Free Ricin A Chain, Proricin, and Native Toxin Have Different Cellular Fates When Expressed in Tobacco Protoplasts*

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Ricin is a cytoxin present in the endosperm of Ricinus communis seeds, where it accumulates in protein bodies (storage vacuoles) to 5% of the total particulate protein (1). Structurally, ricin is a heterodimeric glycoprotein comprising a ribosome-inactivating A chain (RTA)1 and a galactose-binding B chain (RTB) covalently linked by a single disulfide bond. RTA (glycosylated molecular mass of ~32 kDa) catalyzes the removal of a single adenine from a highly conserved loop of 28 residues within the context of a eukaryotic ribosome (2). Ribosomes depurinated in this manner are unable to bind the elongation factor-2-GTP complex, and protein synthesis is blocked at the translocation step of the elongation cycle (3). The precise activity of RTA varies depending on the source of ribosomes. Thus, a single A chain molecule can depurinate 1000–2000 mammalian cell ribosomes/min under physiological conditions (2). This can be measured in vitro as a DC50 (the concentration causing 50% depurination) of ~5 ng/ml under standard conditions. Although ricin A chain is certainly active against tobacco ribosomes, the DC50 value is 650 ng/ml, showing that tobacco ribosomes are ~130-fold less sensitive than mammalian or salt-washed yeast ribosomes (4). Nevertheless, such is the potency of RTA that should it begin to accumulate within the cytosol of tobacco leaf protoplasts, the protein bio-synthetic capacity of the expressing cells would be severely compromised.

When heterodimeric ricin is presented to the surface of mammalian cells, RTB opportunistically binds to membrane components with exposed galactose residues. Toxin molecules are then endocytosed to a specific internal compartment from which RTA translocates to reach the cytosol, where the ribosomes are located. There is now considerable evidence supporting the cytosolic entry of RTA from the endoplasmic reticulum (ER) lumen (5–7). The possibility of studying the retrotranslocation of RTA by directly delivering the protein to the ER lumen has been explored, but attempts to express RTA in eukaryotic cells such as mammalian and yeast cells have failed due to the extreme sensitivity of the ribosomes to the toxins.2 This poses obvious questions concerning the biosynthesis of toxin in planta. If the ER is the compartment for reverse translocation of toxin to the cytosol in eukaryotic cells, how does the synthesizing plant cell avoid intoxication? Cells of R. communis synthesize ricin as a precursor polypeptide (proricin) with a glycosylated molecular mass of ~68 kDa (8). This protein consists of RTA (preceded by a signal peptide) and RTB joined by a short linker peptide (9). From the ER lumen, proricin is transported to storage vacuoles via vesicular transport through the Golgi complex (10). Only upon correct targeting to these storage vacuoles is the linker between RTA and RTB removed to yield mature toxin (10). Ricin then stably accumulates within the low pH environment of these storage organelles. That RTA exists in the Ricinus cell ER as part of a precursor may render it incompetent for reverse translocation across the ER membrane, a possible safeguard against cell suicide. Here we present evidence that the cellular fate of RTA can vary depending on the form of toxin expressed within tobacco protoplasts. Indeed, it is only when RTA is synthesized as part of the proricin molecule that it is delivered, with minimal cytotoxicity, to its normal destination of the vacuoles.

EXPERIMENTAL PROCEDURES

Plasmid Construction—All coding sequences derive from the full-length proricin cDNA clone (GenBank™ accession number X03179) and were cloned in the CaMV35S promoter-driven expression vector pDHA (11). The basic features of the inserts in the different constructs are summarized in Fig. 1. Full-length proricin was subcloned as an XbaI/PstI fragment into XbaI/PstI-cut pDHA. pRTA, encoding pre-RTA (residues 1–302 on the proricin cDNA clone (9)) was constructed by site-directed mutagenesis of pRTA, ricin toxin A chain; RTB, ricin toxin B chain; ER, endoplasmic reticulum; BFA, brefeldin A; PAGE, polyacrylamide gel electrophoresis; pRTA, ricin A chain preceded by a signal sequence; cRTA, cytosolic RTA lacking a signal sequence.

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1 The abbreviations used are: RTA, ricin toxin A chain; RTB, ricin toxin B chain; ER, endoplasmic reticulum; BFA, brefeldin A; PAGE, polyacrylamide gel electrophoresis; pRTA, ricin A chain preceded by a signal sequence; cRTA, cytosolic RTA lacking a signal sequence.

2 L. Frigerio, A. Vitale, J. M. Lord, A. Ceriotti, and L. M. Roberts, unpublished results.
cloning an XbaI/PstI fragment from pGEMRA (12) into the same sites of pDH.A. cRTA, encoding a truncated form of pRTA starting at Met-12, was excised from pGEMRA (13) as an XbaI/PstI fragment and cloned into the same sites of pDH.A.

To obtain pRTB, the sequence encoding the full signal peptide (residues 1–24) of β-phaseolin (SwissProt accession number P02853) (14) was fused to the mature RTB coding sequence via overlapping mutagenic polymerase chain reaction. The fusion protein was then inserted into the XbaI/PstI sites of pDH.A.

The construction of pDHET343F has been described (15). This construct contains the complete coding sequence of a phaseolin variant in which the glycosylation site at position 341 has been destroyed.

Transient Transformation of Protoplasts and Pulse-Chase Labeling—For transient expression of all constructs, protoplasts were prepared from axenic leaves of Nicotiana tabacum cv. Petit Havanna SR1. Protoplasts were subjected to polyethylene glycol-mediated transfection as described (15). Vector pDHNA without inserts was used as a negative control for transfection.

After transfection, protoplasts were allowed to recover overnight in the dark at 25 °C in K3 medium (Gamborg's B5 basal medium with minimal organics (Sigma), supplemented with 750 mg/liter CaCl₂, 250 mg/liter NH₄NO₃, 136.2 g/liter sucrose, 250 mg/liter xylose, 1 mg/liter 6-benzylaminopurine, and 1 mg/liter α-naphthaleneacetic acid, pH 5.5) at a concentration of 10⁶ cells/ml. Protoplasts float in K3 medium and can be kept viable in this medium for days. Protoplasts were radiolabeled by incubation in the dark at 25 °C in K3 medium supplemented with 150 μg/ml bovine serum albumin and 100 μCi/ml Pro-Mix (Amersham Pharmacia Biotech). Chase was performed by adding unlabeled methionine and cysteine to 10 and 5 mM, respectively. In some experiments, before radioactive labeling, protoplasts were incubated for 45 min at 25 °C in K3 medium supplemented with 10 μg/ml brefeldin A (BFA) (Boehringer Mannheim; 2 mg/ml stock solution in 1.5% Triton X-100) supplemented with “complete” protease inhibitor mixture (Boehringer Mannheim). After vortexing, the homogenates were pelleted by centrifugation at 60,000 × g for 5 min. Due to the composition of W5 medium, protoplasts sink without bursting. The supernatant, containing secreted proteins, was removed, leaving 50 μl above the protoplast pellet. Cells and supernatants were frozen in liquid nitrogen and stored at −80 °C.

Preparation of Protein Extracts and Immunoprecipitation of Toxins—The frozen samples were homogenized by adding 2 volumes of protoplast homogenization buffer (150 mM Tris–Cl, pH 7.5, 150 mM NaCl, and 1.5% Triton X-100) supplemented with “complete” protease inhibitor mixture (Boehringer Mannheim). After vortexing, the homogenates were used for immunoprecipitation with rabbit polyclonal antisera against phaseolin or vector alone (control). We then pulse-labeled the protoplasts with [³⁵S]methionine and [³⁵S]cysteine for 1 h and chased them for different times in the presence of unlabeled amino acids. We homogenized protoplasts in the presence of non-ionic detergent to solubilize all the proteins present in the endomembrane system. Immunoprecipitation with anti-RTA antiserum showed that, after the pulse, preproricin-transfected cells synthesized a polypeptide that has an apparent molecular mass of 68–70 kDa (Fig. 2A). This is equivalent to the expected size of the glycosylated ricin precursor. After a 5-h chase, however, most of the precursor had disappeared, and immunoreactive bands were detected with the expected sizes of mature glycosylated RTA (32 kDa) and RTB (34 kDa). Minor polypeptides around 40–46 kDa were present also in mock-transfected protoplasts and therefore do not represent ricin.

Thus, proricin was being proteolytically processed within tobacco cells to release the toxin subunits. The conversion from the precursor form to the mature subunits was particularly evident when samples were analyzed at intermediate chase times (Fig. 2C). These data are in agreement with earlier work tracing the transport of proricin within Ricinus cells (10). Indeed, the processing activity has been shown to reside in the storage vacuoles (10, 20). In the presence of BFA, which prevents transport of storage proteins to the vacuole in transgenic tobacco (15, 21), the appearance of mature subunits was prevented (Fig. 2, A and C), as would be expected if proricin were normally transported to storage vacuoles via vesicular transport through the Golgi stack. Proricin was never detected in the...
methionine and cysteine for 1 h and chased for the indicated periods of time in the presence of unlabeled amino acids. Samples were immunoprecipitated with anti-RTA antiserum and analyzed by SDS-PAGE and fluorography. The SDS-PAGE analyses shown in A and C were performed under reducing conditions; the one shown in B was performed under nonreducing conditions.

extracellular medium (data not shown). That mature RTA and RTB are covalently linked is shown in Fig. 2B, a duplicate of Fig. 2A, except that the samples were run under nonreducing conditions. Prorcin contains five intrachain disulfide bonds, which results in its having an apparently lower molecular mass on gels than when these bonds are broken under reducing conditions (Fig. 2, A and C). The small size difference observed between prorcin and mature holotoxin after the chase in the absence of BFA (Fig. 2B) most likely represents loss of the 12-amino acid linker during toxin maturation.

**RTA Is Proteolytically Degraded in Tobacco Protoplasts**

When experiments similar to the ones described above were performed using the two RTA constructs (viz. with and without the ricin signal peptide), there was a dramatic loss of RTA during the chase period (Fig. 3A). This was particularly noticeable for the RTA possessing a signal peptide (pRTA). Such apparent loss was not prevented by continuous treatment of the protoplasts with BFA, suggesting that RTA was not being secreted or targeted to and degraded within acidic vacuoles. Analysis of the extracellular medium revealed a complete absence of RTA (not shown here, but see Fig 6). By treating protoplasts with tunicamycin (an inhibitor of N-linked glycosylation), it was evident that all the detectable pRTA was glycosylated (Fig. 3B). As expected, tunicamycin did not have any effect on cRTA mobility, confirming the cytosolic localization of this RTA variant. Thus, pRTA was being translocated into the lumen of the ER and becoming glycosylated prior to an apparent degradation that did not depend on Golgi complex-mediated transport to the vacuole. The glycosylated RTA observed after a 1-h pulse in the presence of BFA had a slightly lower molecular mass than that made in the absence of BFA (Fig. 3A, compare lane 3 with lane 1). This is probably the result of oligosaccharide-trimming events catalyzed by Golgi enzymes after their redistribution to the ER following BFA treatment (15). The time course of degradation of cytosolic and ER-segregated forms of RTA was also analyzed (Fig. 3C). Degradation started within the first hour of chase and occurred with comparable kinetics for both RTA forms, with pRTA being degraded at a slightly faster rate than cRTA.

**Microsomal Localization of pRTA in Tobacco Protoplasts**

pRTA glycosylation demonstrates that this protein is initially translocated to the ER. However, the BFA-insensitive degradation suggests that, as must occur in intoxicated mammalian cells, the glycosylated polyepitope is retrotranslocated to the cytosol.

To compare the cellular location of pRTA with that of prorcin and mature toxin subunits, cells were pulse-labeled for 1 h and chased for 4 h. Protoplasts were then homogenized in buffer containing 12% (w/v) sucrose, which is isosmotic with the cytosol and avoids bursting of the microsomes originating from the ER and the Golgi complex; the soluble proteins contained in the vacuoles, which rupture completely during homogenization, are released and remain in the soluble fraction, even when subjected to high speed centrifugation (15, 22). The homogenates were centrifuged through a sucrose pad to separate microsomal pellets from soluble material. Immunoselection of immunoglobulin heavy chain binding protein revealed the integrity of the microsomal fraction (Fig. 4A). Glycosylated RTA was present in the microsomal fraction predominantly after the 1-h pulse, but after a 4-h chase, it had largely disappeared (Fig. 4B). When protoplasts expressing prorcin were similarly labeled and fractionated (Fig. 4C), the precursor was found mainly in microsomes at the 1-h time point, whereas processed subunits were prevalent only in the soluble fraction after 4 h, indicating their likely presence in the vacular fraction. The presence of precursor polyepitopes in the soluble fraction at the end of the pulse probably represents a fraction of

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**Fig. 2. Expression of preproricin in tobacco protoplasts.** Cells were transfected with control (Co) or prorcin (ppricin) constructs. Transfected protoplasts were preincubated for 45 min either in the presence (+) or absence (−) of 10 μg/ml BFA and then labeled with [35S]methionine and [35S]cysteine for 1 h and chased for the indicated periods of time in the presence of unlabeled amino acids. Samples were immunoprecipitated with anti-RTA antiserum and analyzed by SDS-PAGE and fluorography. The SDS-PAGE analyses shown in A and C were performed under reducing conditions; the one shown in B was performed under nonreducing conditions.

**Fig. 3. Synthesis and fate of free ricin A chain in tobacco protoplasts.** Cells were transfected with the secretory (pRTA) or cytosolic (cRTA) RTA constructs. A, pulse-chase analysis in the presence (+) or absence (−) of 10 μg/ml brefeldin A. Cell homogenates were immunoselected with anti-RTA antiseraum and analyzed by SDS-PAGE and fluorography. B, cells pulse-labeled for 1 h in the presence (+) or absence (−) of 50 μg/ml tunicamycin (Tm) and analyzed as described for A. C, time course of A chain degradation. Cells transfected as described for A were pulse-labeled for 1 h and chased for 1, 2, 3, and 4 h, and homogenates were analyzed as described for A. The intensity of the immunoselected bands was measured by densitometry and expressed as the percentage of total RTA immunoselected after the pulse. Results are the average of four independent experiments.
proricin polypeptides that have already reached the vacuole, but have not yet undergone proteolytic maturation.

prRTA Is Toxic to Tobacco Protoplasts—Since RTA has ribosome-inactivating ability, it was of interest to assess the toxicity of these proteins to the tobacco cells. Since the percentage of protoplasts transfected is low, usually on the order of ~10%, a standard measurement of [35S]methionine/cysteine incorporation into protein of the entire population of cells would be meaningless. It was therefore important to examine the transfected cell population alone. This was achieved by cotransfecting protoplasts with DNA encoding toxin together with DNA encoding phaseolin, a nontoxic storage protein normally found in Phaseolus vulgaris seeds. After cotransfection, cells were incubated overnight to allow accumulation of the exogenous mRNAs and then subjected to pulse labeling for 1 h. Radiolabeled phaseolin was immunoselected, and its synthesis was quantified by densitometry of the immunoprecipitated bands, relative to the control transfection in which toxin DNA was excluded. The results are shown in Fig. 5, revealing that proricin was not toxic to cells, whereas both glycosylated RTA and cytosolic RTA showed significant inhibition of phaseolin synthesis. It should be noted that the inhibition measured by pulse labeling is the result of the continuous synthesis of RTA during the overnight incubation that preceded the pulse period. It seems unlikely that glycosylated RTA could inhibit cytosolic ribosomes from the lumen of the ER. Therefore, glycosylated toxin is most probably retrotranslocated from the endomembrane system to the cytosol.

In Vivo Reconstitution of Ricin Holotoxin—To examine whether coexpression of RTB would permit assembly of a mature toxin within the ER and whether such assembly might reduce the toxicity caused by glycosylated RTA, protoplasts were cotransfected with prRTA and prRTB DNAs and pulse-labeled and chased in the standard way. Immunoprecipitates are shown in Fig. 6A. Both prRTA and prRTB, when expressed singly or together, were detectable intracellularly at the end of the pulse, but not after the chase. Examination of the extracellular medium revealed that free prRTB and coexpressed prRTA/prRTB were being secreted from the cells. By contrast, free glycosylated RTA was not found in the medium after the chase period. Thus, prRTB expression leads to the stabilization and secretion of prRTA, which is otherwise degraded intracellularly. The co-immunoprecipitation of prRTA and prRTB with anti-RTA antibodies indicates that prRTA stabilization and secretion are due to the formation of pRTA-prRTB heterodimers. Indeed, when the coexpressed sample was analyzed by nonreducing SDS-PAGE, it was clear that the two subunits are disulfide-bonded, suggesting a post-translational assembly of mature toxin in the ER lumen, followed by secretion rather than a routing to vacuoles. Not all the prRTA was present as a disulfide-linked dimer, as judged by the presence of free prRTA in the nonreduced sample (Fig. 6B).

Interestingly, the synthesis of prRTA was found to be consistently higher in protoplasts coexpressing both subunits. To investigate whether this reflected a prRTB quenching effect on the toxicity of the coexpressed prRTA, incorporation of radiolabeled amino acids into the reporter protein phaseolin was again examined. As shown in Fig. 5, prRTB coexpression partially rescued phaseolin synthesis in pRTA-expressing protoplasts. prRTB coexpression must therefore interfere with step(s) involved in the presentation of the toxic subunit to cytosolic ribosomes.

**DISCUSSION**

Correct Targeting and Lack of Toxicity of Preproricin in Tobacco Protoplasts—In this work, we have compared the in-
tracellular targeting and toxicity of preproricin and the two ricin subunits when expressed in tobacco protoplasts. Preproricin is synthesized, transported, and processed to a heterodimer, reflecting correct vesicular transport to the vacuoles and endoproteolytic processing after deposition, analogous to events within castor bean seeds (10). Indeed, this pathway is taken by many storage proteins and lectins, including those from bean, rice, pea, barley, and pumpkin (15, 23, 24). Vacuoles of leaf cells have a higher hydrolytic activity than those of storage tissues. However, it has been shown for some proteins normally accumulated in storage vacuoles that they are faithfully processed when expressed in vegetative tissues or protoplasts (24, 25). Whereas the ricin precursor is correctly targeted to the vacuole, the reconstituted pRTA-pRTB dimer is secreted, indicating that the targeting information for routing to vacuoles normally requires the 12-amino acid linker.

Expression of preproricin does not affect the synthesis of a marker protein, showing that the precursor form is efficiently targeted to the ER lumen and is absent in the ribosome-containing cytosol (Fig. 5). The presence of RTB within the precursor may render the ER-segregated material incapable of export back to the cytosol. The data suggest that once transported through the Golgi complex and processed intracellularly, the mature toxin can safely accumulate in vacuoles of plant cells. The interchain disulfide bond would be very stable in such a low pH compartment and might be important in maintaining mature ricin incompetent for translocation to the cytosol.

**Free A Chain Is Toxic and Targeted for Degradation in Tobacco Protoplasts**—When cytosolic or ER-targeted RTA was expressed, the proteins were unstable, and we observed a strong reduction in the synthesis of coexpressed phaseolin. Since all detectable ER-targeted RTA was in a glycosylated state, it is likely that toxicity was due to reverse translocation of pRTA from the ER lumen (the site of N-glycosylation) to the cytosol. Alternatively, this toxicity might be due to a tiny fraction of non-segregated, non-glycosylated pRTA, too low in amount to visualize. The latter explanation would appear unlikely in that the level of toxicity observed is equivalent to that seen when RTA is deliberately expressed in the cytosol. It seems more likely that a significant amount of glycosylated pRTA was able to reach the ribosomes to inhibit protein synthesis. This interpretation is also supported by the observation that pRTB coexpression reduces the toxic effect of synthesizing pRTA. This strongly suggests that pRTB is sequestering a fraction of ER-located RTA from the pathway that leads to its presentation to cytosolic ribosomes. The effect of pRTB on pRTA toxicity must be exerted within the ER and cannot be due to an interaction occurring in the cytosol since microinjected RTA-RTB dimers are potently active on ribosomes (26).

The observation that pRTB expression mitigates the toxicity of pRTA is apparently in contrast with the high toxicity of ricin holotoxin to mammalian cells. However, in these cells, ricin might be subjected to activation steps during internalization and retrograde transport, which are essential for its toxicity. Such steps may not occur when newly made holotoxin is assembled in the endomembrane system of plant cells. Recent evidence suggests that, in mammalian cells, the presence of RTB is not essential for RTA to enter the cell (6); rather, RTB increases the efficiency of binding to the cell surface and internalization. RTB is therefore likely to dissociate from RTA at some stage during retrograde transport, although where this might occur is not known.

The observed toxicity of ER-targeted RTA and its rapid degradation might be linked events. Indeed, the ER lumen is a major cellular site of protein folding and oligomerization, and it has been recognized for some time that proteins that do not fold or assemble properly in the ER are rapidly degraded (27). Recent evidence indicates that several defective proteins are not degraded within the ER, but rather by the cytosolic ubiquitin/proteasome system. For example, in the presence of particular viral gene products in mammalian cells, glycosylated major histocompatibility complex class I molecules can be transported from the ER to the cytosol for degradation (28). Malformed secretory proteins have also been shown to exit the yeast ER (29, 30). The ER is therefore able to rapidly and selectively export proteins and glycoproteins back into the cytosol, possibly by reverse translocation through the Sec61p-containing translocons that normally deliver nascent proteins into the ER lumen (31). If this is the fate of pRTA, it may be that, in the absence of pRTB, pRTA is recognized as an unassembled subunit of an oligomeric protein and thus dislocated for degradation in the cytosol. Thus, we might have recreated within plant cells the translocationally competent form of RTA that is not normally found in the ER of the toxin-producing plant cells. We tested the effect of “classical” proteasome inhibitors such as lactacystin (32) and MG-132 (31) on the degradation kinetics of pRTA and cRTA (data not shown). At the concentrations normally effective in mammalian cells, these inhibitors did not prevent RTA degradation when added to tobacco protoplasts. This is apparently the case in yeast cells also (33). Whether this is due to inefficient uptake by the plant cells, to a lower sensitivity of the plant proteasome to the drugs, or to the fact that the degradative pathway of RTA does not involve the proteasome is not clear at present. These findings, however, preclude the experimental approach successfully used with mammalian cells. We should add that no successful application of proteasome inhibitors has been reported in plants so far.

Although a fraction of pRTA must reach the cytosol to exert its toxic effects, we cannot completely exclude the possibility that the bulk of free pRTA is delivered to the vacuole for degradation. However, vacuolar degradation is not supported by results from the BFA experiments. These show that pRTA degradation is not affected by BFA, whereas proricin delivery to the vacuole can be efficiently inhibited by the drug. Thus, if the bulk of pRTA is degraded in the vacuole, the pathway followed during transport from the ER must radically differ from that followed by proricin, which utilizes a route through the BFA-sensitive Golgi stack. An autophagic route to the vacuole has been shown to participate in storage protein deposition in wheat endosperm (34). Overall, the behavior of pRTA is similar to that of an assembly-defective mutant of the trimERIC storage protein phaseolin. In protoplasts from transgenic tobacco, wild-type phaseolin is targeted to the vacuole in a BFA-sensitive manner, whereas the assembly-defective mutant has a prolonged interaction with the ER chaperone immunoglobulin heavy chain binding protein before being degraded in a process that cannot be inhibited by BFA (15). In this case also, the location of the degradation process remains to be established. It is also evident, from the efficient secretion of co-assembled pRTA-pRTB heterodimers, that pRTA does not possess an active vacuolar targeting signal.

In contrast to the fates of pRTA and preproricin, free pRTB is efficiently secreted by protoplasts, indicating that, in plant cells, correct folding of this polypeptide can occur in the absence of RTA. This has been previously observed using *Xenopus* oocytes as an expression system (35). Secretion of pRTB also reveals the absence of a vacuolar targeting signal within the mature polypeptide. It would therefore appear that the signal utilized by ricin for vacuolar targeting resides within the 12-amino acid residue linker that connects RTA and RTB in the proricin precursor.
Tobacco Cells Tolerate RTA Synthesis—Overall, it can be seen that RTA has a number of very different fates depending on the way it is synthesized in tobacco protoplasts. The preproricin precursor is clearly the most effective means of producing ricin in a nontoxic manner. Only when synthesized in this form can eukaryotic cells survive in the long term, as exemplified by the expression of preproricin in transgenic tobacco plants (36). The synthesis of ricin in a precursor form most likely guarantees the perfect stoichiometric balance between the two subunits and the concomitant absence of any free RTA in the ER.

Other efforts to successfully express ricin A chain in eukaryotic cells, including *Xenopus* oocytes, yeast, insect cells, and mammalian cells, have failed, and such work remains largely unpublished. The tobacco protoplast expression system therefore shows a unique feature: it allows the expression of RTA in a nonlethal fashion, providing an unprecedented tool to follow the intracellular fate and a means to measure the toxicity of the expressed protein in *vivo*. In addition to the relative resistance of tobacco ribosomes to RTA action, other factors may allow RTA synthesis in tobacco cells. If RTA is able to cross the ER membrane in tobacco protoplasts, it may arrive in the cytosol in an unfolded or partially folded state. Using mammalian ribosomes *in vitro*, we have evidence for ribosome-facilitated refolding of a partially unfolded RTA.3 This refolding may protect the toxin from degradation. By contrast, we may speculate that the more recalcitrant tobacco ribosomes do not facilitate refolding of RTA to the same degree, leaving a significant fraction of the toxin susceptible to degradation.

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