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Phytochemistry 67 (2006) 1865–1873
PHYTOCHEMISTRY
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Purification, characterization and cloning of antiviral/ribosome inactivating protein from *Amaranthus tricolor* leaves

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Received 25 October 2005; received in revised form 4 June 2006
Available online 21 July 2006

Abstract

An antiviral protein (AVP), imparting high level of resistance against sunnhemp rosette virus (SRV) was purified from the dried leaves of *Amaranthus tricolor*. The purified protein (AAP-27) exhibited ~98% inhibition of local lesion formation at a concentration range of ~30 μg ml^{-1}. The protein was found to be highly basic glycoprotein monomer (pI ~ 9.8) of Mr 27 kDa, with neutral sugar content of 4%. The purified protein exhibited *N*-glycosidase and RNase activities. We have also isolated full-length cDNA clone, encoding this protein designated as *A. tricolor* antiviral protein-1 (AAP-1). Two primers, one designed on the basis of N-terminal sequence of the purified protein and the other from the conserved active peptides of other AVPs/RIPs were used for PCR amplification of double stranded cDNA, isolated from the leaves of *A. tricolor*. The amplified fragment was used as a probe for library screening. The isolated full-length cDNA consisted of 1058 nucleotides with an open reading frame encoding a polypeptide of 297 amino acids. The deduced amino acid sequence of AAP-1 has a putative active domain conserved in other AVPs/RIPs and shows varying homology to the RIPs from other plant species.

Keywords: *Amaranthus tricolor*; Antiviral protein; Protein purification; Basic glycoprotein; *N*-glycosidase activity; RNase activity; cDNA

1. Introduction

Many plants contain proteins that are capable of inactivating ribosomes and accordingly are called ribosome-inactivating proteins or RIPs. These typical plant proteins receive a lot of attention in biological and biomedical research because of their unique biological activities toward animal and human cells. In addition, evidence is accumulating that some RIPs play a role in plant defense and hence can be exploited in plant protection against different viruses, fungi and bacterial pathogens and are also known as antiviral proteins (AVPs) (for reviews, see Stirpe et al., 1992; Tumer et al., 1999; Nielsen and Boston, 2001; Van Damme et al., 2001; Wang and Hudak, 2003; Girbes et al., 2004; Stirpe, 2004). There has been an increasing controversy as to whether or not RIPs possess enzymic activities other than *N*-glycosidase activity. Most of the newly discovered enzymic activities of RIPs are DNase activity on supercoiled DNA (Ling et al., 1994), RNase activity (Hudak et al., 2000) and depurination of capped mRNAs (Hudak et al., 2002). Recently, some RIPs have also been reported to show superoxide dismutase (SOD) activity (Li et al., 1997; Sharma et al., 2004), phospholipase activity (Helmy et al., 1999) and antioxidant activity (Gholizadeh et al., 2004). Recent studies suggest that RIPs are also capable of inducing cell death by apoptosis (Narayanan et al., 2005). But widely accepted mechanism suggests that RIPs possess characteristics *N*-glycosidase activity that inactivates the ribosomes, inhibiting protein synthesis irreversibly (Van Damme et al., 2001). However, much remains to be known about the mechanism through which these AVPs operate to inhibit virus infection (Nielsen and

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doi:10.1016/j.phytochem.2006.06.011

Boston, 2001; Wang and Hudak, 2003; Sang-Woo Park et al., 2004; Stirpe, 2004).

Plants containing these AVPs/RIPs genes are therefore, a potential source of resistant genes that could be isolated and transferred to other economically important crops to develop virus resistant plants (Tumer et al., 1997, 1999; Smirnov et al., 1997; Zoubenko et al., 2000; Vandenbussche et al., 2004).

Plants belonging to the family of Amaranthacea are also potential candidates for the presence of these viral inhibitors (Kwon et al., 1997). A cDNA encoding RIP/AVP of 270 amino acid residues has been isolated and characterized from *Amaranthus viridis* (Kwon et al., 2000). Preliminary screening studies done in our laboratory had indicated that the leaf extracts of *Amaranthus tricolor* are very effective in preventing local lesion formation by sunnhemp rosette virus (SRV) on host *Cyamopsis tetragonaloba*. Here, we report the isolation, purification, characterization and cloning of RIP/AVP from the leaves of *A. tricolor*.

2. Results and discussion

Since extracts from both fresh and dry *A. tricolor* leaves inhibited local lesion formation by sunnhemp rosette virus in its hypersensitive host, *C. tetragonoloba* (Guar) to the same extent, the dried leaves were used to extract the antiviral activity. The antiviral protein (AVP) was purified from the leaves of *A. tricolor* var. Lal Chulai. Sunnhemp rosette virus (SRV) was used as test virus and *C. tetragonoloba* as test plant for bioassay of the antiviral protein. The antiviral activity was expressed in terms of percent inhibition of lesion formation on the test plant leaves by the virus.

2.1. Purification of antiviral protein from amaranthus tricolor leaves

The leaves of *A. tricolor* were harvested, washed with tap water and dried at room temperature or at 40–45 °C. These dried leaves were used to extract the antiviral activity. Approximately 2200 mg of soluble proteins was obtained from 60 g of dried leaves. Bioassay of crude extracts against SRV, on its local lesion host, *C. tetragonoloba* showed very high activity (~95.5% inhibition). The crude extracts were further purified by following sequential purification through ammonium sulphate fractionation followed by dialysis, DEAE-Cellulose anion exchange chromatography, CM-Sepharose cation exchange chromatography and finally Superose-12 size exclusion chromatography. The pooled fractions obtained at each stage of purification were bio-assayed for their antiviral activity. The results of the various purification steps are presented in Table 1. Upon stepwise ammonium sulphate precipitation, most of antiviral activity was recovered in the fraction precipitating between 60% and 80% ammonium sulphate saturation. The 60–80% ammonium sulphate precipitated fraction was redissolved in 20 mM sodium phosphate buffer, pH 6.2 containing 10 mM NaCl and dialyzed against the same buffer at 4 °C for 24 h. The dialyzed protein fraction was subjected to anion-exchange chromatography on DEAE cellulose. The antiviral activity was observed in unbound fraction indicating the basic nature of the protein.

Table 1
Purification of antiviral protein (AAP-27) from the leaf extracts of *Amaranthus tricolor*

Protein fraction	Total protein[a] (mg)	Percent inhibition[b]
Crude extract	2207.0	95.5
Ammonium sulphate fraction 60–80%	320.0	94.1
DEAE-cellusose chromatography		
Unabsorbed fraction	96.4	93.4
Absorbed fraction	156.2	12.0
CM-sepharose chromatography		
0.1 M NaCl		
Fraction I	14.4	88.9
Fraction II	8.2	10.0
0.2 M NaCl		
Fraction III	15.4	98.5
Superose-12 chromatography	1.8	98.3

[a] From 60 g of dried leaves.
[b] Concentration range of protein applied ~30 μg ml^{-1}.

The active unbound fractions were pooled and subjected to cation-exchange chromatography on CM-Sepharose. The bound proteins were eluted with discontinuous linear NaCl gradient (0.1, 0.2 and 0.3 M). When eluted with 0.1 M NaCl, it resulted in two protein fractions separating as peak I and peak II whereas elution with 0.2 M NaCl resulted in only one fraction as peak III. Negligible

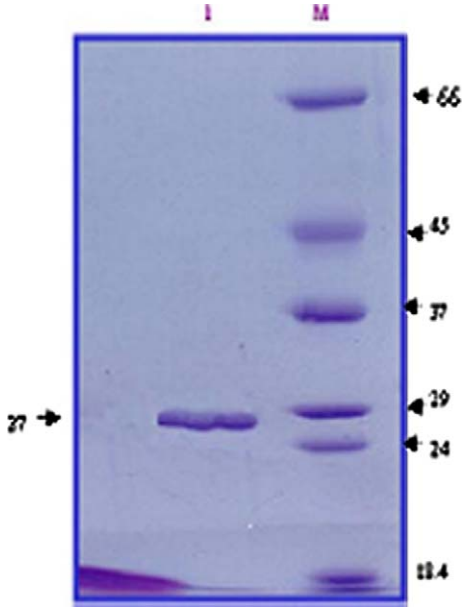

Fig. 1. SDS–PAGE of purified AAP. Purified AAP. (lane 1). Molecular weight markers (M).

amounts of protein were eluted at higher salt concentrations (results not shown). Bioassay of different fractions (I, II, and III) exhibited 88.9%, 10.0% and 98.5% inhibition, respectively (Table 1). The fractions I and III, that exhibited high antiviral activity were separately concentrated by lyophilization and subjected to size exclusion chromatography on Superose-12 column. Fraction I again resolved as a single peak. The percent inhibition caused by this fraction was 31% only. Thus, fraction-I after passing through Superose-12 column lost around 60% of its activity. The exact cause for this loss is unknown, but it is speculated that some physico-chemical (conformational) changes during fractionation might have resulted in such observation. Fraction III when subjected to size exclusion chromatography again resolved into a single peak. This fraction was found to posses the maximum (98.3% inhibition) antiviral activity (Table 1) and when tested for homogeneity exhibited single band on SDS–PAGE (Fig. 1). This purified *Amaranthus* antiviral protein has been designated as AAP-27.

2.2. Molecular weight of AAP-27

Molecular weight (Mr) of the purified AAP-27 as determined by both calibrated gel permeation chromatography and SDS–PAGE was observed to be ~27 kDa (Fig. 1).

2.3. Isoelectric focusing and amino acid composition

The isoelectric focusing pattern showed the protein to migrate towards the cathodic end of the focused gel and the pI was calculated to be ~9.8 (results not given). This confirmed that the protein is basic in nature. The basic nature of protein was further confirmed by amino acid analysis, which shows that lysine (38.4%) and arginine (24.8%) formed the major constituents of the protein.

2.4. Presence of carbohydrates

The AAP protein stained positively for glycoproteins on polyacrylamide gels when periodic acid-Schiff's reagent was used for staining. Total neutral sugar content as determined by the phenol-sulfuric acid method was ~4%. Different antiviral proteins like Dianthin-30 and 32 contain 2.6% and 4.3% sugars, respectively (Stirpe et al., 1992), while *Celosia* AVPs have been reported to be containing very high neutral sugar contents in the range of 26–28% (Balasubrahmanyam et al., 2000).

2.5. Effect of actinomycin D on antiviral activity

AAP-27 imparted resistance to *C. tetragonoloba* against SRV. This resistance could largely be inhibited when actinomycin D (ActD, 20 μg ml^{-1}) was applied immediately after treating the plant with AAP-27. When ActD was applied 6 and 12 h respectively, after AAP-27 treatment, it failed to inhibit the resistance response to a greater extent (Table 2). ActD is known to bind to double helical DNA by intercalating between the bases and thereby inhibiting DNA-directed RNA synthesis. This inhibition as a result of immediate application of ActD, following the AAP-27 treatment, suggests that this protein might be inducing the synthesis of some new virus interfering substances or enhancing the production of already existing ones, thereby altering the susceptibility of the host plant. However, the antiviral property is not reversed if ActD is applied 6 or 12 h after protein treatment. This is because the substances involved in antiviral effect are already present and the late application of ActD does not interfere in their synthesis. This property is quite similar to that exhibited by *C. inerme*, *C. aculeatum* and *C. cristata* antiviral proteins (Verma et al., 1996; Balasubrahmanyam et al., 2000).

Table 2
Effect of actinomycin D (ActD) on antiviral property[a]

Treatment	Average lesion number	% Inhibition
Buffer control	77	–
ActD (20 μg ml^{-1})	69	–
Bougainvillea xbuttiana (BB) extract – positive control	2	97.40
BB + ActD (20 μg ml^{-1} after 0 h)	59	14.49
BB + ActD (20 μg ml^{-1}) after 6 h	5	92.70
BB + ActD (20 μg ml^{-1}) after 10 h	3	95.60
AAP-27	3	95.60
AAP-27 + ActD (20 μg ml^{-1}) after 0 h	47	31.80
AAP-27 + ActD (20 μg ml^{-1}) after 6 h	11	84.00
AAP-27 + ActD (20 μg ml^{-1}) after 10 h	2	97.10

Control sets were treated with buffer alone or with the ActD solutions alone. Dry leaf extract of *Bougainvillea xbuttiana* (BB) was used as a positive control (Narwal et al., 2001).

[a] Purified AAP-27 (30 μg ml^{-1}) was applied on leaves of *Cyamopsis tetragonaloba* (Guar). In one set, actinomycin D (ActD, 20 μg ml^{-1}) was applied immediately after treatment, while in other sets ActD treatment was given after 6 and 12 h of AAP-27 application.

2.6. N-glycosidase activity

Most of the antiviral proteins are known to possess ribosomal inactivating activity and therefore known as ribosomal inactivating proteins (RIPs). As shown in Fig. 2, aniline treatment of rRNA extracted from AAP-27 treated *C. tetragonoloba* ribosomes resulted in generation of specific RNA fragments (lane 2) due to their *N*-glycosidase activity.

2.7. RNase activity

The purified antiviral protein from *Amaranthus tricolor* was also tested for its ribonuclease activity. To determine if AAP could degrade RNA, a method for substrate-based RNase activity assay was adapted. As the gels were stained with toluidine blue, which interacts with RNA to give blue colour, the unstained portion, indicated the position of RNase activity. Although an RNase activity gel assay

indicates that all RNase activity comigrates as a single band with AVP, it cannot be ruled out that a genuine RNase comigrates with AVP after SDS–PAGE. Most plant ribonucleases consist of polypeptide of ~25–30 kDa, and thus can comigrates with AVP. The findings that a commercial preparation of PAP from seeds (PAP-S) is contaminated with a nuclease that can be quantitatively removed by dye-chromatography on Red-Sepharose (Barbieri et al., 2000) cannot but emphasise the need for thorough purity check of all AVP preparations.

Fig. 3 (lanes 2 and 3) shows single unstained band that have been found to comigrate with purified AAP-27 previously passed through Red-Sepharose column, indicating that AAP-27 does posses a strong RNase activity. Presence of only one band with RNase activity corresponding to the position of AAP-27, suggests that there was no other contaminating nucleases with the purified AAP preparation. Lane 1 shows the position of positive control (2 μg RNase A). The present results are in agreement with the earlier findings of Mock et al. (1996).

The ribosome inactivating property of RIPs has been held responsible for inhibition of protein synthesis in eukaryotes (Gessner and Irvin, 1980; Irvin, 1995). Taylor et al. (1994) have shown a positive correlation between the *N*-glycosidase catalysed depurination of tobacco ribosomes and antiviral activities of PAP and dianthin-32. These observations, therefore, support the hypothesis that antiviral proteins work through ribosomal inactivation and thus indirectly prevent the multiplication/replication phase of the virus infection process (Chen et al., 1993; Taylor et al., 1994). However, studies by Tumer et al. (1997) using C-terminal deletion mutant of PAP show that ribosome inactivating property by itself may not be responsible for the antiviral action of RIPs. Based on their study, they concluded that antiviral activity of PAP might be dissociated from its toxicity. More recently, Hudak et al. (2000) proposed a novel mechanism for the inhibition of translation by PAP and concluded that PAP possibly inhibits translation by binding to the cap structure and depurinating the mRNA, and that the depurination of capped viral RNA may be the primary mechanism for the antiviral activity of PAP. According to them, PAP degraded capped luciferase transcripts and behaves as an RNase at high concentrations. RIPs may also act indirectly through the activation of the plant's defense system resulting in a systemic resistance independently of salicylic acid or pathogenesis related protein accumulation (Smirnov et al., 1997; Zoubenko et al., 2000; Gholizadeh et al., 2004). At present it is thus, unclear which of these proposed mechanisms is operative.

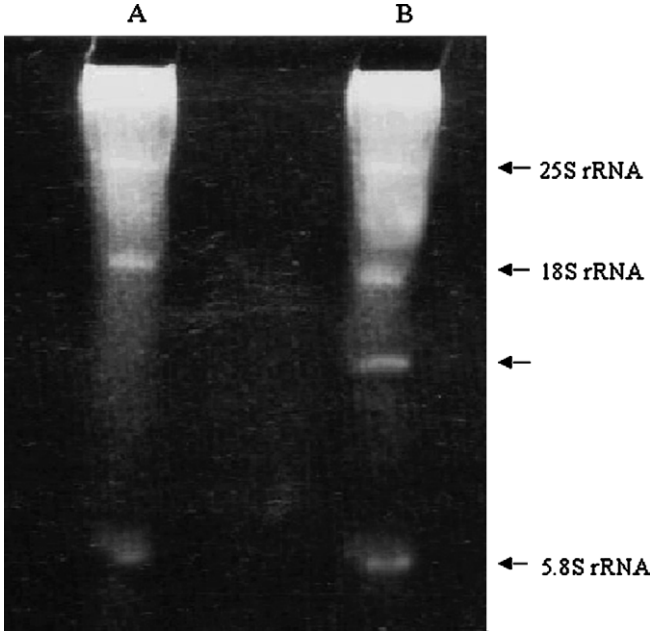

Fig. 2. *N*-glycosidase activity of purified AAP on *Cyamopsis tetragonoloba* rRNA. Control rRNA (lane 1) and AAP treated rRNA (lane 2).

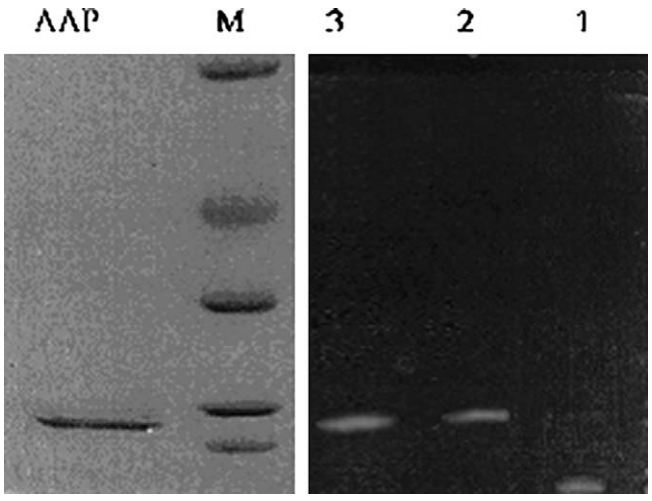

Fig. 3. Degradation of torula Yeast RNA by AAP on RNase activity gel. The unstained portion indicates the position of RNase activity. Positive control, 2 μg of RNase A (lane 1). 2 μg of purified AAP (lane 2). 4 μg of AAP (lane 3). Lanes containing protein markers (M) and AAP were cutoff from the gel and stained with Coomassie blue.

2.8. N-terminal sequencing of purified AAP

The purified AAP-27, after SDS–PAGE run was electroblotted and the blot was processed for N-terminal sequencing. The results showed the following 15 amino acid residues at the N-terminal end.

Ala-Tyr-Leu-Thr-Phe-Ile-Val-Thr-Leu-Glu-Ile-Gly-Asp-Gln-Asp

This N-terminal sequence did not show any significant homology with any of the RIPs known so far.

2.9. Generation of probe

In order to isolate the cDNA encoding the RIP/AVP, a cDNA library was constructed from the leaf tissues of *A.*

tricolor. The cDNA was amplified by using primer (designed on the basis of N-terminal sequence of purified protein) as the forward primer and primer (designed on the basis of conserved region of other RIPs) as the reverse primer. Three prominent bands at around 0.75 kb, 0.4 kb and 0.2 kb were observed when the PCR product was electrophoresed on an ethidium bromide (EtBr) stained 1% agarose gel. However, the band corresponding to 0.75 kb was eluted from the gel and used for further cloning and subsequent sequencing. The choice of this particular single band for cloning was based on the fact that the two primers used were according to N-terminal sequence of the purified AAP and conserved sequence of other RIPs/AVPs, respectively and in most of the RIPs this conserved region is mostly ~225–240 amino acid residues away from the N-terminal region of the proteins. The fragments, ~0.2 and 0.4 kb being very small, were therefore, rejected.

2.10. Screening of cDNA library

To isolate the cDNA clone(s) of AAP-27, PCR amplified fragment, as described earlier, was used as a probe to screen the cDNA library. In the primary screening, 5×10^5 plaques were screened and eight putative positive clones were selected by aligning the plates with auto-radiograms. From the secondary screening, five single well-isolated positive plaques were picked up and excised in *E. coli* BM25.8 to liberate ampicillin resistant pTriplEx2 plasmid to allow insert characterization in plasmid system. Restriction digestion of the plasmids of all the five putative positive clones with *Eco*RI and *Hin*dIII was done but only two of the clones gave the insert cDNA. Restriction pattern of these two clones showed insert sizes of 1.0 kb and 0.70 kb, respectively.

The two, cDNA clones that exhibited the insert DNA were restricted and electrophoresed on a 0.8% gel along with DNA size marker. It was then transferred to a nylon membrane and Southern hybridized with labeled probe (α-^{32}P). However, only the 1.0 kb insert cDNA lighted up prominently. The complete sequencing of the insert of this cDNA clone shows that it is 1058 bp in length. The cDNA was translated in all six reading frames by EMBOSS transeq tool. The longest ORF was present in the first frame and it has 894 nucleotides encoding 297 amino acid residues with translation initiation codon (ATG) at position 40 down stream of the first base and a termination codon (TAG) located after nucleotide 930 (Fig. 4). A polyadenylation signal (AATAA) located at position 968 and poly (A) tail of 26 A residues was also found. The 3′ UTR is 123 base pair long. The sequence of the partial gene used as probe was found to be present between the positions 77 and 770 bp (gene sequence submitted to GenBank with Accession No. AY354205). The amino acid sequence encoded by the ORF of isolated cDNA when blasted using protein–protein blast tool (BLASTP) of NCBI site showed a significant similarity with other RIPs/AVPs. Amaranthin, an RIP from *Amaranthus viridis* showed maximum identity of 120/264 sequences with 45% homology to the isolated cDNA. In addition, this protein also shows strong homology to a number of RIPs/AVPs as regards to its conserved region (EAAR) that is also the active site of the proteins. The Clustal W alignment of the protein encoded by ORF of the isolated clone with several other RIPs/AVPs revealed, its maximum similarity at the active site (EAAR) of the protein (Fig. 5).

The translated amino acid sequence also contains the same amino acid sequence (from position 12 to 26) as that obtained by N-terminal sequencing of the purified protein. However, it does not match from the start amino acid (M) to 11th residue (A). Thus, it is speculated that it might have a signal peptide sequence that has probably undergone post-translational modifications before its transport to target site. Several other RIPs/AVPs have also been reported to have signal peptide at N-terminus. For examples, N-terminal of PAP contains 22 amino acids that are removed in mature protein Lin et al. (1991). Tricohanguin, a RIP from *Trichosanthes anguina* has a signal peptide of 19 residues (Chow et al., 1999). It was also predicted that *D. sinensis* RIP cDNA might have a putative signal peptide of 23 amino acid residues at their N-terminus (Cho et al., 2000).

Thus an antiviral protein (AVP), imparting high level of resistance (98%) against sunnhemp rosette virus (SRV) was

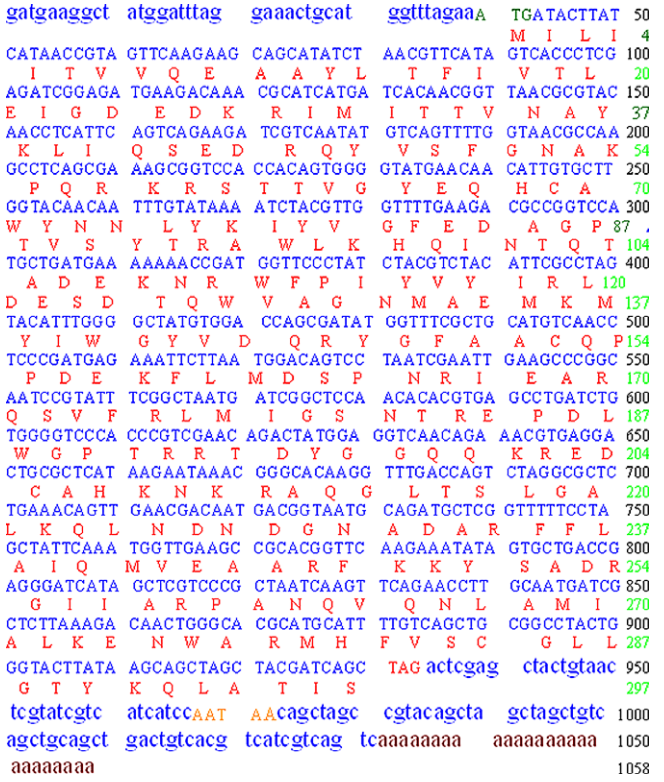

Fig. 4. Nucleotide and deduced aminoacid sequence of *Amaranthus tricolor* cDNA. The stop codon (red), poly(A) adenylation signal (orange) and poly (A) tail (brown) are also shown. Lower case letters represent 5′UTR and 3′UTR regions, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

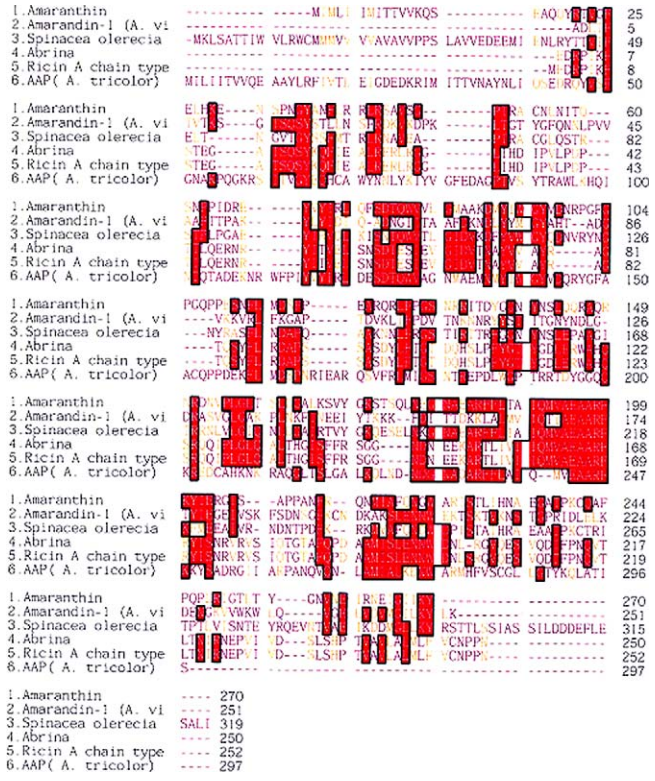

Fig. 5. Clustal W alignment of *Amaranthus tricolor* antiviral protein (AAP) with various RIPs/AVPs. Red shaded areas show the region of similarity. Maximum similarity is observed at the active site region EAAR. Gene Bank Accession Numbers of different RIPs used for analysis are as follows, abrin-a (1406189 A), amarandin-1 (AAB93956), amaranthin (U85255), *Spinacia oleracea* (BAB83507), ricin A chain (CAA38655), *A. tricolor* (AY354205). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

purified from the dried leaves of *Amaranthus tricolor*. The purified protein exhibited *N*-glycosidase and RNase activities but its exact mechanism of action in imparting resistance to plants against viruses is still to be worked out. The isolated full-length cDNA consisted of 1058 nucleotides with an open reading frame encoding a polypeptide of 297 amino acids. The deduced amino acid sequence of AAP1 has a putative active domain conserved in other AVPs/RIPs and shows varying homology to the RIPs from other plant species. It can serve as a potential source of resistant gene that could be transferred to other economically important crops to develop virus resistant plants.

3. Experimental

3.1. Preparation of purified virus inoculum and bioassay

Sunnhemp rosette virus (SRV), used as a test virus, was maintained on its systemic host *Crotolaria juncea*. Virus inoculum was prepared by homogenizing the infected leaves with 20 mM sodium phosphate buffer, pH 7.2. The contents were squeezed through two layers of muslin cloth and the filtrate was centrifuged at 12,000*g* for 10 min. The clear supernatant was used as virus inoculums after proper dilution to give countable number of lesions on test plants. For testing the antiviral activity of the extracts, local lesion host of SRV viz. guar (*Cyamopsis tetragonoloba*) was used. Bioassay was done as described before (Balasubrahmanyam et al., 2000). The percent inhibition was calculated using the formula:

$$\text{Percent inhibition} = \{(C - T)/C\} \times 100$$

where C is the average number of lesion on control plant and T is the average number of lesion on treated plant.

3.2. Purification of antiviral protein

Fresh leaves of *A. tricolor* were collected at vegetative stage, washed sequentially with tap water and distilled water and dried at room temperature. Sixty grams of dried leaf material was taken and homogenized with 5-vol. of extraction buffer, 0.1 M NaOAc buffer (pH 5.2) containing 10 mM β-mercaptoethanol and a pinch of polyvinyl polypyrolidone in a warring blender. The slurry was filtered through muslin cloth and centrifuged at 12,000*g* for 10 min. The clear supernatant was taken for further purification. The supernatant obtained from leaf extracts was subjected to ammonium sulphate fractionation at 0–25%, 25–60% and 60–80% saturation, by slow addition of calculated quantities of solid ammonium sulphate at each step to the supernatant with constant stirring using a magnetic stirrer at 4 °C. The pellet obtained at each step was suspended in 20 mM sodium phosphate buffer, pH 6.2 and dialyzed against the same buffer for 24 h at 4 °C. All the fractions were tested for their antiviral activity. The 60–80% fraction, which exhibited maximum antiviral activity, was then passed through DEAE-cellulose equilibrated with 20 mM sodium phosphate buffer, pH 6.2 containing 10 mM NaCl and 0.01% sodium azide. The unabsorbed proteins were eluted with 0.5 M NaCl in the same buffer. Both absorbed and unabsorbed fractions were then tested for antiviral activity using SRV/*C. tetragonoloba* system. The unabsorbed fractions which retained most of the antiviral activity was passed through CM-sepharose column equilibrated with 20 mM sodium acetate buffer, pH 5.2 containing 10 mM NaCl and 0.01% sodium azide. After washing out the unabsorbed protein, the bound proteins were eluted with a step gradient of NaCl (0.1 M, 0.2 M, 0.3 M, 0.4 M and 0.5 M, respectively) in the equilibration buffer. About 4–5 bed volumes of each concentration were passed through the column at a flow rate of 30 ml h^{-1}, 3.0 ml fractions collected and simultaneously monitored at 280 nm. The fractions corresponding to different peaks were pooled and tested for antiviral activity. The fractions I, II and III obtained from CM-Sepharose column were lyophilized and passed through Superose-12 column equilibrated with 20 mM NaOAc buffer, pH 5.2 containing 0.2 M NaCl and 0.01% sodium azide. The elution was done with the equilibration buffer at flow rate of 0.3 ml min^{-1} and 2.2 ml fractions were collected that were simulta-

neously monitored at 280 nm. The fractions corresponding to different peaks were again pooled and tested for antiviral activity.

3.3. Estimation of soluble proteins

Protein content of leaf extract at each step of purification was estimated by the method as described by Lowry et al. (1951).

3.4. Protein characterization

All the active fractions obtained at different steps of purification starting from crude extract to Superose-12 fractionation were analyzed by polyacrylamide gel electrophoresis under denaturing conditions on 12% separating gel according to Laemmli (1970). The molecular weight of the purified protein was determined under native conditions by calibrated gel-permeation chromatography using MW-GF-70 Kit (contains aprotinin, 6.5 kDa; cytochrome *c*, 12.4 kDa; carbonic anhydrase, 29 kDa and bovine serum albumin, 66 kDa) (Sigma) in a Sephadex G-75 column. The molecular weight of the purified protein was also determined under denaturing conditions on 12% polyacrylamide gel using molecular weight markers (Dalton mark VI, Sigma).

3.5. Carbohydrate estimation

The presence of carbohydrates was detected by Periodic acid-Schiff's reagent staining procedure (Leach et al., 1980) using ovalbumin as positive control and BSA as negative control. The amount of total sugar was determined by the method as described by Dubois et al. (1956).

3.6. Isoelectric focusing

The isoelectric point (pI) of the purified protein was determined by isoelectric focusing under non-denaturing conditions on 5% polyacrylamide gel containing ampholines (pH 8.0–10.5) using the method of Righetti and Chellemi (1975).

3.7. Amino acid analysis

The purified protein was hydrolyzed with 6 N HCl at 110 °C for 24 h. Hydrolyzed sample was derivatized with phenyl-isothiocyanate (PITC) and analyzed by HPLC (PicoTag-amino acid analysis system, Operation manual, Waters, USA).

3.8. Effect of actinomycin D on antiviral activity

Actinomycin D (ActD 20 μg ml^{-1}) was applied at different time intervals (0, 6 and 10 h, respectively) following treatment with purified antiviral protein on the same leaves of *C. tetragonoloba*. An equal number of leaves in control sets were treated with ActD alone, antiviral protein alone and buffer alone. After 24 h, all the leaves were inoculated with SRV and observed for lesion development.

3.9. Isolation of ribosomes and assay for N-glycosidase activity

Ribosomes were isolated from the leaves of *C. tetragonoloba* using the method as described by Tumer et al. (1997). Enzyme assay was carried out using methods of Girbes et al. (1993), with little modifications. Twenty microgram of ribosomes were incubated with 2 μg of purified antiviral protein for 15 min at 37 °C in a 100 μl reaction mixture that contained 20 mM Tris–HCl (pH 7.8), 50 mM KCl, 10 mM DTT and 2 mM $MgCl_2$. Total RNA was extracted, subjected to aniline treatment and then analyzed on 5% denaturing acrylamide gel.

3.10. RNase activity gel assay

To determine if AAP-27 could degrade RNA, a method for a substrate-based RNase activity assay was adapted from Yen and Green (1991). 100 mg^{-1} ml of yeast RNA was dissolved in 1 M Tris–HCl buffer, pH 9.0 and phenol extracted in 1:1 ratio. The contents were spinned for 10 min at 7000 rpm. The aqueous layer was chloroform extracted (1:1) twice and again spinned for 10 min at 7000 rpm. The contents were precipitated with 1/10 the volume of 3 M NaOAc and two volumes of ethanol, spinned for 10 min at 12,500 rpm and the pellet was washed once with 70% ethanol. Air dried the pellet and dissolved it in 0.1 M Tris–HCl, pH 9.0. The absorbance was checked at 260 nm.

AAP-27 samples were prepared in 50 mM Tris–HCl, pH 6.8, 0.1% SDS, and 0.01% bromophenol blue without the addition of reducing agents and further purified by passing through Red Sepharose column as described by Barbieri et al. (2000). As a positive control for RNase activity, 2 μg RNase A was prepared in the same buffer. The sample and control were run on 12% SDS–PAGE containing 2 mg^{-1} ml torula yeast RNA (Sigma) in the resolving gel. Following electrophoresis, the gel was washed with 25% isopropanol in 10 mM Tris–HCl, pH 7.0 to remove SDS. The isopropanol was removed by further washing in 10 mM Tris–HCl, pH 7.0, 2 μM $ZnCl_2$. Protein in the gel was renatured by incubation in 100 mM Tris–HCl, pH 7.0, at 50 °C for 1 h. The gel was stained with 0.2% toluidine blue-O in 10 mM Tris–HCl, pH 7.0 for 10 min and destained in distilled water until transparent bands appeared. Lanes containing protein markers and AAP were cut from the gel and stained with Coomassie blue to indicate location of AAP.

3.11. N-terminal sequencing of purified AAP-27

About 10 μg pure AAP was taken and run on the 12% SDS–PAGE gel. After the run, the gel was soaked with

gentle shaking, in transfer buffer (10 mM CAPS buffer in 10% MeOH) pH 8.3 for 10 min. The PVDF membrane was prepared by immersion in MeOH for 10 s followed by equilibration in transfer buffer for 5 min. A gel/PVDF/blotting paper sandwiched blotting assembly was prepared in AE-6657 (Atto) electro-blotter in the sequence adsorbent papers, PVDF membrane, gel and adsorbent papers. A long glass rod was rolled out at every step before keeping the next item so that no air bubble gets entrapped in between. Finally the apparatus was connected to power supply and run at 20 V for 2 h.

After completion of transfer, the PVDF membrane was removed and washed immediately in water for 10 min with gentle shaking. The membrane was stained with 0.1% Coomassie blue R in 50% MeOH for 1–2 min and destained with several changes (2–5 min each) of 50% MeOH and 10% acetic acid. Finally, the membrane was washed in water for 5 min and air-dried. The membrane was placed in a plastic bag and stored at −20 °C until analyzed for N-terminal sequencing. The blotted AAP-27 sample was analyzed for N-terminal sequence from Indian Institute of Technology, Mumbai, India.

3.12. Cloning of cDNA

3.12.1. Plant materials and bacterial strains

Amaranthus tricolor variety Lalchulai plants were grown in vermiculite in growth chamber at 35 °C with 16 h/8 h light/dark period. The leaves were harvested after 35–45 days at vigorous growth stage. *E. coli* strain, DH5α was used for manipulation of cDNA.

3.13. DNA and RNA preparation

Plasmid DNA was prepared by alkaline hydrolysis mini preparation method as described by Ahn et al. (2000). Total RNA was isolated from the leaves of *A. tricolor* by GTC method as described by Ausubel et al. (1999). Poly A+ RNA was isolated and purified by using the Oligotex poly A+ RNA kit (Qiagen, Catalogue No. 70022).

3.14. Generation of probe for library screening

PCR was conducted to obtain the portion of Amaranthus antiviral protein (AAP-27) cDNA, needed for library screening, by using degenerate primer (GCITAYCTITGYTTYATHGTIAC) based on N-terminal sequence of the purified AAP1 and another primer (CGIGCIGCYTCIGANACCAT) designed on the basis of conserved active site amino acid sequence of other RIPs/AVPs. In order to amplify the partial gene, ds cDNA (synthesized for library construction) was used as a template. The amplified fragment was cloned in $pGEMT_{Easy}$ vector (CaT No. Tm 042, Promega, USA). The amplified partial gene fragment was sequenced and analyzed. The cDNA library was screened with this amplified fragment as probe.

3.15. cDNA library construction, screening and sequence determination

A full-length cDNA library was constructed in λTriplEX2 vector from 0.5 μg mRNA using SMART cDNA library construction kit (Clontech, Cat No. 1015-1). The cDNA library (approximately 1×10^6 pfu) was probed with (α^{32}P) dCTP random labeled partial gene (obtained by PCR amplification) that was labeled by using the protocol as described by the manufacturer in the hexalabel kit (Promega, USA). Five plates (120×120) each containing 20,000 pfu were transferred to nylon membranes as described by Ausubel et al. (1992). The membranes were pre-hybridized and hybridized in sodium chloride/sodium citrate (SSC) at 60 °C. The membranes were then washed sequentially with 2× SSC/0.1%SDS at room temperature for 30 min, in 1× SSC/0.1%SDS at 60 °C for 15 min and finally in 0.1× SSC/0.1%SDS at 60 °C for 15 min and auto radio-graphed. The isolated clones were processed for Southern analysis. Positive λ clones were converted separately to pTriplEX2 in BM25.8 strain of *E. coli* according to conversion protocol of SMART cDNA library construction kit. The positive recombinant plasmid having antiviral cDNA was isolated and restricted with *Eco*RI and *Hin*dIII restriction enzymes to take out the insert DNA. The insert fragment was further confirmed by Southern analysis according to standard protocol (Ausubel et al., 1999). The isolated cDNA was completely sequenced by automated sequencer at South Campus, University of Delhi, India.

References

Ahn, S.C., Back, B.S., Oh, T., Song, C.S., Chatterjee, B., 2000. Rapid mini-scale plasmid isolation for DNA sequencing and restriction mapping. Biotechniques 29, 466–468.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, R. (Eds.), 1992. Short Protoc. Mol. Biol. Wiley, NY, USA.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seiolman, J.G., Smith, J.A., Struuhlk, R. (Eds.), 1999. Curr. Protoc. Mol. Biol. Wiley, NY, USA.

Balasubrahmanyam, A., Baranwal, V.K., Lodha, M.L., Varma, A., Kapoor, H.C., 2000. Purification and properties of growth stage dependent antiviral proteins from the leaves of *Celosia cristata*. Plant Sci. 154, 13–21.

Barbieri, L., Valbonesi, P., Righi, F., Zuccheri, G., Monti, F., Gorni, P., Samori, B., Stirpe, F., 2000. Polynucleotide adenosine glycosidase is the sole activity of ribosome-inactivating proteins on DNA. J. Biochem. 128, 883–889.

Chen, Z.C., Antoniw, J.F., Lin, Q., White, R.F., 1993. Expression of pokeweed (*Phytolacca americana*) antiviral protein cDNA in *Escherichia coli* and its antiviral activity. Physiol. Mol. Plant Pathol. 42, 237–247.

Cho, H.I., Lee, S.J., Kim, S., Kim, B.D., 2000. Isolation and characterization of cDNA encoding ribosome inactivating protein from *Dianthus sinensis* L. Mol. Cells 10, 135–139.

Chow, L.P., Chou, M.H., Ho, C.Y., Chuang, C.C., Pan, F.N., Wu, S.H., Lin, Y., 1999. Purification, characterization and molecular cloning of trichoanguin, a novel type I ribosome-inactivating protein from the seeds of *Trichosanthes anguina*. Biochem. J 338, 211–219.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Coloremetric method for determination of sugars. Anal. Chem. 28, 350–356.

Gessner, S.L., Irvin, J.D., 1980. Inhibition of elongation factor2-dependent translocation by the pokeweed antiviral protein and ricin. J. Biol. Chem. 255, 3251–3253.

Gholizadeh, A., Kumar, M., Balasubrahmanyam, A., Kumar, S., Narewal, S., Lodha, M.L., Kapoor, H.C., 2004. Antioxidant activity of antiviral proteins from *Celosia cristata*. J. Plant Biochem. Biotechnol. 13, 13–18.

Girbes, T., Citores, L., Iglesias, R., Ferreras, J.M., Munoz, R., Rojo, M.A., Arias, F.J., Garcia, J.R., Mendez, E., Calonge, M., 1993. Ebulin I, a nontoxic novel type 2 ribosome inactivating protein from *Sambucus ebulus* L. leaves. J. Biol. Chem. 268, 18195–18199.

Girbes, T., Ferreras, J.M., Arias, F.J., Stirpe, F., 2004. Description, distribution, activity and phylogenetic relationship of ribosome – inactivating proteins in plants, fungi and bacteria. Mini Rev. Med. Chem. 4, 461–476.

Helmy, M., Lombard, S., Pieroni, G., 1999. Ricin RCA60: evidence of its phospholipase activity. Biochem. Biophys. Res. Commun. 258, 252–255.

Hudak, K.A., Wang, P., Tumer, N.E., 2000. A novel mechanism for inhibition of translation by pokeweed antiviral protein; depurination of the capped RNA template. RNA 6, 369–380.

Hudak, K.A., Bauman, J.D., Tumer, N.E., 2002. Pokeweed antiviral protein binds to the cap structure of eukaryotic mRNA and depurinates the mRNA downstream of the cap. RNA 8, 1148–1159.

Irvin, J.D., 1995. Antiviral proteins from Phytolacca. In: Antiviral Proteins from Higher Plants. CRC Press, Boca Raton, FL, pp. 65–69.

Kwon, S., An, C.S., Liu, J.R., Peak, K., 1997. A ribosome inactivating protein from *Amaranthus viridis*. Biosci. Biotech. Biochem. 61, 1613–1614.

Kwon, Y.S., Chung, S.A., Jang, R.L., Sang, S.K., Haeng, S.L., Jeong, K.K., Kyung, H.P., 2000. Molecular cloning of a cDNA encoding ribosome-inactivating protein from *Amaranthus viridis* and its expression in *E. coli*. Mol. Cells. 10, 8–12.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophasge T4. Nature 227, 680–685.

Leach, B.S., Collawn, J.F., Fish, W.W., 1980. Behaviour of glycoproteins empirical molecular weight estimation methods I. In sodium dodecyl sulphate. Biochemistry 19, 5734–5741.

Li, X-D., Chen, W-F., Liu, W-Y., Wang, G-H., 1997. Large scale preparation of two new ribosome inactivating proteins, cinnamomin and camphorin, from the seeds of *Cinnamomum camphora*. Protein Exp. Purif. 10, 27–31.

Lin, Q., Chen, Z.C., Antoniw, J.F., White, R.F., 1991. Isolation and characterization of cDNA clone encoding the antiviral protein from *Phytolacca americana*. Plant Mol. Biol. 17, 609–614.

Ling, J., Liu, W.Y., Wang, T.P., 1994. Cleavage of supercoiled double-stranded DNA by several ribosome-inactivating proteins in vitro. FEBS Lett. 345, 143–146.

Lowry, O.H., Roenbrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with Folin–phenol reagent. J. Biol. Chem. 193, 265–275.

Mock, J.W., Ng, T.B., Wong, R.N., Yao, Q.Z., Yeung, H.W., Fong, W.P., 1996. Demonstration of ribonuclease activity in the plant ribosome-inactivating proteins alpha- and beta-momorcharines. Life Sci. 59, 1853–1859.

Narayanan, S., Surendranath, K., Bora, N., Surolia, A., Karande, A., 2005. Ribosome inactivating proteins and apoptosis. FEBS Lett. 579, 1324–1331.

Narwal, S., Balasubrahmanyam, P., Sadhna, P., Kapoor, H.C., Lodha, M.L., 2001. A systemic resistance inducing antiviral protein with N-glycosidase activity from *Bougainvillea xbuttiana* leaves. Indian J. Exp. Biol. 39, 600–603.

Nielsen, K., Boston, R.S., 2001. Ribosome inactivating proteins: a plant perspective. Ann. Rev. Plant Physiol. Plant Mol. Biol. 52, 785–816.

Righetti, P.G., Chellemi, F., 1975. Isoelectric focusing of peptides. J. Chromatogr. 157, 243–251.

Sang-Woo Park, Vepachedu, R., Sharma, N., Vivanco, J.M., 2004. Ribosome- inactivating proteins in plant biology. Planta 219, 1093–1096.

Sharma, N., Park, S-W., Vepachedu, R., Barbieri, L., Clani, M., Stripe, F., Savary, B.J., Vivanco, J.M., 2004. Isolation and characterisation of an RIP (ribosome inactivating protein) – like protein from tobacco with dual enzymatic activity. Plant Physiol. 134, 171–181.

Smirnov, S., Shulaev, V., Tumer, N.E., 1997. Expression of pokeweed antiviral protein in transgenic plants induced virus resistance in grafted wild-type plant, independent of salicylic acid accumulation and pathogenesis-related protein synthesis. Plant Physiol. 114, 1113–1121.

Stirpe, F., Barbieri, L., Battelli, M.G., Soria, M., Lappi, D.A., 1992. Ribosome-inactivating proteins from plants: present status and future prospects. Biotech. 10, 405–412.

Stirpe, F., 2004. Ribosome inactivating proteins. Toxicon 44, 371–383.

Taylor, S., Massiah, A., Lomonossof, G., Roerts, L.M., Lord, J.M., Hartley, M., 1994. Correlation between activities of tobacco ribosome-inactivating proteins in depurination of tobacco ribosomes and inhibition of tobacco mosaic virus. Plant J. 5, 827–835.

Tumer, N.E., Hwang, D.J., Bonness, M., 1997. C-terminal deletion mutant of pokeweed antiviral protein inhibits viral infection but does not depurinate host ribosomes. Proc. Natl. Acad. Sci. USA 94, 3866–3871.

Tumer, N.E., Hudak, R.D.R., Coetzer, C., Wang, P., Zoubenko, O., 1999. Pokeweed antiviral protein and its application. In: Hammond, J., McGarvey, P., Yusoboy, V. (Eds.), Current Topics in Microbiology and Immunology, Plant Biotechnology: New Products and Applications, vol. 240. Springer-Verlag, Berlin, Heidelberg, pp. 139–158.

Van Damme, E.J.M., Hao, Q., Chen, Y., Barre, A., Vandenbussche, F., Desmyter, S., Roughe, P., Peumans, W.J., 2001. Ribosome-inactivating proteins: a family of plant proteins that do more than inactivate ribosomes. Crit. Rev. Plant Sci. 20, 395–465.

Vandenbussche, F., Desmyter, S., Ciani, M., Proost, P., Peumans, W.J., VanDamme, E.J.M., 2004. Analysis of the in planta antiviral activity of elderberry ribosome-inactivating proteins. Eur. J. Biochem. 271, 1508–1515.

Verma, H.N., Srivastava, S., Kumar, V., Kumar, D., 1996. Induction of systemic resistance in plants against viruses by a basic protein from *Clerodendrum aculeatum* leaves. Biochem. Cell Biol. 86, 485–491.

Yen, X., Green, J., 1991. Identification and the properties of the major ribonucleases of *Arabidopsis thaliana*. Plant Physiol. 97, 1487–1493.

Wang, P., Hudak, K., 2003. Applications of plant antiviral proteins. In: Setlow, J.K. (Ed.), Genetic Engineering, Principles and Methods, vol. 25. Kluwer Academic Publishers, USA, pp. 143–161.

Zoubenko, O., Hudak, K., Tumer, N., 2000. A nontoxic pokeweed antiviral protein mutant inhibits pathogen infection via a novel salicylic acid independent pathway. Plant Mol. Biol. 44, 219–229.