CHAPTER V

RESULTS

V. RESULTS

5.1 Generation of ricin constructs

Expression of ricin under control of three different promoters, Cauliflower mosaic virus 35S promoter (35S), dual enhanced Cauliflower mosaic virus 35S promoter ($35S^{DE}$) and the MeGA™ promoter was investigated. The aim was to determine which promoter provides the highest expression levels of ricin. $35S^{DE}$ is a modified 35S promoter consisting of the core 35S promoter with a dual enhancer and the 5 -untranslated leader sequence from the tobacco etch virus. Construction of this vector was performed in two steps (Figure 4.2). First, a fragment containing 35S and ricin was introduced from pBIR into pBC phagemid. Ricin from this intermediate vector and $35S^{DE}$ from pRTL2 (Kulakova *et al.*, 1995) were then ligated into the binary transformation vector pBIB-HYG (Becker, 1990). Colonies were screened by performing PCR on plasmid DNA with ricin gene specific primers. The presence of the $35S^{DE}$-ricin gene fragment in pBIB-HYG was confirmed in the positive colonies by restriction endonuclease digestion using *Hind*III/*Sst*I (Figure 5.1). The vector construct (clone 8) was verified by sequencing.

Ricin was also fused to MeGA™, a wound inducible promoter. This construct was assembled in a single step. Ricin was excised from pBIR and introduced into pYT-2 behind the MeGA promoter. Positive clones were identified by colony hybridization using a ricin- specific probe. One positive clone was found (Figure 5.2) and the insertion of ricin into pYT-2 was verified by PCR (Figure 5.3). The vector construct was verified by sequencing.

5.2 Introduction of ricin constructs into *Agrobacterium tumefaciens*

The pBIR, $pBIR^{DE}$ and $pBIR^{MeGA}$ plasmids were purified from *E. coli* and transferred into *A. tumefaciens* strain LBA4404. Plasmid DNA was subsequently extracted from LBA4404 and restriction digestion was performed to confirm that the constructs were successfully transferred into *Agrobacterium*.

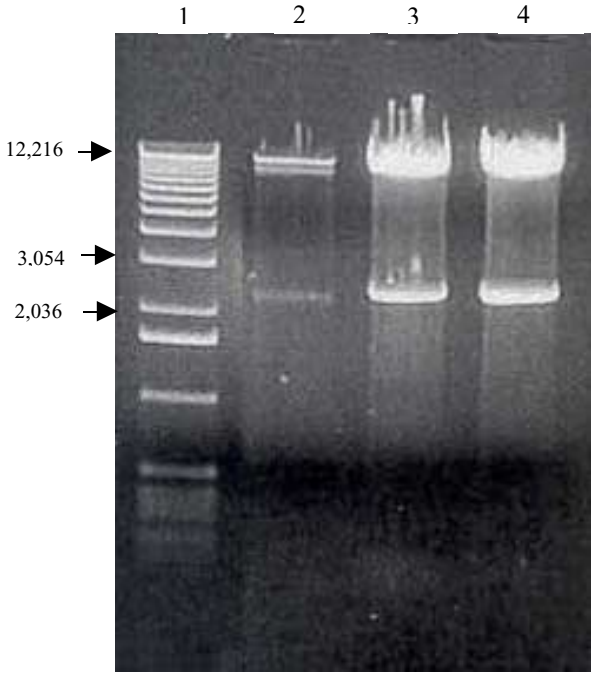

Fig. 5.1. Restriction endonuclease digestion to confirm insertion of the $35S^{DE}$-ricin fragment (2.8 kb) in pBIB-HYG. Plasmid DNA from three colonies was digested with *Hind*III/*Sst*I, fractionated by 0.8% agarose gel electrophoresis and stained with ethidium bromide. Lane 1: Molecular weight standard (1 kb marker); Lane 2: clone 1; Lane 3: clone 8; Lane 4: clone 10

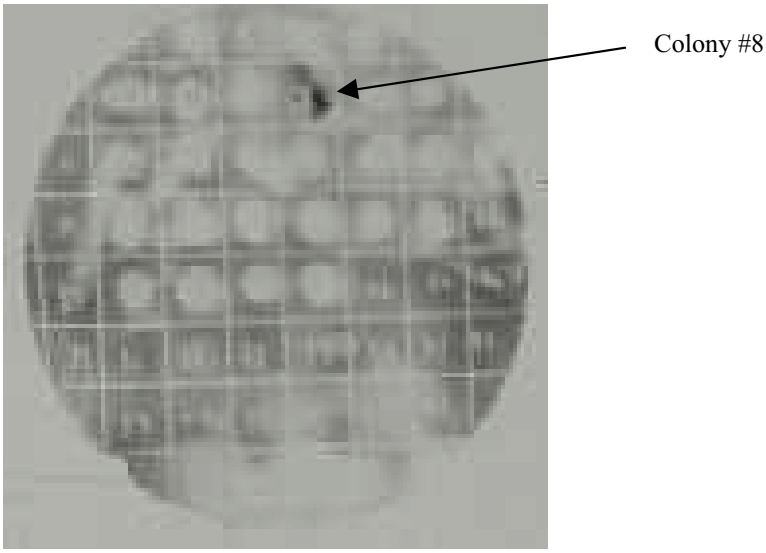

Fig. 5.2. Colony hybridization of MeGA:ricin clones. Colonies generated from transformation with pYT-2 (pBIB-HYG-MeGA) and ricin ligation mix were streaked onto a nitrocellulose membrane and incubated on LB-kanamycin plates. DNA from these colonies was denatured and probed with a DIG-labeled ricin-specific probe. One colony (colony #8) was found positive for ricin hybridization.

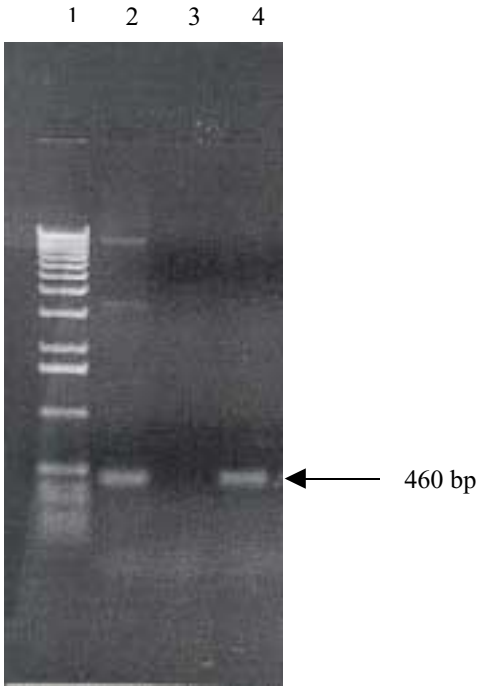

Fig. 5.3. Confirmation of ricin insertion by PCR. Plasmid DNA from colony #8 was isolated and PCR amplification was performed using ricin-specific primers to verify insertion of ricin in pYT-2 vector. Lane 1: molecular weight standard (1kb marker); Lane 2: positive control (35S-ricin in pBC); Lane 3: negative control (no template added); Lane 4: plasmid DNA from colony #8.

5.3 Transient expression of ricin in hairy root cultures

Expression of ricin was shown using *A. tumefaciens* transformation of hairy root cultures. This is a transient expression system that utilizes T-DNA regions that are not inserted in the genomic DNA. Advantages of this system are that it provides rapid assessment of vector construction and comparative expression in the absence of position effects. Transient expression was performed in 14 days old hairy roots (Figure 5.4a and Figure 5.4b) to which *Agrobacterium*, activated with acetosyringone, was added. Two grams of roots were harvested at 24, 48 or 72 hours after *Agrobacterium* addition, ground in protein extraction buffer and the resulting crude protein extract was concentrated. For analyses of roots transfected with pBIR^{MeGA} constructs, the MeGA promoter was induced by cellulase 12 hours after addition of *Agrobacterium.* The levels of ricin in these samples were assayed by immunoblot analysis using anti-ricin B antibodies. Bands were detected at ~30 kDa (Figure 5.4a and Figure 5.4b), representing ricin B, and at ~60 kDa (Figure 5.4b) representing the whole ricin molecule (A and B chain). Both bands are present at a lower molecular weight than ricin purified from castor bean. Tobacco roots transiently expressing 35S:GUS were used as a negative control.

There were no consistent differences seen between the different times of harvesting and expression levels generated from different promoters. Ricin was not detected by western analysis of total soluble protein (data not shown). Thus, ricin expression was very low in this transient system. The system appears to have low reproducibility (e.g. comparison of ricin production behind MeGA promoter in Figure 5.4a and 5.4b) but the results confirmed vector construction: product that cross-reacted with anti-ricin B antibodies was observed at molecular sizes similar to castor bean ricin and showed processing to lower molecular weight forms. The presence of 30 KDa forms suggests that tobacco targets ricin to vacuoles, the site of proteolytic activation in castor beans.

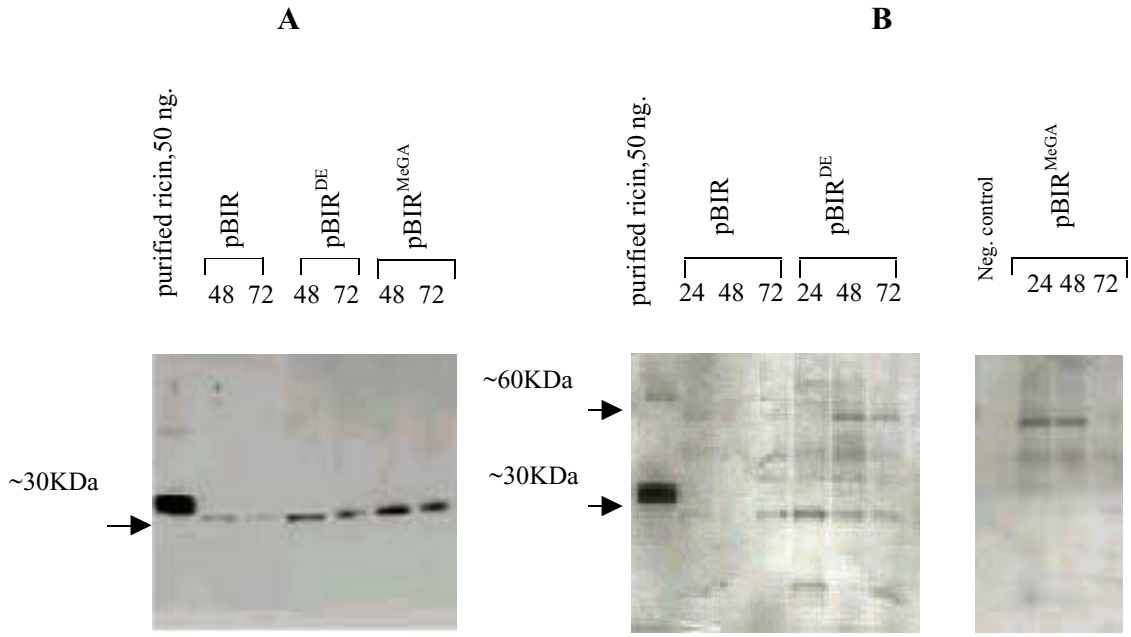

Fig. 5.4. Two independent experiments involving transient expression of ricin in hairy root cultures. Two grams of roots were harvested 24, 48 or 72 hr after co-cultivation with *Agrobacterium*. For the MeGA promoter, the roots were harvested 24, 48 or 72 hr after the addition of cellulase (1µg/ml; added 12 hours after addition of *Agrobacterium*) which induced the promoter. Roots were ground in 4 ml of K-phosphate extraction buffer.The crude protein extract was concentrated to 1 ml, purified using a galactose binding resin, separated on SDS-PAGE and analyzed by immunoblotting using polyclonal anti-ricin antibodies. Negative control = transformation with 35S:GUS vector.

5.4 Immunoblot detection of ricin from transgenic tobacco plants

Following transformation into tobacco, 30 transgenic tobacco lines were regenerated and screened for the presence of the transgene by immunoblot assays. Leaf tissue from individual transgenic plants growing axenically in Magenta jars were harvested for analysis of ricin production. Ricin was purified from soluble leaf extracts using a galactose containing affinity resin. Different amounts of resin (10, 20, 30 and 40 µl/ 4 ml leaf extract) gave similar yields of ricin, indicating that the binding capacity of the resin was not exceeded. Twenty µl of resin were used in all subsequent experiments. Ricin production was visualized by immunoblot analyses (Figure 5.5) of proteins eluted from the galactose resin and fractionated by SDS-PAGE under reducing conditions. As expected, independent transformants containing the same gene construct showed significant variation in ricin accumulation. This is due to position effects commonly observed in transgenic eukaryotes. Depending on the integration site of the transgene, expression levels will vary.

Proteins migrating as 64- and 34-kDa polypeptides were detected in tobacco plants containing the ricin transgene when the blots were probed with anti-ricin B antibodies. These bands were absent from proteins extracted from non-transgenic control plants (e.g. Fig. 5.5 A lane 1 blot A). The 64-kDa band represents unprocessed or non-reduced ricin while the 34-kDa band represents the B chain of ricin. In some cases a 30-kDa band could be distinguished. This band may represent the A chain of ricin or a non-glycosylated or less glycosylated form of ricin B chain. Control ricin B (50 ng/lane) was included as standard. The percentage of control ricin migrating at 64-kDa versus 34-kDa varied from gel to gel suggesting that the 64-kDa form represents the non-reduced two-chain form rather than the single chain form. Sehnke (1994), who also performed immunoblot analysis of tobacco plants expressing a ricin gene under control of 35S promoter using antibodies against ricin A, reported a faint protein band visible in the lane containing the non-transformed plant and suggested the existence of tobacco type I RIP. Figure 5.5 shows six immunoblots, result of screening transgenic plants containing either pBIR, $pBIR^{DE}$ or $pBIR^{MeGA}$. Protein concentrations were in the range of 2-4 mg/g fresh leaf weight.

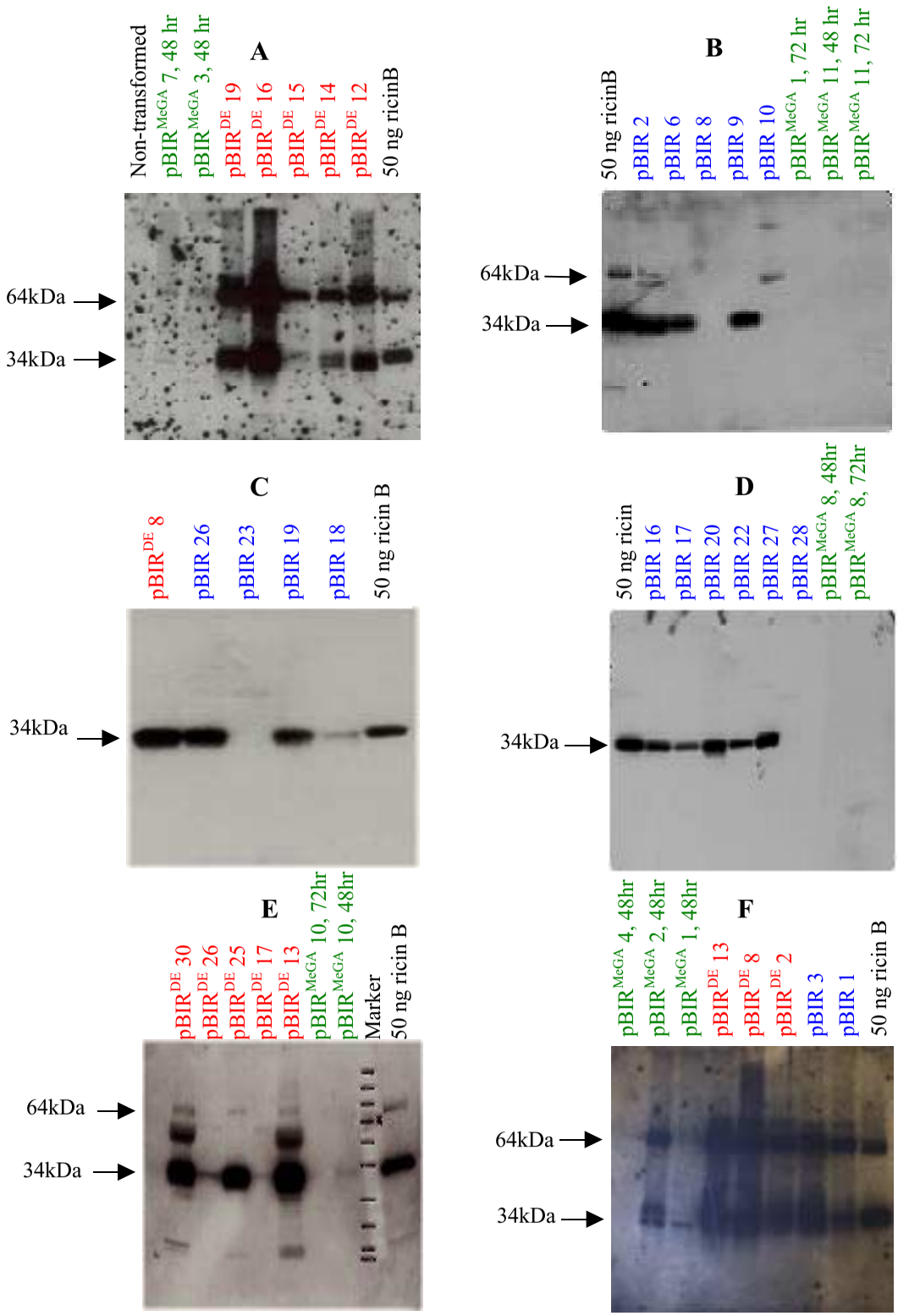

Fig. 5.5. Immunoblot analysis of affinity-purified ricin from transgenic tobacco plants. The blots represent total recovery from 4 ml leaf homogenate (2 g fresh leaf weight) detected with anti-ricin B. For MeGA promoter the time of harvesting after wound induction is indicated (48 or 72 hours). The numbers after the constructs represent plant numbers.

To accurately quantify ricin levels in these tobacco plants, spot densitometry was performed using AlphaEase software (data not shown). Pixels in defined areas were summed to give an integrated density value (IDV). A background was linked to each area. These efforts were unsuccessful due to over-exposure of key samples on the films. We, therefore, catagorized expression (low, intermediate or high) levels based on visual comparisons with the ricin standard (50 ng) present on each gel. Based on intensities of the bands, four categories of expression levels can be distinguished (see table 5.1). Among those plants showing no or low levels (~ 0-25 ng ricin/g fresh leaf weight) of ricin expression, there were 7 plants containing $pBIR^{MeGA}$, 5 containing pBIR and 2 containing $pBIR^{DE}$. Among those showing intermediate levels (~ 25-250 ng ricin/g fresh leaf weight) of ricin production, there were 11 plants containing pBIR, 4 containing $pBIR^{DE}$ and 1 containing $pBIR^{MeGA}$. Lastly, among those showing high levels (~ 250-2500 ng ricin/g fresh leaf weight) of ricin expression there were 7 plants containing $pBIR^{DE}$ and 1 containing pBIR. These high expression levels are probably underestimated because of the detection limits. Transgenic plants containing ricin expressed under the control of the dual enhanced 35S CaMV promoter showed highest expression of ricin. The transgenic plants having ricin driven by the 35S CaMV promoter showed expression in most cases, although some of the plants did not contain detectable levels of the transgene product.

The transgenic plants expressing ricin behind the MeGA promoter showed low expression levels of ricin and in many cases, no ricin was detected. Because MeGA is an inducible promoter, it is possible that lack of ricin detection was due to ineffective induction or analysis at inappropriate times after induction. To find the optimal induction time for the MeGA promoter, a time course of ricin expression was conducted 0, 12, 24, 36 and 48 hours after wounding leaves from plant $pBIR^{MeGA}$ 2 (Figure 5.6). Very low expression levels were seen at all of these time points. The levels were even lower than in the initial experiment (see Figure 5.5 F, lane 2) which suggest that there may be additional factors that affect the effectiveness of induction of the MeGA promoter

Table 5.1. Expression levels of ricin in transgenic tobacco. Using the western immunoblots shown in Figure 5.5, band intensities (64 + 34 kDa) of tobacco-derived ricin were compared to ricin standards (50 ng ricin from castor bean) and classified as no, low, intermediate, or high expressors. The numbers following the construct designation represents the individual plant line.

No expression	Low expression (~ 0-25 ng ricin/g fresh leaf weight)	Intermediate expression (~ 25-250 ng ricin/g fresh leaf weight)	High expression (~ 250-2500 ng ricin/g fresh leaf weight)
PBIR 8	pBIR 10	pBIR 1	pBIR 3
PBIR 23	pBIR 18	pBIR 2	
PBIR 28		pBIR 6	$pBIR^{DE}$ 2
	$pBIR^{DE}$ 17	pBIR 9	$pBIR^{DE}$ 8
$pBIR^{MeGA}$ 4	$pBIR^{DE}$ 26	pBIR 16	$pBIR^{DE}$ 13
$pBIR^{MeGA}$ 8		pBIR 17	$pBIR^{DE}$ 16
$pBIR^{MeGA}$ 10	$pBIR^{MeGA}$ 1	pBIR 19	$pBIR^{DE}$ 19
$pBIR^{MeGA}$ 11	$pBIR^{MeGA}$ 3	pBIR 20	$pBIR^{DE}$ 25
	$pBIR^{MeGA}$ 7	pBIR 22	$pBIR^{DE}$ 30
		pBIR 26	
		pBIR 27	
		$pBIR^{DE}$ 12	
		$pBIR^{DE}$ 14	
		$pBIR^{DE}$ 15	
		$pBIR^{DE}$ 18	
		$pBIR^{MeGA}$ 2	

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Fig. 5.6. Time course for induction of the MeGA promoter. The experiment was performed on pBIR^{MeGA} plant #2. Proteins were extracted from two grams of leaves which had been wounded to induce MeGA promoter. Ricin was affinity purified, fractionated by SDS-PAGE, and detected with anti-ricin antibodies. Lane 1: 48 hours after induction; Lane 2: 36 hours after induction; Lane 3: 24 hours after induction; Lane 4: 12 hours after induction; Lane 5: 0 hours after induction; Lane 6: purified ricin from castor bean (50ng).

5.5 Southern blot analyses

Southern hybridization analysis was performed on the highest expressing plants for each construct to get an idea about copy number in these transgenic tobacco plants. Genomic DNA was digested with *Hind*III, which cuts only once within the introduced T-DNA such that distinct integration events should yield hybridizing fragments of different sizes.

As shown in Figure 5.7, pBIR 3 contains 3 copies of 35S-ricin, while pBIR^{DE} 8 contains only one copy of the 35S^{DE}-ricin fragment. pBIR^{MeGA} 2 may have as many as 5 copies of the MeGA-ricin fragment. All plants, including the non-transgenic Xanthi parent, contained a high molecular weight *Hind*III fragment (~18 kb) that cross-hybridized with the ricin probe. This presumably represents an endogenous tobacco RIP.

5.6 Ribosome inactivating activity of tobacco-synthesized ricin

The transgenic plants were assayed for ribosome inactivating protein activity using a protein translation inhibition assay. Affinity-purified ricin as well as soluble leaf extracts from the ricin-producing plants were tested for characteristic RIP RNA endoglycosidase activity by measuring protein translation inhibition in rabbit reticulocyte lysates. Both purified ricin (Figure 5.8a, lane 2; Figure 5.8b, lane 4, 5) and crude leaf extracts from transgenic plants (Figure 5.8b, lane 3 and 7) showed complete inhibition of translation of the test protein, luciferase, except for pBIR^{MeGA} 2 (Figure 5.8b, lane 6) where purified ricin gave partial inhibition of protein translation. No band was detected at 60 KDa which is the size of luciferase in the positive control. In Figure 5.8a, a GUS-transgenic tobacco plant which also underwent the resin purification step, was included as negative control. In Figure 5.8b, crude leaf extract from a GUS-transgenic plant was included as negative control (lane 8) showed partial inhibition of protein translation which could mean that other RIP s are present in the tobacco extract. Ricin toxicity requires binding to cell surface β-galactoside-terminated oligosaccharides, membrane translocation, and release of the A chain from the B chain.

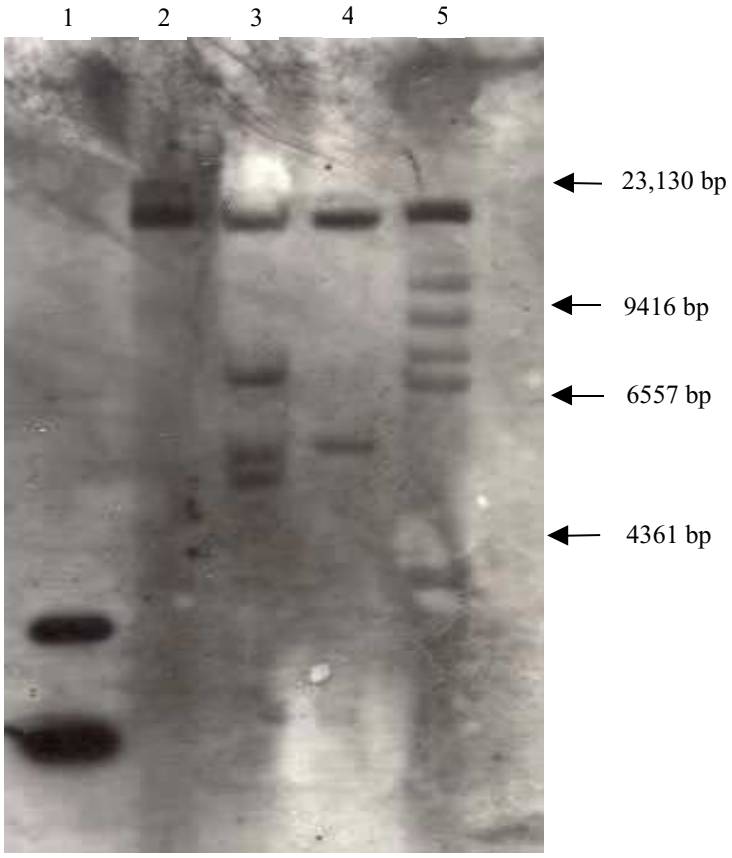

Fig. 5.7. Southern blot of DNA from transgenic tobacco plants. Total genomic DNA (10 µg/lane), digested with *Hind*III, size-separated on 0.8% agarose gel, transferred to nitrocellulose membrane and probed with DIG-labeled ricin cDNA. Migration of molecular weight marker (lambda HindIII) is indicated to the right of gel. Lane 1: positive control, plasmid DNA from pBC containing 35S-ricin digested with *Hind*III and *Sst*I; Lane2: genomic DNA from non-transformed *Nicotiana tabacum* var. Xanthi; Lane 3 genomic DNA from pBIR3; Lane 4: genomic DNA from pBIR^{DE}8; Lane 5: genomic DNA from pBIR^{MeGA}2.

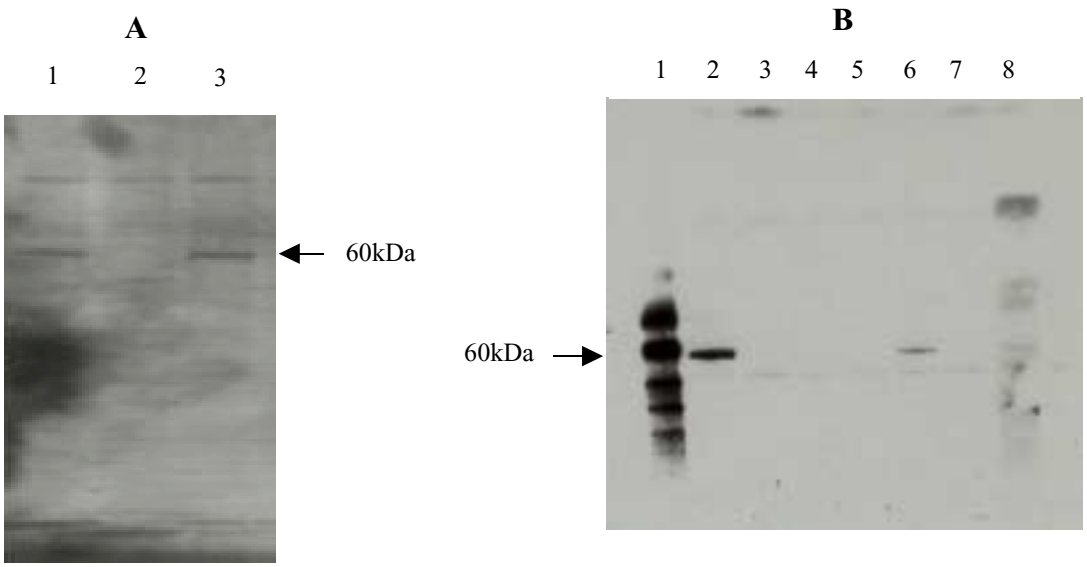

Fig. 5.8. Protein translation inhibition assay. Total protein was extracted from ricin-containing plants and GUS-containing control plants. For those designated the proteins were affinity purified using a ricin-specific resin. 6 µl crude extract or affinity-purified ricin was used in a 50 µl reaction. **A**: Lane1: luciferase protein; Lane 2: affinity-purified ricin from pBIR^{DE} 13; Lane 3: GUS-transgenic tobacco plant. **B**: Lane 1: molecular weight markers; Lane 2: luciferase protein; Lane 3 and 7: crude leaf extract from pBIR^{DE} 13 and 30; Lane 4: affinity-purified ricin from pBIR^{DE} 8; Lane 5: affinity-purified ricin from pBIR 1; Lane 6: affinity-purified ricin from pBIR^{MeGA} 2; Lane 8: GUS-transgenic tobacco plant.

These complex functions necessitate that the ricin molecule contains an active endoglycosidase (A chain) and a functional lectin B-chain, which are linked via a reducible disulfide bond. Our results demonstrate that tobacco is capable of processing the type II RIP preproricin into a fully active mature toxin.