Expression of *Grapevine leafroll-associated virus 3* Coat Protein Gene in *Escherichia coli* and Production of Polyclonal Antibodies

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FAJARDO, T.V.M., BARROS, D.R., NICKEL, O., KUHN, G.B. & ZERBINI, F.M. Expression of *Grapevine leafroll-associated virus 3* coat protein gene in *Escherichia coli* and production of polyclonal antibodies. Fitopatologia Brasileira 32:496-500. 2007.

ABSTRACT

Grapevine leafroll-associated virus 3 (GLRaV-3), the main viral species of the grapevine leafroll complex, causes yield and quality reduction in grapes (*Vitis* spp.). The coat protein gene was RT-PCR-amplified from total RNA extracted from infected grapevine leaves and the amplified fragment was cloned and completely sequenced. The fragment was subsequently subcloned into the pRSET-C expression vector. The recombinant plasmid was used to transform *Escherichia coli* BL21:DE3 and express the capsid protein. The coat protein, fused to a 6 His-tag, was purified by affinity chromatography using an Ni-NTA resin. The identity of the purified protein was confirmed by SDS-PAGE and Western blot. The *in vitro*-expressed protein was quantified and used for rabbit immunizations. The antiserum was shown to be sensitive and specific for the detection of GLRaV-3 in grapevine extracts in Western blot and DAS-ELISA assays, with no unspecific or heterologous reactions against other non-serologically related viruses being observed.

Additional keywords: grapevine, GLRaV-3, recombinant protein, serology, antibodies.

RESUMO

Expressão da proteína capsidial do *Grapevine leafroll-associated virus 3* em *Escherichia coli* e produção de anticorpos policlonais

Grapevine leafroll-associated virus 3 (GLRaV-3), a principal espécie viral do complexo do enrolamento da folha da videira (*Vitis* spp.), causa reduções no rendimento e na qualidade da uva. O gene da proteína capsidial foi amplificado via RT-PCR a partir de RNA total, extraído de folhas de videira infectadas. O fragmento amplificado foi clonado e completamente seqüenciado. Em seguida, o fragmento foi subclonado no vetor de expressão pRSET-C. O plasmídeo recombinante foi utilizado para a expressão da proteína capsidial em *Escherichia coli* BL21:DE3. A proteína capsidial, ligada a uma cauda de 6-His, foi purificada por cromatografia de afinidade em coluna de Ni-NTA. A identidade da proteína purificada foi confirmada em SDS-PAGE e *Western blot* e, após quantificação, foi utilizada para imunizar coelhos. O anti-soro mostrou-se sensível e específico para a detecção do GLRaV-3 em extratos de videira por *Western blot* e DAS-ELISA, não tendo sido observadas reações inespecíficas ou heterólogas contra outros vírus sorologicamente não relacionados.

Palavras-chave adicionais: videira, GLRaV-3, proteína recombinante, sorologia, anticorpos.

Grapevine leafroll occurs in all main grapevine (*Vitis* spp.) growing regions worldwide, reducing the productivity and quality of grapes for consumption *in natura* and for processing, delaying ripening, negatively affecting the number, size and uniformity of bunches and the grape's pigmentation, as well as reducing sugar content. In Brazil, grapevine leafroll is a disease of economic relevance (Fajardo *et al*., 2003).

Nine serologically distinct viral species have been identified and associated with grapevines affected by leafroll, named *Grapevine leafroll-associated virus*, GLRaV-1 to -9. The disease is considered to be caused by a virus complex, although each viral species may occur individually in a plant. GLRaV-1, -2, -3 and -6 have already been detected in Brazil, with GLRaV-3 occuring most frequently (Martelli *et al*., 2002). GLRaV-3, the type-member of the genus *Ampelovirus* (*Closteroviridae*), is the best characterized among the viruses of the grapevine leafroll virus complex. The coat protein (CP), encoded by ORF 6 (Ling *et al*., 1997; Martelli *et al*., 2002), has a predicted molecular mass of approximately 35 kDa (Ling *et al.,* 1997), estimated at 41.6 kDa (Rigotti *et al*., 2006) and 43 kDa (Zimmermann *et al*., 1990) by SDS-PAGE and Western blot. According to Ling *et al*. (1997), the difference between the predicted molecular mass on the basis of the deduced amino acid sequence and estimated values from SDS-PAGE lies within acceptable range.

Generally, molecular methods such as RT-PCR are not suitable as routine tests for indexing large numbers of samples due to costs and the relative complexity of execution. On the other hand, indexing on woody indicators takes a long time for symptoms to be expressed (Nickel

et al., 2004). As a consequence, serology has traditionally been the most used method of plant virus diagnosis in a large number of samples, using ELISA as the method of choice (Zimmermann *et al*., 1990; Ling *et al*., 2000).

Virus purification is usually a labor-intensive procedure with varying, occasionally unsatisfactory results concerning purity and concentration of the final preparation. Specifically for grapevine leafroll, the eight virus species associated with the disease are inseparable from the viral complex by biological means, with the exception of GLRaV-2. Additionally, the lack of adequate herbaceous hosts, the low virus titer in woody plant tissues and the presence of inhibitor compounds such as polyphenols, tannins and polysaccharides are common difficulties that compromise purification of many plant viruses from their woody hosts (Ling *et al*., 2000; Ling *et al*., 2007). Also, antisera produced against different preparations of purified viruses may possess varying titers and specificities (Barbieri *et al*., 2004) which may lead to inconsistent diagnostic results.

With the development of molecular biology techniques, cloning and expressing of viral genes coding for structural (Ling *et al*., 2000; Minafra *et al*., 2000; Ling *et al*., 2007) and non-structural proteins has become an important strategy for obtaining large amounts of antigens with uniform concentration and stable properties among preparations (Targon *et al*., 1997; Barbieri *et al*., 2004; Nickel *et al*., 2004).

This study aimed to produce and characterize specific antibodies against the GLRaV-3-CP, expressing the coat protein in *Escherichia coli*. Where not stated otherwise, procedures were performed according to Sambrook & Russel (2001). The coat protein (CP) gene of GLRaV-3, isolate Pet-4, from grapevine cv. Petite Syrah collected in Petrolina, Pernambuco, Brazil, was amplified by RT-PCR with the oligonucleotides 8504 (5' ATGGCATTTGAACTGAAATT 3', viral sense) and 9445 (5' CTACTTCTTTTGCAATAGTT 3', complementary sense), ligated into the pGEM-T-Easy vector (Promega), cloned in *E. coli* DH5α and completely sequenced (GenBank access number AY753208) (Fajardo *et al.*, 2007). One recombinant clone containing the CP gene of GLRaV-3 (GLRaV-3cp, 942 bp) was cultured for 8 h at 37 °C in LB medium with 100 µg/mL ampicilin (LB/ampicilin), and the plasmid DNA was purified using the Flexi Prep kit (Amersham Biosciences), according to the manufacturer's instructions. The GLRaV-3cp gene was removed from the plasmid pGEM-T Easy by digestion with *Eco*RI and ligated to the expression vector pRSET-C (Invitrogen), previously digested with the same enzyme. For *in vitro* expression, the construct pRSET-C/GLRaV-3cp was transferred to *E. coli* strain BL21:DE3 by a heat shock procedure and one colony cultivated at 37 °C in 200 mL LB/ampicilin until an OD_{600} of approximately 0.5, when expression was induced by addition of IPTG to a final concentration of 2 mM. Six hours post-induction, the bacterial cells were collected by centrifugation (5,000 *g*/10 min) and stored at -80 °C. Total protein extracts were obtained by re-suspension in lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, pH 8.0), lysozyme treatment and sonication as described by Noueiry *et al.* (1994). The CP extract, re-suspended in 1 mL of 100 mM $NaHCO_3$, pH 9.0, plus 0.5% SDS (w/v) was purified by affinity cromatography in Ni-NTA columns (Qiagen), according to the manufacturer's instructions. GLRaV-3cp expression was evaluated by SDS-PAGE and Western blot, using commercial antibodies against the CP of GLRaV-3 (Agritest). Protein quantification was done with the Bradford reagent (Bio-Rad) according to the manufacturer's instructions.

After dialysis in 10 mM phosphate buffer, pH 7.4, plus 0.425% NaCl (w/v), 218, 220, 298, 464 and 789 µg of the *in vitro*-expressed protein were injected intramuscularly into the hind legs of each of two white New Zealand rabbits, approximately 35 days old, at weekly intervals. The first injection was done with complete Freund's adjuvant (1:1 v/v), and the four remaining injections with incomplete Freund's adjuvant (1:1 v/v). Beginning one week after the last injection, seven weekly bleedings were carried out (25-30 mL/bleeding/animal). Blood samples were allowed to coagulate for 1 h at 37°C and 30 min at 4°C, and then centrifuged at 3,000 *g*/10 min. The supernatant (antisera) was aliquoted and stored at -20°C.

Purification of the globuline fraction (IgG) from the antisera was carried out by ion exchange chromatography in a DEAE-Sephacel resin (Sigma) equilibrated with 25 mM sodium acetate buffer pH 5.2, diluting the antisera 1:10 (v/v) in distilled water. An equal volume of saturated ammonium sulphate was used to precipitate proteins, the suspension was centrifuged at 3,000 *g*/10 min, the pellet was re-suspended in 2 mL of half-strength PBS and dialysed for 12 h at 4°C in the same buffer. Quantified globuline fractions were stored at -20°C.

Conjugate was prepared by mixing alkaline phosphatase type VII-S (Sigma) sedimented by centrifugation at 8,000 *g* for 20 min with IgG (1:2 v/v). The mixture was dialysed in half-strength PBS for 12 h. Glutaraldehyde was added at 0.06% (v/v) and the mixture was incubated in the dark at 28 °C for 4 h and dialysed again. Before the DAS-ELISA procedures, PTA-ELISA (plate-trapped antigen-ELISA), using purified IgG from the produced GLRaV-3 antisera (2 µg/µL), was initially performed to evaluate the specific recognition with GLRaV-3-expressed CP (about 5 µg/µL) in this type of indirect ELISA test.

In preliminary assays to determine the antisera titer by DAS-ELISA (Clark & Adams, 1977) using the purified CP (1:250 and 1:500 v/v), IgG was evaluated at 1, 2 and 4 µg/mL and the conjugate was diluted 1:200, 1:400, 1:800, 1:1,000, 1:2,000 and 1:4,000 (v/v) for each IgG concentration. Sensitivity and specificity of the collected antisera were evaluated by DAS-ELISA and by Western blot using healthy and GLRaV-3-infected grapevines maintained in greenhouses, which had been previously biologically indexed. The samples, consisting of leaf veins and petioles, were powdered in the presence of liquid nitrogen and

diluted 1:3 (w/v) in 500 mM Tris-HCl, pH 8.2, containing 0.8% NaCl (w/v), 2% polyvinylpyrrolidone 40,000 (w/v), 1% polyetheleneglycol 6,000 (w/v) and 0.05% Tween 20 (v/v). Samples were considered infected when absorbance at 405 nm was at least twice the average value of the healthy controls.

Additionally, the DAS-ELISA assay was performed to confirm the GLRaV-3 antibody specificity and to demonstrate the lack of serological relationship between produced GLRaV-3 antisera and some antigens, including other grapevine virus [*Grapevine leafroll-associated virus* 1 (GLRaV-1), GLRaV-2, GLRaV-5, GLRaV-7, *Grapevine fanleaf virus* (GFLV), *Grapevine fleck virus* (GFkV), *Grapevine virus A* (GVA), *Grapevine virus B* (GVB)] and *Apple chlorotic leaf spot virus* (ACLSV).

Samples for Western blot were ground in TBS (20 mM tris, 500 mM NaCl), pH 7.5 plus 0.2% sodium sulfite (w/v), 1:5 (w/v). For comparison, commercial antibodies against the GLRaV-3cp (Agritest) were used in both assay.

Expression of the GLRaV-3cp was induced three times in 200 mL cultures, thus obtaining 2.121, 1.966 and 1.578 mg coat protein in each induction. Compared to other results (Targon *et al*., 1997; Ling *et al*., 2000; Ling *et al*., 2007) on fusion protein expression, the average protein yield obtained in this study (10.6 µg/mL culture medium) was considered adequate for the required immunizations.

The expressed coat protein was analyzed by SDS-PAGE in which the presence of a band with a molecular mass corresponding to approximately 44 kDa, an expected value for the fusion protein, was observed (Figure 1A); ca. 41 kDa corresponding to the GLRaV-3cp itself, increased by approximately 3 kDa, of amino acids ligated to the N-terminal of the GLRaV-3cp, including the 6-His tag. The band corresponding to the fusion protein was absent in the sample transformed with the empty vector (Figure 1A). The identity of the expressed protein was confirmed by Western blot (Figure 1B). One band migrating ahead of the fusion protein indicates a common post-purification degradation process (Figures 1A-B).

Testing the antisera for the specific detection of GLRaV-3 by Western blot revealed that it reacted strongly with extracts from virus-infected plants, demonstrating high sensitivity and specificity. The antisera did not show the least reaction to healthy samples (Figure 2). The purified IgG was obtained in a concentration of 1.278 µg/mL, and its positive reactivity to GLRaV-3-expressed CP (A_{405nm} = 1.82; healthy control = 0.14) was also confirmed by PTA-ELISA.

In DAS-ELISA, the antiserum reacted with the *in vitro*-expressed CP as well as with leaf extracts from infected plants in dilutions of up to 1 µg/mL and 1:4,000 of IgG and conjugate, respectively (Table 1). The dilutions finally chosen for IgG and conjugate, 2 µg/mL and 1:1,000, respectively, gave the best and most reproducible reactions. Healthy grapevine absorbance values were compatible with sensitive and reliable GLRaV-3 detection. The antiserum did not show heterologous cross reactions with other non-

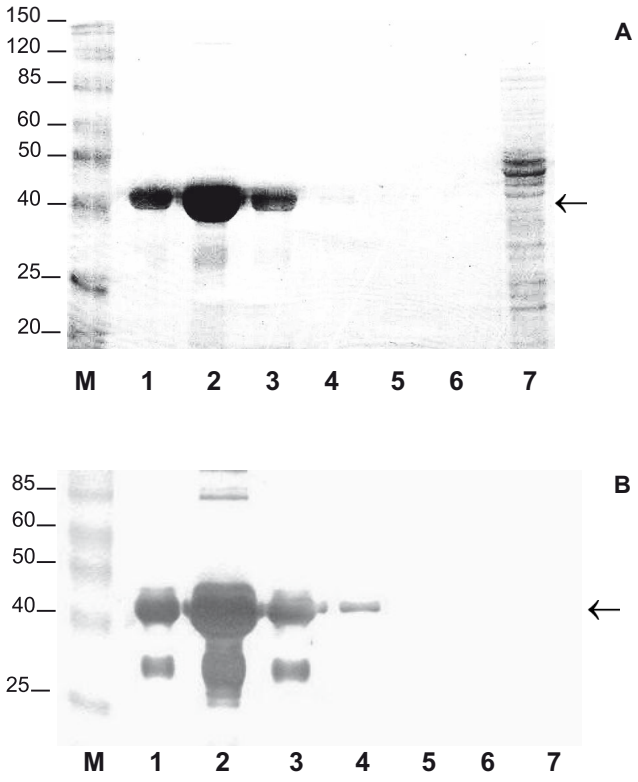

FIG. 1 - A. SDS-polyacrylamide gel electrophoresis, 12%, stained with Coomassie Blue; **B.** Western blot with commercial antiserum (Agritest) against *Grapevine leafroll-associated virus 3* (GLRaV-3) (1:750 v/v). Samples in both gels: Lanes 1 to 6, aliquots (5µL) of sequential fractions of total protein extracts from *Escherichia coli* transformed with the construct pRSET-C/GLRaV-3cp, collected from Ni-NTA-column; Lane 7, aliquot (5µL) of the control extract containing the empty expression vector pRSET-C. M, molecular mass markers (kDa). Arrows on the right indicate the position of the fusion protein.

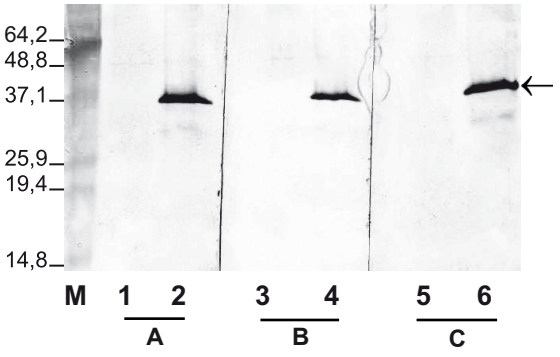

FIG. 2 - Western blot with antisera against the fusion protein obtained from 2nd (A), 3rd (B) and 4th (C) bleedings (1:250 v/v); Lanes 1, 3, 5, extracts from healthy grapevine cv. Itália, 1:3 (w/v) (20 µL); Lanes 2, 4, 6, extracts from GLRaV-3-infected grapevine cv. Petite Syrah, 1:3 (w/v) (20 µL). M, molecular mass markers (kDa). The arrow on the right indicates the GLRaV-3 coat protein position.

TABLE 1 - Results of DAS-ELISA (absorbance values at 405 nm) from grapevine (*Vitis* spp.) samples using antiserum against GLRaV-3 fusion protein, expressed in *Escherichia coli* cells

Samples	Absorbance at 405 nm IgG (µg/mL) / Conjugate (v/v)				
	4 / 1:1000 (30 min)	**4 / 1:2000 (30 min)**	**2 / 1:1000 (50 min)**	**4 / 1:2000 (50 min)**	**2 / 1:500 (40 min)**
Healthy grapevine (cv. Itália 1:3 w/v or Agritest extract)	0.30	0.15	0.30	0.25	0.30
GLRaV-3-infected grapevine cv. Petite Syrah 1:3 (w/v)	0.90	0.51	0.83	0.75	n.d.
Expressed GL RaV-3cp fusion protein 1:500 (v/v)	3.22	1.81	>3.50	2.93	n.d.
Grapevine leafroll-associated virus 1 (Agritest grapevine extract)	n.d.	n.d.	n.d.	n.d.	0.30
Grapevine leafroll-associated virus 2 (Plantest-Sanofi grapevine extract)	n.d.	n.d.	n.d.	n.d.	0.19
Grapevine leafroll-associated virus 5 (Plantest-Sanofi grapevine extract)	n.d.	n.d.	n.d.	n.d.	0.16
Grapevine leafroll-associated virus 7 (Plantest-Sanofi grapevine extract)	n.d.	n.d.	n.d.	n.d.	0.18
Grapevine fanleaf virus (Agritest grapevine extract)	n.d.	n.d.	n.d.	n.d.	0.26
Grapevine fleck virus (Agritest grapevine extract)	n.d.	n.d.	n.d.	n.d.	0.24
Grapevine virus A-infected grapevine (1:3 w/v)	n.d.	n.d.	n.d.	n.d.	0.25
Grapevine virus B-infected grapevine (1:3 w/v)	n.d.	n.d.	n.d.	n.d.	0.18
Apple chlorotic leaf spot virus-infected apple (*Malus* spp.) (1:10 w/v)	n.d.	n.d.	n.d.	n.d.	0.26

n.d., not determined

serologically related viruses (Table 1). Performance of the antiserum was similar to that obtained with commercial antibodies. Absorbance values from grapevine infected samples suggest that the virus titer was low in evaluated grapevine plants. In this work, DAS-ELISA was chosen to check the antiserum because of its low cost, reliability and practicality as a large-scale virus diagnostic method (Zimmermann *et al*., 1990; Ling *et al*., 2000).

Although a number of polyclonal antisera has been raised against recombinant viral proteins, only in a few cases were they effective in detecting the virus by ELISA, as previously reported by Ling *et al*. (2000) to GLRaV-3, using DAS-ELISA and by Ling *et al*. (2007) to GLRaV-2, using PTA-ELISA. In this work, the complete GLRaV-3 capsid protein gene was expressed, while the GLRaV-3 coat protein clone expressed by Ling *et al*. (2000) covered 96% of the coat protein gene, lacking 13 amino acid residues of the C-terminus. Antibodies raised against recombinant viral proteins are more frequently useful for virus detection through other means, including Western blot, immuno-electron microscopy (ISEM), immunocapture RT-PCR or indirect ELISA (Ling *et al*., 2007).

Antibodies produced against recombinant antigens may not be functional when used in non-denaturing procedures such as ELISA (Minafra *et al*., 2000), being more probably functional when used in denaturing assays such as Western blots (Nickel *et al*., 2004). Changes in the antigenic structure of the viral CP expressed in bacteria could be an explanation for the weak recognition of the native CP in infected plant samples (Minafra *et al*., 2000). In the present study the antibodies produced against the *in vitro*-expressed CP recognized the GLRaV-3cp sensitively and specifically in both systems.

The antibodies produced against the recombinant antigen GLRaV-3cp isolate Pet-4 were not tested against GLRaV-3 isolates Pet-1 to Pet-3, all collected in Northeastern Brazil, from which Pet-4 was shown to differ by only 11 amino acids (Fajardo *et al*., 2007). However, these antibodies were tested and recognized GLRaV-3 in other infected samples of different origins, such as distinct accessions of Cabernet Sauvignon (A_{405nm} = 1.02), Centennial (1.04), Chardonnay (1.07) and Clara (0.94) cultivars (healthy grapevine A_{405nm} = 0.26).

Barbieri *et al.* (2004) concluded, based on observations with *Watermelon mosaic virus* (WMV), family *Potyviridae*, genus *Potyvirus*, that antibodies produced against expressed CPs in *E. coli* tend to be more specific, reducing the occurrence of unexpected heterologous reactions. This property was shown in the results of Table 1, as expected, the nine non-serologically GLRaV-3-related

viruses (GLRaV-1, GLRaV-2, GLRaV-5, GLRaV-7, GFLV, GFkV, GVA, GVB and ACLSV) (Ling et al., 2007) did not react with the produced GLRaV-3 antisera.

Multiple infections in grapevines, found frequently under field conditions, make virus diagnosis based on symptoms difficult and misleading. Multiple grapevine virus infections also make conventional purification of *Closteroviridae* viruses for antisera production impracticable, especially when individual virus species diagnosis is required. The availability of antisera of high sensitivity and specificity, such as those obtained in this study from recombinant antigens, will allow reliable virus diagnosis in these woody hosts.

ACKNOWLEDGEMENTS

The authors would like to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq for financial support (Proc. 470000/2005-8). Technical support by Marcos F. Vanni is gratefully acknowledged.

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Received 7 August 2007 – Accepted 11 December 2007 – FB 7084