Polyclonal Antibodies to the Coat Protein of *Apple stem grooving virus* expressed in *Escherichia coli*: Production and Use in Immunodiagnosis

Osmar Nickel[1], Maria L.P.N. Targon[2], Thor V.M. Fajardo[1], Marcos A. Machado[2] & Ana P. Trivilin[3]

[1]Embrapa Uva e Vinho, Cx. Postal 130, CEP 95700-000 Bento Gonçalves, RS, e-mail: nickel@cnpuv.embrapa.br; [2]Centro Avançado de Pesquisa Tecnológica do Agronegócio de Citros Sylvio Moreira, Cx. Postal 4, CEP 13490-970 Cordeirópolis, SP; [3]Grantee, Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul - FAPERGS, Porto Alegre, RS

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Author for correspondence: Osmar Nickel

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ABSTRACT

The coat protein gene of *Apple stem grooving virus* (ASGV) was amplified by RT-PCR, cloned, sequenced and subcloned in the expression vector pMal-c2. This plasmid was used to transform *Escherichia coli* BL21c^{+} competent cells. The ASGV coat protein (cp) was expressed as a fusion protein containing a fragment of *E. coli* maltose binding protein (MBP). Bacterial cells were disrupted by sonication and the ASGVcp/MBP fusion protein was purified by amylose resin affinity chromatography. Polyclonal antibodies from rabbits immunized with the fusion protein gave specific reactions to ASGV from infected apple (*Malus domestica*) cv. Fuji Irradiada and *Chenopodium quinoa* at dilutions of up to 1:1,000 and 1:2,000, respectively, in plate trapped ELISA. The ASGVcp/MBP fusion protein reacted to a commercial antiserum against ASGV in immunoblotting assay. The IgG against ASGVcp/MBP performed favorably in specificity and sensitivity to the virus. This method represents an additional tool for the efficient ASGV-indexing of apple propagative and mother stock materials, and for use in support of biological and molecular techniques.

Additional keywords: recombinant antigen, latent virus, ELISA, *Malus domestica*.

RESUMO

Anticorpos policlonais contra a proteína capsídica de *Apple stem grooving virus* expressada em *Escherichia coli*: produção e uso em imunodiagnose

O gene da capa protéica de *Apple stem grooving virus* (ASGV) foi amplificado por RT-PCR, clonado, seqüenciado e subclonado no plasmídeo pMal-c2. A capa protéica de ASGV foi expressa em *Escherichia coli* como proteína de fusão contendo um fragmento do gene da proteína de ligação a maltose de *E. coli* (MBP). Células bacterianas foram rompidas por sonicação e a proteína de fusão ASGVcp/MBP foi purificada por cromatografia de afinidade em resina de amilose. Anti-soros policlonais de coelhos imunizados com a proteína de fusão reagiram especificamente com extratos de maçãs (*Malus domestica*) cv. Fuji Irradiada e *Chenopodium quinoa* infetadas com ASGV em diluições de 1:1000 e 1:2000, respectivamente, em ELISA indireto. A proteína de fusão ASGVcp/MBP reagiu positivamente com anticorpos comerciais produzidos contra ASGV em testes de "immunoblotting". A imunogamaglobulina contra ASGVcp/MBP teve desempenho favorável em especificidade e sensibilidade ao vírus. Este método representa um instrumento adicional para a eficiente indexagem de ASGV em material propagativo e básico de maçã e como complemento de métodos biológicos e moleculares.

Apple stem grooving virus (ASGV) (family *Flexiviridae*; genus *Capillovirus*) is disseminated worldwide in Rosaceae fruit trees such as apple (*Malus domestica* Borkh.), pear (*Pyrus communis* L.), apricot (*Prunus armeniaca* L.) and cherry (*Prunus serotina* Ehrh.), usually as a latent infection in most commercial cultivars. Citrus (*Citrus* spp.) species and lilies (*Lilium* spp.) may also carry ASGV isolates, formerly known as *Citrus tatter leaf virus* (Magome *et al.*, 1997; Lovisolo *et al.*, 2003). Recently ASGV was detected in a close relative of the commercial kiwifruit, [*Actinidia deliciosa* (A. Chev.) C.F. Liang et A.R. Ferguson], a new and surprising host, intercepted on the New Zealand border (G. Clover, personal communication). The ASGV is one of the most important and destructive of the so-called apple latent viruses. In Brazil, in recent years, it has been associated with syndromes that caused severe damages to apple plant nurseries and adult orchards, especially in connection with Maruba-kaido rootstocks [*Malus prunifolia* (Willd.) Borkh. cv. *ringo*] (Nickel *et al.*, 1999). Symptoms associated with the presence of ASGV in susceptible *Malus* spp. include severe xylem pitting and grooving with pegs protruding on the inner bark face, phloem necrosis, reduced vigour of the canopy and decline of the plant.

Strategies to control it involve heat therapy, *in vitro* meristem culture, and shoot tip grafting *in vivo*. Indexing of plants is required following these procedures. While molecular methods such as RT-PCR (reverse transcriptase-

polymerase chain reaction) are not adequate for mass screening and inoculations of woody indicator plants have a long reaction time, the enzyme-linked immunosorbent assay (ELISA), is a convenient, relatively cheap, simply executed and reliable method. Serological testing requires large quantities of antisera in order to index candidate mother stock of heat treated apple clones. Therefore, the production of large amounts of antigens for immunization purposes is needed. These antigens have usually been produced by labor-intensive, recurrent purifications of the virus increased in herbaceous hosts.

The ASGV, the type-member of the genus *Capillovirus,* has flexuous, filamentous particles with 27 kDa coat protein subunits. The virus particles have relatively low stability and tend to aggregate with plant debris or among the elongated virus particles themselves. This leads to considerable losses of virus during the purification process, thereby requiring only gentle and highly labor-intensive purification methods, with especial attention to removal of plant proteins (Fuchs & Merker, 1985). During the months of the year with higher temperatures, production of infected herbaceous propagation plants for purification is not practical. To overcome these difficulties, molecular biology techniques are currently being used to express the genes of interest in heterologous systems and to produce the antigen when required (Targon *et al.*, 1997).

The coat protein (cp) gene of the Brazilian isolate ASGV UV01 has recently been cloned and sequenced (Nickel et. al., 2001; Genbank access number AF438409), opening the way for the use of molecular biology techniques to produce large quantities of ASGVcp antigen. In this paper, we report the preparation of a fusion protein with the coat protein gene of the Brazilian isolate ASGV UV01 in *Escherichia coli* and the use of the antigen to raise polyclonal virus-specific antibodies for immunodiagnosis. Results of the evaluation of these antibodies from three antisera (out of 14 bleedings) for ASGV detection are presented.

Virus source

The ASGV UV01 was originally isolated from nursery apple trees, established by grafting onto tolerant rootstocks in a greenhouse, and transmitted mechanically to *Chenopodium quinoa* Willd. and *Nicotiana occidentalis* Wheeler cv. 37B.

Nucleic acid extraction and RT-PCR

Total RNA was extracted from infected *C. quinoa* by capture on silica particles and used as template for cDNA synthesis by reverse transcription and for PCR as described previously (Nickel *et al.*, 1999). For PCR amplification, reverse primer ASGV6396 (5' CTG CAA GAC CGC GAC CAA GTT T 3', MacKenzie *et al.*, 1997), complementary to nucleotides 6375 to 6396 and the virus sense primer ASGV5641 (5' ATG AGT TTG GAA GAC GTG CTT C 3'), corresponding to nucleotides 5641 to 5662 were used as described (Nickel *et. al.*, 1999).

Cloning of ASGV CP in an expression vector

The amplified fragment containing the ASGV cp gene was cloned in the pGEM-T Easy vector (Promega) and sequenced (Nickel *et al.*, 2001; GenBank access number AF438409). The pGEM-T Easy vector containing the ASGV coat protein gene was digested with *Eco*RI. The expression vector pMAL-c2 (1.5 µg) (New England Biolabs) was simultaneously digested with *Eco*RI (0.5 unit) and dephosphorilated with alkaline phosphatase (0.5 unit). Both ASGVcp insert and vector were run on 1% LMP agarose, extracted with phenol-chloroform and precipitated with ethanol (Sambrook *et al.*, 1989). Approximately 10 ng of the insert were ligated into pMAL-c2 in-frame with the fragment of maltose-binding protein (MBP), according to manufacturer instructions using T4 DNA ligase, for 2 h at 15 °C and transformed by heat shock into *E. coli* competent cells BL21c+ prepared according to Hanahan (1983). After addition of SOC medium to complete 1 ml, the reaction was incubated for 1 h at 37 °C, plated on Luria-Bertani broth/2% agar, containing 0.2% glucose and 100 µg/ml ampicilin/X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside)/IPTG (isopropyl-beta-D-thiogalactopyranoside) and incubated overnight at 37 °C. The PCR of minipreps of white colonies selected on plates containing X-Gal, was used to detect recombinant clones (Sambrook *et al.*, 1989).

Expression of ASGV CP in *E. coli* and purification of the fusion protein

A total of 250 ml Luria-Bertani broth, with 0.2% glucose and 100 µg/ml ampilicin, was inoculated with 2.5 ml of fresh overnight culture of *E. coli* BL21c+ cells bearing recombinant plasmid pMAL-c2 and incubated at 37 °C with vigorous shaking. When the bacterial suspension reached OD_{600nm} of approximately 0.5, IPTG was added to a final concentration of 0.3 mM and incubation was continued at 37 °C for 3 h. Cells were centrifuged (4000 x g, 20 min), ressuspended in column buffer (CB) (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 1 mM EDTA) and frozen. Cells were disrupted by sonication in an ice bath, the crude cell extract was diluted 1:5 in CB and the fusion protein was purified through a pre-swollen amylose resin packed in 2.5 x 10 cm glass columns. The fusion protein was eluted with CB containing 10 mM maltose. Concentration of the eluted protein was estimated spectrophotometrically at extinction coefficient A_{280nm}=1.44 (New England Laboratories).

Electrophoresis, ELISA and immunoblotting of fusion protein

Expression of induced fusion proteins was analysed in 4%/12% discontinuous polyacrylamide gels (SDS-PAGE) (Laemmli, 1970). Samples were denatured prior to PAGE by boiling for 3 min in the presence of sample buffer (SB) with 2% SDS and 700 mM 2-mercaptoethanol. Gels were stained with coomassie blue. Commercial protein molecular weight markers (Invitrogen) were used. For immunoblotting PAGE-separated proteins were transferred electrophoretically onto a nitrocellulose membrane (Sigma) in transfer buffer according

to Towbin *et al.* (1979) in semi-dry blotter. After transfer, membranes were soaked in Tris-buffered saline (TBS, 100 mM Tris-HCl, pH 7.5, 0.9% NaCl), containing 2% powder milk or 2% bovine serum albumin, and 10 µl Tween-20 (Sigma) and incubated with gentle agitation for 16 h at room temperature with ASGV-specific immunogammaglobulin (IgG) (1 mg/ml) produced against ASGV virions (Bioreba). After 3 x washing with TBS containing 0.1% Tween-20 membranes were incubated in anti-rabbit antibodies-alkaline phosphatase conjugates (Sigma), and fusion proteins were visualized by reaction with NBT/BCIP as a substrate. Purified fusion protein was tested before immunization by standard DAS-ELISA (Clark & Adams, 1977) against commercial ASGV-antibodies, using extracts from non-transformed *E.coli* BL21c+ cells as controls.

Immunization of rabbits, absorption and calibration of antibodies

Immunizations were performed by priming 2 New Zealand female rabbits, approximately 1.5 months old, four times at weekly intervals. Aliquots of purified ASGVcp/MBP fusion protein (0.6 mg) were emulsified with complete Freund's Adjuvant (FA) and injected intramuscularly into the hind legs of the rabbits. After a further five weeks, a booster injection emulsified with incomplete FA (0.6 mg protein) was given and rabbits were bled beginning one week later at weekly intervals for seven weeks. The IgG was extracted from the antiserum (Mishell & Shiigi, 1980) and purified through a DEAE-Sephacel (Sigma) column (10 cm x 0.7 cm I.D.). Antibody concentration was estimated based on the specific extinction coefficient $A_{280, 1cm}$=1.4 (= 1.0 mg/ml). IgG samples were stored frozen at –20 °C in 0.5 ml aliquots. The antisera A1S, J2S and J3S were processed for antibody purification. For the absorption of antisera (AS), total proteins from healthy apple seedlings (HP) were extracted with ELISA sample extraction buffer (1:1,5, weight:volume), acidified with 0.1M acetic acid pH 4.8 and centrifuged for 20 min at 10.000 x g. The supernatant was adjusted to pH 7.0 with 0.1M NaOH and centrifuged at 99,000 x g. Sediments were resuspended in 0.02 M phosphate buffer and stored frozen in 1 ml aliquots. One ml AS was diluted 1:9 with distilled water, mixed with 1 ml HP, incubated overnight at room temperature and centrifuged 20 min at 12,000 x g. Supernatants containing IgG were processed as above.

Sensitivity and specificity of purified IgG against the ASGVcp were evaluated by plate trapped (PTA) ELISA (Clark & Adams, 1977), modified by coating with the antigen. The ELISA plate wells were coated with 200 µl of extracts from apple or *C. quinoa* leaves prepared by grinding plant tissues (1:10, w:v) in 0.2 M sodium carbonate buffer, pH 9.6. After incubation at 4 °C overnight and 3-time washing with PBS/0.1% Tween-20 (PBS-T), 200 µl of the purified ASGVcp/MBP IgG (1ì g/ml) were added and incubated 3 h at 37 °C. Finally plates were loaded with goat anti-rabbit-alkaline phosphatase conjugate (Sigma) (1:1000) and incubated for 3 h at 37 °C. After washing, 200 µl of the enzyme substrate p-nitrophenylphosphate (0.6-1.0 mg/ml) were added and absorbance was recorded using an ELISA plate reader at A_{405nm}. Negative-positive thresholds were set at two times the mean of healthy control sample absorbance.

A 755 bp DNA fragment amplified using primers ASGV5641 and ASGV6396 containing the complete ASGV cp gene (714 bp) was subcloned into the pMAL-c2 plasmid. This expression plasmid contained a fragment of *E.coli* MBP as a tag of 42.7 kDa for the expression of fusion proteins under the control of the inducible *tac* promotor that allows their purification in an amylose resin column. After induction of the expression by IPTG, BL21c+ cells transformed with recombinant pMAL-c2/ASGVcp produced a 69 kDa protein, absent in non-transformed cells (Figure 1). This 69 kDa protein reacted in western blots to commercial antibodies produced against purified ASGV virus particles, and was therefore referred to as ASGVcp/MBP fusion protein. The specificity of the fusion protein showing that it contained the ASGVcp was demonstrated by its strong reaction in western blot analysis with commercial antibodies against ASGV. One strong band migrating ahead of the fusion protein indicates a common post-purification degradation process (Figure 2).

Results of the evaluation of antibodies, J2S and J3S, purified from three different antisera gave very useful absorbance values for infected samples in PTA-ELISA (Table 1). Although it did not compromise the reliability of the results, it was observed that unspecific absorbance of the healthy apple controls was also considerable, also when antisera were absorbed with healthy host proteins (Figure 3). These values, however, remained in the absorbance range of commercially acquired antibodies used in our laboratory for detection of grape and apple viruses.

The ELISA results obtained with three different antisera show that the antibodies produced against ASGVcp/MBP are of good quality and specificity for ASGV in *C. quinoa*. With antibodies J2S the virus was detectable in apple cvs. Fuji Irradiada and Imperial Gala in dilutions of up to 1:1,000 (Figure 3) and up to 1:2,000 in *C. quinoa* (Table 1).

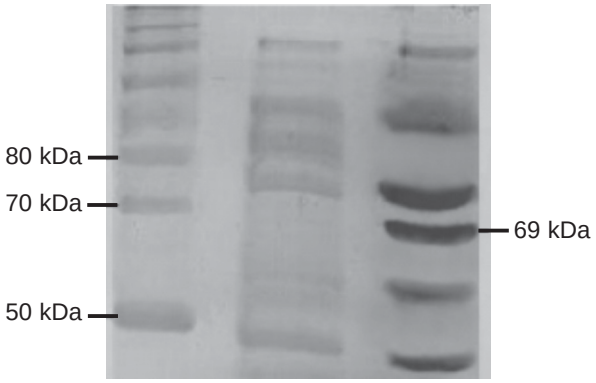

FIG. 1 - Electrophoretic analysis of extracts of *Escherichia coli* transformed with pMALc2/ASGVcp before (2) and after (3) IPTG-induction. Lane 1 contains molecular weight marker proteins (Invitrogen). The gel was stained with coomassie blue.

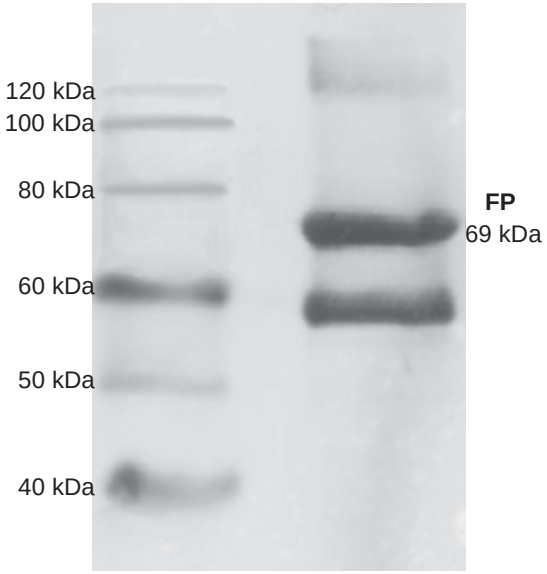

FIG. 2 - Western blot analysis of fusion protein (FP) *Apple stem grooving virus* (ASGV)cp/MBP. Lane 1, molecular weight marker proteins (Invitrogen). Lane 2, fusion protein.

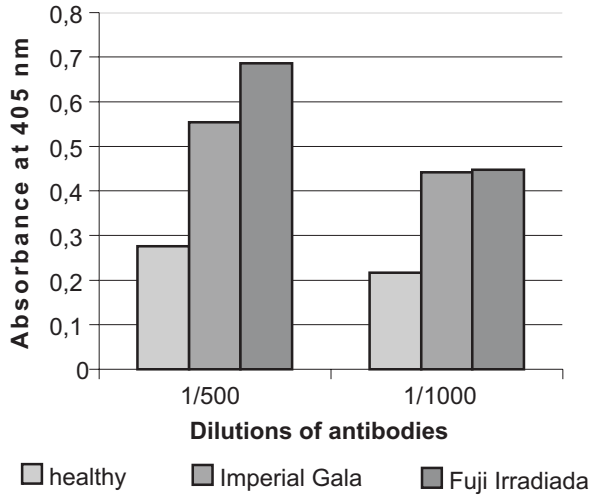

FIG. 3 - Evaluation by ELISA of antibodies raised against *Apple stem grooving virus* (ASGV)cp/MBP fusion protein for detection of ASGV in apples. Young healthy seedlings were used as negative controls.

However, antibodies A1S failed to produce clear positive results with cv. Imperial Gala in the same test, while giving high absorbance values in cv. Fuji Irradiada (not shown). This may be attributed to a low virus titer effect in the cv. Imperial Gala.

The ELISA was selected as the method of choice, for which the antisera were prepared, because it is the most widely used, cost-efficient, reliable and practical plant virus diagnostic instrument for mass analises. Although antibodies raised against recombinant antigens are not generally functional in non-denaturing procedures (Jelkmann & Keim-Konrad, 1997), and are usually tested in denaturing systems (Rubinson *et al.*, 1997) the goal to produce ELISA-functional antibodies against ASGV was achieved. The ELISA diagnosis was in conformity with biological indexing on the woody indicators of the plants used to test the antibodies by ELISA, except for one ELISA test with Imperial Gala. This led to the assumption that a titer effect prevented the detecting of ASGV in Imperial Gala. We conclude that this technology bears great potential for the serological diagnosis of viruses in woody perennial plants and that producing antibodies against ASGV using a recombinant antigen is an advantageous procedure compared to the labor-intensive virus increase in herbaceous hosts and the cumbersome and expensive virus purification. It is expected that development of these ASGVcp antisera will be important in supporting surveys, certification programs and indexing of foundation mother stocks in Southern Brazil.

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TABLE 1 - Evaluation of antibodies produced against the fusion protein *Apple stem grooving virus* (ASGV)cp/MBP for detection of ASGV in *Chenopodium quinoa*

Sample	Test 1	Test 2	Test 3	Test 4	Average$_{1-4}$	2 x average
			Antibodies J2S, 1:1000, absorbed			
Healthy	0.157	0.161	0.186	0.193	0.174	0.348
Chenopodium. quinoa 1	0.397	0.409	0.678	0.641	0.531	
C. quinoa 2	0.455	0.369	0.713	0.554	0.523	
			Antibodies J2S, 1:2000, unabsorbed			
Healthy	0.174	0.194	0.185			0.370
C. quinoa 1	0.853	1.304	1.078			
C. quinoa 2	0.911	0.954	0.932			
			Antibodies J3S, 1:1000, absorbed			
Healthy	0.168	0.162	0.198	0.197	0.181	0.394
C. quinoa 1	-	0.666	-	1.184	0.925	
C. quinoa 2	0.572	0.706	1.017	1.187	0,875	

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