AGROINFILTRATION OF *LYCOPERSICON ESCULENTUM* USING LURIA BERTANI BROTH MEDIUM ON WOUNDED LEAVES

Angela Lok Siew Foong

Bachelor of Science with Honours
(Resource Biotechnology)
2010
Agroinfiltration of *Lycopersicon esculentum* Using Luria Bertani Broth Medium on Wounded Leaves

Angela Lok Siew Foong

This project is submitted in fulfillment of requirement for the degree in Bachelor of Science with Honours

(Resource Biotechnology)

Resource Biotechnology
Department of Molecular Biology
Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2010
ACKNOWLEDGEMENT

First and foremost, I would like to offer my greatest heartfelt gratitude to my supervisor, Miss Safarina Ahmad who has been supporting me with her patience and knowledge upon completing this thesis even as to train me to work in my own way of conducting the research. Without her encouragement and effort, this thesis would have never been possible to be completed. Special thanks to Dr. Hairul Azman Roslan who has been supervising me in laboratory works, giving recommendations on result troubleshooting as well as providing all the materials I ever needed in this research.

I would also like to acknowledge all the master students in Genetic Engineering laboratory for their patience and guidance that I have been aided in running the equipments, obtaining samples and chemicals. Through their generosity to spend their time to analyze the result as well, this thesis was able to be written successfully.

Blessings from all my friendly and cheerful group of fellow friends in my daily work, I thanked them for their continuous encouragement and moral support. Also to my friends at Sarawak Biodiversity Centre, thanks for the suggestion on certain procedures to be done in order to get a successful amplification of DNA.

Finally, I thank my parents for supporting me throughout all my studies in UNIMAS. To their encouragement, I had successfully completed the level of my Degree in Biotechnology.
Agroinfiltration of *Lycopersicon esculentum* Using Luria Bertani Broth Medium on Wounded Leaves

Angela Lok Siew Foong

Resource Biotechnology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Tomato, *Lycopersicon esculentum* is a potential model plant for the studies on transient expression of foreign gene. The main objective of this project is to transform the plant leaves using agroinfiltration technique onto wounded leaves with LB medium as an infiltration medium. Agroinfiltration was carried out by injecting the *Agrobacterium tumefaciens* directly into wounded tomato leaves to allow transformation of gene to occur. Germination of tomato seed was done first followed by the preparation of *Agrobacterium tumefaciens* that carries pBI121 plasmid. Transformation of the young leaves was later then carried out, leaving it 3 to 7 days after the infiltration to allow integration of gene to occur. Analysis of putative transformants by using Polymerase Chain Reaction (PCR) has successfully amplified the sequence of gus gene with the size of 600 bp at 10X and 20X dilution of DNA template. It was reported that young leaves aged 5-8 weeks which has been injected with LB medium gave a better result as compared to the IM-treated leaves.

Keywords: Agroinfiltration, *Agrobacterium tumefaciens*, pBI121 plasmid, tomato, PCR.

ABSTRAK

Tomato, *Lycopersicon esculentum* merupakan tanaman model berpotensi untuk kajian tentang ekspresi sementara gen asing. Objektif utama bagi projek ini adalah untuk mentransformasi daun tomato dengan kaldu LB pada daun luka menggunakan teknik agroinfiltrasi. Dalam kajian ini, suatu kaedah yang dikenali sebagai agroinfiltrasi telah dilakukan dengan menyerapkan *Agrobacterium tumefaciens* terus ke dalam daun tomato yang dilukakan untuk membolehkan transformasi gen berlaku. Percambahan benih tomato dilakukan dahulu diikuti dengan penyediaan *Agrobacterium tumefaciens* yang membawa plasmid pBI121. Seterusnya, transformasi tanaman tomato dilakukan pada daun muda dan dibiarkan selama 3 hingga 7 hari untuk membolehkan integrasi gen berlaku. Analisis PCR yang dilakukan terhadap transforman putatif telah berjaya mengamplifikasi jujukan gen gus bersaiz 600 pb pada pencairan DNA 10X dan 20X. Didapati daun muda berumur 5-8 minggu yang disuntik dengan kaldu LB memberikan keputusan yang lebih baik untuk agroinfiltrasi berbanding dengan daun yang dirawat dengan IM.

*Kata kunci: Agroinfiltrasi, Agrobacterium tumefaciens, plasmid pBI121, tomato, PCR.*
# TABLE OF CONTENTS

ACKNOWLEDGEMENT i

ABSTRACT & ABSTRAK ii

TABLE OF CONTENTS iii

LIST OF FIGURES vii

LIST OF TABLES viii

LIST OF ABBREVIATIONS ix

CHAPTER 1 INTRODUCTION AND OBJECTIVES 1

CHAPTER 2 LITERATURE REVIEW 4

2.1. *Lycopersicon esculentum* 4

2.2. *Agrobacterium tumefaciens* 6

2.3. *In planta* transformation 7

2.3.1. Agroinfiltration 7

2.4. Reporter gene 8
CHAPTER 3  MATERIALS AND METHODS

3.1. Materials

3.1.1. Source of tomato seeds

3.1.2. pBI121 plasmid

3.1.3. *Escherichia coli*

3.1.4. *Agrobacterium tumefaciens*

3.1.5. Chemicals

3.1.6. Enzymes and DNA ladders

3.2. Tomato seeds sowing

3.3. Confirmation of *Escherichia coli* carrying pBI121 plasmid

3.3.1. Overnight growing of DH5α strain of *E. coli* carrying pBI121 plasmid

3.3.2. Isolation of pBI121 plasmid

3.3.3. Restriction enzyme analysis of pBI121 plasmid

3.3.4. Polymerase Chain Reaction (PCR) of pBI121 plasmid

3.3.5. Agarose gel electrophoresis

3.4. Preparation of *Agrobacterium tumefaciens* carrying pBI121 plasmid

3.4.1. Competent *A. tumefaciens* LBA4404 cell preparation

3.4.2. Transformation of positive pBI121 into competent *A. tumefaciens*

3.4.3. Analyses of transformed *A. tumefaciens*

3.5. Transformation of tomato plant

3.5.1. Preparation of inoculums

3.5.2. Agroinfiltration
3.6. Analysis of putative transformants

3.6.1. DNA isolation from infiltrated leaves

3.6.2. Polymerase Chain Reaction

3.6.3. Ethanol precipitation of DNA

3.6.4. 10X and 20X DNA dilution

CHAPTER 4 RESULTS AND DISCUSSION

4.1. Seed germination

4.2. Preparation of pBI121 plasmid in A. tumefaciens

4.2.1. Isolation of pBI121 plasmid by using alkaline lysis method

4.2.2. Isolation of pBI121 plasmid by using TENS solution

4.2.3. Isolation of pBI121 plasmid by using Vivantis Plasmid DNA Extraction Kit

4.3. Restriction enzyme analysis of pBI121 plasmid

4.4. Polymerase Chain Reaction (PCR) of pBI121 plasmid

4.5. Preparation of A. tumefaciens LBA 4404 containing pBI121 plasmid

4.5.1. Analyses of transformed Agrobacterium tumefaciens

4.6. Plant transformation

4.7. DNA plant extraction

4.8. Ethanol precipitation

4.9. Analysis of putative transformants

4.9.1. PCR on 10X and 20X diluted DNA template
### LIST OF FIGURES

| Figure 4.1 | Tomato plants with different ages | 26 |
| Figure 4.2 | Agarose gel electrophoresis (1 %) of the isolated pBI121 from *E. coli* using alkaline lysis method | 27 |
| Figure 4.3 | Agarose gel electrophoresis (1 %) of the isolated pBI121 from *E. coli* using TENS solution | 28 |
| Figure 4.4 | Agarose gel electrophoresis (1 %) of the isolated pBI121 from *E. coli* using Vivantis Plasmid DNA Extraction Kit | 29 |
| Figure 4.5 | Agarose gel electrophoresis (1 %) of the restricted pBI121 plasmid for single digestion | 30 |
| Figure 4.6 | Agarose gel electrophoresis (1 %) of the restricted pBI121 plasmid for double digestion | 31 |
| Figure 4.7 | Agarose gel electrophoresis (1 %) of the PCR products of amplified genes in pBI121 plasmid | 32 |
| Figure 4.8 | Agarose gel electrophoresis (1 %) of the PCR products of amplified genes in pBI121 plasmid for transformed *A. tumefaciens* | 33 |
| Figure 4.9 | Infiltrated leaves with LB medium and IM | 34 |
| Figure 4.10 | Extracted plant DNA from LB-treated leaves of set 1, 2, 3, 4 and 5 | 36 |
| Figure 4.11 | Extracted plant DNA from IM-treated leaves of set 1, 2, 3, 4 and 5 | 37 |
| Figure 4.12 | DNA template precipitated down from 5 tubes for each treatment | 39 |
| Figure 4.13 | PCR products of 10X and 20X dilution of IM and LB-treated leaves of DNA | 41 |
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 3.1</td>
<td>Sowed seeds on 5 polybags</td>
<td>12</td>
</tr>
<tr>
<td>Table 3.2</td>
<td>Restriction enzyme single digestion</td>
<td>16</td>
</tr>
<tr>
<td>Table 3.3</td>
<td>Restriction enzyme double digestion</td>
<td>16</td>
</tr>
<tr>
<td>Table 3.4</td>
<td>Primers sequence for PCR</td>
<td>17</td>
</tr>
<tr>
<td>Table 3.5</td>
<td>The composition of PCR mixture for amplification</td>
<td>18</td>
</tr>
<tr>
<td>Table 3.6</td>
<td>The reaction profile for PCR amplification</td>
<td>18</td>
</tr>
<tr>
<td>Table 3.7</td>
<td>Parameters that were applied on different ages of the plant</td>
<td>21</td>
</tr>
<tr>
<td>Table 3.8</td>
<td>The composition of PCR mixture for amplification of total volume 10 µL</td>
<td>22</td>
</tr>
<tr>
<td>Table 4.1</td>
<td>PCR result of GUS expression on wounded leaves treated with LB medium and IM</td>
<td>47</td>
</tr>
</tbody>
</table>
# LIST OF ABBREVIATION

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial Artificial Chromosome</td>
</tr>
<tr>
<td>CAPs</td>
<td>Cleaved Amplified Polymorphic Sequences</td>
</tr>
<tr>
<td>COS</td>
<td>Conserved Orthologous Set</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ESTs</td>
<td>Expressed Sequence Tags</td>
</tr>
<tr>
<td>GMO</td>
<td>Genetically modified organism</td>
</tr>
<tr>
<td>GUS</td>
<td>Glucuronidase</td>
</tr>
<tr>
<td>IM</td>
<td>Inoculation medium</td>
</tr>
<tr>
<td>LA</td>
<td>Luria Agar</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>MARDI</td>
<td>Malaysian Agricultural Research and Development Institution</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphisms</td>
</tr>
<tr>
<td>SSR</td>
<td>Simple Sequence Repeats</td>
</tr>
<tr>
<td>T-DNA</td>
<td>Transfer-deoxyribonucleic acid</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

Plant transformation defines as a genetic engineering tool for introducing transgenes into plant genomes whereby an insertion of the transgene into plant chromosomal DNA will be done (Latham, 2005). A plasmid is used as a vector to carry the particular foreign gene in order for it to integrate in the plant DNA. Combining genes from different organisms is known as recombinant DNA technology, and the resulting organism is said to be "genetically modified," "genetically engineered," or "transgenic" (Human Genome Project Information, 2008). There are several methods that can be done to achieve GMOs. One of the latest methods that will be discussed throughout this study is the in planta transformation protocol using tomato as a model plant.

According to Deynze et al., 2007, tomato (L. esculentum) with 24 chromosome numbers was chosen as an experimental model plant because it has rich genetic and genomic resources including comprehensive databases of Expressed Sequence Tags (ESTs), Bacterial Artificial Chromosome (BAC) libraries, and genetic and comparative maps which are in the process of being linked to a physical map and eventually the euchromatic genomic sequence. These resources serve as template to study genetic variation and to manipulate agricultural traits. Current genetic maps for tomato include 2,200 Restriction Fragment Length Polymorphisms (RFLPs), Cleaved Amplified Polymorphic Sequences (CAPs), and Simple Sequence Repeats (SSRs), as well as emerging genetic resources which include a comparative map with Arabidopsis of over 500 Conserved Orthologous Set (COS) markers (Deynze et al., 2007).

In the early days back to year 1986, genetic manipulation has been done towards breeding of a tomato genotype which involved in vitro tissue culture technique (Koorneef
et al., 1986). This study used *Agrobacterium*-mediated transformation, leaf disc and protoplast technique transformation as well as tissue culture approach that rendered callus formation. Continuous researches of several other methods have led to the discovery of *in planta* transformation protocol in which this method has not yet clearly elaborated on tomato plant. Advantages of using *in planta* transformation such as time saving, high transformation efficiency, low cost and convenience have been made possible to enhance the propagation of the transgenic tomato (Liu et al., 2002).

Agroinfiltration technique is one of the *in planta* transformation protocols. This is applicable for genetic transformation in plant that mainly uses *A. tumefaciens* for transforming the bacterium gene into the plant DNA without performing tissue culture technique. According to Wroblewski et al., 2005, *Agrobacterium*-mediated transient assay has become the favorite choice in many of the gene functional analyses, particularly the leaf agroinfiltration which represents an easy and not invasive technique. When *A. tumefaciens* cell cultures are infiltrated into a plant, (particularly via the intercellular spaces of leaf parenchyma) the transfer of T-DNA into the plant cell nucleus will give a high efficiency of transformation through this agroinfiltration technique. In order to confirm the integration of T-DNA (that contains foreign gene) into plant DNA, GUS (β-glucuronidase) assay and PCR has to be performed. This is crucial to confirm the presence of integrated *GUS* gene in the tomato plant.

This project has created a very interesting and potential study on the integration of desired gene into tomato plant via infiltrating the *A. tumefaciens* into tomato leaves. Agroinfiltration method somehow does not promise an effective transformation depending on several factors such as the medium used to infiltrate the leaves, age of plants as well as the condition of the leaves (wounded or not). Optimization has to be done upon this method in order to distinguish the best parameter that could allow gene integration to
occur. This research contributes to the identification of the best parameter for agroinfiltration of tomato leaves. The parameters used throughout this research were wounded leaves with LB broth medium and wounded leaves with inoculation medium (IM) as a control.

The objectives of this study are listed as below:

1. To transform *L. esculentum* by using agroinfiltration technique onto wounded leaves with LB medium as an infiltration medium.
2. To identify the best possible age of tomato plant for agroinfiltration.
3. To analyze the putative transformant by using PCR.
CHAPTER 2

LITERATURE REVIEW

2.1 *Lycopersicon esculentum*

*Lycopersicon esculentum* is frequently referred to as a tomato species in the genus of Lycopersicon and the family of Solanaceae, same as potato and eggplant, which preferably chosen as model plant for genetic study (Deynze, 2007). The family is currently consists of around 90 genera (D’Arcy, 1979). The Solanoideae genera have a coiled embryo of more or less uniform diameter. The sub-genera include two groups which are Eulycopersicon (red-fruited species) and Eripersicon (green-fruited species). *L. esculentum* is in the red-fruited species. Its alternative names *Solanum lycopersicum* L. or *Lycopersicon lycopersicum* (L.) Karsten have been appeared in literature (Taylor, 1986). *L. esculentum* for tomato was initially proposed by Miller in 1768, has replaced the earlier Linnean name *Solanum lycopersicum*. This tomato species is thought to have evolved from the cherry tomato (*Lycopersicon esculentum* var. *cerasiforme*) which exists wild in Peru, Ecuador and other parts of tropical America.

The original domestication of tomato is suggested to be in Mexico until it was spread to Europe by the Spaniards and then throughout the Old World in the sixteenth century (Vaughan and Geissler, 1997). Previously, tomato was known as the ‘golden apple’, ‘love apple’, or ‘Peruvian apple’ and they are the only wild tomatoes found outside of South America, latter spread towards various regions such as Zambia, Borneo and Hawaii (TGA Stock List, 1983).

Tomato is one of the most important vegetable or salad plants on a world wide basis in which it is consumed raw, or cooked and processed into various products such as juice, paste, sauce, soup, purée, ketchup or even in powder. Tomato plant shows a wide
climate tolerance, growing in tropical and temperate regions in the field, under plastic shelter or in greenhouses but not frost-tolerate. Even though it is cultivated worldwide, countries like Russia, China, Egypt, Italy and United States still record a large-scale of production. The tomato is a herbaceous plant which is capable of perennial growth but normally cultivated as an annual (Vaughan and Geissler, 1997). Tomato seeds are relatively small and should only be planted about a quarter inch deep into loose and well-drained soil of pH 5.8-6.6. They are susceptible to calcium deficiency even if adequate levels of calcium are present in the soil. Disorder of blossom-end rot is the effect of calcium deficiency and can occur as a result of insufficient or excessive watering or excessive fertilization with ammonium source (Rhodes, 2009).

Tomato has chosen to be a model plant since it contains high genetic and genomic resources (Deynze, 2007). These resources provide a good template to study genetic variation and to manipulate agricultural traits. It takes about five to six weeks in order to obtain a whole plant with complete growing leaves without any fruiting yet. An average tomato plant usually grows up to 1–3 metres (3–10 ft) in height and has a weak, woody stem that often vines over other plants. The leaves are 10–25 centimetres (4–10 in) long, odd pinnate, with 5–9 leaflets on petioles (Acquaah, 2002) each leaflet up to 8 centimetres (3 in) long, with a serrated margin; both the stem and leaves are densely glandular-hairy. The flowers are 1–2 centimetres (0.4–0.8 in) across, yellow, with five pointed lobes on the corolla; they are borne in a cyme of 3–12 together.
2.2 Agrobacterium tumefaciens

Agrobacterium tumefaciens is a Gram-negative, non-sporing, motile and rod-shaped bacterium which can cause crown gall disease (large tumour-like swellings that typically occur at the crown of the plant) of a wide range of dicotyledonous plants (Deacon, 2003). According to Wood and his associates from University of Washington in 2001, A. tumefaciens can be used to insert a piece of DNA in the middle of a plant gene, thus inactivating the gene and mutation can then be analyzed. There are several studies have been done on Arabidopsis thaliana and Oryza sativa L. using the in planta transformation method by infiltrating A. tumefaciens (Bechtold et al., 2000 and Supartana et al., 2005).

The A. tumefaciens genome has a very remarkable structure. Some 5,400 genes reside on four DNA elements which consist of a circular chromosome, a linear chromosome, and two smaller circular structures called plasmids. Many bacteria have circular chromosomes and some have linear chromosomes, but Agrobacteria are the only species known to have both structures together (Allardet-Servent et al., 1993). A. tumefaciens has a very unique and specialized system in which its DNA transfer to eukaryotic cells is the only known example of interkingdom DNA transfer (Dumas et al., 2001). This DNA is known as the transferred DNA (T-DNA) of the tumor-inducing (Ti) plasmid that causes crown gall disease when it is stably integrated into the host genome and transcribed. T-DNA contains two types of genes which are the oncogenic genes that encode for enzymes involved in the synthesis of auxins and cytokinins and responsible for tumor formation and the genes that encode for the synthesis of opines. Opines are a class of chemicals that serve as a source of nitrogen for A. tumefaciens, but not for most other organisms. When interacting with susceptible dicotyledonous plant cells, the virulent strain of A. tumefaciens that contains a large mega plasmid (more than 200 kb) plays a role in tumor induction thus causes crown gall disease. For this reason, it was names as Ti
plasmid. A mobile segment of Ti plasmid is transferred to the plant cell nucleus and integrated into the plant chromosome which associates changes in plant metabolism during infection of T-DNA.

The unique mode of action has enabled this bacterium to be used as a tool for genetic engineering of plants (Winstead, 2001). In any genetic study, a proper preservation of strains is important. The risk of introducing contaminants must be minimized and bacteria cultures should be genetically stable during storage. This could be achieved by storing the culture in sterile distilled water at 10°C. However, storage at low temperature of at least -50°C and freeze-drying are the most reliable techniques, especially for long term storage.

2.3 In planta transformation

In planta transformation methods are now commonly used to transform a plant by A. tumefaciens. So far, Arabidopsis thaliana has been used as an experimental plant for this method in several researches (Bechtold et al., 2000). Inoculation of A. tumefaciens into different stages of plant tissue can be made by agroinfiltration methods and latter then examining the gametophytic expression of an introduced β-glucuronidase marker gene encoding GUS (Bechtold et al., 2000).

2.3.1 Agroinfiltration

Agroinfiltration is a newly developed method in plant biotechnology that induces transient gene expression. The advantage of this technique is that it is simple, rapid, reproducible and much more convenient compared to traditional plant transformation and has been widely used in analysis of foreign gene expression, hypersensitive reaction, gene silencing, promoter activity as well as identification of new disease-resistance genes (Liu et al., 2002). However, biolistic method that needs costly equipment would have more advantage over agroinfiltration in terms of time-saving. There are several types of in planta
transformation method which include the vacuum infiltration, syringe infiltration floral dip and spraying. Research has been done on rice (*Oryza sativa* L.) by using the needled-syringe infiltration in which inoculation has been taken place on the embryo that contains apical meristem (Supartana *et al.*, 2005). Floral dip and vacuum infiltration methods have also been done on the *in planta* transformation of *Arabidopsis thaliana* (Wiktorek-Smagur *et al.*, 2009).

In this research of agroinfiltration on tomato leaves, a suspension of *A. tumefaciens* was injected into a plant leaf by using free-needle syringe, whereby the desired gene is transferred to plant cells. Once the *A. tumefaciens* is inside the leaf, gene of interest will transform to a portion of the plant cells and the gene is then transiently expressed. Any possible effect that can be observed from the phenotype of the plant will then be subsequent for plant DNA isolation. Confirmation of DNA integration can be done by performing polymerase chain reaction or GUS assay.

### 2.4 Reporter gene

A reporter gene allows the study of gene expression in which the gene product is not known or is difficult to identify. It encodes an enzyme that reacts with a substrate to report on the transcriptional activity of a gene of interest. The most commonly used reporter gene is the *gusA* gene that encodes for β-glucuronidase (GUS) enzyme. This enzyme can cleave the chromogenic (color-generating) substrate X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronic acid), resulting in the production of an insoluble blue color in those plant cells displaying GUS activity (Karcher, 2002). Plant cells themselves do not contain any GUS activity, so the production of a blue color when stained with X-gluc in plant cells indicates the activity of the promoter that drives the transcription of the *gus*-chimeric gene in that plant cell.
In bacteria, \textit{lacZ} gene from \textit{E. coli}, which encodes \(\beta\)-galactosidase, is used as a reporter of gene activity. Other reporter genes that are often used in bacteria and animals include \textit{cat} gene which encodes the enzyme chloramphenical acetyl transferase, \textit{fus} gene which encodes the jellyfish green fluorescent protein, and \textit{lux} gene which encodes the enzyme firefly luciferase. \textit{lacZ} is not usually a useful reporter gene for plants because plants contain endogenous \(\beta\)-galactosidase activity. GUS assay is easy to perform, sensitive, relatively inexpensive, highly reliable, safe, requires no specialized equipment, and is highly visual (Jefferson, 1987, Jefferson \textit{et al.}, 1987, and Jefferson and Wilson, 1991).
CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Sources of tomato seeds

In this study, tomato seeds *L. esculentum* cultivar MARDI TOMATO 1 (MT1) was obtained from MARDI, Jalan Kebun, Kelang, Selangor. Seeds were grown on black soil until the plant gives raise to a tomato tree. This took about two weeks. The age of plants that were used for infiltration were 2 weeks, 3 weeks, 4 weeks, 6 weeks, and 7 weeks.

3.1.2 pBI121 plasmid

The pBI121 plasmid in *E. coli* was obtained from the Genetic Engineering lab. This plasmid that carries pBI121 plasmid was cultured overnight in 10 ml of LB broth containing 100 mg/ml kanamycin at incubator shaker of 37 °C. 1 ml of overnight culture was streaked on LA plates (Appendix A) as well containing 100 mg/ml kanamycin and were left to grow overnight at 37 °C. *E. coli* containing pBI121 plasmid was also kept in a glycerol stock of 20 %. A 50 % glycerol was prepared first out of the 100 % for better pipetting. 4 ml of distilled water was added to a 4 ml of 100 % glycerol that makes a 50 % glycerol. Suggested final concentration and volume of stock culture was 20 % and 20 ml, respectively. Thus, 8 ml of 50 % glycerol was added in a 10 ml *E. coli* culture, topped up the whole thing with 2 ml of fresh LB broth (Appendix A). The pBI121 plasmid contains a kanamycin resistance gene (*npt* II) for selection of transgenic cells and a *gus* gene for reporter protein expression (Wang, 2006). The complete sequence of this plasmid size is 14758 bp (Appendix D) which is now available in Gene Bank of accession number AF485783.
3.1.3 *Escherichia coli*

In this study, *E. coli* strain DH5α was used as a recipient host to prepare replicates that carries pBI121 plasmid. These replicates were kept in a glycerol stock as has been mentioned in 3.1.2. Before doing any plasmid extraction, single colony of *E. coli* was picked from the LA plate and grown overnight in 10 ml of LB broth at 37 °C incubator shaker.

3.1.4 *Agrobacterium tumefaciens*

*A. tumefaciens* strain LBA 4404 that was used throughout this study. This strain was obtained from the Genetic Engineering lab and it was kept on LA plates (Appendix A) for any future use. Transformation of pBI121 plasmid was done upon this strain before infiltrating them into the tomato leaves.

3.1.5 Chemicals

Most of the chemicals used were obtained from the Genetic Engineering lab such as ethidium bromide, TAE buffer, 100 % ethanol and isopropanol. 70 % ethanol that was needed for sterilization or disinfection purposes was prepared beforehand by diluting it from the available pure ethanol. For PCR analysis, chemicals used were 5U/µL *Taq* polymerase, 10X Buffer S, and 2mM dNTPs which were all from Vivantis brand as well as the GUS forward and reverse primers from Genetic Engineering lab. Chemicals in powder form supplied by the department were such as nutrients for LB medium which were the Bacto-trypytoe, Bacto-agar, yeast extract and NaCl. The antibiotics used were also obtained from Genetic Engineering lab such as the kanamycin (Amresco brand) and rifampicin (Phytotechnology Laboratories brand).
3.1.6 Enzymes and DNA ladders

All the used enzymes for restriction analysis were from the brand of Fermentas such as \textit{Bam} HI, \textit{Hind} III, \textit{Pst} I, \textit{Bgl} II, \textit{Eco} RI, \textit{Sac} I, and \textit{Sma} I. \textit{Taq} polymerase and 1 Kb DNA ladder as well as enzyme buffers that were used along this study were from the brand of Fermentas and Vivantis.

3.2 Tomato seeds sowing

About two to three seeds of tomato were sowed every week in a polybag containing black soil and were labeled according to the dates and parameters that will be applied for infiltration. They were grouped as A, B and C for easy distinction of infiltration date.

<table>
<thead>
<tr>
<th>Group</th>
<th>Date/age</th>
<th>Set 1</th>
<th>Set 2</th>
<th>Set 3</th>
<th>Set 4</th>
<th>Set 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Date of planting</td>
<td>04/02/10</td>
<td>08/02/10</td>
<td>11/2/10</td>
<td>17/02/10</td>
<td>22/02/10</td>
</tr>
<tr>
<td>A</td>
<td>Date of infiltration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Age of plant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Set 4</td>
<td>10/03/10</td>
<td>10/03/10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Age of plant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 weeks</td>
<td>2 weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Date of infiltration</td>
<td>24/03/10</td>
<td>24/03/10</td>
<td>24/03/10</td>
<td>24/03/10</td>
<td>24/03/10</td>
</tr>
<tr>
<td></td>
<td>Age of plant</td>
<td>7 weeks</td>
<td>6 weeks</td>
<td>6 weeks</td>
<td>5 weeks</td>
<td>4 weeks</td>
</tr>
<tr>
<td>C</td>
<td>Date of infiltration</td>
<td>02/04/10</td>
<td>02/04/10</td>
<td>02/04/10</td>
<td>02/04/10</td>
<td>02/04/10</td>
</tr>
<tr>
<td></td>
<td>Age of plant</td>
<td>8 weeks</td>
<td>7 weeks</td>
<td>7 weeks</td>
<td>6 weeks</td>
<td>5 weeks</td>
</tr>
</tbody>
</table>

Two bags were planted every week up to week 5 and watering of plants was done everyday. Seed details are as follow:

- Type of vegetable: Tomato
- Variety: MT1
3.3 Confirmation of *E. coli* carrying pBI121 plasmid

3.3.1 Overnight growing of DH5α strain of *E. coli* carrying pBI121 plasmid

*E. coli* that carries pBI121 plasmid was provided in a glycerol stock by the Genetic Engineering lab and 50 µL of it was pipetted out into each of the 10 ml LB broth in a conical flask. 10 µL of 100 mg/ml kanamycin was added in the culture and left to overnight growing for 16 hours in an orbital shaker of 150 rpm at 37 ºC.

3.3.2 Isolation of pBI121 plasmid

Thus far, three attempts were done on plasmid isolation by using Solution I, II and III (the alkaline lysis method), TENS solution and Vivantis kit. These three methods were used to identify the best method for plasmid extraction.

a. **Isolation of pBI121 plasmid by using alkaline lysis method**

50 ml were prepared for every solution I, II and III. (Appendix B). Harvested *E. coli* from the 10 ml overnight culture was centrifuged in a falcon tube at 6000 rpm for 5 minutes at room temperature. Supernatant was removed carefully, retaining the pellet and re-centrifuged for another 2 minutes. Supernatant or any traces of liquid were removed completely. Cell pellet was then resuspended using 100 µL of Solution I and vortexed briefly for 10 seconds. Tube was kept on ice. 100 µL of Solution II was added to the cell suspension and mixed gently by inverting the tube 10 times. Tube was left at room temperature for minutes to allow lysis reaction. A clear viscous liquid was observed. 300 µL of Solution III was added and mixed gently by inverting the tube 10 times. A white precipitate was observed. Centrifugation was done at 10000 rpm for 5 minutes to precipitate the pellet. Same volume of 300 µL supernatant was carefully transferred into a new sterile 1.5 ml microcentrifuge tube. DNA was precipitated by adding 2X volume of