Type-1 ribosome-inactivating protein from iris (*Iris hollandica* var. Professor Blaauw) binds specific genomic DNA fragments

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The capacity of IRIP, a type-1 ribosome-inactivating protein (RIP) isolated from the bulbs of *Iris hollandica*, to bind specific DNA sequences from a mixture of approx. 200 bp (average length) fragments of total genomic DNA from *Iris* genome was studied. Fragments that were preferentially bound by IRIP were enriched by several cycles of affinity binding and PCR, and were cloned and sequenced. The selected DNA fragments do not share conserved sequences, indicating that IRIP does not bind DNA fragments in a strictly sequence-specific manner. According to sequence analysis, most IRIP-bound fragments contain one or more possible free energy-stable hairpin structure(s) in their secondary structure, which may be the basis for recognition

between IRIP and these DNA fragments. Some, but not all, DNA fragments moderately lower the RNA N-glycosidase activity of IRIP towards rabbit reticulocyte lysate ribosomes. IRIP does not remove adenines from the binding fragments, which implies that it does not act as a polynucleotide:adenosine glycosidase towards these DNA fragments. The selective binding of IRIP to conspecific DNA fragments is also discussed in view of the novel concept that RIPs may act as DNA-binding proteins with a regulatory activity on gene expression.

Key words: DNA-binding protein, gene regulation, *in vitro* selection, protein–DNA interaction.

INTRODUCTION

Ribosome-inactivating proteins (RIPs) (also known as polynucleotide:adenosine glycosidases; PAGs) are a family of plant proteins with strong inhibitory activity of protein synthesis [1]. Based on their molecular structure, RIPs are divided into three families [2]. Type-1 RIPs, which consist of a single polypeptide chain with a molecular mass of approx. 30 000 Da, show a wide spectrum of activities toward several nucleic acid substrates, such as ribosomal RNA from different sources, DNA and poly(A) [3–5]. They can remove one or more adenines from the nucleic acid substrates, thus damaging the nucleic acids and affecting their biological activities [6]. Type-2 RIPs are built up of one, two or four protomers, each consisting of two functional peptides [7]. The A chain is similar to the type-1 RIPs, whereas the B chain possesses carbohydrate-binding activity. Though not required for enzymic activity, the additional B chain is helpful for type-2 RIPs to recognize and bind specific receptors on the cell membrane and subsequently to enter the cell [8]. Hitherto, only one type-3 RIP has been identified. The so-called JIP60 [9] from barley leaves is a single-chain protein of 60 kDa, consisting of an N-terminal domain similar to type-1 RIPs linked to an unrelated C-terminal domain with unknown function.

The apparent widespread occurrence of RIPs in flowering plants raises the question of their biological role(s) in plants. Though it has been demonstrated that most probably all RIPs can act on conspecific ribosomes *in vitro* [10,11], it is still unknown whether RIPs damage ribosomes *in planta*. If so, the questions have to be addressed as to what the purpose of the *in planta* inactivation of ribosomes is, and how plants can protect themselves against undesired damage of ribosomes by these toxic proteins [12]. One possible mechanism to prevent RIPs killing the

plant cell is to separate the RIPs and ribosomes into different cell compartments. Alternatively, plants can synthesize enzymically inactive forms of RIPs that are activated only under certain conditions [13]. There is also a possibility that some unknown proteins or nucleic acids can bind and regulate the activity of RIPs *in vivo*. A complex form of pokeweed antiviral protein with an unknown protein factor was identified in the leaves of pokeweed, and this complex shows reduced activity as compared with the native pokeweed antiviral protein [14]. Recently developed *in vitro* genetics and binding site selection techniques allow the isolation from a nucleic acid library of those nucleic acid fragments that can bind with specific protein ligands (for review see [15] and references therein). RNA molecules that can specifically bind and inhibit a type-1 RIP from *Cucurbita pepo* and the A chain of a type-2 RIP from *Ricinus communis* have been selected from a pool of random RNA sequences [16,17]. These RNA sequences, which were shown to inhibit the RNA N-glycosidase activity of the respective RIPs, were quite different from each other and showed no sequence homology with the sequence of the toxin-substrate domain (i.e. the so-called sarcin/ricin loop) in rat 28 S rRNA. In contrast with RNA sequences, no evidence has been reported yet that conspecific plant DNA sequences can bind and affect RIP activity.

A few years ago [18] one of the predominant proteins from bulbs of *Iris hollandica* was identified as a highly active type-1 RIP (called IRIP). This abundant, well-characterized type-1 RIP was used as a model to study the *in vitro* interactions between a RIP and DNA fragments from the same plant species. Several DNA fragments that specifically bind to IRIP were selected from a pool of conspecific genomic DNA fragments and sequenced. Some, but not all, selected fragments inhibit the RNA N-glycosidase activity of IRIP. None of the bound DNA fragments

Abbreviations used: RIP, ribosome-inactivating protein; IRIP, *Iris* type-1 RIP; PAG, polynucleotide:adenosine glycosidase.

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can be used as a substrate by IRIP. The selective binding of IRIP to conspecific non-substrate DNA sequences containing stable hairpin structures suggests that RIPs may act as DNA-binding proteins with a regulatory activity on gene expression.

EXPERIMENTAL

Proteins, antibodies and enzymes

IRIP was isolated as described previously [18] and checked for purity by SDS/PAGE. Primary antibodies against IRIP were raised in male New Zealand White rabbits. Rabbits were injected subcutaneously with 1 mg of purified protein dissolved in PBS buffer and emulsified in 1 ml of Freund's complete adjuvant. Four booster injections (with 1 mg of purified protein dissolved in PBS) were given with 10-day intervals. Ten days after the final injection, blood was collected from an ear marginal vein. Serum was collected by centrifugation and the antibodies purified by affinity chromatography on a Sepharose 4B column with immobilized IRIP. The monospecificity of the antiserum was checked by Western-blot analysis as described in [19].

The restriction enzymes, kinase and ligase were obtained either from Roche Diagnostics (Mannheim, Germany) or MBI Fermentas GmbH (St Leon-Rot, Germany).

Isolation of genomic DNA

Total genomic DNA of *Iris* was prepared from young shoots buried within resting *Iris* bulbs. The plant tissue was ground in liquid N₂ with mortar and pestle, and incubated with prewarmed (at 65 °C) DNA isolation buffer [1.0 % sarcosyl, 0.8 M NaCl, 0.022 M EDTA, pH 8.0, 0.22 M Tris/HCl, pH 7.8, 0.8 % cetyltrimethylammonium bromide ('CTAB'), 0.14 M mannitol and 0.014 % 2-mercaptoethanol]. An equal volume of chloroform/isopropylalcohol was added, and the tube was inverted gently to form an emulsion. After incubation for 10 min with occasional gentle inversion, the mixture was centrifuged at 4000 g for 5 min to separate the phases. The aqueous layer was transferred to a new centrifuge tube and an equal volume of isopropanol was added. After standing at room temperature for 5 min, the DNA was precipitated by centrifugation at 4000 g for 5 min and dissolved in double-distilled water. The DNA preparation was then treated with RNase to remove contaminating RNA and extracted with phenol/chloroform to remove any protein. The concentration of DNA was estimated by reading the A₂₆₀. The DNA was stored in small aliquots at -20 °C until use.

Preparation of genomic DNA fragments

The strategy for the preparation of small genomic DNA fragments was based on the methodology of Kinzler and Vogelstein [20]. Genomic DNA was first thoroughly cleaved with *Mbo*I to produce small DNA fragments. The average fragment length after this treatment was approx. 200 bp (ranging from 50–2000 bp as determined by agarose gel electrophoresis). Subsequently, 20 bp of complementary catch-linkers were ligated to the DNA fragments. The linkers were synthesized by Eurogentec (Seraing, Belgium), their sequences are: 5'-GAG TAG AAT TCT AAT ATC TC-3' (oligonucleotide 1) and 5'-GAG ATA TTA GAA TTC TAC TC-3' (oligonucleotide 2). The small DNA fragments were treated with kinase, exposed to the Klenow fragment of DNA polymerase I to generate blunt ends, and ligated to the double-stranded kinase-treated catch-linkers. The catch-linked DNA fragments were incubated with *Xho*I to cleave the self-ligation of the catch-linkers and subsequently used for binding experiments.

Binding of genomic DNA fragments to IRIP

Typical binding reactions contained in a final volume of 10 µl: 2 µM IRIP in binding buffer (25 mM Tris/HCl, pH 7.4, 25 mM KCl, 5 mM MgCl₂, 2 mg/ml BSA and 1 mM dithiothreitol) and 1–5 µg of catch-linked DNA of *Iris*. The amount of DNA was decreased gradually from the first to the fourth selection cycle in order to increase the stringency of the selection. After incubation at room temperature for 30 min, monospecific polyclonal antibodies against IRIP and immunoprecipitation buffer (25 mM Tris/HCl, pH 7.4, 25 mM KCl, 5 mM MgCl₂, 1 % Triton X-100 and 0.05 % SDS) were added. After incubation for an additional hour at 37 °C, IRIP–DNA–antibody complexes were pelleted by centrifugation at 13000 g for 20 min. The pellet and tube walls were carefully rinsed three times with immunoprecipitation buffer. The DNA was then separated from the protein by three–five extractions with phenol/chloroform and precipitated overnight with ethanol and glycogen at -20 °C. This DNA was amplified by PCR and used as the starting material for the next round of binding to IRIP. Four cycles of binding, isolation and amplification were performed. Finally, the selected DNA molecules were either analysed by Southern blotting or cloned into a vector.

PCR, cloning and sequencing of the amplified DNA molecules

PCR reactions were typically performed in a PerkinElmer DNA Thermal Cycler Model 480 in the step-cycle mode. The PCR program consisted of an initial denaturation step at 94 °C for 3 min, followed by 25 cycles of 94 °C for 1 min, 50 °C for 2 min, and 72 °C for 2 min. The final step was an incubation at 72 °C for at least 10 min in order to fulfil the requirement of the TOPO® PCR cloning kit (Invitrogen, Carlsbad, CA, U.S.A.). The amplified DNA from the final PCR reactions was purified by High Pure PCR Product Purification kit (Roche Diagnostics) and cloned into *Escherichia coli* cells using the TOPO® PCR cloning kit according to the manufacturer's instructions. The plasmids were isolated from purified single clones on a miniprep scale using the alkaline lysis method described by Mierendorf and Pfeffer [21]. They were then cleaved with *Eco*RI to check the presence and length of the insertion fragments. Manual sequencing of both ends of the DNA clones was performed by the dideoxy method [22].

Purification of DNA fragments

The plasmids containing the binding sequences were cleaved with *Eco*RI to release the binding sequence, and run on a 1.5 % agarose gel. The gel was stained in 0.5 mg/ml ethidium bromide, and the DNA visualized on an UV transilluminator. DNA fragments were excised from the gel and purified using the Mermaid® kit (for purification of low-molecular-mass DNA fragments) (Bio 101 Inc., La Jolla, CA, U.S.A.).

Sequence analysis

Analysis of sequence similarity between different sequences was performed using the program GenePro (Riverside Scientific, Seattle, WA, U.S.A.). A search for hairpin structures and calculation of the free energy was performed using the HAIRPIN package from the programme PC/GENE (IntelliGenetics, Inc., Mountain View, CA, U.S.A.). The sequences (without catch-linkers) were also compared with entries in GenBank® using a Basic BLAST search (BLAST 2.0 version) [23].

Table 1 Characteristics of the IRIP-binding sequences

Only those matches with an error value < 10 were counted.

DNA-binding sequence	Accession number in GenBank®	Number of bp	Number of possible hairpin structures	Sequence comparison	
				Total number of BLAST hits in database	Number of BLAST hits from plant genome
I1	AF317299	139	0	0	0
I2	AF317308	315	8	44	13
I5	AF317312	89	0	13	1
I7	AF317305	189	2	23	5
I8	AF317306	296	5	9	0
I11	AF317300	146	1	16	0
I12	AF317301	241	7	97	53
I13	AF317302	380	4	35	2
I16	AF317303	154	1	6	0
I17	AF317304	243	4	43	6
I18	AF317307	167	2	7	0
I23	AF317309	194	2	3	0
I25	AF317310	132	3	32	0
I26	AF317311	377	4	35	2

Southern-blot analysis

PCR products of the selected DNA fragments from the first to the fourth selection round were separated by electrophoresis on a 1% agarose gel, transferred to Immobilon N membranes (Millipore, Bedford, MI, U.S.A.) and hybridized at 65 °C. The IRIP-binding DNA sequences isolated from agarose gels were radiolabelled by the random primer method and used as hybridization probes.

Assay for RNA N-glycosidase activity

The RNA N-glycosidase activity of IRIP was determined by the method of Endo et al. [3] with minor modifications. Ribosomes from rabbit reticulocyte lysate were used as a substrate. The purified plasmids containing binding sequences were first cleaved with *Eco*RI to release binding fragments. After the restriction enzyme was inactivated by heating at 75 °C for 5 min, 150 pg of IRIP in 15 µl of water and 10 µl of binding buffer (25 mM Tris/HCl, pH 7.4, 25 mM KCl, 5 mM MgCl₂, 2 mg/ml BSA and 1 mM dithiothreitol) were added to form IRIP–DNA complexes. After standing at room temperature for 30 min, 65 µl of the rabbit reticulocyte lysate and 60 µl of RIP buffer (25 mM Tris/HCl, pH 7.4, 25 mM KCl and 5 mM MgCl₂) were added, and the mixture was incubated at 37 °C for 30 min. SDS (final concentration 0.5%) was added to stop the reaction. RNA was isolated by phenol/chloroform extraction and precipitated by adding 2.5 vol. of ethanol. The recovered RNA was divided into two parts: one part was used as control, and the second part treated with acidic aniline to cleave the so-called Endo's fragment. The Endo's fragment was detected by running the RNA in a 1.2% agarose-formamide gel and visualized by UV detection. To quantify the extent of depurination of the rRNA, the ratio of the Endo's fragment/5.8 S rRNA was determined as described in [24]. Agarose gels were scanned with an AlphaImagerTM 2200 Documentation & Analysis system (Alpha Innotech Corporation, San Leandro, CA, U.S.A.), which allows the total absorbance for each band to be calculated and corrected for the background. This assumes that the 28 S and 5.8 S rRNA are present in equimolar amounts in the ribosomes. A sample from a reaction mixture, in which no DNA fragment was added to

IRIP, was used as a control. The depurination activity of this control sample was defined as 100%. The results presented here are the average of two measurements.

Assay for PAG activity

The possible PAG activity of RIPs on DNA fragments was quantified using the method developed by Zamboni et al. [25], as described by Wang and Tumer [26]. Purified DNA fragments (approx. 2 ng) were incubated with 6 µg of RIP in a 50 µl volume containing RIP buffer at 37 °C for 1 h. After incubation, DNA fragments were precipitated with 2.5 vol. of ethanol. The ethanol-soluble fractions (containing the released adenine residues) were diluted to 1 ml with water, and 0.4 ml of 0.14 M chloroacetaldehyde containing 0.1 M sodium acetate (pH 5) was added to each sample. The samples were incubated at 80 °C for 1 h. After cooling to room temperature, fluorescence was measured in a Spex-25 spectrofluorometer at an excitation wavelength of 310 nm and an emission wavelength of 410 nm. Adenine (Sigma) at concentrations ranging from 1–1200 pmol was used as a standard for the calculation of the adenine concentrations in the samples.

RESULTS

Selection of genomic DNA fragments that bind to IRIP

The strategy used to select DNA fragments that bind to IRIP was essentially the same as that described by Kinzler and Vogelstein [20] for RNA fragments. Genomic DNA from *Iris* was first extensively cleaved with *Mbo*I (recognition sequence GATC) to produce a library of DNA fragments of 50–2000 bp (average length 200 bp). The genomic DNA fragments were then ligated with two linkers containing a half *Xho*I site and one *Eco*RI site to facilitate further amplification and subcloning. Catch-linked DNA fragments were incubated with RIP to generate the RIP–DNA complexes. After incubation, the IRIP–DNA complexes were precipitated by adding specific antibodies against the IRIP to separate the bound DNA fragments from the

Several control experiments were designed to assure the success of this strategy. When catch-linked DNA was incubated with

To confirm the occurrence of the IRIP-binding sequences throughout the selection cycles, the clones encoding I7 and I18 were selected for Southern-blot analysis. The insert encoding I7

Figure 2 Southern-blot analysis of the PCR products of different selection cycles

PCR products from the first to the fourth selection cycle of IRIP-binding sequences (lanes 1–4) were hybridized using the labelled DNA fragment 17.

was purified, labelled with [32 P] α -dCTP and hybridized with the PCR products of the selected DNA fragments from the first to the fourth selection cycle (Figure 2). Clear hybridization signals were observed only with DNA obtained after the third PCR cycle, and became stronger after further selection. When I18 was used as a probe, similar results were obtained except that the hybridization signal appeared only after the fourth selection cycle (results not shown). These results suggest that, at least for some IRIP-binding sequences, the sequences are not abundant and can be isolated only after binding and amplification.

Effect of the binding of DNA fragments on the RNA N-glycosidase activity of IRIP

To assess the possible effect of the binding of DNA fragments on the RNA N-glycosidase activity, the activity of IRIP complexed with the different DNA fragments was compared with that of free IRIP. Only some IRIP-binding DNA fragments moderately reduced the RNA N-glycosidase activity of IRIP (which was quantified by measuring the amount of the generated Endo fragments). DNA fragments I17, I18 and I26, which exhibited the highest inhibitory activity, reduced the activity of IRIP by approx. 30–40%. Other DNA fragments had no effect on the RNA N-glycosidase activity of IRIP. The activity of IRIP was unaffected by an empty vector or a mixture of genomic DNA fragments.

The IRIP-binding DNA fragments cannot serve as a substrate for the PAG activity of IRIP

IRIP not only depurinates ribosomes but also exhibits a high PAG activity towards a whole range of polynucleotides [18]. To check whether IRIP can use the DNA fragments to which it binds as a substrate for its PAG-activity, a fluorimetric assay was used to determine whether adenine is released from the selected DNA fragments after treatment with IRIP. No difference in the amount of free adenine in solution before or after treatment with IRIP was detected, suggesting that the IRIP-binding DNA fragments cannot act as substrate for the PAG activity of IRIP.

The possible PAG-activity can also be detected by treatment of RIP-modified nucleic acid substrates with acidic aniline. When adenine(s) is removed from nucleic acids, the modified site(s) become sensitive to β -elimination, and as a result the nucleic acids are cleaved at the abasic site(s) in the presence of acidic aniline. Several DNA fragments (I7, I12, I18 and I23) were

incubated with IRIP at a 100:1 protein/DNA molar ratio for 1 day at 37 °C, and subsequently treated with acidic aniline. No cleavage products could be detected (results not shown), which confirms that IRIP does not act as a PAG on the IRIP-binding DNA fragments.

DISCUSSION

During the last few years several reports appeared on the DNA-binding activities of RIPs from different plant species. However, all the identified RIP-binding DNA fragments were either derived from plasmid DNA or were oligonucleotides [27–29]. To check whether RIPs have the ability to bind to specific DNA fragments from the plant genome, and, if so, whether these fragments possibly affect the activity of the RIPs, we used a combination of immunoprecipitation and a PCR-associated method to select DNA fragments from the *Iris* genome that have the ability to bind IRIP.

The results of the selection experiments indicate that IRIP selectively binds to some conspecific genomic DNA fragments. Analysis of the RIP-binding DNA fragments revealed little or no conserved DNA sequences, indicating that IRIP does not bind genomic DNA in a strictly sequence-specific manner.

Most of the IRIP-binding DNA fragments have little, if any, effect on the RNA N-glycosidase activity of the enzyme. Only three fragments (I17, I18 and I26) are capable of inhibiting the depurination activity of IRIP on rabbit reticulocyte ribosomes by 30–40%. Interestingly, none of the IRIP-binding DNA fragments serves as a substrate for the PAG activity of IRIP (which has been shown to deadenylate a wide range of nucleic acids) [18]. This implies that the weak inhibition of the RNA N-glycosidase activity of IRIP by some of the DNA fragments is most probably not due to an interference with the catalytic activity of IRIP, but rather due to a binding mechanism, which may cause a steric hindrance or keep the RIP molecule in a low-active or inactive conformation.

The identification of conspecific RIP-binding DNA fragments in the genome of *Iris* has important consequences. First, our results demonstrate for the first time that conspecific genomic DNA fragments are capable of binding and affecting the activity of RIPs. Though no highly conserved motifs could be identified in these DNA fragments, there is no doubt that RIPs bind very selectively to certain fragments, presumably through the recognition of stable hairpin structures in the DNA sequences. Secondly, the binding of IRIP to specific DNA sequences without subsequent deadenylation of the bound sequence may have pronounced effects on the replication and/or transcription of the target DNA. For example, one can imagine that binding of RIP to specific sequences may activate or inactivate specific genes. Such a RIP-mediated regulation of gene expression may explain why some RIPs, such as JIP60 [30] and IRIP (W. J. Peumans, B. Hause and E. J. M. Van Damme, unpublished work), are (partly) located in the nucleus. Moreover, gene inactivation by RIPs may also explain why in many cases the synthesis of RIPs is closely associated with senescence [9,31]. Though these presumed regulatory activities are still speculative, they can explain some biological activities of RIPs that definitely do not rely on their RNA N-glycosidase or PAG activity. For example, the selective toxicity of type-1 RIPs, such as momordica anti-HIV protein-30 ('MAP30') from *Momordica charantia* and gelonin from *Gelonium multiflorum*, towards human/animal cancer cells and cells infected with viruses [32,33], which cannot be explained in terms of a selective enzymic activity [28,34], may rely on the inactivation of crucial genes in these cells. These considerations on a possible novel activity of RIPs justify detailed

studies of RIP–nucleic acid interaction *in vitro*, not only because these studies may give valuable clues to the understanding of the role and mode of action of RIPs *in planta*, but also because they may contribute to an improvement of medical and therapeutical uses of RIPs.

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