Faculty of Resource Science and Technology

Construction of an Inducible Green Fluorescent Protein (GFP) Gene in a Binary Vector

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DECLARATION

I, Noor Adzlina Binti Abidin, hereby declare that this final year project is carried out by me. I had put 80% effort in completing this final year project and report writing, with 10% contributions were from my supervisor who assists me to check and correct my experiment and report writing. Whereas, the other remaining 10% was contributed by the postgraduate students and my course mates who had help me throughout this project.

No portion of this work referred to in 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.

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LIST OF ABBREVIATIONS

bp	base pair
$CaCl_2$	Calcium chloride
DNA	Deoxyribonucleic acid
GFP	Green Fluorescent Protein
GUS	Glucuronidase
kb	kilo base pair
LA	Luria Agar
LB	Luria broth
mg/ml	milligram/milliliter
ml	milliliter
mM	millimolar
OD	Optical density
PCR	Polymerase Chain Reaction
rpm	rotation per minute
sGFP	synthetic Green Fluorescent Protein
Ti	Tumor inducing
UV	ultraviolet
µl	microlitre
°C	degree Celcius

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Construction of an Inducible Green Fluorescent Protein (GFP) Gene in a Binary Vector

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ABSTRACT

Green fluorescent protein (GFP) gene is synthesized by jellyfish *Aequorea victoria* and it is widely used as reporter gene in molecular biology studies. The discovery of this reporter gene contributes to the development in the gene expression, cell development and signal transduction studies. It also leads to the discovery of novel characteristics in a particular organism. The construction of inducible synthetic GFP into a binary vector was carried out by using the restriction enzyme digestion of the DNA fragments and followed by the ligation of the DNA fragments by DNA ligase. Both mechanisms were carried out *in vitro*. The binary vector was used in this research because it facilitates the transfer of the gene into a target organism via *Agrobacterium*- mediated transformation. Meanwhile, for the verification steps, the Polymerase Chain Reaction (PCR) was used to identify the integration of synthetic GFP gene in the binary vector. There were many *Escherichia coli* XL1 Blue colonies grew on the Luria Agar (LA) media containing antibiotic ampicillin. However, the PCR cannot verify the presence of sGFP gene in the pAGS3/sGFP plasmids harbored by the *E. coli* XL1 Blue colonies. The result indicated that there was an absence of pAGS3/sGFP plasmid in the transformed *E. coli* XL1 Blue.

Keywords: green fluorescent protein gene, binary vector, restriction enzyme digestion, *in vitro, Escherichia coli*

ABSTRAK

Gen green fluorescent protein (GFP) dihasilkan oleh ubur- ubur daripada spesies Aequorea victoria dan digunakan sebagai gen reporter dalam kajian biologi molecular. Penemuan gen reporter ini menyumbang kepada perkembangan kajian pengekspresan gen, perkembangan sel dan transduksi signal. Selain itu, gen reporter juga mendorong kepada penemuan ciri-ciri dan potensi baru organisma. Konstruksi GFP sintetik ke dalam vektor biner dilakukan menggunakan teknik sekatan pencernaan enzim ke atas segmen DNA dan diikuti oleh pencantuman segmen DNA oleh DNA ligase. Kedua- dua mekanisma ini dilakukan secara in vitro. Vektor biner digunakan dalam kajian ini kerana vektor tersebut membantu pemindahan gen ke dalam organisma sasaran melalui transformasi menggunakan Agrobacterium. Manakala, analisis PCR telah digunakan untuk mengesahkan pencantuman gen GFP sintetik dalam vektor biner. Terdapat banyak koloni Escherichia coli XL1 Blue tumbuh pada permukaan media Luria Agar (LA) yang mengandungi antibiotik ampisilin. Walau bagaimanapun, PCR tidak boleh mengesahkan kehadiran gen sGFP dalam plasmid pAGS3/sGFP yang dibawa oleh koloni E. coli XL1 Blue. Keputusan ini menunjukkan ketiadaan plasmid pAGS3/sGFP dalam E. coli XL1 Blue yang telah ditransformasi.

Kata kunci: gen green fluorescent protein, vektor biner, sekatan pencernaan enzim, in vitro, Escherichia coli

CHAPTER 1

INTRODUCTION

Genetic engineering is a study area that focused on the genetic transformation or scientific modification of the structure of the genetic materials (Madigan *et al.,* 1997). The modern genetic engineering era had been started in 1953 since James Watson and Francis Crick discovered the three-dimensional double helix structure of DNA and the discovery of the restriction endonucleases in *Escherichia coli* (*E. coli*) by Stewart Linn and Erner Arber (Weaver, 1999). Genetic engineering usually involved the alteration in the nucleotide sequences by using the recombinant DNA techniques. Recombinant DNA resulted from the insertion of DNA fragment of an organism into the DNA of the other organism. Genetic transformation is a vital tool that allows better understanding on the metabolism and regulation of gene expression in an organism (Prabha & Punekar, 2004). It also enables the study of the specific function of a particular gene.

The discovery of the green fluorescent gene from jellyfish *Aequorea victoria* enhanced the development of molecular biology especially in the gene expression, cell development and signal transduction studies as it is one of the important tools in genetic engineering. GFP gene is a reporter gene and it has many advantages compared to another reporter gene. According to Reman *et al.* (1999), the detection of GFP gene can be carried out with a non- destructive manner since the GFP gene expression can be seen directly. Meanwhile, detection of other reporter gene usually involves the destruction of tissues of the target organisms. Furthermore, Reman *et al.* (1999) also stated that it is a useful reporter gene because it does not involves any substrate or cofactors. The discovery of this reporter gene leads to the establishment of

novel characteristics in a particular organism and expand its application in terms of increasing the yield and quality of products.

The construction of inducible synthetic green fluorescent gene in a binary vector is carried out *in vitro*. It involves the restriction enzyme digestion and followed by the ligation by DNA ligase (Chino *et al.*, 2010). Two DNA fragments which have been digested with the same restriction enzyme and have the complimentary of bases in the protruding end of the fragment can be ligated together by connecting a terminal 5' phosphate to a terminal 3' hydroxyl group. This reaction is carried out by DNA ligase (Jain, 1999). The construction of inducible synthetic green fluorescent gene in a binary vector enables the gene to be transferred into the target organisms through the *Agrobacterium*- mediated transformation (Komari *et al.*, 2006).

Plasmid construction also can be carried out by using *in vivo* cloning method. *In vivo* cloning also known as gap repair or gap- filling. The concept of this method is filling the gapped plasmids by using homologous sequences as the templates (Kitazono, 2009). Only short tracts, approximately 20 bp of flanking homology are required to carry out the effective gap- filling cloning in budding yeast (Matsuo *et al.*, 2010). According to Matsuo *et al.* (2010), the DNA fragments with the flanking homology with the target plasmid can be cloned directly into a linearized plasmid without the need of *in vitro* DNA ligation.

The main objective of this study is to construct the inducible synthetic green fluorescent protein gene in a binary vector.

CHAPTER 2

LITERATURE REVIEW

2.1 Inducible synthetic Green Fluorescent Protein (sGFP) Gene

Green fluorescent protein (GFP) is synthesized from jellyfish *Aequorea victoria*. Synthetic green fluorescent protein with a S65T has been engineered and according to Niwa (2003), the sGFP has the ability to produce up to 100- fold brighter fluorescent signals compared to the wild- type jellyfish GFP. It has been widely used as reporter gene in the studies of gene regulation, signal transduction, cell biology and development of variety organisms and *Caenorhabditus elegans* was the first model organism that has been transformed with GFP (Khafagy *et al.*, 2008).

The discovery of GFP gene leads to the development of biotechnology especially in the establishment of novel characteristics in a particular organism. One of the organisms that have been widely studied through the application of GFP gene is *Aspergillus* species. The fungi species is very well known for their importance in the industries (traditional production of wine, beer and cheese), medical (production of secondary metabolites such as antibiotic) and production of industrial enzymes (proteases and amylases) (Fleibner & Dersch, 2010).

In addition, *Aspergillus* sp. also has inducible promoter Alcohol dehydrogenase gene *alcA* in their expression system (Roslan *et al.*, 2001). Therefore, the expression system can be induced by the presence of acetaldehyde, an intermediate derived from ethanol and subsequently increases the yield and the quality of the protein produced (Fleibner & Dersch, 2010). According to the Prabha and Punekar (2004), the inducible promoters also enable a

particular gene being expressed under conditions that they are not normally expressed. The GFP gene facilitates the studies of gene expression in the *Aspergillus* sp.

2.2 Binary Vector

Binary vector is a vector system that contains two separate replicons which refer to the replicon carrying the virulence gene and replicon containing T- DNA (Komari *et al.*, 2006). Binary vectors also can be identified as the small plasmid which consists of a cloning site and a selectable marker gene in between its left and right border (Kiyokawa *et al.*, 2009). Komari *et al.* (2006) also stated that binary vector is the standard tool used to transfer foreign DNA in the *Agrobacterium tumefaciens*- mediated transformation.

Agrobacterium tumefaciens is a gram negative plant pathogen and the bacteria also have the Ti (tumor inducing) plasmid which causes the formation of crown galls once it infected a particular plant (Madigan *et al.*, 1997). In the transformation, the foreign DNA fragment is inserted into the Ti plasmid containing T- DNA (Weaver, 1999). The T-DNA is transferred to the infected plant and integrated into the plant genetic materials together with the foreign DNA (Weaver, 1999).

The components of the binary vector consist of T-DNA region, antibiotic resistance marker, origin of replication and virulence area (facilitate the transfer of T-DNA) (Madigan *et al.*, 1997). The cloned DNA in the vector is firstly inserted into *E. coli* before it is transformed into the target organisms such as *Aspergillus nidulans* and *Arabidopsis thaliana* (Madigan *et al.*, 1997). Some of the common binary vectors used are pGPTV, pGreen and pBIN (Komari *et al.*, 2006).

2.3 Plasmid construction

Plasmid construction is one of the molecular biology techniques that widely used in biotechnology. The plasmid construction allows the cloning of a foreign gene into a plasmid by using restriction enzyme and DNA ligase (Matsuo *et al.*, 2010). Usually the plasmid construction is carried out *in vitro* and involves the digestion of a particular DNA fragment with a specific restriction enzyme and followed by the ligation which is done by DNA ligase (Chino *et al.*, 2010). The constructed plasmid was transformed into *Escherichia coli* in order to allow the amplification of the plasmid.

Restriction enzyme is one of the important components in the plasmid construction. The first restriction enzyme was discovered in bacterium *Haemophilus influenzae* by Hamilton Smith in 1970 (Jain, 1999). The function of restriction enzymes is to cleave the DNA duplex molecule at a particular specific site. Jain (1999) stated that restriction enzyme possess four characteristics which include strain- specific, the restriction enzyme activity can be subjected to other strain of bacteria, the restriction is not absolute which means the DNA molecules might not be cleave by the restriction enzyme under some circumstances and the gene encoded for the restriction enzyme can be carried on the bacterial chromosomes, plasmid or phage DNA. The restriction enzyme digestion might results in either blunt end (cleavage of the two chains occur at the same point) or sticky end sequences (cleavage of the two chains occur at different points) (Lamb, 2000).

After the restriction enzyme digestion, the ligation reaction carried out by DNA ligases take place. The function of DNA ligases is to join two ends of DNA by connecting a terminal 5’ phosphate to a terminal 3’ hydroxyl group. Therefore, two fragmented DNA molecules are readily join by the DNA ligase through the complementary of bases in their protruding ends

when they are mixed together. The DNA ligase is also able to replace the missing covalent bonds between the ligated DNA fragments (Lamb, 2000).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Stock of plasmids

pMCB30 plasmid, pAGS3 plasmid and pGPTV-SRN4/AGS plasmid were given by Associate Professor Dr. Hairul Azman Roslan from Universiti Malaysia Sarawak (UNIMAS).

3.2 Methods

3.2.1 Culture of *Escherichia coli* XL1 Blue

E. coli XL1 Blue was cultured in Luria Bertani (LB) culture medium through the inoculation of 500 µl of *E. coli* XL1 Blue into 20 ml LB broth in the Falcon tube. Then, *E. coli* XL1 Blue was incubated in the incubator shaker overnight at 37°C (250 rpm).

3.2.2 Preparation of *E. coli* Competent Cells

Calcium chloride competent cells method described by Sambrook *et al.* (1989) with some modification was used to prepare the competent cells of *E. coli* XL1 Blue. A volume of 100 µl from *E. coli* XL1 Blue overnight culture was transferred into 10 ml of LB broth without antibiotic. Then, it is allowed to grow at 37°C (250 rpm shaking) for about 1 hour 20 minutes until the OD_{600} reached the range approximately 0.45 to 0.5. After the culture reached the OD_{600} 0.45 to 0.5, the cells were allowed to cool on ice for 20 minutes and followed by the centrifugation at 3500 rpm, 4°C for 5 minutes. The supernatant was discarded and the cells were washed in 25 ml ice cold 100 mM $CaCl_2$. The cells were put in ice for 10 minutes and

centrifuged again. After the centrifugation, the supernatant was discarded to resuspend the cell pellet in 2.5 ml of cold and sterile 100 mM $CaCl_2$. Then, 200 µl of 20% glycerol and 800 µl of 80% $CaCl_2$ solution were added to the cell suspension. Finally, 100 µl aliquots were transferred into 1.5 ml centrifuge tubes and stored at -80°C in freezer.

3.2.3 Construction of plasmid

3.2.3.1 Purification of synthetic green fluorescent protein (sGFP) gene

The purification of sGFP genes were carried out to isolate the gene from the pMCB30 plasmid before the genes were ligated into the vectors. There were two methods used in the purification of sGFP genes which were amplification through Polymerase Chain Reaction and isolation by using restriction enzyme digestion.

3.2.3.1.1 Amplification of sGFP gene by using Polymerase Chain Reaction

PCR was carried out to amplify the sGFP gene in the pMCB30 plasmid. A set of primer was used which were sGFP forward (5' CC ATG GTG AGC AAG GGC GAG 3') with sGFP reverse (5' TTA CTT GTA CAG CTC GTC CAT 3'). The composition of the reaction mixture and the parameter of the PCR are shown in Table 3.1 and Table 3.2, respectively.

Table 3.1: Composition of PCR mixture

Components	Volumes (µl)
DNAenzyme mastermix	12
Double distilled water	5
Plasmid	1
sGFP forward primer	1
sGFP reverse primer	1
Total	20

Table 3.2: Parameters of the PCR

Steps		Temperature (°C)	Time
Step 1	Initial denaturation	94	3 min
Step 2	Denaturation	94	1 min
Step 3	Annealing	60	30 sec
Step 4	Extension	72	30 sec
Step 5	Final extension	72	5 min
Step 6	Incubation	20	1 min

The reaction mixture of the PCR was separated on 1% agarose gel, run at 100 Volt for 25 minutes. The agarose gel was then observed under UV light and the desired sGFP gene fragments were cut for gel extraction. The Gel Recovery DNA kit was used to purify the DNA from the extracted gel.

3.2.3.1.2 Restriction enzyme digestion of pMCB30 plasmids

The restriction enzyme digestion was carried out on the pMCB30 plasmids to isolate the sGFPS65T gene fragment. Restriction enzyme *Sal*I and *Bam*HI were used to cut the plasmid at the 5' end and 3' end, respectively. The restriction enzyme digestion mixture was prepared and incubated overnight at 37°C. The composition of the mixture is shown in Table 3.3. The reaction of the restriction enzyme digestion was deactivated by heating at 65°C for 10 minutes.

Table 3.3: Composition of restriction enzyme digestion for pMCB30 plasmid

Components	**Volumes (µl)**
Plasmids	16
Restriction enzyme *Sal*I	1
Restriction enzyme *Bam*HI	1
Buffer Tango	2
Total	20

The reaction mixture of the restriction enzyme digestion was separated on 1% agarose gel run at 100 Volt for 25 minutes. The agarose gel was then observed under UV light and the desired sGFP gene fragments were cut for gel extraction. The Gel Recovery DNA kit was used to purify the DNA from the extracted gel.

3.2.3.2 Preparation of plasmid vectors

The restriction enzyme digestion was carried out on the pAGS3 and pGPTV plasmids to isolate GUS gene fragment from both plasmids. Restriction enzymes *Sal*I and *Bam*HI were used to cut GUS gene fragment of pAGS3 plasmids and produced sticky end pAGS3 plasmid vector. Blunt end pAGS3 plasmid vector also has been prepared by using the restriction enzyme *Sma*I. The composition of the restriction enzyme digestion mixtures are shown in Table 3.4 and Table 3.5, respectively.

Table 3.4: Composition of restriction enzyme digestion for the preparation of sticky end pAGS3 plasmid vectors

Components	Volumes (µl)
pAGS3 plasmid	16
Restriction enzyme *Sal*I	1
Restriction enzyme *Bam*HI	1
Buffer Tango	2
Total	20

Table 3.5: Composition of restriction enzyme digestion for the preparation of blunt end pAGS3 plasmid vectors

Components	Volumes (µl)
pAGS3 plasmid	16
Restriction enzyme *Sma*I	2
Buffer Tango	2
Total	20

Meanwhile, the pGPTV plasmid was cut by using the restriction enzyme *Hind*III. The composition of the restriction enzyme digestion mixture was shown in Table 3.6. The restriction enzyme digestion mixture was prepared and incubated overnight at 37°C. The reaction of the restriction enzyme digestion was deactivated by heating at 65°C for 10 minutes. The reaction mixture of the restriction enzyme digestion was separated on 1% agarose gel, run at 100 Volt for 25 minutes. The agarose gel was then observed under UV light and the desired gene fragments (plasmid vector) were cut for gel extraction. The Gel Recovery DNA kit was used to purify the DNA from the extracted gel.

Table 3.6: Composition of restriction enzyme digestion for pGPTV plasmids

Components	Volumes (µl)
pGPTV plasmid	16
Restriction enzyme *Hind*III	2
Buffer R	2
Total	20

3.2.3.3 Ligation of sGFP gene into pAGS3 plasmid vectors

3.2.3.3.1 Blunt end ligation of sGFP gene into pAGS3 plasmid vector

The end of the purified sGFP genes that were obtained through the PCR amplification in previous protocol (3.2.3.1.1) was blunt ended before the genes were ligated into the blunt end pAGS3 plasmid vectors (obtained from protocol 3.2.3.2). DNA blunting reaction mixture was prepared and incubated at room temperature for 3 hours. The DNA blunting reaction was deactived by heating at 75°C for 10 minutes. Meanwhile, the reaction mixture for the blunt end ligation was incubated overnight at 4°C. The composition for the DNA blunting and blunt end ligation reaction mixtures are shown in Table 3.7 and Table 3.8, respectively.

Table 3.7: Composition of reaction mixture for DNA blunting

Components	Volumes ((µl)
5× reaction buffer for T4 DNA polymerase	2
PCR product (sGFP gene)	5
dNTP mix, (2 mM each)	1
T4 DNA polymerase	1
Nuclease free water	1
Total	10

Table 3.8: Composition of reaction mixture for blunt end ligation

Components	Volumes (µl)
pAGS3 vector	5
sGFP gene	10
10× T4 DNA ligase buffer	2
50% PEG 4000 solution	2
T4 DNA ligase	1
Total	20

3.2.3.3.2 Sticky end ligation of sGFP gene into pAGS3 plasmid vector

The purified sGFP genes that have been isolated from pMCB30 by using restriction enzyme digestion in previous protocol (3.2.3.1.2) were ligated into the sticky end pAGS3 plasmid vectors that were obtained in previous protocol (3.2.3.2). The reaction mixture for the sticky end ligation was prepared and incubated overnight at 4°C. The composition for the reaction mixture is shown in Table 3.9.

Table 3.9: Composition of the reaction mixture for the sticky end ligation

Components	Volumes (µl)
pAGS3 vector	5
sGFP gene	10
10× T4 DNA ligase buffer	2
T4 DNA ligase	2
Nuclease free water	1
Total	20

3.2.3.4 Transformation of pAGS3/sGFP plasmid into competent *E. coli*

The successful integration of sGFP gene into the pAGS3 plasmid vectors had produced pAGS3/sGFP plasmids. The heat shock method described by Irma *et al.* (1983) with some modifications was used to transform the pAGS3/sGFP plasmid into competent *E. coli***.** Firstly, the competent cells were thawed on ice for 10 minutes. Then, 10 µl of DNA plasmid were added into 50 µl of the competent cells in a 1.5 ml centrifuge tube and incubated on ice for 10 minutes. After 10 minutes incubation on ice, the tube was kept in a water bath that had been set to 42°C for 2 minutes for heat shock and immediately transferred into ice for another 10 minutes. Next, the mixture was transferred into 1 ml of pre-warmed LB broth. Finally, the t ube was incubated in the incubator shaker for 1 hour at 37°C. 250 µl of the *E. coli* culture carrying pAGS3/sGFP plasmids was plated out on LA media containing 100 mg/ml concentration of ampicilin. Then, the inoculated plates were incubated at 37°C overnight.

3.2.3.5 Analysis of transformed *E. coli*

3.2.3.5.1 Colony PCR

E. coli colonies that grew on the selection media were analyzed by using colony PCR and restriction enzyme digestion. The colony PCR was carried out to confirm the *E. coli* that grown on the selection media was harboring the pAGS3/sGFP plasmids. Firstly, half of the colony was picked and put into 60 µl of double distilled water in a centrifuge tube. The tube was vortex and followed by the centrifugation at 6000 rpm for 5 minutes. Then, the supernatant was collected. After that, the reaction mixture of the Colony PCR was prepared. The composition of the Colony PCR mixture and parameter of the Colony PCR are shown in Table 4.0 and Table 4.1.