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Biotechnology activities at CIRAD

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Introduction

The Rubber Program at CIRAD is involved in biotechnology by developing tools to improve rubber tree productivity and resistance to diseases. Hence, research areas such as micropropagation, genetic transformation and molecular genetics were developed for respectively producing clones on their own root part, studying and improving rubber productivity and generating molecular genetic markers to characterise genetic determinism of SALB (South American Leaf Blight) resistance and productivity, traits which could be improved by Marker Assisted Selection.

Micropropagation

Vitroplant field trials were initiated in 1989, in order to evaluate the potential of both growth and production of this new plant material, with several partnerships: CNRA Côte d'Ivoire, the RRI of Thailand and MICHELIN. These trials were implemented with vitroplants produced by microcutting and somatic embryogenesis. This latter technique relies actually on 2 ways of embryo production: (1) Primary embryogenesis using tegument-embryogenic callus and (2) Maintained embryogenic friable callus culture. To date, only the procedure (2) is considered for economically viable large-scale propagation. The largest field trial conducted with PB 260 vitroplants, which were produced from primary embryogenesis in 1992, showed a 10% higher growth (trunk girth at 1m) and 30% higher natural rubber production after 3 years of tapping compared to grafted plants. Nevertheless, the latest field trials established with PB 260 vitroplants from maintained culture revealed a loss in vigour and consequently in growth of trees. These observations led to consider the effect of culture conditions on the quality of plant material at each step of the procedure. Thus, study on the duration of callus proliferation, the selection of callus lines and certain components of culture media such as carbohydrate and growth regulator sources are underway to improve the quality both of embryos and plantlets [Blanc, 2000 #932] [Blanc, 2002 #1610]. Besides, regeneration capacity of maintained callus cultures has been shown variable and hence affects the plantlets production for field evaluation and commercial development. Analysis of molecular markers specifically regulated upon embryogenesis induction has been carried out in order to get an early diagnosis of both embryogenic and regenerant potential of callus lines [Charbit, 2003 #1611]. Four cDNAs were shown differentially expressed at the callus stage before the embryogenesis induction between callus lines with or without the potential to produce embryos and/or plantlets. Pragmatically speaking, cryopreservation of embryogenic callus lines is now prioritized for the preservation of selected regenerant callus lines.

Genetic transformation

Production of transgenic callus lines

Collaboration with RRI of Thailand and Kasetsart University resulted in the setting up of genetic transformation procedure and in the production of transgenic callus lines [Montoro,

2003 #1153] [Rattana, 2001 #966]. So far, optimization of this procedure led to routine and highly efficient technique for the production of transgenic callus lines, which are being evaluated in terms of plantlet regeneration capacity. Recently, 20 independant transgenic lines harbouring the 35S::GUS construct were produced and regenerated more than 200 plantlets. This advance permits now to study the regulation of genes of interest and in particular first experiments were initiated with constructs consisting of fusion between hevea promoter sequences (HEV4 and GS2) with the *gus*A reporter gene.

Functional analysis of the promoter sequence of an hevein gene in model plants

Hevein is a small lectine-like protein pertaining to the group of type IV Pathogenesis-Related proteins. In rubber tree, it is located in latex cells exclusively and participates in the latex coagulation processes. It has antifungal properties *in vitro*. A 1.8 kb promoter sequence fused to the GUS reporter gene was introduced in rice and Arabidopsis, by *Agrobacterium*-mediated transformation. In rice, it appeared to be functional both in leaves, root vessels and anthers. It was activated in response to mechanical wounding in leaves, both directly and systemically. Additionally, a 1.2 to 1.6 fold activation was observed after inoculation with the rice pathogen *Magnaporthe grisea*. These results are in agreement with the presence on the sequence of potential regulatory elements involving biotic and abiotic stress signal transduction hormones. Preliminary analysis in *Arabidopsis* indicates that the promoter is highly expressed in young seedlings. A joint patent on promoter sequences from *Hevea* hevein genes has been filed between MRB and CIRAD

Molecular physiology

Tapping Panel Dryness

The protein pattern from healthy and TPD tree latex cells cytoplasm was compared by electrophoresis [12]. Two polypeptides (P15 and P22) of 15 and 22 kDa respectively were found to accumulate in the cytosol of the TPD-affected trees, whereas a 29 kDa polypeptide (P29) appeared de novo. P15 and P22 were identified as REF (Hev b 1) and SRPP (Hev b 3) respectively, two proteins proposed to be involved in rubber biosynthesis. P29 appeared to be a new member of the patatin-like protein family. REF and SRPP genes were concomitantly up-egulated by tapping. No significant difference in REF and SRPP gene expression was observed between healthy and TPD trees, indicating that their TPD-related accumulation in the cytosol was not transcriptionaly regulated. Western blot analysis demonstrated that osmotic lysis of the sedimentable organelles (lutoids) in vitro caused the release of REF and SRPP from the rubber particle membrane into the cytosol. A mechanism of cellular delocalization as a consequence of the lutoids instability was proposed to explain REF and SRPP accumulation in the cytosol of TPD trees.

Ethylene metabolism

A recent collaboration with the Indonesian RRI resulted in the launch of a PhD research program on the establishment of a model for the characterisation of latex cell metabolism and ethylene biosynthesis in the bark tissue. Isolating of full length cDNA is in progress for the multigenic family encoding enzymes involved in the ethylene biosynthesis pathway such as ACC synthetase and ACC oxidase.

Molecular genetics [Hamon, 2003 #1605] [Seguin, 2003 #1608]

Improvement of resistance to Corynespora cassiicola

Cassiicoline is a small toxic protein produced by *Corynespora cassiicola* and responsible for the pathogenicity of this fungus on rubber tree. It has first been purified and described by Frederic Breton, in the frame of a collaboration between Montpellier University (Pr D'Auzac) and CIRAD. Purified cassiicoline is a useful tool to screen the resistance of various rubber tree clones to *Corynespora*, using the *in vitro* bio-test described by Frederic Breton. However, the purification protocol initially set up is rather time consuming and yields very low amounts of the toxin. We are currently working on a new purification procedure that should allow cassiicoline production with a higher yield and higher degree of purity. Our objective is to characterize the toxin in more details (sequence, structure….) in order to evaluate its diversity among various *Corynespora* populations. Such information may also be valuable in order to investigate the mechanism of action of the toxin and to establish optimized strategies for the creation of rubber trees resistant to *Corynespora*.

Development of M. ulei microsatellite markers [Le Guen, 2003 #1620]

A *M. ulei* genomic library, enriched for CA and GA microsatellite sequences was produced at Biotrop lab (Cirad, Montpellier). A total of 389 bacterial clones were proved to contain a microsatellite region by southern hybridization. From these, 120 were sequenced by the Centre National de Séquençage (CNS, Génoscope, Evry, France) and 14 primer pairs were designed. The corresponding 14 DNA sequences are registered in EMBL/Genbank sequences database under the accession numbers AY228709 to AY228722. PCR amplification efficiency and genetic length polymorphism were tested on a sample of 11 isolated strains of the fungus. The *M. ulei* strains come from the Cirad fungus library conserved in French Guiana and are originating from various locations of French Guiana and Brazil. Eleven of the microsatellite primer pairs revealed genetic polymorphism among the *M. ulei* strains. These preliminary results demonstrated the usefulness of microsatellite markers for further genetic diversity studies on *M. ulei*. In our knowledge, this is the first report of the identification of DNA sequences and genetic markers in this fungus species.

QTL analysis of resistance to Microcyclus ulei in rubber tree

A population of 192 progeny individuals derived from a cross between a resistant clone (FX3899) and a susceptible cultivated clone (PB260) was planted in a field trial in French Guiana in order to evaluate the resistance parameters under real infestation conditions [Le Guen, 2003 in press #5. The same progeny was previously used for QTL detection of SALB resistance in controlled conditions]. In the field, the resistance type (RT), presence of stromata (ST) and level of attack (AT) were observed twenty times on a twenty-two months period, and semi-quantitative evaluation of stromata was registered only once. The search for QTLs was performed using the Kruskal-Wallis test, Interval Mapping and Composite Interval Mapping methods, on a saturated genetic maps encompassing 231 and 158 molecular markers for FX3899 and PB260 respectively. One major QTL localized on linkage group g13 was detected on the FX3899 map, responsible for 36 to 89 % of the phenotypic variance of resistance. This resistance QTL corresponds to one that had previously been detected under controlled conditions of infestation and we called it M13-1bn. Surprisingly, the effect of this QTL was larger under natural conditions of infestation than under controlled inoculation.

Other minor QTLs (four on RO38 map and one on PB 260 map) were also detected. This is the first report of the identification of a major resistance gene in rubber tree.

Microsatellite mapping of Hevea genome

Under a grant from Agropolis (Montpellier, France), a collaborative research work (the GENMAP project, led to the establishement of a microsatellite-based genetic map on a RRIM600xPB217 cross, under a one year course of a RRIT researcher held at Cirad. Total DNA samples of parents and progenies were extracted at CRRC (RRIT, Thailand) and microsatellite genotyping was performed at Cirad-Montpellier lab using either fluorescent labelling in automatic genotyper (LiCOR DNA sequencer) or 33P radiolabelling. The map currently encompasses 139 microsatellite markers, genotyped on 334 progenies. Microsatellite loci are clustered in 15 independant linkage groups. This map constitutes the first fully PCR-based genetic map of rubber tree, but mapping work has to be completed on this progeny by genotyping additional (microsatellite and AFLP) markers in order to reach map saturation with the expected 18 linkage groups.

Conclusions and perspectives

Biotechnology activities on rubber tree at Perennial crops department of CIRAD are focusing on the improvement of the rubber tree productivity and the resistance to leaf diseases.

Vitroplants propagated by somatic embryogenesis and genetically modified plants have strong potential for the improvement of plant material quality. Next is on the one hand to produce embryogenic callus lines from three recommended clones, and on the other hand to apply the genetic transformation procedure to these ones. Molecular characterisation of ethylene metabolism is carried out through IRRI partnership and might lead to isolating candidate genes. Besides, mapping and QTLs analysis of latex production determinism through the characterisation of RRIM 600 x PB 217 offsprings produced by RRIT might be useful to consider genetic improvement by molecular assisted selection.

Studies on resistance to SALB and to Corynespora are concerned by the important threatening of these diseases on the rubber production in South-East Asia. The previous mapping and QTLs analysis of SALB determinism led to enlarge the analysis to other genetic background. Recently, gene tagging through linkage desequilibrium and associated studies were initiated and fine mapping of one major resistance gene by chromosome walking from BAC libraries. With regards to the resistance to Corynespora, an involvement of CIRAD on the analysis of toxin structure is in project.