Transformation of Mungbeans via Stratification of Seeds

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DECLARATION

I hereby declare that this thesis entitled 'Transformation of Mungbean via Stratification of Seeds' is my own work and all sources have been cited and referred to have been acknowledge by means of complete references. It has been submitted and shall not submit to other university or higher learning institute.

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List of Abbreviations

%	percent
µl	microlite
°C	degree Celsius
AGE	Agarose Gel Electrophoresis
DNA	Deoxyribonucloic acid
LB	Luria-Bertani Broth
LA	Luria Agar
T-DNA	Transfer DNA
GUS	β-glucuronidase
rpm	Rotation per minute
E. coli	*Escherichia coli*
OD	Optical density
ml	mililitre
DMSO	Dimethyl sulfoxide
hpt	Hygromycin phosphotransferase
PCR	Polymerase chain reaction
MS	Murashige and Skoog
CIA	Chloroform – Isoamyl Alcohol
SDS	Sodium Docedyl Sulphate
$CaCl_2$	Calcium Chloride

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ABSTRACT

Mungbean (*Vigna radiata* (L.) Wilzeck) is one of the important crops used in daily life. Mungbean is rich in protein and easy to digest. Plant transformation can be conducted in several ways; either through direct DNA introduction or natural transfer mechanism. In this study, transformation was conducted using natural transfer mechanism via use of *Agrobacterium tumefaciens*. The *Agrobacterium* carrying the plamid pGSA1131, contains the antibiotic resistance towards chloramphenicol and a reporter gene, β-glucuronidase (GUS). For this work, the transformation was done via the use of stratified and non-stratified seeds. To facilitate the transformation, the seeds were exposed to vacuum infiltration and also the addition of acetosyringone. Analysis of putative transformants was done via extraction of plant DNA and detection of transgenes via polymerase chain reaction (PCR) technique. The PCR detecting the GUS reporter gene were undertaken and showed the presence of the gene in the putative transformants. It was also shown that stratification of the seeds facilitated the transfer and integration of transgenes.

Keywords: Mungbean, *Agrobacterium*, pGSA1131, vacuum infiltration, acetosyringone.

ABSTRAK

Kacang hijau (*Vigna radiata* (L.) Wilzeck) merupakan salah satu tanaman penting yang digunakan dalam kehidupan seharian. Kacang hijau kaya dengan protein dan mudah untuk dihadam. Transformasi tumbuhan boleh berlaku melalui beberapa cara, antaranya melalu pengenalan terus DNA ataupun mekanisma pemindahan secara semulajadi. Dalam projek ini transformasi dijalankan menggunakan mekanisma pemindahan secara semulajadi iaitu menggunakan *Agrobacterium tumefaciens*. *Agrobacterium* akan membawa plasmid pGSA1131 yang mengandungi rintangan antibiotik Chloramphenicol dan gen β-glucuronidase (GUS). Dalam projek ini, transformasi dijalankan menggunakan biji benih yang distratifikasikan dan juga tidak distratifikasi. Untuk membantu proses transformasi, biji benih tersebut didedahkan kepada infiltrasi vakum dan juga acetosyringone. Proses isolasi dan pengasingan DNA tumbuhan dijalankan bagi mengenal pasti sama ada berlakunya transformasi. Polymerase chain reaction (PCR) juga dijalankan untuk melihat perpindahan GUS reporter gene ke dalam tumbuhan. Transformasi berjaya dilakukan dalam tumbuhan yang biji benihnya distratifikasikan.

Kata kunci: Kacang hijau, *Agrobacterium*, pGSA1131, infiltrasi vakum, acetosyringone..

1.0 Introduction

Mungbean is one of the important crops in agricultural world as it is rich in protein. Islam and Islam (2010) stated that, another importance of mungbean is, it can be used in soil fertility by fixing atmospheric nitrogen. Mungbean is very widely used for its excellent protein quality and high digestibility pulses.

Transformation of plant and animal cell can help to increase the productivity of the animal and plant. For plant transformation, the transfer of the DNA can be divided into two that is the direct DNA introduction and natural transfer mechanism. For direct DNA introduction, it includes PEG-mediated DNA transfer and DNA bombardment. While for natural transfer mechanisms, it can be from viruses or *Agrobacterium*. For this study, the *Agrobacterium tumefaciens* was used as the method to transfer the DNA.

According to Chawla (2009), there are many advantages of using *Agrobacterium* method. One advantages is that *Agrobacterium* is capable of infecting the plant cells, tissues or organ and transformed the plant. Another advantage is that *Agrobacterium* can transfer large DNA fragment efficiently without substantial rearrangement.

The disadvantage of this method is in its limitations of host that can be infected by *Agrobacterium*, especially to cereals (Wheeler, Farrand, & Widholm, n.d). Besides, the cells in tissue that has been regenerate are difficult to transform and may not be suitable for T-DNA transfer.

In this work, mungbean seeds were used as the host for transformation. Two treatments of mungbean seeds were conducted prior to transformation i.e. stratified and non-stratified. Referring to Conner (2008), stratification affects the germination of 'Fry' Muscadine (*Vitis rotundifolia*) seeds. The transformation of the plasmid into the seeds were also assisted by application of vacuum and addition of acetosyringone. The vector used in this transformation is pGSA1131, contains the chloramphenicol antibiotic resistance and β-glucuronidase (GUS) reporter genes.

1.1 Problem Statements

1. Can seeds of mungbean be transformed using *Agrobacterium tumefaciens*?
2. What are the effects of transformation when using wounded and non-wounded seeds?

1.2 Objectives

1. To use the Agrobacterium-mediated technique to transform mungbean seeds
2. To analyze mungbean transformed by wounded seed and non-wounded seeds.

2.0 Literature review

2.1 Mungbean

Mungbean is one of the important crops in the world. It is used in many countries in the world as it is high in protein and easy to digest. The scientific name for mungbean is *Vigna radiata* (L.) Wilzeck. According to Poehlman (1991), mungbean is a leguminous species or pulse crop that is grown for its protein-rich edible seeds. As stated by an article from the Washington State University website, this legume crops have the ability to release nitrogen into soil and play an important role as a rotation crop.

Poehlman (1991) also stated that mungbean is sensitive to low temperature and have suitable temperature range of 20°C to 40°C. In low temperature, mungbean will be easily killed. This explains why the mungbean crops are mostly found in tropical countries. Poehlman (1991) also stated that mungbean can produce crops on a limited soil moisture supply due to its short growth duration.

There are many researches that have been conducted on mungbeans. One of the studies conducted by Tazeen and Mirza (2004) focused on the factors affecting *Agrobacterium tumefaciens*-mediated genetic transformation of mungbean. The study was aimed to standardize the *Agrobacterium* transformation protocol for *Vigna radiata* and the parameters used were; sensitivity of explant towards kanamycin, pH of co-culture media, age of explants, type of explants, co-cultivation time and optical density of *Agrobacterium* culture medium. Tarzeen and Mirza (2004) stated that kanamycin is a suitable selectable marker for *Vigna radiata*.

Another study by Islam and Islam (2010) used the cotyledon attached with embryonal axis (CAEA) and cotyledonary leaf as explants. The result showed that transformations that occur through CAEA are more effective compare to the cotyledonary explants. From this study also, Islam and Islam (2010) stated that the marker genes such as GUS and selectable marker such as neomycin phophotrasnferase assay (nptIII) are applicable for transformation in mungbean.

2.2 Transformation via *Agrobacterium tumefaciens*

The aim of genetic transformation is to introduce foreign gene into host genome. Transformation involves three important steps that are: manipulation of foreign gene, transformation and selection methods, and lastly is the method of analysis. There are many methods in transferring the DNA. First is direct DNA introduction which is divided to two that are: (i) PEG-mediated DNA transfer and (ii) DNA bombardment. While for the second method is natural transfer mechanism that is also divided into two: (i) plant viruses and (ii) *Agrobacterium*. *Agrobacterium* is divided into several types which are; *A. radiobacter*, *A. tumefaciens*, *A. rhizogenes* and *A. rubi* (Gelvin, 2003). According to Gelvin (2003), each of the *Agrobacterium* has it symptoms, the *A. radiobacter* is an 'avirulent' species, *A. tumefaciens* causes crown gall disease, *A. rhizogenes* causes hairy root disease and *A. rubi* causes cane gall disease.

In this study, the *A. tumefaciens* is used for the transformation. According to Chawla (2003), *Agrobacterium* is a natural genetic engineer because it has the unique capacity to transfer its own DNA into nuclear genome of plant cells. Slater et al. (2003) stated that the *A. tumefaciens* is the causative agent of 'crown gall' disease. *A. tumefaciens* is the Gram-negative, rod-

shaped, motile bacterium that found in rhizosphere (region around the roots of the plant). It normally survives on nutrients releases from the plant roots.

However, when the plant is wounded, *A. tumefaciens* can infect the plant at the wound site and causes disease to the plants. Slater et al. (2003) also stated that, formation of the crown-gall disease depended on the presence of the plasmid in the *A. tumefaciens* that known as 'Ti (tumour-inducing) plasmid'.

2.3 Binary vector, pGSA1131

T-DNA is the region which is transferred to the host plant cells. The vector pGSA1131 consists of chloramphenicol resistant gene and β-glucuronidase (GUS) as the reporter gene (Figure 1).

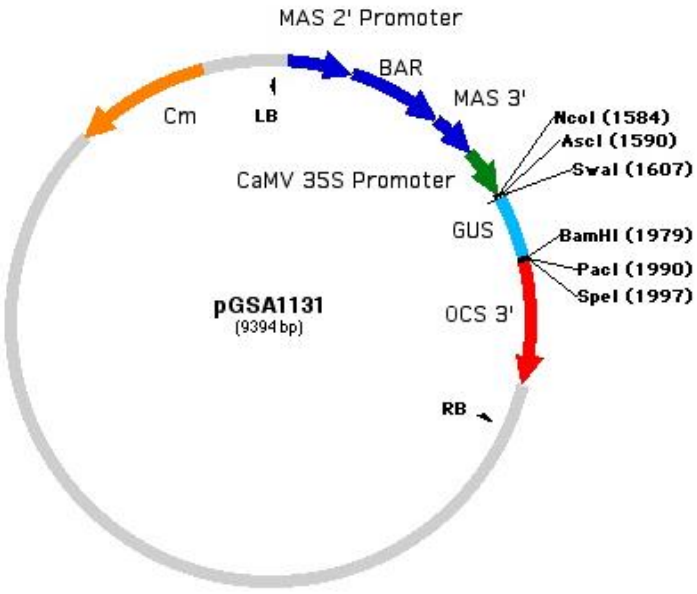

Figure 1. The vector construct consist of chloramphenicol (Cm) and GUS reporter gene. Size of the vector is approximately 9000 bp, and the size of GUS reporter gene is approximately 300 bp.

According to Rao & Rohini (2008), the selectable marker functions as the selection agent of the transformed cell and their capability to grow in the presence of antibiotic or herbicide. While for the reporter gene, it encodes for a gene that can be assayed to allow for the detection of the transformant. GUS gene is a widely used reporter gene for plant transformation and this is due to its easy and sensitive detected in either *in vitro* or *in situ* analyses. It is also strong enough to withstand fixation and enables histochemical localization in cells and tissue sections (Rao & Rohini, 2008).

2.4 Acetosyringone

Acetosyringone is used to increase the effectiveness of the *Agrobacterium* transfer. According to Sheikholeslam and Weeks (1987) acetosyringone increases the efficiency of the transformation in the *Arabidopsis thaliana*. They stated that acetosyringone causes the activation of the *Agrobacterium* and Ti plasmid that promote the transfer of the DNA from *Agrobacterium* into plant cells.

3.0 Material and methods

3.1 Preparation of medium and solutions

Luria agar (LA) and Luria broth (LB) were prepared in volume of 250 ml. Approximately 8.75 g of LA and 6.25 g of LB each were dissolved in distilled water in a 250 ml bottle. Solutions I, II and III for extraction of bacterial plasmid were also prepared in volume of 10 ml and it follows the following table:

Table 1. Solutions I, II, and III use in isolation of bacterial plasmid

Solution I	Solution II	Solution III
0.09 g of Glucose 0.83 g of Formic Acid 0.03 g of Tris-HCl	0.08 g of NaOH 0.1 g of SDS	2.94 g of KAc (Potassium acetate) 0.03 g of EDTA Me

For plant transformation, the media used were the infiltration media solution and agar, and the antibiotics carbenicillin. For infiltration media, the solution contains 0.15 g of Murashige and Skoog (MS) powder, 2 g of sucrose and 0.35 g of MES dissolved in a 70 ml volume. For infiltration agar, 0.22 g of MS powder together with 1 g of agar bactericidal powder dissolved in 100 ml volume. Carbenicillin was prepared by dissolving 1 g of carbenicillin in 10 ml of sterile distilled water and filtered sterilized using 0.22 µM filter. Carbenicillin is used at 1:1000 dilutions in LB and LB agar.

3.2 Preparation of *E. coli* competent cells

Firstly, the fresh culture of *E. coli* was plated on agar and incubated for 16 to 20 hours at 37°C. The next day, a single colony of the *E. coli* was picked and transferred into 13 mL of

Luria broth (LB) medium at 250 mL flask. Then, the LB was incubated for 16 - 18 hours at 37°C with vigorous shaking (250-300 rpm).

The next morning, the optical density (O.D) of the culture was measured at 600 nm. And then allow it to grow at 37 °C with shaking at 350 rpm until the reading reached 0.4 to 0.55. Then, the culture vessel was cooled ice for 10 to 20 minutes. After that, the cells were harvested by centrifugation at 3500 rpm for 5 minutes in 4°C. Then, the supernatant was poured off and the cells was washed gently using 4-5 ml iced-cold 100 mM $CaCl_2$. The cells suspension was kept on ice for 10 minutes and then it was centrifuged again as before. After the centrifugation, the supernatant was discarded again and then the pellet was resuspended gently with cold sterile 100 mM $CaCl_2$. After that, it was incubated on ice for 1 hour until being used. Then, the competent cells were snap freeze in liquid nitrogen and the stock was stored at -80°C.

3.3 Transformation of *E. coli* competent cells

The transformation of competent cell was according to the method described by Sim et al. (2011). It starts with equilibration of the ligation reaction mixture to the room temperature for 1 minute. Then, the mixture was centrifuged at 8, 000 rpm. The ligation mixture was transferred to a sterile 2 ml centrifuge tube that has been pre-cooled on ice. After that, the XL1 blue competent cells were removed from the freezer and placed in a 50% ice/deionized water bath for exactly 5 minutes and flicking it gently to mix. After that, 200 µL of competent cells were added to the Falcon tubes on ice using wide-bore pipette tips and pipetted gently. Then, the mix was incubated on ice for 20 minutes. Subsequently, the cells were heat shock for 45 econds at 42°C water bath and return back onto ice for 2 minutes. Then, 950 ml of LB media

were added to each of the transformation and flicking it gently to mix the mixture. After that, the tubes were incubated in an incubator shaker for 90 minutes at 37°C.

After 60 minutes, LB plates were placed at 37°C incubator oven and were inverted with lids off for 30 minutes to dry. After the plates have dried, the transformation was incubated for 90 minutes in the shaker, the tubes were centrifuged at 10,000 rpm for 2 minutes. After 2 minutes, the supernatant were poured off and the cells were resuspended in 180 µL of LB. Then, two LA were prepared by spreading 100 µL and 80 µL of transformation mixture. The plates were then sealed with parafilm strip and place in 37°C incubator for overnight.

3.4 Isolation of bacterial plasmid

The isolation of bacterial plasmid was performed using the mini-prep isolation method described by Sim et al. (2011). This method uses the Solution I, II and III that have been prepared earlier.

Firstly, the bacteria were harvested from an overnight culture by transferring the culture into a microcentrifuge tube. Then, it was centrifuged at 8000 rpm for 2 minutes at room temperature. The supernatant was removed and the pellet was recentrifuged for 1 minute. Then, the cell pellet was resuspended using 100 µl of solution I, resuspended by vortexing it briefly for 10 seconds and put directly into ice.

After that, 100 µl of Solution II was added to the cell suspension and mix gently by inverting the tube. The tube was left at room temperature allowing lysis reaction to occur for 5 minutes. The lysis reaction produced clear viscous liquid. Next, 300 µl of Solution III was added and mixed by inverting the tube and then white precipitate was observed. The pellet was

centrifuged again at 10,000 rpm for 5 minutes. Then, the supernatant was transferred carefully into sterile microcentrifuge tube.

The DNA was precipitated by adding the cold absolute ethanol. The content was mixed gently by inverting the tube. DNA was pelleted by centrifugation at 13,000 rpm for 5 minutes at room temperature. The supernatant was discarded, washed with 500 µl of 70% ethanol and was centrifuged at 13,000 rpm for 2 minutes. The supernatant was discarded and the pellet was allowed to air dry for 15 minutes at room temperature. Finally, the DNA was resuspended and dissolved in 50 µl of sterile ultra-pure water. The plasmid DNA was checked using agarose gel electrophoresis (AGE).

3.5 Preparation of *Agrobacterium* competent cells

Referring to protocols by Chawla (2003), to prepare the *Agrobacterium* culture, an empty *Agrobacterium* strain was cultured in 10 ml Luria Agar (LA). Exactly 10 ml of LA was poured together with 20 µl rifampicin into petri dish and left for 20 to 30 minutes. After that, *Agrobacterium* was added into two petri dish using volume 5 µl and 10 µl each. Both plates were stored in dark condition at room temperature for 2 days.

After 2 days, single colony from each plate was put picked and transferred into 50 ml Falcon tubes containing 10 ml LB with 20 µl rifampicin. The tubes, sealed with aluminium foil to keep in dark condition, were left on a rotary shaker (160 rpm) for two days at room temperature. The OD of the *Agrobacterium* culture was measured at 550 nm until the required reading was reached. Approximately 3ml of the culture was transferred to 50ml of LB medium and left at 28°C rotating shaker for 1-2 hours and the OD_{550} reaches between 0.6-0.9.

After the culture reached to the required OD, it was transferred to a falcon tube and chilled in ice water for 10 minutes. After that, the culture was centrifuged for 10 minutes at 4,000 rpm (4°C). Then, the supernatant was removed and the pellet was gently resuspended with 1.0 ml ice cold 20 mM calcium chloride. Then, it was mixed with 20% glycerol and aliquoted into individual microcentrifuge tube.

3.6 Transformation of *Agrobacterium tumefaciens.*

The transformation of *Agrobacterium* was started with mixing 10 µl of plasmid DNA together with 100 µl of *Agrobacterium* competent cells (Chawla, 2009). The tube was mixed by gently pipetting up and down a few times and put on ice for 25 minutes. After that, the tube containing plasmid DNA and *Agrobacterium* was frozen using liquid nitrogen for 5 minutes. The tube was then heat shocked by put the tube in 37°C water bath for 5 minutes and returning it to ice for another 5 minutes. Then, 1 ml of LB was added to the tube and incubated on rotating shakers for 3 to 4 hours and overnight.

The next day, 100 µl of the culture was spread on LB plate (LA) containing chloramphenicol and rifampicin. The plate was left to grow for 2 days at 28°C (at bench).

Table 2. Mixture of LB and appropriate selection agent

LB / LB plate (LA)	10 ml
Chloramphenicol	3 µl
Rifampicin	20 µl

A single colony from the plate was picked and transferred to LB supplemented with chloramphenicol and rifampicin. It was left to grow on a rotating shaker (28 °C) with 180 rpm for 2 days.

3.7 Isolation of *Agrobacterium tumefaciens*

The isolation of the *Agrobacterium* was performed using DNA extraction kit. The solution that were used includes Activation Buffer PL, Cell R, Lysis Blue Buffer, Buffer Neutral B (neutralization and binding buffer), Wash PLX 1, Wash PLX2 and Elution Buffer.

The isolation started equilibration of the spin column with 40 µl of activation buffer PL at room temperature. The culture was transferred into a 1.5 ml tubes and centrifuged at 14,000 rpm for 2 minutes. After that, the supernatant is poured off and the tubes are turned upside down to remove remaining media.

It then proceeded with the addition of 250 µl of Cell R and the pellet was resuspended. Then, 200 µl of blue-coloured Lysis Buffer was added into the tubes and gently mixed until uniform blue colour of cell resuspension was obtained. After that, 350 µl of neutralization and binding buffer Neutral B was added and inverted until the blue colour disappears. The tube was the centrifuged at 14,000 rpm for 7 minutes. The supernatant was transferred into spin-column and placed in the receiver tube, spun down again at 12,000 rpm for 1 minute. The spin-column was removed and the supernatant was poured off.

After that, 500 µl of Wash PLX1 was added into the tube and proceeded to centrifugation at 12,000 rpm for 1 minute. The supernatant was poured off. Approximately 650 µl Wash PLX2 was added, centrifuged again at 12,000 rpm for 1 minute and the supernatant was removed

again. The tube was centrifuged again at 12,000 rpm for 2 minutes to remove any traces inside the tube. Lastly, 50 µl of Elution buffer was added and left for incubate for 2 minutes at room temperature. It was then centrifuged again at 12,000 rpm for 2 minutes and then the plasmid DNA was now ready for analysis.

3.8 Preparation of seeds of mungbean

To prepare the seeds, the seeds were washed in running tap to clean it. After the washing, the seed were treated with 70% alcohol for 30 second to 2 minutes in a beaker. to completely removed all the chemical, the seeds thoroughly for 5 times with sterile distilled water. Lastly, the seeds were left in a plate containing sterile distilled water for few hours to soften the seeds.

For the stratification methods, the mungbean was scarified uniformly using sterile needle. Approximately, 5 to 6 holes were made on the seeds and it is performed gently to ensure the seeds do not break.

3.9 Transformation of mungbean

The co-cultivation method started with measuring the O.D of the *Agrobacterium* at 600 nm (Chawla, 2003). A volume of 3 ml of overnight culture (*Agrobacterium*, LB, appropriate antibiotic) was put in 50 ml of LB containing chloramphenicol and rifampicin, and left to grow for 1 to 2 hours at 28°C rotating shaker with speed 180 rpm. The OD was checked in between the incubation time to ensure that it will not exceed between the readings of 0.7 to 0.9.

After reaching the required OD, the culture was poured off into a new falcon tube and centrifuged (28°C) at 3500 rpm for 10 minutes. The supernatant was discarded and thepellet was rinsed using infiltration media. Next, the prepared seeds were kept in a beaker and incubated with infiltration media containing the *Agrobacterium* and 150 µM of acetosyringone and 2 µl of silwet.

Vacuum infiltration was applied according to method described by Weigel and Glazebrook (2002). The plants were transferred into a desiccator. Then, the vacuum was applied until bubbles appeared in the solution. The vacuum was then released quickly. This sudden increase in pressure will force the bacterial cells into the plant tissue. It was then left to rest in the machine for 15 to 20 minutes with pressure 700 mbar. The treated seeds were then transferred on the plate with agar which contain acetosyringone and left to grow for one day. The next day, the seeds were washed with 150 µM of carbenicillin and water to clean the *Agrobacterium* suspension and then directly put in the soil.

3.10 Isolation of plant DNA

To extract plant DNA, the leaves were cut into small pieces and put in 1.5 ml microcentrifuge tube. All the tubes were labeled according to the leaves from respective plant. In the meantime, a tube of β-mercaptoethanol with CTAB buffer was prepared, and heated in water bath (65°C). Then, the liquid nitrogen was put in tube and the leaves were grinded using plastic pestle until slurry. After that, 1 ml of the CTAB and β- mercaptoethanol was added to the tube and vortex until the CTAB and the leaves are mixed. The tubes were then put in 65°C waterbath for 30 minutes and vortex in 10 minutes interval. After 30 minutes, the tubes were removed from the waterbath and left to cool. Approximately, 200 µl of CIA was added into

the tubes and mixture was vortexed again to mix. The tubes were hen centrifuged for 5 minutes at 13,000 rpm. After that, the supernatant was transferred into new tube and added with 500 µl of CIA, vortexed and centrifuge at 13,000 rpm for 5 minutes. The supernatant was transferred into a new tube and 600 µl of ice-cold isopropanol was added. The tube was mixed to precipitate the DNA and stored at -20°C overnight.

The extraction process was proceeds the next day by centrifuging the DNA that has been stored. The supernatant was poured off and the pellet was mixed with 1 ml wash buffer and gently mixed. Then, the tube is stored in -20°C for at least 30 minutes. After that, the tube was centrifuged at 13,000 rpm for 5 minutes, the supernatant was poured off the pellet and left to dry. Finally, the pellet was resuspended with 50 µl of TE buffer and stored at -20°C.

4.1 Agarose gel electrophoresis (AGE) and polymerase chain reaction (PCR) method

To analyse the DNA, polymerase chain reaction (PCR) technique was used. A master mix of the PCR mixture was prepared and used in the analysis (Table 3). The master mix was then aliquoted into individual PCR tubes and mix gently.

To run the PCR, the correct profile should be used according to the ingredients and also the primer used. The suitable temperature and time was needed to ensure the PCR process was done correctly. PCR profile used in this study is listed in Table 4.

Table 3. PCR mixture

Solution	x1
10x Taq Buffer	2.5 µl
dNTP Mix	0.5 µl
Forward Primer	1 µl
Reverse Primer	1 µl
25 mM $MgCl_2$	1.5 µl
Template DNA	1 µl
Taq DNA polymerase	1 µl
Water, Nuclease Free	16.5 µl
Total volume	**25 µl**

Table 4. PCR profile

Step	Temperature (°C)	Time	Number of Cycles
Initial denaturation	95	3 mins	1
Denaturation	95	30 sec	35
Annealing	57.5	30 sec	
Extension	72	45 sec	
Final extension	72	5 mins	1
Final hold	20	∞	1

Agarose electrophoresis gel (AGE) was prepared by addition of 0.3 g of agarose powder in 30 ml TAE buffer. The mixture was heated in the oven for 2 minutes and left to cool before adding ethidium bromide (EtBr). The mix was then poured into a casing tray and let to solidify. The samples were then loaded with loading dye and let run for 30 minutes.