Ribosome-inactivating and Adenine Polynucleotide Glycosylase Activities in *Mirabilis jalapa* L. Tissues*

Received for publication, December 3, 2001
Published, JBC Papers in Press, February 1, 2002, DOI 10.1074/jbc.M111514200

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Several tissues of *Mirabilis jalapa* L. (Nyctaginaceae) were assayed for inhibition of translation by a rabbit reticulocyte lysate (as a signal of ribosome-inactivating activity) and for adenine DNA glycosylase activity, activities that are both due to the presence of a class of enzymes called ribosome-inactivating proteins (RIPs), currently classified as rRNA N-glycosylases (EC 3.2.2.22). These activities were highest in seed; intermediate in flower bud, immature seed, sepal + gynoecium, leaf, and root; and very low in all other tissues. By cation-exchange chromatography, four protein peaks with inhibitory activity on cell-free translation were identified in extracts from seeds, and two proteins were isolated from peaks 1 and 4, all of which have the properties of single-chain type 1 RIP. One is *Mirabilis* antiviral protein (MAP), so far purified only from roots. The second is a new protein that we propose to call MAP-4. The distribution of MAP and MAP-4 in several tissues was determined with a novel experimental approach based on liquid chromatography/mass spectrometry. The direct enzymatic activity of MAP on several substrates is described here for the first time. MAP depurinated not only rRNA in intact ribosomes, thus inhibiting protein synthesis, but also other polynucleotides such as poly(A), DNA, and tobacco mosaic virus RNA. Autologous DNA was depurinated more extensively than other polynucleotides. Therefore, the enzymatic activity of this protein may be better described as adenine polynucleotide glycosylase activity rather than rRNA N-glycosylase activity. Finally, MAP does not cross-react immunologically with other commonly utilized RIPs.

Ribosome-inactivating proteins (RIPs)\(^1\) from plants may be classified as type 1 or 2 according to their single- or double-chain structure (reviewed in Refs. 1–4). Besides the classical type 1 and 2 RIPs, a 60-kDa RIP (called JIP60) has been identified in barley (*Hordeum vulgare*) that consists of an amino-terminal domain closely related to the RIP enzymatic chain linked to an unrelated carboxyl-terminal domain with unknown function (5); this protein may be classified as type 3 RIP (3). The mechanism of action of this class of proteins became clearer when it was found that ricin and subsequently all RIPs tested release a single adenine residue from ribosomes in a precise position (\(A^{4224}\) in the case of rat liver ribosomes) of a universally conserved GAGA sequence in a peculiar stem-loop structure (review in Ref. 2). They were thus classified as rRNA N-glycosidases (EC 3.2.2.22). Subsequently, it was observed that some RIPs release more than one adenine residue from ribosomes, that others act on RNA species apart from ribosomal RNA and on poly(A), and that all RIPs release adenine from DNA. Thus, the enzymatic activity of RIP was defined as polynucleotide:adenosine glycosidase (6), which we propose to change to adenine polynucleotide glycosylase (APG) in analogy with the EC nomenclature of nucleic acid glycosylases.

Two main biological properties of RIPs, viz. (i) inhibition of multiplication of plant viruses (reviewed in Ref. 4) and (ii) extremely potent cytotoxicity (reviewed in Ref. 1), upon entry into eukaryotic cells, led to several applications. In agriculture, plants were transfected with RIP genes, viz. barley RIP, pokeweed antiviral protein (PAP), trichosanthin, and dianthin, to confer resistance to viruses and fungi (reviewed in Ref. 4). In traditional Chinese medicine, trichosanthin and momordin have been used as abortifacient agents (reviewed in Ref. 1). RIPs are currently under study as therapeutic agents against cancer (review in Ref. 7) and possibly HIV infection (8) after linkage to antibodies (immunotoxins) or other specific carrier molecules to make them selectively toxic to a given type of target cells. One of the unsolved problems in the clinical use of immunotoxins is the immune response elicited against both the mouse monocolonal antibody and the toxic moiety that prevents repeated administrations. This problem can be partially circumvented by the use of immunotoxins prepared with human or humanized antibodies and different RIPs that do not cross-react with each other. Thus, the availability of a set of non-cross-reacting RIPs should be highly valuable in this kind of therapeutic strategy.

A potent antiviral activity was found in extracts from a yellow flower cultivar of *Mirabilis jalapa* L. (Nyctaginaceae) in root, leaf, and stem tissues and in *in vitro* cultured cells (9, 10). From the roots of *M. jalapa*, a protein was then purified that was highly effective in preventing viral infection caused by contact-transmitted virus (11). This protein, named *Mirabilis* antiviral protein (MAP), was later identified as a RIP (12, 13) for its activity on the major rRNA in intact ribosomes.

Little is known about the distribution of RIPs in plant organs and tissues (14, 15) and very little in *M. jalapa* (9). Here, we describe the distribution of both translation inhibitory activity...
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(RIP activity) and, for the first time in any plant, APG activity in the different organs of M. jalapa. From the seeds of this plant, which contain the highest levels of both activities, two RIP isoforms were purified, and the most abundant one was characterized. This protein is identical to the isoform purified from root tissue, MAP (12, 16), and has never been characterized for APG activity. In plants, RIPs may be present with many isoforms in several tissues (PAPs in Phytolacca americana (reviewed in Ref. 17), saponins in Saponaria officinalis (14), and luffins in Luffa cylindraca (15)), often with different yields and biological properties. In this study, the distribution of the two major isoforms of MAP in six tissues was determined, and the hitherto unknown APG activity and immunological properties of MAP are described.

EXPERIMENTAL PROCEDURES

Materials—M. jalapa (red flower cultivar) tissues were collected from plants grown in the garden of the Dipartimento di Patologia Sperimentale, Università di Bologna.1-[U-14C]leucine and 1-[4,5-3H]leucine were from Amersham Biosciences (Buckinghamshire, United Kingdom). Materials for low-pressure chromatography, including calibrating sub-

stances, were from Amersham Biosciences (Uppsala, Sweden). Adenine, tRNA, and electrophoresis markers were from Sigma. Poly(A), genomic RNA from tobacco mosaic virus (TMV), and RNA from Escherichia coli (16S + 23S, M = 1.75 x 10^6) were from Roche Molecular Biochemicals (Mannheim, Germany). Cell culture medium and supplements and all other chemicals were as described in previous work (18). Sera against various RIPs were a gift from Dr. P. Strocchi (University of Bologna). Chloroacetaldehyde was prepared according to McCann et al. (19). All other reagents were of analytical or molecular biology grade and, when possible, RNase-free. Milli-Q water (Millipore Corp., Milford, MA) was used when applicable. DNA from herring sperm (hsDNA) (Sigma) was mechanically sheared and made RNA-free by treatment with DNase-

free RNase A (Roche Molecular Biochemicals) for 2.5 h at 37 °C. DNA was then precipitated in ethanol to remove the enzyme. Genomic DNA from M. jalapa leaves, prepared following the general procedure described by Ausubel et al. (20), was precipitated with iso-

propyl alcohol, resuspended in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, twice phenol-extracted, precipitated with ethanol, and re-
suspended in the same buffer. DNA was then subjected to mechanical shearing and RNase treatment as described for hsDNA. Poly(A) RNA from M. jalapa leaves was obtained by extraction on oligo(dT)-

cellulose as described by Ausubel et al. (20).

Preparation of Crude Extracts and Basic Protein Fractions—Fresh plant materials were frozen in liquid nitrogen, ground in a mortar, and homogenized with a Ultraturrax apparatus (Ika, Staufen, Germany) in cold phosphate-buffered saline (0.14 M NaCl and 5 mM sodium phosph-

ate buffer (pH 7.5)). Mature seeds and sepals were homogenized with an Ultraturrax apparatus (Ika, Staufen, Germany) in cold phosphate buffer (pH 7.0). Tissue homogenates were centrifuged at 10,000 x g at 4 °C for 10 min. Each supernatant was applied to an SP-Sephadex Fast Flow column (15 x 2.5 cm) equilibrated with 10 mM sodium acetate (pH 4.5) at room temperature. The column was washed with 1 volume of the equilibration buffer and then extensively with 5 mM sodium phosphate buffer (pH 7.0), and bound proteins were eluted with 1 M NaCl in the same buffer.

Equilibration buffer and then extensively with 5 mM sodium phosphate buffer (pH 7.0), and bound proteins were eluted with 1 M NaCl in the same buffer. Eluted protein was either dialyzed exhaustively against 100 mM NaCl with 1% of purified MAP was then passed through a CM-Sepharose Fast Flow column (42 x 1.6 cm) equilibrated with the same buffer at room temperature. The column was washed with the equilibration buffer until the absorbance at 280 nm was lowered to the base line and was eluted with 1 liter of a linear (0–300 mM) NaCl gradient in the same buffer (see Fig. 1). Portions fractionated in phosphate-buffered saline were assayed for translation inhibitory activity by a rabbit reticulocyte lysate. Active fractions from peaks indicated as MAP, MAP-2, MAP-3, and MAP-4 were pooled, dialyzed extensively against water at 4 °C, and stored frozen at −80 °C. Analytical Methods—Proteins obtained by cation-exchange chromato-

graphy were analyzed for purity and molecular mass by gel filtration, SDS-PAGE under reducing conditions, and reverse-phase HPLC as described (22). Chromatographic conditions were as follows: protein C_4 reverse-phase column (250 x 4.6 mm; Vydac) equipped with the appropriate pre-column and equilibrated and eluted at 20 °C at 1 ml/min; solvent A, 0.1% trifluoroacetic acid in water; and solvent B, 0.1% tri-

fluoroacetic acid in acetonitrile. The column was equilibrated with 90:10 solvent A/solvent B and loaded with 100 μl of protein sample in water. Bound material was eluted with a linear gradient of solvent B up to 40% in 10 min and then to 60% in 50 min. The effluent was split, 5% was analyzed by electropray ionization mass spectrometry (single quadrupole ZMD, Micromass, Manchester, United Kingdom); and molecular mass was calculated by the maximum entropy algorithm (MaxEnt, Micromass). The remaining effluent was analyzed at 214 nm in a Kontron spectrophotometric monitor. The isoelectric point was determined with Phast system and PhastGel IEF 3–9 (Amersham Biosciences) following the manufacturer’s instructions. N-terminal se-

quencing was performed as described (23). The F_200,000s,cm of purified MAP was determined with water solutions of freeze-dried samples. The immunoreactivity of MAP with antibodies against other RIPs was mea-

sured with an enzyme-linked immnosorbent assay as described previ-

ously (22).

Identification of MAP Isomers in Basic Protein Fractions from M. jalapa Tissues—The identification of MAP isomers in basic protein fractions was performed by LC/MS as described above. Protein was applied at ~100 μg in 200 μl of phosphate-buffered saline.

In Vitro Inhibition of Protein Synthesis by Cell Cultures—Murine 3T3 (fibroblasts) and human HeLa (carcinoma), NB100 (neuroblastoma), and BeWo (choriocarcinoma) cells were maintained as monolayer cultures in RPMI 1640 medium supplemented with antibiotics and 10% fetal calf serum in a humidified atmosphere containing 5% CO2 at 37 °C. Subcultures were obtained by trypsin treatment of confluent cultures. The JM cell line (human monocyte-derived) was grown in suspension and treated with phorbol myristate to induce adhesion as described by Bolognesi et al. (22). Protein synthesis by cells was deter-

mined as described by Ferreras et al. (14). Other experimental details are described in the legend to Fig. 4.

In Vitro Translation by a Rabbit Reticulocyte Lysate—The effect of protein from M. jalapa on translation in a cell-free system (a rabbit reticulocyte lysate) was studied essentially as described by Doly et al. (23). Reaction mixtures contained, in a final volume of 62.5 μl, 10 mM Tris-HCl (pH 7.4), 100 mM ammonium acetate, 2 mM magnesium acetate, 1 mM ATP, 0.2 mM GTP, 15 mM phosphocreatine, 3 μg of creatine kinase, 0.05 mM amino acids (minus leucine), 89 nCi of 1-[U-14C]leucine, and 25 μl of rabbit reticulocyte lysate. Incubation was at 28 °C for 5 min.

Determination of Adenine Polynucleotide Glycosylase Activity—The enzymatic activity of the purified protein was determined by measuring adenine released from various substrates by HPLC (24) essentially following the procedure of McCann et al. (25) as described by Barbieri et al. (26). Reactions were run for 40 min at 30 °C in a final volume of 50 μl containing 100 mM KCl, 50 mM sodium acetate (pH 4.0), increasing concentrations of RIPs, and the indicated amounts of polynucleotide substrates. A standard curve of adenine was run with each experiment. The determination of bases other than adenine was performed as described (26). The determination of adenine released by crude extracts or basic protein fractions was done by LC/MS because of interference of compounds present in crude preparations with the derivatization step of the method described above. Briefly, the reaction was stopped in ice by the addition of 100 μl of ice-cold 10 mM ammonium acetate, and reagents were separated by solid-phase extraction on Bond Elute NH2 microcolumns (Varian) equilibrated with 10 mM ammonium acetate as described (26). Adenine was measured by LC/MS on a Waters Alli-

ance/ZQ apparatus. Chromatography to separate adenine was carried out on a Waters X Terra MS C_18 column (2.1 x 50 mm, 2.5-μm beads) equilibrated with 0.1% TFA and eluted with 0.1% aqueous ammonium AcAc and HAc methanol (solvent B) at 0.3 ml/min at 15 °C. Equilibration was in 98:2 solvent A/solvent B; and after sample injection (120 μl), the column was eluted with equilibration solvents for 2 min and then with 90:10 solvent A/solvent B for 5 min. Tightly bound material was eluted with 20:80 acetonitrile/solvent A for 0.6 min, and equilibration was attained with 90:10 solvent A/solvent B for 1.2 min, followed by 98.2 solvent A/solvent B for 9 min. Mass spectrometric analysis was carried out in positive...
electrospray with a single-ion recording (m/z 135 + 1) on a split flow of ~50 μl/min. Parameters were optimized manually for maximum sensitivity under the present column elution conditions. Duplicate chromatograms were combined to reduce noise using Micromass MaxLynx software. Samples containing standard adenine (from 1 to 300 pmol) were incubated and processed together with the experimental samples. A standard curve for adenine was generated (Fig. 2), and the absorbance at 280 nm was recorded (solid line), and the inhibitory activity on translation by a rabbit reticulocyte lysate system of selected fractions (25 μl at a 1:5000 dilution) is reported (dotted line). Pooled fractions containing MAP, MAP-2, MAP-3, and MAP-4 are indicated by horizontal bars.

RESULTS

Tissue Distribution—The tissue distribution of RIP and APG activities was assayed both in crude extracts from most *M. jalapa* tissues and in basic protein fractions from root, mature shelled seed, immature unshelled seed, green leaf, flower bud, and sepal + gynoecium (Table I). The highest activity was found in mature seed.

Purification from Seeds—The basic protein fraction from 100 g of seeds was loaded onto a CM-Sepharose column. Bound proteins were eluted with a linear NaCl gradient, and the inhibitory activity was resolved into four protein peaks (Fig. 1). The first eluted protein, which is identical to MAP from roots described in the literature at only four amino acids, which are not involved in the putative active site (16), and has a pI >9, like that of most other RIPs; and its E_{280 nm} is 7.06.

Immunological Properties—MAP gave no reaction with antisera specific for seven other RIPs (bouganin, dianthin-32, momordin I, momorcochin-S, PAP-R, saporin-S6, and trichokirin).

Effects on Protein Synthesis—The RIP activities of MAP isoforms in a rabbit reticulocyte lysate are reported in Table IV. Inhibition of protein synthesis by various cell lines (Fig. 4) was observed at concentrations of MAP much higher than those effective on cell-free protein synthesis. The effect varied greatly from one cell line to another, with the IC_{50} values ranging by more than an order of magnitude from the most resistant HeLa cells to the most sensitive JM and BeWo cells.

Adenine Polynucleotide Glycosylase Activity—MAP released adenine in a concentration-dependent manner from all substrates tested, viz. DNA from herring sperm and *M. jalapa*, genomic TMV RNA, poly(A)^+ RNA from *B. dioica*, *E. coli* rRNA, and poly(A) (Fig. 5). Under the present experimental conditions, autologous DNA appeared to be the best substrate, with 24.4 mol of adenine released per mol of enzyme/min, followed

### Table I

| Tissue                  | Crude extract RIP activity | Basic protein fraction RIP activity | % of root activity | Crude extract APG activity | Basic protein fraction APG activity | % of root activity |
|-------------------------|----------------------------|------------------------------------|--------------------|---------------------------|-------------------------------------|--------------------|
| Seed (mature)           | 971                        | 426                                | 1610               | 3300                      |                                     |                    |
| Flower bud              | 322                        | 57                                 | 60                 | 270                       |                                     |                    |
| Leaf (apical)           | 141                        | 0.4                                |                    |                           |                                     |                    |
| Sepal + gynoecium       | 130                        | 1.6                                | 60                 | 40                        |                                     |                    |
| Seed (immature)         | 126                        | 61                                 | 140                | 70                        |                                     |                    |
| Root                    | (100)                      | (100)                              | (100)              | (100)                     |                                     |                    |
| Leaf (yellow)           | 64                         | 57                                 |                    |                           |                                     |                    |
| Leaf (intermediate color)| 64                         | 51                                 |                    |                           |                                     |                    |
| Leaf (green)            | 62                         | 36                                 | 330                | 830                       |                                     |                    |
| Petiole                 | 32                         | 10                                 |                    |                           |                                     |                    |
| Petal + stamen          | 27                         | 0.3                                |                    |                           |                                     |                    |
| Stem                    | 24                         | 21                                 |                    |                           |                                     |                    |
by hsDNA (16.3 mol/mol/min), whereas other substrates were less sensitive (0.6 mol/mol/min for TMV RNA, 0.5 mol/mol/min for poly(A)\textsuperscript{+} RNA, 0.4 mol/mol/min for poly(A), and 0.35 mol/mol/min for rRNA) at the lowest enzyme concentration assayed (Fig. 5). The APG activities of MAP isoforms on hsDNA are determined by the maximum entropy algorithm are reported. Determination error is ±2 mass units.

**FIG. 2.** HPLC analysis of active protein peaks from cation-exchange chromatography. MAP, MAP-2, MAP-3, and MAP-4 were analyzed by reverse-phase HPLC on a VyDAC protein C\textsubscript{18} column as described under "Experimental Procedures." Approximately 100 μg of protein was applied in each run. Molecular masses (in daltons) determined by the maximum entropy algorithm are reported. Determination error is ±2 mass units.

**Distribution**—The distribution of RIP activity in the various tissues of a given plant has been described only for very few species. The antiviral properties of some tissue extracts from *M. jalapa* were studied, and the highest activity was found in roots (9), from which a RIP denominated MAP was purified (11). Here, we have described the distribution, in *M. jalapa* anatomical parts, of both main activities attributed to the group of plant enzymes provisionally called ribosome-inactivating proteins and so far classified as rRNA N-glycosylases, viz. inhibition of *in vitro* translation (RIP activity) and APG activity on various polynucleotides. The last activity has not been described so far in partially purified enzyme preparations due to the interference of several substances present in crude plant extracts with the highly sophisticated methodology involving derivatization of released adenine to its fluorescent derivative, ethenoadenine. Both RIP and APG activities were determined in crude extracts and partially purified samples containing basic proteins. These last preparations were chosen for two main reasons. (i) Recovery of RIP activity was often complete after batch-wise cation-exchange chromatography (27), and (ii) inconsistent results were obtained in measuring glycosylase activity in crude extracts due to interference of nucleases that degrade the substrate and other substances that inhibit enzymatic activity. Distribution of RIP activity was similar in crude extracts and basic protein fractions, whereas it was confirmed that glycosylase activity was sometimes hindered in several crude extracts, as could be inferred by the activities found in basic protein fractions (Table I). The distribution pattern in basic protein fractions is similar for both activities. At least in *M. jalapa* tissues, it may be said that all proteins with APG activity are also ribosome-inactivating proteins. The tissue with the highest activities was mature seed, in good agreement with what was found in the unrelated plant *S. officinalis* (family of Caryophyllaceae) (14). The distribution of RIP activity in *M. jalapa* organs is similar to that of saporins in *S. officinalis*, the only other plant in which RIP distribution has been most thoroughly studied (14). The distribution of activity described here differs somewhat from MAP contents estimated by Kubo et al. (9) with an enzyme-linked immunosorbent assay using anti-MAP antibodies. This difference may be due to the cultivar (red versus yellow flower), to the growing environmental conditions of the plants, or to non-homogeneous reaction with the antisera of the different MAP isoforms. No clear difference in activity was observed in senescent leaf tissue, in contrast to what was observed in other species (28).

**Purification**—From seeds, we purified the most abundant protein with RIP and APG activities together with three other isoforms. The most abundant RIP was analyzed by LC/MS, and the molecular mass obtained was very similar to that described for the major form found in and purified from roots (16, 29): only four amino acids were different, none of them in putative N-terminal portion. The identification of this form as a RIP was rather crude extracts, thus without the inevitable artifacts induced by variable purification yields. Furthermore, the possibility of detecting and identifying specific proteins in preparations subjected to a very limited treatment (basic protein fractions) allows for the determination of isoform distribution in many tissues, even if present in very low quantities.
confirmed by the presence of the invariable residues Tyr, Arg, and Phe, which are totally conserved in all amino-terminal sequences of RIPs reported so far (Fig. 6).

**Immunological Properties**—MAP is not recognized by antibodies against several commonly used RIPs. Thus, this protein, which is easily purified in large enough quantities, may be useful to prepare immunologically distinct immunotoxins to overcome the immune response caused by the in vivo administration of these compounds.
Analysis of Isoforms—Several tissues were then assayed for the presence of the two major isoforms, MAP and MAP-4. The substantial difference in N-terminal sequence (Fig. 6) indicates that they are products of different genes and not just minor variations due to non-significant random mutations. Both isoforms were found in all tissues examined, with the exception of root, in which only MAP could be detected. The absolute amount of each protein and their relative proportions varied from tissue to tissue; thus, it may be inferred that the expression of the relative genes is tissue-regulated. Comparison with the RIP isoforms purified from the closely related plant *Mirabilis expansa* shows that there is a strict similarity between pairs of isoforms from the two plants (MAP/ME2 and MAP-4/ME1), as shown in Fig. 6, a similarity that is much greater than that of the isoforms from the same plant (very low apart from the three invariable Tyr, Arg, and Phe residues). This may allow the classification of the two different pairs as different classes of RIPs, as happens in *S. officinalis* (14). All isoforms have little similarity to bouganin, a RIP isolated from *Bougainvillea spectabilis*, another member of the Nyctaginaceae family (18). Work is in progress to verify whether MAP-4 has enzymatic and substrate specificities different from those of MAP. The existence in the same plant of several forms of RIPs, sometimes functionally different, has been frequently observed (e.g. PAP from *P. americana*, reviewed by Irvin (17); and saporins from *S. officinalis* (14)).

Enzymatic Activities—So far, MAP has been assayed only for activity in translation systems and purified animal ribosomes. Here, we have reported the determination of the direct enzymatic activity of MAP on several substrates, including autologous DNA. MAP depurinated all substrates assayed, including deoxy- and ribonucleic acids. It should be noted that autologous DNA was one of the best substrates; this is the first demonstration of activity of a RIP on the DNA of its own plant. Furthermore, no bases other than adenine were released from hsDNA (data not shown), and >1 mol of adenine/mol of enzyme was released from all substrates without the need for any cofactor, required by some other RIPs to act efficiently on ribosomes (30). MAP belongs to the restricted group active on poly(A) (6). These results indicate that MAP acts catalytically and does not require a highly restricted sequence on purified substrates. Moreover, (i) the activity on poly(A), observed so far...
only in RIPs from Caryophyllales (6) and in MAP, may suggest a role in mRNA post-transcriptional changes; and (ii) the activity on TMV RNA suggests a role in the antiviral activity of MAP.

Cytotoxicity—MAP inhibits protein synthesis more efficiently in a cell-free system than in whole cells, like all type 1 RIPs that are internalized with low efficiency. Toxicity varied depending upon the cell type tested, with HeLa cells being the least sensitive, as it is usually with other RIPs, and JM and BeWo cells being the most sensitive. As JM and BeWo cells are derived from monocots and choriocarcinoma cells, respectively, both with a high pinocytic activity, their high sensitivity could be the consequence of a better cellular uptake of the protein, although differences in intracellular routing cannot be excluded. All lethally intoxicated cells showed the morphological features of cell death by apoptosis (data not shown), as previously described for some other RIPs (31, 32). Cytotoxicity of RIPs has long been attributed entirely to protein synthesis inhibition; however, direct damages to DNA and/or RNA other than ribosomal RNA may also have a role in the induction of apoptosis in xenobiotic cells.

Implications for the Biological Role—Some variations in RIP distribution and content in M. jalapa are to be noted. (i) The levels of RIP and APG activities are 8-fold higher in mature as compared with immature seeds, as observed in S. officinalis (14); and (ii) the ratio between MAP and MAP-4 is in favor of MAP in organs with storage tissues (root and seed), whereas MAP-4 is the prevalent isoform in leaf-related tissues. These variations in activity content point to the question of the function of RIPs in plants, which is still not clear. The notion was put forward that they may be storage proteins and/or defense systems (33); and indeed, they may confer some protection against a broad spectrum of viruses and fungal pathogens, as observed in transgenic plants expressing RIPs (reviewed in Ref. 4). Nevertheless, RIP expression rises in mature seeds and in stressed (5, 28, 34) and virally infected (35) plant tissues.

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*J. Biol. Chem.* 2002, 277:13709-13716.  
doi: 10.1074/jbc.M111514200 originally published online February 1, 2002

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