Functional Plant Science and Biotechnology ©2007 Global Science Books

Current Perspectives on Application of Biotechnology to Assist the Genetic Improvement of Rubber Tree (*Hevea brasiliensis* Muell. Arg.): An Overview

P. Venkatachalam[1,2*] • P. Kumari Jayasree[2] • S. Sushmakumari[2] • R. Jayashree[2] • K. Rekha[2] • S. Sobha[2] • P. Priya[2] • R. G. Kala[2] • A. Thulaseedharan[2]

[1] Plant Molecular Biology Lab, Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN 47907, USA
[2] Biotechnology Division, Rubber Research Institute of India, Kottayam-686009, Kerala, India

Corresponding author: * **pvenkat67@yahoo.com, venkat@rubberboard.org.in**

ABSTRACT

Hevea rubber clones are intensively cultivated in tropical regions of the world as main sources for Natural Rubber (NR) production which is one of the important raw materials for many industries. However, the heterozygous nature, long breeding time of trees and the prolonged period required for evaluation of mature traits are strong limitations for conventional breeding and selection methods. The development of methods for *in vitro* culture and genetic engineering has increased the possibility of producing rubber genotypes with improved latex yield, tolerance to tapping panel dryness (TPD) syndrome, growth rate and wood quality or reduction in undesirable traits. The combination of conventional breeding and molecular techniques will help to develop rubber trees with positive effects on the environment. However, the risks associated with the biotechnological applications should be carefully evaluated and field trials are to be performed with transgenic rubber tree. Genomic technologies were taken up by various research groups working on *Hevea* to identify new targets for breeding and/or complementary genetic transformation. In addition, molecular markers can provide simultaneous and sequential selection of agronomically important genes in *Hevea* breeding programs and effectively replace time consuming bioassays in early generation screens. With the advent of molecular techniques, several genes involved in rubber biosynthetic pathway have been characterized. Both HMGR and HMGS are essential enzymes involved in early steps of rubber biosynthesis. Among the genes identified, *REF* is a key rubber biosynthetic gene involved in polymerization of isoprene chain. However, further research is needed to use these identified genes for genetic manipulation of rubber tree. Most recently, a set of genes associated with TPD has been identified by SSH analysis. This review provides a comprehensive picture on rubber biotechnological research achievements in the last two decades.

Keywords: biotechnology, genetic engineering, molecular markers, tapping panel dryness, tissue culture, rubber biosynthesis related genes

CONTENTS

INTRODUCTION

Natural rubber (*cis*-1,4-polyisoprene), an isoprenoid polymer composed of 320 to 35,000 isoprene molecules, is produced in about 2000 plant species with varying degrees of quality and quantity. At present, the Brazilian rubber tree (*Hevea brasiliensis* Muell. Arg., 2n=36) is one of the most important commercial sources due to its high rubber content

Received: 1 March, 2007. Accepted: 1 May, 2007.

Special Feature

and quality (Backhaus 1985). Natural rubber is considered a vital raw material by developed countries and is valued for its high-performance characteristics. Synthetic rubber, derived from petroleum is not as elastic or resilient and does not have the heat transfer properties of natural rubber. Although synthetic rubber is often blended with natural rubber, various products, such as airplane tires require natural rubber. Also, synthetic rubber is obtained from a non-renewable resource, whereas natural rubber is available indefinitely from renewable plant sources. Rubber tree is a perennial crop which belongs to the genus *Hevea* and to the botanical family Euphorbiaceae. The genus *Hevea* encompasses ten species, all originated from the Amazon region and all are strongly outcrossing and monoecious (Schultes 1990). Only one species, *H. brasiliensis*, is cultivated commercially for latex biosynthesis (rubber production). Among the other nine species, only *H. benthamiana* produces latex of reasonable quality, but this species has rarely been used in breeding programs. In *H. brasiliensis*, rubber biosynthesis takes place on the surface of rubber particles suspended in the latex (the cytoplasm of laticifers). The laticifers are specialized vessels that are located adjacent to the phloem of the rubber tree. When severed during tapping, the high turgor pressure inside the laticifers expels latex containing 30-50% (w/w) *cis*-1,4-polyisoprene.

In breeding and selection method of any crop species, one of the most important parameters is to bring together traits of agronomical interest. Genetic improvement of *Hevea* has been very slow and time-consuming, as in many other perennial crops, the major limitations being its very narrow genetic base, non-synchronous flowering, low fruit set, long gestation period, heterozygous nature, insufficient availability of land for field experiments and the absence of fully reliable early selection parameters. Furthermore, conventional breeding is rather difficult due to the heterozygous nature of the crop and its long juvenile phase that includes 6-7 years before latex collection. Cultivated clones are propagated vegetatively by grafting. As a rule, the rubber tree displays inbreeding depression, making it difficult to develop appropriate progenies for classical studies (Lespinasse *et al.* 2000a). Therefore, the most appropriate process is the selection of suitable genotypes. At any rate, the selection or breeding scheme of superior *Hevea* clones addresses important questions regarding the choice of traits, the methodology and how the results should be interpreted in order to provide farmers with new, superior clones. To this end, we should also take into consideration the climatic conditions and the tree variability. In spite of all these difficulties, rubber breeding programmes are in progress to develop improved clones in various rubber growing countries including India. Nowadays, a selection scheme of clones or individuals is underway using rapid genetic selection techniques and variability identification. Even though the number of laboratories working on rubber is restricted mainly to tropical countries, molecular markers have been established in less than a decade to set the basis in order to distinguish, characterize or identify clones, to estimate germplasm variability and to trace *Hevea* origin. Moreover, a number of genes involved in rubber biosynthesis have been recently isolated. This has helped to shed light on the regulation of rubber biosynthesis of this important crop. However, since we are only at the beginning, we need further investigation in order to find clues on how the rubber biosynthesis and the variability of quantitative and qualitative latex yield traits are regulated.

Although, traditional breeding of *Hevea* has limitations, improved clones have been produced by using conventional methods and there is a strong need for additional methods for genetic improvement. In concert with traditional plant breeding practices, biotechnology is contributing towards the development of novel methods to genetically alter and control plant development, plant performance and plant products. Conventional breeding utilizes domestic crops and related genera as a source of genes for improvement of existing cultivars, and this process involves the transfer of a set of genes from the donor to the recipient. In contrast, biotechnological approaches can transfer defined genes from any organism, thereby increase the gene pool available for improvement. The improvement of *Hevea* by biotechnological approaches primarily involves introduction of exogenous genes in a heritable manner, and secondarily, the availability of genes that confer positive traits when genetically transferred into *Hevea*. The development of *in vitro* technologies has thus complemented the conventional methods of *Hevea* breeding in generating elite clones via somatic embryogenesis. The genetic improvement of *Hevea* has received considerable attention over the years from plant breeders with the purpose of increasing the latex yield and to minimize crop loss due to various stresses including tapping panel dryness syndrome. The introduction of foreign genes encoding for useful agronomic traits into commercial clones has resulted in saving precious time required for introgression of the desired trait from the wild relatives by conventional practices (Jayashree *et al.* 2003; Sobha *et al.* 2003a). Therefore, in recent years, *Hevea* improvement efforts have been focused on raising genetically altered plants with high yield potential, capable of producing value added products such as recombinant protein and tolerant to abiotic stresses such as TPD (tapping panel dryness) according to the regional requirement of the crop. Though considerable progress has been made to improve the latex yield in *Hevea* in the recent past, TPD is one of the important physiological factors to limit the latex production in rubber plantations across world. It is estimated that 20-40% of annual rubber production was affected by the TPD syndrome (Chen *et al.* 2003). Therefore, development of new clones tolerant to TPD is one of the prerequisites in *Hevea* improvement.

The ever increasing demand for natural rubber has made it imperative to develop *in vitro* techniques for rapid propagation of elite *H. brasiliensis*. Also tissue culture and genetic manipulation are potential techniques that could be used in selection of elite plants for *Hevea* improvement. Initially attempts were made to propagate *Hevea* via shoot tip culture and haploid plants via anther culture. However, the difficulties associated with plantlet regeneration from microspores have compelled researchers to look for alternate target explants with better regeneration capabilities. Therefore, attention shifted to somatic embryogenesis and embryogenic callus cultures derived from tissues of immature anther and inflorescences. Detailed information on tissue culture, haplogenesis, somatic embryogenesis, protoplast culture of *Hevea* from different explants is available in various reviews and so has not been dealt with in detail here (Carron *et al.* 1989, 1995a, 1995b, 2001, 2005) (**Table 1**). In recent years, with the development of suitable regeneration protocols, somatic embryogenic calli derived from immature anthers and inflorescences are emerging as suitable target tissues for genetic transformation experiments (Kumari Jayashree *et al.* 1999; Sushamakumari *et al*. 2000a). The last two decades have witnessed the widespread use of micropropagation approaches for development of 'true-to-type' clones in *Hevea*. Isolated embryos cultured *in vitro* on suitable solid culture medium could develop into normal plantlets. In *Hevea*, successful plant regeneration from immature anther explants via somatic embryogenesis has been demonstrated by Kumari Jayshree *et al.* (1999). However, there are still some difficulties due to low efficiency of regeneration. To date, the *Agrobacterium*-mediated approach has been the most successful method for delivering foreign genes into *Hevea* (Jayashree *et al.* 2003; Sobha *et al.* 2003a).

In conventional breeding, molecular genetic markers are powerful tools that could enhance the speed and effectiveness of rubber breeding. General advantages of DNA markers include their ability to reveal the sites of variation in DNA segments among many individuals, their abundance and distribution over the whole genome and their independence from the variations of the environment. The process of using such markers as selection criteria is called Marker-Assisted Selection (MAS), the methodology of which is still at research level in rubber. There are several types of molec-

Table 1 Summary of *in vitro* research applied to *Hevea brasiliensis*.

Explants used	Results	References[a]
Micropropagation		
Shoot apices	PD, RD	Paranjothy and Ghandimathi 1975, 1976
Somatic embryos	PD	Carron and Enjalric 1982
Axillary buds from young trees	PD, RD	Enjalric and Carron 1982
Rubber rootstocks	PD	Carron and Enjalric 1983
Shoot tips from immature seedling	PD	Gunatilleke and Samaranayake 1988
Shoot tips, internodes	PD, RD	Te-chato and Muangkaewngam 1992
Apices from mature shoots	PD, RD	Perrin *et al*. 1994
Axillary buds	PD, RD	Seneviratne *et al*. 1995
Nodal explants from juvenile plants	PD, RD	Seneviratne and Flegmann 1996
Shoot tips	PD	Seneviratne 1991
Axillary buds from mature clones	PD, RD	Perrin *et al*. 1997
Axillary buds	PD	Seneviratne and Wijesekara 1996
Axillary buds	PD	Lardet *et al*. 1999
Nodal explants	PD	Lardet *et al*. 1999
Axillary buds	PD, RD	Mendanha *et al*. 1998
Shoot tips	PD	Kala *et al*. 2004
Organogenesis		
Cotyledon, hypocotyls, epicotyl	RD	Paranjothy and Ghandimathi 1975, 1976
Mature embryonic axes	PD	Paranjothy and Ghandimathi 1976
Young stem	CD, RD	Wilson and Street 1974
Stamens	CD, PD	Wilson and Street 1974
Leaves	CD, ED	Carron and Enjalric 1982
Leaves	CD, PD	Mendanha *et al*. 1988
Callus from inner integument	ED	Blanc *et al*. 2002
Anther/microspore culture		
Anther	CD	Satchuthananthabale and Irugalbandra 1972
Anther	CD	Satchuthananthabale 1973
Anther	CD, ED	Paranjothy 1974
Anther	CD, CD	Ghandimathy and Paramjothy 1975; Paranjothy and Ghandimathy 1975, 1976
Anther	CD, ED, PD	Paranjothy and Rohani 1978
Anther	CD, ED, PD	Chen *et al*. 1978, 1979, 1981, 1982; Chen 1984
Anther	CD, ED, PD	Shijie *et al*. 1990
Anther	CD, ED	Das *et al*. 1994
Isolated microspore culture	CD	Jayashree *et al*. 2005
Isolated microspore	CD	Jayashree *et al*. 2005
Somatic embryogenesis		
Anther	CD, ED, PD	Paranjothy 1974
Anther	CD, ED	Paranjothy and Ghandimathi 1975
Anther	CD, ED, PD	Paranjothi and Rohani 1978
Anther	CD, ED	Wang *et al*. 1980, 1984
Anther	CD, ED	Carron and Enjalric 1982
Anther	CD, ED, PD	Wan *et al*. 1982
Integument tissue	CD, ED, PD	Carron and Enjalric 1985
Integument tissue	CD, ED	EI Hadrami *et al*. 1991, 1992
Integument tissue	CD, ED, PD	Etienne *et al*. 1993a, 1993b
Integument tissue	CD, ED	Montoro *et al*. 1993
Integument tissue	CD, ED	Veisseire *et al*. 1994a, 1994b
Integument tissue	CD, ED, PD	Asokan *et al*. 1992
Stamens	CD, ED, PD	Wang and Chen 1995
Immature anthers	CD, ED, PD	Kumari Jayasree *et al*. 1999
Immature inflorescence	CD, ED, PD	Sushamakumari *et al*. 2000a, 2000b
Leaf	CD, ED, PD	Kala *et al*. 2005
Roots	CD, ED, PD	Sushamakumari *et al*. 2006
Protoplast culture		
Immature leaves	PI, PF, FD	Cailloux and Lleras 1979
Stem pith cell suspensions	PI, FD	Rohani and Paranjothy 1980
Stem tissues	PI, FD	Wilson and Power 1989
Anther callus and cell suspension	PI, PF	Haris *et al*. 1993
Embryonic callus	PI, PPD, MCF	Cazaux and d'Auzac 1994
Stem tissues	PI, FD	Cazaux and d'Auzac 1995
Embryogenic cell suspension	PPD	Sushamakumari *et al*. 2000b
Embryogenic cell suspension	PPD	Sushamakumari *et al*. 2002
Genetic transformation		
In vitro and *in vivo* derived seedlings	Developed tumour cells	Arokiaraj and Wan 1991
Anther callus	TPR	Arokiaraj *et al*. 1994
Anther callus	TPR	Arokiaraj *et al*. 1996
Anther callus		Arokiaraj *et al*. 1998
Friable callus from inner integument of immature fruit	TC	Montoro *et al*. 2000
Anther callus	TPR	Arokiaraj *et al*. 2002
Anther callus	TPR	Jayashree *et al*. 2003
Anther callus	TPR	Sobha *et al*. 2003a, 2003b
Embryogenic callus from inner integument of immature fruit	TPR	Montoro *et al*. 2003
Embryogenic callus from inner integument of immature fruit	TPR	Blanc *et al*. 2005
Embryogenic callus from anther	TCD	Rekha *et al*. 2006

[a] For more information refer to reviews by Thulaseedharan *et al*. 2000, Venkatachalam *et al*. 2006a, Clement-Demange *et al*. 2007.

Abbreviations: CD, callus development; ED, embryo development; MCD, micro calli development; PI, protoplast isolation; PD, plant development; PPD, protoplast division; RD, root development; TCD, transgenic callus development; TPP transgenic plant production.

ular genetic markers currently used: Restriction Fragment Length Polymorphism (RFLP), Random Amplification of Polymorphic DNA (RAPD), Amplified Fragment Length polymorphism (AFLP), Single Sequence Repeats (SSR) or Sequence Tagged Microsatellite Sites (STMS), DNA Amplification Fingerprinting (DAF), Microsatellite Primed-PCR (MP-PCR), and Single Nucleotide Polymorphism (SNP).

Recent reviews have been published on agricultural practices (Thulaseedhran *et al*. 2000; Priyadarshan 2003), breeding (Priyadarshan *et al.* 2004), genetics (Clément-Demange *et al.* 2007) and transformation (Venkatachalam *et al*. 2006a) of *Hevea*, so this review will concentrate on biotechnology and molecular biology of this species. An attempt to give an overview on the most important topics related to *Hevea* biotechnology is made, but due to the broad spectrum of the review, some omissions are unavoidable.

APPLICATIONS OF TISSUE CULTURE

Shoot tip culture

As the conventional method of propagation may lead to undesirable stock-scion interactions, the clonal propagation of *Hevea* by tissue culture is a relevant technique to a greater extent. The production of uniform individual plants is one of the main objectives for *in vitro* propagation of *Hevea.* Several reports are available on *in vitro* propagation of *Hevea* using different explants, mostly derived from seedlings (Seneviratne 1991; Seneviratne and Wijeskara 1996). Initially, Paranjothy and Ghandimathi (1975, 1976) made an attempt to culture shoot apices from 2-3–weeks old aseptically grown seedlings and rooted plantlets were obtained within four weeks in liquid MS (Murashige and Skoog 1962) medium. Also, Enjalric and Carron (1982) achieved shoot sprouting from the stem nodes of young green house plants. Carron and Enjalric (1983) also reported that the propagation of elite *Hevea* clonal stock material from stem cuttings was a failure due to inadequate rooting system, which is necessary for the tree stability. Later, Carron *et al.* (1989) obtained full plantlets from seedlings. Gunatilleke and Samaranayake (1988) cultured shoot tip explants from aseptically grown seedlings for micropropagation. Further, Te-Chato and Muangkaewngam (1992) produced multiple shoots from different explants derived from *in vitro* seedlings. Shoot apices collected from mature trees of two *Hevea* genotypes were micro grafted onto 3 week old seedlings grown *in vitro* by Perrin *et al.* (1994). This indicates that the physiological juvenility of the explants is important for successful micropropagation. Seneviratne *et al.* (1995) described that the regenerated shoots did not produce tap root system, however, the growth of these plants was similar to the plants developed by bud grafting or embryo culture. Seneviratne and Flegmann (1996) reported multiple shoot production from the nodal explants of juvenile origin and elongated shoots produced roots. Seneviratne and Wijesekara (1996) also demonstrated that the axillary bud development was enhanced with the application of cytokinins. Mendanha *et al*. (1998) described plant development from the axillary buds cultured on MS medium supplemented with growth hormones. At Rubber Research Institute of India (RRII), *in vitro* propagation of *Hevea* clone was attempted where the shoot tips were cultured on a medium containing indole-3-acetic acid (IAA) (1.5-3.0 mg/l) and Kinetin (0.5-1.5 mg/l) and fully developed plantlets were obtained and rooted plantlets were established in the field (Thulaseedharan *et al.* 2000). Carron *et al.* (2000) evaluated the rooting ability of *in vitro* propagated plants in *Hevea*. The *in vitro* plantlets had a well developed tap root system with lateral roots similar to that of plants obtained from seeds. The effect of different fungicides and antibiotics to control microbial contamination in *Hevea* cultures were also examined (Kala *et al*. 2004).

Organogenesis

In vitro organogenesis from various explants has been attempted by earlier workers, but the success rate was very low. Initial attempt was made to develop callus and suspension cultures in *Hevea* by Wilson and Street (1974). Paranjothy and Ghandhimathi (1975) described a method for full plantlet regeneration from dehusked seeds or freshly fallen seeds. Cotyledon, epicotyl or hypocotyl explants produced compact callus with roots but failed to regenerate plants (Paranjothy and Ghandhimathi 1975). Carron and Enjalric (1982) demonstrated callus induction from the leaf explants but no organogenesis was noticed. Muangkaewngam and Te-Chato (1992) evaluated the effect of low temperature on zygotic embryo culture *in vitro*. Perrin *et al.* (1997) compared the ability for *in vitro* axillary shoot organogenesis and rhizogenesis between mature and rejuvenated clones of *H. brasiliensis*. Mendanha *et al*. (1998) reported abundant callus initiation from the leaf explants however, no regeneration was noticed when they were subcultured onto medium.

Protoplast culture

Protoplast culture is now widely applied in cell biology and in association with molecular characterization of genes. It is a fundamental tool for the genetic improvement of plants by somatic hybridization and genetic engineering. The reports on protoplast culture of *Hevea* are scanty. Initially, Cailloux and Lleras (1979) isolated protoplasts from young leaves and stem tissues but no division was observed. Rohani and Paranjyothi (1980) made an attempt to isolate protoplasts from different tissues of *Hevea* and reported that pith from young shoots and suspension cultures of anther derived calli were the most promising sources for obtaining viable protoplasts. Even though cell wall regeneration was noticed within 7 days, no cell division was observed. Wilson and Power (1989) obtained protoplasts from stem tissues of *Hevea* and they were successful in cell wall regeneration and divisions, but rapid degeneration of the protoplasts was recorded. Haris *et al.* (1993) isolated protoplasts from cell suspensions derived from anther calli while Cazaux and d'Auzac (1994) used embryogenic calli for protoplast isolation and obtained microcalli from *Hevea* protoplasts. Later, Cazaux and d'Auzac (1995) reported that *Hevea* stem protoplasts were found to be recalcitrant to division due to high ethylene production. Sushamakumari *et al.* (1999a) successfully isolated protoplasts from 2 month old embryogenic cell suspensions derived from immature inflorescence. Further, cell division and microcalli formation were also obtained. Subsequently, Sushamakumari *et al.* (2000a) regenerated plants from protoplast derived callus via somatic embryogenesis. Also a detailed study was conducted to examine the effect of nitrogenous compounds on plant regeneration from protoplast derived callus by Sushamakumari *et al.* (2000a). It is reported that the amino acids arginine, proline (50-100 mg/l), glutamine (400 mg/l) stimulated embryo induction while plant regeneration was enhanced by glutamine. They also observed that GA3 increased the percentage of embryogenesis (1.45 µM) as well as plant regeneration (2.9 µM). The addition of glucose along with sucrose had beneficial effect on embryo induction and plant regeneration.

Anther and microspore culture

Haploid plants can play an important role in the study of quantitative traits, in the removal of deleterious recessive genes and in speeding up selection in conventional breeding. Anther culture and isolated pollen culture offers the possibility of easy production of homozygous haploid lines. Microspore culture is an elegant system for gene mapping and genetic transformation. However, the long juvenile phase and the requirement of many generations of inbreeding make this approach impractical. *In vitro* approaches to

induce haploids in *Hevea* have only limited success in comparison with other plant species. Satchuthananthavale and Irugalbandra (1972) cultured *Hevea* anthers for the first time, with the aim of obtaining haploid plants. Satchuthananthavale (1973) and Paranjothy and Gandimathi (1976) noticed callus formation as well as continued growth from anther tissues and pollen grains after 4-5 weeks in culture. Chen *et al.* (1978, 1979, 1981, 1982) and Chen (1984) reported the production of pollen plantlets in *Hevea* and reported the establishment of pollen plantlets in the soil. Das *et al.* (1994) obtained callus induction from several clones on a medium with 6% sucrose. No report is available on the isolated microspore culture of *Hevea*. Jayasree *et al.* (2005) reported microcalli formation from isolated microspores. Though anther culture was the first significant success of *in vitro* culture applied to rubber tree, this approach did not receive much attention in research after the end of the 1980s because of difficulties in achieving new elite clones via haplogenesis.

Somatic embryogenesis

Somatic embryogenesis is one of the powerful tissue culture techniques for mass propagation of elite *Hevea* clones. In recent years, the utilization of this system also opens up new avenues for molecular farming through genetic transformation. *Hevea* somatic embryogenesis was first developed in China and Malaysia, using the anther wall as initial mother tissue explant. Paranjothy (1974) induced embryoids from anther wall derived calli and subsequently shoot development of the embryoids was also achieved (Paranjothy and Ghandimathi 1975; Paranjothy and Rohani 1978). Since then, Wang *et al.* (1980, 1984) and Wan *et al.* (1982) succeeded in plant development via somatic embryogenesis from anther wall calli. Carron and Enjalric (1982) induced calli and embryoids from anther wall. Later, Wang and Chen (1995) developed plantlets from stamen cultures. At CIRAD, the inner integument of immature seeds was chosen as explant for developing somatic embryogenesis through four successive phases: (1) callogenesis (2) differentiation of embryos (3) multiplication of embryos and (4) germination of embryos and development into plantlets (Carron and Enjalric 1982). Carron and Enjalric (1985) used integumental tissue of immature fruits for somatic embryogenesis and regenerated plantlets were successfully established in the soil. The effect of various factors on somatic embryogenesis was studied in detail by EI Hadrami *et al*. (1989, 1991, 1992), Michaux-Ferrier and Carron (1989), Auboiron *et al.* (1990) and Etienne *et al.* (1991). Etienne *et al.* (1993a, 1993b) described that a slow desiccation or maturation with 351 mM sucrose supplemented with 1mM ABA strongly improved germinability and conversion to plantlets. Role of ABA on embryo development was also reported by Veisseire *et al.* (1994a, 1994b). Montoro *et al.* (1993, 1995) reported that a higher level (351 mol m^{-3}) of sucrose and 12 mM calcium promoted embryogenesis. Somatic embryogenesis from integument tissues and subsequent plant regeneration has been reported by Te-Chato and Chartikul (1994). Carron *et al.* (1995a) made a qualitative and quantitative comparison on somatic embryogenesis in 4 different *Hevea* clones (PB260, PR107, RRIM600, and PB235). Cailloux *et al.* (1996) established a long term embryogenic line with recurrent embryogenesis. Etienne *et al.* (1997a, 1997b) also reported enhanced somatic embryogenesis by the use of a temporary immersion technique with addition of higher concentration of $CaCl_2$ in embryo induction medium. Etienne *et al.* (1997b) standardized a pulsed-air temporary immersion system for enhancing embryo production, through culturing embryogenic callus under immersion in an autoclavable filtration unit RITA®. Engelmann *et al.* (1997) observed a rapid regrowth as well as production of somatic embryos with cryopreserved callus using freezing protocols. Wang *et al.* (1998) identified suitable temperature requirement for callus and embryo induction as well as for plant regeneration. Martre *et al.* (2001) explained that temporary immersion generates potentially stressful conditions for explants which may enhance somatic embryo production. Blanc *et al.* (1999) investigated the role of carbohydrates and reported that somatic embryo production was significantly higher with maltose. A comparative study between somatic and zygotic embryos was conducted by Lardet *et al.* (1999). Although plant regeneration via somatic embryogenesis was successful in *Hevea*, this regeneration system is still a fleeting phenomenon because of low germination percentage and plant conversion (Cailloux *et al.* 1996; Linossier *et al.* 1997) as in many other tree species. Charbit *et al.* (2004) identified 28 differentially expressed cDNAs during induction in the embryogenic regenerating line (ER) in *Hevea* by DD-RTPCR analysis. Most recently, Lardet *et al.* (2007) reported that calli pre-cultured on 1 mM $CaCl_2$ displayed better regeneration ability than those pre-cultured on 0 mM $CaCl_2$ medium. Further they showed that some non-regenerant lines became regenerant after cryopreservation, indicating that cryopreservation improves morphogenetic competence of embryos derived from cryopreserved callus. The authors also demonstrated that embryos derived from cryopreserved tissues led to plant recovery with no phenotypic differences between cryopreserved and non- cryopreserved calli.

Substantial progress has been achieved in this direction in the recent years (Thulaseedharan *et al*. 2000). Somatic embryogenesis remains difficult due to the occurrence of low frequency plant regeneration from different *Hevea* genotypes. Futher, reliable embryo formation is limited to only a few genotypes (PB 260, PR 107, PB 235, RRII 105 and RRIM 600) and embryo formation is also fugacious (Carron *et al.* 1989; Kumari Jayasree *et al.* 1999). Molecular mechanism of embryogenic callus formation, embryo induction and differentiation were poorly understood (Etienne *et al.* 1993a). In fact, somatic embryogenesis needs further improvement for the commercial application of the system. There has been an ever-increasing interest in the development of plantlets through somatic embryogenesis especially by the advent of genetic transformation (Kumari Jayasree *et al.* 1999). Since 1990, research has been initiated for *in vitro* plant regeneration via somatic embryogenesis especially for Indian *Hevea* clones at the Rubber Research Institute of India (RRII). Both immature anthers (Kumari Jayasree *et al.* 1999) and inflorescence (Sushamakumari *et al.* 2000b) explants were found ideal for somatic embryogenesis and plant regeneration for the high yielding Indian *Hevea* clone RRII 105 (**Fig. 1A-C, 1G**). In depth studies were carried out to find out the nutritional and hormonal requirements as well as other environmental conditions like light for maximum induction of calli, embryos and subsequent plantlet regeneration (Kumari Jayasree *et al.* 1999). With immature anthers, optimum callus induction was noticed on medium supplemented with 2.0 mg/l 2,4-D combined with 0.5 mg/l KIN. On subsequent culturing to modified MS medium with 0.7 mg/l KIN and 0.2 mg/l NAA induced embryos and plantlet regeneration achieved on hormone-free medium. Also the diploid nature of the regenerated plantlets was further confirmed by cytological analysis. Somatic embryo derived plants were successfully planted in field at Rubber Research Institute of India (**Fig. 1H**). Further, plantlet development was enhanced by induction of multiple shoots from germinative embryos (Sushamakumari *et al.* 1999b). Sushamakumari *et al.* (2000b) studied the role of sucrose and ABA on callus development, embryo induction and plant regeneration and somatic embryo-derived plants were established in RRII experimental field. Later, a detailed study was undertaken with immature anthers for enhancing embryo induction and plant development (Kumari Jayasree *et al.* 2001). Accordingly, initial preculture of anthers in liquid medium for 10 days prior to culturing onto solid medium promoted callus induction as well as reduced the time requirement. Polyamines had no significant effect except with spermidine (0.5 mg/l). Addition of 150-200 mg/l glutamine and 400 mg/l casein hyd-

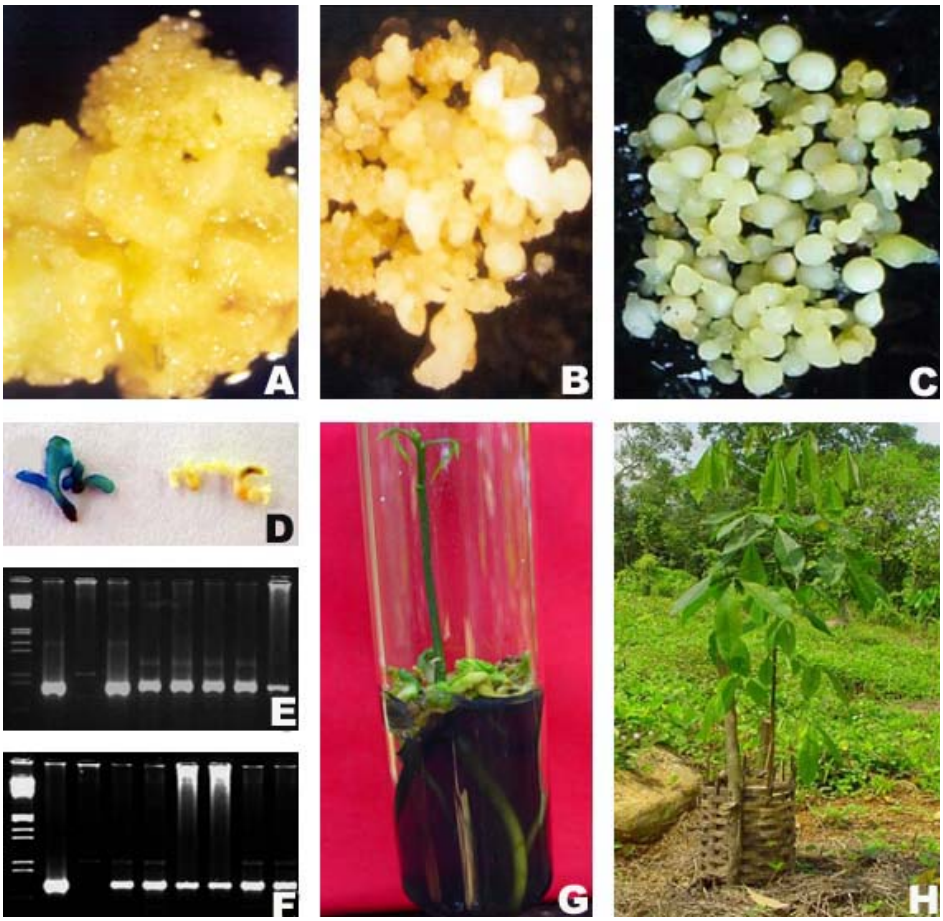

Fig. 1 Rubber propagation via somatic embryogenesis. (**A**) Callus initiation from immature anther explants; (**B**) Embryogenic callus formation; (**C**) Different stages of somatic embryos; (**D**) Transformed embryos with GUS expression (left) and control embryos (right); (**E**) Confirmation of *npt*II gene (800 bp) by PCR; (**F**) Amplification of *uid*A gene fragments (600 bp), (**G**) Embryo germination and plantlet development and (**H**) Somatic embryo derived plant established at RRII experimental field.

rolysate enhanced embryo induction rate. Kumari Jayasree and Thualseedharan (2001) reported that the incorporation of 2.0 mg/l GA_3 increases embryo induction as well as germination percentage. Repetitive embryogenesis was also induced from primary somatic embryos derived from integumental tissue (Asokan *et al.* 2002). An isozyme study during different developmental stages of somatic embryogenesis revealed a marked difference between embryogenic and non embryogenic calli (Asokan *et al.* 2001).

Kumari Jayasree and Thulaseedharan (2004) observed long-term embryogenesis from embryogenic callus and the embryogenic potential was retained for a period of 3 years. Inclusion of 100 mg/l proline increased the regeneration potential and activated charcoal was found to be essential for high frequency plant regeneration up to 80%. Among 4 cytokinins [6-benzylaminopurine (BA), zeatin (ZEA), kinetin (Kn) and thidiazuron (TDZ)] tested, TDZ at 0.25 mg/l was found to be superior for somatic embryogenesis followed by BA and ZEA (0.25 mg/l) (Kumari Jayasree and Thulaseedharan 2005). Recently attempts were also made to induce somatic embryos from leaf and root explants (Kala *et al.* 2005, 2006; Sushamakumari *et al.* 2006). Yellow, friable callus induced from leaf explants on modified MS medium (Kumari Jayasree *et al.* 1999) containing 2,4-D, NAA and BA as growth hormones was differentiated into embryos. Embryo maturation occurred on woody plant medium (WPM) (Lloyd and McCown 1980) supplemented with BA (0.3 mg/l), TZD 0.5 mg/l and GA_3 (1.5 mg/l). Further, organic supplements (100 mg/l malt extract and 150 mg/l banana powder) enhanced embryo maturation and the regenerated plantlets were established in polybags (Kala *et al.* 2006). Friable embryogenic calli derived from root explants were differentiated into somatic embryos on WPM medium fortified with the combination of GA_3 (4.35 µM) and BA (8.84 µM) and the maximum frequency of plant regeneration obtained was 65%. Regenerated plants were successfully initially transferred to small polybags filled with autoclaved soil rite and kept in the glass house at controlled environment. Subsequently, the acclimatized plantlets were transferred to larger poly bags and maintained in the filed under shade before planting in the experimental field (Sushamakumari *et al.* 2006).

Genetic engineering

Conventional rubber breeding takes more than 25 years to obtain a new clone. Genetic transformation offers a potential tool to breeders for adding valuable traits to crop plants, leading to the development of elite clones in a relatively short period of time. The rubber tree is a good candidate for manipulation by genetic transformation due to the long breeding cycle and heterozygous nature. Furthermore, *in vitro* techniques including plant regeneration via somatic embryogenesis have been established. Thus, the basic technology for genetic manipulation of rubber plant at the cellular and molecular levels is available. Many agronomic traits that could be considered for rubber improvement biotechnology programme include TPD tolerance, high yield potential, resistance to diseases, production of recombinant protein and timber wood quality improvement. Genetic engineering of crop plants integrated with specific genes has been achieved either by direct gene transfer or by *Agrobacterium tumefaciens* mediated genetic transformation. Although, genetic transformation and stable integration of foreign genes have been successful in many annual crop plants, the success in perennial tree crops is rather limited in general and *Hevea* in particular. The possibility of genetic transformation in *Hevea brasiliensis* was first explored in 1991 by *Agrobacterium*-mediated transformation (Arokiaraj and Wan 1991). Later Arokiaraj *et al.* (1994) succeeded in developing the first transgenic *Hevea* plants using anther derived calli from the clone GL 1, by the particle gun method. Subsequently, *A. tumefaciens*-mediated transformation was achieved with anther derived calli as initial explant and transgenic *Hevea* plants were produced (Arokiaraj *et al*. 1996, 1998). Montoro *et al.* (2000) reported that the transformation efficiency was significantly enhanced when $CaCl_2$ (9 mM)-treated friable callus was cul-

tured on calcium-free medium. Montoro *et al.* (2003) used callus from inner integument tissues of immature fruits (clone PB260) for genetic transformation. Recently, Blanc *et al.* (2005) developed transgenic plants of *Hevea* (clone PB 260) via *Agrobacterium*-mediated transformation. In order to find out the most suitable explant for genetic transformation, an experiment was performed with different explants such as immature anther and ovule, sixty day old callus and embryogenic callus derived from immature anther and ovule explants and maximum transformation frequency (62%) was obtained with anther derived embryogenic callus (Rekha *et al.* 2006). Further, histochemical GUS assay revealed the expression of the *uid*A gene in the transgenic callus lines. Molecular confirmation of the transgene integration with *Hevea* genome was also confirmed by polymerase chain reaction (PCR).

Arokiaraj *et al.* (1998) used *Agrobacterium* GV2260 (p35SGUSINT) that harboured theβ-glucuronidase (*gus*) and neomycinphosphotransferase (*npt*II) genes for genetic transformation. Subsequently, Montoro *et al.* (2000, 2003) developed transgenic calli cocultivated with *Agrobacterium* (EHA105) containing binary vector pCAMBIA2301 using inner integument tissues of immature fruits (clone PB260) and led to high transient GUS activity in callus. Blanc *et al.* (2005) produced transgenic plants of *Hevea* (clone PB 260) via *Agrbacterium*-mediated transformation where pCAMBIA2301with strain EHA105 was used for all the experiments. Its T-DNA construct harbours both a *uid*A gene interrupted by a Catalase-intron and a *npt*II gene conferring resistance to neomycin, each of them under the control of a CaMV35S RNA promoter. Although, earlier transformation studies in *Hevea* were performed with various marker genes, recent experiments are mainly focused on transferring various agronomically important genes into *Hevea* for the production of transgenic plants with enhanced tolerance to abiotic stresses including TPD, high latex and timber yield and for the production of recombinant proteins. 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 20-40% of rubber trees in almost every rubber growing country. Under normal conditions, active oxygen species (AOS) are efficiently scavenged by detoxifying enzymes such as superoxide dismutase (SOD). 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 increase the SOD enzyme activity by over expression of this gene in *Hevea* transgenic plants. Transgenic plants were produced with the SOD gene under the control of CaMV 35S and FMV 34S promoters (Jayashree *et al.* 2003; Sobha *et al.* 2003a). Two-month-old callus derived from immature anther was co-cultivated with *Agrobacterium* strain EHA 101 harbouring a plasmid vector containing the *HbSOD* gene under the control of CaMV 35S promoter, *uid*A gene for screening and *npt*II for selection. Transformed callus surviving on medium containing 300 mg/l kanamycin was selected and used for somatic embryo development. Subsequently, mature embryos germinated and developed into plantlets. Histochemical GUS assay revealed the expression of *uid*A gene in embryos (**Fig. 1D, 1F**) as well as leaves of transgenic plants. The presence of the *uid*A, *npt*II, and *HbSOD* genes in the *Hevea* genome was confirmed by molecular analysis (**Fig. 1E**). In another study, the *HbSOD* gene fused under the control of the *Figwort Mosaic Virus* (FMV) 34S promoter was used for genetic transformation and biochemical studies indicated that the superoxide dismutase, catalase and peroxidase enzyme activities increased significantly in transgenic embryogenic callus lines as compared to the control (Sobha *et al.* 2003a, 2003b). Transgenic *Hevea* plants have been successfully developed and established in soil at the Rubber Research Institute of India for further evaluation (Jayashree *et al.* 2003). At present, rubber transgenic plants with SOD gene were multiplied by bud-grafting and established in the field. It would be interesting to study the tapping effect on overexpression of SOD enzyme in these plants but it will take 6 years for initiation of tapping.

Venkatachalam *et al.* (2006a) described an efficient protocol for *Agrobacterium*-mediated genetic transformation in rubber. As in the case with most other crops, 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 elite clones. There is no doubt that conventional breeding will lead to increased latex production in rubber trees. However, it is quite possible that the rate of rubber biosynthesis within the tree becomes the limiting factor (Arokiaraj 2000). At this point, latex yield could only be enhanced either by treatments or by over-expression of rubber biosynthesis genes in transgenic *Hevea* plants. It was reported that the constitutive level of HMGR1 enzyme may be a limiting factor in rubber biosynthesis. Based on this hypothesis, Arokiaraj *et al.* (1995) initiated genetic transformation experiment to overexpress HMGR1 in *Hevea* where, *hmgr*1 activity of transformed callus ranged from 70-410% of the value of wild type control and the activity is transformed embryos obtained ranged from 250-300%. 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. As such, REF protein amount is shown to be correlated simultaneously with rubber yield, there is a possibility to enhance rubber yield by over expression of REF gene which is involved in rubber biosynthesis. At present, the author's laboratory has initiated a research project in which rubber biosynthesis genes such as HMGR1, FDP, REF and CIS are cloned into binary vectors and transformation work is in progress to develop transgenic plants. Currently transgenic callus lines were obtained and further multiplication and production of transgenic plants via somatic embryogenesis is under way.

Rapid progress in molecular biology and physiology makes it possible to modify wood quality in tree species. Until now, the most important product of the rubber tree was its latex, however, rubber wood (non-latex product) is increasingly being sought as a commercial commodity for the furniture industry (Arokiaraj 2000). With the depletion of tropical forests leading to a shortage of timber for many industrial and engineering uses, attention has moved on to rubber wood as an alternative source of timber for markets (Arokiaraj *et al.* 2002). As the composition of wood is important for the pulp industry, lignin genetic engineering is a very active area of research that has been stimulated in recent years by the characterization of important genes controlling lignification. The reports of rubber-wood from Malaysia rose from RM900 million in 1993 to RM3.7 billion in 1998 and subsequently to RM5.2 billion in 2001 (Arokiaraj *et al.* 2002). This figure clearly indicates how rubber wood is gaining importance for making furniture, sawn rubber wood and new plywood material. In trees, little is known about the process of differentiation and development that are involved in wood formation. Knowledge on what determines the pathway of differentiation of cambial cells is essential for any attempt to design better wood characteristics. 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.* 2002). However, till date there is no report on transgenic plant development to enhance wood quality in rubber tree.

In many plants, foreign proteins can be produced easily, but their purification is a real problem. Alternatively, the *para* rubber tree, which synthesizes an enormous volume of latex upon tapping could be exploited without any destruction for large-scale production of foreign protein in the latex throughout the year. Depending upon the promoters used, recombinant proteins will be sequestered throughout

the plant or in specific parts of the plants or in specific organelles within a given plant cell. Already, human serum albumin proteins were expressed in transgenic *Hevea* plant (Arokiaraj *et al.* 2002). Two hevein promoters (*Hev1* and *Hev2*) were cloned and promoter-*gus* constructs were introduced into rice callus tissues by *A. tumefaciens* for functional analysis (Pujade-Renaud *et al.* 2005). Also in this respect, the Rubber Research Institute of India has been working on the isolation of laticiferous-specific promoters which will eventually enhance recombinant protein production in transgenic rubber trees. Recently, the promoter sequence of a rubber elongation factor gene was cloned and characterized from high latex yielding Indian *Hevea* clone RRII105 (Priya *et al.* 2006). Further work is in progress to insert this promoter into a binary vector for the production of recombinant proteins in *Hevea*.

Applications of molecular markers

Molecular markers have served as useful aids in understanding the genetics of *H. brasiliensis* in the recent past. They can play an important role in assisting *Hevea* clonal identification and origin. For the last two decades, a large number of molecular markers and techniques have been applied in *Hevea* breeding. Nowadays many types of molecular marker techniques are available, the most widely used include RAPD (random amplified polymorphic DNA), RFLP (restriction fragment length polymorphism), AFLP (amplified fragment length polymorphism), minisatellite fingerprints and microsatellites or SSR (simple sequence repeats) (reviewed in Teixeira da Silva *et al.* 2005). These markers differ in the type and amount of variability they express, in their suitability for each particular question and in the ease and costs of their development and application. Among these, three major molecular marker techniques were applied to cultivar identification: random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP). In addition to these methodologies, microsatellite markers, otherwise known as simple sequence repeat length polymorphisms (SSRLP) are generated by highly specific PCR amplification. Simple sequence repeats (SSR) are regions of short tandemly repeated DNA motifs (generally less than or equal to 4 bp) with an overall length in the order of tens of base pairs. SSR have been reported to be highly abundant and randomly dispersed throughout the genomes of many plant species. Thus, SSRLP may occur even between closely related individuals. Microsatellite markers have been used in plants for fingerprinting, mapping, and genetic analysis. Though RFLPs are powerful for studying genetic diversity and mapping, this technology is not preferred now since it is labour intensive and requires large DNA samples. Its marker index value (expressed as the number of polymorphic products per sample) is also low with only 0.10 compared to PCR based marker systems like RAPDs (0.23), SSRs (0.60) and AFLPs (6.08) (Low *et al.* 1996). Initially, isozymes were utilized for clonal identification (Chevallier 1988); subsequently other tools like minisatellites (Besse *et al.* 1993a), RFLPs (Besse *et al.* 1993b, 1994), mitochondrial and chloroplastic RFLPs (Luo *et al.* 1995), RAPDs and DAFs (Low *et al.* 1996; Varghese *et al.* 1997; Venkatachalam *et al.* 2001, 2002, 2006b), AFLPs (Lespinasse *et al.* 2000a), and SSRs (Besse *et al.* 1993a; Atan *et al.* 1996; Low *et al.* 1996; Bindu Roy *et al.* 2004) were developed and used in detection of molecular markers in *H. brasiliensis*.

Isozyme markers

In conventional plant breeding, many morphological traits were used as markers for analysing genetic traits and identifying cultivars, but specific genetic information on Mendelian traits has been rare in *Hevea*. In the 1980s, isozymes have been used in rubber as genetic markers for cultivar identification, genetic diversity analysis, control of progenies issued from hand pollination, and reproductive biology (Chevallier 1988; Leconte *et al.* 1994; Paiva *et al.* 1994a; Sunderasan *et al.* 1994). Analysis of isozymes was developed at CIRAD with a set of 13 polymorphic isozymic systems to formulate a diagnostic kit associated with a clonal identification database. This kit was able to differentiate a large set of cultivated clones (Leconte *et al.* 1997). Subsequently, a study was carried out to estimate the distance *H. brasiliensis* pollen dispersed under natural conditions by using isozyme markers. Esterase isozyme markers were used to determine if the seeds had been derived from self- or cross-pollination. The incidence of cross-pollination was then examined in relation to the distance from the inter-clonal boundary. A logarithmic model suggested that pollen could travel distances in the order of 0±3 to 1±1 km (Yeang and Chevallier 1999). Moreover, isozyme-based analyses are limited by the rather small number of marker loci available and a general lack of polymorphism for these loci.

RAPD markers

Though considerable progress has been made to increase the yield in *Hevea* clones in the last two decades, satisfactory resistance to biotic and abiotic stress has not been achieved because of limited genetic resources within the *Hevea* gene pool. Wind damage is one of the serious problems in rubber-growing countries, each year a considerable number of rubber trees were lost due to wind damage in rubber plantations. The incorporation of the dwarf character into high-yielding *Hevea* clones would be useful for generating a high-yielding tree with a desirable architecture (dwarf stature) and high density planting (Venkatachalam *et al.* 2004). Using RAPD analysis, Varghese *et al.* (1997) evaluated 24 cultivated *Hevea* clones to estimate genetic distances. Subsequently, Venkatachalam *et al*. (2002) described the genetic relationships for 37 *Hevea* clones by using RAPD markers (**Fig. 2A, 2D**). This molecular information concurs with the reported high morphological variability in *Hevea* and a dendrogram was constructed based on the RAPD markers, which showed that 69% of the bands observed were polymorphic between the 37 *Hevea* clones. The clones were classified into seven major groups based on DNA markers. The phenogram showed that RRII105 (India) and RRIM600 (Malaysia) were nearly identical. Three clones belonging to Thailand have been included in the same cluster. The two clusters formed by the Indian clones were distinct with one group (RRII201, RRII204, RRII205, RRII207, and RRII209) clustering with one Malaysian clone. Clones PB312 and PB314, which were issued from the same cross (RRIM600 × PB235) appear genetically very close. RAPD analysis therefore reflects genetic differences and geographical origin of the *Hevea* clones. The Indian RRII203 clone was very dissimilar to the Malaysian PB255 clone (similarity index: 0.692). From the dendrogram it is intriguing to note that several primary clones developed in different countries, such as TJIR1, Gl1, PB86, Mil3/2, AVROS255, and BD10 were closely clustered. In most cases, clones with common ancestors, such as RRII105, RRIM600, PB311, PB312, PB314, KRS128, KRS163, PB217, PB255, PB260, RRII204, RRII209 were observed to cluster together.

Venkatachalam *et al.* (2004) identified a dwarf genome-specific randomly amplified polymorphic DNA (RAPD) marker in rubber tree. The primer OPB-12 generated a 1.4-kb DNA marker from both natural and controlled F_1 hybrid progenies (dwarf stature) derived from a cross between a dwarf parent and a normal cultivated clone as well as from the dwarf parent; it was absent in the other parent (RRII118). To validate this DNA marker, 22 F_1 hybrids (13 with a dwarf stature and nine with a normal stature) were analyzed; the dwarf genome-specific 1.4-kb RAPD marker was present in all dwarf-stature hybrids and absent in all normal-stature hybrids. This DNA marker was cloned

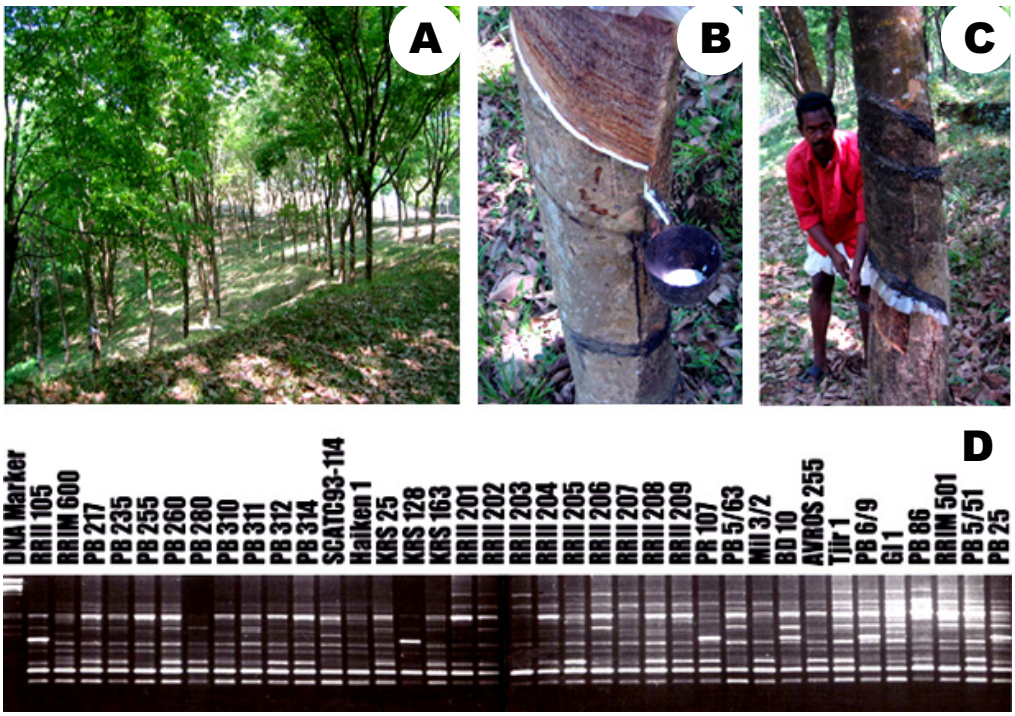

Fig. 2 Rubber plantation and molecular markers. (**A**) A view of rubber plantation at Rubber Research Institute of India (RRII, Kottayam); (**B**) A healthy rubber tree producing latex; (**C**) TPD affected tree with no latex production at tapping panel (tree is being tapped by tapper); (**D**) RAPD marker profile generated by OPC-05 prime.

and its locus specificity was further confirmed by Southern blot hybridization. More recently, Venkatachalam *et al.* (2006b) identified two DNA markers with OPB-12 primer in *Hevea* and the size of these two polymorphic bands was 1.2 kb and 1.4 kb. The 1.4 kb RAPD marker was cloned and nucleotide sequence revealed certain homology with *Saccharomyces cerevisiae* proline-specific permease gene. Further, to validate this RAPD marker, SCAR primers were designed and the SCAR marker was amplified from 14 clones among 37 clones tested. Therefore accumulated data on *Hevea* RAPD analysis from different clones give information on genetic relations, *Hevea* origin and could provide the initial basis for clonal distinction and germplasm evaluation of agronomical interest. The data can be also used as tools in *Hevea* improvement programs.

RFLP markers

In *Hevea*, Low *et al.* (1996) used RFLPs for identification of progenies with two common parents and Besse *et al.* (1993a) reported the ribosomal DNA variations among genotypes. Identification of 73 Wickham clones was carried out with 13 probes associated with restriction enzyme *Eco* RI (Besse *et al.* 1993b). Besse *et al.* (1994) studied the genetic diversity of 92 Amazonian and 73 Wickham clones using RFLP analysis. One clone from Rondonia, RO/C/8/9, showed eight specific restriction fragments and a unique *malate dehydrogenase* (*MDH*) allele indicating its interspecific origin (Besse *et al.* 1994). Application of microsatellites to genetic diversity analysis confirmed that wild accessions were more polymorphic than cultivated Wickham clones. Some accessions such as RO/OP/4 20/16 and RO/A/7 25/133 seem unique since they do not fall under any cluster due to their high level of specific alleles (Lekawipat *et al.* 2003a, 2003b). A high mt DNA polymorphism was found in Amazonian accessions while no such variation was noticed in the Wickham population except GT 1 (Luo *et al.* 1995). Sequencing of a highly polymorphic mt DNA fragment from 23 genotypes showed real potential for phylogenetic analysis in *Hevea* (Luo and Boutry 1995). Chloroplast cp DNA analysis was carried out over 217 accessions representing 126 mitochondrial genotypes and only two cp DNA RFLP profiles were found, thus showing a much lower polymorphism and this indicate the high level of conservation of this chloroplast genome (Luo *et al.* 1995). The mt DNA of Wickham population has lesser variation since their female progenitors are restricted to a very small set of primary clones. Cytoplasmic donors of most of the improved clones were either PB56 or Tjir1. Obviously, this may be one of the reasons for the mt DNA profile showing only two clusters (Priyadarshan and Gonçalves 2003). A possible explanation for greater polymerphism in mt DNA of wild accessions is that they might have been evolved through interspecific hybridization. Seguin *et al*. (2003) proposed a general organization of *H. brasiliensis* germplasm with 6 genetic groups.

AFLP markers

The first comprehensive genetic linkage map of *Hevea brasiliensis* was constructed by using RFLP, AFLP, microsatellites and isozyme markers (Lespinasse *et al.* 2000a). The parents used were PB260 (PB5/51 × PB49) and RO38 (F4542 × AVROS363) and homologous markers segregating in both parents were ascertained for consensus map construction. F4542 was a clone of *H. benthamiana* species. The F_1 synthetic map of 717 markers was distributed in 18 linkage groups corresponding to the 18 chromosomes and consisted of 301 RFLP, 388 AFLP, 18 microsatellite and 10 isozyme markers. The genetic length of the 18 chromosomes was fairly homogeneous with an average map length per chromosome of 120 cM. Many AFLP markers were seen in clusters, which were attributed as reduced recombination frequency regions. Though the RFLP markers were well distributed all over the 18 linkage groups, these were insufficient to saturate the map. AFLPs and few microsatellites together contributed to saturating the map (Lespinasse *et al.* 2000a). A genetic approach developed on the cross PB260 × RO38 was targeted to identify the resistance allele or loci to South American Leaf Blight (SALB) disease (Lespinasse *et al.* 2000b). Eight different QTLs were identified as resistance to different SALB strains in RO38 and it was further rationalized that the resistance (alleles) of RO 38 have inherited from wild grandparent (*H. benthamiana*) and no favourable alleles came from AVROS 363, the Wickham parent (Lander and Botstein 1989). Field evaluation against the pool of *Microcyclus* strains was per-formed under the real infestation conditions and the pre-sence of the predominant QTL in g13 previously found was confirmed by Le Guen *et al.* (2003). Most recently, Le Guen *et al.* (2007) investigated the genetic resistance components of the *H. brasiliensis* × *H. benthamiana* RO 38 cultivar to *Microcyclus ulei* disease by inoculating isolates which succeeded in partially or completely infecting genotypes of a

mapping population. Among eight QTLs tested, only one contributed to the partial resistance against a highly pathogenic isolate, and no QTL was detected for resistance against the most pathogenic isolate.

Microsatellite markers (SSRs)

Simple sequence repeats or microsatellites (SSRs), issued from PCR developed from specifically designed primers, appeared very powerful due to their high polymorphism (between 15 and 20 alleles per marker). Polymorphism in microsatellites was detected also in *H. pauciflora*, *H. guianensis*, *H. camargoana*, and *H. benthamiana* (Low *et al.* 1996). Microsatellite-enriched libraries were produced and led to the identification of large numbers of microsatellite markers (Atan *et al.* 1996; Seguin *et al.* 2003; Saha *et al.* 2005); sequences were deposited in the EMBL/Genbank databases. Microsatellite markers from rubber pathogens can also be used for distinguishing the genetic differences between the races, such as was made with 11 markers for *Microcyclus* (Le Guen *et al.* 2004). For methodological purposes, one seed garden made up with 50 Amazonian genotypes and GT1 clone, planted at CNRA (Ivory Coast) was subjected to the analysis of gene flux and paternity identification with isozymes and microsatellites (Blanc *et al.* 2001; Lidah 2005). A high level of confidence was found for paternity identification carried out with 8 microsatellite markers. Paternity identification with microsatellites was carried out with the Cervus software (Marshall *et al*. 1998).

Genetic mapping was developed with a set of 247 microsatellite markers complemented by 198 AFLPs in order to study the genetic basis of different factors related with latex production (Prapan *et al.* 2004). Lekawipat (2003a, 2003b) performed a genetic diversity analysis of *H. brasiliensis* germplasm over 66 Amazonian and 40 Wickham accessions, by using both non-expressed molecular genetic markers (12 microsatellites) and 17 markers of expressed genes. Bindu Roy *et al.* (2004) described the simple sequence repeats (SSRs) or microsatellites from genomic library of *Hevea*, derived from a clone GT1 and RRII105. Different types of repeat motifs comprising dinucleotides (TG/AC, AG/TC, TA/AT), trinucleotides (AAG, AGG, ATT), tetranucleotides (GAAA, AAGG, ATCC, TAAA, AAAT) and one pentanucleotide (GAAAT) were detected. It was reported that about 67 microsatellites having characteristic simple and compound repeats, out of which 59 were from GT1 and eight from RRII105. Subsequently, Saha *et al.* (2005) used a combination of four microsatellite markers to discriminate uniquely all the 27 *Hevea* clones and some clone-specific allelic profiles were generated. Further, cross-species amplification of the markers developed in *H. brasiliensis* had also been demonstrated with two other *Hevea* species, *H. benthamiana* and *H. spruceana*, indicating a high degree of sequence homology at the flanking regions. Also, sequence analysis of the SSRs at the 3′-UTR of the hydroxymethylglutaryl-coenzyme A reductase gene, revealed the existence of two alleles based on the repeat length polymorphisms. It is obvious that the results obtained from the above mentioned molecular marker techniques have contributed to the understanding of clonal relations and are potential tools in germplasm evaluation and genetic characterization of traits with agronomical interest.

Applications of molecular biology

Hevea rubber tree (*H. brasiliensis*) is the only plant species being cultivated for commercial production of rubber in the world. It is therefore of great interest to study the regulation and the expression of the genes involved in natural rubber biosynthesis. To meet the ever increasing rubber demand, it is necessary to increase the latex production substantially by genetic manipulation (Priya *et al.* 2006). Rubber biosynthesis in *Hevea* has become a major field of research to understand the isoprenoid pathway. The general metabolic pathway of rubber biosynthesis is as follows: Sucrose from photosynthesis is actively transported into laticiferous cells through the plasmalemmic membrane, and is then hydrolyzed into glucose and fructose by invertase. These sugars are then converted into acetyl-CoA through glycolysis. Three molecules of acetyl-CoA are condensed into mevalonic acid which is converted to IPP. Polymerization of thousands of IPP molecules assisted by the action of the enzyme rubber transferase in association with REF, a molecule fixed on the rubber particles membranes leads to the formation of high molecular weight rubber. Genes expressed in the latex of *Hevea* can be divided into three groups based on the proteins they encode: (1) rubber biosynthesis-related proteins such as REF (rubber elongation factor), HMGR (hydroxymethylglutaryl-coA reductase), HMGS (hydroxymethylglutaryl-coA synthase), CIS (*cis*-prenyltransferase), GGPP (geranylgeranyl diphosphate) synthase, SRPP (small rubber particle protein), IPP (isopentenyl diphosphate) isomerase; (2) defense/stress-related proteins such as MnSOD, hevein, chitinase, β-1,3-glucanase and HEVER; and (3) latex allergen proteins such as Hev.b.3, Hev.b.4, Hev.b.5, Hev.b.7 etc.. Biological functions of the allergenic proteins are largely unknown (Oh *et al.* 1999).

Rubber biosynthesis-related genes

Rubber biosynthesis is a side-branch of the ubiquitous isoprenoid pathway (**Fig. 3**). Natural rubber is made almost entirely of isoprene units derived from the precursor isopentenyl pyrophosphate (IPP). Also, *trans*-allylic pyrophosphates are essential for rubber formation as they are used to initiate all new rubber molecules. Both the HMGS (*3-hydroxy-3-methylglutaryl coenzyme A synthase*) and HMGR have been shown to be involved in the early steps of rubber biosynthesis by forming HMG-CoA (*3-hydroxy-3methyl glutaryl coenzyme A*) using HMGS. The HMGS catalyses the condensation of acetyl CoA and acetoacetyl-CoA to form HMG-CoA (Suwanmanee *et al.* 2002, 2004; Sirinupong *et al.* 2005). According to Suwanmanee *et al.* (2002) HMGS mRNA transcripts accumulation was more in laticifers than in leaves. Moreover, a positive correlation has been observed between the activity of HMGR and HMGS and the dried rubber content of the latex from tappings. Two members of HMGS (1.8 Kb and 1.9 Kb cDNAs) from *H. brasiliensis* called *hmgs1*, and *hmgs2*, were cloned and characterized by Suwanmanee *et al.* (2002) and Sirinupong *et al.* (2005). The expression of *hmgs1* was found to be higher in laticiferous cells than in leaves whereas the abundance of *hmgs2* gene transcripts was more in laticifer and petiole than in leaves. In plants, HMG-CoA was reduced by HMGR to mevalonate and was subsequently converted into IPP. It was reported that there were three genes encoding HMGR in *Hevea* namely, *hmg1*, *hmg2* and *hmg3*. The *hmg1* gene is likely to be involved in the rubber biosynthesis, whereas the *hmg3* is possibly involved in isoprenoid biosynthesis of another nature (Chye *et al.* 1992). The first step in rubber biosynthesis is the isomerisation of IPP to DMAPP (dimethylallyl diphosphate) by IPP isomerase. The successive head-to-tail condensation reactions of the five carbon intermediates, catalyzed by the enzyme "rubber transferase", have been assumed to yield rubber.

Moreover, Oh *et al.* (2000) have isolated and characterized a cDNA clone encoding IPP isomerase from *Hevea* and showed by *in vitro* rubber assay that the recombinant IPP isomerase was required for rubber biosynthesis. Subsequently, Takaya *et al.* (2003) described the role of GGPP synthase gene in rubber biosynthesis in *Hevea*. Based on their investigation, GGPP synthase would catalyse the condensation of IPP with allylic diphosphates to give (all-E)-GGPP. Natural rubber is predicted to be made almost entirely of *cis*-isoprene units derived from IPP, and the enzyme responsible for polymerization is believed to have characteristics similar to the *cis*-prenyl diphosphate syntha-

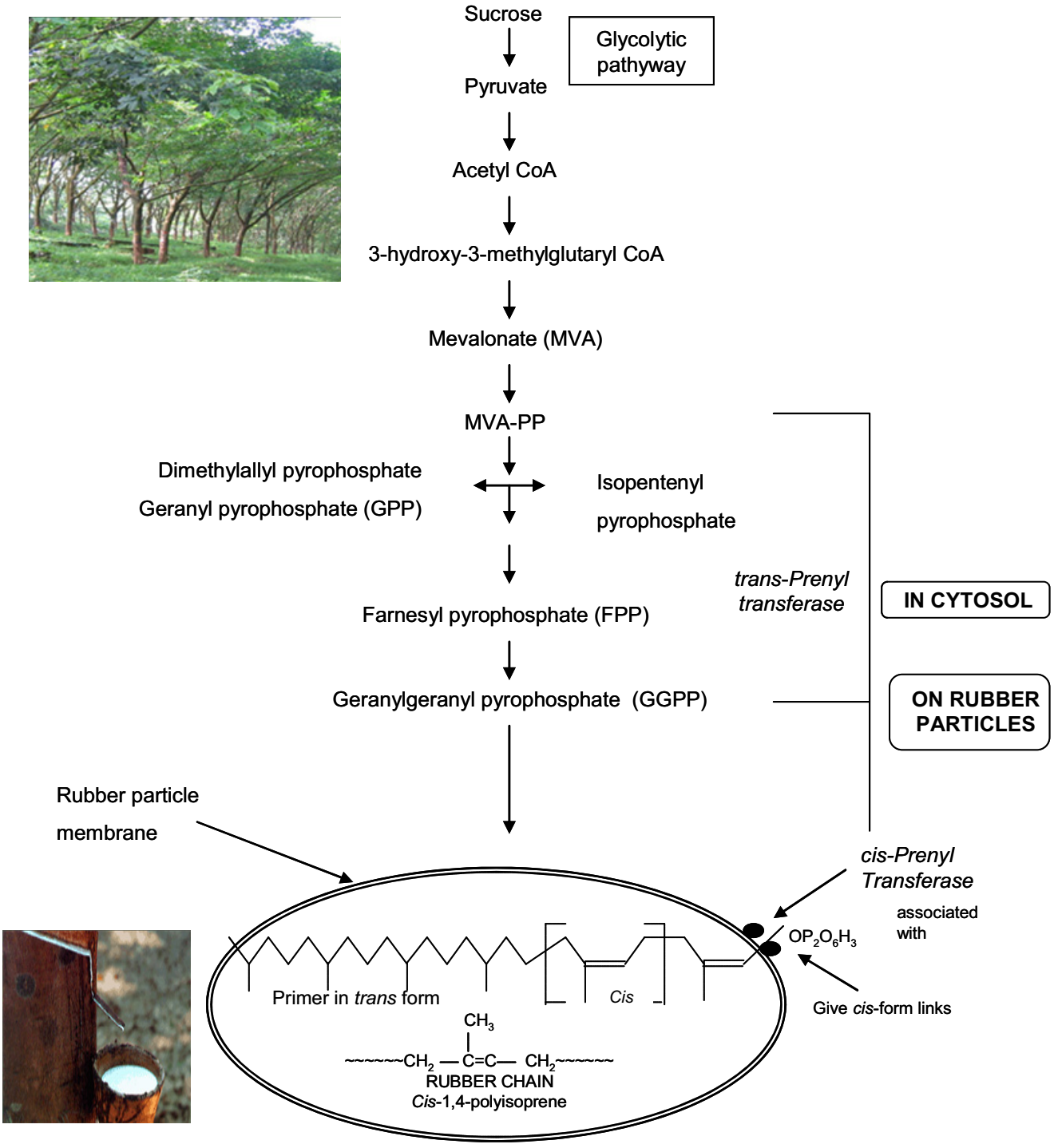

Fig. 3 General rubber biosynthesis pathway.

ses. Further, the gene responsible for the *cis*-1,4-polymerization of isoprene units has been isolated and characterized in *Hevea* by Asawatreratankul *et al.* (2003). It was suggested that rubber biosynthesis in *Hevea* is mediated by the association of a soluble *trans*-prenyltransferase with the REF, a 14.6 kDa protein, tightly bound to the rubber particles in the laticifers (Dennis and Light 1989). FPP (farnesyl diphosphate) is a key intermediate in the biosynthesis of at least 20,000 isoprenoids. FPP is also the allylic diphosphate initiator of rubber biosynthesis in plants. FPP is synthesized by the enzyme FPS (farnesyl diphosphate synthase), which has been cloned and characterized from *Hevea* by Adiwilaga and Kush (1996). REF protein was isolated and studied extensively and the results indicate that the FPS and REF complex was responsible for the *cis*-1,4-polyprenol condensations observed in isolated rubber particles (Light *et al.* 1989). The REF gene was isolated and the involvement of REF on rubber formation was analysed by Attanyaka *et al.* (1991) and Goyvaerts *et al.* (1991). Also, Oh *et al.* (1999) reported a novel *Hevea* cDNA of a protein called small rubber particle protein (SRPP) associated with small rubber particles and the sequence analysis revealed that this protein is highly homologous to the REF. Recently, Priya *et al.* (2006) described cloning and characterization of REF gene from genomic DNA which was isolated from a high yielding clone RRII105. REF gene (1367 bp) has three exons interrupted by two introns and encoded a 138 amino acid peptide containing an open reading frame of 414 bp with a calculated M_W of 14,700 Da. Nucleotide sequence analysis showed that 1.3 kb genomic DNA showed 100% homology to REF cDNA from *Hevea*. RNA blot analysis indicated that REF transcript is highly expressed in high yielding clone than in low yielder. Our ultimate goal is to produce transgenic

Hevea plants with enhanced latex yield by over-expression of REF protein. It is possible that the rubber biosynthesis pathway is coordinately regulated by these enzymes. Transcripts involved in rubber biosynthesis are 20 to 100 times greater in laticifers than in leaves (Kush *et al.* 1990). On the other hand, transcripts for chloroplastic and cytoplasmic forms of glutamine synthetase are restricted to leaves and laticifers respectively (Kush *et al.* 1990), indicating thereby that the cytoplasmic form of glutamine synthetase plays a decisive role in amino acid metabolism of laticifers. According to the reports on EST analysis from *Hevea*, rubber biosynthesis-related genes are highly expressed in latex (Han *et al.* 2000). Subsequently, Ko *et al.* (2003) used cDNA-AFLP technique to analyse the ESTs in *Hevea*. The results revealed that the most abundant ESTs were the genes encoding REF and SRPP, comprising 29% of the total ESTs.

Defense/stress-related genes

Several defense/stress related genes from latex of *Hevea* have also been identified. One of the stress related genes, MnSOD was isolated by Miao and Gaynor (1993). MnSOD transcripts were found in all tissues examined (leaf, petiole, root, latex, callus) and young leaves exhibited the highest levels in intact plants. Also, the transcript level of MnSOD in embryogenic callus was nearly 50-fold higher than in mature leaves. Sivasubramaniam *et al.* (1995) cloned and characterized a novel stress-induced gene, *HEVER* from the rubber tree, which was found to be developmentally regulated and expressed at basal levels in *Hevea* tissues. Chye and Cheung (1995) described the isolation and characterization of β-1,3-glucanase gene from *Hevea* cDNA library prepared from the latex of *Hevea brasiliensis*. Nucleotide sequence analysis revealed that the 1.2 kb cDNA encoding a basic β-1,3-glucanase showed 68% nucleotide homology with other glucanase genes in the database and was expressed at higher levels in latex than in leaf. Te-Chato and Chartikul (1995) identified resistant callus lines to *Phytophthora spp*. culture filtrate in rubber. Subsequently, Thanseem *et al.* (2003, 2005) cloned and characterized β-1,3-glucanase gene from Indian *Hevea* clone. Further, they also demonstrated that a prolonged accumulation of β-1,3-glucanase mRNA transcripts was noticed with abnormal leaf fall (ALF) tolerant RRII105 clone compared with susceptible one. Another defence related protein is *Hevein* (a chitin-binding protein) that play a crucial role in the protection of wound sites from fungal attack and is also involved in the coagulation process. It belongs to a multigene family and the specificity of its expression in the latex is under investigation (Broekaert *et al.* 1990; Pujade-Renaud *et al.* 2005). Chrestin *et al.* (1997) described that over-expression of chitinase (hevein) gene was due to the partial deglycosylation of the hevein receptor and the resulting delay in coagulation. The level of hevein and chitinase expression in the latex is regulated by ethylene.

Laticifer-specific promoters

Recently there has been growing interest in using plants for the production of biopharmaceutical proteins and peptides because they are easily transformed and provide a cheap source of protein. In this aspect the commercial rubber tree has great potential to produce foreign proteins in the latex, which can be easily purified from the serum phase of the latex. The availability of regulatory sequences or promoters to target expression to laticifers is an essential component for engineering traits in transgenic *Hevea* plants as the constitutive expression of foreign proteins is deleterious to the plants. One way to identify such promoters would be to study the upstream region of genes like REF, HMGCoA reductase etc. that are highly expressed in latex. Besides promoters, other regulatory elements like introns and enhancers also play important roles in gene expression. Pujade-Renaud *et al.* (2005) reported the isolation of promoter regions from two hevein genes of *Hevea* and analyzed them in rice. Although both were functional, only the longest promoter sequence (PHev2.1) conferred a high level of expression to the transgene in various tissues of this heterologous host plant. Most recently, promoter sequence of REF gene was cloned and characterized by Priya *et al.* (2006). The cloned 5′ promoter region has a putative TATA element at −150 and CAAT box at the −221 position. To identify the regulatory role of REF promoter, chimaeric fusion between REF promoter sequence and the β-glucuronidase (GUS) coding, *uidA* gene was constructed and used to transform tobacco and *Arabidopsis*. Expression of the *uidA* reporter gene was detected histochemically in the transformed tobacco plants where, GUS activity was detected in the leaf and petiole of transformed plants. The stable integration of *REF*:*uidA* fusion into the tobacco genome was further confirmed by PCR amplification and Southern blot analysis. A histochemical study of stable transformants demonstrated that the 5′ upstream region of REF can drive strong GUS gene expression specifically in the vascular tissues (xylem and phloem) of leaf, stem and midribs of transgenic *Arabidopsis*. GUS staining revealed that REF:GUS expression was also induced by wounding. The results suggested that the cloned REF promoter is capable of directing gene expression.

Tapping Panel Dryness (TPD) syndrome

Tapping panel dryness (TPD) syndrome ultimately leads to the partial or complete stoppage of latex biosynthesis in high latex yielding rubber trees. Different approaches have been used to describe and study the development of the TPD syndrome in the recent past. It had been previously reported that the latex from trees displaying the TPD syndrome exhibited different protein patterns compared to healthy trees (Dian *et al.* 1995). Accumulation of three polypeptides in the latex cytosol of TPD affected trees were also considered as protein markers (Sookmark *et al.* 2002). However these protein markers have been yet utilized neither for early detection nor for diagnosis of TPD syndrome in rubber tree. Subsequently, studies aimed to identify genes associated with TPD have also been attempted. Chen *et al.* (2003) reported that the expression of *HbMyb1* was likely associated with TPD syndrome. Recently, Venkatachalam *et al.* (2005, 2007) described the identification of TPD responsive genes by Suppression Subtractive Hybridization (SSH) technology using mRNA from latex samples of healthy and TPD trees (**Fig. 2B, 2C**). To identify the genes involved in this process, two SSH cDNA libraries were constructed. For forward subtracted cDNA library, healthy RNA was used as 'tester' and TPD RNA served as 'driver', whereas TPD RNA was 'tester' and healthy RNA was 'driver' for reverse subtracted cDNA library. A total of 1079 putatively positive clones were screened from these two libraries; 352 of these clones were found to be positive by differential screening with forward and reverse subtracted probes and were selected for further sequencing analysis. The putative functions of clones were predicted by BLASTX/BLASTN analysis. Among these, 64 clones were genes whose function had been previously identified while the remaining clones were genes with either unknown protein function or insignificant similarity to other protein/DNA/EST sequences in existing databases. Differentially expressed genes selected by subtractions were classified into 12 broad categories according to their putative functions generated by BLAST analysis. Two genes, i.e. *Myb transcription factor* and *TCTP* (*Translationally Controlled Tumor induced Protein*) that were up-regulated in the forward SSH library, were selected for expression analysis. Results from Northern analysis confirmed that the expression of these two genes was down-regulated in TPD trees. Some other known genes identified in this study might provide new insights into the TPD triggered PCD development in rubber tree (Venkatachalam *et al.* 2007).

Risks and concerns

The introduction of transgenic crops into the existing natural system has generated a number of questions about the possible negative consequences. Biosafety considerations, including the impact of transgene dispersion through pollen and unexpected effects on non-target organisms, are now receiving attention. The issues on *Hevea* rubber transgenic plants can be broadly grouped into concerns about:

Damage to human health and natural environment

Since *Hevea* is not used as a food crop, there is no such food safety issue with transgenic *Hevea* plants. Gene flow from transgenic crops to others requires: 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 3) the presence of a pollinating agent such as a bird or an insect unless, the likelihood that transgenes will spread can be different for each crop and wild combination in each area of the world. Unlike other crops, there are no wild relatives of *Hevea* in the nature. If pollen grains from transgenic *Hevea* plants are released in these areas they do not encounter any compatible plants to pollinate, so there is no risk of gene flow. Also rubber seedlings are developed via budgrafting technique, so the risk of gene transfer is much insignificant.

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 an organic 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 neighbour's crop. It is important to ensure that hybridization is not occurring in the field. Many agencies publish recommended minimum separation distances for a variety of crops. These distances have been developed 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 GM pollen pollinates plants in neighbouring 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 *Hevea* transgenic plants are concerned, it takes about 6 years for the first flowering, so the crop suitability can be assessed before pollen production. The main objective of *Hevea* transformation is to produce plants for enhanced latex yield, TPD tolerance, disease resistance and recombinant protein production. Therefore the impact of any gene escape to the environment or neighbor field is expected to be remote with transgenic *Hevea* plants. Also, the ultimate product from transgenic *Hevea* plant is latex with protein and it is to be purified from latex so neither the inserted gene not the selection marker gene is thus available to the consumer.

CONCLUSIONS AND FUTURE PROSPECTS

In *Hevea* rubber tree species, there has been tremendous progress in *in vitro* culture research. In the last decade several protocols have been developed for *in vitro* mass propagation of *Hevea*. Micropropagation of superior rubber clones via somatic embryogenesis has had considerable impact on production of uniform plants. Few protocols are available for micropropagation by using immature anther and inflorescence explants from elite mature rubber trees. In these reports it is evident that there is a possibility to overcome some of the inherent problems of large scale production and establishment of rubber plants. However, in many instances the extent of rejuvenation through tissue culture techniques is insufficient to permit commercial propagation. The importance of selecting the appropriate explants of rubber tree for the initiation of *in vitro* cultures should receive more attention. Moreover, the techniques are not yet sufficiently refined for commercial application. A number of large-scale field trials with *in vitro* derived clones are in progress and it is expected that these will reveal whether commercial application is possible or not. The field trials will determine the current potential of micropropagation techniques for commercial scale cultivation of rubber. *In vitro* approaches are presently being applied to *Hevea* rubber tree to achieve genetic transformations. These techniques are likely to play a vital role in future tree-improvement programs. More refinement in the protocols is essential in understanding the requirements for efficient plant regeneration. With recent research developments, molecular biology provides tools that may allow genetic improvement to make up lost ground. The genetic transformation provides opportunities for creating new clones with important agronomic traits otherwise unavailable, such as TPD tolerance, improvement of growth, latex biosynthesis, and wood quality and a reduction in the expression of endogenous genes that encode undesirable traits. It is obvious that the results obtained from the above mentioned molecular marker techniques have contributed to the understanding of clonal relationships, origin and distribution and are potential tools in germplasm evaluation and genetic characterization of traits with agronomical interest. Molecular investigations provide a basis for understanding the regulation of various genes and their potential involvement in the complex rubber biosynthetic pathway. Using the identified genes, it is possible to determine the rate limiting steps and the regulation points or controlling events in latex biosynthesis at least at the transcriptional level. Although considerable progress was achieved in annual crops during the last decade, many biotechnological approaches are at a preliminary stage in rubber tree. Moreover, the application of biotechnology to rubber tree requires further investigations on the risk of genetic instability in transgenic plants and on potential escapes of transgenes into wild plants.

A combination of tissue culture and molecular biology may synergistically provide great benefit to the improvement of rubber tree. All available biotechnological tools must be employed to meet the challenges of sustainable agriculture and rubber security in the world. Scientists are investigating new biotechniques that may have great applications in the genetic enhancement of rubber tree. However, biotechnology should be integrated along with other existing techniques in current *Hevea* improvement programs. Today, biotechnology offers to *Hevea* improvement programs clean and fast multiplication of clones via micropropagation, and genetic markers for assisted selection. Rubber biotechnology has made a first phase impact. If current progress in *in vitro* culture and genetic transformation combined with molecular biology applications continues, the future may witness superior *Hevea* tree species tailored for special agronomic and economic characteristics.

ACKNOWLEDGEMENTS

We wish to thank Drs. James Jacob, Director, Annamma Vargheese, (Joint Director), RRII for their constant encouragement.

REFERENCES

Adiwilaga K, Kush A (1996) Cloning and characterization of cDNA encoding farnesyl diphosphate synthase from rubber tree (*Hevea brasiliensis*). *Plant Molecular Biology* **30**, 935-946

Arokiaraj P (2000) Genetic transformation of *Hevea brasiliensis* (rubber tree) and its applications towards crop improvement and production of recombinant proteins of commercial value. In: Jain SM, Minocha SC (Eds) *Molecular Biology of Woody Plants*, Kluwer Academic Publishers, The Netherlands, pp 305-325

Arokiaraj P, Jaafar H, Hamzah S, Yeang HY, Wan AR (1995) Enhancement of *Hevea* crop potential by genetic transformation: HMGR activity in trans-

formed tissue. Proc. IRRDB Sym. Physiol. Mol. Aspects of the Breeding of *Hevea brasiliensis*. Penang, Malaysia, pp 74-82

Arokiaraj P, Jones H, Cheong KF, Coomber S, Charlwood BV (1994) Gene insertion into *Hevea brasiliensis*. *Plant Cell Reports* **13**, 425-430

Arokiaraj P, Jones H, Jaafar H, Coomber S, Charlwood BV (1996) *Agrobacterium*-mediated transformation of *Hevea* anther calli and their regeneration into plantlets. *Journal of Natural Rubber Resesarch* **11**, 77-87

Arokiaraj P, Ruker F, Obermayr E, Shamsul Bahri AR, Hafsah J, Carter DC, Yeang HY (2002) Expression of human serum albumin in transgenic *Hevea brasiliensis*. *Journal of Natural Rubber Research* **5**, 157-166

Arokiaraj P, Wan AR (1991) *Agrobacterium*-mediated transformation of *Hevea* cells derived from *in vitro* and *in vivo* seedling cultures. *Journal of Natural Rubber Ressearch* **6**, 55-61

Arokiaraj P, Yeang HF, Cheong KF, Hamzah S, Jones H, Coomber S, Charlwood BV (1998) CaMV 35S promoter directs β-glucuronidase expression in the laticiferous system of transgenic *Hevea brasiliensis*. *Plant Cell Reports* **17**, 621-625

Asawatreratanakul K, Zhang YW Wititsuwannakul D, Wititsuwannakul R, Takahashi S, Rattanapittayaporn A, Koyama T (2003) Molecular cloning, expression and characterization of cDNA encoding *cis*-prenyltransferases from *Hevea brasiliensis*: A key factor participating in natural rubber biosynthesis. *European Journal of Biochemistry* **270**, 4671-4680

Asokan MP, Kumari Jayasree P, Sushamakumari S (1992) Plant regeneration by somatic embryogenesis of rubber tree (*Hevea brasiliensis*). *International Natural Rubber Conference*, Bangalore, India, p 49

Asokan MP, Kumari Jayasree P, Thomas V, Sushamakumari S, Kala RG, Jayashree R, Sethuraj, MR, Thulaseedharan A (2002) Influence of 2, 4-D and sucrose on repetitive embryogenesis in rubber (*Hevea brasiliensis* Muell. Arg. Cv. GT1). *Journal of Tree Sciences* **21**, 18-26

Asokan MP, Narasimhan K, Kumari Jayasree P, Thulaseedharan A, Sushamakumari S, Kala RG, Jayashree R (2001) Isozyme markers for distinguishing embryogenic and nonembryogenic callus during somatic embryogenesis *Hevea brasiliensis* (Muell.) Arg. *Journal of Tree Sciences* **20**, 78-85

Atan S, Low FC, Saleh NM (1996) Construction of a microsatellite enriched library from *Hevea brasiliensis. Journal of Natural Rubber Research* **11**, 247-255

Attanyaka DP, Kekwick RG, Franklin FC (1991) Molecular cloning and nucleotide sequencing of rubber elongation factor gene from *Hevea brasiliensis*. *Plant Molecular Biology* **16**, 1079-1081

Auboiron E, Carron MP, Michaux-Ferriere N (1990) Influence of atmospheric gases particularly ethylene on somatic embryogenesis of *Hevea brasiliensis. Plant Cell, Tissue and Organ Culture* **21**, 31-37

Backhaus RA (1985) Rubber formation in plants - mini review. *Israel Journal of Botany* **34**, 283-93

Besse P, Lebrun P, Seguin M, Lanaud C (1993b) DNA fingerprints in *Hevea brasiliensis* (rubber tree) using human minisatellite probes. *Heredity* **70**, 237-244

Besse P, Seguin M, Lebrun P, Chevallier MH, Nicolas D, Lanaud C (1994) Genetic diversity among wild and cultivated populations of *Hevea brasiliensis* assessed by nuclear RFLP analysis. *Theoretical and Applied Genetics* **88**, 199-207

Besse P, Seguin M, Lebrun P, Lanaud C (1993a) Ribosomal DNA variations in wild and cultivated rubber tree (*Hevea brasiliensis*). *Genome* **36**, 1049-1057

Bindu Roy C, Nazeer MA, Saha T (2004) Identification of simple sequence repeats in rubber (*Hevea brasiliensis*). *Current Science* **87**, 807-811

Blanc G, Baptiste C, Oliver G, Martin F, Montoro P (2005) Efficient *Agrobacterium tumefaciens* mediated transformation of embryogeneic calli and regeneration of *Hevea brasiliensis* Mull. Arg. plants. *Plant Cell Reports* **24**, 724-733

Blanc G, Lardet L, Martin A, Jacob JL, Carron MP (2002) Differential carbohydrate metabolism conducts morphogenesis in embryogenic callus of *Hevea brasiliensis* (Muell. Arg.). *Journal of Experimental Botany* **53**, 1453-1462

Blanc G, Michaux-Ferriere N, Teisson C, Lardet L, Carron MP (1999) Effects of carbohydrate addition on the induction of somatic embryogenesis in *Hevea brasiliensis. Plant Cell, Tissue and Organ Culture* **59**, 103-112

Blanc G, Rodier-Goud M, LidahYJ, Clément-Demange A, Seguin M (2001) Study of open pollination in *Hevea* using microsatellites. *Plant Research and Development* **8**, 68-71

Broekaert I, Lee HI, Kush A, Chua NH, Raikhel N (1990) Wound-induced accumulation of mRNA containing a hevein sequence in laticifers of rubber tree (*Hevea brasiliensis*). *Proceedings of the National Academy of Sciences USA* **87**, 7633-7637

Cailloux F, Guerrier JJ, Linossier L, Coudret A (1996) Long term embryogenesis and maturation of somatic embryos in *Hevea brasiliensis. Plant Science* **120**, 185-196

Cailloux M, Lleras E (1979) Fusão de protoplastos de *Hevea brasiliensise* e *Hevea pauciflore*. *Estabelecimento de Técnica Acta Amazonica* **9**, 9-13

Carron MP, d'Auzac J, Etienne H, EI Hadrami I, Housti F, Michaux-Ferriere N, Montoro P (1992) Biochemical and histological features of somatic embryogenesis in *Hevea brasiliensis. Indian Journal of Natural Rubber Research* **5**, 7-17

Carron MP, Enjalric E (1982) Studies on vegetative micropropagation of *Hevea brasiliensis* by somatic embryogenesis and *in vitro* on microcutting. In: Fujiwara A (Ed) *Plant Tissue Culture*, Maruzen, Tokyo, pp 751-752

Carron MP, Enjalric F (1983) Perspectives du microbouturage de l'*Hevea brasiliensis*. *Caoutchoucs et Plastiques*, pp 627-628

Carron MP, Enjalric E (1985) Somatic embryogenesis from inner integument of the seed of *Hevea brasiliensis* (Muell. Arg.). *Comptes Rendus de L'Academic des Sciences, Paris Series III* **300**, 653-658

Carron MP, Enjalric F, Lardet L, Deschamps A (1989) Rubber (*Hevea brasiliensis* Müll. Arg.). In: Bajaj YPS (Ed) *Biotechnology in Agriculture and Forestry* (Vol 5), Springer-Verlag, Berlin, pp 222-245

Carron MP, Etienne H, Michaux-Ferrière N, Montoro P (1995a) Somatic embryogenesis in Rubber tree (*Hevea brasiliensis* Müll. Arg.). In: Bajaj YPS (Ed) *Biotechnology in Agriculture and Forestry (Vol 30) Somatic Embryogenesis and Synthetic Seed I*, Springer, Berlin, pp 353-369

Carron MP, Etienne H, Lardet L, Campagna S, Perrin Y, Leconte A, Chaine C (1995b) Somatic embryogenesis in rubber tree (*Hevea brasiliensis* Müll. Arg.). In: Jain S, Gupta P, Newton R (Eds) *Somatic Embryogenesis in Woody Plants* (Vol 2), Kluwer, Dordrecht, pp 117-136

Carron MP, Lardet L, Montoro P (2005) Different ways of integrating *in vitro* culture in *Hevea* planting material propagation. IRRDB Conference, Cochin, Nov. 7-11 2005, pp 79-82

Carron MP, Lardet L, Julien J, Boko C (2001) Somatic embryogenesis in *Hevea brasiliensis* (Muell.Arg.) Current advances and limits. In: Sainte-Beuve J (Ed) *Annual IRRDB Meeting*, CIRAD, Montpellier, France, pp 110-118

Carron MP, le Roux Y, Tison J, Dea BG, Caussanel V, Clair J, Keli J (2000) Compared root system architectures in seedlings and *in vitro* plantlets of *Hevea brasiliensis*, in the initial years of growth in the field. *Plant and Soil* **223**, 73-85

Cazuax, d'Auzac J (1994) Micro callus formation from *Hevea brasiliensis* protoplasts isolated from embryonic callus. *Plant Cell Reports* **13**, 272-276

Cazuax, d'Auzac J (1995) Explanation for the lack of division of protoplasts from stems of rubber tree (*Hevea brasiliensis*). *Plant Cell, Tissue and Organ Culture* **41**, 211-219

Charbit E, Legavre T, Lardet L, Bourgeois E, Ferrière N, Carron MP (2004) Identification of differentially expressed cDNA sequences and histological characteristics of *Hevea brasiliensis* calli in relation to their embryogenic and regenerative capacities. *Plant Cell Reports* **22**, 539-548

Chen F, Chen C, Wang Chang S, Hsu H, Ho Y, Li UT (1978) Obtain-ing pollen plants of *Hevea brasiliensis* Muell Arg. *Proc. Symp. Plant Tissue Culture*, Peking, China, pp 11-22

Chen F, Chen C, Wang X, Chang S, Hsu H, Ho Y, Li UT (1979) A process of obtaining pollen plants of *Hevea brasiliensis* Muell Arg. *Scientia Sinica* **XXII**, 81-90

Chen Z, Qian C, Qin M, Wang C, Suo C, Xiao Y, Xu X (1981) Relationship between somatic cells and microspores in the process of of anther culture of *Hevea brasiliensis* Muell. Arg. *Acta Botanica* **1**, 1-7

Chen Z, Qian C, Qin M, Xu X, Xiao Y (1982) Recent advances in anther culture of *Hevea brasiliensis* (Muell. Arg). *Theoretical and Applied Genetics* **62**, 103-138

Chen Z (1984) Rubber (*Hevea*). In: Sharp R, Ammirato DA, Yamada Y (Eds) *Handbook of Plant Cell Culture (Vol 2) Crop Species*, McMillan Publishers, pp 546-571

Chen S, Peng S, Huang G, Wu K, Fu X, Chen Z (2003) Association of decreased expression of a Myb transcription factor with the TPD (tapping panel dryness) syndrome in *Hevea brasiliensis*. *Plant Molecular Biology* **51**, 51-58

Chevallier MH (1988) Genetic variability of *Hevea brasiliensis* germplasm using isozyme markers. *Journal of Natural Rubber Research* **3**, 42-53

Chrestin H, Gidrol X, Kush A (1997) Towards a latex molecular diagnostic of yield potential and the genetic engineering of the rubber tree. *Euphytica* **96**, 77-82

Chye ML, Tan CT, Chua NH (1992) Three genes encode 3-hydroxy-3-methylglutaryl-coenzyme A reductase in *Hevea brasiliensis*: hmg1 and hmg3 are differentially expressed. *Plant Molecular Biology* **3**, 473-484

Chye ML, Cheung KY (1995) β-1,3-glucanase is highly-expressed in laticifers of *Hevea brasiliensis*. *Plant Molecular Biology* **29**, 397-402

Clement-Demange A, Priyadarshan PM, Hoa TTT, Venkatachalam P (2007) *Hevea* rubber breeding and genetics. In: Janick J (Ed) *Plant Breeding Reviews*, John Wiley and Sons Inc., USA, pp 177-283

Das K, Sinha RR, Potty SN, Sethuraj MR (1994) Embryogenesis from anther derived callus of *Hevea brasiliensis* Muell. Arg. *Indian Journal of Hill Farming* **7**, 90-95

Dennis MS, Light DR (1989) Rubber elongation factor from *Hevea brasiliensis.* Identification, characterization and role in rubber biosynthesis. *Journal of Biological Chemistry* **264**, 18608-18617

Dian K, Sangare A, Diopoh JK (1995) Evidence for specific variation of protein pattern during tapping panel dryness condition development in *Hevea brasiliensis. Plant Science* **105**, 207-216

EI Hadrami I, Michaux-Ferriere N, Carron MP, d'Auzac J (1989) Polyamines a possible limiting factor in somatic embryogenesis of *Hevea brasiliensis*. *Comptes Rendus de l'Academic des Sciences, Paris Series* **308**, 205-211

EI Hadrami I, Carron MP, d'Auzac J (1991) Influence of exogenous hormone on somatic embryogenesis in *Hevea brasiliensis. Annals of Botany* **67**,

511-15

EI Hadrami I, d'Auzac J (1992) Effects of polyamine biosynthetic inhibitors on somatic embryogenesis and cellular polyamines in *Hevea brasiliensis. Journal of Plant Physiology* **140**, 33-36

Engelmann F, Lartaud M, Chabrillange N, Carron MP, Etienne H (1997) Cryopreservation of embryogenic calluses of two commercial clones of *Hevea brasiliensis. Cryo Letters* **18**, 107-116

Enjalric F, Carron MP (1982) Microbouturage *in vitro* de jeunes plants d'*Hevea brasiliensis* (Kunth) Muell. Arg. *Comptes Rendus de l'Academic des Sciences, Paris Series* **295**, 259-264

Etienne H, Berger A, Carron MP (1991) Water status of callus from *Hevea brasiliensis* during induction of somatic embryogenesis. *Physiologia Plantarum* **82**, 213-218

Etienne H, Montoro P, Michaux-Ferriere N, Carron MP (1993a) Effects of desiccation, medium osmolarity and abscisic acid on the maturation of *Hevea brasiliensis* somatic embryos. *Journal of Experimental Botany* **44**, 1613-1619

Etienne H, Sotta B, Montoro P, Miginiac E, Carron MP (1993b) Relationship between exogenous growth regulators and endogeous indole-3-aectic acid and abscisic acid in the expression of somatic embryogenesis in *Hevea brasiliensis* (Muell. Arg.). *Plant Science* **88**, 91-96

Etienne H, Lartaud M, Michaux-Ferriere N, Carron MP, Berthouly M, Teisson C (1997a) Improvement of somatic embryogenesis in *Hevea brasiliensis* (Muell. Arg.) using temporary immersion technique. *In Vitro Cellular and Developmental Biology – Plant* **33**, 81-87

Etienne H, Lartaud M, Carron MP, Michaux-Ferriere N (1997b) Use of calcium to optimize long term proliferation of friable calluses and plant regeneration in *Hevea brasiliensis* (Muell. Arg.). *Journal of Experimental Botany* **48**, 129-137

Gandimathi H, Paranjothy K (1975) Anther culture attempts at induction of haploidy in *Hevea* and other plants. *Proceedings of National Plant Tissue Culture Symposium*, Kuala Lumpur, Malaysia, pp 32-35

Goyvaerts E, Dennis M Light D, Chua NH (1991) Cloning and sequencing of cDNA encoding the Rubber Elongation Factor of *Hevea brasiliensis*. *Plant Physiology* **97**, 317-321

Gunatilleke ID, Samaranayake G (1988) Shoot tip culture as a method of micropropagation of *Hevea*. *Journal of Rubber Research Institure of Sri Lanka* **68**, 33-44

Han K, Shin DO, Yang J, Kim IJ, Oh SK, Chow KS (2000) Genes expressed in the latex of *Hevea brasiliensis. Tree Physiology* **20**, 503-510

Haris Ndarussamin A, Dodd WA (1993) Isolation of rubber tree (*Hevea brasiliensis* Muell. Arg) protoplasts from callus and cell suspensions. *Menara-Perkebunan* **61**, 25-31

Jayashree R, Rekha K, Sushamakumari S, Thulaseedharan A (2005) Establishment of callus cultures from isolated microspores of *Hevea brasiliensis*. *National Symposium on Biotechnological Interventions for Improvement of Horticultural Crops*, Trissur, pp 176-178

Jayashree R, Rekha K, Venkatachalam P, Uratsu SL, Dendekar AM, Kumari Jayasree P, Kala RG, Priya P, Sushamakumari S, Sobha S, Asokan MP, Sethuraj MR, Thulaseedharan A (2003) Genetic transformation and regeneration of rubber tree (*Hevea brasiliensis* Muell. Arg) transgenic plants with a constitutive version of an anti-oxidative stress superoxide dismutase gene. *Plant Cell Reports* **22**, 201-209

Kala RG, Leda Pavithran, Thulaseedharan A (2004) Effect of fungicides and antibiotics to control microbial contamination in *Hevea* cultures. *Plant Cell Biotechnology and Molecular Biology* **5**, 51-58

Kala RG, Kumari Jayasree P, Sushamakumari S, Jayashree R, Rekha K, Sobha S, Thulaseedharan A (2005) *In vitro* regeneration of *Hevea brasiliensis* from leaf explants. *ICAR National Symposium on Biotechnological Interventions for Improvement of Horticultural Crops: Issues and Strategies*, Trichur, India, pp 105-106

Kala RG, Kumari Jayasree P, Sobha S, Rajani J, Thulaseedharan A (2006) Plant regeneration from leaf derived somatic embryos of *Hevea brasiliensis*. *International Symposium on Frontiers in Genetics and Biotechnology – Retrospect and Prospect*, Hyderabad, India, p 160

Ko JH, Chow KS, Han KH (2003) Transcriptome analysis reveals novel features of the molecular events occurring in the laticifers of *Hevea brasiliensis* (Para rubber tree). *Plant Molecular Biology* **53**, 479-492

Kumari Jayasree P, Asokan MP, Sobha S, Sankariammal L, Rekha K, Kala RG, Jayashree R, Thulaseedharan A (1999) Somatic embryogenesis and plant regeneration form immature anthers of *Hevea brasiliensis* (Muell. Arg.). *Current Science* **76**, 1242-1245

Kumari Jayasree P, Vinoth Thomas, Thulaseedharan A (2001) Optimization of parameters affecting somatic embryogenesis in *Hevea brasiliensis. Indian Journal of Natural Rubber Research* **14**, 20-29

Kumari Jayasree P, Thulaseedharan A (2001) Gibberllic acid regulated embryo induction and germination in *Hevea brasiliensis* (Muell. Arg.). *Indian Journal of Natural Rubber Research* **14**, 106-111

Kumari Jayasree P, Thulaseedharan A (2004) Initiation and maintenance of long term somatic embryogenesis in *Hevea brasiliensis*. *IRRDB, Biotechnology Workshop*, Kuala Lumpur, Malaysia, 56 pp

Kumari Jayasree P, Thulaseedharan A (2005) *In vitro* germination of *Hevea* somatic embryos: Effect of cytokinins. *Plant Cell Biotechnology and Molecular Biology* **6**, 61-64

Kush A, Goyvaerts E, Chye ML, Chua NH (1990) Laticifer specific gene expression in *Hevea brasiliensis* (rubber tree). *Proceeding of the National Academy of Sciences USA* **87**, 1787-1790

Lander BS, Botstein D (1989) Mapping mendelian factors underlying quantitative traits using RFLP linkage maps. *Genetics* **121**, 185-199

Lardet L, Piombo G, Orioi F, Dechamp E, Carron MP (1999) Relations between biochemical characteristics and conversion ability in *Hevea brasiliensis* zygotic and somatic embryos. *Canadian Journal of Botany* **77**, 1168-1177

Lardet L, Martin F, Dessailly F, Carron MP, Montoro P (2007) Effect of exogenous calcium on post-thaw growth recovery and subsequent plant regeneration of cryopreserved embryogenic calli of *Hevea brasiliensis* (Mull. Arg.). *Plant Cell Reports* **26**, 559-569

Leconte A, Lebrun P, Nicolas D, Seguin M (1994) Electrophoresis application to *Hevea* clone identification. *Plant Research and Dévelopment* **1**, 28-36

Leconte A, le Guen V, Rodier-Goud M, Seguin M (1997) Germplasm characterization and clonal identification through leaves zymogram analysis. *Seminar: The Biochemical and Molecular Tools for Exploitation Diagnostic and Rubber Tree Improvement*, 20-22 Oct., Faculty of Science, Mahidol Univ, Bangkok, Thailand, (Mahidol Univ Rrit, Orstom and Cirad)

le Guen V, Lespinasse D, Oliver G, Rodier Goud M, Pinard F, Seguin M (2003) Molecular mapping of genes conferring field resistance to South American leaf blight (*Microcyclus ulei*) in rubber tree. *Theoretical and Applied Genetics* **108**, 160-167

le Guen V, Rodier-Goud M, Troispoux V, Xiong T, Brottier P, Billotand C, Seguin M (2004) Characterization of polymorphic microsatellite markers for *Microcyclus ulei*, causal agent of South American leaf blight of rubber trees. *Molecular Ecological Notes* **4**, 122-124

le Guen V, Garcia D, Mattos C R R, Doaré F, Lespinasse D, Seguin M (2007) Bypassing of a polygenic *Microcyclus ulei* resistance in rubber tree, analyzed by QTL detection. *New Phytologist* **173**, 335-345

Lekawipat N, Teerawatanasuk K, Vanavichit A, Toojinda T, Tragoonrung S (2003a) Evaluating the genetic relatedness of wild and cultivated *Hevea brasiliensis* accessions with SSCP markers. *SABRAO Journal of Breeding and Genetics* **35**, 123-134

Lekawipat N, Teerawatannasuk K, Rodier-Goud M, Seguin M, Vanavichit A, Toojinda T, Tragoonrung S (2003b) Genetic diversity analysis of wild germplasm and cultivated clones of *Hevea brasiliensis* Muell. Arg. by using microsatellite markers. *Journal of Rubber Research* **6**, 36-47

Lespinasse D, Rodier-Goud M, Grivet L, Leconte A, Legnaté H, Seguin M (2000a) A saturated genetic linkage map of rubber tree (*Hevea* spp.) based on RFLP, AFLP, microsatellite and isozyme markers. *Theoretical and Applied Genetics* **100**, 127-138

Lespinasse D, Grivet L, Troispoux V, Rodier-Goud M, Pinard F, Seguin M (2000b) Identification of QTLs involved in the resistance to South American leaf blight (*Microcyclus ulei*) in the rubber tree. *Theoretical and Applied Genetics* **100**, 975-984

Lidah YJ (2005) Contribution à l'amélioration génétique de l'hévéa (*Hevea brasiliensis* Muell. Arg.) par l'étude du mode de reproduction de populations sauvages en vergers à graines. PhD Thesis, l'Université de Cocody, Abidjan, Ivory Coast

Light DR, Lazarus RA, Dennis MS (1989) Rubber elongation by farnesyl pyrophosphate synthases involves a switch in enzyme stereospecificity. *Journal of Biological Chemistry* **264**, 18598-18607

Linossier L, Veisseire P, Cailloux F, Coudret A (1997) Effect of abscisic acid and high concentration of PEG on *Hevea brasiliensis* somatic embryo development. *Plant Science* **124**, 183-191

Lloyd GB, McCown BH (1980) Commercially feasible micropropagation of mountain laurel (*Kalmia latifolia*) by use of shoot tip culture. *Proceedings of the International Plant Propagators' Society* **30**, 421-437

Low FC, Atan S, Jaafar H, Tan H (1996) Recent advances in the development of molecular markers for *Hevea* studies. *Journal of Natural Rubber Research* **11**, 32-44

Luo H, Boutry M (1995) Phylogenetic relationships within *Hevea brasiliensis* as deduced from a polymorphic mitochondrial DNA region. *Theoretical and Applied Genetics* **91**, 876-884

Luo H, van Coppenolle B, Seguin M, Boutry M (1995) Mitochondrial DNA polymorphism and phylogenetic relationships in *Hevea brasiliensis. Molecular Breeding* **1**, 51-63

Marshall TC, Slate J Kruuk LEB, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology* **7**, 639-655

Martre P, Lacan D, Just D, Teisson C (2001) Physiological effects of temporary immersion on *Hevea brasiliensis* callus. *Plant Cell, Tissue and Organ Culture* **67**, 25-35

Mendanha ABL, de Almeida Torres RA, de Barros Freire A (1998) Micropropagation of rubber tree (*Hevea brasiliensis* Muell. Arg.). *Genetics and Molecular Biology* **21**, 1415

Mia Z, Gaynor JJ (1993) Molecular cloning, characterization and expression of Mn-superoxide dismutase from the rubber tree (Hevea *brasiliensis*). *Plant Molecular Biology* **23**, 267-277

Michaux-Ferriere N, Carron MP (1989) Histology of early somtic embryogenesis in *Hevea brasiliensis:* The importance of the timing of the subculturing.

Plant Cell, Tissue and Organ Culture **19**, 243-256

Michaux-Ferriere N, Grout H, Carron MP (1991) Origin and ontogenesis of somtic embryos in *Hevea brasiliensis* (*Euphorbiaceae*). *American Journal of Botany* **79**, 174-180

Montoro P, Etienne H, Michaux-Ferriere N, Carron MP (1993) Callus friability and somatic embryogenesis in *Hevea* brasiliensis. *Plant Cell, Tissue and Organ Culture* **33**, 331-338

Montoro P, Etienne H, Carron MP (1995) Effect of calcium on callus friability and somatic embryogenesis in *Hevea* brasiliensis Mull. Arg. Relations with callus mineral nutrition, nitrogen metabolism and water parameters. *Journal of Experimental Botany* **46**, 255-261

Montoro P, Tcinscree N, Rattana W, Kongsawadworakul P, Michaux-Ferriere N (2000) Effect of exogenous calcium on *Agrobacterium tumefaciens*-mediated gene transfer in *Hevea brasiliensis* (rubber tree) friable calli. *Plant Cell Reports* **19**, 851-855

Montoro P, Rattana W, Pugade-Renaud V, Michaux-Ferriere N, Monkolsook Y, Kanthapura R, Adunsadthapong S (2003) Production of *Hevea brasiliensis* transgenic embryogenic callus lines by *Agrobacterium tumefaciens*: roles of calcium. *Plant Cell Reports* **21**, 1095-1102

Muangkaewngam A, Te-Chato S (1992) *In vitro* zygotic embryo of rubber (*Hevea brasiliensis* Muell. Arg.) at low temperature. *Journal of Agriculture* **8**, 273-280

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

Oh SK, Kang H, Shin DH, Yang J, Han KH (2000) Molecular cloning and characterization of a functional cDNA clone encoding isopentenyl disphosphate isomerise from *Hevea brasiliensis*. *Journal of Plant Physiology* **157**, 549-557

Oh SK, Kang H, Shin DH, Yang J, Chow K-S, Yeang HY, Wagners B, Breiteneder H, Han K-H (1999) Isolation, characterization and functional analysis of a novel cDNA clone encoding a small rubber particle protein from *Hevea brasiliensis*. *The Journal of Biological Chemistry* **274**, 17132-17138

Paiva JR, Kageyama PY, Vencorsky R, Contel PB (1994a) Genetics of rubber tree *Hevea brasiliensis* (Willd. *ex* Adr. de Juss.) Muell. Arg. 1. Genetic variation in natural populations. *Silvae Genetica* **43**, 307-312

Paranjothy K (1974) Induced root and embryoid differentiation in *Hevea* tissue culture. *3rd International Congress Plant Tissue Cell Culture*, University, Leicester, Abstract No 67

Paranjothy K, Ghandimathi H (1975) Morphogenesis in callus cultures *Hevea* brasiliensis Muell. Arg. *Proc. Natl. Plant Tissue Culture Symposium*, Kuala Lumpur, pp 19-25

Paranjothy K, Gandhimathi H (1976) Tissue and organ culture of *Hevea*. *Proc. Intl. Rubber Conference 1975* (Vol 2), Kuala Lumpur, Malaysia, pp 59-84

Paranjothy K, Rohani O (1978) Embryoid and plantlet development from cell culture of *Hevea*. *4th International Congress Plant Tissue Cell Culture*, University Calgary, Abstract No 134

Perrin Y, Lardet L, Enjalric F, Carron MP (1994) Rajeunissement de clones matures d'*Hevea brasiliensis* (Müll. Arg.) par microgreffage *in vitro*. *Canadian Journal of Plant Science* **74**, 623-630

Perrin Y, Doumas P, Lardet L, Carron M P (1997) Endogenous cytokinins as a biochemical marker of rubber tree (*Hevea brasiliensis* Mull.Arg.) clone rejuvenation. *Plant Cell, Tissue and Organ Culture* **47**, 239-245

Prapan K, Clément-Demange A, Teerawatanasuk K, Rodier-Goud M, Seguin M (2004) Genetic mapping and field study of a full-sib family (RRIM600 x PB217) in *Hevea brasiliensis* (Genmap project). First results. CIRAD Seminar: *Towards the Improvement of the Productivity of the Rubber Tree*, 27-28 May, Kasetsart Univ., Bangkok

Priya P, Venkatachalam P, Thulaseedharan A (2006) Molecular cloning and characterization of the rubber elongation factor gene and its promoter sequence from rubber tree (*Hevea brasiliensis*): A gene involved in rubber biosynthesis. *Plant Science* **171**, 470-480

Priyadarshan PM (2003) Breeding *Hevea brasiliensis* for environmental constraints. *Advances in Agronomy* **79**, 351-400

Priyadarshan PM, Gonçalves P de S (2003) *Hevea* gene pool for breeding. *Genetics Research and Crop Evolution* **50**, 101-114

Priyadarshan PM, Clément-Demange A (2004) Breeding *Hevea* rubber: Formal and molecular genetics. *Advances in Genetics* **52**, 51-115

Pujade-Renaud V, Sanier C, Cambillau L, Arokiaraj P, Jones H, Ruengsri N, Tharreau D, ChrestinH, Montoro P, Narangajavana J (2005) Molecular characterization of new members of the *Hevea brasiliensis* hevein multigene family and analysis of their promoter region in rice. *Biochemica and Biophysica Acta* **1727**, 151-161

Rekha K, Jayashree R, Kumary Jayasree P, Venkatachalam P, Thulaseedharan A (2006) An efficient protocol for *Agrobacterium*-mediated genetic transformation in rubber tree (*Hevea brasiliensis*). *Plant Cell Biotechnology and Molecular Biology* **7**, 155-158

Rohani O, Paranjothi K (1980) Isolation of *Hevea* protoplasts. *Journal of Rubber Research Institute of Malaysia* **28**, 61-66

Sachuthananthavale R, Irugalbandra ZE (1972) Propagation of callus from *Hevea* anthers. *Quarterly Journal of Rubber Research Institute of Ceylon* **49**, 65-68

Sachuthananthavel R (1973) *Hevea* tissue culture. *Quarterly Journal of Rubber Research Institute of Ceylon* **50**, 91-97

Saha T, Roy B, Nazeer MA (2005) Microsatellite variability and its use in the characterization of cultivated clones of *Hevea brasiliensis*. *Plant Breeding* **124**, 86-92

Schultes RE (1990) *A Brief Taxonomic View of the Genus Hevea*. Kuala Lumpur, MRRDB Monograph, p 14

Seguin M, Flori H, Legnaté, Clément-Demange A (2003) Rubber tree. In: Hamon P, Seguin MA, Perrier X, Glaszmann JC (Eds) *Genetic Diversity of Tropical Crops*, CIRAD, France and Science Publ., Enfield, USA, pp 277-306

Seneviratne P (1991) Micropropagation of juvenile and mature *Hevea brasiliensis*. PhD Thesis, University of Bath, UK, 278 pp

Seneviratne P, Wijesekara GAS, De Zoysa GM (1995) Root system of hevea with special reference to micropropagated plants. *Journal of the Rubber Research Institute of Sri Lanka* **76**, 11-20

Seneviratne P, Flagmann A (1996) The effect of thidiazuron on axillary shoot proliferation of *Hevea brasiliensis in vitro*. *Journal of the Rubber Research Institue of Sri Lanka* **77**, 1-14

Seneviratne P, Wijesekara GAS (1996) The problem of phenolic exudates in *in vitro* cultures of mature *Hevea brasiliensis*. *Journal of Plantion Crops* **24**, 54-62

Shijie Z, Zhenghua C, Xueng X (1990) A summary report on anther culture for haploid plants of *Hevea brasiliensis*. In: *Proc. IRRDB Symposium on the Breeding of Hevea brasiliensis*, Kumming, China, pp 69-78

Sivasubramaniam S, Vanniasingham VM, Tan CT, Chua NH (1995) Characterisation of HEVER, a novel stress-induced gene from *Hevea brasiliensis*. *Plant Molecular Biology* **29**, 173-178

Sirinupong N, Suwanmanee P, Doolittle RF, Suvachitanont W (2005) Molecular cloning of a new cDNA and expression of 3-hydroxy-3-methylglutaryl-CoA synthase gene from *Hevea brasiliensis*. *Planta* **221**, 502-512

Sobha S, Sushamakumari S, Thanseem I Kumari Jayasree P, Rekha K, Jayashree R, Kala RG, Asokan MP, Sethuraj MR, Dandekar AM, Thulaseedharan A (2003a) Genetic transformation of *Hevea brasiliensis* with the gene coding for superoxide dismutase with FMV 34S promoter. *Current Science* **85**, 1767-1773

Sobha S, Sushamakumari S, Thanseem I , Rekha K, Jayashree R, Kala RG, Kumari Jayasree P, Asokan MP, Sethuraj MR, Dandekar AM, Thulaseedharan A (2003b) Abiotic stress induced over-expression of superoxide dismutase enzyme in transgenic *Hevea brasiliensis*. *Indian Journal of Natural Rubber Resesarch* **16**, 45-52

Sookmark U, Pujade-Renaud V, Chrestin H, Lacote R, Naiyanetr C, Seguin M, Romruensukharom P, Narangajavana J (2002) Characterization of polypeptides accumulated in the latex cytosol of rubber trees affected by the tapping panel dryness syndrome. *Plant Cell Physiology* **43**, 1323-1333

Sunderasan E, Wickneswari R, Aziz MZA, Yeang HY (1994) Incidence of self- and cross-pollination in two *Hevea brasiliensis* clones. *Journal of Natural Rubber Research* **9**, 253-257

Sushamakumari S, Sobha S, Jayashree R, Asokan MP (1999a) Evaluation of parameters affecting the isolation and culture of *Hevea brasiliensis* (Muell. Arg.). *Currernt Science* **77**, 1580-1582

Sushamakumari S, Rekha K, Thomas V, Sobha S, Jayashree R (1999b) Multiple shoot formation from somatic embryos of *Hevea brasiliensis. Indian Journal of Natural Rubber Research* **12**, 23-28

Sushamakumari S, Asokan MP, Anthony P, Lowe KC, Power JP, Davey MR (2000a) Plant regeneration from embryogenic cell suspension-derived protoplasts of rubber. *Plant Cell, Tissue Organ Culture* **61**, 81-85

Sushamakumari S, Sobha S, Rekha K, Jayashree R, Asokan MP (2000b) Influence of growth regulators and sucrose on somatic embryogenesis and plant regeneration from immature inflorescence of *Hevea brasiliensis. Indian Journal of Natural Rubber Research* **13**, 19-29

Sushamakumari S, Sobha S, Rekha K, Jayashree R, Thulaseedharan A (2006) Plant regeneration via somatic embryogenesis from root explants in *Hevea brasiliensis*. *International Symposium on Frontiers in Genetics and Biotechnology - Retrospect and Prospect*, Hyderabad, India, 160 pp

Suwanmanee P, Suvachittanont, W, Fincher GB (2002) Molecular cloning and sequencing of a cDNA encoding 3-hydroxy-3-methylglutaryl coenzyme A synthase from *Hevea brasiliensis* (HBK) Mull. Arg. *Science Asia* **28**, 29-36

Suwanmanee P, Sirinupong N, Suvachittanont W (2004) Regulation of the expression of 3-hydroxy-3-methylglutaryl-CoA synthase gene in *Hevea brasiliensis* (B.H.K.) Mull. Arg. *Plant Science* **166**, 531-537

Takaya A, Zhang YW, Asawatreratanakul K, Wititsuwannakul D, Wititsuwannakul R, Takahashi S, Koyama T (2003) Cloning, expression and characterization of a functional cDNA clone encoding geranylgeranyl diphosphate synthase of *Hevea brasiliensis*. *Biochimica et Biophysica Acta* **1625**, 214-220

Te-Chato S, Muangkaewngam A (1992) Tissue culture of rubber I: *In vitro* micropropagation of rubber. *Songklanakarin Journal of Science and Technology* **14**, 123-132

Te-Chato S, Chartikul M (1994) Tissue culture of rubber: Somatic embryogenesis induction from integument subsequent to plantlet regeneration. *Songklanakarin Journal of Science and Technology* **15**, 235-241

Te-Chato S, Suranilpong P (1995) Identification of rubber callus resistant to culture filtrate of *Phytophthora* spp. *Songklanakarin Journal of Science and Technology* **17**, 1-6

Teixeira da Silva JA, Nhut DT, Giang DTT, Rashid SZ (2005) The phylogeny, breeding and ecology of trees using molecular markers. In: Thangadurai D, Pullaiah T, Tripathy L (Eds) *Genetic Resources and Biotechnology* (Vol III) Regency Publications, New Delhi, India, pp 279-308

Thanseem I, Venkatachalam P, Thulaseedharan A (2003) Sequence characterization of β-1,3-glucanase gene from *Hevea brasiliensis* through genomic and cDNA cloning. *Indian Journal of Natural Rubber Research* **16**, 106-114

Thanseem I, Joseph A, Thulaseedharan A (2005) Induction and differential expression of β-1,3-glucanase mRNAs in tolerant and susceptible *Hevea* clones in response to infection by *Phytophthora meadii*. *Tree Physiology* **25**, 1361-1368

Thulaseedharan A, Kumari Jayasree P, Venkatachalam P (2000) Biotechnological approaches for crop improvement in rubber. In: Chadha KL, Ravindran PN, Leela Sahijram (Eds) *Biotechnology in Horticultural and Plantation Crops*, Malhotra Publishing House, New Delhi, India, pp 323-351

Varghese YA, Knaak C, Sethuraj MR, Ecke W (1997) Evaluation of random amplified polymorphic DNA (RAPD) markers in *Hevea brasilinesis*. *Plant Breeding* **116**, 47-52

Veisseire P, Cailloux F Coudret A (1994a) Effect of conditioned media on the somatic embrogenesis of *Hevea brasiliensis*. *Plant Physiology and Biochemistry* **32**, 571-576

Veisseire P, Linossier L, Coudret A (1994b) Effect of absicsic acid and cytokinins on the development of somatic embryos in *Hevea brasiliensis. Plant Cell, Tissue Organ Culture* **39**, 219-223

Venkatachalam P, Sailasree R, Priya P, Saraswathyamma CK, Thulaseedharan A (2001) Identification of a DNA marker associated with dwarf trait in *Hevea brasiliensis* Muell. Arg. through random amplified polymorphic DNA analysis. In: Sainte-Beuve J (Ed) Annual IRRDB Meeting. CIRAD, Montpellier, France, pp 71-79

Venkatachalam P, Thomas S, Priya P, Thanseem I, Gireesh T, Saraswathyamma CK, Thulaseedharan A (2002) Identification of DNA polymorphism with the cultivated clones of rubber tree (*Hevea brasiliensis* Muell. Arg.). *Indian Journal of Natural Rubber Research* **15**, 72-181

Venkatachalam P, Priya P, Saraswathyamma CK, Thulaseedharan A (2004) Identification, cloning and sequence analysis of a dwarf genome-specific RAPD marker in rubber tree *Hevea brasiliensis* Muell. Arg. *Plant Cell Reports* **23**, 327-332

Venkatachalam P, Raghothama KG, Thulaseedharan A (2005) Using a suppression subtractive cDNA library-based approach to identify differentially expressed genes from rubber tree (*Hevea brasiliensis*) in response to TPD syndrome. IRRDB workshop on TPD, Rubber Research Institute of India, Nov. 10, Kottayam, India, pp 151-161

Venkatachalam P, Jayashree R, Rekha K, Sushmakumari S, Sobha S, Kumari Jayashree P, Kala RG, Thulaseedharan A (2006a) Rubber tree (*Hevea brasiliensis* Muell. Arg.). In: Wang K (Ed) *Methods in Molecular Biology (Vol 344): Agrobacterium* Protocols (Vol 2), Humana Press Inc., NJ, pp 153-164

Venkatachalam P, Priya P, Gireesh T, Saraswathyamma CK, Thulaseedharan A (2006b) Molecular cloning and sequencing of a polymorphic band from rubber tree [*Hevea brasiliensis* (Muell.) Arg.]: the nucleotide sequence revealed partial homology with proline-specific permease gene sequence. *Current Science* **90**, 1510-1515

Venkatachalam P, Thulaseedharan A, Raghothama KG (2007) Identification of expression profiles of tapping panel dryness (TPD) associated genes from the latex of rubber tree (*Hevea brasiliensis* Muell. Arg.). *Planta* **226**, 499-515

Wan AR, Ghandimathi H, Rohani O, Paranjothy K (1982) Recent developments in tissue culture of *Hevea*. In: Rao AN (Ed) *Tissue Culture of Economically Important Plants*, Costed, Singapore, pp 152-158

Wang Z, Zeng X, Chen C, Wu H, Li Q, Fan G, Lu W (1980) Induction of rubber plantles from anther of *Hevea. brasiliensis* Mulle. Arg. *in vitro*. *Chinese Journal of Tropical Crops* **1**, 25-26

Wang Z, Wu H, Zeng X, Chen C, Li Q (1984) Embryogeny and origin of anther plantlet of *Hevea brasiliensis. Chinese Journal of Tropical Crops* **5**, 9-13

Wang ZY, Chen XT (1995) Effect of temperatue on stamen culture and somatic plant regeneration in rubber. *Acta Agronimics Sincia* **21**, 723-726

Wang ZY, Wu HD, Chen XT (1998) Effects of altered temperatues on plant regeneration frequencies in stamen culture rubber trees. *Journal of Tropical and Subtropical Botany* **6**, 166-168

Wilson HM, Street HE (1974) The growth, anatomy and morphogenetic potential of callus and cell suspension cultures of *Hevea brasiliensis. Physiologia Plantarum* **36**, 399-402

Wilson ZA, Power JB (1989) Elimination of systemic contamination in explants and protoplast cultures of rubber (*Hevea brasiliensis)* Muell. Arg. *Plant Cell Reports* **7**, 622-625

Yeang HY, Chevalier M (1999) Range of *Hevea brasiliensis* pollen dispersal estimated by esterase isozyme markers. *Annals of Botany* **84**, 681-684