DEVELOPMENTS TOWARD THE APPLICATION OF DNA CHIP TECHNOLOGY IN OIL PALM TISSUE CULTURE

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ABSTRACT

In recent years, microarrays have become widely recognized as a significant technological advance to facilitate high throughput analysis of thousands of genes and their interactions simultaneously. Microarray technology can help in the rapid identification of novel genes or gene functions most worthy of detailed characterization. cDNA microarrays were utilized to understand the molecular mechanism associated with oil palm tissue culture. The availability of oil palm expressed sequence tags (ESTs) allowed the development of a DNA chip, with 3806 oil palm gene clones. The basic parameters associated with the DNA microarray experimental technique [RNA extraction, RNA amplification, Cyanine dye (CyDye) labelling, hybridization, scanning and data normalization] were successfully established. This paper will focus on developments of cDNA microarray technique towards understanding oil palm tissue culture, with emphasis on the parameters used for data analysis. We also include specific examples to demonstrate how this technology can be applied to improve our understanding of oil palm tissue culture.

Keywords: microarray, tissue culture, ESTs, oil palm.

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INTRODUCTION

The successful propagation of oil palm through tissue culture (Jones, 1974) was received with much enthusiasm by the oil palm industry. It paved the way for propagation of selected elite material, which the industry hoped would be significantly superior to commercial DxP seedlings. This inspired MPOB (then PORIM) and many commercial organizations to exploit the *in vitro* propagation technique.

The initial enthusiasm was, however, dampened in the mid 1980s when United Plantations first reported abnormal flower development in oil palm clones. Subsequently, several other organizations also reported widespread abnormality among their material propagated via tissue culture. The abnormalities in floral development involve an apparent feminization of male parts in flowers of both sexes, known as the *mantled* phenotype (Corley *et al*., 1986; Rival, 2000; Rival *et al*., 2001). In male inflorescence, no pollen is formed, whereas in female inflorescences a ring of supplementary carpels surrounds the gynoecium, thereby inhibiting the ripening of the fruits (mantled fruits). The resulting bunch failure or absence of pollen can have serious implications, as it will result in plantations suffering yield lost. However, the effect depends very much on the severity of the abnormality. Rival *et al.* (1998) reported that reversions towards the normal phenotype have been found to occur over time, which leads to a 50% recovery of severely mantled individuals to almost complete recovery (100%) of the slightly mantled palms after nine years in the field.

Another stumbling block to the commercialization of mass propagation of the oil palm was the inefficiency of oil palm tissue culture process. The rate of callogenesis of oil palm explants remains very low, at about 19% (Corley and Tinker, 2003), while the rate of embryogenesis from proliferating callus cultures has been reported to be

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only 6% (Wooi, 1995). These reasons motivated the industry and MPOB to redirect their efforts at understanding and improving the efficiency of oil palm tissue culture and at the same time reduce and/or control the abnormality phenomenon.

Almost three decades after the successful propagation of oil palm through tissue culture, reliable tissue culture procedures and extensive culling at the various stages of the process has helped to improve the efficiency of tissue culture and reduce the incidence of abnormality reported. Most plantations now routinely report less then 5% abnormality observed in their field planting, compared to more then 50% in some cases two decades ago. Nevertheless, big plantation companies still remain skeptical about large-scale propagation of tissue culture material, fearing the occurrence of abnormality.

The rising popularity of molecular biology and the advent of molecular marker technology in the 1980s and 1990s provided renewed hope to the industry. It was envisaged that these new sciences would help to unravel the phenomenon of tissue culture efficiency and abnormality at the molecular level. Markers could be developed for detecting callogenesis, embryogenesis and abnormality, making large-scale propagation viable. In this aspect, an EST programme was initiated to isolate genes expressed in oil palm tissue culture and about 20 000 expressed sequence tags (ESTs) have been identified (Elyana *et al*., 2005). These gene sequences, along with ESTs isolated from other programmes were then used to provide the framework for large-scale functional analysis of thousands of genes using the DNA microarray technique.

Generally, cDNA microarrays or cDNA chips consist of amplified cDNA fragments spotted at high density onto a solid substrate (*e.g*. glass slides). Fluorescently labelled cDNAs of tissues of interest are then hybridized together with fluorescently labelled cDNAs of a reference tissue onto a DNA microarray. The cDNAs will hybridize to its complementary strand (amplified cDNA fragments) on the array. By comparing the expression ratios obtained from the spots in the DNA microarray, one can predict the gene profile or degree of expression of the gene in the tissue of interest.

The DNA microarray programme is currently used in generating data to assist in understanding the molecular changes in oil palm tissue culture with the aim of developing a diagnostic tool for tissue culture amenity and abnormality. The cDNA chips were used to differentiate non-embryogenic callus from other stages of tissue culture (*e.g*. embryogenic callus, embryoid) and in the identification of genes modulated during the initial stages of tissue culture. The potential of the technology in unraveling differential expression during the tissue culture process was demonstrated. The same chip was also used to study the floral abnormality phenomenon associated with tissue culture clones and several genes of interest were identified.

MATERIALS AND METHODS

Plant Material

Spear leaf, inflorescence, embryogenic callus, non-embryogenic callus, polyembryoid and shoots from polyembryoids of *Elaeis guineensis* var. *tenera* were used in this study. The plant materials were frozen in liquid nitrogen and stored at -80°C prior to RNA extraction. For the tissue culture abnormality project, inflorescence from four different oil palm clones (P10, P15, P38 and P75) derived from tissue culture were used to generate both the normal and abnormal RNA pools.

RNA Extraction, Purification and Amplification

Total RNA for the tissue culture amenity project was extracted essentially as described by McCarty (1986) whilst the total RNA for the tissue culture abnormality project was extracted as described by Schultz *et al*. (1994). Normal total RNA pool comprised of 73 individual RNA samples (from various stages of development) while abnormal total RNA pool was made up of 83 individual RNA (from various stages of development) samples. Subsequently, the total RNA was further purified using Qiagen RNeasy Mini Kit and on-column RNase-free DNase I digestion according to the manufacturer's instructions (Qiagen USA, Valencia, CA). Prior to CyDye labelling, 5 µg of total RNA was amplified using the MessageAmp™ aRNA Kit (Ambion Inc., Austin, USA) according to the manufacturer's protocol. The yield and purity of the total RNA and antisense RNA (aRNA) generated from the amplification process were analysed using the Nanodrop® ND-1000 Spectrophotometer (Nanodrop Technologies Inc., USA) while the quality was accessed by electrophoretic fractionation on an Agilent 2100 Bioanalyser (Caliper Technologies Corp., USA).

Labelling of aRNA

The fluorescent-tagged (Cyanine 3 [Cy3] and Cyanine 5 [Cy5]) cDNAs were prepared by reverse transcription of aRNA (5 ug) using CyScribe First-Strand cDNA Labelling Kit (GE Healthcare Biosciences, USA; formerly Amersham Biosciences) in the presence of the fluorescent dyes, as recommended by the manufacturer. The labelled probes were purified using the QIAquick PCR Purification Kit (Qiagen, Germany) according to the manufacturer's protocol. Purified probes were

quantified at 260 nm /550 nm (Cy3) or 260 nm /650 nm (Cy5).

Hybridization

Prior to hybridization, the Type 7-star slides were pre-treated for 1.5 hr at 55°C in 5X saline sodium citrate (SSC) buffer, 0.2% sodium dodecyl sulphate (SDS), rinsed in MilliQ water and dried under purified nitrogen gas. For each slide, 40 pmoles of purified fluorescent labelled probes from each sample were co-hybridized to the pre-treated arrayed slides using the Automated Slide Processor (ASP) Hybridization System (GE Healthcare Biosciences, USA; formerly Amersham Biosciences) at 45°C. Post-hybridization stringency washings were performed sequentially at 45°C for 5 min each (1X SSC, 0.2% SDS; 0.1X SSC, 0.2% SDS; 0.1X SSC) on the ASP and then dried.

Post-Hybridization Analysis

Hybridized slides were scanned sequentially for Cy3- and Cy5-labelled probes with Generation III scanner (GE Healthcare Biosciences, USA; formerly Amersham Biosciences) at high resolution under two-colour scanning with PMT voltage set at 500. Image analysis and signal quantification were performed by Array Vision™ 8.0 (Imaging Research Inc., Ontario, Canada). The data generated was converted into MEV file format in Microsoft® Excel (Microsoft Corporation, USA). Data normalization was implemented using a native Java implementation in TIGR Microarray Data Analysis System (MIDAS) Version 2.18, which is freely available through The Institute for Genomic Research (TIGR). Pearson correlation coefficient, *r* was determined in Minitab 12 (Minitab Inc., USA). Data visualization and mining was done using TIGR Multi Experiment Viewer (TMeV) Version 3.03 (TIGR, USA).

RESULTS AND DISCUSSION

Microarray Experiments

In MPOB, the generation of ESTs and the DNA chip programme complements each other by contributing to the better understanding and improvement of the commercial value of oil palm. The oil palm chip (cDNA microarray) is then used to bridge the gap between sequence information and functional genomics and thus, provide information on genome-scale sampling of gene expression patterns. The oil palm cDNA microarray (DNA Chip 8) consist of 3806 gene clones (ESTs) and 474 control spots. The control spots include in-house control spotting samples (dynamic range controls, ratio controls, positive and negative controls) and the Lucidea™Universal ScoreCard™controls (GE Healthcare Biosciences, USA; formerly Amersham Biosciences). Each element is spotted in duplicate. Following the establishment of the oil palm cDNA chip, experiments were carried out to standardize protocols for microarray experiments. Standard Operating Procedures (SOPs) for RNA extraction, RNA amplification, CyDye labelling and microarray hybridization were established.

Duplicated dye-swap microarray experiments were carried out. Dye-swap experiments result in a relative rather than an absolute expression value for each gene on the array. Therefore in order to look at the expression level, the dye-swap results need to start from the same baseline and subsequently become directly comparable. Intensity-dependent measurement was applied in the experiments because it can correct for artifacts caused by differential Cy3 and Cy5 dye incorporation and non-linear rates of fluorescence between the two dyes (Bai *et al*., 2003). After intensity correction, TIGR MIDAS (TIGR, USA) was used to perform normalization and to create the input data for TIGR MeV (TIGR, USA). Lowess normalization was carried out based on the LOCFIT package developed by Bell Labs. The *smoothing* parameter used in this normalization process was set to 33%. The higher the smoothing parameter, more adjustments to the raw intensities of either channel will have to be done (*Figure 1*). Normalization is a crucial step in any microarray experiment as this technology is known to generate background noise and has very high variability. Some sources of variability are random but most are systematic and due to specific features of the particular microarray technology (Workman *et al*., 2002). In microarray analysis, normalization refers to a collection of processes that are used to adjust data means or variances for effects resulting from systematic non-biological differences between array, sub-arrays and dye-label channel (Schuchhardt *et al*., 2000; Quackenbush, 2002).

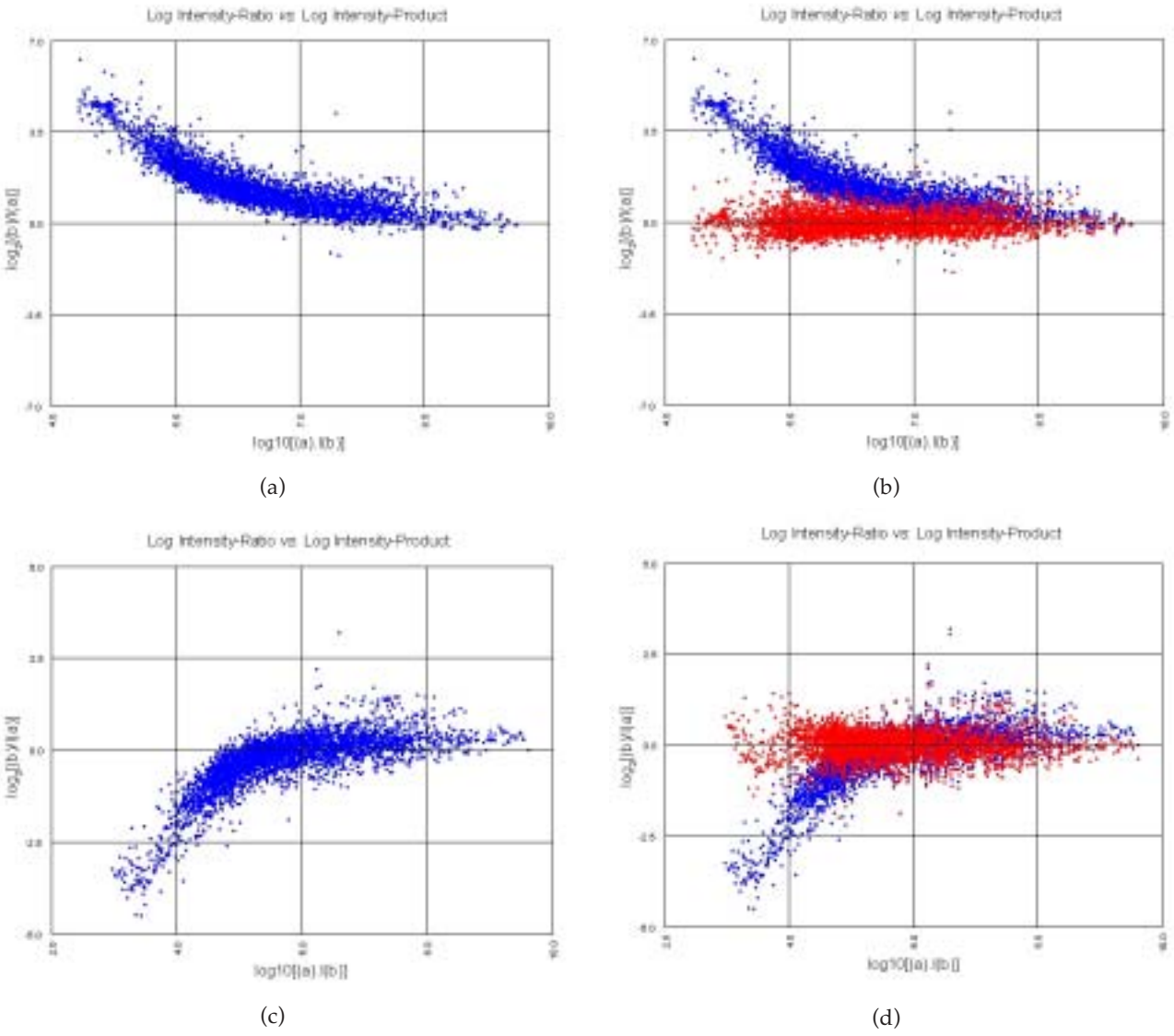


Figure 1. (a) and (c) shows the raw data plot while (b) and (d) shows normalized data. Both plots (a) and (c) represent a dye-swap experiment. Note that the plots are shown as log_{10} *[I(a).I(b)]* vs. log_2 *[I(a)/I(b)], also known as log intensity-ratio* vs. *log intensity-product.*

Following data normalization, TMeV (MultiExperiment Viewer; Version 3.03) was used to visualize processed microarray slide representations and identify genes and expression patterns of interest. A variety of normalization algorithms and clustering analyses allow the user flexibility in creating meaningful views of the expression data (Saaed *et al*., 2003). The normalized data from MIDAS is then taken through a series of analyses pipelines in TMeV such as T-test, Self Organization Tree Algorithm (SOTA) and Significance Analysis of Microarray (SAM). A typical view of the T-test result is shown in *Figure 2*.

T-test is a simple, statistically-based method for detecting differentially expressed genes (Cui and Churchil, 2003). In replicated experiments (as adopted in this experiment), the error variance can be estimated for each gene from the log ratios, and a standard T-test can be conducted for each gene (Callow *et al*., 2000). The resulting *t* statistic can be used to determine which genes are significantly differentially expressed. This gene-specific T-test is not affected by heterogeneity in variance across genes because it only uses information from one gene at one time. Global T-test (applied in this analysis) uses an estimation of error variance, that is pooled across all genes and the assumption made is that the variance is homogenous between different genes (Arfin *et al*., 2000). This is actually a fold-change test because the global *t* test ranks genes in an order that is the same as fold-change; that is, it does not adjust for individual gene variability.

In TMeV, SAM analysis reported by Tusher *et al*. (2001) was also used to identify significant genes based on differential expression between sets of experiment, such as genes affected by different parameters in the experiment. An advantage of SAM analysis is the ability to estimate the false-discovery rate (FDR). This refers to the proportion of false positives amongst all of the genes initially identified as being differentially expressed (rejected null hypotheses – a hypothesis for which the effects of interest are assumed to be absent and commonly used as a basis for constructing statistical test) (Benjamini and Hochberg, 1995; Benjamini and Yekutieli, 2001). SAM also allows users to modify the delta parameter, thereby selecting the threshold limit for the desired significance level.

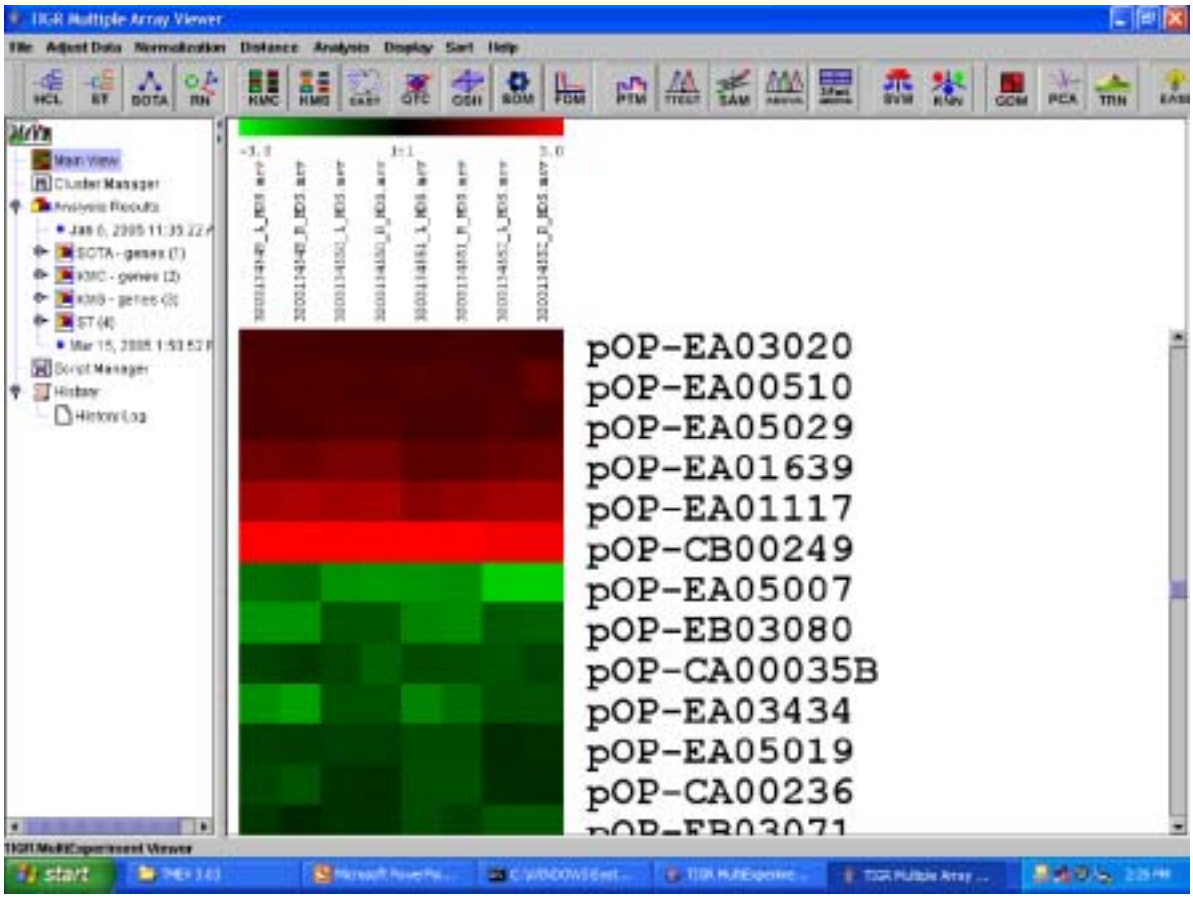


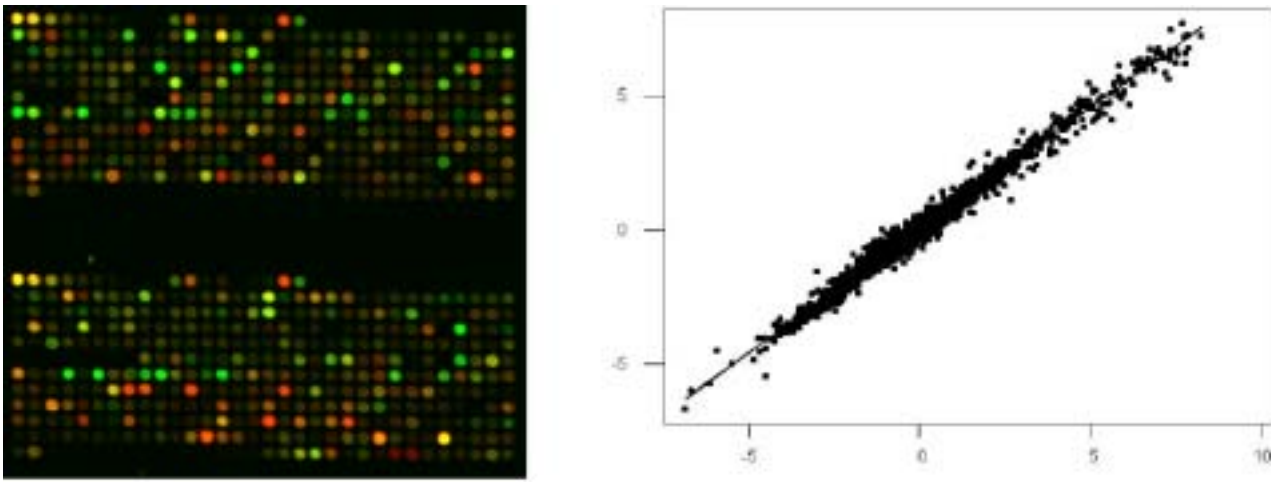
Figure 2. The cluster viewer output from TMeV after a T-test analysis. It is then arranged accordingly based on the hierarchical tree algorithm assigned. T-test design: one-class analysis; alpha (overall threshold p-value): 0.01; P-values based on: T-distribution; significance determined by: just alpha (no correction).

Tissue Culture Amenity

A number of researchers have already investigated methods to improve the efficiency of oil palm tissue culture (Jones, 1974; Nwanko and Krikorian, 1983; Thomas and Rao, 1985). Nevertheless, many difficulties have yet to be overcome. The rate of callogenesis of oil palm explants remains very low, at about 19% (Corley and Tinker, 2003), while the rate of embryogenesis from proliferating callus cultures has been reported to be only 6% (Wooi, 1995). The molecular basis of callogenesis and embryogenesis is still poorly understood. Therefore, the microarray approach was used to provide an insight into the molecular mechanisms associated with oil palm tissue culture.

The cDNA microarrays containing 3806 oil palm gene clones and 474 control spots were used to analyse three sets of tissue culture samples. Each set consisted of one line of polyembryoid, shoots from the polyembryoid, one line of embryogenic callus and one line of non-embryogenic callus. This experiment was carried out to identify biomarkers for the tissue culture process. The control tissue used was *dura* x *pisifera* (DxP) spear leaf. After microarray hybridization and data quantification, linear regression analysis showed that the Pearson correlation coefficient (r) between replicated spots in a single slide was 0.976 ± 0.021. Comparisons between duplicated spots in replicated experiments and reverse colour experiments showed correlation coefficients of 0.951 ± 0.041 and 0.930 ± 0.027 respectively. A scatter plot of the microarray results is shown in *Figure 3.* This shows that the microarray experiments were precise and reproducible.

Once the quality of the microarray data was deem acceptable, statistical analysis was carried out in TMeV. SAM analysis and hierarchical clustering of the tissue culture samples showed that the non-embryogenic callus formed in a separate cluster. The embryogenic callus, polyembryoid and shoots from the polyembryoids also clustered as separate clusters but within one main cluster. An example of a hierarchical cluster of the SAM output for a single line is shown in *Figure 4*. The results indicate that the clustering technique could differentiate non-embryogenic callus from other tissue culture samples tested. Similar results were obtained from the other two sets. This indicates that the microarray profile could distinguish embryogenic callus from non-embryogenic callus. Clones were selected for confirmation using Northern blots and the Northern profiles were consistent with the microarray expression profile (data not shown). This proved that the microarray profiles were reproducible, and as such, the technology will be applicable in developing a diagnostic chip for oil palm tissue culture.



(a) (b)

Figure 3. Reproducibility and preservation of the differential expression levels of microarray expression data among replicate slides. (a) and (b) represent microarray segments and a scatter plot of log_2 average ratio of gene clones between replicate slides.

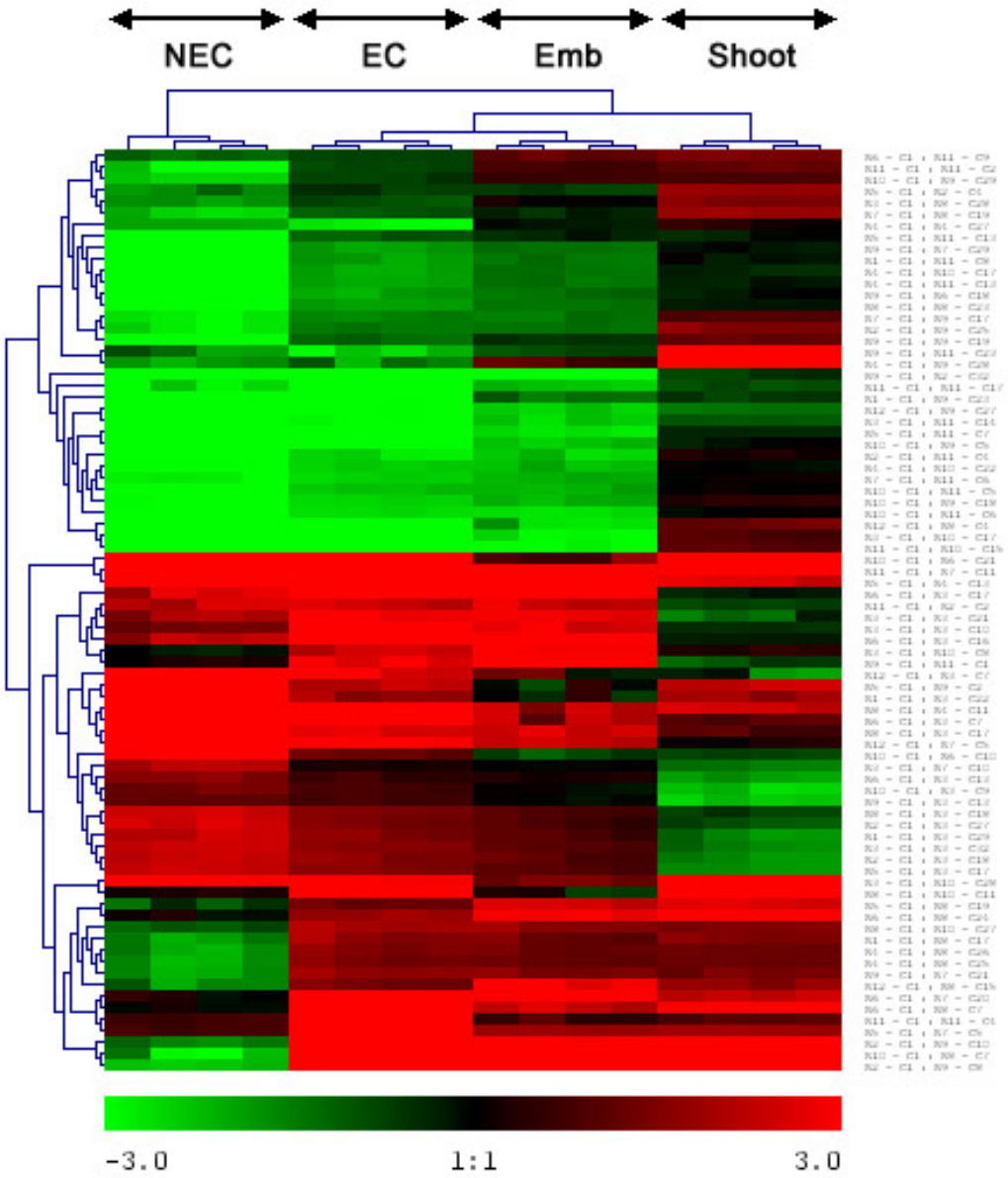


Figure 4. Hierarchical clustering of one set of tissue culture samples [non-embryogenic callus (NEC), embryogenic callus (EC), polyembryoids (Emb) and shoots from the polyembryoids (Shoot)]. Clustering of a subset of gene clones from DNA Chip 8 showed differential gene expression between the non-embryogenic callus and embryogenic material.

Subsequently, the cDNA microarrays were also used in a time course experiment, in which tissue culture samples were frozen at selected time intervals during culture. Day 0 represents the spear leaf used for tissue culture. The cultures were initiated and sampled after one day (Day 1), seven days (Day 7) and 14 days (Day 14) in culture. This was done in order to identify genes that are modulated during the tissue culture process. These genes would be of primary importance in developing a diagnostic assay for tissue culture amenity. *Figure 5* shows an example of the expression patterns of a number of genes at four time points (Day 0, Day 1, Day 7 and Day 14). Genes showing similar expression were clustered together, and the different clusters were identified. A high percentage of the genes printed on the oil palm chip were not differentially expressed during tissue culture. However, about 7%-10% of the gene clones in each time point were found to be differentially expressed. The results showed that genes are being modulated at least in the early stages of culture development.

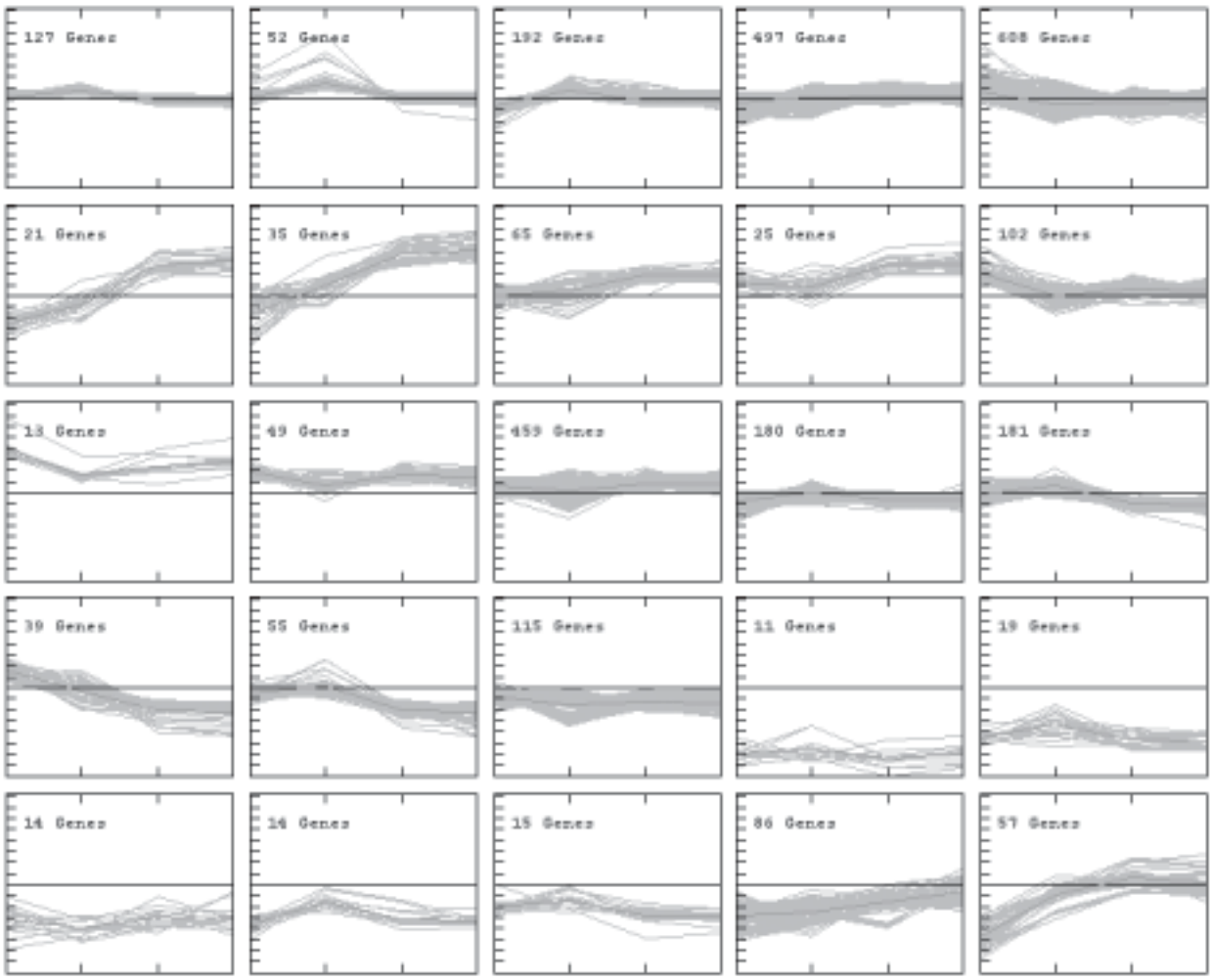


Figure 5. The output from SOTA analysis. Twenty-five clusters were identified, with the number of gene clones belonging to the respective groups stated in the cluster. Each line represents one gene clone.

Floral Abnormality Research

Flower development involves interplay of several types of genes. They are genes that control flowering time, meristem identity, cadastral genes and organ identity (also known as the ABC genes). Extensive studies on flower organogenesis in eudicots have established that the architecture of flowers is largely determined by the activity of a number of homeotic genes containing a conserved MADS box domain. These genes were originally studied in *Arabidopsis thaliana* and *Antirrhinum majus*, which has led to the development of the ABC model of floral organ identity (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). Focusing on MADS box alone may not be sufficient for us to unravel the coordinated regulation of different metabolic pathways that causes abnormality. Therefore, similar genome wide analysis approach applied to the embryogenesis studies was adopted. In relation to studying flower development with microarray technology, the outcome has been very promising as it had led to the identification of downstream genes regulated by MADS-box genes (Zik and Irish, 2003). With this in mind, microarray approaches were formulated and carried out to provide an insight to the abnormality problem.

In the pooled experiment, two total RNA pools (normal RNA and abnormal RNA pools) were generated. Pooling of the samples theoretically helps in reducing the effects of biological variation (Chabas *et al*., 2001; Waring *et al*., 2001; Enard *et al*., 2002; Agrawal *et al*., 2002). This is so that subject-to-subject variation can be minimized and substantive features easier to find (Churchill and Oliver, 2001; Simon and Dobbin, 2003; Churchill, 2002; Hans *et al*., 2004). The hybridization was carried out in eight technical replicates and hybridized slides were scanned and subjected to further analyses. Raw data computed from Array Vision™ 8 was formatted with the *normal pool* as the denominator. Therefore, the observed data would be based on intensities of the abnormal pool over the normal pool. Normalization and statistical analysis were carried out. The T-test analysis showed that from the total of 3806 genes on the array, 580 genes (15%) were found to be significant, while 3226 genes (85%) were non-significant.

In SAM, from the 580 significant genes obtained from the T-test, only 16 genes (3%) were found to be not significant while the remaining 564 genes (97%) were. From the 97% of significant genes, 50% (290 genes) were categorized as positively significant, meaning they were up-regulated in the abnormal palms. The remaining 47% (274 genes) were negatively significant, that is down-regulated in the abnormal palms (*Figure 6*).

In this one-class design, a delta value of 0.4934 was specified, against which the mean expression of each gene is tested. A gene is considered significant if its mean $\log_2$ expression ratio over all included experiments is significantly different from the user-specified mean. SAM analysis is routinely carried out to identify the false-discovery rate (FDR). This refers to the proportion of false positives amongst all of the genes initially identified as being differentially expressed (rejected null hypotheses – a hypothesis for which the effects of interest are assumed to be absent and commonly used as a basis for constructing statistical test) (Benjamini and Hochberg, 1995; Benjamini and Yekutieli, 2001).

The significant genes identified in SAM was further analysed using SOTA analysis. The algorithm was used to cluster the microarray data according to the expression pattern. SOTA uses the structure of a hierarchical tree and the clusters obtained are proportionate to the heterogeneity of the data, instead of the number of items (Herrero *et al*., 2000). This makes SOTA a straightforward way of comparing average patterns of gene expressions at different hierarchical levels. The analysis generated 11 clusters of genes (*Figure 7*) and several probes (genes) of interest were identified (*Table 1*). These genes were selected based on their expression fold-change of two and above. They are currently under going verification with Northern analysis.

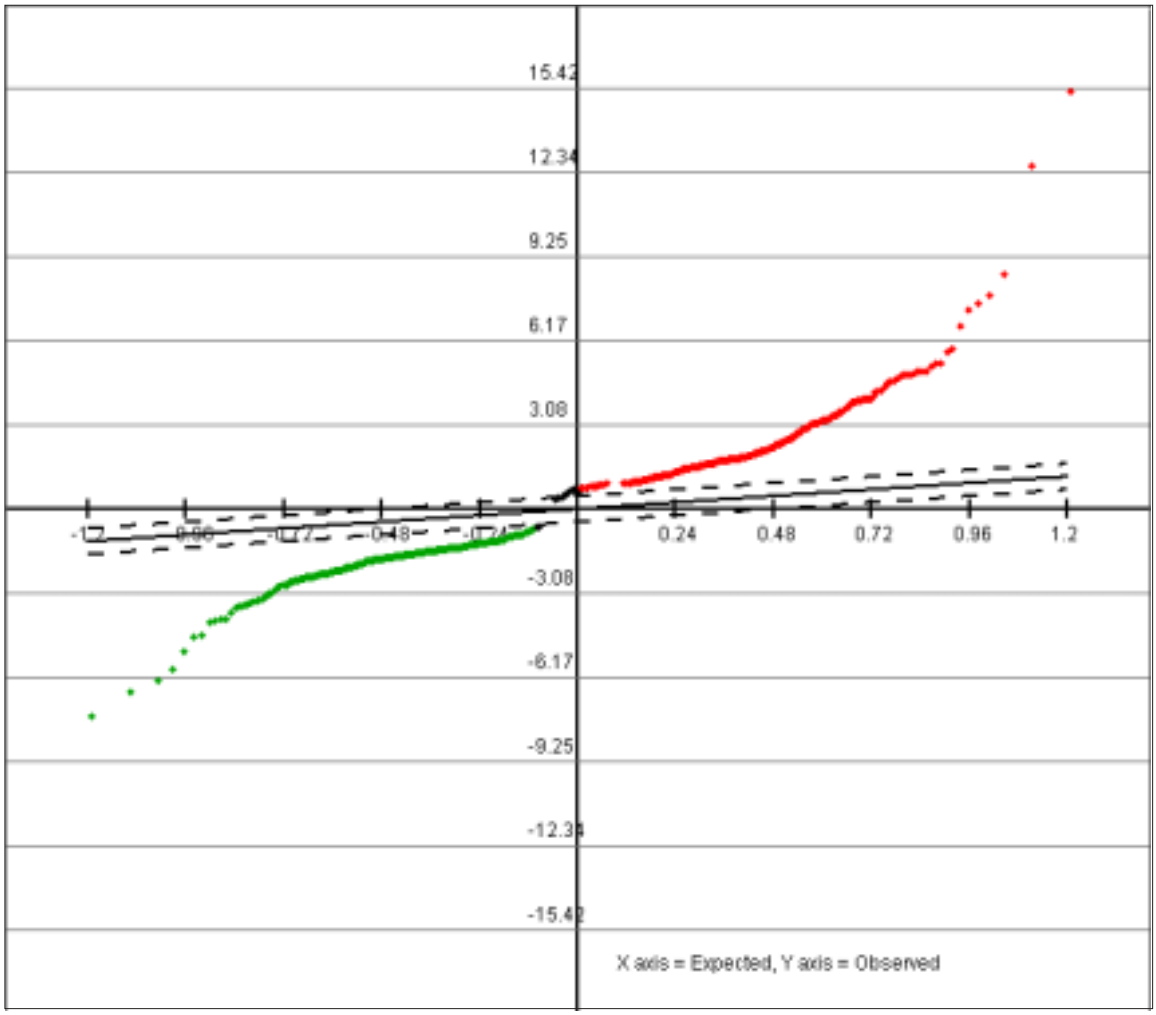


Figure 6. The SAM analysis output in TMeV. Red line indicates the positive significant genes while green line represents negative significant genes. The parameters used were: SAM study design: one-class; imputation engine: K-nearest neighbour; No. of K-nearest neighbour: 10; delta value: 0.4934; upper cut-off point: 0.8647; lower cut-off point: -0.6221.

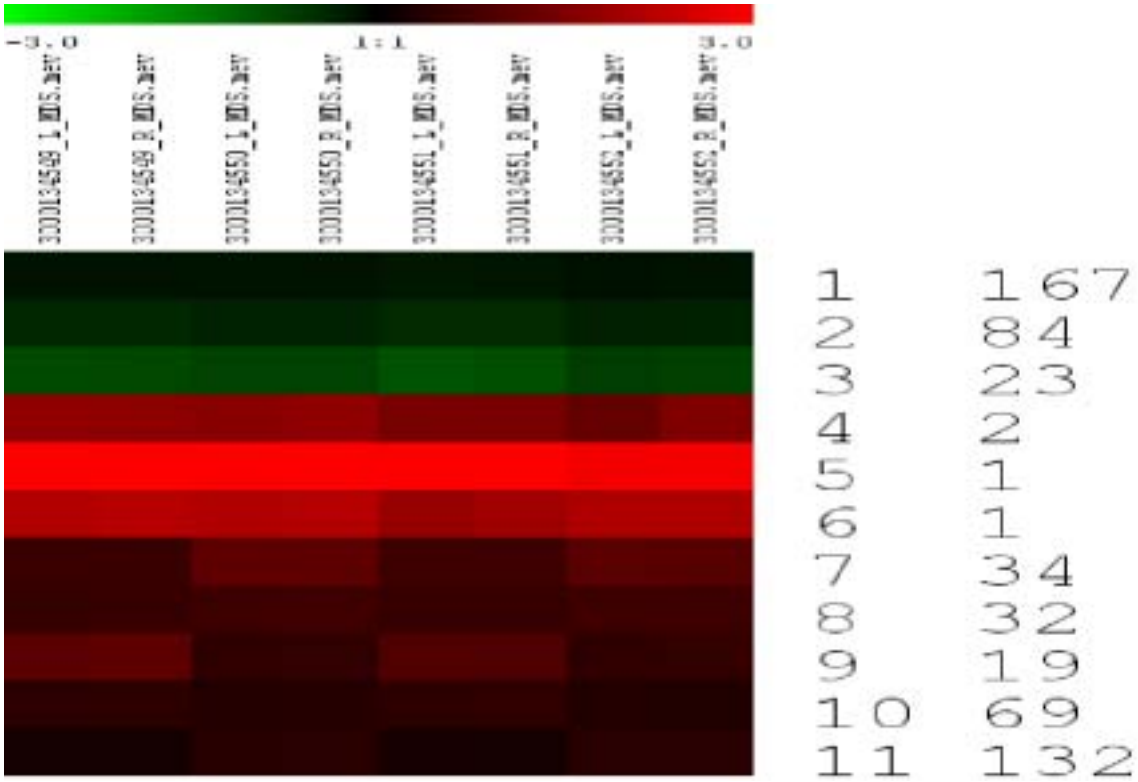


Figure 7. Output from SOTA analysis. Eleven clusters were identified, with the number of genes belonging to the respective groups stated on the side of the cluster.

TABLE 1. SELECTED PROBES FOR VERIFICATION WITH NORTHERNS

Probe ID	Putative function	Regulation (up/down)	Fold-change
pOP-EA01639	Metallothionein-like protein (*Elaeis guineensis*)	Up-regulated in abnormal	3
pOP-CB00249	GAST-like gene product (Fragaria x ananassa)	Up-regulated in abnormal	6
pOP-EB03080	Heat shock protein (*Hevea brasiliensis)*	Down-regulated in abnormal	4
pOP-EA03434	Unknown protein (*Oryza sativa*) [japonica cultivar-group]	Down-regulated in abnormal	2
pOP-CA0035B	Beta 6 subunit of 20S proteasome *Oryza sativa* (japonica cultivar-group)	Down-regulated in abnormal	2

CONCLUSION

This is an initial attempt to study genes associated with tissue culture amenity and abnormality using the DNA microarray technology. Through this exercise, a platform to optimize and streamline protocols had been made available for the oil palm cDNA microarray. Controls and monitoring steps had also been identified. The platform will pave the way for identifying some possible candidate genes that may help unravel the molecular mechanism associated with oil palm tissue culture. The experiments have also shown that this is possible as the results could be confirmed by Northern analysis. However, we also have to take into consideration that the slides used do not have the complete representation of the oil palm genome on them. Nevertheless, the study will help improve our understanding of the underlaying mechanisms of tissue culture and pave the way for the development of a DNA microarray diagnostic chip for oil palm tissue culture.

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