

**DEVELOPMENT OF PINEAPPLE TRANSGENIC  
LINES FOR THE FUNCTIONAL PROFILING OF  
MIR535 GENE FAMILY THROUGH THE USE  
OF ARTIFICIAL MICRORNA TECHNOLOGY**

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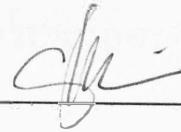
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## ABSTRACT

Recently, artificial microRNA (amiRNA) technology has been widely used as a tool for creating loss-of-function mutants, especially in studies involving functional profiling, as it is able to silence genes or gene families in a specific manner. Artificial microRNA is derived by replacing the native mature miRNA duplex from an endogenous precursor miRNA (pre-miRNA) with synthetic ones. Like mature miRNA, amiRNA is designed with an ability to bind complementarily to its target gene. The aim of the study was to develop amiRNAs using different backbones, which can then be used to silence the endogenous pineapple microRNA *MIR535* family. In order to find the efficient amiRNA silencing in pineapple, stems of precursors were modified, as this will affect their processing efficiency by endogenous miRNA biogenesis. And, in order to silence the *MIR535* family, amiRNA was designed with the ability to bind to this mature region, as this increase the probability of it targeting more than one miR535 member. The amiRNAs were developed from newly discovered pre-miRNA from pineapple, and previously identified ones from *Arabidopsis thaliana* and *Oryza sativa*. The first step involved the identification of the pre-miRNAs from pineapple transcriptomic libraries through *in silico* analysis. The amiRNAs were then designed to target the *MIR535* family, and subsequently inserted into precursors, and synthesized. The sequences of the expression cassette (promoter, enhancer, and terminator) were then fused into it, before transforming it into the plant expression vector, pCambia1303. Transgenes were then inserted into pineapple callus (MD2 hybrid) through *Agrobacterium* mediated transformation. Transgenic lines developed were used for expression profiling of amiRNAs and miR535's through stem-loop RT-qPCR. Three precursors found from pineapple (pre-miR156, pre-miR399, pre-miR2673) were modified (to have 20nt and 50nt stem) and used to carry amiRNA, together with the precursors of *A. thaliana* (pre-miR319) and *O. sativa* (pre-miR528). Here, transgenic lines which have been inserted with these precursors showed the presence of amiRNA. Two pineapple precursors were found to be highly efficient in expressing amiRNA i.e. pre-miR156-50nt stem (Cq value of 20), followed by pre-miR2673 (Cq value of 24.4). The precursors from *A. thaliana* and *O. sativa* were also found to be functional in pineapple, each with the Cq values of 20.8 and 23.8, respectively. Next, the ability of this amiRNA to silence the target gene (mature miR535b) was observed. In conjunction with the expression of amiRNA in transgenic callus, the expressions of target gene was found downregulated, with the highest silencing rate was by amiRNA produced from pre-miR156-50nt and pre-miR319. Also, the expressions of other mature miR535's were also quantified, and were found downregulated. In conclusion, the amiRNA technology was successfully developed for pineapple evidenced by the creation of loss-of-function mutant in the *MIR535* family. The pineapple endogenous precursor was found capable to serve as backbone for amiRNA technology in pineapple. This study suggests that targeting 'common region' when designing amiRNA results in the silencing of several genes of the same family at the same time. Now, two highly efficient amiRNA precursors, pre-miR156 and pre-miR319 can be utilized in gene silencing- programs in pineapple.

## ABSTRAK

### **PENGHASILAN TANAMAN TRANSGENIC UNTUK PEMPROFILAN FUNGSI GEN DALAM KELUARGA MIR535 DENGAN MENGGUNAKAN TEKNOLOGI 'ARTIFICIAL MICRORNA'**

Teknologi 'artificial microRNA' (amiRNA) telah digunakan secara meluas untuk menghasilkan tanaman mutan, terutamanya bagi kajian melibatkan pemprofilan fungsi gen, kerana dapat menghalang ekspresi sesuatu gen atau kesemua gen dalam keluarga yang sama. AmiRNA dihasilkan dengan menggantikan jujukan asal miRNA matang di dalam prekursor miRNA (pre-miRNA) dengan jujukan sintetik. Jujukan sintetik ini menyerupai jujukan miRNA asal, dimana ia direka supaya mempunyai keupayaan melekat pada gen yang disasarkan. Kajian ini dijalankan dengan tujuan membangunkan teknologi amiRNA di dalam nanas, dengan menggunakan prekursor daripada nanas, *A. thaliana*, dan *O. sativa*. AmiRNA ini mensasarkan untuk menghalang ekspresi gen di dalam keluarga 'microRNA' *MIR535*, yang diwakili oleh lebih daripada 50 gen (miRBase, keluaran 21.0). Sebagai miRNA, miR535 ini mempunyai jujukan yang berbeza di bahagian 'stem', tetapi berkongsi jujukan yang hampir sama di 'mature' miRNA. Untuk menghalang ekspresi gen dalam keluarga *MIR535*, amiRNA telah direka dengan keupayaan melekat pada bahagian 'mature' miRNA, kerana ini menambahkan kebarangkalian amiRNA ini untuk melekat pada lebih daripada satu miR535. Kajian ini telah dimulakan dengan mengenalpasti pre-miRNA dari perpustakaan transkrip nanas melalui analisis berkomputer. AmiRNA kemudiannya direka mensasarkan *MIR535*, dimasukkan ke dalam prekursor, dan disintesis. Jujukan 'promoter', 'enhancer', dan 'terminator' digabungkan bersamanya, sebelum dimasukkan ke dalam vektor, pCambia1303. Gen ini kemudiannya dimasukkan ke dalam kalus nanas (MD2 hibrid) melalui transformasi menggunakan *Agrobacterium tumefaciens*. Tanaman transgenik yang terhasil digunakan untuk memprofil ekspresi amiRNAs dan miR535 melalui 'stem-loop' RT-qPCR. Tiga prekursor (pre-miR156, pre-miR399, pre-miR2673) digunakan untuk membawa amiRNA, bersama-sama dengan prekursor oleh *A. thaliana* (pre-miR319) dan *O. sativa* (pre-miR528). Tanaman transgenik yang telah dimasukkan dengan prekursor ini telah menunjukkan kehadiran amiRNA apabila dianalisis dengan q-PCR. Dua prekursor nanas didapati sangat berkesan dalam mengekspresikan amiRNA iaitu pre-miR156 (nilai Cq pada paras 20), diikuti oleh pre-miR2673 (nilai Cq pada paras 24.4). Prekursor dari *A. thaliana* dan *O. sativa* pula telah didapati sangat berkesan dalam nanas, masing-masing dengan nilai Cq pada paras 20.8 dan 23.8. Seterusnya, keupayaan amiRNA untuk menghalang ekspresi gen sasaran (miR535) diperhatikan. Dengan kehadiran amiRNA dalam tanaman transgenik, ekspresi gen sasaran telah menurun, dengan kadar tertinggi adalah dari pra-miR156 dan pra-miR319. Selain itu, ekspresi miR535 yang lain juga diprofil, dan kadar ekspresinya juga didapati menurun. Ini sekali gus menunjukkan bahawa teknologi amiRNA telah berjaya dibangunkan untuk nanas yang telah menyebabkan penghasilan mutan yang ekspresi gen dalam keluarga *MIR535* terhalang. Kesimpulannya, prekursor daripada nanas didapati mampu untuk berkhidmat sebagai tulang belakang untuk teknologi amiRNA dalam nanas. Selain itu, kajian ini menunjukkan bahawa amiRNA yang direka dengan mensasarkan kawasan yang mempunyai tahap perkongsian jujukan yang tinggi dapat menghalang ekspresi beberapa gen dalam keluarga yang sama pada masa yang sama. Kini, dua prekursor yang sangat berkesan, pre-miR156 dan pre-miR319 boleh digunakan dalam program pemadaman gen di dalam nanas menggunakan teknologi amiRNA ini.

# TABLE OF CONTENTS

	Page
<b>TITLE</b>	i
<b>DECLARATION</b>	ii
<b>CERTIFICATION</b>	iii
<b>ACKNOWLEDGEMENT</b>	iv
<b>ABSTRACT</b>	v
<b>ABSTRAK</b>	vi
<b>LIST OF CONTENTS</b>	vii
<b>LIST OF TABLES</b>	xii
<b>LIST OF FIGURES</b>	xiv
<b>LIST OF ABBREVIATIONS AND SYMBOLS</b>	xviii
<b>LIST OF APPENDIX</b>	xxi
<b>CHAPTER 1: INTRODUCTION</b>	
1.1 Research Background	1
1.2 Problem Statement	2
1.3 Research Hypotheses	3
1.4 Research Objectives	3
<b>CHAPTER 2: LITERATURE REVIEW</b>	
2.1 MicroRNA	5
2.1.1 Overview of MiRNA Biogenesis	5
2.1.2 Accuracy of MiRNA Processing by DCL1	8
2.1.3 The Accuracy of Gene Silencing by MiRNA and AGO1	13
2.2 The MiRNA in Pineapple	15
2.2.1 The Expression and Function of <i>MIR535</i> in Plants	15
2.3 Genetic Tools Utilizing MiRNA Theory	17
2.3.1 MiRNA Mimic (MIMIC)	17
2.3.2 Anti-miRNA (AntimiR)	19



2.3.3	Target Mask (MASK)	19
2.3.4	Target Sponge (SPONGE)	20
2.3.5	Artificial MicroRNA (amiRNA)	20
2.4	The Engineering of AmiRNA for Custom Gene Silencing	21
2.4.1	Silencing Efficiency of AmiRNA (Backbone Design)	21
2.4.2	Silencing Efficiency of AmiRNA (The Design of AmiRNA)	28
2.5	The AmiRNA Technology for Gene Silencing in Pineapple	29

### CHAPTER 3 : MATERIAL AND METHODS

3.1	Introduction	31
3.2	Sampling of Plant Materials	31
3.3	Isolation of Total RNA	32
3.4	Purification of RNA Enrichment of Small RNAs	33
3.5	The Expression of the MiR535 in MD2 Pineapple Leaves and Callus using Stem-loop RT-PCR	34
3.5.1	Amplification of MiR535 through Stem-loop RT-PCR	34
3.5.2	The Cloning of Amplicons Amplified	35
3.5.3	Sequencing of MiR535	37
3.5.4	Sequence Analyses	38
3.6	<i>In silico</i> Identification of Endogenous Pre-miRNA from Pineapple and their Presence in Pineapple by PCR	38
3.6.1	<i>In silico</i> Identification of Pre-miRNA from the Pineapple Transcriptomic Library	39
3.6.2	DNA Extraction for Amplification of Pre-miRNA Genes in Pineapple	41
3.6.3	Amplification of Pre-miRNA Genes through PCR Reaction	42
3.7	The Design of Pre-amiRNA Targeting the <i>MIR535</i> Family in Pineapple	42
3.7.1	The Selection and Modification of Pre-miRNA as a Backbone	43
3.7.2	The Design of AmiRNA Targeting for MiR535b, MiR535m, and MiR535s	43
3.7.3	The Design of AmiRNA* for each AmiRNA	45
3.7.4	Insertion of the AmiRNA:amiRNA* Duplex into a Precursor backbone	45
3.8	The Construction of Pre-amiRNA Expression Vector	45
3.8.1	Synthesis of Pre-amiRNA	45
3.8.2	Construction of Pre-amiRNA Expression Cassette	48
3.8.3	Construction of Pre-amiRNA Expression Vector	48

3.9	Transformation of Pre-amiRNA Expression Vector into <i>A. tumefaciens</i>	50
3.10	The Development of MD2 Pineapple Tissue Culture System	52
3.10.1	Planting of Plant Materials	53
3.10.2	Media Preparation, Explant Sterilization, and Culturing of Explants	53
3.10.3	Observation and Data Analyses	57
3.11	The Optimization of the <i>A. tumefaciens</i> Transformation Protocol for MD2 Pineapple	57
3.12	Development of a Pineapple Transgenic Line Transformed with Pre-amiRNA Expression Vector	59
3.12.1	Sample Preparation	59
3.12.2	Preparation of the Suspension Culture ( <i>A. tumefaciens</i> carrying Pre-amiRNA Expression Vector)	59
3.12.3	Transfection and Co-cultivation of MD2 Pineapple Callus with Suspension Culture	60
3.12.4	'Resting' of Callus Transfected with <i>A. tumefaciens</i> carrying Pre-amiRNA Expression Vector	61
3.12.5	Antibiotic Selection of Putative Transgenic Callus	61
3.13	Expression Profiling of AmiRNAs and Three Members of MiR535 in Transgenic Lines using Stem-loop RT-qPCR	62
3.13.1	Amplification of AmiRNAs and their Target Genes through Stem-loop RT-PCR	63
3.13.2	Quantitative Real-time PCR (q-PCR) of AmiRNA and Three Members of MiR535 in Pineapple Transgenic Line	63
3.13.3	Analyses of Q-PCR Data	64

## CHAPTER 4: RESULTS

4.1	Sampling and Planting of Plant Materials	66
4.2	Isolation of Total RNA	66
4.3	Isolation of Genomic DNA	66
4.4	The Amplification of Three <i>MIR535</i> from MD2 Pineapple Leaves and Callus Stem-loop RT-PCR	68
4.5	<i>In silico</i> Identification of Pre-miRNA from Pineapple mRNA Transcripts and their Presence in Pineapple by PCR	74
4.6	The Amplification of Four Newly found Pineapple Pre-miRNA Genes	84
4.7	The Selection of Pre-miRNA to Serve as Backbone for AmiRNA Technology in MD2 Pineapple	84

4.8	The Design of AmiRNA Targeted to three <i>MIR535</i> in MD2 Pineapple	86
4.9	The Design of Specific AmiRNA* for each AmiRNA and Backbone	86
4.10	The Insertion of AmiRNA:amiRNA* Duplex into Precursor Backbone	92
4.11	The Construction of Pre-amiRNA Expression Cassette	92
4.12	The Construction of Pre-amiRNA Expression Vector	120
4.13	Transformation of Pre-amiRNA Expression Vector into <i>A. tumefaciens</i>	120
4.14	The Development of MD2 Pineapple Tissue Culture System (Preliminary Screening)	126
4.15	Callus Induction, Shoot Regeneration, Shoot Proliferation, and Rooting of MD2 Pineapple	128
4.16	The Efficient <i>A. tumefaciens</i> Transformation Protocol for MD2 Pineapple	134
4.17	Transformation of Pre-amiRNA Expression Vector into MD2 Pineapple through <i>A. tumefaciens</i> Mediated Transformation and Antibiotic Selection of Putative Transgenic Lines	137
4.18	The Amplification of AmiRNA from Transgenic Line through Stem-loop RT-PCR	139
4.19	The Amplification of MiR535 Targeted by AmiRNA on Transgenic Lines through Stem-loop RT-PCR	141
4.20	The Expression of AmiRNA by Different Precursor Backbones Quantified through Real-time Quantitative PCR (Q-PCR)	147
4.21	The Expression of MiR535 Targeted by AmiRNA on Transgenic Line Quantified through Real-time Quantitative PCR (Q-PCR)	152
4.22	The Expression of Non-target miR535 on Transgenic Line Quantified through Real-time Quantitative PCR (Q-PCR)	156

## **CHAPTER 5: DISCUSSION**

5.1	The Expression of Three MiR535 in MD2 Pineapple	163
5.2	The Pre-MiRNA of Pineapple	163
5.3	The Micropropagation of MD2 Pineapple	165

5.4	The Pineapple Transgenic Lines	166
5.5	The Expression of AmiRNA in MD2 Pineapple Transgenic Lines	168
5.5.1	The Efficiency of Pineapple Pre-miR156 and Pre-miR399 Backbones in Expressing AmiRNA	168
5.5.2	The Efficiency of Pineapple Pre-miR2673 Backbones in Expressing AmiRNA	170
5.5.3	The Efficiency of Pre-miR528 of <i>O. Sativa</i> and Pre-miR319 of <i>A. thaliana</i> Backbones in Pineapple	171
5.6	Gene Silencing by AmiRNA in MD2 Pineapple Transgenic Lines	174
5.6.1	Silencing of MiR535m by AmiR535m Produced from Pre-miR156 (50 nt stem) and Pre-miR399 (50 nt stem) Backbones	174
5.6.2	Silencing of MiR535b by AmiR535b Produced from Pre-miR156 (20nt stem), Pre-miR399 (20 nt stem), and Pre-miR319 Backbones	175
5.6.3	Silencing of MiR535m by AmiR535b Produced from Pre-miR2673 Backbone	176
5.6.4	Silencing of MiR535b and MiR535m by AmiRNA Produced from Pre-miR528 Backbone	177
5.7	The Silencing of <i>MIR535</i> Family in Pineapple by AmiRNA	178
5.8	The Non-expressed AmiR535s in Pineapple	181
5.9	Research Potential	182
5.10	Research Limitations	184
<b>CHAPTER 6: CONCLUSION</b>		186
<b>REFERENCES</b>		189
<b>APPENDIX</b>		199 - 208

## LIST OF TABLES

	Page
Table 2.1 : List of plants that reported the expression of <i>MIR535</i> , number of member, and its predicted target	16
Table 2.2 : Comparison between five different techniques manipulating the gene silencing pathway by miRNA	22
Table 2.3 : List of amiRNA technology utilized in various plant species and the name of the pre-miRNA used as backbone	25
Table 3.1 : The samples used in the four major parts of the research	32
Table 3.2 : The sequences of the forward and reverse primers used for colony PCR	36
Table 3.3 : Primer set for colony PCR for selection of <i>E. coli</i> transformants inserted with pRI201-ON vector	50
Table 3.4 : Primer set for colony PCR for selection of <i>E. Coli</i> transformants inserted with pCambia1303 vector	50
Table 3.5 : Media for the development of a MD2 pineapple tissue culture system	54
Table 3.6 : Treatments for the optimisation of <i>A. tumefaciens</i> transformation in MD2 pineapple	59
Table 3.7 : The Gus Activity Index (GAI) scoring for transient expression of transgenes on putative transgenic lines	59
Table 3.8 : The list of transgenic line and experimental control	61
Table 3.9 : The selection of pineapple transgenic lines	62
Table 3.10 : The sequences of the forward and reverse primers used for amplification of <i>β-actin</i>	64
Table 4.1 : List of primer designed and used for amplification of three <i>MIR535</i> in pineapple using stem-loop RT-PCR	71
Table 4.2 : The <i>in-silico</i> identification of putative pre-miRNA from pineapple	74
Table 4.3 : Four putative pre-miRNAs identified from pineapple	83

Table 4.4	:	List of primer used for PCR amplification of four pineapple pre-miRNAs	85
Table 4.5	:	The list of pre-miRNA used as backbone for development of amiRNA technology in MD2 pineapple	85
Table 4.6	:	List of sequence for 21 pre-amiRNAs designed from seven pre-miRNA backbones and targeting for three <i>MIR53</i>	86
Table 4.7	:	List of product size for colony PCR and RE digestion reaction of pre-amiRNA inserted into pRI201-ON vector and pCambia303 vector	116
Table 4.8	:	Preliminary screening of 49 media for micropropagation of MD2 pineapple	127
Table 4.9	:	Medium and conditions for callus induction of MD2 pineapple	130
Table 4.10	:	Media and condition for shoot regeneration of MD2 pineapple	131
Table 4.11	:	Media and condition for callus differentiation of MD2 pineapple	131
Table 4.12	:	Media and condition for shoot proliferation of MD2 pineapple	133
Table 4.13	:	Media and condition for rooting of MD2 pineapple plantlets	135
Table 4.14	:	Optimization of <i>Agrobacterium</i> transformation conditions for MD2 pineapple	136
Table 4.15	:	The hygromycin concentration for selection of transgenic pineapple lines against non-transformed callus	138
Table 4.16	:	The number of callus survived after eight series of hygromycin selection during the development of transgenic lines	140
Table 4.17	:	List of primer used for amplification of three amiRNA in pineapple transgenic lines using stem-loop RT-PCR	141
Table 4.18	:	The number of callus survived after series of hygromycin and molecular selection	142
Table 4.19	:	The expression of target gene (miR535b ad miR35m) in pineapple transgenic lines obtained from stem-loop RT qPCR	154

## LIST OF FIGURES

	Page
Figure 2.1 : Schematic pathway representing the biogenesis of miRNA in plants	6
Figure 2.2 : Schematic of the components of DCL1 and the cleavage of dsRNA	9
Figure 2.3 : The size detection of pre-miRNA by the PAZ and helicase domains of DCL1	11
Figure 2.4 : Positions of DCL1 cleavage sites in pri-miR171a and pre-miR167a	12
Figure 2.5 : Binding of miRNA on three domains of AGO1 protein for cleavage of the target transcript	14
Figure 2.6 : Overview of the five techniques based on the concept of gene silencing by endogenous miRNA	18
Figure 2.7 : Overview of the engineering of amiRNA for custom gene silencing	23
Figure 2.8 : The effect of different stem lengths of backbones on their efficiency to produce amiRNA	26
Figure 2.9 : The chimeric precursor and the production of amiRNA in plants	27
Figure 2.10 : The effect on <i>MIRNA (MIR164)</i> gene silencing of targeting different regions associated with the stem, mature and terminal loop regions by amiRNA	30
Figure 3.1 : The bioinformatics pathway for the identification of pre-amiRNA from pineapple transcriptomic library	39
Figure 3.2 : Schematic diagram on the selection and modification of pre-miRNA as backbone	44
Figure 3.3 : Schematic diagram representing the design of amiRNA targeting for mature miR535	46
Figure 3.4 : Schematic diagram on the design of amiRNA*	46
Figure 3.5 : Schematic diagram on the insertion of amiRNA:amiRNA* duplex into precursor backbone	47

Figure 3.6	: Schematic diagram on the construction of pre-amiRNA expression cassette	49
Figure 3.7	: Schematic diagram on the construction of pre-amiRNA expression vector	51
Figure 3.8	: Shoot meristem used as an explant for micropropagation of MD2 pineapple tissue culture	56
Figure 4.1	: Sampling of plant materials (MD2 and Babagon variety)	67
Figure 4.2	: Photo of 1.2% gel electrophoresis of total RNA extracted from leaf and callus tissues for amplification of three members of miR535	68
Figure 4.3	: Photo of 1.2 % gel electrophoresis of total RNA extracted from transgenic calli for amplification of amiRNA and miR535	69
Figure 4.4	: Photo of 1.0 % gel electrophoresis of genomic DNA extracted from leaf of Babagon pineapple for amplification of four pre-miRNA genes	70
Figure 4.5	: Photo of 1.0 % gel electrophoresis of genomic DNA extracted from transgenic calli (eight transgenic lines) for amplification of the CaMV35S promoter	70
Figure 4.6	: The expression of <i>MIR535</i> (miR535b, miR53m, and miR35s) on leaf and callus of MD2 pineapple amplified through stem-loop RT-PCR	72
Figure 4.7	: Colony PCR of <i>MIR535</i> (miR535b, miR53m, and miR35s) on leaf and callus of MD2 pineapple, amplified through stem-loop RT-PCR	72
Figure 4.8	: Alignment of three <i>MIR535</i> sequences amplified from leaves and callus of MD2 pineapple	73
Figure 4.9	: Alignment of miRNA of four putative pre-miRNAs against plant miRNAs	76
Figure 4.10	: The secondary structure of putative pineapple pre-miRNA resides inside the pri-miRNA	77
Figure 4.11	: Structure analyses of putative pre-miRNA found in pineapple	81
Figure 4.12	: PCR amplification of pre-miRNAs in pineapple	85



Figure 4.13	: The design of amiRNA targeted to three <i>MIR535</i> (miR535b, miR535m, and miR535s) in pineapple	87
Figure 4.14	: The design and sequence of antisense strand of amiRNA (amiRNA*) for five pre-miRNAs (backbone)	89
Figure 4.15	: The structure of 21 pre-amiRNAs developed from seven different backbones, each inserted with three amiRNA:amiRNA* duplex targeted to three <i>mir535</i> (miR535b, miR535m, and miR535s)	93
Figure 4.16	: The overview on the development of pre-amiRNA expression cassette	115
Figure 4.17	: The construction of 21 pre-amiRNA expression cassettes	117
Figure 4.18	: The overview on the development of pre-amiRNA expression vector	121
Figure 4.19	: The construction of 21 pre-amiRNA expression vectors	122
Figure 4.20	: Overview of map of pre-amiRNA expression vector	125
Figure 4.21	: RE Digestion of pre-amiRNA expression vector from pre-amiRNA expression vector transformed into <i>A. tumefaciens</i>	126
Figure 4.22	: Callus of MD2 pineapple	130
Figure 4.23	: Shoot regeneration of MD2 pineapple	132
Figure 4.24	: Shoot proliferation of MD2 pineapple from callus	133
Figure 4.25	: Rooting of MD2 plantlet	135
Figure 4.26	: Picture of calli transformed with <i>A. tumefaciens</i> stained through GUS histochemical analysis	136
Figure 4.27	: Molecular selection of transgenic callus	137
Figure 4.28	: Pineapple transgenic lines	143
Figure 4.29	: Expression of amiRNA from pineapple transgenic lines	146
Figure 4.30	: Colony PCR of amiRNA on transgenic calli of MD2 pineapple	147
Figure 4.31	: Expression of target gene (miR535b or miR53m) from eight pineapple transgenic lines	148
Figure 4.32	: Quantification of amiRNA through q-PCR	149

Figure 4.33	:	Accumulation of amiRNA produced by different backbones in eight transgenic lines, quantified through stem-loop RT-qPCR	150
Figure 4.34	:	Expression of amiRNA produced from pre-miR156 and pre-miR399 backbones, both with different length at the stem region	151
Figure 4.35	:	The expression of amiRNA produced from five backbones of pineapple	153
Figure 4.36	:	Four efficient backbones with ability to produce high accumulation level of amiRNA in pineapple transgenic line	153
Figure 4.37	:	Fold changes and relative expression of amiR535m's target gene (miR535m) on transgenic lines quantified through stem-loop RT-PCR	155
Figure 4.38	:	Fold changes and relative expression of amiR535b's target gene (miR535b) on transgenic lines quantified through stem-loop RT-PCR	157
Figure 4.39	:	The expression of target gene on pineapple transgenic line with the highest and the lowest silencing effect	158
Figure 4.40	:	Expression of non-target miR535 in eight transgenic lines	160
Figure 4.41	:	The expression of target and non-target <i>MIR535</i> in two transgenic line with pre-miR399 (50nt stem) and pre-miR2673 backbone	162
Figure 5.1	:	The performance model of seven amiRNA backbones in expressing amiRNA. The performance model of silencing effect by amiRNA expressed from seven backbones	173
Figure 5.2	:	The performance model of silencing effect by amiRNA expressed from seven backbones	179
Figure 5.3	:	The performance model of pre-amiRNA constructs developed from seven precursor backbones	183

## LIST OF ABBREVIATIONS AND SYMBOLS

<b>µl</b>	Microliter
<b>µM</b>	Micromolar
<b>°C</b>	Degree Celsius
<b>%</b>	Percentage
<b>x g</b>	G-force
<b>amiRNA</b>	Artificial microRNA
<b>bp</b>	Base pair
<b>CaCl<sub>2</sub></b>	Calcium chloride
<b>cm</b>	Centimetre
<b>dH<sub>2</sub>O</b>	Water
<b>dsRNA</b>	Double-stranded RNA
<b>dsRBD</b>	Double-stranded RNA binding domain
<b>dsRBD</b>	Double-stranded RNA binding protein
<b>dNTP</b>	Deoxyribonucleotide triphosphate
<b>g</b>	Gram
<b>hr</b>	hour
<b>mg</b>	Miligram
<b>min</b>	Minute
<b>mL</b>	Millilitre
<b>mm</b>	Millimetre
<b>mM</b>	Millimolar
<b>miRNA</b>	MicroRNA
<b>miRNA*</b>	Antisense microRNA
<b>mRNA</b>	Messenger RNA
<b>MgCl<sub>2</sub></b>	Magnesium Chloride
<b>NaCl</b>	Sodium Chloride
<b>nm</b>	Nanometer
<b>nt</b>	Nucleotide
<b>Pre-miRNA</b>	Precursor microRNA
<b>Pri-miRNA</b>	Primary microRNA



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<b>q-PCR</b>	Quantitative polymerase chain reaction
<b>rpm</b>	Rotation per minute
<b>RNAi</b>	RNA interference
<b>siRNA</b>	Small interfering RNA
<b>sRNA</b>	Small RNA
<b>6-BA</b>	6-Benzylaminopurine
<b>A</b>	Adenine
<b>AGO</b>	Argonaute
<b>AMFE</b>	Adjusted minimal folding energy
<b>C</b>	Cytosine
<b>CI</b>	Callus induction
<b>CTAB</b>	Cetyltrimethyl Ammonium Bromide
<b>DCL</b>	Dicer like
<b>DEPC</b>	Diethylpyrocarbonate
<b>DTT</b>	Dithiothreitol
<b>EDTA</b>	Ethylenediamineteraacetic acid
<b>EST</b>	Expressed sequence tag
<b>G</b>	Guanine
<b>GAI</b>	GUS activity index
<b>HCl</b>	Hydrogen chloride
<b>HEN1</b>	HUA ENHANCER 1
<b>HYL1</b>	HYPONASTIC LEAVES 1
<b>IBA</b>	Indole-3-butyric acid
<b>LB</b>	Lysogeny broth
<b>MFE</b>	Minimal folding energy
<b>MFEI</b>	Minimal folding energy index
<b>MS</b>	Murashige and Skoog
<b>NAA</b>	Naphthaleneacetic acid
<b>PCR</b>	Polymerase chain reaction
<b>PME</b>	Pectin methylesterase
<b>R</b>	Rooting
<b>RE</b>	Restriction enzyme

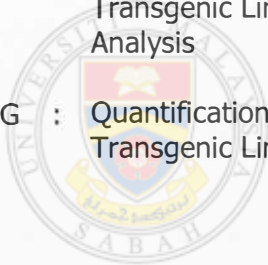
<b>RISC</b>	RNA inducing silencing complex
<b>RT</b>	Reverse transcription
<b>RT-PCR</b>	Reverse transcription polymerase chain reaction
<b>RT-qPCR</b>	Reverse transcription quantitative polymerase chain reaction
<b>SP</b>	Shoot proliferation
<b>SR</b>	Shoot regeneration
<b>TAE</b>	Tris acetate EDTA
<b>TBE</b>	Tris boric EDTA
<b>U</b>	Uracil
<b>UV</b>	Ultra violet
<b>WAC</b>	Week after culture
<b>XET</b>	Xyloglucan endotransglycosylase



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## LIST OF APPENDIX

	Page
Appendix A : Flowchart of the Nine Major Parts of Research Methodology and Analysis	199
Appendix B : MS Medium Composition (Murashige and Skoog, 1962)	200
Appendix C : Sequence of Three Pre-miRNAs of Pineapple Obtained through Sanger Sequencing Analyses	201
Appendix D : Sequence of 21 Pre-amiRNAs Obtained from Expression Vector Inserted into <i>A. Tumefaciens</i> , through Sanger Sequencing Analyses	202
Appendix E : Sequence of CaMV35S Promoter Region Amplified from Transgenic Callus, Obtained through Sanger Sequencing Analysis	205
Appendix F : Sequence of AmiRNA Amplified from Calli of Eight Transgenic Lines, Obtained through Sanger Sequencing Analysis	206
Appendix G : Quantification of MiR535b and MiR535m from Eight Transgenic Lines through Q-PCR Analyses	207



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# CHAPTER 1

## INTRODUCTION

### 1.1 Research Background

*Ananas comosus* or pineapple is one of many commercial fruits available in the global market. Nevertheless, compared with other fruits, pineapple has a distinctive position in that it is ranked second in terms of global production among major tropical fruits (after only the banana) by the United Nations Conference on Trade and Development (UNCTAD). Malaysia was once the world's largest producer of pineapple, but is now ranked fifteenth (as of 2014). According to the Malaysian Pineapple Industry Board (MPIB), there are nine main pineapple cultivars grown in Malaysia at present, namely Moris, N36, Sarawak, Moris Gajah, Gandul, Yankee, Josaphine, Masapine and MD2. Among these, MD2 pineapples have been most successfully commercialised and are traded in about 75% of the European Union market. Indicative of the potential of this cultivar, MD2 has been listed as a key crop under the National Key Economic Area (NKEA) of the Economic Transformation Programme (ETP).

MicroRNAs (miRNA) are a type of small RNA (~21nt) which are processed by the Dicer-like1 (DCL1) protein from a longer sequence of a secondary structure called the *precursor microRNA* (pre-miRNA, or precursor) (Ambros *et al.*, 2003). Since miRNAs are small and single stranded, it can bind complementarily to other single-stranded sequences such as the mRNA transcripts. When this occurs, the translation process of mRNA is disrupted, and no protein is produced (Ambros *et al.*, 2003). This mechanism is called gene silencing by endogenous miRNA. Several miRNAs have been discovered, and have been reported to regulate important genes in plants (Palatnik *et al.*, 2007; Debernardi *et al.*, 2012). However, the functions of many other miRNAs are yet to be profiled, including the function of miR535. This miRNA family has been found in various plants, although its function remains unknown (Yusuf *et al.*, 2015; Pantaleo *et al.*, 2016). This miRNA family have been reported to be expressed in pineapple, while more than 50 members have been found in plants as catalogued in the miRNA database (miRBase, Release 21.0).

AmiRNA is a genetic-based technology developed to mimic gene silencing by miRNA. Its difference, however, also represents an advantage over the use of miRNA, whereby it can be custom-designed to silence a specific target gene within an organism. An amiRNA is a ~21 nt oligonucleotide with a sequence that is complimentary to the targeted mRNA sequence (Schwab *et al.*, 2006). It has been reported that, when the sequence was inserted into the endogenous pre-miRNA (backbone), thus replacing the natural ~21 nt miRNA, it was able to function normally (i.e., able to produce amiRNA). AmiRNA then bound to the target mRNA and silenced it (Schwab *et al.*, 2006).

## 1.2 Problem Statement

Among the constraints faced in the production of pineapple is the cultivation of the seedlings themselves, as the parent plant requires a significant length of time to produce slips or suckers. In the future, focused breeding will be essential to the economy, but the dependency on one cultivar (MD2) indirectly contributes to the limitations in pineapple production. Therefore, the production of new varieties or the improvement of the current MD2 variety through genetic modification may be needed.

Crop improvement and development of new variety is not solely about knocking or inserting one particular gene. It's about knocking or inserting the 'key' gene/genes/gene family of that particular pathway. However, current practice in plant breeding relies on conventional techniques such as conventional crossing or chemically induced mutations. Although these techniques have long been reported as effective, however they are time consuming and occurs in a random manner. This is the limitation that can be addressed by amiRNA technique. The establishment of amiRNA technique holds significant potential for development of new varieties in pineapple through large scale yet specific gene silencing, for this case silencing each gene on one particular pathway (individually or the whole family).

AmiRNA has been established and widely used for the creation of loss-of-function mutants/lines in plants and commercial crops such as *Oryza sativa*, *Arabidopsis thaliana*, *Zea mays* and *Vitis vinifera* (Schwab *et al.*, 2006; Warthmann *et al.*, 2008; Meng *et al.*, 2011). However, since pre-miRNA is known to be species specific, precursors used as amiRNA backbones in these plants may or may not be compatible with pineapple. And up until now, amiRNA system for gene silencing in pineapple has yet to be established.