

**PRELIMINARY ASSESSMENT OF THE EFFECT OF PLANT  
PROTEASE INHIBITORS ON THE EXPRESSION OF  
HETEROLOGOUS GENES IN PLANT CELLS**

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**FACULTY OF SCIENCE  
UNIVERSITI OF MALAYA  
KUALA LUMPUR**

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EXPRESSION OF HETEROLOGOUS GENES IN PLANT CELLS**

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## ABSTRACT

Despite the successful development of plant-based expression systems for the production of a wide range of recombinant proteins in recent years, there remain serious limitations in the final yield accumulation of the foreign protein in the plant cells used. Plant protease activity represents a significant barrier to efficient and hence economical recombinant protein production. Inadequate information is available on specific plant proteases and its role in foreign protein degradation. Some strategies have been reported recently to overcome endogenous plant protease effects on foreign protein integrity and activity in plants including the co-expression of recombinant proteases to reduce the endogenous protease activity. The lack of information in this area suggests that more studies should be carried out to assess different plant protease inhibitors for the improvement of the stability of foreign protein production in particular host plants. The aim of this research was to evaluate the effect of recombinant plant protease inhibitors on the expression of a cloned heterologous ScFv antibody gene in plant cells. The recombinant tomato Cathepsin D Inhibitor (CDI) gene fused to a KDEL sequence was ligated to a previously constructed recombinant antibody (scFv) gene developed against toxoplasmosis epitopes (pCTOXO-CDI-KDEL) and then transferred successfully into *Nicotiana tabacum* cv. BY-2 cell suspension using *Agrobacterium*-mediated transformation method. In addition, the scFv gene was also ligated to a construct containing only the endoplasmic reticulum retention signal, KDEL, (pCTOXO-KDEL) and this was also transferred into tobacco cells as a comparison against CDI-KDEL containing cells. Both constructs contained GUS marker gene. To assess the effect of the CDI gene in putative transformed tobacco cells GUS fluorometric assay was performed. Total soluble protein of the tobacco cells was also analyzed by Bradford protein assay. The tobacco cells carrying the CDI protease inhibitor gene showed higher TSP levels in comparison with plant cells lacking this

gene. This finding suggests the positive effect of co-expression of the CDI gene on the expression of scFv anti-toxoplasmosis in tobacco cells. However, quantitative GUS enzymatic activity expressed in pmol 4-MU/mg protein/min did not show any significant differences between two constructs in paired t-test ( $p>0.05$ ). Overall results suggest that this strategy could be viable for increasing the production of ScFv antibodies in plant cells.

## ABSTRAK

Walaupun perkembangan sistem ekspresi berasaskan tumbuhan dalam penghasilan protin rekombinan beberapa tahun kebelakangan ini telah berjaya, tetapi masih terdapat halangan untuk memastikan pengumpulan produk akhir yang maksima di dalam sel tumbuhan yang digunakan. Di antara faktor penghalang yang utama dalam penghasilan protein rekombinan yang cekap dan produktif adalah aktiviti enzim protease. Walaupun fungsi sesetengah protein protease dalam degradasi protein asing telah diketahui, namun maklumat yang ada tidak mencukupi. Beberapa strategi dilaporkan telah digunakan untuk mengatasi masalah ini termasuklah ekspresi bersama (co-expression) protease rekombinan untuk mengurangkan aktiviti protease dalaman (endogenous) tumbuhan. Kurangnya maklumat dalam bidang ini menunjukkan lebih banyak kajian perlu dijalankan untuk menilai potensi perencat protease tumbuhan (plant protease inhibitor) yang berbeza bagi meningkatkan pengeluaran/penghasilan protein asing dalam tumbuhan. Kajian ini bertujuan untuk menilai kesan perencat (inhibitor) rekombinan protease tumbuhan ke atas pengekspresan klon gen 'heterologous ScFV' antibodi dalam sel tumbuhan. Gabungan antara gen 'Cathepsin D Inhibitor' (CDI) daripada tomato rekombinan dan jujukan KDEL telah dicantumkan ke dalam gen rekombinan antibodi yang dapat mengecam 'toxoplasmosis epitope' (pCTOXO-CDI-KDEL). Fragmen DNA ini (pCTOXO-CDI-KDEL) seterusnya dipindahkan ke dalam cell suspension *Nicotiana tabacum* cv. BY-2 melalui kaedah transformasi berasaskan *Agrobacterium*. Di samping itu, gen scFv turut digabungkan ke dalam konstruk (fragmen DNA) yang hanya mengandungi isyarat pengekal retikulum endoplasmik, KDEL, (pCTOXO-KDEL) dan seterusnya dipindahkan ke dalam sel tembakau untuk dijadikan perbandingan dengan sel yang mengandungi CDI-KDEL. Kedua-dua fragmen DNA ini mempunyai gen penanda GUS. Ujian fluorometrik GUS dilakukan untuk menganalisa/menilai kesan gen CDI dalam sel tembakau tersebut. Jumlah protein larut dalam sel tembakau turut

dianalisa menerusi ujian protin Bradford. Sel tembakau yang mengandungi gen ‘CDI protease inhibitor’ menunjukkan jumlah protein larut yang lebih tinggi berbanding sel tumbuhan yang tidak mempunyai gen ini. Penemuan ini menunjukkan kemungkinan wujudnya kesan positif ekspresi bersama gen CDI ke atas pengekspresan scFv anti-toxoplasmosis dalam sel tembakau. Namun begitu, aktiviti kuantitatif pmol enzim GUS yang dikira berdasarkan t-berpasangan ( $p>0.05$ ) dalam unit pmol 4-MU/mg protein/min di antara kedua-dua DNA tidak menunjukkan perbezaan yang ketara. Secara keseluruhannya, data daripada eksperimen yang dijalankan menunjukkan petanda positif keberkesanan kaedah ini dalam meningkatkan pengeluaran antibodi scFv dalam sel tumbuhan.

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## **ABBREVIATION**

A: Absorbance

Agrob: *Agrobacterium*

anti-toxo: anti-toxoplasmosis

BBTI: Bowman–Birk trypsin inhibitor

bp: base pair

BSA: Bovine serum albumin

BY-2: Bright Yellow–2

CaCl<sub>2</sub>: Calcium chloride

CDI: Cathepsin D Inhibitor

cDNA: Complementary deoxyribonucleic acid

conc: Concentration

cv: Cultivar

DNA: deoxyribonucleic acid

*E.coli: Escherichia coli*

EDTA: Ethylenediaminetetracetic acid

ER: Endoplasmic reticulum

ETOH: Ethanol

Fab: Fragment antigen binding

g: Gram

GAB: GUS assay buffer

GEB: GUS extraction buffer

HDEL: His-Asp-Glu-Leu

HIV: human immunodeficiency virus

Kb: Kilobase

KCl: Potassium chloride

KDEL: Lys-ASP-Glu-Leu

l: Liter

LB: Luria-Bertani

M: Molar

M2: Media 2

mAB: Monoclonal antibody

mg: milligram

min: minute

ml: milliliter

mM: Millimolar

MnCl<sub>2</sub>: Manganese chloride

MOPS: 3-(N-morpholino)propanesulfonic acid

MS: Murashige and Skoog media

MSO: Murashige and Skooge basal medium

MUG: 4-methylumbelliferyl- $\beta$ -D-glucuronide

MW: Molecular weight

Na<sub>2</sub>CO<sub>3</sub>: Sodium carbonate

NaOH: Sodium hydroxide

nM: Nanomolar

OCPI: Oryzacystatin protease inhibitor I

OD: Optical density

PCR: polymerase chain reaction

pCTOXO-BBTI: pCAMBIA 1301-scFv anti -Toxo-BBTI

pCTOXO-CDI-KDEL: pCAMBIA 1304-scFv anti -Toxo-CDI-KDEL

pCTOXO-CDI: pCAMBIA 1301-scFv anti -Toxo-CDI

pCTOXO-KDEL: pCAMBIA 1304-scFv anti -Toxo-KDEL

pCTOXO-OCPI: pCAMBIA 1301-scFv anti -Toxo-OCPI

pCTOXO-PI: pCAMBIA 1301-scFv anti -Toxo-Protease inhibitor

pH: Potential hydrogen

PI: Protease inhibitor

pmol: picomole

pUC-PI: pUC57-Protease inhibitor

RE: Restriction endonuclease

RNA: ribonucleic acid

RNAse A: Ribonuclease A

rpm: Revolutions per minute

scFv: Single chain variable fragment

sdH<sub>2</sub>O: Sterile distilled water

SDS: Sodium dodecyl sulphate

sec: Second

TBE: Tris borate

Ti: Tumor inducing

TOXO: Toxoplasmosis

Tris-Cl: Trisbase-Hydrochloric acid

TSP: Total soluble protein

u: Unit

UV: Ultraviolet

v: Volume

Vir: Virulence

vs: Versus

w: Weight

w/v: Weight over volume

X: Times

2,4-D: 2,4-Dichlorophenoxyacetic acid

4-MU: 4-methylumbelliferone

$\mu$  l: Micro liter

g: Micro gram

$\mu$ M: Micro molar

$^{\circ}$ C: Degree Celsius

%: Percentage

## CHAPTER 1: INTRODUCTION

The demand of producing different recombinant proteins is increasing in recent years. For instance production of useful therapeutic recombinant proteins is highly needed in order to improve the health level of human society. To date, several heterologous expression systems have been designed for the production of useful therapeutic recombinant proteins. In most cases, eukaryotic hosts competent in performing complex posttranslational modifications have been involved. But, plant-based systems compared favorably with alternative expression platforms, both in terms of quality and cost of complex therapeutic proteins. Higher plants are used as versatile expression systems for producing therapeutic foreign proteins. This technology offers unique advantages, including cost reduction, possibility of bulk production of useful foreign proteins as well as the range of plant hosts available and the stability of transgenic lines in seeds that might allow export of transgenic crops (containing, for example, medicinal compounds) to deprived areas of the world .

One major problem of producing recombinant proteins in higher plants is instability of produced recombinant proteins. This system presents serious challenges to protein integrity. That means foreign protein degradation directly impacts on the final yield, homogeneity and overall quality of the resulting protein product. The instability of recombinant proteins is due to activity of plant proteases. The proteolytic enzymes in plant cells, in particular, are often a major hurdle to recombinant protein integrity, both *in planta* at the expression stage and *in vitro* during protein recovery from plant tissues. Therefore, in plant expression systems proteolytic processing, notably, may dramatically alter the structural integrity and overall accumulation of recombinant proteins in plant expression systems.

Although several strategies have been considered to minimize proteolysis in plant protein factories involving the targeting of transgene expression or protein accumulation to specific tissues or cellular organelles, this problem still needs a lot of researches to overcome this negative effect on foreign protein final yield.

A recent solution proposed is the co-expression of companion protease inhibitors interfering with endogenous proteases which are active against specific endogenous proteases. Recombinant protease inhibitors could prove functional to modulate proteolytic activities *in situ* but very few researchers conducted in this area so there is a need to conduct more investigations to improve this area of research to help improving the yield and functionality of foreign proteins.

The lack of sufficient information on different plant proteases existence in plants makes conducting more researches in this area necessary. The *Nicotiana tabacum* plant as a very important target system in producing foreign proteins has shown the proteolytic degradation problem. However many of the proteases responsible for the recombinant protein degradation in this plant remained unknown.

For obtaining the information on type of proteases which exist in tobacco plant cells one way is to assess the co-expression of protease inhibitor genes with recombinant protein gene. The improved level of desired foreign protein translation in plant cells carrying the suitable companion protease inhibitor can make the production of foreign proteins more efficient in tobacco cells as a very useful target system. The Cathepsin D inhibitor gene, Bowman Birk Trypsin I inhibitor and Oryzacystatin protease Inhibitor genes were designed to assess the presence of proteases that can become inactive by the help of co-expression of these inhibitors in tobacco cells.

In this research, the tomato Cathepsin D Inhibitor gene was ligated to a recombinant single chain fragment anti-toxoplasmosis antibody (-scFv). The construct was then transferred and expressed in *Nicotiana tabacum* cultivar BY-2 cells. In addition, the ER retention signal, KDEL, ligated to scFv (obtained from previous studies) was transferred and expressed in tobacco cell suspension in separate experiments to produce putatively transformed tobacco plant cells carrying scFv gene but lacking CDI protease inhibitor. The expression of scFv in the presence of the CDI protease inhibitor in plant cells was investigated and compared to heterologous scFv gene expression in plant cells without CDI protease inhibitor.

The objective of this research was to assess the effect of plant protease inhibitors on the expression of heterologous monoclonal antibody scFv gene of anti-toxoplasmosis in plant cell suspension.

The specific objectives are to develop constructs of scFv-PIs, carry out transformation experiments and then analyze the resulting putative transformed plant cells in terms of total soluble protein (TSP) and GUS Enzymatic activity using Bradford protein assay and GUS Fluorometric Assay.

## **CHAPTER 2: LITERATURE REVIEW**

### **2.1 PRODUCTION OF ANTIBODIES *IN VITRO***

Today antibodies are applied in the diagnosis of diseases and therapeutic uses, by-research institutions and pharmaceutical companies (Joosten et al., 2003). The production of antibodies have increased in quality and quantity in recent years by the help of recombinant DNA technology, so the expression of a whole antibody gene or a fragment of it has become achievable in different living organisms' cells such as bacteria (Nouaille et al., 2003), mammalian cells (Yazaki et al., 2004) and plant cells.

### **2.2 COMPARING DIFFERENT EXPRESSION SYSTEMS**

The production of heterologous proteins is mostly carried out in prokaryotic systems such as cultures of bacterial cells (Lu Yinghua et al., 2004). In many cases the prokaryotic system is preferred because the bacterial cells can be grown rapidly and high cell densities can be achieved easily, besides the media in this system is cheap (Lu Yinghua et al., 2004). However, there are some limitations to use of the prokaryotic systems. Firstly, the lack of post-transcriptional modifications such as phosphorylation, acylation, and N-and O-linked glycosylation which can affect the recombinant protein activity (Lu Yinghua et al., 2004), since the ability to perform post translational modifications does not exist in bacterial expression systems (Jung and Williams, 1997). Secondly, the heterologous proteins may accumulate into insoluble structures because the prokaryotic expression system does not have the ability to secrete the protein product into the extracellular medium, and proteins may remain in structures known as

inclusion bodies. Therefore, the downstream processing of heterologous proteins become very expensive and complicated (Lu Yinghua et al., 2004).

The eukaryotic system was then established to overcome the problem of the prokaryotic system. One of the popular eukaryotic systems is yeast. The proteins produced in yeast system can be secreted to the media. The viruses that can affect humans do not exist in yeasts (Lu Yinghua et al., 2004). Yeast are able to glycosylate the proteins that are produced in their system and they can multiply rapidly. However, the folding of manufactured protein is different from the type seen in mammalian cell cultures. The difference is glycosylation process. Hyper mannosylation is another disadvantage of yeast system. It means that proteins produced in yeast normally contain extra mannose added to the basic oligosaccharide and thus such manufactured proteins are not usually appropriate for the production of human medicines (Jung and Williams, 1997).

A higher eukaryotic system in protein production process in comparison with yeast cells are insect cells. This system also has been used for the production of useful proteins. An example of such expression system is the baculovirus (Rai and Padh, 2001) but, with some limitations. In baculovirus expression system the proteolytic cleavages in lysine or arginine rich sequences is ineffective. This deficiency causes imperfect protein folding. Other disadvantages of this system are expensive media for the production of insect cells and slow growth of the cells (Lu Yinghua et al., 2004).

The other eukaryotic system in protein production process is mammalian cells. The mammalian cells are generally used for high value end products. Performing the most suitable type of post-translational modifications to the protein product is one of the advantages using this expression system. This benefit of the mammalian cellular

expression system makes them appropriate for use in human medication (Rai and Padh, 2001). However, there are some problems associated with the use of this mammalian cell system. This expression system is very hard to maintain and it is a time consuming system. The production and maintenance of this system is a very complex task in comparison with the other biological expression systems and the yield of the protein product is normally low, so only when other systems are incompatible mammalian cells are used as the expression system (Rai and Padh, 2001).

### **2.2.1 Using plants as an expression system for protein production**

An attractive host system for production of recombinant proteins such as biopharmaceuticals is plant-based expression system. Plants offer several important advantages over other heterologous expression systems. As a eukaryote system, plants are able to fold and assemble complex proteins correctly and post-translational modifications like glycosylation can be achieved in plants. The requirement for animal cells, animal derived culture materials or potentially infectious animals is also eliminated with applying the plant host system in the production of foreign proteins. An alternative technique for the production of recombinant proteins is plant cell culture in bioreactors. The control of production environment is easier in plant cultures in comparison with whole plants. Besides, the process cycle is shorter and purification process is simpler and cheaper due to secretion of proteins from plant cells (Doran, 2006).

The first successful animal antibody production was achieved in transgenic tobacco plants in the late 1980's (Hiatt et al., 1989). That successful experiment showed recombinant monoclonal antibodies can be produced in plants as an intact antibody

molecule. In that work cDNAs encoding either the heavy chain or light chain of a catalytic mouse antibody molecule were expressed in the engineered plants. Then, the plants were cross pollinated. As a result both cDNAs were found to be expressed in the progeny, and the fully assembled recombinant antibody expressed in plants. Almost 1% of total soluble protein (TSP) in the plant was found to be comprised of the recombinant antibody. The antibody molecules produced in plant tissue had the same antigen-binding activity like the progenitor antibody. Since then, expression of antibody molecules in plants has been done by several groups for either modification or improvement of plant characteristics and performance or for large-scale production of antibodies using plants as bioreactors (Ma et al., 1995). The techniques for the production of human recombinant proteins in plants have improved a lot and the techniques have become more efficient. In recent years, the production of monoclonal antibodies both in the form of full length or antibody fragments preferably has been done on plant system by the scientific community (Hendy et al., 1999) instead of using traditional microbial systems. The large scale production of commercially important biomolecules can be achieved in plants (Whitelam and Cockburn, 1997).

In general, plant expression systems produce recombinant biomolecules more efficiently, both in terms of quality and cost of complex therapeutic proteins in comparison with other expression platforms (Komarnytsky et al., 2006). The range of plant hosts available and the possibility of bulk production of recombinant therapeutic proteins and reducing costs are some of the advantages of using plant-based technology. The export of transgenic plants to deprived areas of the world is also another advantage of this system. Using stable transgenic lines in seeds containing medicinal compounds is an example for this benefit (Ma et al., 1995). The heterologous production of clinically

useful proteins as therapeutic agents can be achieved in higher plants which represent versatile expression platforms.

### **2.2.1.1 Plant cell cultures expression systems**

Culturing the specific organs or cells that produce the antibody and either isolate the antibody from these cells or tissues, or collect it from the culture medium is an alternative system to whole plants expression systems. A number of different culture systems have been developed, although most research has focused on cell suspension cultures. Suspension cells are individual plant cells and small aggregates thereof growing in liquid medium in a fermenter (Hellwig et al. 2004; Doran 2006). Suspension cell cultures are usually derived from callus tissue by the disaggregation of friable callus pieces in shake bottles, and are later scaled up for fermenter-based production. Recombinant antibody production is achieved by using transgenic explants such as *Nicotiana tabacum* to derive the plant cell cultures, or transforming the cells after disaggregation, usually by co-cultivation with *Agrobacterium tumefaciens* (Twyman et al., 2007).

Many foreign proteins have been expressed successfully in suspension cells, including antibodies, enzymes, cytokines and hormones (Hellwig et al. 2004, Fischer et al. 1999). Tobacco cultivar Bright Yellow 2 (BY-2) is the most popular source of suspension cells for molecular farming, since these proliferate rapidly and are easy to transform (Nagata et al., 1992). Other than tobacco, rice suspension cells have also been used to produce several antibodies (Torres et al., 1999). Recombinant antibodies expressed in plant cell suspension cultures may be secreted into the culture supernatant or retained within the cells. Localization depends on expression construct design and the permeability of the plant cell wall to the antibody.

The inclusion of a C-terminal KDEL sequence results in higher levels of antibody accumulation in cultured cells because the biochemical environment of the endoplasmic reticulum favors stable protein folding and assembly while reducing the level of proteolytic degradation. However, this also makes it necessary to disrupt the cells in order to isolate the protein, which requires additional processing time and causes the release of phenolic molecules that interfere with purification and reduce production yield. Thus, the preferred approach is to secrete the target proteins and capture them from the culture supernatant or release them from the cells by mild enzymatic digestion. (Twyman et al., 2007).

### **2.3 FOREIGN PROTEIN ACCUMULATION LEVEL IN PLANTS**

The accumulation level of foreign protein product in plant system is a very challenging and crucial parameter which affects the economics of protein production. A significant problem that limits the commercial exploitation of recombinant plant as host system is low protein yield (Doran, 2006). The accumulation level of most foreign proteins is much lower than 1% of total soluble protein (TSP) in plant biomass; Commonly, about 0.01–0.1% TSP or less are reported as maximum product concentrations ( Daniell et al., 2001). The low yield of recombinant protein is usually considered inadequate for competition with other heterologous protein production systems and it remains as a major difficulty in this technology (Kusnadi et al., 1997). Therefore, the future development of this technique is directly affected with improving foreign protein accumulation in plant systems.

In recent years, there is an increasing recognition that shows overall yield of foreign proteins in plant cells are crucially determined by protein degradation. At any given time, the amount of recombinant protein found in plant cells expresses a balance

between protein production and protein degradation or loss. As evidence, several recombinant plant systems have been reported in which there is a lack of correlation between foreign mRNA transcript levels and foreign protein product concentration (Richter et al., 2000; Outchkourov et al., 2003). It suggests that foreign protein may be expressed successfully but subsequently degraded. Therefore, high mRNA levels do not guarantee high accumulation levels of foreign protein (Doran, 2006).

## **2.4    PROTEOLYTIC ENZYME**

Higher plants are used as versatile expression systems for producing therapeutic foreign proteins. However, this system presents serious challenges to protein integrity. The instability of recombinant proteins is due to activity of plant proteases. The well-documented importance of proteolytic enzymes in plant cells, in particular, is often a major hurdle to recombinant protein integrity, both *in planta* at the expression stage and *in vitro* during protein recovery from plant tissues (Faye et al., 2005). Foreign protein degradation in plant expression systems directly affects the final yield, homogeneity and overall quality of the resulting protein product. Proteolytic processing may dramatically change the overall accumulation and structural integrity of foreign proteins in plant-based expression systems (Benchabane et al., 2008).

Proteolytic enzymes, or proteases, contribute to the overall control of metabolic and transduction pathways by directing the activation or hydrolysis of proteins implicated in key regulatory processes, or by contributing to the elimination of misfolded proteins and the selective recycling of amino acids from short-lived proteins (Vierstra, 2003; Schaller, 2004). For the efficient processing and quality control of proteins, proteolytic

enzymes are essential and are a fundamental element in the reaction of cells to changing environmental conditions (Doran , 2006).

A significant challenge for producing foreign proteins economically in all heterologous systems, including plants, is countering the effects of protease activity. Loss of recombinant protein after synthesizing, assembly and, in some cases, glycosylation and post-translational modification processes represents a considerable waste of biosynthetic resources in transgenic plant cells. Increasing protein product heterogeneity and lower consistency are other detrimental consequences of proteolysis that can cause regulatory problems. Therefore, more difficult and expensive downstream processing is needed due to the presence of incomplete protein or protein fragments (Stein et al., 2001).

Degrading abnormal or incorrectly processed proteins is an important role of proteases. Therefore, the susceptibility of recombinant proteins to protease attack in plant cells could reflect their improper synthesis or assembly. Folding and quaternary structure of foreign proteins are affected by differences between plant and animal glycans. This issue can make plant-derived proteins more susceptible to protease activity than their animal-derived counterparts (Doran, 2006). Misfolding and lack of proper disulphide crosslinking in plant-derived proteins can also increase the likelihood of protease attack (Smith et al., 2002).

## **2.5 MINIMIZING PROTEOLYSIS OF HETEROLOGOUS PROTEINS IN PLANT CELLS AND TISSUES**

Specific organelles or sub-cellular locations within plant cells and tissues may be associated with proteolytic degradation of recombinant proteins. Plant vacuoles were identified as a possible site of foreign protein degradation in *Arabidopsis thaliana* (Yang et al., 2005) and potato leaves (Outchkourov et al., 2003). Vacuoles contain a variety of proteases that are active under mildly acidic conditions. Particular cleavage of glycosylated antibody in tobacco cells were identified to occur in the secretory pathway between the ER and golgi. The proteases were considered most likely to be responsible for the cleavage (Sharp et al., 2001). Besides, the production of proteolytic fragments in several transgenic plant systems has been reported in apoplast or plant cell wall (Outchkourov et al., 2003; Sharp et al., 2001; Stevens et al., 2000).

### **2.5.1 Targeting of recombinant gene expression in specific plant tissue**

The final yield of foreign protein product and its quality are strongly influenced by specific tissue in which foreign protein is produced. Because the quantity and quality (or overall substrate specificity) of plant proteases, notably, differ from one tissue to another (Schaller, 2004), with a possible differential impact on the integrity of proteins.

An accumulation site with low levels of overall proteolytic activity or with endogenous protease enzymes with no or little specific activity against accessible peptide bonds in the protein of interest is desirable in effective foreign protein production.

To date, green leaves have been the destination of choice for several foreign proteins, given their rapid growth rate, the possibility in some platforms to harvest leaf material

more than once over the growing season, and the availability of numerous regulatory sequences well adapted to transgene expression in the leaf cell environment (Daniell et al., 2001). However, high protein synthesis and turnover rates which are due to the highly active metabolism of leaf tissues may represent a significant hurdle to protein accumulation *in vivo*. In particular, increased protease levels in senescing leaves (Kato et al., 2004, 2005; Lin and Wu, 2004; Otegui et al., 2005; Parrott et al., 2005) represent a potential drawback in leaf-based production systems (Stevens et al., 2000; Birch-Machin et al., 2004).

By contrast with leaf tissues, efficient deposition and post-harvest storage of foreign proteins have been reported in the seeds and storage organs of several crops. In general, seeds appear to be a suitable tissue for recombinant protein production (Stoger et al., 2005). The low abundance of active proteases in seed tissues during dormancy, together with the desiccated nature of mature seeds prevent extensive proteolysis and promote long-term stability of proteins *in planta* (Fiedler and Conrad, 1995; Stoger et al., 2000). Seeds have the appropriate molecular environment to promote protein accumulation, and achieve this through the creation of specialized storage compartments such as protein bodies and storage vacuoles that are derived from the secretory pathway. Antibodies expressed in seeds remain stable for at least three years at ambient temperatures with no detectable loss of activity (Stoger et al., 2005). Similarly, the storage roots and tubers of some plants show reduced metabolic activity, and may represent an interesting solution for foreign protein storage (Artsaenko et al., 1998).

## **2.5.2 Targeting of recombinant protein to specific organelle in plant cells**

Organelles have specific, complementary functions in the cell and therefore harbour their own metabolic machinery, including a protease complement well adapted to their specific enzymatic and physicochemical environment (Callis, 1995). Not surprisingly, the recombinant proteins accumulation rate is strongly influenced by targeting of foreign protein to different organelles using appropriate peptide signals. In practice, the choice of a suitable cellular destination will also depend on the structural characteristics of the foreign protein, which will often dictate specific co- or post-translational modifications essential for adequate activity, stability and/or homogeneity (Faye et al., 2005).

### **2.5.2.1 Targeting and retention of recombinant protein in the endoplasmic reticulum**

Proteins carrying a signal peptide for cellular secretion first enter the endoplasmic reticulum via the ER protein translocation channel (Galili et al., 1998), and then migrate through this compartment and the golgi apparatus until reaching the extracellular medium (default pathway) or the vacuole, if a vacuolar sorting signal is found in the primary sequence. Retention of foreign proteins in this compartment is also achievable for proteins entering the ER by simple apposition of the tetrapeptide ER retention signal (K/H)DEL (Michaud et al., 1998) at the C-terminus. The (K/H)DEL motif is a common ER retrieval signal in eukaryotes, believed to redirect tagged proteins to the ER after their recognition by a (K/H)DEL receptor complex in the golgi apparatus (Pagny et al., 2000). Numerous studies have been published illustrating the positive impact of retaining clinically or industrially useful proteins in the ER compartment of plant cells, using, in most cases, a (K/H)DEL retention signal (Ma et al., 2003). The inclusion of a

C-terminal KDEL sequence results in higher levels of antibody accumulation in cultured cells because the biochemical environment of the endoplasmic reticulum favors stable protein folding and assembly while reducing the level of proteolytic degradation (Twyman et al., 2007). Biologically active mAbs require a number of assembly steps and posttranslational modifications that are carried out in the endoplasmic reticulum (ER) (Deneke, J., 1990).

At the biochemical level, the low abundance of proteolytic enzymes and the presence of molecular chaperones in the ER, together with an oxidizing status favouring disulphide bond formation, make this organelle a suitable destination for several proteins susceptible to rapid turnover or showing a complex folding pathway (Nuttall et al., 2002; Faye et al., 2005). The ER, which constitutes a natural reservoir for some storage proteins in seed cells (Shewry and Halford, 2002), can physically accommodate high levels of foreign protein product *in planta* (Wandelt et al., 1992).

#### **2.5.2.2 Targeting of recombinant protein to the vacuole**

There are two different types of vacuole in plant cells: lytic vacuoles, which are rich in hydrolytic enzymes, so they have an acidic environment; and protein storage vacuoles. The second type shows a slightly acidic or neutral pH which is in favor of protein storage (Robinson et al., 2005). Targeting to the vacuole, although not yet fully understood, is determined by small stretches of amino acids within the protein primary sequence acting as sorting signals to direct the maturing protein towards the vacuole (Mackenzie, 2005; Vitale and Hinz, 2005). In general, lytic vacuoles are not considered as a suitable destination for recombinant proteins *in planta*, owing to their high proteolytic content (Goulet and Michaud, 2006). By contrast, protein storage vacuoles

present a milder environment compatible with protein accumulation (Stoger et al., 2005), especially in seeds, where they are most abundant (Park et al., 2004).

### **2.5.2.3 Targeting of recombinant protein to the chloroplast**

The chloroplast, peroxisome and nucleus have been proposed as other cellular destinations for protein production in plant platforms (Daniell et al., 2002; Hyunjong et al., 2006). In practice, recombinant proteins may be sent to these organelles by the inclusion of an appropriate targeting peptide (or localization signal) in the transgene sequence. Chloroplast transformation offers several advantages over nuclear transformation, including uniform transgene expression rates, multiple copies of the transgene in each cell, co-expression of multiple genes from the same construct, minimal gene silencing and minimal transgene escape in the environment owing to the maternal inheritance of chloroplast DNA in several species (Daniell et al., 2002). The chloroplast stroma supports protein post translational modifications, such as multimerization and disulphide bridge formation (Daniell, 2006), making it a suitable environment for the expression of proteins not relying on complex modifications, such as glycosylation, typical of the cell secretory pathway.

Recombinant protein degradation by chloroplast proteases might appear, however, to be a non-relevant issue in terms of net production yields for some proteins expressed at very high levels (Daniell, 2006). The proteolysis-labile protein human serum albumin, for instance, was found at levels reaching 11% of TSP in transplastomic tobacco lines developed using chloroplast untranslated regions in gene constructs, in sharp contrast with levels below 0.02% of TSP in lines developed using the commonly employed Shine–Delgarno regulatory sequence (Fernandez-San Millan et al., 2003).

However, there are two disadvantages to the chloroplast system – first, chloroplast transformation is not a standard procedure and is thus far limited to a relatively small number of crops (tobacco, tomato, potato, cotton, soybean and most recently lettuce and cauliflower (Daniell et al. 2005b; Lelivelt et al. 2005; Nugent et al. 2006). Secondly, they lack much of the eukaryote machinery for posttranslational modification, i.e. they are unable to synthesize glycan chains. For this reason, they would be suitable for the production of scFvs but not full-size immunoglobulins.

### **2.5.3 Co-expression of protease inhibitors**

Several strategies have been considered recently to minimize proteolysis in plant protein factories. Common strategies to overcome unwanted proteolysis *in planta* involve the targeting of transgene expression or protein accumulation to specific tissues or cellular organelles. Approaches involving the grafting of protein-stabilizing fusion domains to recombinant proteins or the co-expression of companion protease inhibitors interfering with endogenous proteases have also been proposed recently (Benchabane et al., 2008).

The strategy proposed recently to increase recombinant protein production *in planta* involving the use of transgenic hosts with reduced proteolytic capacities is studied in plant made pharmaceuticals. In theory, protease processes affecting recombinant protein accumulation could be contained using antisense or RNA silencing strategies implemented in transgenic host plants (Watson et al., 2005). Alternatively, recombinant protease inhibitors active against specific endogenous proteases could prove functional to modulate proteolytic activities *in situ* (Faye et al., 2005). Recent evidence in the literature has suggested that the ectopic expression of protease inhibitors may have a

positive impact on protein levels in leaves, with negligible effects on growth and development (Benchabane et al., 2008).

### **2.5.3.1 Introduction of plant protease inhibitors**

Monocot plants such as tobacco, potato, tomato, banana, rice and etc, are used as alternative systems for production of pharmaceutical proteins however there are several proteases in these plant cells resulting in low yields of the transgenes. Some of these proteases are known and have shown negative effects on expression of heterologous proteins in these plants. Some are known as Trypsin, Chymotrypsin and Cystatine proteases. The importance of trypsin and Chymotrypsin-like activities in crude protein extracts of potato (*Solanum tuberosum* L.) leaves have been confirmed (Rivard et al., 2006).

The broad-spectrum inhibitor tomato cathepsin D inhibitor (CDI), for instance, has been shown to yield increased TSP levels (by 20%–35%) in leaves of transgenic potato lines accumulating this inhibitor in the cytosolic compartment (Michaud et al., 2005). Likewise, the rice cysteine protease inhibitor oryzacystatin I (OCPI) led to total protein levels higher than expected in tobacco leaf tissues expressing this inhibitor in the cytosol (Van der Vyver et al., 2003). Stabilized recombinant antibodies secreted by the roots of transgenic tobacco plants also expressing (and co-secreting) a Bowman–Birk trypsin inhibitor (BBTI) from soybean was successfully studied (Komarnytsky et al., 2006).

## 2.6 INTRODUCTION TO TOXOPLASMOSIS AND SCFV GENE

Toxoplasmosis is a parasitic disease caused by the protozoan *Toxoplasma gondii*. (Ryan KJ; Ray CG. 2004). The primary host of *Toxoplasma gondii* is the cat family. However, it is able to infect most animals, including humans. Eating infected meat, ingestion of feces of an infected cat and transmission from mother to fetus are the important ways of infection to toxoplasmosis in animals. Although cats are often blamed for spreading toxoplasmosis, contact with raw meat is a more significant source of human infections in many countries, and faecal contamination of hands is a greater risk factor (Torda A 2001). The parasite can cause encephalitis (inflammation of the brain) and neurologic diseases and can affect the heart, liver, inner ears and eyes (chorioretinitis). However, people with a weakened immune system, such as those infected with advanced HIV disease or those who are pregnant, may become seriously ill, and it can occasionally be fatal.

The recombinant single chain fragment anti toxiplasmosis scFv antibody is a recombinant antibody designed to be produced in recombinant host systems. It is important to understand the mechanisms of action of the antibody in designing clinical antibodies for production in heterologous expression systems. Binding to antigen is the primary function of antibodies. Therefore, it can be sufficient to produce the antigen-binding domain of the antibody molecule alone for the recombinant protein to be active. The single-chain Fv molecules, which consist of the variable domains of the light and heavy chains joined by a peptide linker and Fab fragments (fragment antigen-binding) is an example of this approach (Ma et al., 1995).

## 2.7 *AGROBACTERIUM* MEDIATED TRANSFORMATION

*Agrobacterium tumefaciens* is a soil-borne, gram-negative bacterium. This bacteria is the causative agent of crown gall disease, an economically important disease of a wide range of plants. Crown-gall formation depends on the presence of a plasmid in *A. tumefaciens* known as the Ti (tumour-inducing) plasmid. Part of this plasmid (the T-DNA region) is actually transferred from the bacterium and into the plant cell, where it becomes integrated into the genome of the host plant. The T-DNA region of any Ti plasmid is defined by the presence of the right and left-border sequences. These border sequences are 24-bp imperfect repeats. Any DNA between the borders will be transferred into the genome of the host plant (Slater et al., 2003).

The T-DNA contains two types of genes: the oncogenic genes, encoding for enzymes involved in the synthesis of auxins and cytokinins and responsible for tumour formation; and the genes encoding for the synthesis of opines, a product resulted from condensation between amino acids and sugars, which are produced and excreted by the crown gall cells and consumed by *A. tumefaciens* as carbon and nitrogen sources. Outside the T-DNA, are located the genes for the opine catabolism, the genes involved in the process of T-DNA transfer from the bacterium to the plant cell (Zupan et al., 1995). Virulent strains of *A. tumefaciens* contain a large megaplasmid (more than 200 kb) that plays a key role in tumour induction and for this reason it was named Ti plasmid. The transfer is mediated by the co-operative action of proteins encoded by genes determined in the Ti plasmid virulence region (vir genes) and in the bacterial chromosome, *chvA*, *chvB*, *psvA* and *att* which are involved in the binding of bacteria to the injured plant cell wall (Slater et al., 2003). The 30 kb virulence (vir) region is a region organised in six operons that are essential for the T-DNA transfer (*virA*, *virB*, *virD*, and *virG*) or for the increasing of transfer efficiency (*virC* and *virE*) (Zupan et al.,

1995; Jeon et al., 1998). The initial results of the studies on T-DNA transfer process to plant cells demonstrate three important facts for the practical use of this process in plants transformation. Firstly, the tumour formation is a transformation process of plant cells resulted from transfer and integration of T-DNA and the subsequent expression of T-DNA genes. Secondly, the T-DNA genes are transcribed only in plant cells and do not play any role during the transfer process. Thirdly, any foreign DNA placed between the T-DNA borders can be transferred to plant cell, no matter where it comes from. These well established facts, allowed the construction of the first vector and bacterial strain systems for plant transformations (Opabode 2002). *Agrobacterium*-mediated gene transfer into monocotyledonous plants became possible when reproducible and efficient methodologies were established on rice, banana, corn, wheat, and sugarcane (Opabode, 2006).

## **2.8 BINARY VECTORS FOR *AGROBACTERIUM* MEDIATED TRANSFORMATION**

In binary vectors, the transfer apparatus (i.e. the vir genes) and the T-DNA are located on separate plasmids. As only the border sequences are needed to define the T-DNA region and the vir region is absent, binary vectors are relatively small (and therefore easily manipulated) (Slater et al., 2003).

### **2.8.1 pCAMBIA Vector**

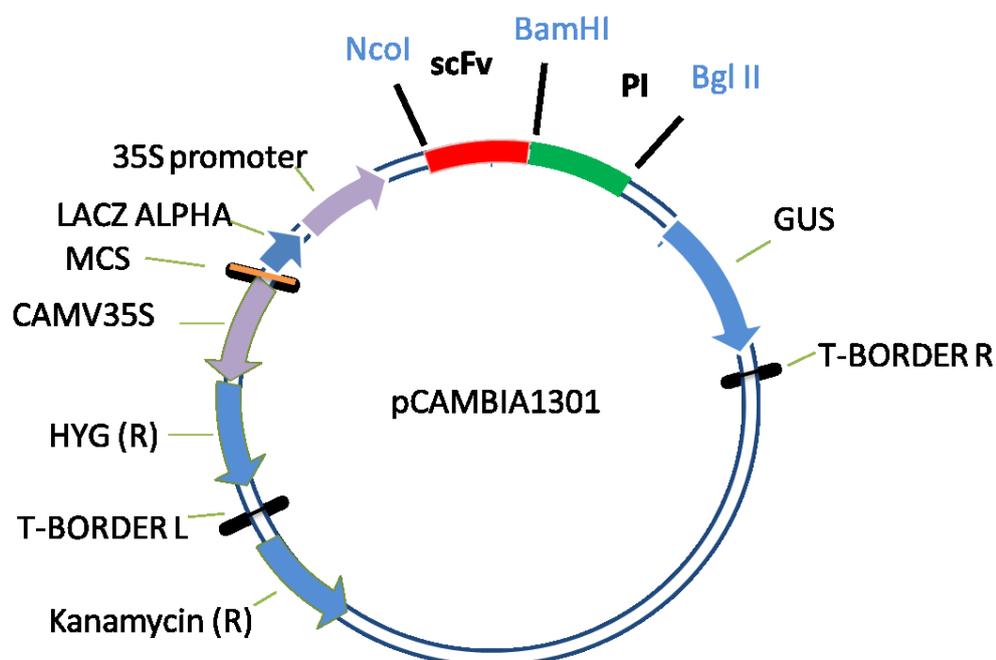
The pCAMBIA vector backbone is derived from the pPZP vectors (Hajdukiewicz et al., 1994). pCAMBIA vectors offer several important and beneficial characteristics.

They can be rapidly multiplied in *Escherichia coli*, therefore their high copy number produce high DNA yields. For high stability of vector in *Agrobacterium* pVS1 replicon was designed in pCAMBIA. They are small in size. Depending on the type of plasmid pCAMBIA they are usually 7-12kb. In these vectors restriction sites have been designed for modular plasmid modifications and there are small but adequate poly-linkers for introducing the DNA of interest. For easy bacterial selection, kanamycin or chloramphenicol resistant genes have been designed in the backbone of these vectors. The selection of transformed plants with the pCAMBIA vectors can be carried out with hygromycin B or kanamycin antibiotics as their resistant genes have been inserted in the pCAMBIA vector backbone. The presence of gusA reporter genes in the pCAMBIA vector makes the preliminary assessment of construct translation simple as the inserted genes in pCAMBIA vector can be fused to the gusA reporter genes. (pCambia vectors, 2010).

## CHAPTER 3: MATERIALS AND METHODS

### 3.1 MATERIALS

In this research, a plasmid construct that contained an anti-toxoplasmosis scFv gene (courtesy of YasminLab) (TOXO), a Protease Inhibitor gene (PI) and a gusA gene was designed and developed as the plasmid pCTOXO-PI. The diagram of the plasmid DNA construct is shown in Figure 3.1.



**Figure 3.1:** pCTOXO-PI construct

Three scFv genes (scFv3, scFv9 and scFv15) were used in the development of the new plasmids. The scFv gene was introduced into the phage display vector pCANTAB5E. The phage display vector pCANTAB5E carrying the scFv gene and the DNA construct pCTOXO-KDEL were developed in an earlier study conducted by Prof.Dr. Rofina Yasmin Othman's laboratory (YasminLab) in University Malaya.

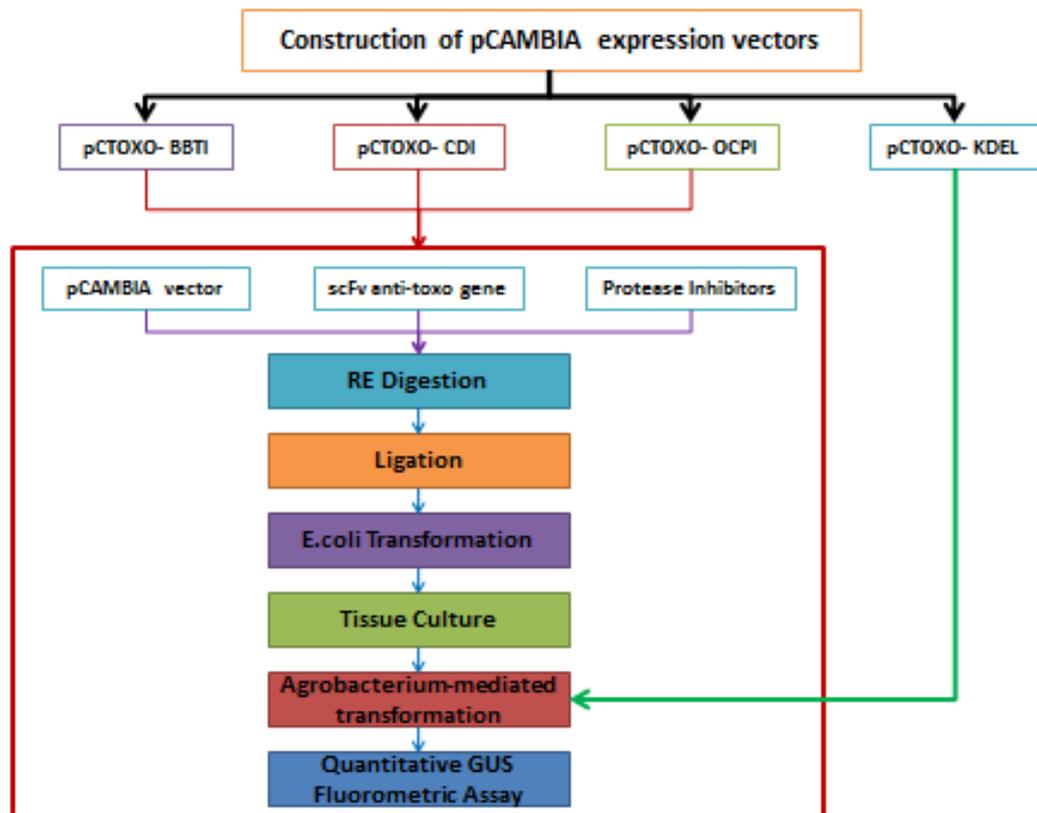
Three protease inhibitor genes (PIs) commercially synthesized, including Bowman-Birk Trypsin Inhibitor (BBTI), Cathepsin D Inhibitor (CDI) and Oryzacystatin Protease Inhibitor (OCPI). The Three PI genes were synthesized and cloned into pUC57 vector by the Biosyntech Company. A BamHI sequence was added at the 5' end of all three PI genes. Additionally a KDEL gene and Bgl II sequence was added at the 3' end of the PI genes.

The plasmid pCAMBIA 1301 was used as the cloning vector for the development of three desired DNA constructs of pCTOXO-PI. The desired plasmid constructs to be produced in this research were designated as pCTOXO-BBTI, pCTOXO-CDI and pCTOXO-OCPI.

The *Escherichia coli* strain JM109 was obtained from the laboratory to conduct the bacteria transformation of desired constructs in this research.

The *Agrobacterium tumefaciens* strain LBA4404 was used for the *Agrobacterium* mediated transformation of the constructs into plant cells.

The general overview of experiments conducted in this research is shown in Figure 3.2.



**Figure 3.2:** General Overview of experiments conducted in this research.

### 3.1.1 Preparation of plasmid pCANTAB5E and pCAMBIA 1301

#### 3.1.1.1 Growing *Escherichia Coli* cells

Fifty  $\mu$ l of frozen *Escherichia coli* bacterial cells harboring pCANTAB5E carrying scFv genes were grown in 10ml LB broth supplemented with 100 $\mu$ g/ml ampicillin, at 37°C for overnight (12-14 hours). Three replicates were prepared for each scFv gene.

Fifty  $\mu$ l of frozen *Escherichia coli* cells harboring pCAMBIA 1301 were also grown in 10ml LB broth supplemented with 100 $\mu$ g/ml kanamycin at 37°C for overnight, shaking at 220 rpm. Four replicates were prepared.

### 3.1.1.2 Plasmid Extraction

The overnight cultures of *Escherichia coli* cells carrying either pCANTAB5E or pCAMBIA 1301 were centrifuged at 6000 rpm for 15 minutes and the supernatant was discarded. Two hundred  $\mu$ l of ice-cold solution **I** [50 mM glucose, 10 mM EDTA and 25 mM Tris-Cl (pH8)] was added into the pellets in each falcon tube. After resuspending the pellets in solution **I** they were transferred into 1.5ml microcentrifuge tubes and 200  $\mu$ l of freshly made solution **II** [0.2 M NaOH, 1% SDS] was added into them and incubated at room temperature for 4 minutes. Next step is to add 200 $\mu$ l of ice-cold solution **III** [3M Potassium Acetate and 1% Acetic Acid] into the mixture and incubate on ice for 15 minutes. After centrifuging the mixture at 13,000 rpm for 10 minutes the supernatant were transferred into new 1.5ml microcentrifuge tubes and 1 $\mu$ l of RNase A was added into each sample and incubated in water bath at 37°C for 2 hours.

The next step was performing phenol-chloroform extraction method to isolate DNA content from aqueous solution. First , 600  $\mu$ l of phenol was added and after centrifuging at 13,000 rpm for 3 minutes the aqueous layer on top was transferred into new tubes. Second, 600  $\mu$ l of chloroform was added into the each collected sample and they were spin down at 13,000 rpm for 3 minutes. Finally, about 400  $\mu$ l of the aqueous phase was collected from each sample tube by pipetting and 40 $\mu$ l of 3M sodium acetate and 1000 $\mu$ l of absolute ethanol were added in to each tube of samples, and then incubated in ice for 20 minutes. After that they were centrifuged at 13,000 rpm for 15 minutes. The supernatant was completely discarded and 1ml of 70% ETOH was added. After centrifuging at 13,000 rpm for 5 minutes the supernatant was discarded and the pellets were dried in a dryer centrifuge machine for 5 minutes. Finally, 50 $\mu$ l of sterile distilled

water (sdH<sub>2</sub>O) was added into each microcentrifuge tubes to resuspend the pellet of plasmids pCANTAB5E and pCAMBIA 1301.

### **3.1.2 Preparation of plasmid pUC-PI**

#### **3.1.2.1 Preparation of competent cells**

One hundred µl of JM109 culture was grown in 10ml fresh LB broth at 37°C for overnight in water bath shaker, shaking at 220rpm to prepare *Escherichia coli* competent cells for the transformation of synthesized plasmid construct of pUC57 carrying PI genes (three pUC-PIs: pUC-BBTI, pUC-CDI, pUC-OCPI). After 12-14 hours of incubation ,500µl of fresh cells from overnight culture was transferred into 10ml fresh LB broth and was shaken for another 1-2 hours at 37°C to achieve the optical density 0.5 at the wavelength of 600 nm.

After reaching the desired optical density (0.5) the culture was transferred into 15ml Falcon tube in sterile condition and then incubated on ice for 30 minutes. The refrigerated centrifuge machine was adjusted to 4°C and the cultures were centrifuged at 3000rpm for 5 minutes at 4°C. The supernatant was removed and 5ml of RF1 solution [100mM KCl, 50Mm MnCl<sub>2</sub>,30mM Potassium acetate, 10mM CaCl<sub>2</sub> and 15% Glycerol (pH5.8)] was added into the pellets and incubated on ice for 20 minutes before centrifuging at 3000 rpm for 5 minutes, 4°C. After centrifugation the supernatant was removed and appropriate volume of RF2 solution [10mM MOPS, 10mM KCl, 75mM CaCl<sub>2</sub>, and 15% Glycerol (pH6.8)] (depending on the pellet) was added into the cells. The pellets were dissolved in RF2 solution within 20 minutes by tapping the Falcon tube while keeping on ice until the pellets were totally dissolved. Finally, 100µl of

competent cells were aliquot to 1.5ml microcentrifuge tubes and frozen in liquid nitrogen prior to storing at -20°C.

### **3.1.2.2 Transformation, analysis and extraction of three plasmid DNA, pUC-PI, in *E.coli* cells**

Three tubes of 100µl frozen JM109 bacteria culture which were prepared as described in Section 3.1.2.1 were thawed in ice for 30 minutes and 3µl of each plasmid DNA, pUC-PI, carrying BBTI, CDI and OCPI was added into them separately to insert the plasmid DNA. The tubes were then heat shocked in a water bath at 42°C for 45 seconds. The tubes were then rapidly transferred to an ice bath for 5 minutes. Then, 900µl of LB broth was added to them and incubated for 2 hours at 37°C in a spin shaker. Subsequently, 100µl culture from each tube was spread on LB agar plates supplemented with 100µg/ml ampicillin and incubated at 37°C for overnight.

Colony selection for the transformed bacteria with pUC-PI plasmid constructs was carried out after 12 to 16 hours incubation. This procedure was carried out under sterile conditions. The single bacteria colonies formed in the plates of the transformed cultures were selected using a sterile toothpick. The single colonies were cultured onto a gridded DNA library master plate containing LB agar supplemented with 100µg/ml ampicillin. The same colonies were also re-suspended in 50µl of sterile distilled water in individual 0.5ml PCR tubes. The DNA library master plate was then incubated at 37°C for overnight. The colony suspensions in the tubes of distilled water were boiled at 99°C for 10 minutes before performing colony confirmation PCR.

After performing colony PCR as will be described in Section 3.2.1 the positive transformed colonies that showed the desired band in gel electrophoresis of colony PCR products were chosen from library plate prepared as described in Section 3.1.2.2 and grown in 10ml LB broth supplemented with 100µg/ml ampicillin. After 12 to 14 hours incubation at 37°C in a water bath shaker the culture of positive colonies were harvested by centrifuging at 6000 rpm for 15 minutes. Then, the plasmids were extracted using the same phenol-chloroform method as described earlier in Section 3.1.1.2.

### **3.1.3 Preparation of plasmid construct pCTOXO-KDEL**

The *Escherichia coli* culture harboring pCTOXO-KDEL plasmid DNA construct obtained from previous studies was grown in 10ml fresh LB broth supplemented with 50µg/ml kanamycin at 37°C for overnight. After 14 hours of incubation the plasmid DNA pCTOXO-KDEL was extracted using the same technique for plasmid preparation as described in Section 3.1.1.2.

## **3.2 POLYMERASE CHAIN REACTION**

### **3.2.1 Colony PCR for pUC-PIs**

The transformation of plasmid DNA pUC-PI constructs into grown bacteria cells was verified by performing the colony PCR method. The colony PCR was carried out by amplifying a segment of the desired PI genes using the Polymerase Chain Reaction (PCR) under these conditions:

Initial denaturation      1 min 94°C

Denaturation	1 min 94°C
Annealing	30 sec 59.3°C
Elongation	1 min 72°C
Repeat step 2	additional 34 cycles
Final elongation	5 mins 72°C
Cooling	10 mins 25°C

The primers that were used for colonies transformed with pUC-BBTI, pUC-CDI and pUC-OCPI are listed in below:

BBTI-Forward primer	5'-TGC ATC TAG AGG ATC CTA CAG CGT G-3'
BBTI-Reverse primer	5'-GGA TCC AGA TCT CTA GAG CTC ATC T-3'
CDI-Forward primer	5'-TAT CGG ATC CGG ATC CTA CAA TAT A-3'
CDI-Reverse primer	5'-GGC CCA GAT CTC TAG AGC TCA TCT T-3'.
OCPI-Forward primer	5'-TAT CGG ATC CGG ATC CTA CGC ATT C-3'
OCPI-Reverse primer	5'-GAC GGG CCC AGA TCT CTA GAG CTC A-3'

### 3.2.2 PCR Confirmation for extracted pUC-PI

The extracted pUC57 plasmids carrying BBTI, CDI and OCPI were subjected to PCR to confirm back the presence of desired PI genes after plasmid preparation. The primers used and the PCR condition was the same as colony PCR for pUC-PI plasmid DNA constructs as described in Section 3.2.1.

### 3.2.3 PCR Confirmation for extracted pCTOXO-KDEL

The extracted pCAMBIA 1304 plasmid carrying scFv116-KDEL gene was subjected to PCR to confirm back the presence of desired gene after plasmid preparation. The annealing temperature for scFv116-KDEL gene Polymerase Chain Reaction was 61.2°C and the primers used were as follows:

scFv-KDEL Forward primer	5'-GCA TTA AGC TTC GCA ATT CCT TTA GTT GTT CC-3'
scFv-KDEL Reverse primer	5'-CGC CAT GGT CTG AGA AAG ATG AGC TCT AGT CCA GAC GTT-3'

### 3.2.4 PCR for anti-toxoplasmosis scFv gene

The extracted pCANTAB5E plasmid carrying scFv anti-toxoplasmosis gene as described in Section 3.1.1.2 was subjected to PCR in order to insert the desired NcoI and BamHI Restriction Endonuclease sites (RE sites) at 5' and 3' end respectively, before performing RE digestion. The annealing temperature was 57.8°C.

The primers used to amplify three scFv genes are listed in below:

NcoI- scFv Forward primer	5'-CCA TGG TTG GCT GCA GAG ACA GTG-3'
BamHI-scFv Reverse primer	5'-GGA TCC GTT CCT TTC TAT GC-3'

After running PCR, the purification of three anti-toxoplasmosis scFv gene PCR products was carried out using Megaquick- spin kit from iNtRON Company. The manufacturer instruction was performed. The PCR products of scFv3, scFv9 and scFv15 genes were purified.

### 3.2.5 PCR for Protease Inhibitor genes (PIs)

Polymerase Chain Reaction was carried out for three PI genes using one set of primer. All PI genes contained BamHI Restriction Endonuclease site (RE site) at 5' end and KDEL-BglII fragment at 3' end so the purpose of performing PCR was not inserting the RE sites. The PCR for PI genes was done to get thicker and brighter band of amplified PI genes that can be easily observed in electrophoresis GEL. The PCR products then can be excised from the GEL by performing the gel extraction method which will be described in Section 3.3.4.

The annealing temperature was 55°C and the primers used were as follows:

PIs-Forward primer	5'-TAA AAC GAC GGC CAG TGA AT-3'
PIs-Reverse primer	5'-ATG ACC ATG ATT ACG CCA AG-3'

Purification of PIs PCR products was carried out using Megaquick- spin kit from iNtRON Company. The manufacturer instruction was performed and BBTI, CDI and OCPI PCR products were purified.

### **3.3 RESTRICTION ENDONUCLEASE DIGESTION**

#### **3.3.1 Double RE digestion for anti-toxoplasmosis scFv gene**

Double RE digestion for scFv genes was carried out with NcoI and BamHI restriction endonuclease enzymes (Conc. 5u/μl) from Fermentas Company. Two μl of each enzyme was added to 5μl of pure PCR product of scFv gene prepared earlier as described in Section 3.2.4 in the presence of 5μl of 10X Tango Buffer. The recommended buffer for double RE digestion with these two enzymes was 1X Tango Buffer according to Fermentas website. Then, 36μl of sterile distilled water was added into each tube to provide total volume of 50μl RE digestion solution. The tubes were incubated at 37°C in a water bath for overnight.

#### **3.3.2 Double RE digestion for Protease Inhibitor genes**

For double RE digestion of PI genes 2μl of each BamHI and BglII enzyme (Conc. 5u/μl) from Fermentas company was added to 5μl of pure PCR products of PI genes prepared earlier as described in Section 3.2.5 in the presence of 10μl of 10X Tango Buffer. The recommended buffer for double RE digestion with BamHI and BglII enzymes was 2X Tango Buffer according to Fermentas website. Then, 31μl of sterile distilled water was added into each tube to provide total volume of 50μl RE digestion solution. The tubes were incubated at 37°C in a water bath for overnight.

#### **3.3.3 Double RE digestion for cloning vector pCAMBIA 1301**

In order to provide the desired cloning site for the insertion of scFv-PI fragment to pCAMBIA 1301 backbone, 2μl of each NcoI and BglII RE enzymes (Conc. 5u/μl) from Fermentas company was added to 5μl of prepared pCAMBIA 1301 plasmid as

described in Section 3.1.1 in the presence of 10 $\mu$ l of 10X Tango Buffer. The recommended buffer was 2X Tango Buffer according to Fermentas website. Then, 31 $\mu$ l of sterile distilled water was added into each tube to provide 50 $\mu$ l of total solution. Finally, the tubes were incubated at 37°C in a water bath for overnight.

### **3.3.4 GEL Extraction**

The digested products of scFv genes, Protease Inhibitor genes and pCAMBIA 1301 vector were loaded in a 1% w/v agarose gel with 1X TBE buffer and run at 90 volts for 1 hour. After achieving the complete separation of digested bands, they were visualized under long wavelength UV lamp in dark room and the desired fragments of DNA were excised using a clean scalpel.

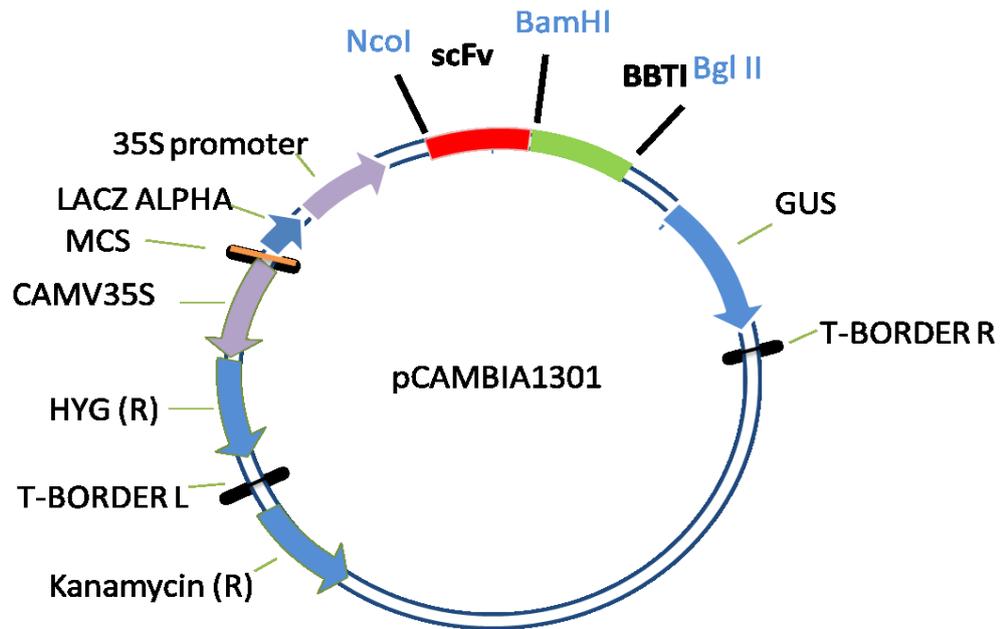
The desired DNA fragment was purified from the excised gel using an Agarose Gel DNA Purification Megaquick spin kit from iNtRON Company. The manufacturer instruction was performed and RE digested scFv genes, PI genes and pCAMBIA 1301 were purified.

## **3.4 CONSTRUCTION OF PCAMBIA EXPRESSION VECTORS**

### **3.4.1 pCTOXO-BBTI construct**

T4 DNA ligase enzyme was applied for the ligation of purified RE digested scFv gene, BBTI gene and pCAMBIA 1301 plasmid as the cloning vector. Total solution of 10 $\mu$ l ligation mixture was prepared by mixing 4 $\mu$ l of digested pCAMBIA 1301, 2 $\mu$ l of digested scFv gene and 2 $\mu$ l of digested BBTI gene as described in Section 3.3, supplemented with 1 $\mu$ l of 10X ligation buffer and 1  $\mu$ l T4 DNA ligase enzyme.

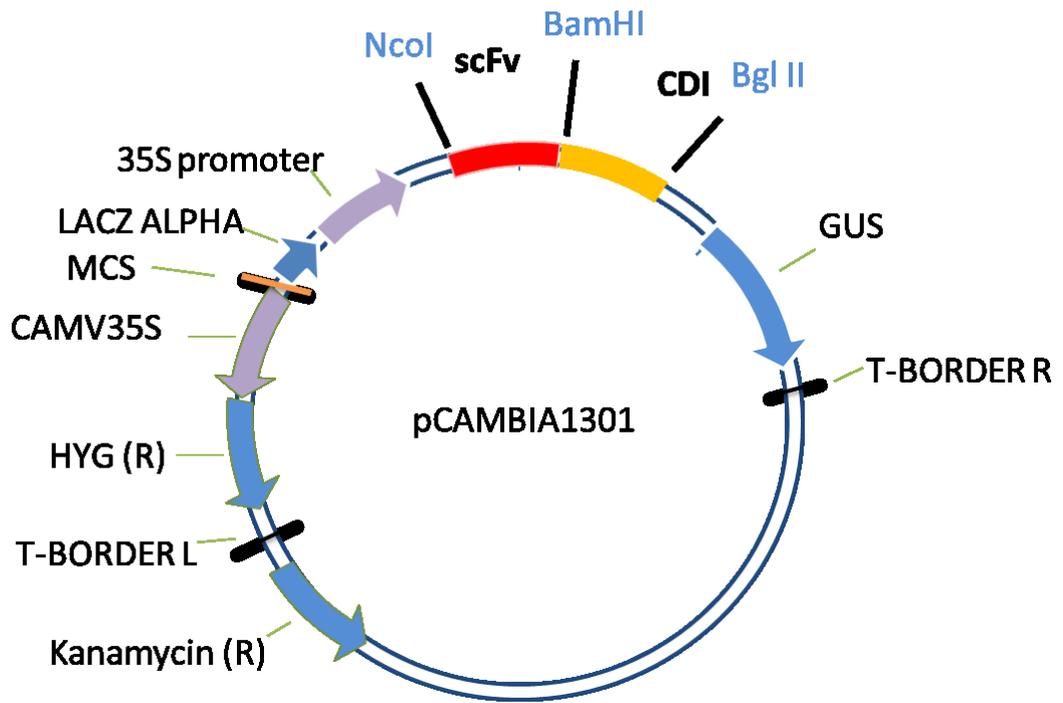
Then, the ligation mixture of plasmid construct pCTOXO-BBTI was transferred into *Escherichia coli* JM109 cells. For this procedure one tube of frozen JM109 competent cell was thawed in ice for 30 minutes and 3µl of pCTOXO-BBTI ligation mixture was added into *Escherichia coli* JM109 cells to insert the plasmid DNA carrying the desired scFv-BBTI genes. The tube was then heat shocked in a water bath at 42°C for 45 seconds. It was then rapidly transferred to an ice bath for 5 minutes. Then, 900µl of fresh LB broth was added to it and incubated for 2 hours at 37°C in a spin shaker. After incubation, the mixture was centrifuged at 1000 rpm for 10 minutes and 800µl of supernatant was removed. The pellet was then resuspended in the remaining culture. Subsequently, 100µl of the culture was spread on LB agar plate supplemented with 100µg/ml kanamycin and incubated at 37°C for overnight. Figure 3.3 shows the expected pCTOXO-BBTI construct.



**Figure 3.3:** pCTOXO-BBTI construct

### 3.4.2 pCTOXO-CDI construct

The procedure for the construction of the pCTOXO-CDI construct was the same as construction of the pCTOXO-BBTI as described in Section 3.4.1 except for one component which was the protease inhibitor gene. Therefore, the RE digested BBTI was replaced with RE digested CDI in the ligation mixture. The expected pCTOXO-CDI construct is shown in Figure 3.4.



**Figure 3.4:** pCTOXO-CDI construct

### 3.4.3 pCTOXO-OCPI construct

The procedure for the construction of the pCTOXO-OCPI construct was the same as construction of two previous constructs as described in Sections 3.4.1 and 3.4.2 except for the protease inhibitor gene. So the RE digested BBTI /CDI was replaced with RE digested OCPI in the ligation mixture. The expected pCTOXO-OCPI construct is shown in Figure 3.5.

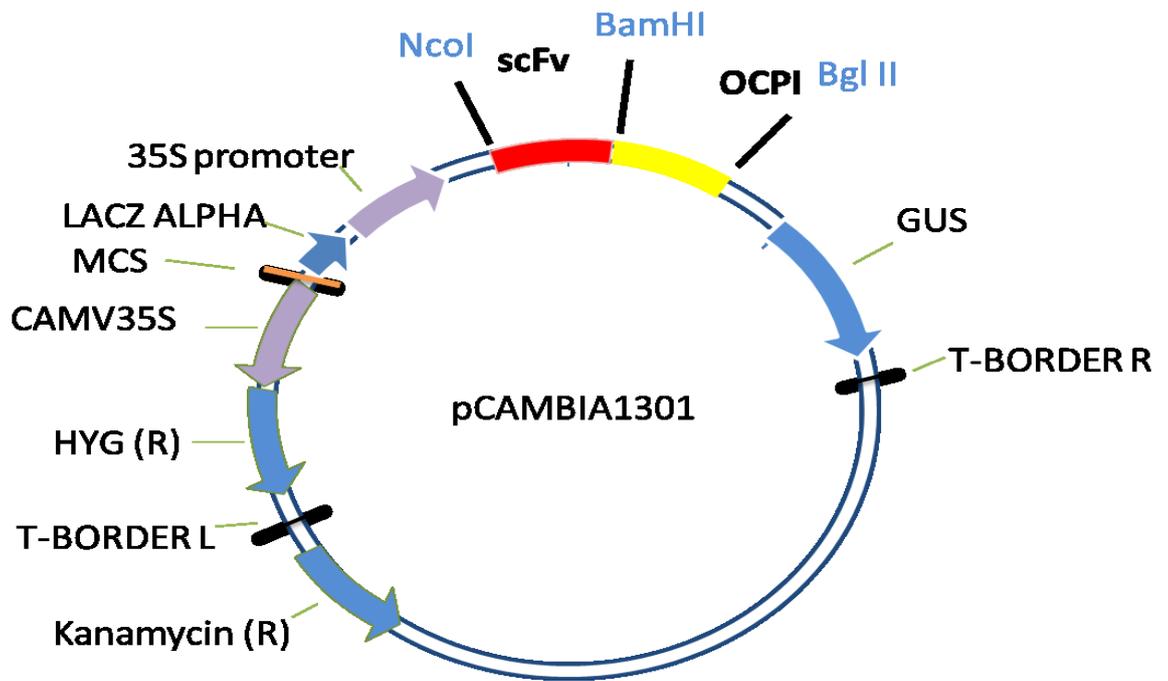


Figure 3.5: pCTOXO-OCPI construct

### 3.5 COLONY PCR FOR PCTOXO-PIS CONSTRUCTS

Colony selection for the transformed JM109 bacteria with three pCTOXO-PIs as described in section 3.4 was carried out after 12 to 16 hours incubation. This procedure was carried out under sterile condition. The single colonies were cultured onto a gridded DNA library master plate containing LB agar supplemented with 100µg/ml kanamycin. The same colonies were also resuspended in 50µl of sterile distilled water in individual 0.5ml PCR tubes. The DNA library master plate was then incubated at 37°C for overnight. The colony suspensions in the tubes of distilled water were boiled at 99°C for 10 minutes before performing colony PCR verification.

First, colony PCR was done with scFv primers to select the positive colonies which at least contained the scFv gene showing correct band size on the electrophoresed gel. The primers used were scFv-Forward and scFv-Reverse primers; the sequence of the primers described in Section 3.2.4. After colony PCR with scFv primers, the positive colonies suspensions which showed the scFv band on electrophoresed gel were chosen to perform colony PCR using scFv-PI flanking primers to verify the presence of the desired DNA fragments of scFv-BBTI, scFv-CDI and scFv-OCPI in pCTOXO-PI constructs of interest in this research. The annealing temperature was 54.9°C and the primers used were as follows:

scFv-PI Forward primer	5'-CAT TTG GAG AGA ACA CGG GGG ACT -3'
scFv-PI Reverse primer	5'-GGG TCC TAA CCA AGA AAA TGA A-3'

The procedure of ligation, transformation and colony PCR with scFv-PI primers were repeated for several times using different purified RE digested scFv3, scFv9, scFv15, BBTI, CDI, OCPI and pCAMBIA 1301.

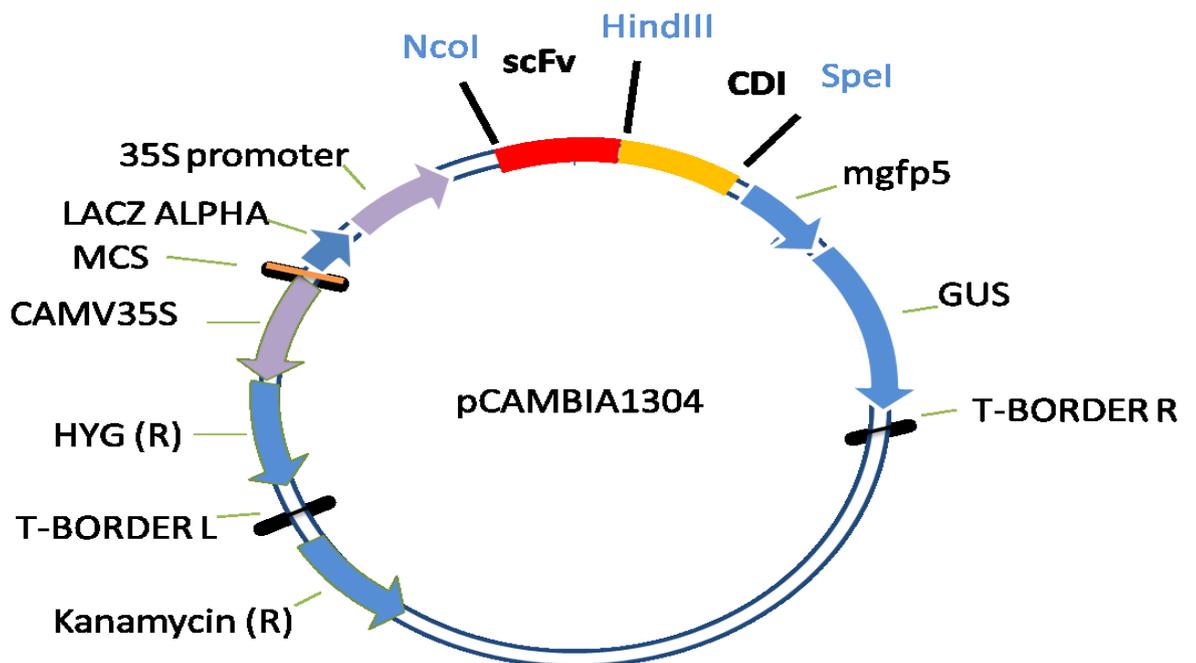
### **3.6 CONSTRUCTION OF A SECOND PCTOXO-CDI-KDEL CONSTRUCT**

A second plasmid construct for pCTOXO-CDI was developed in collaboration with a parallel study (Go Pei See pers. comm.). The cloning vector, scFv gene and restriction endonuclease RE sites were all changed to obtain the new pCTOXO-CDI-KDEL construct containing CDI gene fused to KDEL. Besides, new primers were designed to amplify scFv116 and CDI genes. A new vector pCAMBIA 1304 was used instead of pCAMBIA 1301 as the cloning vector and it was digested using NcoI and SpeI RE enzymes. For the construction of this new construct scFv116 was amplified using new

primers to insert NcoI RE site at 5' end and HindIII RE site at 3' end. Also new PI flanking primers were designed to insert HindII and SpeI RE sites at 5' and 3' end of CDI protease gene respectively. The pure RE digested scFv116 gene was then ligated to pure RE digested CDI protease inhibitor gene and cloned into the pCAMBIA 1304 vector by applying T4 DNA Ligase (Figure 3.6). The plasmid for the new construct of pCTOXO-CDI-KDEL was prepared using the same method as described in Section 3.3.1. The pCTOXO-CDI-KDEL construct is shown in Figure 3.7.



**Figure 3.6:** Ligation product of pCTOXO- CDI-KDEL



**Figure 3.7:** pCTOXO-CDI-KDEL construct

### 3.7 TRANSFORMATION OF CONSTRUCTS INTO *A. TUMEFACIENS*

*Agrobacterium*- mediated transformation method was used to transform the desired gene into the plant cells.

#### 3.7.1 Preparation of *Agrobacterium* competent cell

The *Agrobacterium tumefaciens* strain LBA4404 was streaked onto a fresh solid LB agar plate supplemented with 50µg/ml rifampicin and was incubated at 28°C for 2 to 3 days in dark. The rifampicin antibiotic was filter sterilized prior to addition. After 3 days, a single colony of the *Agrobacterium* was picked from solid LB agar plate with a sterile toothpick for the inoculation and transferred into 3 ml liquid LB broth medium supplemented with 50µg/ml rifampicin and incubated in water bath shaker at 28°C, 200 rpm for overnight (16 to 18 hours). One ml of the overnight *Agrobacterium* culture was then subcultured into 25ml of LB broth in 50ml Falcon tube. The culture was incubated at 28°C in a water bath shaker (200rpm). Every hour, 100µl of the culture was taken for optical density (O.D.) reading at absorption  $\lambda=600\text{nm}$ . The optical density reading was taken until it reached OD<sub>600</sub>~ 0.45 to 0.5.

After achieving the OD<sub>600nm</sub>~0.5, the *Agrobacterium* culture was incubated on ice for 20 minutes. The refrigerated centrifuge machine was set at 4°C. Then, the *Agrobacterium* culture was centrifuged at 5,000 rpm for 10 minutes at 4°C. The supernatant was discarded then the tube was put back on ice . Appropriate volume of LB broth was added to the tube to resuspend the pellets of *Agrobacterium*. Then, 100µl of the prepared *Agrobacterium* competent cells was aliquot to sterile 1.5ml

microcentrifuge tubes. The liquid nitrogen was applied on the tubes prior to storing the LBA4404 competent cells in -20°C. All steps were done in sterile condition.

### **3.7.2 *Agrobacterium* heat shock transformation**

Two tubes of 100µl frozen *Agrobacterium* strain LBA4404 cells were thawed on ice and 5µg of each plasmid construct, pCTOXO-CDI and pCTOXO-KDEL carrying the scFv116-CDI and scFv116-KDEL genes respectively (described in Sections 3.1.3 and 3.6) was added into each tube of LBA4404 competent cells to transform the extracted plasmid constructs into *Agrobacterium*. After add in the plasmids the tubes were directly chilled on ice for 30 minutes. Then, they were frozen in liquid nitrogen for 30 seconds to 1 minute. Heat shock transformation was carried out by transferring tubes into 37°C water bath and incubated for 4 minutes. After that the tubes were transferred into ice for 1 minute. Then, 900µl of fresh LB broth was added into each tube to grow the *Agrobacterium* containing the plasmids, and incubated at 28°C (room temperature) on a rotary shaker (200rpm) and placed in the dark for 2 to 4 hours. Finally, 100µl of each *Agrobacterium* culture carrying pCTOXO-CDI construct and pCTOXO-KDEL construct was spread on 2 LB Agar plates supplemented with 100µg/ml kanamycin and 50µg/ml rifampicin. The Agar plates were incubated in dark at 28°C for 2 to 3 days until colonies appeared.

### **3.7.3 Colony PCR for transformed *Agrobacterium* with plasmid DNA constructs**

Colony selection for the transformed *Agrobacterium* with plasmid constructs was carried out after 3 days of incubation. This procedure was carried out under sterile conditions. The single colonies were cultured onto a gridded DNA library master plate

containing LB agar supplemented with 100µg/ml kanamycin and 50µg/ml rifampicin. The same colonies were also resuspended in 50µl of sterile distilled water in individual 0.5ml PCR tubes. The DNA library master plate was then incubated at 28°C in dark for overnight. The colony suspensions in the tubes of distilled water were boiled at 99°C for 10 minutes.

Two µl of each boiled colony suspension was used as the DNA template to perform the colony PCR for transformed *Agrobacterium* LBA4404 with two constructs to verify the existence of desired scFv116-CDI and scFv116-KDEL genes in *Agrobacterium*.

### **3.7.3.1 Colony PCR verification for transformed *Agrobacterium* with pCTOXO-CDI**

Colony PCR was carried out for *Agrobacterium* containing pCTOXO-CDI construct. The annealing temperature for the PCR was 58.3°C. The PCR products were electrophoresed in a 1% w/v agarose gel with 1X TBE buffer and run at 120 volts for 25 minutes (Figure 4.18).

### **3.7.3.2 Colony PCR verification for transformed *Agrobacterium* with pCTOXO-KDEL**

The primers which were used for the verification of *Agrobacterium* transformation with pCTOXO-KDEL construct were as follows:

Flanking scFv anti-TOXO Forward	5'-TTG GAG AGA ACA CGG GGG ACT C-3'
---------------------------------	-------------------------------------

primer	
Flanking scFv anti-TOXO Reverse primer	5'-TCA CCT TCA CCC TCT CCA CT-3'

The annealing temperature for the PCR was 64.3°C. Then the PCR products were electrophoresed in a 1% w/v agarose gel with 1X TBE buffer and run at 120 volts for 25 minutes (Figure 4.19).

### **3.8 PLANTS CELL SUSPENSION**

#### **3.8.1 Tobacco cell suspension media preparation**

For the subculturing of tobacco cultivar *Nicotiana tabacum* cell suspension M2 media was prepared. The concentration of M2 media components were as follows:

Murashige and Skoog medium including vitamins (MS powder) (4.4g/l), Sucrose (25g/l), 2, 4-dichlorophenoxyacetic acid (2,4-D) (0.5mg/l) and Kinetin (0.2mg/l). Then, one litter of sterile distilled water was added to dissolve the ingredients. The pH of media was adjusted to 5.8 by adding very small amount of 1Molar NaOH solution to the medium. The M2 media was autoclaved at 121°C for 15 minutes. After autoclaving the medium was stored in 4°C.

### **3.8.2 Subculturing of Tobacco cell suspension**

The subculture work of tobacco cell suspension was carried out every 7-14 days depending on the growth of tobacco cells. The procedure of sub-culturing was done in sterile condition. The cells present in each conical flask were divided into two flasks to produce enough tobacco cell suspension for the research. First, 40ml of the used media was removed by pipetting and then 5 to 10ml of the tobacco cell suspension was transferred into two autoclaved 250ml conical flasks. Then, 50ml of the prepared sterilized M2 media was added in each flask. The flasks of plant cells were incubated in a shaker at 80 rpm, 25°C. All procedures were carried out in sterile conditions.

The subculture work was routinely repeated for a period of 30 days. After this period of time enough tobacco cell suspension were established that were needed to perform the next step of the experiment which was *Agrobacterium* transformation of plant cells. This amount of cells was needed for the performance of Quantitative GUS fluorometric assay.

### **3.9 PREPARATION OF PLANT CELL SUSPENSIONS**

For *Agrobacterium*-mediated transformation of pCTOXO-CDI and pCTOXO-KDEL into tobacco cells the plants cell suspension were prepared. For each transformation the cells of 3 conical flasks were mixed by removing the excessive M2 media first and transferring into a Petri dish on laminar flow. Then, 5ml of plant cell suspension was transferred into a 50ml Falcon tube by pipetting. Twenty five ml of sterilized M2 media was subsequently added into them. The ratio volume of cell suspension to M2 media was adjusted to 1:5. Then, 5ml plant cell suspension was resuspended into 25ml M2 media by the use of pipetting. Then, 1000µl of the mixture was aliquot into 4

disposable, polystyrene bottles. Finally, 8000µl of M2 media was added into the cells. This step was done one day earlier of infection .So each bottle contained 9ml of plant cell suspension.

For each colony transformation this step was repeated. So to transform each colony (A1, A2) of *Agrobacterium* carrying the pCTOXO-CDI construct and (B1, B2) carrying the pCTOXO-KDEL construct, 4 replicates of tobacco cell suspension were prepared in disposable, polystyrene bottles. Besides, 4 replicates of tobacco cell suspension were also prepared to perform the control experiment.

### **3.10 PREPARATION OF *AGROBACTERIUM* CULTURE**

Two positive *Agrobacterium* colonies of each construct were grown from the library plate into 5ml LB broth supplemented with 100µg/ml kanamycin and 50µg/ml rifampicin to transform the construct of desired genes into plant cells. The pCTOXO-CDI containing colonies were named as A1 and A2 and the pCTOXO-KDEL containing colonies were named as B1 and B2. Then they were incubated at 28°C in a water bath shaker at 200 rpm for 16 to 18 hours. After 18 hours of incubation, 3ml of each overnight culture was subcultured into 30ml LB broth in a 50ml Falcon tube. The tubes were then incubated at 28°C in a shaker at 200 rpm. Every hour, 100µl of the culture was taken for optical density (OD) reading at absorption  $\lambda=600\text{nm}$ . The optical density reading was taken until the  $\text{OD}_{600\text{nm}} \sim 0.45$  to 0.5. After achieving the  $\text{OD}_{600\text{nm}} \sim 0.5$  the refrigerated centrifuge machine was set at 4°C. Then, the *Agrobacterium* culture was centrifuged at 5,000 rpm for 10 minutes at 4°C. The supernatant was discarded. Then the pellet of *Agrobacterium* was resuspended in 3ml fresh M2 media. All the procedures were carried out in sterile conditions.

### **3.11 AGROBACTERIUM-MEDIATED TRANSFORMATION OF PLANT CELLS**

One ml of the prepared *Agrobacterium* culture in fresh M2 media as described in Section 3.10 was added into the 9ml of plant cell suspension which was prepared earlier as described in section 3.9. Four replicates were prepared for each colony. High efficiency transformation of *Agrobacterium* into plant cells was promoted using acetosyringone with the concentration of 100 $\mu$ M. Then, the infected plant cells were incubated in dark by covering the bottles with aluminum foil to prevent the exposure of light. The bottles were incubated at 25°C for 30 minutes on a shaker shaking at 250 rpm. After 30 minutes all the liquid was removed and only cell suspension was remained in the bottles. The plant cells were washed by sdH<sub>2</sub>O to remove the excessive *Agrobacterium* in the bottles. Finally, the sdH<sub>2</sub>O was removed by pipetting.

#### **3.11.1 Co-cultivation procedure**

The co-cultivation of *Agrobacterium* and plant cells was carried out by adding 10ml of MSO media in the bottles. The cell suspension was then incubated in dark at 80 rpm for 4 days. The MSO media (MS basal media) components for co-cultivation work were as follows:

Murashige and Skoog medium, including vitamins (MS powder) (4.4g/l) and Sucrose (20g/l). Then, one litter of sterile distilled water was added to dissolve the ingredients. The pH of media was adjusted to 5.8 for tobacco cells by adding very small amount of 1Molar NaOH solution to the medium. The MSO media was autoclaved at 121°C for 15 minutes. After autoclaving the medium was stored in 4°C.

### 3.11.2 Killing *Agrobacterium*

After 4 days of co-cultivation, the used MSO media was replaced with 10ml fresh sterilized MSO media supplemented with 50µg/ml cefotaxime on laminar flow. The plant cell suspension was incubated in dark, shaking at 80 rpm for another 2 days to kill the *Agrobacterium*. After killing the *Agrobacterium* using cefotaxime antibiotic, the MSO media was removed and 10ml of fresh MSO media was added into each sample. The plant cells were incubated in dark at 80rpm for another 7 days to give time to the plant cells for growth.

### 3.12 ASSAY OF β-GLUCURONIDASE (GUS) QUANTITATIVE ACTIVITY BY FLUOROMETRY

The *gusA* gene was isolated from *Escherichia coli* (Jefferson et al., 1987) and a number of gene cassettes are available (e.g. from Clontech) which allow the insertion of promoter regions adjacent to the *gusA* gene generating either transcriptional or translational gene fusions. β-glucuronidase can be both assayed fluorometrically (using 4-methylumbelliferone glucuronide, MUG, as substrate) and localized in situ histochemically (using 5-bromo-4-chloro-3-indolyl glucuronide, X-gluc, as substrate). This research involved quantifying enzyme activity units that normally expressed in pmol 4-MU released per mg of protein per minute.

GUS

MUG (non-fluorescent) → glucuronic acid+ 4-MU (fluorescent)

### 3.12.1 Preparation of sample, extraction and fluorescence 4-MU quantification

First, the media of transformed tobacco cells was removed by pipetting and only cells were remained in the bottles. Three replicates for tobacco cells transformed with each colony A1 and A2 containing pCTOXO-CDI=KDEL construct and three replicates for each colony B1 and B2 containing pCTOXO-KDEL construct were prepared. The untransformed tobacco cells of 3 bottles were also harvested to run the control experiment without *Agrobacterium* transformation. The harvested tobacco cells were then frozen by using liquid nitrogen so the cells were made fragile and grinded easily with mortar and pestle. The GUS Extraction Buffer (GEB) [50mM NaHPO<sub>4</sub>, pH 7; 10mM 2-mercaptoethanol; 10mM Na<sub>2</sub>EDTA; 0.1% sodium lauryl sarcosine; 0.1% Triton X-100] was prepared to extract the protein from the ground tobacco cells (Jefferson et al., 1987).

The tobacco cells were lysed using GEB. Total protein was extracted by adding 0.5g of ground tobacco cells to 250µl of GUS extraction buffer. The mixtures were thoroughly vortex and incubated on ice for 10 minutes. Then they were centrifuged at 12000 rpm, for 5 minutes at 4°C. The supernatant was collected from different 1.5ml microcentrifuge tubes separately and transferred into new tubes. Then, 100µl supernatant was added to 700 µl GUS extraction buffer for each sample and the remainder was saved on ice for performing Bradford protein assay. The supernatants were incubated for 5 minutes at 37°C. Then, 200 µl pre-warmed GUS assay buffer (GAB) [2mM 4-Methyl umbelliferyl β-D glucuronide (MUG) in GUS Extraction Buffer] was added to the mixture. Then the reaction mixture was vortex and incubated at 37°C for 90 minutes. After incubation for 90 minutes, the reaction was terminated. One hundred µl of the reaction mixture was added to 900 µl of 0.2 M carbonate stop buffer. The addition of Na<sub>2</sub>CO<sub>3</sub> serves the dual purposes of stopping the enzyme

reaction and developing the fluorescence of 4-MU, which is about seven times as intense at alkaline pH. Finally, the unknown sample fluorescence was measured by using a spectrofluorometer with the emission at 455 nm and excitation at 365 nm and calculated into GUS specific activity by using 4-methyl-umbelliferone (4-MU) as standard. Three replicate assays were carried out for each *Agrobacterium* colony transformed into tobacco cells.

### **3.12.1.1 Standard dilution of 4-Methylumbelliferone and relative Fluorescent**

#### **Intensity**

Standard Dilution of 4-Methylumbelliferone (4-MU) was prepared and Relative Fluorescent Intensity of different 4-MU dilutions was measured to obtain the equation of the curve (4-MU vs. Fluorescent Intensity) (Figure 4.22). The equation was needed to calculate the amount of 4-MU (pmol) in unknown samples.

One mM 4-MU stock solution was prepared by dissolving 19.8mg of 4-methylumbelliferone (sodium salt), MW=198.20 in 100ml deionised distilled water. Then 1 $\mu$ M 4-MU stock solution was prepared from the 1mM 4-MU stock solution and stored at 4° C away from light. Subsequently, 4-MU standard measurements for series of concentrations as shown in table 3.1 were prepared. The Fluorescent Intensity of each sample was recorded (Table 4.4) using a fluorometer and the standard dilution curve for 4-MU was plotted (Figure 4.22). From the obtained equation of 4-MU standard curve the enzyme activity of unknown samples was calculated which expressed in pmol 4-MU released per mg protein per minute (Table 4.8).

0.2M Carbonate buffer (ml)	1 $\mu$ M 4-MU stock ( $\mu$ l)	Sample conc.(X) (nM)
2.0	0	<b>0</b>
1.9	100	<b>50</b>
1.8	200	<b>100</b>
1.7	300	<b>150</b>
1.6	400	<b>200</b>

**Table 3.1:** 4-MU Standard Dilutions

### 3.12.2 Bradford protein determination

Quick Start Bradford Protein Assay Kit from BIORAD was used to perform the Bradford assay. The Quick Start Bradford protein assay is a simple and accurate procedure for determining the concentration of protein in solution. The Bradford assay is a protein determination method that involves the binding of Coomassie Brilliant Blue G-250 dye to proteins (Bradford, 1976). The dye exists in three forms: cationic (red), neutral (green), and anionic (blue) (Compton et al., 1985). Under acidic conditions, the dye is predominantly in the doubly protonated red cationic form ( $A_{\max}=470\text{nm}$ ). However, when the dye binds to protein, it is converted to a stable unprotonated blue form ( $A_{\max}=595\text{nm}$ ). It is this blue protein-dye form that is detected at 595nm in the assay using a spectrophotometer or microplate reader.

In this research, 20 $\mu$ l of the supernatant of unknown samples which was saved on ice as described in Section 3.12.1 to perform the Bradford protein assay was transferred into separate clean 1ml disposable cuvettes and then 1ml pre-warmed 1X Bradford Dye Reagent (included in the kit )was added to each sample. Then, it was mixed gently by inversion and incubated at room temperature for 5 to 10 minutes before OD reading.

The absorbance was read at 595nm for each sample separately using a spectrophotometer. The protein concentration (mg/ml) of samples was determined by using the Bradford protein standard curve (Figure 4.23) as will be described in Section 3.12.2.1.

### **3.12.2.1 Protein standard curve**

In any protein assay, the ideal protein to use as standard is a purified preparation of the protein being assayed. In the absence of such an absolute reference protein, another protein must be selected as a relative standard. The best relative standard to use is one that gives a colour yield similar to that of the protein being assayed. The two most common protein standards used for protein assays are Bovine Serum Albumin (BSA) and gamma-globulin. With the Quick Start Bradford protein assay, dye colour development is significantly greater with BSA than with most other proteins, including gamma-globulin. Therefore, the BSA standard would be an appropriate standard.

The 1X dye reagent was removed from 4°C storage and let it warm to ambient temperature to perform the standard assay. The 1X dye reagent was inverted a few times before use. The 1mg/ml BSA stock solution included in the Quick Start Bradford Protein Assay Kit was used to prepare the standard dilutions. For the diluents the same buffer as in the samples was used. So GEB was applied as the diluents. Nine tubes of diluted protein standard were prepared to perform the 1ml standard assay in disposable cuvettes. The kit instruction was performed to prepare the dilutions. The BSA volume in  $\mu$ l was then converted to BSA concentration in mg as shown in table 3.2.

Then, each standard solution was transferred into separate 1m disposable cuvettes supplemented with 900µl of 1X dye reagent and they were inverted. The standard solutions were incubated at room temperature for 5 to 10 minutes. The absorbance of standard samples was measured using a spectrophotometer which was set at 595nm. The instrument was zeroed using blank sample prior to OD reading. The blank sample was prepared using water and dye reagent. Triplicate standard measurements were carried out (Table 4.6).

Tube	BSA volume (µl)	Diluent(GEB) Volume (µl)	Bradford dye reagent (µl)	BSA Protein conc. (mg)
1	0	100	900	<b>0</b>
2	2.5	97.5	900	<b>0.0025</b>
3	5.0	95	900	<b>0.005</b>
4	7.5	92.5	900	<b>0.0075</b>
5	10.0	90.0	900	<b>0.01</b>
6	15.0	85.0	900	<b>0.015</b>
7	20.0	80.0	900	<b>0.02</b>
8	25.0	75.0	900	<b>0.025</b>
9	30.0	70.0	900	<b>0.03</b>

**Table 3.2:** Bradford Standard Measurements

To determine the protein concentration of unknown samples, standard curve was drawn by plotting the OD<sub>595nm</sub> values (y-axis) versus the BSA protein concentration (x-axis)

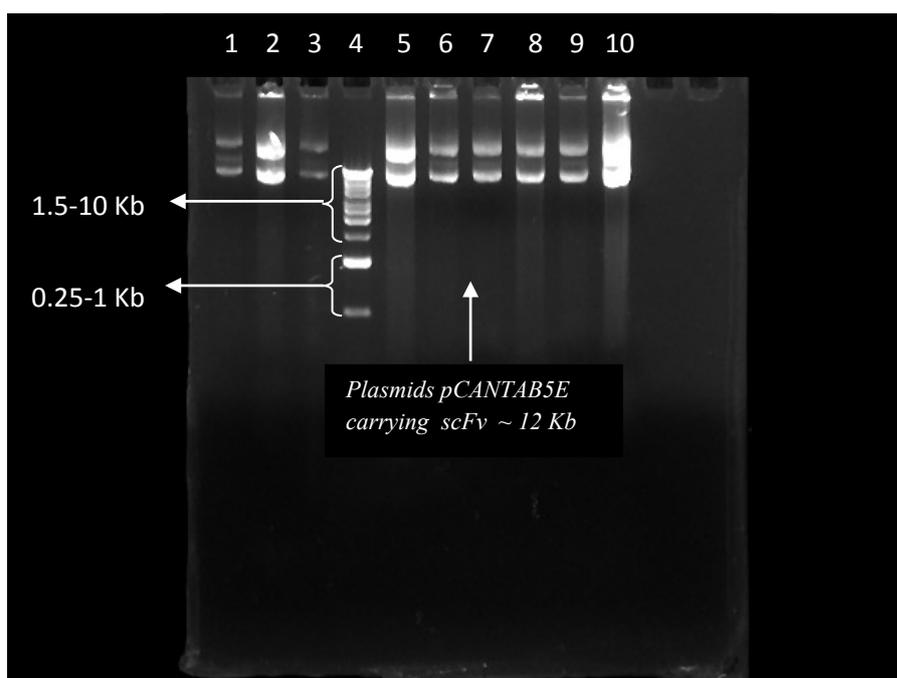
(Figure 4.23). From the obtained equation of the standard curve the protein concentration of unknown samples was calculated (Table 4.7).

## CHAPTER 4: RESULTS

### 4.1 PLASMID PREPARATION

#### 4.1.1 Preparation of plasmid pCANTAB5E and pCAMBIA 1301

The plasmid pCANTAB5E carrying scFv3, scFv9 and scFv15 genes and plasmid pCAMBIA 1301 as the cloning vector were prepared as described in Section 3.1.1. The extracted plasmids were electrophoresed in a 1% w/v (weight in gram over volume in milliliter) agarose gel with 1X TBE buffer and run at 120 volts for 25 minutes. The results obtained showed the presence of the pCANTAB5E and pCAMBIA 1301 plasmid in the electrophoresed gel (Figure 4.1 and 4.2).



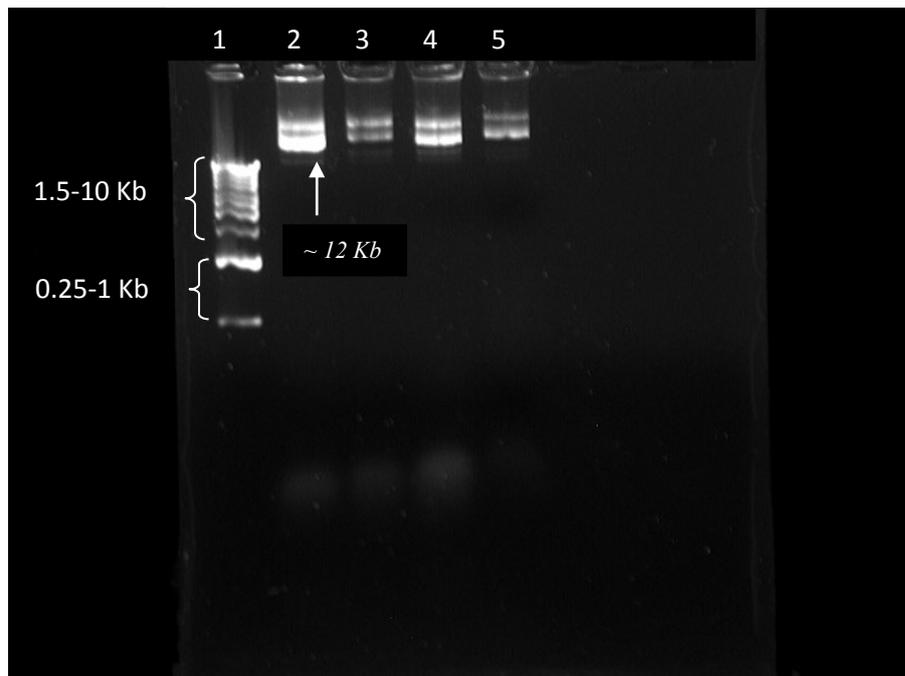
**Figure 4.1:** Plasmid preparations for pCANTAB5E carrying scFv genes

*Lane 1, 2, 3: plasmid sample 1, pCANTAB5E –scFv3*

*Lane 4: 1Kb DNA ladder*

*Lane 5, 6, 7: Plasmid sample 2, pCANTAB5E –scFv9*

*Lane 8, 9, 10: Plasmid sample 3, pCANTAB5E –scFv15*



**Figure 4.2:** Plasmid preparations for pCAMBIA 1301

*Lane 1: 1Kb DNA ladder (Appendix M)*

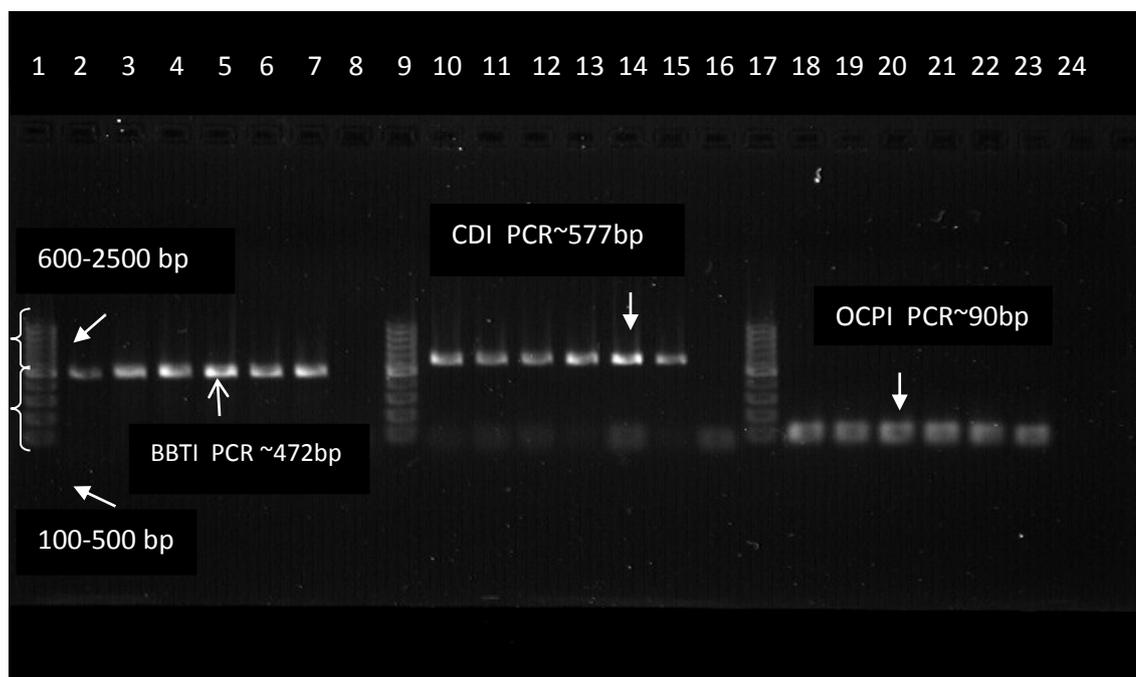
*Lane 2, 3, 4, 5: Plasmid sample, pCAMBIA 1301*

#### 4.1.2 Preparation of plasmid pUC-PI

##### 4.1.2.1 Colony PCR analysis of transformed plasmid pUC-PI into *E.coli* cells

The plasmid pUC57 carrying Protease Inhibitor genes BBTI, CDI and OCPI, (pUC-PIs), were transformed into *Escherichia coli* JM109 cells as described in Section 3.1.2.2. After colony selection the colony PCR was carried out on the colony suspensions of the transformed *Escherichia coli* cells as described in Section 3.2.1. The PCR products were electrophoresed in a 1% w/v agarose gel with 1X TBE buffer and run at 120 volts for 25 minutes. The results obtained showed the presence of the PI

genes as a specific band with the expected size of 472bp for BBTI, 577bp for CDI and 90bp for OCPI gene in the electrophoresed gel (Figure 4.3).



**Figure 4.3:** Colony PCR for pUC-PIs carrying PI genes transformed into JM109

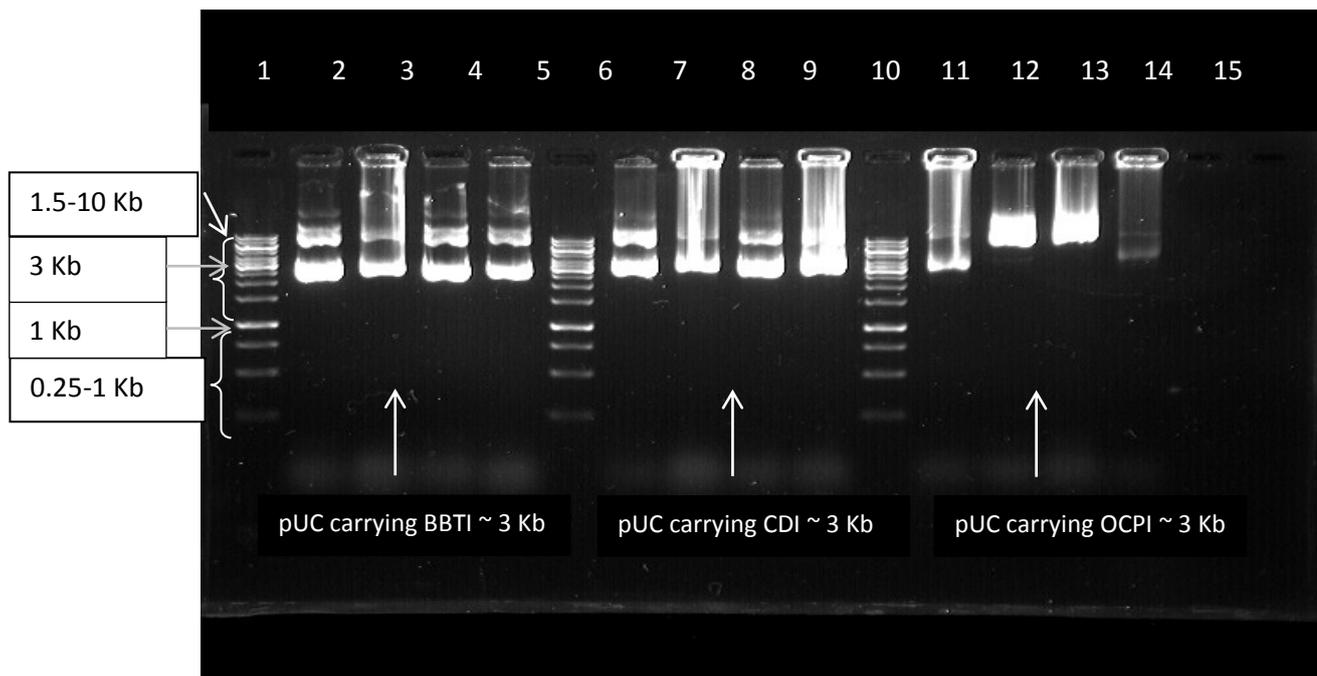
*Escherichia coli* competent cells. Expected size of PCR product for each PI gene was shown in the figure.

*Lane 1: 100 bp DNA ladder (Appendix M)*  
*Lane 2-7: colonies A1-A6, carrying BBTI gene*  
*Lane 8: Negative control*  
*Lane 9: 100 bp DNA ladder*  
*Lane 10-15: colonies B1-B6, carrying CDI gene*  
*Lane 16: Negative control*  
*Lane 17: 100 bp DNA ladder*  
*Lane 18-23: colonies C1-C6, carrying OCPI gene*  
*Lane 24: Negative control*

#### 4.1.2.2 Plasmid pUC-PIs extraction

Four positive colonies of each pUC-PI constructs transformed into *Escherichia coli* cells were grown and the plasmids were extracted as described in Section 3.1.2.2. The

extracted plasmids were electrophoresed in a 1% w/v agarose gel with 1X TBE buffer and run at 120 volts for 25 minutes. The results obtained showed the presence of the plasmid pUC57 in the electrophoresed gel (Figure 4.4). The plasmid pUC57 was used as the cloning vector for PI genes by the Biosyntech company for after synthesising the PI genes as described in Section 3.1.

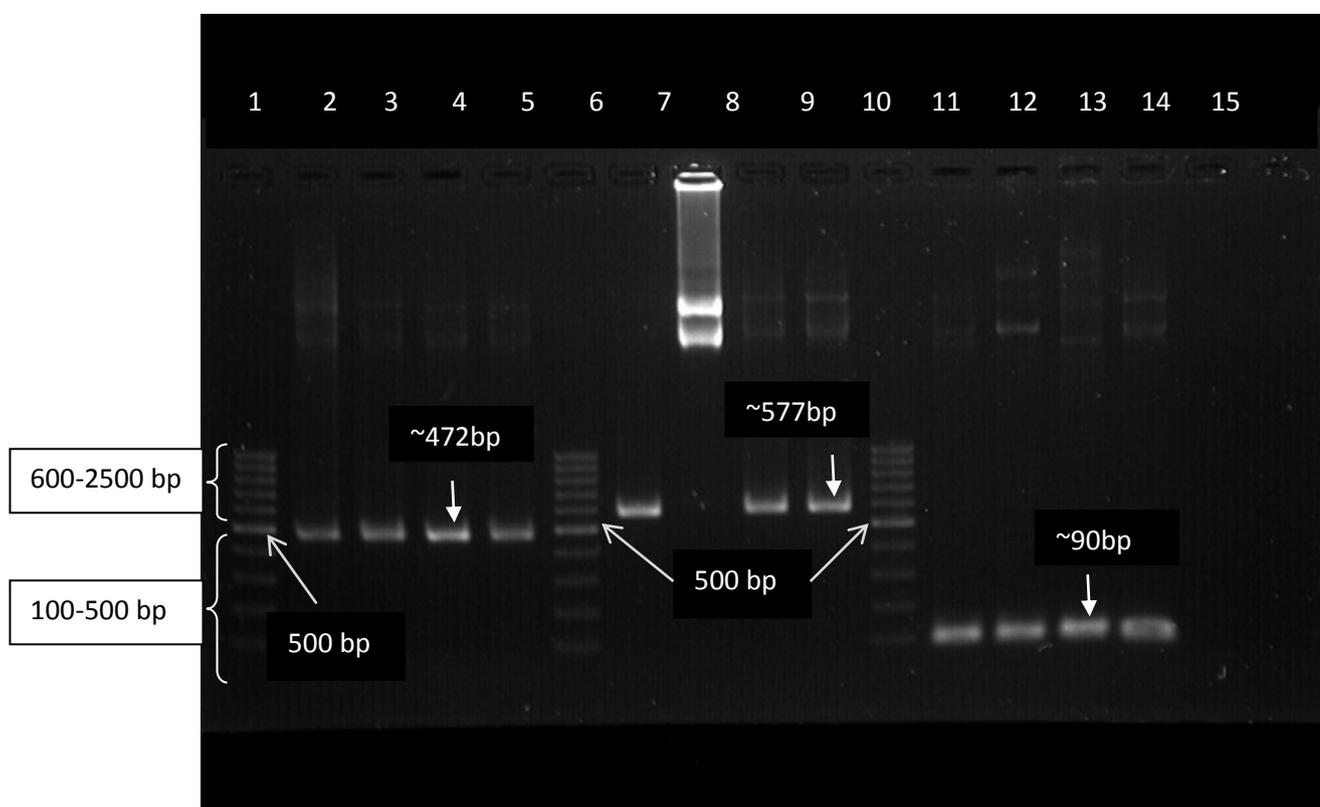


**Figure 4.4:** Plasmid preparation for pUC57 vector carrying PI genes (pUC-PI) from transformed *Escherichia coli*.

*Lane 1: 1 Kb DNA ladder*  
*Lane 2-5: plasmid prep for pUC-BBTI*  
*Lane 6: 100 bp DNA ladder*  
*Lane 7-10: plasmid prep for pUC-CDI*  
*Lane 11: 100 bp DNA ladder*  
*Lane 12-15: plasmid prep for pUC-OCPI*

#### 4.1.2.3 PCR Confirmation for extracted pUC-PI

The extracted pUC57 plasmids carrying BBTI, CDI and OCPI were subjected to PCR to confirm the presence of desired PI genes after plasmid preparation. The primers used and the PCR condition was the same as colony PCR for pUC57 plasmid as described in Section 3.2.1. The PCR products were electrophoresed in a 1% w/v agarose gel with 1X TBE buffer and run at 120 volts for 25 minutes. The results obtained showed the presence of the PI genes as a specific band with the expected size of 472bp for BBTI, 577bp for CDI and 90bp for OCPI gene in the electrophoresed gel (Figure 4.5).



**Figure 4.5:** PCR verification for plasmid pUC57 carrying PI genes after plasmid preparation. Expected size of PCR products: BBTI= 472bp, CDI= 577bp, OCPI=90bp

*Lane 1: 100 bp DNA ladder*

*Lane 7-10: PCR for plasmid prep pUC-CDI*

*Lane 2-5: PCR for plasmid prep pUC-BBTI*

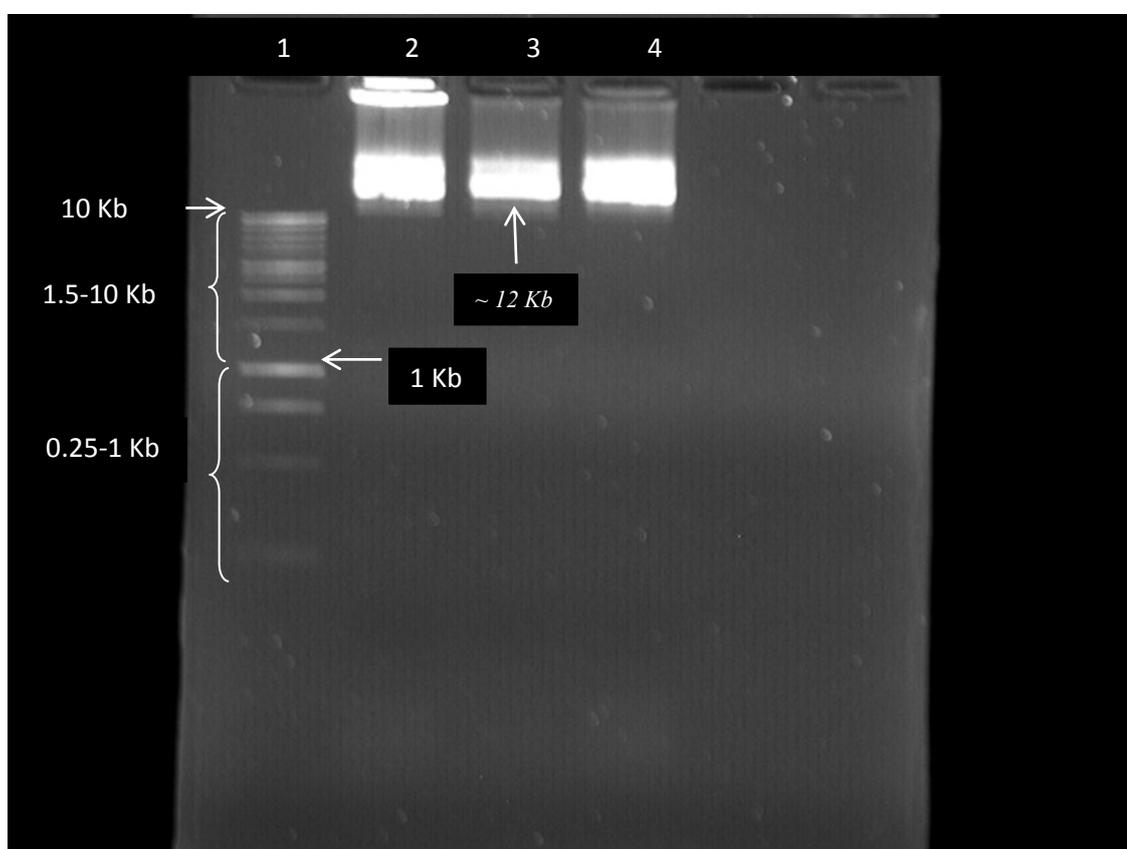
*Lane 11: 100 bp DNA ladder*

*Lane 6: 100 bp DNA ladder*

*Lane 12-15: PCR for plasmid prep pUC-OCPI*

### 4.1.3 Preparation of plasmid construct pCTOXO-KDEL

The plasmid pCAMBIA 1304 carrying scFv116-KDEL genes (pCTOXO-KDEL) were extracted as described in Section 3.1.3. The extracted plasmids were electrophoresed in a 1% w/v agarose gel with 1X TBE buffer and run at 120 volts for 25 minutes. The results obtained showed the presence of the pCAMBIA 1304 in the electrophoresed gel. Expected size of pCTOXO-KDEL plasmid was 12 Kb as shown in Figure 4.6.



**Figure 4.6:** Plasmid preparation for pCTOXO-KDEL plasmid construct.

*Lane 1: 1 Kb DNA ladder*

*Lane 2-4: plasmid prep sample, pCTOXO-KDEL*

#### 4.1.3.1 PCR Confirmation for extracted pCTOXO-KDEL

The extracted pCAMBIA 1304 plasmid carrying scFv116-KDEL gene was subjected to PCR as described in Section 3.2.3 to confirm back the presence of desired gene after plasmid preparation. The PCR product was electrophoresed in a 1% w/v agarose gel with 1X TBE buffer and run at 120 volts for 25 minutes. The results obtained showed the presence of the scFv116-KDEL genes as a specific band with the expected size of 900bp in the electrophoresed gel (Figure 4.7). This construct was selected for use in the subsequent transformation experiments.



**Figure 4.7:** PCR product for pCTOXO-KDEL using scFv-KDEL primers. Expected size of scFv116-KDEL: 900

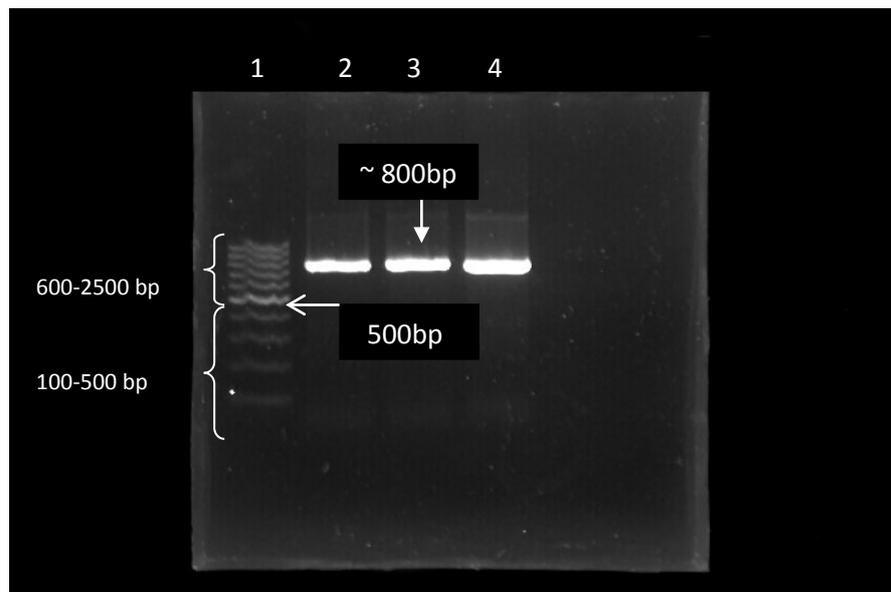
*Lane 1: 100 bp DNA ladder*

*Lane 2-4: PCR for pCTOXO-KDEL*

*Lane 5: Negative control*

## 4.2 PCR FOR ANTI-TOXOPLASMOSIS scFV AND PROTEASE INHIBITOR GENES

The PCR was carried out for scFv3, scFv9 and scFv 15 as described in Section 3.2.4 to insert the desired RE sites. The PI genes were also amplified as described in Section 3.2.5. The PCR products were purified using Megaquick-spin kit. The purified PCR product of scFv3, scFv9 and scFv15 genes and PI genes, BBTI, CDI and OCPI were electrophoresed in a 1% w/v agarose gel with 1X TBE buffer and run at 120 volts for 25 minutes. The results obtained showed the presence of the scFv genes as a specific band with the expected size of 800bp in the electrophoresed gel (Figure 4.8). The BBTI, CDI and OCPI protease inhibitors were also shown very distinct and clear band of 700bp, 950bp and 800bp respectively (Figure 4.9).



**Figure 4.8:** Purified PCR product of anti-toxoplasmosis scFv genes using scFv primers.

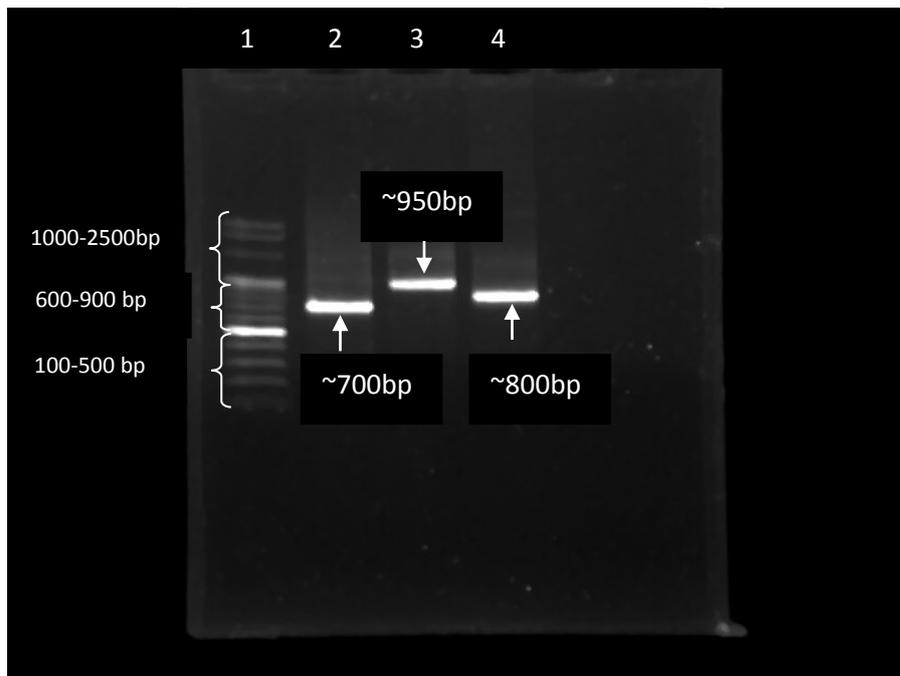
Expected size: scFv=800bp

*Lane 1: 100bp DNA ladder*

*Lane 2: PCR product for scFv3 gene*

*Lane 3: PCR product for scFv9 gene*

*Lane 4: PCR product for scFv15 gene*



**Figure 4.9:** Purified PCR product of PI genes using PI primers.

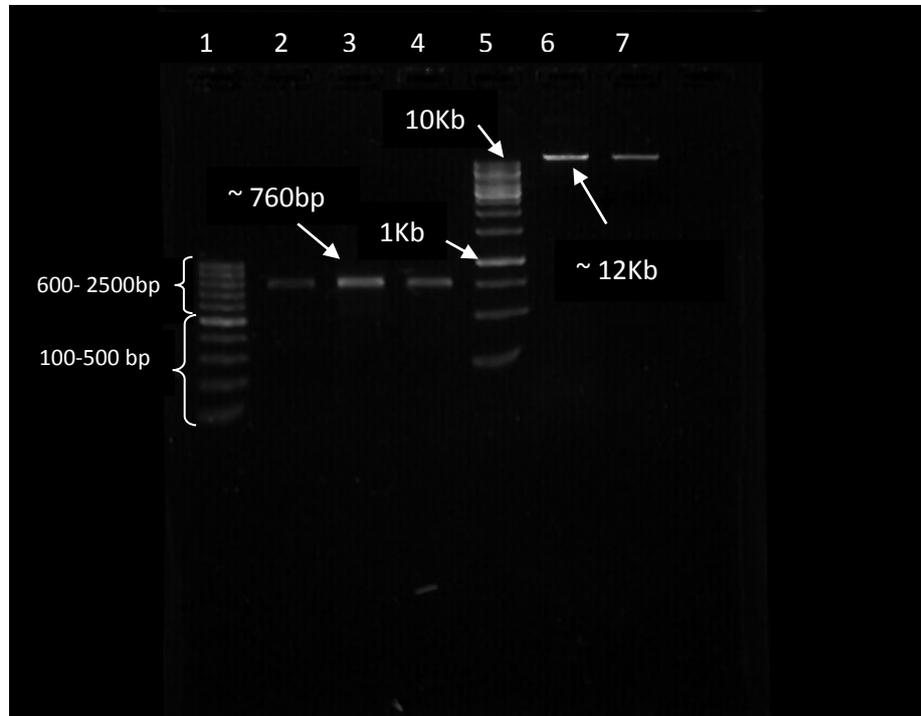
Expected size of PCR products: BBTI= 700bp, CDI= 950bp, OCPI= 800bp.

*Lane 1: 100 bp DNA ladder*  
*Lane 2: PCR product for BBTI gene*  
*Lane 3: PCR product for CDI gene*  
*Lane 4: PCR product for OCPI gene*

### 4.3 DOUBLE RE DIGESTED GENES

Three scFv genes, PI genes and pCAMBIA 1301 cloning vector were digested using restriction endonuclease enzymes to produce desired fragmented DNA as described in Section 3.3. The digested genes were gel extracted and purified as described in Section 3.3.4. After purification process was done each purified DNA fragment was electrophoresed in a 1% w/v agarose gel with 1X TBE buffer and run at 120 volts for 25

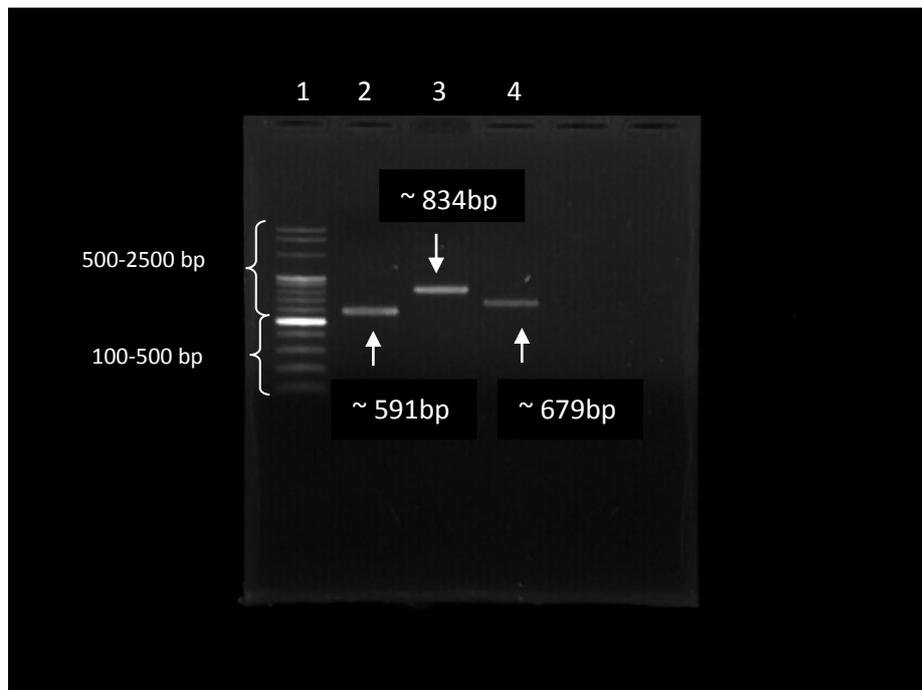
minutes. The results obtained showed the presence of the pure digested scFv genes, PI genes and pCAMBIA 1301 plasmid as a specific band in the electrophoresed gel (Figure 4.10 and 4.11).



**Figure 4.10:** Purified RE digested scFv genes and pCAMBIA 1301

Expected size of RE digested genes: scFv: 760bp, pCAMBIA 1301:12Kb

*Lane 1: 100 bp DNA ladder*  
*Lane 2: RE digested scFv3 gene*  
*Lane 3: RE digested scFv9 gene*  
*Lane 4: RE digested scFv15 gene*  
*Lane 5: 1 Kb DNA ladder*  
*Lane 6-7: RE digested pCAMBIA 1301*



**Figure 4.11:** Purified RE digested Protease Inhibitor genes

Expected size of RE digested PI genes: BBTI=591BP, CDI=834bp, OCPI=679bp

*Lane 1: 100 bp DNA ladder*  
*Lane 2: RE digested BBTI gene*  
*Lane 3: RE digested CDI gene*  
*Lane 4: RE digested OCPI gene*

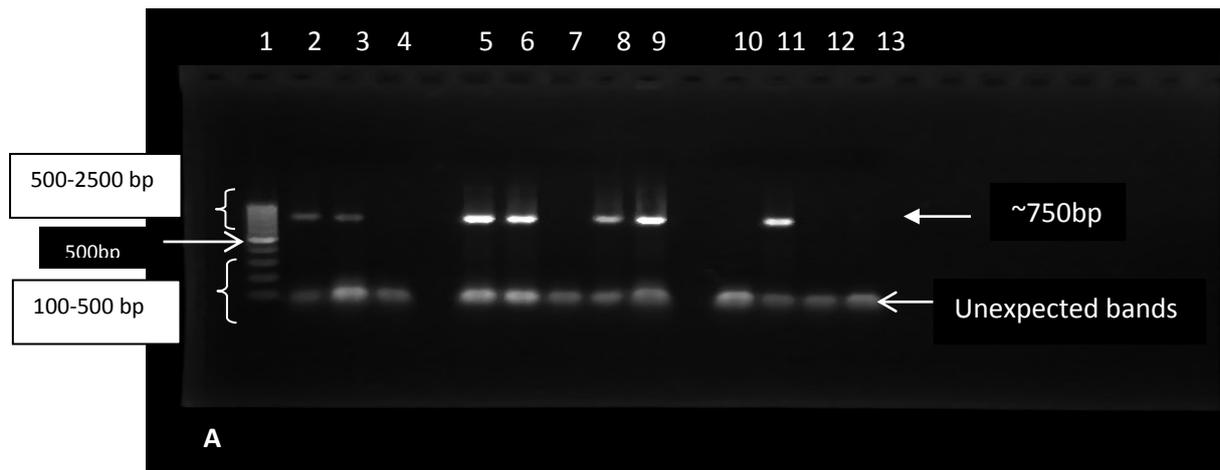
#### 4.4 CONSTRUCT ANALYSIS

##### 4.4.1 Analysis of pCTOXO-PIs

##### 4.4.1.1 PCR analysis of transformed *E.coli* with ligation mixture of pCTOXO-PIs

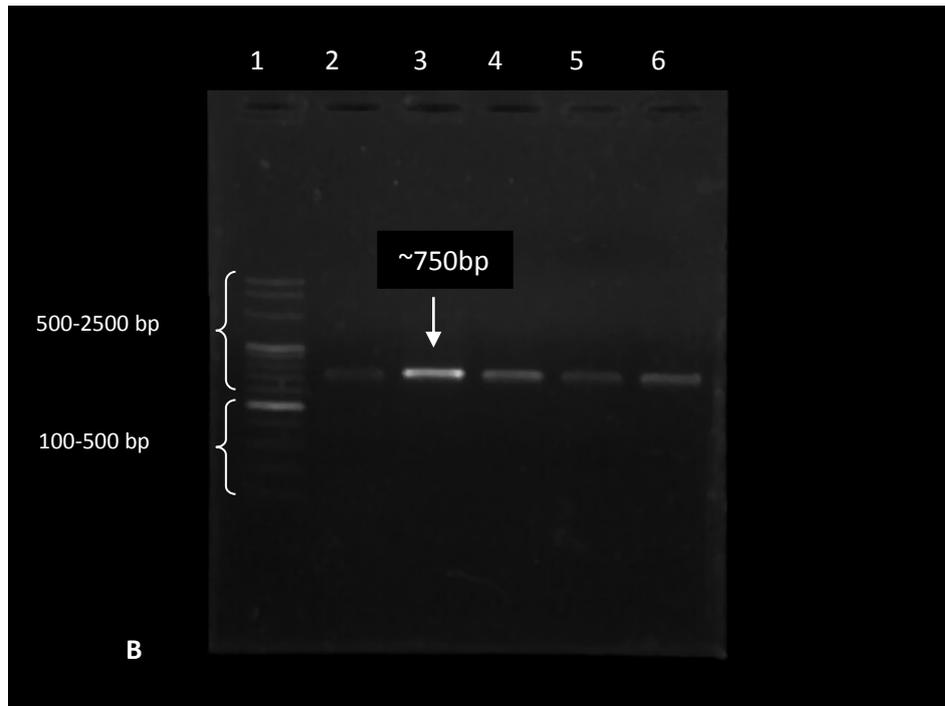
PCR was carried out on colony suspension of transformed JM109 *Escherichia coli* cells with pCTOXO-PIs as described in Section 3.5. First, colony PCR was carried out with scFv primers to confirm wherever the colonies carried scFv gene. The PCR products were electrophoresed in a 1% w/v agarose gel with 1X TBE buffer and run at

120 volts for 25 minutes. The results obtained showed the presence of the scFv genes as a specific band with the expected size of 750bp in the electrophoresed gel (Figure 4.12 and 4.13). The unexpected bands showed as PCR product in GEL electrophoresis result (Figure 4.12) might be due to primer dimer problem. The scFv primers using in this step might have strings of complimentary bases that can attach to each other and lead to a potential by-product in PCR.



**Figure 4.12:** Colony PCR for pCTOXO-PIs using scFv primers.  
scFv15+PIs+pCAMBIA1301

*Lane 1: 100 bp DNA ladder*  
*Lane 2-4: PCR for pCTOXO-BBTI*  
*Lane 5-9: PCR for pCTOXO-CDI*  
*Lane 10-13: PCR for pCTOXO-OCPI*



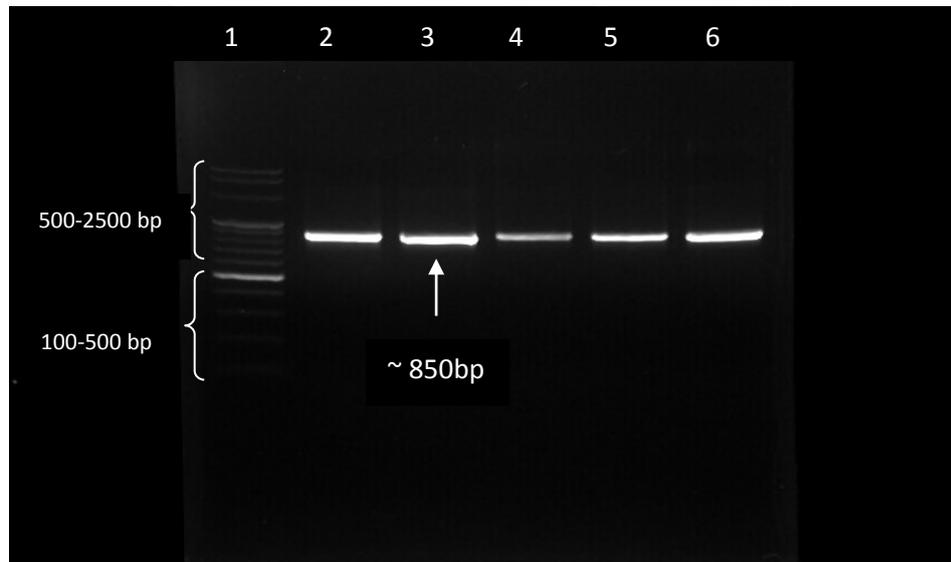
**Figure 4.13:** Colony PCR for pCTOXO-PIs using scFv primers.

scFv9+BBTI+pCAMBIA1301

**Figure 4.13:**  
*Lane 1: 100 bp DNA ladder*  
*Lane 2-6: PCR for pCTOXO-BBTI*

The positive colony suspensions that showed the scFv band on electrophoresis gel were then chosen to perform the colony PCR using scFv-PI flanking primers to confirm the success of ligation process. Then the PCR products were electrophoresed in a 1% w/v agarose gel with 1X TBE buffer and run at 120 volts for 25 minutes. The results obtained did not show the presence of the correct band size for amplified scFv-PI genes. The expected size of PCR product for ligated scFv-BBTI gene was 1400bp but the gel picture showed a band with the size of about 850bp (Figure 4.14). For scFv-CDI the expected size was 1600bp but the obtained result showed a band with the size of 850bp (Figure 4.15). Also for the scFv-OCPI the expected size of the PCR product was

1450bp. However, the result showed a band with 850bp in the electrophoresed gel (Figure 4.16).

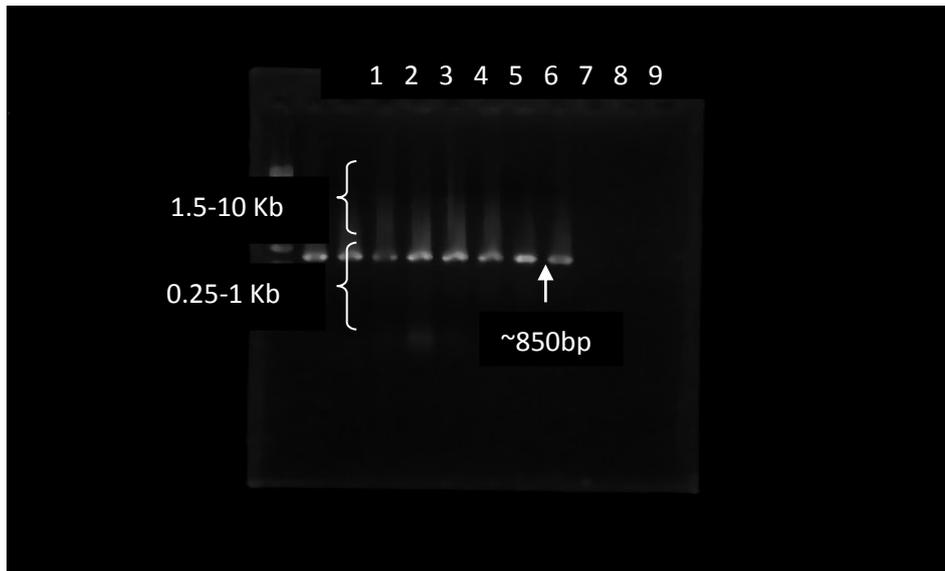


**Figure 4.14:** Colony PCR for pCTOXO-BBTI using scFv-PI flanking primers.

Expected size of pCTOXO-BBTI: 1400bp

*Lane 1: 100 bp DNA ladder*

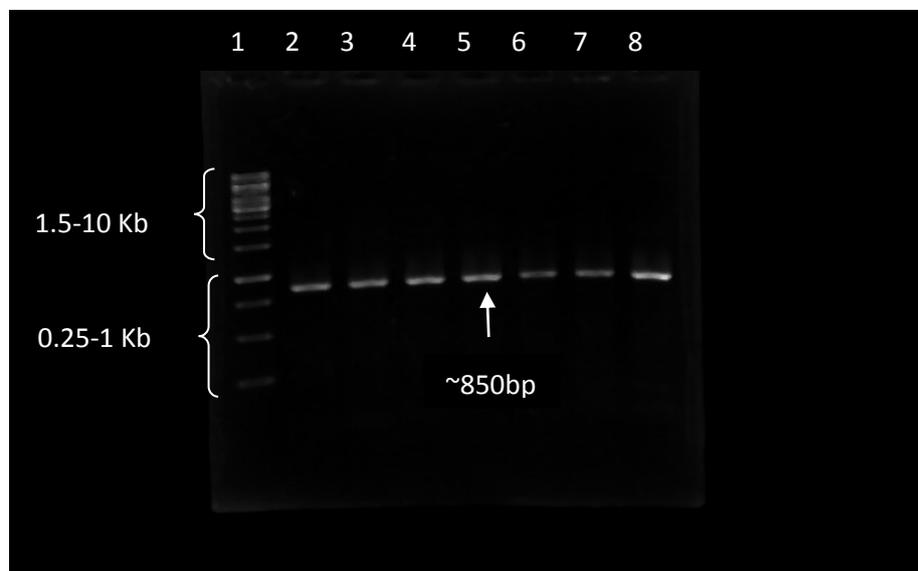
*Lane 2-6: PCR for pCTOXO-BBTI*



**Figure 4.15:** Colony PCR for pCTOXO-CDI using scFv-PI flanking primers.

Expected size of pCTOXO-CDI: 1600bp

*Lane 1: 1 Kb DNA ladder*  
*Lane 2-9: PCR for pCTOXO-CDI*



**Figure 4.16:** Colony PCR for pCTOXO-OCPI using scFv-PI flanking primers.

Expected size of pCTOXO-OCPI: 1450bp

*Lane 1: 1 Kb DNA ladder*  
*Lane 2-8: PCR for pCTOXO-OCPI*

The procedure of ligation, transformation and colony PCR with scFv-PI primers were repeated for several times using different purified RE digested scFv3, scFv9, scFv15, BBTI, CDI, OCPI and pCAMBIA 1301. But the result obtained still did not show the desired correct band size of PCR product for ligated scFv-PI genes.

#### **4.4.1.2 Gene Sequencing Analysis**

The PCR was carried out on two pCTOXO-PIs colony suspensions prepared after transforming the ligation mixture of pCTOXO-BBTI into JM109 cells. The PCR was carried out in large scale, 50µl, for scFv-PI genes using scFv-PI flanking primers. The sequence of primers was described in Section 3.5. The PCR products were purified with the use of Megaquick-spin kit from iNtRON Company. The purified PCR products were then sent for sequencing.

The gene sequencing results were analyzed using scFv-PI flanking primer sequences to search for the desired sequence of scFv-PI gene ligated to the pCAMBIA 1301, but the obtained result showed only scFv anti-toxoplasmosis gene ligated to the pCAMBIA 1301vector without ligating to Protease Inhibitor gene. (Gene Sequencing results in Appendix A).

The analysis of forward DNA sequencing results was carried out (Appendix B) by searching the sequence with the reverse complement sequence of scFv-PI reverse primer. The reverse complement sequence of scFv-PI reverse primer was as follows:

5'-TTCATTTTCTGTTTTAGGAACCCA - 3'

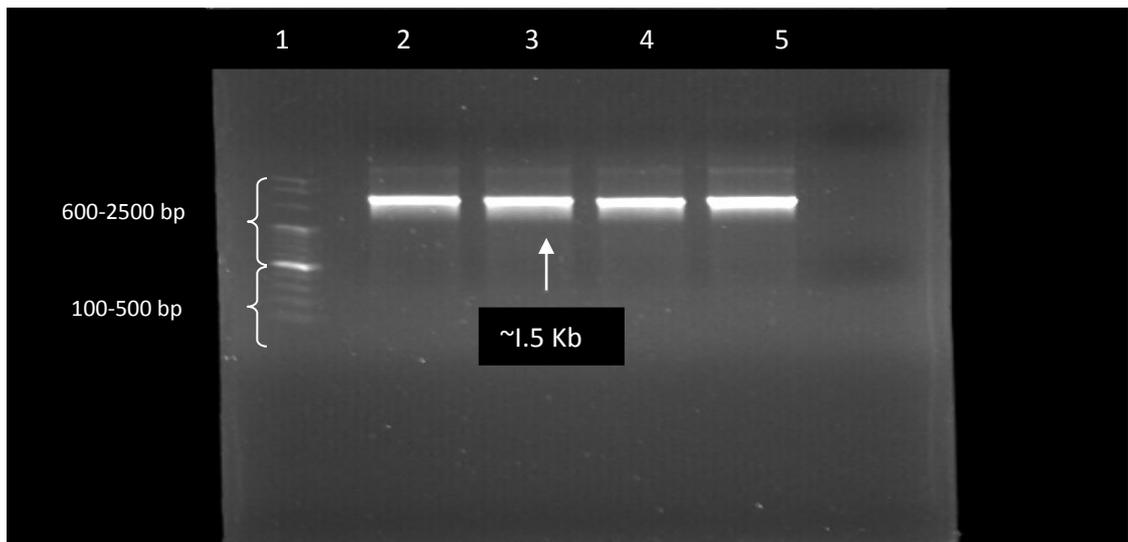
Then, the reverse DNA sequencing result was first reverse complemented to search for the desired scFv-PI gene sequence using forward scFv-PI primer (Appendix C). The two obtained forward DNA sequence and reverse DNA sequence was then combined for searching for the desired scFv-PI sequence ligating to the pCAMBIA 1301vector. The Appendix B shows the forward DNA sequencing result and Appendix C shows the reverse DNA sequencing result. The combination of these two sequences was shown in Appendix D and the scFv sequence and pCAMBIA 1301 sequence were highlighted but the PI gene sequence was not identified as it was not ligated to the vector.

#### **4.4.2 pCTOXO-CDI-KDEL**

As the previous attempts to obtain the recombinant PI constructs did not yield any suitable clone a new pCTOXO-CDI construct containing the scFv116-CDI gene developed with a parallel project in YasminLab (Go Pei See pers.comm. as described in Section 3.6) was used for the transformation experiments. The construct also contained a KDEL element and hence was labeled pCTOXO-CDI-KDEL.

##### **4.4.2.1 PCR analysis of extracted pCTOXO-CDI from *E.coli* cells**

The extracted pCTOXO-CDI-KDEL plasmid was used to perform the *Agrobacterium*-mediated transformation of plant cells in this study. The PCR was carried out on the new pCTOXO-CDI-KDEL plasmid construct using scFv-PI flanking primers to confirm the presence of scFv116-CDI gene after plasmid preparation. The PCR products were electrophoresed in a 1% w/v agarose gel with 1X TBE buffer and run at 120 volts for 25 minutes (Figure 4.17)



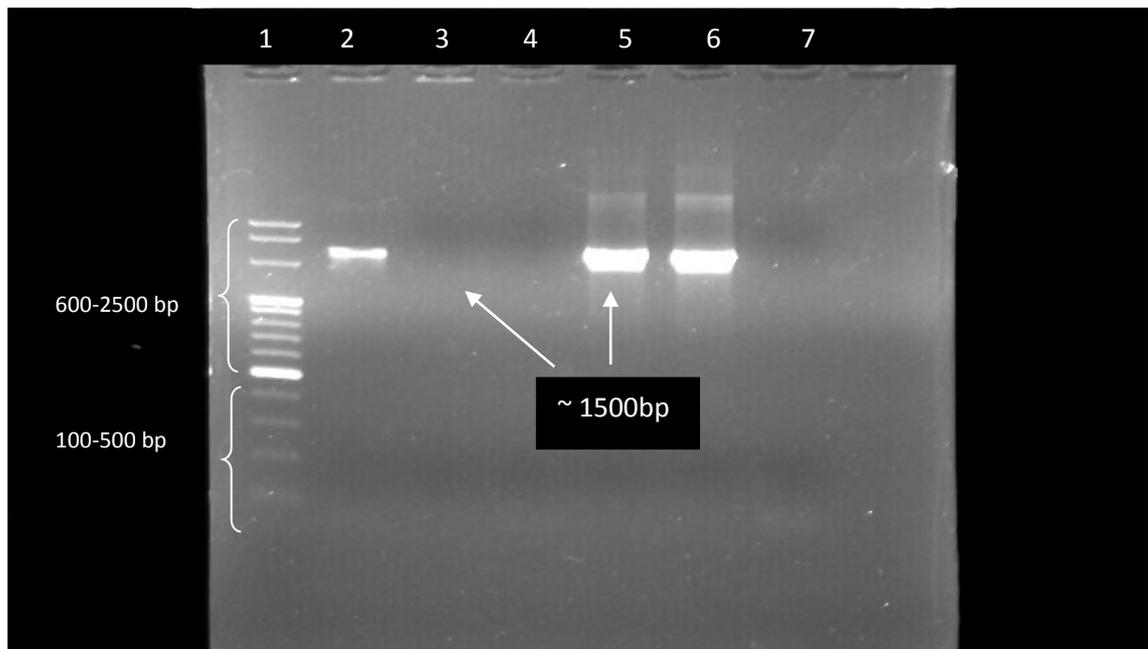
**Figure 4.17:** PCR for ligated plasmid construct pCTOXO-CDI2 using new scFv-PI primers. Expected size of PCR product: 1.5 Kb

*Lane 1: 100 bp DNA ladder*

*Lane 2-5: PCR for pCTOXO-CDI construct*

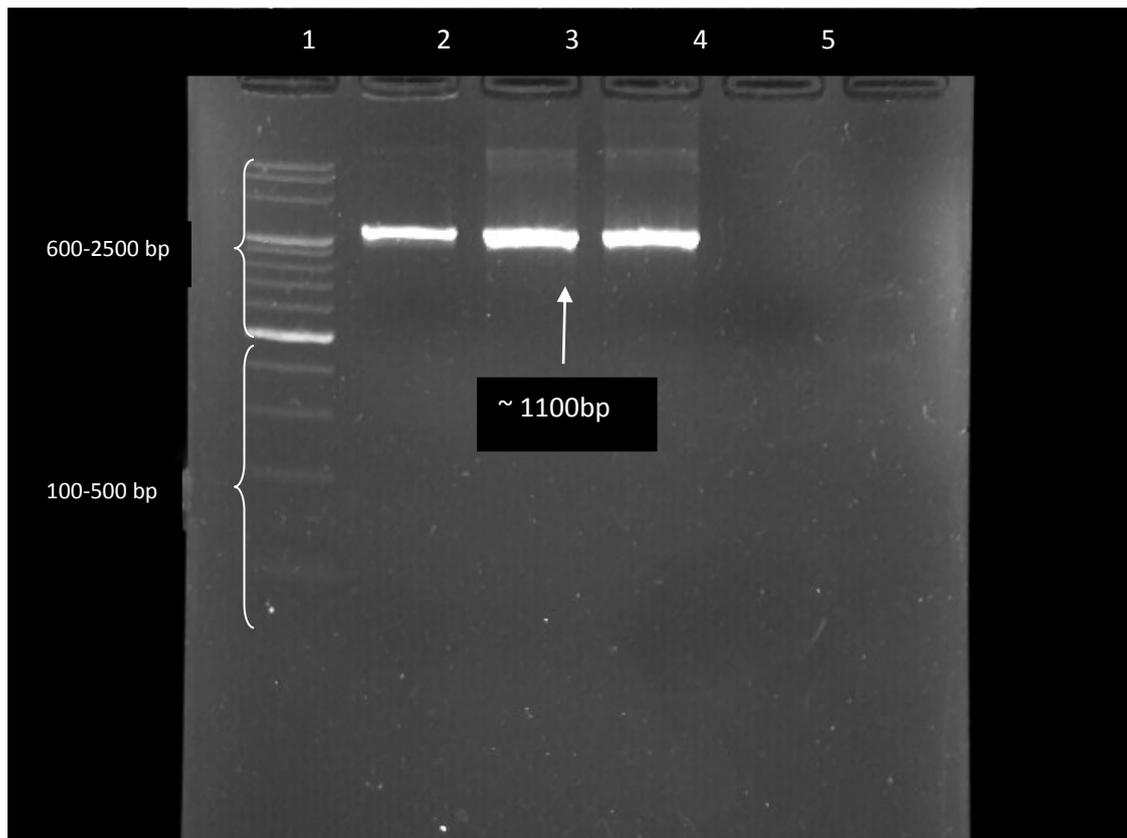
#### **4.5 PCR ANALYSIS OF TRANSFORMED *AGROBACTERIUM* LBA4404 WITH PLASMID CONSTRUCTS**

Colony PCR was carried out for *Agrobacterium* transformed with pCTOXO-CDI and pCTOXO-KDEL to verify the presence of desired constructs. The PCR products were electrophoresed in a 1% w/v agarose gel with 1X TBE buffer and run at 120 volts for 25 minutes. The results obtained showed the presence of the scFv116-CDI gene as a specific band with the expected size of 1500bp (Figure 4.18) and scFv116-KDEL gene as a specific band with the expected size of 1100bp in the electrophoresed gel of some transformed *Agrobacterium* colonies (Figure 4.19).



**Figure 4.18:** Colony PCR for *Agrobacterium* transformed with pCTOXO-CDI plasmid construct. Expected size: 1500 bp

<i>Lane 1: 100 bp DNA ladder</i>	<i>Lane 6: positive control</i>
<i>Lane 2: transformed colony A1</i>	<i>Lane 7: negative control</i>
<i>Lane 3-4: untransformed colonies</i>	
<i>Lane 5: transformed colony A2</i>	



**Figure 4.19:** Colony PCR for *Agrobacterium* transformed with pCTOXO-KDEL plasmid construct. Expected size: 1100 bp

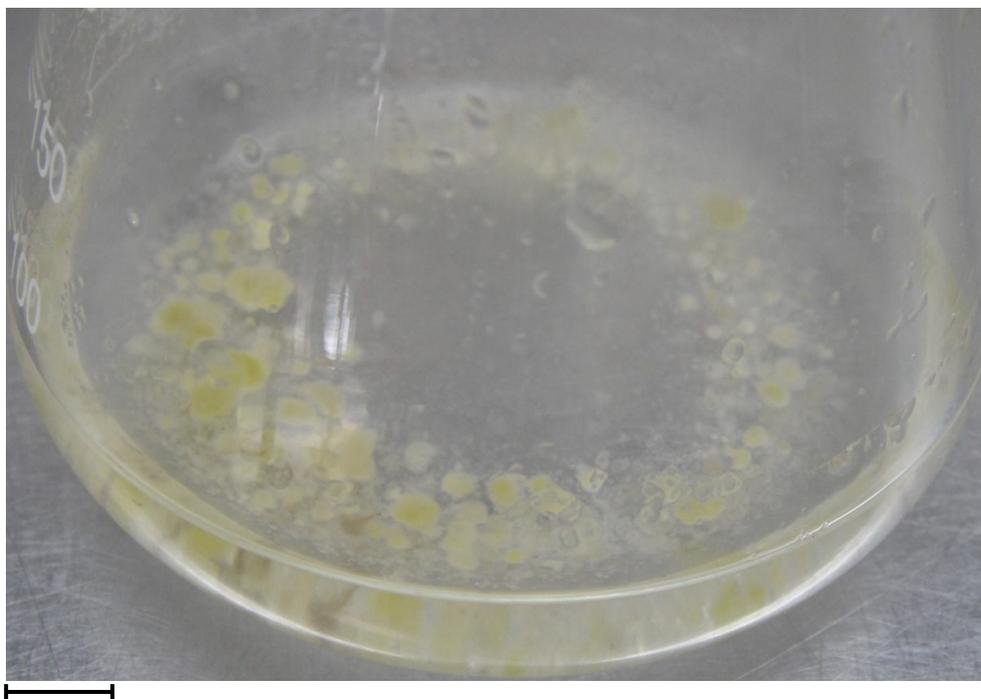
*Lane 1: 100 bp DNA ladder*  
*Lane 2: transformed colony B1*  
*Lane 3: Transformed colony B2*  
*Lane 4: positive control*  
*Lane 5: negative control*

## 4.6 TARGET SYSTEM

### 4.6.1 Tobacco cell suspension culture

In this research, the target system used was cell suspension of *Nicotiana tabacum* cv. BY-2 (cultivar Bright Yellow - 2 of the tobacco plant) for performing the

*Agrobacterium*-mediated transformation of the two constructs pCTOXO-CDI-KDEL and pCTOXO-KDEL. The cell suspension was propagated by subculturing as described in Section 3.8.4. Several flasks of tobacco BY-2 cell suspension were prepared. The typical tobacco cell suspension is shown in Figure 4.20.



**Figure 4.20:** *Nicotiana tabacum* cv. BY-2 cell suspension. Scale bar represents 1 cm.

For each transformed *Agrobacterium* colony carrying the desired plasmid construct 4 replicates of tobacco cell suspension were prepared. Two colonies for each plasmid construct were chosen to be transformed into tobacco cells as described in Section 3.9. The cells were transformed with two plasmid construct pCTOXO-CDI-KDEL and pCTOXO-KDEL using *Agrobacterium*-mediated transformation method as described in Section 3.11. The survival rate of tobacco cell suspension transformed with *Agrobacterium* carrying plasmid construct pCTOXO-CDI-KDEL and pCTOXO-KDEL is summarized in table 4.1.

Condition of cultures	tobacco cell suspension co-cultivated with pCTOXO-CDI-KDEL (number of plates used)		tobacco cell suspension co-cultivated with pCTOXO-KDEL (number of plates used)	
	A1	A2	B1	B2
Agrob colony name				
Living plates	4	3	3	4
Contaminated plates	0	1	1	0
Total plates	4	4	4	4
<b>% survival</b>	<b>100</b>	<b>75</b>	<b>75</b>	<b>100</b>

**Table 4.1:** Number and percentage of surviving and dead tobacco cell suspension cultures after 14 days of co-cultivation with *Agrobacterium* carrying plasmid construct pCTOXO-CDI-KDEL and pCTOXO-KDEL.

To compare the GUS enzyme activity results obtained by performing the Quantitative GUS Fluorometric Assay of transformed tobacco cells (A1 vs. B1 and A2 vs. B2), three living replicate of tobacco cells transformed with each colony were chosen to continue the experiment to the next step, because the number of samples was required to be equal for the analysis of data in paired t-test method. Therefore, 1 plate of tobacco cells transformed with A1 and 1 plate of tobacco cells transformed with B2 were set aside to have 3 replicates in total from each transformation event.

#### 4.7 QUANTITATIVE GUS FLUOROMETRIC ASSAY

$\beta$ -glucuronidase (GUS) is the reporter enzyme of choice for most plant transformation research. Typically GUS activity in solution is determined with the fluorogenic substrate 4-methylumbelliferyl  $\beta$ -D glucuronide (MUG):

GUS

MUG (non-fluorescent) → glucuronic acid+ 4-MU (fluorescent)

The reaction product 4-methylumbelliferone (4MU) is maximally fluorescent at high pH, where the hydroxyl group is ionized. Addition of a basic solution of sodium carbonate simultaneously stops the assay and adjusts the pH for quantitating the fluorescent product.

In this research the unknown sample fluorescence (relative fluorescent intensity of 4-MU) was measured for each sample by using a spectrofluorometer with the emission at 455 nm and excitation at 365 nm as described in Section 3.12.1. The relative fluorescent intensity of unknown samples is listed in Table 4.2. For the calculation of exact fluorescent intensity the average amount of control experiments was subtracted from test samples 'relative fluorescent intensity amounts to reduce the background effect which is due to intrinsic effects of plant cells (Table 4.2).

Sample	Control	Tobacco cells transformed with pCTOXO-CDI-KDEL		Tobacco cells transformed with pCTOXO-KDEL		Exact Fluorescent Intensity			
		A1	A2	B1	B2	A1	A2	B1	B2
colony									
replicate 1	3274	8019	8008	5413	5417	<b>4743</b>	<b>4732</b>	<b>2137</b>	<b>2141</b>
replicate 2	3305	7886	8022	5429	5435	<b>4610</b>	<b>4746</b>	<b>2153</b>	<b>2159</b>
replicate 3	3249	8031	7989	5396	5414	<b>4755</b>	<b>4713</b>	<b>2120</b>	<b>2138</b>
<b>Average</b>	3276	7978.6	8006.3	5412.6	5422	<b>4702.6</b>	<b>4730.3</b>	<b>2136.6</b>	<b>2146</b>
<b>Standard deviation</b>	28.05352	80.47567	16.56301	16.50253	11.35782	<b>80.47567</b>	<b>16.56301</b>	<b>16.50253</b>	<b>11.35782</b>

**Table 4.2:** The relative fluorescent intensity of test samples for three replicates of each colony transformation A1, A2, B1, B2

For calculation of 4-MU amount of the test samples in pmol the Standard Dilution of 4-Methylumbelliferone was carried out as described in Section 3.12.1.1 and the fluorescent intensity of different dilutions of 4-MU was obtained as listed in Appendix I.

The measured fluorescent intensity amounts of 4-MU dilutions were plotted versus different 4-MU concentrations as shown in Appendix J. From the obtained equation of the curve the Relative Fluorescent Intensity of the test samples was calculated in pmol as shown in Table 4.3.

Sample	Exact Fluorescent Intensity				4-MU (pmol)			
	A1	A2	B1	B2	A1	A2	B1	B2
replicate 1	4743	4732	2137	2141	<b>778.79</b>	<b>775.85</b>	<b>352.45</b>	<b>353.11</b>
replicate 2	4610	4746	2153	2159	<b>755.94</b>	<b>778.13</b>	<b>355.06</b>	<b>356.04</b>
replicate 3	4755	4713	2120	2138	<b>779.60</b>	<b>772.75</b>	<b>349.68</b>	<b>352.62</b>
<b>Average</b>	4702.66	4730.33	2136.66	2146	<b>771.44</b>	<b>775.57</b>	<b>352.39</b>	<b>353.92</b>
<b>Standard Deviation (STDEV)</b>					<b>13.43</b>	<b>2.70</b>	<b>2.69</b>	<b>1.84</b>

**Table 4.3:** Relative Fluorescent Intensity of test samples (pmol)

Comparison of the standard deviation of A1 vs. A2 shows higher STDEV for A1 samples (13.43 > 2.70). However the mean values (average) of A1 and A2 are very close (Table 4.3). This indicates the 4-MU measurements of A2 samples in three

replicates have closer values in comparison with three A1 replicates. Thus the 4-MU measurements were more accurate in A2 sample replicates.

Comparison of the standard deviation of B1 vs. B2 shows higher STDEV for B1 samples ( $2.69 > 1.84$ ). However the mean values (average) of B1 and B2 are very close (Table 4.3). This indicates the 4-MU measurements of B2 samples in three replicates have closer values in comparison with three B1 replicates. Thus the 4-MU measurements were more accurate in B2 sample replicates.

#### **4.7.1 Bradford assay**

The protein standard assay was carried out as described in Section 3.12.2.1 to obtain the protein content of the unknown samples. The obtained OD values for different BSA protein concentrations listed in Appendix K. Then, the protein standard curve was plotted to obtain the equation needed to determine the protein content of unknown samples (Appendix L).

The Bradford protein assay was carried out to obtain the amount of soluble protein in the unknown samples. The absorbance was read at 595nm for each unknown sample separately using a spectrophotometer as described in Section 3.12.2. Then, the protein concentration of unknown samples was determined by using the Bradford protein standard curve (Appendix L). The OD reading values and protein concentration of samples are listed in table 4.4. Statistical analysis of total soluble protein concentration in transformed tobacco cells with two plasmid constructs showed significant difference (Table 4.4). The testing of paired differences between TSP of transformed tobacco cells

with pCTOXO-CDI-KDEL and pCTOXO-KDEL were done using paired t-test at P = 0.05.

Sample	OD (A595nm)		OD (A595nm)		Protein conc. mg		Protein conc. mg	
	A1	A2	B1	B2	A1	A2	B1	B2
Replicate 1	0.7310	0.7284	0.6349	0.6382	<b>0.0242</b>	<b>0.0238</b>	<b>0.0104</b>	<b>0.0109</b>
Replicate 2	0.6989	0.7326	0.6412	0.6423	<b>0.0196</b>	<b>0.0244</b>	<b>0.0113</b>	<b>0.0115</b>
Replicate 3	0.7598	0.6983	0.6270	0.6354	<b>0.0337</b>	<b>0.0195</b>	<b>0.0093</b>	<b>0.0105</b>
<b>Average</b>	0.7299	0.7197	0.6343	0.6386	<b>0.0258</b>	<b>0.0225</b>	<b>0.0103</b>	<b>0.0109</b>
<b>Standard Deviation (S.D.)</b>					<b>0.00719</b>	<b>0.00267</b>	<b>0.00100</b>	<b>0.00050</b>
<b>T-TEST protein conc. (mg) A1 vs. B1</b>					<b>0.040846</b>			
<b>T-TEST protein conc. (mg) A2 vs. B2</b>					<b>0.006164</b>			

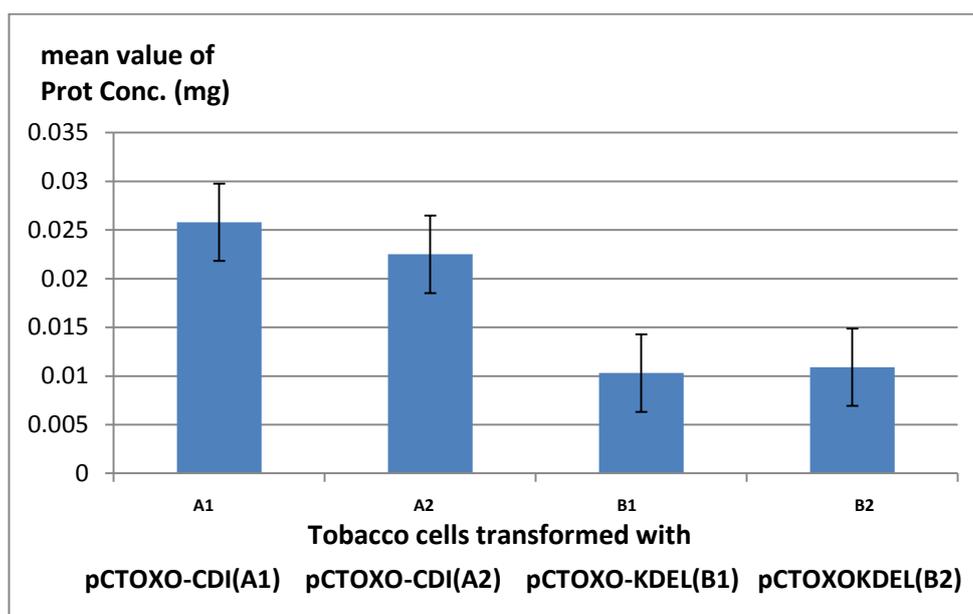
**Table 4.4:** The OD values for test samples and protein conc. of samples

Comparison of the standard deviation of A1 vs. A2 shows higher STDEV for A1 samples ( $0.00719 > 0.00267$ ). However the mean values (average) of A1 and A2 are very close (Table 4.4). This indicates the total soluble protein concentration measurements of A2 samples in three replicates have closer values in comparison with three A1 replicates. Thus the TSP measurements were more accurate in A2 sample replicates.

Comparison of the standard deviation of B1 vs. B2 shows higher STDEV for B1 samples ( $0.00100 > 0.00050$ ). However the mean values (average) of B1 and B2 are very close (Table 4.4). This indicates the total soluble protein concentration

measurements of B2 samples in three replicates have closer values in comparison with three B1 replicates. Thus the TSP measurements were more accurate in B2 sample replicates.

The soluble protein concentration mean values (average) of three sample replicates was plotted in Figure 4-21 and error bars were indicated on the chart.



**Figure 4-21:** Comparison of TSP of tobacco cells transformed with pCTOXO-CDI (A1 & A2 colonies) and pCTOXO-KDEL (B1 & B2 colonies).

#### 4.8 COMPARISON OF ENZYME ACTIVITY

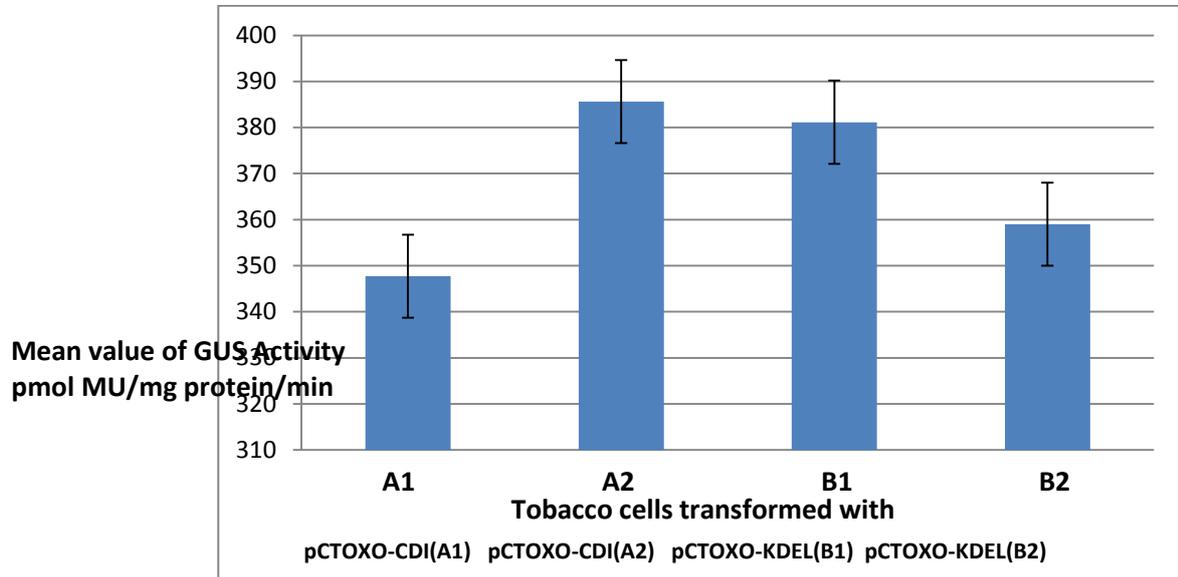
The gusA gene enzyme activity was quantified and expressed in pmol 4-MU released per mg of protein per minute according to 4-MU (pmol) and protein conc. (mg) that were obtained and listed in Tables 4.3 and 4.4 respectively. The 4-MU enzyme activity was calculated for samples after 90 minutes reaction as described in Section 3.12.1 and listed in Table 4.5.

Statistical analysis of GUS activity in transformed tobacco cells with two plasmid constructs did not show any significant difference (Table 4.5). The testing of paired differences between GUS activity of transformed tobacco cells with pCTOXO-CDI-KDEL and pCTOXO-KDEL were done using paired t-test at P = 0.05.

Sample	4-MU (pmol)		4-MU (pmol)		Protein conc. mg		Protein conc. mg		4-MU (pmol)/mg prot/min		4-MU (pmol)/mg prot/min	
	A1	A2	B1	B2	A1	A2	B1	B2	A1	A2	B1	B2
1	778.79	775.85	352.45	353.11	0.0242	0.0238	0.0104	0.0109	<b>357.571</b>	<b>362.208</b>	<b>376.549</b>	<b>359.949</b>
2	755.94	778.13	355.06	356.04	0.0196	0.0244	0.0113	0.0115	<b>428.537</b>	<b>354.339</b>	<b>349.124</b>	<b>344.000</b>
3	779.60	772.75	349.68	352.62	0.0337	0.0195	0.0093	0.0105	<b>257.039</b>	<b>440.313</b>	<b>417.777</b>	<b>373.143</b>
Average	771.44	775.57	352.39	353.92	0.0258	0.0225	0.0103	0.0109	<b>347.71</b>	<b>385.62</b>	<b>381.15</b>	<b>359.03</b>
<b>Standard Deviation</b>									<b>86.172</b>	<b>47.528</b>	<b>34.556</b>	<b>14.593</b>
<b>T-TEST enzyme activity (4-MU: pmol/mg/min) A1 vs. B1</b>									<b>0.339395</b>			
<b>T-TEST enzyme activity (4-MU: pmol/mg/min) A2 vs. B2</b>									<b>0.16136</b>			

**Table 4.5:** Comparison of 4-MU enzyme activity between A1 vs. B1 and A2 vs. B2 samples.. 4-MU enzyme activity expressed in pmol/mg. protein/min for tobacco cells transformed with pCTOXO-CDI-KDEL (A1 and A2 Agrob colonies) and pCTOXO-KDEL (B1 and B2 *Agrobacterium* colonies).

The GUS activity mean values (average) expressed in 4-MU/mg protein/min of three sample replicates was plotted in Figure 4-22 and error bars were indicated on the chart.



**Figure 4.22:** Comparison of the level of GUS Activity of the transformed tobacco cells with two different plasmid constructs.

## CHAPTER 5: DISCUSSION

In this research tobacco cell suspension were selected as host systems for testing of scFv constructs enhanced using protease inhibitor elements. Although no positive results were obtained using the banana cell suspensions, the scFv anti-toxoplasmosis gene ligated to Cathepsin D Inhibitor in pCTOXO-CDI-KDEL and also ligated to KDEL fragment in pCTOXO-KDEL was shown to be putatively transformed and expressed in tobacco cells as demonstrated by GUS fluorometric Assay.

The gene transfer and expression technology in tobacco cells is well-established. The high biomass yield is one of the major advantages of tobacco plant as a host system. So it can be a major source of plant-derived recombinant antibodies. (Twyman et al., 2007).

The effect of Plant Protease Inhibitor (CDI) on the expression of the heterologous scFv anti-toxoplasmosis gene was then investigated by comparing the total soluble protein (TSP) and GUS activity in tobacco cells transformed with two different constructs with protease inhibitor (pCTOXO-CDI-KDEL) or with the ER-targetting element KDEL (pCTOXO-KDEL). The measurement of total soluble protein content in the transformed cells and GUS assay analysis can only assess the preliminary positive effects of CDI protease inhibitor in recombinant protein production in plant cells, but as these methods are preliminary assessments for measuring positive transformation events, presence of higher total soluble protein content (Table 4.4 and Figure 4.21) in cells transformed with TOXO gene may not necessarily resulted from the effect of the CDI protease inhibitor.

## **5.1 PLASMID CONSTRUCT DEVELOPMENT**

In this research the development of three pCTOXO-PI plasmid constructs expressed as pCTOXO-BBTI, pCTOXO-CDI and pCTOXO-OCPI was attempted by ligating RE digested scFv anti-toxoplasmosis and PI genes DNA fragments and cloning into pCAMBIA 1301vector.

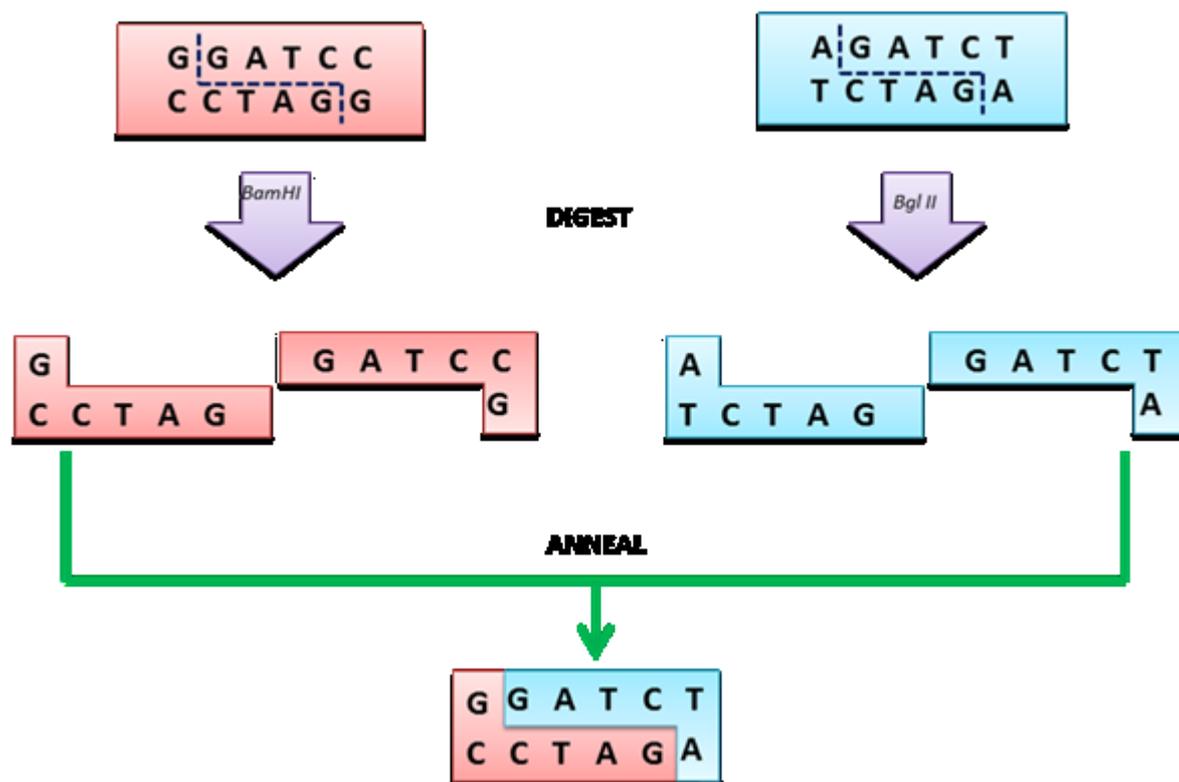
### **5.1.1 Verification of Ligation**

PCR was carried out on ligation mixture using scFv primers and scFv-PI flanking primers as described in Section 3.5. The PCR products amplified using scFv primers confirmed the presence of scFv gene ligated to pCAMBIA 1301 vector on gel electrophoresis (Figure 4.12 and 4.13). However, the results obtained from PCR work with scFv-PI flanking primers unexpectedly did not show the presence of correct band size of ligated scFv-PI product in PCR gel electrophoresis (Figure 4.14, 4.15 and 4.16). The failure of desired plasmid constructs pCTOXO-PI development was also verified by sequencing analysis as was described in Section 4.4.1.2. The sequencing results showed the presence of scFv gene ligated to pCAMBIA 1301 vector but the PI gene sequence was missing in the sequencing result (Appendix D).

#### **5.1.1.1 Failure of plasmid constructs pCTOXO-PI development**

The ligation of scFv gene to pCAMBIA 1301 vector was verified by sequencing as described in Section 4.4.1.2. But no ligation of the PI genes was detected. This failure could be due to mismatching of compatible sticky ends (Clark, 2005). BamHI and BglII generate the same overhanging or sticky ends. It means BamHI and BglII generate

different-but-compatible ends. BamHI recognizes the sequence 5'-GGATCC-3' and cuts after the first 5' G, which generates the 3'-CTAG-5' overhang on the bottom strand. BglII recognizes the sequence 5'-AGATCT-3' and cuts after the first 5' A, which generates a 5'-GATC-3' overhang on the top strand. If these two pieces are allowed to anneal, the complementary sequences will hydrogen bond together, allowing the nicks to be sealed more easily by DNA ligase as shown in Figure 5.1. In this research the scFv gene had BamHI sticky ends at 5' end and the vector pCAMBIA 1301 was digested using NcoI and BglII as described in Section 3.3.3. Therefore, the two BamHI and BglII sticky ends might ligate together despite the fact that BamHI and BglII generate different ends. Missing (deletion or point mutations) of some parts of nucleotide sequences in PI genes or pCAMBIA 1301 backbone could be the other probability that the desired constructs of pCTOXO-PI failed to develop. Because of this another construct was developed in a parallel study (pCTOXO-CDI-KDEL) and was used for comparative analysis with the constructed pCTOXO-KDEL.



**Figure 5.1:** Matching of compatible sticky ends

## 5.2 COMPARISON OF TOTAL SOLUBLE PROTEIN

The Total Soluble Protein (TSP) of *Agrobacterium*-mediated transformed tobacco cells with two plasmid constructs, pCTOXO-CDI-KDEL and pCTOXO-KDEL was measured using Bradford protein assay as described in Section 3.12.2. The paired t-test (at  $P=0.05$ ) results of protein concentration (mg) obtained from Table 4.4 indicates a significant difference ( $P < 0.05$ ) in TSP of tobacco cells transformed with two different constructs. The TSP mean values of transformed tobacco cells with two constructs are illustrated in Figure 4.21 to compare the TSP amounts. The Figure 4.21 indicates that the TSP of tobacco cells transformed with pCTOXO-CDI-KDEL was higher than tobacco cells transformed with pCTOXO-KDEL. The CDI gene in this construct was synthesized with a KDEL fragment at its 3' end. Hence both plasmid constructs contained the KDEL fragment but the difference was in pCTOXO-CDI which contained the Protease Inhibitor gene Cathepsin D Inhibitor. This suggested that the CDI gene had

an effect on the TSP in tobacco cells transformed with pCTOXO-CDI. The CDI gene was suggested to inhibit the endogenous proteases of tobacco cells that degrade the heterologous protein in plant cells leading to higher TSP measurements in cells carrying it. These results concurred with a report by Michaud et al., (2005) and Rivard et al., (2006). Hence suggesting this strategy would be suitable for enhancing the production of anti-toxoplasmosis antibodies in plant cells.

### **5.3 COMPARISON OF ENZYME ACTIVITY**

The GUS reporter gene transformation efficiencies were monitored by plasmid pCAMBIA1304 transformation into cell suspension of tobacco cultivar BY-2 via *Agrobacterium*-mediated transformation. The evaluation of the effect of plant protease inhibitor gene (CDI) on the expression of heterologous gene (scFv anti-toxoplasmosis) in tobacco cells was based on quantitative fluorometric assay analysis of GUS expression.

The  $\beta$ -glucuronidase was visualized as fluorescence blue color in tobacco cell extracts indicating positive transformation event as described in Section 3.12.1. Since the  $\beta$ -glucuronidase was designed as a fusion construct with the gene of interest in the plasmid construct therefore, observation the presence of  $\beta$ -glucuronidase could imply the possible successful expression of scFv-CDI-KDEL and scFv-KDEL genes in the transformed tobacco cells. Reporter genes like  $\beta$ -glucuronidase encode for messages which would clearly indicate the transient or stable expression of the transferred genes in the transgenic cells (Slater et al., 2003).

In this research, the quantitative fluorometric assays of GUS activity demonstrated the transcript level of GUS gene in the putative transformed tobacco cells with plasmid

pCTOXO-CDI-KDEL and pCTOXO-KDEL. The presence of  $\beta$ -glucuronidase enzyme will cleave the fluorogenic substrate 4-methylumbelliferyl  $\beta$ -D glucuronide (MUG) to produce fluorescent 4-methylumbelliferone (4-MU) (Jefferson et al., 1987). The *Agrobacterium* transformed tobacco cells were incubated for two weeks to allow cell growth and stable gusA gene integration before fluorometric assay was performed. Figure 4.22 indicates that the transient gene expression level was detected fluorometrically in tobacco plant cell extracts prepared two weeks after *Agrobacterium*-mediated transformation experiment, regardless of the plasmid construct used. In comparison to the transformed tobacco cells, higher total protein (mg) production level (TSP) was achieved for pCTOXO-CDI-KDEL transformed cells based on Bradford assay (Table 4.4 and Figure 4.21). However, the statistical analysis (paired t-test) of the GUS activity (pmol 4-MU/mg protein/min) for the putative transformed tobacco cells revealed no significant ( $P > 0.05$ ) differences between pCTOXO-CDI-KDEL and pCTOXO-KDEL (Table 4.5). This finding was probably due to inadequate sample replicates for performing paired t-test. Future work should include more replicates to obtain more statistically accurate results.

These preliminary results suggested that protease inhibitor genes may present a viable strategy for enhancing levels of production of scFv proteins in plant cells. Further studies are needed to establish the functionality of scFv protein and the integration of scFv-CDI gene into the plant genome and also improve transformation protocol in banana cells.

## CHAPTER 6: CONCLUSION

The anti-toxoplasmosis scFv gene (TOXO) ligated to CDI plant protease inhibitor gene was putatively transformed into *Nicotiana tabacum* cv. BY-2 cell suspension using pCTOXO-CDI-KDEL. In addition, the scFv anti-toxoplasmosis gene ligated to KDEL fragment was also putatively transformed into tobacco cells using pCTOXO-KDEL by *Agrobacterium*-mediated transformation method.

The effect of co-expression of plant protease inhibitor (CDI) on the expression of scFv heterologous gene in plant cells was investigated by GUS fluorometric assay and Bradford protein assay. The transient gene expression level was detected fluorometrically in tissue extracts of tobacco cells and the TSP of transformed tobacco cells was measured by Bradford assay.

The TSP of tobacco cells transformed with pCTOXO-CDI appeared to be higher than tobacco cells transformed with pCTOXO-KDEL in Bradford protein assay. Therefore, suggesting the positive effect of co-expression of Plant Protease Inhibitor (CDI) on the expression of heterologous gene scFv anti-toxoplasmosis in tobacco cells.

## **6.1 FUTURE STUDIES**

The results obtained from assessment of the GUS assay analysis and total soluble protein contents measurement are considered as preliminary findings for the real effects of protease inhibitor in the transformed plant cells. Therefore, further research has to be conducted to strength the results obtained.

The expression level of scFv anti-toxoplasmosis gene ligated to CDI and KDEL genes was not verified in this research therefore, further studies need to be carried out to determine the expression level of the recombinant protein using two different constructs. The functionality of scFv anti-toxoplasmosis protein was also not studied thus indicating the need for further research in this area.

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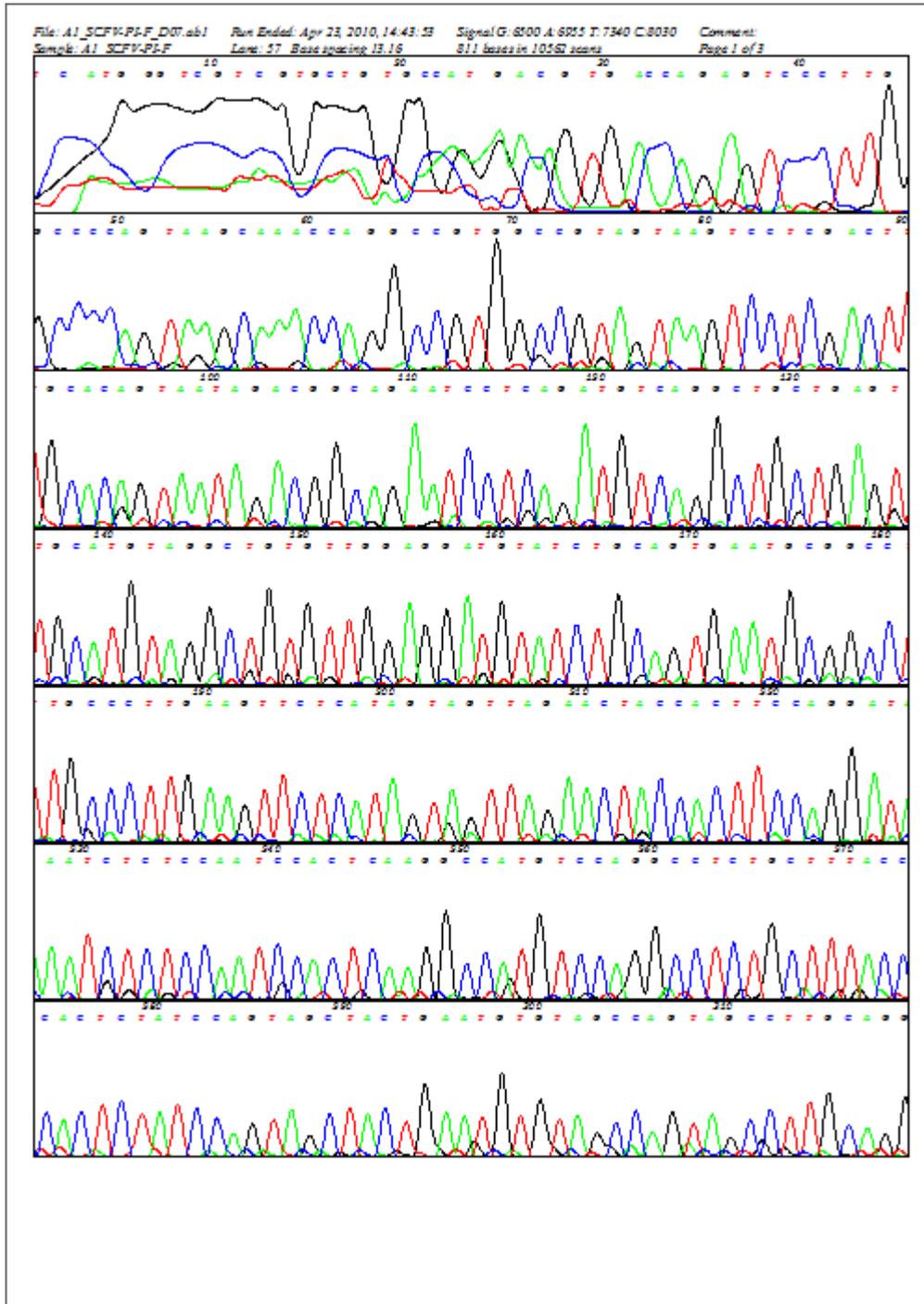
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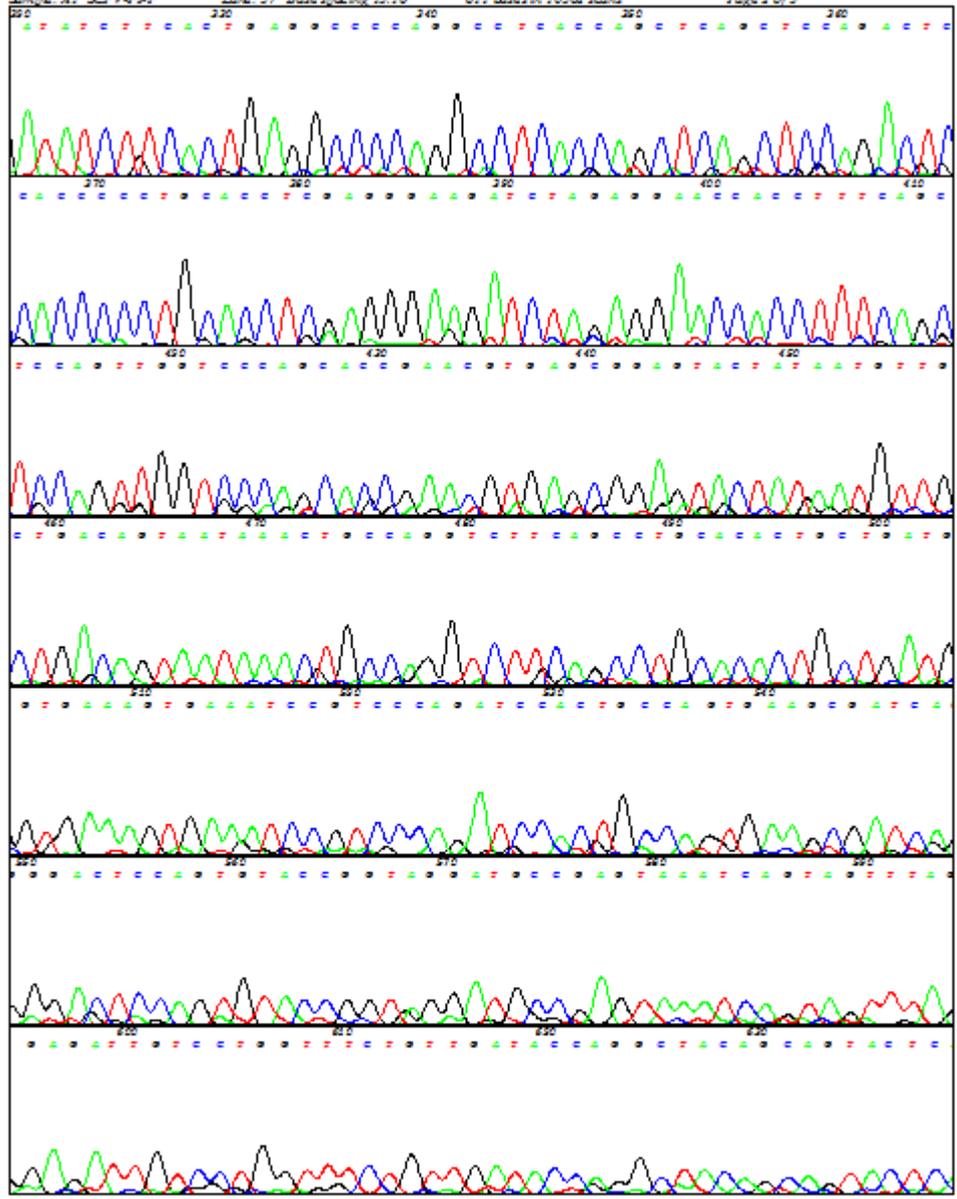
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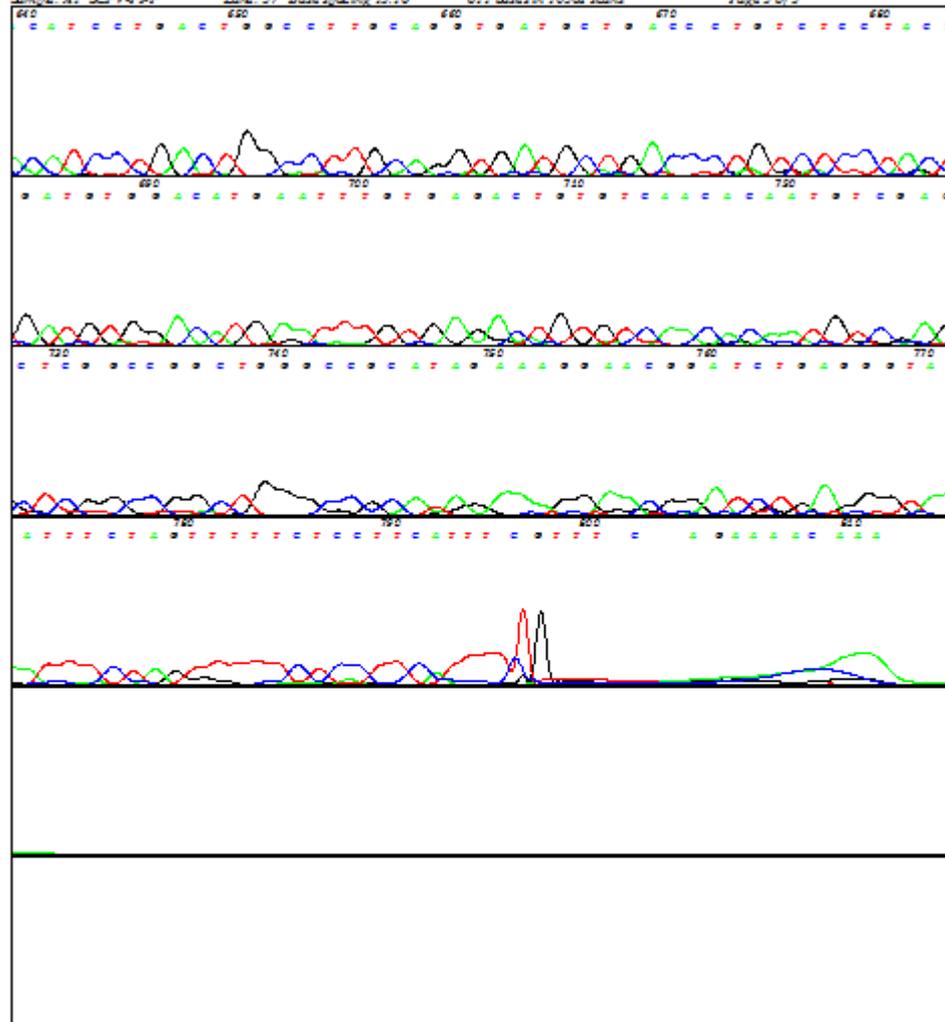
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## APPENDICES

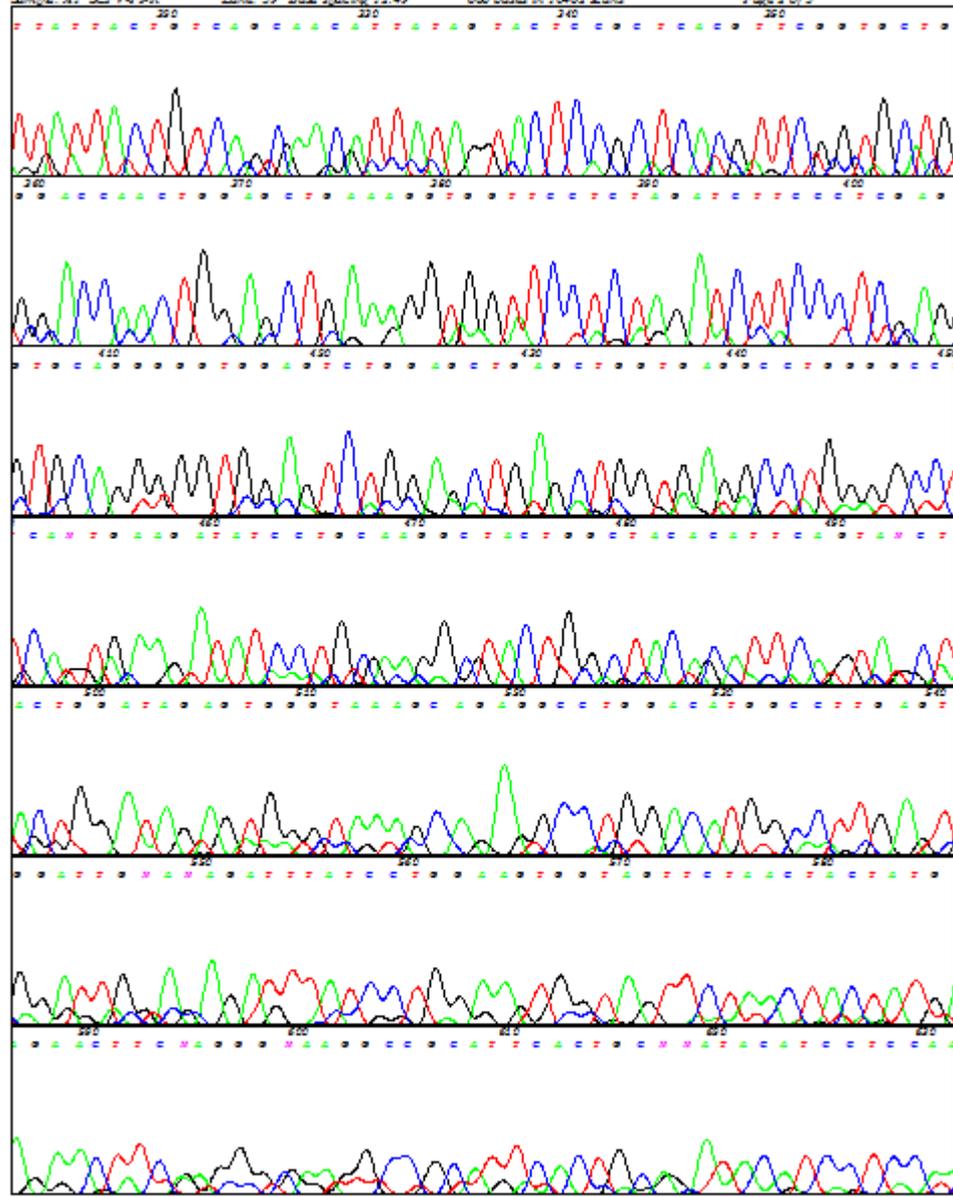
# APPENDIX - A

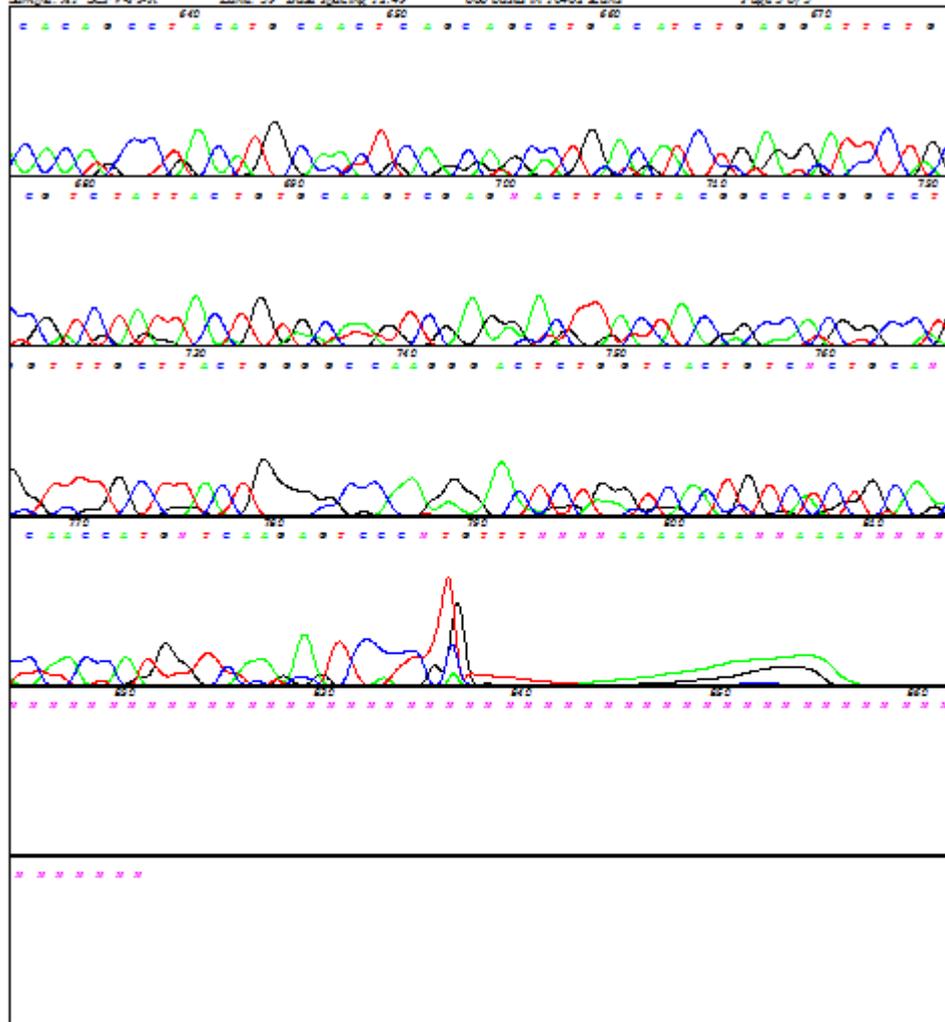




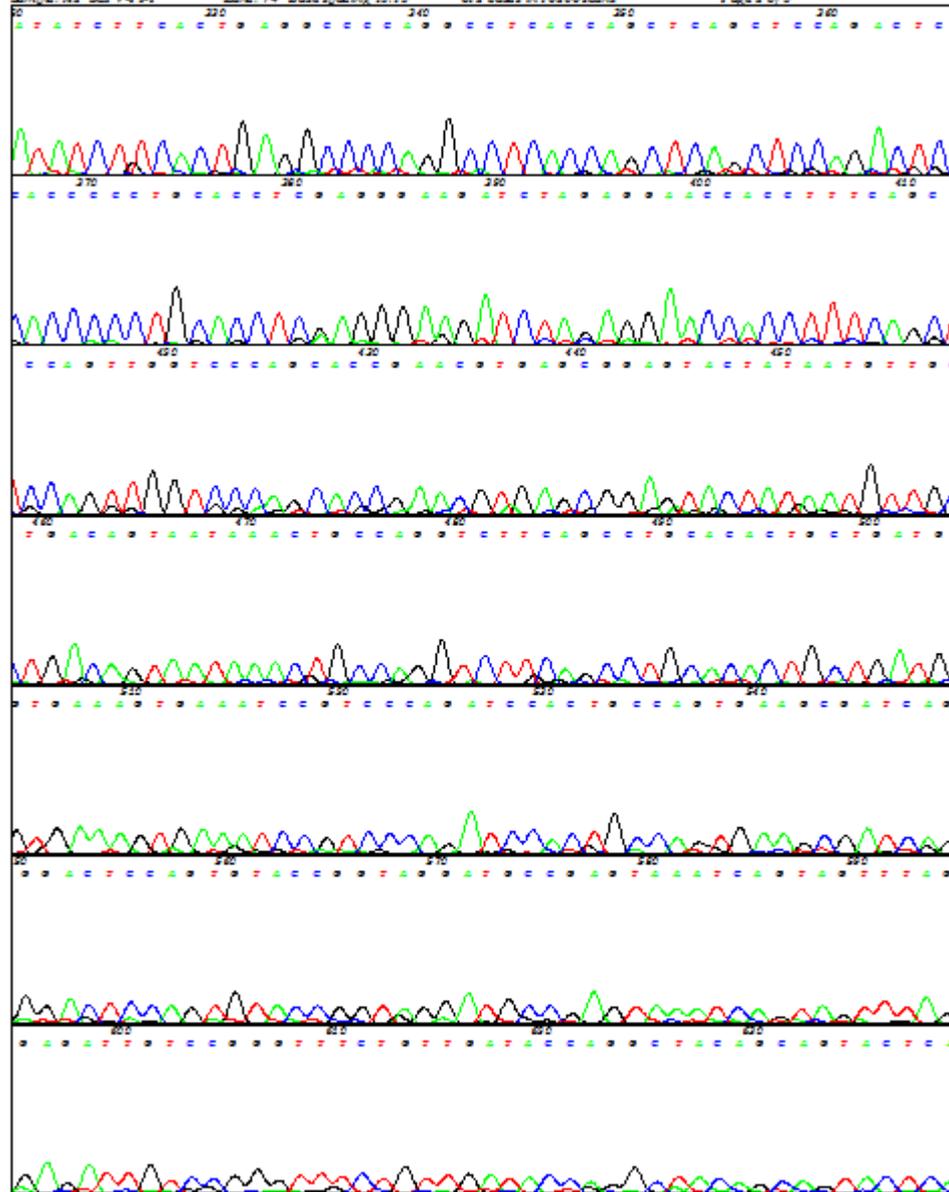


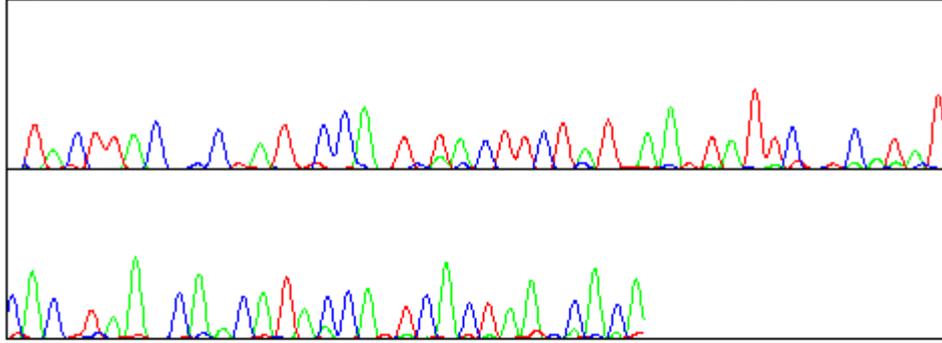


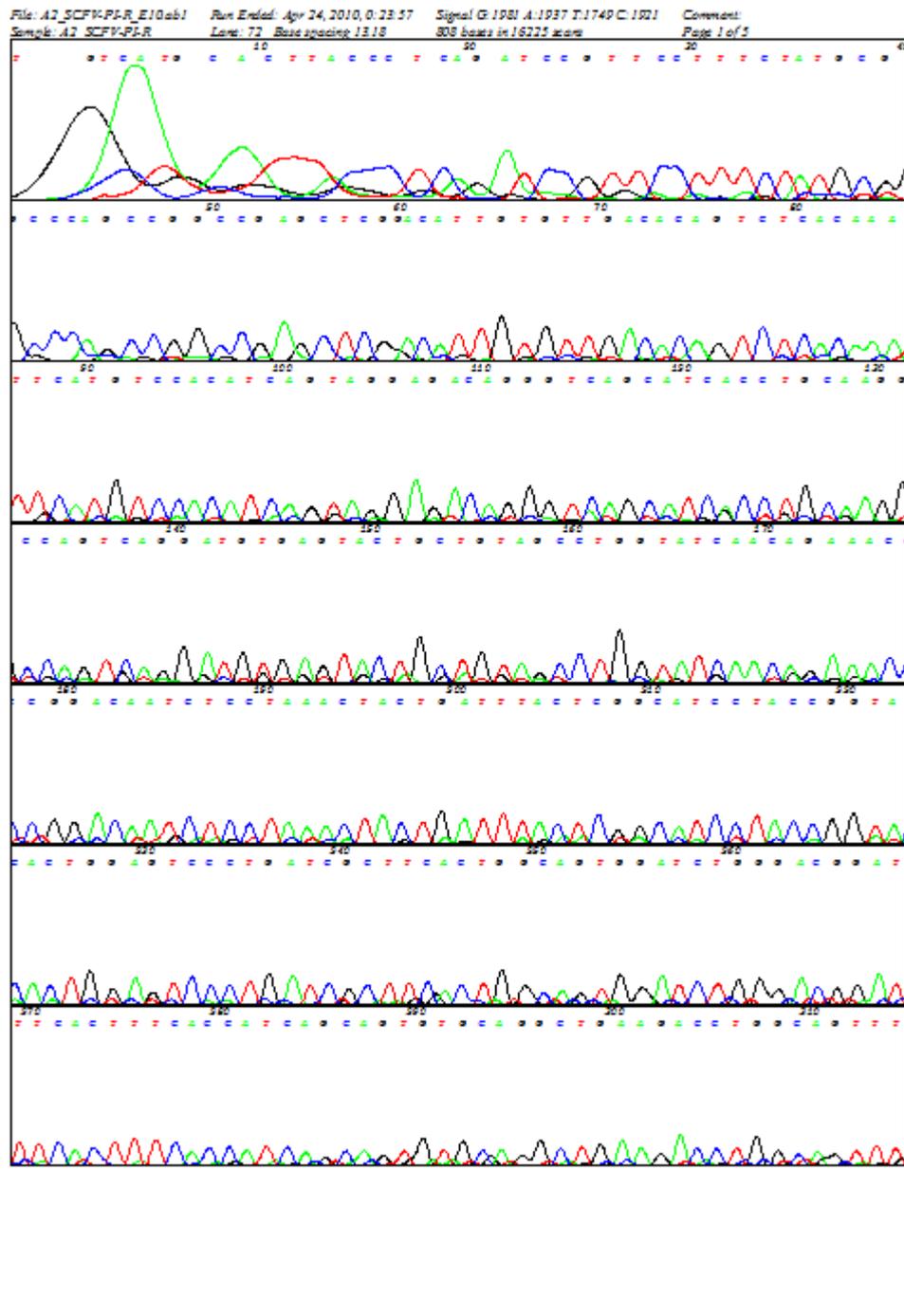


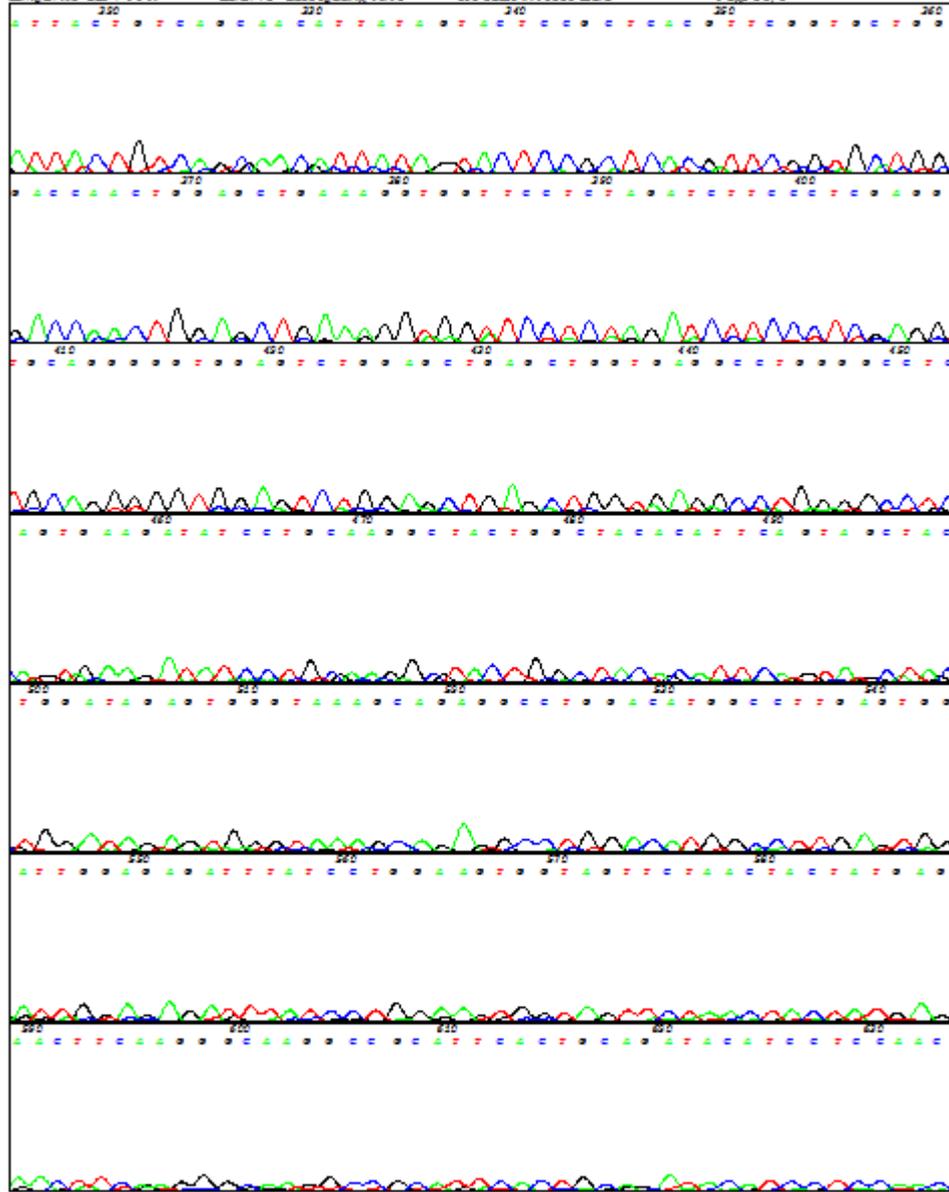


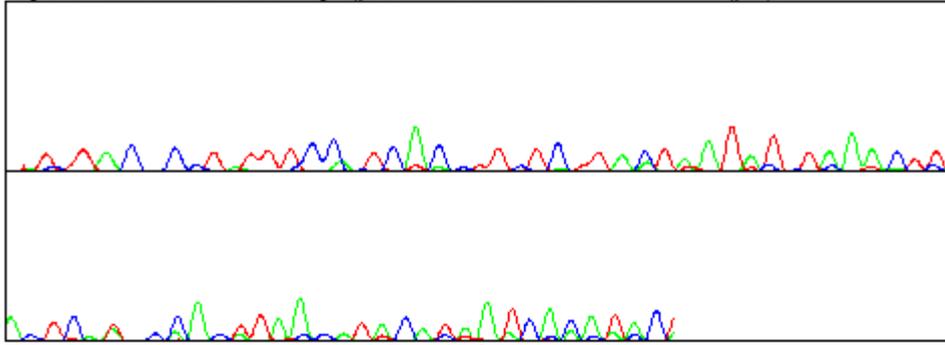












## APPENDIX - B

### FORWARD DNA SEQUENCING RESULT ANALYSIS

```
TCCACTATTTAACTAGCCTGCAGAGAAGTGACCAGAGTCCCTTGGCC
CCAGTAAGCAAACCAGGCCGTGGCCGTAGTAAGTCCTCGACTTGCA
CAGTAATAGACGGCAGAATCCTCAGATGTCAGGCTGCTGAGTTGCAT
GTAGGCTGTGTTGGAGGATGTATCTGCAGTGAATGCGGCCTTGCCCT
TGAAGTTCTCATAGTAGTTAGAACTACCACTTCCAGGATAAATCTCTC
CAATCCACTCAAGGCCATGTCCAGGCCTCTGCTTTACCCACTCTATC
CAGTAGCTACTGAATGTGTAGCCAGTAGCCTTGCAGGATATCTTCAC
TGAGGCCCCAGGCCTCACCAGCTCAGCTCCAGACTCCACCCCCTGC
ACCTCGAGGGAAGATCTAGAGGAACCACCTTTCAGCTCCAGTTGGTC
CCAGCACCGAACGTGAGCGGAGTACTATAATGTTGCTGACAGTAATA
AACTGCCAGGTCTTCAGCCTGCACACTGCTGATGGTGAAAGTGAAAT
CCGTCCCAGATCCACTGCCAGTGAAGCGATCAGGGACTCCAGTGTA
CCGGTAGGATGCCGAGTAAATCAGTAGTTTAGGAGATTGTCCGGGTT
TCTGTTGATACCAGGCTACAGCAGTACTCACATCCTGACTGGCCTTG
CAGGTGATGCTGACCCTGTCTCCTACTGATGTGGACATGAATTTGTG
AGACTGTGTCAACACAATGTTCGAGCTCGGCCGGCTGGGCCGCATAG
AAAGGAACGGATCTGAGGGTAAATTTCTAGTTTTTCTCC
```

1. Orange sequence matches the scFv gene



## APPENDIX - D

### ANALYSIS OF COMBINED FORWARD AND REVERSE DNA SEQUENCING RESULT

```
CTTGANCATGGTTGGNTGCAGNGACAGTGACCAGAGTCCCTTGGCCCCA
GTAAGCAAACCAGGCCGTGGCCGTAGTAAGTNCTCGACTTGCACAGTAA
TAGACGGCAGAATCCTCAGATGTCAGGCTGCTGAGTTGCATGTAGGCTG
TGTTGGAGGATGTATCTGCAGTGAATGCGGCCTTGCCCTTGAAGTTCTC
ATAGTAGTTAGAACTACCACTTCCAGGATAAATCTCTCCAATCCACTCAA
GGCCATGTCCAGGCCTCTGCTTTACCCACTCTATCCAGTAGCTACTGAAT
GTGTAGCCAGTAGCCTTGCAGGATATCTTCACTGAGGCCCCAGGCCTCA
CCAGCTCAGCTCCAGACTCCACCCCCTGCACCTCGAGGGAAGATCTAGA
GGAACCACCTTTCAGCTCCAGTTGGTCCCAGCACCGAACGTGAGCGGA
GTACTATAATGTTGCTGACAGTAATAAACTGCCAGGTCTTCAGCCTGCAC
ACTGCTGATGGTGAAAGTGAAATCCGTCCCAGATCCACTGCCAGTGAAG
CGATCAGGGACTCCAGTGTACCGGTAGGATGCCGAGTAAATCAGTAGTT
TAGGAGATTGTCCGGGTTTCTGTTGATACCAGGCTACAGCAGTACTCACA
TCCTGACTGGCCTTGCAGGTGATGCTGACCCTGTCTCCTACTGATGTGG
ACATGAATTTGTGAGACTGTGTCAACACAATGTTCGAGCTCGGCCGGCTG
GGCCGCATAGAAAGGAACGGATCTGAGGGTAAATTTCTAGTTTTTCTCC
```

1. Red sequence matches the pCambia1301 sequence (GenBank: AF234297.1)
2. Blue sequence is BamHI (ggatcc) sequence which is located at 3' end of scFv gene sequence.
3. Green and orange sequence smatche the scFv gene.
4. PI gene (BBTI) sequence is missing in the sequencing result.

## APPENDIX - E

### SCFV GENE SEQUENCE

5'NNNNACACTTTCACAGTCTATGCGGCACGCGGTTCCAGCGGATCCGGATA  
CGGCACCGGCGCACCTGCGGCCGCCACTAGTGACAGATGGGGCTGTTGTT  
TTGGCTGCAGAGACAGTGACCAGAGTCCCTTGGCCCCAGTAAGTCCGTCCC  
AGTTGTGCACAGTAATAGACTGCAGAGTCCTCAGATGTCAGGCTGCTGAGC  
TGCATGTAGGCTGTGTTGGAGGATTTGTCTACATTCAATGTGGCCTTGTCCT  
TGAACTTCTGATTTAACCTAGTTTCACTATTGGAAGGATCAATCATGCCAAT  
CCACTCAAGGCCTTGTCCAGGCCTCTGTTTCACCCAGTGCATCCAGTAGCTG  
GTGAAGGTATAGCCTGAAGCCTTGCAGGACATCTTCACTGAAGCCCAGGCC  
TCACCAGCTCAGGCCCAGACTCCACCAGCTGCACCTCGAGGGAAGATCTAG  
AGGAACCACCTTTGATTTCCAGCTTGGTCCCCCCTCCGAACGTGTACGGAAT  
CTCCCAACTGTGCTGACAGTAATATGTTGCAGTATCCTCCTCCTCCACAGGA  
TGGATGTTGAGGGTGAAGTCTGTCCCAGACCCACTGCCACTGAACCTGGCA  
GGGACCCAGATTCTAGGTTGGATGCATACTTGATGAGGAGTTTGGGTGGC  
TGTCTGGTTTCTGTTGGTACCAGTGCATATAACTATAGCTAGATGTACTION  
CACTTTGGCTGGCCCTGCATGAGATGGTGGCCCTTGCCCCAGAGATACAGC  
TAAGGAAGCAGGAGACTGTGTCATCACAATGTCGAGCTCGGCCGGCTGGGC  
CGCATAGAAAGGAACAACCTAAAGGAATTGCGAATAATAATTTTTTTCACGTT  
GAAAATCTCCAAAAAAAAGGCTCCAAAGCTTGGCGTAATCATGGTCATAGC  
TGTTTCCTGTGTGAAATTGTTATCCGCTCACAATCCACACAACATACGAGC  
CGGAAGCATAAAGTGTAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCAC  
ATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCCGGC  
CACCTGCATTAATGAATCGGCCAACCCCGCGGGGAAAGGCGGTTTGCAT  
TGGGCGCTCTTTCCGCTTCTCGCCAATGAACCCGTGCCCTCGGTCGTTTCG

CTGCCGGCGAAAGCGGTTAATCCAGCCTCACCTCAGAGGCGGTTATNNNGG  
TTATTCNACCAAGAANNNNNGGGNNTAACCCGCGGAGAGNANGAANNAN  
TTGTGTTGN-3'

## APPENDIX - F

### BBTI GENE SEQUENCE

5' ggatcctacagcgtgcaagcaccgcagaaacaacaaaaaacacccgggaaatccaagcagctgtgcaaccggtcc  
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ccactgggcaaagcgcacgaggaggaggaggaaggggtgaactcgaagccagcaggaggcgggtggccgtgctgcgac  
cagtgcggcattgcaccaggtcgcagccgccgatatgcgagtgaggacacgtcgcagaccggctgccacccggcttg  
caaggcgtgcgccctgtccatctccgacggcctctcgtgtgcaaggacaagatcgtcaactctgcaagcggcgtgcaccc  
gtcgtactgatgatgatgctgactataatcacttaattaggcaataaaccttgccaataaataattccgttcggagctccat  
ttgatctttctcaagatgcagacgacagggtttaataatcaaggcgtgcttgcttgcataaaaaaaaaaaatctgagaaaga  
tgagctctagagatct-3'

## APPENDIX - G

### CDI GENE SEQUENCE

5' ggatcctacaatataccaatcaatgatgaagtgtttatTTTTgttatgtctgtgtttgtttccattgggtgtttcatcaagttca  
ctcccaaatcccattgaattaccgagtgcttctcctaaacctaatccgacttgacacaaatggtaacgaacttaacctaattc  
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gttatgttgaataaacacatgctaactatctatatatTTtagcatgtcttcttaataaaattggagtgtttgcttatataaaaa  
aaaaaaaaatctgagaaagatgagctctagagatct-3'

## APPENDIX - H

### OCPI GENE SEQUENCE

5'ggatcctacgcattcgctagccacgccgctccgctcaggccgagggcgcacgcgcagggggagaaggggaggagaaga  
tgtcgagcgacggaggccggtgcttggcggcgtcgagccggtggggaacgagaacgacctccacctcgtcgacctgcc  
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tagagatct-3'

## APPENDIX - I

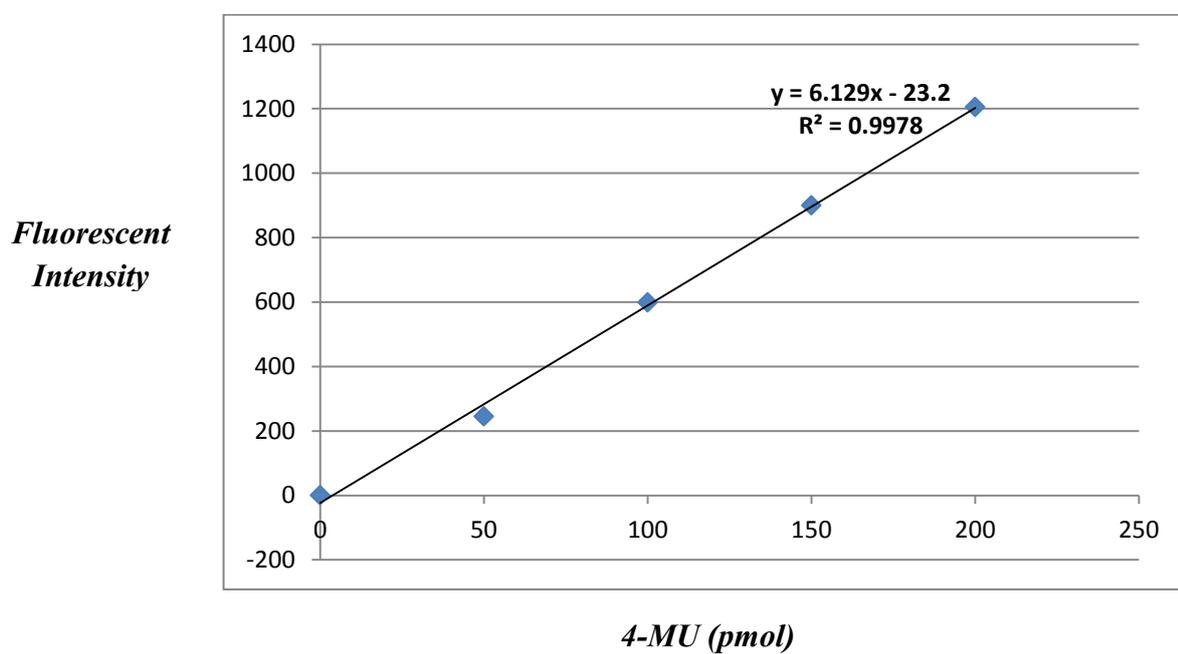
### 4-MU STANDARD MEASUREMENTS

0.2M Carbonate buffer (ml)	1 $\mu$ M 4- MU stock ( $\mu$ l)	<b>Sample conc.(X) (nM)</b>	Fluorescence Reading replicate1	Fluorescence Reading replicate2	<b>Average</b>
2.0	0	<b>0</b>	0	0	<b>0</b>
1.9	100	<b>50</b>	250	240	<b>245</b>
1.8	200	<b>100</b>	600	598	<b>599</b>
1.7	300	<b>150</b>	905	894	<b>899.5</b>
1.6	400	<b>200</b>	1210	1200	<b>1205</b>

## APPENDIX - J

### 4-MU STANDARD CURVE

*Standard Dilution of  
4-Methylumbelliferone and  
Relative Fluorescent Intensity*



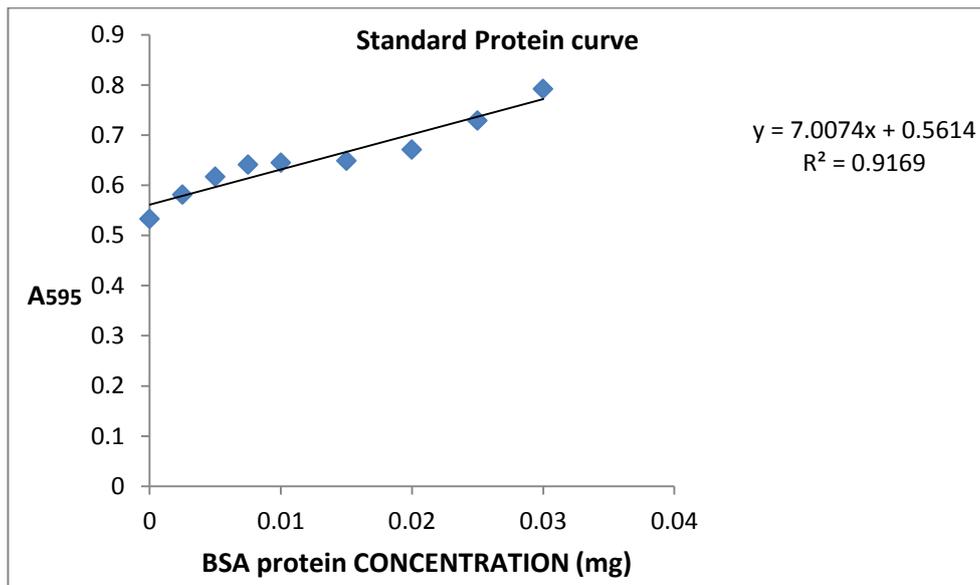
## APPENDIX - K

### OD VALUES FOR DIFFERENT BSA CONCENTRATIONS IN PROTEIN STANDARD CURVE ASSAY

<b>BSA conc. (mg)</b>	<b>OD replicate1</b>	<b>OD replicate3</b>	<b>OD replicate 3</b>	<b>Average</b>
<b>0</b>	0.537	0.534	0.529	<b>0.533</b>
<b>0.0025</b>	0.577	0.581	0.585	<b>0.581</b>
<b>0.005</b>	0.617	0.619	0.615	<b>0.617</b>
<b>0.0075</b>	0.643	0.639	0.642	<b>0.641</b>
<b>0.01</b>	0.645	0.647	0.643	<b>0.645</b>
<b>0.015</b>	0.652	0.649	0.647	<b>0.649</b>
<b>0.02</b>	0.675	0.668	0.671	<b>0.671</b>
<b>0.025</b>	0.725	0.736	0.728	<b>0.729</b>
<b>0.03</b>	0.793	0.799	0.785	<b>0.792</b>

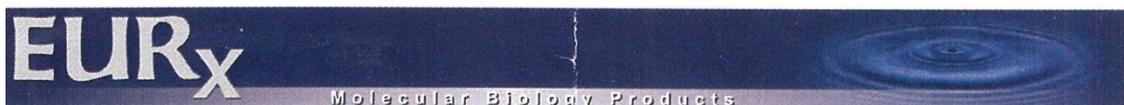
## APPENDIX - L

### BRADFORD STANDARD CURVE



## APPENDIX - M

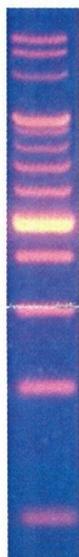
### DNA LADDER 100BP



#### Perfect™ 100 bp DNA Ladder

Cat. No.	Size
E3134-01	50 µg
E3134-02	250 µg

**Storage Conditions:** Store at +4°C.  
For long-term storage, store at -20°C.



DNA ladder with 100 bp increments for sizing small-to-medium DNA fragments.

#### Description:

- Ideal for sizing linear double-stranded DNA fragments from 100 to 2500 bp.
- Contains 13 bands with fragments of the following sizes: 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500 bp.
- Bands at 500 and 1000 bp are three times brighter for easy reference on agarose gels.
- Can be 5'-end labeled with radioisotopes and T4 Polynucleotide Kinase for visualization by autoradiography after a dephosphorylation step.

#### Storage Buffer:

10 mM Tris-HCl (pH 8.0 at 22°C), 1 mM EDTA, dye.

#### Loading:

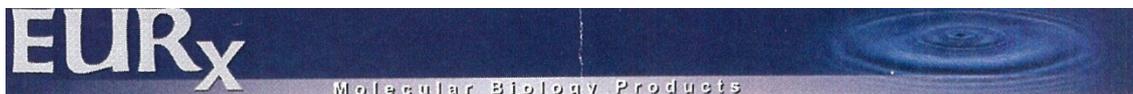
The recommended amount of size marker to load on a gel is 2-5 µl per lane depending on a gel type and size of well.

#### Concentration:

The Perfect™ 100 bp DNA Ladder is supplied at 125 µg/ml in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA.

5 µl load would load 625 ng DNA  
2500 bp 25 ng,  
2000 bp 20 ng,  
1500 bp 15 ng,  
1000 bp 70 ng,  
900 through 600 bp 30 ng per band,  
500 bp 250 ng,  
400 through 100 bp 30 ng per band.

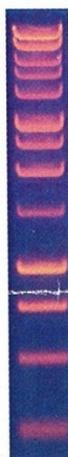
## DNA LADDER 1 KB



### Perfect Plus™ 1 kb DNA Ladder

Cat. No.	Size
E3131-01	100 µg
E3131-02	500 µg

**Storage Conditions:** Store at +4°C.  
For long-term storage, store at -20°C.



DNA ladder with 0.25-1 kb increments for sizing medium-to-large DNA fragments.

#### Description:

- Ready to use.
- Ideal for sizing linear double-stranded DNA fragments from 0.5 to 10.0 kb.
- Contains 13 bands with fragments of the following sizes: 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0, and 10.0 kb.
- Bands at 1.0 and 3.0 kb are three times brighter for easy reference on agarose gels.

#### Storage Buffer:

10 mM Tris-HCl (pH 8.0 at 22°C), 1 mM EDTA, dye.

#### Loading:

The recommended amount of size marker to load on a gel is 5-10 µl per lane depending on a gel type.

#### Concentration:

The Perfect Plus™ 1 kb DNA Ladder is supplied at 100 µg/ml in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA.

5 µl load would load 500 ng DNA.

- 10 kb 60 ng,
- 8 kb 50 ng,
- 6 kb 25 ng,
- 5 kb 15 ng,
- 4 kb 25 ng,
- 3 kb 60 ng,
- 2.5 kb 30 ng,
- 2 kb 35 ng,
- 1.5 kb 20 ng,
- 1 kb 80 ng,
- 0.75 kb 45 ng,
- 0.50 kb 25 ng,
- 0.25 kb 30 ng.