CHAPTER IV

MATERIALS AND METHODS

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4.1 Isolation of plasmid DNA

Isolation of plasmid DNA from *E. coli* and *A. tumefaciens* was done using the Qiaprep Spin Miniprep Kit (Qiagen, Valencia, CA). The procedures were followed as described by Qiagen.

4.2. Purification of DNA fragments

Before performing ligation reactions, all DNA fragments were purified from agarose gel using Qiaquick gel extraction kit (Qiagen, Valencia, CA).

4.3 Transformation of *E. coli* competent cells

Methods for *E. coli* transformation were adapted from Sambrook *et al.* (1989). Approximately 100-200 µl of competent cells (One Shot™ competent *E. coli* cells, Invitrogen, Carlsbad, CA) were transferred to microcentrifuge tubes and 1-2 µl of plasmid were added (approximately 50 ng plasmid per 10 µl of competent cells). The samples were incubated on ice for 30 minutes, followed by incubation in a 42 °C water bath for 2 minutes. Samples were then incubated on ice for 5 minutes and 800 µl of SOC medium (GibcoBRL, Rockville, MD; 10mM sodium chloride, 2.5 mM potassium chloride, 20 mM glucose, 2% tryptone, 0.5% yeast extract, pH 7.0) were added. Next, the samples were incubated in 37 °C water bath for 45 minutes. Two hundred µl of the putative transformed cells were transferred to LB plates containing 100 mg/l kanamycin. Plates were then incubated at 37 °C overnight.

4.4 35S – ricin construct

The full length coding region of ricin, which is 1731bp (Lamb *et al.*, 1985), was generously provided by Sehnke (Department of Horticultural Sciences, University of Florida) in the pBI121 plasmid (Clontech, Palo Alto, CA; Figure 4.1) under the control of the 35S promoter (Sehnke *et al.*, 1994). This vector was called pBIR and stored as a dimethylsulfoxide (DMSO) stock (70µl DMSO/1ml culture) in *E. coli* strain DH5α at

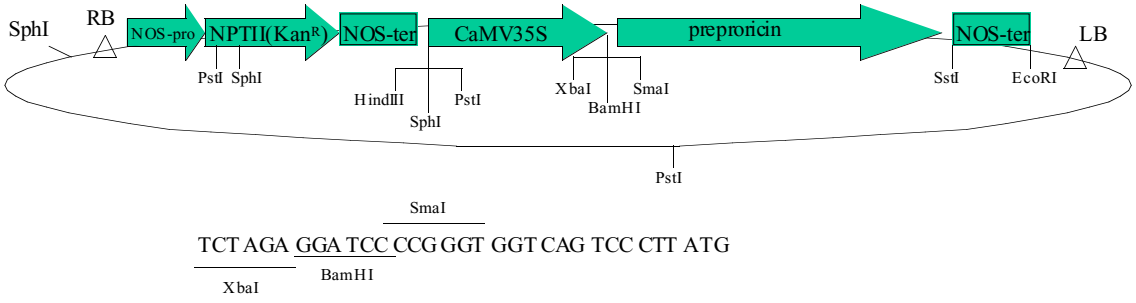

Fig. 4.1. pBI121 vector (13.0 kb, Clontech, Palo Alto, CA)

-70°C. *E. coli* was grown in LB medium containing 100µg/ml kanamycin.

4.5 Construction of $35S^{DE}$ ricin vector

The cloning strategy for $pBIR^{DE}$, which fused ricin to the enhanced 35S promoter, is shown in Figure 4.2. A construct consisting of the preproricin coding region fused to $35S^{DE}$ promoter was produced. The $35S^{DE}$ promoter was provided to the Cramer laboratory by J. Mullet (Texas A & M) as a fusion with the leader sequence of the tobacco etch virus (TEV) in the vector pRTL2 (Kulakova *et al.*, 1995). The 35S-ricin fragment was excised from pBIR using the restriction enzymes *Hind*III and *Sst*I and ligated to pBC vector (Promega, Madison, WI). This intermediate plasmid was linearized with *Xba*I, treated with Mung-bean nuclease (GibcoBRL, Rockville, MD) to create blunt ends and then digested with *Sst*I to yield a *Sst*I/blunt-ended fragment containing the ricin coding region. Similarly pRTL2 containing the $35S^{DE}$ promoter was linearized with *Nco*I, also treated with Mung-bean nuclease to create blunt ends and was then digested with *Hind*III to yield a *Hind*III/blunt ended fragment containing the $35S^{DE}$ promoter. The ricin and $35S^{DE}$ fragments were ligated into pBIB-Hyg (Becker, 1990) resulting in a plasmid referred to as $pBIR^{DE}$. This construct was kept at —70℃ in *E. coli* as a DMSO stock. The junctions of $pBIR^{DE}$ were confirmed by sequencing at the sequencing facility of the University of Chicago.

4.6 Construction of MeGA™- ricin vector

Figure 4.3 shows the cloning strategy for $pBIR^{MeGA}$ vector in which ricin is fused to MeGA™ promoter. Preproricin was excised from pBIR using the restriction enzymes *Xba*I and *Sst*I and was inserted into pYT-2-HYG (Tian and Cramer, unpublished) which contained the MeGA promoter fused to the hemorrhagic enteritis virus (HEV) fiber gene. Preproricin replaced the HEV-fiber in this pBIB-Hyg vector. Colonies containing MeGA-ricin were selected using colony hybridization using a labeled ricin probe (see section 4.12). Colonies were transferred onto a nitrocellulose filter, which was placed on top of LB media containing kanamycin (100mg/l).

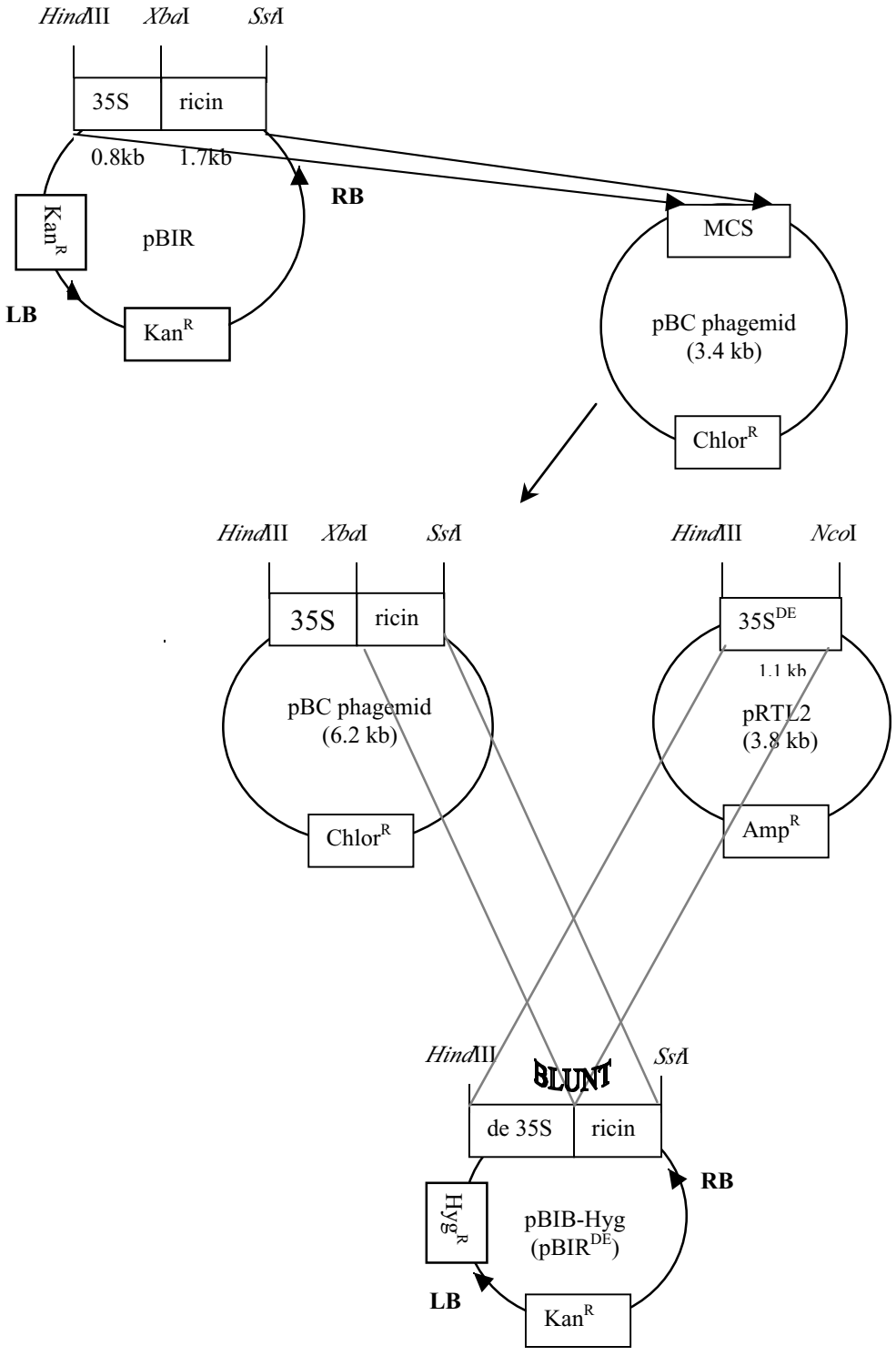

Fig. 4.2. Cloning strategy for pBIR^{DE} vector. Preproricin and the enhanced CaMV 35S promoter (35S^{DE}) were inserted into the binary vector pBIB-Hyg (Becker, 1990). The vector contained a kanamycin resistance gene, plant terminators and the T-DNA border sequences (LB, RB) that define the region to be transferred to the plant genome. (Chlor^R=chloramphenicol resistance gene; Amp^R=ampicillin resistance gene; Kan^R=kanamycin resistance gene; MCS=multiple cloning site)

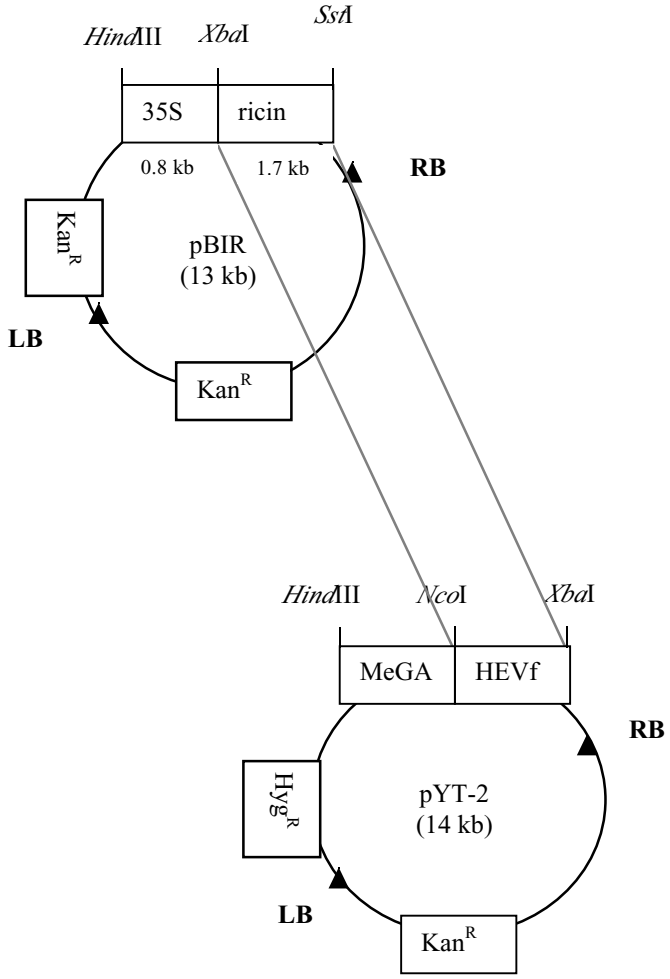

Fig.4.3. Cloning strategy for pBIR^{MeGA} vector. Preproricin was inserted into the binary vector pYT-2-Hyg replacing HEVf fragment. This vector contained the MeGA promoter as well as a kanamycin resistance gene, plant terminators and the T-DNA border sequences (LB, RB) that define the region to be transferred to the plant genome. (Kan^R=kanamycin resistance gene; Hyg^R=hygromycin resistance gene; HEVf=hemorrhagic enteritis virus fiber)

Each colony was also copied onto master plates for recovery after the hybridization. The colonies on the filters were incubated at 37 °C overnight, the DNA from individual colonies was liberated by denaturing solution (0.5 N NaOH, 1.5M NaCl) and bound to the filter by UV-crosslinking, then further treated as described in 4.12. The resulting plasmid was referred to as pBIR^{MeGA} and kept as a DMSO stock in *E. coli* at -70°C. The junctions of pBIR^{MeGA} were confirmed by sequencing (University of Chicago, sequencing facility).

4.7 Transformation of *Agrobacterium tumefaciens*

A. tumefaciens strain LBA4404 was transformed with the pBIR, pBIR^{DE} and pBIR^{MeGA} constructs using the freeze/thaw method (An, G. *et al.*, 1988). LBA4404 contained an additional plasmid (pAL4404) carrying the vir genes necessary for T-DNA transfer into plant cells. *A. tumefaciens* was grown for 48 hours at 28 …C in 5 ml liquid YEP medium containing streptomycin (30 mg/L) and then centrifuged for 5 minutes at 5000 RPM (4 …C). The cells were resuspended in 100µl ice cold 20 mM $CaCl_2$. Twenty µl of plasmid containing the desired construct was added and the mixture was first frozen in liquid N_2 and then thawed in a 37 …C water bath for 5 minutes. One ml of YEP was added and the culture was shaken (170 RPM) at 28 …C for 4 hours. The cells were then centrifuged, resuspended in 100 µl of YEP, plated on YEP containing streptomycin (30 mg/L) and kanamycin (100 mg/L), and incubated at 28 …C. Colonies were screened using PCR with specific ricin primers (see section 4.12). DMSO stocks of *A. tumefaciens* containing the pBIR, pBIR^{DE} and pBIR^{MeGA} plasmids were kept at -70…C.

4.8 Transient expression of ricin in hairy roots

Transient expression was performed by unstable transformation of tobacco hairy root cultures (Figure 4.4) with *A. tumefaciens* containing the different ricin constructs. Previously established hairy-root cultures of *Nicotiana tabacum* cv Xanthi were generated by Dr. L.F. Medina-Bolivar (Medina-Bolivar and Flores, 1995). The root cultures were maintained by transferring every 2 weeks 15 root tips of about 1 cm to 50 ml of new Gamborg s B5 medium. The cultures were incubated with shaking (90 RPM) at 26 °C.

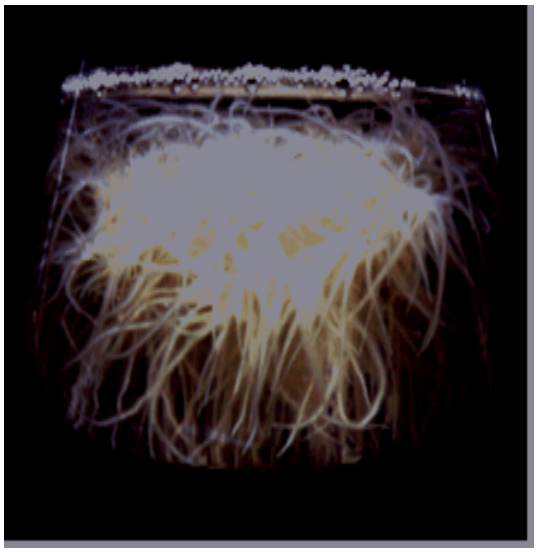

Fig. 4.4. Hairy root culture

A novel hairy root transient expression system was developed by Dr. L.F. Medina-Bolivar, Virginia Tech (personal communication). Ten to fourteen-day old hairy roots in 50 ml Gamborg s B5 medium (GibcoBRL, Rockville, MD) were co-cultivated with *A. tumefaciens* strains carrying each of the pBIR constructs. Acetosyringone (200µM; signal molecule for the *vir* genes induction) was added to the root cultures prior to addition of the appropriate amount of *A. tumefaciens* (700µl of an *A. tumefaciens* culture with OD_{600} = 1 for a 20 ml culture of hairy roots). The root cultures were sonicated for 1 minute, incubated on a shaker (90 RPM) at 26°C and harvested at different times (24, 48 and 72 hours). The MeGA™ promoter was induced by adding cellulase (1µg/ml; Sigma, St Louis, MO) 12 hours after the addition of *Agrobacterium*.

4.9 Generation of transgenic tobacco lines by *Agrobacterium*-transformation

A simplified procedure for *Agrobacterium*-mediated transformation (originally described by Horsch *et al.*, 1985 and modified by Dr. L.F. Medina-Bolivar, Virginia Tech) and regeneration of tobacco was done using the wild-type tobacco line, *Nicotiana tobacum* var. Xanthi nc. A colony of *Agrobacterium* containing the desired vector was taken with a scalpel and a cut was made in the lower part of the steel of excised leaves of axenically grown tobacco seedlings. The leaves were put on MS medium (Murashige and Skoog, 1962; MS salts [GibcoBRL, Rockville, MD]; MS vitamins [Sigma, St Louis, MO], 3% sucrose, 0.4g/L $MgSO_4.7H_2O$) for 2-3 days and were then transferred for shooting and selection to MS plates containing 0.1 mg/L NAA (α-naphthalene acetic acid), 1mg/L BAP (6-benzyl amine purine), 500 mg/L carbenicillin and either kanamycin or hygromycin at a concentration of 100 mg/L. After three weeks plantlets were excised at the stem and transferred to rooting medium (MS medium containing carbenicillin and kanamycin or hygromycin). Thirty plants were generated for each construct and screened for ricin expression. Plants representing independent transformation events were propagated in culture (MS medium + agar in Magenta jars) and leaves were excised for testing. For the MeGA™ promoter, wound induction of the leaf samples involved slicing into 1 mm strips (a specialized machine was developed for reproducible induction) and incubation at room temperature under conditions that maintained moisture levels.

Different times of induction were investigated. Once identified, the best expressors for each construct were planted in soil and grown to seed in a plant growth room.

4.10 Extraction and purification of ricin

For crude protein extraction, two grams of tobacco roots (transient expression system) or leaf tissue (stable transgenic plants) were ground to a fine powder with a mortar and pestle in liquid N_2 or directly in buffer, and transferred to glass corex tubes that were already on ice and contained 4 ml of extraction buffer (0.1M potassium-phosphate, pH7, protease inhibitor [Boehringer-Mannheim, Indianapolis, IN], 5mM dithiothreitol and 1% Triton X-100). Hairy roots co-cultivated with *A. tumefaciens* carrying pBI121 (containing 35S:β-glucuronidase; Clontech, Palo Alto, CA) and non-transformed *Nicotiana tabacum* var. Xanthi leaf tissues grown under the same conditions were used as controls. The extracts were sonicated for 30 seconds and centrifuged for 15 minutes at 12,000 RPM. To eliminate rubisco, the pH of the leaf extracts was lowered to 5-5.5 and the extracts were left overnight at 4 °C. The next day extracts were centrifuged for 15 minutes at 12,000 RPM. The samples were concentrated using a Centricon YM10 filtration unit (Millipore, Bedford, MA).

A galactose containing affinity resin (6-aminohexyl β-D thiogalactopyranoside coupled to agarose resin; Sigma, St. Louis, MO) was used to purify ricin. The standard protocol was as follows: 20 µl beads were added to either 1 ml extract (after concentration with Centricon) or 4 ml extract (no concentration step) and the extracts with added beads were gently shaken for one hour at room temperature. The extracts were centrifuged at 8000 RPM for 5 minutes, the supernatant was removed and the beads were resuspended in 1 ml PBS and centrifuged again at 8000 RPM for 5 minutes. This washing step was repeated and ricin was eluted by resuspending the beads in 35 µl 0.1M galactose, incubating for 15 minutes at room temperature and centrifuging at 13,000 RPM for 5 minutes. The supernatant containing ricin was used. Different amounts of agarose beads (20, 30, 40 µl) and varying levels of galactose (0.1, 0.2 and 0.4 M) were tested to optimize ricin recovery. Protein concentrations were determined using the Bradford assay (Bradford, 1976) and measured at OD_{595} with a Beckman DU640 spectrophotometer.

4.11 Western immunoblot analyses

Western blotting was performed using a polyclonal antibody against the B-chain of ricin that was raised as follows in the Laboratory of Dr. T. Wilkins at Virginia Tech: 200 µg ricin B (Sigma, St. Louis, MO) in 1 ml Complete Freund s adjuvant was injected subcutaneously into rabbits (5 injections, 40 µg/injection) and serum was collected 1 week after the third boost (2-week boost schedule). The western blotting protocol was as described by Sambrook (1989). Five µl 6X SDS-PAGE sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 5% β-mercaptoethanol [v/v], 0.025% bromophenol blue) was added to 25µl of ricin purified as described in section 4.5. The sample was boiled for 5 minutes and loaded on a precast 12% Tris-glycine SDS gel (Novex, San Diego, CA). The gel was run at 150V for 90 minutes. Transfer of proteins to nitrocellulose membrane (0.2µm Optitran, Keene, NH) was done overnight (25V, 4 …C) in transfer buffer (192 mM glycine, 25mM Tris-base, 20% [v/v] methanol, pH 8.3) using a BioRad transfer apparatus. The transfer sandwich consisted of a sponge, then 2 pieces of 3MM Whatman paper, the gel, the nitrocellulose membrane, two more pieces of 3 MM Whatman paper and a second sponge. The resulting filter was used to perform immunodetection of immobilized proteins. The membrane was incubated in blocking solution (5% non-fat dry milk [Bio-Rad] in PBST [80 mM Na_2HPO_4, 100 mM NaCl, 0.3% Tween-20, pH 7.5]) for 2 hours at room temperature with shaking. The membrane was then incubated in rabbit anti-ricin (1/100 dilution in 5% non-fat dry milk/PBST) as primary antibody for one hour at room temperature and washed three times 15 minutes with PBST. Goat anti-rabbit IgG alkaline phosphatase conjugate (Promega, Madison, WI; dissolved 1/3000 in 5% non-fat dry milk/PBST) was used as secondary antibody and the membrane was incubated for one hour at room temperature and was then washed three times in PBST. CDP-Star™ (Boehringer Mannheim, Indianapolis, IN) was the chemiluminescent substrate for alkaline phosphatase. The membrane was incubated (2 times five minutes) in detection buffer (0.1M Tris-HCl, 0.1M NaCl, pH 9.5) and then incubated for 5 minutes in a sealed hybridization bag in 4 ml CDP-Star™ solution (1/100 dilution in detection buffer) containing Nitroblock enhancer II (200µl; Tropix, Bedford,

MA). The membrane was exposed for 4 minutes to Polaroid film (X-OMAT). Spot densitometry was performed on the films using AlphaEase software.

4.12 Southern blotting

Genomic DNA was isolated from transgenic tobacco using a modified CTAB procedure (Rogers and Bendich, 1988). Five grams of leaf tissue were ground to fine powder with a mortar and pestle in liquid nitrogen and the powder was transferred to a 50 ml centrifuge tube that contained 15 ml extraction buffer (2 % cetyltrimethylammonium bromide [CTAB], 100 mM Tris-HCl [pH 8.0], 20 mM EDTA [pH 8.0], 1.4 M NaCl, 0.2% β-mercaptoethanol). The mixture was incubated at 65 °C for 3 hours, kept at room temperature for several hours, and extracted with equal volumes of 1:1 phenol/chloroform-isoamyl alcohol [24:1]. The samples were then centrifuged at 10,000 RPM for 10 minutes. The top layer (aqueous phase) was transferred to new tubes and the phenol/chloroform-isoamyl alcohol extraction was repeated. Ethanol (2.5:1) and 3 M sodium acetate (1:10) were added to the extract that was then kept at -20 °C for at least 30 minutes. The precipitated DNA was recovered by centrifugation at 13,000 RPM for 5 minutes. The pellet was air dried and resuspended in 400 µl TE (10mM Tris-HCl, 1mM EDTA, pH 8.0). RNase (RNace-It™ cocktail, Stratagene, La Jolla, CA) was added to digest RNA. DNA concentration was measured at OD_{260} with a DU640 spectrophotometer (Beckman, Fullerton, CA).

Ten µg of each genomic DNA sample were digested with *Hind*III overnight at 37 °C. The digested samples were separated by electrophoresis in a 0.8% agarose in 1X TAE buffer gel (Southern, 1975). The DNA was transferred by capillary blotting to a nylon membrane (Micro Separations Inc, Westboro, MA) and was cross linked using UV light. A 461 bp DNA probe was prepared from the preproricin N-terminal DNA by PCR amplification (ready to go™ PCR beads, Amersham Pharmacia, Piscataway, NJ) using as primers: 5 -GAACGAGCTCCTATCAAAATAATGG-3 and 5 -TGGTACCACACTTACGGTGC-3 . The probe was labeled with digoxigenin-11-dUTP (Boehringer-Mannheim, Indianapolis, IN) and gel purified using a Qiaquick PCR Purification Kit (Qiagen, Valencia, CA). The membrane was prehybridized for 1 hour at 65° in Church buffer (Church and Gilbert, 1984) and hybridized with the denatured probe

(50 ng in 10 ml Church buffer) overnight at 65°. The membrane was washed several times as described by Church and Gilbert (1984) and the detection procedure was performed according to the manufacturer s specifications (CDP-Star™, Boehringer Mannheim, Indianapolis, IN)

4.13 Detection of enzymatic activity of tobacco-synthesized ricin

The enzymatic activity of ricin was characterized for cell-free translation inhibition using a rabbit reticulocyte lysate translation inhibition assay (TNT® Quick Coupled Transcription/Translation System, Promega, Madison, WI). A plasmid containing luciferase DNA (provided in kit) was used as control. The luciferase gene was transcribed and translated to a 60 kDa protein following Promega protocols. The Transcend™ translation detection system (Promega, Madison, WI) was used for the detection of the proteins synthesized *in vitro*. In this system, biotinylated lysine residues were incorporated into nascent proteins during translation. This biotinylated lysine was added to the translation reaction as a precharged biotinylated lysine tRNA complex. After SDS-PAGE and electroblotting, the biotinylated proteins were visualized by binding streptavidin-horseradish peroxidase followed by chemiluminescent detection. The reactions were set up as follows: 40 µl TNT® Quick Mastermix, 1 µl 1mM methionine, 2 µl luciferase DNA template, 1 µl Transcend™ tRNA and either 6 µl H_2O or 6 µl extract containing ricin. The reactions were incubated for 90 minutes at 30° C. SDS-PAGE was performed with 1 µl of each reaction. After electroblotting, the membrane was blocked for 1 hour in TBS (20 mM Tris-HCl [pH 7.5], 150 mM NaCl) containing 0.5% Tween 20 (TBST). The membrane was incubated in streptavidin-HRP conjugate (1/5000 dilution in TBST) for 60 minutes and then washed three times 5 minutes in TBST and three times 5 minutes in TBS. The membrane was incubated in the chemiluminescent substrate for 1 minute and exposed to Kodak film (X-OMAT) for 15 minutes.