Identification of early molecular markers associated with the mantled phenotype in micropropagated oil palms by subtractive PCR and cDNA array analysis

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ABSTRACT

Tissue culture of oil palm induces somaclonal variation. The main one, known as the mantled abnormality, displays homeotic floral modifications and is associated with a lower oil yield. To detect abnormal cell lines as early as possible during the tissue culture process, studies to identify molecular markers have been carried out. Given the epigenetic nature of the mantled phenotype, differential gene expression has been focused upon rather than genome structure. We investigated gene expression patterns at the transcriptome level for three developmental stages: embryogenic cell suspensions, somatic embryos and leafy shoots. In the first step, the technique of subtractive PCR (SSH) was used to construct six cDNA libraries from variant and normal plant materials allowing enrichment for cDNA corresponding to genes which are up- or down-regulated in association with clonal conformity. In the second step, transcript abundance was monitored by macroarray hybridization. A range of genes altered in their expression in abnormal tissues has been identified and a number of which have been validated by RT-PCR. This approach will provide insights into the biological processes involved in the generation of the mantle phenotype and the identified markers should lead to the development of a clonal conformity test.

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INTRODUCTION

Micropropagation by somatic embryogenesis in oil palm produces a small but significant proportion of off-type individuals displaying a homeotic floral modification known as the mantled phenotype (Corley *et al*, 1986). A transformation into pseudocarpels is observed for the stamens of male flowers and the staminodes of female flowers (Adam *et al*, 2005), as is a reduction of flower fertility which may sometimes involve complete sterility and therefore zero oil production.

As this somaclonal variation phenomenon must be dealt with in the management of clonal production labs, the identification of molecular markers becomes of paramount importance so as to detect as early as possible any abnormal cell lines. Given the epigenetic nature of the mantled abnormality (Jaligot *et al*, 2000), gene expression has been focused upon in the work described here rather than genome structure.

An approach associating the suppression subtractive hybridization (SSH) and macroarray techniques was developed to compare transcriptome patterns between true-to-type and variant tissues at different steps during the *in vitro* process.

SUBTRACTIVE cDNA LIBRARY CONSTRUCTION AND ANALYSIS

The SSH technique (Diatchenko *et al*, 1996), allows the production of cDNAs of genes expressed in oil palm tissue cultures and enrichment for clones corresponding to genes which are up or down-regulated in association with clonal conformity. Three different types of plant material were studied: the embryogenic suspension culture, the somatic embryo and the leafy shoot.

mRNA preparation was carried out as described in Tregear *et al* (2002). Three pairs (true-to-type/variant) of SSH libraries were prepared using a “PCR-Select cDNA subtraction kit” (Clontech). From each library a portion of the 960 cloned cDNAs was sequenced and sequence comparisons performed to estimate the efficiency of the subtraction step.

The comparison of paired libraries reveled relatively low degrees of redundancy as witnessed by the low number of sequences belonging to mixed clusters (*Table 1*). This illustrates the effectiveness of the subtraction step and the significantly different compositions of the libraries.

TABLE 1: CLUSTERING ANALYSIS OF EST SSH LIBRARIES

Libraries	Origin	Number of analysed sequences	% singletons	% of sequences belonging to a cluster	% of sequences belonging to cluster associated with both libraries
S1 and S2	Cell suspension	1820	52.6	47.4	8.2
E3 and E4	Somatic embryo	552	27.2	72.8	2.6
A2 and A3	Leafy shoot	938	82.9	17.1	16.8

ANALYSIS OF GENE EXPRESSION BY MACROARRAY HYBRIDIZATION

Macroarray hybridization allows the monitoring of the expression of several thousand genes simultaneously, thus increasing the probability of finding reliable markers associated with the mantled abnormality.

To date, two sets of macroarrays corresponding respectively to cell suspension libraries and leafy shoot libraries have been prepared by spotting the cDNA inserts onto nylon membranes after PCR amplification.

Macroarray hybridizations have been carried out with radiolabelled reverse transcribed total RNA extracted from normal and abnormal material. Transcript profiling was performed to compare normal and abnormal suspension lines. A similar comparison was performed between normal and abnormal nodular callus (NCC) lines. In order to obtain a broader perspective, the same macroarray membranes were used to study gene expression between fast growing callus (FGC) with normal nodular callus (NCC). Fast growing callus (FGC) is known to regenerate 100 % mantled palms ; this material is therefore of interest for the identification of molecular markers associated with mantled phenotype.

The second macroarray set corresponding to leafy shoot libraries was hybridized with probes synthesized from leafy shoot total RNA samples from material of four different genotypes assembled together in a “bulk” approach.

TABLE 2: MARKERS IDENTIFIED BY MACROARRAY HYBRIDIZATION

	"N" specific	**"A" specific**
Suspension	53	56
NCC	17	52
NCC/FGC	16	32
Leafy shoot	20	6

"N specific": signal greater in normal material
"A specific": signal greater in abnormal material

A total of 252 potential markers discriminating normal and abnormal material at different steps during the *in vitro* process were identified following a statistical analysis of macroarray gene expression patterns (*Table 2*).
Moreover, an analysis comparing the results from the three early stage materials together i.e. cell suspensions, NCC and FCC, allowed us to select 7 markers discriminating normal and abnormal material common to all of these three categories of plant material.

VALIDATION OF MARKERS BY SEMI-QUANTITATIVE RT-PCR

In order to validate the macroarray results, oligonucleotides primers were designed for different marker genes and used in semi-quantitative RT-PCR.
Eighteen of thirty two primer pairs tested using the same RNA as for the macroarray revealed differential expression patterns of the type expected (*Table 3*) (*Figure1*).
The fact that a number of putative markers were not confirmed suggests that either they are not genuine markers or that the primers used were unable to amplify specifically the target cDNA.

TABLE 3: MARKERS TESTED AND VALIDED BY SEMI-QUANTITATIVE RT-PCR ANALYSIS

Origin (material)	**Markers identified**	**Markers validated**
Suspension	8	6
NCC	5	2
Common Susp/NCC/FGC	7	3
Leafy shoot	12	7

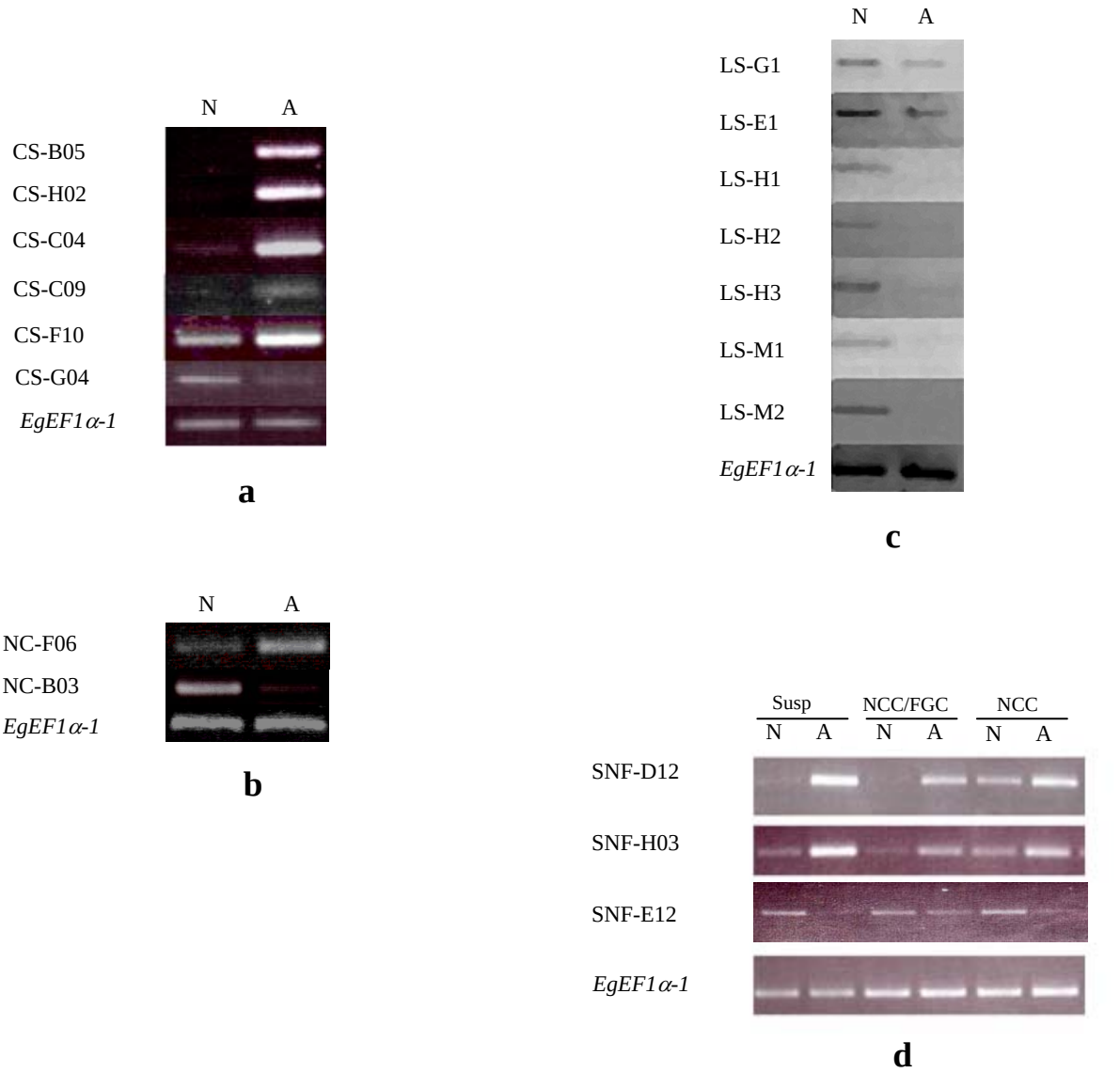

Figure 1: Semi-quantitative RT-PCR profiles of the macroarray markers producing differential signals between normal (N) and abnormal material (A) from suspensions [a] ; NCC [b] ; leafy shoots [c] and three materials in common: suspension, NCC, FCC [d]. The oil palm elongation factor EgEF1-alpha 1 gene was used as a reference.

CONCLUSION

These results illustrate the utility of an approach associating the SSH method with macroarray hybridization to identify differentially expressed genes in relation to the conformity status of oil palm tissue cultures. A number of molecular markers potentially associated with the mantled phenotype have been isolated and they now need to be validated on wider range of materials to assess their reliability with a view to their incorporation into a conformity test. In parallel, these data may provide us with insights into the developmental pathways affected in the mantled palm and to the formulation of new *in vitro* protocols.

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REFERENCES

ADAM, H; JOUANNIC, S; ESCOUTE, J; DUVAL, Y and TREGEAR J W (2005) Reproductive developmental complexity in the African oil palm (*Elaeis guineensis,* Arecaceae). *American Journal of Botany, 92 (11):* 1836-1852

CORLEY, R-H V ; LEE, C H ; LAW, I M L and WONG, C Y (1986) Abnormal flower development in oil palm clones. *Planter, 62*: 233-240.

DIATCHENKO, L ; CHRIS LAU, YF ; AARON, P ; CAMPBELL, A P ; CHENCHIK, A ; MOQADAM, F ; HUANG, B ; LUKYANOV, S ; LUKYANOV, K ; GURSKAYA, N ; SVERDLOV, E D and SIEBERT, P D. (1996) Suppression subtractive hybridization: A method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proceedings of the National Academy of Sciences of the United States of America, 93*: 6025-6030.

JALIGOT, E; RIVAL, A; BEULÉ, T; DUSSERT, S and VERDEIL, J L (2000) Somaclonal variation in oil palm (*Elaeis guineensis* Jacq.): the DNA methylation hypothesis. *Plant Cell Reports, 19*: 684-690.

TREGEAR, J W; MORCILLO, F; RICHAUD, F; BERGER, A; SINGH, R; CHEAH, S C; HARTMANN, C; RIVAL, A and DUVAL, Y (2002) Characterization of a defensin gene expressed in oil palm inflorescences: induction during tissue culture and possible association with epigenetic somaclonal variation events. *J. Exp. Bot. 53*: 1387-1396.