AGROINFEILTRATION OF *LYCOPERSICON ESCULENTUM* WITH LURIA BROTH MEDIUM ONTO UNWOUNDED LEAVES

Sharul Aida Binti Mohd. Shayuti
(19865)

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Agroinfiltration of *Lycopersicon esculentum* with Luria Broth Medium onto Unwounded Leaves

Sharul Aida Binti Mohd. Shayuti

(19865)

This project is submitted in partial fulfillment of requirement for the degree of Bachelor of Science with Honours (Resource Biotechnology)

Supervisor: Miss Safarina Ahmad

Co-supervisor: Dr. Hairul Azman Roslan

Resource Biotechnology Programme
Department of Molecular Biology

Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
2010
DECLARATION

I hereby declare that no portion of this dissertation has been submitted in support of an application for another degree of qualification of this or any other university or institution of higher learning.

(SHARUL AIDA BINTI MOHD. SHAYUTI)

Resource Biotechnology Programme
Department of Molecular Biology
Faculty of Resource Science and Technology
University Malaysia Sarawak
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Sharul Aida Bt Mohd. Shayuti

Resource Biotechnology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Agroinfiltration is an Agrobacterium-mediated transformation technique and acts as a delivery system in introducing a foreign DNA into the host cell. This study aims to transform the L. esculentum by using agroinfiltration technique with Luria Broth medium as infiltration medium onto unwounded leaves, and to analyze the putative transformants by using PCR. In this study, the pBl121 plasmid was extracted from E.coli culture and then introduced into the Agrobacterium tumefaciens strains LBA440. Then, it was resuspend in infiltration medium before injected into the tomato leaves specific in L. esculentum range in 4 to 6 weeks age. Then, the tomato leaves were further analyzed by PCR technique. The plasmid isolation and transformation of pBl121 plasmid into the Agrobacterium tumefaciens were successfully done based on restriction enzyme digestion and PCR analysis. However, based on the PCR analysis of the putative transformants, agroinfiltration of L. esculentum by using Luria Broth medium as infiltration medium onto unwounded leaves was not successful. The amplification failure may be due to PCR inhibition or no transformation occurs in the plant genome.

Keyword: regeneration system; Agrobacterium tumefaciens; Agroinfiltration

ABSTRAK


Kata kunci: system penjanaan semula; Agrobacterium tumefaciens; Infiltrasi agro
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<tr>
<td>μL</td>
<td>Microlitre</td>
<td></td>
</tr>
<tr>
<td>AGE</td>
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</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
<td></td>
</tr>
<tr>
<td>E.coli</td>
<td>Escherichia coli</td>
<td></td>
</tr>
<tr>
<td>GUS</td>
<td>β-glucuronidase</td>
<td></td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
<td></td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
<td></td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
<td></td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
<td></td>
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<tr>
<td>SS-phenol</td>
<td>salt-saturated phenol</td>
<td></td>
</tr>
<tr>
<td>T-DNA</td>
<td>transferred-DNA</td>
<td></td>
</tr>
<tr>
<td>Ti</td>
<td>tumor-inducing</td>
<td></td>
</tr>
<tr>
<td>UV</td>
<td>ultra-violet</td>
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</tr>
</tbody>
</table>
1.1 Background

Transformation is a process in which foreign DNA is taken up by the host cells or recipient from its environment. Bacteria that have taken-up a plasmid are selected by growth on a plate containing an antibiotic which confer to the plasmid vector according to Turner et al. (1997). Transformation technique becomes one of the most valuable tool or method in the production of transgenic crops. One of transformation technique which is applied recently is agroinfiltration technique or *Agrobacterium* mediated transient gene expression in which one of potential and valuable tool in plant genetic engineering. It is aims to induce transient expression of a foreign gene into the plant (Anonymous, 2009).

In this study, agroinfiltration technique was applied in tomato leaf. It is because; agroinfiltration technique is one of the most preferable way or technique in many gene functional analysis nowadays (Kapila et al., 1997). This technique is faster and more convenience compared to the conventional method. Agroinfiltration is often used as a mobile tool for replicons even in the species with limited transfer efficiency (Orzaez et al., 2006). In applying agroinfiltration technique, there were a few steps in which are very essential in this study. The steps were consists of plasmid preparation, introduction of DNA, plant testing, and analysis step.
In agroinfiltration technique, the bacterial suspension containing *Agrobacterium* is injected into the selected plant leaf. This method is aims to transfer the foreign gene to the plant genome. Therefore, the transfer of binary vector or plasmid in which carrying the reporter gene to the plant’s nucleus cell or genome is the most crucial step in this study after the *Agrobacterium* cell cultures are injected to the intracellular space of the leaf parenchyma.

There also risks in using agroinfiltration technique in which should be alert of. One of the problem is the protocols and efficiency of the agroinfiltration technique may be vary or different in each plant species. The different of efficiency is caused by topological factor in which consist of compactness of tissues, and innervations patterns according to Orzaez *et al.* (2006). Plant model in which used in this study was tomato, specific in *Lycopersicon esculentum* species. It is also used as a plant model in *Agrobacterium*-mediated tomato transformation and regeneration of transgenic lines expressing *Tomato leaf curl virus* coat protein gene for resistance against TLCV infection (Raj *et al.*, 2005).

There are a lot of international and global efforts in genomic characterization in tomato plant, including expressed sequences tags and also genome sequencing project. Besides, tomato plant also has been proposed in medical studies such as production of oral vaccine and immunotherapeutic protein in which conducted by Sala *et al.* (2003).
In this study, the tomato leaves was injected with *Agrobacterium tumefaciens* suspension containing pBI121 (shown in Appendix A). Then, the leaves were further analyzed by Polymerase Chain Reaction (PCR) technique in order to determine the efficiency of agroinfiltration as delivery system in this study. In this study, PCR analysis was conducted in order to detect the presence of GUS gene. GUS gene acts as a reporter gene which is functions in delivery system’s efficiency determination in this study. GUS gene was used in this study since it is located at the T-DNA region in pBI121 plasmid vector in which transferred during the transformation process.

1.2 Objectives

The objectives of this study are:

1. To transform the *L. esculentum* by using agroinfiltration technique with Luria Broth medium as an infiltration medium onto unwounded leaves.

2. To analyze the putative transformants by using PCR technique.
CHAPTER 2

LITERATURE REVIEW

2.1 Lycopersicon esculentum

Tomato plants are dicots and the tips of the tomato plants grows from the series of branching stem in which normally from the terminal of the bud. The tip will stop growing when the plant is flowering. There are fine and short hairs in which cover its vines to facilitate the vining process in which turning into roots when the plant is in touch with the ground (soil) and moisture. Most of them also have compound leaves in which called regular leaf plants. In regular leaf pattern, there are a lot of variation in it, such as rugose (deeply groove), variegated and angora leaves (have addition or extra colours) in which caused by genetic mutation that mutation that effect chlorophyll to be excluded in some division or part of the leaves.

Tomato is herbaceous and usually, it reaches 1-3 metres in height and has a weak and hairy stem. Its leaves usually are 10 to 25 centimetres long with odd pinnate and 5 to 9 leaflets on its petioles. Its flowers are yellow in colour and 1 to 2 centimetres across. It is fruits can be yellow, pink, orange, red, or in white colour.

It is also one of most preferred plant model in plant transformation due to its amenability to transformation, easily to grow under containment, short generation time, and also has relatively small genome size (0.7-1.0 pg). There are a lot of researches have been conducted on L.esculentum, for an example in regeneration and Agrobacterium-mediated transformation studies in tomato research which is conducted by Oktem et al. (1999).
2.1.1 *Lycopersicon esculentum* leaves

Tomato plant's consist of compound leaves. This compound leaflets that are distributed along the rachis of the tomato leaf. The leaf is connected by the stem of the plant via a structure in which called petiole. Therefore, petiole acts as a connector between stem and also leaf of the tomato leaf.

Most of the tomato leaves are covered by a thin outer cuticle which's act as a barrier. There is epidermis inside of the cuticle. While in the lower part of the epidermis layer, there stomata inside. In the centre part of the epidermis layer, there are the mesophyll in which consists of two different type of cells. The two different type cells are called palisade mesophyll and also spongy mesophyll. Spongy mesophyll is consists of parenchyma cells in which irregularly shaped and also arranged in a loose structure. While, palisade mesophyll is consists of oblong upright parenchyma cells. Tomato leaves' vascular bundle are arranged in veins in which run throughout the leaf. This type of venation is common type of venation among dicotyledon plants according to Thomas L. R. (1996).

*L. esculentum* leaves are also used as plant material or samples in agroinfiltration transformation technique. In agroinfiltration technique of *L. esculentum* leaves, the infiltration medium is infiltrated onto the underside of the leaves by using needle-free syringe. The infiltration medium containing A. tumefaciens is specifically directed into the epidermis layer which is consists of stomata and parenchyma cells due to its function in regulating or allowing gas exchange. There are also a lot of researches have been onto *L. esculentum* leaves such as in Genetic transformation of a local variety of tomato using gus gene: an efficient genetic transformation protocol for tomato (Paramesh *et al.*, 2010).
2.2 *Agrobacterium tumefaciens*

*Agrobacterium tumefaciens* is gram negative, soil bacteria and rod in shape. It is also a parasite and does not bring any benefits to plant besides cause diseases to plants and one the causal of tumor in dicot plants. *Agrobacterium tumefaciens* is capable in DNA transmission which is applied in biotechnology by inserting its genes into the plant and induce plant's machinery to express gene in which will give nutrient to the bacterium itself. *Agrobacterium* mediated plant transformation is one the method *in planta* transformation. It was also had been applied in grapevine leaves (Zottini et al., 2008).

It is the easiest and simplest mode in transforming plant genomic DNA. It contains DNA molecule in which called Ti (tumor-inducing) plasmid. These DNA molecule will integrate with the plant’s genome once the plant’s cell is transformed (Orzaez et al., 2006).

2.3 Transformation

Transformation is a process by which host organism take a DNA (foreign) from its environment. Transformation is applied in introduction of vector DNA into the host organism. Bacteria in which can undergo transformation naturally are said to have genetic competent. The exact mechanism of transformation or DNA uptake is not known yet but it is possibly caused by the shielding of negative charge of the DNA by the Ca$^{2+}$ ions, which is allow DNA to pass through the cell membrane (host cell). This mechanism has been applied recently in various species such as in *Sesbania drummondii* (Padmanabhan, P. & Sahi, S.V., 2008), *Arabidopsis thaliana* (Trembley et al., 2008) and in *Cynza canadensis (L.) Cronquist* (Scheiberet et al., 2004).
2.3.1 *In planta* transformation

*In planta* transformation is one type of transformation which is the transformed plant's cell or tissues are developed and regenerate in within the plant (*in vivo*). The inoculation and co-cultivation process with the foreign gene take place as the plant developed normally. Some of transformations techniques in which apply *in planta* transformation approach are floral dip method (Clough, S. J., & Bent, A. F., 1999), vacuum infiltration (Bechtold *et al.*, 1993), agroinfiltration (Kapila *et al.*, 1997), and also electroporation technique (Weaver, 1995).

Floral dip method is a method in which based on *in planta* flower infiltration in which not require *in vitro* cell culture technique and also plant differentiation. This method is also based on *A. tumefaciens* mediated transformation. The flower or the sample will be submerged into the *A. tumefaciens* suspension. Floral dip method is much more similar to the vacuum infiltration technique in which involves the dipping method of the plant but floral dip method is conducted with no vacuum. Floral dip method is potential in preventing somaclonal variation from occur since it is one of *in planta* transformation. *In vitro* cell culture technique is one of the major factors in which cause somaclonal variation (genetic change). This problem occurs because of the genetic modification or mutation in which occur during the DNA integration process. The floral dip approach can reduce or avoid DNA changes. Besides, this method is also simple to be carried out and also eliminate labor-intensive process such as vacuum infiltration (Clough, S. J., & Bent, A. F., 1999). However, this method has low efficiency since there is no vacuum used. But then, the vacuum effect can be replaced by adding or increase surfactant level in inoculation medium (Clough, S. J., & Bent, A. F., 1999). Floral dip approach was applied in *Arabidopsis thaliana* (Labra *et al.*, 2004).
Vacuum infiltration is also one of the in planta transformation in which excludes the need or requirement of plant tissue culture. In this technique the plant will be submerged into the solution or suspension containing A. tumefaciens with the presence of vacuum effect in which acts in facilitate the DNA uptake by the plant genome (Bechtold et al., 1993). This method is much similar to the floral dip method. Vacuum infiltration method is also high transformation efficiency since vacuum effect is used in this method. However, vacuum effect used may create plant stress to the plant. Therefore, the plant needed to be warped and incubated in order to maintain its humidity and prevent dehydration (Bechtold et al., 1993). Vacuum infiltration method is labor-intensive and requires specialized equipment such as vacuum chamber compared to other methods.

Agroinfiltration is one of the in planta transformation method or potential tool. This method is based on Agrobacterium tumefaciens mediated transformation. In this technique, Agrobacterium tumefaciens is injected into plant leaves as liquid culture by using needleless syringe. Agroinfiltration method is also has become the most preferred method in gene functional analysis (Kapila et al., 1997). This method is preferred to its simplicity and also its reability. However, this method has limited host range because of not all of the plant species can be infected by Agrobacterium tumefaciens. Besides of tomato (Solanum lycopersicon), there’s also another plant species in which apply this kind of method such as N. benthamiana (Kopertekh, L., & Sciemann, J., 2005).
Electroporation technique is one of the methods in which does not require any cell or tissue culture (*in vitro* method or approach). Besides, only small amount of DNA required in this method compared to the other techniques and have high efficiency in DNA transformation. However, the electroporation method also has possible risk of getting cell damage. If the length and intensity of pulse is wrong, the pores may possibly become larger and fail to close the pores after the membrane discharge and this could leads to cell damage or rupture (Weaver, 1995). Besides, transport material in and out from the membrane during the electro permeability is relatively nonspecific. This may leads to an imbalance of ion in which could leads to improper cell function and death (Weaver, 1995). In this technique, the foreign gene (DNA) will be introduced to the sample such as pollen through electroporation method. Then, it will be able to produce genetically engineered seed. This method has been applied in tobacco (Matthews *et al.*, 1990).
CHAPTER 3
MATERIALS AND METHODS

3.1 Plant materials

*L. esculentum* leaves were the plant materials in which used in this study. Ages of *L. esculentum* plant leaves used in this study were in the range of 4 to 6 weeks since their sizes are much larger compared to the younger leaves. Therefore, they are easier to be injected compared to the younger leaves. Hence, only leaves in range of 4 to 6 weeks were chosen. The *L. esculentum* seeds (variety MT1) were obtained from MARDI, Jalan Kebun, Serdang. There were 30 *L. esculentum* seeds used in this study.

3.2 Germination of *Lycopersicon esculentum* seeds

The *L. esculentum* seeds were ensured fresh, mature, and insect- and disease-free. Then, the seeds pre-treated first before it was germinated. After that, the seeds were ready for sowing. Floating seeds were discarded. Then, the seeds were sprinkled on the surface of the soil. Then, the seeds were covered lightly with sifted sand so that the seed were buried to a depth about equal to its thickness. The plants were watered 2 times in a day in order to prevent the dehydration from occurred.
3.3 Confirmation of *E. coli* harbouring pBI121 plasmid

3.3.1 Preparation of *E. coli* culture harbouring pBI121 plasmid

Luria Broth media containing kanamycin antibiotic was transferred into a sterilized conical flask or bijou bottle for about 10 ml. Then, 50 μL of *E.coli* colony was pipette into the Luria Broth media in conical flask. Then, the media containing *E.coli* was incubated for overnight at 34-37°C with good aeration and shaking until the culture was saturated.

3.3.2 Isolation of double stranded plasmid DNA pBI121 from bacteria culture

In this step, pBI121 plasmid was extracted from *E.coli* by using GF-1 Plasmid Extraction Kit (Vivantis). Falcon tube containing *E.coli* culture was centrifuged at 6000 rpm for 5 minutes in order to harvest the cell pellet. After that the supernatant was discarded completely. Then, the 250 μL of S1 was added into the cell pellet and the cells were resuspended completely by vortexing. After that, the suspension was transferred into a clean 1.5 ml microcentrifuge tube.

Then, 250 μL of S2 was added into the tube. Then, the mixture was mixed gently by inverting the tube several times in order to obtain a clear lysate. Next, the mixture was incubated on ice or at room temperature for not longer than 5 minutes. After that, 400 μL of Buffer NB was added in order to neutralize the lysate and the mixture was gently mixed by inverting the tube several times until a white precipitate formed. Then, the tube was centrifuged at 13000 rpm for 10 minutes. After that, the supernatant was transferred into a column assembled in a clean collection tube. Then, the tube containing supernatant was centrifuge at 10000 rpm for 1 minute. The flow through was discarded.
Then, the column was washed with 700 µL wash buffer and centrifuged at 10000 rpm for 1 minute. The flow through was discarded. After that, the column was centrifuge at 10000 rpm for 1 minute in order to remove residual ethanol. Then, the column was placed into a clean microcentrifuge tube. 60 µL of TE buffer was added directly onto the column membrane and stand for 1 minute. The column was centrifuge at 10000 rpm for 1 minute to elute the DNA. Then, the DNA was stored at -20°C.

3.3.3 **Restriction enzyme digestion analysis of pBI121**

Restriction enzyme digestion analysis of pBI121 plasmid was conducted in order to ensure or confirm the extracted plasmid from *E. coli*. There were two type of restriction enzyme digestion used in this step in which consist of single and double digestion. In single digestion *BamH*I, *Hind*III, *Pst*I and *Bgl*II were used, while for double digestion, *Eco*RI/*Hind*III pair and *Sal*I/*Sma*I pair. All of the items in which shown in Table 3.1 and 3.2 were mixed evenly. Then, it was incubated for overnight. Next, the mixture was heated at 65°C for 20 minutes in order to inactivate the enzymes. The composition of each reagent used in single restriction enzyme digestion and double restriction enzyme digestion as shown in Table 3.1 and 3.2 respectively.

<table>
<thead>
<tr>
<th>Item</th>
<th>Volume (µL)</th>
</tr>
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<tbody>
<tr>
<td>Restriction Enzyme</td>
<td>1</td>
</tr>
<tr>
<td>10X Buffer Tango™</td>
<td>1</td>
</tr>
<tr>
<td>Plasmid mini prep DNA/ DNA sample</td>
<td>8</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>10</strong></td>
</tr>
</tbody>
</table>

Table 3.1: The composition of each reagent in which mixed and used in Restriction Enzyme Digestion (single digestion).
Table 3.2: The composition of each reagent which was mixed and used in Restriction Enzyme Digestion (double digestion).

<table>
<thead>
<tr>
<th>Item</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EcoRI/HindIII</td>
</tr>
<tr>
<td>Restriction Enzyme 1</td>
<td>1</td>
</tr>
<tr>
<td>Restriction Enzyme 2</td>
<td>1</td>
</tr>
<tr>
<td>10X Buffer Tango™ (yellow buffer)</td>
<td>4</td>
</tr>
<tr>
<td>ddH2O</td>
<td>4</td>
</tr>
<tr>
<td>Plasmid mini prep DNA/ DNA sample</td>
<td>10</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20</strong></td>
</tr>
</tbody>
</table>

3.3.4 Polymerase chain reaction (PCR) for *nptII* and GUS gene in pBI121

After the pBI121 plasmid DNA was isolated from the transformed *E. coli* XLI Blue, PCR was carried out for further verification of the extracted plasmid. The PCR analysis was carried out in a thermal cycler (Perkin Elmer) by using *nptII* and GUS primer sets in order to verify the presence of the pBI121 plasmid with GUS and *nptII* fragments. PCR analysis was carried out for 35 cycles in order to obtain optimum amplification product. In this study, there were positive and negative controls used. Negative control contains reaction mixture with no plasmid sample while positive control contains reaction mixture with pBI121 plasmid that was already confirmed. Expected sizes of the amplification products for GUS and *nptII* genes are 600 bp and 250 bp respectively.

The composition of the PCR reaction mixture and parameter for the amplification of *nptII* and GUS fragments are shown in Table 3.3 and 3.4 respectively. The amplified product was visualised by handling 1% agarose gel electrophoresis.