EXPRESSION OF ANTI-*TOXOPLASMA* SCFV ANTIBODIES IN PLANTS

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ABSTRACT

Toxoplasmosis is a disease caused by a single-celled protozoan parasite known as *Toxoplasma gondii*. It infects approximately one-third of the world's population. The definitive hosts of this obligate intercellular parasite are feline family members; but it is widespread in human and many warm-blooded animals, particularly farm animals. The disease causes critical symptoms in infants, pregnant women, and fetuses and there is still an urgent need new more effective therapeutic, diagnostic and bioimaging compounds as well as affordable production methods amenable to upscaling. A plant-based recombinant antibody against *Toxoplasma* antigen may offer a potential approach in developing strategies to produce such molecules. The antibody was designed to specifically target surface protein of the *T.godii* tachyzoites can be further developed as targeting antibodies or diagnostic reagents against the organism. Thus, in this study, anti-*Toxoplasma* scFv antibody genes were introduced into the pCambia1304 vector and transformed into *Musa acuminata* cv. Berangan and *Nicotiana tabacum* L. cv. SR1 leaf discs through *Agrobacterium*-mediated transformation. In addition, a KDEL retention sequence and Bowman-birk Serine proteinase inhibitor genes were added into the construct to stabilize and enhance the scFv antibody production. The transgene was successfully integrated into the tobacco genome. Unfortunately, the desired gene failed to integrate into banana genome despite many attempts and the possible constrains are discussed. Molecular analysis including PCR and Real-time PCR were used to confirm the integration of transgene into the plant genome. From Real-time PCR results, the average mRNA level for the leaves transformed with the construct pToxo130BBI based on the RT-qPCR of three transgenes elements was 4.4 fold when compared to non-transformed tobacco whereas for the construct pToxo130BBIKDEL, it was 3.58 fold higher. The unexpected lower level of mRNA for pToxo130BBIKDEL compared to

pToxo130BBI (0.82 fold) in Real-time PCR may not reflect the actual protein accumulation level in plant cell. Further studies need to be carried out to examine the effectiveness of adding KDEL retention sequence and Bowman-birk Serine proteinase inhibitor genes in the constructs through quantitative analysis of the tobacco-expressed protein. Overall, the study proposes a potentially useful expression platform for production of anti-*Toxoplasma* scFv antibodies.

ABSTRAK

Toxoplasmosis adalah penyakit yang disebabkan oleh parasit protozoa yang bersel tunggal, iaitu *Toxoplasma gondii*. Ia menjangkiti kira-kira satu pertiga daripada populasi dunia. Perumah definitif untuk parasit ini adalah ahli keluarga kucing tetapi ia telah berleluasa di kalangan manusia dan haiwan yang berdarah panas, terutamanya haiwan ternakan. Penyakit ini menyebabkan gejala-gejala kritikal dalam bayi, wanita yang berhamil, dan janin. Oleh itu, therapeutik yang berkesan, diagnostik dan "bioimaging compound"serta kaedah penghasilan yang berpatutan dan berskala besar adalah diperlukan segera. Antibodi rekombinan terhadap antigen *Toxoplasma* yang berasaskan tumbuhan berkemungkinan besar boleh menawarkan satu pendekatan yang berpotensi dalam membangunkan strategi untuk menghasilkan molekul tersebut. Antibodi yang direka khususnya untuk menentang protein di permukaan tachyzoites *T.gondii* boleh diubahsuaikan sebagai antibodi atau reagen diagnostik yang menentang organisma tersebut. Oleh itu, dalam kajian ini, gen antibodi scFv terhadap antigen *Toxoplasma* telah diperkenalkan ke dalam vektor pCambia 1304 dan ditransformasi ke dalam *Musa acuminata* cv. Berangan dan daun *Nicotiana tabacum* L. cv. SR1 dengan menggunakan *Agrobacterium*. Di samping itu, "KDEL retention sequence" dan "Bowman-birk Serine proteinase inhibitor gene" telah ditambah ke dalam konstruk untuk menstabilkan dan meningkatkan penghasilan antibodi scFv. Transgen tersebut telah berjaya diintegrasikan ke dalam genom tembakau, tetapi, gagal untuk diintegrasikan ke dalam genom pisang selepas beberapa kali pencubaan. Analisis molekul seperti PCR dan Real-time PCR telah digunakan untuk mengesahkan integrasi transgen ke dalam genom tumbuhan. Berdasarkan keputusan Real-time PCR, tahap purata mRNA bagi tembakau yang ditransformasi dengan pToxo130BBI konstruk adalah 4.4 kali ganda berbanding tembakau yang tidak-ditransformasi; manakala bagi

pToxo130BBIKDEL konstruk, ia hanya 3.58 kali ganda lebih tinggi. Tahap mRNA adalah di luar jangkaan iaitu, pToxo130-BBI-KDEL lebih rendah berbanding pToxo130BBI (0.82 kali ganda).Walau bagaimanapun, keputusan Real-time PCR tidak mewakili tahap penghasilan protein yang sebenar di dalam sel tumbuhan. Kajian yang selanjutnya perlu dijalankan untuk mengkaji keberkesanan penambahan "KDEL retention sequence" dan "Bowman-birk Serine proteinase inhibitor gene" dalam konstruk melalui analisis kuantitatif terhadap tembakau-ekspres protein. Secara keseluruhannya, kajian ini mencadangkan platform yang berpotensi untuk menghasilkan antibodi scFv terhadap *Toxoplasma*.

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The fulfillment of this thesis was derived with these few people helps.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	percent
µg	microgram
µl	microliter
µM	micromolar
6-BA	6-benzylaminopurine
BBI	Bowman birk Serine proteinase inhibitor
bp	base pair
BSA	bovine serum albumin
cDNA	complimentary deoxyribonucleic acid
Ct	threshold cycle
cv	cultivar
DEPC	diethlypyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease acid I
dNTP	deoxyribonucleoside triphosphates
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
g	gram
H_2O	water
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-y] ethanesulfonic acid
$K_3[Fe(CN)_6]$	potassium ferricyanide
$K_4[Fe(CN)_6]$	potassium ferrocyanide

kb	kilo base pair
KOAc	potassium acetate
l	liter
LB	Luria-Bertani
M	molar
mg	milligram
$MgCl_2$	magnesium chloride
ml	milliliter
mM	milimolar
$MnCl_2$	manganese (II) chloride
MOPS	3-morpholinopropane-1-sulfonic acids
mRNA	messenger ribonucleic acid
MS	Murashige & Skoog
MW	molecular weight
Na_2HPO_4	disodium hydrogen phosphate
NAA	1-naphthylacetic acid
NaH_2PO_4	sodium di hydrogen phosphate
NaOH	sodium hydroxide
$NaPO_4$	sodium phosphate
NCBI	National Center for Biotechnology Information
nm	nanometer
^oC	degree Celsius
PCR	polymerase chain reaction
PMP	plant-made protein
psi	pounds per square inch
rAb	recombinant antibody

$RbCl_2$	rubidium chloride
RE	restriction endonuclease
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	reverse transcriptase
RT-PCR	Reverse-transcriptase polymerase chain reaction
RT-qPCR	Real-time polymerase chain reaction or quantitative polymerase chain reaction
scFv	single chain variable fragment
SDS	sodium dodecyl sulphate
ssRNA	single stranded ribonucleic acid
TMV	Tobacco mosaic virus
TBE	Tris-Borate-EDTA
Tris	tris (hydroxyl methyl) amino methane
U	unit
UV	ultraviolet
v	volt
v/v	volume per volume
w/v	weight per volume

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 General introduction

Toxoplasmosis gondii is an obligate intracellular parasite that is estimated to infect approximately one-third of the world's population. This one-celled parasite was first discovered by Nicolle and Manceaux in 1908 and named a year later (Dubey, 2008). It is classified under the phylum of *Apicomplexa*, subclass *Coccidia*.

The parasites are reproduced in 2 different ways, through sexual and asexual cycles. The sexual cycle occurs in the gut of the cat family both wild and domesticated as its natural hosts. The cat can shed the oocysts for 7 to 12 days. After the sporulation, the oocyst becomes infectious to other animals if ingested. The oocyst can survive in the environment for approximately one year. For the asexual cycle, almost all animals which are intermediate host to this parasite can produce the parasite either in the tachyzoite form or cycts (which are transformed from the tachyzoites). Both tachyzoites and cycsts are also infectious.

The disease is acute and chronic. Currently there are no effective cures for toxoplasmosis. The treatments used are sulfonamides, or a combination therapy of sulfonamide and pyrimethamine, spiramycin and clindamycin. However, all these drug treatments are needed for the entire life, and as yet no effective vaccine against toxoplasmosis has been produced. Another alternative treatment for controlling the disease is using antibody. An effective antibody can prevent the progression of the disease to critical condition. In this study, an anti-*Toxoplasma* scFv was transformed into plant genome for producing antibody against the protozoa.

The plant systems were chosen as bio-factories since they have many advantages compared to mammalian cell cultures. For instance, the mammalian cell cultures that are commonly used in producing antibody have a high risk of human pathogens

contamination. There are two different plant hosts used in this study, i.e. banana cell suspension and tobacco. The tobacco was used as a model (reference) plant because the study on plant made protein (PMP) in tobacco is well established. Additionally, there are several biopharmaceutical products that are produced from tobacco that have been commercialized. For example, the production of antibody against Hepatitis B by the Center for Genetic Engineering and Biotechnology (CIGB), Cuba (Mola et al., 2006).

In this study, the *Agrobacterium*-mediated transformation is used as a method for transforming the transgene into the plant genome because of its single copy gene transformation method and its ability to transfect the plant. Moreover, the insertion of the transgene is more stable compared to particle bombardment method.

1.2 Objectives

The objectives of this study are;

i. To design gene expression cassettes based on a plant expression constructs and pre-cloned genes of a scFv anti-*Toxoplasma* antibody as the gene of interest.

ii. To carry out gene transfer experiments with these cassettes into somatic cell embryos of *Musa acuminata* cv. Berangan and *Nicotiana tabacum* L. cv. SR1 leaf disc.

iii. To determine the levels of expression of the foreign gene in the plants using constructs containing different enhancer elements.

CHAPTER 2: LITERATURE REVIEW

2.1 Toxoplasmosis

Toxoplasmosis is a disease caused by *Toxoplasma gondii*, which is a species of parasitic protozoa in the genus of *Toxoplasma*. This disease is widespread in humans and warm-blooded animals, especially farm animals. Members of the Felidae family are the definitive hosts of the *Toxoplasma gondii*, because they are the only animal that can harbour the adults parasites in their intestinal tract. The sexual life cycle of *Toxoplasma gondii* occurs in the cat family, while the asexual life cycle can occur in any other warm-blooded animal.

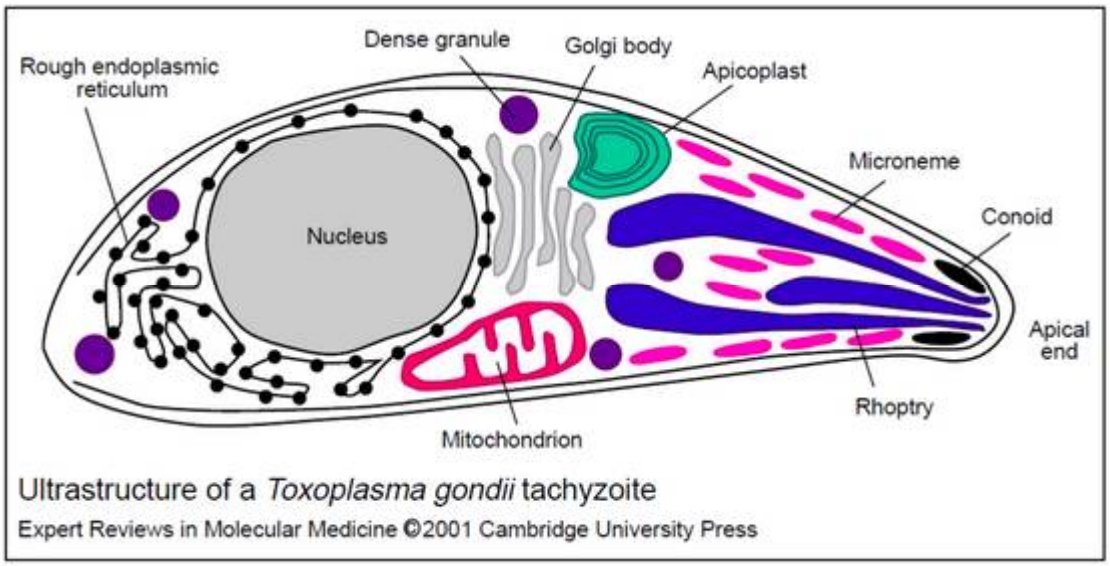


Figure 2.1: Ultrastructure of a *Toxoplasma gondii* tachyzoite (Ajioka et al., 2001).

2.1.1 *Toxoplasma gondii* strains

There are three difference strains of this pathogen (type I, II and III), which are classified into two groups, virulent and non-virulent. Only type I strain is the highly pathogenic; while type II and type III strains were grouped in the non-virulent group. As summarized from Maubon et al. in 2008, type I was rarely been isolated but highly

virulent. The multiplication rate is high but low in interconversion tachyzoite-bradyzoite. The most commonly been isolated strain in Europe and North America is type II which is non-virulent with slow rate of multiplication but easier interconversion tachyzoite-bradyzoite and formation of cysts. While type III is less frequent been isolated if compare to type II.

Among these three major strains, the type I strain RH is the first human strain which is only found in severe cases of human toxoplasmosis. The first isolated RH strain was by Albert Sabin in USA from a six year old boy who died of toxoplasmosis encephalitis in 1939. The RH strain is highly virulent and caused 100% lethality when tessted in laboratory mice (Ajzenberg, 2010).

2.1.2 Sources of infection

Infection occurs worldwide, but the prevalence is higher in warm and wet countries, such as South America, Africa and parts of Asia (Petersen, 2007). The high prevalence may also be related to hygiene standards and eating habits. Eating undercooked meat and drinking unpasteurized milk and untreated water will increase the exposure rate to the infection. Besides this, gardening and husbandry of domestic animals also contribute to infection chances, because oocysts of *Toxoplasma gondii* can survive in the moist shaded soil. Another source of the infection is through contact with the definitive host, i.e. cats. In many families, cats are kept as pets and this will increase the chances of exposure. The sources can be summarized in the Figure 2.2.

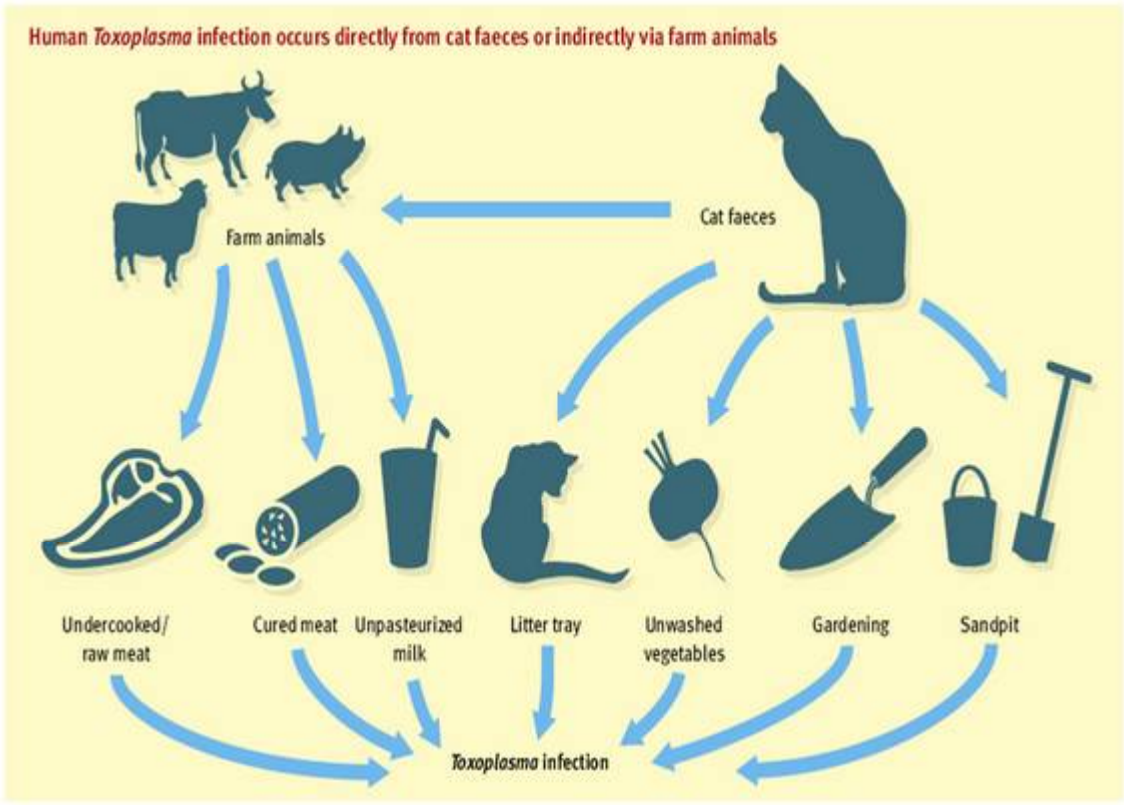


Figure 2.2: Sources of *Toxoplasma* infection (Ho-Yen, 2005).

2.1.3 Symptoms of toxoplasmosis

The symptoms of the infection include swollen lymph nodes, fever, vague ill feeling and sometimes eye pain, blurred vision and sore throat. However, some individuals may not show any symptom after infection if they have a healthy immune system.

The acute symptoms are usually shown by newborn babies, especially those born with congenital toxoplasmosis. They may exhibit inflammatory lesions that can lead to permanent neurological damage, chorioretinis which may lead to blindness, hydrocephalus, mental retardation and enlargement of the liver and spleen (Petersen, 2007).

2.14 Control of toxoplasmosis

There are several means that can be taken in preventing infection by toxoplasmosis, such as taking general hygiene precautions. As an example, avoid eating raw or undercooked meat or drinking unpasteurized milk, washing of hands after handling animal litter trays or gardening. Additionally, reducing contact with cats is also a preventive action, especially for pregnant women who are high risk (Ho-Yen, 2005). There are currently no effective vaccines for this disease. Treatment of toxoplasmosis consists of spiramycin, pyrimethamine and sulfadoxine (Elsheikha, 2008) all of which are associated with side-effects.

One potential approach for treating the disease post-infection is the use of specific therapeutic antibodies. This approach may present an effective way in controlling the spread of the disease. In particular it may be applied to carriers of the disease including animal carriers such as pet cats. Applications of scFvs as therapeutic agents include as reported by Yoshida et. al. in 2003, the bispecific single-chain antibody fragment (biscFv) was targeted to the surface protein of human malignant malaria parasite, *Plasmodium falciparum*, and activated the macrophages to kill the parasite. scFv antibodies were also used as diagnostic and therapeutic agents, i.e. immunosensors, molecular magnetic resonance imaging (MRI), near infrared fluorescence imaging (NIRF), anticoagulants, fibrinolytics and intrabodies (Hagemeyer et. al., 2009). In this study, an scFv gene coding for a recombinant phage derived anti-*Toxoplasma* antibody will be introduced into a plant expression system to potentially produce the antibody in plant cells in large volume.

2.2 Therapeutic proteins

There are two major categories of biotechnology-derived drugs, which are antibodies and vaccines. In the antibodies category, these include several forms

including monoclonal antibodies, single-chain variable fragments antibodies (scFv), and antigen-binding fragments (Fab) all of which are potentially candidates as therapeutic proteins.

Recombinant therapeutic proteins are the proteins that are synthesized in living organisms, such as bacterial, yeast, mammalian cell cultures and plants. Based on a data monitor report, recombinant therapeutics were estimated to contribute $53 billion to the market by 2010 (Datamonitor, 2004).

2.2.1 Monoclonal antibodies

Monoclonal antibodies were first studied by Cesar Milstein and Georges Kohler in 1975 from B cell hybridomas of mice and was a discovery that subsequently brought them the Nobel Prize in Medicine in 1984 (Milstein, 1985; Khöhler, 1985).

Monoclonal antibodies have been used as the antibody-based therapy agent for many diseases due to its monospecificity, homogeneity and consistency (Lipman et al., 2005). However, they are expensive and time consuming in production. Additionally, the risk of toxicity is high as they are produced in mammalian cell cultures which predispose it to potentially cause allergic-type reactions (Peterson et al., 2006).

Hence, several different forms of antibody fragments have been developed to overcome the limitations of monoclonal antibodies. The antibody fragments that have been studied are antigen-binding fragment (Fab), single-chain variable fragments (scFvs) and Third Generation (3G) molecules (Nelson, 2010). Monoclonal antibody types were shown in Figure 2.3.

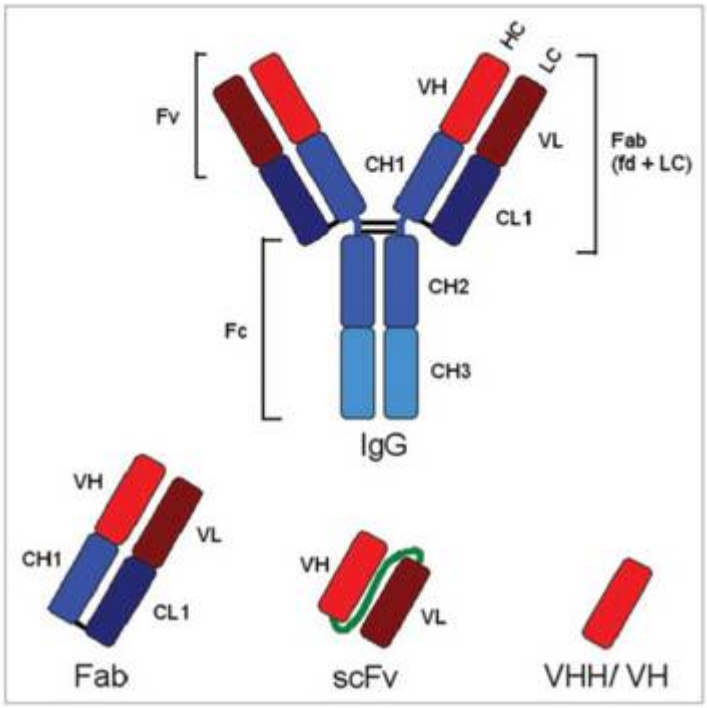


Figure 2.3: Antibody fragment types. Depiction of a full size antibody and various antibody fragment types. CH, constant heavy chain; CL, constant light chain; IgG, immunoglobulin; Fab, antigen-binding fragment; scFv, single chain variable fragment, VH, variable heavy chain; VL, variable light chain. (Nelson, 2010).

2.2.2 scFv gene

Typically, antibodies are the immunoglobulin molecules that consist of two identical heavy and light chains that joined together by disulfide and non-covalent bond. One of the most well researched approaches is in the development of single chain variable fragment (scFv) antibodies. A scFv antibody consists of the variable regions of heavy and light chain of immunoglobulin, which are joined together by a flexible peptide linker. Therefore, the size of the molecule is smaller than the original immunoglobulin but it still retains the intact antigen binding site (Ahmad et. al., 2012). The scFv antibody with its small molecular size is an attractive candidate because of its ease in penetrating the tissue or tumor, its reduced immunogenicity, and also the ease of cloning it in bacteria for genetic engineering (Hagemeyer et. al., 2009; Ahmad et. al., 2012).

2.3 Plant molecular farming

Plant molecular farming refers to the recombinant proteins that are produced by genetically-modified plants for pharmaceutical and industrial uses (Breyer et al., 2009). Plant expression system plays important role in producing recombinant proteins and biopharmaceutical products as it offers several potential advantages.

First, the cost of producing and processing plant-based recombinant proteins is lower compared to the traditional expression system, such as bacterial expression system and mammalian cell cultures. Plants offer more economical platforms because the process of producing required inexpensive equipment, media or skilled personnel, unlike major fermentation-based system (Fischer et al., 2011). The processing technology is also more straightforward.

In addition, plant-based technology could promise safer products compared to mammalian cell cultures-based products. The PMP products are considered lower risk as there is minimal chances for human pathogen contamination arising from the host cell.

Furthermore, plants have the ability to perform post-translation modifications that are required for producing functional mammalian proteins (Stoger st al., 2002; Breyer et al., 2009). As an example, the proper folding and disulphide bond formation are occurred in endoplasmic reticulum (ER) and chloroplast (Daniell et al., 2009).

Using plants as expression platforms also offer the benefit of easy and rapid scaling up. The desired recombinant protein can be produced in large scale, once the transgenic plants that harbour the gene of interest have been developed (Commandeur et al., 2003).

2.4 Factors affecting protein expression in plants

Transgenic approaches are being widely studied compared to the traditional approaches as they present many advantages in terms of production times and costs as described in the previous sections. In addition, production of therapeutic proteins from the plants also more cost efficient and easy to store (Daniell et al., 2001; Ma et al., 2003).

However, there remain some limitations of using plants. One of the major concern is the lower yield of recombinant protein production which can be affected by the stability of recombinant proteins in plant system, the design of expression constructs or system, and the plant host species (Abolade and Ahmadu, 2012; Nagels et. al., 2012). There are several factors that cause the instability of recombinant proteins in the plant system, including the localization of transgene in plant system and the presence of protease naturally in plant system. For example, as reported by Loos et. al. in 2011, the stability and production yield of two monoclonal antibodies (2G12 and HA78) in the seeds of Arabidopsis wild type plants. The KDEL-tagging scFv-Fcs of 2G12 and HA78 were more stable in the plant system compare to the non-subcellular targeting (Loos et. al., 2011).

2.4.1 Strategies for preventing proteolysis or increase foreign protein accumulation

One of the factors that affect the maximum production of heterologous protein in plants is proteolytic activities by the plant defense system. Proteolysis is an important and necessary process in all living organisms, because only with the presence of this process, the damaged, misfolded and harmful proteins will be degraded and release the amino acids for other protein synthesis. Hence, proteolysis poses a significant challenge to recombinant proteins that are introduced into the cells, because the foreign protein

will be treated as harmful to the cells and has been degraded by the proteolytic enzymes. Thus, the yield of the recombinant protein will be decreased as well.

To overcome this challenge and establish or increase the accumulation of the viable transgene expression level; co-expression of a companion protease inhibitors, organelle targeting peptides and tissue targeting peptides have been proposed in recent studies (Benchabane et al., 2008; Deng et al., 2003).

2.4.2 Organelle targeting (ER targeting - KDEL)

There are a number of organelles which are involved in protein synthesis. Hence, organelle targeting is a promising approach for enhancing the yield of recombinant protein production in plants. The subcellular compartments which are involved are apoplast, chloroplast, vacuole, cytosol, and endoplasmic reticulum (ER).

Most of the recent transgenic approaches for expression of foreign proteins in plants have utilised the endoplasmic reticulum retention signal also known as the KDEL retention sequence in their constructs. The KDEL retention sequence is 21 base pairs long, which is 5'-TCTGAGAAAGATGAGCTCTAG-3'. This simple sequence will then target the transgene into the lumen of the endoplasmic reticulum (ER) in the plant cells. The lumen of the endoplasmic reticulum is the first destination of the cell secretory pathway. The protein is later directed to the Golgi apparatus, then transferred to the vacuoles and lastly to the cell surface (Okamoto et al., 2003). In this study, KDEL retention sequence is fused to the gene of interest so that the stability and the accumulation rate of the protein expression will be improved.

2.4.3 Co-expression with protease inhibitor (BBI)

Other than organelle targeting, co-expression with protease inhibitor is another alternative method to maximize the production of protein.

There are many protease inhibitors that have been found in plants. The mechanism of action of plant protease inhibitors still remains unclear. Based on Plant-PI database, plant protease inhibitor can be categorized into 10 families. The families consists of the Bowman-Birk serine proteinase inhibitors (BBI), Cereal trypsin/alfa amylase inhibitors, Cysteine proteinase inhibitors, Metallocarboxypeptidase inhibitors, Mustard trypsin inhibitors, Potato type I inhibitors, Potato type II proteinase inhibitors, Serpin, Soybean trypsin inhibitors(Kunitz) and Squash inhibitors (De Leo, 2002).

Different protease inhibitors have different functions in the plants systems. The endogenous proteinases are involved in the plant development and growth. Therefore, the protease inhibitor that was chosen for the study should not affect the plant growth. There are few studies which showed the successfully of increasing the protein level by co-expression with protease inhibitors. Rivard and co-workers have reported that the level of cathepsin D-like and ribulose 1,5-biphosphate carboxylase/oxygenase hydrolysing activities was decreased in the leaf crude protein of transgenic potato in the presence of either tomato cathepsin D inhibitor (CDI) or bovine aprotinin. Therefore, the recombinant protein that was recovered from the transgenic potato is higher compared to normal (control) potato (Rivard et al., 2006). Another research done by Komarnytsky et al. proved that the Bowman-Birk serine proteinase inhibitor (BBI) was able to stabilize the recombinant antibodies in tobacco roots. Therefore, in this study, we adapted the technique by using BBI to analyse its ability to stabilize the anti-*Toxoplasma* antibody in the banana cells and tobacco plants.

2.5 Plant expression vector

Plant expression vector plays the vital role in transforming the transgene into the plant host. Therefore, a plant expression vector must contain several fundamental features which includes promoter, origin of replication, multiple cloning sites, antibiotic

resistance marker and reporter gene (Slater et al., 2003). In this study, pCambia1304 was used as the backbone of the construct. The reporter genes of pCambia1304 are gusA and mgfp5 (as showed in Figure 2.4).

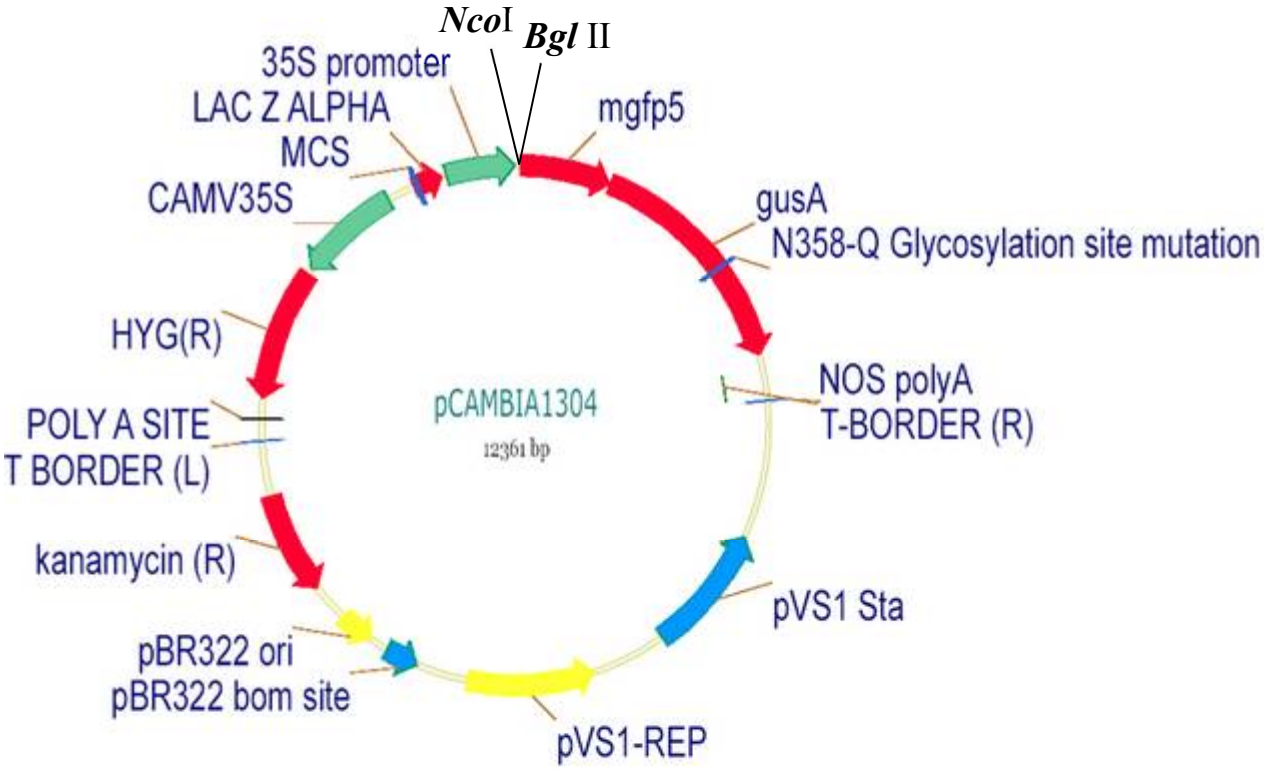


Figure 2.4: Map of pCambia1304 vector (obtained from www.cambia.org).

2.5.1 Reporter genes

Reporter genes or non-selectable marker genes in plant expression vector are the tools that are used for analyzing the host cells ability to take up the gene of interest. Reporter genes were used to improve transformation systems and the efficiency of recovering transgenic plants by allowing the visual detection of transformed tissues (Miki and McHugh, 2004). An ideal reporter gene is easy to assess and non-destructive to the assay system. It will be more excellent if the reporter gene has no or only a little endogenous activity in the plant to be transformed (Slater et al., 2003).There are several reporter genes that are used in plant expression vectors, such as β-glucuronidase, green

fluorescent protein, luciferase genes and chloramphenicol acetyltransferase. In this study, the β-glucuronidase and green fluorescent were used for transient assay.

2.5.1.1 β-glucuronidase (GUS)

β-glucuronidase (GUS) is an enzyme that found in the *Escherichia coli*, which encoded by *uidA* gene (Hull and Devic, 1995). It was first developed in plant systems by Richard Anthony Jefferson in 1987. Since then, it is widely been used as a reporter gene in plant molecular biology. The common assays that were carried out with this reporter gene are fluorometric assay and GUS histochemical staining.

Fluorometric assay is used to analyze the activity of transgene expression through quantitative. The reaction is carried out by adding the substrate, 4-methylumbelliferyl-beta-D glucuronidase (MUG) before measurement with a fluorometer.

GUS histochemical staining is a common assay used for transient expression. In GUS histochemical staining, the substrate, 5-bromo-4-chloro-3-indolyl glucuronidase (X-Gluc) was hydrolyzed and produces an indigo-blue precipitation.

2.5.1.2 Green fluorescent protein (GFP)

Green fluorescent protein is a protein that is found in jellyfish, *Aequorea victoria*, which consists of 238 amino acids. It was first studied by Osamu Shimomura in 1960s, where he found that the jellyfish glows green because the GFP protein had absorbed the blue light from the aequorin, (jellyfish photoprotein, which glows blue with the presence of calcium) and the ultraviolet light. This protein needs only oxygen (which are readily in most living organisms), without other additional substrate or co-factor to react in the fluorophore. Besides this, it is also a non-destructive technique that can be visualized *in vivo* (Hibberd et al., 1998), and expressed shortly after the transformation.

2.6 *Agrobacterium*-mediated transformation

Genetic transformation in plants are the tools that are used for transferring the desired gene into plant cells and allowing it to integrate into the plant's genome enabling it to be passed on to the next generation. There are two categories of the genetic transformation methods, which is direct gene transfer and indirect gene transfer. The direct gene transfer methods include particle bombardment, electroporation, silicon carbide fibres-whiskers, microinjection, polyethylene-mediated transformation and macroinjection. While, the indirect gene transfer method is by *Agrobacterium*-mediated transformation.

In this study, *Agrobacterium*-mediated transformation is used to transfer the transgene into banana cell suspension and tobacco leaf disc, rather than the biolistic technique. This method is an effective indirect gene transfer protocol that has been widely used in recent years compared to biolistic technique because of the problem of losing the inserted DNA randomly.

Agrobacterium-mediated transformation is the most common and efficient method in plant genetic engineering. The main principle in this method is the use of *Agrobacterium tumefaciens*, a soil plant pathogenic bacterium. It utilises its T-DNA region in transferring the foreign DNA into plant cells through homologous recombination. There are several essential steps for transferring the transgene into plant cells. Firstly, *Agrobacterium* colonizes on the plant cell surface, and then induces the bacterial virulence systems in the presence of phenolic compounds, such as acetosyingrone, produced by the wound of the plants. In the presence of phenolic compounds, the vir gene will be activated and therefore generate the T-DNA transfer complex. Thus, the transgene that are placed between the T-DNA borders will be transferred into the plant cell as single strand DNA and integrated into plant genome (De la Riva et al., 1998).

Even though, *Agrobacterium*-mediated transformation is not necessarily suitable to all types of explants and plant species, it is still widely used because of its remarkable advantages over the direct gene transformation methods. *Agrobacterium*-mediated transformation can avoid the formation of mosaic plants because of it is single cell transformation system. Additionally, the transgene that is transferred through *Agrobacterium* is low in copy number and therefore can reduce the problem of co-suppression or instability of the transgene in the cells host.

2.7 The plant host

Plants are popularly used as protein factories in many current studies, because they offer significant advantages over the classical expression system (such as bacterial and animal cell cultures). They especially have the potential to produce the desired proteins more economically. Many proteins were produced by transgenic plants and have been used, such as glucocerebrosidase and granulocyte-macrophage colony stimulating factor (Giddings, 2001). However, some plant hosts have biosafety issues because of their natural promiscuous nature which allow for outcrossing with wild relatives. These plants include tobacco and maize which are the popular plant systems that are often been used. Therefore, a naturally sterile system will be an attractive alternative to overcome this problem.

In this study, banana, *Musa acuminata cv.*Berangan, was chosen as the plant host because of its male sterility which allows for easier biosafety containment design. While, tobacco was used as a conventional model and plant reference for foreign gene expression, since it has been successfully produced heterologous protein in other studies (Llop-Tous et al., 2011; Yakoby et al., 2006).

2.7.1 Banana cell suspensions

The stable integration, ability to regenerate from a single cell to complete plantlet and also the expression of desired transgene in plant host are the fundamental requirements.

In this study, the candidate tissues for genetic engineering of the banana are embryogenic cell suspensions, which regenerated through somatic embryogenesis. This cell has a potential to prevent formation of chimeras. Somatic embryogenesis is an asexual form of plant propagation which can form a complete plant under a mimic of natural condition. The plantlets that are produced from somatic embryogenesis will have the same genetic makeup. Therefore, all transformed cells suspension will be able to express the same gene of interest and may lead to higher protein production.

2.7.2 Tobacco leaf discs

Tobacco is widely used as plant expression system in producing recombinant proteins, because of its unique characteristics. Tobacco is a short life cycle plant; it only takes 3 months to grow from seed until flowering. Besides that, it is a non food/feed crops. This particular trait makes it the choice as a biofactory because there is no food chain contamination issue involved. Thus, it has lowered the risk assessment challenges.

Tobacco is also a well-studied plant and is easily transformed. The first transgenic plant is tobacco which produced antibody targeting gum disease was developed by the company Plant Biotechnology of CaroRx^{TM} (Ganapathi et al., 2004; Wycoff, 2004).

Furthermore, tobacco is a cost effective and economically productive biofactory. It has the largest biomass yield on the earth after sugar cane. The track record in plant-made pharmaceuticals (PMP) research is also positioning it to be the forefront candidate as a PMP biofactory (Stoger et al., 2002).

2.8 Biosafety and risk assessment

Plant expression system provides an alternative platform with several potential advantages over traditional expression systems. However, PMPs lead to the challenges and risks of environmental, animal/human health and safety issues. The challenges are included gene flow, activation of antibiotic resistant genes, spontaneous formation of superweed, toxicity, allergenicity and anti-nutritional effects (Talas-Ogras, 2011). Thus, risk assessments need to be carried out on all transgenic plants in a scientific and transparent manner in monitoring the possible risks.

Since there are a lot of challenges and possible risk issues, biosafety regulations and risk assessment are important in addressing the strategies to minimize and limit the potential environment and animal/human health impacts. For instance, the Cartagena Biosafety Protocol (CBP) of the Convention on Biological Diversity was emphasized on the regulations of transboundary movement between countries, handling and use of living modified organisms among the signatory countries in 2000 (Talas-Ogras, 2011).

In different countries, there are different regulations and organizations that involved. For example, in European Union (EU), the legalization is focus on the genetic engineering processes and products; while, in United States (US), the legalization is only products based (Talas-Ogras, 2011). In Malaysia, PMPs are regulated under the Malaysian Biosafety Act 2007 (www.nre.gov.my).

PMP products will be the trend in the future market. Therefore, more elements must be taking into the account of risk assessment, such as, characteristic of plant host, production area and handling practices (Breyer et al., 2009). The consultation and advice from experts of different fields like molecular biology, toxicology, dietetics and genetics must be considered as well for producing the safer and useful PMP products.

CHAPTER 3: MATERIALS & METHODS

3.1 Materials

The experiments for this study involved the construction of a cassette containing several genes and sequences with the plant transformation vector, pCambia as a backbone. The genes are an anti-*Toxoplasma* scFv, the Bowman-Birk Serine Proteinase Inhibitor (BBI) sequence and the KDEL retention sequence.

The anti-*Toxoplasma* scFv gene sequences, scFv64 (Accession number: JN104603, NCBI gene bank) and scFv130 (Accession number: JN104602, NCBI gene bank) were obtained from a previous study (Lim, 2013). The fragments were amplified from a pCANTAB 5E vector. Briefly, *E.coli* carrying the plasmid pCANTAB 5E containing these scFv fragments were cultured on standard solid media with 100μg/ml ampicillin. A colony PCR screen was carried out to select the positive clones by using pCANTAB 5E_S1 primer and pCANTAB 5E_S6 primer (as stated in Table 3.2).

The Bowman-Birk Serine Proteinase Inhibitor (BBI) gene of the *Musa sp.* and KDEL retention sequence were synthesized based on information from gene bank, NCBI (Accession number: 127893 and AX164111, respectively) by Vivantis (M) Sdn. Bhd.

Two different pCambia plasmids were used in this study, which are the original pCambia1304 (www.cambia.org) for constructing scFv64 and the modified pCambia1304 plasmid, i.e pCambia1304*Avr*II, which was used to construct scFv130 clone. The protocol for modification of the pCambia1304 is as described in Section 3.2.4.3.

The plant hosts for this study were *Musa acuminata* cv. Berangan cell suspensions and *Nicotiana tabacum* L. cv. SR1. *Musa acuminata* cv. Berangan cell suspensions were used to express the scFv64 and scFv130 against *Toxoplasma* antigen. While

Nicotiana tabacum L. cv. SR1 was the platform used for expressing scFv130 against *Toxoplasma* antigen. Both plants were provided by Professor. Dr. Norzulaani Khalid from the Plant Biotechnology Incubator Unit (PBIU), CEBAR, University Malaya.

The overview of the project is as shown in Figure 3.1.

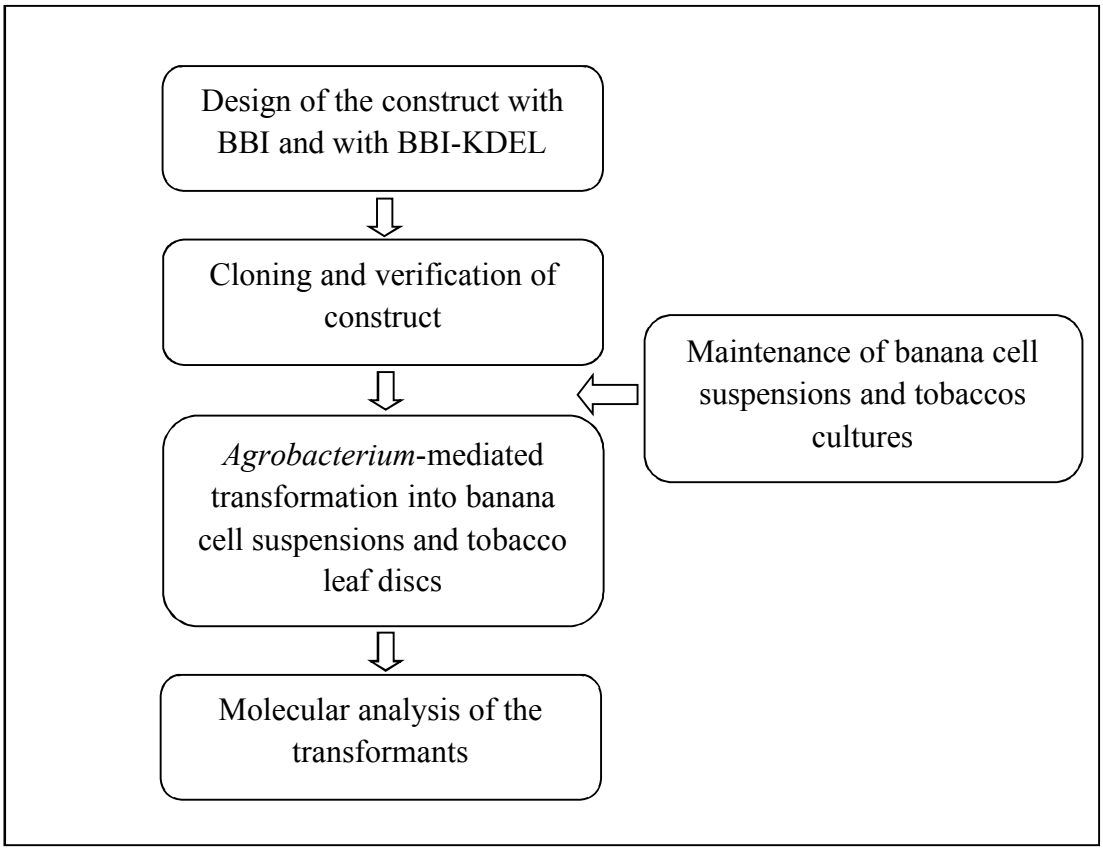


Figure 3.1: Overview of the project.

3.2 Methods

3.2.1 Plasmid extraction

The protocol for plasmid extraction from *E.coli* cells was modified from Sambrook, 2001. Firstly, a single colony was inoculated into 10µl sterile LB broth with 100µg/µl kanamycin and incubated overnight at 37ºC with 220rpm. The overnight culture was transferred into a 15ml falcon tube and centrifuged at 6000rpm for 15 minutes. The supernatant was discarded and 200µl of ice-cold Solution I was added into the tube. After that, 200µl of Solution II were added and incubated at room temperature

for 4 minutes. Next, 200µl of ice-cold Solution III were added and incubated on ice for 15 minutes. Then, the mixture was centrifuged at 13000rpm for 10 minutes. The supernatant layer was transferred to a new 1.5ml microcentrifuge tube.

Then, 1µl of RNase A 20mg/ml were added to the supernatant and incubated at 37ºC for 2 hours. After incubation, 600 µl of phenol was added and vortex. Then, the mixture was centrifuged at 13000rpm for 3 minutes. The aqueous layer was transferred into another new microcentrifuge tube and 600µl chloroform was added. The mixture was vortex and centrifuged at 13000rpm for 3 minutes. Then, 400µl of aqueous layer was transferred to new 1.5ml microcentrifuge tube.

Finally, the DNA was precipitated with 0.1 volume of 3M sodium acetate and 2.5 volumes of ethanol. The mixture was incubated on ice for 20 minutes and centrifuged at 13000rpm for 15 minutes. The supernatant was discarded and 1ml of 70% ethanol was added. The mixture was centrifuged again at 13000rpm for 5 minutes. After that, the pellet was dried by using vacuum centrifuge (DNA PLUS HETO). The dried pellet was dissolved in 50µl sterile distilled H_2O and stored at -20ºC.

The $OD_{260/280}$ assay was carried out by Eppendorf Biophotometer to determine the concentration of DNA sample in solution. The reading at 260nm is indicates the concentration of nucleic acid; while, reading at 280nm indicates the amount of protein in the sample. The ratio of absorbance, $A_{260/280}$, for pure DNA should be in the range of 1.8-2.0; however, the range of 1.7-2.0 was considered sufficient purity.

For measuring DNA concentration, 5µl of samples were mixed with 495µl sterile distilled H_2O. The diluted samples were measured at wavelength of 260nm and 280nm. The readings were taken against a blank of 500µl sterile distilled H_2O.

3.2.2 PCR and primers

The oligonucleotide primers that used in PCR screening, PCR amplification, PCR of RE site insertion and PCR confirmation were lists in the following tables (Table 3.2, Table 3.3 and Table 3.4). They are designed by using Primer 3.0 software. While, oligonucleotide primers that used for Real-time PCR were designed by using Primer Express 3.0 software and listed in Table 3.5.

The regime of all thermal cycling condition is stated in the following table.

Table 3.1: The thermal cycling condition that were used in the experiments.

Condition	Temperature, ºC	Time, minutes	No. of cycle
Initial denaturation	94	4	1
Denaturation	94	1	35
Annealing	Depend on primer set	1	
Extension	72	1	
Final extension	72	10	1
Final hold	25	10	1

PCR screening was carried out on the colonies that formed after transformation. The colonies were selected from overnight cultured LB agar plate and picked up by toothpick to make a library on new LB agar plate. The toothpick was dabbed in a PCR tube that contains 50µl distilled water. Then, the library plate was incubated at 37ºC for overnight; while, the PCR tubes with bacteria were boiled at 99ºC for 10 minutes and used as template for PCR screening.

The PCR products that obtained after amplification and RE site insertions were performed with purification for further use. PCR purification was carried out according to the provided protocol of QIAquick PCR Purification kit from Qiagen.

Five volumes of Buffer PBI were added to one volume PCR product and mix well. Then, the mixtures were transferred to QIAquick spin column which placed into a

provided 2ml collection tube. The mixtures were centrifuged at high speed for 1 minute. The flow-through was discarded and 750µl of the Buffer PE were added to the spin column. Centrifugation for 1 minute at high speed is carried out. The flow-through was discarded and centrifuge for 1 minute again.

After that, the spin column was transferred into a new 1.5ml microcentrifuge tube. Then, 30µl of Buffer EB were added and incubated for 1 minute before centrifuge at high speed for 1 minute. The elution product was stored at -20ºC.

Table 3.2: List of primers that were used for PCR screening.

Primer Set	Sequence	length	Annealing temperature, ºC	Expected size of PCR product, bp
pCANTAB 5E_S1 pCANTAB 5E_S6	5'-CGGGGAGCCGGCCGAGCTCG-3' 5'-GCCTGCGGCCGCACTAGTGACAGA-3'	20 24	56.0	~850
57BBIKDEL_F 57BBIKDEL_R	5'-GTTTTCCCAGTCACGACGTT-3' 5'-AGTGAGCGCAACGCAATT-3'	20 18	59.3	~800
*gfp*_F *gfp*_R	5'-GGCTCGAGGACCATGGTAGATCTGACTAGTA-3' 5'-CTCGAGTCTAGAGGGGTTTCTACAGGACGTAAACT-3'	31 35	58.0	~750
1304SK_F 1304SK_R	5'-GAGAGAACACGGGGGACTC-3' 5'-TCCATGAAAAGTTCTTCTCCTTT-3'	19 23	58.0	~1500

Table 3.3: List of primers that were used in PCR amplification and RE site insertion.

Primer Set	Sequence	Number of nucleotides	Annealing temperature, ºC	Expected size of PCR product, bp
scFv64*Nco*I_F scFv*Hind*III_R	5'- CGAATTGCCATGGTGATGACC -3' 5'- TATGAGGTTAAGCTTTTGCTAAAC -3'	21 24	42.0	~800
scFv130*Avr*II_F scFv*Hind*III_R	5- CGACATCCTAGGTGTATGACCCAG -3' 5'- TATGAGGTTAAGCTTTTGCTAAAC -3'	24 24	42.0	~800
57BBIKDEL_F 57BBI_R	5'- GTTTTCCCAGTCACGACGTT -3' 5'-CGTGAGCGCAACGCAATTA-3'	20 19	59.3	~800
57BBI_F 57BBI_R	5'-GCTCATCTTTCTCAGATCTTT-3' 5'-CGTGAGCGCAACGCAATTA-3'	21 19	59.3	~800
35S*Bam*HI_F 35S*Nco*I_R	5'-GTACCCGGGGATCCTCTAGAGTCG-3' 5'-CTACCATGGCCTAGGTCAAGCGCTAGAGTC-3'	24 30	42.0	~800

Table 3.4: List of primers that were used in PCR confirmation.

Primer Set	Sequence	Number of nucleotides	Annealing temperature, ºC	Expected size of PCR product, bp
1304SK_F 1304SK_R	5'-GAGAGAACACGGGGGACTC-3' 5'-TCCATGAAAAGTTCTTCTCCTTT-3'	19 23	58.0	~1500
*gfp*_F *gfp*_R	5'-GGCTCGAGGACCATGGTAGATCTGACTAGTA-3' 5'-CTCGAGTCTAGAGGGGTTTCTACAGGACGTAAACT-3'	31 35	58.0	~750
NtAct_F NtAct_R	5'-AGTCCTCCTTACTGAAGCGCCT-3' 5'-GAATGGCAACGTACATAGCTGG-3'	22 22	60.0	~400
Actin9_F Actin9_R	5'-CTATTCTCCGCTTTGGACTTGGCA-3' 5'-AGGACCTCAGGACAACGGAAACG-3'	24 23	60.0	~250

Table 3.5: List of primers that were used in Real-time PCR.

Primer Set	Sequence	Number of nucleotides	Expected size of PCR product, bp
Actin9_F Actin9_R	5'-CTATTCTCCGCTTTGGACTTGGCA-3' 5'-AGGACCTCAGGACAACGGAAACG-3'	24 23	~400
TG13090_F TG13090_R	5’-TTCCTCCAGCACAGCCTACA-3’ 5’-TAAGCAAACCCATCCCCTCTT-3’	20 35	~90
*gfp*180_F *gfp*180_R	5’-CAAGCAAAAGAACGGCATCA-3’ 5’-CTCTTTTCGTTGGGATCTTTCG-3’	20 22	~180

3.2.3 Digestion

Different restriction enzymes combinations were used throughout the experiments, (as mentioned in Section 3.2.4) based on the design of the constructs. In general, the reaction was carried out by incubation at 37 ºC for one hour.

Then, the restriction enzyme digestion products were purified using QIAquick Gel Extraction Kit (Qiagen) based on the provided protocol. The electrophoresed DNA fragment was excised from agarose gel under UV light (Vilber Lourmat). The gel slice was weighed and 3 volumes of Buffer QG were added before incubation at 50ºC for 10 minutes. After that, 1 volume of isopropanol was added and mixed well.

Then, the mixture was transferred into the spin column which was placed in the provided 2ml collection tube, and centrifuged at high speed for 1 minute. The flow-through was discarded and 500µl of Buffer QG were added and centrifuged for a further 1 minute at high speed to remove all traces of agarose. Then, 750µl of Buffer PE were added and centrifuged for 1 minute at high speed. After that, the flow-through was discarded and centrifuged for another 1 minute at high speed.

The QIAquick column was placed in a new 1.5ml microcentrifuge tube. Then, 30µl of the Buffer EB were added and incubated for 1 minute before centrifuge at high speed for 1 minute. The purified DNA fragment was stored at -20 ºC.

3.2.4 Preparation of construct

3.2.4.1 pToxo64BBIKDEL

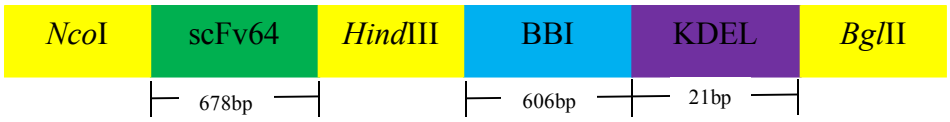


Figure 3.2: Construct of the gene of interest with the restriction enzyme sites, pToxo64BBIKDEL. (Not to scale)

Plasmid pCambia1304 vector was isolated (as mentioned in Section 3.2.1) and treated with restriction enzymes digestion (as showed in Table 3.6).

Colonies of scFv64 antibody fragments from LB agar plate were screened with PCR using pCANTAB 5E_S1 and pCANTAB 5E_S6 primers (referred to Table 3.2). Then, colonies which showed positive results in PCR screening were selected and inoculated in 10ml LB broth with 100µg/ml ampicillin. Plasmid of scFv64 antibody fragment was isolated using the protocol that stated in Section 3.2.1. The plasmid was used as template for PCR optimization and amplification using scFv64*Nco*I_F and scFv*Hind*III_R (referred to Table 3.3). Then, the products were performed with purification (as mentioned in Section 3.2.2) and restriction enzymes digestion.

For BBIKDEL gene fragment, PCR optimisation was carried out to optimise the annealing temperatures (50ºC - 60ºC) of 57BBIKDEL_F and 57BBIKDEL_R primers (referred to Table 3.2), with plasmids containing the BBIKDEL gene fragment that were commercially-synthesized (Vivantis. Sdn. Bhd.). The plasmid with BBIKDEL gene fragment was transformed into *E.coli* competent cells and cultured on agar plate with 100µg/ml ampicillin. Colonies that formed after transformation were screened using 57BBIKDEL_F and 57BBIKDEL_R with 59.3ºC as their optimum annealing temperature. Colonies with positive results were inoculated in 10ml LB broth with 100µg/ml ampicillin and plasmid was isolated based on the protocol as stated in Section

3.2.1. The BBIKDEL gene fragment was then amplified from the plasmid using primers 57BBIKDEL_F and 57BBI_R (stated in Table 3.3) with 59.3ºC as annealing temperature. The PCR amplification products were purified (as stated in Section 3.2.2) and treated with restriction enzymes digestion (see Table 3.6).

Table 3.6: List of restriction endonucleases and buffers that were used to digest components for constructing pToxo64BBIKDEL.

Sample	Enzyme	Enzyme	10 x Restriction buffer
pCambia1304	*Nco* I	*Bgl* II	NEBuffer 3
scFv64 fragment	*Nco* I	*Hind* III	NEBuffer 2
BBI gene with KDEL	*Hind* III	*Bgl*II	NEBuffer 2

Then, the digestion products were purified with QIAquick Gel Extraction Kit (as mentioned in Section 3.2.3) and ligation was performed (referred to Table A6 in AppendixA6). The ligation mixture was incubated at 22ºC for 1 hour before transformation into *E.coli* competent cells (as mentioned in Section 3.2.6.1). Transformation products were cultured on LB agar plate with 100µg/ml kanamycin and incubated at 37ºC overnight. Colonies from overnight cultures were selected and screened with primers 1304SK_F and 1304SK_F (as shown in Table 3.2). Then, plasmids from the colonies which showed expected result in PCR screening was isolated (as in Section 3.2.1).

3.2.4.2 pToxo64BBI

Figure 3.3: Construct of the gene of interest with the restriction enzyme sites, pToxo64BBI.(Not to scale)

The pCambia1304 vector and scFv64 antibody fragment were prepared as stated in Section 3.2.4.1.

The BBIKDEL gene fragment was used as template to amplify BBI gene fragment with primers 57BBI_F and 57BBI_R (as stated in Table 3.3) and the PCR amplification products were purified with the protocol that mentioned in Section 3.2.2 before perform with restriction enzymes digestion.

The digestions were carried out based on combinations in Table 3.7. Then, the experiments were carried out as stated in Section 3.2.4.1.

Table 3.7: List of restriction endonucleases and buffers that were used to digest components for constructing pToxo64BBI.

Sample	Enzyme	Enzyme	10 x Restriction buffer
pCambia1304	*Nco* I	*Bgl* II	NEBuffer 3
scFv64 fragment	*Nco* I	*Hind* III	NEBuffer 2
BBI gene	*Hind* III	*Bgl* II	NEBuffer 2

3.2.4.3 pToxo130BBIKDEL

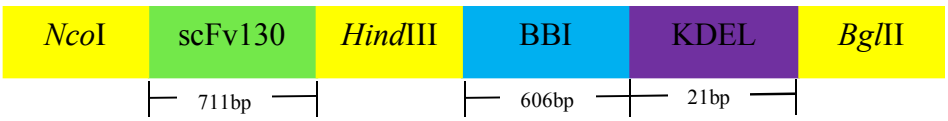


Figure 3.4: Construct of the gene of interest with the restriction enzyme sites, pToxo130BBIKDEL.(Not to scale)

The construct, pToxo130BBIKDEL was prepared as mentioned in Section 3.2.4.1. However, the pCambia1304 vector was modified by PCR with 35S*Bam*HI_F and 35S*Nco*I_R (referred to Table 3.3) to add new restriction enzyme sites, *Afe*I&*Avr*II. The modified pCambia1304 vector was named as pCambia1304*Avr*II. Additionally, the scFv130 antibody fragment was also used in place of the scFv64 antibody fragment. The scFv130*Avr*II_F and scFv*Hind*III_R (referred to Table 3.3) were used to amplify the desired fragment. Then, all the fragments were treated with restriction enzymes digestion. The combination restriction enzymes used is as shown in Table 3.8.

Table 3.8: List of restriction endonucleases and buffers that were used to digest components for constructing pToxo130BBIKDEL.

Sample	Enzyme	Enzyme	10 x Restriction buffer
pCambia1304*Avr*II	*Avr* II	*Bgl*II	NEBuffer 2
scFv130 antibody fragment	*Avr*II	*Hind* III	NEBuffer 2
BBI gene with KDEL	*Hind* III	*Bgl*II	NEBuffer 2

3.2.4.4 pToxo130BBI

Figure 3.5: Construct of the gene of interest with the restriction enzyme sites, pToxo130BBI.(Not to scale)

The construct, pToxo130BBI was prepared as described in Section 3.2.4.1. The combinations of restriction enzymes digestion are as shown in Table 3.9.

Table 3.9: List of restriction endonucleases and buffers that were used to digest components for constructing pToxo130BBI.

Sample	Enzyme	Enzyme	10 x Restriction buffer
pCambia1304*Avr*II	*Avr* II	*Bgl*II	NEBuffer 2
scFv130 antibody fragment	*Avr*II	*Hind* III	NEBuffer 2
BBI gene	*Hind* III	*Bgl* II	NEBuffer 2

3.2.4.5 Verification of construct

The plasmid of the constructs (pToxo64BBIKDEL, pToxo64BBI, pToxo130BBIKDEL and pToxo130BBI) was digested with restriction enzyme, *Nde*I, (referred Table 3.10) and send for sequencing to confirm the integration of the desired genes into the pCambia1304 or pCambia1304*Avr*II vector.

Table 3.10: List of constructs that were digested with *Nde*I.

Constructs	Enzyme	10 x Restriction buffer
pCambia1304	*Nde*I	NEBuffer 4
pToxo64BBI	*Nde*I	NEBuffer 4
pToxo64BBIKDEL	*Nde*I	NEBuffer 4
pToxo130BBI	*Nde*I	NEBuffer 4
pToxo130BBIKDEL	*Nde*I	NEBuffer 4

3.2.5 Competent cell preparation

3.2.5.1 *E.coli*

The bacterial stocks of competent cells (*E.coli* JM109 strain) were cultured in 10ml fresh antibiotic free LB broth at 37ºC with 200rpm for overnight. Then, 1ml overnight culture was sub-cultured in 20ml fresh antibiotic free LB broth. The OD_{600nm} of the culture was taken in the interval times.

When OD_{600nm} of the culture reached ~ 0.5, the culture was transferred to a 50ml centrifuge tube and incubated on ice for 30 minutes. After incubation, the culture was centrifuged at 3000rpm for 5 minutes at 4ºC. The supernatant was discarded and 15ml of TFB I solution were added to resuspend the pellet. The mixture was incubated on ice for 20 minutes before centrifugation at 3000rpm for 5 minutes at 4ºC. Again, the supernatant was removed and the pellet was resuspended in 500µl TFB II solution. Then, 100µl of freshly prepared competent cells were aliquoted to ice-cold sterile microcentrifuge tubes and frozen in liquid nitrogen before store at -80ºC.

3.2.5.2 *Agrobacterium tumefaciens*

The bacterial stock of *Agrobacterium sp.* strain LBA4404 competent cells was streaked onto an LB agar plate with 50µg/ml rifampicin and incubated at 28±1ºC for 2 days.

After 2 days, a single colony of the *Agrobacterium sp.* was picked to inoculate into 5ml sterile LB broth with 50µg/ml rifampicin and incubated in 28ºC for overnight with 200rpm. Then, 2ml overnight culture were transferred into 50ml fresh antibiotic free LB broth and incubated at 28±1ºC with 200rpm until the OD_{600nm} reach ~0.5.

After that, the cultures were transferred into 50ml centrifuge tubes and centrifuged at 5000rpm for 10minutes at 4ºC. The supernatant was discarded and the pellet was resuspended in 20ml ice-cold 1mM HEPES (pH 7.0). Then, the mixture was centrifuged at 5000rpm for 10 minutes at 4ºC. The wash step with 20ml ice-cold 1mM HEPES and centrifugation were repeated for 2 times. Finally, the pellet was resuspended in 500µl ice-cold 10% glycerol. Then, 100µl of freshly prepared competent cells were aliquoted to ice-cold sterile microcentrifuge tubes and frozen in liquid nitrogen to store at -80ºC before use.

3.2.6 Transformation into competent cell

3.2.6.1 *E.coli*

The freshly prepared competent cells, *E.coli* strain JM109 (Section 3.2.5.1) were thawed on ice and the ligation mixture was chilled on ice. After thawing, 2µl ligation mixture was added to 100µl competent cells and mixed gently. The mixture was incubated on ice for 20 minutes before being heat shocked at 42ºC for 45 seconds. After heat shock, the mixture was chilled on ice for 5 minutes. Then, 900µl of fresh LB broth was added to the mixture and incubated for 1 hour at 37ºC. The transformation products were cultured on LB agar plate with 100µg/ml kanamycin and incubated at 37ºC for overnight.

3.2.6.2 *Agrobacterium tumefaciens*

The freshly prepared *Agrobacterium sp.* strains LBA4404 competent cells (Section 3.2.5.2) were thawed on ice and the plasmids of the construct were chilled on ice. Then, 5µl plasmids were added to 100µl competent cells and mixed gently. The mixture was incubated on ice for 30 minutes before freeze in liquid nitrogen for 1minute. After frozen, the mixtures were immediately thawed at 37ºC for 4 minutes. Then, the mixture was chilled on ice for 1 minute before added 900µl of fresh LB broth and incubated at 28±1ºC for 2 hours. The transformation products were cultured on LB agar plate with 50µg/ml rifampicin and 100µg/ml kanamycin. The culture plate was incubated at 28ºC for 2-3 days.

3.2.7 *Agrobacterium*-mediated transformation into plant host

3.2.7.1 Banana cell suspensions

An *Agrobacterium sp*. colony with each construct (pToxo64BBIKDEL, pToxo64BBI, pToxo130BBIKDEL, and pToxo130BBI) was inoculated in 3ml LB

broth with 100µg/ml kanamycin and 50µg/ml rifampicin. The inoculants were incubated at 28±1ºC with 220rpm for overnight. As for experiment control, pCambia1304 vector was used and transformed with the same procedure.

Then, the overnight cultures were sub-cultured into 30ml LB broth and grow at same condition for another 6 hours. After that, the cultures were centrifuged at 4ºC with 5000rpm for 10 minutes. The supernatant was removed and the pellet was resuspended in 1ml M2 medium supplemented with 100µM acetosyringone.

Seven days post subculture cell suspensions were transferred into 50ml Falcon tube to adjust the settled cell volume: liquid M2 medium ratio approximately 1:5. Cells aggregate were then resuspended in M2 medium and 500µl of the cell cultures were aliquoted into 10ml of M2 medium.

The aliquoted cell suspensions were inoculated with 1ml of *Agrobacterium sp.* cultures and incubated at 28±1ºC in dark with 200rpm for 30 minutes. After 30 minutes, the media containing the *Agrobacterium sp.* cultures were removed and replaced with 10ml M2 medium supplemented with 100µM acetosyringone. Then, the inoculated cell suspensions were co-cultivated in the dark at 28±1ºC with gentle shaking at 80 rpm for 3days. After co-cultivation, M2 medium with 50µg/L cefotaxime was used to replace the co-culture media to eliminate *Agrobacterium sp*.

After 48hours, M2 medium with 50µg/L cefotaxime was removed and M3 media was added. The inoculated cells were maintained in the dark with M3 media and the media changed every 2 week intervals until somatic formation was observed. An aliquote of the cells were also transferred into 1.5ml microcentrifuge tube for GUS histochemical staining.

3.2.7.2 Tobacco leaf discs

The tobacco plants were maintained in MSO media and changed at one month intervals. For *Agrobacterium*-mediated transformation, 1month old leaves were cut into ~1cm² and put on TSM media for 2 days. An *Agrobacterium sp*. colony with each construct (pToxo130BBIKDEL and pToxo130BBI) was inoculated in individual 10ml LB broths with 100µg/ml kanamycin and 50µg/ml rifampicin. The inoculants were incubated at 28±1ºC and shaken at 220rpm overnight.

The overnight *Agrobacterium sp.* cultures were centrifuged at 5000rpm for 10minutes. The supernatant was removed and the pellet resuspended in 10ml co-culture media. The pre-culture explants were transferred into the *Agrobacterium sp.* culture and incubated for 10minutes with gentle shaking. After that, the inoculated explants were blotted dry on filter paper before transfer into TSM media and co-cultivation in the dark for 3 days. After co-cultivation, the inoculated explants were transferred to TSM selection media and kept in 16 hours light: 8 hours dark photoperiod. The TSM selection media was changed every week until the formation of the shoots.

The shoots were excised from explants and transferred to TRM selection media. The shoots in TRM media were maintained until root formation. Then, the plantlets were transferred to soil. Some of the excised shoots were assayed with GUS histochemical staining for confirmation of successful transformation.

As an experimental control, pCambia1304 vector was used and transformed with the same procedure.

3.2.8 Analysis of transgenes

3.2.8.1 GUS assay

The inoculated banana cell suspensions were centrifuged at 13000rpm for 1minute. The remaining media was removed by pipetting. Then, the cells were immersed in GUS

histochemical assay reagents (as in Table A7, Appendix A9) and incubated at 37ºC overnight. The samples were then fixed in FAA solution. The stained cells were examined under contrast phase light microscope (Zeiss) and images were captured by using a microscope adapted Nikon digital camera (COOLPIX995).

While, for the tobacco, the excised shoots were stained with the GUS histochemical reagent. Then, the stained shoots were immersed in 70% ethanol (for removing chlorophyll) before fix in FAA solution.

3.2.8.2 DNA extraction

The genomic DNA of inoculated banana cell suspensions and transgenic tobacco leaves was isolated using DNeasy plant mini kit (Qiagen). The experiment was carried out based on the provided protocol and buffers.

Firstly, 100mg of inoculated banana cell suspensions were ground into fine powder by using mortar and pestle with liquid nitrogen. Then, the samples were transferred into the 1.5ml microcentrifuge tubes which contained 400µl AP1 buffer with 4µl 100mg/ml RNase A. The mixture was vortex vigorously before incubate at 65ºC for 10 minutes. After that, 150µl AP2 buffer were added and incubated on ice for 5 minutes to precipitate the detergents, proteins and polysaccharide. Then, the lysate was centrifuged at 14000rpm for 5 minutes. The lysate was pipetted into the QIAshredder mini spin column and centrifuged at 14000rpm for 2 minutes. The flow-through fraction was transferred into a 1.5ml microcentrifuge tube and 1.5 volumes of AP3/E buffer were added and mixed by pipetting.

Then, the mixture was transferred into the DNeasy mini spin column and centrifuged at 8000rpm for 1 minute. After centrifugation, the DNeasy mini spin column was transferred to a new 2.0ml collection tube and 500µl AW buffer were

added and centrifuged at 8000rpm for 1 minute. The flow-through was discarded and another 500µl AW buffer were added before centrifuge at 14000rpm for 2 minutes.

Finally, 50µl AE buffer were added directly onto the membrane and incubated at room temperature for 5 minutes before centrifugation at 8000rpm for 1 minute. The isolated genomic DNA were electrophoresed on agarose gel (referred to Appendix A10 (i)) before stored at -20ºC.

3.2.8.3 PCR

The isolated genomic DNA of inoculated banana cell suspensions and transgenic tobacco was used as template for PCR analysis.

For genomic DNA of inoculated banana cell suspensions, 2 sets of primers, 1304SK_F & 1304SK_R and NtAct_F&NtAct_R (referred to Table 3.4) were used to analysis. Whilst, 1304SK_F & 1304SK_R and Actin9_F & Actin9_R (referred to Table 3.4) were used to analyze transgenic tobacco.

Thermal cycle conditions were set as described in Table 3.1. The PCR products were electrophoresed on agarose gel (referred to Appendix A10(i)).

3.2.9 Verification of transgenic plant via Real-Time PCR analysis

3.2.9.1 RNA extraction

The RNA of transgenic tobacco was isolated using easy-spin^{TM} [DNA free] Total RNA Extraction Kit which purchased from iNtRON Biotechnology. The RNA was isolated based on the provided protocol and buffers.

Firstly, 100mg of fresh transgenic tobacco leaves was ground into fine powder using mortar and pestle with liquid nitrogen. The ground tissue sample was transferred into 1.5ml microcentrifuge tube which contained 1ml lysis buffer (easy-BLUE^{TM} reagent) and vortexed vigorously at room temperature for 10seconds. Then, 200µl of

chloroform were added and vortexed. The mixture was centrifuged at 4ºC with 13000rpm for 10 minutes.

After centrifugation, 400µl of upper fluid were transferred into 1.5ml microcentrifuge tube. Then, 400µl of binding buffer were added and mixed by pipetting. The mixture was loaded into spin column and centrifuged at 13000rpm for 30 seconds. The flow-through was discarded and 700µl of washing buffer A was added. Again, the tube was centrifuge at 13000rpm for 30 seconds. Then, 700µl of washing buffer B was added and centrifuged at 13000rpm for 30 seconds. The flow-through was discarded and the spin column was placed back to the collection tube; and centrifuged at 13000rpm for 2 minutes to dry the membrane.

Lastly, the spin column was placed into a 1.5ml microcentrifuge tube and 50µl of elution buffer were added to the membrane. Then, the membrane was incubated at room temperature for 1 minute before centrifuge at 13000rpm for 1 minute. Then, the elution products were performed with agarose gel electrophoresis (referred to Appendix A10 (ii)).

OD $_{260/280}$ assay also carry out to determine the RNA concentration of the samples. For pure RNA, the ratio of absorbance, $A_{260/280}$, should be in the range of 1.8-2.0; however, the range of 1.7-2.0 was considered sufficient purity. For measuring RNA concentration, 2µl of samples were mixed with 98µl DEPC-treated distilled H_2O. The diluted samples were measured at wavelength of 260nm and 280nm. The readings were taken against a blank of 100µl DEPC treated distilled H_2O.

After measuring the RNA concentration, DNase I (Invitrogen) treatment was carried out on the RNA samples for removing the remaining DNA. The reaction mixture was prepared as showed in the Table 3.11.

Table 3.11: DNaseI treatment reaction mixture.

Component	Working concentration	Volume, µl
RNA sample	1µg	Concentration dependent
10x DNaseI reaction buffer	1x	1
DNaseI (1U/µl)	1U	1
DEPC-treated H_2O to	-	10µl

The tube with reaction mixture was mixed and incubated at room temperature for 15 minutes. Then, the reaction was inactivated with 1µl of 25mM EDTA solution and heated at 65ºC for 10minutes. Then, agarose gel electrophoresis (referred to Appendix A10 (ii)) was carried out on the treated RNA samples.

3.2.9.2 Reverse-transcriptase PCR

The Reverse-transcriptase PCR was carried out using high-capacity cDNA reverse transcriptase kit that purchased from Applied Biosystems.

The reaction mixture was prepared as stated in Table A2 (Appendix A4b).Then, 10µl of reaction mixtures were aliquoted into ice-cold 0.2ml PCR tubes; 10µl of DNaseI treated RNA were added and mixed by pipetting up and down. Then, the tubes were centrifuged briefly before loaded into PCR machine. The thermal cycling condition of Reverse-transcriptase PCR was mentioned as below;

25 ºC	10 minutes
37 ºC	120 minutes
85 ºC	5 minutes
4ºC	∞

3.2.9.3 Real-time PCR

The quantitative PCR was carried out using Applied Biosystems 7500 Real-time PCR System.

Firstly, the examination of primer set (referred to Table 3.5) specificity were performed with dissociation program. The primer set with single peak indicates the higher specificity of primer. The reaction mixtures were prepared as mentioned in Table A3 (Appendix A4c). Four replicates of each primer set and a negative control (without cDNA template) were prepared and assayed with the following dissociation program;

30 cycles	95 ºC for 15seconds
1 cycle	60 ºC for 1minutes
1 cycle	95 ºC for 15seconds

After examining the primer specificity, a relative standard curve with 5x serial dilution of cDNA was constructed. Five series of 5x serial dilution of cDNA was carried out and used as template for performing Relative Quantification PCR. The Real-Time PCR reaction mixture (referred to Table A3 in Appendix A4c) with 3 set primers (as showed in Table 3.5) were prepared. Four replicates of each serial dilution and a negative control (without cDNA template) were used in the experiment. The thermal cycling condition for Relative Quantification PCR, as below:

Stage 1	95 ºC for 10 minutes	1cycle
Stage 2	95 ºC for 1minutes	
	60 ºC for 15seconds }	40 cycles

Then, the collected data was performed with Relative Quantification Study using SDS Software 1.3.1 to generate the Ct value. A standard curve was plotted with the log input amount versus Ct value.

After obtaining the relative standard curve, the relative quantification of gene expression was carried out on cDNA of transgenic tobacco with pCambia1304 vector

(as plant expression vector control) and cDNA of transgenic tobacco with pToxo130BBIKDEL and pToxo130BBI. The cDNA of non-transformed tobacco was used as control for the experiment. Four replicates and a negative control (without cDNA template) were prepared for each sample. The thermal cycling condition for Relative Quantification PCR, as mentioned above was used to quantitate the gene expression.

All data of Relative Quantification plates were used to perform the Relative Quantification Study (SDS Software 1.3.1) for comparing the gene expression level in between transgenic tobacco pToxo130BBIKDEL and pToxo130BBI with non-transformed tobacco.

3.2.10 Flow chart of study

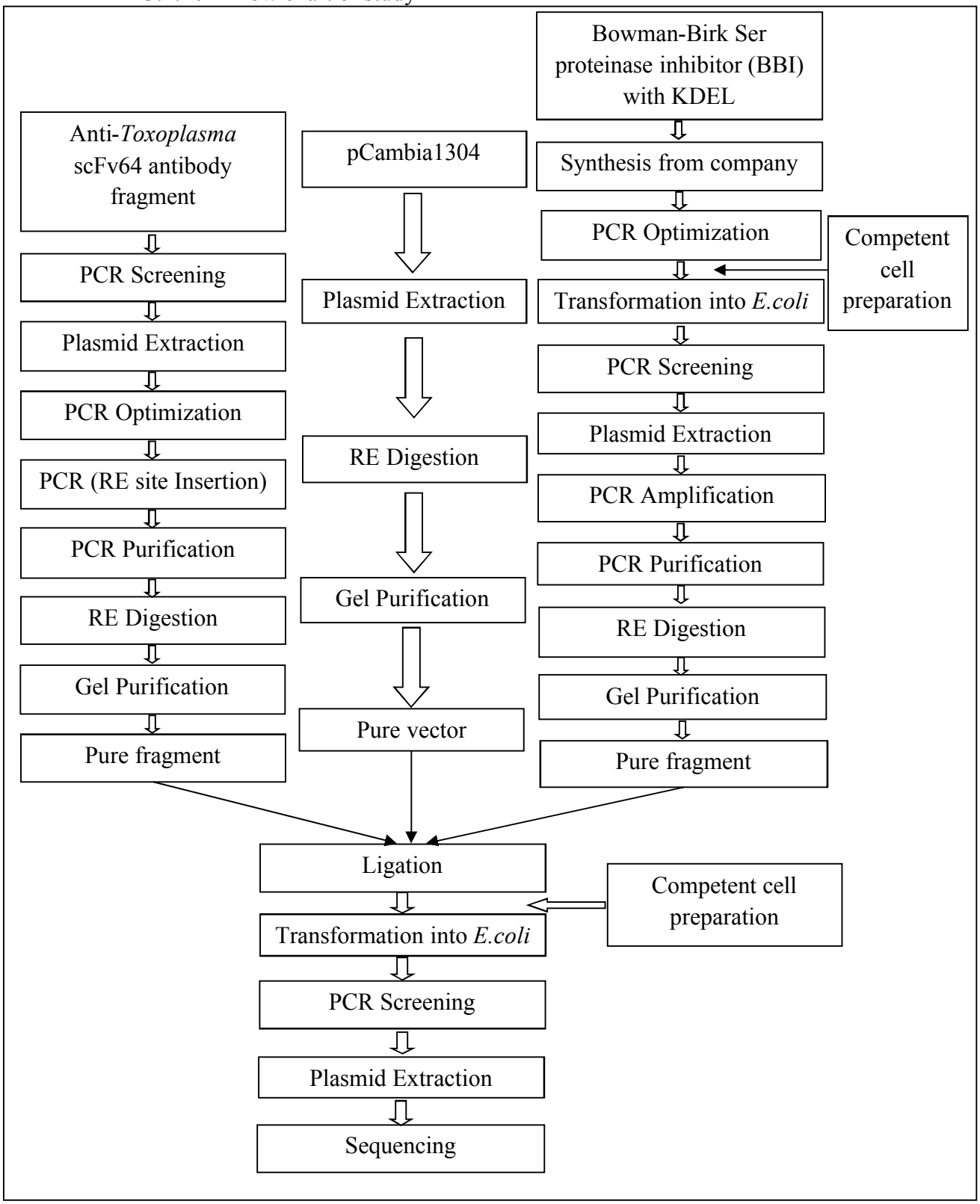


Figure 3.6: Flow chart used in developing the constructs (pToxo64BBI and pToxo64BBIKDEL).

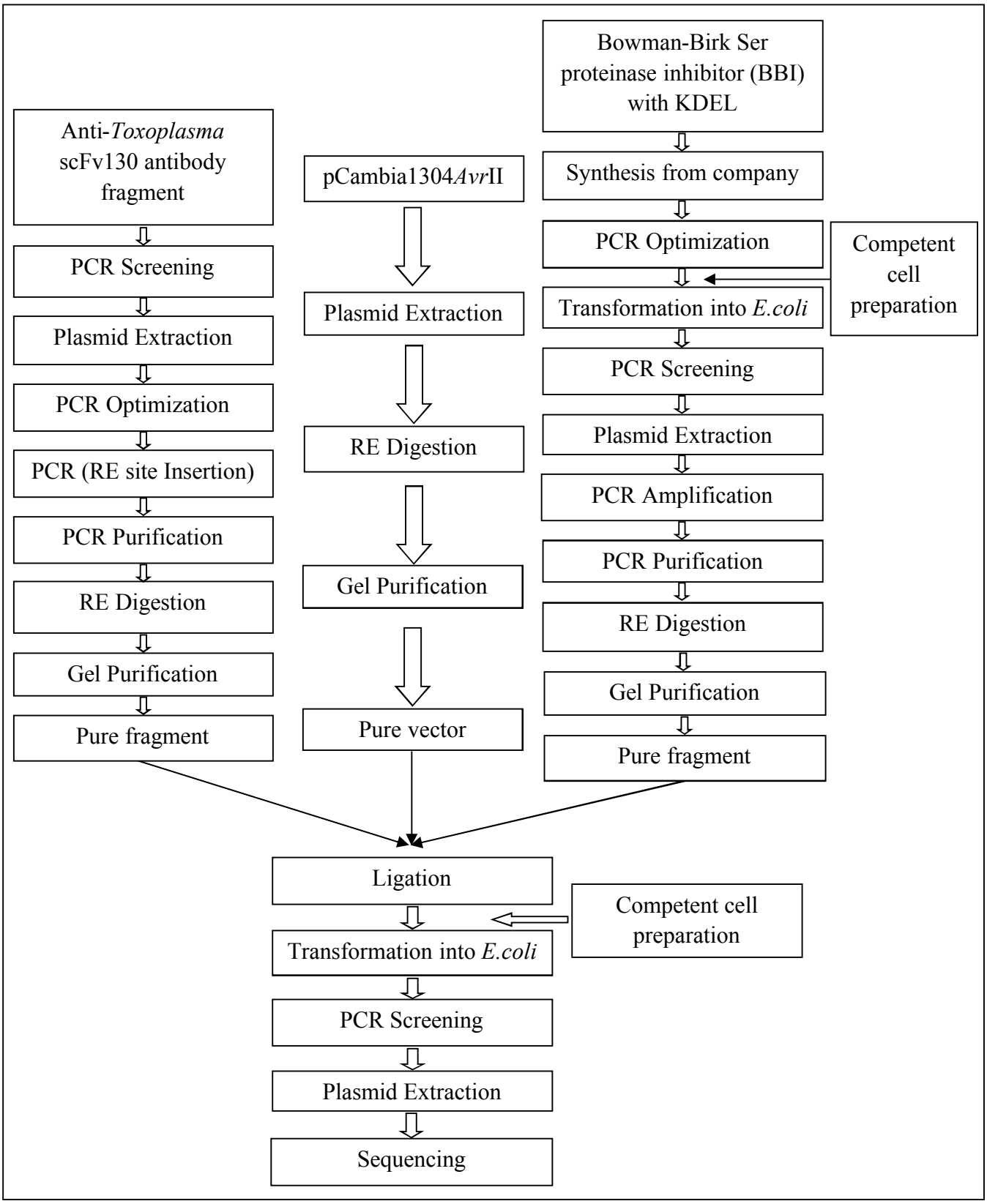


Figure 3.7: Flow chart for the development of the constructs (pToxo130BBI and pToxo130BBIKDEL).

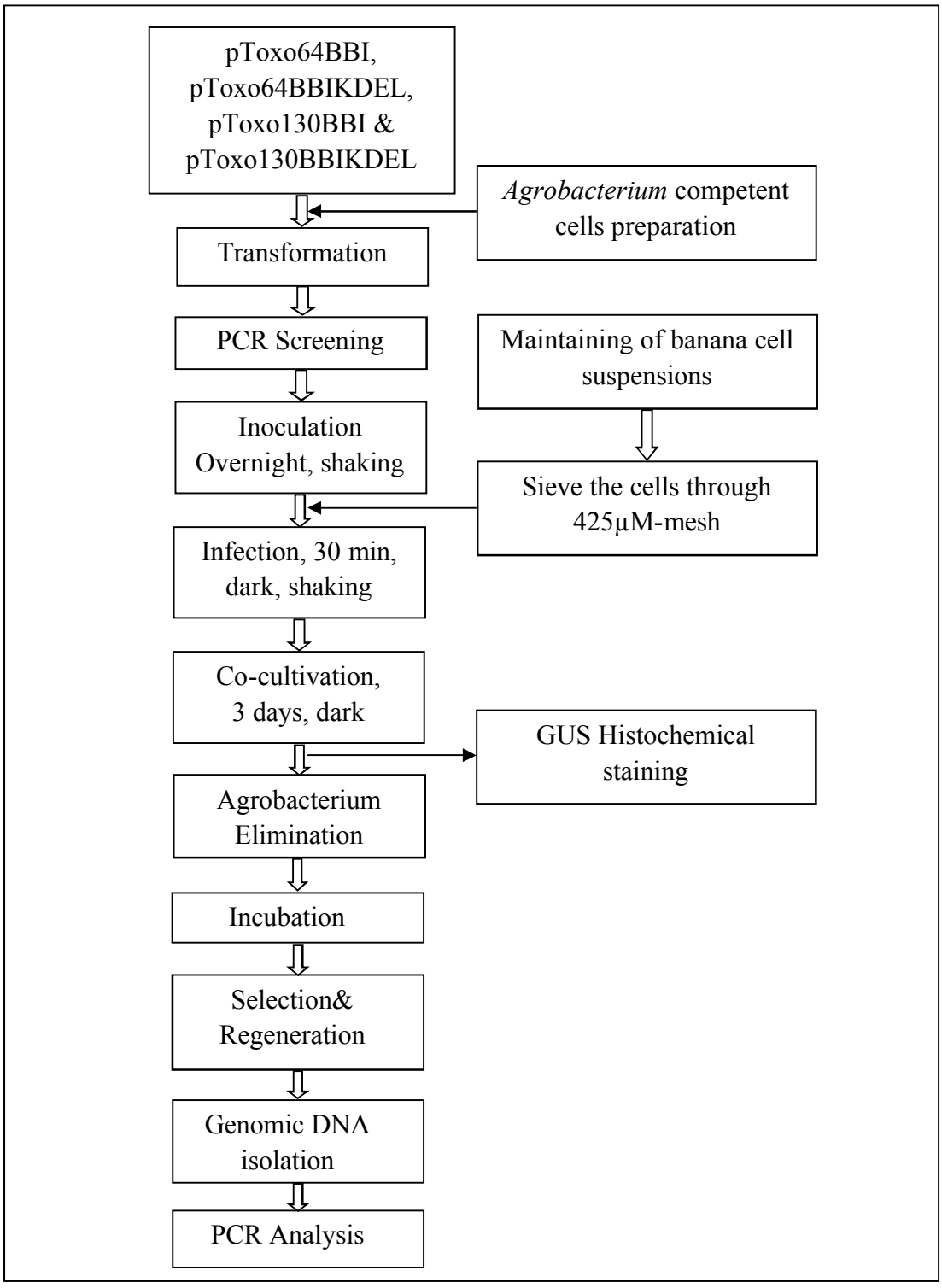


Figure 3.8: Flow chart for *Agrobacterium*-mediated transformation into banana cell suspensions.

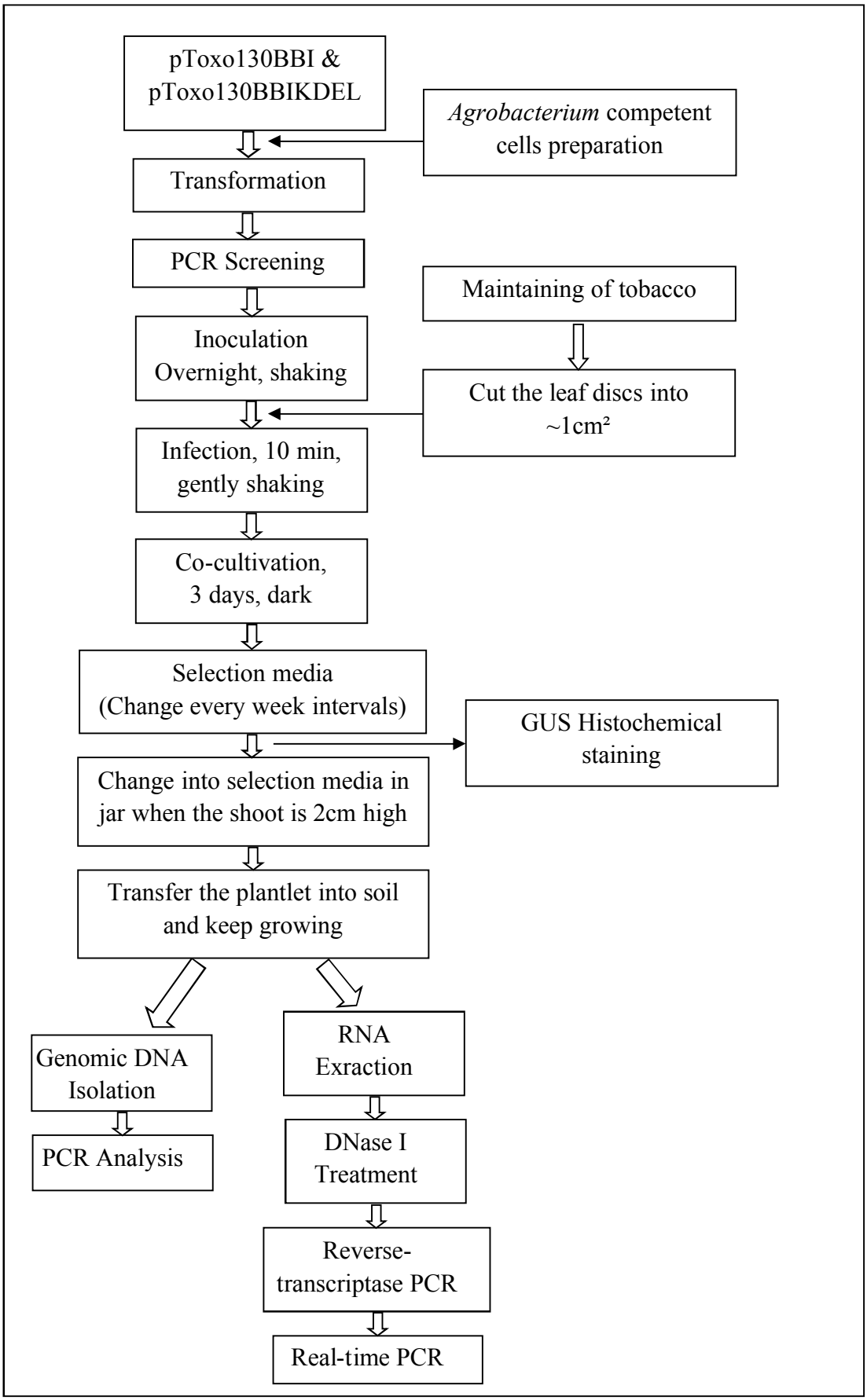


Figure 3.9: Flow chart for *Agrobacterium*-mediated transformation into tobacco leaf discs.

CHAPTER 4: RESULTS

4.1 Preparation of vectors and inserts

4.1.1 pCambia1304 and pCambia1304*Avr*II

The glycerol stock of the *E.coli* strain JM109 harbouring the plant transformation vector pCambia1304 and the *E.coli* strain JM109 harbouring pCambia1304*Avr*II were successfully cultured and the plasmids extracted. The isolated plasmids are as shown in Figure 4.1. The $OD_{A260/280}$ readings of the extracted plasmids are shown in Table 4.1 and 4.2 respectively indicating concentrations attained between 1.4 to 2.4µg/µl with sufficient purity for the subsequent procedures.

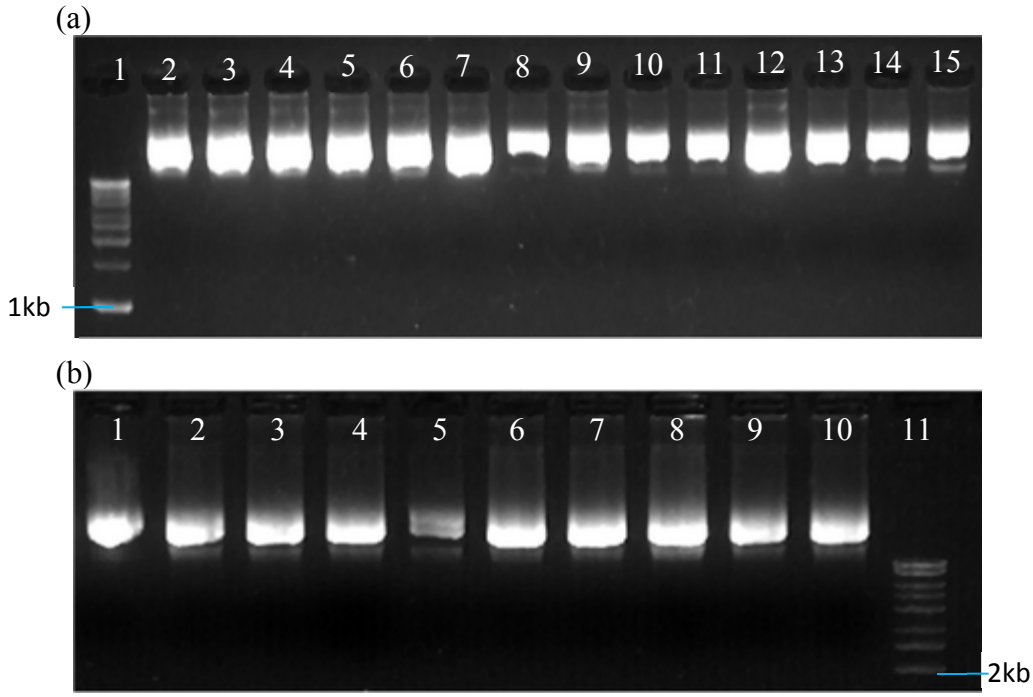


Figure 4.2: Agarose gel electrophoresis of pCambia vectors.

(a) pCambia1304 in the 1% EtBr-stained agarose gel
Lane 1: Marker; 1kb DNA ladder (EUR_X).
Lane 2-15: pCambia1304 from plasmid extraction.

(b) pCambia1304*Avr*II in the 1% EtBr-stained agarose gel
Lane 1-10: pCambia1304*Avr*II from plasmid extraction.
Lane 11: Marker; 1kb DNA ladder (EUR_X).

Table 4.1: Concentration of pCambia1304.

Sample	Concentration µg/µl	Purification	Sample	Concentration µg/µl	Purification
1	2.622	1.87	8	2.401	1.92
2	3.137	1.88	9	1.886	1.91
3	2.962	1.91	10	1.769	1.90
4	1.821	1.91	11	2.513	1.88
5	3.045	1.90	12	1.787	1.90
6	1.984	1.89	13	1.681	1.89
7	1.682	1.87	14	1.584	1.91

Table 4.2: Concentration of pCambia1304*Avr*II.

Sample	Concentration µg/µl	Purification	Sample	Concentration µg/µl	Purification
1	1.166	1.81	6	1.591	1.81
2	1.266	1.81	7	1.635	1.80
3	2.765	1.83	8	1.431	1.80
4	1.128	1.81	9	1.313	1.80
5	0.987	1.78	10	1.323	1.81

4.1.2 scFv64 and scFv130 antibody fragments

As described in Section 3.1, PCR screening was carried out to select the appropriate *E.coli* colony harbouring the scFv64 and scFv130 antibody fragments. Ten colonies for each clone were randomly selected. All colonies selected potentially harbouring the scFv130 antibody fragment showed positive results; while for scFv64 antibody fragment clone only 9 colonies gave positive amplification. The expected size of the PCR products for both fragments was approximately 850bp, as shown in Figure 4.2.

Seven colonies giving positive amplification for each of the clones (scFv64 and scFv130) were subcultured prior to plasmid extraction. The plasmids of scFv64 and scFv130 antibody fragments were successfully extracted from the *E. coli*, as shown in Figure 4.3. The concentration based on $OD_{A260/280}$ results of the plasmid extracts are listed in Table 4.3.

A pairs of specific primers were designed for both clones (scFv64*Nco*I_F and scFv*Hind*III_R for scFv64; scFv130*Avr*II_F and scFv*Hind*III_R for scFv130) with some modifications or additions of nucleotides inserted into the primers to generate new restriction sites for ease of cloning. The amplification resulted in the addition of new terminal restriction enzyme sites, *Nco*I and *Hind*III, for scFv64 antibody fragment and *Avr*II and *Hind*III for the scFv130 antibody fragment. Gradient PCR was carried out to determine the optimal annealing temperature for both clones, as shown in Figure 4.4. The fragments were successful amplified at 42ºC and the expected size for both amplicon was approximately 800bp, as shown in Figure 4.5.

After PCR amplification, the products (scFv64 and scFv130 antibody fragments with the desired restriction enzyme sites) were purified using QIAquick PCR Purification Kit. The sizes of the scFv64 and scFv130 antibody fragments after purification were both approximately 800bp, as shown in Figure 4.6.

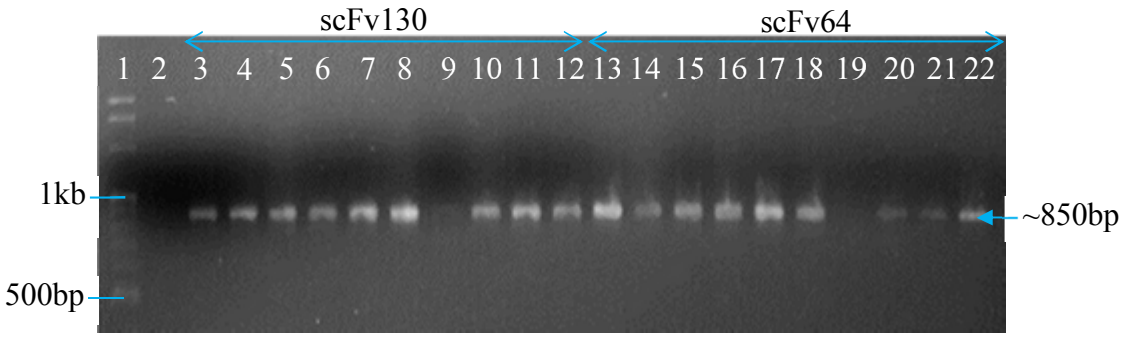


Figure 4.2: PCR screening for scFv64 and scFv130 antibody fragments using pCANTAB 5E_S1 and pCANTAB 5E_S6 primers.
The products were electrophoresed on 1% EtBr-stained agarose gel.
Lane 1: Marker; 100bp DNA ladder (EUR_X).
Lane 2: Negative control; without DNA template.
Lane 3-12: Colonies of scFv130 antibody fragment clone.
Lane 13-22: Colonies of scFv64 antibody fragment clone.

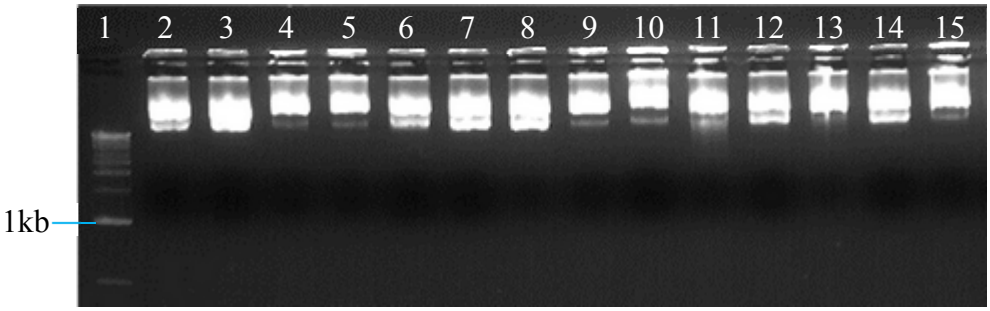


Figure 4.3: Agarose gel electrophoresis of plasmids with scFv130 and scFv64 antibody fragment after extraction.
Lane 1: Marker; 1kb DNA ladder (EUR_X).
Lane 2-8: Plasmid of scFv130 antibody fragment obtained from pCANTAB5E.
Lane 9-15: Plasmid of scFv64 antibody fragment obtained from pCANTAB5E.

Table 4.3: Concentration of scFv64 and scFv130 antibody fragments.

scFv130 antibody fragment			scFv64 antibody fragment		
Sample	Concentration µg/µl	Purification	Sample	Concentration µg/µl	Purification
1	2.473	1.90	8	2.562	1.88
2	2.230	1.85	9	2.765	1.87
3	2.451	1.85	10	2.599	1.83
4	2.167	1.86	11	2.007	1.87
5	2.353	1.89	12	2.606	1.84
6	2.687	1.88	13	2.214	1.78
7	2.851	1.84	14	2.751	1.85

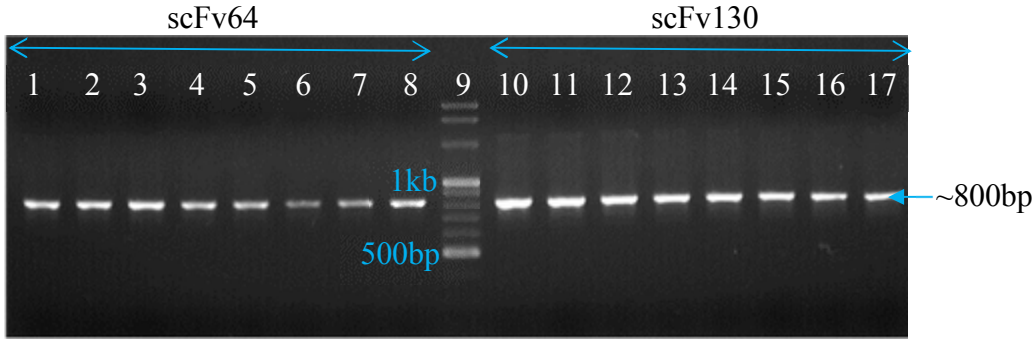


Figure 4.4: PCR optimization of scFv64 and scFv130 antibody fragments.
The products were electrophoresed on 1% EtBr-stained agarose gel.
Lane 1-8: Amplicon of scFv64 antibody fragment using scFv64*Nco*I_F & scFv*Hind*III_R with gradient annealing temperatures (42.0 ºC -58.0ºC).
Lane 9: Marker; 100bp DNA ladder (EUR_X).
Lane 10-11: Amplicon of scFv130 antibody fragment using scFv130*Avr*II_F & scFv*Hind*III_R with gradient annealing temperatures (42.0ºC-58.0ºC).

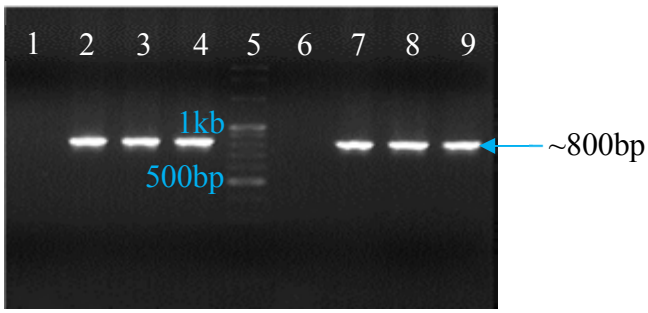


Figure 4.5: PCR amplification with optimum annealing temperature, 42°C**.**
The products were electrophoresed on 1% EtBr-stained agarose gel.
Lane 1 & 6: Negative control, without DNA template.
Lane 2-4: Amplicon of the scFv64 antibody fragment using scFv64*Nco*I_F & scFv*Hind*III_R.
Lane 5: Marker; 100bp DNA ladder (EUR_X).
Lane 7-9: Amplicon of the scFv130 antibody fragment using scFv130*Avr*II_F & scFv*Hind*III_R.

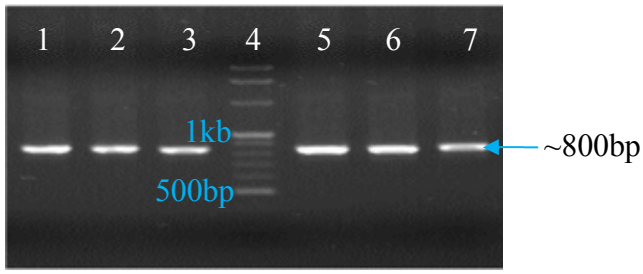


Figure 4.6: PCR purification products of scFv64 and scFv130 antibody fragments.
The products were electrophoresed on 1% EtBr-stained agarose gel.
Lane 1-3: Purification PCR products of scFv64 antibody fragment with *Nco*I and *Hind*III.
Lane 4: Marker; 100bp DNA ladder (EUR_X).
Lane 5-7: Purification PCR products of scFv130 antibody fragment with *Avr*II and *Hind*III.

4.1.3 Amplification of BBIKDEL and BBI gene fragments

PCR optimization was carried out using a synthetic BBIKDEL gene fragment as a template with 57BBIKDEL_F (forward) and 57BBIKDEL_R (reverse) primers. Gradient annealing temperatures, 50.0ºC (Lane2), 50.8ºC (Lane3), 52.1ºC (Lane4), 54.0ºC (Lane5), 56.2ºC (Lane6), 58.0 ºC (Lane7), 59.3 ºC (Lane8), and 60.0 ºC (Lane9) were used in the optimisation of the PCR. The expected size of the amplicon was approximately 800bp (Figure 4.7). The optimum annealing temperature was found to be at 59.3ºC, as shown in Figure 4.7.

The plasmid harbouring the synthetic BBIKDEL gene fragment was transformed into *E.coli* JM109 strain. PCR screening was carried out with a 57BBIKDEL primer set to screen for the positive colony. The expected amplicon (~800bp) from the PCR screening is as shown in Figure 4.8. Out of 22 colonies, 21 colonies gave a positive result (Figure 4.8).

The screened colonies with positive result were inoculated into LB broth prior to plasmid extraction. The extracted plasmids were electrophoresed on 1% agarose gel, as shown in Figure 4.9. The $OD_{A260/280}$ readings of the extracted plasmid are as given in Table 4.4.

PCR amplification with the specific primers (57BBIKDEL_F and 57BBI_R for amplifying the BBIKDEL gene fragment; and 57BBI_F and 57BBI_R for amplifying the BBI gene fragment) was carried out. The expected size for both amplicon, for, BBIKDEL and BBI gene fragment, were approximately 800bp (as shown in Figure 4.10).

Then, the PCR products were purified using QIAquick PCR Purification kit. The size of the purified BBIKDEL and BBI gene fragments were as expected at approximately ~800bp, as shown in Figure 4.11.

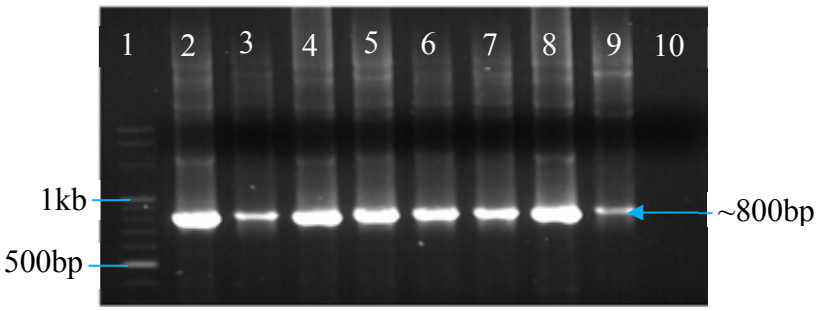


Figure 4.7: PCR optimisation for amplification of BBIKDEL gene fragment with 57BBIKDEL_F and 57BBIKDEL_R primer.
The products were electrophoresed on 1% EtBr-stained agarose gel.
Lane 1: Marker; 100bp DNA ladder (EUR_X).
Lane 2: BBIKDEL gene fragment at different annealing temperature (Lane 2:50.0ºC; Lane 3: 50.8ºC; Lane 4: 52.1ºC; Lane 5: 54.0ºC; Lane 6: 56.2ºC; Lane 7: 58.0ºC; Lane 8:59.3ºC; Lane 9: 60.0ºC).
Lane 10: Negative control; without DNA template.

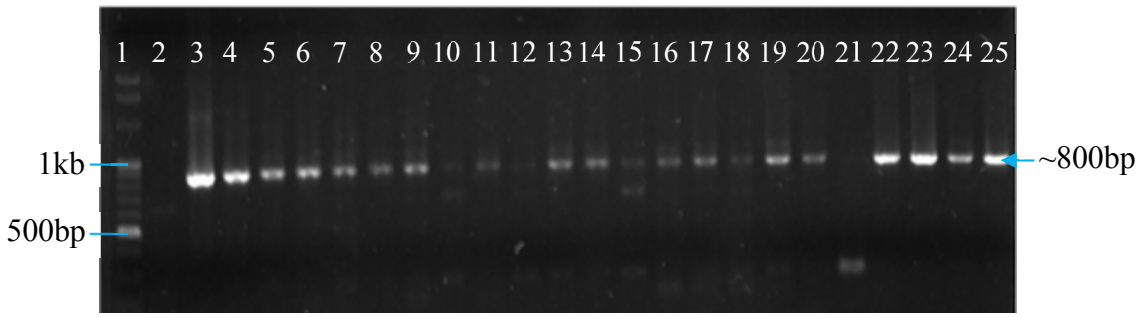


Figure 4.8: PCR screening of BBIKDEL gene fragment.
The products were electrophoresed on 1% EtBr-stained agarose gel.
Lane 1: Marker; 100bp DNA ladder (EUR_X).
Lane 2: Negative control; without DNA template.
Lane 3: Positive control; plasmids harbouring the BBIKDEL gene fragment.
Lane 4- 25: Colonies selected from the agar plate.

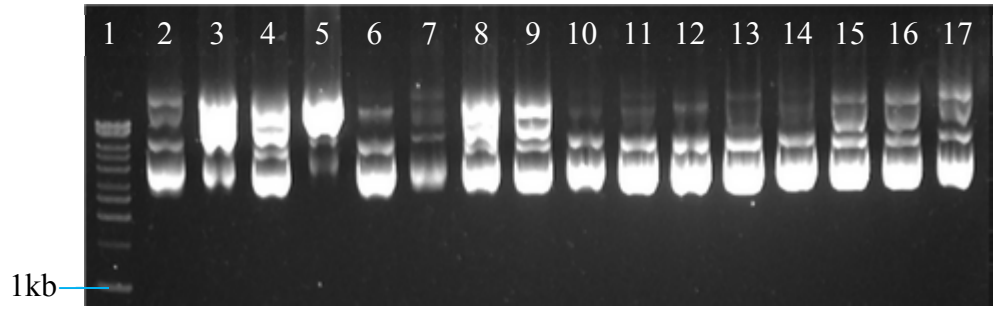


Figure 4.9: Plasmids harbouring the BBIKDEL gene fragment.
The samples were electrophoresed on 1% EtBr-stained agarose gel.
Lane 1: Marker; 1kb DNA ladder (EUR_X).
Lane 2-17: Purified plasmids with the BBIKDEL gene fragment.

Table 4.4: Concentration of BBIKDEL gene fragment.

Sample	Concentration µg/µl	Purification	Sample	Concentration µg/µl	Purification
1	1.285	1.83	9	1.546	1.81
2	1.705	1.85	10	1.644	1.78
3	1.629	1.79	11	1.835	1.81
4	1.124	1.74	12	1.662	1.81
5	1.394	1.75	13	1.570	1.82
6	0.274	1.77	14	1.637	1.81
7	1.361	1.80	15	1.563	1.79
8	1.486	1.83	16	1.228	1.78

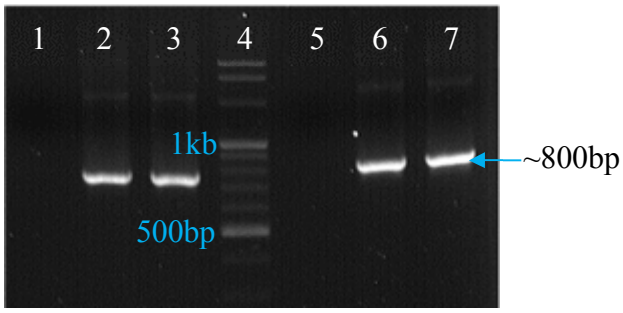


Figure 4.10: PCR amplification for BBIKDEL and BBI gene fragments.
The products were electrophoresed on 1% EtBr-stained agarose gel.
Lane 1 & 5: Negative control; without DNA template.
Lane 2-3: BBIKDEL gene fragment with desired RE sites.
Lane 4: Marker; 100bp DNA ladder (EUR_X).
Lane 6-7: BBI gene fragment with the desired RE sites.

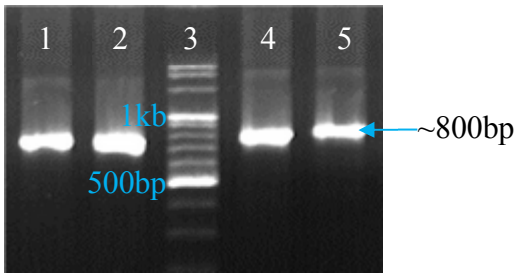


Figure 4.11: PCR purification product of BBIKDEL and BBI gene fragments.
The products were electrophoresed on 1% EtBr-stained agarose gel.
Lane 1-2: Purification PCR products of BBI gene fragment.
Lane 3: Marker; 100bp DNA ladder (EUR_X).
Lane 4-5: Purification PCR products of BBI KDEL gene fragment.

4.2 Restriction enzyme digestion

4.2.1 Digestion of pCambia1304 and pCambia1304*Avr*II

pCambia1304 was digested with *Nco*I and *Bgl*II, while the pCambia1304*Avr*II was digested with *Avr*II and *Bgl*II. The digestion products were electrophoresed on 1% EtBr-stained electrophoresis gel, as shown in Figure 4.12.

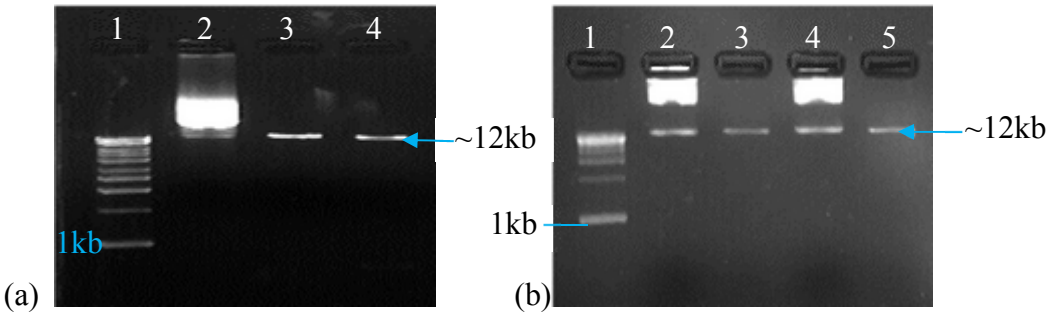


Figure 4.12: Restriction enzyme digestion of vector with *Nco*I and *Bgl*II enzymes. The products were electrophoresed on 1% EtBr-stained agarose gel.

(a) pCambia1304
Lane 1: Marker; 1kb DNA ladder (EUR_X).
Lane 2: Undigested pCambia1304, as control.
Lane 3-4: pCambia1304 digested with *Nco*I and *Bgl*II (expected size: ~12kb).

(b) pCambia1304*Avr*II
Lane 1: Marker; 1kb DNA ladder (EUR_X).
Lane 2 & 4: Undigested pCambia1304 *Avr*II, as control.
Lane 3 & 5: pCambia1304*Avr*II digested with *Avr*II and *Bgl*II (expected size: ~12kb).

4.2.2 Digestion of scFv64 & scFv130 antibody fragments and BBIKDEL & BBI gene fragments

PCR products of scFv64 antibody fragment were digested using *Nco*I and *Hind*III, and scFv130 antibody fragment with *Avr*II and *Hind*III. Enzymes that was used to digest PCR products of BBIKDEL and BBI gene fragments were *Hind*III and *Bgl*II. The digestion products were electrophoresed on 1% EtBr-stained agarose gel with un-digested PCR products as a control.

The digestion product sizes of scFv64 and scFv130 antibody fragments were approximately 800bp, while BBIKDEL & BBI gene fragments were approximately 650bp (as shown in Figure 4.13).

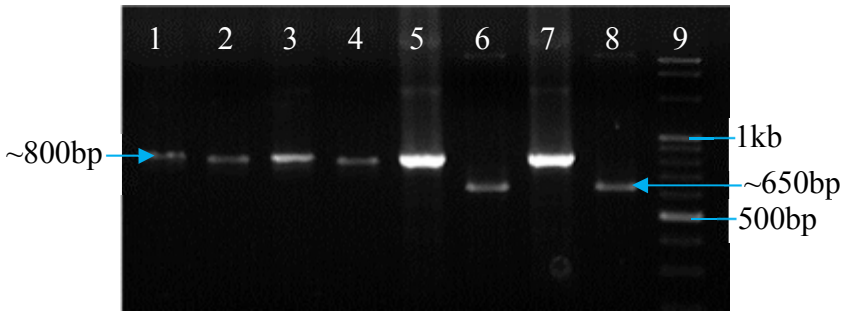


Figure 4.13: Restriction enzyme digestion of scFv64 & scFv130 antibody fragments and BBIKDEL & BBI gene fragments using different restriction enzymes. The products were electrophoresed on 1% EtBr-stained agarose gel.

Lane 1: Undigested scFv64 antibody fragment, ~800bp.
Lane 2: scFv64 antibody fragment digested with *Nco*I and *Hind*III, ~800bp.
Lane 3: Undigested scFv130 antibody fragment, ~800bp.
Lane 4: scFv130 antibody fragment digested with *Avr*II and *Hind*III, ~800bp.
Lane 5: Undigested BBIKDEL gene fragment, ~800bp.
Lane 6: BBIKDEL gene fragment digested with *Hind*III and *Bgl*II, ~650bp.
Lane 7: Undigested BBI gene fragment, ~800bp.
Lane8: BBI gene fragment digested with *Hind*III and *Bgl*II, ~650bp.
Lane 9: Marker; 100bp DNA ladder (EUR_X).

4.2.3 Purification of digested pCambia1304 and pCambia1304*Avr*II

Digestion products of pCambia1304 and pCambia1304*Avr*II were purified using QIAquick Gel Extraction Kit. The purified digested pCambia1304 and pCambia1304*Avr*II were electrophoresed on agarose gel, as shown in Figure 4.14.

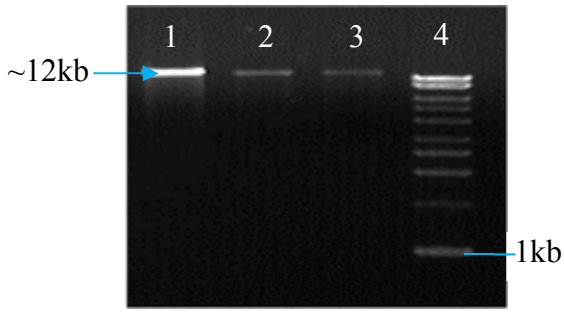


Figure 4.14: Gel purification product of digested pCambia1304 and pCambia1304*Avr*II.
The products were electrophoresed on 1% EtBr-stained agarose gel.
Lane 1: Purification of digested pCambia1304, ~12kb.
Lane 2-3: Purification of digested of pCambia1304*Avr*II, ~12kb.
Lane 4: Marker; 1kb DNA ladder (EUR_X).

4.2.4 Purification of digested scFv64 & scFv130 antibody fragments and BBIKDEL & BBI gene fragments

Follow 4.2.3, the products were electrophoresed on agarose gel and result was showed in Figure 4.15. Expected size of digested scFv64 and scFv130 antibody fragments are approximately 800bp; while BBIKDEL and BBI gene fragment are approximately 650bp.

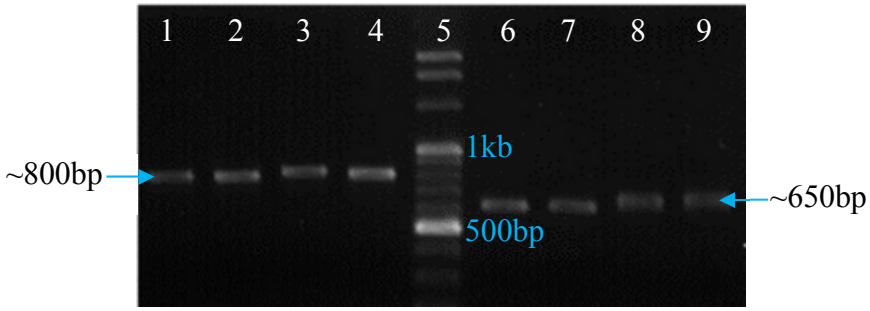


Figure 4.15: Gel purification products of digested scFv64 and scFv130 antibody fragments and BBIKDEL & BBI gene fragments.
The products were electrophoresed on 1% EtBr-stained agarose gel.
Lane 1-2: Purified digested product of scFv64 fragment, ~800bp.
Lane 3-4: Purified digested product of scFv130 fragment, ~800bp.
Lane 5: Marker; 100bp DNA ladder (EUR_X).
Lane 6-7: Purified digested product of BBIKDEL gene fragment, ~650bp.
Lane 8-9: Purified digested product of BBI gene fragment, ~650bp.

4.3 Cloning and transformation into *E.coli*

4.3.1 Construction of pToxo64BBI, pToxo64BBIKDEL, pToxo130BBI and pToxo130BBIKDEL

The purified digestion products of pCambia1304 and pCambia1304*Avr*II with scFv64, scFv130, BBI and BBIKDEL were ligated together, as summarized in Table 4.5. The constructs were then transformed into *E.coli* JM109 competent cells.

Table 4.5: Combination of vector and inserts for 4 different constructs.

Construct	Vector	Insert
pToxo64BBI	pCambia1304	scFv64 and BBI
pToxo64BBIKDEL	pCambia1304	scFv64 and BBIKDEL
pToxo130BBI	pCambia1304*Avr*II	scFv130 and BBI
pToxo130BBIKDEL	pCambia1304*Avr*II	scFv130 and BBIKDEL

4.3.2 Colony selection and PCR analysis

Colony PCR was carried out for confirmation. The colonies were screened with 1304SK_F and 1304SK_R primers. The expected size of amplicon is approximately 1.5kb, as shown in Figure 4.16.

For pToxo64BBI and pToxo64BBIKDEL, 5 colonies of each construct have been selected randomly to analyse with PCR using 1304SK primers. Out of 5 colonies that were selected, only one colony gave a positive result for each construct (refer to Lane 6 and Lane 9 of Figured 4.16a). While, for pToxo130BBI and pToxo130BBIKDEL, out of 6 randomly selected colonies, only one colony of each construct gave positive result for each construct (refer to Lane 3 and Lane 12 of Figure 4.16b).

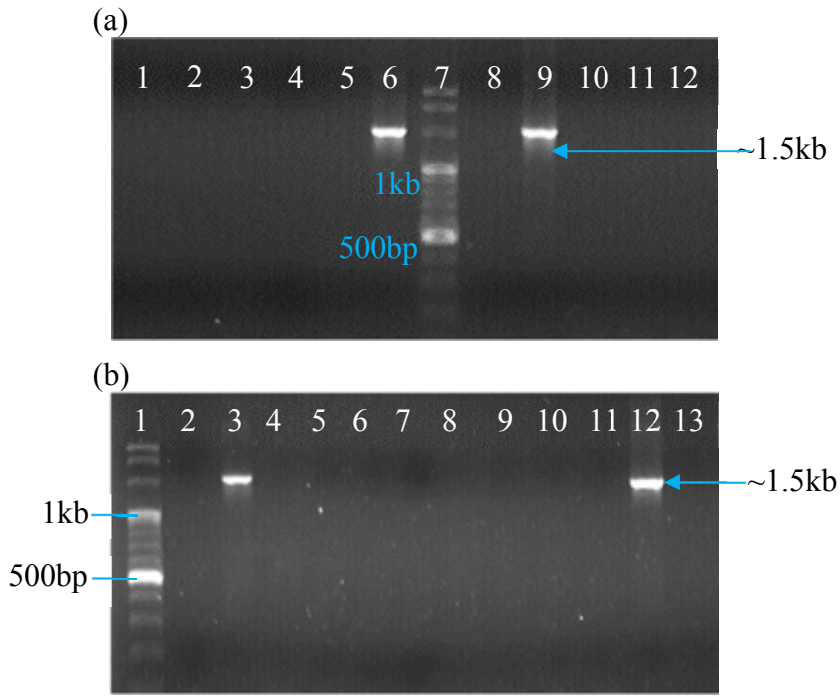


Figure 4.16: PCR screening of *E.coli* colonies with 1304SK primers.
The products were electrophoresed on 1% EtBr-stained agarose gel.

(a) pToxo64BBI and pToxo64BBIKDEL
Lane 1: Negative control; without DNA template.
Lane 2-6: Colony PCR of pToxo64BBI.
Lane 7: Marker; 100bp DNA ladder (EUR_X).
Lane 8-12: Colony PCR of pToxo64BBIKDEL.

(b) pToxo130BBI and pToxo130BBI-KDEL
Lane 1: Marker; 100bp DNA ladder (EUR_X).
Lane 2-7: Colony PCR of pToxo130BBI.
Lane 8-12: Colony PCR of pToxo130BBIKDEL.
Lane 13: Negative control; without DNA template.

4.3.3 Plasmid extraction of the constructs (pToxo64BBI, pToxo64BBIKDEL, pToxo130BBI and pToxo130BBIKDEL)

The positive colony (from Section 4.3.2) was inoculated in LB broth and plasmid extraction was carried out. Bacterial culture was prepared in duplicates for each construct prior to plasmid extraction. The extracted plasmids were electrophoresed on agarose gel (as shown in Figure 4.17) and $OD_{A260/280}$ readings are as listed in Table 4.6.

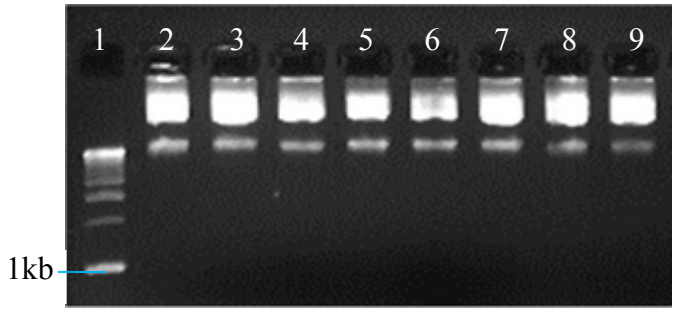


Figure 4.17: Plasmid extraction from *E.coli* JM109 harbouring different constructs. The samples were electrophoresed on 1% EtBr-stained agarose gel.
Lane 1: Marker; 1kb DNA ladder (EUR_X).
Lane 2-3: pToxo64BBI.
Lane 4-5: pToxo64BBIKDEL.
Lane 6-7: pToxo130BBI.
Lane 8-9: pToxo130BBIKDEL.

Table 4.6: Concentration of plasmids for all constructs after extraction.

Sample	Construct	Concentration µg/µl	Purification
1	pToxo64BBI	1.789	1.84
2	pToxo64BBI	1.381	1.81
3	pToxo64BBIKDEL	1.189	1.81
4	pToxo64BBIKDEL	0.914	1.81
5	pToxo130BBI	1.063	1.80
6	pToxo130BBI	1.076	1.78
7	pToxo130BBIKDEL	1.607	1.81
8	pToxo130BBIKDEL	1.627	1.81

4.3.4 PCR confirmation

PCR confirmation was carried out for all constructs (i.e. pToxo64BBI, pToxo64BBIKDL, pToxo130BBI and pToxo130BBIKDEL) with 1304SK primers. All constructs showed positive results (~1.5kb), as shown in Figure 4.18. Purified PCR products were sent for sequencing for further verification.

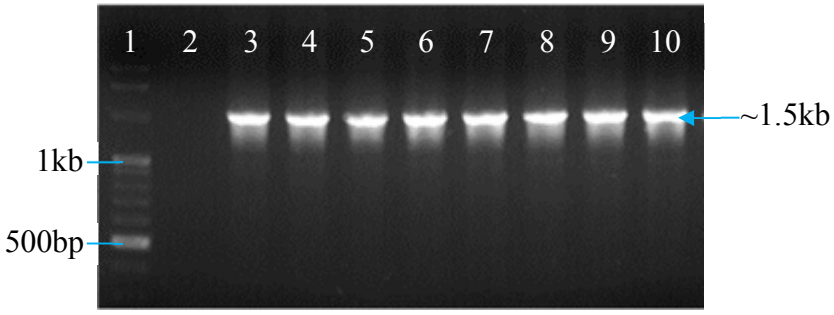


Figure 4.18: PCR confirmation for all constructs using 1304SK primers.
The products were electrophoresed on 1% EtBr-stained agarose gel
Lane 1: Marker; 100bp DNA ladder (EUR_X).
Lane 2: Negative control; without DNA template.
Lane 3-4: PCR product of pToxo64BBI.
Lane 5-6: PCR product of pToxo64BBIKDEL.
Lane 7-8: PCR product of pToxo130BBI.
Lane 9-10: PCR product of pToxo130BBIKDEL.

4.3.5 Restriction enzyme digestion for all constructs

All constructs (pToxo64BBI, pToxo64BBIKDEL, pToxo130BBI and pToxo130BBIKDEL) were digested with *Nde*I to examine the integration of the desired gene. The sizes of the digested fragments of each construct are as shown in Table 4.7. The digestion products were electrophoresed on 1% EtBr-stained agarose gel, as shown in Figure 4.19.

Table 4.7: Fragment sizes after digestion with *Nde*I.

Construct	Expected size of fragment after digestion with *Nde*I, bp			
pCambia1304	4283	3020	2569	2489
pToxo64BBI	4283	3020	3974	2489
pToxo64BBIKDEL	4283	3020	3995	2489
pToxo130BBI	4283	3020	3977	2489
pToxo130BBIKDEL	4283	3020	3998	2489

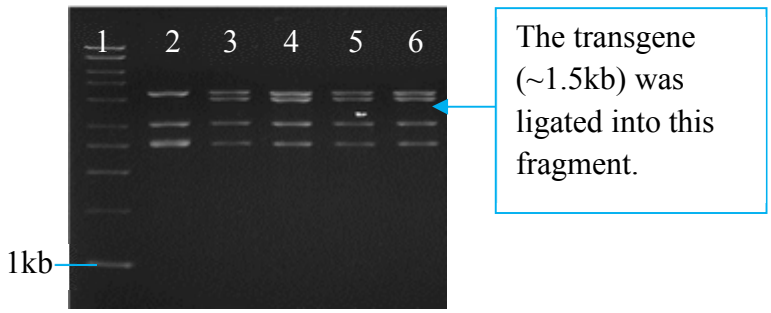


Figure 4.19: Digestion products for all constructs with *Nde*I.
The products were electrophoresed on 1% EtBr-stained agarose gel.
Lane 1: Marker; 1kb DNA ladder (EUR_X).
Lane 2: Digestion product of pCambia1304, size:2569bp.
Lane 3: Digestion product of pToxo64BBI, size:3974bp.
Lane 4: Digestion product of pToxo64BBIKDEL, size:3995bp.
Lane 5: Digestion product of pToxo130BBI, size:3977bp.
Lane 6: Digestion product of pToxo130BBIKDEL, size:3998bp.

4.3.6 Sequencing

The sequencing results confirmed the correct sequences for all constructs (pToxo64BBI, pToxo64BBIKDEL, pToxo130BBI and pToxo130BBIKDEL). The sequencing results are attached in Appendix C.

4.4 Cloning and transformation into *Agrobacterium sp.*

4.4.1 PCR screening on *Agrobacterium sp.* colony after transformation

The four constructs (pToxo64BBI, pToxo64BBIKDEL, pToxo130BBI and pToxo130BBIKDEL) were transformed into *Agrobacterium sp.* strain LBA4404 competent cells. Few colonies were seen on the LB agar plates. Five colonies of each construct were selected randomly and analysed with 1304SK primers and *gfp* primers. All selected colonies showed positive results, as shown in Figure 4.20.

pCambia1304 without transgene was also transformed into *Agrobacterium sp*. strain LBA4404 as a control. Two colonies were picked randomly and analyzed with *gfp* primers. Both colonies showed positive results, amplifying an approximately 750bp band (Lane 1 and Lane 2 in Figure 4.20b).

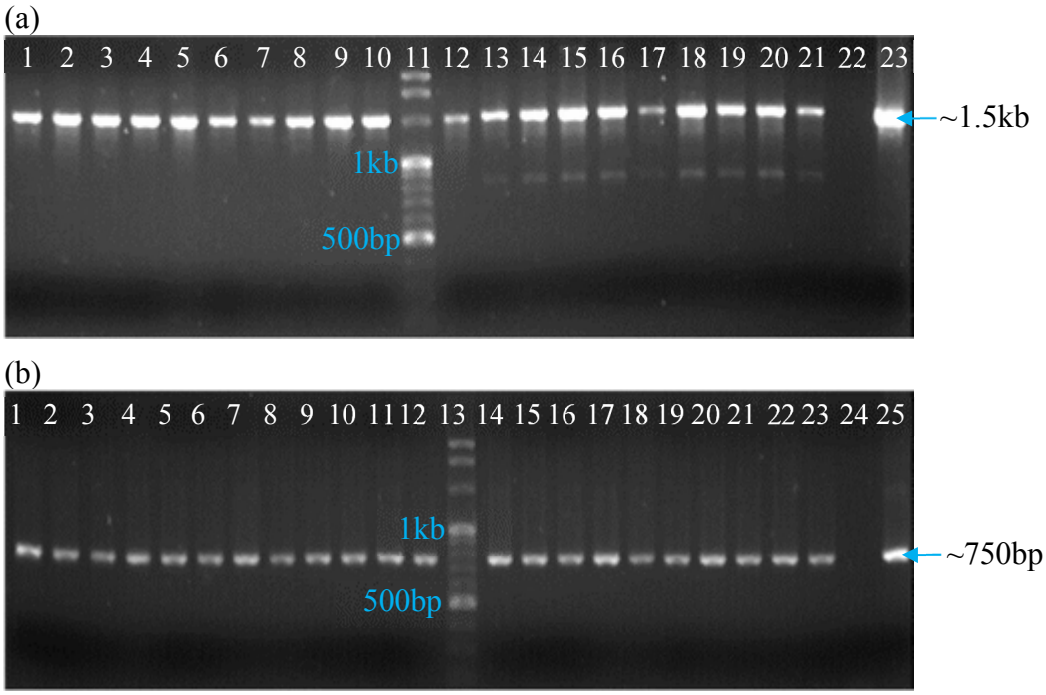


Figure 4.20: PCR screening for *Agrobacterium sp.* colonies after transformation. The products were electrophoresed on 1% EtBr-stained agarose gel.

(a) Analysis with 1304SK primers

Lane 1-5: Colonies of *Agrobacterium sp.* harboured pToxo64BBI.
Lane 6-10: Colonies of *Agrobacterium sp.* harboured pToxo64BBIKDEL.
Lane 11: Marker; 100bp DNA ladder (EUR_X).
Lane 12-16: Colonies of *Agrobacterium sp.* harboured pToxo130BBI.
Lane 17-21: Colonies of *Agrobacterium sp.* harboured pToxo130BBIKDEL.
Lane 22: Negative control; without DNA template.
Lane 23: Positive control; plasmid of construct (pToxo130BBI).

(b) Analysis with *gfp* primers

Lane 1-2: Colonies of *Agrobacterium sp.* harboured pCambia1304.
Lane 3-7: Colonies of *Agrobacterium sp.* harboured pToxo64BBI.
Lane 8-12: Colonies of *Agrobacterium sp.* harboured pToxo64BBIKDEL.
Lane 13: Marker; 100bp DNA ladder (EUR_X).
Lane 14-18: Colonies of *Agrobacterium sp.* harboured pToxo130BBI.
Lane 19-23: Colonies of *Agrobacterium sp.* harboured pToxo130BBIKDEL.
Lane 24: Negative control; without DNA template.
Lane 25: Positive control; plasmid of construct (pToxo130BBI).

4.5 *Agrobacterium*-mediated transformation into banana cell suspensions

4.5.1 GUS histochemical staining on inoculated cell suspensions

GUS assay was carried out on selected cell suspensions (after 4 days co-cultivation with the *Agrobacterium sp.* harbouring transgenes) as a transient assay prior to transformation. The non-inoculated cell suspensions were prepared as negative controls of the experiment.

As shown in Figure 4.21a, no blue colour spot was observed on the non-inoculated cell suspensions. The inoculated cell suspensions gave positive result, where blue colour spots were observed, as shown in Figure 4.21b-f. The stained cell suspensions were visualized under contrast phase light microscope with 400x magnification (refer to Figure 4.22).

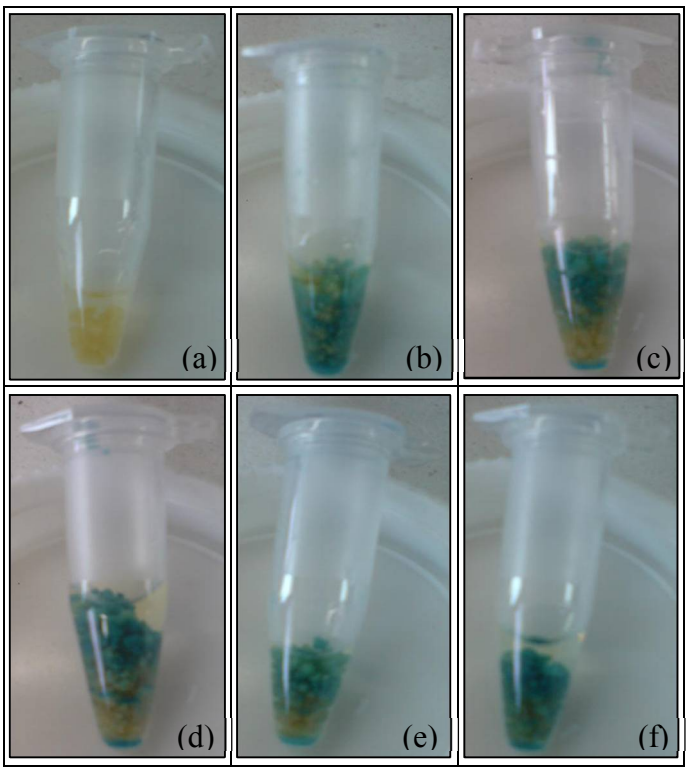


Figure 4.21: GUS histochemical staining of inoculated banana cell suspensions after 4 days co-cultivation with *Agrobacterium sp*.

Figure a: Non-inoculated; as negative control.

Figure b: Inoculated with pCambia1304, as positive control.

Figure c: Inoculated with *Agrobacterium sp*. harboured pToxo64BBI.

Figure d: Inoculated with *Agrobacterium sp*. harboured pToxo64BBIKDEL.

Figure e: Inoculated with *Agrobacterium sp*. harboured pToxo130BBI.

Figure f: Inoculated with *Agrobacterium sp*. harboured pToxo130BBIKDEL.

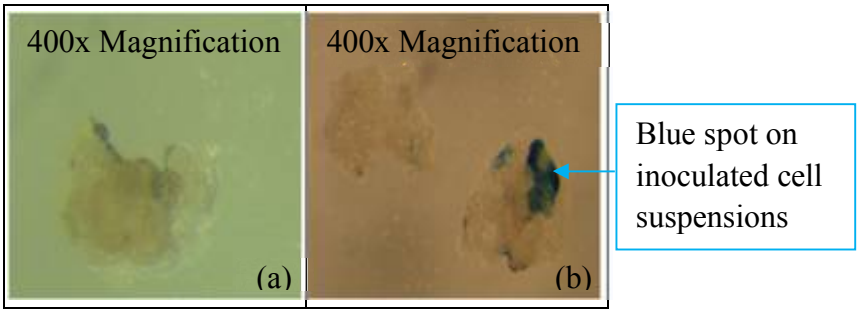


Figure 4.22: Microscopic visualization of non-inoculated and inoculated banana cell suspensions after GUS histochemical staining.

Figure a: Non-inoculated banana cell suspensions; as negative control (400x magnification).

Figure b: Inoculated banana cell suspensions (400x magnification). Blue spots were observed on the inoculated cells.

4.5.2 Molecular analysis of inoculated banana cell suspensions

4.5.2.1 Genomic DNA isolation of inoculated banana cell suspensions

Genomic DNA of inoculated banana cell suspensions was isolated after co-cultivation with *Agrobacterium sp*. elimination media for one month. The genomic DNA was electrophoresed on 1% EtBr-stained agarose gel, as shown in Figure 4.23. The isolated genomic DNA was used as template in PCR analysis.

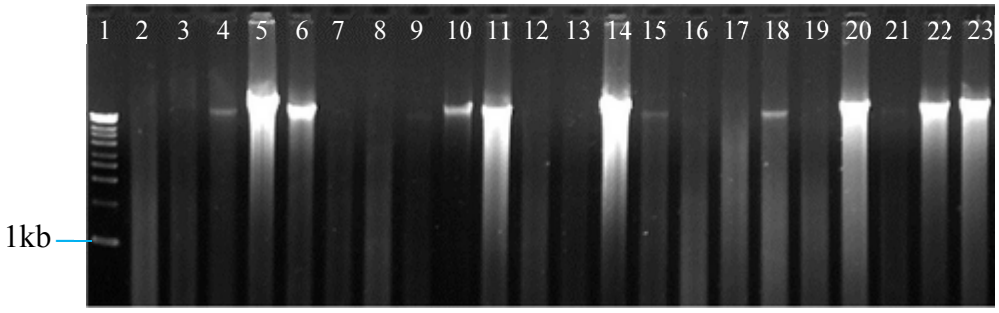


Figure 4.23: Genomic DNA of inoculated banana cell suspensions after 1 month co-cultivation with *Agrobacterium sp*. elimination media.
The samples were electrophoresed on 1% EtBr-stained agarose gel.
Lane 1: Marker; 1kb DNA ladder (EUR_X).
Lane 2-3: Genomic DNA of non-transformed cell suspensions.
Lane 4-8: Genomic DNA of pCambia1304 inoculated cell suspensions.
Lane 9-13: Genomic DNA of pToxo130BBI inoculated cell suspensions.
Lane 14-17: Genomic DNA of pToxo130BBIKDEL inoculated cell suspensions.
Lane 18-20: Genomic DNA of pToxo64BBI inoculated cell suspensions.
Lane 21-23: Genomic DNA of pToxo64BBIKDEL inoculated cell suspensions.

4.5.2.2 PCR analysis of genomic DNA of inoculated banana cell suspensions

PCR was carried out on the isolated genomic DNA. As shown in Figure 4.24, all samples showed positive results (amplification of an approximately 400bp band) when examined with the NtAct_F and NtAct_R primers. Thus, the genomic DNA of banana has been successfully isolated from the cell.

The presence of transgene in the genomic DNA was verified by PCR with 1304SK primers. Unfortunately, no positive results were observed in all the specimens tested.

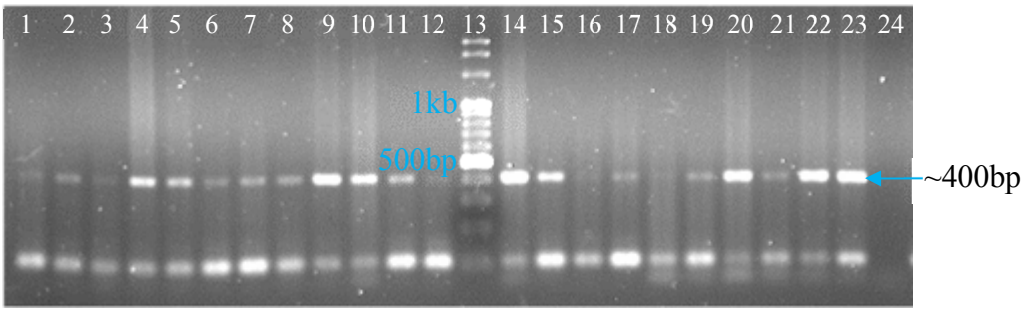


Figure 4.24: PCR analysis for the isolated genomic DNA with actin (NtAct) primers. The products were electrophoresed on 1% EtBr-stained agarose gel.
Lane 1-2: PCR product of non-transformed cell suspensions.
Lane 3-7: PCR product of pCambia1304 inoculated cell suspensions.
Lane 8-12: PCR product of pToxo130BBI inoculated cell suspensions.
Lane 13: Marker; 1kb DNA ladder (EUR_X).
Lane 14-17: PCR product of pToxo130BBIKDEL inoculated cell suspensions.
Lane 18-20: PCR product of pToxo64BBI inoculated cell suspensions.
Lane 21-23: PCR product of pToxo64BBIKDEL inoculated cell suspensions.
Lane 24: Negative control; without DNA template.

4.6 *Agrobacterium*-mediated transformation into tobacco leaf discs

4.6.1 GUS histochemical staining on regenerated tobacco plantlets

The one month old regenerated plantlets were stained with GUS histochemical reagents. In total 2 controls and 2 samples were analysed. The plant vector control and 2 transgenic plantlets both exhibited blue colouration after staining; while no colour changes were seen on non-transgenic plantlets. The results are as shown in Figure 4.25.

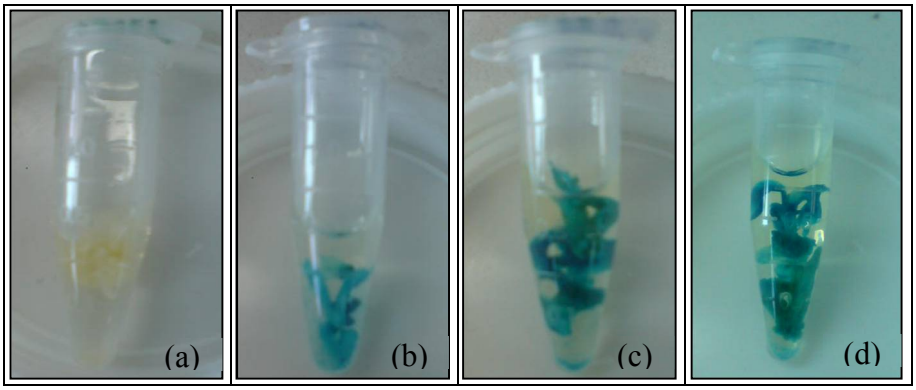


Figure 4.25: GUS histochemical staining of one month old non-transformed tobacco and transgenic tobacco plantlets.
Figure a: Non-transformed tobacco; as negative control.
Figure b: Transgenic tobacco plantlet with pCambia1304; as plant vector control.
Figure c: Transgenic tobacco plantlet with pToxo130BBI.
Figure d: Transgenic tobacco plantlet with pToxo130BBIKDEL.

4.6.2 Molecular analysis of transgenic tobacco plantlets

4.6.2.1 Genomic DNA isolation of transgenic tobacco leaves

Genomic DNA of 6 month old transgenic tobacco leaves were isolated for further verification, as shown in Figure 4.26.

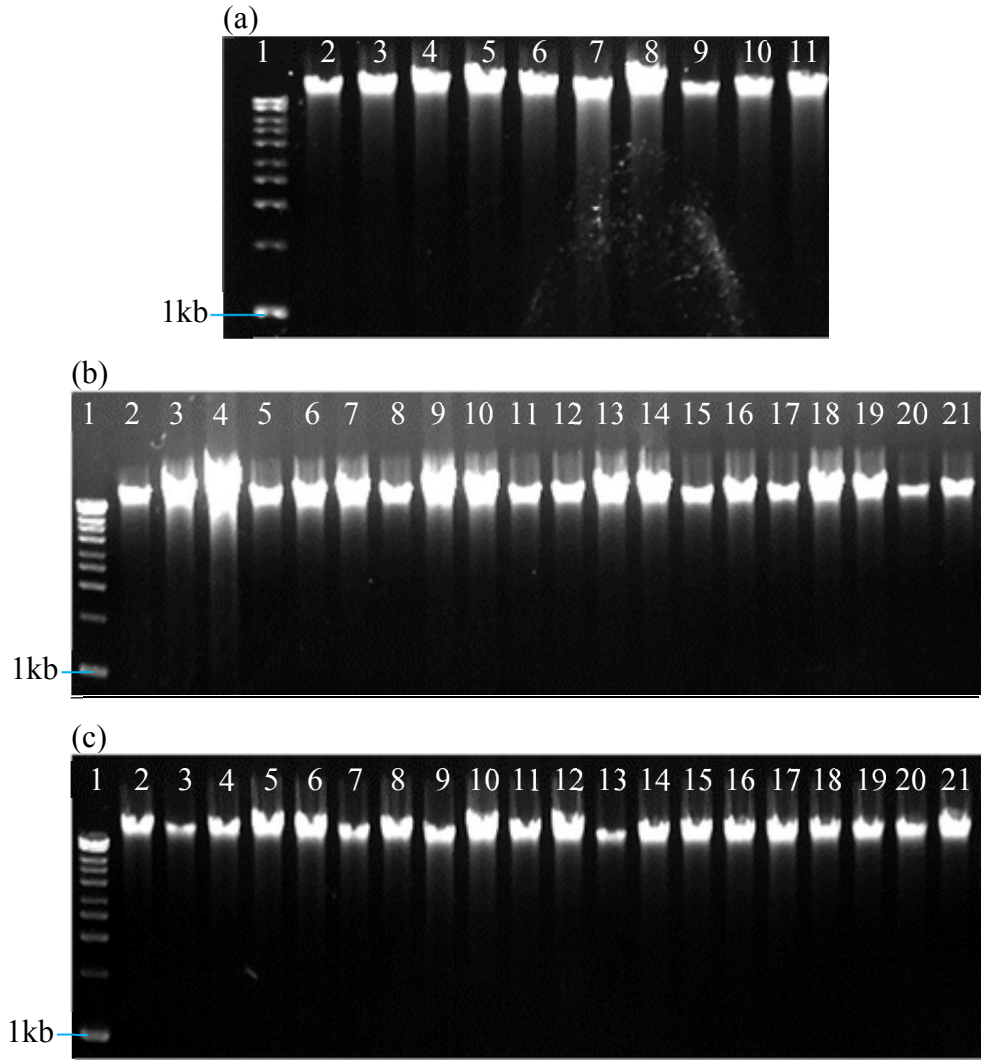


Figure 4.26: Genomic DNA isolation from six month old plantlets.
The products were analyzed on 1% EtBr-stained agarose gel.

(a) Non-transformed and transgenic tobacco with pCambia1304
Lane 1: Marker; 1kb DNA ladder (EUR_X).
Lane 2-5: Genomic DNA of non-transformed tobacco leaves.
Lane 6-11: Genomic DNA of transgenic tobacco with pCambia1304.

(b) Transgenic tobacco with pToxo130BBI
Lane 1: Marker; 1kb DNA ladder (EUR_X).
Lane 2-21: Genomic DNA of transgenic tobacco with pToxo130BBI.

(c) Transgenic tobacco with pToxo130BBIKDEL
Lane 1: Marker; 1kb DNA ladder (EUR_X).
Lane 2-21: Genomic DNA of transgenic tobacco with pToxo130BBIKDEL.

4.6.2.2 PCR analysis on isolated genomic DNA of transgenic tobacco

As showed in Figure 4.27, the isolated genomic DNAs were analyzed using actin gene (Actin9) primers. From the results, all but two of the samples, including non-transformed and transgenic tobacco, showed positive results.

From the PCR analysis, all except three samples of pToxo130BBI showed positive result when examined with 1304SK primers (refer to Lane 2, 4, and 18 in Figure 4.28b) and *gfp* primers (refer to Lane 2, 4, and 18 in Figure 4.29b). For samples of pToxo130BBIKDEL, 14 out of 20 samples showed amplification when analysed with 1304SK primers and *gfp* primers, as shown in Figure 4.28c and Figure 4.29c.

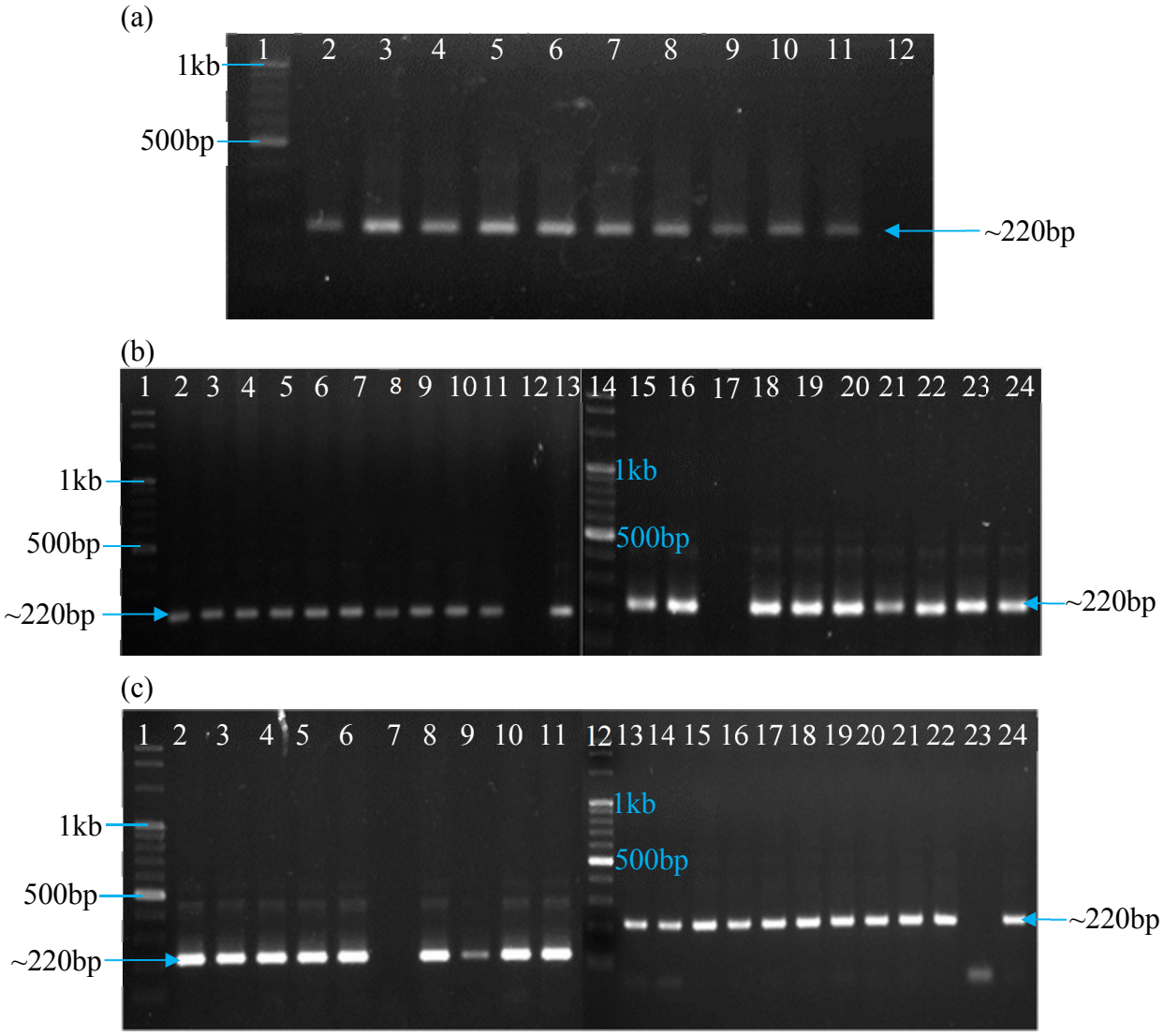


Figure 4.27: PCR analysis for the isolated genomic DNA with Actin9 primers. The products were analyzed on 1% EtBr-stained agarose gel.

(a) Non-transformed and transgenic tobacco with pCambia1304
Lane 1: Marker; 100bp DNA ladder (EUR_X).
Lane 2-5: Genomic DNA of non-transformed tobacco.
Lane 6-11: Genomic DNA of transgenic tobacco with pCambia1304.
Lane 12: Negative control; without DNA template.

(b) Transgenic tobacco with pToxo130BBI
Lane 1 & 14: Marker; 100bp DNA ladder (EUR_X).
Lane 2-11: Genomic DNA of transgenic tobacco with pToxo130BBI.
Lane 12: Negative control; without DNA template.
Lane 13: Positive control; plasmid of pToxo130BBI.
Lane 15-24: Genomic DNA of transgenic tobacco with pToxo130BBI.

(c) Transgenic tobacco with pToxo130BBIKDEL
Lane 1 &12: Marker; 100bp DNA ladder (EURX).
Lane 2-11: Genomic DNA of transgenic tobacco with pToxo130BBIKDEL.
Lane 13-22: Genomic DNA of transgenic tobacco with pToxo130BBIKDEL.
Lane 23: Negative control; without DNA template.
Lane 24: Positive control; plasmid of pToxo130BBI.

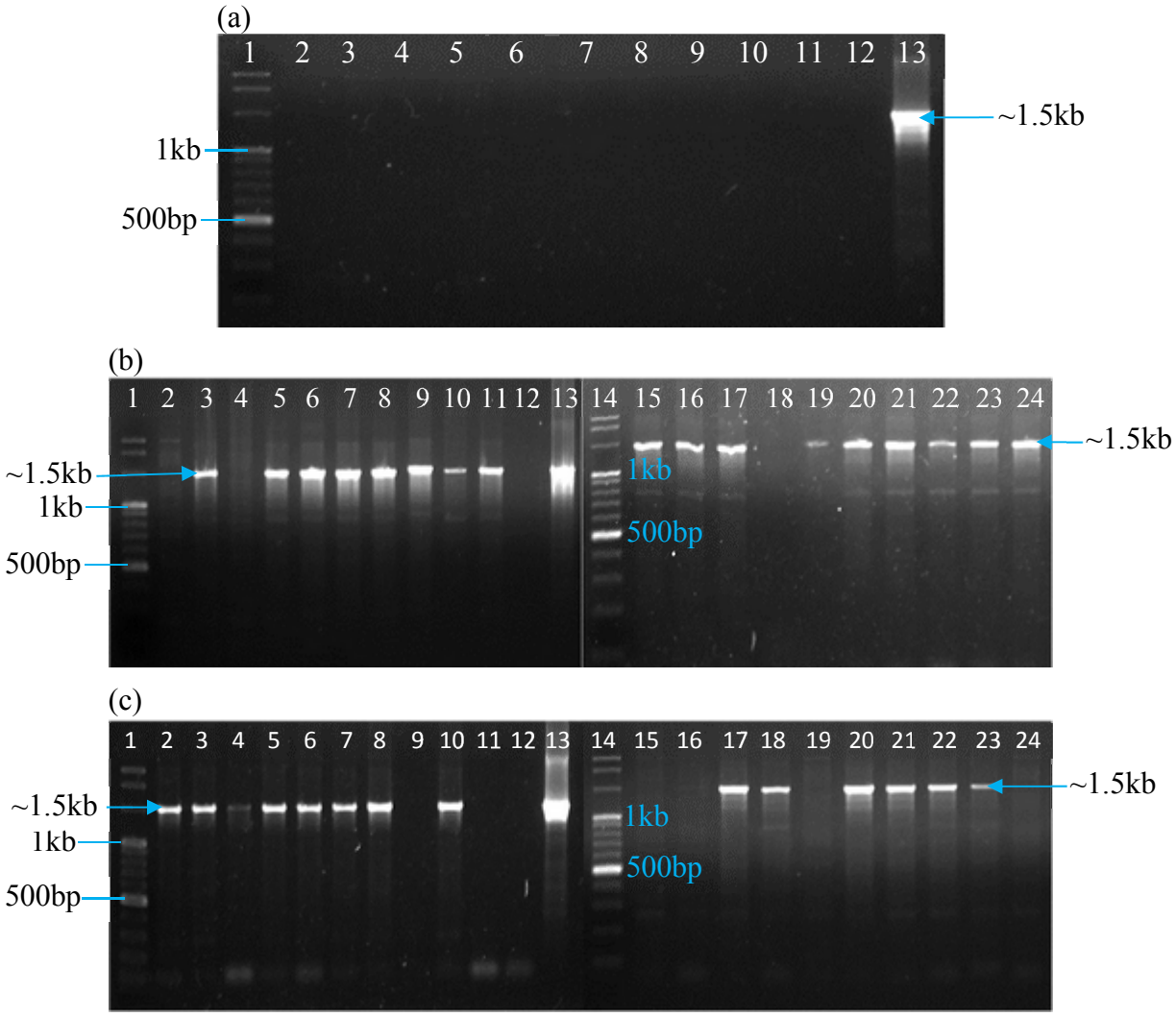


Figure 4.28: PCR analysis for the isolated genomic DNA with 1304SK primers. The products were analyzed on 1% EtBr-stained agarose gel.

(a) Non-transformed and transgenic tobacco with pCambia1304
Lane 1: Marker; 100bp DNA ladder (EUR_X).
Lane 2-5: Genomic DNA of non-transformed tobacco.
Lane 6-11: Genomic DNA of transgenic tobacco with pCambia1304.
Lane 12: Negative control; without DNA template.
Lane 13: Positive control; plasmid of pToxo130BBI.

(b) Transgenic tobacco with pToxo130BBI
Lane 1 & 12: Marker; 100bp DNA ladder (EUR_X).
Lane 2-11: Genomic DNA of transgenic tobacco with pToxo130BBI.
Lane 13-22: Genomic DNA of transgenic tobacco with pToxo130BBI.
Lane 23: Negative control; without DNA template.
Lane 24: Positive control; plasmid of pToxo130BBI.

(c) Transgenic tobacco with pToxo130BBIKDEL
Lane 1 & 14: Marker; 100bp DNA ladder (EUR_X).
Lane 2-11: Genomic DNA of transgenic tobacco with pToxo130BBIKDEL.
Lane 12: Negative control; without DNA template.
Lane 13: Positive control; plasmid of pToxo130BBI.
Lane 15-24: Genomic DNA of transgenic tobacco with pToxo130BBIKDEL.

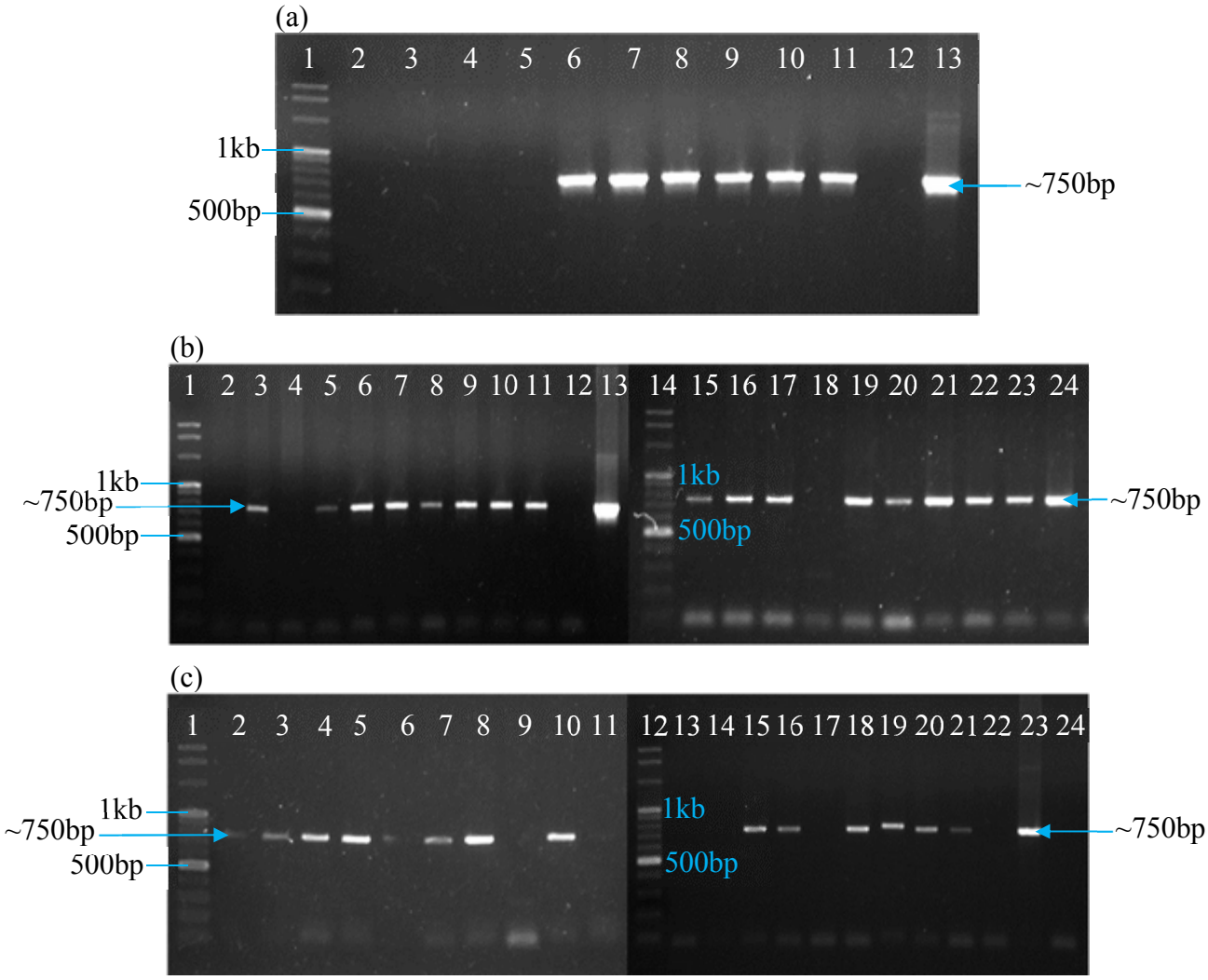


Figure 4.29: PCR analysis for the isolated genomic DNA with *gfp* primers. The products were analyzed on 1% EtBr-stained agarose gel.

(a) Non-transformed and transgenic tobacco with pCambia1304

Lane 1: Marker; 100bp DNA ladder (EUR_X).
Lane 2-5: Genomic DNA of non-transformed tobacco.
Lane 6-11: Genomic DNA of transgenic tobacco with pCambia1304.
Lane 12: Negative control; without DNA template.
Lane 13: Positive control; plasmid of pToxo130BBI.

(b) Transgenic tobacco with pToxo130BBI

Lane 1 & 14: Marker; 100bp DNA ladder (EUR_X).
Lane2-11: Genomic DNA of transgenic tobacco with pToxo130BBI.
Lane 12: Negative control; without DNA template.
Lane 13: Positive control; plasmid of pToxo130BBI.
Lane 15-24: Genomic DNA of transgenic tobacco with pToxo130BBI.

(c) Transgenic tobacco with pToxo130BBIKDEL

Lane 1 & 12: Marker; 100bp DNA ladder (EUR_X).
Lane 2-11: Genomic DNA of transgenic tobacco with pToxo130BBIKDEL.
Lane 13-22: Genomic DNA of transgenic tobacco with pToxo130BBIKDEL.
Lane 23: Positive control; plasmid of pToxo130BBI.
Lane 24: Negative control; without template.

4.7 Real-time PCR analysis

4.7.1 RNA extraction of transgenic tobacco

After PCR analysis on the isolated genomic DNA of transgenic tobacco, the samples which showed positive results were selected for RNA isolation using easy-spin^{TM} [DNA free] Total RNA Extraction Kit (iNtRON Biotechnology,Inc) prior to Real-time PCR. The isolated RNA was quantified and listed in Table 4.8. The RNA isolation products were electrophoresed on 1% EtBr stained agarose gel, as shown in Figure 4.30. DNaseI treatment was carried out to remove the remaining genomic DNA from 1 µg RNA, as shown in Figure 4.31.

Table 4.8: $OD_{A260/280}$ and $OD_{A260/230}$ readings of non-transformed and transgenic tobacco after RNA extraction.

Sample	Concentration µg/µl	Purification		Sample	Concentration µg/µl	Purification	
		$OD_{A260/280}$	$OD_{A260/230}$			$OD_{A260/280}$	$OD_{A260/230}$
Non-transformed (1)	1.106	1.68	2.51	pToxo130BBI (17)	0.380	1.61	2.07
Non-transformed (2)	1.321	1.71	2.54	pToxo130BBI (19)	0.909	1.74	2.00
pCambia1304 (1)	0.429	1.70	2.25	pToxo130BBI (20)	0.796	1.66	2.35
pCambia1304 (2)	0.463	1.69	2.11	pToxo130BBIKDEL (1)	1.049	1.63	2.35
pToxo130BBI (2)	0.407	1.71	2.11	pToxo130BBIKDEL (2)	1.058	1.67	2.56
pToxo130BBI (4)	2.639	1.79	2.55	pToxo130BBIKDEL (3)	0.863	1.75	2.40
pToxo130BBI (5)	0.744	1.75	1.84	pToxo130BBIKDEL (4)	1.400	1.86	2.33
pToxo130BBI (6)	2.165	1.74	2.66	pToxo130BBIKDEL (5)	0.587	1.67	2.51
pToxo130BBI (7)	0.625	1.70	2.46	pToxo130BBIKDEL (6)	0.471	1.66	2.42
pToxo130BBI (8)	0.658	1.76	1.87	pToxo130BBIKDEL (7)	1.178	1.69	2.60
pToxo130BBI (9)	1.467	1.71	2.56	pToxo130BBIKDEL (9)	0.554	1.75	2.30
pToxo130BBI (10)	1.778	1.73	2.64	pToxo130BBIKDEL (13)	0.982	1.70	2.42
pToxo130BBI (11)	0.600	1.65	2.30	pToxo130BBIKDEL (14)	1.472	1.70	2.56
pToxo130BBI (12)	0.437	1.72	1.76	pToxo130BBIKDEL (16)	0.829	1.61	2.16
pToxo130BBI (13)	0.571	1.65	2.28	pToxo130BBIKDEL (17)	1.217	1.65	2.42
pToxo130BBI (15)	0.529	1.66	2.20	pToxo130BBIKDEL (18)	1.065	1.63	2.39
pToxo130BBI (16)	1.329	1.65	2.53	pToxo130BBIKDEL (19)	1.571	1.71	2.47

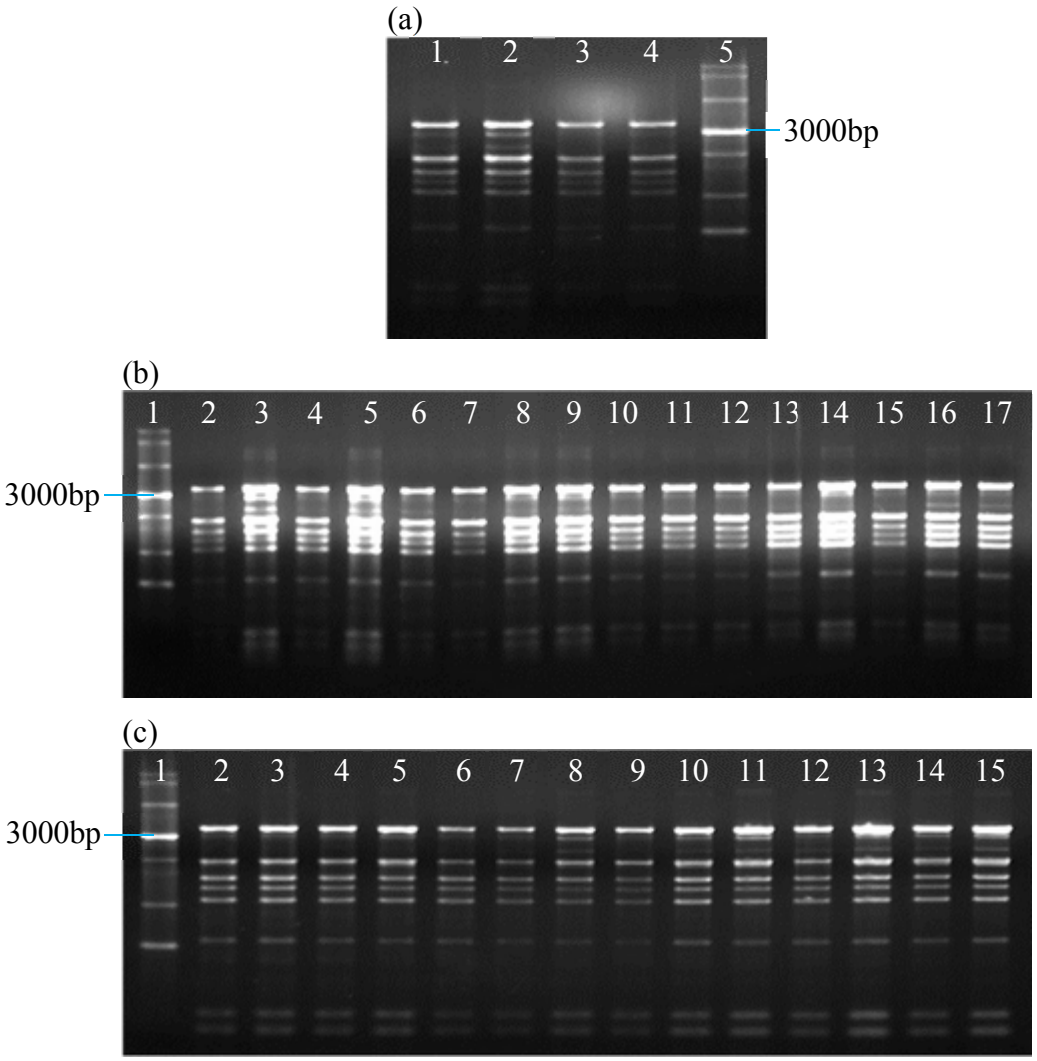


Figure 4.30: RNA of six month old plantlets.
The products were analyzed on 1% EtBr-stained agarose gel.

(a) Non-transformed and transgenic tobacco with pCambia1304
Lane 1-2: RNA of non-transformed tobacco (control).
Lane 3-4: RNA of transgenic tobacco with pCambia1304 vector.
Lane 5: Marker; ssRNA ladder (NEB).

(b) Transgenic tobacco pToxo130BBI
Lane 1: Marker; ssRNA ladder (NEB).
Lane 2-17: RNA of transgenic tobacco pToxo130BBI.

(c) Transgenic tobacco with pToxo130BBIKDEL
Lane 1: Marker; ssRNA ladder (NEB).
Lane 2-15: RNA of transgenic tobacco with pToxo130BBIKDEL.

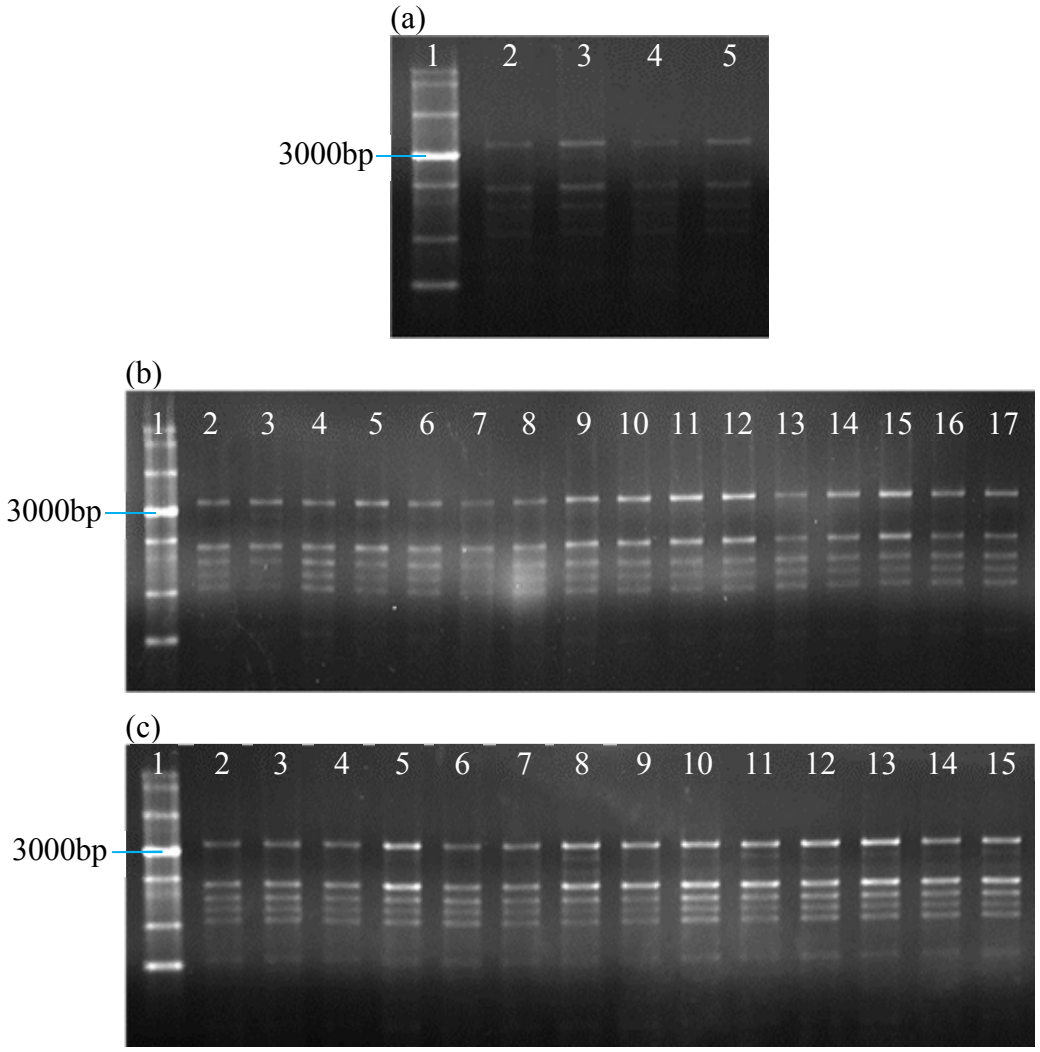


Figure 4.31: RNA after removing genomic DNA with DNaseI treatment.
The products were analyzed on 1% EtBr-stained agarose gel.

(a) Non-tansformed and transgenic tobacco with pCambia1304
Lane 1: Marker; ssRNA ladder (NEB).
Lane 2-3: Treated RNA of non-transformed tobacco.
Lane 4-5: Treated RNA of transgenic tobacco with pCambia1304.

(b) Transgenic tobacco with pToxo130BBI
Lane 1: Marker; ssRNA ladder (NEB).
Lane 2-17: Treated RNA of transgenic tobacco with pToxo130BBI.

(c) Transgenic tobacco with pToxo130BBIKDEL
Lane 1: Marker; ssRNA ladder (NEB).
Lane 2-15: Treated RNA of transgenic tobacco with pToxo130BBIKDEL.

4.7.2 Reverse-transcriptase PCR on RNA of transgenic tobacco

The DNaseI treated RNA was used to synthesis cDNA using Reverse-transcriptase PCR. The synthesized cDNA were prepared prior to Real-time PCR analysis.

4.7.3 Real-time PCR using cDNA of transgenic tobacco

Three pairs of the primers (Actin9, *gfp*180 and TG13090) that designed for the relative quantification were examined with dissociation program for their specificity. The results are shown in Figure 4.32. From the dissociation curves, these three pair of primers showed only single peak, which indicated that they are qualified for Real-time PCR analysis.

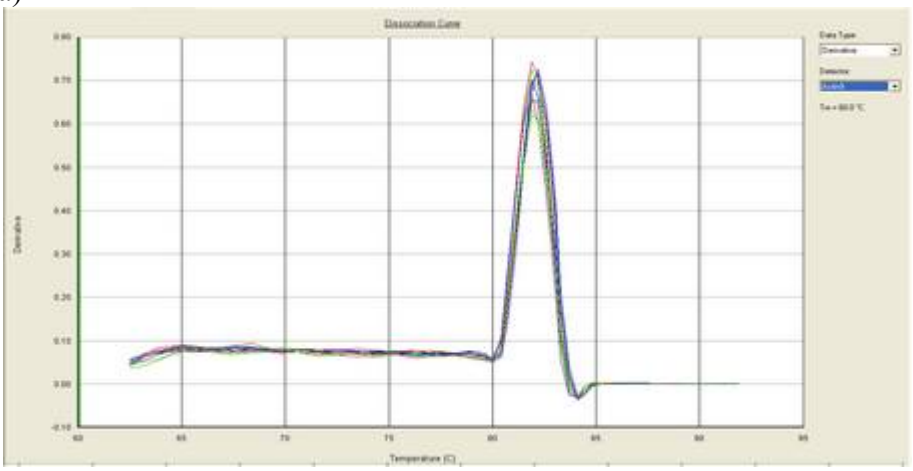
Next, to ensure the equal amount of template is used, a standard curve was constructed based on relative quantification. The standard curve is prepared by 5 series of 5x serial dilution of cDNA. The amplification plot of three different primers sets (Actin9, *gfp*180 and TG13090) are shown in Figure 4.33. The generated average Ct values are summarized in Table 4.9 and graph of average Ct value versus Log cDNA input are shown in Figure 4.34. The linear equation and R square value of 3 pair primers were also included in the Figure 4.34. The values of R square are nearly 0.98 which indicated good confidence in correlating two values.

There are 3 samples of non-transformed (control), 3 samples of transgenic tobacco with pCambia1304 vector, 12 samples of transgenic tobacco with pToxo130BBI and 12 samples of transgenic tobacco with pToxo130BBIKDEL have been studied in relative quantification. The amplification plots for 3 different sets primers with 4 different set samples are shown in Figure 4.35, 4.36 and 4.37.

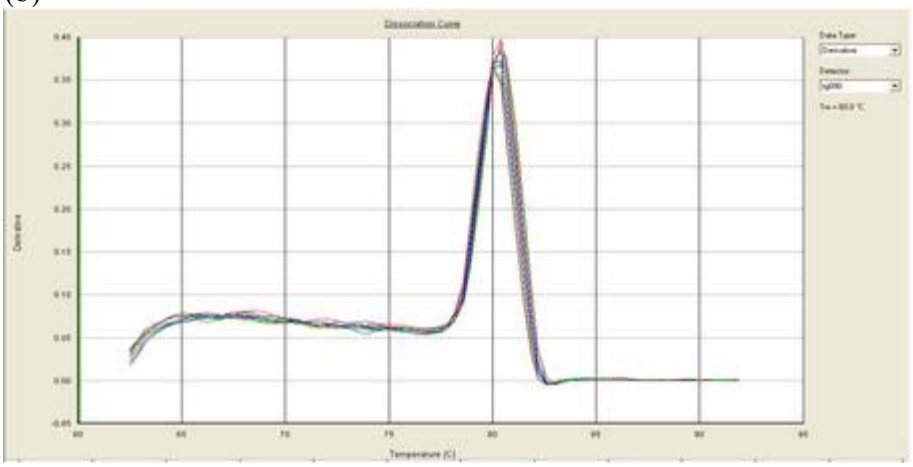
The generated data from the relative quantification was analyzed with SDS Software 1.3.1. The results were summarized in Table 4.10 and bar charts (Figure 4.38

and 4.41). Based on bar chart in Figure 4.41, sample UT(3) is given negative result, it may due to contamination during preparation. Sample pToxo130BBIKDEL, 130BK(14) was not showed any result in both expression, as shown in Figure 4.38 and 4.39. It is likely due to the degradation of cDNA. The average mRNA level for the leaves transformed with the construct pToxo130BBI was 4.4 fold; while pToxo130BBIKDEL is 3.58 fold when compared to non-transformed. The mRNA level of pToxo130BBI was 0.82-fold higher than pToxo130BBIKDEL.

(a)



(b)



(c)

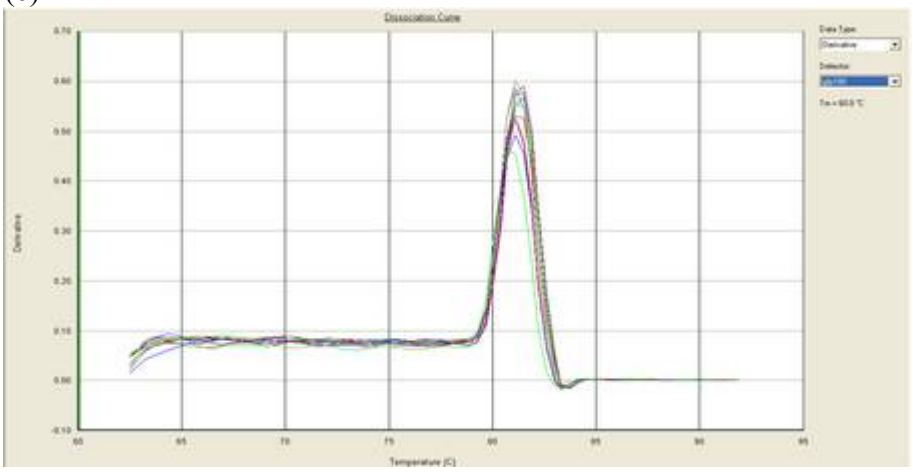
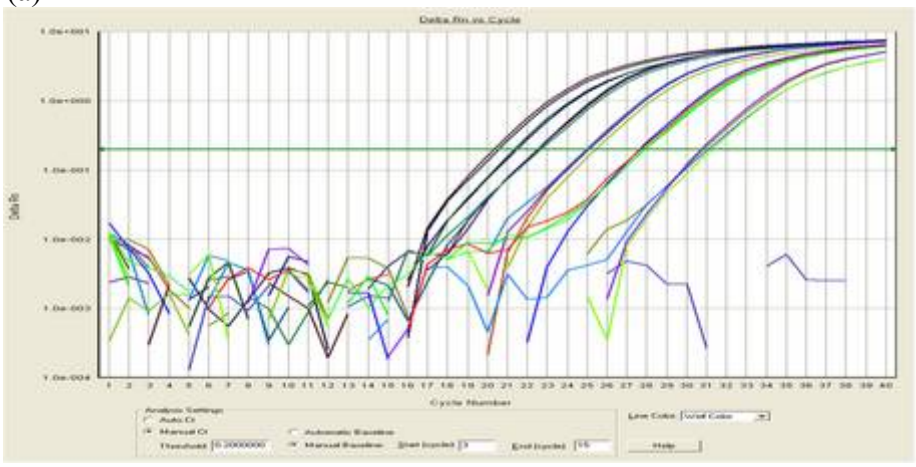


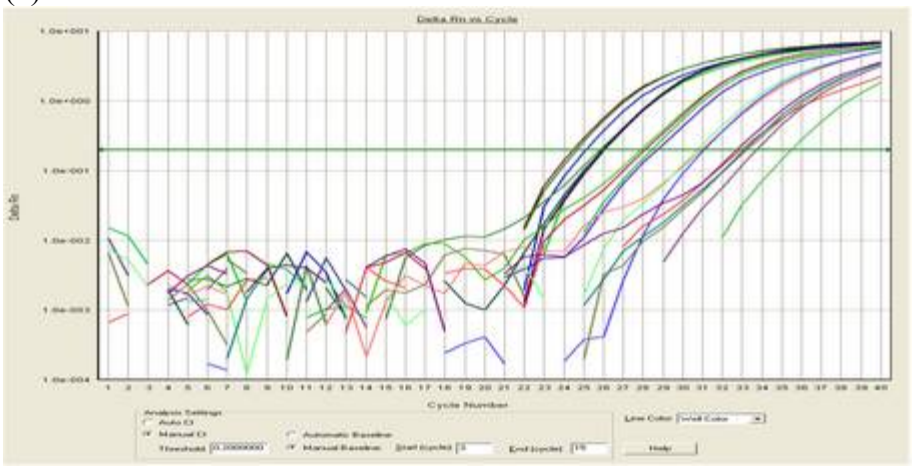
Figure 4.32: Dissociation curve of different primer sets at 60 ºC (Derivative versus temperature).

(a) Actin9 primers
(b) TG13090 primers
(c) *gfp*180 primers

(a)



(b)



(c)

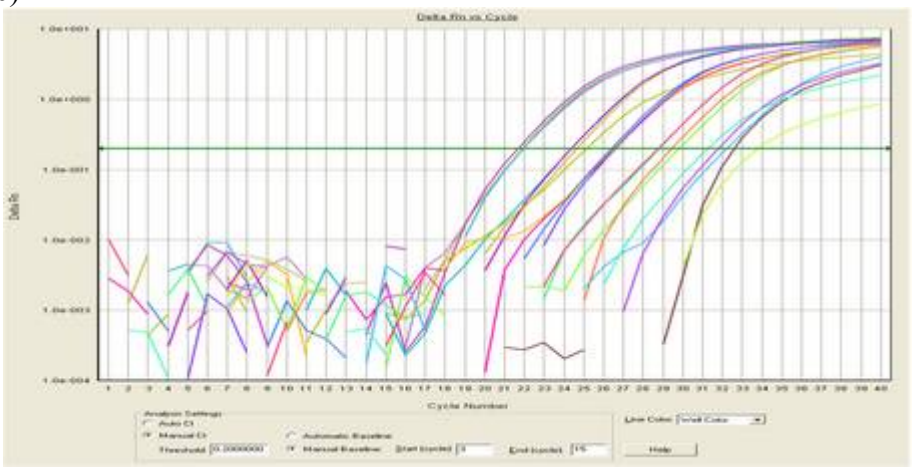


Figure 4.33: Amplification plot of 5x serial dilution cDNA (Delta Rn versus cycle).

(a) Actin9 primers
(b) TG13090 primers
(c) *gfp*180 primers

Table 4.9: Average Ct value of 5 series of 5x serial dilution cDNA.

cDNA Input	Log cDNA Input	Average Ct (Actin)	Average Ct (TG)	Average Ct (*gfp*)
1.0000	0	20.863	25.016	21.850
0.2000	-0.698970004	22.523	25.929	24.560
0.0400	-1.397940009	25.092	28.251	26.444
0.0080	-2.096910013	27.759	30.893	29.142
0.0016	-2.795880017	30.954	33.163	32.223

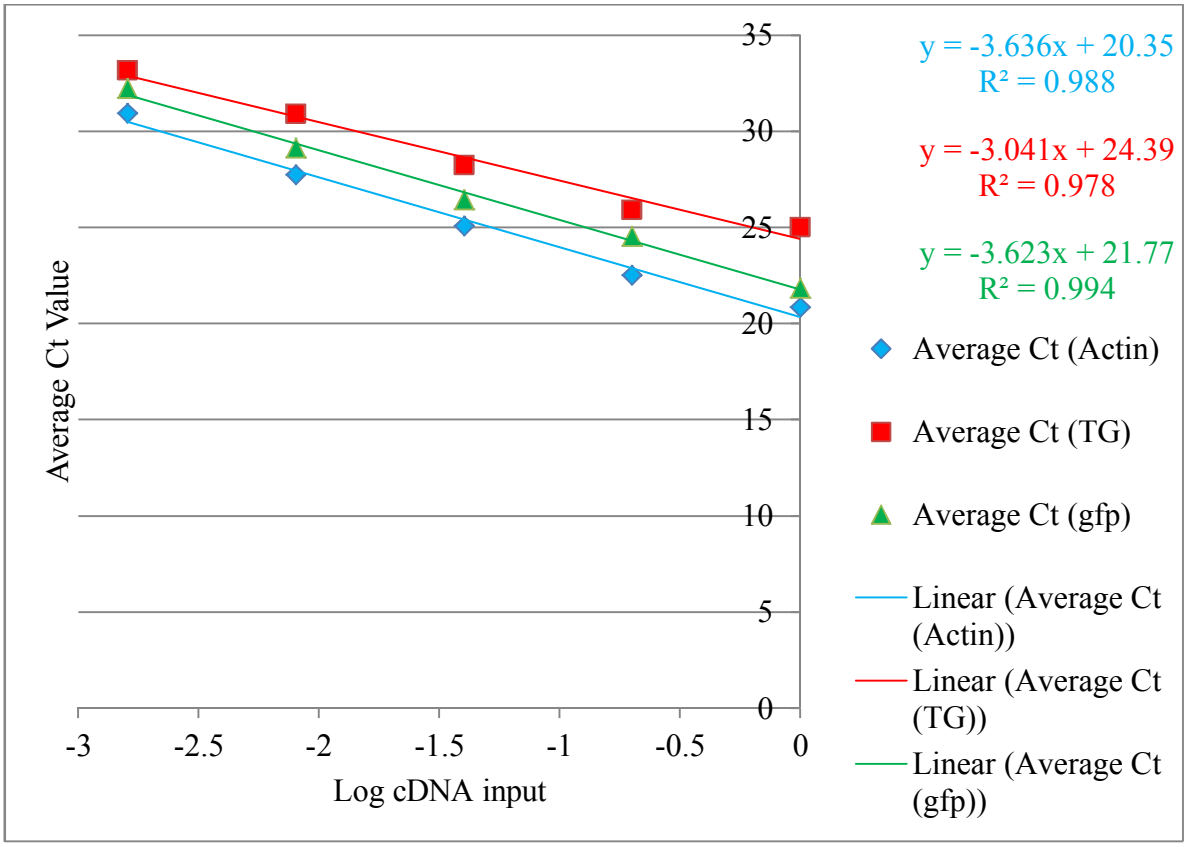
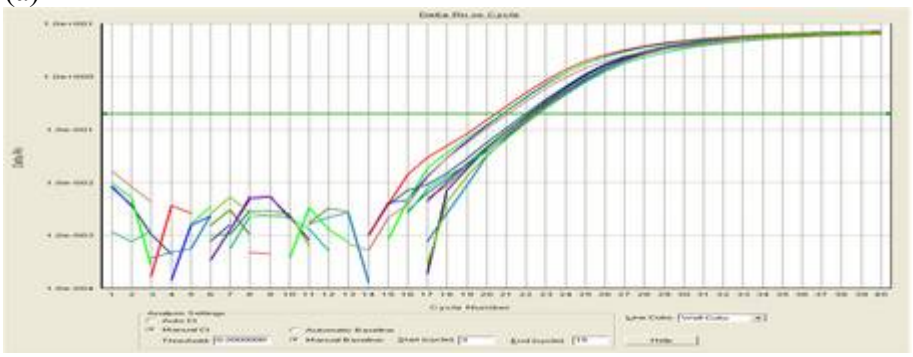
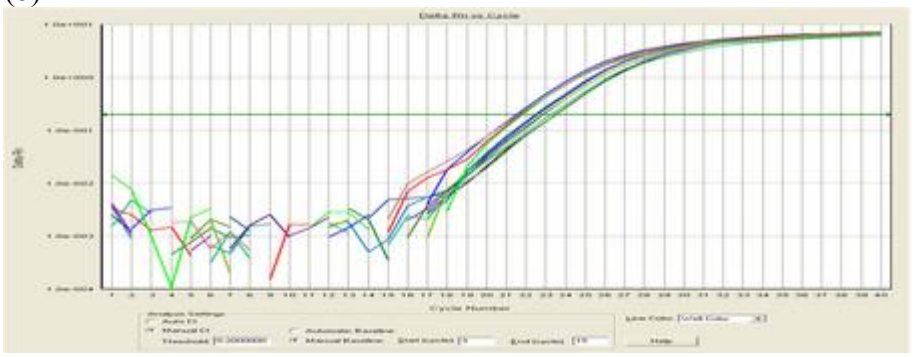


Figure 4.34: Graph of average Ct value of Actin, TG and *gfp* versus Log cDNA input.

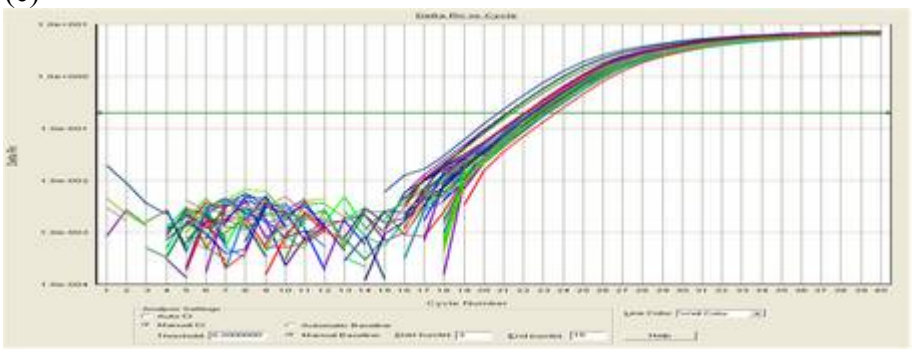
(a)



(b)



(c)



(d)

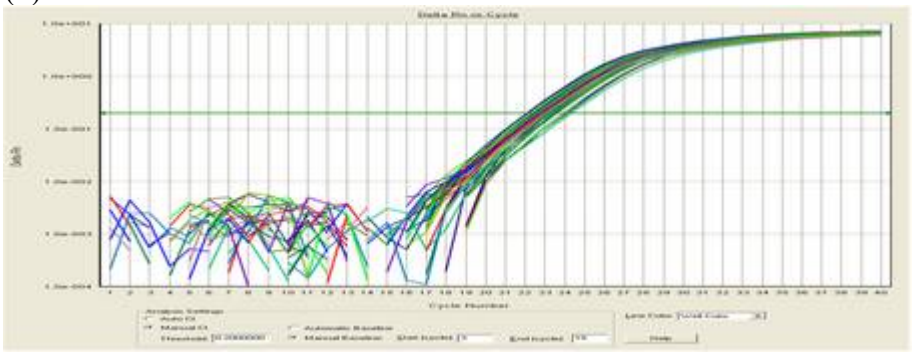
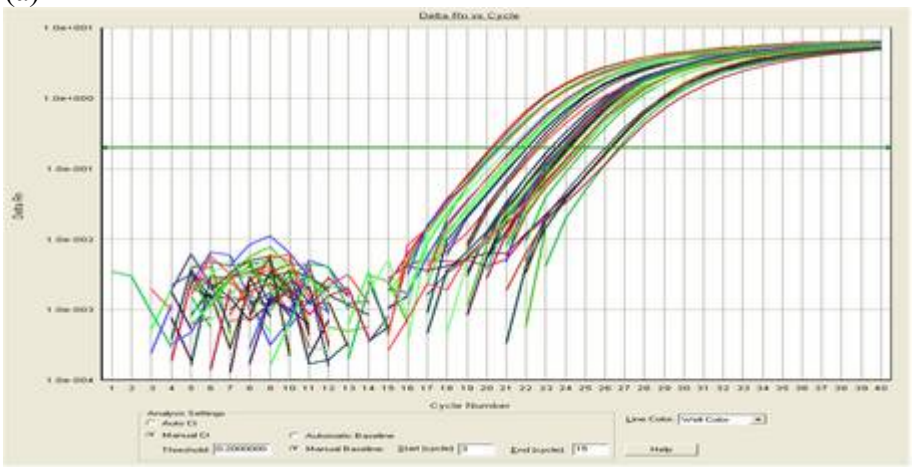


Figure 4.35: Amplification plot of cDNA using Actin9 primers (Delta Rn versus cycle).

(a) Non-transformed tobacco
(b) Transgenic tobacco with pCambia1304
(c) Transgenic tobacco with pToxo130BBI
(d) Transgenic tobacco with pToxo130BBIKDEL

(a)



(b)

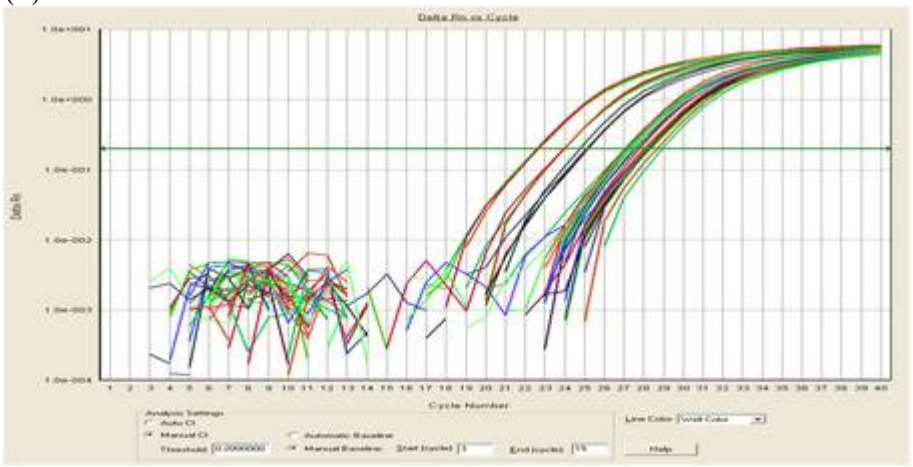
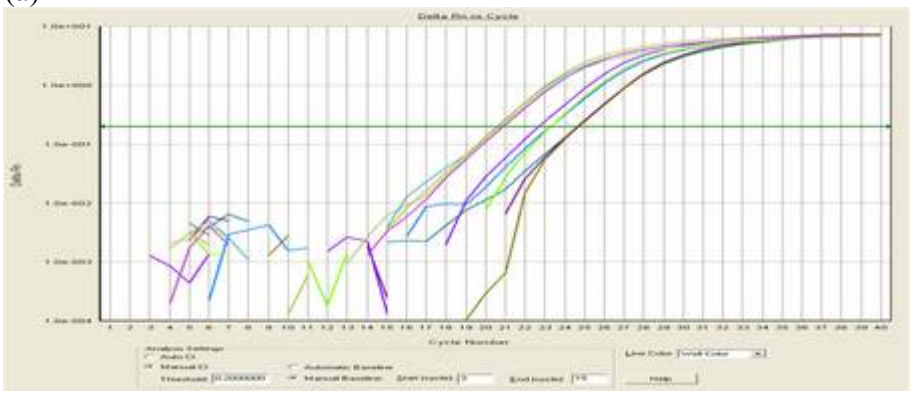


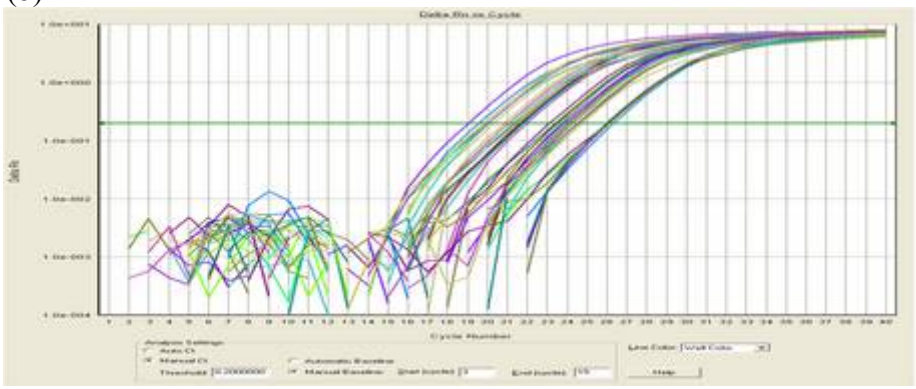
Figure 4.36: Amplification plot of cDNA using TG13090 primers (Delta Rn versus cycle).

(a) Transgenic tobacco with pToxo130BBI
(b) Transgenic tobacco with pToxo130BBIKDEL

(a)



(b)



(c)

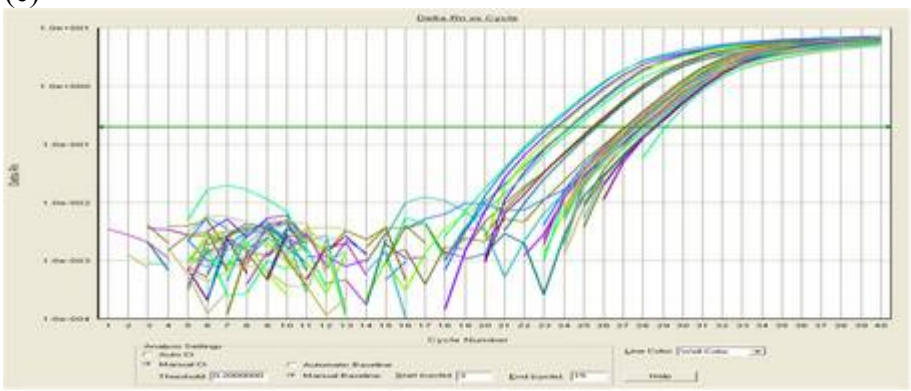


Figure 4.37: Amplification plot of cDNA of transgenic tobacco with pCambia1304 vector by using *gfp*180 primers (Delta Rn versus cycle).

(a) Transgenic tobacco with pCambia1304
(b) Transgenic tobacco with pToxo130BBI
(c) Transgenic tobacco with pToxo130BBIKDEL

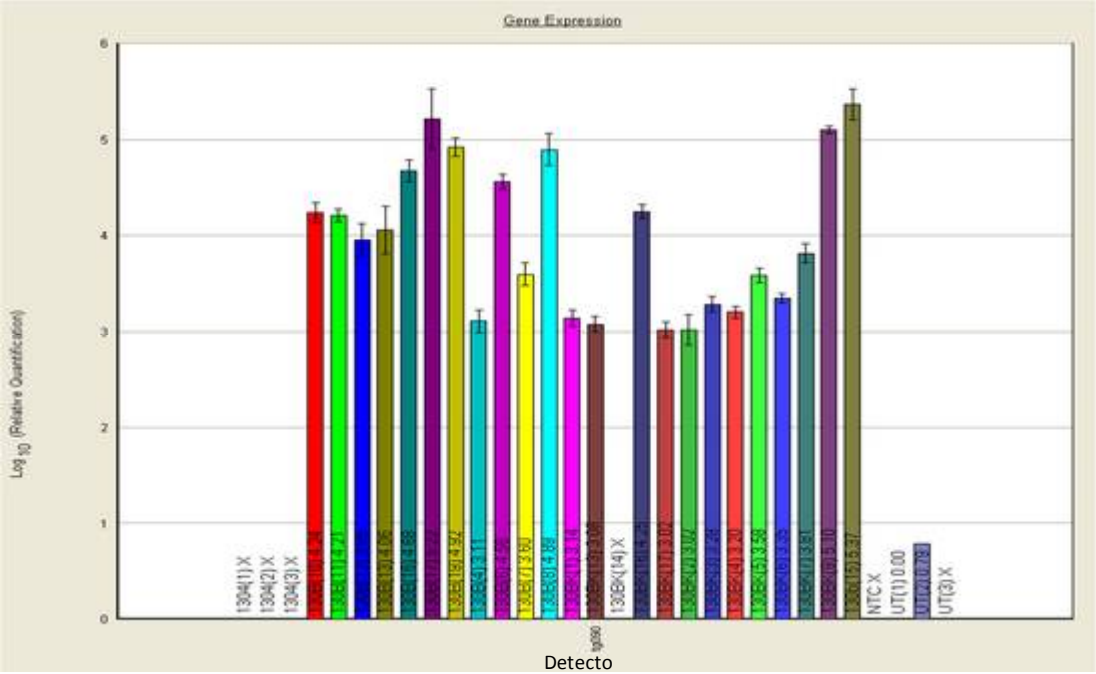


Figure 4.38: Bar chart showing gene expression level of targeted gene (TG) of all samples.

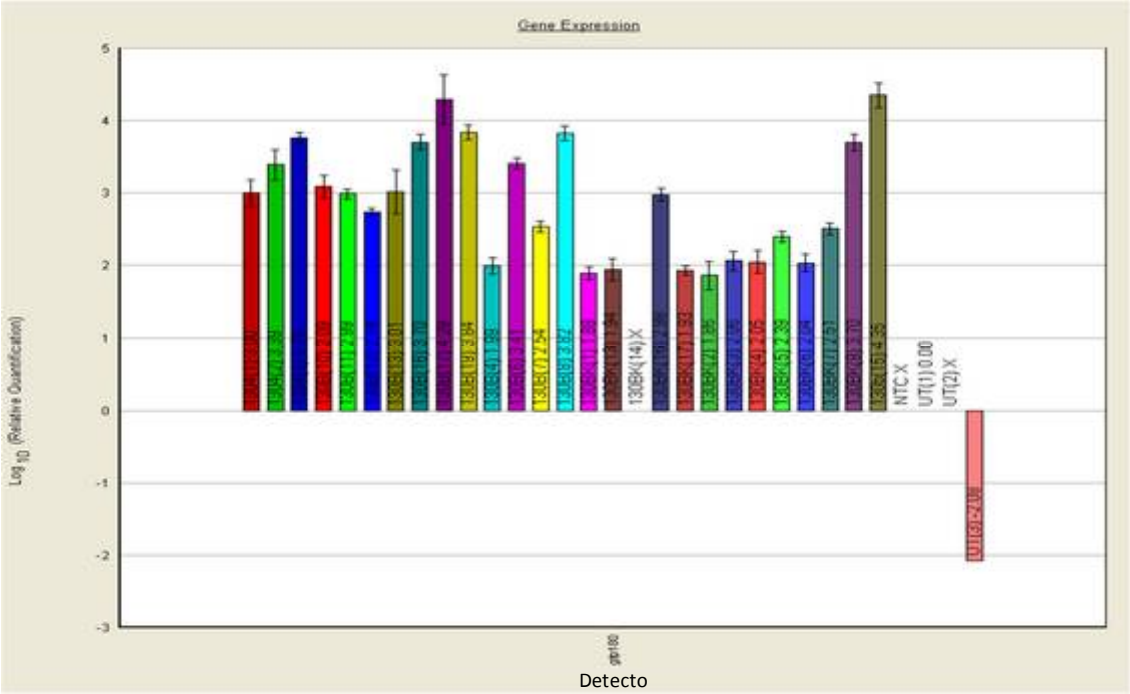


Figure 4.39: Bar chart showing gene expression level of reporter gene (*gfp*) of all samples.

Table 4.10: Summary of Real-time PCR analysis for gene expression in transgenic tobacco and non-transformed tobacco as control. The relative quantitation was obtained with standard curve method. The internal control of this analysis is beta-actin (Actin9) gene. UT(1) was used as the calibrator. CT, threshold cycle and RQ, relative quantitative.

Sample	Average Ct of Actin9	Average Ct of TG	Average Ct of *gfp*	RQ		Expression fold (Log RQ)	
				TG	*gfp*	TG	*gfp*
UT(1)	22.131 (0.058)	Underestimated	Underestimated	-	-	-	-
UT(2)	22.502 (0.045)	Underestimated	Underestimated	-	-	-	-
UT(3)	20.746 (0.139)	Underestimated	Underestimated	-	-	-	-
1304(1)	23.046 (0.236)	Underestimated	24.689 (0.086)	-	946.385	-	2.976
1304(2)	22.767 (0.183)	Underestimated	22.913 (0.182)	-	2672.489	-	3.427
1304(3)	21.626 (0.069)	Underestimated	20.651 (0.084)	-	5811.036	-	3.764
130B(4)	21.105 (0.150)	26.190 (0.050)	26.026 (0.023)	1280.154	97.626	3.107	1.990
130B(6)	23.093 (0.064)	23.355 (0.080)	23.305 (0.074)	36215.426	2553.017	4.559	3.407
130B(7)	23.013 (0.085)	26.469 (0.135)	26.121 (0.048)	3957.225	342.850	3.597	2.535
130B(8)	22.932 (0.089)	22.084 (0.206)	21.771 (0.106)	78205.680	6613.540	4.893	3.820

continued Table 4.10: Summary of Real-time PCR analysis for gene expression in transgenic tobacco and non-transformed tobacco as control. The relative quantitation was obtained with standard curve method. The internal control of this analysis is beta-actin (Actin9) gene. UT(1) was used as the calibrator. CT, threshold cycle and RQ, relative quantitative.

Sample	Average Ct of Actin9	Average Ct of TG	Average Ct of *gfp*	RQ		Expression fold (Log RQ)	
				TG	*gfp*	TG	*gfp*
130B(10)	22.612 (0.107)	23.937 (0.090)	23.879 (0.184)	17330.643	1228.272	4.239	3.089
130B(11)	22.490 (0.085)	23.903 (0.035)	24.208 (0.117)	16303.512	898.877	4.212	2.954
130B(12)	22.104 (0.044)	24.588 (0.037)	24.538 (0.054)	7767.142	547.404	3.890	2.738
130B(13)	22.690 (0.276)	24.615 (0.188)	24.389 (0.271)	11441.140	910.837	4.058	2.959
130B(15)	22.963 (0.217)	20.546 (0.055)	20.030 (0.090)	231946.857	22586.496	5.365	4.354
130B(16)	22.353 (0.116)	22.224 (0.102)	21.590 (0.088)	47514.098	5018.509	4.677	3.701
130B(17)	22.302 (0.414)	20.378 (0.104)	19.572 (0.209)	164826.781	19612.881	5.217	4.293
130B(19)	22.295 (0.093)	21.364 (0.093)	21.083 (0.099)	82828.469	6852.943	4.918	3.834
130BK(1)	23.242 (0.113)	28.217 (0.060)	28.481 (0.025)	1381.332	78.311	3.140	1.894
130BK(2)	23.083 (0.083)	28.460 (0.190)	28.422 (0.252)	1044.651	73.044	3.019	1.864

continued Table 4.10: Summary of Real-time PCR analysis for gene expression in transgenic tobacco and non-transformed tobacco as control. The relative quantitation was obtained with standard curve method. The internal control of this analysis is beta-actin (Actin9) gene. UT(1) was used as the calibrator. CT, threshold cycle and RQ, relative quantitative.

Sample	Average Ct of Actin9	Average Ct of TG	Average Ct of *gfp*	RQ		Expression fold (Log RQ)	
				TG	*gfp*	TG	*gfp*
130BK(3)	22.720 (0.109)	27.222 (0.028)	27.390 (0.146)	1917.380	116.123	3.283	2.065
130BK(4)	22.751 (0.068)	27.521 (0.049)	27.472 (0.202)	1591.326	112.085	3.202	2.050
130BK(5)	23.598 (0.099)	27.115 (0.027)	27.173 (0.036)	3794.330	248.275	3.579	2.395
130BK(6)	23.045 (0.039)	27.329 (0.057)	27.810 (0.151)	2228.900	108.757	3.348	2.036
130BK(7)	22.259 (0.095)	25.095 (0.019)	25.458 (0.056)	6080.688	321.871	3.784	2.508
130BK(9)	24.126 (0.046)	22.585 (0.028)	23.358 (0.144)	126427.961	5035.770	5.102	3.702
130BK(13)	23.142 (0.021)	28.229 (0.039)	28.114 (0.180)	1190.846	87.783	3.076	1.943
130BK(14)	22.625 (0.142)	Underestimated	Underestimated	-	-	-	-
130BK(17)	22.331 (0.023)	27.712 (0.110)	27.455 (0.083)	1042.062	84.816	3.018	1.928

CHAPTER 5: DISCUSSION

5.1 Introduction

Toxoplasmosis is a parasitic disease caused by the one-celled protozoan parasite, *Toxoplasma gondii*, which is widespread in humans and many other warm-blooded animals in particular in cats and farm animals. This disease can cause serious effects in the infants, pregnant women and the unborn fetus.

In this study, a recombinant scFv anti-*Toxoplasma* plant transformation construct was designed using a plant expression vector as a backbone and a pre-cloned mouse derived anti-*Toxoplasma* scFv gene (Lim, 2013) as the target. Single chain antibodies or scFvs are promising therapeutic molecules as they are not only similar to their parental antibodies but are smaller in size and with undemanding folding requirements facilitating their production in recombinant systems while still maintaining their functionality (Smith, 1996). As such the scFv gene has become attractive antibody derivative for various applications including as diagnostic and therapeutic candidates for human and animal applications. Up to now there are many therapeutic antibodies been studied and some were entered the clinical trials. As reviewed by Lotter-Stark et al. (2012), there are four HIV neutralising antibodies (i.e. 2G12, b12, 2F5 and 4E10) have been successfully expressed in plants and the plant-made 2G12 has entered clinical trials in 2011 (Twyman et al., 2012). A plant expression system was chosen, as there have been several studies that have shown that the production of recombinant antibodies has high yield and lower cost when plants are used as biofactories (Sala et al., 2002; Fischer et al., 2004; Gao et al, 2012). Plants as biofactories can also minimise the potential risk of pathogenic contamination that is often associated with animal or bacterial cell culture systems. However, as in any production system, a major focus is in ensuring that the system will express at levels that will be amenable to practical use. A previous study in our laboratory utilising basic plant transformation constructs without

KDEL or any other enhancing sequences showed low and unsatisfactory yields in producing scFv (Wong, 2011). Hence, the experiments also focused on adding elements to the construct that would ensure high expression levels in plant cells. Additionally the study initiated the use of the banana (*Musa acuminata*) plant as a future host for large scale production of antibodies in addition to the model host plant, *Nicotiana tabacum*.

5.2 Development of constructs

The study successfully utilised standard molecular approaches to purify and subclone the gene of interests into the plasmids vector, pCambia1304, pToxo64BBI, pToxo64BBIKDEL, pToxo130BBI and pToxo130BBIKDEL.

The BBIKDEL and BBI gene fragments

The Bowman-Birk Serine proteinase inhibitor (BBI) and KDEL retention sequences were used in this study as enhancements to the constructs which could potentially increase the expression levels of the targeted proteins in the plant cells.

It has been reported previously that transgenes that are targeted for expression within the endoplasmic reticulum showed improvement to the yield of protein (Benchabane et al., 2008). The KDEL retention sequence that was used in this study was Ser-Glu-Lys-Asp-Glu-Leu which was first described by Munro and Pelham (1987) that the presence of retention signal will retain the protein that secreted by transfected COS cells in endoplasmic reticulum only. With this unique characteristic, the retention signal was used in many other studies, as described by Galpin et al in 2010. The human lysosomal enzyme, α-L-iduronidase (IDUA), with C-terminal ER-retention sequence was synthesized with lower concentrations of immunogenic sugars, i.e. fucose and xylose in seeds of transgenic *Nicotiana tabacum* and *Brassica napus* plants and thus increased the protein accumulation levels. Bellucci et. al (2007) also showed that the

production of recombinant zeolin was higher when targeted into the endoplasmic reticulum (ER) as compared to expression in the chloroplast of tobacco. Hence, this strategy was also used in this study to potentially facilitate better expression of the targeted antibody.

A second approach for increasing the yield of recombinant protein production in plant cells is by addition of gene encoding protease inhibitors into the transgene constructs. Co-secretion of protease inhibitors with the transgene has the potential to protect recombinant protein from the host resident proteases activities. This was successfully demonstrated by Komarnytsky and his colleagues (2006) who showed that the production of immunoglobulin complexes in *Nicotiana tabacum*'s roots was higher with the co-secretion of Bowman-Birk Ser protease inhibitor. The Bowman-Birk Ser inhibitor used in this study was synthesised based on a sequence from gene bank (Accession number: EF127893) and originate from the genome of *Musa acuminata* AAA group. The Bowman-birk inhibitor is well known with its unique function in defense against microorganisms, pathogen and insects predation (Koundal & Rajendran, 2003; Yan et al., 2009; Dramé et al., 2013). This protease inhibitor is also involved in regulating the endogenous proteinases during germination and for formation of a sulphur reservoir during dormancy (Yan et al., 2009).

5.3 Transformation of constructs into expression hosts

5.3.1 *Agrobacterium*-mediated transformation into banana cell suspensions

Musa acuminata was chosen as a potential host for expression and production of scFvs due to several attractive properties possessed by the plant. Principally, this is a tropical plant suitable to growing conditions in Malaysia with a physiology and large leaf architecture that potentially provides for large scale expression of proteins. The tissue culture and transformation protocols have also been well developed in our

laboratories (Wong et al., 2008). The banana cultivar used for the study cultivar Berangan is also amenable to large scale production via embryogenic cell suspensions process (Jalil et al., 2003).

In this study, transient assay utilizing GUS histochemical staining carried out on selected cells putatively transformed with *Agrobacterium* harbouring the scFv constructs developed showed initially promising results. The blue spots observed on the inoculated cell suspensions (as shown in Figure 4.21) suggested that the transformation of the constructs into the cell suspensions were successful.

However, PCR analysis on the extracted DNA was negative indicating that the transgenes were not successfully integrated into plant genome. The positive results (blue colour spots) observed from the GUS assay was likely due to residual *Agrobacterium sp.* located on the surface of cell suspensions. Difficulty in removing inoculum from putatively transformed primary cells is a recurring problem in transgenic experiments (Cubero et al., 2005; Dahdi et al., 2012) despite the use of antibiotics. The negative results of the experiments on the other hand could also be attributed to the state of the suspension cells used. The cells had been in culture for more than 24 months possibly rendering them less amenable to transformation (N. Khalid per comm). Banana cell suspensions have been successfully transformed in *Musa acuminata* cv, Mas (Wong, 2007). However, there may also be differences in the efficiency of transformation in different banana cultivars. This possibility has not been reported and may be worth investigating. Future experiment would thus use fresh suspensions but no further work could be carried out for this study due to time constraints. It should be noted also that attempts to transform scFv in other monocots have shown low efficiency. Kamo et al. (2012) recently reported the expression of an scFv against *Cucumber mosaic virus* (CMV) subgroup I and II in *Gladiolus* 'peter Pears' and 'Jenny Lee' where only 7% of

the transgenic plant lines were functional. The remaining work in this study focused on the use of tobacco as the expression host.

5.3.2 *Agrobacterium*-mediated transformation into tobacco

Agrobacterium-mediated transformation is a common tool for transforming foreign gene into tobacco. It is also the principle expression host that have been used for production of scFvs. Among the scFvs that have been produces in tobacco include the scFv anti-*cucumber mosaic virus* (Chua et al., 2006), scFv anti-foot-and-mouth disease (Joensuu et al., 2009) etc. Additionally, there are some scFvs undergoing the clinical tests, i.e. scFv for Melanoma that produce to target the P97 antigen was entered preclinical stage and the bispecific scFv for B-cell tumors was undergoing the Phase I clinical trials (Pucca et al., 2011).

The protocol that was used in this study was modified from the method of Fisher and Guiltinan in 1995. After transferring the gene of interest into tobacco leaf discs, the explants were regenerated in the antibiotic selection media.

The regenerated plantlets were selected and examined with GUS histochemical staining. The transgenic plantlets showed blue colour, while the non-transformed tobacco showed no colour changes after chlorophyll removal (as shown in Figure 4.25). As presented in the Figure 4.27, 4.28 and 4.29 showed that 17 out of 20 transgenic tobaccos harboured the transgene from pToxo130BBI; while 14 out of 20 were positive for transgene from pToxo130BBIKDEL.

The transgenic tobaccos which showed expected results were further studied through RNA isolation and RT-qPCR for determination of the gene expression levels of the transgenes. The results of RT-qPCR as shown in Figure 4.38 and Figure 4.39, revealed that most of the samples of pToxo130BBI showed higher gene expression level of the targeted gene (Toxoplasmosis, TG) when compared to pToxo130BBIKDEL. The

same phenomenon was observed for the gene expression level of the reporter gene, green fluorescent protein (*gfp*) in the respective constructs. The results were unexpected as the dual element enhancer constructs (BBIKDEL) were expected to have higher expression than the single BBI construct.

Theoretically, by the addition of the KDEL retention sequence to the scFv antibody gene is to direct and stabilize the foreign protein into the ideal environment for expression within the endoplasmic reticulum (ER), for higher protein accumulation (Schouten et al., 1996). Additionally, co-expression with a protease inhibitor has been shown to be an effective tool for stabilizing and enhances the yield of the scFv antibody (Komarnytsky et al., 2006). However there have been no reports of both sequences being used in a single construct as attempted by this study. It should be noted that the results obtained from relative quantification studies are based on mRNA levels of the transgene in tobacco cells and not the protein accumulation level as reported by Schouten et al., 1996.

Consequently, while the RT-qPCR results successfully showed the integration and functionality of the transgenes (pToxo130BBI and pToxo130BBIKDEL) to be expressed in tobacco, it did not provide quantitative evidence of protein accumulation. Further studies on the protein expression levels are needed to examine the real effect of additional KDEL retention sequence and co-expression with the Bowman-Birk Serine protease inhibitor.

CHAPTER 6: CONCLUSION

This study was conducted to examine the transgene expression level of different constructs (pToxo64BBI, pToxo64BBIKDEL, pToxo130BBI and pToxo130BBIKDEL) in different plant expression system, banana cell suspension and tobacco. However, transformation into banana cell suspension was not successful and further optimization will have to carry out in future studies taking into account the observation from this study. Therefore, the remaining experiments were carried out using the tobacco system with two candidate constructs pToxo130BBI and pToxo130BBIKDEL.

Relative quantification studies of transgenic tobacco showed successful of transgene expression and transgene expression level and demonstrate the potential of system for production of anti-*Toxoplasma* scFv. However, there remains question the relationship between the transgene expression level and protein accumulation yield which merit further study and investigation. Additionally, further quantitative verification of the effect of KDEL retention sequence and Bowman-birk Serine protease inhibitor on protein expression levels need to be carried out.

The scFv transgenes (pToxo130BBI and pToxo130BBIKDEL) were successfully integrated into tobacco genome and have been expressed. However, further analysis is needed to examine the accumulation level and functionality of the scFv antibody. Western blot can be carried out for examining protein accumulation level; while, functionality of the anti-*Toxoplasma* scFv antibody can be studied using ELISA.

Other than this, further studies such as large scale greenhouse trials for examining the stability of transgene in next generation and observation on the potential gene flow to other crops plants and other risk assessment parameters (Miki and McHugh, 2004) need to be included. The produced scFv antibody also needs to go through the clinical

trials. Only with the successfully field trials and clinical trials, transgenic tobacco with this anti-*Toxoplasma* scFv antibody can be commercially produced.

However, biosafety issues of transgenic plants, especially for plant-produced pharmaceutical needs to be addressed especially in the context of the lack of specific regulations in Malaysia. Therefore, the risk assessment and risk management on this transgenic tobacco needs to be carried out thoroughly before any commercial release.

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APPENDICES

Appendix A: Sterilization

All glassware, micropipette tips, microcentrifuge tubes, PCR tubes, stock solutions and buffers for DNA work were autoclaved at 121ºC with 15psi for 20 minutes; while for RNA works, autoclaving cycle is at 121ºC with15psi for 45 minutes.

The culture media for bacterial and plant tissues/cells were autoclaved at 121ºC with 15psi for 15minutes. The handling of plant tissue cultures were carried out in a laminar flow.

Appendix A1: Stock solutions, buffers and media of bacterial cultures

(i) LB broth

LB broth consists of 1.0g of Tryptone (1%), 0.5g of Sodium Chloride (0.17M), and 0.5g of Yeast Extract (0.5%), were dissolved in 80ml distilled H_2O. Then, the volume was adjusted to 100ml and 10ml of the mixtures were aliquoted into 28ml universal bottle before sterilization.

(ii) LB agar plate

One gram of Tryptone (1%), 0.5g of Sodium Chloride (0.17M), 0.5g of Yeast Extract (0.5%), and 1.5g of agar were dissolved in 80ml distilled H_2O. Then, the volume was topped up to 100ml and sterilized. For colony selection, the sterile mixtures were mixed with 100µg/µl kanamycin. Then, 25ml of the mixture were aliquoted into petri dish. After solidified, the plates were kept at 4ºC.

Appendix A2: Stock solutions, buffers and gel for electrophoresis

(i) 5x TBE buffer for DNA use

Tris base	54g
Boric acid	27.5g
0.5M EDTA (pH 8.0)	20ml
Distilled H_2O to	1000ml

Tris base, boric acid and EDTA were dissolved in distilled H_2O and adjusted to the final volume of l000ml before sterilization. Then, the sterile solution was kept in room temperature. The solution was diluted to 1x working concentration (90mM Tris-borate and 2mM EDTA) for agarose gel electrophoresis.

(ii) 5x TBE buffer for RNA use

The solution was prepared as mentioned above (Appendix A2, (i)) and treated with 1ml DEPC. The treated solution was incubated at room temperature for overnight before autoclaving.

(iii) 6x DNA loading dye

1.0M Tris-Cl (pH7.6)	100µl
bromophenol blue	30mg
xylene cyanol FF	30mg
0.5M EDTA	1.2ml
Glycerol	6ml

The compositions (as stated in Fermentas catalogue) were mixed well and adjusted to final volume of 10ml with sterile distilled H_2O. The loading buffers were kept at room temperature.

(iii) 2x RNA loading dye

The loading dye was purchased from Fermentas with the composition of 95% formamide, 0.025% SDS, 0.025% bromophenol blue, 0.025% xylene cyanol FF, 0.025% ethidium bromide, and 0.5mM EDTA. The loading dye was kept at -20ºC.

(iv) Molecular weight markers (Ladder)

The perfect^{TM} 100bp DNA ladder and perfect^{TM} 1kb DNA ladder were purchased from ERU$_X$; while, ssRNA ladder was purchased from NEB. All the ladders were kept at -20ºC.

(v) Agarose gel

For preparing 1% agarose gel, 0.6g of the agarose were added into 60ml of 1x TBE buffer. Approximately 5µl of ethidium bromide were added into the gel.

Appendix A3: Stock solutions and buffer for plasmid extraction

(i) Alkaline Lysis Solution I

50mM glucose
25mM Tris-Cl (pH 8.0)
10mM EDTA (pH 8.0)

The solution was prepared from the standard stocks and sterilized by autoclaving. The solution was then kept at 4ºC.

(ii) Alkaline Lysis Solution II

0.2N NaOH
1% (w/v) SDS

The solution was prepared freshly from the stock solutions and used at room temperature.

(iii) Alkaline Lysis Solution III

5M potassium acetate	60ml
Glacial acetic acid	11.5ml

Sixty milliliter of 5M potassium acetate were added to 11.5ml glacial acetic acid. The mixture was adjusted to 100ml with sterile distilled H_2O and stored at 4ºC.

(iv) 1M Tris-Cl (pH 8.0)

Tris base	12.11g
Distilled H_2O to	100ml

Tris base was dissolved in 50ml of distilled H_2O and pH was adjusted to 8.0 with 42ml concentrated HCl. Final adjustment to pH was carried out after the temperature of solution is cooled down to room temperature. The solution was adjusted to final volume of 100ml before sterilization. The solution was kept at room temperature.

(v) 0.5M EDTA (pH 8.0)

Disodium EDTA.$2H_2O$	93.05g
Distilled H_2O to	500ml

Disodium EDTA.$2H_2O$ was added in distilled H_2O and stirred continuously while adjusting the pH. The pH is adjusted to 8.0 by using NaOH. (Note: The disodium salt of EDTA will only dissolve when the pH reached 8.0.) After pH adjustment, the solution was adjusted to final volume of 500ml and sterilized by autoclaving.

(vi) 10N NaOH

NaOH	40g
Distilled H_2O to	100ml

The NaOH pellets were dissolved in distilled H_2O in a plastic beaker, as the preparation of 10N NaOH is highly exothermic reaction. The solution was stirred continuously until the pellet dissolved completely into distilled H_2O. The solution was adjusted to final volume of 100ml and kept at room temperature.

(vii) 10% (w/v) SDS

Sodium dodecyl sulphate (SDS)	10g
Distilled H_2O to	100ml

Sodium dodecyl sulphate was added into 80ml distilled H_2O and stirred continuously until dissolve completely. The solution was adjusted to final volume of 100ml and kept at room temperature.

(viii) 3M Sodium acetate (pH 5.2)

Sodium acetate (anhydrous)	40.83g
Distilled H_2O to	100ml

Sodium acetate.$3H_2O$ was dissolved into 80ml and the pH was adjusted to 5.2 with glacial acetic acid. The solution was adjusted to final volume of 100ml before sterilization and kept at room temperature.

(ix) 70% (v/v) ethanol

Absolute ethanol	70ml
Sterile distilled H_2O	30ml

Absolute ethanol was mixed well with the sterile distilled H_2O before store at 4ºC.

Appendix A4: Stock solution and buffers of PCR

Appendix A4a: Stock solutions and buffers of conventional PCR

(i) PCR reaction mixture (11x)

The i-Taq™ DNA polymerase PCR kit was purchased from Intron Biotechnology. The compositions of the kit including 10x PCR buffer (with 20mM $MgCl_2$), 10x $MgCl_2$ free PCR buffer, 10mM dNTPs, 25mM $MgCl_2$ and i-Taq™ DNA polymerase(5U/µl). The PCR reaction mixture was prepared as showed in Table A1.

Table A1: 11x PCR reaction mixture.

Component	Volume (µl) in 1x reaction	Volume (µl) in 11x reaction	Final concentration in 1x reaction
10x PCR buffer (with 20mM $MgCl_2$)	5.00	55.00	1x
10mM dNTPs (2.5mM each)	4.00	44.00	0.2mM each
i-Taq™ DNA polymerase (5U/µl)	0.25	2.75	0.025U/µl
downstream primer (10µM)	5.00	55.00	1µM
upstream primer (10µM)	5.00	55.00	1µM
Template	4.00	44.00	1ng - 1µg
Sterile distilled H_2O	26.75	294.25	-
Total	50	550	-

Appendix A4b: Stock solutions and buffers of Reverse-transcriptase PCR

(i) Reverse-transcriptase PCR reaction mixture (11x)

The High Capacity cDNA Reverse-transcription kit was purchased from Applied Biosystems. The kit consists of 10x RT buffer, 10x RT Random Primers, 25x dNTP mix (100mM), and MultiScribe^{TM} Reverse-transcriptase (50U/µl). The reaction mixture was prepared as shown in Table A2.

Table A2: 11x Reverse-transcriptase PCR reaction mixture.

Component	Volume (µl) in 1x reaction	Volume (µl) in 11x reaction	Final concentration in 1x reaction
10x RT buffer	2.0	22.0	1x
25x dNTP mix (100mM)	0.8	8.8	1x (4mM)
10x RT Random Primers	2.0	22.0	1x
MultiScribe^{TM} Reverse-transcriptase (5U/µl)	1.0	11.0	0.25 U/µl
DEPC treated distilled H_2O	4.2	46.2	-
Total	10	110	-

Appendix A4c: Stock solutions and buffers of Real-time PCR

(i) Real-time PCR reaction mixture (5x)

Power SYBR® Green PCR master mix (2x) was purchased from Applied Biosystems. The reaction mixture was prepared as shown in Table A3.

Table A3: 5x Real-time PCR reaction mixture.

Component	Volume (µl) in 1x reaction	Volume (µl) in 5x reaction	Final concentration in 1x reaction
2x Power SYBR® Green PCR master mix	6.25	31.25	1x
Primer set mix (10µM)	1.25	6.25	1µM
cDNA template	1.0	5.0	-
DEPC treated distilled H_2O	4.0	20.0	-
Total	12.5	62.5	-

Appendix A5: Restriction endonuclease and buffer for digestion

The restriction endonucleases and reaction buffers were purchased from NEB. Combinations of double restriction enzymes with buffer (optimum % activity) were showed in the following table, Table A4.

Table A4: Combinations of double restriction enzymes with optimum buffer.

Enzyme	Enzyme	Optimum assay buffer
Nco I	*Bgl* II	NEBuffer 3
Avr II	*Bgl* II	NEBuffer 2
Nco I	*Hind* III	NEBuffer 2
Avr II	*Hind* III	NEBuffer 2
Hind III	*Bgl* II	NEBuffer 2
Bam HI	*Nco*I	NEBuffer 3 + BSA

Table A5: Restriction enzyme, *Nde*I, with its optimum assay buffer.

Enzyme	Optimum assay buffer
*Nde*I	NEBuffer 4

1x NEBuffer 2 contained:

NaCl	50 mM
Tris-HCl	10 mM
$MgCl_2$	10 mM
DTT (dithiothreitol)	1 mM

1x NEBuffer 3 + BSA contained:

NaCl	100 mM
Tris-HCl	50 mM
$MgCl_2$	10 mM
DTT (dithiothreitol)	1 mM
BSA	0.1mg/ml

1x NEBuffer 4 contained:

Potassium acetate	50 mM
Tris-acetate	20 mM
Magnesium Acetate	10 mM
DTT (dithiothreitol)	1 mM

Appendix A6: Ligase and buffers

T4 DNA Ligase (5U/µl) and assay buffer were purchased from Fermentas.

Table A6: Reaction mixture of ligation.

Component	Volume / Concentration of 1x reaction
Linear DNA vector	1:3 molar ration over insert
Insert DNA	3:1 molar ration over vector
10x T4 DNA Ligase buffer	2µl
T4 DNA Ligase (5U/µl)	1U
Sterile distilled H_2O	Adjust the final volume to 20µl

Appendix A7: Stock solution and buffers for competent cell preparation

Appendix A7a: Stock solutions and buffers for *E.coli* strain JM109 competent cell preparation

(i) TFB I solution

$RbCl_2$	1.2g
$MnCl_2.4H_2O$	0.99g
KoAc	0.3g
$CaCl_2.4H_2O$	0.15g
Glycerol	15ml

The chemicals were dissolved in 80ml sterile distilled H_2O and pH is adjusted to 5.8 with diluted acetic acid. Then, the solution was adjusted to final volume of 100ml and sterilized by filtration. The solution was then stored at 4ºC.

(ii) TFB II solution

$RbCl_2$	0.12g
MOPS	0.21g
$CaCl_2.4H_2O$	1.10g
Glycerol	15ml

All chemical components were dissolved in 80ml sterile distilled H_2O and stirred continuously. Then, the pH was adjusted to 6.8 with NaOH before adjusted to final volume of 100ml. The solution was sterilized by filtration and stored at 4ºC.

Appendix A7b: Stock solutions and buffers for *Agrobacterium* sp. strain LBA4404 competent cell preparation

(i) 1mM HEPES (pH7.0)

HEPES	23.83mg
Sterile distilled H_2O to	100ml

HEPES was dissolved in 80ml sterile distilled H_2O and stirred continuously. Then, the pH was adjusted to 7.0 with NaOH before adjusted to final volume of 100ml. The solution was sterilized by filtration and stored at 4ºC.

(ii) 10% (v/v) glycerol

Glycerol (100%)	10ml
Sterile distilled H_2O	90ml

Glycerol was mixed well with sterile distilled H_2O and filtered sterilization before store at 4ºC.

Appendix A8: Stock solutions, buffers and media for plant tissue cultures

Murashige and Skoog (MS) media powders contained macronutrients, micronutrients and vitamins were purchased from PhytoTechnology Laboratories®.

Appendix A8a: Stock solutions, buffers and media for banana cell suspensions

(i) M2 media

MS media powder	4.4g
2,4-D (lmg/ml)	1.1ml
Sucrose	20g

The chemical components were dissolved in 800ml distilled H_2O and stirred continuously while adjusting the pH to 5.8 with 1N NaoH. The volume was adjusted to 1000ml and sterilized by autoclaving. When the temperature of the media is cooled down to room temperature, 250µl of trans-zeatin (1mg/ml) were added.

(ii) M2 media with 100µM Acetosyringone

The M2 media was prepared as stated as above (Appendix A8a (i)), but in final volume 100ml. The media were stored at 4ºC. In the media, 100µl of 0.1M Acetosyringone were added before use.

(iii) M2 media with 50µg/l cefotaxime

The M2 media was prepared as stated in Appendix A8a (i). The media were stored at 4ºC. In the media, 50µl of 1mg/ml cefotaxime were added before use.

(iv) M3 media

MS media powder	4.4g
L-glutamine	0.4g
Sucrose	20g

The chemical components were dissolved in 800ml distilled H_2O and stirred continuously. The pH was adjusted to 5.8 with 1N NaoH and the volume was adjusted to 1000ml before sterilized by autoclaving. The media were stored at 4ºC.

Appendix A8b: Stock solutions, buffers and media for tobacco tissue cultures

(i) MSO solid media

MS media powder	4.4g
Sucrose	30g
Phytagar	4g

The MS media powder and sucrose were dissolved in 800ml distilled H_2O and stirred continuously. The pH was adjusted to 5.8 with 1N NaOH and the volume was adjusted

to 1000ml. Then, 4g of phytagar were added before sterilized by autoclaving. The sterile media were aliquoted in sterile tissue culture jars and sealed with parafilm before stored at 4ºC.

(ii) Co-culture media

MS media powder	0.44g
Sucrose	3g
NAA (1mglml)	10µl
6-BA (1mg/ml)	100µl

The chemical components were dissolved in 80ml distilled H_2O and stirred continuously. The pH was adjusted to 5.8 with 1N NaOH before adjusted the volume to 100ml and sterilized by autoclaving. The sterile media were stored at 4ºC.

(iii) TSM media

MS media powder	4.4g
Sucrose	30g
NAA (1mglml)	100µl
6-BA (1mg/ml)	1000µl
Phytagar	4g

The chemical components were dissolved in 800ml distilled H_2O and stirred continuously. The pH was adjusted to 5.8 with 1N NaOH before adjusted the volume to 1000ml. Then, 4g of phytagar were added and sterilized by autoclaving. The sterile media were aliquoted in petri dishes and sealed with parafilm before stored at 4ºC.

(iv) TSM selection media

The media are prepared as stated in Appendix A8b (iii). Before aliquoted into petri dished, 250µl of 100mg/ml hygromycin and 2500µl of 100mg/ml cefotaxime were

added and mixed well. The media were stored at 4ºC and need to be used within 2 weeks.

(v) TRM selection media

The media are prepared as stated in Appendix A8b (iv) without 6-BA and aliquoted into sterile tissue culture jars before sealed with parafilm and stored at 4ºC.

Appendix A9: Stock solutions and buffers for GUS histochemical staining reagent

(i) 0.2M $NaPO_4$ buffer (pH 7.0)

0.2M NaH_2PO_4 (Solution A)	39ml
Na_2HPO_4 (Solution B)	61ml

Both solutions are prepared as mentioned in Appendix A9 (ii) and Appendix A9 (iii). After mixed well, the pH of buffer was adjusted to 7.0 with NaoH. The buffer was kept at 4ºC.

(ii) 0.2M NaH_2PO_4 (Solution A)

NaH_2PO_4	3.12g
Sterile distilled H_2O to	100ml

Sodium phosphate was dissolved in 80m sterile distilled H_2O before adjusted to final volume of 100ml. The solution was kept at 4ºC.

(iii) Na_2HPO_4 (Solution B)

Na_2HPO_4	2.84g
Sterile distilled H_2O to	100ml

Disodium phosphate was dissolved in 80m sterile distilled H_2O before adjusted to final volume of 100ml. The solution was kept at 4ºC.

(iv) 0.1M $K_3[Fe(CN)_6]$

$K_3[Fe(CN)_6]$	3.293g
Sterile distilled H_2O to	100ml

Potassium ferricyanide was dissolved in 80ml sterile distilled H_2O before adjusted to final volume of 100ml. The solution was kept at 4ºC.

(v) 0.1M $K_4[Fe(CN)_6].3H_2O$

$K_4[Fe(CN)_6].3H_2O$	3.293g
Sterile distilled H_2O to	100ml

Potassium ferrocyanide was dissolved in 80ml sterile distilled H_2O before adjusted to final volume of 100ml. The solution was kept at 4ºC.

(vi) 20M X-gluc

X-gluc	20mg
DMSO	1ml

X-gluc was dissolved in DMSO and stored at -20ºC before use. It is light sensitive.

(vii) 0.5% Triton X-100

Triton X-100	50µl
Sterile distilled H_2O to	10ml

Triton-X-100 was mixed well sterile distilled H_2O and keep at 4ºC.

(viii) GUS histochemical reagents mixture

Table A7: GUS histochemical reagents mixture for 10ml.

Stock solutions	Working Solution	
	Concentration	Volume, µl
0.2M $NaPO_4$ buffer (pH 7.0)	0.1M	5000
0.1M $K_3[Fe(CN)_6]$	0.5mM	50
0.1M $K_4[Fe(CN)_6].3H_2O$	0.5mM	50
0.5M EDTA (pH8.0)	10mM	200
20M X-gluc	1M	500
0.5% Triton X-100	0.1%	2000
Methanol	20% (v/v)	2000
Sterile distilled H_2O	-	200

(ix) FAA solution

Absolute ethanol	45ml
Glacial acetic acid	5ml
Formaldehyde	5ml
Distilled H2O to	100ml

The chemicals were mixed well and adjusted to final volume of 100ml and stored at 4ºC.

Appendix A10: Agarose gel electrophoresis

(i) Agarose gel electrophoresis for DNA

Agarose gel was prepared as stated in Appendix A2 (vi) and transferred to a gel tank which contained 1x TBE buffer (diluted from 5x TBE).

The samples, each 5µl, were mixed with 1µl of 6x DNA loading dye on parafilm and loaded into the wells. After that, the samples were electrophoresed at 121volt for 30minutes.

The gel was visualized under ultraviolet light transilluminator of AlphaImager^{TM} 2200 and photograph was taken for result.

(ii) Agarose gel electrophoresis for RNA

The same protocol was used as stated in Appendix A10 (i) with the 5x TBE buffer of RNA used. However, there are some differences.

The RNA samples were mixed well with 2x RNA loading dye in equal volume in 1.5ml microcentrifuge tubes. Then, the mixtures were heated at 70ºC for 10 minutes. After that, the heated mixtures were chilled on ice before loading into the gel.

The samples were electrophoresed at 120volt for 25 minutes. Then, the gel was visualized under ultraviolet light transilluminator of AlphaImager^{TM} 2200 and photograph was taken for result.

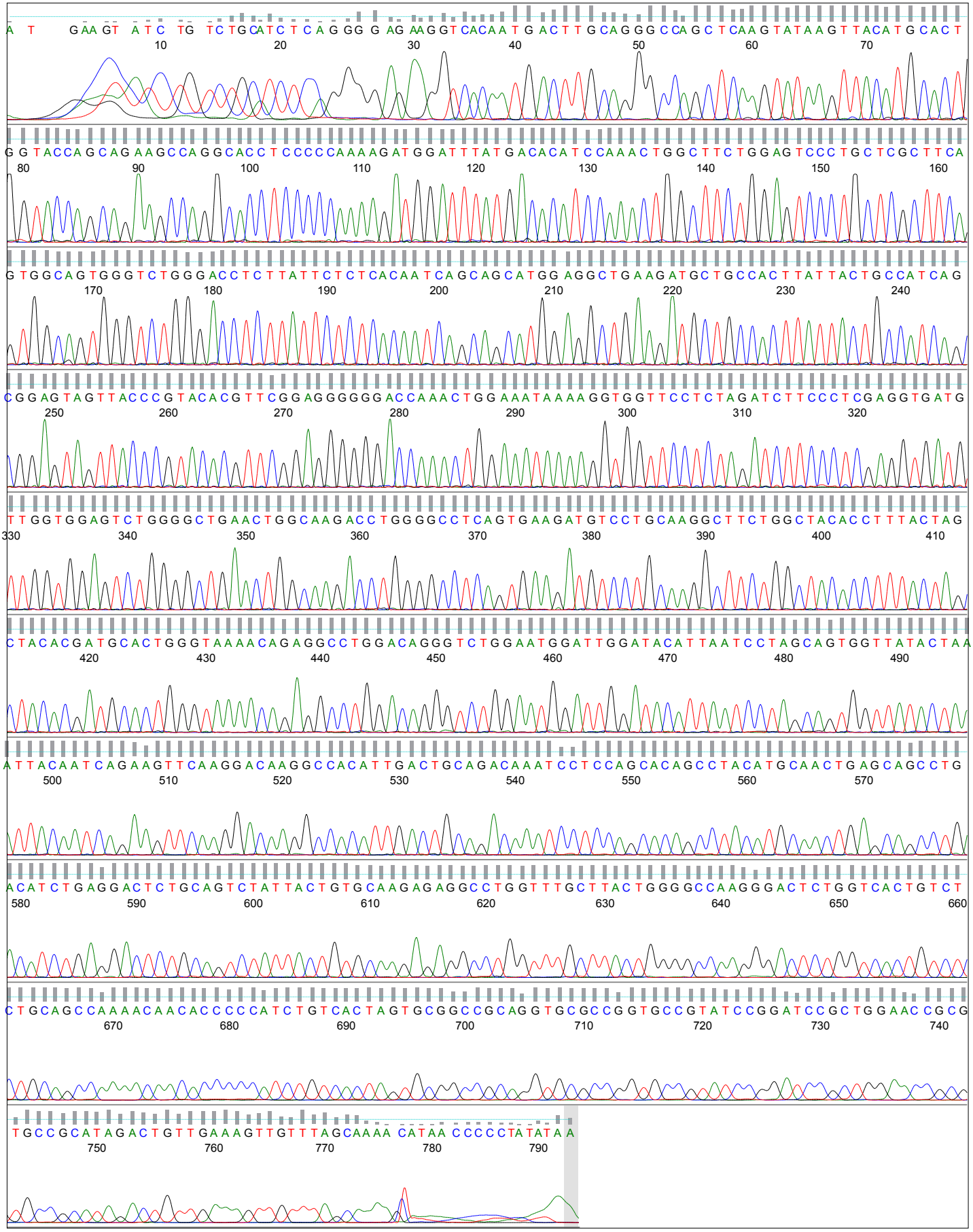
Appendix B: Compositions of Murashige and Skoog (MS)

Essential Element	Concentration in medium (mg/l)
Macroelement	
Ammonium nitrate (NH_4NO_3)	1650
Kalium nitrate (KNO_3)	1900
Calcium chloride ($CaCl_2.2H_2O$)	440
Magnesium sulphate ($MgSO_4.7H_2O$)	370
Potassium phosphate (KH_2PO_4)	170
Microelement	
Potassium Iodide (KI)	0.83
Boric acid (H_3BO_3)	6.2
Manganase sulphate ($MnSO_4.4H_2O$)	22.3
Zinc sulphate ($ZnSO_4.7H_2O$)	8.6
Sodium manganate ($Na_2MnO_4.2H_2O$)	0.25
Copper sulphate($CuSO_4.5H_2O$)	0.025
Cobalt chloride($CoCl_2.6H_2O$)	0.025
Iron source	
Ferum sulphate ($FeSO_4.7H_2O$)	27.8
Ethylenediaminetetraacetic acid, disodium ($Na_2EDTA.2H_2O$)	37.3
Organic supplement	
Myoinositol	100
Nicotinic acid	0.5
Pyridoxine-HCl	0.5
Thiamine-HCl	0.5
Glycine	2
Carbon source	
Sucrose	30000

Appendix C: Sequence
Appendix C1: Sequence of scFv64 antibody fragment

Sample Name: scFv64_scFv64-F
Mobility: KB_3730_POP7_BDTv3.mob
Spacing: 14.0515
Comment: n/a

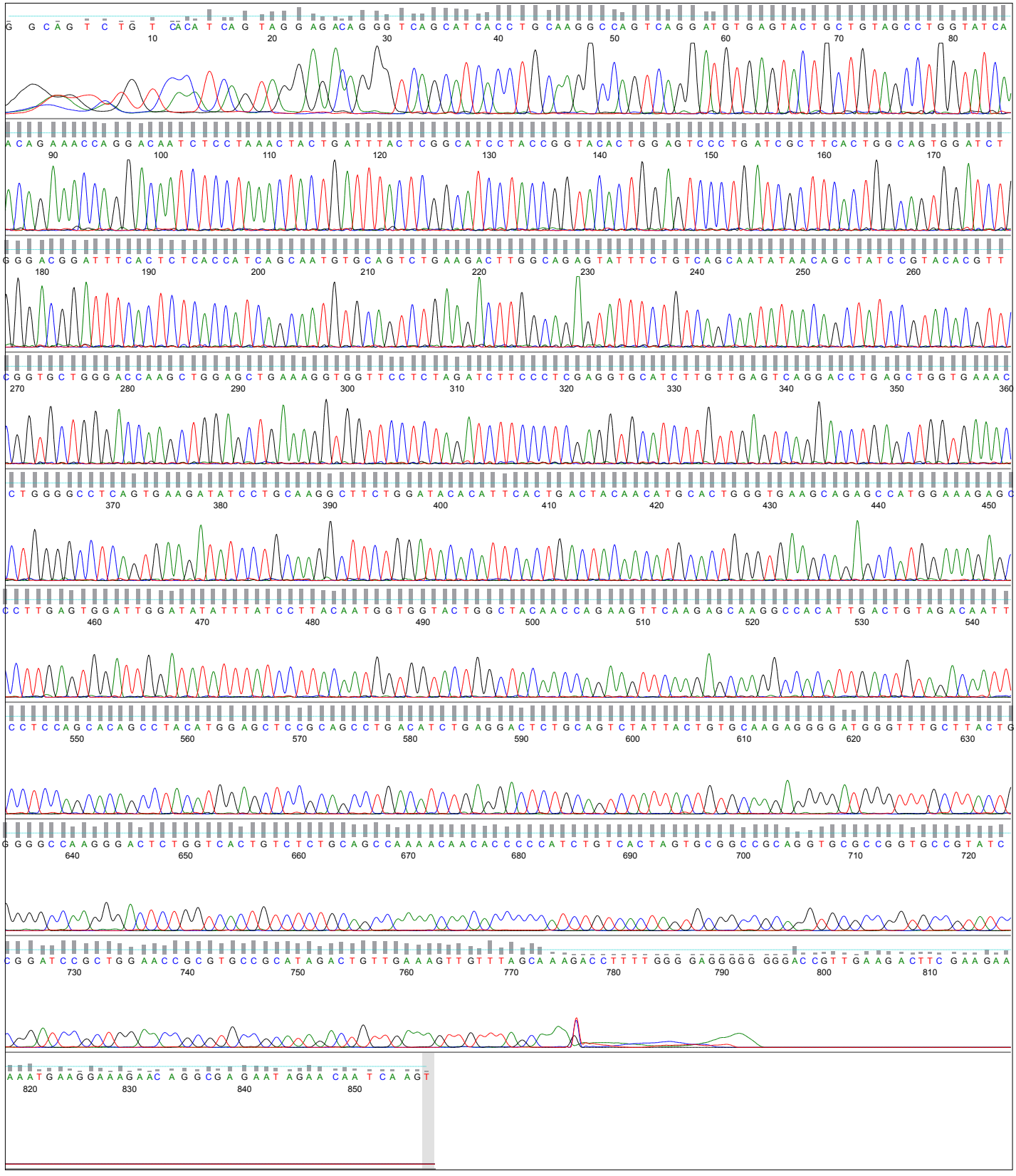
Signal Strengths: A = 1913, C = 1928, G = 2289, T = 1884
Lane/Cap#: 15
Matrix: n/a
Direction: Native



Appendix C2: Sequence of scFv130 antibody fragment

Sample Name: scFv130_scFv130-F
Mobility: KB_3730_POP7_BDTv3.mob
Spacing: 13.9625
Comment: n/a

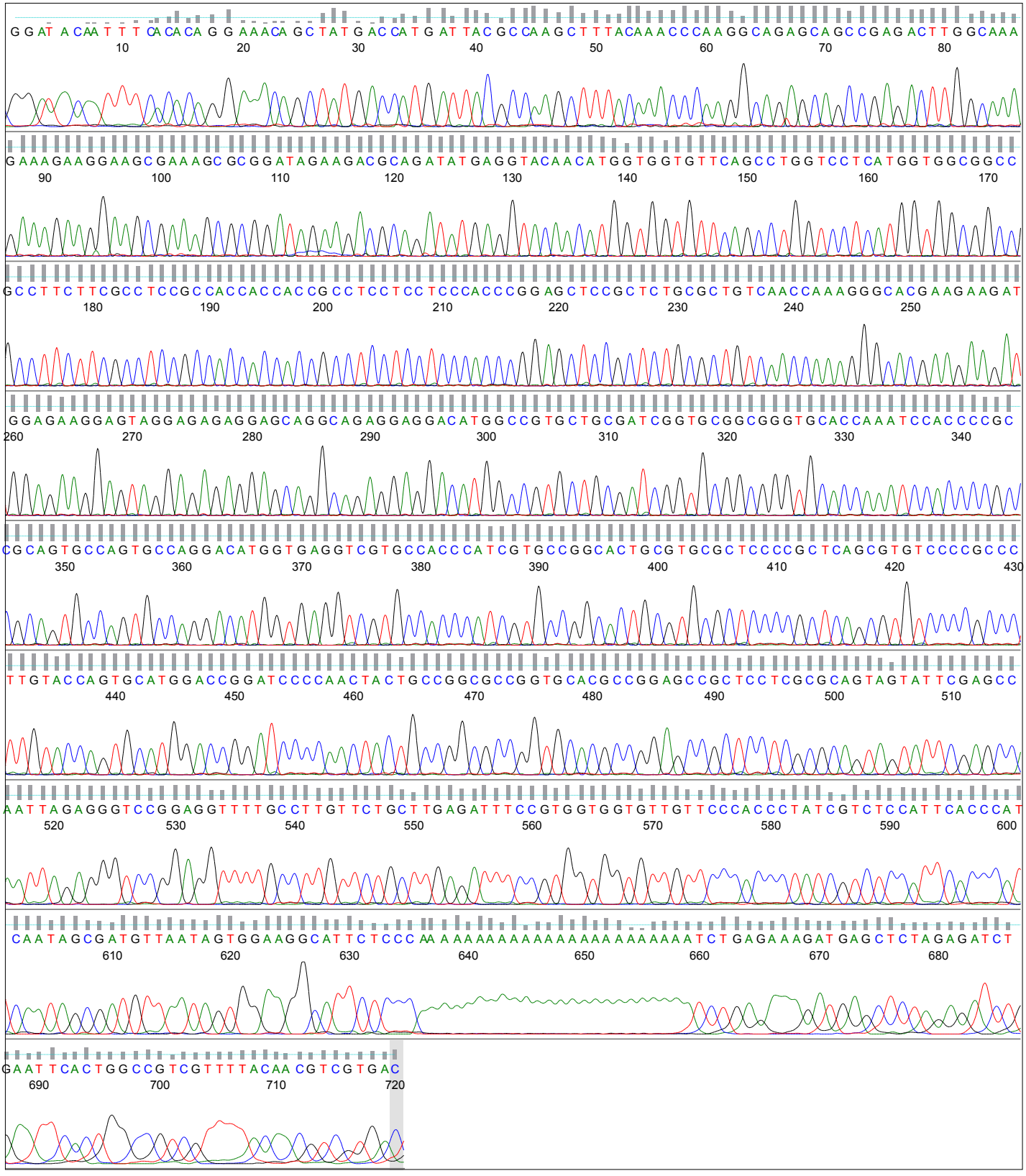
Signal Strengths: A = 3958, C = 3986, G = 3588, T = 3705
Lane/Cap#: 9
Matrix: n/a
Direction: Native



Appendix C3: Sequence of BBIKDEL gene fragment

Sample Name: 090526Q7256-1(Y1209D)M13N-
Mobility: KB_3730_POP7_BDTv3.mob
Spacing: 15.5012
Comment: n/a

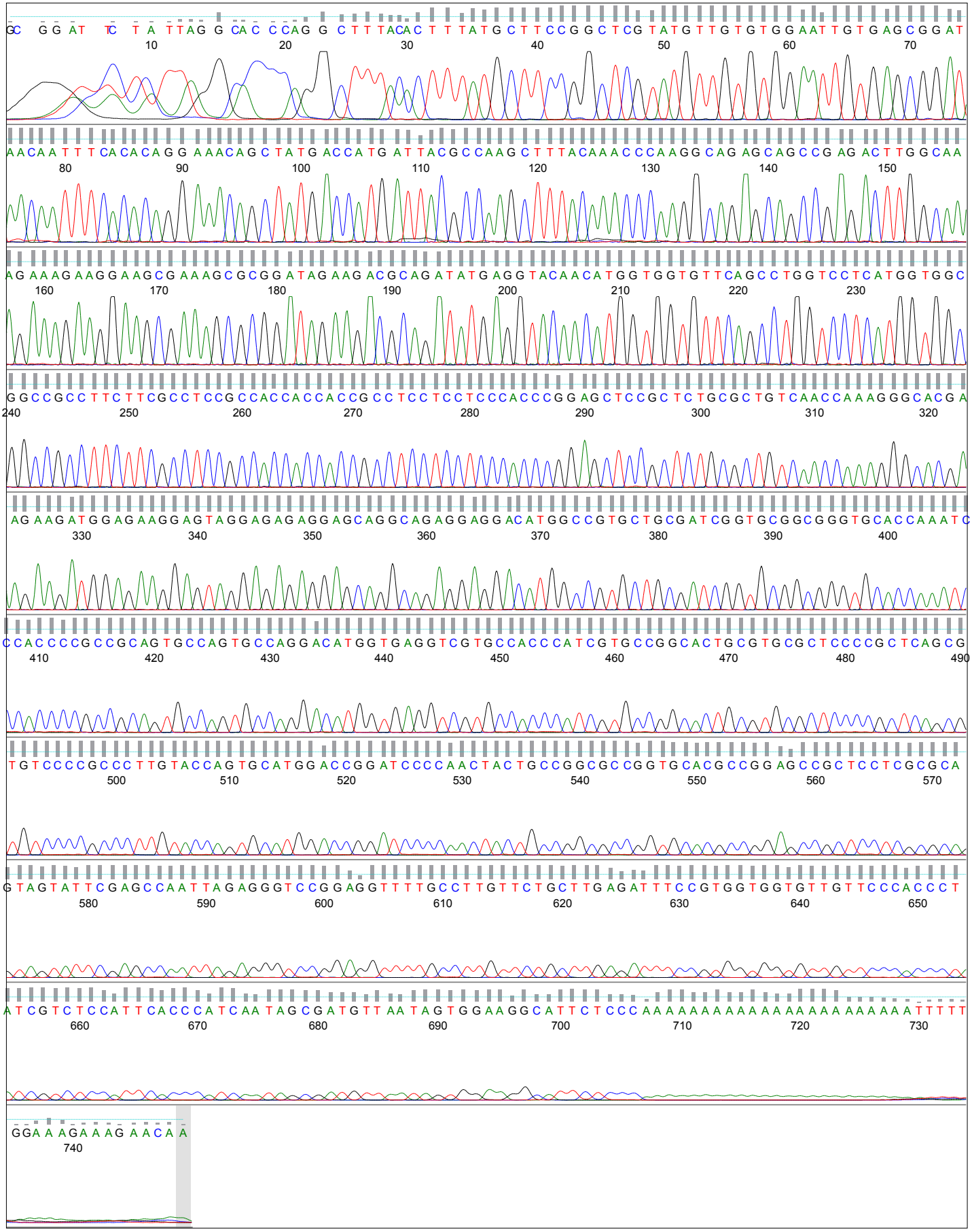
Signal Strengths: A = 5177, C = 4532, G = 5662, T = 3896
Lane/Cap#: 69
Matrix: n/a
Direction: Native



Appendix C4: Sequence of BBI gene fragment

Sample Name: BBI(c5)_57BBI-R
Mobility: KB_3730_POP7_BDTv3.mob
Spacing: 13.084
Comment: n/a

Signal Strengths: A = 2565, C = 2409, G = 2461, T = 1556
Lane/Cap#: 2
Matrix: n/a
Direction: Native



Certificate of Participation

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EXPRESSION OF ANTI-TOXOPLASMOSIS scFv ANTIBODIES IN PLANT CELLS

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INTRODUCTION

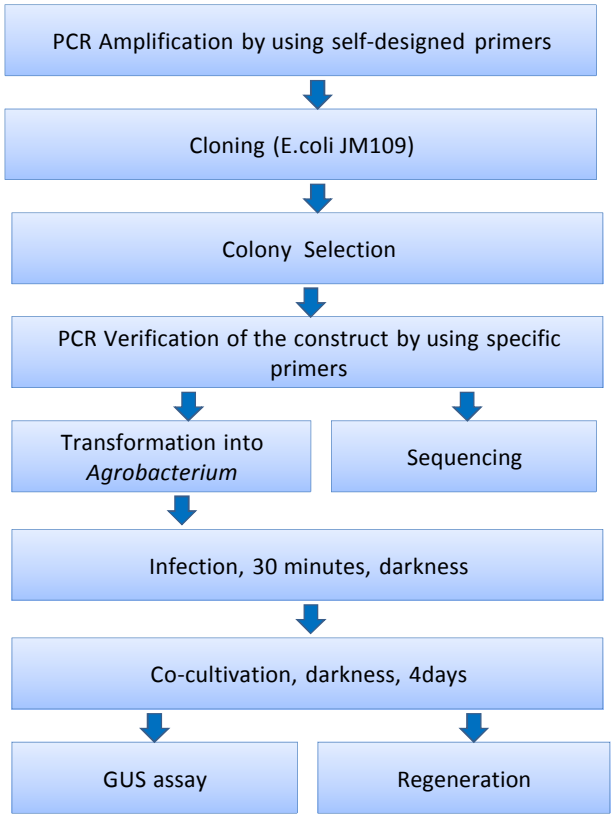
Toxoplasmosis is a parasitic disease caused by single celled protozoa parasite, known as *Toxoplasmosis gondii* which is widespread in human and many other warm-blooded animals, especially, cats and farm animals. This disease causes serious symptoms in infants, pregnant women and fetuses. There are currently no effective vaccines for this disease. One potential approach for treating the disease is the use of specific therapeutic antibodies. In this study, an scFv gene coding for a recombinant phage derived anti-*Toxoplasma* antibody will be introduced into a plant expression system to produce the antibody in the plant cells in large volume. The banana embryogenic cell suspensions were used as plant biofactory in this study, because plantlet that regenerate from somatic embryogenesis can prevent chimerism formation.

OBJECTIVE

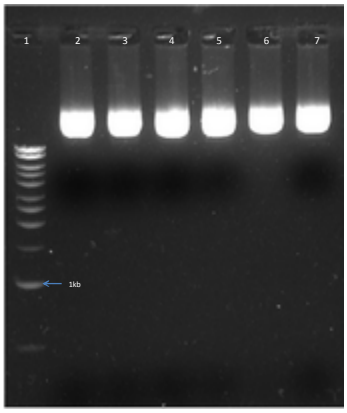
The aim of this study is to develop a recombinant antibody against Toxoplasmosis antigen which can be expressed in plant cells.

MATERIALS AND METHODS

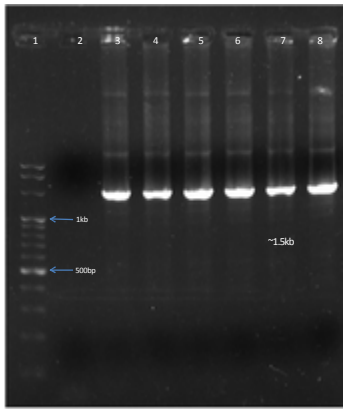
Linear map of construct.
bbi : Bowman-birk Serine protease inhibitor
35S : 35S promoter of Cauliflower Mosaic Virus
hpt : Hygromycin phosphotransferase



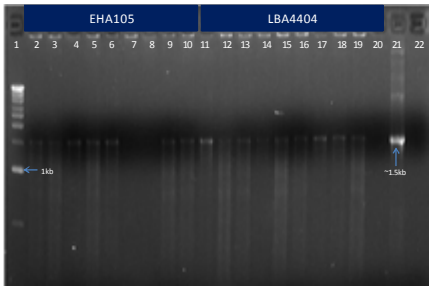
RESULTS AND DISCUSSION



Plasmid analysis of the construct after PCR screening.
Lane 1: Marker; 1kb DNA Ladder.
Lane 2-7: Plasmid of the construct after PCR selection.



PCR Verification of the construct after extracted from *E.coli* by using specific primers.
Lane 1: Marker; 100bp DNA Ladder.
Lane 2: Sterile distilled water as negative control.
Lane 3-8: Different plasmids were used as template.



PCR Verification of the transgene in *Agrobacterium* by using specific primers.
Lane 1: Marker; 1kb DNA Ladder.
Lane 2-20 : Different colonies of the *Agrobacterium*.
Lane21: Plasmid of the construct as positive control.
Lane22: Sterile distilled water as negative control.

Gus activity in banana embryogenic cell suspension after co-cultivation.

In this study, the transgene was successfully introduced into the plant expression system. Therefore, further analysis being carried out on the regenerated plants to confirm the integration of transgene and also the level of expression.

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Certificate of Attendance

This is to certify that

MS. GO PEI SEE

Has participated in the

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Prof. Dr. Mustafa Abdul Rahman
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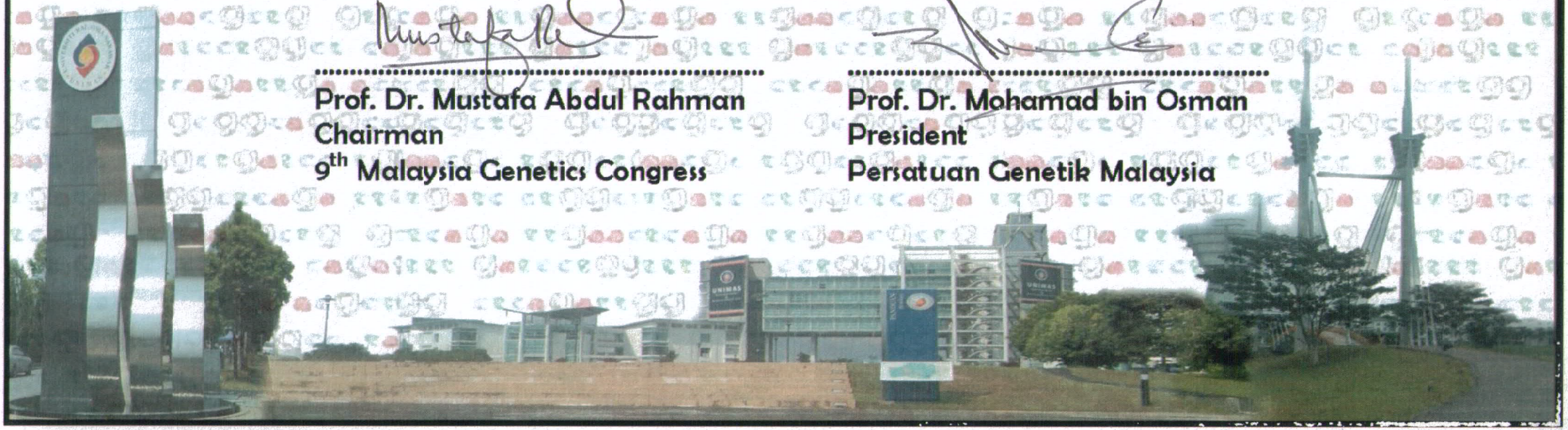

EXPRESSION OF ANTI-TOXOPLASMOSIS scFv ANTIBODIES IN TOBACCO

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INTRODUCTION

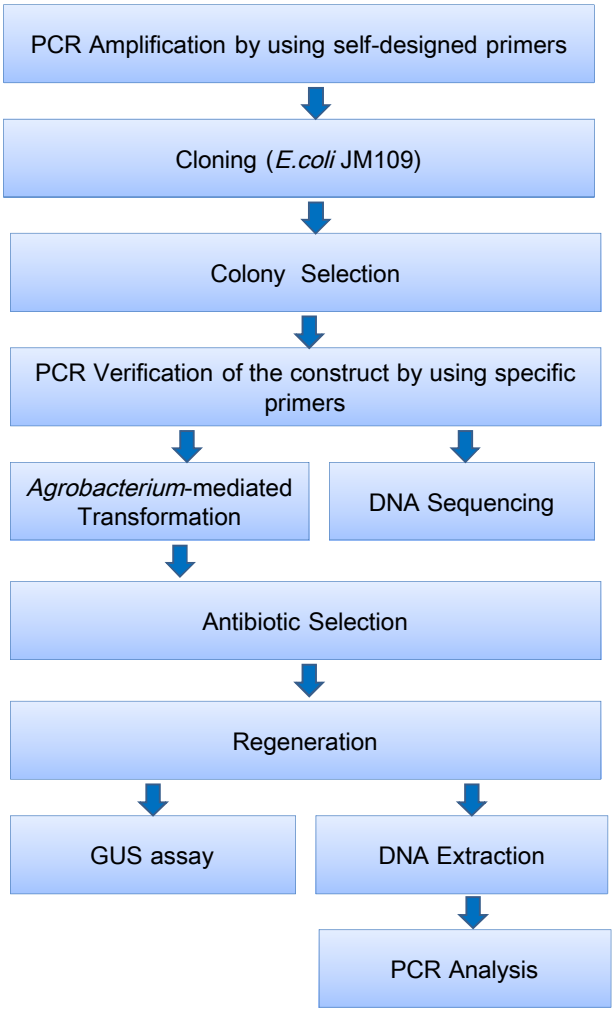
Toxoplasmosis is a parasitic disease caused by single celled protozoa parasite, known as *Toxoplasmosis gondii* which is widespread in human and many other warm-blooded animals, especially, cats and farm animals. This disease causes serious symptoms in infants, pregnant women and fetuses. There are currently no effective vaccines for this disease. One potential approach for treating the disease is the use of specific therapeutic antibodies. In this study, an scFv gene coding for a recombinant phage derived anti-*Toxoplasma* antibody will be introduced into a plant expression system via *Agrobacterium* sp. Plant was chosen as the platform to produce the antibody because of its advantages, such as low risk of human pathogenic contamination, the post-translation modification and protein folding in plant system are similar to human, and the antibody can be produced in large-scale.

OBJECTIVE

The aim of this study is to develop a recombinant antibody against Toxoplasmosis antigen which can be expressed in plant cells.

MATERIALS AND METHODS

Figure 1: Linear map of construct, pToxobbiKdel.
*gus*A: *uid* A gene encoding the β-glucuronidase
mgfp5: green fluorescent protein
bbI : Bowman-birk Serine protease inhibitor
35S : 35S promoter of *Cauliflower Mosaic Virus*
hpt : Hygromycin phosphotransferase



RESULTS AND DISCUSSION

Figure 2 GUS histochemical staining of the regenerated shoots.

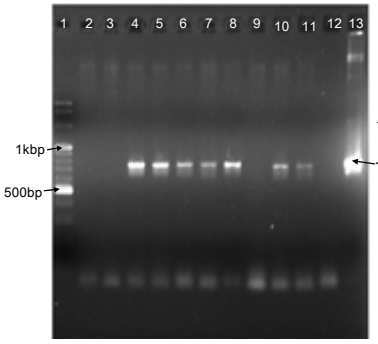


Figure 3 PCR amplification using *gfp* gene primers.
Lane 1: 100bp Marker;
Lane2-3: Untransformed tobacco;
Lane4-11: Transgenic tobacco;
Lane 12: Negative control, without DNA;
Lane 13: Positive control, using construct plasmid as template

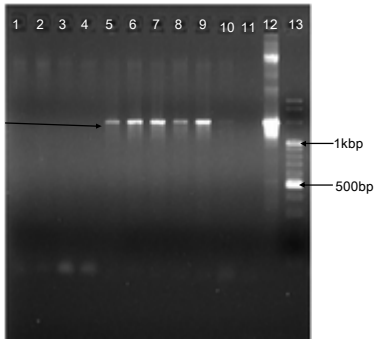


Figure 4 PCR amplification using *gfp* gene primers.
Lane 1-2: Untransformed tobacco;
Lane3-10: Transgenic tobacco;
Lane11: Negative control, without DNA;
Lane 12: Positive control, using construct plasmid as template;
Lane 13: 100bp Marker.

In this study, the gene of interest is successfully integrated into the plant genome. Further analysis need to carry out to study the expression level and its stability in the next generation of the pToxobbiKdel in the tobacco.

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EXPRESSION OF RECOMBINANT ANTI-*TOXOPLASMA* SCFV ANTIBODIES IN TOBACCO

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ABSTRACT

Toxoplasmosis is a disease that widespread not only in humans, but also in animals particularly farm animals. This disease is caused by a single-celled protozoan parasite known as *Toxoplasmosis gondii*. Even though this disease causes critical effect on human health and economic loses; however, there are currently no effective vaccines on the market. A potential approach in developing strategies to control the disease included the use of recombinant antibodies targeting *T.godii* tachyzoites. As part of the development of these antibodies in plant cells is also being explored. In this study, the recombinant antibody was transformed into *Nicotiana tabacum* L. cv. SR1 leaf disks through *Agrobacterium*-mediated transformation. The recombinant antibody was co-expressed with a KDEL retention sequence and Bowman-birk Serine proteinase inhibitor gene. PCR results showed the successful transfer of the transgene integrated into the tobacco genome. Further molecular analysis was carried out to examine the levels of expression of transgene. As shown by the real-time PCR results, the average mRNA levels for transgenic tobacco expressing pToxo130BBI (proteinase inhibitor only) was 4.4-fold higher and only 3.58-fold higher for pToxo130BBIKDEL (proteinase inhibitor plus KDEL) when compared to non-transformed tobacco (control). The unexpected results are being verified by analysis of the effectiveness of adding a KDEL retention sequence and Bowman-birk Serine proteinase inhibitor genes individually in constructs and through further quantitative analysis of the tobacco-expressed protein.

MATERIALS AND METHODS

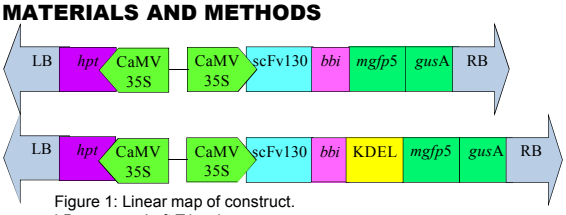
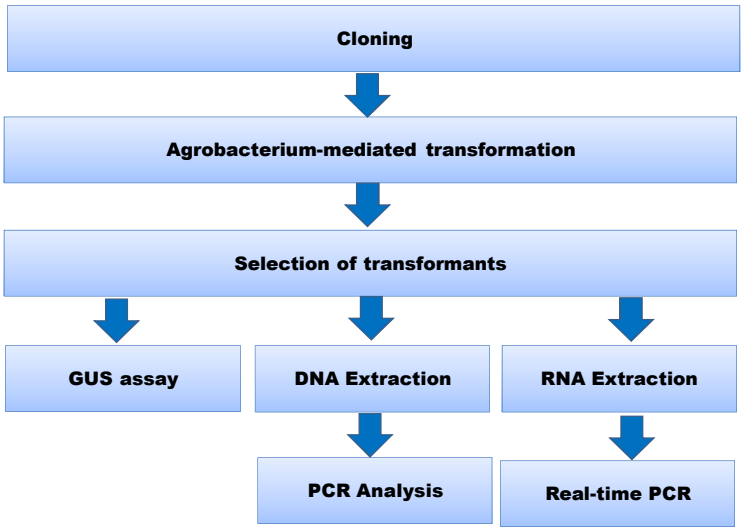


Figure 1: Linear map of construct.
LB: Left T-border
hpt : Hygromycin phosphotransferase
CaMV35S: 35S promoter of Cauliflower Mosaic Virus
scFv130: Recombinant antibody against Toxoplasmosis
bbI : Bowman-birk Serine protease inhibitor
KDEL: KDEL Retention Sequence
Mgfp5: Green Fluorescent protein
*gus*A: β-Glucuronidase gene
RB: Right T-border



RESULTS AND DISCUSSION

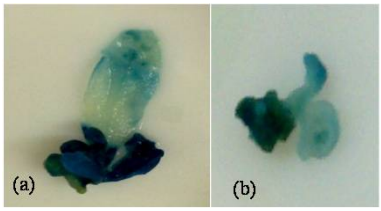


Figure 2.0: GUS histochemical staining of the regenerated shoots.

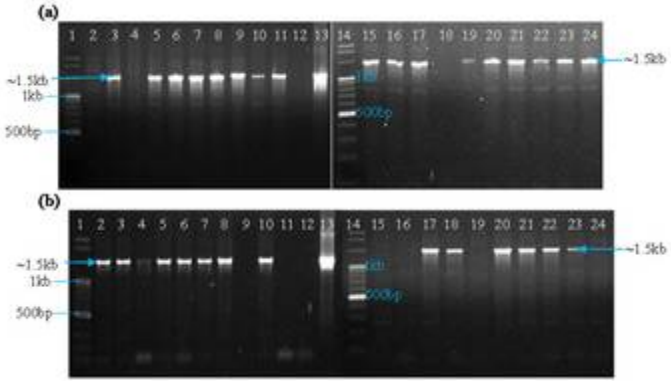


Figure 3.0: PCR amplification using *gfp* gene primers. (a) Lane 1&12: 100bp Marker; Lane2-11 & 13-22: Transgenic tobacco with pToxo130BBI; Lane 23: Negative control, without DNA; Lane 24: Positive control, using construct plasmid as template; (b) Lane 1&14: 100bp Marker; Lane2-11 & 15-24: Transgenic tobacco with pToxo130BBIKDEL; Lane 12: Negative control, without DNA; Lane 13: Positive control, using construct plasmid as template.

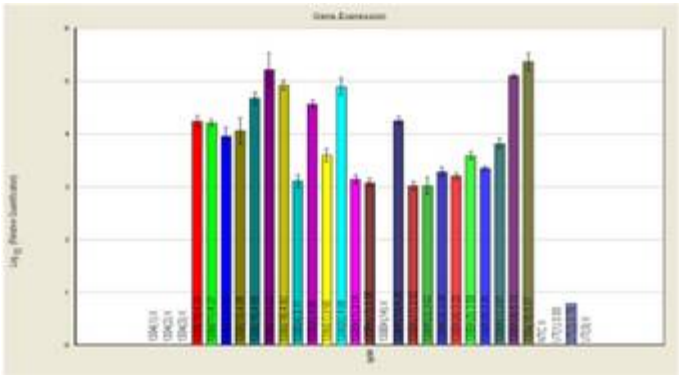


Figure 4.0: Gene expression level of recombinant antibody in tobacco.

Transgenic tobaccos carrying pToxo130BBI and pToxo130BBIKDEL were examined with real-time PCR to study the levels of expression of the transgene. Surprisingly, the expression level of pToxo130BBI was 0.82-fold higher compared to pToxo130BBIKDEL. This result is unexpected as pToxo130BBIKDEL contained two elements for enhancing and stabilizing the recombinant antibody, whilst pToxo130BBI only had one element. One possibility for the lower expression level indicated in pToxo130BBIKDEL is that the relative quantification studies carried out was based on mRNA level of the transgene instead of protein accumulation levels in the transgenic tobacco cells (Schouten, et al., 1996). The actual amount of successfully accumulated protein may not be reflected by the level of transcript. Furthermore, it is possible that the larger transgene cassette may also incur a penalty to the amount of mRNA produced.

Thus, further studies on protein expression level such as by Western Blot analysis needs to be carried out to examine the effectiveness of adding the Bowman-birk Serine proteinase and KDEL retention sequence to the recombinant protein.

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