical inoculation.

RMV isolates. Nucleotide identity of RMV-Ca1 CP gene was 96.4 and 96.0% with RMV-Sh and RMV-Imp isolates, respectively. In amino acids, between RMV-Ca1 and the two isolates, identity was 96.8% equally. According to alignment analysis, RMV-Ca1 gene sequence showed higher sequence identity for the two RMV isolates (-Sh and -Imp) than other crucifer-infecting tobamoviruses (Table 5). In the sequences of 158 amino acids for coat protein, RMV-Ca1 differed with the other two isolates of RMV (-Sh and -Imp) at the position no. 2 (Val → Ser), 66 (Tyr → Ser), 83 (Ser → Ara) and 152 (Ara → Thr) (Plate 5).

In the neighbor joining tree showing sibling and ancestor (Fig. 4), however, RMV-Ca1 was shown closer relationship with YoMV than the RMV-Sh or RMV-Imp. Further, it could be inferred that RMV-Ca1 might have been a progenitor of other RMV isolates, and RMV-Sh and -Imp might have been diverged from a common ancestor.

According to the complete nucleotide of a number of tobamoviruses, it has led to their sub-grouping in different subgroups (Lartey, 1995; 1996). It has been reported that various other tobamovirus isolates could be infected on

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**Table 4. Serological relationships of RMV-Ca1 with antisera of tobamoviruses**

| Antiserum* | Relationship | RMV isolates         |
|------------|--------------|----------------------|
| TMV-Cym    | Homologous   | RMV-Ca1, RMV-Ac1, RMV-Ac3 |
| TMV-O      | Homologous   | RMV-Ca1, RMV-Ac1, RMV-Ac1, RMV-Ac3 |
| PMMoV      | Homologous   | RMV-Ca1, RMV-Ac1, RMV-Ac3 |
| ORSV       | Non relationship | RMV-Ca1, RMV-Ac1, RMV-Ac3 |

*TMV-Cym: Tobacco mosaic virus (TMV) isolated from cymbidium. TMV-O: TMV from tobacco. PMMoV: Pepper mild mottle virus, ORSV: Odontoglossum ringspot virus.

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**Fig. 3.** A single virus band was formed after 10-40% sucrose density gradient centrifugation (A). Virus particles of RMV-Ca1 were rigid rod with 300 nm length (B). Three isolates of RMV were related homogeneously with antisera of TMV-O, however, two isolates of RMV-Ac1 and RMV-Ac4 were different serologically. Center well: antiserum of TMV-O; a: RMV-Ca1; b: RMV-Ac1; c: RMV-Ac4 (C). The virus particles of RMV were observed frequently in all kinds of cells and tissues, especially as compact or scattering in cytoplasm of mesophyll cells (D). In conducting tissues, RMV particles were aggregated densely in xylem (E).
cruciferous plants (Heinze, 2006; Hii, 2002; Wetzel, 2006; Zhu, 2001). The isolates of RMV including RMV-Ca1 and the other tobamoviruses infecting Cricifer plants were analyzed to make sub-grouping among them which has high variation of their sequences. It could not be made any subgroup through comparing only coat protein gene. Further studies should be conducted with the construction of a full-length infectious clone of RMV-Ca1 for gaining

![Fig. 4. Phylogenetic tree generated from coat protein gene sequences of RMV-Ca1 and other tobamoviruses using the Neighbor-joining method.](image)

**Table 5.** Similarities of sequence for RMV-Ca1 and other tobamoviruses

| Tobamovirus* | Plant reported | % similarity of nucleotide | % similarity of amino acid |
|--------------|----------------|---------------------------|---------------------------|
| RMV-Sh       | Cabbage        | 96.4                      | 96.8                      |
| RMV-Imp      | Impatiens      | 96.0                      | 96.8                      |
| YoMV         | Rape           | 96.4                      | 97.5                      |
| TVCV         | Kiwifruit      | 87.6                      | 89.8                      |
| SFBC         | Cape Primrose  | 58.0                      | 61.2                      |

*RMOV; Ribgrass mosaic virus, YoMV; Youcai mosaic virus; TVCV; Turnip vein clearing virus, SFBC; Streptocapus flower break virus.

**Table 6.** Nine primers designed for sequences of RMV encoding coat protein for VC/RT-PCR

| No. | Primer   | Product Size (base pair) |
|-----|----------|--------------------------|
| 1   | RCP-455  | 455                      |
| 2   | RCP-498  | 498                      |
| 3   | RCP-586  | 586                      |
| 4   | RCPF1-712| 712                      |
| 5   | RCPF1-972| 972                      |
| 6   | RCPF-973 | 973                      |
| 7   | RCPF1-1107| 1107                    |
| 8   | RCP-1201 | 1201                     |
| 9   | RCPF-1385| 1385                     |

![Fig. 5. Alignment of the putative amino acid sequences of the coat proteins of RMV-Ca1, RMV-Sh and RMV-Imp, respectively. Identical sequences indicated by point.](image)
deeper insights into the molecular characteristics.

**VC/RT-PCR.** Out of nine primers for RMV in VC/RT-PCR, five kinds of primer pairs made specific detection bands suspected product size using saps of *Brassicaceae,* whole radish and Chinese cabbage which infected with RMV, and saps of those two healthy plants (Table 6, Fig. 6). In saps from the whole radish and Chinese cabbage, VC/RT-PCR was done clearly and specifically with the five specific primers of No. 1, 4, 5, 6 and 7 without the formation of any minor bands. When the nine primers for VC/RT-PCR were tested with the saps from *Nicotiana occidentalis,* three kinds of primer pairs of No. 1, 5 and 7 could be chosen by the specific bands in the infected and healthy *N. occidentalis* (Fig. 7). From the results of selection for specific primers of RMV using species of *Brassicaceae* and *Solanaceae,* three primers of No. 1, 5 and 7 were selected as optimum ones for the detection of RMV through VC/RT-PCR.

**Soil transmission.** Two commercial cultivars of Chinese cabbage ‘Chunhawang’ and ‘Seoul’ cultivated in the RMV infested soils were infected with RMV by the infection rate of 81.0% and 69.0%, respectively (Table 7). The fields of Pyungchang alpine area were infested prevalently with RMV virions with the transmission rate of 75% in average. The main cause of important epidemic of tobamoviruses

| Cultivar     | No. of plant | % infection |
|--------------|--------------|-------------|
|              | observed     | diseased    |              |
| ‘Chunhawang’ | 58           | 47          | 81.0         |
| ‘Seoul’      | 58           | 40          | 69.0         |
| Average      | 116          | 87          | 75.0         |

*chinese cabbages cultivated in net and vinyl house were investigated at 80 days after transplanting of 3-4 leaf stage seedlings at the infested soils collected from Pyungchang alpine area in 1994.
like RMV in fields should be contamination of soil with the virus. TMV, a typical tobamovirus could easily incite with the average infection rate of 30.6% in red peppers (Kim et al., 1989) and sometime 100% in tobacco (Katahira and Kiriyama, 1974) when seedlings were transplanted in the infested soils. However, Tomato mosaic tobamovirus in tomato did not occur soil transmission by transplanting of seedlings (Lanter et al., 1982) or showed low infection rate of 2.0% for TMV in red pepper (Kim et al., 1989). Symptoms occurred on chinese cabbages from Table 7 were mid rib necrotic streak, necrotic spots, leaf necrosis and stunt (Table 8). Major symptoms induced by RMV were double symptom of necrotic spot and mid rib necrotic streak, and mid rib necrotic streak having occurrence rate of 44.6% and 38.4%, orderly. These symptoms were typical by RMV and it can be found as the same in chinese cabbages which cultivated in the natural fields and greenhouses by artificial inoculations.

**Field survey.** The infection rates of RMV on chinese cabbages were ranged from 21.3% for Gimhae area to 85.7% for Youngam area at 10 areas in three provinces in 1995 (Table 9). RMV was prevalent already whole country, Gangwondo in alpine area of northern part, Gyeonggido, Chungcheongnamdo and Gyungsangbukdo in middle part, and Jeollanamdo in southern part of Korea. However, in 2008, the prevalence was diminished to 7.1% at Pyungchang alpine area.

**Resistance screening.** The 57 cultivars in Cruciferae crops including cabbage ‘Early flat Dutch’ were screened against RMV-Ca1 by seedling test with mechanical inoculation (Table 10). The cultivars of cabbage ‘Early Flat Dutch’ and radish ‘Chunchi’ were immune with non infection. There were no resistant cultivars in chinese cabbage, leaf mustard and turnip against RMV.

### Table 8. Symptoms induced by RMV on chinese cabbage cultivated on the infested soils

| Symptom                        | % occurrence in* | Total  |
|--------------------------------|------------------|--------|
| Necrotic ring spot + Midrib necrotic streak | 23.4 21.2 | 44.6  |
| Midrib necrotic streak         | 23.4 15.0        | 38.4   |
| Leaf necrosis                  | 1.1 11.8         | 12.9   |
| Stunt                          | 2.1 2.0          | 4.2    |

*% in 58 plants by visual inspection in Table 7.

### Table 9. Occurrence of RMV on chinese cabbage collected from fields

| Area collected  | % infection b |
|-----------------|---------------|
| Chungcheongnamdo|               |
| Yeosan          | 68.4          |
| Boryung         | 50.0          |
| Seocheon        | 45.5          |
| Jeollanamdo     |               |
| Gochang         | 52.6          |
| Naju            | 65.3          |
| Yeongam         | 85.7          |
| Haenam          | 31.3          |
| Gyungsangbukdo  |               |
| Dalsung         | 42.9          |
| Gimhae          | 21.3          |
| Tongyoung       | 33.3          |
| Gangwondo       |               |
| Pyungchang      | 7.1c          |

a Chinese cabbages at late heading and harvest stage were tested randomly for 90 specimens per area in 1997.

b Viral infection was judged by electron microscopy and ELISA using antisemur of RMV-Ca1 isolate.

c Disease survey was done on chinese cabbage with electron microscopy and RT-PCR in 2008.

### Table 10. Screening of resistant Cruciferae crops to RMV-Ca1

| Crop            | No. of cultivars a | Name of resistant cultivar |
|-----------------|--------------------|----------------------------|
| Chinese cabbage | 42 0               |                            |
| Cabbage         | 3 1                | Early Flat Dutch           |
| Radish          | 2 1                | Chunchi                    |
| Leaf mustard    | 7 0                |                            |
| Turnip          | 3 0                |                            |

*Screening was done on 9 seedlings at 2-3 leaf stage with 3 replications per cultivar by mechanical inoculation.

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