Production and Use of Polyclonal Antibodies to the Coat Protein of *Apple Stem Grooving Virus* Expressed in *Escherichia coli*

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Abstract

The coat protein (cp) gene of *Apple stem grooving Capillovirus* was amplified by RT-PCR, cloned, sequenced and subcloned in the expression plasmid pMal-c2. The ASGV cp gene was expressed in *E. coli* as a fusion protein (fp) containing a fragment of *E. coli* maltose binding protein (MBP). Bacterial cells were disrupted by sonication and the ASGVcp/MBP-fp was purified by amylose resin affinity chromatography. Polyclonal antibodies from rabbits immunized with the fp, gave specific reactions to ASGV from several infected apple cultivars at dilutions of up to 1:2000 in indirect ELISA. The ASGVcp/MBP-fp reacted to antisera raised against it as well as to commercial antisera against ASGV virions in immunoblotting. These results are a fundamental contribution to the development of an efficient ASGV-indexing of propagative and mother stock materials in Southern Brazil.

INTRODUCTION

Apple stem grooving virus (ASGV) is disseminated worldwide in *Rosaceae* fruit trees such as apple, pear, apricot, cherry, and citrus, usually as a latent infection in most commercial cultivars (Magome et al., 1997). Recently ASGV was detected in a close relative of Chinese gooseberry (G. Clover, New Zealand, personal communication). It is one of the most destructive of the known apple latent viruses. In Brazil it has been associated with severe damages to apple plants in nurseries using Maruba-kaido rootstocks (*Malus prunifolia* cv. 'Ringo') (Nickel et al., 1999).

ASGV, the type-member of the genus *Capillovirus* has flexuous, filamentous particles of approximately 600-700 nm (Sequeira & Lister, 1969), with 27 kDa coat protein subunits. Since available strategies to its control (heat therapy, meristem in vitro culture or shoot tip grafting in vivo) do not warrant virus freedom, indexing of plants is required following these procedures. While molecular methods such as RT-PCR are not adequate for mass screening and biological indexing is lenghthy, the enzyme-linked immunosorbent assay (ELISA), although restricted seasonaly, is a convenient, relatively cheap, and reliable method. However, serological indexing candidate mother stock requires large quantities of antisera and large amounts of antigens for immunization purposes, meaning recurrent labor-intensive purifications of the virus increased in herbaceous hosts. ASGV particles have a relatively low stability and tend to aggregate with plant debris or among themselves leading to high losses during purification, so that only very gentle, labour-intensive purification methods, and removal of plant proteins, may be applied. (Sequeira & Lister, 1969; Fuchs & Merker, 1985). 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 gene of Brazilian isolate ASGV-UV01 has recently been cloned and sequenced (Nickel et al., 2001; Genbank access number AF438409), opening the way for the production of large quantities of recombinant ASGVcp antigen. In this paper, we report preparation of a fusion protein with the coat protein gene of isolate ASGV-UV01 in

Escherichia coli as an antigen to raise polyclonal virus-specific antisera for immunodiagnosis.

MATERIALS AND METHODS

Virus Source

ASGV-UV01 was originally isolated from nursery apple trees, established onto tolerant rootstocks, and transmitted mechanically to *Chenopodium quinoa* Willd. and *Nicotiana occidentalis* Wheeler '37B'.

Total RNA was extracted from infected herbaceous hosts by capture on silica particles as described previously (Nickel et al., 1999) and used as template for cDNA synthesis. For PCR amplification, reverse primer ASGV6396 5' CTG CAA GAC CGC GAC CAA GTT T 3' (MacKenzie et al., 1997), complementary to nucleotides 6374 to 6396 and the virus sense primer ASGV5641 5' CGG GAT CCA TGA GTT TGG AAG ACG TGC TTC 3', corresponding to nucleotides 5641 to 5662 with additional 8 nucleotides at its 5' end including a BamHI (underlined, 6 nt) restriction site (Nickel et al., 2001) were used.

RT-PCR Amplification, Sequencing, Cloning of ASGVcp in an Expression Vector

The amplified fragment containing the ASGV coat protein gene was cloned in the pGEM-T Easy vector (Promega) and sequenced (Nickel et al., 2001; Access number AF438409), digested with EcoRI, and subcloned into the dephosphorilated expression vector pMAL-c2 (1.5 µg) (New England Biolabs). Approximately 10 ng insert were ligated into pMAL-c2 in-frame with the fragment of maltose-binding protein (MPB), using T4 DNA ligase, for 2 hours at 15°C and transformed by heat shock into *E. coli* competent cells BL21c+ (Hanahan, 1983). Bacterial suspensions were plated on LB broth (0.2% glucose; 100 µg/ml ampicilin; X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside); IPTG (isopropyl-beta-D-thiogalactopyranoside) and incubated at 37°C (Sambrook et al., 1989).

Expression of ASGV cp in *E. coli* and Purification of the Fusion Protein

250 ml LB broth (0.2% glucose and 100 µg/ml ampilicin) were inoculated with 2,5 ml of fresh overnight cultures of BL21c+ cells/recombinant plasmid pMAL-c2 and incubated at 37°C with vigorous shaking. At OD_{6oo} =0.5, IPTG was added (final conc. of 0.3 mM) and after further 3 hours at 37°C, cells were centrifuged (4000 x g, 20 min.), resuspended (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 1 mM EDTA) and frozen. After thawing, cells were sonicated in an ice bath and the fusion protein purified through a pre-swollen amylose resin/maltose column. Concentration of the eluate was estimated spectrophotometrically at extinction coefficient A_{280nm}=1.44 (according to New England Biolabs information).

Electrophoresis of Fusion Protein and Immunoblotting

Fusion proteins were analysed by SDS-PAGE in 4%/12% gels stained with coomassie blue. Molecular weight (MW) markers (Invitrogen) were used. PAGE-separated proteins were transferred electrophoretically onto a nitrocellulose membrane according to Towbin et al. (1979). After transfer, membranes were "blocked" in Tris-buffered saline (TBS, 100 mM Tris-HCl, pH 7.5, 0.9% NaCl, 2% BSA, 10 µl/10 mlTween-20) for 1 h, and incubated overnight with ASGVcp/MBP-specific antiserum or purified IgG (1:10.000), and commercial ASGV IgG (Bioreba). After washing, first antibody was detected by anti-rabbit IgG-alkaline phosphatase conjugate (Sigma), and fusion proteins were visualized with NBT/BCIP as a substrate. MW markers (Invitrogen; BSA (Sigma) 66 kDa) were used.

Immunization of Rabbits and ELISA Tests

Female New Zealand white rabbits were injected intramuscularly 4 times with ASGVcp/MBP-fp suspension (0.6 mg protein), emulsified 1:1 with complete Freund's Adjuvant (FA) at weekly intervals. After 5 weeks, a booster injection emulsified with incomplete FA was given. Antisera were obtained from seven bleeds beginning one week

later at weekly intervals. IgG was purified through a DEAE-Sephacel (Sigma). Antibody concentration was estimated spectrophotometrically (O.D. 1.4, $A_{280,\ 1cm}$= 1.0 mg/ml). The purified IgG was checked by indirect ELISA (Clark & Adams, 1977) in microtitulation polystyrene plates. Wells were coated with 200 µl of extracts from apple leaves (cvs. 'Fuji Standard', 'Fuji Irradiada', 'Standard Gala', 'Braeburn' and 'Imperatriz' (1:10 in 0.2 M sodium carbonate buffer, pH 9.6), incubated at 4°C overnight, washed (TBS-Tween 20), and 200 µl dilutions of the purified ASGVcp/MBP IgG (1µg/ml) were added for 3 h at 37°C. After washing, plates were loaded with goat-anti-rabbit alkaline phosphatase conjugate (1:1000; 3 h at 37°C). Finally, 200 µl of the enzyme substrate p-nitrophenylphosphate (0.6-1.0 mg/ml) were added. Reactions were recorded at 30 min., 1, 2 and 15 hours using an ELISA plate reader at A_{405nm}.

RESULTS AND DISCUSSION

A 755 bp DNA fragment amplified using primers ASGV5641 and ASGV6396 containing the complete ASGV coat protein gene (714 bp) was subcloned into the pMAL-c2 expression plasmid, which contains a fragment of *E.coli* MBP as a tag of 42.7 kDa for the construction 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, BL21c2+ cells transformed with recombinant pMAL-c2/ASGVcp produced a 69 kDa protein (Figure 1), absent in non-transformed cells (data not shown). This protein reacted in western blots to antibodies raised against it as well as against commercial ASGVcp antibodies and was therefore referred to as ASGVcp/MBP fusion protein. (Figure 2).

The antibodies from both antisera raised against the fusion protein, A-01 and J-01, gave excellent results and low background with healthy control plant tissues when used in indirect ELISA. These antibodies detected ASGV from plants held in water-cooled, unrefrigerated greenhouses in the end of the spring and in the first summer month at dilutions of up to 1:2000 (data not shown).

The specificity of the antibodies produced was demonstrated by western blot analysis of total proteins from ASGV-infected, screenhouse-held, apple cultivars and healthy apple seedlings. The antiserum against the ASGVcp/MBP fusion protein gave a weak but specific reaction with the ASGV coat protein band running at approximately 27 kDa. The weak reaction is explained by the usually low titer of ASGV during hot periods (data not shown). Given the low virus titer in the samples due to seasonal high temperatures, the ELISA results show that the antibodies produced against ASGVcp-fp are of high quality and specificity. 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 as are needed in this case. 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. These preliminary tests of the two antisera show no preferential detection of denatured ASGV coat protein. The diagnosis based on western blots was corroborated by biological indexing on the woody indicator Virginia Crab of the plants used to test the antibodies. 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 labour-intensive virus increase in herbaceous hosts and the cumbersome and expensive virus purification. It is expected that these ASGVcp antisera will be important in supporting surveys, certification programms and indexing of foundation mother stocks in Southern Brazil.

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Figures

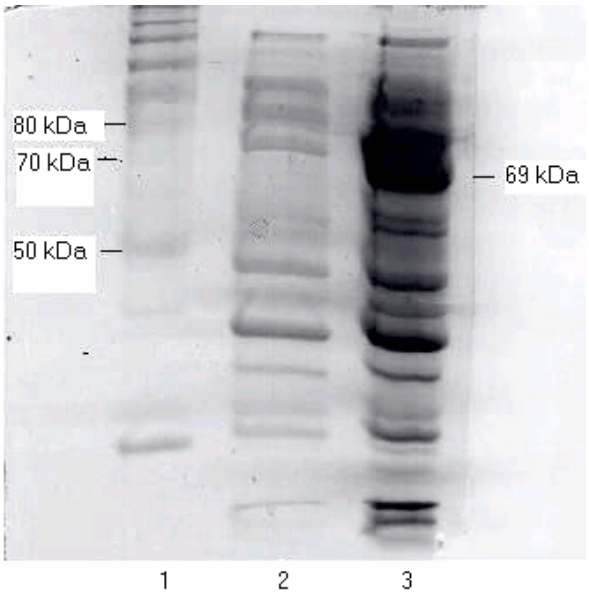

Fig. 1. Electrophoretic analysis of extracts of *E. coli* transformed with pMal-c2/ASGVcp before (lane 2) and after (lane 3) IPTG induction. Lane 1 contains marker proteins (Invitrogen).

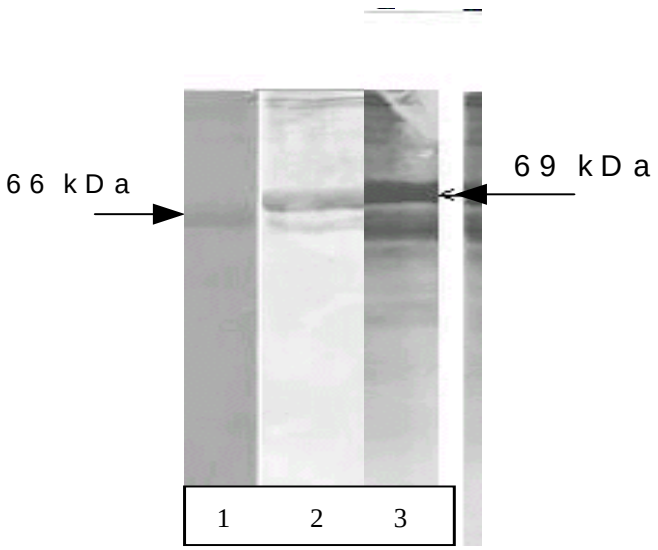

Fig. 2. Western blot analysis of fusion protein (fp) MBP/ASGVcp. Lane 1, molecular weight marker bovine serum albumin (Sigma, 66 kDa); Lanes 2 and 3, MBP/ASGVcp-fp, detected, respectively, by comercial antibodies against ASGV, and antibodies against MBP/ASGVcp-fp.

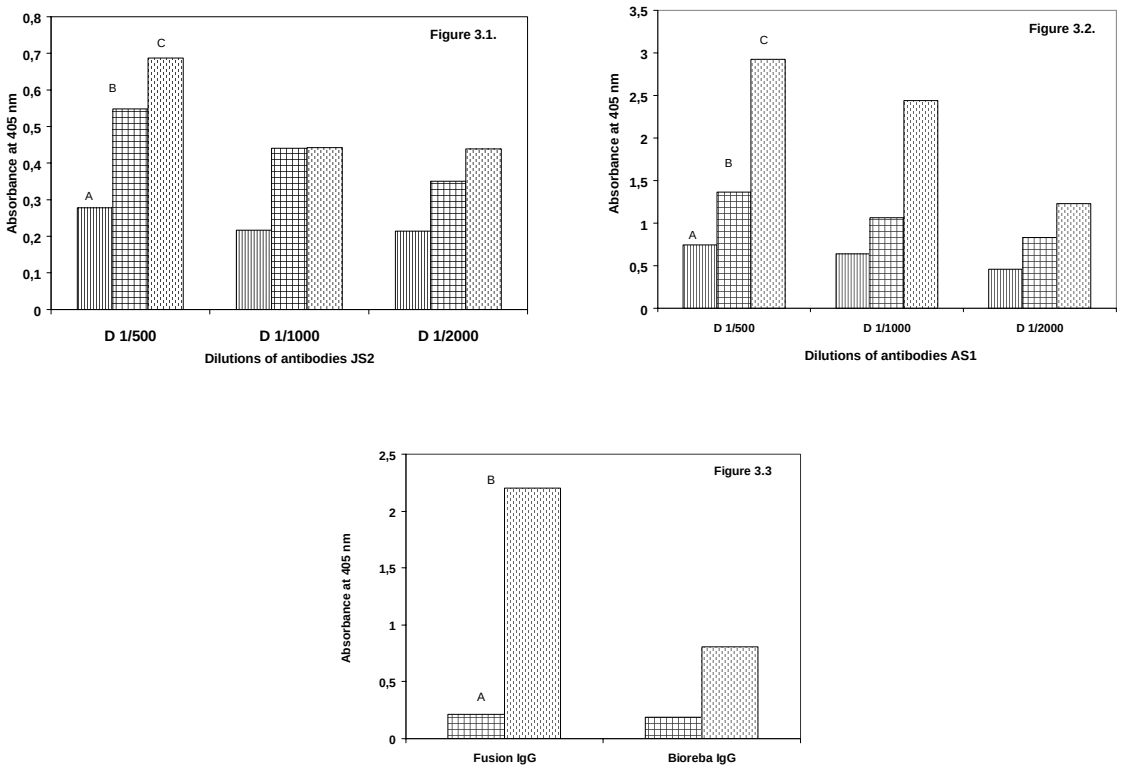

Fig. 3. Evaluation by ELISA of anti-ASGV IgG raised against recombinant antigens. 3.1: Antibodies JS2, A. Healthy control, apple seedlings; B. 'Imperial Gala'; C. 'Fuji Irradiada', B. and C, ASGV-infected. 3.2.: Antibodies AS1, A, B. and C as in 3.1.; 3.3.: Comparison of sensitivity of fusion-IgG and commercial antibodies for ASGV detection, A., Healthy control, cv. 'Braeburn', B. ASGV-infected cv. 'Imperial Gala'.