Transformation of mungbean cotyledon by using

*Agrobacterium tumefaciens*

Syafiqah Diana Binti Subandi
(38954)

Bachelor of Science with Honours
(Resource Biotechnology)
2015
ACKNOWLEDGEMENT

In preparing this thesis, I was in contact with many people, researchers, and academicians. They have contributed towards my understanding and thoughts.

Foremost, I would like to express my sincere gratitude to my supervisor, AP Dr. Hairul Azman Roslan for the continuous support of my final year project, for his motivation, patience and immense knowledge. His guidance helped me in all the time of research and writing of this thesis.

My sincere thanks also goes to almighty, my parents, my dear sister, my lab mates and my friends for their constant encouragement and supporting me spiritually throughout my life. Without them, this project would not be possible.
DECLARATION

I declare that this thesis entitled “Transformation of mungbean cotyledon by using Agrobacterium tumefaciens” is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in the candidature of any other degree.

Signature : ....................................................

Name : Syafiqah Diana Binti Subandi

Date : June 2015
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ACKNOWLEDGEMENT</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>DECLARATION</td>
<td>II</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>III</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>V</td>
</tr>
<tr>
<td>LIST OF TABLES AND FIGURES</td>
<td>VI</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>VIII</td>
</tr>
</tbody>
</table>

1. Introduction 1
   1.1 Problem statement 2
   1.2 Objective 2

2. Literature Review 3
   2.1 *Agrobacterium*-mediated transformation 3
      2.1.1 Transfer of T-DNA to plant cells 4
      2.1.2 Binary vector 5
   2.2 pGPTV 6
   2.3 Mungbean (*Vigna radiata* (L.) Wilezek) 6

3. Materials and Method 7
   3.1 Preparation medium and solution 7
   3.2 Preparation of competent *E.coli* 7
3.3 Calcium chloride preparation of *Agrobacterium tumefaciens* competent cells

3.4 Standard heat-shock transformation of chemically competent bacteria

3.5 Isolation of plasmid DNA from bacterial culture

3.6 Agarose gel electrophoresis

3.7 Polymerase Chain Reaction (PCR)

3.8 Transformation of *Agrobacterium* into plasmid DNA

3.9 Isolation of double stranded plasmid DNA from bacterial culture by using kit

3.10 Plant transformation

3.11 Plant genomic DNA extraction

3.12 Purification of genomic DNA

4. Result and discussion

4.1 Preparation of competent cells and preparation of *Agrobacterium tumefaciens*

4.2 Transformation of competent cells with plasmid pGPTV by using standard heat-shock

4.3 Bacterial plasmid isolation

4.4 Heat shock *Agrobacterium* transformation

4.5 Mungbean transformation I (mungbean at 2 leaves stage)

4.6 Mungbean transformation II (cotyledon attached with embryo)

5. Conclusion

6. References
# LIST OF ABBREVIATION

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>percent</td>
</tr>
<tr>
<td>μL</td>
<td>microliter</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celcius</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>GUS</td>
<td>β-glucuronidase gene</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>LUC</td>
<td>Luciferase gene</td>
</tr>
<tr>
<td>LBA</td>
<td>strain LBA</td>
</tr>
<tr>
<td>CTAB</td>
<td>Hexadecyl trimethyl-ammonium bromide</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>mL</td>
<td>milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>micromolar</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Ri-plasmid</td>
<td>Root inducing plasmid</td>
</tr>
<tr>
<td>rpm</td>
<td>rotation per minute</td>
</tr>
<tr>
<td>T-DNA</td>
<td>transfer DNA</td>
</tr>
<tr>
<td>Ti-plasmid</td>
<td>tumor inducing-plasmid</td>
</tr>
<tr>
<td>vir</td>
<td>virulence gene</td>
</tr>
</tbody>
</table>
LIST OF TABLES AND FIGURES

Table 1: PCR mixture

Figure 1: *E. coli* colonies. The plates consist of agar.
Plate (1) - 5 µL of *E. coli*, plate (2) - 10 µL of *E. coli*,
plate (3) - 20 µL of *E. coli*, plate (4) - 30 µL of *E. coli*

Figure 2: *Agrobacterium tumefaciens* colonies. Plate (1)
- 5 µL *Agrobacterium* added on 20 µL rifampicin with LA
medium. Plate (2) - 10 µL *Agrobacterium* added on 20 µL
rifampicin with LA medium.

Figure 3: Genomic DNA after bacterial isolation. Lane L: 1 kb
DNA Ladder (Fermentas), Lane 1: Sample 1,
Lane 2: Sample 2, Lane 3: Sample 3, Lane 4: Sample 4.

Figure 4: Analysis of PCR for bacterial plasmid isolation,
Lane L: 100 bp DNA Ladder (Fermentas),
Lane 1: Sample negative, Lane 2: Sample 1, Lane 3: Sample 2,
Lane 4: Sample 3, Lane 5: Sample 4.

Figure 5(a) Result after isolation of plasmid from *Agrobacterium*
transformed with plasmid pGPTV. Lane L: 1 kb DNA Ladder
(Fermentas), Lane 1: Sample 1, Lane 2: Sample 2.

Figure 5(b) Analysis of PCR of the samples from plasmid isolation of
*Agrobacterium* transformed with plasmid pGPTV.
Lane L: 1 kb DNA Ladder (Fermentas), Lane 1: Sample negative,
Lane 2: Sample 1, Lane 3: Sample 2.

Figure 6(a) Five bottles contains infiltration media and ten plants
(cotyledon leaves) were put inside the bottles for transformation.
Figure 6(b) Cotyledon leaves (2 leaves stage) were dipped into the agar for overnight.

Figure 7 Genomic DNA after plant extraction. Lane L: 1 kb DNA Ladder (Fermentas), Lane 1-10: Sample 1 to Sample 10

Figure 8(a) Analysis of PCR after the DNA was treated with RNase.
Lane L: 100 bp DNA Ladder (Fermentas),
Lane 1: Negative sample, Lane 2: Positive sample,
Lane 3: Sample 1, Lane 4: Sample 2, Lane 5: Sample 3,
Lane 6: Sample 4, Lane 7: Sample 5

Figure 8(b) Analysis of PCR after the DNA was treated with RNase.
Lane L: 100 bp DNA Ladder (Fermentas), Lane 1: Positive sample, Lane 2: Negative sample, Lane 3: Sample 6,
Lane 4: Sample 7, Lane 5: Sample 8, Lane 6: Sample 9,
Lane 7: Sample 10

Figure 9 Cotyledon attached with embryo were dipped into the agar

Figure 10 (a) Result from the genomic DNA after plant extraction.
Lane L: 1 kb DNA ladder (Fermentas) and Lane 1-8: Sample (1-8).
(b) Result from analysis of PCR. Lane L: 100 bp DNA ladder (Fermentas), Lane 1: Negative sample, Lane 2: Positive sample,
Lane 3-10: sample 1-8
Transformation of mungbean cotyledon by using Agrobacterium tumefaciens

Syafiqah Diana Binti Subandi
Resource of Biotechnology
Faculty of Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Mungbean (Vigna radiata (L.) Wilezek) was transformed by using Agrobacterium tumefaciens strain LBA 4404. Plasmid pGPTV-SRN4/ALS was cloned into the Agrobacterium tumefaciens. Two types of explants were used in this research which are the leaf cotyledon and cotyledon attached with embryonal axis (CAEA). These explants were co-cultivated with Agrobacterium tumefaciens in the presence of acetosyringone. Binary vector harbouring luciferase (LUC) gene (pGPTV), was tested to examine its effect on transformation efficiency. The cotyledons from treated and germinated seedlings analysed via polymerase chain reaction (PCR). The result obtained from the comparison of the efficiency of cotyledon leaf (2-3 stages leaves) and cotyledon attached with embryonal axis were inconclusive. For further studies, the transformation efficiency needs to be increased and also improve on the genomic DNA extraction procedure.

Keywords: Mungbean, A. tumefaciens, explants transformation, cotyledons

VIII
1. INTRODUCTION

Introducing gene of interest into plant genomes is a genetic tool for plant transformation (Latham et al., 2006). Nowadays, plant transformation is broadly used as a technique to understand how plants work and to improve crop plant characteristics (Slater et al., 2003). There are several techniques of plant genetic transformation such as Agrobacterium-mediated transformation (Arora, 2010), particle gene gun or biolistic method, microinjection of DNA and Galiston expansion femtosyringe (Chawla, 2009).

As mentioned by Arora (2010), the frequently used method for plant transformation is Agrobacterium tumefaciens-mediated gene transfer. Naturally, A. tumefaciens will causes the crown gall tumors by infecting the wound sites of dicotyledonous plant (De la Riva et al., 2010). The Ti-plasmid of A.tumefaciens which is a circular DNA segment can be transferred into the host genome to infect the plants. The large plasmids called as tumour-inducing plasmid (Ti plasmid) and root inducing plasmid (Ri plasmid) confer on their hosts the pathogenic capacity. In this research, the binary plasmid pGPTV-SRN4/ALS will be integrated into A. tumefaciens for mungbean transformation.

Mungbean is chosen as the host plant because the seeds are easily available and have a rapid growth (Poehlman, 1991). There are many others leguminous species or pulse crop, such as dry bean (Phaseolus vulgaris L.), broadbean (Vicia faba L.), garden pea (Pisum sativum L.), lima or butterbean (Phaseolus lunatus L.) and cowpea (Vigna unguiculata (L.) Walp.). Mungbean have variety of function such as can be used as a green manure crop, as forage for livestock and also as a human food (as dry beans or fresh sprouts) (Oplinger et al. (1990).
These legumes provide inexpensive source of vegetable dietary protein and regarded as an important world food crops (Poehlman, 1991). Therefore, an efficient transformation of mungbean method would be enable further genetic research of this plant species. The problem statements and objectives of this research are indicated below.

1.1 PROBLEM STATEMENT

- Can mungbean be transformed by using *Agrobacterium*-mediated transformation method?
- Can *Luciferase (LUC)* gene be expressed in this research?

1.2 OBJECTIVES

- To assess the transformation of mungbean using *Agrobacterium tumefaciens*.
- To observe the expression of reporter gene in transformed mungbean.
2 LITERATURE REVIEW

2.1 Agrobacterium-mediated transformation.

In 1983, plant genetic engineering marking a breakthrough with a first successful plant transformation system via Agrobacterium system (Chawla, 2009). Chawla (2009) also mentioned that, plasmid carried by the bacterial plant pathogen A. tumefaciens and A. rhizogenes bring to the discovery of gene manipulation in plants and provide gene expression, natural gene transfer and selection systems as well. Nowadays, A. tumefaciens become the most effective plant genetic engineer.

Slater et al. (2003) stated that A. tumefaciens is a soil-borne and gram-negative bacterium. The capability of Agrobacterium spp. to transfer bacterial genes into the plant genome would induce the tumorous growth or roots to develop on infected plants (McCLean, 1998). Even though there are many other alternatives for plant transformation such as direct transformation, Agrobacterium-mediated transformation has remarkable advantages. The copy number of transgene is reduced and problem with transgene co-suppression and instability is fewer. (De la Riva, 1998). Furthermore, Agrobacterium-mediated transformation is a single-cell transformation system not forming mosaic plants, which are mostly used in direct transformation.

There are several essential steps in the process of gene transfer from Agrobacterium tumefaciens to plant cells. First is the colonization of bacterial. Then, the induction of bacterial virulence and generation of T-DNA transfer complex. After that, T-DNA transfer and lastly integration of T-DNA into plant genome (De la Riva, 1998).
2.1.1 Transfer of T-DNA to plant cells

A particular DNA segment (T-DNA) of the tumor-inducing (Ti) plasmid can transmit into the nucleus of infected cells with the exceptional capability of *A. tumefaciens*. Then, it will cause crown gall diseases when Ti plasmid is stably integrated into the host genome and transcribed. T-DNA consists of two kinds of genes which are the oncogenic genes and the genes encoding for the synthesis of opines. The oncogenic genes are the important genes in the formation of tumor and it is also encode enzyme involved in the synthesis of auxins and cytokinins (De la Riva, 1998). These genes only active in a plant cell and must contain typical eukaryotic controlling sequences to be transcribed. According to McClean (1998), TATA, CAAT and polyadenylation signals were included in those genes. Opines is the genes involved in bacterium-bacterium plasmid conjugative transfer and also genes involved in the process of T-DNA transfer from bacterium to the plant cell. This gene is located outside the T-DNA. (De la Riva, 1998).

According to the Slater et al. (2003), the T-DNA insertions in the plant genome were bordered by small 24 bp, nearly perfect, direct repeats, and border the T-DNA in the Ti plasmid. Ti plasmids from different strains of *A. tumefaciens* usually have some features in common. The genes in the bacterial chromosome and *vir* genes which is Ti plasmid virulence region are the genes that help the process of T-DNA transfer. The *vir* genes consist of six operons that are important for the transfer of T-DNA which are *vir A*, *vir B*, *vir D* and *vir G*. Besides, it also consists of genes for the increasing of transfer efficiency which are *vir C* and *vir E*.

Regarding to the De La Riva et al. (1998), they comes out into the conclusion about the truths on the process of T-DNA transferred into the plant cells. Firstly, the expression, transfer and
integration of T-DNA in the transformation process of plant cells would cause a tumor formation. Second, T-DNA genes not involved during transfer process and only transcribed in plant cells. Lastly, as long recombinant DNA is placed between T-DNA borders it can be transferred to plant cells.

2.1.2 Binary vector

Standard tool used for Agrobacterium-mediated transformation is a binary vector. It is consist of the borders of T-DNA, replication function for Escherichia coli and A. tumefaciens, multiple cloning sites, reporter genes, selectable marker genes and other elements which can develop efficiency and further capability to the system.

Chawla in 2009 stated that binary vector consist of two autonomously replicating plasmid A. tumefaciens which are a shuttle-vector that contains gene of interest between the T-DNA borders and the other one is a helper Ti plasmid that provide a vir gene products for transmitting into plant cells. The binary vector standard components include multiple cloning sites, a broad host range origin of replication functional in both E.coli and A. tumefaciens (e.g. RK2), selectable markers for both bacteria and plants, and T-DNA border sequences. According to Slater et al. (2003), the example of selectable markers that is widely used for transformation is hygromycine phosphotransferase (hpt) which resistance towards antibiotic hygromycin. The function of reporter gene is as an indicator of transformation. The widespread use in plant transformation vectors are β-glucuronidase (GUS), green fluorescent protein (gfp), luciferase genes (LUC) and chloramphenicol acetyltransferase gene (CAT). The example of binary plant transformation vectors is pGPTV.
3 MATERIALS AND METHOD

3.1 Preparation of medium and solution

Luria Broth (LB) and Luria Agar (LA) have been prepared for 250 mL volumes. Three solutions were also prepared, Solution I (10 mL) consisted of 0.09 g of 50 mM glucose, 0.82 g of 1.8 M formic acid, and 0.03 g of 25 mM Tris-HCl; and Solution II (10 mL) consisted of 0.08 g of 0.2 M NaOH and 0.1 g of 1% SDS. Solution III (10 mL) consists of 3 g of 3 M KAc and 0.03 g of EDTA. (Sim et al., 2011).

3.2 Preparation of competent E.coli

About 5 μL, 10 μL, 20 μL, and 30 μL of E.coli have been added on four different agar plates. Then those agar has been incubated for 16-20 hours at 37°C. The day after, the single bacterial colony about 2-3 mm in diameter was picked from a plate that contains 5 μL E.coli. Next, the colony was transferred into 13 mL Luria broth medium. Then the culture was incubated for 16 hours at 37°C with vigorous shaking (350 rpm). The next day, 100 μL cultures were taken out and added into 13 mL Luria Broth. The culture was incubated again for half an hour at 37°C with vigorous shaking (350 rpm).

The OD₆₀₀ of culture was monitored every 45 minutes until it reaches 0.4-0.6. After the required OD was reached, the falcon tube was cool on ice for 10 to 20 minutes. Then the cell suspension was centrifuged at 3500 rpm at 4°C for 5 minutes.

The supernatant was discarded and the cell washed gently by resuspending in 5 mL ice cold of 100 mM CaCl₂. Next the cell suspension was kept on ice for 10 minutes and centrifuged again.
After centrifuge, the supernatant was decant and the cell pellet was resuspend in 2.5 mL of cold sterile of 100 mM calcium chloride (CaCl₂) and incubated on ice for 1 hour until use. At this point, glycerol stocks, which can be stored for long period of time at 80°C, were prepared with the addition of 20% (v/v) pure glycerol to the cell suspensions. Then the solution was mixed, the aliquots of 20 µL was transferred into Eppy tubes, and was snap frozen it in liquid nitrogen before storage at -80°C (Sim et al., 2011).

3.3 Calcium chloride preparation of *Agrobacterium tumefaciens* competent cells

LA plates that contain rifampicin have been streak with 50 µL of *Agrobacterium* and were growing for two days at 28°C (room temperature). About 5 mL of LB which contain rifampicin was prepared and added with a single colony from the plate. The culture was grow overnight at 28°C on rotating shaker (180 rpm). In the next day, 3 mL of overnight culture added to 30 mL of LB medium and incubated at 28°C on rotating shaker (180 rpm) until the culture grows to an OD₆₀₀ of 0.6. After that, the culture was then chill in ice water for 10 minutes (0°C). Then, it was centrifuged for 10 minutes at low speed (4000 rpm) at 4°C. The supernatant removed and gently resuspend the pellet in 5.0 mL ice cold 20 mM CaCl₂. Next, the tubes were again centrifuge for 5 minutes at 4000 rpm at 4°C. The supernatant then has been removed and gently resuspend the pellet in 1.0 mL ice cold 20 mM CaCl. Then, 20% glycerol was added and mixed. 100 µL of mixture were aliquot into pre-chilled microcentrifuge tubes. Then the tubes were snap freeze in liquid nitrogen and stored at -80°C for later use.
3.4 **Standard heat-shock transformation of chemically competent bacteria**

Competent cell was taken out from -80°C and thawed on ice for about 20-30 minutes. Then, agar plates that contain antibiotic kanamycin were taken out from 4°C to warm up to room temperature. About 10 μL of pGPTV plasmid mixed with 50 μL of competent cell in a microcentrifuge. The mixture was gently mixed by flicking the bottom of the tube with finger for a few times. The mixture of plasmid and competent cell placed on ice for 20 minutes. Then, each transformation tubes were heat shock by placing the bottom ½ of the tube into a 42°C water bath for 45 seconds. After that, the tubes were put back on ice for 2 minutes. About 950 μL LB (without antibiotic) were added and grow in 37°C shaking incubator for 45 minutes. Next, all of the transformations were plated onto LB agar plate containing kanamycin. Then, the plates were incubated at 37°C overnight (Addgene, n.d.).

3.5 **Isolation of plasmid DNA from bacterial culture**

The bacterial cell from a 2 mL overnight culture was harvested by transferring the culture into 2 mL microcentrifuge tube and has been centrifuged at 8000 rpm for 2 minutes at room temperature. Then, the supernatant which is the culture media was removed carefully, and the pellet recentrifuged for 1 minute. Any traces of liquid media were removed from the tube. The cell pellet resuspended by using 100 μL of Solution I. Resuspension was done by vortexing the tube briefly for 10 seconds. After that, the tube was kept on ice for 5 minutes. Then, 100 μL of Solution II was added to the cell suspension and mix gently by inverting the tubes ten times. The tube was leave at room temperature to allow the lysis reaction to occur for five minutes. A clear viscous liquid was observed. Next, 300 μL of Solution III was added and mixed by inverting the tube ten times. A white precipitate observed. By centrifuging at 10,000
rpm for 5 minutes, the precipitate appeared as pellet. The supernatant was carefully transferred into a sterile 1.5 mL microcentrifuge tube. Then, the DNA was precipitated by adding 2 volume of cold absolute ethanol. The content was mixed gently by inverting the tube ten times. The DNA was pellet by centrifuging at 13,000 rpm for 5 minutes at room temperature. The supernatant discarded and the pellet washed with 500 μL of 70% ethanol, and recentrifuge at 13,000 rpm for 2 minutes. After that, the supernatant was discarded as much as possible and the DNA pellet was allow to air dry for 15 minutes at room temperature. Then, the DNA pellet was then resuspend and dissolved in 30 μL of sterile ultra-pure water. 5 μL of the plasmid DNA solution was checked by using Agarose Gel Electrophoresis (AGE) method and run on the Polymerase Chain Reaction (PCR) (Sim et al., 2011).

3.6 Agarose gel electrophoresis

To prepare 1.0% agarose gel, 0.3 of agarose powder was dissolved in 30 mL of TAE buffer and heat them in the microwave for 2 minutes. After that, let the solution cool. The solution then mixed with 0.1 μL of EtBr and pour into the container. Gel was kept at room temperature for 30 min to solidify the gel. From each sample, 5 μL of DNA sample mixed with 3 μL of 6X loading dye and loaded into the well and 5 μL of 1 kb DNA ladder (Vivantis) was pipetted into one of the wells. The function of the DNA ladder is as a roller for measuring the weight of the bands. Then, the electrophoresis machine was set on 30 minutes and 100 V to separate the bands and to be compared with DNA ladder. After 30 minutes, the gel was placed on UV machine to visualize the bands under the UV light.
3.7 Polymerase Chain Reaction

DNA sample which have LUC gene was amplified using specific primers: forward (5'-TGG AGA GCA ACT GCA TAA GGC-3') and reverse (5'-ATA GGA TCT CTG GCA TGC GAG-3'). The existence of LUC gene in the studied plant genome is come from the amplification of certain part of LUC gene. PCR mixture was prepared in 25 μL volume as in Table 1. The PCR condition was set up: 3 min at 95°C (denaturation) and 35 cycles of 30 sec at 95°C and 57.5 (annealing temperature), 45 sec at 72°C and final elongation of 5 min at 72°C.

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume (1x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taq buffer</td>
<td>2.5 μL</td>
</tr>
<tr>
<td>dNTPs mix</td>
<td>0.5 μL</td>
</tr>
<tr>
<td>forward primer</td>
<td>1.0 μL</td>
</tr>
<tr>
<td>reverse primer</td>
<td>1.0 μL</td>
</tr>
<tr>
<td>MgCl</td>
<td>1.5 μL</td>
</tr>
<tr>
<td>DNA template</td>
<td>1.0 μL</td>
</tr>
<tr>
<td>Tag DNA polymerase</td>
<td>1.0 μL</td>
</tr>
<tr>
<td>dH₂O</td>
<td>1.0 μL</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>25 μL</strong></td>
</tr>
</tbody>
</table>

3.8 Transformation of Agrobacterium into plasmid DNA

About 10 μL plasmid DNA was added into two tubes of competent cells contains 100 μL per tubes. Then both tubes were mix gently by pipetting up and down three times. After that both tubes were hold on ice for 25 minutes, then have been froze in liquid nitrogen for 5 minutes. Heat shock both tubes by put them in 37°C of water bath for 5 minutes then returned them into ice for 5 minutes. 1.0 mL of LB was added to the both tubes then, incubated in a 28°C rotating shaker for 4 hours (180 rpm) in one tube and another one tube incubated for overnight.
The day after, 100 μL of culture 4 hours tubes and overnight tubes were spread on LA plate containing kanamycin and rifampicin separately. Both plates have been growth for two days at 28°C in room temperature. After two days, pick colony on agar plates and were transferred into falcon tubes which contains 10 mL LB, 20 μL of rifampicin and 10 μL of kanamycin. Both tubes were put in the shaker for 2 days in a room temperature (180 rpm). After the culture turned into cloudy, isolation of plasmid DNA was carry out by using kit.

3.9 Isolation of double stranded plasmid DNA from bacterial culture by using kit.

About 40 μL of activation Buffer PL was applied onto the spin column and was kept at room temperature until transferring lysate to the spin column. In another 1.5 mL microcentrifuge tubes the cells from overnight culture have been pour. Then spin down 1.5 mL microcentrifuges at 14 000 rpm for 2 minutes (repeated 2 times). The supernatant was pouring off and the tubes were blot upside down on paper towel to remove any remaining media. Next, 250 μL of Cell R buffer added and resuspended the pellet completely. Added 200 μL of blue-colored Lyses Blue buffer and mixed them gently until uniform blue color of cell resuspension obtained. Then, 350 μL of neutralization and binding buffer, Neutral B added and mixed by several-fold inverting until blue color was disappeared. Microcentrifuges were spin-down at 13 500 rpm for 7 minutes. The supernatant were pour into spin-column placed in the receiver tube which contains 40 μL of activation Buffer PL, then microcentrifuges were spin-down at 12 000 rpm for 1 minute. Then, spin column removed, and pour-off supernatant and placed back into the receiver tube. After that, 500 μL of Wash PLX 1 buffer were added and spin-down at 12 000 rpm for 1 minute. Again, removed spin column, pour-off supernatant and
replaced back into the receiver tube. About 650 μL of Wash PLX 2 buffer added and spin
down at 12 000 rpm for 1 minute. Again, removed spin column, pour-off supernatant and
replaced back into the receiver tube. After that, spin down again at 12 000 rpm for 2 minutes
to removed traces of the Wash Buffer PLX 2 buffer. Then, spin column were placed into new
receiver tubes. 30 μL of Elution buffer were added to elute bound DNA. Spin-column with the
receiver tubes were incubated in room temperature for 2 minutes. Then, spin down them at 12
000 rpm for 2 minutes. Lastly, spin-column removed and caps the receiver tube and stored at
-20°C temperature. The cultures then have been run on Agarose gel to check the band. After
that, check for PCR analysis.

3.10 Plant transformation.

The Agrobacterium with plasmid DNA cultures have been grown again in LB by taken out
100 μL cultures and added in the another 10 mL LB which contain 10 μL kanamycin and 20
μL rifampicin. Then the cultures were leave in a 180 rpm shaker in room temperature for two
days.

After growth the culture for 2 days, 3 mL of overnight cultures were taken out and inoculate
with 50 mL LB, 50 μL kanamycin and 100 μL rifampicin. Grow them in a shaker 180 rpm for
about two hours until the O.D₆₀₀ reached 0.8. When the O.D₆₀₀ reached 0.8, the culture then
were transferred into falcon tube and spin at 3 500 rpm in 28 °C for 10 minutes. When the
pellet formed, supernatant was thrown away. Then, the pellet resuspended with 70 mL of
infiltration media. Infiltration media consist of 0.15 g of MS media, 2 g of sucrose and 0.035 g
of MES.
• Two leaves stage of mungbean transformation

Acetosyringone and silwet were added in the infiltration media. Then the mixed solution is divided into 5 bottles. 10 mungbeans which have undergone two leaves stage were taken out and incubated inside the bottles that contain mixed solution. Then, leave it for 3 hours in the shaker 120 rpm. After that, take the plants out from the bottles and put inside beaker which contain 70 mL agar and acetosyringone.

Leave it overnight. The day after, all plants were taken out from the beaker and rinsed with carbenicillin. After rinsing, plants were transferred into the soil. About two days after, the leaves were cut and used to extract the genomic DNA.

• Cotyledon attached with embryo

Ten mungbeans cotyledon which have been scarified were taken out and put inside the beaker. Infiltration media was pour inside the beaker slowly. Then, acetosyringone and silwet were poured inside the beaker that contains mungbeans cotyledons. Incubated them for 3 hours in the shaker (120 rpm). After that, take the mungbeans out from the beaker and rinsed with carbenicillin. After rinsing, the mungbeans cotyledons were transferred into the soil to grow. About four days later, the leaves were cut and used to extract the genomic DNA.

3.11 Plant Genomic DNA extraction

The leaves were cut into small pieces and put into the 1.5 mL tube. Ten putative transformed mungbeans samples were prepared in the extraction of genomic DNA. Liquid nitrogen was poured into each tube and grinded them until become powder. In another tube, 20 µL of β-mercaptoethanol was added in 10mL of CTAB. The mixture then heated in water bath (60°C) for 10 minutes. 1 mL of mixed solution was taken out and put in one tube that contains
leaves. The tubes were vortex and incubated them in water bath again for 30 minutes at 65°C (each 10 minutes, vortex). After 30 minutes, all tubes were taken out from water bath and let them cool. Then, 200 µL CIA were added into each tubes and centrifuged them for 5 minutes (13 000 rpm). Supernatant transferred into new tubes and 500 µL CIA added into it and vortex them. Then, all tubes were centrifuge for 5 minutes (13 000 rpm). Again, supernatant was transferred into new tubes and added with 600 µL ice-cold propanol. The tubes mixed gently to precipitate the DNA and stored them at -20 °C overnight. The day after, all tubes centrifuged at 13 000 rpm for 2 minutes. Then, supernatant was thrown away (use pellet) and 1 mL wash buffer added. The tubes mixed gently and store at -20 °C for 30 minutes. After that, centrifuged 13 000 rpm again all the tubes for 5 minutes and thrown away supernatant. The pellet was air-dried. 50 µL of TE buffer added into the tube that contain pellet and stored at -20°C. Leave it for few hours then check the band on agarose gel. After that, PCR analysis has been carried out.

3.12 Purification of genomic DNA

All samples that have been extracted then undergo purification method. DNA samples (20 µL each) mixed with 0.5 µL RNase to each sample. The samples were then incubated at 37°C for 1 hour. The equal volume of P.C.I was added to the DNA sample and inverted gently for 15 min. after that centrifuged 13 000 rpm for 15 min. the aqueous phase then transferred into new tubes. Equal volume of CIA was added and inverted gently for 15 min. Centrifuged 13 000 rpm for min. and transfer aqueous phase to new tube. Cold ethanol (2 volume) was added and
ammonium acetate (7.5, pH 7.5) to final concentration 2.5 M. The tubes were inverted gently for few times. Then precipitate for overnight at -20°C.

The day after, DNA was recover by centrifuge 13 000 rpm for 15 min. the supernatant was remove and used only the pellet. The pellet was washed with 70% ethanol for 1 hour. Centrifugation at 13 000 rpm was applied for 15 min. The supernatant was then pour off and the pellet was air dried. Pellet was resuspended in 50 μL of sterile distilled water and stored at -20°C.