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J. M. Vivanco · H. E. Flores

Biosynthesis of ribosome-inactivating proteins from callus and cell suspension cultures of *Mirabilis expansa* (Ruiz & Pavon)

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Abstract Callus and cell suspension cultures from the little known Andean crop *Mirabilis expansa* were developed and maintained on Murashige and Skoog medium supplemented with 2,4-dichlorophenoxyacetic acid (1 mg/l) and kinetin (0.1 mg/l). Callus and cell suspension cultures were screened with antibodies raised against ME1 (27.5 kDa) and ME2 (27 kDa), two ribosome-inactivating proteins (RIPs) previously found in roots of *M. expansa*. A 29-kDa protein found in callus and cell suspensions reacted strongly with ME1 antibodies. The 29-kDa protein, named MEC, was purified to homogeneity by ammonium sulfate precipitation and cation exchange perfusion chromatography. Amino acid N-terminal sequencing revealed close homology between MEC and ME1. The MEC amino acid sequence examined was highly conserved among RIPs from widely different sources. This new RIP was immunolocalized to the cell walls of callus and cell suspension cultures.

Key words RIPs · Ribosome inactivating proteins · Cell cultures · *Mirabilis expansa* · Andean root crop

Abbreviations *MS*: Murashige and Skoog medium (1962) · *2,4-D*: 2,4-Dichlorophenoxyacetic acid · *SDS-PAGE*: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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J. M. Vivanco
Department of Horticulture and Landscape Architecture,
217 Shepardson, Fort Collins, Colorado 80523-1173, USA
e-mail: jvivanco@lamar.colostate.edu

H. E. Flores (✉)
Department of Plant Pathology and Biotechnology Institute,
315 Wartik, The Pennsylvania State University, University Park,
PA 16802, USA
e-mail: hefl@psu.edu
Fax: 814-863-7217

Introduction

Plant cell cultures have been utilized for the production of valuable secondary metabolites, one example of which is shikonin, a naphthoquinone used as an antimicrobial agent in cosmetics (Tabata and Fujita 1985). Recently, the potential of plant cell cultures to produce plant enzymes such as peroxidases, chitinases and ribosome-inactivating proteins has also been explored (Parkinson et al. 1990; Kurosaki et al. 1990; Stoner et al. 1997). Of particular interest is the class of defense proteins known as ribosome-inactivating proteins (RIPs) (see review by Mehta and Boston 1998). All RIPs inactivate ribosomes by modifying the 28S rRNA, through its N-glycosidase activity, at a specific adenine residue (Endo and Tsurigi 1988). Through this mechanism, the binding of elongation factor 2 is prevented, with the consequent arrest of protein synthesis.

Ribosome-inactivating proteins also display a variety of biological activities such as broad-spectrum antiviral activity against plant and animal viruses (Batelli and Stirpe 1995), antibacterial and antifungal activities (Roberts and Selitrennikoff 1985; Vivanco et al. 1999). A number of RIPs have also been successfully tested as therapeutic agents against HIV (Zarling et al. 1990), cancers (Frankell et al. 1986) and graft versus host disease (Kernan et al. 1988). To satisfy the growing demand for RIPs, recombinant production systems such as the one employing *E. coli* have been established (Shaw et al. 1991). However, the production of RIPs in bacterial systems is rather problematic due to their intrinsic ribosome-inactivating activity and insolubility (Kataoka et al. 1991). On the other hand, plant callus and cell suspension cultures have been shown to produce RIPs at high and consistent levels (Thorup et al. 1994).

We have previously reported the identification of two RIPs with antifungal and antibacterial activities, named ME1 (27.5 kDa) and ME2 (27 kDa), in the

storage roots of the Andean root crop *Mirabilis expansa* (Vivanco et al. 1999). The endogenous production of RIPs in undifferentiated *M. expansa* cells was investigated in callus and cell suspension cultures. In this paper we report the isolation of MEC (29 kDa), a RIP related to ME1 in callus and cell suspension cultures of *M. expansa*. We found that MEC was produced at high levels (20 % of total soluble protein), which is consistent with the levels produced in the roots of *M. expansa*.

Materials and methods

Tissue culture

Shoots from *Mirabilis expansa* were collected from plants grown in the greenhouse and surfaced-sterilized with 10 % (v/v) commercial bleach (5.25 % sodium hypochlorite) for 15 min and then rinsed four times with sterile water. Shoot cuttings of approximately 3 cm long were placed in Magenta GA-7 vessels containing MS basal medium solidified with 0.3 % Phytagel (Sigma) and supplemented with different concentrations of sucrose and kinetin (Table 1). Cultures were kept in a light chamber maintained at 24 °C with a fluence rate of 100 μmole $^{m-2}$ s^{-1} PAR.

Cell culture

Callus cultures were initiated from *in vitro* -rooted cuttings. Calli were placed in MS medium supplemented with 2,4-D and kinetin and solidified with 0.3 % Phytagel. Cultures were placed in dark and light (100 μmole m^{-2} s^{-1} PAR) chambers maintained at 24 °C. Callus cultures were subcultured every 3 weeks.

Suspension cultures were initiated by transferring approximately 0.2 g callus tissue to 20 ml MS liquid medium supplemented with 2,4-D (1 mg/l) and kinetin (0.1 mg/l) in 50-ml flasks. Flasks were placed on a gyratory shaker set at 90 rpm in the dark and light chambers. Suspension cultures were subcultured every 2 weeks by aseptically pipetting 5 ml of cell culture into a new flask containing 20 ml fresh medium. The medium was stored at 4 °C for subsequent protein extraction.

Mirabilis expansa cell suspension growth was monitored on a daily basis for 21 days. Cells were collected and the fresh weight was measured in triplicate on different days. A duplicate set was collected and dried at 75 °C for 72 h to determine the corresponding dry weight. Single cells were cloned from suspension cultures using the double filter paper technique of Horsh and Jones (1980).

Table 1 *Mirabilis expansa in vitro* tissue culture and callus production. Different media formulations were used to generate in vitro plantlets and callus *from M. expansa* stems

Line	Medium	Characteristics
A	MS 1 % sucrose	Good plant growth, one major stem, primary root production, white callus
B	MS 3 % sucrose	Good plant growth, one major stem, primary root production, brown callus
C	MS 6 % sucrose	Stunted, brownish callus
D	MS 6 % sucrose, 1 mg kinetin	Stunted, brownish callus
E	MS 6 % sucrose, 2.5 mg kinetin	Good plant growth, more than five stems, primary root production, small brownish callus
F	MS 6 % sucrose, 5 mg kinetin	Stunted, more than two stems, brownish callus
G	MS 3 % sucrose, 2.5 mg kinetin	Stunted, small brownish callus
H	MS 3 % sucrose, 5 mg kinetin	Stunted, more than two stems, small brownish callus
I	MS 9 % sucrose	Stunted, more than two stems,small brownish callus

Protein extraction

Callus and cell suspension extracts were prepared according to Savary and Flores (1994). The protein solution was concentrated to 1 mg/ml using a Stirred Cell 8050 (Amicon, Beverly, Mas.) with a YM10 membrane. Protein concentration was determined by the Bradford method (1976), using bovine serum albumin (BSA) as a standard and an LKB Ultrascan XL laser densitometer to quantify individual proteins.

Preparation of proteins from liquid media

Cell suspension cultures (21 days old) were vacuum filtered with a 0.2-μm nylon membrane filter (GelmanSciences, Ann Arbor, Mich.). The 50-ml aliquot of MS medium was first stored overnight at 4 °C and then concentrated to 1 ml using a Stirred Cell with a YM 10 membrane 8050 (Amicon, Beverly, Mass.). The samples were precipitated with trichloroacetic acid (TCA) according to the method of Peterson (1977). Briefly, to each 1-ml sample containing approximately 5–100 μg protein, 100 μl of Na deoxycholate was added; the sample was then mixed and incubated for 10 min. Subsequently, 100 μl of 72 % TCA was added and the sample mixed and incubated on ice for 15 min, then centrifuged for 8 min. The supernatants were removed, and the pellets were washed three times with ice-cold 80 % acetone. Pellets were then dissolved in SDS-PAGE buffer (Laemmli 1970).

Chromatography

Cation exchange separation was performed using Waters CM-Accell SepPacks with 20 m*M* sodium phosphate (pH 7.0) as previously described by Savary and Flores (1994). The cell suspension medium was adjusted to pH 7.0 and directly applied to the SepPacks. Basic proteins were eluted with a step gradient of 50 m*M*, 200 m*M* and 600 m*M* NaCl. Target proteins identified in the 200-m*M* fraction were dialyzed and subsequently separated by POROS perfusion chromatography according to Vivanco et al. (1999). Individual peaks were collected and concentrated by ultrafiltration using a Stirring Cell 8050 (Amicon). Protein purity and peak size were confirmed by SDS-PAGE stained with Coomassie brilliant blue G-250 (Calbiochem, La Joya, Calif.).

Electrophoresis and Western blot analysis

SDS-PAGE was performed with 13.5–15 % acrylamide discontinuous gels (Laemmli 1970) using a Mini-Protean II electrophoresis cell (Bio-Rad, Richmond, Calif.). A low-molecular weight (14–66 kDa) protein marker kit was used to determine approximate protein sizes (Sigma, M.), and the proteins were visualized with Coomassie brilliant blue G-250 (Calbiochem, La Joya, Calif.). Proteins were electroblotted onto nitrocellulose membranes (Protran, Keene, N.H.), using a Bio-Rad Mini-Trans electrotransfer cell, for 1 h at 150 V (constant voltage), with 10 m*M* 3-(cyclohexylamino) propanesulfonic acid (pH 11.0 with

NaOH) and 10 % methanol transfer buffer (LeGendre and Matsudaira 1989). Membranes were developed with a secondary antibody-alkaline phosphatase detection system (Promega, Wis.), using polyclonal antibodies produced against ME1 and ME2 (Vivanco et al. 1999). An antiserum titer of 1/5000 was used for all the experiments.

Microscopy and immunolocalization

To study the location of ME1/ME2 and related proteins in cell cultures of *Mirabilis* sp., we carried out immunolocalization using a protocol adapted from Wright and Rine (1989).

Results and discussion

In vitro tissue culture

Shoot cuttings were cultured *in vitro* to generate sterile plantlets and callus. Basal MS medium was supplemented with several concentrations of sucrose and kinetin to promote callus formation. As seen in Fig. 1 and summarized in Table 1, the regenerated plantlets (lines) showed diverse phenotypes according to the different concentrations of sucrose and kinetin supplied to the medium. Plantlets grown at 1 % and 3 % sucrose levels displayed a fast and vigorous consistent growth. In contrast, plants grown in medium with levels above 3 % sucrose and 1 mg kinetin exhibited dwarfness. All regenerated plant lines showed callus production at the bottom of the stem. The callus varied in size and color depending on the treatment. In some cases (line E), microscopic examination of the interior of the callus showed tissue differentiation (data not shown).

The different callus lines (Table 1) showed similar protein patterns. As shown in Fig. 2A, total proteins extracted from the same amount of tissue in the various callus lines exhibited distinct concentration pat-

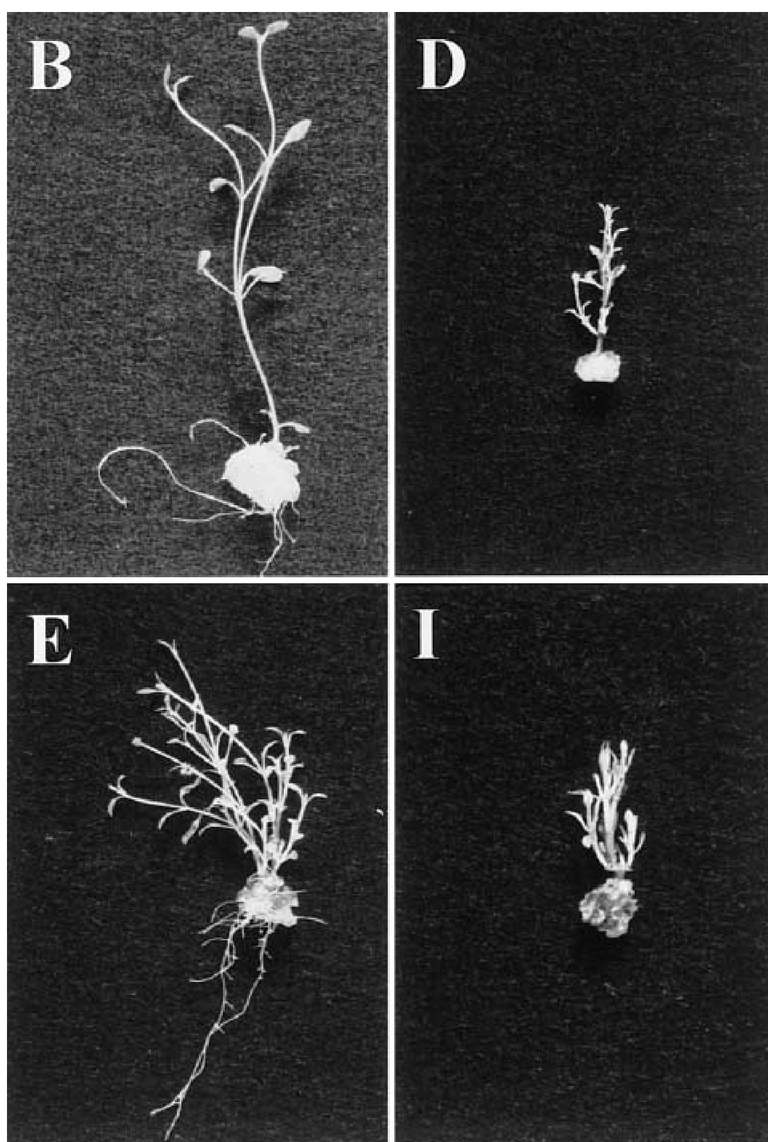


Fig. 1 *Mirabilis expansa in vitro*-grown plants. The different letters correspond to treatments (lines) listed in Table 1

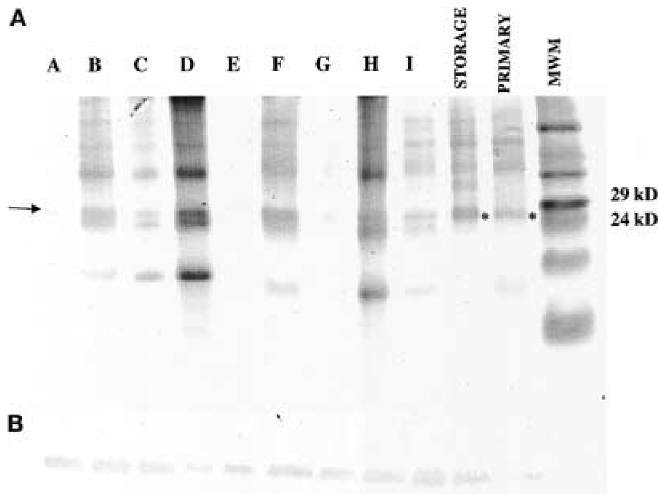


Fig. 2A,B Callus lines and storage and primary roots protein analysis from *M. expansa*. **A** SDS-PAGE of total storage and primary root proteins from the callus lines. Each lane was loaded with protein extracted from 5 mg of callus. The *asterisk* indicates the position of ME1 (27.5 kDa) and ME2 (27 kDa) in *M. expansa* storage and primary roots. **B** Western blot from duplicate gel probed with ME1 and ME2 antibodies (ME1 and ME2: RIPs from *M. expansa*). Western blotting was performed as outlined in the Materials and methods. The *arrow* indicates the position of the callus immunoreactive band in the corresponding SDS-PAGE

terns. Using monospecific antibodies produced against ME1 and ME2, we detected a strong reaction with ME1 antibody in all callus lines tested, which corresponds to a 29 kDa protein (Fig. 2B). Crossreactivity was not observed with the ME2 antibody.

Callus culture

Callus cultures were selected from different lines (Table 1). Line B showed the fastest growth on solid MS (supplemented with 2,4-D and kinetin) and was selected for subculture. Line B callus clones obtained from single cells developed readily, although light-grown calli grew more slowly than dark grown ones (Fig. 3). Certain cells from light- and dark-grown calli produced an unidentified red pigment (Fig. 3). This pigment appeared to be secreted from the cells, probably in response to some type of stress. Red pigment resembling anthocyanin is produced in the leaves of greenhouse-grown plants under drought conditions (Vivanco unpublished observations). While protein profiles were observed in dark and light-grown clones, different protein concentrations were obtained (Fig. 4A). A strong reaction with the ME1 antibody was observed in the 9 clones tested (Fig. 4B). No immunoreactive differences were observed with respect to the production of pigment between light- and dark-grown callus. Light-grown clone 8 showed the highest protein production per tissue weight.

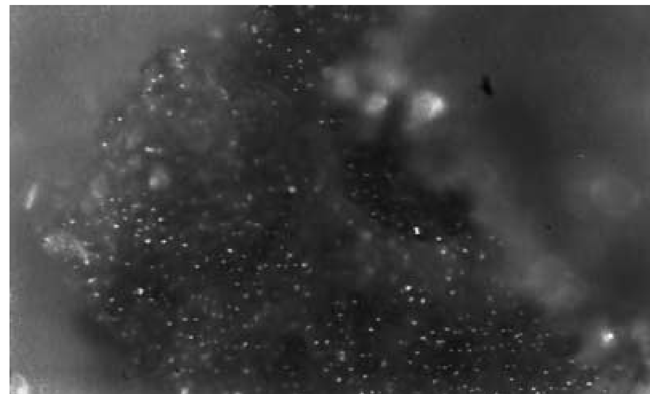


Fig. 3 Red pigment in *M. expansa* callus cultures

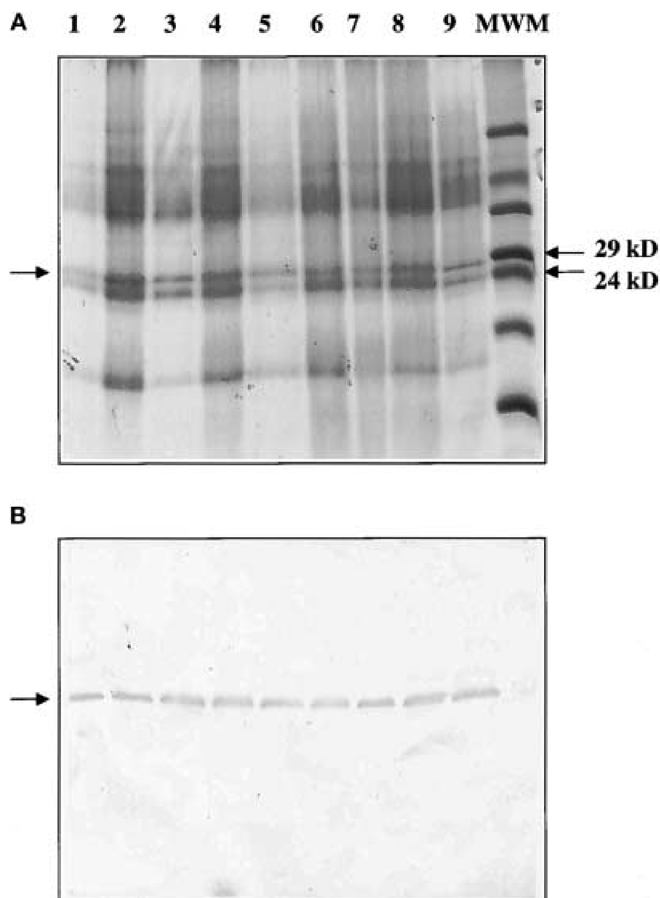


Fig. 4A,B Analysis of total proteins from light- (*lanes 6–9*) and dark-grown (*lanes 1–5*) callus clones generated from *M. expansa* callus (line B). **A** SDS-PAGE of total proteins of callus clones. Each lane was loaded with protein extracted from 5 mg of callus. *MWM* Molecular-weight marker. **B** Western blot from duplicate gel probed with ME1 and ME2 antibodies (ME1 and ME2: RIPs from *M. expansa*). Western blotting was performed as outlined in the Materials and methods

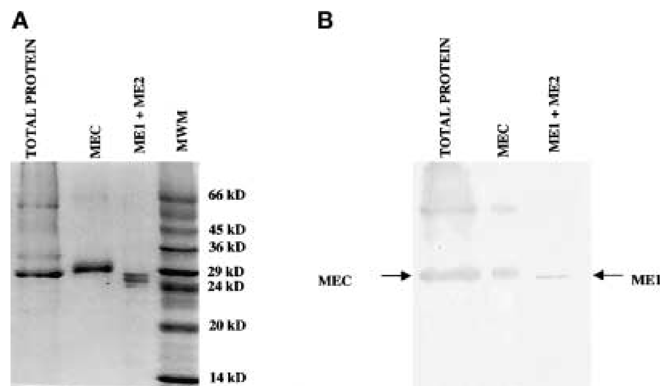


Fig. 5A,B Isolation of MEC from cation-exchange fractions from *M. expansa* cell suspension culture proteins. **A** Coomasie blue staining of a SDS-PAGE of the total proteins and MEC. **B** Western blot analysis from a duplicate gel using ME1-monospecific antibodies. Western blotting was performed as outlined in the Materials and methods

Cell suspension culture and protein purification

A cell growth curve from clone 8 was plotted over 21 days. The *Mirabilis expansa* cell suspension exhibited typical exponential cell growth until day 15, after which time it stabilized (data not shown).

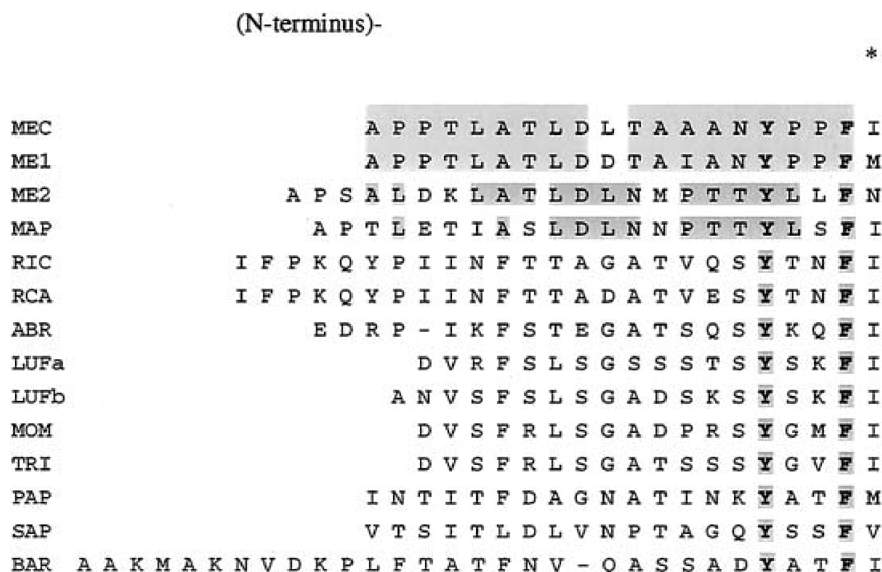
Fig. 6 Comparison of the N-terminal sequences from RIPs. *MEC Mirabilis expansa* cell cultures, *ME1* and *ME2 M. expansa* roots, *MAP M. jalapa*, ricin A-chain (*RIC*) *Ricinus communis*, *R. communis* agglutinin (*RCA*) *Ricinus communis*, *ABR* (abrin A-chain) *Abrus precatorius* , *LUFa* (luffin-a), *LUFb* (luffin-b) *Luffa cylindrica*, *TRI* (trichosanthin) *Trichosanthes kirilowii*, PAP *Phytolacca americana*, *MOM* (momordin) *Momordica charantia*, *SAP* saporin *Saponaria officinalis*, *BAR* (barley translation inhibitor) barley (Funatsu *et al*. 1991). *Shaded boxes* and *bold characters* represent two amino acids, which are conserved in all RIPs reported today. *Shaded boxes* and *normal characters* symbolize homology regions among ME1, ME2 and MAP. The *asterisk* shows the presence of conserved amino acids with nonpolar groups (hydrophobic)

The proteins from cell suspension cultures were separated under non-denaturing conditions by cation exchange (CE) chromatography utilizing a DEAE-MemSep cartridge (see Materials and methods). The 200-m*M* cell suspension fraction reacted with ME1 antibodies and was buffer-exchanged to pH 8, which facilitates the enrichment and separation by CE chromatography of strongly basic proteins, such as RIPs.

A basic 29-kDa protein, named MEC (Fig.5), was resolved by CE perfusion chromatography and subsequently eluted with a linear gradient (0–150 m*M* NaCl). Densitometric determination of total proteins of the cell suspension of *M. expansa* revealed that MEC accounted for almost 20 % of the soluble proteins (data not shown). Cell suspension medium did not show any crossreactivity with ME1 and ME2 antibodies.

Characterization and localization of MEC

The N-terminal amino acid sequence of MEC was determined and compared to those from ME1, ME2 and other RIPs obtained from diverse sources (Fig.6). The MEC amino acids residues Tyr-16 (Y) and Phe-19 (F) aligned well with the consensus sequences found in the same positions for all of the RIPs examined. A significant match was observed between MEC (29 kDa) and ME1 (27.5 kDa). These data suggest that *M. expansa* cell cultures produced a novel RIP with a slightly higher molecular weight that had not previously been found in roots. It has been reported that *Phytolacca americana* cell cultures produce RIPs similar but not identical to whole plant RIPs (Barbieri et al. 1989). We propose that MEC might be a preform of ME1, which is not post-translationally modified under *in vitro* culture conditions. RIPs are often produced as pre-proteins containing a consensus secretory sequence targeted to the endoplasmic reticulum,



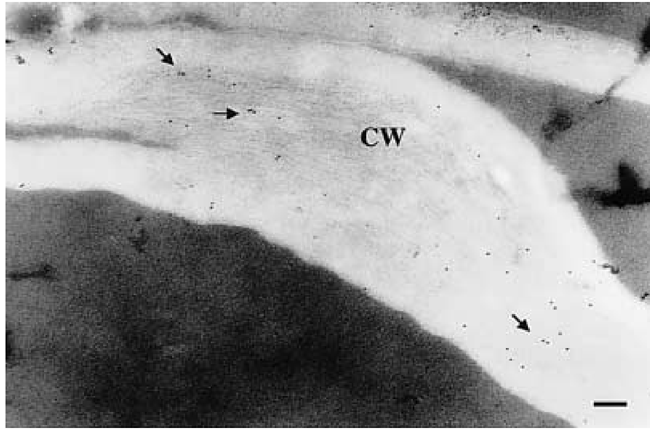


Fig. 7 Ultrastructure and immunogold localization of MEC in *M. expansa* cell cultures. Immunolocalization of MEC in the cell walls (*CW*). The *arrows* point to immunogold particles *Bar*: 200 nm

vacuoles or cell walls that is post-translationally cleaved (Ready et al. 1986; Carzaniga et al. 1994; Stoner et al. 1997). Since cell cultures are unable to differentiate, certain processes may be silenced, such as RIP post-translational modification.

Gold particle labeling using ME1 antibodies was observed in the cell walls (Fig.7). Because RIPs may potentially inactivate the ribosomes in the cells in which they are synthesized, it is believed that these enzymes are targeted to subcellular compartments as a sequestering mechanism. Pre-immune serum was utilized as a negative control without any particle labeling. Accordingly, the type I RIPs from *Phytolacca americana* and *Saponaria officinalis* are localized in the cell wall (Ready et al. 1986) and/or within the vacuole (Carzaniga et al. 1994). Expression of RIPs in cell walls has been found to be consistent with a defense role in *Saponaria officinalis* (Carzaniga et al., 1994). ME1 and ME2 have been located in the cell walls and amyloplasts in *M. expansa* roots (unpublished results). Amyloplasts were not present in cell suspension cultures. Our results thus suggest that amyloplast formation and RIP accumulation in these organelles are regulated upon tissue differentiation.

Mirabilis expansa cell cultures represent a suitable system to obtain and purify considerable amounts of sterile RIPs for pharmaceutical applications. They could also be utilized to study the response of RIPs to several biotic and abiotic stresses *in vitro*.

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