DEVELOPMENT OF MICROPROGATION AND *Agrobacterium*-MEDIATED TRANSFORMATION PROTOCOLS FOR PINEAPPLE (*Ananas comosus*) VAR. MD2

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2018

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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2018

UNIVERSITY OF MALAYA

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Matric No: SGR120098

Name of Degree: Master of Science

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ABSTRACT

In this study, a simple direct in vitro propagation protocol was established for pineapple variety MD2 by using sucker as explants until acclimatization. Shoots were initiated on semi-solid Murashige and Skoog (MS) medium containing 3 mg/L 6-benzylaminopurine (BAP) and 1 mg/L 1-naphathylacetic acid (NAA) before multiplied in MS liquid medium containing 2 mg/L BAP and 1 mg/L NAA. Shoots separated from multiple-shoot clusters started to root after three days on MS medium containing 30 g/L sucrose, 2 g/L gelrite, and 6 g/L charcoal. Significantly different data observed on photosynthesis rate, stomatal conductance, and transpiration rate for plants acclimatized after 42 days compared to 14 and 28 days. Hence, 42 days of acclimatized plants could be the appropriate period for transferring in vitro-derived plants into fields since similar patterns of morphology and physiology observed with field-grown plants. Whilst, transformation of *A. comosus* with *Agrobacterium tumefaciens* strain EHA105 harbouring pkYLX71 plasmid with ABR17 gene was conducted. Polymerase Chain Reaction (PCR) analysis was applied to confirm the presence of ABR17 gene in individual putative transformed *A. comosus*. Studies revealed that explants infected for 10 min and cultured on medium supplemented with 100 µM acetosyringone and co-cultivated for 1 day evoked high number of regenerant line (45) and positive putative transformants (22%). Integration of the gene in the genome *A. comosus* was visualized using Southern Blot analysis.

Keywords: *Acclimatization; Microprogation; Morphology; Physiology; Agrobacterium*

PEMBANGUNAN PROTOKOL MIKROPROPAGASI DAN TRANSFORMASI PERANTARAAN AGROBAKTERIA UNTUK NENAS (*Ananas comosus*) VAR. MD2 ABSTRAK

Dalam kajian ini, satu protokol propagasi secara langsung yang mudah telah dicipta untuk varieti nanas MD2 dengan menggunakan sulur sehingga penanaman. Pertumbuhan pucuk telah digalakkan melalui Murashige dan Skoog (MS) separa pepejal yang mengandungi 3 mg / L 6-benzylaminopurine (BAP) dan 1 mg / L 1-naphathylacetic acid (NAA) sebelum propagasi pucuk dalam medium cairan MS yang mengandungi 2 mg / 1 mg / L NAA. Pucuk yang dipisahkan dari kelompok propagasi pucuk mula berakar selepas tiga hari pada medium MS yang mengandungi 30 g / L sukrosa, 2 g / L gelrite, dan 6 g / L arang. Data yang sangat ketara diperhatikan pada kadar fotosintesis, konduktansi stomatal, dan kadar transpirasi untuk tumbuhan yang disesuaikan selepas 42 hari berbanding 14 dan 28 hari. Oleh itu, 42 hari tumbuhan yang boleh disesuaikan boleh menjadi tempoh yang sesuai untuk memindahkan tumbuhan yang berasal dari in vitro ke ladang kerana corak morfologi dan fisiologi yang serupa diperhatikan dengan tumbuh-tumbuhan yang ditanam. Sebaliknya, transformasi *A. comosus* dengan strain *Agrobacterium tumefaciens* EHA105 telah dijalankan. Analisis Reaksi Rantaian Polimerasi (PCR) digunakan untuk mengesahkan kehadiran gen *ABR17* dalam *A. comosus.* Kajian menunjukkan bahawa eksplant yang dijangkiti selama 10 minit dan ditambah dengan 100 μM acetosyringone dan kala ko-kultivasi selama 1 hari menimbulkan bilangan pertumbuhan semula (45) dan kecekapan transformasi (22%) yang tinggi. Integrasi gen dalam genom *A. comosus* telah dibuktikan dengan Sothern hybridasi.

Kata kunci: *Aklimatisasi; Micropropagation; Morfologi; fisiologi; Agrobakteria.*

ACKNOWLEDGEMENTS

First and foremost, all praise to Allah the Almighty for giving me strength and ability to complete this thesis. I would like to express my deepest thanks and appreciation to my respected supervisors, Prof. Dr. Norzulaani Khalid and Dr. Mahanom Jalil for their endless support, precious advice, guidance and encouragement throughout successful completion of my study. Special thanks id dedicated to Dr. Tan Boon Chin for his willingness to spare his precious time and help in my research write up.

I would like to acknowledge Knowledge Transfer Programme (KTP10-B) for funding partial of this project. I would also like to thank University of Malaya for the financial support and granting me the scholarship of 'Skim Biasiswa Universiti Malaya' (SBUM) and Graduate Research Assistantship Scheme (GRAS).

I am indebted and thanks to the member of Plant Biotechnology Incubator Unit (PBIU) and Plant Biotechnology Research Laboratory (PBRL) for their kind assistance, valuable knowledge, wonderful cooperation and moral support.

I also like to thank my family member especially my father (Hj. Hamid Bin Daud), mother (Hjh. Fatimah Bt Omar) and family in-law for endless love, prayers and understanding. Finally, my highest appreciation is to an amazing man of my life, my husband Nurshamin Bin Nordin and my lovely son, Mohd Luqman Hakimi Bin Nurshamin, the most reason that give me strength in finishing me up my thesis. Thank you for your love, concern, acceptance, patience, motivation, endless prayers and presence during my ups and downs in my thesis writing. Fate has brought us together and I am grateful to have all of you as part of my success story. I really do appreciate it and will always indebted to all of you.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percent
°C	:	Degree
µg	:	Microgram
µL	:	Microliter
2,4-D	:	2,4-dichlorophenoxy acetic acid
BAP	:	6-benzylaminopurine
Bp	:	Basepair
cDNA	:	Complimentary deoxyribonucleic acid
Cm	:	Centimeter
CO_2	:	Carbon dioxide
CTAB	:	Cetyltrimethylammonium bromide
C_i	:	Intercellular CO_2
DNA	:	Deoxyribonucleic acid
E	:	Transpiration rate
EDTA	:	Ethylenediaminetetra acetic acid
EtBr	:	Ethidium bromide
FAO	:	Food and Agriculture Organization
G	:	Gram
GOI	:	Gene of interest
g_s	:	Stomatal conductance
H	:	Hour
H_2O	:	Water
HCl	:	Hydrogen chloride
IAA	:	Indole acetic acid

IBA	:	Indole butyric acid
K	:	Kalium
Kb	:	Kilo basepair
L	:	Liter
LB	:	Luria-Bertani
LB	:	Left border
LD	:	Lethal death
MBC	:	Minimal bactericidal concentration
MIC	:	Minimal inhibitory concentration
Min	:	Minute
Mm	:	Milimolar
MT	:	Million tan
N	:	Natrium
NAA	:	Naphthalene acetic acid
NaCl	:	Sodium chloride
NaOH	:	Sodium hydroxide
NCBI	:	National Centre for Biotechnology Information
Ng	:	Nanogram
O_2	:	Oxygen
OD	:	Optical density
P	:	Phosphorus
PCR	:	Polymerase chain reaction
PGR	:	Plant growth regulator
pH	:	Potential of Hydrogen
P_N	:	Photosynthesis rate
RB	:	Right border

RE	:	Restriction endonuclease
RNA	:	Ribonucleic acid
Rpm	:	Revolutions per minute
SE	:	Somatic embryogenesis
TBE	:	Tris-Borate-EDTA
U	:	Unit
UV	:	Ultraviolet
V	:	Volt
v/v	:	Volume/volume
w/v	:	Weight/volume

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background study

Pineapple [*Ananas comosus* (L.) Merr.] has been listed as a major tropical fruit since 2010 (FAO, 2016). Globally, one of the popular commercial variety is MD2 which generates profits nearly RM 3.6 billion per year in the world production. Since the introduction of this variety by Pineapple Research Institute (PRI) of Hawaii, the production of pineapple has grown by nearly 50% since 1998 (FAO, 2016).

Conventionally, pineapple is propagated using field-collected suckers (Yapo *et al*., 2011). However, this method is slow as only about 3 suckers are produced per plant annually. Moreover, this method can easily transmit numerous diseases, such as fusariosis, butt rot in suckers and black rot, from old to new pineapple plantations. Although chemical fungicides are used to control this problem, it has negative impacts to the environment (Korres *et al*., 2011). Hence, there is a need to establish an alternative method for large-scale production of pineapple plantlets in order to satisfy the increasing demand of pineapple fruit. Previous researched had proved that this technique can produce plant in a large scale (Tekoah *et al*., 2015; Dias *et al.*, 2016).

Plant tissue culture is an important technique to mass produce disease-free and uniform plantlets within a reasonable period (Mengesha *et al*., 2013; Scherer *et al*., 2013). After producing *in vitro*-derived plantlets by implementing plant tissue culture technique, the plantlets were acclimatized in a new environment. This is a crucial process since the *in vitro* plantlets need to adapt and survived in a different environment. Chirinea *et al*. (2012) mentioned that acclimatization protocol needs to be done to ensure high survival rate of the plantlets in a new challenging environment.

In Malaysia, MD2 has been given priority to increase its output production via the National Key Economic Area (NKEA) initiative under the Malaysian Permanent

Food Production Areas (LPNM, 2012). Although many efforts have been made to improve the growth of pineapple, effects of stress due to flooding have significantly decreased its production. Excess accumulation of salts in soil due to flood has prevented plant roots from efficient water and nutrients uptake. Imbalance nutrient composition and toxicity in the soil due to excessive Na and Cl ions may reduce the osmotic potential in the plants and disrupt the cell organelles and their metabolisms. High salinity soil can reduce plant growth and may cause plant death (Evelin *et al.*, 2009; Vickers *et al.*, 2009). About 50% of the arable land is predicted to be covered by salt water by 2050 (Vinocur & Altman, 2005). To address this problem, several measures including the introduction of salt-tolerant genes into plant hosts via genetic transformation could be carried out.

Conventional method using crowns, slips, hapas and suckers were used to propagate pineapple asexually by small scale farmers, but not very promising since pineapple is a self incompatible and highly heterozygous plant to produce consecutive fruit generations (Jackson *et al*., 2016). Hence, genetic engineering such as mediated transformation is a recommended ways by introducing specific traits without altering other agronomic attributes (Smith, 2013). Plant improvement through genetic transformation has been shown to be the most effective strategy to produce salt-tolerant plants. This approach is able to produce new variety after four generations of backcross compared to time-consuming conventional breeding (Wang *et al.*, 2011; Yarra *et al.*, 2012). *Agrobacterium*-mediated genetic transformation is the most commonly used method to produce salt tolerant transgenic plants (Chumakov & Moiseeva, 2012) due to its simplicity, cost effectiveness, and ability to transfer relatively long DNA segments into host systems (Kumar *et al*., 2010).

Danquah *et al*. (2014) reported that abscisic acid-responsive 17 (ABR17), a family member of pathogenesis-related (PR) 10 protein as a central regulator of abiotic stress response in plants such as salt stress. In the past few years, numerous studies indicated the elevated expression of PR10 in response to salt stresses in various plants, including *Brassica napus* (Dunfield *et al.*, 2007), *Arabidopsis thaliana* (Srivastava *et al.*, 2006), and *Oryza sativa* (Hashimoto *et al.*, 2004). Hence, this gene could be used in the future to overcome the salt stress challenge towards pineapples using *Agrobacterium*-mediated technique in order to allow pineapple to grow in the high salt area enabling to fulfill the demands of pineapple var. MD2 in the world market.

This study aimed to establish an efficient *in vitro* regeneration protocol for *Ananas comosus* (L.) Merr. var. MD2, including the optimized acclimatization period by examining the morphological and physiological changes during the process of acclimatization. The protocol for introducing ABR17 gene via *Agrobacterium*-mediated transformation to pineapple variety MD2 has also been established.

1.2 Objectives

1) To optimize an efficient *in vitro* regeneration protocol for *Ananas comosus* (L.) Merr. var. MD2.
2) To optimize an appropriate period of acclimatization before transplanting *in vitro*-derived plants into field based on morphological and physiological parameters.
3) To optimize the optimal parameters for introducing *ABR17* gene into *Ananas comosus* (L.) Merr var. 'MD2' via *Agobacterium*-mediated transformation.

CHAPTER 2: LITERATURE REVIEW

2.1 Pineapple Cultivar

2.1.1 Classification and Nomenclature

Pineapple (*Ananas comosus* (L.) Merr.) is a member of the Bromeliaceae family comprising of about 2,000 species. It is generally known as *pina* in Spanish, *abacaxi* in Portuguese, *ananas* by the Dutch and French, *nanas* in Southern Asia, *po-lo-mah* in China, and *sweet pine* in Jamaica (Morton & Miami, 1987). Figure 2.1 shows a diagram of pineapple plant parts.

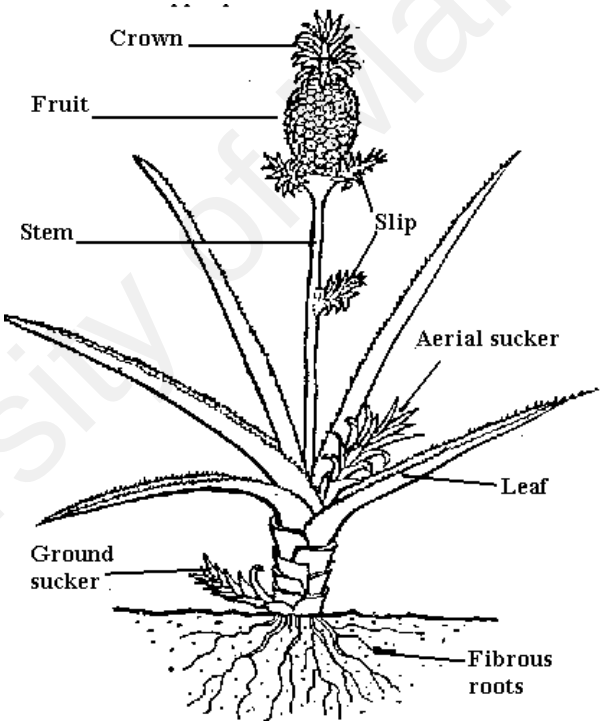


Figure 2.1: Diagram of *Ananas comosus* (L.) Merr. Image reproduced from http://www.uq.edu.au/_School_Science_Lessons/60.2.GIF with permission from University of Queensland.

It is classify taxonomically as below:

Kingdom	Plantae
Order	Poales
Family	Bromeliaceae
Subfamily	Bromelioideae
Genus	Ananas
Species	*Ananas comosus*
Binomial name	*Ananas comosus* (L.) Merr.

2.1.2 Origin of Pineapple

Hawaii was the first country to grow large-scale pineapple plantation in 1900 where Maui Pineapple was the pioneer company. The company had collaborated with Pineapple Research Institute (PRI) in producing a high demand pineapple cultivar '73-114' called MD2 Smooth Cayenne variety (Danso *et al.,* 2008).

In Malaysia, pineapple had been introduced by the Portuguese. It had been planted as cash crop since 1921 starting from Singapore, Johor and Selangor in peat soil. Currently, there are several varieties being planted in Malaysia where MD2 is the variety in high demand due to its characteristic stated in section 2.1.4. This variety had been introduced through Malaysian Pineapple Industrial Board (MPIB) in 2008 by importing 100,000 suckers from the Philippines. The government had listed MD2 pineapple in the National Key Economic Area (NKEA) and targeted its production towards 700,000 MT by 2020. In order to achieve the target, both smallholders and industrial planters were identified as key players. The strategies include recruiting new farmers, supplying suckers and equipment needed for the production (Amar *et al.*, 2015).

2.1.3 Plant Description

According to Marcus *et al*. (2014), pineapple is an herbaceous perennial with a short, stout stem and a rosette of waxy, strap-like leaves, xerophytic, perennial monocotyledonous plant. The leaves can elongate until 72 inches in length, needle tip shaped and generally bearing spikes. The stem extends and expands near the apex and puts forth an inflorescence of small purple or red flowers during flowering time. The flowers are hard and small in size usually pollinated by birds or insects for wild type pineapple. Commercially, pineapple is propagated by ground or aerial suckers. The fruits are compound fruit which develops from many small fruits fused together by fibrous core stem at the centre and are uniformly cylindrical shaped. The fruits can elongate until 30 cm in length and weigh to about 10 kg or more.

2.1.4 Cultivar

There are about 100 pineapple varieties known internationally. Some of the commercially known varieties are shown in Table 2.1. MD2 pineapple variety is included as one of the highly successful commercial varieties after it had been introduced to the European market in 1996 (Wardy *et al.*, 2009). The variety has increasingly gained more attention than other commercial varieties such as smooth cayenne and sugarloaf due to its sweet aroma, blemish-free flesh, and high sugar content (14% Brix) with enrichment of vitamins A, B and C (Bhui *et al.*, 2010; Upadhyay *et al.*, 2011). In addition, 'MD2' has been reported to have even ripening and longer shelf life (Wardy *et al.*, 2009) as well as exhibiting antioxidant and antimicrobial properties (Upadhyay *et al.*, 2011; Tortoe *et al.*, 2014).

Table 2.1: Description of popular commercial pineapple varieties.

Cultivar	Description of the fruit	Resource
Smooth cayenne	10 – 12 kg, suitable size for canning, pale yellow to yellow flesh. Cylindrical in shape with high sugar and acid content. Leaves without spine.	Bartolome *et al.* (1995)
Abacaxi	1 – 5 kg, white or very pale yellowish flesh, suitable for commercial handling but very low yield in production. Spiny leaves.	Reinhardt *et al.* (2002)
Sugarloaf	2 – 6 kg, closely related to 'Abacaxi'. White to yellow, very sweet and juicy flesh and suitable for shipping. Less conical, sometimes round. Spiny leaves.	Norman (1976)
Red Spanish	4 – 8 kg, suitable for shipping as fresh fruit to remote markets, pale yellow flesh with pleasurable aroma; squarish in shape. Spiny leaves.	Bartolome *et al.*(1995)
Queen	2 – 5 kg, deep yellow, less fibrous than 'Smooth Cayenne'. Suitable as fresh fruit and not adapted for shipping. The plant is dwarf, compact and more disease resistant than 'Smooth Cayenne'.	Gangopadhyay *et al.*(2009)

2.1.5 Important uses

Unprocessed pineapple fruit can be eaten directly without cooking and contains high vitamin C (87 mg/g). Some of its functions are to cure wound or injuries and helps the body to absorb iron. In addition, vitamin B1 (Thiamin) and Vitamin B3 (niacin) are very important to maintain a healthy digestion system (Ancos *et al.*, 2017).

Bromelain is a useful enzyme from pineapple fruit which works against thickening and suppress cancer cells. Studies have proven the use of pineapple consistently helps battle against connective tissue disorder, inhibit allergic sensitization and anti cancer agent (Secor *et al*., 2013; Amini *et al*., 2016; Rubin *et al*., 2016). Pineapple is an excellent source of trace mineral such as manganese, which is an essential cofactor enzyme in energy production and antioxidant defenses (Ancos *et al*., 2017).

2.1.6 Economic value

Pineapple has been listed in the top ten of most demanded tropical fruit in the world. In Malaysia, the pineapple productions and demand are increasing. Therefore, Malaysian Institute of Pineapple Board (MIPB) was established in 1957 to ensure high quality pineapple production to cope with the local and international demand. Pineapple variety MD2 has been listed as priority fruit in the National Key Economic Area (NKEA) under agriculture division based on its demand internationally. The Agriculture sector aimed to contribute about RM11.6 million to Malaysia's gross national income (GNI) with 9,075 projected jobs in 2020.

According to Food and Agriculture Organization Standard Division (FAOSTAT, 2016), more than 91 countries produce nearly 24.8 Million Tonnes (MT) of pineapple annually. Pineapple world production and harvested area can be seen increasing nearly 2 folds in 1994 (13 MT) to 2014 (25.4 MT) (Figure 2.2). Thailand (2.17 MT), Philippines (1.89 MT), India (1.26 MT), Indonesia (0.98 MT) and China (0.89 MT) are listed in top ten as the main pineapple producers making Asia (48.2%) as the most productive region for pineapple production. Malaysia is the 21th top producer of pineapple internationally with 0.34 MT of production (FAOSTAT, 2014).

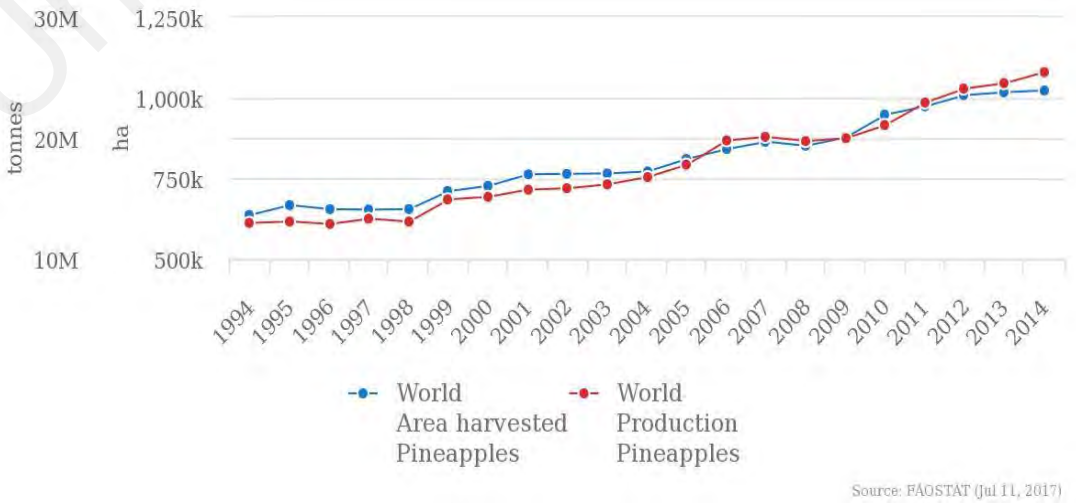


Figure 2.2: World production of pineapple (1994 – 2014). Image was reproduced from FAOSTAT (2016), with permission from FAOSTAT's website.

2.2 Plant Tissue Culture

2.2.1 Introduction

Plant tissue culture is an aseptic technique for mass propagation of *in vitro* plantlets from cells, tissues and organs of plants. The technique requires controlled light, temperature and humidity as well as chemical and physical conditions (Thorpe, 2012). Plasticity and totipotency are central in understanding the technique. Plasticity allows plants to be able to alter their metabolism in order to adapt and survive in a particular environment. The ability of regenerating an entire organism from other type of tissue or organ into whole organisms based on a correct precursor called totipotency (Fehér *et al*., 2016). Hence, there are three main precursors to maintain the regeneration into whole organism which are suitable culture environment, culture media and growth regulators.

There are three basic components for *in vitro* cultivation of plant culture media; essential elements supplying minerals; organic elements supplying vitamins or amino acid and sucrose as a source of carbon. The well-known medium is Murashige-Skoog (MS) developed by Murashige and Skoog (Murashige & Skoog, F., 1962). The major plant growth regulators (PG) are auxin, cytokinins, ethylene, gibberellins, abscisic acid, brassinosteroids, jasmonic acid and salicylic acid (Wang & Irving, 2011; Hou *et al.*, 2013) which are essential components in the culture medium.

After *in vitro* induction and multiplication, the plants are acclimatized in the greenhouse with suitable conditions for a certain period of time before transferring to the field. Acclimatization is a very crucial process to ensure that the plant can adapt to the discrete changes towards the environment.

2.2.2 Application of tissue culture

Through conventional propagation, some plants produce low number of planting materials which limits production. This situation may cause problems to meet high demand for products from plants grown on commercial basis. Hence, to overcome this problem, plant tissue culture can be used for propagation of a large amount of planting materials within a comparatively short period of time (Lynch, 2014; Dias *et al*., 2016).

An efficient plant tissue culture is also a pre-requisite for crop improvement through genetic modification. This method will enable the production of new breeds of plants with new and improved agronomic characteristics (Dias, *et al.*, 2016; Huang *et al.*, 2016). In conventional method, agronomist adopts breeding technique to produce new breeds which could take more than a decade to ensure the genes are stable inherited (Schaart *et al.*, 2016).

2.2.3 Tissue culture of pineapple

Pineapple is a vegetative propagated crop and practically depending on the suckers for its self-sterility. It produces very few suckers per year, so its multiplication rate is lags behind other seed propagated crops (Ma *et al.*, 2012). Hence, tissue culture is a very useful tool to solve this problem and fulfil the market demand (Escalona *et al.,* 1999) since it can increase the production of pineapple more efficiently (Sales *et al.*, 2015). It can also provide a crucial adjunct not only to conventional breeding but also for the propagation and genetic improvement of pineapple, since cultivar improvement requires at least 5 years by sexual hybridization and selection (Sripaoraya *et al.*, 2003).

Plant regeneration of pineapple through tissue culture can be achieved through organogenesis and embryogenesis (Ika *et al.*, 2004). Both processes are very important

in propagating plantlets using a suitable Murashige and Skoog medium containing cytokinin, auxin, or a combination of both.

According to Pardal *et al*. (2012), organogenesis is a process of forming and developing shoots from meristematic tissue. Plant regeneration in pineapple can be developed either via direct or indirect organogenesis (Firoozabady & Moy, 2014). Direct organogenesis occurred by using pineapple parts such as sucker, slip, and crown (Sripaoraya *et al.* 2008). Most researchers used a combination of 6-benzylaminopurine (BAP) and other plant growth regulators like naphthalene acetic acid (NAA) or indole acetic acid (IAA) or indole butyric acid (IBA). Propagation of pineapple can be obtained *in vitro* with BAP alone (Be & Debergh, 2012), mixture of PGR like BAP and NAA (Firoozabady & Gutterson, 2013), IBA (Boxus *et al*., 1991), IAA (Hamad & Taha, 2008), 2,4-dichlorophenoxy acetic acid (2,4-D) (Pohanish, 2015), combination of BAP and two auxins such as NAA and IAA (Mathews & Rangan, 1979), IAA and IBA (Teixeira *et al*., 2006) and NAA and IBA (Soneji *et al*., 2002).

Indirect organogenesis undergo initial nodular globular structures followed by shoot regeneration using leaf base explants (Raju *et al*., 2015, Li *et al*., 2016 & Stanisic *et al*., 2015). Somatic embryogenesis (SE) is more efficient than organogenesis, because this technique can produce a large number of planting materials in a relatively short period of time. It can be used for plant improvement and genetic manipulation (Fernando *et al*., 2016). SE is defined as a developmental process of somatic cells that resemble zygotic embryos (Ma *et al.,* 2012). In pineapple, embryogenic cell suspension cultures have been established for propagation of planting materials (Ma *et al*., 2016).

Furthermore, acclimatization is lastly a crucial procedure in order to enhance pineapple production since the *in vitro* pineapples undergo dramatic changes of environment (temperature, humidity and light). Previous studies reported less than 100

% of pineapple plantlets survive through acclimatization (Jackson *et al*., 2016; Scherer *et al*., 2015). Hence, physiology and morphology of pineapple from *in vitro* until field was investigated in this study in order to get 100 % survival rate and reduce labour cost at the same time.

2.3 Plant Genetic Engineering

Plant genetic engineering is a useful tool in agronomic realm where it can produce new traits of planting material in a short period of time without altering other agronomic attributes (Smith, 2013). Previous research proved that genetic traits in plants can be altered through genetic modification (Huang *et al.*, 2016; Jacobs *et al.*, 2015; Trigiano & Gray, 2016). It has been shown in pineapple that this technique had been applied by inserting gene of interest such as biotic and abiotic stress to produce a pineapple with new traits without altering its physiological and morphological characteristic (Yabor *et al.*, 2016; Yabor *et al.*, 2017). Hence, the productions of the fruits can be optimised since the plants can tolerate biotic and abiotic stress.

2.3.1 Methods of gene transfer to plants

Gene transfer is a well known method for crop improvement. The gene of interest (GOI) is able to produce plants with disease resistance, stress tolerance, and enhance plant improvement by expressing valuable added traits after being inserted into plants through genetic transformation (Jacobs *et al.*, 2015). Transfer of GOI into host plant is made by constructing a vector that consists of GOI flanking with suitable controlling sequences such as promoter and terminator. Promoter determines where and when the GOI is expressed in the host, and a "marker gene" that allows breeders to determine which plants restrain the inserted gene by screening or selection. For

example, marker genes may render plants resistant to antibiotics that are not used medically (e.g., kanamycin, ampicilin and hygromycin), whereas terminator verify where and when the GOI should stop expressing. GOI can be transferred via direct and indirect gene transfer methods.

2.3.2 Direct gene transfer

In this method, GOI are being transferred directly into plant host without the help of any organism such as particle bombardment (biolistic), electroporation and chemical mediated gene transfer. Table 2.2 shows the list of advantages and disadvantages of commonly used direct gene transfer techniques.

2.3.3 Indirect gene transfer (*Agrobacterium*-mediated transformation)

For indirect gene transfer, *Agrobacterium*-mediated gene transfer is favoured because it could produce high transformation efficiency comparatively (Xu *et al*., 2014; Pantazis *et al*., 2013). *Agrobacterium tumefaciens* is a soil borne bacteria that causes crown gall in dicotyledonous plant, and has the ability to introduce new genetic material into the plant cell (Hu *et al.*, 2012). It is classified in genus *Rhizobium* and in family *Rhizobiaceae*. *A. tumefaciens* capable of transferring GOI by integrating into the host genome introduced in the T-DNA (transferred DNA) region which is located on a tumour-inducing (Ti) plasmid (Christie & Gordon, 2014; Lang *et al.*, 2013).

During transformation, several components of the Ti plasmid enable effective transfer of GOI into the plant cell namely T-DNA border sequences, *vir* genes, modified T-DNA region. The T-DNA border sequence, consist of at least the right border, that initiates the integration of the T-DNA region into the plant genome with the help of *vir*

genes. *Onc* genes (crown-gall formation) are removed in a modfied T-DNA region by genetic engineering and replaced by foreign genes (GOI). Hence, the regenerated transformed plants are in normal appearance. Figure 2.3 shows a schematic diagram of wild type Ti plasmid from *Agrobacterium tumaefaciens*.

Agrobacterium-mediated transformation has been remarkably advantages over direct transformation methods due to low copy number of GOI associated with transgene silencing, co-suppression and instability (Andrieu *et al*., 2012; Jackson *et al*., 2013; Zalewski *et al*., 2012). This method has been reported in pineapple varieties such as smooth cayenne (Soneji *et al.*, 2002); shenwan (Ma *et al.*, 2012) and queen (Gangopadhyay *et al.*, 2009).

Table 2.2: Advantage and disadvantage of commonly use direct gene transfer.

Method	Process	Advantage	Disadvantage	Reference
Particle bombardment	Transfer GOI using microprojectile, DNA are coated with Gold tungsten and shot with pressure into target cells.	- Specialized vectors are not required (Standard E. Coli vectors can be function as expression cassettes. - No selectable marker is needed if apply in high-efficiency transient expression of GOI in plant. - Unlimited host range.	- Extensively rearranged of GOI in stable transformants (high copy number, extensive recombination and other alteration can lead to chimerism) - Expensive technique	Chen *et al.* (2014), Jackson *et al.* (2013), Liu *et al.* (2014); Sparks and Jones (2014).
Electroporation	Transfer GOI with the help of intense electrical pulse into plant cell.	- Specialized vectors are not required (Standard E. Coli vectors can be function as expression cassettes. - No selectable marker is needed if apply in high-efficiency transient expression of GOI in plant.	- can be only utilized with protoplasts (requires successful regeneration of plants from protoplasts to be used for establishing transgenic plants) - frequency of stable transformation is low (0.001 or less)	Gothelf and Gehl (2014) and Hayden *et al.* (2012).
Chemical mediated gene transfer	Binding of highly branched organic compounds such as calcium phosphate with the precipitated plasmid DNA carrying GOI and transferred into plant cell by engulfing the cell through endocytosis.	- Low cost technique. - Stable and can be transfer specifically into target cell.	- This method only suitable for developed cell in monolayer and not for suspension cultures.	Jin *et al.* (2012); Oyane *et al.* (2012).

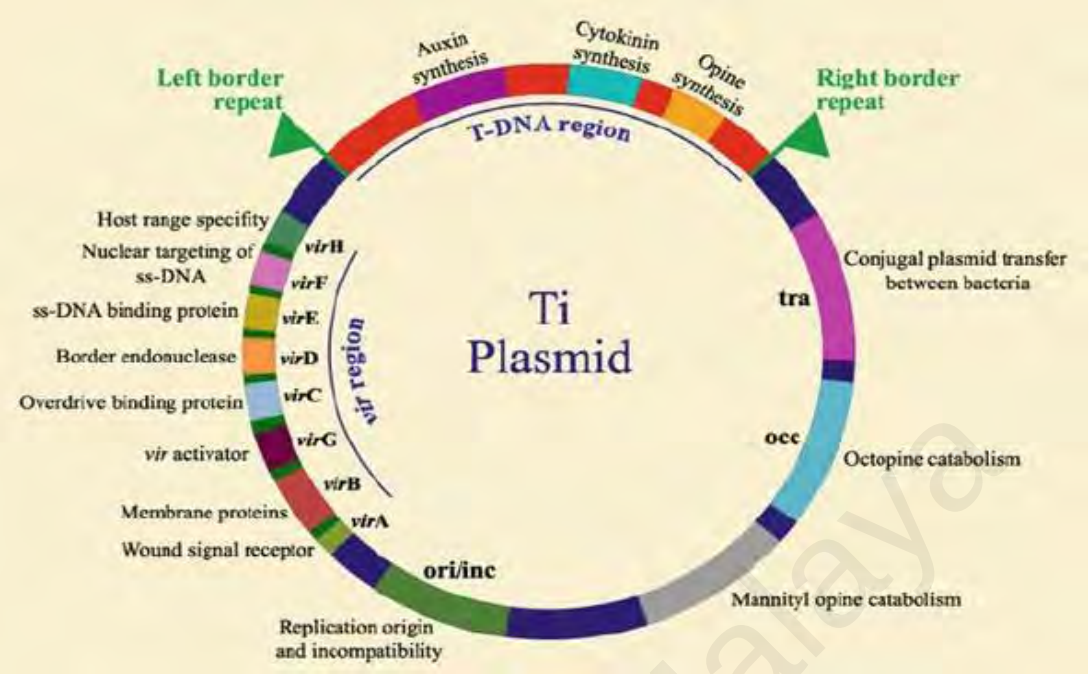


Figure 2.3: Schematic diagram of wild type Ti plasmid from *Agrobacterium tumaefaciens*. Image reproduced from Mahmoudpour, 2016 with permission from EC microbiology.

2.3.3.1 Transferation of *vir* genes

The transfer of T-DNA into plant nucleus is activated by the virulence protein controlled by *VirA, VirB, Vir C1, Vir D1/D2, Vir E,* and *Vir G* genes. When a plant is wounded, it will release phenolic compounds that will act as a signal to attract *A.tumefaciens*. Besides, the *A.tumefaciens* contains chromosomal virulence (*chv*) genes required for bacterial chemotaxis. Acetosyringone will activate vir region by binding to vir A protein (acetosyringone receptor). Subsequently phosphorylating and thereby activating Vir G that works as a transcriptional activation factor by dimerising and activating the expression of all vir operons. Many sugars, but in particular glucose, galactose and xylose, enhance *vir genes* induction. The induced VirD1 and VirD2 act together like endonucleases that recognize and cut the left and right T-DNA borders repeats of the T-DNA bottom strand and release a single-stranded (ss) T-DNA molecule (T-strand) (Lee & Gelvin, 2008). After nicking, VirD2 becomes covalently attached to

the 5' end of the displaced single-stranded T-DNA strand. Repair synthesis replaces the displaced strand. VirC1 may assist in the process. VirB operon and VirD4 encode protein to compose membrane-channel secretory system in bacterial cell by a 'T-pilus' hence producing the T-DNA/VirD2 complex. VirE2 are also exported from the bacterial cell and acts as a coat protein which envelops the T-DNA and protecting it from nuclease attack when it enters the plant cell. The T-strand, together with several proteins, is then exported to the host cell cytoplasm through a membrane-channel formed by the VirB and VirD4 proteins. Once inside the host cell cytoplasm, the T-strand presumably exists as a mature nucleoprotein complex (T-complex). Finally, the T-complex is integrated into the nucleus of the host cell with the assistance of VirD2, VirE2 and host factors (Tzfira *et al*., 2003). Figure 2.4 shows Illustration of the integration of T-DNA from plasmid of *A. tumefaciens* into the nucleus of plant host cell.

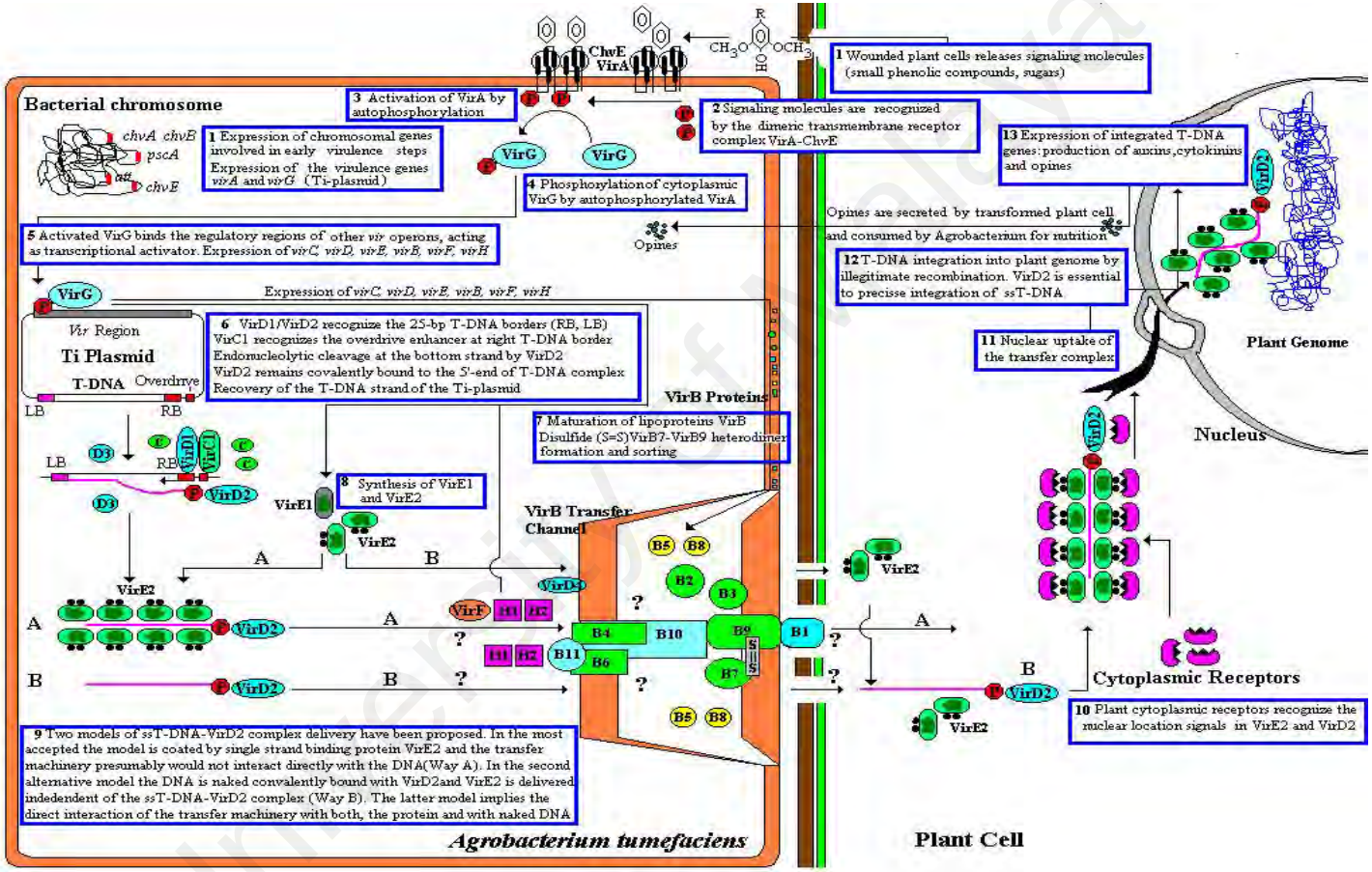


Figure 2.4: Illustration of the integration of T-DNA from plasmid of *A. tumefaciens* into the nucleus of plant host cell. Image was reproduced from Riva *et al.* (1998), with permission from Elsevier.

2.4 Vectors for *Agrobacterium* – mediated transformation

Plant transformation vectors are plasmids that have been particularly intended to encourage the era of transgenic plants. The modification of Ti plasmid play important role in developing *Agrobacterium* – mediated transformation efficiency. Non-essential original regions of the plasmid including cytokinin and auxin synthesis were also isolated to prevent tumor formation on the transformed plants (Gohlke & Deeken, 2014). Bevan, (1988) reported that disarmed plasmid able to prevent intractable attempts of regeneration into whole plant. Furthermore, gene of interest (GOI) and recombinant plasmid construction were added by introducing unique insertion sites. Important elements were also included between left border (LB) and right border (RB) borders of T – DNA such as reporter genes, desirable plant promoters and additional selectable markers (Ishizaki *et al*., 2015; Nguyen *et al*., 2016). *Agrobacterium* – mediated transformation in plant consists of two main types of vectors that have been developed – The binary vectors and co-integrate vectors.

2.4.1 Binary vectors

The most commonly utilized plant transformation vectors are named binary vectors as a result of their capability to imitate in both *E. coli*, a typical lab bacterium, and *A. tumefaciens*, a bacterium used to insert the recombinant (customized) DNA fragment into plants chromosome.

There are two distinguish plasmid employed in the binary vector – a "wide-host-range small replicon" and a "helper Ti plasmid". The binary vector carrying GOI in place of T-DNA, left border (LB) and right border (RB) of T-DNA, markers for selection and maintenance in both *E. Coli* and *A. tumefaciens* and a selectable marker for plants. The tumour-inducing genes located in the T-DNA have been removed

making the plasmid disarmed. The "helper plasmid" of "virulence plasmid" contains *vir* less region compared than the entire T-DNA region (Hoekema *et al*., 1983). The helper plasmid is functions in *cis* orientation with the binary vectors. In transformation, the binary or the virulence plasmid unable to worked if they are not working simultaneously.

Since Bevan, (1984) construct the first binary vector pBIN19, many new modified vectors have been invented to insure their stability, efficiency and utility. Numbers of different *Agrobacterium vir* helper strains with new binary vector have been reported in plant transformation such as *Dunaliella salina* consist of pSM358 binary vector and virE:LacZ fusion vector (Simon *et al*., 2015), *Rhizobium etli* consist of p42a binary vector and homologous *vir* plasmid (Lacroix & Citovsky, 2016) and *Nicotiana benthamiana* consist of binary plasmid pCAM-*virE2* and Ti plasmid (Resmi *et al*., 2015). Our study used PKYLX71 gene as the gene had been studied before by Schardl *et al.* 1987.

2.4.2 The pKYLX71 vector

The pKYLX71 was constructed by Schardl *et al*. (1987) consisting of CaMV 35S promoter and multiple unique cloning sites such as HindIII, XhoI, ScaI and XbaI. This plasmid consists of tetracycline and kanamycin resistance genes for selection of recombinant *E.coli* and *A. tumefaciens* respectively. The vectors offer several advantageous features such as can be replicated stably in both *E. coli* and *A. tumefaciens* whereby allows subsequent transfer without intermediate genetic modifications, produce high copy number and due to its small size, DNA fragment(~35 kbp) able to be transferred into plant chromosome.

The expression cassette transcription and kanamycin resistance gene directions can be observed based on arrows in Figure 2.5. Previous research have been on various types of plant such as tobacco (Ma *et al.*, 2013); potato (Batelli *et al.*, 2012), canola (Verma *et al.*, 2012) and etc. This vector that carry GOI may produce plant that tolerate towards abiotic stress (Jacobs *et al.*, 2015).

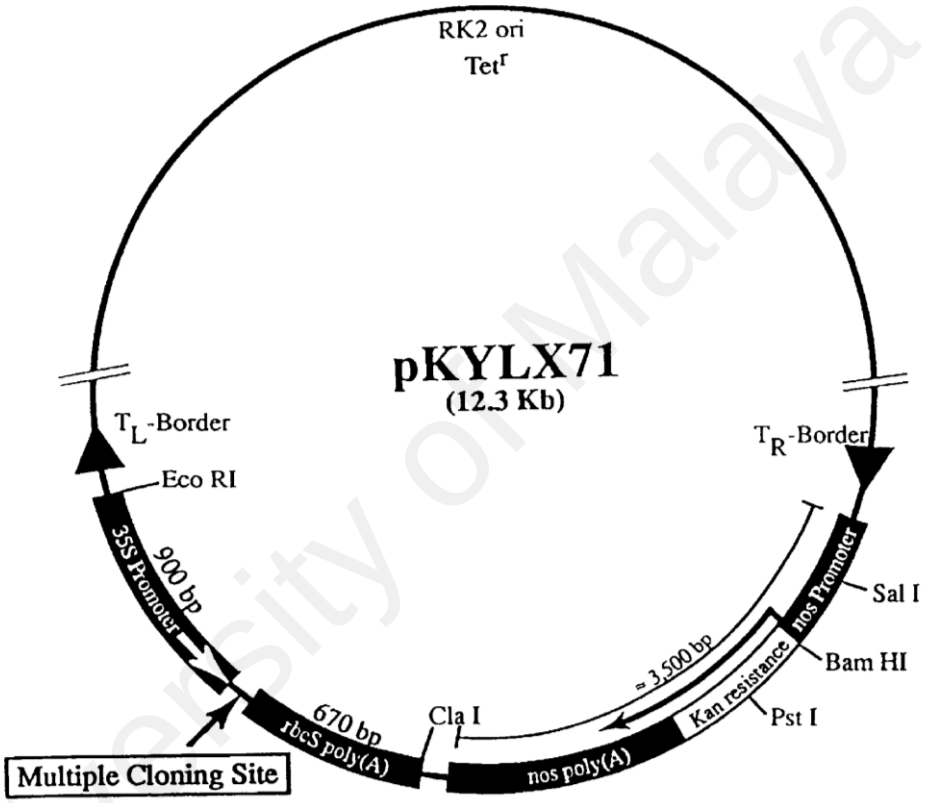


Figure 2.5: Illustration of pKYLX71 vector. Image was reproduced from Schardl *et al.* (1987) with permission from Elseveir.

2.5 Abiotic stress

Abiotic stress is defined as environmental conditions such as flood, drought, temperature and salinity that reduce growth and yield below optimum levels (Cramer *et al.*, 2011). Figure 2.6 shows mechanism effects to the plants from series of abiotc stress. Vickers *et al*. (2009) mention that reactive oxygen species (ROS) productions are the

main cause of all abiotic stress. ROS are critical important for initiating sign falls that activate protection and adaptable reactions. Phytohormones are involved to ensure the pathway working in cascade.

Recently, studies have been done on plant to overcome abiotic stress from reducing yield through genetic modifications in rice (Chen *et al*., 2014), banana (Xu, *et al*., 2014) and maize (Shi *et al*., 2016). In pineapple, abiotic stress is one of the common known factors that can kill the plant. This is due to pineapple characteristic that need continuous water supply and soil with absence of salt in order to enhance its production.

2.5.1 Salt Stress

Salinity, one of the abiotic stresses, has been reported to significantly affects the plant growth, development and survival by water stress, ion toxicity, nutritional disorders, oxidative stress, alteration of metabolic processes, membrane disorganization and reduction of cell division (Xiong & Zhu, 2002; Zhu, 2002). In 2050, about 50% of the arable land has been predicted to be covered by salt water (Vinocur & Altman, 2005; Roy *et al*., 2014). Salt interfere osmotic activity in roots where water and nutrients migrate from low to high concentration. High salinity levels in soil cause imbalanced nutrient composition as well as toxicity, leading to the disruption of cell organelles and their metabolisms. These ultimately affect the plant growth and reduce the yield (Evelin *et al.*, 2009; Vickers *et al.*, 2009).

The improvement of pineapple through genetic modification has been reported as one of the most effective strategies to solve salinity problems. This method is faster than conventional breeding which requires four generations of backcross to produce new varieties (Kato *et al.*, 2004; Anthar & Ashraf, 2009; Wang *et al.*, 2011; Yarra *et al.*, 2012).

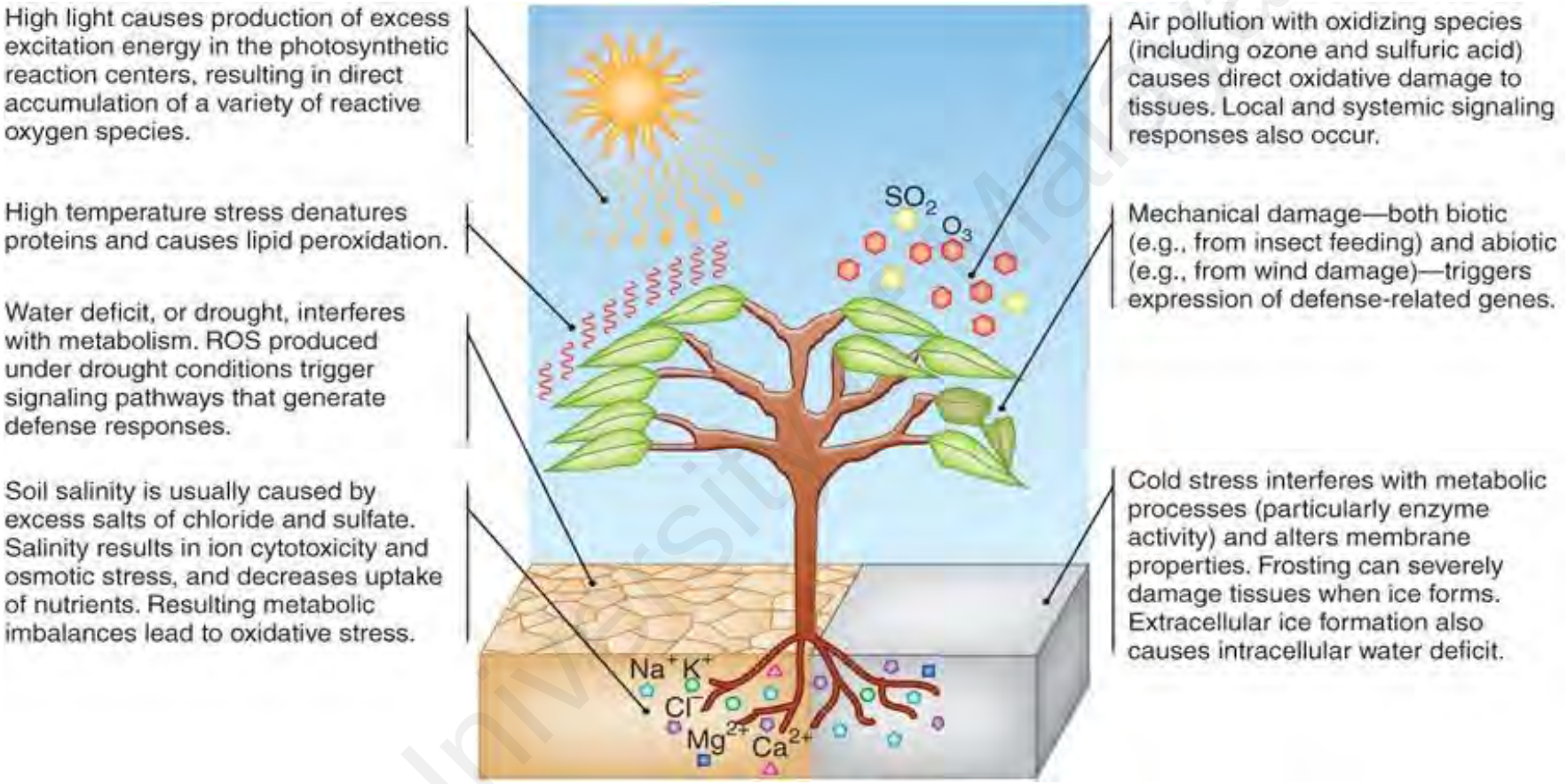


Figure 2.6: The effects of complex abiotic stress that can cause to the plant. Image was reproduced from Vickers *et al*. (2009) with permission from Springer Nature.

2.6 Pathogenesis Related Proteins (PR)

Pathogenesis related (PR) proteins are produced by plants and function as defense mechanisms in response to biotic and abiotic stress (Cristobal *et al.*, 2015; Kothari, *et al.*, 2015). Currently, there are 17 classes of PR protein, based on their primary structure, serological and biological activity in monocot and dicotyledonous plants (Sels *et al.*, 2008; Pan *et al.*, 2018). Among many types of proteins, PR-10 proteins are mostly reported in response to biotic and abiotic stresses, diverse roles in plant growth and development. Biotic stress refers to response to living organisms such as bacteria, fungi, viruses and insects. Abiotic stress is due to environmental factors such as drought, salinity and temperature that adversely affect plant growth and production (Pandey *et al.*, 2014; Shankar *et al.*, 2017). To date, over 100 PR-10 members have been identified in 70 monocotyledonous and dicotyledonous flowering plants (Jain & Kumar, 2015; Xu *et al.*, 2014). All PR-10 proteins are small (154-163 amino acids), slightly acidic and resistance to proteases.

PR10 has been detected to be constitutively expressed in certain parts of plants throughout growth and development (Jain & Kumar, 2015) as well as exhibited inducible expression in response to various abiotic stresses, such as cold (Jain & Kumar, 2015; wounding (Casanal *et al.*, 2013; Chakravarthi *et al.*, 2016) and phytohormones (Ruszkowski *et al.*, 2014).

Several PR10 genes have recently reported increasing in the proteome of pea (*Pisum sativum*) roots exposed to salinity stress (Srivastava *et al.*, 2004). One of the isoforms, PR 10.1, was found to enhance germination and early seedling even though under high salt concentration (275 mM NaCl) in transgenic *Brassica napus* constitutively expressing the PR 10.1 cDNA (Srivastava *et al.*, 2004).

2.6.1 Abscisic acid-related (ABR) 17 gene

Abscisic acid-responsive 17 (*ABR17*), a family member of pathogenesis-related (PR) 10 protein has been implicated in the defense of plants against pathogen and several environmental stresses. The *ABR17* protein in pea exhibits sequence similarities with pea PR10 proteins and for this reason has been grouped within the PR10 family (Dunfield *et al.*, 2007).

2.6.2 Genetic engineering of *ABR 17*

Moons *et al.* (1997) showed that high NaCl concentration caused rapid and transient increase of endogenous ABA protein eventually regulated antagonistically the expression of salt stress-inducible proteins.

The constitutive expression of pea *ABR17* cDNA in *Arabidopsis thaliana* resulted in an increase in germination under salinity and cold conditions. In addition, these seedlings demonstrated freezing tolerance, indicating that *ABR17* may provide multiple abiotic stress tolerance in other plant as well (Srivastava *et al.*, 2006). Srivastava *et al.* (2007) reported that *ABR17* gene has positively integrated into wild-type *A. thaliana* and enhance its germination under high salt by enhancing endogenous cytokinins levels in the plant.

Recently, Verma *et al*. (2014) discovered that ABR17-transgenic *Arabidopsis thaliana* consists of nine miRNAs that control physiological, biochemical, and stress signaling cascades trigger during salt stress. However, no study has been reported on PR10 protein in response to salt stress condition for pineapple.

CHAPTER 3: MATERIALS & METHODS

3.1 Materials

3.1.1 Sucker

One hundred disease-free pineapples (*Ananas comossus* (L.) Merr.) var. MD2 suckers (approximately 30.0 cm in length) with an approximately 20 green leaves were used as source of explants for shoot regeneration. The suckers obtained from plantation of JTP Trading Sdn. Bhd. in Johor, Malaysia were used as explants for shoot initiation (Figure 3.1). The suckers were trimmed into different sizes and prepared as 3.2.1.

Figure 3.1: Pineapple sucker. (= 1 cm).

3.1.2 *Agrobacterium tumefaciens*

Agrobacterium tumefaciens strain EHA 105 harbouring plasmid pKYLX71 containing *ABR17* gene was supplied by the Plant Biotechnology Research Laboratory, University of Malaya, Malaysia. The map of the plasmid vector was described in Section 2.5.

3.2 Plant tissue culture

3.2.1 Explant preparation

The method for explant preparation was conducted using a protocol from Hamid *et al.* 2012. The outer leaves were removed until the suckers were 2.0 cm wide and 3.0 cm length and were washed under running tap water for 1 min followed by 70% (v/v) Clorox™ (commercial bleach with 5.25% sodium hypochlorite) solution containing 3 drops of Tween 20 (Systerm, Malaysia). The explants were continuously placed on an orbital shaker (Protech-Model 722, Malaysia) for 20 min at 70 rpm. The explants were further excised until 0.5 cm wide and 1.25 cm length under sterile condition. They were then sterilized in 10% (v/v) Clorox™ solution with continuous shaking on a gyratory shaker for 10 min. The explants were trimmed into small pieces (0.5 cm wide and 1.25 cm length) before rinsing three times with sterile distilled water. The cleaned explants were blot dried on a sterile tissue paper for 30 min and cultured on various initiation media as stated in Section 3.1.2.

3.2.2 Initiation of shoots

The explants obtained from Section 3.1.1 were cultured on Murashige and Skoog (1962) (MS) (Appendix A) medium containing 3% (w/v) sucrose, 2 g/L gelrite (Sigma, USA) and supplemented with different concentrations of 6-benzylaminopurine (BAP) (Duchefa Biochemie, Netherlands) and 1-naphthaleneacetic acid (NAA) (Duchefa Biochemie, Netherlands) (Table 3.1). The media were adjusted to pH 5.7 ± 0.2 and autoclaved at 121°C for 20 min. All cultures were incubated at 25 ± 2 °C under a photoperiod of 16 hours daylight with a light intensity of 30 μmol m^{-2} s^{-1} provided by cool-white fluorescent lamps. The experiments were conducted with a total of ten

explants per treatment and were repeated thrice. The number of shoots per explants was recorded after one month of cultured.

Table 3.1: Table of different media formulation and concentrations of BAP and NAA for shoot initiation.

Media	BAP (mg/L)	NAA (mg/L)
MB0N0	0	0
MB0N1	0	1
MB0N2	0	2
MB1N0	1	0
MB1N1	1	1
MB1N2	1	2
MB3N0	3	0
MB3N1	3	1
MB3N2	3	2
MB5N0	5	0
MB5N1	5	1
MB5N2	5	2

3.2.3 Multiplication of shoots in semi solid and liquid media.

After one month of culture, shoots generated from the explants were separated and transferred to multiplication media (Table 3.2) for shoot multiplication. Elongated shoots were subculture onto the fresh media every four weeks. The number of shoots per explants was recorded after 2 months of culture. All cultures were maintained at the same conditions as stated on Section 3.2.2.

The optimum combination of BAP and NAA employed in shoot multiplication medium was used in MS liquid medium (without gelrite). The explants were cultured in 250 mL flask with 50 mL liquid and placed on a gyratory shaker (WiseCube, Wisd, Korea) at 70 rpm for 1 month. Liquid media were changed at 2 week-intervals.

Table 3.2: Different concentrations and combinations of BAP and NAA of explants for shoot multiplication.

Media	BAP (mg/L)	NAA (mg/L)
MB0N0	0	0
MB1N0	1	0
MB2N0	2	0
MB3N0	3	0
MB0N1	0	1
MB1N1	1	1
MB2N1	2	1
MB3N1	3	1
MB0N2	0	2
MB1N2	1	2
MB2N2	2	2
MB3N2	3	2

3.2.4 Rooting media

Hundred *in vitro* pineapple plantlets from multiplication media were cultured in MS media supplemented with 30 g/L sucrose, 2 g/L gelrite and 8 g/L charcoal to enhance root formation. All plantlets (4-6cm) were used to determine the root length for this experiment for two weeks. All cultures conditions were maintained as described in Section 3.2.2.

3.2.5 Acclimatization

Rooted plantlets were dipped in 1 % (v/v) Imas-Thiram 80 (Imaspro Resources Sdn. Bhd., Malaysia), rinsed with water and transferred to peat soil in a 7 × 13 cm polyethylene bag. Plantlets were grown in a greenhouse at the University of Malaya (60 % relative humidity, 24 ± 2ºC with photosynthetic photon flux of 400-500 µmol $m^{-2}s^{-1}$) for 3 months. Mixed fertilizer containing 15 N: 15 P: 15 K (Tesco, Malaysia) were applied around each plantlet. Plantlets were irrigated for 5 min with approximately 250 mL water per session at 9.00 am each day using an automatic dripper irrigation system embedded in the soil.

3.3 Morphological and physiological assessments

Morphological and physiological assessments were carried out on tissue culture plants being acclimatised during 0, 14, 28, 42 days. Field grown plants waere used as a comparison to the acclimatised tissue culture plants.

3.3.1 Morphological examination

Plant growth parameters for morphological examination was recorded, namely number of leaf (N), length of a leaf (L), width of a leaf (W), number of roots (R), plant height (H), leaf area (A), fresh weight (F) and dry weight (D) of a leaf (Oven dry at 70 ºC until constant weight was attained), after 0, 14, 28, 42, 56 and 84 days of acclimatization and compared with the field grown plant. The data was recorded in an average for length, width, leaf area, fresh weight and dry weight was taken from three different leaves of the same plant.

3.3.2 Physiological examination

Photosynthetic rate (P), stromata conductance (S), intercellular CO_2 content (C_i), and transpiratation rate (T) of thirty *in vitro*-derived plantlets were recorded using a Photosynthetis Measurement System (Li-COR Biosciences LI6400 Lincoln, Nebraska) according to Villalobo *et al*. (2012) after 0, 14, 28, 42, 56 and 84 days of acclimatization. Light was fixed at 400 µmol/mol. Field-grown plants (308 days) were used as a control.

3.3.3 Measurement of chlorophyll

Chlorophyll was extracted from five randomly selected young and mature leaves harvested after 0, 14, 28, 42, 56 and 84 days of acclimatization. Leaves were incubated at 65 ºC for 1 h until submerged in (~3 mL) dimethyl sulfoxide (Sigma-Aldrich,

Germany). Chlorophyll content was measured at 645 and 663 nm according to Aragon *et al*. (2012) and calculated using the equations described by Porra *et al*. (2002) in mg Chl cm^{-2} leaf area (Richardson *et al*., 2002).

3.3.4 Scanning electron microscope (SEM)

Stomata diameter were observed under Field Emission Scanning Electron Microscope (FESEM), model FEG Quanta 450, EDX-OXFORD. Samples were excised into approximately 1 cm and vacuumed using high pressure on each side to characterize the morphology structure for both abaxial and adaxial sides of the leaves.

3.3.5 Histological examination

Histological examination was carried out on leaves harvested at 0, 14 and 42 days during acclimatization according to Jalil *et al*. (2008). Field-grown plants were used as a control. Slides were mounted with mounting medium for preservation and allowed to dry thoroughly before being examined under an inverted Olympus IX73PS2F (Japan) and photographed using DP-71 image capturing system.

3.4 Preparation of *Agrobacterium tumefaciens*

Before undergo *Agrobacterium*-mediated transformation protocol, the gene of interest (GOI) need to be conformed present inside of the transformed *Agrobacterium* since the GOI (*ABR17* gene) had already been inserted into *Agrobacterium* harboring PKYLX71 plasmid from Plant Biotechnology Research Laboratory (PBRL), UM. Molecular assessment need to be conducted by obtaining the *Agrobacterium*'s growth curve (section 3.4.1) until the *ABR17* gene confirmed to be present by blasting the gene

sequence using NCBI's website with other *ABR17* gene sequences stored in the website (section 3.9).

3.4.1 *Agrobacterium* growth curve

Agrobacterium tumefaciens were prepared by inoculating 100 µL of *Agrobacterium tumefaciens* harboring pKYLX71 gene with *ABR17* gene supplied from PBRL, UM in 10 mL fresh Luria-Bertani (LB) broth (Merck, Germany) supplemented with 50 mg/L kanamycin in a universal container. The bacterial culture was incubated at 28°C in a refrigerated shaker incubator with constant shaking of 150 rpm in the dark. The optical density (OD) of the bacterial culture was measured using a spectrophotometer (Eppendorf Biophotometer, Germany) at wavelength 600 nm at one hour interval until death phase of bacterial growth was achieved.

3.4.2 Determination of *Agrobacterium* cell density

Fresh bacterial culture was prepared by inoculating 100 µL of the overnight bacterial culture into 10 mL LB broth containing 50 mg/L kanamycin in a universal container and maintained as described in Section 3.4. *Agrobacterium* culture was harvested at 5-hours intervals.

3.4.3 Identification of Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of antibiotics towards *Agrobacterium tumefaciens*

The combination of carbenicilin and cefotaxime were used as bacteriostatic and bactericidal agents in *Agrobacterium*-mediated transformation experiment to determine the lowest concentration of antibiotic that can suppress the bacterial growth. A fresh

overnight bacterial culture (100 µL) was inoculated into 10 mL LB broth supplemented with different combinations and concentrations of carbenicilin (0, 50, 100, 150, 200, 250 mg/L; Duchefa laboratories, Netherlands) and cefotaxime (0, 50, 100, 150, 200, 250 mg/L; Duchefa laboratories, Netherlands) and maintained as described in Section 3.2.2. The MIC was determined by observing the turbidity of LB broth containing *Agrobacterium*, whereas the minimal bactericidal concentration (MBC) experiment was carried out by spreading 20 µL culture obtained from each universal container onto LB agar plates and incubated at 28ºC in the dark. After 3 days of culture, the LB agar plates were examined for bacterial colonies. The lowest concentration of antibiotic which completely inhibited the bacterial growth was considered as MBC.

3.4.4 Preparation of *Agrobacterium* glycerol stock

Single colony of *A. tumefaciens* from overnight bacterial culture from section 3.4.3 was harvested at mid-log phase was cultured in LB broth. To prepare the glycerol stock, 850 µL fresh bacterial broths was mixed with 150 µL sterilized glycerol in 1.5 mL microcentrifuge tubes. The glycerol stock was then stored at -80 ºC until use. The purpose of preparing glycerol stock of *A. tumefaciens* harboring PKYLX71 vector with ABR17 gene is to ensure we have the materials for back up purposes.

3.5 Extraction of plasmid DNA from *Agrobacterium*

Plasmid DNA of *A. tumefaciens* strain EHA 105 carrying *ABR17* gene was extracted using the Plasmid Mini Kit (QIAGEN, USA). *A. tumefaciens* grown overnight in 10 mL LB broth containing 100 µL kanamycin was harvested by centrifugation at 10,000 rpm for 1 min. Pellets were resuspended in 300 µL Buffer P1 to degrade RNA. Then, 300 µL Buffer P2 was added and mixed vigorously by inverting the tube for six

times. The samples were incubated at room temperature for 5 min. Pre-chilled P3 buffer (300 µL) was added to the tube and mixed vigorously. After incubation at -4 ºC for 5 min, the samples were centrifuged at 13,000 x g for 10 min. The supernatant was applied into a Qiagen-tip 20 column which was equilibrated by 1 mL Buffer QBT. Buffer QC (2 mL) was added into the column to wash the DNA and this step was repeated to eliminate contaminants. DNA was then eluted with 800 µL Buffer QF in a new 1.5 mL microcentrifuge tube and precipitated with 700 µL isopropanol before centrifuged at 10,000 rpm for 30 min. Pellet was washed with 1 ml of 70 % (v/v) ethanol followed by centrifugation at 10,000 rpm for 10 min. Pellet was air-dried for 30 min and dissolved again in 30 µL TE buffer.

3.5.1 PCR analysis

PCR reactions were carried out according to Sambrook *et al*. (1989) using 58°C as the optimal annealing temperature suitable for *ABR17* gene. Table 3.3 showed a list of primers designed flanking *ABR17*, endogenous genes (*Bromelain*, *Cystatin* and *Actin*) and *Tetr* gene designed using software Primer 3.0. The 20 µL PCR reaction contained 1X PCR buffer (iNtRON BIOTECHNOLOGY, Korea), 1 mM dNTPs, 0.25 µM forward primers, 0.25 µM reverse primers, 1 U of i-Taq^{TM} polymerase and 100 ng of DNA template. Amplification was performed in a PCR thermal cycler machine (Biometra, USA). The cycle conditions were as follows: initial denaturation at 94 ^{o}C for 2 min, followed by 30 cycles of 94 ^{o}C for 30 s, 58.5 ^{o}C for 30 s, 72 ^{o}C for 30 s followed by a final extension at 72 ^{o}C for 10 min.

Table 3.3: List of primers designed flanking *ABR17*, endogenous gene (*Bromelain*, *Cystatin* and *Actin*) and *Tetr* gene.

Primers name	Sequences	Length	GC contents (%)	Melting Temperature (Tm)
ABR-F	5'-GTG GTC GAA GCT TAT GGG TGT CTT TGT TTT TGA TGA ATA C-3'	40 mer	40	54
ABR-R	5'-TAT ATA GCT CGA GTT AGT AAC CAG GAT TTG CCA AAA CGT AAC C-3'	43 mer	49.5	52.4
BRO-F	5'- CAA GGA CAA CGA CGA GAA GA-3'	20 mer	50	56
BRO-R	5'- CAA ATG ACA CCA CTG GCT C-3'	19 mer	52.6	54.5
AcCYS 1-F	5'- CCC CTC AAT AAC GAG AAC G-3'	19 mer	52.6	54.5
Accys1-R	5'- TCT TCT TGC CAG CAT CAT TCA-3'	21 mer	47.6	58
Act-F	5'- GTG GCA CTT GAC TTT GAG CA-3'	20 mer	50	54.3
ACT-R	5'- CTT CCT GAT ATC CAC ATC GC-3'	20 mer	50	56.7
Tetr-F	5'- GTA CGT TGG AGC CGC ATT AT – 3'	20 mer	50	59.9
Tetr-R	5'- TGC AGA GCC AGC CTT CTT AT – 3'	20 mer	50	50.1

3.6 Electrophoresis

The amplified PCR products were analyzed by electrophoresis on a 1.5% (w/v) agarose gel. The gel was prepared by dissolving 0.6 mg of agarose powder (Invitrogen, USA) into 40 ml of 1 × TBE solutions (Tris-borate-EDTA: 90 mM Tris, 90 mM borate and 1 mM EDTA). Then, the gel was heated for 1 min in a microwave. A total of 1.0 µL Ethidium Bromide (EtBr) was added into the gel solution before being poured into a gel cast. After 30 min, the comb was removed and the gel was placed in a gel electrophoresis tank containing 1 × TBE buffer. Subsequently, 5 µL of PCR products was gently mixed with 1 µL of 6 X GLD with pipette and loaded into each well along with the 2.5 µl of 100 bp ladder (Vivantis, Malaysia) and electrophoresis at 125 V and 400 A for about 23 min. The gel was observed under ultraviolet light using UV trans-illuminator (Gel-Pro Imager, USA) and viewed using Gel Pro Analyzer 3.1 software.

3.7 DNA Purification

DNA purification was conducted to ensure that the amplified DNA fragment is *ABR17* gene through gene sequencing. Fragments of ~500 bp were purified from 1.5% preparative agarose gels using Gel Extraction Kit (Axygen, USA). The tube containing the excised gel fragment was centrifuged for 30 seconds at 12,000 × *g*. A total of 300 µl buffer DE-A was added into the tube and vortex before being immersed in a water bath at 75˚C for 6-8 min. After the gel was completely dissolved, 100 µl of isopropanol was added for DNA precipitation. The mixture was transferred to a new 2 ml microcentrifuge tube with a miniprep column before centrifugation at 12,000 × *g* for 1 min. After the filtrate was discarded, 500 µl of Buffer W1 was added to the miniprep column and centrifuged at 12,000 × *g* for 30 s. The filtrate was discarded. Buffer W2 (500 µl) that had been pre-mixed with 95% ethanol was added at once on the miniprep column and centrifuged at 12,000 × *g* for 30 s. After the filtrate was discarded, 700 µl of Buffer W2 was added on the miniprep column and centrifuged at 12,000 × *g* for 1 min. This step was repeated once. After being transferred to a new 1.5 ml microcentrifuge tube, 30 µl of eluent buffer (pre-warmed at 65 ˚C) was added into the miniprep column. The mixture was incubated at room temperature for about 30 min before centrifugation at 12,000 × *g* for 1 min.

3.8 Electrophoresis of purified DNA

The purified DNA was separated by electrophoresis on a 0.8 % agarose gel. The gel was prepared by dissolving 0.32 g agarose powder in 20 ml of 1 × TBE. A total of 5 µl purified sample was mixed with 1 µl loading dye before being loaded into the gel. Electrophoresis was performed as described in Section 3.6.

3.9 Sequence analysis

The 493 bp nucleotides sequence of *ABR17* gene amplified from the pKYLX71 plasmid was sequenced by First Base Sdn. Bhd., Malaysia and analyzed using *blastn* and *blastp* programs in National Center for Biotechnology Information (NCBI) website. A number of 9 outgroups of *ABR17* nucleotides were downloaded from NCBI (Figure 4.19) and aligned with *ABR17* gene obtained from *pKYLX71* plasmid using MEGA (Molecular Evolutionary Genetics Analysis, Version 6.0, Tamura *et al*. 2013). Cluster W option was used to align the sequences in MEGA software before constructing a phylogeny analysis to analyze the relationship between the out groups of *ABR17* gene and the *ABR17* obtained from the plasmid.

3.10 *Agrobacterium*-mediated plant transformation

3.10.1 Determination of minimal inhibitory concentration (MIC) of kanamycin (Km)

Leaf base explants (1 cm wide and length) were cultured on CM medium (MS medium with 2 g/L sucrose, 30 g/L sucrose supplemented with 2 mg/L BAP and 1 mg/L NAA) containing different concentrations of kanamycin (0, 25, 50, 75, 100 and 150 mg/L) to determine the minimum kanamycin concentration for the selection of untransformed and transformed plants. All cultures were maintained as described in Section 3.2.2. The media where 50 % of the leaf base explants respond towards kanamycin concentration by producing shoot will be known as LD_{50} and choose for further experiment.

3.10.2 Determination of 100% Lethal dose (LD_{100}) towards sodium chloride (NaCl)

The natural tolerance of pineapple plants towards salinity stress was conducted by culturing regenerated shoots on MB0N0 media (Table 3.1) containing different concentrations of sodium chloride (NaCl; Sigma-Aldrich, USA) at 0, 100, 200, and 300 mg/L. All culture conditions were maintained as described in Section 3.1.2. The numbers of survival shoots and LD_{100} were recorded after one month of culture. A number of 10 explants was used in each treatment and performed in triplicate. The media where 100 % of the leaf base explants respond towards sodium chloride concentration by not producing shoot will be known as LD_{100} and choose for further experiment.

Prior to transformation, *Agrobacterium* culture was initiated from the glycerol stock in 10 mL LB broth supplemented with 50 mg/L kanamycin and incubated at 28 ºC with constant shaking on an incubator gyratory shaker overnight. Mid-log phase culture was used for plant transformation experiment.

3.10.3 Infection and co-cultivation of explants with *Agrobacterium tumefaciens*

In vitro leaf explants (1 cm × 1 cm) obtained from Section 3.2.3 was precultured on the multiplication medium for three days before transformation. All explants were wounded, incubated in the *Agrobacterium* suspension containing different concentrations of acetosyringone (0, 100, 200, and 300 µM) and incubated at 28^{o}C for various infection times (0, 10, 20, and 30 min) under continuous shaking condition of 150 rpm in the dark. The inoculated explants were blot dried on a sterile tissue paper for

15 min before being co-cultivated for either one, two or three days in the dark. Each treatment consisted of 10 explants and the experiment was repeated.

3.10.4 Selection of putative transformants

After co-cultivation, the explants were washed three times with 30 mL sterile distilled water containing 250 mg/L cefotaxime and 50 mg/L carbenicilin for 10 min to eliminate *A. tumefaciens*. The leaf bases were blot dried on a sterile tissue paper and transferred to multiplication media (MS medium supplemented with 30 g/L sucrose, 2 g/L gelrite, 2 mg/L BAP, 1 mg/L NAA, 250 mg/L cefotaxime, 50 mg/L carbenicilin and 50 mg/L kanamycin) for three months. Shoots more than 2 cm were counted as positive putative transformants after three month of culture. Explants were transferred to new media every 2 weeks.

3.11 Verification of putative transformants

3.11.1 DNA extraction

DNA was extracted using CTAB method (Doyle & Doyle, 1987) with some modifications. Approximately 0.1 g fresh pineapple leaf tissue from shoot regenerated in section 3.4.4 was ground into fine powder in the presence of liquid nitrogen using mortar and pestle and subsequently transferred into 2 mL microcentrifuge tube. Extraction buffer (497 µL CTAB (Sigma, Germany) and 3 µL 2-mercaptoethanol (Sigma, Germany) was added to the powder and vortexed for 1 min before incubated at 65 ºC in a Thermocell cooling and heating block (Fisher Scientific, USA) for 1 h. A total of 500 µL Chloroform: Isoamylacohol (24:1) was added to each sample and inverted slowly for 1 min. After centrifugation at 10,000 rpm for 20 min, three layers

namely aqueous phase (top layer), debris and protein (middle layer), and chlorofom (bottom layer) were formed. The aqueous layer was carefully transferred into a new 1.5 mL microcentrifuge tubes before adding 500 µL Chloroform: Isoamylacohol (24:1). The mixture was mixed by inverting slowly for 1 min followed by centrifugation at 10,000 rpm for 15 min. The aqueous layer was transferred into a new 1.5 mL microcentrifuge tube and mixed with 266 µL isopropanol. The mixture was then stored at -20 ºC fridge for overnight. After centrifugation at 12,000 rpm for 20 min, 700 µL 70 % ethanol was added to wash the pellet. The tube was centrifuged again at 12,000 rpm for 10 min. Pellet was air dried for 30 min and dissolved in 30 µL TE buffer. To eliminate RNA, 1 µL RNase (10 µg/mL) was added into each sample and incubated at 37 ºC for 20 min. The RNase treated samples were used for PCR amplification or stored at -20 ºC freezer.

3.11.2 DNA quantification

The extracted DNA was quantified using a NanoDrop spectrophotometer (Implen, Germany) at wavelength of 260 nm (OD_{260}) and 280 nm (OD_{280}) using the equation below:

$$\text{DNA Concentration } (\mu g/\mu L) = \frac{OD_{260} \times \text{Dilution Factor} \times 50\ \mu g/mL}{1000}$$

The purity of DNA samples were indicated by the ratio of OD_{260} to OD_{280} (OD_{260} / OD_{280}) within the range of $1.7 \leq x \leq 2.0$. The integrity of DNA samples were checked by gel electrophoresis as described in Section 3.6.

3.11.3 PCR analysis for *Tetr* and housekeeping genes

The present of the genes were scored by the gel observed under ultraviolet light using UV trans-illuminator (Gel-Pro Imager, USA) and viewed using Gel Pro Analyzer 3.1 software. The presence of the band with expected sizes according to the table 3.3.

3.11.4 Southern blot analysis

Southern blot analysis been conducted to verify DNA integration and gene copy number into the plant genome.

3.11.4.1 Probe efficiency

Approximately 1 µg of DNA template was added to 1 µL of sterile distilled water to a final volume of 16 µL in a reaction vial. Then, the DNA was denatured by heating in a boiling water bath for 10 min and quickly chilling in an ice/water bath. Subsequently, 5 µL of DIG-High Prime was mixed with 4 µL of the denatured DNA, and centrifuged briefly before being incubated for 20 h at 37 ºC. Lastly, the reaction was stopped by heating the mixture to 65 ºC for 10 min. After the probe has been synthesized, 1 µL of the labeled probe and the control were spotted onto the nylon membrane. Then, the nucleic acid was fixed to the membrane by baking for 30 min at 120 ºC. The membrane was then transferred into a plastic container with 20 mL Maleic acid buffer and incubated under shaking condition for 2 min at room temperature. Maleic acid buffer was then resuspended with 10 mL blocking solution for 30 min, followed by adding 10 mL antibody solution for 30 min in shaking conditions. After the buffer was resuspended, the membrane was washed two times with 10 mL washing buffer for 15 min. The membrane was then equilibrating in 10 mL detection buffer for 5 min. Lastly, the membrane was incubated in 2 mL freshly prepared colour substrate solution in the dark overnight before the reaction was stopped. The desired spot was

observed by washing the membrane for 5 min with 50 mL of PCR grade water. Results were documented by viewing under white light.

3.11.4.2 DNA digestion

DNA digestion using an appropriate digestion enzyme will form fragmented DNA. A combination of various amount of EcoRI FastDigest enzyme, HindIII, plasmid DNA, 10X FastDigest Green Buffer (Thermo Scientific, USA) and nuclease-free water were prepared and mixed thoroughly at various concentration of genomic DNA of plasmid and plantlets for DNA digestion (Appendix E). Following that, both mixtures were mixed by vortexing prior to incubation at 37 ºC in a heat block for 5, 15 and 25 min. The samples were observed by using electrophoresis as mentioned in Section 3.6. Undigested sample was also loaded into the gel as a control. The optimum DNA digestion concentration was used to undergo Southern Blot experiment.

3.11.4.3 Hybridization

The digested DNA was hybridized with the selected probe which was synthesized in Section 3.11.4.1. After electrophoresis, the gel was stained in 1% EtBr for 30 min before destaining in sterilled distilled water for 30 min. Presence of both DNA were then viewed under white light with UV Transilluminator (AlphaInnoTech, Germany). After viewing, the gel was soaked in depurination solution for 15 min before being washed with sterile distilled water for three times at 3 min each. The gel must be agitated on a belly dancer with 4x speed to ensure all gel surface were soaked with the solutions. Then, the gel was soaked in alkaline transfer buffer for 15 min, and then resuspended into a new alkaline transfer buffer for 20 min. Meanwhile, N membrane

was cut into 1mm larger than the gel before soaking into sterile distilled water for 1 min, followed by alkaline transfer buffer for 5 min. Vacuum infilteration was setup as in Figure 3.2 After arranging the vacuum infilteration, the pump was set for 100 mbar. After 2 h, the N membrane was soaked in Neutralization buffer II for 15 min before destaining the gel with 1 % EtBr for 30 min before viewing under UV light. Then, the N membrane was soaked and agitated at 93 rpm in pre-warmed hybridization buffer for 30 min at 42 ºC. Designed labelled probe was denatured at 100 ºC for 5 min and transferred immediately to ice. Then, the membrane was soaked and agitated in 9.2 µL of denatured probe with 5 mL pre-warmed Hybridization buffer overnight.

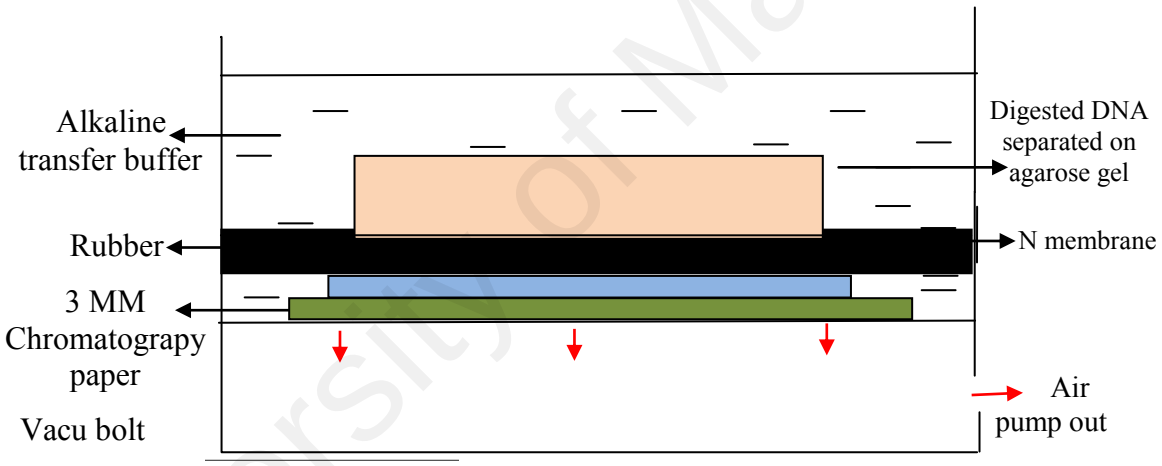


Figure 3.2: An illustration of vacuum infilteration set-up for southern transfer. Arrow represents the flow of the air pumping out.

3.11.4.4 Stringency washes

A stringency wash of the N membrane is to remove any unhybridized probe. After overnight incubation, the N membrane was washed with 2X SSC and 0.5% SDS for 5 min at room temperature. Then, the solution was resuspended into a new 2X SSC and 0.1% SDS for 15 min at room temperature, followed by 1X SSC and 0.1% SDS for 1 h at 65 ºC. Lastly, the N membrane was washed with 0.1X SSC for 5 min at room temperature under shaking condition.

3.11.4.5 Immunological detection

Immunological detection is to detect immunological process of the hybridized probe and the genomic DNA of the sample. After hybridization and stringency washes, detection of immunological procedure was done by rinsing the N membrane briefly for 5 min in washing buffer. Then, the N membrane was incubated for 30 min in 10 mL blocking solution followed by resuspending the membrane with antibody solution and incubated for 30 min. The N membrane was repeatedly washed in 10 mL Washing buffer before equilibrating the N membrane in 10 mL detection buffer for 5 min. Finally, the N membrane was incubated in 10 mL freshly prepared colour substrate solution in an appropriate container in the dark overnight. The container could not be shaken during colour development to obtain optimal result. The reaction was stopped by washing the N membrane with 50 mL of PCR grade water. The results were documented by photographing the N membrane under white light.

3.12 Statistical analysis

The data were recorded and statistically analyzed by one-way analysis of variance (ANOVA) followed by Duncan multiple range test (DMRT) at a significance level of $p < 0.05$ using SPSS software version 22 for window operating system.

CHAPTER 4: RESULTS

4.1 Plant tissue culture

4.1.1 Effects of different sizes of explants on shoot initiation

The explants with about 0.50 × 0.50 cm in size did not showed any contamination, whereas the highest percentage of contamination (43 %) was detected for explants with about 1.25 × 1.25 cm in size (Table 4.1). Most of the bacterial contamination was observed at the base of the meristem explants. The contamination was confirmed as bacteria since no spores were observed. Explants of about 1.0 × 1.0 and 1.25 × 1.25 cm sizes showed 100% shoot formation, whereas no shoots were formed from the explants with about 0.50 × 0.50 cm in size (Table 4.1). Given that explants of 1.0 × 1.0 cm (Figure 4.2a) were able to produce the highest number of shoots with the lowest contamination, this size of explants was selected for the subsequent experiments.

Table 4.1: Effects of various sizes of explants on shoot formation cultured on MS media (MB3N1) based on the percentage of explants forming shoots, mean number of shoots per explants and the percentage of contaminated explants.

Sizes (cm)	Explants forming shoot (%)	Contamination frequency (%)	Mean no. of shoots/explant
0.50 x 0.50	0 ± 0.00^{bc}	0 ± 0.00^{c}	1 ± 0.01^{b}
0.75 x 0.75	18.3 ± 0.01^{b}	2.8 ± 0.01^{c}	2 ± 0.10^{ab}
1.00 x 1.00	100 ± 0.00^{a}	11.3 ± 0.03^{b}	5 ± 0.02^{a}
1.25 x 1.25	100 ± 0.00^{a}	43.1 ± 0.06^{a}	4 ± 0.01^{a}

- Shoots shorter than 2 mm were not counted
- Results followed by different letters are significantly different at $p < 0.05$ according to Duncan multiple range test (DMRT)
- Means of the treatment in the same column followed by different letters are significantly different from each other at the 5 % level

4.1.2 Effects of different culture medium on shoot initiation

Shoot bud initiation was recorded from explants cultured on different media with various combination and concentration of BAP and NAA after one month of culture (Figure 4.1; Table 4.2). The highest number of shoot buds (6.70 ± 0.3) was recorded when cultured on MB3N1 (MS medium containing 30 g/L sucrose, 0.1 g/L Myo-inositol, 2 g/L gelrite supplemented with 3 mg/L BAP and 1 mg/L NAA), whereas no shoot buds were initiated on media without BAP (B0): MB0N0 (control), MB0N1 (1 mg/L NAA) and MB0N2 (2 mg/L NAA). The combinations of PGR in medium favoured shoot bud formation although BAP alone was able to induce shoot buds. Increased BAP from 3 mg/L to 5 mg/L, however, affected the shoot initiation. Roots emerged from media supplemented with only NAA (Figure 4.2c).

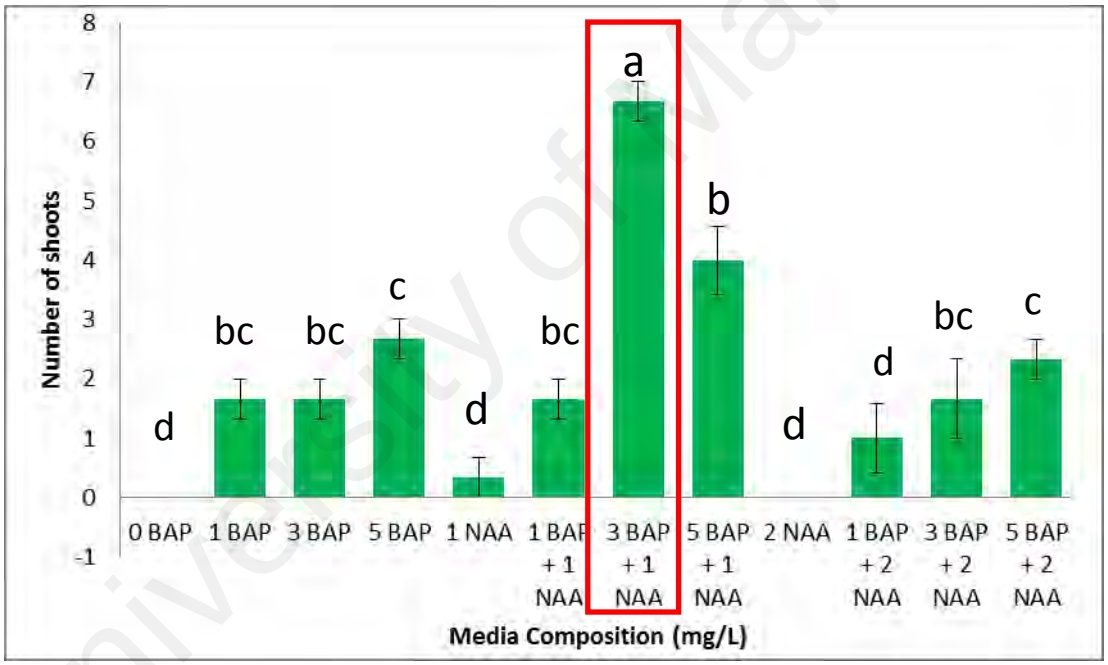


Figure 4.1: Effect of different concentrations of BAP and NAA on shoot initiation of pineapple.

Table 4.2: Effects of different concentrations and combinations of BAP and NAA of explants. The number of shoots produced from explants cultured in direct shoot initiation medium.

Media	BAP (mg/L)	NAA (mg/L)	Number of shoots
MB0N0	0	0	0.00 ± 0.00^{d}
MB0N1	0	1	0.00 ± 0.00^{d}
MB0N2	0	2	0.00 ± 0.00^{d}
MB1N0	1	0	1.71 ± 0.15^{bc}
MB1N1	1	1	1.72 ± 0.12^{bc}
MB1N2	1	2	1.21 ± 0.11^{bc}
MB3N0	3	0	2.25 ± 0.14^{b}
MB3N1	3	1	6.70 ± 0.3^{a}
MB3N2	3	2	1.52 ± 0.22^{c}
MB5N0	5	0	2.82 ± 0.21^{b}
MB5N1	5	1	4.02 ± 0.11^{b}
MB5N2	5	2	2.41 ± 0.12^{b}

- Shoots shorter than 2 mm were not counted
- Treatment means in a column followed by different letters are significantly different at $p < 0.05$ according to Duncan multiple range test (DMRT)
- Means of the treatment in the same column followed by different letters are significantly different from each other at the 5 % level

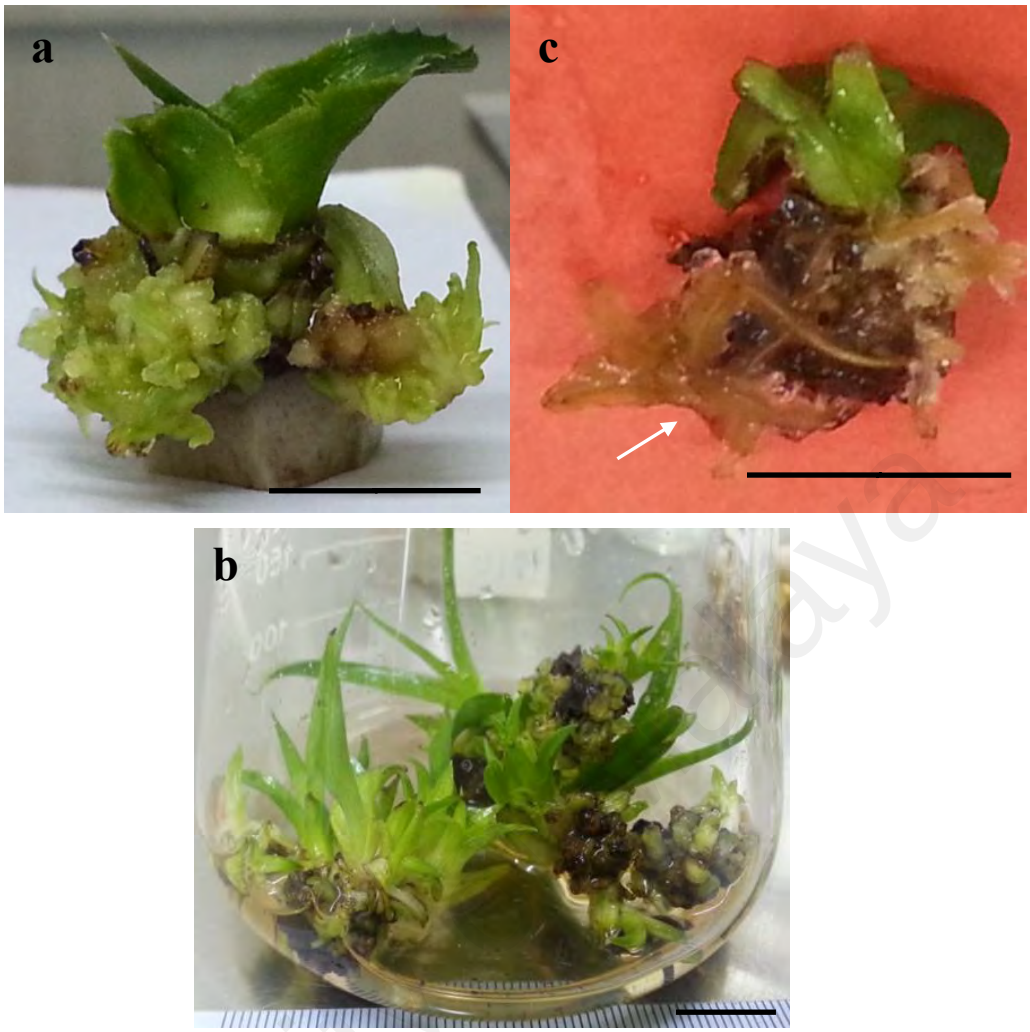


Figure 4.2: *In vitro shoot multiplications* after one month of culture. **a**: Initiation of shoots on MS medium supplemented with 3 mg/L BAP and 1 mg/L NAA in semi-solid medium. **b**: Multiplication of shoots on MS medium supplemented with 2 mg/L BAP and 1 mg/L NAA in liquid medium. **c**: Initiation of roots (arrows) on MS medium supplemented with only NAA. (Bar = 1cm).

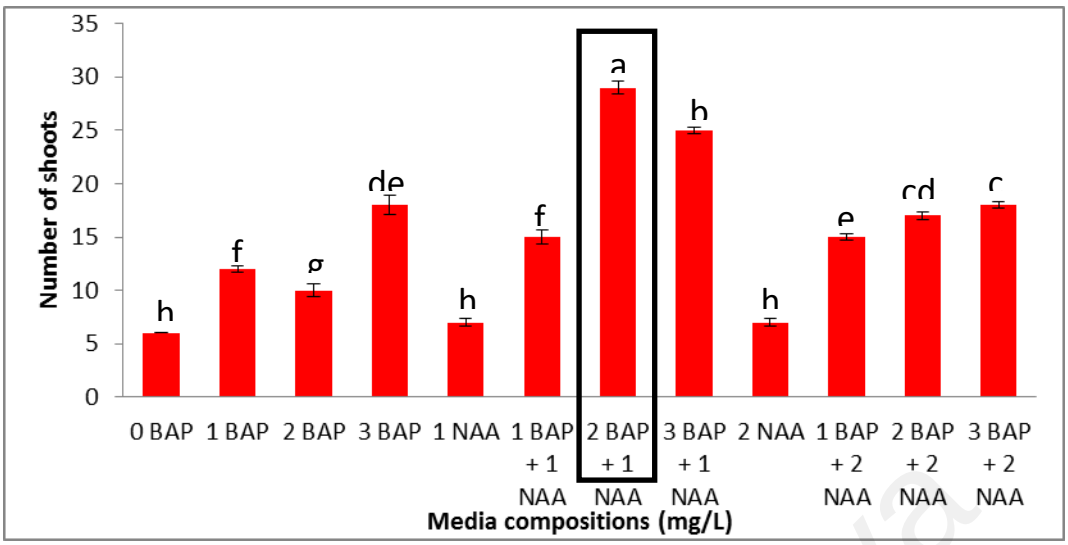


Figure 4.3: Effect of different concentrations of BAP and NAA on shoot multiplication of pineapple.

Table 4.3: Effects of different concentrations and combinations of BAP and NAA of explants. The number of shoots produced from explants cultured in direct shoot multiplication solid medium.

Media	BAP (mg/L)	NAA (mg/L)	Number of shoot	Shoot length (cm)
MB0N0	0	0	1.10 ±0.2^{c}	0.20 ±0.21^{bc}
MB1N0	1	0	12.50 ±0.1^{b}	0.80 ±0.01^{b}
MB2N0	2	0	10.00 ±0.2^{b}	0.50 ±0.23^{b}
MB3N0	3	0	17.20 ±0.1^{b}	0.60 ±0.21^{b}
MB0N1	0	1	1.51 ±0.0^{c}	0.20 ±0.11^{bc}
MB1N1	1	1	15.00 ±0.2^{b}	1.47 ±0.01^{ab}
MB2N1	2	1	30.10 ±0.1^{a}	1.53 ±0.21^{a}
MB3N1	3	1	25.10 ±0.1^{ab}	1.56 ±0.15^{a}
MB0N2	0	2	1.10 ±0.2^{c}	0.20 ±0.11^{c}
MB1N2	1	2	15.00 ±0.1^{b}	1.41 ±0.01^{a}
MB2N2	2	2	15.90 ±0.1^{b}	1.34 ±0.21^{a}
MB3N2	3	2	16.20 ±0.3^{b}	1.39 ±0.22^{a}

- Shoots shorter than 2 mm were not counted
- Treatment means in a column followed by different letters are significantly different at $p< 0.05$ according to Duncan multiple range test (DMRT)
- Means of the treatment in the same column followed by different letters are significantly different from each other at the 5 % level

4.1.3 Multiplication of shoots

To reduce arbitrary influence, single source of shoot buds which originated from the best initiation media (MB3N1) was used to determine the best multiplication media after one month of culture (Figure 4.3). The highest number of shoot buds (30.10 ±0.1) was found in semi-solid MS medium containing 2 mg/L BAP and 1 mg/L NAA

(MB2N1) (Table 4.3). The number of shoot buds in culture media in the presence of NAA but without BAP (MB0N1 and MB0N2) were not significantly different from the control (MB0N0). In general, the combination of BAP and NAA enhanced the number of shoot bud proliferation. Increasing BAP from 2 mg/L to 3 mg/L in medium supplemented with 1 mg/L NAA showed similar pattern with the previous shoot bud initiation experiment (4.1.2).

4.1.4 Effects of semi-solid and liquid media on shoot multiplication

The effects of semi-solid and liquid media for shoot multiplication were determined using the best multiplication media (MB2N1) obtained from Section 4.1.3. The number of shoots multiplied in liquid medium was 1.7-fold higher than semi-solid medium after one month of culture (Table 4.4; Figure 4.2b). The shoot length in both types of media, however, was not significant due to the container shape.

Table 4.4: Effect of multiplication medium in solid and liquid medium. Explants were obtained from optimal multiplication treatment above.

Media	BAP (mg/L)	NAA (mg/L)	Number of shoot	Shoot length (cm)
MB2N1 (SOLID)	2	1	30.0 ± 0.06^{b}	1.67 ± 0.21^{a}
MB2N1 (LIQUID)	2	1	52.1 ± 0.04^{a}	1.73 ± 0.21^{a}

- Shoots shorter than 2 mm were not counted
- Treatment means in a column followed by different letters are significantly different at $p < 0.05$ according to Duncan multiple range test (DMRT)
- Means of the treatment in the same column followed by different letters are significantly different from each other at the 5 % level

4.1.6 *In vitro* rooting

In vitro-derived shoots generated from the optimized shoot multiplication medium were transferred to rooting medium. The highest number of roots (11 roots per shoot) was achieved on the medium containing 4 mg/L activated charcoal (AC) with a mean length of about 8.6 cm (Table 4.5; Figure 4.4). About 2.5 roots per shoot was recorded when plantlets were rooted on the medium without AC (control). Medium containing high concentrations of AC (6, 8 and 10 mg/L) produced significantly higher fresh weight than other concentrations (2 and 4 mg/L). The morphology of the control showed fine and friable roots (Figure 4.4a) compared to thick and strong roots obtained from shoots on media containing AC.

Table 4.5: Effects of different concentrations of charcoal in rooting medium.

Medium	Charcoal (g/L)	Number of root (cm)	Length of root (cm)	Dry weight (g)
MC0	0	2.5 ± 0.01^{b}	3.0 ± 0.01^{b}	0.00 ± 0.02^{b}
MC2	2	7.0 ± 0.01^{b}	7.5 ± 0.02^{ab}	0.00 ± 0.01^{b}
MC4	4	11.1 ± 0.01^{a}	8.6 ± 0.03^{ab}	0.01 ± 0.03^{ab}
MC6	6	9.3 ± 0.02^{ab}	8.8 ± 0.01^{ab}	0.02 ± 0.01^{a}
MC8	8	9.0 ± 0.01^{ab}	11.1 ± 0.02^{a}	0.02 ± 0.01^{a}
MC10	10	8.0 ± 0.01^{b}	10.3 ± 0.01^{a}	0.02 ± 0.02^{a}

- Roots shorter than 1 cm were not counted
- Treatment means in a column followed by different letters are significantly different at $p< 0.05$ according to Duncan multiple range test (DMRT)
- Means of the treatment in the same column followed by different letters are significantly different from each other at the 5 % level

Figure 4.4: *In vitro* rooting on MS medium containing 30 g/L sucrose, 2 g/L gelrite and (a) 0, (b) 2, (c) 4, (d) 6, (e) 8, and (f) 10 g/L of activated charcoal. (Bar = 1 cm).

4.1.7 Survival rates, plant growth and leaf characteristics

In vitro plantlets survived (100%) through acclimatization in a green house were healthy with uniform growth and phenotipically normal compared to field-grown derived plants (Appendix B; Figure 4.5). All parameters tested for *in vitro* derived plantlets showed were general increased over 84 days of transfer, but were lower than field-grown derived plants (Appendix B). It is noteworthy that the chlorophyll content and length of leaf for field-grown plants were 2.3- and 2-fold, respectively, higher than the *in vitro*-derived plantlets.

Figure 4.5: Stages of acclimatization for *in vitro*-derived pineapple plant. (a) 0 (b) 14 (c) 28 (d) 42 and (e) 56 weeks of acclimatization period. (Bar = 1 cm).

4.1.8 Salinity Treatment

In this study, transformed plant that can tolerated towards appropriate salt concentration is the main objective. Hence, preliminary experiment on transformed plantlet was conducted in order to identify the minimum concentration of salt that the non-transformed can tolerate with. In the present investigation of the different (0, 100, 200 and 300 mg/L) concentration of NaCl tested, 200 mM NaCl showing the plantlet was starting to undergo necrosis due to half of the leaf colour changes from green to brown (red arrows on Figure 4.6c). Table 4.6 shows no root elongation for plantlets in 200 & 300 mM indicates no plant growing happened. Whereby all leaves for plantlet in 300 mM NaCl undergo necrosis. Plant capability was score due to the number of leaves undergo necrosis. Hence, 200mM NaCl was chosen as MIC to select the transformed plantlets since it was score as susceptible towards salinity treatment.

Figure 4.6: Plantlet undergoes salinity treatment in 0, 100, 200 and 300 mM of NaCl after one month of culture. (Bar =1 cm).

Table 4.6: Plant capability in different salinity treatment.

Treatment	Shoot length	Root length	Number of necrosis leaf	Plant capability
Control	1.5 ± 0.05^{a}	0.23 ± 0.01^{a}	0	Tolerant
100 mM NaCl	0.6 ± 0.07^{b}	0.11 ± 0.00^{b}	1	Tolerant
200 mM NaCl	0.2 ± 0.08^{c}	0 ± 0.00^{c}	4	Susceptible
300 mM NaCl	0.01 ± 0.01^{d}	0 ± 0.00^{c}	7	Susceptible

- Means of the treatment in the same column followed by different letters are significantly different from each other at the 5 % level

4.2 Plant physiology

4.2.1 Leaf gas exchange

Leaf gas exchange was recorded for *in vitro*-derived and field-grown plants. The results showed that the photosynthetic rates (P_N) for plants at 0 d during day and night were negative (Figure 4.7), reflecting a negative balance between photosynthesis and respiration (dark plus photorespiration rates). Photosynthetic rate at day time for *in vitro*-derived and field-grown plants started to increase after 14 days of acclimatization and began to decrease after 42 days and stabilized after 84 days. The rates for Intercellular CO_2 concentration (C_i) were not significantly increased during the day but decreased at night. During the day, the stomata were completely opened after 14 days of culture and started to close within 42 days. The changes in stomatal conductance (g_s) were matched in parallel to transpiration rates.

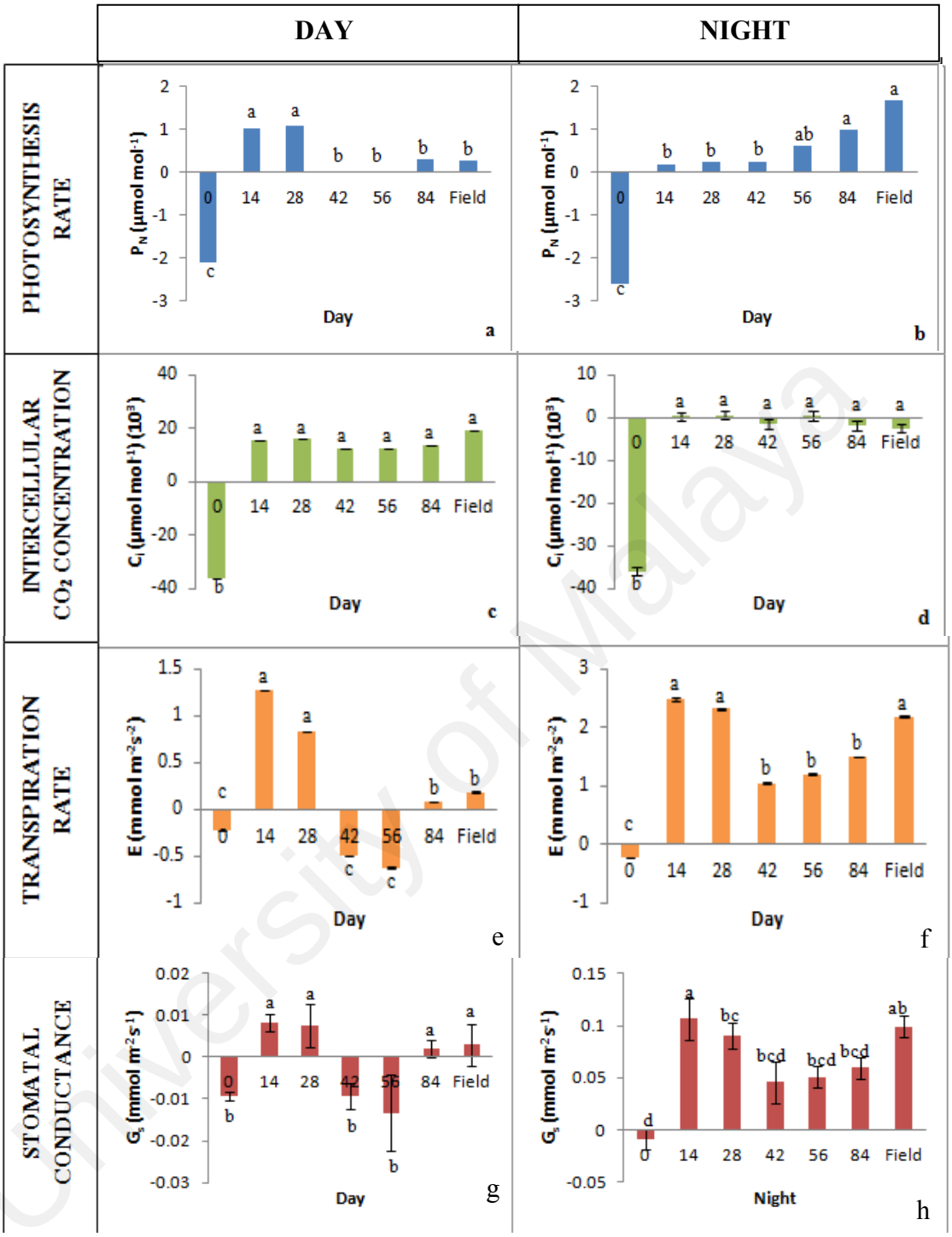


Figure 4.7: Specific properties of leaves from micropropagated *A. comosus* during different acclimatization periods compared with field-grown plants (308 days). a&b. photosynthesis rate (P_N); c&d. intercellular CO2 (C_i); e&f: transpiration rate (E); g&h. stomatal conductance (g_s); c.; and d. Data present mean ± SE (n=10), and different letters show significant differences ($p < 0.05$) according to the Turkey test.

4.2.2 Scanning electron microscope (SEM)

The trichomes and stomata on the upper and lower sides of the leaves of *in vitro*-derived plants and field-grown plants (control) on 0, 14, and 42 days of acclimatization stages were harvested and examined under a SEM. Stomata were seen on the abaxial (lower) side of the leaves at every stages, but the trichomes were negligible on day 0 which gradually developed from day 14 to day 42 (Figures 4.8a-c). Stomata covered by large multicellular trichomes on day 42 (Figure 4.8c) were comparable to field-grown plants (Figure 4.8d). On the other hand, trichomes started to develop on adaxial part of the leaves at the early stage of acclimatization on day 0 but in low numbers. However, large multicellular trichomes were observed on day 42. The edge of trichome became protrude upward due to the abundance growth of trichomes in field-grown plants (Figure 4.8d). For adaxial, stomata were hardly be seen since day 0 (Figure 4.8e). The growing pattern of trihomes was much faster than adaxial part where protruding upward pattern of trichomes started to develop since 42 day.

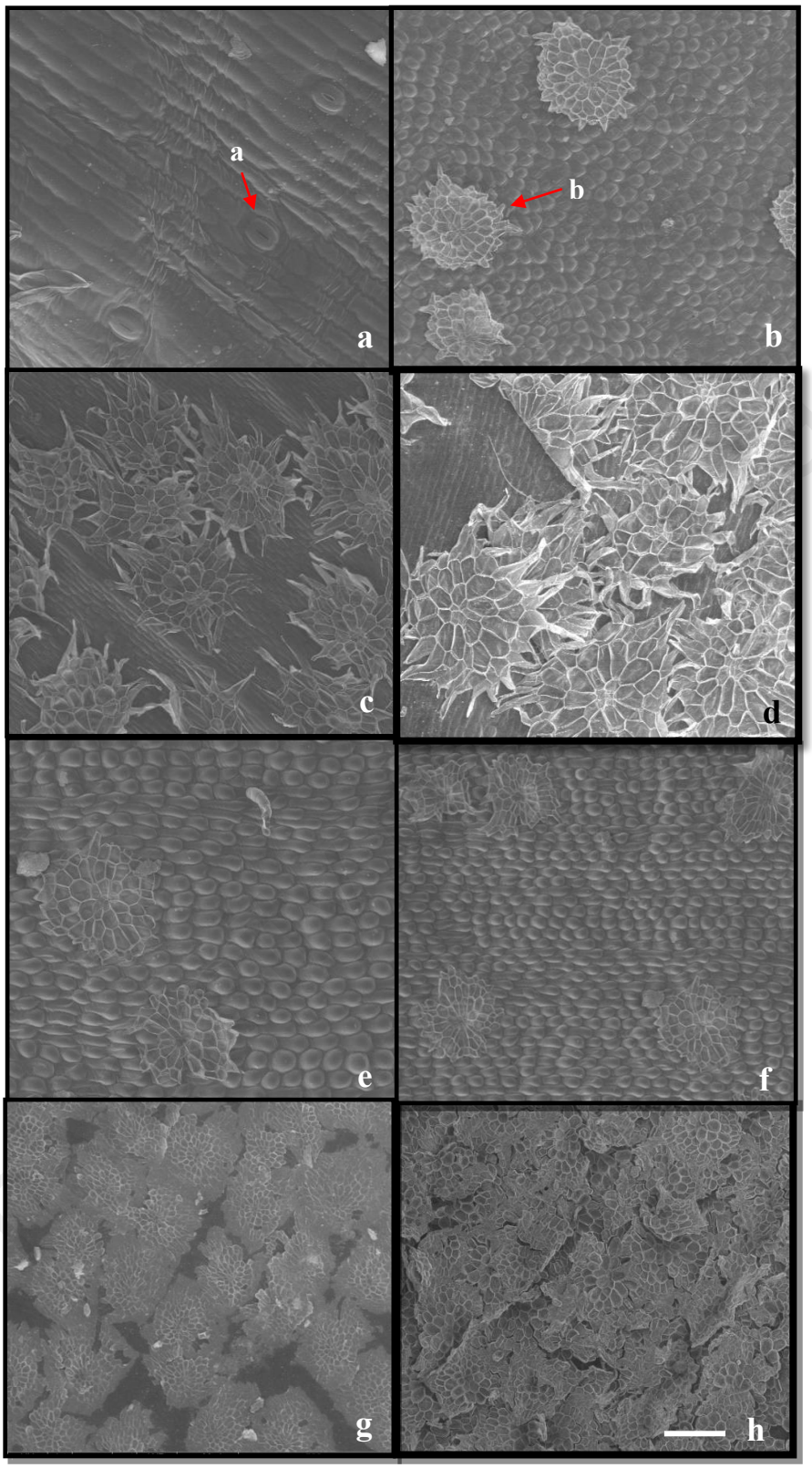


Figure 4.8: Upper view of adaxial leaf (upper) and abaxial leaf using Field Electron Microscope (FESEM) after 0 (a&e, 14 (b&f), 42 (c&g) days and field-grown (d&h) plants of acclimatization. a, b, c, d (abaxial) e, f, g, h (adaxial). (a-stomata; b- trichome). (Bar = 100µM).

4.2.3 Histological examination

Histological examination on the leaf of *in vitro*-derived plantlets and field-grown plants at 0, 14 and 42 days of acclimatization were carried out. Each stage of leaves consisted important basic structures of monocots, namely upper epidermis (A), mesophyll (B), vascular bundle consisting of xylem (C) and phloem (D) and lower epidermis (G). Stomata (E) and guard cells (F) were found on the abaxial section of the leaves at days 14 (Figure 4.9b), 42 (Figure 4.9c) as well as field-grown plant (Figure 4.9d). The leaf structure of 42 days and field-grown plants were similar, where palisade parenchyma cells started to develop during 42 days (Figure 4.9c). However, the size of palisade parenchyma and the number of vascular bundles were abundantly found in field-grown plants. Hence, in this study, 42 days could be considered as the optimal acclimatization period for pineapple.

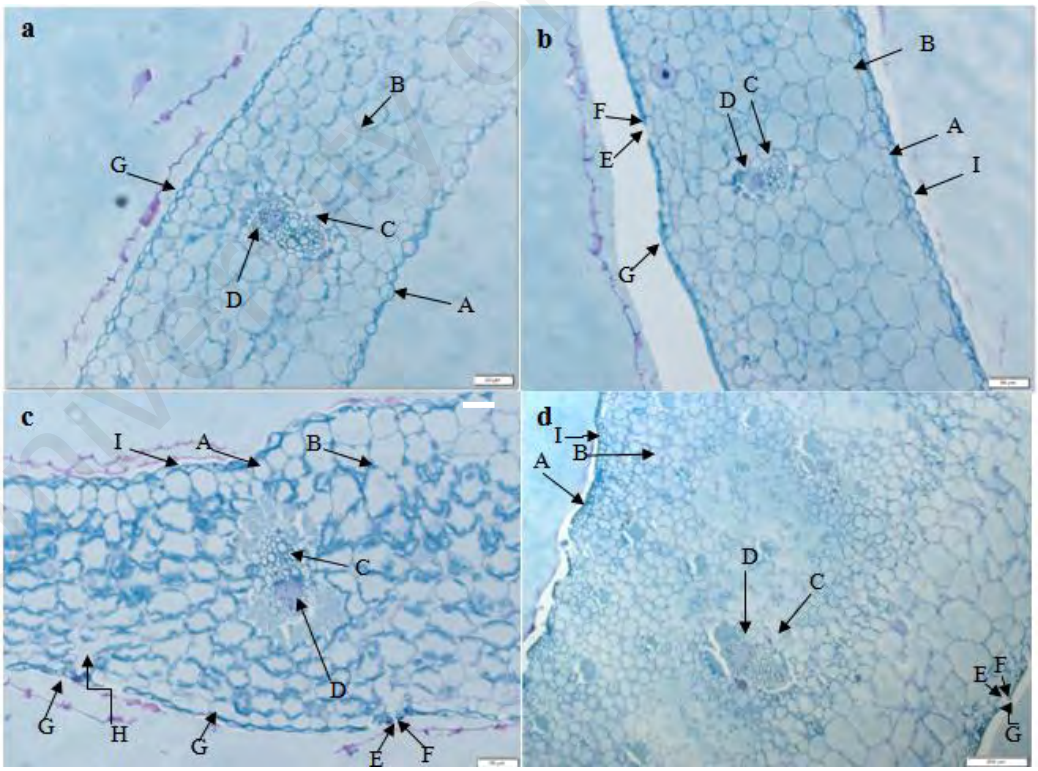


Figure 4.9: Cross section of histology of leaves of *A. comosus* during acclimatization process at: a) 0 day; b) 14 days; c: 42 days and d: field-grown plant. A-Upper epidermis; B- mesophyll; C- xylem; D- phloem (C&D- vascular bundle); E- stomata; F- guard cell; G- lower epidermis; H- air space; I-cuticle. (Bar=40x magnification).

4.3 Determination of kanamycin (Km) concentration for transformant selection

Leaf base explants were excised and cultured on multiplication media (MB2N1) containing different concentrations of Km to determine the natural tolerance of explants towards Km. Shoot buds were generated from medium without Km (Figure 4.9a), while explants on CM medium containing 50 mg/L Km (Figure 4.9b) and 100 mg/L Km (Figure 4.9c) showed LD_{50} and LD_{90} respectively. All leaf bases cultured in media supplemented with 150 mg/L (Figure 4.9d) and 200 mg/L km (Figure 4.9e) became bleached. The inhibition effect of Km on shoot buds was directly proportional to the concentration of Km used. To obtain high transformation frequency in non-stringent selection, M2N1 media containing 50 mg/L km was selected. A more stringent selection using 100 mg/L Km was not selected because this concentration affected the shoot growth.

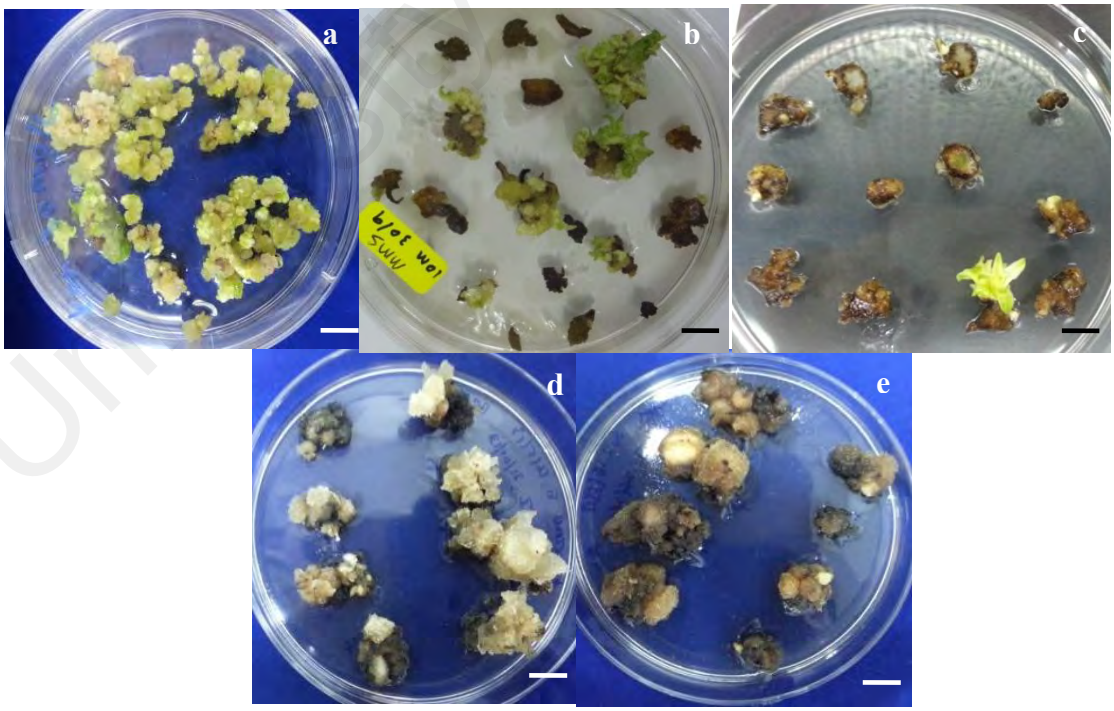


Figure 4.10: Shoots proliferated on CM media containing different concentration of Kanamycin (Km); a: 0 mg/L Kanamycin; b: 50 mg/L Kanamycin; c: 100 mg/L Kanamycin; d: 150 mg/L Kanamycin; e: 200 mg/L Kanamycin. (Bar = 1 cm).

4.4 *Agrobacterium tumefaciens* strain EHA105 harbouring the binary vector pKYLX71

4.4.1 Growth of *Agrobacterium*

The growth of *A. tumefaciens* strain EHA105 harboring the binary vector pKYLX71 was recorded based on the optical density (OD) measurement (Appendix C; Figure 4.11). The results showed that lag phase was between 0 to 8 hours before achieving log phase at 8 to 18 hours. The growth of bacteria started to decline after 44 hours. Therefore, mid-log phase at 14 hours was used for plant transformation.

4.4.2 *Agrobacterium* cell density count

Serial dilution spread–plate method was carried out to determine single colony forming unit (CFU) (Figure 4.12). *Agrobacterium* was adjusted to about 5×10^8 CFU using Luria-broth medium before plant transformation.

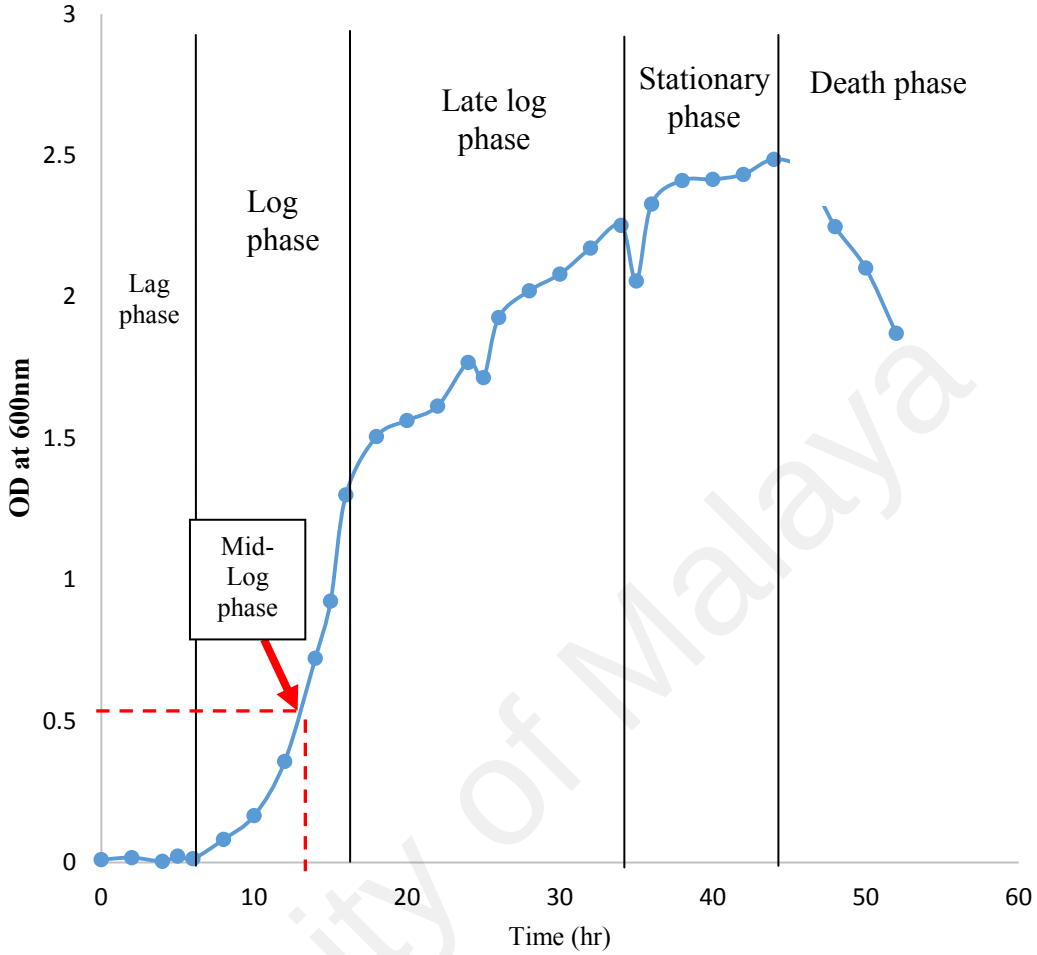


Figure 4.11: Growth curve of *A. tumefaciens* strain EHA105 harbouring the binary vector pKYLX71 plasmid with ABR17 gene.

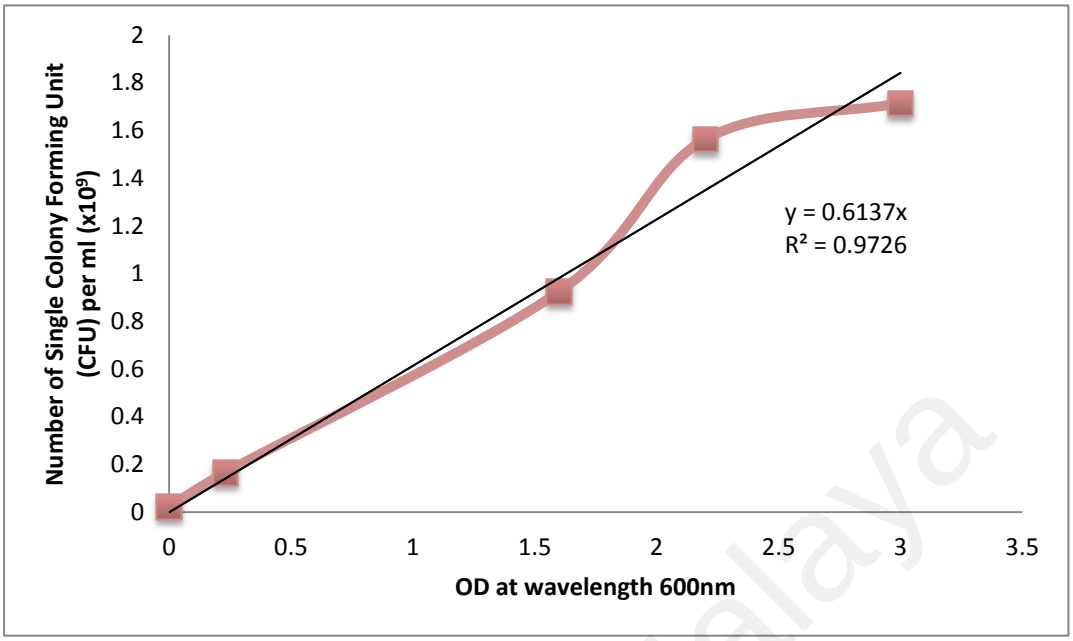


Figure 4.12: Viable cell count of *A. tumefaciens* strain EHA105 harbouring pKYLX71. Cell density was counted based on CFU per ml which represents the bacterial colony derived from a single ancestor of *A. tumefaciens* in one ml broth culture.

4.4.3 Determination of minimal inhibition concentration of antibiotics towards *Agrobacterium*

Thirty six combinations of cefotaxime and carbenicilin at different concentrations were assessed to determine Minimal Inhibition Concentration (MIC) towards *Agrobacterium* (Table 4.7). Seventeen combinations of cefotaxime and carbenicilin (columns shown in yellow) showed bacteriostatic to *A. tumefaciens* strain EHA105 harbouring pKYLX71, whereas five combinations (pink columns) have bactericidal effects where no bacterial growth was observed on the antibiotic-free agar plate. Eighteen combinations of cefotaxime and carbenicilin exhibited bacteriostatic effect but not bacteriocidal to the *A. tumefaciens* (white columns). Taken together, Luria-broth medium with 250 mg/L cefotaxime and 50 mg/L carbenicilin was selected (Table 4.7).

Table 4.7: MIC of *A. tumefaciens* strain EHA105 harbouring pKYLX71 with *ABR17* gene.

Cefotaxime concentration (mg/L)	Carbenicilin concentration (mg/L)					
	0	50	100	150	200	250
0	++++	++++	++++	+++	+	+
50	++++	++++	+++	++	+	+
100	+++	+++	++	+	+	+
150	+++	++	+	+	+	+
200	++	+	+	+	+	+
250	+	-	-	-	-	-

* ++++ = Vigorous growth of bacteria in broth

* +++ = Moderate growth of bacteria in broth

* ++ = Large number of bacteria colony on antibiotic-free agar plate

* + = Limited colony of bacteria on antibiotic-free agar plate

* - = No growth of bacteria on antibiotic-free agar plate

Bacteriostatic – Broth culture of *A. tumefaciens* without visible turbidity
Bactericidal – No growth of *A. tumefaciens* on antibiotic agar plate

4.5 Molecular assessment

4.5.1 Plasmid extraction

The integrity of total plasmid DNA was examined by run an aliquot 5 µL of DNA sample with 2 µL of 6 x Loading Dye to weight the DNA product on 1 % agarose gel. The thickness of the bands indicates the quality of DNA sample. Figure 4.13 showed two bands can be seen. The upper band is linear plasmid DNA and the lower band is super coiled plasmid DNA. Linear plasmid DNA occurs due to nuclease contamination or harsh treatment during purification and the DNA helix is cut in both strands at the same place. Linear DNA generally migrates between the nicked circle

and the super coiled forms. However, it may also migrate the same distance as nicked circle. Supercoiled DNA migrates faster than predicted in an agarose gel due to its conformation.

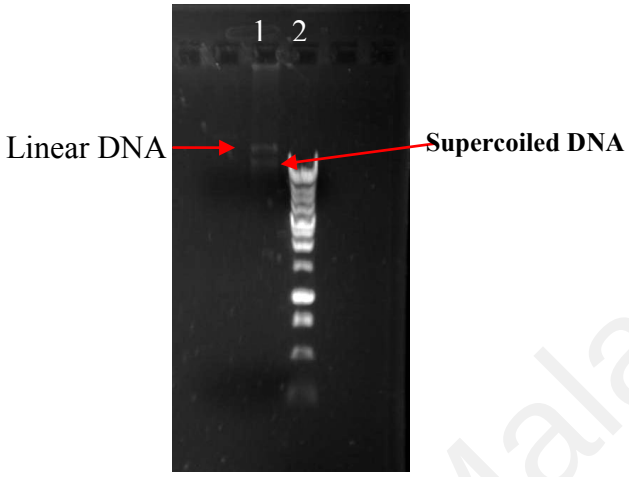


Figure 4.13: Plasmid DNA extraction on 1% agarose gel. Lane 1: Plasmid DNA extraction product; Lane 2: 1 kb ladder.

4.5.2 PCR optimization of *ABR17* gene in *A. tumefaciens*

A gradient PCR was carried out to identify the suitable annealing temperature for amplifying the *ABR17* gene. A single band of amplified *ABR17* gene with a size of approximately 500 bp (lanes 1-12) was detected on agarose gel after electrophoresis (Figure 4.14). The size of this PCR optimization was similar to the expected size of *ABR17* gene fragment amplified using a pair of ABR17 primers. This result indicated the presence of the gene cassette, pKYLX71 (with *ABR17* gene) in the *Agrobacterium tumefaciens* strain EHA105. From the optimization, the best annealing temperature is 58.5 °C. This temperature will be used for the subsequent PCR protocol.

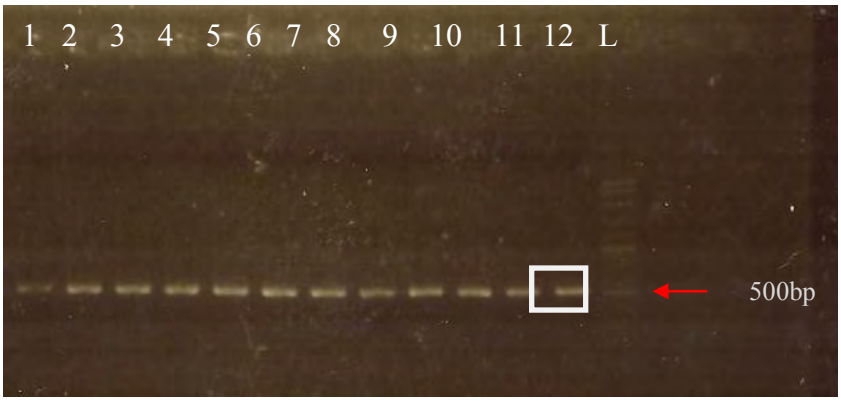


Figure 4.14: A gradient PCR optimization on 1% agarose gel. Lane 1: 50.6 °C; 2: 50.6 °C; 3: 51.6 °C; 4: 53.4 °C; 5: 55.8 °C; 6: 58.5 °C; 7: 61.4 °C; 8: 64.2 °C; 9: 66.7 °C; 10: 68.7 °C; 11: 69.9 °C, 12: 70.1 °C; L: 100bp.

4.5.3 Analysis of *ABR17* gene

The pKYLX71 plasmid carrying *ABR17* gene extracted from *A. tumefaciens* strain EHA105 was verified the presence of the *ABR17* gene using PCR and sequencing. The amplicon of *ABR17* gene with the size of 471 bp was confirmed by 1% agarose gel and sequencing (Figure 4.15). The isolated gene was 100% identical with 95% coverage with *Pisum sativum*. *ABR17* DNA sequence was found to consist of 157 amino acids belonging to SPRBCC superfamily with Bet_v1-like domain, indicating its origin from *P. sativum* (Iturriaga *et al.*, 1994). Hydrophobic ligand binding sites were also found through the sequences alongside glycine-rich loops from Blastx in NCBI (Figure 4.16).

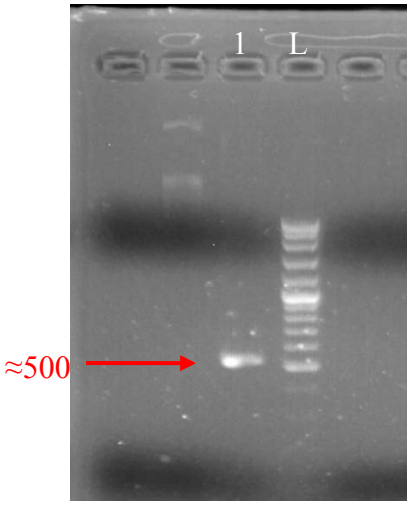


Figure 4.15: PCR amplification of *Agrobacterium tumefaciens* colony containing *ABR17* gene. Lane 1: Positive colony, L; 100 bp ladder.

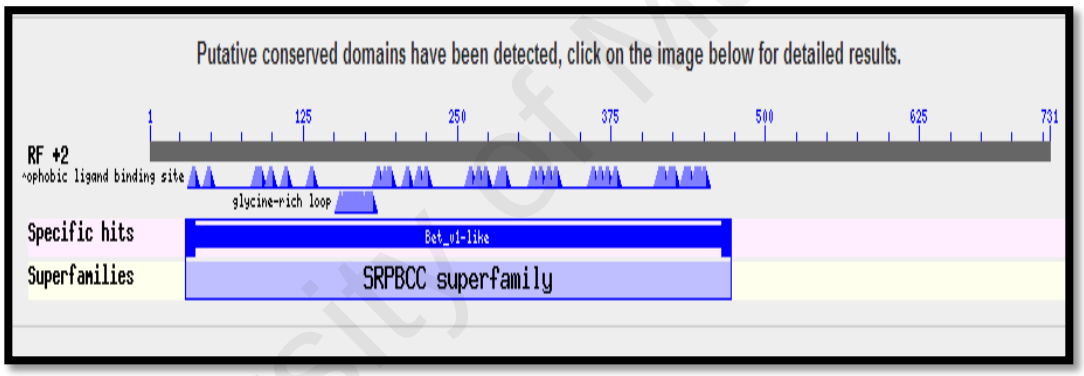
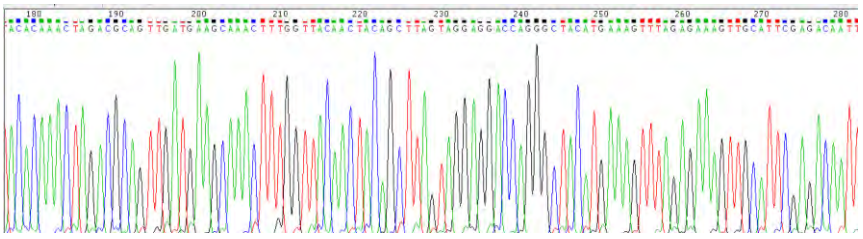


Figure 4.16: Amplified *ABR17* gene has a functional domain known as glycine rich loop and hydrophobic ligand binding-sites.

4.5.4 Verification of *ABR17* gene

An approximately 471 bp in length of *ABR17* gene with high quality sequence reads was obtained from sequencing (Figure 4.17). There was no background noise signals or ambiguities present. The template and primer sequences (forward and reverse) were stringent and the full length *ABR17* ORF sequence was detected.

ABR17 F



ABR17 R

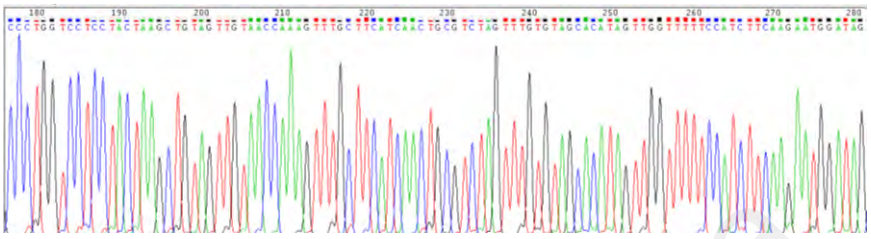


Figure 4.17: Both sequences for forward and reverse of *ABR17* gene harboring in plasmid DNA were assembled showing high quality of the sequence.

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

	Description	Max score	Total score	Query cover	E value	Ident
☐	P.sativum mRNA for ABA-responsive protein	819	819	95%	0.0	100%
☐	Medicago truncatula ABA-responsive protein ABR17 (MTR_2g035190) mRNA, complete cds	486	486	95%	7e-134	87%
☐	Medicago truncatula ABA-responsive protein ABR17 (MTR_2g035210) mRNA, complete cds	472	472	93%	2e-129	86%
☐	Medicago sativa harvest-induced protein mRNA, complete cds	459	459	95%	1e-125	85%
☐	Medicago sativa pprg2 gene	449	449	95%	9e-123	85%
☐	Medicago truncatula clone JCVI-FLMt-9M5 unknown mRNA	448	448	95%	3e-122	85%
☐	Medicago truncatula ABA-responsive protein ABR17 (MTR_2g035220) mRNA, complete cds	448	448	95%	3e-122	85%
☐	Trifolium repens cold responsive protein TRVSP mRNA, complete cds	442	442	95%	1e-120	85%
☐	Vicia faba cultivar Giza 3 ABA-17 responsive protein mRNA, partial cds	422	422	60%	2e-114	94%
☐	Medicago truncatula strain A17 clone mth2-1b1, complete sequence	348	1024	64%	3e-92	88%
☐	Vicia faba cultivar Giza 3 ABA-17 responsive protein mRNA, partial cds	294	294	44%	4e-76	93%

Figure 4.18: Sequence producing significant alignments from NCBI databank. (Blastn result).

The *ABR17* gene was 100 % homology identical with *Pisum sativum* (Figure 4.18) and has a strong relationship (100%) between both sequences (Figure 4.19). Whereas the phylogenetic tree's branch also show the *ABR17* gene was identical more that 80% with other *ABR17* gene in other plant species.

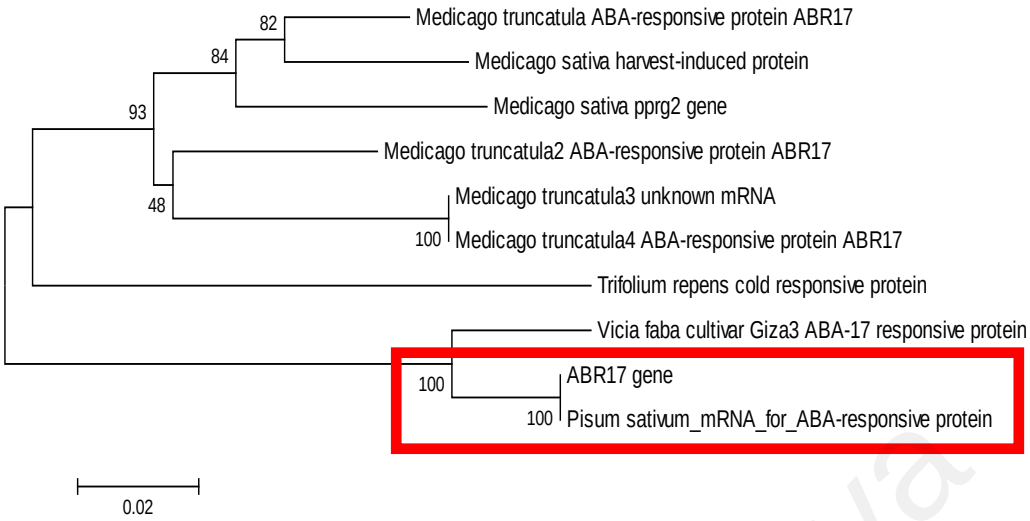


Figure 4.19: Phylogenetic tree analysis of *ABR17* gene. Phylogenetic trees were inferred using neighbor-joining using kimura 2 parameter analysis in the MEGA6 package. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was tested together. The trees were drawn to scale, and the scale bars indicate the branch length corresponding to 0.02 substitutions per site. The Gene of Interest (GOI) used in this study showing close relationship (100%) to *ABR17* gene from origin *Pisum sativum.*

4.6 DNA extraction

The integrity of total DNA extracted from all positive putative transformant samples is shown in Figure 4.20 and the DNA purity (A260/A280) is shown in Appendix D. All of the bands showed high intensity consistent with the DNA concentration and purity. The ratio of the DNA absorbance at wavelength 260 divided 280 for all samples were in the was in the range of $1.7 \leq x \leq 2.0$ and the concentration showed more than 100 ng/µL showing good quality result to undergo PCR experiment.

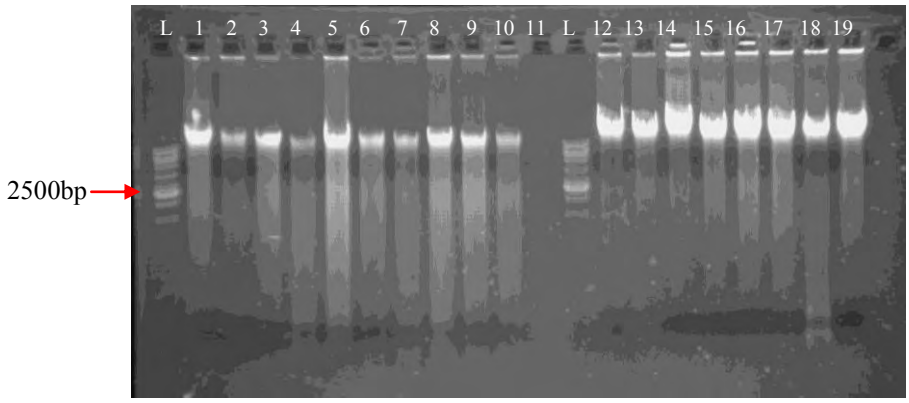


Figure 4.20: Genomic DNA samples extracted from pineapple leaves. Lanes 1 and 13: 1 kb Ladder. Lanes 2 – 10: Non-transformed DNA. Lanes 12 – 19: Positive putative transformed DNA.

4.7 Determination of optimal infection times, acetosyringone concentrations and co-cultivation periods for plant transformation

Prior to transformation, infection times, acetosyringone concentrations and co-cultivation periods were investigated. Results were shown in Table 4.8. For all acetosyringone concentration and co-cultivation period was investigated, the number of regenerant lines that survived on selection media for 10, 20 and 30 min infection time was between 18 to 50, 4 to 25, 9 – 30 respectively. Hence, 10 min infection was the best for transformation of pineapple.

Subsequently, transformation efficiency was determined based on PCR screening for *ABR17* gene (Table 4.9). PCR was obtained for selected parameter combinations. The lowest transformation efficiency (5%) was recorded when explants were infected with *Agrobacterium* for 30 min, co-cultivated on medium supplemented with 300 µM acetocyringone for 1 day.

Table 4.8: Number of line of infection times, acetosyringone concentrations and co-cultivation periods of transformation efficiency of *Agrobacterium tumefaciens* EHA105 harbouring pKYLX71 gene with *ABR17* gene on *Ananas comosus* var. MD2. Ten of samples were used for each parameter combination and repeated five times (p<0.05).

Infection time (min)	Acetosyringone (µM)	Co-cultivation (day)	Number of line
10	100	1	45
10	100	2	29
10	100	3	50
10	200	1	20
10	200	2	35
10	200	3	19
10	300	1	22
10	300	2	18
10	300	3	34
20	100	1	23
20	100	2	25
20	100	3	11
20	200	1	8
20	200	2	7
20	200	3	4
20	300	1	10
20	300	2	10
20	300	3	5
30	100	1	11
30	100	2	11
30	100	3	9
30	200	1	10
30	200	2	16
30	200	3	15
30	300	1	19
30	300	2	30
30	300	3	34

Table 4.9: PCR positive putative transformants and transformation efficiency for the effects of infection times, acetosyringone concentrations and co-cultivation periods of transformation efficiency of *Agrobacterium tumefaciens* EHA105 harbouring pKYLX71 gene with *ABR17* gene on *Ananas comosus* var. MD2. Ten of samples were used for each parameter combination and repeated five times (p<0.05).

Infection times (min)	Acetosyringone conc (μM)	Co-cultivation period (day)	Number of regenerant line	PCR Positive putative transformants	Transformation efficiency
10	100	1	45	10	22±4.04[a]
10	200	2	35	6	17±3.4[a]
20	200	2	7	1	14±0.5[a]
30	300	1	19	1	5±0.5[b]
30	300	2	30	7	23±3.4[a]
30	300	3	34	11	32±4.6[a]

4.8 Molecular analysis and regeneration of transformed explants

Putative transformed *A. comosus* plantlets were assessed individually by PCR (Figures 4.21a and b). Eight (Figure 4.20a) and nineteen (Figure 4.21b) samples showed the presents of *ABR17* gene of with amplified fragment of approximately 500 bp. All single bands were scored as positive putative transformants as determinant for transformation efficiency in Table 4.8.

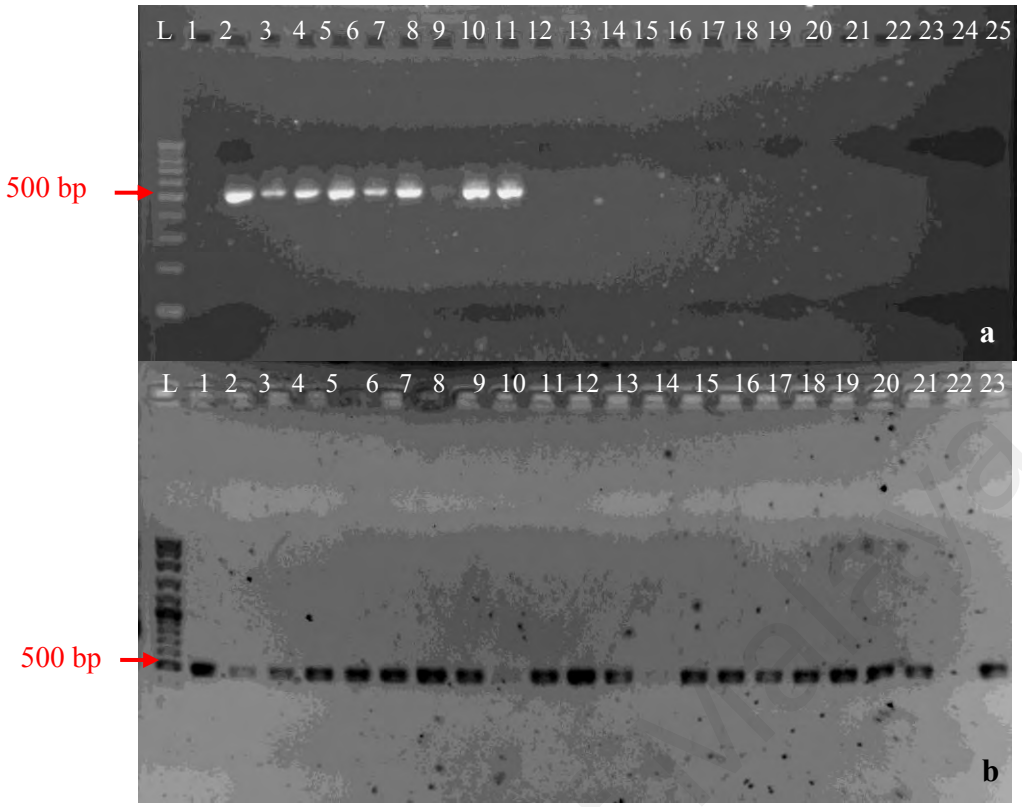


Figure 4.21 a: PCR confirmation of transformed plants using *ABR17* primers with an approximate expected size.of 500 bp. L: 100 bp Ladder; Lane 1: Negative PCR product (SdH_2O); Lane 2: Postive PCR product: plasmid pKYlx71 with *ABR17* gene. Lanes 3 - 25: Amplicon of *ABR17* in putitative transformant. **b**; PCR product of transformed DNA. L: 100 bp Ladder; Lanes 1-20: Amplicon of *ABR17* in putitative transformant; Lanes 21: Negative PCR product (SdH_2O); Lane 22: Postive PCR product: Plasmid pKylx71 with *ABR17* gene.

4.9 PCR Tetracycline

PCR with *Tetr* primers was conducted on positive putative transformed plants with *ABR17* genes to ensure no remaining contamination of *Agrobacterium tumefaciens* left after transformation. Absence of *Tetr* gene amplification in all sample tested after gene transmitted are shown in Figure 4.22. Outer layer of T-DNA region of *PKYLX71* gene was identified as Tetr gene.

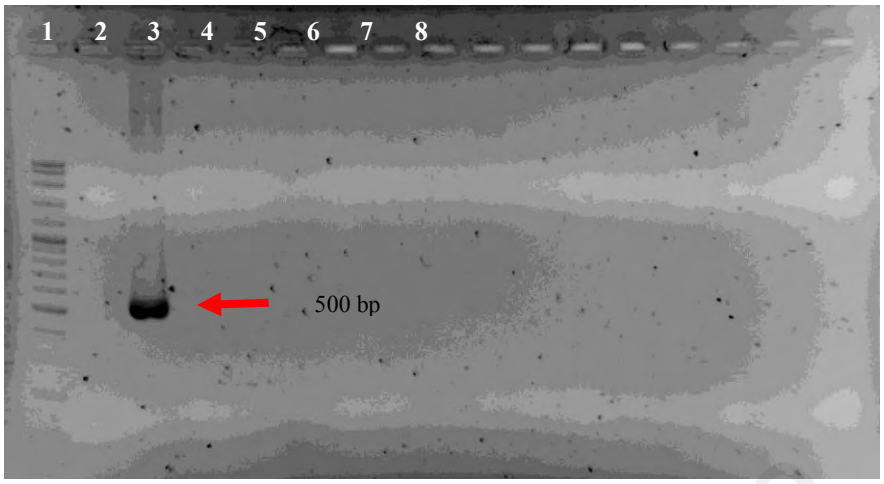


Figure 4.22: PCR confirmation of transformed plants using *Tetr* primers with an approximate expected size of 500 bp. Lane 1: 100bp Ladder; Lanes 2: Negative control (SdH_2O); Lane 3: Postive PCR product: plasmid *pKYlx71* with *ABR17* gene. Lane 4-8: Positive putative transformants.

4.10 PCR housekeeping genes

Determination of the present of housekeeping genes in both transformed, non-transformed plants and pKYLX71 plasmid was carried out. Both transformed and non-transform plantlets indicated the presence of *Bromelain* and *Actin* gene with amplified approximately fragment 200 bp using Bromelain and Actin primers. No band was seen on negative control samples and PKYLX71 plasmid. Multiple bands were detected in pKYLX71 plasmid by using *Bromelain* primer. *ABR17* gene in pKYLX71 plasmid displayed the expected amplified band at approximately 500 bp in size. However, *Cystatin* gene was not amplified in samples tested except in PKYLX71 plasmid (Figure 4.23).

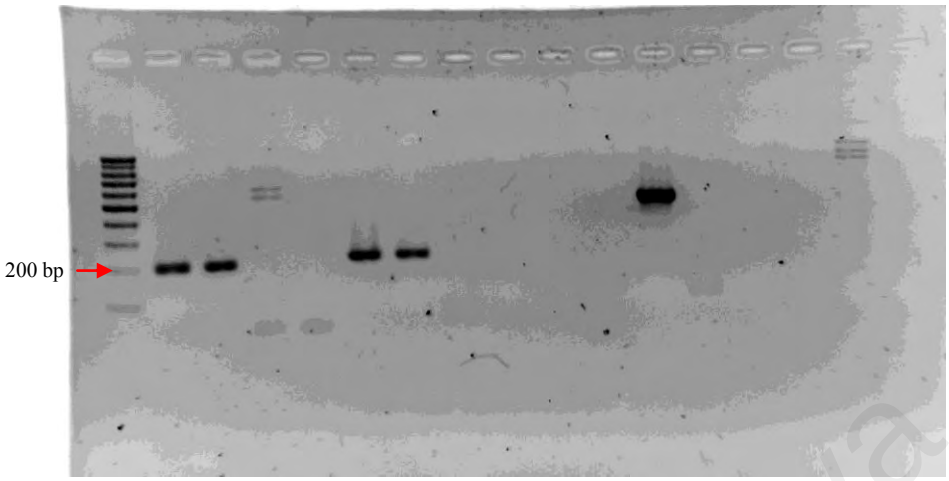


Figure 4.23: PCR on housekeeping gene for positive putative transformants & Non-transformed plantlet), pKYLX71 plasmid with *ABR17* gene, negative control (sdH_2O), *Bromelain* (BRO), *Actin* (Act), *ABR17* and *Cystatin* primer. Lane 1: 100bp Ladder; Lanes 2 – 5: BRO set of primer; Lanes 6 – 9: Act sets of primer; Lanes 10 – 13: *ABR* sets of primer; Lanes 14 – 17: AcCys1 sets of primer; Lanes 2,6,10&14: Putative positive transformants; Lanes 3,7,11&15: Untransform plantlet (control); Lanes 4,8,12&16: pKYLX71 plasmid with *ABR17* gene Lanes 5,9,13&17: Negative control (sdH_2O) gene.

4.11 Southern blot analysis

4.11.1 Probe synthesis

The plasmid and genomic DNA with RE enzymes, EcoR1 and HindIII was digested (Figure 4.24). For plasmid, lane 10 was used since it's the thickest band among others that consist of 0.1 µg plasmid DNA concentration, 20 µL of total volume and 15 min of incubation time. For genomic DNA, Lane 14 is the thickest smearing that consists of 15 µg of genomic positive putative transformant, 20 µL of total volume and 15 min of incubation time. This parameter was used for next southern blot experiments.

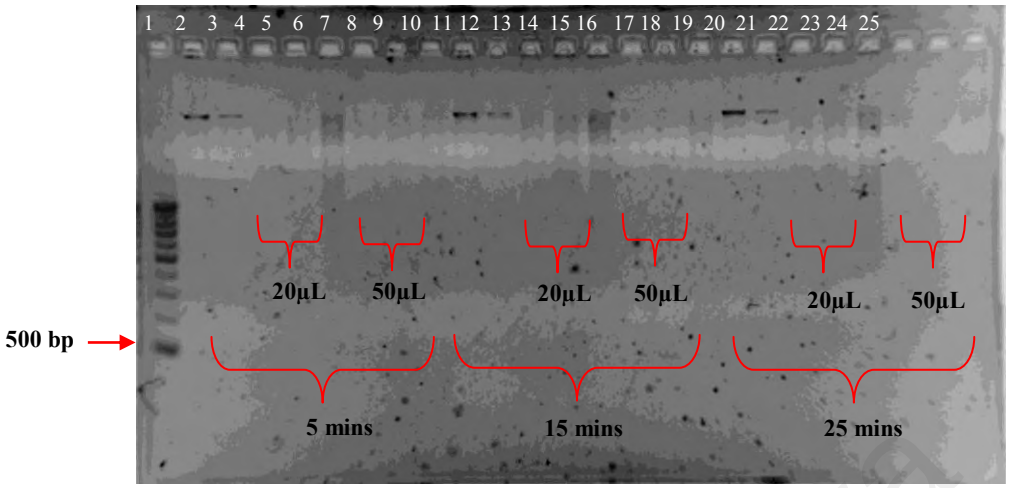


Figure 4.24: DNA digested of plasmid DNA and genomic DNA. Lane 1: 100 bp ladder; Lanes 2-9: Plasmid and DNA digested with EcoR1 and HindIII with 5 min of incubation time. Lanes 10-17: Plasmid and DNA digested with EcoR1 and HindIII with 15 min of incubation time; Lanes 18-25: Plasmid and DNA digested with EcoR1 and HindIII with 25 min of incubation time.

4.11.2 DNA digestion

The digested plasmid and genomic DNA with EcoR1 and HindIII RE enzyme (Figure 4.25). Both plasmids DNA are showing very thick band indicated a very high concentrated DNA. (➔) line in figure is showing that the plasmid DNA has not finished digested yet. Hence, the plasmid DNA concentration need to reduce from 0.1 µg to 0.05 µg so that all plasmid can finished digested. For genomic DNA, the smear on lane 4 is thicker than lane 5 because genomic (0.5 µg) on lane 4 is higher than genomic (0.6 µg) lane 5. Smear on genomic DNA lane showing that the DNA is finish digested with the appropriate RE enzyme. Since the smear is very thin, genomic DNA concentration need to be increase from 5 µg to 10 µg for next southern blot procedure.

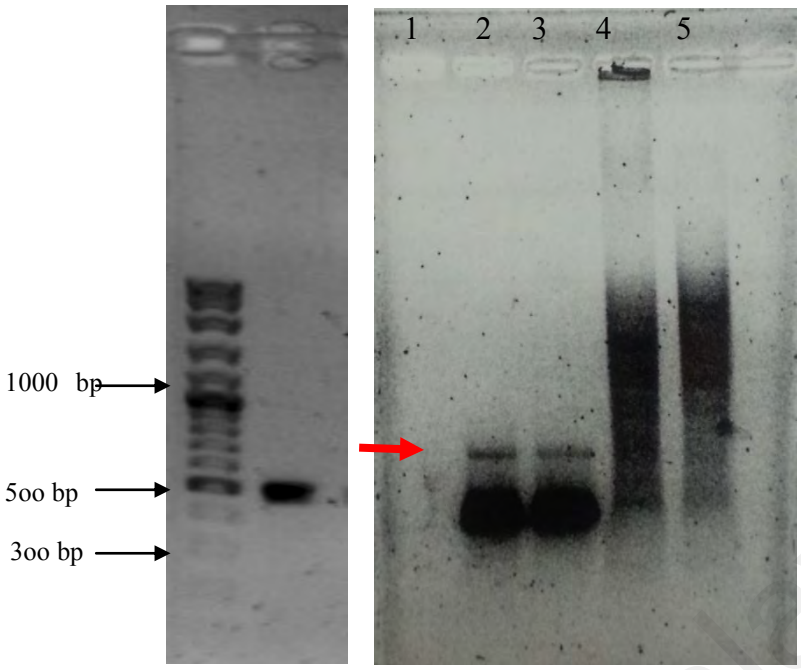


Figure 4.25: Plasmid DNA and genomic DNA. Lanes 2 and 3: Plasmid digested with EcoR1 and HindIII. Lanes 4 and 5: Genomic DNA digested with EcoR1 and HindIII.

4.11.3 Immunological detection

Both plasmid DNA contain 2 copy number of gene of interest (*ABR17* gene) was observed (figure 4.26), whereas genomic DNA in line 4 is showing 2 fine band (➡) indicated 2 copy number of *ABR17* gene successfully integrated into putative transformed pineapple DNA. The bands are very fine maybe because the DNA concentration been used is very low. This may be due to very low concentration of DNA. DNA concentration needs to increase for next genomic DNA.

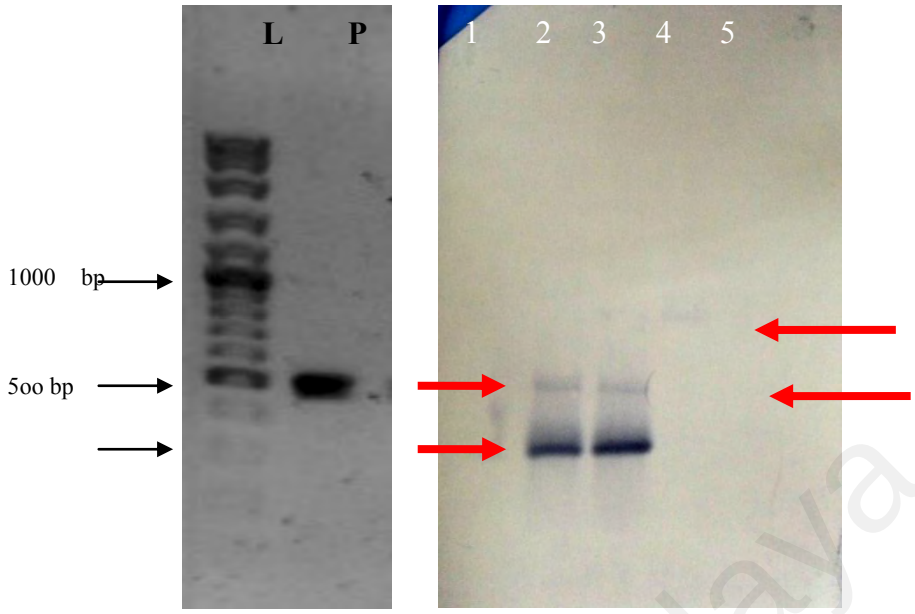


Figure 4.26: Detection of *ABR17* gene using Southern Hybridization. L: 100 bp DNA Ladder; P: PCR products of *ABR17* gene positive control, Lane 1: Non-transformed control *A. comosus*; Lane 2: Plasmid digested with *EcoR1* and *HindIII*. Lanes 3-5: Genomic DNA digested with *EcoR1* and *HindIII*.

CHAPTER 5: DISCUSSION

5.1 Plant Tissue Culture

5.1.1 Initiation and multiplication of *in vitro* shoots

Traditionally, pineapple is propagated commercially through sucker. However, this technique is slow, time consuming and labour intensive. Therefore, establishment of an efficient and simple alternative propagation method, such as plant tissue culture, is crucial. Plant tissue culture is an aseptic technique that allows mass propagation of *in vitro* shoots on a nutrient culture medium to produce offspring that are similar to mother plant through organogenesis (Bhojwani & Dantu, 2013).

In this study, parameters for shoot initiation and multiplication of pineapple var. 'MD2' were investigated using axillary buds (AB) as explants to initiate *in vitro* pineapple cultures. Acheampong *et al*. (2015) and Zhang *et al*. (2016) reported that AB was the suitable explants for organogenesis in pineapples compared to crown and leaves since it produced the highest number of shoots. The size of explants is another important factor to influence shoot initiation. Small size (< 1cm^2) of explants might easily cause cell death during surface sterilization process due to toxicity from disinfectants such as sodium hyplochlorite solution and ethanol. The ability of small size explants to undergo differentiation and division might be low due to less meristematic cells that can uptake the required nutrients (Smith, 2013). Large size explants (> 1cm^2) showed higher contamination rate than small size explants, probably due to incomplete washing especially the inner part of the explants. Explants with approximately 1 cm^2 in size has been considered as appropriate size for culturing in other plant species, including *Freesia sp.* (Pourkhaloee & Khui, 2015), *Withania coagulans* (Valizadeh & Valizadeh, 2011) and *Eleusine coracana (L.) Gaertn.* (Satish *et al*., 2015). Hence, approximately 1 cm^2 explants was used in the current investigation.

Shoot induction medium containing different plant growth regulators (PGR) showed differential explant responses. Cytokinin (BAP) and NAA as an auxin in the range of 0 – 5 mg/L and of 0 – 2 mg/L respectively were used in this experiment. BAP promoted shoot induction even in the lowest amount in all treatments (MB1N0, MB1N1 and MB1N2). BAP is a commonly used cytokinin for shoot induction (Tan *et al.*, 2011; Chauhan *et al.*, 2015). Low concentration of BAP (≤3 mg/L) has been reported to induce low number of shoots and fresh weight (Ibrahim *et al.*, 2013). By combining BAP and NAA, the number of new shoots produced was higher than BAP alone. Higher concentration of BAP (≥3 mg/L), however, has been reported to cause mutation, delay fruit senescence and inhibit plant growth (Zhang *et al.*, 2015). Consequently, the optimized media adopted for this study was MS media supplemented with 3mg/L BAP and 1 mg/L NAA (MB3N1).

The explants from the optimized culture medium (MB3N1) were transferred to shoot multiplication medium containing lower concentration of BAP and NAA since prolonged exposure to high concentration of BAP might trigger abnormalities and affect plant growth (Nelson *et al.*, 2015). The results showed that high number of shoots was found in culture medium containing 2 mg/L BAP and 1 mg/L NAA (Table 4.3). Inclusion of BAP and NAA in culture medium has been reported in different varieties of pineapple (Danso *et al*., 2008; Saif *et al*., 2011; Usman *et al*., 2013; Sripaoraya *et al*., 2003) as well as other plant species, such as *Stevia rebaudiana* (Deshmukh & Ade, 2012), *Eucalyptus globules* (Cordeiro *et al*., 2014), *Phalaenopsis* sp. (Mariani *et al*., 2014), and *Psoralea corylifolia* (Nafi & Shrivastava, 2015). Without BAP (MB0N0, MB0N1, and MB0N2), less number of shoots (1-1-1.5 per explants) was obtained. Zuzarte *et al.* (2015) reported similar results for *Lavandula* sp.

MS medium containing NAA (MB0N1 and MB0N2) had induced adventitious root without shoot formation (Figure 4.2c). Similar findings have been reported in *A. comosus* cultivar Smooth Cayenne and Morris (Hamad *et al*., 2013), *Ceropegia culbosa* (Phulwaria *et al.*, 2013) and *Carica papaya* (Setargie *et al.*, 2015). In this work, *in vitro* shoot propagation was improved in liquid medium, 1-7-fold higher than semi solid media. Liquid media helped cells to aggregate evenly and the whole meristem of the explants was able to absorb nutrients more effectively (Mehta *et al*., 2014; Scherer *et al.*, 2013). Similar results have been demonstrated in *Malus domestica* (Mehta *et al*., 2014) and *Asparagus officinalis* (Chen, 2015).

Addition of AC in rooting medium has shown positive effect in obtaining 100 % survival of *in vitro* plants during acclimatization due to vigorous formation. Vigorous adventitious root for monocotyledon such as pineapple are very crucial since it can spawn efficiently near soil surface to uptake even from very small drip of water. The medium containing 4 mg/L AC showed the highest number of adventitious roots (11 roots per shoot) with a mean length of 8.6 cm. High concentration of AC (> 6 mg/L) in the medium compared to lower concentration on AC (< 6 mg/L). In contrast, medium without AC only recorded 2.5 roots per shoot and brittle root structure. AC has been shown to promote cell growth and development (Khodakovskaya *et al*., 2013), absorb accumulated inhibitory substances in the culture medium (North *et al*., 2012; Ekinci, 2013; Doern *et al*., 2014), promote and enhance root formation (Daud *et al*., 2013). According to Jones and Saxena (2013), the presence of AC could inhibit oxidation of phenolic compounds released from tissues liberated during tissue culture.

5.1.2 Acclimatization, survival rates of plantlets, leaf morphology and physiology of *in vitro*-derived pineapple

High mortality of *in vitro*-derived plantlets is usually observed during *in vitro* rooting and acclimatization due to non-functional stomata, weak or poor root systems (Tan *et al*., 2011; Osorio *et al*., 2013). In this study, 100% *in vitro* plantlets survived in the green house (Appendix B; Figure 4.5) and appeared healthy, grew uniformly and phenotypically normal as field-grown (FG) plants during the observation period. In ensuring 100% success rate of field transplantation, survival of plantlets is very crucial (Deshmukh & Ade, 2012).

In general, all parameters related to acclimatization tested for *in vitro*-derived plantlets showed increasing trend towards morphological and physiological adaptation as in FG plants over 84 days of transfer (Appendix B). Leaves are important since they enable plants to generate energy through photosynthesis. Healthy leaves help plants to generate sufficient energy to survive and fasten growth in order to protect the plants from any abiotic and biotic factors. Width of leaf (W) and dry mass/fresh mass ratio (DM/FM) of *in vitro*-derived plantlets and field-grown plants after 42 days of transplanting were similar. Thus, *In vitro* plants were considered ready for field transplantation after 42 days. Whereas, chlorophyll content (C), length of leaf (L), number of roots (R) and plant height (H) for *in vitro*-derived plantlets were significantly lower than field grown plants. This was in agreement with previous studies on *Tuberaria major* (Osorio *et al.*, 2013), *Triticum aestivum* L. (Dong *et al.*, 2015) and *Lactuca sativa* L. (Poulet *et al.*, 2014) due to growth of the plants to accommodate their needs. Photosynthesis rate is influenced by leaf chlorophyll (Appendix B) and pigment content (Figure 4.5) (Gitelson *et al.*, 2003).

During acclimatization of *IV* plants in the green house, another adaptive visible trait developed was waxy layer with succulent spiny leaves (Figure 4.5) which are crucial for CAM plant such as pineapple. This enables the plant to store water longer making the plants able to survive under drought season.

The morphological and physiological changes for these *IV* derived plantlets in this study were analyzed to determine the suitable time for transplanting into the field. A balanced relationship between photosynthesis and respiration (dark plus photorespiration rates) were observed when P_N at 0 day of *IV*-derived plant for day and night were negative net rates (Figure 4.7). Transmission of CO_2 from the surrounding through stomata into the mesophyll tissue controlling photosynthesis rate eventually producing O_2 that also exits through stoma opening. Stomata, an important organelle, were controlled by guard cells to prevent excessive water loss. When opened, they allow gas exchange of CO_2 and water transpiration.

Stomata and guard cells were observed on the abaxial section of the leaves after 14 and 42 days of planting as seen in fg plants. Since gaseous exchange happened when stomata were opened, the changes of stomatal conductance (g_s) were in parallel to the transpiration rate. In our observation, upper and lower epidermises were progressively thicker from 0 to 42 days. Palisade and spongy mesophyll cells, emerge after 42 days of acclimatization to accommodate the abundance of chloroplast for photosynthesis but not as developed as FG plant. The *IV* plants were able to photosynthesize with the presents of chloroplast in the palisade and spongy mesophyll cells. The presences of vascular system since day 1 enable the *IV* plantlets to absorb water and nutrient uptake which is crucial for the photosynthesize process.

Many stomata were seen clearly when trichome on the adaxial and abaxial part of the leaves were not fully developed at 0 and 14 days after acclimatization. Eventually,

stomata were hardly visible on both parts of the leaves after 42 days of acclimatization. This was due to the presence of expanding mushroom-like trichomes. However, the trichomes were more fully developed on the abaxial than the adaxial part of the leaves after 42 days of acclimatization. Gucci *et al*. (1997) discovered relationship between trichomes presence on abaxial leaves of *Phillyrea latifolia* with high tolerance of the plants towards salinity due to thick cuticle and cell wall of the trichome. Trichome prevents water loss and protects plants from pathogens and abiotic stress (Kolb & Muller, 2004; Nefzaoui *et al*., 2014). Hence, abundance growth of trichomes covering stomata after 42 days decrease transpiration rate and might provide protection against stress preparing the *IV*-derived plants to adapt to surrounding field conditions. The pattern of transpiration rate of the 42 days of acclimatize *IV* plants is comparable to the FG plants.

Originally, pineapple is a Crassulacean acid metabolism (CAM) plants. CAM is plant using Carbon fixation pathway for the plant to adapt the environment. During the day, the stomata will remain closed due to evapotranspiration meanwhile closed during night to accumulate CO_2 for photosynthesis (Cushman, 2001). In contradict, to C3 plant where the stomata opened during day and closed during night. Eventually, Aragon *et al*. (2012), *IV* pineapple plantlets which are C_3 plant change to CAM in response to environmental changes, such as humidity, temperature, and light intensity. In this study, the patterns of photosynthesis rate, stomatal conductance and transpiration rate during day showed high reading proved that stomata were opened before IV plantlets acclimatized for 42 days. This proved that C3 plantlets begin with C3 due to high humidity, controlled temperature and light intensity. Eventually, the patterns were contradicting after 42 days of acclimatization parallel to Aragon *et al*. (2012) findings proved that the *IV* plantlets had switched to CAM plant.

5.2 Transformation parameters

Optimization of *A. comosus* var. MD2 transformation parameters carried out in this study using various concentrations of acetosyringone, co-cultivation period and infection time have influenced transformation efficiency. This is imperative because there was no previous data concerning transformation on *A. comosus* var. MD2 harbouring pKYLX71 plasmid with *ABR17* gene. Pineapple transformation of various variety of pineapple has been established using different plasmid harboring various genes prior to this study. However, it has been shown that different type of plasmid influenced the transformation parameters (Xu *et al*., 2014; Chetty *et al*., 2013).

Explants infected with *Agrobacterium* showed positive responds to all parameters tested, producing between 4 - 50 regenerant lines. The highest data recorded was using 100 µM acetosyringone supplementation for 10 min explants infection time and co-cultivated for 3 days prior to culture on selection media. The lowest number of line recorded was when explants were infected for 20 min with bacteria culture added with 200 µM acetosyringone and co cultivated for 3 days. The different responses recorded showed that it is important to optimise the transformation parameters to obtain the best results.

In this study, lowest acetosyringone concentration (100 µM) showed higher number of putative transgenic lines compared to higher concentration (200 & 300 µM). Addition of optimized acetosyringone concentration is crucial for some monocotyledons plants since they are not able to produce phenolic phytochemicals through wounding unlike dicots. Li *et al*. (2016) showed that this substance has an important role as a signal, attracting and transforming unique *A. tumefaciens* as plant-pathogen recognition. The substance was used to infect plants by encoding receptors to detect *virA* gene on the Ti plasmid from *A. tumefaciens*. Naseri *et al*. (2012) suspended the *Agrobacterium* cells

in a medium containing 100µM of acetosyringone and produced 24% transformed rice plantlets. Mayavan *et al*. (2013) also reported 100 µM of acetosyringone is the best concentration in producing transformed sugarcane seedling (3.8%). Similarly acetosyringone concentration supplementation favoured the *Agrobacterium* infection and improved the transformation efficiency in *Medicago truncatula*, *Brassica napus* and cotton (Iantcheva *et al.*, 2013; Li *et al.*, 2010; TianZi *et al.*, 2010).

Infection time of explants by *A. tumefaciens* is also one of the key parameters for transformation procedure for many plant species (Duan *et al*., 2013). An appropriate *Agrobacterium* infection times could influence the transformation efficiency. In this study, 10 min of infection time showed higher putative shoots formation compared to those incubated for 30 min. The efficiency of T-DNA transfer into explants is affected by infection time, where a short period is insufficient for the transfer and over infection might cause necrosis to the explants (Yang *et al*., 2016; Khan *et al*., 2015).

Co-cultivation period is another important element in transformation to allow the completion of the transformation process (Carlos & Christopher, 2015; Mishra *et al*., 2016). The gene transfer and integration into the plant genome through *Agrobacterium* is a lengthy process and varies widely from a few hours to a few days depending on the species, explants and culture conditions. In this study, it was shown that the optimal period for co-cultivation was 1 day and explants became necrotic on longer duration period. Decreased transformation efficiency might happened by necrosis of explants and overgrowth of bacteria due to unsuitable co-cultivation condition (Nanasato *et al*., 2013).

All positive putative transformed *A. comosus* lines cultured on selection media were assessed for molecular confirmation by PCR using *ABR17* gene primers. PCR was carried out to confirm *ABR17* gene transfer into the plant genome. However, the PCR

results obtained did not corelate with the number of regenerant lines from explants. The highest number of individuals from the regenerant lines obtained did not show any positive results. This might be due to escapes or false positives. Furthermore, these cells might be very competent where they had survived on selection media supplemented with 50 mg/L kanamycin. Smith (2013) also proved that competent cells could grow in any severe condition including stress. In order to overcome false positive results, transgene selections could be made on media with higher kanamycin concentration. In a study conducted by Arshad *et al*. (2014), 100 mg/l kanamycin was used in tomato transformation as stringent selection based on the use of higher concentration of kanamycin than the explant minimum tolerance level.

Subsequently, T_1 transgenic plants were multiplied from the PCR positive T_0 regenerant lines and subjected to PCR screening for the presence of *ABR17* gene. It was found that none of the T_1 plants showed the expected 471bp band indicating absence of *ABR17* in T_1 transgenic plants.This result might indicate the possibility of the amplification of *ABR17* gene from the *Agrobacterium* plasmid and not the plant genome.

In order to confirm that there was no contamination of the *ABR17* gene from the bacteria in the T_0 transgenic plants, PCR amplification for *Tetr* gene was carried out. Tetr gene is in the vector backbone which confers resistance towards tetracycline for bacterial selection. Since *Tetr* gene was not amplified from isolated genomic DNA of the transformed shoots, this suggested that there was no *Agrobacterium* contamination in the T_0 transgenic shoots tested.

Bromelain & *actin* were identified as house-keeping genes which could be used for future PCR analysis conducted on transformed and control plants. Both genes

present in the transformed and non-transformed plantlets indicated that introduction of *ABR17* gene into the genome did not interfere with the house keeping genes tested.

Subsequently, Southern blot was then performed to confirm the integration of *ABR17* gene in the transformed *A.comosus*. Southern blot is more informative than PCR analysis in transgenic plant as a proof of the integration and could reveal the pattern of inheritance. Besides, false positive results that might occur from PCR analysis due to contamination, mis-match and non-specific amplification could be ruled out in Southern blot analysis. PCR amplification of DNA template might not be able to differentiate between plant and *Agrobacterium* origins.

Optimization of Southern Blot parameters had been carried out to ensure best results. In order to detect stable integration of the *ABR17* genes in transformed *A. comosus*, Southern blot analysis of *EcoR1* and *HindIII* restriction enzyme digested genomic DNA was carried out in this study. *EcoRI* cuts on multiple sites of T-DNA region, whereas *HindIII* cuts on specific site of T-DNA region in transformed plants.

Southern blot analysis was performed randomly on three positive putative T_0 transformants lines for *ABR17* gene. The results showed copies of T-DNA integration by dot blot for two lines. Only one sample was found to have stable integration events with two bands observed in correspondence with control PCR product. This indicated that there were two copies of *ABR17* genes in sample 2 (Figure 4.26). The integration frequency of the *ABR17* gene in transformed *A. comosus* was found to be low. This could be due to chimeric characteristic of the plant obtained from the shoot tips culture wherein the transformants were regenerated from a group of cells. It was suggested that somatic embryogenesis is the best solution to produce stable transgene integration by producing non-chimeric transgenic plant (Sivamani *et al*., 2015; Srivastava *et al*., 2015).

Degradation of non-integrated fragment of DNA in the plant genome by autonomous plant system might have caused this false positive results obtained in PCR analysis where the presence of *ABR17* gene was detected in some samples but its integration failed to be verified via Southern hybridization analysis.

5.3 Future study

Somatic embryogenesis is a process where a single somatic cell able to evolve into plant or embryo. The process will produce embryogenic callus (EC) by manipulating PGRs in the tissue culture medium. Hence, EC can be used as plant source to stabilize the transformed genes for transformation process since false positive transformants in this study might happen due to chimerism. Furthermore, EC can enhance the transformation efficiency since it can produce higher number of planting period in a relatively short period of time.

Transformation protocol of this study should also be upgrade by optimizing the parameters such as adding novel compounds, quorum sensing and quorum quenching, different type of plasmid, range of concentration. Optimizing the parameters is very crucial since it can enhance the transformation efficiency.

Then, the positive putative transformants generated from this study need to be challenge towards abiotic stress such as salt and temperature. This procedure need to be done to ensure the functional of the gene and to get a stable transformation plant. Transgenic generation should be propagated and backcrossing of the T1 generation should be done to get new generation of offspring. Furthermore, functional studies on the genes need to be carried out by studying the expression level of RNA and protein.

CHAPTER 6: CONCLUSION

In this study, the optimal shoot initiation was recorded on MS medium supplemented with 0.1 g/L Myo-inositol, 3 mg/L BAP and 1 mg/L NAA. Subsequently for shoot multiplication, the optimal media was similar to shoot initiation but with reduced BAP concentration of 2 mg/L. Rooting of plantlets were best achieved on MS medium containing 6 mg/L activated charcoal (AC) with a mean number of 9.3 and length of about 8.8 cm. It was also found that the photosynthesis rate, stomatal conductance, and transpiration rate of plantlets after 42 days of transfer were significantly different compared to 14 and 28 days. These results indicated that *in vitro* plantlets were initially C3 plants but switched to CAM plants after 42 days of acclimatization. Besides, our findings also indicated that *in vitro*-derived pineapple plantlets started to develop morphological and physiological characteristics comparable to the field-grown plants after 42 days of acclimatization, suggesting that this could be the suitable period for acclimatization before transferring *in vitro*-derived plants into fields.

Efforts have been made to improve the production of economically important pineapple (*A. comosus*) varieties through molecular and transgenic approaches. In this study, transformation parameters including infection time, concentration of acetosyringone and co-cultivation period were optimized for future plant transformation work using *A. tumefaciens* mediated method. The best infection time for transformation of pineapple was 10 min since it produced the highest number of regenerant lines that survived on the selection media (29-50) Explants supplemented with 100 µM acetosyringone for 1 day co-cultivation period and 10min infection time were chosen as the transformation protocol since it produced high number of regenerant lines (45) and positive putative transformants (22%). PCR analysis showed the presence of *ABR17*

gene in regenerated T_0 transformed plantlets of *A.comosus*. The integration of *ABR17* gene in the plant genome was detected through Southern Blot analysis with two copies of ABR17 genes integrated in one of the samples. However, T1 transgenic lines did not show any positive PCR results on the *ABR17* gene. Nonetheless, further studies are required for gene expression and challenge assays.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publication

1. **Hamid, N. S**., Bukhori, M. F. M., & Jalil, M. (2013). Direct and indirect plant regenerations of pineapple var. MD2 *Ananas comosus* L.). *Malaysia Applied Biology, 42*(1), 61-66.

Conference

Nur Syazwani Hamid, Mahanom Jalil, Boon Chin Tan and Norzulaani Khalid. Development of an efficient *Agrobacterium*-mediated transformation protocol for pineapple var. MD2 (*Ananas comosus* L. Merr.). Poster presenter at 21st Malaysian Society for Molecular Biology & Biotechnology, 1st – 3rd October, 2014, Monash University, Selangor, Malaysia.

Nur Syazwani Hamid, Mahanom Jalil, Boon Chin Tan, Normaniza Osman and Norzulaani Khalid. Physiological changes of *In vitro*-derived pineapple plantlets var. MD2 (*Ananas comosus* (L.) Merr.) during acclimatization. Poster presenter ar 25th Malaysian Society of Plant Physiology Conference, 18th – 20th August 2015, Sunway Lost World, Tambun, Ipoh, Perak.