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Inducible antiviral activity and rapid production of the Ribosome-Inactivating Protein I from *Phytolacca heterotepala* in tobacco

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Abstract

We studied the *in vitro* and *in planta* antiviral activity of the PhRIP I, a type 1 Ribosome-Inactivating Protein originally purified from leaves of the *Phytolacca heterotepala*. This protein inhibited protein translation in a cell-free assay and limited the local lesion formation from PVX infection on tobacco leaves. We used a transient expression system based on leaf infiltration with recombinant Agrobacteria to show that tobacco can produce a correctly processed PhRIP I enzyme that retains its antiviral activity. Hence, it is possible to rapidly yield in plants a type 1 RIP by means of this transient expression system. To analyse the possible increase of virus resistance in plants, *Nicotiana tabacum* lines that were transformed with the *PhRIP I* coding sequence under the control of the wound-inducible PGIP promoter were challenged by PVX. A significantly lower number of viral lesions compared to untransformed plants was observed only after the induction of the transgene, indicating that the controlled gene expression of an antiviral protein can increase virus resistance.
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1. Introduction

Ribosome-Inactivating Proteins (RIPs) are a family of RNA *N*-glycosidases (EC 3.2.2.22) with a high site-specific deadenylation activity towards the large subunits of ribosomal RNA [1–3]. Following the removal of a single adenine, protein synthesis is inhibited as ribosomes are no longer able to bind Elongation Factors. Therefore, when RIPs succeed in penetrating the cytoplasm, protein synthesis is arrested and the cell eventually dies [1]. Besides rRNA, RIPs deadenylate other substrates such as DNA, and many of them also depurinate natural or synthetic polynucleotides [4]. Consequently, the name polynucleotide adenine glycosylase was proposed for these proteins [5].

RIPs are classically subdivided in three groups according to their molecular structure [2,3]. Many RIPs exist as monomers of around 25–30 kDa (type 1 RIPs), highly active towards ribosomes *in vitro*. Nonetheless, their cytotoxicity is limited by their reduced ability to bind to and enter cells. Type 2 RIPs, present in some plants, have an N-terminal RNA *N*-glycosidase domain similar to type 1 RIPs (the A chain) that is joined to a C-terminal carbohydrate-binding domain (the B chain) through a single disulphide bond. These proteins can easily enter target cells and among them there are some of the most potent cytotoxins. Type 3 RIPs are proteins composed by an N-terminal RNA N-glycosidase domain and an extended C-terminal domain, whose function has not been completely

Abbreviations: ABA, abscisic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetracetic acid; MS, Murashige and Skoog; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate.

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clarified. Type 3 RIPs are synthesised in plants as larger precursors and require proteolytic removal of an internal peptide to be active.

Although different RIPs have been characterized, their biological role in plants is not fully defined [6]. It is believed that these proteins are mainly involved in plant defence [1] as their constitutive expression in transgenic plants increases resistance against different biotic stresses. However, ectopically expressed RIPs (e.g.: PAP, PAPII and trichosanthin) caused an abnormal phenotype in the transgenics, with the highest expression levels resulting in mottled plants with yellow, smaller leaves [7,8]. Such constraint is not universal, as the constitutive expression in plants of some other RIPs is not associated to phenotypic abnormalities [9,10]. Additionally, some mutant RIPs that lack ribonuclease activity proved to be effective as antiviral proteins [11], although their stability and usefulness in different plant species has been questioned by some authors [9,12,13].

A defensive role for RIPs is also supported by the fact that these proteins are present in large quantity in storage organs such as seeds, roots or bulbs [1], because such accumulation should provide an essential advantage against different pathogens. Moreover, RIPs genes are differentially expressed in relation to various conditions and treatments related to stress [1]. For instance, in sugar beet the expression of beetins is induced by viral infection and pathogenesis-related signalling molecule [14]. Among *Phytolacca* plants, it has been reported that the *PIP2* (*Phytolacca insularis* antiviral protein) gene is developmentally regulated and systemically induced in leaves by wounding, jasmonic acid and ABA [15]. However, it has not been determined if the inducible expression of RIPs in plants is able to protect themselves from infecting viruses [15].

Type I RIPs are also of significant interest because of their anti-HIV activity and more generally of their potential for cancer therapy, especially after conjugation with antibodies or other carrier moieties (immuno-toxins) [16]. Although the potential pharmacological properties of RIPs are well-known, further improvements are still needed [17] and to this aim it would be valuable to have suitable heterologous systems to express and purify variant proteins. Unfortunately, owing to their intrinsic cytotoxicity, RIP expression may be problematic in both bacteria and yeasts [18,19]. Despite some known advantages [20], the possibility to use plants as a biosystem for RIP production has been poorly explored [21], probably because high RIP levels are correlated with phytotoxic effects.

The objectives of this work were to characterise the antiviral properties of a type 1 RIP isolated from *Phytolacca heterotepala* (Mexican pokeweed) *in vitro* and *in planta* and to test the feasibility of a rapid transient expression system to produce an active type 1 RIP in tobacco leaves.

2. Materials and methods

2.1. Determination of translation inhibitory activity

Experimental conditions for activity determinations were as follows: reaction mixtures contained, in a final volume of 62.5 μl: 10 mM Tris/HCl buffer (pH 7.4), 100 mM ammonium acetate, 2 mM magnesium acetate, 1 mM ATP, 0.2 mM GTP, 15 mM phosphocreatine, 0.63 μM creatine kinase, 0.05 mM amino acids (minus leucine) (all from Sigma, St. Louis, MO, USA), 89 nCi of L-[^{14}C]-leucine (GE Healthcare, Milano, Italy), scalar concentrations of protein and 25 μl of rabbit reticulocyte lysate. Incubation was at 28 °C for 5 min. The reaction was arrested with 1 ml of 0.1 M KOH, and two drops of H_2O_2 were then added to eliminate possible interferences in β-counter measuring. Proteins were precipitated by adding 1 ml of trichloroacetic acid (20% w/v). Precipitated proteins were collected on glass-fibre discs and the incorporated radioactivity was measured with a β-counter after the addition of 5 ml of Ready Gel scintillation cocktail (Beckman, Milano, Italy) containing 0.7% acetic acid. Data are media (±S.D.) of two experiments carried out in triplicate

2.2. Local lesion assay with the PhRIP I protein

Virus suspension inocula (100 μl) containing 0.5 μg/ml of potato virus X (PVX) and various concentrations of PhRIP I in 50 mM Na–phosphate buffer (pH 7.2) were rubbed on to half leaves of *Nicotiana tabacum* cv. Samsun NN using carborundum (300 mesh) as an abrasive. A PVX inoculum in 50 mM Na–phosphate buffer solution (pH 7.2) was used on each opposite half leaf as a control and rubbed as before. Each treatment was replicated 10 times and randomized on the leaves of the test plants. Lesions were counted 7 days post-inoculation and the percentage inhibition of local lesion formation was calculated using the equation: percent of inhibition = 1 − (number of lesions on RIP + PVX half leaf/number of lesions on PVX control half leaf) × 100 [22]. The statistical significance of the results was evaluated by a *t*-test.

2.3. Construction of plant expression vectors

For transient expression experiments the PhRIP I cDNA was cloned into a high copy number binary vector of the pGreen series [23] using standard molecular techniques [24]. Unless stated otherwise, all enzymes were purchased from Promega (Milano, Italy) and were used according to the manufacturer's recommendations. Firstly, to mutagenise the *Hin*d III site of the pGreen0029 binary vector, this plasmid was cut with the *Hin*d III restriction enzyme, the ends filled-in by the Klenow fragment of DNA I polymerase, and the resulting molecules self-ligated by a T4 DNA ligase treatment, yielding the pG0029M. Subsequently, the 5′- and 3′-regulatory sequences of the 35S RNA CaMV gene were excised from p35S [23] using *Eco*RV and ligated into a similarly digested pG0029M, yielding the pG2935S. The PhRIP I cDNA coding sequence was amplified using the *Pfu* DNA polymerase (Stratagene, Milano, Italy) adding an *Hin*d III restriction site (bold face) to the 5′ end of both primers, 5HIIIRIP (5′-CTC **AAG CTT** ATG CTT GTG GTG ACA ATA TTC) and 3RIPIII (5′-CTC **AAG CTT** TTA AGA ATT CTT CAA ATA GAT). After *Hin*d III digestion, the PCR fragments were gel purified and cloned into a similarly digested pG2935S, yielding the pG2935SRIP vector. The

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