J. Trop. Agric. and Fd. Sc. 29(1)(2001): 53–59

Construction of a mango (*Mangifera indica* L.) fruit cDNA library

[Pembinaan perpustakaan cDNA buah mangga (*Mangifera indica* L.)]

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Key words: *Mangifera indica*, cDNA library, fruit ripening, mRNA, protein extraction

Abstract

Fruit ripening is a complex process involving changes in colour, flavour and texture of the fruits. These changes have economic values and are used as an eating quality index. The changes are regulated at the gene level. The genes that cause such changes can be cloned and further studied. In this report, the construction of a cDNA library from the mango mesocarp tissues at different stages of ripening was described. The cDNA library consists of approximately 5.32 x 10^5 of phage recombinants. This will create more research opportunities into the mechanism of ripening in mango at the molecular level.

Abstrak

Pemasakan buah merupakan suatu proses yang amat kompleks dan melibatkan beberapa perubahan. Antara perubahannya ialah warna, perisa dan tekstur buah. Perubahan ini mempunyai nilai ekonomi serta digunakan sebagai indeks kualiti pemakanan. Perubahan yang berlaku diatur pada peringkat gen. Gen yang menghasilkan perubahan ini boleh diklon dan dipencilkan untuk kajian selanjutnya. Dalam laporan ini pembinaan sebuah perpustakaan cDNA dari tisu mesokarpa buah mangga pada beberapa tahap pemasakan dibincangkan. Perpustakaan ini mengandungi lebih kurang 5.32 x 10^5 rekombinan faj. Kejayaan ini akan membolehkan lebih banyak kajian dijalankan terhadap mekanisme pemasakan buah mangga pada peringkat molekul.

Introduction

Mango (*Mangifera indica* L.) is one of the most economically important tropical fruit crops. Despite the obvious importance, there is constraint in the international trade of fresh mango due to the highly perishable and short shelf life of the fruit. Mangoes are universally consumed as fresh fruits.

The biochemical events that occur during the ripening of mango result in important changes which affect fruit quality. These changes include the loss of cell wall structure causing softening, the accumulation of yellow/orange carotenoid pigments and the production of volatile flavour compounds. These biochemical events are regulated at the gene level. Several mRNA, declined and others reported to be increased during ripening (Rattanapanone et al. 1978). In tomato, this has been shown to involve the production of many different messenger RNA species, which are specifically synthesized during ripening (Gray et al. 1994). Furthermore,

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there is evidence to show that the tomato is active in protein synthesised throughout ripening and a number of ripening related changes of protein synthesis were observed (Grierson et al. 1985). Using biochemical methods, it is often difficult to isolate ripening related enzyme due to the high background of similar enzymes that are already present in the cell or perhaps the proteins are being produced at a low level.

A useful approach to investigate such changes is through the construction of the cDNA library. This molecular technique will enable the cloning of a large range of cDNA clones representing genes that show expression during ripening. The use of differential screening will facilitate the isolation of the cDNA clones encoding proteins associated with ripening from the library. Understanding the fruit ripening process is of utmost importance in improving fruit quality and storage potential. In this paper, the construction of a cDNA library from mango mesocarp tissues obtained from different stages of mango fruit ripening was reported.

Materials and methods

Plant material

Unripe mango fruits of variety 'Tommy Atkins' were obtained from a commercial source. Only mature and good quality fruits were chosen for experimental work. Fruits of known weight and volume were placed in a sealed container and 10 ppm ethylene was injected to induce fruit to ripen for 24 h and then held at 20 °C in ethylene free air to ripen. The ripening stage of mango fruit was assessed by monitoring the rate of ethylene production as previously described (Zainal et al. 1999).

Total protein extraction

Proteins were extracted from mango mesocarp tissue at all ripening stages according to the method of Meyer et al. (1988). The protein concentration was determined by method of Essen et al. (1978) using bovine serum albumin (BSA, Sigma) as a standard. An amount of 50 mg of protein from each sample was loaded onto SDS-PAGE and electrophoresis at 150–200 V until the dye had reached the bottom of the gel. The gel was stained with Coomassie brilliant blue R and then destained for 1 h. The gel was then photographed.

Extraction of total RNA from mango mesocarp

The method used for RNA extraction was a slight modification of that by López-Gómez and Gómez-Lim (1992). Frozen mesocarp tissue (30 g) was ground to a fine powder using a coffee grinder and transferred to a chilled mortar containing 50 mL extraction buffer (2% w/v SDS, 1% v/v mercapthoethanol, 5% v/v phenol, 50 mM EDTA and 150 mM tris-borate pH 7.5). The mixture was homogenized and 0.25 volumes of absolute ethanol and 0.11 volumes of 5 M potassium acetate were added. The homogenate was then extracted once with chloroform/isoamyl alcohol (49:1 v/v), once with phenol/chloroform (1:1 v/v) and a second time with chloroform/isoamyl alcohol (49:1 v/v).

The RNA was then precipitated from the aqueous phase with LiCl (3 M final concentration) at –20 °C overnight and collected by centrifugation at 13 000 rpm for 90 min at 4 °C. The pellet was washed with 3 M LiCl and resuspended in water and reprecipitated by the addition of potassium acetate (0.3 M final concentration) and 2.5 volumes of absolute ethanol. Following an overnight incubation at –20 °C the RNA was pelleted for 10 min in a micro centrifuge at 13 000 rpm, washed with 70% ethanol, dried in a vacuum desiccator and resuspended in sterile distilled water. Poly (A^+) rich RNA was prepared with the Poly A Tract mRNA isolation system (Promega) as described by the manufacturer.

Construction of mango cDNA library

The cDNA library was constructed in bacteriophage λ ZAP (ZAP-cDNA synthesis kit, Stratagene USA). cDNA was

synthesised from 5 µg of a pooled sample of Poly (A^+) mRNA isolated from pericarp tissue of ripening mango at 24 h, 72 h, 120 h and 168 h after start of the 24 h incubation with ethylene. Approximately 100 ng of the double stranded cDNA was directionally cloned into λ ZAP arms, packaged in gigapack II gold extract (Stratagene) and used to infect *E. coli* XLI-Blue MRF'.

Results and discussion

Colour changes of the pulp and peel of mango

During mango fruit ripening, changes in colour of the peel and pulp occurred. The peel changed from green to yellow or orange, while the pulp changed from white to orange *(Plate 1)*. These changes occur due to the degradation of chlorophyll and an increase in the synthesis of carotenoids (Medlicott et al. 1986).

Changes in protein synthesis during ripening

Before embarking on the construction of the cDNA library, total protein extraction was carried out to get an idea of which stage should be chosen for library construction. The analysis of the protein patterns revealed several differences between stages of ripening *(Plate 2)*. The level of the majority of the polypeptides remained relatively constant but several exhibited notable increase or decrease *(Table 1)*. This observation support the phenomena of ripening which involves new or enhanced protein changes as well as repression of other proteins.

RNA extraction from mango fruit

Many attempts were made to isolate total RNA from mango fruit but most have failed. This was presumably because mango fruit contained a lot of carbohydrate and polyphenol compounds, which co-precipitate with nucleic acid. The use of a method developed by López-Gómez and Gómez-Lim (1992) was successful. A homogenising buffer containing high concentration of Tris-borate was able to eliminate the polyphenol compounds. For precipitation of RNA, LiCL with the final concentration of 3 M was used. This step removed carbohydrate as well as DNA and protein from the preparation.

Table 2 shows the yield and degree of purity of total RNA at each stage of ripening and also the yield of poly (A^+) RNA following isolation of the latter with a PolyAtract mRNA from Promega. For a pure sample, the ratio for OD 260:280 is 2 and 0.42 for 230:280. From these data, it

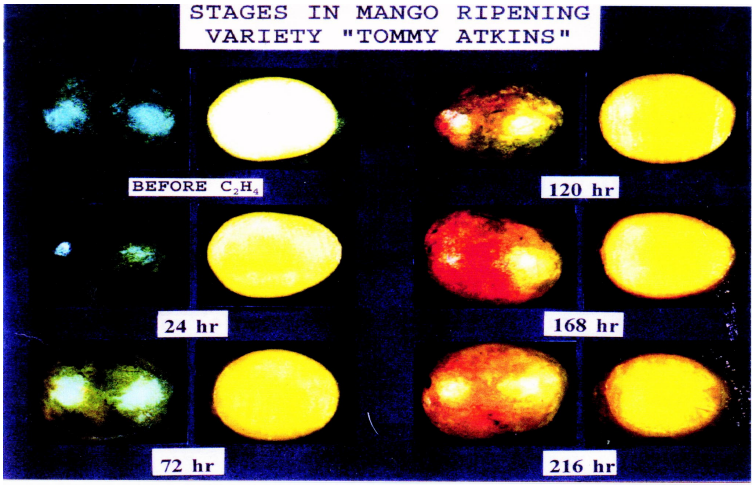

Plate 1. Mango fruit at different stages of ripening after application of ethylene

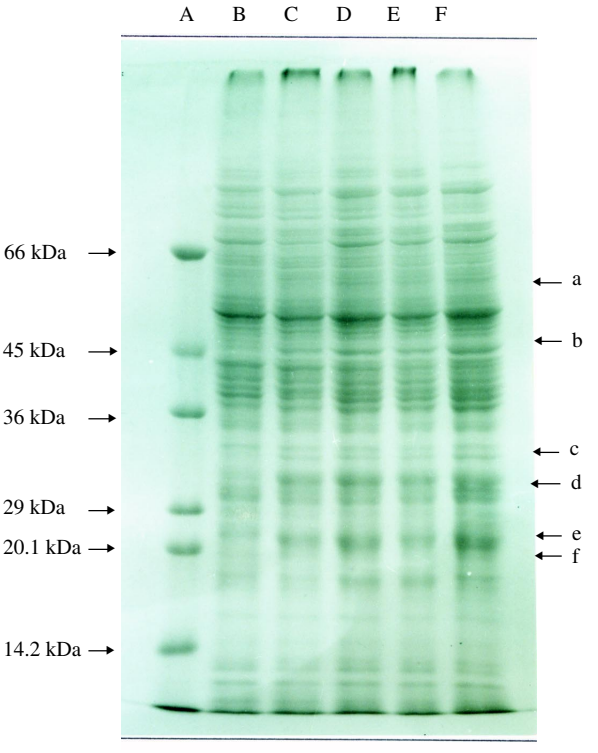

Plate 2. Total protein extracted from mango fruit at different stages of ripening. Protein was extracted from mango mesocarp and separated on a 12% PAGE-SDS gel. Standard molecular weight marker (lane A), minus ethylene treatment (lane B), 24 h (lane C), 72 h (lane D), 120 h (lane E) and 168 h (lane F). Arrows indicate proteins appearing and disappearing during ripening

Table 1. Protein changes during mango fruit ripening. Letters refer to bands indicated in *Plate* 2

Proteins increasing during ripening	Proteins decreasing during ripening
a: 54.0 kDa b: 44.5 kDa c: 34.6 kDa d: 31.6 kDa f: 22.4 kDa	e: 26.4 kDa

was likely that very little carbohydrate or polyphenol was present in the preparation. About 10 µg of total RNA from different stages of ripening was resolved on a 1% (w/v) agarose to determine the quality and integrity of the isolated RNA. Two discrete bands, which correspond to the sizes of 25S and 18S rRNA, were clearly visible suggesting that degradation was not significant during RNA isolation *(Plate 3)*.

cDNA library construction

The poly (A^+) RNA samples isolated from mesocarp tissues of mango that had ripened for 24, 72, 120 and 168 h were pooled. The purpose of using the pooled RNA was to ensure that the library would be well represented with ripening specific transcripts. First strand cDNA was generated from 5 µg of poly (A^+) RNA and this was used to make double stranded cDNA. Result in *Plate 4* shows the quality of the first and second strands cDNA that were synthesised. From this autoradiograph it seemed that the first strand represents a mixture of cDNAs with the size ranged from 0.4 kb to 2.5 kb. This range of sizes indicates the presence of

Table 2. RNA yield, concentration, purity and poly $(A)^+$ mRNA yield from mango fruit before and after ethylene treatment

Hour	Total RNA/g tissue (µg)	OD 260/280	OD 230/280	Poly $(A)^+$mRNA from 1 mg total RNA (µg)
$-C_2H_4$	50.6	1.8	0.40	7.3
24	43.5	1.8	0.44	6.8
72	45.3	1.7	0.38	3.4
120	38.3	1.7	0.42	6.5
168	20.9	1.8	0.36	2.6

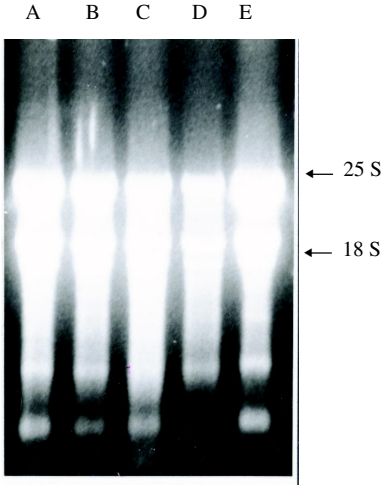

Plate 3. Total RNA extracted from different stages of ripening. Minus ethylene treatment (lane A), 24 h (lane B), 72 h (lane C), 120 h (lane D) and 168 h (lane E)

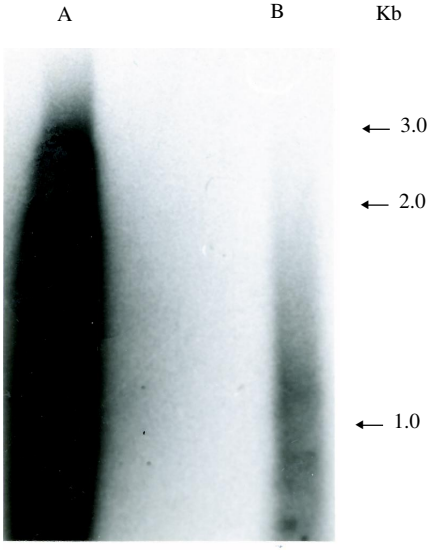

Plate 4. Autoradiograph of first strand (lane A) and second strand (lane B) cDNA synthesised from 5 µg of poly $(A)^+$ mRNA

the full-length cDNAs, which are suitable for cloning into a vector.

Titreing of the cDNA library

The library was titred to estimate the number of recombinant phage by incubating 1 µL of the final packaging reaction (phage stock containing cDNA) with 200 µL of XLI-Blue MRF'. The reactions were plated on NZY agar plate containing IPTG and X-gal to differentiate between recombinant and non-recombinant plaques. The plaques without inserts gave blue colonies while recombinant plaques gave a white colour. From the titre, it was calculated that package 1 had 2.3 x 10^5 clones, package 2 had 1.54 x 10^5 clones and package 3 had 1.59 x 10^5 clones. The total number of clones in the library was approximately 5.49 x 10^5 while the percentage of blue (non-recombinant) is about 3%. Thus the number of recombinant clones was approximately 5.32 x 10^5. Size of the cDNA inserts was determined by randomly selecting 10 single plaques. The plaques were subjected for in vivo excision and the sizes were determined by digesting the plasmids with the *Eco* R1 and *Xho* 1. *Plate 5* shows that the size was estimated to be between 0.4 kb and 1.1 kb.

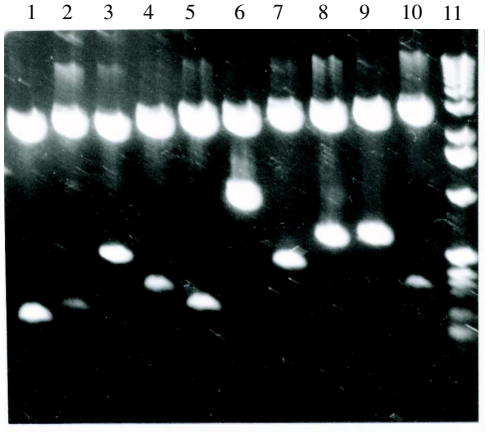

Plate. 5. Electrophoresis gel of 10 randomly selected cDNA clones. Plasmid digestion product from randomly selected plaques (lane 1-10)

Conclusion

Fruit ripening is characterised by a series of coordinated biochemical and physiological changes, which ultimately affect the colour, texture and flavour of the fruit. These changes are controlled by specific gene expression. The application of molecular techniques to understand fruit ripening has enabled the isolation and characterization of a large range of cDNA clones, which show increased expression during ripening. The authors used cDNA cloning techniques in order to investigate the control of gene expression during mango fruit ripening. A cDNA library was successfully constructed from ripening mesocarp tissues of mango fruits. The size of the library is about 500 000 recombinants. The size of the library can be considered small and perhaps is not well represented in the entire mRNA species during ripening. Nonetheless, several ripening-related cDNA clones have been isolated from the library by differential screening technique (Zainal et al. 1996; Lycett et al. 1997). The cDNA library will open up more opportunities in order to understand the basis for the onset of ripening particularly on mango. The isolation of those genes involved in ripening process is of paramount importance for future crop improvements.

Acknowledgement

The author gratefully acknowledges a studentship from Universiti Kebangsaan Malaysia.

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Accepted for publication on 19 April 2001