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Production of cocktail of polyclonal antibodies using bacterial expressed recombinant protein for multiple virus detection

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Cocktail of polyclonal antibodies (PAb) were produced that will help in multiple virus detection and overcome the limitation of individual virus purification, protein expression and purification as well as immunization in multiple rabbits. A dual fusion construct was developed using conserved coat protein (CP) sequences of *Cucumber mosaic virus* (CMV) and *Papaya ringspot virus* (PRSV) in an expression vector, pET-28a(+). The fusion protein (~40 kDa) was expressed in *Escherichia coli* and purified. Likewise, a triple fusion construct was developed by fusing conserved CP sequences of CMV and PRSV with conserved nucleocapsid protein (N) sequence of *Groundnut bud necrosis virus* (GBNV) and expressed as a fusion protein (~50 kDa) in pET-28a(+). PAb made separately to each of these three viruses recognized the double and triple fusion proteins in Western blot indicating retention of desired epitopes for binding with target antibodies. The fusion proteins (~40 kDa and ~50 kDa) were used to produce cocktail of PAb by immunizing rabbits, which simultaneously detected natural infection of CMV and PRSV or CMV, PRSV and GBNV in Cucurbitaceous, Solanaceous and other hosts in DAC-ELISA. This is the first report on production of a cocktail of PAb to recombinant fusion protein of two or three distinct viruses.

1. Introduction

Of more than 300 virus diseases described in India, majority are caused by potyviruses followed by begomoviruses. Besides these viruses, tospo- and cucumo-viruses are emerging as serious pathogens (Varma et al., 2002; Mandal et al., 2012a). Among the tospoviruses recorded from India, *Groundnut bud necrosis virus* (GBNV), is the most prolific tospovirus affecting a variety of crops in the Indian subcontinent. It has become a major constraint to the cultivation of several Fabaceous and Solanaceous hosts such as cowpea, groundnut, mungbean, pea, potato, soybean and tomato (Mandal et al., 2012a). *Cucumber mosaic virus* (CMV) is another economically important viral pathogen of crop plants with a wide host range. CMV infects more than 1000 species of plants, including monocots and dicots, herbaceous plants, shrubs and trees (Gallitelli, 2000). CMV isolates have been classified into two major subgroups (Subgroups I and II) on the basis of serological properties and nucleotide sequence homology (Palukaitis et al., 1992) and their genomes share approximately 75% sequence identity at nucleotide levels. Phylogenetic analysis has further divided Subgroup I into Subgroups IA and IB (Roossinck et al., 1999), that share 92% to 95% nucleotide sequence identity (Roossinck, 2002). *Papaya ringspot virus* (PRSV), a definitive member of the genus *Potyvirus* and family *Potyviridae* (King et al., 2012), causes widespread and devastating disease in papaya and cucurbits. PRSV was first reported in western India in 1958 (Capoor and Varma, 1958) and since then it has spread to different geographical locations becoming a major threat to papaya cultivation throughout India resulting in significant yield losses (Jain et al., 2004). Both papaya infecting (P) and non-papaya infecting (W) pathotypes, which are serologically indistinguishable, are prevalent in India (Roy et al., 1999).

Among the various diagnostic techniques, immuno-based detection has been routinely used for virus detection (Hull, 2002). A major limitation of immuno-diagnosis is the difficulty in producing quality polyclonal antibodies (PAb) to viruses, which are difficult to purify in reasonable quantities. To overcome this problem, the viral protein expressed as a recombinant fusion protein has been used as an antigen for raising PAb against individual virus (Raikhy et al., 2007; Lee and Chang, 2008; Gulati-Sakhuja et al., 2009; Rani et al., 2010; Rana et al., 2011; Khatabi et al., 2012; Mandal et al., 2012b). Although quality PAb against the recombinant coat protein (CP) of PRSV and CMV and nucleocapsid protein (N) of GBNV have been individually developed previously (Jain et al., 2005; Agarwal et al., 2009; Sreenivasulu and Sai Gopal, 2010; Khan et al., 2012),

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Table 1
Primer sequences used in the study.

Primer	Sequence	Restriction sites	Expected product (bp)	Annealing temperature (°C)	Used for amplification of
RKJ 85F RKJ 86R	5′-**GCTAGC**ATGCTTTCGCGACTTAATAAG-3′ 5′-**GGATCC**GAGGACGGCGTACTTTCT-3′	*Nhe*I *Bam*HI	~444	54	CMV Tr-CP for dual and triple construct
RKJ 87F RKJ 88a R	5′-**GGATCC**CCGCAGCAAATTGAC-3′ 5′-**GCGGCCGC**TGTGTGTCTCTCCGTGTT-3′	*Bam*HI *Not*I	~528	52	PRSV Tr-CP for dual construct
RKJ 87 FRKJ 88b R	5′-**GGATCC**CCGCAGCAAATTGAC-3′ 5′-**GTCGAC**TGTGTGTCTCTCCGTGTT-3′	*Bam*HI *Sal*I	~528	52	PRSV Tr-CP for triple construct
RKJ 111FRKJ 112R	5′-**GTCGAC**ATGGAATTGCCATTAGTG-3′ 5′-**GCGGCCGC**TTTGAATTCAATCTGTGAAGC-3′	*Sal*I *Not*I	~252	52	GBNV Tr-N for triple construct

no attempt has been made to develop diagnostic reagents that can simultaneously detect GBNV, CMV and PRSV. Since mixed infections of GBNV, CMV and PRSV are common in horticultural crops (Varma et al., 2002), there is a need for development of diagnostic reagents capable of multiplex virus detection. In view of this, an attempt has been made to develop cocktail of polyclonal antibodies using fusion constructs derived from viral gene sequences of two or three different viruses for multiple virus detection.

2. Materials and methods

2.1. Viral clones

Clones derived from coat protein (CP) genes of *Cucumber mosaic virus* (CMV, *Cucumovirus*) and *Papaya ringspot virus* (PRSV, *Potyvirus*) and nucleocapsid protein (N) gene of *Groundnut bud necrosis virus* (GBNV, *Tospovirus*) were received as gift from Advanced Centre of Plant Virology, Indian Agricultural Research Institute, New Delhi and used in the study.

2.2. Cloning of conserved sequences of CP/N

The conserved sequences in CP of CMV (Tr-CP; ~444 bp) and PRSV (Tr-CP; ~528 bp) were identified from the available CP sequences of cucumoviruses/potyviruses in GenBank. Similarly, the conserved sequences in the N protein of GBNV (Tr-N; ~252 bp) were identified from the available N gene sequences of tospoviruses in GenBank. Four sets of primers were designed for the development of dual fusion construct comprising of conserved CP sequences of CMV and PRSV and a triple fusion construct comprising of conserved CP sequences of CMV, PRSV and conserved N sequences of GBNV (Table 1). The strategy used for the development of dual and triple fusion constructs has been shown in Fig. 1. For dual construct, Tr-CP of CMV and PRSV was amplified from the full length respective CP clones using primers RKJ 85F/RKJ 86R and RKJ 87F/RKJ 88aR respectively. The PCR conditions used were: one cycle of initial denaturation at 94 °C for 4 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C (CMV) and 52 °C (PRSV) for 1 min and extension at 72 °C for 1 min, and one cycle of final extension at 72 °C for 10 min. The expected amplicons of PRSV-CP (~528 bp) and CMV-CP (~444 bp) were PCR purified using the SV wizard PCR clean up system (Promega, Madison, USA) and recloned in the pGEM-T Easy vector (Promega, Madison, USA) following standard molecular biology procedures (Sambrook and Russell, 2001). Similarly, for triple construct, the Tr-CP/N of PRSV and GBNV was amplified from the full length CP/N clones using primers RKJ 87F/RKJ 88bR and RKJ 111F/RKJ 112R respectively. The PCR conditions followed were: one cycle of initial denaturation at 94 °C for 4 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C (PRSV/GBNV) for 1 min and extension at 72 °C for 1 min, and one cycle of final extension at 72 °C for 10 min. The expected amplicons of PRSV-CP (~528 bp) and GBNV-N (~252 bp) were PCR purified and recloned in the pGEM-T Easy vector.

2.3. Construction of dual construct using Tr-CP of CMV and PRSV

The Tr-CP of CMV (~444 bp) was further subcloned in pET-28a(+) vector (5369 bp, Novagen, San Diego, USA) at *Nhe*I and *Bam*HI site. The positive pGEM-T Easy clone containing the Tr-CP of CMV and the expression vector pET-28a(+) (1 μg) were simultaneously digested with *Nhe*I and *Bam*HI (Fast digest, Fermentas, Burlington, Canada). The released Tr-CP fragment (~444 bp) from pGEM-T Easy was purified and ligated into pET-28a(+) in frame to create expression plasmid CMV-CP-pET-28a(+), followed by transformation in *Escherichia coli* BL21(DE3) cells (Novagen, San Diego, USA). The expression construct CMV-CP-pET-28a(+) and the positive pGEM-T Easy clone containing the Tr-CP of PRSV were then digested with *Bam*HI and *Not*I (Fast digest, Fermentas, Burlington, Canada). The released Tr-CP fragment of PRSV (~528 bp) from pGEM-T Easy was purified and ligated into CMV-CP-pET-28a(+) construct in frame to create dual construct CMV-PRSV-CP-pET-28a(+), followed by transformation in *E. coli* BL21(DE3) cells. Recombinant clones were screened by kanamycin (30 μg/ml) selection and confirmed by plasmid PCR as well as by restriction digestion.

2.4. Construction of triple construct using Tr-CP of CMV, PRSV and Tr-N sequence of GBNV

The Tr-CP of PRSV (~528 bp) amplified using another set of primer (RKJ 87F/RKJ 88bR) was mobilized into CMV-CP-pET-28a(+) expression construct at *Bam*HI and *Sal*I site. The CMV-CP-pET-28a(+) expression construct developed previously and the positive pGEM-T Easy clone containing the Tr-CP of PRSV were simultaneously digested with *Bam*HI and *Sal*I (Fast digest, Fermentas, Burlington, Canada). The released PRSV-Tr-CP fragment (~528 bp) was purified and ligated into CMV-CP-pET-28a(+) in frame to create dual construct CMV-PRSV-CP-pET-28a(+). The dual construct CMV-PRSV-CP-pET-28a(+) and the positive pGEM-T Easy clone containing the Tr-N sequence of GBNV were then digested with *Sal*I and *Not*I (Fast digest, Fermentas, Burlington, Canada). The released Tr-N fragment of GBNV (~252 bp) from pGEM-T Easy was purified and ligated into CMV-PRSV-CP-pET-28a(+) dual construct in frame to create a triple construct CMV-PRSV-CP-GBNV-N-pET-28a(+), followed by transformation in *E. coli* BL21(DE3) cells (Novagen, San Diego, USA). Recombinant clones were screened by kanamycin (30 μg/ml) selection and confirmed by plasmid PCR as well as by restriction digestion.

2.5. Expression of recombinant protein from dual/triple constructs

Expression of the recombinant protein from transformed *E. coli* BL21 (DE3) host cells with dual/triple constructs was carried out in LB medium supplemented with 30 μg/ml kanamycin. The culture was grown at 37 °C until the optical density (OD600) value reached 0.5 and induced by isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 1.0 mM for 3 h at 37 °C. The expressed