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ADVANCES AND CHALLENGES IN *HEVEA* BIOTECHNOLOGY

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Taken in the broad sense to mean the use of biological processes to generate a product or specific information, biotechnology encompasses many sub-disciplines of research undertaken at the RRIM. Some of this is of an applied nature, being of direct relevance to the rubber industry, while other aspects are undertaken in recognition to their strategic importance to the industry. Some topics of the RRIM's biotechnology research that are related to *Hevea* breeding and agronomy are outlined below.

Rubber Biosynthesis: increasing rubber content in the latex

The rubber content in latex can vary from more than 40% in trees that are left untapped to below 28% in trees that are tapped intensively or that have been subjected to sustained yield stimulation. With the advent of ethylene-based yield stimulation systems (RRIMFLOW AND REACTORRIM) and the long flow duration that ensues, occurrences of low rubber content in the latex are becoming more frequent. There is a mounting need, therefore, to investigate approaches towards increasing the rate of rubber biosynthesis with the view of raising rubber content in latex harvested from ethylene-stimulated trees.

A proteinaceous stimulator of rubber biosynthesis has been found in the latex C-serum. Several *Hevea* genes showing sequence homology to the eukaryotic initiation factor 5A (eIF5A) of *Medicago sativa* encode for closely related soluble proteins that stimulate the incorporation of isopentenyl diphosphate into rubber. Seventeen cDNAs that were isolated showed more than 90% identity between themselves. At the protein

level, the seventeen cDNAs could be grouped into seven different isoforms of eIF5A proteins of 16 to 18 kDa. The native protein, termed the rubber biosynthesis stimulator (RBS) is slightly smaller at 13 kDa. The size discrepancy may be the result of post-translational modifications. Both the native protein and its recombinant counterparts have been shown to stimulate rubber biosynthesis *in vitro*.

Hevea genetic transformation to enhance crop productivity

There are two main goals in the *Hevea* genetic transformation programme at the RRIM. One research objective is to insert into the rubber tree genes that encode proteins of commercial value. Recombinant foreign proteins synthesised in the latex can then be extracted continually and non-destructively by tapping the tree, which becomes a natural 'factory' for their production. The second objective, currently receiving increasing attention at RRIM, is to improve the productivity of the plant by inserting into its genome genes that may enhance the production of rubber or timber. The rubber biosynthesis stimulator (RBS) protein mentioned above is one of such candidates for genetic transformation to increase rubber content in the latex. To maximize such heterologous gene expression in the latex, the use of gene promoters that direct expression in the latex could be advantageous. Other genes that might beneficially be inserted into *Hevea* include those that have the potential to increase wood volume or latex vessel density to augment timber and latex output respectively.

Latex-specific (hevein) gene promoter

The CaMV 35S constitutive promoter is often used to over-express genes in transgenic plants. In *Hevea* genetic transformation, the CaMV35S promoter facilitates strong GUS expression in the latex, although expression of the protein is also evident in other *Hevea* tissues. It may not always be beneficial for transgenic *Hevea* to have recombinant proteins expressed unselectively in all its tissues as some of these proteins could be

toxic or otherwise deleterious to the plant. Hence, it is desirable to isolate promoters that facilitate latex vessel-specific expression. A gene upstream sequence identified for this purpose is the hevein promoter. Hevein is among the most abundant proteins found in *Hevea* latex. The hevein controlling sequences direct expression of hevein to the lutoids that are organelles in the latex. In work carried out at RRIM, the 5'-flanking region of *hevein* (1.3kb) was isolated by genome walking. The transcriptional start site of the gene was determined by primer extension analysis. The sequences of the gene promoters revealed the consensus eukaryotic TATA and CAAT box sequences.

Modification of* Hevea *anatomy for improve latex and timber production

In trees, the cambium differentiates into the vascular elements comprising the xylem and the phloem. Xylem conducts water and, as wood, its serves the tree also in structural support. The phloem plays a parallel role in conducting nutrients required for growth and development. In the special case of *Hevea,* latex vessels are interspersed in the secondary phloem region of the bark. Hence, morphological aspects of phloem differentiation may also have important implications in the productivity of the rubber tree in terms of latex vessel density in the bark. A study has been initiated to investigate how wood and phloem/laticifer development in *Hevea* might be influenced by homeotic genes and structural genes thought to be associated with wood and phloem differentiation. The cambial region of *Hevea* (clone RRIM 2025) was separated out between the bark and the wood and cDNA libraries ($9x10^5$ Pfu) from tissue representing the cambium and early differentiating cambial tissue adjacent to either the xylem or the phloem were constructed. Useful genes that are identified can be candidates for *Hevea* genetic transformation.

Expressed sequence tags

A project has been initiated to build a catalogue of genes expressed in latex or Expressed Sequence Tags (ESTs) by sequencing random cDNA clones derived from

latex. This approach provides a useful tool for profiling latex gene expression, identifying new genes and generating a pool of genes available for further study. Of particular interest are genes encoding enzymes of and proteins related to the rubber biosynthetic pathway and genes relating to defence and stress tolerance. Plasmid preparation for a total of 2370 clones from the latex cDNA library has been completed. About 84% of the plasmids were found to contain cDNA inserts of more than 0.5 kbp and these were sent for single-pass DNA sequencing. Assignment of gene function of each EST was done by matching each clone with sequences deposited in public databases (BLAST analysis). About 78% of the ESTs matched with known sequences in the databases while the remaining 22% were classified as genes with no known function. Two biosynthesis-related proteins, the rubber elongation factor (REF) and the small rubber particle protein (SRPP), made up a substantial proportion (8%) of the ESTs of known function.

Reproductive biology

Fruit-set in the rubber tree is generally low, the success rate with artificial (hand) pollination being no more than between 3 to 8% in Malaysia. Pollen germination and pollen tube growth following pollination could be important factors determining fruit-set success. These parameters were therefore studied in pollinations involving female flowers from three clones: a good seeder (PB 5/51), a moderate seeder (RRIM 600) and a poor seeder (PR 107). The flowers were then examined by fluorescence microscopy for tube growth and pollen tube penetration of the ovules.

Differences in fruit-set success reputed to exist between *Hevea brasiliensis* genetic clones were verified by an analysis of breeding records for the clones PB 5/51, RRIM 600 and PR 107. Although the best fruit-set success was obtained with PB 5/51 following hand pollination, this clonal trait was not reflected in terms of greater

numbers of pollen tubes developing it the styles of hand-pollinated PB 5/51 female flowers. It was observed, however, that more pollen tubes reached the ovules in PB 5/51 than in RRIM 600 or PR 107. Significantly, PB 5/51 female flowers required fewer pollen tubes to effect penetration of all three of its ovules. As fruit formation in *Hevea* is dependent on all three ovules of the flower being successfully fertilised, PB 5/51 female flowers have hence a greater propensity for successful fruit-set. In all the three clones studied, the frequency distribution of female flowers with 0, 1, 2 or 3 of the ovules penetrated by pollen tubes did not conform to binomial expectations. Flowers with no ovule penetrated and flowers with all three ovules penetrated were greatly over-represented. One explanation for this non-random ("all-or-none") distribution is the existence of 'receptive' female flowers that favour successful fertilisation whereas 'non-receptive' flowers tend to remain unfertilised even when hand-pollinated.