

Molecular priming of a ribosome-inactivating protein sequence and identification of its structural homology with RNA-binding proteins

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ABSTRACT

A partial cDNA containing putative conserved sequence of ribosome-inactivating proteins was primed from *Celosia cristata* leaf cDNA population and its structure was compared with that of RNA-binding proteins by using bioinformatics tools. As an initial report, the significant homology was identified between the primary and tertiary structures of the isolated RIP domain containing cDNA fragment and RBP. Based on the structural homology results as well as the functional similarity between RIP and RBP in relation to RNA molecule processing and translational gene regulation and inhibition, it was proposed that RIPs may be a specific group of RBPs which target 23s ribosomal RNA and inhibit translation process possibly through the overlapping mechanisms or by exhibiting the different mode of actions but with the same outcomes.

The amplified cDNA sequence was submitted to EMBL databases under accession number HF562933. © 2014 Trade Science Inc. - INDIA

KEYWORDS

Ribosome-inactivating protein;
RNA-binding protein;
Translation.

INTRODUCTION

In eukaryotes, transcription and translation processes are highly controlled by RNA-binding proteins (RBP). These proteins influence every aspects of RNA metabolism including pre-mRNA splicing, mRNA trafficking, stability and its translation to protein. Besides these, there are many RBP that are associated with other classes of RNA such as SnRNA^[1,2].

RBP bind RNA molecules with different RNA-sequence specificities and affinities. They usually exhibit a high degree of modularity and repeated domains at their structural level and so create the RNA-binding and functional diversity within the RBP super-family. All of the RNA-RNP interactions are found to be more impor-

tant to cell physiology and biological situations^[3].

Translational regulation system usually provides a rapid mechanism to control cell physiology during different stages of development or in response to various environmental cues. Trading translation with RNA-binding proteins that globally modify the translational efficiency is remarkable in eukaryotic cells^[1,4]. Recent progress on translational control highlights the complexity and versatility of regulation by RNA-binding proteins. Multi-step overlapping mechanisms are often used to keep the translation process silenced^[4].

Keeping this in view, many plants contain proteins that are able to inactivate ribosomes and accordingly are called ribosome-inactivating proteins (RIP). It has been found that RIPs are RNA N-glycosidases that

inactivate ribosomes and so inhibit translation process through a site-specific deadenylation of the large ribosomal RNA^[5,6]. They are also known to interact with different types of RNA molecules rather than ribosomal RNA^[7]. Different enzymatic and biological activities have been attributed to plant RIP^[8-10]. In addition, intensive efforts are going to provide a structural basis for the known and presumed activities of these proteins. For example: Based on the available data, site-specific RNA N-glycosidase activity toward rRNA, polynucleotide:adenosine glycosidase activity toward tRNA and viral RNA, polynucleotide:guanosine glycosidase activity toward rRNA and the enzymatic ribonuclease activity of ribosome-inactivating proteins are reported^[7]. All of these activities share RNA-binding properties of RIP that is of interest.

Our aim was to unravel the structures, RNA-binding properties and the mechanism of action of the plant RIPs and compare them to the structures and the mode of action of the RBP super-family with regard to translational regulation and inhibition. To date more different classes of RNA-binding proteins are known^[11-14]. But, it is certain that many of them still remain to be characterized. Thus far, several methods have been developed to identify the RBP and the related RNA molecules^[15-17]. Among these methods, bioinformatics approaches as well as the reverse genetic approaches are used as simple and very easy methods to identify the RBP and their target RNA molecules^[18].

In the present work, as a part of our studies using an specific priming procedure and performing simple bioinformatics analysis we propose that RIP are different group of RBP that bind specifically to ribosomal RNA and regulate translational gene expression process.

MATERIALS AND METHODS

Bacterial strains and chemicals

E. coli strain DH5 α was used for bacterial transformation. Plasmid vector pGEM-T easy (Cat. no. A1360; Promega) was used for PCR product cloning. Trizol reagent (Cat. no. RN7713C; RNXTM; CinnaGen) was used for total RNA isolation. mRNA purification kit was provided by QIAGEN, USA (Cat. No.70022).

AcessQuick™ RT-PCR System was purchased from Promega (Cat. no. A1701). Fermentas DNA extraction kit (Cat. no. K0513) was used for the purification of the PCR product from the agarose gel. All of the other chemicals used in this research work were of molecular biology grades.

Plant materials

The seeds of *Celosia cristata* were from our laboratory stock. Test plants were allowed to grow under normal laboratory conditions. Experimental materials were collected from leaf tissues and proceeded for the RNA isolation step and RT-PCR reactions.

Total RNA isolation and mRNA purification

Total cellular RNA was separately isolated from the leaves of test plant using Trizol reagent. About 0.2 g of leaf material was fine powdered using liquid N₂ and 2 ml of Trizol reagent was added to homogenize it at room temperature (RT). 200 μ l of chloroform was added to the mixture, mixed for 15 second, incubated on ice for 5 min and centrifuged at 13000g for 15 min. The upper phase was transferred to another tube and RNA was precipitated with an equal volume of isopropanol. The pellet was washed in 1 ml of 75% ethanol, dried at RT and dissolved in 30 μ l RNase-free water. The integrity of the RNA was tested on 1% non-denaturing agarose gel using TBE running buffer. Poly (A⁺) RNA was purified from total RNA using oligo dT-columns according to the provided kit protocol. The integrity of the purified mRNA was also analyzed by electrophoresis using 1% non-denaturing agarose gel. The quantity of the RNA in the starting materials for the next experiments was measured spectrophotometrically^[19].

Primer designing and RT-PCR amplification

Specific degenerate primer (5'TNC/AC/TC/AATT/CCAAAT/GGGTTGCA/TGAAGCAGCTCGA3') for the amplification were designed based on the putative active site of ribosome-inactivating proteins.

For priming the cDNA fragment, RT-PCR reaction was performed using one-step AccessQuick™ RT-PCR System (Cat. no. A1701; Promega). For this, about 0.5 μ g of mRNA sample was mixed with 25 μ l Master Mix (2x) and 1 μ l of correspondent primer set. The mixtures were adjusted to a final volume of 50 μ l using nuclease-free water. The reaction mixtures were incu-

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bated at 45°C for 45 min and proceeded with PCR cycling. PCR was carried out after a pre-denaturation stage at 95°C for 3 minutes in 25 cycles. The PCR step was performed 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. In the next step, the amplified product was extracted from the agarose gel, cloned in pGEM-T easy cloning vector^[19]. The cloned fragment proceeded for the sequencing in Microsynth DNA sequencing center at Switzerland.

Computational analysis

The nucleotide and deduced amino acid sequences of the isolated cDNA were analyzed by BLAST (Basic Local Alignment Search Tool) server of NCBI at <http://www.ncbi.nlm.nih.gov/blast/>. Conserved domains were identified using the NCBI conserved domain architecture retrieval tool (CDART). Sequence alignments were generated by CLUSTALW software at <http://www.genome.jp/> and *ExPasy* proteomic tools at <http://www.expasy.org/tools/>. Tertiary structure predictions were made using internet-based Phyre v 2.0 server

RESULTS

Priming of RIP cDNA sequence

To clone a cDNA fragment containing the conserved ribosome-inactivating site, RT-PCR reaction was performed linearly from *Celosia cristata* leaf cDNA population using a degenerate oligonucleotide designed from the conserved active sequence (AIQMVAEAAR) of the plant RIPs. This sequence order is the highly conserved region among all the plants ribosome-inactivating proteins. Our comparative sequence analysis on different RIP cDNAs revealed that this site has more greater homology than the other parts which shows about 15-30% homology scores (sequence alignment results not presented). Analysis of the RT-PCR end product on 1% agarose gel revealed a detectable amplified band having molecular size of about 250 bp (Figure 1). For further confirmation, the amplified cDNA was cloned on pGEM-T easy vector and followed by nucleotide sequencing.

Bioinformatics analysis

Primary structure analysis of the isolated clone

Sequence analysis of the amplified fragment from

Celosia cristata leaves showed that it is 267 bp in length and contains the conserved peptide of RIPs (Figure 1). By using CDART (conserved domain architecture tool) at NCBI site, the sequence order “VAEAARHPER” was detected to be the homologue of the putative active site peptide of the plant RIPs in the isolated cDNA.

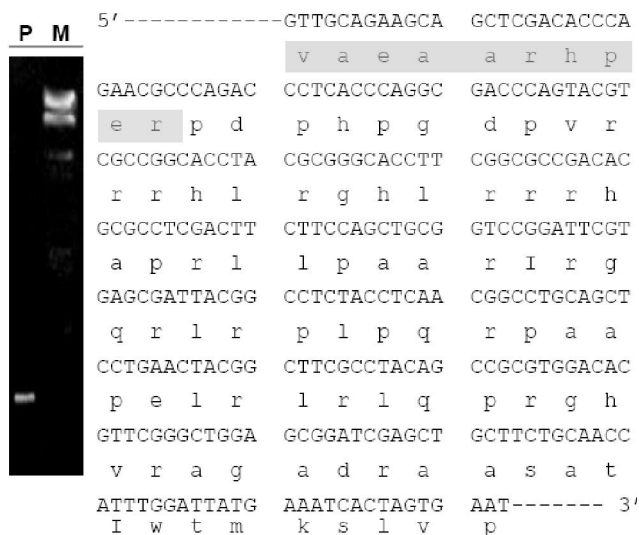


Figure 1 : Analysis of RT-PCR end product. Left: the presence and the size of the amplified cDNA fragment were analyzed on 1 % agarose gel. M: *Eco*RI and *Hind*III double digested lambda DNA marker; P: product of RT-PCR reaction. Right: the nucleotide and deduced amino acid sequences of the isolated RIP peptide containing cDNA is presented. The highlighted conserved RIP sequence is predicted by CDART server at NCBI.

In addition to the existence of conserved RIP motif, our BLAST search data also revealed that the isolated cDNA has got considerable sequence homology with RNA-binding proteins (RBP). The homologous regions were detected to be located at the both termini of *Oryza sativa* RNA-binding protein (Figure 2a). The putative RNA-binding site (RBS) / RNA recognition motif (RRM) of *O. sativa* RNA-binding protein is detected to be located at the N-terminus part while the other conserved peptides were predicted to be located at the C-terminal position of this protein (Figure 2a). The protein domain / motif predictions were made by CDART tool at NCBI. CLUSTALW sequence comparison results between the homologous parts of the isolated *Celosia* cDNA and *O. sativa* RBP showed that the cloned cDNA is more homologue to the carboxyl terminus of *Oryza* RNA-binding protein (Figure

b and c). The homology scores at the N and C-termini were found to be about 23 % and 29 %, respectively.

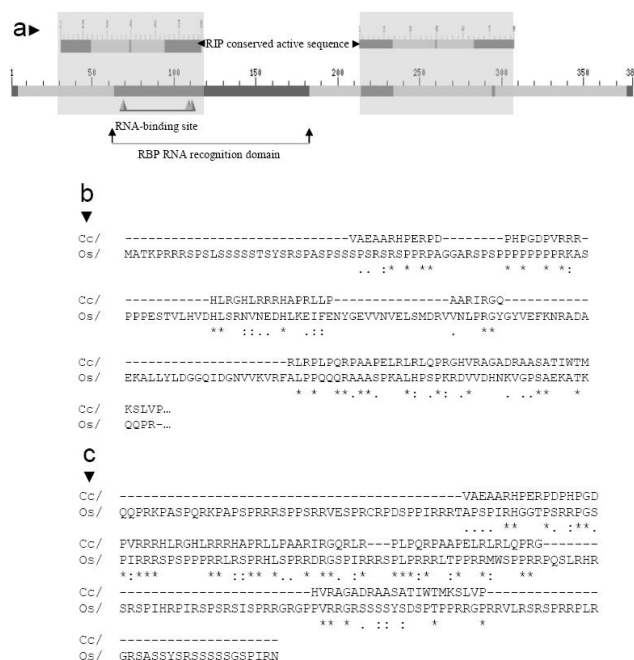


Figure 2 : Comparative analysis of RIP and RBP sequences. a) Conserved regions of *Celosia* RIP cDNA and *O. sativa* RBP (acc. no. NP_001054414) sequences detected by CDART server. The overlapping regions were highlighted, b) Sequence homology between *Celosia* RIP cDNA and the N-terminus region of *Oryza* RBP performed by CLASTALW, c) Sequence comparison between *Celosia* RIP cDNA and the C-terminus region of *Oryza* RBP performed by CLASTALW.

Sequence alignment between RIP and RBP

By using internet-available CLASTALW software, the putative amino acid sequence of the isolated cDNA fragment of *Celosia* was aligned with the RNA-binding protein sequences from different organisms including *Physcomitrella patens* (acc. no. XP_001769794), *Selaginella moellendorffii* (acc. no. XP_002985933), *Ricinus communis* (acc. no. XP_002513413), *Populus trichocarpa* (acc. no. XP_002317618), *Arabidopsis lyrata* (acc. no. XP_002892916), *Oryza sativa* (acc. no. NP_001054414), *Saccoglossus kowalevskii* (acc. no. XP_002732284), *Ixodes scapularis* (acc. no. XP_002406762), *Brugia malayi* (acc. no. XP_001902324). This sequence alignment result furthermore highlighted that the predicted ribosome-binding site of the isolated fragment is homologous to the RNA recognition motif of the RBP (Figure 3). The alignment result clearly showed that there is no additional homologous region between the multiple pro-

tein sequences, except for the known RIP and RBP sites. Analysis of the phylogenetic tree also revealed that *Celosia* fragment is similar to the RBP of different organisms having different evolutionary pathways (Figure 4). The averages of the similarity scores in each evolutionary pathway are shown on the phylogenetic graph.

Comparison of Tertiary structures

To help understand more about the homology between the RIP and RBP proteins, their tertiary structures were predicted and compared to each other. For the better comparison, the likely three dimensional structure of a full-length RIP protein from *Beta vulgaris* (acc. no. 3421353E) was predicted using the internet-based Phyre v 2.0 server and compared to the similarly predicted RBP 3D structure from *Oryza sativa* (acc. no. NP_001054414) (Figure 5). The best templates for the queries were found to be the plant ribosome inactivating protein (PDB Id: d1j1qa) and RNA / export factor binding protein (PDB ID: c2f3ja), respectively. Our comparative observation result showed that the four-stranded beta-sheet package of the RNA recognition motif in RBP protein is homologue to the beta-stranded region of RIP protein. Detailed comparison of these regions showed that the sizes, patterns and the orientations of the beta-strands are similar between two proteins (Figure 5). In this homologous region the Beta-strands are located between two short Alpha-helices that are well detectable and highlighted on the figure.

DISCUSSION

In order to clone the RIP cDNA fragment, we chose *Celosia* plant as experimental material because of its high potential to exhibit RIP activity^[20,21]. *Celosia cristata* is an ornamental plant belonging to the family of *Amaranthaceae*, and its leaf extract has already been shown to contain two growth dependent ribosome-inactivating proteins (RIP; namely CCP-25 and CCP-27)^[20]. Besides this, one small fragment containing putative active site of plant RIPs has also been cloned from the leaves of this plant and exhibited strong antiviral activity towards tobacco mosaic virus (TMV)^[22].

Our BLAST search data as well as the sequence alignment result and phylogenetic graph revealed that

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the isolated cDNA has got considerable sequence homology with RNA-binding proteins. The obtained re-

sults supported the presence of a good enough homology between RIP and RBP sequences, as the overall



Figure 3 : Sequence alignment between RIP and RBPs. Alignment of deduced amino acid sequence of *Celosia* RIP cDNA with the RBP sequences from different organisms were performed using CLASTALW. 1: *Physcomitrella patens* (XP_001769794); 2: *Selaginella moellendorffii* (XP_002985933); 3: *Ricinus communis* (XP_002513413), 4: *Populus trichocarpa* (XP_002317618); 5: *Arabidopsis lyrata* (XP_002892916); 6: *Oryza sativa* (NP_001054414); 7: *Saccoglossus kowalevskii* (XP_002732284); 8: *Ixodes scapularis* (XP_002406762); 9: *Brugia malayi* (XP_001902324); 10: *Celosia cristata*.

sequence similarity scores among different RIPs / RBPs have been observed to be very low (about 15-30 %), except in the region that corresponds to the proposed conserved RNA / ribosome-binding sites with a homology score of about 60 %.

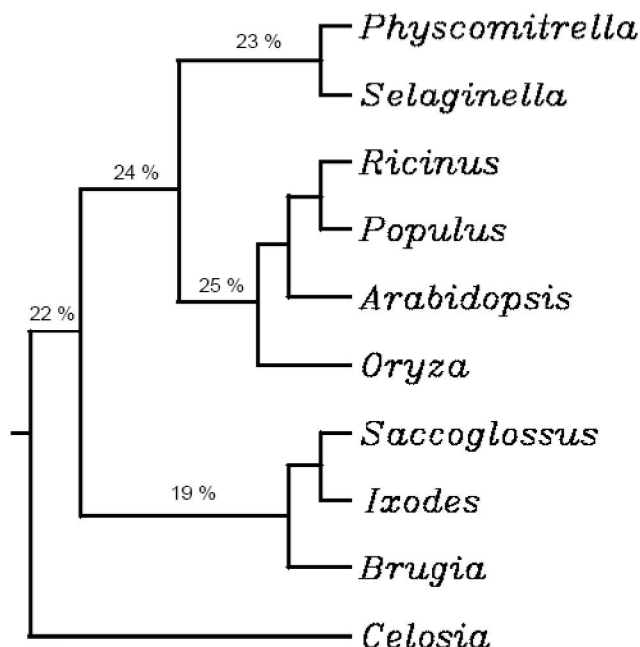


Figure 4 : Analysis of the phylogenetic tree. The phylogenetic tree of the isolated RIP and RBP proteins from different organisms were predicted by CLASTALW software. The averages of the similarity scores in each evolutionary pathway are shown.

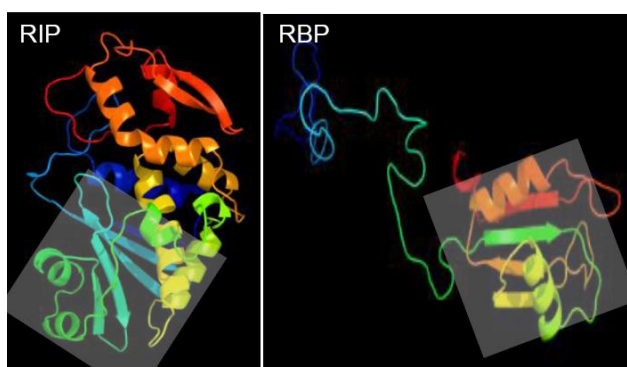


Figure 5 : 3D Structure prediction and comparative analysis. The three dimensional structures of an RIP protein from *Beta vulgaris* (acc. no. 3421353E) and RBP from *O. sativa* (acc. no. NP_001054414) were predicted based on the crystal structures of a plant RIP (PDB ID: c3h4zc) and RNA / export factor binding protein (PDB ID: c2f3jA) by using online Phyre v 2.0 sever. The Overlapping regions are highlighted.

On the bases of the tertiary structural comparison result between typical RRM and RIP domains, we predicted a well homologous part between RNA-binding

and Ribosome-inactivating protein families. Usually, identification of proteins with homologous tertiary structures can provide the strong clues about their possible overlapping functions and mechanisms of action. Therefore, our question now is that whether the understanding the likely structures of RIP and RBP enable us to compare and propose the potential similarities with regard to their mechanisms of action? It is to our knowledge that RRM (RNA recognition motif), also known as RBD (RNA binding domain) or RNP (ribonucleoprotein domain), is a highly abundant domain in eukaryotes found in proteins involved in post-transcriptional gene expression processes including mRNA and rRNA processing, RNA export, and RNA stability^[2,3]. This domain is 90 amino acids in length and consists of a four-stranded beta-sheet packed against two alpha-helices. RRM usually interacts with ssRNA, but is also known to interact with ssDNA as well as proteins.

On the other hand, Ribosome-inactivating domain is found in a typical plant protein family called ribosome-inactivating proteins (RIP). These proteins are known to be RNA N-glycosidases that inactivate ribosomes through a site-specific deadenylation of the large ribosomal RNA. They make susceptible ribosomes impaired in translational elongation processes and so are a group of translational inhibitors^[23,24]. It has become evident that RIP are also capable of inactivating many non-ribosomal nucleic acid substrates and hence has got polynucleotide:adenosine glycosidase activity towards non-ribosomal RNA and DNA molecules^[6,7].

As a first report, by using sequence priming and simple bioinformatics analysis tools, we suggested that there is a structural homology between ribosome-inactivating and RNA-binding proteins. Since these two protein families interact with the structurally similar substrates and they may possess the similar functional process including the RNA cleavage and translational inhibition, therefore their structural / functional homology could be expectable. In this regard, the one explanation is that RBP and RIP use the overlapping mechanisms to influence their homologous substrates. Besides this, they can exhibit the different mode of actions but with the same outcomes that may depend on the binding context and the composition of the ribonucleo-protein particle at the time of binding. Therefore, it is recommended to be much learned about the understand-

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ing on how RBP and RIP affect ribonucleic acids and ribosomal scanning and translational process. It needs to be identified that whether they share their mechanism of actions or not? The multi-step mechanisms of RNA molecules binding, their processing and translation is predicted to be shared between RNA-binding and ribosome-inactivating proteins (Figure 6).

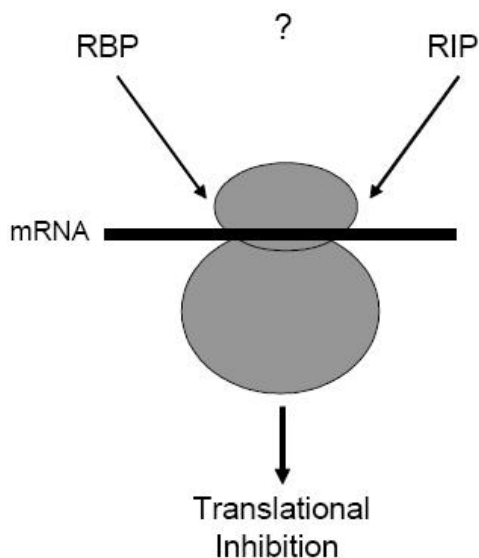


Figure 6 : Schematic representation of similar functional outcomes between RIP and RBP.

Despite our initial report, the more research on these aspects will help us to understand about the molecular structures and the functions of RBP and RIP in the biological systems in the future investigations.

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