Construction of cDNA libraries from ripe and unripe Eksotika 2 papaya

(Pembinaan perpustakaan cDNA daripada buah betik Eksotika 2 yang masak dan mengkal)

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Abstrak

Untuk membolehkan pengasingan gen-gen berguna dalam proses kemasakan buah betik, dua perpustakaan cDNA telah dibina daripada buah betik Eksotika 2 yang mengkal dan masak. Kedua-dua perpustakaan dibina menggunakan kit λZAP cDNA sintesis dan λZAP XR vektor. Perpustakaan cDNA buah betik hijau telah menghasilkan faj rekombinan 7.5 x 10^4 pfu/mL dan digandakan kepada 9.0 x 10^9 pfu/mL manakala perpustakaan cDNA buah betik masak telah menghasilkan 3.19 x 10^5 pfu/mL dan digandakan kepada 4.4 x 10^{10} pfu/mL. Perpustakaan cDNA buah betik masak telah berjaya digunakan dalam pengasingan beberapa gen yang berguna daripada buah betik Eksotika 2 yang masak.

Abstract

To enable the isolation of useful genes involved in the ripening process of Eksotika 2 papaya, two cDNA libraries were constructed from the ripe and unripe papaya. Both libraries were constructed using the λZAP cDNA synthesis kit and the λZAP XR vectors. The green fruit library yielded recombinant phage of 7.5 x 10^4 pfu/mL which was amplified to 9.0 x 10^9 pfu/mL while the ripe fruit library yielded recombinant phage of 3.19 x 10^5 pfu/mL and amplified to 4.4 x 10^{10} pfu/mL. The ripe fruit library was successfully used for the isolation of several important genes from ripe Eksotika 2 papaya.

Introduction

Ripening of fruits involves changes in gene expression resulting in the synthesis of new mRNAs in the tissues (Tucker and Grierson 1987). These mRNAs are called ripening related mRNAs as they encode all proteins required in the normal ripening process of the fruit.

To study each of the ripening gene in greater detail, the mRNA present in the ripening fruit have to be cloned first. cDNA library construction is the most effective method of cloning the mRNAs present in a particular process. In a cDNA library construction, all mRNAs present during the process is extracted from the tissue and purified. The mRNA is then converted to a more stable form DNA by cDNA synthesis. The cDNA is then modified and joined to a vector. These are then encapsulated into

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phages or transformed into bacteria. The phages and bacteria are respectively called phage and plasmid library.

To date, several fruit ripening cDNA libraries have been constructed from which many useful ripening-specific genes have been isolated (Gray et al., 1992, 1994). These genes have been successfully used in genetic manipulation for improvement of fruit quality or for delayed ripening.

Here we report the construction of phage cDNA libraries from ripe and green Eksotika 2 papaya. This is to enable isolation of important genes involved in the ripening process of the Eksotika 2 papaya. Once the genes have been isolated and identified, they will be subsequently used in the improvement of Eksotika 2 papaya through genetic engineering.

Materials and methods
Plant material
Papaya (*Carica papaya* var Eksotika 2) were harvested from MARDI orchard, Serdang, Selangor, Malaysia. Unripe mature fruits of full green skin and ripe fruit having more yellow than green skin were harvested, washed in ethanol and distilled water. The pericarp tissue was cut into small pieces, frozen in liquid nitrogen and were either used immediately in RNA extraction or kept at –80 °C.

RNA extraction
Total RNA was extracted using the method of Chirgwin et al. (1979). Frozen fruit pericarp, average from three different fruits at the same stage was ground to a fine powder using a coffee grinder. The frozen powder was homogenised in extraction buffer (4 M guanidin thiocyanide, 100 mM EDTA, 0.07% β-mercaptoethanol and 0.05 volume sodium sarkosyl) using a mortar and pestle. The final homogenate was overlayed on 6 mL of CsCl cushion (5.7 M CsCl, 100 mM EDTA) and centrifuged in a Beckman SW 28 rotor at 25 000 rpm for 18 h. The RNA pellet was resuspended in water and

was quantified spectrophotometrically as described by Sambrook et al, (1989).

mRNA isolation
PolyA$^+$ RNA was isolated using the polyA Tract Kit (Promega) following the manufacturer's protocol.

cDNA libraries construction
cDNA was synthesised from approximately 5 μg poly(A) mRNA using a λZAP cDNA synthesis kit (Stratagene). The cDNA was then ligated into λUni-ZAP XR vector, encapsulated in vitro and amplified immediately in *Escherichia coli* strain XL1-Blue MRF' according to manufacturer's instruction (Stratagene).

Results and discussion
The fruit characteristic of the two stages of Eksotika 2 papaya used in the library construction is shown in *Plate 1*. The unripe fruit, mature green stage, is characterised by black seeds, white mesocarp and full green skin whereas ripe fruit is characterised by black seeds, edible red mesocarp and more yellow than green skin. cDNA libraries were constructed from the green and the ripe fruits mRNA to enable the isolation of genes specifically active in fruit development before and during the ripening process, respectively.

Plate 2 shows the ethidium bromide stained denaturing gel of total RNA isolated from the mature green and ripe fruit samples indicating that good intact RNAs were used in the library construction. *Plate 3* shows the cDNA that was obtained using the extracted RNA samples as template. The smear ranging from 0.9–5 kb indicates that the majority of the mRNAs have been fully converted to cDNAs. The cDNAs obtained were further separated on size fractionation column, Sepharose CL-2B. This is to remove small molecules such as adaptors and the unincorporated radioactive nucleotides from the samples. *Plate 4* shows the size range obtained from each of the fraction collected. Fractions containing sizes
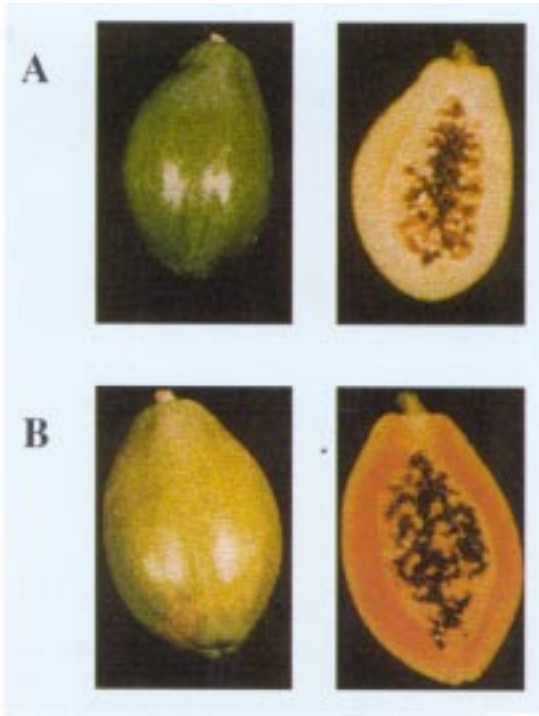
Plate 1. Characteristics of the cross-section of the unripe and ripe Eksotika 2 papaya used in the cDNA library construction. A: unripe fruit (mature green stage); B: ripe fruit
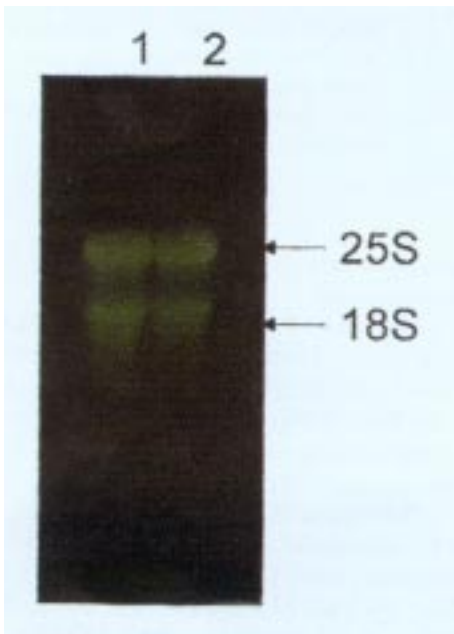

Plate 2. Agarose gel electrophoresis of the total RNA extracted from green and ripe fruits that were used in the construction of the two cDNA libraries. (Lane 1: RNA from green fruit and 2: RNA from ripe fruit. 25S and 18S indicate the two major ribosomal bands)

above 0.9 kb (fraction no. 2 to 4) were pooled and the cDNA concentration of the pooled fraction of 100 ηg/μL was obtained using the ethidium bromide plate assay *(Plate 5)*.

The cDNA obtained was then ligated to the λ phage vector arms and encapsulated into λ phage. *Plate 6* shows the plaques that were formed after infection of the host *E. coli* cells. The recombinant plaques appeared white due to the interruption of the *lac*Z gene expression from the insertion of the cDNA into the multiple cloning sites of the vectors. The nonrecombinant plaques appeared blue resulting from the complete expression of the *lac*Z gene to produce β-galactosidase which then cleaved the X-gal present in the media to produce a blue colour. This blue-white selection of the phagemids is a useful feature to detect immediately the ratio of successful recombinants to nonrecombinants obtained from the library construction. The total number of recombinant phagemids obtained from the library was 75 000 pfu (plaque
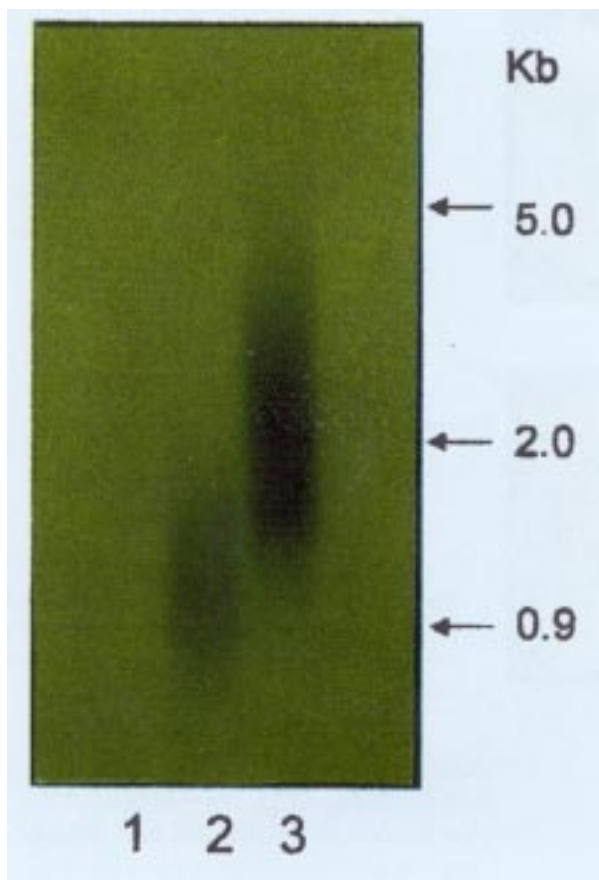
Plate 3. Autoradiograph of the first and second strands of cDNA synthesis. Lane 1: Second strand cDNA synthesis of test RNA; 2: first strand cDNA synthesis of RNA from ripe fruits; 3: second strand cDNA synthesis of RNA from ripe fruits. Numbers on the right indicate molecular weights in kilobases (kb)

forming units) per/mL for the green fruit and 319 000 pfu/mL for the ripe fruit samples. The green and ripe library was then amplified to 9×10^9 pfu/mL and 4.4×10^{10} pfu/mL, respectively. The Uni-ZAP XR vectors used in the library construction allow the phagemids to be converted to recombinant plasmids, in pBluescript, directly through in vivo excision process. Other advantages of using the Uni-ZAP XR vectors include blue-white colour selection of recombinant phagemids or plasmids, availability of 21 unique cloning sites flanked by T3 and T7 RNA promoters which are useful for the in vitro synthesis of RNA from cDNA inserts to make riboprobes, a choice of six different primers sites for sequencing or amplification of cDNA insert using PCR and the presence of

*lac*Z promoters for in vitro expression of fusion proteins.

The sizes of inserts determined by PCR from selected phagemids which were *in vivo* excised from the library ranged from 0.6 to 2 kb *(Plate7)*. This suggested that the recombinant phagemids from the library were successfully converted to plasmids.

The ripe cDNA library has been used in the isolation of near full-length metallothionein-like protein gene cDNA clone (Lam and Abu Bakar 1996), the ACC oxidase cDNA clone (Abu Bakar and Lam 1996) and a cDNA encoding glutathione S-transferase (Lam and Abu Bakar 1998, EMBL accession number AJ000923). A partial cDNA encoding a major intrinsic protein was also isolated (Lam and Abu Bakar 1997, EMBL accession number
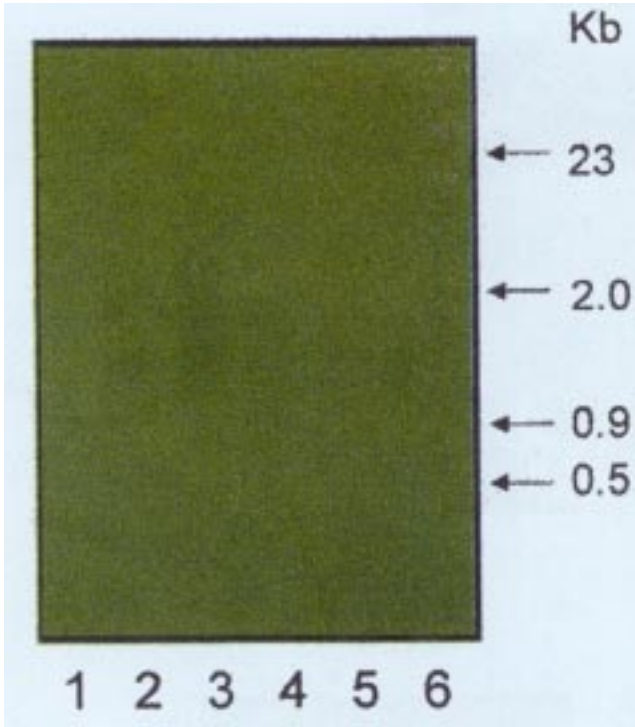
Plate 4. Autoradiograph of cDNA fractions after size fractionations on Sepharose   CL-2B column of the ripe fruit sample. Lane 1–6: 0.5 mL each of collected fractions number   1– 6. Numbers on the right indicate molecular weights in kilobases (kb)
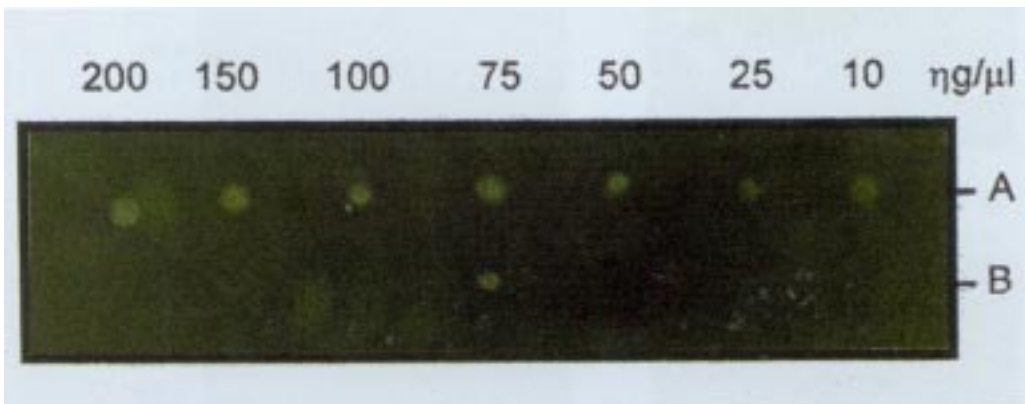

Plate 5. Ethidium bromide plate assay of ripe fruit cDNA. A: 10–200 ηg/uL of λDNA as concentration standard. B: 1 μL of pooled cDNA fraction of ripe fruit sample

AJ000031) and later this cDNA was used as a probe to rescreen the library and a full-length cDNA was obtained.

Conclusion

The library construction using the cDNA synthesis kit and the Uni-ZAP XR unidirectional vector cloning kit (Stratagene) has successfully produced two cDNA libraries from the green and ripe fruits of the Eksotika 2 papaya. The amplified libraries contain 9 x 10^9 pfu/mL and 4.4 x 10^{10} pfu/ mL recombinant phagemids for the green and the ripe fruits, respectively. The ripe cDNA library has been successfully used in the isolation of important fruit ripening cDNA clones and therefore can be used in the isolation of other papaya fruit
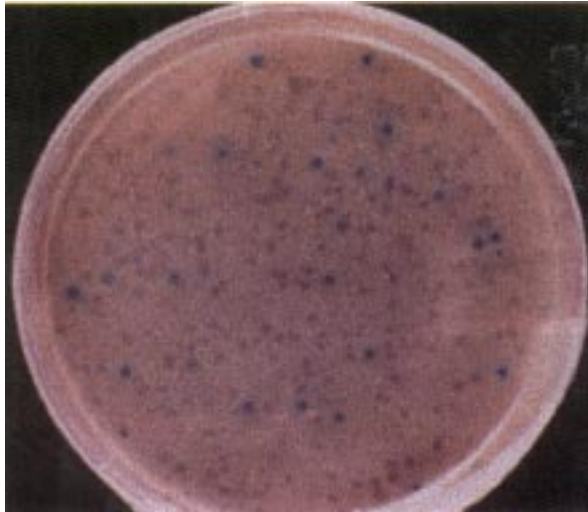
Plate 6. Plaques formed after infection of the host cells, XL1-Blue strain of E.coli with 1 mL of the ripe fruit cDNA library. Blue plaques: nonrecombinant plaque (without cDNA insert); clear plaques: recombinant plaques (containing cDNA inserts)
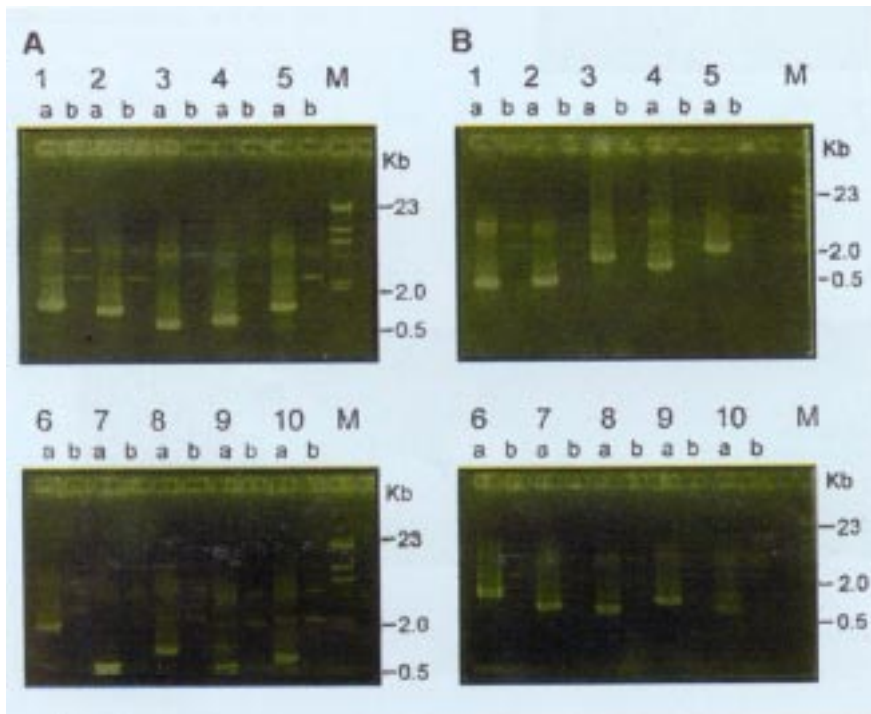

Plate 7. Agarose gel electrophoresis of PCR products of randomly selected cDNA clones from the green and ripe cDNA libraries. Panel A: green fruit library; B: ripe fruit library; Lanes 1a–10a : PCR products of cDNA clones 1–10; Lanes 1b–10 b: plasmid DNA isolated from cDNA clones 1– 10, used as template in PCR. M: **Hind** III digested λ DNA as molecular weight markers in kilobases (kb)

developmental and ripening genes. These genes will be useful in the overall understanding of the Eksotika 2 papaya developmental or ripening process and ultimately utilised in the improvement of papaya through genetic engineering.

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