SHORT COMMUNICATION

Production of polyclonal antibodies to the coat protein gene of Indian isolate of *Apple stem grooving virus* expressed through heterologous expression and its use in immunodiagnosis

Pooja Bhardwaj^{1,2} · Anuradha Negi² · Mahesh Sukapaka³ · Vipin Hallan^{1,2}

Received: 31 August 2019 / Revised: 9 September 2019 / Accepted: 12 December 2019 / Published online: 18 December 2019
© Indian Phytopathological Society 2019

Abstract

Apple stem grooving virus (ASGV) belonging to the genus *Capillovirus* under family *Betaflexiviridae* is one of the widely distributed latent viruses mainly on pome fruit trees. It infects apple causing considerable economic losses that is a threat to the apple industry. To study the occurrence and incidence of ASGV disease, sensitive antisera based diagnostic tool has been developed, which would be helpful in large scale indexing, certification and quarantine programmes. Coat protein gene of ASGV was cloned into pET-32a(+) and pHIS-Parallel expression vectors. Expression characteristics of the proteins expressed from both the systems were compared and taken for raising the antisera. Sensitivity and specificity of the antiserum against virus infection was compared by Double Antibody Sandwich (DAS-ELISA). It was observed that the antisera raised from the protein expressed from pHIS-Parallel expression system (smaller ~ 3 kDa His tag) was more sensitive as compared to the antisera raised from the protein expressed from pET-32a(+) expression system (~ 18 kDa His tag) and the commercially available antisera. The antisera could also be successfully used for western blotting of infected samples and in DAS-ELISA. Thus, this study presents the first report on production of polyclonal antiserum against recombinant coat protein of ASGV from India, for reliable detection of the virus.

Keywords ASGV · DAS-ELISA · pET-32a(+) · pHIS-Parallel · Polyclonal antibody

Introduction

Apple is one of the most widely grown fruit crop worldwide. China is the leading producer of apples in the world, followed by United States, while India occupies the seventh position with an average production of around 7.24 tonnes per hectare (Thamaraikannan et al. 2010).

However, viral diseases significantly reduce both yield and quality of apples (Cembali et al. 2003). The major viruses infecting apple are *Apple chlorotic leaf spot virus*

(ACLSV; genus *Trichovirus*), *Apple mosaic virus* (ApMV; genus *Ilarvirus*), *Apple stem pitting virus* (ASPV; genus *Foveavirus*) and *Apple stem grooving virus* (ASGV; family: *Betaflexiviridae* genus *Capillovirus*). ASGV is one of the widely distributed latent viruses infecting pome fruits (Khan and Dijkstra 2006) and also infects Apricot (Takahashi et al. 1990), Cherry (Kinard et al. 1996), Kiwifruit (Clover et al. 2003) and causes Pear Black necrotic leaf spot disease (PBNLS) in Pear, bud union disorder in citrus (syn: *Citrus tatter leaf virus*; CTLV) (Shim et al. 2004) and damage to trifoliate orange (Miyakawa and Ito 2000). Recently, *Nandina domestica* (heavenly bamboo) has been reported to be a new host for ASGV (Tang et al. 2010).

ASGV is known to cause severe pitting and grooving of the xylem. In addition to this, it is also associated with brown line and graft union abnormalities and an overall decline in susceptible *Malus* species. On an average the incidence of ASGV on apple is as high as 40%. The virus has monopartite, positive sense ss RNA (~ 6.5 kb) genome consisting of two overlapping open reading frames (ORF's): ORF1 (6.3 kb) and ORF2 (1.0 kb) (Yoshikawa et al. 1992).

✉ Vipin Hallan
hallan@ihbt.res.in; rnaivi@gmail.com

¹ Academy of Scientific and Innovative Research (AcSIR), New Delhi, India

² Plant Virology Lab, CSIR-Institute of Himalayan Bioresource Technology, Palampur 176061, Himachal Pradesh, India

³ Regulatory Research Facility, CSIR-Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh 176061, India

The larger ORF1 encodes for a 241 kDa polyprotein and a 27 kDa coat protein (CP), while ORF2 encodes for 36 kDa movement protein (MP) and viral proteases (Yoshikawa et al. 1992).

During the last two decades much advancement has been made in the development of diagnostic system for the detection of viral diseases. Molecular method such as Reverse transcription-polymerase chain reaction (RT-PCR) is considered to be very effective but is expensive for the mass screening of plant stocks.

On the other hand virus indexing of mother stocks and tissue culture raised plants on woody indicators is very time consuming as one has to wait for results till symptom expression (Nickel et al. 2004). Among all the serological detection techniques used for the detection of plant viruses, ELISA one of the most suitable technique which being relatively cheap and reliable, is easy to handle and used for the mass screening of the virus infected samples (Lee and Chang 2006). Serological testing requires a large amount of specific antisera which is produced by injecting the purified virus particles in the blood stream of rabbits (Hull 2002). But the conventional methods used for purification of the virus are often associated with large number problems with regard to the purity, virus titer, presence of inhibitory compounds (like polyphenols, polysaccharides and tannins) and specificities which may not give consistent diagnostic results (Ling et al. 2000; Barbieri et al. 2004; Ling et al. 2007). Therefore, in order to overcome these problems, recombinant approach is currently being employed for production of large amount of antigens with uniform concentration and stability. The genes of interest are expressed in prokaryotic systems viz. *E.coli* and the antigens are being produced as and when required (Targon et al. 1997; Hull 2002; Alkowni et al. 2011). Earlier studies also reveal the production of polyclonal antibodies using the structural proteins for several plant viruses which were expressed in bacteria (Gulati-Sakhuja et al. 2009; Khatabi et al. 2012).

Relatively high sequence conservation (around 90–100%) in the coat protein of ASGV isolates makes it an ideal candidate for the development of diagnostics. Present study describes the preparation of CP fusion proteins of Indian ASGV apple isolate and the comparative analysis of antisera produced. Thus effort has been made to develop antisera based on CP gene of Indian isolate of ASGV using recombinant DNA technology.

Materials and methods

Virus source

The leaf sample displaying severe virus like symptoms viz. necrotic spots, chlorosis and chlorotic spots was collected

from Starkrimson variety of apple (Ap-SK), growing at the apple germplasm field of (CSIR-IHBT) Palampur, Himachal Pradesh.

Characterization of complete coat protein gene

For characterization of complete CP gene of ASGV, RNA was extracted through CTAB method (Zeng and Yang 2002) and then RT-PCR was performed using complete CP primers (Nickel et al. 2001). For cDNA synthesis a reaction mixture of 25 µl was prepared which contained 1 µg RNA, 1 µl (10 pmol/µl) of downstream primer (ASGV 6396 R: 5'-CTG CAAGACCGCGACCAAGTTT-3'), 5 µl of M-MLV reverse transcription buffer (5X) (USB, Cleveland, Ohio, USA), 0.5 µl (40 U/µl) RNase inhibitor (USB, USA), 1.5 µl dNTP mix (40 mM) and 0.5 µl (200 U/µl) of Mu-MLV reverse transcriptase (USB). Then RT reaction was incubated at 37 °C for 75 min followed by enzyme inactivation at 70 °C for 5 min.

Further, PCR was carried out in 9700 Thermal Cycler (Applied Biosystems, USA) using reaction mixture of 50 µl which contained 5 µl of *Taq* DNA polymerase buffer (10X) (Merck Biosciences, India), 10 pmol each of upstream and downstream primers (ASGV 5641 F: 5'-ATGAGTTTGGAA GACGTGCTTC-3' and ASGV 6396 R: 5'-CTGCAAGAC CGCGACCAAGTTT-3'), 5 µl cDNA, 1.5 µl dNTP mix (10 mM) and 0.5 µl (3u/µl) *Taq* DNA polymerase (Merck Biosciences, India). Denaturation was performed at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 40 s; primer annealing at 62 °C for 1 min; extension at 72 °C for 1 min and a final elongation at 72 °C for 8 min. Then amplified PCR products were analyzed on 1% agarose gel and visualized under UV transilluminator after staining with ethidium bromide. Amplicons were purified from the gel using GeneJET Gel Extraction kit (Thermo Scientific, Waltham, Massachusetts, USA) and cloned into pGEM-T easy vector (Promega, Madison, Wisconsin, USA). Then recombinant plasmids were purified using Plasmid DNA Miniprep kit (MDI, Ambala Cantt, India) and sequencing was done in an automated DNA sequencer (ABI PRISM®3130xl Genetic Analyzer) using ABI prism Big Dye™Terminator v3.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems, USA), sequencing both strands. The sequence of the complete CP gene of ASGV (714 bp) was submitted to Genbank, NCBI accession no. LN627002.

Amplification of CP gene using expression primers

The above sequence of complete CP gene of ASGV characterized from apple (LN627002) was used for designing the primer. Specific primer pairs containing restriction enzyme sites (BamHI and EcoRI for pET-32a(+)) and EcoRI and XhoI for pHIS-Parallel) in the upstream and downstream

primers were designed for the inframe cloning of the insert in two expression vectors (Table 1).

ASGV CP gene was amplified using already cloned CP as template and above mentioned primers. PCR was performed with Denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 53 °C for 1 min; extension at 72 °C for 1 min and a final extension at 72 °C for 10 min for pET-32a(+), whereas in case of pHIS-Parallel the same PCR conditions were used except for the primer annealing which was performed at 58 °C for 1 min. The amplified complete CP gene (using both primer pairs) was sub-cloned in pGEM-T easy vector (Promega, Madison, WI, USA) and sequenced.

Cloning of ASGV CP in expression vectors

The pGEM-T easy vectors containing cloned CP genes (with restriction enzyme sites) and the expression vectors [pET-32a(+) and pHIS-Parallel] were simultaneously digested with their respective restriction enzymes [BamHI and EcoRI for pET-32a(+) and EcoRI and XhoI for pHIS-Parallel] and separated on 1% agarose gel. The double digested ASGV CP fragments and the expression vectors were subjected to elution using GenElute Gel Extraction kit (Sigma Aldrich, USA). Approximately 50 ng of eluted products were ligated into their respective vectors using T4 DNA ligase (Fermentas, Lithuania) at 16 °C overnight as per manufacturer instructions and then transformed into *E. coli* DH5 α competent cells. Several clones were checked by boiling prep plasmid isolation and restriction digestion methods as per Sambrook et al. (1989) and out of them two were confirmed through sequencing to ascertain inframe cloning. The recombinant clones were then again transformed into *E. coli* BL21 competent cells as per Hanahan (1983).

Standardization of optimal expression conditions for ASGV CP

Single positive colonies (BL21) containing chimeric pET-32a(+) and pHIS-Parallel vectors were grown overnight at 37 °C in 5 ml of Luria broth (LB) supplemented with ampicillin (100 μ g/ml). For protein expression, 2X yeast tryptone broth (YT) supplemented with ampicillin was inoculated with overnight grown cells, and incubated at 37 °C on a rotary shaker till the OD₆₀₀ reached 0.5. Various IPTG

concentrations (ranging from 0.5 to 1.5 mM) and different temperature (28–37 °C) were used for standardization of optimal expression at different time intervals (1, 2, 3 and 4 h).

Then centrifugation was carried out at 10,000 rpm for 2 min and cell pellets obtained were suspended in 800 μ l of 1X PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄ and 2 mM KH₂PO₄, pH 7) and finally subjected to sonication (in an ice bath) for cell disruption. After sonication, samples were again centrifuged at 12,500 rpm for 10 min at 4 °C. Pellets were resuspended in 800 μ l PBS while the supernatant were collected separately. Then resuspended pellets (10 μ l) and supernatant (from each of the tubes) were analyzed on 12% SDS-PAGE and stained with Coomassie Brilliant Blue R-250 (0.2%).

Purification of fusion proteins for antibody production

Expressed fusion proteins (showing maximum expression at standardized conditions) from pET-32a(+) to pHIS-Parallel expression systems were purified using Maxwell[®] 16 Poly-histidine Protein Purification Kit (Promega, USA). Purity and integrity of the fusion proteins were checked on 12% SDS-PAGE and concentration was determined by Bradford method (Bradford 1976).

Immunoblotting of fusion proteins

In order to confirm the identity of fusion proteins, Western blotting was carried out by immunoblotting SDS-PAGE separated fusion proteins according to the protocol of Towbin et al. (1979). The fusion proteins separated on gel were electrophoretically transferred onto a PVDF membrane (Bio-rad, USA) using transfer buffer in a miniVE blotter apparatus (GE Healthcare USA) according to manufacturer's instructions. The membranes were soaked in 1X TBST (50 mM Tris/HCl, 150 mM NaCl, 0.05% Tween 20 (V/V); pH 7.5) for 1 h with constant shaking in a gel rocker and then incubated overnight at 4 °C in 1% blocking solution (skimmed milk powder). Washed the membranes with washing buffer (TBST), 3–5 times and then incubated with ASGV specific immunoglobulin globulin (IgG) (1 mg/ml) produced against ASGV virions (Bioreba, USA) for 2 h with constant shaking. The membranes were washed three times with TBST

Table 1 Primers with restriction sites (underlined) for expression of ASGV CP in pET-32a(+) and pHIS-Parallel expression system

Sr no.	Expression system	Primer name	Primer sequence
1	pET-32a(+)	ASGVexp F	5'- <u>GGATCC</u> ATGAGTTTGAAGACGTGC-3'
		ASGVexp R	5'- <u>GAATTC</u> CTAACCCCTCCAGTTCC-3'
2	pHIS-Parallel	pHIS F	5'- <u>TTCTGAATTC</u> ATGAGTTTGAAGACGTGCTTC-3'
		pHIS R	5'- <u>TCACTCGAGCTA</u> ACCCTCCAGTTCCAGATT-3'

for 5 min each and incubated in anti-rabbit antibodies horse raddish peroxidase (HRP) conjugates (Roche, USA) with constant shaking. The fusion proteins were visualized by reaction with Super Signal West Femto Chemiluminescent Substrate (Thermo scientific Pierce, USA). Membranes were exposed to X-ray film for 1 min to several minutes depending on the intensity of signals and minimal background and finally developed.

Immunization of rabbits and purification of antibodies

The purified fusion proteins were used as antigens and injected in two healthy New Zealander Albino male rabbits (approximately 6 weeks old). In order to raise the hyper-immune sera against ASGV CP the rabbits were injected six times at weekly intervals. For the first injection purified fusion proteins ASGV-CP/HIS (500 µg/injection) were emulsified with Freund's complete adjuvant (FA) (Genei, India) (1:1 v/v) and injected subcutaneously into the hind legs of rabbits at eight different sites. In the subsequent injections purified fusion proteins of ASGV CP were emulsified with the incomplete Freund's adjuvant and one week after booster injection the rabbits were bled from the marginal ear vein.

The blood was collected into falcon tube (50 ml) and allowed to clot at room temperature for an hour and then at 4 °C (overnight). Serum collected from the clotted blood was centrifuged at 5000 rpm for 10 min at 4 °C and stored at 4 °C after adding sodium azide to a concentration of 0.02% (w/v). Finally IgG was purified from the serum using Protein A antibody purification kit (Genei, India) as per manufacturer's instructions. The concentration of IgG was determined by Bradford method (Bradford 1976) and stored at –20 °C.

Preparation of antibody-enzyme conjugates and checking of indigenously raised antibodies

A part of separated ASGV antibodies 2 ml (1 mg/ml concentration) were conjugated with alkaline phosphatase 2 mg (Sigma, USA) according to manufacturer's instructions as per Avrameas (1969). The sensitivity and activity of the prepared antibodies was tested by double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) according to the method of Clark and Adams (1977). The activity of indigenously raised antibodies (IgG) and their enzyme conjugates were checked at various dilutions (1:100, 1:200, 1:300, 1:500 1:1000 and 1:2000) for identification of maximum dilution of antibodies at which the virus could be detected successfully. In order to check these dilutions Indian ASGV positive apple sample (Ap-SK; apple), purified fusion proteins, healthy and ASGV infected herbaceous

host (*Chenopodium quinoa*, maintained in the greenhouse) inoculated with the positive apple sample were screened.

Field screening for efficacy of the indigenously raised kits

To estimate the strength of raised antiserum, leaf samples from different pome and stone fruits were analyzed for the presence of ASGV by DAS-ELISA as per Clark and Adams (1977) using developed antibodies (IgG and enzyme conjugates). At the same time these samples were also checked and compared with the reference kit (Standard commercial kit, Bioreba, Switzerland) using manufacturer's instructions.

Results and discussion

Complete CP gene of ASGV was amplified using Starkrimson cultivar of apple (Ap-SK) and complete CP primers. An amplification of ~750 bp which includes 714 bp of complete CP was obtained and confirmed through sequencing. Further ASGV CP was sub-cloned into the pET-32a(+) and pHIS-Parallel expression vectors, using primers flanked by appropriate restriction sites for directional and inframe cloning, which was also confirmed through sequencing. The positive clone (one each, for pET-32a(+) and pHIS-Parallel expression vectors) was used for the standardization of optimal expression conditions for ASGV CP gene in vitro.

The best expression level of ASGV CP was observed at 1 mM IPTG concentration after 3 h induction at 30 °C for pET-32a(+) vector (Fig. 1a) and at 1 mM after 4 h induction at 28 °C for pHIS-Parallel vector (Fig. 1b). The expressed protein of ~45 kDa fused with His tag along with TRX and S tags (~18 kDa) in pET-32a(+) vector and ~30 kDa fused with His tag of ~3 kDa in pHIS-Parallel vector were observed on SDS-PAGE gel. The fusion proteins of ASGV were expressed in the pellet in form of inclusion bodies (insoluble inactive aggregates of target protein) which were isolated and used for the production of the antiserum. This procedure has an added advantage that protein can be easily purified by centrifugation in highest yield and the protein is also protected from the proteolytic degradation in the host cell. Similar results have been reported for *Faba bean necrotic yellows virus*, *Pelargonium zonate spot virus* and *Potato mop-top virus* (Kumari et al. 2001; Cerovska et al. 2003, 2006; Gulati-Sakhuja et al. 2009).

Purified proteins were checked for purity and integrity by electrophoresis on 12% SDS-PAGE gel. After coomassie staining, one distinct band of expected size ~45 kDa for pET-32a(+) and ~30 kDa for pHIS-Parallel vector was observed (Fig. 2a–b). The maximum concentration of purified proteins (by Bradford method) in best fractions

Fig. 1 Expression of ASGV CP as His Tag fusion protein in pET-32a(+) and pHIS-Parallel expression systems. **a** Lane 3: ~45 kDa His tag fusion protein (in inclusion bodies: insoluble fraction) expressed after 3 h of induction at 1 mM IPTG concentration and at 30 °C, Lane 1: Corresponding protein profile in soluble fraction, Lane 2: Control

with no IPTG. **b** Lane 3: ~30 kDa His Tag fusion protein (in inclusion bodies) expressed after 4 h of induction, at 1 mM IPTG concentration and at 28 °C, Lane 2: Corresponding protein profile in soluble fraction, Lane 1: control with no IPTG. Lane M: Prestained protein marker (Fermentas, Lithuania)

Fig. 2 Purified ASGV CP as His Tag fusion protein expressed in **a** pET-32a(+) and **b** pHIS-Parallel vector from Maxwell 16 polyhistidine protein purification system (Promega USA). Lane M: Prestained protein marker (Fermentas, Lithuania)

was found to be 4 mg/ml for pET-32a(+) and 6 mg/ml for pHIS-Parallel expression system.

The specificity of the (~45 and ~30 kDa) fusion proteins was evaluated by their strong reaction in western blotting with commercial antibodies (produced against purified ASGV virus particles), which indicates that it contained ASGV CP and is specific for the virus (Fig. 3a, b).

For immunization of the rabbits, the fractions of eluted proteins were diluted to a final concentration of 1 mg/ml and injected in the form of 6 weekly injections, the serum was collected and IgG was extracted. The concentration of IgG extracted from the total serum was determined, which was found to be 6 mg/ml in 5 ml of elution buffer for pET-32a(+) and 10 mg/ml in 5 ml of elution buffer in case of pHIS-Parallel. The antibodies were diluted to final concentration of 1 mg/ml and evaluated using DAS-ELISA.

It was observed that the produced antibodies reacted positively with purified fusion proteins, known ASGV (Indian isolate) positive apple samples and infected *Chenopodium quinoa* plants at 1:1000 (2 µg/ml) dilutions in DAS-ELISA. An intense reaction was observed in case of the antibodies raised in pHIS-Parallel as compared to the antibodies raised in pET-32a(+). The maximum titer to which the antibodies could be diluted and gave confirmed positive results for both indigenously raised antibodies (IgG and the conjugate) was 1:1000. However, no reaction was observed with the extract from healthy *Chenopodium quinoa* plant (used as a control) thereby demonstrating the specificity of the indigenously raised antibodies.

To check the efficacy of indigenously raised kits (at 1:1000 dilution of IgG and the conjugate), total 60 leaf samples from different pome and stone fruits were assayed with DAS-ELISA. At the same time they were

Fig. 3 Western blot confirming the identity of ASGV (CP) protein expressed in **a** pET-32a (+) and **b** pHIS-Parallel system

also analyzed by a reference kit (i.e. commercial ASGV kit, Bioreba, Switzerland). Twenty out of 60 samples were found positive for ASGV by indigenously raised kits in pET-32a(+) and pHIS-Parallel, while 14 samples were found positive with commercial kit (Table 2). Further, it was observed that in nine samples [WC2 (Wild Cherry); RD3 (Royal Delicious); T1 (Tydeman); G1, G5 (Gala); CR3 (Crytonian); SC5 (Starkrimson); BE2 (Bright N Early); and GH1 (Golden Horner)] ASGV was diagnosed better. In all these samples, an intense signal was observed with indigenously raised antisera in pHIS-Parallel as compared to pET-32a(+) raised protein and reference kit. Moreover, six samples [T4 (Tydeman); CR2 (Crytonian); SC3 (Starkimson); GS3 (Gold Spur); RC1 (Red Chief) and RF2 (Red Fuji)] which tested negative with the reference (commercial) kit were found positive with indigenously raised antisera [in pHIS-Parallel and pET-32a(+)] and gave strong intense reactions with pHIS-Parallel raised antisera. All the positive samples were also confirmed by RT-PCR (data not shown). It was observed that some of the samples which were negative with commercial kit but positive with indigenously raised antisera were positive in RT-PCR, which indicates that the indigenously raised antisera were more sensitive as compared to commercially available antisera.

The DAS-ELISA results clearly indicates that the antiserum prepared in the present study was more sensitive for the detection of Indian isolates of ASGV in comparison to the commercial kit. Moreover, as the antiserum was raised using Indian isolate and testing was done for the Indian isolates,

this could be another reason for better results obtained with indigenously raised antisera as compared to the commercial kit.

The sensitivity of antiserum depends upon the antigenicity of protein, which in turn depends on the interference due to the affinity tag (Wu and Filutowicz 1999). Small size of the affinity tag ensures that the protein activity is rarely affected (Terpe 2003). As pHIS-Parallel vector has relatively smaller tag size (~3 kDa) than the pET-32a(+) vector (~18 kDa), this could be the most probable reason for the higher sensitivity of pHIS-Parallel raised antibody.

However, if we rely only on the commercial antisera for indexing of the plants, some of the infected material could pass unnoticed, as some of the samples [like T4 (Tydeman); CR2 (Crytonian); SC3 (Starkimson); GS3 (Gold Spur); RC1 (Red Chief) and RF2 (Red Fuji)] which were found negative with the commercial kit turned out to be positive when tested with indigenously raised antiserum in pHIS-Parallel (strong intense reactions) expression system.

It is expected that this specific antisera will be important in certification programs and indexing of the mother stock for raising virus free plants. Moreover, the use of purified and expressed fusion protein as antigen gives consistent results in terms of quantity and quality of the antigen.

To the best of our knowledge this is a first report from India on the production of polyclonal antibodies against ASGV CP and its application in mass screening of samples by DAS-ELISA. This specific polyclonal antiserum would be helpful for the serological detection of ASGV infection.

Table 2 Comparison of OD values at 405 nm of DAS-ELISA for different pome and stone fruits using diagnostic antisera developed for ASGV and the commercial kit (reference)

Sr. no.	Plant tested	Sample code	OD values of ELISA using antisera obtained from		
			pHIS-parallel	pET-32a(+)	Commercial antisera
1	Wild cherry	WC1	0.339 (–)	0.311 (–)	0.297 (–)
2	Wild cherry	WC2	1.827 (++++)	0.820 (++)	0.608 (+)
3	Wild cherry	WC3	0.347 (–)	0.290 (–)	0.219 (–)
4	Wild cherry	WC4	0.326 (–)	0.312 (–)	0.333 (–)
5	Wild cherry	WC5	0.205 (–)	0.198 (–)	0.197 (–)
6	Wild cherry	WC6	0.286 (–)	0.269 (–)	0.258 (–)
7	Royal delicious	RD1	1.614 (++)	0.832 (++)	0.676 (+)
8	Royal delicious	RD2	0.166 (–)	0.146 (–)	0.145 (–)
9	Royal delicious	RD3	2.370 (++++)	1.099 (++)	0.695 (+)
10	Royal delicious	RD4	0.239 (–)	0.229 (–)	0.230 (–)
11	Royal delicious	RD5	0.111 (–)	0.110 (–)	0.111 (–)
12	Vance delicious	VD1	1.345 (++)	0.839 (++)	0.643 (+)
13	Vance delicious	VD2	1.342 (++)	0.812 (++)	0.801 (++)
14	Vance delicious	VD3	0.228 (–)	0.216 (–)	0.212 (–)
15	Vance delicious	VD4	0.310 (–)	0.307 (–)	0.294 (–)
16	Vance delicious	VD5	0.220 (–)	0.199 (–)	0.197 (–)
17	Vance delicious	VD6	0.160 (–)	0.127(–)	0.118 (–)
18	Tydemman	T1	1.776 (++++)	0.730 (+)	0.689 (+)
19	Tydemman	T2	0.222 (–)	0.212 (–)	0.201 (–)
20	Tydemman	T3	0.167 (–)	0.154 (–)	0.145 (–)
21	Tydemman	T4	1.876 (++++)	0.982 (++)	0.345 (–)
22	Tydemman	T5	0.239 (–)	0.220 (–)	0.219 (–)
23	Tydemman	T6	0.278 (–)	0.269 (–)	0.264 (–)
24	Gala	G1	2.604 (++++)	1.091 (++)	0.916 (++)
25	Gala	G2	0.153 (–)	0.145 (–)	0.132 (–)
26	Gala	G3	1.166 (++)	0.888 (++)	0.624 (+)
27	Gala	G4	0.170 (–)	0.156 (–)	0.145 (–)
28	Gala	G5	1.674 (++)	0.756 (+)	0.632 (+)
29	Crytonian	CR1	0.304 (–)	0.302 (–)	0.298 (–)
30	Crytonian	CR2	1.166 (++)	0.788 (+)	0.349 (–)
31	Crytonian	CR3	2.101 (++++)	1.966 (++++)	0.624 (+)
32	Crytonian	CR4	0.159 (–)	0.125 (–)	0.119 (–)
33	Crytonian	CR5	0.313 (–)	0.309 (–)	0.303 (–)
34	Starkrimson	SC1	0.187 (–)	0.155 (–)	0.145 (–)
35	Starkrimson	SC2	0.188 (–)	0.161 (–)	0.157 (–)
36	Starkrimson	SC3	1.152 (++)	0.916 (++)	0.346 (–)
37	Starkrimson	SC4	0.238 (–)	0.222 (–)	0.213 (–)
38	Starkrimson	SC5	0.942 (++)	0.710 (+)	0.602 (+)
39	Bright N Early	BE1	0.150 (–)	0.148 (–)	0.149 (–)
40	Bright N Early	BE2	1.933 (++++)	0.728 (+)	0.527 (+)
41	Bright N Early	BE3	0.158 (–)	0.134 (–)	0.127 (–)
42	M4 (RS)	M41	0.169 (–)	0.158 (–)	0.155 (–)
43	M4 (RS)	M42	0.959 (++)	0.843 (++)	0.623 (+)
44	M4 (RS)	M43	0.213 (–)	0.209 (–)	0.210 (–)
45	M4(RS)	M44	0.155 (–)	0.145 (–)	0.123 (–)
46	M4 (RS)	M45	0.163 (–)	0.160 (–)	0.157 (–)
47	Gold Spur	GS1	0.110 (–)	0.109 (–)	0.108 (–)
48	Gold Spur	GS2	0.108 (–)	0.101 (–)	0.103 (–)
49	Gold Spur	GS3	1.791 (++++)	0.904 (++)	0.343 (–)

Table 2 (continued)

Sr. no.	Plant tested	Sample code	OD values of ELISA using antisera obtained from		
			pHIS-parallel	pET-32a(+)	Commercial antisera
50	Gold Spur	GS4	0.288 (–)	0.220 (–)	0.217 (–)
51	Golden Hornet	GH1	1.214 (++)	0.688 (+)	0.576 (+)
52	Golden Hornet	GH2	0.346 (–)	0.310 (–)	0.305 (–)
53	Golden Hornet	GH3	0.176 (–)	0.156 (–)	0.155 (–)
54	Red Chief	RC1	1.282 (++)	0.874 (++)	0.352 (–)
55	Red Chief	RC2	0.339 (–)	0.320 (–)	0.319 (–)
56	Red Chief	RC3	0.187 (–)	0.169 (–)	0.159 (–)
57	Red Chief	RC4	0.326 (–)	0.312 (–)	0.333 (–)
58	Red Fuji	RF1	0.239 (–)	0.229 (–)	0.230 (–)
59	Red Fuji	RF2	1.809 (+++)	0.890 (++)	0.352 (–)
60	Red Fuji	RF3	0.173 (–)	0.160 (–)	0.156 (–)
	Positive control		2.649	1.923	1.910
	Negative control		0.177	0.176	0.169

Three times the value of negative control was taken as positive. The samples in bold are positives identified by indigenously raised antisera's but negative by commercial

(+++; > 1.76)= Strong reaction, (++; 0.8–1.75)= Mild reaction, (+; 0.5–0.75)= Weak reaction (–)= Negative reaction

Acknowledgements The authors are thankful to the Director, CSIR-Institute of Himalayan Bioresource Technology Palampur, HP (India), for providing necessary facilities. Department of Biotechnology (DBT), Government of India is duly acknowledged for financial support. Academy of Scientific and Innovative Research (AcSIR) New Delhi is also acknowledged. This is CSIR-IHBT publication number 3616.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

References

- Alkowni R, Zhang YP, Rowhani A, Uyemoto JK, Minafra A (2011) Biological, molecular, and serological studies of a novel strain of grapevine leafroll-associated virus 2. *Virus Genes* 43:102–110
- Avrameas S (1969) Coupling of enzyme to proteins with glutaraldehyde. *Immunochemistry* 6:43–52
- Barbieri MR, Carvalho MG, Zambolim EM, Zerbini FM (2004) Expressão de *Escherichia coli* da proteína capsidial do *Watermelon mosaic virus* e produção de anti-soro. *Fitopatol Bras* 29:215–219
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Cembali T, Folwell RJ, Wandschneider P, Eastwell KC, Howell W (2003) Economic implications of a virus prevention program in deciduous tree fruits in the US. *Crop Prot* 22:1149–1156
- Cerovska N, Moravec T, Rosecka P, Dedic P, Filigarova M (2003) Production of polyclonal antibodies to a recombinant coat protein of *Potato mop-top virus*. *J Phytopathol* 151:195–200
- Cerovska N, Filigarova M, Pecenkova T (2006) Production of polyclonal antibodies to a recombinant *Potato mop top virus* non-structural triple gene block protein. *J Phytopathol* 154:422–427
- Clark MF, Adams AN (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J Gen Virol* 34:475–483
- Clover GR, Pearson MN, Elliot DR, Tang Z, Smales TE, Alexander BJR (2003) Characterization of a strain of *Apple stem grooving virus* in *Actinidia chinensis* from China. *Plant Pathol* 52:371–378
- Gulati-Sakhuja A, Sears JL, Nunez A, Liu HY (2009) Production of polyclonal antibodies against *Pelargonium zonate spot virus* coat protein expressed in *Escherichia coli* and application for immunodiagnosis. *J Virol Methods* 160:29–37
- Hanahan D (1983) Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* 166:557–580
- Hull R (2002) Matthews' plant virology, 4th edn. Academic Press, London
- Khan JA, Dijkstra J (2006) Handbook of Plant Virology. Food Products Press, London
- Khatabi B, He B, Hajimorad MR (2012) Diagnostic potential of polyclonal antibodies against bacterially expressed recombinant coat protein of *Alfalfa mosaic virus*. *Plant Dis* 96:1352–1357
- Kinard GR, Scott SW, Barnett OW (1996) Detection of *Apple chlorotic leaf spot* and *Apple stem grooving viruses* using RT-PCR. *Plant Dis* 80:616–621
- Kumari SG, Makkouk KM, Katul L, Vetten HJ (2001) Polyclonal antibodies to the bacterially expressed coat protein of *Faba bean necrotic yellows virus*. *J Phytopathol* 149:543–550
- Lee SC, Chang YC (2006) Multiplex RT-PCR detection of two orchid viruses with an internal control of plant nad5 mRNA. *Plant Pathol Bull* 15:187–196
- Ling KS, Zhu HY, Jiang ZY, 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. *Eur J Plant Pathol* 106:301–309
- Ling KS, Zhu HY, Petrovic N, Gonsalves D (2007) Serological detection of *Grapevine leafroll virus 2* using an antiserum developed against the recombinant coat protein. *J Phytopathol* 155:65–69
- Miyakawa T, Ito T (2000) Tatter leaf-citrus stunt. In: Timmer LW, Garnsey SM, Graham JH (eds) Compendium of citrus diseases, 2nd edn. American Phytopathological Society, St. Paul, p 60

- Nickel O, Fajardo TVM, Jelkmann W, Kuhn GB (2001) Sequence analysis of the capsid protein gene of an isolate of *Apple stem grooving virus*, and its survey in Southern Brazil. *Fitopatol Bras* 26(3):655–659
- Nickel O, Targon MLPN, Fajardo TVM, Machado MA, Trivilin AP (2004) Polyclonal antibodies to the coat protein of *Apple stem grooving virus* expressed in *Escherichia coli*: production and use in immunodiagnosis. *Fitopatol Bras* 29:558–562
- Sambrook J, Fritsch EF, Maniatis T (1989) In *Molecular cloning: A laboratory manual*, 2nd edn. Cold Spring Harbor Laboratory Press, USA
- Shim H, Min Y, Hong S, Kwon M, Kim D, Kim H, Choi Y, Lee S, Yang J (2004) Nucleotide sequences of a Korean isolate of *Apple stem grooving virus* associated with black necrotic leaf spot disease on pear (*Pyrus pyrifolia*). *Mol Cells* 18:192–199
- Takahashi T, Saito N, Goto M, Kawai A, Namba S, Yamashita S (1990) *Apple stem grooving virus* isolated from Japanese apricot (*Prunus mume*) imported from China. *Res Bull Plant Prot Serv Japan* 26:15–21
- Tang J, Olson JD, Ochoa- Corona FM, Clover GRG (2010) *Nandina domestica*, a new host of *Apple stem grooving virus* and *Alternanthera mosaic virus*. *Australas Plant Dis Notes* 5:25–27
- Targon MLPN, Nikolaeva O, Manjunath KKK, Lee RF, Machado MA (1997) Cloning and expression in *E. coli* of CTV coat protein gene and production of polyclonal antisera. *Fitopatol Bras* 22:99–102
- Terpe K (2003) Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. *Appl Microbiol Biotechnol* 60:523–533
- Thamaraikannan M, Palaniappan G, Sengottuval C (2010) Can indian apples beat imports in quality and price? Facts for you, pp 7–11
- Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 76:4350–4362
- Wu J, Filutowicz M (1999) Hexahistidine (His6)-tag dependent protein dimerization: a cautionary tale. *Acta Biochim Pol* 46:591–599
- Yoshikawa N, Sasaki E, Kato M, Takahashi T (1992) The nucleotide sequence of *Apple stem grooving capillovirus* genome. *Virology* 191:98–105
- Zeng Y, Yang T (2002) RNA isolation from highly viscous samples rich in polyphenols and polysaccharides. *Plant Mol Biol Rep* 20:417a–417e

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.