Biotechnology Oil palm biotechnology: progress and prospects

Oléagineux, Corps Gras, Lipides. Volume 8, Numéro 4, 295-306, Juillet - Août 2001, La filière

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Résumé : Les stratégies d'amélioration génétique du palmier à huile (Elaeis guineensis Jacq.) font désormais appel à tout un ensemble d'approches biotechnologiques, allant de l'embryogenèse somatique à la biologie moléculaire. Micropropagation clonale. Les méthodes de clonage par culture in vitro ont conduit au développement d'une technique de micropropagation qui a été testée à l'échelle pilote sur des génotypes d'élite, permettant ainsi la production de clones hautement producteurs en huile. Cette phase a révélé un certain nombre de facteurs limitants liés au changement d'échelle, en particulier les productivités nécessaires pour satisfaire la demande des planteurs et la fidélité génétique du matériel végétal régénéré. Ces deux problèmes ont conduit les chercheurs à analyser les mécanismes physiologiques et/ou moléculaires impliqués dans l'embryogenèse somatique et les variations somaclonales induites par le clonage in vitro. Génomique structurale et fonctionnelle. La sélection assistée par marqueurs constitue, pour le palmier à huile, un projet à long terme, articulé sur plusieurs phases : l'analyse moléculaire de la diversité génétique dans le germplasm d' E. guineensis et d' E. oleifera ; le développement à grande échelle par PCR de marqueurs microsatellites, et le développement en parallèle de trois projets de cartographie génétique et de détection de QTLs s'adressant à des caractères agronomiques majeurs. Post-génomique. Afin de comprendre le phénomène d'anomalie florale « mantled », induite au cours du procédé de micropropagation, des études sur l'expression du génome ont été menées sur des cultures de tissus, dans le but de mettre au point un test de conformité précoce. Il importe d'évaluer quelle méthodologie est la plus adéquate pour ce test, en comparant des approches basées sur l'analyse des ARN, des protéines ou de l'ADN (PCR). Les recherches menées en parallèle sur les changements de niveaux de méthylation de l'ADN génomique induits par culture in vitro suggèrent une implication décisive de ce phénomène dans le déterminisme de l'anomalie « mantled ».

Mots-clés : Elaeis guineensis Jacq., épigénétique, embryogenèse somatique, génomique, marqueurs moléculaires, sélection assistée par marqueurs.

Summary : Today, a range of biotechnological approaches, from somatic embryogenesis to biomolecular research, play an increasingly important role in breeding strategies for oil palm (Elaeis guineensis Jacq.). Clonal micropropagation. Methods of cloning by in vitro culture led to the development of a micropropagation technique for oil palm based on somatic embryogenesis which was tested at the pilot stage on elite genotypes, thus enabling the production of high oil yielding

Article disponible sur le site http://www.ocl-journal.org ou http://dx.doi.org/10.1051/ocl.2001.0295

clones. This phase allowed the identification of limiting factors associated with scaling-up, with respect in particular to the scale of mass production required to meet the needs of planters and to the problem of ensuring genetic fidelity in the regenerated plant material. These two concerns led researchers to look further into the underlying physiological and/or molecular mechanisms involved in somatic embryogenesis and the somaclonal variation events induced by the in vitro cloning procedure. Structural and functional genomics. Marker-assisted breeding in oil palm is a long-term multi-stage project including: molecular analysis of genetic diversity in both E. guineensis and E. oleifera germplasms; large scale development of PCR-based microsatellite markers; and parallel development of three genome mapping and QTL detection projects studying key agronomic characters. Post-genomics. In order to tackle the problem of the mantled flowering abnormality, which is induced during the micropropagation process, studies of gene expression have been carried out in tissue cultures as a means of establishing an early clonal conformity testing procedure. It is important to assess what kind of methodology is the most appropriate for clonal conformity testing by comparing RNA, protein and DNA (PCR) based approaches. Parallel studies on genomic DNA methylation changes induced by tissue culture suggest that the latter may play an important role in the determination of the mantled abnormality.

Keywords : Elaeis guineensis Jacq., epigenetics, genomics, marker-assisted breeding, molecular markers, somatic embryogenesis.

ARTICLE

Challenges in oil palm biotechnology

Due to the increasing demand for palm oil, the lack of plantable land and the foreseeable increases in cultivation costs, it is necessary to make available to the planters planting material with high genetic potential.

Nowadays, planting material consists solely of *tenera* hybrids (fruits with shell of intermediate thickness), originating from crosses between *dura* (thick shell) and *pisifera* (thin shell) types, the thickness character being controlled by a monofactorial gene [1].

The breeding strategies developed by seed companies are aimed at producing *dura* x *pisifera* hybrids with a high productivity of oil containing a high proportion of unsaturated fatty acids, a low growth rate and, in certain cases, resistance/tolerance to diseases such as vascular wilt caused by *Fusarium oxysporum elaeidis* in Africa [2]. Breeding schemes now incorporate the exploitation of genetic resources able to provide tolerance/resistance to diseases which are confined to specific parts of the oil palm cultivation area: *Ganoderma* disease in South East Asia; and also bud rot in Latin America.

Since each selection cycle lasts for around 10 years, genetic improvement is very slow, even if much progress has been achieved over the last 50 years [3]. A very high heterogeneity is still observed among hybrids, some palms producing 60% more oil than the average of the progeny of a given cross [4].

These characteristics must be considered along with the low planting density (generally *ca* 143 palms per hectare) and the necessity of establishing seed orchards for the production of commercial planting material; thus it can be seen that oil palm improvement is labour intensive, time consuming and therefore expensive.

Biotechnological approaches applied to oil palm breeding have considerably increased in their importance, not only in micropropagation, but also in marker-assisted breeding, and more generally, in physiological and molecular studies of the expression of genes of paramount agronomic value (flowering, abscision, etc.).

Clonal propagation

The constraints which exist in oil palm breeding make desirable the development of a vegetative propagation technique which would allow:

- the exploitation of the variability existing among the tenera hybrids by cloning elite individuals [4];

- an increase of the production of high quality seeds by cloning the best male parents *(pisifera),* since pollen production can be a limiting factor [5, 6];

- the exploitation of *(E. guineensis x E. oleifera)* interspecific hybrids, a limited number of which are fertile, but which can show a good tolerance to pests and diseases, notably in South America [7];

- the production of biclonal seeds from somatic embryogenesis-derived parents [5];

- the true-to-type regeneration of future genetically-engineered materiel bearing useful agronomic traits [8].

The biological characteristics of the oil palm do not allow its vegetative propagation by conventional horticultural means, therefore, the only possible way to clonally propagate elite oil palms is by means of somatic embryogenesis. Cloning of oil palm *(Elaeis guineensis* Jacq.) is performed by inducing somatic embryogenesis on calli derived from various tissue sources, using tissue culture protocols recently reviewed in detail [8].

Current protocols

Approximately 25 years ago, two major groups initiated research programmes for oil palm micropropagation: in the UK and in Malaysia, the Unilever Plantations and Harrison and Crossfields Plantations group [9, 10] and in France and Côte d'Ivoire, the IRHO/Orstom group (which became Cirad-CP/IRD in the 80's) [4, 11]. These programmes were initiated to complement in-house breeding strategies and were aimed at multiplying the elite germplasm available in the plantations for commercial use. In a recent review [8] a description was given of different regeneration protocol applied to oil palms from various different genetic origins.

Large scale propagation through embryogenic suspension cultures

Progress has been made in plant production through somatic embryogenesis, particularly by developing systems based on the artificial seed concept [12] in which the somatic embryogenesis process is used to produce individual embryos with relatively synchronous development.

The initiation of embryogenic oil palm cell suspensions has been reported by several authors [13, 14] thus demonstrating the feasibility of this technique for a rather "recalcitrant" model. Research has been carried out [13, 15, 16] in order to develop new methods of large scale micropropagation for oil palm (see also a review [17]).

In oil palm, embryogenic suspensions are established from friable, nodular calli, which are isolated from nodular compact calli [13]. The production scheme currently used at Cirad/IRD involves 4 distinct stages, as shown in *Figure 1*.

To date, embryogenic suspensions have been successfully isolated for more than 20 different clonal lines. The average concentration was found to be *ca* 10^5 cell clusters per litre with a multiplication factor reaching 4x per month. These characteristics allow mass propagation. For oil palm, Sondahl (pers. com.) estimated the price of an encapsulated embryo produced by bioreactor technology in the USA at *ca* 0.20 USD, to which has to be added the cost of *in vitro* culture for germination, rooting and acclimatisation. Field trials are under way for the assessment of the clonal fidelity of plantlets originating from cell suspension cultures. The first results obtained from suspension culture-derived clones are quite encouraging, and various research groups from the oil palm industry in Malaysia have now developed embryogenic suspension cultures growing on 2,4-D free or even auxin-free media. These protocols are presently under assessment and initial observations of trueness-to-type have revealed a generally very low percentage of abnormal palms. A management strategy, based on the sequential cryopreservation of embryogenic lines [18] could be followed in order to lower the risk of losing embryogenic capacity in suspensions during multiplication.

Embryo maturation

Oil palm somatic embryos produced from embryogenic suspension cultures exhibit an incomplete maturation and develop directly towards germination without passing through a quiescent phase [19]. Studies have been carried out in the aim of understanding the biology of embryonic development and to identify factors involved in maturation, a critical step in the determination of somatic embryo quality for several species [20]. Changes in the abundance of compounds involved in the determination of desiccation tolerance (such as oligosaccharides and abscisic acid [ABA]), or in the vigour of regenerated plantlets (such as storage proteins) were investigated.

Acquisition of desiccation tolerance

Zygotic embryos of oil palm can withstand complete desiccation and dehydrated seeds can be stored for two to three years. Dry matter, water content, sugar and ABA contents were investigated throughout zygotic embryo development in relation to the acquisition of desiccation tolerance. The acquisition of desiccation tolerance between the 3rd and the 4th month was associated with sugars and ABA synthesis, underlying the role of these compounds in the phenomenon [21, 22].

In *Figure 2* are summarised on a single graph the main changes in biochemical and physiological parameters occurring *in planta* throughout oil palm embryogenesis.

Taking these results in account, the effects of sugars and ABA on desiccation tolerance, soluble sugar content and germination rate of oil palm somatic embryos were investigated [22]. The enrichment of the medium with sucrose and ABA improved somatic embryo maturation and desiccation tolerance which remains however incomplete as compared to zygotic embryos.

Accumulation of storage proteins

Storage proteins accumulated during oil palm embryo development were extracted, purified and characterised [23]. Only water- and low salt-soluble proteins, with respective sedimentation coefficients of 2S and 7S, were detected in mature embryos. After purification by gel filtration, the various protein classes identified were characterised by electrophoresis and amino acid composition analysis.

The accumulation of 7S globulins was studied in zygotic and somatic embryos [24]. The amounts of soluble protein and 7S globulins in somatic embryos were found to increase rapidly during the early stages of development, but were almost 80 times lower than in zygotic embryos. The *in vitro* production of 7S globulins (and more generally salt-soluble proteins) was improved by the addition to the culture medium of glutamine, arginine, sucrose and ABA, the effects of these components being additive [25].

To investigate further the regulation of 7S globulin gene expression in both zygotic and somatic embryos of oil palm, a cDNA clone, GLO7A, has been isolated for use as a probe in northern hybridisation studies [26].

In summary, it is possible to identify markers of maturity (sugars, ABA and storage proteins) in zygotic embryo which can be used to evaluate somatic embryo quality. The results achieved on somatic embryo allow to define *in vitro* culture conditions for improved tolerance to desiccation and accumulation of storage proteins. Further investigations will be undertaken to improve maturation protocols.

These results open the way for the use of strategies based on the "artificial seeds" concept for oil palm. The latter could be obtained from embryos produced at a high rate from embryogenic suspensions, which would then be correctly treated to achieve maturation (enrichment in storage proteins). Single somatic embryos would be encapsulated and stored (either at room temperature or at 0-4°C) before delivery at the appropriate time, according to the planting season, to tissue culture laboratories or nurseries situated close to the plantations, where germination either *in vitro* or *ex vitro* could be carried out.

Physiology of vitroplants

Physiology of in vitro rooting

Following induction by auxin treatment, the *in vitro* rooting performance of oil palm shoots varies considerably. A study [27] has described a preliminary evaluation of gaiacol-peroxidase activity as a marker of *in vitro* rooting, which has enabled major improvements in the standard rooting protocol, which initially involved two separate induction/expression phases. A single rooting step is now used, with an auxin treatment applied over a much longer time (8 weeks) using lower NAA (Naphthalene Acetic Acid) concentrations (0.5-1.0mg.L^{-1}). This quite long induction/expression phase probably acts as a buffer stage, which is able to stabilise the physiological status of shoots (peroxidase activity, levels of endogenous auxins) before the inductive treatment by auxin may act.

In vitro *photosynthesis and acclimatisation*

Several studies have been conducted in oil palm in order to reduce acclimatisation losses [28-32]. The *in vitro* photosynthetic parameters of somatic seedlings have been measured throughout somatic embryogenesis-based cloning procedure, with the aim of characterising the physiological status of the *in vitro* regenerated plants and thus optimising success rates during acclimatisation.

All the studied photosynthetic parameters (photochemical activities, CO_2 exchange and carboxylase enzymatic activities) indicate that, in oil palm, photosynthetic activity could be measured as early as during the first caulogenesis step, showing a noticeable increase during the second step. Subsequently, photosynthesis decreased during root growth.

With respect to Grout's classification [33], it can be assumed that the oil palm belongs to the class of plants in which *in vitro*-grown leaves can contribute to autotrophy and then play an active part in acclimatisation. It is therefore highly probable that acclimatisation losses could be due principally to poor or incomplete rooting and/or to a poor management of the environment of vitroplants (especially concerning RH) during this very critical step.

Molecular analysis of somaclonal variation

Approximately 5% of somatic embryo-derived oil palms show abnormalities in their floral development, involving an apparent feminisation of male parts in flowers of both sexes, called the *«mantled»*phenotype [8, 34]. This somaclonal variation phenomenon may result in partial or complete flower sterility, thus directly affecting oil production, depending on the severity of the abnormality. Interestingly, reversions to the normal phenotype over time have been found to occur, leading to a complete recovery of the normal phenotype for 100% of the slightly mantled individuals, and for 50% of the severely *mantled* ones after 9 years in the field [35].

Several potential biochemical markers of the *mantled* abnormality in oil palm have been investigated: polypeptide patterns [36] and endogenous cytokinins content [37] have been studied comparatively in normal and *mantled* plant material. Nevertheless, it has been very difficult to assess the validity of such markers on a large number of samples, because of the lack of reproducibility (in the case of proteins) or the high cost (in the case of endogenous cytokinins) of such estimations.

Flow cytometric analyses performed on seedlings and on normal/*mantled* plant material demonstrated a uniform 2C ploidy level [38]. Extensive Random Amplified Polymorphic DNA (RAPD) experiments, involving the examination of 8,900 markers, also failed to show banding patterns discriminating either the mother palm genome from its clonal offspring, or true-to-type regenerants from somaclonal variants [39].

Previous studies have shown that the *mantled* abnormality is epigenetic in nature. Firstly, it has been observed that reversion to a normal floral phenotype may occur in the field [8]; secondly, although the *mantled* abnormality is strongly transmitted through tissue culture, only a weak non-Mendelian transmission occurs via seeds [40]. Thirdly, our previous studies did not allow us to identify any major alterations in genomic DNA structure that could be linked with the *mantled* phenotype.

The DNA methylation hypothesis

In recent years, evidence has been accumulating that DNA methylation at the genome wide level plays a key role in regulating plant development [41].

Changes in DNA methylation on deoxycytidine (dC) residues have been shown to be involved in the regulation of gene expression at the transcriptional level, particularly during the differentiation/dedifferentiation processes and as a response to a variety of environmental stresses [42, 43]. Furthermore, micropropagation protocols often involve growth regulator treatments, which might affect the level of DNA methylation. Genetic and phenotypic variation found among regenerated plants has been termed somaclonal variation [44]. It is now clear that a diversity of genetic and epigenetic changes are underlying this instability, which may not be the result of a single causal mechanism [45, 46].

We firstly chose to use a global approach for the investigation of DNA methylation rate, aimed at revealing differences between normal and variant plant material. Using two genome-wide quantification methods (HPLC estimation of total 5 mdC concentrations, and *in vitro* saturation of CG sites with methyl groups by the SssI Methylase-Accepting Assay), we demonstrated [47] the occurrence of a significant genomic hypomethylation in abnormal calli (-4.5%; $p < 10^{-5}$) and leaves (-1.2%; $p < 10^{-5}$) from *"mantled"* regenerants, compared with their normal counterparts *(Figure 3)*.

This study on global methylation rates provides us with a first glimpse of the molecular changes associated with the *mantled* abnormality and it is consistent with the epigenetic characters observed, including reversion. Despite the highly significant decrease in methylation rate observed in *mantled* palms, it is likely that very few of the corresponding cytosines play a direct role in the triggering of the somaclonal variant phenotype.

Global DNA hypomethylation associated with local genetic or epigenetic defects has already been documented in several cases of developmental abnormalities, in plants [43] as well as in animals [48].

Therefore, we considered it necessary to target more precisely those sequences which, when misregulated, could potentially account for the "*mantled*" phenotype, or which could be used as markers for the early detection of DNA methylation perturbation in the regeneration process. To this end, we have been carrying out methylation-sensitive RFLP and AFLP studies, involving the isoschizomeric enzymes *Msp*I and *Hpa*II. Methylation-sensitive RFLPs were envisaged primarily in

order to screen a pool of oil palm cDNA clones for methylation-dependent polymorphism, while the aim of MSAP (methylation-sensitive amplified polymorphism) investigations was to generate a large number of relevant markers, exhibiting a differential methylation pattern depending on the normal/*mantled* phenotype but independent of the genetic origin of clones.

In parallel, we addressed the question of a possible link between the observed hypomethylation and chromatin rearrangement, as DNA methylation often parallels the compaction of a genomic domain [49]. As a continuation of this work, we hope that by isolating oil palm relatives of the *Arabidopsis thaliana METI* DNA-methyltransferase gene, we will obtain useful information to help explain how the *mantled* abnormality is generated, dysfunctions of genes of this family having been found to be linked to a number of developmental abnormalities.

Analysis of differential gene expression

The fact that the *mantled* abnormality involves a characteristic homeotic modification of oil palm floral architecture implies that the activity of a specific subset of genes has been altered in somaclonal variant plants, at least within the flower and fruit tissues. Attempts are made to identify floral homeotic genes of the "MADS box" transcription factor family which might be affected by the chain of events resulting in the *mantled* abnormality. Most of our efforts to date have however been concentrated on investigating whether *mantled*-dependent gene expression patterns may also exist during the *in vitro* stages of the micropropagation procedure. At the vegetative stages of development, no distinct morphological criteria are available for screening out abnormal palms. The identification of "fingerprint" genes displaying *mantled*-related expression at pre-planting stages would thus be of great benefit by providing us with the means to formulate molecular tests for clonal conformity.

Extensive studies have been carried out on gene expression in oil palm tissue cultures as a means of identifying putative early markers for clonal conformity testing. The differential display *(ddRT-PCR)* technique [50] was used for this purpose. The latter technique exploits the rapidity of the polymerase chain reaction (PCR) to compare mRNA abundance between two or more RNA samples extracted from the plant material of interest. The isolation, validation and characterisation of expression markers of the *mantled* abnormality involves a number of stages summarised in *Figure 4*.

The differential display stage provides candidate markers which must be validated and characterised using a range of different molecular techniques. Initially, northern blotting is used to confirm differential expression using the same material as for the differential display experiment. Next, markers confirmed as being differential are tested on various other cultures to assess whether gene expression is consistently associated with *mantled* status. Any markers considered sufficiently promising after this stage are characterised in detail by the isolation of the cDNA coding sequence, Northern studies of tissue specificity and genomic 5' flanking region isolation. The latter allows to investigate whether any conserved cis-acting elements are present in the promoter region which might shed light on the regulation of the gene in question.

An illustration of the results obtained in the various stages of marker isolation and validation is shown in *Table 1*.

In this case, data obtained for leafy shoot material is presented. We have identified and characterised putative expression markers of the *mantled* abnormality for several different stages of the micropropagation procedure (callus, embryoids, leafy shoots and greenhouse-harvested leaves).

It is now of paramount importance to test the reliability of the markers on a wider range of genotypes. This will provide a clearer picture of their reliability and enable the selection of specific markers of interest for pilot scale clonal conformity testing.

The latter might be achieved by a number of different techniques, including northern hybridisation, immunodetection (see below) or PCR. The final choice will depend on a number of factors, notably the nature of the marker gene and the developmental stage and tissue chosen for the testing. This choice of marker will also have to integrate the real cost of the technique, in comparison with the production costs of oil palm vitroplants.

In parallel with the work described above, the Cirad-CP/Ird group is also currently establishing a collection of systematically sequenced EST (Expressed Sequence Tag) cDNA clones. This work is being carried out as part of a French government-funded *Genopole* project. A key part of our EST project is centred on studying the functioning of the oil palm shoot apical meristem. We thus plan to build up a collection representing genes expressed in the oil palm shoot meristem within specific tissues, at certain developmental stages or in response to environmental conditions of interest. Our aim is to assemble an extensive catalogue of oil palm genes in this way, which will be screened either on the basis of their sequence affinities (similarity to known genes of interest) or by using high throughput macro- or microarray screening to examine their expression patterns.

Protein studies

In order to improve our understanding of the differential gene expression phenomena underlying the appearance of the *mantled* abnormality and to develop strategies for the implementation of simple and inexpensive clonal conformity testing, we are in the process of initiating protein studies as a complementary approach to the transcriptome-based work described above. Initial work will be aimed at characterising variations in the abundance of individual polypeptides in relation to clonal conformity using two dimensional SDS polyacrylamide gel electrophoresis (2D SDS-PAGE).

Although the latter technique has been practised for many years, it originally suffered from problems of reproducibility which have been considerably improved thanks to recent efforts in this area [51]. Thus there has emerged the new field of proteomics which enables global comparisons to be made between mRNA and protein accumulation. We will be using this approach in an attempt to identify early protein markers of normal or variant tissues. One inherent advantage of a protein-based approach is that it allows faster progression towards an antibody test compared with RNA studies.

Genetic engineering studies

Transient expression and stable transformation of oil palm tissues has already been demonstrated [52, 53].

As a further complementary approach to understand the molecular phenomena underlying the *mantled* abnormality, we are currently in the process of preparing promoter: GUS constructions that will be initially tested for their activity by transient expression analysis using particle bombardment. This may provide indications as to whether DNA methylation plays a direct role in determining the differential expression pattern of a given gene, since the activity of the gene promoter carried in an exogenous plasmid-derived DNA will be probably be unaffected by clonal conformity in this case. Conversely, if the differential expression pattern is determined by signalling elements further upstream in a regulatory cascade, the activity of the bombarded promoter would be expected to be influenced by tissue conformity.

For our transformation work, we have chosen to concentrate on the use of embryogenic suspensions [13] as the starting plant material, since this should simplify the process of regeneration from tissue culture: low size, abundant and very homogenous. A typical example of results obtained is shown in *Figure 5*.

Marker assisted breeding

Oil palm breeding programmes are all based on a major Sh gene determining three variety types [1]: *dura* (homozygous loci Sh^+/Sh^+) with thick-shelled fruits, *pisifera* (homozygous loci Sh^-/Sh^-) which is generally sterile female and produce shell-less fruits, and *tenera* (heterozygous loci Sh^+/Sh^-) with medium-thickness shells.

The originality of oil palm lies in its high capacity for multiplication from seed, which allows breeders to build up families of full-sib individuals which enable an effective evaluation of the general and specific combining abilities of their parents, if tested in appropriate structures.

Oil palm breeding strategy and update on molecular markers

Further progresses in oil palm breeding are hampered by several factors, notably:

* the duration of a generation for the crop and the long selection cycles - ten to twelve years - that have to be set up on vast experimental areas;

* the limited knowledge of the genetic diversity and degree of heterozygosity of the material tested;

* the complex phenotypic expression of the main quantitative characters which have been selected, such as:

- oil production, which depends on relatively heritable but negatively correlated characters, or on characters influenced by the environment;

- tolerance of vascular wilt *(Fusarium oxysporum* f.sp. *elaeidis)*; a major disease in Africa;

- tolerance to But Rot disease in South-America;

- vegetative development: the aim being to reduce vertical growth in order to prolong the economic lifespan of the plantations;

* the impossibility of determining at the nursery stage the variety of individuals to be planted, which results in not testing *tenera* x *tenera* individuals whose 25% *pisifera* individuals are too numerous, and not planting plots of pure male pisifera chosen for commercial *dura* x *pisifera* seed production.

In short, in addition to the perennial nature and dimensions of the plant, it is our poor knowledge of the oil palm genome that prevents optimum conventional breeding.

We now have a clear idea of the requirements relating to better knowledge of the genetic structure of populations, checks on the identity and heterozygosity of progenies, more effective studies of the genetic links between selected characters and, above all, the provision of early selection aids [54-56].

The use of oil palm genetic markers was initiated in Malaysia and the UK at the start of the 1990s, for clonal identification and genetic mapping [57, 58]. It developed prospectively, with the appearance of new techniques: genetic diversity studies of *E. guineensis* using RFLP or RAPD markers [59, 60]; RFLP genotyping of *E. guineensis* and *E. oleifera* accessions [56, 61]; search for cDNA markers of genes involved in floral development [62, 63] or genes coding for fatty acid biosynthesis enzymes [64, 65]; search for RAPD markers of somaclonal variants [39]; and RAPD detection of Bud Rot tolerance [66]. The Plant Breeding Institute in UK published [58] the first RFLP genetic map of oil palm in 1997. Although it is incomplete, with 860 cM mapped and 24 linkage groups compared to 16 chromosome pairs, the map was a first step towards a rational use of molecular markers.

Furthermore, molecular analysis of *E. oleifera* germplasm has been realised using cDNA and genomic RFLP probes [67] and the joint use of selection indexes and molecular markers was employed to optimise the breeding scheme [68, 69].

Marker-assisted breeding strategy for oil palm

Biomolecular research applied to marker-assisted oil palm breeding is a long-term plan. The different stages identified for oil palm [70] are detailed in this chapter.

Mass development of PCR based-markers in oil palm

Simple Sequence Repeats (SSRs), also called microsatellites, are tandem arrays of simple nucleotide motifs that are ubiquitous components of eucaryotic genomes [71]. Inherited in a Mendelian fashion [72] their hypervariable length polymorphism is simply revealed by the Polymerase Chain Reaction (PCR) using flanking primers that generate co-dominant markers. Development of oil palm microsatellite markers has been undertaken since 1998, with the view to developing genetic diversity studies, to variety identification, to pedigree analysis as well as to genome mapping and QTL detection towards marker-assisted selection activities.

A quick and simple technique for building microsatellite-enriched libraries was developed [73], from a hybridisation-based capture methodology using biotin-labelled microsatellite oligoprobes and streptavidin-coated magnetic beads. About 200 functional SSR primer pairs have already been developed for oil palm from microsatellite clones which have been sequenced in collaboration with the French National Sequencing Centre in Evry France *(Genoscope)*.

The characterisation of 21 SSR loci was published [74] together with primer sequences, estimates of allele size range as well as expected heterozygosity in *E. guineensis* and in the closely related species *E. oleifera* where an optimal utility of the SSR markers was observed. Multivariate data analyses showed the ability of SSR markers to efficiently reveal the genetic diversity structure of the genus *Elaeis* in accordance with known geographical origins and measured genetic relationships based on previous molecular studies *(Figure 6)*. High levels of allelic variability indicated that *E. guineensis* SSRs will be a powerful tool for genetic studies of the *Elaeis* genus including variety identification and intra- or inter-specific genetic mapping *(Table 2)*. PCR amplification tests, from a subset of 16 other palm species, and allele sequence data showed that *E. guineensis* SSRs are putative transferable markers across palm taxa. In addition, phenetic information based on SSR flanking region sequences makes *E. guineensis* SSR markers a potentially useful molecular resource for any researcher studying the phylogeny of palm taxa.

Establishing a reference genetic map

In addition to providing further knowledge of the genome, the aim of drawing up reference maps is a rational choice of markers and parents to be used later in the plan. A control *dura* Deli x *tenera* La Mé F1 progeny widely used in the breeding scheme was chosen (cross DA10D x LM2T). The homologous and heterologous markers mapped will primarily be co-dominant, locus-specific and easily transferable from one population to another (mostly microsatellites but also genomic RFLP (Restricted Fragment Length Polymorphism) or cDNA markers, and EST (Express Sequenced Tag) markers). Highly polymorphic microsatellite markers will make up the web of the reference genetic map; these markers are effective in genetic diversity studies notably in oil palm [75], and highly suitable for genome mapping as they provide a wide genome coverage [76]. These markers will be topped up with RFLP and cDNA "anchor points". Finally, AFLP (Amplified Fragment Length Polymorphism) markers will be used to saturate the reference linkage map.

Establishment of a multi-parent consensus map

The theoretical work performed by Muranti [77, 78] showed that a multi-parent consensus map, established with several parents (at least four to six) and full-sib families linked within a diallel or factorial design is the most effective in the search for QTL suitable for use in marker-assisted breeding. In addition to ensuring more accurate detection compared to a single two-parent map, it also enables an evaluation of the effects of QTL depending on their type (additive, dominance) and on different genetic backgrounds. Such a multi-parent map will be established by Cirad in collaboration with PT SOCFIN Indonesia, using microsatellite and cDNA markers on an existing factorial genetic design already observed for most vegetative and production characters. The map population consists of about 600 palms and several *dura* x *tenera* full-sib families, including the reference map population, obtained from different La Mé, Deli and Yangambi parents. All individual maps of the system will be connected between themselves by common parents and by co-segregating microsatellite or cDNA/EST markers.

Search for worthwhile QTL or major genes

QTL for vegetative and yield characters, as well as of vascular wilt tolerance are detected by studying the correlation existing between the markers and the phenotypic characters of the individuals chosen for the individual or consensus maps. The variability of QTL effects due to the environment will be assessed on a control progeny duplicated under different ecological conditions.

At the present time, two complementary approaches have been followed in the aim of identifying AFLP or microsatellite markers linked to the Sh gene, governing shell thickness: i) by BSA (Bulk Segregant Analysis) of segregant groups [79], an efficient methodology for detecting major genes and ii) by genetic mapping [80]. A total of 124 *Eco*RI/*Mse*I AFLP primer pairs and 88 microsatellite primer pairs was used to analyse 3 *dura*, *tenera* and *pisifera* segregant groups, each consisting of the DNA of 8 individuals obtained by selfing of a tenera parent LM2T. Out of a total of 9,330 loci screened by the AFLP technique, an AFLP-BSA candidate marker AggCAA20 was identified *(Figure 7)* then validated by individual analyses of the DNA making up the mixes. Microsatellite screening did not reveal any marker on these mixes.

A F1-type progeny (*dura* x tenera) of 90 individuals obtained from a DA115D x LM2T cross was chosen to carry out genetic mapping of the oil palm and its Sh gene, in the LM2T parent. The genetic map of LM2T, constructed with MAPMAKER at LOD score = 5.0 and r = 0.3 (apart from minor exceptions), distributed a set of 149 AFLP or microsatellite markers in 15 linkage groups, 3 pairs and 6 unlinked markers *(Figure 8)*. The number of linkage groups, close to the number n = 16 pairs of chromosomes of the plant, and a total map size of 1,355 cM already indicate relatively good coverage of the genome. The AFLP-BSA marker, which had revealed a co-dominance reading of the Sh^{+} gene on the *dura* and *tenera* phenotypes, was likewise mapped in LM2T. The gene Sh and its AFLP-BSA marker were mapped onto the longest linkage group of the map (219.5 cM), at 7.2 cM or 12.6 cM according to the respective JOINMAP or MAPMAKER genetic mapping software.

These results demonstrated the pertinence of BSA analysis in the search for molecular markers of the Sh gene, and the paramount interest of genetic mapping as a complementary approach. The AFLP-BSA marker and the genetic map of LM2T are an important step towards marker-assisted selection of the Sh gene, and of other genes of agronomic interest in oil palm.

Useful applications of genetic mapping

* The molecular dissection of the chromosomal region surrounding major gene Sh, coding for the existence or lack of a shell, with a view to cloning and (intragenic) labelling of the related genes responsible for fruit morphology and fertility (degree of shell lignification, kernel volume in *dura* and *tenera* varieties, female sterility of *pisifera* genotypes).

* The characterisation, cloning and tagging of vascular wilt tolerance genes.

* Molecular studies of genetic diversity in oil palm, which will be based partly on QTL markers and will determine the potential for exploiting natural diversity, particularly to predict heterosis.

* The search for plant material tolerant to Bud Rot in Latin America, through the genetic mapping of tolerance genes in the American species *E. oleifera* [81] with a view to their introgression into *E. guineensis* by marker-assisted backcrossing. This latter project is based on the genetic mapping of an

inter-specific back-cross of first generation (BC1) for which the resistance to Bud Rot of each genotype will be evaluated through the field test of clonal descendants planted in an area strongly affected by the disease.

Validation of molecular markers

Field Trial Systems (FTS) consisting of several populations to be used for future marker-assisted breeding programmes will be established using new factorial genetic designs. Trials will be planted at different locations consisting of 5,000 genotypes and involving at least 20 parents (full-sib families).

The objectives are to test the accuracy of molecular markers on multi-parent and connected full-sib families issued from extended genetic backgrounds and dedicated to a wider identification of intra- and inter-population QTL diversities. Also, the project results will integrate into current oil palm breeding programmes in the form of an applied multi-characters marker-assisted breeding strategy for worthwhile genes. Various parents will be used including palms suspected or known to present genetic resistance factors to the lethal diseases caused by *Ganoderma* or *Fusarium oxysporum*.

Some trials will test inter-specific back-crosses of first or second generation for the marker-assisted detection and fast introgression of important *Elaeis oleifera* characters into oil palm: low height increment and high oil quality, but also genetic resistance factors to the lethal diseases *Ganoderma*, *Fusarium* wilt and Bud Rot.

Perspectives in marker-assisted breeding of oil palm

Molecular markers data will be associated with selected phenotypic characters to define and apply the marker-assisted breeding scheme. Such use of molecular markers will increase the efficiency of conventional breeding in terms of accuracy and time saving. It is an early selection tool. It will be easier to determine the genotype of material to be tested at an early stage, rather than measuring certain phenotypic characters at each generation, for ten years or more. This will limit the time lapse between selected generations, fasten genetic gain and so increase the expected quality of commercial seed.

Major applications will concern the following areas:

* variability management (germplasm, combining ability groups),

* genotyping for characters highly influenced by the environment or costly to measure,

* control and monitoring of recombinations,

* predicting genotypic values for complex characters,

* predicting the value of a cross from information about the parents.

Genotype identification and legitimacy analyses (determination of paternity, reproduction schemes) are one major application. In oil palm, molecular markers will be used to select *dura*, *tenera* or *pisifera* individuals at the nursery stage. In the same way, it will be possible to detect and then plant *pisifera* individuals in male parent plots for tenera seed production. It will be partly possible, using gene markers, to evaluate the genetic value of these *pisifera* individuals, which was impossible in the

field until now due to their female sterility. Lastly, effective management of the entire variability of the *E. guineensis* species will be a possibility, with field testing of *tenera* x *tenera* progenies, of which only tenera individuals will be retained.

Automatic genotyping will concern the main worthwhile parents and the survey populations will be integrated into the oil palm breeding scheme. Here, biomolecular results, in terms of favourable gene detection and evaluation with molecular tools, will be applied to the whole breeding scheme.

* Results presented in the present article are part of an oral communication given at PIPOC 2001 (MPOB International Palm Oil Congress), 20-23 August 2001, Kuala Lumpur, Malaysia.

CONCLUSION

Biotechnological approaches applied to oil palm breeding have considerably increased in their importance, not only in micropropagation, but also in marker-assisted breeding, and more generally, in physiological and molecular studies of the expression of genes of paramount agronomic value (flowering, abscision, disease resistance, etc.).

Recent results in micropropagation using embryogenic suspension open the way for the use of strategies based on the "artificial seeds" concept for oil palm.

Studies on global methylation rates provided a first glimpse of the molecular changes associated with the *mantled* abnormality and it is consistent with the epigenetic characters observed, including reversion. Methylation-sensitive RFLP and AFLP studies are under way, involving the isoschizomeric enzymes *Msp*I and *Hpa*II, in order to identify relevant markers, exhibiting a differential methylation pattern depending on the normal/*mantled* phenotype but independent of the genetic origin of clones.

In order to tackle the problem of the *mantled* flowering abnormality, studies of gene expression have been carried out in tissue cultures as a means of establishing an early clonal conformity testing procedure. A number of genes whose expression appears to be modulated in tissue cultures according to their *mantled* status have been identified and characterised. Efforts will now be directed towards the development of early clonal conformity tests. Markers will be tested on a wide range of genotypes to assess their reliability and studies will be performed to identify when in the micropropagation procedure testing should best be carried out. It is important to assess what kind of methodology is the most appropriate for clonal conformity testing by comparing RNA, protein and DNA (PCR) based approaches.

Molecular markers data will be associated with selected phenotypic characters to define and apply the marker-assisted breeding scheme. Such use of molecular markers will increase the efficiency of conventional breeding in terms of accuracy and time saving.

Acknowledgements

Research work described in this chapter was conducted under a joint research programme between IRD (ex-Orstom; Institut de recherche pour le développement) and Cirad-CP (Centre de coopération internationale en recherche agronomique pour le développement - Département cultures pérennes).

Research on somaclonal variation was partially funded with the aid of a grant from the Malaysian Palm Oil Board (formerly PORIM) through a Collaborative Research Project (n°CRB-96-001).

The authors thank colleagues involved in the Oil Palm Project: Jean-Luc Verdeil at IRD-GeneTrop; Tristan Durand-Gasselin, Philippe Amblard and Françoise Potier at Cirad-CP.

Biotechnology research would not have been possible without the excellent collaboration of our colleagues working in palm oil producing countries : CNRA at La Mé in Côte d'Ivoire, INRAB at Pobé in Benin, SOCFINDO and IOPRI in Indonesia, and FELDA and MPOB in Malaysia.

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Illustrations

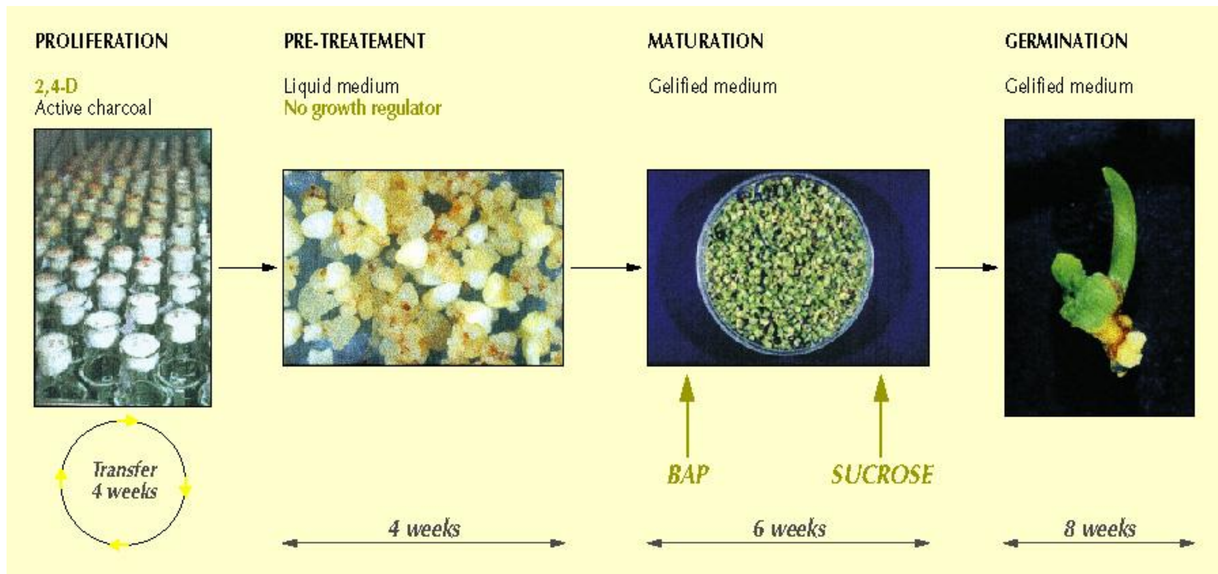

Figure 1. *Diagram of oil palm micropropagation through embryogenic suspensions (Cirad-CP/IRD protocol).*

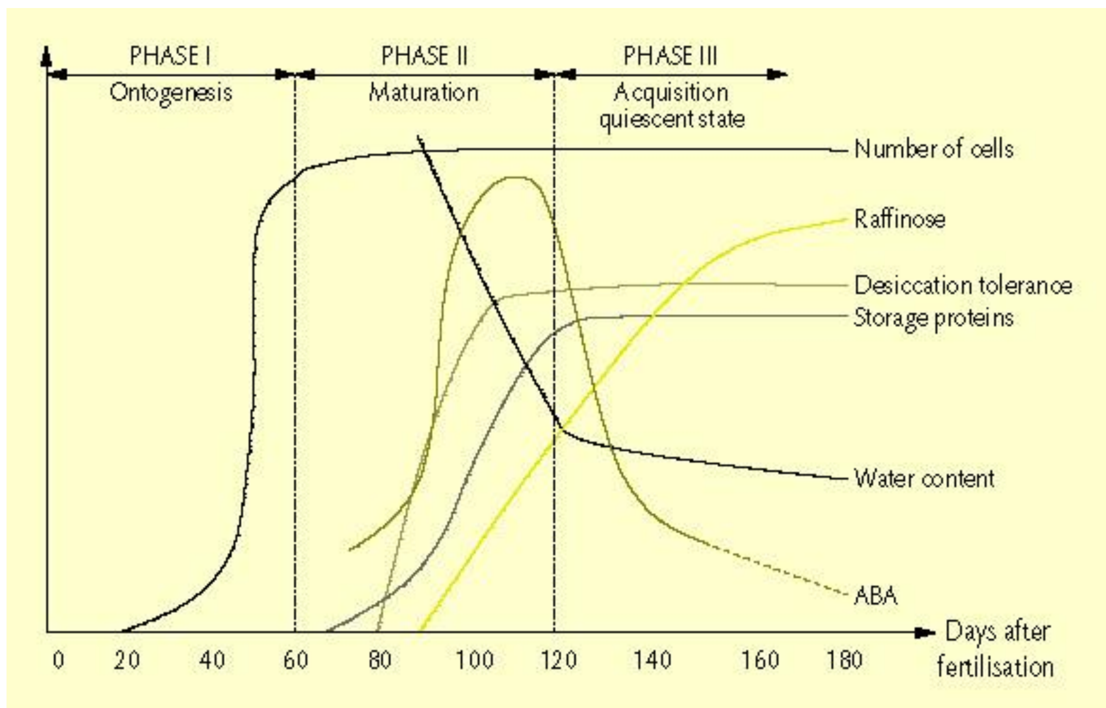

Figure 2. *Changes in biochemical and physiological parameters occurring in planta throughout oil palm embryogenesis.*

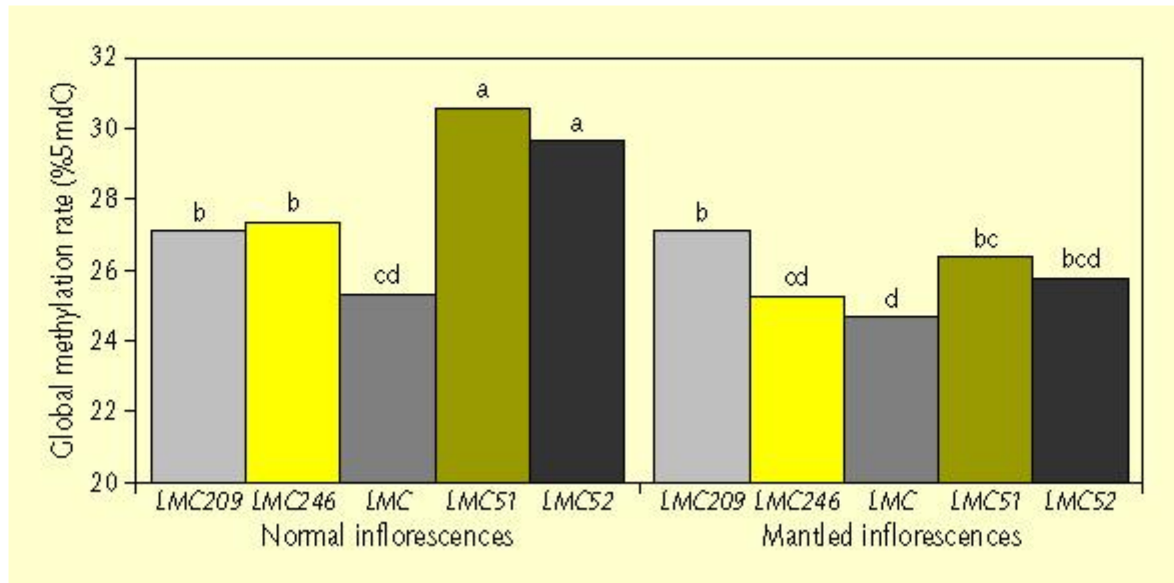

Figure 3. *Comparison of Global Methylation Rates (%) of genomic DNA in mature leaves from normal and* mantled *variants. 7 sampled palms/clone/type. 3 hydrolyses. Interaction: $F(3,113) = 35.40$; $p < 0.0000$.*

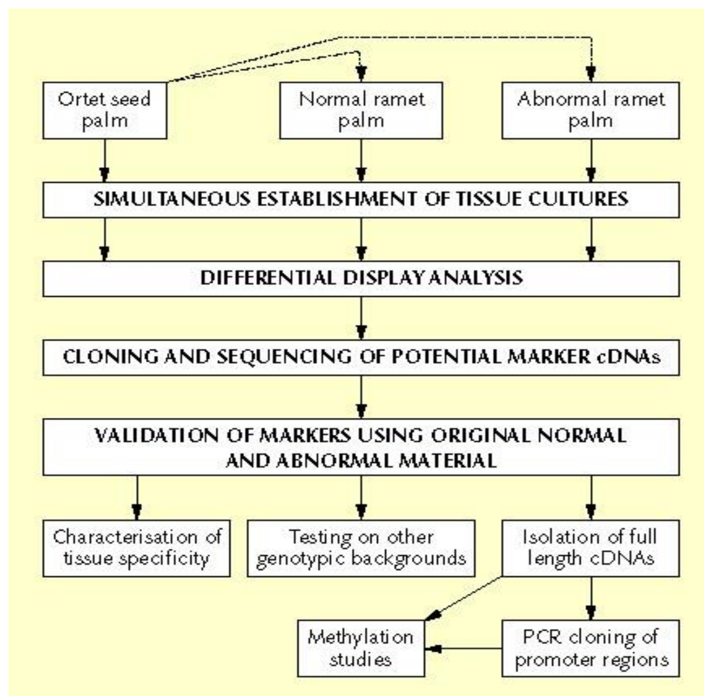

Figure 4. *Experimental strategy for the identification and characterisation of genes displaying a* mantled-*dependent expression pattern.*

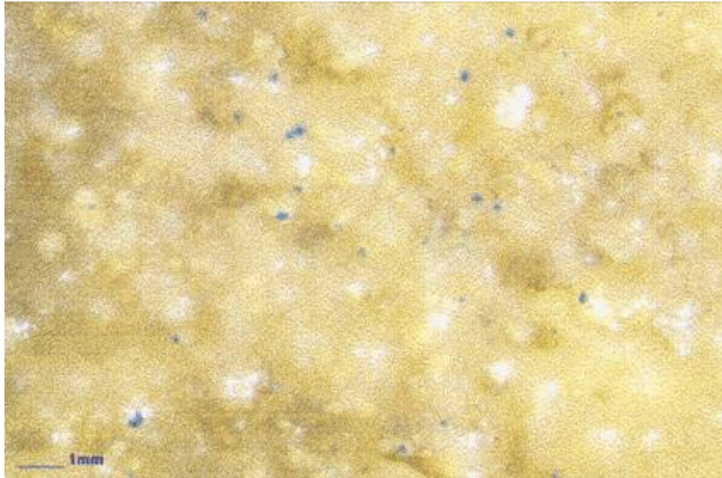

Figure 5. *Cell suspension calibrated at < 1 mm showing transient expression of 35S: gus three days after biolistic transformation. 172 ± 38 spots were counted on 500mg FW of plated cells (10 replicates).*

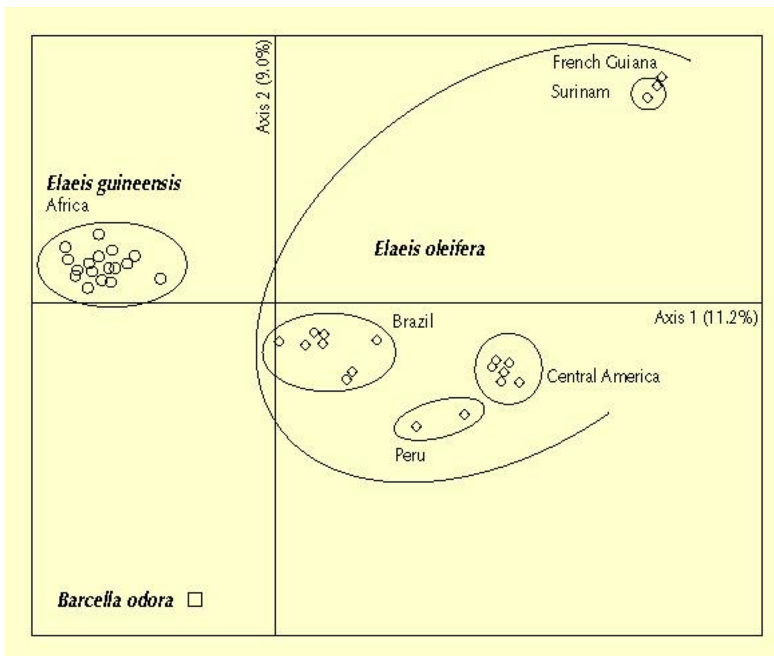

Figure 6. *Factorial analysis of correspondences performed on 20 single-locus microsatellite markers over 18 accessions of* E. guineensis *(Africa), 19 accessions of* E. oleifera *(Brazil, Central America, French Guyana, Peru and Surinam) and 1 accession of* B. odora. *Note: Axis 1 and 2 represent 20.2% of the total molecular variability.*

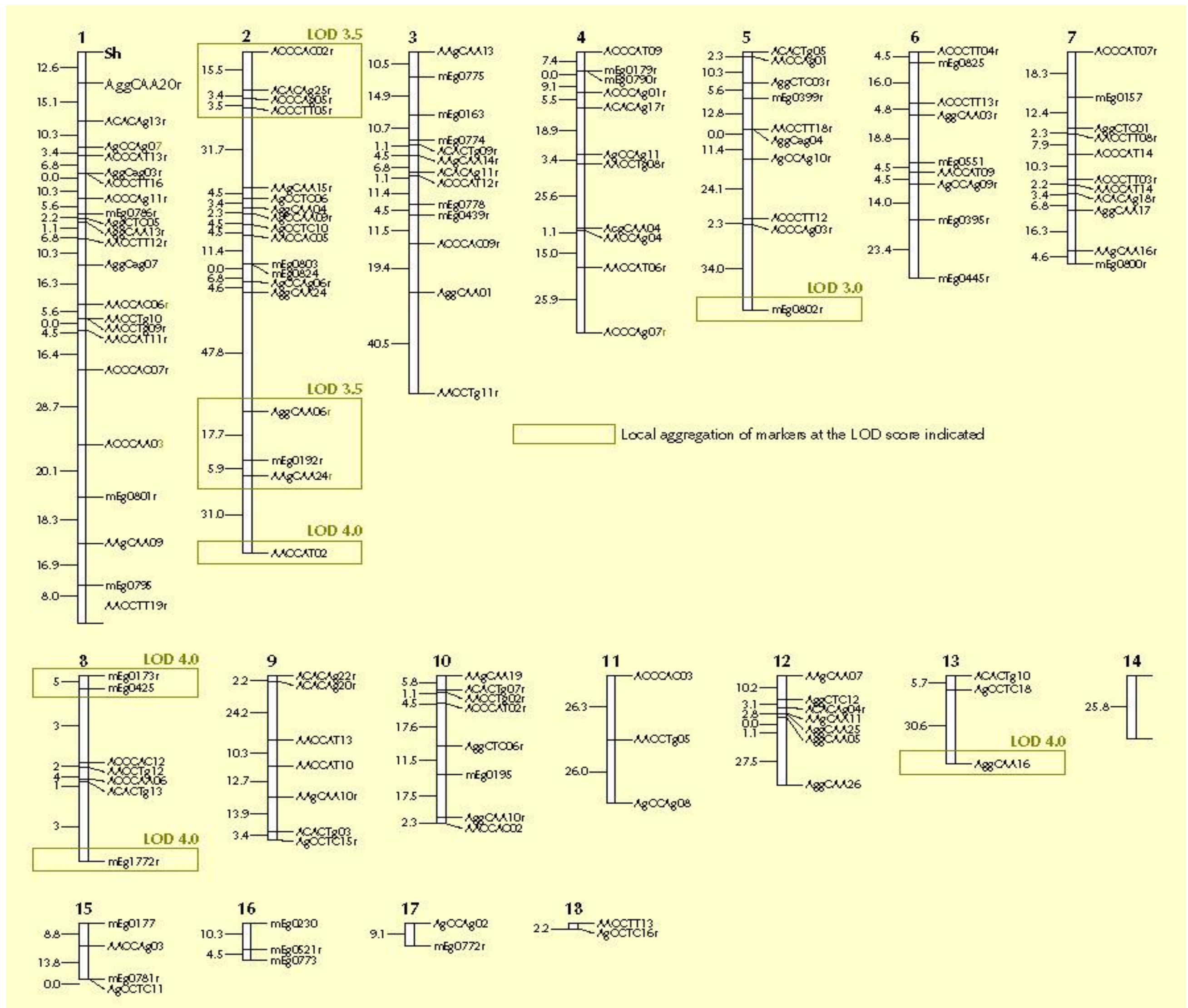

Figure 7. *Candidate AFLP marker of the Sh+ allele evidenced by bulk segregant analysis of groups issued from the selfing of a* tenera *LM2T parent. Note: D =* dura *DNA bulk; T =* tenera *DNA bulk; P =* pisifera *DNA bulk.*

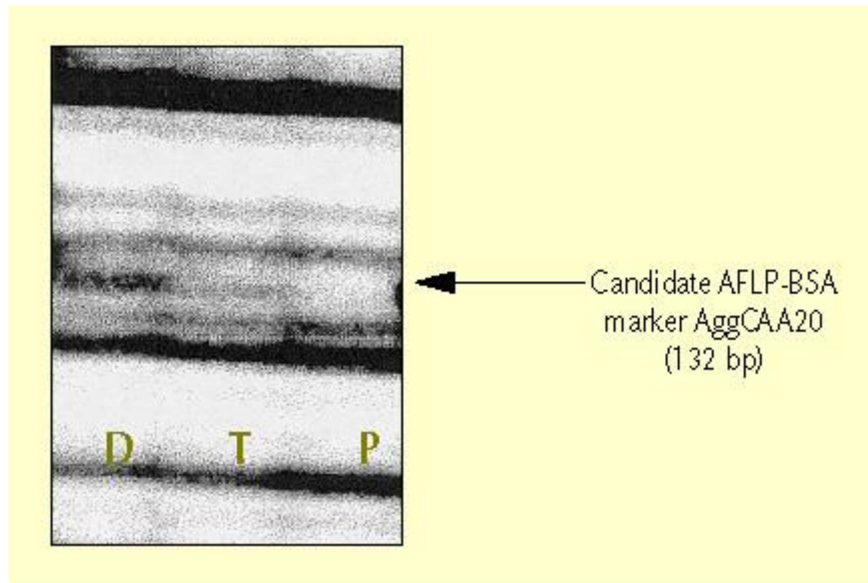

Figure 8. *AFLP and microsatellite oil palm linkage map of the tenera LM2T parent, built with the MAPMAKER 3.0 software (LOD score = 5.0; r = 0.3). Note: the Sh gene and its AFLP-BSA marker are mapped on the linkage group number 1.*

Tableau 1. *Summary of differential display marker data for leafy shoot material.*

No of potential markers identified by differential display	46
No of individual cloned cDNA fragments obtained	58
No of cDNAs producing differential signals in first stage of northern re-testing	13
No of cDNAs producing stronger northern signal in normal tissue	6
No of cDNAs producing stronger northern signal in abnormal tissue	7
No of cDNAs producing consistent signals for several genotypes (confirmed to date)	3

Tableau 2. *Type of repeats and average values of SSR allele numbers, expected heterozygosity (He) and probability of identity (PI) of 20 E.* guineensis *SSR loci in E.* guineensis *and E.* oleifera.

Sample	SSR motif	Repeats Number	# Alleles E.g.	# Alleles E.o.	# Shared alleles	Total # alleles	He E.g.	He E.o.	He E.g. + E.o.	PI E.g.	PI E.o.
9	(GA)n	17	7.1	7.3	3.0	11.4	0.7	0.7	0.8	0.02-0.15	0.02-0.38
7	(GT)n	10	4.3	3.9	1.6	6.6	0.4	0.4	0.6	0.02-1.00	0.09-1.00
4	(CCG)n	6	2.7	3.5	1.7	4.5	0.4	0.6	0.6	0.11-0.40	0.12-0.23
Average	–	–	5.2	5.3	2.2	8.4	0.5	0.6	0.7	0.3	0.2
Polymorphic markers							80%	95%	100%		