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BIOTECHNOLOGICAL APPROACHES FOR CROP IMPROVEMENT IN NATURAL RUBBER AT RRII - PRESENT STATUS

17

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The recent developments in agricultural biotechnology offer great potential for the genetic improvement of *Hevea*. The goals of biotechnology research in RRII include development of efficient micropropagation systems, characterisation of important genes and their expression, development of transgenic plants for increased yield, tolerance to diseases and various environmental stresses and production of recombinant proteins.

Earlier, a method for the production of tissue culture plants through shoot tip culture with a low efficiency was developed. A field evaluation progarmme with shoot tip derived tissue culture plants is now going on at RRII farm to understand the possible advantages of these plants in their field performance. The preliminary tapping data shows an increase in yield with certain clones over their bud grafted control. Since the multiplication rate in shoo- tip cultures was very poor attempts were made to propagate rubber plants through other methods and a system was developed to produce plants through somatic embryogenesis in a large scale using immature anther and inflorescence as explants of *Hevea* clone RRII 105. The initial survival rate and field establishment after transplantation were found to be better with somatic embryogenesis derived plants compared with the bud grafted control.

In the ongoing molecular biology programme, genetic (DNA) markers for tapping panel dryness, abnormal leaf fall disease caused by *Phytophthora* and for dwarf trait in *Hevea* were identified. The gene coding for rubber elongation factor involved in the rubber biosynthesis was isolated and cloned. The β-1,3-glucanase gene, which is giving tolerance to many fungal diseases has been isolated, cloned and characterized. The role of this gene in combating abnormal leaf fall disease in *Hevea* is confirmed through gene expression studies. Transgenic plants over expressing this gene may give tolerance to many fungal diseases.

Genetic transformation was carried out with the genes coding for sorbitiol-6-phosphate dehydrogenase, isopentnyl transferase, superoxide dismutase and antisense ACC synthase. The gene coding for sorbitiol-6-phosphate dehydrogenase would confer drought tolerance and the other three genes are for conferring tolerance to tapping panel dryness. The superoxide dismutase gene would also confer tolerance to elevated temperature and light. Transgenic plantlets developed with the gene coding for superoxide dismutase, were hardened and transferred to polybags. The preliminary biochemical studies revealed that the cells transformed with superoxide dismutase gene was over-expressed when subjected to water stress. The stable incorporation of the introduced genes was confirmed through molecular methods and over-expression of the enzymes through enzyme assay. With the other three genes, the transgenic tissues are under different developmental stages.

INTRODUCTION

Genetic improvement of *Hevea* through conventional breeding and selection is very slow due to the long juvenile period which increases the time gap between two generations. The applications of biotechnology have made it possible to circumvent many of these barriers. The goals of biotechnology research in RRII include development of efficient micropropagation

systems, characterisation of important genes and their expression, development of transgenic plants for increased yield, tolerance to diseases and various environmental stresses and production of recombinant proteins.

MICROPROPAGATION

Soon after establishment of the Biotechnology Division at Rubber Research Institute of India a major effort was directed towards development of efficient micropropagation systems through shoot tip culture and somatic embryogenesis. Earlier a method for the production of tissue culture plants through shoot tip culture with a low efficiency was developed. Tissue culture is the technology that deals with growing cells or tissues like leaves, stems, axillary buds or shoot apices etc. in a specially prepared sterilised nutrient medium and developing many identical plantlets. This has been commercially established in many ornamental plants as well as in annual crop plants (Govil and Gupta, 1997). However, the progress in laticiferous, perennial tree crops like rubber is rather slow.

Shoot tip culture

The shoot tip culture technique comprises of mainly three steps. 1) establishment of healthy shoot tips in culture, 2) induction of roots and 3) transfer and establishment of tissue culture plants to field conditions. At RRII the application of shoot tip culture was initiated in 1986. Since then extensive experiments were conducted to understand the requirement of various nutrients and growth hormones in the culture medium, method of sterilisation of the initial plant material and other cultural conditions like light, temperature etc. for the successful production of plantlets. It was observed that the requirements of nutritional and growth regulator concentrations vary from clone to clone. The culture conditions were also found to vary with the physiological stage of the explant and also with the season. After optimising these parameters several important cultivated *Hevea* clones have been propagated at RRII and established in the field (Sobhana *et al.*, 1986; Asokan *et al.*, 1988)

A field evaluation progarmme of shoot tip culture derived tissue culture plants is now going on at RRII farm. This programme was initiated to study the possible advantages of these plants over their bud grafted controls in field performances. The preliminary tapping data shows 10-20 % increase in yield with tissue culture plants of clones RRII 105, RRIM 600 and PB 311 over their bud grafted controls. No yield difference was observed between the tissue culture derived plants and bud grafted controls of clone RRII 208. However, extensive field trials are to be conducted to evaluate the stability of yield and other parameters over the years before releasing such plants to the farmers.

In *Hevea* also like many other tree crops the multiplication rate of shoots in the culture is very low. *i.e.*, culturing of one shoot tip will produce only one plantlet. Moreover, the mortality rate while transferring the plantlets from the laboratory to the field is also very high. Therefore, the cost involved in the production of shoot tip culture derived tissue culture plants is very high and thus the technology has not reached a commercial scale. Therefore extensive research has been carried out to induce multiple shoots from the axillary buds and as a result a system was developed to induce growth of the axillary buds in the shoots in culture. Work is in progress for the elongation of axillary buds and to produce multiple shoots to make the shoot tip culture system in *Hevea* more cost effective.

Somatic embryogenesis

Since the multiplication rate in shoot tip culture was very poor attempts were made for the micropropagation of *Hevea* through other methods like somatic embryogenesis. This is

the technique of converting any plant cells other than the reproductive cells into an embryo and eventually developing into normal plants. This pathway consists of three stages. In the first stage the cells of explant undergo profuse division and form a mass of cells called callus. In the second stage the callus divide and develop into embryos called somatic embryos. In the third stage the embryos germinate into plantlets. At RRII extensive studies were carried out to identify suitable explants and to develop efficient and reproducible methods for producing elite *Hevea* clones through somatic embryogenesis. As a result a system was developed to produce plants through somatic embryogenesis in a large scale using immature anther and inflorescence as explants of *Hevea* clone RRII 105 (Jayasree *et al.*, 1999; Jayasree and Thulaseedharan, 2001; Sushamakumari *et al.*, 2000).

Good callus production was obtained from very immature anther tissues in a nutrient medium supplemented with the growth regulators 2,4-dichlorophenoxy acetic acid (2.0 mg/l) and kinetin (0.5 mg/l). Subsequent culturing of callus on medium containing various concentrations and combinations of auxin/cytokinin resulted in embryo induction (Jayasree *et al.*, 1999). The embryo induction efficiency was promoted by supplementing 200 mg/l glutamine and 400 mg/l casein hydrolysate in embryo induction medium. Dark incubation favoured induction and proliferation of callus as well as induction of embryogenesis whereas plant regeneration was found to be light dependent (Jayasree *et al*, 2001). Incorporation of gibberlic acid upto 2.0 mg/l increased the embryo induction frequency. Embryo germination frequency was also considerably enhanced by higher concentration of GA_3. However, further plant development was affected by increasing GA_3 concentration (Jayasree and Thulaseedharan, 2001). A detailed investigation on the response of various cytokinins on maximum germination of embryos and production of healthy plantlets were also carried out. Maximum germination of embryos and production of healthy plantlet development was obtained when the medium was supplemented with the growth regulator thidiazuron (Jayasree *et al.*, 2001). In the callus derived from immature inflorescence a higher sucrose concentration was found to be essential for the effective embryo induction as well as maturation (Sushamakumari *et al.*, 2000).

After standardising all the factors required for the production of maximum healthy plantlets, many tissue culture plants of *Hevea* clone RRII 105 were produced through somatic embryogenesis. Morphologically all these plants were uniform. The preliminary molecular analysis carried out with these plants also revealed genetic uniformity. Compared to the shoot tip culture, the plantlet production rate in somatic embryogenesis is very high. Certain advantages are expected for the plantlets of a particular clone produced through somatic embryogenesis over the bud grafted plants of the same clone. Since this type of plants have the same genetic constitution of the shoot and root, the stock-scion interaction encountered when buds of uniform genetic constitutions were grafted to unselected seedling stock could be avoided. In the filed, all the plants are expected to have similar and vigorous growth, low immaturity period and higher yield. A large scale field evaluation of somatic embryo derived plants of the clone RRII 105 has been laid out at the Central Experimental Station of RRII at Chethakkal. The initial survival rate and field establishment after transplantation were found to be better with somatic embryogenesis derived plants compared with bud grafted control plants. In addition to using this system for micropropagation of elite *Hevea* clones, this system could also be used for the development of transgenic plants with specific genes and

also to understand the effect of stock-scion interaction in field performances.

DEVELOPMENT OF TRANSGENIC PLANTS

Genetic transformation offers a viable approach for the introduction of specific agronomically important genes into crop plants. Initial focus was to develop transgenic plants tolerant to abiotic stresses like elevated temperature and light, drought and tapping panel dryness. With the above objectives four genes such as gene coding for sorbitiol-6-phosphate dehydrogenase, isopentenyl transferase, superoxide dismutase and antisense ACC synthase were identified. The gene coding for sorbitiol-6-phosphate dehydrogenase would facilitate the biosynthesis and accumulation of sorbitol in *Hevea* tissues, which in turn confer drought tolerance. The other three genes are for conferring tolerance to tapping panel dryness. It is reported that tapping panel dryness (TPD) syndrome is always associated with high level of free radical generation and it is also found that clones which are tolerant had higher levels of scavenging enzymes such as superoxide dismutase (SOD). Therefore, to reduce the free radical level in the bark, SOD gene was selected. Clones that are tolerant seem to have higher levels of cytokinin, a plant growth regulator. Therefore, for the overproduction of cytokinins, the gene coding for isopentenyl transferase, an enzyme involved in the biosynthesis of cytokinin was chosen. Generation of endogenous ethylene is closely associated with the latex flow process and it is reported that there is a climacteric rise in ethylene production at the onset of TPD syndrome. Therefore, to regulate the ethylene biosynthesis process, the antisense gene coding for ACC synthase was also selected. The superoxide dismutase gene would also confer tolerance to elevated temperature and light. The vectors with the above genes were developed for the introduction into plant cells using the bacterium, *Agrobacterium tumefaciens* as the mediator. Gene constructs for transformation were developed at university of California. At RRII after standardizing the suitable conditions for *Agrobacterium tumefaciens* mediated genetic transformation, the genes were introduced in to *Hevea* cells. The cells transformed with the foreign gene were selected over an antibiotic kannamycin and the somatic embryogenesis technique was followed for further transgenic plant regeneration. Transgenic plantlets were developed with the gene coding for superoxide dismutase, hardened and transferred to polybags. The preliminary biochemical studies reveled that the cells transformed with superoxide dismutase gene were over-expressed when subjected to water stress. The stable incorporation of the introduced genes was confirmed through molecular methods and over-expression of the enzymes through enzyme assay under various levels of stress. With the other three genes, the transgenic tissues are under different developmental stages.

IN VITRO FERTILISATION

In *Hevea* the major constraints in conventional breeding programme are low fruit set, seasonal flowering and lack of synchrony in flowering among clones. The inaccessibility of the flowers is another problem. The brief and periodic nature of flowering impedes the progress of pollination programmes. Very often it is impossible to attempt sufficient number of artificial pollinations to obtain families large enough to conduct effective seedling selection in the nursery. Low fruit set also reduces the size of legitimate families on which selection is to be applied for the evolution of new clones with the desired attributes. The inaccessibility of flowers makes the programme labour intensive and limits the number of crosses that can be performed in a season. In this context, extensive work has been carried out and the

conditions for effective *in vitro* pollination and *in vitro* fertilisation were standardised. The system could be further utilised for interspecific and intergeneric hybridisation, genetic transformation and induction of haploid plants.

MOLECULAR BIOLOGY PROGRAMMES

In the ongoing molecular biology programme, genetic (DNA) markers for tapping panel dryness, abnormal leaf fall disease caused by *Phytophthora* and for dwarf trait in *Hevea* were identified. The gene coding for rubber elongation factor and farnasyl diphosphate synthase involved in the rubber biosynthesis were isolated and cloned. The β-1,3-glucanase gene, which is conferring tolerance to many fungal diseases has been isolated, cloned and characterised. The role of this gene in combating abnormal leaf fall disease in *Hevea* is confirmed through gene expression studies.

Two random amplified polymorphic DNA markers were identified which are linked to tapping panel dryness. DNA from *Hevea* lines differing in tolerance to tapping panel dryness was screened for random amplified polymorphic DNA (RAPD) markers using 200 random decamer primers. Two populations of *Hevea* plants derived from polyclonal seedlings with each individual representing a genotype, planted in 1969 and 1971 were selected for this study. One additional band of 1.3 kb size was detected with one primer in tolerant plants derived from the population planted in 1969, with the same primers the tolerant plants from the population planted in 1971 showed the presence of another polymorphic band with 1.6 kb size. On further amplification of 1.6 kb polymorphic band as template with the same primer, the 1.3 kb fragment was also amplified. In a southern blot the 1.3 kb band hybridised with the 1.6 kb fragment and to the restriction digested genomic DNA of tolerant as well as susceptible plants. The 1.3 kb polymorphic band has been cloned and sequenced. This DNA fragment hybridised with the RNA isolated from the bark tissues in a northern blot indicating that this fragment is part of a functional gene. Now work is in progress to characterise the full functional gene. RAPD analysis was also carried out to identify DNA markers linked to abnormal leaf fall disease caused by *Phytophthora*. DNA was isolated from clones tolerant and susceptible to abnormal leaf fall disease and screened with 100 random 10-mer primers. Two DNA markers 1.6 kb and 2.5 kb in size associated with abnormal Leaf Fall disease were amplified, isolated and cloned.

DNA markers have also been developed to tag introgresed gene and dwarf character in *Hevea* through RAPD analysis. Progenies developed through controlled as well as natural hybridisation between a natural dwarf variant and cultivated clone RRII 118 were used for RAPD analysis. Random primers (85 nos.) were used to amplify genomic DNA by PCR. One primer produced one DNA marker (1.2 kb), which was introgressed from natural dwarf parent and found in natural hybrid lines. However, it was absent in RRII 118 parent as well as controlled hybrid lines. This indicates that the 1.2 kb marker is linked to dwarf character.

RAPD were also used to group 37 clones representing variability for several morphological, physiological and other characters. Among eighty random sequence 10-mer primers used, 8 primers produced polymorphic amplification products between 300 to 4000 base pairs in size sufficient to distinguish between the clones. The total number of bands from the 8 primers was 132 of which 68 (51.5 %) were polymorphic in the clones studied.

Attempts have been initiated to study the molecular mechanism controlling rubber biosynthesis. Two genes coding for important enzymes, farnasyl diphosphate synthase and

rubber elongation factor in the rubber biosynthetic pathway were amplified and cloned. Primers were designed based on the already reported cDNA sequences of the above two genes. The genomic DNA sequences were amplified by polymerase chain reaction (PCR) with the total genomic DNA and the cDNA sequences were amplified from the total RNA after reverse transcriptase polymerase chain reaction (RT-PCR).

The genomic and cDNA sequences encoding β-1,3- glucanase gene, which is conferring tolerance to many fungal diseases, were amplified from *Hevea* with gene specific primers. Under optimal PCR conditions a 968 bp DNA fragment was amplified from genomic DNA. Reverse transcription and amplification of the cDNA also yielded a 968 bp fragment. These bands were cloned and sequenced. No intron was present in the coding region of 316 amino acid final functional protein. Southern hybridisation confirmed the presence of the gene. Gene expression studies were conducted with *Phytophthora* tolerant and susceptible clones after infection. Northern hybridization and RT-PCR analysis showed that the timing and magnitude of induction of β-1,3- glucanase gene in the infected tissues were different in tolerant and susceptible clones (Thanseem *et al.*, 2002).

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