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BRIEF REPORT

Engineered single-chain variable fragment antibody for immunodiagnosis of groundnut bud necrosis virus infection

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Abstract Few studies have been done on engineered antibodies for diagnosis of tospovirus infections. The present study was undertaken to develop a single-chain variable fragment (scFv) for specific diagnosis of infection by groundnut bud necrosis virus (GBNV), the most prevalent serogroup IV tospovirus in India. Heavy chain (372 nucleotide [nt]) and light chain (363 nt) variable region clones obtained from a hybridoma were used to make an scFv construct that expressed a ~29-kDa protein in *E. coli*. The scFv specifically detected GBNV in field samples of cowpea, groundnut, mung bean, and tomato, and it did not recognize watermelon bud necrosis virus, a close relative of GBNV belonging to tospovirus serogroup IV. This study for the first time demonstrated the application of a functional scFv against a serogroup-IV tospovirus.

Polyclonal antibodies (PAbs) and monoclonal antibodies (MAbs) are key reagents for the immunodiagnosis of plant viruses. PAbs are produced by immunizing animals with purified virus or bacterially-expressed recombinant coat protein. MAbs are generally produced by hybridoma technology. Selection and maintenance of MAb-producing hybridomas is time- and labor-intensive, and it requires a cell culture system. This limitation has been overcome with the cloning and expression of immunoglobulin (IgG)-encoding genes in *Escherichia coli* (*E. coli*) [10]. Full-length IgG is not essential for serodiagnosis, as the paratopes that recognize the epitopes in the viral antigen are located in the N-terminal end of the IgG, known as the variable region (V). Heavy (H) and light (L) chains combined as a single-chain variable fragment (scFv) have been used to detect plant viruses, and the sensitivity of such assays is fully comparable and often higher than that achieved with traditional antibodies [4, 9].

Groundnut bud necrosis virus (GBNV) is the most prevalent tospovirus on the Indian subcontinent [8]. GBNV belongs to serogroup IV, and detection of GBNV by enzyme-linked immunosorbent assay (ELISA) has been demonstrated using PAbs [5], although, these antibodies could not distinguish among the different tospoviruses in serogroup IV, such as watermelon bud necrosis virus (WBNV) and capsicum chlorosis virus. MAbs have been produced against GBNV through hybridoma technology and have been shown to be useful for detecting isolates of GBNV [11]. However, these MAbs were not able to distinguish among the members of serogroup IV tospoviruses present in India. Few studies have been done on the application of scFv or recombinant antibody for diagnosis of tospovirus infection [3]. So far, no scFv has been produced against a tospovirus belonging to serogroup IV. The present study was undertaken to produce an scFv against GBNV and to evaluate its usefulness for detection of GBNV by ELISA or immunocapture (IC) RT-PCR.

GBNV was isolated from a groundnut plant showing bud necrosis and maintained by sap inoculation on *Nicotiana benthamiana*. The identity of the virus isolate was confirmed by sequencing of the nucleocapsid protein (NP) gene. An expression construct using 830 nucleotides (nt) of the NP gene was made using pET28a (+) vector (Novagen, Darmstadt, Germany) and used to express as 30-kDa protein in *E. coli* strain BL21 (DE3). A BALB/c mouse was injected intraparitoneally with 50 μg of the recombinant NP emulsified with complete Freund's adjuvant (Sigma, USA).

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A booster injection was given after 2 weeks using 25 μg of the recombinant NP emulsified with incomplete Freund's adjuvant, and two more boosters were administered at one-week intervals. The spleen was removed aseptically from the immunized mouse three days after the third booster injection, and a single-cell suspension was prepared, followed by fusion with mouse myeloma cells. Three hybridoma clones, 1C8, 4D11 and 5F4, were selected based on their reactivity in an ELISA with purified GBNV NP. The MAb from clone 4D11 showed the highest absorbance in ELISA with the purified NP as well as with GBNV from infected peanut samples. MAb 4D11 was isotyped as IgG1 and kappa light chain, using a mouse monoclonal antibody isotyping kit (Thermo Scientific, MA, USA).

For the cloning of V_H and V_L genes, total RNA was isolated from 4D11 hybridoma cells using an EZ Total RNA Isolation Kit (Biological Industries, Israel). The first-strand cDNA was synthesized in a 20-μl mixture containing 4 μl of 5x first strand buffer, 1.0 μl of 10 mM dNTP mix, 1.0 μl of 20 mM DTT, 2.0 μl of 10 μM reverse primers BM254R ggactcgagaggagacgg tgaccgtggtcccttg for V_H and BM451R ggtctcgagggatacagttggtgcagcatc for V_L (primer sequences from Wang et al. [12]), 1 μl of SMARTScribe^{TM} III reverse transcriptase enzyme (Clontech, USA) (100 units/μl), 10 μl of RNA template (500-600 ng), and nuclease-free water. The mixture was incubated at 42 °C for 90 min, followed by inactivation at 70 °C for 15 min using a thermal cycler (Biometra *T* Personal). PCR reactions were conducted in 50-μl reaction mixtures containing 2.0 μl of cDNA, 5.0 μl of 10x Ex-Taq PCR buffer, 4.0 μl of 2.5 mM dNTPs, 2.0 μl of each primer at a concentration of 10 μM (BM443F [cttccggaattc-sargtnmagctgsagsagtc] and BM254R for V_H and BM450F [gggagggattcgayattgtgmtsacmcarwctmca] and BM451R for V_L), 1.25 U of Ex Taq DNA polymerase (Takara, Japan) and nuclease-free water to make up the volume. The mixtures were subsequently subjected to 35 PCR cycles, each consisting of denaturing at 98 °C for 10 s, primer annealing at 55 °C for 45 s, and extension at 72 °C for 2 min with a final extension at 72 °C for 10 min. The PCR products (~400 bp) were cloned into the pT&A vector (RBC, Taiwan). Ten selected clones were sequenced and aligned using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html), which showed that all of the clones were identical in sequence. The translation frame of the clones was determined using ORF Finder software (http://www.ncbi.nlm.nih.gov/gorf), and one clone each for V_H (372 nt, JX088123) and V_L (363 nt, JX088124) was used for making the scFv expression construct. Three complementarity-determining regions (CDRs) and four framework regions (FRs) were identified in the sequences by using the IgBLAST (http://www.ncbi.nlm.nih.gov/igblast/) tool.

New primers were designed based on the sequences of these clones for the insertion of the scFv-encoding sequence in the pET28a (+) expression vector. The primers V_HA (*cttcc*gctagcatgcaagtgaagctggaggagtctgg) and V_HB (*gga*ggatcctccacctcctgaaccgcctccaccatagacagatcggggtgtcgt tttggc) containing *Nhe*I and *Bam*HI sites, respectively, were used for amplifying the V_H gene. The primers V_LA (*cttcc*ggatccggcggtggcggttcggatattgtgctcacacagtctcca) and V_LB ±(*gga*ctcgagggatacagttggtgcagcatc), containing *Bam*HI and *Xho*I sites, respectively, were used for amplifying the V_L gene. A $(Gly_4Ser)_3$-linker sequence (underlined in the primer sequence) was added to the heavy chain 3′ primer (V_HB) and to the light chain 5′ primer (V_LA) for the assembly of V_H and V_L into a scFv form. The 372-nt of V_H and 363-nt V_L sequence were joined with a 45-nt $(Gly_3Ser)_4$ linker, resulting in a 780-nt scFv construct, which was expressed in *E. coli* strain BL21 (DE3) after induction with 1.0 mM IPTG at 37 °C for 4 h. The expressed scFv antibody fragment (~29 kDa) was purified from the insoluble fraction using an Ni-NTA column under denaturing conditions using a His-bind Protein Purification System (Novagen, Darmstadt, Germany) (Fig. 1a). The purified scFv antibody was then dialyzed using a dialysis membrane (MWCO 12,000–14,000 Da) in PBS buffer containing decreasing concentrations of urea (6 M, 4 M, 2 M, 0.5 M) at 4 °C, changing the buffer every 12 to 24 h. The renatured scFv was finally dialyzed against PBS (pH 7.4) for 12 h, and 3-4 mg protein per liter of bacterial culture was obtained. For Western blot analysis, the renatured scFv was resolved by 12 % sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (NCM) using an Iblot® gel transfer device (Invitrogen, Bangalore, India). The membrane was blocked with 5 % skimmed milk powder overnight at 4 °C. The presence of the scFv was detected using rabbit polyclonal anti-His-tag antibody (ABM, Canada) at 1:500 dilution and an Fc-specific goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma, USA) at 1:20000 dilution. A protein band of ~29 kDa was visualized by adding BCIP/ NBT (Genei, Bangalore, India), which indicated that the scFv was successfully expressed (Fig. 1b). The sensitivity of scFv detection was determined in a Western blot using bacterial lysate, sonicated pellet, and purified GBNV NP protein (1000 ng, 100 ng, 10 ng and 0.1 ng). The results showed that the scFv antibody (50 μg/ml) could be used successfully for detection of GBNV NP in Western blot analysis, and as little as 10 ng of antigen could be detected (Fig. 1c).

Initially, the scFv was utilized for the detection of GBNV in a triple antibody coating ELISA using rabbit anti-his-tag antibody and goat anti-rabbit antibody to detect scFv-bound viral antigen. The indirect ELISA gave a high background reading with healthy samples and low

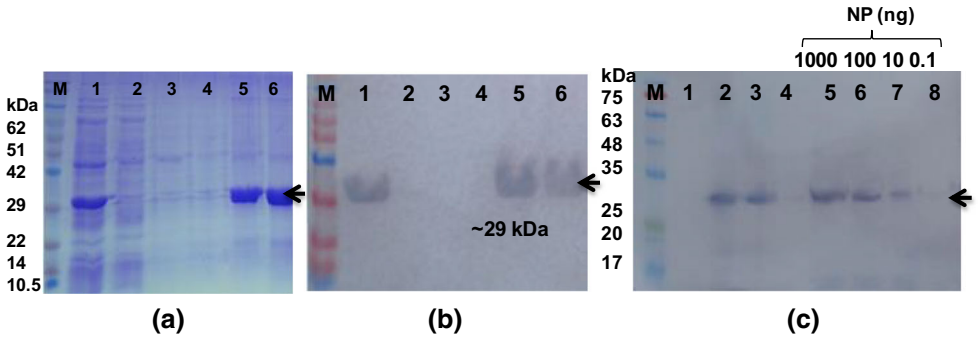

Fig. 1 Expression and purification of recombinant single-chain variable fragment (scFv) antibody from *E. coli*. (a) 12 % SDS-PAGE gel showing expression of scFv (pET28a.scFv) after induction by treatment with 1.0 mM IPTG and purification using Ni-NTA column chromatography. (b) Western blot of the expressed and purified samples in panel a using anti-His-mouse monoclonal antibody. Lane M, prestained protein marker (Fermentas); lane 1, protein crude extract; lane 2, flowthrough; lane 3, fraction after treatment with binding buffer; lane 4, wash fraction; lanes 5 and 6, first and second eluted fractions. (c) Western blot showing detection of NP of GBNV using alkaline-phosphatase-conjugated scFv. Lane 1, uninduced NP; lane 2, induced NP; lane 3, sonicated pellet of expressed NP; lane 4, sonicated supernatant; lane 5-8, 1000 ng, 100 ng, 10 ng and 0.1 ng, respectively, of purified NP

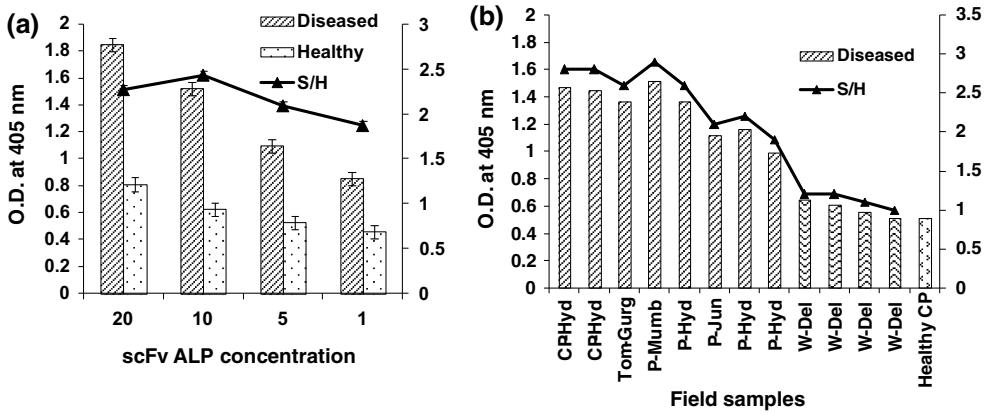

Fig. 2 Direct ELISA showing reactivity and specificity of alkaline phosphatase (ALP)-conjugated single-chain variable fragment (scFv) in detecting groundnut bud necrosis virus (GBNV). (a) Effect of varying the amount of scFv-ALP in the assay (1-20 µg). (b) Detection of field samples using 10 µg scFv. CP, cowpea; Tom, tomato; P, peanut; W, watermelon; Hyd, Hyderabad; Gur, Gurgaon; Mumb, Mumbai; Jun, Junagarh. The leaf sap was used at a 1:5 dilution (w/v). The ELISA was conducted in duplicate. Absorbance at 405 nm was read 1 h after the addition of substrate (p-nitrophenyl phosphate, 0.5 mg/ml), and the standard error was ±0.05

efficiency of detection of GBNV in crude sap. To overcome this problem, a direct ELISA was performed by conjugating scFv with alkaline phosphatase enzyme (ALP). In order to prepare scFv-conjugated ALP, the purified scFv protein (5.0 mg) was mixed with 2.5 mg of ALP and 0.2 % (V/V) glutaraldehyde. The mixture was stirred at room temperature for 2 h and then dialyzed overnight using a membrane with a cutoff of 12-14 kDa (Spectrum labs, Rancho Dominguez, CA) at 4 °C. The scFv-ALP was used to detect GBNV infection in plant samples in direct antigen-coating (DAC) ELISA [1]. To determine the optimum quantity of ScFv-ALP conjugate required, the assay was carried out using different concentrations of scFv-ALP (20.0 µg, 10.0 µg, 5.0 µg and 1.0 µg/200 µl per well). The detection of GBNV with a sample/healthy (S/H) ratio of >2.0 was achieved with an ScFv-ALP concentration of ≥5.0 µg, wherein 10 µg of scFv-ALP showed the highest S/H ratio (Fig. 2a). The scFv-ALP (10 µg/200 µl) was further tested in ELISA with GBNV- or WBNV-infected field samples from peanut, cowpea, tomato and watermelon collected from different places in India during 2012. GBNV was detected in the field samples of cowpea, tomato and peanut (except in one peanut sample from Hyderabad) with an S/H ratio >2.0 (OD range, 0.989 to 1.519). In contrast, WBNV-infected watermelon leaf samples showed an S/H ratio of ~1.0 (OD, 0.513 to 0.650) (Fig 2b). The ELISA results showed that the ScFV could detect GBNV, but not WBNV.

To develop a more sensitive serodiagnosis test using scFv, immunocapture (IC) RT-PCR was performed. The PCR tubes were coated with various concentrations of scFv antibody (5.0 µg, 2.5 µg, 1.0 µg, 0.5 µg and 0.25 µg)

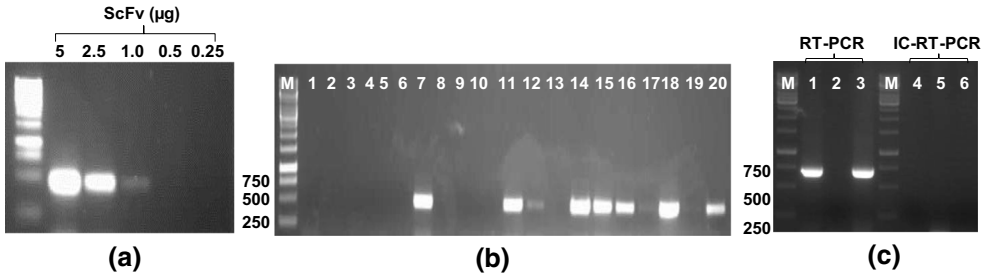

Fig. 3 Immunocapture (IC) RT-PCR with scFv showing differential detection of groundnut bud necrosis virus and watermelon bud necrosis virus (WBNV). (a) Detection of GBNV in infected mung bean leaf tissues with different amounts of scFv. (b) GBNV detection using GBNV-specific NP-region primers (Gs1F and GWs1R) with different field samples. Lanes 1-19, field samples of mung bean; lane 20, positive control. (c) Amplification of WBNV in watermelon samples by RT-PCR, but not by IC-RT-PCR, with scFv. Lanes 1-3, RT-PCR with WBNV-specific primers from the M RNA (Wm67 F and GWm68 R); lanes 4-6, IC-RT-PCR of the same samples with the same primers as in lanes 1-3. Lane M, 1 kb Plus DNA Ladder (Fermentas)

diluted in 100 µl of carbonate coating buffer and incubated at 37 °C for 2 h. Sap was prepared by grinding mung bean leaf samples infected with GBNV at 1:5 dilution (w/v) in carbonate buffer containing 2 % PVP (MW 40,000), followed by centrifugation at 12,000 g for 2 min. About 100 µl of sap was added to PCR tubes pre-coated with scFv, which were then incubated for 2 h at 37 °C. The PCR tubes were washed three times using PBS-Tween-20. The IC-RT-PCR was carried out using a one-step RT-PCR kit (Takara, Japan) with primers specific for GBNV (Gs1F [atggttgaaaagagcaagaatgatgc] and GWs1R [cttcttwgartgthcaccatartcatcc]). The IC-RT-PCR with 2.5 to 5.0 µg of scFv resulted in strong amplification of GBNV; however, only weak amplification was observed with 1.0 µg of scFv, and no amplification was seen with less than 1.0 µg of scFv (Fig. 3a). The IC-RT-PCR performed using 2.5 µg of scFv detected GBNV in field samples of mung bean and watermelon collected from the vegetable field of IARI, New Delhi, during 2013. Initially, these samples were subjected to ELISA using PAbs to GBNV, which showed that 8 out of 19 mung bean samples and 2 out of 3 watermelon samples were positive (data not shown). IC-RT-PCR showed amplification of GBNV in all eight positive samples of mung bean (strong amplification of a ~477-bp band in six samples and faint amplification in two samples) (Fig. 3b). However, when IC-RT-PCR with ScFV was carried out using primers specific for WBNV (Wm67F [caaagttaattttctccactcaac] and GWm68 R [ctttctgtaaggttgtctggtg] with the two watermelon samples that were positive with PAbs to GBNV, there was no positive amplification (Fig. 3c). RT-PCR with the WBNV-specific primers, however, showed clear amplification of a ~620-bp fragment in the watermelon samples (Fig. 3c). This showed that IC-RT-PCR with scFv specifically detected GBNV, but not WBNV, in field samples.

There are about two dozen tospoviruses known all over the world; however, recombinant antibody has been made only to TSWV. The scFv against TSWV originated from a hybridoma cell producing a MAb that could react with the serogroup I-III tospoviruses TSWV, GRSV, TCSV and INSV but did not react with the serogroup IV tospovirus WSMV [2]. In another attempt, a panel of scFvs against TSWV was retrieved from a human combinatorial scFv antibody library, which could react with TSWV, TCSV and GRSV but did not recognize the more distantly related tospoviruses INSV, WSMV and IYSV [3].

GBNV and WBNV, which cause similar bud necrosis disease in several crops, are the two most common serogroup-IV tospoviruses present in India. It was previously thought that GBNV is restricted to legume crops; and WBNV, to cucurbits [6]. However, GBNV has recently been reported in watermelon and WBNV in tomato [7]. This indicates that while both the viruses can cross-infect a common host, specific immunodiagnosis is not available for differentiating these two closely related serogroup-IV tospoviruses. Although a MAb to GBNV was described previously, it was not shown to differentiate between GBNV and WBNV [11]. In our study, a scFv to GBNV was shown to differentiate between GBNV and WBNV in both ELISA and IC-RT-PCR. The performance of scFv in detecting GBNV in ELISA was moderate, with an S/H ratio between 2.0 to 3.0. However, the IC-RT-PCR with scFv was highly effective in detecting GBNV and differentiating between it and WBNV. The IC-RT-PCR with scFv will be highly useful in studying the natural occurrence of GBNV in different crops.

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