Cloning and expression of antiviral/ribosome-inactivating protein from *Bougainvillea* x*buttiana*

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A full-length cDNA encoding ribosome-inactivating/antiviral protein (RIP/AVP) from the leaves of *Bougainvillea* x*buttiana* was isolated. The cDNA consisted of 1364 nucleotides with an open reading frame (ORF) of 960 nucleotides encoding a 35.49 kDa protein of 319 amino acids. The deduced amino acid sequence has a putative active domain conserved in RIPs/AVPs and shows a varying phylogenetic relationship to the RIPs from other plant species. The deduced protein has been designated BBAP1 (*Bougainvillea* x*buttiana* antiviral protein1). The ORF was cloned into an expression vector and expressed in *E. coli* as a fusion protein of ~78 kDa. The cleaved and purified recombinant BBAP1 exhibited ribosome-inhibiting rRNA *N*-glycosidase activity, and imparted a high level of resistance against the tobacco mosaic virus (TMV).

1. Introduction

Many higher plant species belonging to various taxonomic families are known to produce endogenous, non-stress induced inhibitor proteins called antiviral proteins (AVPs). Many of these AVPs have ribosome-inhibiting rRNA *N*-glycosidase activity and are known as ribosome-inactivating proteins (RIPs). RIPs are subdivided into holo-RIPs and chimero-RIPs based on the structure of both proteins and their corresponding genes. Holo-RIPs consist exclusively of a single RNA *N*-glycosidase domain, and correspond to the classical type-1 RIPs, which are single-chain proteins of approximately 30 kDa. The chimero-RIPs correspond to the classical type-2 RIPs consisting of an *N*-glycosidase domain similar to type-1 RIPs and an unrelated C-terminal domain with carbohydrate-binding activity (VanDamme *et al* 2001). The gene encoding the jasmonate-induced protein of 60 kDa (JIP60), the only known type-3 RIP, consists of an N-terminal domain resembling type-1 RIPs, tandemly arrayed to an unrelated C-terminal domain with unknown function (VanDamme *et al* 2001).

The RIPs/AVPs have also been found to possess a number of enzymatic properties. To name a few, these include DNase activity on supercoiled DNA (Wang and Tumer 1999; Bhatia and Lodha 2005; Begam *et al* 2006; Choudhary *et al* 2007), RNase activity (Hudak *et al* 2000; Bhatia and Lodha 2005; Begam *et al* 2006; Roy *et al* 2006; Choudhary *et al* 2007) and depurination of capped mRNAs (Hudak *et al* 2002). Recently, some RIPs/AVPs have also been reported to show superoxide dismutase (SOD)

(Sharma *et al* 2004), phospholipase (Hemly *et al* 1999) and antioxidant activity (Gholizadeh *et al* 2004), and induce cell death by apoptosis (Narayanan *et al* 2005). However, much remains to be known about the mechanisms through which these AVPs operate to inhibit virus infection *in vivo* without affecting the host ribosomes (Wang and Hudak 2003; Park *et al* 2004; Stirpe 2004).

Bougainvillea, a popular ornamental plant, is a member of the family Nyctaginaceae. Two highly basic AVPs designated as BAP-28 and BAP-24 have been purified from *B.* x*buttiana* cv. Enid Lancester leaves (Narwal *et al* 2001a). Similarly, AVP consisting of two polypeptide bands (33 kDa and 28 kDa) was purified from the leaves of *B.* x*buttiana* cv. Mahara (Bhatia *et al* 2004). Like other AVPs, these were classified as RIPs owing to their *N*-glycosidase activity. Further, bougainvillea RIPs/AVPs have been suggested to be multifunctional proteins capable of inducing systemic resistance, and their virus inhibitory activity may be directly correlated to their enzymatic activities, such as *N*-glycosidase, RNase and DNase (Narwal *et al* 2001b; Bhatia and Lodha 2005), and antioxidant properties (Bhatia *et al* 2004). Barbieri *et al* (1993) reported that bougainvillea AVPs are the least toxic and a similar observation has also been made by Hartog *et al* (2002) in respect of a recombinant AVP, bouganin, encoded by a cDNA isolated from the leaves of *Bougainvillea spectabilis.* Development of transgenic lines with genes encoding RIPs/AVPs may be a promising strategy to develop virus-resistant crop plant species (Tumer *et al* 1999; Zoubenko *et al* 2000; Wang and Hudak 2003; Vandenbussche *et al* 2004). In the present study, we report the isolation and characterization of a cDNA encoding an AVP/RIP from *B.* x*buttiana* leaves and its expression in *Escherichia coli.* The expressed recombinant protein, BBAP1, has already been shown to have RNase activity against *Torula* yeast RNA, DNase activity against pBSK⁺

supercoiled DNA, and antiviral activity against sunnhemp rosette virus which infects *Cyamopsis tetragonoloba* (Choudhary *et al* 2007).

2.   Materials and methods

2.1   *Plant materials, viral and bacterial strains*

Fresh, tender leaves of *B.* x*buttiana* cv. Mahara (non-host plant) were collected from the Bougainvillea garden of the Division of Floriculture and Landscaping, IARI, New Delhi. Seeds of *Nicotiana glutinosa* (host plant) were obtained from the Central Tobacco Research Institute, Rajamundry and grown in a greenhouse at the National Phytotron Facility, IARI, New Delhi. Tobacco mosaic virus (TMV) inoculum was obtained from the Advanced Centre for Plant Virology, IARI, New Delhi. *E. coli* strains DH5α and TB1 were used for cloning and expression of the cDNA, respectively.

2.2   *Isolation of cDNA encoding BBAP1*

Total RNA was isolated from fresh, tender bougainvillea leaves (0.1 g) using TRI reagent extraction buffer (Cat # TR-118, MRC, USA). The purity of the isolated RNA was checked spectrophotometrically. The isolated RNA was dissolved in nuclease-free water and stored at –80°C until further use for cDNA synthesis. First-strand cDNA was synthesized as per the manufacturer's protocol (Sensiscript RT Kit, Cat # 205211, Qiagen). cDNA sequence data of the conserved region of RIPs/AVPs available in the GenBank were used to design gene-specific primers for PCR amplification of the cDNA. The programme used in a thermocycler was 4 min at 94°C, 35 cycles each of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C. Subsequently,

Table 1   Primers used for isolating the cDNA encoding BBAP1

Primer no.	Nucleotide sequence	Nucleotides
1	FP 5′ CAGATGTGTATGTTGTGGGTTAT 3′	23
2	RP 5′ GCTGCCTCTGAAACCATTTG 3′	20
3	FP 5′ CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG 3′	35
4	FP 5′ GCTGATGGCGATGAATGAACACTG 3′	24
5	RP 5′ GCAAGTGGAGGAGCATCTTTAAGC 3′	24
6	FP 5′ AAAACGTGGCCAGGTTCTTCC 3′	21
7	RP 5′ CGCGGATCCGAATTAATACGACTCACTATAGG 3′	32
8	FP 5′ ACGGAAATCAAAATCATAAAAAC 3′	23
9	RP 5′ TAATTAACGTGATTATTGCATTATAT 3′	26
10	FP 5′ CGGAATTCCGATGAA GCCACTAGGGGGGCTTG 3′	32
11	RP 5′ TGCACTGCAGTGCATTAGCCAATGATTGTTTCTAGTTT 3′	38

FP, forward primer; RP, reverse primer.

the PCR products were analysed on 1% agarose Tris/borate/EDTA gels and cloned into the pDrive vector (Cat # 231122, Qiagen). *E. coli* DH5α was transformed with the clone and the positive clone was sequenced at South Campus, University of Delhi. The sequence similarity analysis of the 261 bp cDNA fragment thus obtained was done by performing a basic local alignment search tool (BLAST) search at NCBI (*www.ncbi.nlm.nih.gov*; Altschul *et al* 1997).

For isolation of the full-length cDNA encoding BBAP1, seven primers were used (table 1, S. nos.3–9). Using the RNA ligase-mediated rapid amplified cDNA ends (RLM-RACE) technique (First Choice® RLM-RACE kit, Ambion), the 5′ untranslated region (UTR) and 3′ UTR missing regions of the obtained fragment were determined as per the manufacturer's protocols. By using primers 3, 4 and 5 (table 1), the 5′ missing region was amplified, and primers 6 and 7 (table 1) were used to amplify the 3′ missing region. The full-length cDNA was amplified by a one-step reverse transcription polymerase chain reaction (RT-PCR kit, Cat # 210210, Qiagen) using leaf mRNA as the template, and forward and reverse primers (table 1, S. nos. 8 and 9), specific to the 5′UTR and 3′UTR ends, respectively. mRNA was isolated from the total RNA as per the manufacturer's protocol provided along with the Oligotex mRNA mini kit (Cat # 70022, Qiagen). All the PCR products were analysed, cloned and sequenced as mentioned above. Phylogenetic analysis of the cloned cDNA sequences was performed using the Mega 3.0 software (*http://www.megasoftware.net*).

2.3 In vitro *expression of cDNA and western blotting*

To amplify only the open reading frame (ORF), PCR was carried out with specific primers of the 5′ and 3′ ends of the coding region. To do directional cloning of the PCR product in the pMAL-c2X expression vector, *Eco*RI and *Pst*I restriction sites were incorporated at the 5′ ends of the forward and reverse primers, respectively. The PCR reaction mixture (25 μl) contained 0.5 μg full-length cDNA, 2.5 μl 10x *Pfu* buffer, 10 mM dNTPs mix, 10 mM MgSO$_4$, 100 pmol each of forward and reverse primers (table 1, primers 10 and 11), and 1.25 U *Pfu* DNA polymerase. The thermocycler programme used was 4 min at 94°C, 35 cycles each of 1 min at 94°C, 1 min at 77°C and 1 min at 72°C. The amplified products were digested with *Eco*RI and *Pst*I restriction enzymes, cloned into the pMAL-c2X vector and used for transformation of TB1 cells for protein expression using the pMAL™ Protein Fusion and Purification kit, as per the manufacturer's protocol (New England BioLabs, UK Cat # E8000S). Western blotting of the expressed fusion protein was performed as described by O'Donnell *et al* (1982), and Rybicki and Von wechmar (1982) using a nitrocellulose membrane and alkaline peroxidase-conjugated antibodies (Bangalore Genei Cat #105498).

2.4 *Purification of recombinant BBAP1 protein and* N-*glycosidase assay*

The fusion protein was purified from the crude extract by affinity column chromatography as per the manufacturer's instructions (New England BioLabs Cat # E8000S). It was done on a 10 × 2.5 cm column packed with amylose resin specific for maltose-binding protein (MBP). The eluted protein was checked for its purity on 12% SDS-PAGE, and cleaved with factor Xa. The cleaved protein sample was dialysed against 20 mM sodium acetate pH 5.2 containing 0.01% sodium azide, and loaded on to a Sephadex G-100 column pre-equilibrated with column buffer (20 mM sodium acetate pH 5.2, 0.01% sodium azide) with a flow rate of 40 ml h^{-1}. The protein was eluted and 2 ml fractions were collected using an LKB Fraction Collector system. Protein homogeneity was checked on 12% SDS-PAGE.

Ribosomes were isolated from *Nicotiana tabacum* leaves according to the method of Tumer *et al* (1997). Ribosomes (10 μg) were treated with 2 μg of purified expressed BBAP1 to check the *N*-glycosidase activity (Girbes *et al* 1993). In the positive control, saporin RIP was added in place of the BBAP1. Ribosomal RNA was extracted by the standard method (Sambrook and Russel 2001) and treated with freshly prepared 10 μl of 2 M aniline/acetate (pH 4.5). These tubes were incubated on ice for 10 min in the dark. The reaction was stopped by dilution with 200 μl of water. Aniline was removed by two extractions with ether. RNA was again recovered by ethanol precipitation and resuspended in 15 μl of loading buffer. It was then incubated at 90°C for 30 s and immediately chilled on ice. Samples were then electrophoresed on 5% polyacrylamide gel and stained with ethidium bromide (0.5 μg ml^{-1} in 1× TBE) for 45 min. The presence of a released fragment on the gel was recorded as rRNA *N*-glycosidase activity.

2.5 *Antiviral bioassay of BBAP1*

The antiviral activity of purified recombinant BBAP1 was tested by the local lesion assay using *Nicotiana glutinosa* plants and TMV. Test plants with the same height and vigour were selected and, for each treatment, five plants with 3–4 leaves of equal size were used. BBAP1 was applied on the test plant leaves at the rate of 50 μg leaf^{-1}. After 1 h, the protein-treated leaves were washed with distilled water and gently blotted dry.

The leaves were then rubbed very lightly with 600 mesh carborundum powder and inoculated gently and uniformly with the virus inoculum. For the control, test plant leaves were initially treated only with buffer and then with virus inoculum. Ten minutes after inoculation, the leaves were washed with distilled water. Plants were observed for the development of lesions for 3–4 days. The inhibitory

activity of the protein was calculated in terms of percentage inhibition using the following formula: % inhibition = (C–T) × 100/C; where C = average number of lesions in control plants and T = average number of lesions in treated plants.

3. Results and discussion

3.1 *Isolation and sequence analysis of the cDNA fragment encoding BBAP1*

Total RNA was isolated from *Bougainvillea* x*buttiana* leaves and its reverse transcript (RT). cDNA was used in the PCR reaction as a template. Using gene-specific primers, a ~261 bp cDNA fragment was obtained. However, the isolated sequence lacked the 5′ and 3′ regions, which were amplified using the RLM-RACE technique. Both the 5′ and 3′ missing regions were amplified using gene-specific reverse and forward primers designed on the basis of the nucleotide sequence of the 5′ and 3′ ends of the cDNA fragment, respectively.

A full-length cDNA encoding bougainvillea AVP was obtained using forward and reverse primers specific to the 5′ and 3′ ends, and bougainvillea leaf mRNA as template. The full-length cDNA sequence was found to be 1364 bp long, and has been submitted to the NCBI GenBank (Accession No. DQ013264), wherein the deduced AVP has been designated as BBAP1 (*Bougainvillea* x*buttiana* antiviral protein1).

3.2 In silico *analysis of isolated gene/cDNA*

The nucleotide sequence of the full-length cDNA and deduced amino acids are shown in figure 1. The cDNA has a translation initiation codon (ATG) after the 120th position, downstream of the first base, and is terminated by the stop codon (TAA) located after the 1077th nucleotide. Two polyadenylation signals (AATAA) were located after positions 1161 and 1252, respectively. In addition, a poly (A) tail of 12 adenine nucleotides was also detected. The 5′ and 3′ UTRs have been found to be 120 and 284 bp long, respectively. The nucleotide composition of the cDNA as analysed by the BioEdit software was found to be: 473 adenine (A), 233 cytosine (C), 257 guanine (G) and 401 thymine (T). The total G + C and A + T content was 35.92% and 64.08%, respectively. Thus, based on the nucleotide composition, the isolated cDNA was found to be AT rich.

A BLAST N search did not show any significant homology of the isolated cDNA with other known sequences of RIPs/AVPs, except 88.83% and 88.16% homology with cDNAs encoding the AVP of leaves (bouganin) and roots (Ap1) of *Bougainvillea spectabilis,* respectively. However, Clustal W alignment showed a strong homology with the nucleotide sequence from 715 to 753 of cDNAs of nine different RIPs/AVPs (results not shown). The full-length cDNA was translated (*www.expasy.com*) and it was found that the ORF of 960 bp encodes 319 amino acid residues. The theoretical amino acid composition using the software BioEdit revealed that the protein was rich in leucine (10%), followed by valine (9.4%) and lysine (9.1%). The molecular weight of the protein was predicted to be 35.49 kDa. The BLAST P ORF-encoded amino acid sequence of BBAP1 showed an identity range of 30–57% with different RIPs/AVPs, being maximum with both bouganin and Ap1. The ClustalW alignment as well as conserved domain BLAST showed a strong homology in the conserved region (IQIVSEAARFKYI) (figure 2), which is incidentally the active site of the protein.

The phylogenetic relationship of BBAP1 with ten other RIPs/AVPs constructed on the basis of ClustalW alignment is shown in figure 3. The BBAP1, bouganin and Ap1 proteins could be grouped into one cluster with 98% bootstrap values. This may be due to the fact that all these AVPs are from two species of bougainvillea only. The BBAP1 cluster was also related to another cluster of four AVPs (*M. jalapa*, MAP, ME1 and amaranthin). This may be due to the fact that BBAP1 and other AVPs except amaranthin belong to the same family Nyctaginaceae. The BBAP1 cluster showed a distant relationship with PAP and PAP1; and was unrelated to AAP1 and CCP.

3.3 *Characterization of a putative signal sequence*

The homology studies with a signal sequence at the N-terminal of bouganin (results not shown) suggested that BBAP1 might have a signal peptide sequence of 36 amino acids (3.89 kDa) as against 26 amino acids in case of bouganin (Hartog *et al* 2002). Several other RIPs/AVPs have also been reported to have a signal peptide at the N-terminus (Lin *et al* 1991; Chow *et al* 1999; Cho *et al* 2000; Kwon *et al* 2000; Vepachedu *et al* 2003; Begam *et al* 2006).

3.4 *Cloning and expression of the gene in* E. coli

E. coli TB1 cells containing cDNA encoding for AVP from the leaves of *B.* x*buttiana,* cloned in an expression vector (pMAL-c2X), were grown in LB medium containing 0.3 mM IPTG to induce protein expression.

When the optical density of the culture was measured at 600 nm every 45 min, the bacterial cells with non-recombinant (control) as well as recombinant vectors showed normal growth even after IPTG induction. A linear growth was observed up to 135 min, and thereafter no further growth was observed (results not shown). This pattern of growth of *E. coli* TB1 cells indirectly indicates that the translational product BBAP1 of the cloned ORF has no toxic effect on the

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1      acg gaa atc aaa atc ata aaa aca cat cct tta cct aca att ttc   45

46     att aca agc agt cca cca agt tta att aca gct gtt aca aat tac   90

91     aat tgt gtc ttt tat cga tca ggc aca agt ATG AAG CCA CTA GGG   135
1                                              M   K   P   L   G     5

136    GGG CTT GTA ATA ACG TGT ATT TGG TGG GCT ATT ATA GTT GAA CTA   180
6      G   L   V   I   T   C   I   W   W   A   I   I   V   E   L     20

181    GTG GTT ATA AAG CCA TCG ATA ATC ACC AAA GAA ACA GCC TCA CTT   225
21     V   V   I   K   P   S   I   I   T   K   E   T   A   S   L     35

226    GGT CAC AAC ACT GTG TCA TTT AAC CTG GAA GAT GCT ACT GAA CAA   270
36     G   H   N   T   V   S   F   N   L   E   D   A   T   E   Q     50

271    GTG TAC CGC TCT GTT ATA AAA TCT TTG CGC AAC GAA TTA CAA ACT   315
51     V   Y   R   S   V   I   K   S   L   R   N   E   L   Q   T     65

316    AGC ACA CTA GTA TGT AAA ATT CCA GTG ACA CCA CAA AGC CTA GCC   360
66     S   T   L   V   C   K   I   P   V   T   P   Q   S   L   A     80

361    GTT GAT AAA AAC CAA TTT GTT GTG GTT AGT CTC ACT TGG AAG TCA   405
81     V   D   K   N   Q   F   V   V   V   S   L   T   W   K   S     95

406    AAG GAA GTT CAA CTT GGT ATA GAT GTG ACG AAT GCG TAT GTG GTG   450
96     K   E   V   Q   L   G   I   D   V   T   N   A   Y   V   V     110

451    GGT TAT CAA GAC AAA TTG AAG AAC CAA GAT TAT CGA GCC TTT TTG   495
111    G   Y   Q   D   K   L   K   N   Q   D   Y   R   A   F   L     125

496    CTT AAA GAT GCT CCT CCA CTT GCA AAA GAT AAA CTT TTC CCT GGA   540
126    L   K   D   A   P   P   L   A   K   D   K   L   F   P   G     140

541    GTG AAA AGT GAA ACA TTA CCG TTT GGA GGC ACC TAT CCA AAC CTT   585
141    V   K   S   E   T   L   P   F   G   G   T   Y   P   N   L     155

586    GTA AAA GCT GGC AAT GTC GAT CGT AGA GAA CAA TTC GAA CTG GGT   630
156    V   K   A   G   N   V   D   R   R   E   Q   F   E   L   G     170

631    GTT GAA AAA CTC GGA CAT GCC ATT GAT TCA ATT TTT GGT AAA CCA   675
171    V   E   K   L   G   H   A   I   D   S   I   F   G   K   P     185

676    AAA AAT GAT CAA AAC GTG GCC AGG TTC TTC CTC AAT GCC ATC CAG   720
186    K   N   D   Q   N   V   A   R   F   F   L   N   A   I   Q     200

721    ATA GTT TCA GAG GCT GCA CGG TTT AAA TAC ATC GAG AAT GAG GTG   765
201    I   V   S   E   A   A   R   F   K   Y   I   E   N   E   V     215

766    GTT CGT GGA GGA TTA AAT TCT GGA TTA TCT AAA CCC AAC GGC AAA   810
216    V   R   G   G   L   N   S   G   L   S   K   P   N   G   K     230

811    ATG TTG AAC TTG GAG AAC AGT TGG GGA CAT ATC TCT GAT GCC ATT   855
231    M   L   N   L   E   N   S   W   G   H   I   S   D   A   I     245

856    CAC AAC TCA AAT CAA AAA TAT AAA TGT GAA ACT ATT TCA CCA GCA   900
246    H   N   S   N   Q   K   Y   K   C   E   T   I   S   P   A     260

901    CTT GAG TTA CAA AAC GCG GAT TAT AGC AAA TGG GTT GTG AAT AAA   945
261    L   E   L   Q   N   A   D   Y   S   K   W   V   V   N   K     275

946    GTG AGT GAA ATT AGT CCT GAT TTG GGT ATC CTT AAG TAT AAA AGC   990
276    V   S   E   I   S   P   D   L   G   I   L   K   Y   K   S     290

991    TCT AAA TTA ACA CAG TCT ACA ACA ATA ATG AGG TCT ATT GTA GTT   1035
291    S   K   L   T   Q   S   T   T   I   M   R   S   I   V   V     305

1036   GAA GAT AGT GAT GAA CTT GAA AAA CTA GAA ACA ATC ATT GGC TAA   1080
306    E   D   S   D   E   L   E   K   L   E   T   I   I   G   *     319

1081   tca cct taa ttc cat cac ctc aaa gcc aga tct ttt ata tat cca   1125

1126   gaa gtt act taa att aat ggg atg ccc acg gct aag aat aat gat   1170

1171   tat gca tgg tgc att tgt gtt act gtt tta tgt atg aat aat gta   1215

1216   tgt ggg tat gta ctt tta tga gga att gtt tat ggt taa taa tta   1260

1261   tat cct aaa agg ttc cca tcc ttg taa gat gca tat cta aag ttc   1305

1306   aat aat atg taa tgt aat tta tat ata atg caa taa tca cgt taa   1350

1351   tta aaa aaa aaa  aa 1364
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Figure 1. Nucleotides (pink) and deduced amino acids (black) sequences of full-length cDNA encoding RIP/AVP of *Bougainvillea* x*buttiana* (GenBank accession no. DQ013264). In lower case, the 5′ UTR (blue) corresponds to nucleotide positions from 1 to 120 and the 3′ UTR (green) sequence from positions 1081 to 1364. The coding region nucleotide sequence contains positions 121–1080. Two polyadenylation signals (AATAA) are present after the 1161th and 1252th positions. The conserved region sequence is represented by blue (nucleotides) and light green (amino acids).
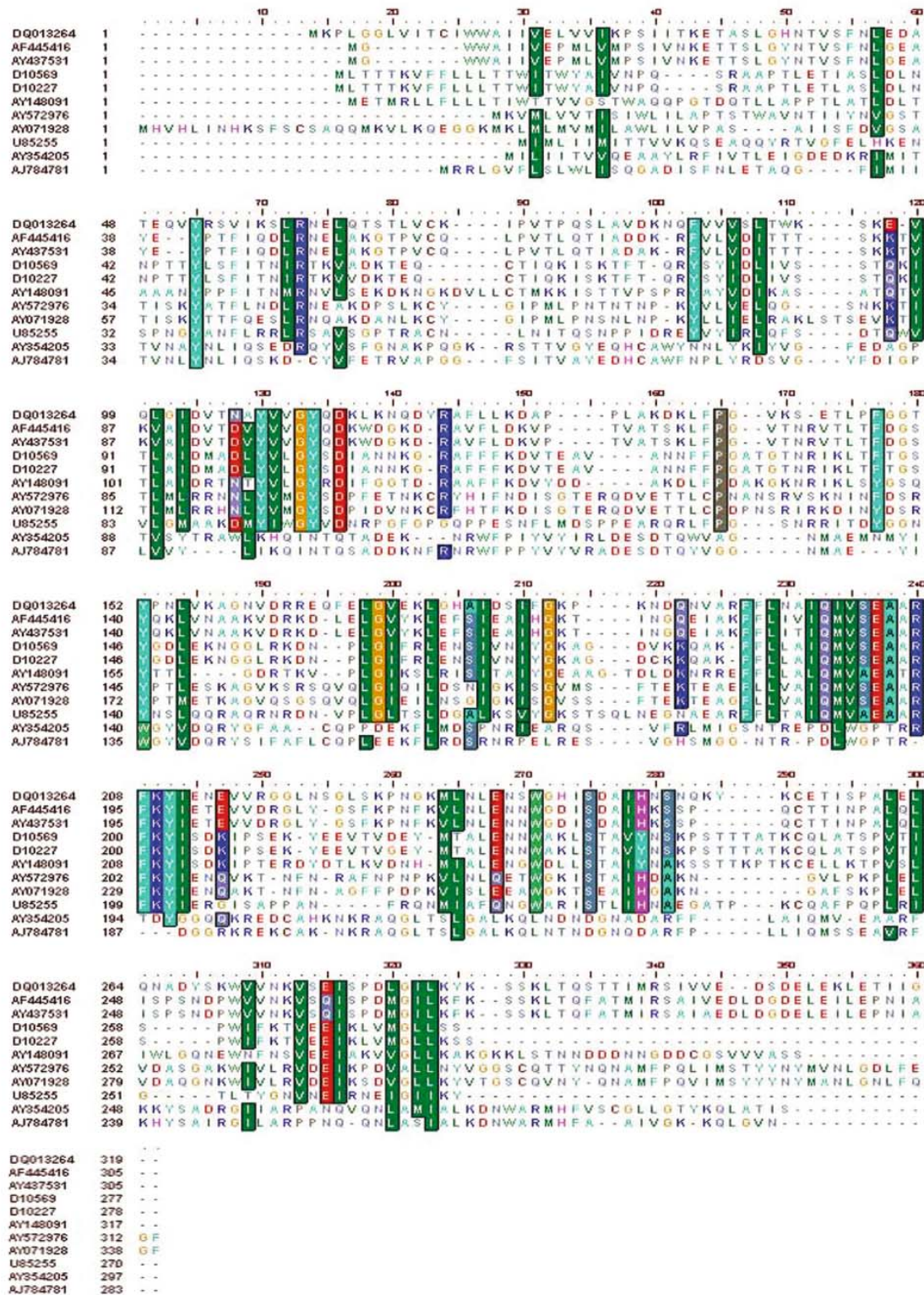
Figure 2. ClustalW alignment of the deduced amino acid sequence of BBAP1 with other RIPs/AVPs. GenBank accession numbers of RIPs used for analysis are as follows: BBAP1 (DQ013264); bouganin (AF445416); Ap1 (AY437531); MAP (D10569, D10227); ME1 (AY148091); PAP (AY572976); PAP1 (AY071928); amaranthin (U85255); AAP1 (AY354205) and CCP-27 (AJ784781). Identical residues are highlighted in boxes.

bacterial cells. Similar results were also obtained in the case of expression of recombinant AVP/RIP from pre-flowering stage *Celosia cristata* (Gholizadeh *et al* 2005) and ME1 RIP from *Mirabilis expansa* (Vepachedu *et al* 2005).

3.5 *Western blotting of expressed fusion protein*

Total protein from uninduced and induced *E. coli* TB1 cells containing recombinant plasmid pMAL-c2X and from control cells, i.e. TB1 cells with non-recombinant plasmid (with and without IPTG), was extracted and analysed on 12% SDS-PAGE (results not shown). A band corresponding to ~78 kDa was found only in the induced cells containing recombinant plasmid. This band consisted of maltose-binding protein (MBP) fused to the expressed AVP BBAP1. To confirm the presence of a fusion protein, the protein extracted from induced recombinant cells was subjected to western blot analysis. As shown in figure 4, a prominent protein band corresponding to ~78 kDa was detected, thereby confirming that the expressed protein is a fusion product (lanes 1 and 2). In addition, a faint protein band of MBP (~42.5 kDa) was detected, which might have been released due to partial degradation of the fusion protein.

3.6 *Purification of expressed BBAP1*

As shown in figure 5, the affinity chromatography-purified protein showed a single band of ~78 kDa (lane 1), confirming that it was a fusion protein. The fusion protein cleaved with factor Xa showed two bands (lane 2), indicating the presence of MBP (~42.5 kDa) and recombinant BBAP1 (~35 kDa). Further, on gel filtration using Sephadex G-100, a single band corresponding to ~35 kDa (lane 3) was obtained. Theoretically, the ORF-deduced protein was also found to be 35.49 kDa, confirming the purity of the expressed recombinant BBAP1. Earlier in our laboratory, the purified native BBAP1 was found to be of ~33 kDa (Bhatia *et al* 2004). Thus, the expressed BBAP1 (~35 kDa) is larger than the native protein. As discussed earlier (section 3.3), this might be due to the presence of a signal peptide. Similar results have been reported for MAP (Kataoka *et al* 1991), PAP II (Poyet *et al* 1994), bouganin (Hartog *et al* 2002) and *Celosia cristata* AVP, CCP-27 (Begam *et al* 2006).

3.7 N-*glycosidase activity*

Most of the AVPs are known to possess ribosome-inhibiting *N*-glycosidase activity and are therefore known as ribosome-inactivating proteins (RIPs). As shown in figure 6, tobacco ribosomes treated with recombinant BBAP1 released a fragment of rRNA, which was observed in the presence of

acidic aniline treatment (lane 1). A positive control, in which saporin RIP was used, also showed such a released fragment (lane 4). The released fragment is diagnostic of an RIP. However, in the case of negative controls (without aniline treatment), no such released fragment was observed when rRNA was treated with BBAP1 or saporin (lanes 2 and 3). Due to *N*-glycosidase activity, the position of the 25S rRNA also shifted and its mobility became slightly faster (figure 6, lanes 1, 4) than that of the respective controls (lanes 2, 3). These results corroborate well with most of the other results reported for RIPs/AVPs from different plant species (Honjo *et al* 2002; Park *et al* 2004; Begam *et al* 2006). The faint band observed in case of the positive control (lane 4) may be due to the low *N*-glycosidase activity of saporin.

3.8 *Antiviral activity of recombinant BBAP1*

The purified recombinant BBAP1 was tested for its antiviral activity using local lesion assay wherein tobacco (*Nicotiana glutinosa*) plants were infected with TMV (figure 7). The average number of lesions per leaf in case of BBAP1-treated plants (50 μg) was only 2 as against 49 lesions in control plants; thus, ~94% inhibition was exhibited. A similar level of inhibition was observed when BBAP1 was tested against sunnhemp rosette virus (Choudhary *et al* 2007). These results are in conformity with our earlier results obtained with native bougainvillea AVPs (Narwal *et al* 2001a; Bhatia *et al* 2004).

In addition to having broad-spectrum antiviral activity, a few RIPs have been shown to have antifungal and antibacterial activity, including some plant pathogenic microorganisms. Thus, such RIPs have a potential to be used as biopesticides. Two root-specific RIPs of the Andean crop *Mirabilis expansa* have been found to be active against a number of plant pathogens, including root-rot fungi and bacteria (Vivanco *et al* 1999; Park *et al* 2002). More recently, an RIP isolated from tobacco leaves has been shown to inhibit fungal as well as bacterial growth (Sharma *et al* 2004). Previous studies also have shown that expression of PAP mutants in transgenic tobacco plants leads to resistance against a broad spectrum of viruses and fungal pathogens (Lodge *et al* 1993; Zoubenko *et al* 1997). Guo *et al* (1999) transformed tomato cultivars with cDNA encoding trichosanthin (TCS), and the transgenic plants showed resistance against tomato mosaic virus and cucumber mosaic virus.

The present study and earlier results (Choudhary *et al* 2007) suggest that BBAP1 is a multifunctional protein having antiviral activity. One way of utilizing the cloned gene encoding BBAP1 is to develop transgenic crop plants with resistance against plant pathogens, including viruses. Alternatively, the gene can be expressed in a prokaryotic system and the expressed recombinant AVP could be used as
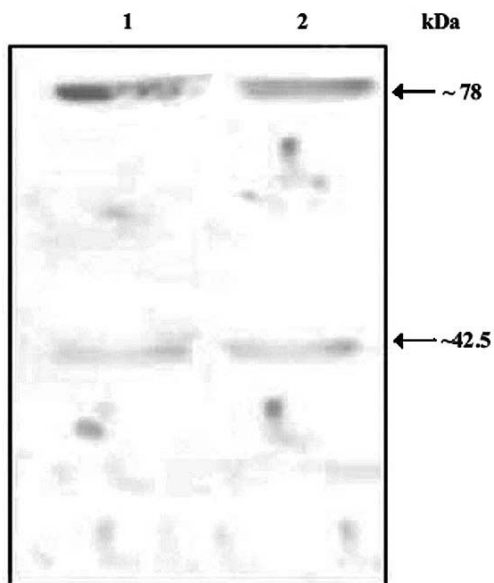
a biopesticide for spraying on the leaves of crops to prevent or control infection due to pathogens. However, further studies on BBAP1 are required for a better understanding
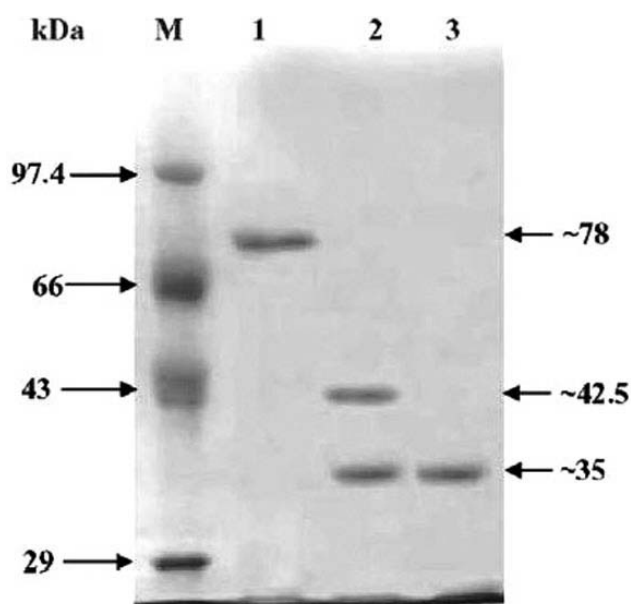
of the mechanism of its antiviral/antimicrobial action, which should facilitate the development of biopesticides and virus-resistant plants.
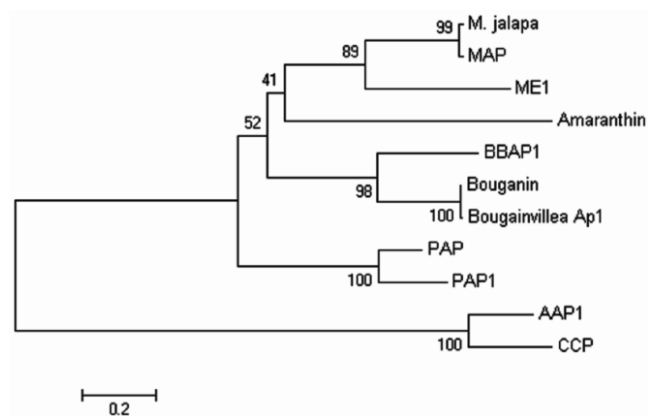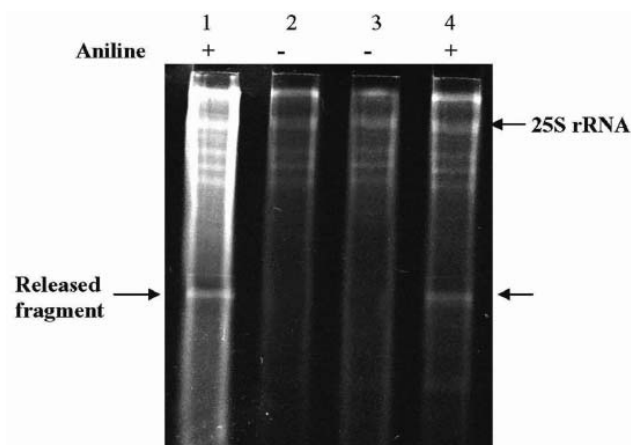

Figure 3. Phylogenetic relationship of BBAP1 to different RIPs/AVPs. The amino acid sequences of BBAP1 (DQ013264); bouganin (AF445416); Ap1 (AY437531); PAP (AY572976); PAP1 (AY071928); M. jalapa (D10227); MAP (D10569); ME1 (AY148091); amaranthin, (U85255); AAP1 (AY354205) and CCP-27 (AJ784781) were used. Values given on the nodes are bootstrap values of replications.


Figure 5. SDS-PAGE analysis of the purified expressed protein BBAP1. Lane 1: affinity chromatography purified fusion protein; lane 2: fusion protein on cleavage with factor Xa; lane 3: purified protein after gel filtration; and lane M: molecular weight marker.

Figure 4. Western blot analysis of fusion protein (lanes 1 and 2) using antimaltose-binding protein as the primary antibody and rabbit antiglutathione-S-transferase conjugated with horseradish peroxidase as the universal conjugate. *E. coli* expressed BBAP1 protein (20 μg) fractionated on 12% SDS-PAGE and electroblotted onto a nitrocellulose membrane. One prominent band of ~78 kDa for the fusion protein and another faint band of ~42.5 kDa for maltose-binding protein (MBP) were detected. MBP might have been released due to partial degradation of the fusion protein. Non-specific signal may be due to further degradation of fusion protein.

Figure 6. *N*-glycosidase activity of expressed BBAP1. Recombinant BBAP1 (2 μg) tested for its *N*-glycosidase activity on rRNA of ribosomes (10 μg) isolated from tobacco leaves. Lane 1: rRNA treated with BBAP1 (+); lane 2: rRNA treated with BBAP1 (−); lane 3: rRNA treated with saporin RIP (−); and lane 4: rRNA treated with saporin RIP (+). '+' and '−' signs indicate, respectively, with and without aniline treatment of rRNA. Saporin RIP (+) served as the positive control, while treatment without aniline was the negative control.
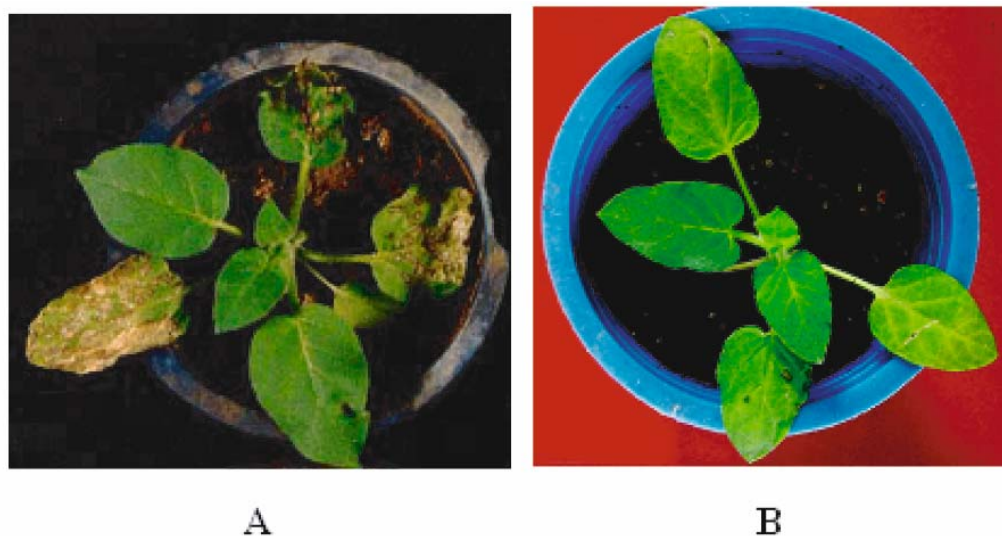
Figure 7. Antiviral bioassay of purified recombinant protein BBAP1 by a local lesion test using tobacco (*Nicotiana glutinosa*) as the test plant and tobacco mosaic virus (TMV) for infection. (**A**) Control: Plant treated with buffer only and then infected with the virus. (**B**) Plant treated with recombinant protein BBAP1 (50 μg leaf^{-1}) dissolved in the buffer, and subsequently infected with the virus as explained in Materials and methods.

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