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## Molecular characterization and post-transcriptional regulation of ME1, a type-I ribosome-inactivating protein from *Mirabilis expansa*

Received: 3 October 2002 / Accepted: 12 February 2003 / Published online: 15 May 2003  
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**Abstract** Ribosome-inactivating proteins (RIPs) are N-glycosidases that remove a specific adenine from the sarcin/ricin (S/R) loop of the large rRNA, thus arresting protein synthesis at the translocation step. In the present study, ME1, a type-I RIP, was cloned and sequenced from storage roots of *Mirabilis expansa* (Ruiz & Pavon). The full-length cDNA sequence of ME1 has 1,129 nucleotides with an open reading frame of 951 nucleotides representing 317 amino acids. Nucleotide analysis revealed that the N-terminal region of ME1 was cleaved, and the mature protein started at amino acid 34. ME1 showed very close similarities to MAP and MAP-4 from *Mirabilis jalapa*. Southern blot analysis revealed the presence of two homologous genes for ME1 cDNA in *M. expansa*. Northern blot analysis showed high levels of ME1 transcripts in primary and storage roots. Interestingly, jasmonic acid induced ME1 transcript expression in cell suspension cultures of *M. expansa*; however, the production of ME1 protein was not enhanced as observed by Western blot analysis. Our data suggest that ME1 has the ability to depurinate its own mRNA, thus inhibiting its translation. These observations suggest a possible mechanism by which ME1 protein levels are post-transcriptionally regulated.

**Keywords** Depurination · ME1 · *Mirabilis* · Ribosome-inactivating protein

**Abbreviations** JA: jasmonic acid · MAP: *Mirabilis* antiviral protein · PAP: pokeweed antiviral protein · RACE: rapid amplification of cDNA ends · RIP: ribosome-inactivating protein

### Introduction

Ribosome-inactivating proteins (RIPs) are a family of cytotoxic enzymes widely distributed in the plant kingdom (Nielsen and Boston 2001). RIPs are polynucleotide adenosine glycosidases that cleave the glycosidic bond of an adenosine base in an evolutionarily conserved sequence (GAGA) located in the  $\alpha$ -sarcin/ricin (S/R) loop in both prokaryotic and eukaryotic ribosomes (Endo et al. 1987; Endo and Tsurugi 1988). Depurination of the S/R loop prevents binding of elongation factor 2 to the ribosome, and results in protein synthesis inhibition (Stirpe et al. 1992). Apart from the depurination of rRNA, some RIPs exhibit the ability to depurinate DNA, poly(A) and viral RNA (Barbieri et al. 1994, 1997; Bolognesi et al. 2002). Many RIPs possess antimicrobial and antiviral activities. *Mirabilis* antiviral protein (MAP), an RIP isolated from the roots and leaves of *Mirabilis jalapa*, inhibits the infection of plant viruses such as tobacco mosaic virus, potato virus X, potato virus Y and potato spindle tuber viroid (Kubo et al. 1990; Kataoka et al. 1991). Similarly, pokeweed antiviral protein (PAP), an RIP from *Phytolacca americana*, has antiviral activity against different human and plant viruses such as poliovirus (Ussery et al. 1977), herpes simplex virus (Aron and Irvin 1980) and tobacco mosaic virus (Irvin 1983). Despite the extensive enzymatic and biological characterization of RIPs, the exact role of these enzymes in plants is not yet known.

Generally, RIP genes have isoforms that are differentially expressed in plant tissues. Expression of RIP genes is also influenced by age and by chemical, physical and environmental conditions. The expression of the PIP2 gene, an RIP from *Phytolacca insularis*, is developmentally regulated in leaves and roots and expressed only in plants 6 weeks or older (Song et al. 2000). PIP2 is also inducible by mechanical wounding, jasmonic acid (JA) and abscisic acid (ABA) treatment but not with salicylic acid elicitation (Song et al. 2000). JIP60, a type-III RIP, is induced in barley by treatment with JA, ABA or due to

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desiccation, wounding and senescence of leaves (Becker and Apel 1993; Chaudhry et al. 1994; Reinbothe et al. 1994). *P. americana* exhibits differential expression of RIPs depending on season and stage of development. Accordingly, different isoforms of PAP are produced in spring leaves (PAP), early summer leaves (PAP-II) and in late summer leaves (PAP-III), while PAP-S is seed specific (Barbieri et al. 1982; Irvin 1975; Irvin et al. 1980, Irvin and Uckun 1992). Different RIP isoforms of *M. jalapa*, MAP and MAP-S, have been found in roots and seeds, respectively, and show slight amino acid differences (Habuka et al. 1989; Di Maro et al. 2001). Recent studies have shown that there are at least four RIP isoforms, MAP, MAP-2, MAP-3, MAP-4, in the seed extracts of *M. jalapa* (Bolognesi et al. 2002). In *M. expansa*, two RIP isoforms, ME1 and ME2, have been found in roots (Vivanco et al. 1999; Vivanco and Flores 2000). Despite detailed protein expression studies regarding the effect of physical and environmental conditions, to our knowledge there is no information linking RIP transcript turnover with its protein expression in plants. In the present study, we have isolated and characterized the cDNA clone of ME1 from *M. expansa*. The distribution, localization and enzymatic activities of ME1 were examined. Furthermore, our studies indicate that induction of the ME1 transcript, but not the expression of the ME1 protein, occurs in response to exposure to JA in cell cultures, which indicates a possible post-transcriptional regulation.

## Materials and methods

### Plant material

Seeds of *Mirabilis expansa* (Ruiz & Pavon) (CIP accession 208001, ARB 5395) were obtained from Dr. M.K.V. Zant, Southern Illinois University, USA.

### Callus and suspension culture initiation

Seeds were washed in running tap water and surface-sterilized using sodium hypochlorite (0.3% v/v) for 10–15 min, followed by three to four washes in sterile distilled water. Surface-sterilized seeds were placed on static MS (Murashige and Skoog 1962) basal media (4.4 g of MS salts, 30 g of sucrose and 8 g of agar per liter) for germination. Callus cultures were initiated using primary roots from 10-day-old seedlings grown in vitro and subsequently cultured in media containing MS salts and vitamins supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D; 0.90  $\mu\text{M}$ ), kinetin (0.11  $\mu\text{M}$ ) and sucrose (30 g l<sup>-1</sup>). Plant growth regulators were obtained from Sigma (St. Louis, MO, USA). Cultures were maintained at 25  $\pm$  2 °C. The light intensity under a continuous mode was 4.4117 J m<sup>-2</sup> s<sup>-1</sup>. Cell suspension cultures were established from the callus cultures, and were maintained in 125-ml Erlenmeyer flasks with 50 ml of nutrient medium containing 2,4-D (0.90  $\mu\text{M}$ ), kinetin (0.11  $\mu\text{M}$ ) and sucrose (30 g l<sup>-1</sup>) by biweekly sub-culturing on a rotary shaker at 90 rpm maintained at 25  $\pm$  2 °C.

### Preparation and addition of JA

An initial inoculum of 200 mg (fresh weight basis) of callus mass was used for all growth and elicitation experiments. The cell suspension cultures of *M. expansa* were analyzed at 3-day intervals for

growth. Cell suspension cultures of *M. expansa* grown in 40-ml MS flasks were elicited with jasmonic acid (JA). JA was dissolved in ethanol (20  $\mu\text{l}$ ) and added on the sixth day to cell cultures in MS flasks to final concentrations of 50, 100 and 200  $\mu\text{M}$ . A time-course study of the influence of JA on RIP expression was conducted by harvesting the cells at 1 h and 24 h after the addition of JA; a non-elicited control was also harvested during the same period for biomass and RIP content.

### Isolation of ME1 cDNA

The nucleotide sequence of ME1 was obtained by using RACE (rapid amplification of cDNA ends) amplification. The 5' and 3' RACE-ready cDNA was prepared using the Smart RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol, and the total RNA was obtained by Qiagen RNeasy Plant mini kit (Valencia, CA, USA) from storage roots of *M. expansa*. The primer for 3' RACE, 5'-ACA CTTGCAACACTTG, was designed based on the common N-terminal sequence TLATLD of the ME1 protein (Vivanco and Flores 2000) using the codon preference of *M. jalapa* known sequences. The RACE PCR was performed for 30 cycles with denaturation for 30 s at 94 °C, annealing for 30 s at 42 °C and extension for 3 min at 72 °C with Advantage2 Polymerase mix (Clontech) followed by a 10-min extension at 70 °C. The PCR products were cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) by TA cloning and for subsequent sequencing. The primer for 5' RACE, 5'-GCTTTAATGCTCTACGTACGCG TATCG-3', was designed from the sequence obtained from the 3' RACE, and PCR was performed as above with an annealing temperature of 72 °C for the first 7 cycles followed by 30 cycles at 65 °C annealing temperature. The PCR products were cloned into pGEM-T Easy (Promega) and sequenced at the Macromolecular Resources Facility—Colorado State University. The sequence was analyzed using Vector NT software (InforMax, Bethesda, MD, USA). The entire open reading frame of ME1 was obtained from the cDNA library by PCR using 5'-GGCATGGAAAC TATGAGTTTGCTCTTCC-3' as the forward primer, and 5'-TCAATTTTATTTAATTAATAATTACAAGATGGCC-3' as the reverse primer using either the 5' or 3' end of the RACE-ready cDNA as template.

### SDS-PAGE and Western blot analysis

The calli harvested from growth and induction experiments were frozen in liquid nitrogen and ground in a mortar to powder. The protein was extracted from the powder samples with 20 mM phosphate buffer (pH 7.0) with 0.01% Tween 20 (Bio-Rad, Hercules, CA, USA). The protein concentration was estimated with Bio-Rad Protein Assay Dye Reagent. For Western blots, 20  $\mu\text{g}$  of the crude protein extract was separated on a 12% SDS-PAGE gel and transferred to PVDF membranes (Millipore, Bedford, MA, USA) and the membranes were blocked with 0.5% non-fat dry milk in TBST for 2 h at room temperature. The membranes were then incubated in anti-ME1 antibodies (Vivanco and Flores 2000) at 1:1,000 dilutions for 1 h. The membranes were washed 3 times with TBST and incubated in horseradish peroxidase-conjugated goat anti-rabbit IgG (Immun-Blot Assay Kit; Bio-Rad) at 1:3,000 dilution, and labeled bands were detected according to the manufacturer's protocol.

### Northern analysis

The total RNA was extracted from the callus powder using Qiagen RNeasy plant mini kit (Valencia). Ten  $\mu\text{g}$  of total RNA was separated on a 1% agarose gel containing formaldehyde and transferred to nylon membranes (Pall Gelman, Ann Arbor, MI, USA) overnight. The membranes were probed with <sup>32</sup>P-labeled

ME1 sequence according to standard procedures (Sambrook et al. 1989). The probe sequence was obtained by PCR from ME1 cDNA clones using the same primers used to obtain the entire open reading frame (see above). The PCR product was separated on an agarose gel and was extracted from the gel using the Gel Slice Kit (Bio-Rad). The DNA was then radio-labeled with [<sup>32</sup>P]ATP using random primers from DECAprime II Random Priming DNA labeling kit (Ambion, Austin, TX, USA). This labeled ME1 sequence was used as a probe for both Northern and Southern blots.

#### Southern analysis

The genomic DNA was obtained from storage roots frozen in liquid nitrogen and ground in a mortar to powder. The DNA was extracted by CTAB buffer (Woodhead et al. 1998). Ten µg of genomic DNA was digested with *EcoRI*, *SalI*, *NorI*, *NcoI* and *HindIII* separately overnight. The digests were separated on a 1% agarose gel, transferred to nylon membranes and probed with <sup>32</sup>P-labeled ME1 cDNA sequence.

#### Localization and electron microscopic examination of ME1

To study the cellular location of ME1 in root tissues of *M. expansa*, we performed immunogold localization using a protocol adapted from Wright and Rine (1989). Root tissues were cut into 1- to 2-mm segments and fixed for 1 h at 4 °C in 3% paraformaldehyde and 0.5% glutaraldehyde. Tissues were washed 3 times in 0.1 M PO<sub>4</sub> buffer (pH 7.4) and incubated for 15 min at room temperature in 1% sodium metaperiodate. Tissues were washed once in 0.1 M PO<sub>4</sub> buffer (pH 7.4), incubated 15 min at room temperature in 50 mM NH<sub>4</sub>Cl, and washed once in 0.1 M PO<sub>4</sub> buffer (pH 7.4). Samples were dehydrated in a graded ethanol series (20 min in 25, 50, 70, 95 and 100% ethanol). After an additional 20 min in 100% ethanol (EM grade), samples were progressively infiltrated with LR White resin in ethanol, for 8–18 h in steps of 25, 50, 75 and then 100% LR White resin. Infiltration of tissues in 100% LR White resin was repeated twice. Samples were placed in gelatin capsules containing fresh LR White resin and overlaid with one or two drops of mineral oil by gently pipetting into each capsule. Trays with gelatin capsules containing the samples were incubated for 24 h at 50 °C to polymerize the resin. After 24 h, samples were removed from the capsules, and ultra-thin sections were cut on a Reichert ultra-microtome and placed on Formvar- and carbon-coated nickel grids. The ultra-thin sections on the grids were incubated in 50 mM glycine for 15 min. Samples were then placed for 30 min in PBS blocking buffer (10 ml of 0.1 M PO<sub>4</sub> buffer, pH 7.4, containing 5% bovine serum albumin, 0.1% cold fish skin gelatin, and 5% normal cold fish gelatin, followed by incubation overnight with primary antibody diluted in PBS incubation buffer (1:2,500) containing 0.8% bovine serum albumin, 0.1% cold water fish skin gelatin and 20 mM NaN<sub>3</sub>. The antibodies used for localization studies were developed against ME1 (Vivanco et al. 1999). The antibodies gave specific labeling to RIP and no background reaction was observed. Negative control samples were incubated in pre-immune serum diluted in incubation buffer (1:2,500). After 17 h, the sections were washed 6 times for 5 min, then incubated 3 h in goat anti-rabbit IgG (H+L) secondary antibody conjugated to colloidal gold (Goldmark Biologicals, Bradford, NJ, USA) diluted in incubation buffer (1:50). Sections were washed 6 times for 5 min each in incubation buffer, and then washed 3 times for 5 min in PBS. Final washes consisted of 2% glutaraldehyde in PBS for 5 min, and three washes with water for 1 min. Sections were stained with 2% uranyl acetate and 12% lead citrate, blotted, dried and examined with JOEL 1200EXII transmission electron microscope (JOEL, Peabody, MA, USA). All steps were carried out on Parafilm in a petri dish by floating grid sections side down on a droplet of the reagent. All incubations were at room temperature except the overnight incubation (4 °C) in

primary antibody. Scanning electron microscopy examination of *M. expansa* was carried out according to Humphreys (1978) in a JOEL 5400 SEM.

#### Depurination assay and translation studies

Depurination of mRNA was assayed by a modification of the method of Tumer et al. (1997). The ME1 was purified from storage roots as described previously (Vivanco et al. 1999). The protein was further passed through an anti-ME1 antibody affinity column. The purity of the protein was checked on an SDS-PAGE gel by silver staining. The amount of protein was estimated by Bio-Rad Protein Assay Dye Reagent concentrate. ME1 mRNA was in vitro-transcribed using T7-polymerase (Promega) as per the manufacturer's instructions from linearised plasmid DNA (pGEM-T Easy; Promega) containing the ME1 open reading frame. Luciferase mRNA was obtained from Promega. Both ME1 and luciferase mRNAs were incubated with 10 ng ME1 protein for 60 min at 30 °C. The reaction was stopped and mRNA was extracted by phenol extraction followed by ethanol precipitation. The ME1-treated mRNA was used to check the depurination by aniline as well for translation studies. One µg of ME1-treated mRNA was incubated on ice for 30 min with 1 M aniline (pH 4.5) and precipitated with ethanol. RNA was resolved in a urea-polyacrylamide gel and stained with ethidium bromide.

The effect of ME1 protein on mRNAs was further studied by translating the ME1-treated mRNAs with the Flexi Rabbit Reticulocyte Lysate System (Promega). The translation was done as per the manufacturer's protocol in the presence of [<sup>35</sup>S]methionine to label the products. Five µl of reaction mix was added with 25 µl of protein sample loading dye and boiled for 3 min to denature the proteins. The sample was resolved by SDS-PAGE and the gel was dried on a gel dryer and exposed to film.

We studied the effect of ME1 on rabbit reticulocytes from the Flexi Rabbit Reticulocyte Lysate System, which were incubated with 10 ng of ME1 for 30 min at 30 °C, then used to translate control luciferase mRNA (Promega) as per the manufacturer's instructions.

## Results

### Cloning of ME1

The ME1 gene sequence was obtained by RACE from the cDNA libraries constructed using total RNA from *M. expansa* storage roots and cell cultures. Gene-specific primers were designed using common sequences from the N-terminal region of ME1 (Vivanco et al. 1999) for 3' RACE. The RACE from both storage roots and cell cultures resulted in a similar sequence and that sequence matched the N-terminal sequence of the ME1 protein. The full length of the cDNA sequence is 1,129 nucleotides with an open reading frame of 951 nucleotides representing 317 amino acids. In the deduced amino acid sequence the start (ATG) codon was found 30 bases downstream of the first base and the polyadenylation signal (AATAAA) was found 29 bases upstream of the putative polyadenylation site (Fig. 1). We found 33 upstream amino acids that were not present in the N-terminal amino acid sequence of the mature protein, and amino acid residues 34–53 matched the N-terminal amino acid sequence of the mature ME1 (Vivanco et al. 1999), suggesting that the first 33 amino acids are deleted in the mature protein. The N-terminal amino acid

**Fig. 1** Nucleotide and predicted amino acid sequences for *Mirabilis expansa* ME1 (numbered on the right). The arrow indicates the start of the mature ME1 protein sequence at amino acid residue 34. The N-terminal amino acid (amino acids 34–53) sequence of the mature protein is in **bold**. The regions encoding the polyadenylation signal (AATAAA) and poly(A) tail are in **bold** and underlined

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catcaatttaaatcatcaattaattccggc 30
atggaaactatgaggttgctcttctctattgctgacaatatggacgacagtcgtgggttcaacctgggccaacaaccgggtaccgatcag 120
M E T M R L L F L L L T I W T T V V G S T W A Q Q P G T D Q 30
acacttttggcaccaccaacctcgcaacccttgatctcacggcgccccaattaccctccatttatcacaacaatcgcaaatgattg 210
T L L A P P T L A T L D L T A A A N Y P P F I T N M R N V L 60
tcggaaaaagataagaatggaaaagatgtactgctatgtaccatgaaaaaataagcaccacagtcaccatccccgcgatcgcgtacgta 300
S E K D K N G K D V L L C T M K K I S T T V P S P R Y A Y V 90
gacattaaagcctctgcaacacaacggtaacattagcaatagatcgaactaatacttatgtgtgggttatcgtgatattcgggtggg 390
D I K A S A T Q T V T L A I D R T N T Y V L G Y R D I F G G 120
actgatcgtgcccctctctttaaagacgtgtacgatgacgcaaaccttttccagatgccaaggggaaaaatcgaaataaattgagt 480
T D R A A F F K D V Y D D A K D L F P D A K G K N R I K L S 150
tatggctcacaataactactccttaggagataggactaaagttccattaggaattaaaagcctaaggataagcataactgctatctatggg 570
Y G S Q Y T T L G D R T K V P L G I K S L R I S I T A I Y G 180
gaagcagccggtacagacctcgataaaaaccggcgcgagttttctcttgcattgcagatggtagcagaagcaacacgattcaaatat 660
E A A G T D L D K N R R E F F L L A L Q M V A E A T R F K Y 210
atctctgacaagatccccacagaaagggactacgacactttaaggtagataatcacatgatagcattggagaatggtgggactatata 750
I S D K I P T E R D Y D T L K V D N H M I A L E N G W D L L 240
tcaactgctatttacaacgtaagtcttccacaactaaacctactaagtgtgaactacttaaacaccctgtttcattaatcgtgcttgggt 843
S T A I Y N A K S S T T K P T K C E L L K T P V S L I W L G 270
caaatgaatggaacttcaactctgtggaggaaatgccaagtcgtgngctcctaaaggcaaaaggcaaaagtgtcgacgaataat 930
Q N E W N F N S V E E I A K V V X L L K A K G K K L S T N N 300
gacgacgacaacaacggcgatgattgtgtagtgtttagtgcattcttcttaaatataattatgtcgtttaatcctgccttaataattat 1020
D D D N N G D D C G S V V V A S S * 317
gaataaaatataatcatgttgcaccatggtgacattctgtaattattaataaataaaatgaataagaggttatgctgttattttca 1110
aaaaaaaaaaaaaaaaaaaa 1129

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sequences of ME1 and deduced gene sequence are similar except at three positions (ME1<sub>D10, I13, M20</sub>; Fig. 2B), which could be due to the amino acid sequencing error reported in our previous studies (Vivanco et al. 1999). Analysis of the ME1 amino acid sequence with TargetP V1.0 (Emanuelsson et al. 2000) showed the presence of a signal peptide sequence in the first 24 amino acids. The expected size of the mature protein is 284 amino acids and the sequence analysis indicates that the protein has a molecular weight of 31.4 kDa with a pI of 8.55. The MALDI/TOF mass spectroscopy showed the size of the ME1 as 29,208, which is in close proximity to the expected size of the gene product.

Sequence comparison of ME1 with other RIPs showed that MAP has the highest amino acid homology (42%) through the entire length of the protein (Fig. 2A), and accordingly the ME1 nucleic acid sequence showed 49.9% similarity to the MAP cDNA sequence. The C-terminal sequence of ME1 is longer and does not show similarity to the C-terminal extension sequences from other RIPs like PAP, PIP2 and bouganin (Lin et al. 1991; Song et al. 2000; Den Hartog et al. 2002). However, a comparison of a short stretch of the N-terminal sequences of various RIPs showed a very close similarity between ME1 and MAP-4, with approximately 85% identity in the first 37 amino acids of the mature proteins with only three amino acid variations (Fig. 2B). ME1 and ME2 have 31.2% homology in the N-terminal amino acid sequence. The signal sequence of ME1 has very close homology with the MAP signal sequence.

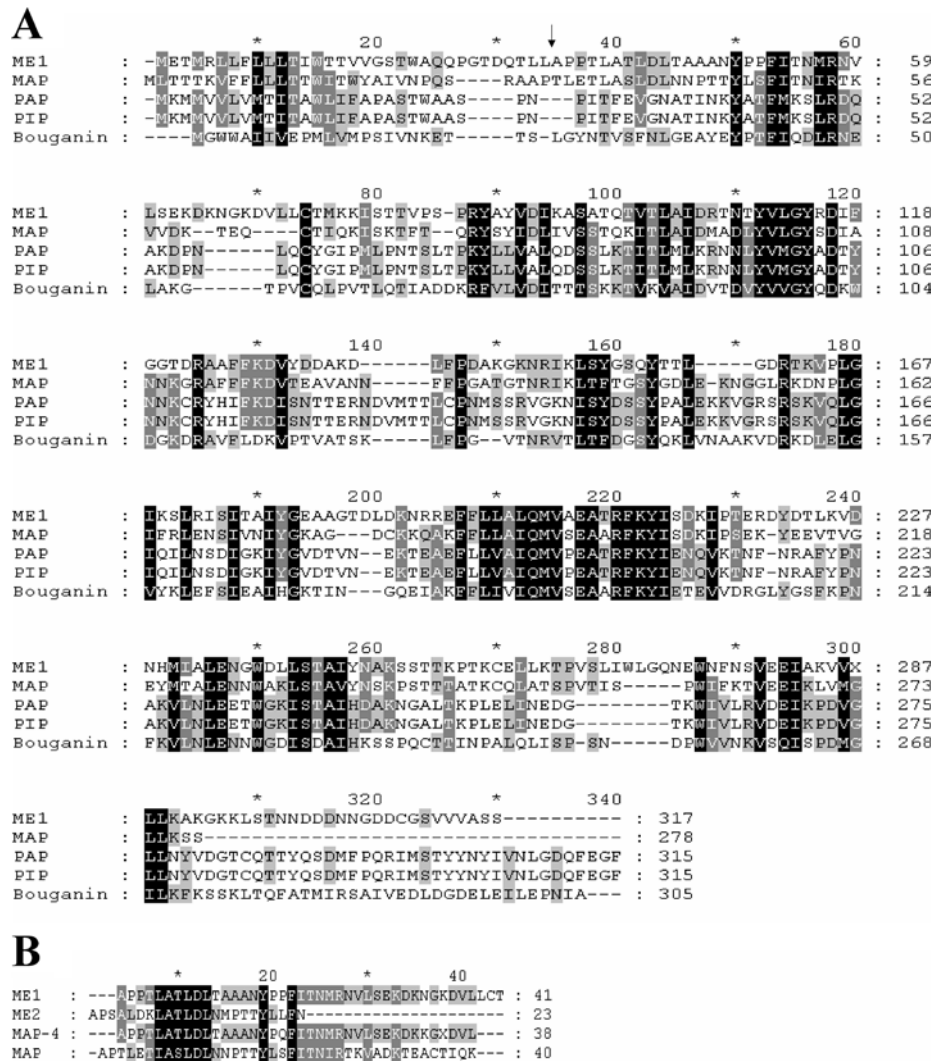
## Southern analysis

Earlier studies showed two RIPs (ME1 and ME2) present in *M. expansa* roots. To study the total number of genes homologous to the ME1 cDNA sequence, *M. expansa* DNA was digested with *EcoRI*, *SalI*, *NcoI*, *NotI* and *HindIII*, resolved on an agarose gel, blotted, and probed with <sup>32</sup>P-labeled ME1 sequence. None of the enzymes used for digestion have any cutting sites in the ME1 cDNA sequence, and two bands appeared in all digestions (Fig. 3A). All the lanes showed one band with stronger hybridization. The presence of two bands in the Southern blot could suggest that there is an intron in the ME1 gene with restriction sites for the enzymes used, or that there are two RIP genes in the *M. expansa* genome.

## Tissue-specific expression of ME1

To study the expression patterns of ME1 at the transcript level, total RNA was extracted from primary roots, storage roots, stems and leaves of *M. expansa*. The Northern blot analysis of total RNA from the different parts of *M. expansa* indicated high RNA transcript levels of the ME1 gene in primary and storage roots (Fig. 3B). A faint but clear band could be seen in the RNA from stems but ME1 gene expression was not detectable in the leaves of *M. expansa* under our experimental conditions.

**Fig. 2A, B** Comparison of the deduced amino acid sequences of ME1 with closely related RIPs. *Shading* highlights conserved residues. *Black shading* indicates typically highly conserved residues, *dark and light gray shading* represent relatively less conserved groups of amino acids. **A** Amino acid sequence of ME1, MAP (D10227), PAP (X55383), PIP2 (AF141331) and bouganin (AF445416). The *arrow* indicates the start of the mature ME1 protein sequence at amino acid residue 34. **B** Comparison of the N-terminal amino acid sequence of RIPs from *M. expansa* and *M. jalapa*

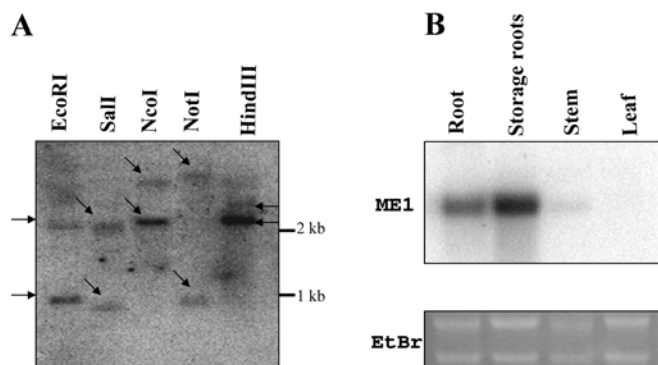


### Cellular localization of RIP in *M. expansa* roots

We studied the cellular localization of ME1 in the roots of *M. expansa*. Localization of ME1 in *M. expansa* roots was studied by immunocytochemistry in combination with light and electron microscopy. Previous studies have shown ME1 localization in the cell wall of *M. expansa* cell cultures (Vivanco and Flores 2000). In *M. expansa* roots, the cells corresponding to the cortex and core of the mature storage roots were completely filled with starch granules as seen by the scanning electron microscopy (Fig. 4A) and the starch granules were found to be stored in amyloplasts (Fig. 4B). Immunolocalization studies showed ME1 localization in the cell walls and starch granules in the storage roots (Fig. 4C). Our results suggested that ME1 might be localized in the cell walls and amyloplasts. Although we used ME1 antibodies in these studies, at this point we cannot rule out the possibility that ME2 cross-reacted with the antibodies. In no experiments did control samples incubated with pre-immune serum react with any protein in the cells.

### Expression of ME1 in cell cultures

The growth curve of *M. expansa* cell cultures showed an initial lag phase for the first 3 days followed by rapid growth up to 15 days (Fig. 5A). We tested whether JA can induce ME1 gene expression, as it does in the case of some other RIPs (Song et al. 2000). For the induction studies, JA was added to the cell suspension culture on day 6 when cell growth was at its peak. The expression of the ME1 gene was analyzed by extracting total RNA and total protein, and resolving them on agarose gels and SDS-PAGE for Northern and Western blot analysis, respectively. The results indicated that JA induces ME1 transcript expression in cell cultures (Fig. 5B). In the control where no JA was included and at 1 h after induction with JA, the ME1 band could not be detected. Very thin bands appeared 24 h after induction using 50 and 100  $\mu$ M JA. A clear band representing ME1 mRNA was observed at 24 h and 200  $\mu$ M JA. Although appreciable ME1 transcript expression was observed upon JA treatment, similar induction could not be detected at the protein level on Western blots probed



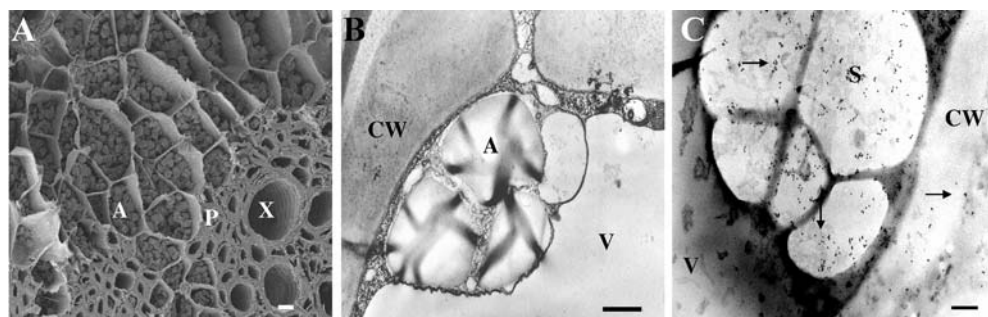
**Fig. 3A, B** Southern blot and Northern blot analyses. **A** DNA was extracted from callus culture of *M. expansa* and 10  $\mu$ g of DNA was digested with *EcoRI*, *SalI*, *NotI*, *NcoI* and *HindIII* separately overnight. The digests were separated on a 1% agarose gel and transferred to nylon membranes. The blot was hybridized with  $^{32}$ P-labeled ME1 cDNA sequence. The hybridization bands are highlighted with arrows. **B** Total RNA was extracted from roots, storage roots, stems and leaves of a 2-month-old *M. expansa* plant. Ten  $\mu$ g of each of the RNAs was separated on a 1% formaldehyde/agarose gel under denaturing conditions and blotted. Labeled ME1 cDNA sequence was used for hybridization. Bottom panel Ethidium bromide (EtBr) staining of the gel before blotting to show equal loading

with polyclonal antibodies raised against ME1 (Fig. 5C). In contrast to non-induced and JA-induced levels of ME1 mRNA, ME1 protein levels remained unaffected by JA. Interestingly, in the non-induced condition, where ME1 mRNA was very low and undetectable, the Western blots show a fair amount of protein (Fig. 5C). At 200  $\mu$ M JA induction, the ME1 protein levels remained the same even though the RNA showed a many-fold increase. These results suggest that the translation of ME1 mRNA may be post-transcriptionally regulated.

#### Depurination of mRNA

Although JA induced ME1 transcript expression in *M. expansa* cell cultures, ME1 protein was not induced,

**Fig. 4A–C** Ultrastructure and immunogold localization of ME1 in *M. expansa*. **A** Scanning electron micrograph of cortex and vacuolar cells of mature storage roots. Bar = 10  $\mu$ m. **B** Transmission electron micrograph of a storage parenchyma cell with amyloplasts. Bar = 500 nm. **C** Immunolocalization of ME1 (arrows) in storage roots. Bar = 200 nm. A Amyloplast, CW cell walls, P phloem, S starch, V vacuole, X xylem

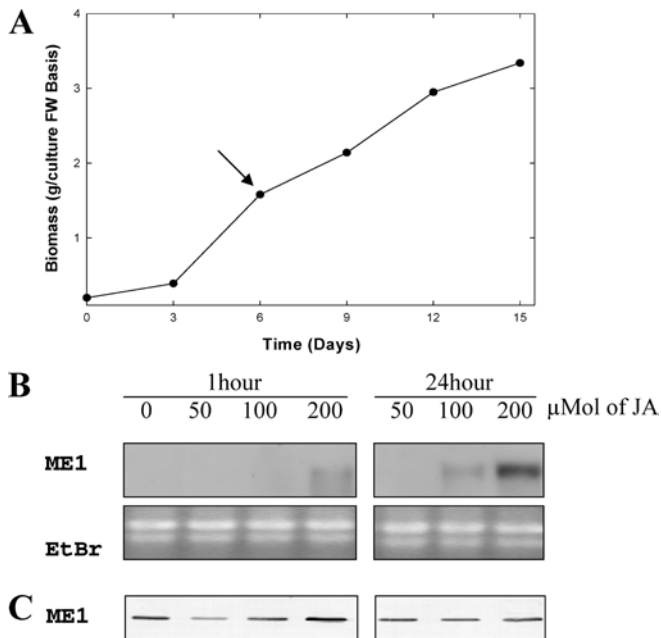


indicating that the over-induced ME1 transcript was either defective or untranslatable. It has been shown that PAP expressed in yeast is able to selectively depurinate its own mRNA (Parikh et al. 2002). Accordingly, we hypothesized that ME1 may depurinate its own transcripts or regulate its translation by affecting its own ribosomes. To test this hypothesis we checked the depurination effect of ME1 on ME1 mRNA and on rabbit reticulocyte translation.

Luciferase (control) and ME1 mRNAs were used to study the depurination effect of ME1 protein on these RNAs. In these studies, pure ME1 was used as indicated in Materials and methods (Fig. 6A). The mRNAs of ME1 and luciferase were treated independently with ME1 protein for 60 min; subsequently the mRNAs were phenol-extracted. The mRNAs of luciferase and ME1 remained intact after ME1 treatment. However, aniline treatment of these mRNAs indicated a reduced amount of the mRNAs of luciferase and ME1 (Fig. 6B), which indicated that ME1 depurinated these transcripts. The ME1-treated mRNAs (without aniline treatment) were used for translation in rabbit reticulocytes using untreated mRNAs as a control. Our results showed that the depurination of mRNAs results in inhibition of translation (Fig. 6C). The translation of luciferase mRNA was inhibited by depurination with ME1 protein. In the case of untreated ME1 transcript the translation product is low, indicating the toxic nature of the translation product. However, the ME1 protein-treated transcript failed to give any translation product.

#### Inhibition of translation of rabbit reticulocytes

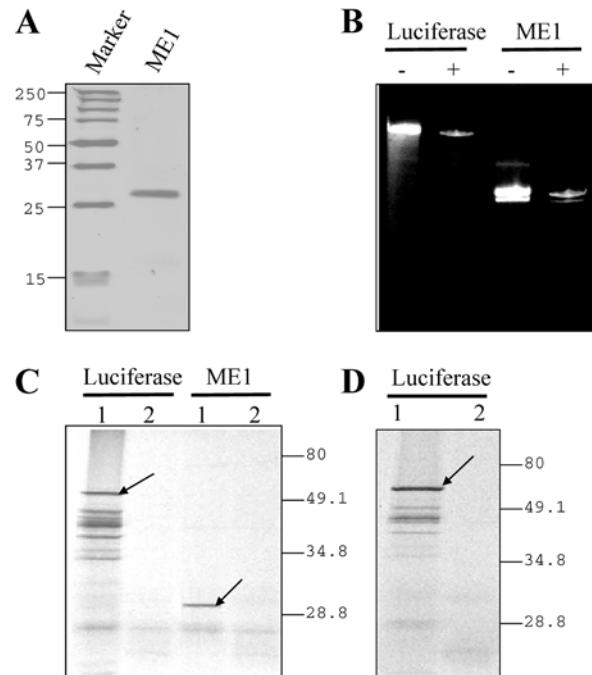
To observe if ME1 is capable of inhibiting protein translation, we performed *in vitro* translation using rabbit reticulocytes treated with ME1 protein. The reticulocytes were incubated for 30 min with 10 ng of ME1 for 30 min before the translation and the translation reaction was started by adding luciferase mRNA to the ME1-treated reticulocytes. The translation was observed by the incorporation of [ $^{35}$ S]methionine into the translation products. The translation products were resolved by 12% SDS-PAGE and the gel was dried and exposed to film. The results show that translation by rabbit reticulocytes is inhibited by a 30-min pretreatment with ME1 (Fig. 6D); in contrast, translation of luciferase mRNA can be seen in the control where no ME1 was added.



**Fig. 5A–C** Growth and elicitation studies of *M. expansa* cell cultures. **A** Growth curve of ME1 callus. An initial inoculum of 200 mg (fresh weight) of callus mass was grown in 40 ml of culture medium. The cell suspension cultures of *M. expansa* were analyzed at 3-day intervals for growth during the culture period of 15 days. The results presented are an average of three experiments. The time point that was used for JA induction studies is indicated (arrow). **B** Upper panel Induction of ME1 by JA included in the media of 6-day-grown callus cultures. Cultures were harvested at 1 and 24 h, and total RNA was extracted. Ten µg of total RNA was resolved on a 1% formaldehyde/agarose gel under denaturing conditions and blotted. Labeled ME1 cDNA sequence was used for hybridization. Lower panel Ethidium bromide (EtBr) staining of the gel before blotting to show equal loading. **C** Western analysis of ME1 induction. Protein extracts from the same cultures were extracted and 20 µg of crude protein was separated by 12% SDS-PAGE and blotted onto PVDF membrane. Polyclonal antibodies raised against ME1 were used in the immunoreaction

## Discussion

In the present study, we report the cDNA cloning of ME1, an RIP from *M. expansa* storage roots. Two RIPs have been previously reported in *M. expansa* roots (Vivanco et al. 1999; Vivanco and Flores 2000), ME1 and ME2. The full-length ME1 sequence shows that this RIP has a signal sequence, which is removed in the mature protein. Due to their N-terminal signal sequence, many RIPs are compartmentalized and secreted to avoid damage to the host cell ribosomes (Nielsen and Boston 2001). The signal sequence of ME1 has a hydrophobic region composed of 22 amino acids, which forms part of the signal motif in the N-terminal signal peptides of eukaryotes (Emanuelsson and von Heijne 2001). The signal peptide of ME1 (33 amino acids) is larger than the signal peptides of MAP, PAP, PIP2 and bouganin (Kataoka et al. 1991; Lin et al. 1991; Song et al. 2000; Den Hartog et al. 2002).



**Fig. 6A–D** Effect of ME1 protein on translation. **A** Purification of ME1 from root tubers. The affinity-column-purified ME1 protein was electrophoresed on a 12% SDS-PAGE gel and silver-stained. The positions of protein size markers (kDa) are shown on the left. **B** Luciferase and ME1 mRNAs were treated with 10 ng of ME1 protein at 30 °C for 60 min and phenol-extracted. One set of samples was further treated with aniline for 30 min at pH 4.5. The samples were resolved on an acrylamide gel (with 7 M urea). (–) ME1 protein-treated mRNA without aniline treatment, (+) with aniline treatment. **C** Translation of ME1-treated mRNA. The mRNA was treated with ME1 and then translated: 1 translation of control mRNA, 2 translation of ME1-treated mRNA. Arrows indicate translation products. The positions of protein size markers (kDa) are shown on the right. **D** Effect on translation of luciferase mRNA in rabbit reticulocytes that were previously incubated with 10 ng ME1 for 30 min at 30 °C. 1 Control reticulocyte translation, 2 ME1-treated reticulocyte translation. Arrow indicates translation products. The positions of protein size markers (kDa) are shown on the right

Southern analysis of ME1 indicates a possible homologous gene to ME1 in *M. expansa*. ME1 and ME2 show 32% homology in the N-terminal amino acid sequence. It is possible that the second gene appearing on Southern blots is ME2. However, the presence of an intron in the ME1 gene with restriction sites for the enzymes used may also give two bands on the Southern blot. Thus, our results suggest that the ME1 gene may contain an intron, or may indicate the presence of two RIPs, ME1 and ME2, in *M. expansa*. In our previous studies we have purified two RIPs (ME1 and ME2) by chromatography (Vivanco et al. 1999). Thus the presence of two bands in the Southern blot indicates the possibility of both genes being shown in the Southern blot analysis. The possibility of restriction sites for all five enzymes in the intron is unlikely unless it is a very large intron. The Northern blots show the distribution of ME1 as being restricted to primary and storage roots.

We have found that ME1 is compartmentalized in the cell walls and amyloplasts in the primary and storage roots by electron microscopy. The amyloplasts were abundant in the parenchyma of the storage root, which explains the widespread expression of RIPs in this organ. In the case of cell cultures, which express ME1 (originally referred as MEC) and do not have amyloplasts, the labeling was restricted to the cell walls (Vivanco and Flores 2000). However, in storage roots ME1 is localized in the cell walls and amyloplasts. Expression of RIPs in the walls of the root cells is consistent with its defensive role (Carmona et al. 1997). Previous reports on the sub-cellular localization of RIPs show that ricin, a type-II RIP, accumulates solely in the protein bodies of developing castor beans (Tully and Beevers 1976). PAP is present in the cell wall matrix of pokeweed leaves (Ready et al. 1986). However, the accumulation of ME1 in amyloplasts is quite unexpected and raises some questions, such as the possible resistance to RIPs in the ribosomes associated with the amyloplasts.

Plant growth regulators like JA are known to induce defense or stress-related genes in plants by activating signal transduction pathways (Sembdner and Parthier 1993). For instance, induction of RIP transcripts in the leaves of *P. insularis* is increased by both JA treatment and mechanical wounding, suggesting that wounding induces expression of the PIP2 gene via the JA-dependent pathway (Song et al. 2000). In barley, methyl jasmonate (MeJa) treatment of leaf segments caused JIP60 protein, a type-III RIP, to be induced (Chaudhry et al. 1994). To investigate the possible induction of ME1 by JA in *M. expansa* cell cultures, we studied the RNA transcript levels as well as protein levels of ME1. After JA application, the level of ME1 transcript increased but the ME1 protein levels remained uninfluenced by JA induction. We observed ME1 protein expression even when the ME1 transcripts were below the Northern blot detection levels, and the ME1 protein concentration was not appreciably changed even though the ME1 mRNA transcripts increased many fold after JA induction. This finding raises the intriguing possibility that the higher levels of ME1 transcript were not translated into ME1 protein immediately or that the excess ME1 mRNA was untranslatable. In contrast, the protein expression of JIP60 increased upon MeJa induction, representing a corresponding increase in the transcript levels (Reinbothe et al. 1997). Interestingly, jasmonates suppress protein synthesis while selectively inducing the transcripts of some proteins (Reinbothe et al. 1993). It is possible that in *M. expansa* cell cultures the induced ME1 transcripts are not translated because of the toxic nature of ME1. There are two possible ways by which ME1 may affect its own translation. It can either depurinate ribosomal RNA or depurinate its own mRNA transcript. To check the possibility of ME1 protein affecting its own transcript's translation, in vitro-synthesized ME1 transcripts were translated using rabbit reticulocytes. The in vitro translation of ME1 yielded a very low amount of ME1 protein. Since ME1

can depurinate the rRNA of rabbit reticulocytes and inhibit translation, the newly synthesized ME1 protein may inhibit further translation of its own transcript. Accordingly, ME1 can depurinate rabbit reticulocyte rRNA as seen by its ability to inhibit protein synthesis of ME1 pre-treated reticulocytes. The other possibility could be that ME1 protein may be depurinating its own transcript apart from ribosomal RNA depurination and thus affecting its translation. Treating ME1 transcripts with ME1 protein and subjecting them to aniline treatment results in loss of transcripts, indicating the depurination of mRNA at various places. A similar treatment using luciferase mRNA also resulted in failure of translation due to depurination of the transcript. Thus it is possible that ME1 may inhibit cellular translation by depurinating rRNA and its own transcript. Previous reports on PAP show that PAP can distinguish between capped and uncapped mRNA of luciferase and specifically depurinated capped mRNA of luciferase (Hudak et al. 2000). A recent finding shows that PAP can selectively destabilize its own mRNA by depurination, which is independent of rRNA depurination and translation in yeast cells (Parikh et al. 2002). Our studies indicate that ME1 can depurinate luciferase and its own transcript. Interestingly, in both cases the transcripts are not capped, indicating the selective mechanism is different in RIPs from diverse plant species. The ability of ME1 to depurinate mRNA and the failure of large amounts of ME1 transcripts to translate into protein under JA induction suggests that ME1 may regulate its own translation in *Mirabilis expansa* cell cultures.

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