PARTIAL MOLECULAR CHARACTERIZATION OF A CHINESE ISOLATE OF *GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 2* AND PRODUCTION OF ANTISERA TO RECOMBINANT VIRAL PROTEINS

Z.Y. Xu[1], N. Hong[1,3], B. Xing[1] and G.P. Wang[1,2]

[1] College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, Hubei 430070, P.R. China
[2] Key Lab of Horticultural Plant Biology, Ministry of Education, Wuhan, Hubei 430070, P.R. China
[3] National Indoor Conservation Center of Virus-free Gemplasms of Fruit Crops, Huazhong Agricultural University, Wuhan, Hubei 430070, P.R. China

SUMMARY

The genes encoding RNA-dependent RNA polymerase (RdRp) and coat protein (CP) of a Chinese isolate of *Grapevine leafroll-associated virus 2* (GLRaV-2-CF) were cloned in the vector pET-28a (+) and sequenced. Comparisons of these sequences with those of other GLRaV-2 isolates available from databases revealed homology levels with GLRaV-2-PN and GLRaV-2-Sem higher than 98% at the nucleotide and amino acid levels. GLRaV-2-CF had lower homology with GLRaV-2-RG, another distinct strain, with similarities of 78% (RdRp) and 79% (CP) at the nucleotide level. The cloned RdRp and CP were successfully expressed in *Escherichia coli* BL-21 (DE3 plysS) and the resulting recombinant proteins were used to raise antisera in rabbits. Tests using these antisera readily detected the recombinant proteins in Western blots and the native virus proteins in infected grapevine samples.

Key words: Grapevine, Grapevine leafroll-asociated virus 2, gene cloning, sequencing, recombinant proteins, PAS-ELISA, Western blotting.

INTRODUCTION

Grapevine leafroll- associated virus 2 (GLRaV-2), one of the nine viruses associated with grapevine leafroll disease (Gugerli, 2003; Martelli *et al.*, 2005), is also implicated in the aetiology of other serious grapevine disorders known as graft incompatibility (Greif *et al.*, 1995), young vine decline (Golino *et al.*, 2000), and rootstock stem lesion (Uyemoto *et al.*, 2001).

This virus is a member of the genus *Closterovirus* (family *Closteroviridae*) and occurs in nature as a number of biological and molecular variants (Goszczynski *et al.*, 1996; Abou Ghanem-Sabanadzovic *et al.*, 2000; Rowhani *et al.*, 2000; Bonfiglioli *et al.*, 2003; Angelini

and Borgo, 2004; Meng *et al.*, 2005) that have recently been grouped into four distinct strains, based on their coat protein (CP) gene sequences (Meng *et al.*, 2005). GLRaV-2 has a positive-sense single-stranded RNA genome that consists of eight open reading frames (ORFs), of which ORF1b encodes the RNA-dependent RNA polymerase (RdRp, Mr 52,000) and ORF6 encodes the CP (Mr 22,000) (Zhu *et al.*, 1998).

Serology is a widely used tool for the routine detection of grapevine viruses. However, production of high quality virus-specific antisera to GLRaVs may not be easy, due to the frequency of complex infections in the field and the low yields of virus particles that can be obtained from naturally infected grapevine tissues (Hu *et al.*, 1990; Choueiri *et al.*, 1996).

Nevertheless, production of virus-specific antibodies by using recombinant proteins from cloned virus genes expressed in *Escherichia coli* can overcome these difficulties (Vaira *et al.*, 1996; Rubinson *et al.*, 1997; Ling *et al.*, 2000; Valerie *et al.*, 2003; Sukhacheva *et al.*, 2004).

Grapevine leafroll occurs in most grape-growing regions of China (Li *et al.*, 1989). GLRaV-2, -3, and -4 were identified serologically by Cai *et al.* (1997). More recently it was shown that GLRaV-1, -2, -3, and -7 occur in Northern and Southern China with the rate of GLRaV-2 infection in symptomatic vines being close to 36% (Hong *et al.*, 2005). However, of the Chinese GLRaVs, only an isolate of GLRaV-3 has been partially sequenced until now (Zhang *et al.*, 2000).

A study was therefore undertaken to clone and sequence the RdRp and CP genes of a Chinese isolate GLRaV-2 denoted CF, to express the cloned genes in *E. coli*, and to test the possible use of the antisera raised to recombinant virus proteins for the detection of GLRaV-2 infection in diseased grapevines.

MATERIALS AND METHODS

Virus sources. Grapevine canes from fourteen varieties showing leafroll symptoms were collected from vineyards in Northern China, rooted and grown in a greenhouse for further observations. All accessions were tested for the presence of GLRaV-1, -2, -3, -4, and -7 by

---
Corresponding author: G.P. Wang
Fax: +86.27.87397735
E-mail: Gpwang@mail.hzau.edu.cn

DAS-ELISA using commercial kits (Bioreba, Reinach, Switzerland), and a vine of cv Cabernet Franc found to be infected only by GLRaV-2 was used as virus source for gene cloning. Seven additional accessions infected or not by GLRaV-2 were used for testing the obtained antisera and a healthy LN33 was used as a negative control.

dsRNA extraction and cDNA synthesis. About 10 g of cortical shavings from dormant canes were ground to a fine powder in liquid nitrogen and dsRNA was obtained by phenol extraction and purification through cellulose CF-11 column as described by Hu *et al.* (1990). Approximately 600 ng of dsRNA were denatured with dimethylsulfoxide, primed with random 6-mer nucleotide primers (TaKaRa, Dalian, China), and reverse-transcribed with M-MLV reverse transcriotase (Promega, Madison, USA) at 37°C for 3 h.

Cloning and sequencing. Two sets of primers were designed on the basis of the published nucleotide sequence of GLRaV-2 (AF039264). Primer sequences for amplification of the RdRp cistron were GLV2-RdRp-L1: 5'-AAG-GATCCATGAGCGTAGTTCGGTCG-3' and GLV2-RdRp-R2: 5'-ATGAGCTCGTCACGAACAAAGAAAC TGC-3' with the BamHI and SacI restriction sites underlined. Primer sequences for amplification of the complete CP citron were GLV2-CP-L1: 5'-AAGAATTCGC-TATGGAGTTGATGTCC-3' and GLV2-CP-R2: 5'-ATC TCGAGGATTCGGATTTTTCTTCGTAA-3' with the EcoRI and XhoI restriction sites underlined.

Polymerase chain reactions (PCR) were made in a 96-well block PCR Thermal Cycler (Model PTC-100, MJ Research, Ramsey, MN, USA). The amplified products were gel purified, digested with restriction endonucleases, and cloned into pET-28a(+). Plasmids with the inserted target DNA fragments were transferred into *E. coli* DH5α. Recombinant plasmids found positive for RdRp and CP by enzymatic digestion were denoted 28a-RdRp and 28a-CP, respectively. Sequences of cloned fragments were determined by a commercial sequencing service (Shanghai Sangon Biological Engineering & Technology and Service, Shanghai, China).

Protein expression in *E. coli* and antisera preparation. *E. coli* BL-21 (DE3 plysS) was transformed with the recombinant plasmids 28a-RdRp and 28a-CP by electroporation. Proteins were induced in *E. coli* by the addition of 1mM isopropylthio-β-D-galactoside (IPTG) at 30°C and induction periods of 4-6 h were evaluated by 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by staining with 0.25% Coomassie Blue G250 solution or by transferring the separated proteins onto HybondTM-P membranes (Amersham Biosciences, Little Chalfont, England) for Western blot analysis using commercial kits as described by Valerie *et al.* (2003).

For immunization, induced bacteria were disrupted by sonication, centrifuged at 12,000 rpm for 10 min, and proteins in the supernatant and pelleted debris fractions were separated by 12% SDS-PAGE. The bands corresponding to the recombinant RdRp and CP were excised from the gels and homogenized in a small volume of chilled 0.1M phosphate-buffered saline (PBS, pH 7.4, 0.14 M NaCl). Hypodermic and intramuscular injections at two-week intervals were used for immunization of rabbits. Approximately 1 ml suspension containing 0.5 mg protein was emulsified in Freund's complete adjuvant for the first injection, which was followed by five injections each with 1 mg protein in Freund's incomplete adjuvant. Blood was collected two weeks after the last injection.

Western blotting and ELISA. Western blotting was used to detect proteins expressed in *E. coli* cells and to assess the specificity of antisera as described by Valerie *et al.* (2003). PAS-ELISA and indirect dot-blot immunobinding assay (IDBIA) were done to evaluate the potential of the antisera raised for the detection of GLRaV-2 in diseased grapevine samples.

Crude extracts were prepared from petioles of diseased and healthy grapevines by grinding in 0.05M Tris-HCl buffer (pH 8.2) containing 2% polyvinylpyrrolidone, 1% polyethylene glycol 6000, 0.45% sodium diethyldithiocarbamate trihydrate, and 0.05% Tween 20. PAS-ELISA was done as described by Edwards and Cooper (1985), with first and second antibodies diluted 1:500 and 1:250 for RdRp detection, and 1:1,000 and 1:500 for CP detection. For IDBIA, 2 μl aliquots of crude extracts were spotted onto Hybond[TM]-P membranes, which were air dried and blocked in 0.5% BSA-PBST solution at room temperature for 3 h.

Membranes were then incubated with antisera at dilution of 1:500 (RdRp) or 1:1,000 (CP) for 2 h, followed by alkaline phosphatase-conjugated goat anti-rabbit IgG (1:5,000; Promega, Corp., Madison, WI, USA) for 2 h. Reaction was visualized by incubation in a freshly prepared substrate solution containing nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

RESULTS

Amplification and sequence analysis. Products with the expected size of ca. 1.4 kb (RdRp) and 0.6 kb (CP) were amplified from tissue extracts infected with isolate GLRaV-2-CF, but not from negative controls (Fig. 1). The cloned cDNA fragments corresponding to the CP and RdRp genes were 597 bp and 1,380 bp respectively, and the sequences were deposited in GenBank database with accession numbers DQ 116440 (RdRp) and AY842932 (CP).
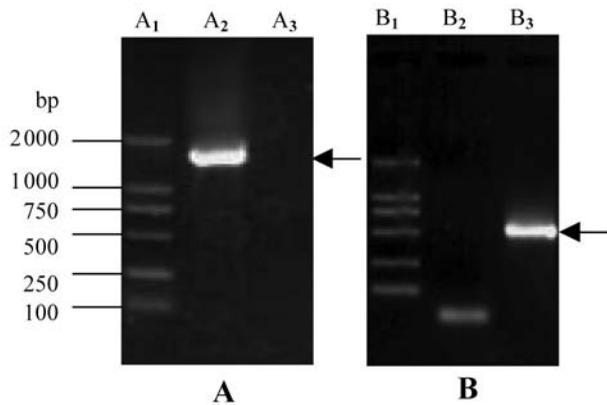
Fig. 1. Amplified products with primer sets GLV2-RdRp-L1/R2 (A) and GLV2-CP-L1/R2 (B). DNA Marker (DL2000) (A$_1$, B$_1$); Cabernet Franc (A$_2$, B$_3$); LN33 (A$_3$, B$_2$).

Comparison of these sequences with those of other GLRaV-2 isolates revealed extensive homology with GLRaV-2-PN and GLRaV-2-Sem (Meng *et al.*, 2005) and some differences from GLRaV-2-H4 (Abou Ghanem Sabanadzovic *et al.*, 2000), GLRaV-2-RG (Rowhani *et al.*, 2002) and GLRaV-2-93/955 (Meng *et al.*, 2005). In particular, the CP of GLRaV-2-CF had identities with comparable genes of the five above isolates, ranging from 78% to 97% at the nucleotide level and from 90% to 99% at the amino acid level. Identity of GLRaV-2-CF RdRp with the same cistron of the other isolates was in the order of 98% (GLRaV-2-PN and GLRaV-2-Sem), 79% (GLRaV-2-RG) and 92% (GLRaV-2-93/955) at the nucleotide level and 98% (GLRaV-2-PN and GLRaV-2-Sem), 94% (GLRaV-2-RG) and 97% (GLRaV-2-93/955) at the amino acid level.

No amino acid change was found in the C termini of the CP of the six isolates, and the amino acid residues N, R, G and D conserved in all closterovirus CPs (Zhu *et al.*, 1998) were also present in CP of GLRaV-2-CF. By

contrast, variations of amino acid sequence in the RdRp among isolates GLRaV-2-PN, GLRaV-2-Sem, GLRaV-2-RG and GLRaV-2-93/955 occurred in a variety of positions.

In phylogenetic trees constructed with the nucleotide sequences of CP and RdRp of the six GLRaV-2 isolates under comparison and those of three other members of the genus Closterovirus, i.e. *Beet yellow virus* (BYV), *Citrus tristeza virus* (CTV) and *Beet yellow stunt virus* (BYSV), GLRaV-2-CF clustered next to GLRaV-2-Sem and GLRaV-2-PN (Fig. 2).

Analysis of *E. coli*-expressed products. The expression of the RdRp and CP in *E. coli* resulted in the production of proteins with relative Mr of about 52000 and 25000, respectively. The size of *E. coli*-expressed CP was slightly larger than that estimated from the amino acid sequence (22,000 Da), thus resembling results reported for the movement protein of *Grapevine virus* A (Rubinson *et al.*, 1997). The amounts of recombinant proteins did not significantly differ after induction for 4 h or 6 h (Fig. 3). When 2% glucose was added to the bacterial cultures during protein induction, the yield of recombinant CP increased but that of recombinant RdRp did not (data not shown). Western blot analysis showed that the recombinant CP reacted strongly with a commercial anti-GLRaV-2 antiserum raised against purified virus (data not shown).

Effectiveness of antisera to recombinant proteins. In western blots, antisera against recombinant RdRp diluted 1:5,000 or CP diluted 1:10,000 reacted strongly with homologous proteins expressed in *E. coli*. No signal was observed with the induction products of pET-28a that lacked inserted cDNA (Fig. 4).

Both antisera were used for testing grapevine samples by PAS-ELISA and IDBIA. The results showed that the
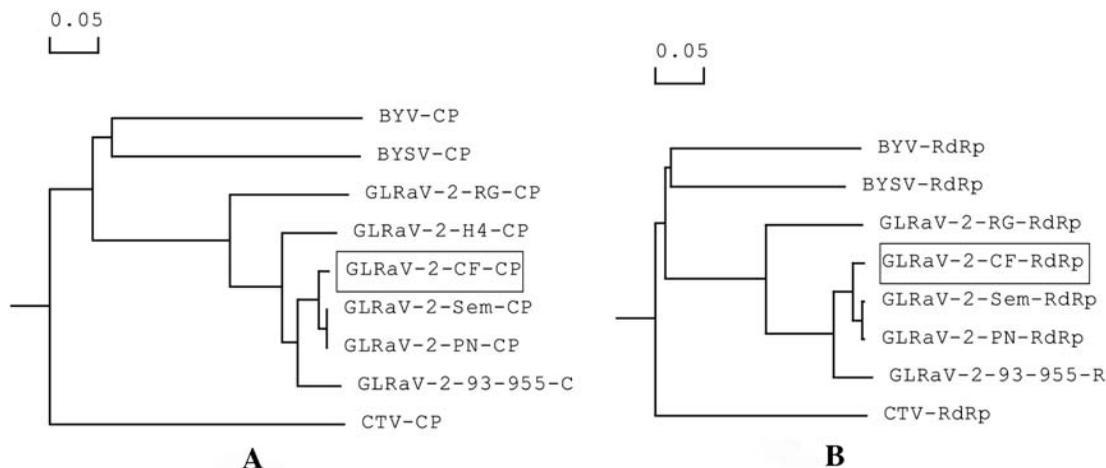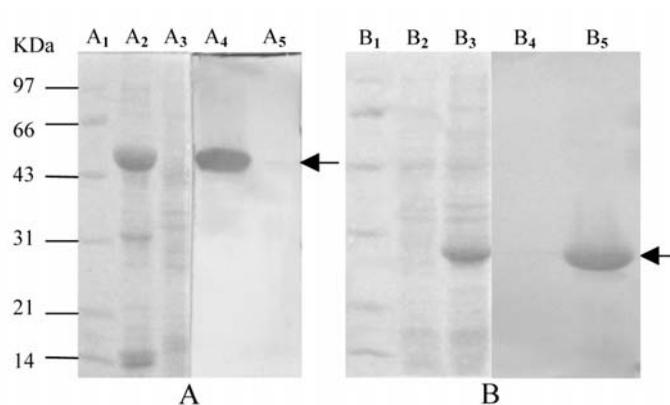

Fig. 2. Phylogenetic analysis of the sequences of coat protein gene (**A**) and RNA-dependent RNA polymerase gene (**B**) of several GLRaV-2 isolates and other three closteroviruses, *Beet yellow virus* (BYV, X73476), *Beet yellow stunt virus* (BYSV, U51931), *Citrus tristeza virus* (CTV, U16304). The trees were generated by DNAMAN software (Lynnon Biosoft, DNAMAN Windows version).
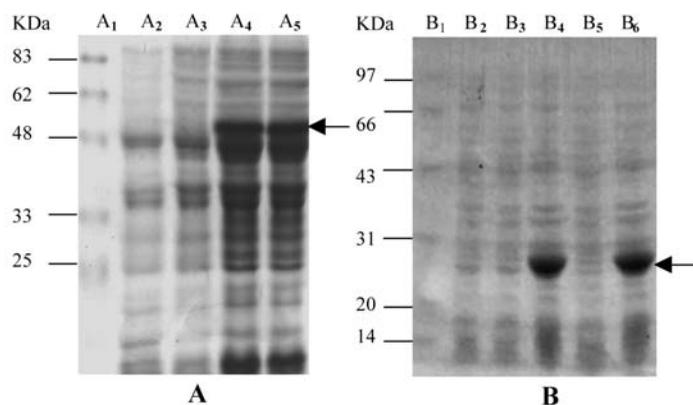
Fig. 3. SDS-PAGE analysis of the expressed recombinant RdRp (**A**) and CP (**B**) in *E. coli*. Protein marker (A_1, B_1); total protein from *E. coli* transformed with BL/pET-28a-*RdRp*: non-induced by IPTG (A_2), induced by IPTG for 4 h (A_4), induced by IPTG for 6 h (A_5); total protein from *E. coli* transformed with BL/pET-28a-*CP*: non-induced by IPTG (B_2), induced by IPTG for 4 h (B_4), induced by IPTG for 6 h (B_6); total protein from *E. coli* transformed with BL/pET-28a: induced by IPTG for 4 h (A_3, B_3), induced by IPTG for 6 h (A_5, B_5).

Fig. 4. SDS-PAGE analysis and Western-blot analysis of expressed recombinant RdRp (**A**) and CP (**B**). Low molecular protein marker (A_1, B_1); total protein from induced *E. coli* transformed with BL21/pET-28a-*RdRp* (A_2, A_4) and with pET-28a-*CP* (B_3, B_5) after sonication and centrifugation; total protein from induced *E. coli* transformed with BL/pET-28a (A_3, A_5, B_2, B_4) after sonication and centrifugation.

antiserum to recombinant CP could be used effectively to detect GLRaV-2 by either method; the results were comparable to those obtained by DAS-ELISA using a commercial kit (Table 1). When the recombinant RdRp-based antiserum was used to analyze the same samples, somewhat different results were obtained. Accessions JingXiu and Semillon both gave positive reactions in DAS-ELISA, but JingXiu was negative with both PAS-ELISA and IDBIA whereas Semillon was positive in IDBIA and negative in PAS-ELISA (Table 1).

For use in PAS-ELISA, suitable dilutions of the antiserum to RdRp were 1:500 and 1:250 for trapping and revealing, respectively; those for the antiserum to CP were 1:1,000 and 1:500.

DISCUSSION

Two major results were achieved in this study, i.e. the assignment of a Chinese GLRaV-2 isolate to a specific virus strain, based on molecular data, and the production of virus-specific antisera from recombinant CP and RdRp proteins, that can be used for diagnosis.

Sequence comparisons revealed identity levels of 78 to 97% at the nucleotide and 90 to 99% at the amino acid sequence among CPs of the Chinese GLRaV-2-CF isolate and those of five other isolates of GLRaV-2. Homology levels of RdRps ranged from 79 to 97% at the nucleotide level and from 94 to 98% at the amino acid level. Proteins of both GLRaV-2-CF genes had the high-

Table 1. Response of eight grapevine accessions with leafroll symptoms to GLRaV-2 antisera by DAS-ELISA, PAS-ELISA and IDBIA.

	Antiserum source	LN33[a]	Cabernet Franc	Luochaliao	Semillon	Dawanhong No.2	Kyoho	Jingxiu	Yan73	Beizhi 34
DAS- ELISA	Commercial kit	-	++	++	+	-	-	+	-	-
PAS- ELISA	Against recombinant RdRp	-	+	+	-	-	-	-	-	-
	Against recombinant CP	-	++	++	++	-	-	+	-	-
IDBIA	Against recombinant RdRp	-	+	+	+	-	-	-	-	-
	Against recombinant CP	-	++	++	++	-	-	+	-	-

[a] healthy control; - = no reaction; + = positive reaction (A_{405} was 2-5 times higher than the mean value of the healthy control); ++ = strong positive reaction (A_{405} was more than five times higher than the mean value of the healthy control).

est homology with comparable products of GLRaV-2-PN and GLRaV-2-Sem, two isolates with which GLRaV-2-CF grouped in the phylogenetic tree. GLRaV-2-PN and GLRaV-2-Sem are both associated with typical leafroll disease, and are members of a strain of their own that Meng *et al.* (2005) regard as distinct from three other GLRaV-2 strains typified by GLRaV-2-93/95, GLRaV-2- H4, and GLRaV-2-RG..

The production of GLRaV-free material relies on sensitive and specific methods of virus detection. CP and RdRp proteins of GLRaV-2-CF were efficiently produced in transformed bacteria. Antisera raised to these proteins were able to recognize the corresponding recombinant proteins in Western blots and were able to detect infection by GLRaV-2 in all (CP antiserum) or in most (RdRp antiserum) plant extracts tested by ELISA and IDBIA.

It seems, then, that the antiserum to recombinant CP had no difficulty in recognizing native virus CP and has a greater potential than antisera to RdRp for use in the routine detection of GLRaV-2 in field infected vines. However, the antiserum to RdRp may be more appropriately used in the study of virus-host relationships at the cellular level.

ACKNOWLEDGMENTS

REFERENCES

Abou Ghanem-Sabanadzovic N., Sabanadzovic S., Castellano M.A., Boscia D., Martelli G.P., 2000. Properties of a new isolate of grapevine leafroll-associated virus 2. *Vitis* **39**: 119-121.

Angelini E., Bertazzon N., Borgo M., 2004. Diversity among grapevine leafroll-associated virus 2 isolates detected by heteroduplex mobility assay. *Journal of Phytopathology* **152**: 416-422.

Bonfiglioli R., Edwards F., Pantaleo A., 2003. Molecular studies on a graf incompatibility syndrome in New Zealand vineyards yields another probable variant of *Grapevine leafroll-associated virus 2*. In: *Extended Abstracts 14th Meeting of ICVG, Locorotondo* 2003, 141.

Cai W.Q., Xu S.H., Mang K.Q., 1997. Purification, serological research of grapevine leafroll-associated virus and use for detecting grapevine free of virus. *Acta Microbiologica Sinica* **37**: 385-392.

Choueiri E., Boscia D., Digiaro M., Castellano M.A., Martelli G.P., 1996. Some properties of a hitherto undescribed filamentous virus of the grapevine. *Vitis* **35**: 1-3.

Edwards M.L., Cooper J.I., 1985. Plant virus detection using a new form of indirect ELISA. *Journal of Virological Methods* **11**: 309-319.

Golino D.A., Sim S., Rowhani A., 2000. Identification of the latent viruses associated with young vine decline in California. In: *Extended Abstracts 13th Meeting of ICVG, Adelaide* 2000, 85-86.

Goszczynski D.E., Kasdorf G.G.F., Pietersen G., van Tonder H., 1996. Detection of two strains of grapevine leafroll-associated virus 2. *Vitis* **35**: 133-135.

Greif C., Garau R., Boscia D., Prota V.A., Fiori M., Bass P., Walter B., Prota U., 1995. The relationship of grapevine leadfroll-associated closterovirus 2 with a graft incompatibility condition of grapevines. *Phytopathologia Mediterranea* **34**: 167-173.

Gugerli P., 2003. Grapevine leafroll and related viruses. In: *Extended Abstracts 14th Meeting of ICVG, Locorotondo* 2003, 25-31.

Hong N., Xu Z.Y., Xiang B., Wang G.P., 2005. Gene cloning of etiological viruses causing grapevine leafroll in China. In: *Proceedings 2nd Asia Conference on Plant Pathology, Singapore* 2005, 89.

Hu J.S., Gonsalves D., Teliz D., 1990. Characterization of Closterovirus-like particles associated with grapevine leafroll disease. *Journal of Phytopathology* **128**: 1-14.

Li Z., Martelli G.P., Prota V., 1989. Virus and virus-like diseases of the grapeviner in the People's Republic of China, a preliminary account. In: *Proceedings of the 9th Meeting of ICVG, Kyriat Anavim* 1987, 31-34.

Ling K.S., Zhu H.Y., Jiang Z.Y., Gonsalves D., 2000. Effective application of DAS-ELISA for detection of grapevine leafroll associated closterovirus-3 using a polyclonal antiserum developed from recombinant coat protein. *European Journal of Plant Pathology* **106**: 301-309.

Martelli G.P., Agranowsky A.A., Bar-Joseph M., Boscia D., Candresse T., Coutts R.H.A., Dolja V.V., Falk B.W., Gonsalves D., Jelkmann W., Karasev A.V., MinafraA., Namba S., Vetten H.J., Wisler G.C., Yoshikawa N., 2005. Family *Closteroviridae*. In: Fauquet C.M., Mayo M.A., Maniloff J., Desselberger U., Ball L.A. (eds.). Virus Taxonomy. Eighth Report of the International Committee on Taxonomy of Viruses, pp. 1077-1087. Elsevier Academic Press, San Diego, USA.

Meng B.Z., Li C.H., Goszczynski D.E., Gonsalves D., 2005. Genome sequences and structures of two biologically distinct strains of grapevine leafroll-associated virus 2 and sequence analysis. *Virus genes* **31**: 31-41.

Rowhani A., Zhang Y.P., Golino D.A., Uyemoto J.K., 2000. Isolation and partial characterization of two new viruses from grapevine. In: *Extended Abstracts 13th Meeting of ICVG, Adelaide* 2000, 82.

Rubinson E., Galiakparov N., Radian S., Sela I., Tanne E., Gafny R., 1997. Serological detection of grapevine virus A using antiserum to a nonstructural protein, the putative movement protein. *Phytopathology* **87**: 1041-1054.

Sukhacheva E.A., Fomicheva V.V., Efimova N.A., Shubert J., 2004. Immunodection of RNA-Dependent RNA Polymerase from Turnip yellow luteovirus using monoclonal antibodies. *Russian Journal of Bioorganic Chemistry* **30**: 25-29.

Uyemoto J.K., Rowhani A., Luvisi D., Krag C.R., 2001. New closterovirus in 'Redglobe' grape causes decline of grafted plants. *California Agriculture* **55**(4): 28-31.

Vaira A.M., Vecchiati M., Masenga V., Accotto G.P., 1996. A polyclonal antiserum against a recombinant viral protein combines specificity with versatility. *Journal of Virological Methods* **56**: 209-219.

Valerie H., Emmanuel J., Maryse G., Yves L.H., Daniele G.D.,

2003. Production of recombinant potato mop-top virus coat protein in *Escherichia coli* and generation of antisera recognizing native virus protein. *Journal of Virological Methods* **110**: 91-97.

Zhang Y.M., Li Y., Tian Y.T., La P., Cai W.Q., 2000. Cloning, sequencing and expression of coat protein gene of grapevine leafroll associated closterovirus. *Acta Phytopathologica Sinica* **30**(3): 232-236.

Zhu H.Y., Ling K.S., Goszcynski D.E., McFerson J.R., Gonsalves D., 1998. Nucleotide sequence and genome organization of grapevine leafroll-associated virus-2 are similar to Beet yellow virus, the closterovirus type member. *Journal of General Virology* **79**: 1289-1298.