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Chapter 9
Genomics of *Hevea* Rubber

Thakurdas Saha and P.M. Priyadarshan

Abstract *Hevea brasiliensis* is the most recent domesticated tree species from Amazonian rain forest producing latex of commercial utility. Major hurdles for genetic improvement of rubber tree were attributed to its perennial nature, long juvenile period, and a narrow genetic base. Further, the limited availability of *Hevea* genomic resources/information is another impediment to genomics-assisted crop improvement. Improvement of rubber tree in terms of latex production through breeding was the major focus of the scientific community dealing with the crop. Due to unidirectional selection for yield, other secondary attributes of rubber plants were lost during the process of developing high-yielding clones. Work on plant genomics gained momentum only after whole genome sequencing of *Arabidopsis thaliana* in 2000 (Arabidopsis Genome Initiative, 2000) followed by rice (International Rice Genome Sequencing Project, 2002) and poplar, the first tree genome (International Populus Genome Consortium, 2004). However, rubber genomics is still in its infancy. Initial molecular work started in the 1990s with cloning and characterization of latex biosynthesis genes followed by the studies on gene expression influenced by various biotic and abiotic stresses, tapping panel dryness (TPD), and ethylene stimulation of latex production. Simultaneously, different genetic markers were established in rubber for understanding the inheritance and diversity of natural variation existing among the Wickham and wild populations. Genetic markers were used successfully to generate linkage map for QTLs involving disease tolerance. During the last decade, transgenic research also progressed significantly with the development of transgenic *Hevea* clones with overexpressed

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MnSOD gene effective against TPD and drought stress. In recent years, with the advent of new-generation sequencing techniques, large-scale EST generation in rubber had been possible, which provided insights into genomic architecture and helped to elucidate genes involved in biological processes like latex production. In the absence of whole genome sequence information, the available transcriptome sequences form a potential resource to be utilized in genetic enhancement of rubber tree. The future challenge is to translate and integrate available genomic knowledge into appropriate methodologies, which we believe will revolutionize future *Hevea* breeding program.

Keywords Genomics • *Hevea brasiliensis* • Latex transcriptome • Linkage map • Para rubber tree

Introduction

Hevea (the Para rubber tree), which produces natural rubber, is a deciduous tree of 30–40 m high in the Amazonian forest (its natural habitat), belonging to the family Euphorbiaceae. One of the species, *Hevea brasiliensis* (Willd. ex Adr. de Juss.) Muell. Arg., is exclusively cultivated over 11.33 million hectares in the world for providing the industry with natural rubber (10.4 million tons in 2010)*. Natural rubber 1,4 *cis*-polyisoprene is a renewable ("green") elastomer being used mainly in tire sector (70%), in latex products (12%), and in many other industrial applications.

Natural rubber is produced in Southeast Asia (92%), Africa (6%), and Latin America (2%). The main natural rubber producing countries are Thailand (3.25 million tons in 2010), Indonesia, Malaysia, India, Vietnam and Côte d'Ivoire, China, Sri Lanka, Brazil, Philippines, Liberia, Cambodia, Nigeria, Cameroon, Guatemala, Myanmar, Ghana, Democratic Republic of Congo, Gabon, and Papua New Guinea. Natural rubber, collected by tapping the bark of the tree, was used by the native people of West Indies. It was brought to Europe by Columbus in 1493, Cortes in 1528, la Neuville in 1723, la Condamine in 1736, Fresneau in 1751, and Fusée Aublet in 1775. Its industrial use was first developed by Charles Macintosh in 1823 and reached its full potency with the invention of vulcanization by Charles Goodyear in 1839. Natural rubber was increasingly associated with transport with the invention and development of tires by Dunlop in 1888, Michelin in 1895, and Ford in 1910.

As per directions of Sir Clements Markham, Sir Henry Wickham collected 70,000 seeds from Rio Tapajoz region of Upper Amazon (Boim district) and transported it to Kew Botanic Gardens during June 1876 (Wycherley 1968; Schultes 1977; Baulkwill 1989). Of the 2,700 seeds germinated, 1,911 were sent to Botanical Gardens, Ceylon, during 1876, and 90% of them survived. During September 1877, 100 *Hevea* plants specified as "Cross material" were sent to Ceylon. Earlier, in June 1877, 22 seedlings, not specified either as "Wickham" or "Cross", were sent from Kew to Singapore, which were distributed in Malaya and formed the prime source of 1,000 tappable trees found by Ridley during 1888. An admixture of "Cross" and

Source: International Rubber Study Group (IRSG), Singapore.

"Wickham" materials might have occurred, as the 22 seedlings were unspecified (Baulkwill 1989). One such parent tree planted during 1877 was available in Malaysia even after 100 years (Schultes 1987). Seedlings from Wickham collection of Ceylon were also distributed worldwide. As a matter of fact, rubber trees covering millions of hectares in Southeast Asia are derived from a very few plants of Wickham's original stock from the banks of the Tapajoz (Imle 1978).

The first commercial planting with bud-grafted plants was undertaken during 1918 in Sumatra's east coast. Ct3, Ct9, and Ct38 were the first clones identified by Cramer (Dijkman 1951; Tan et al. 1996). Commercial ventures gradually spread to China, Thailand, and Vietnam, and rubber became an integral part of the economy of Southeast Asia towards latter half of the twentieth century.

Breeding was initiated with a very strict mass selection among the trees at the beginning of the twentieth century. With the introduction of bud grafting, "generative" and "vegetative" selection methodologies were simultaneously used that resulted in seedlings and grafted clones (Dijkman 1951). Around 1950, the advantages of grafted clones proved to be overwhelming for yield potential compared to genetically improved seedlings, and the focus shifted to derivation of clones for latex productivity. Progress in yield improvement in *Hevea* resulted in a gradual increment, from 650 kg/ha in unselected seedlings during the 1920s to 1,600 kg/ha in best clones during the 1950s. The yielding potential was further enhanced to 2,500 kg/ha in PB, RRIM, RRII, RRIC, IRCA, BPM, and RRIV clones during the 1990s. During these 70 years of rigorous breeding and selection, notable clones like RRIM 501, RRIM 600, RRIM 712, PB 217, PB 235, PB 260, RRII 105, RRIC 100, IRCA 18, IRCA 230, IRCA 331, and BPM 24 were derived (Tan 1987; Simmonds 1989; Clément-Demange et al. 2001; Priyadarshan 2003a, b). Some of the primary clones like PB 56, Tjir 1, Pil B84, Pil D65, Gl 1, PB 6/9, and PB 86 selected during the aforesaid period became parents of improved clones. It must also be highlighted that primary clones like GT 1 and PR 107 are still widely used, although their identification traces back to the 1920s.

In Latin America, every breeding effort is focused on derivation of clones having acceptable yield together with durable resistance to South American leaf blight (SALB) (Dean 1987). It must be emphasized that this disease represents a permanent threat for the whole rubber industry (Davies 1997). In the more humid areas of Asia, susceptibility to Corynespora leaf fall disease has become important for the breeders and rubber industry.

Rubber is currently planted in the form of grafted trees, at a density of about 450 trees per hectare (Fig. 9.1). The buds are collected from budwood grown in the budwood gardens, which are developed for the recommended clones. The plants produced in the nurseries can be budded stumps grown in the soil or budded plants grown in plastic bags. Rootstocks can be also grown directly in the plantation field at standard density, with budding carried out at field level. Rubber tree experiences an immature phase that may vary from 5 to 9 years, depending on climate, soil conditions, and management. When the trunk girth of the trees reaches 50 cm, tapping is initiated that may last between 15 and 30 years. The tapping, a periodically renewed cut incised in the bark of the trunk, generates latex (cell cytoplasm containing rubber

Fig. 9.1 Rubber plantation at the Rubber Research Institute of India, Kerala

particles) throughout the year (Jacob et al. 1995). The tapping intensity is a result of the combination of tapping frequency (tapping every 2, 3, 4, or 5 days) and of chemical stimulation intensity by the application of ethephon, an ethylene releaser (Abraham et al. 1968). In Asia, rubber wood has become an increasingly important economic product, and it represents a new challenge for breeders, which was first addressed by RRIM (Othman et al. 1995). Many of the clones issued from the RRIM 2000 series claim to be latex-timber clones. The trunk and the branches are used for varied transformation (furniture, plywood, particle wood, fuel wood). Latex and wood are two complementary ways for atmospheric carbon sequestration (d'Auzac 1998).

The genus *Hevea* is basically composed of ten species: *H. brasiliensis, H. guianensis, H. benthamiana, H. pauciflora, H. spruceana, H. microphylla, H. rigidifolia, H. nitida, H. camporum,* and *H. camargoana* (Webster and Paardekooper 1989; Wycherley 1992; Schultes 1990). Seven species are found in the upper Rio Negro region, considered to be the center of origin of the genus. *Hevea brasiliensis* is found in southern areas outside this center, in the upper Rio Madeira, where five other species are represented. It has generally been assumed that the species are freely intercompatible (Baldwin 1947). Pires (1981) observed natural hybrids of *H. camargoana*×*H. brasiliensis*, and Gonçalves et al. (1982) analyzed progenies issued from hand pollination from this type of crossing. Consequently, *Hevea* species might be considered as a species complex due to the absence of a strict barrier to recombination between species. Many efforts led to the identification of certain types, which were formerly presented as other possible species. *H. paludosa* was identified in Brazil by Ule in 1905 and is often considered as an 11th species (Gonçalves et al. 1990; Priyadarshan and Gonçalves 2003).

All *Hevea* species have $2n=36$ chromosomes, with the exception of one triploid clone of *H. guianensis* ($2n=54$) and the existence of one genotype of *H. pauciflora* with $2n=18$ (Baldwin 1947; Majumder 1964). Although *Hevea* behaves as a diploid, it is believed to be an amphidiploid ($2n=36$; $x=9$) that stabilized during the course of evolution. This contention is supported by the observance of tetravalents during meiosis (Raemer 1935; Wycherley 1976). *In situ* hybridization studies revealed two distinct 18S-25S rDNA loci and one 5S rDNA locus, suggesting a possible allotetraploid origin with the loss of 5S rDNA during the course of evolution (Leitch et al. 1998). But locus duplications are infrequent in *Hevea* genome, and they could have occurred due to chromosomal modifications posterior to the polyploidization event (Seguin et al. 2003); consequently, the two unknown ancestral genomes of *Hevea* would have strongly diverged.

Low and Bonner (1985) characterized *Hevea* nuclear genome as containing 48% of slowly annealing DNA (putative single copy) and 32% middle repetitive sequences with remaining highly repetitive or palindromic DNA. Estimated haploid genome size of *H. brasiliensis* is 4×10^9 base pairs considering its disomic nature (Roy et al. 2004). Mean molecular size of chloroplast DNA (cpDNA) is predicted to be 152 kb (Fong et al. 1994). Differentiation of the genus into species appears to be linked with the evolution of the Amazonian forest over the last one hundred thousand years. Alternations of humid and semiarid periods responsible for the forest extension or fragmentation resulted in the formation of forest islets. These are assumed to have become zones of protection and differentiation under local selection pressures.

Genetic as well as genomic resources play equally important role in crop improvement. In conventional breeding, genetic resources contribute significantly to develop improved varieties. In the last century, *Hevea* breeding made significant strides in enhancing its productivity. However, the progress in manipulating quantitative traits is still far behind with limited success. The major constraint is the lack of knowledge about the genetic interactions and complex biochemical pathways that are involved in plant responses to varying environmental stresses in traditional and nontraditional rubber-growing regions. During the past two decades, there has been an exponential increase in genomic data acquisition such as gene sequences, DNA markers, linkage maps, ESTs, and knowledge about gene expression profiles. Researchers are now better equipped to apply genomics to understand complex biological processes by combining high-throughput genomic techniques with innovative bioinformatic tools, which facilitate development of superior clones suited to different agroclimatic conditions. This chapter gives an overview of the genomic work carried out in rubber during the last two decades, which have far-reaching impact on *Hevea* improvement towards plant health, productivity, and enhanced stress tolerance.

Genetic Resources

Allied species of *Hevea* make up a gene pool for breeding purposes, especially for the identification and introduction of genes of resistance to leaf diseases (Priyadarshan and Gonçalves 2003). Within *Hevea brasiliensis*, the basic species for natural rubber

production, a very clear distinction need to be made between "Wickham" population and the series of wild accessions from the Amazonian forest, usually called "Amazonian" population. "Wickham" population has been the basis for rubber domestication and has evolved through a breeding history of one century, with a current high level of adaptation to modern rubber cropping, except in SALB-affected areas (many areas of Latin America). Conversely, the Amazonian populations, still under evaluation, have not been much modified by human selection. They display an average latex yield of around 12% of the level of currently developed Wickham clones (Clément-Demange et al. 2001) and a fairly high resistance to leaf diseases such as *Microcyclus* or *Corynespora cassiicola* (Berk. & Curt.) Wei.

Different expeditions for the collection and transfer of allied species and Amazonian accessions have been organized since 1890. During 1951–1952, 1,614 seedlings of five *Hevea* species (*H. brasiliensis, H. guianensis, H. benthamiana, H. spruceana, and H. pauciflora*) were introduced to Malaysia (Tan 1987). In Sri Lanka, 11 clones of *H. brasiliensis* and *H. benthamiana* and 105 hybrid materials were imported during 1957–1959 through triangular collaboration of USDA, Instituto Agronomico do Norte (IAN) (Brazil), and Liberia. Many of these clones were later given to Malaysia (Tan 1987). Introductions to the germplasm collection of CNRA in Côte d'Ivoire, with CIRAD cooperation, are made of 40 accessions from the French-Brazilian collection of 1974 from Acre and Rondonia, 19 accessions from a Firestone collection in the Madre de Dios basin in Peru (MDF accessions), 24 accessions given by the Brazilian Research Centre, Embrapa, in Manaus (CNSAM accessions), and 10 accessions from allied *Hevea* species. Part of the collections made by R.E. Schultes has also been rescued from two conservation sites in Columbia, thanks to a France-Columbia agreement, with 302 accessions from Calima site and 41 accessions from Palmira site, which were transferred to Côte d'Ivoire in 1987 after a quarantine period in Martinique island. Between 1945 and 1982, collections from Brazil (mostly Rondonia) have been undertaken at least ten times (Goncalves et al. 1983).

During 1981, due to initiative taken by IRRDB, 63,768 seeds, 1,413 m of budwood from 194 high-yielding trees, and 1,160 seedlings were collected from Brazilian Amazonia (Tan 1987; Simmonds 1989). This collection was performed over three states, namely, Acre, Rondonia, and Mato Grosso, in 16 different districts and in 60 different locations overall. Of this, 37.5% of the seeds were sent to Malaysia and 12.5% to Côte d'Ivoire. Half of the collections were maintained in Brazil. The accessions from budwood collection were brought to Malaysia and Côte d'Ivoire after quarantine against SALB. After the establishment of two IRRDB germplasm centers in Malaysia and in Côte d'Ivoire, other IRRDB member countries were supplied with material according to their request.

Crosses between Wickham and Amazonian accessions are relevant due to possible introgression of more variation. A specific program was undertaken by Côte d'Ivoire and France (CIRAD and CNRA) for the characterization and utilization of Amazonian accessions from 1985 to 1997. Evaluation of the wild Amazonian germplasm for latex yield showed that the wild origins annually produce around 10% of the currently used Wickham clones, which means about 200–300 kg/ha. It was found that the average latex yield in Wickham × Amazonian crosses was rather low,

ranging between 30% and 50% of the level of GT1, probably due to the important gap lying between the two populations. Conversely, a wide variability was found within these crosses for growth, with probable heterotic effects enabling the selection of very vigorous Wickham × Amazonian clones. However, the breeders of Rubber Research Institute of India could show significant yield increase (14–82%) in Wickham × Amazonian hybrids (Sankariammal and Mydin 2011). In 1997, a hybridization program was conducted by the breeders of Rubber Research Institute of India to broaden the narrow genetic base of cultivated rubber incorporating two popular Wickham clones, namely, RRII 105 and RRIM 600, as females and seven wild accessions as males. Out of 27 hybrid clones generated, 5 hybrids showed higher yield than the RRII 105 and other superior secondary attributes. These promising hybrids were selected for the next phase of evaluation in participatory trials.

Genomic Resources

Molecular Markers

Application of molecular tools in rubber tree improvement was lagging behind because of limited knowledge of the genome. The genetic base of the cultivated rubber tree, *Hevea brasiliensis,* is assumed to be narrow. It is from the "Wickham gene pool" that a spectacular yield improvement of about ten times has been achieved. The genetic variability of *H. brasiliensis* is high at the center of origin, and knowledge on such variability is fundamental for conservation, breeding, and commercial production of this species. Most often, specific phenotypes of discrete variation have been used as morphological markers. In addition, molecular markers could be highly beneficial as a tool in assisting genetic characterization and breeding (Nodari et al. 1997; Brondani et al. 1998). The perennial nature, the long breeding and selection cycle, and the difficulties in raising F_2 progeny make conventional genetic analysis in *Hevea* difficult (Varghese et al. 1998). Thus, the genetics of rubber tree has been poorly investigated.

Since the development of DNA marker technology in the 1980s, tremendous advancement has taken place in terms of marker development, genetic map construction, functional and comparative genomic linkages, genome sequencing, and development of low-cost technologies. Consequently, a variety of molecular techniques were introduced to study the extent of genetic relationship between wild and cultivated *Hevea* clones.

Restriction Fragment Length Polymorphisms (RFLPs)

Initially, hybridization-based RFLP markers providing codominant information were used to characterize *Hevea* germplasm. RFLP technique was proved to be useful for

genetic diversity study in wild and cultivated *Hevea* accessions using low copy number nuclear probes (Besse et al. 1994). Wild populations appeared more polymorphic than the cultivated clones. Rondonian and Mato Grosso populations were found more variable than Acre. RFLP analysis of organelle genomes of *Hevea* was also performed for establishing evolutionary relationships as these two genomes could reflect true evolution because of their uniparental inheritance (Luo et al. 1995). RFLP analysis with mitochondrial DNA probes revealed considerable variations among the accessions from Brazil, Colombia, Peru, and cultivated clones from Wickham collections. Chloroplast DNA-RFLP analysis revealed low level of genetic variation, indicating conserved nature of chloroplast genome than the mitochondrial genome.

Random Amplified Polymorphic DNAs (RAPDs)

The random amplified polymorphic DNA (RAPD) technique, described by Williams et al. (1990), has provided a useful approach for evaluating population genetic differentiation. Using RAPD analysis, Varghese et al. (1998) analyzed 24 cultivated *Hevea brasiliensis* clones to estimate genetic distance. Subsequently, Venkatachalam et al. (2002) described the genetic relationships for 37 *Hevea clones* using RAPD markers, and the clones were classified into seven major groups. Venkatachalam et al. (2004) identified a dwarf-specific RAPD marker and studied inheritance pattern among F1 hybrid progenies. Mathew et al. (2005) studied the phylogenetic relationship among three species of rubber, *Hevea brasiliensis, H. benthamiana,* and *H. spruceana,* employing different molecular marker techniques, namely, RAPD, chloroplast DNA PCR-RFLP, and heterologous chloroplast microsatellites. RAPD analysis clearly indicated a high degree of polymorphism among the three species. Analysis of interrelationships among the species clearly revealed that the clones of *H. brasiliensis* (>50% genetic dissimilarity) are closer to *H. benthamiana* than to *H. spruceana* (>70% genetic dissimilarity). Species-specific RAPD markers were identified for each species, and their locus specificity was proved through hybridization. Venkatachalam et al. (2006) identified two DNA markers in *Hevea* sequencing, and one of them (1.4-kb RAPD marker) revealed homology with *Saccharomyces cerevisiae* proline-specific permease gene. RAPD analysis was used to examine the genetic diversity and structure of the IRRDB'81 germplasm (Lam et al. 2009). A total of 59 accessions from 13 different districts of the Brazilian states, namely, Acre, Rondonia, and Mato Grosso, were studied using few primers. However, low interdistrict differentiation was noticed.

Microsatellite Markers

Microsatellites are known also as simple sequences or simple sequence repeats and are of 1–6 nucleotides. These repeats are subject to a high rate of single-motif

insertion and deletion mutations through the process of replication slippage (Levinson and Gutman 1987). They appear to be ubiquitous in higher organisms, although the frequency of microsatellites varies between species. These are abundant, dispersed throughout the genome, and show higher levels of polymorphism than other genetic markers (Schlotterer and Tautz 1992). These features coupled with their ease of detection through PCR using flanking primers have made them useful molecular markers. Their potential for automation and their inheritance in a codominant manner are additional advantages (Morgante and Olivieri 1993; Thomas and Scott 1993). Microsatellite markers are found throughout both the transcribed and nontranscribed regions of a genome (Varshney et al. 2005). Their role in gene regulation and genome evolution has also been discussed widely (Aishwarya and Sharma 2007). The genotyping results can be used for the registration of clones and the protection of breeders' rights (Bocharova et al. 2009). Microsatellite analysis helps in better germplasm management and for devising strategies for identifying core selection (Upadhyay et al. 2010).

There are two approaches for the identification of SSR-containing sequences: (1) molecular and (2) computational. The molecular approach for the development of SSRs is to construct genomic libraries (with or without enrichment for SSRs), screen the libraries, sequence candidate clones, and identify SSR motifs either manually or using computer programs. The computational or bioinformatics approaches take advantage of the available sequences such as those in the public databases by scanning through them and then identifying the ones that contain SSRs. They supplement the molecular approaches by identifying SSR repeats in candidate sequences derived from the libraries.

DNA fingerprints in *H. brasiliensis* using heterologous minisatellite probes from humans were reported by Besse et al. (1993). Low et al. (1996), for the first time, detected microsatellites in the *Hevea* genome through the database search of some *Hevea* gene sequences. The construction of a microsatellite-enriched library in *Hevea brasiliensis* was reported by Atan et al. (1996). Studies to identify SSRs in *Hevea* by Roy et al. (2004) revealed the presence of 67 microsatellites having characteristic simple and compound repeats. They showed the prevalence of $(AG)_n$ and $(AC)_n$ repeats in *Hevea* genome. Besides dinucleotide repeat motifs such as TG/AC, AG/TC, and TA/AT, trinucleotides (AAG, AGG, ATT), tetranucleotides (GAAA, AAGG, ATCC, TAAA, AAAT), and pentanucleotide (GAAAT) repeats were also found. AG repeat motifs occurred at higher frequency as a component repeat. Microsatellite markers developed from the above study were successfully used to identify 27 *Hevea brasiliensis* clones (Saha et al. 2005). The polymorphic microsatellite loci isolated and characterized from an enriched genomic library of *H. brasiliensis* were highly useful in understanding genetic diversity and gene flow among *Hevea* species (Souza et al. 2009). Using 15 highly polymorphic microsatellite loci, Le Guen et al. (2009) assessed genetic diversity of 307 clonally propagated individuals of the wild *Hevea brasiliensis*. Moderate differentiation among wild population was explained based on a subsample of 220 individuals from 14 populations. Among the wild *Hevea* population, Mato Grosso populations were genetically more distant from all other populations. Three population clusters that match the boundaries of hydrographical

basins of the main Amazon River tributaries were identified. For genomic studies in *H. brasiliensis*, Le Guen et al. (2010) introduced 296 new polymorphic microsatellite markers through screening of an enriched genomic library. More than 100 *Hevea* microsatellite sequences were registered with the NCBI GenBank by Genome Analysis Laboratory of the Rubber Research Institute of India, and markers were also generated for linkage mapping in rubber (unpublished results).

EST-SSR Markers

Data mining of microsatellites from ESTs has proven effective for generating markers for fingerprinting, genetic mapping, and comparative mapping among species (Varshney et al. 2005). Large-scale SSR-mining projects in plants have aimed at developing microsatellite markers, especially in economically important crop plants. Sequences from many genomes are continuously made freely available in the public databases, and mining of these sources using computational approaches permits rapid and economical marker development. Expressed sequence tags (ESTs) are ideal candidates for mining SSRs not only because of their availability in large numbers but also due to the fact that they represent expressed genes.

By analysis of 10,018 ESTs out of 10,829 for *Hevea brasiliensis,* available in public domain DNA databases, 799 SSR loci were found in the 643 nonredundant SSR-containing ESTs (Feng et al. 2009). Out of 799 SSRs in these ESTs, 84.2% contained simple repeat motifs while 15.8% represented compound motif types, and among the total EST-SSRs, 42.2% were dinucleotide repeats. Genetic variability among 60 *Hevea* genotypes consisting of Asiatic, Amazonian, African, and IAC clones was estimated with 68 selected polymorphic SSRs generated through data mining of 470 reads from GenBank by Gouveia et al. (2010). Recently available next-generation transcriptome sequencing data set (NCBI database – accession number GSE26514), submitted by Xia et al. (2011) was analyzed in the author's laboratory for large-scale SSR mining. The repeat number threshold was designated as more than five for dinucleotide, four for trinucleotide, and three for tetranucleotide repeat motifs. Consequently, 698 dinucleotide, 867 trinucleotide, and 72 tetranucleotide repeat sequences were identified from 48,768 unigenes (unpublished).

Genic Microsatellites

In rubber, SSRs were also identified at the 5′ and 3′ UTR of mRNA sequences. Gene sequences like HMG-CoA reductase (*HMGR*), MnSOD, and β-1,3-glucanase also contain repeat sequences at the untranslated region of mRNA or in the introns in the genomic sequences. Dinucleotide $(CT)_n$ repeats detected in MnSOD had been used as SSR markers for genetic relationship studies by Lespinasse et al. (2000a) and Lekawipat et al. (2003). HMG-CoA reductase encoded by the gene *HMGR* is a key

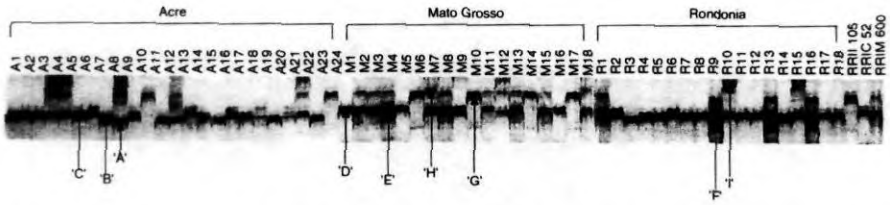


Fig. 9.2 Autoradiogram showing the allelic variation at the locus *HMGR* among wild *Hevea* germplasm accessions from three different provinces of Brazil: Acre, Rondonia, and Mato Grosso. Three cultivated popular clones, RRII 105, RRIC 52, and RRIM 600, were also genotyped. Nine microsatellite alleles ("*A*" to "*I*") were identified at this locus (Saha et al. 2007)

enzyme involved in latex biosynthesis in rubber. It was detected with dinucleotide repeats $(AG)_n$ at the 3′ UTR of mRNA (Saha et al. 2005). SSR polymorphism at this locus was successfully used for studying the allelic diversity in wild accessions of rubber by Saha et al. 2007 (Fig. 9.2). Cross-species amplification of the markers developed for *H. brasiliensis* was also found successful in the wild *Hevea* species *H. guianensis, H. rigidifolia, H. nitida, H. pauciflora, H. benthamiana, and H. camargoana* (Saha et al. 2005; Souza et al. 2009), revealing a high degree of sequence homology at the microsatellite flanking regions of these species. The SSR loci developed are considered as potential tool for studies of population genetics, genetic diversity, and gene flow among *Hevea* species.

Single Nucleotide Polymorphisms

Single nucleotide polymorphisms (SNPs) are the most abundant form of DNA polymorphism in a genome and a resource for mapping complex traits (Rafalski 2002). Bini et al. (2010) identified SNPs in popular *Hevea brasiliensis* clones at the 3′ untranslated regions (3′ UTRs) of 12 genes responsible for complex biochemical traits including latex biosynthesis (Fig. 9.3). Out of 12 loci, 5 loci, (1) geranylgeranyl diphosphate synthase, (2) farnesyl diphosphate synthase, (3) mevalonate kinase, (4) ubiquitin precursor, and (5) latex patatin homolog, were detected with 40 nucleotide substitutions and four indels. Average frequency of SNPs was found to be one in every 90 bases. Heterozygosity for SNPs could also be detected in some of the genotypes/clones.

Retroelements

Retroelements are dispersed as interspersed repetitive sequences throughout the host genome and exploited as genetic tools for plant genome analysis. A reverse transcriptase (*RT*) gene fragment of *Hevea* was cloned indicating the presence of

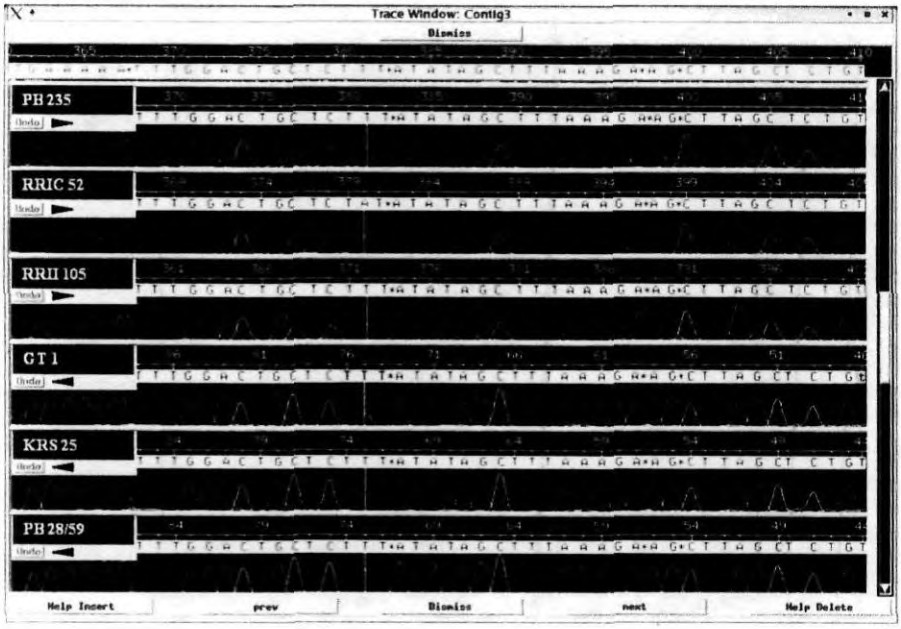


Fig. 9.3 Screen-shot illustration of Consed window showing alignment of partial chromatograms of the locus geranylgeranyl diphosphate synthase from *Hevea* genotypes for identifying SNP at the nucleotide position 381. Heterozygosity could clearly be detected in GT1

retrotransposons – a class of mobile genetic elements in the *Hevea* genome for the first time (Saha et al. 2006) (Fig. 9.4). A *Hevea* genomic library was screened for retroelements using reverse transcriptase (*RT*) gene fragment as the probe, and consequently 23 positive clones were identified. Sequence analysis of positive clones, screened for retroelements, showed homology of eight clones with nucleotide sequences of putative non-LTR retrotransposon *RT* in *Arabidopsis thaliana*, *RT* in *Medicago truncatula*, Ty3-Gypsy type of retrotransposons in *Oryza sativa*, viral gag/pol polyprotein from *Pisum sativum*, and polyprotein of *Ananas comosus* suggesting abundance of retroelements in rubber genome.

Genetic Linkage Mapping

Genetic linkage map presents the linear order of markers (genes and other identifiable DNA sequences) in their respective linkage groups depicting the relative chromosomal locations of DNA markers by their patterns of inheritance. The linkage map allows revelation of more and more restricted segments of the genome and undoubtedly enhances our understanding in many areas of plant systematics. A genetic map for *Hevea* spp. was constructed using a population derived from an interspecific

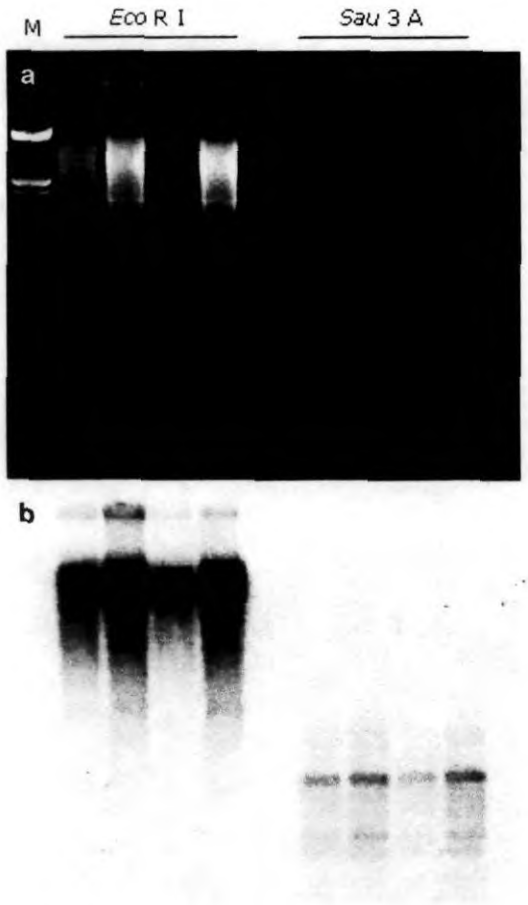
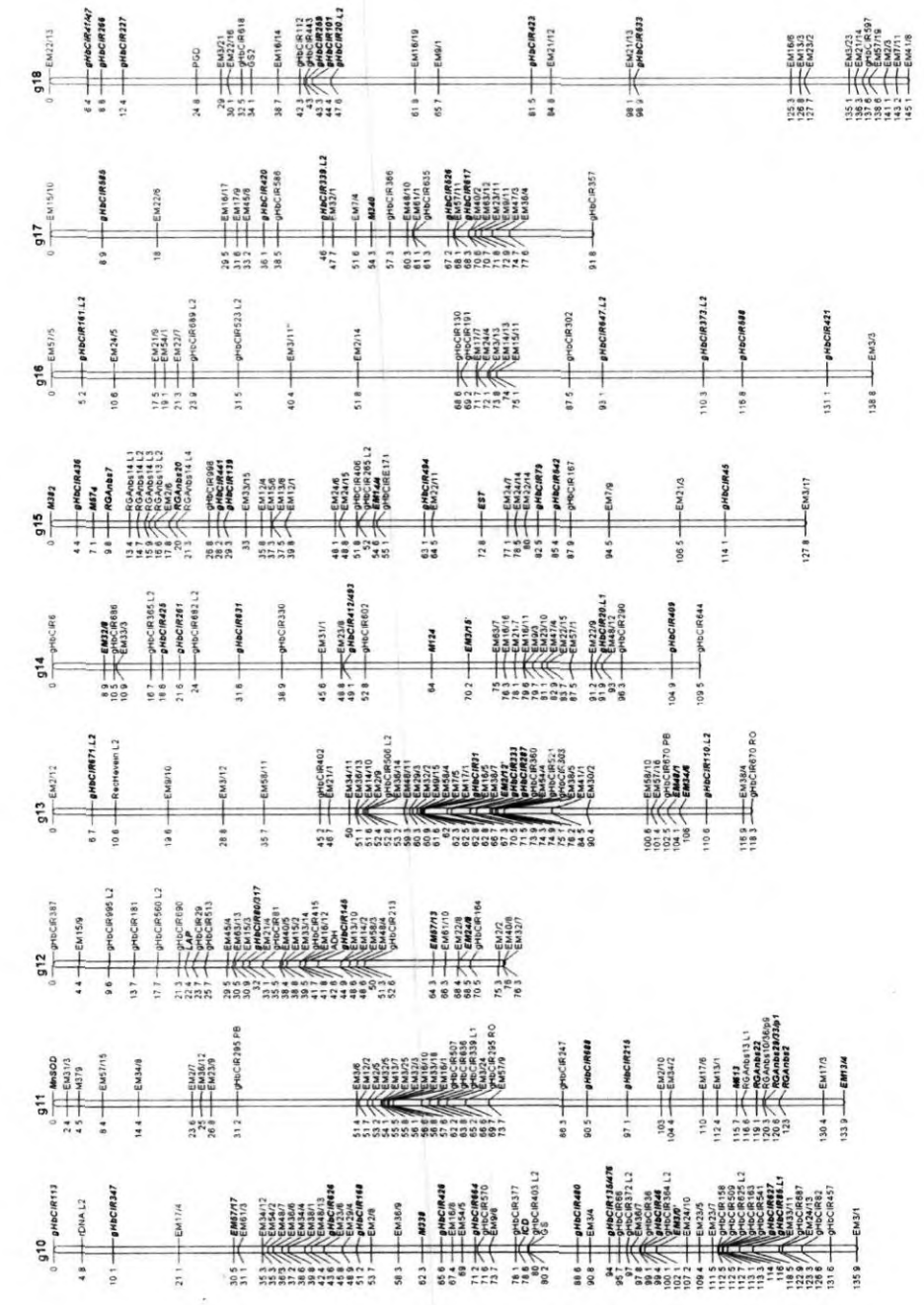
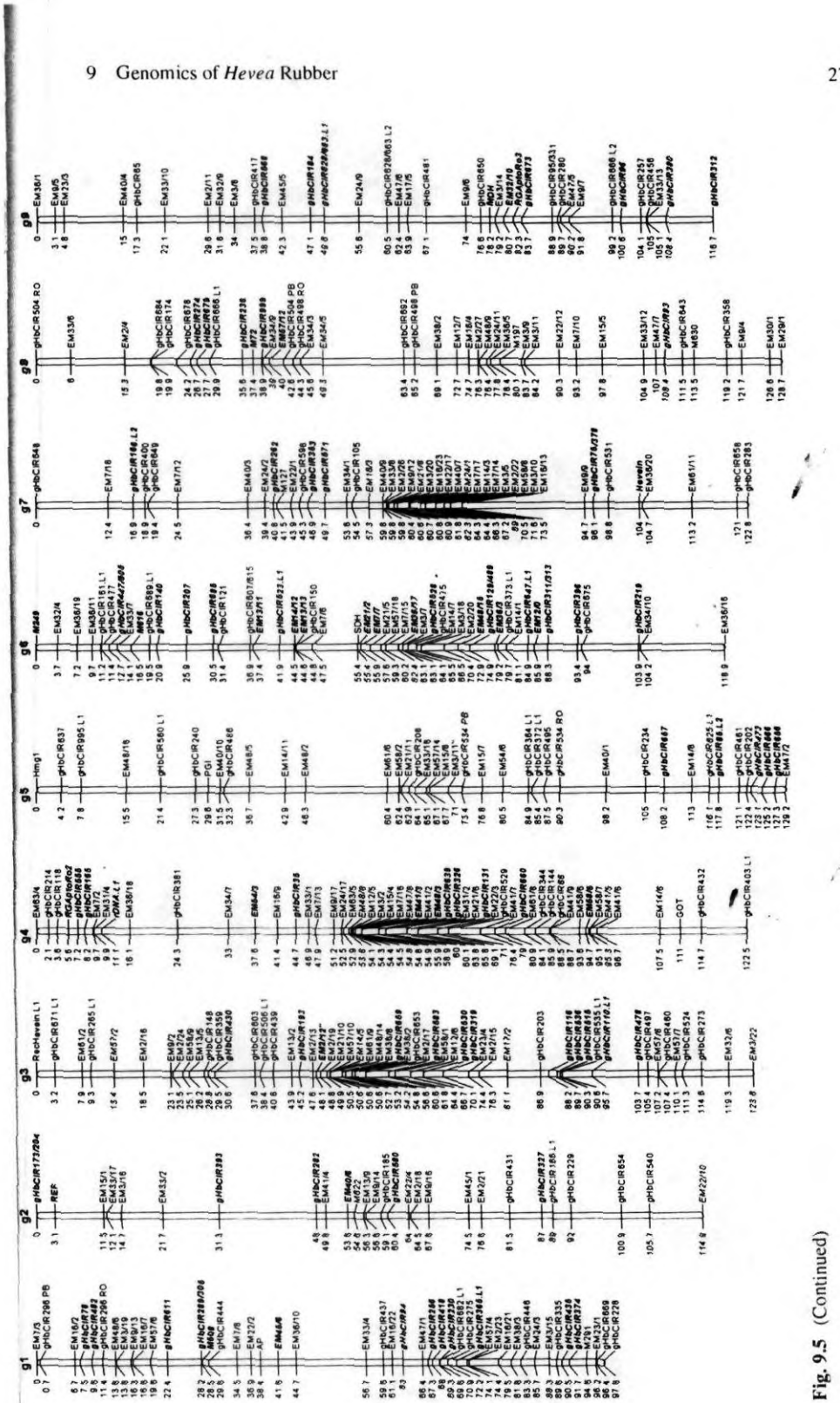


Fig. 9.4 Abundance of retrotransposons in *Hevea* genome was confirmed through Southern hybridization of genomic DNA digested with *Eco*RI and *Sau*3AI against partial reverse transcriptase gene (*RT*) probe generated from rubber. (**a**) Digested DNA on agarose gel; (**b**) autoradiogram of the hybridized blot; *M* molecular weight marker (Lambda DNA/*Eco*RI+*Hin*dIII)

cross between PB 260 (*H. brasiliensis*) and RO 38, an interspecific hybrid clone (*H. brasiliensis*×*H. benthamiana*), following the pseudotestcross strategy (Lespinasse et al. 2000a). The markers were assembled into 18 linkage groups (Fig. 9.5), thus reflecting the basic chromosome number, and covered a total distance of 2,144 cm. A total of 717 loci constituted the synthetic map, including 301 restriction fragment length polymorphisms, 388 amplified fragment length polymorphisms, 18 microsatellites, and 10 isoenzymes. Homologous linkage groups between the two parental maps were merged using bridge loci. Average marker density was 1 per 3 cm. Lespinasse et al. (2000b) mapped quantitative trait loci (QTL) for resistance to South American leaf blight (SALB), a disease of the rubber tree caused by the fungus *Microcyclus ulei* using the same cross combination (PB 260, a susceptible clone, and





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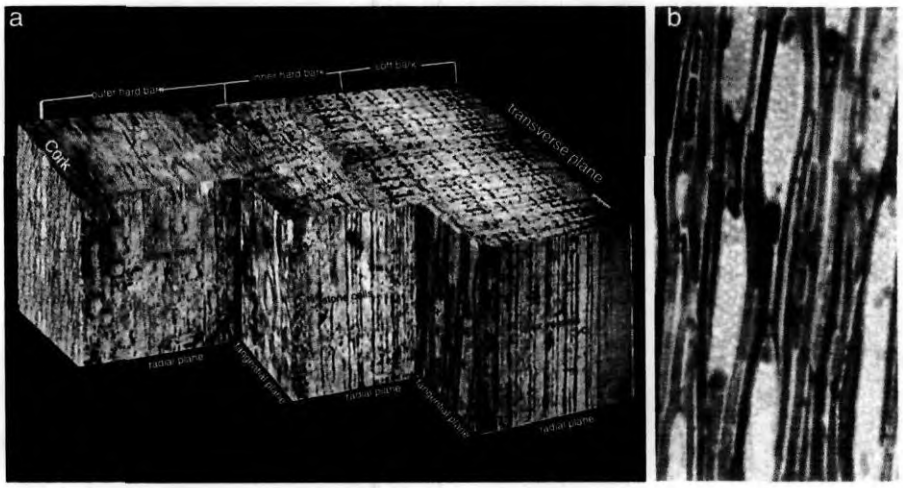

Fig. 9.6 (**a**) Three-dimensional picture of *H. brasiliensis* bark anatomy; (**b**) Enlarged view of the laticiferous system in tangential section of soft bark tissue stained with Oil Red O (Omman and Reghu 2003)

RO 38, a SALB-resistant clone). Eight QTLs for resistance were identified on the RO 38 map, whereas only one QTL was detected on the PB 260 map.

In the author's laboratory, also an effort was made to construct a linkage map of rubber using a segregating progeny population obtained from a cross between two popular cultivated *Hevea* clones: RRII 105 and RRII 118. A total of 227 markers comprising of 96 RAPD, 79 AFLP, 47 SSRs, and five SNP-based markers were utilized for the construction of a genetic linkage map (unpublished).

Gene Discovery

Latex Biosynthesis Genes

Latex is produced in specialized cells known as laticifers or latex vessels, located adjacent to the phloem of the rubber tree. These laticifers form a very complex laticiferous system by anastomosis between tubular cells in the tree (Fig. 9.6). Isoprenoid biosynthesis is brought about through the mevalonate-dependent metabolic pathway (Hepper and Audley 1969; Gronover et al. 2011) (Fig. 9.7). Although it is known that biosynthesis of natural rubber takes place by a mevalonate pathway, molecular biological characterization of related genes has not been adequate. Initial understanding on the regulation of gene expression in the laticifers of *H. brasiliensis* came from the study of Kush et al. (1990), who demonstrated for the first time that transcript levels of genes involved in rubber biosynthesis and genes induced by wounding and ethylene treatment were higher in laticifers than in leaves. Rubber particle

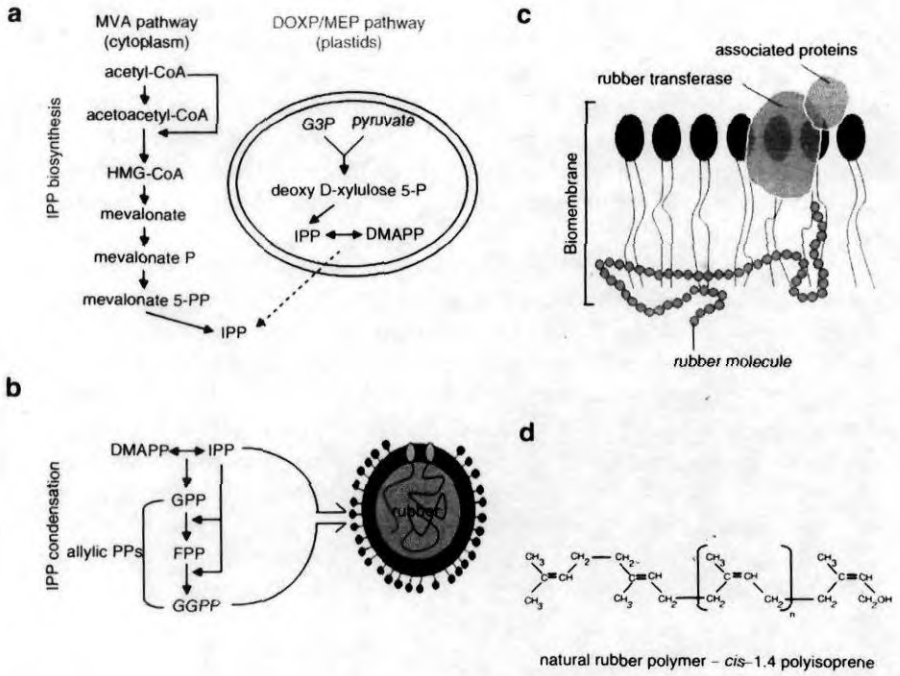

Fig. 9.7 Schematic representation of *cis*-1,4-polyisoprene biosynthesis in plants. (**a**) The monomeric subunit of natural rubber IPP is synthesized by MVA pathway from acetyl CoA in cytosol and chloroplastic DOXP/MEP pathway from G3P and pyruvate. (**b**) IPP condensation to allylic diphosphates for natural rubber synthesis. Each new molecule of dis-1,4-polyisoprene requires an allylic diphosphate initiator before the isoprene units from IPP are polymerized in rubber particles. (**c**) Natural rubber is synthesized by the activity of rubber transferase and other associated proteins at the monolayer biomembrane surface of rubber particles. (**d**) *cis*-1,4 polyisoprene. IPP isopentenyl diphosphate, MVA mevalonate, DMAPP dimethyallyl diphosphate, DOXP/MEP 2-1-deoxy-D-xylulose-5-phosphate/2-C-methyl-D-erythritol-4-phosphate, G3P glyceraldehyde-3-phosphate, GPP geranly diphosphate, FPP farnesyl diphosphate, GGPP geranylgeranyl diphosphate (modified after Gronover et al. 2011)

in the laticifers is the site of rubber (*cis*-1,4-polyisoprene) biosynthesis. A 14-kilodalton protein, rubber elongation factor (REF), is associated with the rubber particle. To obtain more information concerning the function of REF and its synthesis and assembly in the rubber particle, Goyvaerts et al. (1991) isolated cDNA clones encoding REF and characterized the same. Biosynthesis of natural rubber is known to take place biochemically by a mevalonate pathway including six steps catalyzed by corresponding enzymes (Sando et al. 2008). A key enzyme involved in rubber biosynthesis is HMG-CoA synthase, which catalyzes the condensation of acetyl-CoA with acetoacetyl-CoA to form HMG-CoA (Suwanmanee et al. 2002, 2004; Sirinupong et al. 2005). Reduction of HMG-CoA to mevalonic acid, catalyzed by HMG-CoA reductase, is considered as a rate-limiting factor in rubber biosynthesis and thereby regulating the biosynthesis of natural rubber. These two enzymes possibly function in concert in response to the supply of substrates for rubber biosynthesis (Suwanmanee

et al. 2002). HMGS mRNA transcript accumulation was found to be more in laticifers than in leaves. A positive correlation was also observed between the activity of *HMGS* and dry rubber content of the latex. Two members of *HMGS* from *Hevea brasiliensis hmgs-1* and *hmgs-2* were cloned and characterized. *hmgs-1* was found to be higher in laticiferous cells than in leaves, whereas the abundance of *hmgs-2* was more in laticifer and petiole than in leaves. In the case of *HMGR*, three genes, *hmg-1*, *hmg-2*, and *hmg-3*, were identified of which *hmg-1* was reported to be involved in rubber biosynthesis (Chye et al. 1991, 1992).

The main precursor for IPP in the pathway is phosphorylated mevalonate and is synthesized by mevalonate kinase (Archer and Audley 1987). Oh et al. (2000) isolated and characterized a cDNA clone encoding IPP isomerase from *H. brasiliensis* and showed involvement of IPP isomerase in rubber biosynthesis through *in vitro* assay. The major enzymes involved in the condensation of IPP are geranylgeranyl diphosphate synthase (GGPP synthase) and farnesyl diphosphate synthase (FDP synthase). Takaya et al. (2003) identified GGPP synthase catalyzing the condensation of IPP with allylic diphosphates to produce (all-E)-GGPP. Adiwilaga and Kush (1996) isolated a full-length cDNA encoding a 47-kDa FDP synthase from *Hevea* and suggested a dual role for FDP synthase in the biosyntheses of rubber and other isoprenoids. Its expression level increased with the regeneration of latex due to tapping.

The gene responsible for the *cis*-1,4 polymerization of isoprene units was isolated and characterized by Asawatreratanakul et al. (2003). Predominance of the transcripts of *Hevea cis*-prenyltransferase was detected in latex as compared with other *Hevea* tissues examined.

Sando et al. (2008) isolated full-length cDNA of genes encoding enzymes catalyzing the six steps of MVA pathway. They characterized three acetyl-CoA acetyltransferase genes, two HMG-CoA synthase genes, and four HMG-CoA reductase genes and one each of mevalonate kinase (*MVK*), phosphomevalonate kinase (*PMK*), and mevalonate diphosphate decarboxylase (*MVD*), which were highly expressed in latex. According to Sando et al. (2008), *MVK* and *PMK* were found to be involved in other isoprenoid biosynthesis in addition to MVA pathway, as their expression level was found to be the same in both laticifers and xylem (which contains no rubber). Venkatachalam et al. (2009) also cloned and characterized a full-length cDNA as well as genomic fragment for *hmgr1* gene from an elite rubber clone RRII 105. The nucleotide sequence of a genomic clone comprised of four exons and three introns, giving a total length of 2,440 bp. The sequence of 42 bp 5′ UTR and 69 bp of 3′ UTR was also determined.

Rubber biosynthesis could also follow a mevalonate-independent pathway as evidenced by transcriptome studies (Ko et al. 2003). The alternative metabolic pathway for IPP synthesis is 1-deoxy-D-xylulose-5-phosphate/2-C-methyl-D-erythritol-4-phosphate (DOXP/MEP) pathway, which is located in the plastid (Rohmer et al. 1996). Both the pathways coexist in laticifers and need sucrose as a precursor for rubber synthesis (d'Auzac 1964; Chow et al. 2007). Therefore, sucrose should cross the plasma membrane through specific sucrose transporters before being metabolized in the laticifers. Two isoforms of the sucrose transporter SUT1, *HbSUT1A*, and

HbSUT2A cloned from latex-specific cDNA library were found to play an essential role in sucrose import into laticifers of virgin *Hevea* trees (Dusotoit-Coucaud et al. 2009), although the relative importance of these sucrose transporters in determining latex yield is unknown. These genes were upregulated by ethylene application (essential for ethylene-stimulated latex production), and their localization in the latex cell was confirmed by *in situ* hybridization. Tang et al. (2010) functionally characterized another *Hevea* SUT member, *HbSUT3*. This isoform was found to be the predominant member expressed in the rubber-containing cytoplasm (latex) of laticifers compared to other *Hevea* SUT genes.

Ethylene-Regulated Genes for Latex Production

Biosynthesis of natural rubber, like other secondary metabolites, is affected by various plant hormones. The latex flow rate and duration are the first intrinsic factors known to limit rubber yield – the faster and the longer the latex flow, the higher the yield (d'Auzac et al. 1989). In the extensively studied plant hormones, only ethylene was identified to stimulate the latex production, which is applied as ethephon (an ethylene releaser). Bark treatment with ethephon is known to increase the latex yield by 1.5–2-fold in rubber tree (Coupé and Chrestin 1989; Pujade-Renaud et al. 1994). Even though the exact mechanism of ethylene action is poorly understood on the rubber tree, progress has been made in physiological and biochemical aspects. Compared with the physiology and biochemistry of ethylene stimulation on latex production, the progress in understanding the molecular mechanism is at a slow pace. Till date, only a few genes responding to ethylene have been characterized in *H. brasiliensis*.

Kush et al. (1990) reported laticifer-specific genes induced by ethylene in *H. brasiliensis*. Hevein, a lectin-like protein involved in the coagulation of latex, was mediated by ethylene (Broekaert et al. 1990; Sivasubramaniam et al. 1995; Gidrol et al. 1994). Ethylene could upregulate activity of glutamine synthetase (GS), a key enzyme of nitrogen metabolism and its transcript levels in *H. brasiliensis* latex cells, suggesting involvement of GS in stimulation of rubber production with ethylene (Pujade-Renaud et al. 1994). Higher expression of MnSOD, regulated by ethephon application, prevented lutoid disruption by superoxide radicals (Miao and Gaynor 1993), leading to increased rate of latex flow.

Both the HMGS and HMGR were known to be involved in early steps of rubber biosynthesis. Of the three HMGR genes, *hmg1*, *hmg2*, and *hmg3*, only *hmg1*, responsible for rubber biosynthesis, was induced by ethylene (Chye et al. 1991, 1992); *hmg2* was involved in defense reactions against wounding and pathogens, and *hmg3* was possibly involved in other isoprenoid biosynthesis for housekeeping purposes (Chye et al. 1992; Wititsuwannakul 1986). Ethephon also influenced the expression of the HMG-CoA synthase gene activity (Suwanmanee et al. 2004; Sirinupong et al. 2005). However, the expression level of FDP synthase, catalyzing the synthesis of the last common substrate isopentenyl pyrophosphate (IPP) in the isoprenoid biosynthesis, was not affected by ethylene treatment (Adiwilaga and

Kush 1996). Luo et al. (2009) and Zhu and Zhang (2009) documented that ethephon had no effect on the gene expression and the activity of RuT (a cis-prenyltransferase) needed for rubber biosynthesis.

A specific and significant activation of the cytosolic glutamine synthetase (GS) in the laticiferous cells after ethylene treatment parallels the increase of latex yield. A marked accumulation of the corresponding mRNA was found, but in contrast, a slight and variable increase of the polypeptide level was noticed (Pujade-Renaud et al. 1994). The GS response to ethylene might be mediated by ammonia that increases in latex cytosol following ethylene treatment.

Zhu and Zhang (2009) reported prolonged latex flow and acceleration of sucrose metabolism due to ethylene stimulation and considered the main reasons for enhanced latex yield. The rapid water exchanges with surrounding liber cells probably occur via the aquaporin pathway as the mature laticifers are devoid of plasmodesmata. Two full-length aquaporin cDNAs (*HbPIP2;1* and *HbTIP1;1*, for plasma membrane intrinsic protein and tonoplast intrinsic protein, respectively) known to facilitate water and/or small neutral solute fluxes across cell membranes were cloned and characterized from rubber (Tungngoen et al. 2009). Through their study, it was evident that aquaporin *HbPIP2;1* was effective in increasing plasmalemma water conductance than HbTIP1;1, and their expression was noticed in all liber tissues in the young stem, including the laticifers. *HbPIP2;1* was upregulated in both liber tissues and laticifers, whereas *HbTIP1;1* was downregulated in liber tissues but upregulated in laticifers in response to bark Ethrel treatment. Increase in latex yield in response to ethylene was related with water circulation between the laticifers and their surrounding tissues as well as with the probable maintenance of liber tissue turgor, which together favor prolongation of latex flow. Ethylene stimulation of latex production results in high sugar flow from the surrounding cells of inner bark towards the latex cells. Dusotoit-Coucaud et al. (2010) studied the expression pattern of sugar transporters (*HbSUTs*) and hexose transporter (*HbHXT1*) in two *Hevea* clones, PB 217 and PB 260, under different physiological conditions. The *HbSUT1*, one of the most abundant isoforms, displayed the greatest response to ethylene treatment. Ethylene treatment led to a higher accumulation of *HbSUT1B* in latex cells than in the inner bark tissues of the high-yielding PB 217 clone. Tang et al. (2010) reported induced expression of *HbSUT3* by the latex stimulator Ethrel and positive correlation with latex yield. Application of ethylene also enhanced transcription of cysteine protease *HbCP1* isolated from rubber (Peng et al. 2008).

Defense/Stress-Related Genes

Defense/stress-related genes, namely, MnSOD, *HEVER*, hevein, chitinase, and β-1,3-glucanase, are expressed in laticifers of *Hevea*. Miao and Gaynor (1993) isolated MnSOD gene, which was found to express in all tissues, *i.e.*, leaf, petiole, root, latex, and callus, and highest level expression was noticed in young leaves through northern analysis. A novel stress-induced gene, *HEVER* (*Hevea* ethylene-responsive),

from the rubber tree was isolated and characterized (Sivasubramaniam et al. 1995). A multigene family encodes HEVER. *HEVER* transcript and protein were induced by stress treatment with salicylic acid and ethephon. β-1,3-glucanase gene was identified from a cDNA library derived from latex by Chye and Cheung (1995), and its higher expression level was noticed in latex compared to leaf. Thanseem et al. (2003, 2005) also cloned and characterized the same gene from Indian *Hevea* clones and demonstrated prolonged accumulation of β-1,3-glucanase transcripts in abnormal leaf fall-tolerant RRII 105. Hevein, a lectin-like protein, belonging to a multigene family was found to play a crucial role in the protection of wound sites from fungal attack through latex coagulation (Broekaert et al. 1990; Pujade-Renaud et al. 2005). Overexpression of chitinase was noticed during fungal infection and by ethylene stimulation. In addition to the role in defense responses, expression of hevein and chitinase is linked to the characteristics of the latex flow. The products of hevein ("procoagulant") and chitinase ("anticoagulant") genes, which compete for the same site (the N-acetyl-glucosamine moiety) of the hevein receptor to induce or inhibit the process of coagulation, could be used as molecular markers for assessing yield potential of rubber clones. Such markers are of help in early selection of high-yielding and stimulation-responsive rubber clones (Chrestin et al. 1997). The full-length cDNA encoding a cysteine protease, designated *HbCP1*, was isolated for the first time from *Hevea brasiliensis* (Peng et al. 2008). The predicted HbCP1 protein possessed a putative repeat in toxin (RTX) domain at the N-terminal and a granulin (GRAN) domain at the C-terminal. In plants, cysteine proteases are involved in diverse physiological and developmental processes including biotic and abiotic stresses. Transcription pattern analysis revealed that *HbCP1* had high transcription in laticifer and low transcription in bark and leaf.

Resistance Gene Analogues (RGAs)

Isolation and characterization of the NBS-LRR-encoding genes are of significance in understanding plant-pathogen interactions for effective management of diseases. Sequences analogous to plant resistance genes of NBS-LRR class (Fig. 9.8) were cloned from the genomic DNA of two species of rubber: *Hevea brasiliensis* (clones RRII 105 and RRIM 600) commercially cultivated for latex production and *H. benthamiana*, a noncultivated species known for its tolerance to fungal diseases (Saha et al. 2010a). From the genomic RGA library of rubber, different RGAs were identified, structurally each having an open reading frame and characteristic motifs. Sequence analysis revealed that RGAs are highly diverged in rubber. Functional RGAs from *Corynespora*-challenged leaf samples of RRII 105 were identified through RT-PCR using degenerated primers. A comparison of these two types of RGAs revealed that a large group of closely related genomic RGAs, except a few, had no function against *Corynespora* leaf disease as they did not show perfect homology with any of the RT-RGAs on the basis of deduced amino acid sequences. Characterization of these RT-RGAs is a significant step towards understanding plant response to *Corynespora* infection in rubber.

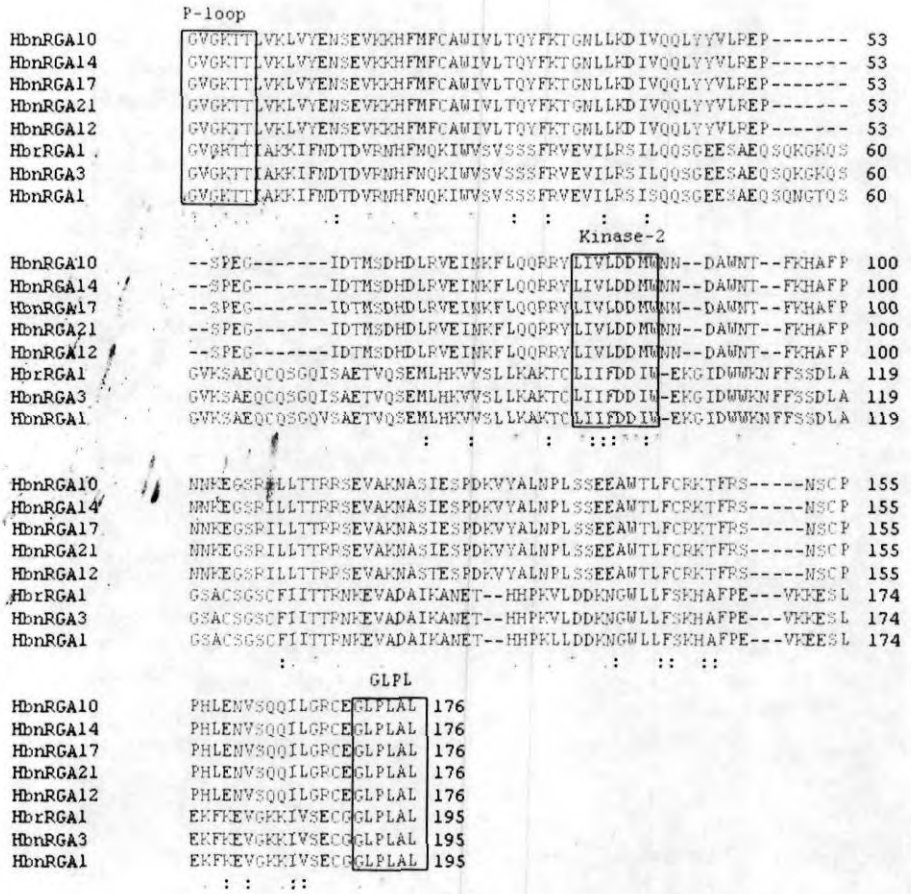

Fig. 9.8 Multiple alignment of representative amino acid sequence (conceptual translation) of resistance gene analogues (RGA) isolated from rubber showing three conserved motifs: P-loop, Kinase-2, and GLPL in NBS region. "HbnRGA" and "HbrRGA" refer to RGAs derived from *Hevea benthamiana* and *H. brasiliensis*

Genes for Flowering

To understand the genetic and molecular mechanisms underlying the reproductive process in rubber trees, Dornelas and Rodriguez (2005) characterized FLORICAULA/LEAFY (FLO/LFY) orthologue *HbLFY* from *H. brasiliensis* (RRIM 600), regulating flower and inflorescence development. Expression patterns of *HbLFY* were analyzed during vegetative and reproductive development. *HbLFY* is expressed in lateral meristems that give rise to inflorescence and in all flower meristems, consistent with a role in reproductive development.

Genes Involved in Signaling Pathways

Coronatine-insensitive 1 protein (COI1) is essentially involved in jasmonic acid signaling pathway regulating defense responses against stress in plants. A cDNA encoding coronatine-insensitive 1 protein (HbCOI1) from rubber was cloned by Peng et al. (2009) to study jasmonic acid signaling for the genes involved in latex biosynthesis. Transcription of *HbCOI1* in latex was induced by jasmonate and tapping. Three MADS-box genes from *Hevea brasiliensis*, *HbMADS1*, *HbMADS2*, and *HbMADS3*, encoding polypeptides consisting of 245, 217, and 239 amino acids, respectively, were cloned and characterized by Li et al. (2011). All of them contained conserved MADS-box motifs at N-terminus. Transcript abundance of all these three genes was noticed in the laticifer cells. The transcriptions of *HbMADS1* and *HbMADS3* were induced by jasmonic acid. Ethephon was not effective in inducing their expression. It was observed that these three genes were differentially expressed during somatic embryogenesis of rubber tree.

Promoter Research

Interests in promoter research for tissue-specific expression of desired gene are increasing in rubber as rubber tree has great potential to produce foreign proteins in the latex, which can easily be purified from the serum of the latex. Therefore, main interest was to identify the promoter region at the upstream of the genes involved in latex biosynthesis. Pujade-Renaud et al. (2005) isolated promoter regions of two *hevein* genes and analyzed in rice through transgenic approach. They showed that the longest promoter sequence (*PHev2.1*) regulated high level of expression of the transgene. Priya et al. (2006) characterized promoter sequence of *REF* gene. A gene construct containing *REF* promoter sequence and the GUS coding *uidA* as reporter gene were transformed to tobacco and *Arabidopsis* to understand regulatory role of *REF* promoter. Results suggested that isolated promoter sequence was capable of regulating gene expression. The promoter sequences of two aquaporin genes were cloned and found to harbor ethylene-responsive and other chemical-responsive (auxin, copper, and sulfur) elements known to increase latex yield (Tungngoen et al. 2009).

EST Sequencing

Expressed sequence tags (ESTs), short partial cDNA sequences, are currently the most widely sequenced nucleotide element from the plant genome with respect to the number of sequences and the total number of nucleotide available to researchers. EST provides a robust sequence resource that can be exploited for gene discovery, genome annotation, and comprehensive genomics. In rubber, EST-sequencing

approach has enabled laticifer gene expression analysis on a large scale. Chow et al. (2007) reported a collection of 10,040 ESTs from latex to analyze genes involved in latex biosynthesis. Among these ESTs, they identified 1,380 consensus sequence and 2,061 singletons through progressive assembly. Functional analysis revealed that 26.2% of the 3,441 unique transcripts could be assigned known gene identities. Among these sequences, there was a dominance of ESTs involved in rubber biosynthesis, *i.e.*, *REF* and *SRPP*, followed by latex abundant protein, and ring-zinc finger protein. Very recently, Xia et al. (2011) adopted the next-generation massively parallel sequencing technologies to gain a comprehensive overview of the *H. brasiliensis* transcriptome. They reported 48,768 unigenes through *de novo* transcriptome assembly – the most comprehensive sequence resource available for the study of rubber trees. In total, 37,432 unigenes were successfully annotated, of which 24,545 (65.5%) aligned to *Ricinus communis* proteins.

Stress-Related ESTs

Understanding of stress adaptation process at molecular level is essential for improvement of abiotic stress tolerance in rubber tree for extending its cultivation to nontraditional cold-prone areas. Genes that are differentially expressed during cold acclimation in rubber were identified (Saha et al. 2010b, c). Transcript profiling in two relatively stress-tolerant *Hevea* clones PR 261 and RRII 208 in relation to cold stress was performed (Fig. 9.9). Sequencing of 131 cDNA sequences (59 downregulated and 72 upregulated cDNAs) revealed 110 unique sequences comprising of 13 clusters/contigs and 97 singletons. However, several differentially expressed genes, *i.e.*, catalase, phosphatidylinositol/phosphatidylcholine transfer protein, NADH dehydrogenase, MYB transcription factor, downward leaf-curling protein, epimerase/dehydratase, Na^+/H^+ antiporter, chloroplast *ycf2*, and chloroplast *ftsH* protease, involved in cold adaptation process were also identified along with the unique transcripts.

A subtracted cDNA library was constructed from the cold-stressed leaf sample (Saha et al. 2010b). High-quality sequences of 156 subtracted cDNA clones (ScDNA) were subjected to "contig analysis" to assemble similar sequences in groups. Thirty-one contigs containing 90 clones (2–8 clones per contig) and 66 singletons (single sequences) were identified. All sequences were subjected to BLASTX search to know about the homology with the gene sequences existing in GenBank from rubber or other plant species. Transcripts/clones were assigned to the category based on the shared structural elements and (or) inferred functions. All these ESTs, except a few with unknown functions, relevant to cold responsiveness were grouped into the following categories for which interesting functions in relevance to stress response could be inferred. These groups are (1) osmoprotection/detoxification, (2) oxidoreductases, (3) cell wall and polysaccharide metabolism, (4) protein/amino acid metabolism, (5) transport and secretion, and (6) transcription

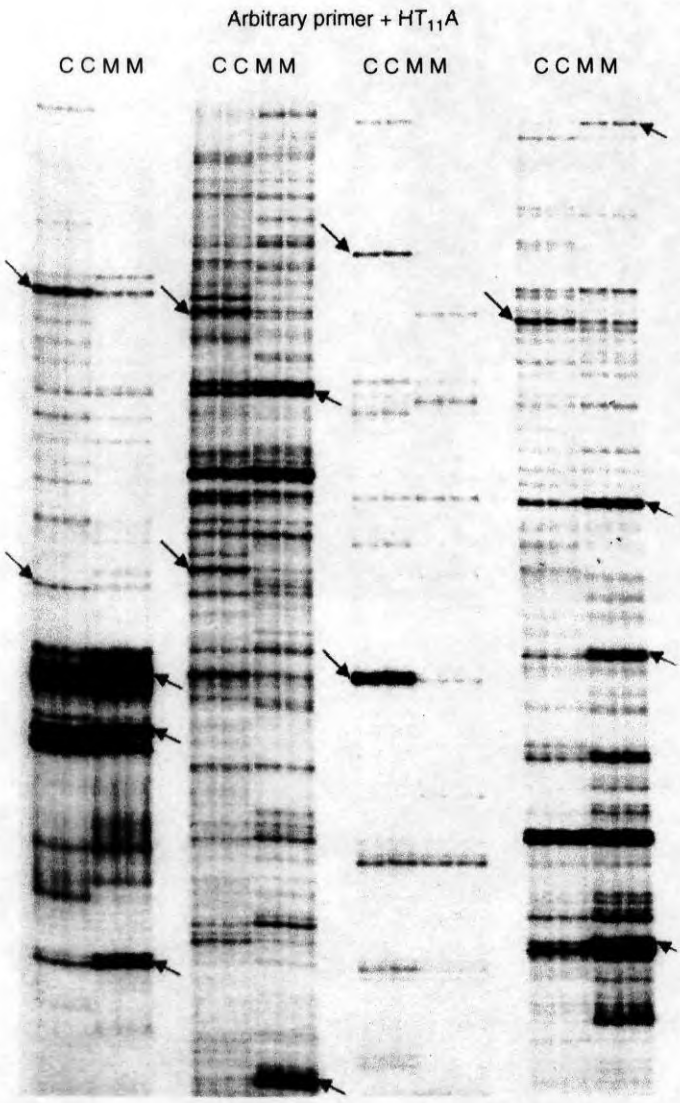

Fig. 9.9 Autoradiogram of transcript profiling through DDRT-PCR of stress-tolerant clone RRII 208 of *Hevea brasiliensis* grown at Kottayam (*C: control*) and at Munnar (*M*), a cold-prone high-altitude area to identify differentially expressed stress-responsive genes. Samples were loaded twice to avoid loading error. Blue and brown arrows indicate down-regulation and up-regulation of transcripts under low temperature, respectively. Four arbitrary primers were used in combination with $HT_{11}A$

factors. Ninety-six stress-responsive cDNA clones (31 contigs + 65 singletons) were subjected to reverse northern dot-blot analysis to screen for truly differentially expressed cDNA fragments. Duplicate blots of the 96 stress-responsive cDNA clones (subtracted) were hybridized with labeled cDNA probes from cold-treated and control RNA samples to screen for truly differentially expressed cDNA

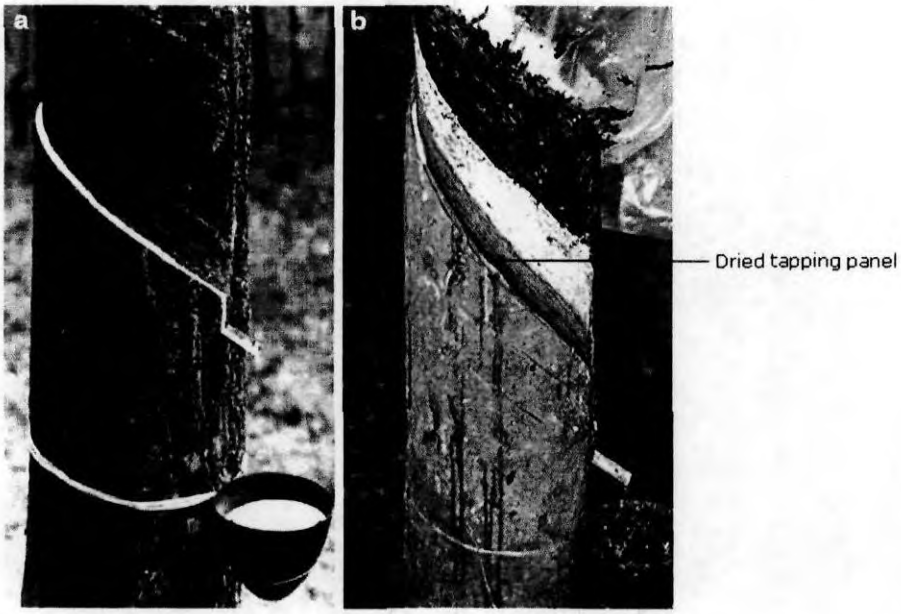

Fig. 9.10 (**a**) Healthy tapping panel of rubber tree showing latex flow; (**b**) cessation of latex flow due to tapping panel dryness (TPD)

fragments. Out of 96 clones, 56 gave quantitatively more signals with the cDNA probe from cold-treated PR 261 plants indicating overexpression of the respective genes under cold stress. Among these genes, carbonic anhydrase, glutathione peroxidase, metallothionein, chloroplastic Cu/Zn SOD, serine/threonine protein kinase, transcription factor, DNA-binding protein, etc., showed significant increase in expression levels.

Genes Involved in Tapping Panel Dryness (TPD)

TPD is the appearance of partial dry zones without latex flow along the tapping panel (Fig. 9.10). In later stage, the tapping panel becomes completely dry, and other symptoms such as browning, thickening, and flaking of bark can occur (Sookmark et al. 2002). A great deal of work was performed to reveal the nature and molecular mechanisms leading to TPD. It was initially presumed that TPD might be caused by pathogens (Zheng and Chen 1982; Soyza 1983), which could not be confirmed (Nandris et al. 1991a, b). Later involvement of viroid in etiology of TPD was reported (Ramachandran et al. 2000).

Research on the physiological aspects suggested that the TPD syndrome was a complex physiological disorder resulting from excessive tapping and overexploitation/overstimulation with ethylene (Chrestin 1989; de Faÿ and Jacob 1989; Faridah et al. 1996). In TPD tree, the contents of protein, nucleic acid, thiols, and ascorbic

acid decreased (Fan and Yang 1995), whereas the activities of RNase and proteinase increased in general (Tupy 1969; Fan and Yang 1995; Zeng 1997). In addition, the levels of variable peroxidase and superoxide dismutase (SOD) also decreased (Xi and Xiao 1988). Krishnakumar et al. (1997) reported reduced level of cytokinin in bark tissues of TPD-affected trees. Increased bark respiration due to TPD was demonstrated by Krishnakumar et al. (2001). Proteins related to TPD were identified by comparing the expression patterns between healthy and TPD-affected trees (Dian et al. 1995; Lacrotte et al. 1995; Sookmark et al. 2002), but their functional relations with TPD still remain unknown. Therefore, it is necessary to identify the genes associated with TPD to unravel the molecular mechanisms involved.

Chen et al. (2003) reported lower expression of *HbMyb1*, a key transcription factor in bark and latex of TPD trees compared to healthy trees. Functional analyses further indicated that *HbMyb1* negatively regulated programmed cell death (PCD) in transgenic tobacco plants. In another study, Venkatachalam et al. (2007) identified 134 genes associated with TPD in rubber tree by SSH method. They analyzed expression patterns of partial genes and discussed the relationship between differentially expressed genes and TPD. Later, the same group through mRNA differential display technique claimed involvement of a gene *HbTOM20*, which played an important role in the alteration of mitochondrial metabolism, resulting in impaired latex biosynthesis (Venkatachalam et al. 2009).

Li et al. (2010) also identified the genes associated with TPD using suppression subtractive hybridization (SSH) method. Among 237 unique genes, 205 were reported to be related to TPD in rubber tree. Of different functional categories, the large numbers of genes related to TPD were associated with transcription and post-transcription, metabolism and energy, protein metabolism, or stress/defense response. Systematic analyses of the genes related to TPD suggested that the production and scavenging of reactive oxygen species (ROS), ubiquitin proteasome pathway, programmed cell death, and rubber biosynthesis play important roles in TPD. However, much more information is needed for understanding TPD in rubber tree at the molecular level.

Genome Sequencing

The past several years have witnessed major advances in our understanding of plant genomes and genomic information through whole genome sequencing. The increasing availability of data from several plant genome-sequencing projects provides a promising direction for investigating genes and their functional and sequence homologs involved in plant development (Avraham et al. 2008). Although genome-sequencing projects lead to the identification of the complete catalogue of genes of an organism, they do not consider the gene expression patterns. Large-scale end sequencing of cDNA library generates ESTs, representing genes expressed in particular tissues or under particular developmental or environmental conditions. They have also been the target of sequencing in many of the projects and found invaluable

for genome assembly and annotation. Whole genome sequence information helps in many aspects of plant-trait improvement through gene discovery to transgenesis and use of molecular markers in breeding. *Hevea* genome-sequencing project had already been launched jointly by Tun Abdul Razak Research Centre (TARRC) of the Malaysian Rubber Board in the UK and newly established. The Genome Analysis Centre (TGAC) at Norwich, UK. Although completion of whole genome sequencing of a Malaysian latex/timber clone RRIM 928 performed jointly by TARRC and TGAC was announced in 2010 by the Malaysian Rubber Development Board, sequence information has not been released in the public domain till date. International Rubber Research and Development Board (IRRDB) also proposed to do whole genome sequencing of rubber in biotechnology meeting at CIRAD in France in 2009. However, quantum of *Hevea* genome-sequencing work is a monumental task as the haploid genome size is enormous (~4×10^3 Mbp as per our calculation based on the DNA content measured by Leitch et al. 1998), and also rubber possesses a high-complexity genome with >60% repetitive sequences making the sequencing effort more challenging.

Methylation Dynamics in *Hevea* Genome

Cytosine methylation is a fundamental epigenetic mechanism for gene expression regulation and development in plants. Identification of DNA methylation patterns and their putative relationship with abiotic stress in *H. brasiliensis* was reported by Uthup et al. (2011). Significant variations in the methylation pattern were observed at core DNA-binding motifs within the regulatory sequences of four major genes involved in the mevalonate pathway and one general defense-related gene of three high-yielding popular rubber clones, RRII 105, RRIM 600 and PB 260, grown at two different agroclimatic conditions. Several consistent clone-specific and location-specific methylation patterns were identified (Fig. 9.11).

Transgenesis

Transgenesis is referred to as the introduction of heterologous or homologous DNA into plant genome resulting in its stable integration and expression. The technology has played a critical role in defining the *in vivo* functions of plant genes. In recent years, with the rapid increase in gene sequence information, systematic transgenic approaches have been adopted to characterize large number of genes in both reverse and forward genetic studies. As one of the experimental methods in functional genomics, transgenesis has the advantage of revealing the direct link between gene sequence and function; such results not only provide further understanding of basic biological question but also facilitate exploitation of genomic information for crop improvement (Dixon et al. 2007). There are many variations of gene transfer methods

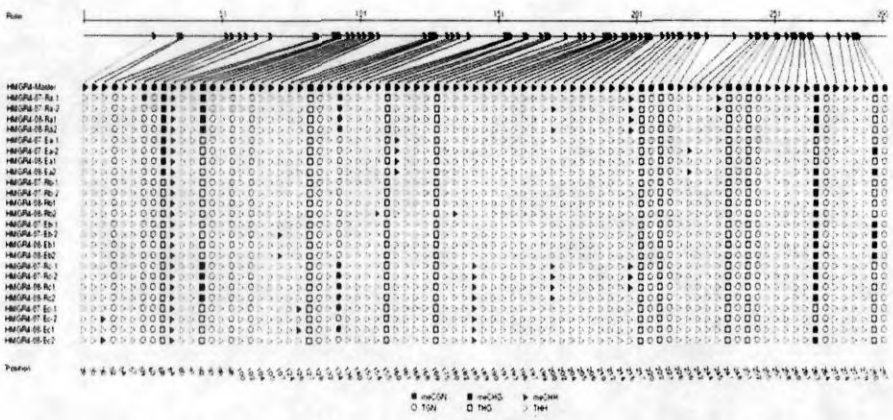

Fig. 9.11 DNA methylation pattern of a 288-bp partial promoter region of HMGR gene from control and test plants belonging to the three different clones having differential responses toward cold stress. The data was generated through bisulfite sequencing followed by CyMATE analysis of the region of interest. Clone and location wise groupings of each class of methylation are identified at the positions: 68, 92, 134, 160, 185, and 200 bp. The figure shows all the possible sites of methylation in the given sequence under the three categories. The blocked symbols represent the sites where actual methylation has taken place. R: control plants grown at RRII campus, E: test plants grown at Elappara (cold-prone area), year of sampling is suffixed to the gene (*HMGR*4-07 and *HMGR*4-08). Clone designation – a: RRII 105, b: RRIM 600, c: PB 260

to introduce transgenes into the plant genome. Genetic transformation offers a potential tool to breeders for introducing valuable traits to crop plants, leading to the development of elite clones in a relatively short period of time. The most widely used methods are *Agrobacterium*-mediated gene transfer and biolistic transformation.

Conventional rubber breeding takes more than 25 years to develop a new clone. The first transformation report in *Hevea brasiliensis* was published in 1991 (Arokiaraj and Wan 1991) through *Agrobacterium*-mediated transformation. The first transgenic *Hevea* plants, using anther-derived calli as the explant of the clone Gl 1, were successfully developed by Arokiaraj et al. (1994) following biolistic transformation method. Subsequently, transgenic plant was developed using *Agrobacterium*-mediated gene transfer of anther-derived calli (Arokiaraj et al. 1996, 1998). Transformation efficiency could significantly be enhanced when the friable callus was treated with calcium chloride and cultured on calcium-free medium prior to transformation (Montoro et al. 2000). Inner integument tissue of the immature fruit of the clone PB 260 was used as the explant for genetic transformation (Montoro et al. 2003). Transgenic plants of *H. brasiliensis* PB 260 were developed through *Agrobacterium*-mediated transformation by Blanc et al. (2005). It was further reported that anther-derived embryogenic callus was the most suitable explant for genetic transformation (Rekha et al. 2006). Earlier transformation events were only with various marker genes. Later, the experiments were focused on transferring various agronomically important genes into *Hevea* with enhanced tolerance to abiotic stresses, production of recombinant proteins, etc. Subsequently, attempts were made to increase the SOD enzyme activity by overexpression of the same genes in

Hevea. Transgenic plants were developed with SOD gene under the control of CaMV 35S and FMV 34S promoters (Jayashree et al. 2003; Sobha et al. 2003a). Biochemical analysis of the transgenic embryogenic calli of *Hevea* with SOD indicated significant increase in the activity of superoxide dismutase, catalase, and peroxidase as compared to the control (Sobha et al. 2003a, b). Jayashree et al. (2003) reported successful development and establishment of transgenic rubber plant with SOD gene for their further evaluation. Genetic transformation experiment to overexpress *hmgr1* gene, involved in latex biosynthesis, in *Hevea* was performed by Arokiaraj et al. (1995). They could generate transgenic embryos, which failed to produce any transgenic plant. However, they showed enhanced *hmgr* activity in the transformed calli.

Experiments were also undertaken for the production of foreign proteins in the latex of *Hevea*. The Para rubber tree, which produces enormous volume of latex upon tapping, could easily be exploited without any destruction for the production of foreign proteins in the latex throughout the year. Recombinant protein may be expressed in the specific parts of the plants or in specific organelle within the plant cell using tissue-specific promoters. Human serum albumin protein was expressed in transgenic *Hevea* plants by Arokiaraj et al. (2002). To characterize tissue-specific promoters derived from latex biosynthesis genes, transgenic approaches were adopted by Priya et al. (2006). They cloned and characterized promoter sequence of the rubber elongation factor gene. A significant achievement towards antibiotic marker-free *Hevea* transgenic development avoiding the constraints of GMO regulations was made by Leclercq et al. (2010). They developed an efficient genetic transformation procedure in the clone PB 260 using a recombinant green fluorescent protein (GFP). They showed GFP selection is less time-consuming in terms of callus subculturing and offered the possibility of producing antibiotic-resistant marker-free transgenic plant.

Conclusion

The past decade has witnessed tremendous advancement in our understanding of plant genomics. Genomic information available is being utilized for accelerating crop improvement program through gene discovery, transgenesis, and use of molecular markers. The accelerating pace of technology development promises much more than can easily be applied into traditional plant improvement programs. Therefore, the genomic information is not exploited fully as anticipated earlier. The major challenge of the postgenomic biology is to translate and integrate the knowledge into application tools and methodologies for the full understanding of gene functions at the organismal level.

Although significant achievements in the area of genomics were made in other crops including some tree crops, very little work on the genomics of *Hevea* was initiated till the end of the twentieth century. This is evident with the availability of limited number of GenBank submissions (12,385 ESTs, 1,499 nucleotide sequences,

and 246 GSS as on August 15, 2011) compared to other tree crops. However, a large quantum of genomic information is expected to be available in the public domain soon due to the initiatives taken by various laboratories with regard to transcriptome sequencing and whole genome sequencing using next-generation sequencing platform. In fact very recently, next-generation transcriptome sequencing data generated using Illumina RNA-Seq technology by Xia et al. (2011) was made available in the public domain (NCBI database – GSE26514; public on Sept. 01, 2011). The enormous information thus generated recently will hopefully be translated by the concerted efforts from all the rubber-growing countries for the improvement of rubber tree in terms of crop health and productivity.

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