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Molecular cloning and functional identification of a ribosome inactivating/antiviral protein from leaves of post-flowering stage of *Celosia cristata* and its expression in *E. coli*

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Abstract

A full-length cDNA clone, encoding a ribosome inactivating/antiviral protein (RIP/AVP) was isolated from the cDNA library of post-flowering stage of *Celosia cristata* leaves. The full-length cDNA consisted of 1015 nucleotides, with an open reading frame encoding 283 amino acids. The deduced amino acid sequence had a putative active site domain conserved in other ribosome inactivating/antiviral proteins (RIPs/AVPs). The coding region of the cDNA was amplified by polymerase chain reaction (PCR), cloned and expressed in *Escherichia coli* as recombinant protein of 72 kDa. The expressed fusion product was confirmed by Western analysis and purification by affinity chromatography. Both the recombinant protein (reCCP-27) and purified expressed protein (eCCP-27) inhibited translation in rabbit reticulocytes showing IC_{50} values at 95 ng and 45 ng, respectively. The native purified nCCP-27 has IC_{50} at 25 ng. The purified product also showed *N*-glycosidase activity towards tobacco ribosomes and antiviral activity towards tobacco mosaic virus (TMV) and sunnhemp rosette virus (SRV).

Keywords: Antiviral; cDNA; *Celosia cristata*; Expression; Ribosome inactivating

1. Introduction

Antiviral proteins also referred as ribosome inactivating proteins (RIPs/AVPs) are an extended, fairly heterogeneous group of plant proteins which confer resistance against different viruses when applied exogenously or expressed in transgenic lines (Barbieri et al., 1993; Nielsen and Boston, 2001; Girbes et al., 2004; Stirpe, 2004; Iglesias et al., 2005). These have been identified in a number of plant species viz. *Phytolacca americana* (Lin et al., 1991), *Mirabilis jalapa* (Kataoka et al., 1991), *Dianthus caryophyllus* (Legname et al., 1991), *Clerodendrum aculeatum* (Kumar et al., 1997), *Trichosanthes kirilowii* (Guo et al., 1999), *Amaranthus viridis* (Kwon et al., 2000), *Celosia cristata* (Balasubrahmanyam et al., 2000), *Bougainvillea spectabilis* (Hartog et al., 2002), edible plants (Barbieri et al., 2006). However, their antiviral mechanism has not been precisely understood. The most widely accepted mechanism of action of RIPs is the inhibition of protein synthesis through inactivation of host ribosomes because of their RNA *N*-glycosidase activity (Van Damme et al., 2001; Hartley and Lord, 2004a,b; Stirpe, 2004). Most of the other discovered enzymic activities of RIPs are DNase activity on supercoiled DNA (Ling et al., 1994; Begam et al., 2006), RNase activity (Hudak et al., 2000; Begam et al., 2006) and depurination of capped mRNAs (Hudak et al., 2002). Recently, some RIPs have also been reported to exhibit super oxide dismutase (SOD) activity (Li et al., 1997; Sharma et al., 2004), phospholipase activity (Helmy et al., 1999) and antioxidant activity (Gholizadeh et al., 2004). However, much remains to be known about the mechanism through which these RIPs/AVPs operate to inhibit virus infection (Nielsen and Boston, 2001; Wang

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and Hudak, 2003; Park et al., 2004). RIPs/AVPs encoding genes, may be a potential candidates for developing virus resistant economically important transgenic plants (Hong et al., 1996; Tumer et al., 1997; Wang et al., 1998; Guo et al., 1999; Zoubenko et al., 2000; Vandenbussche et al., 2004). Recently these have also aroused considerable interest due to their therapeutic value as immunotoxins (Stirpe et al., 1992; Girbes et al., 2004).

Celosia cristata, an ornamental plant of *Amaranthaceae* family is one of the potential candidates for the presence of RIP/AVP. Its leaf extract, as well as two growth stage dependent purified antiviral proteins (CCP-25 and CCP-27), have been shown to inhibit lesion formation caused by virus infection (Baranwal and Verma, 1992; Balasubrahmanyam et al., 2000). CCP-25 is predominant at its pre-flowering stage while, CCP-27 is predominant at its post-flowering stage. Baranwal et al. (2002) reported the depurination of ribosomal RNA and inhibition of viral RNA translation by CCP-25. Cloning and characterization of a cDNA encoding cysteine-proteinase inhibitor (Celostatin) from pre-flowering stage of *C. cristata* has also been reported (Gholizadeh et al., 2005). In this report, we describe the cloning and characterization of a RIP/AVP gene from leaves of *C. cristata* at its post-flowering stage and its expression in *Escherichia coli*.

2. Results and discussion

2.1. Screening of C. cristata leaf cDNA library

The cDNA library was screened with 42 bases long synthetic degenerate oligonucleotide probe. In primary screening, 10 putative positive plaques were selected. From secondary screening, three well-isolate positive plaques were picked up and excised in *E. coli* BM25.8 strain. On Southern analysis, only one insert of ~1.0 kb was lighted up. The entire sequence and deduced amino acid sequence of this cDNA has been submitted to GenBank with Accession No. AJ784781. The largest ORF of 849 bases was present in the third reading frame, which encodes a polypeptide of 283 amino acid residues. It has translation initiation codon (ATG) at position 56 down stream of the first base and terminates by stop codon TAA located after 905 nucleotides. Additionally, a polyadenylation signal (AATAA), located after position 945 and a poly (A) tail of 30 adenine nucleotides was also detected. The 3′ UTR was found to be 110 base pair long. Pair wise matrices, comparing amino acid sequences of RIPs/AVPs from a series of plant species, including *C. cristata* (AJ784781) is shown in Fig. 1. The upper matrix specifies identity values between the sequences, while the lower matrix is a comparison of the similarity values between those sequences. MatGAT (Campanella et al., 2003) was employed to generate this matrix. AAP-1 (AY354205), a RIP/AVP from *Amaranthus tricolor* showed maximum similarity of 69% and maximum identity of 59% with the isolated cDNA. Similarity values are supposed to be higher than those of identity as they additionally include similar amino acids as e.g. aromatic or hydrophobic amino acids. In addition, this protein also shows strong similarity to a number of RIPs/AVPs as regards to its conserved region (EAAR) which, is also the active site of the proteins (Fig. 2). RIP/AVP from *A. tricolor* is incidentally a close relative of *C. cristata*. CCP-27 (AJ784781) and AAP1 (AY354205) thus can be grouped together. The translated amino acid sequence also contains the same amino acid sequence (from position 16 to 27) as that obtained by N-terminal sequencing of the

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.
1. AJ784781	----	**18.8**	**18.3**	**16.9**	**18.6**	**17.3**	**59.1**	**21.5**	**30.9**	**18.1**	**16.9**	**19.9**	**18.1**	**29.1**
2. AY148091	**37.5**	-----	22.1	26.3	24.8	25.7	21.6	28.3	24.9	22.5	26.3	24.4	29.5	30.5
3. AF219236	**35.5**	**40.9**	-----	**23.6**	**56.6**	**27.2**	**15.7**	**27.6**	**19.4**	**22.9**	**23.6**	**89.9**	**30.8**	**23.2**
4. AF219237	**33.9**	46.7	44.0	-----	27.0	27.1	18.1	31.3	21.2	67.1	100.0	25.2	28.0	24.9
5. S57638	**37.5**	**44.3**	**66.0**	**47.0**	-----	**26.6**	**17.7**	**32.0**	**20.5**	**24.7**	**27.0**	**61.9**	**29.0**	**24.1**
6. AY071928	**33.7**	44.1	46.7	43.8	43.5	-----	18.8	32.9	19.7	26.5	27.1	27.0	59.8	23
7. AY354205	**69.0**	**38.4**	**32.7**	**36.0**	**35.4**	**36.4**	-----	**17.8**	**40.0**	**16.3**	**18.1**	**16.2**	**15.2**	**38.7**
8. AF230812	**38.5**	47.4	46.2	53.8	49.5	49.1	37.7	-----	25.3	26.9	31.8	28.6	35.5	28.3
9. U70215	**43.8**	**41.6**	**38.4**	**38.4**	**42.7**	**33.4**	**52.2**	**46.2**	-----	**19.5**	**20.8**	**21.9**	**21.2**	**80.6**
10. AF000937	**31.6**	42.6	42.8	79.4	43.6	44.4	34.7	47.1	33.7	-----	67.1	23.4	27.2	31.8
11. AF004389	**33.9**	**46.7**	**44.0**	**100.0**	**47.0**	**43.8**	**36.0**	**54.5**	**38.7**	**79.4**	-----	**25.2**	**28.0**	**24.9**
12. AF219238	**38.4**	44.6	91.2	48.3	72.1	45.9	33.3	48.6	41.8	44.6	48.3	-----	30.3	25.0
13. AF141331	**35.2**	**47.9**	**53.8**	**47.3**	**46.3**	**74.6**	**35.2**	**52.1**	**37.5**	**47.3**	**47.3**	**52.1**	-----	**25.2**
14. U85255	**45.9**	49.5	43.4	44.1	47.4	39.9	53.2	51.3	85.2	46.4	44.1	46.6	42.9	-----
15. AF228508	**40.6**	**48.1**	**45.6**	**54.8**	**49.1**	**48.2**	**41.4**	**96.1**	**43.4**	**47.8**	**54.8**	**48.0**	**51.1**	**49.1**

Fig. 1. Pair wise matrices comparing amino acid sequences of antiviral/ribosome inactivating proteins from a series of plant species, including *Celosia cristata* (AJ784781). The upper matrix specifies identity values between the sequences, while the lower matrix is a comparison of the similarity values between those sequences. MatGAT (Campanella et al., 2003) was employed to generate matrix. Amarandin-2 (AF004389); Amarandin-1 (AF000937); *Phytolacca insularis*, PIP2 (AF141331); *Chenopodium album*, CAP30A (AF228508); *Chenopodium album*, CAP30B (AF230812); *Dianthus chinensis*, protein 3-precursor (AF219238); *Dianthus chinensis*, Protein 2-precursor (AF219237); *Dianthus chinensis* protein 1-precursor, Rip 1 (AF219236); *Amaranthus tricolor* (AY354205); *Mirabilis expansa*, ME1 (AY148091); *Phytolacca americana* type I (AY07192); Amarandin S (U70215); *Amaranthus viridis* (U85255); Saporin-6 (S57638).

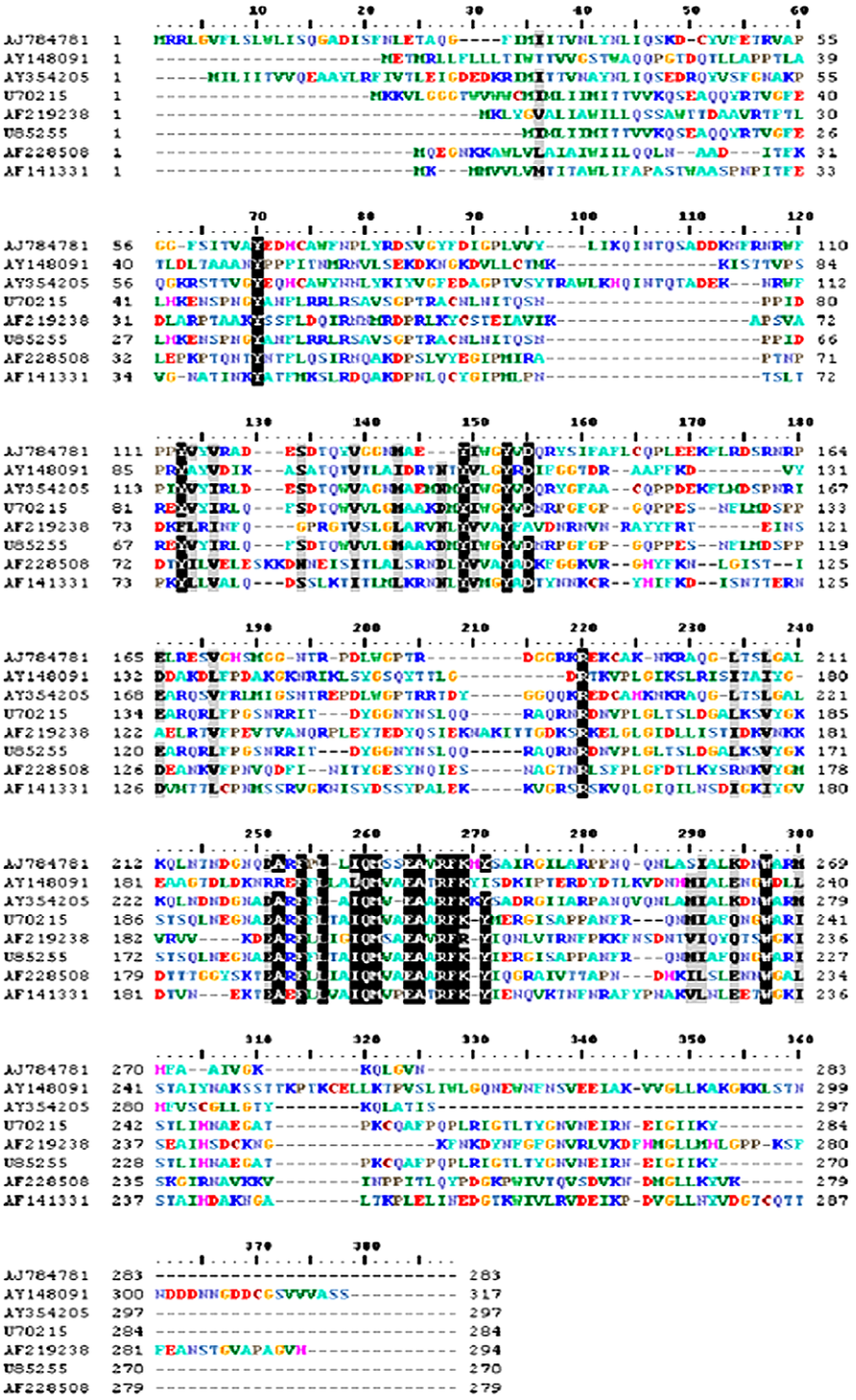

Fig. 2. Clustal W alignment of *Celosia cristata* RIP/AVP with various RIPs/AVPs. This protein shows strong similarity to different RIPs/AVPs, as regards to its conserved region (EAAR), the active site of the proteins. The gene names corresponding to different accession numbers are given in the caption of Fig. 1.

purified native (nCCP-27). However, it does not match from the start amino acid (M) to 15th residue (Q). Thus, it is predicted that it might possess a signal peptide sequence, which has undergone post-translational modification before its transportation to target site. Several other RIPs/AVPs have also been reported to have signal peptide at N-terminus. For example, Lin et al. (1991) showed that N-terminal of PAP contains 22 amino acids, which are absent in mature protein. Tricohanguin, a RIP from *Trichosanthes anguina* has a signal peptide of 19 residues (Chow

et al., 1999). Cho et al. (2000) predicted that *Dianthus sinensis* RIP cDNA might have a putative signal peptide of 23 amino acid residues as their N-terminus. The analysis of the amino acid composition of the predicted protein performed using the ProtParam tool of ExPasy revealed the number of amino acid residues to be 283. The amino acid composition reveals that it is rich in leucine (8.83%), followed by glycine and arginine (7.77% each). The total number of negatively charged residues (Asp + Glu) was found to be 27 and total number of positively charged residues (Arg + Lys + His) is 39. Thus, overall it shows its basic nature. This protein has an isoelectric point (p*I*) of 9.02, which further confirms its basic nature like many other RIPs/AVPs (Barbieri et al., 1982; Takanami et al., 1990). The molecular weight of the protein was predicted to be 30 kDa. This is little higher than the actual observed value of 27 kDa of purified nCCP-27. It is expected, as most of the reported RIPs/AVPs undergo post-translational modifications after their synthesis (Lin et al., 1991; Chow et al., 1999; Cho et al., 2000).

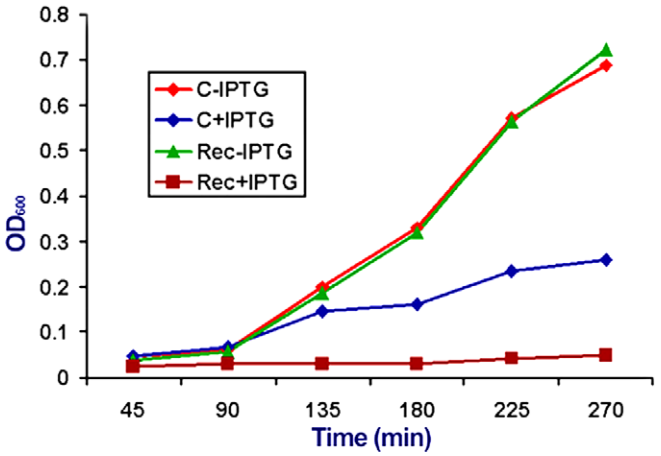

Fig. 3. Growth curves of *E. coli* TB1 control and *Celosia* RIP-expressing cells. *E. coli* was grown in LB media containing ampicillin 100 μg ml^{-1} at 37 °C. A pre-culture was used to initiate a culture in LB medium containing 0.3 mM IPTG. The OD_{600} of the culture was measured every 45 min. The bacterial cells with non-recombinant vector showed normal growth even after IPTG induction, while the RIP gene containing cells grew very slowly after IPTG induction.

2.2. Expression of the cDNA in E. coli

The coding region of the cDNA clone was amplified by PCR and cloned directionally in pMAL-c2X *E. coli* expression vector. In order to express the cloned gene, TB1 cells were grown under inducible conditions. A pre-culture was used to initiate a culture in LB medium containing 0.3 mM IPTG. The OD_{600} of the culture was measured every 45 min. It was interesting to note that the bacterial cells with non-recombinant vector showed normal growth while, those containing the RIP gene grew very slowly after IPTG induction (Fig. 3). This growth inhibition of TB1 cells indirectly indicated that the translational product of the cloned RIP gene has a toxic effect on bacteria, and that a functional product is produced in TB1 cells. Similar results were also obtained in case of expression of RIPs from *M. jalapa* (Kataoka et al., 1991), *P. americana* (Chen et al., 1993), *D. sinensis* (Cho et al., 2000), *A. viridis* (Kwon et al., 2000) and *Beta vulgaris* (Iglesias et al., 2005) in *E. coli* cells under inducible conditions. All these studies showed that the transformants with RIP gene were unable to grow upon

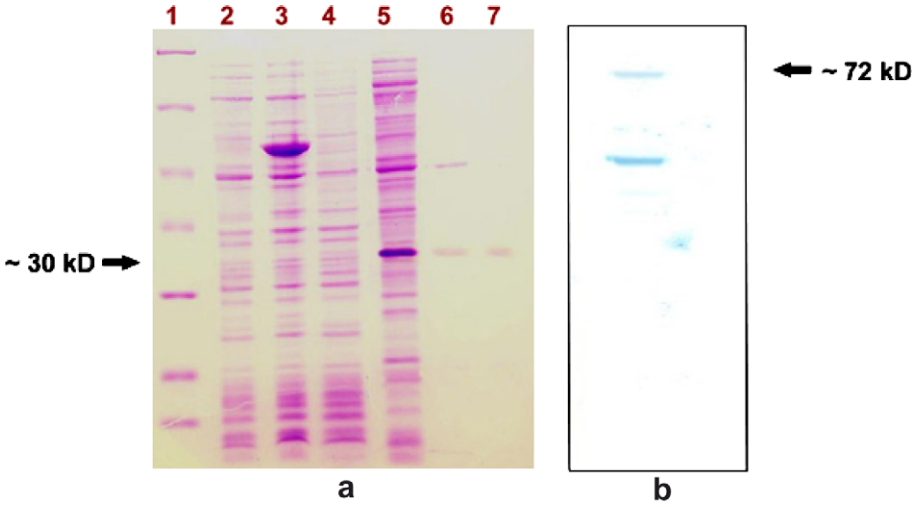

Fig. 4. (a) SDS–PAGE analysis of induced recombinant protein. Lane 1: protein molecular weight marker; lane 2: TB1 transformed with expression vector (−IPTG); lane 3: TB1 transformed with expression vector (+IPTG); lane 4: TB1 transformed with recombinant expression vector (−IPTG); lane 5: TB1 transformed with recombinant expression vector (+IPTG); lane 6: purified fusion protein after treatment with factor Xa; and lane 7: purified 30 kDa expressed protein. (b) Western blot analysis of the recombinant protein using anti-Maltose-Binding Protein as primary antibody and alkaline phosphatase conjugated Goat-Anti Rabbit (GAR^{XAP}) as universal conjugate. *E. coli* cells were grown under inducible condition with IPTG and total proteins were extracted by sonication. 10–15 μg of total protein sample was fractionated on 12% SDS–PAGE and blotted as indicated in the materials and methods. Two bands were detected on the membrane, ~72 kDa band for recombinant fusion protein and ~ 42 kDa band for maltose binding protein released by partial degradation of the recombinant fusion protein.

IPTG induction, suggesting that the expression of RIP gene renders cytotoxicity to *E. coli*. Total protein from uninduced, induced TB1 with recombinant plasmid and also from control cells (TB1 with non-recombinant plasmid) were extracted from the culture of bacterial cells and analyzed on 12% SDS–PAGE (Fig. 4a). A band corresponding to ~72 kDa, consisting of the maltose-binding protein with molecular weight of 42 kDa fused to the product of 283 amino acid residues of cDNA encoding region was found in the gel. In addition, there were two more induced bands corresponding to ~42 kDa and ~30 kDa, respectively. It may be due to partial degradation of recombinant protein. To confirm the presence of the fused recombinant protein, the sample from recombinant cells (transformed with plasmid containing RIP cDNA) was subjected to Western analysis using anti-MBP as primary antibody. A protein band corresponding to ~72 kDa was detected on the membrane (Fig. 4b). At the same time another band of ~42 kDa was also detected which might be the maltose-binding protein released by the degradation of the recombinant protein. The recombinant protein was purified by maltose-affinity chromatography. The purity of the sample was tested by SDS–PAGE analysis and the fused protein was cleaved with factor Xa to separate the protein encoded by cloned cDNA from the maltose-binding protein (Fig. 4a, lane 6). The cleaved proteins were separated by gel filtration chromatography on Superose-12 column (Fig. 4a, lane 7).

2.3. Translation inhibition of rabbit reticulocytes

Inhibition of protein synthesis by native (nCCP-27), expressed purified (eCCP-27) and recombinant (reCCP-27) was compared, using rabbit reticulocyte system. Rabbit reticulocytes were incubated with increasing concentrations of these protein preparations (10–1000 ng) each for 30 min at 30 °C before the translation studies. Untreated rabbit reticulocytes served as negative control. CCP-25 was taken as a positive control (Baranwal et al., 2002). The IC_{50} for the nCCP-27 was found to be 25 ng while higher values were shown by eCCP-27 and reCCP-27, being 45 ng and 95 ng, respectively. The recombinant protein and expressed purified protein were found to be ~75% and ~50% less efficient, than the native purified protein, respectively. The inhibitory activity of a RIP may vary with the structural confirmation of the isolated RIP. The reCCP-27 has a fused maltose binding protein while; the expressed purified CCP-27 has an extra signal peptide sequence at its N-terminal end. This indicated that the cDNA expressed CCP-27 products (fused and Xa-cleaved) need further post-translational modifications to give optimum levels of translational inhibition. However, the results clearly suggest the RIP-like property of expressed CCP-27 gene products. CCP-25, the positive control showed almost the same inhibitory effect as that observed with nCCP-27 indicating that both the growth dependent proteins have the same translational inhibitory activities. The IC_{50} value for tobacco ribosome inactivating protein (TRIP) was found to be 30 ng for the wheat germ and 100 ng for rabbit reticulocytes (Sharma et al., 2004). Studies of inhibition by BE27 (sugarbeet RIP) of protein synthesis indicated that the most sensitive cell-free system was that of rabbit reticulocyte lysate ($IC_{50} \sim 1.15$ ng ml^{-1}) while for *Vicia sativa* L. and *Triticum aestivum* L. cell-free systems IC_{50} were 68 and 1318 ng ml^{-1}, respectively (Iglesias et al., 2005).

2.4. N-Glycosidase activity of protein expressed in E. coli

The ribosome inactivating activity of the purified protein was tested by its *N*-glycosidase activity towards tobacco rRNA. Results depicted in Fig. 5, clearly show that aniline treatment of rRNA extracted from expressed protein treated tobacco ribosomes resulted in generation of a specific RNA fragment due to its *N*-glycosidase activity. On the contrary, the negative control, without AVP treatment showed no such released fragment. These results corroborate well with most of the other reported RIPs/AVPs from different plant species. The antiviral proteins isolated from *D. caryophyllus* (Stirpe et al., 1981), *P. americana* (Taylor and Irvin, 1990), *M. jalapa* (Kataoka et al., 1991), *B. spectabilis* (Bolongnesi et al., 1990), *Bougainvillea xbuttiana* (Narwal et al., 2001), all have been reported to have ribosome inactivating property. The ribosome inactivating property of RIPs has been held responsible for inhibition of protein synthesis in eukaryotes.

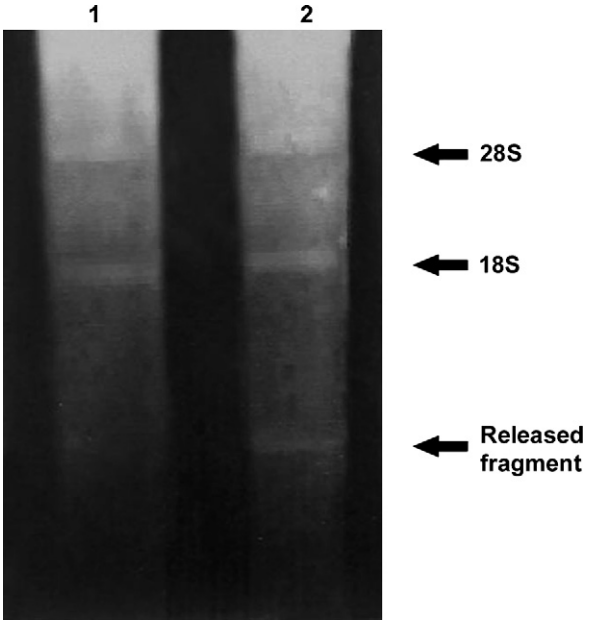

Fig. 5. *N*-Glycosidase activity of purified expressed protein on tobacco rRNA. Lane 1: control rRNA, without protein treatment showed no released fragment; lane 2: rRNA treated with 2 μg of expressed protein resulted in generation of specific RNA fragments due to their *N*-glycosidase activity.

2.5. Antiviral activity of expressed protein

Antiviral property of the purified protein expressed in *E. coli* cells was tested using local lesion assay by infecting tobacco (*Nicotiana glutinosa*) leaves with tobacco mosaic virus (TMV) and guar (*Cyamopsis tetragonoloba*) leaves with sunnhemp rosette virus (SRV) (Fig. 6a and b, respectively). It showed 95% reduction of local lesion in both systems when applied at a concentration of as low as 100 μg ml^{-1}.

These observations, therefore, propose that CCP-27 is a multifunctional protein. It may act as a RIP-like protein under certain conditions and thus indirectly prevent the multiplication/replication phase of the virus infection process.

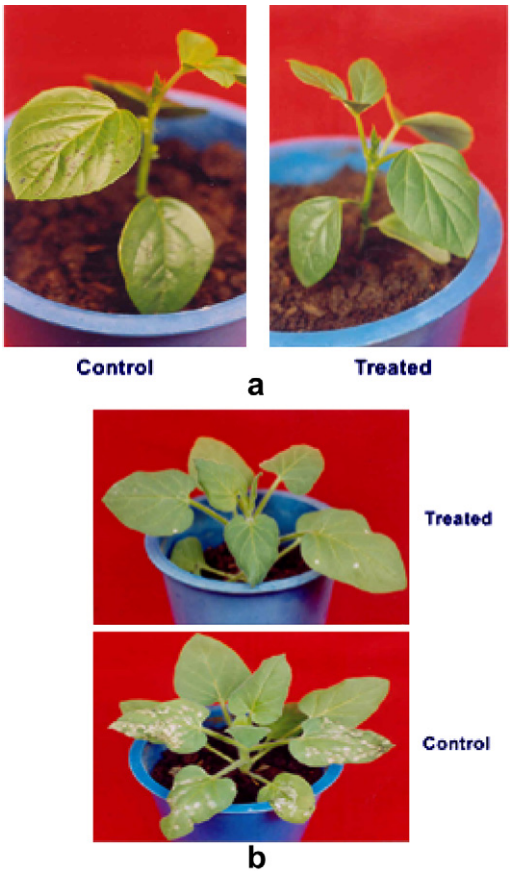

Fig. 6. (a) Antiviral bioassay of purified expressed protein by a local lesion test using *Cyamopsis tetragonoloba* as test host plant and sunnhemp rosette virus for infection. Percentage of lesion inhibition was calculated by the formula described in materials and methods. Average number of lesions in control plants (plants treated with crude extract of protein isolated from *E. coli* cells containing non-recombinant plasmid) = 165. Average number of lesions in treated plants (plants treated with purified protein) = 9. Percent inhibition = 94.5. (b) Antiviral bioassay of purified expressed protein by a local lesion test using *Nicotiana glutinosa* as test host plant and tobacco mosaic virus for infection. Percentage of lesion inhibition was calculated by the formula described in materials and methods. Average number of lesions in control plants (plants treated with crude extract of protein isolated from *E. coli* cells containing non-recombinant plasmid) = 128. Average number of lesions in treated plants (plants treated with purified protein) = 6. Percent inhibition = 95.3.

3. Experimental

3.1. Plant materials and bacterial strains

Celosia cristata (non-host plant), *N. glutinosa* (host plant) and *C. tetragonoloba* (host plant) were grown in the green house. *E. coli* strains, BM25.8 and TB1 were used for cloning and expression of the cDNA, respectively. Tobacco mosaic virus and sunnhemp rosette virus inoculums were obtained from tobacco and sunnhemp leaves, respectively, and used for local lesion assay.

3.2. cDNA library construction

Leaves at post-flowering stage of *C. cristata* were used for isolation of total RNA, using guanidium thiocyanate reagent as described by Ausubel et al. (1999). Full-length cDNA library was constructed in λTriplEx2 vector using SMART cDNA library construction kit (CLONTECH, Cat. No. 1015-1). The leaf cDNA library was made with unamplified and amplified titres of 2×10^5 and 1.3×10^8 pfu/ml, respectively, with about 90% recombinants.

3.3. Designing of heterologous probe for screening of cDNA library

Homology search data of protein sequences of different RIPs/AVPs show a conserved region common to all. This conserved protein sequence is also the functionally active sequence of the RIPs/AVPs. Glutamic acid, arginine and alanine are the conserved amino acids, which are involved in the active site of RIPs/AVPs in EAAR order. A synthetic oligonucleotide of 42 bases long corresponding to this RIP/AVP active site was used as a heterologous probe to isolate the RIP/AVP encoding gene of *C. cristata*.

3.4. Isolation of cDNA clone

The cDNA library (approximately 1×10^5 pfu) was probed with [γ-^{32}P]-ATP end-labelled 42 bases synthetic degenerate oligonucleotide designed, based on conserved active site of the RIPs/AVPs. The positive plasmids were isolated by alkaline hydrolysis method described by Ahn et al. (2000). The insert fragment was further confirmed by Southern analysis according to standard protocol (Ausubel et al., 1999). The isolated cDNA clone was completely sequenced by automated sequencer at South Campus, University of Delhi, India.

3.5. Expression of C. cristata RIP cDNA in E. coli

For expression of RIP cDNA, the coding region of the isolated clone was amplified by polymerase chain reaction (PCR) using two specific oligonucleotides, 5′GAATTCATGCGTCGTTTAGGT3′ and 5′CTGCAGTTAGTTGACACCTAA3′ as the forward and reverse primers, respectively. In order to do directional cloning in pMAL-

c2X expression vector, *Eco*RI and *Pst*I restriction sites were designed at the 5′ ends of the forward and reverse primers, respectively. PCR was conducted using recombinant pTriplEx2 DNA as template (94 °C, 1 min; 55 °C, 1 min; 72 °C, 2 min). The amplified fragment was digested with *Eco*RI and *Pst*I and cloned into pMAL-c2X *E. coli* expression vector using pMAL-c2X protein fusion and purification system kit (Cat. No. E8000S; NEW ENGLAND, Biolab). The *E. coli* cells (strain TB1) were transformed with the expression vector. A single colony harboring the recombinant plasmid was used for expression. The overnight culture was then inoculated into 50 ml of Lauria Broth containing ampicillin (100 μg ml^{-1}). IPTG (0.3 mM) was added to the culture at 0.3 of A_{600}. *E. coli* growth was monitored by measuring A_{600}. Total soluble protein was extracted by sonication as described by manufacturer. Total protein fractions were then subjected to SDS-polyacrylamide gel electrophoresis analysis on 12% running gel (Laemmli, 1970).

3.6. Western analysis

To confirm the expressed recombinant product, Western blot analysis was conducted on total protein extract of induced sample using anti-MBP as primary antibody and alkaline phosphatase conjugated goat-anti rabbit (GAR^{XAP}) as universal conjugate. *E. coli* cells were grown under inducible condition with IPTG and total proteins were extracted by sonication. About 10–15 μg of total protein sample was fractionated on 12% SDS–PAGE and transferred onto nitrocellulose membrane using blotting buffer containing 48 mM Tris–Cl (pH 8.3), 39 mM glycine, 0.037% SDS and 20% methanol. After transfer, the blot was kept in blocking solution (20 mM Tris–Cl, 150 mM NaCl, 5% BSA and 0.1% Tween-20) overnight at 4 °C and then incubated for 2 h with specific antiserum (anti-MBP) at 1:500 ratios in the same buffer at 37 °C for 1 h. After washing with TBS-T (TBS + 0.1% Tween-20) the membrane was incubated for 1 h with alkaline phosphatase conjugated goat-anti rabbit (GAR^{XAP}) at 1:20,000 dilution in TBS-BSA buffer. After exclusive washing, the signal band was visualized with a substrate solution containing nitroblue tetrazolium chloride (NBT, 0.33 mg/ml) and 5-bromo-1-chloro-indolyl phosphate (BCIP, 0.165 mg/ml) in 0.1 M Tris–Cl buffer containing 0.1 M NaCl and 5 mM $MgCl_2$.

3.7. Purification of recombinant protein

The fusion protein was purified from crude extract of induced bacterial culture by maltose-affinity column chromatography in a 2.5 × 10 cm column packed with amylose resin specific for the maltose-binding protein (MBP), which forms a part of the fusion protein. The recombinant protein was eluted out from the column with elution buffer containing 10 mM maltose. The recombinant protein was then cleaved with factor Xa to release the protein of interest from the maltose-binding protein. The cleaved proteins were separated by gel filtration chromatography on Superose-12 column and checked on SDS–PAGE to confirm its purity.

3.8. rRNA N-glycosidase activity test

Polysomes of *N. glutinosa* leaves were isolated (Tumer et al., 1997) and treated with 2 μg of purified expressed protein (Girbes et al., 1993). Ribosomal RNA was extracted by standard method (Sambrook et al., 1989) and treated with 2 M aniline/acetate, pH 4.5 (prepared freshly by addition of 1.049 ml glacial acetic acid and 1.820 ml of aniline in total volume of 10 ml made with sterile DEPC treated water) for 10 min in darkness. The reaction was stopped by dilution with 200 μl of sterile water. Aniline was removed by two extractions with petroleum ether and RNA was recovered by ethanol precipitation and electrophoresed on 5% polyacrylamide/urea gel.

3.9. In vitro protein synthesis inhibition

Native CCP-27 (nCCP-27), expressed recombinant protein (reCCP-27) and expressed purified (eCCP-27) were tested for their in vitro protein synthesis inhibition activity using Flexi rabbit reticulocytes in vitro translation system (Promega) (Sharma et al., 2004). The rabbit reticulocytes were treated with different concentrations of different proteins (10–1000 ng) at 30 °C for 30 min. The treated rabbit reticulocytes were incubated with Bromo mosaic virus RNA. The ^{35}S-labelled Met was used to label the protein product. The reaction was carried out at 30 °C for 1.5 h. The reaction was terminated by adding 0.25 ml of 1 N NaOH containing 0.2% (v/v) H_2O_2, followed by incubation at 37 °C for 10 min. The protein was precipitated with 25% (w/v) trichloroacetic acid (TCA) on ice for 30 min and collected on glass fiber filter. The filters were washed with 5% chilled TCA and finally with chilled acetone. The filters were dried with hair drier at room temperature and counts taken using liquid scintillation. CCP-25 was used as a positive control (Baranwal et al., 2002).

3.10. Antiviral bioassay of the purified expressed protein

Antiviral activity of purified protein was tested by the local lesion assay using *N. glutinosa* plants and TMV as well as *C. tetragonoloba* plant and SRV. Percentage of lesion inhibition was calculated by the following formula:

$$\% \text{ Inhibition} = C - T/C \times 100,$$

where C is the average number of lesions in control plants (plants treated with crude extract of protein isolated from *E. coli* cells containing non-recombinant plasmid); T is the average number of lesions in treated plants (plants treated with purified protein).

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