CONSTRUCTION OF A PLANT TRANSFORMATION VECTOR CARRYING A MARKER GENE FOR EXPRESSION IN PLANTS

Ngien Leh Nah

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CONSTRUCTION OF A PLANT TRANSFORMATION VECTOR CARRYING A MARKER GENE FOR EXPRESSION IN PLANTS

NGIEN LEH NAH

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

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Construction of a plant transformation vector carrying a marker gene for expression in plants

Ngien Leh Nah

Resource Biotechnology
Faculty of Resource Science and Technology
University Malaysia Sarawak (UNIMAS)

ABSTRACT

The construction of plant transformation vector, a binary vector is important especially in plant transformation involving Agrobacterium tumefaciens. The resultant binary vector is transformed into Agrobacterium tumefaciens as a carrier to be further transformed into the plants. In this study, the transferred-DNA or T-DNA (~5.5 kb) originated from binary vector, pBI121 supplied by Arabidopsis Biological Resource Center (Columbus) was PCR-amplified in order to be subcloned into pUC19 vector. The complete sequence of pBI121 (Accession number: AF485783) was derived and primer designed for amplification of T-DNA and non-T-DNA regions. T-DNA was successfully PCR-amplified by using forward primer (5' – GCATACTAGTTACCCGCCAATATATCCTGTCA – 3') and reverse primer (5' – GCATATGGCAGGATATTGTGGTGAAC – 3'). Several trials with different subcloning strategies were carried out to subclone the PCR fragment (T-DNA) into pUC19. The ligation reaction mixture was eventually transformed into E. coli strain JM109. Less than 50% of white colonies were observed by using adaptors with BamHI site which were added during ligation together with BamHI-cut pUC19. However, the gene cloning was failed as no insert was detected both with restriction enzyme and PCR analyses. The problem was mainly due to failure in ligation reaction. The work to form a helper plasmid from PCR-amplified non-T-DNA could not be carried out due to time constraint.

Key words: plant transformation vector, binary vector pBI121, Agrobacterium tumefaciens, T-DNA, PCR

ABSTRAK

Pembinaan vektor transformasi tumbuhan, iaitu vektor binari sangat penting terutamanya dalam transformasi tanaman yang melibatkan Agrobacterium tumefaciens. Vektor binari yang berjaya dibina akan ditransform ke dalam Agrobacterium tumefaciens yang bertindak sebagai pembawa untuk ditransform ke dalam tumbuhan. Dalam kajian ini, 'transferred-DNA' atau T-DNA (~5.5 kbp) berasal daripada vektor binari, pBI121 yang didelajakan oleh Arabidopsis Biological Resource Center, Columbus telah diamplifikasi melalui Tindak Balas Berasal Polimerase (PCR) untuk disubklonkan ke dalam vektor pUC19. Jujur pemah pBI121 (Nombor akses: AF485783) diterbitkan dan pencen diherabentuk untuk amplifikasi bahagian T-DNA dan bukan T-DNA. Bahagian T-DNA berjaya diamplifikasikan dengan menggunakan pencen ke hadapan (5' – GCATACTAGTTACCCGCCAATATATCCTGTCA – 3') dan pencen ke belakang (5' – GCATATGGCAGGATATTGTGGTGAAC – 3'). Beberapa percubaan dengan pendekatan strategi pengsubklonan yang berbeza telah diambil untuk subklon fragmen PCR (T-DNA) ke dalam pUC19. Akhirnya, campuran reaksi ligasi ditransformkan ke dalam E. coli strain JM109. Koloni-koloni putih (kurang daripada 50%) telah diperhatikan di mana 'adaptors' yang mempunyai tempat pembatasan BamHI telah ditambahkan semasa proses ligasi bersama-sama dengan pUC19 yang telah dibatas oleh BamHI. Walau bagaimanapun, pengklonan gagal kerana tiada fragmen sebesar 5.5 kbp dikenali dengan menggunakan enzim pembatasan dan analisis PCR. Masalah ini disebabkan kerana terdapat oleh kegagalan dalam ini reaksi ligasi. Kerja pembinaan plasmid pembantu daripada hasil amplifikasi PCR untuk bahagian bukan T-DNA tidak dapat dijalankan kerana kesulitan masa.

Kata kunci: vektor transformasi tumbuhan, vektor binari pBI121, Agrobacterium tumefaciens, T-DNA, PCR
CHAPTER I
INTRODUCTION

Plasmid is a small, circular extrachromosomal double stranded DNA that can replicate itself and is not important in the survival of a bacterium (Tortora et al., 2004). However, linear plasmids have been identified in both gram positive and gram negative bacteria (Towner and Cockayne, 1993; Ausubel et al., 1999). If a bacterial cell has plasmid, it can survive under certain environment such as the toxic environment. For example, Pseudomonas spp. contain plasmid which encode enzymes capable of degrading an enormous range of both natural and synthetic organic compounds such as toluene and camphor (Glover, 1984). Therefore, it is an additional advantage for the bacteria to survive in very diverse and challenging environments.

Plasmid is an important biotechnological tool and serves as a cloning vector. Molecular biologists had genetically engineered naturally occurring plasmids which have these features: unique restriction sites, small size, replicates in host and selectable marker and/or reporter genes (Haddock, 2004). The unique restriction sites are essential for introducing foreign DNA. Sambrook et al. (1989) revealed that the construction of smaller plasmid vector will increase the bacterial transformation efficiency. The origin of replication (ori) in plasmid will ensure autonomous replication within its host. Selectable marker (eg. antibiotic resistance gene) and/or reporter genes (eg. β-glucuronidase or GUS gene) will ease the identification of recombinant bacterial cells.
According to Schepple et al. (2000), several transformation methods had been developed for gene delivery in plant transformation process. They are the Agrobacterium tumefaciens Ti (Tumor-inducing) plasmid system, chemical permeabilization of the plasma membranes of protoplasts, microinjection, electroporation, and biolistics. Biolistics or microprojectile bombardment is useful in transforming monocots (eg. wheat, rice and maize) while A. tumefaciens Ti plasmid system is most successful in transforming dicots (eg. soybeans and tomatoes). However, recent studies have demonstrated the successful transformation of recalcitrant crops such as rice (Raineri et al., 1990; Afolabi et al., 2005). Agroinfection which is essential in delivering viral genomes to graminaceous hosts were also reported by Ellis (1993).

Agrobacterium-mediated plant transformation is the easiest system by which the crown gall-producing soil bacterium A. tumefaciens are modified with the removal of nonessential regions (Schepple et al., 2000). Wu (1988) stated that the binary vector system will overcome the problem of the big-sized (200 kb) Ti plasmid which cannot be used directly for cloning purpose.

In the binary vector system, the A. tumefaciens should have two plasmids; one plasmid carrying a marker gene within the T-DNA region, another plasmid is the disarmed plasmid or helper plasmid (no T-DNA region but has the virulence, vir genes). Once the plant is infected by A. tumefaciens, the vir genes of the helper plasmid will direct the incorporation of the T-DNA into the plant genome (Guerineau and Mullineaux, 1993; Schepple et al., 2000). Reengineering of binary vector, pBI121 by using PCR approach to construct a smaller binary vector and a helper
plasmid was attempted in this study. The knowledge of the complete sequence of the binary vector pBI121 (Chen et al., 2003) was applied in this research.

According to Xiang and co-workers (1999), many useful and versatile vectors have been constructed in the past for the production of transgenic plants but those vectors are fairly large (more than 10 kb) and offer fewer unique restriction sites for cloning gene of interest. This research rationale focuses on the reduction of plasmid vector size with more unique restriction sites to accommodate larger fragment of foreign gene or gene of interest.

The first aim of this research is to verify the plasmid pBI121 and pUC19 from the stock collection and to construct a smaller binary vector carrying a marker gene and a helper plasmid derived from pBI121. Subsequently, series of plant transformation vectors will be constructed based on the newly-constructed binary vector.
CHAPTER II
LITERATURE REVIEW

2.1 pBI121 and pUC19

In this research, a mini binary vector and a helper plasmid derived from pBI121 (Figure 2.1) will be constructed by using Polymerase Chain Reaction (PCR) approach. T-DNA region (Figure 2.2) will be amplified by PCR and subcloned into pUC19 vector at multiple cloning site, MCS (Figure 2.3). The mini binary vector will have essential elements in plant transformation process which are an *Escherichia coli* origin of replication (*ori*) and the T-DNA region (RB-right border, expression cassettes for NPTII-neomycin phosphotransferase II as plant-selectable marker, MCS-multiple cloning site and the LB-left border).

The non-T-DNA region (Figure 2.2) of pBI121 containing origin of replication from plasmid RK2 *oriV*, NPTIII 5', NPTIII CDS, and RK2 *TrfA* will also be amplified and recircularized to form a helper plasmid to be transformed into *A. tumefaciens* strain LBA4404. RK2 *oriV* is the vegetative and broad host-range plasmid origin of replication while RK2 *TrfA* encodes trans-acting functions necessary for replication of plasmid in its host cells (Guerineau and Mullineaux, 1993; Ausubel *et al.*, 1999).
Figure 2.1. Restriction map of pBII21, based on Brusslan (2000).

Figure 2.2. Restriction map of a binary vector pBII21 showing the T-DNA region and non-T-DNA region (Adapted from: Plant Molecular Genetics: Lecture 2, part 3 of 4. (n.d.). Retrieved July 8, 2005, from http://www.umanitoba.ca/afs/plant_science/COURSES/39-768/102/102.3.html).
pUC19 plasmids (Figure 2.3) will be used to carry the foreign gene because they have a high copy number (500-700). pUC19 vectors express the amino-terminal fragment of the lacZ gene product (β-galactosidase) and show α-complementation in appropriate hosts (Sambrook et al., 1989). Polycloning site within the lacZ gene allows the insertion of foreign DNA. Disruption of the lacZ gene will ease the selection of recombinant bacterial cells through histochemical staining.

Figure 2.3. Restriction map of pUC19. The restriction enzyme sites within the multiple cloning site are not shown in the figure. Adapted from: Fermentas Description and Restriction Maps: pUC18, pUC19 (2005). Retrieved July 13, 2005, from http://www.fermentas.com/techinfo/nucleicacids/mappuc1819.htm
2.2 Alkaline lysis method in plasmid minipreparation or miniprep

Alkaline lysis miniprep takes advantage of the fact that plasmid DNA is much smaller than genomic DNA (Scheppler et al., 2000). Thus, smaller molecules such as RNA and plasmid will escape the lysis reaction once the genomic DNA and proteins are denatured and precipitated with high-salt-concentration buffers.

As explained by Birnboim and Doly (1979), 50 mM glucose and 25 mM Tris-HCl act as buffer solution for pH control. Lysis solution (0.2 N NaOH and 1% sodium dodecyl sulphate, SDS) is then added. 0.2 N NaOH is used to denature linear, chromosomal DNA while 1% SDS, a detergent is added to break open the cell membranes and denatures the proteins (Turner et al., 1997; Scheppler et al., 2000).

The preparation is then neutralized by adding Solution III (Turner et al., 1997). Potassium acetate (KOAc) is used to cause the precipitation of protein-SDS complexes and low molecular weight RNA (Birnboim and Doly, 1979). Following centrifugation after phenol extraction, three phases can be observed, namely the lower phase containing hydrophobic cell components, a middle layer containing protein and an upper phase with plasmid DNA (Walker and Rapley, 1997).

Cold absolute ethanol is used to precipitate and concentrate the DNA. Finally, the DNA is pelleted by centrifugation; the pellet is retained and washed with 70% ethanol to remove unwanted salts from the DNA. The DNA pellet is air-dried to allow the evaporation of ethanol.
Hence, ethanol contamination which will interfere with subsequent analysis can be avoided (Scheppler et al., 2000).

Finally, the purified DNA is suspended in Tris-EDTA (TE) buffer. As explained by Turner et al. (1997), the ethylene diamine tetraacetic acid (EDTA) is added to chelate the cation, Mg$^{2+}$ which is the cofactor of the bacterial endogenous nucleases, the DNases. Thus, DNases are inactivated and plasmid will not be degraded whilst the Tris-HCl will buffer the solution at pH8.0.

2.3 Plasmid conformation

In 2003, Hayes reported that plasmid DNA is mostly maintained in a covalently closed circular, supercoiled form or Form I. Plasmid converts to an open circular form or Form II when a nick is introduced on one of the strands of the double-stranded plasmid DNA. Another form is the linearized or Form III in which nicks are introduced at opposite positions on both DNA strands. Supercoiled plasmid migrates the fastest on an agarose gel when subjected to electrophoresis followed by linearized plasmid and the slowest migrating form is the open circular plasmid (Boffey, 1986; Towner and Cockayne, 1993; Martin, 1996). According to Martin (1996), the accurate size of certain plasmid can only be determined based on the migration of its linear form compared to a set of linear markers.
2.4 Restriction endonucleases

Restriction endonucleases or restriction enzymes (REs) are enzymes that can cleave or digest certain specific sequences of nucleotides in DNA (Turner et al., 1997; Scheppler et al., 2000). EcoRI was the first isolated restriction enzyme in 1962 by Werner Arber from E. coli. Naturally, REs serve as protective weapons in bacterial cells which will kill the invaders especially the bacteriophage or foreign DNA by digesting the unmethylated DNA. According to Williams and co-workers (1993), the bacterial DNA is protected from its RE activity as its own DNA is chemically modified (methylated).

REs have been used as an important tool in genetic engineering. Restriction enzymes can be divided into two types, which are the endonucleases and exonucleases. Brown (1999) revealed that endonucleases digest at the middle of DNA molecule while exonucleases digest either from end of 5’ to 3’ or the end of 3’ to 5’ DNA molecule. REs cleavage that produce sticky ends (are not directly opposite each other) in the two strands of a DNA are featured in most of the REs (eg. BamHI or EcoRI). In 1996, Dawson et al. reported that some enzymes simply cleave DNA with a double-stranded break in the center of the recognition sequence which produces DNA fragments with termini called blunt ends (eg. AulI or HaellIII). This feature of either sticky or blunt ends made it possible to create new recombinant DNA molecules.

According to Sambrook and co-workers (1989), REs have been classified into three groups namely Type I, II and III. Type I and Type III restriction endonucleases are less useful in molecular cloning because the sequences of the resulting fragments they cut are not precisely
known (Brown, 1999). Type II restriction enzymes are routinely used in molecular cloning which are mostly six-base cutters (Sambrook et al., 1989).

2.5 The construction of binary vectors used in Agrobacterium-mediated plant transformation

The natural gene transfer system by using Agrobacterium has been used to produce transgenic plants in floricultural industry like Dendrobium orchid (Yu et al., 2001) and for crops improvement such as cotton (Ul-Haq, 2004), canola (Basu et al., 2001), sorghum (Zhao et al., 2000) and Acacia hybrid (Pang et al., 2005) and flax plants (Dong et al., 1993).

Sambrook et al. (1989) explained that the trend for the future was to streamline vectors to reduce the size to the minimum while increasing the capacity to accept large foreign DNA. The advantages of construction of smaller plasmid vectors are to gain higher transformation efficiency, easier characterization by restriction mapping and they can replicate to higher copy numbers.

As described by Bevan (1984), a binary vector called pBIN19 (approximately 10 kbp) was constructed by utilizing the trans-acting functions of the vir region inside tumor-inducing, Ti resident plasmid in A. tumefaciens to transfer T-DNA (Transferred-DNA) sequences into the nuclear genome of plants. pBIN19 was relatively small size which has large number of restriction sites and requires no recombinational steps for incorporation into the Ti plasmid.
An improved binary vector, pBIN20 was designed to contain a large multi-cloning site (MCS) derived from the superlinker found in pSL301 and selectable kanamycin resistance gene, nptII between the Ti border (Hennegan and Danna, 1998). pBIN20 was derived from pBI121 and has been proven to create lines of transgenic tobacco plant.

As explained by Xiang et al. (1999), pBIN19 binary vector was streamlined because of the availability of its complete sequence. The nonessential sequences (i.e. KIlaC gene and isl transposable element) were eliminated by using PCR-based approach. The newly-constructed mini binary vector, pCB301 (3.5 kb) was tested fully functional in transforming Arabidopsis plants. As a result, its series (pCB301-based vectors) were also constructed for various plant transformation tasks.

According to Zhao and co-workers (2000), Agrobacterium strain LBA4404 carrying a ‘super-binary’ with a bar gene as a selectable marker for herbicide resistance in the plant cells was used to transform sorghum. It was the first successful report on the Agrobacterium-mediated transformation in producing stable transformants of sorghum plants.

In 2003, Chen et al. reported on the reconstruction of the widely used expression vector pBI121 by applying the knowledge of the complete sequence of pBI121 (accession number AF485783). Chen and co-workers (2003) had cloned the three different plant genes into pBI121 by replacing the GUS coding region within T-DNA. Then, they PCR-amplified the region and subcloned the PCR products into pGEM-T Easy vector.
In recent study conducted by Afolabi et al. (2005), marker-free or clean gene transgenic rice was produced by using multiple T-DNA approach. This approach utilizes the novel pGreen/pSoup dual-binary vector system. Both pGreen and pSoup vector would carry different transgenes in its T-DNA and were co-transformed into *E. coli* and *Agrobacterium*. 
CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1 Plasmids

pBI121 supplied by Arabidopsis Biological Resource Center, Columbus and pUC19 (MBI Fermentas, U.S.A) were used in this study.

3.1.2 Bacterial cell

The *Escherichia coli* strain JM109 (Promega, U.S.A) were used as host cell for propagation of plasmids.

3.2 Plasmid recovery of pBI121

3.2.1 Preparation and maintenance of *E. coli* strain JM109 cultures carrying pBI121

*E. coli* strain JM109 from stock collection (CD3-388) was streaked on Luria-Bertani (LB) agar with 20 μl of kanamycin (50 μg/ml) and LB agar without kanamycin as negative control. The inoculated plates were then incubated at 37°C for overnight.

3.2.2 Large scale isolation of pBI121 from overnight *E. coli* strain JM109 culture.

Large scale preparations of pBI121 were carried out manually for restriction analysis. Half of a bacterial colony was transferred into 100 ml of Luria-Bertani (LB) broth containing 100 μl of
kanamycin (50 µg/ml) in a 250-ml conical flask. The cultures were incubated overnight at 37°C with vigorous shaking (200 rpm) in the innova™ 4000 incubator shaker. 50 ml of the culture was transferred each into two 50-ml Falcon tube and centrifuged at 8,000 rpm for 5 minutes at room temperature in the rotor centrifuge (KUBOTA 8800). The LB broth was removed by decanting and recentrifuged at 8,000 rpm for 1 minute. Traces of liquid media were completely removed from the tube.

The bacterial pellet was resuspended in 1 ml of ice-cold Solution I (Appendix A) by vortexing briefly for 10 seconds and kept on ice. 1 ml of freshly prepared Solution II (Appendix A) was added and the mixture was mixed gently by inverting the tube ten times. The tube was left at room temperature and the lysis reaction was allowed to occur for 5 minutes without exceeding 5 minutes. 2 ml of Solution III (Appendix A) was added and mixed by inverting the tube ten times. The mixture was centrifuged at 8,000 rpm for 5 minutes to pellet the precipitate. The supernatant was transferred to a new Falcon tube. Two volume of cold absolute ethanol was added to precipitate the DNA and the content was mixed gently by inverting the tube at least ten times. The DNA was pelleted by centrifuging at 8,000 rpm for 10 minutes at room temperature. The supernatant was discarded and washed with 1 ml of 70% ethanol, and recentrifuged at 8,000 rpm for 1 minute. The supernatant was discarded as much as possible and air-dried. The DNA pellet was resuspended in 35 µl of TE buffer. Four replicates of large-scale plasmid preparation were done and transferred into a 1.5 ml Eppendorf tube by pipetting.
3.2.3 Restriction enzyme analysis of pBI121

Several restriction enzymes were used for single and double digestions of pBI121, such as EcoRI, HindIII, BamHI, Ndel, Eco81I, SmaI, NotI and XbaI. Both single digestions and double digestions of pBI121 were performed in 10 µl reaction with 4 µl of pBI121 added. All double digestions were done by using Universal Tango™ Buffer (MBI Fermentas, New York). The reaction mixtures were incubated at 37°C overnight inside LABTECH® water bath. The enzyme activity was stopped by incubating at 65°C for 20 minutes. 5 µl of undigested and digested samples were loaded with 1 µl of 1X gel loading dye and electrophoresed at 80V for 2 hours in 0.8% agarose gel with ethidium bromide. The agarose gel was then visualized under U.V transilluminator and photographed by using Polaroid 667.

3.2.4 PCR for GUS gene of pBI121

Besides the restriction analysis performed on the isolated pBI121 to confirm the desired plasmid, the DNA samples were used as DNA template for amplification of GUS gene by using the forward and reverse primers (Table 3.1). The composition of PCR reaction mixture is shown in Table 3.2 while parameters used in the PCR is shown in Table 3.3. The PCR was carried out by using PCR thermocycler (Biometra). The annealing temperature, 59°C was referred from previous PCR work done by Mohamed Ali, 2005. Positive control (pSRN/pAGS which contains GUS gene) and negative control (without DNA samples) were included.