ANALYSIS OF HISTONE DEACETYLATION AS AN EPIGENETIC FACTOR IN SOMACLONAL VARIATION OF *Ananas comosus* VARIETY MD2

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ANALYSIS OF HISTONE DEACETYLATION AS AN EPIGENETIC FACTOR IN SOMACLONAL VARIATION OF *Ananas comosus* VARIETY MD2

ABSTRACT

The removal of acetyl groups ($O=C-CH_3$) from ε-N-acetyl lysine amino acid on histone tails (histone deacetylation) plays a significant role for plants in response to stress conditions, leading to epigenetic modification related to somaclonal variation phenomenon. In spite of its importance, information about histone deacetylation activities in major tropical crops, such as pineapple (*Ananas comosus* variety MD2), remains obscure. Therefore, the main aim of this study was to investigate the changes of deacetylation in response to abiotic stresses in pineapple. Leaf base of the *A. comosus* var. MD2 was used as explants for shoot initiation. The regenerated plantlets were then used to create somaclonal variants. In this study, optimum regeneration media (OM) with the highest number of microshoots (6 ± 0.4) produced from leaf bases was Murashige and Skoog (MS) medium supplemented with 1.0 mg L^{-1} indole-3-butyric acid (IBA) and 2.0 mg L^{-1} 6-benzylaminopurine (BAP). Somaclonal variants were induced from *in vitro A. comosus* through hormonal induction, NaCl, and abscisic acid (ABA) supplementation. Dwarfism was observed in regeneration media (RM) containing either 2.0 mg L^{-1} IBA and 4.0 mg L^{-1} BAP, 1.0 % (w/v) NaCl or 1.0 mg L^{-1} ABA. The histone deacetylase (HDAC) enzyme activity for dwarf and non-dwarf plantlets was also analyzed. The highest HDAC activity (109,333.33 ± 4.40 ng/min/mg) was recorded for dwarf plantlets grown on RM supplemented with 1.0 mg L^{-1} ABA. The dwarf variants underwent phenotypic recovery within 8 months after transferred to MS basal medium. No ploidy alteration was detected in these dwarf plantlets by a flow cytometry. The relative expression level of endogenous *Ananas comosus* histone deacetylase gene (*AcHD2)* was also evaluated to determine the transcripts level among the plantlets. Data analysis showed that the expression level of *AcHD2* in the variants

grown on RM 1.0 % (w/v) NaCl and RM supplemented with 1.0 mg L^{-1} ABA were higher compared to phenotypically normal plantlets grown on MS basal media and optimum regeneration media (OM). To confirm the dwarfism is caused by epigenetic modification, *AtHD2* gene from *Arabidopsis thaliana* was synthesized and expressed in *A. comosus*. Morphological observation of the transgenic based on its height shows significant reduction (dwarf) as compared to the wild type. The histone deacetylase (HDAC) enzyme activity for the transgenic is also higher (5,902.00 ± 25.10 ng/min/mg) compared to the wild type (666.67 ± 22.07 ng/min/mg). Ploidy evaluation of transgenic shows no change occurred in the sample and remained as 2n. The relative expression level of *AtHD2* in the transgenic plant was higher compared to the wild type plants. These evidences have supported the results of the incidence of somaclonal variation in the media after treated with different abiotic stresses. Collectively, these results depict the histone deacetylation activities in *A. comosus* and implied that the incidence of somaclonal variation in response to abiotic stress in *A. comosus* was epigenetically regulated.

Keywords: pineapple, somaclonal variant, histone deacetylase, dwarf, gene repression

ANALISIS DEASETILASI HISTON SEBAGAI FAKTOR EPIGENETIK TERHADAP VARIASI SOMAKLONAL PADA *Ananas comosus* VARIETI MD2

ABSTRAK

Penyingkiran kumpulan asetil ($O=C-CH_3$) daripada hujung histone asid amino ε-N-asetil lisin (deasetilasi histone) memainkan peranan yang penting kepada tumbuhan dalam tekanan yang menyebabkan pengubahsuaian epigenetik terkait dengan fenomena variasi somaklonal. Walaupun ianya penting, maklumat mengenai deasetilasi histone pada tanaman tropika utama; seperti nanas (*Ananas comosus* varieti. MD2) masih kurang jelas. Oleh itu, tujuan utama kajian ini adalah untuk menyelidik perubahan deasetilasi sebagai tindak balas kepada tekanan abiotik di dalam nanas. Asas titik tumbuh daun *A. comosus* var. MD2 digunakan sebagai tisu eksplan untuk inisiasi pucuk baru. Anak pokok yang diregenarasi kemudiannya digunakan untuk menghasilkan varian somaklonal. Di dalam pembelajaran ini, media optimum untuk regenerasi (OM) dengan bilangan pucuk mikro tertinggi didapati pada medium Murashige dan Skoog (MS) yang ditambah dengan 1.0 mg L^{-1} indole-3-butyric acid (IBA) dan 2.0 mg L^{-1} 6-benzylaminopurine (BAP). Dalam kajian ini, kami menginduksi variasi somaklonal pada pokok nanas *in vitro* dengan induksi hormon, NaCl dan suplementasi asid abscisic (ABA). Kekerdilan telah diperhatikan pada regenerasi media (RM) yang mengandungi sama ada 2.0 mg L^{-1} IBA dan 4.0 mg L^{-1} BAP, 1.0 % NaCl atau 1.0 mg L^{-1} ABA. Aktiviti enzim histon deasetilas (HDAC) untuk anak pokok kerdil dan tak kerdil juga dianalisis. Aktiviti HDAC yang paling tinggi (109,333.33 ± 4.40 ng/min/mg) telah direkodkan pada anak pokok nanas yang kerdil ditanam di media yang ditambah dengan 1.0 mg L^{-1} ABA. Varian-varian yang kerdil juga menjalani pemulihan fenotip selama 8 bulan selepas dipindahkan ke media asas MS. Tiada perubahan ploidi dikesan ke atas tumbuhan kerdil selepas dianalisis oleh sitometri aliran. Tahap ekspresi relatif gen endogen *Ananas comosus* histon deasetilas juga turut dievaluasi untuk mengenalpasti

tahap transkrip di antara semua anak pokok. Analisis data menunjukkan tahap ekspresi *AcHD2* pada varian-varian yang tumbuh pada RM 1.0 % NaCl dan RM disumplementasi dengan 1.0 mg L^{-1} ABA adalah lebih tinggi berbanding anak-anak pokok normal yang tumbuh pada media MS dan media OM. Untuk mengesahkan kekerdilan tersebut adalah disebabkan oleh modifikasi epigenetik, gen *AtHD2* daripada *Arabidopsis thaliana* disintesis dan diekspresi di dalam *A. comosus.* Pemerhatian morfologi pada transgenik berdasarkan ketinggian menunjukkan pengurangan yang signifikan (kerdil) berbanding nanas liar (wild type). Activiti enzim histon deasetilas (HDAC) pada nanas transgenik (5,902.00 ± 25.10 ng/min/mg adalah tinggi berbanding nanas liar (666.67 ± 22.07 ng/min/mg). Evaluasi ploidi pada transgenik menunjukkan tiada perubahan berlaku pada sampel dan kekal sebagai 2n. Tahap relatif ekspresi pada *AtHD2* pada nanas transgenik adalah lebih tinggi berbanding nanas liar. Bukti-bukti ini telah menyokong hasil-hasil di atas insiden variasi somaklonal pada di dalam media yang dirawat dengan tekanan abiotik yang berbeza-beza. Secara keseluruhannya, keputusan-keputusan ini menggambarkan aktiviti deasetilasi histon *A. comosus* dan menunjukkan kejadian variasi somaklonal nanas dalam tindakbalas terhadap tekanan abiotik adalah dikawal secara epigenetik.

Kata kunci: nanas, varian somaklonal, histon deasetilas, kerdil, gen penindasan

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bp	:	Base pairs
cm	:	Centimeter
ºC	:	Degree celcius
g	:	Gram
µg	:	Microgram
µg/ml	:	Microgram per milliliter
µl	:	Microliter
%	:	Percentage
ABA	:	Abscisic acid
ACC	:	Aminocyclopropane-1-carboxylic acid synthase
AFLP	:	Amplified fragment length polymorphism
BAP	:	6-benzylaminopurine
cpDNA	:	Chloroplast DNA
CAM	:	Crassulacean Acid Metabolism
DNA	:	Deoxyribonucleic acid
ELISA	:	Enzyme-linked immunosobent assay
EST	:	Expressed sequence tag
EST-SSR	:	Expressed sequence tag- simple sequence repeat
FAO	:	Food Agriculture Organization
FAOSTAT	:	FAO Statistics
gfp	:	Green fluorescence protein gene
HAT	:	Histone acetyltransferases
HDAC	:	Histone deacetylase enzyme
HD2	:	Histone deacetylase gene

hpt	:	hygromycin phosphotransferase gene
IBA	:	Indole-3-butyric acid
LB	:	Luria-Bertani
MAS	:	Marker-assisted selection
MPIB	:	Malaysian Pineapple Industrial Board
mRNA	:	Messenger RNA
NAA	:	Naphthalene acetic acid
NKEA	:	National Key Economic Area
OM	:	Optimum regeneration media
PBIU	:	Plant Biotechnology Incubator Unit
PCR	:	Polymerase chain reaction
PGR	:	Plant growth regulator
PRI	:	Pineapple Research Institute of Hawaii
RAPD	:	Random amplified polymorphic DNA
RCBD	:	Randomized complete block design
RE	:	Restriction enzyme
RFLP	:	Restriction-fragment length polymorphism
RM	:	Regeneration media
RNA	:	Ribonucleic acid
ROS	:	Reactive oxygen species
RT-qPCR	:	Real time quantitative PCR
SE	:	Standard error
SPSS	:	Statistical Package for the Social Sciences
SV	:	Somaclonal variation
TAIR	:	The Arabidopsis Information Resource
YEB	:	Yeast extract beef

OF APPENDICES

CHAPTER 1: INRODUCTION

Pineapple [*Ananas comosus* (L.) Merr.], a cultigen species of a cluster from hundreds of fruitlets, is believed to be derived from two wild species: *A. comosus* and *A. fritz-muelleri* (Van Wyk, 2005). After 6,000 years of its discovery, pineapple has now become one of the most appreciated tropical fruits due to its delicious taste and nutritional benefits (de Ancos et al., 2017). Pineapple fruits are juicy, tangy, sweet and rich in vitamins, including vitamins A, B6, B12, C and D, as well as minerals like calcium, iron and magnesium (Lobo & Paull, 2017). It contains bromelain, a powerful proteolytic-digesting enzyme, which is important for protein digestion (Amini et al., 2016). At present, pineapple has become one of major contributors for world's global wellness after its initial domestication in the Americas and Europe (Lobo & Siddiq, 2017).

MD2 which is also known as the Golden Juanita or Yankee are the most preferred pineapple fruit (Wardy et al., 2009). Malaysian government has listed pineapple variety MD2 as one of the focused fruits under the Eleventh Malaysia Plan, aiming to increase its production to meet the high demand in local and foreign markets (Mansor et al., 2017). However, the production of MD2 pineapple is low due to many constraints, such as the weather fluctuations, pest and disease attacks (Akhilomen et al., 2015; Baruwa, 2013; Richardson et al., 2013).

To address these challenges, an alternative viable approach to produce planting material through plant tissue culture is essential. Plant tissue culture is a powerful technique not only for micropropagation, but to explore potential genetic improvement, for biodiversity conservation of elite clones and selection of novel variants (Opabode, 2017).

Although plant tissue culture has been used in propagation of various commercial crops, this technique can result in a unique phenomenon known as somaclonal variation (Larkin & Scowcroft, 1981). Somaclonal variation is the occurrence of somaclones that is usually observed by phenotypic alteration either due to tissue culture protocols (Yaacob et al., 2013) or happen naturally (Jain et al., 2013; Nwauzoma & Jaja, 2013). Numerous factors have been reported to cause somaclonal variation, such as initiation of tissue culture plants from organized meristematic growth, genetic constituents (genotype, ploidy) of the explant source, utilization of plant growth regulators (type and concentration), source of explants, and chromosome rearrangements.

Plants are sedentary organisms that are incapable to hinder external stresses, therefore, have unique defense systems to ensure resistance, tolerance and avoidance towards the unfavourable stimuli. Abiotic stresses, such as involuntary damages, climatic changes, chemical and physical adjustment to the environment, and biotic factors, such as pathogens attack, can also trigger plant adaptation leading to phenotypic and genotypic alterations (Ben Rejeb et al., 2014).

Example of tissue culture-induced somaclonal variation include the mantled phenotypes in oil palm, where they have finger-like fruits and a thick outer coating, thus minimizing the seed size and affects oil production. However, somaclonal variation has now become a worthy tool for plant breeding wherein the variants regenerated from somatic cells might have novel traits that can be used for crop improvement (Cardoza & Stewart, 2004a).

The molecular facets of the occurrence of somaclonal variation have not yet been fully examined, but one of the most likely factors is gene expression (Miguel &

Marum, 2011). There are various factors that can trigger gene repression such as DNA methylation, histone methylation and histone deacetylation.

Acetylation and deacetylation of the nuclear histone proteins interchangeably regulate the transcription in eukaryotic cells (Xu et al., 2007). The acetylation progression of histone and non-histone protein is determined by histone acetyltransferases (HAT) and histone deacetylases (Bannister & Kouzarides, 2011). Histone deacetylases (HDAC) participate in eukaryotic gene regulation by catalysing the acetyl groups' removal from the lysine residues on histone, thus HDAC transcriptionally repress gene to be expressed (Coombs, 2013; Ma et al., 2013; Miguel & Marum, 2011). Principally, acetylation of histone stimulates a more relaxed structure of chromatin, allowing transcriptional activation. In contrast, HDACs act as repressor during histone deacetylation and consequently trigger chromatin condensation.

This study aimed to analyse the effect of histone deacetylation as an epigenetic factor behind the occurrence of somaclonal variation in *in vitro* pineapple plants. Formation of somaclonal variants were induced with abiotic stress (salinity and high abscisic acid concentration; a phytohormone that involves in abiotic stress signalling) and manipulation of high auxin and cytokinin concentrations.

To verify the occurrence of somaclonal variation due to histone deacetylation, *Arabidopsis thaliana* histone deacetylase gene (*AtHD2*), was transformed into *A. comosus* plants. The variants were subjected to histone deacetylase enzyme activities assay by using an ELISA technique. In addition, real time quantitative PCR (RT-qPCR) was also performed to determine the mRNA transcript level of the transformed gene (*AtHD2*) as well as the expression of endogenous gene (*AcHD2* derived from *A. comosus*).

Somaclonal variation of *A. comosus* var. MD2 observed from *in vitro* micropropagation which may provide new insights related to *HD2* epigene studied in this research were acquired through the following objectives;

(i) To optimize tissue culture conditions by choosing the best concentration and/or combination of plant growth regulators such as IBA and BAP hormones.

(ii) To investigate the effects of abiotic stress factors, such as plant growth regulators, salinity and abscisic acid in generating somaclonal variants in *A. comosus* var. MD2.

(iii) To evaluate the effects of transformation with *AtHD2* gene in phenotypically normal *A. comosus* var. MD2 plants.

(iv) To determine the histone deacetylase enzyme activity in somaclonal variants.

CHAPTER 2: LITERATURE REVIEW

2.1 Importance of Pineapple

Pineapple [*Ananas comosus* (L). Merr.], one of the most valuable fruits, belongs to the family of Bromeliaceae which originated from South America such as Brazil, Venezuela and Paraguay It has been widely planted in major tropical and sub-tropical regions in the world, mainly for its fruit (Iwuchukwu et al., 2017). Pineapple ranked third for global production after banana and citrus (Joy & Anjana, 2016; Ko et al., 2008; Paull & Duarte, 2011).

Food Agriculture Organization (FAO) Statistics (FAOSTAT) showed that pineapple production worldwide has increased from 3,831,533 metric tons in 1961 to 25,809,038 metric tons in 2016 (FAOSTAT, 2017). Currently, Brazil, China, Thailand and the Philippines are the prime pineapple exporters in the world producing almost 50 % of the total worldwide production (Hossain et al., 2015).

In Malaysia, pineapple was first introduced to Johor in 1888 and was then expanded to Selangor and Perak (Musa, 2012). Varieties cultivated in Malaysia are N36, Gandul, Maspine, MD2, Yankee, Josapine, Moris, Sarawak and Moris Gajah. These cultivars had made Malaysia as the top three global producers during 1960s to early 1970s.

About 70% of pineapple fruit is freshly consumed in top producing countries, such as Brazil (2.7 million tonnes), China (1.6 million tonnes), Thailand (1.8 million tonnes) and the Philippines (2.6 million tonnes) (Aragón et al., 2013; d'Eeckenbrugge et al., 2011; FAOSTAT, 2017). In Malaysia, Josapine, Moris and Sarawak were planted for fresh consumption whereas variety Gandul are grown for canning and juices. Josapine and Maspine were used for both consumptions.

2.1.1 Morphology and botanical classification

A. comosus is a herbaceous perennial monocotyledonous plant. Pineapple is Crassulacean Acid Metabolism (CAM) plant which grows and dies annually with vegetative propagule parts surviving for continuous plantation. The main morphological components of pineapple are the stems, leaves, peduncle, crown, slip, roots and sucker (Figure 2.1). The matured plants are 1-2 metres high with stem length of 25-50 cm (Bartholomew et al., 2002). Its aerial part is straight and erect, while the shape of the earthed part depends on the materials used for planting. It is markedly curved when coming from a slip, as the stems of these propagules are comma-shaped, less curved when coming from a stem shoot and erect when coming from crown.

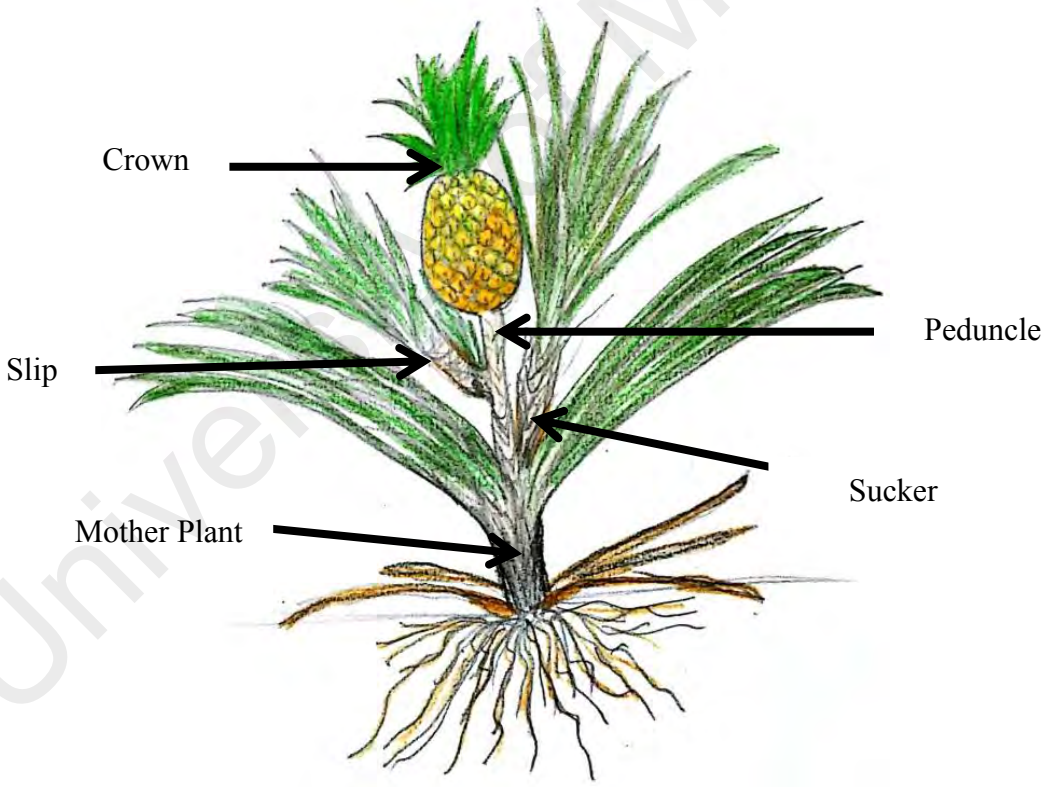


Figure 2.1: Structure of *A. comosus* plant showing sequence of vegetative cycles and different types of starting materials for field propagation (Slip, crown and sucker). Picture was drawn by the writer of this thesis.

Leaf number varies between cultivars and generally about around 40-80 leaves. The lower ones, originating from the planting material or produced soon after planting, are smaller (5-20 cm) compared with the younger ones, which can reach more than 1.6 m in length and 7 cm in diameter width, depending on the cultivar and ecological conditions.

The leaves are waxy and arranged spirally on the stem. The strap-like long-pointed green or red striped leaves are usually needle-tipped and generally bear sharp, up-curved spines on the margins. Many cultivars, such as the „Smooth Cayenne" show a partial or complete absence of spines on the leaves. The leaves also have trichomes mostly on the underside surface. Trichomes are tiny, hair-like structures that surround the stomata to further limit water loss by increasing the boundary layer thickness.

Table 2.1: Botanical description of *A. comosus*

Kingdom:	Plantae (Plants)
Subkingdom:	Tracheobionta (Vascular plants)
Superdivision:	Spermatophyta (Seed plants)
Division:	Magnoliophyta (Flowering plants)
Class:	Liliopsida (Monocotyledons)
Subclass:	Zingiberidae
Order:	Bromeliales
Family:	Bromeliaceae (Bromeliad family)
Genus:	*Ananas* Mill. (Pineapple)
Species:	*Ananas comosus* (L.) Merr. (Pineapple)

The pineapple fruit is formed from the fusion of hundreds inflorescence of florets (Figure 2.2) and has been classified in Magnoliophyta division (Table 2.1). Each fruitlet has small naked, winged or plumose seeds. The flowers are actinomorphic (radially symmetrical) and trimerous with differentiated floral parts. They produce a coenocarpium (a multiple fruit derived from ovaries, floral parts and receptacles of many coalesced flowers) (Paull & Duarte, 2011).

Figure 2.2: The MD2 pineapple florets grown at University of Malaya Biotechnology Research Centre, Glami Lemi, Jelebu, Negeri Sembilan, Malaysia

2.1.2 Nutritional value and health benefits

The fruit of pineapple contains high nutritive value, which benefits for human consumption. The nutritive analysis of 100 g of pineapple is shown below.

Table 2.2: Proximate analysis of nutrient content in pineapple (de Ancos et al., 2016)

	Nutrient value	**Percentage of RDA**
Proximate		
Energy	50 kcal	2.5 %
Carbohydrates	13.52 g	10 %
Proteins	0.54 g	1 %
Total Fat	0.1 g	< 1 %
Cholesterol	0 mg	0 %
Dietary fibre	1.40 g	4 %
Vitamins		
Folates	18 µg	4.5 %
Niacin	0.500 mg	4 %
Pyridoxine	0.112 mg	9 %
Riboflavin	0.018 mg	1.5 %
Thiamin	0.079 mg	6.5 %
Vitamin A	58 IU	2 %
Vitamin C	47.8 mg	80 %
Vitamin E	0.02 mg	<1 %
Vitamin K	0.07 µg	0.5 %
Electrolytes		
Potassium	1 mg	0 %
Sodium	109 mg	2.5 %
Minerals		
Calcium	13 mg	1.3 %
Copper	0.110 mg	12 %
Iron	0.29 mg	3.5 %
Magnesium	12 mg	3 %
Manganese	0.927 mg	40 %
Phosphorus	8 mg	1 %
Selenium	0.1 µg	<1 %
Zinc	0.12 mg	1 %
Phytonutrients		
Carotene-β	35 µg	-

2.1.3 Pineapple varieties in Malaysia

In Malaysia, several varieties have been farmed and commercialized in the market (K. Y. Lee, 2010). These include:

MD2

This variety originates from the Philippines. It is now planted on a commercial scale Johor Bharu. The cylindrical and square-shouldered fruit has large, flat "eyes" that turn from orange to yellow when it starts to ripen. The flesh is rich golden colour with an intense floral aroma and is less fibrous, very juicy and sweet. It has a luscious taste similar to mango.

Moris

This variety, also known as Mauritius or Queen, is commonly found all the year round. The fruit is elongate in shape with small, prominent eyes that require a thick cut to be removed. When it is bitten into, it has a crisp crunch, unique flavor and sometimes sharp taste. This highly fibrous fruit is commonly used for jam making, cooked in curries or tossed in a rojak. Other sweeter varieties have made this variety less popular as an eating pineapple.

Josapine

This is a hybrid pineapple that was developed by the Malaysian Agricultural Research and Development Institute in August 1996. The name originated from its parents, the "JOhor" (Red Spanish) and "SArawak" (Smooth Cayenne) PINEapple varieties. It has small, roundish, cylindrical shape and turns an appealing bright from orange to red upon ripening. The flesh is deep golden yellow, sweet and just a bit fibrous, with a strong, sweet, floral aroma emanating from the fruit. In recent years, it

has gained a local popularity and thus, can be found in most supermarkets and local markets throughout the year.

Sarawak

This cylindrical-shaped pineapple, also known as Smooth Cayenne, has large and shallow eyes. The flesh is pale yellow and juicy, and when totally ripen, it has a sweet and tangy taste. Once a premium variety and was sought-after for its sweetness and smoothness, but now been substituted by cheaper and better tasting hybrids such as the Josapine. It is available throughout the year, but in smaller quantities.

Yan Kee

The Yan Kee reflects out for its rather odd shape. The long and tapering pineapple is planted in Klang and Sungai Pelek. It is also known as Selangor Sweet. The flesh is pale and translucent when entirely ripe. Its eyes are not deeply embedded and required only shallow cuts in order to remove them. The Yan Kee has the highest juiciness level compared to other types and least fibrous of the pineapples. Its elegant, sweetish fragrance is reminiscent of melon. The season for harvesting Yan Kee is May-June and October-November.

2.1.4 Origin and importance of MD2 pineapple

MD2 pineapple (Figure 2.3), has dominated the international markets and maintained as the highest export demand commodity (Ding & Syazwani, 2016). The fresh MD2 pineapple consumption has shown a four-fold increase within 13 years in the North American and European Union (Loeillet et al., 2010). This Hawaiian hybrid was developed by Del Monte Corporation from a cross between two PRI hybrids, 58-1184 and 59-443 (Greig, 2004).

Figure 2.3: A ripened MD2 pineapple grown at University of Malaya Biotechnology Research Centre, Glami Lemi, Jelebu, Negeri Sembilan, Malaysia.

MD2 pineapple was named after Millie, the wife of the Del Monte General Manager, Frank Dillard. Two selections from the PRI hybrids mentioned earlier was renamed as MD1 (as internal use and later was patented as CO2) and MD2 (for export purposes), respectively (Joy & Anjana, 2016). MD2 has several trade names in Malaysia such as "Golden Ripe", "Super Sweet", "Rompine" or "Gold".

MD2 variety has dominated most pineapple production and fresh markets in many countries over the Smooth Cayenne, which a popular variety before MD2 (Reid & Jiang, 2011). It has lustrous, green golden shell colour, a regular and appealing cylindrical shape which made it to be known as "golden pineapple" (Janick, 2003).

In Malaysia, the MD2 bred cultivar is the leading pineapple in its production due to the most preferred attributes (aroma, sweetness and colour) by consumers and enlarged market internationally (Ankrah & Dorward, 2015; Halim et al., 2017; Syahrin, 2010). To meet the high demand of MD2 fruits in local and foreign markets, for instance China, Korea, Singapore and Middle East, MD2 has been included in the Eleventh Malaysia Plan and National Key Economic Area (NKEA), which aims to increase its production. (Mansor et al., 2017; Ng et al., 2015).

Malaysian Pineapple Industrial Board (MPIB) to set up a new hub for pineapple plantation and production in Sarawak to meet the demand from China (Ahmad, 2018). Research targeting variety improvements (Chan et al., 2002), physiological attributes (Wardy et al., 2009) as well as the genetic improvements of MD2 pineapple (Hidayat et al., 2012; Ming et al., 2015), have been carried out to ensure sustainability and improvement of the traits and the quality of MD2.

2.2 Improvement of pineapple

2.2.1 Conventional breeding of pineapple

The three most economically important traditional pineapple cultivars in the world are „Cayenne", „Singapore Spanish", and „Queen" (d'Eeckenbrugge et al., 1997). Three other cultivars („Red Spanish", „Perola", and „Manzana") are cultivated in their regions of origin for local markets (d'Eeckenbrugge et al., 1997). „Smooth Cayenne" is the most important cultivar owing to its high yield potential and favorable characteristics as a fresh fruit and for processing. Therefore, „Smooth Cayenne" is the principal cultivar used around the world for pineapple breeding (Leal and d'Eeckenbrugge 1996). The first pineapple breeding program was carried out in Florida, USA, with the goal of obtaining cultivars that were better adapted to local conditions and that had better fruit quality. Subsequently, similar programs were developed in

Hawaii (USA), South Africa, India, Malaysia, Côte d'Ivoire, and Brazil (d'Eeckenbrugge et al., 1997, Leal and d'Eeckenbrugge 1996).

The objective of the world's major breeding programs has been the improvement of „Smooth Cayenne". However, as markets for fresh fruit developed, fruit quality became an increasingly important characteristic. Therefore, the objective of breeding in most programs gradually shifted from high yield and processability to high quality of the fresh fruit. The most successful breeding program was conducted by the Pineapple Research Institute (PRI) in Hawaii, with the goal to breed cultivars as alternatives to „Smooth Cayenne" for use as fresh and processed fruit (Williams and Fleisch, 1993). Two hybrids originated from this program. For example, „MD2" replaced „Smooth Cayenne" in many production areas around the world. Pineapple breeding in many other countries has produced new cultivars, including „Tainung 17" and „Tainung 21" from Taiwan (Bartholomew et al., 2010, Tang et al., 2014); „Josapine" from Malaysia (Bartholomew et al. 2010); „Aus Jubilee", „Aus Carnival", and „Aus Festival" from Australia (Bartholomew et al., 2010, Sanewski, 2014); and „Imperial" from Brazil (Bartholomew et al., 2010, Cabral and de Matos, 2009).

2.2.2 Biotechnology improvement of pineapple

New biotechnology techniques offer substantial opportunities for improving pineapple breeding. These include genetic engineering, the development of molecular markers, evaluation of genetic diversity, development of DNA markers linked with traits of interest and their use in marker-assisted selection (MAS), construction of genetic linkage maps, and also genome and transcriptome analyses (d'Eeckenbrugge et al., 2011, Smith et al., 2003, Zhang et al., 2014). While genetic transformation allows small targeted changes to the recipient pineapple plant's genome through direct gene transfer and *Agrobacterium*-mediated transformation (Ko et al., 2008).

The genetic diversity among pineapple germplasms was initially investigated using isozyme markers. DeWald et al. (1988) found that 15 out of 27 pineapple cultivars could be identified by using markers for five enzymatic systems: two peroxidases, and three phosphoglucomutases. Besides, DNA-based markers have been used to study phylogenetic and genetic relationships in *Ananas*; these include amplified fragment length polymorphism (AFLP), randomly amplified polymorphic DNA (RAPD), restriction-fragment length polymorphism (RFLP), chloroplast DNA (cpDNA) polymorphism, PCR-RFLP, and simple sequence repeat (SSR) markers.

Raus et al. (1995) evaluated four pineapple cultivars („Perola", „Smooth Cayenne", „Primavera", and „Perolera") using RAPD markers. Kato et al. (2004) evaluated 162 accessions, including 148 of *A. comosus* and 14 of related species, using AFLP markers, and found abundant genetic variation within them. Their data suggested that discrete DNA fingerprints for commercial cultivars could be detected and used for cultivar protection. Wohrmann and Weising (2011) identified 696 SSRs among 3389 expressed sequence tag (EST) unigenes, and developed primer sequences and described the locus characteristics for 18 selected EST-SSR markers. Feng et al. (2013) defined genetic diversity of 48 pineapple germplasms using 18 newly developed SSR markers.

Another possible method could be applied in pineapple improvement is somactic hybridization. However, somatic hybridization have been applied for pineapple (Davey et al., 2004; Soneji & Rao, 2009), are not yet successful due to the failure of reproducible protoplasts-to-plant system (Ruhlman & Daniell, 2007).

Biolistics, a direct gene transfer method has been used to deliver genes that confer herbicide resistance in „Smooth Cayenne" (Sripaoraya et al., 2001). The introduction of aminocyclopropane-1-carboxylic acid synthase (ACC synthase) genes via *Agrobacterium* inoculation has been carried out to control ripening (Firoozabady et

al., 2006). Field trials have been performed with pineapples genetically modified for viral and nematode resistance, delayed maturation, modified sugar composition, and flowering time (Hanke and Flachowsky, 2010). Del Monte company has obtained red-fleshed „Rosé‟ pineapple by combining overexpression of a gene derived from tangerine and suppression of other genes to increase the accumulation of lycopene.

In addition pineapple could be improved through somaclonal variation. This phenomenon occurred as a result of tissue culture techniques to result in genetic variability which may be useful and valuable for crop improvement (Ebrahimi et al., 2018).

2.3 Propagation of pineapples

2.3.1 Methods of propagation

Propagation of pineapple usually was initiated from its vegetative parts such as slips, crowns, hapas and suckers (Firoozbady & Young, 2015). In commercial plantation, pineapples are grown from crowns and slips (Jackson et al., 2016). Slips formed from buds in axillary of leaves, grow upwards and are curved at the base. Slips are taken from peduncle, dried and planted within one month of harvest. Macro-propagation by crown is by far less complicated. It involves removing the crown from the harvested fruit, allowing it to dry for a minimum of 2 weeks after which it is replanted. However, crowns grow at a slower rate compared to slips and are less resistant to drought. The turn over time for new propagules when using either method is approximately 18 months which is a slow route for commercial purposes.

An alternative to traditional methods of propagation is the application of tissue culture techniques. The tissue culture process can be summarized in four stages; initiation, multiplication, rooting and acclimatization (Al-Saif et al., 2011; Davey, 2010). The initiation stage involves selecting the mother plant and surface sterilization

of the explant. In a sterile condition, the explant is placed on the nutrient medium for establishment of the plant in culture. The next stage is multiplication, which involves proliferation and multiplication of shoots. This stage is normally repeated until the desired numbers of plantlets are obtained. Subsequently, rooting stage prepares the micro-propagated plants for planting out. The plantlets are often transferred to rooting medium, which serves the purpose of inducing root formation. The last stage is acclimatization, where the plants are transferred from culture medium to soil and acclimatized to the external conditions. The plants are kept in shade or green house where they become adapted to the external environment.

The assessment on the effectiveness of utilizing tissue culture techniques to produce pineapple plants and comparison of their productivity, nutritional profile and maturation period with those grown traditionally has been reported (Jackson et al., 2016). The study discovered tissue cultured pineapples had similar physical and chemical properties when compared to traditionally grown plants. The pineapples produced fruits at the same time. The heights were also the same at the time of fruiting. The fruit weight and nutrient profiles were statistically similar with no significant difference.

Although plant tissue culture has been used in propagation of various commercial crops, this technique can result in a unique phenomenon known as somaclonal variation. Larkin and Scowcroft were the first researchers to define the term somaclonal variation (SV) as a variation originating in cell and tissue cultures (Larkin & Scowcroft, 1981). SV is a phenomenon in which the phenotypes of somaclones were altered, either caused by tissue culture protocols and techniques (Yaacob et al., 2013) or happen naturally that may pre-exist in the population from field assemblage or genebank (Castillo et al., 2010; Jain et al., 2013; Nwauzoma & Jaja, 2013). SV is a

worthy tool in plant breeding wherein variation in tissue culture regenerated plants from somatic cells can be used in the improvement of crops with novel traits (Cardoza & Stewart, 2004b).

2.3.2 Somaclonal variation

In recent years, few studies have detected SV using molecular markers (Aversano et al., 2009; Bairu et al., 2011). SV is sometimes considered as an undesirable by-product due to the stress caused in tissue culture procedures (Mgbeoji & Benda, 2016). However, somaclonal manipulations in cultured plant cells can also generate variants that are useful for breeders (Vasil, 1988).

SV can be induced by several factors explants, media and tissue culture procedures. For example, type of tissue or starting materials used, genotype (ploidy level) of the explant source and establishment of tissue culture plants from organized meristematic growth. As for media factors, type and concentration of plant growth regulators used would contribute to SV, wounding practices during explant preparation the frequency and duration of subculture and effect of stress could cause stress in tissue culture practice (Bairu et al., 2011; Yaacob et al., 2013). The occurrence of SV has been researched in depth where at molecular level, it is believed to be controlled or orchestrated by gene expressions (Miguel & Marum, 2011). Some examples of SV are listed as follows indicating the origin of variation and their outcomes. on the previous factor mentioned are outlined in Table 2.3 (Bairu et al., 2011):

Table 2.3: Occurrence of somaclonal variation from different species of plants (Bairu et al., 2011)

Species (Common name)	Origin of variants	Desirable outcome
Allium tuberosum Rottl. ex. Spreng (Garlic)	Callus culture	Yes
Ananas comosus (L.) Merr. (Pineapple)	Callus culture, colchicine	No

Table 2.3, continued

Species (Common name)	Origin of variants	Desirable outcome
Arabidopsis thaliana (L.) Heynh. (Thale cress)	Callus culture, 2,4-D	No
Asparagus officianalis L. (Garden asparagus)	Callus culture, 2,4-D, duration in culture	No
Avena sativa L. (Oat)	Embryogenic culture, microprojectile bombardment	No
Beta vulgaris L. (Sugar beet)	Callus culture, explant	Yes, No
Camellia sinensis (L.) O. Kuntze (Tea)	Embrogenic culture, number of subcultures	No
Capsicum annuum L. (Chili pepper)	Genotype, BAP	Yes
Citrus lemon (L.) Burm (Lemon)	Callus culture, gamma rays	Yes, No
Coffea Arabica L. (Coffee)	Callus culture, genotype, duration in culture	No
Cucumis sativus L. (Cucumber)	Explant, duration in culture	No
Daucas carota L. (Carrot)	Callus culture	Yes
Elaeis guineensis Jacq. (Oil palm)	Genotype, hormonal imbalance	No
Eleusine coracana (L.) Gaertn (Finger millet)	Callus culture	Yes
Fragaria L. (Strawberry)	BAP	Yes
Fragaria x ananassa Duch. (Strawberry)	Explant, genotype, duration in culture	Yes
Glycine max (L.) Merr. (Soybean)	Embryogenic culture	No
Glycine max (L.) Merr (Soybean)	TDZ, number of subcultures	Yes
Hordeum vulgare L. (Barley)	Callus culture	No
Hordeum vulgare L. (Barley)	Callus culture, 2,4-D, genotype	No
Lycopersicon esculentum L. (Tomato)	Chimeric effect, ethyl methane sulphonate, duration in culture	No
Malus pumila Mill. (Apple)	Number of subcultures	No
Musa acuminate L. (Dessert banana)	Genotype, explant source, number of subcultures	Yes
Musa spp. (Plaintain)	Shoot tip culture	No

Table 2.3, continued

Species (Common name)	Origin of variants	Desirable outcome
Musa acuminata L. (Dessert banana)	Cytokinins, IAA, ABA metabolism	No
Musa acuminata L. (Dessert banana)	IAA, IBA, activation of transposable element	No
Musa acuminata L. (Dessert banana)	BAP, number of subcultures	Yes
Nicotiana tabacum L. (Tobacco)	Protoplasm culture, genotype	No
Oryza sativa L. (Rice)	Duration in culture	Yes
Oryza sativa L. (Rice)	Callus culture, genotype, duration in culture	Yes
Oryza sativa L. (Rice)	Callus culture, 2,4-D, duration in culture	Yes , No
Oryza sativa L. (Rice)	Protoplast-derived callus culture	No
Oryza sativa L. (Rice)	Embryogenic culture	Yes, No
Oryza sativa L. (Rice)	Explant source	No
Phalaenopsis Hsiang Fei (Orchid)	Embryogenic culture	Yes, No
Saccahrum L. hybrid (Sugarcane)	Callus culture	Yes, No
Solanum tuberosum L. (Potato)	Callus culture, duration of culture	No
Triticum aestivum L. (Wheat)	Callus culture, 2,4-D, genotype	Yes
Triticum aestivum L. (Wheat)	Gamma rays, *in vitro* salt treatment	Yes
Triticum aestivum L. (Wheat)	Callus culture, duration in culture	No
Tulipa gesneriana L. (Tulips)	Duration in culture	Yes
Zea mays L. (Maize)	Embryogenic culture, 2,4-D	Yes , No
Zea mays L. (Maize)	Callus culture, mannitol, polyethylene glycol	Yes

2.3.3 Molecular basis of somaclonal variation

In plant growth, gross changes in the genome may occur during somatic differentiation, including endopolyploidy, polyteny and amplification or diminution of DNA sequences (Leva et al., 2012). Somaclonal variability often arises in tissue culture as a manifestation of epigenetic control or changes in the genome of differentiating vegetative cells (Soniya et al., 2001). The processes of de-differentiation and re-differentiation of cells may involve both qualitative and quantitative changes in the genome, and different DNA sequences may be amplified or deleted during cell reprogramming. In addition, this process is closely related to the source of tissue and the regeneration system. Gross and cryptic chromosomal changes or extensive changes in the chromosome number occur during early induction in an *in vitro* culture (Mujib et al., 2007).

Variations in chromosome numbers and structures, and chromosome irregularities (such as breaks, acentric and centric fragments, ring chromosomes, deletions and inversions) are observed during *in vitro* differentiation and among regenerated somaclones (Nwauzoma & Jaja, 2013). Such rearrangements in chromosomes may result in the loss of genes or function, the activation of genes that are previously silent and the expression of recessive genes, when they become haploid (Chen, 2007). The irregularities in the chromosomes may be lost during plant regeneration and result in the production of normal plants, or appear in the regenerated somaclones (Snyman et al., 2011).

Generally, chromosomal changes are frequently observed in regenerated plants than chromosome number changes (Kaeppler et al., 2000). Chromosome breakage and rearrangements usually occur during *in vitro* culture techniques (Neelakandan & Wang, 2012), whereas chromosome breakpoints usually happened between inter

heterochromatic knobs and the centromere (Lee & Phillips, 1987) or within centromeric heterochromatin (Johnson et al., 1987).

The tissue culture environment may result in the modification of DNA methylation patterns histone modifications may be associated with the physiological responses of the plant cells to the conditions *in vitro* (Feil & Fraga, 2012) (Cedar & Bergman, 2012). Global methylation levels and methylation of specific sites are documented in several crops such as oil palm, grapevine and apple (Baránek et al., 2010; Nwauzoma & Jaja, 2013; Smulders & De Klerk, 2011). Rival et al. (2013) observed that *in vitro* proliferation induces DNA hypermethylation in a time-dependent fashion and changes in DNA methylation is involved in modulating the expression of embryogenic capacity of oil palm during tissue culture. In addition, alteration in genomic DNA methylation rate hasattributed to the development of „mantled" somaclonal variant in oil palm (Eeuwens et al., 2002; Jaligot et al., 2011).

In vitro regeneration, proliferation and propagation caused variation in ploidy level represented gross alterations to the genome. (Roy et al., 2010; Wang & Wang, 2012). Ploidy referred to the number of chromosomal sets in a cell (Rieger et al., 2012). Polyploid organisms have several sets of chromosomes in the genome compared to normal diploid organisms (Leal et al., 2006). This is due to the endogenous reduplication where the nuclear genome continues to replicate abnormally during cell division, (Weber et al., 2008). When an extra or missing chromosome number could occur (aneuploidy) (Sheltzer et al., 2012). The structural changes during abnormal cell division may be associated with deletions, duplications, inversions or translocations of specific chromosomal segments (Sybenga, 2012).

Tissue culture has also been reported to activate silent transposable elements, resulting in somaclonal variations. Insertions of transposable elements and

retrotransposons can function as insertional mutagens in plant genomes, whereas widespread activation may result in a wide gamut of chromosomal rearrangements (Tanurdzic et al., 2008). In turn, these rearrangements can lead to misregulation of genes, aneuploidy and new transposon insertions (Smulders & de Klerk, 2011).

2.3.4 Somaclonal variation in *A. comosus*

Somaclonal variants in *A. comosus* has been produced from *in vitro* pineapple axillary bud explants cultured in naphthalene acetic acid (NAA) and 6-benzyladenine (BAP) for micropropagation; kinetin for callus induction; and indole-3-butyric acid (IBA) and gibberellin for plant regeneration (Pérez et al., 2011). Two variants were successfully produced from *in vitro* culture of cv. Red Spanish Pinar and named as P3R5 and Dwarf based on morphological characters. The number of shoots of Dwarf, P3R5 and donor plants was also significantly different. Dwarf plants produced two shoots per plant whereas P3R5 type and donor did not produce any shoots. P3R5 was separated from cv. Red Spanish Pinar by 2.83 units based on Euclidean distance, whereas Dwarf was separated by 3.00 units. However, the phenotype indicators revealed higher differences from donor 3.74 in P3R5 and 4.71 in Dwarf.

Pérez et al. (2012) determined the possibility of genetic alterations in the plant growth regulator (PGR)-induced plantlets. The amplified fragment length polymorphism (AFLP) analysis revealed that genetic distance among the somaclonal variants and the donor plant existed. Among the 44 phenotype indicators tested, one variant (P3R5) differed from the donor (original mother plant) in 19 variables (19/44; 43.18%), while Dwarf variants showed in 31 indicators (bands) (31/44; 70.45%).

2.4 Histone modifications and chromatin remodeling

Epigenetic changes in somaclonal variants occurred due to DNA methylation and histone modification such as histone acetylation and deacetylation, ubiqutination

and sumoylation. All these modifications are cause by chromatin structural changes. Chromatin sequence is a complex structure of 145 bp of DNA repeating unit known as nucleosomes that are wrapped around octameric histones. It is a group of basic proteins that consists of two molecules for each histone H2A, H2B, H3 and H4 (Grunstein, 1997). Chromatin structures are very dynamic and are essential in gene regulations that are important for cellular functions, such as gene transcription, DNA replication, DNA repair and also in DNA recombination (Orphanides & Reinberg, 2002).

Chromatin modification is controlled precisely by a range of transcriptional regulators that responds to both the cellular and environmental stimuli, hence reducing the proper temporal and spatial development in eukaryotic organisms (Barrera & Ren, 2006; Li et al., 2007; Orphanides & Reinberg, 2002). These chromatin structures in turn are regulated by post-translational modifications of the histone proteins, which involve various enzymes to modify the histones and chromatin remodeling machines (Habu, 2017; Li et al., 2007; Probst et al., 2004; Wang et al., 2015).

These modifications include methylation, acetylation, ubiquitination, carbonylation, biotinylation, sumoylation, phosphorylation, ADP ribosylation and glycosylation as shown in Figure 2.4 (Lillycrop, 2017; Loidl, 2004; Probst et al., 2004; Vanzan et al., 2017). These modifications can result in structural and functional reorganization of the chromatin complex and thus, on the important elements that builds the „epigenetic histone code", which is recognized and interpreted by the transcriptional regulators and chromatin remodeling machines that are involved (Baroux & Autran, 2015; Varriale, 2017).

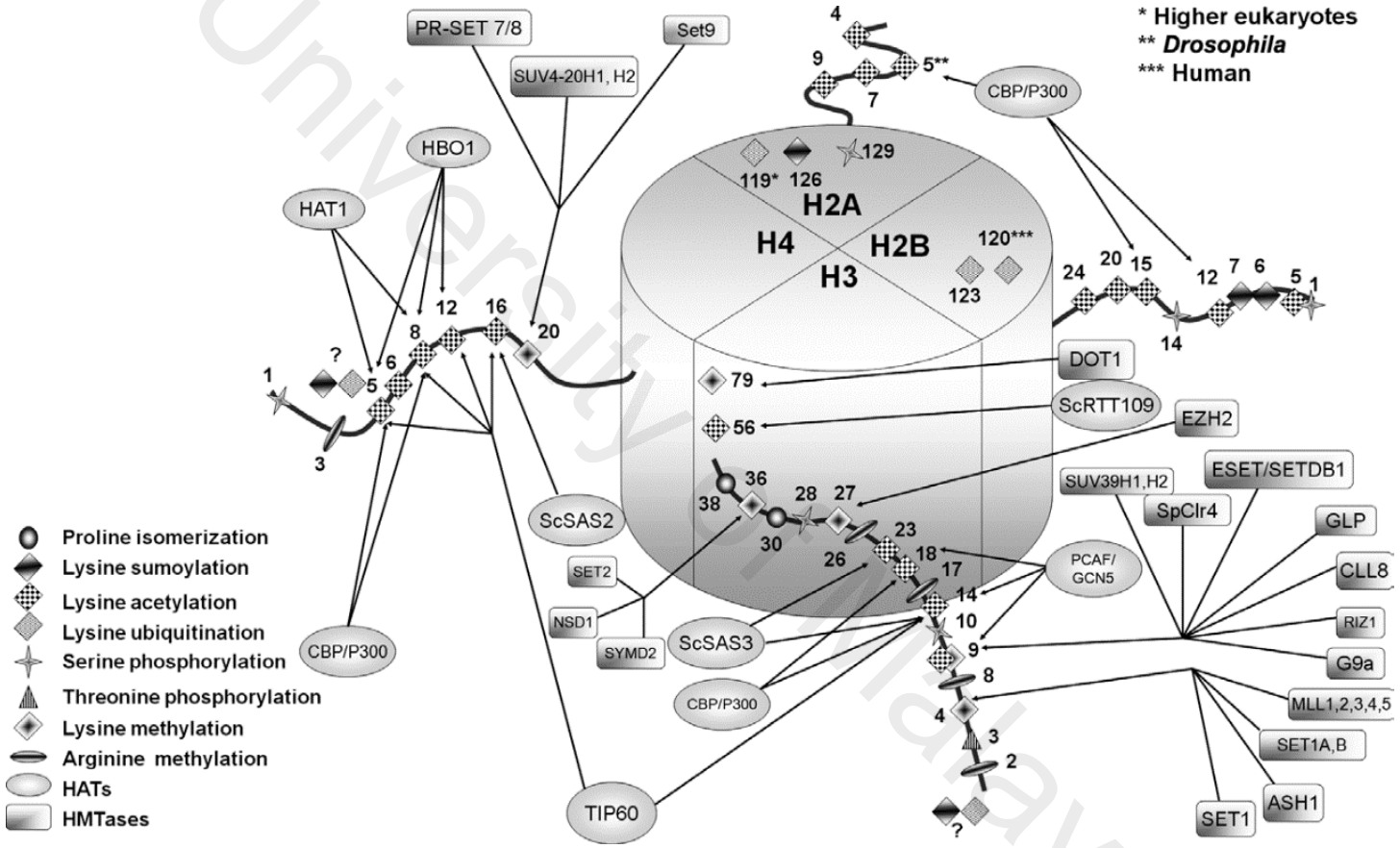


Figure 2.4: Schematic representation of a nucleosome and major histone modifications. Post-translational modifications of histones occur primarily on N-terminal tails of core histones (H2A, H2B, H3, and H4) and include acetylation, methylaphosphorylation, and ubiquitination. Note that several lysines (e.g. Lys 9) can be either acetylated or methylated (Vanzan et al., 2017).

Each modification has a specific effect on gene regulation and other processes involving DNA, such as lysine methylation that can lead to gene activation and gene repression, where both effects are dependent on the site and the extent of methylation (Ren et al., 2006; Latham & Dent, 2007). Extensive knowledge had been obtained for both the methylation and acetylation of histones, as compared to the other modes of modification (Loidl, 2004). The genome contains all the information needed to build an organism. However, during differentiation and development, additional epigenetic information determines the functional state of cells and tissues. This epigenetic information can be introduced by cytosine methylation and by marking nucleosomal histones. The code written on histones consists of post-translational modifications, including acetylation and methylation.

2.4.1 Histone deacetylation mechanism and regulation

In histone acetylation, the ε-Amino groups of lysines in the N-terminal domain of core histones are acetylated by histone acetyltransferases (HATs) with acetyl-CoA as the co-substrate (Loidl, 2004). This type of modification can be reverted back by histone deacetylases (HDACs) (Gallusci et al., 2017), suggesting that histone acetylation affects gene expression, whereas histone deacetylation yields the opposite outcome (Tollefsbol, 2017). Four HATs families have been identified through sequence analysis, namely the GNAT-MYST family, the p300/CBP co-activator family, the nuclear receptor co-activator family and the $TAF_{II}250$-related family (Li et al., 2007; Loidl, 2004). All eukaryotic organisms have all four HAT families except for the nuclear receptor co-activator family, which is only present in vertebrates (Li et al., 2007; Loidl, 2004; Sterner & Berger, 2000).

HAT action has been shown in *Arabidopsis*, where HAT GCN5 was found to be linked with a transcriptional adaptor protein called ADA2 (Stockinger et al., 2001).

HAT GCN5 is a catalytic element in the multiprotein complexes that contained ADA2, where HAT GCN5 is involved in the regulation of chromatin structure and to modulate gene expression (Ciurciu et al., 2006). When ADA2 or GCN5 is mutated, prominent effects affecting gene expression patterns, growth and development are produced (Li et al., 2007; Loidl, 2004).

In an experiment conducted using transgenic maize (*Zea mays*) lines expressing the GCN5 antisense transcript or in a cell line treated with trichostatin A, which is a strong HDAC inhibitor, the relationship between the extent of acetylation of histones and how it affects histone synthesis and degradation was shown (which is naturally in an equilibrium) (Becker et al., 1999; Bhat, 2002). Besides, histone acetylation also affects cell differentiation. Previous studies showed that an increase in H3 and H4 acetylation at the promoter of a light-regulated plastocyanin gene in pea (*Lathyrus aphaca*) was interrelated with the transcriptional activity produced in response to the light stimuli (Chua et al., 2001; Chua et al., 2003). From this, it is deduced that transcription is activated by the association of the transcriptional enhancer of the pea plastocyanin gene with the nuclear matrix, therefore yielding the histone acetylation reaction on the promoter and hence modifying the chromatin structure (Cloix & Jenkins, 2008; Li et al., 2007).

HDACs play the opposite role of HATs (Figure 2.5), whereby it is related to transcriptional repression and involved in gene silencing (Asensi-Fabado et al., 2017; Ma et al., 2013; Saha & Pahan, 2006). In plants, there are three families of HDAC, namely the RPD3/HAD gene family, the HD2 enzymes family (maize histone deacetylases) and the sirtuin family that is associated with yeast SIR2 (Li et al., 2007; Luo et al., 2017). The SIR2 proteins are eukaryotic NAD+ dependent protein

deacetylases that are involved in many important biological processes, such as DNA repair, transcriptional modulation and life span control (Avalos et al., 2002).

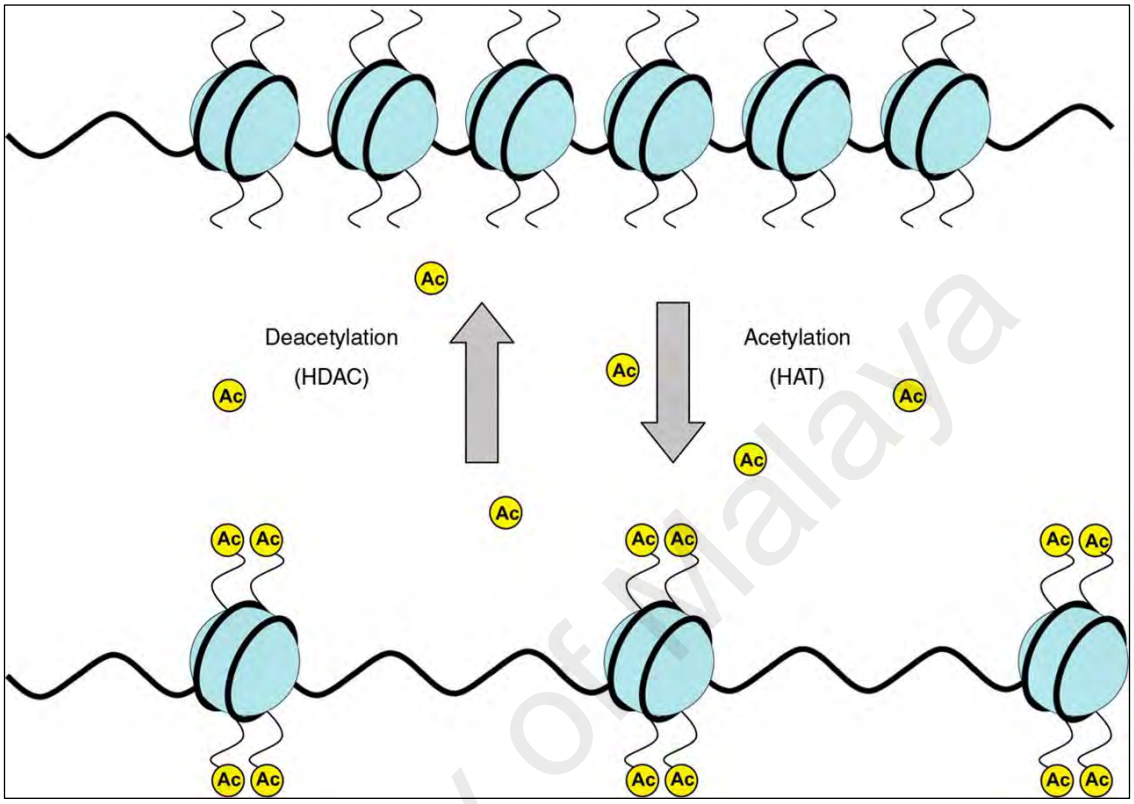


Figure 2.5: Histone deacetylation mechanism (Pons et al., 2009)

Plants have another HDAC type called the HD2-type deacetylases that is only unique to plants only, and is unrelated to the other three HDAC types (Bourque et al., 2016; Grandperret et al., 2014). HDACs often work together with DNA methyltransferases and HMTs in its action (Bannister & Kouzarides, 2011; Vriet et al., 2015). Examples on the effects of HDACs reaction include the experimental study on overexpression of rice HDAC1 that resulted in a boost of growth rate and striking phenotypic change in rice (Yaacob et al., 2013). In *Arabidopsis*, mutations of the genes encoding for Rpd3-type HDAC HDA6 showed that they were involved in gene silencing, while antisense inhibition of HD2-type HDAC leads to seed abortion (Tanaka et al., 2008). There are also other examples on HDACs activity that have been observed in other plants, but all of them also imply that HDACs repress gene transcription and

hence also repress gene expression (Ikeuchi et al., 2015; Pazin & Kadonaga, 1997; Tang et al., 2017).

Perrella et al. (2013) reported that overexpression of HDACs would often produce pleiotropic morphological abnormalities (Perrella et al., 2013). Histone deacetylase HDA6 and HDA19 coupled with DNA methylation were found to yield silencing of transposons and repetitive elements (Kim et al., 2012; To et al., 2011). Nevertheless, HDA6 and HDA19 also involve in silencing of embryonic gene after germination and HDA6 induces flowering in mature plants by repressing flowering inhibitor Flowering Locus C, FLC (Tanaka et al., 2008; Yu et al., 2011). Therefore, it can be deduced that histone deacetylation regulates gene expression during plant stress responses, making it an interesting target to allow for manipulation of epigenetic regulation related to stress sensitivity in plants (Perrella et al., 2013).

2.4.2 Epigenetic regulation of plants in response to stress

Plants are sedentary organisms. The prolonged nature of environmental conditions that continuously influence plant growth (sometimes over many generations of plants) poses significant challenges to the plant defense mechanism/systems (Boyko & Kovalchuk, 2011). Unlike majority of higher eukaryotes that can leave their environment, many plants cannot use escape-avoidance tactics to minimize the damaging influence of biotic and/or abiotic stresses (Altman, 2003; Barnabás et al., 2008). Thus, it is natural that plants possess both short-term response systems and long term defense strategies allowing them to cope with acute and chronic stresses (Agrawal, 2007).

In recent years, ample progress has been made in understanding the role of epigenetic mechanisms in regulating plant responses to stress. Since the response to stress involves transcriptional activation and repression at various genomic loci,

changes in the chromatin structure play the most active role in this process (Boyko & Kovalchuk, 2011). Chromatin de-condensation involves the action of ATP-dependent remodeling complexes, covalent modifications of histones, the deposition of histone variants, and/or changes in cytosine methylation (Gutzat & Scheid, 2012). In fact, it is not the changing of genetic information but the manipulating of the expression of the existing gene pool via epigenetic regulatory pathways that allows plants to survive stress and adapt to new growth conditions (Chinnusamy et al., 2008; Chinnusamy & Zhu, 2009).

The main strength of plant response to stress is the ability to rapidly alter homeostasis (Hirschi, 1999). This may require the following massive changes in the number and the amount of metabolites produced as well as changes in their de novo synthesis and re-compartmentalization; dynamic alterations in mRNA and protein synthesis and turn over; and balancing salt concentrations, pH levels and levels of hormones (Gesteland & Atkins, 1996). The majority of these events are controlled by epigenetic mechanisms operating in somatic cells, including changes in DNA methylation and histone modifications as mentioned earlier.

It is possible that the effects of various environmental cues on somatic and meristem cells of plants can be reflected in the form of various epigenetic marks that form a molecular basis for epigenetic memory (Sung & Amasino, 2004). This could mediate the transmission of environmental memories from ancestral plants to their progeny, thereby preparing them for new growth conditions (Boyko & Kovalchuk, 2011; Sano & Kim, 2013). For example, changes in genome-wide DNA methylation forms in response to biotic and abiotic stress interventions (pathogen, herbivore, high salt, low nutrients) occur in asexually reproduced dandelions (*Taraxacum officinale*) (Verhoeven et al., 2010).

Epigenetic mechanisms responsible for transgenerational responses to stress may include DNA methylation and various histone modifications regulated by a number of physiological and developmental stimuli including stress (Boyko & Kovalchuk, 2011). In the perspective of environmental challenges, such epigenetic modifications may be thought of as relatively "plastic" yet heritable marks that allow for rapid feedbacks and adaptations as well at the same time might avoid excessive genetic diversification (Golldack et al., 2011; Lira-Medeiros et al., 2010).

CHAPTER 3: METHODOLOGY

3.1 Plant material, media and culture conditions

Leaf base of *in vitro A. comosus* var. MD2 plantlets (approximately 1 cm in length) from Plant Biotechnology Incubator Unit (PBIU) was used as starting materials and as source of explants for shoot initiation. The leaf base was cultured on solid Murashige and Skoog (MS) media (Murashige & Skoog, 1962) containing 3 % sucrose and 2 g L^{-1} Gelrite (Duchefa Biochemie) for 2 months. The media were adjusted to pH 5.8 ± 0.2 and autoclaved at 121ºC for 15 min. All cultures were maintained at 25 ± 1ºC under a photoperiod of 16 h light and 8 h dark with an illumination of 1000 lux.

3.2 Induction of somaclonal variation

Somaclonal variation was induced by culturing *in vitro A. comosus* plantlets on semi-solid MS media containing different concentrations and combinations of plant growth regulators, namely indole-butyric acid (IBA) and 6-benzylaminopurine (BAP), and/or in the presence of sodium chloride (NaCl) or abiotic stress signalling hormone; abscisic acid (ABA). The occurrence of somaclonal variation was observed among the *in vitro* regenerants based on morphology.

3.2.1 Hormonal induction

3.2.1.1 Regeneration of *A. comosus* for optimum micropropagation

Different concentration of auxin and cytokinin within the range of 0.0-2.0 mg L^{-1} of IBA and 0.0-2.0 mg L^{-1} BAP were used to induce *in vitro* shoots of *A. comosus.* The fastest and highest shoot formation produced was recorded. Growth with the best propagation plantlets produced also examined (to be used as the explants for the next phase). Cultures were maintained at described in Section 3.1.

3.2.1.2 Hormonal induction for somaclonal variants

Somaclonal variants were induced by increasing cytokinin at different concentrations and combinations, within the range of 0.0-2.0 mg L^{-1} of IBA and 0.0-4.0 mg L^{-1} BAP. Variants were recorded based leaf height and leaf diameter, while the shoots grown from section 3.1.1.1 were also observed for any formation of somaclonal variants. Cultures were maintained at described in Section 3.1.

3.2.1 Salt induction

In vitro plantlets were propagated on the optimum media before salt induction experiment. Somaclonal variants in medium supplemented with different concentrations of NaCl (0.1-3.0 %). Cultures were maintained at described in Section 3.1.

3.2.2 Abscisic acid (ABA) induction

Induction of somaclonal variants was induced by subjecting the *in vitro* plantlets to different concentrations of ABA (0.5-6.0 mg L^{-1}). Cultures were maintained as described in Section 3.1.

3.3 Transformation of *AtHD2* gene into *A. comosus*

The *HD2* gene from *Arabidopsis thaliana*, *AtHD2* (based on The Arabidopsis Information Resource (TAIR) accession no: 4010741337; locus tag: AT5G22650) was inserted into the *in vitro A. comosus* plantlets to determine the role of *HD2* gene in the formation of somaclonal variants. Morphology (based on height) of *AtHD2*-transformed plantlets was then observed and recorded.

3.3.1 Synthesis of *HD2* gene and plasmid construct

The verified *AtHD2* gene sequence (Appendix B) of *A. thaliana* was synthesized and inserted into pCambia 1304 plasmid (Appendix A) by Next Gene Scientific Sdn. Bhd. (Kuala Lumpur, Malaysia) before the plasmid construct (Figure 3.1) transformed into *A. tumefaciens* cells.

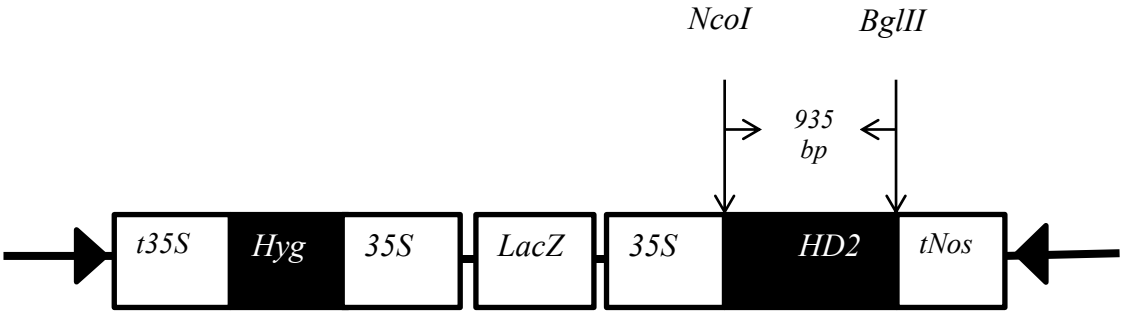


Figure 3.1: Plasmid construct of pCambia 1304 harboring *HD2* gene

3.3.2 Preparation of *A. tumefaciens* for *A. comosus* transformation

Agrobacterium cells containing the gene cassette were revived from a glycerol stock by growing on YEB medium (Oxoid, England) supplemented with 50 mg L^{-1} rifampicin (Duchefa Biochemie) and incubated at 28 °C for 2-3 days. A single colony was picked and allowed to grow in a 5 mL YEB medium containing 50 mg L^{-1} rifampicin, at 28 °C on an incubator shaker (120 rpm) overnight. Ten mL of YEB media was added to 4 mL overnight culture and further incubated at 28 °C for 4 hours (120 rpm). The culture was then centrifuged at 10 000 rpm for 5 min to harvest the cells pellet and its supernatant was discarded. The pellets were re-suspended in 50 µL LB media before plating on LB media supplemented with 50 mg L^{-1} streptomycin (Sigma, USA) and 50 mg L^{-1} kanamycin (Sigma, USA). The cultures were incubated at 28°C.

3.3.3 Verification on presence of *AtHD2* in *A. tumefaciens* by colony PCR

Successful integration of *AtHD2* gene into the pCAMBIA1304 plasmid was verified by colony polymerase chain reaction (PCR) before *A. comosus* transformation was carried out.

All single colonies grown on the selection plate were subjected to colony PCR following standard method as shown in Table 3.1. The PCR reagents used were listed in Table 3.2, while *gfp* primers (Table 3.3) were used to verify successful integration of the gene and its presence in the *Agrobacterium* cells.

Table 3.1: PCR amplification reaction thermal cycling protocol to amplify *gfp* gene

Thermal cycling step	Temperature °C	Time	Cycle
Initial denaturation	94	2 min	1
Denaturation	95	30 s	
Annealing	47-57	60 s	30
Extension	68	1 min	
Final extension	72	7 min	1
Cooling	10	Indefinite	1

Table 3.2: The PCR reagents for amplification of DNA template (EURx, Poland)

PCR reagents	Volume (µL)	Final concentration
10 X buffer	2.5	1x
25 mM $MgCl_2$	3.5	2.5 mM
dNTP	1	0.2 mM
Upstream primer	1	0.5 mM
Downstream primer	1	0.5 mM
Taq DNA polymerase 5U/ µL	0.1	1.25 U
Template DNA	1	0.5 µg/µL
Sterile double distilled water	14.9	-
Total volume	25	-

Table 3.3: *gfp* gene primers used for sequence amplification

Forward sequence (5'- 3')	Reverse sequence (5'-3')	Product size
CTGGTCGAGCTGGACGGCGAC G	CATGGTCCTGCTGGAGTTCG TG	716 bp

3.3.4 Verification of sequence orientation by restriction enzyme (RE) Digestion

Two types of RE, *NcoI* and *BglII*, were used to verify orientation the sequence after gene insertion. The component for RE digestion is shown in Table 3.4. The first digestion was carried out using RE with the lowest salt concentration, NcoI-HF. The reaction was incubated at 37°C for 5-15 min. Sufficient NaCl (100 mM) was added according to the manufacturer's protocol (NEB, BioEngland). About 10 units or 1 µL of *BglII* then was added and incubated at 37°C for 5–15 min.

Table 3.4: The component and reactions protocol used for RE digestion

Component	50 µL reaction
DNA	up to 1 µg
10X CutSmart Buffer	5 µL (1X)
NcoI-HF	1.0 µl (or 10 units)
Nuclease-free Water	to 50 µL

3.3.5 *A. comosus* transformation

Agrobacterium cultures (5 mL) containing the pCAMBIA1304-*HD2* were grown in LB broth overnight before mixing with fresh 45 mL LB broth containing 100 µg/mL streptomycin and 50 µg/mL kanamycin. The cultures were placed on an incubator shaker at 28°C, 120 rpm until the OD_{600} reached 0.8-1.0. The cells were harvested by centrifugation at 5000 rpm for 10 min. The pellet was re-suspended in MS medium and re-shaken in an incubator until the OD_{600} reached 0.8-1.0. The leaf base

explants (longitudinally-dissected producing two explants per plantlet) were immersed in 50 mL MS medium containing the *Agrobacterium* for 10 min.

After infection, the explants were blot dried on a sterilized tissue paper. The explants were then transferred to solid co-cultivation medium containing 1.0 mg L^{-1} IBA and 2.0 mg L^{-1} BAP and 200 µM acetosyringone and 500 µM vanillin (in separate experiment) for three days in dark condition. All 100 infected explants were incubated under light for until new shoot tips emerged or regenerated. The shoot tips were transferred to selection medium supplemented with 20 mg L^{-1} hygromycin (Sigma, USA), 250 mg L^{-1} cefotaxime (Sangon Biotech) and 100 mg L^{-1} timentin (Sangon Biotech) for shoot regeneration.

3.3.6 Verification of successful transformants using *hpt* and *HD2* gene

After transformation was conducted, confirmation of the presence of the gene in the putative transformants was verified by PCR amplification of *hpt* and *HD2* genes. The primers sequences used for both genes are shown below (Table 3.5). While PCR components and reaction protocol were the same as mentioned in previous sections 3.3.3 (Table 3.1 and 3.2).

Table 3.5: *hpt* and *HD2* gene specific primers used for sequence amplification

Type of primers	Forward sequence (5'- 3')	Reverse sequence (5'-3')	Product size
hpt	CGCACAATCCCACTATC CTTCGCAA	GGCAGTTCGGTTTCAG GCAGGTCTT	514 bp
HD2	TTACCATGGAATGGAGT TCTGG	GAAGATCTCTTAAGCT CTACCCTT	940 bp

3.4 Flow cytometry

The protocol was according to Doležel et al. (2010). About 20 mg of fresh *in vitro* leaf tissues of *A. comosus* were placed in the centre of a plastic Petri dish. 1 mL of ice-cold nuclei isolation buffer (LB01) was added to the Petri dish on top of the leaves. The tissues were chopped immediately in the buffer with a new razor blade or a sharp disposable scalpel. The homogenate was gently mixed by pipetting up and down for several times to avoid air bubbles. The homogenate was filtered through a 42 µm nylon (Sartorius) mesh into a labelled sample tube.

A stock solution containing 50 µg/mL DNA fluorochrome (propidium iodide; Sigma-Aldrich, USA) and 50 µg/mL RNase A (Sigma -Aldrich, USA) was added simultaneously and shaken gently. The sample was incubated on ice for a few minutes to 1 h with occasional shaking before flow cytometry analysis. The suspension of stained nuclei was introduced into a flow cytometer (BD FACS Calibur, Germany) for ploidy analysis and determination of nuclear DNA content. The DNA ploidy and amount of nuclear DNA of the unknown sample is calculated as follows:

$$\text{Sample Ploidy (integer)} = Reference\ \ ploidy\ x\ \frac{mean\ position\ of\ the\ G1\ sample\ peak}{mean\ position\ of\ the\ G1\ reference\ peak}$$

$$\text{Sample 2C value (DNA pg/Mbp)} = Reference\ \ 2C\ value\ x\ \frac{sample\ 2C\ mean\ peak\ position}{reference\ 2C\ mean\ peak\ position}$$

3.5 Phenotypic reversion

Somaclonal variants that exhibited dwarf phenotype were allowed to recover by transferring to fresh MS basal media without any plant growth regulator, NaCl and ABA until 100 % of the variants had reverted to normal phenotype (compared to the control plants).

3.6 Nuclear Protein extraction and Bradford assay

The transformed and non-transformed plantlets were analyzed for their histone deacetylase activity using phenotypically normal plantlets as control. Nuclear protein was extracted from all samples using Plant Nuclei Isolation/Extraction Kit (Sigma-Aldrich, USA). The bovine serum albumin, BSA (Bio-Rad) standard curve was prepared by using Quick Start™ Bradford Protein Assay (Bio-Rad, USA).

Nuclear proteins were quantified using Bradford's reagent based on a 96 well plate assay protocol. Prior to dispensing into separate wells (5 uL each) (BSA) protein extracts and the blank were aliquoted (5 uL) into the plates and mixed with protein buffer before added including the blank well (5 µL each). The protein extracts were added in other wells. Bradford reagent (250 µL) was added into each well and mixed thoroughly by shaking the plate for 30 min. The samples were incubated at room temperature for 45 min before the absorbance reading was measured at 594 nm using Sunrise^{TM} microplate reader (Tecan, Austria). The protein concentrations of the samples were determined based on the standard curve.

3.7 Histone deacetylase (HDAC) enzyme activity analysis

The *EpiQuik*™ HDAC Activity or Inhibition Assay Kit (Colorimetric) was designed to measure the total HDAC activity or inhibition. In this experiment, the unique acetylated histone substrate was captured on the strip wells. Active HDACs bind and deacetylate histone substrate. The remaining un-deacetylated substrate could be recognized with a high affinity acetylated histone antibody. The ratio or amount of the un-deacetylated histone, which was inversely proportional to the HDAC enzyme activity, was colorimetrically quantified through an ELISA-like reaction. Histone deacetylase activities depicted by all samples were compared and the level of histone deacetylation represents the degree of gene repression.

In this assay, acetylated histone HDAC substrate was stably coated onto the microplate wells. The nuclear protein extracts (10 µg) were incubated at 37 °C with acetylated substrate (1 µL) and assay buffer (49 µL) for 90 min by using 96-well microplate. The active HDACs present in the nuclear extracts would bind to the substrate and effectively removed the acetyl groups from the substrate. The wells were then washed with 150 µL wash buffer before subsequent addition of capture antibody (50 µL) provided by the manufacturer (Epigentek, USA). The wells were washed again and added with detection antibody solution (50 µL). Developing solution was then added for color development and the absorbance was subsequently measured. The amounts of deacetylated products were colorimetrically measured using Sunrise™ microplate reader (Tecan, Austria) at 450 nm.

A deacetylated histone standard curve was prepared and used to determine HDAC quantity and enzyme activity. The value of the slope was used to calculate the amount of deacetylated product (ng). The following formulas were used for calculation:

$$Deacetylaed\ product\ (ng) = \frac{(\text{Sample OD} - \text{Blank OD})}{\text{Slope}}$$

$$HDAC\ activity\ ((ng/min)/mg) = \frac{\text{Deacetylated product (ng)}}{\text{Protein Amount (µg)}\ x\ incubation\ time\ (\text{min})}\ x\ 1000$$

3.8 Real time-quantitative PCR (qPCR)

Total RNA from approximately 100mg of leaf samples from each treatment were extracted by using RNeasy Plant Mini Kit (Qiagen, Germany) following the manufacturer's protocol (Qiagen). In brief, the leaf samples were ground using mortar and pestle in liquid nitrogen. Buffer RLT together with QIAshredder column were used in the extraction process. RNA was eluted in 50 µL RNase-free water. Removal of any remaining genomic DNA was carried out by DNase digestion by RQ1 RNase-free

DNase (Promega, USA) according to the manufacturer's protocol (Promega). 20 µL of each RNA sample was subjected to DNase digestion in 30 µL reactions. cDNA synthesis was conducted for all DNase-digested RNA samples using RevertAid Reverse Transcriptase (Thermo Scientific, USA) according to the manufacturer's protocol (Thermo Scientific) for First Strand Synthesis. Reverse transcription was carried out with 11.5 µL of each RNA sample and 0.2 µg of random hexamer per 20 µL reaction.

Real-time PCR (qPCR) was carried out using SensiFAST™ SYBR® Hi-ROX Kit (Bioline, UK) according to the manufacturer's protocol (Bioline, USA) in an Applied Biosystems StepOne Real-Time PCR System (Thermo Fisher Scientific, USA) and analyzed using StepOne Software v2.3 (Thermo Fisher Scientific, USA). Fast ramping was used, data was collected on the SYBR Green I channel, with ROX as the passive reference.

Optimizations for annealing temperature, cDNA input and primer input were carried out using mixed cDNA samples to obtain cycle threshold (Ct) values of between 10-35, with single melt-curve peaks. Total reaction of 20 µL containing cDNA template, 2X SensiFAST™ SYBR® Hi-ROX Master Mix buffer, PCR-grade water and forward and reverse primers (10 µM) were used for RT-PCR amplification. The thermal cycling conditions were optimized to 2 min for polymerase activation (95 °C) and 40 cycles for denaturation (95°C, 15 sec), annealing (52°C, 15 sec) and elongation (72°C, 15 sec). The data were normalized to the geometric means of the two reference genes and analysed by $2^{\hat{}\Delta\Delta CT}$ method according to algorithm (Vandesompele et al., 2002).

The reference genes used were β-actin and 18sRNA, with NCBI Accession Number: HQ148720 and JN129389, respectively. The primer sequences for the HDAC gene were designed based on *Ananas comosus* histone deacetylase 2 gene (NCBI Accession Number: OAY83945.1). Design of the primers was done using Primer

Premier 6 and the primers were manufactured by IDT Technologies (IDT, USA). The primers used in this analysis were listed in Table 3.5.

Table 3.6: List of primer sequences used to amplify the specific genes for qPCR

Gene	Primer Sequences (5'-3')
HD2-F	GAGAGACCGTCAAGTGCGAC
HD2-R	AGCCAAGGAAGTAGACGCTG
β-actin-F	CTGGCCTACGTGGCACTTGACTT
β-actin-R	CACTTCTGGGCAGCGGAACCTTT
18srRNA-F	ATGGTGGTGACGGGTGAC
18srRNA-R	CAGACACTAAAGCGCCCGGTA

3.9 Statistical analysis

All experiments (inductions of somaclonal variation) were carried out in triplicates following a randomized complete block design (RCBD). Statistical analysis was done by one-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) at 5% significance level using SPSS version 22. All results were reported as mean ± SE of three replicated experiments.

CHAPTER 4: RESULTS

4.1 Shoot proliferation and induction of somaclonal variations

4.1.1 Optimization of tissue culture conditions protocol for *in vitro* regeneration of *A. comosus*

In this study, shoot proliferation was initiated from *A. comosus* leaf base explants by culturing the explants on MS media added with 0.5 – 2.0 mg L^{-1} IBA and BAP, either in combination or singly applied. The optimum media that results in the fastest growth and highest number of plantlets produced was determined. Leaf base explants showed differential responses to the different concentrations of PGRs in the culture media after two months of culture. After 2 months of culture, the highest number of microshoots per explant (6 microshoots) was observed on MS media containing 1.0 mg L^{-1} IBA and 2.0 mg L^{-1} BAP, as shown and shaded in Table 4.1. In contrast, the least number of microshoots per explant (1 microshoot) was recorded on MS media supplemented with only 2.0 mg L^{-1} IBA.

The use of high concentration of BAP (> 2 mg L^{-1}) either alone or combined with IBA did not improve shoot regeneration. However, low concentration of BAP (0.5 mg L^{-1}) produced the tallest plantlet (7.5 ± 0.3 cm) with high number of leaves (9 ± 0.2 leaves), compared to other treatments. Plantlets grown on MS media supplemented with 0.5 mg L^{-1} BAP also showed the widest leaf diameter (1.0 ± 0.0 cm). Moreover, the addition of 0.5 mg L^{-1} BAP to the media, even when combined with 2.0 mg L^{-1} IBA also results in efficient production of leaves (9 ± 0.3 leaves), indicating that BAP is an excellent PGR for induction of leaf growth, when it is used at low concentrations. On the other hand, MS media added with 2.0 mg L^{-1} IBA and 2.0 mg L^{-1} BAP combined was found to be the least responsive media for *in vitro* regeneration of MD2 pineapple, producing plantlets with the least number of leaves (3 ± 0.2 leaves), the shortest leaf length (3cm ± 0.03) and the smallest leaf diameter (0.5cm ± 0.02). Therefore, MS media

added with 1.0 mg L^{-1} IBA and 2.0 mg L^{-1} BAP has been identified as the optimum regeneration media (OM) of this species, yielding the highest mean number of microshoots per explant.

Table 4.1: Effects of different concentrations and combinations of plant growth regulators on the number and diameter of leaves, height of plantlets and number of microshoots per explant

Plant growth regulator (mg L^{-1})	Number of microshoots per explant	Plantlet height (cm)	Number of leaves	Diameter of leaves (cm)
Control (MS basal)	1.7 ± 0.3^{b}	5.1 ± 0.3^{cd}	7.7 ± 0.3^{de}	0.5 ± 0.0^{ab}
1.0 IBA	1.9 ± 0.3^{b}	5.3 ± 0.2^{cd}	6.8 ± 0.2^{c}	0.6 ± 0.0^{ab}
2.0 IBA	1.2 ± 0.2^{a}	5.2 ± 0.2^{cd}	7.3 ± 0.2^{cd}	0.6 ± 0.0^{bc}
0.5 BAP	2.8 ± 0.2^{c}	7.5 ± 0.3^{f}	9.5 ± 0.2^{g}	1.0 ± 0.0^{g}
1.0 BAP	3.7 ± 0.5^{d}	5.0 ± 0.2^{cd}	8.4 ± 0.2^{ef}	0.8 ± 0.0^{e}
2.0 BAP	3.7 ± 0.3^{d}	4.8 ± 0.6^{c}	5.7 ± 0.5^{b}	0.8 ± 0.0^{e}
1.0 IBA + 0.5 BAP	4.0 ± 0.2^{d}	6.4 ± 0.2^{e}	8.4 ± 0.3^{ef}	0.8 ± 0.0^{e}
2.0 IBA + 0.5 BAP	3.7 ± 0.2^{d}	5.4 ± 0.1^{d}	8.9 ± 0.3^{fg}	0.7 ± 0.0^{de}
1.0 IBA + 1.0 BAP	2.0 ± 0.3^{b}	5.0 ± 0.1^{cd}	8.1 ± 0.3^{def}	0.7 ± 0.0^{e}
2.0 IBA + 1.0 BAP	2.0 ± 0.3^{b}	4.2 ± 0.1^{b}	5.7 ± 0.3^{b}	0.5 ± 0.0^{ab}
1.0 IBA + 2.0 BAP	6.0 ± 0.4^{e}	5.0 ± 0.2^{cd}	8.4 ± 0.3^{ef}	0.8 ± 0.1^{e}
2.0 IBA + 2.0 BAP	2.0 ± 0.2^{b}	3.5 ± 0.1^{a}	3.4 ± 0.2^{a}	0.5 ± 0.0^{a}

Data represents mean values ± standard error (SE) with 30 replicates in each treatment. Means with different letters in the same column are significantly different at $p < 0.05$ according to ANOVA and Duncan's multiple range test (DMRT).

4.1.2 Induction of somaclonal variants through addition of high cytokinin concentration

Plant tissue culture technique usually causes undesirable effects known as somaclonal variation. This effect can be easily detected by macro-morphological changes, such as plant height and leaf characteristics. Somaclonal variation was induced by increasing the concentration of cytokinin (BAP) to up to 4.0 mg L^{-1} (Appendix C). Data analysis showed that the height of the plantlets became shorter as BAP concentration increased. Supplementation of high concentration of BAP (4.0 mg L^{-1}),

either with IBA or alone, was observed to produce significantly shorter plantlets (dwarf) compared to other treatments (Figure 4.1). As depicted in Figure 4.1, the shortest plantlets (1.9 ± 0.1 cm) were recorded on MS media containing 2.0 mg L^{-1} IBA and 4.0 mg L^{-1} BAP.

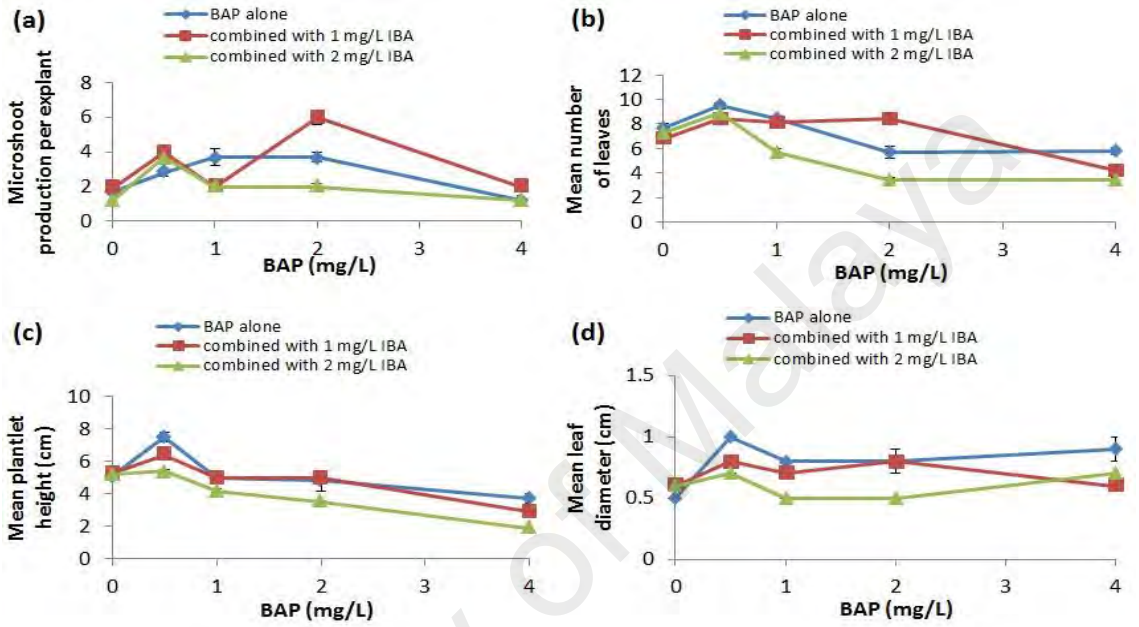


Figure 4.1: Effects of high BAP concentrations (either singly applied or in combination with IBA) on the number and diameter of leaves, plantlet height and number of microshoots per explant after two months of culture.

4.1.3 Induction of somaclonal variation through salinity stress

In this study, sodium chloride (0.1 % to 3.0 % NaCl) was used as the stress agent to yield somaclonal variants. Optimum regeneration media (OM) added with 0.2 % NaCl was found to produce plantlets with the highest number of microshoots (4 microshoots). It was observed that plantlet height was significantly affected as higher NaCl concentrations were used. For example, after two months of culture, the shortest plantlets were observed on the OM added with 1.0 % NaCl, with mean plantlet height of 1.4 ± 0.3 cm (shaded in Table 4.2). There were no significant observations that can be seen from other parameters such as number and diameter of leaves. At the same time, further increase in NaCl concentration did not result in subsequent organogenesis from

the explant. Based on the findings of this study, it could be deduced that addition of 1.0 % NaCl to OM produced the shortest plantlets, thus was chosen as the somaclonal variants maintained for subsequent analysis.

Table 4.2: Effects of different concentrations of sodium chloride (NaCl) concentration on the number and diameter of leaves, height of plantlets and number of microshoots per explant

Treatment	Number of microshoots per explant	Plantlet height (cm)	Number of leaves	Diameter of leaves (cm)
Control (MS basal)	1.0 ± 0.2^{bc}	3.4 ± 0.03^{e}	5 ± 0.3^{d}	0.4 ± 0.02^{b}
OM + 0.1 % NaCl	2.0 ± 0.3^{c}	1.9 ± 0.2^{cd}	6 ± 0.5^{d}	0.3 ± 0.02^{b}
OM + 0.2 % NaCl	4.0 ± 0.5^{d}	1.7 ± 0.1^{bc}	5 ± 0.5^{d}	0.4 ± 0.03^{b}
OM + 0.3 % NaCl	3.0 ± 0.5^{c}	2.3 ± 0.2^{d}	6 ± 0.3^{d}	0.7 ± 0.05^{d}
OM + 0.4 % NaCl	2.0 ± 0.3^{bc}	1.5 ± 0.1^{bc}	6 ± 0.4^{d}	0.6 ± 0.04^{c}
OM + 0.5 % NaCl	2.0 ± 0.5^{bc}	1.6 ± 0.1^{bc}	6 ± 0.4^{d}	0.8 ± 0.05^{d}
OM + 1.0 % NaCl	1.0 ± 0.3^{ab}	1.4 ± 0.3^{ab}	4 ± 0.2^{b}	0.4 ± 0.02^{b}
OM + 2.0 % NaCl	0.0 ± 0.0^{a}	1.0 ± 0.0^{a}	0 ± 0.0^{a}	0.0 ± 0.00^{a}
OM + 3.0 % NaCl	0.0 ± 0.0^{a}	1.0 ± 0.0^{a}	0 ± 0.0^{a}	0.0 ± 0.00^{a}

Data represents mean values ± standard error (SE) with 15 replicates in each treatment. Means with different letters in the same column are significantly different at $p < 0.05$ according to ANOVA and Duncan's multiple range test (DMRT).

4.1.4 Induction of somaclonal variation through ABA stress

Abscisic acid (ABA) is a plant hormone that is involved in stress signaling pathways. In this part of the study, 0.5 mg L^{-1} to 6.0 mg L^{-1} ABA was supplemented into OM media to induce formation of somaclonal variants. According to Table 4.3, OM added with 1.0 mg L^{-1} ABA produced the shortest shoot length (1.7 ± 0.1 cm). Data analysis revealed no significant differences between the number of microshoots produced, number of leaves and the diameter of the leaves. Additionally, it was observed that at concentration of 6.0 mg L^{-1} and above, the inhibitory effect of ABA became more prominent, whereby the leaves became brown and necrotic, thus

eventually causing the plantlets to die. Therefore, the media that produced the shortest plantlets was identified (OM added with 1.0 mg L^{-1} ABA) and the resulting dwarf plantlets were used for further investigations.

In this study, dwarfism is the most consistent and prominent variation being observed and recorded. Figure 4.2 depicts the differences of plant stature exhibited by the different treatments. Thus, dwarfism was chosen as the phenotype to be studied in subsequent analysis.

Table 4.3: Effects of different concentrations of abscisic acid (ABA) concentration on the number and diameter of leaves, height of plantlets and number of microshoots per explant

Treatment	Number of microshoots per explant	Plantlet height (cm)	Number of leaves	Diameter of leaves (cm)
Control (MS basal)	1 ± 0.2^{b}	3.3 ± 0.03^{d}	5 ± 0.2^{cd}	0.4 ± 0.02^{d}
OM + 0.5 mg L^{-1} ABA	1 ± 0.3^{b}	2.2 ± 0.2^{c}	5 ± 0.6^{bcd}	0.4 ± 0.08^{d}
OM + 1.0 mg L^{-1} ABA	0 ± 0.0^{a}	1.7 ± 0.1^{a}	2 ± 0.4^{a}	0.2 ± 0.04^{ab}
OM + 1.5 mg L^{-1} ABA	0 ± 0.0^{a}	1.8 ± 0.1^{ab}	6 ± 1.2^{d}	0.3 ± 0.04^{abc}
OM + 2.0 mg L^{-1} ABA	0 ± 0.0^{a}	1.8 ± 0.1^{ab}	3 ± 0.5^{ab}	0.2 ± 0.04^{ab}
OM + 3.0 mg L^{-1} ABA	0 ± 0.0^{a}	2.2 ± 0.05^{bc}	5 ± 0.3^{bcd}	0.4 ± 0.03^{d}
OM + 4.0 mg L^{-1} ABA	1 ± 0.3^{b}	2.3 ± 0.09^{c}	5 ± 0.3^{bcd}	0.3 ± 0.03^{d}
OM + 5.0 mg L^{-1} ABA	1 ± 0.3^{b}	2.0 ± 0.1^{abc}	4 ± 0.3^{bc}	0.3 ± 0.05^{bcd}
OM + 6.0 mg L^{-1} ABA	0 ± 0.0^{a}	1.7 ± 0.2^{a}	2 ± 0.5^{a}	0.2 ± 0.06^{ab}

Data represents mean values ± standard error (SE) with 15 replicates in each treatment. Means with different letters in the same column are significantly different at $p < 0.05$ according to ANOVA and Duncan's multiple range test (DMRT).

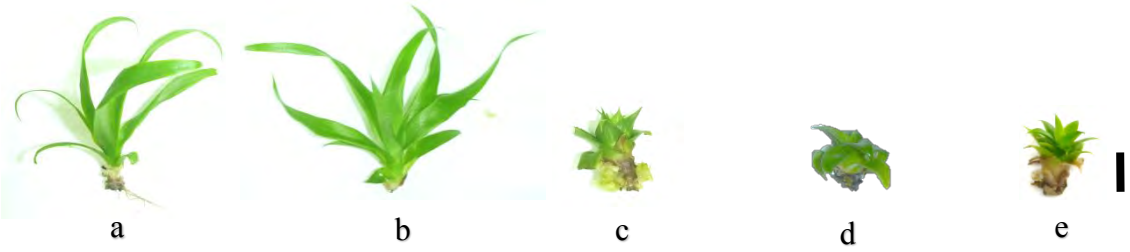


Figure 4.2: Phenotype observations of all samples (a) Plantlet exhibiting normal phenotype (grown on MS basal media), (b) Plantlet exhibiting normal phenotype (grown under optimum growth media; MS media added with 2 mg L^{-1} BAP plus 1 mg L^{-1} IBA), (c) Dwarf plantlet produced on MS media supplemented with 4.0 mg L^{-1} BAP combined with 2.0 mg L^{-1} IBA, (d) Dwarf plantlet produced on MS media supplemented with 1.0 % (w/v) NaCl, (e) Dwarf plantlet produced on MS media supplemented with 1.0 mg L^{-1} ABA. Bar = 1 cm

4.2 Phenotype reversion (recovery of somaclonal variants)

The dwarf plantlets were removed from the stress media and allowed to undergo phenotype reversion, to determine whether the dwarfism phenotype observed in this study arise due to either genetics or epigenetics mechanism. Phenotype recovery of the *in vitro* plantlets was assessed within 8 months of culture. In this study, more than 85% of dwarf plantlets induced from TC, TD and TE (Table 4.4) were observed to recover and assume normal morphology within the first 3 months after being subcultured on MS basal media. Meanwhile, the slowest recovery rate of dwarf plantlets was recorded on TE (Table 4.4), where full phenotype recovery (100 %) to normal stature occured after 8 months, compared to TC and TD. These results indicated that the occurence of dwarfism in the stressed plantlets may be due to the epigenetic regulation, thus the variants could undergo phenotype recovery when the stressful environments were removed.

Table 4.4: Phenotype recovery of the plantlets after 8 months of culture

Treatment	Normal (%)	Dwarf (%)	Reversion to normal after 3 months (%)	Reversion to normal after 4 months (%)	Reversion to normal after 6 months (%)	Reversion to normal after 7 months (%)	Reversion to normal after 8 months (%)
TA	100	0	N/A	N/A	N/A	N/A	N/A
TB	100	0	N/A	N/A	N/A	N/A	N/A
TC	0	100	100 ± 0.00^{b}	100 ± 0.00^{b}	100 ± 0.00^{b}	100 ± 0.00^{b}	100 ± 0.00^{b}
TD	7	93	96.43 ± 1.11^{b}	96.43 ± 1.11^{b}	100 ± 0.00^{b}	100 ± 0.00^{a}	100 ± 0.00^{a}
TE	0	100	86.67 ± 1.92^{a}	86.67 ± 1.92^{a}	92.22 ± 1.11^{a}	95.56 ± 1.11^{a}	100 ± 0.00^{a}

Means with different letters in the same column are significantly different at $p<0.05$ according to ANOVA and Duncan's multiple range test (DMRT)

TA: MS basal media; TB: MS media added with 1.0 mg L^{-1} IBA and 2.0 mg L^{-1} BAP; TC: MS media added with 2.0 mg L^{-1} IBA and 4.0 mg L^{-1} BAP; TD: MS media added with 1.0% (w/v) NaCl; TE: MS media added with 1.0 mg L^{-1} ABA; N/A: not applicable

4.3 HDAC enzyme activity

Following successful identification of somaclonal variants and subsequent phenotype reversion, the histone deacetylase (HDAC) enzyme activity level in the samples were evaluated.

4.3.1 Bradford assay for protein quantification

In this study, the amount of nuclear protein in the samples was measured via Bradford assay because it is simpler, faster and more sensitive (Kruger, 2009). The absorbance values of the protein standard (0.0 – 1.0 mg/ml of BSA) were measured at 595 nm and used to generate a standard curve (Figure 4.3). The fitted linear curve resulted in R^2 of 0.9906. The concentration of the extracted proteins in the samples (Appendix D) was then calculated based on the equation obtained from the fitted linear curve.

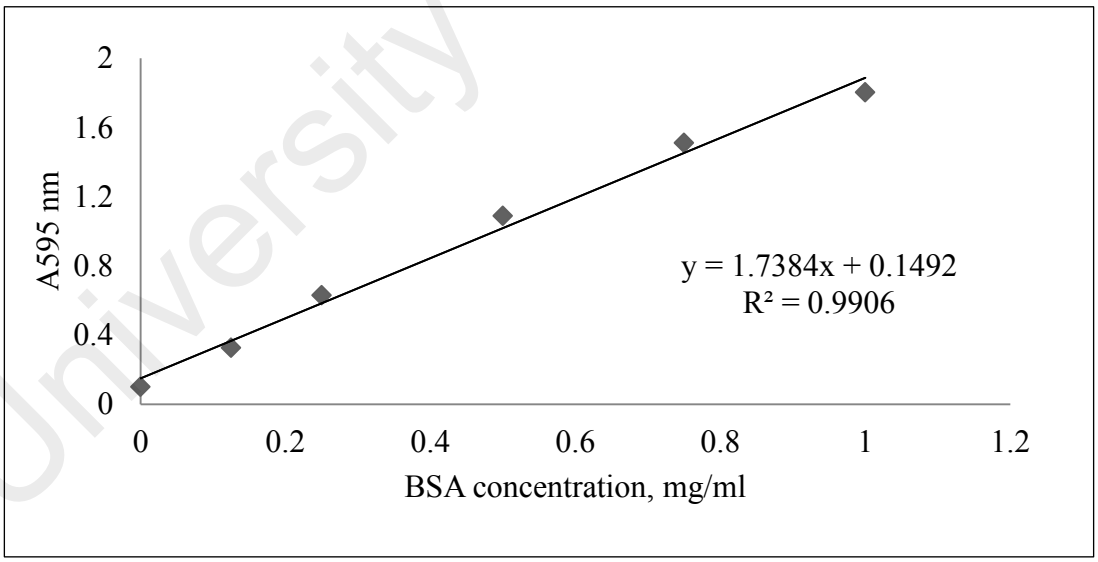


Figure 4.3: Bradford standard curve based on BSA concentration (mg/ml) versus absorbance at 595 nm

4.3.2 Determination of HDAC activity before and after phenotypic recovery

The quantification of HDAC activity by an ELISA-like reaction in this study is trivial to prove that the formation of somaclonal variation observed in this study was due to epigenetic regulation, such as through histone deacetylation. Based on the morphological data recorded, it was observed that addition of stress factors such as high BAP concentration (4 mg L^{-1}) combined with 2 mg L^{-1} IBA (TC), 1% (w/v) NaCl (TD) and 1 mg L^{-1} ABA (TE) produced dwarfed plantlets, with significantly reduced plant stature compared to normal (Figure 4.2).

Deacetylation activity of the histone proteins in the selected plantlets was determined as shown in Table 4.6. In general, plantlets from TB, TC, TD and TE showed significantly higher HDAC activity compared to control (TA) (Table 4.6). The highest HDAC activity (109,333.33 ng/min/mg) was recorded in TE compared to TD (16,629.78 ng/min/mg) and TC (5,074.22 ng/min/mg). These values were observed to correlate with the time taken by the dwarfed plantlets to undergo complete phenotype reversion, where TE plantlets were the slowest to fully recover, possibly due to its very high HDAC activity level that interferes with phenotype recovery.

Table 4.5: Histone deacetylase (HDAC) enzyme activity of variants before and after reversion

Treatment	Before reversion		After reversion	
Sample name	Phenotype	HDAC activity (ng/min/mg)	Phenotype	HDAC activity (ng/min/mg)
TA	Normal	666.67 ± 22.07^{a}	Normal	686.56 ± 13.50^{a}
TB	Normal	$4{,}370.22 \pm 46.55^{b}$	Normal	2452.00 ± 162.50^{b}
TC	Dwarf	$5{,}074.22 \pm 1.37^{c}$	Normal	2407.56 ± 221.37^{b}
TD	Dwarf	$16{,}629.78 \pm 3.15^{d}$	Normal	2911.11 ± 33.75^{c}
TE	Dwarf	$109{,}333.33 \pm 4.40^{e}$	Normal	3229.78 ± 72.81^{c}

Means with different letters in the same column are significantly different at $p < 0.05$ according to ANOVA and Duncan's multiple range test (DMRT).

4.4 Relative *AcHD2* gene expression

The relative expression level of endogenous *Ananas comosus* histone deacetylase gene (*AcHD2)* was evaluated to determine whether the transcripts level was significantly different among the plantlets. Data analysis showed that the expression level of *AcHD2* in the variants (TD and TE) was higher compared to phenotypically normal plantlets (TA and TB) (Figure 4.4). However, the expression level of *AcHD2* in TC was comparable to TB. These findings were in line to the observations of HDAC activity of the variants recorded previously (Table 4.6).

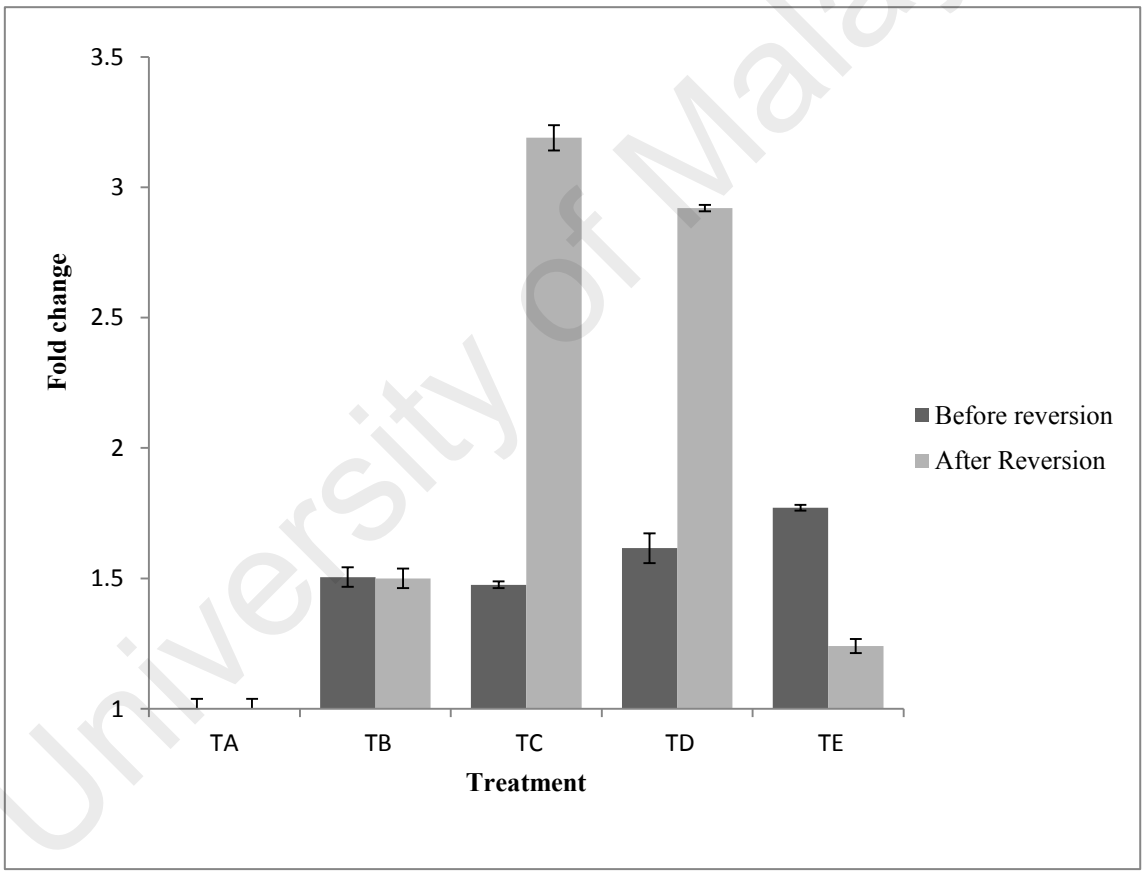


Figure 4.4: Gene expression of *Ananas comosus* histone deacetylase 2 (*AcHD2*) in the variants before and after phenotypic reversion, expressed as fold change compared to control (TA). The fold change was represented by $2^{-\Delta\Delta CT}$ where ΔΔCT is the difference between reference ΔCT with target ΔCT.

The expression level of *AcHD2* in the plantlets after phenotype recovery was also assessed (Figure 4.4). Interestingly, it was observed that *AcHD2* expression in TC and TD was dramatically increased, but decreased in TE after phenotype recovery. These observations implied that the correlation between histone deacetylase mRNA expression with HDAC enzyme activity obtained in this study was poor. However, it has been well established in scientific literature that the correlation between mRNA level and protein abundance in biological samples is usually very poor (Maier et al., 2009).

Nevertheless, the level of HDAC inhibition measured in the samples (Figure 4.5), to yield further information with regards to HDAC enzyme regulation in relation to its gene expression. Based on data analysis, it was revealed that there was a marked increase in HDAC inhibition in the variants (TC, TD and TE) after phenotype recovery. At this juncture, it could be argued that despite the high level of *AcHD2* gene expression in the variants during phenotype recovery, the significant reduction in their HDAC enzyme activity (Table 4.6) could be attributed by the increase in HDAC inhibition (Figure 4.5).

4.5 Measurement of HDAC inhibition

In this study, the levels of HDAC inhibition were also determined to to yield further information with regards to HDAC enzyme regulation in relation to its gene expression. Based on data analysis, it was revealed that there was a marked increase in HDAC inhibition in the variants (TC, TD and TE) after phenotype recovery. At this juncture, it could be argued that despite the high level of *AcHD2* gene expression in the variants during phenotype recovery, the significant reduction in their HDAC enzyme activity (Table 4.6) could be attributed by the increase in HDAC inhibition (Figure 4.5).

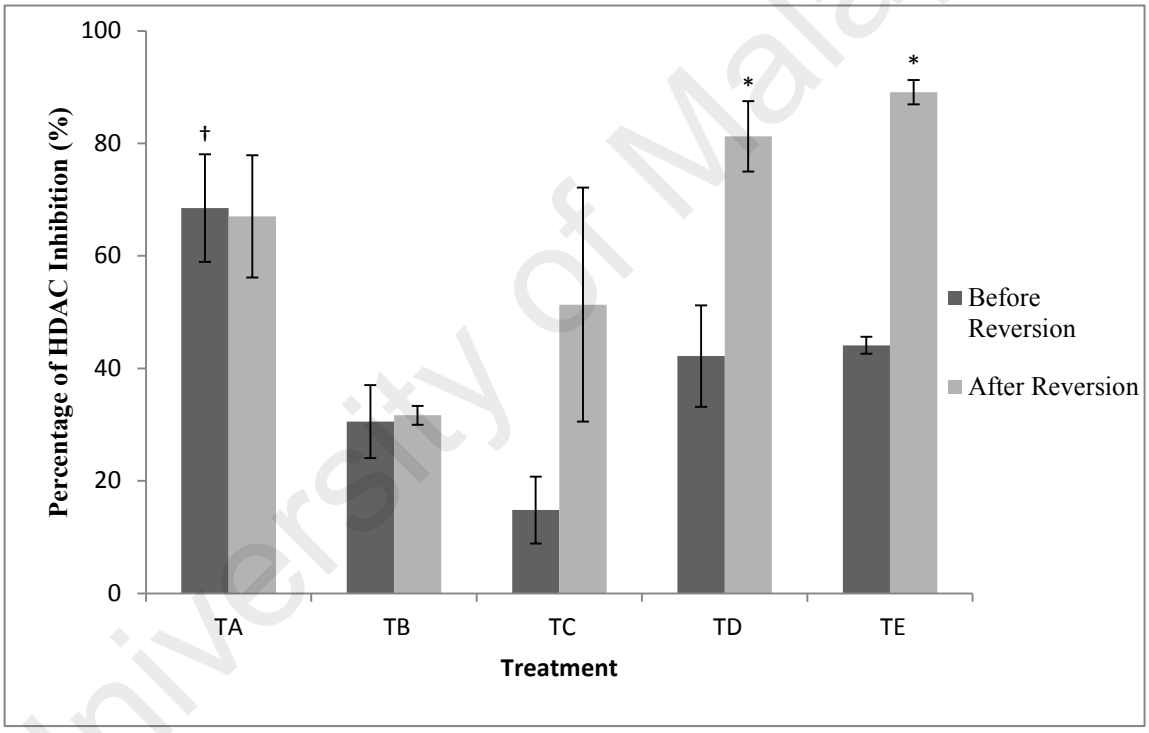


Figure 4.5: Histone deacetylase (HDAC) enzyme inhibition of variants before and after phenotypic reversion

*† indicates the highest percentage of HDAC inhibition, subject to before/after reversion (significant at $p < 0.05$).

4.6 2C DNA content and ploidy estimation

Flow cytometry was used to investigate the influence of somaclonal variation events on ploidy level of the plantlets. In this study, soybean (*Glycine max* cv Polanka) leaves with known genome size (2.50 picogram) and ploidy number (2n), was used as

the reference standard. The results showed that the ploidy level of the dwarf variants before (C, D and E) and after (F, G and H) phenotype recovery was the same as the control as shown in Table 4.6 as follows:

Table 4.6: Determination of 2C DNA content and ploidy number for dwarf variants (before and after recovery) and phenotypically normal samples

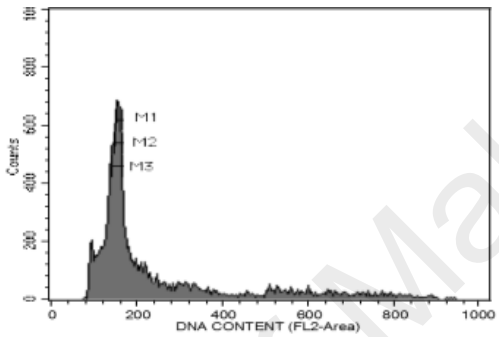
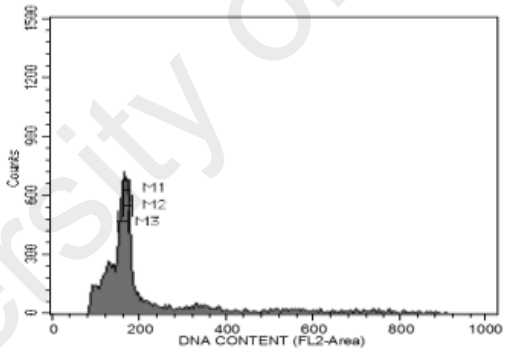
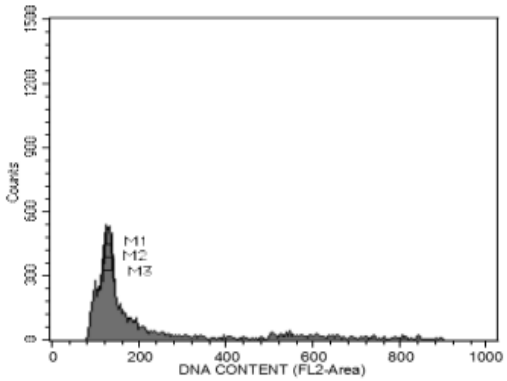
Sample	Phenotype	Histogram	2C DNA content (pg DNA)	Ploidy level
A	Normal		2.40 (2.347 x 10^9 bp)	2n
B	Normal		2.59 (2.533 x 10^9 bp)	2n
C	Dwarf		2.00 (1.956 x 10^9 bp)	2n

Table 4.6, continued

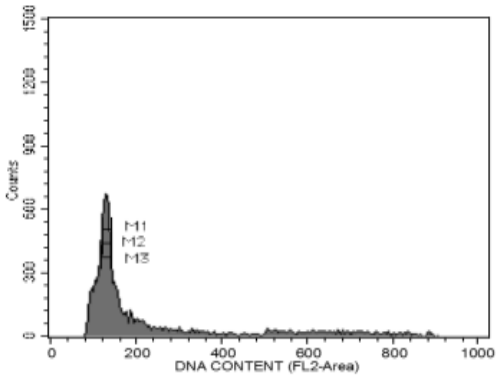
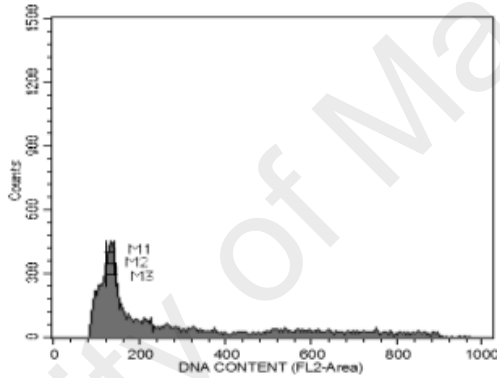
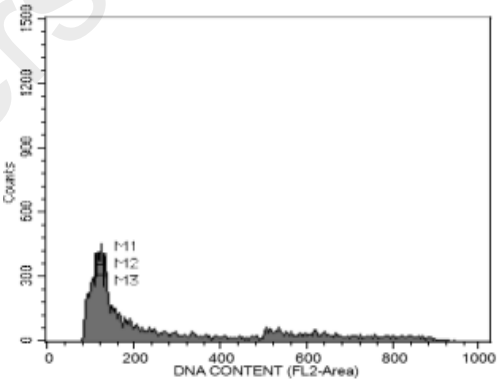
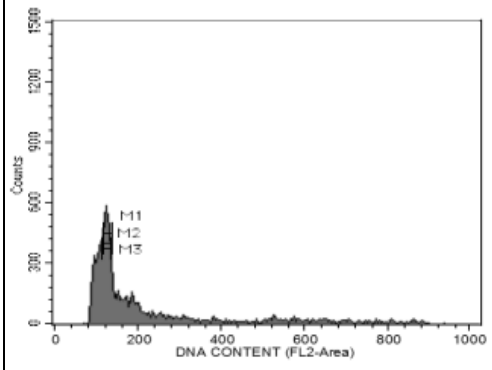
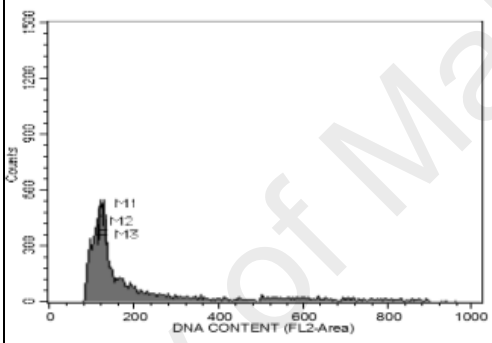
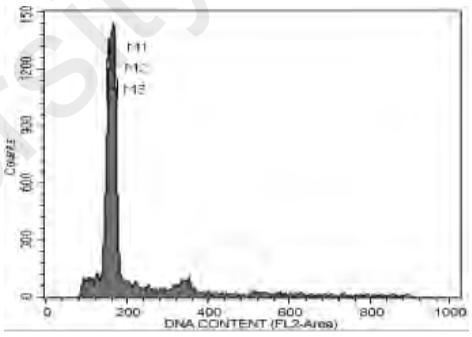
Sample	Phenotype	Histogram	2C DNA content (pg DNA)	Ploidy level
D	Dwarf		2.02 (1.976 x 10^9 bp)	2n
E	Dwarf		2.04 (1.995 x 10^9 bp)	2n
F	Normal		1.88 (1.839 x 10^9 bp)	2n

Table 4.6, continued

Sample	Phenotype	Histogram	2C DNA content (pg DNA)	Ploidy level
G	Normal		1.95 (1.907 x 10^9 bp)	2n
H	Normal		1.91 (1.868 x 10^9 bp)	2n
Reference standard			2.50 (2.445 x 10^9 bp)	2n

(A) Peak histogram of *A. comosus* plantlets grown on MS basal media; (B) Peak histogram of *A. comosus* plantlets grown on optimum regeneration media; (C) Peak histogram of *A. comosus* plantlets grown on MS media added with 2 mg L^{-1} IBA and 4 mg L^{-1} BAP; (D) Peak histogram of *A. comosus* plantlets grown on MS media supplemented with 1.0 % (w/v) NaCl; (E) Peak histogram of *A. comosus* plantlets grown on MS media supplemented with 1.0 mg L^{-1} ABA; (F) Peak histogram of *A. comosus* plantlets grown on MS media added with 2 mg L^{-1} IBA and 4 mg L^{-1}, after phenotype recovery on MS basal media; (G) Peak histogram of *A. comosus* plantlets grown on MS media supplemented with 1.0 % (w/v) NaCl, after phenotype recovery on MS basal media; (H) Peak histogram of A. comosus plantlets grown on MS media supplemented with 1.0 mg L^{-1} ABA, after phenotype recovery on MS basal media.

4.7 Verification of *AtHD2* gene in the plasmid *of A. tumefaciens*

In this study, *AtHD2* gene cassette was designed and transformed into the *A. tumefaciens* (section 3.3.1). *AtHD2* gene was synthesized based on *A. thaliana* (*AtHD2*) genome sequence.

The *A. tumefaciens* cultures were maintained and grown on a selection media to produce single colonies. Five successful forming colonies were screened by colony PCR to confirm the plasmid vector harboring the *AtHD2* gene. The orientation of the plasmid construct was determined using RE digestion followed by gel electrophoresis. This was carried out prior to *AtHD2* gene transformation into *A. comosus*.

4.7.1 Verification of *AtHD2* gene by PCR amplification

Prior to verification of *AtHD2* gene, the annealing temperature was optimized to obtain the best PCR products with clear and distinct bands. Based on Figure 4.6, the best annealing temperature (AT) was 50.8°C producing the most intense band. The *AtHD2* gene primer pair used in this study has amplified the target gene with an approximate size of 940 bp (Figure 4.6). This indicated the presence of *AtHD2* gene in the plasmid construct of *A. tumefaciens*.

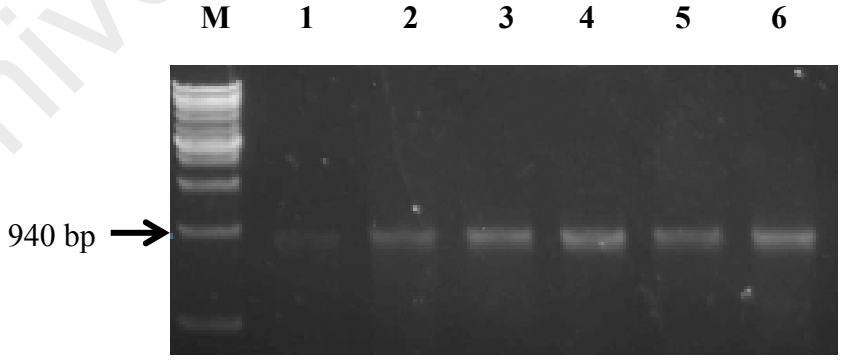


Figure 4.6: Gel showing DNA bands obtained after gradient PCR amplified by *HD2* gene primers. The most intense band on lane 4 was obtained with annealing temperature of 50.8 °C.

Lane M: 1kb DNA markers (Vivantis Technologies Sdn Bhd); Lane 1: Amplified DNA with AT of 49.0°C; Lane 2: Amplified DNA with AT of 49.3 °C; Lane 3: Amplified DNA with AT of 49.9°C; Lane 4: Amplified DNA with AT of 50.8°C; Lane 5: Amplified DNA with AT of 52.1 °C; Lane 6: Amplified DNA with AT of 53.4°C

4.7.2 Verification orientation of the plasmid construct

Restriction enzymes (NcoI and BglII) digestions were carried out to ensure the correct plasmid construct orientation and no formation of sequence mutation in the plasmid vector. Expression vector pCambia 1304 is approximately 12 362 bp while gene of interest is approximately 935 bp.

Figure 4.7 showed two fragments from the RE digestion. The DNA bands depicted the correct sizes of both plasmid backbone (~12 362 bp) and DNA insert (~935 bp).

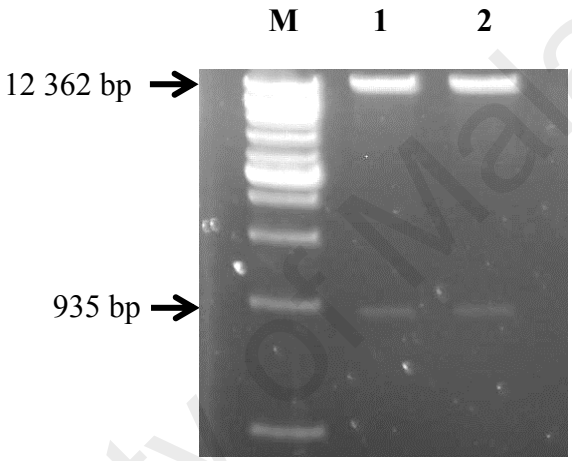


Figure 4.7: Gel showing DNA bands obtained after restriction enzymes digestion

Lane M: DNA markers 1kb (Vivantis Technologies Sdn Bhd); Lane 1: DNA bands cut by NcoI and BglII for plasmid extracted from colony; Lane 2: DNA bands cut by NcoI and BglII for plasmid extracted from colony 4

4.8 Confirmation of *gfp* gene in the plasmid construct

From the five colonies formed (Appendix E), only three showed positive bands using *gfp* primers as shown in Figure 4.8. The primer used amplified the green fluorescence gene with PCR product size of about 716 base pairs. The positive PCR band has showed a successful transformation of the bacterial plasmid harboring the *gfp* gene. However, lane 5 showed the presence of additional non-specific bands. For

subsequent transformation experiments, only colonies with specific bands (lane 1 and 4) were used.

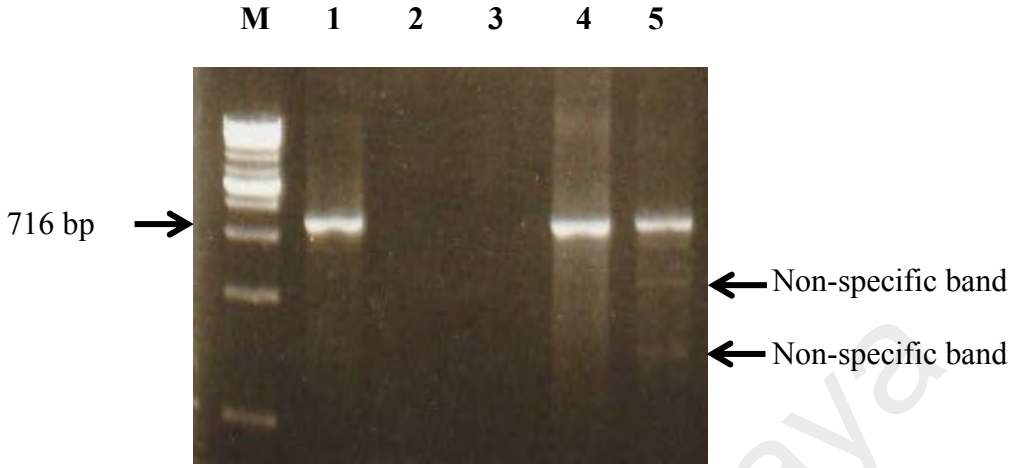


Figure 4.8: Gel showing amplified DNA fragments from each colony by PCR

Lane M: 100 bp DNA markers (Vivantis Technologies Sdn Bhd); Lane 1: Amplified DNA band showing positive/successful transformation from *Agrobacterium* colony 1; Lane 2: No DNA band showing negative/unsuccessful transformation from an *Agrobacterium* colony 2; Lane 3: No DNA band showing negative/unsuccessful transformation from an *Agrobacterium* colony 3; Lane 4: Amplified DNA band showing positive/successful transformation from an *Agrobacterium* colony 4; Lane 5: Amplified DNA band showing positive/successful transformation from an *Agrobacterium* colony 5 with unspecific bands

4.9 Transformation of *AtHD2* gene into *A. comosus* mediated by *A. tumefaciens*

In this study, *AtHD2* gene was transformed into phenotypically normal *A. comosus* plants to determine its ability to induce somaclonal variation with its role as epigenetic regulator for the occurrence of dwarfism. Moreover, a protocol for transformation of *A. comosus* variety MD2 to ascertain better transformation efficiency was also studied.

4.9.1 Comparison of transformation efficiency using acetosyringone and vanillin

In order to improve the transformation efficiency**,** acetosyringone and vanillin was used during transformation experiments. The efficiency was determined by recording number of transformed explants producing shoots over the total number of explants inoculated on a selection media.

According to the analyzed data in Figure 4.9, the transformation efficiency recorded in selection media added with 200 µM of acetosyringone (about 44.4 %) was higher compared to 500 µM vanillin (33.3 %)**.**

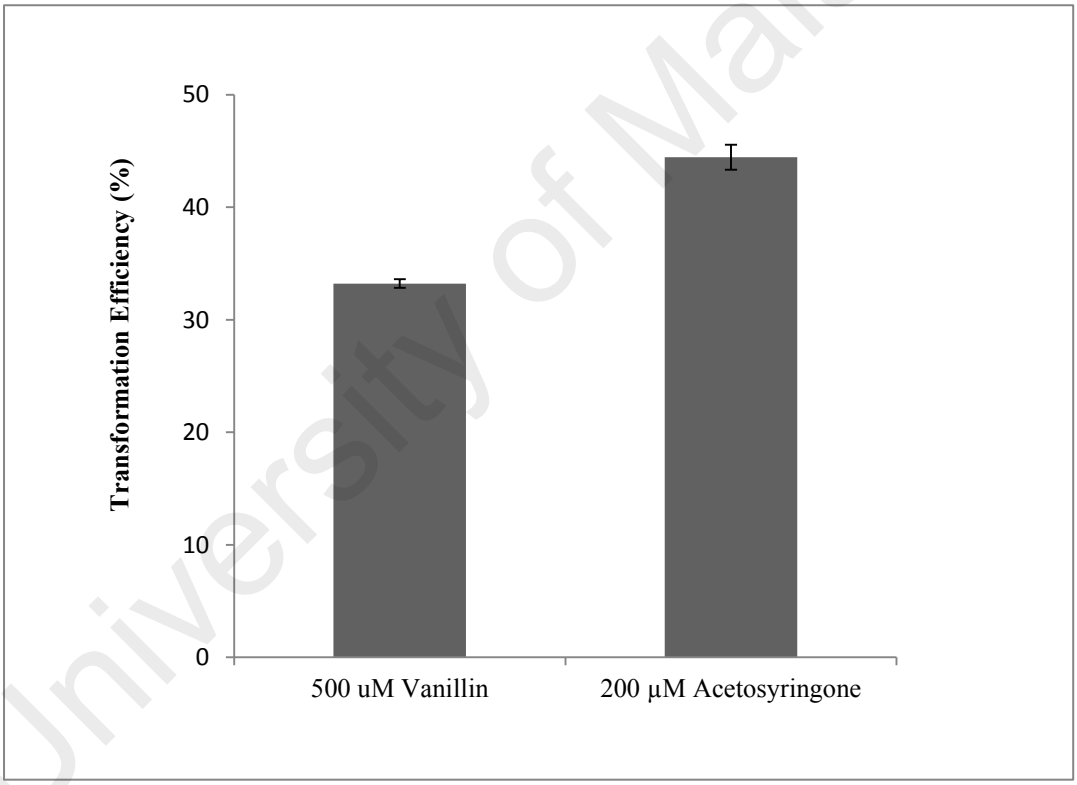


Figure 4.9: The percentage of transformation efficiency for two types of phenolic compounds, vanillin (500 µM) and acetosyringone (200 µM). The efficiency calculated from the number of putative transgenic lines (survived plantlets) over total number of inoculated samples used for *Agrobacterium* transformations

4.9.2 PCR screening after transformation of *A. comosus* transformed with acetosyringone

Acetosyringone is well known for its function in promoting high efficiency transformation in various plants (Yang et al., 2016). Putative transformants in co-cultivation media with 200 µM acetosyringone were screened for *HD2* and *hpt* genes. The screening was carried out through the amplification of *hpt* gene (Figure 4.10) by *hpt* primer conferring hygromycin phosphotransferase gene as well as *HD2* specific primer (Figure 4.11) for target gene amplification.

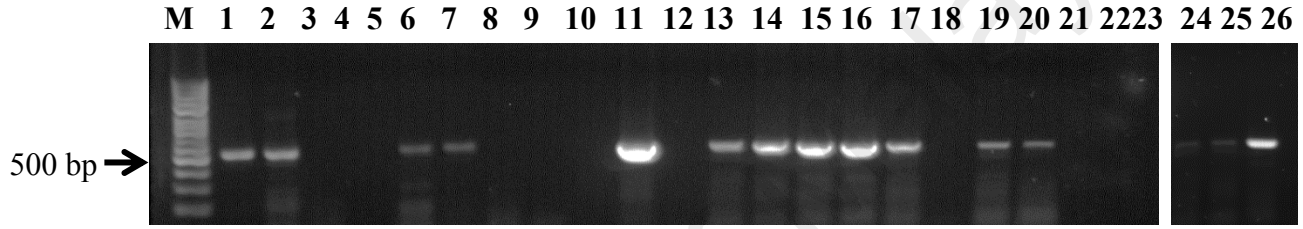


Figure 4.10: Gel electrophoresis image showing putative transgenic lines (15 lanes) by using *hpt* primer producing 514 bp size of PCR product. Lane 23 and 26 are negative (WT) and positive control, respectively. M: 100 bp DNA marker (GENESTA™)

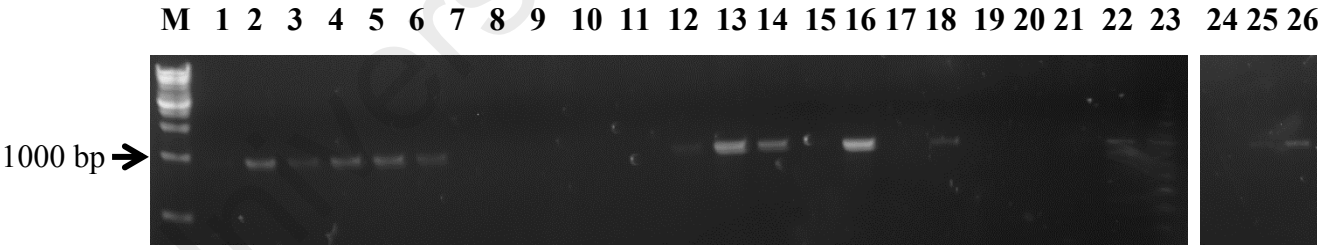


Figure 4.11: Gel electrophoresis image showing putative transgenic lines (13 lanes) by using *HD2* primer producing 935 bp PCR product size. Lane 25 and 26 are negative (WT) and positive control, respectively. M: 1kb DNA marker (Vivantis)

4.9.3 PCR screening after transformation of *A. comosus* transformed with vanillin

Other than acetosyringone, vanillin was also has been studied to enhance transformation efficiency in many plants. Thus, this experiment sights to compare its efficiency in producing transgene regenerants. Aziz et al. (2012) have discovered high transformation efficiency in *A. comosus* variety N36 and was improved by addition of 500 µM of vanillin. Hence, the same concentration was used in this study resulted in presence of successful transformants. The screening was carried out by amplification of *hpt* gene (Figure 4.12) by *hpt* primer as well as *HD2* specific primer for target gene (Figure 4.13).

Figure 4.12: Gel electrophoresis image showing putative transgenic lines (8 lanes) by using *hpt* primer producing 514 bp size of PCR product. Lane 25 and 26 are negative (WT) and positive control, respectively. M: 100 bp DNA marker (GENESTA™)

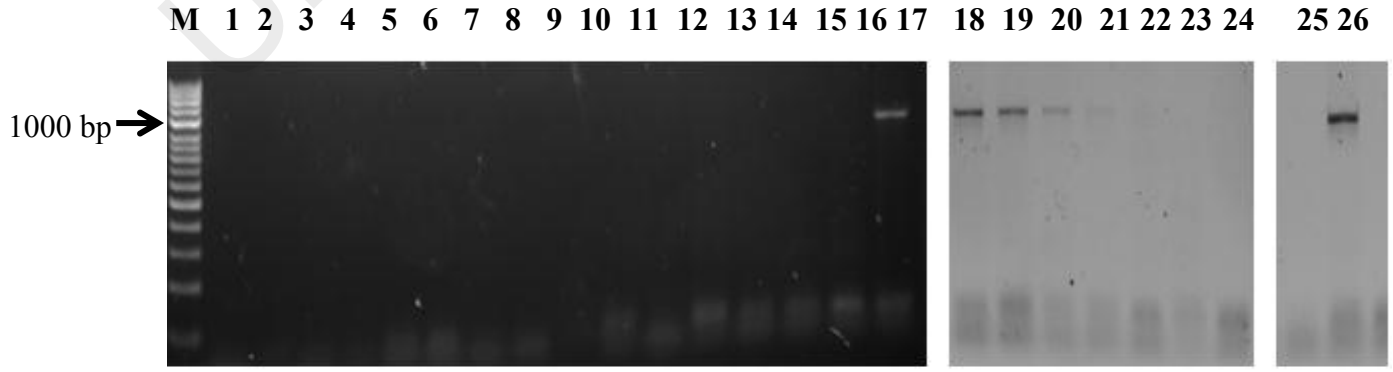


Figure 4.13: Gel electrophoresis image showing putative transgenic lines (5 lanes) by using *HD2* primer producing 940 bp size of PCR product. Lane 25 and 26 are negative (WT) and positive control, respectively. M: 1kb bp DNA marker (Vivantis)

4.10 Relative gene expression of *AtHD2*

The expression level of *AtHD2* was evaluated to determine whether the transcripts level was significantly different among the transgenic and the wild type samples. According to Figure 4.14, the relative expression level of *AtHD2* in the transgenic plants (TF) was higher (3.2-fold up-regulation) compared to the wild type plants (TA and TB).

These results further support the data obtained in previous experiments, where the transgenic plants were observed to exhibit dwarfed phenotypes and showed higher HDAC enzyme activity level compared to the wild type plants. Therefore, it can be suggested that the mRNA transcripts of *HD2* gene in the transgenic sample was higher, which further resulted in the higher HDAC enzyme activity level and formation dwarf phenotype.

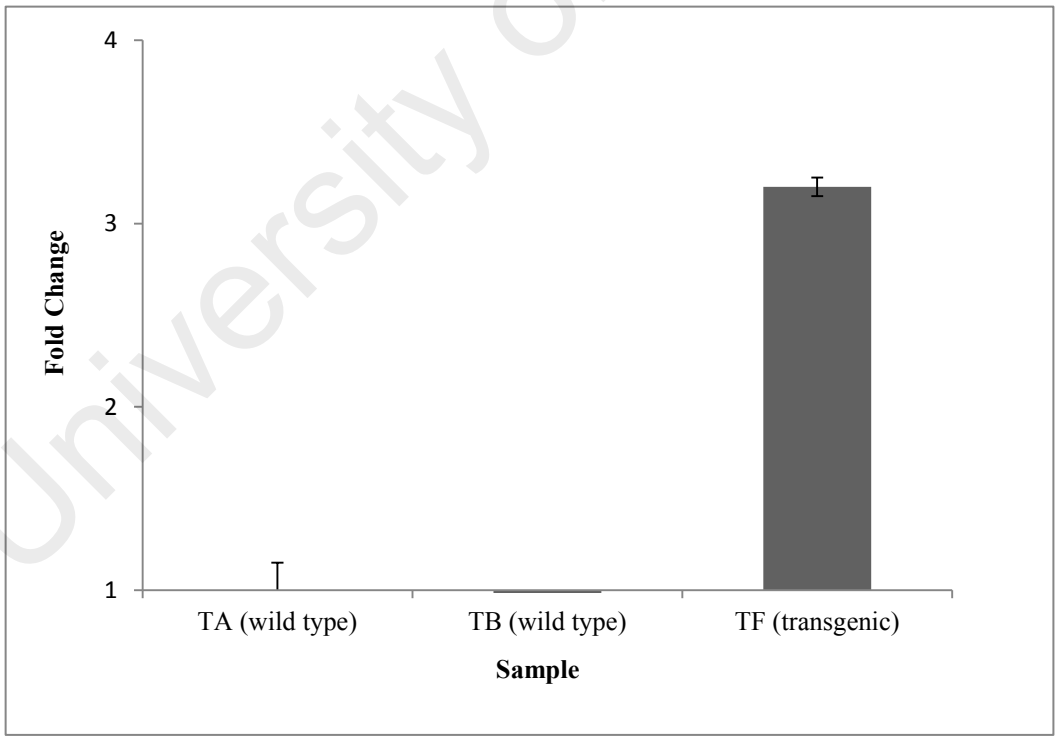


Figure 4.14: Gene expression of *Arabidopsis thaliana* histone deacetylase 2 (*AtHD2*) in the transgenic samples (TF). TA: Plantlets grown on MS basal media; TB: Plantlets grown on optimum regeneration media. The fold change was represented by $2^{\wedge\Delta\Delta CT}$ where ΔΔCT is the difference between reference ΔCT with target ΔCT.

4.11 Height morphology of transgenic *A. comosus*

Upon transformation of *AtHD2* gene, the putative transgenic plantlets were allowed to grow on the selection media containing hygromycin for about three months preceded by observation of height morphology. It can be observed the height of the transgenic plantlet (about 2 cm) is shorter than the wild types (about 4 cm) as shown in Figure 4.15. The short structure of the transgenic has preliminary revealed it may due to the *AtHD2* gene epigenetically controlled the plantlet growth. This results also in line with previous findings on the occurrence of dwarfism phenomenon which strongly presume caused by the HDAC regulatory mechanism.

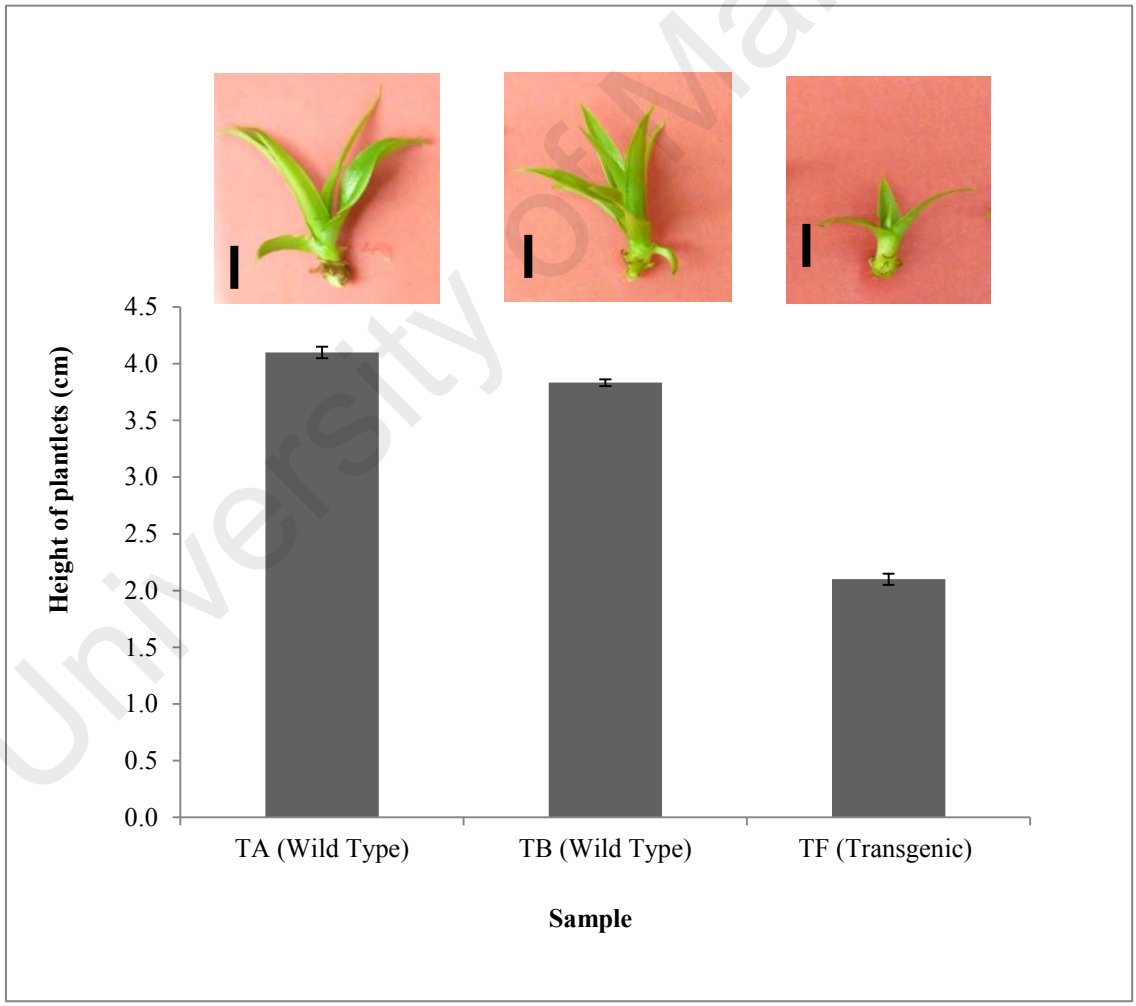


Figure 4.15: The graph showing height of transgenic lines (TF) compared to wild types (TA and TB). The images on the top of the bar chart showing the plantlets for each respective treatment. TA: Plantlets grown on MS basal media; TB: Plantlets grown on optimum regeneration media. Bar = 1 cm

4.12 HDAC enzyme activity of transgenic *A. comosus*

In this part, determination of HDAC enzyme activity for the transgenic samples is crucial to support the outcome from the previous section. The concentration of nuclear protein of the samples (Appendix D) was determined after extraction. Based on the results shown in Table 4.8, the HDAC enzyme activity level of the transgenic plants (TF) was significantly higher (5,902.00 ng/min/mg), compared to the wild type TA (666.67 ng/min/mg) and wild type TB (4,370.22 ng/min/mg).

Data analysis revealed that transformation of the plantlets with an exogenous *Arabidopsis* HD2 gene (*AtHD2*) had resulted in formation of dwarfed plantlets, with significantly higher HDAC activity level. These results further confirmed that the occurrence of somaclonal variation (dwarfism) observed in this study was due to histone deacetylation events. It has been reported that the *HD2* gene is involved in regulating the compaction of nucleosomes and therefore gene expression, where deacetylated histones are associated with subsequent decrease in gene transcription.

Table 4.7: Histone deacetylase (HDAC) enzyme activity of transgenic and phenotypically normal plantlets (wild types)

Sample name	Phenotype	HDAC activity (ng/min/mg)
TA (wild type)	Normal	666.67 ± 22.07^{a}
TB (wild type)	Normal	$4{,}370.22 \pm 46.55^{b}$
TF (transgenic)	Dwarf	$5{,}902.00 \pm 25.10^{c}$

Means with different letters in the same column are significantly different at $p < 0.05$ according to ANOVA and Duncan's multiple range test (DMRT).

4.13 2C DNA content and ploidy estimation

In this part, determination of ploidy number and 2C DNA content were carried out to verify whether any ploidy alteration had occured in the transgenic samples. Data analysis showed that there was no ploidy change occured in the transgenic samples.

Table 4.8: Determination of ploidy number and 2C DNA content for transgenic and wild type samples

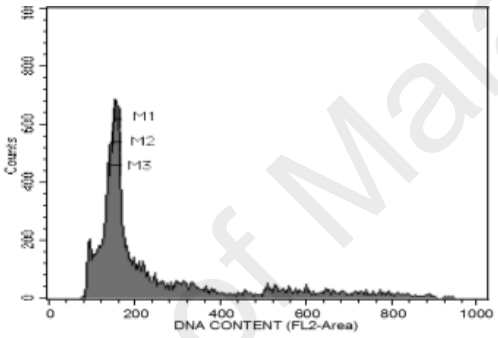
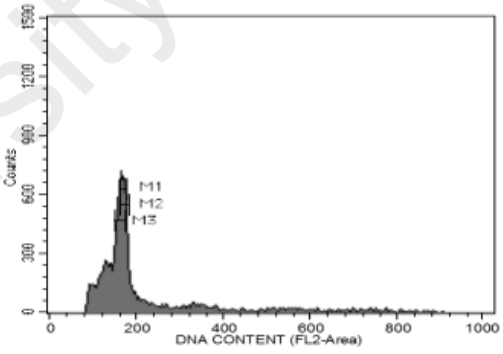
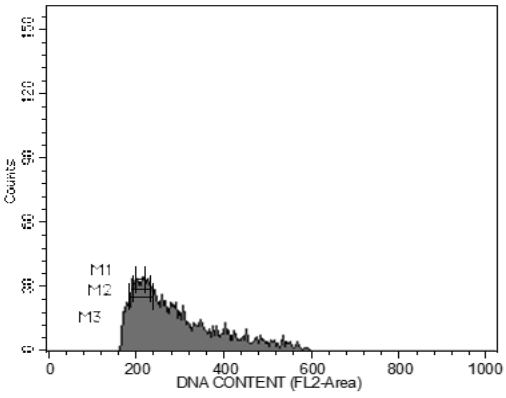
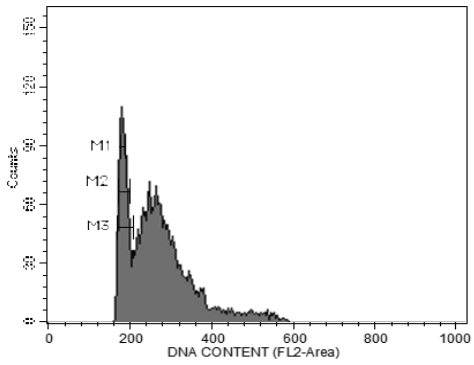
Sample	Phenotype	Histogram	2C DNA content (pg DNA)	Ploidy level
Wild type (TA)	Normal		2.40 (2.347x 10^9 bp)	2n
Wild type (TB)	Normal		2.59 (2.533x 10^9 bp)	2n
Transgenic (TF)	Dwarf		2.30 (2.251 ×10^9 bp)	2n

Table 4.8, continued

Sample	Phenotype	Histogram	2C DNA content (pg DNA)	Ploidy level
Reference standard			2.50 (2.445×10^9 bp)	2n

Wild type (TA) Peak histogram of non-transformed *A. comosus* plantlets grown on MS basal media; Wild type (TB) Peak histogram of non-transformed *A. comosus* plantlets grown on optimum regeneration media; Transgenic (TF) Peak histogram of transformed *A. comosus* plantlets grown on selection media

CHAPTER 5: DISCUSSION

5.1 Shoot proliferation and optimum regeneration media of *A. comosus*

Optimum regeneration conditions especially growth media is crucial in *in vitro* micro-propagation to maintain the highest number and health of the proliferated plantlets (Nelson et al., 2015). It is also essential to attain genetically identical and stable regenerants (Faisal et al., 2017). In this study, selection of optimum regeneration medium is based on the production of favorable number of regenerants that are phenotypically normal and healthy as their mother plants. The optimum regeneration media producing the highest number of microshoots per explants was found in MS media supplemented with 1.0 mg L^{-1} IBA and 2.0 mg L^{-1} BAP.

Various studies have been conducted on application of plant growth regulators (PGRs) for pineapple regeneration. Pineapple shoot multiplication has been studied in medium enriched with BAP alone (Be & Debergh, 2006), combination of two BAP added with naphthalene acetic acid (NAA) (Firoozabady & Gutterson, 2003), indole acetic acid (IAA) (Hamad & Taha, 2008) and 2,4-dichlorophenoxy acetic acid (2,4-D) (Liu et al., 1989) as well as supplementation of BAP combined with two auxins such as NAA and IBA (Soneji et al., 2002), NAA and IAA (Mathews & Rangan, 1979) and IAA and IBA (Teixeira et al., 2006). However, limited number of studies was found on pineapple shoot regeneration media by employing the use of various concentrations of IBA and BAP, either singly applied or in combination.

In a previous study by Hassan and Zayed (2018), supplementation of *Jatropha curcas* culture medium with IBA (0.25 mg L^{-1}) and BAP (0.5 mg L^{-1}) has induced the highest number of shoots after 30 days (Hassan & Zayed, 2018). In addition, it was also reported that single application of 1.0 mg L^{-1} IBA promoted shoot elongation of *J. curcas*. Also, supplementation of 2.22 mg L^{-1} BAP in olive culture media has been

reported to yield the highest proliferation number with an average of 3.4 new shoots after 30 days (Peixe et al., 2007). Furthermore, culture media supplemented with 2.0 mg L^{-1} BAP and 0.5 or 1.0 mg L^{-1} IBA induced the highest number of regenerated plantlets of Nemaguard and Okinawa rootstock (Hassan & Zayed, 2018). These findings were consistent with the findings of this study, where MS media added with 1.0 mg L^{-1} IBA and 2.0 mg L^{-1} BAP yielded the highest number of proliferated plantlets with an average of 6 new microshoots.

5.2 Induction of somaclonal variations by PGR, Salt and ABA

Plant tissue culture provides a broad and remarkable platform for plant research, yet it can cause undesirable effects known as somaclonal variation. This effect can be easily detected by macro-morphological changes, such as plant height and leaf characteristics (Rastogi et al., 2015). Supplementation of chemical compounds in the culture media is one of the methods to induce phenotypic changes in plantlets or explants as well as influence the gene and protein expression levels (Fraga et al., 2016). In this study, somaclonal variation was induced by three stress factors, PGRs, NaCl, and ABA.

5.2.1 Plant growth regulator

Leaf base explants showed differential response to the different concentrations of PGRs in the culture media after two months of culture. The use of high concentration of BAP (more than 2.0 mg L^{-1}) either alone or combined with IBA did not improve shoot regeneration. The height of the plantlets became shorter as BAP concentration increased. The shortest plantlets (1.9 cm) were recorded on MS media containing 2.0 mg L^{-1} IBA and 4.0 mg L^{-1} BAP.

BAP is a strong cytokinin that is always associated with epigenetic aberrations (Smulders & De Klerk, 2011). Exogenous application of plant growth regulators like

BAP often disturbs cell cycle, which may lead to variability (Bairu et al., 2011). For instance, high concentration of BAP (15 mg L^{-1}) increased the number of chromosome in the banana cultivar 'Williams' (Giménez et al., 2001). Similarly, combination of high levels of BAP (5 mg L^{-1}) with NAA (0.5 mg L^{-1}) resulted in diminished pollen viability (Sun et al., 2013).

The incidence of unwanted somaclonal variation can also lead to dire financial loss, for example in 'fruit mantling' phenomenon in oil palm, where the flowers developed a second whorl of carpels instead of stamens (Alwee et al., 2006; Smulders & De Klerk, 2011). It was reported that this disorder was due to epigenetic change, and caused by short subculture periods as well as supplementation of tissue culture media with high levels of cytokinin combined with low levels of auxin (Eeuwens et al., 2002). These are in line with the current results of this study, where supplementation of high concentration of BAP (4.0 mg L^{-1}), either with IBA or alone, produced significantly shorter plantlets (dwarf) compared to other treatments.

5.2.2 Salinity

Induction of somaclonal variation by different concentrations of NaCl was also carried out. Data analysis after two months of culture in the stress media showed that in MS media containing 1% NaCl, plantlets were found to produce less number of leaves, smaller leaf size and shorter in height. Various reports in literature have indicated that somaclonal variation could be influenced by stressing agents. For example, addition of NaCl in culture medium has been shown to influence the growth characteristics and increase the variability of *in vitro* regenerated rice plantlets (Lutts et al., 2001). Plants' tolerance to salinity differs greatly, yielding different growth responses. Among cereals, rice (*Oryza sativa*) is the most sensitive and barley (*Hordeum vulgare*) is the most tolerant (Munns et al., 2006). Moreover, bread wheat (*Triticum aestivum*) is more

tolerant to salt than durum wheat (*Triticum turgidum* ssp. *durum*) (Munns et al., 2006). Meanwhile, pineapple has moderate tolerance to salt (Grieve et al., 2012).

In this study it was show that high concentration of NaCl (>2%) significantly (p<0.05) affected shoot development, plantlet height and leaf formation of *in vitro* grown MD2 pineapple. High concentration of NaCl might reduce water uptake and leaf expansion as well as damaging the root system, which leads to growth reduction, creating salt-specific or ionic effect of salinity (Greenway & Munns, 1980; Zhang et al., 2016). In addition, various plant studies have reported on the increment of reactive oxygen species (ROS) level under saline conditions (Hasanuzzaman et al., 2013). Moreover, ROS mediated membrane damage was observed to be a major cause of the cellular toxicity by saline stress in rice, tomato, citrus, pea and mustard (Acosta-Motos et al., 2017; Hameed et al., 2014; Mittova et al., 2015; Parihar et al., 2015).

5.2.3 Abscisic acid

Other than salinity stress, exogenous application of ABA was also used to induce somaclonal variation. In this study, MS media containing 1.0 or 6.0 mg L^{-1} ABA produced the shortest plantlets (1.7 cm). However, high concentration of ABA (6.0 mg L^{-1}) caused browning effect on the plantlets. ABA accumulation may induce imbalance of water potential which later disrupts the biological processes and physiological activities, such as regulation of stomatal conductance (Verslues & Bray, 2006). It has also been demonstrated that ABA can accumulate in plant tissues subjected to drought, salt, desiccation, and cold, to act as a signal for the initiation of acclimation toward the stressors (Hare et al., 1999; Rock & Sun, 2005; Yu & Ho, 2015).

The aberrant phenotype observed after ABA treatment might be due to the adaptation strategy of plants to cope with stress. This finding is in line with a previous report by

Zaffari et al. (1998), where it was observed that endogenous ABA levels in green and yellow sectors of variegated leaves of *Musa acuminata* L. was significantly higher than in normal leaves (Bairu et al., 2011; Zaffari et al., 1998).

5.3 Morphological recovery of somaclonal variants

The restoration of the dwarf phenotype to normal plant stature could be due to phenotypic plasticity, which is the ability of a genotype to express different phenotypes in different environments (Pigliucci, 2005). It was reported that stress which occurred in periods shorter than a plant's life span could strongly trigger reversible phenotypic plasticity (Gabriel, 2005). It has also been established that phenotypic plasticity is an adaptive strategy for plants to cope with variable environments (Schlichting, 1986). This unique mechanism is usually achieved by employing rapid genome-wide changes in gene expression and altering its metabolome composition (Cook et al., 2004).

Furthermore, Li and Qu (2010) has reported that somaclonal variants demonstrated unstable phenotypes due to epigenetic effects (Li et al., 2010). For example, DNA methylation variants (epiallelles) for naturally occurring single-locus DNA that are transgenerationally stable and independent DNA sequence variation have been reported (Rakyan et al., 2003). Moreover, natural epigenetic variation have also been observed among ecotypes of *A. thaliana* and other plant species (Zhang et al., 2013). Han et al. (2016) reported that high level of HD2D in transgenic *A. thaliana* may cause higher plasticity in response to abiotic stressors. This is implied to the results obtained in the variants (TC, TD, TE) with higher HDAC and ability to perform phenotype recovery. The increasing HDAC activity has cause phenotypic plasticity and thus allowing the plants to recover and revert its phenotype to the normal after a period of time.

5.4 HDAC enzyme activity/inhibition before and after phenotype recovery

The HDAC enzyme activities in the dwarf plantlets were found to be significantly high, compared to plantlets with normal phenotype. Deacetylation mediated by HDACs is involved in regulating gene expression and various biological processes, such as cell differentiation, growth arrest, cytotoxicity and induction of apoptosis (Tsaftaris et al., 2005). This has also led to epigenetic modulation of morphological traits such as plant height, leaf shape and colour, flower shape and colour, plant maturity as well as disease resistance (Semal, 2013). This is in line with the current findings where the HDAC activity level of the dwarf variants was significantly reduced following phenotype reversion to normal plant stature, after the stress factors were removed.

In plants, almost all the intermediary enzymatic metabolisms are mediated by acetylation, hence it involves indirect metabolism route control through reversible lysine acetylation and deacetylation. Acetylated proteins have been reported to regulate a range of cellular activities such stress function, biotic and abiotic stimulus proteins, transcriptional factors, signal transduction, structural proteins and enzymatic reactions in cytoplasm and organelles (Choudhary et al., 2009; Finkemeier et al., 2011). Moreover, plant tissue culture has been reported to cause increased expression of various histone modifying enzymes such as histone deacetylases (HDACs) as well as de novo DNA methylases (Law & Suttle, 2005; Tanurdzic et al., 2008). Plants adapt to stressful environment and new growth conditions not by changing genetic information, but by manipulating the expression of existing gene pool through epigenetic regulatory pathways (Boyko & Kovalchuk, 2013).

After allowing for recovery, the HDAC activity of the plantlets was measured and found to be significantly lower than before recovery. The decrease in HDAC activity level during phenotype recovery may be due to the high HDAC inhibition exhibited by

the samples. It has been previously reported that HDAC inhibition would largely result in hyperacetylation and consequently gene expression (Bassett & Barnett, 2014; Hebbes et al., 1988; Lopez-Atalaya et al., 2013; Miguel & Marum, 2011), to aid in phenotype reversion. Plants could synthesize HDAC inhibitory molecules for fungal disease resistance as well as for self-regulation in response to both biotic and abiotic stressors (Cuperlovic-Culf & Culf, 2014). For example, products of glycolysis such as lactate and pyruvate have been reported as potent and ubiquitous HDAC inhibitors (Latham et al., 2012; Thangaraju et al., 2006).

5.5 Ploidy analysis of somaclonal variants

Somaclonal variations can be somatically and genetically stable (Meyer, 2015). The genetically stable somaclonal variants can be termed as mutations (Meyer, 2015). On the other hand, due to the possibility of reversible epigenetic variations, the term 'variations' is widely used instead of 'mutations'. Epigenetic changes are temporary and reversible but not heritable (Meyer, 2015). However, genetically stable somaclonal variants could be induced due to point mutations, alteration in chromosome number and structure, deletions and tranpositons of DNA sequences in nuclear genomes. In this study, the ploidy level of the dwarf variants before and after phenotype recovery was the same as the control. The results of flow cytometry analysis revealed the lack of ploidy alteration hence suggested that the variation occurred could be due to epigenetic orchestration.

Although the type of explants and synthetic additives such as auxins and cytokinins have been shown to affect the chromosome number and ploidy levels in some species and genotypes, the results of this study indicated that there was no alteration of ploidy level in stress-treated MD2 pineapple plantlets. These synthetic stress additives are not considered as a direct mutagenic agent compared to antimitotic substances such as

colchicine, but they may affect the cell cycle controlling DNA synthesis and endoreduplication, as well as cell growth.

Besides, the type, concentration and combination of synthetic substitutes of auxins and cytokinins have been shown to affect chromosome number and ploidy levels in selected species and genotypes (Smulders & De Klerk, 2011). Synthetic auxins such as 2, 4-D, which is not transportable out of the cells, is believed to facilitate a meristematic state by altering the endogenous auxin gradient (Morris, 2000). High concentrations of 2, 4-D resulted in the generation of mixoploids and tetraploids in cucumber suspension culture (Ładyżyński et al., 2002). Although these chemicals may not be directly mutagenic, they may affect ploidy level by triggering unorganized cell growth, disturbing cell cycle control leading to DNA synthesis and endoreduplication.

5.6 Gene expression studies of *AcHD2* in somaclonal variants

The expression level of endogenous *A. comosus* histone deacetylase gene, *AcHD2* was evaluated to determine whether the transcripts level was significantly different among the plantlets. Data analysis showed that the expression level of *AcHD2* in the variants (TD and TE) was higher compared to phenotypically normal plantlets (TA and TB). The expression level of *AcHD2* in the plantlets after phenotype recovery was also assessed where, *AcHD2* expression in TC and TD was found to be dramatically increased, but decreased in TE after phenotype recovery. These observations implied that the correlation between histone deacetylase mRNA expressions with HDAC enzyme activity obtained in this study was poor. However, it has been well established in scientific literature that the correlation between mRNA level and protein abundance in biological samples is usually very poor (Maier et al., 2009).

Nevertheless, the level of HDAC inhibition in the samples was also measured to yield further information with regards to HDAC enzyme regulation in relation to its gene expression. Based on data analysis, it was revealed that there was a marked increase in HDAC inhibition in the variants (TC, TD and TE) after phenotype recovery. At this juncture, it could be argued that despite the high level of *AcHD2* gene expression in the variants during phenotype recovery, the significant reduction in their HDAC enzyme activity could be attributed by the increase in HDAC inhibition.

ABA has been known as a stress hormone by generations of researchers where it regulates the plant water level through guard cells and growth through induction of genes that code for enzyme and proteins involved during water scarcity (Zhang et al., 2006). The involvement of ABA and NaCl in modulation of HDAC activity during seed germination has been studied in great detail. It was reported that NaCl and ABA repressed the expression of *HD2A*, *HD2B*, *HD2C*, and *HD2D* during *Arabidopsis* seed germination (Luo et al., 2012). Specifically, ABA and NaCl were found to cause HDACs gene activation by interaction of two types of HDACs; HD2C and HDA6 (Luo et al., 2012). This is in line with the findings obtained in this study, where it was shown that the dwarf variants produced through treatments with 1.0% (w/v) NaCl (TD) and 1.0 mg L^{-1} ABA (TE) exhibited significantly higher *AcHD2* expression than the phenotypically normal plantlets (TA and TB).

In *Arabidopsis* mutants, a few types of HDACs have been found to be hypersensitive to ABA and NaCl (Colville et al., 2011). They reported that *Arabidopsis* seed germination was enhanced in *hd2a* mutants but decreased in *hd2c* mutants compared to wild-type seeds. On the other hand, germination potential was restored in *hd2a/hd2c* double mutants, suggesting that *HD2A* and *HD2C* played opposing functions during seed germination (Colville et al., 2011).

In contrast, little information was found on the role of ABA and NaCl during post-germinative growth, especially in relation to modulation of HDAC activity. In an earlier report, *Arabidopsis sax1* (hypersensitive to abscisic acid and auxin) mutants were shown to have dwarf stature and increased sensitivity to ABA, but the phenotypic traits could be partially restored via exogenous application of brassinosteroids (Ephritikhine et al., 1999). This is further validated by a recent study by Qiu et. al. which reported that ABA acts antagonistically with brassinosteroids (BRs) during seed germination and post-germinative growth (Qiu et al., 2017).

However, the implications of ABA and NaCl on histone acetylation during post-germinative growth have much to be studied. Recently, Li et al. reported that in *Arachis hypogaea*, a histone deacetylase 6-like gene was up-regulated by ABA and water stress (Li et al., 2014). These findings were supported by Su et al., which showed that the expression of *AhHDA1*, an RPD3/HDA1-like superfamily of histone deacetylase (HDAC) gene in *Arachis hygogaea* was up-regulated by ABA and osmotic stress (Su et al., 2015). These are in agreement with our results, which showed that supplementation of NaCl and ABA significantly reduced plantlet height in *A. comosus* var. MD2, producing plantlets with severe dwarf stature. The HDAC enzyme activities in the dwarves were also significantly high, compared to plantlets with normal phenotype.

5.7 Integration and expression of *AtHD2* in transgenic *A. comosus*

Genetic transformation was carried out to determine the role of *HD2* gene in the formation of somaclonal variants. Thus, to test the function of the gene, the synthesized *AtHD2* gene was first inserted into pCambia 1304 plasmid vector and was transformed into the *A. comosus* plant system using the *A. tumefaciens*.

Transformation by *A. tumefaciens* is more efficient compared to other techniques to exchange gene, due its efficacy to result in transgenes of a more stable manner in the plant system (Hofmann, 2016).

5.7.1 Transformation efficiency using acetosyringone and vanillin

The lack of signalling molecules such as phenolic compounds in monocot plants including pineapple (*A. comosus*) has caused limitation in ensuring a high success rate to transfer foreign genes (Rani et al., 2013). Thus, the application of widely used phenolic compounds such as acetosyrinone, cinnamic acid, coumaric acid and vanillin are crucial for plant transformation, especially in major monocot plants such as rice, wheat, pineapple and others (Rani et al., 2013). To date, transformation protocols of dicot and monocot include exogenous addition of phenolic compounds to enhance *A. tumefaciens* mediated transformation since this compound is not naturally produced (Rani et al., 2013). In this study, two types of phenolic compounds (acetosyringone and vanillin), have been used to compare the transformation efficacy of *HD2* gene into *A. comosus*. Acetosyringone has been reported to be widely used by researchers and is known for decades due to its important function in inducing the *Agrobacterium-vir* gene, enabling the transfer of T-DNA into the host plants (Subramoni et al., 2014). Based on the results obtained in this study, the transformation efficiency using 200 µM of acetosyringone was better than 500 µM vanillin by 10 %.

In a previous study on almond transformation, the presence of 150 µM of acetosyringone has resulted in 12.3 % transformation efficiency (Costa et al., 2006). Study conducted by Wu et al. (2003) concurred with the current work where 200 µM of acetosyringone applied during wheat transformation has increased the efficiency by 3.3 %. On the other hand, 100 µM of acetosyringone was used in pineapple (*A. comosus*) transformation but at a lower frequency (20.6 %) compared to the current study

(Gangopadhyay & Mukherjee, 2015). Based on these previous studies, acetosyringone has been regularly selected as the phenolic compound used for *Agrobacterium* transformation in many plants.

On the other hand, it has been reported that the addition of 500 µM vanillin yielded 55.5 % of transformation efficiency in transgenic N36 pineapple (Aziz et al., 2012). This result was contrastingly lower in this study where the transformation efficacy of 33.3 % was obtained. Vanillin has also improved the *Agrobacterium*-mediated transformation for microalgae (Pratheesh et al., 2014). It has been reported that the success and efficiency of plant transformation vary immensely according to the plant species, the genotype and the plant tissue types that are used (Poloniova et al., 2013).

5.7.2 Gene expression of *AtHD2* and morphology of transgenic *A. comosus*

The transformation of *A. thaliana* histone deacetylase gene, *AtHD2* (based on The Arabidopsis Information Resource (TAIR) accession no: 4010741337; locus tag: AT5G22650) into *A. comosus* plantlets was studied to determine the relative expression level of the gene and to verify its function in the occurence of somaclonal variation. Data analysis revealed a higher relative expression of *AtHD2* in the transgenic sample (TF) compared to the control/wild type (TA). This reflected a successful transformation of *AtHD2* in *A. comosus.* This was supported by the morphology of the resulting transgenic plantlets was shown to be phenotypically dwarf. These showed that the expression of *AtHD2* had directly caused a change in the stature of the transgenic plantlet.

It has been reported that the *AtHD2A*, *AtHD2B* and *AtHDC* (homologies of *Arabidopsis* histone deacetylase gene) were associated with diverse developmental

aberrations such as early senescence, ectopic expression of silenced gene, apical dominance disturbance, homeotic imbalance and flower defects (Tian & Chen, 2001).

Furthermore, *AtHD2* expression has been observed to cause development of a short silique, abortion of seeds, created a mutant phenotype, with narrowing and curling of leaves and slow down blooms (Han et al., 2016; Lagacé et al., 2003). In brief, the *AtHD2* expression has been identified to be associated with phenoypte instability, thus verified its function in generating somaclonal variant as demonstrated by the generation of dwarf plantlets in the treatments (PGR, salt and ABA).

5.8 HDAC enzyme activity of transgenic *A. comosus*

Measurement HDAC enzyme activity of the transgenic plantlets was conducted to determine the activity of HDAC enzyme presents in the transgenic samples. The HDAC enzyme activity in the transgenic samples was found to be higher compared to the control. Histone deacetylation activities were found to be involved in many plant metabolic pathways, including signalling pathways in response to environmental stresses, transcriptional co-activator, protein folding and various enzymatic reactions (Fisher & Franklin, 2011).

The identification of HATs and HDACs as modulators of transcription has largely focused the attention of molecular biologists on roles of its involvement in the regulation of gene expression (Lusser et al., 2001). *HDA6* gene from a family of histone deacetylases known as Reduced Potassium Dependency3 (RPD3) has been reported to mediate histone deacetylation therough transcriptional gene silencing (TGS) caused by jasmonic acid (JA) and ethylene in *A. thaliana* (Chinnusamy & Zhu, 2009). Environmental and endogenous signals can repress the target genes through reduction in histone acetylation levels. Overexpression of *HDA19* gene from the same family RPD3

in transgenic *A. thaliana* has been observed to decrease histone acetylation levels and increase deacetylation (Zhou et al., 2005)

5.9 Ploidy analysis of Transgenic *A. comosus*

The ploidy analysis was carried out in this research to investigate if there is any ploidy change occurred in the transgenic sample. In this study, the ploidy level of the transgenic plantlets was also found to be the same with the wild type plant. This shows that no chromosomal aberrations caused by expression of *AtHD2* gene had occurred in the transgenic plants, thus the variation observed in this study was probably epigenetically controlled by HDAC.

Ploidy level changes in plants have been reported to be associated with chromosomal aberration and gene mutation that affect the growth of plants (Olhoft & Philips, 2018). These results also further supported the previous findings of this study, where the ploidy level of the somaclonal (TC, TD and TE) was the same with the controls (TA and TB).

CONCLUSION AND RECOMMENDATION OF FUTURE WORKS

In this concluding remark, the specific objectives in this research have been succesfully acquired with the optimisation of tissue culture conditions using 1.0 mg L^{-1} IBA and 2.0 mg L^{-1} BAP in optimum regeneration media of *A. comosus* var. MD2, investigation of the effects of PGRs, NaCl and ABA in generating somaclonal variants with dwarf phenotypes in *A. comosus* var. MD2, evaluation of transformation of *AtHD2* gene in normal phenotype plants of *A. comosus* var. MD2 and determination of histone deacetylation activity in the somaclonal variants of *A. comosus* var. MD2.

Somaclonal variation has been successfully induced in *A. comosus* var. MD2 using high concentration of BAP combined with IBA, NaCl and ABA. The generated dwarf variants showed high HDAC enzyme activity compared to control, suggesting that the dwarf phenomena might be due to epigenetic modifications through histone deacetylation. The ploidy level of the dwarf variants remained as 2n, before and after phenotype recovery, albeit the significant elevation of HDAC activity in the dwarf variants. The increase in *AcHD2* expression in the variants after phenotype recovery despite its low HDAC activity indicated that plants can modulate relative HDAC/HAT activities by synthesizing HDAC inhibitory molecules, which in turn would induce histone hyperacetylation to aid in gene expression during phenotype recovery.

Genetic transformation of exogenous *AtHD2* gene in phenotypically normal plants which has been carried out and suggested the somaclonal variation demonstrated by the dwarf phenotype was probably due to the *HD2* gene expression was repressed and controlled in epigenetic manners. Besides, HDAC enzyme activity, height morphology observation and ploidy analysis are reflecting to the results obtained in the samples treated with PGR, salt and ABA. These findings imply that the same mechanism for the occurence of somaclone variability may be operating under the same strategy displayed

by the transgenic sample. HDAC/HAT activity is involved in stress and somaclonal variation which led to phenotypic modification resulting from epigenetic changes in plant regeneration which exhibited by the dwarf plantlets. However, more research are needed to further elucidate the mechanism of HDAC and how it responses to stress stimuli is the current limitation in this study.

This research can be further continued by investigating histone acetylation (HAT) activity in the somaclonal variants. In addition, it is recommended to study the histone deacetylation/acetylation behavior in the variants at *ex vitro* stage where the somaclonal variants are planted on field. *Ex vitro* study may provide understanding of the mechanism of HDAC and how it response to external environmental stimuli/stresses.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

PUBLICATION

1. **Halim, N. A. A**., Tan, B. C., Midin, M. R., Madon, M., Khalid, N., & Yaacob, J. (2017). Abscisic acid and salinity stress induced somaclonal variation and increased histone deacetylase (HDAC) activity in *Ananas comosus* var. MD2. *Plant Cell, Tissue and Organ Culture*, *133*(1), 123-135.

PAPERS PRESENTED

1. Evaluation of Somaclonal Variation in *Ananas comosus* var. MD2 grown *in vitro*. 3rd International Conference Sustainable Agriculture, Food and Energy (SAFE 2015), Nong Lam University of Ho Chi Minh, Vietnam, 16 November 2015 -19 November 2015. Oral presentation.

2. Evaluation of Somaclonal Variation in *Ananas comosus* var. MD2 grown *in vitro*. 20th Biological Student Graduate Congress (BSGC 2015), Chulalongkorn University, Thailand 08 December 2015 -11 December 2015. Poster presentation.

3. The Epigenetic Interplay in Somaclonal Variation of *Ananas comosus* var. MD2 Grown *in vitro* and Its Histone Deacetylation Perspective. 4th International Conference Sustainable Agriculture, Food and Energy (SAFE 2016). Ruhuna University, Colombo, Sri Lanka, 20 December 2016 - 23 December 2016. Oral presentation.