Compendium of Transgenic Crop Plants

Transgenic Plantation Crops, Ornamentals and Turf Grasses

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5

Rubber Tree

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1. INTRODUCTION

Natural rubber, cis-1,4-polyisoprene, produced in the milky cytoplasm (latex) of specialized cells, called laticifers, of certain plants, is one of the most important biological macromolecule, used as industrial raw material for the manufacture of a variety of products. Natural rubber has been found in the latex of over 2000 plant species belonging to 311 genera of 79 families. However, *Hevea brasiliensis* (Para rubber tree) remains as the only cultivated species as a commercial source of natural rubber because of its abundance in the latex, high quality, and convenience of harvesting. Nature has not provided any other industrial raw material of plant origin as flexible as natural rubber. Rubber has a number of applications and there is hardly any segment of life that does not make use of rubber-based material. All along this chapter, *H. brasiliensis* will be mentioned as rubber or rubber tree, unless otherwise a specific mention is required.

1.1 History, Origin, and Distribution

H. brasiliensis is a native of Amazon River basin of South America. Rubber is one of the recently domesticated crops in the world. The successful transfer of *H. brasiliensis* to Asia and the subsequent establishment of commercial rubber plantations were in response to the growing demand for this raw material. *H. brasiliensis* was introduced to Tropical Asia in 1876 through Kew Gardens from the seeds brought from the Rio Tapajós region of the upper Amazon region of Brazil by Sir Henry Wickam (Dijkman, 1951). Kew Gardens, UK, played a special role in the domestication of wild plants. It was in the Kew Gardens where the planting materials were assembled from the native land, propagated, and then distributed to other botanical gardens around the world (Baulkwil, 1989). *H. brasiliensis* is now commercially cultivated in the tropical regions of Asia, Africa, and South America in countries like Indonesia, Thailand, Malaysia, India, China, Sri Lanka, Philippines, Vietnam, Nigeria, Cameroon, Ivory Coast, Liberia, Brazil, Mexico, etc. However, the major share comes from Tropical Asia.

1.2 Botanical Descriptions

1.2.1 Taxonomy

H. brasiliensis belongs to the family Euphorbiaceae. Ten species of *Hevea* have been identified in the genus, namely, *H. benthamiana, H. brasiliensis, H. camargoana, H. camporum, H. guianensis, H. microphylla, H. nitida, H. pauciflora, H. rigidifolia,* and *H. spruceana* (Schultz, 1990).

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1.2.2 Botany

Rubber tree (*H. brasiliensis*) is a sturdy, quick-growing tree, which attains a height of 25–30 m. The tree has a straight trunk with light gray bark. Branches are usually developed to form an open leafy crown. The bark of the trunk is the part from where rubber is exploited (Figure 1). The young plants show a characteristic growth pattern of alternating periods of rapid elongation and consolidation. The tree is deciduous with annual leaf fall. Refoliation and flowering follow wintering. The leaves are arranged in groups or storeys. From each storey, a cluster of spirally arranged trifoliate glabrous leaves is produced. The petioles are long, usually about 15 cm, with extra floral nectaries present in the region of insertion of the leaflets (Premakumari and Saraswathyamma, 2000).

Rubber tree is a monoecious tree with diclinous flowers arranged in a pyramid-shaped panicle. The flowers are short stalked and fragrant. The flower has one whorl of bell-shaped yellow-colored perianth with five lobes. Male flowers are smaller in size but more in number than the female flowers. In the male flower, there are 10 sessile anthers arranged on a slender staminal column in two whorls of five each. Each anther contains two pollen sacs that split longitudinally on dehiscence. Pollen grains are tricolpate, smooth, and sticky. Female flowers are seen at the tip of the panicle and its branchlets. When fully developed, they are recognizable by their relatively bigger size and the green torus basal disc. The gynoecium is tricarpellary and syncarpous with an ovule in each locule. The stigma is short styled and three lobed. Pollination is mediated by insects. Sticky pollen and stigmatic surfaces indicate the typical entomophilous nature of the flower. After fertilization the ovary will develop into a three-lobed dehiscent capsule, regma, with three large mottled seeds. Fruits ripen 5–6 months after fertilization (Kavitha and Saraswathyamma, 2005).

Figure 1 A rubber plantation (RRII 105 clone) at RRII with an enlarged view of the tapping region at the inset (arrow indicates latex flow into a cup from rubber tree after tapping)

1.2.3 Habitat

Rubber tree could be predominantly grown in the tropics where an equatorial monsoon climate prevails. The optimum climatic conditions for the successful growth of rubber are as follows: (1) rainfall of 2000 mm or more, evenly distributed without any marked dry season, and with 125 to 150 rainy days per annum; (2) maximum temperature of about 29–34 °C; (3) high atmospheric humidity in the order of 80% with moderate wind; and (4) bright sunshine amounting to about 2000 h per annum at the rate of 6 h per day throughout all the months (Watson, 1989; Rao and Vijayakumar, 1992).

1.2.4 Cytology

The chromosome counts made by various investigators showed variations and reported as $2n = 16$, 34, and 36. However, detailed cytological investigations have confirmed the chromosome complement of rubber tree in the somatic cells as $2n = 2x = 36$ (Ramaer, 1935; Saraswathyamma *et al.*, 1984). The chromosomes are small and vary in length and the total chromosome length of the species is 89.7 µm. Meiotic division is regular and pollen fertility is over 80%. Critical analysis of karyomorphology revealed significant differences between clones with reference to centromeric position and total chromosome

length (Saraswathyamma, 1990). There are no chromosomal or genetic barriers between the 10 *Hevea* species. Triploid plants with $2n = 3x = 54$ (Nazeer and Saraswathyamma, 1987) and induced tetraploids with $2n = 4x = 72$ by the application of colchicine (Saraswathyamma, 1990) were also reported. Wide range of meiotic abnormalities was noticed in the triploids and tetraploids (Saraswathyamma, 1997). The cytophotometric determination of DNA content of various cytotypes revealed 44.2 pg (picogram) in the diploids, 62.4 pg in the triploid, and 89.37 pg in the tetraploids (Saraswathyamma, 1990).

1.2.5 Harvesting and processing

In rubber tree, natural rubber is synthesized in highly specialized cells called latex vessels, present in all parts of the tree except the heartwood. These latex vessels originate from the cells produced by the cambium and they are articulated and anastomosing (Hebant and de Fay, 1980). The milky cytoplasmic content (latex) is collected by the controlled wounding of the bark, called tapping. The rubber is separated from latex upon coagulation and further processing.

1.3 Economic Importance

The global area under rubber cultivation is about 9.6 million hectares producing 9.2 million tons annually valuing about US$18 billion as raw material alone (IRSG, 2007). The major rubber producing countries are Thailand, Indonesia, India, Malaysia, China, Vietnam, etc. Chemically, natural rubber is cis-1,4-polyisoprene, having molecular weight of 200 000 to 8 000 000 and with viscoelastic properties. The flexibility of natural rubber to undergo vulcanization with sulfur under high temperatures is an important attribute. The higher strength, low heat build up, and better resistance to wear and flex cracking made natural rubber a suitable raw material for the manufacture of automobile tires. A major quantity of natural rubber produced is consumed by the automobile tire industry. Natural rubber is a good insulator and can be easily manipulated. Being water resistant, it finds use in the manufacture of water proofing materials. More than 35 000 rubber-based products such as hand gloves, toys, balloons, hoses, footwear, etc. are manufactured. Besides, rubber is also useful in soil stabilization, in vibration absorption, road surfacing, etc.

1.4 Traditional Breeding

The global consumption of natural rubber is steadily increasing and the production has also to be increased so as to meet the demand. The major objective of rubber tree breeding is to develop potential clones with high rubber yield combined with desirable secondary characters such as high initial vigor, smooth and thick bark with good latex vessel system, good bark renewal, high growth rate after initiation of latex harvest, tolerance to major diseases and wind, etc. (Annamma *et al.*, 1990; Varghese *et al.*, 1992). Recently, importance has also been given to develop clones with tolerance to abiotic stresses such as drought, high temperature, cold, etc. (Thulaseedharan *et al.*, 2000). Clones attaining early tapping girth and high initial yields are preferred to clones with higher yields in a later stage (Lim *et al.*, 1973). In countries where labor is cheap and the small-holding sector is predominant, clones capable of withstanding high tapping intensities are preferred. Besides high rubber yield, superior technological properties of rubber, timber and its quality (latex–timber clones), and low incidence of tapping panel dryness (TPD) are also major breeding objectives.

The conventional methods of genetic improvement are introduction, selection, and hybridization. The elite clones developed in one country are introduced to other countries. Popular clones introduced from other countries are evaluated under local agroclimatic conditions and the promising ones are recommended for large-scale planting. Introductions are effected under bilateral and multilateral clone exchange programs. Considerable yield variability in seedling populations was first observed by the Dutch workers in Java and Sumatra in the second decade of the last century (Whitby, 1919). Simultaneously, the technique of bud grafting was perfected, which facilitated the fixation of desired characters and the development of early primary clones through ortet selection. Ortet selection, mother tree selection or plus tree selection, is the oldest selection method

adopted in rubber trees. Ortet is, therefore, the original tree from which members of clone have descended. Hybridization programs are aimed at combining genes for desirable characters found dispersed in different clones. Hand-pollination between selected parent clones, evaluation of F_1 hybrids, selection of promising recombinants from the progeny, and multiplication by bud grafting are still the most important methods of conventional breeding (Varghese and Maydin, 2000).

Rapid progress made in the ortet selection between 1919 and 1926 in Indonesia and Malaysia resulted in the development of many classical primary clones commercially very promising (Marattukalam *et al.*, 1980). Controlled pollination was started in 1918 in Malaysia, 1920 in Sumatra, 1937 in Brazil, 1939 in Sri Lanka, 1948 in China, and 1954 in India, using the primary clones developed through ortet selection. Progress in yield improvement in rubber tree resulted in gradual increment, from 650 kg ha^{-1} in unselected seedlings during 1920s to 1600 kg ha^{-1} in the best clones during the 1950s. The yielding potential was further enhanced to 2500 kg ha^{-1} in PB, RRIM, RRII, RRIC, IRCA, BPM, and RRIV clones during 1990s. During these 70 years of rigorous breeding and selection, notable clones including RRIM 501, RRIM 600, RRIM 712, PB 217, PB235, PB 260, RRII 105, RRIC 100, IRCA 18, IRCA 230, IRCA 331, and BPM 24 were derived (Tan, 1987; Simmonds, 1989; Clement-Demangne *et al.*, 2001; Priyadarshan, 2003). Some of the primary clones, like PB 86, Tjir 1, Pil D65, Gl 1, PB 6/9, and PB 86, selected during the aforesaid period, became parents of improved clones. It must also be acknowledged that primary clones like GT 1 and PR 107 are still widely used, although their identification traces back to the 1920s.

1.5 Limitations of Conventional Breeding and Rationale for Transgenic Breeding

As a perennial tree species, genetic improvement through conventional breeding in rubber tree is rather a slow and difficult process. The major constraints in rubber tree breeding are long juvenile period, seasonal flowering, lack of any early selection parameters especially for estimating traits such as latex yield, susceptibility to TPD and wind damage, low fruit set, very long period of field experimentation, and pronounced interaction of genotype and environment. The major advantages are monoecious nature of the tree, which makes hybridization easy and the amenability to vegetative method of propagation (Saraswathyamma, 2002). The recent developments in recombinant-DNA and *in vitro* plant regeneration techniques could provide a direct route for the introduction of specific genes controlling agronomic traits into crop plants. The transfer of selected genes in a single generation by genetic transformation is especially interesting for this species, since its improvement is limited by long breeding cycles and high levels of heterozygosity. The major objectives of genetic transformation of rubber tree in different laboratories are (a) improvement of agronomic traits of elite rubber clones and (b) production of pharmaceuticals and other valuable recombinant proteins in the transgenic rubber tree. In the improvement of agronomic traits, the underlying direction is toward development of transgenic rubber tree with increased rubber biosynthesis, improved quality and volume of timber, tolerance to various diseases as well as abiotic stresses, etc. (Thulaseedharan, 2002; Yeang, 2004). A unique feature of rubber tree is the presence of latex vessels with the continuous production and harvesting of latex, which contains about 30–40% rubber and remaining with a serum containing all the machinery for protein synthesis, provided the gene is integrated through transgenic technology. Hence, the rubber tree could be exploited as a biological factory for the production of desired recombinant proteins by the integration of the desired genes.

2. PROGRESS IN THE GENETIC TRANSFORMATION OF RUBBER TREES

2.1 Donor Genes Employed and Design of Transgenics

2.1.1 Marker and antibiotic selection genes

In spite of the economic importance of the rubber trees, transgenic approaches for crop improvement have started only a little more than a decade ago. Two major factors that helped to lay the foundation for modern plant biotechnology are the capacity to regenerate whole plants from single

Table 1 *Different genes, promoters, and antibiotic-resistant genes used for genetic transformation of rubber trees*

Genes tried	*Agrobacterium* strain	Antibiotic used	Promoter	Selectable marker gene	References
GUS	Microprojectile	Kanamycin	CaMV 35S	*npt*II	Arokiaraj *et al.*, 1994
GUS	LBA 4404, GV2260	Kanamycin	CaMV 35S	*npt*II	Arokiaraj *et al.*, 1996
GUS	GV2260	Kanamycin	CaMV 35S	*npt*II	Arokiaraj *et al.*, 1998
GUS	C58p MP 90, C58p GV2260, AGL1, LBA 4404, EHA 105	Kanamycin	CaMV 35S	*npt*II	Montoro *et al.*, 2000
HAS	GV2260	Kanamycin	CaMV 35S	*npt*II	Arokiaraj *et al.*, 2002a
GUS	C58p MP 90, C58p GV2260, AGL1, LBA 4404, EHA 105	Paramomycin	CaMV 35S	*npt*II	Montoro *et al.*, 2003
SOD	EHA 101	Kanamycin	CaMV 35S	*npt*II	Jayashree *et al.*, 2003
SOD	EHA101	Kanamycin	FMV 34S	*npt*II	Sobha *et al.*, 2003b
GUS	EHA 105	Paramomycin	CaMV 35S	*npt*II	Blanc *et al.*, 2006
SOD	EHA101	Kanamycin	CaMV 35S	*npt*II	Rekha *et al.*, 2006
IPT	EHA101	Kanamycin	mas'	*npt*II	Kala *et al.*, 2003
TB antigen	LBA4404	Kanamycin	CaMV 35S	*npt*II	Kala *et al.*, 2006
ScFv	GV2850	Kanamycin	CaMV 35S	*npt*II	Yeang *et al.*, 2002

cells without changing the original genetic features of the cell (Birch, 1997) and successful gene transfer into plant genomes with stable expression of an introduced foreign gene (Zambryski *et al.*, 1983) through the natural genetic transformation mechanism by *Agrobacterium tumefaciens*. The first transgenic rubber tree was developed in 1994 incorporated with *GUS* (β-glucurinidase) gene (Arokiaraj *et al.*, 1994). Rubber plants being very recalcitrant and rubber cultivation being restricted to specific locations around the world, only few laboratories are actively involved in rubber research. A list of genes successfully tried for rubber tree transformation is presented in Table 1. In order to optimize the *Agrobacterium*- and biolistic-mediated genetic transformation systems in rubber trees, different workers employed *GUS* as the marker gene (Arokiaraj *et al.*, 1994, 1996, 1998; Montoro *et al.*, 2000, 2003; Blanc *et al.*, 2006). The *GUS* gene used is from *Escherichia coli* and the expression is controlled by the cauliflower mosaic virus (CaMV) 35S promoter and the selectable marker gene used was the sequence coding for neomycin phosphotransferase II (*npt*II).

2.1.2 Genes for recombinant protein production

The advent of genetic engineering in the early 1970s and its application to plant biotechnology have revolutionized agriculture. Recent years have seen a dramatic increase in application of biotechnology for the production of a variety of recombinant proteins in plants. Transgenic rubber trees that secrete human serum albumin (HSA) in the serum fraction of rubber latex were generated by Arokiaraj and coworkers in 2002. They constructed a binary vector pLGMR, which contained a 1.8-kb polymerase chain reaction (PCR)-amplified sequence coding for HSA fused with the CaMV 35S promoter and polyA tail to control the expression. *npt*II gene complementary DNA (cDNA) under the control of *nos* (nopaline synthase) 5′ promoter and *nos* 3′ polyA tail were used as the antibiotic selection. Further, Yeang *et al.* (2002) reported the expression of a functional recombinant antibody fragment in the latex of transgenic rubber. The binary vector used for the genetic transformation contained the gene for a mature immunoglobulin single chain variable fragment (ScFv) with specificity for the dental bacterium *Streptococcus gordonii*, together with the coding region of the tobacco pathogenesis-related protein signal sequence for targeting the recombinant fragment antibody. The CaMV 35S promoter and the *nos* terminator sequence were used for the expression of the gene. *npt*II gene was used as the selectable marker gene. Kala *et al.* (2006) used a cDNA sequence coding for a 10.8-kDa (kilodalton) TB antigen protein isolated from *Mycobacterium tuberculosis*.

2.1.3 Genes for tolerance to stress and TPD

Transgenic breeding for tolerance against environmental stress as well as TPD were initiated at the Rubber Research Institute of India (RRII). Transgenic rubber plants were developed using the functional gene coding for superoxide dismutase (Jayashree *et al.*, 2003). The 702 nucleotide rubber (*H. brasiliensis*) superoxide dismutase (Hb.SOD) cDNA was obtained by the reverse transcription PCR from the mRNA (messenger-RNA) isolated from the bark tissues of the trunk region, using forward and reverse sequences corresponding to a previously reported sequence of Hb.SOD (Mia and Gaynor, 1993). The Hb.SOD coding sequence was inserted into the binary vector pDU92.3103 (Tao *et al.*, 1995) at the unique *Bam*HI site between the CaMV 35S promoter and 3′ polyadenylation sequences, thereby creating the binary vector pDU96.2144 (Jayashree *et al.*, 2003). Similarly, *SOD* gene containing binary vector were developed under the control of figwort mosaic virus (FMV) 34S promoter and transgenic plants were obtained (Sobha *et al.*, 2003a). Rekha *et al.* (2006) reported genetic transformation of rubber tree with *SOD* gene in the antisense orientation in order to understand the role of *SOD* in the normal growth and development as well as environmental stress tolerance, including TPD in rubber. In order to enhance the cytokinin level in the bark to confer tolerance against TPD, attempts were also made to develop transgenic plants integrated with gene coding for isopentenyl transferase (*ipt*). The plasmid vector used was pDU.970612, which contained the sequence coding for *ipt* from *Agrobacterium* under the control of its own 5′ promoter and 3′ polyadenylation signal (Kala *et al.*, 2003).

2.2 Status of Genetic Transformation in Rubber Trees

2.2.1 Optimization of transformation techniques

The possibility of genetic transformation in rubber trees was first explored by Arokiaraj and Rahaman (1991). They employed co-cultivation of *in vitro*- and *in vivo*-propagated plantlets with *A. tumefaciens* (Strain 541) and cultured in the Murashige and Skoog (MS) medium without growth regulators. Co-cultivated explants developed tumors and produced octopine indicating effective transformation. Subsequently transformations were developed for direct DNA delivery through microprojectile bombardment as well as through *A. tumefaciens*-mediated methods (Arokiaraj *et al.*, 1994, 1996). In their studies, anther-derived calli were used as explants and binary vectors harboring *GUS* as reporter gene and either *npt*II or chloramphenicol acetyl transferase (*CAT*) as the selection gene. Genetic transformation was confirmed by histochemical staining and fluorometric assay for GUS activity, ELISA (enzyme-linked immunosorbent assay) for detecting expression of the *npt*II gene, and direct enzyme assay for detection of *CAT* gene expression were carried out. The presence of foreign gene in the transformed calli, embryoids and transgenic plants was further confirmed by PCR analysis. Arokiaraj *et al.* (1998) studied the constitutive promoter (CaMV 35S) directed β-glucuronidase expression in the laticifer system of transgenic rubber and the stability of expression of the transgene in successive vegetative generation raised through bud grafting of the original transformants. Anther calli were genetically transformed using *Agrobacterium* strain GV2260 harboring the β-glucuronidase (*uidA*) and *npt*II genes. β-glucuronidase protein was expressed in the leaves of transformed plants. GUS was also expressed in the serum fraction of latex. Transverse sections of the leaf petiole from a transformed plant revealed GUS expression, especially an enhanced expression in the phloem and laticifers. GUS expression was detected in three successive vegetative cycles propagated from the original transformants.

Studies were also undertaken to enhance the efficiency of *Agrobacterium*-mediated genetic transformation of rubber callus. It is reported that the virulence capacity of the *Agrobacterium* strain and the combination of the *Agrobacterium* strain and the type of binary vector used significantly influenced the transient expression of the GUS (Montoro *et al.*, 2000). Out of the five *A. tumefaciens* strains, C58pMP90, C58 pGV2260, AGL1, LB4404, and EHA 105 and the two binary vectors, pGIN and pCAMBIA 2301 tested the combination of EHA 105 and the binary plasmid vector pCAMBIA 2301 showed the highest transient expression. It is also reported

that transfer of friable callus from a maintenance medium containing 9.0 mM $CaCl_2$ to a calcium-free medium before *Agrobacterium* infection as well as use of a calcium-free *Agrobacterium* resuspension medium to inoculate friable calli significantly enhanced the transformation efficiency. Paramomycin was proved more effective than kanamycin for the selection of transformed cells (Montoro *et al.*, 2003). The influence of cryopreservation of explants, co-cultivation temperature, and duration of co-cultivation of the *Agrobacterium*-mediated genetic transformation of rubber callus was studied by the above group. It is reported that, the transformation efficiency and competence of the embryogenic calli were improved after two cycles of cryopreservation. When the co-cultivation temperature was reduced from 27 °C to 20 °C and the duration of this phase was increased up to 7 days, the GUS activity was increased (Blanc *et al.*, 2006).

2.2.2 Development of transgenic plants for environmental stress tolerance

After developing efficient plant regeneration protocols through somatic embryogenesis, RRII has initiated active research for the development of transgenic plants integrated with genes for desirable agronomic traits. An efficient *Agrobacterium*-mediated genetic transformation system for transgenic plant regeneration has been developed in the recent past (Jayashree *et al.*, 2000, 2003; Sobha *et al.*, 2003b). Initial focus was to develop transgenic plants tolerant to abiotic stresses like elevated light and temperature, drought, and TPD. In nature, plants encounter a wide range of environmental stresses that detrimentally affect their growth and development. Plants exposed to environmental stress generate excess reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) (Asada and Takahashi, 1987). Superoxide dismutase (SOD) is the first enzyme involved in the detoxifying process of reactive oxygen species (Fridovich, 1986). Significant yield loss occurred in plantations across the rubber-growing countries due to TPD, a physiological disorder, characterized by the browning of bark followed by the cessation of latex flow. Since, no pathogens are unequivocally proved as the causative organism yet, this is considered as a physiological disorder. It is reported that the TPD-affected bark shows an increased free radical accumulation and a reduced level of SOD enzyme as well as cytokinin (Das *et al.*, 1998). Therefore, attempts were made at RRII to develop transgenic rubber plants tolerant to a variety of environmental stresses as well as TPD, by incorporation of the genes coding for *SOD* and *ipt*.

Agrobacterium-mediated genetic transformation was carried out with *Hb.SOD* gene under the control of CaMV 35S promoter. Two-month-old immature anther calli were used as the initial explants in the transformation experiments. The plasmid used was pDU96.2144, which contains *uidA* as the reporter gene and *npt*II as the selectable marker gene. Transgenic plants integrated with the *SOD* gene under the control of different promoters such as CaMV 35S and FMV 34S were developed separately from different cell lines obtained through independent transformation events. The stable integration and copy number of the transgene were confirmed through molecular analysis (Jayashree *et al.*, 2000, 2003; Sobha *et al.*, 2003a). The preliminary biochemical studies conducted with SOD-transformed callus cultures showed enhanced activity of SOD and related enzymes such as catalase and peroxidase (Sobha *et al.*, 2003b).

Genetic transformation with *ipt* gene for the overproduction of cytokinin was also attempted. Rubber (Clone RRII 105) anther calli were transformed through *A. tumefaciens* (strain EHA 105) harboring the binary vector carrying the gene coding for *npt*II as the selectable marker gene, *GUS* as the reporter gene, and the sequence coding for *ipt*. The putatively transformed calli were able to grow hormone autotrophically and showed increased cytokinin levels compared with the controls. The embryos showed developmental abnormalities and most of the transformants were severely deformed. The constitutive expression of *ipt* gene by its promoter elevated the endogenous cytokinin levels, which might have enhanced ethylene production leading to phenotypic abnormalities hampering the plant development (Kala *et al.*, 2003).

2.2.3 Transgenic plants for enhanced rubber biosynthesis

Natural rubber is cis-1,4-polyisoprene. The biosynthesis of rubber from sucrose is through a

series of reactions catalyzed by various enzymes. Few enzymes involved in the conversion of acetate into rubber in rubber tree latex have been analyzed, which indicates that the activity of 3-hydroxy-3-methyl glutaryl Coenzyme A reductase (hmgr1) is low compared to the other enzymes (Lynen, 1969) suggesting that the constitutive level of this enzyme may be a limiting factor in rubber biosynthesis. Arokiaraj *et al.* (1995) overexpressed *hmgr*1 in transgenic rubber, where HMGR activities of transformed anther derived callus ranged from 70 to 410% of the value of wild-type control and the activity in the transformed embryos ranged from 250 to 300%.

In order to enhance the rubber biosynthesis, experiments were initiated at RRII to develop transgenic plants integrated with the genes coding for important enzymes involved in this pathway. Initially, the genes coding for hmgr1, farnesyl-diphosphate synthase (FDP) and rubber elongation factor (REF) were selected. The *hmgr1* and *REF* genes were cloned into the binary vector pBIB 121 for the genetic transformation which contains the antibiotic genes *npt*II and *hpt* (hygromycin phosphotransferase). The *FDP* gene was cloned into the binary vector pCAMBIA under the control of CaMV 35S promoter. Three *Agrobacterium* strains such as EHA 105, LBA 4404, and pGV 3101 were used for infecting the tissue for the transformation of *hmgr1* gene while EHA 105 alone is used for *FDP* and *REF* genes. Two-month-old calli were infected with different *Agrobacterium* strains carrying the above genes and the transgenic lines were selected. Transgenic embryos and few plantlets integrated with the *FDP* gene were regenerated. Further work in this direction is in progress at RRII.

2.2.4 Transgenic plants for recombinant protein production

For commercial production of diagnostic and therapeutic products, the pharmaceutical industry essentially depends upon microorganisms involving sophisticated bioreactors. Since plants are easy to maintain, require only sunlight, water, and agricultural inputs, they are cheaper compared to microorganisms involving bioreactors. As protein-manufacturing factories, they are also ecologically friendly. Production of diagnostic and therapeutic products using plant biotechnology has become well recognized in the field of pharmaceutical industry. Recently, various recombinant proteins have been expressed in crop plants making them living factories for the production of commercially valuable proteins (Nilesh *et al.*, 2004).

Natural rubber from rubber tree is continuously extracted through tapping. The rubber tree has many unique advantages for biopharming. Rubber tapping is a systematic and controlled wounding of the bark. The bark of the rubber tree contains a complex network of articulated laticifers or latex vessels, notably in the soft bark of the trunk from which rubber is collected. The latex is a cytoplasm that contains rubber particles, microvacuoles known as lutoids, and double-membraned organelles rich in carotenoids assimilated to plastids, the Frey-Wysling particles (Paradkooper, 1989). All the organelles of nonphotosynthetic plant cells: vacuoles (the lutoids), plastids, mitochondria, nuclei, and endoplasmic reticulum are also present (de Faiÿ *et al.*, 1989). It also contains various types of RNAs (Priyadarshan and Clemant-Demange, 2004), polysomes, and ribosomes as well as other ingredients required for the protein synthesis. It means latex contains all the machinery for the protein synthesis, if the desired gene is inserted into rubber plants.

The disadvantage of plants in general as biological factories for recombinant protein production is that the proteins may be accumulated in certain plant portions and the harvesting is mainly by destructive methods. After each harvest, it takes time for new growth to take place before the next harvest, making the harvest batchwise, rather than continuous processes. The ideal plant for recombinant protein production would be one that is cheaper to maintain and easy to multiply clonally, while allowing for continual harvesting of the protein (Arokiaraj, 2000). In rubber tree, latex harvesting is by nondestructive method and a continuous process (every alternate days or once in 3 days) throughout the year and the latex replenishment after each tapping is rapid. Moreover, once the tree is genetically transformed, the trait could be fixed in the T_1 generation itself with large-scale clonal propagation. Therefore, among the plants, rubber tree is having the unique advantage as the most suitable candidate for biopharming.

The expression of a recombinant protein, the HSA in the transgenic rubber tree was reported

for the first time by Arokiaraj *et al.* (2002b). An attempt was made to express the 68-kDa HSA protein in transgenic rubber. Transgenic rubber plants that secrete HSA in the serum fraction of rubber latex were generated using a binary vector pLGMR.*HSA* (13.8 kb) containing *HSA* cDNA (1.8 kb) with its native leader sequence in the *A. tumefaciens* (GV2260) containing a supervirulent plasmid (pToK 47). In this vector (pLGMR.*HSA*), the inserted *HSA* cDNA with its leader sequence is placed under the control of a CaMV 35S promoter. Rubber anther callus (clone GL1) was used as the explant for the infection using *Agrobacterium* GV2260. The presence of the inserted gene was confirmed by PCR using the primers of *npt*II and *HSA*. Detection of HSA in the leaf and latex extracts was carried out using an antibody coupled to a protein chip array. Western blot analysis was also done for the immuno detection of the HSA. An expression level in the latex serum of about 24 μg of HSA per milliliter of latex extract in young plants was also detected.

Yeang (2004) reported the expression of a functional recombinant antibody fragment in the latex of transgenic rubber. The plasmid for the genetic transformation contained the gene for a mouse immunoglobulin ScFv with specificity for the dental bacterium *Streptococcus gordonii*. The CaMV 35S promoter and the *nos* terminator regulatory sequences and the selectable marker *npt*II, together with the coding region of the tobacco pathogenesis-related protein pathogenesis related protein 1a (PR-1a) signal sequence regulated the expression of the recombinant antibody fragment. The gene construct was transformed into *A. tumefaciens* (GV 2850). The presence of the inserted genes in the putative transgenic plants was analyzed by PCR. The ScFv protein concentration detected in the latex was about 6 $\mu g\,ml^{-1}$ latex serum for the most productive plant.

Recently, attempts were initiated at RRII for the production of a recombinant TB antigen protein in rubber. The embryogenic calli derived from the leaf explants were transformed with the *A. tumefaciens* strain LB 4404 carrying the binary plasmid vector having the gene coding for a 10-kDa TB antigen and the *npt*II as the selectable marker gene under the control of CaMV 35S promoter. Co-cultivated tissues were transferred onto a selection medium containing kanamycin ($350\,mg\,l^{-1}$) and cefotaxime ($500\,mg\,l^{-1}$). High-frequency transformation was obtained and embryos were developed from the transgenic tissues on the modified MS medium with benzylamino purine (BA) ($2.0\,mg\,l^{-1}$), gibberellic acid A3 (GA3) ($1.2\,mg\,l^{-1}$), α-naphthalene acetic acid (NAA) ($0.2\,mg\,l^{-1}$) and 2,4-dichlorophenoxyacetic acid (2,4-D) ($0.1\,mg\,l^{-1}$). The gene integration was confirmed by PCR analysis. Work is in progress to develop plantlets (Kala *et al.*, 2006).

2.3 Methods of Genetic Transformation

Genetic transformation is a potential tool in different areas such as manipulation and understanding of the biochemical processes, knowledge of genome regulation, and integration of the genes, which is not feasible through conventional breeding. Different techniques for gene transfer into plant systems have been developed, which includes *A. tumefaciens*- and *A. rhizogenes*-mediated transformation, microprojectile bombardment, tissue and protoplast electroporation, polyethylene glycol (PEG)-mediated direct gene transfer to protoplasts, microinjection, and fiber-mediated transformation. The possibility of genetic transformation in rubber tree was first explored in 1991 by *A. tumefaciens*-mediated transformation of callus derived from *in vitro* and *in vivo* seedling cultures (Arokiaraj and Rahaman, 1991). The first transgenic rubber plant was produced by the integration of *GUS* and *npt*II genes into callus cultures by particle gun method. However, because of the efficiency and convenience, *A. tumefaciens*-mediated genetic transformation system remains as the most widely used method for rubber genetic transformation.

2.3.1 *Agrobacterium*-mediated gene transfer in rubber trees

2.3.1.1 A. tumefaciens—a natural vector

The natural capacity of the gram-negative soil bacterium, *A. tumefaciens* to introduce a segment of oncogenic DNA present in the tumor-inducing (Ti) plasmid makes it an efficient vector system in genetic transformation. In rubber, *Agrobacterium*-mediated genetic transformation has been used widely and effectively for genetic transformation

(Arokiaraj *et al.*, 1996, 1998; Jayashree *et al.*, 2003; Sobha *et al.*, 2003a; Kala *et al.*, 2003). Strains of *Agrobacterium* used commonly for transformation include C58, EHA101, EHA 105, LBA 4404, pGV2260, and pGV3850. These have been reported to show high efficiency in rubber genetic transformation. Presence of phenolic compounds in rubber cells might have contributed to the success of *Agrobacterium*-mediated transformation since this act as *vir* gene inducers aiding in transfer-DNA (T-DNA) transfer. In most of the reports on rubber genetic transformation using *Agrobacterium*, *npt*II has been used as the selection marker and qualitative marker is the commonly used GUS reporter. In recent reports on transformation, most vectors were found to avoid the qualitative markers since these were found to hamper plant regeneration.

2.3.1.2 Agrobacterium *protocols*

A detailed protocol of *Agrobacterium*-mediated genetic transformation developed at the RRII is described here (Figure 2). *Agrobacterium* strain EHA 101 was transformed with the binary vector pDU 96.2144, which contained *uidA* as the reporter gene, *npt*II as the selectable marker gene, and the 702-nucleotide *Hb.SOD* cDNA under the control of CaMV 35S promoter (Jayashree *et al.*, 2003). *Hb.SOD* gene under the control of FMV 34S promoter were also tried separately (Sobha *et al.*, 2003a). The bacteria harboring the vector was grown in the AELB medium overnight at 28 °C in the presence of 50 mg l^{-1} kanamycin and 20 mg l^{-1} gentamycin until an A_{600} of 0.5. The bacterial cell density was adjusted to 5×10^8 cells ml^{-1} in this medium and used for tissue infection.

Approximately 2.0 g of calli were precultured on a callus proliferation medium prior to infection with *Agrobacterium*. The explants were immersed in the *Agrobacterium* cultures for 10 min and were blotted dry in a sterile filter paper and transferred to a co-cultivation medium (MS + acetosyringone 20 mg l^{-1} + glycine betaine·HCl 153.6 mg l^{-1} + proline 113 mg l^{-1}). After 3 days of co-culture the explants were subcultured on a selection medium containing 500 mg l^{-1} cefotaxime and 300 mg l^{-1} kanamycin for 50 days and maintained at 25 ± 2 °C in the dark. After 8 weeks of

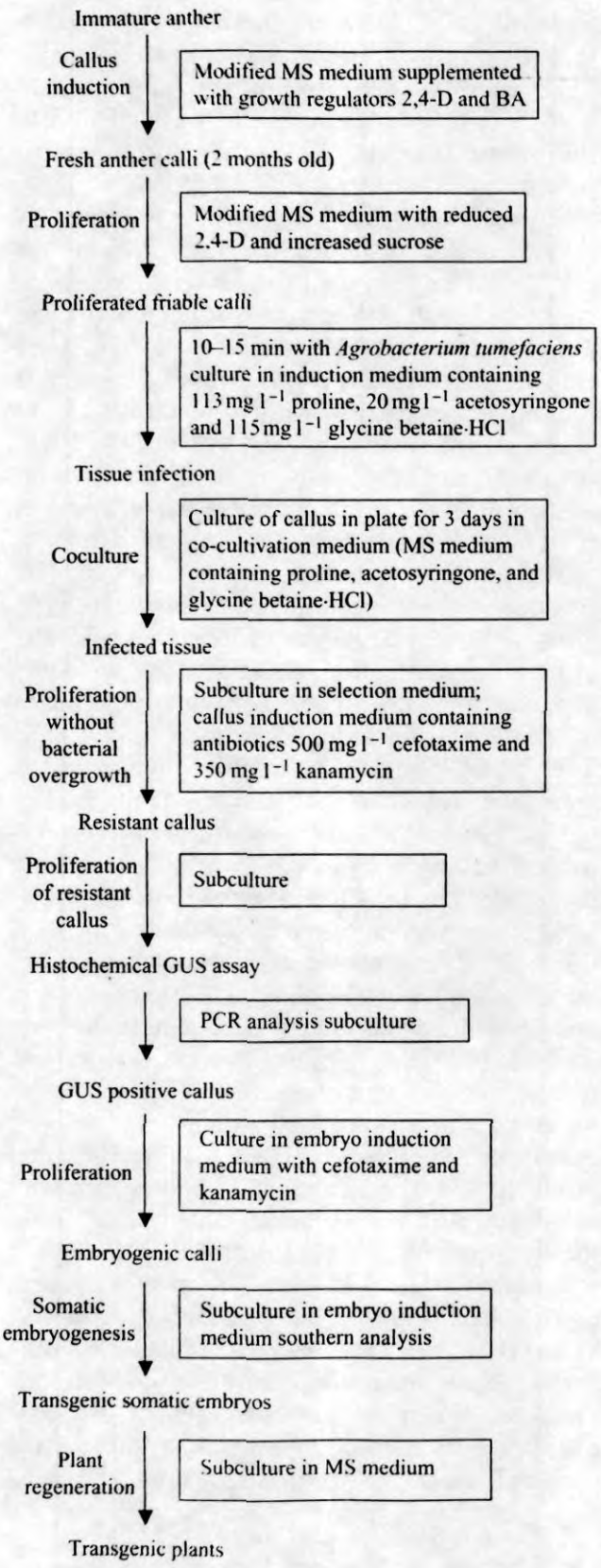

Figure 2 Flow chart of *Agrobacterium*-mediated genetic transformation and plant regeneration protocol in rubber

culture, kanamycin-resistant callus lines were subjected to histochemical GUS assay according to Jefferson (1987). The transformed cells were incubated overnight at 37 °C in 2 mm X-gluc (5-bromo-4-chloro-3-indolyl β-D glucuronide) in phosphate buffer (pH 7.0) containing 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 0.1% (v/v) Triton X-100, and were observed under microscope.

The transformed callus lines were subcultured into a fresh medium for proliferation. After 2 months of culture, the embryogenic calli obtained were subcultured to an embryo-induction medium. The gene integration in the transgenic plants was confirmed by PCR analysis and Southern hybridization. The different hybridization patterns obtained for the transgenic plants indicate random integration and multiple insertions of the T-DNA in the genome of the plants.

In order to understand the effect of the physiological state and sources of explants on transformation efficiency, Rekha *et al.* (2006) examined six different explants, i.e., intact explants (immature anther, ovule), 2-month-old callus and embryogenic callus of the anther and ovule for *Agrobacterium* infection. Results suggested that the target tissue is one of the important factors that determine the transformation efficiency. Among the different explants used, maximum transformation frequency (62%) was obtained with the embryogenic callus of the anther. Similar results were also observed when embryogenic callus derived from leaf explants was used for *Agrobacterium* infection (Kala *et al.*, 2006). A detailed investigation has been carried out by Montoro *et al.* (2000) on the effect of exogenous calcium on *Agrobacterium*-mediated gene transfer in rubber friable callus. It is demonstrated that the exogenous $CaCl_2$ reduces the efficiency of *Agrobacterium*-mediated gene transfer. The use of Ca-free media, both in bacteria and plant tissue preparations, dramatically increased the number of transformation events and consequently the positive effect of acetosyringone on transformation efficiency. Further, this group has done extensive studies to improve the transformation efficiency by manipulating the competence of the explants and the co-cultivation temperature (Blanc *et al.*, 2006). They observed a drop in transformation efficiency as the age of callus increases. Reducing the temperature and lengthening the co-cultivation period with the *Agrobacterium* suspension increased the transformation frequency significantly. Rattana *et al.* (2000) reported that co-cultivation at 20 °C slowed down the *Agrobacterium* proliferation and callus browning by which duration of co-cultivation could be extended. In that way, contact between the plant cells and the bacteria was favored, making gene transfer more efficient (Blanc *et al.*, 2006).

2.3.2 Biolistic transformation system

Gene delivery into intact tissues by DNA-coated microprojectiles allows genetic transformation of several recalcitrant species. Usually, this technique results in transient gene expression and chimera formation. Effective gene transfer using this system is found to be influenced by several factors. Nature and size of particles and target tissues usually influence gene expression. Biological factors such as growth stage and media supplements that help cell survival are also detrimental. Acceleration of a microcarrier was achieved by a shock wave generated from gunpowder charge or sudden release of compressed air. In rubber, this technique was used by Arokiaraj *et al.* (1994) for transforming anther-derived callus with vectors harboring the *gus* gene, *npt*II, and *cat* gene. Plasmid DNA was precipitated on to tungsten particles, loaded on to a microprojectile, and accelerated toward the target placed 5 cm below the stopping plate with a biolistic particle gun. The calli were then dark incubated for 24 h after which they were transferred to the incubation medium containing antibiotics from which kanamycin-resistant transformants were obtained. Optimization of parameters that would influence DNA delivery such as microprojectile velocity, coating mixture, and particle dispersal were carried out, which proved that microprojectiles can deliver DNA into rubber cells and helped recovery of kanamycin-resistant transformants.

2.4 Regeneration of Whole Plant

An efficient plant regeneration pathway through somatic embryogenesis is an essential prerequisite for crop improvement through transgenic approaches besides using this as a micropropagation

system (Thulaseedaran *et al.*, 2004). Wang *et al.* (1980) and Wan *et al.* (1982) successfully regenerated rubber plants through somatic embryogenesis from anther walls. Carron (1981) used inner integument tissue of seeds for somatic embryogenesis and was successful in plantlet development. Extensive experiments were carried out by many researchers to enhance the frequency of somatic embryo induction and plant regeneration. Studies were also conducted to optimize cultural conditions, nutritional and hormonal requirements during somatic embryogenesis. The parameters include the effect of polyamines (El Hadrami *et al.*, 1989), hormone balance (Michaux-Ferriere and Carron, 1989), water status of the medium and explant (Etienne *et al.*, 1991a), mineral and carbohydrate nutrition (Etienne *et al.*, 1991b), interaction of growth regulators, sucrose, and calcium on callus friability (Montoro *et al.*, 1993, 1995), role of sucrose and ABA on embryo induction (Veisseire *et al.*, 1994a, b; Cailloux *et al.*, 1996; Linossier *et al.*, 1997), and carbohydrate types (Blanc *et al.*, 2002). In spite of all these studies, the plant regeneration frequency remains very low. In most of the above studies inner integument tissue was used as the explant. It is reported that the calli obtained from the integuments of immature seeds frequently display browning leading to tissue degeneration and a loss of embryogenic competence (Housti *et al.*, 1991).

There has been a renewed interest in rubber for development of techniques for plant regeneration through somatic embryogenesis especially for use in genetic transformation (Kumarijayasree *et al.*, 1999). In order to identify the suitable explant source, a variety of explants such as leaf, tender shoots, integumental tissues of immature fruit, immature anther, immature inflorescence, etc., were tried at the RRII. Extensive optimization experiments were carried out to improve the plant regeneration efficiency through somatic embryogenesis for the Indian clones of rubber. Attempts were also made to standardize the optimum growth regulator concentration and the balance between different growth regulators, the nutritional requirements, and the physical factors such as light and temperature for maximum callus proliferation, embryo induction, and subsequent regeneration of normal and healthy plantlets. As a result, initially immature anthers (before microsporogenesis) (Kumarijayasree *et al.*, 1999), immature inflorescence (Sushamakumari *et al.*, 2000) and leaf (Kala *et al.*, 2005, 2006) were identified as suitable explant source and protocols were developed for high-frequency somatic embryo induction and plant regeneration for RRII-105, the most popular Indian rubber clone. Kumarijayasree *et al.* (1999) reported a standardized protocol for the induction of friable embryogenic callus, somatic embryogenesis, and further plant regeneration from the immature anthers. Optimum callus induction was obtained in the modified MS medium supplemented with 2.0 mg l^{-1} 2,4-D and 0.5 mg l^{-1} kinetin. Somatic embryo induction was found to be better with 0.7 mg l^{-1} kinetin and 0.2 mg l^{-1} NAA. Further development of the embryos into plantlets was achieved on a hormone-free medium (Figure 3).

Culture conditions and other nutritional requirements for improving the efficiency of somatic embryo induction and germination were investigated by Kumarijayasree *et al.* (2001). Immature anthers precultured in a liquid medium for 10 days followed by 25-day culture in a solid medium were found to promote callus induction. The embryo induction efficiency was promoted by supplementing 200 mg l^{-1} glutamine and 400 mg l^{-1} casein hydrolysate in the embryo-induction medium. Dark incubation favored callus induction and proliferation as well as induction of embryogenesis, whereas plantlet regeneration was found to be light dependent. A study on the gibberellic acid requirement on embryo induction and maturation revealed that incorporation of GA3 up to 2.0 mg l^{-1} increased the embryo-induction frequency. Germination percentage was also significantly enhanced by higher concentrations; however, further plant development was affected by increasing GA3 levels (Kumarijayasree and Thulaseedharan, 2004). A detailed investigation was also done on the response of various cytokinins such as BA, zeatin (ZEA), kinetin and thidiazuron (TDZ) on germination of somatic embryos derived from immature anther explants. TDZ was proved to be superior to BA and ZEA while kinetin showed the least response. Maximum percentage of embryo germination and plantlet regeneration was 80% and 82%, respectively, when the medium was supplemented with TDZ (Kumarijayasree and Thulaseedharan, 2005).

Sushamakumari *et al.* (2000) studied the role of sucrose and abscisic acid (ABA) on embryogenesis

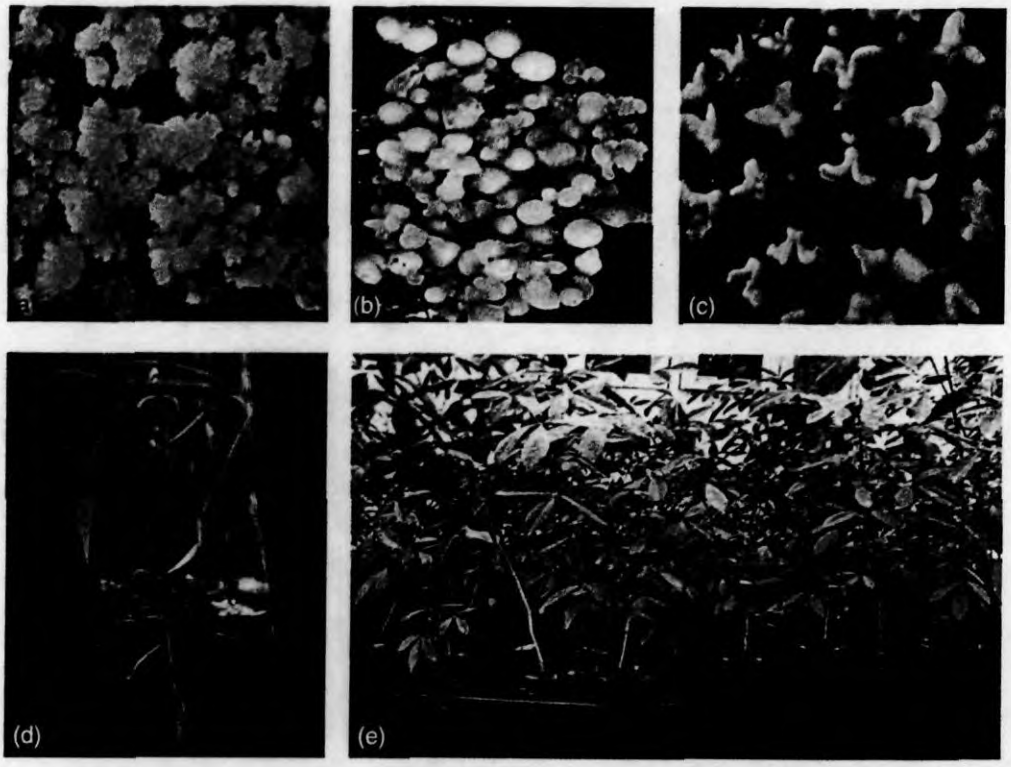

Figure 3 Somatic embryogenesis and whole plant regeneration from immature anther explants of *H. brasiliensis* (RRII 105 clone): (a) embryogenic callus; (b) globular embryos; (c) bipolar embryos; (d) full-plantlet development; and (e) somatic embryo-derived plants were established in poly bags and ready for field planting

from immature inflorescence-derived explants. A higher sucrose level was found to be essential for effective embryo induction as well as maturation. However, lower level was found to be beneficial for plant regeneration. Further, efforts have been made to enhance the embryo induction and plant regeneration frequency by the manipulation of the nutrients and hormonal combinations. Recently a system was also standardized for plant regeneration from leaf tissues of rubber where the explants are available throughout the year. Callus induction could be obtained in the MS medium with enhanced calcium nitrate (850 mg l^{-1}) and supplemented with casein hydrolysate (1.0 mg l^{-1}), B_5 vitamins, sucrose 20 g l^{-1}, and phytohormones BA (1.0 mg l^{-1}) 2,4-D, (1.5 mg l^{-1}), and NAA (0.2 mg l^{-1}). Embryo induction was obtained in the modified MS medium by the addition of amino acids, glutamine (300 mg l^{-1}), proline (100 mg l^{-1}), and arginine (37 mg l^{-1}), and organic supplements like casein hydrolysate and coconut water with phytohormones, 2.0 mg l^{-1} BA, 1.0 mg l^{-1} GA_3, 0.2 mg l^{-1} NAA and 0.1 mg l^{-1} 2.4-D. Maturation and apex induction of embryos could be obtained in woody plant medium containing coconut water 10%, malt extract 100 mg l^{-1}, casein hydrolysate (400 mg l^{-1}) and hormones BA (0.3 mg l^{-1}) and GA_3 (1.2 mg l^{-1}). The hormone-free 1/2 MS medium helped plant regeneration (Figure 4). All media except callus induction needed the presence of activated charcoal (Kala *et al.*, 2005, 2006).

With minor modifications, the transgenic rubber callus integrated with different genes could be regenerated. At RRII protocol for the regeneration of transgenic plants integrated with the *SOD* gene under the control of different promoters were developed (Jayashree *et al.*, 2003; Sobha *et al.*, 2003a). The proliferation of the transgenic callus and embryo induction could be obtained as reported earlier (Kumarijayasree *et al.*, 1999). A combination of ABA (0.1 mg l^{-1}) and phytagel (0.4%) promoted the frequency of embryo induction. A high sucrose level was beneficial for both embryo induction as well as maturation in rubber. Addition of organic supplements and polyamines played a significant role in the induction and maturation of the embryos. Inclusion of spermine (2.0 mg l^{-1}) in the embryo-induction medium had

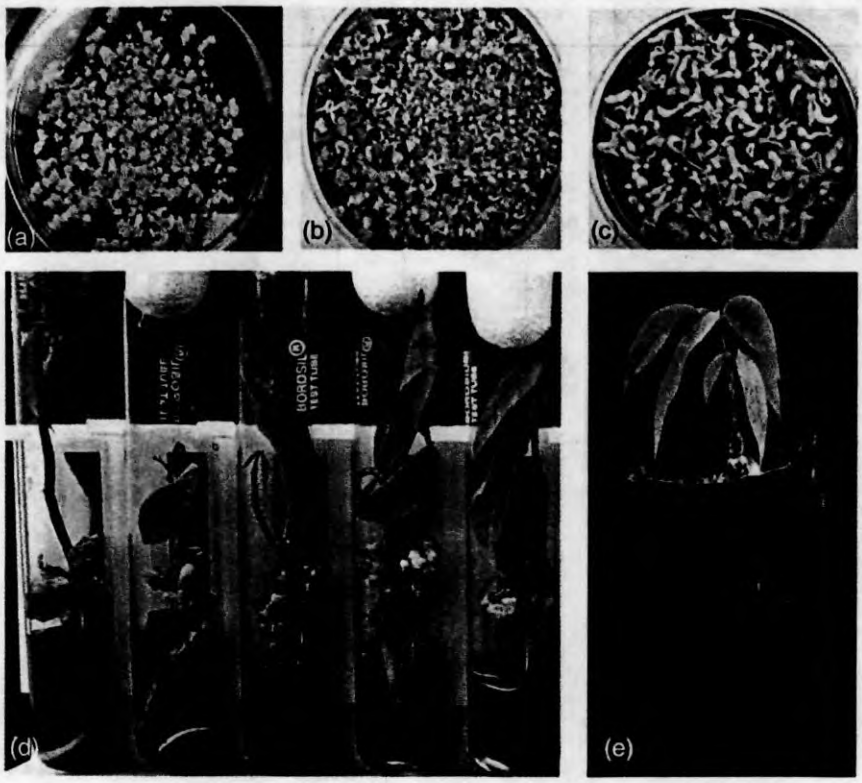

Figure 4 Somatic embryo induction and plant regeneration from leaf explants of RRII 105 clone of *H. brasiliensis*: (a) callus initiated from leaf explants; (b) embryogenic callus with embryos; (c) bipolar embryos; (d) development of full plants; and (e) a plantlet established in poly bag

a positive effect on embryogenesis. Although, casein hydrolysate (200 mg l^{-1}) was good for the embryo induction, maturation was favored by the addition of 150 mg l^{-1} banana powder. Addition of amino acids like glutamine and proline influenced the maturation frequency dramatically. Plant regeneration was promoted in the medium with reduced levels of sucrose (20 g l^{-1}) and phytagel (0.2%) (Figure 5).

3. FUTURE ROAD MAP

3.1 Expected Improved Products through Genetic Engineering

Biotechnology would play an important role in the future of the rubber industry. Conventional rubber breeding is a long process involving various methods such as selection of population followed by hybridization, progeny evaluation, and backcrossing. It takes more than 25 years to release a new clone by using any of these methods. Rubber tree is a good candidate for transgenic manipulation due to the long breeding cycle and heterozygous nature. *In vitro* techniques including plant regeneration via somatic embryogenesis have been established in this tree species. Thus, the basic technology for genetic manipulation of rubber plant at the cellular and molecular levels is available, making rubber a suitable crop for genetic engineering. Many agronomic traits could be considered for a rubber biotechnology program, namely, high yield potential, tolerance to TPD, tolerance to environmental stresses and diseases, production of recombinant proteins, improvement in wood quality, etc.

3.1.1 Enhancement of latex yield in transgenic rubber trees

As in the case with most other crops, rubber biotechnology has focused much effort on

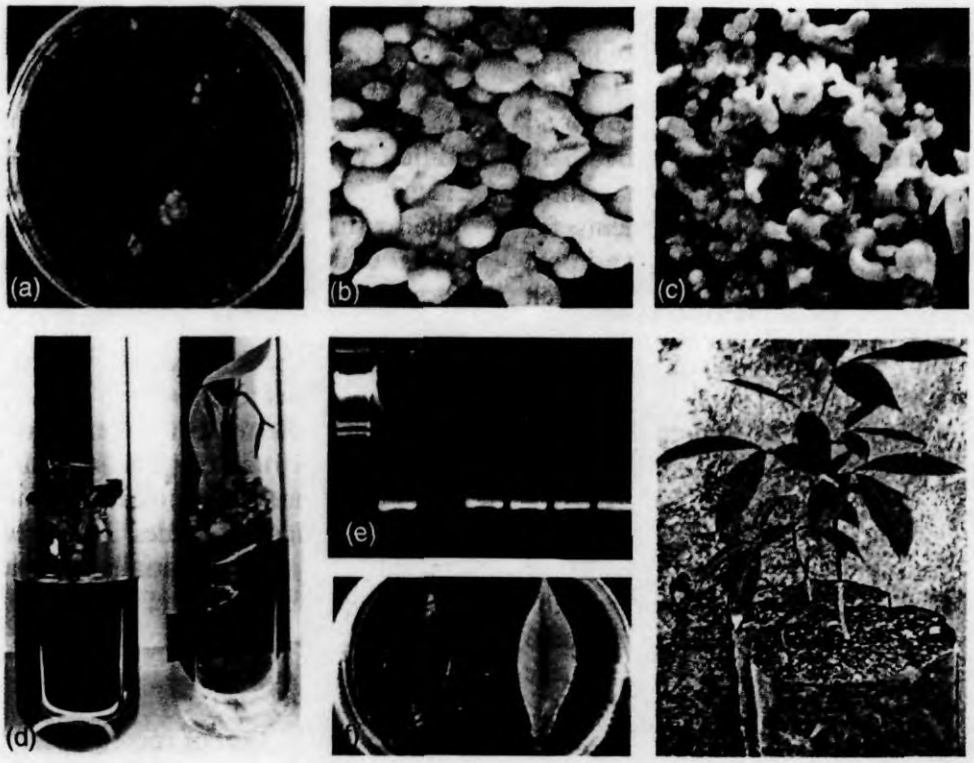

Figure 5 Development of *Hevea* transgenic plants for the overexpression of *Hb.SOD* gene using immature anther-derived callus as initial explants: (a) emergence of new transgenic callus lines on selection medium containing kanamycin (350 mg l^{-1}) and cefotaxime (500 mg l^{-1}); (b) globular embryos from transgenic callus; (c) putatively transformed bipolar embryos; (d) germination of transgenic plants; (e) PCR confirmation of transgenic plants using *npt*II gene-specific primers; (f) histochemical GUS expression analysis; and (g) transgenic plants growing in poly bags

increased latex yield. In any crop improvement program, the overall objective is to increase yield and adaptation to environmental conditions. To meet this objective, the genetic variation must be exploited by conventional breeding methods. The major limitation to crop improvement is likely to be the availability of germplasm from which desirable genes can be readily introgressed into the plant of interest. However, major improvements have been made over the last century in the productivity of rubber, as the yield of dry rubber per acre have been increased significantly by releasing better clones. There is no doubt that the conventional breeding will continue to increase the latex production in rubber trees, but a stage would eventually come when the rate of rubber biosynthesis with the tree becomes the limiting factor (Arokiaraj, 2000). At this stage, latex yield will be enhanced only by treatments designed to affect the rate of rubber biosynthesis. It was reported that the constitutive level of hmgr1 enzyme may be a limiting factor in rubber biosynthesis. On the basis of this hypothesis, Arokiaraj *et al.* (1995) initiated genetic transformation experiment to overexpress *hmgr1* in rubber, where hmgr1 activity of transformed callus ranged from 70 to 410% more value of wild-type control and the activity in transformed embryos obtained ranged from 250 to 300%. However, they failed to produce transgenic plants. Among the latex associated proteins, the amount of REF in the whole latex is proportional to the rubber content (Priya *et al.*, 2006). As such, if REF protein amount is shown to be correlated simultaneously with rubber yield, there is a possibility that this relationship could be used to select for clones that are able to sustain high rubber biosynthetic efficiency. It could be possible to enhance rubber yield by overexpression of *REF* gene involved in rubber biosynthesis. At present, the authors' laboratory had undertaken a research project in which rubber biosynthesis genes such as *hmgr1*, *FDP*, *REF*, and *cis-prenyl transferase* were cloned into binary vectors and

transformation works are in progress to develop transgenic plants.

3.1.2 Transgenic plants for TPD tolerance

Plant stress is one of the major problems for crop production. In rubber tree, TPD syndrome is considered to be a physiological disorder, which greatly affects latex yield. Once the TPD occurs, the tapping incision is partly or entirely blocked and the amount of latex production is significantly decreased or stops completely. The incidence of TPD occurs in 12–50% of rubber trees in almost every rubber-planting country. Under normal conditions, active oxygen species (AOS) are efficiently scavenged by detoxifying enzymes such as SOD. Nevertheless, during TPD stress conditions this defense system becomes saturated and cellular damage is inevitable (Chrestin, 1989). The presence of excessive AOS results in severe latex yield losses. It has been reported that SOD activity protected plants from oxidative and other stresses. In order to overcome this TPD problem, an attempt was made to overexpress *SOD* gene in transgenic rubber plants. Transgenic rubber plants have been successfully developed and established in soil at the RRII (Jayashree *et al.*, 2003). Further evaluation is in progress.

3.1.3 Transgenic trees with improved wood quality

Until now, the most important product of the rubber tree was its latex and considerable efforts have been made to improve the latex yield. With the depletion of tropical forests leading to a shortage of timber for many industrial and engineering uses, attention has moved on rubber wood as an alternative source of timber for markets (Arokiaraj *et al.*, 2002b). As the composition of wood is important for the pulp industry, genetic engineering to modify lignin content in rubber is a very active area of research that has been stimulated in recent years by the characterization of important genes controlling lignification. The export of rubber wood from Malaysia rose from RM 900 million in 1993 to RM 3.7 billion in 1998 and subsequently to RM 5.2 billion in 2001 (Arokiaraj *et al.*, 2002b). These figures clearly indicate how rubber wood industry is growing. For tree species, only limited information is available about the process of differentiation and development that are involved in wood formation. Knowledge of what determines the pathway of differentiation that cambium cells undergo is essential to any attempt to design better wood characteristics and improve latex yield. In rubber tree, the homeobox (*HB*) gene has been isolated and it is presumed that *HB* genes may be involved in differentiation of cambium cells to form latex vessels (Arokiaraj *et al.*, 2002b). Research in this direction is important because it clearly demonstrates that modifying specific genes in the wood-forming process can also potentially influence important tree characteristics and hence improve timber production.

3.1.4 Production of recombinant proteins in transgenic rubber plants

Currently, numerous immunotherapeutic proteins, antibodies, and vaccines have been produced. Recent work suggests that plants will be a facile and economic bioreactor for large-scale production of industrial and pharmaceutical recombinant proteins (Kusnadi *et al.*, 1997). Genetically engineered (transgenic) plants have several advantages as sources of proteins compared with human or animal fluids or tissues, recombinant microbes, transfected animal cell lines, or transgenic animals. These include the following: (1) efficiency of the transformation events at large scale; (2) correct assembly of multimeric antibodies (unlike bacteria); (3) increased safety, as plants do not act as hosts for human pathogens, such as human deficiency virus (HIV), prions, and hepatitis viruses; (4) production of raw materials on an agricultural scale at low cost; and (5) reduced capitalization costs relative to fermentation technology. Other than latex and timber, useful products such as serum proteins, sugars, lipids, carotenoids, inositols, organic acids, and minerals in the latex can be exploited profitably with a new research strategy. Commercial-scale production of recombinant proteins from rubber plants will also benefit from the technology and equipment

commonly used in the food and beverage industry. Already, HSA proteins were expressed in a transgenic rubber plant (Arokiaraj *et al.*, 2002b). Rubber trees synthesize enormous volume of latex upon tapping and the trees could be exploited without any destruction for large-scale production of the foreign protein throughout the year. Depending upon the promoters used, transgenic proteins will be sequestered throughout the plant or in specific parts of the plants or specific organelles within a given plant cell. A laticifer cell-specific hevein promoter has been reported earlier (Pujade-Renaud *et al.*, 2005). In this respect, the RRII has been working on isolation of laticiferous specific promoters, which will eventually enhance the recombinant protein production in transgenic rubber trees. Recently, laticiferous-specific promoter sequences for rubber elongation factor (Priya *et al.*, 2006), hevein (Saleena *et al.*, 2006), β-1,3-glucanase (AY325498), and hmgr1 (DQ785798) have been isolated and characterized. Further work is in progress to develop transgenic plants for recombinant protein production as well as for enhanced rubber production by inserting the appropriate genes under the control of laticifer cell-specific promoters.

3.2 Risks and Concerns

The introduction of transgenic crops into the existing natural system has generated a number of questions about possible negative consequences. The issues on transgenic plants can be of various groups of concerns as delineated below.

3.2.1 Damage to human health and natural environment

The possibility that we might see an increase in the number of allergenic reactions to food as a result of genetic engineering has a powerful emotional appeal. Since rubber is not used as food crop, there is no such food safety issue with transgenic rubber plants. Gene flow from transgenic crops to others requires the following: (1) the presence of sexually compatible wild relatives close to the crop; (2) an overlap of flowering times between the crop and wild relatives and the presence of a pollinating agent such as a bird or an insect unless the likelihood that transgenes spread can be different for each crop and wild combination in different area of the world. If pollen grains from transgenic rubber plants are released in these areas they do not encounter any compatible plants to pollinate and therefore the risk of gene flow is remote. Also rubber-planting materials are developed via bud grafting, which further reduces the risk.

3.2.2 Concerns about damage to current farming practices

Hybridization of transgenic crops with nearby conventional crops raises concerns on several fronts. Movement of pollen from a transgenic field to a conventional field involves farmers in discussions about the distance required between fields to ensure purity of a crop, and about who must pay if unwanted genes move into a neighbor's crop. It will be important to ensure that hybridization is not occurring in the field. Many agencies publish recommended minimum isolation distances for a variety of crops. These distances have been decided to maintain a level of purity that has been acceptable to the agricultural community in the past. When there is a danger of gene flow to nearby fields, it is possible to prevent contamination of nearby crops by planting tall barrier plants to physically block the flow of pollen. If genetically modified (GM) pollen pollinates plants in neighboring field, then the issue of genetic trespass may arise. These issues have already prompted several lawsuits and they will continue to be a factor in the development and use of transgenic plants for years to come. As far as transgenic rubber plants are concerned, it takes about 6 years for first flowering, so the crop suitability can be assessed before pollen production. The main objective of rubber transformation is to produce plants for enhanced latex yield, TPD tolerance, disease resistance, and recombinant protein production. Also, the ultimate product from transgenic rubber plant is latex with protein and it is to be purified from latex, so neither the inserted gene nor the selection marker gene is thus available to the consumer.

3.3 Intellectual Property Rights for Transgenic Rubber Plants

The historical context of overincreasing protection for plant innovations, coupled with ongoing efforts to achieve international harmonization, provides an optimistic backdrop to some of the uncertainties that surround present efforts to obtain strong intellectual property protection for the full scope of innovations in the area of transgenic plants. An important objective of intellectual property law is to provide a reliable framework of rules, so that important commercial decisions about investigations in research may be made with a degree of certainty about how the products of the research will be protected. On the basis of this requirement for predictability, intellectual property laws are typically drafted in general terms and applied to new technology on the basis of established principles.

The requirements for patentability are generally judged with respect to the claimed invention. In addition, an invention must be within the categories of subject matter deemed patentable in a particular jurisdiction. There is also a generally recognized requirement that the description of an invention in a patent must be provided to allow a skilled technical person to make and use the invention, without the need for further innovation. Also a patentee is free to define the invention broadly in the claims and to cover various aspects of the invention, provided the requirements for patentability can be met with respect to each claimed aspect of the invention. To be new, an invention generally must not previously have been made available to the public, either through human activities or by virtue of its occurrence in nature. Many inventions are combinations of pre-existing knowledge in a way that yields an unexpected and hence patentable result. Typically, genetic inventions must be carefully characterized in claims in ways that distinguish them from a naturally occurring product. Genomic innovation in the filed of transgenic plants may include genes that are novel in the sense that they have not previously been identified, genes that are novel only in the sense that their function has not previously been identified, new combinations of genetic material, such as a recombinant gene made up of a tissue-specific promoter, and a coding sequence with which it would not normally be associated with expressed sequence tags. The exclusive right conferred by a patent in most jurisdictions includes the right to exclude others from making, using, and selling the invention defined by the claims for the term of the patent. Generally, patents have a term of 20 years from the filing date of the patent application. The innovation protectable under a plant patent can be seen as the combination of a three-step process: (a) cultivation or discovery of the plant, (b) identification of the new and distinct characteristics of the plant, and (c) asexual reproduction of the plant. Patents clearly offer the most flexible form of intellectual property protection for transgenic plants. Currently, RRII has undertaken various projects for development of transgenic plants and expected to produce soon. Once the transgenic plants are available then efforts will be taken to protect those transgenic plants intellectual property rights (IPR) by filing patent.

3.4 Public Perceptions of Transgenic Rubber Plants

Transgenic plants will continue to be developed and grown in the future, provided that a number of social and political constraints can be overcome. To understand public perceptions about transgenic plants, it is helpful to understand public perception of technology and educators have long been interested in how people perceive and understand risks associated with health and environmental issues (Wilson and Crouch, 1987). Risk perception must be considered in its social and cultural context. Many problems associated with risk management and communication result from the differences between scientists and the public (Freudenburg, 1988). The technical concept of risk is too narrow and ambiguous to serve as the crucial yardstick for policy making. Public perceptions, however, are the product of intuitive biases, economic interests, emotions, and cultural values. Technical risk must, therefore, be viewed in combination with psychological, social, and cultural processes that can heighten or reduce public perceptions of risk.

GM ingredients in food are perceived as more risky because they are considered to pose an involuntary risk. Many people believe they have very little control over food production

and processing. Furthermore, people are often concerned with secondary effects that the experts are unable to assess. Because political leaders have many of the same perceptions as other citizens, they are likely to base policy decisions on subjective factors as well. Public perception of risk is also influenced by public attitudes toward science and technology in general (Freudenburg, 1988). These problems are particularly serious as related to agriculture, because many people are no longer personally familiar with farming. Most have little understanding of how food is produced. If people are genuinely interested in a subject, such as biotechnology, they talk about it. The surveys in Europe, Canada, and the United States asked consumers to evaluate different applications of biotechnology (Hoban and Katic, 1998). The results are actually quite encouraging. The opponents of biotechnology in Europe have had the chance to tell their side of the story for several years without much balance. Educational efforts are starting to take hold among European leaders and consumers. Overall, we need to increase consumer understanding of food production and processing. However, the products from transgenic rubber plants are not consumed directly as food so there is no risk factor involved. Therefore, it could be easily explained to public how their understanding about transgenic rubber products.

3.5 Industry Perspectives

The success of GM organisms (GMOs) in the fresh-produce market in the world hinges on several factors: (1) the existence of a benefit from any GMO that is evident or can be made evident to the buyer; (2) the education of the buyer (whether a trade buyer or a consumer) about the development (process) and benefits of GMOs (this included the labeling debate); and (3) successful interaction with opponents of GMOs. The ultimate buyer is the consumer, but there are many buyers along every distribution chain. In the food business, seed companies buy materials they need to produce their seeds. Growers buy seeds and plants from the seed companies. Genetic engineering offers many potential benefits to the producer including the ability to grow a crop in hostile conditions, time savings, economic savings, economic gain, and environmental benefits. Where the growers see that the benefit of a GMO is worth it, he will pay more for it: (1) genetically engineered drought resistance would allow production in hostile climates as well; (2) genetically engineered disease resistance can permit production in areas infested with a given disease and can reduce crop protection inputs, savings the grower money, time, and environmental impact; and (3) genetically engineered rubber plant for recombinant protein production would give additional income to the growers along with rubber.

Consumers may see some of the on-farm benefits as beneficial to society at large and a certain percentage of them might pay more for a product that can be grown with fewer crop protection chemicals. The produce industry, as any other industry, reacts to its customer's actual or potential desires. The desire for greater availability of produce led to breeding new clones that can be cultivated for high yield potential. Growers and researchers have worked to bring value-added products to the market place. Genetic engineering allows breeding to take a huge step toward specificity in trait exchange: whether the genetic enhancement benefited the industry or consumers, labeling was an issue. Labeling on the marketing side was something else entirely. It became a major consumer issue and any major consumer issue usually surfaces as a major trade issue. However, rubber is not consumed as food, so there is no such issue to the industry. When genetically engineered rubber trees are in the small-scale trial, it is quite possible that consumer issues may be discussed before entering into market.

3.6 Political and Economic Consequences

Biotechnology is increasingly affecting the competitive base for much of that industry. As with most revolutionary technological changes, biotechnology has generated both economic and political responses. The new technologies have fundamentally altered the innovation process itself. The advent of biotechnology did two things: first, it created the potential to target the research more finely to specific market needs; second, it made the research process far more complex, with no one individual or small group of individuals able to undertake the entire process. The fragmentation of the innovation system into different structures of knowledge development and

use has fundamentally affected the economics of scale and scope in the industry. Most of the fundamental patentable technologies developed have come from desperate research programs around the world and have then been assembled and commercialized by private companies. The introduction of biotechnology has also participated in a major industrial restructuring in the agrifood sector. The opportunity presented by biotechnology to manage the research process to deliver custom products has created investment potential for some private companies while creating a threat or risk to many existing business. However, rubber is produced mainly from a few Asian countries and then traded globally. As a result, there is increasing potential that traditional net exporters may both export and import. All these trends in economics of scale and scope, product attributes, and consumer demand increase the dependence of the industry on international trade. The fundamental economic changes underway in the rubber sector are creating both winners and losers. Economic studies done over the years show that research in rubber has yielded relatively high private returns and even higher public returns but that farmers tend to get a smaller share of the returns on innovations that improve yield rather than quality and their share is depressed when the related processing sector is imperfectly competitive.

Economic change often is precipitated by or compels political responses. This is especially true for changes in the rubber sector, which is traditionally viewed as a politically important constituency that produces a strategic rubber of value as a geopolitical tool. The most significant government response to the introduction of biotechnology was the extension of intellectual property rights to processes and products of biotechnology. Governments around the world have been urged to regulate biotechnology-based research and production. However, rubber biotechnology research is in the infant stage and it will take more time for practical reality to trigger political responses.

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