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Identification of *Grapevine leafroll-associated virus 3* Ampelovirus from Grapevines in Korea

Hyun Ran Kim*, Sin Ho Lee, Bong Choon Lee[1], Yeong Tae Kim[2] and Jin Woo Park[3]

Horticultural Environment Division, National Horticultural Research Institute, Rural Development Administration (RDA), Suwon 441-440, Korea

[1]National Yeongnam Agricultural Research Institute, RDA, Suwon 441-440, Korea

[2]National Institute of Agricultural Biotechnology, RDA, Suwon 441-440, Korea

[3]National Institute of Agricultural Science and Technology, RDA, Suwon 441-440, Korea

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***Grapevine leaf roll-associated virus 3* (GLRaV-3) is one of the most important viral diseases of grapevine in the world. In this study, GLRaV-3 *Ampelovirus* was identified from grapevines in Korea by analyzing viral coat protein size, nucleotide, and amino acid sequences. The molecular weight of viral coat protein from virus-infected in vitro plantlets was determined by western blot using a commercial GLRaV-3 polyclonal antibody. Western blot analysis showed a coat protein of about 43 kDa. RT-PCR product of about 942 bp which encoded the coat protein (CP) gene was amplified with specific primers. When the viruses existed at low titers in the host plant, the dsRNA had very specific template in RT-PCR amplification of fruit tree viruses. Especially, small-scale dsRNA extraction method was very reliable and rapid. Sequence analysis revealed that the CP of the GLRaV-3 Ko consisted of 942 bp nucleotide, which encoded 314 amino acid residues. The CP gene of GLRaV-3 Ko had 98.9% nucleotide sequence and 98.7% amino acid sequence identities with earlier reported GLRaV-3. This is the first report on molecular assay of GLRaV-3 *Ampelovirus* identified from Korea. The GLRaV-3 Ko CP clone would be very useful for breeding of virus resistant grapevines.**

Keywords : *Ampelovirus*, coat protein, grapevine, GLRaV-3, identification, RT-PCR

Leaf roll disease is one of the most important and widely distributed viral diseases in grapevines. The leaf roll symptom is not lethal to grapevines, but it causes erratic bearing, and lowers sugar content about 25-50% of the fruits (Goheen, 1970; Goheen and Cook, 1959; Hewitt, 1968).

Numerous reports have shown that flexuous, filamentous closterovirus-like particles ranging from 1400 to 2200 nm in length are associated with grapevine leaf roll diseases (Faoro et al., 1981; Milne et al., 1984; Namba et al., 1979; Zimmermann et al., 1990). These are serologically distinct; thus, these are designated as Grapevine leafroll-associated virus 1 (GLRaV-1) to Grapevine leaf roll-associated virus 8 (GLRaV-8) (Bosica et al., 1995; Choueiri et al., 1996; Hu et al., 1990; Zimmermann et al., 1990; Martelli et al., 2002).

Among the eight GLRaVs, grapevine leaf roll-associated virus 3 (GLRaV-3) is transmitted by mealybugs and classified as *Closterovirus*. However, in the meeting of the International Committee on Taxonomy of Viruses (ICTV) in July 2002, GLRaV-3 was separated from *Closterovirus* group and was accommodated in a new genus named *Ampelovirus* (from Greek, ampelos for grapevine) under the family *Closteroviridae* (Martelli et al., 2002).

The virions of *Ampelovirus* are of one size(usually 1400-2200 nm in length) and contain a single molecule of linear, positive sense single-stranded RNA of 16.9-19.5 kb. The genus contains viruses that infect only dicotyledonous hosts, and those are transmitted semi-persistently by coccid or pseudococcid mealybugs. None of the members is transmissible by sap inoculation. GLRaV-1, GLRaV-3, GLRaV-4, GLRaV-5, GLRaV-6, and GLRaV-8 were included in the genus *Ampelovirus*, and GLRaV-3 was designated as type species of the genus.

In Korea, GLRaV-3 is the most prevalent viral disease according to ELISA screening of grapevines in major cultivated areas (Kim et al., 2003). In this paper, characteristics of the viral dsRNA pattern, virus specific protein analysis, and CP region amino acid sequence of GLRaV-3 isolated from commercial grapevines in Korea are reported.

Materials and Methods

Virus isolates. GLRaV-3 infected vines were selected through ELISA using commercial antiserum (BIOREBA Co., Switzerland). *Vitis vinifera* cv. Rubi Okuyama, showing symptoms of leaf roll and leaf reddening, was selected from germplasms in National

*Corresponding author.
Phone) +82-31-290-6221, FAX) +82-31-295-9548
E-mail) kimhr0@rda.go.kr

Horticultural Research Institute, Suwon, Korea.

Virus propagation. For propagation of the virus, chip graft inoculation was done on sensitive indicator cultivar 'LN33' (Couderc 1613 × *Vitis berlandieri*) and then cultured in vitro to get virus-infected lateral buds. The inoculated 'LN33' plants were grown in plastic pots under a glasshouse, and in vitro plantlets were cultured in a constant conditioned culture room.

Virus coat protein characterization. The virus-infected in vitro plantlets and healthy plantlets were ground with liquid nitrogen; suspended in 1.5 mL sample buffer (0.125 M Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), and 5% 2-mercaptoethanol (2-ME); and centrifuged in 6,000 g for 3 min. The supernatants of virus source were diluted from 2^0 to 2^3, and degraded by heating at 100°C for 5 min. Molecular weight of the virus coat protein was determined by SDS-PAGE using 12.5% separated gel and 3% stacking gels at 90-120 V for 2 h in Mighty Small™ SE245 (Hoefer Co.). Prestained molecular marker, See Blue™ Prestained Marker (Novex Co.), was used.

After the electrophoresed gel was transferred to nitrocellulose membrane at 130 V for 40 min, the membrane was washed with TBS buffer (10 mM Tris, pH 7.5, 150 mM NaCl). The membrane was pre-blotted by soaking in 4% dry milk powder in TBS buffer for 1 h, and then incubated overnight at 4°C in preparation of polyclonal GLRaV-3 IgG (1:1000 in TBS, Bioreba Co.).

The membrane was then washed three times, 15 min each in TBS buffer, added with 0.01% Tween 20, and incubated with goat-anti-rabbit IgG-horseradish peroxidase conjugate (1:1000 in TBS) for 2 h at 37°C. The membrane was incubated in a substrate (PIERCE Supersignal West Pico. Chemiluminescent Substrate kit) for 1-2 min in darkroom, exposed directly to X-ray film for 2 min, and developed into photo.

RT-PCR and nucleotide sequence analysis. dsRNA was extracted from GLRaV-3-infected grapevine leaves by using the small-scale dsRNA extraction method (Zhang et al., 1998). The dsRNA was used as template in reverse transcription-polymerase chain reaction (RT-PCR). A pair of primers for detection of GLRaV-3 Korean isolate (GLRaV-3 Ko) was designed based on the nucleotide sequences of the earlier reports (GenBank U82937).

The primers 1 (P1, 5'GCGG ATCCATGGCATTTGAACTG-AA3') and 2 (P2, 5'CTACTTCTTTTGCAATAG TTGGAAG3') were designed to detect and amplify the CP gene of GLRaV-3 Korean isolate. DsRNA was mixed with 1 μL of 10 pmol reverse primer (P1) and incubated at 95°C for 8 min to denature the RNA and primer annealing. The tubes were quickly chilled in ice for 2 min, and 5 μL of the reactions were used for one-step RT-PCR. One-step RT-PCR was accomplished by using commercial kit (Promega, USA), with 33 pmol of reverse and forward primers in a thermocycler (MJ Research PTC-220, USA). RT-PCR was carried out with one cycle at 48°C for 45 min, and 94°C for 2 min. A total of 40 cycles of PCR amplification program (94°C, 30 sec; 60°C, 1 min; and 72°C, 2 min) was carried out, followed by a final extension at 72°C for 7 min.

The amplified product underwent electrophoresis in 1.5% agarose gel, stained with ethidium bromide, illuminated with UV light, and photographed. The cDNA of the CP gene of GLRaV-3 Korean isolate was cloned into the pGEM-T-Easy vector (Promega, USA). The nucleotide sequence was determined by using the BigDye DNA sequencing kit (Perkin-Elmer Corp. Norwalk, CT, USA) on the ABI 377 DNA sequencer. The sequences were analyzed by using the DNASTAR program (USA). Homology test of the nucleotide and amino acid sequences were performed by using the BLAST in the Gene Bank database.

Results and Discussion

Virus-infected grapevine 'LN33' leaves showed symptoms of leaf roll and reddening in early July (Fig. 1). This leaf roll and reddening were typical symptoms in sensitive red-fruit cultivars. Leaf roll symptom initially appeared in 2 months after inoculation, but typical diagnostic symptoms appeared in mature leaves in the following year. Leaf roll and reddening were observed in lower leaves of vines in July. These became more intense and progressed in the upper leaves.

At early stage, the leaf blades turned into bright red and

Fig. 1. Leafroll and reddening symptoms of GLRaV-3 infected grapevine leaves.

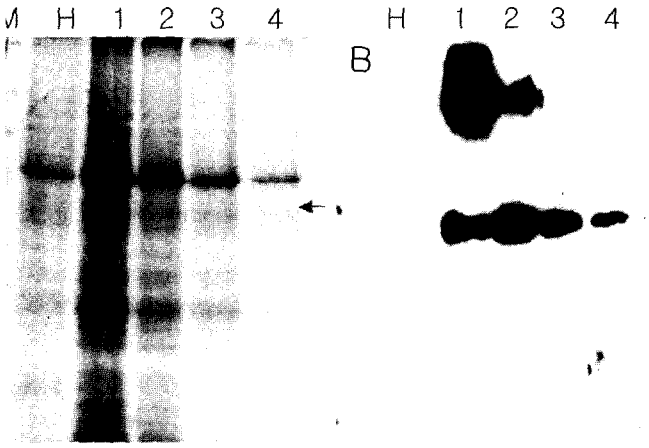

Fig. 2. Western blot analysis of viral coat protein extracted from GLRaV-3 infected grapevine. A, Total protein pattern; B, Specific viral protein bands reacted with GLRaV-3 antiserum; M, SeeBlue™ prestained marker; H, Healthy sap; Lane 1-4, Sap dilution series 2^0, 2^1, 2^2, 2^3.

Fig. 3. Agarose gel electrophoresis of RT-PCR product of GLRaV-3 coat protein gene. M, size marker (100 bp ladder). G, GLRaV-3.

leaf margins rolled downward. Symptoms were also manifested in the margins of upper leaves being rolled and leaves turning dark red with green main veins.

Western blot analysis showed a coat protein of about 43 kDa (Fig. 2) in virus-infected sample, while there was no reaction in the healthy sample. These results were similar to those derived from the earlier reports by Bosica et al. (1995) and Hu et al. (1990). Ling et al. (1997) described different results in their earlier report about molecular weight of coat protein of GLRaV-3.

However, they agreed with the results of the earlier work done by Hu et al. (1990). Our results were similar to those

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1    M  A  F  E  L  K  L  G  Q  I  Y  E  V  V  P  E  N  N  L  R  V  R  V  G  D  NCBI-GLRaV-3
1    .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  GLRaV-3 Ko
1    .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  GLRaV-3 Ko. seq

26   A  A  Q  C  K  F  S  K  A  S  F  L  K  Y  V  K  D  G  T  Q  A  E  L  T  G  NCBI-GLRaV-3
76   .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  GLRaV-3 Ko
76   .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  GLRaV-3 Ko. seq

51   I  A  V  V  P  E  K  Y  V  F  A  T  A  A  L  A  T  A  A  Q  E  P  P  R  Q  NCBI-GLRaV-3
151  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  L  .  .  GLRaV-3 Ko
151  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  CTT  .  .  GLRaV-3 Ko. seq

76   P  P  A  Q  V  A  E  P  Q  E  T  D  I  G  V  V  P  E  S  E  T  L  T  P  N  NCBI-GLRaV-3
226  .  .  .  .  .  V  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  GLRaV-3 Ko
226  .  .  .  .  .  GTG  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  GLRaV-3 Ko. seq

101  K  L  V  F  E  K  D  P  D  K  F  L  K  T  M  G  K  G  I  A  L  D  L  A  G  NCBI-GLRaV-3
301  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  T  .  GLRaV-3 Ko
301  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  ACG  .  GLRaV-3 Ko. seq

126  V  T  H  K  P  K  V  I  N  E  P  G  K  V  S  V  E  V  A  M  K  I  N  A  A  NCBI-GLRaV-3
376  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  GLRaV-3 Ko
376  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  GLRaV-3 Ko. seq

151  L  M  E  L  C  K  K  V  M  G  A  D  D  A  A  T  K  T  E  F  F  L  Y  V  M  NCBI-GLRaV-3
451  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  K  .  .  .  .  .  .  GLRaV-3 Ko
451  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  AAA  .  .  .  .  .  .  GLRaV-3 Ko. seq

176  Q  I  A  C  T  F  F  T  S  S  S  T  E  F  K  E  F  D  Y  I  E  T  D  D  G  NCBI-GLRaV-3
526  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  GLRaV-3 Ko
526  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  GLRaV-3 Ko. seq

201  K  K  I  Y  A  V  W  V  Y  D  C  I  K  Q  A  A  A  S  T  G  Y  E  N  P  V  NCBI-GLRaV-3
601  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  GLRaV-3 Ko
601  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  GLRaV-3 Ko. seq

226  R  Q  Y  L  A  Y  F  T  P  T  F  I  T  A  T  L  N  G  K  L  V  M  N  E  K  NCBI-GLRaV-3
676  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  GLRaV-3 Ko
676  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  GLRaV-3 Ko. seq

251  V  M  A  Q  H  G  V  P  P  K  F  F  P  Y  T  I  D  C  V  R  P  T  Y  D  L  NCBI-GLRaV-3
751  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  GLRaV-3 Ko
751  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  GLRaV-3 Ko. seq

276  F  N  N  D  A  I  L  A  W  N  L  A  R  Q  Q  A  F  R  N  K  T  V  T  A  D  NCBI-GLRaV-3
826  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  GLRaV-3 Ko
826  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  GLRaV-3 Ko. seq

301  N  T  L  E  N  V  F  Q  L  L  Q  K  K                                      NCBI-GLRaV-3
901  .  .  .  .  .  .  .  .  .  .  .  .  .                                      GLRaV-3 Ko
901  .  .  .  .  .  .  .  .  .  .  .  .  .  TAG                                 GLRaV-3 Ko. seq

Decoration 'Decoration #1': Hide (as '.') residues that match GLRaV3 CP.PRO
 exactly.J
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Fig. 4. Alignment of the amino acid and nucleotide sequences for coat protein gene of GLRaV-3 Ko isolate and GLRaV-3 (U82937).

reported by previous researchers. There is, however, a need for more discussions on this area.

In dilution series test for Western blot assay, 2^1-2^3 dilutions were better than the non-diluted sap. RT-PCR product of about 942 bp fragment which encoded the CP gene was amplified with specific primers (Fig. 3). Especially, the use of dsRNA extracted from grapevine leaves by small-scale dsRNA extraction method was very efficient and reliable for RT-PCR detection. This dsRNA extraction method was previously reported as an efficient method for RT-PCR detection of ACLSV and ASGV in apple or pear leaves (Lee et al., 2003).

The nucleotide sequences of GLRaV-3 Ko CP gene and their deduced amino acid sequences are shown in Fig. 4. The sequence analysis revealed that the CP of GLRaV-3 Ko consisted of 942 bp nucleotide, which encoded 314 amino acid residues. The CP gene of GLRaV-3 Ko had 98.9% nucleotide sequence and 98.7% amino acid sequence identity which was previously reported by Ling et al., GLRaV-3 (U82937). However, the differences of the two isolates should be further analyzed in succeeding experiments.

GLRaV-3 Ko isolate was identified as *Ampelovirus*. Cytopathological characteristics of the GLRaV-3 isolated from Korea had already been reported (Kim et al., 2002). This is the first report on molecular assay of GLRaV-3 *Ampelovirus* identified from Korea and the GLRaV-3 Ko CP clone would be very useful for breeding of virus-resistant grapevines.

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