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Production of a polyclonal antiserum against recombinant nucleocapsid protein and its application for the detection of fig mosaic virus

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ABSTRACT

Mosaic disease (MD), caused by *Fig mosaic emaravirus* (FMV), is the most important and devastating virus disease of fig trees worldwide. The detection of FMV in infected plants is possible only through the use of molecular techniques, i.e. RT-PCR and LAMP, which both offer high sensitivity of detection, but are also considered laborious when dealing with a large number of samples. To cope with this restriction, a polyclonal antiserum through the immunization of a rabbit by injecting the recombinant nucleocapsid protein (NP) of FMV was raised and evaluated for its efficacy in Western Blot, Dot immuno-binding and DAS-ELISA. The results obtained showed that the raised antiserum was able to identify the nucleocapsid protein of FMV (p3) which was found to have an estimated molecular weight of ca. 35 KDa. In addition, the antiserum, when used in the three serological assays, was able to detect the p3 of FMV in protein extracts of infected plants with different levels of efficacy. Dot immuno-binding, using denatured plant protein extract, proved to be the most efficient serological assay for detecting FMV in samples collected from different fig orchards. This is the first report on an antiserum raised against FMV that could be used for immunological detection of the virus.

1. Introduction

Fig mosaic syndrome was first reported in California in the 1930's (Condit and Horne, 1933) and is now found where figs are grown. The disease is characterized by a range of symptoms of varying severity, i.e. leaf chlorosis, deformation, mosaic or discoloration patterns (Blodgett and Gomec, 1967; Martelli et al., 1993). The year 2009 was a turning point in the identification of the etiology of this disease, when the causal agent was found to be a negative-sense single-stranded RNA virus that belongs to the genus *Emaravirus* and family *Fimoviridae* (Elbeaino et al., 2009a, 2018). The virus is transmitted in nature by the eriophyid mite *Aceria ficus* and by vegetative propagation through infected cuttings (Blodgett and Gomec, 1967; Elbeaino et al., 2009a).

The genome of FMV is composed of six RNA segments which encode the RNA-dependent RNA polymerase (RdRP, p1), a putative glycoprotein (GP, p2), a putative nucleocapsid protein (NP, p3), a putative movement protein (MP, p4) and two proteins with unknown functions (RNA-5, p5 and RNA-6, p6) (Elbeaino et al., 2009a, b; 2012; Ishikawa et al., 2012). Two molecular assays (RT-PCR and RT-LAMP) are available for its detection in infected plants (Elbeaino et al., 2009a; Ishikawa et al., 2015). However, the inability to transmit FMV to herbaceous hosts, the difficulty in purifying the virus particles from infected plants and the presence of high amounts of contaminants and inhibitors (polyphenols, polysaccharides, latex, resins, etc.) in figs are aspects that restrain the development of biological (mechanical inoculation of FMV to herbaceous hosts and\or to indicator plants) and serological tools needed for its detection and\or further characterization. Although RT-PCR and RT-LAMP assays are sensitive and specific for detecting FMV in infected material, they remain precarious when a large variation of sequences exists among FMV isolates and the time needed by both techniques to analyze large numbers of samples. Therefore, the production of recombinant virus proteins through gene amplification and expression in a bacterial host, to be used for antibody production for serological assays, could further assist in the detection of FMV. For this purpose, the NP of FMV was chosen, similarly to *European mountain ash ringspot-associated emaravirus* (EMARaV) which has a genomic

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characteristic similar to that of FMV (Mielke et al., 2008), to develop a serological detection tool. Furthermore, the advantage from choosing the NP resides in its high sequence conservation among FMV isolates (El Air et al., 2016) and immunogenic response to express a stable polyclonal antiserum for immunodiagnostic tests [Double-Antibody Sandwich Enzyme-Linked Immuno-Sorbent Assay (DAS-ELISA), Dot immuno-binding assay (DIBA) and Western Blot (WB)] for which the results are reported hereinafter.

2. Materials and methods

2.1. Source of plant material

An Iranian fig accession (ES3) infected with FMV and showing 97% of identity at the amino acid level (sequence deposited in the GenBank under the accession number MG880755) with the FMV Italian isolate (accession number FM991954) was used as a source material to produce a recombinant NP. Forty-seven samples, which consist of leaves taken from mosaic-diseased and apparently healthy fig plants, were collected from commercial orchards in five main fig-growing provinces of Iran [Gilan and Mazandaran (North), Tehran and Markazi (Centre), Fars (South)], in summer 2016. These samples were firstly screened by RT-PCR to identify FMV-infected and healthy fig accessions and used afterward as negative and positive controls to evaluate the immunodiagnostic efficacy of the antiserum produced.

2.2. Cloning of FMV-NP fragment and construction of pET-28a(+)-FMV-NP expression plasmid

Total nucleic acids (TNA) were extracted from leaf vein tissues using Total HiYield™ mini RNA extraction kit (Real-Biotech, Taiwan) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using 0.5 μg of RNA extract mixed with 0.1 μg of random primers, in the presence of 200 units of *Moloney murine leukaemia virus* (M-MLV) reverse transcriptase enzyme, according to the manufacturer's instructions (ThermoFisher Scientific, USA). RT-PCR was carried out using 15 pmol of sense (FMV-NP-s: 5′-*CCATGG*CACCT AAGAGTAAGACTAC-3′) and antisense primers (FMV-NP-a: 5′-*CTCGA G*AACATGAGCACTTGCAATC-3′), designed to amplify the complete coding region of RNA-3 of FMV (947 nt). Two stretches of nucleotide sequences (underlined), cleavable by *Nco*I and *Xho*I endonucleases, were introduced at sense and antisense primers, respectively to facilitate the directional cloning of the PCR product. RT-PCR consisted of an initial denaturation at 94 °C for 4 min followed by 35 cycles at 94 °C for 45 s, annealing at 57 °C for 45 s, elongation at 72 °C for 45 s and a final extension at 72 °C for 5 min. Expected PCR amplicon (947 bp) was excised from the agarose gel using the Ultra-Clean purification kit (Fermentas, Germany) and ligated into a TA cloning vector pTZ57R/T (ThermoFisher Scientific). The viral nature of the ligated PCR product was ascertained by bidirectional sequencing (Macrogen, Rep. of Korea). The PCR fragment was subcloned into the pET-28a(+) by digesting both the pTZ57R/T and pET-28a(+) vectors with *Nco*I and *Xho*I; the vector carrying the FMV-NP gene was transformed into *E. coli* SHuffle® T7 Express competent cells, according to the manufacturer's instructions (New England Biolabs, USA). The clone chosen for gene expression was re-sequenced to confirm that it contains the NP selected sequence and in-frame with the 5′ sequence of the His-tag protein gene. The transformed *E. coli* SHuffle® T7 Express bearing a single clone with pET-28a(+)-FMV-NP was incubated for 20 h in 500 μl LB. The grown bacterial cells were used as source to inoculate 50 ml LB at 37 °C. At OD_{600} = 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM for the induction of recombinant FMV-NP protein expression and bacterial culture was allowed to grow at 37 °C for 4 h. Both induced and non-induced cell cultures were grown in incubator under the same conditions mentioned before. The bacterial pellet was then re-suspended in lysis buffer (NaH_2PO_4 50 mM, NaCl 300 mM, Imidazole 10 mM; pH 8) containing 1 mg/ml of lysozyme. The suspension was sonicated six times in short pulses of 30 s (150 W with 30 s interval). The purification of expressed fusion protein was carried out under native conditions through immobilized metal ion affinity chromatography (IMAC), following the manufacturer's protocol (Qiagen, Netherlands). The quality of purified recombinant FMV-NP was determined by coomassie-stained discontinuous SDS-PAGE system (Laemmli, 1970), as well as by immuno-blotting with anti- 6 × His tag monoclonal antibody (Ausubel et al., 1992).

2.3. Immunization, IgG purification and conjugation

Immunization was performed using a New Zealand White female rabbit with five subcutaneous injections, at intervals of two weeks. One hundred μg of purified recombinant FMV-NP were emulsified with an identical volume of Montanide complete adjuvant preparation and injected into the rabbit. Test bleeds were taken one week after the last injection and IgG purification was performed by affinity chromatography column prepared by coupling the immunogenic antigen to CNBr-activated sepharose 4B (GE Healthcare, Uppsala, Sweden). The bound protein was eluted with 0.1 M Glycine/HCl pH 2.5 and dialyzed against Phosphate-buffered saline solution (1X PBS) overnight. The purified anti-FMV-NP antibodies were labeled with alkaline phosphatase (AP) from calf intestinal mucosa using 1% glutaraldehyde solution as homobifunctional reagent (Sigma-Aldrich, USA), as described by Harlow and Lane (1988). The preparation was kept for 4 h at room temperature, and dialyzed for three times against 1X PBS to eliminate glutaraldehyde residues. Blocking of reaction was performed by adding 5 mg/ml of bovine serum albumin (BSA) and the labeled antiserum was stored at 4 °C.

2.4. WB, DIBA and DAS-ELISA

The immunodiagnostic efficacy of the produced polyclonal antiserum was evaluated by WB, DIBA and DAS-ELISA.

(i) WB was conducted according to Towbin et al. (1979); thus 0.2 g of leaf vein tissues from healthy and infected fig plants were homogenized in 5 vol of extraction buffer (Tris-HCl 0.5 mM, pH 8.8, 4% SDS, 40% glucose and 4% of 2-mercapthoethanol) for the extraction of proteins from plant sap. Extracts were heated for 5 min. at 100 °C, centrifuged at 6000 g for 3 min. and 30 μl were loaded onto 10% SDS-PAGE. Proteins were electro-blotted onto Millipore polyvinylidene difluoride (PVDF) membrane and further blocked with 5% non fat dry milk and incubated overnight at 4 °C. Ten μg/ml anti-IgG-FMV-NP were added for 1 h with slow agitation. After washing with Tris-buffered saline solution (1X TBS), blots were incubated for 30 min in anti-rabbit (Sigma) antibodies conjugated with AP at 1:500 dilution in 1X TBS [140 mM NaCl, 25 mM Tris, 3 mM KCl, pH 7.4]. Target proteins were finally revealed by adding 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) substrate and nitro blue tetrazolium (NBT). The membrane was washed in deionized water, air-dried and photographed.

(ii) DIBA was conducted with the same homogenized protein extracts used in the WB. Protein extracts were centrifuged at 5700 g for 30 min and 30 μl from the supernatant were spotted onto nitrocellulose membrane (Hawkes et al., 1982). Blocking of free binding sites on membrane was done with 5% nonfat dry milk. The AP-labeled anti-NP IgGs were added at a dilution of 1:500 for 2 h at room temperature with slow agitation. Finally, the membrane was developed as previously described.

(iii) DAS-ELISA was performed according to Clark and Adams (1977). Proteins from infected and healthy figs were extracted using PBS buffer (1X PBS, pH 7.4), 1% polyvinylpyrrolidone and loaded in 100 μl wells with two repetitions. The absorbance value of each well was measured at 405 nm. Reactions with mean absorbance values twice higher than healthy controls (mean of absorbance value 0.495) were considered positive.

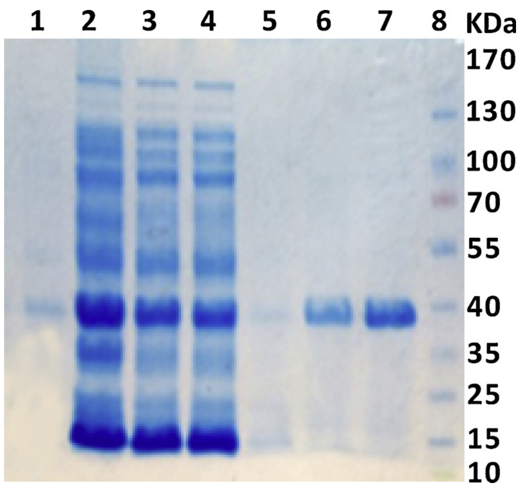

Fig. 1. SDS-PAGE showing the expression of fusion pET-28a(+)-FMV-NP in *E. coli* SHuffle® T7 Express and inducing a protein band with a molecular weight ranging between 35 and 37 kDa. Lane 1: non-induced pET-28a(+)-FMV-NP in *E. coli*, lanes 2-4: induced pET-28a(+)-FMV-NP in *E. coli*, lanes 5-7: purified pET-28a(+)-FMV-NP protein. Lane 8: PageRuler Prestained Protein Ladder (ThermoFischer).

3. Results and discussion

3.1. Induction of the fusion protein and antibody preparation

Upon induction with IPTG at a final concentration of 1 mM, *E. coli* cells harboring pET-28a(+)- FMV-NP, but not those with non-induced cells, produced a polypeptide of *ca.* 35 kDa, which was identified as the expected recombinant nucleoprotein product of FMV. The size of the induced protein was in harmony with that previously predicted using computer-assisted Bioinformatics (Elbeaino et al., 2009b). After purification through IMAC in column containing NiNTA agarose beads, the aspecific proteins were eliminated and a homogenous preparation of the fusion protein was obtained (Fig. 1). Quantitative analysis of SDS-PAGE using ImageJ software estimated the total yield of purified protein to be about 600 μg/ml whereas the concentration of IgGs after purification from serum was calculated to be *ca.* 2 mg/ml (Fig. 2).

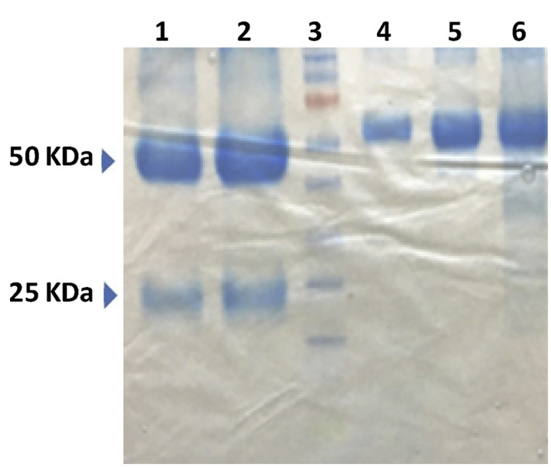

Fig. 2. SDS-PAGE analysis of purified IgG by affinity chromatography using CNBr-activated sepharose 4B column. Lanes 1- 2: purified antiserum showing two bands, 50 kDa (heavy chain), 25 kDa (light chain); lanes 4-6: different concentration of BSA (500, 750 and 1000 μg/ml); lane 3: PageRuler Prestained Protein Ladder (Thermo Fischer).

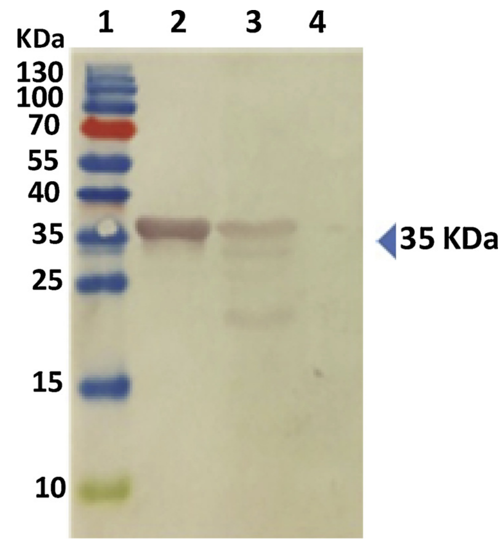

Fig. 3. Crude saps of FMV-infected and FMV-free plants (lanes 3 and 4, respectively), together with purified FMV-NP fusion protein (lane 2), were separated by SDS-PAGE and blotted onto nitrocellulose membrane that was probed with FMV-NP antiserum (1:500), followed by 1:4000 goat anti-rabbit IgGs-alkaline phosphatase (AP) conjugate as a secondary antibody. Bands of interest (ca. 35KDa) were visualized by reaction with freshly prepared BCIP/NBT substrate. Lane 4: PageRuler Prestained Protein Ladder (ThermoFischer).

3.2. Use of polyclonal antiserum in WB, DIBA and DAS-ELISA for FMV detection

Western blotting, which was used as a preliminary assay to evaluate the immuno-efficacy of polyclonal antibodies raised against the recombinant FMV-NP, showed to be a proficient assay to detect both FMV-NP purified recombinant protein and the native viral NP in infected plants by probing a distinct band of *ca.* 35 kDa in both cases (Fig. 3). The optimal concentrations of anti-NP polyclonal antibody used in ELISA and WB were dilutions of 1:500 and 1:1000 of IgG in 1X PBS, respectively; whereas a dilution of 1:500 of AP-labeled anti-FMV-NP was adopted for both assays. Similarly, DIBA was found effective to detect the presence of FMV-NP in the infected leaf tissue of figs. It is noteworthy mentioning that the antiserum (IgG) was more efficient to detect denatured proteins extracted from infected plants rather than using non-denatured native proteins. Accordingly, a denaturing step of extracted proteins before spotting onto membrane was adopted (Fig. 4).

3.3. Comparative detection of FMV by DIBA, DAS-ELISA and RT-PCR

Initially, all samples collected from fig orchards were assayed by RT-PCR for the presence of FMV (Elbeaino et al., 2009a), in order to

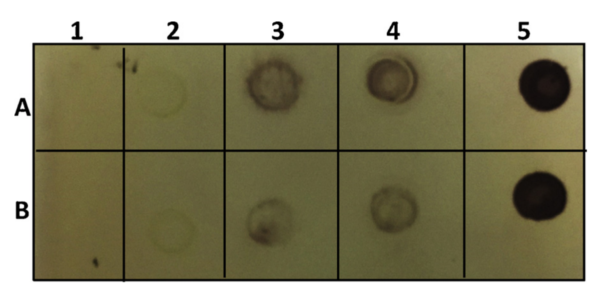

Fig. 4. Detection of FMV-NP in several FMV-infected fig accessions by dot immuno-binding assay, using purified FMV-NP-IgG, conjugated with alkaline phosphatase. A1-2, B1-2: FMV-free fig accessions; A3-4 and B3-4: different fig accessions infected with FMV; A5 and B5: purified recombinant FMV-NP used as positive control reactions. Before spotting, all protein extracts were denatured by boiling.

Table 1
Results of RT-PCR, DIBA and DAS-ELISA assays conducted on fig samples for the detection of FMV.

Province	Collected and tested samples	RT-PCR	DIBA	DAS-ELISA
Gilan	13	4	4	1
Mazandaran	9	7	7	7
Tehran	3	2	–	–
Markazi	5	3	3	3
Fars	17	14	14	14
Total	47	30 (63.8%)	28 (59.5%)	25 (53.1%)

identify infected and healthy fig accessions to be used as controls to evaluate the immunodiagnostic efficacy of the produced polyclonal antiserum in DIBA and DAS-ELISA assays, and also to assess the reliability of the latter to detect FMV at a large scale spectrum. The three diagnostic techniques, *i.e.* DIBA, DAS-ELISA and RT-PCR, showed different levels of sensitivity in the detection of FMV. In fact, RT-PCR results showed that 30 samples were infected with FMV, whereas those of DIBA and DAS-ELISA yielded 28 (96% of sensitivity) and 25 (85.7% of sensitivity) FMV-infected samples, respectively (Table 1). Conversely, they were all highly specific (100%), since there was no discrepancy in results regarding the FMV-free samples that were all equally negative.

4. Discussion

In the case of fig, there are a lot of limitations in obtaining purified virus particles from infected plants and a successful antiserum to be used in different serological assays. This is due to (i) the multiple viral infections regularly found in figs, (ii) the non-transmissible nature of all fig-infecting viruses for being inoculated onto herbaceous hosts, (iii) the low virus concentration in infected plant tissue, (iv) the high content of inhibitors, phenols, latex and contaminants, (v) the irregular distribution of the virus inside the plant organs; all aspects that make it quite difficult to produce an immunological assay and to apply it in the detection of fig viruses. Notwithstanding these difficulties and in order to provide a serological tool that could be exploited in diagnostics of FMV, the choice of producing a recombinant viral protein of FMV (nucleocapsid protein, FMV-NP) in bacterial host was considered as an alternative approach to avoid the production of antibodies against host proteins normally occurring within a viral preparation from plants (Nikolaeva et al., 1995).

In this study, among all serological and molecular assays applied, RT-PCR showed to remain a technique of choice in the detection of FMV. Also, the Western Blot assay was found to be consistent and reliable to probe FMV-NP in infected plants; accordingly, it could be useful for nucleocapsid protein characterization of FMV isolates. DIBA was not performing as RT-PCR, since two out of thirty (6.5%) RT-PCR positive samples were not detectable by this assay. However, this technique, if used in a large-scale detection, could give a preliminary insight into the sanitary status of fig samples, in a short lapse of time with minimum sample handling, and consequently would bypass its partial incompetency of detection. Similarly to DIBA, the partial inefficacy of the antiserum produced to detect the native viral protein in DAS-ELISA (*ca.*16.5%) is most likely due to a major immunological response throughout the production of antibodies against the linearized epitopes of the injected recombinant nucleocapsid protein (detected in WB and DIBA tests using denatured proteins), more than to the secondary structure of the injected recombinant proteins (not detected in DAS-ELISA). Nevertheless, the partial failure of DAS-ELISA to detect the viral protein is most likely due to the low sensitivity of this technique, notoriously known, rather than to the immunogenic response of the antiserum to the injected protein. This study constitutes the first approach to the production of a specific antibody against FMV that could be largely exploited in WB, DIBA and ISEM applications.

Declarations of interest

None.

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