DISCOVERY, TARGET PREDICTION AND EXPRESSION OF MICRORNAS IN PINEAPPLE (*Ananas comosus var. comosus*)

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ABSTRACT

DISCOVERY, TARGET PREDICTION AND EXPRESSION OF MICRORNAS IN PINEAPPLE (*Ananas comosus var. comosus*)

Pineapple (*Ananas comosus* var. *comosus*) is a non-climacteric fruit, of which the underlying mechanism of fruit ripening is still unknown. The non-ethylene induced ripening of non-climacteric fruit is very much different from climacteric ripening as revealed by the model organism of flesh-fruit i.e. tomato. Recently discovered gene regulators, i.e. miRNAs, are known to silence the translation of mRNA transcripts, leading to a silencing effect on proteins and affecting various phenotype changes in plants throughout plant development. Thus, this study aims to determine the mechanism of non-climacteric ripening through the differential expression of miRNA genes. Orthologous miRNAs were first amplified through stem-loop RT-PCR to prove for the existence of the presence of miRNAs in pineapples. Large scale mining of miRNA from small RNA libraries (fruit and leaf) constructed through the use of high-throughput Solexa technology was then carried out and the target mRNA transcripts were subsequently predicted. For this purpose, the miRNA registry (miRBase) and EST database (NCBI) were referred to and a bioinformatics pipeline was developed and utilized. The regulation of miRNAs in regulation of fruit ripening was postulated through correlating the target protein predicted with the pattern of expression of miRNAs during pineapple ripening obtained through stem-loop RT-qPCR. As a result, 12 orthologous miRNAs were amplified and showed the presence of miRNAs in pineapple, while finding of 153 miRNAs, 41 miRNA families, 20 miRNA*s, and 20 target transcripts obtained showed the conservation of miRNAs in pineapple. The miRNAs were then characterized. Finally, differential regulation was postulated where 7 miRNAs (miR165, miR166, miR164, miR171, miR444, miR1088, and miR396) are believed to be involved in fruit development and 4 miRNAs (miR172, miR156, miR535, miR319) in phase transition from flowering to senescence. Besides that, throughout pineapple development, 5 miRNAs (miR159, miR167, miR390, miR393 and miR394) control hormone signaling, 4 miRNAs (miR395, miR397, miR399, and miR893) control nutrient uptake and homeostasis, and 2 miRNAs (miR162 and miR168) control the overall homeostasis of miRNAs. Thus, the preliminary putative of pineapple regulated by miRNAs is postulated and revealed.

ABSTRAK

Nenas (Ananas comosus) berada dalam kategori buah bukan klimakterik dimana mekanisma pemasakannya masih tidak diketahui. MiRNA memainkan peranan dalam menghalang penterjemahan gen-gen lain kepada protein dengan mensasarkan transkrip-transkrip untuk dihancurkan atau disekat dari diterjemahkan kepada protein, lalu meyebabkan perubahan fenotip pada tumbuhan. Oleh itu, kajian ini adalah bertujuan mempelajari pemasakan buah bukan-klimakterik melalui miRNA. Ortolog miRNA pada mulanya diamplifikasi bagi membuktikan kewujudan miRNA pada nenas menggunakan kaedah stem-loop RT-PCR. Kajian diteruskan dengan pengenalpastian miRNA dari perpustakaan sRNAs (daun dan buah) yang dijujuk menggunakan teknologi berimpak tinggi yang dipanggil Solexa. MiRNA dikenalpasti dari sRNA dengan membandingkan jujukan sRNAs dengan jujukan miRNA yang terdapat dalam pusat pengumpulan miRNA (miRBase) dan ia kemudiannya dibandingkan pula dengan jujukan EST dan protein yang terdapat di NCBI bagi tujuan pengenalpastian transkrip dan protein sasarannya. Kaedah bioinformatik diaplikasikan. Corak ekspresi bagi miRNA yang baru dijumpai semasa dua peringkat pemasakan buah (pre-pemasakan dan pasca-pemasakan) telah dibandingkan dengan menggunakan kaedah stem-loop RT-qPCR. Hasilnya, amplifikasi 12 ortolog miRNA membuktikan kehadiran miRNA pada nenas. Selain itu, penemuan 153 miRNA, 41 keluarga miRNA, 20 miRNA, dan 20 transkrip yang disasarkan oleh miRNA menunjukkan bahawa miRNA adalah terpulihara dalam nenas. MiRNA yang dijumpai telah dikaji dan dicirikan. Akhir sekali 7 miRNA (miR165, miR166, miR164, miR171, miR444, miR1088, and miR396) dipercayai terlibat dalam pembentukan buah, dan 4 miRNA (miR172, miR156, miR535, miR319) terlibat dalam peralihan fasa nenas bermula dari pertumbuhan bunga hingga peranuman buah. Selain itu 5 miRNA (miR159, miR167, miR390, miR393 and miR394) dipercayai mengawal hormon di sepanjang proses pembentukan buah nenas manakala 4 miRNA (miR395, miR397, miR399, and miR893) mengawal pengambilan dan keseimbangan nutrient pada nenas. Akhir sekali 2 miRNA (miR162 dan miR168) terlibat dalam pengawalan keseluruhan rangkaian pemasakan nenas yang dikawal oleh miRNA dangan memnyeimbangkan kadar miRNA.*

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LIST OF ABBREVIATION

µl	Microliter
µM	Micromolar
bp	Base pair
$CaCl_2$	Calcium chloride
cm	Centimetre
dNTP	Deoxyribonucleotide triphosphate
g	Gram
hr	hour
min	Minute
mL	Millilitre
mM	Millimolar
miRNA	MicroRNA
miRNA*	Antisense microRNA
mRNA	Messenger RNA
$MgCl_2$	Magnesium Chloride
NaCl	Sodium Chloride
nm	Nanometer
nt	Nucleotide
Pi	Phosphorus
PPi	Phospholipids
Pre-miRNA	Precursor microRNA
Pri-miRNA	Primary microRNA
q-PCR	Quantitative polymerase chain reaction
rpm	Rotation per minute
RNAi	RNA interference
siRNA	Small interfering RNA
sRNA	Small RNA
ABA	Abscisic acid
ADP	Adenosine diphosphate
AGO	Argonaute

ALDH	Aldehyde dehydrogenase
AMP	Adenosine monophosphate
AP2	Apetala2
ARF	Auxin response factor
ATP	Adenosine-5'-triphosphate
CTAB	Cetyltrimethyl Ammonium Bromide
CUC	Cup-shaped cotyledon
DCL	Dicer like
DEPC	Diethylpyrocarbonate
DTT	Dithiothreitol
EDTA	Ethylenediamineteraacetic acid
EST	Expressed sequence tag
GA	Gibberellic acid
GIF	GRF-interacting factor
GRF	Growth regulating factor
HCl	Hydrogen chloride
HEN	Hua enhancer
LB	Lysogeny broth
NGS	Next generation sequencing
PCR	Polymerase chain reaction
PME	Pectin methylesterase
RISC	RNA inducing silencing complex
ROS	Reactive oxygen species
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SAM	Shoot apical meristem
SCR	Scarecrow
SPL	Squamosa
SULTR	Sulfate transporter
TAE	Tris acetate EDTA
TBE	Tris boric EDTA

TIR	Transport inhibitor response
U	Unit
UV	Ultra violet
XET	Xyloglucan endotransglycosylase

LIST OF SYMBOLS

°C	Degree Celsius
%	Percentage

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Pineapple (*Ananas comosus*) is a tropical fruit cultivated mainly for its fleshy fruit and it is a model organism of non-climacteric fruit. Non-climacteric fruits do not undergo an increase in respiration and ripening is not accelerated by the application of endogenous enzyme ethylene, as it is in climacteric fruit (Giovannoni, 2004; Moyle *et al*., 2005). In fact, even the application of exogenous ethylene fails to accelerate the rate of ripening (Dull *et al*., 1967). Thus, although the ripening mechanism of fleshy fruit, studied through the model fruit-bearing crop tomato, is shared among different fruit, it can't be readily assumed to be the case for pineapple, as tomato is a climacteric fruit. Lack of information regarding the non-climacteric ripening has suppressed the crop improvement of their kind. This is the reason that urges study into mechanism of non-climacteric ripening.

MicroRNA (miRNAs) are a new class of gene regulators whose mode of action is through gene silencing. As gene silencers, miRNAs suppress the translational process of proteins coding mRNA transcripts through translational repression or cleavage (Dugas and Bartel, 2004). As a result, proteins coded by these mRNAs are silenced for good and later, when needed, miRNAs will be downregulated through their own mechanism and the target transcripts will be released for translation. Therefore up- and downregulation of miRNAs regulates the expression of specific proteins, leading to phenotypic changes in plants.

MiRNAs are commonly classified as being conserved or non-conserved. Conserved miRNAs are found in most plant species and are functionally proven to play the same common, yet major role in plant development (Jung *et al*., 2009). On the other hand, the less conserved miRNAs, as they are more sample-specific (species, tissues, organs, development stage), play more specific roles in the particular samples in which they are found. Therefore, identification of both

miRNAs is crucial in providing a full picture of the developmental program (common and specific programs) in an organism.

One of the ways to identify and discover miRNAs is through the direct cloning method. This method tends to miss non-conserved miRNAs because of low expression in samples in which they may not play a significant role. However, through the use of high-throughput sequencing, both conserved and non-conserved miRNA will be captured even to the extend of miRNAs with as low as one copy number (Yao *et al*., 2007). The availability of mature miRNA sequences and plant ESTs sequences in the miRNA registry (miRBase) and EST database (NCBI) make comparative genomics possible in identifying miRNAs and their target mRNA transcripts. The bioinformatics algorithm and tools available also provide a fast track for these processes.

Since miRNAs are highly conserved in the plant kingdom and they regulate plant development through differential expression at different developmental stages (Song *et al*., 2010), this study seeks to investigate the roles of miRNAs in the ripening process of pineapples. It is hypothesized that microRNAs are present in pineapple and are differentially expressed in different stages of fruit ripening. In order to prove this hypothesis, a set of objectives will be undertaken. These are stated as given below:

1. To detect for the presence of miRNAs in pineapple through the amplification of orthologous miRNAs by using stem-loop transcription polymerase chain reaction (RT-PCR).
2. To isolate and characterize miRNAs present in pineapple from libraries (pre-ripening fruit and pre-ripening leaf) of small RNAs derived from high-throughput sequencing.
3. To identify the target mRNA transcripts and proteins of pineapple miRNAs through *in silico* analysis.
4. To determine the level of expression of pineapple miRNAs at two different developmental stages (pre-ripening and post-ripening).

CHAPTER 2

LITERATURE REVIEW

2.1 Pineapple

Ananas comosus commonly known as pineapple, might not be as widely consumed as other staple plant crops such as rice or maize, but for the fruit cropping and processing industry, pineapples are widely cultivated in most tropical and subtropical countries and pineapple ranks third in the world production of tropical fruit after bananas and citrus fruits (Botella and Smith, 2008).

Pineapple is a herbaceous perennial of Liliopsida (monocotyledon). Taxonomically it was simplified to one genus, Ananas, with two species of *A. comosus* (L.) Merr and *A. macrodontes* Morren. Among these two, *A. comosus* is widely used for cultivation and includes five botanical varieties including *comosus*, *ananassoides*, *paraguazensis*, *erectifolius*, and *bracteatus* (Carlier *et al*., 2012; Smith and Downs, 1979).

In terms of its industrial value, pineapple is cultivated mainly for fresh or canned fruit and juice, but in the world of research, the key to the non-climacteric ripening mechanism resides in pineapple genetics, as pineapple is the model of non-climacteric fruit.

2.1.1 Pineapple fruit development

Principally the growth of the pineapple plant is a cycle, where the plant materials (commonly used are the crown and shoot/sucker) are planted to produce a fruit, and the fruit will grow, carrying the plant materials with it for future production (D'Eeckenbrugge and Leal, 2003). After the maturation of the first fruit, the new shoot and crown, which are capable of producing another fruit, are developed. Figure 2.1 shows the growth of the pineapple with planting materials (crown and shoot).

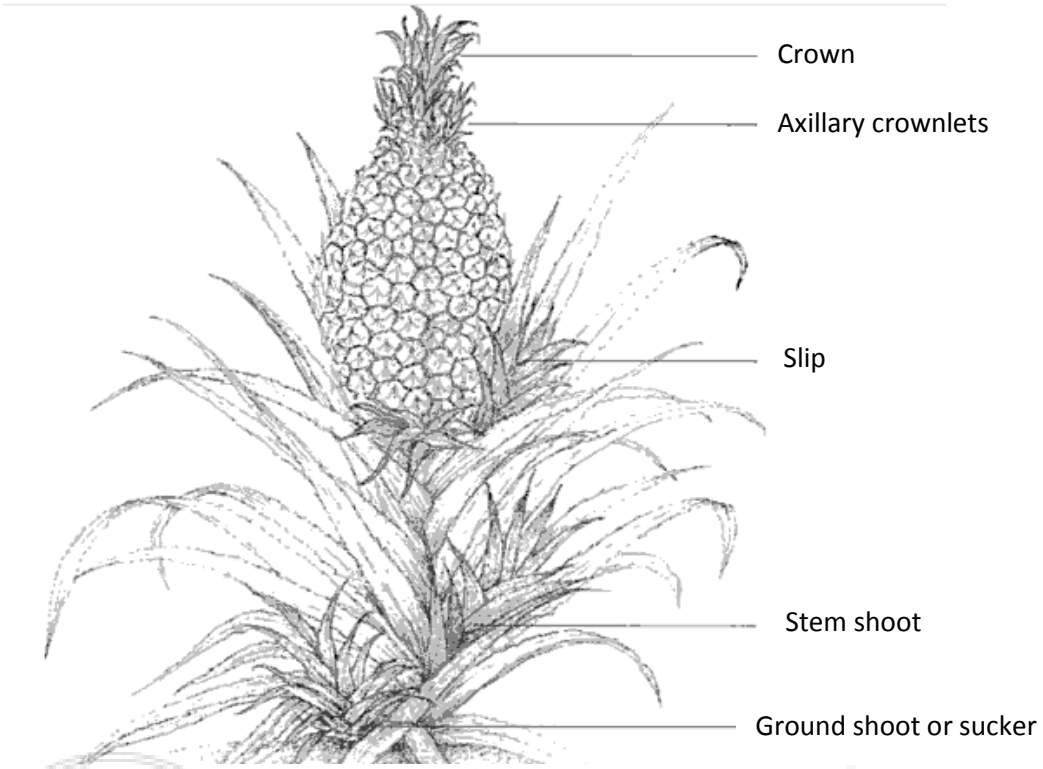

Figure 2.1: Pineapple plant materials. Picture indicates the pineapple crown and shoot used as planting materials still attached to the plant (D'Eeckenbrugge and Leal, 2003).

The shoot apical meristem (SAM) located at the tip of the crown will actively divide and differentiate to develop peduncles and inflorescence (Paull and Duarte, 2011). The inflorescence emerges with a few reddish peduncle bracts at its base, thus the process is called the 'red heart'. The small purplish flower will then open from the inflorescence, carrying an ovary in it, and the peduncle elongates after flower formation (D'Eeckenbrugge and Leal, 2003). Figure 2.2 shows the inflorescence and the open flower of the pineapple. The maturation of the ovary will form a part of the fruit while the bract will widened and become fleshy (D'Eeckenbrugge and Leal, 2003; Paull and Duarte, 2011). The inflorescence is capped by a crown composed of numerous shoot leaves on a shoot stem.

The essential elements and the concentration of a nutrient in plant tissue is necessary to provide good growth. Principally, the concentration of nutrients in plant tissue is parallel with its requirement for growth. According to Salisbury and Ross (1992), the concentration of nutrient in plant tissue throughout plant growth