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Cloning and Expression of Small cDNA Fragment Encoding Strong Antiviral Peptide from *Celosia cristata* in *Escherichia coli*

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Abstract—A small cDNA fragment containing a ribosome-inactivating site was isolated from the leaf cDNA population of *Celosia cristata* by polymerase chain reaction (PCR). PCR was conducted linearly using a degenerate primer designed from the partially conserved peptide of ribosome-inactivating/antiviral proteins. Sequence analysis showed that it is 150 bp in length. The cDNA fragment was then cloned in a bacterial expression vector and expressed in *Escherichia coli* as a ~57 kD fused protein, and its presence was further confirmed by Western blot analysis. The recombinant protein was purified by affinity chromatography. The purified product showed strong antiviral activity towards tobacco mosaic virus on host plant leaves, *Nicotiana glutinosa*, indicating the presence of a putative antiviral determinant in the isolated cDNA product. It is speculated that antiviral site is at, or is separate but very close to, the ribosome-inactivating site. We nominate this short cDNA fragment reported here as a good candidate to investigate further the location of the antiviral determinants. The isolated cDNA sequence was submitted to EMBL databases under accession number of AJ535714.

Key words: *Celosia cristata*, antiviral protein, ribosome-inactivating protein, cloning, expression

Many naturally virus-resistant higher plants contain proteins/glycoproteins in their crude extracts that inhibit virus infection when applied exogenously on host plant leaves or when expressed endogenously in transgenic plants [1-13]. These inhibitors are known as antiviral proteins (AVPs). Most of the well-characterized plant AVPs belong to type-1 ribosome-inactivating proteins (holo-RIPs, which usually consist of single functional polypeptide, RNA N-glycosidase). But the antiviral determinants and whether the ribosome-inactivation is responsible for the antiviral mechanism need to be understood.

Analysis of the antiviral activity in a number of pokeweed (*Phytolacca americana*) antiviral protein (PAP) mutants in transgenic plants resulted in the new findings in this area. Analysis of transgenic plants expressing PAPc, a C-terminal deletion mutant with an intact RNA N-glycosidase site, showed that they were resistant to virus infection [14]. An active site mutant of pokeweed antiviral protein, PAPx, did not protect transgenic plants against viruses [15]. In both cases, ribosomes from the transgenic plants were intact and their rRNAs had not been depurinated [15]. Therefore, it was suggested that the ribosome inactivation by PAP or other ribosome-inactivating proteins is not responsible for their antiviral activities. Similarly, a PAP mutant with two amino acid changes in the N-terminal part of the protein exhibited antiviral activity in transgenic tobacco plants without inactivation of the host ribosomes [8]. Another mutant of PAP with the single amino acid substitution Gly75—Asp, PAPn, offered transgenic tobacco plants resistance against infection with viruses but did not depurinate tobacco rRNAs *in vitro* [16]. Generation of pokeweed antiviral protein mutations in *Saccharomyces cerevisiae* showed that ribosome depurination is not sufficient for cytotoxicity [17] and also for antiviral activity [14]. All these results together indicate that the documented functionally active RIP sequence may not be sufficient for antiviral activity or, on the other hand, the ribosome-inactivation could not be the only mechanism responsible for antiviral activity. Keeping this in view, we supposed that theses suggestions could be confirmed by cloning a short DNA fragment containing the functionally active site of ribosome-inactivating proteins and analyzing its antiviral activity.

Celosia cristata is an ornamental plant belonging to the family Amaranthaceae, and its leaf extract had been shown to contain antiviral agents and two growth-dependent AVPs (CCP-25 and CCP-27) have been puri-

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fied earlier [18, 19]. But no antiviral DNA sequences have been identified from this plant. We cloned one small cDNA fragment from the leaves of this plant that exhibits strong antiviral activity towards tobacco mosaic virus (TMV) being expressed as recombinant protein without affecting the bacterial growth. Our experiment revealed that the antiviral determinants might be at or is very close to the ribosome-inactivating functional peptide. We also expressed the cloned cDNA fragment in *Escherichia coli* and purified it as a fused protein.

MATERIALS AND METHODS

RNA isolation and cDNA synthesis. Total cellular RNA was isolated from the leaves of *Celosia cristata* grown in a growth chamber using Trizol reagent according to the manufacturer's instructions (Gibco BRL, USA, Cat. No. 15596-013). Poly(A)$^+$ RNA was purified from total RNA using a Qiagen (USA) mRNA purification kit (Cat. No. 70022). From the mRNA population the double-stranded cDNA were synthesized according to the protocol of Promega (USA) cDNA synthesis kit (Cat. No. C4360).

PCR-amplification of cDNA fragments by a RIP specific primer. Polymerase chain reaction was conducted linearly to obtain cDNA fragment(s), which contain(s) active peptide of RIPs/AVPs, using a degenerate primer (5′-TNC/AC/TC/AATT/CCAAAT/GGGTTGCA/TGAAGCAGCTCGA-3′) designed from the partially conserved functional peptide sequence of RIPs/AVPs and cDNA population as template. PCR reaction was carried out by mixing the following components in a 0.2 ml PCR tube; primer, 100 pmol (1 μl); cDNA, 50 ng (1 μl); PCR reaction buffer (10×), 2.5 μl; 10 mM dNTP, 1 μl; H_2O, 18.5 μl; and *Taq* polymerase, 1 μl. The reaction mixture was processed in thermocycler (PTC 5000, USA) under the following cycling program: denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 2 min. The PCR product was analyzed on a 1.2% agarose gel. The amplified fragments were cloned in pGEM-T Easy Vector (Cat. No. TM 042, Promega) and transformed to *E. coli* JM109. Transformants were grown in isopropyl β-D-thiogalactopyranoside (IPTG)/X-gal media and single recombinant colonies were selected and processed for plasmid isolation using the alkaline lysis method [20]. The isolated plasmids were digested with *Eco*RI restriction enzyme and separated on 0.8% agarose gel.

Sequencing of the amplified fragments. Sequencing was done according to Sanger method and the reaction was carried out using Reader^{TM} DNA sequencing protocol (Fermentas, USA, Cat. No. K1811). The reaction mixture was run on 8% acrylamide/urea gel using a sequencing apparatus (BRL, USA). The sequenced cDNA fragments were then searched for similarity with other databases in Gene Bank by computing at the BLAST server developed by NCBI (USA).

Expression of the amplified fragment in *E. coli*. The cDNA fragment was taken out from the pGEM-T Easy Vector by *Eco*RI and cloned in the *Eco*RI site of the pMALc2X expression vector using the pMAL protein fusion and purification system (Cat. No. E8000S; NEB). The recombinant plasmid was transformed to *E. coli* (TB1). To extract crude protein from *E. coli* lysate, 500 ml of rich broth + glucose media containing ampicillin was inoculated with 5 ml of overnight grown culture of single colony transformants and allowed to grow until A_{600} reached ~0.5 (uninduced cells). A 1-ml uninduced sample was taken and to the rest of the culture IPTG was added at a final concentration of 0.3 mM, and the culture was allowed to grow for 2 h more (induced cells). Crude protein was extracted from uninduced, induced, and control (transformants containing initial vector) samples by the freeze-thaw method as described by the kit and using extraction buffer containing 20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM azide, and 10 mM β-mercaptoethanol. The crude protein samples were subjected to SDS-PAGE analysis [21] on 10% gel to analyze the induced recombinant protein.

Western blot analysis. Proteins in crude extracts of control and induced samples were separated on 10% polyacrylamide gel and transferred to nitrocellulose membrane using transfer buffer containing 0.025 M Tris-HCl, 0.192 M glycine, and 20% methanol, pH 8.3. After the transfer, the blot was kept in TBS-BSA buffer (0.02 M

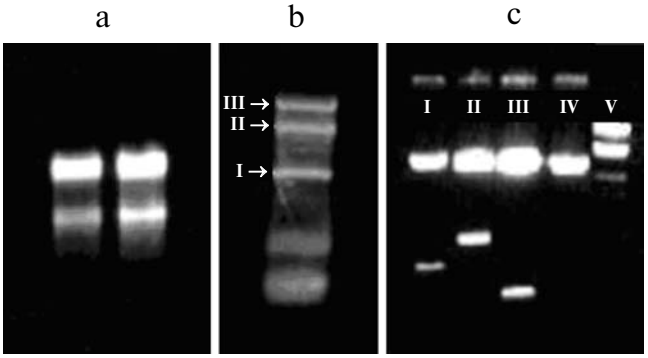

Fig. 1. Isolation of cDNA fragments containing RIP active site. Total RNA was isolated from the leaves of *Celosia* using Trizol reagent (a) and cDNAs were synthesized from the purified mRNAs using a Promega cDNA synthesis kit. PCR reaction was conducted using leaf cDNA as template and degenerate primer designed based on partially conserved functional sequence among different RIP/AVP. Three amplified bands (I, II, and III) were detected on the gel (b). DNA marker was not photographed due to smaller size of the fragments than marker DNA. PCR-amplified partial cDNAs were cloned in pGEM-T Easy Vector and transformed to *E. coli* JM109. Recombinant plasmids were analyzed on 0.8% agarose gel (c): I, II, III) recombinant plasmids; IV) initial plasmid; V) *Eco*RI and *Hind*III digested DNA marker.

Tris-HCl, pH 7.5, 0.5 M NaCl, 1% BSA) overnight at 4°C and then incubated for 2 h with specific antiserum (anti-maltose-binding protein provided by pMALc2X expression and purification system kit; Cat. No. E8000S, NEB) at 1 : 500 ratio in the same buffer at 37°C. After washing with TBS-T (TBS + 0.05% Tween 20) the membrane was incubated for 1 h with alkaline phosphate conjugated goat-rabbit antibody (GAR^{XAP}) which was diluted 1 : 20,000 in TBS-BSA buffer. After extensive washing, the signal band was visualized with a substrate solution containing nitro blue tetrazolium chloride (NBT, 0.33 mg/ml) and 5-bromo-1-chloro-3-indolyl phosphate (BCIP, 0.165 mg/ml) in 0.1 M Tris-HCl buffer, pH 8.5, containing 0.1 M NaCl and 5 mM $MgCl_2$ as described earlier [22].

Purification of recombinant protein. To test the biological activity of the expressed recombinant protein, it was purified from the crude extract of induced sample by maltose-affinity chromatography using an amylose resin column (2.5 × 10 cm). The bound fusion protein was eluted from the column using protein extraction buffer + 10 mM maltose at the flow rate of 1 ml/min. Detailed protocol used for purification has been described in the pMAL protein fusion and purification kit.

Antiviral bioassay. Antiviral activity of the recombinant protein was tested by a local lesion assay using *N.*

Fig. 2. Nucleotide sequence and deduced amino acid sequence of PCR-cloned cDNA fragments. RIP documented active sequence is given in bold.

glutinosa as test plant and tobacco mosaic virus (TMV) for infection. Protein and virus treatments were done as described by Xu et al. [23]. About 50 ng of purified fused product was applied on the test plant leaves with same age, size, and vigor. After 1 h, the treated leaves were washed with distilled water and then inoculated gently with TMV inoculum. As a control, test plant leaves were treated with only buffer and TMV inoculum. Plants were observed for the development of lesions after 3-4 days. The percentage of the lesion inhibition was calculated by the following formula:

$$\% \text{ inhibition} = [1 - \text{No. lesions (induced protein + TMV)}/\text{No. lesions (control + TMV)}] \times 100,$$

where control is crude extract of protein isolated from *E. coli* cells containing non-recombinant plasmid.

RESULTS AND DISCUSSION

Isolation and characterization of cDNA fragment. To clone cDNA fragments containing the conserved ribosomal inactivating site, polymerase chain reaction was performed linearly from *C. cristata* leaf cDNA population using a degenerate oligonucleotide designed from the conserved active sequence (AIQMVAEAAR) of RIPs/AVPs. Three amplified fragments were obtained (Fig. 1). These fragments were cloned in pGEM-T Easy Vector and sequenced. Nucleotide and deduced amino acid sequences data showed that they are 150, 268, and 576 bp in length and contain functionally conserved active peptide of ribosome inactivating proteins (Fig. 2). They did not show any homology with other RIPs/AVPs except in the putative active site region. It is well known that there is low homology (11-15%) among characterized RIPs/AVPs except at the putative active site [24].

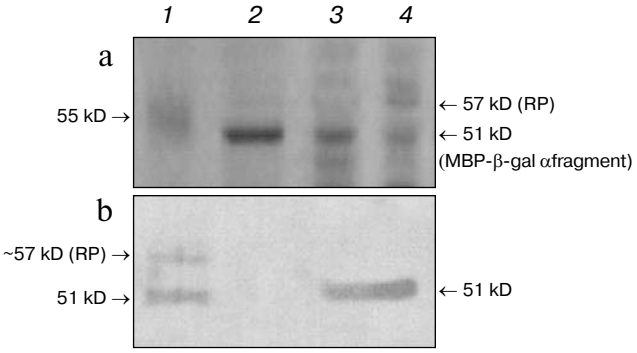

Fig. 3. SDS-PAGE analysis (a) and Western blotting (b) of induced recombinant protein (RP). a) Total protein was extracted from the *E. coli* lysate by the freeze-thaw method. Protein extracts from induced (*4*), uninduced (*3*), and control samples (*2*) (extract of *E. coli* cells containing non-recombinant protein) along with protein molecular weight marker (*1*) were electrophoresed on 10% gel to screen the expressed product. b) The control (transformed cells carrying plasmid without insert) and induced protein samples were subjected to Western blot analysis. The proteins after electrophoresis were blotted onto a nitrocellulose membrane for 2 h at 20 V. The blot was probed with anti-MBP (maltose-binding protein) at 1 : 10,000 dilution and developed by alkaline phosphatase mediated enzyme reaction. A single band corresponding to about 57 kD was detected on the membrane. Left lane, induced sample; right lane, uninduced sample.

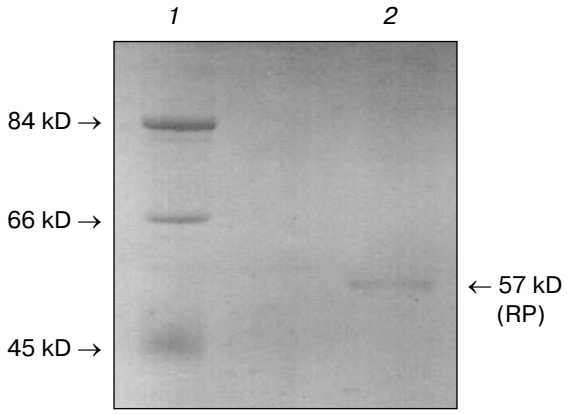

Fig. 4. Purification of recombinant protein (RP). The fusion protein was purified from crude protein extract from *E. coli* TB1 lysate by affinity chromatography using an amylose resin column. The bound fusion protein was eluted from the column by extraction buffer containing 10 mM maltose; 10 μg of purified sample was loaded on 10% polyacrylamide gel and electrophoresed at 150 V for 4 h. Its molecular weight was found to be about 57 kD. Lanes: *1*) molecular weight markers; *2*) the purified recombinant protein sample stained with Coomassie Brilliant Blue R250.

Expression of isolated small cDNA fragment in *E. coli*. To show the functionality of the isolated cDNA fragments, the smallest fragment was selected and expressed in *E. coli* as described in "Materials and Methods". By comparison of induced and non-induced protein patterns on 10% SDS-PAGE, no well detectable band was observed on the gel (Fig. 3). However, the presence of the expressed protein was confirmed by Western blot analysis using antibody against maltose-binding protein (Fig. 3). The molecular weight of expressed recombinant product was found to be about 57 kD. Because the molecular weight of the maltose-binding protein is about 51 kD, therefore the net weight of the isolated cDNA product was considered as an about 6 kD polypeptide, which is consistent with the size of the cloned cDNA (150 bp).

Purification and biological activity test of recombinant protein. The fusion protein was purified from the crude extract using maltose-affinity chromatography. However, the yield of the purification was very low. About 0.05 mg protein per liter of bacterial culture was obtained. The purity of the sample was confirmed by SDS-PAGE analysis (Fig. 4). The probable reasons for low yield of purification could be poor expression of recombinant protein or instability of expressed product

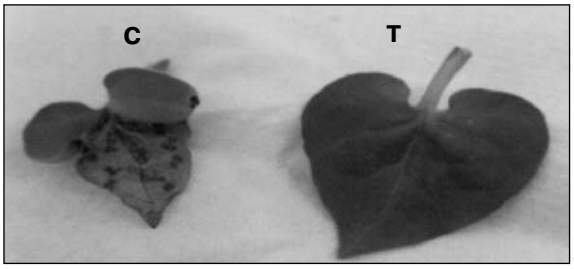

Fig. 5. Antiviral bioassay of recombinant protein. Purified fusion protein was subjected to antiviral bioassay by a local lesion test using tobacco (*N. glutinosa*) as test host plant and tobacco mosaic virus (TMV) for infection. Treatment procedure and formula for calculation of the percentage of the lesion inhibition are described in "Materials and Methods". Leaf C, treated with TMV inoculums and *E. coli* protein lysate carrying initial plasmid; leaf T, treated with TMV inoculums and 50 ng of recombinant protein.

that might have been degraded during protein extraction (Fig. 4).

Biological activity of the purified recombinant protein was tested by local lesion assay on tobacco plant (*N. glutinosa*) using TMV. The leaves treated with purified recombinant protein and TMV showed strong antiviral activity. Serial dilution experiment with purified product showed that this protein at a concentration of 50 ng/ml could results in up to 98% reduction in local lesions (Fig. 5). The inhibitory test was conducted at different concentrations (10, 50, 100 ng/ml) of the purified product. At the concentrations less than 50 ng/ml no significant inhibitions were detected, but at concentrations more than 50 ng/ml of the purified fusion protein the results remained the same. This experiment was repeated 3-4 times to confirm the results. Protein lysate from *E. coli* cells carrying initial plasmid, taken as control, was found to show no inhibitory effect on virus infection.

The fusion protein did not affect the *E. coli* growth and the bacteria grew normally for 5-6 h after addition of IPTG. This indicates that the recombinant protein does not affect *E. coli* ribosomes or may not have ribosome-inactivating property.

If the recombinant protein does not have RIP activity, it is speculated that its antiviral property might be independent of RIP activity. So, our experiment shows that it might be very close to the RIP active sequence. Tumer et al. [14] and Zoubenko et al. [15, 16] expressed different mutants of PAP in transgenic tobacco plants that confer antiviral but not RIP activities, indicating that depurination of host rRNA (ribosome inactivation) is not responsible for inhibition of virus infection. They also concluded that the intact active site of RIPs is pre-requisite for antiviral activity, but it is not essential.

Secondly, if the recombinant protein does not affect the *E. coli* ribosomes but affects the host plant ribosomes, there is a possibility that its RIP activity on tobacco ribosomes is responsible for its antiviral activity. So, in this case the antiviral functional site is the same for as the ribosome-inactivating one. However, our sequence result showed that the isolated cDNA fragment contain RIP active site and exhibit strong antiviral activity as recombinant product without affecting the bacterial growth. Usually ribosome-inactivating proteins have been reported to affect both prokaryotic and eukaryotic ribosomes [23, 25-29]; therefore, we suppose that the antiviral determinants might be different from those of RIPs and these determinants are present in the short cDNA fragment reported here. It is speculated that the present cDNA fragment may be a good candidate for further investigations to find the difference between RIPs and AVPs and their determinants.

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