

CHAPTER VI  
DISCUSSION

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There is significant interest in using ricin, a plant toxin from castor bean, for co-expression as a mucosal adjuvant in the production of an edible vaccine against entamoebic dysentery in plants. In the present study, we focused on expression of ricin in tobacco as a model system. Our data showed that transgenic tobacco plants provide an effective system for processing the type II RIP preproprotein precursor, normally found exclusively in the endosperm of castor seeds, into a fully active mature toxin. In castor bean, preproricin is synthesized, transported to the vacuoles and processed to a heterodimer. Detection of ricin B as a 34 KDa fragment in transgenic tobacco plants suggests correct vesicular transport to the vacuoles and endoproteolytic processing after deposition, analogous to events within castor bean seeds. The production of ricin was also found not to be detrimental for the tobacco plants.

As expected, the expression levels varied significantly among the different transgenic lines, presumably due to position effects. The position of the transgene with respect to neighboring genes may affect functional transcription of the transgene, such that transgene expression may be reduced or enhanced. Three different promoters, namely the CaMV 35S promoter, the stronger plant constitutive promoter CaMV 35S<sup>DE</sup>, and the inducible MeGA<sup>TM</sup> promoter, were compared for their ability to express ricin in tobacco plants. Use of CaMV 35S<sup>DE</sup> was expected to increase the expression of the ricin transgene compared to CaMV 35S. Indeed, overall, the ricin protein expression levels in the CaMV 35S<sup>DE</sup>:ricin-containing plants were higher than the expression levels in the CaMV 35S:ricin transgenic plants generated in this study. The idea of using the inducible MeGA promoter is that ricin expression can be activated in harvested tobacco tissues and thus would minimize ricin production during normal growth and development. Our study showed low expression levels of ricin after induction of the MeGA promoter. This result is surprising based on high levels of transgene expression for several human genes linked to MeGA (Cramer *et al.*, 1999). This could be due to several different reasons: 1) It could be that our method of wound-induction was not optimal for activation of the promoter. Future experiments need to focus on elaborating an effective induction protocol. 2) The studies here were performed on young tissues from plantlets grown in plastic jars

containing artificial growth media. It is possible that these conditions suppress wound—inducibility of MeGA. Next, one needs to look at expression in mature tissue of plants grown in soil which has been shown to be higher for transgenes linked to the MeGA promoter. 3) The Southern blot analysis showed integration of multiple copies of the transgene in the tobacco genome. This can result in post-transcriptional silencing of the transgene (Kooter *et al.*, 1999). Other plants need to be checked on their number of copies of the transgene and plants containing one copy should be used for further experiments. 4) It is possible that ricin produced in one cell is transported via the wounding area to neighboring cells where it inhibits ribosomes and shuts down protein production, thus also its own expression.

Western blot analyses of the tobacco-synthesized (and castor bean-synthesized) ricin showed a high molecular weight form of about 64 kDa, representing the whole ricin molecule, and a low molecular weight form of about 34 kDa, representing only the B chain of ricin. The high molecular weight form may represent either the single chain proricin or processed but non-reduced ricin. Because the 64 kDa form is not always observed and the ratio of 64 to 34 kDa forms varies in the purified ricin standard, this may reflect differences in reduction of the ricin molecule. Since ricin consists of an A-chain and a B-chain linked by disulfide bond, inefficient reduction of the intermolecular disulfide bond would result in proteins 64 kDa and 34 kDa in size. Complete reduction would yield only the B-chain of ricin migrating as a 34 kDa band (as seen in Fig. 5.5C) The use of additional reducing agent and longer boiling times before SDS-PAGE could test this possibility. Alternatively, recovering the 64 kDa band from gels, re-reducing, and generating 34 kDa product would also support our interpretation that the 64 kDa form reflects incompletely reduced ricin. The 30 kDa band (e.g. Fig. 5.5A, lane 7) which was sometimes observed may represent either the A-chain of ricin or a less glycosylated form of the B-chain. This will be investigated by expression of a construct that contains only the B-chain of ricin.

The plants containing ricin under control of the dual enhanced 35S promoter showed the highest expression of ricin. It is estimated that the highest expressor produced ~ 4 µg ricin / gram fresh leaf weight. This is an underestimation since the films were overexposed. To be able to have an accurate quantification we need to develop an Elisa

assay. Using pBIR, Sehnke *et al.* (1994) identified tobacco plants producing 0.25% of total soluble protein in their leaves. We estimate that our levels in the high expressors are about the same. It is likely that yields will vary dependent upon age, tissue and growth conditions.

Production of ricin was confirmed using transient expression in tobacco hairy root cultures. The construct enters the cell but is not integrated into the genome. This system provides a quick answer about the constructs and position effects are avoided. Again two molecular weight forms were observed. Ricin was detected around 60 kDa, while ricin B was around 30 kDa. Both forms were of a lower molecular weight than the forms seen for purified ricin from castor bean. The molecular sizes of ricin found in transgenic leaves was 64 and 34 kDa although they may migrate slightly faster than the castor bean ricin standard. Ricin A and ricin B contain one and two N-linked glycans, respectively. Differences in molecular size may reflect differences in degree of glycosylation or composition of the glycans. This could be tested by treating ricin molecules (e.g. galactose affinity resin purified) with endo F/N glucanase. This will remove all glycans and allows one to compare ricin from hairy root, tobacco leaf and castor bean.

Our experiments also uncovered evidence for the existence of an endogenous RIP in tobacco. Ricin sequences cross-hybridized with a tobacco sequence present in non-transformed Xanthi in genomic Southern analyses. Partial inhibition of protein translation was also observed in the *in vitro* protein translation assays. It would be interesting to investigate if this activity is a type I (~30 kDa) or type II (~60 kDa) RIP based on its molecular weight.

We are currently designing constructs of ricin B fused to mutated and thus non-toxic ricin A, as well as fused to the CRD antigen of *Entamoeba histolytica*. Both will be expressed in tobacco. Ricin B and CRD antigen will also be expressed separately in tobacco.

Future experiments will address the practical use of ricin as an adjuvant. Tobacco-synthesized ricin will be nasally administered in mice to test its adjuvancy. Fusion of ricin B to green fluorescent protein (GFP) will be used as model to test 1) if ricin B increases the immunogenicity of fused versus co-administered GFP or if ricin A is

needed for adjuvancy and 2) the efficacy of ricin B to enhance the delivery of fused adjuvants, thus to see if GFP enters the NALT cells in the nasal mucosa of mice.