Oil Palm Biotechnology

*Recent achievements and prospects*

Dr Alain RIVAL

CIRAD
French Agricultural Research Centre for International Development
Montpellier, France

The Agricultural Research Centre for International Development, CIRAD, is a French agricultural research centre working for development in developing countries and the French overseas regions. Most of its research is managed under collaborative projects.

CIRAD has chosen sustainable development as the cornerstone of its operations worldwide. Research at Cirad takes account of the long-term ecological, economic and social consequences of changes in developing communities and countries.

CIRAD contributes to development through research and training, dissemination of information, innovation and appraisals. Its expertise spans life sciences, human sciences and engineering sciences and their application to agriculture and food, natural resource management and society.

CIRAD employs 1825 people, including 1047 senior staff members of whom 856 are scientists, and has an annual operating budget of 203 million euros.
The oil palm

◆ A giant perennial grass: Monocotyledoneous, Arecaceae (Palmaceae) 
Coconut palm, date palm, rattan, edible palms,.. 

◆ Two cultivated species:
  • Elaeis guineensis 
  • Elaeis oleifera 
    (enriched in unsaturated FAs)
  • Interspecific hybrid

The oil palm

✓ 8 millions ha planted in intertropical regions
✓ The first world source of edible vegetable oil (ahead soya)
✓ A 8.3 billions USD business
✓ Imports in EU-15 : 1.6 billions USD

A strategic crop for tropical countries
### Major Vegetable Oil: World Supply (Million Metric Tons)

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean</td>
<td>30.57</td>
<td>29.97</td>
<td>32.28</td>
<td>34.11</td>
<td>34.94</td>
</tr>
<tr>
<td>Palm</td>
<td>27.71</td>
<td>29.59</td>
<td>33.88</td>
<td>35.37</td>
<td>37.37</td>
</tr>
<tr>
<td>Sunflowerseed</td>
<td>8.12</td>
<td>9.13</td>
<td>9.01</td>
<td>10.11</td>
<td>10.10</td>
</tr>
<tr>
<td>Rapeseed</td>
<td>12.21</td>
<td>14.14</td>
<td>15.73</td>
<td>17.07</td>
<td>17.61</td>
</tr>
<tr>
<td>Cottonseed</td>
<td>3.51</td>
<td>3.83</td>
<td>4.72</td>
<td>4.56</td>
<td>4.74</td>
</tr>
<tr>
<td>Peanut</td>
<td>4.62</td>
<td>5.01</td>
<td>5.06</td>
<td>5.18</td>
<td>5.00</td>
</tr>
<tr>
<td>Coconut</td>
<td>3.16</td>
<td>3.29</td>
<td>3.44</td>
<td>3.54</td>
<td>3.26</td>
</tr>
<tr>
<td>Olive</td>
<td>2.51</td>
<td>3.00</td>
<td>2.74</td>
<td>2.28</td>
<td>2.85</td>
</tr>
<tr>
<td>Palm Kernel</td>
<td>3.36</td>
<td>3.67</td>
<td>4.13</td>
<td>4.31</td>
<td>4.48</td>
</tr>
</tbody>
</table>

Source: USDA-FAS 08-2006

### Major vegetable oils: world supply 2006/2007

- **Soybean**: 31%
- **Palm**: 31%
- **Palm Kernel**: 4%
- **Soybean**: 29%
- **Cottonseed**: 4%
- **Oilive**: 2%
- **Coconut**: 3%
- **Rapeseed**: 15%
- **Sunflower**: 8%

Source: USDA-FAS 08-2006
Oil palm in Colombia

✓ Colombia is the world’s fifth producer of palm oil and the leading producer in Latin America

✓ During the 1980’s, the number of hectares of oil palm in Colombia has tripled.

✓ By now, oil palm was the country’s most important raw material in the production chain of oil seeds and oils and fats.

✓ The areas with the highest number of hectares of oil palm are (in order): The departments of Meta (1), Cesar (2), Santander (3), Magdalena (4), Nariño (5), Casanare (6), Bolívar (7), Cundinamarca (8), Chocó (9) and Norte de Santander (10).

Facing the global context ...

✓ The last 10 years have been marked by:
  - a significant increase in demand for fat: + 50 %
  - a twofold increase in the production of oil palm and palm kernel, which now account for one third of total vegetable fat production.

✓ This trend is likely to continue over the next few years:
  In addition to traditional uses for vegetable fat, there is an increase interest and forecasted demand for bio-fuels.
Facing the global context ...

Meeting these demands will be extremely difficult, if not impossible, unless there is a considerable increase in oil palm production.

The necessary increase in oil palm production will involve extending plantations but also improving yields.

This will only be possible if planters can rely on quality plant material.

In order to achieve this goal, it is important to establish production centres for high quality planting material, located in oil-producing regions.

Overall genetic progress from 1960 to date

Genetic value of Cirad Seeds

Water deficit = 0 mm/year

1,2 % /year

55 Kg/year
Biotechnologies are impacting breeding strategies

Molecular breeding

- Molecular analysis of genetic diversity in *E. guineensis* and *E. oleifera* germplasms
- Large scale development of PCR-based microsatellite markers
- Development of a reference high-density linkage map
- Genome mapping and QTL (Quantitative Trait Loci) detection:
  - Resistance to *Fusarium* wilt
  - Increased and stable oil palm production
  - Detection and introgression of *E. oleifera* genetic factors conferring resistance to Bud Rot.

A network of field trials is being established in order to validate QTL markers, to implement marker-assisted breeding strategies and to pursue physical mapping towards the characterisation, cloning and tagging of useful genes.

Biotechnologies are impacting breeding strategies

Structural and Functional genomics:

**Aims**
Cloning genes of agronomic interest such as the *Sh* major gene responsible of the fruit variety or
Cloning a gene coding for a lipase responsible of the palm oil acidity in the pulp of mature fruits.

**Tools**
(i) EST (Expressed Sequence Tag) of cDNA sequences,
(ii) Physical mapping of BAC clones,
(iii) cDNA-AFLP mapping
(iv) Differential display of cDNAs

The aim is to assemble an extensive catalogue of oil palm genes, which can 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 monitor their expression patterns.
Oil palm micropropagation

- Feasibility of SE-based process
  - 2 millions vitroplants
  - Sizeable genetic progress

- Transferred in producing countries:
  Indonesia, Malaysia, Côte d'Ivoire, Costa Rica, Colombia

- Bottlenecks from scaling-up:
  - Production costs: 2 to 4 US$ per vp (5 to 7 x seeds)
  - Genetic fidelity

Oil production in clonal palms
(with reference to standard cross L2T x D10D)

<table>
<thead>
<tr>
<th></th>
<th>3-5 years period*</th>
<th>Adult period**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FFB extraction rate</td>
<td>Oil</td>
</tr>
<tr>
<td></td>
<td>FFB extraction rate</td>
<td>oil</td>
</tr>
<tr>
<td>Average</td>
<td>105 % 108 % 114 %</td>
<td>98 % 108 % 105 %</td>
</tr>
<tr>
<td>Selection 1/5</td>
<td>124 % 112 % 136 %</td>
<td>114 % 112 % 128 %</td>
</tr>
</tbody>
</table>
Bottlenecks to commercial development

1. Production costs

To set up an improved production process for oil palm
- large scale production (x 10^5 vitroplants / year / clonal line)
- significant reduction in manpower costs

2. Clonal fidelity

To set up a set of DNA/RNA/serological markers
- monitoring of SE process
- discard off-type lines as early as possible

Oil palm embryogenic suspensions
The “mantled” somaclonal variant phenotype
Characteristics of the “mantled” somaclonal variation

- Inter clonal variability: 0 to 85%
- Intra clonal variability: between production batches
- Variable expression on a given palm:
  - from: one fruit on one single bunch
  - to: all the fruits from all the bunches
- Expression varying with time
  - 100% of the slightly mantled palms reverted to the normal phenotype after 10 years in the field
  - 50% of severely mantled reverted to normal
- Non-Mendelian sexual transmission

Impact of the “mantled” somaclonal variation

<table>
<thead>
<tr>
<th></th>
<th>Observed palms</th>
<th>Normal palms</th>
<th>Slightly abnormal</th>
<th>Severely abnormal</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDEFOR Côte d’Ivoire</td>
<td>29,415</td>
<td>90.3%</td>
<td>3.7%</td>
<td>6.0%</td>
</tr>
<tr>
<td>FELDA Malaysia</td>
<td>18,935</td>
<td>92.0%</td>
<td>5.6%</td>
<td>2.4%</td>
</tr>
<tr>
<td>IOPRI Indonesia</td>
<td>6,771</td>
<td>87.3%</td>
<td>5.3%</td>
<td>7.4%</td>
</tr>
</tbody>
</table>
Reversion of somaclonal variation in the field

A few things that we know ... (from the field)

Recent in-depth analysis of phenotypic characters suggested that “normal” regenerants may show reversible developmental abnormalities:

- Flower sex-ratio
- Flower abortions
- Vegetative growth

The impact of somaclonal variation in oil palm clones is wider than the “mantled” phenotype alone ...
A few things that we know ... (from the field)

- The "mantled phenotype occurs very rarely in progenies originating from sexual reproduction (a handful of individuals in 500 millions commercial seeds sold yearly...)
- One spontaneous ecotype of *Elaeis guineensis* showing a stable "mantled" phenotype has been described and named "poissonii"
- Several different SE protocols gave rise to the same variant phenotype
- Recloning from leaf explant sampled on variant somaclones always gives rise to variant somaclones
- Somaclonal variants in Date Palm (*Phoenix dactylifera*) originating from SE are reported to show supernumerary carpels

MOLECULAR DETERMINISM OF THE "MANTLED" SOMACLONAL VARIATION IN OIL PALM

- Genome Structure
  - Flow Cytometry
  - RAPD Markers
  - RFLP Markers
  - AFLP Markers
- Genome Expression
  - DNA Methylation
  - Differential Display RT-PCR
  - Expression of MADS Box genes
  - Proteomics
  - Flow Cytometry
  - RAPD Markers
  - RFLP Markers
  - AFLP Markers
  - DNA Methylation
  - Differential Display RT-PCR
  - Expression of MADS Box genes
  - Proteomics
  - Flow Cytometry
  - RAPD Markers
  - RFLP Markers
  - AFLP Markers
  - DNA Methylation
  - Differential Display RT-PCR
  - Expression of MADS Box genes
  - Proteomics
A few things that we know ... (from the tissue culture lab)

Oil Palm embryogenic calli

Nodular Compact Callus  Fast Growing Callus

In vitro regeneration via Somatic Embryogenesis

SOMACLONAL VARIANTS

< 5%  100%

What is (are) the underlying molecular mechanism(s)?

- Genome size
- DNA markers
  - RAPDs
  - AFLPs
- Gene expression markers
  - ddRT-PCR
  - Homeotic MADS Box RFs
- DNA Methylation studies
  - Global methylation rates
  - Methylation-sensitive RFLP/AFLP
  - DNA methyltransferases
  - Chromatin remodelling
Flow cytometric analysis

<table>
<thead>
<tr>
<th>Plant material</th>
<th>qDNA (pg/nucleus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seed derived palms</td>
<td>3.786 ± 0.125b</td>
</tr>
<tr>
<td>Acclimatized vitroplants</td>
<td>3.701 ± 0.223b</td>
</tr>
<tr>
<td>In vitro rooted plantlets</td>
<td>3.790 ± 0.164b</td>
</tr>
<tr>
<td>Fast Growing Calli</td>
<td>3.295 ± 0.379a</td>
</tr>
<tr>
<td>Nodular Compact Calli</td>
<td>3.290 ± 0.432a</td>
</tr>
<tr>
<td>Friable Calli</td>
<td>3.212 ± 0.223a</td>
</tr>
</tbody>
</table>

Strategy for the identification of expression markers
Differential Display analysis of shoot apices

N = ortet-derived material
A = abnormal ramet-derived material
- = plantlets grown on hormone-free medium
+ = plantlets grown on medium containing $10^{-5}$ M BAP

Results of the ddRT approach

- 5 expression markers potentially exploitable in clonal conformity testing (4 A-specific, 1 N-specific)
- Potential markers for all developmental stages (callus, somatic embryo, leafy shoot (apex), plantlet leaf
- Quantitative difference between N & A varies
- Identification of possible factors of importance, including wound response (*EGADI*)
Search for markers through MacroArrays

- 2000 cDNAs /filter
- cDNAs originating from SSH libraries (variant/normal in vitro cultivated material)
- Tissues from key stages of regeneration through embryogenic suspensions
- 48 putative markers in embryogenic suspensions
- 12 putative markers in SE-derived shootlets

Egad1 Patent. MPOB/CIRAD

Northern blot analysis of a “mantled” specific probe
Flower MADS Box homeotic genes

The "mantled" abnormality involves a homeotic modification of the floral organs (cf apetala3) and genes potentially regulating flower development are being isolated using PCR and cDNA library screening with heterologous probes.

- Identification of 15 MADS Box genes from 7 different groups (SQUA, DEF, GLO, AG et AGL2) in oil palm
- Changes in expression related to the expression of the « mantled » phenotype

B function mutations affect flower development in Whorl 2 and 3

A normal
sepal sepal carp carp

Sepals normal
petals Sepals or absent

Stamen carpelloid
carpel normal
The DNA methylation hypothesis

- Epigenetic nature of the *mantled* abnormality
  - Field results
  - Standard DNA markers (RAPDs, AFLPs ....)

- DNA methylation rates changes with developmental stages

- Tissue culture induced instability

- Growth regulators (2,4-D) affect DNA methylation rates

- Defects in DNA methylation (anti MET1) generated abnormal flower phenotypes in *Arabidopsis*

Estimation of Global DNA Methylation Rates

\[
\frac{5\text{mdC}}{\text{dC} + 5\text{mdC}}
\]
Enzymatic hydrolysis of genomic DNA

HPLC separation of nucleosides
Global Methylation Rates in embryogenic calli

No Clone effect; Type effect: F(1,11) = 58.19; p<0.0000

LMC458  LMC464  LMC458  LMC464
Normal Compact Calli  Fast Growing Calli

Global Methylation Rate (% $\text{mC}$)

SssI-Methylase Accepting Assay

Saturation of CG methylatable sites

Radioactivity $\alpha = \frac{1}{\% \text{CpG Meth}}$
**SssI-Methylase Accepting Assay**

Normalised Methylation Index (NMI) calculated from SssI-MAA analysis of DNA extracted from embryogenic calli.

<table>
<thead>
<tr>
<th>Clonal line</th>
<th>Callus type</th>
<th>NMI ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMC458</td>
<td>NCC</td>
<td>0.60 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>FGC</td>
<td>0.99 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>LMC464</td>
<td>NCC</td>
<td>0.73 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>FGC</td>
<td>1.17 ± 0.43&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Each NMI is the mean of three independent measurements. Data followed by the same letter are not significantly different at the 5% level.

---

**Search for MS-RFLP Markers**

- Oil palm cDNA probes from immature inflorescences and/or calli
- Isoschizomeric restriction enzymes (MspI/HpaII)
- Search for differential genomic DNA Methylation patterns
Isoschizomeric restriction enzymes

The restriction enzyme Mspl cleaves all CCGG sequences whether or not they are methylated at the second C, but HpaII cleaves only nonmethylated CCGG tetramers.

Search for MS-RFLP Markers

LMC 458

LMC 464

Southern blot experiments involving RFLP digestion products hybridised with the cDNA probe GPH062.
RFLP: MspI / HpaII restriction. Homologous cDNA Probe CPH07

MSAP: Methylation Sensitive Amplified Polymorphism

MSAP banding pattern obtained on DNA extracts from 3 normal (N) and 3 abnormal (AN) individuals originating from the same clone.
VARIOMETH
EXPLORING THE ROLE OF DNA METHYLATION IN EPGENETIC VARIATION IN HIGHER PLANTS

• The VARIOMETH fellowship will focus on the role of DNA methyltransferases on the determinism of somaclonal variation and on the exploration of the relationship between DNA methylation and chromatin remodelling.

• Both approaches will be developed in parallel with the aim of describing specific molecular events which could be used for the development of markers of epigenetic instability in plants.

• These markers will be integrated in a strategy aimed at the identification of in vitro treatments which are prone to generate epigenetic variability in somatic embryogenesis-based micropropagation processes.

### Table: Homologies of oil palm DNA Methyltransferases with available accessions from model plants

<table>
<thead>
<tr>
<th>Model plant</th>
<th>Sequences</th>
<th>MET</th>
<th>CMT</th>
<th>DRM</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Oryza sativa</em></td>
<td>% Identity</td>
<td>65</td>
<td>62</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>% Positives Accession #</td>
<td>79</td>
<td>75</td>
<td>70</td>
</tr>
<tr>
<td><em>Zea mays</em></td>
<td>% Identity</td>
<td>67</td>
<td>64</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>% Positives Accession #</td>
<td>81</td>
<td>76</td>
<td>68</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>% Identity</td>
<td>57</td>
<td>55</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>% Positives Accession #</td>
<td>71</td>
<td>70</td>
<td>69</td>
</tr>
<tr>
<td><em>Nicotiana tabacum</em></td>
<td>% Identity</td>
<td>62</td>
<td>59</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>% Positives Accession #</td>
<td>76</td>
<td>73</td>
<td>74</td>
</tr>
</tbody>
</table>

---

Seminario de Investigación en Ciencias Agrarias, 2008/06/02, Bogotá, Colombia.
Conclusions

1. Full lengths cDNAs coding for three different DNA (cytosine-5)-methyltransferases families (namely MET, CMT and DRM) were isolated from oil palm (*Elaeis guineensis* L) and the corresponding EgMET, EgCMT and EgDRM products were studied.

2. Global DNA hypomethylation which was previously measured in variant calluses is not related with any decrease in expression of any of the three isolated METases.
Establishment and Maintenance of DNA methylation

Establishment pathway:
- siRNA-generating pathway
- RDR2
- DCL3
- AGO4
- RDR6
- SDE2
- SDE3
- AGO1
- OTHER?

Maintenance pathway:
- CG
- MET1
- CNG
- CHH
- DRM1/DRM2
- CMT3
- siRNAs
- DRM1/DRM2
- KYP
- Histone H3K9 methylation
- Unknown protein
- Histone H3K9 methylation
- DDM1
- Chromatin remodelling
- HDMA
- Histone deacetylation
- DDM1
- Chromatin remodelling

Gene regulation:
- Methyltransferases are known to be involved in sRNA-mediated gene regulation
- Methyltransferases are part of active DNA-protein complexes
- The abundance of transcripts is not sufficient to prove the activity of Methyltransferases
- Post-transcriptional / translational regulation are involved

Seminario de Investigación en Ciencias Agrarias, 2008/06/02, Bogotá, Colombia.
The **ABC** model involves genes governing flower structure in higher plants, known as MADS box genes.

Oil palm MADS box genes have been found to be affected in *mantled* material.

Present research work is focusing on the study of DNA methylation in and around MADS box genes (promoter/intron/coding sequences).

Recent advances have facilitated the mapping of DNA methylation across the entire genome in model plants (*Arabidopsis*, *rice*).

Tools are now in hand to determine whether tissue specific or developmental patterns of gene expression are dictated by changes in DNA methylation.

Deep sequencing technology will be applied for the discrimination of differentially methylated sequences in mantled vs normal material.

Our final aim is to isolate candidate sequences which can be used for the development of Methylation Sensitive detection kits for the early identification of variant cell lines.
Relevant literature


Acknowledgements

- FELDA Malaysia
- MPOB Malaysia
- SOCFINDO Indonesia
- INRAB Benin
- CNRA Ivory Coast
- ASD de Costa Rica
- Hacienda La Cabaña (Colombia)
- Floramerica (Colombia)
- FMI Basel (Switzerland)
- University of Leicester (UK)
- Cirad/IRD Oil Palm Biotechnology Group, Montpellier, France:
  - Estelle Jaligot, Thierry Beulé
  - James Tregear, Fabienne Morcillo, Stefan Jouannic, Helene Adam, Frédérique Richaud
- CSIRO Plant Industry
  - Jean Finnegan, Liz Dennis, Jim Peacock
Muchas gracias por su atención ...