DEVELOPMENT OF PROTOPLAST ISOLATION AND DNA-FREE CRISPR-CAS9 GENOME EDITING PROTOCOLS FOR BANANA (*Musa acuminata* cv Berangan)

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UNIVERSITY OF MALAYA
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ABSTRACT

DEVELOPMENT OF PROTOPLAST ISOLATION AND DNA-FREE CRISPR-CAS9 GENOME EDITING PROTOCOLS FOR BANANA (*Musa acuminata* cv Berangan)

Banana is an important crop in the tropics and subtropics and is ranked the fourth most important crop after rice, wheat and maize. In Malaysia, *Musa acuminata* cultivar Berangan (with an AAA genome) is the most widely cultivated banana cultivar due to its sweet taste and high nutritional value. However, banana production is seriously affected by environmental stress. Soil salinity has become an emerging challenge for banana farmers as most banana plants are grown in marginal soil with high salinity. Hence, developing a cultivar with enhanced salinity tolerance in bananas is imperative. *Sugar Transport Protein 13* (*STP13*) maybe a valuable target for banana improvement due to its essential stress response role in plants. However, improving bananas via modern breeding and conventional genetic engineering is often limited by the recalcitrance and complexity of the genetic system of bananas. Hence, this study aimed to establish efficient protoplast isolation and DNA-free CRISPR/Cas9 ribonucleoprotein-mediated transformation protocols for banana cultivar Berangan. In this study, immature male flowers and bracts of bananas were used for protoplast isolation. The results showed that immature male flower buds produced more protoplasts (1.54×10^7 protoplasts/g FW) than bracts in an enzymatic mixture of 1% cellulase RS, 1% macerozyme and 0.15% pectolyase. Therefore, immature male flower buds were used for the subsequent experiments. The efficiency of isolating protoplasts under different vacuum infiltration times and mannitol concentrations were then determined. The application of 10 minute-vacuum infiltration twice and 0.5 M mannitol significantly increased the number of protoplasts. To optimise the protoplast transfection procedures, the isolated protoplasts were transfected with

pCAMBIA1304-GFP using polyethylene glycol solution for different incubation times. About 76.89% of the pCAMBIA1304-GFP-transformed protoplasts were GFP-positive after 15 minutes of transfection. Next, *STP13* digested by different molar ratios of CRISPR/Cas9 ribonucleoprotein complex targeting the banana *STP13* gene. A 3:1 ratio resulted in the highest percentage of cleavage at 12.5%. The protoplasts were transfected with 3:1 Cas9: STP13gRNA were then sampled for targeted deep sequencing. The results showed that mostly indels (-1 bp) occurred at the target sites of *STP13* with a mutation rate of 4.40-4.90%, indicating that the established protocols can efficiently modify the genomes of recalcitrant plants like bananas.

Keywords: Banana, CRISPR/Cas9, DNA-free gene editing, PEG-mediated protoplast transfection, protoplast isolation, STP13

ABSTRAK

PEMBINAAN SISTEM PENGASINGAN PROTOPLAS DAN SISTEM PENEDITAN GENOM CRISPR-CAS9 TANPA PENGGUNAAN DNA ASING UNTUK PISANG (*Musa acuminata* cv Berangan)

Pisang ialah tanaman penting di kawasan tropika dan subtropis dan disenaraikan sebagai tanaman keempat terpenting selepas beras, gandum dan jagung. Di Malaysia, *Musa acuminata* cultivar Berangan (dengan genom AAA) ialah kultivar pisang yang paling banyak ditanam kerana manis dan mempunyai nilai pemakanannya yang tinggi. Walau bagaimanapun, pengeluaran pisang terjejas teruk oleh tekanan alam sekitar. Kemasinan tanah telah menjadi cabaran baru bagi petani pisang kerana kebanyakan tanaman pisang ditanam di tanah marginal yang amat masin. Oleh itu, kultivar pisang yang mampu toleransi terhadap kemasinan perlu diperkenalkan. *Sugar Transport Protein 13* (*STP13*) mungkin ialah sasaran penting untuk meningkatkan penghasilan pisang kerana ia berperanan dalam mekanisme tindak balas tekanan yang penting. Walau bagaimanapun, peningkatan hasilan pisang melalui pembiakan moden dan kejuruteraan genetik konvensional sering dihadkan oleh sifat rekalsitran dan kerumitan sistem genetik pisang. Oleh itu, kajian ini bertujuan untuk mewujudkan sistem pengasingan protoplas berefektif dan menentukan kaedah sistem transformasi CRISPR/Cas9 ribonucleoprotein tanpa DNA asing untuk pisang kultivar Berangan. Dalam kajian ini, kelompok bunga jantan muda dan bract pisang digunakan untuk pengasingan protoplas. Kelompok bunga jantan muda menghasilkan lebih banyak protoplas (1.54×10^7 protoplast / g FW) daripada bract dengan campuran enzim 1% selulase RS, 1% macerozim dan 0.15% pektinase. Oleh itu, kelompok bunga jantan muda digunakan untuk eksperimen berikutnya. Kecekapan sistem pengasingan protoplas telah dicuba dengan masa vakum infiltrasi dan kepekatan mannitol yang berbeza. Penggunaan vakum infiltrasi dengan 10 minit dua kali dan 0.5 M mannitol

dibuktikan boleh meningkatkan bilangan protoplas. Untuk mengoptimumkan prosedur transfeksi protoplas, masa inkubasi protoplas dan pCAMBIA1304-GFP di dalam polietilena glikol telah diuji. Terdapat 76.89% protoplas yang berjaya ditransfeksi oleh pCAMBIA1304-GFP dan menunjukkan signal positif GFP selepas 15 minit transfeksi. Seterusnya, ribonukleoprotein yang mempunyai nisbah molar yang berbeza telah diujikan untuk memotong gen STP13 pisang. Nisbah 3:1 menghasilkan peratusan pemotongan tertinggi pada 12.5%. Protoplas yang telah ditrasfeksi dengan 3:1 Cas9 dan *STP13*gRNA kemudiannya disampelkan untuk penjujukan DNA sasaran mendalam. Keputusan menunjukkan bahawa kebanyakan penyuntingan DNA (-1 bp) berlaku di tapak sasaran *STP13* dengan kadar mutasi 4.40 - 4.90%, menunjukkan bahawa protokol yang ditubuhkan dapat mengubahsuaikan genom tumbuh-tumbuhan rekalsitran seperti pisang.

Kata kunci: Pisang, CRISPR/Cas9, penyuntingan gen tanpa DNA asing, transfeksi protoplas melalui PEG, pengasingan protoplas, *STP13*

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LIST OF SYMBOLS AND ABBREVIATIONS

List of Symbols

Symbol	Full name
× g	relative centrifugal force
°C	degree Celsius
µL	microlitre
bp	base pair
cm	centimetre
h	hour
ha	hectare
mg	milligram
Min	minute
mL	millilitre
mm	millimetre
mM	millimolar
MT	megatonne
rpm	rotation per minute
s	second
sp./spp.	species
V	volume
V	Voltage
W	weight

List of Abbreviations

Abbreviation	Full name
Cas	CRISPR-Associated Systems
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DNA	Deoxyribonucleic Acid
et al.	et alia
G	Guanine
Mac-miR6	*Musa acuminata-microRNA6*
N	Nucleotide
PAM	Protospacer Adjacent Motif
RNA	Ribonucleic acid
STP13	*Sugar Transport Protein 13*
PEG	Polyethylene glycol

CHAPTER 1: INTRODUCTION

Bananas (*Musa* spp.) are economically significant cultivated fruit throughout areas that are tropical and sub-tropical (Amah et al., 2019). It is the second most widely cultivated fruit in Malaysia, with a total plantation area of 29,000 ha and a total production of 294,000 metric tons in 2015 (Din et al., 2018). Berangan (with an AAA genome) is the banana cultivar most widely grown in Malaysia (Hui et al., 2012). This cultivar is sensitive to salinity stress (Mazumdar et al., 2019) and could be an investigation model for the genetic causes of salt sensitivity (Lee et al., 2015). When analysing the control and salt-stressed banana roots using a transcriptomics approach, Lee et al. (2015) discovered that salt-stressed banana roots had down-regulated mac-miR6, whilst its target *STP13* (Gudimella et al., 2018) had increased expression. This finding suggests that *STP13* may play a role in stress response in bananas and may be a useful target for banana improvement.

Developing an effective biological system and delivery method is indispensable to elucidate the function of a target gene. However, the current methods, such as conventional breeding and classical genetic approaches, are time-consuming and often have a low success rate (Tripathi et al., 2019; Woo et al., 2015). Moreover, the parthenocarpic nature of bananas is an impediment to breeding. Hence, developing an efficient biological system through tissue culture is imperative (Jafari et al., 2015). Banana plantlets have been propagated through tissue culture techniques using immature male flower (Chin et al., 2014; Jafari et al., 2015; Rayis & Abdallah, 2015) and protoplast culture (Haicour et al., 2009).

Protoplasts are naked cells whose cell walls have been removed by either enzymatical digestion or mechanically. The isolated protoplasts are capable of uptake foreign substances via polyethylene glycol (PEG)-mediated or electroporation transformation

systems (Matsumoto et al., 2010). Protoplast transfection is a single cell event, so the plants regenerated from transformed protoplast are not chimeric (Matsumoto et al., 2010). Although optimisation and establishment of protoplast-based transformation systems has been reported for many plant species, there is no protoplast isolation and transformation protocol for banana cultivar Berangan which is a barrier to transformation for this cultivar of banana cultivar.

The recently developed *Agrobacterium*-mediated clustered regularly interspaced short palindromic repeats-CRISPR-associated protein 9 (CRISPR-Cas9) genome editing method has been widely used to investigate the role of target genes. A gene of interest is targeted by sequence-specific nucleases to induce double-strand breaks (DSBs) (Schmidt et al., 2019). Plants have two DNA repair mechanisms to repair DSBs, which are nonhomologous end-joining (NHEJ) and homology-directed repair (HDR) (Budhagatapalli et al., 2015). NHEJ is the dominant DNA repair pathway in plants, leading to frameshift mutation that often creates genetic knockouts (Schmidt et al., 2019). CRISPR-Cas9 system comprises a bacterial monomeric DNA endonuclease Cas9 and an easily engineered 20 base pair RNA guide sequence (gRNA). When introduced to plant cells, the Cas9-sgRNA complex can specifically bind to the target gene located upstream of PAM sequence 5'-NGG-3' to generate a site-specific DSB (Jinek et al., 2012). The CRISPR-Cas9 system is currently the most straightforward to use (Shen et al., 2017). Furthermore, CRISPR-Cas9 has a higher targeted mutation efficiency than TAL effector nucleases (TALENs) (Liang et al., 2014) and zinc finger nucleases (ZFNs) (Liu et al., 2018). Hence, the CRISPR-Cas9 system could be used to study banana genes and produce genome-edited banana plants from lab to field (Pua et al., 2019).

The use of *Agrobacterium*-mediated CRISPR-Cas9 genome editing in bananas faces some challenges (Tripathi et al., 2019). The T-DNA cannot be eliminated from the banana

genome by back-crossing due to its seedless property (Tripathi et al., 2019). Besides, the integration of transgenes Cas9 along with gRNA and the selectable marker into the banana genome might lead to the continuous action of editing machinery, gene disruptions, and off-target mutations (Tripathi et al., 2019). Moreover, the *Agrobacterium*-mediated CRISPR-Cas9 genome editing approach remains debatable for its regulation status and public acceptance. In response to this concern, optimising CRISPR/Cas9 mediated genome editing to avoid transgene integration is imperative.

Many efforts are being made to optimise the CRISPR-Cas9 system to avoid transgene integration. For example, Woo et al. (2015) introduced a combination of purified Cas9 enzyme and gRNA, which comprise a ribonucleoprotein (RNP) complex, to produce stable nucleotide alterations in rice, tobacco, lettuce, and *Arabidopsis thaliana*. Since the DNA-free CRISPR-Cas9 via RNPs does not require intracellular transcription and translation, this technique is faster than a plasmid-based transformation in editing plant genome (Park et al., 2019). Furthermore, the chimeric event will not occur as RNP components will self-degrade after the mutation (Tripathi et al., 2019). A DNA-free CRISPR-Cas9 system of bananas was recently established by introducing RNP components into banana protoplasts through PEG-mediated transformation (Wu et al., 2020). However, the protoplast transfection frequency and mutation efficiency were lower than those reported for cabbage (Park et al., 2019) and Chinese cabbage (Murovec et al., 2018).

Hence, developing an efficient protoplast isolation method and improved DNA-free CRISPR-Cas9 system is critical to increasing the mutation efficiency for banana gene studies and improving banana crops. Therefore, this study aimed to establish an improved DNA-free CRISPR-Cas9 genome editing system for banana with the following specific objectives:

1. To optimise a protocol for protoplast isolation and transformation from banana cultivar Berangan,
2. To optimise the ratio of RNP component by *in vitro* digestion using the banana *STP13* as a target for gene editing,
3. To establish the CRISPR-Cas9 system in banana cells using the titrated molar ratio of RNP.

CHAPTER 2: LITERATURE REVIEW

2.1 Botanical Description of Banana Plants

Bananas are commercial fruit and a major export commodity in the tropics. They are commercially farmed from the equator to 30 degrees latitude or above, in warm areas with at least 100 mm precipitation or irrigation. The banana plant is a huge flowering herbaceous plant (Brisibe et al., 2019). The height of "Dwarf" Cavendish is around 2 m. The plant grows from a rhizome called a corm, and the "trunk" is a pseudo-stem. A petiole and a lamina make up the leaves of banana plants (Deka & Neog, 2021). When mature, banana plants produce only one inflorescence, known as the banana heart. The inflorescence consists of two rows of flowers separated by a bract. A row of female flowers is above the bract, and the male flower row is at the bottom of the bract. Female flowers mature into fruits, forming a big hanging cluster with numerous tiers ("hands"), each with around 20 fruits (Figure 2.1) (Deka & Neog, 2021).

Bananas (*Musa* spp.) belong to the family of Musaceae and the order of Zingiberales (Brisibe et al., 2019). Banana breeders classify accessions or varieties of banana according to "ploidy", the number of chromosome sets they contain, and the relative proportion of *Musa acuminata* (A) and *Musa balbisiana* (B) in their genome (Brisibe et al., 2019). The edible seedless *Musa acuminata* Colla exists in two forms, a diploid and a triploid, which was actively picked by the farmers. The triploid 3n has 33 chromosomes, while the diploid 2n has 22. The letters AA and AAA are used to represent these cultivars. In contrast, the *Musa balbisiana* Colla is inedible, contains seeds and is diploid. After breeding from these two Colla, the most familiar, seedless, cultivated varieties (cultivars) of bananas are triploid hybrids (AAA, AAB and ABB) (Brisibe et al., 2019). According to the World Checklist of Selected Plant Families, there are currently 70 species of banana and this genus is divided into five sections, as shown in (Amah et al., 2019).

M. acuminata has been classified into nine subspecies (*banksii, burmannica, burmannicoides, errans, malaccensis, microcarpa, siamea, truncata,* and *zebrina*) and three variations (chinensis, sumatrana, and tomentosa) based on morphology and geographic distribution (Brisibe et al., 2019). At least four subspecies of *M. acuminata* have contributed to the genesis of cultivated bananas. These include *M. acuminata* ssp. *banksii* (originated in New Guinea), *M. acuminata* ssp. *burmannica* (originated from Myanmar), *M. acuminata* ssp. *malaccensis* (originated from the Malay peninsula), and *M. acuminata* ssp. *zebrina* (originated from Indonesia) (Brisibe et al., 2019).

Banana is considered of the greatest significant fruit crops throughout the globe (Amah et al., 2019). In Malaysia, banana is the second most widely cultivated fruit, with a total plantation area of 29,000 ha and a total production of 294,000 MT in 2015 (Din et al., 2018). Many banana cultivars are cultivated at a commercial scale, including 'Dwarf' (Vinson III, 2016), 'Cavendish' (Someya et al., 2002), 'Berangan' (Mia et al., 2010), and 'Grande Naine' (Bello-Bello et al., 2019). Malaysia is reported to grow about 50 banana cultivars, such as 'Berangan', 'Mas' and 'Cavendish' (Jalil et al., 2003). 'Cavendish' (AAA) and 'Berangan' (AAA) are widely planted due to their flavour and storage qualities (Chai et al., 2004). 'Berangan' (AAA genome) is popular due to its market value, high bunch weight and availability of planting materials (Chai et al., 2004).

Banana cultivar Berangan has a synonym names of "Lakatan" in Philippines, "Pisang Barangan Kuning" in Indonesia, and "Kluai Hom Maew" in Thailand (Valmayor et al., 2000). Berangan is a dessert cultivar that is best consumed raw rather than cooked. When ripe, the fruit is around 10–12 cm long and 5–7 cm wide, with a sweet taste, a faint sourness, and a little dry and starchy texture (Singh et al., 2016). Berangan has a bunch weight range of 15 to 25 kg and sweet and aromatic pulp. It is also a banana cultivar for exporting to Southeast Asian neighbouring nations (Omar et al., 2014). Berangan, like

Cavendish and Gros Michel, is a triploid A genome banana having haplotype lineages to *Musa acuminata* subspecies *malaccensis*, *zebrina*, and *banksii* wild bananas (Omar et al., 2012).

Figure 2.1: Structure of mature banana plant. Adapted from Deka & Neog, 2021.

2.2 Importance of Banana Farming

The banana is a crucial yet underappreciated food crop that is grown all over the world. It has the largest global production after rice, wheat, and milk (127.3 million tonnes) and the fourth-highest agricultural commodity value (USD63.6 billion) (Ploetz & Evans, 2015). Although bananas are mostly grown in Latin America and the Philippines (Voora et al., 2020), India accounts for almost a third of global output (29%) despite the fact that bananas are primarily farmed in Latin America and the Philippines (Voora et al., 2020), followed by China, the Philippines, Brazil, Indonesia, and Ecuador (Figure 2.2). In 2015, the Philippines had surpassed Indonesia as the world's second-largest banana exporter but lost its position due to El Nio's prolonged drought (Santos et al., 2018).

In 2013, global banana production was predicted to be around 145 million tonnes (Ploetz & Evans, 2015). The Great Lakes region of East Africa has the greatest banana consumption rates, with an average consumption of 200-250 kg per person per year (Nyombi, 2010). This indicates that bananas are a substantial component of the diet in

this region. In comparison, European and North American countries only recorded about 8-18 kg per person per year (Nyombi, 2010).

Banana is mostly grown in gardens and smallholdings in about 120 countries throughout the tropics and subtropics. Farmers in poor countries find bananas to be an appealing perennial crop (Achterbosch et al., 2014). Most bananas are for domestic use and cultivated in various soils and under different conditions (Valmayor et al., 2000). Some countries use almost 10% of their total land area for banana production, for example Columbia, Uganda and Philippines (Onderwater, 2020). The fruit can be harvested annually, resulting in a consistent cash flow and/or a stable supply of healthful food.

Banana is famous for its traditional, medicinal, and nutritional uses (Onderwater, 2020). Banana fruits are high in carbohydrates (22.84 g/100 g), providing 370 kJ/100 g of energy. It is one of the greatest sources of potassium (358 mg/100 g), supplying 8% of the daily required requirement. Apart from that nutritional values, bananas have excellent therapeutic capabilities. In many regions, the entire plant is used for traditional medicinal purposes. For example, banana has traditionally been used to treat heart disease, diabetes, ulcerative colitis, diarrhoea, dysentery, nephritis, gout, hypertension, sprue, uremia, inflammation, pain, and snakebite (Khare, 2004). The root is used to treat blood abnormalities and anthelmintic (Khare, 2004). Banana leaf ash is used to treat eczema and as a cool dressing for burns and blisters (Okoli et al., 2010). Cholera, haemoptysis, otalgia, dysentery, and diarrhoea are all treated with stem juice (Okoli et al., 2010). Menorrhagia, diabetes, and dysentery are all treated with flower (Khare, 2003).

Bananas can be eaten fresh, roasted, baked, steamed, or fermented, among other preparations. In addition, the entire fruits and peel can be processed into banana chips, powder, biscuits, and, most often, banana juice (Onderwater, 2020). As a result, bananas and plantains are have become entwined with human society's culture and livelihoods.

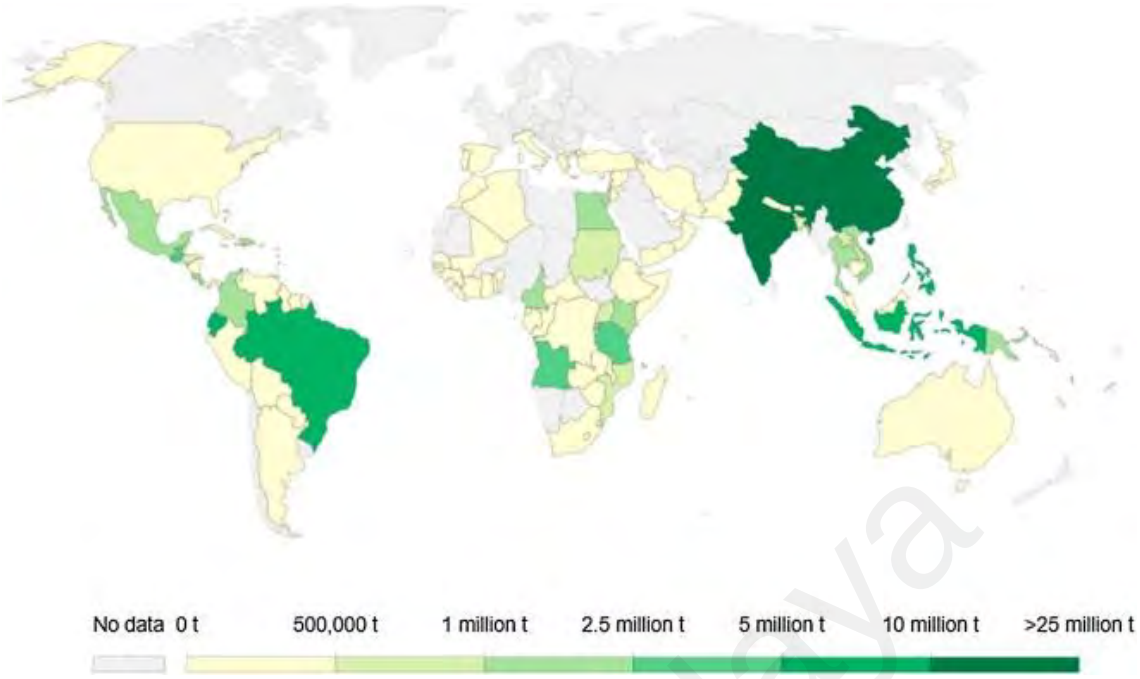

Figure 2.2: Global banana production (million tonnes) in 2014. (Adapted from Onderwater, 2020)

2.3 Drought Threatens Banana Farming

Drought strains are expected to worsen due to climate change and human activities, heightening the threats to global food security. Drought reduces the banana yield by up to 65% when rainfall is below 1,100 mm per annum (van Asten et al., 2011). Bananas are generally salt-sensitive, especially *Musa acuminata*. This is because banana plants are highly sensitive to water shortages because of their features, such as their shallow root system and huge canopy, which lead to high transpiration rates and evergreen nature (van Asten et al., 2011).

Drought can be classified as meteorological, agricultural, hydrological, and socioeconomic droughts (Ravi et al., 2013). Meteorological drought is defined as a lack of precipitation, whereas agricultural drought is defined as a lack of soil moisture. Hydrological drought is defined as a reduction in streamflow and groundwater. Socioeconomic drought can be defined as a gap between water demand and supply.

Multiple climatic process interactions and land-atmosphere responses, as well as human activities could influence the cause/linkages of droughts (Mustaffa et al., 2015).

In natural circumstances, drought is invariably linked to extreme temperature stress and, frequently, soil salinity (Ravi et al., 2013). As a result, focusing on a single method to combat drought will not yield results.; it must be studied from a multi-trait approach. When dealing with a recalcitrant crop, such as bananas, it becomes more challenging because (1) it is a long-term crop that can last anywhere between 12 and 18 months depending on the cultivar, and (2) It has a minimum of four crucial crop growth phases spaced out during that period, consisting of the stage of juvenile, the development of flower bud, stem, and ultimately bunch. Apart from that, the broad leaf area of banana plants with roots that are not too deep make it vulnerable to water shortages (Ravi et al., 2013). Apart from that, although banana requires at least 1,300 mm of annual rainfall to thrive (Mustaffa et al., 2015), the majority of banana cv. Berangan production systems in Malaysia are small-scale, in which irrigation is not used due to the high cost of production and lack of sufficient land (Nansamba et al., 2020). The growers are entirely dependent on rainfall.

Early vegetative stage banana growth, floral primordium initiation, flowering, and bunch/finger development can all be impacted by drought (Mahouachi, 2007). However, the severity of the damage is determined by the plant's growth stage and the length of exposure time to the stress (Ravi et al., 2013). Drought-sensitive banana varieties display distinct outward and interior symptoms (Uma & Sathiamoorthy, 2002). Internal symptoms include physiological and biochemical alterations at the cellular level (Uma & Sathiamoorthy, 2002). In comparison, external symptoms, such as leaf wilting and/or drying due to overheating and cell dehydration (Ravi et al., 2013), as well as a considerable drop in bunch yield, could be observed (van Asten et al., 2011). For example, in cultivars 'Robusta,' 'Karpuravalli,' and 'Rasthali,' a drop in bunch weight, fruit length,

and circumference were observed at harvest when banana trees were denied water during blooming for four weeks (van Asten et al., 2011). The decrease in crop output can be ascribed to a decrease in the plant's photosynthetic rate, which is controlled substantially by leaf chlorophyll content and stomata closure (Figure 2.3) (Nansamba et al., 2020).

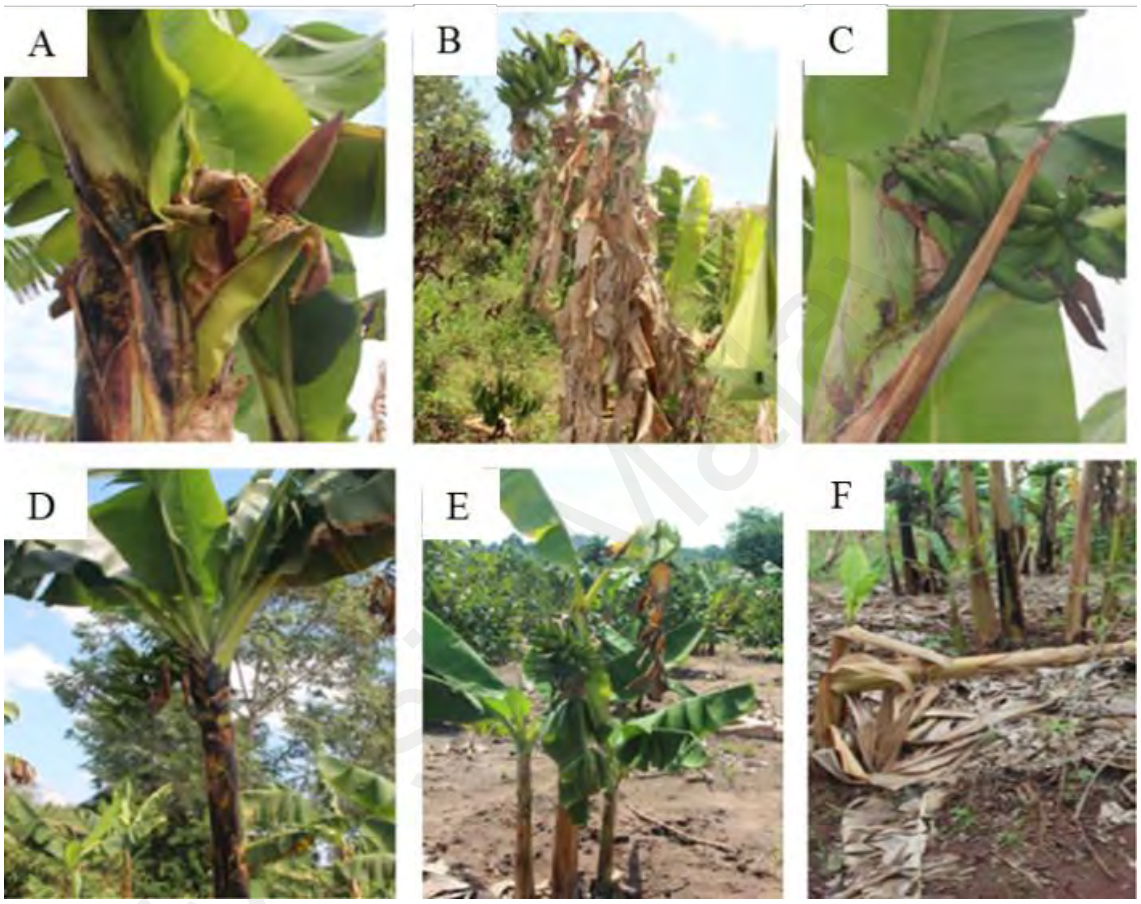

Figure 2.3: Symptoms of drought stress on banana plant: (a) strangled birth, (b) wilting, (c) reduced biomass, (d) formation of petiole rosette, (e) stunted growth and (f) weak pseudo-stem. Adapted from Nansamba et al. (2020).

2.4 Difficulty of Banana Breeding for Producing Climate-Resistance Banana Plants

Because of the tremendous variability of bananas with regard to ploidy (diploid, triploid, and tetraploid) and genomic makeup, bananas are no largely though a product of a specific crop (De Langhe, 2002). Bananas today are descended from two important ancestors, *M. acuminata* and *M. balbisiana*, which contributed to the A and B genomes,

respectively. *M. acuminata* and its varieties are thin and fragile plants that thrive in the shade and under the right climatic circumstances, whereas *M. balbisiana* has evolved and been bred in hostile conditions. *M. balbisiana* has been shown to resist abiotic stresses, such as drought and temperature extremes. In contrast, varieties of banana with the B genome have better abiotic stress resistance compared to banana cultivars with A genome. For example, traditional AAB and ABB types grown in rural regions of Egypt are more drought-tolerant than Cavendish variants (De Langhe, 2002). Banana cultivar "Sugar" ABB Pisang Awak has also been reported to be drought-tolerant (De Langhe, 2002). A number of dessert banana cultivar ABB have good wholesomeness, which is valuable for breeding. Breeding edible AB varieties and wild banana (*M. balbisiana*) might produce new productive dessert ABB types that are drought weather resilient, presenting promising future prospects.

Banana breeding programmes have been initiated by several research institutes, such as the International Institute for Tropical Agriculture (IITA) (Nigeria & Tanzania, http://www.iita.org/), the National Research Council of India (NRCB) (India, https://nrcb.icar.gov.in/), and the Centro de Investigación Cientifica del Yucatán (CICY) (Mexico, http://www.cicy.mix/). In Uganda's semi-arid zones, IITA has propagated a huge number of banana genotypes. Landraces, East-African highland bananas, plantains, and their triploid and tetraploid hybrids are among the material being evaluated for drought tolerance. The *Musa* International Transit Centre (ITC) in Belgium collected over 1,500 *Musa* accessions in *in vitro*. Similarly, a total of 340 Musa accessions are being maintained and tested by NRCB for their ability to set seeds and their responses to biotic stress, fertility in both sexes, capable of cross-pollinating with various groups, and biotic stress response. The researchers in NRCB tested 112 genotypes for drought stress resistance from 340 germplasm. It has also been attempted to undertake systematic screening of aban wide variety of genotypes for specific characteristics such as leaf water

retention capacity. (Ravi and Uma, 2009). However, banana breeding faces bottlenecks in producing bananas with elite traits (Tripathi et al., 2019).

Selection for desirable characters is time-consuming and may take up to 12 years to develop a new cultivar (Pillay et al., 2012). In most cases, banana breeding only yields 1 to 1.5 seeds per bunch. As a consequence, obtaining huge quantities of seeds is time-consuming and labour-intensive (Ssebuliba et al., 2009). Apart from that, wild species have a number of unfavourable characteristics, such as low yield and non-parthenocary, which require multiple backcrosses to be removed, which prolongs the breeding process. *Musa* breeding is also difficult due to the germplasm's limited genetic diversity and a lack of knowledge of wild species with useful agronomic properties (Nyine & Pillay, 2011).

On the other side, undesired gene co-inheritance can be reduced by using genetic modification techniques, in which only desirable genes are put into the genome of the genotype that needs to be improved (Tripathi et al., 2010). As a result, there is a growing interest in employing molecular breeding tactics such genetic engineering (Ding et al., 2016). As a consequence, identifying the genes implicated in stress tolerance is critical (Gao et al., 2021).

2.4.1 Genes related to abiotic stress tolerance in bananas

The adoption of semi-dwarf rice, wheat, and maize varieties considerably enhanced agricultural productivity globally during the first "green revolution" in the middle of the twentieth century (Davey et al., 2013). The development of crops with perfect plant design, according to proponents of "the second Green Revolution," will boost food yields. Currently, ideal plant architecture banana types have great lodging resistance, optimal photosynthesis, and efficient water absorption. These characteristics could aid in increasing banana yield (Nyine & Pillay, 2011). Due to the sterility of most cultivars,

genetic engineering is useful for the generation of banana types with drought resistance and optimum plant architecture (Nyine & Pillay, 2011).

Depending on the genetics, different banana cultivars may react differently to drought. For example, better drought resistance is facilitated by the "B" genome (Santos et al., 2018). The full genome of DH-Pahang, a doubled-haploid genotype of the *Musa acuminata* subspecies malaccensis accession 'Pahang', (an "A" genome) was sequenced (D'Hont et al., 2012). The genome sequencing of the wild diploid *M. balbisiana* variety 'Pisang Klutuk Wulung' (PKW) was generated (Davey et al., 2013). More than 20 candidate genes have been suggested as possible indicators of banana drought resistance. as reviewed by Santos et al. (2018). These include *MaAGPase*, *MaAQP*, *MabZIP*, *DEG*, *MaSWEET*, *PYL-PP2C-SnRK2* and cuticular wax biosynthetic genes (*FATB* and *KCS11*).

2.4.2 Sugar Transport Protein 13 (STP13) is Expressed in Response to Stress

Sugar Transport Protein 13 (*STP13*) belongs to the family of large monosaccharide transporters (MST) (Schofield et al., 2011). The STP subfamily comprises H^+ symporters, which can transfer diverse hexoses and pentoses but not sucrose (Schofield et al., 2021). When the phloem transports sucrose to the apoplasm of sink tissues, the sucrose is hydrolysed into hexoses, which can be taken into cells by hexose transporters. This process is essential in maintaining plant source-sink strengths under stress. So far, many STPs are high-affinity hexose transporters (Milne et al., 2019).

STP13 is highly expressed in *Arabidopsis* in response to abiotic stress, such as high salinity and drought (Yamada et al., 2011). STP13 also has been shown to contribute to plant defence mechanisms by limiting sugar flux toward pathogens in *Arabidopsis* (Lemonnier et al. 2014). Yamada et al. (2016) demonstrated that the phosphorylation of STP13 by BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 enhanced the defence responses and monosaccharide uptake in *Arabidopsis*. In banana,

STP13 is believed to be involved in stress responses. As shown by Lee et al. (2015), the *STP13*-targeting mac-miR6 was significantly down-regulated in 300 mM NaCl salt-treated banana roots. *Musa*-specific mac-miR6, was first identified in the version-1 genome of *Musa acuminata* (Davey et al., 2013). This mac-miR6 was located within 1377251..1377323 in chromosome 7 of the Locus A-genome (Davey et al., 2013).

Drought and other abiotic conditions have been demonstrated to influence gene expression involved in sugar transport (Lee & Seo, 2021). The link between sugar transporter genes and drought has long been known. In comparison to normal conditions, the involvement and role of STP13 in plants for monosaccharide uptake increased under high salt stress conditions (Yamada et al., 2011). This gene is the sole one described as an abiotic stress inducible gene (Lee & Seo, 2021). During salt stress, neither stp1-1 nor stp13-1 have shown an increase in monosaccharide absorption. The expression of STP1 in epidermal cells is responsible for this decline (Yamada et al., 2011). Taken together, these studies indicate that *STP13* has a significant impact in biotic and abiotic stress in plants (Lee & Seo, 2021).

2.5 Banana Biological System Required for Studying the Function of a Target/Candidate Genes of Banana

Once candidate genes with functions related to abiotic stress have been identified, such as by bioinformatics or associative studies, there is a need to validate the function in a biological system. Developing an effective biological system and gene delivery method is indispensable to elucidate the function of a target gene (Jafari et al., 2015). Banana plantlets have been propagated through tissue culture technique, which included developing methods for the establishment of embryogenic cell suspension via embryogenic callus (Chin et al., 2014; Jafari et al., 2015; Rayis & Abdallah, 2015) and protoplast culture (Haicour et al., 2009). There are several advantages of plant tissue culture, which includes: a small size of explant (reproduction component) could be used

as starting material, the ability to produce large numbers of plants without the use of seeds or pollinators, the ability to grow mature plants in a limited amount of area and time, cultivation takes place in sterile circumstances with carefully managed environmental parameters (sufficient temperature and light) to reduce the risk of disease, pests, or pathogens being transmitted, plant production from seeds with a low germination rate, plant regeneration using genetically engineered cells, easy to care, and these plants can be moved and stored until needed, regardless of the season or weather (Rayis & Abdallah, 2015).

2.5.1 Plant tissue culture

Tissue culture can be defined as the aseptic culture of cells, tissues, organs or whole plants under controlled nutritional and environmental conditions, and the resultant clones are true-to-type of the selected genotype (Hussain et al., 2012). The development of tissue culture techniques rest upon two properties of plant cells: cell totipotency (Su et al., 2021) and cell plasticity (Fehér, 2015). To adapt to environmental stresses, plants have a remarkable developmental plasticity, and plant cell totipotency is an example of the plant plasticity (Bidabadi & Jain, 2020). Haberlandt (1902) proposed cell totipotentiality is the ability of living cells to kept its genetic information as an origin to divide and differentiate into new genetically identical cells, followed by developing tissues, organs, systems, and complete individuals. It is possible that only a few specific types among these embryonic cells do acquire totipotency to develop into somatic embryos (Su et al., 2021). Plant regeneration is the morphogenic response of tissues towards the stimulus and produce organs or complete plants (Sugimoto et al., 2019). Plant tissue culture, uses explants from donor plants which can be regenerated via organogenesis or somatic embryogenesis.

Organogenesis is the formation of a vegetative tissue, such as shoot and root, to form complete plants, characterized by being polar, which means that only one aerial organ or

root is emitted and from this a new complete plant is regenerated (Laxmi & Giri, 2003). A developed plant component, such as a root, stem, or leaf, can be used to obtain the cells for an explant (the plant part to be used in culture). The explant, or plant portion to be cultured, has highly differentiated cells, which is the foundation for this approach. When an explant is put in artificially supplemented nutritive medium, the differentiated cells begin to dedifferentiate (revert to an undifferentiated state) and generate a clump of disorganised cells called as "callus." In response to specific growth regulators (plant hormones) introduced to the medium, the callus cells redifferentiate and generate the desired tissue embryo (García-Gonzáles et al., 2010). The tissue then grows into an organ. Single cells may be cultured then coaxed to generate shoot as well as root after the other using the correct plant hormone combinations. The relative amounts of plant growth regulators (hormones) play an important role in differentiation (García-Gonzáles et al., 2010). Auxins and cytokinins are two significant classes of plant hormones that play critical functions in plant tissue culture. Root differentiation is aided by auxins like naphthalene acetic acid (NAA) and indole-3-acetic (IAA), while shoot differentiation is aided by cytokinins like adenine and kinetin (Laxmi & Giri, 2003). A callus is normally formed when auxin and cytokinin levels are balanced. The ratio or numerical relationship of auxins and cytokinins is known to affect root and shoot differentiation. This idea is used to *in vitro*-grown plant cells and tissues. To induce shoot and root development, the *in vitro* culture is introduced with kinetin and IAA successively in the proper ratios and quantities (Laxmi & Giri, 2003).

Somatic embryogenesis is the production of embryos from somatic plant cells (any non-sexual cell) to obtain a complete plant, which is a polar process where the aerial structures and roots of the plants are obtained from the somatic embryo (García-Gonzáles et al., 2010). Unlike zygotic embryos, somatic embryos can be developed into whole plants without undergoing the process of sexual fertilization (Hussain et al., 2012). Both

organogenesis and somatic embryogenesis may be direct, if the process originates from the initial explants, or indirect if through callus induction (García-Gonzáles et al., 2010). A collection of solitary cells or a tissue developed on a semi-solid nourishing surface is a starting point for the creation of a somatic embryo. A cell divides indefinitely, resulting in a cell aggregate. The clump of cells goes through a number of phases, such as globular, heart-shaped, and torpedo. The mature phase refers to the torpedo phase. The start of the culture is on a semi-solid nourishing surface, and the callus is then moved to a liquid media that is aerated and well stirred. The callus has been divided into cells, each of which will grow into a somatic embryo. To obtain a regenerated plant, a semi-solid nourishing surface is used to grow mature somatic embryos after being selected at their mature phases (Hussain et al., 2012).

The formation of embryogenic cell cultures is the most choice of *in vitro* culture of bananas (Strosse et al., 2006) (Figure 2.4). When an embryogenic callus is produced on a solid medium with high auxin concentrations, it can be moved to a liquid medium and produce embryogenic cell suspensions. These high-regeneration-capacity suspensions are the source of regenerable protoplasts in bananas and can be exploited for bulk clonal propagation. More importantly, they are the primary target material for genetic engineering and induced mutations (Strosse et al., 2006).

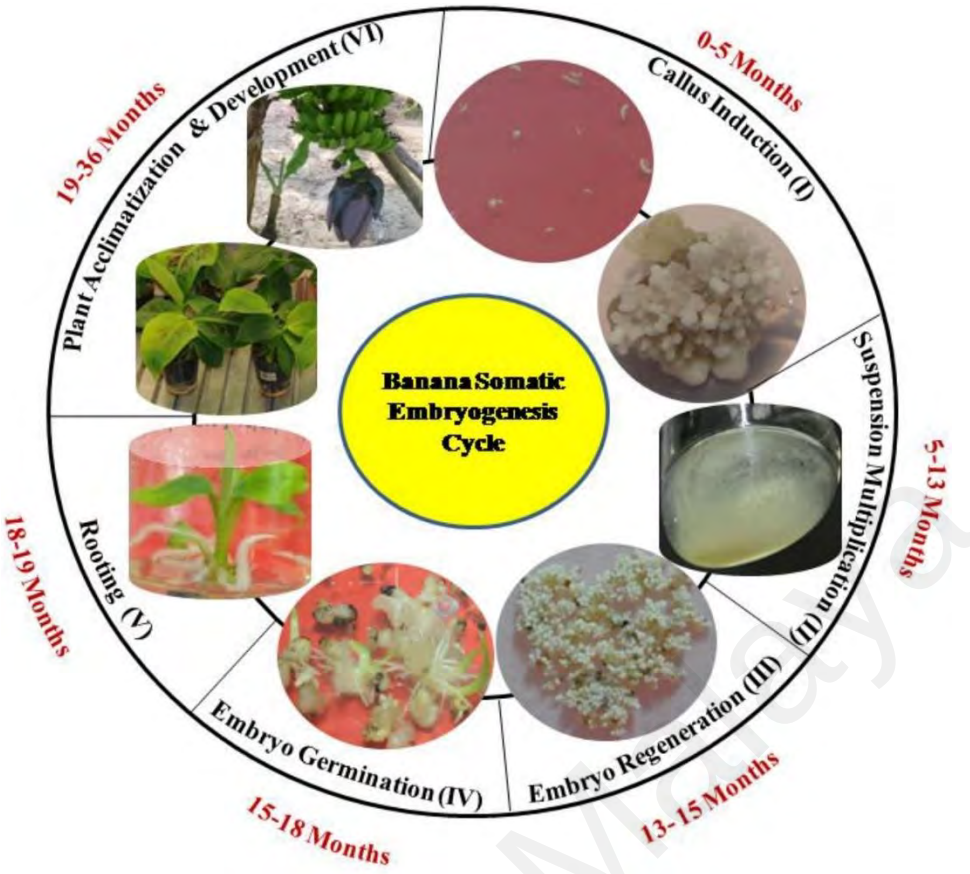

Figure 2.4: Stages of somatic embryogenesis in bananas. The following are the stages of the banana somatic embryogenesis cycle: (I) Induction of calluses (0–5 months) (II) Multiplication and Suspension (5–13 months) (III) Regeneration of the embryo (13–15 months) (15–18 months) (IV) Embryo germination (V) Rooting (18–19 months) (VI) Plant growth and acclimatisation (19–36 months). (Adapted from Strosse et al., 2006).

2.5.2 Callus Culture of Banana

Callus is defined as disorganised cell masses (Ikeuchi et al., 2013). Callus forms when there is the presence of cell or tissue differentiation constraints, such as hormone gradients, chromatin regulation or a cell division block. In order to regenerate from callus into the complete plant body, the plant cells will undergo transdifferentiation to increase developmental potency and/or cell proliferation. However, only rooty, shooty or embryogenic calli can display organ regeneration, friable or compact callus do not have the ability for regeneration (Figure 2.5) (Ikeuchi et al., 2013).

To identify the difference of embryogenic callus and non-embryogenic callus, Jafari et al. (2015) studied the morpho-histological profiles during the developmental stages of somatic embryogenesis of *Musa aciminata* cv. Berangan (Figure 2.6). After 5–6 months of culture, transparent pro-embryos were formed from yellow globular callus in the banana cultivar Berangan. The pro-embryos then matured into somatic embryos that were globular and torpedo-shaped. With the formation of starch reserves, the somatic embryos had a visible presence of protoderm encircling the highly meristematic embryogenic cells (Jafari et al., 2015).

In vitro banana callus culture has been established using a variety of explants. The regularly utilised explant includes the shoot tip and inflorescence (Table 2.1). The apical meristem of banana suckers, for example, can be employed to promote direct regeneration, while male inflorescence can generate both direct and indirect regeneration (Kulkarni et al., 2006). However, banana suckers are heavily contaminated with soil microorganisms, as a consequence it is proned to produce clean culture from inflorescence (Resmi & Nair, 2007; Darvari et al., 2010). Apart from that, when male floral meristems were employed, no somaclonal variation was discovered (Harirah & Khalid, 2006).

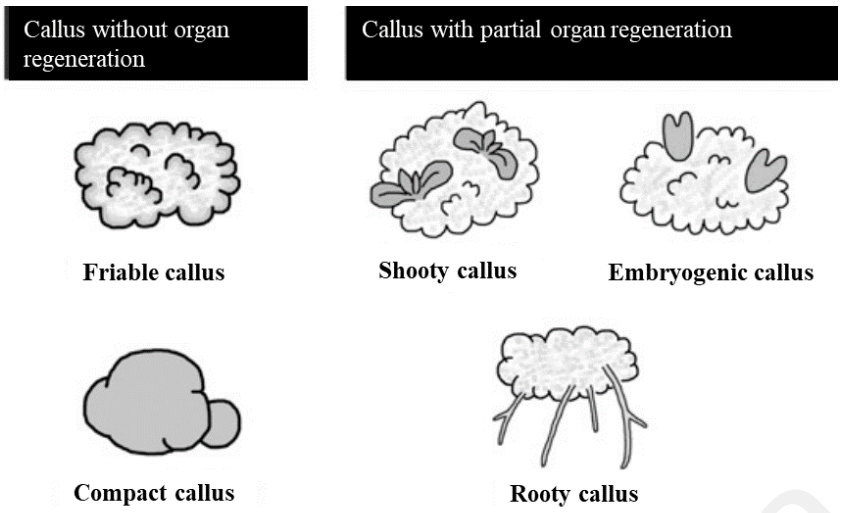

Figure 2.5: Calli phenotypic characterization. Calli without any obvious organ regeneration are friable or compact callus depending on their tissue characteristics. Calli with some degrees of organ regeneration are often called rooty, shooty, or embryonic callus depending on the organs they form. Adapted from Ikeuchi et al. (2013).

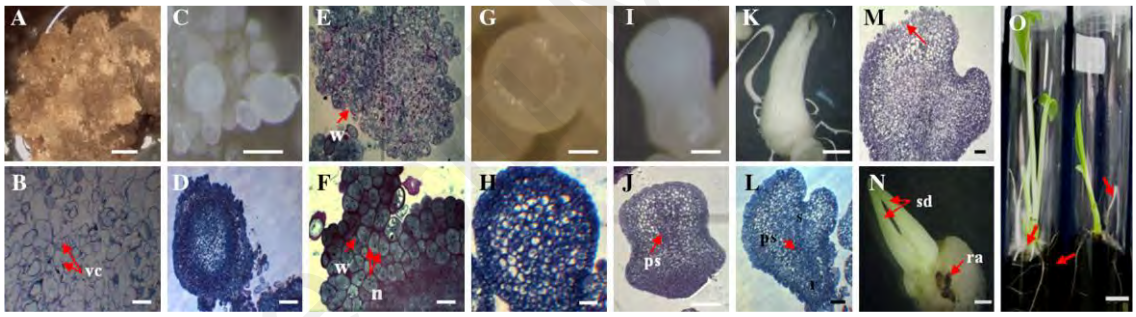

Figure 2.6: The morpho-histological alterations that occur throughout somatic embryogenesis. a Callus that is non-embryogenic (bar 1 mm). Non-embryogenic callus histology (bar 25 lm). c Male inflorescence Embryogenic callus (bar 200 lm). d Embryogenic callus histology (bar 50 lm). e Cells cultivated in M2b media have unclear nuclei and poor protein storage (bar 50 lm). f Meristematic cells grown in M2a medium with distinct nuclei (bar 50 lm). g Embryo globular (bar) (Lm: 250). h Globular embryo histology (bar 100 lm). I Torpedo stage of maturity (bar 500 lm). j A mature torpedo embryo's histology (bar 200 lm). j Embryo that has germinated (bar 1 mm). l Germinated embryo histology (bar 200 lm). m Protodermal layer with irregularities (bar 100 lm). n A germinated mature embryo (bar 1 mm). o Plantlets with roots that are generated from

somatic embryos (bar 1 cm). vc vacuolated cells, w wall, n nucleus; ps procambial strand, s shoot pole, r root pole; ps procambial strand, s shoot pole, r root pole. (Adapted from Jafari et al., 2015)

Table 2.1: Banana callus induction methods.

Type of explants	Variety of *Musa*	Media composition for callus induction	References
Immature male flowers	*Musa* (ABB group) 'Kluai Namwa' and *Musa* (ABB group) 'Kluai Hakmuk'	MS media supplemented with 4 mg L-1 2,4-D incorporated with 1 mg L-1 NAA, 1 mg L-1 biotin and 30 g L-1 sucrose.	Karintanyakit et al. (2011)
Leaf and stem	*Musa sapientum* cv. Anupam	Murashige and Skoog (MS) medium containing 2,4-Dichlorophenoxyacetic acid [2,4-D] (3mg/L) + 1-Naphthaleneacetic acid [NAA] (0.5mg/L)	Pervin et al. (2013)
Sword sucker	*Musa paradisiaca L.* cv. Udhayam	MS media supplemented with 2,4-D (4.00 mg/L)	Shukla et al. (2020)
Immature male flowers	*Musa acuminata* Cavendish	MS media supplemented with glutamine (400 mg/L), sucrose: maltose (20:20 g/L) in maturation media	Nandhakumar et al. (2018)
Male flowers	Banana cultivars cv. Sabri, Gine and Ranginsagar	Murashige and Skoog media supplemented with 2 mg/l 2,4-D + 1mg/l NAA + 1mg/l IAA + 1mg/l Biotin + 1mg/l glutamine and 3% (w/v) glucose	Hossain et al., (2009)

2.5.3 Banana protoplast

Protoplasts are naked cells without cell walls, which can occur naturally in some organisms, but in plant research is achieved when cell walls have been removed by either enzymatical digestion or mechanically (Rahmani et al., 2016). Mechanical or enzymatic isolation of plant protoplasts is possible. Mechanical isolation, which involves cutting plant components and releasing protoplasts from the cut surface, is a historically significant technique, although it is rarely employed due to a lack of isolated protoplasts.

Its advantage, however, is that it eliminates the unknown effect of enzymes on protoplasts. Because protoplasts are obtained in large quantities, cells are not harmed, and osmotic conditions may be adjusted, enzymatic isolation is favourable. The level of thickness of cell walls, temperature, time of enzyme incubation, pH of enzyme solution, agitation, and type of the osmoticum are all factors that influence protoplast release efficiency during the isolation process (Sinha et al., 2003).

Protoplasts are totipotent, meaning they may make a new cell wall, divide to create colonies, and mature into calluses from which plants can be regenerated (Rahmani et al., 2016). Protoplast fusion has been utilised to create new cultivars with desirable characteristics from essential plants, particularly those with incompatibility barriers in sexual hybridization (Rahmani et al., 2016). Apart from that, because protoplasts can directly take up foreign genetic materials, the use of protoplast-based technology for genetic improvement of commercially important crops, such as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9-mediated genome editing, has increased (Rahmani et al., 2016).

Depending on the types of tissues and variances in cell wall thickness, enzyme concentration and digestion time influence protoplast release (Davey et al., 2005; Rahmani et al., 2016). The clear fact that some organ elements, such as mature xylem elements and phloem sieve tubes, lack cellular structures that can develop as protoplasts, is one of the protoplast generation challenges linked with organ type and structure. The cell wall of plants is made up of a complex blend of cellulose, hemicellulose, pectin, proteins, and lipids (Davey et al., 2013). The stiffness of the cell wall is caused by cellulose and hemicellulose, while pectin holds adjacent cells together. Pectinases and cellulases make up an enzyme combination used to dissolve the cell wall. The first type detaches cells from one another, while the second breaks down the cell wall's cellulose and hemicellulose (George et al. 2008). As a result, it is important to break down the cell

wall using suitable quantities of cell wall-degrading enzymes (such as cellulase, macerozyme, pectinase, and driselase), which vary depending on the plant species and source tissue (Davey et al., 2013). Cellulases and hemicellulases are commonly used in enzyme digestion (e.g. beta-glucanases, xylanases, protopectinases, polygalacturonases, pectin lyases, and pectinesterases). Cellulase R-10, Macerozyme R-10, and Pectolyase Y-23 are some of the most regularly utilised enzymes or enzyme combinations. Apart from that, digestion time varies depending on enzyme concentration, which is important in protoplast isolation to avoid incomplete digestion (Davey et al., 2013).

Because protoplast cells lack a protective cell wall, osmotica is required to keep them from rupturing or shrinking (Davey et al., 2013). All media that are used to suspend protoplasts, such as the enzyme combination, media used during purification, and the culture medium, typically contain 0.4–0.7 M mannitol or sorbitol. Cells are usually cultured in a preplasmolytic solution for a brief length of time (1 hour) before being switched to enzyme medium. Scarification or slicing of the plant material is usually accompanied with incubation in such a media, which is often treated with salts, sugars, or sugar alcohols. This improves preplasmolytic solution infiltration and, as a result, enzyme infiltration (Davey et al., 2013).

Vacuum infiltration of plant tissue with the enzyme solution, in addition to plasmolysis, can be utilised to ensure that the enzymes reach intracellular cells, potentially increasing protoplast output (Osakabe et al., 2018). Plant tissues are first soaked in an enzyme solution before being vacuum infiltrated. In a vacuum chamber, the submerged plants are then exposed to negative atmospheric pressure. The vacuum sucks air out of the submerged plant tissues' interstitial spaces. When the vacuum is released, the space formerly held by air is filled with enzyme solution, resulting in vacuum infiltration treatment (Osakabe et al., 2018). Vacuum infiltration was used to optimise the

protoplast isolation technique in both apple (*Malus domestica*) and grapevine to achieve the most viable protoplasts per gramme of fresh weight (Osakabe et al., 2018).

The buildup of a positive signal by viable protoplasts can be used to determine viability, for example, by staining with fluorescein diacetate (FDA). The standard and most reliable fluorochrome for measuring protoplast/cell viability is fluorescein diacetate (Widholm, 1972). Protoplast viability should be assessed using fluorescence staining procedures at all stages. The cytoplasmic esterases hydrolyse FDA, which is non-fluorescent and permeable to the plasma membrane, to create fluorescein, which is extremely fluorescent and substantially less permeable to the plasma membrane than FDA. As a result, living protoplasts produce fluorescence (Widholm, 1972).

The following are reports on getting banana protoplasts (Table 2.2). According to previous studies, cell suspension culture is the most generally reported material for banana protoplast isolation, with cellulase, macerozyme, and pectinase being the most commonly employed enzymes. Bakry (1984) reported the first effective isolation of live banana protoplast from Cavendish banana (AAA) inflorescence. Megia (1992) induced banana callus from protoplasts, which was followed by plant regeneration directly from protoplasts (Matsumoto et al., 2010). *In vivo* bracts, leaf explants, slices of shoot tissue, roots (Cronauer, 1986), and immature female flowers were used by Matsumoto et al., (2010) to obtain protoplasts.

Table 2.2: Banana protoplast isolation methods (Matsumoto et al., 2010).

Type of explants	**Variety of *Musa***	**Enzyme composition for isolation**	**References**
Leaf and callus	Various (AA; BB; AAA; AAB; ABB)	2.5% Cellulase R10, 0.2% Hemicellulase (Sigma), 0.3% Pectolyase Y23, 0.6% Macerozyme	Bakry (1984)
Youngest leaf	Cavendish (AAA); *acuminate* (AA)	0.5% Cellulysin. 0.5% Rhozyme HR-150, 0.125% Pectolyase Y23	Chen and Ku (1985)

Bracts	Maca (AAB); Nanica (AAA); *balbisiana* (BB)	0.2% Cellulase R10, 0.2% Macerozyme R 10, 0.2% Driselase	Matsumoto et al. (1988)
Suspension cells	Long Tavoy (AA)	5% Cellulase RS, 2% Pectolyase Y23	Megia et al. (1992)
	Bluggoe (AAB)	1% Cellulase R10, 1% Macerozyme R10, 1% Pectinase	Panis et al. (1993)
	Bluggoe (AAB)	1.5% Cellulase RS, 0.15% Pectolyase Y23, 0.2% Hemicellulase (Sigma)	Megia et al. (1993)
	Maca (AAB)	1.5% Cellulase RS, 0.2% Pectolyase Y23	Matsumoto and Oka (1997)
	Grande Naine (AAA)	2% Cellulase RS, 0.5% Macerozyme (Sigma), 0.2% Hemicellulase (Sigma), 0.25% Pectolyase Y23	Assani et al. (2001)
	Various (AAA); (AAB); (AA)	1.5% Cellulase RS, 0.15% Pectolyase Y23	Assani et al. (2002)
	Mas (AA)	3.5% Cellulase R10, 1% Macerozyme R10, 0.15% Pectolyase Y23	Xiao et al. (2007)

2.6 Banana Transformation Systems Applied in Functional Gene Studies

2.6.1 Protoplast transformation with DNA

The isolated plant protoplasts are capable of uptake foreign substances via polyethylene glycol (PEG)-mediated or electroporation transformation systems (Matsumoto et al., 2010). Protoplast transfection is a single cell event, so the plants regenerated from a transformed protoplast are not chimeric (Matsumoto et al., 2010). The direct introduction of DNA into individual plant cells using PEG or electroporation is known as protoplast transformation (Weeks & Yang, 2017). The DNA might be transiently expressed or permanently integrated into the genome once it enters the nucleus. The most extensively utilised transformation approach is polycation treatment, such as PEG (Weeks & Yang, 2017).

PEG has a significant impact on DNA conformation chimaera (Matsumoto et al., 2010). The neutralisation of the DNA and the membrane charges is thought to induce the polycation to condense the DNA and for DNA to be connected with the membrane (Lenaghan & Neal Stewart 2019). The polycation solution has a high osmotic pressure, which sucks water out of protoplasts while also encouraging protoplast adhesion (Lenaghan & Neal Stewart 2019). The method by which DNA travels from the cell to the nucleus (or chloroplast) is still unknown (Lenaghan & Neal Stewart 2019). Plants that have been grown from altered protoplasts are not chimaera (Matsumoto et al., 2010). The use of protoplasts as a transformation platform has a major benefit over intact tissues or cell suspension cultures since the lack of a cell wall should obviate one barrier to DNA entrance (Lenaghan & Neal Stewart 2019).

Following protoplast transfection or the production of stable transformed lines, additional fluorescent tags can be included into the experimental design via transgenic expression of fluorescent macromolecules (Petersen et al., 2019). Transfection is a faster, more efficient, direct, and well-established for some specific cell types, such as mesophyll protoplasts, than the generation of stable transgenic lines (Petersen et al., 2019). In recent years, *Aequorea Victoria* green fluorescent protein (GFP) has become one of the most widely researched reporter proteins in biochemistry, cell biology, and industry (Ren et al., 2020) In plant systems, GFP has been utilised as an *in vivo* fluorescent marker (Ren et al., 2020). When expressed by living cells, GFP emits a persistent and unique green fluorescence that requires no cofactors or subtracts other than oxygen. GFP-based selection is a positive process because it does not involve the death of non-desirable cells, as does antibiotic resistance-based selection, reducing the risk of cell death and the release of toxic compounds, which could affect the hybrids' viability and regeneration capacity, invalidating the results. As a result, GFP is widely utilised as a reporter in plant biology research and as a scoreable marker in plant genetic transformation (Petersen et al., 2019).

The transitory expression of the gusA reporter gene under control of derivatives of the 35S RNA promoter of the cauliflower mosaic virus (CaMV 35S) was the first demonstration of successful genetic transformation in banana in electroporated protoplasts (Sági et al., 1994). When protoplasts from 1-week-old embryogenic cell suspensions were electroporated with a 960 F capacitor, an electric field strength of 800 V/cm, ASP electroporation buffer, a PEG concentration of 5% (w/v), and a heat shock of 45oC for 5 min before the addition of PEG, approximately 2% of the protoplast population expressed the -glucuronidase (gusA) gene, according to Matsumoto et al. (2010) used a green fluorescent protein (GFP) reporter gene in electroporated protoplasts to perform transient gene expression studies. Matsumoto et al. (2010) found that when protoplasts were electroporated with 40 g/mL of plasmid DNA, an electric field strength of 1250 V/cm for 50 s, and three pulses in ASP electroporation buffer supplemented with 3% (w/v) PEG, more than 25% of the protoplasts survived the protoplast transformation.

2.6.2 *Agrobacterium*-mediated transformation

The hunt for the causal agent of plant diseases such as crown gall, cane gall, and hairy root sparked first scientific interest in the genus *Agrobacterium* (Krenek et al., 2015). *Agrobacterium tumefaciens*, which carries the tumor-inducing (Ti) plasmid, causes galls on the roots and crowns of many dicot angiosperm species as well as some gymnosperms, whereas *Agrobacterium rhizogenes*, which carries the root-inducing (Ri) plasmid, causes abnormal root production on the host plants. The expression of oncogenes found in transferred-DNA (T-DNA) transmitted from these bacteria into the plant nucleus and incorporated into the plant genome causes neoplastic tumor-like cell development (Lacroix et al., 2019).

The presence of two genetic components on the bacterial Ti-plasmid is required for the *Agrobacterium*-mediated plant genetic transformation process (Lacroix et al., 2019).

The T-DNA is the first and most important component, which is characterised by border sequences, which are conserved 25-base pair imperfect repeats at the extremities of the T-region. The virulence (vir) region, which is made up of at least seven main loci (virA, virB, virC, virD, virE, virF, and virG) that encode components of the bacterial protein machinery that mediates T-DNA processing and transfer, is the second. The two-component regulators VirA and VirG stimulate the expression of additional vir genes on the Ti-plasmid. T-DNA from A. tumefaciens is processed, transferred, and integrated into a plant cell by the VirB, VirC, VirD, VirE, and maybe VirF (Krenek et al., 2015).

2.7 CRISPR-Cas9 Genome Editing System

2.7.1 Genome Editing Systems

Genome editing (also called gene editing) methods were evolved to lessen the amount of "foreign" DNA added by genetic transformation system (Yin et al., 2017). Gene editing methods comprise ZFNs, TALENs and CRISPR-Cas9. In 1996, protein domains "zinc fingers" coupled with FokI endonuclease domains were discovered to act as site-specific nucleases [zinc finger nucleases (ZFNs)], which are able to cleave the DNA *in vitro* in strictly defined regions by recognising one triplet of nucleotides by each of the "zinc finger" domains (Kim et al., 1996). The quest for efficient and selective manipulation of target genomic DNA led to the identification of unique transcription activator-like effector (TALE) proteins (Yin et al., 2017).

The CRISPR-Cas9 genome editing system is more efficient, less expensive and simpler compared to other genetic editing systems, such as TALENs and ZFNs (Wu et al., 2019). CRISPR and its associated proteins (cas) have critical roles in developing adaptive immunity to viruses and plasmids in prokaryotes and archaea. CRISPR, which relies on the existence of loci in bacteria, was first found in *E. coli*. Operons for the synthesis of the Cas9 protein (nuclease) and repetitive spacer sequences make up CRISPR

loci (Chen et al., 2019). The protospacer is a small piece (20 bp) of foreign DNA (viral or plasmid) that integrates into the bacterial genome. PAM (protospacer adjacent motif) sequences are detected by bacteria in the invading microbe in the first stage, and the invading microbe then integrates a portion of their genome into its CRISPR locus (Ran et al., 2017). CRISPR expresses the complete locus in the second stage and creates crRNA (short for CRISPR-RNA), an RNA molecule that, along with Cas9 and other essential proteins, forms a hybrid with invading genomes and Cas9 chops the genome. CRISPR functions in prokaryotes to protect bacteria from outside invaders in this way. This natural system of prokaryotes has been used in higher organisms, including plants, for targeted genome editing (Xu et al., 2020).

Generally, CRISPR-Cas9 genome editing in plant genomes consists of several steps. The approach uses a bacterial monomeric DNA endonuclease, Cas9 and a 20 base pair RNA guide sequence, gRNA, to target a specific genomic sequence (Belhaj et al., 2015). The target site contains a protospacer adjacent motif (PAM) sequence, 5'-NGG-3', wherein N can be any nucleotide and G represents guanine, which is involved in triggering the transition between Cas9 target binding and cleavage conformations (Hsu et al., 2014). The binding of the PAM and a matching target triggers Cas9 nuclease activity by activating the HNH and RuvC domains (Nishimasu et al., 2014). After sequence recognition, Cas9 nuclease will cleave the double stranded DNA to form a double-strand break (DSB) (Jinek et al., 2012). The formation of DSBs triggers endogenous DNA repair mechanisms, either nonhomologous end-joining (NHEJ) or homology-directed repair (HDR) (Liu et al., 2017), resulting in the introduction of small indels at the target site (Jiang et al., 2013).

Modified sequence-specific nucleases are created to produce DNA double-strand breaks (DSBs) at specified gene loci, allowing for desired genomic alterations via one of

two DNA repair pathways: non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Schiml & Puchta, 2016). Because it does not require a homologous template sequence and frequently forms indels, NHEJ is the most error-prone repair process in response to DSBs, and as a consequence can create functional gene knockouts. It can also insert homology-independent DNA sequences from a donor, making it a viable technique for gene integration into a pre-determined locus for crop improvement. NHEJ is evolutionarily conserved and active throughout the cell cycle, but it is especially crucial during the G1 cell phase, when no homologous template for recombination is available (Gehrke et al., 2022). NHEJ is ten times faster than HDR at repairing most sorts of breaks. As a result, NHEJ is the primary repair route for the breaks caused by CRISPR/Cas9 mutagenesis. NHEJ can also be utilised to construct donor DNA sequence insertions in a homology-independent way, making it an effective approach for crop improvement gene stacking. NHEJ can be used to accomplish sequence substitution or modifications in plant genomes (Gehrke et al., 2022).

While NHEJ is a capable and effective technique for large-scale gene knockout research, HDR-dependent targeted gene editing is still an unrivalled method for precise genomic modification that been extensively used in plants (Malzahn et al., 2017). Homology-directed repair makes precise sequence modifications or replacements, allowing for precise gene editing (gene targeting). Broken DNA ends are repaired in the case of HDR using sequence homology regions from the genome. In theory, HDR can produce gain-of-function point mutations, which can be useful in genetic investigations and disclose gene function. In somatic cells, HDR is a modest DSB repair pathway that is active mostly during the S and G2 phases of the cell cycle. Even while HR-mediated CRISPR-Cas9 editing has been successful in rice and maize, HDR has shown to be more difficult in higher plants, inhibiting its widespread use, because the target DSB and a repair template must co-exist (Malzahn et al., 2017)

2.7.2 CRISPR-Cas9 Genome Editing System of Plants

The CRISPR-Cas9 technology has been applied effectively in many crops, such as barley (Kapusi et al., 2017), rice (Zhou et al., 2017), maize (Liang et al., 2014), tomato (Brooks et al., 2014), potato (Butler et al., 2015) and banana (Naim et al., 2018; Tripathi et al., 2019). It was discovered in citrus by altering the carotenoid biosynthesis pathway's Phytoene desaturase (CsPDS) gene (Jia & Wang, 2014). CRISPR/Cas9 has also been used to generate citrus canker-resistant plants by knocking out the susceptibility gene CsLOB1 (Jia et al., 2017). The effector binding element (EBEPthA4) is found in several alleles of the CsLOB1 gene found in citrus, and it is recognised by the primary effector PthA4 of Xcc to activate CsLOB1 expression. In addition to citrus, CRISPR/Cas9 genome editing has been demonstrated in watermelon (Tian et al., 2017) and grape (Nakajima et al., 2017) by targeting the Phytoene desaturase gene in both crops.

The *Agrobacterium*-mediated CRISPR-Cas9 system was recently developed to generate targeted gene mutations and corrections in plants (Weinthal & Gürel, 2016) (Figure 2.7a). The use of *Agrobacterium*-mediated CRISPR-Cas9 genome editing in bananas faces some challenges (Naim et al., 2018; Tripathi et al., 2019). In most edible banana cultivars, the T-DNA cannot be eliminated from the banana genome by back-crossing due to its seedless property (Tripathi et al., 2019). Apart from that, the integration of Cas9 transgenes along with gRNA and the selectable marker into the banana genome can lead to the continuous action of editing machinery, gene disruptions, and off-target mutations (Tripathi et al., 2019). Moreover, US Department of Agriculture (USDA) does not impose any GMO regulations to plants with targeted mutagenesis generated by self-repair mechanisms, if they are free from Agrobacterium or any transgenic or foreign genetic materials (Malnoy et al., 2016).

In Europe, there are three classes of site-directed nuclease (SDN) and one class of oligonucleotide-directed mutagenesis (ODM) genome editing experiment according to EU (EFSA Panel on Genetically Modified Organisms, 2012; EFSA Panel on Genetically Modified Organisms, 2020). In SDN-1 applications, only the SDNs are introduced (stably or transiently), generating site-specific mutations by non-homologous end-joining (NHEJ). In SDN-2 applications, homologous repair DNA (donor DNA) is introduced together with the SDN complex to create specific nucleotide sequence changes by homologous recombination (HR). In the SDN-3 technique a large stretch of donor DNA (up to several kilobases) is introduced together with the SDN complex to target DNA insertion into a predefined genomic locus by HR or by NHEJ. Oligonucleotide-directed mutagenesis (ODM) is an approach that uses oligonucleotides to introduce targeted mutations on one or a few adjacent nucleotides in the genome, resulting in genetic changes including substitutions, insertions, or deletions. (EFSA Panel on Genetically Modified Organisms, 2020).

According to the classification of the genome editing system, mostly SDN-1 application is accepted in many countries as Table 2.3. In response to these concerns, optimizing CRISPR-Cas9 mediated genome editing to avoid transgene integration is imperative and regulation-accepted. The proposal emerged to adapt genome-editing strategies such that the synthetic nuclease is provided in its active form, analogous to chemical mutagens in traditional breeding, rather than being expressed in the transformed cell from a recombined nucleic acid (Figure 2.7b) (Wolter & Puchta, 2017).

recombined nucleic acid (Figure 2.7b) (Wolter & Puchta, 2017).

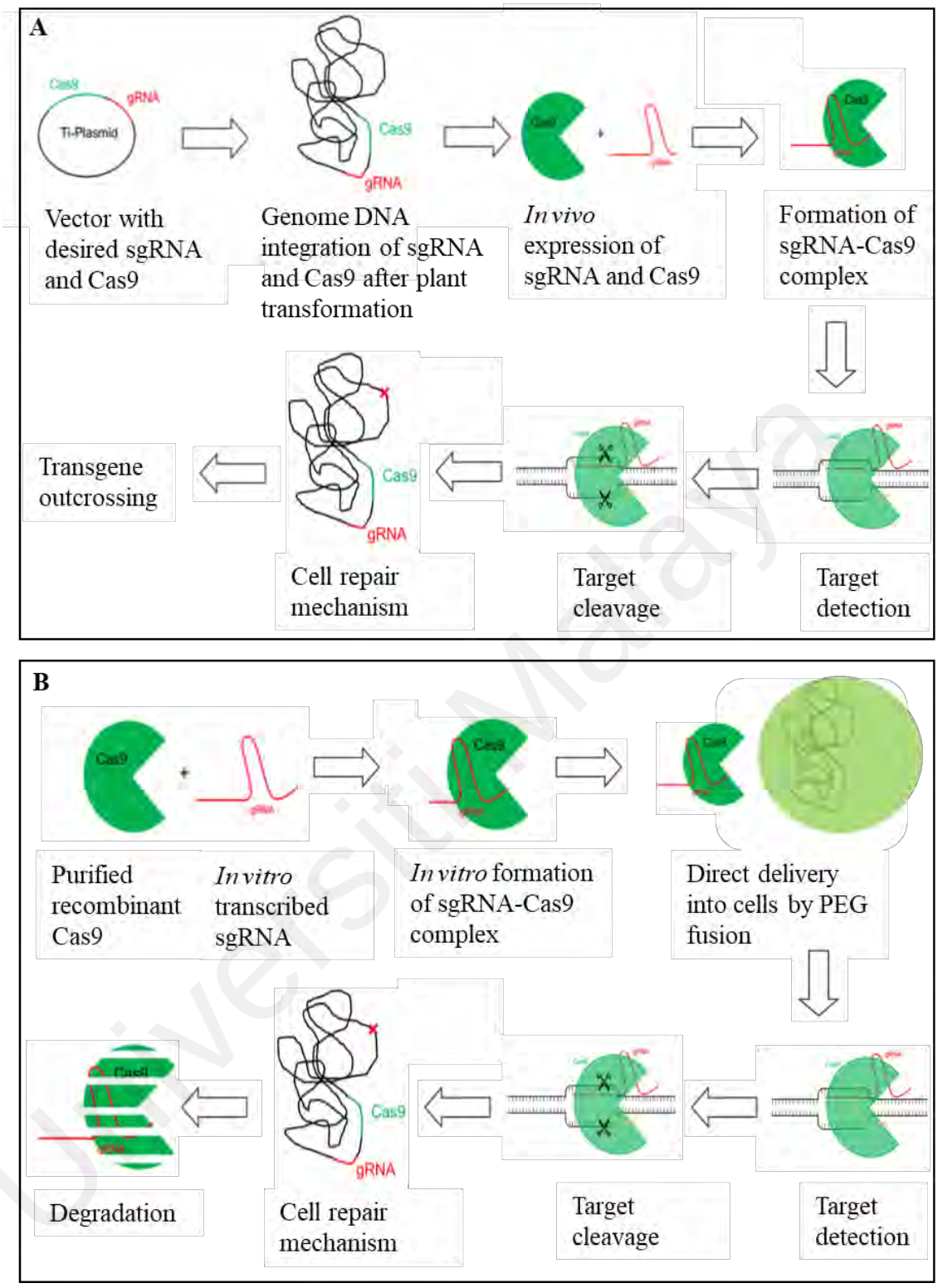

Figure 2.7: Common approaches to delivering components of the CRISPR-Cas9 gene editing system into plant cells include: (a) the *Agrobacterium*-mediated CRISPR-Cas9 gene editing method and (b) the DNA-free CRISPR-Cas9 system. Adapted from Metje-Sprink et al. (2018).

Table 2.3: Classification of products (GMOs) obtained through oligonucleotide-mediated genome modification (ODM), site-directed nuclease 1 (SDN1), SDN2 and SDN3 in different countries. SDN-1 application = only SDNs are introduced stably or transiently to generate site-specific mutations by non-homologous end-joining (NHEJ); SDN-2 application = donour DNA and SDN are introduced to generate site-specific mutations by homologous recombination (HR); SDN-3 application = donor DNA with several kilobases and SDN are introduced to generate site-specific DNA insertion by HR or by NHEJ; ODM application: oligonucleotides are introduced to generate targeted mutations on one or a few adjacent nucleotides; "+ve" = GMO; "-ve" = non-GMO. Adapted from Hamburger, 2019.

Countries	ODM	SDN-1	SDN-2	SDN-3
Argentina	-ve	-ve	-ve	-ve
Australia	+ve	-ve	+ve	+ve
The EU	+ve	+ve	+ve	+ve
Japan	-ve	-ve	-ve	+ve/-ve
The USA	-ve	-ve	-ve	+ve/-ve

2.7.3 The DNA-Free CRISPR-Cas9 Genome Editing System of Plants

Many efforts are being made to optimise the CRISPR-Cas9 system to avoid transgene integration (Table 2.4). For example, Woo et al. (2015) introduced a combination of purified Cas9 enzyme and gRNA, which comprised a ribonucleoprotein (RNP) complex, to produce stable nucleotide alterations in rice, tobacco, lettuce, and *Arabidopsis thaliana*. Since the DNA-free CRISPR-Cas9 via RNPs does not require intracellular transcription and translation, this technique is faster than a plasmid-based transformation approach for editing plant genomes (Park et al., 2019). There is no need for codon optimization or specific promoters for expression in plant cells when RNP is directly used (Yu et al., 2020). Also, the direct RNP method involves of shorter time of enzymatic action (Park et al., 2019). RNP cleaves chromosomal target sites immediately after transfection, as shown in cultured human cells, and is rapidly destroyed by endogenous proteases in cells, potentially reducing mosaicism and off-target effects in regenerated entire plants (Sung et al., 2014). In addition, *in vitro* prescreening on gRNA could be carried out in DNA-free CRISPR-Cas9 system (Sung et al., 2014). The gene editing results in a permanent change in the genome, the CRISPR-Cas9 reagents do not need to be produced or survive in the cell once the gene editing event has happened (Sung et al., 2014).

DNA-free CRISPR-Cas9 genome edited grapevine and apple was established, which the preassembled Cas9 protein-gRNA ribonuleoproteins (RNPs) was directly delivered into plant cells (Malnoy et al., 2016). In this way, the RNPs edit the target sites immediately after delivery and are then degraded by endogenous proteases in cells (Malnoy et al., 2016). Tripathi et al. (2019) suggested that preassembled RNPs can be directly delivered into banana embryogenic cells by particle bombardment or can be delivered to banana protoplasts through PEG, and the regenerated edited banana plants would bypass GM legislation (Figure 2.8).

For regulating economically relevant features in crop plants, fidelity in genome editing is vital (Park et al., 2019). Any unwanted or off-target mutation could result in large alterations in the plant genome, raising various regulatory difficulties. All genome editing methods with a broader applicability in biology, medicine, or agriculture must have target specificity. Genome editing techniques can efficiently cut on-target locations in the genome, causing site-specific double stranded breaks (DSBs), but they can also cause off-target changes at homologous sites. Off-target mutations can cause cytotoxicity, apoptosis, and chromosomal inversions, deletions, and translocations (Park et al. 2019). As a consequence, when trying to generate faultless DNA-free genetically modified crop plants, the target sequence, different versions of sgRNAs, nickases and paired nickases, Cas9 protein and nuclease delivery directly are the main considerations (Liu et al., 2018).

Before the sequences to be targeted, in 20-nt sequences, it's critical to create a unique target sequence that differs from every other place in the genome by at least 2 or 3 nucleotides (Woo et al., 2015). With a perfect match in the 12-nt long PAM proximal sequence (seed sequence), CRISPR/Cas9 discriminates efficiently against putative off-target locations。

Using the Cas9 recombinant protein instead of the Cas9 encoding plasmid reduces off-target mutations even more (Kim et al., 2014; Liu et al., 2018). Cas9-sgRNA ribonucleoprotein (RNP) complexes delivered directly cause mutation at target locations soon after delivery and breakdown quickly by endogenous proteases, minimising off-target mutations without compromising on-target efficiency.

A DNA-free CRISPR-Cas9 system of bananas was recently established by introducing RNP components into banana protoplasts through PEG-mediated transformation (Wu et al., 2020). However, the protoplast transfection frequency and mutation efficiency were lower than those reported for cabbage (Park et al., 2019) and Chinese cabbage (Murovec et al., 2018). As a consequence, an efficient and improved DNA-free CRISPR-Cas9 genome editing system of banana should be established to study the *STP13* gene of bananas.

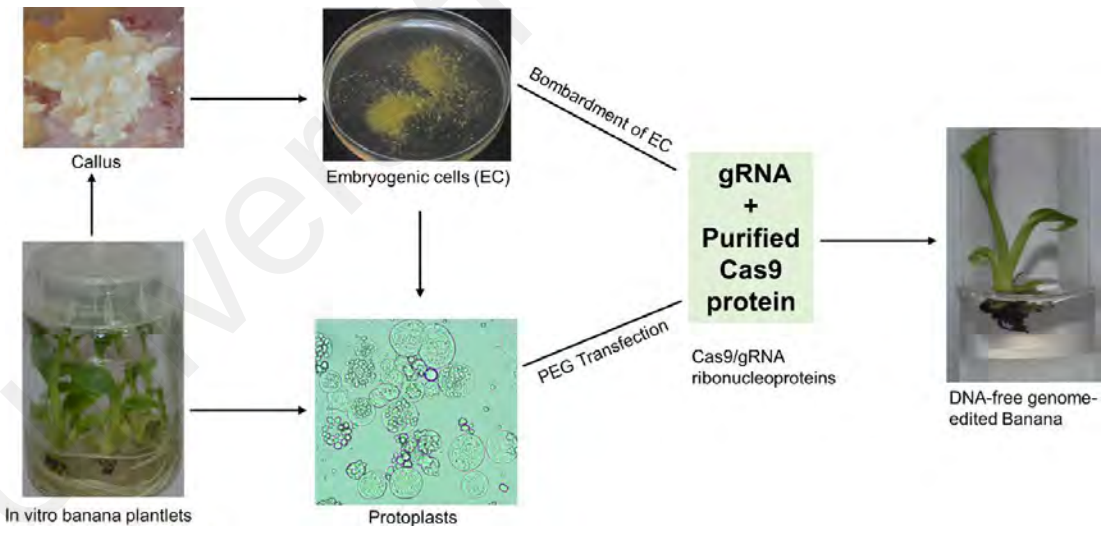

Figure 2.8: Schematic diagram showing generation of DNA-free genome-edited banana by delivering preassembled Cas9 protein-gRNA ribonucleoproteins (RNPs) directly into embryogenic cells or protoplasts and regeneration into complete plants (Tripathi et al., 2019).

Table 2.4: Summary of mutation efficiency of protoplast-mediated DNA-free CRISPR-Cas9 system in plants that analysed with targeted deep sequencing.

Plant	Target gene	Gene function	Indel percentage (%)	Reference
Apple	*DIPM-1, DIPM-2, and DIPM-4*	Increase resistance to fire blight disease.	0.50-6.90	Malnoy et al., 2016
Arabidopsis	*PHYB (PHYTOCHROME B)*	Photoreceptors	16.00	Woo et al., 2015
Banana	*PDS (phytoene desaturase)*	Key enzyme in the carotenogenic pathway	0.19-0.92	Wu et al., 2020
Cabbage	*FRI*	Vernalization determinant FRIGIDA	0.09-2.25	Murovec et al., 2018
Cabbage	*GIGANTEA (GI)*	Flowering-time regulator	2.00	Park et al., 2019
Chinese cabbage	*FRI*	Vernalization determinant FRIGIDA	1.15-24.51	Murovec et al., 2018
Grapevine	*MLO-7*	Increase resistance to powdery mildew	0.10	Malnoy et al., 2016
Petunia	*F3H*	Flower colour	9.99-26.27	Yu et al., 2020
Potato	*GBSS*	Encode granule bound starch synthase	9.00	Andersson et al., 2018
Rice	*Cytochrome P450*	Controlling plant organ growth	19.00	Woo et al., 2015
Rice	*DWD1*	Heat stress response	8.4	Woo et al., 2015
Tobacco	*AOC (allene oxide cyclase)*	Key enzyme in the jasmonate biosynthetic pathway	44.00	Woo et al., 2015

CHAPTER 3: METHODOLOGY

3.1 Experimental Design

The experimental design was arranged into three major phases: (I) establishing the plant culture and optimisation of a protoplast transfection system for *Musa acuminata* (*M. acuminata*) cv. Berangan; (II) confirmation of the guide RNA (gRNA) targeting *STP13* (*STP13*gRNA) of *M. acumintata* cv. Berangan through *in vitro* gene cleavage by ribonucleoprotein (RNP) complex; (III) editing of *STP13* and confirmation of the gene edited product (Figure 3.1).

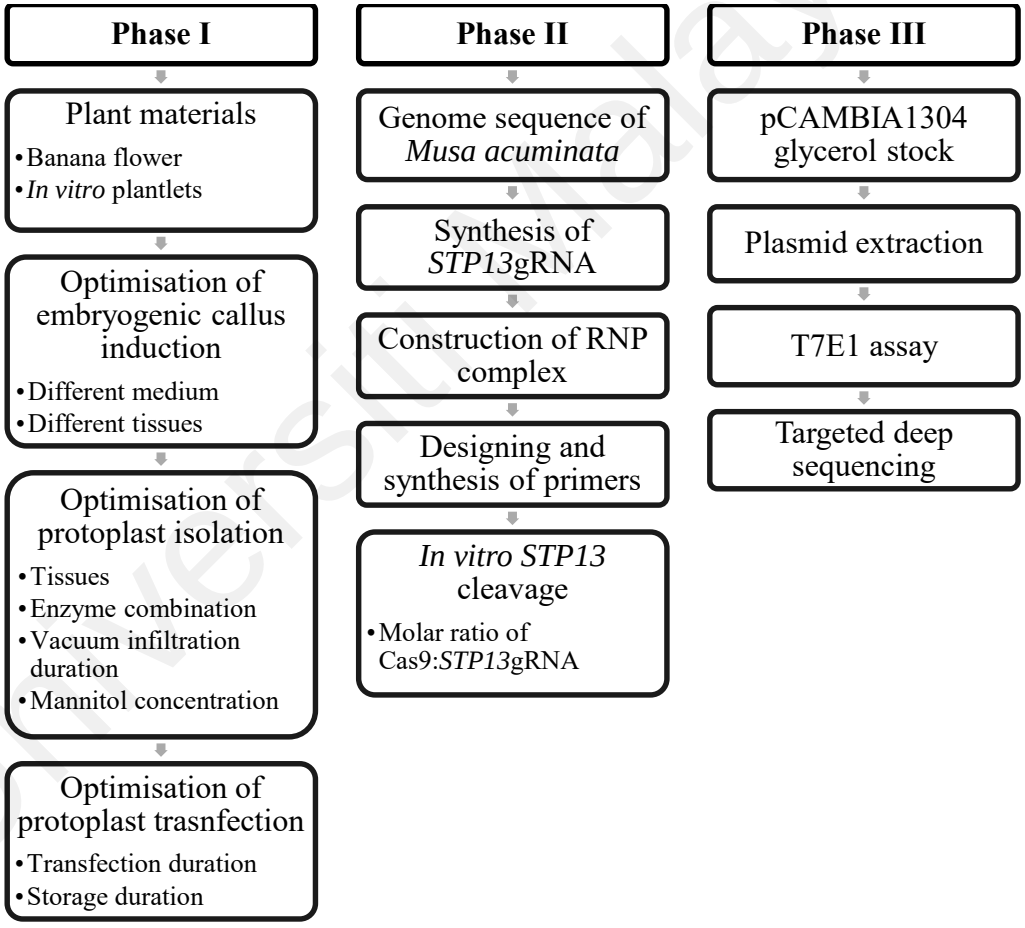

Figure 3.1: Experimental design. Phase I: Establishing the plant culture and optimisation of a protoplast transfection system for *M. acuminata* cv. Berangan; Phase II: Confirmation of the guide RNA "*STP13*gRNA" through *in vitro* gene cleavage by RNP complex; Phase III: Gene editing of the *STP13*.

3.2 Establishment of Embryogenic Callus and Protoplast Isolation and Transformation Procedures

3.2.1 Induction of Embryogenic Callus

Male flowers of *M. acuminata* cv. Berangan (AAA), obtained from a local market at Jalan 17/1a, Seksyen 17, Petaling Jaya, Selangor Darul Ehasan, Malaysia, were used for optimisation of embryogenic callus induction and protoplast isolation. Buds from male flowers of banana cv. Berangan were cut into 5 cm × 5 cm pieces and surface sterilised in 70% (v/v) ethanol for 5 minutes with agitation in a laminar air flow cabinet (Esco, Singapore). The flowers were rinsed thrice with sterile distilled water (2 minutes each) before trimming into a size of 2.0-3.0 cm in length. Immature male flower clusters were further excised from positions 1 to 15 (where 1 is the flower cluster closest to the meristematic dome) and placed on callus induction media M1, M2, M3, M4 and M5 that modified from Chin et al. (2014) as listed in Table 3.1. The explants were incubated at 25 ± 2°C in the dark until callus was induced.

In vitro plantlets of *M. acuminata* cv. Berangan that purchased from Granatech, Universiti Malaya were maintained on culture media containing Murashige and Skoog (MS) basal salts (Duchefa Biochemie, The Netherlands) and 3% (w/v) sucrose (Duchefa Biochemie, The Netherlands). Buds from *in vitro* plantlets of banana cv. Berangan were sliced into pieces of 2-3 mm and cultured on M1 medium as formulated in Table 3.1. The cultures were incubated at 25 ± 2°C in the dark and observed weekly.

Table 3.1: Culture media for embryogenic callus induction of banana cv. Berangan.

The highlighted rows indicated the optimised parameters.

Components (Manufacturing company, country)		**Media**				
		M1 media	M2 media	M3 media	M4 media	M5 media
1-naphthaleneacetic acid (Duchefa Biochemie, The Netherlands)		1 mg/L	1 mg/L	1 mg/L	1 mg/L	1 mg/L
2,4-dichlorophenoxyacetic acid (Duchefa Biochemie, The Netherlands)		4 mg/L	4 mg/L	4 mg/L	4 mg/L	4 mg/L
Ascorbic acid (Nacalai Tesque, Japan)		10 mg/L	10 mg/L	10 mg/L	10 mg/L	10 mg/L
Biotin (Nacalai Tesque, Japan)		1 mg/L	1 mg/L	1 mg/L	1 mg/L	1 mg/L
Gelrite (Duchefa Biochemie, The Netherlands)		2 g/L	2 g/L	2 g/L	2 g/L	2 g/L
Murashige and Skoog basal salts (Duchefa Biochemie, The Netherlands)		4.2 g/L (Full-strength)	2.1 g/L (Half-strength)	1.05 g/L (Quarter-strength)	4.2 g/L (Full-strength)	2.1 g/L (Half-strength)
Myo-inositol (Nacalai Tesque, Japan)		100 mg/L	100 mg/L	100 mg/L	100 mg/L	100 mg/L
Sucrose (Duchefa Biochemie, The Netherlands)		30 g/L	30 g/L	30 g/L	30 g/L	30 g/L
Dhed'a Vitamins	Glycine (Duchefa Biochemie, The Netherlands)	0.002 mg/mL	0.002 mg/mL	0.002 mg/mL	-	-
	Nicotinic acid (Duchefa Biochemie, The Netherlands)	0.005 mg/mL	0.005 mg/mL	0.005 mg/mL	-	-
	Pyridoxine HCl (Duchefa Biochemie, the Netherlands)	0.005 mg/mL	0.005 mg/mL	0.005 mg/mL	-	-
	Thiamine HCl (Duchefa Biochemie, the Netherlands).	0.005 mg/mL	0.005 mg/mL	0.005 mg/mL	-	-

3.2.2 Optimisation of Protoplast Isolation

The isolation of protoplasts was done according to Haïcour et al. (2009) with modifications. Immature male flowers and bracts (one gram each) were cut into 1mm × 1 mm pieces. S1 buffer [67 mM $CaCl_2$ (Merck, Germany), 204 mM KCl (Duchefa Biochemie, The Netherlands), 100 mg/L ascorbic acid (Duchefa Biochemie, The Netherlands), 0.4, 0.5 or 0.6 M mannitol (Duchefa Biochemie, The Netherlands), pH5.6] were used for plasmolysing the tissues for 45 minutes. The S1 buffer was discarded. The tissues were incubated with freshly-prepared enzyme solution as listed in Table 3.2. The mixture was subjected to different vacuum infiltration treatment as listed in Table 3.3. Next, the mixture was incubated in the dark, agitated at 35 rpm, at 25°C for 2.5, 4 or 15 hours. The mixture was filtered through a pre-wet 40 µm cell strainer (Biologix, China). The filtered mixture was topped-up with S1 buffer to 10 mL and centrifuged at 150 × g for 10 minutes at 18°C in a fixed-angle rotor centrifuge (Eppendorf, Germany). The pellet was resuspended gently in 10 mL S2 buffer and centrifuged at 150 × g for 10 minutes in a fixed-angle rotor centrifuge machine. After discarding 8 mL of the supernatant, the protoplast pellet was resuspended gently in the remaining 2 mL buffer and overlaid gently on top of 8 mL S1-25S buffer [67 mM $CaCl_2$, 204 mM KCl, 25%(w/v) sucrose, pH5.6]. The mixture was centrifuged at 500 × g for 5 minutes in a swing-bucket rotor centrifuge (Centurion, United States). The protoplast layer from the interface of the solutions was carefully pipetted into a clean tube and placed on ice. The cell staining was made by mixing 49 µL of purified protoplast suspension and one µL of 5 mg/mL fluorescein diacetate (FDA) (EMD Millipore, United State). A total of 10 µL of stained protoplast suspension was loaded into an Improved Neubauer hemocytometer (Weber, England). Protoplasts at the four outer squares and in the middle square of the grid were counted under a bright field or fluorescence light inverted microscope (Olympus IX73, Japan) at a magnification of 10×. The protoplast yield and the viability rate of protoplasts was

calculated (n=3) using the Equation 1 and Equation 2 respectively as described by Wen et al. (2020).

$$\text{Protoplast yield (cell/g fresh weight)} = \frac{\text{Protoplast count in} \times 10^4 \times \text{Protoplast volume} \times \text{Dilution factor}}{5} \quad (1)$$

$$\text{Viability of protoplast (\%)} = \frac{\text{Number of protoplast stained green in view} \times 100\%}{\text{Number of protoplast in view}} \quad (2)$$

The microscope images were captured with digital microscope camera EM-CCD (Hamamatsu Photonics K.K., Japan) and processed using Adobe Photoshop CC software (Adobe Inc., United State) by adjusting merely the brightness/contrast and exposure. The results were analysed using SPSS software (SPSS Inc., Chicago, IL, United State). The significant difference was analysed by two-tailed *t*-test. Multiple comparisons were performed with the Tukey HSD test, where $P \leq 0.01$ was considered as significant difference (Tukey, 1949). The graphs were produced by using SPSS software.

Table 3.2: Enzyme treatments for optimisation of protoplast isolation. Enzyme solution was prepared by dissolving enzyme powder in 6 mL of S1 buffer, warmed at 50°C for 10 min, agitated on an orbital shaker for 20 -30 min at 50 rpm and filter sterilized through a 0.45 µm sterile syringe filter (Membrane Solutions, China).

Enzyme treatment	Percentage (w/v) % of the enzymes			Digestion time (h)
	Cellulase RS (Duchefa Biochemie, The Netherlands)	Macerozyme R-10 (Duchefa Biochemie, The Netherlands)	Pectolyase Y23 (Yakult Pharmaceutical, Japan)	
1	1.0	2.0	-	2.5
2	1.0	2.0	-	4.0
3	1.0	2.0	-	15.0
4	1.0	1.0	0.5	2.5

Table 3.3: Vacuum infiltration treatments for protoplast isolation. For two-step vacuum infiltration, the vacuum of the first session was released fully by opening the desiccator for one minute then closing before starting the second session of vacuum infiltration.

Vacuum infiltration treatment	Vacuum infiltration time (min)	Number of treatments
A	0	0
B	20	1
C	10	2
D	30	1
E	15	2

3.3 Construction of RNP Complex and *In Vitro* Cleavage Assay

3.3.1 Confirmation of the Design of *STP13*gRNA

*STP13*gRNA was designed by the research committee from CEBAR molecular lab as 5'-CGAGGCGAAGATAACGCCGG-3'. The sgRNA was designed 3 bp upstream of the PAM site (5'-NGG-3') in the *STP13* DNA sequence (Malnoy et al., 2016). The potential off-target mutations for *STP13*gRNA were predicted using the CRISPR web-tool Breaking-Cas (http://bioinfogp.cnb.csic.es/tools/breakingcas).

3.3.2 Formation of Ribonucleoprotein (RNP) Complex

The designed crRNA, tracrRNA, and Cas9 protein were synthesized by Integrated DNA Technologies, Inc. (IDT, Singapore). A total of 10 µM of the designed crRNA and 10 µM of tracRNA were mixed in nuclease-free duplex buffer (IDT inc., United State). The mixture was heated at 95ºC for 5 minutes before cooling to room temperature to obtain 10 µM *STP13*gRNA duplex. Three combinations of *STP13*gRNA duplex and Cas9 were used to form RNP complex, i.e., one µM of *STP13*gRNA duplex and one µM Cas9; one µM of *STP13*gRNA duplex and three µM Cas9; or three µM of *STP13*gRNA duplex and one µM Cas9.

3.3.3 DNA Isolation From Banana Protoplasts

The genomic DNA of the protoplasts was isolated using sodium dodecyl sulfate (SDS) method (Dellaporta et al., 1983) with modifications. The isolated protoplasts were centrifuged at 150 × g for 5 minutes. The pellets were diluted with MMG buffer [0.5 M mannitol, 30 mM $MgCl_2$ (Sigma-Aldrich, United State) and 4 mM MES hydrate (Sigma Aldrich, United State), pH5.6] to a final concentration of about 1×10^6 cells. A total of 200 µL SDS extraction buffer [200 mM Tris (Duchefa Biochemie, The Netherland), pH8.0; 10 mM EDTA (Fisher Scientific, United State), pH8.0; 25 mM NaCl (Duchefa Biochemie, The Netherland); 0.1% (w/v) SDS (Amresco, United State) and 20 mg RNase A (Bioteke, China)] was added to the mixture. The mixture was then incubated at 60°C for 20 minutes with a gentle inversion at every 10 minutes. A total of 200 µL phenol/chloroform/isoamyl alcohol (P: C: I; 125:24:1, v/v/v, pH ≥ 7.8) (Solarbio, China) was added to the mixture, mixed and centrifuged at 13,000 × g for 10 minutes at 4ºC. The upper aqueous phase from three tubes was randomly pooled and transferred into a new tube. The mixture was mixed with C:I (24:1, v/v, pH 8.0) in a ratio of 1:1 before centrifuging at 13,000 × g for 10 minutes at 4ºC. The upper aqueous phase was collected and mixed with pre-chilled ethanol solution (95% v/v) in a ratio of 1:3. The mixture was incubated at -20°C for 2 hours and centrifuged at 13,000 × g for 20 minutes at 4°C. The pellet was washed with pre-chilled ethanol (70% v/v) and centrifuged at 13,000 × g for 10 minutes at 4°C. The pellet was air dried for 10 minutes before dissolving with 15 µL milliQ water. All samples were kept at -80°C until further use. The concentration of DNA (substrate) was quantified using a NanoDrop spectrophotometer (Implen, Germany) at the absorbance of 230 nm (A_{230}), 260 nm (A_{260}) and 280 nm (A_{280}). The sample was mixed with 1 × DNA loading buffer (TransGen Biotech, China) and loaded into wells of 1% (w/v) agarose gel along with 100 ng of 1 kb ladder (TransGen Biotech, China). The agarose gel was placed in a gel electrophoresis tank and immersed with 1× TBE buffer

(First Base, Malaysia). The electrophoresis was run at a constant 100 V for 35 minutes in a gel electrophoresis tank (Major Science, United State). The agarose gel was viewed by UV light and photographed with a gel documentation system (Alpha Innotech Corporation, United State).

3.3.4 Primer Design for Amplification of the Target Gene Sequence *STP13*

Primers were designed to target *STP13* of *Musa acuminata* (NCBI acession number: 103993067) containing PAM site using PrimerQuest™ Tool (https://sg.idtdna.com/pages/tools/primerquest (Owczarzy et al., 2008) as listed in Table 3.4. Primer S1 was used for *in vitro* cleavage with RNP complex and T7E1 assay. Primer S2 was designed by adding Illumina MiSeq adapter sequence at the 5' end as shown in Table 3.4 for the use of Illumina MiSeq barcoded library preparation. Desalted S1 primers was synthesised by Integrated DNA Technologies, Inc. (IDT), Singapore, whereas HPLC-graded S2 primers was synthesized by Bioneer Corporation, South Korea,

Table 3.4: Sequences of primers to amplify *STP13* of *M. acuminata*. Desalted Primer S1 was used for *in vitro* cleavage with RNP complex and T7E1 assay. HPLC-graded Primer S2 was used for targeted deep sequencing. Underlined text indicates adapter sequences. The amplicon size did not include the size of adapter sequences.

Primers	Primers' sequences (5' to 3')	Primer size (bp)	Amplicon size (bp)	Annealing temperature (°C)
Forward	ACA AAT CCA TCG AGC CCT CC	20	359	60
Reverse	TGT TTC TTC AGC ATG TAG TCC T	22		
Forward	TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG GCA ATC TCC TCT CTC TCT CT	20	332	60
Reverse	GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGG TCT GTC TGT CTG TGT TTC	20		

3.3.5 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) was conducted to amplify *STP13* using an Aeris™ PCR Thermal Cycler (Esco, Singapore). A 20 µL-reaction mixture consisting of 20 ng DNA was mixed with 0.2 µM desalted forward primer S1, 0.2 µM desalted reverse primer and 1 × of *TransTaq*® High Fidelity (HiFi) PCR SuperMix II (TransGen Biotech, China). The mixture was amplified according to the programmed thermal cycling condition as 94^0C for 5 min; 30 cycles of 94^0C for 30 s, 60^0C for 30 s and 72^0C for 50 s; and a final extension of 72^0C for 5 min. The sample was mixed with 1 × DNA loading buffer (TransGen Biotech, China) and loaded into wells of 1% (w/v) agarose gel along with 100 ng of 1 kb ladder (TransGen Biotech, China). The agarose gel was placed in a gel electrophoresis tank and immersed with 1× TBE buffer (First Base, Malaysia). The electrophoresis was run at a constant 100 V for 35 minutes in a gel electrophoresis tank (Major Science, United State). The agarose gel was viewed by UV light and photographed with a gel documentation system (Alpha Innotech Corporation, United State).

3.3.6 Gel/PCR Purification

The desired size band was excised from agarose gel using a clean scalpel and purified using FavorPrep GEL/PCR Purification Mini Kit (Favorgen, Taiwan) following the manufacturer's protocol. The gel piece was mixed with 500 mL of FAPD1 buffer and incubated at 55ºC until completely melted. The solution was filtered through silica membrane of a column tube, followed by centrifugation at 1,1000 × *g* for 30 seconds. After discarding the pass-through solution, 750 µL of Washing Buffer with 95% (v/v) ethanol was added to the same column tube and centrifuged at 1,1000 × *g* for 30 seconds. The column was centrifuged again at 1,8000 × *g* for 3 minutes and transferred to a new 1.5 mL microcentrifuge tube. A total of 20 µL of milliQ water was added to the silica membrane of the column tube and left to stand for 2-5 minutes. The centrifuge tube-capped-with-column tube was centrifuged at 1,8000 × *g* for 1 minute. The concentration

of DNA (substrate) was quantified using a NanoDrop spectrophotometer at the absorbance of 230 nm (A_{230}), 260 nm (A_{260}) and 280 nm (A_{280}). The sample was mixed with 1 × DNA loading buffer (TransGen Biotech, China) and loaded into wells of 1% (w/v) agarose gel along with 100 ng of 1 kb ladder (TransGen Biotech, China). The agarose gel was placed in a gel electrophoresis tank and immersed with 1× TBE buffer (First Base, Malaysia). The electrophoresis was run at a constant 100 V for 35 minutes in a gel electrophoresis tank (Major Science, United State). The agarose gel was viewed by UV light and photographed with a gel documentation system (Alpha Innotech Corporation, United State).

3.3.7 *In Vitro STP13* Cleavage by RNP Complex

In vitro STP13 cleavage by RNP complex was modified from Mehravar et al. (2019) as shown in Figure 3.2. A total of 10 µL reaction mixture containing one µM RNP complex, 100 ng purified wild type (WT) *STP13* and 1 × Cas9 nuclease reaction buffer [1 M NaCl; 0.1 M $MgCl_2$; 1 mg/mL BSA (Sigma-Aldrich, United State) and 0.5 M Tris (Duchefa Biochemie, The Netherlands), pH 8.0] was incubated at 37ºC for 2 hours, followed by 65ºC for 20 minutes. The mixture was then mixed with 1 × DNA loading buffer and loaded into wells of 2% (w/v) agarose gel together with 100 ng of 100 bp ladder. The agarose gel was gel electrophoresed at a constant 80 V for 65 minutes. The sample was mixed with 1 × DNA loading buffer (TransGen Biotech, China) and loaded into wells of 1% (w/v) agarose gel along with 100 ng of 1 kb ladder (TransGen Biotech, China). The agarose gel was placed in a gel electrophoresis tank and immersed with 1× TBE buffer (First Base, Malaysia). The electrophoresis was run at a constant 100 V for 35 minutes in a gel electrophoresis tank (Major Science, United State). The agarose gel was viewed by UV light and photographed with a gel documentation system (Alpha Innotech Corporation, United State). The intensity of bands was measured based on the band fluorescence intensity (pixel number) using ImageJ (Rueden et al. 2017). The results

were analysed using SPSS software. The significant difference was analysed by two-tailed *t*-test. Multiple comparisons were performed with the Tukey HSD test, with $P \leq 0.01$ considered as a significant difference (Tukey, 1949). The graphs were produced by using SPSS software.

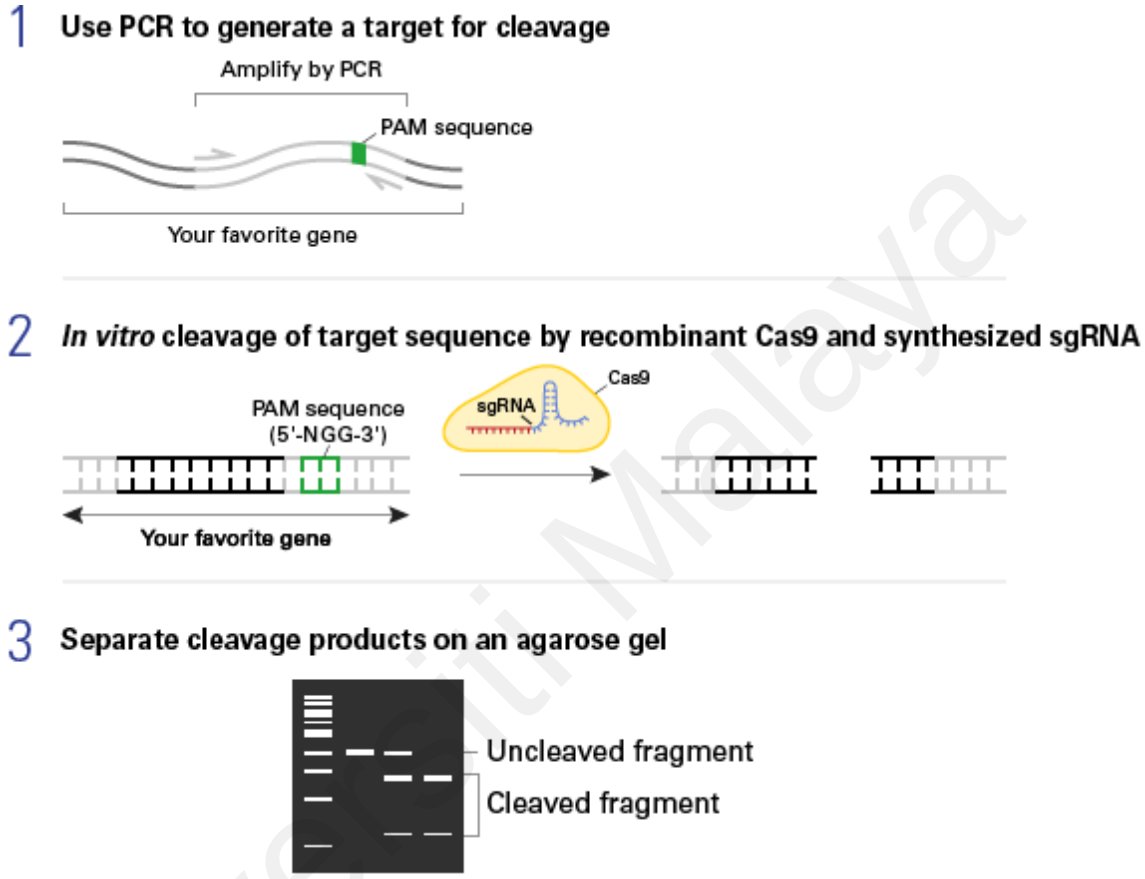

Figure 3.2: *In vitro* gene cleavage method. A reaction mixture containing one µM RNP complex, 100 ng purified PCR product and Cas9 nuclease reaction buffer was incubated at 37ºC for 2 hours. The mixture was gel electrophoresised and visualised. Adapted from Takara Bio USA Inc. (2018).

3.4 Gene Editing of *STP13*

3.4.1 Competent Cell Preparation

Escherichia coli (E. coli) DH5α strain was allowed to grow in LB broth for 16 hours at 37°C, 200 rpm. A total of 1 mL of cloudy overnight culture was pipetted to 50 mL of LB broth, and allowed to grow at 37°C, 200 rpm for 2.5 hours. The optical density (OD)

of the culture was measured at a wavelength of 600 nm to ensure the reading was 0.6. A total of 10 mL of culture was centrifuged at 6000 rpm, 4°C for 20 minutes. The supernatant was removed, and cell pellet was resuspend in 5 mL of ice-cold 0.1 M $CaCl_2$ and incubated in ice for 30 minutes. Centrifugation of the mixture was carried out at 6000 rpm, 44°C for 20 minutes. The cells were resuspended in 1 mL of a mixture consisting of 85 mM $CaCl_2$ and 1 mL of 100% glycerol. A total of 100 µL of the mixture was aliquoted into chilled centrifugation tubes and stored at -80°C until further use.

3.4.2 Cloning of pCAMBIA1304 into DH5-Alpha

A total of 100 ng of the pCAMBIA1304 was mixed with 200 µL of 8×10^8 cells/ml competent*E. coli* DH5α strain using a heat-shock method (Chang et al., 2017). The mixture was left in ice for 15 minutes then was heated at 42°C for 2 minutes. Following that, the mixture was put on ice for 20 minutes. A total of 20 µL mixture was inoculated into 800 µL LB broth, and shaken at 90 rmp, 37°C for 1 hour. A total of 1 mL of the mixture was centrifuged at 5000 rpm, 5 minutes, 4°C. A total of 990 µL supernatant was removed. The remaining 10 µL supernatant was mixed with pellet. The mixture was then plated on 20 mL LB agar (Conda, Spain) supplemented with 100 ng/mL ampicillin (Duchefa Biochemie, The Netherland). The plate was incubated at 37°C and colony selection was carried out after 16 hours incubation. The single colonies were picked using a sterile pipette tip and cultured onto a gridded DNA library master plate containing LB agar supplemented with 20 µL of 100 ng/mL ampicillin. The DNA library master plate was then incubated at 37°C for 16 hours.

3.4.3 Preparation of Glycerol Stock of DH5-Alpha pCAMBIA1304

Colonies were picked by toothpick and cultured in 4 mL of LB broth with 100 ng/mL ampicillin. The liquid culture was grown at 37°C at 48 rpm for 15 hours. A total of 800

µL of cloudy overnight liquid culture was mixed with 200 µL of sterile 100% glycerol. The glycerol stock was stored at -80°C until further use.

3.4.4 Plasmid Extraction

E. coli bacterial strain DH5α containing pCAMBIA1304 plasmid (Cambia et al., Australia) provided by CEBAR team, Universiti Malaya, was used. The plasmid was extracted using a FavorPrep^{TM} Plasmid DNA Extraction Mini Kit (Favorgen® Biotech Corporation, Taiwan) with modifications as suggested by Pronobis et al. (2016). A total of 50 mL of overnight bacterial culture was centrifuged. The pellet was resuspended in 200 µL of FAPD1 buffer. Next, 200 µL FAPD2 buffer was added into the mixture. The tube was inverted 10 times before incubating at room temperature for 2 minutes. Next, 300 µL pre-chilled FAPD3 buffer was added and mixed by inverting the tube 10 times. The solution was centrifuged at 13,500 rpm for 5 minutes at 4ºC. The supernatant was transferred to a 15 mL centrifuge tube. Absolute ethanol was added to the supernatant in a ratio of 1:1 and mixed for 5 seconds. The mixture was transferred into an FAPD Column. After centrifugation, the column was placed in a collection tube. A total of 400 µL of W1 Buffer was added to the mixture in the FAPD Column. The mixture was centrifuged for 1 minute. The flow-through was discarded. The column was then placed in a new collection tube. A total of 600 µL of wash buffer with ethanol was added to the column. The mixture was centrifuged for 1 minute. After discarding the flow-through, the column was placed back to the collection tube. The column was centrifuged again for 3 minutes before transferring to a new 1.5 mL microcentrifuge tube. A total of 35 µL distilled water was added to the column and left to stand for 2 minutes. The column was centrifuged at 13,500 rpm for 1 minute. The extracted plasmid was digested as shown in Table 3.5. The mixture was incubated at 37°C for three hours. Next, the mixture was mixed with 1 × DNA loading buffer and loaded into wells of 1% (w/v) agarose gel along with 100 ng of 100 bp ladder. The sample was mixed with 1 × DNA loading buffer

(TransGen Biotech, China) and loaded into wells of 1% (w/v) agarose gel along with 100 ng of 1 kb ladder (TransGen Biotech, China). The agarose gel was placed in a gel electrophoresis tank and immersed with 1× TBE buffer (First Base, Malaysia). The electrophoresis was run at a constant 100 V for 35 minutes in a gel electrophoresis tank (Major Science, United State). The agarose gel was viewed by UV light and photographed with a gel documentation system (Alpha Innotech Corporation, United State).

Table 3.5: Restriction digestion of pCAMBIA1304 plasmid.

Components	**Stock concentration**	**Working concentration**		**Volume (µL)**
		Single digestion	**Double digestion**	
Template	Variable	100 ng	200 ng	Variable
CutSmart® Buffer (NEB)	10 ×	1 ×	1 ×	2.0
Hind III-HF (NEB)	10 U/µL	5 U	5 U	0.5
Spe I (NEB)	10 U/µL	-	5 U	0.5
Top-up of nuclease-free water to final volume of 20 µL				

3.4.5 Polyethylene Glycol (PEG)-Mediated Protoplast Transfection

Protoplast transfection was carried out according to Yoo et al. (2007) with modifications. The isolated protoplasts were centrifuged at 150 × *g* for 5 minutes in a swing-bucket rotor centrifuge, the supernatant was removed and the pellet was mixed with 100 µL of MMG buffer. Ten µL of one µg/mL pCAMBIA1304 or 10 µL of one µM RNP complex mixed with 1 × PBS (Sigma-Aldrich, United State) was added to 100 µL of 2 × 10^6 viable protoplasts in a 1.5 mL Spinwin™ Micro Centrifuge Tube (Tarsons, India) and incubated at room temperature for 15 minutes. Next, 110 µL freshly prepared protoplast transfection buffer [200 mM mannitol, 100 mM $CaCl_2$ and 40%(w/v) PEG-4000 (Sigma-Aldrich, United State)] was added to the mixture and incubated in the dark for 10, 15 or 20 minutes. After adding 600 µL of S1 buffer, the mixture was centrifuged at 150 × *g* for 5 min in a fixed-rotor centrifuge. The pellet was resuspended in 1 mL of

S1 buffer solution and incubated at 25°C in the dark for 3-9 hours. The mixture was centrifuged at 150 × *g* for 5 minutes at fixed-rotor centrifuge machine. About 800 µL supernatant was removed. The pellet was resuspended in the remaining S1 buffer solution. A total of 10 µL of protoplast suspension was loaded into an Improved Neubauer hemocytometer. Protoplasts at the four outer squares and in the middle square of the grid were counted under a bright field or fluorescence light inverted microscope (Olympus IX73, Japan) at a magnification of 10×. The protoplast transfection efficiency was calculated following the Equation 3 (Wen et al., 2020).

$$\text{Protoplast transfection efficiency (\%)} = \frac{\text{Number of green protoplasts in the dark under fluorescence light} \times 100\%}{\text{Number of protoplasts in bright view}} \quad (3)$$

The microscope images were captured with digital microscope camera EM-CCD (Hamamatsu Photonics K.K., Japan) and processed using Adobe Photoshop CC software (Adobe Inc., United State) by adjusting merely the brightness/contrast and exposure. The transfection efficiency and analysed using SPSS software. The significant difference was analysed by two-tailed *t*-test. Multiple comparisons were performed with the Tukey HSD test, where $P \leq 0.01$ was considered as significant difference (Tukey, 1949). The graphs were produced by using SPSS software.

3.4.6 T7E1 Mismatch Assay for Confirmation of Gene Editing Events

For the T7E1 assay, (Figure 3.3) 150-200 ng purified WT and MU *STP13* were denatured at 95 °C for 5 minutes and annealed by ramping down the temperature to 85°C at 2°C s^{-1}, further ramped down to 25°C at 0.1°C s^{-1} and then stored at 4°C. The annealed PCR products were then digested with 5U of T7E1 (NEB, United States) for 2 hours at 37°C (Figure 3.3). A total of 0.75 µL of 0.5 M EDTA was added after the incubation. The sample was mixed with 1 × DNA loading buffer (TransGen Biotech, China) and loaded into wells of 1% (w/v) agarose gel along with 100 ng of 1 kb ladder (TransGen Biotech,

China). The agarose gel was placed in a gel electrophoresis tank and immersed with 1× TBE buffer (First Base, Malaysia). The electrophoresis was run at a constant 100 V for 35 minutes in a gel electrophoresis tank (Major Science, United State). The agarose gel was viewed by UV light and photographed with a gel documentation system (Alpha Innotech Corporation, United State).

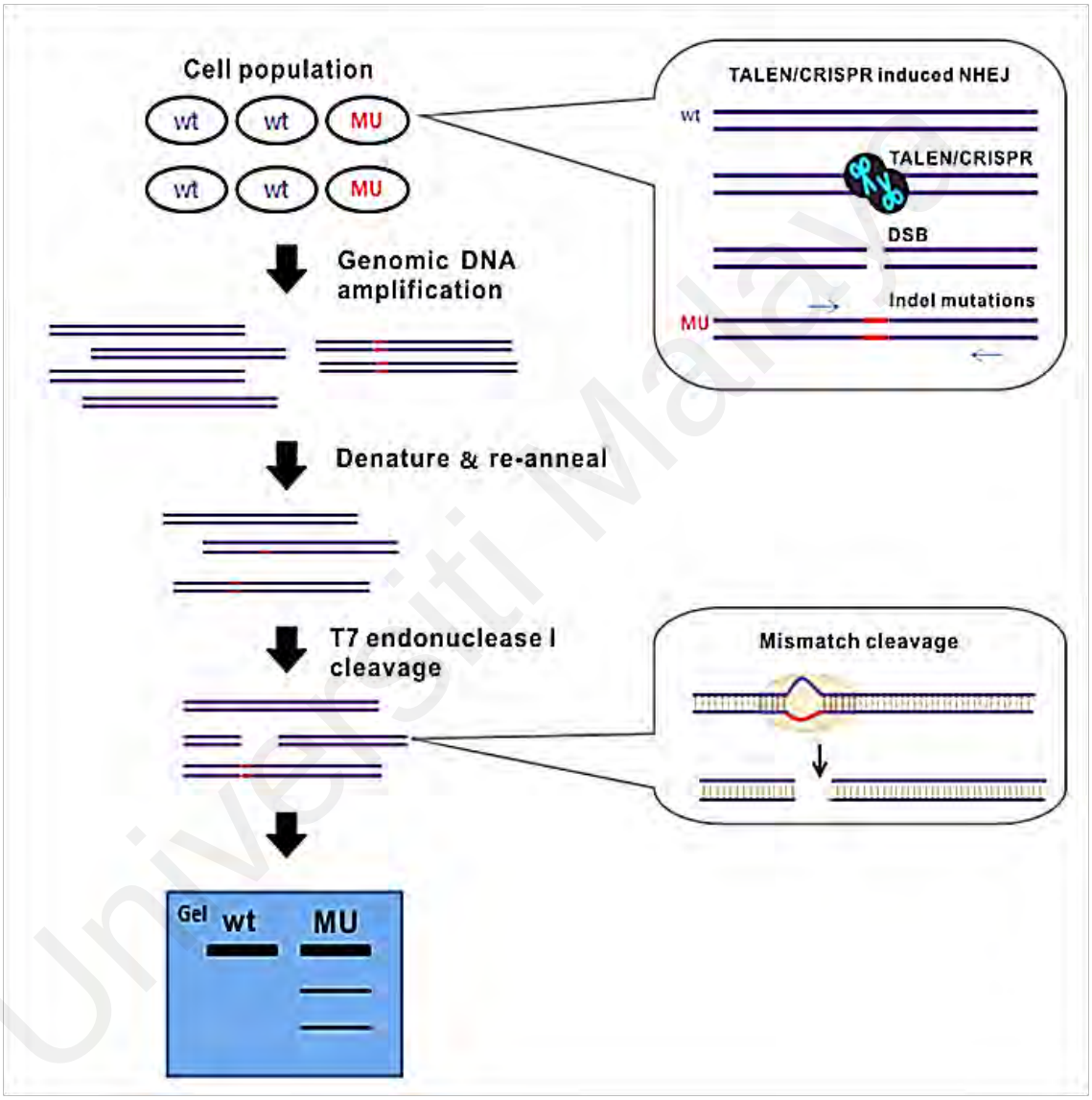

Figure 3.3: The T7E1 assay. PCR product was denatured at 95 °C for 5 minutes and annealed by ramping down the temperature to 85°C at 2°C s−1. Annealed PCR products were digested with 5U of T7E1 for 2 hours at 37°C. The digested product was gel eletrophoresised and visualized with a gel documentation system. WT: wild-type; MU: mutated; DSB: double-strand break. Adapted from GeneCopoeia Inc (2021).

3.4.7 Targeted Deep Sequencing

Targeted deep sequencing was carried out using Illumina MiSeq platform by the First Base Company (Malaysia). Illumina MiSeq Nano flow cell 250 bp paired-end run was carried out. Sample QC for Amplicons (with Illumina overhang adaptors) was carried out. Library preparation and QC was prepared by First Base Company. A total of 1.5 M raw data provided by First Base Company was analysed. Firstly, the quality of the FastQ file was screened by FastQC software. Then, the paired-end sequences were merged by Fastq-join feature of Cas-Analyzer (http://www.rgenome.net/cas-analyzer/) and the mutation frequencies was analysed by Cas-Analyzer. The relative frequency of the mutated position was calculated by using the Equation 4.

$$\text{Relative frequency of the mutated position} = \frac{\text{Frequency of indel sequence at certain position} \times 100\%}{\text{Total frequency of indel sequence}} \quad (4)$$

The data were analysed using SPSS software. The significant difference was analysed by two-tailed *t*-test. Multiple comparisons were performed with the Tukey HSD test, where $P \leq 0.01$ was considered as significant difference (Tukey, 1949). The graphs were produced by using SPSS software.

CHAPTER 4: RESULTS

4.1 Induction of Callus as a Source for Banana Protoplasts

Male flowers (Figure 4.1 a, b) and shoot buds from *in vitro* banana plantlets (Figure 4.1 c) were used as explants to induce callus. No embryogenic callus was observed on any of the media (Table 4.1). In contrast, white and yellowish non-embryogenic calli were formed in these media after three months of culture (4.1 d). Of these, M2 produced the highest percentage of non-embryogenic callus (22%) after five months of culture. However, some calli turned black and brown after five months of culture (Figure 4.1 e).

No callus was formed from shoot buds on any of the MS media. The shoot buds turned black after one week of culture (Figure 4.1f). Hence, other starting materials, such as bract, were explored for protoplast isolation.

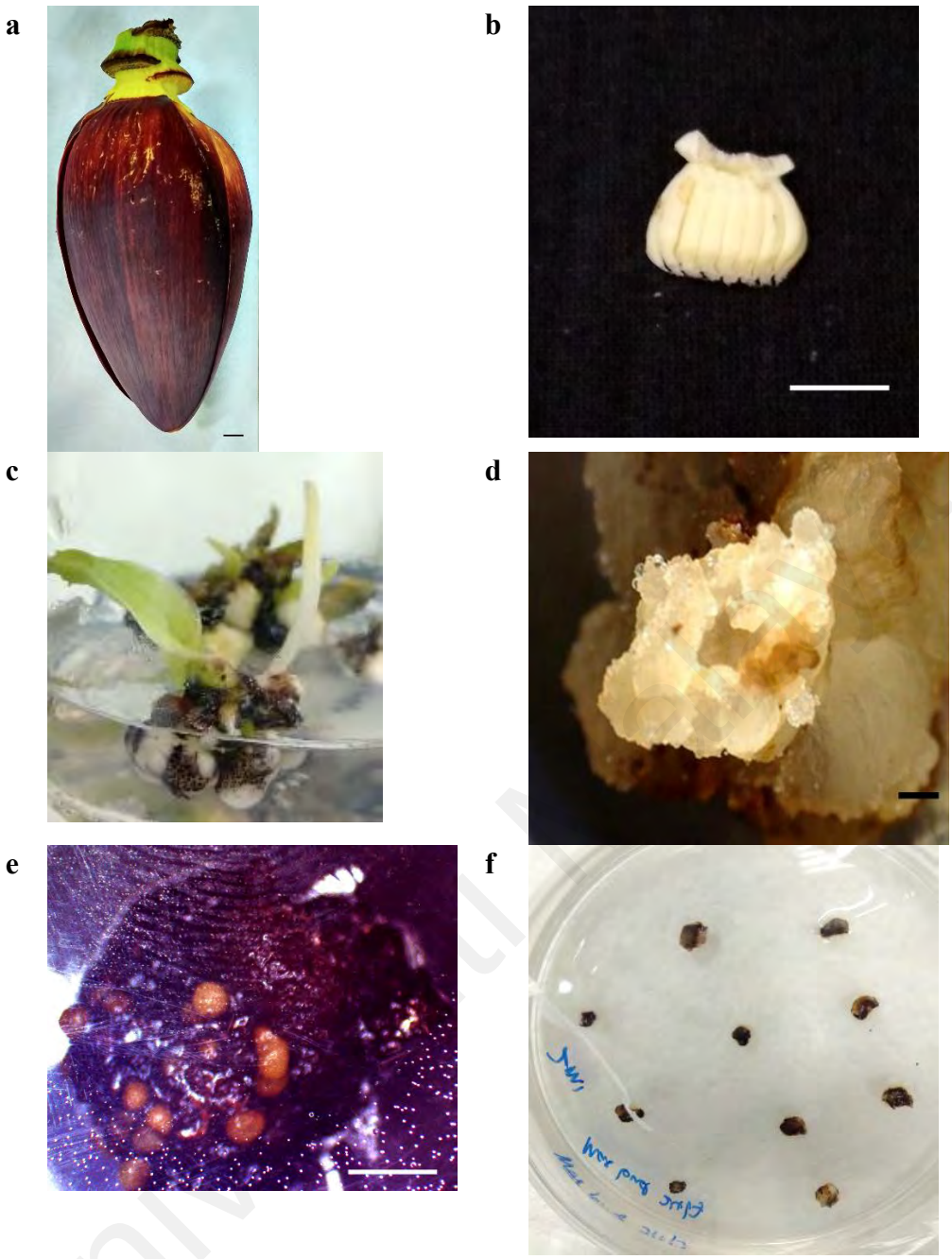

Figure 4.1: Callus induced from banana flower and buds. (a) Male banana flower; Scale bar = 1 cm; (b) Immature male flower; Scale bar = 1 cm; (c) Buds from in vitro plantlets were used for callus induction; (d) Non-embryogenic callus induction on MS media after three months of culture; Scale bar = 1 mm; (e) The callus turned black after five months of culture; Scale bar = 1 mm; (f) Buds turned black after one week of inoculation.

Table 4.1: The percentage of male flowers forming embryogenic and non-embryogenic callus on different types of media. A total of 423 male flowers were cultured on different strengths of MS with Dhed'a vitamins, namely M1 (full-strength), M2 (half-strength) and M3 (quarter-strength), and MS without Dhed'a vitamins, namely M4 (full-strength) and M5 (half-strength) for 52 weeks.

Media	Number of flowers	Number of immature male flower buds	Percentage of male flowers forming embryogenic callus (%)	Percentage of male flowers forming non-embryogenic callus (%)
M1	50	480	0	5.34
M2	150	1500	0	22.07
M3	50	500	0	14.33
M4	123	570	0	6.83
M5	50	500	0	2.60
Total	423	3550	0	12.05

4.1.1 Optimisation of Protoplast Isolation Parameters

4.1.1.1 Effect of different digestion enzyme treatment towards protoplast yield and viability

Protoplasts were isolated from immature male flower (Figure 4.2 a) and bract (Figure 4.2 b) of banana. The protoplasts from immature male flower and bract were spherical and appeared green after staining with FDA and viewing with a UV filter under the fluorescent microscope (Figure 4.2 c-f).

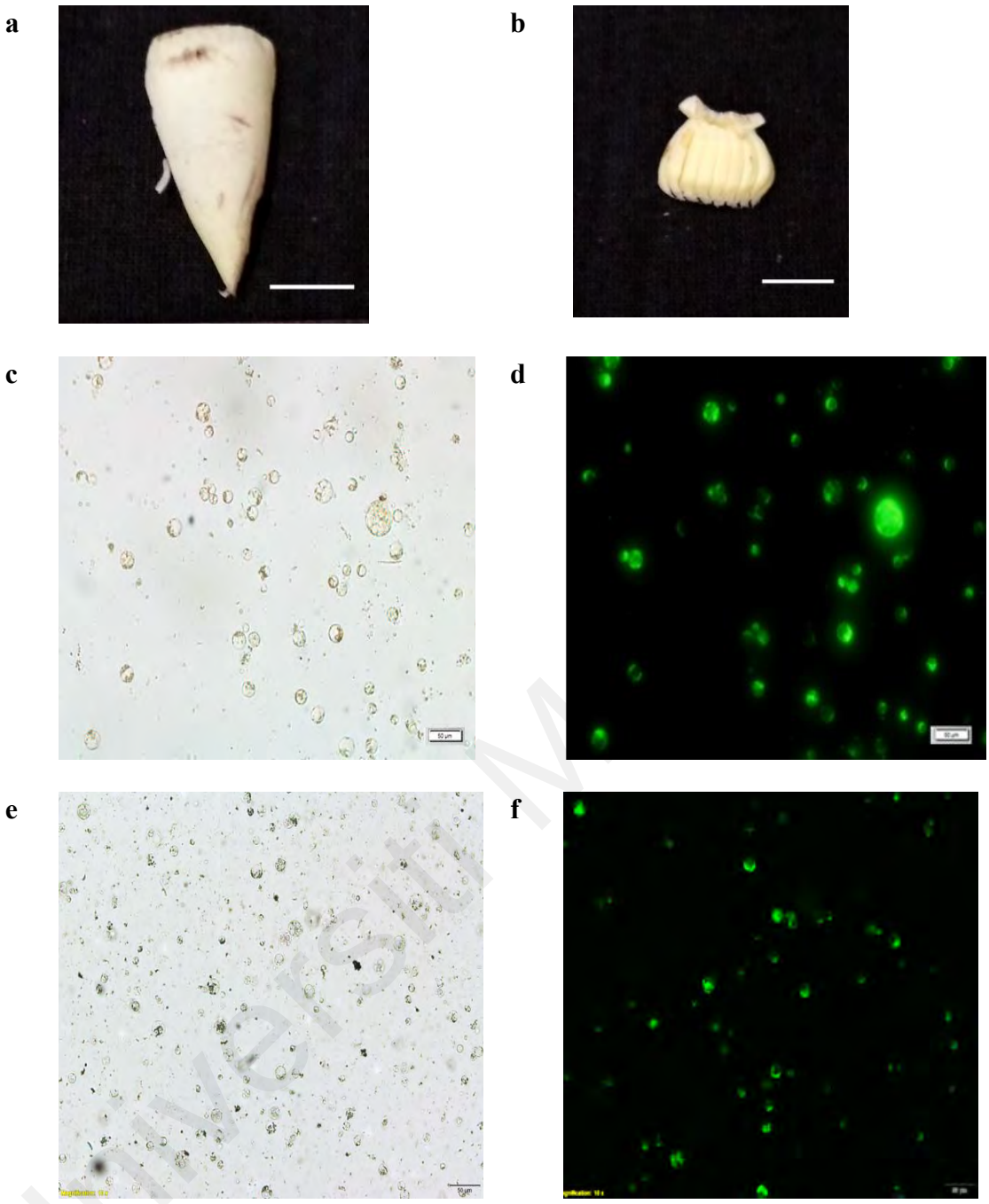

Figure 4.2: Banana protoplast isolation. (a) Banana bract; Scale bar = 1 cm; (b) Immature male flower; Scale bar = 1 cm; (c) Protoplasts isolated from bracts viewed under brightfield; Scale bar = 50 µm; (d) Fluorescein diacetate (FDA)-stained bract protoplasts; Scale bar = 50 µm; (e) Protoplasts isolated from immature male flowers viewed under bright field; Scale bar = 50 µm; (f) Fluorescein diacetate (FDA)-stained immature male flower protoplasts; Scale bar = 50 µm.

Optimisation of enzyme treatment on immature male flower and bract were carried out by using four different enzyme treatments without any vacuum infiltration treatment after plasmolysis of tissues with 0.5 M mannitol (Figure 4.3). Among the four enzyme treatments tested, the immature male flower digested with 1% cellulase RS, 1% macerozyme, and 0.15% pectolyase for 2.5 hours (Treatment 4) produced the highest yield of protoplasts [1.21 ± 0.18) × 10^7 protoplasts/g tissue] with a viability of 91.33% ± 9.29%. At the same treatment, (1.07 ± 0.22) × 10^7 protoplasts/g tissue was obtained from bract with a viability of 88.27% ± 23.34%. Taken together, immature male flowers and enzyme treatment 4 were selected for the subsequent experiments.

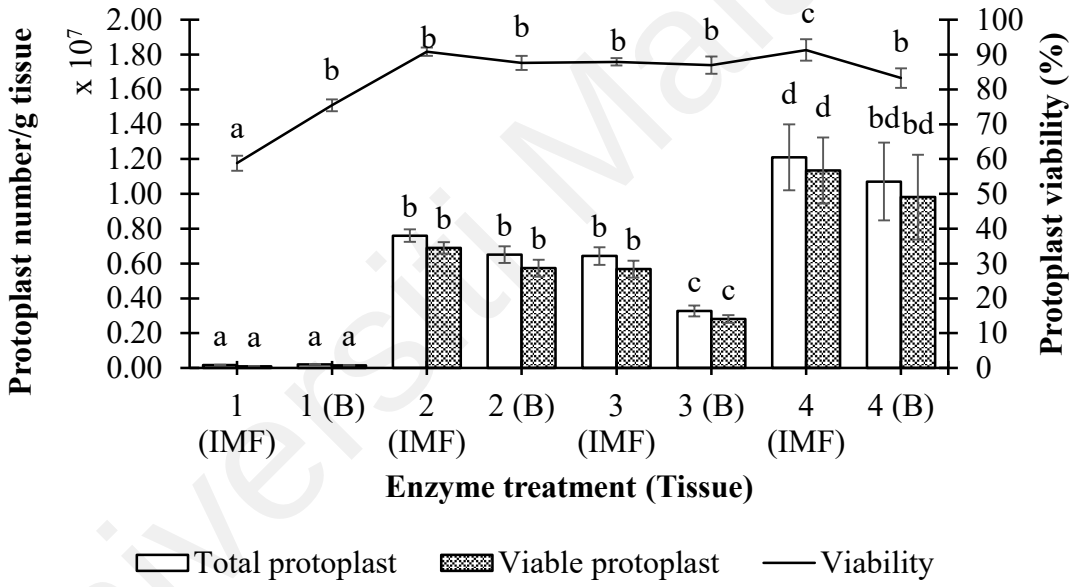

Figure 4.3:The protoplast yield and viability from different enzyme treatments. Data represents mean value ± standard error (SE) in each treatment n = 3. Means with different letters in the same column are significantly different at $p<0.01$ according to Tukey's HSD test. Treatment 1: 1% cellulase RS and 2% macerozyme R10, 2.5 h digestion; Treatment 2: 1% cellulase RS and 2% macerozyme R10, 4 h digestion; Treatment 3: 1% cellulase RS and 2% macerozyme R10, 15 h digestion; Treatment 4: 1% cellulase RS, 1% macerozyme R10 and 0.15% pectolyase, 2.5 h digestion. IMF = immature male flower; B: bract.

4.1.1.2 Effect of different vacuum infiltration periods towards protoplasts yield and viability

A total of five vacuum infiltration treatments were tested with enzyme treatment 4 after plasmolysis of tissues with 0.5 M mannitol (Figure 4.4). In general, Treatment C (vacuum infiltration with enzymatic mixtures for 10 min twice) produced the highest protoplast yield (1.54 ± 0.47) × 10^7 protoplasts/g tissue with 93.2% ± 1.34% viability. Treatment D (vacuum infiltration with enzymatic mixtures for 30 min once) yielded the lowest number of protoplasts [(4.30 ± 0.14)× 10^6 protoplast/g tissue). Based on these findings, Treatment C vacuum infiltration was selected for protoplast isolation.

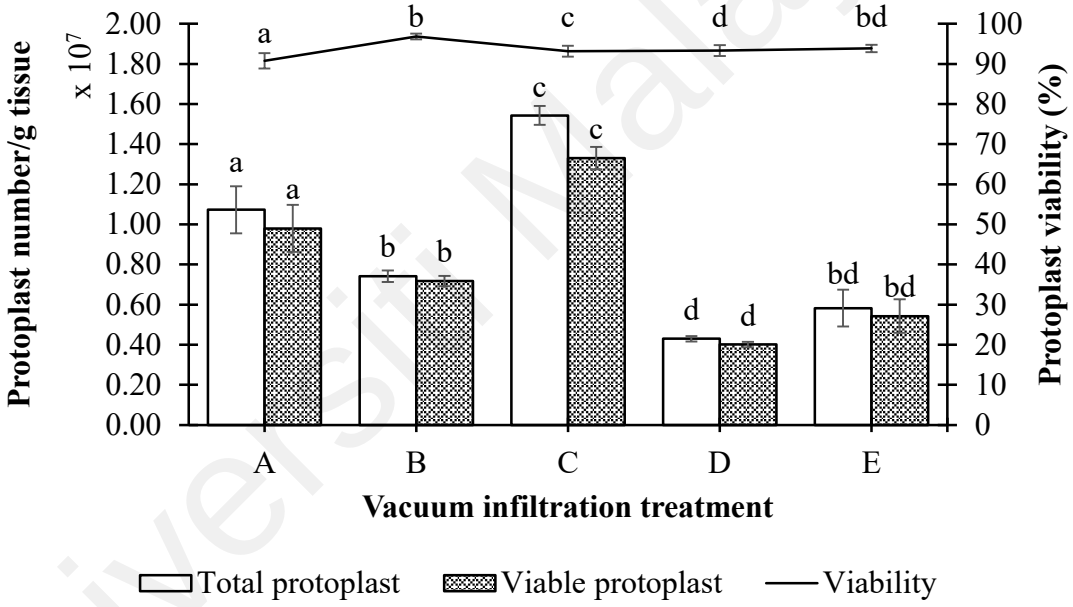

Figure 4.4: The effect of vacuum infiltration periods on the protoplast yield and viability. Data represents mean value ± standard error (SE) in each treatment n = 3. Means with different letters are significantly different at $p<0.01$ according to Tukey's HSD test. Treatment A (no vacuum infiltration), Treatment B (vacuum infiltration of the sample with enzyme for 20 min once), Treatment C (vacuum infiltration of the sample with enzyme for 10 min twice), Treatment D (vacuum infiltration of the sample with enzyme for 30 min once), and Treatment E (vacuum infiltration of the sample with enzyme for 15 min twice).

4.1.1.3 Effect of using different concentrations of mannitol to plasmolyse tissue prior to protoplast isolation

Different concentrations of mannitol were tested with enzyme treatment 4 and vacuum infiltration treatment of 10 min twice (Figure 4.5). The results showed that S1 buffer containing 0.5 M mannitol produced the highest number of protoplasts (1.54 ± 0.07) × 10^7 protoplasts/g tissue] and the highest viability with 96.3% ± 0.67%. In contrast, when the tissue was plasmolused in 0.4 M mannitol, the lowest number of protoplasts [(7.40 ± 0.25) × 10^6 protoplast/g tissue] was produced.

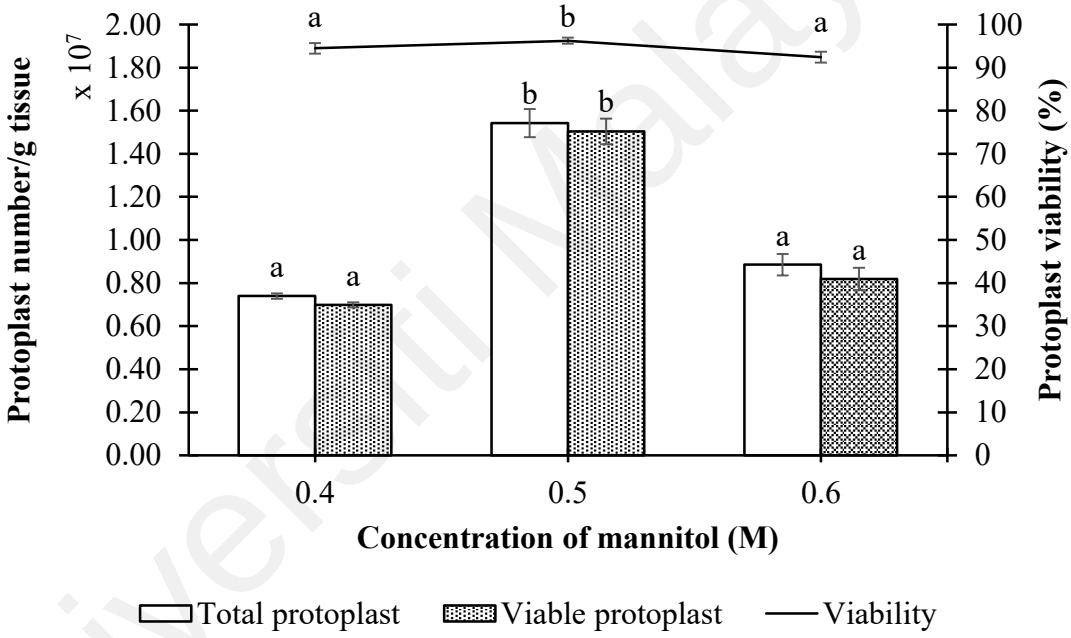

Figure 4.5: The effect of different concentrations of mannitol on the protoplast yield and protoplast viability. Data represents mean value ± standard error (SE) in each treatment n = 3. Means with different letters are significantly different at $p<0.01$ according to Tukey's HSD test.

4.1.2 Effect of Different of Protoplast Transfection Duration Towards Protoplast Transfection Efficiency

After PEG-mediated transfection of the isolated banana protoplasts with pCAMBIA1304 plasmid a GFP signal detected under UV light of the fluorescent microscope indicated that transfection was successful (Figure 4.6 a-b).

a

b

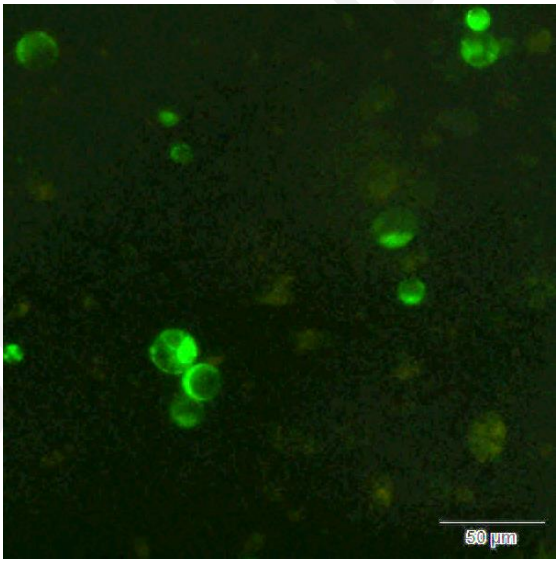

Figure 4.6: GFP signal of pCAMBIA1304 plasmid inside the transfected banana protoplasts. (a) Bright field view of transfected banana protoplast; Scale bar = 50 µm; (b) UV view of transfected banana protoplast; Scale bar = 50 µm.

The highest transfection efficiency of protoplasts (65.28% ± 1.43%) was recorded when protoplasts were incubated with pCAMBIA1304 in PEG solution for 20 minutes. However, differences between the treatments were not significant (Figure 4.7 a). On the other hand, transfected protoplasts could remain viable for up to 3 hours (Figure 4.7 b).

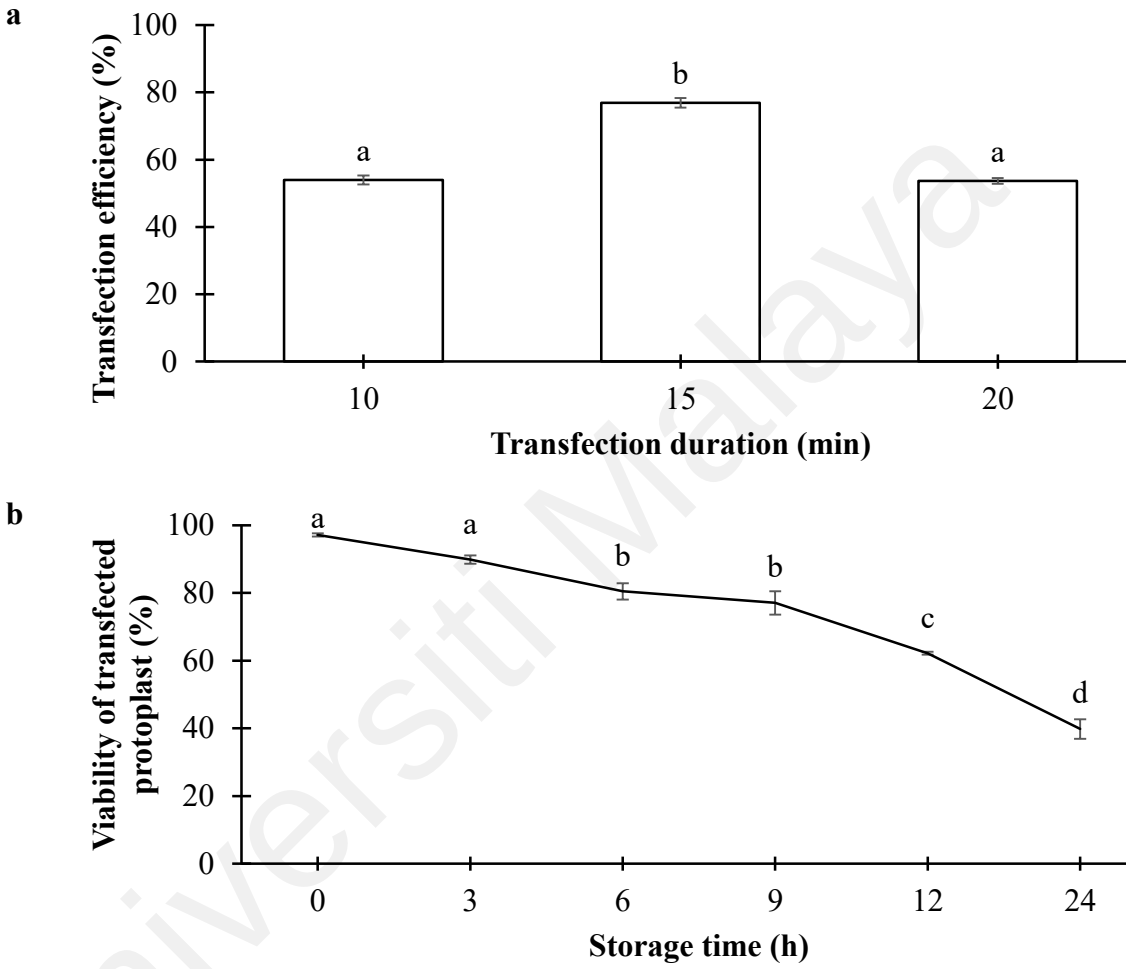

Figure 4.7: Studies of banana protoplast transfection on (a) the effect of different transfection time towards banana transfection efficiency; (b) the effect of culture time of transfected protoplast towards the protoplast viability. Data represents mean value ± standard error (SE) in each treatment n=3. Means with different letters are significantly different at $p<0.01$ according to Tukey's HSD test.

4.2 *In Vitro* Cleavage Experiments for the Designing of Guide RNA

4.2.1 Confirmation of the design of *STP13*gRNA

There was no other similar sequence or reverse complement sequence. There was no potential off-target for *STP13*gRNA (Table 4.2).

Table 4.2: No potential off-target for *STP13*gRNA.

Target position			Target sequence	Number of mismatch	Mismatch position	Score
Chromosome	Start	End				
7	34855608	34855630	CGAGGCGAAGATAACGCCGGTGG	0	[]	100

4.2.2 Amplification of *STP13*

The genomic DNA from intact banana protoplasts was extracted using SDS extraction method. An intact single band was detected on electrophoresis gel (Figure 4.8).

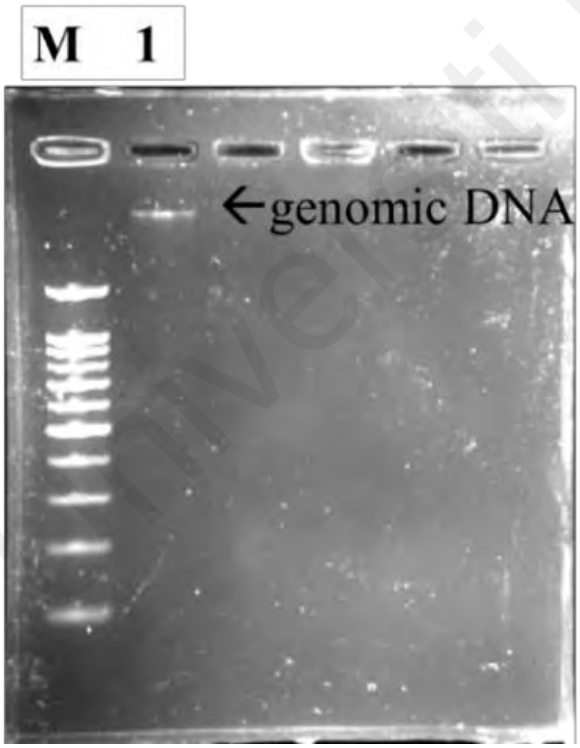

Figure 4.8: DNA extracted from banana protoplast. Lane M: 100 bp ladder; Lane 1: DNA extracted from 10^5 banana protoplasts.

Primers S1 and S2 were designed as shown in Figure 4.9 a-b. The extracted DNA was amplified using Primer S1 and S2 (Figure 4.10 a-b). After gel purification, a single distinct band was obtained for each primer pair (Figure 4.11 a-b).

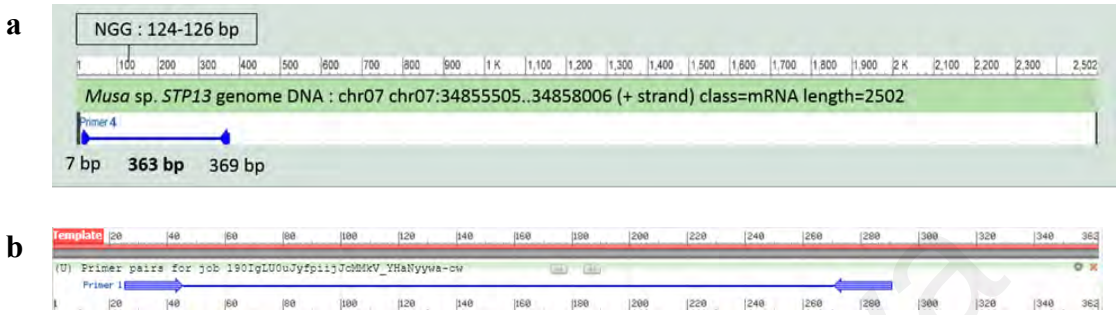

Figure 4.9: Position of primers (a) S1 and (b) S2 on *STP13*.

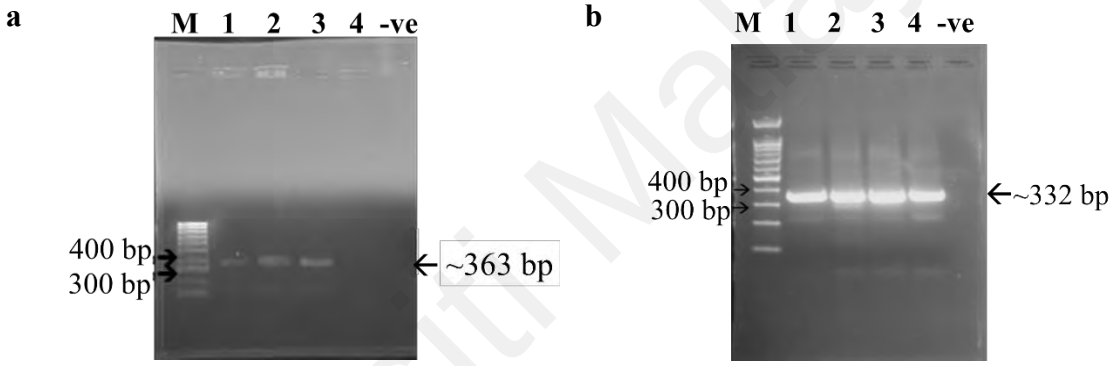

Figure 4.10: Amplification of *STP13* by (a) Primer S1 and (b) S2. Lane M: 100 bp ladder; Lane 1-4: PCR of *STP13*; Lane -ve: PCR without *STP13*.

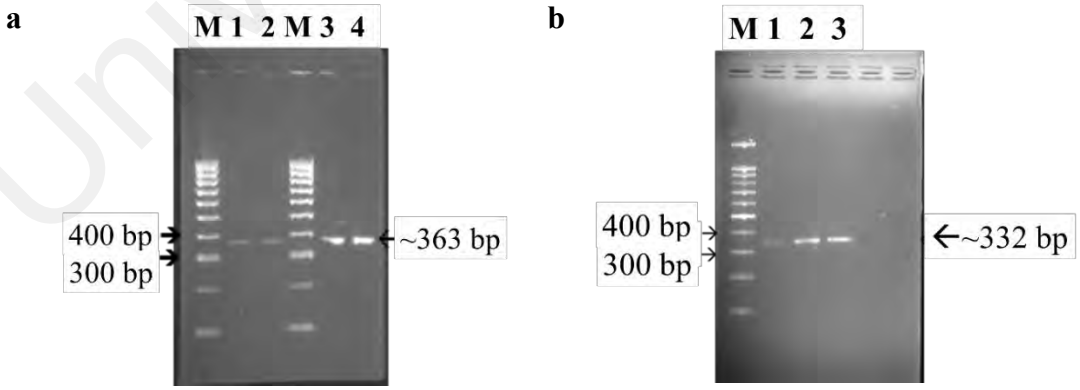

Figure 4.11: Purified *STP13* after amplified by (a) Primer S1 and (b) S2. Lane M: 100 bp ladder; Lane 1-4: Purified *STP13*.

4.2.3 *In Vitro* Ribonucleoprotein Digestion to Evaluate the Cutting Efficiency of RNP on *STP13*

In vitro digestion was carried out to evaluate the ability of the designed *STP13*gRNA in editing *STP13.* The purified *STP13* was digested by the RNP complex. Digestion of the 363bp product from amplification with primer pair S1 resulted in additional bands corresponding to the expected sizes of 246 bp and 117 bp were detected (Figure 4.12).

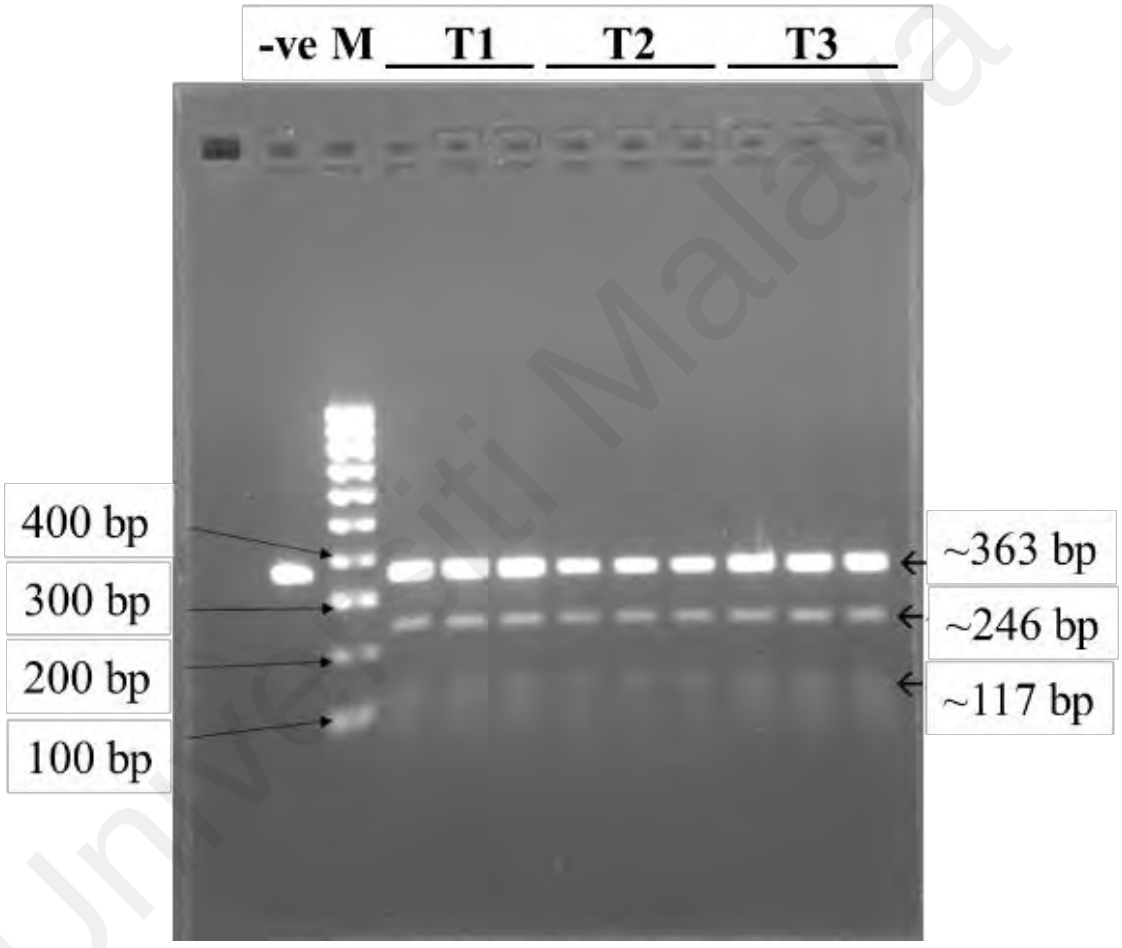

Figure 4.12: *In vitro* RNP digestion with 1 molar Cas9 to 3 molar *STP13*gRNA on purified intact *STP13* gene fragments on 1.5% (w/v) agarose gel. M: 100 bp ladder; Lane -ve: *in vitro* digestion products of *STP13* with ultrapure water; Lanes T1: *in vitro* digestion products *STP13* with RNP complex; Lanes T2: *in vitro* digestion products of *STP13* sample 2 with RNP complex; Lanes T3: *in vitro* digestion products of *STP13* sample 3 with RNP complex.

The cleavage efficiency of different molar ratios of Cas9 to *STP13*gRNA was also analysed (Figure 4.13 a-b). The results showed that Cas9 and *STP13*gRNA in a ratio of 3:1 showed the highest efficiency (12.47% ± 1.10%).

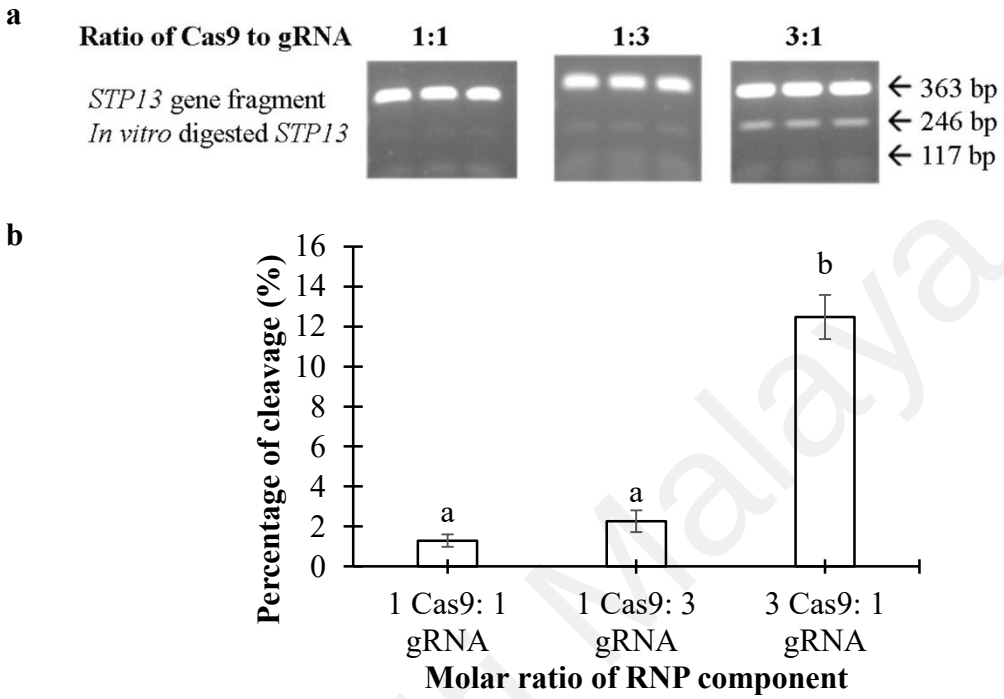

Figure 4.13: The efficiency of different molar ratios of Cas9 and STP13gRNA. (a) *In vitro* digestion of the PCR amplified *STP13* DNA with three different molar ratios of Cas9 and *STP13*gRNA, which were 1:1, 1:3 and 3:1. The uncleaved *STP13* DNA was 363 bp. Band sizes of 246 bp and 117 bp are the result of cleavage by Cas9. (b) Relative band intensity analysis of uncleaved: Cas9 cleaved product of *STP13*. Data represent mean values ± standard error (SE) for each treatment (n=3). Different letters above error bars indicate significant differences at $p<0.05$ according to Tukey's HSD test.

4.3 Gene Editing of *STP13*

4.3.1 T7E1 Assay

A T7E1 assay was carried out to determine the presence of mutation in transfected protoplasts (Figure 4.14). T7E1 digested DNA products were successfully detected from a RGEN transfected protoplasts samples (Lane 1 and 2). In addition, the expected uncleaved and cleaved band sizes were observed, confirming that the presence of RGEN-induced mutant sectors at target sites within the *STP13* genomic locus.

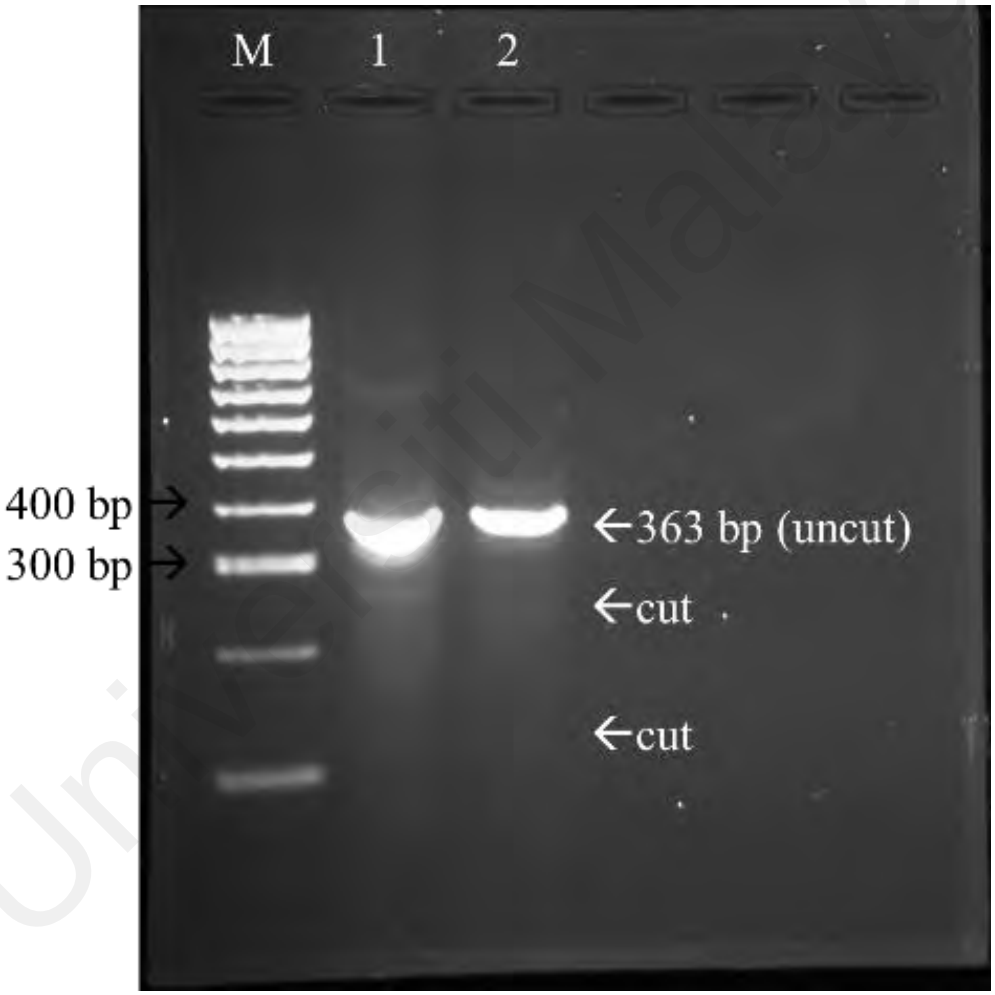

Figure 4.14: Mutation screening by T7E1 assay. M: 100 bp ladder; Lane 1 and 2: T7E1 treated transfected protoplast.

4.3.2 The Frameshift Mutation on *STP13* was Obtained

STP13 amplified from Primer S2 was sequenced to study the mutations resulting from gene editing of *STP13*. The quality of the Illumina sequences showed that less than 10% of the sequence length scored less than 20 Phred scale (Figure 4.15).

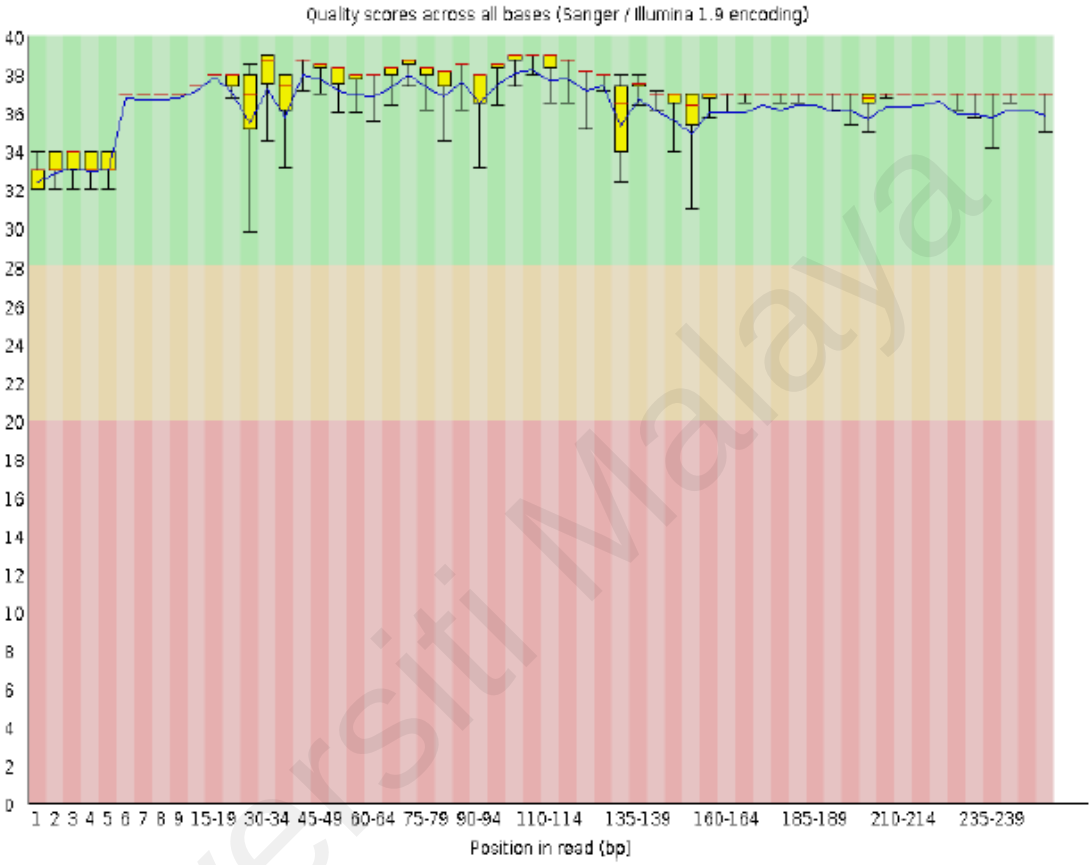

Figure 4.15: The quality of the FastQ file was screened by FastQC software. There was less than 10% of the sequence length that was lower than 20 Phred scale (Pink region).

A total of 289799 to 313463 sequences were obtained and the number of indel sequences ranged from 12917 to 14618 (4.40% to 4.90%) (Table 4.3). One or three deletions were detected at the mutation sites, but no insertions were observed at the mutation sites, mostly the number of deletion was one base pair.

Table 4.3: Percentage of indels of *STP13*.

Mutated position (bp)	Sequences	Number of sequences		
		P1	P2	P3
WT	CGAGGCGAAGATAACGCCGGTGG	299671	276758	284654
12th	CGAGGCGAAGA-AACGCCGGTGG	1128	1065	1206
13th	CGAGGCGAAGAT-ACGCCGGTGG	1334	1261	1427
16th	CGAGGCGAAGATAAC-CCGGTGG	1360	1285	1454
17th	CGAGGCGAAGATAACG-CGGTGG	1652	1561	1766
19th	CGAGGCGAAGATAACGCC-GTGG	4437	4191	4743
20th	CGAGGCGAAGATAACGCC--GG	594	561	635
Total sequences		313463	289799	299321
Total mutation rate (%)		4.40	4.50	4.90

The average relative freqeuency percentage of mutation position of all mutated sequences was analysed (Figure 4.16). The highest average relative frequency percentage of mutation position of all mutated sequences was at position 19 (36.79% ± 2.79%), followed by positions 17, 16, 13, and 12. The position 20 showed the least average relative frequency percentage of mutation position of all mutated sequences (4.34% ± 0.27%). The positions 1-11, 14, 15 and 18 showed no mutation at all. The deleted three nucleotides would produce a point mutation, and the deletion of one nucleotide would produce a frameshift mutation (Figure 4.17).

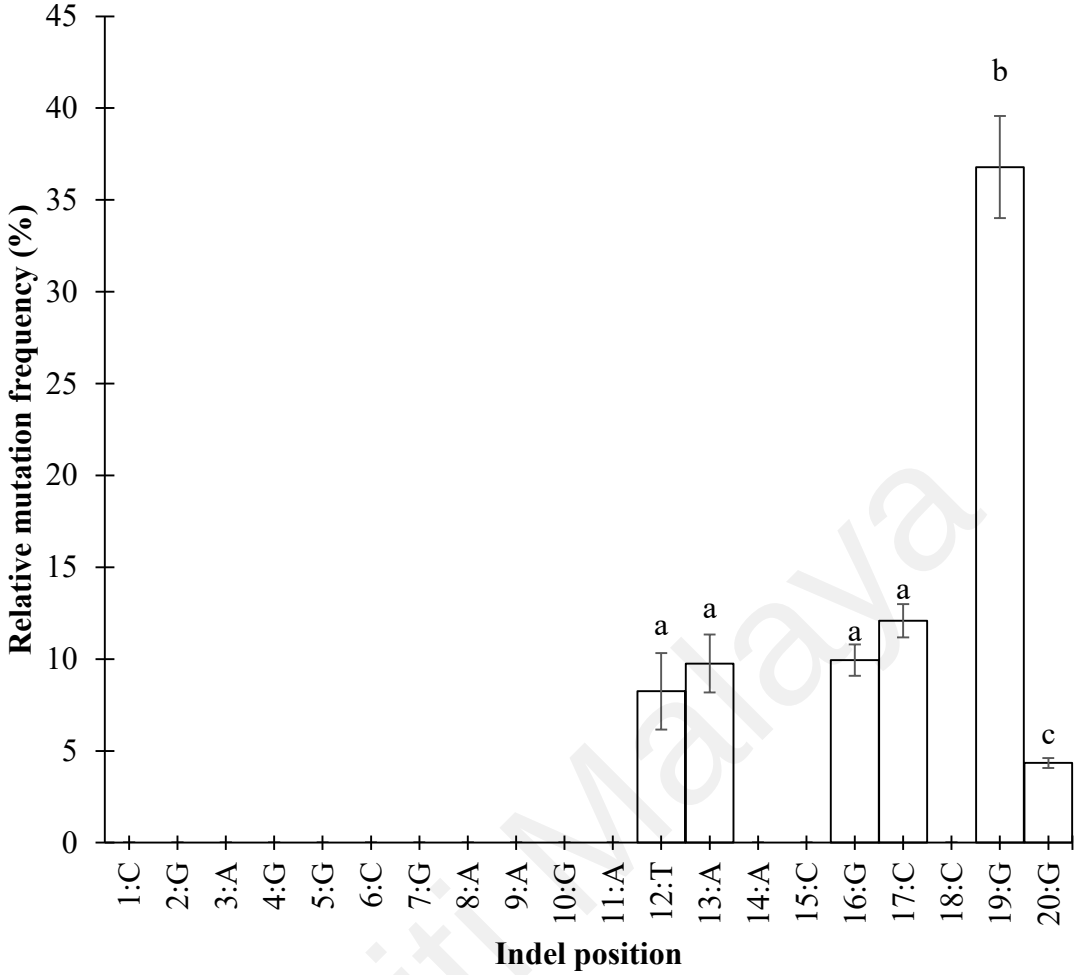

Figure 4.16: Relative frequency of each indel position at target site.

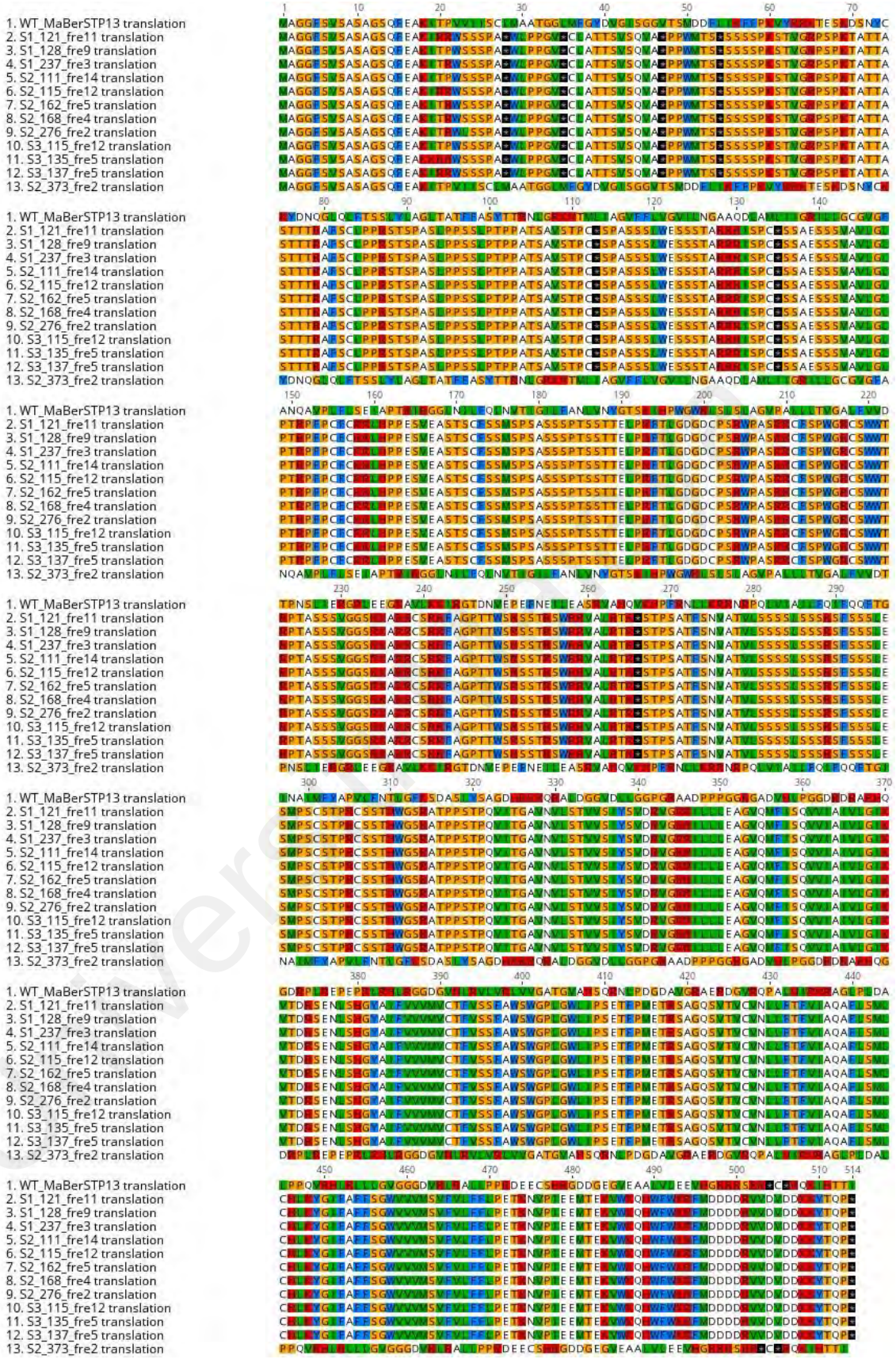

Figure 4.17: Translated mutated protein.

CHAPTER 5: DISCUSSION

Commercially important banana cultivars are seedless. They are propagated vegetatively, resulting in uniform planting material. However, this method produces low diversity of cultivars, particularly with abiotic stress-resistant and -tolerant materials (Tripathi et al., 2019). The plant protoplast system is ideal for studying gene function to produce abiotic stress-resistant and -tolerant traits (Tripathi et al., 2019). The development of a banana protoplast system is necessary as protoplast material is the only source that allows the introduction of a DNA-free RNP complex into plants (Zhang et al., 2021). Gene editing methods that leave transgenes in the edited plant are challenging because banana is difficult to cross-breed to remove unwanted gene editing constructs. Therefore, an efficient alternative system using DNA-free gene editing of bananas should be explored. In this study, the isolation and purification protocols of banana protoplasts have been optimised. The generated protoplasts were used for DNA-free CRISPR-Cas9 editing.

5.1 Induction of Embryogenic Callus

An embryogenic callus is a valuable material for the genetic modification of bananas since the embryogenic callus has regeneration ability (Chin et al., 2014). It can be the direct source of protoplasts or suspended in cell culture, which could be the source of protoplasts (Assani et al., 2002). Nevertheless, there was no embryogenic callus in the current lab. Consequently, optimising embryogenic callus induction procedures is crucial in the laboratory. Several factors greatly influence the induction of embryogenic callus, including the explant used and the medium (Khalid & Tan, 2016). It is also important to obtain at least 1 g of embryogenic callus for each protoplast isolation or at least 2 g for each embryogenic cell suspension (Dai et al., 2010). Therefore, the current research

determined if different explants used for callus induction and the culture media affect the embryogenic callus induction.

Banana cells, tissues and organs, such as leaves, roots, and cells from suspension culture, can be used to induce callus (Khalid & Tan, 2016). In the current study, immature male flowers and *in vitro* shoot buds were used to induce embryogenic callus. Despite multiple attempts over several months with many batches of banana explant materials, no embryogenic callus was produced from the immature male flower and *in vitro* shoot bud of *M. acuminata* cv. Berangan, regardless of the culture media used. This is comparable to the embryogenic callus induction percentage range of 0% to 1% from the immature male flower of banana cultivar Cavendish (AAA group) (Xu et al., 2004) and *in vitro* shoot bud of banana cultivar Calcutta 4 (AA group) (Torres et al., 2012). The low rates of embryogenic callus are due to the banana being a recalcitrant crop with an extremely low embryogenic response in somatic embryogenesis (Xu et al., 2008). However, about 100% callus induction percentage have been recorded using *in vitro* shoot bud of banana cv. Gros Michel (AAA group) (Srangsam & Kanchanapoom, 2003) and 11.1% to 30% from immature male flowers of banana cv. Berangan (Chin et al., 2014; Jafari et al., 2015), which is in contrast to the current study results. The different frequencies of embryogenic callus formation might be due to the genotype and cultivar used (Xu et al., 2008). The frequency of forming embryogenic callus might differ from one experiment to another due to the source of the explant used, mother plants and environmental conditions or climates (Xu et al., 2008). Since the induction of embryogenic callus was unsuccessful, other sources of explants were then explored for protoplast isolation.

There are several challenges to the induction of banana embryogenic callus. Firstly, the browning effects present in the culture media which likely due to phenolic compound exudation. After two weeks of culture, the explants turned brown and swelled at the base.

This might be due to the presence of phenolic chemicals, whereby the excessive accumulation of phenolic compounds might harm the cells and result in cell death (Chin et al., 2014). On the other hand, the duration of forming embryogenic callus in *M. acuminata* cv. Berangan (8 months) is longer than other banana cultivars, such as Mas (Jalil et al., 2003) and Grande Nain (5-6 months) (Côte et al., 1996). This observation indicates that different cultivars might respond differently to media conditions, with banana cultivar AAA less responsive to somatic embryogenesis than AAB and ABB cultivar groups. Besides, during the cutting of explant from the sterile banana flower, purple fluid was observed on the surface of the sterile metal scalpel blade and the banana flowers. It is believed that the purple fluid is the result of unknown stress on the banana flowers, either from the oxidation or the reaction of the metal blade on the banana flowers. The harmful effect of purple fluid on the embryogenic callus induction is also unknown.

In the future, there are several steps that might be done to obtain embryogenic callus. Callus cultures are suggested to be kept on solid agar medium with appropriate nutrients, salts, vitamins, and minerals added (e.g., nitrogen, phosphorus, and potassium) (Ikeuchi et al., 2013). Various culture media is suggested to test for the embryogenic responses of banana. This is due to the fact that occasionally MS medium components were so inadequate that optimization was not possible. Besides, MS medium does contain high levels of ammonium ions, which may not favoured by all plants. The White's medium, and the woody plant medium are the other well-known culture mediums for the callus induction for other plants due to has less ammonium than MS (Phillips & Garda, 2018). Hence, The White's medium amd the woody plant medium are suggested to test for the embryogenic callus induction of banana cv. Berangan. Besides the types of media used to test the embryogenic callus response, to induce callus growth, certain phytohormones must usually be given to the medium. A two-medium method is preferred to improve

secondary metabolite production: one medium for optimum cell growth and another for good secondary metabolite generation (Ikeuchi et al., 2013).

Plant hormones, such as auxins, cytokinins, and gibberellins, are responsible for callus formation, or somatic embryogenesis (Evans et al., 2020). Plant growth hormones are created spontaneously by the plants, however some additional growth hormones are required for better growth and to enrich metabolite synthesis, notably in the case of plant bioactive molecules. Auxins, cytokinins, and gibberellins are common plant growth hormones used for embryogenic callus induction. Indole-3-acetic acid, indole-3-butyric acid, 2, 4 dichlorophenoxyacetic acid, naphthalene acetic acid, 2, 4, 5-trichlorophenoxyacetic acid, and naphthoxyacetic acid are some of the primary auxins that aid in cell division, elongation, and root differentiation (Evans et al., 2020). Cytokinins, such as benzyl amino purine, isopentenyl adenine, kinetin, and 4-hydroxyl-3-methyl-trans-2- butenylaminopurine, are important for the induction, development, and proliferation of shoots (Loyola-Vargas et al., 2018). Gibberellins aid blooming and elongation in plants (Loyola-Vargas et al., 2018).

Besides testing various hormones to induce embryogenic callus induction, vitamins are suggested to test the embryogenic responses of the banana. Vitamins are nitrogenous compounds that are required in trace amounts in enzyme systems to perform catalytic actions during embryogenic callus induction (Chandran et al., 2020). Clearly, highly managed plant *in vitro* cells may produce the same important natural compounds in many circumstances. However, plant cells cultivated *in vitro* can only synthesise important vitamins in little amounts, as a consequence, vitamins are frequently added to culture conditions to boost development. Myoinositol and thiamine-HCl are key cofactors that drive plant cell development, as well as their involvement in stimulating bioactive metabolites as precursors (Loyola-Vargas et al., 2018). Myoinositol is a natural

component of plants that plays a role in the permeability of cell membranes, cell production and metabolism. When added to the culture media at low quantities, it encouraged cell division (Loyola-Vargas et al., 2018). The optimisation of the vitamin is suggested because the vitamin makeup of several conventional media formulations and alterations reveals significant variances (Chandran et al., 2020).

The purpose of establishing embryogenic callus in this study was that is important for regeneration of gene edited banana because it has the ability to grow vigorously in the form of suspension culture, which multiple identical plants can be grown from the active cells. It is important to have large number of identical regenerated plants for the study of the effect of the gene editing in the banana field despite of in the laboratory only (Pua et al., 2019). Embryogenic callus is the only genetic material that can be kept for the regeneration of the gene edited banana as banana is seedless plant (Tripathi et al., 2019). Apart from that, there is no genetic variation in the regenerated banana plants from embryogenic callus (Tripathi et al., 2019), which the effect of the gene editing will not be affected.

5.2 Optimisation of Banana Protoplast Isolation

Protoplasts (naked cells) are valuable materials for plant transformation especially DNA-free CRISPR-Cas9 system since the regenerated plants will not be chimeric due to the single-cell process (Matsumoto et al., 2010). Nevertheless, there is no universal method for protoplast isolation. As a consequence, optimising protoplast isolation procedures is vital for different plant species or cultivars (Khalid & Tan, 2016). Several factors greatly influence the success of protoplast isolation and transformation. These include tissue type, enzyme combination, vacuum infiltration, and mannitol concentration (Ren et al., 2020). Moreover, it is critical to obtain a large number of homogeneous and viable protoplasts (at least 2×10^6 protoplasts/g FW) to ensure successful protoplast

regeneration (Khalid & Tan, 2016). Therefore, the current research determined if different tissue types, enzyme combinations, vacuum infiltrations, and mannitol concentrations affect protoplast production and viability.

The tissues from the flower organ are generally the priority selection for protoplasts isolation in herbaceous flowering plants, such as flower petals of Rosa rugose (Hirata et al., 2012), the flower buds of Freesia hybrida cultivar 'Red River®' (Shan et al., 2019) and flower pedicels of Cymbidium orchids (Ren et al., 2020). In this study, immature male flowers and bracts from banana male flower organ had been digested with cellulase RS, macerozyme R-10s and pectolyase Y23 enzymes. The immature male flower produced a higher protoplast yield than the bract. This is probably because immature male flowers are rich in active meristem cells (Khalid & Tan, 2016). The current protoplast yield of immature male flower (1.21×10^7 protoplasts/g tissue) is comparable to the protoplast yield of immature male flower-derived suspension culture of banana cv. "Da Jiao" (*M. paradisiaca* ABB Linn.) which ranged from 5×10^6/mL to 6×10^6/mL packed cell volume of protoplasts (Dai et al., 2010). Previously, a relative low yield of protoplasts (8.6×10^5 protoplasts/g) had been isolated from bracts of banana cultivars Maca (AAB), 'Nanica' (AAA) and wild *M. balbisiana* (BB), which is not sufficient for regeneration (Matsumoto et al., 1988). The fresh protoplast viability from the fresh tissue of immature male flower of the current study (91.33%) is comparable to the protoplast yield of immature male flower-derived cell suspension of cv. Gros Michel (AAA group), which has 89.4% viability. and higher than callus-derived protoplast of cv. Klutuk (B group) with 30% viability and leaves-derived protoplast of cultivars Tani (BB group) with 40% viability (Assani et al., 2002). The difference in protoplast viability may be due to the difference in genome group, tissue or cultivar used. In future work, cell suspension culture might be used for establishing protoplasts since it is the most generally reported material for banana protoplast isolation (Xiao et al., 2007; Assani et al., 2002; Assani et al., 2001).

As cell suspension culture has a high regeneration capacity that can be used for mass clonal propagation, it should be further explored as an alternative platform for genetic engineering research. Other banana cultivars with the AAA genome could also be used for protoplast isolation. These include banana cv. “Jahaji”, banana cv. “Zelig” and banana cv. Ardhapuri. This is because there is no established protoplast isolation protocol for these banana cultivars. Apart from that, other banana genome groups, such as Mas (AA) and Rasthali (AAB), could be tested with a similar enzyme treatment to verify the consistency of the protoplast isolation efficiency.

Banana cell walls are made up of cellulose, hemicellulose, and pectin. As a consequence, cellulase RS, macerozyme R-10, and pectolyase Y23 enzymes are commonly used to isolate banana protoplasts (Khalid and Tan, 2016). The current experiment showed the importance of pectolyase Y23 in improving banana protoplast yield as it contains pectin lyase and polygalacturonase that can digest pectin (Tayi et al., 2016). This observation is similar with Matsumoto et al. (2010), whereby cellulase and pectinase could produce 1×10^7 protoplasts from the cell suspension culture of banana cv. Embrapa/CNPMF 2803-01 (AA group). Macerozyme R-10 was not used in their study as embryogenic suspensions are single cell cultures and do not require a maceration activity for separation of tissue into single cells. The suitable enzymatic digestion time for the current enzyme combinations 1% cellulase RS, 1% macerozyme R-10 and 0.15% pectolyase Y23 is 2.5 h. A digestion time of 2.5 hours is shorter than the 10-12 hours for embryogenic cell suspension of banana cv. Da Jiao” (*M. paradisiacal* ABB Linn.), when digested by 3.5% cellulase R-10, 1% macerozyme R-10 and 0.15% pectolyase Y23 (Dai et al., 2010). The current approach has the advantage of shortening the digestion time required to isolate protoplasts, allowing protoplast to stay viable before depletion of nutrients. Regardless of the tissue and the cultivar used, cellulase RS is a vital enzyme for banana protoplast isolation, such as the corm tissue of banana cv. Basrai (AAA group)

and derived embryogenic suspensions of banana cv. Embrapa/CNPMF 2803-01 (AA group). This likely is due to the composition of cellulose, which contains exoglucanases, endoglucanases and β-glucosidase, allowing the dissolution of the banana cell wall (Jayasekara & Ragnayake, 2019). In the future, other cell wall-degrading enzymes, such as driselase, xylanases and hemicellulose, could be used to isolate protoplasts. This is because the cell wall of plants is made up of a complex blend of cellulose, hemicellulose, pectin, proteins, and lipids (Davey et al., 2005). Driselase is a degrading cell wall enzyme that contains cellulase, hemicellulase, and pectinase. Driselase has been used for the protoplast isolation of olive (Sahouli et al., 2022), oil palm (Fizree et al., 2021) and potato (Wang et al., 2018). Hemicellulase is a group of enzymes that catalyzes the breakdown of heteropolysaccharides. It includes glucanases that act on glucan. Hemicellulase has been used in the protoplast isolation of guava (Rezazadeh et al., 2015), mango (Rezazadeh et al., 2011) and apricot (Ortin-Parraga & Burgos, 2003).

Vacuum infiltration is a common pre-treatment for protoplast isolation in monocotyledonous plants, such as *Alstroemeria* VV024 (Kim et al., 2005), barley (Saur et al., 2019), and oil palm (Fizree et al., 2021). However, vacuum infiltration only has been reported to increase the efficiency of *Agrobacterium*-mediated transformation of banana cv. Grand Nain (AAA group) (Acereto-Escoffié et al., 2005) and banana cv. Rasthali (AAB group) (Subramanyam et al., 2011), and has not been reported for banana protoplast transfection. In this study, the highest protoplast number and viability percentage were recorded when vacuum infiltrated with enzymatic mixtures for 10 minutes twice. The banana protoplast yield of two-step vacuum infiltration treatment is higher than previous studies of protoplast isolation from the leaf of banana cv. Dwarf Cavendish without vacuum infiltration (1.9×10^4 protoplast/g FW) and callus of Pisang Klutuk cultivars without vacuum infiltration (1.7×10^6 protoplast/g FW) (Partovi et al., 2017). Vacuum infiltration eliminates gases from the plant tissue's intercellular gaps,

allowing for better enzyme penetration and digestion of interior tissue (Razdan, 2003). The use of a two-step vacuum treatment with a one-minute resting time in between allows oxygen to be refilled into the tissues, preventing explant asphyxiation, and has previously been demonstrated to improve the effectiveness of *Agrobacterium*-mediated cucumber transformation (Nanasato et al., 2013). Because of the short rest period, the banana tissue was exposed to vacuum pressure for a longer time, allowing for optimal protoplast release while limiting damage to their viability. In the future, different vacuum infiltration durations could be tested to improve the protoplast isolation efficiency for banana samples. For example, the vacuum infiltration for 10 minutes once, for 15 minutes once, vacuum infiltration for 20 minutes twice, vacuum infiltration for 30 minutes twice, vacuum infiltration for 5 minutes once and twice, etc. In addition, different frequency numbers of the vacuum infiltration treatment to apply to the banana should be tested. For instance, vacuum infiltration for 10 minutes three times, vacuum infiltration for 15 minutes for three times, vacuum infiltration for 20 minutes three times or vacuum infiltration for 30 minutes three times. In this way, the effect of vacuum infiltration treatment on protoplast isolation can be studied systematically.

Mannitol and/or sorbitol are often added to the enzymatic mixture as osmotic stabilizers as they infuse into the protoplasts allowing the protection of the cytoplasm while releasing protoplasts from tissues (Eriksson et al., 2018). Although both 0.4 M mannitol and sorbitol had been used simultaneously to isolate protoplast of diploid *Musa ornata* Roxb (Krikorian et al., 1988), the usage of mannitol alone is sufficient to produce regenerable banana protoplast for triploid cultivars, such as cultivars Grande Naine (AAA group) (Assani et al., 2006), Dwarf Cavendish (AAA group) and Valery (AAA group) (Partovi et al., 2017). As a consequence, mannitol alone was used in this research. The current findings revealed that 0.5 M mannitol produced more protoplasts than other concentrations, implying that this concentration is sufficient to create an osmotic

condition conducive to extracting banana protoplasts from immature male flowers. Similarly, the enzymatic mixture containing 0.5 M mannitol produced protoplast from suspension cultures of banana cv. 'Bluggoe' (ABB group) (8.6×10^6 protoplast/mL) reported by Panis et al. (1993) after digestion for 4 hours by three enzyme combinations. In contrast, Matsumoto et al. (1998) reported that 0.6 M mannitol is required for producing protoplast yield ranged from 2×10^6 to 2×10^7 protoplasat/g FW calli of banana cv. "maca" (AAB group). This seems that the optimal mannitol concentration is cultivar dependent. Reports using the lower mannitol concentrations of 0.4 M produced relatively low banana protoplast yields, ranging from 2.5×10^5 to 8.7×10^5 protoplasts/g FW from corm tissue of banana cv. Basrai (AAA group), William hybrid (AAA group) and Grand Nain (AAA group) (Siddiqui et al., 1995). In the future, sorbitol alone or the combination of sorbitol and mannitol may be tested to improve the protoplast isolation efficiency of bananas. This is because sorbitol is another type of sugar alcohol that is commonly used as an osmotic stabilizer in protoplast isolation (Chong et al., 2010). Sorbitol alone had been used in the protoplast isolation of *Dendrobium crumenatum* (Chong et al., 2010). This is because the usage of sugar alcohol is species-dependent. The combination of sorbitol and mannitol had been used in the protoplast isolation of guava (Rezazadeh et al., 2015). By testing different sugar alcohol, the collection of more information enables the protoplast isolation to become better.

There are challenges to the protoplast isolation of banana cv. Berangan. Firstly, the quality of the protoplast could not be maintained for more than one hour regardless of room temperature or stored in the ice before proceeding to further downstream processes, such as protoplast culture and transfection. This is because the protoplasts are fragile and susceptible to stresses. On the other hand, maintaining protoplast cultures is also challenging, like *in vitro* cultures, such as contamination, somatic variation, and labor-intensiveness. Furthermore, viable protoplasts rebuild wall-like components and

structures rapidly after isolation, often within a few hours (Wang et al., 2012), resulting in the creation of daughter cells. As a result, an efficient storage mechanism for either source materials or protoplasts is required, allowing the regeneration process to be paused while retaining viability (Wang et al., 2012). Cryopreservation appears to meet the aforementioned criteria for long-term storage of in vitro source cultures and viable protoplasts. Carrot protoplasts were cryopreserved successfully using vitrification (Chen et al., 2003). Apart from that, although the viability of the protoplast could be observed and identified under the microscope, there is no established protoplast to isolate the viable protoplast apart from the dead protoplast. The toxic compound, such as reactive oxygen species released from the dead protoplast (Bethke et al., 2001). The effect of the toxic compound is believed to have a negative effect on downstream processes, such as protoplast transfection and protoplast culture. It is believed that the separation of the viable protoplast from the dead protoplast could maintain the viability of the viable protoplast and increase the rate of protoplast transfection and increase the chance of protoplast culture.

The established protoplast isolation protocol can contribute to various research related to genetic engineering. Technology for plant genetic improvements, such as somatic hybridisation and DNA microinjection, are made easier by established and optimised banana protoplast isolation protocol (Rezazadeh and Niedz, 2015). Matsumoto et al. (2010) proposed a protoplast fusion strategy for producing somatic hybrids between triploid and diploid bananas. The method is beneficial for introducing disease resistance from wild relatives or other species into cultivated varieties. Microinjection is one of the most precise procedures for delivering foreign DNA into particular compartments of protoplasts. Microinjections could be used to introduce not only plasmids but whole chromosomes into plant cells (Rezazadeh & Niedz, 2015).

5.3 Optimisation of PEG-Mediated Protoplast Transfection

PEG-mediated protoplast transfection system is a beneficial system for direct transfer of plasmid or RNP into protoplast and PEG-treated protoplasts of banana cv. Gros Michel (AAA group) has been showed the presence of mitotic cell activities, allowing the regeneration via somatic embryogenesis (Assani et al., 2005). Nevertheless, there is no established PEG-mediated protoplast transfection system for banana cv. Berangan. As a consequence, optimising PEG-mediated protoplast transfection procedures is important for different crops (Yoo et al., 2007). The transfection time greatly influences the efficiency of PEG-mediated protoplast transfection (Yoo et al., 2007). Apart from that, it is important to obtain stable transfected protoplast for plant regeneration (Matsumoto et al., 2010). As a consequence, the current research aimed to determine if different transfection times affected the efficiency of PEG-mediated protoplast transfection and the duration of the transfected protoplast keeping its viability.

In this research, the PEG-mediated transfection method was used to introduce pCAMBIA1304-GFP. Different transfection times (10, 15, and 20 minutes) were optimised since it is a parameter affecting protoplast transfection efficiency (Ren et al., 2020). The current study showed that incubation of banana protoplasts in PEG for 15 minutes produced the highest number of protoplasts with intense GFP signals. In comparison, the transfection efficiency in the current work was higher than that reported by Wu et al. (2020), who found 5.6% transfection efficiency in the banana cultivar Baxi (AAA group) after 30 minutes of incubation in PEG. This is because a prolonged incubation of protoplasts in polycation PEG solution can lead to shrinking of the protoplast membrane reducing the uptake of plasmid DNA. When utilising the banana cultivar Bluggoe (ABB group), protoplast incubation in PEG for 3 minutes resulted in low protoplast transfection efficiency (1.03% to 1.35%) (Sagi et al., 1994). Shorter incubation durations in a polycation PEG solution are hypothesised to prevent complete

neutralisation of plasmid DNA and the membrane charge required for DNA entry into the protoplast (Yoo et al., 2007), although this may be due to the transfection time is cultivar dependent. To increase the protoplast transfection frequency, different concentrations of PEG might be tested, as shown by several studies (Burris et al. 2016; Huo et al. 2017). PEG is a polycation solution that has high osmotic pressure. Different concentrations of PEG might affect the volume of the water being sucked out from protoplasts (Lenaghan & Neal Stewart 2019). In addition, the molecular size of the DNA and CRISPR-RNP could also influence the efficiency of PEG in the withdrawal of water (Malnoy et al., 2015). As a consequence, it is likely that the protoplast transfection methods for plasmid DNA by PEG could be further developed and improved.

The amount and quality of transfected protoplasts are as well as important considerations in gene editing and regeneration of crop plants. If protoplasts are truly stressed and dying, the general gene expression program is shut down (Asai et al., 2000). The viability percentages of transfected protoplasts in this study were stable for at least 3 hours. For banana cv. Bluggoe (ABB group), protoplast vitality was observed to be similar (90%) for 4 hours following transfection in PEG solution, with viability dropping to 67% after 24 hours (Sagi et al., 1994). A lack of nutrients could affect the protoplast viability (Matsumoto et al., 2010). In contrast, the viability of the transfected protoplasts was stable for 24 hours in cassava protoplast (Chatukuta & Rey, 2020) and 48 hours in tobacco protoplast (Vanitharani et al., 2003) after by PEG-mediated transfection. The viability of transfected protoplast may be species dependent (Chatukuta & Rey, 2020). In the future, the viability of the protoplasts could be increased by maintaining the protoplast cultures for a longer time, which enables other downstream activities. In banana cv. Basrai, the protoplasts were cultured at a density of 2-3 $\times 10^5$ protoplast/ml in culture media containing SH salts, Staba vitamins and growth hormone with 13% mannitol and maintained at 25°C in the dark (Chatukuta & Rey, 2020). Besides, nurse

cells could also be used to improve protoplast formation, such as the rapidly growing banana, rice or *Lolium prenne* suspension cells (Matsumoto et al., 2010; Haicour et al., 2009).

There is a challenge for the PEG-mediated protoplast transfection of bananas. The recently established protoplasts are suitable for small-scale production when the manpower is sufficient. However, an automation system is needed to perform large-scale production. For instance, an integrated robotic system was recently used to automate protoplast generation, transfection, and fluorescence analysis (Qi et al., 2019). The Orbitor, MultiFlo FX reagent dispenser, Teleshake plate shaker, and InHeco Peltier plate heater/chillers are mostly used for protoplast isolation. All equipment is utilised for transformation, with the exception of the Synergy H1 plate reader, which is used for screening. The Agilent Bravo is the major piece of equipment utilised for protoplast transfer between containers as well as dispensing reagents 70 L. Plates are examined using a microplate reader after transfection and incubation for more than 18 hours to determine transformation efficiency (Qi et al., 2019).

The established and optimised PEG-mediated protoplast transfection system can contribute to other application related to crops improvement. subcellular localisation, *in vivo* protein-protein and protein-DNA interaction, The settings in which proteins work are determined by their subcellular localisation. As a result, protein activity is influenced by subcellular localization by restricting access to and availability of all types of molecular interaction partners. As a consequence, understanding protein localisation is frequently important in describing the physiological function of hypothesised and newly discovered proteins (Shen et al., 2014). Yeast two-hybrid analysis using protoplast is commonly used to study in vivo protein-protein interactions (Strotmann & Stahl., 2022). The binding of GAL4 to the upstream activation region in the Y2H system causes the

transcription of an enzymatic reporter gene, such as lacZ, which codes for -galactosidase. The separation of these two domains and their fusing to two distinct POIs enables testing of their interaction with a simple read-out, such as a colour response produced by the presence of a suitable substrate for -galactosidase (Ehlert et al., 2006). Chromatin immunoprecipitation (ChIP) is a vital technique for studying the in vivo binding of DNA-binding proteins (e.g., transcription factors) to their target sequences. The fast and straightforward nature of the ChIP test utilising Arabidopsis mesophyll protoplasts allows researchers to investigate in vivo interactions between transcription factors and their target genes (Lee et al., 2017).

5.4 DNA-Free CRISPR-Cas9 Gene Editing of *STP13*

The DNA-free CRISPR-Cas9 gene editing system is suitable for crops that are seedless, clonally propagated and have difficulty in cross-breeding, because the system can edit the target gene without the introduction of foreign genes, allowing the production of edited crops that can fall outside of GMO regulation (Park et al., 2019). However, there is no established DNA-free CRISPR-Cas9 protocol for banana cv. Berangan. Optimising DNA-free CRISPR-Cas9 genome editing system is crucial for different plant species or cultivars (Osakabe et al., 2015). The ratio of RNP components greatly affect the success of DNA-free CRISPR-Cas9 genome editing. Additionally, it is necessary to obtain double strand break and indels near the PAM site to ensure accurate gene knock-out (Park et al., 2019). Therefore, the current research determined if the ratio of RNP components influence DNA-free gene editing of *STP13*, although the result is limited to the analysis of the target site.

In this study, T7 endonuclease 1 (T7E1) was used to pre-screen the presence of mutation of the protoplast after being transfected with CRISPR-RNP by amplified PCR products (Vouillot et al., 2015). Double-strand breaks (DSBs) are formed when a cell

experiences CRISPR/Cas9-mediated editing, and the endogenous DNA repair machinery responds quickly. In most circumstances, cells immediately ligate gaps after DSBs are produced, using the error-prone non-homologous end-joining (NHEJ) mechanism. The T7E1 assay can detect insertion or deletion alterations in this low-fidelity repair pathway (Vouillot et al., 2015). To do so, genomic DNA from the samples will be first amplified using PCR. Subsequently, the PCR products will be denatured and progressively annealed at room temperature to allow heteroduplexes to develop. This process will result in a DNA bulb that T7E1 recognises and cleaves. The frequency of mutations will then be determined by comparing the gel patterns of T7E1-cutting products in control and experimental groups. However, this method can only provide information on the likely frequency of mutations after editing (Sentmanat et al., 2018).

The development of sequence-specific nucleases like TALENs and ZFNs, as well as the potent CRISPR/Cas9 system, has transformed genome editing. The CRISPR/Cas9 system is extremely adaptable, having been used in a wide range of organisms from many walks of life. Regardless of the organism being studied, all sequence-specific nuclease researchers encounter the same difficulties, which is the confirmation of the desired mutation on-target. On-target mutation detection is the target sequence edited by editing endonucleases is known (Chen et al., 2019). To do so, corresponding paired primers are created, then polymerase chain reaction (PCR) and subsequent sequencing or other high-resolution technologies are used to discern tiny variations between wild-type and mutant PCR fragments. Sanger sequencing is simple and requires no special equipment or technology; nevertheless, when the sample size is big, it is time consuming and costly (Belhaj et al., 2015). Next-generation sequencing (NGS) is more expensive than Sanger sequencing, but it allows for larger sample sizes. Apart from that, NGS gives a mutation-to-wild-type gene ratio. As a result, PCR combined with NGS sequencing can reveal the exact amount of on-target locus change before and after editing (Belhaj et al., 2015).

The ratio of Cas9 to gRNA is critical to producing a high mutation percentage in crops when utilising CRISPR RNP (Malnoy et al., 2016). Despite the fact that a 1:1 Cas9:gRNA ratio can be used for RNP construction (Kim et al., 2014), the current findings revealed that a 3:1 Cas9:gRNA ratio could produce a relatively high indel frequency (4.1-4.9%), implying that this ratio allows the process that gRNA recognises a PAM site, allowing Cas9 to follow the gRNA and cleave the target site to produce a double-strand break. Similarly, 3:1 Cas9:gRNA ratio produced indel in DIPM1 of apple (6.7%) reported by Malnoy et al. 2016 and IPK of maize (5.85%) reported by Sant'ana et al. 2020. In contrast, Kim et al. (2020) reported that 1:6 Cas9:gRNA ratio is required for producing 17.6% mutation frequency in *CaMLO2* of *Capsicum annuum.* This seems that the varied targets and species have different optimum ratios for nuclease proteins and guide RNAs, which could be due to target specificity. Reports using the 1:1 ratio of Cas9 and gRNA produced relatively low indel frequency (0.42%) in *PDS* of banana cv. Baxi (Wu et al., 2020). In the future, a higher amount of Cas9 can be tested as it allows a higher chance of the target site being cleaved (Holmgaard et al., 2021). Holmgaard et al. (2020) reported that the indel frequencies increased as a result of increased amounts of Cas9 in mice cells. The indel frequency of 3,000 ng Cas9 RNP produced 36.9% indel, which was higher than the indel frequency of 250 ng Cas9 RNP, which produced 12.5%. Apart from that, different concentrations of CRISPR RNP should be tested in the future to increase the CRISPR-Cas9 editing efficiency. For example, the mutation efficiency of that *A. niger gaaX* gene when treated with 90 nM of CRISPR RNP, was higher than treated with 54 nM of CRISPR RNP (Kuivanen et al., 2019).

To detect the mutation efficiency generated by the CRISPR-RNP, Miseq of the target site was carried out. This is because, for NGS or targeted deep sequencing, the MiSeq is one of the commonly used techniques (Choudhary et al., 2020). MiSeq, Illumina's integrated next-generation sequencing device, provides end-to-end sequencing solutions

using reversible-terminator sequencing-by-synthesis technology. In a single run, the MiSeq device can perform onboard cluster creation, amplification, genomic DNA sequencing, and data processing, including base calling, alignment, and variant calling (Choudhary et al., 2020). It can run single-end, and paired-end runs with reading durations ranging from 136 base pairs to 2,300 base pairs. In as little as 4 hours of runtime, a single run can produce output data of up to 15 GB and up to 25 million single reads and 50 million paired-end reads. As a result, MiSeq is an excellent platform for quick turnaround. MiSeq is also a cost-effective technique for targeted gene sequencing (amplicon sequencing and target enrichment), metagenomics, and gene expression investigations, among other things. MiSeq has become one of the most extensively utilised next-generation sequencing technologies as a result of these factors (Liang et al., 2018).

Targeted deep sequencing results showed that two mutation patterns, including one and two nucleotide deletions, were induced at the 19th and 20th position of the target sites in banana protoplasts. These mutations in the protein-coding regions are predicted to produce frameshift or premature stop, and as a consequence disrupt the functions of the *STP13* gene in banana cells. Similarly, CRISPR-Cas9 system-induced deletions were templated by the 19th position of the target site of the *PDS* gene of banana cultivar Rasthali (AAA group) by Kaur et al. (2018), Gonja Manjaya (AAB group) and Sukali Ndiizi' (AAB group) by Ntui et al. (2019). The deletions present near the PAM site indicate that the spacer sequence of the designed gRNA matches the genomic sequence immediately upstream of the PAM sequence, allowing the cleavage of genomic DNA by Cas9 to produce double-strand breaks at the recognise position, and NHEJ pathway repairs the DSB. CRISPR-Cas9 gene editing system has been applied in various plants to inactivate salinity-related gene functions, for example, the GmMYB118 of soybeans and *Arabidopsis* (Du et al., 2018), SlARF4 (Auxin Response Factor 4) of tomato (Bouzroud et al., 2020) and drought and salt tolerance (DST) gene of indica rice cv. MTU1010

(Kumar et al., 2020). Targeted deep sequencing is precise in identifying the mutation pattern at the target site, but analysing off-target might be inaccurate as this approach misses potential off-target sites elsewhere in the genome. In contrast, Digenome-seq could be used for studying the off-target effect in the whole banana genome. Furthermore, Digenome-seq is a reproducible and sensitive approach for unbiasedly profiling nuclease off-target effects (Kim et al., 2015). Rather than binding, Digenome-seq relies on DNA cleavage. In addition, unlike in vitro selection methods, Digenome-seq is carried out within a genomic context. Digenome-seq captures potential off-target locations with a DNA/RNA bulge. Besides, Digenome-seq is sensitive enough to discover off-target sites with a frequency of 0.1 percent or lower, which is close to the detection limit of high-throughput sequencing systems (Kim et al., 2015).

The recently established DNA-free CRISPR-Cas9 system for banana can enable further study of *STP13* in bananas, especially if plants could be regenerated from gene edited banana protoplasts in the future. The data and methods generated from this study should be useful for the future DNA-free CRISPR-Cas9 genome editing of bananas for the development of lines with improved stress resistance or other agronomically useful traits. In the future, if the *STP13*-edited banana plants are regenerated, there are several studies can be done to study the effect of mutated on *STP13* on banana plants. The growth of the mutated banana plants can be tested on sea salt. This is because sea salt reduced root length (2.0-6.0-fold), plant height (1.2-1.6-fold), leaf number (2.0-2.3-fold) and leaf area (3.3-4.0-fold) of banana cv. Berangan (Mazumdar et al., 2019). The degradation of pigments (total chlorophyll increased by 1.3–12.3-fold, chlorophyll a by 1.3–9.2-fold, chlorophyll b by 1.3-6.9 fold, and carotenoids by 1.4–3.7-fold) revealed the susceptibility of photosystems to salt stress. Salt stress resulted in a maximum 1.5-fold reduction in relative water content. Sea-salt exposure results in 2.3–3.5-fold increased lipid peroxidation, according to MDA analyses. When compared to control plantlets, roots

exposed to excessive salinity had a 73-fold higher Na value, according to a metal content analysis. When compared to control plantlets, the antioxidant enzymes SOD (3.2-fold), CAT (1.7-fold), and GR (six-fold) were more active at moderate salinity levels, but less active at severe (SOD: 1.3-fold; CAT: 1.5-fold; GR: 2-fold lower) and extreme seawater salinity (SOD: 1.5; CAT: 1.9; GR: 1.3-fold lower).

There are several identified drought-related gene in banana but systematic study has no yet been studied, such as *Calmodulin-binding transcription activator (CAMTA)* (Meer et al., 2019) and Leucine zipper (bZIP) transcription factor gene (Liu et al., 2019). Apart from that, distinct *Musa* genotypes have different intrinsic mechanisms for drought resistance. A number of researchers have studied the effect of water scarcity on commercial cultivar (Mazumdar et al., 2019), diploid acuminata clones (Ismail et al., 2000; Shamsuddin et al., 2000), and Cavendish clone (Ismail et al., 2000). There are, however, very few publications on drought responses across genotypes and their varying. As a consequence, the established DNA-free CRISPR-Cas9 could be applied to study the effect of drought to different cultivars of bananas.

Lastly, DNA-free CRISPR-Cas9 genome editing system also could be applied in improving the regeneration efficiency of banana. It has been discovered that the generation of altered plants utilising embryogenic cell suspension is genotype-dependent. One approach to overcoming this difficulty in banana is to use morphological regulator genes such as *Baby boom* (*Bbm*) and *Wuschel2* (*Wus2*) to improve transformation efficiency (Tripathi et al., 2022). This increases the possibility for banana gene editing in generating resistance to bacterial illnesses in farmers' favoured cultivars. Using morphological regulators, the genetic transformation efficiency of numerous resistant crops such as maize, sorghum, and wheat has been enhanced. For example, the insertion of maize *Bbm* and *Wus2* in addition to a gene of interest facilitated the transformation of

hitherto non-transformable commercial maize lines (Gordon-Kamm et al., 2019). These maize morphogenic genes also promoted embryo metamorphosis in sorghum, sugarcane, rice, and wheat (Gordon-Kamm et al., 2019). Overexpression of a chimeric protein containing growth-regulating factor 4 (GRF4) and its cofactor, growth-interacting factor 1 (GIF1), increased transgenic plant regeneration efficiency and extended transformation and regeneration to known refractory genotypes in wheat (Debernardi et al., 2020). The STM is necessary for the creation of proposer meristems. STM expression boosted somatic embryogenesis by two-fold in *Brassica oleracea*, but expression of maize STM ortholog KNOTTED1 (KN1) increased shoot organogenesis by threefold in *Nicotiana tabacum* (Gordon-Kamm et al., 2019). These findings suggest that morphogenic genes are promising options for improving plant transformation and could be used in banana transformation. These genes may be studied by the recent established DNA-free CRISPR-Cas9 system of banana.

CHAPTER 6: CONCLUSION

The main goal of this study was to establish an efficient host system for banana genome editing work. Finding a suitable host system is critical in ensuring the success of genome editing. In this study, different explant types (immature male flowers and *in vitro* shoot buds) and media were evaluated for embryogenic callus induction. Unfortunately, no embryogenic callus was formed from the juvenile male flowers and *in vitro* shoot bud of banana cv. Berangan, despite many attempts over several months with several batches of banana explant materials. This might be due to the recalcitrance of banana cv. Berangan and the phenolic compounds produced from the explant over a longer period. Thus, optimising callus medium and plant growth regulators is indispensable.

Protoplasts (naked cells) are useful for plant transformation, particularly in the DNA-free CRISPR-Cas9 system. Since there was no standard approach for isolating protoplasts, optimising protoplast isolation procedures for bananas is critical. Several factors, such as tissue type, enzyme combination, vacuum infiltration, and mannitol concentration, could influence protoplast formation and viability. In this study, protoplasts were isolated from the immature male flower of banana cv. Berangan with 1.21×10^7 protoplasts/g tissue with 91.33% viability after 2.5 hours of digestion in enzyme solution consisting of 1% cellulase RS, 1% macerozyme R-10 and 0.15% pectolyase Y23. To increase the protoplast yield, several parameters were optimised, including mannitol concentration and vacuum infiltration treatment. The results revealed that the highest protoplast number (1.54×10^7 protoplasts/g tissue) and viability percentage (96.3%) were recorded when plasmolysing the tissues using 0.5 M mannitol and vacuum infiltrated with enzymatic mixtures for 10 minutes twice. In future, other banana cultivars with the AAA genome could also be evaluated since some cultivars produce a higher percentage of embryogenic callus. Other cell wall-degrading enzymes,

such as driselase, xylanases, and hemicellulose, should also be explored to increase cell wall digestion. Besides, different frequencies of the vacuum infiltration treatment should be tested on the banana. To increase banana protoplast isolation efficiency, sorbitol alone or combined with mannitol may be explored. The challenges of protoplast isolation of *M. acuminata* cv. Berangan is the quality of the protoplast that could not be maintained for more than one hour, and the viable protoplast could not be separated apart from the dead protoplast. The established protoplast isolation methodology can help with numerous genetic engineering research projects. The developed protoplast isolation system may make plant genetic improvement technologies such as somatic hybridization and DNA microinjection easier.

The PEG-mediated protoplast transfection technique is useful for direct transfer of plasmid or RNP into banana cv. Berangan protoplasts. The isolated protoplasts were used to establish PEG-mediated protoplast transfection protocol. The protoplasts transfected with the pCAMBIA1304-GFP plasmid in PEG-4000 for 15 minutes produced green fluorescent signal, indicating successful protoplast transfection. To increase transfection efficiency without losing cell viability, several parameters were optimised. These included the transfection time and the storing time for the transfected protoplasts. The highest protoplast transfection efficiency (76.89%) was recorded when incubating banana protoplasts and pCAMBIA1304-GFP plasmid in PEG for 15 minutes. In addition, the transfected protoplasts were stable for at least 3 hours with 89.9% viability. PEG protoplast transfection methods for plasmid DNA could be developed by determining the optimal PEG concentration. The current PEG-mediated protoplast transfection system is suitable for small-scale production but not for large-scale production. The created and optimised PEG-mediated protoplast transfection technique can contribute to further crop enhancement applications. Proteins' subcellular localisation determines the environments

in which they function. Understanding protein localisation is widely used to describe the physiological function of newly identified proteins.

STP13, a salt-responsive gene from banana cv. Berangan, was selected as the target gene for genome editing. The isolated cDNA of *STP13* was *in vitro* digested by the RNP complex to ensure the designed gRNA can target the *STP13*. After digestion, two bands of about 246 bp and 117 bp were observed, indicating the designed gRNA's ability to cleave *STP13*. The molar ratio of Cas9:*STP13*gRNA was then optimised to improve the indel efficiency. The results showed that a 3:1 Cas9: *STP13*gRNA ratio produced the highest percentage of cleavage at 12.47%. This ratio was then used for the CRISPR-Cas9 gene editing of *STP13*. After being transfected with Cas9 and gRNA at a 3:1 ratio, the protoplasts were harvested for the T7E1 assay. The digested DNA products showed the expected uncleaved and cleaved band sizes, indicating the successful editing of *STP13*. Finally, targeted deep sequencing was performed to determine the mutation frequency. The results showed that mutation frequency ranged from 4.1% to 4.9%. Two mutation patterns, including one and two nucleotide deletions, were induced at the 19th and 20th nucleotide positions of the target sites in banana protoplasts. A greater amount of Cas9 can be tested in the future. This is because a larger concentration of Cas9 increases the likelihood of the target site being cleaved. Digenome-seq is recommended for future off-target studies since it is a reproducible and sensitive method for profiling nuclease off-target effects.

In conclusion, optimal protoplast isolation and transfection protocols have been established. These protocols could benefit future DNA-free CRISPR-Cas9 genome editing for other crops to establish lines with increased stress tolerance or other agronomically important features. In future work, the transfected banana protoplast cultures can be regenerated into whole plants to further analyse the role of *STP13* in

alleviating salt stress in banana plants. If the STP13-edited banana plants are regenerated, further investigations can be conducted to determine the effect of STP13 mutations on banana plants. The mutant banana plants' development can be examined in sea salt. Aside from that, other known drought-related genes in banana that have yet to be examined could be studied using the existing DNA-free CRISPR-Cas9 to aid in the advancement of banana drought resistance.

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