EVALUATION AND APPLICATION OF SCAR MARKERS ON BAP-INDUCED DWARF OFF-TYPES OF *MUSA ACUMINATA* CV. BERANGAN

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ABSTRACT

Micropropagation has been widely used to produce better crops at a rapid rate. Plant growth regulators are used to increase shoot proliferation, with benzylaminopurine (BAP) being the most commonly used cytokinin. However, various studies have shown that BAP concentration affects the frequency of abnormality in banana micropropagation. For this study, the effects of BAP concentrations on *in vitro* cultures of *Musa acuminata* cv. "Berangan" were investigated and it was found that on Murashige and Skoog (MS) media supplemented with concentrations of $9mgL^{-1}$ and $12mgL^{-1}$ BAP, cultures took a longer time to regenerate as compared to concentrations of $3mgL^{-1}$ and $6mgL^{-1}$. Abnormalities such as having abnormal shoot clusters and remaining in an undifferentiated callus state were also observed on cultures of the higher BAP concentrations.

Dwarf SCAR markers developed by Damasco *et al*. (1996) were used to analyze the genomic changes of the *in vitro* cultures via polymerase chain reaction. A single band of about 1.6kb present only on normal plants and absent in dwarfs would have been expected. However, instead of the predicted 1.6kb single band, two bands of 662bp and 438bp in length were observed regardless of the morphology of the regenerants produced (normal or stunted). A BLAST analysis of the 662bp sequence did not reveal any homologous fragment but the 438bp sequence was found to be homologous to fragments of the *Musa acuminata* clone BAC MA4-3F3.

Initially found to be hypothetical proteins, a further BLAST analysis using the banana genome revealed that these proteins would presumably be present in chromosome 6 and identified as the ubiquitin-fold modifier 1.

ABSTRAK

Teknik kultur tisu telah digunakan secara meluas untuk menghasilkan anak pokok yang berkualiti dalam masa yang singkat. Untuk meningkatkan pertumbuhan tunas, hormon telah digunakan dan BAP merupakan hormon yang paling kerap digunakan. Bagaimanapun, kepekatan BAP boleh meningkatkan kebarangkalian penghasilan anak pokok pisang yang tidak normal. Kesan kepekatan BAP ke atas teknik kultur tisu pisang Berangan telah diuji bagi kajian ini. Didapati bahawa kultur pisang yang dicambahkan pada media Murashige dan Skoog (MS) dengan kepekatan BAP $9mgL^{-1}$ dan $12mgL^{-1}$ mengambil masa lebih lama untuk bercambah berbanding dengan kultur pisang yang dicambahkan pada media kultur $3mgL^{-1}$ dan $6mgL^{-1}$. Tunas yang tidak normal serta kultur yang terbantut turut diperhatikan pada kultur yang mengandungi kepekatan hormon BAP yang tinggi.

Penanda molekul SCAR bagi pisang kerdil yang telah digunakan oleh Damasco dan rakan-rakan (1996) turut digunakan bagi menganalisa perubahan genomik dalam kajian ini. Satu jalur bersaiz 1.6kb diramalkan pada kultur yang normal dan tidak akan kelihatan pada kultur kerdil. Walaubagaimanapun, dua jalur bersaiz 662bp dan 438bp telah terhasil dan jalur-jalur tersebut muncul tanpa mengira keadaan kultur tisu (normal dan tidak normal).

Kajian BLAST mendapati bahawa jalur bersaiz 662bp tidak menunjukkan persamaan pada sebarang jujukan DNA tetapi jalur bersaiz 438bp didapati mempunyai persamaan dengan jujukan klon *Musa acuminata* BAC MA4-3F3.Pada mulanya jalur tersebut hanya dikenali sebagai protein yang belum dikenalpasti. Akan tetapi kajian BLAST yang dilakukan pada genom pisang mendapati bahawa jujukan

438bp tersebut berkemungkinan berada pada kromosom 6 dan adalah pengubahsuai lipatan ubiquitin 1.

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic acid
BAP	6-Benzylaminopurine
RAPD	Random Amplified Polymorphic DNA
kb	kilobase
SCAR	Sequence Characterized Amplified Regions
cm	Centimeter
RNA	Ribonucleic acid
rRNA	ribosomal RNA
PCR	Polymerase Chain Reaction
cv.	Cultivar
%	Percentage
INIBAP	International Network for the Improvement of Banana and Plantain
IPGRI	International Plant Genomic Research Institute
CIRAD	Centre de coopération Internationale en Recherche Agronomique pour
le	Développement
2,4-D	2,4-Dichlorophenoxyacetic acid
2iP	N^6-(2-isopentyl)adenine
GA_3	Gibberellin A3
ABA	Abscisic acid
mg	miligram
ISSR	Inter Simple Sequence Repeat
MS	Murashige and Skoog
RFLP	Restriction Fragment Length Polymorphism

STM	Sequenced Tagged Microsatellites
MP-PCR	Microsatellite-primed PCR
SNP	Single-nucleotide polymorphism
AFLP	Amplified Restriction Fragment Length Polymorphism
PBIU	Plant Biotechnology Incubator Unit
CEBAR	Center for Research in Biotechnology for Agriculture
g	Grams
L	Liter
°C	Degree Celsius
psi	Pounds per square inch
CTAB	Cetyl trimethylammonium bromide
µ	Micro
NaCl	Sodium chloride
Tris	Tris(hydroxymethyl)aminomethane
EDTA	Ethylenediaminetetra acetic acid
PVP	Polyvinylpyrrolidone
rpm	Rotations per minute
mM	Milimolar
M	Molar
TE	Tris-EDTA
HCl	Hydrochloric acid
UV	Ultraviolet
nm	nanometer
$OD_{260/280}$	Optical density ratio of UV light absorbed by nucleic acids at a wavelength of 260 nm vs. 280nm

$MgCl_2$	Magnesium chloride
KCl	Potassium chloride
dNTP	deoxynucleotriphosphate
TBE	Tris/Borate/EDTA
NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment Search Tool
BAC	Bacterial Artificial Chromosome
Ubls	Ubiquitin-like proteins
SUMO	Small ubiquitin-like modifier
NEDD8	Neuronal-precursor-cell-expressed developmentally downregulated protein-8
ATP	Adenosine triphosphate
Aux	Auxin
IAA	Indole-3-acetic acid
SCF	Skp1p, Cdc53p/cullin, and F-box protein
UPL	Ubiquitin protein ligase
UFM	Ubiquitin-fold modifier
CNSs	Conserved non-coding sequences
WGD	Whole genome duplication
GTP	Guadenosine triphosphate
RDA	Representational difference analysis
QTL	Quantitative traits loci
MAS	Marker-assisted selection
FISH	Fluorescence *in situ* hybridization

CHAPTER 1

INTRODUCTION

1.0 INTRODUCTION

1.1 Introduction

Bananas are herbaceous plants with tall and fairly sturdy pseudostems. These pseudostems would produce inflorescence which would eventually develop into banana fruits. Bananas can be found growing in more than 100 countries in the tropical and sub-tropical regions; having a wide range of varieties including both cooking and dessert types (Escalant and Panis, 2002). Sterility and polyploidy affect breeding technology development, thus tissue culture and molecular biology techniques are the way to go (Escalant *et al.*, 1994).

DNA segments characterizing genomic level differences that may not correlate with phenotypic traits are molecular markers. These markers are stable and detectable in tissues irrespective of growth, differentiation, development, or defense status of the cell and are unaffected by the environment, pleiotropic and epistatic effects (Agarwal *et al*., 2008). They can be made up of biochemical constituents such as secondary metabolites and macro molecules like proteins and DNA (Jonah *et al*., 2011).

In bananas, dwarfism occurs in high frequencies and is only able to be detected in the late stages of development (Ramage *et al*., 2004). Some of the ways to overcome dwarfism are using lower concentrations of cytokinin BA, choosing auxiliary shoot over adventitious shoots for multiplication, and limiting the number of multiplication cycles from explants (Ramage *et al*., 2004). Although the incidences of dwarfism have been reduced, the problem was never eliminated. Implementing early identification is difficult as it is only effective at 7 weeks post-deflasking; when the plants reached 18-20cm in height and are ready for field transplantation. These are

only effective if the plants were uniformly grown under vigorous conditions (Ramage *et al*., 2004).

Damasco *et al*. (1996) have used 66 arbitrary RAPD markers to detect dwarf banana off-types in "Cavendish". In their preliminary examinations, they discovered that 19 of the 66 markers revealed polymorphism between the normal and dwarf plants – a single band was present in the normal but not in the dwarf plants (Suprasanna *et al*., 2008). The RAPD marker producing the single polymorphic band, 1.6kb in size, was identified and upon further investigation revealed partial homology with known chloroplast DNA sequences from approximately 6 plant species (Ramage *et al*., 2004).

The OPJ-04 marker was then regarded as a SCAR marker (Sequence Characterized Amplified Region), able to detect dwarf off-types via PCR (Suprasanna *et al*., 2008). The SCAR marker in itself has a drawback as it could only amplify the product from normal DNA and not dwarf off-types. An additional positive internal PCR control, the 18S rRNA gene of *Musa acuminata* (GeneBank Accession No. U42083.1), amplifying both normal and dwarf off-types is required to avoid false positives (Ramage *et al*., 2004).

1.2 OBJECTIVES

Somaclonal variations during micropropagation, especially dwarf off-types are difficult to identify at an early stage. This research, if applicable, would successfully serve:

i. To determine whether an established SCAR marker can be applied to detect the dwarf trait in *Musa acuminata* cv. "Berangan"

ii. To apply the SCAR markers to detect and identify micropropaged off-types of *Musa acuminata* cv. "Berangan" culture

CHAPTER 2

LITERATURE REVIEW

2.0 LITERATURE REVIEW

2.1 Bananas

Musa paradisiaca Linn. was the first scientifically published term given to bananas by Karl Linnaeus in his book "Species Plantarum" in 1753 (Valmayor *et al*., 2000). Bananas are herbaceous plants with tall and fairly sturdy pseudostems. These pseudostems would produce inflorescence which would eventually develop into banana fruits. Bananas can be found growing in more than 100 countries in the tropical and sub-tropical regions; having a wide range of varieties including both cooking and dessert types (Escalant and Panis, 2002).

Bananas are monoecious plants with male flowers at the tip of their inflorescence and female flowers at the back. The fruit is a product of parthenocarpy and characterized as berry with a leathery outer peel containing much collenchyma (Daniells et al., 2001). Banana fruits are formed in layers called combs or hands, consisting of 10-20 bananas with 6-15 combs per stalk; weighing up to 40-50 kilograms per stalk (Arvanitoyannis *et al*., 2008). Unripened bananas and plantain fruits have high starch and low sugar levels with high amounts of bitter-tasting latex. As the fruit ripens, the starch is converted to sugar, eventually containing up to 25% of total sugars, and the latex decomposed. Bananas are harvested unripe and green as they ripen quickly; ehtylene produced by ripe fruits also encourages the ripening of the greener fruits (Arvanitoyannis *et al*., 2008).

2.2 The genus *Musa* and its classification

The genus *Musa* has been classified into four sections, with which includes seeded (wild) and non-seeded or parthenocarpic edible types (Ortiz, 1997). *Callimusa* and *Australimusa* contain species with chromosome number of 2n=2x=20 while species in the *Eumusa* and *Rhodochlamys* have a basic chromosome number of n=11 (Arvanitoyannis *et al*., 2008). Bananas and plantains belong to the *Eumusa* section of the genus *Musa*, family Musaceae, and order Zingiberales (Gill, 1988). The *Eumusa* is the most widely spread in the world and is found throughout South East Asia. Simmonds and Shepherd in 1955 have proposed genomic groups to classify the edible clones: AA, BB, AAA, AAB, ABB, AAAA, and ABBB (Arvanitoyannis *et al*., 2008). A majority of cultivars are a derivation of *Musa acuminata* (AA) and *Musa balbisiana* (BB), with *Musa acuminata* being the most widespread among the *Eumusa* species (Osuji *et al*., 1997; Arvanitoyannis *et al*., 2008).

2.3 Banana classification in Southeast Asia

Triploid (AAA) banana cultivars originated from diploids, from crosses between edible diploids and wild *M. acuminata* subspecies (Arvanitoyannis *et al*., 2008). The triploid cultivars have replaced the original diploids (AA) in most part of Southeast Asia. Via natural hybridization of diploid and triploid *M. acuminata* cultivars with *M. balbisiana*, native to areas like India, Myanmar, Thailand, and the Philippines, hybrid progenies of (AB), (AAB), and (ABB) were formed (Daniells & Smith, 1991). Asia, being the center of *Musa* diversity has a variety of local cultivars possessing characteristics that goes beyond the normal specifications used to differentiate bananas from plantains (Valmayor *et al*., 2000). Besides that, the same cultivars could be known by different names in different countries.

Since bananas are made up of two natural species and a hybrid complex, a three tier system based on species, genome group, and cultivar has been developed to classify and identify cultivars synonymous to the region. The expression of 15 characters such as pseudostem color, ovules, bract apex, and assigning scores for these characteristics according to wild *acuminata* or *balbisiana*, identification of species and genomic groups were possible. Once this has been achieved, highly discriminating descriptors such as on plant stature, pseudostem and leaf characteristics, and so forth were referred to "Descriptors for Banana (Musa spp.) and Musa Germplasm Information System (MGIS)" published by INIBAP/IPGRI and CIRAD to further ascertain the banana varieties (Valmayor *et al*., 2000).

2.4 Status of banana industry in Malaysia

In the Third National Agricultural Policy (1998-2010), bananas have been selected as one of fifteen fruit types for prioritized commercial cultivation (Nik Mohd. Masdek, 2002). From 1992-2001, banana acreage has stabilized to about 31,000 hectares, about 10-12% of total fruit acreage in Malaysia. Major producers are Johor, Pahang, and Sarawak consisting mostly smallholders - grown as mono-crop, and mixed or inter-cropped with perennial industrial crops (Mokhtarud-din and William, 2011).

About half of the 31,000 hectares were of Berangan and Cavendish cultivars for local consumption and exportation while the other cultivars such as the Mas, Rastali, Nangka, Raja, Awak, Abu, and Tanduk were mainly cultivated for the domestic market (Nik Mohd. Masdek, 2002). Dessert cultivars include the Berangan, Mas, Cavendish, and Rastali while Nangka, Raja, Lang, Relong, Tanduk, Nipah, and Awak are examples of cooking bananas. Banana production in 2009 was 279,762

metric ton with an average yield of 10.2 metric ton per hectare (Mokhtarud-din and William, 2011).

In this study, the Berangan cultivar was used. The Berangan has fruit bunches weighing about 12-22 kilograms, with about 8-12 hands and each hand bearing about 12-20 fruits or fingers (Perak Agricultural Department, 2010). *Musa acuminata* cv. "Berangan" is generally a tall variant. Support brackets made out of wood or bamboo needed to be used in order to prevent the plant from falling over and to prevent the pseudostem from breakage due to the weight of the fruit bunches (Perak Agricultural Department, 2010). Hence, a shorter variant of the plant would be more cost-efficient and offer an easier way of retrieving the fruit bunches.

2.5 Banana micropropagation and plant growth regulators

Tissue culture techniques range from recombinant DNA methods, genome characterization, gene transfer techniques, aseptic growth of cells, tissues, organs, and *in vitro* regeneration of plants (Brown and Thorpe, 1995). In bananas, propagation was always done via vegetative means due to its reputation of being the most conspicuously sterile crop of the world (Khatri *et al.*, 1997). Conventional propagation methods were initially used but due to it being laborious, time consuming, and having a low multiplication rate (4-5 plants per year from a single sucker), a more modern approach were developed for rapid propagation (Khatri *et al.*, 1997). Micropropagation produces plantlets that establish faster, healthier, stronger, with shorter production cycles and produces higher yields than conventionally produced plantlets - making banana the most intensely micropropagated crop (Darvari *et al.*, 2010).

Plant hormones or growth regulators are small, diverse, non-protein molecules that control aspects of plant growth and development (Hay *et al*., 2004). There are five classes of plant growth regulators: auxins, cytokinins, gibberellins, abscisic acid, and ethylene (Slater *et al*., 2008). Auxins promote cell division and cell growth and includes indole-3-acetic acid (IAA) and 2,4-Dichlorophenoxyacetic acid (2,4-D). Cytokinins promote cell division in plants and comprise of zeatin, N^6-(2-isopentyl)adenine (2iP), and 6-benzylaminopurine (BAP). However, zeatin and 2iP are not commonly used as they are not cost-efficient and are fairly unstable (Slater *et al*., 2008). GA_3 is the most common gibberellin, involved in regulating cell elongation while abscisic acid (ABA) inhibits cell division and is usually used to promote somatic embryogenesis. Finally, ethylene is a naturally occurring gas associated with fruit ripening and is not typically used in plant tissue culture (Slater *et al*., 2008). Cytokinins are normally used for banana micropropagation. Shoot proliferation rates are affected by cytokinin types, its concentration and the type of banana cultivars. Benzylaminopurine (BAP) is the most commonly used cytokinin (Shirani *et al*., 2009).

In 2007, Venkatachalam *et al*. micropropagated dessert banana cv. "Nanjanagudu Rasabale" (AAB) and tested various concentrations of BA or kinetin (0-10mgL^{-1}) in M2 media. After three subcultures, morphological observations were recorded and the shoots were taken for molecular analyses. They found that the number of shoot buds increase with increasing BAP concentration up to 5mgL^{-1}. However, at higher concentrations, shoot development was suppressed. At BAP concentrations of 6-10mgL^{-1}, the numbers and lengths of shoot buds occurred and exudation of phenolics were observed. A BAP concentration of 5mgL^{-1} was considered optimum for shoot multiplication. However, ISSR and RAPD analyses revealed that in banana cv. "Nanjanagudu Rasabale" (AAB), high concentrations of

plant growth regulators did not induce somaclonal variations (Venkatachalam *et al*., 2007).

A study done by Shirani *et al*. (2009) on micropropagated banana cultivars "Berangan Intan" (AAA), "Berangan" (AAA), "Rastali" (AAB), and plantain cultivars "Baka Baling" (AAB) and "Nangka" (AAB), has shown that although the number of shoots increased with increasing BAP concentrations, it also caused a higher abnormality index; shoot regeneration was also decreased. At 7.5mgL^{-1} of BAP treatment, Bairu *et al*. (2006) noticed a variation rate of 72% at the 10th multiplication cycle; indicating that somaclonal variation increased with an increase in multiplication cycle and BAP concentration.

2.6 Somaclonal variation in bananas

As a highly economically important crop, tissue culture would be able to increase production exponentially. Shoot tips were often used for *in vitro* culture of bananas which sometimes resulted in genetic defects known as somaclonal variants (Bairu *et al*., 2006). In 1981, Larkin and Scowcroft defined somaclonal variation as variation originating in cell and tissue cultures. A universally accepted term, it can also be known as protoclonal, gametoclonal, and mericlonal variation have been used to describe variants from protoplast, anther, and meristem cultures (Bairu *et al*., 2011). There are many factors contributing to somaclonal variations such as culture medium composition, rate of multiplication, primary explant origin, formation of adventitious shoots, increased culture duration, and certain banana genotypes (Bairu *et al*., 2006).

Detection of variations in micropropagated bananas are laborious, time consuming, and expensive as it is done visually and is only possible 3-4 months after field establishment. Although early detection is probable, it is also laborious as it

requires individual inspection and an optimal and uniform growth condition for all plants (Bairu *et al*., 2006). Damasco *et al*. have tried using GA_3 to detect dwarf off-types; however, 5-10% misclassification still occurred even under the most stringent screenings (Bairu *et al*., 2006). Thus the need for efficient and reliable methods of detection is critical and a molecular alternative could prove useful.

2.7 Dwarfism in bananas

Dwarfism occurs in high frequency in bananas and can only be detected in later stages of development (Ramage *et al*., 2004). Alternative cultural management practices to overcome dwarfism were proposed such as using lower concentrations of cytokinin BAP, selection of auxiliary shoots over adventitious shoots for multiplication, and limiting the number of multiplication cycles from any explants (Ramage *et al*., 2004). These have reduced the occurrence of dwarfism but the problem was never eliminated. Early identification is difficult and effective at 7 weeks post-deflasking when the plants reach 18-20cm in height and ready for field implantation. However, this is only effective if the plants were grown under uniform vigorous growing conditions (Ramage *et al*., 2004).

In 1996, Damasco *et al*. used 66 arbitrary RAPD markers to detect dwarf banana off-types. In the early screening, they discovered that 19 of the markers revealed polymorphism between normal and dwarf plants with the presence of a single band in the normal, but not in the dwarf types (Suprasanna *et al*., 2008). A single polymorphic 1.6kb RAPD marker was identified and further investigation revealed partial homology with known chloroplast DNA sequences from at least six plant species (Ramage *et al*., 2004).

This marker (OPJ-04) was consequently characterized into a SCAR marker (Sequence Characterized Amplified Region) to be used as a PCR based detection system for dwarf off-types (Suprasanna *et al*., 2008). However, the SCAR marker by itself has a limitation as it only amplifies the product from the DNA of normal and not dwarf off-types. The inclusion of a positive internal PCR control (18S rRNA gene of *Musa acuminata*; GeneBank Accession No. U42083.1) amplifying regions of both normal and dwarf off-type is required (Ramage *et al*., 2004).

2.8 Molecular markers

Markers can be defined as any trait of an organism that can be identified and followed in a mapping population and these can be associated with economically important traits under the control of polygenes (Bhat *et al*., 2010). Genetic markers are categorized into three broad classes: visually assessable traits (morphological and agronomic traits), based on gene products (biochemical markers), and those that rely on DNA assays (molecular markers) (Semagn *et al*., 2006). Morphological markers can be detected visually or a difference in physical or chemical properties of the macromolecules (Bhat *et al*., 2010).

Genetic markers can be further characterized into two types: morphological markers and non-morphological markers. Morphological markers are traits that can be visualized with the naked eye. These include plant height, disease response, fruits or seeds, and so forth. Although morphological markers are easily distinguished, effects of linked minor genes are often overlooked (Bhat *et al*., 2010). Morphological characteristics vary with environmental situations and plants would need to grow to full maturity to accurately identify their discriminating traits (Jonah *et al*., 2011).

Non-morphological markers are also known as molecular markers. Breeding programs have often relied on phenotypic or morphological traits. With the rise of new molecular techniques, molecular markers were introduced within the last two decades and have become an important tool in genetic crop improvement (Bhat *et al*., 2010). Molecular markers are DNA segments that represent differences at a genomic level and may not correlate with the phenotypic traits. However, they are stable and detectable in tissues regardless of growth, differentiation, development, or defense status of the cell and are not affected by the environment, pleiotropic and epistatic effects (Agarwal *et al*., 2008). Molecular markers can consist of biochemical constituents such as secondary metabolites and macromolecules like proteins and DNA (Jonah *et al*., 2011).

Protein based markers are some examples of biochemical markers. Proteins are primary products of structural genes, thus a change in the coding base sequence will result in changes to the primary structure of proteins. The earliest protein based markers to be used was isozyme which is an enzyme exhibiting the same catalytic activity but differ in charge and electrophoretic mobility (Bhat *et al*., 2010). Plant extracts are subjected to electrophoresis using starch or polyacrylamide gels, and the enzymes of interest are detected by treating the gels with specific activity stains. Inconsistencies among the bending patterns between individual samples were then able to be acknowledged and sorted out (Bhat *et al*., 2010).

Genes are organized in a linear order on chromosomes and if they are linked together, a combination of genes can be inherited in a group. Individual genes flanking within a defined close interval are known as molecular DNA markers. Identifiable DNA sequences found at specific locations of the genome and are associated with the inheritance of a trait or linked gene can be defined as molecular

DNA markers (Jonah *et al*., 2011). Plant DNA markers were first developed in 1985-86 by researchers at native plants incorporated, U.S.A. and Cornell University Ithaca, U.S.A. DNA markers are identifiable sequences found at specific locations on the chromosomes and passed on via inheritance; they should not be considered as normal genes as they typically do not have any biological effect (Bhat *et al*., 2010). A good DNA marker should be highly polymorphic, codominantly inherited and expressed, selectively neutral, easily accessible and assayable, follows Mendelian inheritance, reproducible, allows easy exchange of data between laboratories, genetically linked to a particular trait, and is not affected by pleiotropism and epistatic interactions (Bhat *et al*., 2010; Jonah *et al*., 2011).

However, it is rather impossible to acquire a genetic marker possessing all the aforementioned traits and thus a marker system can be identified by fulfilling a few of those characteristics (Jonah *et al*., 2011).

2.8.1 Non-PCR based genetic markers

Restriction Fragment Length Polymorphism (RFLP) was first developed in the early 1980s by Botstein *et al*. (Jonah *et al*., 2011; Bhat *et al*., 2010). The technique is based on southern blotting hybridization technique. A chemically labeled DNA probe hybridizing to a southern blot of DNA digested by restriction endonucleases would detect polymorphisms, resulting in differential DNA fragment profile (Agarwal *et al*., 2008). It is these variations that are called Restriction Fragment Length Polymorphism. DNA digested by restriction enzymes would produce differing fragment sizes. These fragments would have a higher mobility when electrophoresed on a gel and these fragments are then transferred onto nitrocellulose membranes making the DNA permanently immobilized. Chromosomal DNA fragments prepared

as probes would then be labeled and these probes would hybridize to complementary DNA bands on the membranes; the bands would finally be visualized via autoradiography (Bhat *et al*., 2010).

RFLP markers are highly polymorphic, codominantly inherited, highly reproducible, and are considered superior because of its high heritability and locus specificity in the plant genome (Agarwal *et al*., 2008). However, this technique does have its share of limitations. RFLP requires high quantity and quality DNA, results depend on the development of specific probe libraries for the species, time consuming, usually requiring radioactively labeled probes, and this technique is not able to be automated (Semagn *et al*., 2006). It is with these limitations that a new, less technically complex methods such as the PCR-based techniques was realized.

2.8.2 PCR-based genetic markers

The discovery of polymerase chain reaction (PCR) by Mullis *et al*. in 1985 has provided a technological breakthrough that enabled the development of various PCR-based techniques genetic marker techniques. PCR involves two oligonucleotide primers flanking the DNA fragment of interest, and through a series of repeated cycles of heat denaturation of the DNA, primer annealing to their complimentary sequences, and the extension of the annealed primers via thermophilic DNA polymerase, that fragment could then be continually synthesized and thus amplified exponentially (Jonah *et al*., 2011).

PCR-based genetic markers consist of Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSRs), Inter Simple Sequence Repeat (ISSR), Sequence Characterized Amplified Regions (SCAR), and Amplified Restriction Fragment Length Polymorphism (AFLP).

2.8.2.1 Random Amplified Polymorphic DNA (RAPD)

RAPD was developed by William *et al*. in 1990 (Bhal *et al*., 2010). A single arbitrary nucleotide primer sequence of mostly ten bases long, called a decamer, is used for amplification. These amplified DNA fragments are randomly selected thus providing random samples of DNA markers (Jonah *et al*., 2011; Bhat *et al*., 2010). This technique does not require any prior knowledge of the analyzed genome and thus can be used across species using universal primers. However, profiling is dependent on the reaction conditions and it may vary within different laboratories; since several distinct loci are amplified by each primer, heterozygous and homozygous individuals are indistinguishable (Agarwal *et al*., 2008). Moreover, RAPD polymorphisms are inherited as dominant or recessive - causing a loss of information relative to markers showing co-dominance, and since the primers are short, a mismatch of a single nucleotide can prevent annealing thus losing a band (Jonah *et al*., 2011).

Despite its limitations, RAPD analyses do not require technically complex or laborious methods such as blotting or hybridization, radioactive assays and species specific probe libraries. The small amount of DNA used also makes it possible for populations that are inaccessible via RFLP. Additionally, RAPD analyses are fast and efficient (Jonah *et al*., 2011).

2.8.2.2 Simple Sequence Repeats (SSR)

Simple Sequence Repeats (SSR) is also known as short tandem repeats or microsatellites (Jonah *et al*., 2011). These microsatellites are present in all eukaryotic genomes, making them ideal DNA markers for genetic mapping and population studies (Bhat *et al*., 2010). Variations in the number of tandem repeat units are largely due to strand slippage during DNA replication where these repeats allow matching via

excision or addition of repeats. Since slippage in replication is possibly due to point mutations, microsatellite loci are hypervariable and thus able to show extensive inter-individual length polymorphisms during PCR analyses of exclusive loci using specific primer sets (Agarwal *et al*., 2008).

SSR primers are developed by cloning random DNA segments from the target species. These are then inserted into a cloning vector for replication. The colonies are developed, screened, and the DNA is sequenced and PCR primers are constructed from sequences flanking regions determining a specific locus. Microsatellite repeats must be predicted and randomly isolated primers may not display polymorphism (Semagn *et al*., 2006). Choosing the best candidate markers and optimizing its conditions is a way to obtain a balance of high specificity and high intensity of the amplified products.

With that in mind, it can be seen that it is costly to produce such primers as a lot of rather complex and laborious methods have to be employed. However, these markers are both dominant fingerprinting markers and codominant sequence tagged microsatellites (STMs) makers. These markers are also highly reproducible and polymorphic (Jonah *et al*., 2011; Bhat *et al*., 2010).

2.8.2.3 Inter Simple Sequence Repeats (ISSR)

Inter Simple Sequence Repeats (ISSRs) are semi arbitrary markers amplified by PCR in the presence of a complementing primer to a target microsatellite (Bhat *et al*., 2010). The primers used in ISSR are microsatellites targeting multiple genomic loci amplifying inter simple sequence repeats of different sizes. These primers can be either anchored or unanchored at the 3' or 5' end with 1 to 4 degenerate based extended into the flanking sequences (Semagn *et al*., 2006).

Unanchored primers are called microsatellite-primed PCR, or MP-PCR (Bhat *et al*., 2010). ISSR amplifications do not require genome sequence information - multilocus and highly polymorphic patterns would arise. Each of those bands would match up to a DNA sequence delimited by two inverted microsatellites (Bhat *et al*., 2010). ISSRs are fast and easy but its reproducibility, dominant inheritance, and homology of co-migrating amplified products limits its use (Semagn *et al*., 2006).

2.8.2.4 Amplified Restriction Fragment Length Polymorphism (AFLP)

AFLP is capable of "genome representation" which is a simultaneous screening of DNA regions distributed at random throughout the genome (Semagn *et al*., 2006). AFLP involves three steps: DNA is first subjected to restriction enzymes and oligonucleotide adapters are ligated to them; sites of restriction fragments are selectively amplified; and finally the fragments are analyzed via gel electrophoresis. A rare restriction endonuclease such as Eco*RI* or Pst-*I* is normally used as those enzymes reduce the number of DNA fragments to be amplified, while a common endonuclease would be used to generate small DNA fragments to be used as amplification primers (Bhat *et al*., 2010). At first the DNA fragment is amplified by the adapter ligated smaller fragments, and then these amplified products are later used as a template for the subsequent PCR amplification. The products were then analyzed via gel electrophoresis and visualized by silver staining; radioactive probes or fluorescent dyes can also be used (Bhat *et al*., 2010). In this manner, AFLP somewhat combines techniques of RFLP with the use of restriction enzymes and RAPD via PCR amplification (Jonah *et al*., 2011).

Prior knowledge of DNA sequence isn't necessary as this technique would produce fingerprints of any DNA irrespective of its source (Agarwal *et al*., 2008).

AFLP is a sensitive technique and with the addition of fluorescent probes, it can also be automated. The advantages of AFLP are: polymorphism can be easily generated in huge amounts, no sequence information is required, and PCR is fast and with a high multiplex ratio (Jonah *et al*., 2011).

2.8.2.5 Sequence Characterized Amplified Regions (SCAR)

SCAR markers are PCR-based markers that characterize genomic DNA fragments at defined loci that are determined using a sequence specific primer (Jonah *et al*., 2011; Agarwal *et al*., 2008). SCAR markers arise from RAPD analyses whereby an arbitrary marker is later defined as being able to identify a certain trait. The RAPD marker associated terminals are sequenced and long primers are designed for the specific locus. Amplification is followed by gel electrophoresis for locus specific band detection (Bhat *et al*., 2010).

Polymorphism is identified either with a presence or absence of the amplified band, or it could appear as length polymorphisms convert dominant arbitrary primed marker loci into codominant SCAR marker (Agarwal *et al*., 2008). SCAR markers can be used as physical maps in the genome or as genetic markers. The use of codominant SCAR markers would provide information for genetic mapping than dominant arbitrary-primed markers as these SCAR markers can be used to screen genomic libraries for physical mapping, to define locus specificity, and as a comparative mapping for homology studies among related plants species (Agarwal *et al*., 2008).

CHAPTER 3

MATERIALS AND METHODS

3.0 MATERIALS AND METHODS

3.1 Micropropagation of banana cultures

3.1.1 Plant starter cultures

Banana starter cultures, *Musa acuminata* cv. Berangan (AAA), were obtained from the Plant Biotechnology Incubator Unit (PBIU), Center for Research in Biotechnology for Agriculture (CEBAR), University of Malaya. Shoot meristems were excised and transferred onto Murashige and Skoog (MS) media (1962), containing $30gL^{-1}$ sucrose and $2gL^{-1}$ gelrite supplemented with benzylaminopurine (BAP) at $3mgL^{-1}$. The media pH was adjusted to 5.7 and autoclaved at 14.5psi, 120°C for 20 minutes. The cultures were maintained twice; 2 weeks interval each at 26°C under a 24-hour photoperiod.

3.1.2 Maintenance on concentration gradient of benzylaminopurine (BAP).

The micropropagated cultures were excised and transferred onto Murashige and Skoog (MS) media (1962), containing $30gL^{-1}$ sucrose and $2gL^{-1}$ gelrite supplemented with benzylaminopurine (BAP) at concentration gradients of 3, 6, 9, and $12mgL^{-1}$. Shoots were also grown on MS media with no BAP ($0mgL^{-1}$) as a control. Media pH was adjusted to 5.7 before being autoclaved at 14.5psi, 120°C for 20 minutes. The cultures were incubated at 26°C under a 24-hour photoperiod. A total of 120 micropropagated cultures were grown, in triplicates of 10 explants per concentration. Morphological observations in regenerated shoots were recorded and the lengths of time required for regeneration were noted.

3.2 Molecular approach

3.2.1 Genomic DNA extraction

After being maintained for three subcultures at 2 weeks interval each subculture, shoots and young leaf samples were collected and stored at -80°C until further use. Total genomic DNA extraction was done according to a modified CTAB method as describes by Doyle and Doyle (1990). About 100-200mg of the plant samples were macerated using mortar and pestle in addition with liquid nitrogen into a fine powder. The powder was then transferred into microfuge tubes with 500µL of pre-warmed CTAB buffer (2% (w/v) hexadecyltrimethylammonium bromide, 1.4mM NaCl, 100mM Tris (pH 8.0), 20mM EDTA, and 1% (w/v) PVP) and incubated at 65°C for about an hour. 1% (v/v) of β-Mercaptoethanol (Sigma-Aldrich, Germany) was added into the mixture and the tubes were mixed every 10-15 minutes.

The microfuge tubes were then centrifuged at 13,000rpm for 5 minutes to spin down cell debris. After centrifugation, 250µL of chloroform:isoamyl alcohol (24:1; Merck, Germany:Amresco, Ohio) were added into the tubes and were mixed by inversion. The tubes were then spun at 13,000rpm for 1 minute. The upper layers of the supernatants were transferred into clean microfuge tubes. Into the tubes, 50µL of 7.5M ammonium acetate were added, followed by 500µL of ice cold absolute ethanol.

The tubes were inverted to mix the solution and precipitate the DNA. After precipitation, the tubes were centrifuged at 13,000rpm for 1 minute to form a pellet. The supernatant was removed and the DNA pellets were washed with two changes of ice cold 70% ethanol. DNA pellets were allowed to dry for about 15 minutes; the pellets resuspended in 50µL of TE buffer (10mM Tris-HCl, 0.1mM EDTA), and 2µL of RNase A (10µg/mL; Amresco, Ohio) was added. After resuspension, DNA was kept at -20°C until further use.

Some of the DNA extractions were also carried out using DNeasy Plant Mini Kit (QIAGEN, Germany) as a means of comparison. The extraction was done according to the supplied manual.

3.2.2 DNA quality and quantity confirmation

Extracted DNA was diluted to 100X by mixing 990µL of TE buffer and 10µL of the DNA suspension. The mixtures were inserted into cuvettes (Eppendorf, Germany) and measured with a biophotometer (Eppendorf, Germany). DNA purity was determined by the ratio of wavelength absorbances at 260nm/280nm ($OD_{260/280}$). A ratio between 1.8 and 2.0 denotes pure DNA. A manual calculation can also be done by using the following equation to confirm the quantity of the DNA:

$$\text{DNA concentration } \left(\frac{\mu g}{mL}\right) = \frac{OD_{260} \times 50 \frac{\mu g}{mL} \times \text{Dilution factor}}{1000}$$

3.2.3 SCAR investigation in a multiplex PCR

Dwarf specific primers were synthesized based on Damasco *et al.*, (1996) and as highlighted by Ramage *et al.* (2004), and Suprasanna *et al.* (2008). The primers were: Dw1 (5' CTG TGG TTG CAT TCT CAT AC 3') and Dw2 (5' GTG AAT CAT ACT CGC GAA CC 3'). The SCAR primers were used together with two primers amplifying a 500bp region of the 18S rRNA gene of *Musa acuminata* (GeneBank Accession No. U42083.1) in a multiplex PCR. This is to ensure that the absence of a band was a result of the dwarf condition and not from a failure in the PCR (Ramage *et al*., 2004).

DNA amplifications were executed in volumes of 20µL with 1X PCR buffer (10mM Tris-HCl, 4.5mM $MgCl_2$, 50mM KCl, pH 8.3), 0.25mM dNTPs, 500nM of Dw1/Dw2 SCAR primers, 125nM 18S rRNA primers, and 3 units of *Taq* DNA

polymerase. The PCR reactions were performed with an initial denaturation of 94°C for 3 minutes, followed by 30 cycles of each of the following: denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 2 minutes, and a final extension at 72°C for 3 minutes.

3.2.4 Agarose gel electrophoresis

PCR products were separated by gel electrophoresis on a 1.5% (w/v) agarose gels (0.6g agarose; Amresco, Ohio in 30mL 1X TBE buffer; 89mM Tris base, 89mM boric acid, and 2mM EDTA pH8.0). 1µL of ethidium bromide (10mg/mL) was added into the melted gels before being poured into the gel casting apparatus and left to solidify.

Solidified gels were placed in an electrophoresis chamber filled with sufficient volumes of 1X TBE to cover the entire surface of the gel. Samples were mixed with 6X loading buffer (Fermentas, U.S.A.) before being loaded into the gel wells. Electrophoresis was done at 75 volts for about 30 minutes or until the dye has almost reached the end of the gel. A gel documentation system, (Geldoc AlphaImager TM2200; Alpha Innotech, U.S.A.) was used to analyze the gel under UV illumination.

CHAPTER 4

RESULTS

4.0 RESULTS

4.1 Micropropagation of banana cultures

4.1.1 Maintenance and propagation of plant samples

Excised shoot meristems from the starter cultures grew within 5 days of sub-culture (*Figure 1*). By 14 days, the cultures grew multiple shoots (*Figure 2*) and these shoots were further excised and re-cultured again before being subjected to concentration gradients of benzylaminopurine (BAP).

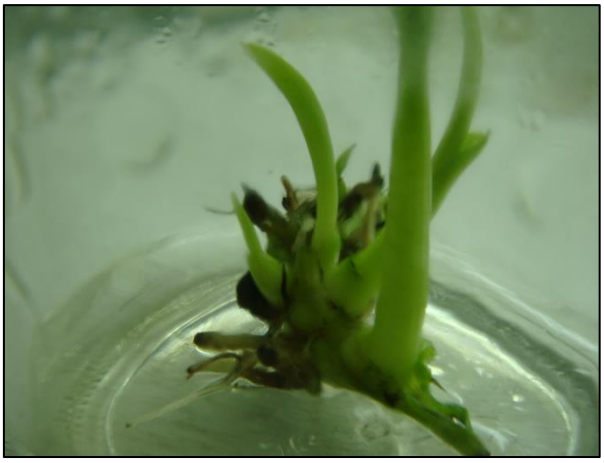


Figure 1: 5-day old plant culture.

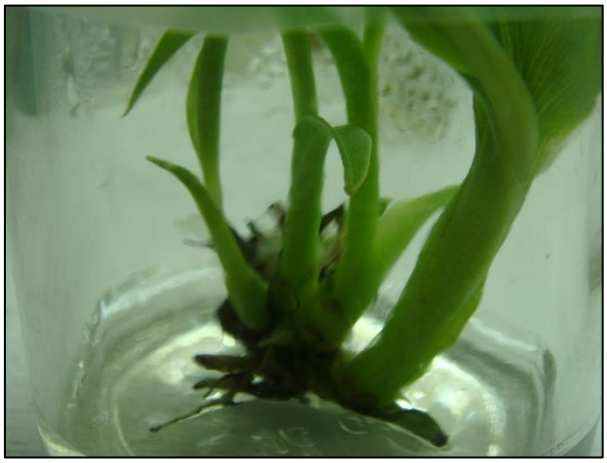


Figure 2: 14-day old plant culture.

4.1.2 Effects of benzylaminopurine (BAP) on shoot cultures

Shoot meristems maintained for 2 weeks were excised and grown on MS media supplemented with a concentration gradient of benzylaminopurine (BAP): 3, 6, 9, and 12mgL^{-1}. The shoot meristems were also grown on MS media with no BAP (0mgL^{-1}) as a control. However, explants grown on 0mgL^{-1} did not regenerate and thus 3mgL^{-1} BAP was used as the controlled condition. After three subcultures at two week intervals for each subculture, shoots and roots started to regenerate from the shoot meristems of the source cultures (*Table 1*).

Table 1: Percentage of regenerated explants based on their morphologies.

Concentration of BAP (mgL^{-1})	**Morphology of regenerants (%)**	
	Normal	**Stunted**
3	100.0	0
6	100.0	0
9	93.3	6.7
12	60.0	40.0

Explants grown on 3mgL^{-1} BAP took 5 days to regenerate shoots; as do the explants on 6mgL^{-1} BAP. At 9mgL^{-1} of BAP, the explants took an average of 7-10 days to regenerate and produce shoots. On 12mgL^{-1} BAP, regeneration was the longest at about 14-21 days. Cultures grown on BAP concentrations of 3 and 6mgL^{-1} did not show any signs of morphological abnormalities. As compared with the cultures with the higher BAP concentrations, these cultures grew at a much faster rate. However, cultures grown on 9 and 12mgL^{-1} BAP required a much longer time for regeneration than the ones with lower concentrations.

Cultures grown on 9mgL^{-1} BAP started to show some signs of stress such as browning of the leaves and stems, hyperhydricity, and having abnormal serrated leaves. On 12mgL^{-1} BAP, cultures took a much longer time to regenerate. More cultures did not completely regenerate and remained stunted, having abnormal shoot clusters and remained in an undifferentiated callus state. For this study, the abnormal shoot clusters and undifferentiated calli are considered stunted. Normal regenerants are plantlets with elongated, healthy leaves and roots (*Figure 3*).

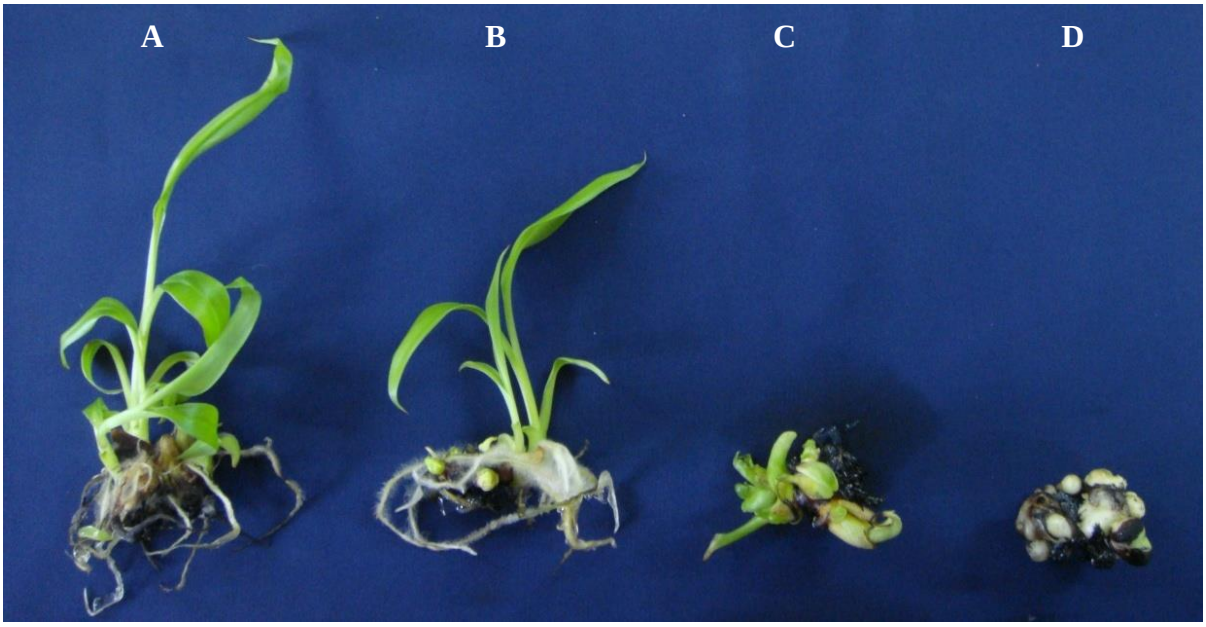


Figure 3: Morphologies of the micropropagated cultures; A and B: normal regenerants, C: abnormal shoot clusters, and D: undifferentiated callus.

After 2 months of culture, explants subjected to 3 and 6mgL^{-1} of BAP, produced only normal regenerants (*Figure 4*). As the BAP concentration increased to 9mgL^{-1}, stunted regenerants began to emerge and increases as it reached 12mgL^{-1}.

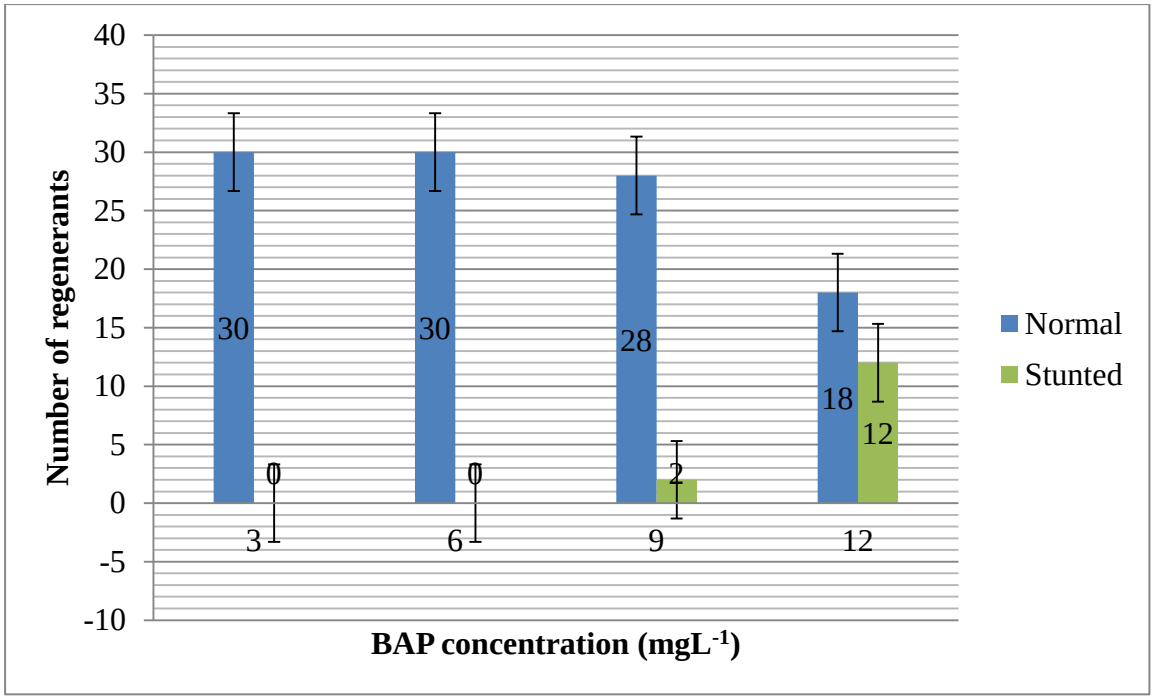


Figure 4: Number of regenerants in media of varying concentrations of BAP after 2 months of culture.

4.2 Molecular genetic studies

4.2.1 Genomic DNA extraction

4.2.1.1 A comparison between conventional extraction method and a commercial extraction kit

To compare the efficacy of banana DNA extraction between the conventional CTAB method and commercial extraction kit (DNeasy Plant Mini Kit, Qiagen, Germany), 5 samples were used. The results of the comparison are tabulated in *Table 2*. From the results, the CTAB method produced samples with purity range of 1.19-1.81. It can be seen that the CTAB method gave a higher purity score as compared to the samples extracted using the kit, which gave a purity range of 0.80-1.24. DNA concentrations were also higher by using the CTAB method than that of the kit. By using CTAB, the concentrations were between 3.43-8.93µgmL^{-1}. A concentration range of 0.5-3.23µgmL^{-1} was observed from using the kit. Oh *et al*. (2007) have stated that modifications were made during DNA extractions of "Cachaco", "Figue Rose",

"Prata", and "Cavendish" banana genome using this kit. As per that, the kit was not necessarily suitable to be used straight out of the box for banana DNA extraction.

Table 2: A comparison of DNA extraction between CTAB and DNeasy Plant Mini Kit.

DNA Extraction Method	**Purity ($OD_{260/280}$)**	**Concentration (μgmL^{-1})**
CTAB	1.72	8.80
	1.63	3.90
	1.19	5.73
	1.81	8.93
	1.52	3.43
DNeasy Plant Mini Kit	0.80	0.50
	1.02	1.60
	1.24	3.23
	0.99	1.20
	1.14	2.13

4.2.2.2 Total genomic DNA extraction of micropropagated samples

Results of DNA purity and concentrations were tabulated and presented in *Table 3*. Although an $OD_{260/280}$ of between 1.80 to 2.00 has been widely accepted as pure, Ramage *et al*., (2004) have demonstrated that by using a crude treatment to extract genomic DNA (Alkaline leaf treatment), their SCAR PCR analyses were able to produce results. The only discerning concern was that the plant material must be of young actively growing tissue. In alkaline leaf treatment, the young banana tissues were placed on ice in a tube containing sodium hydroxide supplemented with β-mercaptoethanol, incubated at 94°C and placed on ice again. The mixture was neutralized with hydrochloric acid and Tris-HCl. Finally, the banana tissues were incubated at 94°C, placed on ice and macerated with a pipette tip in potassium hydrochloride. Meenakshi *et al*. (2011) have also stated that SCAR markers are less sensitive to reaction conditions, thus the purity would not have been a concern.

Table 3: DNA purity and concentration of all the extracted plant materials.

	3mgL^{-1}		**6mgL^{-1}**		**9mgL^{-1}**		**12mgL^{-1}**	
	Purity ($OD_{260/280}$)	**Concentration (µgmL^{-1})**	**Purity ($OD_{260/280}$)**	**Concentration (µgmL^{-1})**	**Purity ($OD_{260/280}$)**	**Concentration (µgmL^{-1})**	**Purity ($OD_{260/280}$)**	**Concentration (µgmL^{-1})**
1	1.40	1.33	1.98	2.83	1.28	6.63	1.03	3.50
2	1.91	2.50	1.11	7.80	1.52	0.57	0.80	0.50
3	1.42	1.40	2.06	5.07	1.63	0.63	1.02	1.60
4	1.33	3.50	2.18	3.70	1.34	4.97	1.08	1.83
5	1.27	0.37	1.51	1.77	2.10	2.60	0.99	1.20
6	1.22	0.20	1.30	4.40	1.24	8.57	1.72	8.80
7	1.83	3.40	2.08	4.23	1.16	3.97	1.14	2.13
8	1.12	1.80	2.09	4.63	1.70	5.00	1.74	4.87
9	1.90	2.60	1.69	1.57	1.18	11.67	1.63	3.90
10	1.80	2.63	1.94	1.87	1.68	2.97	1.19	5.73
11	1.61	1.33	1.26	1.77	1.82	4.30	1.60	2.50

Table 3, continued.

	3mgL^{-1}		6mgL^{-1}		9mgL^{-1}		12mgL^{-1}	
	Purity ($OD_{260/280}$)	**Concentration (μgmL^{-1})**	**Purity ($OD_{260/280}$)**	**Concentration (μgmL^{-1})**	**Purity ($OD_{260/280}$)**	**Concentration (μgmL^{-1})**	**Purity ($OD_{260/280}$)**	**Concentration (μgmL^{-1})**
12	1.77	1.70	1.37	2.10	2.91	2.20	1.31	5.20
13	1.31	1.57	2.19	6.45	1.83	2.33	1.18	2.13
14	1.15	1.33	2.11	5.10	1.81	3.07	1.81	8.93
15	1.62	2.77	2.07	4.95	2.00	4.00	1.04	2.93
16	1.30	1.33	2.20	1.70	1.49	3.10	1.73	5.83
17	1.45	0.97	1.92	2.80	1.49	1.23	1.52	3.43
18	1.26	0.70	2.22	4.20	2.44	2.13	1.24	3.23
19	1.92	3.10	2.07	3.75	2.18	3.73	1.78	8.23
20	1.84	3.30	1.92	1.00	2.26	2.90	1.44	3.37
21	1.86	2.60	1.18	3.50	1.82	11.73	1.30	2.27
22	1.82	2.70	1.29	3.80	1.38	12.77	1.16	2.22

Table 3, continued.

	3mgL^{-1}		6mgL^{-1}		9mgL^{-1}		12mgL^{-1}	
	Purity ($OD_{260/280}$)	**Concentration (µgmL^{-1})**	**Purity ($OD_{260/280}$)**	**Concentration (µgmL^{-1})**	**Purity ($OD_{260/280}$)**	**Concentration (µgmL^{-1})**	**Purity ($OD_{260/280}$)**	**Concentration (µgmL^{-1})**
23	1.64	1.30	1.95	1.30	3.60	0.90	1.22	5.56
24	1.79	1.70	2.20	9.40	1.98	8.07	1.64	2.50
25	1.78	2.25	1.32	10.80	1.31	4.63	1.81	9.00
26	1.72	1.15	2.39	4.70	3.18	2.83	1.21	2.20
27	2.07	4.20	1.19	4.00	0.86	12.90	1.54	3.40
28	2.09	4.55	2.17	3.70	2.00	0.80	1.86	0.60
29	1.99	1.85	2.02	2.85	2.04	7.80	2.12	2.60
30	1.95	2.75	1.48	1.67	2.16	4.00	1.92	3.10

4.2.3 Evaluation of dwarf SCAR markers in a multiplex PCR

To test the integrity of the extracted DNA, a PCR with the 18S rRNA internal control was run using samples randomly chosen from the $3mgL^{-1}$, $6mgL^{-1}$, $9mgL^{-1}$, and $12mgL^{-1}$ BAP concentrations. A distinct band of about 500bp in size was detected (*Figure 5*).

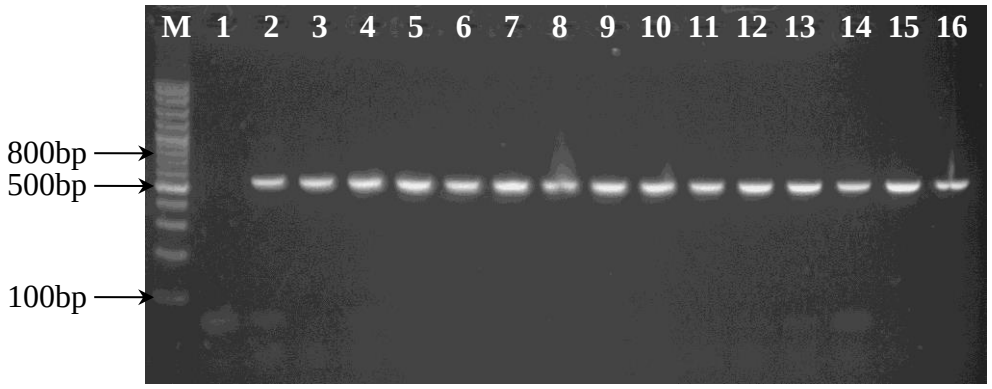


Figure 5: A gel visualization of the PCR using the 18S rRNA primers of the random samples. Lanes from left to right: M, 100bp DNA ladder, 1, negative control, 2-5, $3mgL^{-1}$ samples, 6-9, $6mgL^{-1}$ samples, 10-13, $9mgL^{-1}$ samples, and 14-16, $12mgL^{-1}$ samples.

Preliminary multiplex PCR using the protocols established by Ramage *et al*. (2004), yielded multiple unspecific bands (*Figure 6*). The multiplex PCR was done using DNA samples with the best purity from each of the differing BAP concentration samples. Further optimization by increasing the annealing temperature to 56°C revealed no visible bands other than the positive internal control 18S rRNA at 500bp (*Figure 7*).

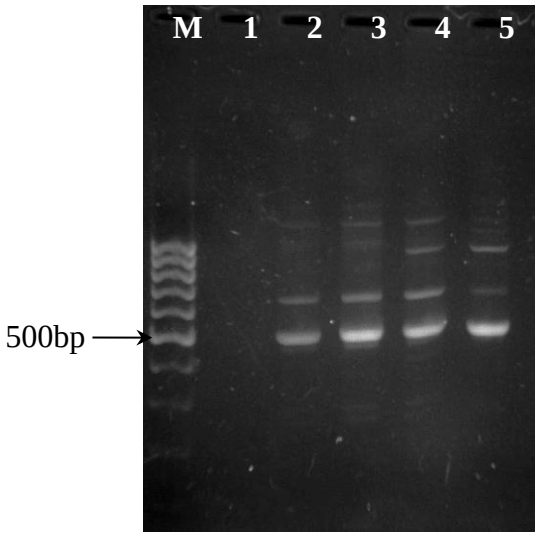


Figure 6: A gel visualization of the preliminary results of the multiplex PCR using the SCAR markers and the positive internal 18S rRNA control. The lanes from left to right: M, 100bp DNA ladder, 1, negative control, 2, 3mgL^{-1} BAP sample, 3, 6mgL^{-1} BAP sample, 4, 9mgL^{-1} BAP sample, and 5, 12mgL^{-1} BAP sample.

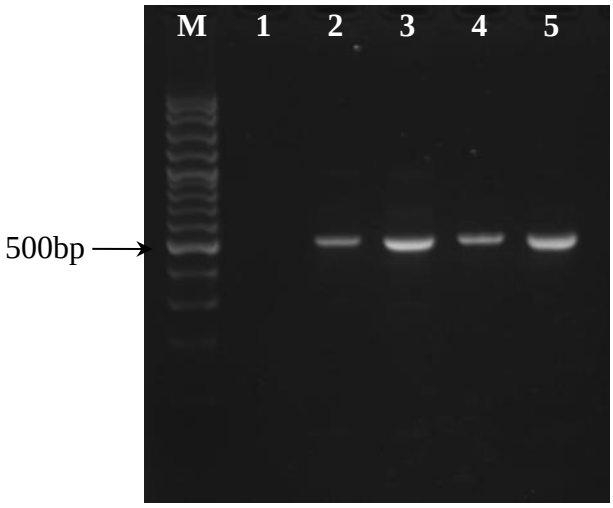


Figure 7: A gel visualization of the second optimization of the multiplex PCR of the SCAR marker and the positive internal 18S rRNA control. The lanes from left to right: M, 100bp DNA ladder, 1, negative control, 2, 3mgL^{-1} BAP sample, 3, 6mgL^{-1} BAP sample, 4, 9mgL^{-1} BAP sample, and 5, 12mgL^{-1} BAP sample.

To further support the notion that DNA purity did not influence the results, a multiplex PCR and SCAR marker PCR were done retaining 56°C as the annealing temperature but increasing the annealing time for 35 seconds. Samples used were of

the 3mgL^{-1} cultures with high and low purities. From the gel visualization, only the 18S rRNA control bands at 500bp were visible (*Figure 8*).

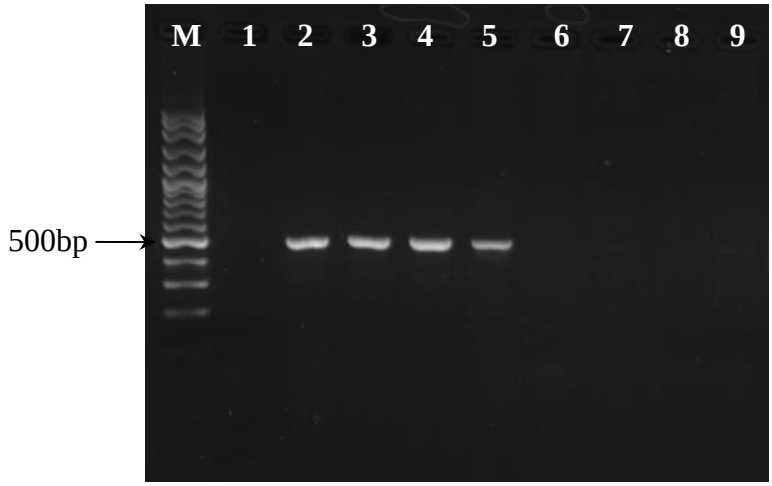


Figure 8: A gel visualization of the multiplex PCR with the SCAR and 18S rRNA positive internal control (Lanes 2-5) and of the SCAR marker only PCR (Lanes 6-9). The lanes from left to right, M, 100bp DNA ladder, 1, negative control, 2, 3mgL^{-1} BAP sample (high purity), 3, 3mgL^{-1} BAP sample (low purity), 4, 3mgL^{-1} BAP sample (high purity), 5, 3mgL^{-1} BAP sample (low purity), 6, 3mgL^{-1} BAP sample (high purity), 7, 3mgL^{-1} BAP sample (low purity), 8, 3mgL^{-1} BAP sample (high purity), and 9, 3mgL^{-1} BAP sample (low purity).

A gradient PCR with only the SCAR markers was then optimized to detect for any mistakes in the synthesized primers. DNA sample with the best purity from the 3mgL^{-1} BAP concentration was used. A distinct band of about 500bp in size was present (*Figure 9*).

Since a single distinct band was present with the SCAR marker primers, another multiplex PCR was done with 53°C as the annealing temperature. A DNA sample for each of the differing BAP concentrations were used in this multiplex PCR. However, the results were unsatisfactory and again, multiple bands were present (*Figure 10).*

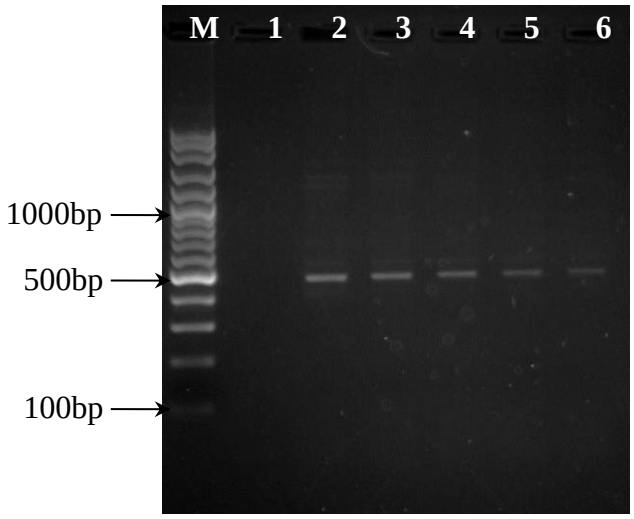


Figure 9: A gel visualization of the gradient PCR of the SCAR marker primers. Annealing temperatures from left to right: M, 100bp DNA ladder, 1, negative control, 2, 50°C, 3, 51°C, 4, 52°C, 5, 53°C, and 6, 54°C.

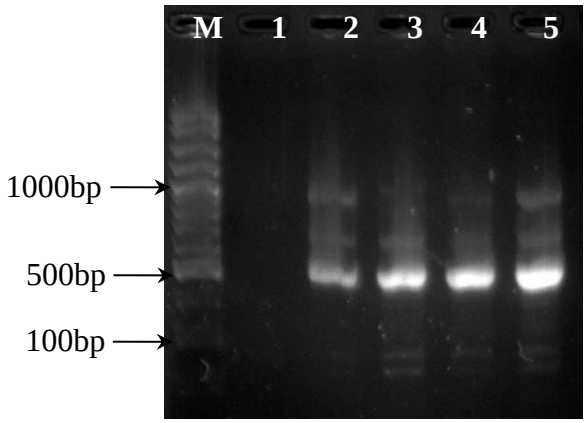


Figure 10: A gel visualization of the multiplex PCR of the SCAR markers and 18S rRNA internal control primers. From left to right: M, 100bp DNA ladder, 1, negative control, 2, 3mgL^{-1} BAP sample, 3, 6mgL^{-1} BAP sample, 4, 9mgL^{-1} BAP sample, and 5, 12mgL^{-1} BAP sample.

Samples from each BAP concentration gradient with varying DNA purities were subject to a SCAR marker primer only PCR with an annealing temperature of 53°C. Bands of about 500bp and 700bp were visible during visualization (*Figure 11*).

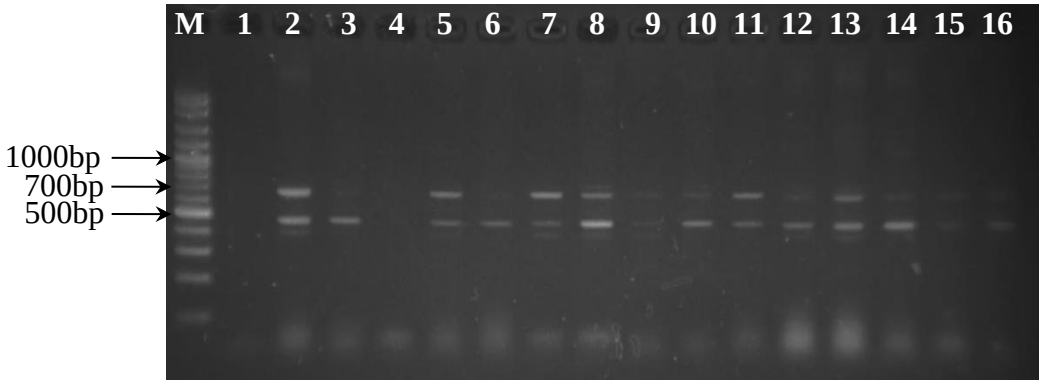


Figure 11: A gel visualization of the SCAR marker primer PCR from samples of each BAP concentration with varying purities. Lanes from left to right: M, 100bp DNA ladder, 1, negative control, 2-4, 12mgL-1 BAP samples (high purity, low purity, high purity), 4-7, 9mgL-1 BAP samples (high purity, low purity, high purity), 8-11, 6mgL-1 BAP samples (high purity, low purity, high purity, low purity), and 12-16, 3mgL-1 BAP samples (high purity, low purity, high purity, low purity, high purity).

All of the DNA samples were then subjected to a PCR of only the SCAR marker primers. The 18S rRNA primers were used as a positive control to the PCR runs. The gels were visualized under UV and presented in *Figures 12-29*, along with their diagrammatic representations.

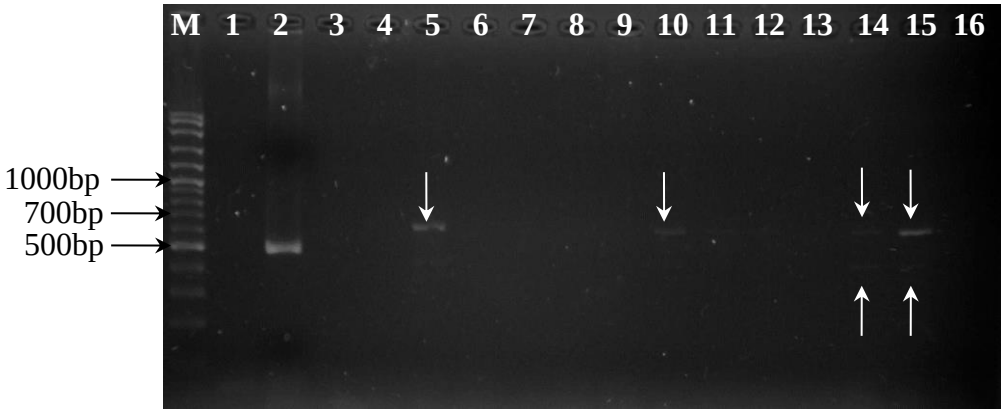


Figure 12: Gel 1 - A gel visualization of the SCAR marker PCR on 14 DNA samples of 3mgL^{-1} BAP concentration. Lanes from left to right: M, 100bp DNA ladder, 1, negative control, 2, positive control (18S rRNA), and 3-16, 3mgL^{-1} BAP culture DNA samples. The white arrows indicate the presence of a band.

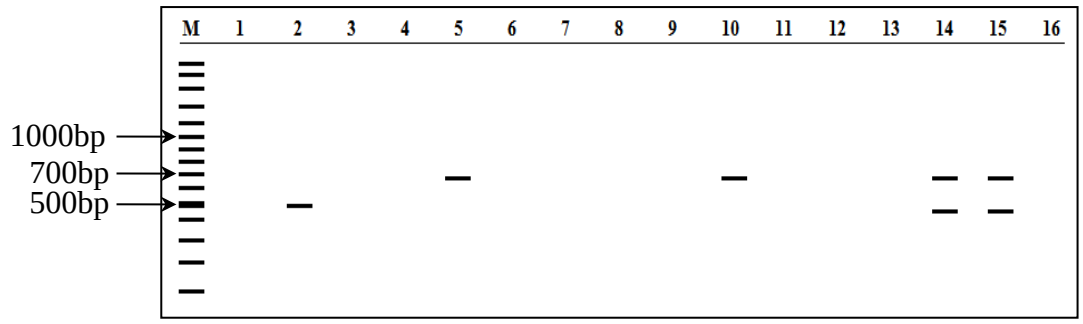


Figure 13: Diagrammatic representation of Gel 1.

These 14 samples were regenerants grown on 3mgL^{-1} BAP media; each having normal morphology. Only lanes number 5 and 10 produced single bands while lanes 14 and 15 produced 2 bands (*Table 4*).

Table 4: Correlation between number of bands present and morphologies of regenerants in Gel 1. N represents normal morphology and the numbers represent bands present.

	Lane													
	3	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Morphology	N	N	N	N	N	N	N	N	N	N	N	N	N	N
No. of band	-	-	1	-	-	-	-	1	-	-	-	2	2	-

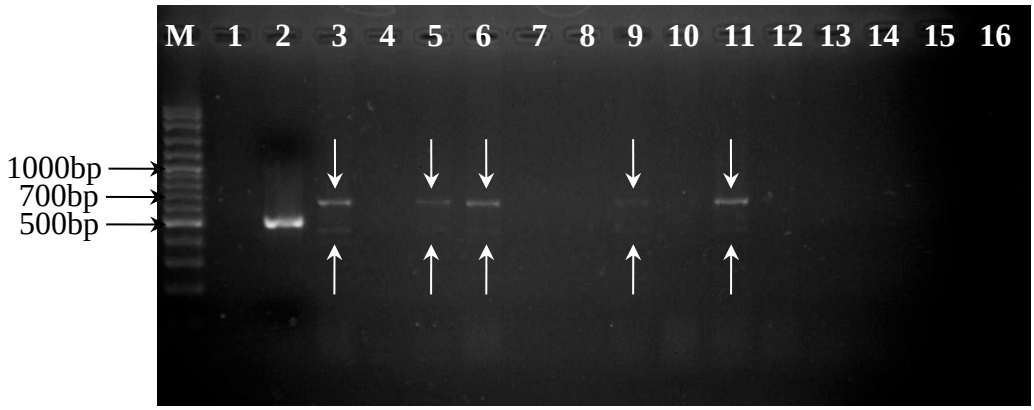


Figure 14: Gel 2 - A gel visualization of the SCAR marker PCR on the subsequent 14 DNA samples of 3mgL^{-1} BAP concentration. Lanes from left to right: M, 100bp DNA ladder, 1, negative control, 2, positive control (18S rRNA), and 3-16, 3mgL^{-1} BAP culture DNA samples. The white arrows indicate the presence of a band.

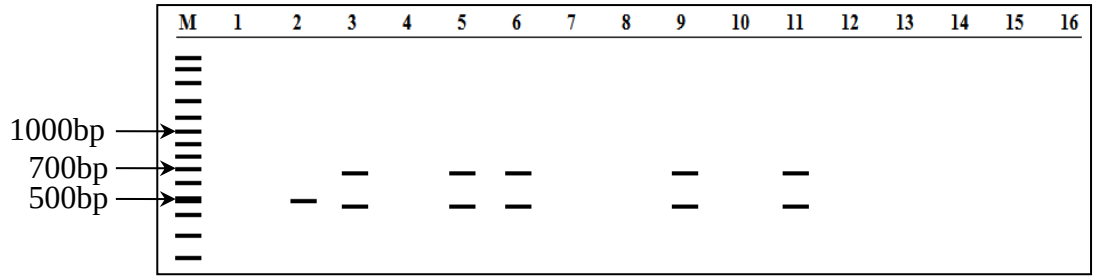


Figure 15: Diagrammatic representation of Gel 2.

As with the samples of Gel 1, these following 14 samples were also normal in morphology and grown on 3mgL^{-1} BAP media. Lane numbers 3, 5, 6, 9, and 11 all produced 2 bands from the PCR analysis (*Table 5*).

Table 5: Correlation between number of bands present and morphologies of regenerants in Gel 2. N represents normal morphology and the numbers represent bands present.

	Lane													
	3	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Morphology	N	N	N	N	N	N	N	N	N	N	N	N	N	N
No. of band	2	-	2	2	-	-	2	-	2	-	-	-	-	-

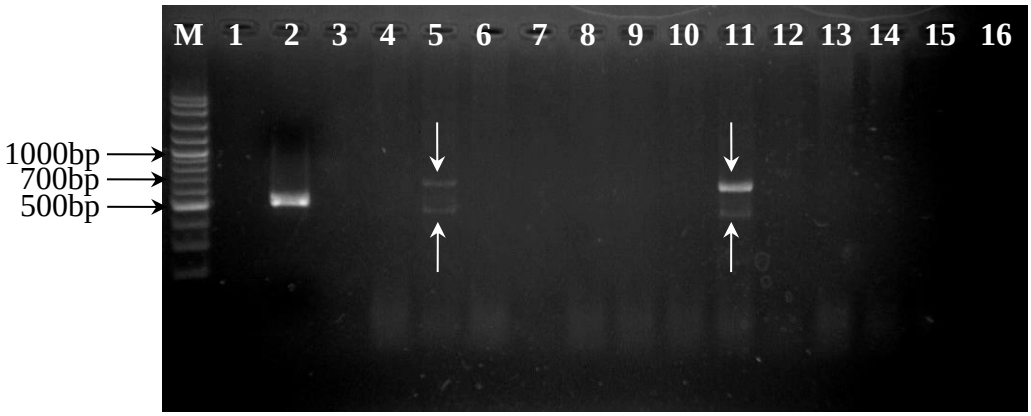


Figure 16: Gel 3 - A gel visualization of the SCAR marker PCR on 14 DNA samples of 6mgL^{-1} BAP concentration. Lanes from left to right: M, 100bp DNA ladder, 1, negative control, 2, positive control (18S rRNA), and 3-16, 6mgL^{-1} BAP culture DNA samples. The white arrows indicate the presence of a band.

In gel 3, the 14 samples were grown on 6mgL^{-1} media. All of them were of normal morphology. Lanes 5 and 11 were the only samples producing 2 bands (*Table 6*).

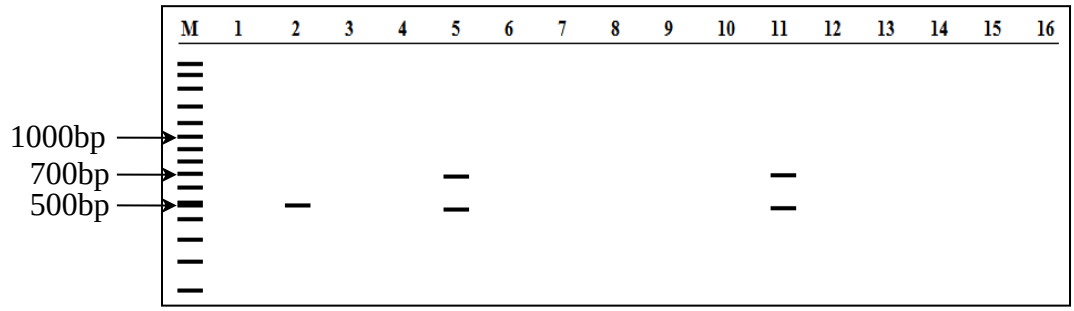


Figure 17: Diagrammatic representation of Gel 3.

Table 6: Correlation between number of bands present and morphologies of regenerants in Gel 3. N represents normal morphology and the numbers represent bands present.

	Lane													
	3	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Morphology	N	N	N	N	N	N	N	N	N	N	N	N	N	N
No. of band	-	-	2	-	-	-	-	-	2	-	-	-	-	-

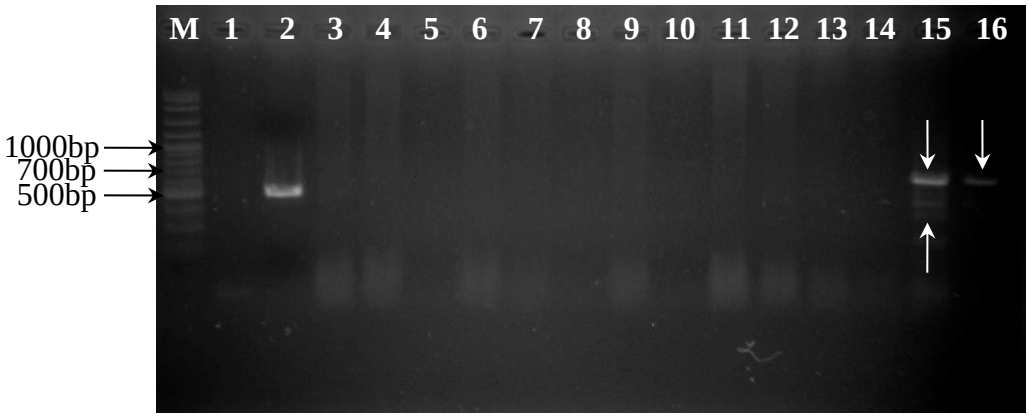


Figure 18: Gel 4 - A gel visualization of the SCAR marker PCR on the subsequent 14 DNA samples of 6mgL^{-1} BAP concentration. Lanes from left to right: M, 100bp DNA ladder, 1, negative control, 2, positive control (18S rRNA), and 3-16, 6mgL^{-1} BAP culture DNA samples. The white arrows indicate the presence of a band.

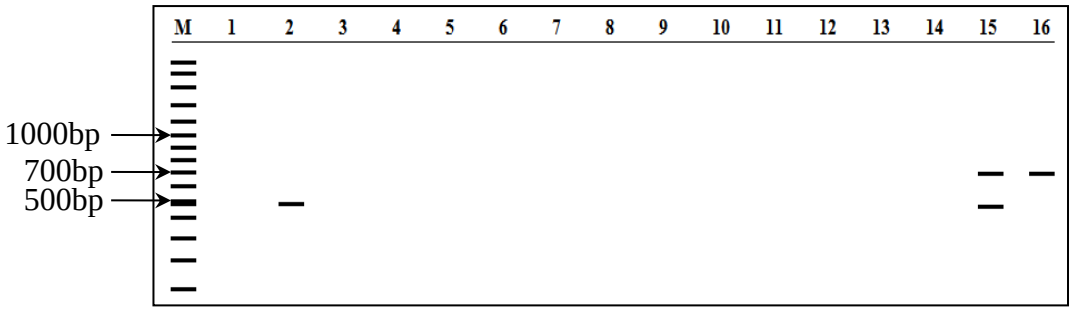


Figure 19: Diagrammatic representation of Gel 4.

For the next 14 samples of the 6mgL^{-1} BAP regenerants, all were of normal morphology and only lanes 15 and 16 produced bands: lane 15 with 2 bands and lane 16 with 1 band (*Table 7*).

Table 7: Correlation between number of bands present and morphologies of regenerants in Gel 4. N represents normal morphology and the numbers represent bands present.

	Lane													
	3	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Morphology	N	N	N	N	N	N	N	N	N	N	N	N	N	N
No. of band	-	-	-	-	-	-	-	-	-	-	-	-	2	1

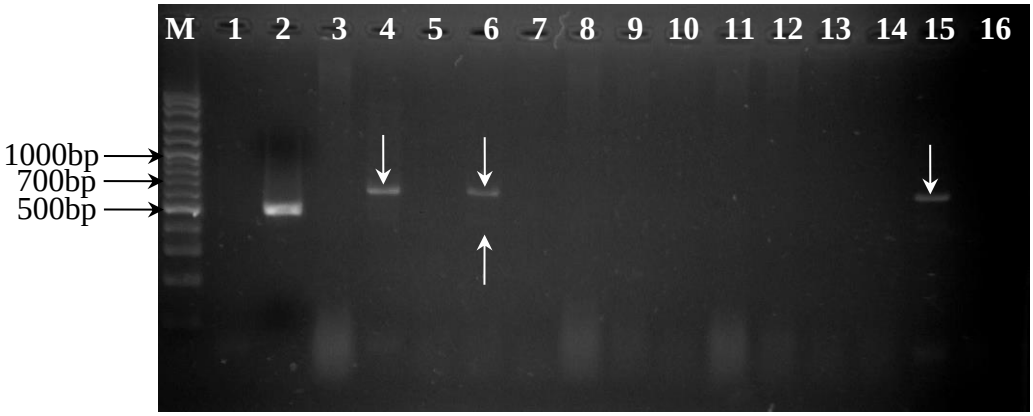


Figure 20: Gel 5 - A gel visualization of the SCAR marker PCR on 14 DNA samples of 9mgL^{-1} BAP concentration. Lanes from left to right: M, 100bp DNA ladder, 1, negative control, 2, positive control (18S rRNA), and 3-16, 9mgL^{-1} BAP culture DNA samples. The white arrows indicate the presence of a band.

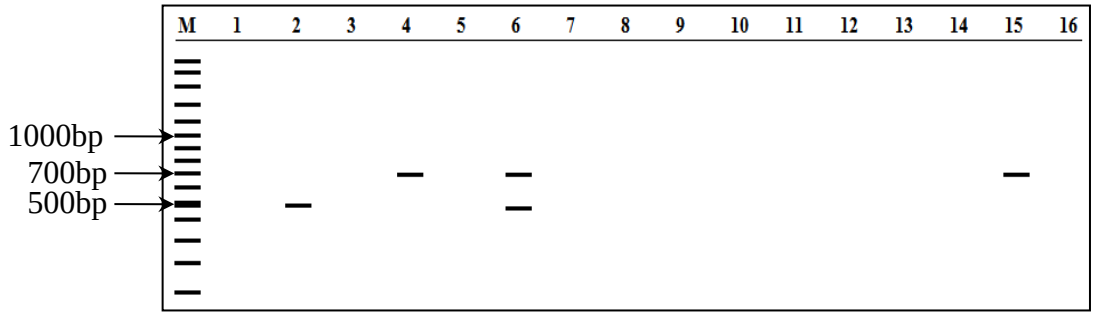


Figure 21: Diagrammatic representation of Gel 5.

For regenerants grown on 9mgL^{-1} BAP media, only lane 8 was with stunted while the rest of the samples were normal. However, only lanes 4 and 15 (normal morhphology) produced a single band while lane 6 (normal morphology) produced 2 bands (*Table 8*).

Table 8: Correlation between number of bands present and morphologies of regenerants in Gel 5. N represents normal morphology, S represents stunted morphology, and the numbers represent bands present.

	Lane													
	3	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Morphology	N	N	N	N	N	S	N	N	N	N	N	N	N	N
No. of band	-	1	-	2	-	-	-	-	-	-	-	-	1	-

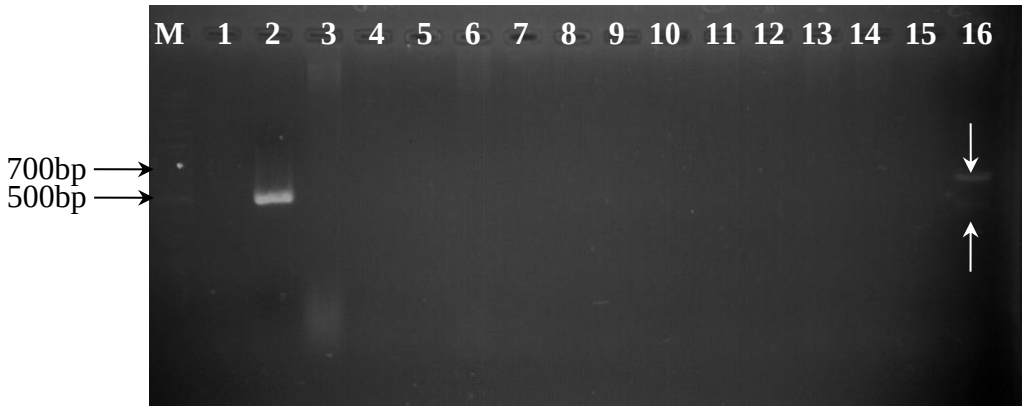


Figure 22: Gel 6 - A gel visualization of the SCAR marker PCR on the subsequent 14 DNA samples of 9mgL^{-1} BAP concentration. Lanes from left to right: M, 100bp DNA ladder, 1, negative control, 2, positive control (18S rRNA), and 3-16, 9mgL^{-1} BAP culture DNA samples. The white arrows indicate the presence of a band.

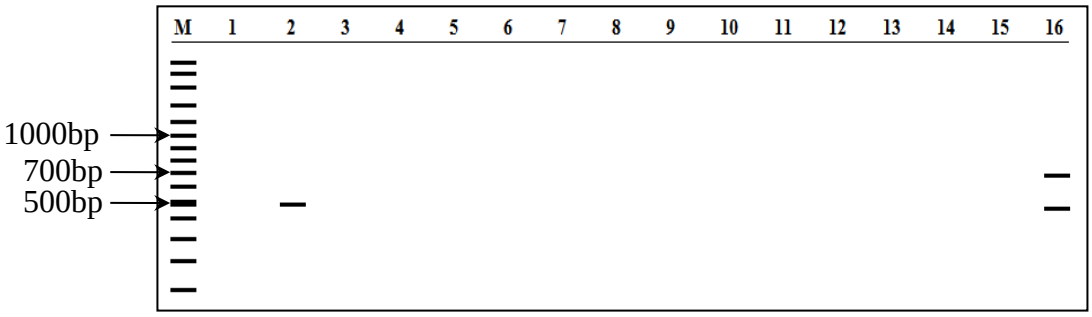


Figure 23: Diagrammatic representation of Gel 6.

The following 14 samples of the 9mgL^{-1} BAP concentration were normal in morphology except lane 16 that was stunted. Only lane 16 produced 2 bands (*Table 9*).

Table 9: Correlation between number of bands present and morphologies of regenerants in Gel 6. N represents normal morphology, S represents stunted morphology, and the numbers represent bands present.

	Lane													
	3	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Morphology	N	N	N	N	N	N	N	N	N	N	N	N	N	S
No. of band	-	-	-	-	-	-	-	-	-	-	-	-	-	2

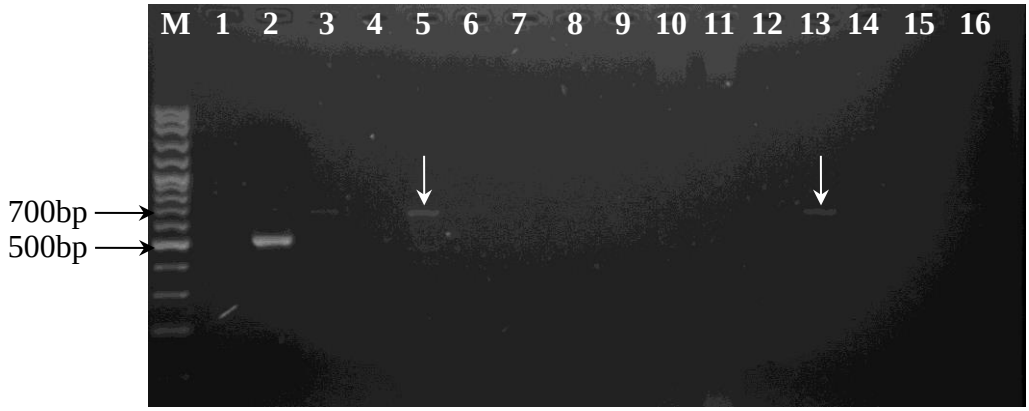


Figure 24: Gel 7 - A gel visualization of the SCAR marker PCR on 14 DNA samples of 12mgL^{-1} BAP concentration. Lanes from left to right: M, 100bp DNA ladder, 1, negative control, 2, positive control (18S rRNA), and 3-16, 12mgL^{-1} BAP culture DNA samples. The white arrows indicate the presence of a band.

In gel 7, the samples were regenerants grown on 12mgL^{-1} BAP media. Lanes 3, 4, 5, 9, 10, and 12 were normal in morphology. The remaining lanes, 6, 7, 8, 11, 13, 14, 15, and 16 were stunted. Lanes 5 (normal) and 6 (stunted) produced 1 band each (Table *10*).

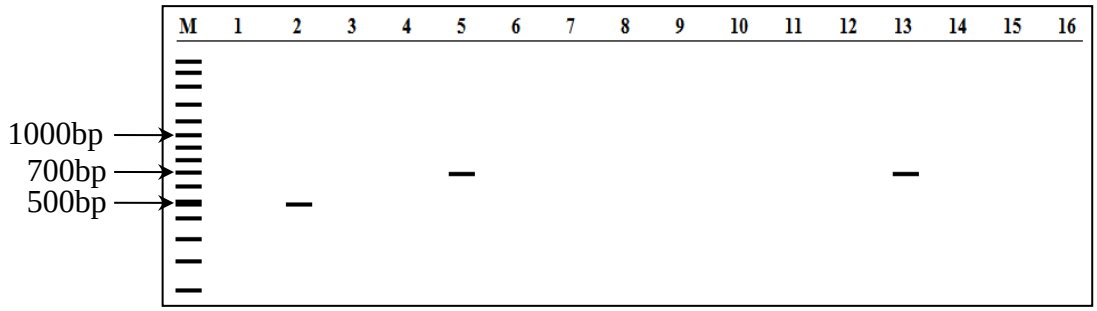


Figure 25: Diagrammatic representation of Gel 7.

Table 10: Correlation between number of bands present and morphologies of regenerants in Gel 7. N represents normal morphology, S represents stunted morphology, and the numbers represent bands present.

	Lane													
	3	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Morphology	N	N	N	S	S	S	N	N	S	N	S	S	S	S
No. of band	-	-	1	-	-	-	-	-	-	-	1	-	-	-

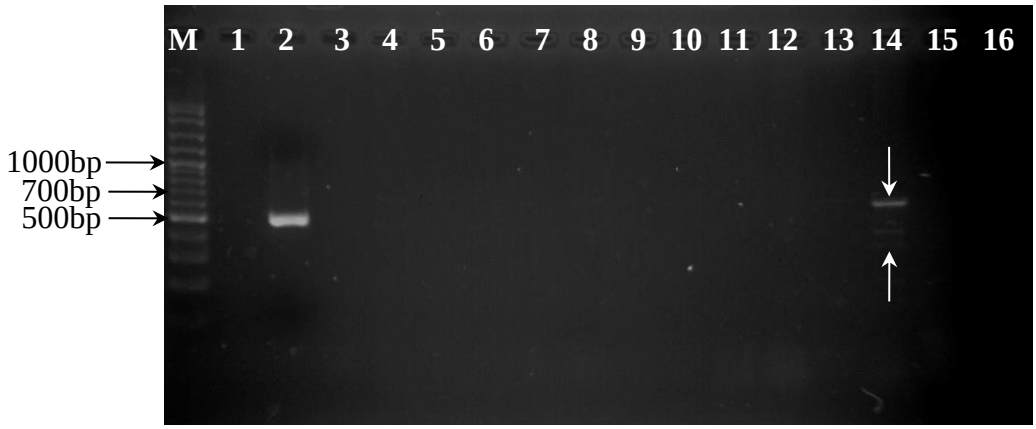


Figure 26: Gel 8 - A gel visualization of the SCAR marker PCR on the subsequent 14 DNA samples of 12mgL^{-1} BAP concentration. Lanes from left to right: M, 100bp DNA ladder, 1, negative control, 2, positive control (18S rRNA), and 3-16, 12mgL^{-1} BAP culture DNA samples. The white arrows indicate the presence of a band.

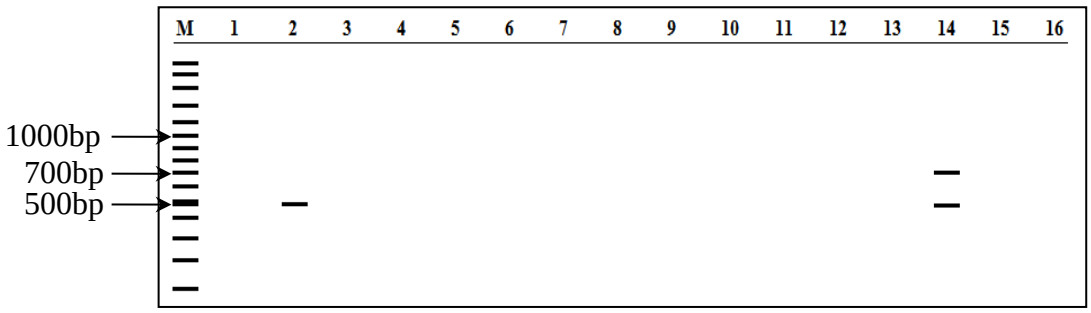


Figure 27: Diagrammatic representation of Gel 8.

For the following 14 DNA samples of the 12mgL^{-1} concentration, lanes 3, 4, 5, 9, 10, 11, 12, 13, 15, and 16 were of normal morphology while lanes 6, 7, 8, and 14 were stunted. However, only lane 14 (stunted) produced 2 bands (*Table 11*).

Table 11: Correlation between number of bands present and morphologies of regenerants in Gel 8. N represents normal morphology, S represents stunted morphology, and the numbers represent bands present.

	Lane													
	3	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Morphology	N	N	N	S	S	S	N	N	N	N	N	S	N	N
No. of band	-	-	-	-	-	-	-	-	-	-	-	2	-	-

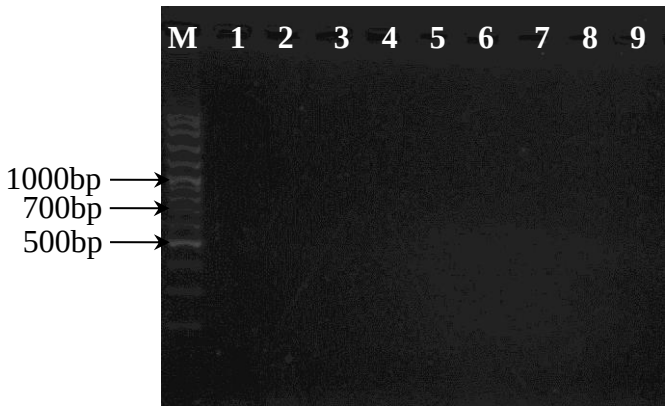


Figure 28: Gel 9 - A gel visualization of the SCAR marker PCR on the remaining 2 DNA samples for each of 3, 6, 9, and 12mgL^{-1} BAP concentrations. Lanes from left to right: M, 100bp DNA ladder, 1, negative control, 2-3, 3mgL^{-1}, 4-5, 6mgL^{-1}, 6-7, 9mgL^{-1}, and 8-9, 12mgL^{-1} BAP culture DNA samples.

The final remaining 2 DNA samples of each 3, 6, 9, and 12mgL^{-1} BAP were normal in morphology but none of them produced any bands with the SCAR marker (*Table 12*).

Table 12: Correlation between number of bands present and morphologies of regenerants in Gel 9. N represents normal morphology, and the numbers represent bands present.

	Lane							
	2	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Morphology	N	N	N	N	N	N	N	N
No. of band	-	-	-	-	-	-	-	-

The presence and absence of bands in the electrophoresis is summarized and correlated with the morphologies of the samples. They are presented in *Table 13*. It can be seen from the summary that the presence of a band is random and irrespective of the regenerants' morphology (i.e.: normal or stunted). However, out of the 120 regenerants, 6 of them exhibited one band of about 700bp in size, and 13 showed two bands of about 700bp and 500bp in size (*Figure 29*).

In contrast to the longer bands, the shorter bands observed were rather faint in some samples. The DNA samples obtained from regenerants grown on 3mgL^{-1} BAP produced 2 samples with 1 single band and 7 samples with 2 bands out of the 30 DNA extracted (*Table 4*). For regenerants grown on 6mgL^{-1} BAP, 1 sample gave a single band while 3 samples gave 2 bands out of 30. 2 samples were observed to produce 1 single band, and 2 samples also produced 2 band from DNA extracted from the 9mgL^{-1} BAP plant cultures. Finally, in regenerants grown on 12mgL^{-1} BAP, 2 samples produced 1 single band, and 1 sample gave 2 bands.

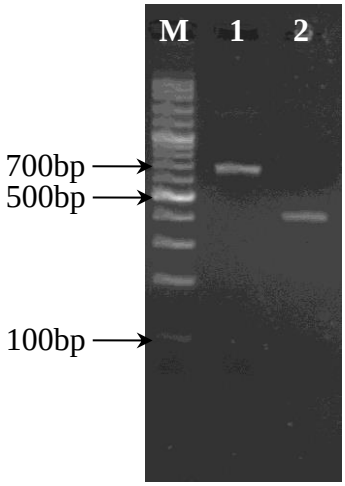


Figure 29: A gel visualization of the bands purified after gel electrophoresis. Lanes from left to right: M, 100bp DNA ladder, 1, the ≈700bp sized band, and 2, the ≈500bp sized band.

These bands were excised from the agarose gel after electrophoresis and then purified. The excised bands were obtained from both the $3mgL^{-1}$ and $12mgL^{-1}$ BAP concentration samples, to verify any differences or similarities between the bands. They were chosen from these two concentrations as the morphology of the regenerants produced was starkly contrasting (i.e.: normal and stunted regenerants). The purified DNA samples were then sent for sequencing.

Table 13: Presence of bands from SCAR PCR of the extracted DNA from regenerants cultured on different concentrations of BAP.

Concentration of BAP (mgL^{-1})	**No. of bands observed according to their estimated sizes**	
	500bp	**500 and 700bp**
3	2	7
6	1	3
9	2	2
12	2	1

The sequenced results were aligned among the fragments from each concentration using National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST) webpage (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome). The alignment scores for both of the fragments were 95-99% aligned which meant that both fragments from the $3mgL^{-1}$ and $12mgL^{-1}$ BAP concentrations were identical. The longer fragment is about 662bp in length. The full sequence of the band is as follows:

AAAAAATATTACTCGCCTACTTGACTTGAAAGTGGAGTTCAGACCT
ATGTCTTTGATTCTGACGTTACTACTAGTGCTAGGTTTACAAGATAGGATA
GAATTTCTTTGTTGATAGCCTCATTCTGAGTTTCGAGTTAAGGATTGGATG
AAGGAGAGGCAGACCAAGGGATAGGATATTAAGACGAAAAAGCCAGGA
ATGGTAGTTTTGTAGTTGGGGGTAGAAGGCTCACTTATGTGTTTAGGTCAC
AACTGCTTTTGAGGTTCATTCTTCGGCTTATTATTCTTAGCCTTACCCCTAT
ATAAGTAAGAGTCCCCCTCTTATCCTTCCACTAGCGTATTTCTTCTTTAATG
GTAAGTCCTTGCATTCCTACTTTATTGTTTGAAGTCTTTAGTTGCGTCTTCT
CATTCTTTAGGGCTTACTCGCTTGGTTCTTGTGCTACTGAGTTCGATCTTTT
GTATTAGATTAGATTNAGATTAGGNAGTCGCTTGGGGAGCACTANGTATC
ATTTATTGTACCTTCCGNGTGTCGAGTCTAGGGGTTCTATGTTCCAGTGGG
AAAGGTCCCTGCCTGCTTCGCTCCTNGTTCGCCTATTGGGTAAATCATTCA
ATTTCTTGCNTTGGTTGAGTTTGGNGGTTCNGCGGAGTTATTGATTTCAAC
AAAA.

The shorter fragment is about 438bp in length with a full sequence is as follows:

CCGGACGGCTACCCCGCCATGACGCATCAGTTCATCAACAATTACT
ACTTGTTTATCATGGTCATCCCAAAAGTCAAACAAGTACATGCACAGGAA

TTGACATGTCGGTGCTTCGAGAATAACTATCATGTTCAACTGATGACCACA

AGAAAAATATCCTTGCATTTGGAGTTTTGACATATAGAACAGGTGCAATG

AATATTCACTTGCATTCTCGATTGAACAAACAGGGTAACAGTTAAATTTAT

TAGGATCATGTAAAATGTGATAATCGATGTTATTTATCTTATCATGCGAGG

TAGAATAACAAGTTCAGAGAGAAAGCTAATAAACATAATGGCAAACATA

AAACCCAATAGCTCTGGATATGGTAGTACTAAACATCCAATAAGCTTCCT

CCAACTCTGTCACGAGGTATGAGAATGCAACCACAGAANN.

The sequenced products were then analyzed again using the BLAST-N program; in order to detect for nucleotide sequence alignment within their database. The 662bp sequence did not reveal any homologous products but a fragment of the 438bp sequence revealed some homology with a 124,825bp *Musa acuminata* clone BAC MA4-3F3 sequence (GeneBank Accession No. AC226038.1) with an E value of 0.17. An Expect (E) value closest to 0 was identified as the most significant match.

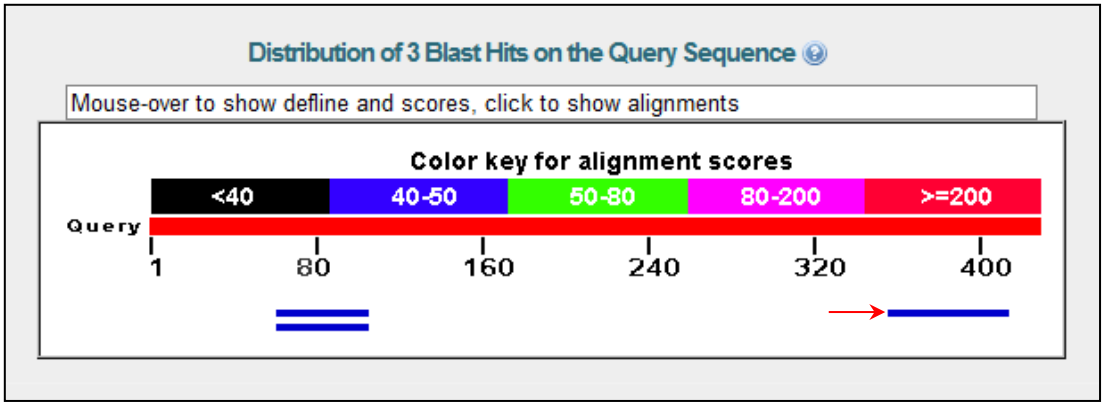
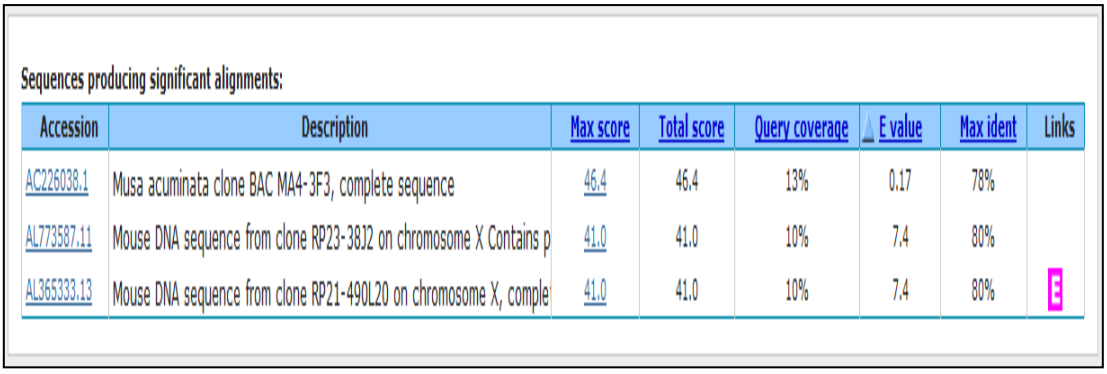


Figure 30: Alignment score of the 438bp sequence with other gene bank sequences in the NCBI database. The arrow shows the region of similarity closest to *Musa acuminata* clone BAC MA4-3F3.



Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
AC226038.1	Musa acuminata clone BAC MA4-3F3, complete sequence	46.4	46.4	13%	0.17	78%	
AL773587.11	Mouse DNA sequence from clone RP23-383J2 on chromosome X Contains p	41.0	41.0	10%	7.4	80%	
AL365333.13	Mouse DNA sequence from clone RP21-490L20 on chromosome X, comple	41.0	41.0	10%	7.4	80%	

Figure 31: Significant alignment ratios homologous to the 438bp sequence. *Musa acuminata* clone BAC MA4-3F3 being the most significant out of the three.

Checks with the protein database did not reveal any homology to significant proteins. However, three hypothetical *Musa acuminata* proteins were detected when the 438bp sequence was aligned with the BAC MA4-3F3 sequence. These proteins were located within the 75,781-76,260bp region of the sequence.

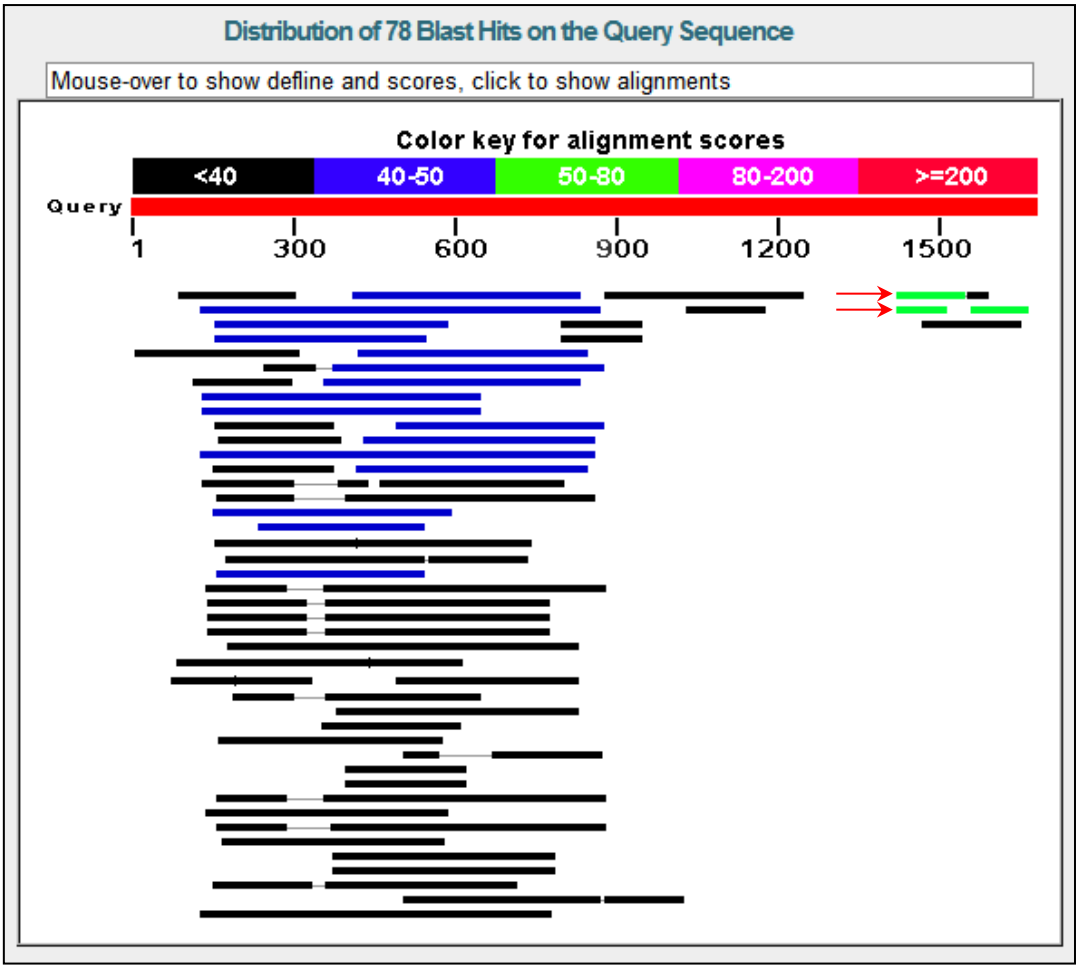


Figure 32: Alignment score of the 438bp sequence with the *Musa acuminata* clone BAC MA4-3F3 sequence in the NCBI database. The areas highlighted in green and accentuated by the red arrows denote the hypothetical proteins.

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
ABF70144.1	hypothetical protein MA4_112I10.6 [Musa acuminata]	66.2	99.0	10%	3e-12	93%	
ABF70038.1	hypothetical protein MA4_78I12.72 [Musa acuminata]	55.1	55.1	6%	2e-05	83%	
ABF70039.1	hypothetical protein MA4_78I12.80 [Musa acuminata]	50.4	50.4	5%	1e-04	78%	

Figure 33: The three hypothetical proteins detected in the sequence.

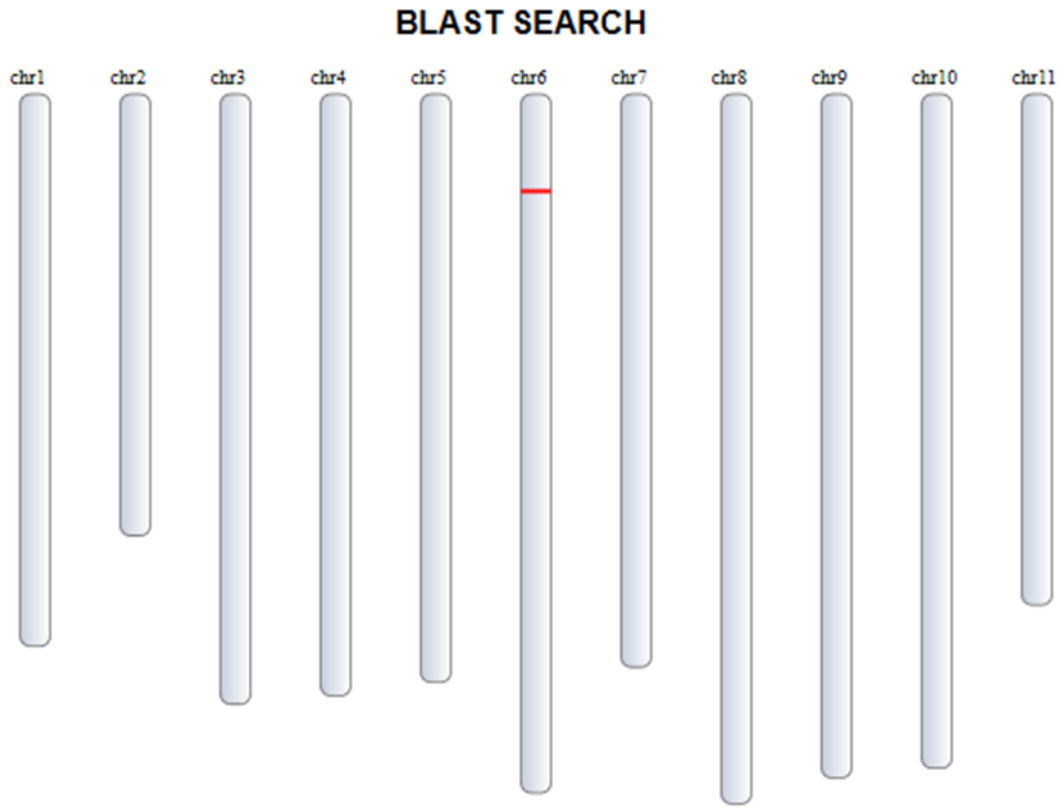


Figure 34: Possible location of the 438bp fragment in the banana genome.

Further analysis of the 438bp sequence by "blasting" the fragment to the banana genome provided by CIRAD at http://banana-genome.cirad.fr/blast.html; selecting the "blastn" program and "pseudochromosome" database revealed that the fragment would have been present in chromosome 6. The possible position of the fragment would be between the 4,853,352-4,853,752bp positions of chromosome 6 of the banana genome.

Query name	Chromosome	Start	End	Strand	Evalue	% identity	Link
Query_1	chr6	4853352	4853752	-	0.0	97.01	View result on gbrowse

Figure 35: The possible position of the fragment, between the 4,853,352-4,853,752bp positions of chromosome 6 of the banana genome.

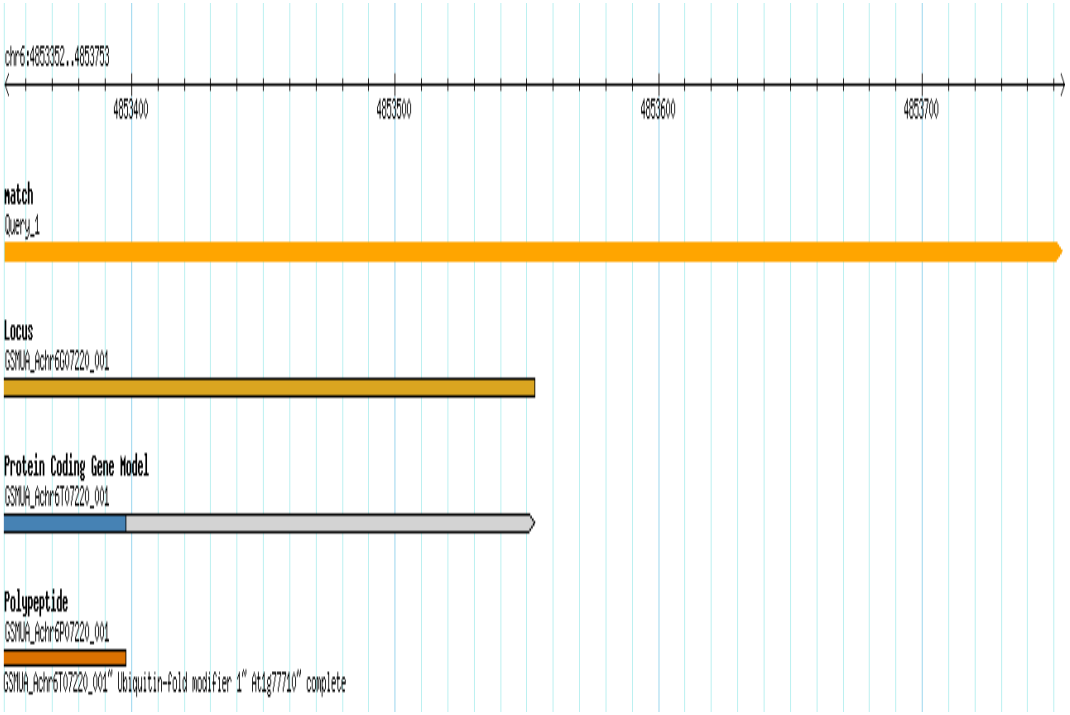


Figure 36: The fragment identified as "ubiquitin-fold modified 1".

Viewing the results on gbrowse, the fragment was later identified as the ubiquitin-fold modifier 1.

CHAPTER 5

DISCUSSION

5.0 DISCUSSION

5.1 The effects of benzylaminopurine (BAP) during micropropagation

For propagation of plant samples in this study, MS media containing $3mgL^{-1}$ were used. Shoot meristems did not propagate on hormone-free media. This formulation was also used in other works (Bairu *et al*., 2006; Buah *et al*., 2010). This shows the importance of plant growth regulators in promoting shoot growth.

In this study, varying concentrations of BAP during micropropagation have shown to produce variations in morphology of the regenerants. Bairu *et al*. (2006) stated that plant growth regulators could indirectly increase multiplication rate, thus inducing adventitious shoots. In their study, at $7.5mgL^{-1}$ of BAP, a high variation rate was observed, possibly due to increased adventitious shoot proliferation. Bairu *et al*. (2006) have also stated that according to Pierik's study in 1987, the chance of mutation was increased if adventitious shoot formation occurred due to the use of growth regulators. According to Israeli *et al*. in 1995, somaclonal variation happened when a high rate of proliferation was accomplished.

It was also observed in this study that the regeneration efficiency in terms of regeneration duration and number of shoots decreased in cultures of $12mgL^{-1}$ BAP as compared to the other concentrations investigated. This result concurs with Sheidai *et al*. (2008) who observed that at high concentrations of BAP, the number of proliferated shoots was significantly reduced.

The percentage of stunted regenerants was increased with higher BAP concentrations (*Table 1*). From the experiments conducted, on media containing $9mgL^{-1}$ BAP, stunted regenerants were initially observed. However, at $12mgL^{-1}$ BAP, the percentage of abnormality increased 6 times more than the former. Shirani *et al*.

(2009) observed a high rate of abnormal shoot production in banana cultivars "Berangan Intan", "Berangan", "Rastali", "Baka Baling", and "Nangka" when BAP concentration was at 44.4µM (10mgL^{-1}). They have also stated that higher levels of BAP, 22.2-44.4µM (5-10mgL^{-1}), increased the exudation of phenolic compounds, causing morphological aberrations. The results of this study are consistent with observations by Shirani *et al*. (2009). According to George and Sherington (1984), BAP has a mutagenic effect when used as a plant regulator in tissue culture media. Furthermore Buah *et al*. (2010) reported that BAP could easily induce shoots as it is not easily broken down and thus persists in the medium. They also reported that BAP would be readily absorbed by plant tissues as free, ionized forms in the medium.

According to the findings of Teisson and Cote in 1985 and Okole and Schultz in 1996, an increased exposure to high levels of cytokinin could lead to vitrification and poor shoot formation in three *Musa* cultivars (Buah *et al*., 2010). A study done by Kalimutha *et al*. in 2007 have shown that high concentrations of cytokinins led to profuse callusing and reduced shoot multiplication (Buah *et al*., 2010). In this study, the most prevalent abnormality was abnormal shoot clusters on media containing 9mgL^{-1} BAP. But at 12mgL^{-1} BAP, undifferentiated callus with no shoot formation was observed. This showed that at high concentrations, BAP severely affects shoots elongation.

The explant source used could also contribute to the increase in phenotypic variations. Explant sources with highly differentiated tissues such as roots, leaves, and stems typically produce more variations as compared to explants with pre-existing meristems such as axillary buds and shoot tips (Sahijram *et al*., 2003). However, according to Schukin *et al*. in 1997, somaclonal variation was higher in shoot-tip derived cultures of "Grand Naine" and this was further confirmed by Sahijram *et al*.,

(2003). Hence the contributing factor to increased variations in this study was possibly due to the use of shoot meristems in this study

Although it is uncertain whether the stunted regenerants would become dwarfs in the field, it is clear from this experiment that high concentrations of BAP affect growth of banana cultures during micropropagation.

5.2 Molecular analysis of somaclonal variants

Phenotypic or somaclonal are variations that occurred in regenerants from plant tissue culture. This aberration could induce a variety of physiological changes. These changes could either be beneficial or detrimental to the plant. While easily observed under micropropagation, variants can arise naturally in plant somatic and reproductive tissues (Oh *et al*., 2007). Dwarfism has been the most common variant in banana tissue culture. Although typically an undesirable trait, it could prove useful for banana cultivars such as the "Berangan" which are typically tall in stature.

For this study, varying concentrations of BAP were used to create somaclonal variations in *Musa acuminata* cv. "Berangan" via micropropagation. Dwarf SCAR markers developed by Damasco *et al*. in 1997 were used to analyze the genomic changes. These markers used by Ramage *et al*. (2004) were used in this study. Molecular markers were used in this study as it is able to identify a DNA fragment or sequence that is associated to a specific part of a genome and a comparison can be easily made by the presence of absence of a DNA band (Bairu *et al*., 2011).

According to Ramage *et al*. (2004), the expected results would be a 500bp band for the positive internal control 18S rRNA and a band of about 1,500bp for the SCAR marker, present only on normal plants and absent in dwarfs. From these results, instead of a single band of about 1.6kb as predicted by Ramage *et al*., two

distinct band sizes were observed. The two bands present were of a longer, 662bp length and a shorter band, 438bp in length. In addition to that, it can be seen that the morphology of the regenerants (i.e.: normal or stunted) did not affect the number of bands present. Furthermore, out of the 120 regenerants, only 6 exhibited the 662bp band while 13 exhibited both the 662bp and 438bp bands. These fragment bands of the two different sizes were further isolated and sequenced and the 2 bands obtained from regenerants grown on both the $3mgL^{-1}$ and $12mgL^{-1}$ BAP were homologous with each other.

From the BLAST-N analysis, the 662bp band did not reveal any homology but the 438bp sequence was closely homologous to a fragment of the *Musa acuminata* clone BAC MA4-3F3. Upon further analysis of the fragment, it was discovered that the 438bp sequence was aligned with fragments of three hypothetical proteins of *Musa acuminata*. This suggested that the SCAR markers may have exposed some conservation between the three cultivars of *Musa* spp. (Cavendish, Berangan, and *Musa acuminata*). This would also explain the differences in band sizes between the "Cavendish", as demonstrated by Ramage *et al*. (2004) and in "Berangan" cultivars.

A BLAST-N analysis of the fragment with the banana genome using the "pseudochromosome" database revealed that these hypothetical proteins would presumably be present in chromosome 6, between the 4,853,352-4,853,752bp positions. The fragment was then identified as the ubiquitin-fold modifier 1. Ubiquitin was first found in the mid-1970s and since then, small proteins related to ubiquitin (ubiquitin-like proteins or Ubls) were defined and more are being added. Ubls may not possess the same high sequence similarities but they all have the three-dimensional structure of ubiquitin or the β-grasp fold (Kerscher *et al*., 2006). Ubiquitins and ubiqutin-like modifiers are involved in post-translational modifications

of proteins (Welchman *et al*., 2005; Park *et al*., 2011). Besides that, they are also involved in DNA repair, transcription, signal transduction, endocytosis, and sorting (Welchman *et al*., 2005).

Protein degradations are regulated by the ubiquitin system. Ubiquitin, a highly conserved 76-amino acid protein, marks and modifies target proteins for degradation by the 26S proteasome. It is via this pathway cell division, metabolism, immune response, and apoptosis is regulated (Gray and Estelle, 2000). Glycine is located at the C-terminal of the Ubl and the carboxyl group of this glycine forms the attachment site to substrates. The most common target sites are lysine side chains, forming an amide or isopeptide bond between the Ubl and substrate (Kerscher *et al*., 2006). In plants, ubiquitin-based post-translational modifications such as sumoylation (with SUMO: small ubiquitin-like modifier) and neddylation (involving NEDD8: neuronal-precursor-cell-expressed developmentally downregulated protein-8) are present (Welchman *et al*., 2005; Park *et al*., 2011).

ATP-dependent, ubiquitin-activating enzyme-1 (E1) activates ubiquitin and it is then transferred to a ubiquitin-conjugatin enzyme (E2). With the help of the ubiquitin-protein ligase (E3), this ubiquitin-enzyme complex attaches to a specific target protein through the ε-amino group of a lysine residue. This E2 and E3 enzyme-ubiquitin chain can also include an accessory factor (E4); able to attach to four sequentially attached ubiquitins allowing the ubiquitylated target protein be recognized and degraded by the 26S proteasome. Ubiquitins can also be removed by deubiquitylating enzymes (Welchman *et al*., 2005).

Ubiquitin and Ubls offer a wider and more chemically varied surface as compared to small molecule modifiers like the phosphoryl or methyl groups and hence they are more flexible in altering protein conformation or protein-protein

interactions (Schwartz and Hochstrasser, 2003). Various proteins can be created just by tagging them with ubiquitin or Ubls, and these can be identified by downstream protein receptors and interactors used to control regulatory pathways in the cell (Welchman *et al*., 2005). Moreover, modifications of the Ubl-encoding genes via duplication and diversification could give rise to many molecules with new functions that could play a role in distinct cell regulatory mechanisms (Schwartz and Hochstrasser, 2003).

Studies with *Arabidopsis thaliana* have shown that the ubiquitin proteolytic system is pivotal in the auxin-response pathway (Gray and Estelle, 2000). Aux/IAA proteins are ubiquitylated by the SCF (Skp1p, Cdc53p/cullin, and F-box protein) ubiquitin ligase using the TIR1 F-box protein as the substrate receptor. The TIR1 then binds to the Aux/IAA proteins and polyubiquitylated by SCF^{TIR1} which will then be degraded by the proteasome (Kerscher *et al*., 2006).

In *Arabidopsis*, seven HECT-E3s (large proteins containing ubiquitin-binding and E2-binding sites) named UPL1-UPL7 (ubiquitin protein ligase) were found; all having shown E3 ligase activities. A study done in 2003 by Downes *et al*. and Refy *et al*. have shown that the *upl3* and *kaks* (KAKTUS) mutants of *Arabidopsis* developed branched trichomes and the *upl3* mutants developed longer hypocotyls than the wild type plants when grown on gibberellic acid-3 (GA_3) containing media (Park *et al*., 2011). Moreover, these *upl3* plants were hypersensitive to gibberellic acid (GA).

Ubiquitin-fold modifier 1 (UFM1) is synthesized as a precursor that is processed by the UFSP2 protease, exposing the functional C-terimnal Glycine. Studies with mammalian cells have found that a limited number of UFM1 substrates, the only valid one being the endoplasmic reticulum-resident protein UFBP1, is modified at one or more Lysine residues by isopeptide linkages (Vierstra, 2012).

Ufymylation pathways which include the UFBP1 target can be found encoded in other metazoans and in plants like *Arabidopsis* but they were absent in yeast or *Schizosaccharomyces pombe* - signifying a probable unique role for UFM1 in multicellular organisms (Vierstra, 2012). A study by Tatsumi *et al*. and in Lemaire *et al*. in 2011 have shown that *Uba5*$^{-/-}$ mice are embryonic lethal, caused by defective erythroid development and cultured human cells silenced for *Ufm1* and *Ufl1* showed accelerated apoptosis due to heightened endoplasmic reticulum stress (Vierstra, 2012). However, the functions of the UFM1 pathway in plants are relatively unknown.

Point mutations, gene duplication, chromosomal rearrangements, chromosome number changes, transposable element movement, and changes in DNA methylation through small interfering RNA are the possible reasons for giving rise to somaclonal variants (Oh *et al*., 2007). Interestingly, almost half of the *Musa acuminata* genomic sequence is composed of transposable elements - long terminal repeat retrotransposons accounting for the largest part (D'Hont *et al*., 2012). Alteration in DNA methylation might be due to *de novo* formation and/or activation of enzymes catalyzing methylation reactions, decrease in formation and/or inhibition of enzymes catalyzing methylation reactions or concentration changes of substances or co-factors participating in the methylation reactions (Sahijram *et al*., 2003).

As stated earlier, somaclonal variation is not unique to *in vitro* propagation but could also arise as a trigger to genomic shocks or plasticity after the plant has exhausted its physiological responses to environmental stress; the genomic shock response may incur genomic reorganization in which it is limited to a sub-fraction of the genome (Oh *et al*., 2007).

Regulatory elements embedded in the non-coding regions of the genome control gene expressions (Reineke *et al*., 2011). Nevertheless, identifying the

embedded putative gene expression regulators in conserved non-coding sequences (CNSs) is a challenge as universal highly conserved motifs are limited and it is an even greater challenge in plants as their genomes are more diverse due to duplication events, polyploidy, increased recombination, transposable elements, and gene silencing (Rineke *et al*., 2011).

D'Hont *et al*. (2012) have noted that whole-genome duplications (WGD) play an important role in angiosperm genome evolution. They stated that 65.4% of genes included in the *Musa* α/β ancestral blocks are singletons and 10% are retained in four copies - in line with the loss of most gene-duplicated copies after WGD. Genes involved in transcription regulation (transcription factor activity), signal transduction (small GTPase-mediated signal transduction and protein kinase), and translational elongation were retained - suggesting that genes involved in multi-proteic complexes or regulatory genes are dosage sensitive and are more prone to be co-retained or co-lost after WGD (D'Hont *et al*., 2012).

Inada *et al*. (2003) stated that DNA coding regions for proteins are predicted to exhibit sequence conservation between related species due to the functional constraints of the protein structure. These conserved non-coding DNA regions are described as "phylogenetic footprints". Inada *et al*. (2003) went on citing works by Kaplinsky *et al*. in 2002, that CNSs represented DNA sequences that were conserved during selection while the surrounding non-conserved sequences undergo randomization via mutation. According D'Hont *et al*. (2012), genes associated with deeply conserved CNSs are retained as duplicates at a higher frequency than genes with less deeply conserved CNSs. A comparison of *Musa*, rice, sorghum, *Brachypodium*, date palm, and *Arabidopsis* proteomes have uncovered 7,674 common gene clusters suggesting ancestral gene families (D'Hont *et al*., 2012).

Representational difference analysis (RDA) technology, with its ability to scan about $5x10^8$ base pairs of DNA in each sub-fraction - greater than other differentiation techniques like RAPDs or AFLPs, can be applied to detect genomic losses, rearrangements, amplifications, point mutations, and pathogenic organisms between two genomes (Oh *et al*., 2007). The DNA regions obtained in this study could be considered unique regions as it appears to be conserved among three banana genomes.

5.3 Developing future investigations

To successfully investigate the morphological variations among regenerants grown on varying concentrations of BAP, the study could be extended to include greenhouse trials as to truly identify their phenotypic characteristics. *In vitro* aberrants could revert to normal in a greenhouse environment (i.e.: transient or permanent). The mature plants could then be reassessed and characterized based on their stable phenotype. After a more distinct abnormality was reached, fluorescence *in situ* hybridization (FISH) can be done to locate the aberrant trait. RAPD markers could be used to detect polymorphisms, which would subsequently be used as a probe to detect the sequence on a chromosome.

The choice of explants used could also be a co-factor to somaclonal variations. The effects of explants source on somaclonal variations could be further investigated. Different explant sources could be used to further correlate the emergence of variations caused other than the use of high concentrations of BAP. More studies on "Berangan" cultures must be done to effectively reach a conclusion as most studies were done on somatic embryogenesis and not micropropagation.

The SCAR markers used in this study were specific for "Cavendish" banana cultivars. A specific SCAR marker for "Berangan" could be designed to investigate

the variations induced. Representational difference analysis (RDA) technology could be used to detect genomic changes within the variants. When further investigated, these changes could be further identified and developed into DNA markers for diagnosis in banana. The markers can be cloned, sequenced, and mapped to specific locations in the chromosome. Quantitative traits loci (QTL) can be used to identify traits such as yield and disease resistance. Furthermore, closely linked markers flanking both sides of the genes could be identified and used as a starting point for gene isolation using the map-based cloning approach; selection criteria based on these markers can be used in breeding programs to monitor the transfer of genes, referred to as marker-assisted selection (MAS) (Escalant and Panis, 2002).

The discovery of the hypothetical proteins which was later presumably the ubiquitin-fold modifier 1 on chromosome 6 of the banana genome could be further investigated to identify its significance. These proteins could be important in determining the morphologies of the regenerants. Moreover, if the roles of these proteins are identified, they could possibly be involved in other complex functions as they were seen to be conserved among the three cultivars of bananas (*Musa*, “Cavendish”, and “Berangan”). Furthermore, candidate gene mapping could be done as the region of the Ubl has been identified on chromosome 6, thus quantitative trait loci (QTL) or genome-wide association study (GWAS) can be done on that region to detect specific mutations or single-nucleotide polymorphisms (SNPs) that could impact the phenotypic characteristics of the cultures.

CHAPTER 6

CONCLUSION

6.0 CONCLUSION

High concentrations of BAP have been shown to increase the probability of somaclonal variation. In this study, BAP concentrations of 9-12mgL^{-1} induced morphological aberrations in the micropropagated cultures of *Musa acuminata* cv. "Berangan". At lower concentrations, the micropropagated cultures exhibited fewer variations which were in accordance to the findings of Bairu *et al*. (2006) and Shirani *et al*. (2009).

The dwarf SCAR markers for "Cavendish" used in this study, although did not successfully ascertain specific dwarf variants in "Berangan", did provide an indication that among the three cultivars of bananas ("Cavendish", "Berangan", and *Musa acuminata*), existed conserved regions. However, as with most plant biotechnology experiments, the *in vitro* cultures would have to be transplanted to a greenhouse environment to ascertain the true phenotypic traits (e.g.: normal or abnormal) of the mature plant. A reassessment of the SCAR markers then would provide a more conclusive outcome.

RDA analysis could be used to identify the hypothetical proteins that may be involved in epigenetic differences. Moreover, the downstream region of the BAC MA4-3F3 sequence could be further analyzed to locate conserved regulatory elements. As the hypothetical protein discovered is presumably an Ubl, further investigations are needed to obtain a clearer picture of its functions as it is still unknown in plants. It will be interesting to identify and rectify whether these proteins are responsible for altering the phenotypic characteristics of *in vitro* cultured *Musa acuminata* and plants in general under duress or as a means of adaptation.

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