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Isolation of fruit ripening genes from *Carica papaya* var. Eksotika 1 cDNA libraries

(Pemencilan gen pemasakan *Carica papaya* var. Eksotika 1 daripada perpustakaan cDNA)

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Abstract

As an initiative to discover genes that are associated with the fruit ripening process, the expressed sequence tags (ESTs) analysis was conducted on the local papaya variety Eksotika I. Single pass sequencing was performed on a total of 2,150 ESTs from early ripe and ripe *Carica papaya* var. Eksotika I cDNA libraries. Homology search on ESTs with BLASTX algorithm against Swiss-Prot non-redundant amino acid database revealed 71.1% of the papaya ESTs sequences matched the registered proteins with a cut off value of 1 x $10E^{-5}$. Analysis of KEGG and UniProt showed that there were 129 transcripts or 22 unigenes involved in fruit ripening processes such as ethylene biosynthesis pathway, fruit softening and aroma. The ESTs profiling data revealed a general picture of papaya gene expression and regulation pattern particularly of genes related to fruit ripening. The results obtained in this study can facilitate the follow up on gene expression studies using DNA microarray and contribute to the development of new markers for comparative and functional genomics studies of other tropical fruit crops.

Introduction

Cultivated papaya, *Carica papaya* L. is a fast-growing tree-like herbaceous plant belonging to the family Caricaceae (Badillo 2000). It is a dicotyledonous, polygamous, diploid species with nine pairs of chromosomes (Bennett and Leitch 2005). Papaya is now commonly grown in most tropical countries and many sub-tropical regions of the world. *Carica papaya* var. Eksotika 1 is a unique variety developed and released by MARDI in 1987 from a backcross breeding programme involving the local Subang 6 variety and the Sunrise Solo variety introduced from Hawaii (Chan 1987). With its sweet taste and pleasant aroma, the Eksotika is now recommended as a table variety for local market as well as for export to countries like Hong Kong, China, Singapore, the Middle East and Europe. The export revenue of this particular papaya variety climbed steadily from RM3 million in 1987 to a peak of RM120 million by 2004 (Anon. 2006).

Fruit ripening is a complex developmental process that includes changes in color, aroma, metabolism of sugars and organic acids, texture and softening (Brady 1987). All biochemical and physiological changes that take place

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during fruit ripening are driven by the coordinated expression of fruit ripening-related genes including the breakdown of chlorophyll, degradation of the cell wall, conversion of starch to sugars, alteration in pigment biosynthesis and the accumulation of flavour, aromatic compounds and lipid peroxides (Giovannoni 2001). These genes are also the regulatory proteins that participate in the signaling pathways and in the transcriptional machinery which regulate gene expression and set in motion the ripening developmental program (Bouzayen et al. 2010).

Expressed sequence tag (EST) is generally a single-read sequence produced from complementary DNA (cDNA) and collection of ESTs could represent a snapshot of gene expression under a certain developmental stage following some specific biotic and abiotic challenges. They provide a robust sequence resource that can be exploited for gene discovery, genome annotation and comparative genomics (Gibson and Muse 2002).

ESTs is always the first approach to study an unknown organism partly because it is the simplest and rapid tool used for gene discovery and transcriptome profiling as well as its relative cheapness as compared to other methods such as DNA microarray or genome sequencing. Hence, this study aimed to discover genes that are related to the fruit ripening processes from *Carica papaya* var. Eksotika 1 and to obtain a general transcriptome profile in order to study the underlying ripening mechanism in papaya.

Materials and methods

Plant materials

Papaya fruits from the Eksotika 1 variety grown at the MARDI Papaya Germplasm located at the Headquarters of MARDI, Serdang were collected at four developmental stages: breaker, less than 50% ripe, more than 50 % ripe and fully ripe. The seeds and placenta were removed from the fruits. Both the skin and mesocarp were cut into 1–2 cm cubes and immediately frozen in liquid nitrogen before storing at –80 °C.

Total RNA extraction, mRNA isolation

Total RNA was extracted using the Cetyltrimethylammonium bromide (CTAB) method as described by Chang et al. (1993). The extracted total RNA was then purified using Qiagen RNeasy columns (Qiagen, Valencia, CA). Messenger RNA (mRNA) was later isolated from total RNA using the Ambion Poly(A) Purist mRNA Purification Kit (Ambion, Inc.) according to the manufacturer's protocol. The purified mRNA was ethanol precipitated and resuspended in Diethlpyrocarbonate (DEPC) treated water.

Papaya cDNA libraries construction

The first and second strand cDNAs of each papaya cDNA library was synthesized using 5 μg of mRNA and the Gateway CloneMiner™ cDNA library construction kit (Invitrogen, Carlsbad, CA, USA) according to manufacturers' protocols. Subsequent to the second-strand cDNA synthesis, blunt end products were ligated with an attB1 adapter using T4 DNA ligase. After size fractionation, cDNA fragments in the range of 0.5–1.5 kb and 1.5–4.0 kb were cloned separately into the cloning vector pDONR 222 with the BP clonase enzyme. Recombinant plasmids were used to transform ElectroMax DH10B competent cells via electroporation, and random cDNA clones were picked for quality control analysis.

cDNA clone propagation, DNA isolation and DNA sequencing

White colonies from Luria Broth (LB) agar media plates were tooth-picked into a 96-well block containing 1 ml LB culture media (supplied with kanamycin 50 mg/ml) before incubation overnight at 37 ºC with shaking at 200 rpm. Plasmid DNA extraction was performed using the Montage™ Plasmid Miniprep 96 Kit (Millipore, Billerica, MA) and bacterial glycerol stocks of individual cDNA clones were stored in 96-well

microtitre plates at –80 °C. Individual recombinant plasmids containing papaya cDNA inserts were subjected to 5' end single-pass sequencing using M13 Forward primer (5'- GTAAAACGACGGCCAG -3') and the BigDye ® Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing products were resolved and analysed on a 3730 DNA Analyzer (Applied Biosystems).

Sequence processing and analysis

The papaya ESTs sequences were processed to remove vector derived sequences, ambiguous sequences with low quality and sequences shorter than 100 bps using Lucy version 1.18 – sequence alignment software tool (Chou and Holmes 2001). The trimmed EST sequences were then clustered and assembled into groups using the base-calling PHRED (Ewing et al. 1998) and contig assembly PHRAP (http://bozeman.mbt.washington.edu/phrap.docs/phred.html) computer programmes. For the unigenes identification, CAP3 programme was used to assemble ESTs into contigs using the parameters of 60 bp overlap length and 95 % overlap identity (Huang and Madan 1999).

All EST nucleotide sequences were then subjected to Basic Local Alignment Search Tool (BLAST)X homology search for sequence similarity against the UniProt/Swiss-Prot annotated protein database. ESTs which showed significant matches (E-value ≤ 10^{-5}) to registered unknown function proteins in the public database were grouped into unknown proteins. There was no homology of ESTs with an E-value of >1.0E^{-5} to the registered protein sequences in the Swiss-Prot database.

Functional analysis and pathway assignments

Functional analysis of papaya ESTs was performed using Blast2GO software (http://www.blast2go.de/) (Conesa et al. 2005). Enzyme commission (EC) numbers were assigned to ESTs sequences that had BLASTX scores with a cut-off value of E ≤ 10^{-6} upon searching the protein databases. The sequences were mapped to Kyoto Encyclopedia of Genes and Genomes (KEGG) biochemical pathways according to the EC distribution in the pathway database.

Results and discussion

cDNA library construction and EST sequencing

Two high quality papaya cDNA libraries namely early ripe (C1) and ripe (C2) were constructed. C1 library was constructed by pooling the messenger RNA of breaker and ≤50% yellow fruits whilst C2 library was constructed from messenger RNA of ≥50% yellow and fully yellow fruits. This approach was chosen to enable a wider representation of genes expressed during fruit ripening. The titers of the papaya C1 and C2 cDNA libraries were 4.662 x 10^7 cfu and 3.789 x 10^7 cfu respectively. PCR screening on the cDNA clones revealed >95% recombinants for each cDNA library constructed and the insert size ranged from 500 bp to 3 Kbp.

EST sequencing and clustering

Single-pass sequencing from 5'end was subjected to a total of 2,150 ESTs from both cDNA libraries (883 ESTs from C1 library and 1,267 ESTs from C2 library). The average read length of ESTs sequences was 700 bp with 46% of the average Guanine-cytosine (GC) content. After low quality and vector trimming, there were 94.3% (2,027/2,150) of high quality sequences with more than 100 bp. There were 732 unigenes representing different putative

Table 1. *Carica papaya* var. Eksotika I unigene set statistics

	No. of EST sequences
Total number of ESTs sequenced	2,150
ESTs after trimming	2,027
Total contigs	536
Total singletons	196
Total unigenes	732

papaya Eksotika 1 transcripts consisting of 536 contigs and 196 singletons (*Table 1*). BLASTX results against Swiss-Prot database revealed 71.1% of the high quality ESTs sequences matching to registered proteins with a cut off value of ≤1x10E^{-5}.

Seven most abundant EST unigenes were found from C1 and C2 cDNA libraries with more than 15 transcripts encoding metallothionein-like protein type 3 (MT-3), basic endochitinase CHB4 precursor, abscisic stress-ripening protein, 1-aminocyclopropane-1-carboxylate (ACC) oxidase 3, polygalacturonase precursor (PG), taxadien-5-alpha-ol O-acetyltransferase and ACC oxidase (*Table 2*). Most of the abundantly found transcripts are associated with fruit ripening and softening, such as abscisic stress-ripening protein, ACC oxidases and polygalacturonase.

MT-3 was the most abundant transcript and accounted for 332 transcript (16%) of all the papaya ESTs. Majority of the metallothionein transcripts were found from C1 cDNA library (248 transcripts) as compared to C2 cDNA library (84 transcripts). MT proteins are generally known for their metal-binding capacities and predicted to have a defense or stress function. The prevalence of this transcript in papaya libraries could be associated with an earlier study by Cobbett and Goldsbrough (2002) who found that MT-3 was normally expressed at high levels in leaves and ripening fruits.

Data mining of papaya ESTs via KEGG and Uniprot from both cDNA libraries revealed a total of 129 ESTs or 22 unigenes with their putative biochemical functions associated with fruit ripening process e.g. ethylene biosynthesis process (8 unigenes), fruit softening (8 unigenes) and synthesis of fruit aroma (6 unigenes) (*Table 3*).

Table 2. Highly expressed transcripts in both of the Eksotika 1 papaya fruit cDNA libraries, C1 and C2

Transcript	Number of ESTs	Consensus E-value	Swiss-Prot. Accession No	Organism
Metallothionein-like protein type 3 (MT-3)	332	1.00E-35	Q96386	*C. papaya*
Basic endochitinase CHB4 precursor	199	1.00E-106	Q06209	*B. napus*
Abscisic stress-ripening protein	31	1.00E-10	Q08655	*S. lycopersicum*
1-aminocyclopropane-1-carboxylate oxidase 3 (ACC oxidase 3)	23	1.00E-114	Q08507	*P. hybrida*
Polygalacturonase precursor (PG)	22	1.00E-60	P48979	*P. persica*
Taxadien-5-alpha-ol O-acetyltransferase	18	1.00E-36	Q8S9G6	*T. chinensis*
1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase)	16	1.00E-86	Q9MB94	*P. mume*

Table 3. Eksotika 1 papaya EST sequences with similarity to proteins associated with fruit ripening processes

Predicted gene function	E-value	Swiss-Prot Accession No.	Organism	ESTs from Papaya cDNA libraries	
				Early Ripe (C1) (%)	Ripe (C2) (%)
Ethylene biosynthesis and regulation					
S-adenosylmethionine synthetase 1 (EC:2.5.1.6)	1.00E-147	Q96551	*C. roseus*	6 (0.7)	3 (0.2)
S-adenosylmethionine synthetase 2 (EC:2.5.1.6)	1.00E-142	Q96552	*C. roseus*	0 (0)	1 (0.1)
ACC synthase 1 (EC:4.4.1.14)	1.00E-118	Q9MB95	*P. mume*	0 (0)	1 (0.1)
ACC oxidase (EC 1.14.17.4)	1.00E-86	Q9MB94	*P. mume*	5 (0.6)	11 (0.9)
ACC oxidase 3 (EC 1.14.17.4)	1.00E-114	Q08507	*P. hybrida*	10 (1.1)	13 (1.0)
ACC oxidase 4 (EC 1.14.17.4)	1.00E-32	P24157	*S. lycopersicum*	1 (0.1)	0 (0)
Ethylene-responsive transcription factor 7	1.00E-38	Q9LDE4	*A. thaliana*	1 (0.1)	0 (0)
Ethylene-responsive transcription factor RAP2-4	1.00E-41	Q8H1E4	*A. thaliana*	0 (0)	1 (0.1)
Fruit softening					
Polygalacturonase precursor (EC:3.2.1.15)	1.00E-95	P48979	*P. persica*	1 (0.1)	21(1.7)
Putative beta-D-xylosidase (EC:3.2.1.37)	1.00E-37	P83344	*P. persica*	0 (0)	2 (0.2)
Beta-fructofuranosidase1 (EC:3.2.1.26)	1.00E-94	P26792	*D. carota*	2 (0.2)	1 (0.1)
Beta-fructofuranosidase 3 (EC:3.2.1.26)	1.00E-89	Q39693	*D. carota*	2 (0.2)	0 (0)
Endo-1,4-beta-D-glucanase (EC:3.2.1.4)	1.00E-104	Q9ZT66	*D. carota*	2 (0.2)	0 (0)
Probable xyloglucan endotransglucosylase (EC:2.4.1.207)	1.00E-89	Q38908	*Z. mays*	0 (0)	3 (0.2)
Expansin-A8 precursor	1.00E-106	O22874	*O. sativa*	1 (0.1)	3 (0.2)
Expansin-like B3 precursor	1.00E-27	Q9ZV52	A. thaliana	1 (0.1)	1 (0.1)
Plastid lipid associated protein	1.00E-101	Q9ZWQ8	*C. unshiu*	8 (0.9)	11 (0.9)
Fruit aroma					
Ester biosynthesis pathway					
Putative lipoxygenase 5 (EC:1.13.11.12)	1.00E-44	Q7XV13	*O. sativa*	0 (0)	1 (0.1)
Alcohol dehydrogenase-like 5 (EC 1.1.1.1)	1.00E-78	Q0V7W6	*A. thaliana*	3 (0.3)	7 (0.6)
Phenylpropanoid biosynthesis pathway					
Cinnamic acid 4-hydroxylase (EC:1.14.13.11)	1.00E-98	Q43054	*P. kitakamiensis*	0 (0)	1 (0.1)
Caffeic acid 3-O-methyltransferase (EC:2.1.1.68)	1.00E-127	Q00763	*P. tremuloides*	0 (0)	1 (0.1)
Cytochrome P450 84A1 (EC:1.14.-.-)	1.00E-100	Q42600	*A. thaliana*	0 (0)	3 (0.2)
Terpene biosynthesis pathway					
Hydroxymethylglutaryl-CoA synthase (EC:2.3.3.10)	1.00E-132	P54873	*A. thaliana*	1 (0.1)	0 (0)

Genes controlling fruit ripening process

Ethylene biosynthesis process By positioning transcripts related to ethylene biosynthesis process onto the ethylene biosynthesis pathway (Adam and Yang 1979), it was clear that we managed to retrieve genes encoding for all the important enzymes in that pathway from our EST studies. These include ESTs for encoding S-adenosylmethionine synthetase 1 (AdoMet synthetase 1), S-adenosylmethionine synthetase 2 (AdoMet synthetase 2), ACC synthase 1 and ACC oxidases as shown in *Figure 1*. In terms of their predominance in the libraries, it was noticed that majority of the transcripts had low gene frequency except ACC oxidases. There were a total of 39 ESTs encoding for papaya ACC oxidase and ACC oxidase 3 from both libraries and they were two of the most abundant transcripts as mentioned above (*Table 2*).

From the analysis, it was observed that gene expression of ACC oxidases was found up-regulated as the number of transcripts increases from 16 transcripts at early ripe (C1 library) to 24 transcripts at ripe stage (C2 library) (*Table 3*). This may be a good indication that relatively more 1-aminocyclopropane-1-carboxylic acids were being converted to ethylene by ACC oxidases at the later stages of fruit ripening process. In fact, the increased activity of ACC oxidases could always be linked with the onset of fruit ripening following a surge of ethylene production in climacteric fruits. For instance, an earlier study by Fonseca et al. (2004) reported that the burst of ethylene is an autocatalytic process and it accelerates during the ripening phase resulting in changes in transcription and translation of many ripening related genes.

Two genes encoding transcription factors involved in ethylene regulation and signaling pathways, ethylene-responsive transcription factor 7 and ethylene-responsive transcription factor RAP2-4 were found in this study. Ethylene-responsive transcription factor 7 is involved in the regulation of gene expression by abscisic acid which is one of the major stress factors during the fruit ripening process according to Song et al. (2005). On the other hand, a recent study by Lin et al. (2008) reported that ethylene-responsive transcription factor RAP2-4 regulates multiple plant developmental processes and stress responses including fruit ripening process via light and ethylene signaling pathways.

Fruit softening From the ESTs analysis, there were six papaya unigenes which showed high similarity to cell wall hydrolases (*Table 3*). Fruit softening is well associated with cell wall hydrolases as these enzymes resulted in loss of tissue integrity and changes in cell wall structure during fruit ripening (Fischer and Bennett 1991). Most of the hydrolases listed in *Table 3* have not been previously reported from papaya except for polygalacturonase (Chan and Tam 1982). Recently, Fabi et al. (2009) reported the discovery of polygalacturonase sequence in papaya Golden cultivar which showed high silimilarity (90% homology) to our polygalacturonase sequence. Interestingly, the gene expression of this particular

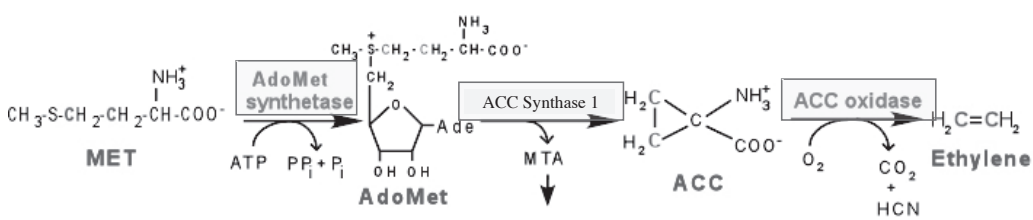

Figure 1. Papaya ESTs encoding enzymes (in boxes) in the Ethylene Biosynthesis Pathway. The enzymes catalyzing each step are shown above the arrows. AdoMet: S-adenosylmethionine; ACC: 1-aminocyclopropane-1-carboxylic acid; MTA: methylthioadenine

enzyme was found up-regulated from only one transcript in the early ripe library to 21 transcripts in the ripe library (*Table 3*). Perhaps it is to be expected that the role of polygalacturonase is essential in fruit cell wall softening particularly at the later stage of fruit ripening.

The papaya ESTs also revealed two isoenzymes namely beta-fructofuranosidase 1 and 3. These are invertase enzymes that breakdown sucrose into fructose and glucose and their expression is highly induced during tomato fruit ripening (Klann et al. 1993). However, there was no marked increase in frequency of this transcript from early ripe to ripe stage from the papaya ESTs data (*Table 3*).

From the papaya ESTs, two types of expansins were discovered, expansin-A8 precursor and expansin-like B3 precursor. Rose and Benette (1999) had shown the significant role of expansins in fruit softening as these enzymes contributed to cell wall polymer disassembly or degradation and fruit softening by increasing access of specific cell wall polymers to hydrolase action. Analysis of the ESTs data showed that both expansins were expressed in early ripe as well as ripe libraries (*Table 3*) which may suggest their roles in facilitating the softening of papaya fruit and fruit cell wall degradation during the ripening process.

Fruit aroma A total of 17 papaya ESTs sequences from the study showed significant similarity to enzymes associated with fruit volatile biosynthesis. Fruit aromas are generally compounds derived from lipids, sugars and amino acids such as alcohols, aldehydes, ketones, sesquiterpenes, polypropanoids and esters (Schaffer et al. 2007). Analysis via KEGG revealed six unigenes from the papaya ESTs with their putative functions involved in the ester biosynthesis pathway, phenylpropanoid biosynthesis pathway and terpene biosynthesis pathway (*Table 3*).

According to Pino et al. (2003), methyl and ethyl ester derivatives of lipid catabolism have been identified as strong contributors to aroma in tropical fruits including papaya. In this study, two unigenes were discovered which are involved in the ester biosynthesis pathway, namely putative lipoxygenase 5 and alcohol dehydrogenase-like 5. Lipoxygenase has been reported as a key enzyme responsible for the formation of hydroperoxides from unsaturated fatty acids and hydroperoxide lyase which ensures the generation of the volatile aldehydes (Blee et al. 1993).

Only one transcript was found encoding this enzyme in the ripe stage of papaya library. In comparison, a more pronounced up-regulated expression of transcripts encoding for alcohol dehydrogenase-like 5 was observed. Three to seven transcripts from early ripe to ripe stage of papaya were encoding this enzyme respectively. The increasing alcohol dehydrogenase2 (ADH2) activity during ripening process is probably due to the decreasing oxygen concentration within the ripening fruit as reported by Speirs et al. (2002).

There were three unigenes encoding enzymes in the phenylpropanoid biosynthesis pathway which were responsible for synthesis of fruit aromatic compounds among the sequenced ESTs. These genes, cinnamic acid 4-hydroxylase, caffeic acid 3-O-methyltransferase and cytochrome P450 84A1 were only detected in the later stages of ripening in the ripe library. Among these genes, expression of the gene encoding for cytochrome P450 84A1 was relatively higher. On the other hand, the only gene involved in terpene biosynthesis pathway, hydroxymethylglutaryl-CoA synthase was expressed only in the early ripe papaya library.

Conclusion

The results of the study revealed a general transcriptome profile and numerous key

functional genes involved in the papaya fruit ripening process using the expressed sequence tags approach. Like fruits of most plant species, the papaya undergoes sequential and rapid events during development and ripening. From the analysis of KEGG and Uniprot, 22 unigenes were discovered with their putative functions related to fruit ripening process such as ethylene biosynthesis process, fruit softening and aroma.

The findings from this study are of valuable agronomical interest to the local papaya Eksotika fruit industry. The data can also be used as primary knowledge for future genetics and genomics studies on papaya. Furthermore, this data can facilitate subsequent gene expression studies using DNA microarray and also in the development of new markers for comparative and functional genomics studies in other tropical fruit crops. With the completion of the transgenic papaya genome sequencing project (Ming et al. 2008), the results that we report here may contribute to the current work of papaya genome annotation.

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Abstrak

Sebagai inisiatif untuk menemui gen yang dikaitkan dengan proses pemasakan buah, analisis tag jujukan ekspresi (EST) telah dijalankan ke atas varieti betik tempatan Eksotika I. Penjujukan DNA sehala telah dijalankan ke atas sejumlah 2,150 EST daripada dua perpustakaan cDNA betik varieti Eksotika I. RNA diekstrak daripada buah di peringkat awal pemasakan dan semasa masak sepenuhnya. Pencarian homologi pada ESTs dengan menggunakan algoritma BLASTX ke atas pangkalan data asid amino yang tidak berulang, Swiss-Prot, untuk pengenalpastian fungsi gen mendapati 71.1% jujukan ESTs berpandanan dengan protein berdaftar dengan had nilai 1 x $10E^{-5}$. Analisis KEGG dan UniProt menunjukkan terdapat 129 transkrip atau 22 unigen terlibat dalam proses pemasakan buah-buahan seperti laluan biosintesis etilena, pelembutan buah-buahan dan aroma. Profil ESTs betik ini telah mendedahkan gambaran umum ekspresi gen betik dan corak kawalan terutamanya gen yang berkaitan dengan pemasakan buah-buahan. Keputusan yang diperoleh daripada kajian ini dapat memudahkan kajian susulan ekspresi gen menggunakan mikroatur DNA dan menyumbang kepada pembangunan penanda DNA baru untuk kajian perbandingan dan fungsi genomik tanaman buah-buahan tropika yang lain.

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