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Ribosome-inactivating and adenine polynucleotide glycosylase activity in *Mirabilis jalapa* L. tissues

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Running title: RIP from *Mirabilis jalapa*

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SUMMARY

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, 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 activity 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 ribosome-inactivating protein (RIP). One is MAP (*Mirabilis* antiviral protein) 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 upon LC/MS. The direct enzymatic activity of MAP on several substrates is here described 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 TMV 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 (APG) activity rather than rRNA N-glycosylase activity. Finally, MAP does not cross-react immunologically with other commonly utilised RIP.

INTRODUCTION

Ribosome-inactivating proteins (RIP) from plants may be divided into type 1 or type 2 according to their single- or double-chain structure (reviewed in Refs. 1-4). Besides the classical type 1 and 2 RIP, 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 RIP tested, release a single adenine residue from ribosomes in a precise position (A_{4324} in the case of rat liver ribosomes) of a universally conserved GAGA sequence in a peculiar stem and loop structure (review in Ref. 2). They were thus classified as rRNA N-glycosidases (EC 3.2.2.22). Subsequently it has been observed that some RIP release more than one adenine from ribosomes, others act on RNA species apart from ribosomal, and on poly(A), and all RIP 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 acids glycosylases.

Two main biological properties of RIP, namely (i) inhibition of multiplication of plant viruses (reviewed in Ref. 4) and (ii) extremely potent cytotoxicity (reviewed in Ref. 1), when they gain entry into eukaryotic cells, led to several applications. In agriculture, plants were transfected with RIP genes, namely barley RIP, pokeweed antiviral protein (PAP), trichosanthin and dianthin, to confer them resistance to viruses and fungi (reviewed in Ref. 4). In traditional Chinese medicine, trichosanthin and momordin have been used as abortifacient agents in (reviewed in Ref. 1). RIP

are currently under study as therapeutic agents against cancer (review in Ref. 7) and possibly HIV (human immunodeficiency virus) 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 monoclonal 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 RIP that do not cross-react with each other. Thus the availability of a set of non cross-reacting RIP should be highly valuable in this kind of therapeutic strategy.

A potent anti-viral 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* anti-viral protein (MAP), was later identified as a RIP (12,13) for its activity on the major rRNA in intact ribosomes.

Little is known on the distribution of RIP in the plant organs and tissues (14,15) and very little in *M. jalapa* (9). Here we describe the distribution of both translation inhibitory activity (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 level 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, *Mirabilis* antiviral protein (MAP) (12,16), and never characterized for APG activity. In plants, RIP may be present with many isoforms in several tissues (PAPs in *Phytolacca americana*, (reviewed in Ref. 17); saporins in *Saponaria officinalis*

(14); luffins in *Luffa cylindrica* (15)), often with different yields and biological properties. With present studies the distribution in 6 tissues of the two major isoforms of MAP 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. L-[U-^{14}C]leucine and L-[4,5-^{3}H]leucine were from Amersham International, Bucks., U.K. Materials for low-pressure chromatography, including calibrating substances, were from Pharmacia (Uppsala, Sweden). Adenine, tRNA and electrophoresis markers were from Sigma Chemical Co. (St. Louis, MO, USA). Poly(A), genomic RNA from tobacco mosaic virus (TMV) and rRNA from *Escherichia coli* (16S + 23S, mol.wt. 1.75×10^{6}) were from Boehringer GmbH (Mannheim, Germany). Cell culture medium and supplements and all other chemicals were as in previous work (18). Sera against various RIP were a gift from Dr. P. Strocchi, 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, Milford, Mass, USA) was used when applicable. DNA from herring sperm (hsDNA) (Sigma) was mechanically sheared and made RNA-free by treatment with DNase-free RNase A (Boehringer) for 2.5 h at 37°C. DNA was then repeatedly precipitated in ethanol to remove the enzyme. Genomic DNA from *M. jalapa* leaves, prepared following the general procedure described in Ausubel *et al*. (20), was precipitated with isopropylic alcohol, resuspended in 10 mM Tris/HCl, pH 8.0, containing 1 mM EDTA, twice phenol extracted, precipitated with ethanol and resuspended in the same buffer. DNA was then subjected to mechanical shearing and RNase

treatment as described for hsDNA. Poly(A)$^+$RNA from Bryonia dioica leafwas obtained by extraction and oligo(dT)cellulose as described by Ausubel et al. (20).

Preparation of crude extracts and basic protein fractions—Fresh plant material were frozen in liquid nitrogen, ground in a mortar and homogenized with an Ultraturrax apparatus (Ika, Staufen, Germany) in cold phosphate-buffered saline (0.14 M NaCl, 5 mM Na phosphate buffer, pH 7.5, PBS). Mature seeds and sepals+gynoecium were homogenized in 8 ml and all other tissues in 5 ml of cold PBS per g of starting material. The slurries were extracted overnight at 4°C with magnetic stirring, filtered through cheesecloth and clarified by centrifugation at 10000×*g* for 30 min at 4°C (crude extracts). When appropriate, crude extracts were adjusted to pH 4.0 with glacial acetic acid and centrifuged again at 10000×*g* for 30 min at 4°C. The supernatant was applied to an SP Sepharose™ Fast Flow column (15×2.5 cm) equilibrated with 10 mM Na acetate, pH 4.5 at room temperature. The column was washed with 1 vol of the equilibration buffer, then extensively with 5 mM Na 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 water at 4°C (for further preparative processing) or concentrated at 4°C in an Amicon concentrator equipped with a PM 10 membrane under nitrogen pressure (4 bar) and magnetic stirring, and then desalted by chromatography on Sephadex™ G25. These preparations are referred to as basic protein fractions and contain most, if not all, RIP present in the starting material in all plant species and tissues assayed (21).

Purification of ribosome-inactivating protein—The basic protein fraction from 100 g of *M. jalapa* seeds was adjusted to 5 mM phosphate buffer, pH 7.0, and applied to a CM Sepharose™ Fast Flow column (42×1.6 cm) equilibrated with the same buffer at room temperature. The

column was washed with the equilibration buffer until the A_{280} was lowered to baseline and was eluted with 1 l of a linear (0-300 mM) gradient of NaCl in the same buffer (Fig. 1). Fractions appropriately diluted in PBS 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 from cation exchange chromatography were analysed for purity and molecular mass by gel filtration, SDS-PAGE in reducing conditions, and by HPLC reverse phase chromatography as described in Ref. (22). Chromatographic conditions were as follows: Protein C4 (250×4.6 mm) reverse phase column (Vydac), equipped with the appropriate pre-column, equilibrated and eluted at 20°C at 1 ml/min; solvent A: 0.1% trifluoroacetic acid (TFA) in water; solvent B: 0.1% TFA in acetonitrile (ACN). The column was equilibrated with 90/10 (A/B), loaded with 100 µl of protein sample in water. Bound material was eluted with a linear gradient of buffer B up to 40 % in 10 min and then to 60 % in 50 min. The effluent was split, 5% was analysed by ESI^+-MS (single quadrupole ZMD from Micromass, U.K.) and molecular mass calculated by maximum enthropy algorithm (MaxEnt, Micromass), whilst the remaining effluent was analyzed at 214 nm in a Kontron spectrophometric monitor. Isoelectric point was determined with Phast System and PhastGel IEF 3-9 (Pharmacia), following the manufacturer's instructions. N-terminal sequence was performed as described in (23). The $E^{1\%}_{280nm,1cm}$ of purified MAP was determined with water solutions of freeze-dried samples. The cross-reactivity of MAP with antibodies against other RIP was measured with an ELISA assay as described previously (22).

Identification of MAP isoforms in basic protein fractions from M. jalapa *tissues*—Identification of MAP isoforms in basic protein fractions was performed by LC/MS as described above. Protein applied was approximately 100 µg in 200 µl of PBS.

In vitro inhibition of protein synthesis by cell cultures—Murine 3T3 (fibroblasts), and human HeLa (carcinoma), NB100 (neuroblastoma) and BeWo (chorioncarcinoma), were maintained as monolayer cultures in RPMI 1640 medium supplemented with antibiotics and 10% foetal calf serum, in a humidified atmosphere containing 5% CO_2, 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 determined 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 Parente *et al*. (23). 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, 3 µg of creatine kinase, 0.05 mM amino acids (minus leucine), 89 nCi of L-[U-^{14}C]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 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 Na acetate, pH 4.0, increasing concentrations of RIP and the indicated amount of polynucleotide substrates. A standard curve of adenine was run with each experiment. Determination of bases other than adenine was performed as described before (26). 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 derivatisation 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 NH_2 Bond Elute mini columns (Varian), equilibrated with 10 mM ammonium acetate as described in (26). Adenine was measured by LC/MS on a Waters Alliance/zq apparatus. Chromatography to separate adenine was on a Waters XTerra MS C18 column (2.1× 50 mm, 2.5 µm beads), equilibrated and eluted in 10 mM ammonium acetate (solvent A)/methanol (solvent B) at 0.3 ml/min at 15 C. Equilibration was in 98/2 (A/B), and after sample injection (120 µl) the column was eluted (2 min) with equilibration solvents, and then with 90/10 (A/B) for 5 min. Tightly bound material was then eluted with 20/80 (acetonitrile/A) (0.6 min) and equilibration was obtained with 1.2 min of 90/10 followed by 9 min of 98/2 (A/B). MS analysis was in positive electrospray with single ion recording (135 +1 m/z) on a splitted flow of approximately 50 µl/min. Parameters were optimised manually for maximum sensitivity in 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 run with each experiment was fitted by linear-regression analysis and released adenine from experimental samples was determined by plotting against the standard curve.

RESULTS

Tissue distribution—Tissue distribution of RIP and APG activities was assayed both in crude extracts of most *M. jalapa* tissues and in basic protein fractions from root, mature shelled seed, immature unshelled seed, green leaf, flower bud, sepal + gynoecium (Table 1). The highest activity was found in mature seed.

Purification from seeds—The basic protein fraction from 100 g of seeds was loaded on the 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 (see later), and the fourth (MAP-4) appeared to be more than 98% homogeneous by reverse phase HPLC (FIG. 2) and both gave a single band on SDS-PAGE (results not shown). MAP was also pure when analysed by gel filtration in non-denaturing conditions (results not shown).

The inhibitory activity and protein yield at the various steps of purification are summarized in Table 2. It should be noticed that the total activity recovered after acidification and SP Sepharose™ steps was higher than that present in the original crude extract. This was observed already with other RIP (27) and possibly is due to removal of an inhibitor or to "activation" of the *M. jalapa* ribosome-inactivating proteins.

Identification and quantification of MAP and MAP-4 in tissue protein extracts—MAP and MAP-4 were identified by LC/MS in all basic protein extracts examined, with the exception of root in which only MAP was detected (FIG. 3 and Table 3). MAP-2 and MAP-3, identified in preparative ion exchange chromatography fractions, were below detection limits in this assay.

Physico-chemical properties—At LC/MS molecular masses (± 2) were: 27,788 (MAP), 30,412 (MAP-2), 29,771 (MAP-3) and 29,339 (MAP-4). The first isoform eluted from CM Sepharose™ differs from MAP from roots described in the literature for only 4 amino acids, not involved in the putative active site (16), has a pI greater than 9, as that of most other RIP, and its $E^{1\%}_{280nm}$ is 7.06.

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

Effects on protein synthesis—RIP activity in a rabbit reticulocyte lysate of MAP isoforms is reported in Table 4. 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 a cell line to another, the IC_{50}'s ranging by more than one order of magnitude from the most resistant HeLa to the most sensitive JM and BeWo cells.

Adenine polynucleotide glycosylase activity—MAP released adenine in a concentration-dependent manner from all substrates tested, namely DNA from herring sperm and *M. jalapa*, genomic TMV RNA, poly(A)$^+$RNA from *B. dioica*, *E. coli* rRNA, and from poly(A) (FIG. 5). In present experimental conditions, autologous DNA appeared the best substrate, with 24.4 moles of adenine released per mole of enzyme/min, followed by hsDNA (16.3 mol/mol/min), whilst other substrates were less sensitive (mol/mol/min: 0.6 for TMV RNA, 0.5 for poly(A)$^+$RNA, 0.4 for poly(A) and 0.35 for rRNA at the lowest enzyme concentration assayed (FIG. 5). APG activities of MAP isoforms on hsDNA are reported in Table 4, MAP-4 showing the highest activity.

DISCUSSION

Methodology—Two innovations in the methodological approach were fundamental to obtain the results here described: the use of LC/MS (i) for the determination of adenine and (ii) for the identification and quantification of enzyme isoforms in partially purified extracts. These different approaches allow for a direct measurement of RIP, as protein or as activity, in rather crude extracts, thus without the inevitable artefacts 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.

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 of *M. jalapa* were studied and the highest activity was found in roots (9), from which a RIP denominated MAP was purified (11). Here we describe 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: namely inhibition of in vitro translation (RIP activity) and adenine polynucleotide glycosylase activity (APG activity) on various polynucleotides. The last activity has never been described so far in partially purified enzyme preparations due to the interferences 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), (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 enzyme 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 1). 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 highest activities was mature seed, in good agreement with what 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 was most thoroughly studied (14). The distribution of activity here described differs somewhat from MAP contents estimated by Kubo et al. with an ELISA assay using anti-MAP antibodies (9). 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 antiserum of the different MAP isoforms. No clear difference in activity was observed in senescent leaf tissue in contrast with what observed in other species (28).

Purification—From seeds we purified the most abundant protein with RIP and APG activities together with 3 other isoforms. The most abundant RIP was analysed by LC/MS and the molecular weight obtained was very similar to that described for the major form found and purified from roots (16,29): only 4 amino acids were different, none of them in putative regions implicated in enzyme activity (reviewed in 1). This result could be due either to the presence in seeds of a different isoform or to a microvariability between subspecies (i.e. yellow versus red flower cultivar) of the same protein gene. To solve this problem we purified by reverse-phase

HPLC the main RIP from roots from the same cultivar we utilised for seeds. Retention time and molecular mass were absolutely identical to those of the protein purified from seeds. Thus we demonstrated that the main isoform of MAP was present also in seed, and that there are minor subspecies variations in the sequence of the same isoforms. The second abundant isoform of seed (MAP-4) was isolated also from leaf tissue by reverse-phase HPLC and identified by retention time and molecular mass. This highly purified protein was sequenced in the N-terminal portion. The identification of this form as a RIP was confirmed by the presence of the invariable residues Y, R, F which are totally conserved in all amino-terminal sequence of RIP reported so far (FIG. 6).

Immunological properties—MAP is not recognized by antibodies against several commonly used RIP. Thus this protein, which is easily purified in large enough quantities, may be useful to prepare immunologically distinct immunotoxins, in order 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 where 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. The comparison with the RIP isoforms purified from the closely related plant *M. 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 which is much greater than that (very scarce apart from the three invariable Y, R, F residues) of the isoforms from the same

plant. This may allow to classify the two different pairs as different classes of RIP as happens in *S. officinalis* (14). All isoforms have little similarity with bouganin, a RIP isolated from *Bougainvillea spectabilis*, another member of the Nictagynaceae family (18). Work is in progress to verify whether MAP-4 has enzymatic and substrate specificity different from those of MAP. The existence in the same plant of several forms of RIP, sometimes functionally different, was frequently observed (e.g. PAP from *Phytolacca americana*, review in Irvin (17); saporins from *S. officinalis* (14)).

Enzymatic activities—So far MAP has been assayed only for activity on translation systems and purified animal ribosomes. Here we report the determination of the direct enzymatic activity of MAP on several substrates among which autologous DNA. MAP depurinates all substrates assayed including deoxy- and ribonucleic acids. It should be noted that autologous DNA is 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 more than one mole of adenine per mole of enzyme was released from all substrates, without the need for any cofactor, required by some other RIP 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 RIP from Caryophyllales (6) and in MAP, may suggest a role in mRNA post-transcriptional changes, and (ii) the activity on TMV RNA suggest 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 RIP that are internalised with low efficiency. Toxicity varied depending upon the cell type tested, HeLa cells being the least, as it is usually with other RIP, and JM and

BeWo cells the most sensitive cells. As JM and BeWo cells are derived from monocytes and chorioncarcinoma cells, respectively, both with a high pinocytotic activity, their high sensitivity could be the consequence of a better cellular uptake of the protein, although differences in intra-cellular routing cannot be excluded. All lethally intoxicated cells showed the morphological features of cell death by apoptosis (results not shown), as previously described for some other RIP (31,32). Cytotoxicity of RIP has long been attributed entirely to protein synthesis inhibition, however, direct damages to DNA and/or RNA either than ribosomal 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 of APG activities, are 8-fold higher in mature as compared with immature seeds, as observed in *S. officinalis* (14); (ii) the ratio between MAP and MAP-4 is in favour 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 RIP in plants, which is still not clear. The notion was put forward that they may be storage proteins and/or defence systems (33), and indeed they may confer some protection against a broad spectrum of viruses, and fungal pathogens, as observed in transgenic plants expressing RIP (reviewed in Ref. 4). Nevertheless, RIP expression rises in mature seeds, in stressed (34-36) and virally infected plant tissues (37), conditions that may all require programmed cell death activation. RIP may have a primary role in the mechanisms leading to apoptosis. This could be through the effect on ribosomes, which occurs *in vivo* (38). However, as discussed above, MAP and other RIP release adenine from DNA more efficiently than from RNA. Should this occur in vivo, it could be a very efficient mechanism to kill a cell, since the few alterations sufficient to induce lethal damage to DNA could be caused more rapidly than the

inactivation of the relatively high number of ribosomes required to significantly impair protein synthesis.

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Figure Legends

FIG. 1. Cation-exchange chromatography of extract from *M. jalapa* seeds. Experimental details are given in Experimental Procedures. The A_{280} was recorded (continuous line) and the inhibitory activity on translation by a rabbit reticulocyte lysate system of selected fractions (25 µl at a 1:5,000 dilution) is reported (dotted line). Pooled fractions, containing MAP, MAP-2, MAP-3 and MAP-4, are indicated by horizontal bars.

FIG. 2. **HPLC analysis of active protein peaks from cation exchange chromatography.** MAP. MAP-2, MAP-3 and MAP-4 were analysed by HPLC reverse-phase chromatography on a Vydac Protein C4 column as described in Experimental Procedures. Approximately 100 µg of protein was applied in each run. Molecular masses, determined by maximum enthropy algorithm, are reported. Determination error is ± 2 mass units.

FIG. 3. **Identification of MAP isoforms in basic protein fractions of selected *M. jalapa* tissues.** Experimental details are described in the Experimental Procedures section. Molecular masses determined by maximum enthropy algorithm (Max Ent) are given.

FIG. 4. **Inhibition of protein synthesis by cell lines.** Cells (10^5/well) were incubated with MAP for 18 h, followed by a 2 h pulse with L-[4,5-^{3}H]leucine (125 nCi/0.25 ml). Results are mean values of two experiments performed in triplicate, with a S.D.<15%. The concentration giving 50% inhibition (IC_{50}) was calculated by linear-regression analysis. Incorporation of L-[4,5-^{3}H]leucine by control cells was (d.p.m. ± S.D.): BeWo 61440 ± 6716, HeLa 18600 ± 2206, JM 14727 ± 1909, NB100 8103 ± 531 and 3T3 14120 ± 978.

FIG. 5. **Adenine polynucleotide glycosylase activity of MAP.** Reactions were run for 40 min at 30° C in a final volume of 50 µl containing 100 mM KCl, 50 mM Na acetate (pH 4.0), increasing concentrations of RIP and 20 µg of polynucleotide substrates. A standard curve of adenine was run with each experiment. Lines were obtained by regression analysis. The substrates tested were in panel A plant related DNA from *M. jalapa* (○), poly(A)$^+$RNA from *B. dioica* (Δ) and TMV RNA (□) in panel B plant unrelated DNA from herring sperm (●), rRNA from *E. coli* (▲) and poly(A) (■).

FIG. 6. **Amino-terminal sequence comparison between RIP from Nyctaginaceae.** Residue numbering refer to MAP sequence. Sequences of MAP from seeds are from (16), of ME1 and ME2, RIP from *Mirabilis expansa* roots, are from (33), of bouganin, RIP from the leaves of *Bougainvillea spectabilis*, are from (18). Alignment is for the three black shaded residues that are invariant in all amino-terminal sequences of RIP.

Tables

TABLE 1

Distribution of RIP and APG activities in M. jalapa tissues

Experimental details are given in Experimental procedures. One unit of RIP activity is defined as the amount of protein necessary to reduce by 50% translation in 1 ml of rabbit reticulocyte lysate system in present experimental conditions. One unit of APG activity is defined as the amount of protein necessary to release 1 μmol of adenine from DNA in present experimental conditions. Values have been normalised to activity in roots taken as 100. Values for root tissue were for crude extract 9261 ± 1177 (SD) and 1.55 ± 0.18 (SD) U/g of starting tissue for RIP and APG activity, respectively, and for basic protein fraction 12400 and 1.06 for RIP and APG activity, respectively. Values refer to the mean of the results of three different crude extracts or to a single basic protein fraction. Samples were all run in duplicate and controls with all reagents but no incubation were run in each experiment.

	Crude extract		Basic protein fraction	
	RIP activity	APG activity	RIP activity	APG activity
Tissue	*% 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 colour)	64	51		
Leaf (green)	62	36	330	830
Petiole	32	10		
Petal+stamen	27	0.3		
Stem	24	21		

TABLE 2

Purification of RIP from seeds of M. jalapa

Results refer to 100 g of seeds. Activity was determined as inhibition of translation by a rabbit reticulocyte lysate as described in Experimental Procedures. IC_{50} is the concentration of protein, which inhibits protein synthesis by 50% in a rabbit reticulocyte lysate system. One unit (U) is the amount of protein causing 50% inhibition in 1 ml of reaction mixture. The CM Sepharose™ elution profile is shown in FIG. 1.

Purification step		Total protein	IC_{50}	Specific activity	Total activity	Yield
		mg	*ng/ml*	*10^{-3}×U/mg of protein*	*10^{-6}×U*	*%*
Crude extract		10986	1096	0.9	9.99	(*100*)
Acidified extract		3637	224	4.5	16.22	162
Basic protein fraction		1118	83	12.1	13.47	134
CM Sepharose™ eluate:	MAP	22.6	5.4	183.8	4.16	42
	MAP-2	2.5	41.4	24.2	0.06	1
	MAP-3	3.5	13.3	75.2	0.26	3
	MAP-4	5.5	15.3	65.4	0.36	4

TABLE 3

Distribution of MAP and MAP-4 proteins in M. jalapa tissues

Determinations of MAP and MAP-4 content were performed on basic protein fractions analysed by LC/MS (FIG. 3). Peak areas were determined by MassLynx software and are expressed as arbitrary area units.

	MAP	MAP-4	Ratio MAP/MAP-4
	Arbitrary A_{214} units/g of starting tissue		
Seed (mature)	7020	3950	1.8
Seed (immature)	744	105	7.1
Root	427	<2[a]	-
Leaf	26	89	0.29
Flower bud	11	17	0.62
Sepal and gynoecium	7	21	0.33

[a] Resolution limit in present experimental conditions

TABLE 4

Activity of MAP isoforms from seeds

Inhibition of translation (RIP activity) was determined in a rabbit reticulocyte lysate as described in the legend to Table 3. APG activity was determined on hsDNA as described in Experimental procedures and in the legend to FIG. 5.

Isoform	RIP activity	APG activity
	IC_{50}	Adenine released
	nM	*pmol/pmol of enzyme*
MAP	0.19	651
MAP-2	1.36	1250
MAP-3	0.45	833
MAP-4	0.52	1417

Abbreviations: ACN, acetonitrile; APG, adenine polynucleotide glycosylase; HIV, human immunodeficiency virus; hsDNA, herring sperm DNA; IC_{50}, concentration giving 50% inhibition; MAP, *Mirabilis* antiviral protein; PAP, pokeweed antiviral protein; PBS, phosphate-buffered saline; RIP, ribosome-inactivating protein; TFA, trifluoroacetic acid; TMV, tobacco mosaic virus.

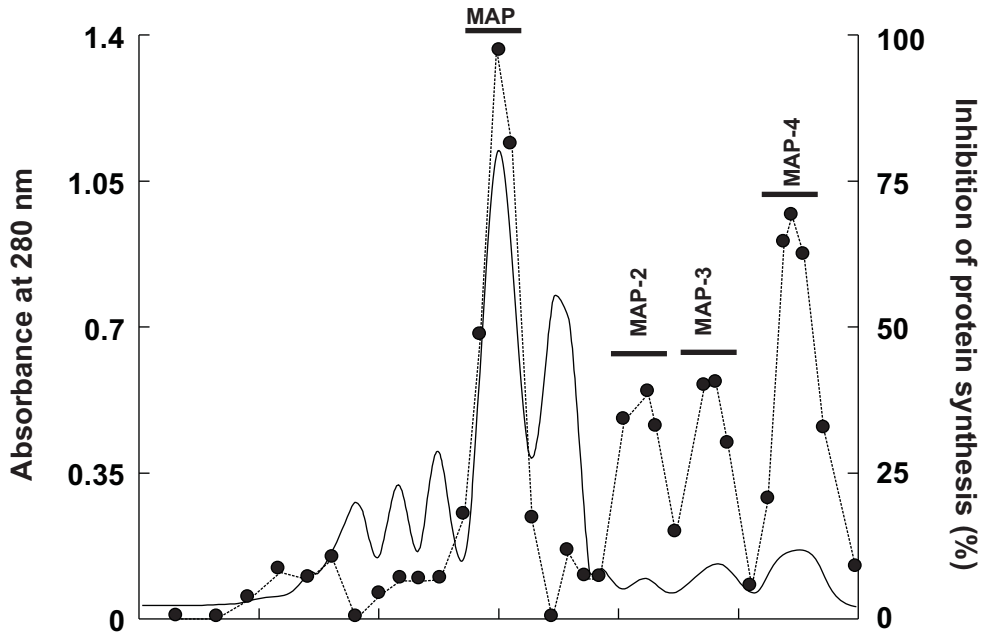

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Fig. 1

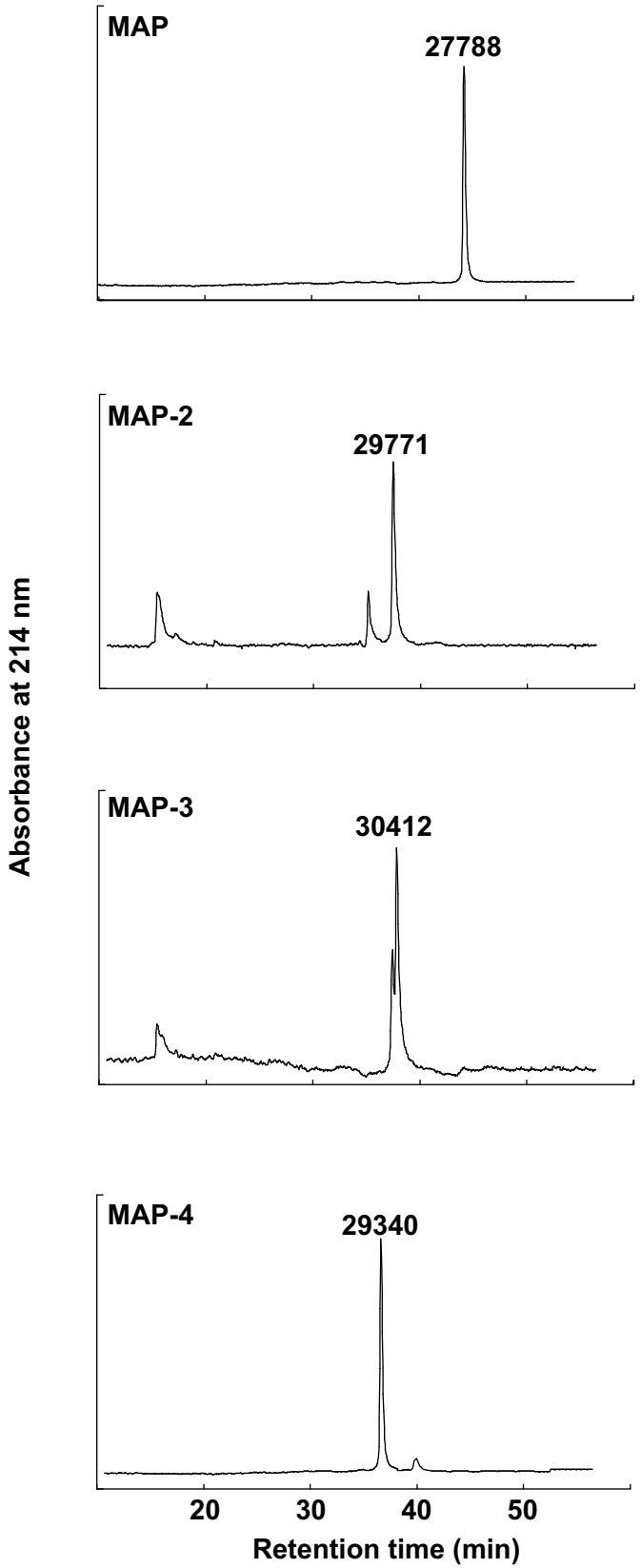

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Fig. 2

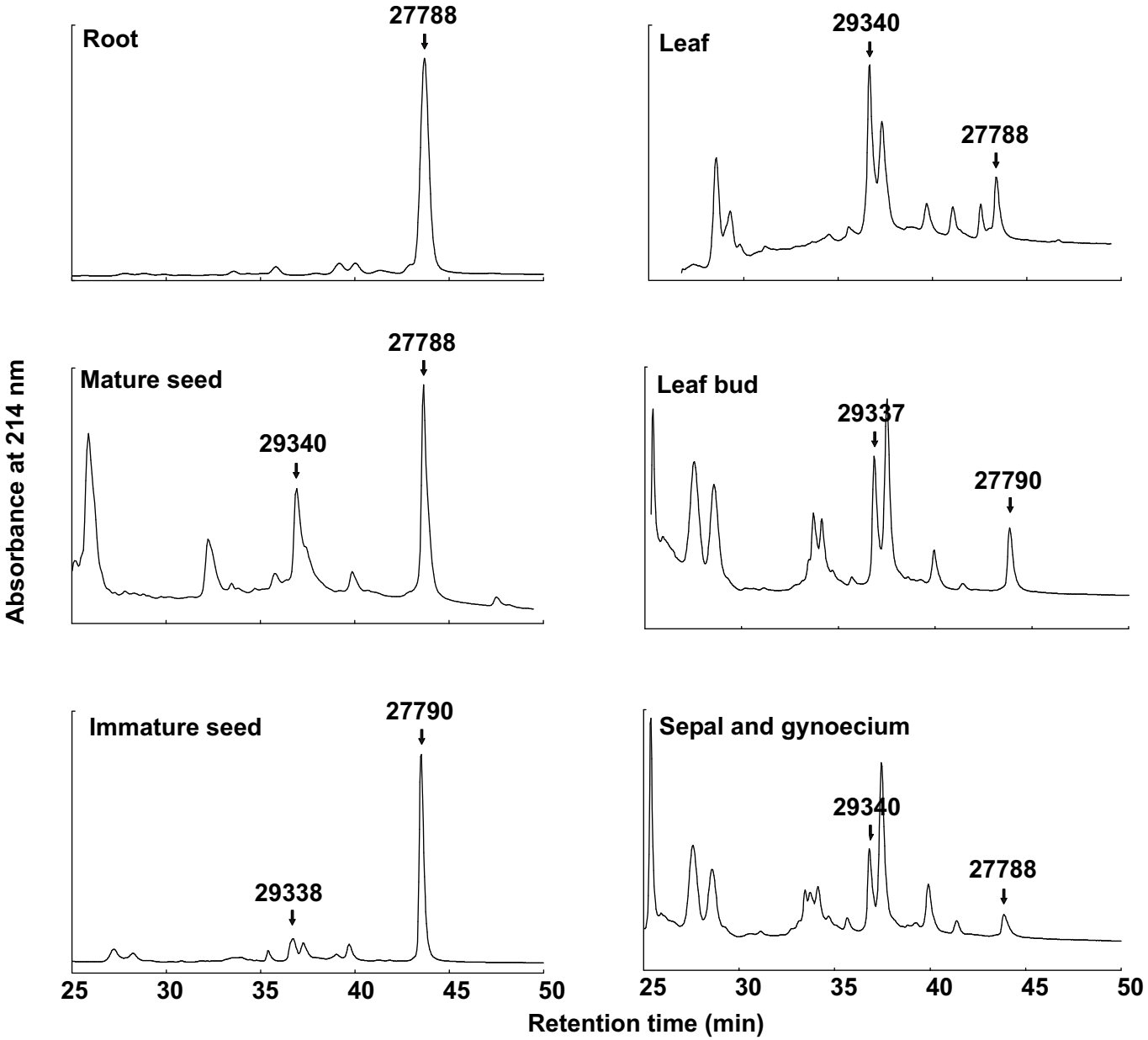

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Fig. 3

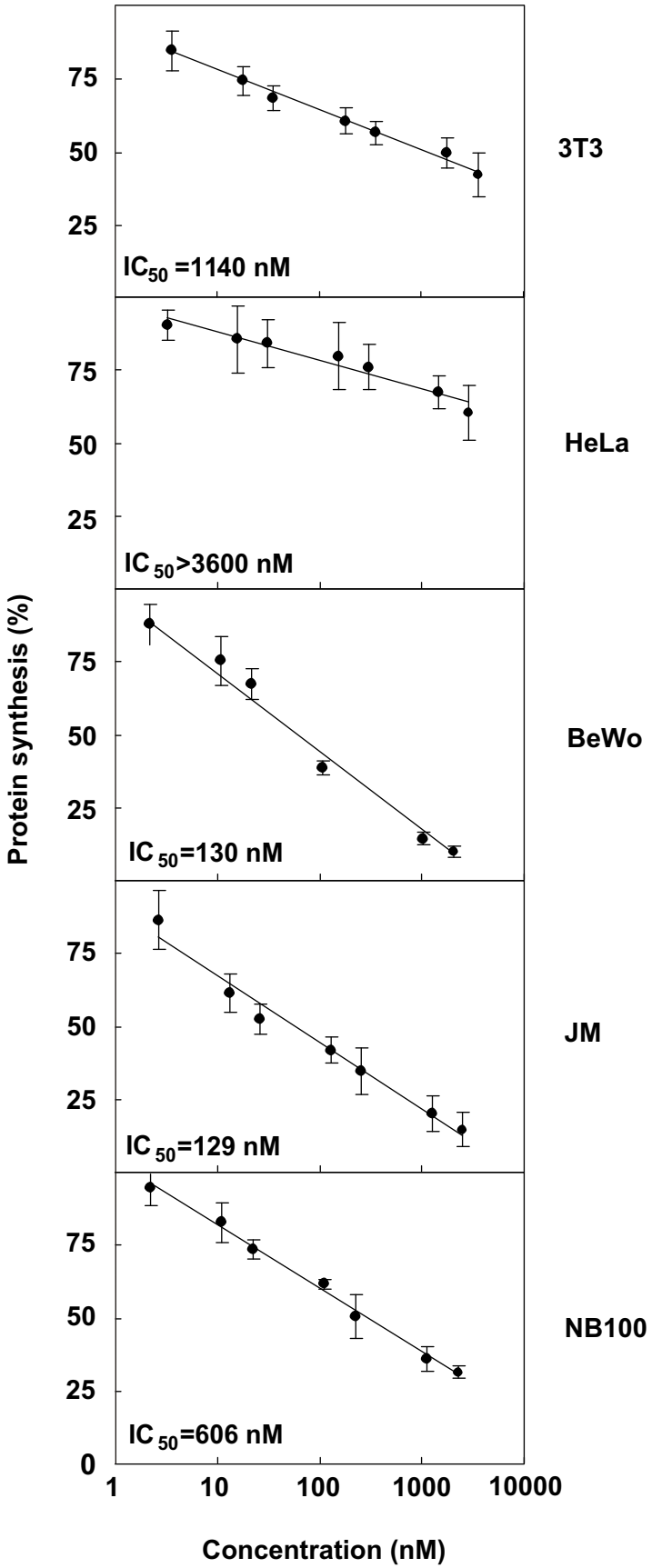

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Fig. 4

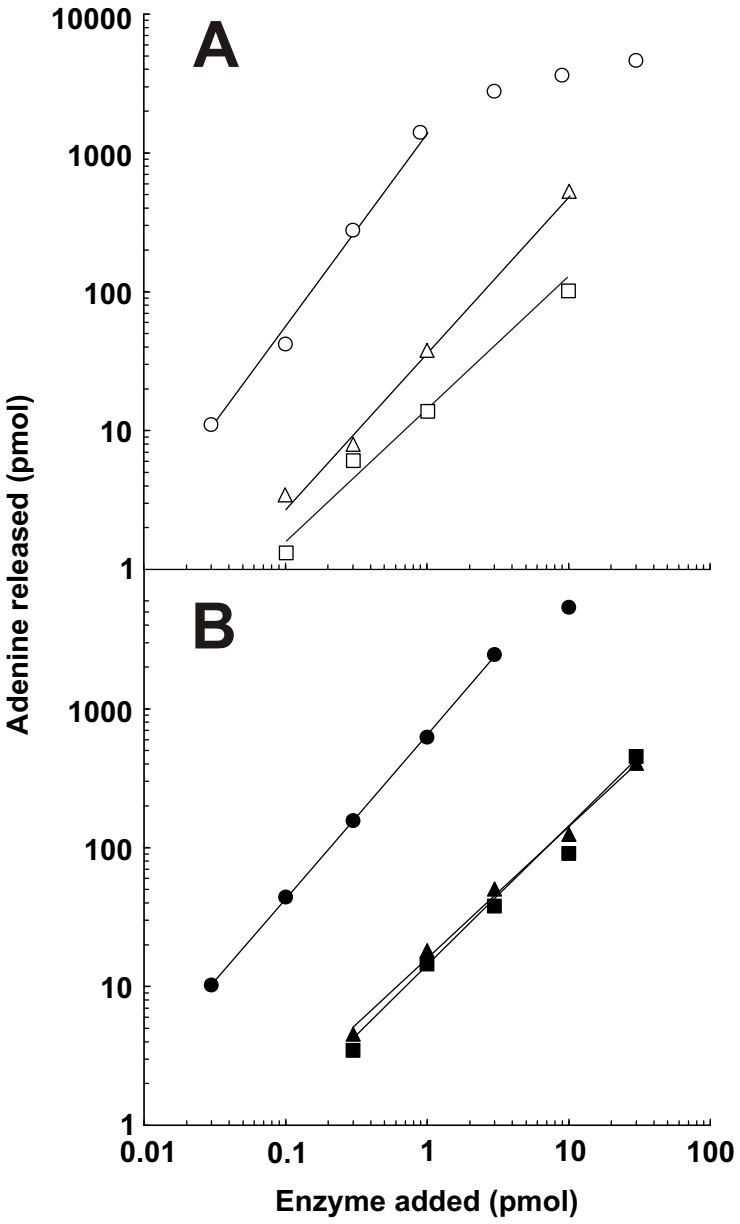

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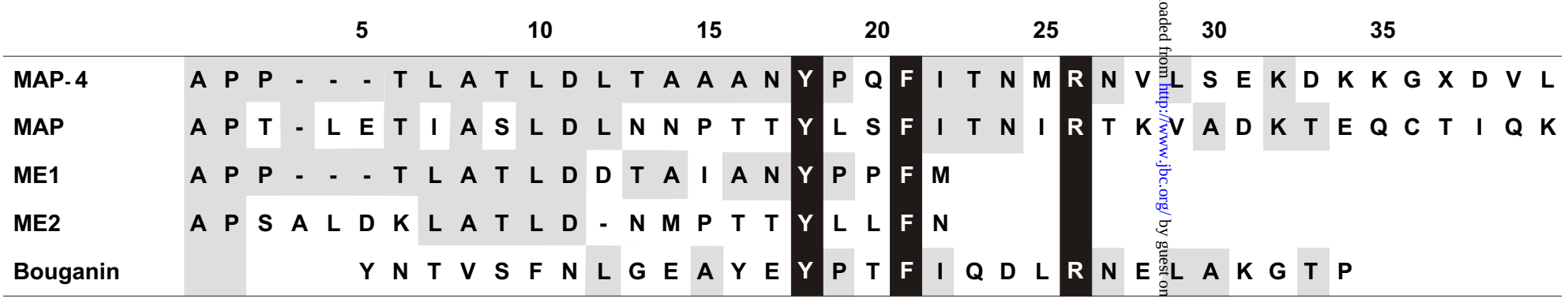
Fig. 5



Homology (%)

	MAP-4	MAP	ME1	ME2
MAP	38			
ME1	80	38		
ME2	41	62	43	
Bouganin	23	20	19	10

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Fig. 6

Ribosome-inactivating and adenine polynucleotide glycosylase activity in Mirabilis jalapa L. tissues

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