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Immunodiagnosis of episomal *Banana streak MY virus* using polyclonal antibodies to an expressed putative coat protein

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A B S T R A C T

A cryptic *Badnavirus* species complex, known as banana streak viruses (BSV) poses a serious threat to banana production and genetic improvement worldwide. Due to the presence of integrated BSV sequences in the banana genome, routine detection is largely based on serological and nucleo-serological diagnostic methods which require high titre specific polyclonal antiserum. Viral structural proteins like coat protein (CP) are the best target for *in vitro* expression, to be used as antigen for antiserum production. However, in badnaviruses precise CP sequences are not known. In this study, two putative CP coding regions (p48 and p37) of *Banana streak MY virus* (BSMYV) were identified *in silico* by comparison with caulimoviruses, retroviruses and *Rice tungro bacilliform virus*. The putative CP coding region (p37) was *in vitro* expressed in pMAL system and affinity purified. The purified fusion protein was used as antigen for raising polyclonal antiserum in rabbit. The specificity of antiserum was confirmed in Western blots, immunosorbent electron microscopy (ISEM) and antigen coated plate–enzyme linked immunosorbent assay (ACP–ELISA). The antiserum (1:2000) was successfully used in ACP–ELISA for specific detection of BSMYV infection in field and tissue culture raised banana plants. The antiserum was also utilized in immuno-capture PCR (IC-PCR) based indexing of episomal BSMYV infection. This is the first report of *in silico* identification of putative CP region of BSMYV, production of polyclonal antiserum against recombinant p37 and its successful use in immunodetection.

1. Introduction

Banana streak viruses (BSV) of the genus *Badnavirus* are a major constraint in banana (*Musa* spp.) production and genetic improvement worldwide (Dahal et al., 1998a,b; Hull et al., 2000; Fargette et al., 2006). Badnaviruses have nonenveloped bacilliform virus particles of 30 × 130–150 nm with a double stranded circular DNA genome of about 7.2–8.8 kb which is non-covalently closed (King et al., 2011). Badnaviruses typically encode three open reading frames (ORFs); function of ORF1 is unknown and that of ORF2 is virion associated (Stavolone et al., 2001). The ORF3 encodes a multifunctional polyprotein which is cleaved post-translationally to movement protein (MP), coat protein (CP), aspartic protease (AP), reverse transcriptase (RT) and ribonuclease H (RNase H) (King et al., 2011).

At least eleven genetically diverse cryptic BSV species have been reported to be associated with streak complex of banana worldwide (James et al., 2011b; King et al., 2011; Zhuang et al., 2011; Adam and Carstens, 2012). *Banana streak MY virus* (BSMYV), one of the many badnaviruses infecting banana worldwide, originally described from Mysore banana cultivar (Geering et al., 2005b) has been widely associated with leaf streak disease in India (unpublished results). Management of streak disease largely relies on the production and use of virus free planting material. In spite of a wide array of serological and nucleic acid based detection techniques available for plant viruses, indexing for BSV has still been a real challenge due to high genetic and serological heterogeneity (Geering et al., 2000; Harper et al., 2002, 2005). Integrated counterparts having significant sequence homology to the cognate episomal BSV species has been found in the banana genome (Harper et al., 1999; Geering et al., 2005a,b; Gayral et al., 2008, 2010). Both *Musa acuminata* (A) and *Musa balbisiana* (B) genome of banana contain integrated sequences, but the activable endogenous viral genome has only been detected in the B genome, which are expressed in hybrids (Gayral et al., 2008, 2010). Banana interspecific hybrids having genetic constitution of AB, and have the tendency to show reactivated BSV infections in the tissue

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culture raised plants (Dallot et al., 2001; Gayral et al., 2008; Cote et al., 2010). These integrated *Badnavirus* like sequences found in the host genome makes the PCR based diagnosis unreliable due to its inability to distinguish between integrated and episomal viral sequences. Immunocapture-PCR (IC-PCR) combined with immunosorbent electron microscopy (ISEM) is currently used as standard method for BSV indexing worldwide (Le Provost et al., 2006; Iskra-Caruana et al., 2009). Success and application of both these techniques have prerequisites of availability of BSV specific antiserum.

Conventionally antisera for badnaviruses have been raised using purified virus preparations (Lockhart, 1986, 1988; Ndowora and Lockhart, 2000; Agindotan et al., 2003). However, badnaviruses usually occurs at low concentration in their respective hosts (Dahal et al., 1998a,b; Thottappilly et al., 1998), thus achieving good concentration of purified virus preparation without contamination is a major challenge. Use of *in vitro* expressed coat protein as antigen is a widely used method for production of polyclonal antisera against a number of plant viruses (Gulati-Sakhuja et al., 2009; Tatineni et al., 2013). In badnaviruses the precise coat protein sequences are not identified in the ORF3 polyprotein. Present study reports *in silico* identification of putative coat protein coding region of BSMYV and development of polyclonal antiserum using *in vitro* expressed p37 protein. Further, by using high titre polyclonal antibodies, immuno-diagnostics have been developed for the specific detection of BSMYV, and their applicability in routine indexing of banana has been demonstrated.

2. Material and methods

2.1. In silico identification of putative coat protein of BSMYV

The known amino acid motifs in BSMYV (Geering et al., 2005b), were identified in the present sequence of an Indian isolate; BSMYV-IN1 (isolated from Mysore banana in India: GenBank accession KF724854). The conserved domains in the ORF3 polyprotein were identified by Conserved Domain Database (CDD; Marchler-Bauer et al., 2011) and Conserved Domain Architecture Retrieval Tool (CDART; Geer et al., 2002). The sequences were also analyzed in Predict protein server (http://www.predictprotein.org/). The domains showing homology to coat proteins of other sequences in the database were considered. In the polyprotein sequences, the hits showing similarity with the coat protein sequences of other caulimoviruses, viruses from other families and retroviruses were first aligned in pairwise and then in multiple fashion with BSMYV polyprotein. All the sequence hits were then manually aligned by taking BSMYV polyprotein as reference. The sequence alignment was done with the C-terminus of putative coat protein fragments of *Commelina yellow mottle virus* (ComYMV; GenBank accession NC_001343) and *Cacao swollen shoot virus* (CSSV; GenBank accession NC_001574) (Cheng et al., 1996; Jacquot et al., 1999). To identify the N-terminal end, the mapped region in the polyprotein was finally aligned with *Rice tungro bacilliform virus* (RTBV; GenBank accession AF076470) coat protein (Marmey et al., 1999).

2.2. Cloning of two putative coat protein regions (p48 and p37)

Based on the alignment with RTBV coat protein, two putative CP regions (p48 kDa and p37 kDa) were identified in the BSMYV polyprotein. For amplification of p48 region, specific primer pair: BSV-p48-*Xba*I 5′ *GGC*TCTAGA**ATG**CCCATGTTCCGAGAACTTG 3′ (forward primer with *Xba*I site underlined) and BSV-p48-*Sal*1 5′ *CGC*GTCGAC**TCA**TCTTGGGTTTCGGCATTCAC 3′ (reverse primer with *Sal*1 site underlined) was designed for inframe cloning in to pMAL-c2X expression vector (New England Biolabs, UK). For the p37 region, a primer pair: BSV-p37F-*Xba*I (5′ *GGC*TCTAGA**ATG**TCCCAGTACAGACCTGCAG 3′) and BSV-p37R-*Sal*1 (5′ *CGC*GTCGAC**TCA**TCTTGGGTTTCGGCATTCAC 3′), was designed for inframe cloning in to pMAL-c2X expression vector. Additional primer pairs (with same sequences as above but forward with *Sal*I and reverse with *Xho*I restriction site and lacking stop codon in reverse primer so as to have His tag at both the ends of expressed proteins) targeting p48 and p37 regions were also designed for cloning in to pET-28a (+) expression vector (Novagen, San Diego, CA, USA). The p48 and p37 regions were amplified from the full length pUC18-BSMYV-IN1 clone, using these primers. The amplified fragments of 1272 bp and 981 bp, respectively, were separated on 1.0% agarose gel and eluted using Qiagen Gel Extraction Kit (Hilden, Germany). Both the gel purified p48 and p37 fragments were sub cloned into pET28a (+) and pMAL-c2X after double digestion of both insert and vector. The ligated product was transformed in to *Escherichia coli* DH5α using standard protocols (Sambrook and Russel, 2001) and confirmed by restriction digestion of recombinant plasmids. The correct orientation and frame of inserts were further confirmed by sequencing. The pET28a and pMAL-c2X cloned p37 and p48 constructs were transferred into *E. coli* strain BL21 (DE3) (Stratagene, LaJolla, CA, USA) and TB1 (New England Biolabs, UK).

2.3. In vitro expression and purification

As expression of only p37 protein was obtained in pMAL, it was used for further study. The p37 coding region was *in vitro* expressed as a fusion protein with maltose-binding protein (MBP) of *E. coli* under the control of the *lac* promoter and suppressed by the *lac* repressor. The MBP-p37 fusion protein was induced by adding 0.4 mM isopropyl β-D 1, 5 thiogalactopyranoside (IPTG) to the *E. coli* TB1 containing recombinant plasmid grown at 37 °C for 3 h. The over expressed fusion protein was examined in 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the Laemmli buffer system (Laemmli, 1970). To purify sufficient quantity of fusion protein (MBP-p37), TB1 cell line having pMAL-c2X-p37 clone was grown in 2 l of Luria-Bertani broth containing 0.2% glucose and antibiotic (100 mg/ml of ampicillin). Bacterial cells were harvested by centrifugation, resuspended in 70 ml column buffer (20 mM Tris–HCl, 200 mM NaCl, 1.0 mM EDTA, 10 mM β-mercaptoethanol and 10% glycerol) and disrupted by sonication. Purification of MBP-p37 was done from supernatant on a one step affinity column containing amylose resin according to manufacturer's instructions (New England Biolabs, UK). After a thorough washing, the MBP-p37 fusion protein was eluted in different fractions with the column buffer containing 10 mM maltose. The purified protein was analyzed in 12% SDS-PAGE (Laemmli, 1970), which reacted positively with a polyclonal antiserum to BSMYV (prepared against purified virus preparations, kindly provided by Dr. A.D.W. Geering) in Western blot following standard procedure (Jacquot et al., 1999; Sambrook and Russel, 2001). The expressed fusion protein was cleaved with protease factor *Xa* at a weight ratio of 80:1. The purified fusion protein (mixture of partially cleaved and uncleaved) was dialyzed in dialysis buffer (20 mM Tris–HCl, 10% glycerol and 0.2 mM PMFS) and concentrated by lyophilisation. The samples were withdrawn at different steps and analyzed by SDS-PAGE gel using Coomassie Brilliant Blue visualization. Protein quantification was done with Bradford assay and nanodrop (Nanodrop ND1000).

2.4. Production of polyclonal antiserum

The concentrated mixture of partially cleaved and uncleaved MBP-p37 protein was used for injection after dialysis. 800 μg of dialyzed protein emulsified with an equal volume of Fruend's