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IDENTIFICATION OF FLOWER SPECIFIC PROTEINS IN THE OIL PALM (*Elaeis guineensis*) BY TWO-DIMENSIONAL GEL ELECTROPHORESIS

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Total RNA was isolated from young etiolated oil palm seedlings, leaves and inflorescences at different stages of development. The mRNA was subsequently extracted by oligo-dT cellulose chromatography and in vitro *translated using a rabbit reticulocyte system. The translated products were examined by SDS-PAGE gel electrophoresis as well as a combination of SDS-PAGE and isoelectric focusing (two-dimensional polyacrylamide gel electrophoresis). Both methods revealed differences in banding pattern between the inflorescence (flower) and vegetative (leaf and young etiolated seedlings) tissues, indicating differential gene expression. Some changes were also observed in the pattern of* in vitro *translation products for mRNA extracted from the different stages of flowering.*

INTRODUCTION

Flowering in higher plants is a complex process that involves the interplay of environmental and genetic factors. Flowering basically begins with a series of events that lead to the conversion of vegetative meristems to floral meristems (Drews and Goldberg, 1989). The molecular process that controls flower development is being unraveled in many plant species such as *Arctbidopsis,* tomato and almond (Okamuro *et al., 1993;* Gasser *et al., 1989;* Suelves and Puigdomenech, 1998). This has led to the isolation and characterization of several flower specific genes, proteins and cDNA clones that have provided invaluable insight into the molecular control of flower development.

One of the experimental approaches used to understand the molecular basis of flower development is analysis of the complex protein patterns of flowering tissues by means of two-dimensional polyacrylamide gel electrophoresis @D-PAGE). ZD-PAGE as originally described

by Patrick O'Farell in 1975, is a method in which proteins are separated according to their isoelectric point by isoelectric focusing in the first dimension and then according to their molecular weight by SDS-PAGE in the second dimension. Since these two parameters are unrelated, it is possible to obtain an almost uniform distribution of a large amount of spots across a two dimension gel. The protein spots from different tissues can then be rapidly and directly compared for differentially expressed genes.

Studies using BD-PAGE to search for changes in the pattern of total accumulated proteins or translatable RNA populations (in *vitro* translated products) during flowering have been reported for several plant species. For example, in *Lolium temulentum* L. Ceres, several *in vitro* translated polypeptides were found to be highly expressed under flower inductive conditions (Perilleux *et al.,* 1996). Jones and Thomas (1994) reported that increases in two apical proteins (between the range of 14kDa to 20kDa) were responsible for the switch from vegetative to floral development. Similarly, other workers using BD-PAGE have also detected changes in protein spectra under conditions favouring flower development (Taylor *et al.,* 1990; Cremer *et* al., 1992; Kuboyama *et al.,* 1997).

This paper describes the changes in the profiles of *in vitro* translated proteins of mRNA extracted from oil palm inflorescences at different stages in their development and from the vegetative tissues. The study seeks to gather some basic knowledge on the molecular aspects of flowering in the oil palm. This is of special relevance as the commercial products of the palm are in the fruit.

MATERIALS AND METHODS

Plant Material

The oil palm materials used were from the *teneru* variety. The inflorescence samples, once removed from the axils of their subtending fronds, were quickly frozen in liquid, nitrogen and stored at -80°C prior to RNA extraction. The ages of the inflorescences were taken as those of their subtending fronds. The youngest inflorescence sampled was InfF9 (from Frond 9) and the oldest InfF19 (from Frond 19).

Leaf tissues were also frozen as soon as they were harvested from the palm. Young etiolated seedlings (roots and shoots) were excised from germinated seeds and frozen as above.

RNA Extraction

Two methods were employed for RNA extraction. The first method as described by Rochester *et al.* (1986) was used for extraction of total RNA from large amounts of tissue, while a second method (Chang *et* al., 1993) was found to be more suitable when smaller amounts of tissue were available (less than 2 g).

The integrity of the RNA extracted was examined by electrophoresing an approximate 10 µg aliquot in 1% agarose gel in Tris-borate (TBE) buffer, pH 8.5. The RNA was heat denatured at 65°C for 15 min before loading onto the gel with 1/5 volume tracking dye gel solution (0.1 M EDTA, 25% Ficoll, 0.1% Orange G and 50% glycerol).

mRNA Extraction

This was performed using oligo dT-cellulose affinity chromatography. Oligo dT (Type 7, Pharmacia LKB Biotechnology AB) was suspended in 2 ml of high salt buffer (HSB) (0.5 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA and 0.5% SDS). This was then packed into a siliconized sterile pasteur pipette, plugged with sterile siliconized glass wool.

The RNA samples were dissolved in low salt buffer (LSB) (10 mM Tris-HCl, pH7.5, 1 mMEDTA, O.l%SDS) heat treated at 65°C for 5 min. One ninth (1/9) volume of 5 M NaCl was added before the sample was loaded onto the oligo dT-cellulose at room temperature. The eluate was loaded three more times to ensure binding of the mRNA to the column. The mRNA was then eluted from the column with LSB. A second round oligo-dT chromatography was then performed with the mRNA obtained.

The mRNA eluted from the second round was ethanol precipitated at -80°C overnight. The pellet obtained was dissolved in sterile water. Adsorption readings were taken at 260,280 and 350 nm to determine the yield and purity.

For mRNA extraction from total RNA of less than 5 mg, only one round of oligo-dT chromatography was performed.

In vitro Translation

The mRNA was translated in nuclease treated rabbit reticulocyte lysate (Promega Corporation) in the presence of ^{3}H-Leucine (Amersham Pharmacia Biotech). The 'reaction mixture contained 25 µl ^{3}H-Leucine which was dried by centrifuging under vacuum., To this was added 35 µl rabbit reticulocyte lysate, 1 µl mRNA (1µg µl^{-1}), 1µl leucine minus amino acid mixture and 13 µl sterile water. The reaction mixture was incubated at 30°C for 60 min. At the end of the incubation, a 5 µl sample was removed into an eppendorf tube for SDS-PAGE and two-dimensional gel electrophoresis.

Total incorporation into protein was determined by trichloroacetic acid (TCA) precipitation, as described in Sambrook et al. (1989).

SDS-PAGE Gel Electrophoresis

The *in vitro* translated sample was mixed with an equal volume of sample buffer (20% glycerol, 4% SDS, 125 mM Tris-HCl pH 6.8 and 0.05% bromophenol blue). The mixture was boiled for 2 min and used for SDS polyacrylamide gel electrophoresis as described by Laemmli (1970).

After electrophoresis, the proteins were fixed with 30% ethanol and 12% acetic acid. Fluorography was done using 0.5% 2,5-Diphenyloxazole (PPO), 30% xylene, 58% acetic acid and 15% ethanol. The gel was then dried and exposed to X-ray film (Kodak XK-1) at -80°C for about one week.

Two-dimensional Gel Electrophoresis

Two dimension separation of proteins was implemented by a combination of isoelectric focusing (O'Farrell, 1975) and SDS gel electrophoresis (Laemmli, 1970). Electrophoresis was performed using the SE-600 system (Hoeffer, U.S.A).

For IEF, gel containing 9 M urea, 4% (w/v) polyacrylamide (acrylamide: bisacrylamide = 28.4:1.6), 2% (v/v) Nonidet P-40, 5% (v/v) mixture of LKB ampholines of pH 3.5-10, pH 4-6, pH 6-8 in a proportion of 1:2:2, and 0.02% (w/v) ammonium persulphate was prepared in a glass capillary tube (1.5 mm inside diameter). The upper reservoir was filled with 0.02 M NaOH and the lower with 0.01 M phosphoric acid. The gels were prerun at 200V for 15 min, 300V for 30 min and 400V for 1 hr. *In vitro* translation mixture in 5 µl lysis buffer [9 M urea, 2% (v/v> ampholines pH 3.5-10 and 5% (v/v> 2-mercaptoethanoll were loaded and electrophoresis was carried out at 400V for 18 hr.

After the first dimension electrophoresis, the rod gels were equilibrated in buffer containing *62.5* mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol and 5% 2-mercaptoethanol. The second dimension electrophoresis was performed in SDS polyacrylamide slab gel comprising a 5% stacking gel and 15% acrylamide resolving gel, run at 10 mA/1.5 mm gel for 15 hr.

The proteins in the second dimension gel were fixed and prepared for viewing as described above. Molecular weight markers used were [^{14} Cl methylated protein mixtures (Amersham Pharmacia Biotech). The experiment was carried out twice for each of the sample tested.

RESULTS AND DISCUSSION

Total RNA Extraction

Table 1 shows the yield and purity of the RNA extracted from leaf, young etiolated seedlings and inflorescences at various stages of development.

TABLE 1. RNA YIELDS FROM OIL PALM TISSUES

Tissue	RNA yield (mg g^{-1} tissue)	A_{260}:A_{280}
'Leaf	0.20	1.8 : 1
'Young etiolated seedlings	0.40	1.9 : 1
[1]Inf F19	0.40	1.7 : 1
[2]Inf F18	0.40	1.8 : 1
[2]Inf F17	0.30	1.8 : 1
[2]Inf F14	0.80	1.9 : 1
[2]Inf F9	0.70	1.7 : 1

Notes:

'RNA isolated using the method described by Rochester et *al.* (1986).

[2]RNA isolated using the method of Chang et al. (1993).

Inf F:Inflorescence at Frond.

From the results, both extraction methods yielded RNA of good purity indicated by the A_{260}:A_{280} ratio which ranged from 1.7:1 to 1.9:1. This was encouraging, as it is has long been acknowledged that isolation of RNA from plant tissues is difficult due to high content of polyphenols, polysaccharides and ribonucleases associated with tissues.

Comparing both methods of extraction for inflorescences at about the same stage of development (that is inflorescences at Fronds 17, 18 and 19), the yield obtained was quite similar in all the three samples. The younger inflorescence tissues at Fronds 9 and 14, however yielded much higher RNA compared to mature inflorescence tissue.

The quality of the RNA was analyzed by gel electrophoresis using agarose gel. The integrity of the RNA extracted was confirmed by the appearance of distinct bands of the 25S and 18S ribosomal RNA (rRNA) *(Figure 1)*. The results obtained showed that there was little nuclease mediated degradation, an indication of the good quality of the RNA from both the methods of extraction.

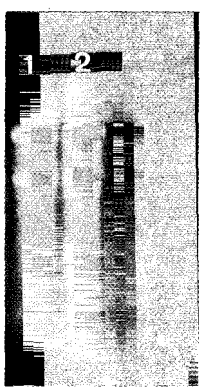

Figure 1. Gel analysis of total RNA from Inf F19 and Inf F18, isolated using two separate methods as described in Materials and Methods (Lane l-10 μg of Inf F18 RNA and Lane 2-10 μg of RNA from If F19).

mRNA Extraction

Figure 2 shows an example of the elution profile of total RNA applied to an oligo dT-column. Here, total RNA extracted from leaf tissue was applied to the column. Fractions 0 to 30 represent the rRNA and transfer RNA (tRNA) that did not bind to the column in high salt buffer and which passed straight through. Fractions 32 to 38 represent the mRNA with poly $(A)^+$ tails that bound to the column. The latter was eluted by lowering the ionic strength of the wash buffer.

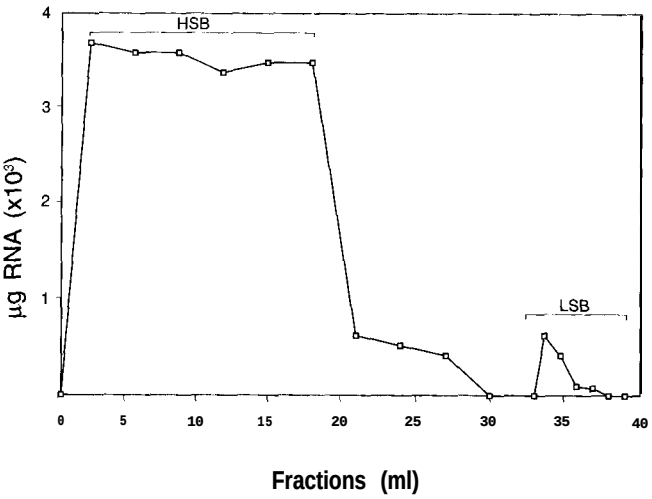

Figure 2. Oligo dT-cellulose chromatography of oil palm leaf RNA. High salt buffer (HSB) elutes rRNA while low salt buffer (LSB) elutes mRNA.

The yields obtained for mRNA extraction are shown in *Table 2.*

TABLE 2. mRNA YIELD FROM OIL PALM TISSUES

Tissue	Total RNA loaded (mg)	mRNA recovered (μg)	Purity A_{260}:A_{280}
Leaf	30.0	90	1.8 : 1
Young etiolated seedlings	13.1	34	1.7 : 1
Inf F19	15.0	60	2.0 : 1
Inf F18	0.7	30	2.0 : 1
Inf F17	0.9	38	**1.9** : 1
Inf F14	1.5	50	1.7 : 1
Inf F9	0.7	17	1.9 : 1

In our study, it was possible to isolate mRNA even when low amounts of starting total RNA was available, as was the case for inflorescences at Fronds 9, 14, 17 and 18. In this case, the mRNA eluted was not put through a second round of oligo dT- chromatography as was done for leaf, young etiolated seedlings and inflores-

Generally, polypeptides ranging in size from 16kDa to 97kDa were synthesized, indicating that both high and low molecular weight proteins were obtainable. Differences in the banding pattern between the inflorescences and vegetative tissues were apparent, indicating, as expected, there was differential gene expression in these tissues. For the inflorescence samples, two protein bands at about 48kDa and 61kDa appear to be highly expressed in the *in* vitro translated protein profile. The 48kDa protein also appeared in the translation profile of young etiolated seedlings. A group of proteins at about 36kDa appeared in the translated products of inflorescence mRNA but not those of the vegetative tissues. An interesting observation was made on the inflorescence samples (lanes 4 to 8). A protein of about 34kDa (indicated by an arrow in *Figure* 1) appeared only in the translation products of mRNA from inflorescence at Fronds 14,17,18 and 19 but not that at Frond 9. This protein appears to be specific for the later stages of flowering. This flower specific protein is thus also phase specific.

Using SDS-PAGE gel electrophoresis, differences in gene expression between vegetative and inflorescence tissues were apparent but the differences observed between the various stages of flowering were minimal. In order to get a more comprehensive analysis of gene expression during flowering, we attempted to further characterize the translated products using 2D-PAGE.

Two-dimensional Gel Electrophoresis

The 2D-PAGE was attempted in an effort to increase the sensitivity of detection of differences in the protein profiles of the leaf and inflorescence tissues. The *in vitro* translated profiles of mRNA isolated from leaf and inflorescence (Inf F9, Inf F14 and Inf F19) tissues were compared. The 2D-PAGE patterns obtained are shown in *Figures 4a* to *4d*.

The number of polypeptides resolved by electrophoresis was higher in leaf (about 50) than in flowering tissue (about 30). Several polypeptides specific only for flowering tissue were observed. The most pronounced of these were two polypeptides of about 50kDa (basic isoelectric point) and another polypeptide at about 50kDa (acidic isoelectric point) (marked by black arrows, *Figures* 4a to *4d*). On the other hand, the patterns obtained for the three stages of flowering were very similar. Nevertheless, three flower specific polypeptides which were also phase specific, were identified. Spots 1 and *2 (Figures 4b* and *4c*) (molecular weights of 25 kDa and 66 kDa respectively) appeared only in Inf F9 and Inf F14 samples, but was absent in the Inf F19 samples. Spot *3 (Figure 4d*), (about 80 kDa) was observed only in the Inf F19 samples and not in the Inf F9 and Inf F14 samples. Spots 1 and 2 therefore represent early flowering polypeptides while spot 3 is a late flowering polypeptide. These results indicate that differences exist in the mRNA population at the various stages of flower development.

A high proportion of the polypeptides observed were common for the three stages of flowering, although the relative amounts varied in certain cases. The most prominent of these were two polypeptides of about 46 kDa and 48 kDa *(Figures 4b, 4c* and *4d*, marked by a rectangle) which appeared to be more highly expressed in Inf F19 compared to the two earlier stages of flowering. Differences in intensity of identical spots are attributable to change in the level of synthesis of proteins relative to each other. Many common polypeptides were also observed by comparing the flowering and leaf tissues. These common polypeptides may be the products of constitutive gene expression.

Although the 2D-PAGE method is capable of analyzing a complex set of protein patterns, it is important to note that only proteins synthesized at relatively high levels are detected (Cremer et al., 1992). As thousands of genes are expressed in the cells, the observed protein profile only represents a fraction of the whole pattern. Very rare proteins or proteins expressed at very low levels may not be detected. This could explain the limited number of flower phase specific polypeptides observed in this experiment. Other studies using BD-PAGE to study flower development also reported very few qualitative changes. For example, the work done on BD-PAGE on the short day plant *Pharbitis* nil *Chois.*, strain violet by Ono *et* al. (1988) found few differences occurring in the protein pattern during transition to flowering. Similar results

lated even when small amounts of starting total RNA (as little as 0.7 mg) were available. The mRNA population was subsequently identified by means of *in vitro* translation assays. The mRNA successfully stimulated the incorporation of ^{3}H-Leucine into protein. The *in vitro* translated proteins were then identified by SDS-PAGE and BD-PAGE.

SDS-PAGE analysis showed more differences in the *in vitro* translated protein profile between flowering and vegetative tissue than between the various stages of flowering. Analysis by BD-PAGE also showed a very high degree of similarity in the polypeptide patterns between the different stages of flowering, although relative amounts (intensity) of some polypeptide spots varied in certain cases. Similarity in the protein patterns of the different developmental stages of flowering is, however, indicative that the patterns are highly reproducible between independent experiments. At least three flower phase specific polypeptides were identified in the BD-PAGE analysis suggesting that new translatable mRNAs are present at the different stages of flower development in the oil palm.

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