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Serological detection of *Grapevine leafroll-associated virus* 4 in grapevine growing areas of India using polyclonal antiserum raised against the recombinant coat protein

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ABSTRACT

Grapevine leafroll disease (GLD), caused by a complex of serologically and genetically distinct or related viral species termed as Grapevine leafroll associated viruses (GLRaVs), is a serious threat to the grape production worldwide. Recently, *Grapevine leafroll-associated virus* 4 (GLRaV-4) has been reported in the Indian vineyards, mostly as a mixed infection with GLRaV-3 and GLRaV-1. In order to screen the planting material, a polyclonal antiserum against the *in vitro* expressed coat protein (CP) of GLRaV-4 was produced. The antiserum, purified IgGs and conjugated IgGs detected GLRaV-4 in crude saps of infected grapevines and purified coat protein when used in antigen coated plate enzyme-linked immunosorbent assay (ACP-ELISA), double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), Western blotting and dot immunobinding assay (DIBA). GLRaV-4 could be detected in 54% (39 out of 72) of symptomatic grapevine samples from different grape growing regions of India using DAS-ELISA. The validation of DAS-ELISA results was done by RT-PCR using GLRaV-4 CP-specific primers. To the best of our knowledge, this is the first report of a polyclonal antiserum against the *in vitro* expressed CP of GLRaV-4 and its successful application in diagnosis of the virus in grape vineyards of India.

1. Introduction

Grapevine is a host for several viruses. So far, more than 60 viruses and 5 viroids were reported from grape (Martelli, 2014). Commercially, grapevines are vegetatively propagated by grafting on rootstocks or direct planting of wood cuttings into the soil that could ultimately results in mixed infections of viruses and viroids in a single vine, causing serious diseases (Zhang et al., 2011; Maliogka et al., 2015). Grapevine leafroll disease (GLD), one of the most widespread and economically important viral diseases, is caused by several monopartite, phloem-limited viruses of the family *Closteroviridae* and are referred to as Grapevine leafroll -associated viruses (GLRaVs) (Martelli and Boudon-Padieu, 2006). They have long flexuous virus particles that contain a positive sense RNA genome ranging from 12.5 to 18.6 Kb (Dolja et al., 2006; Martelli et al., 2012). GLRaVs pose a major constrains for the grape production, and reduce the yield of the vines by 15–20% (Martelli and Boudon-Padieu, 2006; Martelli, 1993).

Grapevine leafroll-associated virus 4 (GLRaV-4), is one of the members of the genus *Ampelovirus* subgroup II of family *Closteroviridae* that also includes strains of GLRaV-4 formerly known as GLRaV-5, -6,-9, -Pr, -De, and -Car. The genome size is 13.83 Kb and consists of seven ORFs encoding six genes: the replication-associated polyprotein (expressed via +1 ribosomal frameshift of two partially overlapping ORFs), a small hydrophobic protein (p5), the HSP70 homologue (HSP70h), the 60 kDa protein (p60), the viral coat protein (CP), and a protein of unknown function with a molecular mass of 23 kDa (p23) (Martelli et al., 2012; Thompson et al., 2012). The complete genome sequence of GLRaV-4 has been determined (Abou Ghanem-Sabanadzovic et al., 2012). Recently, we have characterized GLRaV-4 from Indian vineyards based on CP, HSP 70 h and p23 regions (Rai et al., 2017).

Currently, leafroll disease is mainly managed through the use of healthy planting material or managing vector populations (Almeida et al., 2013). Various serological and nucleic acid based methods are used for the detection of GLRaVs, but nucleic acid-based methods such as RT-PCR cannot be performed for large scale screening due to their

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higher cost, need for skilled manpower and well-equipped laboratory facilities (Fajardo et al., 2007). On the other hand serological methods such as ELISA and its variants are robust and cost effective and have been widely used for rapid and large scale screening of infected grapevines (Abou Ghanem-Sabanadzovic et al., 2012; Kumar et al., 2015). There is always a need of species-specific detection using homologous antibodies (Good and Monis, 2001). Purification of individual GLRaVs as "pure" antigen is tedious and has a limitation due to the possibility of mixed infections of GLRaVs and the changes in the virus titre (Constable et al., 2012; Tsai et al., 2012; Cogotzi et al., 2009). To circumvent these problems, *in vitro* expressed viral structural protein such as coat protein could be used as an immunogen for the production of antiserum which could reduce cross-reaction with other serologically related members (Fajardo et al., 2007; Hull, 2013). Here, we describe the use of recombinant CP of GLRaV-4 for generating species-specific antiserum, standardization of a DAS-ELISA based detection and its application in virus diagnostics.

2. Material and method

2.1. Virus source

GLRaV-4 infected grapevine cv. Sharad Seedless (SS-N) collected from Pune, Maharashtra and maintained in a glasshouse facilities at the Advanced Centre for Plant Virology, Indian Institute of Agricultural Research Institute, New Delhi, was used as the source material of GLRaV-4.

2.2. RNA isolation

Total RNA was isolated from 100 to 150 mg of mid ribs and petioles from leaves of GLRaV-4 infected plant material. Samples were frozen in liquid nitrogen and finely pulverized with a pestle-mortar, followed by addition of 1 ml lysis solution containing 2% and polyvinylpyrrolidone (PVP) and 10 µl β-mercaptoethanol (ME). Spectrum™ Plant Total RNA Kit was used for RNA isolation. RNA was quantified using Nanodrop™ (Thermo Scientific, Wilmington, USA) and stored at −80 °C.

2.3. Gene amplification and construction of recombinant plasmid

A pair of expression primers was designed based on GLRaV-4 SS-N CP gene (GenBank Accession No. KJ542647) F- GGCGAATTCATGGCA AATCTCGGTGGTAACG (forward primer) and GLRaV-4 CP R-CGCCTC GAGTCATCTCCTGTTGCCCAAGAAAAT (reverse primer) with *Eco*R1 and *Xho* I (underlined) restriction sites. The first strand cDNA synthesis and PCR amplification were carried out according to Rai et al. (2017). The amplified CP gene was gel-purified using the QIAquick Gel Extraction kit (Qiagen GmbH, Germany), cloned into the TA cloning vector (RBC, Taiwan) and sequenced bidirectionally at the Department of Biochemistry, University of Delhi, and GCC Biotech Kolkata, India. The target fragment (GLRaV-4 CP) was released from GLRaV-4-CP-TA by *Hind* III digestion. The purified expression vector [pET 28a (+)] and the insert (GLRaV-4 CP) were subjected to restriction digestion with corresponding enzymes *Eco*RI and *Xho* I and ligated with pET 28 a (+) expression vector at 16 °C for 18 h using *T4* DNA ligase (5 U/µl) (MBI Fermentas, Germany). Positive transformants in colony PCR were further confirmed through restriction digestion. The correct orientation and frame of inserts were also confirmed by sequencing. The recombinant pET-GLRaV-4-CP (r-CP) construct was transferred into *E. coli* strain BL21 (DE3) expression cell line (Stratagene, La Jolla, CA, USA).

2.4. Standardization of optimal expression of r-CP in expression cell lines BL21 (DE3)

The r-CP was *in vitro* expressed as a fusion protein with Histidine binding protein (His_6BP) at its N terminus in *E. coli* strain BL21 (DE3). Transformed BL21-r-CP cells were grown overnight at 37 °C with 200 rpm in the Luria-Bertani (LB) broth containing kanamycin (100 µg/ml) and chloramphenicol (100 µg/ml). 10% of overgrown culture was re-inoculated in 100 ml LB with same antibiotic selection, incubated at 37 °C for 1 h to obtain an OD_{600} of 0.6 followed by addition of isopropyl β- D 1, 5 thiogalactopyranoside (IPTG) at 0.4 mM, 0.6 mM, 0.8 mM and 1 mM, concentrations for induction. The cells were incubated at 28 °C, 30 °C and 37 °C in a shaker at 200 rpm for 1 h, 2 h, and 3 h each, to standardize optimal expression of protein in insoluble form. Induced cells were pelleted at 6000 rpm and the pellets were re-suspended in sample loading buffer. Overexpression of fusion protein (His_6BP-r-CP) was confirmed on 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie brilliant blue R-250 (Laemmli, 1970).

2.5. Western blotting and DIBA

The specificity of the expressed protein was confirmed through Western blotting and dot immunobinding assay (DIBA) using commercially available DAS-ELISA Kit of GLRaV-4 strains at 1:1000 dilutions following standard procedure (Sambrook and Russell, 2001). The induced and uninduced bacterial cultures were resolved in 15% SDS and then transferred onto nitrocellulose membrane (NCM, 0.22 µm, MDI, Germany) using wet blotting system (Biorad, USA) at 22 mA for 4 h. In case of DIBA, bacterial cultures induced for 3 h and uninduced bacterial cultures were blotted on NCM. Both the trans-blotted NCMs were kept for blocking with 5% skimmed milk in 1× phosphate buffer saline (PBS) for overnight at 4 °C. The trans-blotted NCMs were washed with phosphate buffer saline twin-20 (PBST) and later on probed with GLRaV-4 strains conjugate (Bioreba, Switzerland) at 1:1000 in 5% skimmed milk in 1× phosphate buffer saline twin-20-polyvinylpyrolidone ovalbumin (PBST-PO) for 2 h at 37 °C and 75 rpm. After washing 500 µl of 5-bromo-4-chloro-3-indolylphosphate- nitro blue tetrazolium (BCIP-NBT) were added and kept at 37 °C until a desired band (dot in case of DIBA) of clear intensity was obtained.

2.6. Protein purification

To obtain sufficient quantity of fusion protein (His_6BP-r-CP), BL21 strain of *E.coli* transformed with r-CP was grown in 1.5 L of Luria-Bertani broth containing antibiotics kanamycin (100 µg/ml) and chloramphenicol (100 µg/ml). The expression cells were pelleted, re-suspended in 1× binding buffer [5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9] and disrupted by sonication. The pellet containing insoluble fusion protein (His_6BP-r-CP) was again resuspended in 75 ml of 1× binding buffer containing 6 M urea. The fusion protein was purified using nickel-charged nitrilotriacetic acid (Ni-NTA) column and His.Bind Purification Kit (Novagen, San Diego, USA) following manufacturer's protocol. Protein of interest was eluted in several fractions from Ni-NTA column. These eluted protein fractions were analysed on 12% SDS-PAGE (Laemmli, 1970). The purified fusion protein was further dialyzed to remove salt impurities in PBS, lyophilised and quantified. The specificity of purified protein was confirmed in Western blotting and DIBA as mentioned earlier.

2.7. Production of polyclonal antiserum

Dialyzed fusion protein (His_6BP-r-CP) was used for priming one New Zealand white male rabbit. 500 µg of concentrated fusion protein was emulsified with Freund's incomplete adjuvant (Genie, Bangalore, India) at 1:1 (v/v) ratio and injected intramuscularly into the rabbit weekly for five weeks where a fifth injection was booster dose with 1 mg of protein. The Rabbit was bled after one week after the last immunization three times at weekly intervals and were named as 1st, 2nd and 3rd bleed, respectively. Blood samples were kept tilted at 4 °C for overnight coagulation followed by centrifugation at 6000 rpm for