

Synthesis and Cloning of cDNA Encoding an Antiviral Protein from the Leaves of *Bougainvillea spectabilis* Willd. (Nyctaginaceae)

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Abstract: Total RNA was extracted from the mature leaves of *Bougainvillea spectabilis* Willd. and messenger RNA (mRNA) was separated out. Using mRNA as template, complementary DNA (cDNA) was synthesized and amplified by Reverse Transcription coupled PCR using gene specific primer. A product of 750 base pair plus was selected based on the size of *Bougainvillea* antiviral protein (BAP). After elution the product was purified and cloned into pGEM-T Easy vector and mobilized into *E. coli* strain, JM 109 and sequenced. The partial cDNA sequence of 893 base pairs contained three open reading frames. The sequence of a most possible ORF (297 bp) was translated and its homology with already reported sequences of antiviral proteins was analyzed.

Key words: Antiviral proteins % BAP % cDNA synthesis % RT-PCR % Cloning % Homology search % CLUSTAL analysis

INTRODUCTION

Many higher plants have developed a variety of defense systems to combat pathogen attack which is essential for their survival. Some of these plants possess endogenous proteins that act as virus inhibitors [1]. These antiviral proteins have been purified and characterized [2 - 4]. They are generally basic proteins with molecular weight ranging from 24 to 32 kDa and effective against a wide range of plant viruses. The viral inhibitors are well studied in *Phytolacca americana* [5], *Gelonium multiflorum* [6], *Dianthus caryophyllus* [7], *Mirabilis jalapa* [8], *Bougainvillea spectabilis* [2], *Celosia cristata* [9] and *Pandanus amaryllifolius* [10]. These viral inhibitors are most effective when mixed with the virus inoculum or when they are applied one day before or shortly after mechanical inoculation. The leaves of *Bougainvillea spectabilis*, a member of the family Nyctaginaceae, was selected for this study as it was found to contain an endogenous virus inhibitor which conferred resistance to tospovirus, tobacco mosaic virus (TMV), cucumber mosaic virus (CMV) and cowpea aphid borne mosaic virus (CAMV) in their respective susceptible plants. The viral inhibitor in *Bougainvillea*

spectabilis is very potent, stable and is a 28kDa basic protein (BAP) [2]. In the present study, partial cDNA encoding the *Bougainvillea* antiviral protein was synthesized from the leaf mRNA of *Bougainvillea spectabilis*, cloned and sequenced. Homology with other reported antiviral proteins was also studied.

MATERIALS AND METHODS

Materials: Mature leaves of *Bougainvillea spectabilis* Willd. was obtained from Botanic Gardens, Tamil Nadu Agricultural University campus, Coimbatore. TRIzol reagent, Message Maker assembly Kit and restriction enzymes were purchased from Life technologies Inc., USA. *Taq* DNA polymerase, oligo dT primer, DNA markers and RT-PCR kits were obtained from Bangalore Genei Pvt. Ltd. pGEM-T Easy vector, T4 DNA ligase and JM 109 competent cells were purchased from Promega Inc., USA. Gene specific primer was synthesized based on the N-terminal amino acid sequence of BAP using BACKTRANSLATE program of GCG package. All other biochemicals used were obtained from commercial sources and used according to manufacturer's recommendations.

Isolation of Poly (A)⁺RNA: Total RNA was extracted from the fresh leaves of *Bougainvillea spectabilis* using TRIzol reagent supplied by Life technologies, USA, which is a modified method of RNA isolation from tissues [11]. The quality of the RNA was checked by electrophoresis using 1% agarose gel made at 50 V, in 1.0% Agarose (Genei), containing 2 μ L of 10 g mL⁻¹ ethidium bromide in TBE buffer (0.89 M Tris; 0.89 M Borate, 0.02 M EDTA, pH 8.25-8.29) (Fig. 1). Poly (A)⁺ RNA was subsequently isolated from total RNA using Message maker assembly kit following the protocol supplied by the (Life Technologies, USA). About 200 μ g of total cellular RNA was allowed to bind with oligo dT cellulose and incubated at 37°C for 10 min for annealing. The bound RNA was washed with wash buffers. Finally single stranded poly (A)⁺ RNA was dissolved in preheated sterile distilled water.

Synthesis of BAP cDNA: Fifty ng of poly (A)⁺ RNA was hybridized with 30 pmol of oligo dT and RNAs were reverse transcribed in reaction volume of 20 μ L containing 50 mM Tris-HCl, pH 8.3, 40 mM KCl, 10 mM DTT, 6 mM MgCl₂, 7.5 mM of each dNTP, 20 U of RNasin, 5 U of AMV reverse transcriptase. The reaction products were incubated for 1 h at 42°C. Forward primer was designed based on the N-terminal amino acid sequence of BAP. Reverse transcription reaction mixture (2 μ L) was added to 48 μ L of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 40 pmol of forward and reverse primer (oligo dT), 15 mM of each dNTP and 1.5 U of Taq DNA polymerase. The reaction was performed through 35 cycles of 1 min at 94°C, 1 min. at 60°C and 1.5 min at 70°C.

Cloning of BAP cDNA: The amplified products were analyzed on 1% agarose gel [12]. Three products of around 750 bp plus were eluted separately and reamplified. All gave an 880 bp product. This was further eluted, purified and used for cloning. The 880 bp cDNA fragment obtained was cloned into linearised pGEM-T Easy vector. The *E. coli* strain JM 109 was transformed by pGEM-T Easy vector with a BAP-cDNA insert. The transformants were grown at 37°C in LB broth containing ampicillin (100 μ g mL⁻¹) to an optical density of 0.4 at 600 nm. The recombinants were isolated by alkali miniprep from a number of transformants and the insert was confirmed by restriction digestion and PCR amplification.

Sequencing and Homology Studies: The insert flanking the T7 and SP6 promoter sites was sequenced at

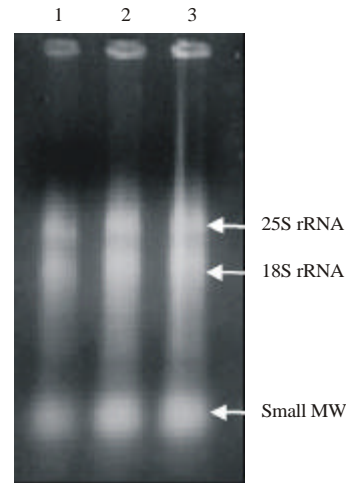


Fig. 1: Analysis of Total RNA from mature leaves of *Bougainvillea spectabilis* on 1% agarose gel and detected by ethidium bromide staining. Lanes 1, 2 and 3 loaded with 1, 2 and 2.5 μ g total RNA, respectively

Microsynth Inc., Switzerland using automated DNA sequencer which is based on the dideoxy chain termination method using fluorescent dyes. Homology search for the cDNA sequence obtained after sequencing was performed through NCBI and EBI BLAST programmes like Nucleotide BLAST, Protein BLAST (SWISSPROT).

RESULTS AND DISCUSSION

Cloning the cDNA of BAP: Synthesis of cDNA by RT-PCR from mRNA has become a routine method for gene cloning and expression studies. Using cDNA as a probe, many antiviral protein genes have been isolated. Using a single gene specific oligonucleotide primer, full-length cDNAs were produced from rare transcripts [13]. The isolation and sequencing of partial cDNA clone encoding an AVP from *Saponaria officinalis* has also been reported [14]. Similarly cDNA was used to isolate PAP gene [15, 16]. In the present study, the total RNA was isolated using TRIzol reagent. The RNA profile on 1% agarose gel (Fig. 1) indicated the intactness of different subunits of RNA. cDNA corresponding to BAP mRNA was synthesized by RT-PCR using gene specific primer and oligo dT as 5' and 3' primers, respectively. The cDNA product of around 800 bp is shown in Fig. 2.

Earlier studies in our laboratory revealed the molecular weight of BAP to be 28 kDa with around 250

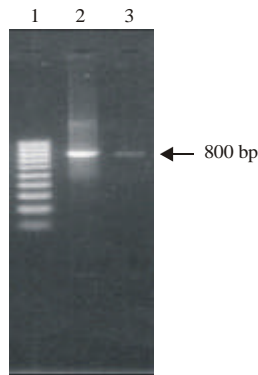


Fig. 2: cDNA resolved on 1.5% agarose gel and stained with ethidium bromide. Lanes 1-100 bp ladder, 2-eluted and reamplified cDNA, 3-eluted cDNA)

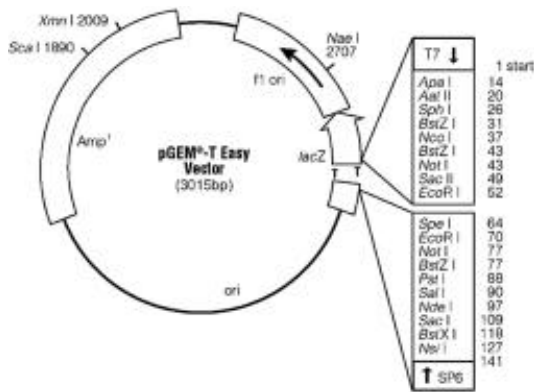


Fig. 3: Physical map of pGEM-T Easy vector

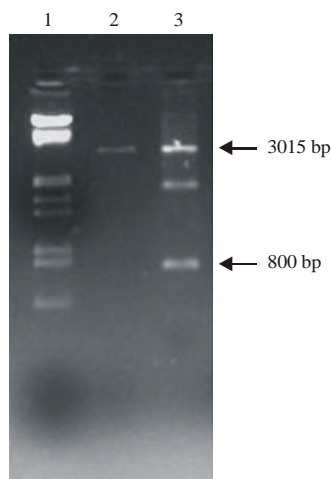


Fig. 4: Restriction digestion of plasmid DNA on 1% agarose gel. Lanes 1- Lambda double digest marker, 2- undigested plasmid DNA, 3- restricted plasmid DNA)

amino acids [2]. Hence the expected cDNA product of about 800 bp was eluted from the gel, purified and further amplified. Presence of the product after was further confirmed by agarose gel electrophoresis. An aliquot of the amplified product was cloned in a pGEM-T Easy vector (Fig. 3) and mobilized into JM 109 *E. coli* competent cells. Transformants were selected by blue-white screening. Restriction digestion (Fig. 4) and PCR amplification of recombinants confirmed the presence of insert.

Sequence homology: Homology search was performed using NCBI BLAST program. The putative region was chosen among the three open reading frames for further homology studies. Alignment of the peptide sequences with known antiviral / ribosome inactivating protein sequences revealed weak homology of BAP-cDNA sequence with the reported AVP / RIPs sequences viz., *Mirabilis* antiviral protein [17], Pokeweed antiviral protein [18] and *Clerodendrum aculeatum* AVP [19]. The results of the sequence homology analyses infer that the cDNA may be specific to *Bougainvillea spectabilis*.

CONCLUSIONS

Plant disease management using antiviral proteins (AVPs) of plant origin holds promising to combat viral diseases. Many of these viral inhibitory proteins possess ribosome inactivating property, the most widely accepted mechanism of action as a direct result of their ribosome specific N-glycosidase activity. The present study attempted synthesizing partial cDNA of *Bougainvillea* antiviral protein. Further research on isolation of full length cDNA will be useful in constructing plant transformation vectors for evolving transgenic plants resistant against selected plant viruses.

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