Studies on reduction of toxic endosperm-specific proteins, ricin and RCA in castor (*Ricinus communis* L.) through post-transcriptional gene silencing (PTGS) approaches

Thesis submitted to the University of Hyderabad for
the award of the degree of Doctor of Philosophy in Plant Sciences

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Certificate

This is to certify that I, **Mohd Ashraf Ashfaq** have carried out the research work embodied in the present thesis entitled "**Studies on reduction of toxic endosperm-specific proteins ricin and RCA in castor (*Ricinus communis* L.) through post-transcriptional gene silencing approaches"** and submitted for the degree of **Doctor of Philosophy** was accomplished for the full period prescribed under Ph.D ordinances of the University, under the supervision of Prof. P.B. Kirti, Department of Plant Sciences at the Department of Plant Sciences, School of Life Sciences, University of Hyderabad and under the co-guidance of Dr. V. Dinesh Kumar at the Directorate of Oilseeds Research, Rajendranagar, Hyderabad and I declare to the best of my knowledge that no part of this thesis was earlier submitted in part or in full, for the award of any research degree or diploma of any university.

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Dedicated to my beloved parents...

When I count my blessings, I always count you twice

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Abbreviations and Acronyms

bp	:	base pair
BSA	:	Bovine Serum Albumin
BAP	:	Benzyl Amino Purine
BLAST	:	Basic Local Alignment Search Tool
$CaCl_2$	:	Calcium chloride
CAMBIA	:	Center for the Application of Molecular Biology to International Agriculture
CaMV	:	Cauliflower Mosaic Virus
CTAB	:	Cetyl Trimethyl Ammonium Bromide
DNA	:	Deoxy Ribo Nucleic Acid
dNTP	:	deoxy Nucleotide Tri-Phosphate
DOR	:	Directorate of Oilseeds Research
dsRNA	:	double-stranded RNA
EDTA	:	Ethylene Diamine Tetra Acetic Acid
g	:	gram
gus	:	glucuronidase
h	:	hours
HCl	:	Hydrochloric acid
HEPES	:	N- (2-Hydroxyethyl) piperazine-1-ethanesulfonic acid
$HgCl_2$	:	Mercuric Chloride
hpt	:	hygromycin phosphate transferase
hpRNA	:	hairpin RNA
IBA	:	Indole Butyric Acid
ihp	:	intron-spliced hairpin
IPTG	:	Isopropyl-β-D-thiogalactopyranoside
kDa	:	kilo Dalton
KCl	:	Potassium Chloride
*lac*Z	:	β-galactosidase
LA	:	Luria Agar
LB	:	Luria Broth

Leu	:	Leucine
M	:	Molar
MCS	:	Multiple Cloning Site
$MgCl_2$	:	Magnesium Chloride
min	:	minutes
ml	:	millilitre
mM	:	millimolar
MS	:	Murashige and Skoog
MUG	:	4-methyl umbelliferyl β-D glucuronide
NAA	:	Naphthalene Acetic Acid
NaCl	:	Sodium Chloride
NaOH	:	Sodium hydroxide
NCBI	:	National Center for Biotechnology Information
ng	:	nano gram
OD	:	Optical Density
O/N	:	Over night
PCR	:	Polymerase chain reaction
pH	:	pussancea hydrogen (potential hydrogen)
PTGS	:	post transcriptional gene silencing
PUFA	:	Polyunsaturated fatty acids
RES	:	Restriction Enzyme Sites
rpm	:	revolutions per minute
SDS	:	Sodium Dodecyl Sulphate
siRNA	:	small interfering RNA
RISC	:	RNA induced silencing complex
Taq	:	*Thermophilus aquaticus*
$T_{10}E_1$	:	10 mM of Tris.Cl, 1 mM of EDTA
T_{10}	:	10 mM of Tris.Cl
TAE	:	Tris.Cl, Acetic Acid, EDTA
T-DNA	:	Transferred DNA
TDZ	:	Thiadiazuron
TE	:	Tris.Cl, EDTA
T_m	:	Temperature of melting
tRNA	:	transfer Ribonucleic Acid

Tris.Cl	:	Tris (hydroxy methyl) aminomethane hydro chloride
U	:	units
UTR	:	untranslated region
V	:	volts
X-Gal	:	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-Gluc	:	5-bromo-4-chloro-3-indolyl-β-D-glucuronide
°C	:	Degree Celsius
µg	:	micro gram
µl	:	microlitre
µM	:	micro Molar
µF	:	micro Faraday

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CHAPTER I

INTRODUCTION

India is the largest producer of castor seed and oil in the world. India earns a large chunk of foreign exchange approximately (Rs 2100 crores) through export of castor oil. The whole seeds yield about 40% oil, which has a lot of industrial and pharmaceutical uses. The de-oiled meal, 0.6 m tonnes produced annually, consists of about 25% high value protein, but cannot be used as protein supplement for animal or human consumption due to the presence of two highly toxic endosperm proteins *viz*., ricin and *Ricinus communis* agglutinin (RCA) to a tune of 5% of the total protein. Therefore, this highly valuable meal is being used as organic manure, instead of a proteinaceous animal feed. Also, the presence of ricin and RCA pose a serious health hazard to the people involved in cultivation and processing of this very important industrial and pharmaceutical oilseed crop. It is interesting to note that to avoid these health hazards and the accidental ingestion of the castor seeds by humans or animals, the cultivation of castor was abandoned in the Texas region of the USA.

The shortage of protein sources, as animal feed, in India has necessitated a search for alternative sources. Beneficiation of castor seed meal for use in animal feedstuffs, instead of its current use as manure, would be of national economic importance (Gandhi *et al.*, 1994). In particular, this will help farmers of Andhra Pradesh where, castor is one of the major rain-fed crops and in Gujarat, where it is grown as a commercial crop. If farmers could use the castor meal as an animal feed, it is expected to improve the health of farm animals.

The major problem of using castor oil meal as animal feed is the presence of toxic proteins ricin and RCA. The genes encoding these proteins have been well characterized at the nucleotide sequence level. Till date, no genotype (variety) devoid of these toxins is identified and efforts in this direction following traditional breeding methodologies have yielded little success. In this light, adopting genetic engineering techniques to reduce the toxins in castor seems the only plausible way for utilizing 600 thousand tonnes of castor oil meal produced in the country as a good animal feed. This can boost the farm production by improved health of farm animals. Therefore, it is surmised that these proteins could be eliminated or reduced to acceptable levels using powerful PTGS techniques.

Efforts to reduce both ricin and RCA content using conventional breeding approaches have yielded little success due to lack of sufficient genetic variability and because of complex genetic control of the genes encoding these proteins. Other methods, both physical and

chemical treatment procedures to detoxify the castor meal are not economically viable and they reduce the availability/ digestibility of the proteins in the meal.

No work in the proposed lines is being carried out in India. However, attempts have been made to detoxify the castor oilmeal of ricin and RCA using several treatment methods (Gandhi et al., 1994, Srinivas and Nagaraj, 2000). So far, no attempts have been made to either block the biosynthesis of ricin and RCA or to search for the genotypes, which are naturally devoid of these toxic proteins.

Considering this background, it was proposed to block the synthesis of both ricin and RCA proteins using post-transcriptional gene silencing approaches. It was proposed to develop and use three different *viz*., antisense RNA, intron hairpin RNA (ihpRNA) and silencing by heterologous 3' untranslated region (SHUTR) PTGS approaches to reduce/eliminate the levels of ricin and RCA proteins in the developing seeds of castor. Ricin and RCA transcripts express and accumulate in the endosperm of castor. This warranted isolation and characterization of ricin promoter which is expected to be seed (endosperm) specific. Tobacco was used a model system to validate the functionality of the isolated ricin promoters. As ricin and RCA genes share very high degree of homology at their nucleotide sequence level, it was propounded that by choosing appropriate gene segments conserved among their genes, it would be possible to target the transcripts of both for degradation. Based on this premise, PTGS constructs were developed both under CaMV35S promoter and ricin full-length promoter (FP).

A meristem-based transformation and regeneration protocol using embryo axis from mature seeds, developed for realizing transgenic castor resistant to lepidopteran pests (Sujatha et al., 2005) has been used for executing the genetic transformation of castor for reduced/nil levels of ricin and RCA.

With this background, the present study was undertaken with the following envisaged objectives:

1. Isolation and characterization of ricin promoters
2. Development of generic and gene–specific PTGS constructs for silencing ricin and RCA in castor
3. Genetic transformation of castor with the PTGS construct.

CHAPTER II

REVIEW OF LITERATURE

Castor is a commercially important non-edible oilseed crop in the Euphorbiaceae with spurges and croton forming very large genera (Hutchinson, 1964). Castor (*Ricinus communis* L. 2n=20) whose origin is believed to be Abyssinia is industrially important for production of non-edible oil. It is distributed throughout the tropics and subtropics, and is well adapted to the temperate regions. The major castor producing countries are India, China, Brazil, USSR, and Thailand, while the major importing countries are the USA, the USSR, the EEC and Japan. India is the leading producer of castor in the world (FAOSTAT, 2008) with an annual production of about 1.1 Mt. India accounts for nearly73% of the world's production of castor (FAOSTAT, 2008). India has virtual monopoly in world castor oil market earning foreign exchange of Rs. 800 to Rs. 1200 crores annually by export of castor oil and its derivatives, castor seed and cake.

2.1 Economic importance of castor

Castor is important as a source of vegetable and medicinal oil and has numerous benefits to humanity. The oil has many industrial uses; dehydrated castor oil is used in the paint and varnish industry, manufacture of a wide range of sophisticated products like nylon fibers, jet engine lubricants, hydraulic fluids, plastics, artificial leather, manufacture of fiber optics, bullet proof glass and bone prostheses and as an antifreeze for fuels and lubricants utilized in aircraft and space rockets (Scarpa and Guerci, 1982; Ogunniyi, 2006). Castor oil is unique among vegetable oils because of the presence of ricinoleic acid, a hydroxy fatty acid up to 90%. The presence of long (18 C) fatty aliphatic carbon chain, a carboxyl group, a hydroxyl group, a double bond and the proximity of the latter two offer exciting possibilities of many chemical reactions and production of a variety of oleo chemicals. The demand for castor oil and its derivatives continues to be high and India is likely to continue to be the major player in world castor seed production as Brazil and China being the only competitors, turning to soybean and coffee. India is yet to leverage the potential of its monopoly as mostly raw oil and a few value added products are exported.

Castor cake is a by product of milling industry, which accounts for 60%of the crushed seed. It is rich in protein (25-40%), sugar (25%) and minerals (20%). Castor cake is a very useful organic manure (N-6.0%, P_2O_5-2.5%, K_2O-2.5%). Presence of many toxic constituents render castor cake non-edible and make it less useful. The most toxic constituent is ricin, which is present to the extent up to 200-mg/l00 g of cake. The cake is also very rich

in allergens (upto13%) because of which its cultivation is avoided in some countries of Europe and USA. Further, castor cake is a good source of vitamins like thiamine, beta-carotene and tocopherols. The detoxified castor cake can be an excellent animal feed. Protein isolates obtained from castor cake are useful for manufacture of surfactants, fibers, plastics and wall distempers. The toxic constituents in castor provide nematicidal and termiticidal properties due to which castor cake is used as organic manure in plantation crops.

2.2 Ricin and RCA

Ricin and *Ricinus communis* agglutinin (RCA) are the two highly toxic endosperm specific proteins of castor bean. These are essentially glycoproteins and belong to the plant lectin family. Lectins are proteins, which specifically bind carbohydrates and frequently agglutinate cells. Ricin is a heterodimeric polypeptide consisting of a 32 KDa. A chain - a potent inhibitor of protein synthesis by 80 S ribosomes and a 32 KDa carbohydrate binding B chain, linked by single disulfide bond. Ricin is one of the most toxic compounds produced in nature. It belongs to type II ribosome inactivating protein (RIP) family. RCA is a four-chained polypeptide comprising of two A chains and two B chains, slightly less toxic than ricin and causes agglutination of RBCs in mammals. The two heterodimers in RCA are held together by noncovalent forces. Ricin is a potent cytotoxin but a weak haemagglutinin, whereas RCA is a weak cytotoxin and a powerful haemagglutinin.

2.2.1 Occurence

Ricin is produced in tissue specific manner in the endosperm tissue during the post-testa maturation of the castor seed (Roberts & Lord, 1981a). Ricin, its isoforms, storage albumins and crystalloid proteins are usually targeted to an organelle called the protein body for storage in the mature seed (Tulley & Beevers, 1976; Youle & Huang, 1976). The protein body is analogous to any other vacuolar compartment in the cel1. Protein bodies are also present in the cotyledons, where again ricin and RCA, are part of the albumin fraction. Cotyledons contain much less lectins than the endosperm. Within these protein bodies, ricin accumulates to around 5 per cent of total particulate protein and is usually degraded within few days after germination of the seeds. (Frigerio & Roberts, 1998).

Barnes *et al.*,(2009) have shown that ricin is present in significant amounts in castor seed after 28 days post-pollination, and ricin disappears from the plant approximately 6 days after radicle emergence. This information provides insight into when castor is ultimately most

safe to handle and when precautions need to be taken to avoid ricin exposure. It also explains the biological function of ricin in the castor lifecycle. Because of its late synthesis in the seed and early disappearance form the seedling, ricin likely does not play a role in the defense of the mature plant, nor in the nematicidal effects of the plant. Thus, it appears that ricin only provides protection from predators of the mature seed. This knowledge will help in further research involving the potential effects of silencing the ricin gene in castor in order to provide a more industrially acceptable crop.

2.2.2 Biochemistry of ricin and RCA

Several isoforms of ricin including ricin D, ricin E and RCA reported in castor are encoded by a multi gene family consisting of approximately eight members, some of which were shown to be non-functional (Tregear & Roberts, 1992). Ricin, one of the members of plant's lectin family, is composed of two subunits of about 30,000 daltons each, namely the A and B chain (Rutenber, 1991). In general, ricin is referred to as lectin, although only the B-chain has the features of a lectin. Ricin A-chain is composed of 267 amino acid residues, and has 28SrRNA N-glycosidase enzymatic activity. The active A chain is approximately 30 per cent helical and contains 7 alpha helices. It also contains about 15 per cent beta structure, which consists of a free stranded beta sheet (Montford *et al.,* 1987).

The B-chain is composed of 262 amino acid residues and is a lectin (Katzin, 1991). The B-chain has affinity for galactoside binding (Katzin, 1991) and possesses two galactose-binding sites that are attracted to galactose containing glycoproteins at the cell surface (Wiley and Oeltman, 1991). The A and B chains are linked by a disulfide bond located at residue 259 of the A chain and residue 4 of the B chain (Montford *et al.,* 1987).

Being a glycoprotein, ricin possesses mannose rich N-linked oligosaccharides as carbohydrate side chains. These functional side chains help in binding of the toxin to certain cell types with mannose receptors. In particular, ricin binds to mannose receptors of the cells of reticuloendothelial system (Wiley & Oeltman, 1991). Ricin has sites with potential for binding of high mannose carbohydrate chains at asparagines 10 and 236 of the A-chain and asparagines 95 and 135 of the B-chain (Rutenber, 1991).

Ricin is not a membrane-bound protein, as it shows no distinct pattern of hydrophobic and hydrophilic regions. There are few alpha helices and the main structure is in beta sheets with many coil and turn regions.

X-ray crystallography has been employed to work out the three dimensional structure

of ricin and is refined and resolved at 2.5 A^o (Montford *et al.,* 1987; Rutenber *et al* 1991). This ribbon-shaped model detailed an accurate description of both the A-chain and the B-chain. Also, the structure of recombinant ricin toxin-A chain (RTA) has been resolved to 2.3 A^0. RTA is a 267-residue globular protein with a prominent and conspicuous binding site cleft; ricin toxin-B chain (RTB) a 262-residue elongated dumbbell shaped protein with galactose binding sites at both ends.

RTA possesses eight alpha helices (A-H) and eight strands of beta sheet (a-h). The 117 residues amino-terminal, which is about 40 per cent of the chain, form a compact folding unit. The bottom of the molecule is represented by an extended mixed sheet (beta strands 1-f) together with helices A and B. The next 40 percent or so of the sequence is largely helical containing helices C-G, all packing in a group resting on the sheet structure. Helix E, more than five turns in length, running through the center of the molecule, is largely non-polar, but contains two crucial active site residues, Glu 177 and Arg 180 near its carboxyl terminus. These lie on consecutive helical turns and face into the solvent of the active site. The remaining or rest 20 per cent of the sequence of RTA folds as a compact unit but is anchored to helix A. The RTA fold is archetypal of the family of plant and bacterial derived ribosome inactivating proteins (RIPs).

About eight conserved amino acids are of common occurrence in the family of RIPs. It is interesting to note that these conserved amino acids form the active site. These include Tyr 80, Tyr 123, Glu 177, Arg 180 and Trp 211. With respect to investigations into substrate binding, it has come to the fore that the substrate purine ring is stacked between the rings of the two conserved tyrosines. Arg 180 bonds to N3 of the ring and Glu 177 is near the ribose. Trp 211 makes no specific contacts with the adenine but may be important for the active site conformation or may interact with a larger polynucleotide substrate.

RTB is a two-domain structure, comprising of domain 1- the amino-terminal half of the molecule and domain 2- the carboxyl-terminal half. These two domains are homologous and must have arisen by gene duplication. Each domain is formed from four sub domain units. Each contains a 17-residue linking peptide, lambda, and three homologous units, a, b, and g, of a roughly 40-residue core peptide. An in depth analysis has hinted that these would have arisen from multiplication and fusion of DNA encoding an ancient galactose binding peptide, which could self assemble around a hydrophobic core into a trimeric structure. Of the six potential galactose binding sites, only 1 a and 2 g are functional. The refined galactose lending pocket is quite shallow and interacts with only about half the sugar. The bottom of

the pocket is formed by a three-residue kink in the peptide chain. The top is an aromatic side chain, Trp 37 and Tyr 248 in 1 a and 2 g, respectively, which contacts the hydrophobic face of the sugar. Specific hydrogen bonds are made between the sugar and the two RTB sites that accounts for the epimeric specificity of binding. Asp 22 in site 1 and Asp 234 in site 2 make the primary interaction. They lie between the C3 and C4 hydroxyls and hydrogen bond to both. The crucial aspartates are locked in position by bonds from GIn 47 in site 1 and GIn 256 in site 2. The C3 hydroxyl of bound galactose also forms a strong hydrogen bond with Asn 46 in site 1 and Asn 255 in site 2. These interactions with galactose were supposed to be the strongest. Site-directed mutagenesis confirmed that the Asn residues are important to galactose lending, but suggest that RTB can still bind to dense polysaccharides, like asialofetuin, even if they are disrupted. Few, about 4 or 5 RTB amino acid residues are required to interact with galactose.

2.2.3 Ripping Apart: Closing in on the mechanism of ricin action

Ricin is a cytosolic heterodimeric plant lectin and belongs to the family of type II ribosome inactivating proteins (RIPs). RIPs are toxic N-glycosidases that depurinate large rRNAs and render them incapable of sustaining further translation. RIPs are found in most of the genera throughout the plant kingdom and also in some fungi and bacteria.

RIPs are classified into three groups based on their physical properties.

Type I : Eg. PAP (Pokeweed antiviral protein); Saporin (from soapwort) *(Saponaria officinalis* L.) and Barley *(Hordeum vulgare).*

These include monomeric enzymes, each with an appropriate M_r of 30,000.These are basic proteins which share a number of highly conserved active self residues and secondary structure within the active site region. They are distinctly different in overall sequence homology and post-translational modifications.

Type II : Eg. Ricin and abrin.

Highly toxic heterodimeric proteins with enzymatic and lectin properties in separate polypeptide subunits, each of approximately M_r of 30,000 constitute type II. They have been shown to be useful for studies of endocytosis and intracellular transport in mammalian cells.

Type III : Eg. Maize and barley.

This type is synthesized as inactive precursors (pro RIPs) that require proteolytic processing events to occur between amino acids involved in formation of the active site. They are much less prevalent than type 1 or type 2.

Endo and co-workers (1987) showed that the enzymatic activity of RIPs was an N-glycosidation to remove a specific adenine corresponding to residue A 4324 in rat 28S rRNA. This adenine lies within a 14-nucleotide region known as a-sarcin loop and is conserved in large rRNAs from bacteria to humans. A GAGA substrate forms the core of a putative tetraloop surrounded by a short base paired stem. Irreversible modification of the target A residue blocks elongation factor (EF-1) and EF-2 dependent GTPase activities and renders the ribosome unable to bond EF-2, thereby blocking translation.

At the ER, RTA is a 28S rRNA N-glycosidase (Endo *et al.,* 1987), inactivating the cell ribosomes by depurinating adenine in a hairpin containing the tetranucleotide loop GAGA of 28S rRNA (Lord *et al.*,1994). A single A-chain molecule is capable of inactivating every ribosome in the cell thus, halting protein synthesis and culminating in cell death (Wiley & Oeltman, 1991). Ricin has a Michaelis constant (K_m) of 0.1mol/L for ribosomes and an enzymatic constant (K_{cat}) of 1500/min.

It is likely that the susceptible adenine base binds between tyrosine residues 80 and 123 while forming specific hydrogen bonds with the backbone carboxyl group and amino nitrogen atom of Val 81 and with the carboxyl group of Gly 12. In the hydrolysis, the leaving adenine is at least partially protonated by Arg 180, and GIn 177 may stabilize a putative oxycarbonium transition state, or more likely, act as a base to polarize the attacking water.

RTA catalyzes irreversible removal of a specific adenine in the large rRNA of the 60 S ribosomal subunit (A 4324 in rat liver 28 S rRNA). This conserved adenine is a part of a region of 28 S rRNA that is critical for the lending of EF 2 ternary complex in the translocation phase of a protein synthesis elongation cycle. Its removal therefore, causes an immediate cessation of elongation cycles and quickly leads to a halt in all cytosolic protein synthesis.

The sensitivity of ribosomes from different sources to the action of RTA varies significantly. Thus, while mammalian ribosomes are most sensitive to the action of RTA, yeast ribosomes are slightly less sensitive and prokaryotic ribosomes are resistant (Lord *et al.*,1991).

A hairpin loop on the 28 S rRNA containing the tetranucleotide loop GAGA is the most likely target for attack by ricin on the ribosome. However, it is thought that the substrate (ribosome) conformation is an important factor in recognition by the protein (Lord *et al.*,.,1991). Therefore, ricin is not a nucleotide sequencespecific protein (Wiley and Oeltman, 1991).

2.2.4 Biogenesis of ricin

Genes encoding ricin are temporally and spatially regulated. They are transcribed only in the developing endosperm. The transcript encodes prepropolypeptide possessing both A and B chains. Biogenesis of ricin involves both co-translational and post-translational modifications.

2.2.4.1 Co-translational modifications

The protein biogenesis starts as preproricin. The NH_2-terminal signal peptidase, after Ser 22 in the 35-residue preproricin leader sequence targets the nascent polypeptide across the ER membrane into the ER lumen. It has been surmised that the resulting proricin's short amino acid sequenced at the new NH_2 terminus might be a protein body targeting sequence. The proricin gets N-glycosylated once it enters the ER lumen. Proricin possesses four N-glycosylation sites, two within the A-chain and two within the B-chain. Finally, protein disulfide isomerase catalyzed formation of four disulfide bonds occurs within the sequences of RTB, while the remaining one links the C- terminus of RTA, with the N-terminus of RTB.

2.2.4.2 Post-translational modifications

The cotranslated proricin is transported from the ER lumen through the Golgi complex to the protein bodies for proteolysis and storage. Transport through the Golgi complex is accompanied by oligosaccharide trimming and addition of fucose and xylose to oligosaccharides on RTA. These modifications confer partial endoN-acetyl glucosaminidase H resistance on the proricin sugar side-chains. Golgi complex carrying proricin fuse with the vacuolar membrane and discharge their contents into the protein body matrix.

In the protein body, the N-terminal propeptide and the 12-residue linker peptide are proteolytically removed, releasing the mature, disulfide linked RTA-RTB heterodimer. Since the cleavage occurs within a disulfide loop connecting the A and B chains, the disulfide bond still remains intact. A vacuolar cysteine protease from castor bean endosperm was shown to convert proricin into its mature form (Hiraiwa *et al.,* 1997; Hara-Nishimura *et al.,* 1991). Recent evidence suggests that the signal for sorting of preproricin to the protein storage vacuoles could reside in the linker peptide (Frigerio *et al.,* 1998).

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2.2.5 How castor bean (*Ricinus communis* L.) avoids poisoning its own ribosomes?

Since ricin is not active when synthesized in precursor form (as proricin), the castor bean protects its own ribosomes from the toxicity of ricin (Richardson *et al.,* 1989). This could be due to the structural constraints generated due to the linker peptide, causing the active site cleft of RTA to be in close contact with RTB, thus rendering it unavailable for catalysis. Also, maturation and activation of the precursor into the active form takes place in the vacuolar compartment, from which mature ricin is unable to retrotranslocate.

2.2.6 Genomics of ricin

Ricin gene was the first plant ribosome inactivating protein (RIP) encoding gene to be cloned. As is characteristic of all plant lectin genes, ricin gene does not contain any introns. Experimental data from molecular hybridization using a ricin cDNA probe revealed that ricin and RCA belong to a multigene family of lectins consisting of eight members (Tregear *et al.,* 1992). cDNA clones encoding preproricin and preproagglutinin have revealed extensive homologies, the A chains being 93 per cent identical at the amino acid level and the B chains being 84 per cent homologous. Expression of these toxic proteins appears to be tissue specific. At least three members of the castor bean gene family are non-functional. The sequence data for these members revealed the presence of frame shift mutations, hinting at the fact that they encode pseudogenes. It was deduced that the lectin precursor encodes a 35 amino acid residue presequence, a 267 amino acid residue A chain, a 12 amino acid linker peptide and 262 amino acid B chain.

The ricin gene uses more than one transcription start site; the most common 5' position determined occurring 60 bp upstream from the ATG codon. There is a TATTAA sequence at -22 (with respect to the most 5' putative transcription start point), which might function as a TATA box. A CAAGT element representing a CAAT box analogue is also present. The AGGA box is represented by ATTGA motif but there is no evidence to support its functional role as an expression signal. RNase protection analysis was used to predict the polyadenylation site (Tregear *et al.,* 1992). Two putative polyadenylation signals, with sequences AATAAA and AATAAG, have been previously identified (Lord *et a1.*, 1985) in the 3' untranslated region of a ricin cDNA and a ricin genomic clone (Halling *et a1.*, 1985). Investigation at the transcriptional level of the expression pattern of ricin and RCA revealed that mRNA accumulates during the post-testa stages of seed development.

2.2.7 Developmental expression of ricin and RCA

The developmental expression of ricin and RCA was studied in castor seed using RT-PCR assay and Northern analysis (Chen *et al.*, 2005). They carefully determined seed development age using two visual markers, seed coat colour, endosperm volume, and three phases for the whole course of castor seed development. Seeds do not develop endosperm tissue until about 26 day after pollination (DAP) and then the endosperm undergoes development until 54 DAP. At 61 DAP, seeds mature and desiccate. The results from this study indicate that the expression of ricin and RCA genes corresponds to the developmental profile of endosperm. The mRNA signal is not detectable before the endosperm begins to develop (12 and 19 DAP) but becomes significant between 26 and 54 DAP when the endosperm expands to occupy most of the seed volume. When seeds enter the desiccation stage (61 DAP), the expression of ricin and and RCA genes drops to a trace level.

In castor seed, the ricin and RCA mRNA levels are correlated with the dimension of endosperm, suggesting that they are also spatially and temporally regulated. Castor endosperm is the site for synthesis and storage of various seed proteins including ricin and RCA (Roberts *et al.*, 1981). A study by Tregear *et al*. (1992) examined ricin and RCA mRNA levels during castor seed development using Northern analysis but it could not distinguish ricin or RCA transcripts due to their high degree of sequence identity.

The RT-PCR assay/restriction fragment analysis reported by Chen *et al*. (2005) is definitive and a highly sensitive method to detect the expression of ricin or RCA genes at the transcriptional level. The technique provides a method to screen the effectiveness of different silencing constructs in individual transformants for the level of success in suppressing expression of ricin and RCA genes in developing castor seed.

2.2.8 Estimation/Quantification of ricin and RCA concentration in castor bean

Pinkerton *et al*. (1999) adopted a radial immunodiffusion (RID) assay to allow screening of castor lines for reduced levels of ricin and RCA concentration. The RID assay is based on incorporating a specific antibody against *Ricinus communis* lectin into a thin layer of agar. Seed extracts containing mixtures of antigens were placed into wells cut into the agar layer. As the antigens in the wells diffused outwardly, they reacted with the antibody forming a precipitation halo around the well with the diameter of the halo providing an estimate of the ricin + RCA concentration. The RID assay had a minimum sensitivity of 1.0 mg of total ricin

+ RCAI g of seed.

An enzyme linked immunosorbent assay (ELISA) with a sensitivity of 0.01 mg/g is being developed to measure the levels of ricin, RCA and ricin + RCA in castor lines with very low concentrations of toxins (Poli *et al.*, 1994). This assay is more expensive to perform than RID assay, but its sensitivity and the ability to perform many assays on a single micro titre plate make it ideal for measuring the concentrations of ricin + RCA in castor lines with extremely low levels of toxins. Rabbit antisera against ricin will be coated onto the plastic walls and seed extracts added. Ricin will bind to the specific antibody on the walls of the microtitre wells and anti-ricin horse radish peroxidase conjugate will be used to quantify bound ricin + RCA. Toxin concentration will then be estimated spectrophotometrically/colorimetrically.

2.2.9 Approaches to eliminate/reduce ricin and RCA

2.2.9.1 Physical and chemical methods

Several attempts have been made to detoxify the castor seed meal by destroying the ricin and RCA using a number of physical and chemical methods. The physical methods included soaking (Anandan *et al.,* 2005), steaming (Anandan *et al.,.,* 2005), boiling (Anandan *et al.,*2005; Barnes *et al.,* 2009), autoclaving (Anandan *et al*., 2005; Barnes *et al*., 2009) and heating (Anandan *et al*., 2005) of the oilcake. The chemical methods consisted of treatment of the cake with ammonia (Anandan *et al*., 2005), formaldehyde (Anandan *et al*., 2005), lime (Anandan *et al*., 2005), sodium chloride (Anandan *et al*., 2005), tannic acid (Anandan *et al*., 2005), sodium hydroxide (Anandan *et al*., 2005), calcium hydroxide (Barnes *et al*., 2009), urea (Barnes *et al*., 2009) and guanidine (Barnes *et al*., 2009). Of all the methods employed, autoclaving and lime treatment destroyed the toxins. Solvent extraction alone is less desirable as it would leave the ricin as a functional toxin within the meal unless solvents were heated to drive them from the meal to be recycled (Barnes *et al*., 2009). Treatment of the meal with urea and guanidine showed no substantial reduction in the presence of the ricin as visualized by the antibody reaction (Barnes *et al*., 2009).

In another approach, it has been suggested that the ricin and RCA proteins could be inactivated by use of sal seed meal, which contains very high levels of tannins. However, the treated meal shows reduced protein content and also shows the presence of substantial levels

of tannins (Gandhi *et al*., 1994). Tannins, which non-selectively bind to the proteins and destroy their enzymatic activities, also reduce the net protein availability of the treated castor oilmeal

In an industrial setting involving larger quantities, care would have to be taken to ensure every part of the seed or seed product was exposed to the treatment. These methods are possibly not commercially feasible because these reactions which are drastic / harsh while destroying the ricin and RCA also destroy the useful proteins. The treated oilmeal impairs the digestibility of the proteins in animals, as the digestive enzymes are also complexed by tannins. Apart from these disadvantages, all the detoxifying methods need additional knowledge and inputs from the end user and also the extent of detoxification might vary subject to the method adopted and followed, thus always having a fair amount of risk on farmers' side.

2.2.9.2 Conventional genetics

Down-regulating the genes responsible for toxic substances, which would result in lines with reduced levels of toxins has been an objective in several breeding programmes. Traditionally, this has been achieved by careful selection of the genotypes from the available variability. Alternatively, new variations could be generated through use of mutagens. Both these processes are slow and costly, and most often, not successful. Efforts to develop lines with reduced levels of ricin and RCA in castor through conventional breeding strategies have not been successful. This has been ascribed to the non-availability of sufficient genetic variability (*Ricinus* is a monotypic species), complex genetic regulation of ricin and RCA concentrations (ricin and RCA abounds in multiple copies of the gene located on different chromosomes as shown by southern data (Tregear *et al*., 1992)), with minor and major genes and substantial environmental influence) and difficulty in selection of the plants or lines with low ricin quantities due to the technical problems of estimating the concentration of ricin. Due to these problems, some trials done in the USA have not led to identification of low ricin stable lines in F_6 generation (Pinkerton *et al*., 1999; Auld *et al*., 2001). Also, this approach is a slow and costly process requiring several generations and considerable effort to develop elite lines with the desired phenotype.

In collaboration with ARS-USDA, Texas Tech University scientists have developed a transformation protocol in castor and currently they are trying to develop transgenic castor with low / no ricin and RCA content using antisense technology (Auld *et al*., 2001).

Using classical mutagenesis techniques, random mutations are introduced into the castor genome and screened to determine the mutations that have the ricin genes knocked out. The screening is accomplished through Targeting Induced Local Lesions In Genomes (TILLING) technology for identification of single nucleotide polymorphisms and the desired mutants are crossed with desired castor germplasm (www.arcadiabio.com).

2.2.9.3 Biotechnological interventions

Use of post-transcriptional gene silencing (PTGS) to improve crop value by eliminating undesirable traits can potentially address many problems in a faster and more precise way than conventional breeding. Multiple genes sharing stretches of identical sequence can be effectively silenced with a single RNAi construct. This feature is especially important in crops harbouring multiple copies of the target gene (as in case of ricin and RCA in castor) and in polyploidy crops like wheat (Fu *et al*., 2007), which has multiple homoeologous copies for each gene. Therefore, RNAi is coming of age as a useful and flexible tool to study gene function in polyploid wheat species, although more research is necessary to establish the additional factors that determine a construct's silencing efficiency in addition to the ''21-nt rule.''

Reported examples include silencing of polyphenol oxidase, to prevent enzymatic browning of potato (Wesley *et al.*, 2001), silencing of ACC oxidase, which produces the ripening compound ethylene in tomato, resulting in altered ripening and prolonged shelf-life (Xiong *et al.*, 2005), and silencing of the Mal d 1 family of allergenic proteins in apple (Gilissen *et al., 2005).*

A particularly interesting example is provided by Sunilkumar *et al*. (2006), who used tissue-specific expression of a hairpin transgene to reduce the levels of the toxic insecticidal terpenoid gossypol in cottonseed. Gossypol is toxic to humans and monogastric animals, and its accumulation in seed limits the use of cottonseed protein. However, as gossypol is a beneficial insecticidal protein elsewhere in the plant, its systemic elimination results in strongly increased susceptibility to insect attack. The enzyme δ-cadinene synthase (encoded by a family of genes) is required to divert terpenoid compounds into the gossypol synthesis pathway. Sunilkumar *et al.* (2006) silenced this target using a conserved hairpin expressed from the seed-specific cotton *α-globulin B* promoter. As a result, seed gossypol was reduced by up to 99%, while levels elsewhere in the plant were not measurably changed.

The accumulation of a vast array of plant metabolites is an important determinant of

value for many agricultural crops, and engineering of metabolic pathways is another potential use for hairpin RNAi. This approach has been used both as a tool to probe biosynthetic pathways (for example, caffeine biosynthesis enzymes in coffee, Ogita *et at.,* 2003) and as a means of changing the properties of plant-derived products (for example, increasing the nutritional value of maize by changing amino acid accumulation, Huang *et at.,* 2006). Two further interesting examples are the modification of fatty acid profiles in cottonseed oil, and the manipulation of alkaloid profiles in opium poppy latex.

Stoutjesdijk *et at.* (2002) and Liu *et at.* (2002) have shown that manipulation of fatty acid biosynthetic genes by RNAi can result in altered oil profiles in seeds of *Arabidapsis* and cotton, respectively. As a demonstration of the technology, Stoutjesdijk *et at.* (2002) silenced a fatty acid desaturase gene in *Arabidapsis* seeds using hairpin RNAi, resulting in increased accumulation of monounsaturated oleic acid at the expense of its polyunsaturated derivatives. Cottonseed oil typically contains a relatively high proportion of palmitic acid (26%), an unhealthy saturated fatty acid, while it is low in the desirable oleic and stearic fatty acids (15% and 2%, respectively, Liu *et at.,* 2002). Liu *et at.* (2002) showed that hairpin RNAi against two fatty acid desaturase genes which act upon stearic acid and oleic acid can strongly increase the proportion of these fatty acids in cottonseed oil. They found a 95% reduction in oleic acid desaturase activity, resulting in an increase in oleic acid content from 13.2% in the parental line to 78.2% in the strongest hairpin line, and a halving of stearic acid desaturase activity, resulting in an increase in stearic acid content from 2.3% in the parental line to 39.8% in the strongest hairpin line. Both fatty acid desaturases targeted for silencing are members of gene families, and residual activity of some family members is thought to account for the desaturase activity found in hairpin lines. For example, the oleic acid desaturase enzyme targeted is thought to be the most active of a family of at least five members, with more divergent family members showing only 70% homology. In comparison to the conserved hairpin experiments discussed previously, this is probably a sufficient level of sequence divergence to prevent strong cross-silencing.

While attempts to engineer metabolic pathways may result in the predicted changes in metabolite profiles, as reported by Liu *et at.* (2002), in other cases feedback regulation controlling activity of other enzymes in a pathway may give surprising results. This was the case when Allen *et at.* (2004) used a combination of the approaches described above to silence the seven-member family of codeinone reductase (COR) enzymes, which catalyse the

final step of morphine biosynthesis in opium poppy. The family is encoded by six highly related members, *Cor1.1-6,* and the more divergent *Cor2,* which were targeted for silencing by a chimeric hairpin sequence containing 336nt of *Cor1.1* and a 242nt sequence of *Cor2.* Specific downregulation of *Cor* transcripts was achieved; however, resultant reduction in COR enzyme activity vastly changed the profile of alkaloids produced in poppy latex in an unexpected way. From the compound reticuline, one branch of the alkaloid biosynthetic pathway leads to morphine accumulation after eight enzymatic steps. Downregulation of this whole branch in response to silencing of its final enzymatic step was observed, resulting in diversion of alkaloids to a group of products derived from reticuline which do not normally accumulate to significant levels.

As these examples demonstrate, hairpin RNAi is a highly effective tool for the modification of gene function, and its application to a wide range of target genes in different plant species has been accomplished.

2.3 Post-transcriptional gene silencing (PTGS)

Gene silencing phenomenon was first observed by scientists working with plants (Matzke *et al*., 1989; Jorgensen 1990; Napoli *et al*., 1990; Smith *et al*., 1990; van der Krol *et al*., 1990). They found that transgenes, introduced into plants for over expression of a certain gene, induced the opposite phenotype of what was expected. This was called co-suppression; the expression of a transgene was suppressed and that further induced suppression of the endogenous gene. In the follow-up experiments, it was shown that co-suppression could be the consequence of transcriptional and/ or posttranscriptional gene silencing mechanisms. Transcriptional gene silencing (TGS) involves changes at the DNA level which leads to loss of transcription, e.g., methylation of the promoter sequences and chromatin remodeling (Matzke and Matzke 1991; Meyer 1993). TGS occurs in the nucleus, inherited stably after meiosis and is not involved in systemic silencing. On the other hand, post-transcriptional gene silencing (PTGS) does not affect transcription but involves sequence specific mRNA degradation (de Carvalho *et al*., 1992; van Blokland *et al*., 1994). PTGS occurs in the cytosol, is reset every generation, and involves systemic silencing, although the exact nature of the movement and extent of spread of the signal has not been established. Methylation of the protein-coding region is often associated with PTGS. The major triggers of PTGS are transgenes and viruses (Ruiz *et al*., 1998).

PTGS in plants is analogous to RNA interference (RNAi) in animals and quelling in fungi, and the formation of short interfering RNA molecules that are produced by degradation of long dsRNA molecules, mediate all these three phenomenon. RNAi was discovered first in C. Elegans (Fire *et al*., 1998) and subsequently in many different organisms of other kingdoms. Though originally RNAi was defined as gene silencing induced by directly introduced/injected double-stranded RNA (dsRNA) designed to target mRNA of a specific gene (Kusuba 2004; Tang and Galili 2004; Chicas and Macino 2001; Voinnet 2002), the term is being used as a generic term these days to denote any gene silencing initiated by dsRNA molecules which subsequently give rise to small interfering RNA molecules. When dsRNA corresponding to the sequence of an endogenous mRNA is either introduced into the cell or it is produced in the cell, the cognate mRNA is degraded. Though known by different terms in different organisms, these processes are in unison with respect to the mechanism of induction of gene silencing and the triggers that bring about induction. PTGS is closely related to the natural processes such as RNA-mediated virus resistance and cross protection in plants. Therefore, PTGS acts as protective measure against viral infection in plants. PTGS phenomenon, which was first identified as co-suppression in petunia plants transformed with chalcone synthase genes, has been subsequently used to silence specific gene transcripts to produce desirable traits in crop plants.

2.3.1 Mechanisms of post-transcriptional gene silencing

Results obtained with different experimental systems have led to a unified concept regarding the mechanism of PTGS. The common opinion is that, homologous genes, dsRNA and small interfering RNAs (siRNAs) are the usual triggers for the PTGS phenomena in different organisms. This implies a conserved mechanism across different kingdoms. However, subtle differences in the mechanisms between different organisms and between different triggers are also observed. For instance, some genes needed for PTGS are organism specific or specific for a subset of organisms e.g. SGS3 (encodes a protein of unknown function containing a coil-coiled domain) and MET1 (encodes a DNA-methyltransferase) are essential for PTGS in plants and are absent in *C. elegans* (Vaucheret *et al*., 2001). These genes could be involved in organism-specific fine-tuning of the mechanism. Alternatively, these genes are dispensable in some organisms because other genes or processes fulfill their task. PTGS mediated gene

silencing exploits cellular mechanism that recognizes double-stranded RNA and subjects the corresponding mRNA to a sequence-specific degradation.

Path-breaking studies with Drosophila extracts showed an activity capable of processing long dsRNA substrates into small RNAs (Zamore *et al*., 2000). Immunoprecipitates of a candidate protein degraded dsRNA into the same kind of small RNAs as observed in vivo and were called small interfering RNAs or siRNAs. These siRNAs were typically 21-25 nt long, double-stranded molecules with 2 nt 3' overhangs, and 5' phosphates. The candidate protein was an RNAse III enzyme that was called DICER. Decreased DICER levels in vivo correlated with decreased gene silencing indicating the central role of the DICER enzyme in RNAi. Several other studies in different organisms using different techniques have lead to cobbling up of pieces of information together to arrive at the common mechanism involved in all PTGS.

Two of the primary steps involved in the process are dsRNA introduction/formation and subsequent degradation of target mRNAs (Waterhouse and Helliwell 2002).

The first step could be achieved through different means. The direct insertion of dsRNA or siRNA into a cell can be accomplished by bombardment, viral-mediated dsRNA transfer or by infiltration (Sijen and Kooter, 2000). Recently, the use of cationic oligo peptides for delivering dsRNA into plant cells has been reported (Unnamalai *et al*., 2004). The methods, which allow the formation of the dsRNA in the cell, utilize vectors designed to produce stable or transient dsRNA in vivo. In other words, the plant must be transformed with a vector that produces dsRNA.

The path to mRNA degradation begins when a piece of dsRNA present in the cytosol, recruits Dicer. This recruitment initiates chopping of the long dsRNA into a number of siRNAs. Soon after their formation, the siRNAs are incorporated into Ribosome Inactivating Silencing Complex (RISC), initiating the process of mRNA degradation (Agrawal *et al*., 2003; Kuznetsov, 2003; Arenz and Schepers, 2003). It is believed that a physical interaction occurs between Dicer and RISC through a common PAZ domain. RISC, then utilizes the siRNA as a targeting sequence seeking the complementary mRNA. Successful docking of the RISC-siRNA complex at the targeted mRNA site initiates the degradation process. The mRNA degradation is executed by the slicing (RNase III) activity of RISC. A representation of common mechanisms of interference is provided in Figure 2.1.

A not yet fully understood mechanism seems to enhance the effectiveness of RNAi, through the amplification of siRNAs by an RdRP (RNA-dependent RNA polymerase).

Lipardi *et al*. (2001) reported that siRNA might be involved in the synthesis of long dsRNA. Sijen *et al*. (2001) revealed the presence of secondary siRNAs by their insight into RdRP-mediated amplification. Secondary siRNAs, not detectable in the introduced dsRNAs are derived from siRNAs that complement the targeted mRNA. These secondary siRNAs actively participate in the degradation of the complementary mRNA.

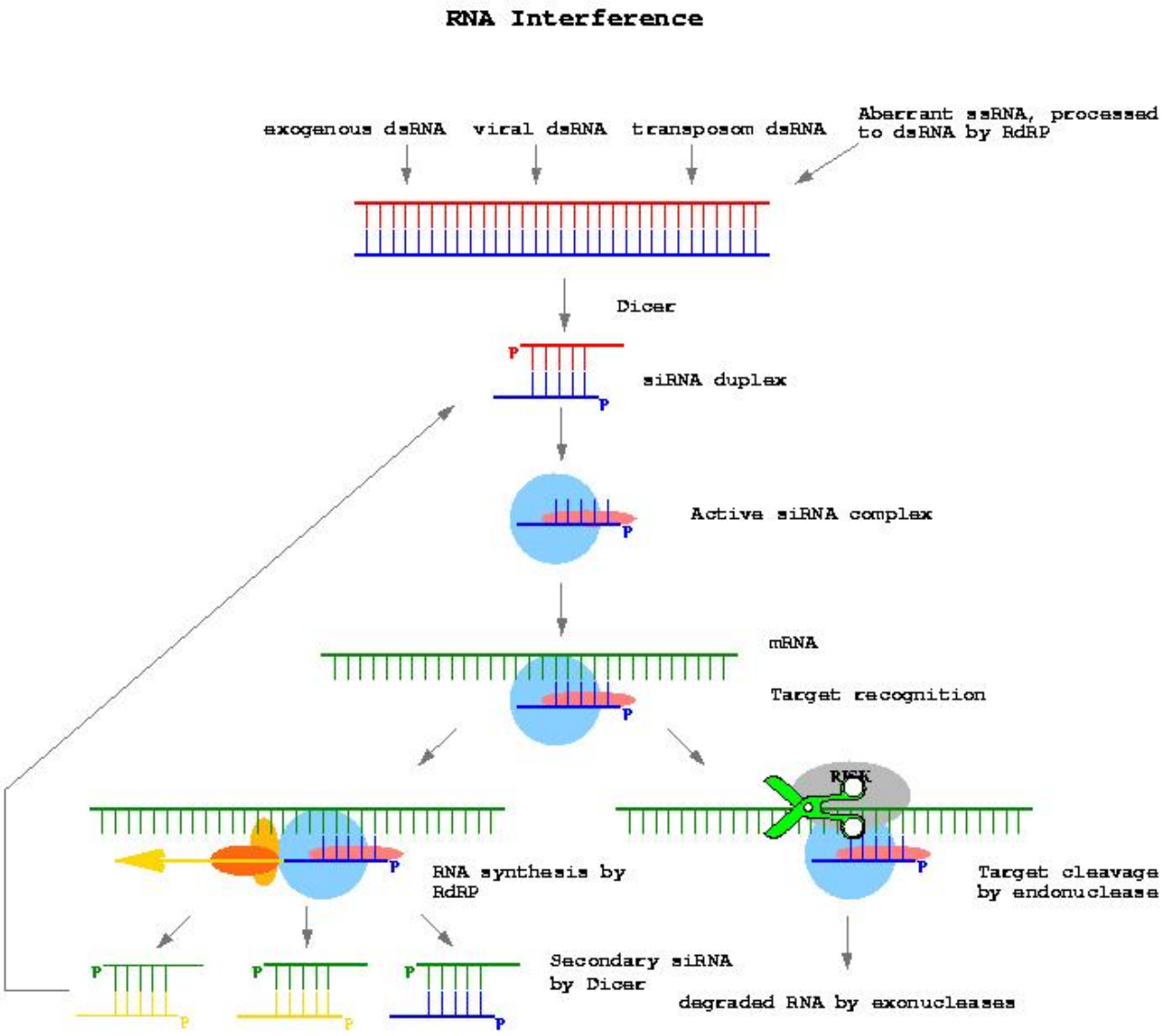


Fig 2.1: Schematic representation of the mechanisms of PTGS

(Adapted from an internet file)

2.3.2 Requirements for induction of post-transcriptional gene silencing in crop plants

The following components/issues are to be considered carefully before working towards induction of post-transcriptional gene silencing in crop plants.

a) Production of double –stranded small RNA as the trigger

Waterhouse *et al.* (1998) observed that transgenes capable of duplex formation gave rise to stronger and more frequent gene silencing than sense or antisense transgenes alone, whether the duplexes were from sense and antisense transgenes brought together by crossing, sense and antisense expressed separately from one transgene locus, or as a single self-complementary transcript. These authors proposed that the previously observed phenomena of antisense suppression and co-suppression were in fact largely induced by the formation of dsRNA from inverted-repeat transgene integration loci (as observed by Starn *et al.,* 1997). A strikingly similar dsRNA-induced silencing phenomenon, termed RNA interference, was observed by Fire *et al.* (1998) in the nematode worm *Caenorhabditis elegans.*

To express dsRNA more efficiently *in planta,* hairpin transgene constructs were designed with sense and antisense copies of the target gene separated by a spacer, usually under the control of a strong constitutive promoter such as cauliflower mosaic virus 35S (35S). Using these early hairpin constructs, silencing was directed at targets including potato virus Y and a *GUS* transgene (Waterhouse *et al.,* 1998), and the endogenous flowering genes *AGAMOUS, CLAVATA3, APETALA1* and *PERIANTHA* (Chuang and Meyerowitz, 2000).

A spacer sequence to separate hairpin arms was initially included to increase stability of the self-complementary sequence during cloning; however, Smith *et al.* (2000) discovered that the use of a splicable intron as the spacer sequence dramatically increased the frequency of silencing phenotypes to near 100%. The means by which intron splicing increases the effectiveness of hairpin RNAi is unknown.

b) Sequence identity

When choosing the RNAi trigger, it is important to consider the presence of continuous stretches of perfect identity between the trigger sequence and its target genes. During the RNAi response in plants, cleavage of dsRNA produces siRNAs of 21–26 nt (Hamilton *et al*. 2002; Llave *et al*. 2002; Tang *et al*. 2003; Qi *et al*. 2005). Therefore, the presence of a continuous stretch of at least 21 identical nucleotides between the trigger and the target gene

is required, although it is not always sufficient to produce efficient silencing (Miki *et al*. 2005; Yue *et al*. 2007; McGinnis *et al*. 2007).

Several studies report overall sequence identity as a criterion for RNAi trigger selection (as opposed to stretches of contiguous identical sequence), but this parameter should be interpreted with caution. Both measurements are related, because the higher the overall sequence identity, the higher the chance of encountering at least one identical contiguous stretch of 21-nt. For example, homoeologous genes in wheat share at least 95% sequence identity, which translates into an average of one polymorphism every twenty nucleotides. The likelihood that all polymorphisms within the target sequence would be perfectly spaced every 20-nt is almost zero. Therefore, in a normal sized trigger of 200–500 bp, wheat homoeologues will naturally have uninterrupted identical stretches of at least 21-nt among them. For this reason, it is safe to predict that using one homoeoalelle as the trigger sequence should usually suffice to silence all three wheat homoeologues simultaneously. This has been confirmed in recent wheat studies where a unique construct was sufficient to down-regulate all three homoeologues (PDS, EIN2 and NAM) and even closely related paralogues (NAM). However, exceptions have been reported for the HMW-glutenin x-type subunits (Yue *et al*. 2007). This may be related to the unusually large divergence between the homoeologous glutenin genes (86–91% sequence identity) or their highly repetitive structure.

The silencing of the NAM paralogous copies provides an example where identical stretches of 40, 31, and 23-nt between the RNAi trigger and the target region were sufficient to effectively reduce transcript levels. On the other hand, RNAi down-regulation of the *VRN2* vernalization gene showed no effect on transcript levels for the paralogous *ZCCT2*, which was more divergent and showed identical contiguous stretches of only 18-nt or less with the VRN2 trigger. Results from the previous two studies indicate that in order to trigger RNAi the two sequences need to share a continuous stretch of identical nucleotides longer than 18-nt, which is consistent with the ‘‘21-nt rule’’ that indicates that at least 21-nt of continuous perfect identity are required to trigger RNAi (Miki *et al*. 2005; McGinnis *et al*. 2007).

Even though an uninterrupted stretch of 21 identical nucleotides may be necessary to trigger RNAi, the presence of these stretches alone is not always sufficient. Regina *et al*. (2006) used RNAi triggers for *SBEII-a* and *SBEII-b*, which each have an identical 21-nt stretch to the paralogous gene. The *SBEII-a* transgenic plants showed reduced levels of both proteins, whereas *SBEII-b* transgenic plants showed reduced protein levels only for SBEII-b. In addition, two recent studies in wheat (Yue *et al*., 2007) and maize (McGinnis *et al*., 2007)

presented examples of genes whose transcript levels were not significantly affected by RNAi in spite of including 21-nt of perfect identity with the RNAi trigger sequence. Other factors, such as GC content and predicted melting temperature of the RNA hairpin, have been suggested to play a role in determining the efficacies of RNAi triggers (Reynolds *et al*. 2004). However, these characteristics were not significantly correlated to non-target silencing efficiency in a comprehensive study in maize (McGinnis *et al*. 2007), suggesting that additional experiments will be required to identify the factors that affect RNAi silencing efficiency beyond the ‘‘21-nt rule.’’ For example, it would be interesting to create artificial RNAi triggers with high sequence identity to the different homoeologues, but with mutations spaced out evenly at intervals of 19–23-nt. An alternative approach would be to create several different RNAi triggers with a unique 19–23-nt identical continuous stretch to the target gene, but lacking sequence identity throughout the rest of the trigger. These unique identical stretches could be shifted to complement different regions along the target sequence to better understand how the trigger sequence itself affects the RNAi efficiency. These experiments would help define the limits of the RNAi silencing response and would aid future users to design more effective RNAi triggers, especially for genes with conserved domains or with duplications elsewhere in the genome. This is especially important in polyploidy wheat, which has been shown to have a high rate of gene duplication (Dubcovsky *et al*., 1996; Akhunov *et al*. 2003), and therefore a high chance of targeting undesired (or unknown) paralogous genes.

c) Promoter

It is well established that the transgenes introduced into plants, should be under the most appropriate promoter to re*g*ulate the expression of the introduced gene(s) spatially and temporally to suit the purpose. In the case of inducing PTGS for a targeted trait, the expression of the construct should preferably be restricted to only the specific cell types where their role is implicated. Therefore, it is very essential to identify and choose promoters that will drive the expression of selected genes in the desired tissues or cell types. PTGS need not be limited to producing single-gene constitutive silencing lines. There are also reports of tissue-specific hairpin RNAi, when hairpins are expressed under the control of tissue-specific promoters. Byzova *et al.* (2004) aimed to modify flower architecture by altering the expression of MADS genes which control floral organ identity in the four whorls of

organs present in flowers - sepals, petals, stamens and carpels. The *APETALAl* promoter, which is specific to sepals and petals, was used to control expression of a hairpin construct against *APETALA3,* which is normally expressed in petals and stamens. Conversion of petals to sepals in *Arabidopsis* and *Brassica nap us* transformants confirmed that *APETALA3* was silenced in the organ whorl normally producing petals; however, normal development of stamens showed that *APETALA3* was correctly expressed in the neighbouring whorl of organs. While this example of highly tissue-specific hairpin RNAi apparently contradicts the known spread of silencing signals, it may be due simply to lower levels of the silencing signal being expressed by the floral-organ-specific promoter used.

Different promoters used in the various approaches for inducing PTGS are constitutive (*e.g.* CaMV35S, ubiquitin, figwort mosaic virus(FMV), subterranean-clover stunt virus (S4S4)), seed or endosperm-specific (*e.g.*, napin, soybean lectin, patatin B33, wheat glutenin, P-B32) and embryo specific (*e.g* soybean β-conglycinin).

d) Gene sequences and constructs

Plant hairpin expression vectors require an inverted repeat of a fragment of the gene of interest behind an appropriate promoter, along with a plant-selectable marker, typically within the borders of a T-DNA binary vector for *Agrobacterium-mediated* transformation. A spacer fragment between the arms of the inverted repeat is useful for increasing the stability of the vector in *Escherichia coli,* and using a splicable intron as the spacer has been shown to dramatically increase the frequency of strong silencing phenotypes (Smith *et al., 2000).*

These features can be constructed from standard binary expression vectors; however, to simplify hairpin cloning, a variety of specifically designed hairpin expression vectors are available. Predominant considerations in vector selection are the choice of appropriate promoter sequences and selectable markers and consideration of the desired cloning strategy. For example, the 35S promoter is suitable for high-level constitutive expression in dicots, whereas the maize ubiquitin promoter is preferable for roughly equivalent expression in monocot species.

Wesley *et al.* (2001) described a pair of hairpin vectors for conventional cloning of PCR fragments into hairpin expression cassettes (pHANNIBAL and pKANNIBAL,

with bacterial ampicillin- and kanamycin-resistance marker genes respectively), and a third vector, pHELLSGATE, for high-throughput cloning using Gateway recombination, which was later refined by Helliwell *et al.* (2002) and Helliwell and Waterhouse (2003). All three vectors are designed for efficient production of intron-spliced hairpin RNAs.

The conventional hairpin cloning vectors pHANNIBAL and pKANNIBAL contain a 35S-driven inverted repeat cassette with restriction sites for directional cloning of fragments from the target gene on either side of a *PDK* intron. The gene of interest is PCR-amplified using PCR primers containing restriction sites matching those in the hairpin vectors, and cloned into the hairpin cassette, which is then cloned into a binary vector. The pHELLSGATE Gateway cloning vector series are binary vectors in which the T-DNA includes a plant-selectable marker and a hairpin cassette containing Gateway recombination sites for efficient directional insertion of gene fragments into the hairpin cassette.

pHELLSGATE was designed to facilitate the construction of large numbers of hairpin expression binary vectors, as the extremely efficient Gateway recombination system (Hartley *et al.,* 2000) replaces conventional cloning techniques. Gene fragments PCR amplified with appropriate sequence tags are recombined into a shuttle vector and then into pHELLSGATE. With two inserts being recombined on either side of an intron fragment, reversal of the intron sequence rendered half of clones non-splicable in early versions of pHELLSGATE (Helliwell *et al.,* 2002). To address this, pHELLSGATE12, the most recent version of the vector, contains two introns in opposite orientations, ensuring that all successful recombinations contain an intron in a splicable orientation (Helliwell and Waterhouse, 2003).

In addition to versions of pHANNIBAL and pHELLSGATE12, versions of these vectors suitable for use in monocot plants are now available from the Commonwealth Scientific and Industrial Research Organisation (CSIRO, Australia, see http://www.pi.csiro.au/rnai). These constructs carry the maize ubiquitin promoter in place of 35S. Vectors developed by other groups include pANDA, a binary vector utilizing Gateway recombination sites which expresses hairpins from the maize ubiquitin promoter (Miki and Shimamoto, 2004), and pKNOCKOUT, a conventional cloning vector with hairpin expression under control of the 35S promoter (Cazzonelli and Velten, 2004).

The region of the gene selected as the RNAi trigger depends on the objective of the RNAi experiment. If the aim is to down-regulate only the targeted gene and its homoeologous copies, but not other members of the gene family, it is important to exclude the conserved domains from the RNAi trigger to avoid silencing a large number of paralogous genes. Both coding sequence and untranslated regions may be effectively used in hairpin constructs (Helliwell and Waterhouse, 2003) and, with untranslated regions typically being poorly conserved even within highly related gene families, they can be particularly useful in designing gene-specific hairpin-silencing constructs (Miki *et al., 2005).*

However, if the objective is to silence all members of a particular multigene family, the conserved region would be preferred for the RNAi trigger in order to target multiple members of the family simultaneously. This was recently accomplished for seven members of the rice OsRac gene family (Miki *et al*. 2005) whose transcript levels were all simultaneously down-regulated using a single RNAi construct targeting a conserved region. These types of constructs are still difficult to design in wheat because its complete genome sequence is still not available.

In addition to sequence similarity considerations, as discussed above, several other factors can influence hairpin insert design. Published reports suggest that hairpin insert sizes of 50-1000nt can effectively induce silencing in stably transformed plants (Helliwell and Waterhouse, 2003). However, hairpin inserts at the shorter end of this range should be avoided, as they may result in weaker and less frequent silencing. Inserts of 300-600nt generally give reliably strong and frequent silencing and are very manageable to clone and manipulate.

e) Reducing off-target effects

In designing siRNA, one must also consider off-target effects that can occur. These include silencing of non-targeted genes or proteins by either siRNA or miRNA mechanisms, and interferon response. Though little is known about how to completely eliminate these effects, the field is rapidly emerging and there are some steps that can be taken to reduce these effects.

A major type of off-target effect is the silencing of unwanted genes by either an siRNA or an miRNA-type mechanism. The use of BLAST analysis can be used to

eliminate target sites that have identity to sequences within other genes. Imperfect matches are, however, more difficult to predict, although criteria for identifying sites of imperfect match are beginning to emerge. A web tool is now available for calculating the number of matches between the 'seed' region within target sequences and the 3' UTR (Birmingham *et al.*, 2006). Evaluation of these seed region matches may help to reduce off-target silencing.

An additional type of off-target effect is the activation of type I interferons. The evident solution to the problem is to avoid sequences that are known to be stimulatory.

f) Transformation protocols

Availability of a reliable and efficient transformation system is a prerequisite in any transgenic development programme. This assumes paramount importance with respect to transgenic silenced lines, as one has to ensure relative if not complete in the T_0 transgenic plants preferably carrying only one insert. This demands analyses of a large number of independent transformants to select the plants expressing the introduced gene in the required cell type at the effective level to ensure effective silencing. Therefore, it is essential that an efficient protocol for transformation be developed in the crop of interest to increase the probability of obtaining the desired trait in the transgenic plant.

2.3.3 Strategies for inducing PTGS in plants

In plants, PTGS has been induced either through the use of DNA construct encoding dsRNA or co-suppression constructs producing siRNA or by introducing DNA constructs which would lead to the production of anti sense RNA. One of the earlier classical examples of inactivation of a gene product by PTGS technology has been the reduction of polygalacturonase enzyme to delay the onset of ripening in tomatoes where the levels of the protein was reduced up to 99% (Gray *et al*., 1992) through the antisense technology. Similarly, by using antisense technology, fatty acid profile of oil seeds has been successfully altered through the silencing of the Δ9-desaturase (Knutzen *et al*., 1992) and Δ12-desaturase (Kinney 1996) genes. Later, PTGS has been induced in plants using other strategies like co-suppression of the endogenous gene by introducing sense copies of the target gene(s). However, the relatively low frequency of PTGS achieved with anti-sense and co-suppression requires that large populations of transgenic plants be produced in order to obtain an acceptable number of transgenic lines exhibiting sufficient degrees of target gene

suppression. This can present a major limitation, particularly in species that have low transformation and regeneration frequency.

To overcome these limitations, alternative strategies for gene silencing have been developed in plants. Gene constructs encoding intron-spliced RNA with a hairpin structure have been shown to induce PTGS with almost 100% efficiency when directed against viruses or endogenous genes (Smith *et al*., 2000). In these constructs, it has been shown that when a functional intron is cloned in between sense and antisense sequences of the target gene, it results in a stable hairpin-RNA after the splicing process and also results in silencing of the target gene in almost 100% of the transgenics obtained. When the functional intron, was replaced with any other spacer sequence or non-functional intron, then the efficiency of gene silencing was reduced significantly. So, in this elegant experiment, they have demonstrated that stable, dsRNA against a target gene results in highly efficient suppression or down regulation of the target gene.

In another study, Wesley *et al*. (2001) compared the efficiency of different types of constructs for gene silencing and have identified intron hairpin RNA strategy as the best for abolishing the gene activity. They have designed a generic vector, which could be used for gene silencing in any plant system. The authors have also opined that these vectors be used for silencing whole gene families if a common conserved region among the target genes is chosen for making hpRNA construct. Recently, it has been demonstrated that if a portion of the target gene is cloned upstream of an inverted repeat of 3' untranslated region (3' UTR) and subsequently introduced into the plant, a high efficiency gene silencing of the target gene is achieved (Brummell *et al*., 2003). This type of gene silencing called silencing by heterologous 3' untranslated regions (SHUTR) has the advantage of ease and rapidity in preparation of the constructs, since a gene of interest can be inserted into a binary vector already containing the promoter and the inverted repeat of the 3'-UTR, in a single cloning step, and does not require any knowledge of the DNA sequence. The authors have demonstrated the utility of this technique for silencing many genes and transcription factors.

2.4 Need for endosperm specific promoter from dicots

When transgene expression should be directed to certain tissues or time points during plant development, regulated promoters are required. A great number of tissue-specific genes have been characterized from many different plant species. Again specificity and amplitude of gene expression is controlled by an interplay of *cis*- and *trans*-acting elements. Promoter

elements conferring specificity for seeds, tubers, vegetative organs, and green tissues have been studied most extensively. Sequence elements controlling specificity are apparently quite conserved; therefore, such promoters can often be used in heterologous systems either as complete promoters or as isolated elements fused to an unspecific core promoter. However, even if specificity is conserved, the expression levels can vary drastically and, in many cases, particularly with dicot elements in monocots or vice versa, regulation will not work properly in a heterologous system. The use of a promoter from the recipient plant, therefore, may be preferable, although this may cause problems of gene silencing.

Despite these potential difficulties, a variety of such promoters have been used to specifically express transgenes in homologous or heterologous plants. Particular targets have been the storage tissues in seeds (e.g., using the napin gene promoter of *Brassica napus* or the 2S albumin gene promoter of *Arabidopsis thaliana*) and tubers (promoter of a potato patatin gene and the vegetative organs (tapetum-specific promoter from tobacco). For gene expression in monocot seeds, the promoters of a maize zein gene and a rice glutelin gene may prove useful.

Constitutive overexpression of transgenes that interfere with normal processes in a plant underscore the need for refinement of transgene expression. The development of tissue-specific promoters to drive transgene expression has helped fulfill that need. Targeted expression has become particularly important for the future development of value-added crops because the public may be more likely to accept ‘less intrusive’ expression of the transgene. For example, confinement of an insecticidal transgene product to tissue besieged by insect pests instead of harvestable material could have potentially defused the Starlink corn fiasco (Bucchini and Goldman, 2002).

The development of tissue-specific promoters for genetic engineering can be complex. The size of the promoter fragment isolated can have an effect on expression, as more distant cis-acting enhancer elements can be eliminated during promoter isolation. Potential trans-interactions that occur based on chromatin structure or location within the genome could also be lost by random integration of the transgene into the host genome. There may not be effective interaction of promoter cis-elements with the heterologous trans-acting factors present in the transgenic host plant species. Additional knowledge on the functionality of both homologous and heterologous promoters in target host plants is essential in efforts to improve the efficiency of transgene expression.

2.5 Tissue culture studies

Earlier studies on tissue culture in Euphorbiaceae including castor were mostly with endosperm cultures (Thomas and Chaturvedi, 2008). The interest in endosperm culture was mainly due to the large endospermic seeds that enabled easy culturability. The objectives were to obtain triploid plants and to understand the metabolism of glyoxylate pathway. Triploids will be useful for obtaining trisomic lines for genetic mapping. This can be accomplished through endosperm culture or through chromosome doubling (in vitro and in vivo) and crossing the resultant autotetraploids with diploids (Gmitter *et al*.,1990; Sikdar and Jolly,1994; Chaturvedi *et al*., 2003). Attempts to grow endosperm tissue in cultures began in the 1930's and immature and mature endosperms of various angiosperm taxa have been successfully cultured (Johri *et al*., 1980). La Rue (1944) had reported organogenic differentiation in endosperm cultures of castor bean. However, extensive investigations by subsequent workers on maize and castor bean could not confirm La Rue's claim of achieving organogenesis.

The ability of cells from mature endosperm to divide was first demonstrated by Mohan Ram and Satsangi (1963) followed by successful establishment of tissue cultures from mature endosperm (Satsangi and Mohan Ram, 1965). White's medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin, and yeast extract (YE) supported optimal growth of the endosperm tissues of castor (Srivastava,1971a; Johri and Srivastava, 1972). In castor, the ‘embryo factor’ was an absolute necessity and mature endosperm required the initial association of an embryo for its proliferation (Satsangi and Mohan Ram, 1965; Johri and Bhojwani, 1965). Organogenesis, however, has not been achieved in this crop. Studies madeby Brown *et al*. (1970) in *R.communis* indicated that the cultured endosperm tissue provides possibilities for detailed studies on enzyme changes that take place during culture but does not appear to provide suitable material for the detailed study of the capabilities of the endosperm *in vivo.*

In castor, callus initiation and plantlet regeneration from vegetative explants are restricted to young seedling tissues (Athma and Reddy, 1983; Sarvesh *et al*., 1992; Ganesh Kumari *et al*., 2008; Table 1). Callus can be initiated from the seedling explants but the ability to regenerate plants has been limited. Athma and Reddy (1983), assessed the differences in callusing ability and organogenic potential of the various seedling explants and obtained shoots from the shoot tips and rhizogenesis from root explants, shoot and leaf

tissues. Similarly, plant regeneration that has been reported to occur in the seedling explants was reported to involve pre-existing meristematic centers (Reddy *et al*., 1987b; Sangduen *et al*., 1987; Khumsub, 1988; Sujatha and Reddy, 1998; Sujatha and Sailaja, 2005; Malathi *et al*., 2006). Multiple shoot induction reported by Sangduen *et al*. (1987) and Khumsub (1988) was from embryo explants. Further, Reddy and Bahadur (1989b) reported the genotypic response for shoot tip multiplication in castor.

The shoot multiplication rates obtained from meristematic explants in most of these studies varied between 1 and 5.2 (Athma and Reddy, 1983; Sangduen *et al*., 1987; Reddy and Bahadur, 1989b; Molina and Schobert, 1995). A significant improvement of meristematic explant proliferation was the assessment of a wide range of cytokinins on different meristematic explants (Sujatha and Reddy, 1998). Shoot proliferation rates reported in this investigation were high and were 40 and 81.7 from the embryo axis and 46.7 and 22.0 from shoot tips on induction and subculture media, respectively. The study clearly revealed the differential effects of cytokinins in different meristematic explants and the carryover effect of thidiazuron (TDZ) in shoot proliferation cultures for 2–3 cycles. Meristematic explants responded differently with different types of cytokinins and TDZ gave the maximum number of shoots (37.8–40.0) from embryo axes, while benzyl adenine (BA) promoted highest number of shoots (46.7) from the shoot apex. Excessive proliferative ability of castor meristematic tissues on medium supplemented with TDZ has been successfully used for genetic transformation of castor (Sujatha and Sailaja, 2005; Malathi *et al*., 2006). Thidiazuron was found to promote direct shoot regeneration from hypocotyl segments (Ahn *et al*., 2007) and callus mediated regeneration from seedling leaf tissues (Ganesh Kumari *et al*., 2008).

Callus mediated regeneration is reported from hypocotyl sections (Reddy *et al*., 1987a), young stem segments (Genyu, 1988), young leaves (Reddy and Bahadur, 1989a) and epicotyl/ cotyledons (Sarvesh *et al*., 1992). However, differentiation of callus into shoots and shoot buds was reported to be either occasional or low. Reddy and Bahadur (1989a) reported 3–4 buds per leaf explant but the overall caulogenic response was not described. In studies by Sarvesh *et al*. (1992), 20% of the cultures with shoot buds induced on B5 (Gamborg, 1968) medium supplemented with 2.5 mg l− 1 BA, 0.1 mg l− 1 naphthaleneacetic acid (NAA) and 0.4 mg l−1 gibberellic acid (GA3), on transfer to the same medium produced 6–8 shoots per callus. Sujatha and Reddy (2007) assessed the morphogenic competence of castor tissues on several basal media supplemented with a large number of growth regulators individually and in combination and in a broad range experiment according to De Fossard *et al*. (1974) having

81 combinations at three levels of minerals, sucrose +growth factors+amino acids besides growth regulators, which revealed low caulogenic response of castor explants for direct as well as callus mediated shoot regeneration.

In most of the tissue culture studies using seedling explants of castor, Murashige and Skoog (MS) basal medium has been found to be ideal except in callus mediated regeneration from epicotyl/cotyledonary tissues (Sarvesh *et al*., 1992; Sujatha and Reddy, 2007; Ganesh Kumari *et al*., 2008) where B5 basal media or B5 vitamins proved to be superior. Multiple shoots could be proliferated when cytokinin was supplemented singly, such as BA at 0.5–2.0 mg l−1 (Athma and Reddy, 1983), 1.0−2.0 mg l−1 (Reddy *et al*., 1986), 4.0 mg l−1 (Sangduen *et al*., 1987; Khumsub, 1988), 0.2 mg l−1 TDZ or 4.0 mg l−1 BA (Ahn *et al*., 2007) or in combination with an auxin [1.0 mg l−1 BA+0.5 mg l−1 NAA or 0.5 mg l−1 kinetin+1.0 mg l−1 NAA] (Reddy *et al*., 1986), 2.0 mg l−1 BA+0.8 mg l−1 NAA (Ganesh Kumari *et al*., 2008). Callus cultures of *R. communis* are usually initiated in the presence of both an auxin and cytokinin. The cytokinins, kinetin (2.0 mg l−1) and BA (1.0, 2.0 or 4.0 mg l−1) with NAA (1.0mg l−1) or indoleacetic acid (IAA) (0.5 mg l−1) were most often used (Table 1). GA3 (0.2 mg l−1) is sometimes added to shoot regeneration medium although no requirement for GA3 has been demonstrated (Sarvesh *et al*., 1992) while it proved effective in shoot elongation (Sujatha and Reddy, 1998; Ganesh Kumari *et al*., 2008). Root formation has been initiated when regenerated shoots were transferred to medium supplemented with auxin (IBA, NAA) alone (Athma and Reddy,1983; Genyu,1988; Sujatha and Reddy,1998; Ahn *et al*., 2007), in combination with cytokinin (Reddy and Bahadur, 1989a,b), on the shoot proliferation medium itself (Sangduen *et al*., 1987) or with silver nitrate (Ganesh Kumari *et al*., 2008).

Despite research efforts over the last three decades, whole plants still could not be regenerated with reproducible frequencies from friable callus cultures of castor. The sporadic appearance of shoots from callus cultures of castor implies that the calli contain at least a few morphogenic cells interspersed in several non-morphogenic tissues. Failure to isolate a competent cell line might result in its suppression by the overgrowth of non-competent cells. Alternatively, the occasional appearance of shoots could be owing to the activation of recalcitrant calli to undergo caulogenesis caused by a rare inductive stimulus resulting from the interaction between exogenous and endogenous conditions. Recent investigations showed stimulatory effect of amino acids on organogenesis in castor (Sujatha and Reddy, 2007; Ganesh Kumari *et al*., 2008). Incorporation of 0.1% lysine monohydrochloride in B5 medium

supplemented with BA and indolebutyric acid (IBA) improved the morphogenic competence of castor tissues (Sujatha and Reddy, 2007). Ganesh Kumari *et al*. (2008) evaluated the influence of amino acids like alanine, glutamine, proline and serine and 15 mg l−1 glutamine showed the best response for multiple shoot proliferation and maximum number of shoots (22) per regenerating callus. The study of Ganesh Kumari *et al*. (2008) also revealed the beneficial effect of polyvinyl pyrrolidone (10–15 mg l−1) in controlling phenolic secretion.

Although there is considerable potential to improve the agronomic performance of castor using recombinant DNA and gene transfer techniques, the lack of a protocol for plant regeneration has restricted the development of transgenic cultivars. Until very efficient and reproducible system of plant regeneration is available, genetic transformation experiments would continue with meristem explants (Sujatha and Sailaja, 2005; Malathi *et al*., 2006). Recently, protocols for highly efficient protocols of shoot regeneration and somatic organogenesis have been reported (Ahn *et al*., 2007; Ganesh Kumari *et al*., 2008). The frequency of callus-mediated shoot regeneration reported by Ganesh Kumari *et al*. (2008) is high (85%) but shoots appear 16 weeks after culture establishment. As genetic transformation involves several manipulations for gene introduction followed by selection for 2–3 subculture cycles, the efficiency of these regeneration systems for genetic transformation of castor needs to be established.

2.6 Genetic transformation of castor

2.6.1 Production of transgenic castor resistant to biotic stresses

Transgenic research in castor has been undertaken for development of insect resistant and ricin free genotypes (Auld *et al*., 2001; Malathi *et al*., 2006). The other interest in castor is for production of epoxy oil which offers all the advantages of a premium oil based paint without the liberation of volatile pollutants (Mc Keon and Chen, 2001). The chemical structure of epoxy oil is very similar to that of castor oil and only minor modifications are needed to cue the castor plant to make epoxy oil instead of castor oil. Castor plant also has the ability to produce industrially useful fatty acids (Ogunniyi, 2006).

Recalcitrance in vitro has been a major problem for undertaking plant transformation experiments in castor. Mc Keon and Chen (2003) obtained genetically engineered plants by employing the method of *Agrobacterium*-mediated transformation through vacuum

infiltration of wounded flower buds (US Patent No 6.620.986). The first successful attempt to develop a stable transformation system for castor using vegetative explants has been described by Sujatha and Sailaja (2005). In this protocol, co-cultivated explants were initially subjected to expansion and proliferation on Murashige and Skoog (MS) medium with 0.5 mg l−1 thidiazuron followed by 3 cycles of selection on medium with 0.5 mg−l BA and increasing concentrations of hygromycin (20–40–60 mg l− 1). Selected shoot clusters were transferred to medium with 0.5 mg−ll BA for proliferation and 0.2 mg l−1 BA for shoot elongation. Elongated shoots were rooted on half-strength MS medium with 2.0 mg-1l NAA. With this protocol, primary transformants could be developed within 5 months from cultured embryo axes with an overall transformation efficiency of 0.08% (Fig. 2). As the protocol does not involve an intervening callus phase, no abnormal phenotypes are expected through this procedure. This protocol has been extensively used for development of transgenic lines of castor resistant to major foliage feeders through deployment of Cry1Ec, Cry1Aa and Cry1Ab genes (Sujatha and Sailaja, 2007). The same procedure with minor modifications was used for production of semilooper resistant transgenic castor by incorporating Cry1Ab gene (Malathi *et al*., 2006). The transformation protocol, in this study, has been optimized with constructs harbouring hpt gene as selectable marker, while the construct harbouring insect resistance gene carried the herbicide resistance gene (bar) for selection of putative transformants. The major biotic threats to castor cultivation in India are capsule borer and botrytis grey rot. Screening procedures are in place for botrytis grey rot but suitable candidate genes have to be identified against this pathogen. For capsule borer, the major challenge lies in optimizing a rearing technique prior to testing effective agents (Lakshminarayana and Raoof, 2005).

2.6.2 Ricin-free castor

The major toxic compound in the seed meal of castor is ricin, an alkaloid ricinine and an allergenic protein polysaccharide CB-1A (Weiss, 2000; Audi *et al*., 2005). This makes the high protein meal to be used as fertilizer or is incinerated (Ogunniyi, 2006; Gressel, 2008). Ricin, a lethal toxalbumin, inhibits protein synthesis by inactivating ribosomes and is deadly when inhaled, ingested or injected. The major objective of castor genetic transformation in USA is to develop ricin free castor plant varieties with the potential to create new economic opportunities for farmers and processors. The problem is tackled through a multiple approach. Through selection and traditional breeding methods, advanced generation lines

(F6) with 70–75% reduction in ricin and *Ricinus communis* agglutinin (RCA120) toxins have been developed (Auld *et al*., 2001, 2003). The second strategy is to develop transgenics using antisense genes for blocking ricin and allergen production that are highly expressed during seed development. The ricin gene is isolated and sequenced, and using mRNA expression of ricin, allergen and numerous lipid biosynthetic enzymes during seed development, promoters useful in expression of genes to suppress toxin and allergen production were identified. However, ricin and *Ricinus communis* agglutinin production are controlled by multiple genes and hence, transgenic strategies to completely eliminate the toxic components is necessary. Efforts are focused upon "knocking out" the genes responsible for ricin production as well as genes responsible for the production of ricinine and CB-1A (Auld *et al*., 2001). Using classical mutagenesis techniques, random mutations could be introduced into the castor genome and screened to determine the mutations that have the ricin genes knocked out. The screening is accomplished through Targeting Induced Local Lesions In Genomes (TILLING) technology for identification of single nucleotide polymorphisms and the desired mutants are crossed with desired castor germplasm (www.arcadiabio.com). Ricin-free castor meal will certainly have significant value for the production of animal feeds and rations.

Table 2.1 : Morphogenic response from cultured tissues of castor (*R. communis* L.)

Explants	**Type of Morphogenic response**	**Medium used (mg l^{-1})**	**Frequency of morphogenesis**	**Reference**
Endosperm	Regeneration of roots	–	–	La Rue, 1944
Mature seed	Proliferation of endosperm	2, 4-D or Kn	–	Mohan Ram and Satsangi, 1963
Decoated mature seed	Proliferation of endosperm and establishment of tissue cultures; differentiation of tracheidal cells in callus	2,4-D+Kn+YE	–	Satsangi and Mohan Ram, 1965
Endosperm from germinated seed	Proliferation of endosperm (endosperm from dried seeds	2,4-D+Kn+YE	Callus −82%	Brown *et al*., 1970

	failed to grow)			
Fresh decoated seed	No organogenesis	2,4-D+Kn+YE	–	Srivastava, 1971a
Fresh decoated seed	Proliferation of endosperm producing callus that could be continuously subcultured	2,4-D+Kn+YE	Callus −56%	Johri and Srivastava, 1972
Shoot, cotyledon, hypocotyl, root, endosperm, embryo	Only callus except endosperm. Multiple shoots from embryo	BA 4.0	–	Khumsub, 1988
Cell suspensions	Nutrient transport and uptake	—	—	Cho and Choi, 1990
Cotyledonary callus cultures	Xylogenesis	NAA 2.0+BA 0.5	–	Bahadur *et al*., 1991
Epicotyl and cotyledonary explants	Multiple shoots/roots	BA 2.5+NAA 0.1 - GA3 0.2 BA 2.0+NAA 1.0	96.5% callus with shoot buds	Sarvesh *et al*., 1992
Root, shoot and cotyledonary leaf	Shoots from pre-existing meristems, roots	BA 0.5-2.0 NAA 0.5 - roots	Callus - 90-98% Shoot regeneration - 25-30% with 1 shoot per explant	Athma and Reddy, 1983
Young stem	Bud differentiation	NAA 1.0+BA 1.0 or IAA 0.5+ BA 2.0 or 4.0 – shoot buds NAA 0.5 – roots	–	Genyu, 1988
Seedling explants	Shoots from pre-existing Meristems	BA 2.0	Shoot proliferation - 25-30%	Athma and Reddy, 1989
Leaf	Adventitious buds	Kn 2.0+IAA	–	Reddy and Bahadur,

		1.0		1989a
Shoot apex	Multiple shoots	Kn 2.0+IBA 1.0	79.1% with a mean of 5.2 shoots	Reddy and Bahadur, 1989b
Hypocotyl, leaf and shoot tips	Shoot tip proliferation	BA 1.0 or 2.0, NAA 1.0+ Kn 0.5; NAA 0.5+BA 1.0	–	Reddy *et al*., 1986
Shoot tip callus	Shoot buds	Kn 2.0+NAA 1.0	–	Reddy *et al*., 1987b
Seed and seedling explants	Shoot bud proliferation	BA 4.0	–	Sangduen *et al*., 1987
Shoot tip	Multiple shoots	BA 0.25	79% with 4.4 shoots per explant	Molina and Schobert, 1995
Embryo axis and shoot tips	Shoot proliferation	TDZ 0.5-10.0	100% shoot proliferation with 81.7 shoots from embryo axis and 22.0 shoots from shoot tips	Sujatha and Reddy, 1998
Hypocotyl from zygotic embryo axis	Adventitious shoots	TDZ 0.25 or BA 4.5	24.2 shoots per explant	Ahn *et al*., 2007
Hypocotyl	Adventitious shoots	BA 2.0+IBA 0.5-1.0+0.1% lysine monohydrochloride	22.3-25.0% shoot bud induction	Sujatha and Reddy, 2007
Seedling explants – cotyledons, hypocotyls, epicotyl, leaf	Callus mediated organogenesis	BA 2.0+NAA 0.8 – callus TDZ 2.5+NAA 0.4+glutamine 15	85.0% callus induction with a shoot mean of 22 shoots per callus	Ganesh Kumari *et al*., 2008

2.6 Analysis of silencing transformants

Hairpin silencing constructs following the guidelines described will usually yield populations of transformants showing a high frequency of strong silencing. Selection of 10-20 independent transformants is suggested as a starting point for likely identification of several strongly silenced lines. However, an important point to note is that the efficiency and frequency of silencing varies considerably between targets. This probably reflects the impact that a number of variables have on gene silencing, including how well target and hairpin expression coincide, and how far the target transcript level must be knocked down for a phenotype to result (reflecting whether the transcript is normally over-abundant or is present at a rate-limiting amount in its relevant biological pathway).

The extent of silencing can be monitored in a variety of ways, with phenotypic observation being of primary interest. Importantly, indications that phenotypes observed are dependent upon target knockdown include the observation of phenotypes is common between multiple independent transformants (perhaps of varying severity), and that phenotypes co-segregate with the hairpin T-DNA. At the molecular level, target transcript levels can be monitored by quantitative real-time PCR (qRT-PCR) or Northern blots, and the production of siRNAs from the hairpin can be monitored with small RNA blots. siRNA levels provide a good indication of how effectively a hairpin construct is expressed and processed; however, the correlation between siRNA level and mRNA knockdown is known to be imperfect for a variety of hairpin-targeted genes.

Finally, quantitative measurements of phenotypes may be an appropriate way of analysing the effect of hairpins upon gene activity and comparing hairpin effectiveness in independent transformants. For example, silencing of *CHS* has a direct effect upon anthocyanin levels, which can be measured by anthocyanin extraction from plant tissues followed by spectrophotometric quantification (Dunoyer *et at.,* 2004). Silencing of the flowering suppressor *flowering tocus* C *(FLC)* can be measured by monitoring the number of days to flower from germination, as this is proportional to the activity of *FLC* (Wesley *et at.,* 2001). To measure the effectiveness of hairpins targeted to viral sequences, viral titre in virus-inoculated hairpin transformants can be measured by ELISA (Fusaro *et at.,* 2006). Such assays can be particularly useful in determining how consistent a phenotype is by measuring a population of plants derived from a single transformant.

CHAPTER III

MATERIALS AND METHODS

The goal of the present study was to develop transgenic castor with reduced or nil levels of ricin and RCA using different PTGS (post transcriptional gene silencing) approaches. Towards the realization of this goal, constructs have been developed on three different canonical principles to selectively target degradation of ricin and RCA transcripts in castor. The constructs were initially developed under CaMV35S promoter and later under ricin full-length promoter (FP). As a prelude to this, ricin promoters were isolated from castor using inverse PCR (IPCR). Castor was transformed with two constructs, LBA4404:: pCAMBIA 1300 ricin FP ihp RA and LBA4404:: pCAMBIA 1300 ricin FP ihp RA RB. Putative transgenic castor plants have been obtained only with LBA4404:: pCAMBIA 1300 ricin FP ihp RB. The materials used and the methods followed to accomplish the present investigation are briefed here.

3.1 Materials

3.1.1 Plant Material

Castor (*Ricinus communis* L.) cultivar DCS-9 (Jyoti) from the Directorate of Oilseeds Research (DOR), Hyderabad and tobacco cultivar *Nicotiana tabacum* L. Xanthi cv. Petit Havana SR were used in the present study.

3.1.2 Equipments/Instruments

Equipment	**Company/Suppliers**
Autoclave	Sanyo, Lab-tech
Bio-imaging system	Syngene
Biolistic PDS-1000/He Particle Delivery System	Biorad
Centrifuges	Heraeus, Sorvall
Electrophoresis units	Biorad, Consort
Electroporator	Eppendorf
Freezer (-20 ºC)	Sanyo
Hot air oven	JSR
Incubators	JSR
Laminar air flows	Klenzaids, Klenz Flo
Micro oven	Kenstar, LG, Bajaj
Microscopes	Leitz/Leica
Milli Q unit	Millipore
pH meter	Eutech
Pipetman	Eppendorf
Water bath or Incubator	Julabo
Spectrophotometer	Genway

UTL freezer (-80 ºC)	Thermo Electron Corporation
Vacuum pump	Millipore
Vortex mixer	Genei
Water bath	Haake

3.1.3 Media

3.1.3.1 Plant tissue culture

Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) was used as the basal medium for tissue culture and transformation of safflower as well as tobacco. For preparation of medium, either the readymade media devoid of hormones (Himedia, India) or stock solutions of component chemicals were made and used for making required working medium (by mixing them in required proportions in distilled water to get the desired concentrations). The composition of MS medium is given in Table 3.1. The basal MS medium with full strength or half strength of the components with suitable hormones, antibiotics/selection agents were used during different stages of transformation and regeneration.

3.1.3.2 Culture and maintenance of bacterial clones and strains

Luria Bertani (LB) liquid medium and solidified medium (LA) were prepared from the products supplied by Himedia Company and used for culture of *E. coli* strains. Teriffic Broth (TB) containing bacto-tryptone 1.2%, bacto-yeast extract 2.4%, glycerol 0.4%, potassium dihydrogen orthophosphate 0.231%, potassium hydrogen phosphate dibasic 1.25%, TA (TB medium supplemented with 0.8-1.5% agar) media as well as YEP [0.5% yeast extract, 0.5% bacteriological peptone, 0.3% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, 2% glucose] were used for culturing as well as maintaining *Agrobacterium* strains. Keto-Lactose medium (Lactose 1%, yeast extract 0.1% and bacto agar 2%) was also used to reconfirm the *Agrobacterium* clones.

3.1.4 Hormones

The details of the hormones used in the present investigation are given in Table 3.2.

3.1.5 Antibiotics / Herbicide

The details of the antibiotics used in the present investigation are given in Table 3.3.

Table 3.1 Composition of MS medium

Stock	Constituents	Actual conc. (mg l^{-1}) × magnificant	Vol. of stock solution (ml)	Vol. of stock solution used (ml/l)
A	**Macronutrients**			
	NH_4NO_3	1650 × 10	500	50
	KNO_3	1900 × 10		
	$MgSO_4.7H_2O$	370 × 10		
	KH_2PO_4	170 × 10		
B	**Calcium Chloride**		100	10
	$CaCl_2.2H_2O$	440 × 10		
C	**Micro-nutrients**			
	KI	0.83 × 50	100	2
	H_3BO_3	6.20 × 50		
	$MnSO_4.4H_2O$	22.3 × 50		
	$ZnSO_4.7H_2O$	8.6 × 50		
	$Na_2MoO_4.2H_2O$	0.25 × 50		
	$CuSO_4.5H_2O$	0.025 × 50		
	$CoCl_2.6H_2O$	0.025 × 50		
D	**Iron-EDTA**		100	10
	$Na_2EDTA.2H_2O$	37.3 × 10		
	$FeSO_4.7H_2O$	27.8 × 10		
E	**Vitamins**		100	1
	Glycine	2.0 × 100		
	Nicotinic Acid	0.5 × 100		
	Pyridoxine-HCl	0.5 × 100		
	Thiamine-HCl	0.1 × 100		
F	**Myo-Inositol**		100	10
	Myo-Inositol	100 × 10		
	Sucrose	30,000	Added directly	Added directly
	Agar	7,000		
	pH	5.8		

Table 3.2 Details of the hormones used for tissue culture

Hormone	Purpose	Working concentration	Stock	Supplier
Benzyl amino purine (BAP)	For shoot multiplication and elongation	0.2-0.5 mg /l	1 mg/ml	Sigma
Naphthalene acetic acid (NAA)	For shoot induction and root induction	0.2 mg /l	1 mg/ml	Sigma
Thidiazuron (TDZ)	For shoot induction	0.2 mg /l	1 mg/ml	Sigma
Kinetin	For elongation	1-2 mg /l	1 mg/ml	Sigma
Indole-3 butyric acid (IBA)	For root induction	1-2 mg /l	1 mg/ml	Sigma
Phloroglucinol	For root induction	1-2 µg/ml	1 mg/ml	Sigma

Table 3.3 Details of the antibiotics/herbicide used for tissue culture

Antibiotic/ hormone/ herbicide	Purpose	Working concentration (mg/ml)	Stock (mg/ml)	Supplier
Ampicillin	For selection of ampicillin resistant *E. coli* strains	100	100	USB
Kanamycin	For selection of binary vector	50	50	Duchefa
Streptomycin	Marker for helper plasmid of *Agrobacterium*	25	25	Synbiotics
Rifampicin	Marker for chromosome of *Agrobacterium*	25	25	Duchefa
Hygromycin	Transgenic plant selection	5-20	20	Duchefa
Cefotaxime	Elimination of *Agrobacterium*	250	250	Alkem
Phosphinothricin	Transgenic plant selection	0.5-1.0	10	Duchefa

3.1.6 Chemicals and consumables

The chemicals used in the present study were procured from Amersham Biosciences, Bangalore Genei, Duchefa, Genetix, Himedia Chemicals, InVitrogen, Life Technologies, Fermentas, Promega Life Science, Sigma, SD Fine Chemicals, USB, Qiagen, Qualigens fine chemicals. Consumables like glassware, plastic ware, scalpel blades, forceps, scalpels, filter papers, pipette tips, eppendorf tubes, etc., were obtained from Axygen, Borosil, Tarsons, etc. Other standard solutions, buffers and reagents were prepared according to the procedures given by Sambrook and Russel (2001).

3.1.7 Vectors and Strains

Salient features of the commercial vectors used in the present study are given in Table 3.4. The physical maps and the features of T/A, pRT100, pCAMBIA1305.2, pCAMBIA2300, pCAMBIA1300 and pCAMBIA1391Z vectors are shown in Figures 3.1, 3.2, 3.3, 3.4, 3.5 and 3.6 respectively. Standard bacterial strain DH5α of *Escherichia coli* was used for maintenance of all the recombinant plasmids. The developed recombinant vectors were mobilized into *Agrobacterium tumefaciens* strain LBA4404, which was further used for plant transformation.

Table 3.4 Salient features of the commercial vectors used

Plasmid	Size (bp)	Multiple cloning site (MCS)	Bacterial selection marker	Blue/ white selection (*lac*Z)	Purpose of use
pTZ57R T/A	2886	*Eco*RI,*Sac*I,*Kpn*I, *Bsp*681, *Mph*I, *Xba*I, *Bam*HI, *Sma*I, *Sma*I, *Apa*I, *Sal*I, *Eco*I, *Pae*AI, *Hin*dIII	Ampicillin	Present	Initial cloning of catalase intron, polyA, RA and RB chains, truncated and full-length ricin promoters and ricin full-length gene
pRT100	3340	*Xho*I, A*pa*I, *Nco*I, *Sst*I, *Kpn*I, *Sma*I, *Bam*HI, *Xba*I	Ampicillin	Absent	Preliminary development of all PTGS constructs
pCAMBIA1305.2	11921	*Eco*RI, *Sac*I, *Kpn*I, *Sma*I, *Bam*HI, *Xba*I, *Sal*I, *Pst*I, *Sph*I, *Hind*III	Kanamycin	Absent	As a source of isolation of catalase I intron
pCAMBIA1391Z	11214	*Xmn*I, *Bst*XI, *Hind*III, *Pst*I, *Sal*I, *Bam*HI, *Sma*I, *Eco*RI, *Nco*I, *Bal*II, *Spe*I, *Nhe*I, *Pml*I, *B*stEII	Kanamycin	Absent	For cloning the gene cassettes developed for characterization of ricin promoters
pCAMBIA 2300	8742	*Eco*RI, *Sac*I, *Kpn*I, *Sma*I, *Bam*HI, *Xba*I, *Sal*I,	Kanamycin	Present	For cloning the gene cassettes developed for

		*Pst*I, *Sph*I, *Hin*dIII			expression of ricin under ricin promoters
pCAMBIA 1300	8958	*Eco*RI, *Sac*I, *Kpn*I, *Sma*I,*Bam*HI, *Xba*I, *Sal*I, *Pst*I, *Sph*I, *Hin*dIII	Kanamycin	Present	For cloning the gene cassettes developed for construction of PTGS constructs

3.2 Methods

3.2.1 Primer designing

In the present investigation several primer sets were designed and used for the present investigation. Some primers available already at DOR were also used.

The primers were designed with appropriate restriction enzyme sites. While designing primers (not only for IPCR, but for other clonings), care was taken to add only those RES (restriction enzyme sites), which were not present in the promoter(s)/gene(s) sequence(s) but present in the multiple cloning site (MCS) of the binary vector employed for cloning. All the primers used in the present study along with their details are given in Table 3.5. The T_m values of primers were calculated using the formula as given by Sambrook and Russell (2001).

$$T_m = 2\ (A+T) + 4\ (G+C)$$

A set of three nested primers was designed to effect the nested PCR reactions of the inverse PCR. These include 5R(EspR1), 6R(EspR2) and 7R(EspR3). 5R starts just one base before ATG codon of the ricin gene. These three primers span a region of 310 bases and are located atleast 100 bases from each other. Based on the sequence information of the clones obtained in the inverse PCR, primers were designed to amplify the TP and FP and clone the same in appropriate vectors to study their tissue-specificity.

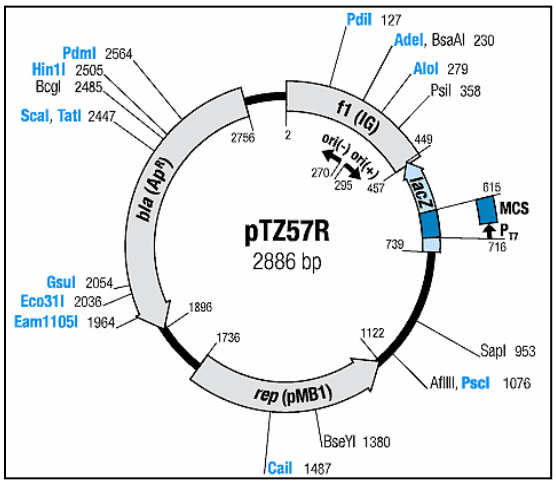


Fig: 3.1 Schematic diagram of T/A cloning vector (MBI Fermentas)

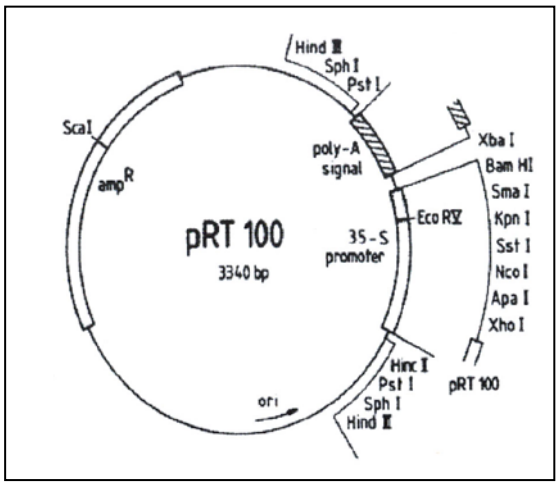


Fig: 3.2 Schematic diagram of pRT100 vector

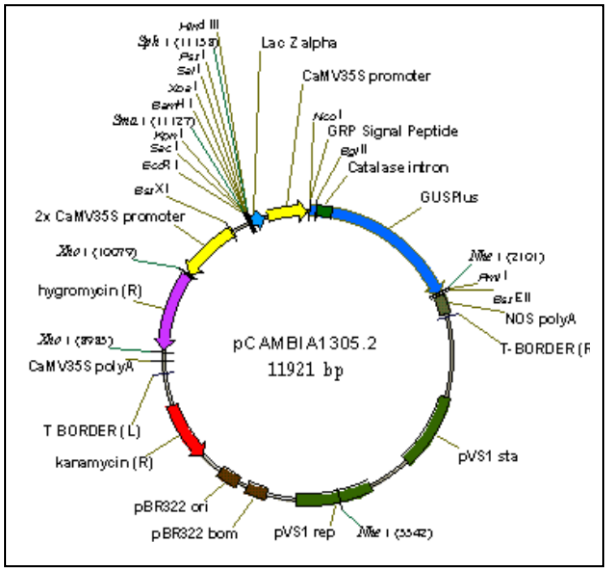


Fig: 3.3 Schematic diagram of pCAMBIA1305.2 vector

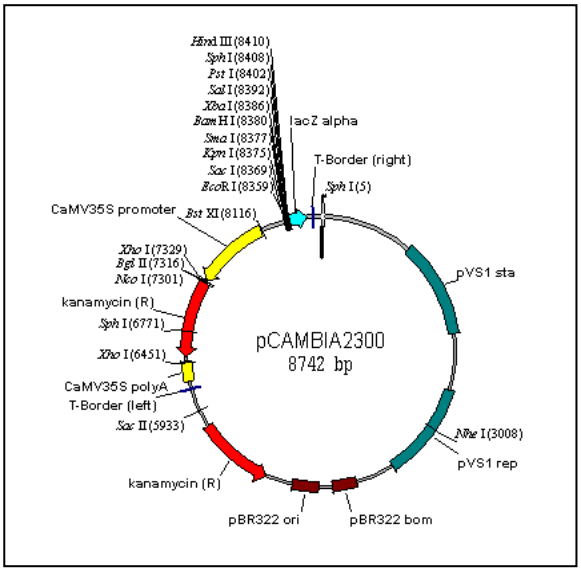


Fig: 3.4 Schematic diagram of pCAMBIA2300 vector

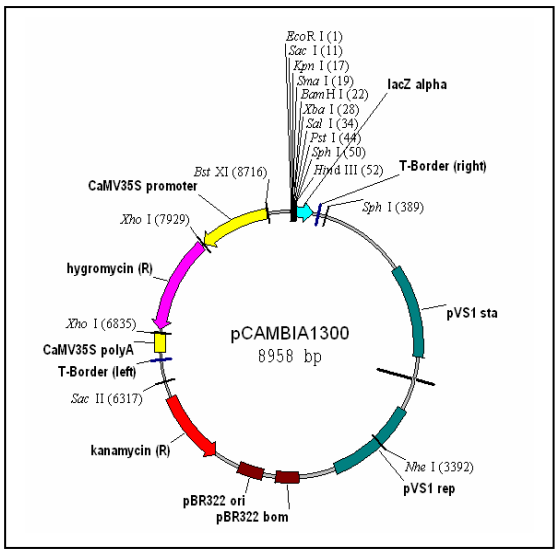


Fig: 3.5 Schematic diagram of pCAMBIA1300 vector

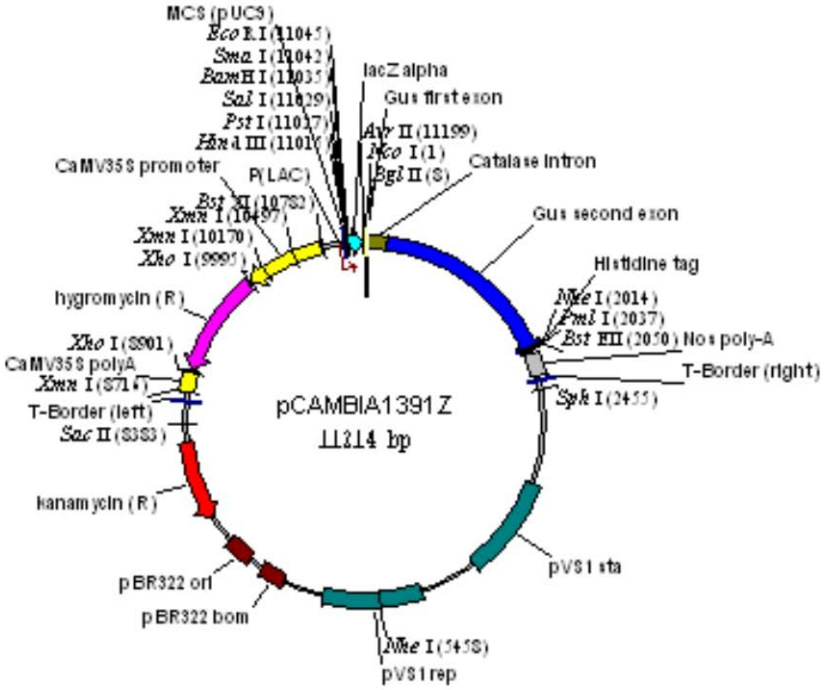


Fig: 3.6 Schematic diagram of pCAMBIA1391Z vector

Table 3.5 Sequence of the primers used, their melting temperature, purpose for which they were used and the PCR product size realized

Primer	Sequence	Tm (^{0}C)	Purpose of primers	Size of PCR product (bp)
SstI ricin Forward	5'TA gagctc atg aaa ccg gga gga aat act att g 3'	70	For amplification of full-length ricin sequence(for antisense construct)	1300
XhoI XbaI ricin Reverse	5'AT ctcgag tctaga atc tgt cta tca aaa ata atg gta acc 3'	68		
*Nco*I *BamHI* Ricin A chain Forward	5' AT cca tgg gga tcc ccc aat tat aaa ctt tac cac ag 3'	62	For amplification of selected portion of ricin/RCA A chain.	357
*Xba*I *Xho*I Ricin A chain Reverse	5' AT tct aga ctc gag cta tca taa tta cct cca aag g 3'	60		
*Nco*I *BamHI* Ricin B chain Forward	5' AT cca tgg gga tcc gtg caa acc aac att tat gcc 3'	60	For amplification of selected portion of ricin/RCA B chain.	307
*Xba*I *Xho*I Ricin B chain Reverse	5' AT tct aga ctc gag cca tca ttc ttg aac ttc cat c 3'	60		
SstI Catalase Intron Forward	5' A gag ctc agg gta aat ttc tag ttt ttc tc 3'	60	For amplification of catalase intron from binary vector pCAMBIA1305.2 and also from castor genome.	210
KpnI Catalase Intron Reverse	5' AT ggt acc gtt ctg taa cta tca tca tca tc 3'	62		
SstI PolyA Forward	5' A gag ctc gtc cgc aaa tca cca gtc tc 3'	60	For amplification of PolyA sequence.	220
NcoI PolyA Reverse	5' AT cca tgg gtc act gga ttt tgg ttt tag 3'	58		
ApaI ricin Forward (complete coding sequence)	5' gggccc **aac aat ggc** atg aaa ccg gga gga aat 3'	52	For amplification of full length coding ricin gene.	1732
SstI ricin Reverse (complete coding sequence)	5' gagctc tca aaa taa tgg taa cca tat ttg 3'	60		
*pRT Sequencing primer	5'cct tcc tct ata taa gga agt tc 3'	64	For confirmation of the clones	-
Ricin Sequencing Primer	5' ata tcg agt tgg gaa atg gtc 3'	64	For confirmation of the clones.	
5R(EspR1)	5' ctt tga ttg cag caa ttc tgg 3'	60	For isolation of endosperm specific ricin promoter	-
6R(EspR2)	5' taa aga aaa cca caa ata ctt g 3'	56	For isolation of endosperm specific ricin promoter	-

7R(EspR3)	5’ ata atg tcg att aga gta att tac 3’	60	For isolation of endosperm specific ricin promoter	-
RFLP(ricin full-length promoter) Forward	5’ ctg cag tca ac gat ctt ctt ttt ctt ttc ttt	52	For cloning full-length ricin promoter	932
RFLP(ricin full-length promoter) Reverse	5’ gtc gac tcg agc ttt gat tgc agc aat tct tg 3’	60		
RTP(ricin truncated promoter) Forward	5’ ctg cag tca ac cct aag aaa ggt aaa tta ctc 3’	56	For cloning truncated ricin promoter	310
*hpt*II Forward Primer	5’ caa aat ccc act atc ctt cgc 3’	62	For confirmation of the hygromycin phosphotransferase II gene conferring hygromycin resistance in transgenic plants	1094
*hpt*II Reverse Primer	5’ gca gtt cgg ttt cag gca ggt 3’	66		
*npt*II Forward Primer	5’ gag gct att cgg cta tga ctg 3’	64	For confirmation of the neomycin phosphotransferase II gene conferring kanamycin resistance in transgenic plants	700
*npt*II Reverse Primer	5’ atc ggg agc ggc gat acc gta 3’	68		
*uid*A Forward Primer	5’ ggt ggg aaa gcg cgt tac aag 3’	62	For confirmation of *gus* gene in transgenic plants	1200
*uid*A Reverse Primer	5’ ggt tac gcg ttg ctt ccg cca 3’	68		

Note:

- The restriction enzyme sites added ′at 5 end of primers are mentioned in the nomenclature.
- Restriction enzyme sites in the primer sequence are underlined.
- T_m was calculated excluding restriction enzyme sites
- **aac aat ggc** - consensus sequence for plant ribosome binding site (RBS)

3.2.2 Inverse PCR

1 μg of high molecular weight castor genomic DNA was digested with *Bgl*II and *Bam*HI in separate tubes in a total volume of 25μL. The reaction was incubated at 37^0C for 3 hrs. A 2μL aliquot was taken from the total reaction volume and loaded to check for smearing and ensuring complete digestion. To the remaining 23μL of the digested reaction, 20μL of 3M potassium acetate(pH 5.5) and the reaction volume was made up to 160 μL with $T_{10}E_1$ (Tris10 mM ; EDTA1mM). This was to minimize the loss of the digested DNA. The restriction enzyme was inactivated by extraction with phenol-chloroform followed by chloroform extraction twice. The reaction was precipitated with 100% alcohol and washed with 75% alcohol. The pellet in reaction tubes were then dried and later dissolved in 88μL of MQ water. A 100 μL self-ligation reaction consisting of approximately 0.2 μg(contained in 88μL of MQ water) of ethanol-precipitated digested DNA,10μL of 10X ligation buffer and 2μL of T4 DNA ligase (5U/μL) was worked out and allowed to self-ligate at 16^0C for overnight. Following overnight ligation, a 10μL aliquot was removed from each tube and added directly to a 100μL PCR reaction containing the primers (i.e. 3F and 6R in case of *Bgl*II and 2F and 6R in case of *Bam*HI digested DNA). A nested PCR was set up with diluted PCR product (2:50 dilution) of the previous reaction with 4F and 7R in case of *Bgl*II and 2F and 7R in case of *Bam*HI digested DNA. Controls for the PCR reaction included 4F alone and 7R alone (in case of *Bgl*II) and 2F alone and 7R alone (in case of *Bam*HI). The resultant amplicons were then eluted from the gel using QIAGEN gel extraction kit and cloned in InsT/A vector. The clones were then subjected to restriction with *Eco*RI and *Hind*III to confirm the cloning. The clones were sequenced with M13 Forward and M13 Reverse primers and then analysed.

3.2.3 Plasmid DNA isolation

Plasmid DNA of required plasmid/vectors was isolated from 5 ml of overnight grown culture. The bacterial cells were spun down at 12000 g for 1 min. The pellet was resuspended in 100 μl sterile double distilled water. 100 μl of lysis buffer (1% SDS, 10 mM EDTA, 0.1 N NaOH of final concentration) was added and boiled at 85-100^oC for 2 min. This was followed by addition of 50 μl of 1.0 M $MgCl_2$ and incubation on ice for 2 min and centrifugation at 12000 g for 2 min. To the same tubes, 50 μl of 5 M potassium acetate (pH 5.2) was added, mixed by tapping and left on ice for 2 min and spun at 12000 g for 2 min.

The supernatant was transferred to fresh tubes and the DNA was precipitated with absolute ethanol and pelleted by centrifugation. The pellet was washed with 70% ethanol, air dried and dissolved in 50 µl $T_{10}E_1$ buffer. For sequencing purpose, the plasmid DNA from selected clones was isolated using QIAprep® Spin Miniprep Kit (Qiagen) as per the suppliers' protocol.

3.2.4 PCR amplification

During development of constructs, whenever the inserts were isolated by PCR, a preparative PCR (10 or 20 µl reaction volume) was carried out with primers specific to each of the components with the respective DNA or RNA. Once the expected size fragment was obtained a large volume PCR (60 or 80 µl reaction volume) was carried out with the same PCR conditions. The PCR reaction mixture consisted of 6-10 ng of DNA, 0.2 µM each of the forward and reverse primers, 100 µM of each dNTP, 1x concentration of *Taq* DNA polymerase buffer (10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin), 1 unit of *Taq* DNA polymerase and milliQ water to a total volume of 60 µl. The product thus generated was used for cloning. The clones generated were confirmed by PCR and/or restriction digestion. Confirmation of the transgenic plants was done using PCR. Appropriate primers for confirming the transgene constructs were utilized in the PCR reaction. The general PCR program utilized for this study is given below. Only the annealing temperature (based on T_m of the primers) and/or the extension time varied (according to the length of the amplicons). The cycling parameters adopted for PCR were:

Initial denaturation - 94^0C for 4 min

Denaturation - 92^0C for 25 sec

Annealing - 56-60^0C for 30 sec

Extension for - 45 sec- 2 min

Final extension - 72^0C for 7 min

3.2.5 Restriction analysis

Restriction analysis was used to digest inserts (from vectors) and also vectors (to linearize them) for cloning and also for confirmation of the developed clones (whether the expected product size is seen on digestion). As a general rule, 1 µg DNA was used. The reaction mixture consisted of 5-10 units of enzyme, 1x final concentration of appropriate buffer, BSA

to a final concentration of 100 µg/ml and sterile water. For cloning purpose, a large volume of restriction digestion was set up (60 or 80 µl) so as to get enough digested vector and insert concentrations for efficient ligation.

3.2.6 Quantification of DNA

Quantification of genomic DNA, plasmid DNA, *etc* was done by running them on 0.8% agarose gels. Approximate DNA concentration was determined by visual comparison against a known amount of standard molecular weight marker (λ DNA/50 bp ladder/100 bp ladder/ λ DNA *Eco*RI + *Hin*dIII double digest).

3.2.7 Cloning

The general procedure followed for cloning is outlined below.

3.2.7.1 Preparation of vector and insert

The vectors as well as insert (to be cloned), were first isolated by setting up large volume PCR or restriction with appropriate enzymes (as mentioned earlier). The products were resolved on 0.8 % agarose gel and the desired fragments were identified on the gel using standard marker (50 bp ladder or 100 bp ladder or λ DNA *Hin*dIII + *Eco*RI double digest). The agarose gel slabs containing the required fragments (cut vector as well as insert) were excised out from the gel using a sharp sterile scalpel blade. DNA was eluted from the excised gel slabs using Qiagen gel elution kit as per manufacturers' instructions. For each gel slab, three volumes of QG buffer was added to 1 volume of gel and incubated at 50°C for 10 min (by vortexing intermittently). After bringing it to room temperature, one volume of isopropanol was added (optional) and the whole mix was transferred to the supplied column. After centrifugation, the flow through was discarded and the column was washed twice with PE buffer. The DNA from the column was collected by adding 25-30 µl of elution buffer. The vector and insert DNA thus prepared were quantified by visual examination on 0.8% agarose gel.

3.2.7.2 Ligation

For all ligation reactions, the ratio of molar concentrations of insert to plasmid DNA used was 3:1. The amount of insert DNA needed for a known quantity of the vector DNA was calculated according to the following formula:

$$\text{Insert DNA (ng)} = \left\{ \frac{(50/100 \text{ ng of vector DNA}) \times (\text{size of the insert in bp})}{\text{Size of the vector in bp}} \right\} \times 3$$

The ligation reactions consisted of 50 or 100 ng of vector DNA, insert DNA (as calculated from the formula mentioned above multiplied by 3 (for a 3:1 insert:vector ratio), 1x final concentration of the buffer, 40 units of T_4 DNA ligase and sterile water and the reaction mixture was incubated at 22^{o}C for 5 hrs.

3.2.7.3 Preparation of *E.coli* competent cells

The competent cells were prepared following the protocol as given by Ausubel *et al*. (1999). *E. coli* strain DH5α was grown at 37^{o}C in liquid LB medium (Himedia) overnight. An aliquot of the culture was transferred to 200 ml LB medium (Himedia) and grown to an OD_{590} of 0.375. The culture was distributed into four 50 ml pre-chilled sterile polypropylene tubes and left on ice for 5-10 minutes. The cells were pelleted by centrifugation at 1600 g for 7 min (4^{o}C). Each pellet was resuspended in 10 ml ice-cold $CaCl_2$ solution (15 % glycerol, 0.2 M $CaCl_2$, 0.1 M PIPES) and spun for 5 minutes at 1100 g (4^{o}C). The pellet was resuspended in 10 ml ice-cold $CaCl_2$ solution and left on ice for 30 minutes and spun at 1100g for 5 minutes (4^{o}C). The resultant pellet was completely resuspended in 2 ml of ice-cold $CaCl_2$ solution and 100 µl was aliquoted into pre-chilled sterile 1.75 ml eppendorf tubes and immediately frozen at -80^{o}C.

3.2.7.4 Transformation *E. coli* competent cells

Heat shock method given by Ausubel *et al*. (1999) was followed for the transformation of competent cells with the plasmid DNA or ligation mix (which was incubated at 22^{o}C for a minimum of 5 h). Frozen *E. coli* competent cells were thawed on ice, to which 1 ng of plasmid DNA (for control reaction) or 100 ng of ligation mix was added. The suspension was carefully mixed with pipette tip and incubated on ice for 15 min followed by heat shock at 42^{o}C for 2 min. Cells were incubated on ice for another 2 min, 1 ml LB medium was added and incubated at 37^{o}C for 1 h with shaking. Aliquots of the suspension were spread evenly on LA medium supplemented with appropriate antibiotic (ampicillin or kanamycin). The plates were incubated at 37^{o}C overnight (14-16 h). The following day single colonies were picked up and inoculated in 3 ml LB medium for plasmid isolation.

3.2.7.5 Confirmation of cloning

The cloning at each step was confirmed by colony PCR, PCR with the isolated DNA and restriction analysis or/and PCR. The plasmid DNA of the new clones were isolated and used for this purpose. When PCR was used for confirmation of cloning, the procedure was same as mentioned above with the use of specific primers so as to confirm the cloning and the orientation of the insert. During restriction analysis for confirmation of cloning, suitable restriction enzymes were used so as to confirm the presence of the insert as well as its orientation in the vector background. The procedure was followed as mentioned previously. Colony PCR was also done for a preliminary confirmation of the clones even before their plasmid DNA was isolated. For this, instead of plasmid DNA, the bacterial cells as such were used in the PCR reaction mixture. Also, the PCR reaction mixture with the bacterial cells was first heated at 95^{o}C for 5 min. The cycling parameters were the same as that for the respective gene fragments but the annealing temperature and extension time varied according to the primers used and the size of the amplicons expected.

3.2.8 Mobilization of the recombinant binary vectors into *Agrobacterium tumefaciens*

All the constructs developed (as mentioned above) were mobilized into the *Agrobacterium* strain LBA4404 and further confirmed before they were used for plant transformation. The general procedures followed are explained below.

3.2.8.1 Preparation of LBA4404 competent cells

Saturated culture of *Agrobacterium* strain LBA4404 was grown overnight. 2 ml of this overnight grown culture was added to 50 ml of YEP medium without any antibiotics and allowed to grow upto an OD of 0.5 at A_{600}. The cells were pelleted down at 3000 rpm for 5 min at 4˚C in a sterile falcon tube. The cells were sequentially washed in 10 ml of 0.15 M sodium chloride and repelleted. The resultant pellet was resuspended in 1 ml of 20 mM ice-cold $CaCl_2$. 100 µl of aliquots were frozen and stored at -80˚C.

3.2.8.2 *Agrobacterium* Transformation by Electroporation

The five PTGS constructs in the binary vector (pCAMBIA1300) under CaMV35S promoter were mobilized into *Agrobacterium* strain LBA4404 by electroporation. Frozen *Agrobacterium* competent cells were thawed on ice to which 1-2 µl binary plasmid DNA (50-100ng) was added. The suspension was carefully mixed with pipette tip and transferred to a

pre-chilled 2mm Cuvette (Eppendorf). Electroporation was carried out at 2.5KV voltage, 25μF capacitance and 201 ohms resistance for nearly 5msec. Immediately after electroporation, 1ml of growth medium (LB), without antibiotic selection, was added and the bacterial suspension was incubated at 28^{o}C for 1hr. The cells were pelleted by a brief spin and re-suspended in 100 ul of TB before plating onto culture plates with appropriate antibiotic selection. The putative transformed colonies were picked up and cultured for plasmid isolation and confirmation of the mobilization of the binary plasmid into the bacteria.

3.2.8.3 Freeze-thaw transformation of *Agrobacterium tumefaciens*

All the five PTGS constructs in the binary vector (pCAMBIA1300) under ricin promoter and all the constructs for promoter characterization were mobilised into *Agrobacterium* strain LBA4404 by freeze-thaw method (Chen et al. 1994). The frozen *Agrobacterium* competent cells were thawed on ice and 1 μg of the recombinant binary plasmid DNA was added to competent cell mixture and mixed well. The entire mix was frozen in liquid nitrogen then the frozen mix was thawed the cells ay 37^{0}C for 5-25 min. Immediately 1 ml of YEP was added and kept back on ice. This culture was kept for shaking at˚C28for 3 -4 h. The cells were pelleted by giving a brief spin, the supernatant was decanted and the pellet was resuspended in the left out medium ~100 μl and spread on YEP agar plates supplemented with kanamycin, streptomycin and rifampicin. After 2-3 days, the colonies were inoculated in 5-10 ml of YEP by adding appropriate antibiotics and plasmid DNA were isolated.

3.2.8.4 *Agrobacterium* plasmid DNA isolation

Plasmid DNA of LBA4404 strains harbouring binary recombinant plasmids were isolated from a starter culture of 10 ml each grown on rifampicin, streptomycin and kanamycin antibiotics. Fully-grown culture was incubated on ice for 5-10 min and the cells were pelleted at 3000 rpm for 15 min. The pellet was resuspended in 500 μl of GET (50 mM glucose, 25 mM Tris-Cl pH 5.8, 10 mM EDTA) and 250 μl lysozyme (@ 4 mg/ml) and incubated on ice for 10-15 min. 1 ml of NSW (0.2 N NaOH, 1% SDS) was added and incubated at room temperature for 10 min. To the same tube, 750 μl of 3 M potassium acetate (pH 5.2) was added and incubated on ice for 15 min. Centrifugation was done at 15000 rpm for 15 min at ˚C4. To the supernatant, equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added and mixed well and spun at 15000 rpm for 10min. The aqueous phase containing plasmid DNA was precipitated with double volume of ethanol and kept at -20˚C for 10 min. The pellet was washed with 70% ethanol, air-dried and dissolved in 50 μl of T_{10}.

3.2.8.5 Confirmation of constructs in *Agrobacterium*

3.2.8.5.1 3-Ketoglycoside test for confirmation of *Agrobacterium* strain

This test was performed to check whether the strain was *Agrobacterium* or any other contaminant. *Agrobacterium* species grown on lactose make an enzyme called hexa pyranoside cytochrome C oxido-reductase that converts lactose to 3-keto-lactose, which in turn converts cupric oxide of Benedict's solution to cuprous oxide, a yellow colored precipitate. *Agrobacterium* strains (with recombinant DNA) streaked on 3-ketolactose medium (1% lactose, 0.1% yeast extract, 2% bacto-agar) were flooded with Benedicts reagent (17.3% sodium citrate, 10% sodium carbonate anhydrous, 1.74% copper sulphate). Yellow hallow of Cu_2O appears wherever 3-ketolactose is produced by *Agrobacterium*. By using this test all the *Agrobacterium* cultures were confirmed as *Agrobacterium* strains.

3.2.8.5.2 Confirmation by restriction endonuclease digestion or PCR

The constructs were finally confirmed at *Agrobacterium* level also since recombinations are known to take place during transformation of *Agrobacterium*. Whenever the plasmid DNA was of good quality (as ascertained by viewing on agarose gels) as well as good quantity (at least 200 ng/µl), restriction digestion was used for confirmation. The reaction mixture consisted of 0.8 to 1.0 µg of plasmid DNA, 10-20 units of restriction enzyme, 1x final concentration of appropriate buffer, BSA to a final concentration of 100 µg/ml and milliQ water to make up the final volume. When plasmid DNA concentration was very low (less than 200 ng/µl), PCR (as per the procedure mentioned in section above) was used for confirmation of the clones.

3.2.8.5.3 Confirmation by reverse mating

For further confirmation of orientation of cloning of the required gene cassette in binary vector, reverse mating (transfer of the binary plasmid back into *E. coli*) was done. Chemically competent *E. coli* cells of DH5α were transformed with ~50 ng of *Agrobacterium* plasmid DNA using the standard heat shock method described earlier. The plasmid DNA from the *E. coli* was subjected to appropriate restriction analysis or PCR.

3.2.9 Tissue culture and Transformation

3.2.9.1 Transformation of Tobacco

3.2.9.1.1 Media preparation

Readymade mix of Murashige and Skoog (1962) medium supplemented with different hormones and 0.8% agar as gelling agent was used. The required media at various stages of explant growth in tissue culture work are as listed in the table 3.6.

3.2.9.1.2 Plant growth conditions

The culture plates with the medium and explants were incubated in growth room at temperature of 27±1 ºC under 16/8 hr photoperiod provided by cool fluorescent lights at an intensity of 30 μmol m^{-2} s^{-1}.

Table 3.6 Media used at various stages in tissue culture work

Medium	**Ingredients with hormones**	**pH to be maintained**
Basal medium	MS powder+1x $CaCl_2.2H_2O$	5.8
Half MS	Half the strength of MS	5.8
Shoot induction medium	BN (MS powder +1x $CaCl_2$+BAP 1mg/l+ NAA 0.1 mg/l	5.8
Shoot elongation medium	MS powder + 1x $CaCl_2$+ BAP 0.2mg/l	5.8
Shoot multiplication medium	MS + 1x $CaCl_2$+ BAP 0.5mg/l	5.8
Rooting medium	Half MS	5.8

Antibiotics and hormone stocks were filter sterilized and stored at -20˚C. All media with required hormones were autoclaved for 20 min at 121˚C, 15 lbs pressure and stored at 25˚C. Prior to use, required antibiotics were added to melted and cooled media.

3.2.9.1.3 Surface sterilization of seed for obtaining aseptic seedlings

Seed treatment is a must for obtaining healthy seedlings for further successful downstream tissue culture work. Good bold and healthy of tobacco (*Nicotiana tabacum* L. Xanthi cv.Petit Havana SR) seeds were selected and washed under running water for 2-3 hr after adding 2-3 drops of tritonx-100. Wetted seeds were soaked in 70% ethanol for 1 min. Later, seeds were surface sterilized with 0.1% $HgCl_2$ (mercuric chloride) for 1min followed by 5 min washing with sterile distilled water thrice with continuous shaking. The seeds were re-suspended in 0.5 ml of sterile water and inoculated on half strength MS media tubes at 3-4 seeds per tube.

3.2.9.1.4 Inoculation of LBA4404 strains of *Agrobacterium*

Agrobacterium strains harbouring full length ricin gene for tobacco transformation were inoculated into YEP/TB medium, with respective antibiotics and incubated at 28ºC for 12 hrs.

3.2.9.1.5 Co-cultivation

Standard leaf-disc transformation method for tobacco was followed. Sterile young tobacco leaves were cut out from aseptic tobacco seedlings (which are being routinely maintained under half strength MS medium in the laboratory). A 1:10 dilution of *Agrobacterium* culture with liquid MS medium was taken into sterile petridish under laminar hood. The leaves cut into small pieces, were placed in this mixture for 5 minutes. They were dried for a while on sterile blotting papers and placed on shoot induction medium. The plates were incubated in dark for 48 hrs.

3.2.9.1.6 Selection and shoot regeneration

The explants were then transferred to plates with shoot induction medium, kanamycin (100mg/l) and cefotaxime (250 mg/l) for full length ricin gene expression constructs and shoot induction medium with hygromycin (30mg/l) and cefotaxime(250 mg/l) (selection I) in case of *Agrobacterium* mediated transformation (AMT) with constructs for promoter characterization and incubated for 15 days. The explants, which showed callusing, were then sub-cultured on to plates with shoot elongation medium with kanamycin (100mg/l) and cefotaxime (250 mg/l) for full length ricin gene expression constructs and shoot elongation medium with hygromycin (30mg/l) and cefotaxime (250 mg/l) in case of constructs for promoter characterization for 15 days (selection II). Shoots obtained were excised and sub-

cultured to plates with shoot proliferation and elongation media with kanamycin (100mg/l) and cefotaxime (250 mg/l) for full length ricin gene expression constructs and shoot proliferation and elongation media with hygromycin (30mg/l) and cefotaxime (250 mg/l) in case of constructs for promoter characterization for 15 days (selection III). At each level of selection, the dried and darkened explants (untransformed) were discarded. Simultaneously, some control (untransformed) explants sub-cultured to the first selection medium were also taken onto second and third cycle of selection even though the explants failed to produce callus in the first selection medium. Remaining control explants were maintained and sub-cultured on media similar in all aspects except that they did not have kanamycin and cefotaxime or hygromycin and cefotaxime. The shoots obtained were maintained as controls for experiments aimed at molecular confirmation of the putative transgenics.

3.2.9.1.7 Rooting and maintenance

Well-established and healthy shoots were transferred and maintained in tubes with rooting medium and respective antibiotics. The time course for tobacco transformation and regeneration is shown in table 3.7.

3.2.9.2 Confirmation of transgenic tobacco obtained

3.2.9.2.1 Isolation of tobacco genomic DNA

DNA was isolated from putative transgenic as well as control tobacco plants by following the modified CTAB procedure based on the protocol of Doyle and Doyle (1987). Fresh tobacco leaves (70-100 mg from tissue culture maintained plantlets) were harvested in to 1.5 ml microcentrifuge tubes, frozen in liquid nitrogen and ground to fine powder using pre-chilled micropestle. 400 µl of pre-warmed (60^{o}C) isolation buffer [2% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0), 0.2% β-mercaptoethanol (added just before use)] was added and incubated at 60^{o}C for 30-60 minutes. The slurry was extracted twice with equal volume of chloroform-iso-amyl alcohol (24:1). The final aqueous phase was transferred to a fresh tube and DNA was precipitated with 0.6 volumes of ice-cold iso-propanol. The DNA pellet obtained after centrifugation was washed with 70% ethanol, air-dried and dissolved in 50 µl $T_{10}E_1$ buffer [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)].

3.2.9.2.2 Quantification of DNA

The DNA samples were electrophoresed on a 0.8% agarose gel .The approximate DNA concentration was determined by visual comparison against a known amount of standard molecular weight marker (uncut λ DNA or λ DNA *Eco*RI + *Hin*dIII digest).

3.2.9.2.3 PCR reaction

Putative transgenics obtained were confirmed using PCR amplification of specific component sequences present in the constructs. The primers used and their sequence information is presented in the table 4. The PCR reaction mixture consisted of 20 ng of DNA, 0.2 μM each of the forward and reverse primers specific for the gene fragment, 1x final concentration of *Taq* DNA polymerase buffer [10 mM Tris-HCl (pH 9), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin], 100 μM of each dNTP, 1 unit of *Taq* DNA polymerase, and sterile water (to make up the volume).

All PCR reactions were carried out with the following cycling parameters.

Step	Temperature (^oC)	Time	Cycles
Initial denaturation	94	4 min	1
Denaturation	92	30 sec	35
Annealing	*	45 sec	
Extension	72	*	
Final extension	72	7 min	1
Hold	4		1

* The annealing temperature and extension time were determined based on the primers used and product size expected.

3.2.9.2.4 GUS assay

GUS histochemical assay was performed to confirm the putative transformants (Jefferson, 1987). X-GlcA (5-bromo-4-chloro-3-indolyl-_-D-glucuronic acid) is a substrate for _-D-glucuronidase (GUS) encoded by the *gusA* gene. The substrate is used as a qualitative histochemical marker of specific GUS expression in cells and tissues. X-GlcA is cleaved by GUS at the β1 glucuronic bond between glucuronic acid and the 5-bromo-4-chloro-3-indolyl part of X-GlcA via hydrolysis. This enzymatic cleavage of X-GlcA results in the precipitation of a water insoluble blue dichloro-dibromo-indigo precipitate. This reaction was used to confirm the presence of the transgene. X-GlcA solution was prepared by dissolving 5 mg of

X-GlcA cyclohexylammonium salt in 50 µl dimethyl formamide (DMF) and 10 ml of autoclaved 0.05 M phosphate buffer solution at pH 7.0 (Sambrook and Russel, 2001). To this, 30 µl of Triton X 100 was added. Different tissues obtained from transgenic tobacco (for promoter study) like leaf, sepal, petal, stamen and carpel were immersed in GUS staining solution and incubated overnight in dark at 37 ºC. Before analysis, chlorophyll was bleached by extraction in ethanol. Untransformed explants (control) which were cultured under identical conditions served as control.

Table 3.7 Time course for transformation (tobacco) protocol

Steps	Description	Materials	Time (days)
1.	Inoculation of *Agrobacterium* culture	TB or YEP+ rifampicin+ kanamycin+ streptomycin	Overnight
2.	Co-cultivation	O/N grown culture (A600nm=0.1) leaf explants on shoot induction medium in petri plates with no antibiotics	2
3.	Selection I for shoot induction	BN+ kanamycin + cefotaxime / BN+ hygromycin + cefotaxime	15
4.	Selection II for shoot proliferation	BA0.5 (mg/l)+kanamycin + cefotaxime/BA0.5(mg/l)+ hygromycin+ cefotaxime in petri plates	15
5.	Selection III for shoot elongation	BA0.2 (mg/l)+kanamycin + cefotaxime/BA0.2(mg/l)+ hygromycin+ cefotaxime in petri plates	15
6.	Selection IV for rooting	½ MS+ kanamycin + cefotaxime/1/2MS hygromycin+ cefotaxime in tubes	15

3.2.9.2 Transformation of Castor

3.2.9.2.1 *Agrobacterium*-mediated transformation of castor

3.2.9.2.1.1 Media preparation

Readymade mix of Murashige and Skoog medium supplemented with different hormones and 0.8% agar as gelling agent was used. The required media at and the time course of various stages of explant growth in castor tissue culture work are as listed in the table 3.8

Table 3.8 Media and time course of various stages in castor transformation and plant regeneration.

Medium	**Ingredients with hormones and antibiotics**	**Time course (days)**
Embryo axes growth medium	MS powder+1x $CaCl_2.2H_2O$+ BAP 0.1mg/l	7
Co-cultivation medium	Full strength MS (MS powder+1x $CaCl_2.2H_2O$)	5
Post co-cultivation medium	MS + 1x $CaCl_2$+ BAP 0.1mg/l + cefotaxime 250mg/l	10
Expansion and proliferation medium	MS + 1x $CaCl_2$+ TDZ 0.5mg/l + cefotaxime 250mg/l	10
Selection I medium	MS + 1x $CaCl_2$+ BAP 0.5mg/l + cefotaxime 250mg/l + hygromycin 20mg/l	15
Selection II medium	MS + 1x $CaCl_2$+ BAP 0.5mg/l + cefotaxime 250mg/l + hygromycin 40mg/l	15
Selection III medium	MS + 1x $CaCl_2$+ BAP 0.5mg/l + cefotaxime 250mg/l + hygromycin 60mg/l	15
Acclimatization (without selection) medium	MS + 1x $CaCl_2$+ BAP 0.5mg/l + cefotaxime 250mg/l	15
Shoot proliferation medium	MS + 1x $CaCl_2$+ BAP 0.5mg/l	15
Shoot elongation medium	MS + 1x $CaCl_2$+ BAP 0.2mg/l	15
Rooting medium	Half MS + NAA 2mg/l	15

The pH of various media used in castor transformation and plant regeneration was maintained at 5.8. Antibiotics and hormone stocks were filter sterilized and stored at -20˚C. All media with required hormones were autoclaved for 20 min at 121˚C, 15 lbs pressure and stored at 25˚C. Prior to use, required antibiotics were added to melted and cooled media.

3.2.9.2.1.2 Explant preparation

Mature seeds were decoated and rinsed in running tap water for 30 min. The decoated seeds were surface sterilized with 0.1% (w/v) aqueous mercuric chloride solution for 12 min and subsequently rinsed 5 times with sterile distilled water. The seeds were blotted dry on sterile filter paper. The endosperm was carefully dissected to expose the embryos. Leaving the

papery cotyledons on the endosperm, the embryo axes were excised and implanted on the culture medium, BA 0.1mg/l.

3.2.9.2.1.3 Inoculation of LBA4404 strains of *Agrobacterium*

Agrobacterium strain LBA4404 harbouring PTGS construct (pCAMBIA1300 FP ihp-RB) was inoculated into YEM with respective antibiotics and incubated at 28ºC for 12 hrs.

3.2.9.2.1.4 Transformation and plant regeneration

Embryo axes isolated from mature seeds were pre-cultured on medium supplemented with 0.1mg/l BA for 7 days prior to infection to *Agrobacterium*. The seedlings were injured with two strokes of a sharp pointed blade (no.11) in the meristematic region, which is distinguishable by its characteristic swelling. The radicular regions and cotyledonary extensions were removed from the germinated embryo axes. The processed explants were immersed in bacterial suspension and subjected to vacuum infiltration for 30 min. Subsequently, the explants were blotted dry on sterile filter paper and co-cultivated for 5 days on full strength MS and incubated in dark. To enhance the penetration of the *Agrobacterium* containing the construct, into the target tissues, explants were subjected to shaking (180 r.p.m) for 10 min during the bacterial incubation. Following co-cultivation, the explants were washed with 250mg/l cefotaxime for 10 min, rinsed with sterile distilled water 3 times for 5 min each with constant stirring, and blotted dry on sterile filter paper. The procedure followed for castor transformation and plant regeneration is given below, schematically. The rooted shoots were transferred to sterile vermiculite and maintained under high humidity for 10 days. Established plantlets were transferred to soil and grown to maturity.

3.2.9.2.2 Particle gun-mediated transient studies in castor (for ricin promoters characterization)

3.2.9.2.2.1 Media preparation

Readymade mix of Murashige and Skoog (MS) medium supplemented with 3% sucrose constituted the pre-osmoticum and post-osmoticum media. All media were prepared according to the standard procedures and pH was adjusted to 5.6 ± 0.2 prior to autoclaving at 121^0C for 20 min.

3.2.9.2.2.2 Explant preparation

Decoated seeds were surface sterilized with 0.1% (w/v) of mercuric chloride for 8.0 min and rinsed thoroughly four times in sterile distilled water. Whole endospermic seeds collected at 47 days after pollination (DAP) were aseptically pre-incubated on pre-osmoticum medium and stored in the dark.

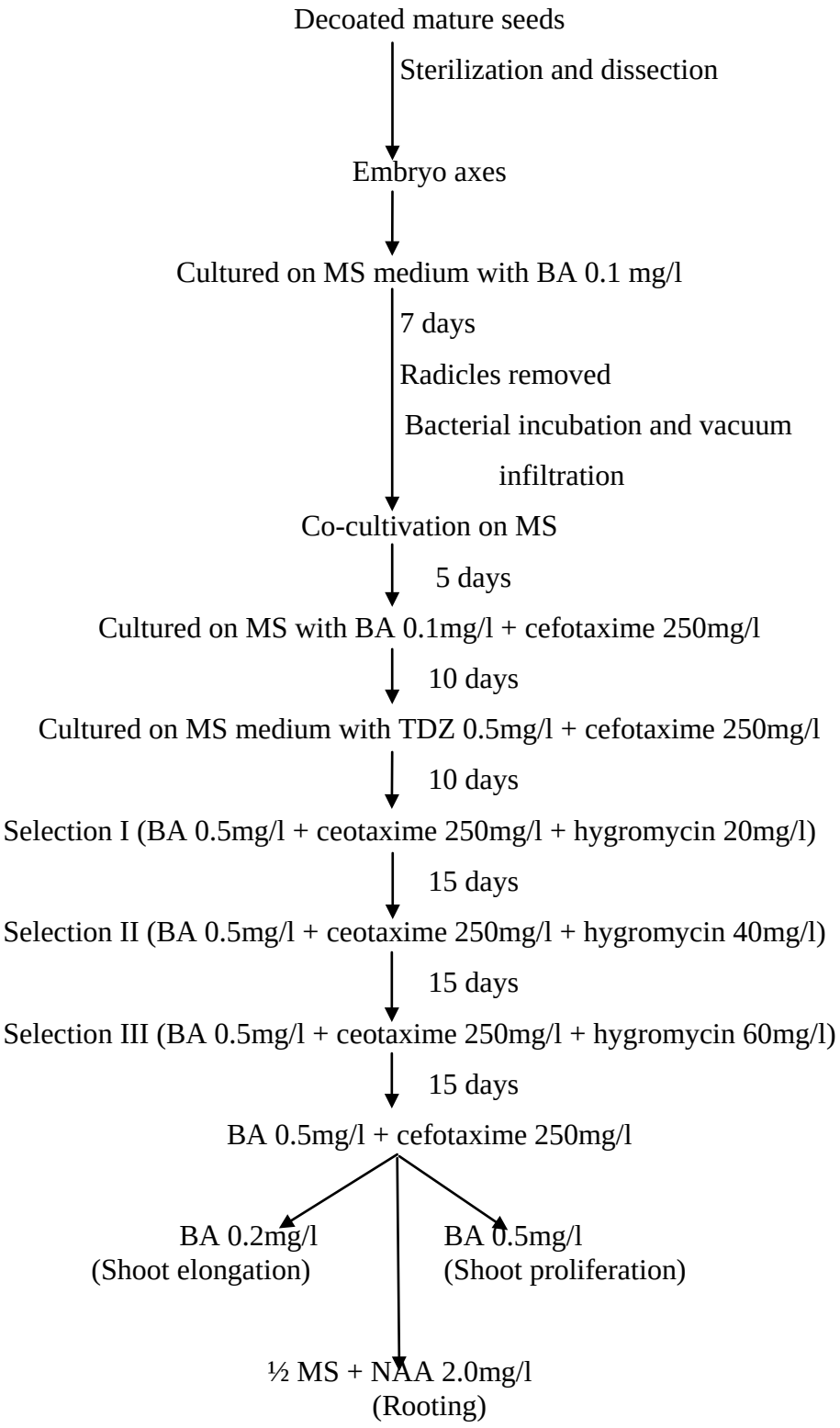


Fig. 3.7: Protocol for castor transformation and plant regeneration

3.2.9.2.2.3 Transformation with particle gun

Following pre-incubation, whole seeds of castor were selected for bombardment with the biolistic PDS-1000 He system (BioRad Hercules, CA). About 9 endosperms were arranged on Whatman No.1 filter paper in a circle of 1.5 cm diameter in the centre of a 9.0 cm petriplate with osmoticum (0.2 M each of sorbitol and mannitol) and plasmolysed for 2 h prior to bombardment. The constructs, pCAMBIA1391Z FP, pCAMBIA1391Z TP, pCAMBIA1391Z CaMV35S gus and the basal vector, pCAMBIA1391Z were used for the experiments.

3.2.9.2.2.4 Preparation of microcarriers

Microcarriers were coated with 6 μg of plasmid DNA per 50 μl particle preparation (3 mg microcarrier/50 μl) using the CaCl2 (50 μl, 2.5 M) and spermidine (20 μl, 0.1 M) precipitation method according to the manufacturer's instructions to give a DNA concentration of 2 μg/mg of microcarriers. Following precipitation, the supernatant was removed and pellet was washed with 300 μl of absolute ethanol. After washing, the particle DNA pellet was resuspended in 50 μl of absolute ethanol for 6 bombardments. Care was taken to ensure uniform particle distribution and minimize agglomeration. Bombardments were done under a vacuum of 27 inches of Hg, a 25 mm distance from rupture disc to macrocarrier and a 10 mm macrocarrier flight distance for all the bombardments. The parameters tested included rupture disc pressures (1,100 psi), microprojectile travel distance (6 cm) and microcarrier (gold particle size 1.0). Non-bombarded embryo axes and embryo axes bombarded with uncoated microcarriers were used as controls. Following bombardment, the explants were kept in dark at 25^0C for 2 h and then transferred to shoot proliferation on MS medium containing 0.5 mg/l BA and after 2 days, the explants were checked for GUS activity.

3.2.9.2.2.5 Histochemical GUS assay

Transient GUS assays were performed 48 h after bombardment by incubating the tissues in assay buffer (0.05 M NaH2PO4 with 500 mg/l X-Gluc and 30% Triton X-100) for 12 h at 37^0C, after which they were destained and stored in 70% alcohol (Jefferson et al. 1987).

CHAPTER IV

RESULTS

The present investigation, carried out at the Directorate of Oilseeds Research, Hyderabad, aimed at developing castor lines that will have reduced levels of ricin and RCA, the toxic endosperm-specific proteins of castor, through PTGS approaches. The main hypothesis of the investigation was that RNA interference or PTGS could be exploited to realize transgenic castor with reduced or nil levels of ricin and RCA. Hence different PTGS constructs under the control of full-length ricin promoter (FP) could be employed to induce PTGS by targeting the transcripts of ricin and RCA for degradation. The main objectives included isolation and characterization of ricin promoter, development of generic and ricin and RCA-specific PTGS constructs and *Agrobacterium tumefaciens* mediated genetic transformation of castor with the developed constructs. The results obtained during the course of this investigation are presented here.

4.1 Isolation and characterization of ricin promoter

4.1.1 Isolation of ricin promoter

A 310 bp upstream of the 5' ricin gene was already available in the database and this served as a platform to 'fish' out another 622 bp upstream of the already known 310 bp, using inverse PCR (IPCR). Other strategies like single oligonucleotide PCR was tried to trap 5' regulatory elements but in vain!!!

IPCR involved the digestion of genomic DNA with appropriate restriction endonucleases, intra-molecular ligation to circularize the DNA fragments and PCR amplification. PCR used primer pairs that originally pointed away from each other but would face each other after ligation of the ends leading to circular DNA.

Castor genomic DNA of VP-1P5 @1μg was digested individually with two selected restriction enzymes, *Bgl*II and *Bam*HI. The choice of the enzyme for digestion was based on the fact that they would digest within the known DNA region (ricin gene sequence) only once. It was proposed to use two enzyme digests, to increase the probability of having the target sequence (the promoter) within 2kb since the efficiency of the subsequent PCR amplification decreases rapidly for fragment sizes above 2kbp in size. Following inactivation of the restriction enzyme, an aliquot was retained for gel analysis and the remainder of the restriction digest reaction was diluted five-fold in ligation mixture (ligation buffer, water, ligase) and incubated for 12 hours at 16^0C.

Using the outermost primers (3F and 6R in case of *Bgl*II digest and 2F and 6R in case of *Bam*HI digest), a standard PCR amplification was performed under high-stringency conditions (55-60^{0}C) using a relatively long extension time (3 min) and allowing the reaction to proceed to 40 cycles. The use of 40 cycles ensured that even extremely rare templates are subjected to amplification. A proofreading DNA polymerase (*Pfu*) was used to minimize the error rate. When the PCR product was fractionated on the gel, it indicated a bit of smearing which could be due to a high degree of non-specific amplification resulting from either too much of template or unsuccessful restriction digestion and ligation.

The second-round PCR, which is a way of 'fishing' out the specific first- round amplification product from the background of non-specific amplification products, was used with the nested primers. A titration series of the first-round PCR was used to ensure specific amplification and to reduce the generic smearing due to template saturation. The second-round PCR was performed with a nested primer pair (4F and 7R in case of *Bgl*II digest and 2F and 7R in case of *Bam*HI digest), at a high annealing temperature (55^{0}C) using an extension time of 2 min and 30 sec for 35 cycles using the proofreading DNA polymerase. A single strong amplification product was observed by agarose gel electrophoresis. In case of *Bgl*II, the amplicon size was ~900 bp and in case of *Bam*HI digest, it was ~1400 bp (Fig.4.1). A preparatory PCR was set with the specific nested primers to realize a ~900 bp in case of *Bgl*II and ~1400 bp in case of *Bam*HI. The specific amplicon bands were eluted from gel and were cloned in InsT/A vector. The clones obtained were then confirmed for the presence of specific amplicons by restriction with *Eco*RI and *Hind*III (Fig.4.2). The desired clones were then sequenced with M13 F and M13R primers and the sequences were analyzed. From sequence analysis (BLAST), it turned out that the ~900 bp fragment (*Bgl*II) showed perfect match with preproricin. Unfortunately, the *Bam*HI fragment did not show any homology to any ricin sequences. Thus it was concluded that only the *BglII* fragment corresponded to the promoter of ricin gene. Based on the sequence of this genomic fragment, primers were designed to isolate the ricin promoter. The entire length of the obtained sequence (~900 bp) was designated as full-length promoter and the earlier known ricin promoter sequence (~315 bp) was designated truncated promoter. When PCR was carried out with ricin full-length specific primers using castor genomic DNA as the template, three bands were realized. These included a ~1100 bp, a ~1000 bp and a ~350 bp fragment. All the bands were gel eluted and cloned in InsT/A vector and sequenced. From sequence analysis, it was evident that the 1100 bp and 1000 bp fragments were identical, although the sequence of 1100 bp fragment contained a lot of background noise. The ricin truncated promoter-specific primers gave an

amplicon of ~310 bp and it's sequence was identical to the 350 bp fragment obtained using ricin full-length promoter-specific primers (Fig.4.4).

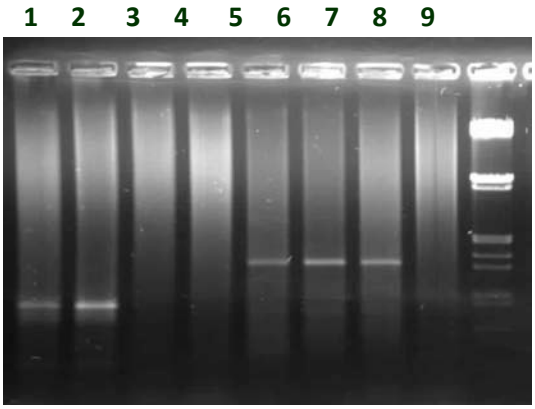


Fig. 4.1: Re-amplification of *BglII* and *BamHI* restricted castor genomic DNA (Inverse PCR)

Lane 1-2 : *BglII* restricted reamplified amplicons (with primers 4F and 7R)
Lane 3-4 : *BamHI* restricted reamplified amplicons (with primers 2F and 7R)
Lane 5 : *BglII* restricted reamplified amplicon (with 4F)
Lane 6 : *BamHI* restricted reamplified amplicon (with 2F)
Lane 7 : *BglII* restricted reamplified amplicon (with 7R)
Lane 8 : *BamHI* restricted reamplified amplicon (with 7R)
Lane 9 : *EcoRI* + *HindIII* λ DNA

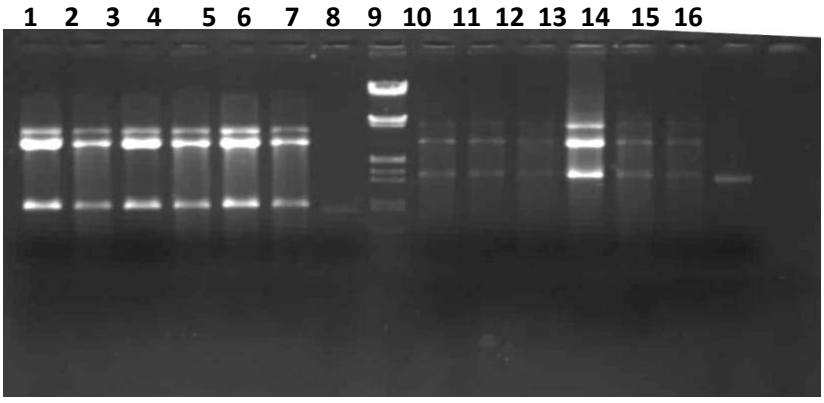
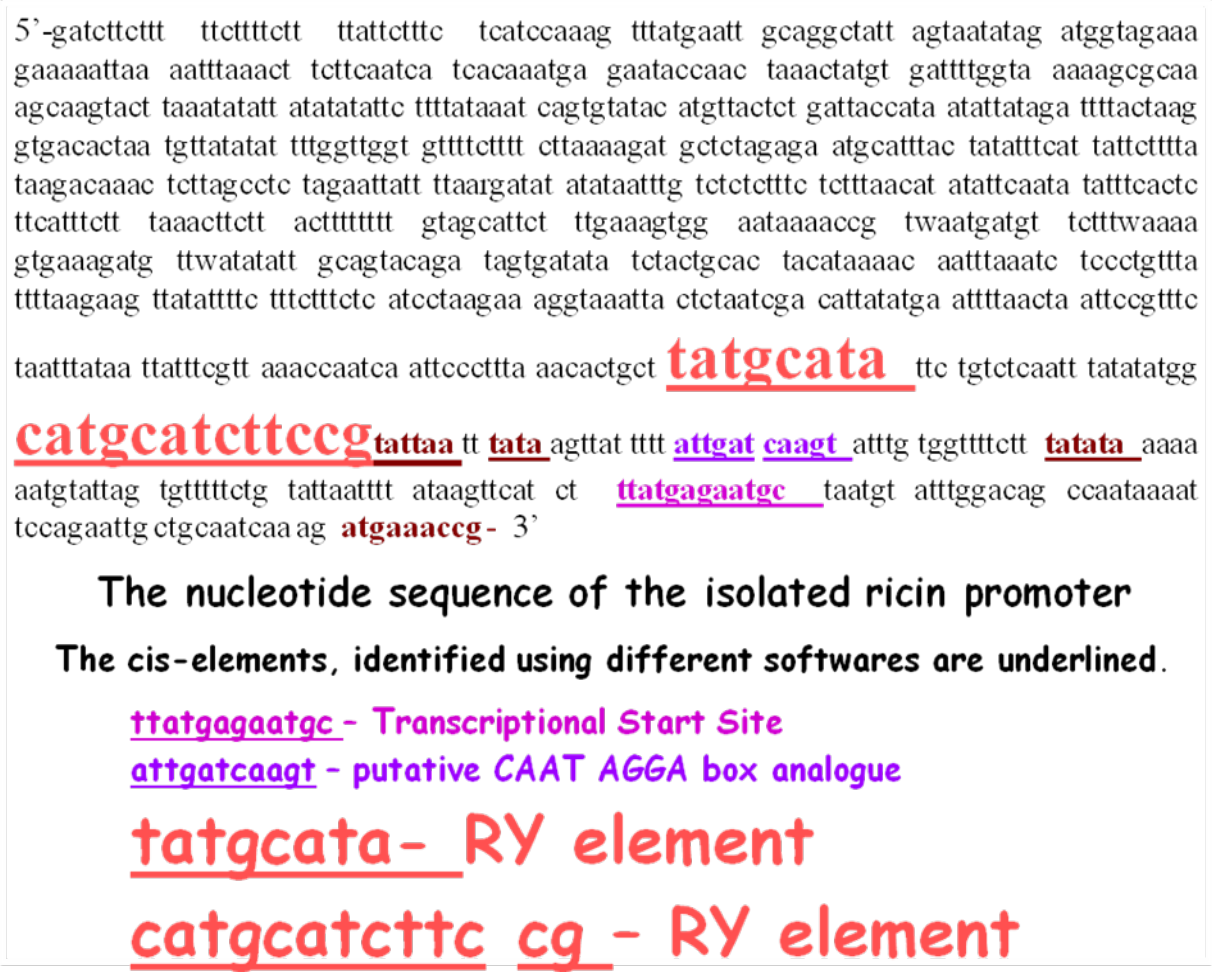


Fig. 4.2: Confirmation of *BglII* and *BamHI* restricted castor genomic DNA in T/A vector by restriction with *EcoR*I and *Hind*III

Lane 1-6 : *BglII* restricted reamplified amplicon (with primers 4F and 7R) clones 1-6 in T/A
Lane 7 : *BglII* restricted reamplified amplicon – eluted product
Lane 8 : *EcoRI* + *HindIII* λ DNA
Lane 9-14 : *BamHI* restricted reamplified amplicon (with primers 2F and 7R) clones 1-6 in T/A
Lane 15 : *BamHI* restricted reamplified amplicon – eluted product

Sequencing and Primer Designing



Ricin Full-length promoter – 932 bp
Ricin Truncated promoter – 310 bp

Fig. 4.3: Nucleotide sequence of the isolated ricin full length promoter

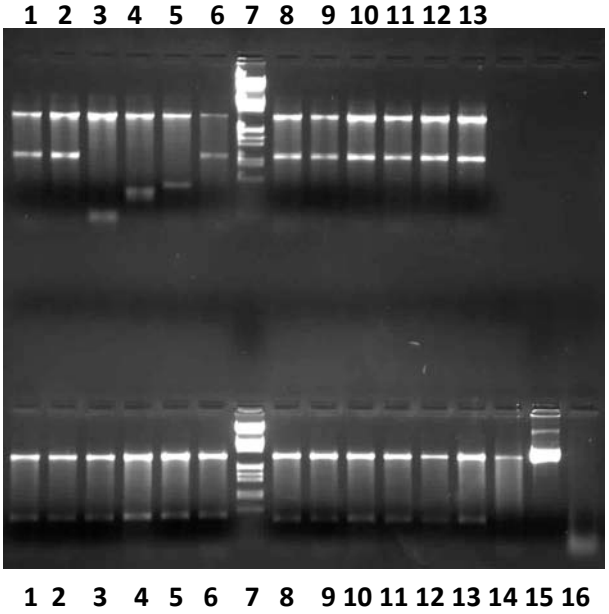


Fig. 4.4: Restriction of T/A Full length ricin promoter band 1,2,3 clones and T/A Truncated ricin promoter clones with *EcoRI* and *HindIII*

Upper Panel
Lane 1-6 : Restricted clones of T/A Full length ricin promoter Band 1 (~1.0 kb)
Lane 7 : *EcoRI* + *HindIII* λ DNA
Lane 8-13 : Restricted clones of T/A Full length ricin promoter Band 2 (~0.9 kb)
Lower Panel
Lane 1-6 : Restricted clones of T/A Full length ricin promoter Band 3(~0.4 kb)
Lane 7 : *EcoRI* + *HindIII* λ DNA
Lane 8-13 : Restricted clones of T/A Truncated ricin promoter (~0.3 kb)
Lane 14 : T/A p TZ57R restricted
Lane 15-16 : T/A p TZ57R uncut

4.1.2 Characterization of ricin promoters

4.1.2.1 *In silico* analysis

The sequence of the *Bgl*II fragment (Fig.4.3) was scanned for the presence of endosperm-specific elements or motifs using a host of on-line software programs like PLACE, PlantCARE, TRANSFAC, PAINT and PlantProm. The sequence was subjected to PLACE Signal Scan available at http://www.dna.affrc.go.jp/htdocs/PLACE/signalup.html. Although the newly fished out promoter sequence (622 bp) did not contain any endosperm-specific motifs, nevertheless, the already known 310 bp of the promoter which is immediately upstream of ricin gene, possesses endosperm-specific elements like RY elements (Fig.4.3). The newly fished out promoter sequence (622 bp) contains sequences which are recognised by DNA One Finger binding (DOF) proteins. DOF proteins are essentially endosperm-specific in nature.

4.2 Development of constructs to assess the tissue-specificity of the isolated ricin full-length and truncated promoters

The 932 bp genomic region upstream of ricin gene designated as full length (FP) promoter and 310 bp designated as truncated (TP) ricin promoter, were cloned in InsT/A vector. The directionality of the clones obtained was confirmed and the clones cloned in the 5'-3' direction were selected for cloning them upstream of *gus* in a promoter-less vector (pCAMBIA 1391Z). The resulting constructs pCAMBIA1391Z TP (Fig.4.5) and pCAMBIA 1391Z FP (Fig.4.6) were confirmed by restriction with *Eco*RI and *Bam*HI (Fig.4.7 and 4.8). pCAMBIA 1391Z TP gave 310 bp and pCAMBIA 1391Z FP gave 932 bp on digestion with *Eco*RI and *Bam*HI. The confirmed clones were then mobilized into *Agrobacterium tumefaciens* strain LBA4404. The mobilization was confirmed by restriction of the constructs in LBA 4404 with *Eco*RI and *Bam*HI. The constructs yielded the expected 310 bp fragment (pCAMBIA 1391Z TP) and 932 bp fragment (pCAMBIA 1391Z FP).

The final constructs in the *Agrobacterium* were designated as follows:

1). LBA4404:: pCAMBIA 1391Z TP

2). LBA4404:: pCAMBIA 1391Z FP

Apart from the above two, yet another two constructs (control) namely LBA4404:: pCAMBIA 1391Z CaMV35S Gus and LBA4404:: pCAMBIA 1391Z (basal vector) have been used for the transformation of tobacco and the transgenic plants realized have been assessed for the presence of the introduced gene cassette.

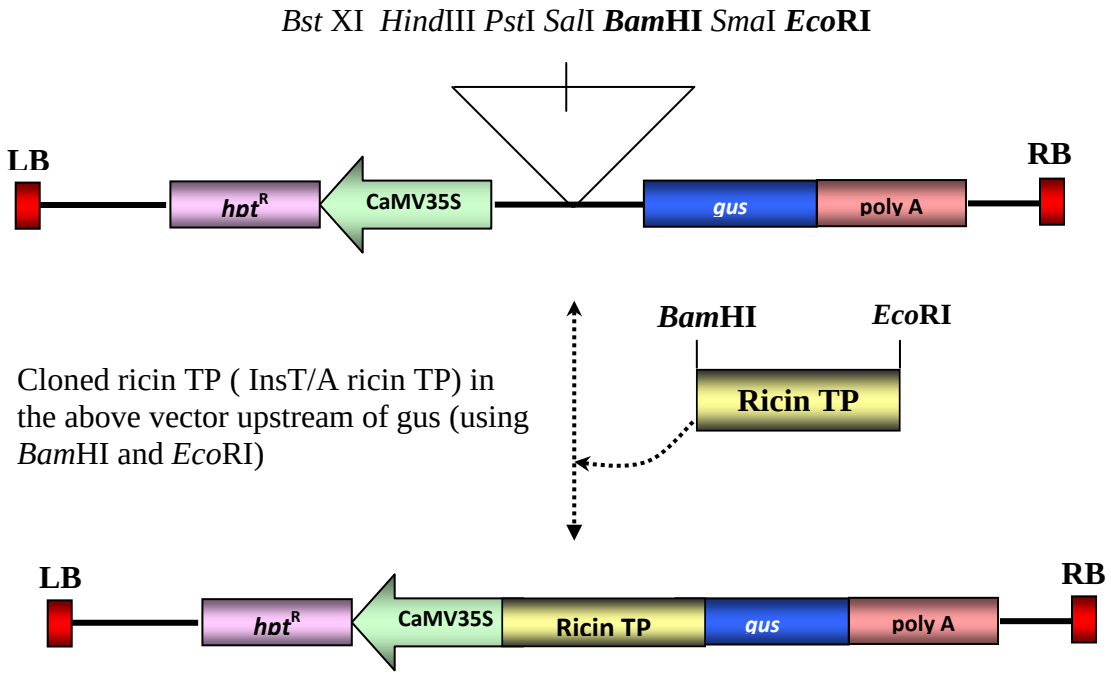


Fig. 4.5. Schematic representation of cloning of ricin truncated promoter upstream of *gus* in pCAMBIA 1391Z

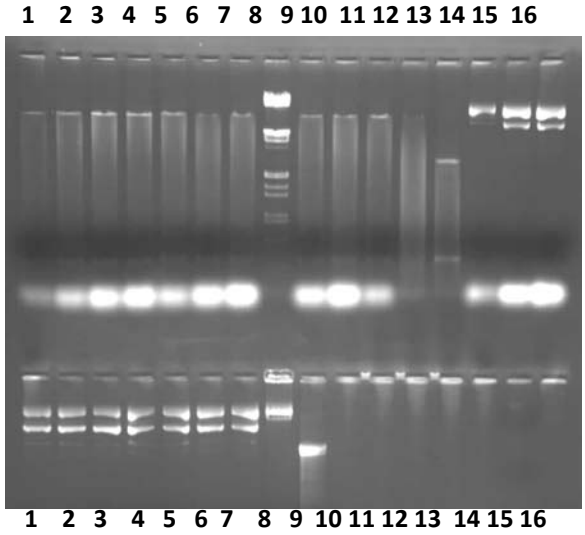


Fig.4.7. Confirmation of cloning of truncated ricin Promoter(TP) in pCAMBIA 1391Z TP clones by restriction with *Eco*RI and *Bam*HI

Upper Panel
Lane 1-7 : Digested clones (1-7)
Lane 8 : (E+H) digested ë DNA marker
Lane 9-11 : Digested clones (8-10)
Lane 12 : Digested pCAMBIA 1391Z
Lane 13 : Digested T/A TP clone 3
Lane 14-16 : Uncut clones (1-3)

Lower Panel
Lane 1-7 : Uncut clones (4-10)
Lane 8 : Uncut pCAMBIA 1391Z
Lane 9 : Uncut T/A TP clone 3

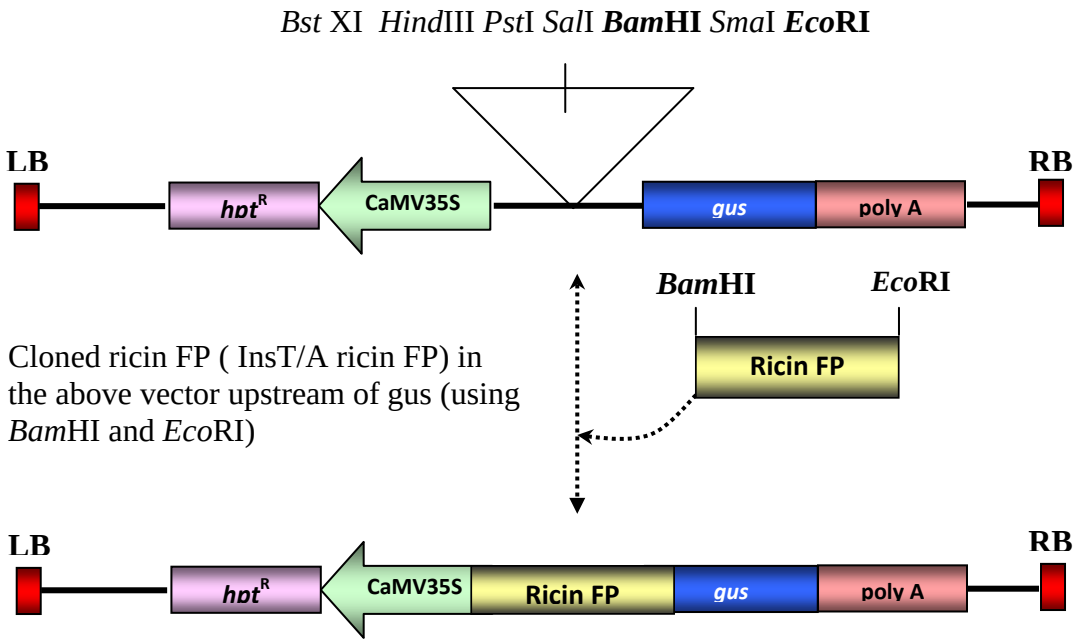


Fig. 4.6. Schematic representation of cloning of ricin full-length promoter upstream of *gus* in pCAMBIA 1391Z

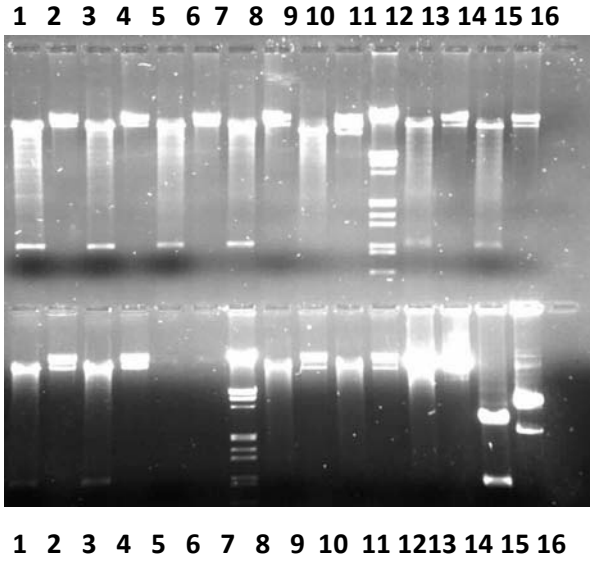


Fig. 4.8. Confirmation of cloning of full length ricin Promoter(FP) in pCAMBIA 1391Z FP clones by restriction with *Eco*RI and *Bam*HI

Upper Panel
Lane 1-10 : Digested clones alternates with uncut (1,2,3,4,5)
Lane 11 :(E+H) digested λ DNA marker
Lane 12-15 : Digested clones alternates with uncut (6,7)
Lower Panel
Lane 1-6 : Digested clones alternates with uncut (8,9,10)
Lane 7 : (E+H) digested λDNA marker
Lane 8-11 :Digested blue clones alternates with uncut
Lane 12-13 : Digested alternates with uncut pCAMBIA 1391Z
Lane 14-15 : Digested alternates with uncut T/A FP clone 3

The ricin gene was cloned under its own promoters and the constructs developed were used for transformation of tobacco for further confirmation of tissue specific expression. The ricin full-length gene (1732 bp) from the InsT/A vector was excised out with *ApaI* and *Sst*I, gel eluted and ligated into pRT100 vector digested with *ApaI* and *Sst*I. Since the *ApaI* site is 5' to the *Sst*I site in the pRT100 vector, the sequence was supposed to be cloned in sense orientation with respect to the 35S promoter. Colony PCR was carried to confirm amplification of full-length ricin gene using '*Apa*I ricin Forward' and '*Sst*I ricin Reverse' primers. The clones were restricted with the cloning enzymes, *Apa*I and *Sst*I, to excise out the cloned fragment. All the clones gave expected fragment of 1730 bp, confirming the cloning of full-length sense sequence. Also, the clones were restricted with *Hind*III to confirm cloning of 1732 bp downstream of CaMV35S promoter. The clones gave the expected 1732 bp of the ricin gene together with 700 bp of the *Hind*III cassette.

To enable directional cloning of the ricin gene in pRT100 TP and pRT100 FP, the ricin gene from the respective T/A vector was excised out using *Apa*I and *Sst*I whose sites were deliberately introduced in primers and the fragments were cloned in pRT100 TP (version of pRT100 in which CaMV35S was replaced with TP) and pRT100 FP (version of pRT100 in which CaMV35S was replaced with FP). Thus, this exercise generated pRT100 TP Ricin 1.7kb and pRT100 FP Ricin 1.7kb constructs.

The above constructs developed in pRT100 backbone were then cloned into a plant transformation vector to enable downstream transformation of tobacco. Since, the constructs were flanked by *Hind*III sites in pRT100, the gene cassettes were restricted with the same and cloned into *Hind*III site of binary vector, pCAMBIA 2300. The entire gene cassette including TP/FP promoters, cloned full-length ricin gene and the polyA signal were excised out from the pRT100 vector backbone by restricting with *Hind*III. After transformation, the colonies were selected based on blue-white screening. Plasmid DNA was isolated from the clones and was restricted with *Hind*III. The clones gave the expected fragment of 2318 bp (310 bp of TP + 1732 bp of ricin gene + 276 bp of the pRT100 backbone) in case of pCAMBIA 2300 TP Ricin 1.7kb (Fig.4.9); and 2940 bp (932 bp of FP + 1732 bp of ricin gene + 276 bp of the pRT100 backbone) in case of in case of pCAMBIA 2300 FP Ricin 1.7kb (Fig.4.10) and thus confirmed the cloning.

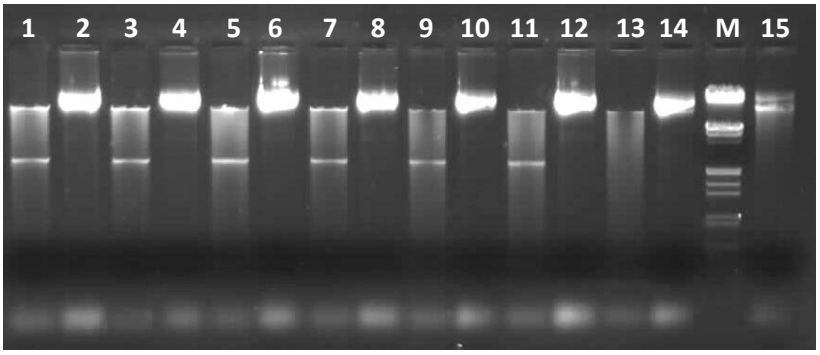


Fig. 4.9. Confirmation of cloning of TP R1.7 kb in pCAMBIA 2300 TP R1.7 kb clones by restriction with *Hind*III

Lane 1-12 : Digested clones alternates with uncut (1,2,3,4,5,6)
Lane 13-14 : Digested blue clone alternates with uncut (negative control)
Lane M : (E+H) digested λ DNA marker
Lane 15 : Uncut pRT TP R1.7 kb clone 8

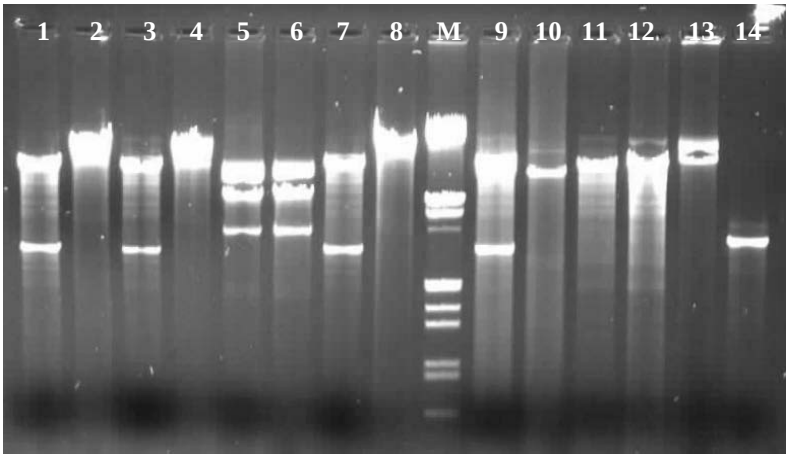


Fig. 4.10. Confirmation of cloning of FP R1.7 kb in pCAMBIA 2300 FP R1.7 kb clones by restriction with *Hind*III

Lane 1-8 : Digested clones alternates with uncut (1,2,3,4)
Lane M : (E+H) digested λ DNA marker
Lane 9-10 : Digested clones (5,6)
Lane 11-12 :Digested blue clones
Lane 13 : Digested pCAMBIA 2300 (negative control)
Lane 14 : Digested pRT FP R1.7 kb clone 12 (positive

The binary constructs harboured in *E.coli* were then mobilized into *Agrobacterium* strain LBA4404 to carry out plant transformation. The final constructs in the *Agrobacterium* were designated as follows:

1). LBA4404:: pCAMBIA 2300 CaMV35S Ricin 1.7kb

2). LBA4404:: pCAMBIA 2300 TP Ricin 1.7kb

3). LBA4404:: pCAMBIA 2300 FP Ricin 1.7kb

The above constructs in *Agrobacterium* strain LBA 4404 were further confirmed by following one or all methods of restriction analysis, PCR, reverse mating and keto-lactose test.

4.2.1 Transformation of Tobacco

4.2.1.1.Stable *Agrobacterium*-mediated genetic transformation of tobacco with the developed constructs

The following account sums up the tobacco transformation with the five constructs developed for the envisaged objectives 1 and 2. The constructs included:

1). LBA4404:: pCAMBIA 1391Z TP

2). LBA4404:: pCAMBIA 1391Z FP

3). LBA4404:: pCAMBIA 1391Z CaMV35S

4). LBA4404:: pCAMBIA 1391Z

5). LBA4404:: pCAMBIA 2300 TP Ricin 1.7kb

6). LBA4404:: pCAMBIA 2300 FP Ricin 1.7kb

For characterization of ricin promoters

The confirmed constructs were transferred into tobacco using *Agrobacterium*-mediated leaf disc transformation method. Leaf explants derived from 45 day old axenic seedlings of tobacco were co-cultivated with *Agrobacterium* culture for two days. The explants were subsequently transferred and maintained on shoot induction medium containing the plant selection agent hygromycin (@ 30mg/l) and bacteriostatic antibiotic cefotaxime (@250mg/l) in case of pCAMBIA 1391Z TP/FP/35S transgenics and kanamycin (@ 100mg/l) and bacteriostatic antibiotic cefotaxime (@250mg/l) in case of TP/FP ricin gene transgenics for 15 days for selecting the shoots initiated with transformed cells in the explants. Putative transformants, which showed callusing was transferred to shoot elongation (SE) medium (BAP 0.5mg/l) supplemented with hygromycin/kanamycin and cefotaxime and were maintained for another 15 days. Putative transformants that formed shoots were sub-cultured onto SE medium with selection pressure for the third cycle of selection. Completely

necrotised explants from the co-cultivated explants were eliminated at each level of selection. The shoots obtained on selection plates, were further maintained on ½ MS medium with hygromycin/kanamycin and cefotaxime for rooting. Simultaneously, some control (untransformed) explants sub-cultured onto the first selection medium were also taken onto second and third cycle of selection, even though the explants failed to produce callus in the first selection medium. Remaining control explants were maintained and sub-cultured on media similar in all aspects except that they did not have hygromycin/kanamycin and cefotaxime. The shoots obtained were maintained as controls for experiments aimed at molecular confirmation of the transgenics. Plate 1 gives a general view of the different stages of tobacco transformation and regeneration followed using all the above six constructs.

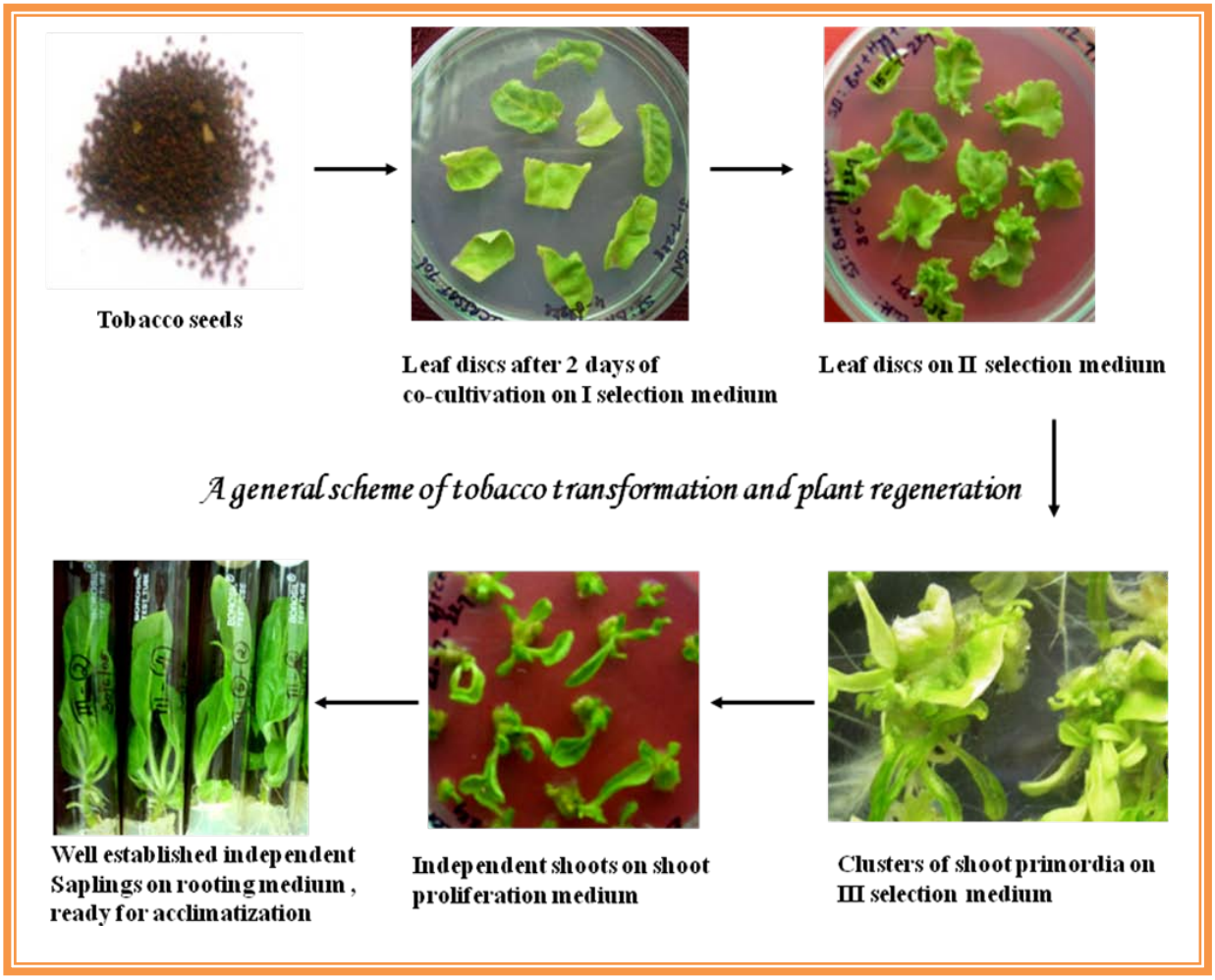


Plate 1: Steps involved in tobacco transformation and plant regeneration

In the present investigation, transgenic tobacco plants carrying *gus* gene under the control of TP, FP and CaMV35S and expressing full-length ricin gene under ricin truncated and full-length promoters have been developed. This method produced large number of putative transgenic shoots on selection medium.

The transgenic shoots obtained were maintained on elongation medium and finally elongated shoots were transferred to half MS medium with selection pressure. There was no morphological difference between control and transformed shoots. In earlier reports also, it has been concluded that the expression of ricin gene in tobacco did not have deleterious effects on the phenotype of the plant as morphologically normal transgenic tobacco plants expressing full-length ricin were realized (Frigerio *et al.*, 1998). On the shoot induction medium, multiple shoots were obtained from a single explant as a cluster and so it was difficult to ascertain whether the shoots had resulted from independent transformations or whether there were copies of the same transformation event. Detailed molecular analysis can reveal the ontogeny of such shoots.

4.2.1.2 Molecular confirmation of the putative transgenic tobacco plants

(A). harbouring *gus* under TP/FP/35S/ promoter-less basal vector (pCAMBIA) in pCAMBIA 1391Z

(a). PCR analyses

Putative tobacco transgenics were analyzed for transgenicity by confirming the presence of

(a). TP (310 bp) (Fig. 4.11A)

(b). TP (310 bp) and *uid*A (*gus*) gene (1200 bp) (Fig. 4.11B)

(c). plant selection marker i.e. *hpt*II conferring hygromycin resistance (1094 bp) (Fig. 4.11C)

This construct yielded twenty plants which could root well and only three plants were analyzed. Genomic DNA extracted from putative transgenics was subjected to PCR analysis. Out of the three analyzed, only two gave consistent amplification across different sets of primers and thus confirmed presence of the component sequences of the construct used for transformation. Both positive and negative controls and the blank gave the expected results indicating that the PCR conditions were ideal.

Putative tobacco transgenics harbouring *gus* under FP in pCAMBIA 1391Z were analyzed for transgenicity by confirming the presence of

(a). FP (932 bp) (Fig.4.12A)

(b). *uid*A (*gus*) gene (1200 bp) (Fig.4.12B)

(c). plant selection marker i.e. *hpt*II conferring hygromycin resistance (1094 bp) (Fig. 4.12C)

This construct yielded twenty plants which could root well and all could be analyzed. Genomic DNA extracted from putative transgenics was subjected to PCR analysis. Out of the twenty analyzed, only fifteen gave consistent amplification across different sets of primers and thus confirmed presence of the component sequences of the construct used for transformation. Both positive and negative controls and the blank gave the expected results indicating that the PCR conditions were ideal.

Putative tobacco transgenics harbouring *gus* under 35S in pCAMBIA 1391Z were analyzed for transgenicity by confirming the presence of

(a). *uid*A (*gus*) gene (1200 bp) (Fig. 4.13A)

(b). plant selection marker i.e. *hpt*II conferring hygromycin resistance (1094 bp) (Fig. 4.13B)

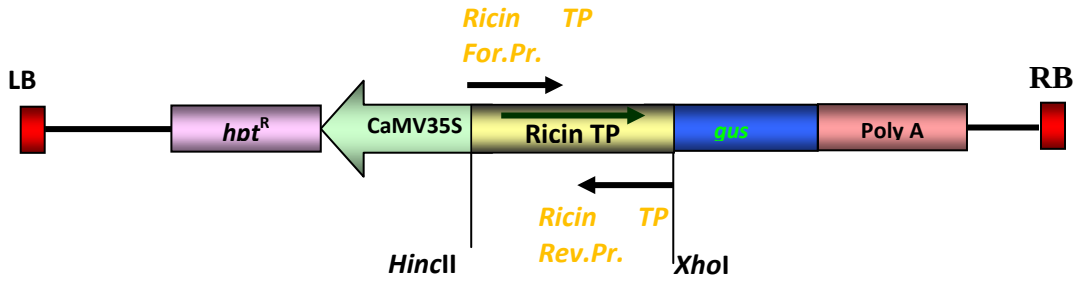
This construct yielded fifteen plants which could root well , twelve of them survived the transfer to soil and could be analyzed. Genomic DNA extracted from putative transgenics was subjected to PCR analysis. Out of the twelve analyzed, only eleven gave consistent amplification across different sets of primers and thus confirmed presence of the component sequences of the construct used for transformation. Both positive and negative controls and the blank gave the expected results indicating that the PCR conditions were ideal.

Putative tobacco transgenics harbouring *gus* under 35S in pCAMBIA 1391Z were analyzed for transgenicity by confirming the presence of

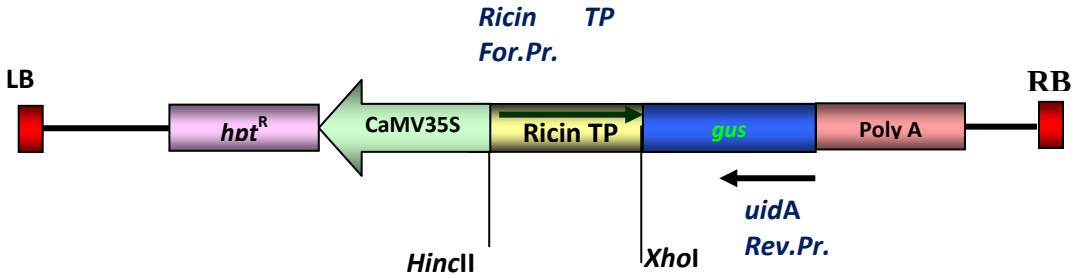
a). *uid*A (*gus*) gene (1200 bp) (Fig. 4.14A)

(b). plant selection marker i.e. *hpt*II conferring hygromycin resistance (1094 bp) (Fig. 4.14B

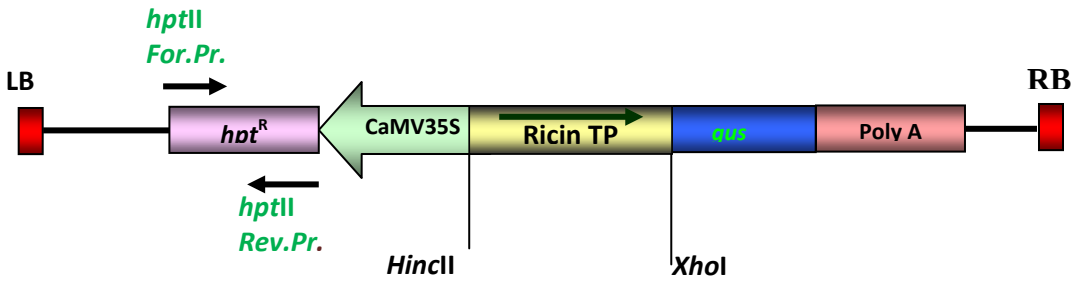
This construct yielded fifteen plants which could root well and eight could be analyzed. Genomic DNA extracted from putative transgenics was subjected to PCR analysis. Out of the eight analyzed, all eight gave consistent amplification across different sets of primers and thus confirmed presence of the component sequences of the construct used for transformation. Both positive and negative controls and the blank gave the expected results indicating that the PCR conditions were ideal.



Panel A: pCAMBIA 1391Z ricin truncated promoter (TP)



Panel B: pCAMBIA 1391Z ricin truncated promoter (TP)



Panel C: pCAMBIA 1391Z ricin truncated promoter (TP)

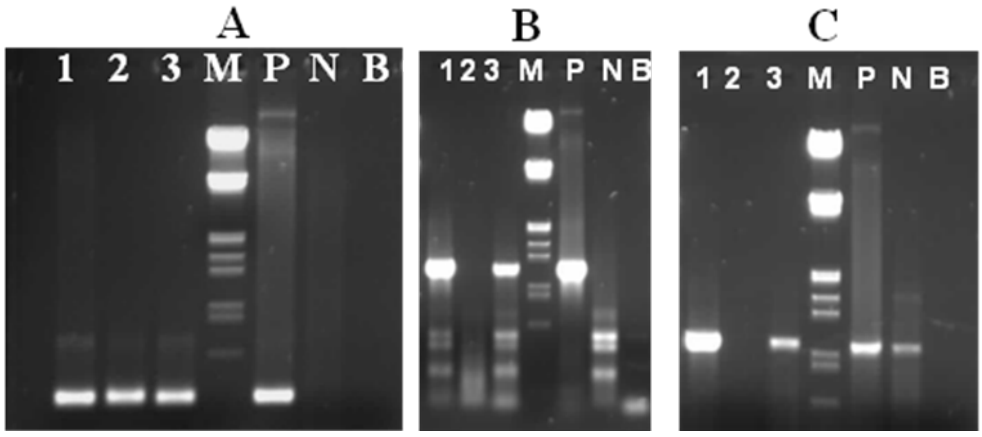


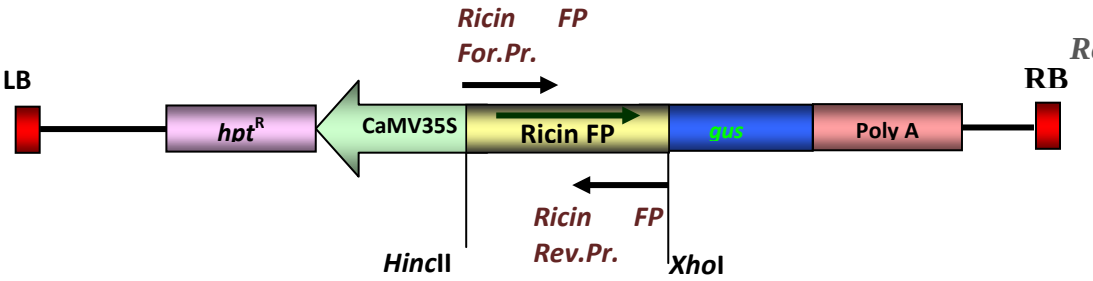
Fig.4. 11. Panel A – Confirmation of the presence of truncated ricin promoter in tobacco transgenics

Panel B – Confirmation of the presence of truncated ricin promoter and *uidA* gene in tobacco transgenics

Panel C – Confirmation of the presence of *hptII* gene in tobacco transgenics

For panels A, B and C :

Lane 1-3 : Tobacco transgenics harbouring 1391Z TP2 construct
M : *Eco*RI & *Hind*III digested λ DNA
P : Positive control (construct in *Agrobacterium* strain LBA4404)
N : Negative control (untransformed or wild or normal tobacco)
B : Blank (without template DNA)



pCAMBIA 1391Z ricin full-length promoter (FP)

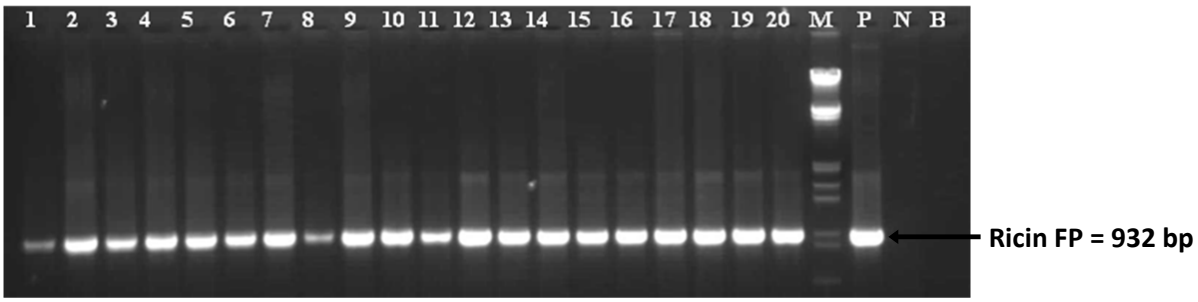
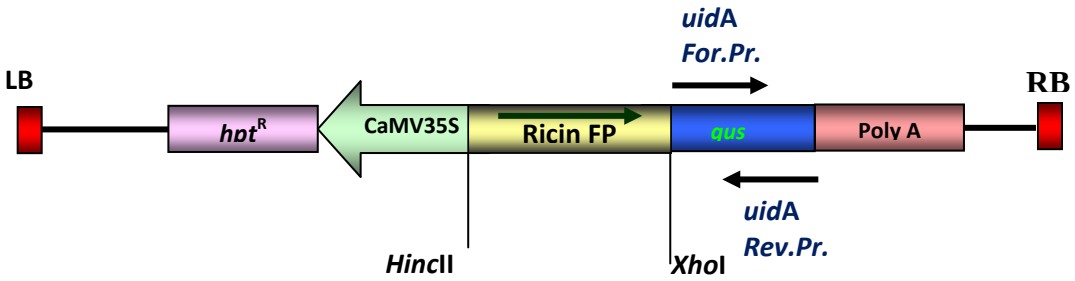


Fig. 4.12A. Confirmation of the presence of full-length ricin promoter through PCR in transgenic tobacco harbouring 1391Z FP2

Lane 1 – 20 : Transgenic tobacco showing presence of full-length ricin promoter
P : Positive control (construct in *Agrobacterium* strain LBA4404)
N : Negative control (untransformed or wild or normal tobacco)
B : Blank (without template DNA)



pCAMBIA 1391Z ricin full-length promoter (FP)

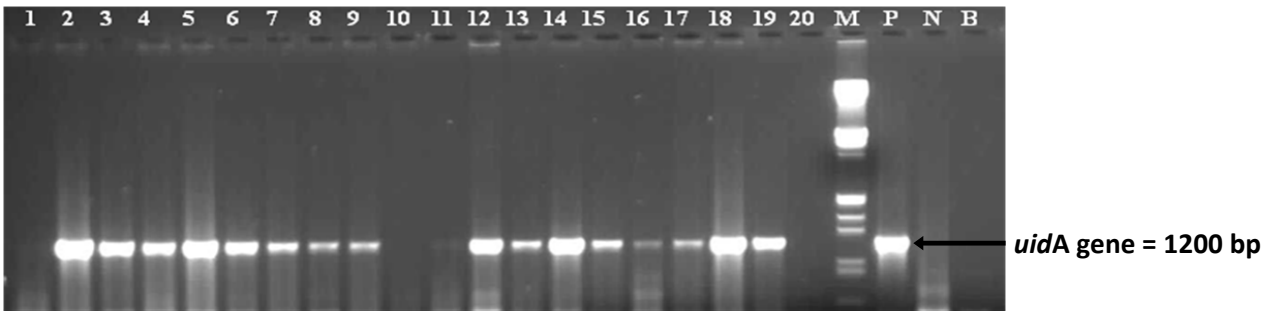
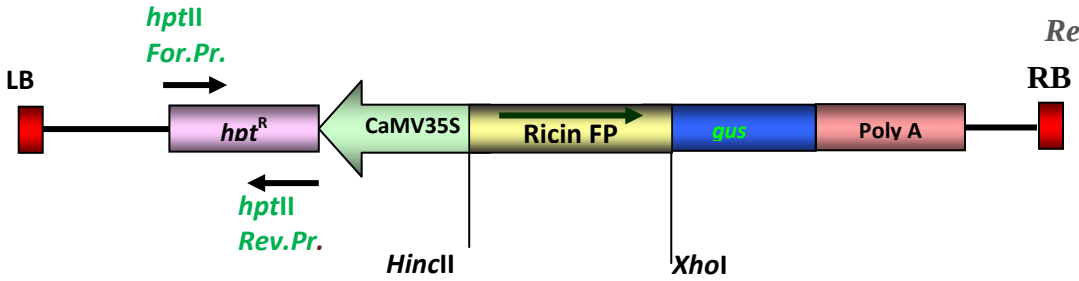


Fig. 4.12B. Confirmation of the presence of *uid*A gene through PCR in transgenic tobacco harbouring 1391Z FP2

Lane 1 – 20 : Transgenic tobacco showing presence of *uid*A gene
P : Positive control (construct in *Agrobacterium* strain LBA4404)
N : Negative control (untransformed or wild or normal tobacco)
B : Blank (without template DNA)



pCAMBIA 1391Z ricin full-length promoter (FP)

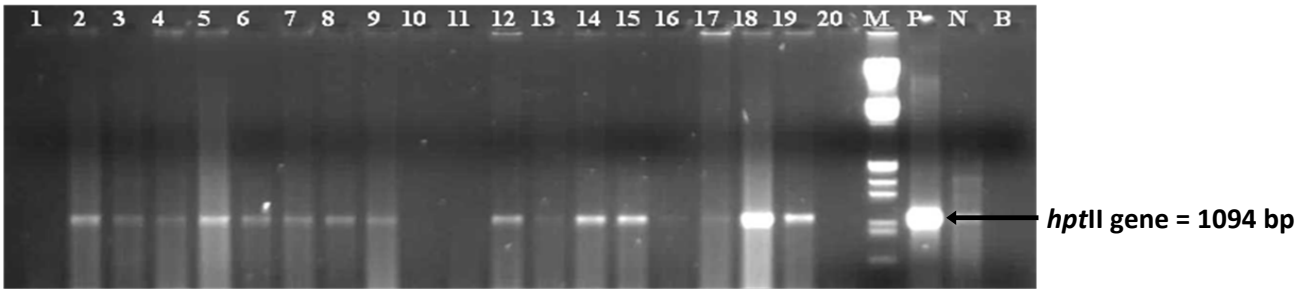
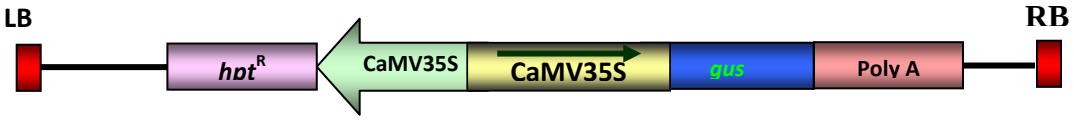


Fig. 4.12C. Confirmation of the presence of *hpt*II gene through PCR in transgenic tobacco harbouring 1391Z FP2

Lane 1 – 20 : Transgenic tobacco showing presence of *hpt*II gene
P : Positive control (construct in *Agrobacterium* strain LBA4404)
N : Negative control (untransformed or wild or normal tobacco)
B : Blank (without template DNA)



pCAMBIA 1391Z CaMV35S

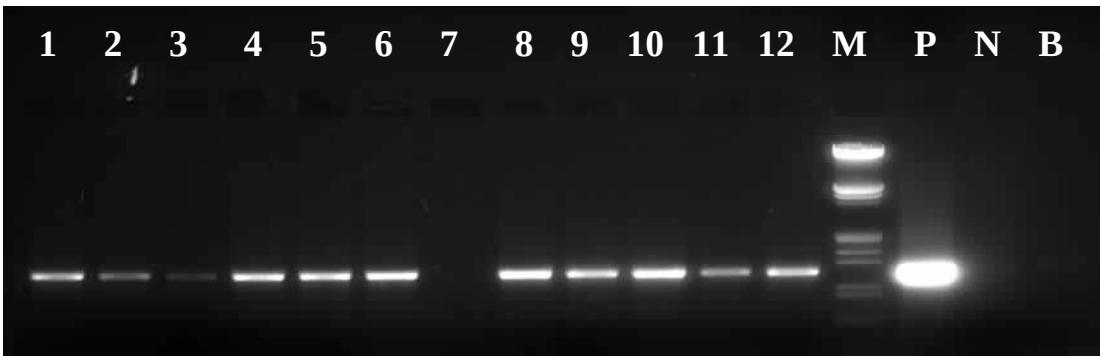
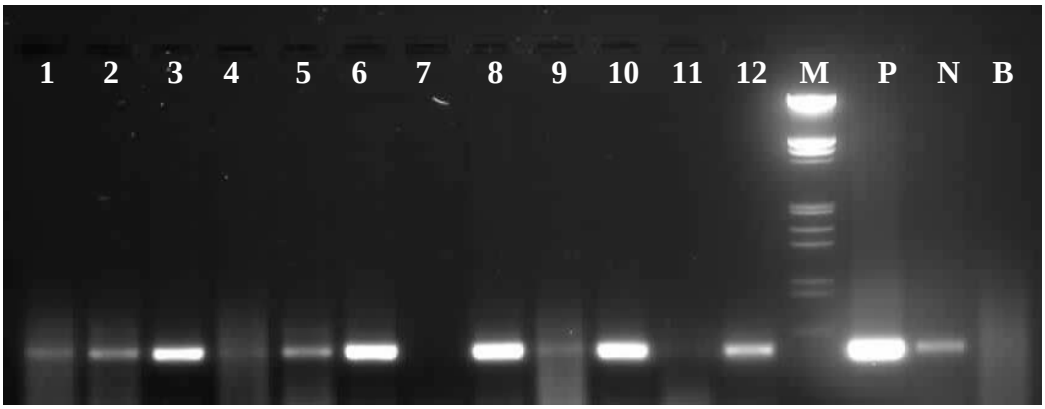


Fig. 4.13A. Confirmation of the presence of *uidA* through PCR in transgenic tobacco harbouring 1391Z CaMV35S

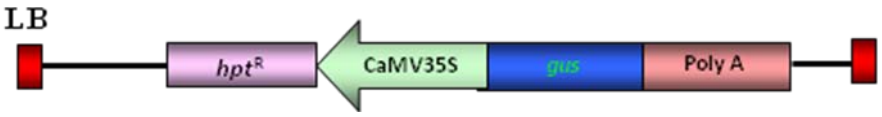
Lane 1 – 20 : Transgenic tobacco showing presence of *hpt*II gene
Lane M : E+H digested λ DNA
P : Positive control (construct in *Agrobacterium* strain LBA4404)
N : Negative control (untransformed or wild or normal tobacco)
B : Blank (without template DNA)



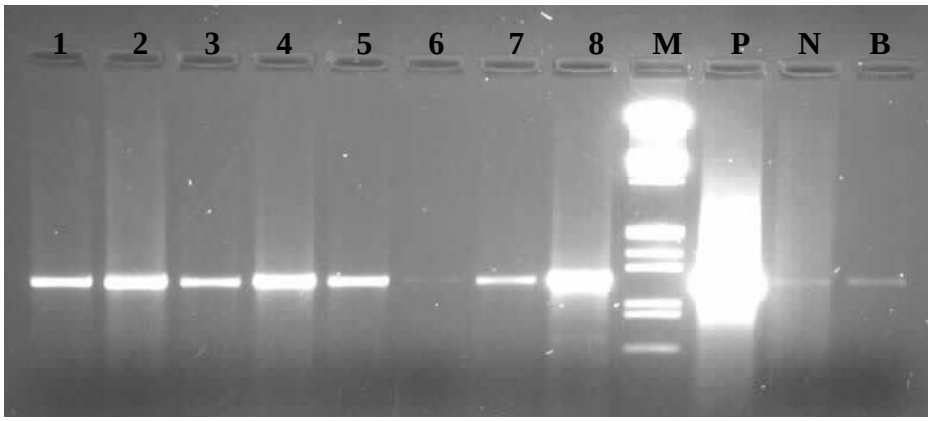
***hpt* gene = 480 bp**

Fig. 4.13B. Confirmation of the presence of *hpt* gene through PCR in transgenic tobacco harbouring 1391Z CaMV 35S

Lane 1 – 20 : Transgenic tobacco showing presence of *hpt*II gene
Lane M : E+H digested λ DNA
P : Positive control (construct in *Agrobacterium* strain LBA4404)
N : Negative control (untransformed or wild or normal tobacco)
B : Blank (without template DNA)



pCAMBIA 1391Z



***uidA* gene = 1200 bp**

Fig. 4.14A. Confirmation of the presence of *uidA* gene through PCR in transgenic tobacco

Lane 1 – 20 : Transgenic tobacco showing presence of *uidA* gene
Lane M : E+H digested λ DNA
P : Positive control (construct in *Agrobacterium* strain LBA4404)
N : Negative control (untransformed or wild or normal tobacco)
B : Blank (without template DNA)

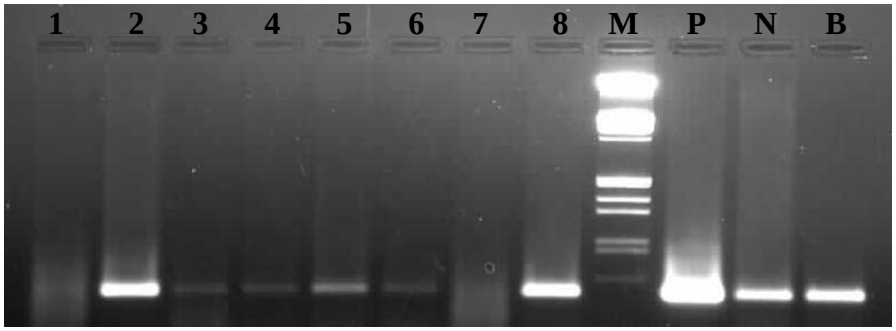


Fig. 4.14B. Confirmation of the presence of *hpt* gene through PCR in transgenic tobacco harbouring 1391Z

Lane 1 – 20 : Transgenic tobacco showing presence of *hpt*II gene
Lane M : E+H digested λ DNA
P : Positive control (construct in *Agrobacterium* strain LBA4404)
N : Negative control (untransformed or wild or normal tobacco)
B : Blank (without template DNA)

(B). expressing full-length ricin (FL) gene under TP/FP through PCR analyses

Putative tobacco transgenics harbouring ricin full-length (FL) gene under TP in pCAMBIA 2300 were analyzed for transgenicity by confirming the presence of

(a). plant selection marker i.e. *npt*II conferring kanamycin resistance (700 bp) (Fig. 4.15)

(b)TP (310 bp) (Fig.4.16)

(c). Ricin full-length gene (1732 bp) (Fig.4.17)

This construct yielded eighteen plants which could root well and all could be analyzed. Genomic DNA extracted from putative transgenics was subjected to PCR analysis. Out of the eighteen analyzed, only nine gave consistent amplification across different sets of primers and thus confirmed presence of the component sequences of the construct used for transformation. Both positive and negative controls and the blank gave the expected results indicating that the PCR conditions were ideal.

Putative tobacco transgenics harbouring ricin full-length (FL) gene under FP in pCAMBIA 2300 were analyzed for transgenicity by confirming the presence of

(a). plant selection marker i.e. *npt*II conferring kanamycin resistance (700 bp) (Fig.4.18)

(b) Ricin full-length gene (1732 bp) (Fig.4.19)

(c). FP (932 bp) (Fig.4.20)

(d). FP (932 bp) and Ricin full-length gene (2664 bp) (Fig.4.21)

This construct yielded 59 plants which could root well and all could be analyzed. Genomic DNA extracted from putative transgenics was subjected to PCR analysis. Out of the fifty nine analyzed, only twenty gave consistent amplification across different sets of primers and thus confirmed presence of the component sequences of the construct used for transformation. Both positive and negative controls and the blank gave the expected results indicating that the PCR conditions were ideal.

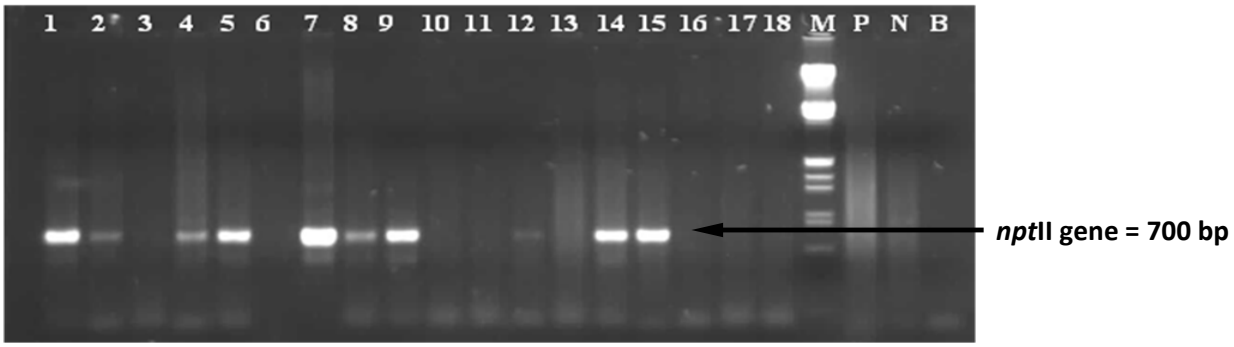
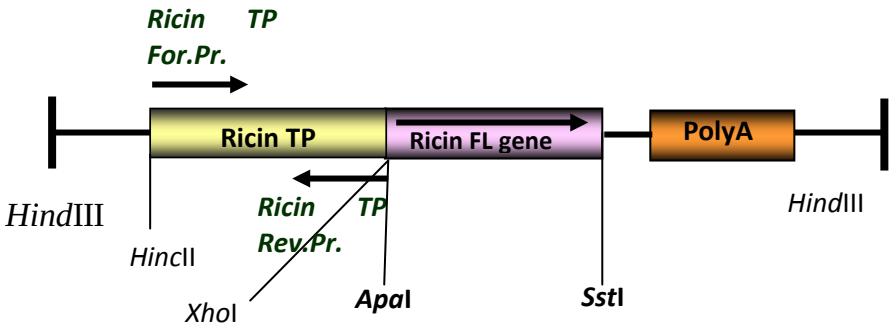


Fig. 4.15: Confirmation of the presence of *nptII* gene through PCR in tobacco transgenics harbouring 2300 TP R1.7 kb construct

Lane 1-18 : Putative transgenic tobacco harbouring the said gene construct
M : *Eco*RI & *Hind*III digested λ DNA
P : Positive control (construct in *Agrobacterium* strain LBA4404)
N : Negative control (untransformed or wild or normal tobacco)
B : Blank (without template DNA)



pRT100 Ricin TP FL ricin gene construct

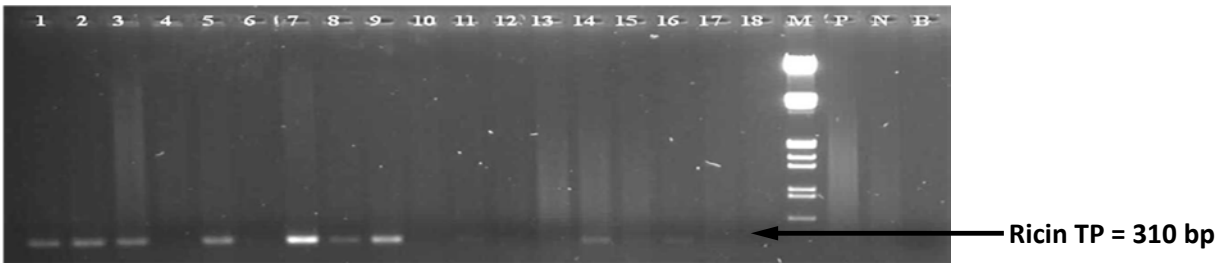
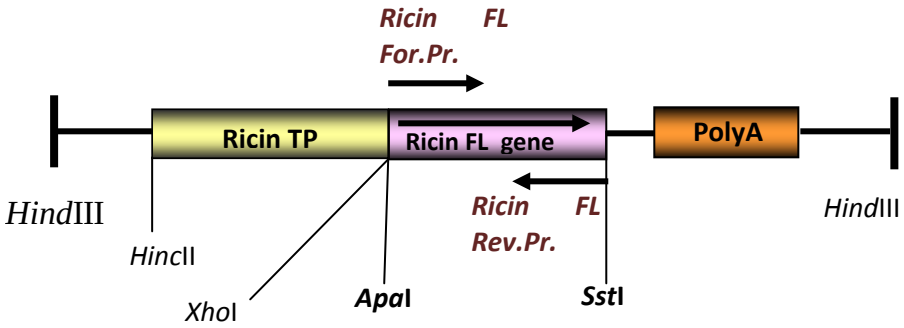


Fig. 4.16: Confirmation of the presence of truncated promoter through PCR in transgenic tobacco harbouring TP R1.7 kb

Lane 1 – 20 : Transgenic tobacco showing presence of ricin truncated promoter
P : Positive control (construct in *Agrobacterium* strain LBA4404)
N : Negative control (untransformed or wild or normal tobacco)
B : Blank (without template DNA)



pRT100 Ricin TP FL ricin gene construct

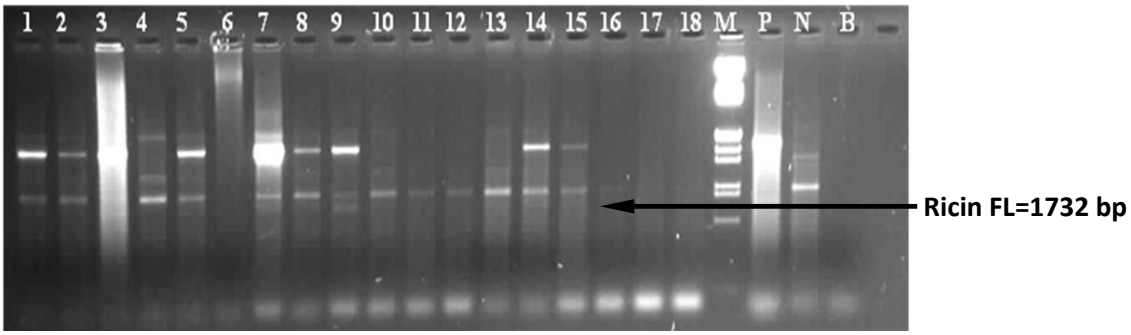


Fig. 4.17: Confirmation of ricin 1.7 kb gene through PCR in transgenic tobacco

Lane 1 – 20 : Transgenic tobacco showing presence of ricin 1.7 kb gene
P : Positive control (construct in *Agrobacterium* strain LBA4404)
N : Negative control (untransformed or wild or normal tobacco)
B : Blank (without template DNA)

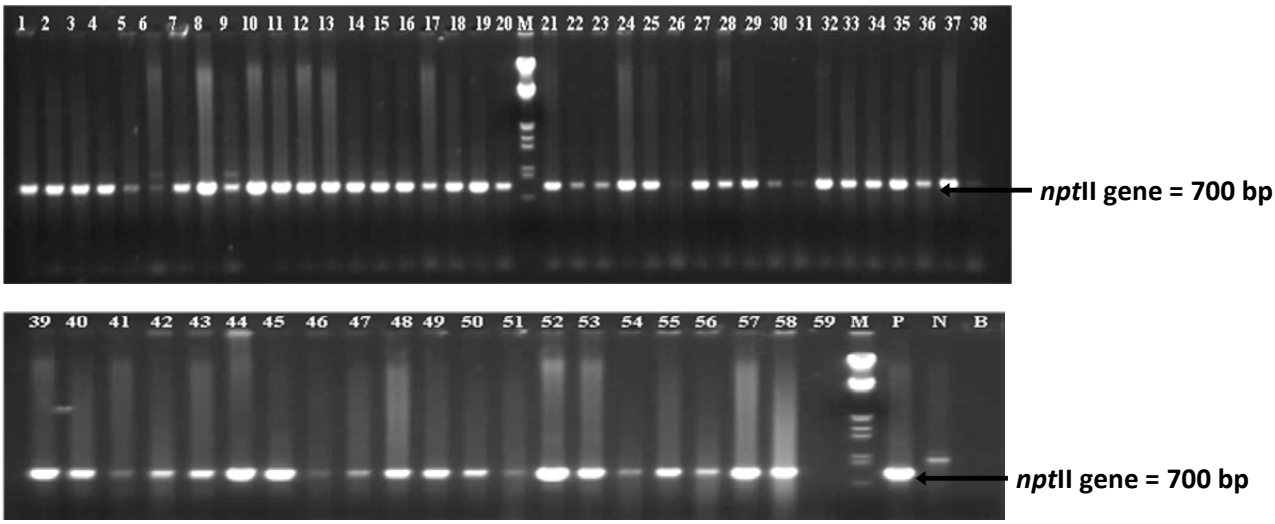
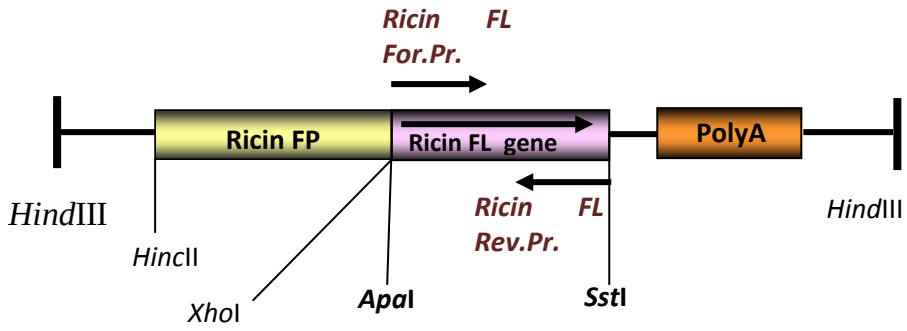


Fig. 4.18: Confirmation of presence of *npt*II gene through PCR in transgenic tobacco harbouring 2300 FP R1.7 kb construct

Lane 1 – 20 : Transgenic tobacco showing the presence of *npt*II gene
M : *Eco*RI & *Hind*III digested λ DNA
Lane 21- 59 : Transgenic tobacco showing the presence of *npt*II gene
P : Positive control (construct in *Agrobacterium* strain LBA4404)
N : Negative control (untransformed or wild or normal tobacco)
B : Blank (without template DNA)



pRT100 Ricin FP FL ricin gene construct

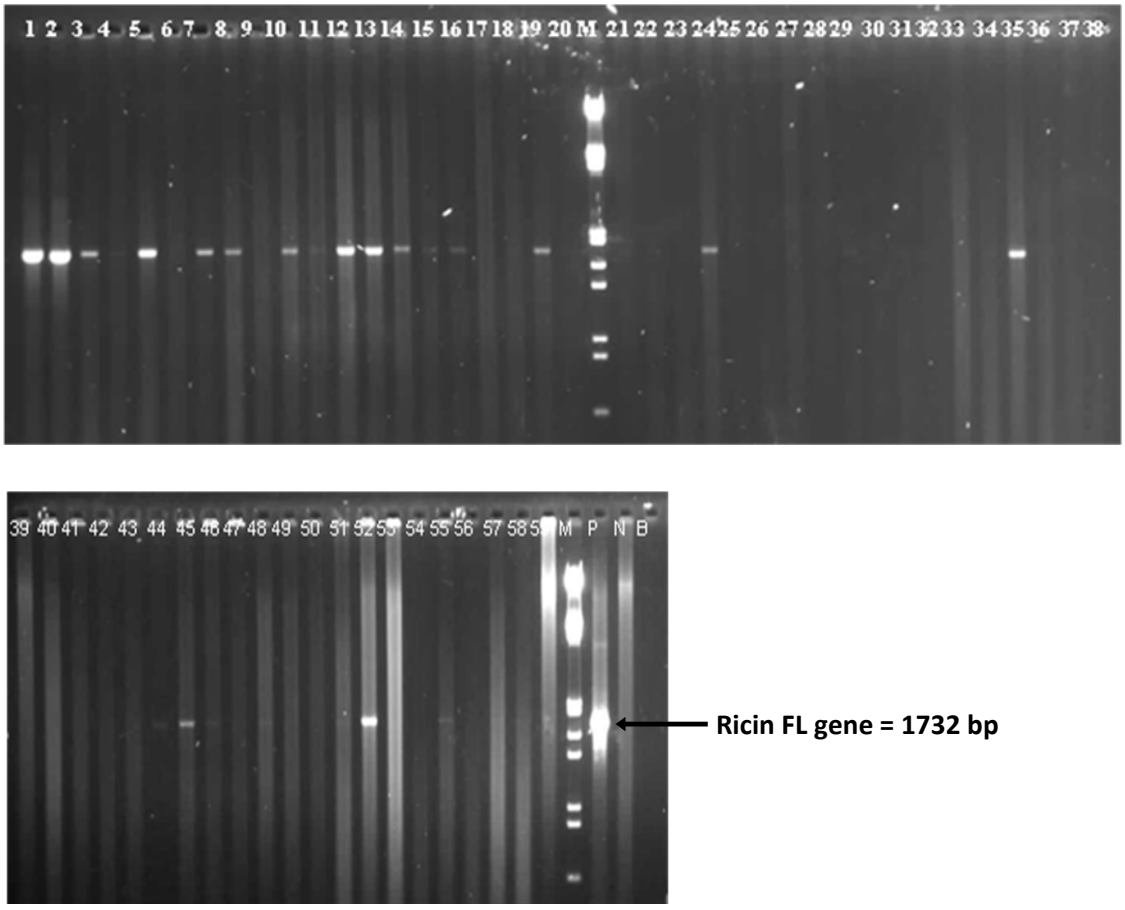
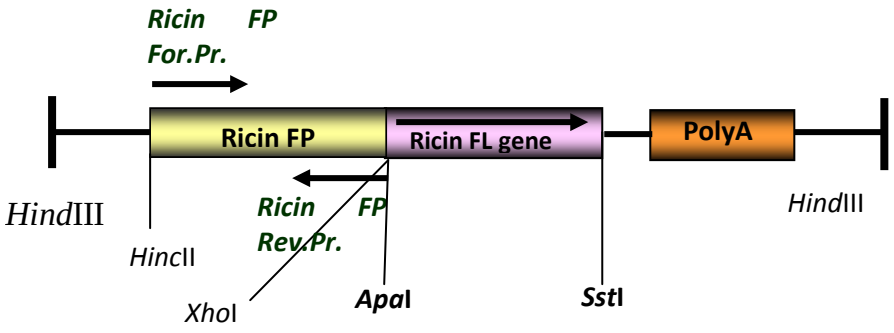
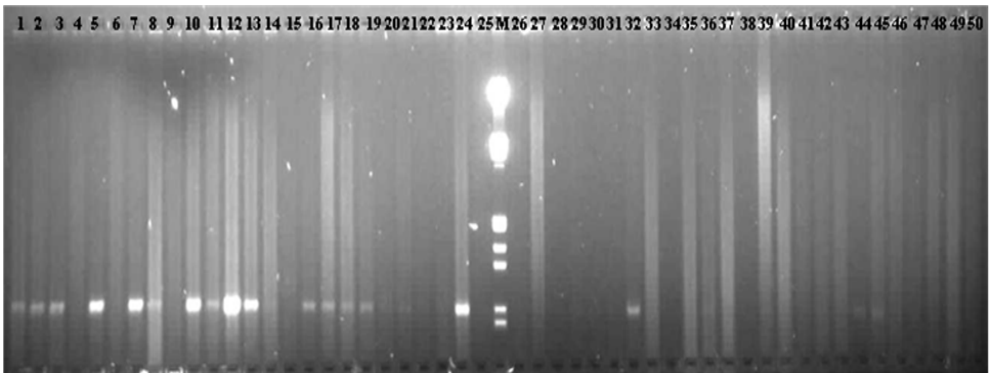


Fig. 4.19: Confirmation of the presence of ricin 1.7 kb gene through PCR in transgenic tobacco carrying FP R1.7 kb construct

Lane 1 – 20 : Transgenic tobacco harbouring full length ricin gene
M : *Eco*RI & *Hind*III digested λ DNA
Lane 21 – 59 : Transgenic tobacco harbouring full length ricin ricin promoter
P : Positive control (construct in *Agrobacterium* strain LBA4404)
N : Negative control (untransformed or wild or normal tobacco)
B : Blank (without template DNA)



pRT100 Ricin FP FL ricin gene construct



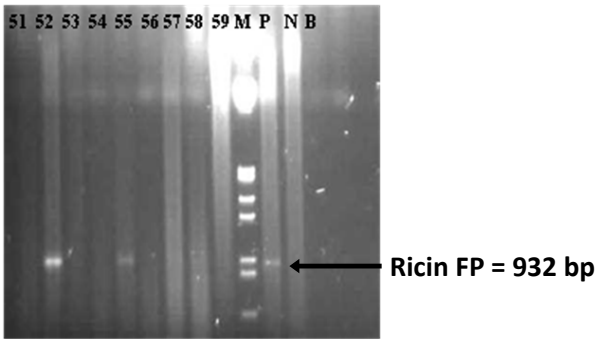
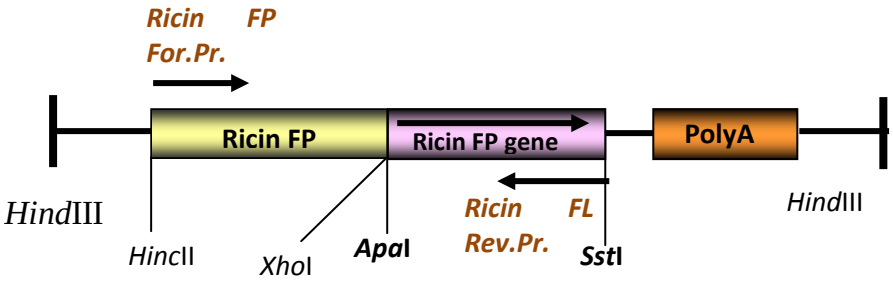
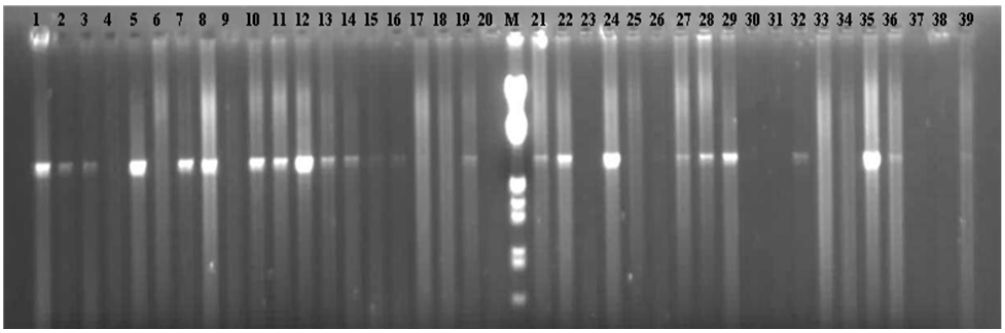


Fig. 4.20: Confirmation of the presence of ricin full-length promoter through PCR in transgenic tobacco carrying FP R1.7 kb construct

Lane 1 – 25 : Transgenic tobacco harbouring full length ricin promoter
M : *Eco*RI & *Hind*III digested λ DNA
Lane 21 – 59 : Transgenic tobacco harbouring full length ricin ricin promoter
P : Positive control (construct in *Agrobacterium* strain LBA4404)
N : Negative control (untransformed or wild or normal tobacco)
B : Blank (without template DNA)



pRT 100 Ricin FP FL ricin gene construct



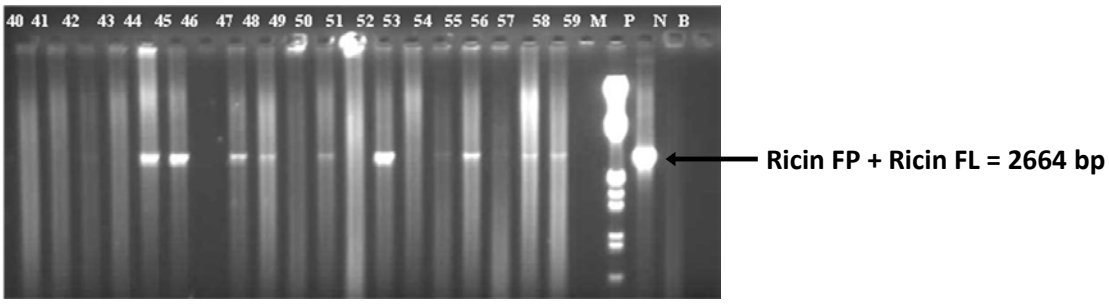


Fig. 4.21: Confirmation of the presence of full length ricin promoter and full-length 1.7 kb ricin gene through PCR in transgenic tobacco harbouring 2300 FP R1.7 kb construct

Lane 1 – 20 : Transgenic tobacco harbouring the full length ricin gene downstream of ricin FP
M : *Eco*RI & *Hind*III digested λ DNA
Lane 21 – 59 : Transgenic tobacco harbouring the full length ricin gene downstream of ricin FP
P : Positive control (construct in *Agrobacterium* strain LBA4404)
N : Negative control (untransformed or wild or normal tobacco)
B : Blank (without template DNA)

4.2.1.3 Confirmation of the putative transgenic tobacco plants through GUS histochemical assay

The presence of the transgene in the putative transformants for constructs

1). LBA4404:: pCAMBIA 1391Z TP

2). LBA4404:: pCAMBIA 1391Z FP

3). LBA4404:: pCAMBIA 1391Z CaMV35S

4). LBA4404:: pCAMBIA 1391Z

was confirmed by the blue colouration observed after GUS staining of the different parts of the acclimatized plants like leaf and flower (different parts of the flower were separated and tested for GUS activity).

The GUS activity though very pronounced and intense for the endospermic seeds of the putative transgenic tobacco (construct 1 followed by construct 2 in terms of the expected intensity), could not be documented. Nevertheless, GUS activity was documented for other parts of the plant. The intensity of blue colouration was very prominent for various parts of the plant including leaf and flower, in case of plants transformed with LBA4404:: pCAMBIA 1391Z CaMV35S and LBA4404:: pCAMBIA 1391Z. This was as expected as 35S is known to have varying activity across different tissues. The control (untransformed tobacco) did not show any blue colouration across different tissues assayed (Plate 2).

4.2.2 Transient assays with the developing seeds of castor

Since the main aim of the present investigation was to effect PTGS of ricin and RCA in the endospermic tissue of castor, transient assays were performed with the above four constructs to validate the tissue-specificity of the ricin promoters in castor. The assays included microprojectile bombardment of developing seeds of castor (47 DAP) and *Agrobacterium*-mediated transformation of developing seeds of castor (47 DAP). The transient assays were performed with developing seeds of castor *i.e.,* 47 DAP since the transcripts of ricin and RCA are known to peak at 47 DAP (Chen et al., 2005).

4.2.2.1 Particle gun-mediated bombardment of castor seeds

Seeds of the cultivar DCS-9 (Jyothi) were used in all the experiments. The developing seeds of castor were bombardment using gene gun model “Biolistic PDS-1000/He Particle Delivery System”. The plasmid DNA concentration of each of the above four constructs used for bombardment was 2 μg/μl. The parameters tested included Helium pressure of 1100psi, target distance of 6cm and a total of 3 replications for each of the four constructs. A total of 9

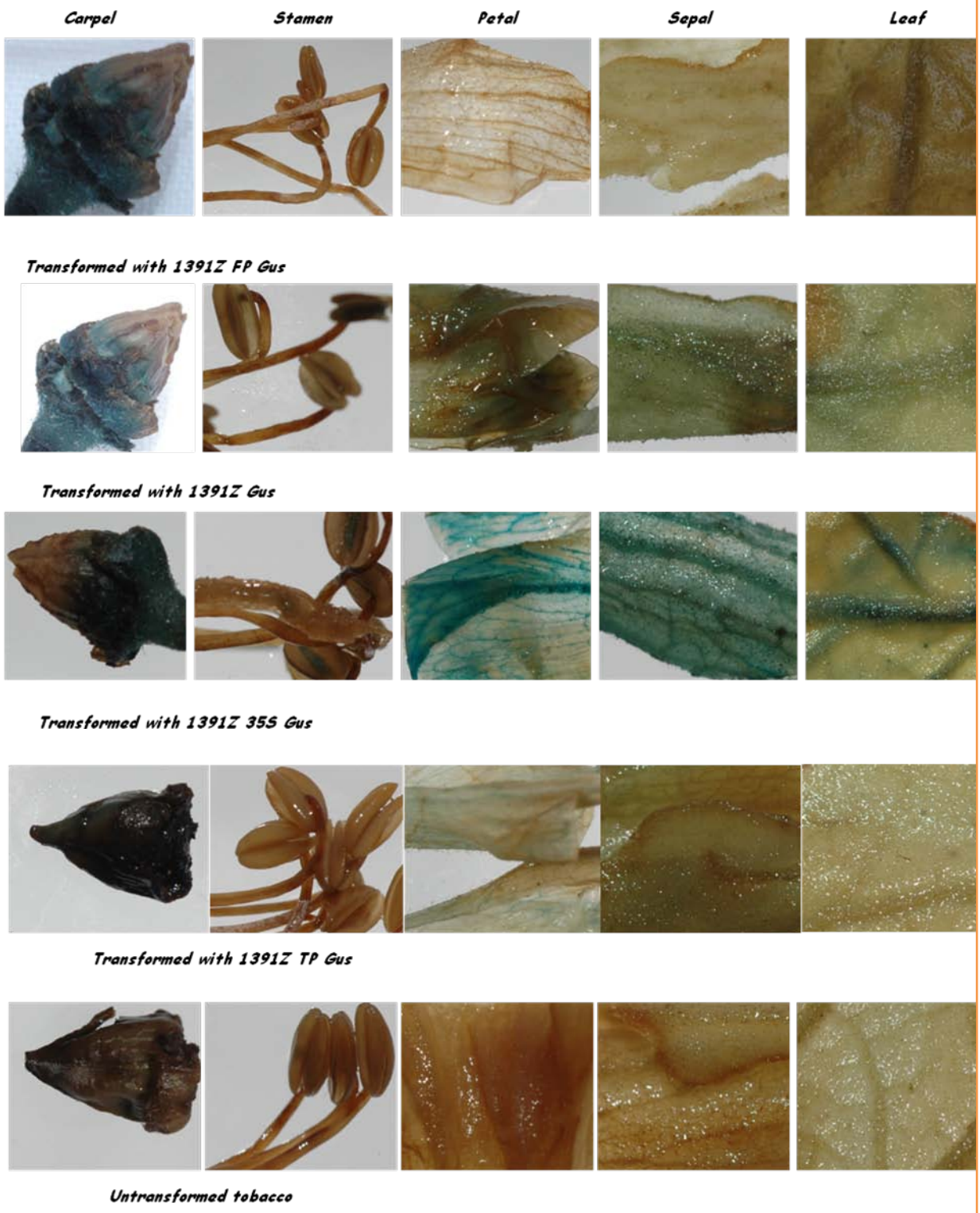


Plate 2: Assessing the tissue specific expression of ricin promoter through gus assays

seeds were bombarded per replication. Two controls were also included in the study namely the uncoated (without DNA) bombardment seeds and unbombarded seeds.

The seeds bombarded with 1391Z FP yielded blue colouration all along the endosperm, but prominent, thick, concentrated blue foci could not be visualized. With 1391Z TP construct, the intensity of blue colour of endosperm was little less when compared with the one bombarded with 1391Z FP. Construct 1391Z 35S gus yielded blue colour which was diffused all along the endosperm. Same was true in case of the basal promoter-less binary vector, 1391Z, though the intensity being less compared to 1391Z 35S gus. The control, uncoated but bombarded yielded very faint blue colouration. The unbombarded control did not show any colouration.

Though three replications were performed for each of the constructs, yet there appears to be lot of inconsistency in the patterns of blue colouration as a result of bombardment. An attempt has been made to assess relative expression levels, qualitatively by observing the density of GUS staining rather than rigorously quantitate numbers of stained spots (Plate 3).

4.2.2.1 *Agrobacterium*-mediated transformation of castor seeds

Decoated, sterilized seeds of castor (47 DAP) of variety DCS-9 were subjected to wounding followed by co-cultivation with each of the four constructs. After co-cultivation, they were assayed for gus activity. The controls included unwounded & untreated (without co-cultivation) seeds and wounded & untreated seeds.

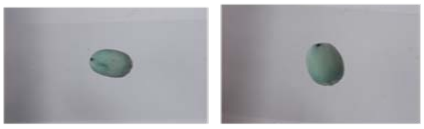
The seeds wounded and co-cultivated with 1391Z FP yielded thick blue colouration all along the endosperm. With 1391Z TP construct, the intensity of blue colour of endosperm was similar to the one bombarded with 1391Z FP. Construct 1391Z 35S gus yielded blue colour which was diffused all along the endosperm. Same holds true in case of the basal promoter-less binary vector, 1391Z, though the intensity being less compared to 1391Z 35S gus. The control, wounded and untreated, yielded very faint blue colouration. The unwounded and untreated control resisted any change and was true to its type (Plate 4).

Though this approach could be a very incipient and unsophisticated approach to address the tissue-specificity of constructs carrying a variety of regulatory regions, nevertheless, it is a quick and easy method to assess promoter activity.

Coated & bombarded castor seed with pCAMBIA 1391Z gus

Coated & bombarded castor seed with pCAMBIA 1391Z P gus

Coated & bombarded castor seed with pCAMBIA 1391Z 35S gus



Coated & bombarded castor seed with pCAMBIA 1391Z TP gus

Controls

47 DAP Unbombarded castor seed

47 DAP Uncoated bombarded castor seed

Plate 3: Particle gun-mediated bombardment of developing seeds of castor

Unwounded & untreated

Wounded & untreated

1391Z FP gus

1391Z TP gus

1391Z

1391Z 35 gus

Plate 4: Agrobacterium-mediated transformation of seeds of castor

4.3 Development of generic PTGS and PTGS constructs for silencing ricin and RCA in castor

The present investigation was undertaken to develop transgenic castor with reduced/nil levels of ricin and RCA. Towards this end, gene constructs, which could induce posttranscriptional gene silencing of ricin and RCA, have been developed under the control of CaMV 35S and ricin FP. The development of constructs involved three different strategies utilizing the gene sequence of A chains and B chains of ricin and RCA. One of the strategies included the conventional antisense technology, in which the full-length antisense version of ricin/RCA was cloned between constitutive promoter (CaMV 35S/ ricin FP) and 35S polyA tail. The other two strategies, aimed at silencing the ricin/RCA through the production of dsRNA which triggers targeted degradation of ricin/RCA transcripts through 'dicer' and 'RISC' mediated mechanisms. This exercise involved cloning of inverted repeats of the selected parts of A chain (RA) and B chain (RB) of ricin/RCA (380 bp in case of ricin/RCA RA and 330 bp in case of ricin/RCA RB) separated by the catalase intron I. This is surmised to result in a hairpin double-stranded RNA transcript which would eventually be acted upon by 'dicer' and 'RISC' yielding siRNAs targeted against cognate transcripts for degradation. The other strategy was executed by cloning antisense version of selected part of A chains and B chains of ricin/RCA upstream of an inverted repeat of a heterologous 3'-UTR (untranslated region) *viz.*, 35S polyA, separated by catalase intron, so that when expressed in plants, the resultant transitive RNAi could induce sequence specific degradation of ricin/RCA transcripts.

The appropriate sequences of A and B chains of ricin/RCA, for effective posttranscriptional silencing of ricin/RCA were selected, based on the standard guidelines and criteria. The primers were designed based on the sequence information of ricin/RCA and the PCR amplified using the specific primers were cloned in InsT/A (T/A cloning vector).

The gene sequences were excised out from InsT/A vector and cloned into pRT100 and its derived generic vectors. The full-length ricin/RCA sequence was cloned in antisense orientation in pRT100 vector using *Sst*I and *XhoI* enzymes. In case of pRT 100 SHUTR RA / pRT 100 SHUTR RB, a selected portion of gene sequence was cloned in pRT 100 catalase intron PolyA, a pRT100 derived vector, with inverted repeat of 3'UTR (35S PolyA) separated by a functional catalase intron, using *Nco*I and *Xba*I restriction enzymes. The constructs pRT 100 ihp RA and pRT 100 ihp RB were cloned using *XbaI* for 'sense' cloning and *Nco*I and *XhoI* for 'antisense' cloning. The construction of pRT 100 ihp RA / pRT 100 ihp RB involved a two step strategy where ricin/RCA's RA and ricin/RCA's RB were first cloned in antisense orientation upstream of catalase intron in pRT 100 catalase intron vector

followed by cloning of sense ricin/RCA's RA and sense ricin/RCA's RB downstream of the catalase intron.

All the clones in the pRT100 vector were confirmed by PCR and restriction analyses. The confirmed clones were excised out from pRT100 and pRT100 derived generic vectors with *Hind*III digestion, and they were cloned in plant transformation vector, pCAMBIA 1300, respectively. The resultant clones of binary vector were confirmed for the gene constructs using PCR and restriction analyses.

4.3.1 Isolation of catalase I intron from pCAMBIA 1305.2

The castor specific catalase I intron was isolated from pCAMBIA 1305.2 using *Sst*I forward primer and *KpnI* reverse primer. While designing primers for the amplification of catalase I intron, care was taken to include the intron-exon junction and two bases of exon on either side of intron. This is highly indispensable as exon-intron splice junctions are very crucial for the process of splicing. The amplification of catalase I intron gave the expected size amplicon of 210 bp.

4.3.2 Development of generic ihp vector (pRT100 35S catalase intron)

The T/A catalase intron (from above) was cloned in pRT100 using *SstI* and *KpnI*. This led to a **generic ihp spliced RNA** silencing vector (pRT100 35S catalase intron) i.e. a ready-to-use vector wherein one can clone their choice of target gene for the purpose of silencing. The resultant clones were confirmed by restriction with *HindIII*. This gave the expected fragment of 910 bp.

4.3.3 Isolation of polyA from pRT100 35S

Poly A was isolated from pRT100 35S by PCR amplification with *Nco*I forward primer and *Sst*I reverse primer. A 220 bp amplicon was realized. The fragment was then cloned in T/A vector.

4.3.4 Development of generic SHUTR vector (pRT 100 35S poly A catalase intron)

After confirmation of cloning in T/A vector with *EcoRI* and *HindIII* (this digestion gave 300 bp), poly A amplicon was directionally cloned in pRT100 in antisense orientation in frame with catalase intron and sense poly A. The resultant construct was designated as pRT100 35S poly A catalase intron- **generic SHUTR vector**. This generic vector facilitates one-step cloning of the target gene in antisense orientation and exploits the basic principles of

transitive RNA silencing. The cloning of the generic SHUTR vector was confirmed by restriction with *HindIII*. This yielded the expected 1130 bp fragment. Further confirmation of the construct was through PCR and it confirmed cloning of poly A upstream of catalase intron in pRT100 35S backbone.

4.3.5 PCR amplification of ricin/RCA sequence

The ricin/RCA gene sequence of full length and selected part of it were PCR amplified using the respective primers designed for them. The full-length sequence (for antisense construct) was amplified by '*SstI* ricin Forward' and '*XhoI XbaI* Reverse' and the full-length coding ricin/RCA sequence (for sense expression in tobacco) was amplified by '*ApaI* Forward' and '*SstI* Reverse' primers. Portions of ricin A chain- (RA)-380 bp and ricin B chain- (RB)-330 bp were PCR amplified from castor genomic DNA using '*Nco*I *BamH*I Forward' and '*Xba*I *Xho*I Reverse' primers.

Once the expected amplicons were realized through a preparative PCR, a large volume PCR was carried out. The amplification of full-length ricin/RCA (both for antisense and sense expression constructs) sequence gave the expected size amplicon of 1730 bp. The size was confirmed as seen against a standard size maker.

4.3.6 Cloning of sequences in T/A cloning vector

The PCR products were first cloned in T/A cloning vector and then directional cloning was carried out in pRT100.

The PCR products of all the sequences of the ricin/RCA gene were purified from agarose gel by using QIAquick® gel extraction kit (QIAGEN). The eluted DNA was quantified by standard electrophoresis and ligated into T/A cloning vector. Chemically competent DH5α *E. coli* cells were transformed with the ligation mix and the transformed cells were plated on LA medium containing ampicillin, X-gal and IPTG. The positive clones were selected based on blue/white screening.

For confirmation of cloning of the sequences in T/A vector, plasmid DNA was isolated from the putative positive clones and subjected to restriction analysis using specific restriction enzymes whose sites were introduced into the primers and/or the restriction enzymes sites present in the MCS of T/A vector. The confirmation was also done by PCR using specific primers for each of the sequences.

4.3.7 Full-length (antisense) sequence

The full-length sequence of ricin, for antisense expression construct, cloned in InsT/A vector (InsT/A- antisense full length ricin) was confirmed by the restriction analysis. Based on blue-white screening, colonies were put for culture and the plasmid DNA was isolated. Plasmid DNA clones of each construct was loaded on agarose gel to check for the plasmid DNA. All the clones of both the constructs were double digested with *Eco*RI and *Hind*III to excise out the fragment including both ricin/RCA sequence and the MCS of InsT/A vector. The clones gave an expected fragment of 1810 bp (1300 bp of ricin/RCA and 80 bp of MCS).

4.3.8 Selected portion of ricin/RCA-RA and RB sequence

The total length of the ricin coding sequence is 1730 bp. Since, it is well known that

1. for the induction of gene silencing using ihp RNA or SHUTR strategy, only a portion of the gene is required; one need not target the entire gene as in the case of conventional antisense technology;
2. targeting either 5' portion or 3' portion of the gene is sufficient to induce silencing, we designed individual constructs harbouring 5' portion (ricin/RCA- A chain- (RA)) and 3' portion (ricin/RCA- B chain-(RB)) of ricin to study their efficacy in silencing the target genes; and
3. the binary vectors from CAMBIA have a limitation on the size of the T-DNA to be transferred by *Agrobacterium*-mediated transformation, therefore, we designed separate constructs specific for A chain and B chain for the two proposed strategies (ihp-RNA and SHUTR).

Portions of ricin A chain (357 bp) and ricin B chain (307 bp) were PCR amplified from castor genomic DNA using *Nco*I *BamHI* forward primers and *Xba*I *Xho*I reverse primers. The ricin A chain and B chain specific amplicons were cloned in T/A vector. The cloning was confirmed through colony PCR and through restriction with *EcoRI* and *HindIII*. To confirm the cloning of the selected portions of A and B chains of the ricin/RCA sequence in InsT/A vector, colony PCR was carried out with the putative clones (white colonies). All the putative colonies turned out to be positive when amplified using sequence specific primers. The primers used were '*Nco*I *BamHI* Forward' and '*Xba*I *Xho*I Reverse' primers. The colony PCR gave amplicons of expected size (357 bp in case of RA and 307 bp in case of RB). This is inclusive of restriction enzyme sites incorporated.

Further confirmation of the clones, which were earlier confirmed by colony PCR, was done by restriction analysis. The selected clones were cultured and plasmid DNA was

isolated and digested with *Eco*RI and *Hin*dIII (the enzyme sites at the extremes of the MCS of InsT/A vector) and the digested samples were run on 1.5% agarose gel. The restriction yielded a fragment of the expected size (437 bp for RA and 387 bp for RB), confirming the cloning.

4.3.9 Confirmation of the orientation of the selected sequences of ricin A and B chains in InsT/A

Since the PCR product could be cloned in either orientation in T/A vector, the orientation of the sequence was confirmed by the restriction analysis. This would enable selection of the right clone for further cloning in pRT100. To check the orientation of the cloned sequence in InsT/A vector, *Xba*I restriction enzyme was used to digest the clones. The digestion yielded the fragment from those clones in which the gene fragment was cloned in the sense orientation with respect to the InsT/A. The *Xba*I site present in both the vector and the primer used for amplification of the gene segment enabled the confirmation of the orientation of cloning.

4.3.10 Directional cloning in pRT100

To enable directional cloning of the gene sequences in pRT100, the individual fragments from the respective T/A vectors were excised out using cloning enzymes whose sites were deliberately introduced in primers and the fragments were cloned in pRT100. From the InsT/A vector, the required sequences were selectively excised out for different strategies mentioned below. The clones in pRT100 35S for each of the strategy were designated for the convenience as follows.

i) Full-length antisense ricin as '**pRT 100 35S- Ricin Full-Length Antisense**'.

ii) Inverted repeat of the selected part of ricin/RCA interspersed by catalase intron as **pRT 100 35S-ihp RA/RB** (intron hairpin RNA).

iii) Antisense portion of ricin/RCA in front of an inverted repeat of nos polyA interspersed by catalase intron as **pRT 100 35S SHUTR RA/RB** (SHUTR-Silencing induced by heterologous 3'-untranslated region).

4.3.11 Construction of pRT 100 35S ricin full-length antisense construct

The full-length ricin sequence from the InsT/A vector was excised out with *Sst*I and *XhoI*, gel eluted and ligated into pRT100 vector digested with *Sst*I and *XhoI*. Since the *XhoI* site is 5' to the *Sst*I site in the pRT100 vector, the sequence was supposed to be cloned in antisense

orientation with respect to the 35S promoter. The clones were restricted with the cloning enzymes, *Sst*I and *XhoI*, to excise out the cloned fragment. All the clones gave expected fragment of 1300 bp, confirming the cloning of full-length antisense sequence. Also, the clones were restricted with *HindIII* to confirm cloning of 1300 bp downstream of CaMV35S promoter. The expected clones gave 1300 bp of the ricin/RCA gene together with 700 bp of the *HindIII* cassette.

4.3.12 Construction of pRT 100 35S ihp RA/RB constructs

This exercise involved cloning of the selected part of RA/RB in both sense and antisense orientation on either side of the catalase intron that was already cloned in pRT100 35S (pRT 100 35S catalase intron). The strategy adopted included cloning of the ricin/RCA specific RA/RB fragment first in sense orientation downstream of catalase intron and later cloning of the fragment in antisense orientation upstream of catalase intron.

4.3.13 Cloning of the selected fragment of ricin/RCA–RA/RB in antisense orientation

The fragment of RA/RB was to be cloned upstream of catalase intron in antisense orientation to effect the construction of pRT ihp RA/RB. To clone the fragment in antisense orientation, InsT/A RA/RB clones were restricted with *Nco*I and *Xho* I. The presence of the above two restriction sites in pRT100 facilitated the directional cloning. Since the *Xho*I site is 5' to the *Nco*I site in the pRT100 vector, the sequence is supposed to get cloned in antisense orientation with respect to the 35S promoter. The colonies obtained after ligation and transformation were randomly selected and screened through colony PCR using 'pRT Sequencing primer' and '*Nco*I *Bam*HI RA/RB Rev' primers to give the expected amplicons of 427bp and 377bp for RA and RB respectively (including portion of 35S and antisense fragment).

Restriction analysis was done to confirm the cloning. The individual clones screened through colony PCR were digested with *Hind*III, which was supposed to take out the *Hind*III cassette from pRT100. The expected clones (designated as pRT 100 35S antisense RA/RB catalase intron) gave the expected size fragment of 1.15 kb and 1.1 kb for RA and RB respectively.

4.3.14 Cloning of the selected fragment of RA/RB sequence in sense orientation

The way primers were designed, for facilitating both sense and antisense cloning from the same amplicon, warranted cloning of the antisense fragment first followed by the antisense fragment. The InsT/A RA/RB clones were initially digested with *XbaI*. Only those InsT/A RA/RB clones were selected in which the fragment could be excised out with *XbaI*. This is possible only when these clones are in the sense orientation (5'-3'). The sense InsT/A RA/RB clones were restricted with *XbaI* and cloned downstream of catalase intron in 'pRT antisense RA/RB catalase intron'.

Colony PCR was carried out using '*SstI* Catalase Intron Forward' and '*Xba*I *Xho*I RA/RB chain Reverse' primers so that only the clones, which had the RA/RB in sense orientation, could give the amplification. The colonies gave prominent amplicons of the expected size 567 bp and 517 bp for RA and RB respectively. The clones were also confirmed by restriction with *Hind*III. The clones gave the expected fragment of 1267 bp and 1217 bp for RA and RB respectively. The directionality of cloning of pRT 100 35S ihp RA and pRT 100 35S ihp RB was confirmed by restriction with *Xho*I. With the clones in 5'-3' direction, a fragment of 924 bp was expected for RA and 824 bp for RB. If the clones were anticipated to be in 3'-5' orientation, a fragment of 567 bp was expected for RA and 517 bp for RB.

4.3.15 Construction of pRT 100 35S SHUTR RA/RB constructs

This clone was constructed with a simple cloning of the selected portion of the RA/RB gene sequence upstream of the antisense polyA in the generic SHUTR vector- pRT 100 polyA-catalase intron. The sequence was excised out from the InsT/A clone with the restriction enzymes whose sites were incorporated in the sequence at the time of primer designing. *Nco*I and *Xba*I were the restriction enzymes used to clone the RA/RB sequence in antisense orientation in the generic vector.

Restriction analysis was carried out with *Hind*III in which it was expected to take out the antisense RA/RB fragment along with antisense polyA and catalase intron. This restriction gave 1487 bp in case of pRT SHUTR RA and 1437 bp for pRT SHUTR RB. PCR was carried out with the clones to confirm the cloning of antisense RA/RB with '*Xba*I *Xho*I RA/RB chain Reverse' and '*Kpn*I Catalase Intron Reverse' primers. This gave the expected amplification of 567 bp and 517 bp for pRT 100 35S SHUTR RA and pRT 100 35S SHUTR RB.

4.3.16 Cloning into plant transformation vector

The final constructs developed in pRT100-35S had to be cloned into a plant transformation vector. Since, the final constructs were flanked by *Hind*III sites in pRT100-35S, the entire gene cassette was restricted with the same and cloned into *Hind*III site of binary vector, pCAMBIA 1300.

The constructs in the binary vector were designated as follows:

1**. pCAMBIA 1300-FLA/S R**: Binary vector with *HindIII* fragment from 'pRT 100 35S Ricin **F**ull **L**ength **A**nti**s**ense'

2. **pCAMBIA 1300-ihp RA/RB**: Binary vector with *HindIII* fragment from 'pRT 100 35s ihp RA/RB'

3. **pCAMBIA 1300-SHUTR RA/RB**: Binary vector with *HindIII* fragment from 'pRT 100 35S SHUTR RA/RB'

4.3.17 Cloning of 'FLA/S R fragment' in binary vector

The entire gene cassette including 35S promoter, cloned full-length antisense ricin gene and the polyA signal was excised out from the pRT100 35S vector by restricting with *Hind*III. The restricted fragment was cloned into a binary vector, pCAMBIA 1300, which was restricted with *Hind*III. After transformation, the colonies were selected based on blue-white screening. The DNA was isolated from the clones and was restricted with *Hind*III. The clones gave the expected fragment of 1300 bp of the ricin gene and 700 bp of the *Hind*III cassette confirming the cloning (Fig.4.22).

4.3.18 Cloning of 'ihp RA/RB fragment' in binary vector

The construct from the pRT100 was excised out with *Hind*III and ligated with *Hind*III digested pCAMBIA 1300. The ligation mix was used for transformation. The colonies were selected through blue-white selection. The isolated clones were analysed by restriction to confirm the cloning in binary vector with *HindIII*. The clones gave the expected fragment of 1624 bp and 1524 bp for RA and RB respectively (Fig.4.22 & Fig.4.23).

4.3.19 Cloning of 'SHUTR RA/RB fragment' in binary vector

The gene cassette from pRT100 35S was excised out with *Hind*III and cloned into the *Hind*III site of pCAMBIA1300 binary vector. To confirm the cloning, restriction analysis was done with *Hind*III, which was supposed to take out the entire gene cassette from the binary vector. The clones gave the expected fragment of 1707 bp in case of SHUTR RA and 1657 bp for

SHUTR RB (Fig.4.24). The directionality of the gene cassette in binary vector was checked since it was cloned by single digestion with *Hind*III. Restriction was done with *Kpn*I whose site was there both in MCS of 1300 and the gene cassette. In case of RA, the clones in 5'-3' orientation gave 1277 bp and those in 3'-5' direction yielded 286 bp. In case of RB, the clones in 5'-3' orientation gave 1257 bp and those in the 3'-5' direction gave 286 bp.

4.3.20 Mobilization of constructs into *Agrobacterium tumefaciens* Strain LBA4404

The binary constructs harboured in *E.coli* had to be mobilized into an *Agrobacterium* strain LBA 4404 to carry out further plant transformation. The constructs in the *Agrobacterium* were designated as follows.

1) LBA4404::1300 35S FLA/S R - with **pCAMBIA 1300-FLA/S R**

2) LBA4404::1300 35S ihp RA/RB - with **pCAMBIA 1300-ihp RA/RB**

3) LBA4404::1300 35S SHUTR RA/RB - with **pCAMBIA 1300-SHUTR RA/RB**

LBA4404::1300 35S FLA/S R

The construct in binary vector maintained in *E.coli* was mobilized into *Agrobacterium* through electroporation method. The colonies were randomly selected, cultured and the DNA was isolated. The mobilization was confirmed by PCR using construct specific primers, 'pRT 100 35S Seq' and '*SstI* ricin For' primers, which gave, expected size amplicon of 1.3 kb. The clones in LBA4404 were restricted with *HindIII* and they gave the expected fragment of 2.0 kb (Fig.4.25).

The mobilization of the construct into *Agrobacterium* was further confirmed by reverse mating of a clone in LBA4404 into *E.coli.* The reverse mated clones were confirmed by restriction with *HindIII*. The reverse mated clones gave a fragment of 2.0 kb.

LBA4404::1300 35S ihp RA/RB

Electroporation was done to transform the *Agrobacterium* with pCAMBIA 1300-ihp RA/RB and the colonies were randomly selected and the DNA was isolated. The mobilization of pCAMBIA 1300-ihp RA/RB into *Agrobacterium* was confirmed by both PCR and restriction analysis. PCR was carried out with construct specific primers, 'pRT 100 35S Sequencing primer' and '*NcoI* PolyA Reverse'. The clones gave the expected amplicon of 1260 bp for RA and 1160 bp, in case of RB (Fig.4.25).

The mobilization of the construct into *Agrobacterium* was further confirmed by reverse mating of a clone in LBA4404 into *E.coli.* The reverse mated clones were confirmed by restriction with *HindIII*. The reverse mated clones gave a fragment of 1624 bp for RA and 1524 bp for RB.

LBA4404::1300 35S SHUTR RA/RB

The pCAMBIA 1300-SHUTR RA/RB clones in binary vector were mobilized into *Agrobacterium* by electroporation and the colonies were chosen randomly. The DNA was isolated and the clones were confirmed through PCR and restriction analysis. PCR was carried out with construct specific primers, 'pRT 100 35S Sequencing primer' and '*KpnI* Catalase Intron Reverse' which gave an expected amplicon of 880 bp for RA and 830 bp for RB.

Restriction was carried out with *Hind*III to excise out the entire gene cassette cloned in binary vector. The clones gave expected fragment of 1510 bp for RA and 1460 bp for RB, confirming the mobilization of the construct (Fig.4.25).

The mobilization of the construct into *Agrobacterium* was further confirmed by reverse mating of a clone in LBA4404 into *E.coli.* The reverse mated clones were confirmed by restriction with *HindIII*. The reverse mated clones gave a fragment of 1510 bp for RA and 1460 bp for RB. The selected clones in Agrobacterium strain were again confirmed using restriction analysis with *Hind*III .

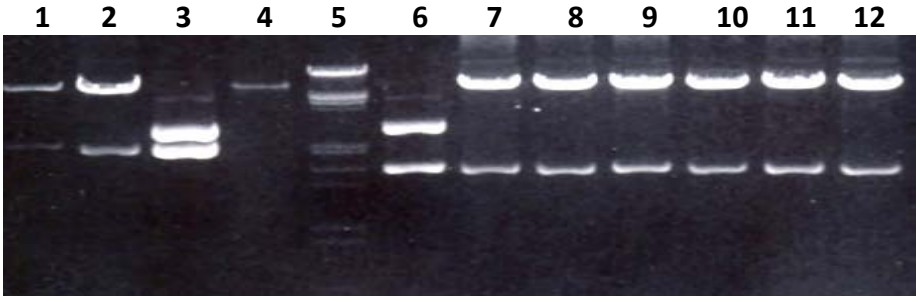


Fig.4.22.Restriction/digestion of pCAMBIA 1300 Ricin (A/S) full length & pCAMBIA 1300 ihp RA with *HindIII*

1. pCAM Ricin clone 1
2. pCAM Ricin clone 2
3. pRT Ricin clone 2
4. pCAM 1300 (-ve control)
5. Mr- λ DNA *Hind III EcoRI* digest.
6. pRT ihp RA clone 1(+ve control)
7. pCAM ihp RA clone 1
8. pCAM ihp RA clone 2
9. pCAM ihp RA clone 3
10. pCAM ihp RA clone 4
11. pCAM ihp RA clone 5
12. pCAM ihp RA clone 6

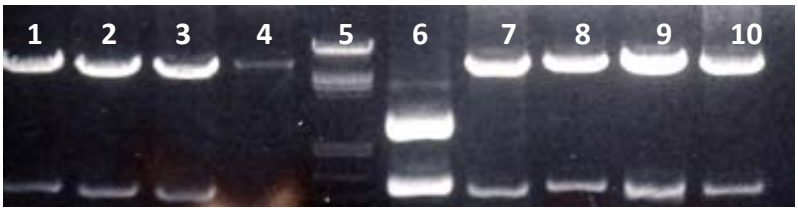


Fig. 4.23. Restriction/digestion of pCAMBIA 1300 ihp RB with *HindIII*

1 .pCAM ihp RB clone 1
2. pCAM ihp RB clone 2
3. pCAM ihp RB clone 3
4. pCAM 1300(-ve control)
5. Mr- λ DNA *Hind III EcoRI* digest.
6. pRT ihp RB clone 5(+ve control)
7. pCAM ihp RB clone 4
8. pCAM ihp RB clone 5
9. pCAM ihp RB clone 6
10. pCAM ihp RB clone 7

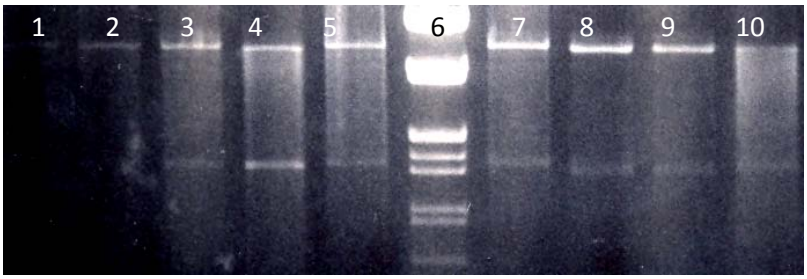


Fig.4.24. Restriction/digestion of pCAMBIA 1300 SHUTR A & B with *Hind*III

1. LBA pCAM SHUTR A 40 clone 2
2. LBA pCAM SHUTR A 40 clone 4
3. LBA pCAM SHUTR A 40 clone 7
4. pCAM SHUTR A 40 control
5. LBA pCAM SHUTR A 40 clone 8
6. Mr- λ DNA *Hind III EcoRI* digest
7. LBApCAM SHUTR A 40 clone 14
8. LBA pCAM SHUTR B6 clone 4
9 .LBA pCAM SHUTR B 6 clone 5
10. pCAM SHUTR B 6

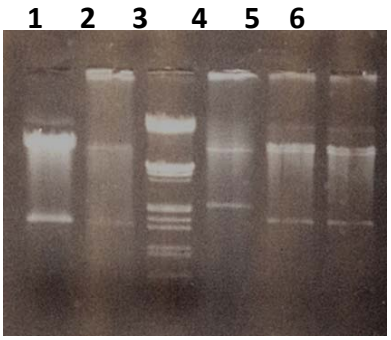


Fig. 4.25. Restriction of the PTGS constructs under 35S in *Agrobacterium* LBA4404 with *Hind III*

Lane1: ihp-RA PTGS construct(released fragment of 1624bp);
Lane2: ihp-RB PTGS construct(1524bp);
Lane3: *EcoRI* + *HindIII* λ DNA;
Lane4: FL-A/S-Ricin PTGS construct(2000bp);
Lane5: SHUTR-RA PTGS construct(1487bp);
Lane6: SHUTR-RB PTGS construct(1437bp)

4.4 Development of PTGS constructs under ricin full-length promoter

Since 35S promoter is known to weakly express in endosperm (where the transcripts of ricin and RCA express and accumulate), the isolated ricin full-length promoter was cloned in place of CaMV35S promoter in all the developed PTGS constructs to enable efficient transformation of castor.

4.4.1 Development of pRT 100 Ricin TP/FP Vector

The available pRT 100 vector was restricted with *Hinc*II and *Xho*I to excise out CaMV35S promoter. The ricin FP (InsT/A ricin TP/FP) was cloned in the above vector in place of CaMV 35S promoter to obtain pRT 100 Ricin TP/FP Vector .The cloning was confirmed both by PCR and restriction analysis. The following vectors were developed and confirmed and designated as:

pRT 100 Ricin FP ihp RA/RB construct
pRT 100 Ricin FP SHUTR RA/RB construct
pRT 100 Ricin FP FLA/S ricin construct

The clones in pRT100 FP, developed for each of the strategies, were designated as follows.

i) Full-length antisense ricin/RCA as '**pRT 100 FP Full Length Antisense Ricin**'.

ii) Inverted repeat of the selected part of ricin/RCA interspersed by catalase intron as **pRT 100 FP ihp RA/RB** (intron hairpin RNA).

iii) Antisense portion of ricin/RCA in front of an inverted repeat of 35S polyA interrupted by catalase intron as **pRT 100 FP SHUTR RA/RB** (SHUTR-Silencing induced by heterologous 3'-untranslated region.

A diagrammatic representation of the cloning strategies followed for developing these constructs has been provided in figures 4.26-4.29.

4.4.2 Cloning into plant transformation vector

The final constructs developed in pRT100 FP had to be cloned into a plant transformation vector. Since, the final constructs were flanked by *Hind*III sites in pRT100, the entire gene cassette was released as *Hind*III fragment and cloned at the *Hind*III site of binary vector, pCAMBIA 1300.

The constructs in the binary vector were designated as follows:

1. **pCAMBIA 1300 FP FLA/S R**: Binary vector with *HindIII* fragment from 'pRT 100 FP **F**ull **L**ength **A**nti**s**ense Ricin' (Fig.4.30)
2. **pCAMBIA 1300 FP ihp RA/RB**: Binary vector with *HindIII* fragment from 'pRT 100 FP - ihp RA/RB' (Fig. 4.30 and 4.31)
3. **pCAMBIA 1300 FP SHUTR RA/RB**: Binary vector with *HindIII* fragment from 'pRT 100 FP - SHUTR RA/RB' (Fig.4.32)

A summary of the various constructs / clones generated during the present investigation is outlined in table 4.1.

4.4.3 Mobilization of the developed gene cassettes into *Agrobacterium tumefaciens* strain LBA4404

The binary constructs harboured in *E.coli* were mobilized into the *Agrobacterium* strain LBA 4404 to carry out further plant transformation. The constructs in the *Agrobacterium* were designated as follows.

1) LBA4404:: pCAMBIA 1300 ricin FP ihp RA/RB (Fig.4. 33)
2) LBA4404:: pCAMBIA 1300 ricin FP SHUTR RA/RB (Fig.4.34 & 4.35)
3) LBA4404:: pCAMBIA 1300 ricin FP Full Length antisense (FLA/S) ricin (Fig.4.34).

All these clones were confirmed using restriction analysis with the plasmid DNA isolated from *Agrobacterium* and also from the reverse mated clones in *E.coli* (Fig.4.36, 4.37 & 4.38).

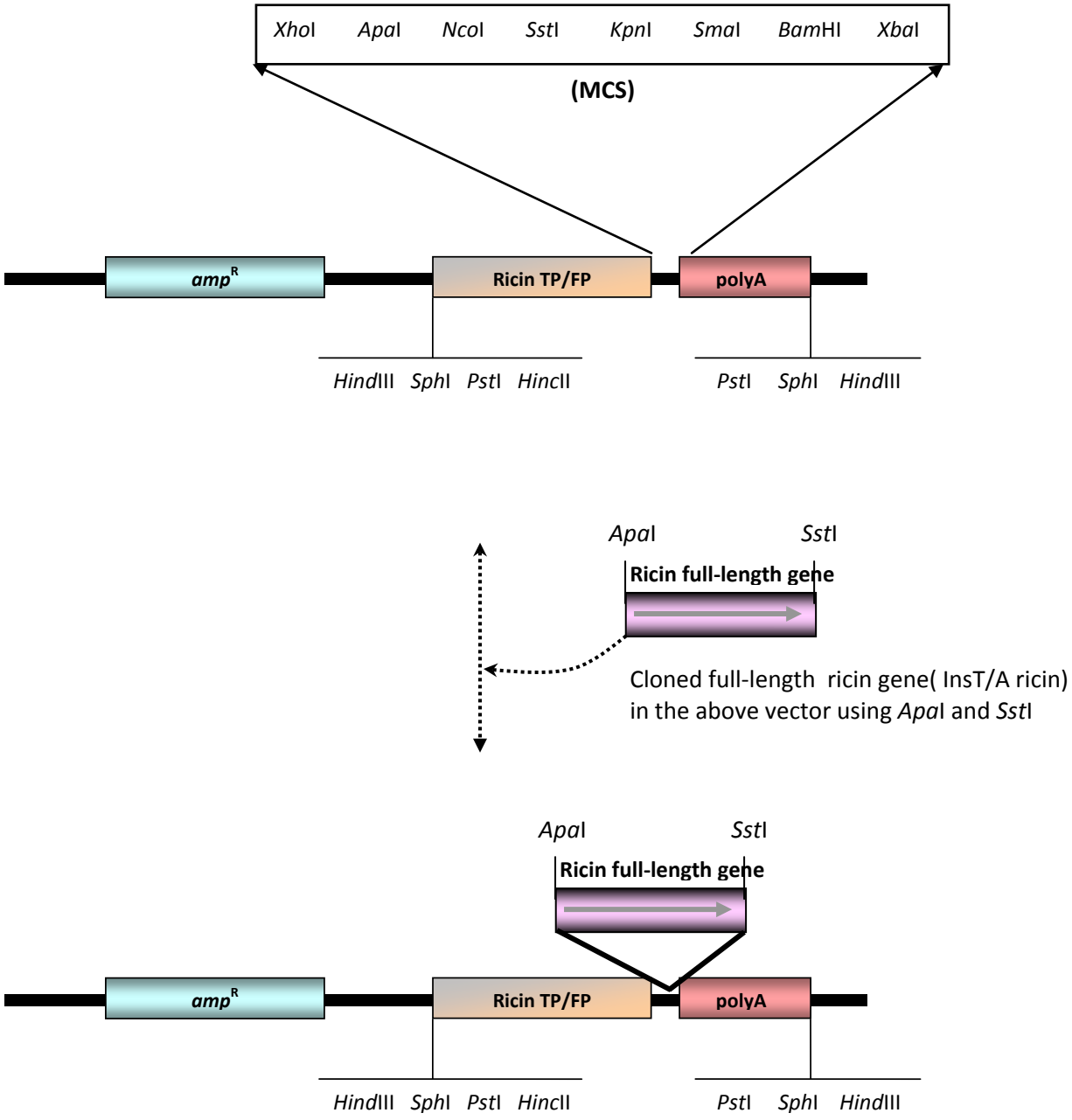
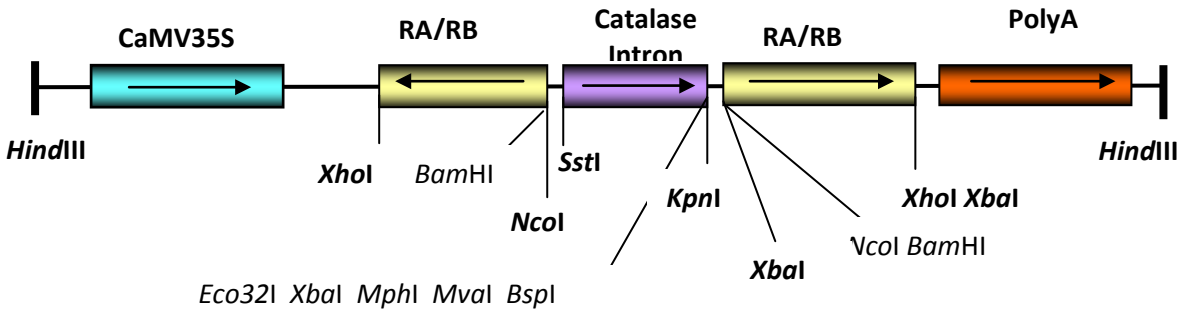


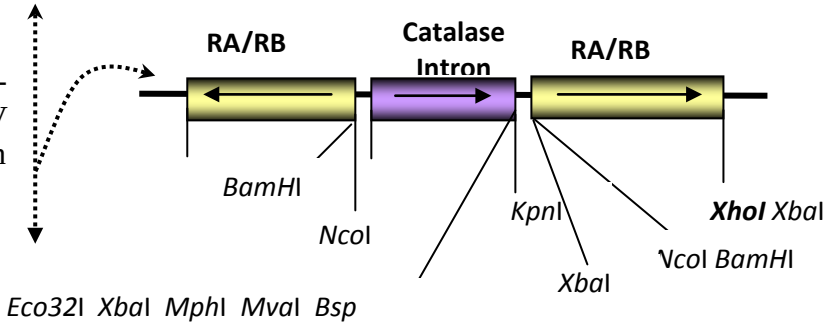
Fig. 4.26. Schematic representation of the steps involved in the development of pRT 100 Ricin TP/FP full-length ricin gene expression constructs

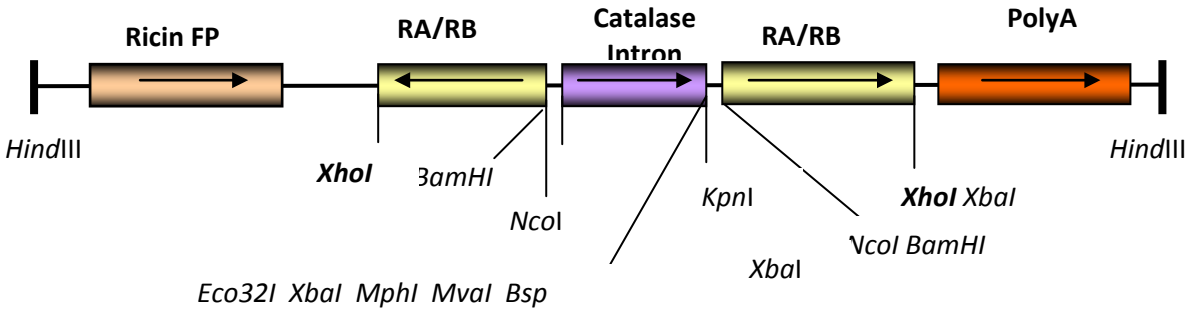


pRT100 CaMV 35S ihp RA/RB

Restricted 'antisense RA/RB-catalase intron-sense RA/RB'cassette from pRT 100 CaMV 35S ihp RA/RB construct (reported earlier in JRF report) with *Xho*I

Cloned the above cassette in pRT 100 Ricin FP vector using *Xho*I





pRT100 Ricin FP ihp RA/RB

Fig. 4.27: Schematic representation of the steps involved in the development of pRT100 Ricin FP ihp RA/RB

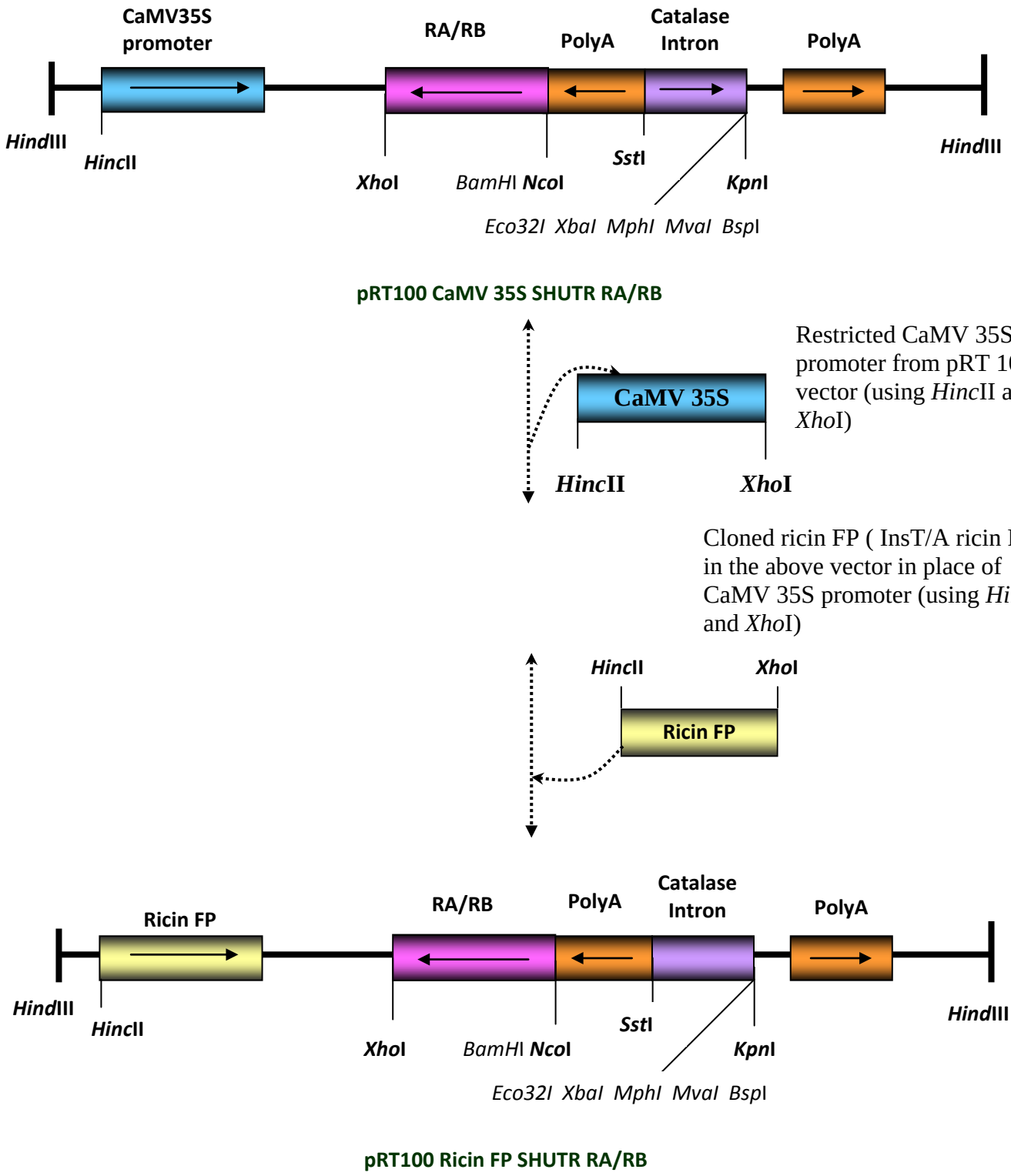
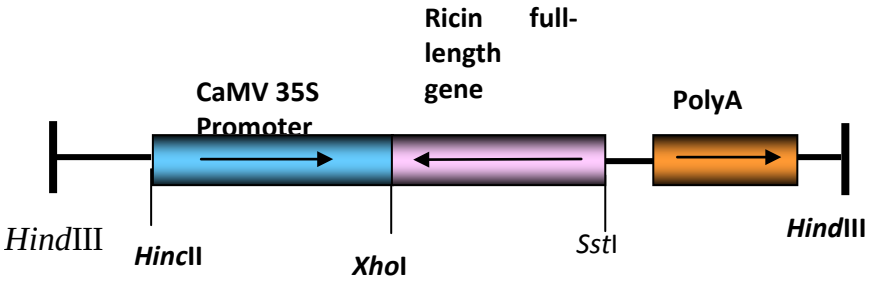
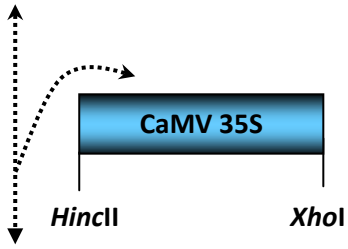


Fig. 4.28: Schematic representation of the steps involved in development of pRT100 Ricin FP SHUTR RA/RB

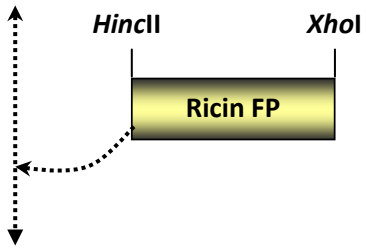


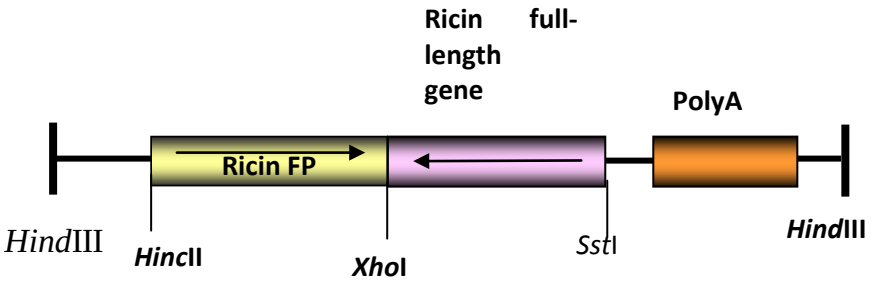
pRT100 CaMV 35S FLA/S ricin



Restricted CaMV 35S promoter from pRT 100 vector (using *Hinc*II and *Xho*I)

Cloned ricin FP (InsT/A ricin FP) in the above vector in place of CaMV 35S promoter (using *Hinc*II and *Xho*I)





pRT100 Ricin FP FLA/S ricin

Fig. 4.29: Schematic representation of the steps involved in the development of pRT100 Ricin FP FLA/S ricin

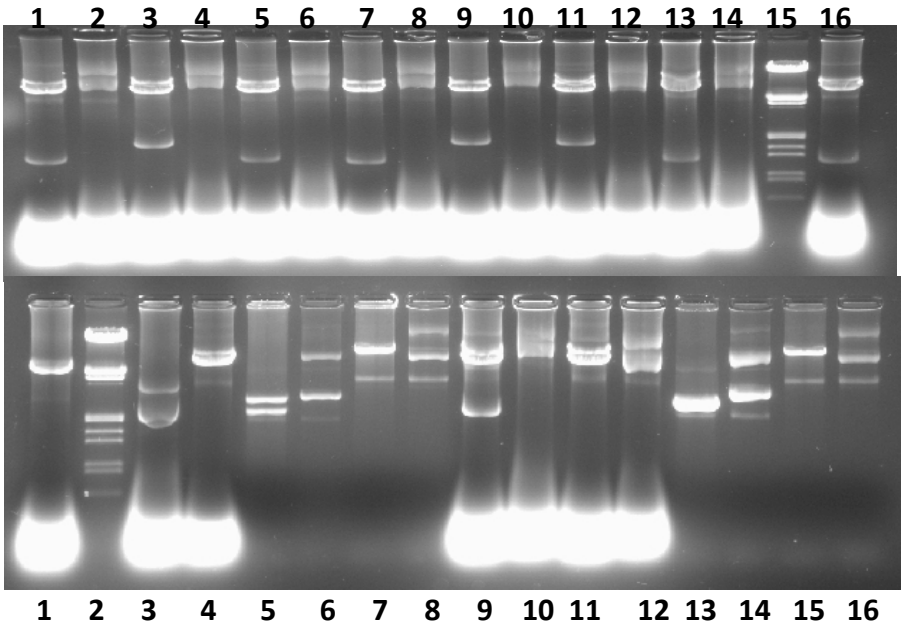


Fig.4.30: Confirmation of cloning of FP ihpRA cassette (upper panel) in pCAMBIA 1300 and FP FLA/S ricin in pCAMBIA 1300 (lower panel) by restriction with *Hind*III

Upper Panel
Lane 1 – 14 : pCAMBIA 1300 FP ihpRA 4 clones1-7(digested alternates with undigested)
Lane 15 : E+H digested λDNA
Lane 16 : pCAMBIA 1300 FP ihpRA 4 clone 8(digested)

Lower Panel
Lane 1 : pCAMBIA 1300 FP ihpRA 4 clone 9(digested)
Lane 2 : E+H digested λ DNA
Lane 3 : pCAMBIA 1300 FP ihpRA 4 clone 10(digested)
Lane 4 : pCAMBIA 1300 FP ihpRA 4 Blue clone (digested)
Lane 5 : pRT100 FP ihpRA 4 (+ ve control) (digested)
Lane 6 : pRT100 FP ihpRA 4 (+ ve control) (undigested)
Lane 7 : pCAMBIA 1300(-ve control) (digested)
Lane 8 : pCAMBIA 1300(-ve control) (undigested)
Lane 9 : pCAMBIA 1300 FP FLA/S ricin clone 1(digested)
Lane 10 : pCAMBIA 1300 FP FLA/S ricin clone 1(undigested)
Lane 11 : pCAMBIA 1300 FP FLA/S ricin clone 1 Blue clone (digested)
Lane 12 : pCAMBIA 1300 FP FLA/S ricin clone 1 Blue clone (undigested)
Lane 13 : pRT100 FP FLA/S ricin 1 (+ ve control) (digested)
Lane 14 : pRT100 FP FLA/S ricin 1 (+ ve control) (digested)
Lane15 : pCAMBIA 1300(-ve control) (digested)
Lane16 : pCAMBIA 1300(-ve control) (undigested)

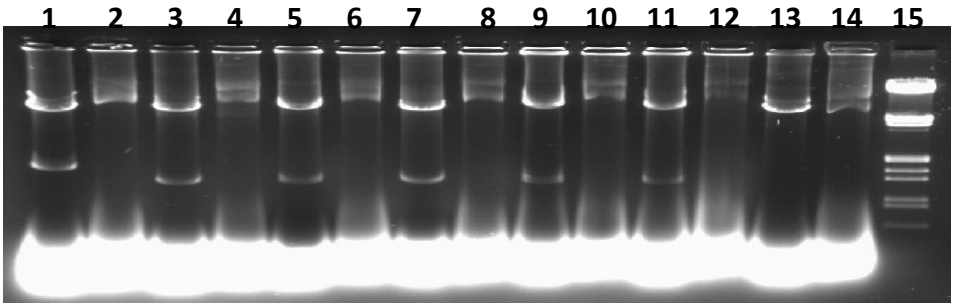


Fig. 4.31: Confirmation of cloning of FP ihp RB cassette in pCAMBIA 1300 by restriction with *Hind*III

Lane 1- 14 : pCAMBIA 1300 FP ihpRB clones 1-7 (digested alternates with undigested)
Lane 15 : E+H digested λ DNA

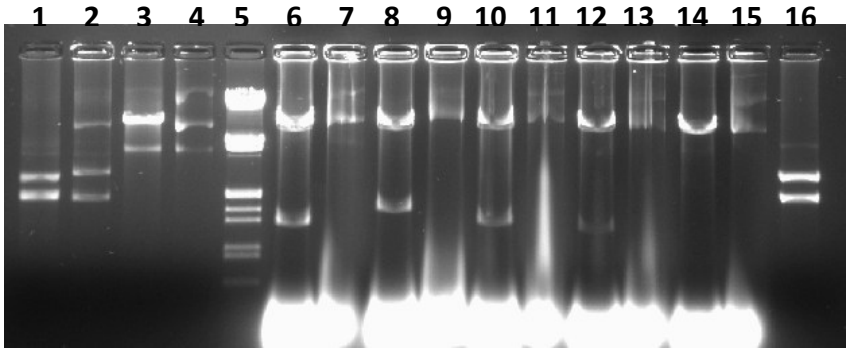


Fig. 4.32 : Confirmation of cloning of FP SHUTR RB 11 cassette in pCAMBIA 1300 by restriction with *Hind*III

Lane 1 : pRT100 FP ihpRA 4 (+ ve control) (digested)
Lane 2 : pRT100 FP ihpRA 4 (+ ve control) (undigested)
Lane 3 : pCAMBIA 1300(-ve control) (digested)
Lane 4 : pCAMBIA 1300(-ve control) (undigested)
Lane 5 : E+H digested λ DNA
Lane 6 – 15: pCAMBIA 1300 FP SHUTR RB 11 clones 1-5 (digested alternates with undigested)
Lane 16 : pRT100 FP SHUTR RB 11 (+ ve control) (digested)

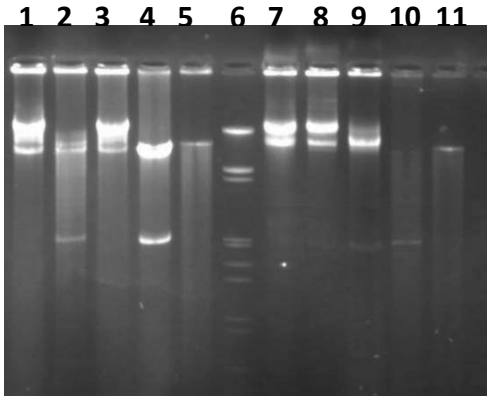


Fig. 4.33: Confirmation of mobilization of pCAMBIA 1300 FP ihp RA 6 and pCAMBIA 1300 FP ihp RB 1 clones in to *Agrobacterium tumefaciens* strain LBA 4404 by restriction with *Hind*III

Lane 1-3 : LBA 4404 pCAMBIA 1300 FP ihp RA 6 clones 1-3
Lane 4 : pCAMBIA 1300 FP ihp RA 6
Lane 5 : pCAMBIA 1300
Lane 6 : E+H digested λ DNA
Lane 7-9 : LBA 4404 pCAMBIA 1300 FP ihp RB 1 clones 1-3
Lane 10 : pCAMBIA 1300 FP ihp RB 1
Lane 11 : pCAMBIA 1300

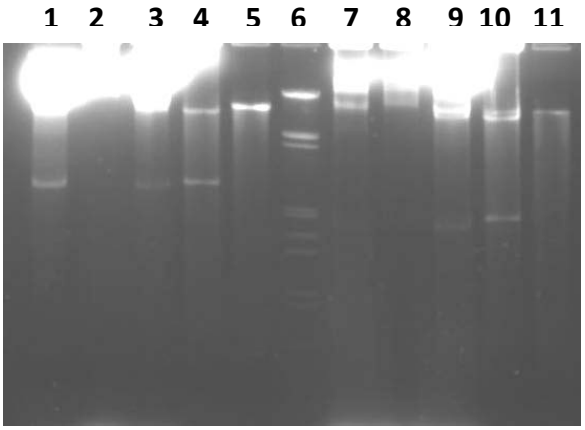


Fig. 4.34: Confirmation of mobilization of pCAMBIA 1300 FP FLA/S R 1 and pCAMBIA 1300 FP SHUTR RA 3 clones in to *Agrobacterium tumefaciens* strain LBA 4404 by restriction with *Hind*III

Lane 1-3 : LBA 4404 pCAMBIA 1300 FP FLA/S R 1 clones 1-3
Lane 4 : pCAMBIA 1300 FP FLA/S R 1
Lane 5 : pCAMBIA 1300
Lane 6 : E+H digested λ DNA
Lane 7-9 : LBA 4404 pCAMBIA 1300 FP SHUTR RA 3 clones 1-3
Lane 10 : pCAMBIA 1300 FP SHUTR RA 3
Lane 11: pCAMBIA 1300

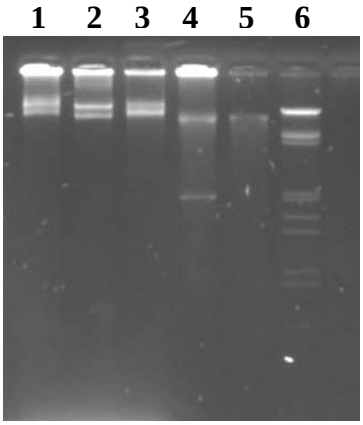


Fig. 4.35: Confirmation of mobilization of pCAMBIA 1300 FP SHUTR RA 3 clones in to *Agrobacterium tumefaciens* strain LBA 4404 by restriction with *Hind*III

Lane 1-3 : LBA 4404 pCAMBIA 1300 FP SHUTR RB 2 clones 1-3
Lane 4 : pCAMBIA 1300 FP SHUTR RB 2
Lane 5 : pCAMBIA 1300
Lane 6 : E+H digested λ DNA

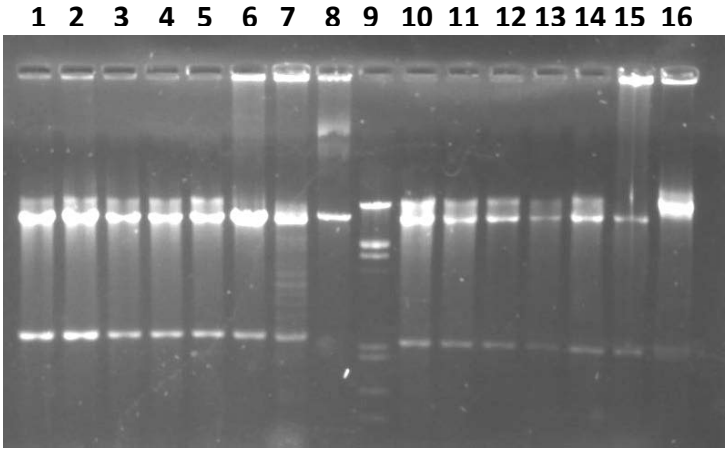


Fig.4.36: Confirmation of mobilization of pCAMBIA 1300 FP ihp RA 6 and pCAMBIA 1300 FP ihp RB 1 in to *Agrobacterium tumefaciens* strain LBA 4404 by restriction with *Hind*III (reverse mating)

Lane 1-5 : RM clones of LBA 4404 pCAMBIA 1300 FP ihp RA 6 clone 2
Lane 6 : pCAMBIA 1300 FP ihp RA 6(+ve control)
Lane 7 : LBA 4404 pCAMBIA 1300 FP ihp RA 6 clone 2(+ve control)
Lane 8 : pCAMBIA 1300
Lane 9 : E+H digested λ DNA
Lane 10-14 : RM clones of LBA 4404 pCAMBIA 1300 FP ihp RB 1 clone 3
Lane 15 : pCAMBIA 1300 FP ihp RB 1(+ve control)
Lane 16 : LBA 4404 pCAMBIA 1300 FP ihp RB 1 clone 3

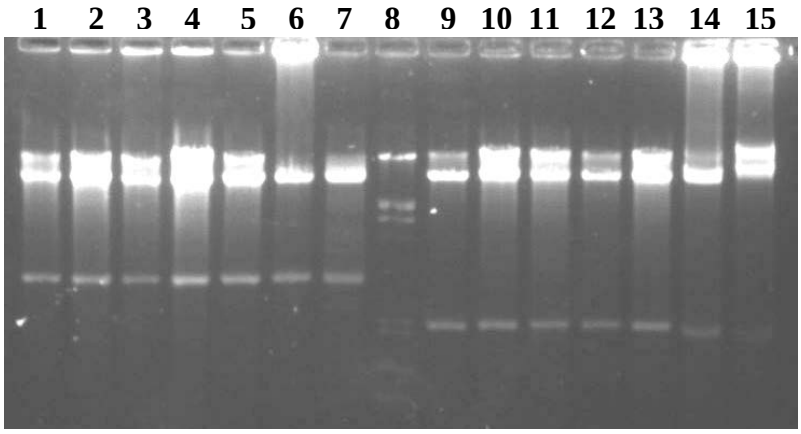


Fig. 4.37: Confirmation of mobilization of pCAMBIA 1300 FP FLA/S R 1and pCAMBIA 1300 FP SHUTR RA 3 in to *Agrobacterium tumefaciens* strain LBA 4404 by restriction with *Hind*III (reverse

Lane 1-5 : RM clones of LBA 4404 pCAMBIA 1300 FP FLA/S R 1 clone 1
Lane 6 : pCAMBIA 1300 FP FLA/S R 1(+ve control)
Lane 7 : LBA 4404 pCAMBIA 1300 FP FLA/S R 1 clone 1 (+ve control)
Lane 8 : E+H digested λ DNA
Lane 9-13:RM clones of LBA 4404 pCAMBIA 1300 FP SHUTR RA 3 clone 3
Lane14 : pCAMBIA 1300 FP SHUTR RA 3 (+ve control)
Lane 15 : LBA 4404 pCAMBIA 1300 FP SHUTR RA 3 clone 3(+ve control)

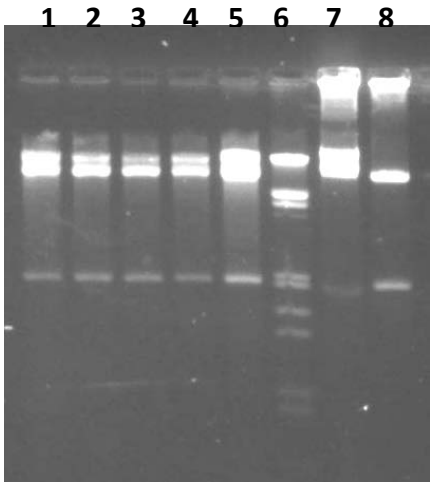


Fig. 4.38: Confirmation of mobilization of pCAMBIA 1300 FP SHUTR RB 2 in to *Agrobacterium tumefaciens* strain LBA 4404 by restriction with *Hind*III (reverse mating)

Lane 1-5 : RM clones of LBA 4404 pCAMBIA 1300 FP SHUTR RB 2 clone 2
Lane 6 : E+H digested λ DNA
Lane 7 : pCAMBIA 1300 FP SHUTR RB 2 (+ve control)
Lane 8 : LBA 4404 pCAMBIA 1300 FP SHUTR RB 2 clone 2 (+ve control)

4.5 Genetic transformation of castor

Though ten PTGS constructs based on three different canonical principles (full-length antisense, intron-spliced hairpin-mediated RNAi and silencing by 3'heterologous untranslated region) were developed and confirmed. However, for castor transformation in the present study, only two constructs *viz*., LBA4404:: pCAMBIA 1300 ricin FP ihp RA and LBA4404:: pCAMBIA 1300 ricin FP ihp RB, were used owing to the poor amenability of castor transformation and regeneration. The choice of the PTGS constructs for castor transformation is based on the enormous potential of the intron-spliced hairpin-mediated RNAi strategy at down regulating undesired proteins/toxic substances as evident by the published successful stories (e.g., gossypol-free cotton). The transformation protocol adopted was the meristem based transformation reported by Sujatha and Sailaja, 2005. Plate 5 shows different stages involved in castor transformation and regeneration.

Since putative transgenic castor plants have been obtained only with LBA4404:: pCAMBIA 1300 ricin FP ihp RB, the results obtained with the use of this construct are presented below.

4.5.1 Transformation and regeneration of castor

A meristem-based protocol for transformation and regeneration of castor (Sujatha and Sailaja, 2005) was followed to realize castor transgenics in the present study. Seeds of the cultivar DCS-9 (Jyothi) were used in all experiments. All the experiments were carried out with *Agrobacterium tumefaciens* strain LBA4404 harbouring the intended constructs.

A total of 37350 embryos were isolated from castor and were subjected to co-cultivation with the construct. *Agrobacterium* strain LBA4404 harbouring the intended construct was grown overnight and the next day, it was diluted and the culture with an OD of 0.2 was used for all the experiments. Co-cultivated explants were initially subjected to expansion and proliferation on MS medium with 0.5 mg/l TDZ followed by three cycles of selection on medium with 0.5 mg/l BA and increasing concentrations of hygromycin (20–40–60 mg/l). Selected shoot clusters were transferred to medium with 0.5 mg/l BA for proliferation and 0.2 mg/l BA for shoot elongation. Elongated shoots were rooted on half-strength MS medium with 4.0 mg/l IBA. This is a deviation from the published protocol, where NAA @ 2.0 mg/l was used instead.

When the explants were subjected to expansion and proliferation on MS medium with 0.5 mg/l TDZ, lot of flakiness was observed on the explants. The flakiness was a major

deterrent for successful transfer of explants to the selection regime. The flakiness was addressed by maintaining the explants on TDZ for a period of only 10 days. This brought down the flakiness of explants to minimum.

The explants were then subjected to the first selection regime which consisted of BA 0.5 mg/l + cefotaxime 250 mg/l + hygromycin 20 mg/l. There was a drastic decrease in the number of explants (4298 explants) from the previous culture. This was very evident even in the co-cultivation control where the explants started turning necrotic. The regeneration control was true to its type and consisted of green explants. The number of explants decreased further from the first to the second (2350 explants) and ultimately to the third (1372 explants) selection regime. The protocol included incremental increase in the concentration of the selection agent i.e., hygromycin. While in the second selection regime, the concentration of hygromycin was maintained at BA 0.5mg/l + ceotaxime 250 mg/l + hygromycin 40 mg/l, the third selection regime consisted of BA 0.5 mg/l + ceotaxime 250 mg/l + hygromycin 60 mg/l. The gradual increase in hygromycin was essential to ensure successful acclimatization of the explants. After the selection regimes, the putative shoots were subjected to six proliferation cycles of 15 days duration each on BA 0.5 mg/l.

4.5.2 Elongation of the putative transformed shoots

A total of 1500 putative transformed shoots were obtained after six cycles of proliferation on BA 0.5 mg/l. The selection agent, hygromycin showed carry-over effects up to third post-selection III proliferation cycle. For ensuring healthy recovery of putative shoots, hygromycin was withdrawn from the first proliferation cycle onwards. Fairly elongated shoots (236 shoots), were directly transferred into rooting media.

A general observation during this study was that very few shoots showed higher elongation (i.e 3-4 nodes or with longer internodal distance) (Plate 6). Most of the shoots showed less number of nodes and shorter internodal length. However for the purpose of rooting, only those shoots that put forth at least two nodes (even upon several subcultures on the maintenance/elongation medium) were identified as elongated and were subjected to rooting.

4.5.3 Rooting of the transformed shoots

The elongated shoots were subjected to rooting on basal MS medium supplemented with NAA (@ 1.0 – 2.0mg/L). No rooting was observed when the shoots were incubated on NAA 1.0 mg/L. The elongated shoots gave a sturdy thick root system on NAA 2.0 mg/L after a

period of one month (from the end of last cycle of elongation). The root system seemed to lack fine ramifications of the root-hairs. Thus, elongated shoots were rooted on half-strength MS medium supplemented with 4.0 mg/l IBA (Plate 6). Though shoots on both the media gave rise to roots, the frequency of root induction was higher when IBA was used, instead of NAA. A total of 87 putative shoots (out of 236) rooted.

4.5.4 Hardening and Acclimatization

All the 87 rooted shoots were acclimatized in plastic pots filled with peat mass. A peculiar feature of the root system of castor is the presence of two to three main supporting type of roots. Only when the supporting, mechanical root system strikes lateral roots with profuse system of thin root hairs, the castor plant could be successfully acclimatized. (Plate 7). Owing to this fact, only 25 rooted shoots could be successfully acclimatized in the tissue-culture laboratory (Plate 8). When the putative rooted transgenic castor plants from the lab were acclimatized in the green-house, only 8 survived out of 25 rooted plants (Plate 9). Many unconfirmed putative shoots are still to be put on rooting media and some of the shoots have already struck roots. They could not be acclimatized and thus were not analyzed for the presence of transgene. In future, they would also be subjected to thorough characterization at the molecular level.

Thus, it becomes very evident from the above account that each and every stage of castor transformation and regeneration entails highly laborious, time-taking long-drawn procedures with lot of inputs in the form of large number of embryos isolated and co-cultivated to realization of very few numbers of putative castor transgenics.

The number of putative shoots obtained at different stages of transformation and regeneration and ultimately the rooted plants have been summarily tabulated in **Table 4.2.**

4.5.5 Molecular analysis of the putative castor transgenics

All the acclimatized putative transgenic castor plants were observed for any differences with respect to vegetative traits like plant height, number of leaves, number of flowers *etc*. Two types of control safflower plants were raised; one type included untransformed plants, which originated from tissue culture explants and the other type included those from seeds sown directly in pots in the green house. There was no difference in the morphological traits between the controls and the transformed plants with the constructs.

4.5.5.1 PCR Analysis for the presence of the transgenes

All the castor plants that were acclimatized were analyzed for the presence of the transgene construct by PCR. Different PCRs were performed that aimed at checking the presence of different component sequences of the LBA4404:: pCAMBIA 1300 ricin FP ihp RB in the putative castor transgenics.

The presence of catalase I intron and antisense version of RB chain transgenes in all the eight plants were confirmed by using 'catalase I intron reverse' and 'RB chain reverse' primers. An amplicon size of 517 bp was realized for the presence of intact catalase I intron and antisense RB downstream of ricin full-length promoter (Fig.4.40). Further, presence of catalase I intron and sense version of RB chain transgenes was confirmed by using 'catalase I intron forward' and 'RB chain reverse' primers. This also yielded the expected amplicon size of 517 bp, thus confirming the presence of sense version of RB chain downstream of catalase I intron (Fig.4.39). Use of just the gene specific primers for PCR was not taken up as that would amplify the endogenous/native RB chain and catalase I intron.

The presence of hygromycin phosphotransferase gene (*hpt*) and hygromycin phosphotransferase gene (*hpt*II) was confirmed by using respective *hpt* forward and reverse primers. In case of PCR with hygromycin phosphotransferase (*hpt*) gene-specific primers, an ampilcon of 480 bp was obtained across all the eight putative plants (Fig.4.41). So was the case with hygromycin phosphotransferase (*hpt*II) gene-specific primers, PCR amplicon of 1094 bp size was obtained (Fig.4.42). All the PCR reactions were repeated twice to confirm the transgenic nature of the plants.

Thus, PCR analyses of the putative castor plants with different sets of primers have confirmed the presence of intact ihp-RB construct.

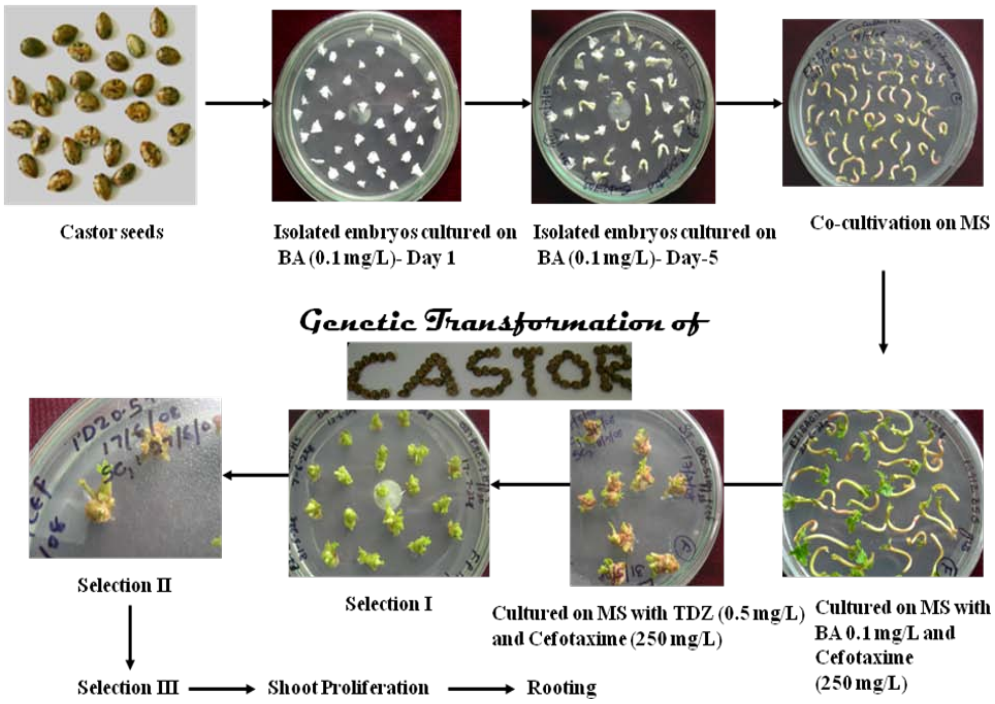
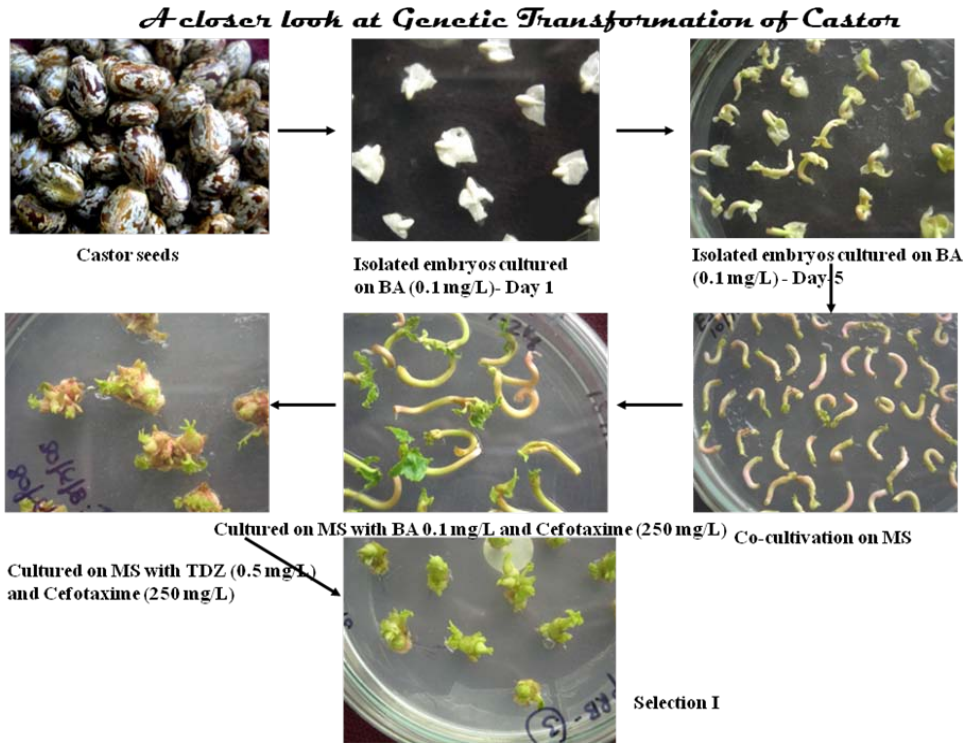


Plate 5: Steps involved in castor transformation and plant regeneration



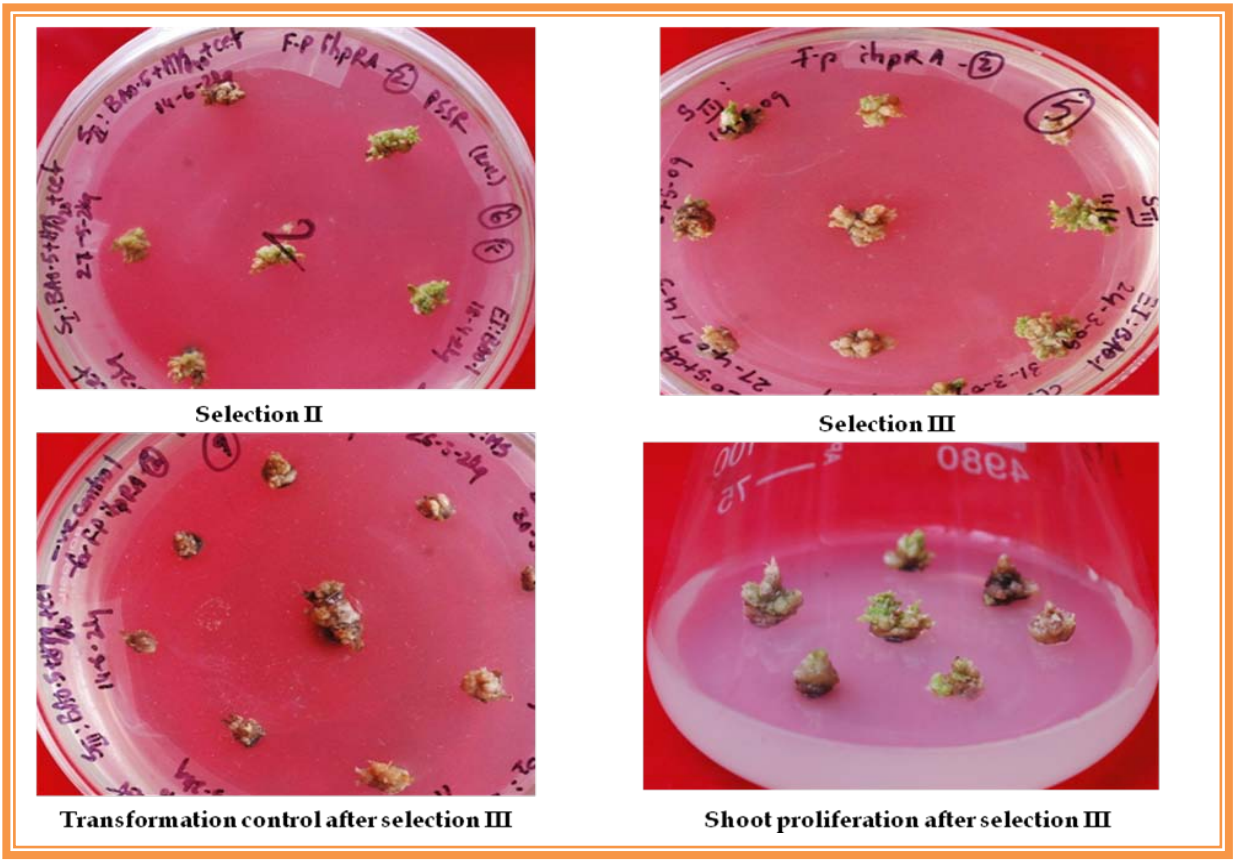


Plate 6: Upper panel : Putative shoots on Selection II ,III and post Selection III
Lower panel : Elongation (A&B) and rooting (C&D) of putative castor

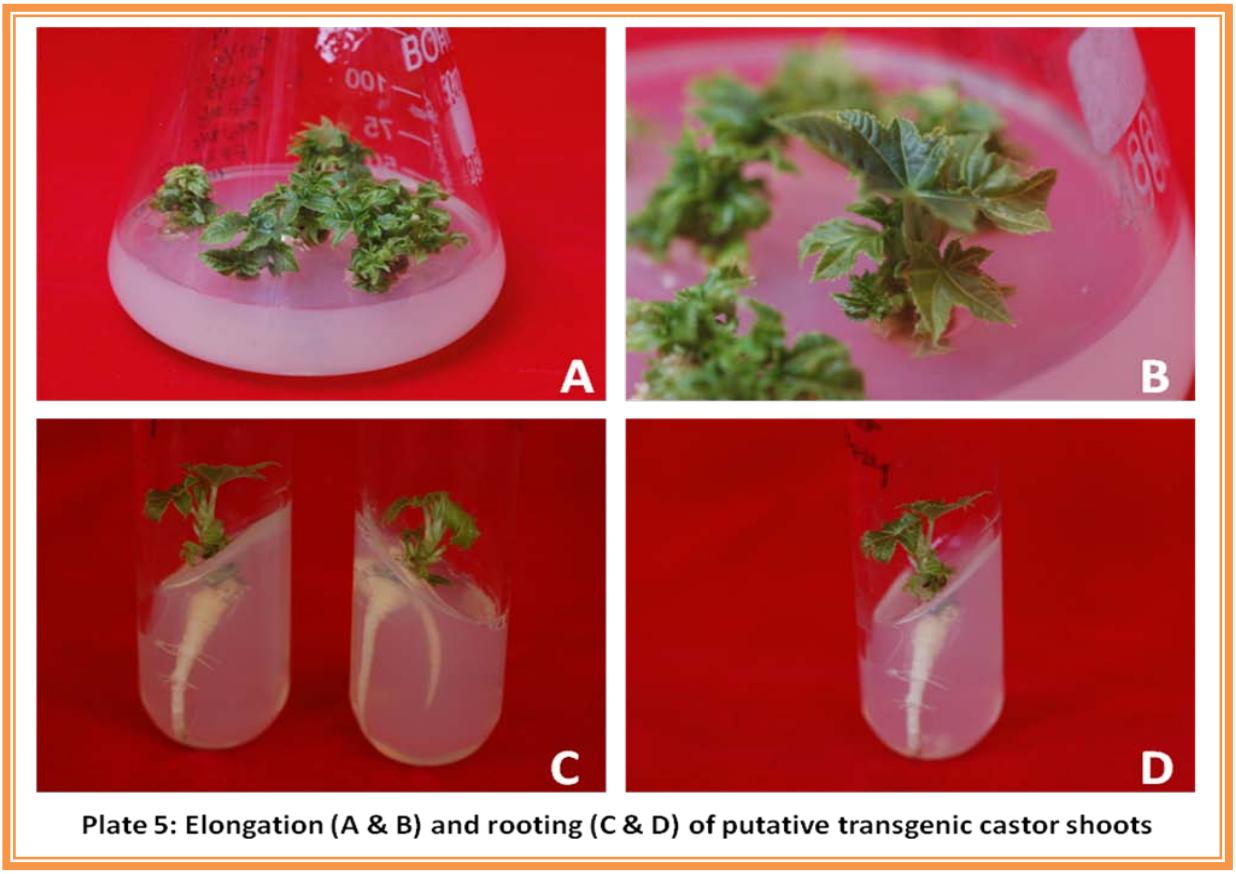


Plate 5: Elongation (A & B) and rooting (C & D) of putative transgenic castor shoots

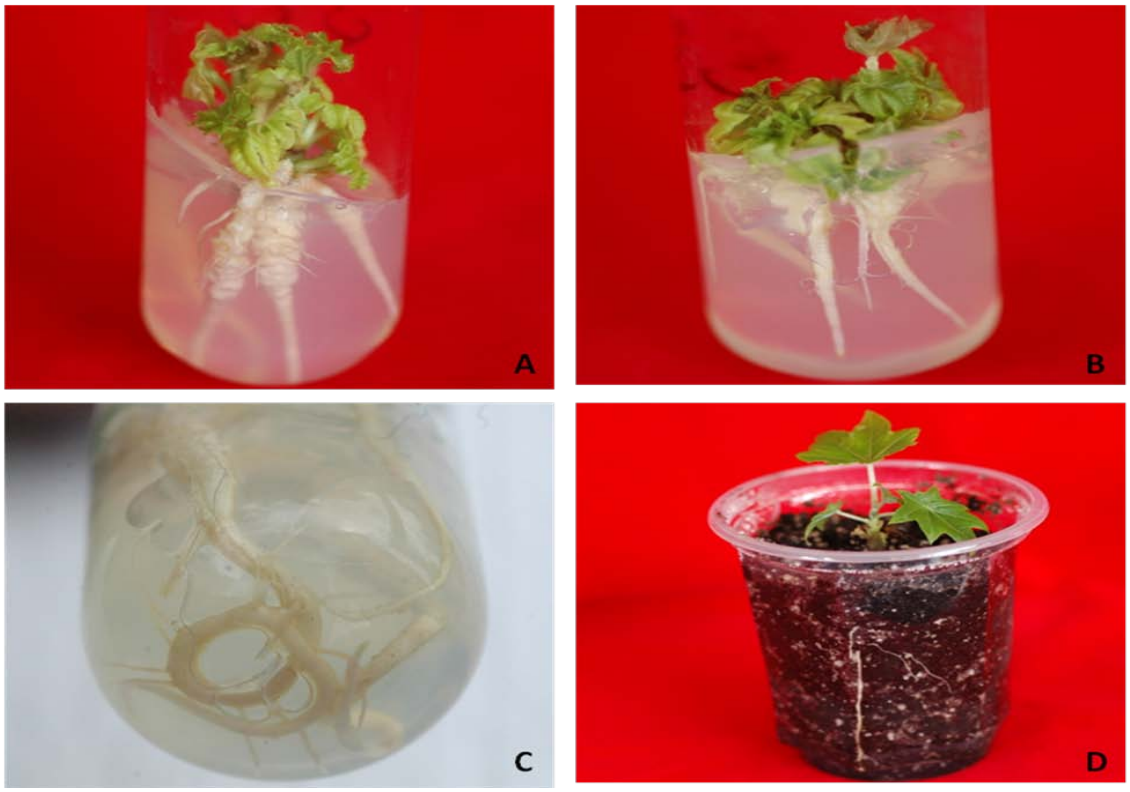
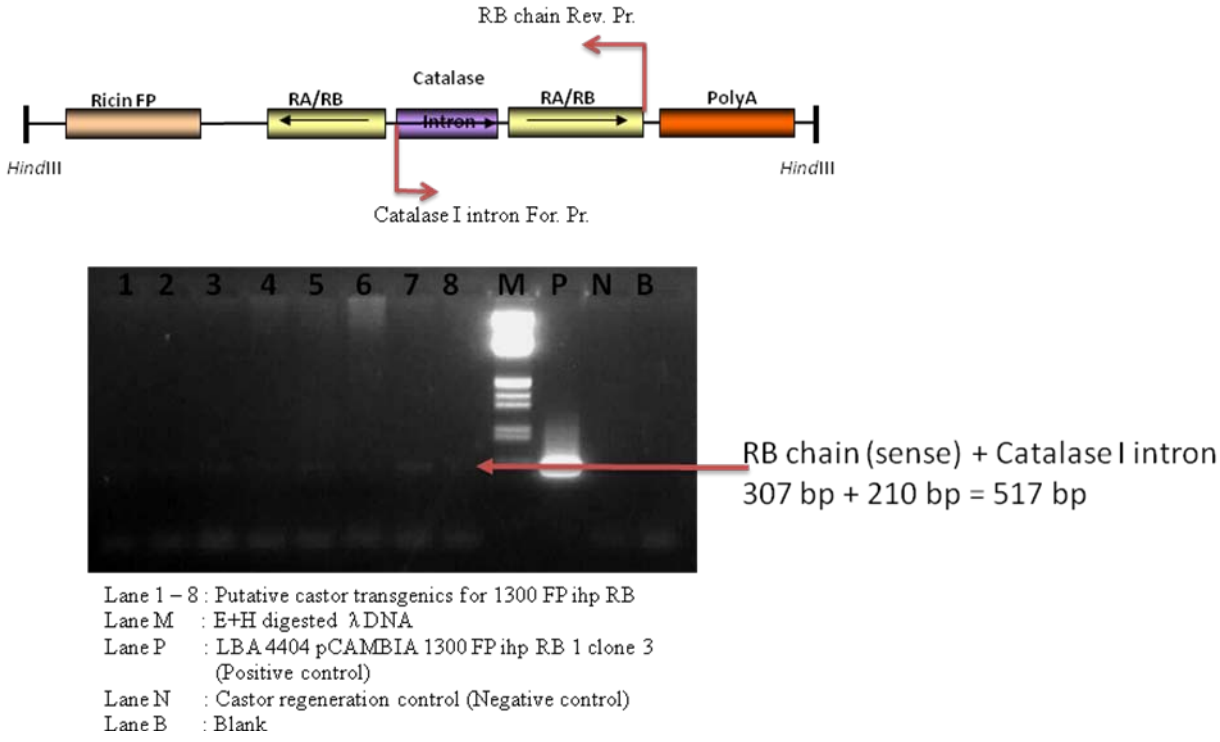


Plate 7: Rooted putative castor plants (A & B) showing presence of lateral roots with root-hairs (C) and acclimatized in plastic containers with peat mass (D)

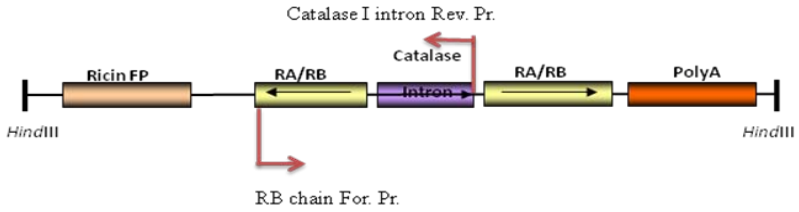
Plate 8: Different stages in acclimatization of putative castor plants in the lab (A,B & C) and in green-house (D)

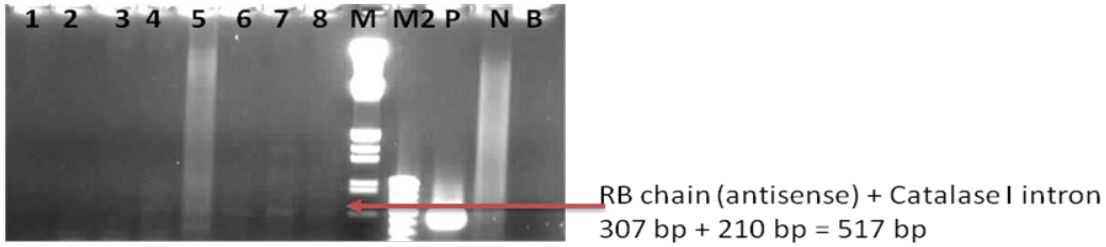
Plate 9: Successfully acclimatized putative castor transgenics in green-house (A). Also seen is acclimatized regeneration control (B)



Lane 1 – 8 : Putative castor transgenics for 1300 FP ihp RB
Lane M : E+H digested λ DNA
Lane P : LBA 4404 pCAMBIA 1300 FP ihp RB 1 clone 3 (Positive control)
Lane N : Castor regeneration control (Negative control)
Lane B : Blank

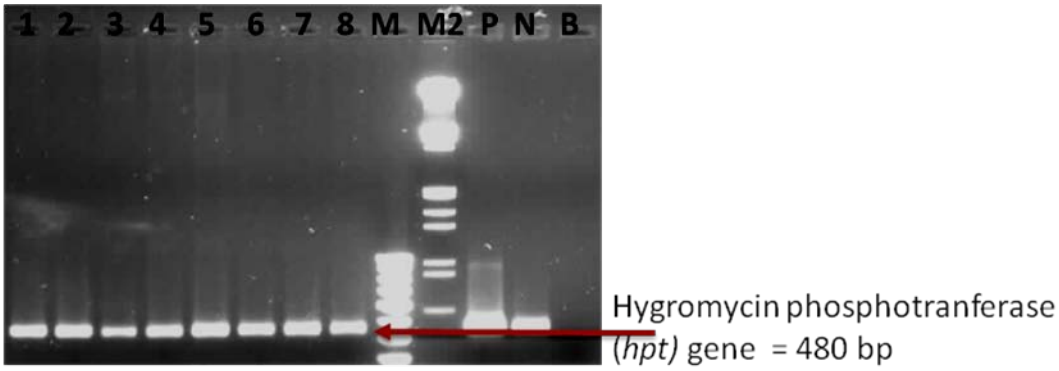
Fig. 4.39. Confirmation of presence of catalase I intron and sense version of RB chain in putative castor transgenics harbouring FP ihp-RB silencing construct through PCR





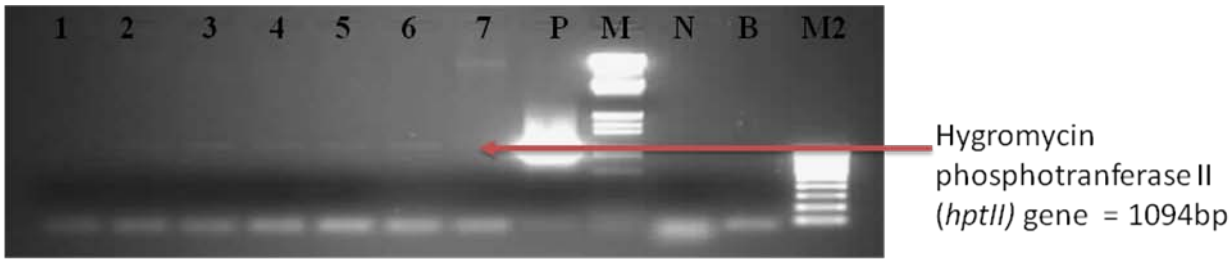
Lane 1 – 8 : Putative castor transgenics for 1300 FP ihp RB
Lane M : E+H digested λ DNA
Lane M2 : 100 bp ladder
Lane P : LBA 4404 pCAMBIA 1300 FP ihp RB 1 clone 3 (Positive control)
Lane N : Castor regeneration control (Negative control)
Lane B : Blank

Fig 4.40. Confirmation of presence of catalase intron and antisense version of RB chain in putative castor transgenics harbouring FP ihp-RB silencing construct through PCR



Lane 1 – 8 : Putative castor transgenics for 1300 FP ihp RB
Lane M : 100 bp ladder
Lane M2 : E+H digested λ DNA
Lane P : LBA 4404 pCAMBIA 1300 FP ihp RB 1 clone 3 (Positive control)
Lane N : Castor regeneration control (Negative control)
Lane B : Blank

Fig 4.41.Confirmation of presence of hygromycin phosphotransferase (*hpt*) gene in putative castor transgenics harbouring FP ihp-RB silencing construct through PCR



Lane 1-7 : PCR product of transgenic castor plants harbouring FP ihp-RB silencing construct
Lane P: PCR product of the positive control (LBA4404::pCAM1300 FP ihp-RB)
Lane M : E+H digested λ DNA
Lane N: PCR product of the untransformed castor (regeneration control)
Lane B: Blank(without DNA)
Lane M2: 100bp ladder

Fig 4.42. Confirmation of presence of hygromycin phosphotransferase (hptII) gene in putative castor transgenics harbouring FP-ihpRB silencing construct through PCR

Table 4.1 : Details of constructs/clones developed during the present investigation

Insert	Source for insert isolation	Vector into which insert was cloned	Enzymes used for cloning	Size of Insert (bp)	Constructs / Clones generated
Catalase intron	T/A catalase intron	pRT 100	*Sst*I and *Kpn*I	207	pRT 100 35S Catalase Intron (Generic ihp vector)
PolyA (antisense)	T/A PolyA	pRT 100 Catalase Intron	*Nco*I and *Sst*I	220	pRT 100 35S antisense PolyA Catalase Intron (Generic SHUTR)
Ricin A chain (antisense)	T/A Ricin A chain	pRT 100 35S Catalase Intron	*Nco*I and *Xho*I	380	pRT 100 35S Ricin A chain (antisense) Catalase intron
Ricin A chain (sense)	T/A Ricin A chain	pRT 100 Ricin A chain (antisense) Catalase intron	*Xba*I	380	pRT 100 35S intron hairpin interrupted RNA (ihp RNA) RA construct
Ricin B chain (antisense)	T/A Ricin B chain	pRT 100 35S Catalase Intron	*Nco*I and *Xho*I	331	pRT 100 35S Ricin B chain (antisense) Catalase intron
Ricin B chain (sense)	T/A Ricin B chain	pRT 100 35S Ricin B chain (antisense) Catalase intron	*Xba*I	331	pRT 100 35S intron hairpin interrupted RNA (ihp RNA) RB construct
Ricin A chain (antisense)	T/A Ricin A chain	pRT 100 35S Antisense PolyA Catalase Intron (Generic SHUTR)	*Nco*I and *Xho*I	380	pRT 100 35S SHUTR RA
Ricin B chain (antisense)	T/A Ricin B chain	pRT 100 35S Antisense PolyA Catalase Intron (Generic SHUTR)	*Nco*I and *Xho*I	331	pRT 100 35S SHUTR RB
Ricin 1.3 kb sequence	T/A ricin 1.3 kb	pRT 100 35S	*Xho*I & *Sst*I	1300	pRT 100 35S Full length antisense ricin
Ricin full length coding sequence	T/A ricin full-length	pRT 100 35S	*Apa*I & *Sst*I	1732	pRT 100 35S Full length sense ricin
Ricin	T/A ricin TP	pRT 100	*Hinc*II and	322	pRT 100 TP

Truncated Promoter (TP)		35S Catalase Intron (Generic ihp vector)	*Xho*I		Catalase Intron (Generic ihp vector)
Ricin Full-length (FP) Promoter	T/A FP	pRT 100 35S Catalase Intron (Generic ihp vector)	*Hinc*II and *Xho*I	944	pRT 100 FP Catalase Intron (Generic ihp vector)
Ricin Truncated Promoter	T/A TP	pRT 100	*Hinc*II and *Xho*I	322	pRT 100 TP
Ricin Full-length Promoter	T/A FP	pRT 100	*Hinc*II and *Xho*I	944	pRT 100 FP
Ricin full length coding sequence	T/A ricin full-length	pRT 100 TP	*Apa*I & *Sst*I	1732	pRT 100 TP Full length sense ricin
Ricin full length coding sequence	T/A ricin full-length	pRT 100 FP	*Apa*I & *Sst*I	1732	pRT 100 FP Full length sense ricin
Ricin Truncated Promoter	T/A TP	pCAMBIA 1391Z	*EcoRI* and *BamHI*	331	pCAMBIA 1391Z TP
Ricin Full-length Promoter	T/A FP	pCAMBIA 1391Z	*EcoRI* and *BamHI*	944	pCAMBIA 1391Z FP
ihpRA cassette	pRT 100 35S ihp RA	pRT 100 FP	*Xho*I	970	pRT 100 FP ihpRA
ihpRB Cassette	pRT 100 35S ihp RB	pRT 100 FP	*Xho*I	930	pRT 100 FP ihpRB
Ricin Full-length Promoter	T/A FP	pRT 100 35S Full length antisense (FL A/S) ricin	*Hinc*II and *Xho*I	944	pRT 100 FP FL A/S R
Ricin Full-length Promoter	T/A FP	pRT 100 35S SHUTR RA	*Hinc*II and *Xho*I	944	pRT 100 FP SHUTR RA
Ricin Full-length Promoter	T/A FP	pRT 100 35S SHUTR RB	*Hinc*II and *Xho*I	944	pRT 100 FP SHUTR RB

Table 4.2. Logistics of the castor transformation work carried out during the investigation

No. of embryos	No. of explants co-cultivated	No. of explants on recovery medium	No.of explants on Proliferation medium	No.of explants on sel I	No.of explants on sel II	No.of explants on sel III	No.of explants on 1st cycle of proliferation	No.of explants on2nd cycle of proliferation	No.of explants on3rd cycle of proliferation	No.of explants on 4th cycle of proliferation
37,350	**24,510**	**19,426**	**8,685**	**4,298**	**2,350**	**1,372**	**310**	**93**	**52**	**23**

No.of explants on elongation medium	No.of shoots kept for rooting	No.of shoots rooted	No.of shoots acclimatized	No.of shoots survived after acclimatization	No.of shoots transplanted to pots	No.of shoots survived in pots
1500	236	87	87	25	25	**8**

CHAPTER V

DISCUSSION

Genetic engineering is being applied to eliminate or substantially reduce plant-derived substances that can be harmful to human or animal health in otherwise nutritious foods. Post-transcriptional gene silencing (PTGS), has been used in efforts to remove allergens from rice, soybean, apple, tomato and peanut. RNA silencing has the potential to simultaneously alter expression of all members of a multi-gene family in a tissue-specific manner with little collateral change in the plant.

The strategy used to reduce plant food allergens has relied on post-transcriptional gene silencing that result in sequence-specific mRNA degradation thereby preventing translation. The first attempts to eliminate plant allergens were in rice (Tada et al. (1996)) and soybean (Herman et al. (2003)) where seed-specific promoters drove antisense or sense constructs, respectively. Although the rice transgenics and their progeny had substantially reduced levels of the targeted 14-16 kDa allergens, none of them were allergen-free probably due to insufficient sequence homology between the antisense constructs and members of the multi-gene family that encoded these allergens (Tada et al. (1996)). By contrast, complete knockdown of the soybean allergen, GlymBd 30K also known as P34, was obtained in one co-suppression line (Herman et al. 2003). Significantly, P34 elimination was maintained over three generations and there were no observable plant morphological or reproductive differences between transgenic plants and wild type. Thus, genetic engineering can produce food crops that are hypoallergenic or are rendered harmless owing to detoxification. The advantage of this approach is that it can make foods that were once unsafe available as good sources of nutrition and calories while also paving the way for better consumer understanding and acceptance of genetically modified foods. Genetic engineering technologies have advantages over classical breeding, not only by enlarging the scope of genes and the types of mutations to be manipulated, but also by the ability to control the spatial and temporal expression patterns of the genes of interest. Additionally, with the current swift advancement of plant genomics, proteomics and metabolomics, there will be both a store of novel genes to evaluate and improved genetic engineering strategies for continued food crop improvement.

To reduce the levels of undesirable gene products, two general approaches are commonly used: recessive gene disruption and dominant gene silencing. In gene disruption approaches, the target sequence is mutated to eliminate either expression or function, whereas dominant gene silencing methods induce either the destruction of the gene transcript or the inhibition of transcription. The advantages of the dominant gene silencing methodologies

over the gene disruption approach are 2-fold. First, dominant gene silencing is easier to manipulate genetically and to screen for subsequent transgenic plants. Second, dominant gene silencing can be manipulated in a spatial and temporal manner, using specific promoters. Among the dominant gene silencing approaches, dsRNA triggered RNAi is the most powerful method (Smith et al. 2000); it is the most efficient in terms of the extent of gene silencing and the resulting silencing is almost as complete as in a gene knockout approach. It has been observed that dsRNA triggered RNAi directly bypasses the requirement for dsRNA synthesis via RdRP, which is likely the rate-limiting step in the plant RNAi pathway.

RNAi has been used successfully to target a wide range of genes including those encoding transcription factors, starch biosynthetic enzymes, storage proteins, and proteins involved in signalling and developmental processes. The RNAi response has been documented in different tissues (seeds and leaves) and developmental stages (vegetative, grain filling, and senescence) and has been shown to be stably inherited. The phenotypic series obtained in most RNAi experiments provide valuable information on the effect of quantitative differences in transcript levels of the target gene, allowing a more precise understanding of the gene's function. In addition, the variable quantitative response generated by RNAi is an advantage when the complete knockdown of the target genes might result in lethality or extreme phenotypes with multiple pleiotropic effects. In these cases, intermediate levels of transcript down-regulation could have less severe effects facilitating a better definition of gene function.

The major problem of using castor oil meal as animal feed is the presence of toxic proteins, ricin and RCA. The genes encoding these proteins have been well characterized at the nucleotide sequence level. Therefore, it is surmised that these proteins could be eliminated or reduced to acceptable levels using powerful PTGS techniques. Efforts to reduce both ricin and RCA content using conventional breeding approaches have yielded little success due to lack of sufficient genetic variability and because of complex genetic control of the genes encoding these proteins. Other methods including physical and chemical treatments to detoxify the castor meal are not economically viable and they reduce the availability/ digestibility of the proteins in the meal.

Ricin and RCA proteins are encoded by a family of genes, which share a high percentage of homology, and these genes have been cloned and completely characterized. Multiple genes sharing stretches of identical sequence can be effectively silenced with a single RNAi construct. This feature is especially important in species like castor, which has

multiple copies of ricin and RCA genes. These genes are shown to be very tightly regulated at the transcriptional level with their expression clearly restricted to the developing endosperm. These proteins are shown to start accumulating at stage D (beginning of the testa formation) reaching a maximum by stage F (the completion of testa formation and beginning of seed maturity). Both transcriptional and translational regulations have been shown to tightly regulate the accumulation of these proteins (Tregear and Roberts, 1992; Lord *et al.,* 1994; Frigerio and Roberts, 1998; Pinkerton *et al.,* 1999). Therefore, if these genes are to be post transcriptionally silenced, the trigger for silencing also must be present in the cells at the exact time. In other words, the trigger molecules of the silencing must be present in the developing endosperm tissue. Thus, the siRNA molecules must be produced in the endosperm tissue. Unfortunately, the commonly used constitutive CaMV35S promoter does not express well in the endosperm tissue (Benfey and Chua, 1990). Hence, if silencing molecules are to be expressed in endosperm tissues, the gene construct for the same must be driven by endosperm specific promoter. Therefore, this has warranted isolation of the ricin promoter, which is expected to express specifically in the endosperm tissue of castor. Thus availability of non-constitutive, highly specific seed promoters will be useful in transgenic strategies aimed at the elimination of ricin and RCA

In the present investigation, attempts have been made to silence ricin and RCA genes in castor. This involved three main activities i) isolation and characterization of the endosperm specific promoter from castor, ii) development of appropriate vectors for PTGS silencing of ricin and RCA and iii) development of transgenic castor plants with the chosen vectors. The results obtained during the course of this investigation are discussed here in the light of the literature available and the interpretations that could be made based on the empirical results obtained during experimentations.

5.1 Isolation and characterization of ricin promoter from castor

The technique of inverse polymerase chain reaction (IPCR) (Triglia *et al.,* 1988; Ochman *et al.,* 1988) was employed to isolate the upstream sequence of the ricin gene whose sequence was already known. IPCR leads to the amplification of previously unknown sequences upstream of downstream of a known sequence and is based on the principle that circular DNA could act a template for PCR. In IPCR, primers are designed in such a way that they face away from each other on a linear template DNA but will face each other when the linear DNA is circularised. Thus, the linear template DNA that includes the target sequence, will be

circularized and primers designed based on the known sequence will be used to amplify the unknown region. Further, amplification with nested primers ensures the integrity of the final product, which can be sequenced directly.

The nucleotide sequence for a 310 bp upstream of the ricin gene was already available in the NCBI database (Tregear and Roberts, 1992). This served as a template for fishing out the upstream sequence. Using inverse PCR, a 622 bp fragment upstream of the 5' of 310 bp of the ricin gene was isolated. The already known 310 bp was designated as the truncated ricin promoter (TP) and this together with the isolated 622 bp was designated as the full-length ricin promoter (FP).

Searches for the putative cis-acting elements in the promoter region were performed using PLACE. This analysis indicated the presence of several cis-elements like two TATA boxes, two RY repeat motifs, twelve CAAT boxes, four MYB binding sites, twenty-eight DOFCORE and two E boxes in the sequence of the isolated promoter. The RY repeat element (or Legumin box) is one of the significant sequence motifs required for seed-specific expression. In fact, this is the one which gives endosperm-specific expression. Some functional elements in the promoters, such as DOFCORE (Yanagisawa 2000), and E box (Hartmann et al. 2005), are related to specific regulations in endosperm. Moreover, the E box is supposed to participate in the regulation of expression of the storage protein. Other functional elements of the promoter, such as the MYB binding site are influenced by light, phytohormone, pathogen inductions, and also respond to dehydration.

The DOFCORE (AAAG) motif is known for endosperm-specific expression in maize, which is bound to Dof proteins. Dof proteins are DNA binding proteins existing uniquely in plants and they can enhance transcription (Yanagisawa 2000). PBF, isolated from maize, is one type of Dof proteins bound to Prolamin box (Wu et al. 2000). But it is more efficient for PBF to bind with four tandem repeated DOFCORE than with single DOFCORE (Yanagisawa and Schmidt 1999).

The isolated putative promoter sequences had to be experimentally validated using appropriate model expression assays. Therefore, appropriate expression vectors were developed using *gus (uidA)* gene and two approaches namely the stable *Agrobacterium*-mediated transformation of tobacco and transient studies with developing seeds of castor (47DAP) were followed. The isolated promoter sequences were cloned upstream of the promoterless *gus* gene in pCAMBIA1391Z. These vectors were used for tobacco transformation. As control vectors, 35S promoter cloned upstream of *gus* gene in

pCAMBIA1391Z as well as the basal vector (pCMBIA1391Z) were used for transforming tobacco. Also, to assess the effect of expressing ricin gene under the isolated promoters, two additional vectors were developed in pCAMBIA2300 where FP and TP were cloned upstream of the full length ricin gene so that the expression levels of ricin transcript could be assessed in the developing seeds. These two constructs were also used to transform tobacco. Thus totally the following six constructs were developed and used for tobacco transformation.

1). LBA4404:: pCAMBIA 1391Z TP
2). LBA4404:: pCAMBIA 1391Z FP
3). LBA4404:: pCAMBIA 1391Z CaMV35S
4). LBA4404:: pCAMBIA 1391Z
5). LBA4404:: pCAMBIA 2300 TP Ricin 1.7kb
6). LBA4404:: pCAMBIA 2300 FP Ricin 1.7kb

Reports of expressing ricin gene in tobacco are already available (Lord et *al.,* 1985; Sehnke *et al.,* 1994; Tagge *et al.,* 1996; Frigerio *et al.,* 1998). From the results obtained in these studies, it has been concluded that the introduced preproricin expressed without any deleterious effects on the transgenic plant. It has also been shown that in these transgenic plants, the ricin protein biosynthesis as well as folding and other downstream processes like transport through sub-cellular compartments and further localization into protein bodies followed exactly the same pathway as seen in castor plant. Thus, it has been established that the ricin protein could be successfully expressed in tobacco plant without any consequences. Moreover, tobacco, being an endospermic plant offers a good model system to study the expression patterns of endosperm specific promoters. Therefore, the approach of assessing the isolated ricin promoters through development of transgenic tobacco plants was adopted. Tobacco transgenics were developed with each of the constructs using the standard leaf disc transformation protocol.

The transgenic plants obtained with each of the constructs were confirmed for the presence of intact gene cassettes. All the component sequences in the cassette were confirmed by carrying out PCRs specific to each of the components. The transgenics which gave consistent amplification across different sets of primers used were then taken for further characterization. Towards this, RT-PCR assays would have provided us with the right cue about the actual developmental stage of expression and accumulation of ricin and RCA

transcripts within the endosperm of tobacco. But the expression studies of the transcripts of ricin and RCA across different stages of tobacco seed development could not be carried out as good quality RNA could not extracted from the developing seeds of these tobacco plants in spite of several attempts made using different protocols. This would be one of the future lines of work to be undertaken. However, GUS assays were performed with the different tissues of transgenic tobacco harbouring constructs 1 through 4 (above). The different tissues tested for the GUS assay inlcuded leaf, sepal, petal, stamen and carpel. Constructs 1391Z FP, 1391Z CaMV35S *gus* and 1391Z (basal vector) yielded consistent blue colouration in the carpel tissue. Since the intact carpel was put for GUS staining, it was difficult to ascertain whether the evident blue colouration was contributed by the entire tissue or was due to the ovules. There was no blue staining of any other tissue from the plants carrying either FP-*gus* or TP-*gus* which demonstrated the tissue specific nature of the isolated ricin promoters though it did not ascertain the seed specific nature of the isolated promoters. There was blue coloration observed across different tissues of plants obtained with 1391Z CaMV35S *gus* and this clearly indicated the constitutive expression pattern of 35S promoter. Interestingly, there was a faint blue colour seen with different tissues in the plants transformed with just the basal vector (pCAMBIA1391Z). Such an observation has already been made by other workers also (Thomas et al., 1990**,** Chileh et al., 2010). All the tissues namely the leaf, sepal, petal, stamen and carpel stained deep blue in case of construct 1391Z CaMV35S *gus* and light and faint blue in case of construct 1391Z.

Transient gene expression studies were also carried out to ascertain the tissue-specificity of the isolated ricin promoters. Transient gene expression systems are often used because they offer a number of advantages over analysis of stable expression: gene expression can be measured very shortly after the DNA delivery, the expression is not biased by position effects, and gene transfer can be assayed independently from regeneration of a transformed cell into a transgenic plant, which is especially advantageous for species recalcitrant to regeneration. In addition to its utility in analyzing promoters and other gene regulatory regions, the transient assay is a quick and easy method for testing reporter gene constructs prior to stable genetic transformation. In the present investigation, two approaches, particle gun-mediated bombardment and *Agrobacterium*-mediated transformation of the developing seeds of castor (47 DAP), were followed to characterize the promoter activity. Based on a preliminary report (Chen et al., 2005), the definite stage of castor seed (when ricin and RCA transcripts peak to the maximum) to be used for transient study was realized as 47

DAP. Both these methods have been employed for the transient studies of gene expression pattern in many different crops (Chileh et al., 2010; Qin et al., 2010).

The results obtained with bombardment studies were not consistent and inconclusive. There was a problem in getting the seeds of the right stage (47DAP) and just based on the morphology of the seeds it was difficult to decide on the exact age of the developing seeds. Also, the blue colouration observed was more of a diffused type than the colour foci that is normally reported. Even though a few parameters such as different DNA coating methods, different batches of chemicals, different duration of incubation were tried, the deep blue coloured foci were not noticed in these bombardment exercises. However, there was a difference in the (blue) colour intensity of the gus-stained castor seeds bombarded with different constructs. The seeds bombarded with FP-gus and TP-gus showed deeper blue colouration compared to those bombarded with either the basal vector or the one with pCAMBIA1391Z-35S *gus*. This expression pattern was consistent across the three replications and thus showed that the castor ricin promoter was functional in castor and expressed in the developing seeds.

The other transient approach of addressing the functionality and tissue-specificity of ricin promoters was the *Agrobacterium*-mediated transformation of developing seeds of castor. A rapid 3-day *Agrobacterium*-mediated transient assay system to test the functionality of ricin promoter in developing seed of castor (47 DAP) was undertaken. Castor seeds of 47 DAP were injured with a fine needle and co-cultivated with *Agrobacterium* harbouring the different constructs. Post-co-cultivation, the seeds were stained for GUS activity 3-5 days following infiltration. Appropriate controls were also included in the exercise to validate the study. Again the results of this experiment were similar to the results obtained with bombardment. Taken together, it was concluded that the isolated ricin promoters expressed in the developing seeds of castor.

5.2 Development of silencing vectors

As discussed in the Review of Literature (Chapter 2), ricin and RCA are the two toxic proteins which render the deoiled meal of castor unfit for animal consumption. The levels of these proteins should be reduced by 94 % to ensure safety of the deoiled meal for animal consumption. Therefore, the silencing of ricin and RCA genes at the transcript level must be substantial. To ensure this, appropriate silencing technology/technologies should be empirically tested and the most efficient strategy should be adopted to develop ricin and RCA

free castor. Several approaches have been adopted for post-transcriptional gene silencing and there are evident differences in the efficiencies of these approaches. Though antisense RNA technology has been the first approach adopted for silencing the genes at the RNA level, this approach is shown to be less efficient and leaky in silencing the targeting gene(s) and necessitates the production of a large number of independent transgenics in order to select the appropriate plant that can bring about the desired levels of silencing the target gene(s). This would be a major limitation especially in species such as castor, where the transformation and regeneration are still a problem. To overcome these limitations, alternate strategies for gene silencing have been developed. Gene constructs encoding intron-spliced RNA with a hairpin structure have been shown to induce PTGS with almost 100% efficiency when directed against viruses or endogenous genes (Smith et al., 2000). In these constructs, it has been shown that when a functional intron is cloned in between sense and antisense sequences of the target gene, it results in a stable hairpin-RNA after the splicing process and also results in silencing of the target gene in almost 100% of the transgenics obtained. When the functional intron, was replaced with any other spacer sequence or non-functional intron, then the efficiency of gene silencing was reduced significantly. So, in this elegant experiment, they have demonstrated that stable, dsRNA against a target gene results in highly efficient suppression or down regulation of the target gene.

In another study, Wesley et al. (2001) compared the efficiency of different types of constructs for gene silencing and have identified intron hairpin RNA strategy as the best for abolishing the gene activity. They have designed a generic vector, which could be used for gene silencing in any plant system. The authors have also opined that these vectors be used for silencing whole gene families if a common conserved region among the target genes is chosen for making hpRNA construct. Recently, it has been demonstrated that if a portion of the target gene is cloned upstream of an inverted repeat of 3’ untranslated region (3’ UTR) and subsequently introduced into the plant, a high efficiency gene silencing of the target gene is achieved (Brummell et al., 2003). This type of gene silencing called silencing by heterologous 3’ untranslated regions (SHUTR) has the advantage of ease and rapidity in preparation of the constructs, since a gene of interest can be inserted into a binary vector already containing the promoter and the inverted repeat of the 3’-UTR, in a single cloning step, and does not require any knowledge of the DNA sequence. The authors have demonstrated the utility of this technique for silencing many genes and transcription factors.

In the present investigation, vectors were developed to reduce the levels of ricin and RCA proteins in castor. Three different PTGS approaches have been adopted to develop these vectors. These vectors were developed in a two step manner, in the first step, the generic vectors were developed and in the second step, the specific vectors for silencing ricin and RCA gene were developed.

5.2.1 Development of generic silencing constructs

Generic vector refers to such vectors which allow cloning of any desirable gene(s) in its backbone already having some component sequences needed to fulfil the feature of the desired vector. In our case, the generic vectors have the property of producing whether ihpRNA or bringing about silencing by heterologous 3'UTR of the target mRNA if representative sequences of the target gene(s) are cloned in these vectors. These generic vectors for induction of PTGS have been developed under the control of CaMV35S promoter in the backbone of pRT100 vector. The choice of the vector pRT100 was based on the presence of CaMV35S promoter, a suitable MCS and a polyA signal.

The development of generic ihpRNA vector involved cloning a 210 bp of functional catalase intron (from castor bean) downstream of CaMV35S and upstream of Poly A in pRT100 backbone. Later, the sense and antisense versions of the desirable gene(s) could be cloned on either sides of the catalase intron so that when this vector is introduced in the plant, a hairpin RNA molecule against the target transcript would be produced constitutively. Catalase intron was chosen for developing this generic ihp vector as this intron has been shown to be functional in several heterologous plant systems and is being used in all the pCAMBIA binary vectors. While selecting the catalase intron sequence to be cloned, deliberately the intron-exon junction was included on both the sides to facilitate appropriate splicing during transcript processing in the host plant. Thus, by cloning the functional catalase intron in pRT100 background, a generic ihpRNA vector (pRT100-ihp) was developed.

The generic SHUTR (silencing by heterologous 3'untranslated region) vector was essentially an extension of the generic ihpRNA (pRT100-ihp) vector developed as described above. This vector is expected to be functional in the host plant through the phenomenon called transitive RNAi where the silencing signals (dsRNA) targeting the sequences both downstream as well as upstream of the targeted transcript region will be produced using the RNA dependent RNA polymerase (RdRP) enzyme of the host plant. This kind of vector has

been developed by Brummell et al. (2003). Developing the generic SHUTR vector in the present study involved cloning antisense version of CaMV35S-Poly A (220 bp – the same sequence found in pRT100) between the catalase intron and CaMV35S promoter. Here, the catalase intron is sandwiched between the upstream antisense Poly A and downstream sense Poly A (already present in pRT100 vector backbone). The generic SHUTR vector facilitates cloning either sense or antisense version of the desirable gene upstream of antisense version of Poly A and downstream of CaMV35S promoter in pRT100 vector backbone.

As ricin and RCA expression is restricted to the endosperm of the developing castor seeds, to silence these genes, the silencing signal (siRNA) must be produced in the endosperm tissue of castor. However, the expression levels of CaMV35S promoter in the endosperm tissue has shown to be lower (Benfey et al., 1990). Therefore, it was decided that the 35S promoter present in the developed generic vector would not be ideal to achieve silencing of ricin and RCA genes and so should be replaced by the endosperm specific ricin promoter. In the above developed generic vectors, the CaMV35S promoter was excised out and full length ricin promoter was cloned in its place to enable induction of PTGS in the endosperm tissues. Thus, this exercise generated generic endosperm-specific PTGS vectors,

1. pRT 100 Ricin Full length promoter (FP) Intron hairpin interrupted RNA (ihpRNA) vector
2. pRT 100 Ricin Full length promoter (FP) Silencing by heterologous 3' untranslated region (SHUTR) vector.

It is postulated that these generic endosperm specific vectors could be used for silencing any endosperm specific genes in heterologous plant systems.

5.2.2Development of PTGS constructs for silencing ricin and RCA in castor

Development of ricin and RCA specific silencing constructs involved three different strategies utilizing the gene sequence of A chain and B chain of ricin and RCA. One of the strategies included the conventional antisense technology, in which the full-length antisense version of ricin/RCA was cloned between constitutive promoter (CaMV 35S/ ricin FP) and 35S polyA tail. The other two strategies, aimed at silencing the ricin/RCA through the production of dsRNA which triggers targeted degradation of ricin/RCA transcripts through 'dicer' and 'RISC' mediated mechanisms. This exercise involved cloning of inverted repeats of the selected parts of A chain (RA) and B chain (RB) of ricin/RCA (380 bp in case of ricin/RCA RA and 330 bp in case of ricin/RCA RB) separated by the catalase intron I. This is surmised to result in a hairpin double-stranded RNA transcript which would eventually be

acted upon by 'dicer' and 'RISC' yielding siRNAs targeted against cognate transcripts for degradation. The other strategy was executed by cloning antisense version of selected part of A chain and B chain of ricin/RCA upstream of an inverted repeat of a heterologous 3'-UTR (untranslated region) *viz.*, 35S polyA, separated by catalase intron, so that when expressed in plants, the resultant transitive RNAi could induce sequence specific degradation of ricin/RCA transcripts.

Both the PTGS and antisense constructs were cloned in the background of pRT100 vector (a plant expression vector). Using *Hind*III, the entire cassettes were taken out and cloned in the binary vector, pCAMBIA1300. The constructs in the binary were then mobilized into *Agrobacterium tumefaciens* strain LBA4404 for effecting castor transformation. The component sequences and the constructs obtained at different stages of construct development were confirmed by methods like colony PCR, PCR with the isolated plasmid, restriction analysis and sequencing. Whenever it was essential that the orientation of a particular component sequence to be cloned in a construct be checked, it was carried out. The mobilization of all the developed constructs into LBA4404 strain of *Agrobacterium* was confirmed by colony PCR, PCR with the isolated plasmid, restriction analysis, reverse mating and β-ketose lactose test. The confirmed PTGS and antisense constructs were then used for tobacco and castor transformation.

For selecting the appropriate sequence of ricin and RCA for inclusion in the generic vectors for effective posttranscriptional gene silencing of ricin/RCA through PTGS, all the guidelines as envisaged (Wesley et al.(2001); Heliwell et al.(2002)) were followed. It has been suggested by many research workers that a part of the gene would be enough to suppress the activity of the target gene.

Trigger size may place a major limitation on the design of the RNAi construct when the target gene contains only short pieces of non-conserved regions. In these cases it is important to know the minimum trigger size that can be used to induce effective gene silencing. Gene fragments ranging from 300-400 bp have been used to successfully silence them. Two factors can influence the choice of length of the fragment. The shorter the fragment, the lesser will be the effective silencing. Very long hairpins increase the chance of recombination in bacterial host strains. The effectiveness of silencing also appears to be gene dependent and could reflect the accessibility of target mRNA or the relative abundances of the target mRNA and the hairpin RNA in cells in which the gene is active. A fragment length

of 300-1000 bp as a suitable size to maximize the efficiency of silencing obtained has been recommended.

Successful RNAi studies in wheat have used trigger regions ranging in size from ~200 to 550-bp, although trigger dsRNA as short as 23-bp have been shown to induce the degradation of target mRNAs in *Nicotiana benthamiana* (Thomas et al. 2001). Transient RNAi studies using VIGS showed that a trigger of 120-bp was sufficient to produce significant gene silencing (Scofield et al. 2005). In the same experiment, trigger sequences of 80-bp were less effective for gene silencing and no detectable silencing was seen when using trigger sequences of 40-bp. Assuming that the VIGS data can be extrapolated to stable RNAi transformed plants, RNAi trigger sizes of at least 120-bp are desirable.

The upper limit for RNAi trigger size is less defined, with successful examples in wheat of up to 683 bp (Li et al. 2005) and in barley VIGS of up to 1,215 bp (Holzberg et al. 2002). The determination of the upper limit for RNAi trigger size may be also related to the number of targets that can be effectively included in a single RNAi construct to knockdown multiple target transcripts. For example, Miki et al. (2005) reported a progressive decrease in silencing efficiency in rice when increasing the size of a chimeric construct from 553 (two targets) to 1,089-bp (four targets).

Researchers have also reported that the use of either 5'UTR or coding region or 3'UTR fragments gave considerable degree of silencing. Since the mechanism of silencing depends on sequence homology, there is a potential for cross silencing of related mRNA sequences. Where this is not desirable, a region with low sequence similarity to other sequences, such as a 5' or 3' UTR, should be chosen. The rule for avoiding cross-homology silencing appears to be to use sequences that do not have blocks of sequence identity of over 20 bases between the construct and the non target gene sequences. This could be achieved when the chosen sequences for RNAi construct development is analysed for sequence homology with all the known gene sequences of the target plant. Usually a BLAST analysis carried out with 20 bp contiguous sequence (of all combinations) of the chosen DNA fragment (used in RNAi vector) against the sequences available of the target host(s) will reveal the off-target effects, if any.

In the present investigation, as the aim was to silence both ricin and RCA genes (present as multi-copy genes in the genome) simultaneously, a sequence with perfect homology between the ricin and RCA was to be chosen. After aligning all the available sequences of the ricin family, care was taken to search for such portions of the Ricin and

RCA genes which were conserved across all the sequences (Appendix - I). This was essential to knock down all the members of the gene family.

After careful consideration of all the factors discussed, a fragment of 357 bp of A chain and 307 bp of B chain was chosen for developing the silencing vectors. By employing BLAST analysis with every 20 bases of the chosen A and B chain fragments, those fragments that would have least off-target effects were chosen. Care was taken not to include regions which could fold-back on their own, thus unamenable to the access of the PTGS biochemical machinery. This has been achieved by subjecting the chosen fragments of ricin and RCA to a bioinformatics tool, M-fold.

The castor specific catalase I intron isolated from pCAMBIA 1305.2 has been included in the PTGS constructs. Catalase I intron is one of the functional and thoroughly characterized introns and this is evident by its inclusion across different plant transformation vectors. While designing primers for the amplification of catalase I intron, care has been taken to include the intron-exon junction and two bases of exon on either side of intron. This is highly indispensable as intact exon-intron splice junctions are very crucial for the process of splicing. Although intronless genes clearly are expressed, insertion of introns has improved expression efficiency in some systems. The positive effect of introns is, however, not universal. In monocots such as *Lolium* and sugarcane, the same introns have no or even a negative effect (Last et al., 1991; Rathus et al., 1993). It is possible that introns in a heterologous context are spliced inefficiently (Kyozuka et al., 1991) or that different splice sites are used than in their natural setting (Tanaka et al., 1990). Although stimulatory effects were also obtained with the first intron of a castor bean catalase gene in rice (Tanaka et al., 1990), monocot introns in dicot plants are usually inhibitory (Xu et al., 1994; Rathus et al., 1993, Tanaka et al., 1990) because splicing is inefficient (Keith et al., 1986; Goodall et al., 1989; Peterhans et al., 1990). Taken together, it was considered prudent to include the catalase intron of castor for developing the silencing vectors in the present investigation.

Thus based on logic and the known information, 5 different PTGS vectors were developed for using them to silence ricin and RCA genes in castor. However, owing to the problems of lower transformation frequencies in castor, it was decided at the Biotechnology laboratory of Directorate of Oilseeds Research, Hyderabad that these vectors be validated using tobacoo as a model system. To achieve this, tobacco was to be transformed to express the ricin gene under 35S promoter and these transgenic plants were to be retransformed with PTGS vectors (against ricin) producing the siRNA molecules constitutively. Alternatively, it

was planned that transgenic tobacco plants harbouring the PTGS vectors (under 35S promoter) would be developed and the resultant plants would be crossed to plants expressing ricin gene, and the progeny would be analysed for the efficient silencing of the ricin gene. Once the efficient PTGS vector(s) was identified through this approach, castor was to be transformed only with the selected vector(s). Though this work was simultaneously initiated at DOR by another research scholar, the set objective of identifying the efficient PTGS approach using tobacco as model system could not be achieved. The transgenic tobacco plants expressing ricin did not flower and also these plants could not be retransformed with the PTGS vectors as many transformation attempts failed to yield retransformed plants. This was unexpected because there are reports of successful transformation of tobacco reported (Schenke et al., 1983). Failure of identifying the efficient PTGS approach using tobacco as model system, led to the option of choosing the appropriate PTGS approach based on the available literature. It has been proven that ihpRNAi approach is better than transitive RNA approach (Filichkin et al., 2007). Also, in another project at DOR, it has been observed that RNAi mediated gene silencing was efficient in reducing the transcript levels of *orf*H522 compared to SHUTR approach when these two approaches were used for restoring the fertility in the transgenic tobacco male sterile plants carrying *orf*H522 gene (Narasimha Rao et al., 2010, unpublished data). Based on these observations, it was decided that castor could be transformed with ihpRNAi vectors.

5.3 Genetic transformation of castor

Castor is extremely recalcitrant to *in vitro* regeneration. Most of the early studies using vegetative tissues as explants have proven to be either inefficient or difficult to reproduce (Reddy et al., 1987; Reddy and Bahadur, 1989; Sarvesh et al., 1992). Then, researchers started to focus on meristematic tissues to improve regeneration efficiency (Molina and Schobert, 1995; Lakshmi and Bahadur, 1997; Sujatha and Reddy, 1998). Sujatha and Reddy (1998) reported the first reliable protocol using embryonic tips and shoot apex. However, all the shoots were derived from the preexisting meristem, and adventitious shoot formation was not observed. When applied in *Agrobacterium*-mediated transformation, this protocol resulted in a low rate of putative transformant recovery (0.08%, one putative transformant in 1,200 embryonic tips used; Sujatha and Sailaja, 2005). The first success at stable transformation of meristematic tissues of castor through *Agrobacterium*-mediated transformation has been reported (Sujatha and Sailaja 2005). Subsequently, transgenic castor

resistant to castor semilooper through deployment of Cry1Ab gene has been developed (Malathi et al. 2006). McKeon et al. (2003) also developed a meristem-based transformation system, *Agrobacterium*-mediated transformation using vacuum infiltration of flower buds, but the efficiency is also very low. To obtain the number of transgenic castor plants needed to screen for virtually complete suppression of ricin toxin and RCA, a high-frequency regeneration protocol is required. As a first step to achieving this goal, Ahn et al., (2007) have evaluated the effectiveness of several plant growth regulators and environmental conditions in plant regeneration from hypocotyl tissue in castor. This nonmeristem-based regeneration protocol is an improved alternative to the previous meristem-based method, which resulted in a very low level of putative transformant recovery when applied in *Agrobacterium*-mediated transformation (Sujatha and Sailaja, 2005). Despite its generally high-regeneration efficiency, meristem-based transformation often presents limitations, such as 1) low rate of transformants, 2) chimerism of primary transformants, and 3) escape of regenerants from selection conditions (reviewed in Sticklen and Oraby, 2005). Recently, protocols for adventitious shoot regeneration and somatic organogenesis have been reported (Ahn et al. 2007; Ganesh Kumari et al. 2008). However, the efficiency of these regeneration systems for transformation of castor needs to be established before exploiting them for transformation of castor with agronomically desirable genes.

A meristem-based protocol for transformation and regeneration of castor (Sujatha et al., 2005) was followed to realize castor transgenics in the present study. Seeds of the cultivar DCS-9 (Jyoti) were used in all experiments. This genotype was selected as it is cultivated as a variety under rainfed conditions and also used as pollen parent for a hybrid (DCH-177). All the experiments were carried out with *Agrobacterium tumefaciens* strain LBA4404 harbouring the intended construct.

Owing to the poor amenability of castor transformation and regeneration, only two constructs have been tried in castor. These include LBA4404:: pCAMBIA 1300 ricin FP ihp RA and LBA4404:: pCAMBIA 1300 ricin FP ihp RB. Putative transgenic castor plants were obtained only with LBA4404:: pCAMBIA 1300 ricin FP ihp RB.

Responding embryo axes showed shoot proliferation during the first cycle of selection. The explants produced white excessive flaky callus on hygromycin selection medium. On medium with hygromycin for selection, the explants turned brown at the base with green proliferating shoots. On hygromycin selection medium, shoot proliferation was concomitant with base expansion. With increasing selection pressure, untransformed shoots

turned brown while transformed sectors remained dark green with proliferation. After three cycles of selection, actively growing green shoot clusters were separated carefully from the surrounding brown/bleached tissues and transferred to shoot multiplication medium devoid of the selection agent. Following withdrawal of selection pressure, shoot proliferation was vigorous in explants recovered from hygromycin selection medium. However, in the subsequent subculture cycle, differences in shoot growth and proliferation were not significant. During every subculture cycle, 2–3 shoots were harvested from each shoot cluster either for elongation or for rooting. The frequency of rooting was 60-65% and around 32% of the rooted shoots were successfully acclimatized and grown to maturity in the green-house. Considering the literature available, this frequency of rooting is very high and this was a positive aspect of the results obtained. Though, putative transgenic plants have been realised in the present study, the very low frequency of obtaining the transgenic plants has been a deterrent in producing more number of plants. From over 30 thousand embryos used for co-cultivation only 8 plants that survived transplantation into soil were obtained. This low frequency (0.02%) is a major limiting factor in realizing more transgenic castor plants. Also, the protocol employed in the present study is a very long duration one and about 12 months are needed to get sufficiently elongated shoots ready for transfer to the rooting medium. This long duration of tissue culture makes the regenerating shoots more liable for contaminations due to frequent sub-culturing. Thus, many shoots were lost during the course of the investigation. At the time of writing this thesis, there were still 32 putative shoots with good root system in the culture vessel and they were to be transferred to the soil.

As meristem-based system for transformation has been used in the present study, there is a possibility of sectorial chimeras. Unless the progeny plants of these T_0 plants are analysed, it would not be possible to ascertain the inheritance of the transgenes.

All the castor plants that were acclimatized were analyzed for the presence of the transgene construct by PCR analysis. Different PCRs were performed that aimed at checking the presence of different component sequences of the LBA4404:: pCAMBIA 1300 ricin FP ihp RB in the putative castor transgenics. Almost all the independent primary transformants recovered from hygromycin selection medium showed the presence of the all the component sequences of the construct used for transformation indicating that the gene cassettes were intact in these putative transgenic plants. At the time of writing this thesis only two of the eight transgenic plants had flowered and only one of them had set seeds. As these plants are grown in the pots, the spikes produced are small and so the seeds obtained would be fewer in

number. Therefore, though one of the plants has produced seeds, these seeds could not be used for assessing the ricin content as these seeds are needed for raising the next generation plants.

In conclusion, in the present investigation, putative transgenic castor shoots have been developed using the developed PTGS vectors. This is a major step in realising ricin-free castor. There are still many researchable issues that need to be addressed to accomplish the aim of obtaining ricin and RCA-free castor.

The future line of work includes, further characterization of the isolated ricin promoters using both heterologous and native plant systems, obtaining more number of castor transgenic plants, analysis of the progeny plants of the obtained castor transgenic plants, identification of transgenic castor line(s) with reduced ricin and RCA content and crossing such castor lines to derive lines with further reduced ricin and RCA content.

LITERATURE CITED

Agrawal N, Dasaradhi PVN, Mohammed A, Malhotra P, Bhatnagar RK, Mukherjee SK (2003) RNA Interference: Biology, mechanism and applications. Microbiological and Molecular Biological Reveiws **67**: 657-685

Ahmad M, Mirza B (2005) An efficient protocol for transient transformation of intact fruit and transgene expression in *citrus*. Plant Molecular Biology Reporter **23:** 419a-419k

Ahn YJ, Vang L, McKeon TA, Chen GQ (2007) High-frequency plant regeneration through adventitious shoot formation in castor (*Ricinus communis* L.). In Vitro Cell Developmental Biology-Plant **43**:9–15

Akhunov ED, Goodyear JA, Geng S (2003) The organization and rate of evolution of the wheat genomes are correlated with recombination rates along chromosome arms. Genome Research **13**: 753–763

Allen RS, Millgate AG, Chitty A, Thisleton, Miller AC, Fist A, Gerlach WL, Larkin P (2004) RNAi-mediated replacement of morphine with the non narcotic alkaloid reticuline in opium poppy. Nature Biotechnology **22**: 1559-1566

Anandan S, Anil Kumar GK, Ghosh J, Ramachandra KS (2005) Effect of different physical and chemical treatments on detoxification of ricin in castor cake. Animal Feed Science and technology **120**:159-168

Arenz C, Schepers U (2003) RNA interference: from an ancient mechanism to a state of the art therapeutic application?. Naturwissenschaften **90**: 345-359

Athma P, Reddy TP (1983) Efficiency of callus initiation and direct regeneration from different explants of castor (*Ricinus communis* L.). Current Science **52**:256–257

Audi J, Belson M, Patel M, Schier J, Osterloh J (2005) Ricin poisoning-a comprehensive review. Journal of American Medical Association **294**:2342–2351

Auld DL, Pinkerton SD, Boroda E, Lombard KA, Murphy CK, Kenworthy KE (2003) Registration of TTU-LRC castor germplasm with reduced levels of ricin and RCA120 Crop Science **43**:746–747

Auld DL, Rolfe RD, McKeon TA (2001) Development of castor with reduced toxicity. Journal of New Seeds **3**:61–69

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith A, Streehl K (1999) Short Protocols in Molecular Biology. **4th edition** John Willey and Sons, Inc., New York

Barnes DJ, Baldwin BS, Braasch DA (2009) Ricin accumulation and degradation during castor seed development and late germination. Industrial crops and products, doi:10.1016/j.indcrop.2009.04.003

Benfey PN, Chua NM (1990) The cauliflower mosaic virus 35S promoter: Combinatorial regulation of transcription in plants. Science **250**: 959-966

Birmingham A, Anderson EM, Reynolds A, Ilsley-Tyree D, Leake D, Fedorov Y, Baskerville S, Maksimova E, Robinson K, Karpilow, Marshau WS, Khvorova A (2006) 3' UTR seed matches, but not overall identity, are associated with RNAi off-targets. Nature Methods **3**: 487

Brown DJ, Canvin DT, Zilkey BF (1970) Growth and metabolism of *Ricinus communis* endosperm in tissue culture. Canadian Journal of Botany **48**:2323–2331

Brummell D A, Peter J. Balint-Kurti, Mark H. Harpster, Joseph M. Palys, Paul W. Oeller, Neal Gutterson (2003) Inverted repeat of a heterologous 3'-untranslated region for high-efficiency, high throughput gene silencing. The Plant Journal **33**: 793-800

Bucchini L, Goldman LR (2002) Starlink corn: a risk analysis. Environmental Health Perspectives **110**: 5-13

Byzova M, Verduyn c, De Brouwer D, De Block M (2004) Transforming petals into sepaloid organs in *Arabidopsis* and oilseed rape: implementation of the hairpin RNA-mediated gene silencing technology in an organ-specific manner. Planta **218**: 379-387

Cazzonelli CR, Velten (2004) Analysis of RNA-mediated gene silencing using a new vector (pKNOCKOUT) and an in planta *Agrobacterium* transient expression system. Plant Molecular Biology Reporter **22**: 347-359

Chaturvedi R, Razdan MK, Bhojwani SS (2003) An efficient protocol for the production of triploid plants from endosperm callus of neem, *Azadirachta indica* A. Juss. Journal of Plant Physiology **160**:557–564

Chen GQ, He X, McKeon TA (2005) A simple and sensitive assay for distinguishing the expression of ricin and *Ricinus communis* agglutinin genes in developing castor seed (*Ricinus communis* L.). Journal of Agriculture and Food Chemistry **53**:2358-2361

Chicas A, Macino G (2001) Characteristics of post-transcriptional gene silencing. European Molecular Biology Organization Reporter **21**: 992-996

Chileh T, Esteben Garcia B, Alonso DP, Garcia Marota F (2010) Characterization of the 11S globulin gene family in the castor plant *Ricinus communis* L. Journal of Agriculture and Food Chemistry **58**: 272–281

Cho BH, Choi YS (1990) Sugar uptake system and nutritional change in suspension cells of *Ricinus communis* L. Korean Journal of Plant Tissue Culture **17**: 167–73

Chuang CF,Meyerowitz EM (2000) Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences USA **97**: 4985-4990

de Carvalho F, Gheysen G, Kushnir S, Van Montagu M, Inze D, Castresana C (1992) Suppression of beta-1,3-glucanase transgene expression in homozygous plants. European Molecular Biology Organization Journal **11**: 2595-2602

De Fossard RA, Myint A, Lee ECM (1974) A broad spectrum tissue culture experiment with tobacco (*Nicotiana tabacum*) pith tissue callus. Physiologia Plantarum **30**:125–130

Dubcovsky J, Luo MC, Zhong GY, Bransteitter R, Desai A, Kilian A, Kleinhofs A, Dvorak J (1996) Genetic map of diploid wheat, *Triticum monococcum* L, and its comparison with maps of *Hordeum vulgare* L. Genetics **143**: 983–999

Dunoyer P, Lecellier CH, Parizotto EA, Himber C, Voinnet O (2004) Probing the microRNA and small interfering RNA pathways with virus-encoded suppressors of RNA silencing. Plant Cell **16**: 1235-1250

Endo Y, Tsurugi K (1987) RNA N-glycosidase activity of ricin A-chain: Mechanism of action of the toxin lectin ricin in eukaryotic ribosomes. Journal of Biological Chemistry **262**: 8128 – 8130

FAO (2006) online http://faostat.fao.org, (Accessed on 06 November 2007)

Filichkin SA, DiFazio SP, Brunner AM, Davis JM, Yang ZK, Kalluri UC, Arias RS, Etherington E, Tuskan GA, Strauss SH (2007) Efficiency of gene silencing in Arabidopsis: Direct inverted repeats vs. transitive RNAi vectors. Plant Biotechnology Journal **5**: 615-626

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabdtis elegans.* Nature **391**: 806-811

Frigerio L, Roberts L M (1998) The enemy within: ricin and plant cells. Journal of Experimental Botany **49**: 1473-1480

Frigerio L, Vitale A, Lord JM, Ceriotti A, Roberts LM (1998) Free ricin A-Chain, proricin and native toxin have different cellular fates when expressed in tobacco protoplasts. Journal of Biological Chemistry **273**: 14194 – 14199

Fu D, Uauy C, Blechl A, Dubcovsky J (2007) RNA interference for wheat functional gene analysis. Transgenic Research **16**: 689–701

Fusaro AE, Matthew L, Smith NA, Curtin S, Dedic-Hagan, Ellacott GA, Watson M,

Wang MB, Brosnan C, Carroll B, Waterhouse PM (2006) RNA interference-inducing hairpin RNAs in plants act through the viral defence pathway. European Molecular Biology Organization Reports **7**: 1168-1175

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Experimental Cell Research **50**:151–158

Gandhi V M, Cherian K M, Mulky M J (1994) Detoxification of castor seed meal by interaction with sal seed meal. Journal of the American Oil Chemists' Society 71: 827-831

Ganesh Kumari K, Ganesan M, Jayabalan N (2008) Somatic embryogenesis and plant regeneration in *Ricinus communis*. Biologia Plantarum **52**:17–25

Genyu Z (1988) Callus formation and plant regeneration from young stem segments of *Ricinus communis* L. Genetic Manipulation in Crops. IRRI, Cassell Tycooly: p. 393

Gilissen L, Bolhaar STH, Matos CR, Rouwendal, GA, Boone M, Krens EA, Zuidmeer L, van Leeuwen A, Akkerdaas, Hoffmann-Sommergruber K, Knulst AC, Bosch D, van de Weg WE and van Ree R (2005) Silencing the major apple allergen Mal d 1 by using the RNA interference approach. Journal of Allergy and Clinical Immunology **115**: 364-369

Gmitter FG, Ling XB, Deng XX (1990) Induction of triploid plants from endosperm calli in vitro. Theoretical and AppliedGenetics **80**:785–790

Goodall GJ and Filipowicz W (1989) The AU-rich sequences in the introns of plant nuclear pre-mRNAs are required for splicing. Cell **58**: 473

Gray J, Picton S, Shabeer J, Schuch W, Grierson D (1992) Molecular biology of fruitripening and its manipulation with antisense genes. Plant Molecular Biology **9**: 69-87

Gressel J (2008) Transgenics are imperative for biofuel crops. Plant Sciences **174**:246–263

Hamilton A, Voinnet O, Chappell L, Baulcombe D (2002) Two classes of short interfering RNA in RNA silencing. European Molecular Biology Organization Journal **21**: 4671–4679

Hara-Nishimura I, Inoue K, Nishimura M (1991) A unique vacuolar processing enzyme responsible for conversion of several proprotein precursors into the mature forms. FEBS Letters **294**: 89 – 93

Hartley L, Temple GE, Brasch MA (2000) DNA cloning using in vitro site-specific recombination. Genome Research **10**: 178 8-1795

Hartmann U, Sagasser M, Mehrtens F, Stracke R, Weisshaar B (2005) Differential combinatorial interactions of cis-acting elements recognized by R2R3-MYB, BZIP, and BHLH factors control light-responsive and tissue-specific activation of phenylpropanoid biosynthesis genes. Plant Molecular Biology **57**: 155–171 doi:10.1007/ s11103-004-6910-0

Helliwell C, Waterhouse P (2003) Constructs and methods for high-throughput gene silencing in plants. Methods **30**: 289-295

Helliwell CA, Wesley SV, Wielopolska A, Waterhouse PM (2002) High-throughput vectors for efficient gene silencing in plants. Functional Plant Biology **29:** 1217-1225

Herman EM, Helm RM, Jung R, Kinney AJ (2003) Genetic modification removes an immunodominant allergen from soybean. Plant Physiology **132**:36-43

Hernandez GG, Berzunza EA, Concha LC, Miranda-Ham ML (2006) *Agrobacterium*-mediated transient transformation of marigold (*Tagetes erecta*).Plant Cell, Tissue and Organ Culture **84**:365-368

Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant cis-acting regulatory DNA elements (PLACE) database. Nucleic Acids Research Vol.27 No.1 pp. **297-300**.

Hiraiwa N, Kondo M, Nishimura M, HaraNishimura I (1997) An aspartic endopeptidase is involved in the breakdown of storage proteins in protein storage vacuoles of plants. European Journal of Biochemistry **246**: 133 – 141

Holzberg S, Brosio P, Gross C, Pogue GP (2002) Barley stripe mosaic virus-induced gene silencing in a monocot plant. The Plant Journal **30**:315–327

Holzberg S, Brosio P, Gross C, Pogue GP (2002) Barley stripe mosaic virus-induced gene silencing in a monocot plant. Plant Journal **30**: 315–327

Huang S, Frizzi A, Florida CA, Kruger DE, Luethy MB (2006) High lysine and high tryptophan transgenic maize resulting from the reduction of both 19- and 22-kD alphazeins. Plant Molecular Biology **61**: 525-535

Hutchinson J (1964) Tribalism in the family Euphorbiaceae. American Journal of Botany **56**:738–758

Jefferson RA (1987) Assaying chimeric genes in plants, the GUS gene fusion system. Plant Molecular Biology Reporter **5**: 387-405

Johri BM, Bhojwani SS (1965) Growth responses of mature endosperm in cultures. Nature (London) **208**:1345–1347

Johri BM, Srivastava PS (1972) In vitro growth responses of mature endosperm of *Ricinus communis* L. In: Murthy YS, Johri BM, Mohan Ram HY, Verghese TM, editors.

Advances in Plant Morphology - V. Puri Commemoration Volume Sarita Prakashan, Meerut, India: p. 339–358

Johri BM, Srivastava PS, Raste AP (1980) Plant tissue culture and crop improvement. Indian Journal of Agriculture Sciences **50**:103–127

Jorgensen R (1990) Altered gene expression in plants due to trans interactions between homologous genes. Trends in Biotechnology **8**: 340-344

Kapila J, Rycke RD, Montagu MV, Angenon G (1997) An *Agrobacterium*-mediated transient gene expression system for intact leaves. Plant Science **122**: 101-108

Katzin BJ, Collins EJ, Robertus JD (1991) The structure of ricin A chain at 2.5 A^0. Proteins: Structure, Function and Genetics **10**: 251 – 259

Keith B, Chua NH (1986) Monocot and dicot pre-mRNAs are processed with different efficiencies in transgenic tobacco. European Molecular Biology Organisation Journal **5**: 2419

Khumsub S (1988) Tissue culture of castor bean (*Ricinus communis* L.). Dissertation. Kasetsart University, Bangkok: pp. 1-61

Kinney AJ (1996) Development of genetically engineered soybean oils for food applications. Journal of Food Lipids **3**: 273-292

Knutzen D S, Thomson G A, Radke S E, Johnson W B, Knauf V C, Kridl J C (1992) Modification of Brassica seed oil by antisense expression of a stearoyl-acylcarrier protein desaturase. Proceedings of National Academy of Sciences, USA **89**: 2624-2628

Kusaba M (2004) RNA interference in crop plants. Current Opinion in Biotechnology **15**: 139-143

Kuznetsov VV (2003) RNA Interference: An approach to produce knockout organism and cell lines. Biochemistry (Moscow) **68**: 1301-1317

Kyozuka J, Fujimoto H, Izawa T, Shimamoto K (1991) Anaerobic induction and tissue-specific expression of maize *Adh1* promoter in transgenic rice plants and their progeny. Molecular and General Genetics **228**: 40

La Rue CD (1944) Regeneration of endosperm of gymnosperms and angiosperms. American Journal of Botany **31**:45

Lakshminarayana M, Raoof MA (2005) Insect pests and diseases of castor and their management. Directorate of Oilseeds Research, Hyderabad, India: p.78

Last DI, Brettell RIS, Chamberlain DA, Chaudhury AM, Larkin PJ, Marsh EL, Peacock WJ, Dennis ES, pEMU (1991) An improved vector for gene expression in cereal cells. Theoretical and Applied Genetics **81**: 581

Li JR, Zhao W, Li QZ, Ye XG, An BY, Li X, Zhang XS (2005) RNA silencing of Waxy gene results in low levels of amylose in the seeds of transgenic wheat (*Triticum aestivum* L.). Acta Genetica Sinica **32**:846–854

Li JR, Zhao W, Li QZ, Ye XG, An BY, Li X, Zhang XS (2005) RNA silencing of Waxy gene results in low levels of amylose in the seeds of transgenic wheat (*Triticum aestivum* L.). Acta Genetica Sinica **32**: 846–854

Lipardi C, Wei Q, Paterson BM (2001) RNAi as random degradative PCR: siRNA primers convert mRNA into dsRNAs that are degraded to generate new siRNAs. Cell **107**: 297-307

Liu Q, Singh SP, Green AG (2002) High-stearic and high-oleic cottonseed oils produced by hairpin RNA-mediated post-transcriptional gene silencing. Plant Physiology **129**: 1732-1743

Llave C, Kasschau KD, Rector MA, Carrington JC (2002) Endogenous and silencing-associated small RNAs in plants. Plant Cell **14**: 1605–1619

Lord JM (1985) Precursors of ricin and *Ricinus communis* agglutinin Glycosylation and processing during synthesis and intracellular transport. European Journal of Biochemistry **146:** 411-416

Lord JM (1985) Synthesis and intracellular transport of lectin and storage protein precursors in endosperm from castor bean. European Journal of Biochemistry **146:** 403-409

Lord JM, Roberts LM, Robertus JD (1994) Ricin: structure, mode of action and some current applications. The FASEB Journal **8**: 201 – 208

Malathi B, Ramesh S, Rao KV, Reddy VD (2006) *Agrobacterium*-mediated genetic transformation and production of semilooper resistant transgenic castor (*Ricinus communis* L.). Euphytica **147**:441–449

Matzke MA, Matzke AJ (1991) Differential inactivation and methylation of a transgene in plants by two suppressor loci containing homologous sequences. Plant Molecular Biology **16**: 821-830

Matzke MA, Primig M, Trnovsky J, Matzke AJM (1989) Reversible methylation and inactivation of marker genes in sequentially transformed tobacco plants. European Molecular Biology Organization Journal **8**: 643-649

Mc Keon TA, Chen GQ (2001) High-tech castor plants may open door to domestic production. ARS Magazine **49**:12–13

Mc Keon TA, Chen GQ (2003) Transformation of *Ricinus communis*, the castor plant. (US Patent No 6,620,986)

McGinnis K, Murphy N, Carlson AR, Akula A, Akula C, Basinger H, Carlson M, Hermanson P, Kovacevic N, McGill MA, Seshadri V, Yoyokie J, Cone K, Kaeppler HF, Kaeppler SM, Springer NM (2007) Assessing the efficiency of RNA interference for maize functional genomics. Plant Physiology **143**: 1441–1451

McIntosh KB, Hulm JL,Young LW, Bonham-Smith PC (2004) A rapid *Agrobacterium*–mediated *Arabidopsis thaliana* transient assay system. Plant Molecular Biology Reporter **22**: 53-61

Meyer P, Heidmann I, Neidenhof I (1993) Differences in DNA-methylation are associated with a paramutation phenomenon in transgenic petunia. Plant Journal **4**: 89-100

Miki D, Itoh R, Shimamoto K (2005) RNA silencing of single and multiple members in a gene family in rice. Plant Physiology **138**:1903–1913

Miki D, Itoh R, Shimamoto K (2005) RNA silencing of single and multiple members in a gene family in rice. Plant Physiology **138**: 1903–1913

Miki D, Shimamoto K (2004) Simple RNAi vectors for stable and transient suppression of gene function in rice. Plant and Cell Physiology **45**: 490-495

Mohan Ram HY, Satsangi A (1963) Induction of cell divisions in the mature endosperm of *Ricinus communis* during germination. Current Science **32**:28–30

Molina SM, Schobert C (1995) Micropropagation of *Ricinus communis*. Journal of Plant Physiology **147**:270–272

Montford W, Villafranca JE, Monzingo AF, Ernst SR, Katzin B, Rutenber E, Nuyhen HX, Hamlin R, Robertus JD (1987) The three-dimensional structure of Ricin at 2.8 A^0. Journal of Biological Chernistry **262**: 5398 – 5403

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum **15** : 473-497

Napoli C, Lemieux C, Jorgensen RA (1990) Introduction of a chimeric chalcone synthase gene into *Petunia* results in reversible co-suppression of homologous genes *in trans*. Plant Cell **2**: 279-289

Ochman H, Gerber AS, Hartl DL (1988) Genetic applications of an inverse polymerase chain reaction. Genetics **120**: 621-625

Ogita S, Uefuji H, Yamaguchi Y, Koizumi N, Sano H (2003) RNA interference producing

decaffeinated coffee plants. Nature **423** :823

Ogunniyi DS (2006) Castor oil: a vital industrial rawmaterial. Bioresource Technology **97**:1086–1091

Peterhans A, Datta SK, Datta K, Goodall GJ, Potrykus I, Paszkowski J (1990) Recognition efficiency of Dicotyledoneae-specific promoter and RNA processing signals in rice. Molecular and General Genetics **222**:361

Pinkerton SD, Rolfe RD, Auld DL, Ghetie V, Lauterbach BF (1999) Selection of castor with divergent concentrations of ricin and *Ricinus communis* agglutinin. Crop Science **39**: 353-357

Poli MA, Rivera VR, Howetson JF, Merrill GA (1994) Detection of ricin by colorimetric and chemiluminescence ELISA. Toxicon **32**: 1371-1377

Prestridge DS (1991) **SIGNAL SCAN*: A computer program that scans DNA sequences for eukaryotic transcriptional elements*. CABIOS 7,** 203-206

Qi Y, Denli AM, Hannon GJ (2005) Biochemical specialization within Arabidopsis RNA silencing pathways. Molecular Cell **19**: 421–428

Qin X, Zhang J, Shao C, Lin S, Jiang L, Zhang S, Xu Y and Chen F (2010) Isolation and characterization of a curcin promoter from *Jatropha curcas* L. and its regulation of gene expression in transgenic tobacco plants. Plant Molecular Biology Reporter DOI 10.1007/s11105-008-0078-8

Rathus C, Bower R, Birch RG (1993) Effects of promoter, intron and enhancer elements on transient gene expression in sugarcane and carrot protoplasts. Plant Molecular Biology **23**: 613

Reddy KRK, Bahadur B (1989) Adventitious bud formation from leaf cultures of castor (*Ricinus communis* L.). Current Science **58**:152–154

Reddy KRK, Bahadur B (1989a) Adventitious bud formation from leaf cultures of castor (*Ricinus communis* L.). Current Science **58**:152–154

Reddy KRK, Ramaswamy N, Bahadur B (1987b) Cross incompatibility between *Ricinus* and Jatropha. Plant Cell Incompatability Newsletter **19**:60–65

Reddy KRK, Rao GP, Bahadur B (1986) In vitro studies on castor (*Ricinus communis* L.). J Swamy Botany Cl **3**:119–122

Reddy KRK, Rao GP, Bahadur B (1987a) In vitro morphogenesis from seedling explants and callus cultures of castor (*Ricinus communis* L.). Phytomorphology **37**:337–340

Reddy KRK. Bahadur B (1989b) In vitro multiplication of castor. In: Farook SA, Khan IA, editors.Recent Advances in Genetics and Cytogenetics. Hyderabad: Premier : p. 479–482

Reddy KRK. Bahadur B (1989b) In vitro multiplication of castor. In: Farook SA, Khan IA, editors. Recent Advances in Genetics and Cytogenetics. Hyderabad: Premier: p. 479–482

Regina A, Bird A, Topping D, Bowden S, Freeman J, Barsby T, Kosar-Hashemi B, Li Z, Rahman S, Morell M (2006) High-amylose wheat generated by RNA interference improves indices of large-bowel health in rats. Proceedings of National Academy of Sciences USA **103**: 3546–3551

Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A (2004) Rational siRNA design for RNA interference. Nature Biotechnology 22: 326–330

Richardson PT, Westley M, Roberts LM, Gould JH, Colman A, Lord JM (1989) Recombinant proricin binds galactose but does not depurinate 28S ribosomal RNA. FEBS Letters **255**: 15 – 20

Roberts LM, Lord JM (1981) Protein biosynthetic capacity in the endosperm tissue of ripening castor bean seeds. Planta **152**:420-427

Ruiz MT, Voinnet O, Baulcombe DC (1998) Initiation and maintenance of virus-induced gene silencing. Plant Cell **10**: 937-946

Rutenber E, Katzin BJ, Collins EJ, Mlsna D, Ernst SE, Ready MP, Robertus JD (1991) The crystallographic refinement of ricin to 2.5 A^0. Proteins: Structure, Function and Genetics **10**: 240 – 250

Rutenber E, Robertus JD (1991) The structure of ricin B-Chain at 2.5 A^0 resolution. Proteins **10**: 260 269

Sambrook J, Russell D M (2001) Molecular Cloning: A laboratory Manual. **3rd edition** Volume I-III. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Sangduen N, Pongtongkam P, Ratisoontorn P, Jampatas R, Suputtitada S, Khumsub S (1987) Tissue culture and plant regeneration of castor (*Ricinus communis* L.). SABRAO Journal **19**:144

Sarvesh A, Ram Rao DM, Reddy TP (1992) Callus initiation and plantlet regeneration from epicotyl and cotyledonary explants of castor (*Ricinus communis* L.). Advances in Plant Sciences **5**:124–128

Satsangi A, Mohan Ram HY (1965) A continuously growing tissue culture from the mature endosperm of *Ricinus communis* L. Phytomorphology **15**:26–30

Scarpa A, Guerci A (1982) Various uses of the castor oil plant (*Ricinus communis* L.) a review.Journal of Ethnopharmacology **5**:117–137

Scofield SR, Huang L, Brandt AS, Gill BS (2005) Development of a virus-induced gene-silencing system for hexaploid wheat and its use in functional analysis of the Lr21-mediated leaf rust resistance pathway. Plant Physiology **138**: 2165–2173

Sehnke PC, Pedrosa L, Paul AL, Frankel AE, Ferl RJ (1994) Expression of active, processed ricin in transgenic tobacco. Journal of Biological Chemistry **269**: 22473-22476

Sijen T, Fleenor J, Simmer F, Thijssen KL, Parish S, Timmons L, Fire A, Plasterk RHA (2001) On the role of RNA amplification in dsRNA-triggered gene silencing. Cell **107**: 465-476

Sijen T, Kooter JM (2000) Post-transcriptional gene-silencing: RNAs on the attack or on the defense?. Bioassays **22**: 520-531

Sikdar AK, Jolly MS (1994) Induced polyploidy in mulberry (Morus spp.) I. Induction of tetraploids. Sericologia **34**:105–116

Smith C, Watson C, Bird C, Ray JWS, Grierson D (1990) Expression of a truncated tomato polygalacturonase gene inhibits expression of the endogenous gene in transgenic plants. Molecular and General Genetics **224**: 477-481

Smith N A, Singh S P, Wang M, Stoutjesdijk P A, Green A G and Waterhouse P M (2000) Total silencing by intron-spliced hairpin RNAs. Nature **407**: 319-320

Srinivas CVS, Nagaraj G (2000) Factors influencing ricin, the toxic protein in castor and its detoxification. Journal of Oilseed Technology Association of India **3(21)**: 21-23

Srivastava PS (1971a.) In vitro growth requirements of mature endosperm of *Ricinus communis* L. Current Science **40**:337–339

Starn M, de Bruin R, Kenter S, Van der Hoorn RAL, Van Blokland R, Mol JNM, Kooter JM (1997) Post-transcriptional silencing of chalcone synthase in *Petunia* by inverted transgene repeats. Plant Journal **12:** 63-82

Stoutjesdijk PA, Singh SP, Liu Q, Hurlstone CJ, Waterhouse PA, Green AG (2002) hpRNA-mediated targeting of the *Arabidopsis FAD2* gene gives highly efficient and stable silencing. Plant Physiology **129**: 1723-1731

Sujatha M, Reddy TP (1998) Differential cytokinin effects on the stimulation of in vitro shoot proliferation from meristematic explants of castor (*Ricinus communis* L.). Plant Cell Reports **7**:561–566

Sujatha M, Reddy TP (2007) Promotive effect of lysine monohydrochloride on morphogenesis in cultured seedling and mature plant tissues of castor (*Ricinus communis* L.). Indian Journal of Crop Sciences **2**:11–19

Sujatha M, Sailaja M (2005) Stable genetic transformation of castor (*Ricinus communis* L.) via *Agrobacterium tumefaciens*-mediated gene transfer using embryo axes from mature seeds. Plant Cell Reports **23**:803–810

Sujatha M, Sailaja M (2007) Development of transgenic castor for insect resistance. Extended Summaries of the National Seminar on Changing Global Vegetable Oils Scenario: Issues and Challenges before India, Hyderabad, India; January 29-31: p. 7–8

Sunilkumar G, Campbell LM, Puckhaber L, Stipanovic RD, Rathore KS (2006) Engineering cottonseed for use in human nutrition by tissue-specific reduction of toxic gossypol. Proceedings of the National Academy of Sciences USA **103**: 18054-18059

Tada Y, Nakase M, Adachi T, Nakamura R, Shimada H, Takahashi M, Fujimura T, Matsuda T (1996) Reduction of 14-16 kDa allergenic proteins in transgenic rice plants by antisense gene. FEBS Letter **391**: 341-345

Tagge EP, Chandler J, Harris B, Czako M, Marton L, Willingham MC, Burbage C, Afrin L and Frankel AE (1996) Preproricin expressed in *Nicotiana tabacum* cells in vitro is fully processed and biologically active. Protein Expression and Purification **8**: 109-118

Tanaka A, Mita S, Ohta S, Kyozuka J, Shimamoto K, Nakamura K (1990) Enhancement of foreign gene expression by a dicot intron in rice but not in tobacco is correlated with an increased level of mRNA and an efficient splicing of the intron. Nucleic Acids Research **18**: 6767

Tang G, Galili G (2004) Using RNAi to improve plant nutritional value: from mechanism to application. Trends in Biotechnology **22**: 463-469

Tang G, Reinhart BJ, Bartel DP, Zamore PD (2003) A biochemical framework for RNA silencing in plants. Genes and Development **17**: 49–63

Thomas CL, Jones L, Baulcombe DC, Maule AJ (2001) Size constraints for targeting post-transcriptional gene silencing and for RNA-directed methylation in *Nicotiana benthamiana* using a potato virus X vector. Plant Journal **25**: 417–425

Thomas MS, Flavell RB (1990) Identification of an Enhancer Element for the Endosperm-Specific Expression of High Molecular Weight Glutenin. The Plant Cell **2**: 1171 - 1180

Thomas TD, Chaturvedi R (2008) Endosperm culture: a novel method for triploid plant production. Plant Cell Tissue and Organ Culture **93**:1–14

Tregear JW, Roberts LM (1992) The lectin gene of *Ricinus communis* : cloning of a functional gene and three lectin pseudogenes. Plant Molecular Biology **18**: 515-525

Triglia T, Peterson, MG, Kemp DJ (1988) A procedure for in vitro amplification of DNA segments that lie outside the boundaries of known sequences. Nucleic.Acids.Research **16**: 8186

Tulley RE, H Beevers (1976) Protein bodies of Castor bean endosperm: Isolation, fractionation and characterization of protein components. Plant Physiology **58**: 710 – 716

Unnamalai N, Bong GK, Woo SL (2004) Cationic oligopeptide delivery of dsRNA for post transcriptional gene silencing in plant cells. FEBS Letter **566**: 307-310

Van Blokland R, Van der Geest N, Mol J, Kooter J (1994) Transgene-mediated suppression of chalcone synthase expression in *Petunia hybrida* results from an increase in RNA turnover. Plant Journal **6**: 861-877

Van der Krol AR, Mur LA, Beld M, Mol JN, Stuitje AR (1990) Flavonoid genes in *Petunia*: addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell **2:** 291-299

Vaucheret H, Beclin C, Fagard M (2001) Post-transcriptional gene silencing in plants. Journal of Cell Science **114**: 3083-3091

Voinnet O (2002) RNA silencing: small RNAs as ubiquitous regulators of gene expression.

Waterhouse PM, Graham HW, Wang MB (1998) Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proceedings of the National Academy of Sciences USA **95**: 13959-13964

Waterhouse PM, Helliwell CA (2002) Exploring plant genomes by RNA-induced gene silencing. Nature Reviews Genetics **4**: 29-38

Weiss EA (2000) Castor. Oilseed Crops. Oxford, UK: Blackwell Science: p. 13–52

Wesley S V, Helliwell C A, Smith N A, Wang M, Rouse D T, Liu Q, Gooding P S, Singh S P, Abbot D, Stoutjesdijk P A, Robinson S P, Gleave A P, Green A G, Waterhouse P M (2001) Construct design for effective and high throughput gene silencing in Plants. The Plant Journal **27(6)**: 581-590

Wesley SV, Helliwell CA, Smith NA, Wang M, Rouse DT, Liu Q, Gooding PS, Singh

Wiley RG, Oeltman TN (1991) Ricin and related plant toxins: Mechanisms of action and neurobiological applications: 665. In Handbook of Natural Toxins, **Volume 6**; Toxicology of Plant and Fungal Compounds (eds) RF Keeler and AT Tu. Marcel Dekker, Inc, New York

Wu C, Washida H, Onodera Y, Harada K, Takaiwa F (2000) Quantitative nature of the Prolamin-box, ACGT and AACA motifs in a rice glutelin gene promoter: minimal cis-element requirements for endosperm-specific gene expression. Plant Journal **23**: 415–421 doi:10.1046/j.1365-313x.2000.00797.x

Xiong AS, Yao QH, Peng RH, Li X, Han PL, Fan HQ (2005) Different effects on ACC oxidase gene silencing triggered by RNA interference in transgenic tomato. Plant Cell Reports **23**:639-646

Xu Y, Yu H, Hall TC (1994) Rice triosephosphate isomerase gene 5' sequence directs ß-glucuronidase activity in transgenic tobacco but requires an intron for expression in rice. Plant Physiology **106**: 459

Yanagisawa S (2000) Dof1 and Dof2 transcription factors are associated with expression of multiple genes involved in carbon metabolism in maize. Plant Journal **21**: 281–288 doi:10.1046/j.1365- 313x.2000.00685.x

Yanagisawa S, Schmidt RJ (1999) Diversity and similarity among recognition sequences of Dof transcription factors. Plant Journal **17**: 209–214 doi:10.1046/j.1365-313X.1999.00363.x

Youle R, A Huang (1976) Protein bodies from the endosperm of Castor beans, subfractionation, protein components, lectins and changes during germination. Plant Physiology **58**: 703

Yue SJ, Li H, Li YW, Zhu YF, Guo JK, Liu YJ, Chen Y, Jia X (2007) Generation of transgenic wheat lines with altered expression levels of 1Dx5 high-molecular-weight glutenin subunit by RNA interference. Journal of Cereal Science doi: 10.1016/j.jcs/2007.03.006

Zamore PD, Tuschl T, Sharp PA, Bartel DP (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell **101**: 25-33

CHAPTER VI

SUMMARY

The present investigation aimed at reduction of toxic endosperm-specific proteins, ricin and RCA in castor using different PTGS approaches. Towards this, ricin promoter(s)-truncated and full-length have been isolated from castor through inverse-PCR (IPCR) and were characterized them by cloning them upstream of *gus* gene in a promoterless binary vector, pCAMBIA 1391Z. This was to facilitate studying tissue-specificity of the isolated promoters in the model crop, tobacco. Ricin and RCA express and accumulate in endosperm. Therefore, if these genes are to be post-transcriptionally silenced, the trigger for silencing also must be present in the cells at the exact time. In other words, the trigger molecules of the silencing must be present in the developing endosperm tissue. Thus, the siRNA molecules must be produced in the endosperm cells. Unfortunately, the common constitutive CaMV35S promoter does not express well in the endosperm tissue (Benfey and Chua, 1990). Hence, if silencing molecules are to be expressed in endosperm tissues, the gene construct for the same must be driven by endosperm-specific promoter.

The nucleotide sequence for a 310 bp upstream of the ricin gene is already available in the NCBI database (Tregear and Roberts, 1992). This served as a template for fishing out and going upstream of the 5' region of the already known 310 bp. Using inverse PCR, a 622 bp fragment upstream of the 5' of 310 bp of the ricin gene was isolated. The already known 310 bp was designated as the truncated ricin promoter (TP) and this together with the isolated 622 bp was designated as the full-length ricin promoter (FP) (932 bp).

Tobacco has been employed as a model to facilitate the study of the tissue-specificity of the isolated ricin promoters. Tobacco was transformed with six constructs to assess the tissue-specificity of the promoters by stable *Agrobacterium*-mediated transformation. Developing seeds of castor (47DAP) were subjected to microprojectile bombardment with four constructs for promoter characterization. Also transient (wounding and infiltration) *Agrobacterium*-mediated transformation of seeds was undertaken for promoter characterization.

Suitable PTGS vectors (ihpRA, ihpRB, SHUTR RA, SHUTR RB, FLA/S R) have been developed under CaMV35S promoter by adopting three different *viz*., intron hairpin RNA (ihpRNA) (Smith 2000, Wesley 2001, Stoutjesdijk 2002), silencing by heterologous 3' untranslated region (SHUTR) (Brummell et al., 2003) PTGS and antisense RNA (Gray, 1992) technologies to reduce/eliminate the levels of ricin and RCA proteins in the developing seeds of castor. Three approaches have been envisaged, as literature is replete with the

information that different strategies of silencing differ in their efficiency in silencing the target gene transcript and the best one will have to be identified empirically.

Also, different PTGS constructs were developed under the full-length ricin promoter, which are expected to express specifically in the endosperm of castor. These were used for transforming castor to obtain transgenic castor with reduced/nil levels of ricin and RCA.

Though two PTGS constructs (LBA4404::pCAMBIA 1300 FP ihp RA and LBA4404::pCAMBIA 1300 FP ihp RB) have been used in the present study to realize transgenic castor with reduced /nil levels of ricin and RCA, only one of them yielded the putative castor transgenics. Though a number of PTGS constructs have been developed during the course of work, only two were used owing to the poor amenability of castor transformation and regeneration. The choice of the PTGS construct for castor transformation is based on the enormous potential of the intron-spliced hairpin-mediated RNAi strategy at downregulating undesired proteins/toxic substances/chemicals as evident by the published successful stories, globally.

A meristem-based protocol (Sujatha and Sailaja, 2005) was followed to realize castor transgenics. Seeds of the cultivar DCS-9 (Jyoti) were used in all the experiments. All the experiments were carried out with *Agrobacterium tumefaciens* strain LBA4404 harbouring the intended constructs. Transgenicity of putative transgenic castor plants was confirmed using PCR.

Thus, during the period of research, the following were accomplished.

- developed the generic PTGS vectors (already being tried in other crops);
- cloned and sequenced the ricin promoter;
- developed PTGS constructs for silencing ricin and RCA under CaMV35S and ricin promoter;
- cloned and sequenced the full length preproricin gene;
- transgenic tobacco plants expressing ricin gene under ricin promoters have been developed;
- transgenic tobacco plants driving *gus* gene under ricin promoters have been developed; and
- putative castor transgenics have been realized and confirmed by carrying out PCRs for different component sequences.;

Appendices

I. Multiple sequence alignment of sequences of ricin, RCA and their isoforms

The highlighted (in red and blue colours) consensus sequence corresponds to the selected chosen sequence of A chain and B chain of ricin and RCA for inclusion in the PTGS constructs.

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S40366.1|              --------------------------------------------------
M17631.1|RCCRICA       --------------------------------------------------
X02388.1|              AAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGTGGCAACATG 50
S40368.1|              --------------------------------------------------
M32614.1|SYNRICA       --------------------------------------------------
X03179.1|              --------------------------------------------------
X52908.1|              --------------------------------------------------
M12089.1|RCCAGG        --------------------------------------------------
S40367.1|              --------------------------------------------------

S40366.1|              --------------------------------------------------
M17631.1|RCCRICA       --------------------------------------------------
X02388.1|              GCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAGAGGATAACA 100
S40368.1|              --------------------------------------------------
M32614.1|SYNRICA       --------------------------------------------------
X03179.1|              --------------------------------------------------
X52908.1|              --------------------------------------------------
M12089.1|RCCAGG        --------------------------------------------------
S40367.1|              --------------------------------------------------

S40366.1|              --ATATTCCCCAAACAATACCCAATTATAAACTTTACCACAGCGGGTGCC 48
M17631.1|RCCRICA       --------------------------------------------------
X02388.1|              ACATATTCCCCAAACAATACCCAATTATAAACTTTACCACAGCGGGTGCC 150
S40368.1|              --------------------------------------------------
M32614.1|SYNRICA       --------------------------------------------------
X03179.1|              --------------------------------------------------
X52908.1|              --------------------------------------------------
M12089.1|RCCAGG        --------------------------------------------------
S40367.1|              --------------------------------------------------

S40366.1|              ACTGTGCAAAGCTACACAAACTTTATCAGAGCTGTTCGCGGTCGTTTAAC 98
M17631.1|RCCRICA       --------------------------------------------------
X02388.1|              ACTGTGCAAAGCTACACAAACTTTATCAGAGCTGTTCGCGGTCGTTTAAC 200
S40368.1|              --------------------------------------------------
M32614.1|SYNRICA       --------------------------------------------------
X03179.1|              --------------------------------------------------
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X52908.1|            ------------------------------------------------
M12089.1|RCCAGG      ------------------------------------------------
S40367.1|            ------------------------------------------------



S40366.1|            AACTGGAGCTGATGTGAGACATGATATACCAGTGTTGCCAAACAGAGTTG 148
M17631.1|RCCRICA     ------------------------------------------------
X02388.1|            AACTGGAGCTGATGTGAGACATGATATACCAGTGTTGCCAAACAGAGTTG 250
S40368.1|            ------------------------------------------------
M32614.1|SYNRICA     ------------------------------------------------
X03179.1|            ------------------------------------------------
X52908.1|            ------------------------------------------------
M12089.1|RCCAGG      ------------------------------------------------
S40367.1|            ------------------------------------------------

S40366.1|            GTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCAAATCATGCA 198
M17631.1|RCCRICA     ------------------------------------------------
X02388.1|            GTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCAAATCATGCA 300
S40368.1|            ------------------------------------------------
M32614.1|SYNRICA     ------------------------------------------------
X03179.1|            ------------------------------------------------
X52908.1|            ------------------------------------------------
M12089.1|RCCAGG      ------------------------------------------------
S40367.1|            ------------------------------------------------

S40366.1|            GAGCTTTCTGTTACATTAGCCCTGGATGTCACCAATGCATATGTGGTCGG 248
M17631.1|RCCRICA     ------------------------------------------------
X02388.1|            GAGCTTTCTGTTACATTAGCCCTGGATGTCACCAATGCATATGTGGTCGG 350
S40368.1|            ------------------------------------------------
M32614.1|SYNRICA     ------------------------------------------------
X03179.1|            ------------------------------------------------
X52908.1|            ------------------------------------------------
M12089.1|RCCAGG      ------------------------------------------------
S40367.1|            ------------------------------------------------

S40366.1|            CTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACAATCAGGAAG 298
M17631.1|RCCRICA     ------------------------------------------------
X02388.1|            CTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACAATCAGGAAG 400
S40368.1|            ------------------------------------------------
M32614.1|SYNRICA     ------------------------------------------------
X03179.1|            ------------------------------------------------
X52908.1|            ------------------------------------------------
M12089.1|RCCAGG      ------------------------------------------------
S40367.1|            ------------------------------------------------

S40366.1|            ATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAATCGATATACA 348
M17631.1|RCCRICA     ------------------------------------------------
```

```
X02388.1|            ATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAATCGATATACA 450
S40368.1|            ---------------------------------------------------
M32614.1|SYNRICA     ---------------------------------------------------
X03179.1|            ---------------------------------------------------
X52908.1|            ---------------------------------------------------
M12089.1|RCCAGG      ---------------------------------------------------
S40367.1|            ---------------------------------------------------
S40366.1|            TTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGCTGGTAATCT 398
M17631.1|RCCRICA     ---------------------------------------------------
X02388.1|            TTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGCTGGTAATCT 500
S40368.1|            ---------------------------------------------------
M32614.1|SYNRICA     ---------------------------------------------------
X03179.1|            ---------------------------------------------------
X52908.1|            ---------------------------------------------------
M12089.1|RCCAGG      ---------------------------------------------------
S40367.1|            ---------------------------------------------------

S40366.1|            GAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGGCTATCTCAG 448
M17631.1|RCCRICA     ---------------------------------------------------
X02388.1|            GAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGGCTATCTCAG 550
S40368.1|            ---------------------------------------------------
M32614.1|SYNRICA     ---------------------------------------------------
X03179.1|            ---------------------------------------------------
X52908.1|            ---------------------------------------------------
M12089.1|RCCAGG      ---------------------------------------------------
S40367.1|            ---------------------------------------------------

S40366.1|            CGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAACTCTGGCTCGT 498
M17631.1|RCCRICA     ---------------------------------------------------
X02388.1|            CGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAACTCTGGCTCGT 600
S40368.1|            ---------------------------------------------------
M32614.1|SYNRICA     ---------------------------------------------------
X03179.1|            ---------------------------------------------------
X52908.1|            -----------------------------------CCTAAAAAAAGTA  13
M12089.1|RCCAGG      ---------------------------------------------------
S40367.1|            ---------------------------------------------------

S40366.1|            TCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAGATTCCAATA 548
M17631.1|RCCRICA     ---------------------------------------------------
X02388.1|            TCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAGATTCCAATA 650
S40368.1|            ---------------------------------------------------
M32614.1|SYNRICA     ---------------------------------------------------
X03179.1|            -------TCGACATTATATGATTTTAAATCAATTCCGTTTCTAATTTATA  43
X52908.1|            AATTACTCTAATCGACATTATATGAATTTTAACTAATTCCGTTTCTAATT  63
M12089.1|RCCAGG      ---------------------------------------------------
S40367.1|            ---------------------------------------------------

S40366.1|            TATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGAGATCTGCAC 598
M17631.1|RCCRICA     ---------------------------------------------------
```

```
X02388.1|             TATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGAGATCTGCAC 700
S40368.1|             ------------------------------------------------
M32614.1|SYNRICA      ------------------------------------------------
X03179.1|             ATTATTTCGTTAAACCAATCAATTCCCTTTAAACACTGCTTATGCATATT 93
X52908.1|             TATAATTATTTCGTTAAACCAATCAATTCCCTTTAAACACTGCTTATGCA 113
M12089.1|RCCAGG       ------------------------------------------------
S40367.1|             ------------------------------------------------
S40366.1|             CAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGACTTTCCACT 648
M17631.1|RCCRICA      ------------------------------------------------
X02388.1|             CAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGACTTTCCACT 750
S40368.1|             ------------------------------------------------
M32614.1|SYNRICA      ------------------------------------------------
X03179.1|             CTGTCTCAATTTATATATGGCATTGCATTCTTCCGTATTAATTTATAAGT 143
X52908.1|             TATTCTGTCTCAATTTATATATGGCATGCATCTTCCGTATTAATTTATAA 163
M12089.1|RCCAGG       ------------------------------------------------
S40367.1|             ------------------------------------------------

S40366.1|             GCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAATTCAACTGCA 698
M17631.1|RCCRICA      ------------------------------------------------
X02388.1|             GCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAATTCAACTGCA 800
S40368.1|             ------------------------------------------------
M32614.1|SYNRICA      ------------------------------------------------
X03179.1|             TCACTTTTTATTGATCAAGTATTTGTGGTTTTCTTTATATAAAAAAATGT 193
X52908.1|             GTTATTTTTATTGATCAAGTATTTGTGGTTTTCTTTATATAAAAAAATGT 213
M12089.1|RCCAGG       ------------------------------------------------
S40367.1|             ------------------------------------------------

S40366.1|             AAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTATATTAATCC 748
M17631.1|RCCRICA      ------------------------------------------------
X02388.1|             AAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTATATTAATCC 850
S40368.1|             ------------------------------------------------
M32614.1|SYNRICA      ------------------------------------------------
X03179.1|             ATTAGTGTTTTTCTGTATTAATTTTATAAGTTCATCTTTATGAGAATGCT 243
X52908.1|             ATTAGTGTTTTTCTGTATTAATTTTATAAGTTCATCTTTATGAGAATGCT 263
M12089.1|RCCAGG       ------------------------------------------------
S40367.1|             ------------------------------------------------

S40366.1|             CTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCATCGTCACAG 798
M17631.1|RCCRICA      ------------------------------------------------
X02388.1|             CTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCATCGTCACAG 900
S40368.1|             ------------------------------------------------
M32614.1|SYNRICA      ------------------------------------------------
X03179.1|             AATGTATTTGGACAGCCAATAAAATTCCAGAATTGCTGCAATCAAGGATG 293
X52908.1|             AATGTATTTGGACAGCCAATAAAATTCCAGAATTGCTGCAATCAAAGATG 313
M12089.1|RCCAGG       ------------------------------------------------
S40367.1|             ------------------------------------------------

S40366.1|             TTTTCTTTGCTTATAAGGCCAGTGGTACCAAATTTTAATGCTGATGTTTG 848
M17631.1|RCCRICA      ------------------------------------GCTGATGTTTG 11
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```
X02388.1|             TTTTCTTTGCTTATAAGGCCAGTGGTACCAAATTTTAATGCTGATGTTTG 950
S40368.1|             ---------------------------------------------------
M32614.1|SYNRICA      ---------------------------------------------------
X03179.1|             AAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGTGGCAACATG 343
X52908.1|             AAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGTGGCAACATG 363
M12089.1|RCCAGG      ---------------------------------------------------
S40367.1|             ---------------------------------------------------
S40366.1|             TATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATGGTCTATGTG 898
M17631.1|RCCRICA     TATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATGGTCTATGTG 61
X02388.1|             TATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATGGTCTATGTG 1000
S40368.1|             ---------------------------------------------------
M32614.1|SYNRICA      ----------------------GAATTCGCGAATCCGGA--GTGTAACAT 26
X03179.1|             GCTTTGTTTTGGATCCACC-TCAGGGTGGTCTTTCACATTAGAGGATAAC 392
X52908.1|             GCTTTGTTTTGGATCCACC-TCAGGGTGGTCTTTCACATTAGAGGATAAC 412
M12089.1|RCCAGG      ---------------------------------------------------
S40367.1|             ---------------------------------------------------
                                  Selected RA portion
S40366.1|             TTGATGTTAGGGATGGAAGATTCCACAACGGAAACG--CAATACAGTTGT 946
M17631.1|RCCRICA     TTGATGTTAGGGATGGAAGATTCCACAACGGAAACG--CAATACAGTTGT 109
X02388.1|             TTGATGTTAGGGATGGAAGATTCCACAACGGAAACG--CAATACAGTTGT 1048
S40368.1|             ---ATATTCCCCAAACAATACCCAATTATAAACTTTACCACAGCAGATGC 47
M32614.1|SYNRICA      ATGATATTTCCCAAACAATACCCAATCATAAACTTTACCACTGCAGGTGC 76
X03179.1|             AACATATTCCCCAAACAATACCCAATTATAAACTTTACCACAGCGGGTGC 442
X52908.1|             AACATATTCCCCAAACAATACCCAATTATAAACTTTACCACAGCGGGTGC 462
M12089.1|RCCAGG      ---------------------------------------------------
S40367.1|             ---------------------------------------------------
                                  Selected RA portion
S40366.1|             GGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTTGAAAAGA 996
M17631.1|RCCRICA     GGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTTGAAAAGA 159
X02388.1|             GGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTTGAAAAGA 1098
S40368.1|             CACTGTGGAAAGCTA-CACAAACTTTA-TCAGAGCTGTGCGCAGTCATTT 95
M32614.1|SYNRICA      CACTGTGCAAAGCTA-CACAAACTTCA-TCAGAGCTGTTCGCGGCCGCTT 124
X03179.1|             CACTGTGCAAAGCTA-CACAAACTTTA-TCAGAGCTGTTCGCGGTCGTTT 490
X52908.1|             CACTGTGCAAAGCTA-CACAAACTTTA-TCAGAGCTGTTCGCGGTCGTTT 510
M12089.1|RCCAGG      ---------------------------------------------------
S40367.1|             ---------------------------------------------------
                                  Selected RA portion

S40366.1|             GACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACGGGTACAG 1046
M17631.1|RCCRICA     GACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACGGGTACAG 209
X02388.1|             GACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACGGGTACAG 1148
S40368.1|             AACAACTGGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAAACAGAG 145
M32614.1|SYNRICA      AACAACTGGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAAACAGAG 174
X03179.1|             AACAACTGGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAAACAGAG 540
X52908.1|             AACAACTGGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAAACAGAG 560
M12089.1|RCCAGG      ---------------------------------------------------
S40367.1|             ---------------------------------------------------
                                  Selected RA portion
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S40366.1|            TCCGGGAGTCTATG---TGATGATCTATGATTGCAATACTGCTGCAACTG 1093
M17631.1|RCCRICA     TCCGGGAGTCTATG---TGATGATCTATGATTGCAATACTGCTGCAACTG 256
X02388.1|            TCCGGGAGTCTATG---TGATGATCTATGATTGCAATACTGCTGCAACTG 1195
S40368.1|            TTGGTTTGCCTATAAGCCAACGGTTTATTTTAGTTGAACTCTCAAATCAT 195
M32614.1|SYNRICA     TTGGTTTGCCTATAAACCAACGGTTCATACTAGTTGAACTCTCAAATCAT 224
X03179.1|            TTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCAAATCAT 590
X52908.1|            TTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCAAATCAT 610
                                Selected RA portion

M12089.1|RCCAGG      --------------------------------------------------
S40367.1|            --------------------------------------------------
S40366.1|            ATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCCCAGA 1143
M17631.1|RCCRICA     ATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCCCAGA 306
X02388.1|            ATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCCCAGA 1245
S40368.1|            GCAGAGCTTTCTGTTACATT---AGCACTGGA--TGTCACCAATGC-ATA 239
M32614.1|SYNRICA     GCAGAGCTTTCTGTTACATT---AGCTCTAGA--TGTCACCAATGC-GTA 268
X03179.1|            GCAGAGCTTTCTGTTACATT---AGCGCTGGA--TGTCACCAATGC-ATA 634
X52908.1|            GCAGAGCTTTCTGTTACATT---AGCGCTGGA--TGTCACCAATGC-ATA 654
M12089.1|RCCAGG      --------------------------------------------------
S40367.1|            --------------------------------------------------
                                Selected RA portion

S40366.1|            TCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACACTTAC 1193
M17631.1|RCCRICA     TCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACACTTAC 356
X02388.1|            TCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACACTTAC 1295
S40368.1|            TGTGGTC-GGCTGCCGCGCTGGAAATAGCGCCTATTTCTTTCATCCTGAC 288
M32614.1|SYNRICA     TGTGGTC-GGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGAC 317
X03179.1|            TGTGGTC-GGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGAC 683
X52908.1|            TGTGGTC-GGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGAC 703
M12089.1|RCCAGG      --------------------------------------------------
S40367.1|            --------------------------------------------------
                                Selected RA portion

S40366.1|            GGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACTAATA 1243
M17631.1|RCCRICA     GGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACTAATA 406
X02388.1|            GGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACTAATA 1345
S40368.1|            AAT-CAAGAAGATGCAGAAGCAATCACTCAT------CTTTTCACGGATG 331
M32614.1|SYNRICA     AAT-CAGGAAGACGCCGAAGCAATCACTCAT------CTTTTCACTGACG 360
X03179.1|            AAT-CAGGAAGATGCAGAAGCAATCACTCAT------CTTTTCACTGATG 726
X52908.1|            AAT-CAGGAAGATGCAGAAGCAATCACTCAT------CTTTTCACTGATG 746
M12089.1|RCCAGG      --------------------------------------------------
S40367.1|            --------------------------------------------------
                                Selected RA portion

S40366.1|            ATACACAACCTTTTGTTACAACCATTGTTGGGCTATATGGTCTGTGCTTG 1293
M17631.1|RCCRICA     ATACACAACCTTTTGTGACAACCATTGTTGGGCTATATGGTATGTGCTTG 456
X02388.1|            ATACACAACCTTTTGTTACAACCATTGTTGGGCTATATGGTCTGTGCTTG 1395
S40368.1|            TTCAAAATTCATTTACATTCGCCTTTGGTGGTAATTATGATAG--ACTTG 379
M32614.1|SYNRICA     TCCAAAATCGATATACATTCGCCTTTGGTGGTAATTACGATAG--ACTCG 408
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X03179.1|             TTCAAAATCGATATACATTCGCCTTTGGTGGTAATTATGATAG--ACTTG 774
X52908.1|             TTCAAAATCGATATACATTCGCCTTTGGAGGTAATTATGATAG--ACTTG 794
M12089.1|RCCAGG       ---------------------------------------------------
S40367.1|             ---------------------------------------------------

S40366.1|             CAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAAAGGC 1343
M17631.1|RCCRICA     CAAGCAAATAGTGGAAAAGTATGGTTAGAGGACTGTACCAGTGAAAAGGC 506
X02388.1|             CAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAAAGGC 1445
S40368.1|             --AACAACTTGGAGGT---CTGAGAGAAAATATTGAGTTGGGAACTGGTC 424
M32614.1|SYNRICA     --AGCAACTTGCTGGTAATCTGAGAGAAAATATCGAGTTGGGAAACGGTC 456
X03179.1|             --AACAACTTGCTGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTC 822
X52908.1|             --AACAACTTGCTGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTC 842
M12089.1|RCCAGG       ---------------------------------------------------
S40367.1|             ---------------------------------------------------
S40366.1|             TGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAGCAAA 1393
M17631.1|RCCRICA     TGAACAACAATGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAGCAAA 556
X02388.1|             TGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAGCAAA 1495
S40368.1|             CA--TTAGAGGACGCTATCTCAGC----GCTTTATTA-----TTATAGTA 463
M32614.1|SYNRICA     CG--CTAGAGGAGGCTATCTCAGC----GCTTTACTA-----TTACAGTA 495
X03179.1|             CA--CTAGAGGAGGCTATCTCAGC----GCTTTATTA-----TTACAGTA 861
X52908.1|             CA--CTAGAGGAGGCTATCTCAGC----GCTTTATTA-----TTACAGTA 881
M12089.1|RCCAGG       ---------------------------------------------------
S40367.1|             ---------------------------------------------------

S40366.1|             ACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGTTGTT 1443
M17631.1|RCCRICA     ACCGCGATAATTGCCTTACAACTGATGCTAATATAAAAGGAACAGTTGTC 606
X02388.1|             ACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGTTGTT 1545
S40368.1|             CTTGTGGCACTCAGATTCCAACTCTGGCTCGTTCCTTTATGGTTTGCATC 513
M32614.1|SYNRICA     CTGGTGGTACCCAGCTTCCAACTCTGGCTCGTTCCTTCATAATATGCATC 545
X03179.1|             CTGGTGGCACTCAGCTTCCAACTCTGGCTCGTTCCTTTATAATTTGCATC 911
X52908.1|             CTGGTGGCACTCAGCTTCCAACTCTGGCTCGTTCCTTTATAATTTGCATC 931
M12089.1|RCCAGG       ---------------------------------------------------
S40367.1|             ---------------------------------------------------

S40366.1|             AAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGTTCAA 1493
M17631.1|RCCRICA     AAGATCCTCTCTTGTGGCCCTGTATCCTCTGGCCAACGATGGATGTTCAA 656
X02388.1|             AAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGTTCAA 1595
S40368.1|             CAAATGATTTCA-GAAGCAGCAAGATTCCAGTACATTGAGGGA------- 555
M32614.1|SYNRICA     CAAATGATTTCA-GAAGCAGCAAGATTCCAATATATTGAGGGA------- 587
X03179.1|             CAAATGATTTCA-GAAGCAGCAAGATTCCAATATATTGAGGGA------- 953
X52908.1|             CAAATGATTTCA-GAAGCAGCAAGATTCCAATATATTGAGGGA------- 973
M12089.1|RCCAGG       ------AAACCGGGAGGAAATACTATTGTAATATG--GATGTATGCGGTG 42
S40367.1|             ---ATGAAACCGGGAGGAAATACTATTGTAATATG--GATGTATGCAGTG 45
                                 *  *  *                    ** * *

S40366.1|             GAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGATGTGA 1543
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M17631.1|RCCRICA      GAATGATGGAACCATTTTAAATTTGTATAATGGATTGGTGTTAGATGTGA 706
X02388.1|             GAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGATGTGA 1645
S40368.1|             GAAATGCGGACGAGAATTAGGTACA-ACCGGAGATCTGCACCAGATCCTA 604
M32614.1|SYNRICA      GAAATGCGCACGCGTATTAGGTACA-ACCGGAGATCTGCACCAGATCCTA 636
X03179.1|             GAAATGCGCACGAGAATTAGGTACA-ACCGGAGATCTGCACCAGATCCTA 1002
X52908.1|             GAAATGCGCACGAGAATTAGGTACA-ACCGGAGATCTGCACCAGATCCTA 1022
M12089.1|RCCAGG       GCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAGA 92
S40367.1|             GCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAGA 95
                                * *    *        **   *          *         * **   *


S40366.1|             G-GCGATCGGATCCGAGC---CTTAAACAAATCATTCTTTACCCTCTCCA 1589
M17631.1|RCCRICA      G-GCGATCGGATCCGAGCAGCCTTAAACAAATCATTGTTCACCCTTTCCA 755
X02388.1|             G-GCGATCGGATCCGAGC---CTTAAACAAATCATTCTTTACCCTCTCCA 1691
S40368.1|             GCGTAATTACACTTGAG--------AATAGTTGGGGG---AGACTTTCCA 643
M32614.1|SYNRICA      GCGTGATCACACTTGAG--------AATAGTTGGGGG---AGACTGTCGA 675
X03179.1|             GCGTAATTACACTTGAG--------AATAGTTGGGGG---AGACTTTCAA 1041
X52908.1|             GCGTAATTACACTTGAG--------AATAGTTGGGGG---AGACTTTCCA 1061
M12089.1|RCCAGG       GGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACAG 142
S40367.1|             GGATAACAACATA-------------------------------------- 108
                              *    *    *


S40366.1|             TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT 1639
M17631.1|RCCRICA      TGGAAACCTAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT 805
X02388.1|             TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT 1741
S40368.1|             CTGCAATTCAAG---AGTCTAACCAAGGAGCCTTTGCTAGTCCAATTCAA 690
M32614.1|SYNRICA      CTGCAATTCAAG---AGTCTAACCAAGGAGCCTTTGCTAGTCCAATTCAA 722
X03179.1|             CTGCAATTCAAG---AGTCTAACCAAGGAGCCTTTGCTAGTCCAATTCAA 1088
X52908.1|             CTGCAATTCAAG---AGTCTAACCAAGGAGCCTTTGCTAGTCCAATTCAA 1108
M12089.1|RCCAGG       CAGATGCCACTGTGGAAAGCTACACAAACTTTATCAGAGCTGTGCGCAGT 192
S40367.1|             ---------------------------------------------- 


S40366.1|             CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA 1689
M17631.1|RCCRICA      CTCTTGCAGTGTGTATGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA 855
X02388.1|             CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA 1791
S40368.1|             CTGCAAAGACGTAACGGTTCCAAATTCAATGTGTACGATGTGAGTATATT 740
M32614.1|SYNRICA      CTGCAAAGACGTAATGGTTCGAAATTCAGTGTGTACGATGTGAGTATATT 772
X03179.1|             CTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTATATT 1138
X52908.1|             CTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTATATT 1158
M12089.1|RCCAGG       CATTTAACAACTGGAGCTGATGTGAGACATGAAATACCAGTGTTGCCAAA 242
S40367.1|             ---------------------------------------------- 
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S40366.1|            GGAC---ATTGTAAATTTTGTAACTGAAAGGACAGCAAGTT-ATTGCAGT 1735
M17631.1|RCCRICA     GGAC---ATTGTAAATTTTGTAACTGAAAGGACAGCAAGTT-ATTGCAGT 901
X02388.1|            GGAC---ATTGTAAATTTTGTAACTGAAAGGACAGCAAGTT-ATTGCAGT 1837
S40368.1|            AATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCGTCGT 790
M32614.1|SYNRICA     AATCCCTATCATAGCTCTCATGGTGTATAGATGCGCGCCTCCACCGAGCT 822
X03179.1|            AATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCATCGT 1188
X52908.1|            AATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCATCGT 1208
M12089.1|RCCAGG      CAGAGTTGGTTTGCCTATAAGCCAACGGTTTATTTTAGTTGAACTCTCAA 292
S40367.1|            --------------------------------------------------


S40366.1|            CCAGTATCTAATAAGAGCACAACTATTGTCTTGTGCATTCTAAATTT--- 1782
M17631.1|RCCRICA     CCAGTATCTAATAAGAGCACAACTATTGTCTTGTGCATTCT--------- 942
X02388.1|            CCAGTATCTAATAAGAGCACAACTATTGTCTTGTGCATTCTAAATTT--- 1884
S40368.1|            CACAGTTTTCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAATGCTGAT 840
M32614.1|SYNRICA     CACAGTTTTAGTAAGGATCCAAGCTT----------------------- 848
X03179.1|            CACAGTTTTCTTTGCTTATAAGGCCAGTGGTACCAAATTTTAATGCTGAT 1238
X52908.1|            CACAGTTTTCTTTGCTTATAAGGCCAGTGGTACCAAATTTTAATGCTGAT 1258
M12089.1|RCCAGG      ATCATGCAGAGCTTTCTGTTACATTAGCACTGGATGTCACCAATGCATAT 342
S40367.1|            --------------------------------------------------

S40366.1|            --------------------------------------------------
M17631.1|RCCRICA     --------------------------------------------------
X02388.1|            --------------------------------------------------
S40368.1|            GTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATGGTCT 890
M32614.1|SYNRICA     --------------------------------------------------
X03179.1|            GTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATGGTCT 1288
X52908.1|            GTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATGGTCT 1308
M12089.1|RCCAGG      GTGGTCGGCTGCCGCGCTGGAAATAGCGCCTATTTCTTTCATCCTGACAA 392
S40367.1|            --------------------------------------------------

S40366.1|            --------------------------------------------------
M17631.1|RCCRICA     --------------------------------------------------
X02388.1|            --------------------------------------------------
S40368.1|            ATGTGTTGATGTTACAGGTGAAGAATTCTTCGATGGAAACCCAATACAAT 940
M32614.1|SYNRICA     --------------------------------------------------
X03179.1|            ATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATACAGT 1338
X52908.1|            ATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATACAGT 1358
M12089.1|RCCAGG      TCAAGAAGATGCAGAAGCAATCACTCATCTTTTCACGGATGTTCAAAATT 442
S40367.1|            --------------------------------------------------

S40366.1|            --------------------------------------------------
M17631.1|RCCRICA     --------------------------------------------------
X02388.1|            --------------------------------------------------
S40368.1|            TGTGGCCATGCAAATCTAATACAGATTGGAATCAGTTATGGACTTTGAGA 990
M32614.1|SYNRICA     --------------------------------------------------
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X03179.1|             TGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTTGAAA 1388
X52908.1|             TGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTTGAAA 1408
M12089.1|RCCAGG       CATTTACATTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGGA 492
S40367.1|             --------------------------------------------------

S40366.1|             --------------------------------------------------
M17631.1|RCCRICA      --------------------------------------------------
X02388.1|             --------------------------------------------------
S40368.1|             AAAGATAGCACTATTCGATCTAATGGCAAGTGTTTGACCATTTCCAAGTC 1040
M32614.1|SYNRICA     --------------------------------------------------
X03179.1|             AGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACGGGTA 1438
X52908.1|             AGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACGGGTA 1458
M12089.1|RCCAGG       GGTCTGAGAGAAAATATTGAGTTGGGAACTGGTCCATTAGAGGACGCTAT 542
S40367.1|             --------------------------------------------------
S40366.1|             --------------------------------------------------
M17631.1|RCCRICA      --------------------------------------------------
X02388.1|             --------------------------------------------------
S40368.1|             CAGTCCAAGACAGCAGGTGGTGATATATAATTGCAGTACCGCTACAGTTG 1090
M32614.1|SYNRICA     --------------------------------------------------
X03179.1|             CAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCAACTG 1488
X52908.1|             CAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCAACTG 1508
M12089.1|RCCAGG       CTCAGCGCTTTATTATTATAGTACTTGTGGCACTCAGATTCCAACTCTGG 592
S40367.1|             --------------------------------------------------

S40366.1|             --------------------------------------------------
M17631.1|RCCRICA      --------------------------------------------------
X02388.1|             --------------------------------------------------
S40368.1|             GTGCCACCCGTTGGCAAATATGGGACAATCGAACCATCATAAATCCCCGA 1140
M32614.1|SYNRICA     --------------------------------------------------
X03179.1|             ATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCCCAGA 1538
X52908.1|             ATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCCCAGA 1558
M12089.1|RCCAGG       CTCGTTCCTTTATGGTTTGCATCCAAATGATTTCAGAAGCAGCAAGATTC 642
S40367.1|             --------------------------------------------------

S40366.1|             --------------------------------------------------
M17631.1|RCCRICA      --------------------------------------------------
X02388.1|             --------------------------------------------------
S40368.1|             TCTGGTCTAGTTTTGGCAGCCACATCAGGGAACAGTGGTACCAAACTTAC 1190
M32614.1|SYNRICA     --------------------------------------------------
X03179.1|             TCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACACTTAC 1588
X52908.1|             TCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACACTTAC 1608
M12089.1|RCCAGG       CAGTACATTGAGGGAGAAATGCGGACGAGAATTAGGTACAACCGGAGATC 692
S40367.1|             --------------------------------------------------
                                Selected RB portion
S40366.1|             --------------------------------------------------
M17631.1|RCCRICA      --------------------------------------------------
X02388.1|             --------------------------------------------------
S40368.1|             AGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACTAATA 1240
M32614.1|SYNRICA     --------------------------------------------------
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X03179.1|            AGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACTAATA 1638
X52908.1|            AGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACTAATA 1658
M12089.1|RCCAGG      TGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGACTTT 742
S40367.1|            --------------------------------------------------

S40366.1|            --------------------------------------------------
M17631.1|RCCRICA     --------------------------------------------------
X02388.1|            --------------------------------------------------
S40368.1|            ATACACAACCTTTTGTGACAACCATTGTTGGGCTATATGGCATGTGCTTG 1290
M32614.1|SYNRICA    --------------------------------------------------
X03179.1|            ATACACAACCTTTTGTGACAACCATTGTTGGGCTATATGGTCTGTGCTTG 1688
X52908.1|            ATACACAACCTTTTGTGACAACCATTGTTGGGCTATATGGTCTGTGCTTG 1708
M12089.1|RCCAGG      CCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAATTCAA 792
S40367.1|            --------------------------------------------------
S40366.1|            --------------------------------------------------
M17631.1|RCCRICA     --------------------------------------------------
X02388.1|            --------------------------------------------------
S40368.1|            CAAGCAAATAGTGGAAAAGTATGGTTAGAGGACTGTACCAGTGAAAAGGC 1340
M32614.1|SYNRICA    --------------------------------------------------
X03179.1|            CAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAAAGGC 1738
X52908.1|            CAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAAAGGC 1758
M12089.1|RCCAGG      CTGCAAAGACGTAACGGTTCCAAATTCAATGTGTACGATGTGAGTATATT 842
S40367.1|            --------------------------------------------------
                                Selected RB portion

S40366.1|            --------------------------------------------------
M17631.1|RCCRICA     --------------------------------------------------
X02388.1|            --------------------------------------------------
S40368.1|            TGAACAACAATGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAGCAAA 1390
M32614.1|SYNRICA    --------------------------------------------------
X03179.1|            TGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAGCAAA 1788
X52908.1|            TGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAGCAAA 1808
M12089.1|RCCAGG      AATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCGTCGT 892
S40367.1|            --------------------------------------------------
                                Selected RB portion

S40366.1|            --------------------------------------------------
M17631.1|RCCRICA     --------------------------------------------------
X02388.1|            --------------------------------------------------
S40368.1|            ACCGCGATAATTGCCTTACAACTGATGCTAATATAAAAGGAACAGTTGTC 1440
M32614.1|SYNRICA    --------------------------------------------------
X03179.1|            ACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGTTGTC 1838
X52908.1|            ACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGTTGTC 1858
M12089.1|RCCAGG      CACAGTTTTCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAATGCTGAT 942
S40367.1|            --------------------------------------------------
                                Selected RB portion

S40366.1|            --------------------------------------------------
M17631.1|RCCRICA     --------------------------------------------------
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X02388.1|             ------------------------------------------------
S40368.1|             AAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGTTCAA 1490
M32614.1|SYNRICA     ------------------------------------------------
X03179.1|             AAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGTTCAA 1888
X52908.1|             AAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGTTCAA 1908
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Selected RB portion

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M12089.1|RCCAGG       GTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATGGTCT 992
S40367.1|             ------------------------------------------------

S40366.1|             ------------------------------------------------
M17631.1|RCCRICA      ------------------------------------------------
X02388.1|             ------------------------------------------------
S40368.1|             GAATGATGGAACCATTTTAAATTTGTATAATGGATTGGTGTTAGATGTGA 1540
M32614.1|SYNRICA     ------------------------------------------------
X03179.1|             GAATGATGGAACCATTTTAAATTTGTATAGTGGGTTGGTGTTAGATGTGA 1938
X52908.1|             GAATGATGGAACCATTTTAAATTTGTATAGTGGGTTGGTGTTAGATGTGA 1958
M12089.1|RCCAGG       ATGTGTTGATGTTACAGGTGAAGAATTCTTCGATGGAAACCCAATACAAT 1042
S40367.1|             ------------------------------------------------
S40366.1|             ------------------------------------------------
M17631.1|RCCRICA      ------------------------------------------------
X02388.1|             ------------------------------------------------
S40368.1|             GGCGATCGGATCCGAGCCTTAAACAAATCATTGTTCACCCTTTCCATGGA 1590
M32614.1|SYNRICA     ------------------------------------------------
X03179.1|             GGGCATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCATGGT 1988
X52908.1|             GGGCATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCATGGT 2008
M12089.1|RCCAGG       TGTGGCCATGCAAATCTAATACAGATTGGAATCAGTTATGGACTTTGAGA 1092
S40367.1|             ------------------------------------------------

S40366.1|             ------------------------------------------------
M17631.1|RCCRICA      ------------------------------------------------
X02388.1|             ------------------------------------------------
S40368.1|             AACCTAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACTCTCT 1640
M32614.1|SYNRICA     ------------------------------------------------
X03179.1|             GACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACTCTCT 2038
X52908.1|             GACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACTCTCT 2058
M12089.1|RCCAGG       AAAGATAGCACTATTCGATCTAATGGCAAGTGTTTGACCATTTCCAAGTC 1142
S40367.1|             ------------------------------------------------

S40366.1|             ------------------------------------------------
M17631.1|RCCRICA      ------------------------------------------------
X02388.1|             ------------------------------------------------
S40368.1|             TGCAGTGTGTATGTCCTGCCACTAAAATAGATGGCTTAAATAAAAAGGA- 1689
M32614.1|SYNRICA     ------------------------------------------------
X03179.1|             TGCAGTGTGTATGTCCTGCCATGAAAATAGATGGCTTAAATAAAAAGGAC 2088
X52908.1|             TGCAGTGTGTATGTCCTGCTATGAAAATAGATGGCTTAAATAAAAAGGAC 2108
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M12089.1|RCCAGG       CAGTCCAAGACAGCAGGTGGTGATATATAATTGCAGTACCGCTACAGTTG 1192
S40367.1|             --------------------------------------------------

S40366.1|             --------------------------------------------------
M17631.1|RCCRICA      --------------------------------------------------
X02388.1|             --------------------------------------------------
S40368.1|             --------------------------------------------------
M32614.1|SYNRICA      --------------------------------------------------
X03179.1|             ATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATTGCAGTCCAGTATC 2138
X52908.1|             ATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATTGCAGTCCAGTATC 2158
M12089.1|RCCAGG       GTGCCACCCGTTGGCAAATATGGGACAATCGAACCATCATAAATCCCCGA 1242
S40367.1|             --------------------------------------------------

S40366.1|             --------------------------------------------------
M17631.1|RCCRICA      --------------------------------------------------
X02388.1|             --------------------------------------------------
S40368.1|             --------------------------------------------------
M32614.1|SYNRICA      --------------------------------------------------
X03179.1|             TAATAAGAGCACAACTATTGTCTTGTGCATTCTAAATTTATGGATGAATG 2188
X52908.1|             TAATAAGAGCACAACTATTGTCTTGTGCATTCTAAATTTATGGATGAATT 2208
M12089.1|RCCAGG       TCTGGTCTAGTTTTGGCAGCCACATCAGGGAACAGTGGTACCAAACTTAC 1292
S40367.1|             --------------------------------------------------
S40366.1|             --------------------------------------------------
M17631.1|RCCRICA      --------------------------------------------------
X02388.1|             --------------------------------------------------
S40368.1|             --------------------------------------------------
M32614.1|SYNRICA      --------------------------------------------------
X03179.1|             TATGAATAAAGCTAATTATTTTGGTCATCAGACTTGATATCTTTTTGAAT 2238
X52908.1|             GTATGAATTAAGCTAATTATTTTGGTCATCAGACTTGATATCTTTTTGAA 2258
M12089.1|RCCAGG       AGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACTAATA 1342
S40367.1|             --------------------------------------------------

S40366.1|             --------------------------------------------------
M17631.1|RCCRICA      --------------------------------------------------
X02388.1|             --------------------------------------------------
S40368.1|             --------------------------------------------------
M32614.1|SYNRICA      --------------------------------------------------
X03179.1|             AAAATAAATAATAATGTTTTTTCAAACTTATAAAACTAATGAATGATATG 2288
X52908.1|             TAAAATAAATAATATGTTTTTTCAAACTTATAAATCTATGAATGATATGA 2308
M12089.1|RCCAGG       ATACACAACCTTTTGTGACAACCATTGTTGGGCTATATGGCATGTGCTTG 1392
S40367.1|             --------------------------------------------------

S40366.1|             --------------------------------------------------
M17631.1|RCCRICA      --------------------------------------------------
X02388.1|             --------------------------------------------------
S40368.1|             --------------------------------------------------
M32614.1|SYNRICA      --------------------------------------------------
X03179.1|             AATATAAATGCGGAGACTAGTCAATCTTTTATGTAATTCTATGATGATAA 2338
X52908.1|             ATATAATGCGGAGACTAGTCAATCTTTTATGTAATTCTATGATGATAAAA 2358
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M12089.1|RCCAGG       CAAGCAAATAGTGGAAAAGTATGGTTAGAGGACTGTACCAGTGAAAAGGC 1442
S40367.1|             --------------------------------------------------

S40366.1|             --------------------------------------------------
M17631.1|RCCRICA      --------------------------------------------------
X02388.1|             --------------------------------------------------
S40368.1|             --------------------------------------------------
M32614.1|SYNRICA      --------------------------------------------------
X03179.1|             AAGCTT------------------------------------------ 2344
X52908.1|             GCTT-------------------------------------------- 2362
M12089.1|RCCAGG       TGAACAACAATGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAGCAAA 1492
S40367.1|             --------------------------------------------------

S40366.1|             --------------------------------------------------
M17631.1|RCCRICA      --------------------------------------------------
X02388.1|             --------------------------------------------------
S40368.1|             --------------------------------------------------
M32614.1|SYNRICA      --------------------------------------------------
X03179.1|             --------------------------------------------------
X52908.1|             --------------------------------------------------
M12089.1|RCCAGG       ACCGCGATAATTGCCTTACAACTGATGCTAATATAAAAGGAACAGTTGTC 1542
S40367.1|             --------------------------------------------------


S40366.1|             --------------------------------------------------
M17631.1|RCCRICA      --------------------------------------------------
X02388.1|             --------------------------------------------------
S40368.1|             --------------------------------------------------
M32614.1|SYNRICA      --------------------------------------------------
X03179.1|             --------------------------------------------------
X52908.1|             --------------------------------------------------
M12089.1|RCCAGG       AAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGTTCAA 1592
S40367.1|             --------------------------------------------------

S40366.1|             --------------------------------------------------
M17631.1|RCCRICA      --------------------------------------------------
X02388.1|             --------------------------------------------------
S40368.1|             --------------------------------------------------
M32614.1|SYNRICA      --------------------------------------------------
X03179.1|             --------------------------------------------------
X52908.1|             --------------------------------------------------
M12089.1|RCCAGG       GAATGATGGAACCATTTTAAATTTGTATAATGGATTGGTGTTAGATGTGA 1642
S40367.1|             --------------------------------------------------

S40366.1|             --------------------------------------------------
M17631.1|RCCRICA      --------------------------------------------------
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X02388.1|             --------------------------------------------------
S40368.1|             --------------------------------------------------
M32614.1|SYNRICA      --------------------------------------------------
X03179.1|             --------------------------------------------------
X52908.1|             --------------------------------------------------
M12089.1|RCCAGG       GGCGATCGGATCCGAGCCTTAAACAAATCATTGTTCACCCTTTCCATGGA 1692
S40367.1|             --------------------------------------------------


S40366.1|             --------------------------------------------------
M17631.1|RCCRICA      --------------------------------------------------
X02388.1|             --------------------------------------------------
S40368.1|             --------------------------------------------------
M32614.1|SYNRICA      --------------------------------------------------
X03179.1|             --------------------------------------------------
X52908.1|             --------------------------------------------------
M12089.1|RCCAGG       AACCTAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACTCTCT 1742
S40367.1|             --------------------------------------------------



S40366.1|             --------------------------------------------------
M17631.1|RCCRICA      --------------------------------------------------
X02388.1|             --------------------------------------------------
S40368.1|             --------------------------------------------------
M32614.1|SYNRICA      --------------------------------------------------
X03179.1|             --------------------------------------------------
X52908.1|             --------------------------------------------------
M12089.1|RCCAGG       TGCAGTGTGTATGTCCTGCCACTAAAATAGATGGCTTAAATAAAAAGGA 1791
S40367.1|             --------------------------------------------------
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S40366.1 proricin A chain [*Ricinus communis*=castor oil plant, seeds, mRNA, 1782 nt
M17631.1 RCCRICA *Ricinus communis* ricin E beta chain mRNA, 3' end
X03179.1 Ricinus communis gene for ricin precursor
X02388.1 Castor bean mRNA for ricin precursor
S40368.1 agglutinin I=galactose-specific lectin [*Ricinus communis*=castor oil plant, seeds, mRNA, 1689 nt
M32614.1 SYNRICA Synthetic ricin A-chain gene, complete cds
X52908.1 *Ricinus communis* ricin gene
M12089.1 RCCAGG Castor bean (*R.communis*) agglutinin mRNA, complete cds
S40367.1 preproricin [*Ricinus communis*=castor oil plant, seeds, mRNA Partial, 108 nt

Publications

Journals

Hegde DM, Ashfaq MA and Dinesh Kumar V (2005). Castor endosperm toxic protein, ricin : some insights and developments. **The Botanica 55:61-73**

Ashraf Ashfaq M, Narasimha Rao N, Kirti PB and Dinesh Kumar V (2009). Isolation of ricin promoters from castor. **Journal of Oilseeds Research 26:208-210**

Somasekhar Reddy P, Ashraf Ashfaq M, Anil Kumar Ch and Dinesh Kumar V (2009).Development of transgenic tobacco model system as a prelude to identify the efficient PTGS technology for silencing ricin and RCA in castor. **Journal of Oilseeds Research 26:199-202**

Dinesh Kumar V, Haritha B, Anusha S and Ashraf Ashfaq M (2009). SCAR and RAPD markers for genetic purity assessment of sunflower hybrid DRSH1. **Journal of Oilseeds Research 26:192-194**

Book Chapters

Hegde DM, Sujatha M, Dinesh Kumar V and Ashfaq MA (2006). Transgenic in oilseeds: Achievements and prospects. In: Plant Tissue Culture and Biotechnology Eds PC Trivedi, Pointer Publishers, Jaipur-302003 (Rajasthan), p46-104

Ashfaq MA, Dinesh Kumar V and Sujatha M (2007). Application of gene silencing in plants. In: Recent Advances in Plant Biotechnology and its Applications (eds:) Ashwani Kumar and Sudhir K. Sopory, IK International Pvt Ltd, pp.105-130

Papers presented in symposia

Harinath D, Rajender Reddy K, Rao NN, Yamini KN, Ashfaq MA, Sujatha M and Dinesh Kumar V (2006) Development of gene constructs for induction of male sterility and fertility restoration in safflower (Carthamus tinctorius L.). National seminar on gene constructs held at IIHR, Bangalore, May 17-18, 2006

Hegde DM, Sujatha M, Dinesh Kumar V and Ashfaq MA (2006). Transgenics in oilseed crops. Internationsl workshop on fostering the next green revolution: Role of biotechnology in advancing Indian agriculture, organized by ANGRAU, Hyderabad on June 2, 2006

Harinath D, Rajendar Reddy R, Narasimha Rao N, Yamini KN, Ashfaq MA, Sujatha M and Dinesh Kumar V. Development of Gene Constructs for Induction of Male Sterility and Restoration of Fertility in Safflower. ISOR, 2007. *Ibid* pp 19-21. (**the poster adjudged "the first best poster" of the seminar)**

Mohd Ashraf Ashfaq, Kirti P.B.and Dinesh Kumar, V. Development Of Generic PTGS and PTGS Constructs For Silencing Ricin and RCA Genes In Castor (*Ricinus communis* L.). ISOR, 2007. *Ibid* pp 9-12

Pasula Soma Sekhar Reddy, Mohd Ashraf Ashfaq, Sivaramakrishnan, S and Dinesh Kumar, V. Expression of ricin gene in tobacco. ISOR, 2007.*Ibid*. pp 23-26