ISOLATION AND CHARACTERIZATION OF MICRORNAS FROM TWO DIFFERENT FRUIT RIPENING STAGES IN PINEAPPLES

YEW CHEE WEI

BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH
2011

ISOLATION AND CHARACTERIZATION OF MICRORNAS FROM TWO DIFFERENT FRUIT RIPENING STAGES IN PINEAPPLES

YEW CHEE WEI

THESIS SUBMITTED IN FULFILLMENT FOR THE DEGREE OF MASTER OF SCIENCE

BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH
2011

PUMS 99:1

UNIVERSITI MALAYSIA SABAH

BORANG PENGESAHAN TESIS

JUDUL : _____
_____
_____

IJAZAH : _____
_____

SAYA : _____ **SESI PENGAJIAN** : _____
(HURUF BESAR)

Mengaku membenarkan tesis *(LPSM/Sarjana/Doktor Falsafah) ini disimpan di Perpustakaan Universiti Malaysia Sabah dengan syarat-syarat kegunaan seperti berikut:-

1. Tesis adalah hak milik Universiti Malaysia Sabah.
2. Perpustakaan Universiti Malaysia Sabah dibenarkan membuat salinan untuk tujuan pengajian sahaja.
3. Perpustakaan dibenarkan membuat salinan tesis ini sebagai bahan pertukaran antara institusi pengajian tinggi.
4. Sila tandakan (/)

☐ SULIT (Mengandungi maklumat yang berdarjah keselamatan atau kepentingan Malaysia seperti yang termaktub di AKTA RAHSIA RASMI 1972)

☐ TERHAD (Mengandungi maklumat TERHAD yang telah ditentukan oleh organisasi/badan di mana penyelidikan dijalankan)

☐ TIDAK TERHAD

Disahkan oleh:

_____ _____
(TANDATANGAN PENULIS) (TANDATANGAN PUSTAKAWAN)

Alamat Tetap: _____
_____
_____
_____

_____
(NAMA PENYELIA)

TARIKH: _____ TARIKH: _____

Catatan:
*Potong yang tidak berkenaan.
*Jika tesis ini SULIT dan TERHAD, sila lampirkan surat daripada pihak berkuasa/organisasi berkenaan dengan menyatakan sekali sebab dan tempoh tesis ini perlu dikelaskan sebagai SULIT dan TERHAD.
*Tesis dimaksudkan sebagai tesis bagi Ijazah Doktor Falsafah dan Sarjana Secara Penyelidikan atau disertai bagi pengajian secara kerja kursus dan Laporan Projek Sarjana Muda (LPSM).

DECLARATION

I hereby declare that the material in this thesis is of my own effort, except for quotations, excerpts, equations, references and summaries which have been duly acknowledged and cited clearly as sources.

14 February 2011

_____

Yew Chee Wei
PB20078431

CERTIFICATION

NAME : **YEW CHEE WEI**

MATRIC NO. : **PB20078431**

TITLE : **ISOLATION AND CHARACTERIZATION OF MICRORNAS FROM TWO DIFFERENT FRUIT RIPENING STAGES IN PINEAPPLES**

DEGREE : **MASTER OF SCIENCE IN BIOTECHNOLOGY (MOLECULAR GENETICS)**

VIVA DATE : **26 MAY 2011**

DECLARED BY

Signature

1. SUPERVISOR
Assoc. Prof. Dr. Vijay Kumar _____

ABSTRACT

ISOLATION AND CHARACTERIZATION OF MICRORNAS FROM TWO DIFFERENT FRUIT RIPENING STAGES IN PINEAPPLES

Plant microRNAs are single-stranded small RNAs with approximately 21-24 nucleotides in size. They play an important role in regulating gene expression at the post-transcriptional level through complementary binding to endogenous mRNA targets which subsequently induces gene silencing. Although pineapple is an important tropical non-climacteric fruit, the regulatory roles of miRNAs in fruit ripening remains unknown. Thus, this study aims to isolate miRNAs from an unripe pineapple fruit, to predict the targeted genes, and to profile the expression levels of selected miRNAs between unripe and ripe fruits. MicroRNAs were isolated through the construction of a small RNA library. The target genes of the isolated miRNAs were then identified with online bioinformatics tools. Subsequently, the expression profiles between unripe and ripe fruits of randomly selected miRNAs were determined through quantitative stem-loop real-time PCR. The construction of sRNA library yielded 1,706 redundant sRNAs. This includes 13 miR/miR* families, namely miR156, miR157, miR159, miR162, miR164, miR166, miR167*, miR171, miR319, miR395, miR396, miR529 and miR827. Among these, miR157/miR157* were the most abundant miRNAs, constituting of 10.9% of the isolated sRNAs. Expression analysis showed that miR157 is 1.6x fold depressed in the ripe fruit, as compared to the unripe fruit. Meanwhile, miR395 showed the most significant induced expression (20x fold) while miR529 is depressed 33.3x fold, in the ripe fruit. miR157 was predicted to target SPL gene which is involved in phase transition from the unripe to ripe stage of the fruit. miR395 was predicted to target ATP-sulfurylase which may facilitate the accumulation of glucosinolates as a defence mechanism against the opportunistic infection by microbes and fungus during fruit ripening. Whereas, miR529 was postulated to target AP2/ERF, a ethylene-response transcription factor that regulates the expression of ethylene-responsive chitinase and defensins, as a defense against pathogenic infections. Overall, miR157, miR395 and miR529 were postulated to be key regulators during the fruit ripening of pineapples, which respectively involved in phase transition and defence against pathogenic infections. The information provided here will be useful in understanding the roles of miRNAs in the process of ripening, particularly in non-climacteric fruits.

ABSTRAK

MikroRNA tumbuhan ialah RNA kecil dengan saiz lebih kurang 21-24 nt. Ia memainkan peranan yang penting dalam pengawalan ekspresi gen pada peringkat pasca-transkripsi melalui ikatan saling melengkapi pada sasaran mRNA dalaman dan seterusnya menimbulkan 'gene silencing'. Walaupun nanas adalah buah tropikal bukan klimak yang penting, peranan pengawalan miRNA dalam pemasakan buah belum ditentukan. Oleh sebab itu, kajian ini bertujuan untuk mengasingkan miRNA daripada buah nanas yang belum masak, untuk menjangkakan sasaran gen, dan untuk memprofil aras ekspresi miRNA terpilih antara buah yang masak dan belum masak. MikroRNA diasingkan melalui pembentukan perpustakaan RNA kecil. Gen yang disasarkan oleh miRNA yang diasingkan adalah ditentukan dengan alat bioinformatik 'online'. Seterusnya, profil ekspresi miRNA yang dipilih secara random ditentukurkan secara 'quantitative stem-loop real-time PCR'. Pembentukan perpustakaan RNA kecil telah mengasingkan 1,706 sRNAs tidak unik. Ini termasuk 13 keluarga miR/miR, iaitu miR156, miR157, miR159, miR162, miR164, miR166, miR167*, miR171, miR319, miR395, miR396, miR529 dan miR827. miR157/miR157* adalah miRNA yang paling banyak dan mendirikan 10.9% daripada jumlah sRNA yang diasingkan. Analisis ekspresi menunjukkan bahawa miR157 adalah 1.6x ganda dikurangkan dalam buah masak. Selain itu, miR395 pula menunjukkan peningkatan ekspresi yang paling signifikan (20x ganda), sementara itu, miR529 adalah dikurangkan 33.3x ganda dalam buah masak. miR157 dijangkakan menyasarkan gen SPL yang terlibat dalam transisi fasa daripada peringkat belum masak ke masak. miR395 pula menyasarkan 'ATP-sulfurylase' yang membantu pengumpulan 'glucosinolate' yang berperanan dalam mekanisme pertahanan terhadap jangkitan daripada mickrob dan fungi semasa pemasakan buah. Sementara itu, miR529 dijangkakan menyasarkan AP2/ERF, satu ethylene-response factor transkripsi yang mengawal ekspresi 'ethylene-responsive chitinase' dan 'defensins', juga sebagai pertahanan terhadap jangkitan patogen. Ringkasannya, miR157, miR395 dan miR529 adalah dijangkakan sebagai pengawal terpenting semasa pemasakan buah nanas, di mana ia masing-masing terlibat dalam transisi fasa dan pertahanan terhadap jangkitan patogen. Maklumat yang diperoleh dalam kajian ini adalah berguna dalam pemahaman peranan mikroRNA dalam proses pemasakan buah, terutamanya dalam buah-buahan bukan klimak.*

ACKNOWLEDGEMENT

I wish to express my deepest gratitude and appreciation to my supervisor, Associate Professor Dr. Vijay Kumar, who has been patient and supportive to advise, guide and supervise me throughout the study. His efforts had encouraged me to complete the research. At the same time, he had given opportunities for me in participating a number of activities such as conferences, competitions and application of scholarships, which had enhanced my soft skills in addition to the laboratory skills and knowledge of the research subjects.

Besides, I would like to thank the Director of Biotechnology Research Institute, Professor Datin Seri Panglima Dr. Ann Anton for giving me the opportunity to further my study in a well-equipped laboratory. In addition, I also would like to thank the Ministry of Higher Education of Malaysia for supporting this research through the Fundamental Research Grant Scheme (grant no. FRG164-SG-2008). I am very thankful to the Ministry of Science, Technology and Innovation for supporting my expenses through a scholarship under National Science Fellowship Scheme.

Lastly, I would like to express my appreciation to all BRI's staffs for their efforts in helping my works in the institute. I would also like to thank my family and my friends especially Mr. Foong Choon Pin and Ms. Ong Wen Dee for their advise and support. Thank you!

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PHOTOGRAPH

LIST OF ABBREVIATIONS

ABA	-	Abscisic acid
bp	-	base pair
BLAST	-	Basic Local Alignment Search Tool
cDNA	-	complementary DNA
DEPC	-	diethylpyrocarbonate
GA	-	gibberellin
µg	-	microgram
µL	-	microlitre
µM	-	micromolar
m	-	mili
miR	-	microRNA
mM	-	milimolar
mol	-	moles
n	-	nano
nt	-	nucleotide
p	-	pico
RNA	-	ribonucleotide acids
RT-PCR	-	reverse transcription - polymerase chain reaction
siRNA	-	small interfering RNA
sRNA	-	small RNA
TAE	-	tris-acetate-EDTA
U	-	Unit
% (w/v)	-	percent in weight per volume

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Research Background

MicroRNAs are small RNAs with approximately 21-24 nucleotides in size. It is endogenously transcribed from *MIR* genes and then processed into the small, mature form of functional miRNAs. It functions as a negative regulator of gene expression. MicroRNAs work post-transcriptionally, by targeting the transcription product of the target genes i.e. mRNAs. Through near-perfect complementary binding to the mRNA, it mediates either the cleavage of the mRNA or inhibits the translation process. The mechanism of regulation is highly specific and occurs in a transient manner. As a result, the targeted gene is down-regulated (Bartel, 2004; Voinnet, 2009).

In plants, microRNAs (miRNAs) have been proven to play an important role in regulating every aspect of development and environmental responses. These involve embryogenesis, organogenesis, phase transition, signal transduction and various abiotic and biotic responses to environmental stimulations. The precise regulation of plant development by miRNAs determines the normal plant growth and development (Yang *et al.*, 2007).

In spite of the vital role it plays in plant development, there is lack of information on the regulatory roles of microRNA in mediating the mechanism of fruit ripening, particularly in non-climacteric fruits. Fruit ripening is characterized as the biochemical and molecular changes of fruit tissues from unfavourable hard and bitter towards favourable soft, sweet and other properties that add values for human consumption (Gillaspy, 1993; Pech *et al.*, 2008). During the transition of unripe fruit to ripe stage, fruits undergo a series of strictly regulated gene expression which involve the storage of sugar, softening of cell wall, production of aromatic compounds and enhancement of pathogen defence (Fei *et al.*, 2004; da Silva *et al.*, 2005).

Fleshy fruits are divided into two categories which are climacteric and non-climacteric (Lelievre *et al.*, 1997). Generally, the regulation of fruit ripening in climacteric fruits is characterized to be dependent of ethylene regulation. In contrast, the non-climacteric fruits are insensitive to ethylene and the ripening process is regulated by some gene expression regulators such as transcription factors, which are generally known as developmental factors (Giovannoni, 2004). Examples for climacteric fruits are tomato, banana and papaya whilst for non-climacteric fruits are pineapple, grape and citrus. Due to different physical structure, this may indicate different pathways of gene expression and its regulation.

Pineapple is an important tropical non-climacteric fruit. In comparison to climacteric fruits, non-climacteric fruits are characterized with no further ripening once the fruit is detached from its mother plant or harvested. This results in no increase in flavours, sweetness, aroma, texture and decreased acidity. Although pineapple is one of the world's most traded fruits, but strikingly the underlying genetics of fruit ripening in pineapples is vastly unknown. Till today, there are only <3000 fruit-specific expressed sequence tags (ESTs) deposited on GenBank (Moyle *et al.*, 2005). In addition, there have been no published reports on pineapple microRNAs.

In contrast, both grapes and citrus, which are non-climacteric fruits have been extensively studied. A number of conserved miRNAs among important plant models such as *Arabidopsis*, rice and moss (Pantaleo *et al.*, 2010; Song *et al.*, 2010) have been identified. So far, there are only several studies on miRNAs in tomato fruit, which is a model plant for studying fruit ripening of climacteric fruit (Giovannoni, 2007; Itaya *et al.*, 2008; Moxon *et al.*, 2008). Interestingly, Moxon *et al.* (2008) discovered two miRNAs, known as sly-miR157 and sly-miR1919, that had validated targets of SPL-CNR and LeCTR4sv1, respectively. LeCTR4sv1 is a key regulator of ethylene response while SPL-CNR is a key developmental factor that regulates the ethylene-independent pathway during fruit ripening. Thus, it was proven that microRNAs do play an upstream regulatory role in both ethylene-dependent and independent pathways in fruit ripening.

As microRNA mediated regulation is specific and occurs in a spatial and transcient manner (Valoczi *et al*., 2006), it is important to isolate and characterize the miRNAs which are expressed in different stages of fruit ripening. This will subsequently help to identify specific target genes and characterize the mechanism that is mediated by miRNAs. Since different sets of genes will be expressed and differentially up- or down-regulated during different stages of fruit ripening, thus it is hypothesized that different sets of microRNAs are correspondingly expressed and regulate the differentially expressed genes in each specific stage of fruit ripening.

As such, literature search as abovementioned shows that the genetics of fruit ripening in the commercially important tropical non-climacteric pineapples remains unknown. Studies on tomato proved that specific microRNAs are expressed in order to act as an upstream regulator to the currently known fruit ripening regulatory pathways. Additionally, a number of conserved miRNAs identified in citrus and grape also proved that miRNAs are ubiquitously expressed for fine-tuning gene expression in fruits. Relatively, there is no miRNA has been identified and characterized before, rendering the dearth of information regarding the regulatory roles of miRNA in fruit ripening of pineapples.

To address these research problems, this project aims to isolate and characterize the expressed microRNAs in the fruit tissue of the unripe fruit. Subsequently, the regulatory roles are characterized through observing the directly targeted genes. Next, the regulatory roles are further characterized in the transition of unripe to ripe stage, which signifies the initiation and termination of the fruit ripening process, through expression profiling of the isolated microRNAs in both stages.

1.2 Objectives

The objectives of this research are:

1. To construct a microRNA library from unripe pineapple fruit tissue through the cloning of small RNAs.

2. To identify sets of target genes regulated by microRNAs based on complementary sequence search on expressed sequence tags (ESTs) databases.
3. To profile the expression levels of microRNAs between pineapple in unripe and ripe stages through quantitative real-time PCR.

CHAPTER 2

LITERATURE REVIEW

2.1 Pineapples

Pineapple (*Ananas comosus* var. *comosus*) is a terrestrial herb of 0.75-1.5m height. The plant consists of a stout stem with a rosette of waxy, long-pointed, tapered leaves. It grows well on slightly acidic peat or mineral soil. The plant is tough and very resistant to drought. Pineapple belongs to the family of Bromeliaceae, order Bromeliales and is a monocotyledon. It originates from the southeast of Brazil, northeast of Argentina and Paraguay. The genus *Ananas* is diploid (2n=50), although some triploid and tetraploid plant do occur naturally (Coppen d'Eeckenbrugge *et al*., 1997; Morton, 1987).

Ananas and the *Pseudonanas* are the only genera in the family which form inflorescence that consist of 50-200 flowers. Upon fruit formation, the flowers are fused and develope into a sorose-type fruit. The fusion merges the whole inflorescence, flowers, bracts and all into one massy fruit. Pineapple is self-incompatible and within the same cultivar, due to inhibition of pollen tube growth. Thus the fruit is parthenocarpic and generally produces no seed (Coppen d'Eeckenbrugge *et al*., 1997). The plant is commercially cultivated by vegetative propagation via suckers, slips and crown, based on the phenotypes of large fruit size, low acidity, reduced seediness and smooth leaves.

The numerous pineapple cultivars are grouped in four main classes i.e. Smooth Cayenne, Red Spanish, Queen and Abacaxi (Morton, 1987). Smooth Cayenne and Queen are the major cultivars worldwide. However, the classification remains chaotic due to new names being given to the same cultivars. For example Smooth Cayenne is known as Sarawak in Malaysia but as Giant Kew in Britain. In the past 2-3 years, a new breed known as MD-2 (Del Monte Gold™), which is more resistant to diseases, sweeter, resistant to internal browning and contains vitamin C

with four fold higher than the Smooth Cayenne, has replaced other cultivars and become the dominant fresh fruit cultivar worldwide (Clark and Finn, 2010).

2.2 Pineapple Industry in Malaysia

Pineapples are ranked the world's third most-traded fruits after banana and citrus. It is cultivated for its fruits which are consumed fresh or canned. Due to increasing demand of world market, pineapple production promises a higher income in Malaysia. In five years, production of pineapple has doubled, from 196,689 metric tonnes in 2004 to 400,070 metric tonnes in 2009. The generated income tripled, from RM19.7 millions to RM59 millions within the same period (Buku Perangkaan Agro-Makanan, 2009).

Pineapple cultivation in Malaysia is mainly on peat soil as some cultivars adapt well with the acidic soil. The cultivars vastly planted for its fresh fruits are N36 and Josapine from the Spanish group, and Moris from the Queen group. Meanwhile, Gandul (Spanish) is planted for canning. Under the plant breeding programme conducted by Malaysian Agricultural Research Division Institute (MARDI), a new cultivar known as Josapine was released in 1996. It is a hybridization of Sarawak (Smooth Cayenne) and Spanish (Johor). It contains sugar levels at 16-17˚Brix and is resistant to cold storage (Chan, 2000). In contrast, the Sarawak cultivar is cultivated on mineral soil in Titi (Negeri Sembilan), Babagon/Penampang (Sabah) and Sarawak. It has a lower sugar content (12-16˚Brix) and is susceptible to internal browning due to cold storage. Recently, MD-2 is now widely planted for the export of fresh pineapple fruits (Ismail, 2008).

2.3 Biotechnology in Pineapples

The extremely low fertility of pineapples hampers the crop improvement via traditional breeding programs (Chan *et al.*, 2003). Since the fruits rarely produce seeds, the crop is propagated vegetatively. The characteristics of low fertility and high incompatibility between cultivars have prevented natural gene transfer which helps to produce desired traits. Therefore, biotechnology approaches such as genetic transformation are being utilized to improve the crop (Firoozabady *et al.*, 2006; Gangopadhyay *et al.*, 2009).

The focal aspects in biotechnology of pineapples are inhibition of precocious flowering control, extending shelf-life, pathogen resistance and herbicide resistance (Botella and Fairbairn, 2005). Control of flowering time is important to harvest fruits uniformly and minimize costs. This was conducted by Trusov and Botella (2006) who transformed the pineapple plant with an antisense construct of the ACC synthase gene. This gene is essential in the biosynthesis of ethylene for induction of floral development. Inhibition of ethylene synthesis with antisense ACC synthase gene thus facilitate uniform flowering via artificial induction of flowering.

Besides, Ko *et al*. (2006) produced transgenic pineapple which is resistant to blackheart injury. This injury is due to the activity of polyphenol oxidase (PPO) that oxidizes phenolic compounds that accumulate as the result of low temperature storage condition. Introduction of antisense PPO in transgenic pineapple has been tested in field. However, the availability of reproducible transformation and *in vitro* regeneration methods are pre-requisite to facilitate these crop improvement methods. As such, many efforts have been put on the development of tissue culture protocols such as callus induction, organogenesis and somatic embryogenesis that are used for *Agrobacterium*-mediated gene transformation (Firoozabady *et al*., 2006; Gangopadhyay *et al*., 2009).

At the same time, the transcriptome of fruit ripening was analyzed through construction of expressed sequence tags (ESTs) libraries by Moyle *et al*. (2005). These include transcriptomes of unripe and ripe fruits, and interaction of nematode with the root of the crop. Their study revealed that the ripe fruits have high expression level of MADS-box genes, which was found to be an important gene family that regulates fruit ripening in tomato (Vrebalov *et al*., 2002). High-throughput analysis of gene expression by microarray analysis was being conducted to reveal details gene expression and its regulation in the fruit ripening mechanism (Moyle *et al*., 2005).

2.4 The Characteristics of Plant MicroRNAs

The small RNA regulatory networks in plants are complex. Basically, this consists of two main classes of small RNA (sRNA) namely, microRNAs (miRNA) and small