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Short communication

Immunodiagnosis of groundnut and watermelon bud necrosis viruses using polyclonal antiserum to recombinant nucleocapsid protein of *Groundnut bud necrosis virus*

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Abstract

In vitro gene expression strategy was used for the production of polyclonal antiserum to the nucleocapsid protein (NP) of *Groundnut bud necrosis virus* (GBNV). The GBNV NP gene from cowpea isolate was cloned into 6x His-tagged UA cloning vector and expressed in *Escherichia coli* [M15] cells. The fusion protein was detected in insoluble fraction and was purified by using Ni-NTA agarose resin. The purified 6x His-fusion protein (∼32 kDa) was used for immunisation to produce a high titre polyclonal antiserum. The antiserum to the NP of GBNV at 1:4000 dilution detected successfully natural infection of GBNV and *Watermelon bud necrosis virus* in a wide range of cucurbitaceous, leguminous and solanaceous hosts from different locations.
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Groundnut bud necrosis virus (GBNV) is the most prolific *Tospovirus* (family *Bunyaviridae*) affecting a variety of crops in the Indian sub-continent. The virus was first recorded in groundnut in 1968 (Reddy et al., 1968). Since then, it has become a major constraint to the cultivation of several leguminous and solanaceous hosts such as cowpea, groundnut, mungbean, potato, soybean and tomato (Bhat et al., 2002; Thien Xuan et al., 2003; Umamaheswaran et al., 2003; Jain et al., 2004). *Watermelon bud necrosis virus* (WBNV) is another *Tospovirus*, which has emerged as a serious pathogen of cucurbits in India (Jain et al., 1998; Mandal et al., 2003). Both the viruses are ascribed currently to *Watermelon silver mottle virus* (WSMoV) serogroup on the basis of nucleocapsid protein (NP) relatedness (Moyer, 1999). Natural infection of both viruses has been detected by enzyme-linked immunosorbent assay (ELISA) using polyclonal antiserum to NP of GBNV as their NP genes shared 82% amino acid sequence identity (Jain et al., 1998). Serological diagnosis, however, is limited by the difficulty in producing quality polyclonal antiserum to NP, which is difficult to purify in reasonable quantities. In this paper, the expression and purification of GBNV recombinant NP, the production of polyclonal antiserum and its use for diagnosis are described.

The GBNV NP gene of cowpea isolate (GenBank Accession No. AF515819), cloned in pGEM-T Easy vector, comprised of 831 nucleotides encoding 276 amino acids, was amplified using primers 5′ATGTCTAACGT (C/T) AAGCA (A/G)CTC3′ and 5′TTACATTTCCAGCGACGAAAGGACC3′. The amplified product was purified by using QIAGEN Gel Elution kit, sub-cloned into 6x His-tagged UA cloning vector (Qiagen) and transformed into *E. coli* [M15] cells containing the pREP4 repressor plasmid by following standard molecular biology protocols (Sambrook and Russell, 2001). Transformants were screened by colony PCR and the potential recombinants were maintained on Luria agar plate containing suitable antibiotics (25 μg/ml kanamycin and 200 μg/ml ampicillin).

Maximum fusion proteins were expressed at 1 mM IPTG and purified using Ni-NTA column as described in the QIAexpressionist manual (Anonymous, 1997). Fractions were

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analysed by SDS–PAGE (Laemmli, 1970) using 5% stacking and 12% resolving gels. Like GBNV, WBNV NP gene from watermelon (GenBank Accession No. AF04507) was also expressed in *E. coli*. The specificity of the fusion proteins was determined using polyclonal antiserum to WSMoV NP (1:1000) in Western blots as described by O'Donell et al. (1982).

Purified fusion protein (1 mg) was emulsified with an equal volume of Freunds incomplete adjuvant and injected intramuscularly into a NewZealand White rabbit up to 5 weeks at 1-week interval. The rabbit was first bled 14 days after the last immunization and the crude antiserum was mixed with glycerol (1:1, v/v) and stored at −20 °C. The antiserum specificity was determined by Western blots (O'Donell et al., 1982). The recombinant proteins of GBNV and WBNV were electro-blotted (30v/3h) onto nitrocellulose membrane (Schleicher and Scheull) using TBE buffer containing Trisborate EDTA and the membrane was kept in blocking solution (3% BSA in TBS) for 1h probed with the antiserum at 1:4000 dilution for 1.30 h. The bound antibodies were detected with antirabbit IgG alkaline phosphatase conjugate (Sigma, 1:20,000 dilution) and 0.33 mg/ml NBT and 0.175 mg/ml BCIP (Sigma) as substrate.

The resulting polyclonal antiserum was used to detect GBNV and WBNV infection in plant samples, which were identified previously on the basis of NP sequences (Bhat et al., 2002; Mandal et al., 2003; Thien Xuan et al., 2003; Umamaheswaran et al., 2003). In addition, a few plant samples with unidentified tospovirus infection were also tested. Direct antigen coated (DAC)-ELISA was performed on polystyrene plates (Corning) using the protocol described by Clark and Bar-Joseph (1984). Sap preparation included grinding infected leaves at 1:1 dilution (w/v) in coating buffer containing 2% polyvinyl pyrrolidone (PVP, MW 40,000) followed by centrifugation at 5000 rpm for 5 min. Sap extracted from healthy leaves of respective plant species was used as negative control. Antiserum was used at 1:4000 dilution after cross absorption with respective healthy sap (in PBS-TPO at 1:100 for 1 h at 37 °C. Anti-rabbit IgG alkaline phosphatase conjugate (Sigma) was used at 1:20,000 dilution. The reactions of ELISA were read at 405 nm 1h after addition of substrate (*p*-nitrophenyl phosphate, 0.5 mg/ml, Sigma) by using a Tecan Sunrise (version 1.2) ELISA reader.

This study is the first report on the production of polyclonal antiserum against GBNV NP using recombinant technology. The expression of the entire NP coding region of GBNV as a fusion protein with 6xHistidine tag was achieved by induction with 1 mM IPTG. As observed in other plant virus systems (Rodoni et al., 1999; Hema et al., 2003), the fusion protein in the present study was detected only in the insoluble fraction from transformed *E. coli*. The apparent molecular weight of the 6x His-GBNV NP fusion protein as expected was 32 kDa (Fig. 1a, lane 1). The fusion protein reacted with antibodies to the NP of *Watermelon silver mottle virus* by Western blots (data not shown), suggesting its viral specificity. The purified fusion protein yielded high titre antiserum upon immunisation. The antiserum at 1: 4000 dilution reacted strongly with purified fusion proteins of GBNV and WBNV in Western blots (Fig. 1b, lanes 1 and 2).

The resulting polyclonal antiserum detected efficiently natural infection of GBNV in leguminous and solanaceous hosts collected from different locations in DAC-ELISA test. GBNV infection was detected from cowpea, mungbean, soybean, tomato and Urdbean. WBNV, antigenically related to GBNV (Jain et al., 1998) was detected from cucumber, muskmelon, ridge gourd and watermelon (Table 1). Antiserum has also been used successfully to detect tospovirus infection related antigenically to GBNV from chilli and weed hosts such as *Acanthospermum, Physalis, Portulaca* and *Cassia* spp. (Table 1).

These results suggest that recombinant technology is a useful approach for raising polyclonal antibodies against NP of Tospoviruses. The procedure is advantageous as it does not encounter the problems associated with tospovirus purifi-

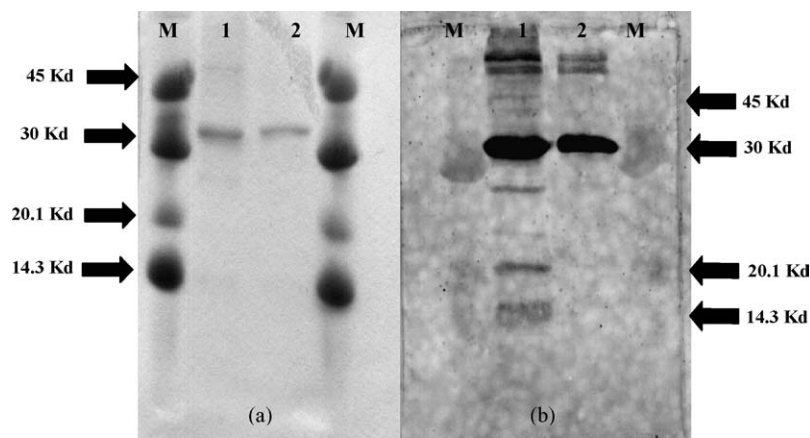

Fig. 1. Detection of recombinant nucleocapsid protein (NP) of *Groundnut bud necrosis virus* (GBNV) in SDS–PAGE (a) and Western blots (b) by probing with polyclonal antiserum to NP of GBNV (1:4000). Lane M: low range SDS marker; 1 and 2: purified recombinant NP of GBNV and Watermelon bud necrosis virus.

Table 1
Detection of tospoviruses in naturally infected plant species by direct antigen-coated enzyme-linked immunosorbent assay using polyclonal antiserum to recombinant nucleocapsid protein of *Groundnut bud necrosis virus*

Virus isolate[a]/source	Origin	A405 nm
Groundnut bud necrosis virus (GBNV)		
Arachis hypogaea (groundnut)	Bangalore	1.75
Glycine max (soybean)	Bangalore	1.50
	Coimbatore	0.82
Lycopersicon esculentum (tomato)	Bangalore	1.96
	Coimbatore	2.61
Vigna mungo (urdbean)	Bangalore	1.50
Vigna radiata (mungbean)	Bangalore	1.90
Vigna unguiculata (cowpea)	Bangalore	1.60
Watermelon bud necrosis virus (WBNV)		
Citrullus lanatus (watermelon)	Bangalore	1.40
	Varanasi	1.28
Cucumis melo (muskmelon)	Bangalore	1.20
Cucumis sativus (cucumber)	Bangalore	1.83
	Varanasi	0.72
Luffa acutangula (ridge gourd)	Coimbatore	0.99
Unidentified tospovirus		
Acanthospermum sp.	Bangalore	1.98
Capsicum annuum (chilli)	Bangalore	1.64
	Faizabad	1.62
	Raichur	1.05
Cassia tora	Bangalore	1.27
Physalis minima	Bangalore	1.40
Portulaca sp.	Bangalore	1.64

[a] Tospovirus infection identified as GBNV and WBNV on the basis of nucleocapsid protein gene sequence (Bhat et al., 2002; Mandal et al., 2003; Thien Xuan et al., 2003; Umamaheswaran et al., 2003 and unpublished observations).

cation and maintenance of live virus culture. The resulting polyclonal antiserum against NP can be applied successfully for efficient detection of GBNV and WBNV infection. The antiserum detected successfully GBNV infection in dot-immunobinding assay also (data not shown). The antiserum showed a low degree of non-specific reaction (A405 nm: 0.20–0.45), suggesting the presence of antibodies against the bacterial proteins having homology with healthy plant proteins. The non-specific reaction could be corrected by cross-absorption with healthy sap (A405 nm: 0.07–0.16) (Table 1).

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