TEMPORAL EXPRESSION OF MICRORNAS ON DIFFERENT STAGES OF PINEAPPLE (*Ananas comosus* var. *comosus*) FRUIT DEVELOPMENT

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ABSTRACT

MicroRNAs are a class of small non-coding RNA that play an important role in gene regulation either by cleaving or repressing the translational process of mRNA. This study focuses on the temporal expression of three miRNAs, namely miR156, miR159, and miR535, on different developmental stages of the pineapple fruit. Total RNA was extracted from six different developmental stages of pineapple (week 0, week 4, week 12, week 16 and week 20). Subsequently, the miRNAs were amplified using stem-loop mediated RT-PCR and the level of microRNAs gene expression was quantified using RT-qPCR. The C_t values were analyzed by using the $2^{-\Delta\Delta Ct}$ method and the expression pattern of miRNAs was examined. The result shows that the expression pattern of miR156 and miR535 fluctuates during maturation with, both miRNAs being highly expressed in early development stages and downregulated during ripening. On the other hand the expression of miR159 gradually declines upon the onset of ripening. This pattern is in agreement with the hypothetical target genes of the three miRNAs where miR156 targets *Squamosa Binding Protein*, miR159 targeting *GAMYB-like* genes, miR535 is predicted to target xyloglucan endotransglucoxylase. The temporal expression pattern of these miRNAs revealed their importance during the pineapple fruit development.

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LIST OF ABBREVATIONS AND SYMBOLS

°	Degree
°C	Degree Celcius
µg	Microgram
µL	Microlitre
nt	Nucleotide
ddH_2O	Double Distilled Water
RNA	Ribonucleic acid
miRNA	MicroRNA
RNase	Ribonuclease
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotides-triphosphate
EDTA	Ethylenediaminetetraacetic acid
SDS	Sodium dodecyl sulphate
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
$MgCl_2$	Magnesium Chloride
TBE	Tris/Borate/EDTA buffer
TAE	Tris/Acetate/EDTA buffer
Tris-HCl	Tris-hydrochloride
DTT	Dithiotheritol
g	Gram
M	Molar
mM	Milimolar

rpm	Rotation per minute
PCR	Polymerase Chain Reaction
RT-PCR	Reverse-Transcription Polymerase Chain Reaction
RT-qPCR	Real-Time Quantative Polymerase Chain Reaction
min	Minute
sec	Second
V	Volt
X	Times
C_T	Threshold cycle

CHAPTER 1

INTRODUCTION

1.1 Background

Pineapple is the third most important tropical fruit in world production after banana and citrus (Bartholomew *et al.*, 2003). Pineapple belongs to the order *Bromeliales*, family *Bromeliaciae*, subfamily *Bromelioideae* with 2,794 species among 56 genera (Coppens d'Eeckenbrugge & Leal, 2003; Luther & Sieff, 1998). Among the *Bromeliaceae*, pineapple is the most important economic plant (Coppens d'Eeckenbrugge & Leal, 2003).

Pineapple was discovered by Christopher Columbus in 1493 when he landed on an island in the Lesser Antilles of the West Indies (Collins, 1960; Gene Technology Regulator, 2003). From South America, the pineapples have been brought and planted all around the world over a wide range of latitudes from approximately 30°N in the northern hemisphere (Hayes, 1960; Gene Technology Regulator, 2003) and 28°30' in the Canary Islands (Galan Sauco *et al.*, 1988; Gene Technology Regulator, 2003) to 33°58'S in South Africa (Bartholomew and Kadzimin, 1977; Gene Technology Regulator, 2003).

According to the Linnaeus taxonomical order, pineapple is differentiated into several species, which are *Ananas macrodonters, Ananas comosus, Ananas comosus* var. *bracteatus, Ananas comosus* var. *parguazensis, Ananas comosus* var. *comosus, Ananas comosus* var. *ananassoides* and *Ananas comosus* var. *erectifolius* (Coppens

d'Eeckenbrugge and Leal, 2003). In Malaysia, several varieties of pineapple have been planted such us Maspine (Spanish), Sarawak (Smooth Cayenne), Moris (Queen), Hybrid (Spanish) and Josapine (Spanish) (Malaysia Pineapple Industry Board).

The data of pineapple world production in 2010 shows that Philippines was the most productive country in producing pineapple with 2,169,230 MT production followed by Brazil (2,120,030 MT), Costa Rica (1,976,760 MT), Thailand (1,924,660 MT) and China (1,519,072 MT) (Statistical Data Base, FAO, 2010). In 2010, Malaysia was ranked 11th in pineapple world production with 416,070 MT production. According to the Malaysian Pineapple Board, the pineapple production in Malaysia decreased approximately 24% from total production in 2010.

Most of the harvested pineapples are processed to be canned which the total production from 1983 to 1992 passing one million tonnes (Rohrbach *et al.*, 2003; Gene Technology Regulator, 2003) and Malaysia has become one of the most productive country for canned pineapple production. Beside canned fruits, pineapple is been as a source of bromelain in pharmaceautical market (Gene Technology Regulator, 2003).

Looking at the world ranking for pineapple production, Malaysia needs to put more effort on it. Not only the diseases and pests can lower down the production of the pineapple but the fact that pineapple is one of non-climacteric fruit also another reason of giving lost on the pineapple production. Being non-climacteric fruit means there is no sudden rise in ethylene upon ripen and these kinds of fruits do not change significantly after harvest. They will soften a little, lose green color and develop rots when they come old but unfortunately they do not change to improve their eating characteristic (Jobling, 2000). Thus, it is very difficult to farmer in Malaysia to judge when the right time to harvest the pineapple since size and color changes are not fully reliable indicators (Gene Technology Regulator, 2003).

In order to increase the production of pineapple, the further research on pineapple fruit ripening is needed. It is still lack of information on non-climacteric fruit ripening and development especially mediated by microRNAs. MicroRNAs (miRNAs) are 19-23 nucleotides long non-coding RNAs that controlling the gene expression at the post-transcriptional level, either by endonucleolytic cleavage or by transitional inhibition.

In the plants, microRNAs are responsible for plant development and their response to environmental stresses (Song *et al.*, 2010). The profiling of expression of miRNAs on different stages of pineapple fruit ripening may become fundamental mechanisms of non-climacteric fruit ripening mediated by microRNAs. Thus in the future, the ripening of non-climacteric fruit such as pineapple can be controlled and the quality of pineapple can be increased.

1.2 Objective of Research

The aim of this research is to study the expression of miRNAs on different stages of pineapple fruit ripening. The result from this experiment can serve as the fundamental knowledge of pineapple fruit ripening. The objectives of this research are :

1. To amplify the microRNAs on six different pineapple fruit stages through the use of stem-loop RT-PCR.
2. To profile the expression of microRNAs on six different stages of pineapple fruit ripening using Real Time-qPCR.
3. To determine the expression pattern of microRNAs on six different stages of pineapple fruit ripening.

CHAPTER 2

LITERATURE REVIEW

2.1 Taxonomy and Morphology of The Pineapple

The pineapple is a vegetative crop and is a common food in the diet of the people in Orinoco, Amazon, Coastal Brazil around Rio de Janeiro and the Carribean (Rohrbach *et al.*, 2003). As a native fruit of South America, mostly the people of South America called pineapple as 'ananas' or 'nanas' (Rohrbach *et al.*, 2003). The pineapple itself is the member of family of *Bromeliaceae.*

Basically, *Bromeliaceae* is divided into three subfamilies, which are *Picarnoideae*, the *Tillandsoioideae* and the *Bromelioideae*. The *Pitcarnioideae* can be found in terrestrial with the characteristic of armed leaf margins, hypogenous or epigenous flowers and dry dehiscent capsules containing naked or appendaged seeds adapted to wind dispersal (Ranker *et al.*, 1990; Terry *et al.*, 1997). The subfamilies *Tillandsioideae* and *Bromelioideae* are considered as monophyletic with sooth leaf margins, flowers usually hypogenous and dry dehiscent capsules containing many plumose seeds adapted to wind dispersal (Coppens d'Eeckenbrugge & Leal, 2003).

Unlike other monocots, the *Bromeliaceae* is very unique due to the present of stellate or scale-like multicellular hairs and unusual conduplicate, spiral stigmas (Gilmartin & Brown, 1987). Moreover, they are being recognized by the present of short stem, a rosette of narrow stif leaves, terminal inflorescence in the form of racemes or

panicles, hermaphroditic and actinomorpic trimerous flowers with well-differentiated calyx and corolla, six stamens and superior to inferior trilocular ovary, with axile placentation and numerous ovules (Coppens d'Eeckenbrugge & Leal, 2003).

The fruit of *Bromeliaceae* are capsules or berries and contain small naked, plumose seeds, with reduce of endosperm and small embryo (Coppens d'Eeckenbrugge & Leal, 2003).

2.2 Pineapple Cultivars

The pineapple classification is regrouped into *Ananas* genus, characterized by the unique feature of their inflorescence, which is fused into a syncarp. Today, there are seven valid *Ananas* cultivars proposed by Smith and Downs (1979), respectively, *Ananas macrodontes*, *Ananas comosus*, *Ananas comosus* var. *bracteatus*, *Ananas comosus* var. *parguazensis*, *Ananas comosus* var. *comosus*, *Ananas comosus* var. *anasoides* and *Ananas comosus* var. *erectifolius*.

Ananas macrodontes it is native to coastal and southern Brazil, up to Pernambuco, and to the drainage of the Paraguay and Parana rivers, from southeastern Paraguay and north-eastern Argentina up to Mato Grosso (Coppens d'Eeckenbrugge *et al.*, 1997). *A. macrodontes* exhibit forest areas, under semidense shade and it is subjected to a rainy season (Coppens d'Eekenbrugge & Leal, 2003). *A. macrodontes* can be easily known by the lack of crown at the top of the syncarp. It reproduces vegetatively by stolons (Coppens d'Eekenbrugge & Leal, 2003). Unfortunately, today it is very hard to find *A. macrodontes* due to the loss of its habitat.

Ananas comosus is highly distributed on the east of Andes, from northern South America to northern Argentina and Paraguay (Coppens d'Eekenbrugge & Leal, 2003). Unlike *A. macrodontes*, *A. comosus* doest not reproduce by stolons but by axillary shoots. It can be easily recognized by the formation of inflorescence densely strobiliform

and usually crowned with one or several rosettes of foliaceous (Coppens d'Eeckenbrugge & Leal, 2003).

Ananas comosus var. *bracteatus* is distributed on the same area with *A. macrodontes*. It is cultivated for the purpose of living hedge, fibre and fruit juice (Baker & Collins, 1939; Duval *et al.*, 1997). The plant is characterized by wide and long leaves and produce big number of shoots (Coppens d'Eeckenburgge & Leal, 2003). The bright pink color of its long bracts made them to easily characterized.

Ananas comosus var. *parguazensis* is originally from Orinoco and Rio Negro basins, eastern Colombia and in the nor-eastern Amazon (Coppens d'Eeckenbrugge *et al.*, 1997). *A. comosus* var. *parguazensis* is quite similar with *Ananas comosus* var. *anasoides*, the retrorse orientation of some spines and a wider leaf giving the differences between two cultivars (Coppens d'Eeckenbrugge, 2003).

Ananas comosus var. *comosus* is native cultivar from Brazil. It can be easily distinguished by its large fruit, borne on a wide and strong peduncle (Coppens d'Eeckenbrugge, 2003). This cultivar the formation of seeds is very rare because of the present reduced fertility combined with self-incompability (Coppens d'Eeckenbrugge, 1993). Some of the cultivar is spiniy and some partially spiny such as 'Smoot Cayenne'.

Ananas comosus var. *anasoides* is easily found in all tropical areas of South America east of the Andes. Generally, this cultivar grows well in the savannahs or in low-shaded forest with limited water-holding capacity (Coppens d'Eeckenbrugge, 2003). This cultivar is the most common of wild pineapple and it shows highest genetic diversity among the botanical variety, thus it is posited that *A. comosus* var. *anasoides* is the origin of cultivated pineapple (Leal & Coppens d'Eeckbrugge, 1996; Coppens d'Eeckenbrugge *et al.*, 1997; Duval *et al.*, 1998). It is characterized by long spiny leaves, globular to cylindrical syncarp, seedy fruit with white or yellow pulp (Coppens d'Eeckenbrugge, 2003).

Ananas comosus var. *erectifolius* is originated from Guinanas, including Orinoco basin, and in the north of the Amazon basin (Leal & Amaya, 1991). This cultivar never be found in the wild. *A. comosus* var. *erectifolius* is quite the same with *A. comosus* var.

anasoides, but it can be distinguished by its smooth leaves and the absence of leaf margin (Coppens d'Eeckenbrugge, 2003).

2.3 Differences Between Climacteric and Non-Climacteric fruit

According to their respiration pattern, fruit is divided into two groups which are climacteric and non-climacteric fruit. Climacteric fruits face a distinct increase in respiratory rate which it is associated with elevated ethylene production (Hoffman & Yang, 1980; Azzolini *et al.*, 2005). On the other hand, non-climacteric fruits do not face the increasing in ethylene respiration, but undergo a gradual decline in respiration during ripening.

Table 2.1 A list of representative climacteric and non-climacteric fruit (Watkins, 2002)

Climacteric Fruits	Non-Climacteric Fruits
Apple (*Malus domestica*)	Asian pear (*Pyrus serotina*)
Apricot (*Prunus armeniaca*)	Cactus pear (*Opuntia amycleae*)
Avocado (*Persea americana*)	Carambola (*Averrhoa carambola*)
Banana (*Musa sapientum*)	Cashew (*Anacardium occidenta*)
Cherimoya (*Annona cherimola*)	Cherry (*Prunus avium*)
Corossol (*Annona muricata*)	Cucumber (*Cucumis sativus*)
Durian (*Durio zibethinus*)	Grape (*Vitis vinifera*)
Feijoa (*Feijoa sellowiana*)	Grapefruit (*Citrus grandis*)
Fig (*Fics carica*)	Lime (*Citrus aurantifolia*)
Guava (*Psidium guajava*)	Limon (*Citrus limonia*)
Kiwifruit (*Actinidia sinensis*)	Litchee (*Litchi sinensis*)
Mango (*Mangifera indica*)	Mandarin (*Citrus reticulata*)
Melon Cantaloup (*Cucumis melo*)	Mangoustan (*Garcinia mangostana*)
Passion fruit (*Passiflora edulis*)	Olive (*Olea europaea*)
Peach (*Pyrus persica*)	Orange (*Citrus sinensis*)

Pear (*Pyrus communis*)	Pepper (*Capsicum annuum*)
Persimmon (*Diospyros kaki*)	**Pineapple (*Ananas comosus*)**
Physalis (*Physalis peruviana*)	Pomegranate (*Punica granatum*)
Plum (*Prunus domestica*)	Rambutan (*Nephelium lappaceum*)
Sapota (*Manilkara achras*)	Raspberry (*Rubus idaeus*)
Tomato (*Solanum lycopersicum*)	Strawberry (*Fragaria* sp.)
	Tamarillo (*Cyphomandra betaccea*)
	Watermelon (*Citrullus lanatus*)

Moreover, climacteric and non-climacteric fruits are distinguished based on the production of autocatalytic synthesis, an auto-stimulated massive ethylene production (McMurchie *et al.*, 1972; Alexander & Grierson, 2002). For the non-climacteric fruits, they do not have the autocatalytic synthesis.

The ripening of climacteric fruit is initiated with the production of ethylene. The ethylene biosynthesis has been established by Hoffman & Yang (1984). The ethylene is synthesized from methionine via S-adenosyl-L-methionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC). There are two enzymes that involve in the ethylene biogenesis, ACC synthase (ACS) which converts SAM into ACC, and ACC oxidase (ACO), which converts ACC into ethylene.

As the result to the response of ripening, ethylene is being produced in climacteric fruit. Ethylene will activate ethylene-responsive genes that encode for putative regulatory proteins that involved in transduction pathways and transcriptional or post-transcriptional regulation indicate that ethylene control of ripening process by operating in a complex multilevel way (Bouzayen *et al.*, 2010). As described by Jones *et al.* (2002), the ethylene will transduce the cascade leads to the activation of *EIN3-Like* (*EIL*) genes, which activates primary target genes (ethylene-response factors, ERFs). ERFs in turn activate the expression of secondary ripening-related genes. This will result on the color changing, flavor changing, softening of the pericarp.

The color changing from unripe to ripe, involves a transition of chloroplasts into chromoplasts. The thylakoid membranes and chlorophyll pigments are broken down, and the new carotenoid pigment is accumulated in the plastids (Srivastava, 2002).

The sugar level also reaches the peak during ripening. It is due to the hydrolysis of starch. There several enzymes involves in the conversion of starch, which are sucrose phosphate synthase and acid invertase. These enzymes allow the hydrolysis of sucrose into fructose and glucose (Srivastava, 2002. There two types of sugar accumulation during ripening, gradual sugar accumulation and rapid sugar accumulation (Ezura & Hiwasa-Tanase, 2010). On gradual sugar accumulation, sugar is accumulated until the fruits reach the maturity stage. On the other hand, on the rapid sugar accumulation, the sugar is accumulated at later developmental stage of the fruit.

During the maturation, cell wall also being degraded. It involves various hydrolases such as expansins, xyloglucan endotransglucoxylases, endo-1,4-β-glucanase, and α-and β-galactosidases (Srivastava, 2002). These enzymes will work by disrupting the hemicellulose-cellulose network. Furthermore, enzymes that disrupt the pectin network of the cell wall also involve in the cell wall loosening such as polygalacturonases and pectin methylesterases (Srivastava, 2002).

The ripening process in the climacteric fruit was well established, on the other hand, the ripening process on the non-climacteric fruits has not been studied to the same extent as in the climacteric fruits (Srivasta, 2002). For non-climacteric fruit, ethylene is generally not required for fruit maturation and ripening (Ezura & Hiwasa-Tanase, 2010). Nevertheless, it has been reported by Purvis & Balmore (1981) that the exposure of non-climacterics fruit to exogenous ethylene leads to the chlorophyll degradation and carotenoid biosynthesis. Moreover, the treatment using ethylene antagonists 2,5-norbornmadiene and silver nitrate prevented the de-greening process (Glodschmidt *et al.*, 1993).

The mechanism of non-climacteric fruit ripening is not clear, but it is posited that it is associated with changes in the concentration of auxin, GA, and abscisic acid (ABA) (Ezura & Hiwasa-Tanase, 2010). It has been reported that in the grape, the ripening process was regulating by ABA (Inaba *et al.*, 1976

2.4 MicroRNAs in Plants

MicroRNAs (miRNAs) are 19-23 nucleotide long non-coding RNAs that involve in gene regulation at post-transcriptional level, by endonucleolytic cleavage or by translational inhibition (Song *et al.*, 2010). It was first found in *Caenorhabditis elegans* through forward genetic screens of the *lin*-4 and *let*-7 mutants (Lee *et al.*, 1993; Reinhart *et al.*, 2000). Due to the intensive study in genetic of various organisms, it has revealed that miRNAs are nearly universally present and major key component of various gene regulatory pathways in higher eukaryotes (Jung *et al.*, 2009).

The first plant miRNAs were reported in early 2002 (Llave *et al.*, 2002a; Park *et al.*, 2002; Reinhart *et al.*, 2002). Plant miRNAs have the same structure as animal miRNAs. They composed of 20-24 nt and are processed from longer precursor transcripts. Even though they are alike, there are some differences between plant and animal miRNAs. The precursor of plant miRNAs are ranging from 50 nt to longer than 350 nt, while animal miRNAs precursor usually have a length of 70-80 nt (Jung *et al.*, 2009). Moreover, plant *MIRNA* genes located in the intergenic regions, on the other hand, animal *MIRNA* genes tend to locate in the intragenic regions (Jung *et al.*, 2009). Lastly, animal *MIRNA* genes very often being clustered within a single precursor, while plant miRNAs are derived from individual precursor RNAs (Bartel, 2004; Kim, 2005; Bonnet *et al.*, 2006; Vaucheret, 2006)

Generally, the miRNAs involve in regulate plant growth, metabolism and control the response to stresses (Allen *et al.*, 2005; Chen, 2005; Berzikov *et al.*, 2006; Carthew *et al.*, 2009; Song *et al.*, 2010). It also has been reported miRNAs play role in fruit ripening in tomato, pineapple, and grape (Moxon *et al.*, 2008; Zuo *et al.*, 2011, Xu *et al.*, 2010; Carra *et al.*, 2009; Yusuf & Kumar, 2011). Since miRNA is highly conserved within kingdom (Arazi *et al.*, 2005), is suggested that the miRNA may control the expression of the same gene within kingdom.

2.4.1 Structure and Biogenesis

The miRNAs are easily recognized by its structure. As describe by Krol *et al.* (2004), structure of miRNAs consist of hairpins, secondary structure as well as hairpin stems.

The biogenesis of miRNAs as described in fig. 1 by Kim & Nam (2006), the miRNAs are derived from the expression of miRNAs genes. Transcription of miRNA genes by RNA polymerase II generates long primary transcripts (pri-miRNAs) that contain local foldback structure. Then the stem-loop structure is cleaved by the nuclear RNase III Drosha to release the precursor of miRNA (pre-miRNA). Later, the pre-miRNA is exported from nucleus into the cytoplasm by Ran-dependent nuclear transport receptors, exportin-5 (Exp5). Exp5 will recognize the pre-miRNA by the 'minihelix motif' consists of a > 14-bp stem and a short 3' overhang. In the cytoplasm, pre-miRNA will be further processed by the cytoplasmic RNase III Dicer to miRNA duplexes. The duplex is separated and one strand is selected as mature miRNA and the other strand is degraded. Finally, the mature miRNAs will incorporated into the effector complexes, miRNA-containing RNA-induced silencing complex (miRISC). This complex helps miRNAs to bind into their complementary site on the target mRNA induced mRNA cleavage or inhibit the translational of mRNA.

2.4.2 MicroRNAs Function in Plant

In plants, miRNAs plays an important role in phase transition, floral development, shoot apical meristem (SAM) development, leaf development, vascular development, root development, stress responses and growth hormone signaling (Jung *et al.*, 2009).

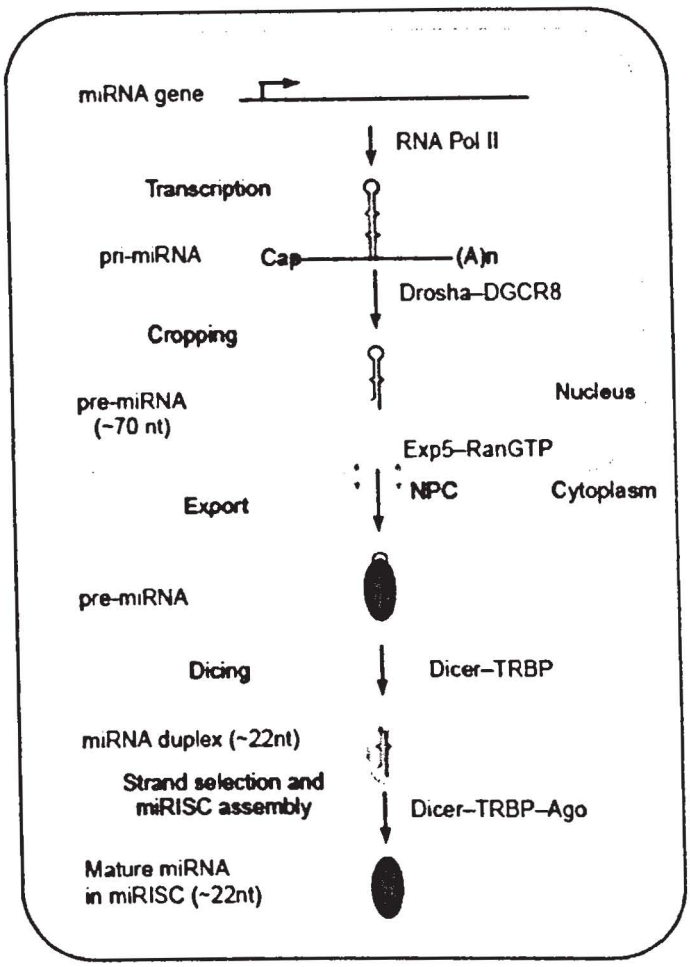

Figure 2.1 MicroRNAs Biogenesis (Kim & Nan, 2006). MiRNA genes are transcribed by an RNA polymerase II in order to generate the primary transcripts (pri-miRNAs). Then pri-miRNAs are cleavage by Drosha–DGCR8 to form pre-miRNA. Later pre-miRNAs will be recognised by Exportin-5 (Exp5) and transported form nucleus in cytoplasm. Upon export, the cytoplasmic RNase III Dicer participates in the second processing step (dicing) to produce miRNA duplexes. MiRNA duplex will be corporated with miRISC and the duplex is separated and usually one strand is selected as the mature miRNA, whereas the other strand is degraded. The miRNA-RISC complex then will be integrated into complementary mRNA to initiate mRNA cleavage or translation inhibition.

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