Biotechnology

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The attainment of yield plateau and prevalent intra-clone variations in yield of *Hevea* prompted researchers to tackle these problems through employing the modern tools of biotechnology. However, higher yield alone would not encourage cultivation of *Hevea*, since the species is sensitive to biotic and environmental attributes and physiological disorders. The long breeding cycle and the large size of the crop also make breeding time-consuming. Biotechnology applied to *Hevea* can be discussed under two headings: (i) in vitro culture and (ii) molecular breeding. While in vitro culture deals mainly with regeneration and propagation, molecular breeding includes identification, characterisation, introduction and expression of novel genes (see Chap. 13). The emergence of genomics has overshadowed the relevance of in vitro culture. The conspicuous reason for this is that in vitro culture was attempted by several laboratories worldwide, but progress had been very minimal as the techniques envisaged could not be commercialised successfully. On the other hand, genomics worked meticulously that could make inroads into the intricacies of *Hevea* culture and development faster than any other branch of science.

12.1 In Vitro Culture

Experimentation with in vitro culture of rubber commenced during the 1960s with Chua (1966) attempting to derive callus cultures from the plumule tissues of seedlings. The effects of osmotic concentration, carbohydrates and pH of the culture media were also studied. Later, the RRIM took the initiative of undertaking large-scale tissue culture work through maintaining callus cultures from various explants (Paranjothy and Gandhimathi 1976). It expanded to somatic embryogenesis and micropropagation through stem explants. While anther culture was employed to achieve pure lines first and exploitation of heterosis thereafter, micropropagation and somatic embryogeny were used to generate homogeneous populations. Although research on in vitro culture commenced nearly 45 years ago, even after rigorous experimentation, these areas are still in their infancy due to shortcomings towards commercial applicability. Expectations of better performance of these multiplication techniques are based on three considerations: (i) cloning the root system would generate new and more homogeneous rootstocks or monogenetic clones; (ii) selection of clonal roots would improve the exploitation of existing genetic variability; and (iii) use of rejuvenated clonal plant material would potentially provide important agricultural attributes towards higher growth, latex yield and resistance to wind and dryness.

Carron et al. (1989, 1995a, b, 2001, 2005) has amply reviewed in vitro approaches applied to the rubber tree (including tissue culture, haplogenesis, microcutting, somatic embryogenesis, protoplast culture, germination of immature embryos and cultivation of laticiferous tissue).

P.M. Priyadarshan, *Biology of* Hevea *Rubber*, DOI 10.1007/978-3-319-54506-6_12

Microcuttings and somatic embryogenesis were studied in *Hevea* in order to achieve rapid clonal propagation as an alternative to the drawbacks of the use of cuttings and bud grafting techniques. Somatic embryogenesis as a means of regeneration opens up possibilities for transgenic technology. In vitro culture is made up of the application of many laboratory protocols involving hormones, nutrients and culture medium and of histocytological controls; details can be found in the works of Chen et al. (1982), Chen (1984), El Hadrami et al. (1991), Etienne et al. (1991, 1993, 1997a), Carron et al. (1992), Housti et al. (1992), Montoro et al. (1993), Veisseire et al. (1994a, b), Wang and Chen (1995), Seneviratne and Wijesekara (1996), Cailloux et al. (1996), Linossier et al. (1997), Wang et al. (1998), Sushamakumari et al. (2000a) and Kumari Jayashree et al. (2001). Bouychou (1953), Chua (1966), Wilson and Street (1975), Paranjothy and Gandhimathi (1976) and Audley and Wilson (1978) were the first rubber researchers to develop callus and tissue cultures derived from epicotyl, green stem or plumule tissues of young seedlings. The aim was to use calli to study the laticiferous system and the action of ethephon, but they encountered problems of ploidy instability. The RRIM took the initiative of maintaining callus cultures from various explants that later expanded to somatic embryogenesis and micropropagation through stem explants (Paranjothy and Gandhimathi 1976).

12.2 Anther Culture

The Rubber Research Institute of Ceylon (RRIC) was the first to carry out the culture of anthers to raise haploid plants (Satchuthananthavale and Irugalbandara 1972). However, the first plants from *Hevea* pollen were made available during 1977 at the Baoting Institute of Tropical Crops, Hainan, China (Chen et al. 1979). Since then, at least four laboratories in China took the lead in researching the production of haploid plants in vitro (Carron et al. 1989). In addition, attempts were made to produce plants through gynogenesis (Guo et al. 1982; Yang and Fu 1997).Carron et al. (1989) enumerated three phases for the production of haploids from anther culture. In the first phase, production of callus and embryos takes nearly 50 days. Here, the media formulation is vital since the balance between callus development and initiation of embryos needs to be maintained (Chen 1983). The modified MB (microbouturage) medium (Chen 1984) is widely used with the addition of naphthalene acetic acid (NAA) and coconut water, which regulate development of microspores, and a judicial concentration of sources of N, K and sugar leads to the production of calli and embryos. The somatic callus then degenerates and the embryos develop from microspores. Subculture must be carried out at this stage into differentiation medium in order to avoid degeneration of embryos (Chen et al. 1982). Maturity of embryos is the crucial factor in the second phase. The cultures need 2–3 months for the apical bud to develop. Coconut water at this stage will be substituted with gibberellic acid (GA3) for better development of cotyledons. In the third phase, progressive increment of GA3, gradual withdrawal of other growth regulators, addition of 5-bromouracil and reduction of sugar result in the development of plants from embryos. Cytological investigations of callus, embryos and plantlets showed mixoploidy (Qin et al. 1979). However, when the plants develop in vitro, there is a progressive tendency towards diploidy (Carron et al. 1989). Above all, the developmental stage of the anther is vital for the right results. The anthers from male flowers that have a yellow corolla should not be selected, for the microspores will be in a binucleate stage. Such anthers will repress callus development and embryogenesis. Only uninucleate pollen is ideal for haplogenesis, which can be obtained from greenish-yellow flowers (Chen 1984; Shije et al. 1990). (see Chap. 4 for somatic embryogenesis and meristem culture).

12.3 Protoplast Culture and Embryo Rescue

Attempts towards protoplast culture and fusion were carried out using young immature leaves (Cailloux and Lleras 1979; Wilson and Power 1989), using discs of pith in the apical part of young green shoots or anther calli (Othman and Paranjothy 1980). Subsequently, Cazaux and d'Auzac (1994) obtained microcalluses from embryogenic callus-derived protoplasts of *H. brasiliensis*, but without plant regeneration. Recently, Sushamakumari et al. (2000b) reported successful plant regeneration from embryogenic cell suspension-derived protoplasts of *Hevea*. Protoplasts were isolated from immature inflorescence-derived cell suspensions and produced microcolonies on 'KPR' medium (Kao and Michayluk 1975). Protoplast-derived cell colonies proliferated, upon subculture on MS-based regeneration medium, with 40% of the protoplast-derived calli developing somatic embryos. Subsequently, they germinated into plants on the same medium. Fusion of protoplasts was aimed at hybridising different *Hevea* species for breeding resistance to SALB.

In vitro germination of mature and immature zygotic embryos issued from hand pollination has been considered as a way of improving the success rate of genetic recombination in rubber (Muzik 1956; Paranjothy and Gandhimathi 1976; Carron 1981). Good results (90% success in germination) were achieved only for immature embryos that were at least 3–3.5 months old after fertilisation. It also appeared that immature seeds of this age could be germinated in vivo under controlled conditions. However, this procedure, which is expensive, did not appear to guarantee increased efficiency, nor was it a means for rescuing rare progenies.

12.4 Direct Gene Transfer

Somatic embryogenesis in rubber is becoming standardised in different laboratories worldwide as an efficient system for plant regeneration from cells. At the same time, efforts have been made to transform *Hevea* cells in order to increase genetic variation in a targeted way (a new form of mutagenesis) and complement plant breeding efforts with the possibility of modifying already selected high-performing clones with specific genes (addition or inactivation) while avoiding meiotic recombination. However, in the short term, genetic transformation is becoming a powerful tool for investigating how the rubber genome functions with the assistance of targeted mutations.

The first transgenic rubber trees were reported by Arokiaraj et al. (1994, 1996, 1998), who used the particle bombardment method and then the *Agrobacterium tumefaciens* system on anther-derived calli from clone GL 1, with a view to in vivo production of high-value recombinant proteins (Yeang et al. 1998). The first experiments were carried out with plasmid vectors harbouring the strong and non-specific cauliflower mosaic virus (CaMV) 35S promoter and β-glucuronidase and *nptII* reporter genes encoding neomycin phosphotransferase. Plant regeneration rates are strongly affected by genetic modification and require improvement. However, fluorometric assays and ELI-SAs were performed to prove the expression of *gus* and *nptII* genes, respectively, in calli and embryoids (Arokiaraj et al. 1996). The expression of foreign proteins in *Hevea* latex was demonstrated by Arokiaraj et al. (1998). This transformation appeared to be stable even after three vegetative generations with no chimeras, indicating that a single transformed plant is sufficient to achieve a population through bud grafting. Lately, a gene for an antibody fragment against the coat protein of the bacterium *Streptococcus sanguis* (Yeang et al. 2002), a gene coding for 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR, involved in rubber biosynthesis) and a gene for human serum albumin (Arokiaraj et al. 2002) have been expressed in rubber latex through genetic transformation. At the present time, these transformation experiments are based on a limited number of regenerated trees.

Stable transgenic callus lines starting from integument-derived friable embryogenic calli of the clone PB 260 have been transformed by *A. tumefaciens* (Montoro et al. 2000, 2003),

and more than 200 transformed plantlets, incorporating the *uidA* reporter gene, have been transferred to soil in a greenhouse (Pujade-Renaud et al. 2005). Recently, Jayashree et al. (2003) achieved *A. tumefaciens*-mediated genetic transformation and the regeneration of transgenic plants in *H. brasiliensis*. This was the first report of the production of transgenic plants with a superoxide dismutase gene (*HbSOD*) under the CaMV 35S promoter using an *A. tumefaciens*-mediated gene transfer system for *H. brasiliensis*. The morphology of the transgenic plants was similar to that of untransformed plants. Histochemical *gus* assay revealed the expression of the *uidA* gene in embryos as well as leaves of transgenic plants. The presence of the *uidA*, *nptII* and *HbSOD* genes in the *Hevea* genome was confirmed by PCR amplification and genomic Southern blot hybridisation analyses. Subsequently, an efficient and reproducible protocol for *A. tumefaciens*-mediated genetic transformation and plant regeneration of *Hevea* with the gene coding for superoxide dismutase under the control of figwort mosaic virus (FMV) 34S promoter has been reported by Sobha et al. (2003a, b). Further, the authors claimed that the transgenic plants are being grown in poly bags and they will be bud grafted for the evaluation of oxidative stress later. Plants of RRII 105 were integrated with the Manganese superoxide dismutase (MnSOD) gene through *Agrobacterium*-mediated genetic transformation events. The SOD activity was 35% and 31% higher under normal and drought conditions, respectively, in one of the transgenic plants than that of bud-grafted plants. Derivation of transgenic plants integrated with genes like MnSOD is a significant step forward to cultivate rubber under marginal conditions (Jayashree et al. 2011).

Evidence on the functionality of introgressed genes and identification of genes of interest for research investigation or for clonal improvement are issues relating to the field of genomics (discussed in the next section). To complement this process, research has been under way for identifying, cloning and characterising specific promoters to be associated with genes of interest in a plasmid vector, in order to optimise the gene expression, especially at the level of the latex cells of the tapped rubber tree. Cloning of ethylene-inducible and/or laticifer-specific promoters from the rubber tree has been undertaken (Pujade-Renaud et al. 2000, 2001). Glutamine synthetase (*gs*) and hevein (*hv*) gene promoters were targeted, based on the fact that *gs* overexpression was observed in latex after ethylene treatment (Pujade-Renaud et al. 1997), and the *hevein* protein has been found only in laticifers (Broekaert et al. 1990). Genomic clones of genes were obtained and partially sequenced (*hv1*, *hv2* and *gs1*, *gs2*, *gs3*). Unfortunately, the promoter region of *gs1* was lacking. It was not possible to distinguish *gs2* from *gs3*, or *hv1* from *hv2*, as these genes were highly homologous, including in their non-coding regions. Gene expression analysis revealed that (i) both *gs1* and *gs2*/*gs3* were responsive to ethylene in latex, with *gs1* apparently strictly induced and *gs2*/*gs3* overexpressed; (ii) *hv* gene expression in latex was very strong but not significantly responsive to ethylene; (iii) *gs1* and *gs2*/*gs3* were differentially expressed in tissues derived from in vitro culture at various stages of development; (iv) both *gs* and *hv* genes were highly expressed in undifferentiated tissue; and (v) *hv* gene expression increased with embryo development, according probably to the laticifer differentiation stage. Sub-cloning of *hv1*, *hv2*, *gs2* and *gs3* promoter regions in a vector for transformation, in fusion with the *gus* reporter gene, was undertaken in order to analyse the functionality of these promoters. As a preliminary result, the *gs3* promoter-*gus* construct was introduced into rubber tree callus tissue by particle gun bombardment.

Transient *gus* activity was detected, which demonstrated functionality of the isolated *gs3* promoter. As *gs* genes revealed differential expression during the embryogenesis process, isolated *gs* promoters combined with a fluorescence reporter gene could become, under non-destructive conditions, a potential marker of embryogenesis. As *hevein* belongs to a multigene family, different *hevein* precursor genes were cloned and compared by sequence alignment, revealing a divergence between two groups (*Hev1* and *Hev2*) in their promoter regions. One representative in each group was chosen

(*Hev1.1* and *Hev2.1*) and promoter-*gus* constructs were introduced into rice callus tissues by *A. tumefaciens* for functional analysis in a heterologous host (Pujade-Renaud et al. 2005). The two promoters were found to be functional and, to some extent, inducible, but *Hev1.1* expression level was very low, adding to other observations (P. Arokiaraj, unpublished results; P. Montoro, unpublished results), suggesting that the range of tissues and organs expressing the *hevein* promoters may be larger and not restricted to latex cells. *Hev2.1* was activated by wounding in rice, confirming Northern blot expression profiles observed in rubber, and was also induced by pathogen infection (*Magnaporthe grisea*) in rice. Functional analysis of these promoters is now continuing in the rubber tree itself; however, the *Hev2.1 hevein* gene promoter is assumed to be able to drive efficient overexpression of genes transferred to the rubber tree at the level of latex cells. New molecular constructs have recently been prepared with promoter *Hev2.1* and genes *Cu*/Zn-*SOD* and *GCL* (codes for glutamyl cysteine ligase, involved in resistance to oxidative stress).

Conventional rubber breeding takes more than 25 years to develop a new clone, but genetic transformation is the quick alternate method to introduce desirable genes. 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 callus as the explant of the clone Gl1, 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). Inner integument tissue of the immature fruit of the clone PB260 was used as the explant for genetic transformation (Montoro et al. 2003). Transgenic plants of *H. brasiliensis* PB260 were developed through *Agrobacterium*-mediated transformation by Blanc et al. (2005). 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 callus 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. 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 for PB260 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. Unfortunately, till date, none of these transformed genotypes has been taken to the planter's field for commercial utilisation.