STABILITY, FUNCTIONALITY AND BIOSAFETY OF *NICOTIANA TABACUM* EXPRESSING ANTI-CMV ANTIBODIES

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Abstract:

Cucumber Mosaic Virus (CMV) is a significant plant pathogen affecting various crops and plants in Malaysia. A single chain variable fragment (scFv) anti-CMV antibody was successfully developed via a scFv library constructed with mRNA from the spleen cells of a CMV coat protein-immunized mouse and transformed by *Agrobacterium tumefaciens* into tobacco plants (*Nicotiana tabacum* L. cv. White burley).

In this study, the performance of primary transformants and 3 successive generations of *Nicotiana tabacum* expressing anti-CMV scFv were evaluated. An overall of 20% reduction in seed germination was observed as compared to wild type tobacco. All 4 generations did not exhibit any unusual phenotype other than delayed flowering times. The presence of anti-CMV scFv transgene in all 4 generations was detected by polymerase chain reaction (PCR) and confirmed via southern hybridization. Western Blot analysis showed low levels of detectable expressed anti-CMV scFv transgene in T_1 and T_2 generations. The binding activities of the expressed scFv were then evaluated using ELISA and Dot Blot Assay. Almost no functional activity of trasngenes and no expressed genes were detected in T_3 generation.

In a challenge assay, early disease symptoms including leaf mosaic and chlorosis were observed on wild type and sensitive transgenic plants 2 weeks after inoculation with CMV.

A computer simulation study was carried out via the AutoDock program to reveal the potential binding interaction of anti-CMV scFv to CMV.

In compliance with the Malaysian Biosafety Act, a pilot framework for risk assessment and risk management protocol was developed in this study.

Abstrak:

Virus Mosaik Ketimun merupakan patogen tumbuhan yang menjangkiti pelbagai tumbuhan di Malaysia. Antibodi scFv Virus Mosaik Ketimun berjaya dihasilkan melalui perpustakaan scFv yang dibina menggunakan mRNA bahagian limpa tikus yang telah diimunkan dengan protein kot Virus Mosaik Ketimun. Antibodi tersebut kemudian dibawa oleh *Agrobacterium tumefaciens* ke dalam pokok tembakau *Nicotiana tabacum* L. cv. White burley) melalui proses transformasi.

Dalam kajian ini, prestasi tumbuhan induk *Nicotiana tabacum* yang mengekspreskan antibodi scFv Virus Mosaik Ketimun dan 3 generasi seterusnya diuji. Secara keseluruhan, didapati bahawa terdapat pengurangan 20% dalam percambahan benih jika berbanding dengan tembakau kawalan (jenis liar). Keempat-empat generasi tidak menunjukkan sebarang fenotip luar biasa kecuali masa berbunga dilambatkan. Kehadiran antibodi scFv Virus Mosaik Ketimun pada semua generasi telah dikesan dengan Polymerase Chain Reaction (PCR) dan pengesahan gen dibuat dengan Southern Hybridization. Analisa Western Blot menunjukkan antibodi scFv Virus Mosaik Ketimun yang diekspres adalah rendah pada generasi T_1 dan T_2. Aktiviti pengikatan antibodi yang diekspres diuiji dengan ELISA and Dot Blot. Didapati bahawa tiada aktiviti berfungsi ataupun gen yang diekspres dikesan dalam generasi T_3.

Apabila tumbuhan transgenik dicabar dengan Virus Mosaik Ketimun, gejala- gejala penyakit seperti daun mosaik dan klorosis telah dikesan.

Kajian simulasi komputer dibuat malalui program AutoDock untuk menguji potensi interaksi antara antibodi scFv Virus Mosaik Ketimun dengan Virus Mosaik Ketimun.

Untuk mematuhi Akta Biokeselamatan, rangka kerja untuk protokol taksiran risiko dan protokol pengurusan risiko telah dibentangkan dalam eksperimen ini.

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List of abbreviations:

ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
BVA	Biological Variation Analysis
B.C	Before Christ
cDNA	Complementary DNA
cm	Centimetre
CTAB	Cetyl trimethylammonium bromide
dH_2O	Distilled water
DIG	Digoxigenin
dNTP	Deoxynucleotide triphosphate
Dr.	Doctor
DTT	Dithiothreitol
E-value	Expectation value
EDTA	Ethylenediaminetetraacetic acid
g	Gram
kg	Kilogram
L	Litre
m	Metre
MALDI	Matrix-assisted laser desorption/ionisation
mRNA	Messenger RNA
MW	Molecular weight
No.	Number

PR	Pathogen-related
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

1.0 Introduction

1.1 General Introduction

The cloning and expression of plantibodies has enormous potential for producing transgenic, pathogen resistant plant varieties (Whitelam and Cockburn, 1997). The first instance of successful research where part or whole antibodies were expressed in model species such as tobacco resulted in the transgenic plants having improved resistance to artichoke mottle crinkle virus (Tavladoraki *et al.*, 1993). Many local economically important plant viruses are sufficiently well studied. As such, the essential genetic sequence information for these viruses is readily available for application using this technology. Additionally, in the case of some viruses which are easily purified, such information may not be necessary for the production of antibodies.

The production, cloning and analysis of these antibodies in local agronomically important plants will not only potentially produce resistant varieties and will also enhance the development of antibody and transgenic plant technology. In addition, insights into mechanisms of viral pathogenecity and plant resistance in plants can be obtained. Transgenic plants expressing scFv antibodies are also a potential means of antibody production for use in plant pathogen diagnostics. This has the advantage of being less controversial and more cost effective in comparison to the use of laboratory animals or cell cultures.

A combinatorial scFv library against the coat protein of cucumber mosaic virus, a major pathogen of *Solanaceous* in Malaysia was successfully developed by Chua *et al.* (2003). A functional recombinant scFv antibody was isolated and characterised by sequencing and molecular modeling. The construct was then cloned into a plant expression vector and transformed via *Agrobacterium tumefaciens* into tobacco plants.

A series of analytical tools are available to characterize transgenic plants at the DNA, RNA and protein level (Sambrook *et al.*, 1989). Every detection method has its own advantages and limitations.

1.2 Objectives of study

The overall aim of this study was to evaluate the stability and functionality of the *Nicotiana tabacum* expressing anti-CMV scFv antibodies. This study proposed to test the hypothesis that anti-CMV scFv antibody is stable, functional and confer protection against CMV. Additionally, biosafety framework can be developed to ensure future application of the technologies in the field.

The specific objectives of the study included,

1. To generate of T_1, T_2 and T_3 trangenic plants
2. To perform molecular analysis of transgenic *Nicotiana tabacum* plants at DNA and protein level
3. To carry out Bioassay test of anti-CMV scFv antibodies for resistance to CMV
4. To perform molecular Docking studies on the interaction between the recombinant scFv and CMV
5. To development of a framework for Risk Assessment and Risk Management Protocols for transgenic plants expressing anti-CMV scFv antibodies

2.0 Literature Review

2.1 History of Plant Pathology

Plant diseases have been a constant fixture that has plagued mankind since the dawn of agriculture. Historical luminaries like Aristotle wrote about plant diseases in 350 B.C. and Shakespeare mentions wheat mildew in one of his plays. Naturally there was great interest to discover the cause of plant diseases, but no breakthroughs were made until the 19th century. The fundamental shift in understanding began with the publication of a book by Heinrich Anton de Bary (1887), who was widely considered to be the founding father of modern plant pathology. There had been description of fungal diseases and nematodes known to cause plant diseases in the 18th century, but the prevailing belief was that plant diseases arose spontaneously from decay (Kutschera and Hossfeld, 2012). De Bary's contributions disproved the spontaneous generation theory and introduced the germ theory of disease. The Great Irish Potato Blight in the 19th century further spurred scientists to research plant pathology. Armed with this new insight from de Bary, the field of plant pathology took off and eminent scientists like Louis Pasteur and Robert Koch made important discoveries regarding crop diseases. In the 20th century, further advances were made. One of the crowning achievements during this period was the Nobel Prize that was awarded to W.M. Stanley for his work on the tobacco mosaic virus (Lucas *et al.,* 1992).

Currently, 11 organism groups have been identified to cause catatrophic plant diseases: parasitic angiosperms, fungi, nematodes, algae, oomycetes, plasmodiophromycetes, trypanosomatics, bacteria, phytoplasmas, viruses and viroids (Strange 2005). With this wide range of pathogens it is therefore unsurprising that even with the advent of modern methods of plant disease control, plant diseases remain a major threat to world food security and to the economy of countries dependent on agriculture.

Viruses in particular play a significant role in plant diseases. Each year, plant viruses cause an estimated USD60 billion loss in crop yields worldwide (Plant virus, Wikipedia). To date, there are approximately 800 species of plant viruses that have been discovered (Brown *et al.*, 2012). Recently, the *Plant Molecular Pathology* journal published a review of the 10 most scientifically or economically important viruses. They are, in rank order, (1) Tobacco mosaic virus, (2) Tomato spotted wilt virus, (3) Tomato yellow leaf curl virus, (4) Cucumber mosaic virus, (5) Potato virus Y, (6) Cauliflower mosaic virus, (7) African cassava mosaic virus, (8) Plum pox virus, (9) Bromemosaic virus and (10) Potato virus X (Scholthof *et al.*, 2011).

2.2 Cucumber Mosaic Virus

In nearly a century since its first discovery (Doolittle, 1916), the Cucumber Mosaic Virus (CMV) has been reported to infect over 1200 species of hosts, including members of 100 plant families (Mochizuki and Ohki, 2012). It possesses one of the broadest host ranges of any known virus. This is due to its ability to adapt rapidly and successfully to new hosts and environments (Roossinck, 2002). Recently, CMV has been nominated by the international plant virology community as one of the top ten most scientifically/economically important plant viruses (Scholthof *et al.*, 2011).

CMV belongs to the genus Cucumovirus of family Bromoviridae (Roossinck *et al.*, 1999) with a molecular weight in the range of 5.8 to 6.7 million (18 percent RNA and 82 percent protein). It consists of three single-stranded messenger sense RNAs (RNA 1, 2 and 3) and two subgenomic RNAs (RNA 4A and RNA 4). RNA 1 and RNA 2 encode components of the replicase complex with 1a and 2a proteins, associated with putative helicase and polymerase activities respectively. The bicistronic RNA 3 encodes the movement protein (MP) and coat protein (CP), and the latter is expressed from subgenomic RNA 4. In addition, the 2b protein is expressed from 3′-proximal sequences

of RNA 2 via subgenomic RNA 4A. This protein determines its pathogenicity and plays a role in the long-distance movement of CMV (Gal-On *et al.*, 2000; Roossinck, 2001). Complete nucleotide sequences of 15 strains and more than 60 sequences of coat protein (CP) are available in GenBank. The phylogenetic analysis study on the entire genome of CMV has revealed important information about the evolutionary history of this group of viruses (Roossinck, 2002).

Numerous strains of CMV have been characterised from all parts of the world with different properties and characteristics such as the diversity of affected hosts, manifestation of symptoms and a variety of transmission methods (Agrios, 1978; Francki *et al.*, 1979). Recently the M-strain of Cucumber mosaic virus (M-CMV) has been shown to be highly virulent to tobacco plants (Lu *et al.*, 2012). In most host plants, CMV causes systemic infection with symptoms of leaf mosaic or mottling, chlorosis, ringspots, stunting, reduction of leaf laminae, and leaf, flower and fruit distortion (Kaper and Waterworth, 1981). These symptoms do not affect tissues and organs that have developed prior to infection; only newly developed cells and tissues after the viral infection are affected (Agrios, 1978). Once the plant is infected it will not recover. In 1997, Kaplan *et al.* observed a phenomenon known as cyclic mosaic symptom expression in tobacco plants. Also documented by Hull (2002), CMV causes mosaic diseases. Lu *et al.* (2012) discovered that photosynthesis, pigment metabolism and plant-pathogen interaction were involved in systemic symptom development in tobacco plants.

CMV disease is spread primarily by aphids, cucumber beetles, humans (during the cultivation and handling of the affected plants) and also mechanically (Francki *et al.*, 1979). In the field, CMV is transmitted by aphids in a non-persistent manner (Jacquemond, 2012). In a recent study using electrical penetration graph methodology,

it is found that higher proportions of aphids showed sustained phloem ingestion on CMV-infected plants when compared to mock-inoculated plants. CMV infection has also been demonstrated to foster aphid survival (Ziebell, 2011). Transmission through seeds and parasitic plants occur at varying degrees. The virus has been reported to stay dormant in certain perennial weeds, flowers and crop plants during winter, only to be transmitted by aphids to susceptible crop plants when spring arrives (Agrios, 1978). In most cases of CMV transmission from seeds of infected plants, the presence of virus was detected during symptom development by the germinated plants (Jacquemond, 2012), though it has been reported that plants infected through seeds can be asymptomatic (Ali and Kobayash, 2010).

CMV attacks a great variety of vegetables, ornamentals and other plants, making it one of the most important viruses for its impact on the economy. Each year, additional hosts of CMV and new diseases are discovered. Prevention of CMV infection in plants has been challenging. It is perhaps justified that CMV is one of the highest placed in terms of scientific importance, as a search of the ISI WEB of Science database in 2011 yielded counts of 1258 for papers with CMV viruses. Though resistance has been found in some varieties of vegetables and flowers, the effectiveness of management methods against all isolates of CMV leaves much to be desired (Agrios, 1978). From a broad perspective, CMV weed hosts should be eradicated from cultivated crop fields to reduce the incidences of infection (Rist and Lorbeer, 1989). The use of chemical controls such as pesticides on hosts and weeds and insecticides on aphids has shown to be only mildly effective in certain situations. Coupled with the fact that management methods are still wanting, there is considerable scientific interest in finding effective ways to control CMV. With the advancement of genetic engineering, the creation of transgenic plants which are resistant to CMV offers the best hope for durable resistance to CMV.

2.3 Current strategies in development of CMV resistant plants

2.3.1 Introduction

In 1983, the successful incorporation of alien genes into plant cells opened up new horizons for the development of virus resistance in plants. Since then, a variety of approaches have been applied to confer virus resistance in plants and some have proven to be remarkably successful. The concept of pathogen derived resistance (PDR) was introduced by Sanford and Johnson in 1985, immediately after the announcement of stable transformation of a plant with a viral CP (Bevan *et al.*, 1985). It was proposed that a portion of a pathogen's own genetic material could be used for host defence against the pathogen itself, as it was believed that non-functional forms of certain pathogen-derived molecules could interfere with virus replication, assembly or movement (Sanford and Johnston, 1985). Over the years, several effective strategies of PDR have been exploited; namely resistance mediated by viral coat protein (CP), viral replicase, post-translational gene silencing (PTGS) and satellite RNA (Lin *et al.*, 2007).

Apart from that, some efforts have also been devoted to resistance derived from non-pathogen sources, including ribosome inactivating proteins, ribonucleases, ribozymes, pathogenesis related proteins and plant expressing antibodies against viral proteins (Morroni *et al.*, 2008). Of these, the expression of antiviral antibodies has been the most prominent. Current knowledge on PDR and non-PDR strategies to combat CMV is briefly described below.

2.3.2 Coat Protein-mediated resistance (CPMR) to CMV

The feasibility of coat protein-mediated resistance (CPMR) was first demonstrated by Powell-Abel *et al.* (1986). CPMR to CMV was reported in the following year (Tumer *et al.*, 1987). This technique, which relies on the expression of CP to block the progression of viral infection processes, has been widely used to create

CMV resistance in tobacco, cucumber, tomato, melon, squash and pepper; some of which have been further tested in both laboratory and field for resistance levels (Fuchs *et al.*, 1996; Gielen *et al.*, 1996; Jacquemond *et al.*, 2001; Tricoli *et al.*, 1995). Numerous studies have been carried out on CMV-CP resistant transgenic plants for the past twenty years. As summarised in the review by Morroni *et al.* (2008), the range of resistance obtained is dependent on the donor strain, the challenging strains and the plant species (Table 2.1).

Two decades of research have yet to provide a total understanding on the molecular mechanism that governs CPMR. The most widely accepted hypothesis so far is that transgenic CP prevents viruses from undergoing co-translational disassembly, the early event of infection (Shaw *et al.*, 1986). In the case of CMV, Okuno *et al.* (1993) documented a blockage by transgene-derived CP at a somewhat later stage in the infection cycle, resulting in the inhibition of the viral transit through the plant. Most recently, Pratap *et al.* (2012) has developed transgenic tomato plants containing the coat protein (CP) gene of CMV of subgroup IB through *Agrobacterium*-mediated transformation. They discovered that the CP of CMV subgroup IB strain showed a significant level of resistance in transgenic tomato plants against the CMV strain.

To date, it is still difficult to identify the ideal CP gene that is most effective to combat the virus, due to the inconsistency in the results shown. This is probably due to the involvement of multiple mechanisms. Therefore, more studies are needed in order to have a clear picture of the effectiveness of any transgene in conferring CP resistance.

Table 2.1: Summary of transgenic plants for CMV-CP resistance

Host species	Donor virus strain (subgroup)	Challenging virus strain (subgroup)	Degree of resistance (%)[1]	Reference
Tobacco	D (I)	D (I)	40 to 90	Tumer *et al.* (1987)
	D (I)	C (I)	0 to 100	Cuozzo *et al.* (1988)
	WL (II)	C Chi (I) WL (II)	100 65 to 85 75 to 85	Namba *et al.* (1991)
	C (I)	C Chi (I) WL (II)	100 85 55	Quemada *et al.* (1991)
	O(I)	B	40	Yie *et al.* (1992)
	O (I)	O, Y (I), TAV[2]	0 to 100	Nakajima *et al.* (1993)
	Y (I)	Y, O (I), Pepo, CF, FT	0 to 100	Okuno *et al.* (1993)
	CP91/367, SB91/366 (I)	CP91/367, SB91/366 (I) LU91/166 (II)	0 to 66 37 to 83	Rizos *et al.* (1996)
	C (I)	3 strains (I) 5 strains (II)	0 to 80 0 to 80	Singh *et al.* (1998)
	R (II)	R (II)	Recovery	Jacquemond *et al.* (2001)
Tomato	WL (II)	Chi (I) WL (II)	80 to 100 91 to 100	Xue *et al.* (1994)
	WL (II)	9 strains (I) 3 strains (II)	100	Provvidenti and Gonsalves (1995)
	ZU (I)	CMV-117F(I), CMV-ARN5(I), CMV-A (II) NI	50 to 100 45 to 100	Gielen *et al.* (1996)
	WL (II)	Fny (I)	100	Fuchs *et al.* (1996)
	D, 22 (I), PG (II)	22 (I), PG (II)	70 to 100	Kaniewski *et al.* (1999)
	D, 22 (I), PG (II)	22 (I), NI	0 to 100	Tomassoli *et al.* (1999)
Cucumber	C (I)	Cat (I), NI	86 to 100	Gonsalves *et al.* (1992)
Melon	WL (II)	Fny (I)	Delay	Gonsalves *et al.* (1994)
Squash	C (I)	C (I)	92 to 100	Tricoli *et al.* (1995)
Pepper	Kor	Kor	10 to 100	Shin *et al.* (2002)

NI – natural infection in the field.
[1]Percentage of the number of non-infected plants over total number of inoculated plants.
[2]Tomato aspermy virus (TAV) i.e. reported as Chrysanthemum mild mottle cucumovirus (CMMV) in the original paper.

Adapted from Reports of plants transgenic for Cucumber Mosaic virus (CMV) coat protein (CP) where resistance were analysed (Morroni *et al.,* 2008).

2.3.3 Replicase-Mediated Resistance (RMR) to CMV

Groundbreaking research using RNA dependent RNA-polymerases (RdRps) or RNA replicase to engineer virus resistance in plants was documented in 1990 by Golemboski *et al.* This technique relies on the expression of the polymerase gene or another viral gene which is associated with virus replication in transgenic plants that evoke the resistance mechanism, as explained by Palukaitis and Zaitlin (1997). In the case of resistance to cucumber mosaic virus (CMV), most studies have centered on truncated 2a protein (Anderson *et al.*, 1992). The mechanisms involved were targeting the virus replication at the single-cell level and limiting the virus from spreading cell-to-cell (Hellwald and Palukaitis, 1995).

The report by Morroni *et al.* (2008) detailed the degree of resistance for the CMV replicase component. In most cases, the resistance obtained was promising (Zaitlin *et al.*, 1994; Hellwald and Palukaitis, 1994; Gal-On *et al.,* 1998). In the case of RMR engendered by either CMV RNA 1 (Canto and Palukaitis, 1998) or a defective 2a polymerase gene encoded by CMV RNA 2 (Carr *et al.*, 1994), virus replication was greatly reduced but not totally suppressed.

With the RMR technique, CMV replication and movement in plants may be restricted, but the mechanism involved may not be immediately apparent (Canto and Palukaitis, 1999). The interpretation of the role of (modified) replicase proteins or their transcripts are still a topic of ambiguity despite derived transgenes being potent sources of resistance. In 2011, Azadi *et al.* reported that the CMV-GDD replicase gene confers effective protection against CMV as their results implied increase levels of resistance to CMV-O strain in *Lilium*.

2.3.4 Resistance to CMV mediated by PTGS

Post-transcriptional gene silencing (PTGS) is a RNA degradation mechanism that can be induced by viruses. In plants, PTGS is required for innate immunity regulating virus accumulation. Two types of small RNAs, i.e. small interfering RNA (siRNA) and microRNA (miRNA) have been characterised in PTGS. Both are believed to be involved in conferring virus resistance (Lin *et al.*, 2007). In 2000, Wang *et al.* reported that the inverted-repeat transgene encoding hairpin RNA could enhance PTGS-mediated resistance. Promising results have also been obtained in studies using inverted-repeat CP or RNA 2 sequence of CMV (Kalantidis *et al.*, 2002; Chen *et al.*, 2004). A new study by Kavosipour *et al.* (2012) has confirmed that 2b- derived PTGS is an effective plant defence mechanism against CMV and can be used in breeding programs.

A more recent attempt to engineer resistance to CMV focused on transgenic artificial miRNA. This may opens up the possibility of developing even smaller transgenes that target specific pathways of the small RNA regulatory network (Niu *et al.*, 2006). A study by Qu *et al.* (2007) discovered that the transgenic expression of artificial miRNA target sequence of the 2b protein effectively reduced the expression and activity of 2b protein and conferred resistance to CMV. In 2012, Qu *et al.* reported that artificial miRNA-mediated virus resistance is efficient and superior to the long viral RNA-based antiviral approaches. They discovered that properly selected artificial miRNA sequences would have little chance to target the host plant genes or to complement or recombine with other invading viruses.

To date, PTGS-mediated is by far the most successful method to confer resistance, despite the fact that mechanisms of activation and maintenance are still not well understood.

2.3.5 CMV satellite RNA (satRNA) mediated resistance

Satellite RNAs (satRNAs) are viral parasites that depend on their helper virus for replication, encapsidation and dispersion (Roossinck *et al.*, 1992). CMV satellite RNAs (satRNAs) was first discovered in the 1970s as a result of the lethal tomato necrosis outbreaks in southern Europe (Simon *et al.,* 2004). So far**,** more than 100 CMV satRNA variants have been found to be associated with over 65 CMV isolates (Palukaitis and Garcia-Arenal, 2003). It was understood that the presence of satRNA attenuates the symptoms induced by CMV infection, and the presence of CMV-satRNA usually reduces the titer of helper virus (Gal-On *et al.*, 1995). These symptoms vary with the helper virus, host plant, and satellite. The technique of pre-inoculating plants with satRNA prior to infection with CMV has been widely used to protect plants from severe symptoms (Sayama *et al.,* 1993). Several reports were published on the case of CMV satRNA attenuated symptoms in some plants (Harrison *et al.*, 1987; Kim *et al.*, 1995; Kim *et al.*, 1997; McGarvey *et al.*, 1994).

The overall safety of such techniques were questioned in 1996 when Palukaitis and Roossinck reported spontaneous shifting of satRNAs from benign to necrogenic and the later phenotypes could rapidly dominate the satRNA population within the host. A subsequent report by Jacquemond and Tepfer (1998) on minor sequence difference distinguishing necrogenic from benign satRNAs was confirmation of the safety problems of this technique. The use of transgenic satRNAs has been in steep decline from that point onwards (Jacquemond and Tepfer, 1998).

Since then, due to the inherent dependence on helper virus, majority of studies have focused on characterizing various strains of satRNAs and their relationship to helper virus' symptom expression and origin (Hajimorad *et al.*, 2009; Hu *et al.*, 2009; Smith *et al.*, 2011). Satellite RNAs' replication was thought to be completely dependent on their helper virus until a recent report proved otherwise. Choi *et al.* (2012) revealed

that a variant of satellite RNA (satRNA) associated with Cucumber mosaic virus strain Q (Q-satRNA) has a propensity to localize in the nucleus and be transcribed, generating genomic and anti-genomic multimeric forms when expressed autonomously in the absence of helper virus.

2.4 Plantibodies

2.4.1 Introduction

Antibodies are products of the vertebral immune system whose primary function is the assist in eliminating pathogens from the body. Antibodies perform this function by recognizing and binding to pathogen-specific antigens. Plantibodies are defined as plant-produced antibodies and was first produced by Hiatt and colleagues during the late 1980s (Hiatt *et al.*, 1989). This was a major breakthrough as it was proven that plants could express and assemble functionally active antibodies. Plants have several advantages versus other methods of producing antibodies such as no culture media and bioreactors are required and less possibility of microbial contamination when compared to antibodies derived from animal systems.

Plantibodies can be used to provide antibody-mediated resistance to pathogenic infections and to function as bioreactors to produce antibodies for medical or industrial use (Stoger *et al.*, 2002). To create plants that are resistant to pathogens, the following criteria have to be fulfilled: cloning of the desired antibody, efficient expression of the antibody, antibody stabilization and targeting to the appropriate cellular compartments (Schillberg *et al.*, 2001). In terms of using plants as biofactories, the first recombinant protein to be synthesized *in planta* was the human growth hormone (Barta *et al.*, 1986). Since then, many other proteins have been produced by plant systems and some of them have been commercialized (Hood *et al.*, 1997; Witcher *et al.,* 1998). There are several methods to introduce antibody genes into plants – transformation by *Agrobacterium* and

particle bombardment have been successfully used (Stoger *et al.*, 2002). The recombinant protein can be deposited throughout the plant or in specific organs. The deposition and storage of antibody molecules in seeds of various crop plants has been demonstrated (Chester and Hawkins, 1995; Fielder *et al.*, 1997; Stoger *et al.*, 2002).

In 2011, Safarnejad *et al.* published a review on the methods used to create and express pathogen-specific antibodies and experiments that have established and developed the principle of antibody-mediated disease resistance in plants. Table 2.2 details the recombinant antibody-mediated disease resistance in plants from 1993 – 2011 (Safarnejad *et al.*, 2011).

2.4.2 Single chain Fv (scFv) antibodies

Single chain Fv (scFv) antibodies consist of variable light chain and variable heavy chain domains of an antibody molecule fused by a flexible peptide linker (Bird *et al.*, 1988). scFv antibodies retain full antigen-binding activity but lack specific assembly requirements. Uses of scFv antibodies include diagnostics and therapeutics (Fielder *et al.*, 1997). scFv antibodies have been successfully synthesized in plants and plant cells as well as in bacteria. The less stringent requirements for folding and assembly, and also the ability to penetrate tissues effectively due to their small size make them suitable for expression in various intracellular compartments of plant cells (Owen *et al.*, 1992; Safarnejad *et al.*, 2011)

Table 2.2: Recombinant antibody-mediated resistance against plant diseases

Year	Disease agent	Targeted protein	Transformed species	rAb format	Cellular localization
1993	ACMV	Coat protein	Nicotiana benthamiana	scFv	Cytosol
1995	TMV	Coat protein	N. tabacum cv. Xanthi	full size IgG	Apoplast
1997	BNYVV	Coat protein	N. benthamiana	scFv	ER
1998	TMV	Coat protein	N. tabacum cv. Xanthi	scFv	Cytosol
1998	Stolbur phytoplasma	IMP	N. tabacum	scFv	Cytosol
1998	Maize stunt spiroplasma	IMP	Zea mays	scFv	Cytosol
2000	TMV	Coat protein	N. tabacum cv. Petite Havana SR1	scFv	Plasma membrane surface
2000	PVY strains Y&D CYVV strain 300	Coat protein	N. tabacum cv. W38	scFv	Apoplast, cytosol
2001	TMV	Coat protein	N. tabacum cv. Samsun NN	scFv	Cytosol
2004	Fusarium oxysporum f. sp. matthiolae	Cell-wall bound proteins	Arabidopsis thaliana	scFv-AFP	Apoplast
2004	TBSV, CNV, TCV, RCNMV	RdRp	N. benthamiana	scFv	Cytosol, ER
2005	Stolbur phytoplasma	IMP	N. tabacum	scFv	Apoplast, cytosol
2005	CMV	Coat protein	N. benthamiana	scFv	Cytosol
2005	TSWV	Nucleoprotein	N. benthamiana	scFv	Cytosol
2006	PVY	NIa protein	Solanum tuberosum	scFv	Cytosol
2008	PLRV	P1 protein	S. tuberosum	scFv	Cytosol
2008	TSWV	Movement protein	N. tabacum cv. Petit Havana SR1	scFv	Cytosol
2008	F. asiaticum	Cell-wall bound proteins	Triticum aestivum	scFv-AFP	Apoplast
2009	GFLV, ArMV	Coat protein	N. benthamiana	scFv	Cytosol
2009	PVY	NIa protein	S. tuberosum	VH	Cytosol
2009	TYLCV	Rep	N. benthamiana	scFv-GFP	Cytosol
2010	CTV	p25 major coat protein	Citrus aurantifolia	scFv	Cytosol
2010	Sclerotinia sclerotiorum	Hyphal proteins	Brassica napus	scFv	Cytosol
2011	PPV	NIb protein	N. benthamiana	scFv	Cytosol, ER, nucleus

Adapted from Table 1 Recombinant antibody-mediated resistance against plant diseases (Safarnejad *et al.*, 2011)

2.4.3 Cloning, expressing and targeting of scFv antibodies in plants

Monoclonal antibodies are now widely used in disease diagnosis and therapy ever since hybridoma technology was developed in 1975 (Kohler and Milstein, 1975). Hybridoma technology results in the production of highly specific monoclonal antibodies; however the process is labour intensive and requires the use of expensive equipment. By comparison, phage display of antibodies has several advantages over hybridoma technology. Rapid antibody cloning and flexibility in selecting and modifying specific antibodies can be achieved using phage display. Apart from this, during library generation, all cloned heavy and light chain gene fragments from a donor are recombined and this permits the generation of novel specificities that cannot be found in the original donor. Phage display and panning process was first described in 1985 by Smith to prepare antibody displays used for isolating antibodies that bind with the greatest affinity to the target. In 2003, Chua *et al.* detailed an anti-CMV scFv gene produced from biopanning M13 phage display library. Later, libraries of scFvs that are pre-selected for cytosolic stability were constructed and used to generate stable scFvs that bind to Cucumber mosaic virus (CMV) (Villani *et al.*, 2005).

Expression systems should be established to allow high-level accumulation of antibodies in transgenic plants. Furthermore, if the harvested material is to be transported before processing, stable storage of antibodies in plant material is important. One method to optimize plant expression is to reduce degradation and improve folding conditions for antibody fragments in leaves and seeds. Known issues with scFv expression include very low or no expression of scFvs in the cytoplasm of plant cells. Directing the scFv through the secretory pathway into the extracellular space or retain them in the lumen of the ER is a potential way to overcome this issue. Research has also shown that when a KDEL sequence was included in the antibody gene construct, accumulation of scFv was increased significantly (Schouten *et al.*, 1996). This held true

for both the secreted (ER targeted) and cytosolic forms of the scFv. The KDEL sequence is hypothesized to protect the cytosolic scFv from proteolytic degradation or may confer protection to the protein through an interaction with the cytosolic side of the ER (Schouten *et al.*, 1996). In 2005, Villani *et al.* reported a high level of accumulation of anti-CMV scFv antibodies in *Nicotina benthamiana* plants, resulting in a high level of protection against CMV. The high accumulation level in cytosol may be due to the fact that certain scFvs have framework regions that contain important determinants of folding efficiency in the cytosol (Safarnejad *et al.*, 2009; Zhang *et al.*, 2008).

Fiedler and Conrad (1995) demonstrated that active scFv molecules can be targeted to other sections of the plant than only leaves. scFv was shown to accumulate in developing and ripe tobacco seeds. The antibody accumulated to 0.67% of total soluble protein in the seeds, and was stably stored for one year at room temperature (Fiedler and Conrad, 1995). This system therefore offers high expression levels along with long-term storage of the protein and does not appear to influence plant growth rate or seed development. Exact cellular location was not determined for the antibody, although the authors felt it may have accumulated in protein bodies of the seeds (Fiedler and Conrad, 1995). Determining the exact cellular location of the stored antibody and transferring the system to another crop, such as corn, would make this strategy valuable for the commercial production of scFvs (Fiedler and Conrad, 1995). In fact, this system would be even more valuable if long-term stable expression could also be achieved for full length antibodies. Specifically, such a system would be valuable for delivering large quantities of full length antibodies for passive immunization. For instance, it has been shown that full length antibodies can be assembled and accumulated in the roots of transgenic tobacco (Van Engelen *et al.*, 1994). If this technology could be utilized to obtain stable accumulation of these antibodies in edible tissues such as potato tubers, it

might allow for long-term storage and easy delivery of antibodies for immunotherapeutic applications.

2.5 Molecular and genetic analyses of transgenic plants

Various characterizations of transgenic plants at DNA, RNA or protein level are well documented in Sambrook *et al*. (1989). Several detection methods for transgenic plants are discussed in the following section. In molecular analysis, standard polymerase chain reaction (PCR) is one of the simplest and most convenient approaches. Standard polymerase chain reaction (PCR) methods have been utilized to detect the presence of recombinant DNA in transformed plants (Ingham, 2005). PCR amplification of transgenes are often taken as an indication of transgenic status of re-generants (Potrykus, 1991). Several conventional or multiplex PCR methods have been reported for qualitative analysis of transgenic samples (Padgette *et al*., 1995; Zimmermann *et al*., 1998; Matsuoka *et al*., 2001). Other PCR-derived technologies such as competitive PCR (Garcia-Canas *et al*., 2004) or real-time PCR (Terry and Harris, 2001; Rønning *et al.,* 2003; Windels *et al*., 2003; Hernandez *et al.,* 2004) allow the quantification of transgenes in a sample. Multiplex PCR has also been proposed as a test for several transgenic plants (Permingeat*, et al*., 2002; Germini *et al.,* 2004; Hernandez *et al*., 2005).

PCR-derived amplifications are the methods of choice to detect the presence of transgenes. Microarrays, also known as DNA chips, allow the analysis of multiple sequence targets in one single assay (Leimanis *et al*., 2006). The main advantages of DNA microarray technology are miniaturization, high sensitivity and screening throughput. Different DNA microarray approaches have been used in combination with multiplex PCRs: a multiplex DNA array-based PCR allowing quantification of transgenic maize in food and feed (Rudi *et al.,* 2003); a ligation detection reaction coupled with an universal array technology that allows for the detection of Bt176

transgenic maize (Bordoni *et al*., 2004) or five transgenic events (Bordoni *et al.,* 2005). A peptide nucleic acid array approach was developed for the detection of five transgenic events and two plant species (Germini *et al*., 2005). The use of fluorescent probes in these methods are costly and photosensitive, thus limiting the common use of microarrays for transgene detection. To avoid the drawback of fluorescent probes, a cost effective, highly sensitive, easy to use assay with reagents was developed (Leimanis *et al*., 2006). The arrays are solid glass supports containing, on their surface, a series of discrete regions bearing capture nucleotide probes that are complementary to the target nucleotide sequences to be detected (Zammatteo *et al*., 2000). After target DNA amplification in the presence of biotinylated nucleotides, amplicons are allowed to directly hybridize onto the arrays and are subsequently detected by a colorimetric system (Alexandre *et al.,* 2001).

For further confirmation of transgene presence, PCR-Southern hybridization can be performed. A labelled specific probe is hybridized with PCR products to check for the existence of a complementary sequence in amplified product with the transgene. Apart from this, Southern hybridization is useful to assess the number of independent insertions of transgene (Southern, 1975). However, this method is difficult to apply in the high-throughput screening of putative transformants (Ingham, 2005). The development of real-time quantitative PCR (qRT-PCR) methods for determining transgene copy number has overcome the limitations of standard PCR-Southern analysis (Beer *et al.*, 2001; Mason *et al*., 2002). qRT-PCR methods provide an accurate, quantitative and high-throughput approach for estimating transgene copy number from small amounts of sample. These assays can be conducted while putative transgenics are still in tissue culture. This allows for the selection of desirable transgenic events prior to expending the cost and resources required for transplantation to soil and propagation to maturity under greenhouse conditions (Ingham, 2005).

Many techniques exist for the analysis of gene expression at RNA level, in particular the quantification and localization of mRNA transcripts. This includes reverse transcription-polymerase chain reaction (RT-PCR) and northern hybridization. The technique most often used for detection of the transcript is Northern blot hybridization, which employs a transgene-specific labelled probe and a variety of detection mechanisms depending on the label used. Although this approach does not distinguish between translationally active and inactive messages, it is often used reliably to study the expression levels of various transcripts (Dean *et al*., 2002). Although northern blot analysis is effective for quantifying gene expression, reverse transcription-polymerase chain reaction (RT-PCR) is found to be more sensitive. RT-PCR reflects the transcription level of the introduced gene in transgenic plants. It uses standard PCR techniques but permits the comparison of transcript quantities between samples by co-amplifying the gene of interest with a housekeeping gene that acts as an internal control (Dean *et al.,* 2002). The accuracy of the results obtained by this method strongly depends on accurate transcript normalization using stably expressed genes, known as references. Statistical algorithms have been developed to help validate reference genes (Gutierrez *et al*., 2008).

Immunoassay is used for the detection and quantification of proteins introduced through genetic transformation of plants. It is based on the specific binding between an antigen and an antibody. Immunoassays can be highly specific and samples often need only a simple preparation before being analysed. Moreover, immunoassays can be used qualitatively or quantitatively over a wide range of concentrations (Tripathi, 2005). Western blot and ELISA (Enzyme-Linked Immunosorbent Assay) are typical protein-based immunoassays methods. These techniques are employed to assess the expression of the introduced gene.

Western blotting combines the resolving power of protein electrophoresis and the specificity of immunology in a rapid and sensitive format for the identification of expressed proteins (Lough *et al.,* 1998). Proteins resolved by electrophoresis are transferred to a nitrocellulose membrane. A primary antibody is bound to a specific antigen on the membrane and this antibody is detected using an enzyme-linked antibody. One-dimensional sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE) is most commonly used separation technique in Western Blotting (Smith, 1994).

The ELISA technique has been widely applied for evaluating, at the experimental stage, the expression level and functionality of the transgene. Most commonly used ELISA methods are the classical plate-based ELISA and the membrane-based lateral flow strips. In plate-based ELISA, the antigen-antibody reaction takes place on microtiter plates. The antigen and antibody react and produce a stable complex which can be visualised by the addition of a second antibody linked to an enzyme. The results can then can be measured photometrically (Tripathi, 2005). Bindler *et al*. (1999) highlighted the advantages and drawbacks of immunoassay methods used to detect transgenic plants. One of the major drawbacks of this technique is that it often fails to detect transgenic proteins expressed at a very low level or those that are degraded and denatured by thermal treatment (Laura *et al.*, 2002). It has been revealed that the accuracy and precision of ELISA can be adversely affected in complex matrixes. Commercially available antibodies have been reported to display poor binding affinity for the protein of interest (Laura *et al.*, 2002). Lateral flow strips are marketed as 'dipstick' procedure. It uses strips as opposed to microtiter plates to detect the presence of transgenic protein. This technique offers rapid and relatively ease of use and low cost, but it cannot quantify the protein of interest (Kole *et al*., 2010).

2.6 Biosafety Issues of Transgenic Crop Plants

2.6.1 Introduction

According to projections by the United Nations, world population exceeded the 7 billion mark in 2011 and is expected to reach 7.77 billion by 2020 (http://esa.un.org/wpp/Excel-Data/population.htm). This has obvious implications on food security as questions on whether food production can keep pace with population growth will inevitably be asked. This is not a new argument as Malthus (1826) has put forward his theory in the early 19th century that mankind would essentially starve once population growth outgrew agricultural output. The Green Revolution, which increased crop yields in the 1960s onwards through use of pesticides and fertilizers, modern irrigation and improved crop varieties, put paid to this theory as food output increased substantially. However, the world food crisis in 2007 – 2008 which sparked riots and unrest in many developing countries raised the spectre of food insecurity again. As Asia accounts for more than half of the world's population, the demand for food in this region is expected to be increasingly difficult to meet; governments and policy makers are now turning to biotechnology as one of the tools to alleviate this problem.

The manipulation of plant reproduction to propagate favourable traits has been going on for thousands of years in the form of selective and controlled breeding in crops and domesticated animals. The advent of modern biotechnology has resulted in a quantum leap in terms of accelerating and refining the genetic modification of organisms – giving rise to the creation of genetically modified organisms (GMOs) via recombinant DNA (rDNA) and other techniques. GMOs or transgenic organisms have the potential of giving higher yields through selection of favourable traits, reduced pesticide requirements due to improved pest resistance, increased utilization of marginal land, decreased water requirements as drought-resistant GMOs are engineered, enhance the shelf-life of crops, increase cost-effectiveness of production, improved nutritional

value of crops and function as vehicles to produce new pharmaceuticals and industrial products (Bhatia and Mitra, 1998; Singh *et al.*, 2006; Hug, 2008). These benefits could translate into sustainable food production and usher in a new Green Revolution.

2.6.2 Why do we need to regulate transgenic crop plants?

As with any new and rapidly advancing technology, there are always concerns on any potential adverse impacts, particularly with regards to its impact on human health and the environment. There is an ethical aspect to consider as well as genetic modification can be viewed as unnatural (Grumet and Gifford, 1998).

Several threats have been proposed: weediness, gene flow, threats to food safety, adverse impact on non-target organisms, pest resistance, negative impact on crop diversity and unknown long-term negative impact on the ecosystem (Bhatia and Mitra, 1998; Krainin, 2004). Weediness occurs when GMOs become more invasive and hardy than it would normally be in nature due to the novel traits that they possess. Gene flow occurs when there is a movement of genes from one population to another (Slatkin, 1985). This may have unpredicted consequences in the wild. In terms of threats to food safety, increased allerginicity due to introduction of novel proteins has been hypothesized (Bhatia and Mitra, 1998). Non-target organisms, i.e. organisms which were not intended to be affected by the genetic modification, could be impacted in the wild as well; for example predators of pests being affected by GMOs that produce insecticides. Apart from this, increased pest resistance due to selection pressure elicited by the novel traits presented by GMOs could cause increased pest resistance (Bhatia and Mitra, 1998). There is a case for diversity in the wild as different varieties of crops can respond differently to different stresses. Due to the unique nature and newness of biotechnology, the predictability of the effects of GMOs are not certain to be accurate, which is why rigorous testing systems and regulations have been put in place in many

countries. For the purposes of this review, the discussions that follow will concentrate on the regulation of transgenic plants.

2.6.3 Cartagena Protocol on Biosafety (agriculture)

In view of some of the risks proposed above, the United Nations' Parties to the Convention on Biological Diversity initiated negotiations to draft an agreement in 1995 to identify and mitigate the potential risks posed by GMOs. In 2000, the Cartagena Protocol on Biosafety was adopted (http://www.cbd.int) and came into force in September 2003. The Protocol comprises of 40 articles and is a comprehensive regulatory system which governs the safe transfer, handling and use of GMOs, with a particular focus on transboundary movements (Cartagena Protocol on Biosafety 2000). Some important aspects of the protocol are the Advanced Informed Agreement (AIA) contained in Article 7, risk assessments (Article 15) and capacity building (Article 22).

With regards to the AIA, any country planning to export GMOs needs to provide the importing country with detailed information regarding the GMO. The competent National Authority of the importing country has the option of either authorizing the shipment or rejecting it. An important point to note is that absence of consent does not denote approval. This AIA mechanism ensures that countries have the possibility of assessing any risks prior to importation.

Additionally the Protocol places strong emphasis on risk assessments. The aim of risk assessments is to identify and evaluate any potential negative effects of GMOs. The principles governing risk assessments are that risk assessment should be carried out in a scientifically sound and transparent manner and that risks should be assessed on a case-by-case basis. An important point to note is that lack of scientific data or consensus must not be interpreted as indicating acceptance of particular level of risk. (http://bch.cbd.int/protocol).

The Protocol demonstrates a level of pragmatism towards its implementation as developing countries, unlike developed countries, have resource constraints in terms effectively implementing all articles successfully. As such, Article 22 states that international cooperation should be in place to cooperate and share resources towards establishing a national biosafety system (Cartagena Protocol on Biosafety 2000).

To be successful, the Cartagena Protocol, like any global protocol, needs the active participation of each signatory country. Governments are crucial in terms of ensuring GMOs are sustainably developed and safely used. To this end, many countries have developed national biosafety regulations – unsurprisingly developed countries like the US and EU have led the way and many developing countries have followed suit. It is also important to note that while the US participated in the Cartagena Protocol negotiations, it is not a party to CBD, so the Cartagena Protocol does not apply to the US (Nuffield Council on Bioethics 2004). The following sections will review the biosafety regulations of some countries.

2.6.4 US, Canada and European Union Regulations on Biosafety (agriculture)

United States

The US, being at the forefront of developing biotechnology, was one of the first countries to establish biosafety protocols to control the use of GMOs. The US framework for regulating GMOs was established in 1986 and is called the Coordinated Framework. Instead of developing brand new legislation, the policy makers decided that the existing laws covering organisms developed via traditional genetic manipulation processes would be sufficient to address the risks posed by GMOs. It was felt that the existing laws would provide immediate protection and certainty for the biotechnology industry as opposed to developing new legislation (Jaffe, 2004). The task of regulating GMOs is mainly divided between three federal agencies: the United States Department

of Agriculture (USDA), the US Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA). The Federal Insecticide, Fungicide and Rodenticide Act grants the EPA authority over plants that produce their own pesticides like Bt corn. The EPA regulates pesticidal GMOs the same way chemical pesticides are regulated (Office of Science and Technology Policy, 1986). The USDA has authority over all other types of GMOs via the Plant Protection Act. One important point is that the Plant Protection Act does not extend to the commercialization stage – i.e. once the crop is commercialized, it is exempted from further regulation. This is an important difference with other countries (Cranor, 2004). The FDA's role is governed by the Federal Food, Drug and Cosmetic Act (FFDCA). The FDA is responsible for reviewing the potential health effects of non-pesticidal transgenic plants that produce pharmaceuticals. The USDA's Animal and Plant Health Inspection Service (APHIS) has authority to assess "the potential effects of non-pesticidal transgenic plants on other plants and animals in both agricultural and non-agricultural environments" (NRC, 2002). This creates a scenario where transgenic plants will not be regulated by all three agencies, but any of these three agencies have the power to issue a withdrawal order if the transgenic crop or its products pose a safety risk to consumers or to the environment (Lemaux, 2009).

The US principle in regulating transgenic plants is to screen the plants prior to environment release and commercial production. The reasoning behind this is that harmful substances are identified and prevented from mass exposure. The burden of proof that the GMO is safe for use would fall upon on the manufacturer or originator of the transgenic organism.

Under APHIS regulations, there are three different processes available to manufacturers to seek approval: "notification", "permitting" and "petitions for non-regulated status" (Jaffe, 2004). For a plant to qualify under the notification procedure,

the plant must not be deemed a "weed" according to a federal list, the DNA functions must be known and not result in disease; plus the DNA must be stable so the trait is inherited in a Mendelian fashion for at least 2 generations. What this means is that notification is for plants that do not cause environmental problems.

The second pathway, the "permitting" pathway, is meant for the "movement, importation and field testing of transgenic plants that do not quality for notification". Examples include commercial pharmaceutical producing plants or plants that pose greater risks to the environment versus plants undergoing the "notification" route.

The third and final procedure, which is "petition for non-regulated status", is one where APHIS determines that a particular transgenic plant s not a regulated article. This procedure is extremely important as this is the only route to commercialize transgenic plants (e.g. transgenic soybean seed) and one of the main paths to commercialization for transgenic plant products (e.g. protein extract from the transgenic plant) (NRC, 2002).

Canada

Canadian regulations, similar to the US regulations, focus on the end product rather than the process used to create the transgenic product. The Canadian regulations govern plants with novel traits (PNT). The classification of PNTs includes those modified transgenically or via mutagenesis. This is a major difference when compared to the US, where the regulations only cover transgenic plants and not plants created via mutagenesis. Another difference is that not all transgenic plants manifest novel traits – this means that not all transgenic plants are subject to regulations.

The Canadian Food Inspection Agency (CFIA) is the main agency responsible for the regulation of GMOs, being responsible for plants, animal feeds, fertilizers and animal biologics. 2 other government agencies, Environment Canada and Health

Canada share certain responsibilities according to Canadian Law (McHughen and Smyth, 2008).

Europe

Directive 2001/18/EC, Regulation No. 1829/2003 and Regulation No. 1830/2003 are the main regulations governing the control of GMOs in Europe. Directive 2001/18/EC outlines the principles regulating the deliberate release of GMOs into the environment, while Regulation 1829/2003 covers genetically modified food and feed and specifies the authorization procedures and labelling requirements. Regulation 1830/2003 deals with the traceability and labelling of GMOs and their food and feed derivatives (Alderborn *et al.*, 2010). The following measures are outlined in the regulations: principles for environmental risk assessment, compulsory post-marketing monitoring, and mandatory disclosure of information to the public and strict labelling /traceability of the products (Directive 2001/18 /EC of the European Parliament and of the Council of 12 March 2001). An important point to note is that potential benefits are to be excluded from the assessment, which means that the risk assessment does not weigh any potential benefits of the transgenic crop (McHughen, 2012). Environmental risk assessment is conducted on a case-by-case basis and this causes data requirements for each individual submission to vary substantially (Garcia-Alonso *et al.*, 2006).

The EU authorization process has 2 phases – risk assessment followed by risk management. In the first risk assessment phase, the European Food Safety Authority (EFSA) conducts a scientific assessment of the risks posed to human health, animal health and the environment. The second phase, risk management, is performed by the European Commission and Member States. The authorization process takes into account EFSA's scientific opinions together with other legitimate factors. The approval lasts for

10 years and a renewal application is to be submitted at the end of the authorization period (EuropaBio, 2011).

The EU regulations follow a "precautionary approach", which means GMOs are considered inherently unsafe. The EU regulations are considered to be the strictest in the world (Nuffield Council on Bioethics 2004). An indication of this strictness is that the average time it takes for a GMO to obtain approval in the EU is 45 months compared to 25 months in the US and 30 months for Canada. In terms of GMOs for cultivation, only 2 products have been authorized in the past 13 years as of October 2011 (EuropaBio 2011).

Summary

There are several notable differences regarding the US, Canada and European regulations. The first is that the US and Canada regulations are product-based whereas the EU regulations are process-based (Ramessar *et al.*, 2009). The triggers to initiate risk assessment are also different: in Canada the focus is on the novelty of the traits presenting in the GMOs, irrespective of whether the product is derived from genetic modification or "normal" breeding procedures like mutagenesis. In the US, even if the plant was developed using rDNA techniques, this does not automatically trigger the requirements for approval under US biosafety regulations (Paoletti *et al.*, 2008). There is a difference in terms of the labelling requirements for food produced from or derived from GMOs. EU regulations require mandatory labelling whereas the US and Canada implements voluntary labelling for GM food. Furthermore the threshold to mandate disclosure that the food is genetically modified is different: food and feed can be classed as non-GM even if they contain up to 5% GM material. The EU has much lower limits, which is 0.9%. This has real implications for the international trade of food and feed as what is permissible in the US and Canada will not be in compliance with EU

regulations. There is already ongoing dispute between EU and the US with regards to the restriction of trade that this mandatory labelling imposes (Carter and Gruère, 2003).

In summary, even though the ultimate aim of biosafety regulations remain the same, i.e. protection of human, animal and environmental health, the numerous differences in terms of regulations and implementation even in developed countries are still cause for concern and form a stumbling block for acceptance of the fruits of biotechnology and the potential benefits it would bring to the world.

2.6.5 Biosafety regulation of transgenic crop plants in Asia pacific

As can be expected from a region ranging from developed countries like Australia and Singapore to the developing economies of Indonesia and Philippines, the biosafety regulatory systems are at different stages of development and implementation. The approaches taken are also different: some countries are developing their biosafety systems from scratch while others are making modifications to existing regulatory systems (Gupta *et al.*, 2008). In many developing countries, this was in response of the ratification of the Cartagena Protocol – unlike the regulations in developed countries which were in place when the Cartagena Protocol came into force in 2003 (McLean *et al.*, 2012). Apart from the adoption of legislation, administrative systems have to be developed in order to enforce and implement the legislation. Developing countries also are at different stages of development in this regard (FAO ITC, 2010). The United Nations recognized that developing countries would lack the necessary resources and know-how to develop national biosafety regulations. In response to this, the Global Environment Facility of the United Nations Environment Programme (UNEP-GEF) was set up with the aim of assisting these countries in developing national biosafety framework (NBFs).

A country's NBF is a combination of legal and administrative tools that are used to ensure the safe and proper use of GMOs and consists of 5 main components: national biosafety policy, regulatory regime, administrative system, public awareness and participation, and system for follow up. (UNEP-GEF, 2006). Some of the Asia Pacific countries who were assisted by UNEP-GEF include China, Indonesia, South Korea, the Philippines, Vietnam and Malaysia (UNEP-GEF, 2008). Countries like Indonesia and Philippines already had some form of legislation in place before joining the NBF (Gupta *et al.*, 2008).

The regulatory systems of different Asia Pacific countries are reviewed below.

Australia

Australia has one of the most developed regulatory systems in Asia Pacific. The Office of the Gene Technology Regulator (OGTR), set up under the Gene Technology Act, is the main GMO-regulating body. The regulations are share many similarities with the EU system: it is process-based rather than product based and labeling/traceability are mandatory. The tolerance threshold to trigger labeling is 1%, making Australia one of the stricter countries in this regard. All intentional environmental releases (field trials and commercial releases) must be licensed. Field trials are restricted to certain limit such as size, location and duration. The trigger for regulation is the use of gene technology. Licensing decisions are based on case-by-case science-based risk assessments. Marketing and trade impacts are outside the scope of assessments required by the Act (OGTR, 2008).

China

Prior to regulations being implemented in 2001, China had approved several GM crops but this has slowed down with the implementation of biosafety legislation

(Baumueller, 2003). According to the "Implementation Regulations on Safety Assessment of Agricultural Genetically Modified Organisms 2004", transgenic plants are classified into classes according to their potential risk to human beings, other organisms and the environment. Class I signifies no known risk, Class II (low risk), Class III (medium risk) and Class IV denotes high risk. The regulations contain definitions of each class, for example organisms can only be classified as Class I if no negative effects on human health or the environment has been demonstrated or if the organism has little possibility of evolving into a hazardous organism. Article 5 states that a National Biosafety Committee (NBC) shall be set up to conduct safety assessments on GMOs. There are three stages of testing: 1) restricted field-testing (small-scale test conducted within a contained system), 2) enlarged field-testing (medium-scale test conducted under natural conditions) and 3) productive testing (large-scale test prior to commercial production and application) (Implementation Regulations on Safety Assessment of Agricultural Genetically Modified Organisms, 2004).

Japan

The aptly named "Cartagena Law" was promulgated in 2004 as Japan's biosafety legislation. Four ministries are involved the Ministry of Agriculture, Forestry and Fisheries (MAFF), the Ministry of Health, Labor and Welfare (MHLW), Ministry of Environment (MOE) and the Ministry of Education, Culture, Sports, Science and Technology (MEXT).The regulations differentiate between field experiments, import and cultivation (type 1 use) and use in laboratories, green house and R&D use (type 2 use). MEXT is responsible to approve type 2 uses while MAFF & MOE are responsible to approve type 1 uses. MEXT needs to grant approval before performing early stage agricultural biotech experiments in laboratories and greenhouses – this usually constitutes the first step of the approval process. Once this type 2 step is complete, the

next step towards approval is planting in an isolated field to assess the transgenic plant's effect on diversity. If the scientific data generated from this field trial is deemed satisfactory, risk assessment procedures will begin via field trials (Type 1 use). A joint MAFF and MOE expert panel carries out the environmental safety evaluations (http://www.bch.biodic.go.jp/english/law.html; accessed on 26 Aug 2012). MHLW is responsible for transgenic plants used as food. Upon the directive of the MHLW minister, the Genetically Modified Foods Expert Committee in the Food Safety Commission (FSC) conducts scientific risk assessments.

Taiwan

Taiwan's regulatory system is product based and mandatory labelling of GMOs is required. The Department of Health (DOH) and Council of Agriculture (COA) are the responsible governing agencies. The DOH conducts food safety risk assessment, while the Council of Agriculture (COA) has oversight on environmental risk assessments. One major difference is that the current Taiwanese GMOs are only applicable to soybeans and corn; this is expected to change to cover all GMOs in the future (USDA Foreign Agricultural Service 2009).

India

GMO regulation is contained in regulations in the Environmental (Protection) Act 1956. Three bodies deal with the approval of GMOs: the Institutional Bio Safety Committee (IBSC), the Review Committee of Genetic Manipulation (RCGM) and the Genetic Engineering Approval Committee (GEAC). The IBSC is established at every institution engaged in GMO research. The RCGM is tasked with reviewing the approval of ongoing R&D projects on GMOs. The GEAC functions under the purview of Department of Environment as the statutory body for review and approval of GMOs and

their products in R&D or environmental release and field applications (Gupta *et al.*, 2008).

Philippines

Philippines was the first country in South-East Asia to establish a regulatory system for GMOs. The first guidelines for biosafety were promulgated in October 1990 as Executive Order (EO) 430 EO 430. EO 430 established the National Committee on Biosafety of the Philippines. These and other subsequent guidelines were incorporated into the Philippines NBF, which was issued as EO 514 in April 2006 (UNEP-GEF Biosafety Unit, 2006).

In the Philippines, risk assessments are carried out on a case-by-case basis. This is based on the nature of the genetic modification and may vary in nature and level of detail depending on the GMO concerned, its intended use and the receiving environment (Gupta, 2008).

Thailand

In 1992, Thailand was one of the first in the South-East Asia to adopt biosafety guidelines. These guidelines were developed under the Ministry of Science, Technology and Energy (MOST) by the National Center for Genetic Engineering and Biotechnology (BIOTEC). Following this, the National Biosafety Committee (NBC) was established in 1993 and BIOTEC served as the coordinating body and secretariat (http://www.biotec.or.th/EN/).

In 2001, the Thai Ministry of Agriculture banned the field testing for transgenic plant products. This ban effectively meant that all GM research conducted must be conducted in laboratories or greenhouses (Teng, 2008).

In 2008, the Thai government approved the National Biosafety Act and the principles governing risk assessments is that the risk assessment procedure should be grounded in science and conducted on a case-by-case basis (Technical Biosafety Committee, 2010).

Indonesia

In Indonesia, a decree was issued by the Minister of Agriculture 1997 containing provisions on biosafety of transgenic plants. An amendment in 1999 expanded the decree's oversight to both biosafety and food safety aspects for all GMOs and not just plants.

A National Biosafety and Food Safety Commission was formed to implement the regulations. This Commission is assisted by a Technical Team for Biosafety and Food Safety which is responsible to assess GMO applications in biosafety containment or in a confined field. All transgenic crops must pass through the assessment and evaluation in the laboratory of biosafety containment and confined field testing (NBF Indonesia 2004).

2.6.6 Malaysian Biosafety Act

Malaysia is a signatory to the Cartagena Protocol and ratification occurred in Sep 2003. To comply with the Cartagena Protocol, the Biosafety Act 2007 was approved on 11 July 2007 and was entered into force in 1 Dec 2009. The Biosafety Act is meant to regulate the release, importation and contained use of living modified organisms (LMOs) and the products of such organisms. It only covers "modern biotechnology", which means products of mutagenesis will not be subject to this Act. The "precautionary approach" is followed – lack of full scientific evidence does not

mean lack of harm and preventive steps should be taken to prevent any negative effects. (http://www.biosafety.nre.gov.my/).

The Act mandates the creation of a National Biosafety Board (NBB). The regulatory and the decision-making functions of the NBB are to decide on GMO approval matters and to monitor activities related to GMOs. The Act also mandates the creation of the Genetic Modification Advisory Committee (GMAC), which is tasked with providing scientific assessment of applications for approval and notifications and gives recommendations to the NBB. The Act makes a distinction between "contained use" and "release and import" activities involving GMOs. Contained use means any work on GMOs that is performed in such a manner that contact and impact on GMOs on the external environment is prevented. For contained use purposes, only notification to the NBB is required and work can commence after an acknowledgment from the Director-General of Biosafety is received. In parallel to this, the GMAC and NBB will assess the notification and a decision (revoke the approval, make a temporary cessation order, impose additional terms and conditions, order the approved person to make rectifications or make any other order as the Board thinks fit in the interest of biosafety) will be made in 90 days.

For release and import activities, submission for approval is required. The NBB will make a decision within 180 days and decisions can range from approval, approval with terms and conditions or rejection. There is also opportunity to solicit public opinion during the approval process via publication in media subject to certain conditions like data confidentiality. Public opinion will be taken into account during the decision-making process. Consistent with the Cartagena Protocol, a detailed and scientific risk assessment process forms the cornerstone of the decision of the NBB. In tandem with risk assessment, part V of the Act states that emergency response plans are required to mitigate against harm and damage that may arise from GMOs. Section 61

states that GMOs and all items containing GMO products must be identified and clearly labelled (Biosafety Act 2007).

The Biosafety (Approval and Notification) Regulations 2010 complements the Biosafety Act and part II of the Regulations are about the establishment of Institutional Biosafety Committees (IBCs). IBCs are set up to ensure that use of GMOs at the institutional level is in compliance to the Biosafety Act (Biosafety (Approval and Notification) Regulations 2010).

The Ministry of Natural Resources and Environment (NRE) is responsible to develop the risk assessment procedures as stated in the Biosafety Act. The risk assessment procedures are broadly in line with the principles of the Cartagena Protocol. Applicants are required to submit information on risk assessment, risk management and emergency response plans. NBB makes a decision with input from GMAC and the Department of Biosafety. [http://www.biosafety.nre.gov.my/, accessed 26 Aug 2012].
The Biosafety Act does not contain sections on socio-economic and ethical considerations and this could potentially complicate public acceptance of biosafety decisions by the NBB (Latifah *et al.*, 2011).

2.7 Potential risks associated with virus-resistant transgenic plants

2.7.1 Introduction

With the increasing sophistication of genetic engineering techniques and subsequent prioritization of biotechnology on national and private research agendas, the safety aspects of this burgeoning field has been in focus over the years. Whilst promising enormous benefit for agriculture, the potential impacts of virus-resistant transgenic plants on the environment and human health have also been raised and reviewed regularly since the 1990s. Topics perceived to be of concern include

recombination, synergism, gene flow, impact on non-target organisms and allergenicity in food safety.

2.7.2 Recombination

It has been widely acknowledged that recombination between a viral transgene mRNA and the genomic RNA of a non-target virus during replication could possibly lead to the creation of a novel virus.

A few cases of recombination experiments were documented by Allison *et al.* (1996) revealed results which were in contrast to this as recombination was not detected in some of CP gene-expressing transgenic plants in the field (Capote *et al.*, 2007; Vigne *et al.*, 2004; Lin *et al.*, 2001). Research report by Turturo *et al.* (2008) showed that a similar population of recombinant viruses appeared in transgenic plants expressing a CMV CP gene infected by another cucumovirus and equivalent non-transgenic ones infected simultaneously with two cucumoviruses. Recently, Zagrai *et al.* (2011) also confirmed that transgenic plums expressing plum pox virus coat protein gene do not assist in the development of virus recombinants under field conditions.

This may suggest that transgenic plants do not contribute to the generation of recombinant viruses that would not have been generated in natural double infections (Turturo *et al.*, 2008). As such, the significance of recombination between transgenes and viruses appears to be very limited with regards to adverse environmental effects since there is no clear evidence that this phenomenon constitutes a risk of virus emergence.

2.7.3 Synergism

Synergism can happen in plants when one virus enhances the severity of infection by a distinct or unrelated virus (Latham and Wilson, 2008). Vance (1991)

reported the occurrence of a synergistic viral interaction between potato virus X (PVX) and a potyvirus which led to high accumulation of PVX and associated worsening symptoms.

In VRTPs, numerous studies have showed that synergism may result from the inhibition of the plant's post-transcriptional gene silencing (PTGS) defence response to viral infection (Vance *et al.*, 1995; Pruss *et al.*, 1997). In 2011, Siddiqui *et al.* detailed the synergistic interaction between CMV and TMV plus the induction of severe leaf malformation in 2b-transgenic tobacco plants.

It is possible that viral genes expressed in transgenic plants for the purpose of protecting the plant could actually confer sensitivity to a synergistic disease. However, the significance of synergism is limited as it could be simply avoided by not using viral genes in interference with PTGS for pathogen-derived resistance.

2.7.4 Effects on non-target organisms

VRTPs could potentially influence the diversity and population dynamics of non-target organisms, such as insect vectors, bacteria or fungi. An investigation on the influence of papaya ringspot virus (PRSV) resistant transgenic papaya showed limited impact on soil microorganisms (Hsieh and Pan, 2006). In a more recent article, Capote *et al.* (2008) reported that no significant differences were found in the genetic diversity of Plum pox virus (PPV) populations and aphids in PPV-resistant transgenic and conventional plums. A report published the following year summarised that transgenic *Zucchini yellow mosaic* virus resistance in cultivated squash affects pollinator behavior (Prendeville and Pilson, 2009). Even so, there has been no concrete evidence to prove that VRTPs have a significant impact on non-target organisms so far.

2.7.5 Allergenicity

The question of whether proteins encoded by viral sequences that are expressed in transgenic plants have a tendency to provoke allergic reaction has always been at the centre of attention. So far, no allergic effects have been attributed to Genetically Modified (GM) foods currently on the market. Moreover, no adverse effects have been observed in the long history of virus-infected plants as a part of the human and domestic animal food supply.

In 1994, the U.S. Food and Drug Administration (FDA) announced that ZW-20 squash appeared to be as safe as its non-engineered counterpart (NBIAP News Report, 1995). In the feature story published online at APSnet, Gonsalves *et al.* (2004) detailed the successful performance of this commercially grown transgenic papaya crop in Hawaii. This GM papaya was the first tree to be deregulated by The Animal and Plant Health Inspection Service (APHIS) in 1996. At present, VRTPs have been not found to pose a risk to allergenic safety in human health, as none of them contain known allergens. Reports of allergenicity assessment on virus resistant transgenic tomato, papaya, pepper and cabbage have shown negative results on food allergy. (Lin *et al.*, 2010; Fermin *et al.*, 2011)

2.7.6 Gene flow

One of the original concerns that has remained intractable is whether transgene escape from a VRTP to its non-transgenic counterpart (crop-to-crop) and wild or weedy relatives (crop-to-wild) via gene flow could have a significant impact on the wild plant's fitness and invasiveness/ weediness (Prins *et al.*, 2008). This gene-flow phenomenon has been known for decades and is well documented for major conventional crop species (Ellstrand *et al.*, 1999). The potential environmental consequences created by VRTPs are probably one of the most contested issues

internationally. In 1996, Bartsch *et al.* reported transgenically introduced resistance to Rizomania disease in cultivated beet showed no adverse effect on wild sea beet populations. Recently, a report of gene flow assessment on transgenic *Solanum tuberosum* spp. *tuberosum* (cv. Spunta) selected for PVY resistance in Argentina suggested that there is an extremely low probability for such an event to occur (Bravo-Almonacid *et al.*, 2012).

Though it seems that many wild type plant species are not susceptible to virus infection, there are still some exceptional cases where introduction of a new plant virus into the environment has had a significant impact on ecosystem structure. In 2005, Malmstrom *et al.* published an article to reveal a true story in California where the introduction of cereal yellow dwarf viruses led to a large-scale replacement of native perennial grasses by non-native annual species. Another recent study has also predicted that transgenic clover harbouring potyvirus resistance genes could also have an effect on invasiveness (Godfrey *et al.*, 2007).

It is essential to remember that environmental safety issues concerning recombination and synergism are relevant for both transgenic crops expressing virus-derived gene constructs and conventional crops alike. To put this in perspective, it is important to ensure that any increased risk posed by VRTPs is more than what one would normally expect from natural background events. The same can be said for gene flow; as there seems to be limited differences between both genetically engineered or bred by conventional techniques. This is due to the fact that it is the resistance trait that is being investigated as opposed to the actual crop development strategy itself. The likelihood and consequences of gene flow must be assessed on a case-by-case basis.

3.0 Materials and Methods

3.1 Materials

3.1.1 General chemicals, buffer, solutions

All chemicals used in this project were of Analytical Grade. The buffers and solutions were prepared as described in the methods section or in accordance with the manufacturer's protocol.

3.1.2 Single chain variable fragments (scFv) antibodies

The positive control used throughout this project was pUMSCFV-CMV1 construct in *E. coli* H2151. (from Dr Chua Kek Heng, UM).

3.1.3 Cucumber Mosaic virus

The local isolate of cucumber mosaic virus was obtained from infected tobacco plants courtesy of Dr Mohd. Roff, MARDI.

3.1.4 Tobacco plants and transgenic plants

The tobacco plants used in this project were *Nicotiana tabacum* L. cv. White Burley. The parent transgenic plants expressing anti-CMV scFv antibodies were the products from Dr Chua Kek Heng's PhD project. T_1, T_2 and T_3 generations of transgenic plants were produced from self-pollination. Wild type tobacco plants were grown from purchased seeds.

3.2 Methods

3.2.1 Sterilization

In general, reusable glassware, non-disposable plastic ware, aqueous solutions and soil were decontaminated by autoclaving at 121^{o}C (15 psi) for 20 minutes. Non-

autoclavable substances were filter-sterilized. Planting materials such as flower pots, seedling trays, watering cans and gardening tools were sterilized by using 10% bleach solution. All solutions and reusable glassware or plastic ware used in RNA work were treated with diethyl pyrocarbonate (DEPC) and autoclaved at 121°C (15 psi) for 20 minutes.

3.2.2 Growth and propagation of tobacco plants

Wild type and transgenic tobacco seeds were soaked in distilled water overnight prior to being sown in seedling trays containing humus-rich soil for germination. Healthy 2-week old seedlings were selected and transplanted into flowering pots with humus-rich soil. They were grown in an enclosed room at 27°C with 16-hour day length at low light intensity, with top watering every day.

The T_1 generations of transgenic tobacco plants were produced by self - pollinating positive T_0 plants. T_1 transgenic tobacco plants expressing anti-CMV antibodies were identified and self-crossed to obtain T_2 generations. T_2 progenies were evaluated for the presence of the desired transgene and were self-pollinated to produce T_3 generations. A small paint brush was used to gently brush the inside of the flowers to increase the chances of pollination. In order to prevent cross pollination amongst the transgenic plants, the flowers were covered with plastic bags during flowering time.

3.2.3 Small scale isolation of plasmid DNA

10µl of bacteria stock culture was grown in 10ml of LB broth (Appendix A) containing an appropriate antibiotic. After 16 hours growth at 37°C, 1ml of overnight culture was transferred into a 1.5ml microcentrifuge tube. The bacterial cells were harvested by spinning for a minute at 10,000xg. The supernatant was discarded and the pellet was dried. The pellet was then resuspended in 200µl ice-cold Solution 1

(Appendix A) and left at room temperature for 5 minutes. Freshly prepared 200µl Solution 2 (Appendix A) was then added to the suspension to lyse the cells. This mixture was then inverted a few times and incubated on ice. After 5 minutes, 150µl of ice-cold Solution 3 (Appendix A) was added and the mixture was inverted a few times. Again, the content was incubated on ice for 5 minutes. Following that, the microcentrifuge tube was spun at full speed for 5 minutes. The cleared lysate was transferred to a new microcentrifuge tube. Equal volume of phenol was added to the content and mixed by vortexing. The mixture was spun at full speed for 2 minutes. The aqueous phase was pipetted into a new 1.5ml tube and an equal volume of chloroform was added to it. The aqueous phase was collected as described previously and was transferred to a new 1.5ml tube. After that, 2V of absolute ethanol was added to it to precipitate the DNA. The mixture was then vortexed and left at room temperature for 2 minutes before being spun at full speed for 5 minutes. The supernatant was discarded and 1ml of 70% ethanol was added to wash the pellet. Again the supernatant was removed after centrifugation at full speed for 5 minutes. After vacuum drying, the pellet was resuspended in 50µl TE (pH8.0) (Appendix A) containing 20µg /ml RNase A. The resultant DNA was kept at -20^{o}C until further usage.

3.2.4 Molecular characterization of transgenic plants expressing scFv antibodies

3.2.4.1 Genomic DNA extraction from transgenic plants

The extraction of genomic DNA was performed using DNeasy® Plant Mini Kit (QIAGEN). The extraction protocol was derived from the instructions in the kit. 100 mg of transgenic tobacco leaves were ground in liquid nitrogen. The plant tissue powder was then transferred to a 1.5ml microcentrifuge tube (Eppendorf®, Germany). To lyse the cells, 400µl of Buffer AP1 and 4µl of RNAase A solution (100mg/ml) were added to the plant material. The mixture was vortexed vigorously prior to incubation at 65^{o}C for

10 minutes in an incubator (Memmert, Germany). Next, a 130µl of Buffer AP2 was added to the lysate and the mixture was incubated on ice for 5 minutes to precipitate detergents, proteins and polysaccharides. The lysate was then centrifuged for 5 minutes at full speed using the Mini Spin Plus Centrifuge (Eppendorf®, Germany). The supernatant was applied to a QIAshredder spin column sitting in a 2ml collection tube. After centrifugation at full speed for 2 minutes, 450µl of flow-through was transferred to a 1.5ml microcentrifuge tube. 675µl of Buffer AP3/E was pipetted directly onto the cleared lysate for mixing. From the mixture, 675µl was applied to the DNeasy mini spin column sitting in a 2ml collection tube and centrifuged for 1 minute at 8000rpm. The flow-through was discarded. This step was repeated with the remaining sample. Following this, the DNeasy column was placed in a new 2ml collection tube. 500µl Buffer AW was added to the DNeasy column and centrifuged for 1 minute at 8000rpm. The flow-through was discarded and the washing step was repeated to dry the membrane. The DNeasy column was then transferred to a 1.5ml microcentrifuge tube. 100µl of preheated Buffer AE was pipetted directly onto the DNeasy membrane and left for 5 minutes at room temperature. Finally, the column sitting in the microcentrifuge tube was spun for a minute at 8000rpm to elute the DNA.

3.2.4.2 Detection of scFv gene by Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR) reaction mixture, with a total volume of 25 µl, consisted of 1x reaction buffer, 0.3mM dNTP mix, 1U Taq polymerase (NEB), 1.0 mM $MgCl_2$, 1µM upstream and downstream primers, 20 ng templates and nuclease-free water (Table 3.1). The PCR conditions used in this study was as follows: 94 ^{0}C – 2 min (1 cycle); 94 ^{0}C – 1 min, 69.7 ^{0}C – 30 s, 72 ^{0}C – 1 min (30 cycles); 72 ^{0}C – 10 min (1 cycle) (Table 3.2). The reaction was performed on MJ Research PTC200 Peltier thermalcycle.

Table 3.1: Optimized conditions of PCR to amplify scFv transgene

Reagent	Volume (µl)
dH_2O	13.8
10x Rxn Buffer with $(NH_4)_2SO_4$	2.5
25 mM $MgCl_2$	1.5
2 mM dNTP	4.0
10 µM Primer forward	1.0
10 µM Primer reverse	1.0
Taq Polymerase (5 µ/µL)	0.2
DNA (1 µg)	1.0
Total Volume	25.0

Table 3.2: PCR cycling conditions to amplify scFv transgene

	Conditions
Initial denaturation	94 ^{0}C – 2 min (1 cycle)
Denaturation Annealing Extension	94 ^{0}C – 1 min 69.7 ^{0}C – 30 s (30 cycles) 72 ^{0}C – 1 min
Final extension	72 ^{0}C – 10 min (1 cycle)

3.2.4.3 Agarose gel electrophoresis

1µl of 6X Loading Buffer (New England, BioLabs Inc.) (Appendix B) was first mixed with 5µl of DNA samples. The mixture was then loaded onto 1% agarose gel (Appendix B) pre-stained with 1µg /ml ethidium bromide. The gel was run in 1X TBE Buffer (Appendix B) for 40 minutes at 100V or until the dye had migrated at least 6.0 cm. Electrophoresis was carried out using Wide Mini Horizontal Gel Electrophoresis System (Major Science, USA) with Power Pac 300 (Bio-Rad, USA). The PCR products were analysed along with 100bp DNA Ladder (New England, BioLabs Inc.). The gel was then viewed using AlphaImager 2200 Gel Documentation System (Alpha Innotech, USA).

3.2.4.4 Purification of PCR products

The purification of PCR products was performed using GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, USA). 300 µl Capture Buffer (Appendix C) was added to 300mg of agarose gel slice in a 1.5ml microcentrifuge tube. The tube was mixed by vortexing vigorously prior to incubation at 60ºC for 5 minutes. The dissolved gel was then transferred to the GFX column and placed at room temperature for a minute. Following this step, the column was centrifuged at full speed for 30 seconds. The flow-through collected was discarded and the collection tube was reused. 500µl of Wash Buffer (Appendix C) was added to the column. Similarly, the tube was spun at full speed for 30 seconds. The GFX column was then placed in a fresh 1.5ml microcentrifuge tube. 50µl of autoclaved distilled water was applied directly to the glass fiber matrix in the column. After l minute, the column was centrifuged at full speed for a minute to recover the purified DNA.

3.2.4.5 Confirmation of scFv gene by Southern hybridization

The specific probe used to hybridize the transgene was labeled according to instructions in the DIG DNA Labeling and Detection Kit (Roche) manual. As the first step, 15µl of purified DNA plant sample and 15µl Control Labeling Reaction (Appendix D) were denatured by heating in boiling water bath for 10 minutes then cooled down immediately on ice. Following this, 2µl of Hexanucleotide Mix, 2µl of dNTP Labeling Mix and 1µl of Klenow enzyme were mixed into the denatured probe and control DNA. The mixtures were incubated overnight at 37^{o}C. The reaction was stopped by heating the mixture to 65^{o}C for 10 minutes

To quantify the labeled probes, a series of dilutions of DIG-labeled DNA was applied to a Hybond-N^{+} nylon membrane (Amersham Biosciences, USA), with defined dilutions of DIG-labeled control DNA as standards. The nucleic acids on the membrane were fixed with 1.5 J/cm^{2} UV light for 3 minutes. The membrane was then transferred into a container containing 20ml Maleic Acid Buffer (Appendix D) and incubated by gently shaking at room temperature for 2 minutes. It was then incubated with agitation in 10ml Blocking Solution (Appendix D) for 30 minutes. Next, the membrane was placed in 10ml Antibody Solution (Appendix D) for 30 minutes. It was then washed twice, each for 15 minutes. Finally the membrane was equilibrated for 5 minutes in Detection Buffer (Appendix D) prior to incubation in the dark with freshly prepared Color-substrate solution (Appendix D). The reaction was stopped when the desired spot intensity was achieved.

Total genomic DNA was first digested with *Hha 1 /Nco 1 /Pml 1* restriction enzymes (New England BioLabs) according to the manufacturer's protocol before being separated on agarose gel. Electrophoresis of DNA samples was then carried out together with DIG-labeled DNA Molecular Weight Marker as standards, for 40 minutes

at 100V. To avoid uneven background problems, ethidium bromide was not used during the preparation of agarose gel.

Next, the gel was agitated in Depurination Solution (Appendix D) for 10 minutes, until the bromophenol blue dye turned yellow. The Depurination Solution was then discarded and the gel was rinsed 2 times with distilled water. After this, the gel was immersed in Denaturation Solution (Appendix D) with constant shaking for 30 minutes to return the bromophenol to blue color. Again, the solution was discarded and the gel was washed with distilled water several times. It was then placed in Neutralization Solution (Appendix D) for 30 minutes with agitation.

The DNA transfer process was carried out immediately. Southern blot was set up as described in the following passage. A pre-wetted Hybond-N^+ nylon membrane (Amersham Biosciences, USA) was immersed into 10X SSC Solutions (Appendix D) for 10 minutes before being placed on top of the gel. Previously, the gel was equilibrated for at least 10 minutes in 20X SSC (Appendix D). A container with a glass plate supporting platform was filled with 20X SSC. A soaked Whatman paper was placed atop the glass plate, functioning as a wick resting in 20X SSC. The gel was placed atop of the wick, with the membrane placed over it. The blot assembly was completed with 3 sheets of soaked Whatman 3MM paper, a stack of paper towels, a glass plate, and a 500g weight. The blot was left overnight at room temperature. Upon completion, the membrane was fixed with 1.5J/cm^2 UV light for 3 minutes and was used immediately for prehybridization.

10ml of DIG Easy Hyb (Roche) solution in hybridization tube was preheated to 68^{o}C in a hybridization oven. The membrane in the DIG Easy Hyb solution was also pre-hybridized at 68^{o}C with gentle agitation for 30 minutes. Approximately 25ng/ml of DIG-labeled DNA probe was denatured by boiling for 5 minutes and cooled rapidly in ice. The denatured probe was then mixed to the pre-warmed DIG Easy Hyb. The pre-

hybridization solution containing the membrane was discarded and replaced with the probe/hybridization mixture. The membrane was then incubated overnight with gentle shaking at 68°C. After the hybridization step, the membrane was washed 2 times, each time for 5 minutes with 2X SSC/0.1% SDS (Appendix D), at room temperature. Next, the membrane was washed twice again, each time for 15 minutes in 0.5X SSC (Appendix D) at 68°C before undergoing immunological detection.

All the incubation steps described in the following passage were performed at room temperature with gentle agitation. Immediately after the stringency washes, the membrane was rinsed briefly in Washing Buffer (Appendix D). Next, the membrane was incubated for 30 minutes in Blocking Solution (appendix D), followed by 30 minutes in Anti-DIG-AP Conjugation solution (Appendix D). The membrane was then washed 2 times for 15 minutes with Washing Buffer (Appendix D). The membrane was equilibrated 3 minutes in Detection Buffer (Appendix D) before incubation in the dark using freshly prepared Color-substrate Solution. (Appendix D). The process took 16 hours to complete. The reaction was stopped with distilled water when the desired bands were obtained.

3.2.4.6 Total RNA extraction from transgenic tobacco plants

The isolation of RNA from plant tissues was performed using RNeasy® Plant Mini Kit (QIAGEN). All apparatus and glassware used in this experiment were treated with DEPC water. 100mg of plant material were ground in liquid nitrogen and transferred to 1.5ml microcentrifuge tube. 450µl of Buffer RLT (with β-mercaptoethanol) was added immediately to the tissue powder. The mixture was then mixed by vortexing vigorously. The lysate was transferred to QIAshredder spin column sitting in a 2ml collection tube before undergoing centrifugation for 2 minutes at full speed. The flow through was then transferred to a 1.5ml microcentrifuge tube. To the

cleared lysate, 0.5 volume of absolute ethanol (Sigma-Aldrich, USA) was added and mixed by pipetting. 650µl of the sample was transferred to an RNeasy Mini spin column sitting in a 2ml collection tube and centrifuged for 15 seconds at 10,000rpm. The flow through was discarded. Next, 700µl Buffer RW1 was added to the spin column and centrifuged at 8000rpm for 15 seconds. The flow through was discarded. 500µl buffer RPE was added to the spin column twice. Both were centrifuged at 8000rpm for 15 seconds and 2 minutes, respectively. The spin column was centrifuged again for a minute at full speed to dry the membrane before being placed in a 1.5ml microcentrifuge tube. Finally, 50µl RNase-free water was added directly to the spin column membrane and centrifuged for 1 min at 8000rpm to elute the RNA.

3.2.4.7 Detection of transcribed scFv gene by Reverse Transcription (RT-PCR)

RT-PCR was performed to detect the transcription level of transgene in the tobacco plants. The generation of cDNA from total RNA was carried out according to the protocol stated in TaqMan® Reverse Transcription Reagents (Invitrogen™). A master mix with a total volume of 10µL was prepared. It consisted of 2µg of template RNA, 1x Taqman RT buffer, 5.5 mM $MgCl_2$, 500µM of each dNTP, 2.5µM random hexamers, 0.4U/µL of RNase inhibitor, 1.25U/µL of Reverse Transcriptase and RNase-free water. The mixture was mixed gently, followed by being briefly spun before incubation in the thermacycler. A pre-incubation step for 5 minutes at 25^{o}C was carried out to maximize the primer-RNA template binding. Reverse transcription was then performed at 48^{o}C for 30 minutes. Finally, the reaction was inactivated at 95^{o}C for 5 minutes. The cDNAs were used as templates for subsequent PCR reactions.

3.2.4.8 Total protein extraction from transgenic plants

1g of tobacco leaves were homogenized in liquid nitrogen. 10ml ice-cold PBS buffer was added to the samples upon transfer to a 15ml centrifuge tube (BD, Falcon™, USA). Following incubation on ice for 20 minutes, centrifugation was performed at 14,000rpm for 20 minutes at 4ºC. The supernatant was transferred to a 50ml centrifuge tube (BD, Falcon™, USA) and 3V of ice-cold acetone was added into it. The mixture was incubated overnight at -20ºC prior to centrifugation at 14,000rpm for 30 minutes at 4ºC. The supernatant was discarded and the pellet was dried. 1ml of ice-cold PBS buffer was added to dissolve the protein pellet.

3.2.4.9 Sodium Dodecyl Sulfate Polyacrylamide Gel (SDS-PAGE) Electrophoresis and Staining

SDS-PAGE gel with 12% resolving gel (Appendix E) and 4% stacking gel (Appendix E) were prepared according to standard protocol in Mini-PROTEAN® Cell (Bio-Rad, USA). Samples in Sample Buffer (pre-added β-mercaptoethanol) (Appendix E) were denatured by heating at 100ºC for 4 minutes and cooled immediately on ice before being loaded into sample wells. The samples to be separated, together with 5µl of the Broad Range protein marker (New England Biolabs), were run in 1X SDS Running Buffer (Appendix E) at 180V for 50 minutes. Following electrophoresis, the gel was placed in a container covered with Fixing Solution (Appendix E) and shaken for 2 hours at room temperature. The Fixing solution was then poured off and replaced with Coomassie blue staining solution (Appendix E). Again, the gel was incubated by shaking for 2 hours at room temperature. Next, the gel was immersed in Destaining solution (Appendix E) until the bands on gel were clearly visible. Finally, the gel was viewed using AlphaImager 2200 Gel Documentation System (Alpha Innotech, USA).

3.2.4.10 Western blot

The samples previously separated by polyacrylamide electrophoresis, together with Kaleidoscope Prestained standards (Bio-Rad), were transferred to a Hybond-P polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, USA). The membrane which was pre-wetted with distilled water, gel, Whatman filter paper and fiber pads were soaked in Transfer Buffer (Appendix F) for 30 minutes. The gel sandwich in the cassette was set up according to the instructions stated in the Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad). Ice-cold Transfer Buffer (Appendix F) was then added into the tank and the blotting process was carried out at 100V for an hour with constant stirring. Upon completion, the membrane was immersed in Blocking Solution (Appendix F) for an hour accompanied with gentle shaking at room temperature. Following that, the membrane was washed 3 times, each for 5 minutes with TBS-T (Appendix F). The membrane was then incubated by shaking for an hour at room temperature using Blocking Solution (Appendix F) containing anti-FLAG® antibody (Sigma-Aldrich). Again, the membrane was washed 3 times, each for 5 minutes with TBS-T. For detection, the membrane was immersed in blocking Solution (Appendix F) with Alkaline Phosphatase (AP) conjugated anti-mouse IgG (Promega) for an hour at room temperature with agitation. 5 times washing with PBS-T (Appendix F) was carried out, each for 5 minutes. Finally, the membrane was developed using BCIP/NBT Colour Development solution (Promega) (Appendix F). The reaction was stopped with distilled water when the bands on the membrane became visible.

3.2.4.11 Dot blot

5µl of sample was spotted slowly on the pre-wetted Hybond-P polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, USA) and allowed to dry at room temperature. The membrane was then incubated in Blocking Solution containing

CMV CP probe for an hour with gentle shaking. After washing 3 times (5 minutes each) with TBS-T buffer, the membrane was immersed into Blocking Solution with anti-FLAG® antibody (Sigma-Aldrich) for 30 minutes. The membrane was washed again as described previously. Then Alkaline Phosphatase (AP) conjugated anti-mouse IgG (Promega) was added into blocking solution which covered the membrane for 30 minutes. Next, washing was done 3 times, 10 minutes each time with TBS-T buffer. Finally, the membrane was incubated in BCIP /NBT solution for colour development. The reaction was stopped by distilled water. All the steps described were performed at room temperature.

3.2.4.12 ELISA

ELISA (enzyme-linked immunosorbent assay) was carried out to study the antibody-antigen specific recognition and interaction to detect the target proteins. The following details the protocol according to the instructions in the Agdia Reagent Set (Agdia, USA). 100µl anti-CMV coating antibody (Agdia, USA) was diluted (1:1000) in coating buffer (Appendix G) and coated in the walls of a 96-well microtiter plate (Costar). The plate was incubated in a humid box overnight at 4°C. After this, the wells were emptied and then filled to overflowing with 1X PBST solution (Appendix G). This step was repeated 7 times. After washing, the plate was held upside down for drying on towel paper. 100µl blocking solution (Appendix G) was then added to all wells and incubated in a humid box at room temperature for an hour. Plant samples from infected plants were ground using general extraction buffer (Appendix G) at a ratio of 1:10 (tissue weight: buffer volume). 100µl of prepared extracts, 100µl of positive control (Agdia, USA), 100µl of negative control (Agdia, USA) and 100µl of extraction buffer were dispensed into designated wells, respectively. The sealed plate was set aside in a humid box at room temperature for 2 hours. After washing for 8 times with 1X PBST

(Appendix G), the plate was dried as previously described. Next, 100µl of prepared anti-CMV alkaline phosphatase enzyme conjugate in ECI buffer (1:1000) (Appendix G) was dispensed into each well and the sealed plate was incubated in a humid box at room temperature for 2 hours. The washing steps were performed as described earlier. Following that, 100µl of PNP solution (Appendix G) was dispensed into each well and the plate was incubated in a humid box for an hour at room temperature. The reaction was stopped using 3M sodium hydroxide (50µl /well) (Appendix G) prior to measurement at wavelength 405nm using Fisher Scientific Multiskan® MCC/340 Microplate Reader.

3.2.5 Bioassay studies

Experiments were carried out at a greenhouse facility in the Malaysian Agricultural Research and Development Institute (MARDI), under the supervision of Dr Mohd. Roff.

3.2.5.1 Growing the test plants

Transgenic and wild type tobacco seeds were sown in seedling trays containing humus rich soil. Upon germination, they were transferred to individual flowering pots. All test plants were grown in a greenhouse and watered twice daily. Inoculations were performed when they were at 4 leaves stage.

3.2.5.2 Procedure for mechanical inoculation

To prepare virus inoculums, approximately 5g of CMV-infected tobacco leaves were ground in 20ml 0.05M PBS (pH7.0) (Appendix H) in pre-chilled sterilized mortar. 0.1% thioglycolic acid was added as a stabilizing agent. The extract was filtered twice through cheesecloth. The suspension was then added with 1% (w/v) of 600-mesh

Carborundum. Healthy tobacco leaves were macerated and prepared as described previously for mock inoculation.

Immediately, the inoculums were applied on the surface of transgenic young leaves by rubbing softly with fingers. The inoculated leaves were then rinsed with distilled water. All test plants were placed in a green house. Control plants which were not rubbed with inoculums were grown separately under the same environment.

3.2.5.3 Symptoms development and monitoring

All plants were monitored and observed for the development of virus symptoms. Leaf samples were collected from each plant at 20 days post-inoculation (DPI). They were evaluated for the presence of cucumber mosaic virus using ELISA. The inserted transgenes were also confirmed by PCR.

3.2.6 Studies of protein-protein binding with Autodock

3.2.6.1 Homology Modeling of anti-CMV scFv antibodies

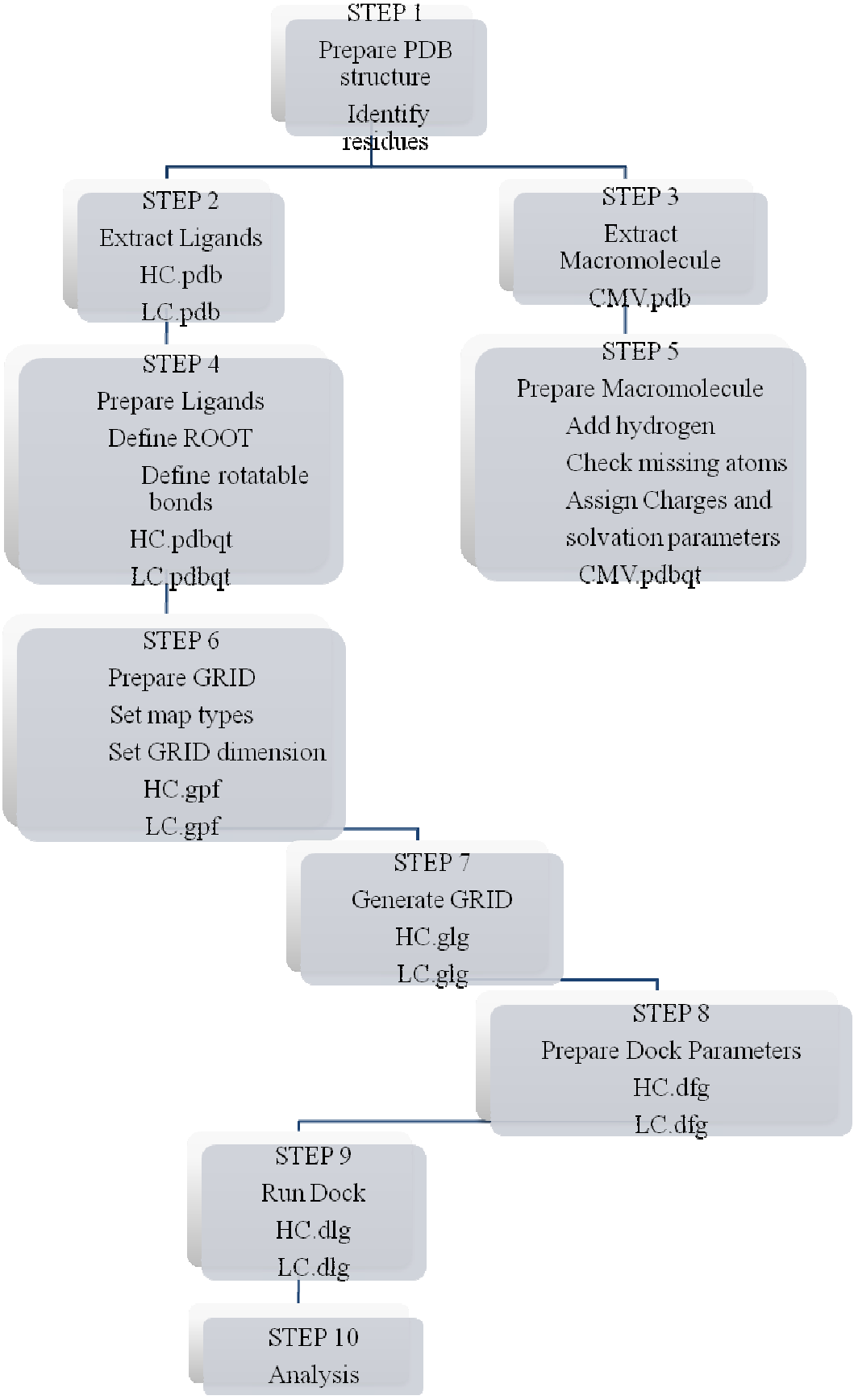
The structures of V_H chain and V_L chain of scFv antibody together with cucumber mosaic virus were predicted via homology modelling method using SWISS-MODAL program (Peitsch, M. C., 1995; Guex, N. and Peitsch, M. C., 1997; Schwede, *et al.*, 2003; Arnold, K. *et al.*, 2006; Kiefer, F. *et al.*, 2009). This step prepared the molecules in pdb files to be used in docking experiments later.

3.2.6.2 Autodock

In this study, the Autodock program package developed by Morris *et al.* (1998) was used to find possible binding sites on proteins. The docking experiments of the protein molecules were performed using AUTODOCK 4.2 software package introduced by Morris *et al.* (2009). The molecules were prepared according to the instructions in

AutoDockTools (ADT) written by Huey, R. and Morris, G. M. (2008). To begin with, the CMV (protein), scFv light chain (ligand 1) and scFv heavy chain (ligand 2) molecules were added with polar hydrogen atoms. Next, the non-polar hydrogens were merged after the Gasteiger charges and atom types were assigned to both molecules. The resulting pdbqt files were saved and were subsequently worked on using AutoGrid and AutoDock. Calculation of affinity maps was done by AutoGrid after parameters were set. Both ligands were set to be rotatable prior to being run on AutoDock. Docking was performed using Lamarckian Genetic Algorithm (LGA) and local search methods. Initially, a hundred searches of blind docking with the protein molecule were performed on heavy and light chains, respectively. Clustering histograms from both runs were analysed and the best 5 confirmations showing the lowest docked energy was chosen. Specific dockings were then performed based on the blind docking results. The detail process of Autodock is shown in Figure 3.1. The binding interactions were analysed using Viewelite 4.2 (Accelrys Software Inc).

Figure 3.1: Flow chart of protein-protein blind /specific docking with Autodock:



4.0 Results

4.1 Generation of transgenic tobacco lines

Five T_0 successfully transformed plants carrying anti-CMV scFv transgene and 2 wild type *Nicotiana tabacum* L. cv. white burley plants were grown in an environmentally-controlled room with a temperature of 27°C and duration of 16-hour day length. The plants carrying the transgenes started to flower at about 5 months old and were allowed to self-pollinate. A total of 4,689 T_1 generation seeds were collected from 5 T_0 transgenic plant lines in a month. Of the 500 T_1 seeds sown, 362 seeds were successfully germinated. Details of the seed germination rate in 5 different parental lines are presented in Table 4.1. 1,986 seeds were collected from 2 wild type tobacco plants which served as negative control plants (Table 4.2). An 87% successful germination rate in C_1 control plants was observed. Statistical analysis indicated that the germination rate for C_1 control plants was significantly higher than T_1 transgenic plants (Table 4.7).

Parental plant lines	Total of T_1 seeds collected	Total of T_1 seeds sown	Total of T_1 seeds germinated	Percentage germination
T_{0A}	968	100	71	71%
T_{0B}	873	100	76	76%
T_{0C}	954	100	68	68%
T_{0D}	987	100	77	77%
T_{0E}	898	100	70	70%
Total	4,689	500	362	72%

Table 4.1: T_1 progenies resulting from 5 T_0 transgenic plants expressing anti-CMV scFv antibodies

Control Plant	Total of C_1 seeds collected	Total of C_1 seeds sown	Total of C_1 seeds germinated	Percentage germination
$C1_0$	975	15	12	80%
$C2_0$	1,011	15	14	93%
Total	1,986	30	26	87%

Table 4.2: Successfully germinated C_1 wild type tobacco plants

4.2 Transgene inheritance in successive generations

One hundred positive T_1 transgenic plants from 5 different lines were allowed to grow and flower to produce the T_2 generation. 25,581 T_2 seeds were collected from these 100 positive T_1 transgenic plants. Table 4.3 details the number of T_2 seeds collected from 5 different T_1 lines. 1,783 C_2 seeds were collected from 2 C_1 wild type tobacco plants which served as negative control plants (Table 4.4).

Of the 500 T_2 seeds randomly picked and sown in soil, 360 T_2 seeds were successfully germinated. Overall, 72% of T_2 seeds and 93% of C_2 seeds were successfully germinated, as detailed in Table 4.3 and Table 4.4 respectively. Unpaired t-test was performed and a highly statistically significant P value of 0.0043 obtained (Table 4.7). The germination rate for the C_2 control plants was significantly higher when compared to T_2 transgenic plants.

Plant lines	Total of positive T_1 transgenic plants grown to flower	Total of T_2 seeds collected	Total of T_2 seeds sown	Total of T_2 seeds germinated	Percentage germination
T_{1A}	20	4,998	100	68	68%
T_{1B}	20	5,230	100	69	69%
T_{1C}	20	4,892	100	75	75%
T_{1D}	20	5,094	100	72	72%
T_{1E}	20	5,367	100	76	76%
Total	100	25,581	500	360	72%

Table 4.3: T_2 progenies resulting from 5 T_1 transgenic plant lines expressing anti-CMV scFv antibodies

Control Plant	Total of C_2 seeds collected	Total of C_2 seeds sown	Total of C_2 seeds germinated	Percentage germination
$C1_1$	866	15	13	87%
$C2_1$	917	15	15	100%
Total	1783	30	28	93%

Table 4.4: Successfully germinated C_2 wild type tobacco plants

A total of 100 positive T_2 transgenic plants from 5 different lines were allowed to grow and flower to produce T_3 generation (Table 4.5). 26,025 T_3 seeds were collected from these self-pollinated T_2 transgenic plants. 1,852 seeds were collected from 2 C_2 wild type tobacco plants which served as negative control plants, C_3 (Table 4.6).

Of the 500 T_3 seeds randomly picked and sown in soil, 356 T_3 seeds were successfully germinated. Table 4.5 shows a germination rate of 71% for T_3 seeds and 90% for C_3 control plants as shown in Table 4.6. Unpaired T test showed the two-tailed P value equals 0.0031 (Table 4.7). By conventional criteria, this difference between C_3 control plants and T_3 transgenic plants is considered to be highly statistically significant.

Plant lines	Total of positive T_2 transgenic plants grown to flower	Total of T_3 seeds collected	Total of T_3 seeds sown	Total of T_3 seeds germinated	Percentage germination
T_{2A}	20	5,388	100	70	70%
T_{2B}	20	5,069	100	69	69%
T_{2C}	20	5,177	100	66	66%
T_{2D}	20	4,989	100	76	76%
T_{2E}	20	5,402	100	75	75%
Total	100	26,025	500	356	71%

Table 4.5: T_3 progenies resulting from 5 T_2 transgenic plant lines expressing anti-CMV scFv antibodies

Control Plant	Total of C_3 seeds collected	Total of C_3 seeds sown	Total of C_3 seeds germinated	Percentage germination
$C1_2$	909	15	13	87%
$C2_2$	943	15	14	93%
Total	1852	30	27	90%

Table 4.6: Successfully germinated C_3 wild type tobacco plants

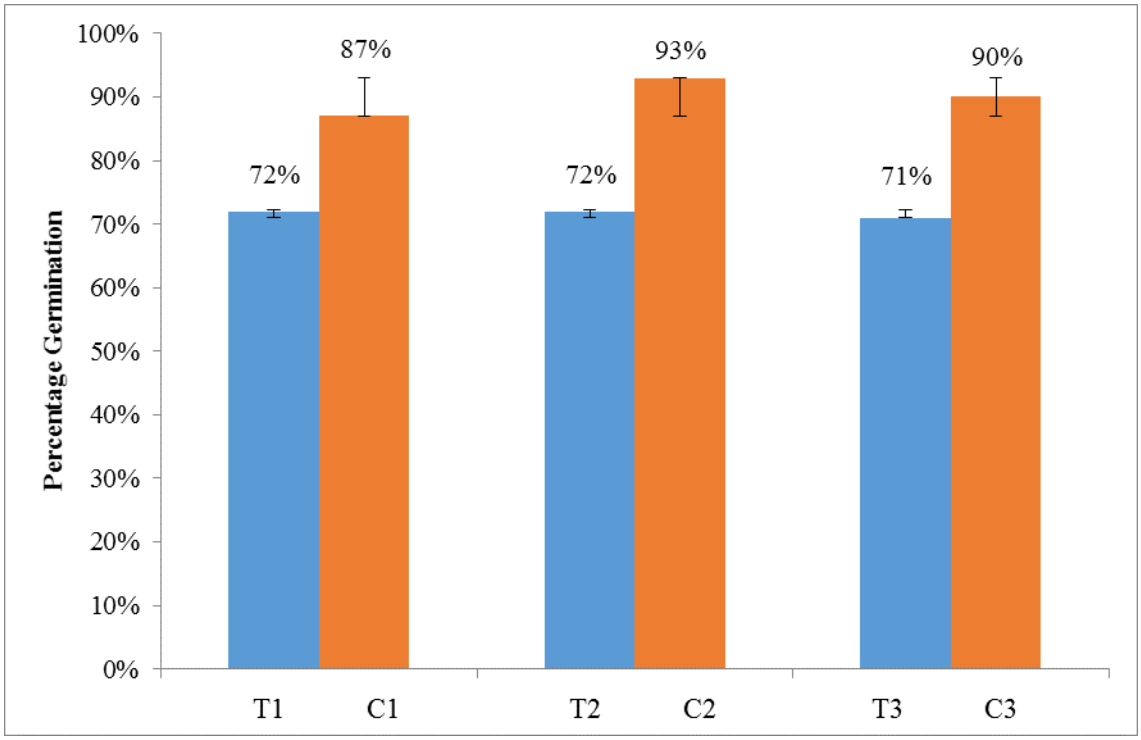


Figure 4.1: Summary of seed germination percentage for T_1, T_2, T_3 and control plants

As presented in Figure 4.1, higher seed germination rates for wild type tobacco plants was achieved as compared to putative transgenic plants for all 3 generations. Statistical analysis was performed using GraphPad Software and the details are shown in Table 4.7.

The details of seed germination rate in 3 generations for individual parental lines are illustrated in Figure 4.2 and Figure 4.3. Line T_{0D} in general achieved the highest germination percentage amongst all 3 generations.

Table 4.7: Unpaired *t* test results of T_1, T_2 and T_3 compared with control plants

(A)

	T_1	C_1
Total Event Numbers	5	2
Mean Event (Average)	0.724	0.865
Standard deviation	0.03912	0.09192
Variance (Standard deviation)	0.00153	0.00845

P value and statistical significance:
The two-tailed P value equals 0.0262
By conventional criteria, this difference is considered to be statistically significant.
Confidence interval:
The mean of T1 minus C1 equals -0.1410000
95% confidence interval of this difference: From -0.2571013 to -0.0248987
Intermediate values used in calculations:
t = 3.1219, df = 5
standard error of difference = 0.045

	T_2	C_2
Total Event Numbers	5	2
Mean Event (Average):	0.72	0.935
Standard deviation	0.03536	0.09192
Variance(Standard deviation):	0.00125	0.00845

P value and statistical significance:
The two-tailed P value equals 0.0043
By conventional criteria, this difference is considered to be very statistically significant.
Confidence interval:
The mean of T2 minus C2 equals -0.2150000
95% confidence interval of this difference: From -0.3265492 to -0.1034508
Intermediate values used in calculations:
t = 4.9545, df = 5
standard error of difference = 0.043

	T_3	C_3
Total Event Numbers	5	2
Mean Event (Average):	0.712	0.9
Standard deviation	0.04207	0.04243
Variance(Standard deviation):	0.00177	0.0018

P value and statistical significance:
The two-tailed P value equals 0.0031
By conventional criteria, this difference is considered to be very statistically significant.
Confidence interval:
The mean of T3 minus C3 equals -0.1880000
95% confidence interval of this difference: From -0.2786355 to -0.0973645
Intermediate values used in calculations:
t = 5.3320, df = 5
standard error of difference = 0.035

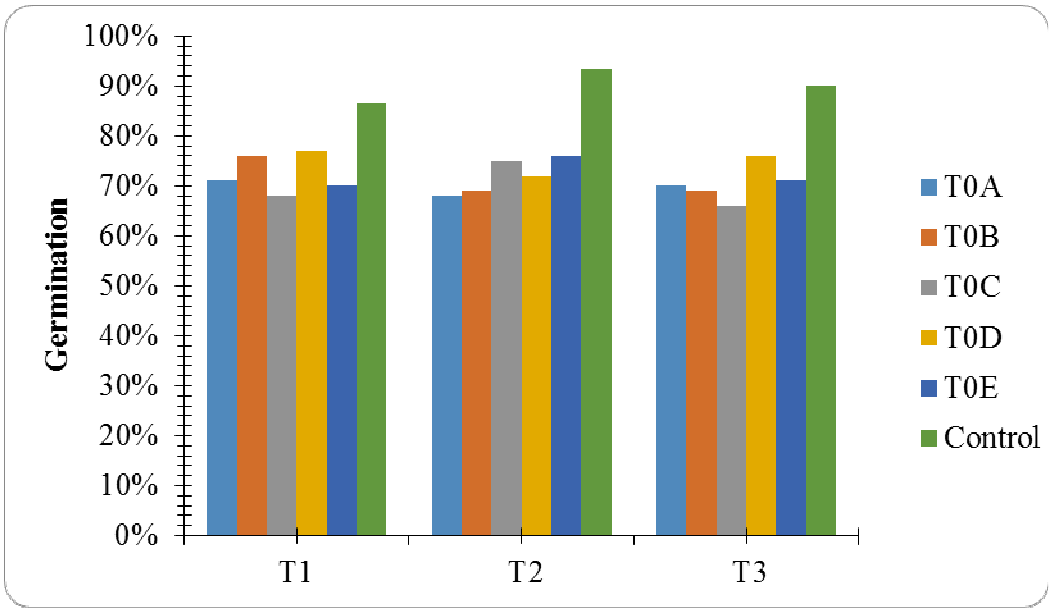


Figure 4.2: Percentage of seed germination in 3 generations for individual parental lines

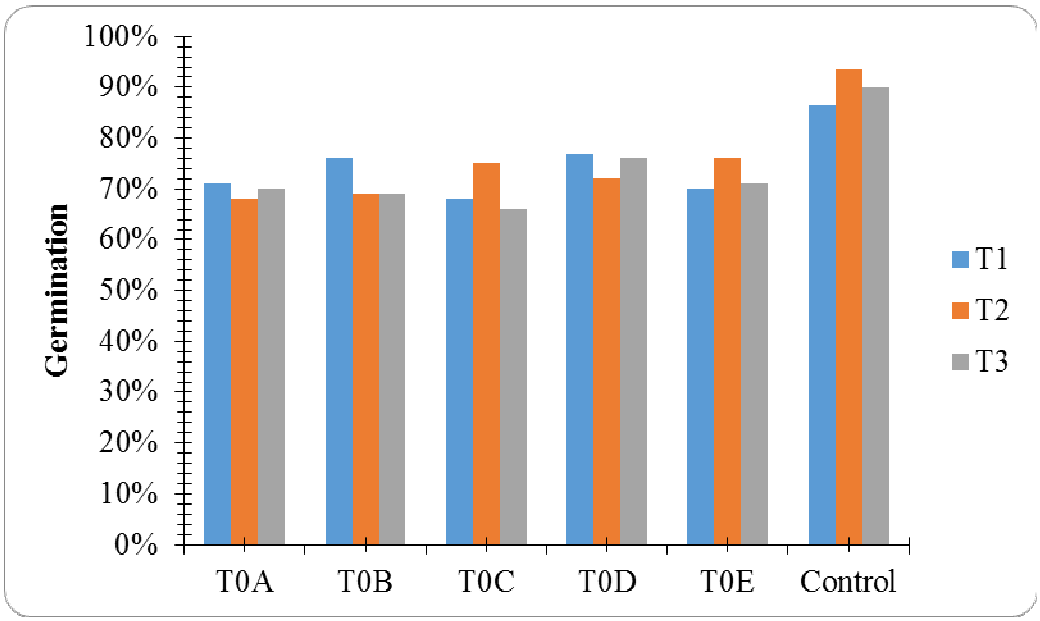


Figure 4.3: Percentage of seed germination in individual parental lines for 3 generations

4.3 Phenotyping of transgenic tobacco plants

All test plants were observed daily. The mature transgenic plants did not show any morphological differences compared to the wild tobacco plants. First generation of wild type tobacco plants are shown in Figure 4.4. Figure 4.5 shows T_1 putative transgenic plants at 3 months old and figure 4.6 illustrates the T_2 generation at 2 months old.

Figure 4.4: T_1 wild type *Nicotiana tabacum* L. cv. white burley grown in different containment areas under the same environmental condition

Figure 4.5: Healthy putative T_1 generation transgenic plants

Figure 4.6: The development of different putative T_2 transgenic lines in growth room

The flowering time of all test plants was recorded when the first open flower appeared. A delayed flowering phenomenon was observed in transgenic plants as compared to control plants. The control plants started to flower at about 4 months old. The plants carrying the transgenes showed first open flower at about 5 months old (Figure 4.7).

Figure 4.7: T_1 transgenic plant flowering at 5 months old

The flowering time for 5 parental lines in 4 generations are recorded in Table 4.8. Table 4.9 shows the flowering time for control plants. The details of flowering time across generations are recorded in Table 4.11. Unpaired t-test results yielded a two-tailed P value of less than 0.0001 in all 4 generations (Table 4.10). By conventional criteria, the flowering time difference between transgenic plants and control plants is considered to be highly statistically significant. Figure 4.8 illustrates the flowering time of transgenic plants and control plants for 4 generations.

	Days until first flower					
Parental line	T_{0A}	T_{0B}	T_{0C}	T_{0D}	T_{0E}	Mean
T_0 test plants	150	153	151	152	150	151
T_1 test plants	150	150	149	150	149	150
T_2 test plants	154	155	155	156	157	155
T_3 test plants	158	159	158	158	159	158

Table 4.8: Summary of flowering time for transgenic plants

	Days until first flower		
Parental line	C1	C2	Mean
C_0 test plants	123	124	124
C_1 test plants	121	120	121
C_2 test plants	120	121	121
C_3 test plants	120	123	122

Table 4.9: Summary of flowering time for control plants

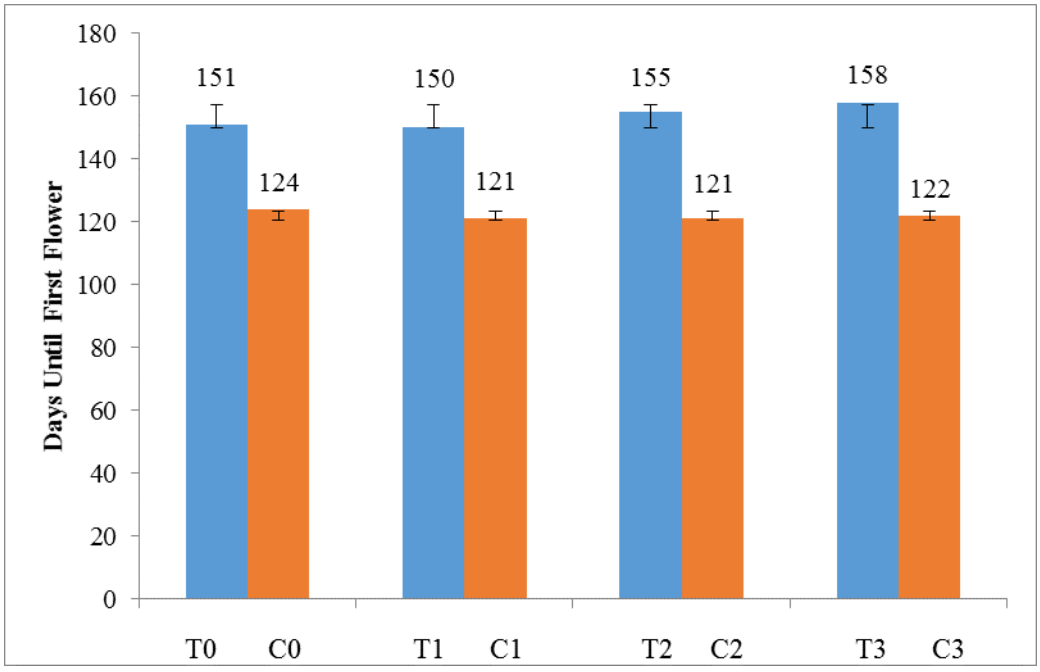


Figure 4.8: Flowering time of transgenic plants compared to control plants

Table 4.10: Unpaired *t* test results of transgenic plants compared to control plants

(A) Parental test plants

	T_0	C_0
Total number events	5	2
Mean events	151	124
Standard deviation	1.30384	0.70711
Variance (Standard deviation)	1.7	0.5

P value and statistical significance:
The two-tailed P value is less than 0.0001
By conventional criteria, this difference is considered to be highly statistically significant.

Confidence interval:
The mean of T0 minus C0 equals 27.0000000
95% confidence interval of this difference: From 24.4012953 to 29.5987047

Intermediate values used in calculations:
t = 26.7078, df = 5
standard error of difference = 1.011

(B) T_1 and C_1 test plants

	T_1	C_1
Total number events	5	2
Mean events	150	121
Standard deviation	0.54774	0.7071
Variance (Standard deviation)	0.3	0.5

P value and statistical significance:
The two-tailed P value is less than 0.0001
By conventional criteria, this difference is considered to be highly statistically significant.

Confidence interval:
The mean of T1 minus C1 equals 29.0000000
95% confidence interval of this difference: From 27.8417803 to 30.1582197

Intermediate values used in calculations:
t = 64.363, df = 5
standard error of difference = 0.451

(C) T_2 and C_2 test plants

	T_2	C_2
Total number events	5	2
Mean events	155	121
Standard deviation	1.14018	0.7071
Variance (Standard deviation)	1.3	0.5

P value and statistical significance:
The two-tailed P value is less than 0.0001
By conventional criteria, this difference is considered to be highly statistically significant.

Confidence interval:
The mean of T2 minus C2 equals 34.0000000
95% confidence interval of this difference: From 31.7545912 to 36.2454088

Intermediate values used in calculations:
t = 38.9238, df = 5
standard error of difference = 0.874

(D) T_3 and C_3 test plants

	T_2	C_2
Total number events	5	2
Mean events	158	122
Standard deviation	0.54772	2.12132
Variance (Standard deviation)	0.3	4.5

P value and statistical significance:
The two-tailed P value is less than 0.0001
By conventional criteria, this difference is considered to be highly statistically significant.

Confidence interval:
The mean of T3 minus C3 equals 36.0000000
95% confidence interval of this difference: From 33.7036797 to 38.2963203

Intermediate values used in calculations:
t = 40.2997, df = 5
standard error of difference = 0.893

Table 4.11: Flowering time for transgenic plants

(A)

Parental line T_1 test plants	Days until first flower				
	T_{1A}	T_{1B}	T_{1C}	T_{1D}	T_{1E}
1	149	153	145	149	148
2	150	147	151	151	150
3	149	146	149	147	151
4	148	151	146	149	149
5	153	152	150	153	149
6	152	151	150	152	150
7	155	147	151	150	148
8	146	149	145	149	148
9	146	150	148	146	149
10	148	153	149	150	149
11	145	153	149	152	149
12	151	148	150	147	151
13	150	149	152	150	150
14	146	151	149	150	150
15	152	147	151	154	149
16	154	150	152	153	150
17	151	150	151	150	149
18	147	151	149	148	151
19	153	153	150	151	148
20	150	152	150	148	148

	Days until first flower	
Parental line C_1 Test plants	$C1_1$	$C2_1$
1	119	120
2	123	120
3	120	122
4	119	118
5	124	121
6	120	119
7	118	119
8	119	117
9	125	121
10	119	123

(B)

Parental line T_2 Test plants	Days until first flower				
	T_{2A}	T_{2B}	T_{2C}	T_{2D}	T_{2E}
1	155	156	155	157	158
2	153	155	151	155	157
3	156	156	157	157	159
4	150	153	158	158	159
5	153	154	154	153	158
6	155	153	157	156	157
7	155	157	155	154	158
8	152	153	155	157	154
9	158	156	158	156	156
10	156	153	154	155	155
11	153	156	152	155	159
12	154	153	156	157	154
13	156	154	154	155	155
14	150	156	155	156	156
15	152	157	152	154	158
16	154	153	156	155	157
17	153	152	158	158	154
18	155	156	153	158	157
19	153	153	152	154	158
20	152	155	156	155	158

Parental line C_2 test plants	Days until first flower	
	$C1_2$	$C2_2$
1	118	122
2	120	121
3	120	123
4	117	119
5	122	121
6	121	120
7	120	124
8	119	117
9	123	118
10	122	124

(C)

	Days until first flower				
Parental line T_3 test plants	T_{3A}	T_{3B}	T_{3C}	T_{3D}	T_{3E}
1	160	158	155	159	162
2	156	157	155	161	157
3	159	156	159	157	161
4	158	161	156	160	159
5	157	162	160	156	159
6	160	161	160	157	160
7	155	157	156	160	159
8	156	159	155	159	160
9	156	160	158	156	155
10	158	155	159	157	158
11	158	155	159	155	159
12	159	158	160	157	156
13	154	159	159	160	160
14	161	157	156	159	159
15	159	157	161	154	159
16	157	160	162	158	155
17	156	160	154	158	159
18	157	161	159	160	161
19	159	159	160	157	160
20	160	162	157	158	158

	Days until first flower	
Parental line C_3 test plants	$C1_3$	$C2_3$
1	118	123
2	117	119
3	121	122
4	119	124
5	120	124
6	122	123
7	120	125
8	119	121
9	123	123
10	120	125

4.4 Genomic DNA extraction of transgenic plants

Genomic DNA extraction was successfully performed using QIAGEN DNeasy Plant Mini Kit. Figure 4.9 shows the quality of genomic DNA extracted from the T_0 primary transformant plants and wild type tobacco plants on 1% agarose gels. Genomic DNA of T_1, T_2 and T_3 putative transgenic plants are detailed in Figures 4.10, 4.11 and 4.12 respectively.

1 2 3 4 5 6 7

Land 1: T_{0A}
Lane 2: T_{0B}
Lane 3: T_{0C}
Lane 4: T_{0D}
Lane 5: T_{0E}
Lane 6, 7: T_0 control plants

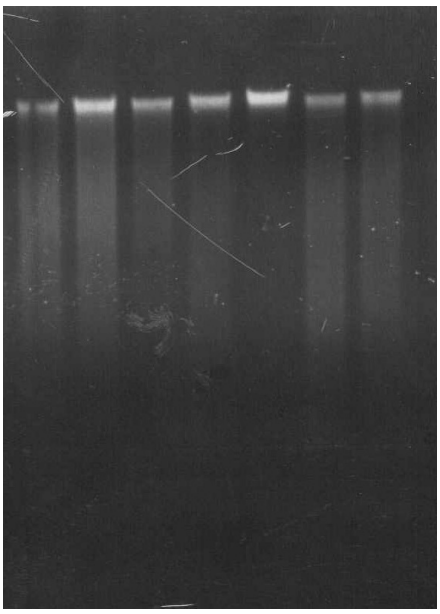


Figure 4.9: Genomic DNA of T_0 putative transgenic plants and wild type tobacco plants

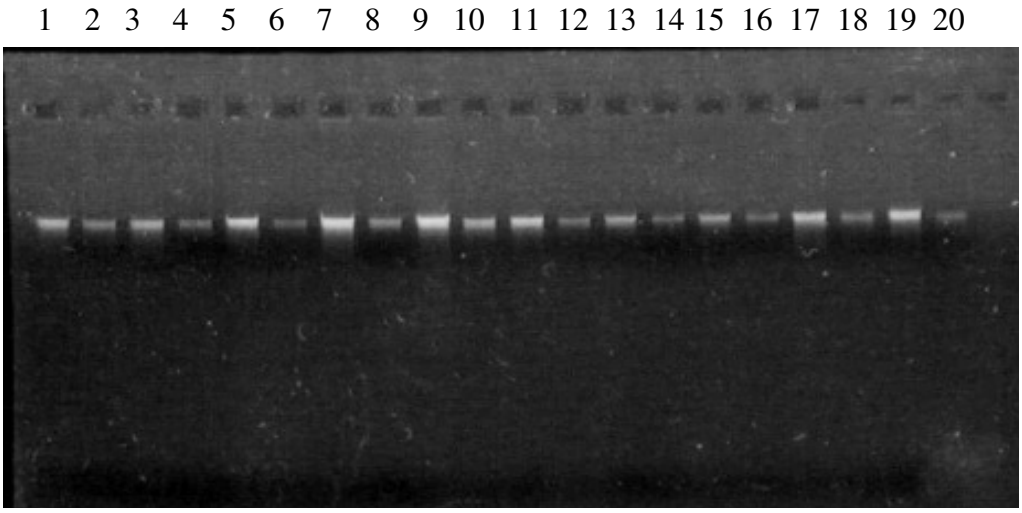


Figure 4.10: Genomic DNA of T_1 putative transgenic plants and control plant

Lane 1: T_1 control plant
Lane 2- 20: T_{1A1} – T_{1A19}

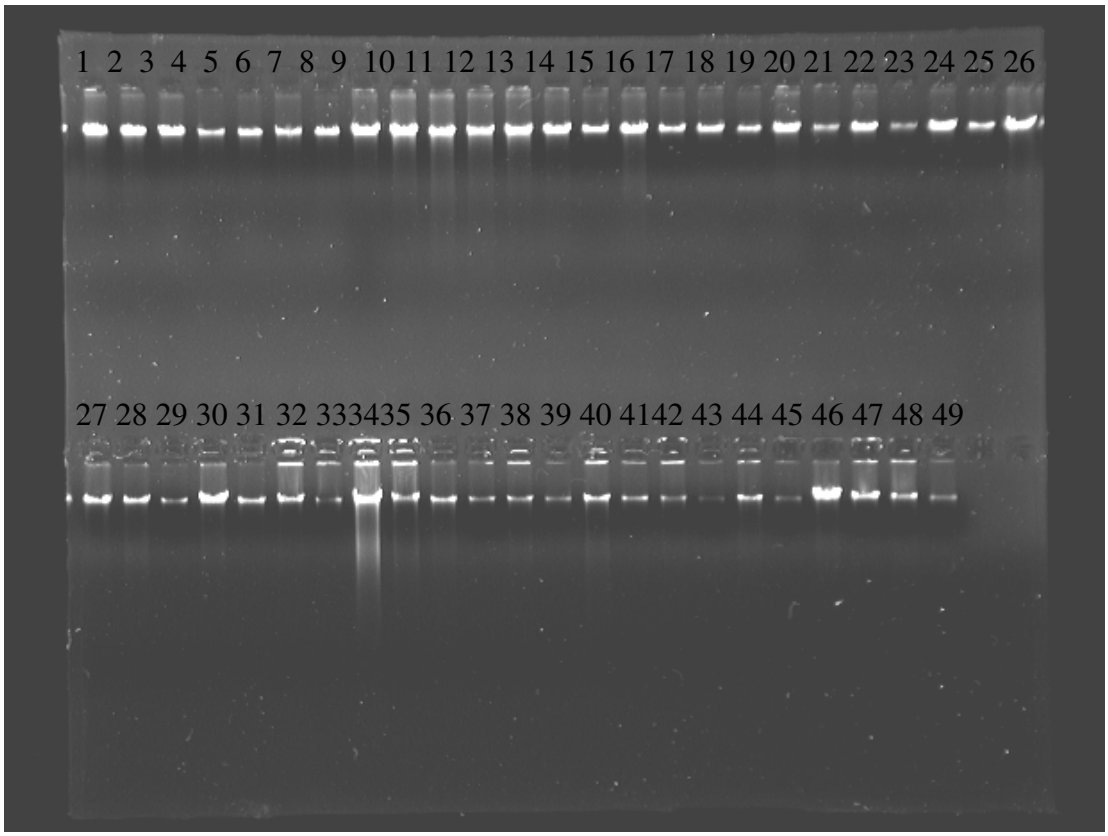


Figure 4.11: Genomic DNA of T_2 putative transgenic plants and control plant

Lane 1: T_2 control plant
Lane 2- 26: T_{2A} putative transgenic plants
Lane 27 – 49: T_{2B} putative transgenic plants

1 2 3 4 5 6 7 8 9 10 11 12 13 14

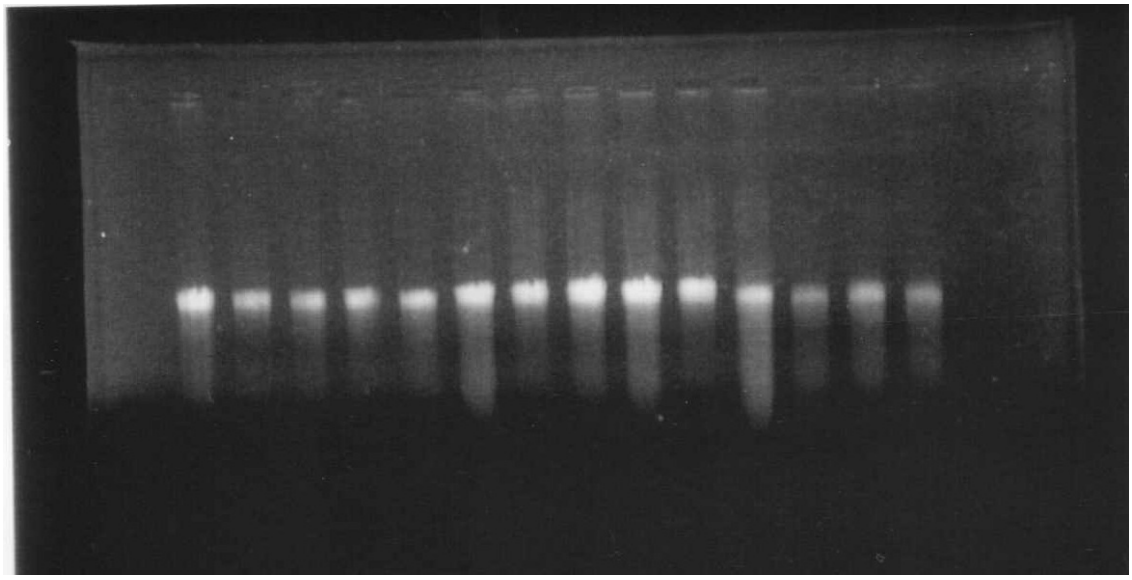


Figure 4.12**:** Genomic DNA of T_3 putative transgenic plants and control plant

Lane 1: T_3 control plant
Lane 2-14: T_{3A} putative transgenic plants

4.5 Detection of scFv transgene by PCR

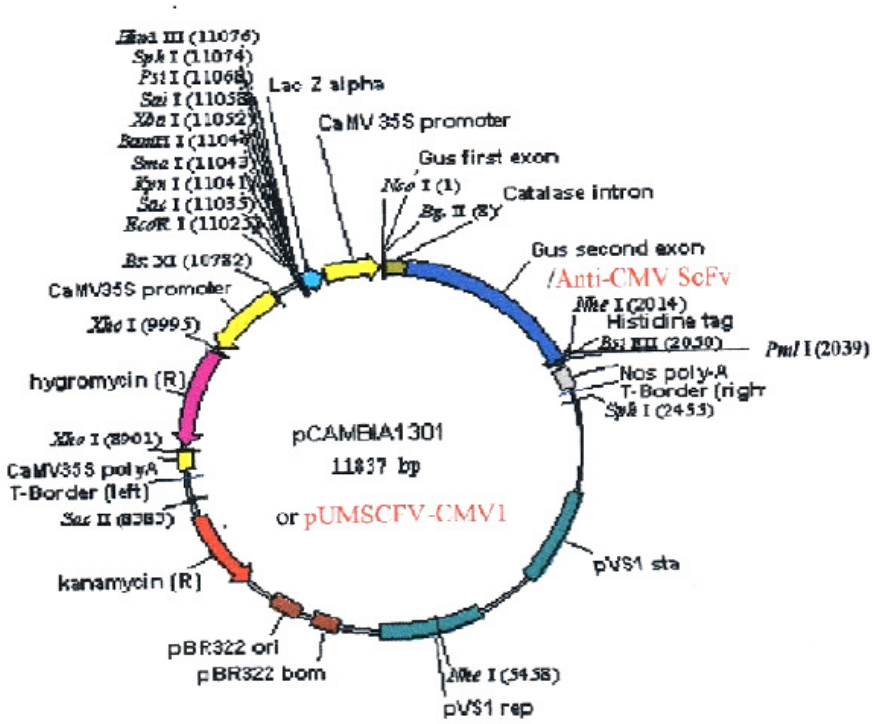
PCR analysis was carried out with scFv forward (5'-GTG CAG CTG CAG GAG TCA GCA ACT- 3') and scFv reverse (5'-CCG TTT GAG CTC CAG CTT GGT GCC-3') primers respectively. All reactions were replicated to reconfirm the results. The genomic DNA of wild type plant was isolated and used in PCR analysis as negative control. A blank control containing all the elements of a typical PCR reaction but containing only distilled water as DNA sample was also used.

For the positive control, pure plasmid pUMSCFV-CMV1 was isolated and used. Figure 4.13 (A) outlines the vector. After double digestion using *Nco* 1 and *Pml* 1 endonucleases, the Gus second exon cassette was removed and replaced with anti-CMV scFv fragment to form pUMSCFV-CMV1 construct. The expected size ~797 bp of PCR products amplified using scFv forward and scFv reverse primers as shown in (B). Figure 4.16 demonstrates the presence of transgene in the positive control samples.

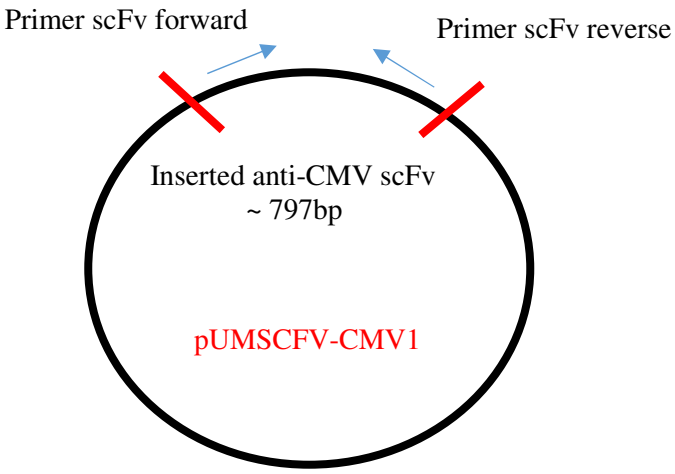
PCR analysis confirmed the presence of the transgene in T_0, T_1 and T_2 transgenic plants with an expected ~797 bp DNA fragment (Figure 4.14). PCR analysis of T_3 transgenic plants detected positive by scFv primers is shown in Figure 4.15.

Figure 4.13: pCAMBIA 1301 vector and pUMSCFV-CMV1 Construct and PCR analysis of inserted anti-CMV scFv.

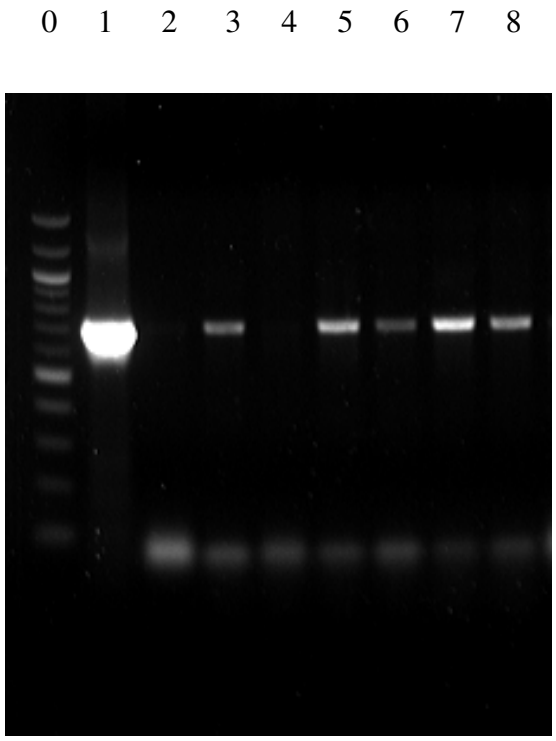
(A)



(B)

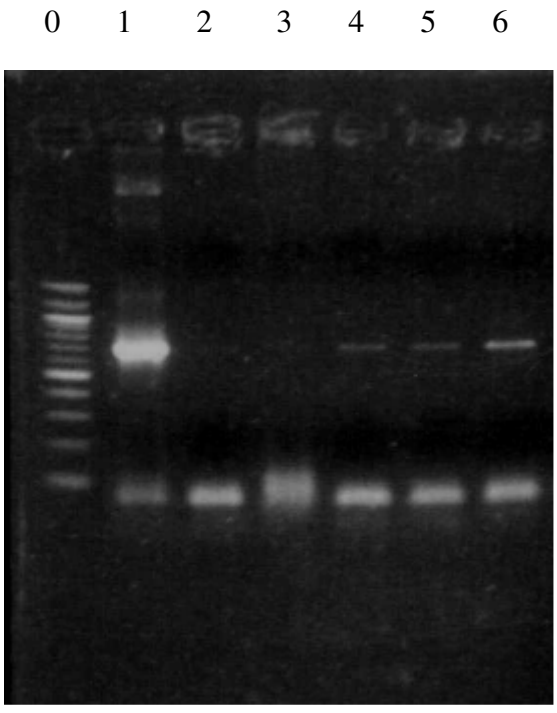


Source: Chua, 2002



Lane 0: 100 bp DNA ladder
Lane1: positive control
Lane 2: negative control
Lane 3: T_0 sample
Lane 4: blank control
Lane 5, 6: T_1 samples
Lane 7, 8: T_2 samples

Figure 4.14: PCR analysis of T_0, T_1 and T_2 transgenic plants



Lane 0: 100 bp DNA ladder
Lane 1: positive control
Lane 2: negative control
Lane 3: blank control
Lane 4-6: T_3 samples

Figure 4.15: PCR analysis of T_3 transgenic plants

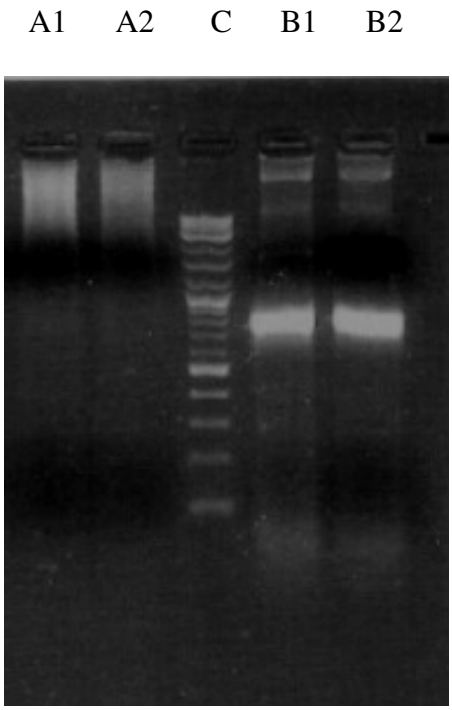


Figure 4.16: The presence of scFv transgene in the positive control samples

A1 and A2: Plasmid isolation of *E. coli* H215 containing pUMSCFV-CMV1
B1 and B2: scFv transgenes in *E. coli* H2151
C: 100 bp DNA ladder

It was observed that in the selected transgenic lines, 797 bp of scFv fragments were amplified as expected and this showed that scFv transgenes have been successfully integrated and inherited into the genome of selected T_1, T_2 and T_3 lines. Table 4.12 summarizes the percentage of detectable anti-CMV scFv transgenes in transgenic primary transformant and progenies. 58% of T_1 tobacco plants were detected positive with an expected ~797 bp DNA fragment using scFv primers. In T_2 transgenic plants, 64% were found positive and 53% of T_3 transgenic plants were confirmed to be carrying the anti-CMV scFv gene. All wild type tobacco plants did not show the presence of transgene. The summary of detectable gene via PCR in 4 generations is presented in Figure 4.17. The details of the PCR analysis of T_1, T_2 and T_3 transgenic plants are tabulated in Table 4.14.

Generation	Number of plants analysed	PCR analysis		Percentage
		Positive	Negative	
T_0	5	5	0	100%
T_1	362	211	151	58%
T_2	200	127	73	64%
T_3	100	53	47	53%
Total	667	396	271	59%

Table 4.12: Summary of PCR analysis of scFv in transgenic primary transformant and progenies

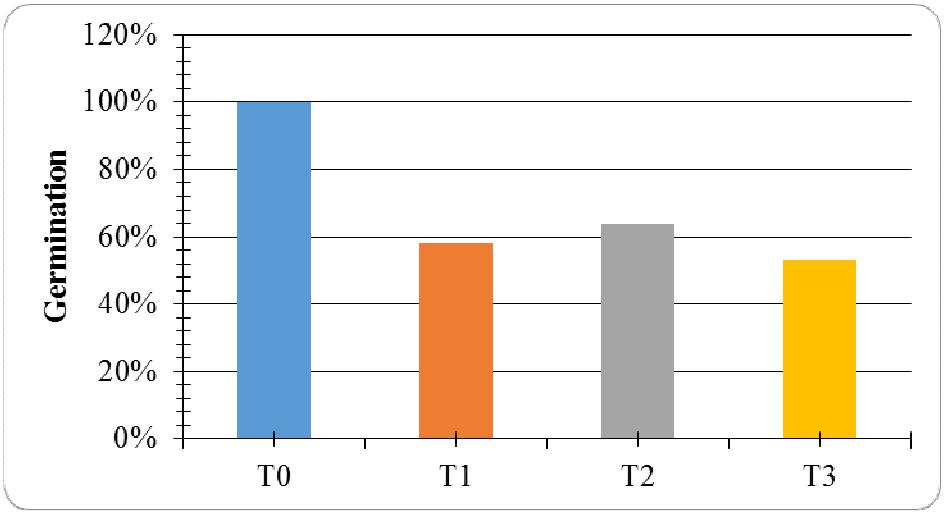


Figure 4.17: Summary of detectable gene via PCR in 4 generations

The inheritance of transgene over the generations is presented in Figure 4.18 and Figure 4.19. In general, we observed the lowest detectable percentage via PCR in line T_{0A} for 3 generations. Line T_{0E} appeared to be the most promising line with the highest percentage for detectable gene (Table 4.13).

	Percentage anti-CMV scFv transgene detected via PCR				
Parental line / Progenies	T_{0A}	T_{0B}	T_{0C}	T_{0D}	T_{0E}
T_1	44%	67%	47%	61%	71%
T_2	48%	64%	59%	74%	65%
T_3	47%	53%	53%	56%	55%

Table 4.13: Summary of PCR analysis of scFv for Parental line T_{0A} - T_{0B}

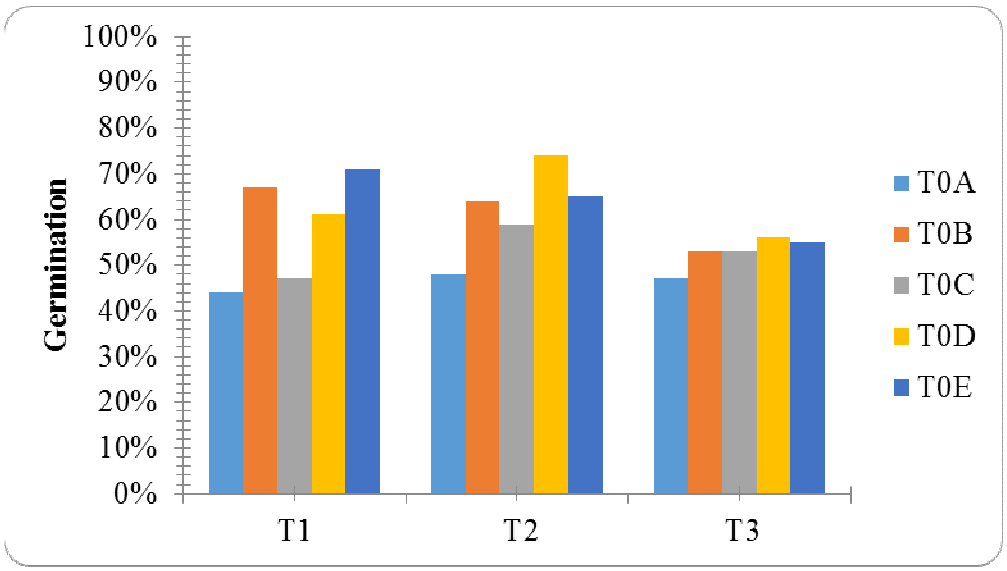


Figure 4.18: Percentage of detectable gene via PCR for 5 parental lines in 3 generations

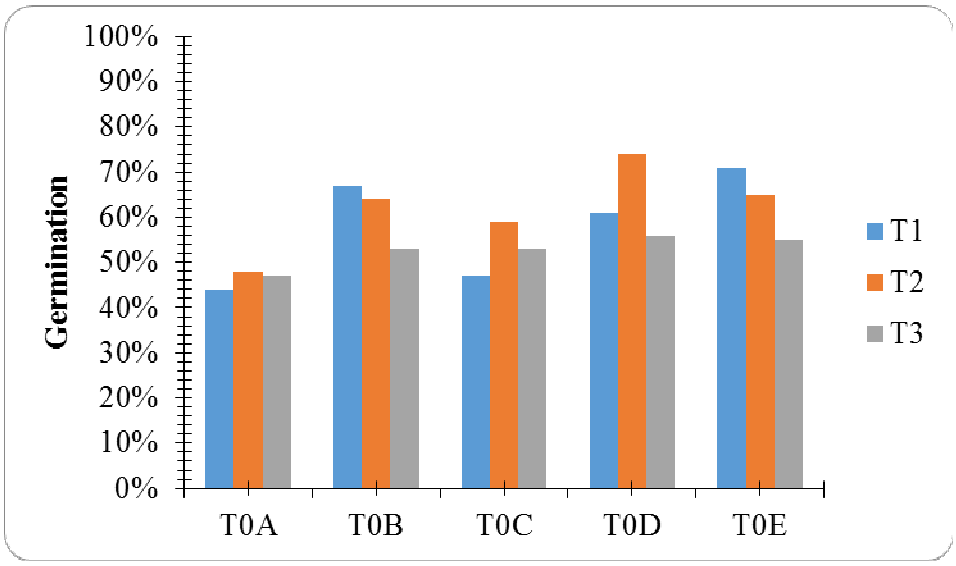


Figure 4.19: Percentage of detectable transgene via PCR for 3 generations in 5 parental lines

Table 4.14: PCR analysis of progenies from parental lines T_{0A} - T_{0E}

Parent Line T_{0A}	Anti-CMV scFv	Parent Line T_{0B}	Anti-CMV scFv	Parent Line T_{0C}	Anti-CMV scFv	Parent Line T_{0D}	Anti-CMV scFv	Parent Line T_{0E}	Anti-CMV scFv
T_{1A1}	-	T_{1B1}	+	T_{1C1}	-	T_{1D1}	+	T_{1E1}	+
T_{1A2}	+	T_{1B2}	+	T_{1C2}	-	T_{1D2}	+	T_{1E2}	-
T_{1A3}	+	T_{1B3}	+	T_{1C3}	-	T_{1D3}	+	T_{1E3}	-
T_{1A4}	+	T_{1B4}	-	T_{1C4}	-	T_{1D4}	+	T_{1E4}	+
T_{1A5}	+	T_{1B5}	-	T_{1C5}	-	T_{1D5}	+	T_{1E5}	+
T_{1A6}	+	T_{1B6}	+	T_{1C6}	+	T_{1D6}	+	T_{1E6}	+
T_{1A7}	-	T_{1B7}	-	T_{1C7}	+	T_{1D7}	+	T_{1E7}	+
T_{1A8}	+	T_{1B8}	+	T_{1C8}	+	T_{1D8}	+	T_{1E8}	+
T_{1A9}	+	T_{1B9}	+	T_{1C9}	+	T_{1D9}	+	T_{1E9}	-
T_{1A10}	+	T_{1B10}	+	T_{1C10}	+	T_{1D10}	+	T_{1E10}	-
T_{1A11}	+	T_{1B11}	-	T_{1C11}	+	T_{1D11}	-	T_{1E11}	+
T_{1A12}	+	T_{1B12}	+	T_{1C12}	+	T_{1D12}	-	T_{1E12}	+
T_{1A13}	+	T_{1B13}	+	T_{1C13}	-	T_{1D13}	-	T_{1E13}	+
T_{1A14}	-	T_{1B14}	-	T_{1C14}	+	T_{1D14}	-	T_{1E14}	+
T_{1A15}	-	T_{1B15}	-	T_{1C15}	+	T_{1D15}	-	T_{1E15}	+
T_{1A16}	-	T_{1B16}	+	T_{1C16}	+	T_{1D16}	+	T_{1E16}	+
T_{1A17}	-	T_{1B17}	+	T_{1C17}	-	T_{1D17}	+	T_{1E17}	+
T_{1A18}	-	T_{1B18}	+	T_{1C18}	+	T_{1D18}	+	T_{1E18}	+
T_{1A19}	-	T_{1B19}	+	T_{1C19}	+	T_{1D19}	+	T_{1E19}	-
T_{1A20}	+	T_{1B20}	+	T_{1C20}	-	T_{1D20}	+	T_{1E20}	-
T_{1A21}	-	T_{1B21}	+	T_{1C21}	+	T_{1D21}	-	T_{1E21}	-
T_{1A22}	+	T_{1B22}	+	T_{1C22}	+	T_{1D22}	+	T_{1E22}	+
T_{1A23}	-	T_{1B23}	+	T_{1C23}	-	T_{1D23}	+	T_{1E23}	+
T_{1A24}	-	T_{1B24}	-	T_{1C24}	-	T_{1D24}	+	T_{1E24}	+
T_{1A25}	+	T_{1B25}	+	T_{1C25}	-	T_{1D25}	-	T_{1E25}	+
T_{1A26}	+	T_{1B26}	+	T_{1C26}	-	T_{1D26}	-	T_{1E26}	+
T_{1A27}	+	T_{1B27}	+	T_{1C27}	-	T_{1D27}	-	T_{1E27}	+
T_{1A28}	+	T_{1B28}	+	T_{1C28}	+	T_{1D28}	+	T_{1E28}	-
T_{1A29}	+	T_{1B29}	+	T_{1C29}	+	T_{1D29}	+	T_{1E29}	-
T_{1A30}	+	T_{1B30}	+	T_{1C30}	+	T_{1D30}	+	T_{1E30}	-
T_{1A31}	-	T_{1B31}	-	T_{1C31}	+	T_{1D31}	-	T_{1E31}	+
T_{1A32}	-	T_{1B32}	+	T_{1C32}	+	T_{1D32}	+	T_{1E32}	+
T_{1A33}	-	T_{1B33}	+	T_{1C33}	+	T_{1D33}	+	T_{1E33}	+
T_{1A34}	-	T_{1B34}	+	T_{1C34}	+	T_{1D34}	+	T_{1E34}	+
T_{1A35}	-	T_{1B35}	-	T_{1C35}	+	T_{1D35}	+	T_{1E35}	+
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T_{1A39}	-	T_{1B39}	+	T_{1C39}	-	T_{1D39}	-	T_{1E39}	+
T_{1A40}	-	T_{1B40}	+	T_{1C40}	-	T_{1D40}	+	T_{1E40}	+
T_{1A41}	-	T_{1B41}	+	T_{1C41}	-	T_{1D41}	+	T_{1E41}	+
T_{1A42}	-	T_{1B42}	+	T_{1C42}	-	T_{1D42}	+	T_{1E42}	+
T_{1A43}	-	T_{1B43}	+	T_{1C43}	+	T_{1D43}	+	T_{1E43}	+
T_{1A44}	-	T_{1B44}	-	T_{1C44}	-	T_{1D44}	-	T_{1E44}	-
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T_{1A46}	+	T_{1B46}	-	T_{1C46}	+	T_{1D46}	-	T_{1E46}	+

T_{1A47}	+	T_{1B47}	+	T_{1C47}	-	T_{1D47}	-	T_{1E47}	+
T_{1A48}	+	T_{1B48}	-	T_{1C48}	+	T_{1D48}	-	T_{1E48}	+
T_{1A49}	+	T_{1B49}	-	T_{1C49}	-	T_{1D49}	-	T_{1E49}	+
T_{1A50}	+	T_{1B50}	+	T_{1C50}	-	T_{1D50}	-	T_{1E50}	+
T_{1A51}	-	T_{1B51}	-	T_{1C51}	-	T_{1D51}	-	T_{1E51}	-
T_{1A52}	-	T_{1B52}	+	T_{1C52}	+	T_{1D52}	+	T_{1E52}	+
T_{1A53}	-	T_{1B53}	+	T_{1C53}	+	T_{1D53}	+	T_{1E53}	+
T_{1A54}	-	T_{1B54}	-	T_{1C54}	-	T_{1D54}	-	T_{1E54}	-
T_{1A55}	-	T_{1B55}	-	T_{1C55}	+	T_{1D55}	-	T_{1E55}	+
T_{1A56}	+	T_{1B56}	+	T_{1C56}	+	T_{1D56}	+	T_{1E56}	-
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T_{1A58}	-	T_{1B58}	-	T_{1C58}	-	T_{1D58}	+	T_{1E58}	+
T_{1A59}	-	T_{1B59}	+	T_{1C59}	+	T_{1D59}	-	T_{1E59}	-
T_{1A60}	-	T_{1B60}	+	T_{1C60}	+	T_{1D60}	+	T_{1E60}	+
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T_{1A66}	+	T_{1B66}	+	T_{1C66}	-	T_{1D66}	-	T_{1E66}	-
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		T_{1B73}	+			T_{1D73}	+		
		T_{1B74}	-			T_{1D74}	-		
		T_{1B75}	-			T_{1D75}	-		
		T_{1B76}	+			T_{1D76}	+		
						T_{1D77}	+		
T_{2A2}	-	T_{2B1}	+	T_{2C6}	+	T_{2D1}	-	T_{2E1}	+
T_{2A3}	+	T_{2B2}	+	T_{2C7}	+	T_{2D2}	+	T_{2E4}	-
T_{2A4}	+	T_{2B3}	-	T_{2C8}	+	T_{2D3}	-	T_{2E5}	-
T_{2A5}	-	T_{2B6}	+	T_{2C9}	-	T_{2D4}	+	T_{2E6}	+
T_{2A6}	-	T_{2B8}	+	T_{2C10}	-	T_{2D5}	+	T_{2E7}	+
T_{2A8}	-	T_{2B9}	+	T_{2C11}	+	T_{2D6}	+	T_{2E8}	+
T_{2A9}	-	T_{2B10}	-	T_{2C12}	+	T_{2D7}	+	T_{2E11}	+
T_{2A10}	+	T_{2B12}	+	T_{2C14}	+	T_{2D8}	+	T_{2E12}	+
T_{2A11}	+	T_{2B13}	-	T_{2C15}	-	T_{2D9}	+	T_{2E13}	-
T_{2A12}	+	T_{2B16}	+	T_{2C16}	+	T_{2D10}	-	T_{2E14}	-
T_{2A13}	+	T_{2B17}	-	T_{2C18}	-	T_{2D16}	-	T_{2E15}	+
T_{2A20}	+	T_{2B18}	-	T_{2C19}	+	T_{2D17}	+	T_{2E16}	-
T_{2A22}	-	T_{2B19}	+	T_{2C21}	-	T_{2D18}	-	T_{2E17}	+
T_{2A25}	+	T_{2B20}	-	T_{2C22}	+	T_{2D19}	+	T_{2E18}	-
T_{2A26}	-	T_{2B21}	-	T_{2C28}	+	T_{2D20}	+	T_{2E22}	+
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T_{2A28}	-	T_{2B23}	+	T_{2C30}	+	T_{2D23}	-	T_{2E24}	+
T_{2A29}	+	T_{2B25}	+	T_{2C31}	-	T_{2D24}	-	T_{2E25}	-
T_{2A30}	+	T_{2B26}	+	T_{2C32}	+	T_{2D28}	+	T_{2E26}	+

T_{2A37}	-	T_{2B27}	-	T_{2C33}	-	T_{2D29}	+	T_{2E27}	+
T_{2A45}	+	T_{2B28}	-	T_{2C34}	+	T_{2D30}	+	T_{2E31}	-
T_{2A46}	+	T_{2B29}	-	T_{2C35}	+	T_{2D32}	-	T_{2E32}	-
T_{2A47}	-	T_{2B30}	+	T_{2C43}	+	T_{2D33}	+	T_{2E33}	+
T_{2A48}	-	T_{2B32}	+	T_{2C46}	+	T_{2D34}	+	T_{2E34}	+
T_{2A49}	-	T_{2B33}	+	T_{2C48}	-	T_{2D35}	+	T_{2E35}	+
T_{2A50}	-	T_{2B34}	+	T_{2C52}	+	T_{2D36}	+	T_{2E36}	-
T_{2A56}	-	T_{2B37}	+	T_{2C53}	-	T_{2D38}	+	T_{2E37}	+
T_{2A62}	-	T_{2B38}	+	T_{2C55}	-	T_{2D40}	-	T_{2E38}	-
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T_{2A64}	+	T_{2B40}	-	T_{2C59}	-	T_{2D42}	-	T_{2E40}	+
T_{2A66}	+	T_{2B41}	-	T_{2C60}	-	T_{2D43}	+	T_{2E41}	-
		T_{2B42}	+	T_{2C63}	+	T_{2D52}	+	T_{2E42}	+
		T_{2B43}	+			T_{2D53}	+	T_{2E43}	+
		T_{2B45}	-			T_{2D56}	+	T_{2E45}	+
		T_{2B47}	+			T_{2D57}	+	T_{2E46}	+
		T_{2B50}	-			T_{2D58}	+	T_{2E47}	+
		T_{2B52}	-			T_{2D60}	+	T_{2E48}	+
		T_{2B53}	+			T_{2D61}	+	T_{2E49}	+
		T_{2B56}	+			T_{2D62}	+	T_{2E50}	+
		T_{2B59}	+			T_{2D63}	-	T_{2E52}	-
		T_{2B60}	+			T_{2D64}	+	T_{2E53}	-
		T_{2B61}	+			T_{2D65}	+	T_{2E55}	-
		T_{2B62}	+			T_{2D70}	+	T_{2E57}	-
		T_{2B63}	+			T_{2D72}	+	T_{2E58}	+
						T_{2D73}	+	T_{2E60}	+
						T_{2D76}	+	T_{2E61}	+
						T_{2D77}	-		
T_{3A3}	+	T_{3B1}	+	T_{3C6}	-	T_{3D2}	-	T_{3E1}	+
T_{3A4}	+	T_{3B2}	-	T_{3C7}	-	T_{3D4}	+	T_{3E6}	+
T_{3A10}	+	T_{3B6}	+	T_{3C8}	-	T_{3D5}	+	T_{3E7}	+
T_{3A11}	-	T_{3B8}	-	T_{3C11}	-	T_{3D6}	+	T_{3E8}	-
T_{3A12}	-	T_{3B9}	+	T_{3C12}	-	T_{3D7}	-	T_{3E11}	-
T_{3A13}	-	T_{3B12}	+	T_{3C14}	-	T_{3D8}	-	T_{3E12}	-
T_{3A20}	+	T_{3B16}	-	T_{3C16}	+	T_{3D9}	-	T_{3E15}	+
T_{3A25}	+	T_{3B19}	+	T_{3C19}	+	T_{3D17}	+	T_{3E17}	+
T_{3A27}	+	T_{3B22}	-	T_{3C22}	+	T_{3D19}	+	T_{3E22}	+
T_{3A29}	-	T_{3B23}	-	T_{3C28}	+	T_{3D20}	+	T_{3E23}	-
T_{3A30}	-	T_{3B25}	-	T_{3C30}	+	T_{3D22}	+	T_{3E24}	-
T_{3A45}	+	T_{3B26}	+	T_{3C32}	-	T_{3D28}	+	T_{3E26}	-
T_{3A46}	-	T_{3B30}	+	T_{3C34}	+	T_{3D29}	+	T_{3E27}	-
T_{3A64}	-	T_{3B32}	+	T_{3C35}	-	T_{3D30}	-	T_{3E33}	+
T_{3A66}	-	T_{3B33}	-	T_{3C43}	-	T_{3D33}	-	T_{3E34}	-
		T_{3B34}	-	T_{3C46}	+	T_{3D34}	-	T_{3E35}	+

+ anti-CMV scFv transgene detected
- anti-CMV scFv transgene not detected

4.6 Confirmation of anti-CMV scFv transgenes by Southern Blot

A total of 155 transgenic plants were analysed by Southern Blot hybridisation to further confirm insertion of the anti-CMV scFv transgene. Anti-CMV scFv transgene was detected in 60% of transgenic plants from T_0, T_1, T_2 and T_3 generations (Table 4.15). The detailed results of the Southern analysis are shown in Table 4.17. A higher detectable percentage was observed in T_2 progenies as compared to T_1 and T_3 (Figure 4.20). As for individual line performance, Line T_{0A} was confirmed to have the lowest percentage which was consistent with the PCR analysis results. Line T_{0D} achieved the highest percentage of detectable gene (Figure 4.21 and Figure 4.22). The details of the Southern analysis of T_1, T_2 and T_3 transgenic plants are tabulated in Table 4.17.

Generation	Number of plants analysed	Southern analysis		Percentage
		Detectable	Undetectable	
T_0	5	5	0	100%
T_1	50	29	21	58%
T_2	50	33	17	66%
T_3	50	26	24	52%
Total	155	93	62	60%

Table 4.15: Summary of Southern Blot Hybridization analysis for T_0, T_1, T_2 and T_3 transgenic plants

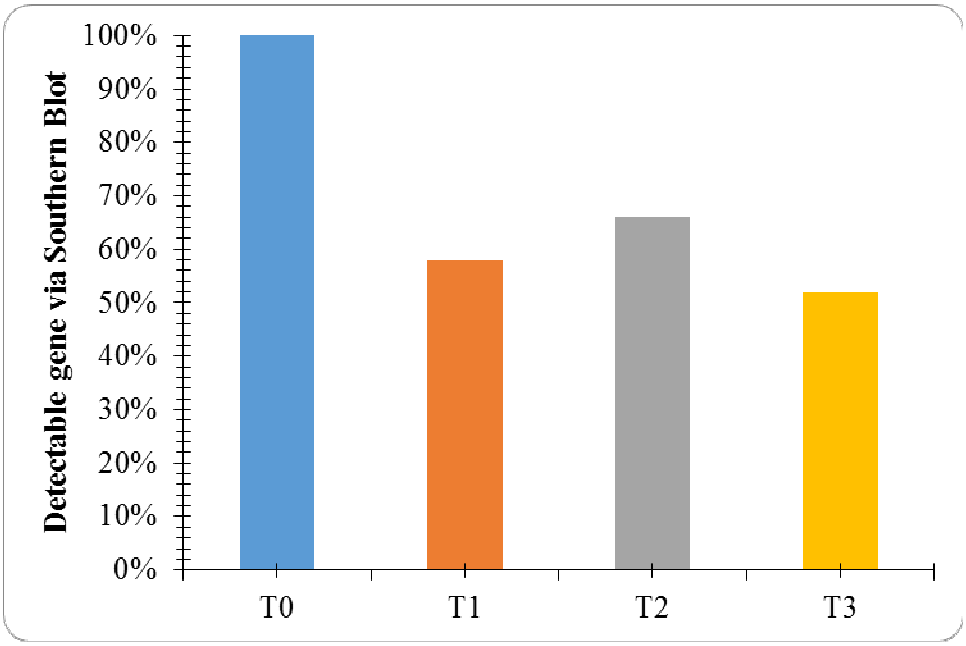


Figure 4.20: Summary of detectable gene via Southern Blot analysis in 4 generations

	Percentage anti-CMV scFv transgene detected via Southern Blot				
Parental line / Progenies	T_{0A}	T_{0B}	T_{0C}	T_{0D}	T_{0E}
T_1	30%	50%	60%	70%	80%
T_2	40%	80%	60%	80%	70%
T_3	14%	64%	30%	73%	64%

Table 4.16: Summary of Southern Blot analysis for Parental line T_{0A} - T_{0B}

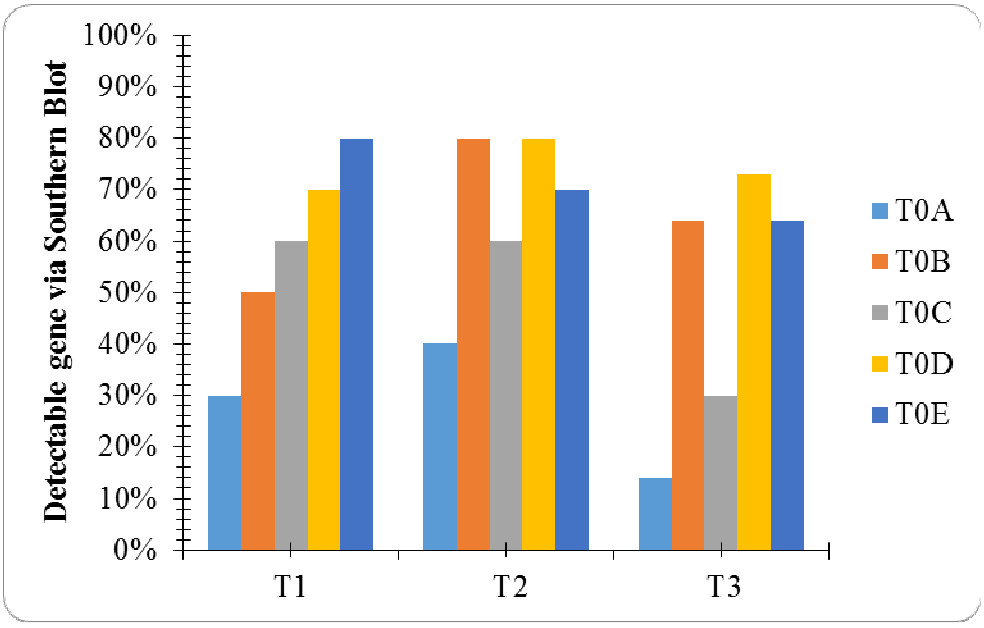


Figure 4.21: Percentage of detectable transgene via Southern analysis for 3 generations in 5 parental lines

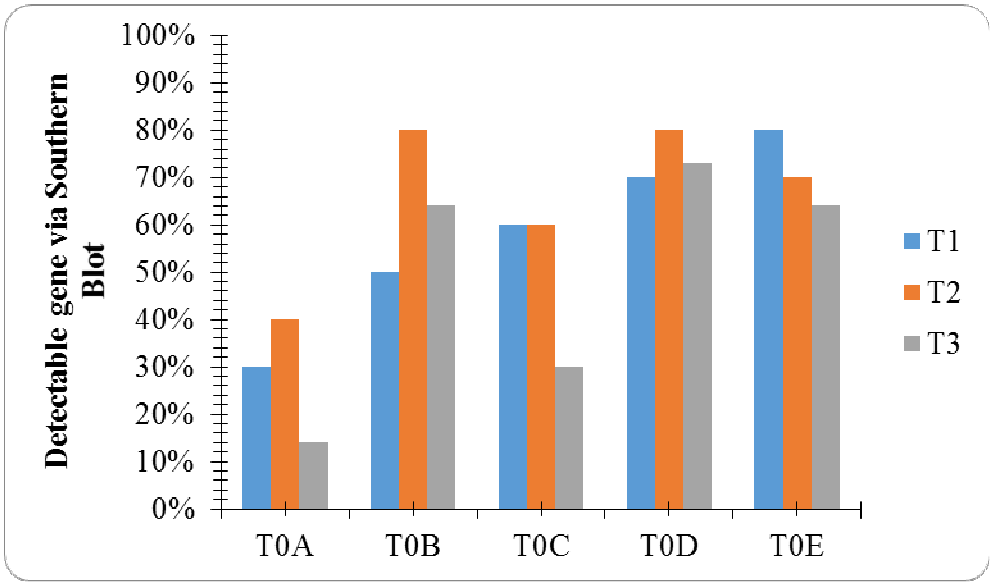


Figure 4.22: Percentage of detectable gene via Southern analysis for 5 parental lines in 3 generations

Table 4.17: Southern analysis of progenies from parental lines T_{0A} - T_{0E}

Parent Line T_{0A}	Anti-CMV scFv	Parent Line T_{0B}	Anti-CMV scFv	Parent Line T_{0C}	Anti-CMV scFv	Parent Line T_{0D}	Anti-CMV scFv	Parent Line T_{0E}	Anti-CMV scFv
T_{1A2}	+	T_{1B1}	-	T_{1C6}	-	T_{1D1}	+	T_{1E1}	+
T_{1A3}	+	T_{1B2}	+	T_{1C7}	-	T_{1D2}	-	T_{1E4}	+
T_{1A4}	-	T_{1B3}	+	T_{1C8}	-	T_{1D3}	-	T_{1E5}	-
T_{1A5}	-	T_{1B6}	+	T_{1C9}	+	T_{1D4}	-	T_{1E6}	+
T_{1A6}	-	T_{1B8}	-	T_{1C10}	+	T_{1D5}	+	T_{1E7}	+
T_{1A8}	-	T_{1B9}	+	T_{1C11}	+	T_{1D6}	+	T_{1E8}	+
T_{1A9}	-	T_{1B10}	-	T_{1C12}	-	T_{1D7}	+	T_{1E11}	+
T_{1A10}	+	T_{1B12}	+	T_{1C14}	+	T_{1D8}	+	T_{1E12}	+
T_{1A11}	-	T_{1B13}	-	T_{1C15}	+	T_{1D9}	+	T_{1E13}	-
T_{1A12}	-	T_{1B16}	-	T_{1C16}	+	T_{1D10}	+	T_{1E14}	+
T_{2A3}	+	T_{2B1}	+	T_{2C6}	+	T_{2D2}	+	T_{2E1}	+
T_{2A4}	+	T_{2B2}	+	T_{2C7}	+	T_{2D4}	+	T_{2E6}	+
T_{2A10}	-	T_{2B6}	-	T_{2C8}	-	T_{2D5}	+	T_{2E7}	+
T_{2A11}	+	T_{2B8}	-	T_{2C11}	-	T_{2D6}	-	T_{2E8}	-
T_{2A12}	-	T_{2B9}	+	T_{2C12}	-	T_{2D7}	-	T_{2E11}	+
T_{2A13}	-	T_{2B12}	+	T_{2C14}	+	T_{2D8}	+	T_{2E12}	-
T_{2A20}	-	T_{2B16}	+	T_{2C16}	+	T_{2D9}	+	T_{2E15}	+
T_{2A25}	+	T_{2B19}	+	T_{2C19}	+	T_{2D17}	+	T_{2E17}	-
T_{2A27}	-	T_{2B22}	+	T_{2C22}	-	T_{2D19}	+	T_{2E22}	+
T_{2A29}	-	T_{2B23}	+	T_{2C28}	+	T_{2D20}	+	T_{2E23}	+
T_{3A3}	-	T_{3B1}	+	T_{3C16}	-	T_{3D4}	+	T_{3E1}	+
T_{3A4}	+	T_{3B6}	+	T_{3C19}	+	T_{3D5}	+	T_{3E6}	+
T_{3A10}	-	T_{3B9}	+	T_{3C22}	-	T_{3D6}	+	T_{3E7}	-
T_{3A20}	-	T_{3B12}	-	T_{3C28}	-	T_{3D17}	-	T_{3E15}	+
T_{3A25}	-	T_{3B19}	-	T_{3C30}	-	T_{3D19}	+	T_{3E17}	+
T_{3A27}	-	T_{3B26}	+	T_{3C34}	+	T_{3D20}	-	T_{3E22}	-
T_{3A45}	-	T_{3B30}	+	T_{3C46}	-	T_{3D22}	+	T_{3E33}	+
		T_{3B32}	+	T_{3C52}	+	T_{3D28}	+	T_{3E35}	-
		T_{3B37}	-	T_{3C56}	-	T_{3D29}	-	T_{3E39}	+
		T_{3B38}	-	T_{3C63}	-	T_{3D36}	+	T_{3E40}	-
		T_{3B42}	+			T_{3D41}	+	T_{3E42}	+

+ anti-CMV scFv transgene detected
- anti-CMV scFv transgene not detected

Figure 4.23 (A) illustrates the total genomic DNA (2µg) of transgenic plant samples that were digested with *Hha 1/Nco 1 /Pml 1* restriction enzymes. They were then separated on 1% agarose gel before being blotted to a positive charge nylon membrane. Southern hybridisation was done using DIG-labelled anti-CMV scFv probe. The expected ~ 797 bp fragment was detected in the transgenic plants as shown in Figure 4.23 (B).

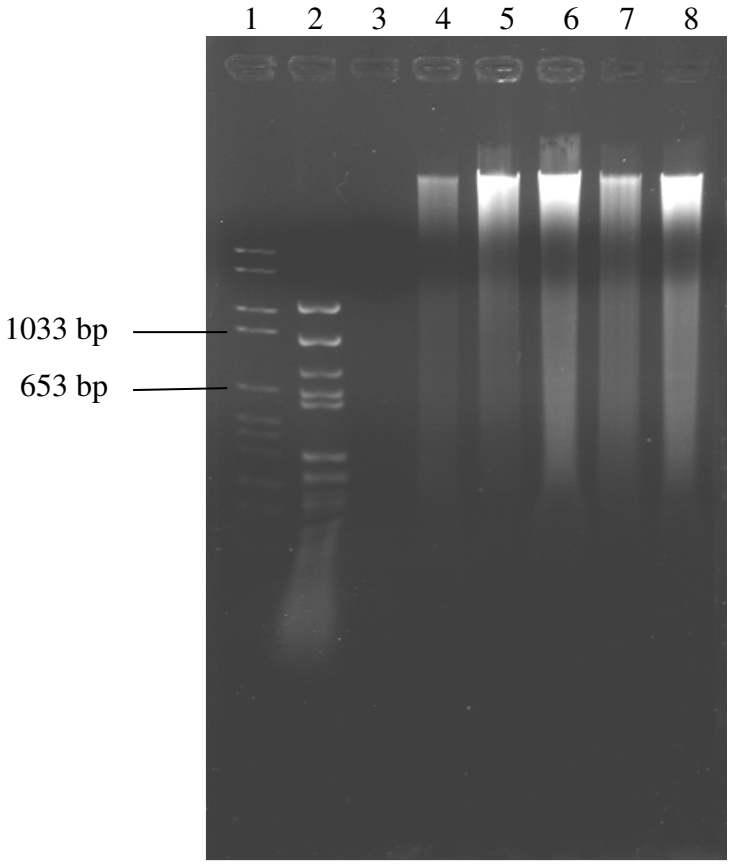


Figure 4.23 (A) *Hha 1/Nco 1 /Pml 1* digested genomic DNA in test plants

Lane 1: DIG- labelled marker VI
Lane 2: Pure plasmid pUMSCFV-CMV1 (positive control)
Lane 4, 5, 6 and 7: T_0, T_1, T_2 and T_3 transgenic samples
Lane 8: Wild type tobacco (negative control)

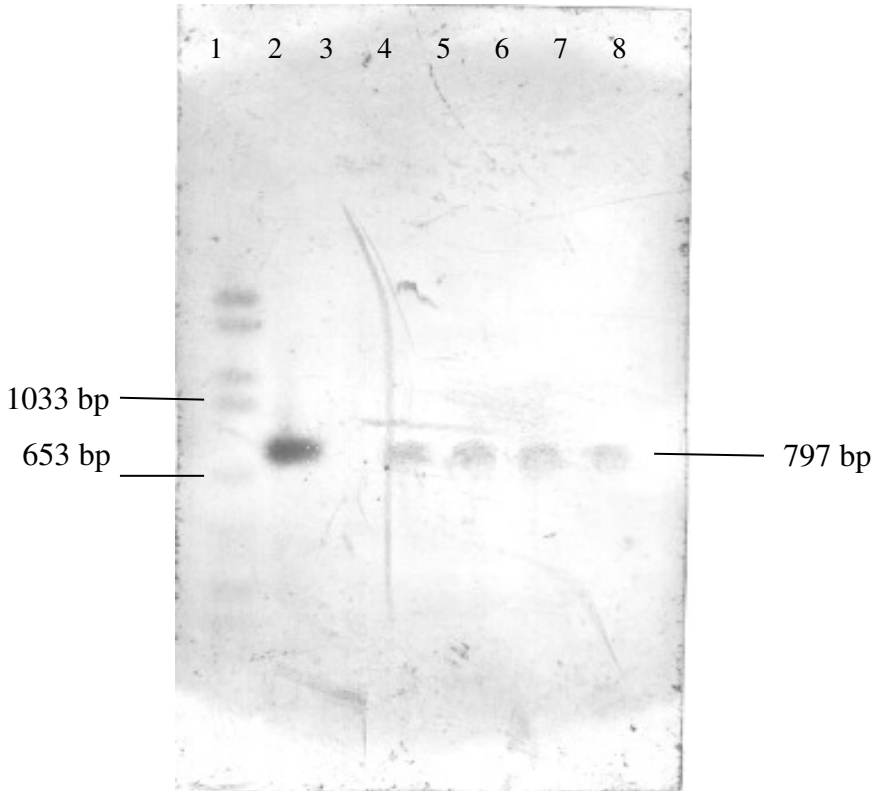


Figure 4.23 (B): ~ 797 bp fragment detected via Southern Blot in the transgenic plants

Lane 1: DIG- labelled marker VI
Lane 2: Pure plasmid pUMSCFV-CMV1 (positive control)
Lane 4, 5, 6 and 7: T_0, T_1, T_2 and T_3 transgenic samples
Lane 8: Wild type tobacco (negative control)

4.7 Detection of expressed anti-CMV scFv by Western Blot

The expressed plant-derived anti-CMV scFv antibodies were detected by Western Blot. Only 30% of the 155 transgenic plants were found to express anti-CMV antibodies to a detectable level over the 4 generations (Table 4.18). The percentage was reduced from 80% in T_0 generation to 26% in T_2 generation. We did not find detectable expressed gene in T_3 generations for all 5 lines. Figure 4.24 represents the detectable percentage of antibody in T_0, T_1, T_2 and T_3 plants. The details of the results are tabulated in Table 4.20. From Figure 4.25 and Figure 4.26, we conclude that T_{0D} is the best performing line. As expected, T_{0A} had the lowest detectable percentage amongst the generations. Table 4.19 shows the summary of Western Blot results for Parental line T_{0A} - $T_{0E.}$

Generation	Number of plants analysed	Western analysis		Percentage
		Detectable	Undetectable	
T_0	5	4	1	80%
T_1	50	29	21	58%
T_2	50	13	37	26%
T_3	50	0	50	0%
Total	155	46	109	30%

Table 4.18: Summary of Western analysis for $T_{0,}$ T_1, T_2 and T_3 transgenic plants

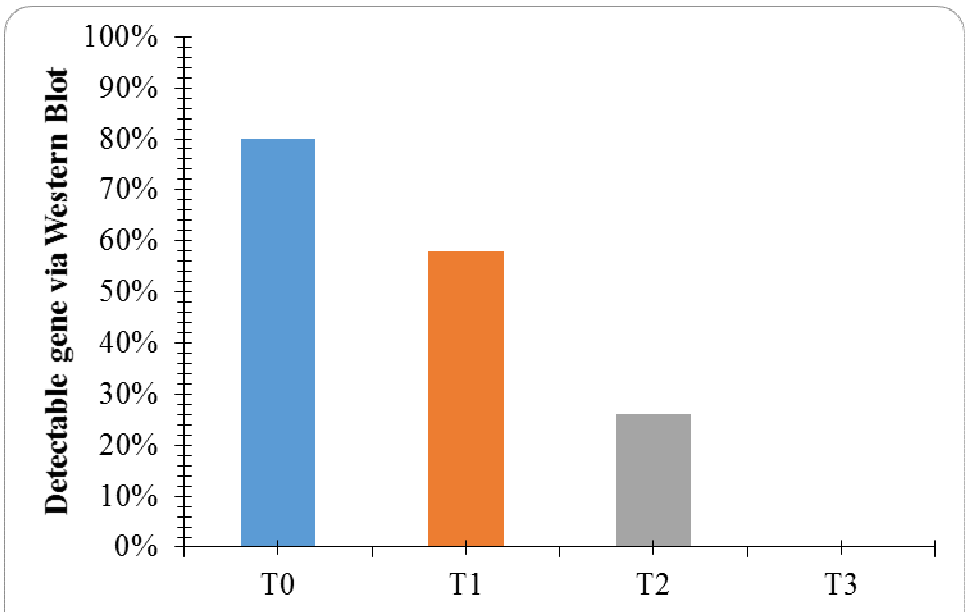


Figure 4.24: Summary of detectable gene via Western analysis in 4 generations

	Percentage anti-CMV scFv transgene detected via Western Blot				
Parental line / Progenies	T_{0A}	T_{0B}	T_{0C}	T_{0D}	T_{0E}
T_1	30%	50%	60%	80%	70%
T_2	10%	30%	20%	40%	30%
T_3	-	-	-	-	-

Table 4.19: Summary of Western Blot analysis for Parental line T_{0A} - T_{0E}

- not detectable

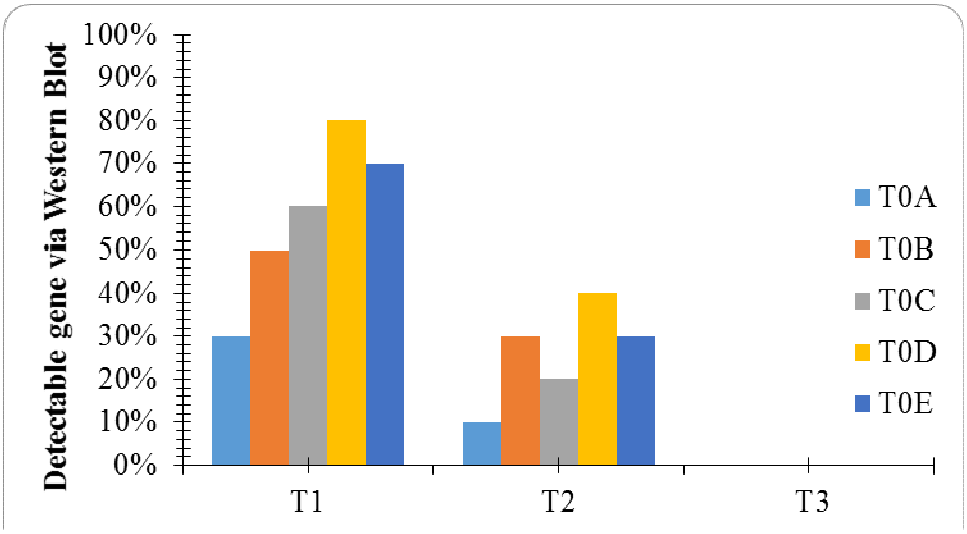


Figure 4.25: Percentage of detectable gene via Western analysis for 5 parental lines in 3 generations

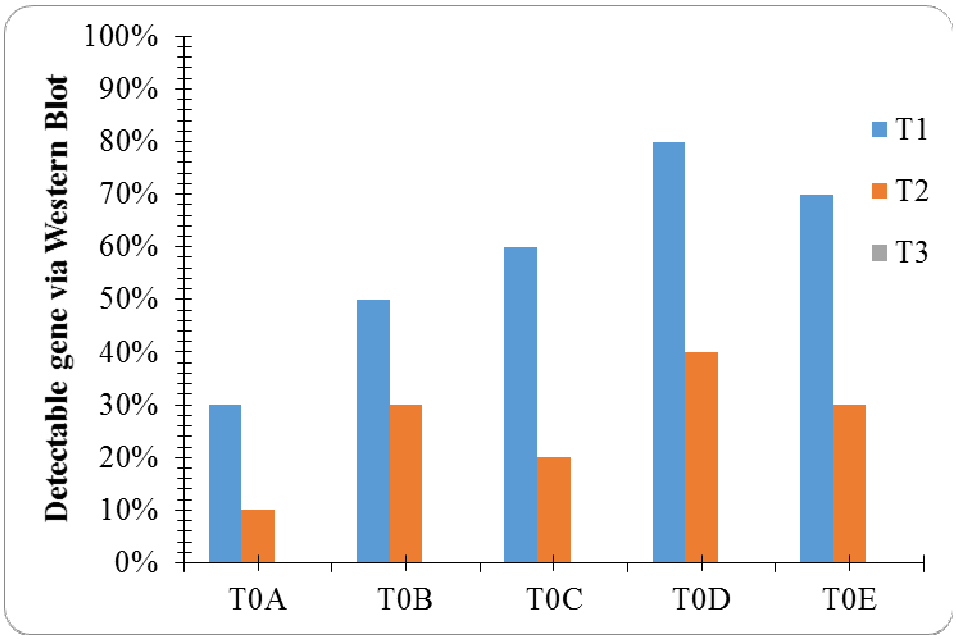


Figure 4.26: Percentage of detectable transgene via Western analysis for 3 generations in 5 parental lines

Table 4.20: Western analysis of progenies from parental lines T_{0A} - T_{0E}

Parent Line T_{0A}	Anti-CMV scFv	Parent Line T_{0B}	Anti-CMV scFv	Parent Line T_{0C}	Anti-CMV scFv	Parent Line T_{0D}	Anti-CMV scFv	Parent Line T_{0E}	Anti-CMV scFv
T_{1A2}	+	T_{1B1}	-	T_{1C6}	-	T_{1D1}	+	T_{1E1}	+
T_{1A3}	+	T_{1B2}	+	T_{1C7}	-	T_{1D2}	+	T_{1E4}	-
T_{1A4}	-	T_{1B3}	+	T_{1C8}	-	T_{1D3}	-	T_{1E5}	-
T_{1A5}	-	T_{1B6}	+	T_{1C9}	+	T_{1D4}	-	T_{1E6}	+
T_{1A6}	-	T_{1B8}	-	T_{1C10}	+	T_{1D5}	+	T_{1E7}	+
T_{1A8}	-	T_{1B9}	+	T_{1C11}	+	T_{1D6}	+	T_{1E8}	+
T_{1A9}	-	T_{1B10}	-	T_{1C12}	-	T_{1D7}	+	T_{1E11}	+
T_{1A10}	+	T_{1B12}	+	T_{1C14}	+	T_{1D8}	+	T_{1E12}	+
T_{1A11}	-	T_{1B13}	-	T_{1C15}	+	T_{1D9}	+	T_{1E13}	-
T_{1A12}	-	T_{1B16}	-	T_{1C16}	+	T_{1D10}	+	T_{1E14}	+
T_{2A3}	+	T_{2B1}	+	T_{2C6}	-	T_{2D2}	+	T_{2E1}	+
T_{2A4}	-	T_{2B2}	-	T_{2C7}	-	T_{2D4}	-	T_{2E6}	-
T_{2A10}	-	T_{2B6}	-	T_{2C8}	-	T_{2D5}	+	T_{2E7}	-
T_{2A11}	-	T_{2B8}	-	T_{2C11}	-	T_{2D6}	-	T_{2E8}	-
T_{2A12}	-	T_{2B9}	-	T_{2C12}	-	T_{2D7}	-	T_{2E11}	+
T_{2A13}	-	T_{2B12}	-	T_{2C14}	-	T_{2D8}	-	T_{2E12}	-
T_{2A20}	-	T_{2B16}	+	T_{2C16}	-	T_{2D9}	-	T_{2E15}	-
T_{2A25}	-	T_{2B19}	-	T_{2C19}	+	T_{2D17}	+	T_{2E17}	-
T_{2A27}	-	T_{2B22}	+	T_{2C22}	-	T_{2D19}	-	T_{2E22}	+
T_{2A29}	-	T_{2B23}	-	T_{2C28}	+	T_{2D20}	+	T_{2E23}	-
T_{3A3}	-	T_{3B1}	-	T_{3C16}	-	T_{3D4}	-	T_{3E1}	-
T_{3A4}	-	T_{3B6}	-	T_{3C19}	-	T_{3D5}	-	T_{3E6}	-
T_{3A10}	-	T_{3B9}	-	T_{3C22}	-	T_{3D6}	-	T_{3E7}	-
T_{3A20}	-	T_{3B12}	-	T_{3C28}	-	T_{3D17}	-	T_{3E15}	-
T_{3A25}	-	T_{3B19}	-	T_{3C30}	-	T_{3D19}	-	T_{3E17}	-
T_{3A27}	-	T_{3B26}	-	T_{3C34}	-	T_{3D20}	-	T_{3E22}	-
T_{3A45}	-	T_{3B30}	-	T_{3C46}	-	T_{3D22}	-	T_{3E33}	-
		T_{3B32}	-	T_{3C52}	-	T_{3D28}	-	T_{3E35}	-
		T_{3B37}	-	T_{3C56}	-	T_{3D29}	-	T_{3E39}	-
		T_{3B38}	-	T_{3C63}	-	T_{3D36}	-	T_{3E40}	-
		T_{3B42}	-			T_{3D41}	-	T_{3E42}	-

+ anti-CMV scFv transgene detected
- anti-CMV scFv transgene not detected

In Figure 4.27 (A), total protein samples from 4 selected T_0 (Lane 1 and 2) and T_1 (Lane 3 and 4) transgenic plants and wild type tobacco plant (Lane 5) were extracted and separated on 12% SDS-PAGE followed by Coomassie Blue staining. Protein sample from *E.coli* was isolated to serve as positive control (Lane 6). Broad range protein marker (NEB) (Lane 7) was used as ladder. 32 kDa anti-CMV scFv antibodies were detected as shown in Figure 4.27 (B).

(A)

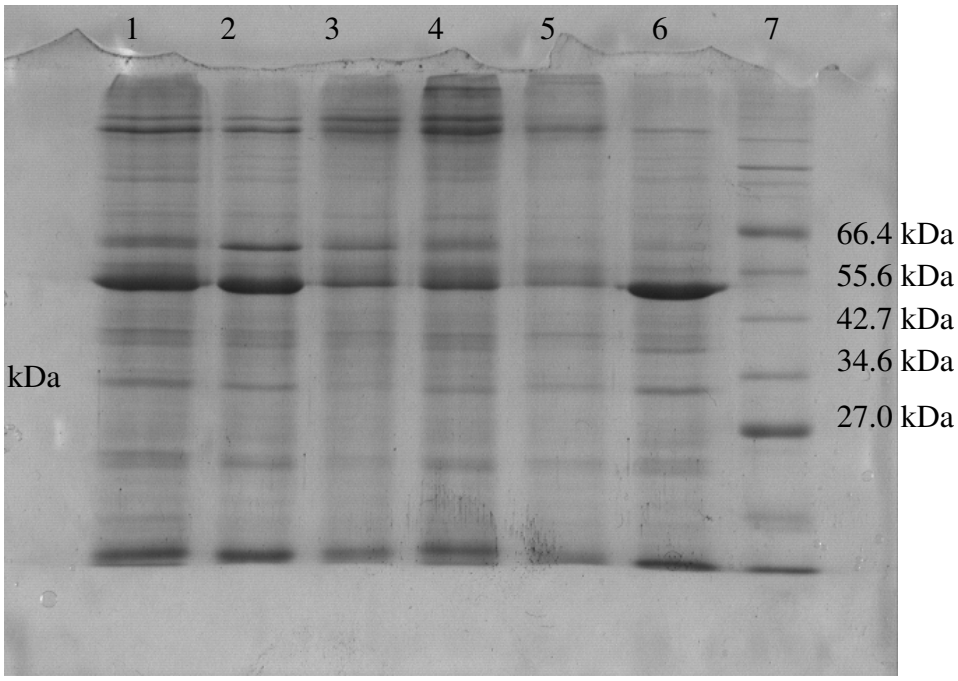


Figure 4.27 (A): Total protein samples separated on 12% SDS-PAGE

Lane 1, 2: T_0 transgenic plants
Lane 3, 4: T_1 transgenic plants
Lane 5: Wild type tobacco plant
Lane 6: Positive control
Lane 7: Broad range protein marker (NEB)

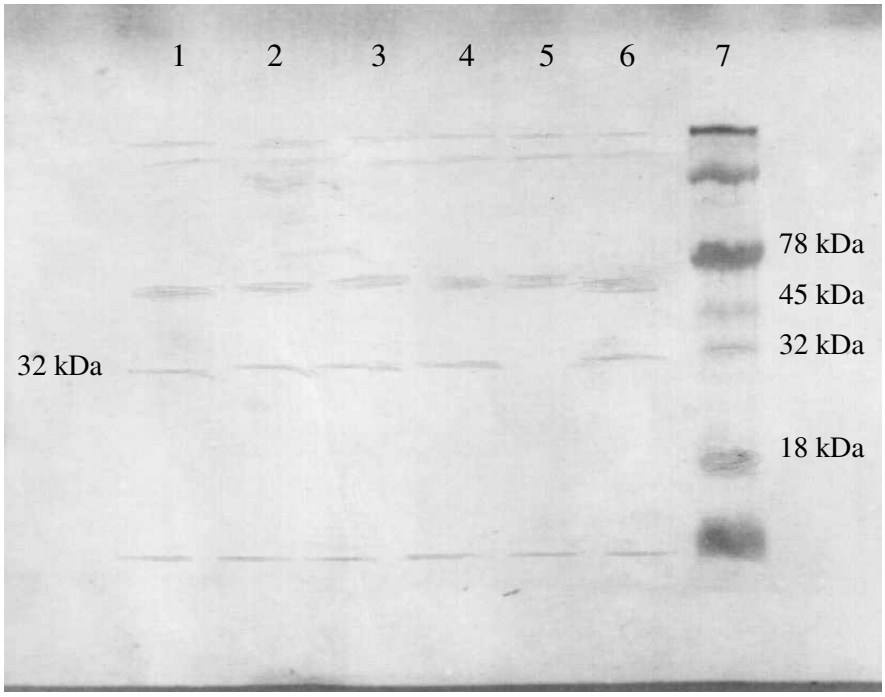


Figure 4.27 (B): 32 kDa anti-CMV scFv antibodies detected on membrane

Lane 1, 2: T_0 transgenic plants
Lane 3, 4: T_1 transgenic plants
Lane 5: Wild type tobacco plant
Lane 6: Positive control
Lane 7: Kaleidoscope Pre-stained Standards (Bio Rad)

4.8 Functionality Test with Dot Blot Assay

A more sensitive dot blot test was performed on all 155 plant protein samples to test the functionality of the expressed transgenes. CMV coat protein from bacteria was used as a probe to bind to the test samples. Anti-FLAG antibody (Sigma-Aldrich) and Alkaline Phosphatase (AP) conjugated anti-mouse IgG were used to detect the anti-CMV scFv antibodies. Anti-CMV scFv derived from *E.coli* as positive control showed high signal intensity. Figure 4.28 demonstrates the functionality of expressed anti-CMV scFv in transgenic plants.

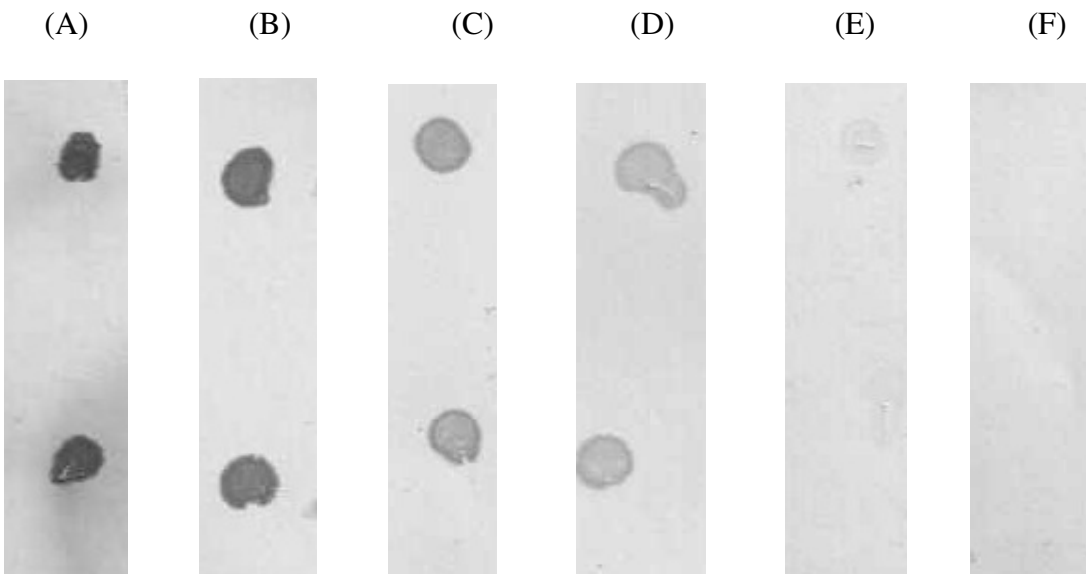


Figure 4.28: Different intensity signals indicate the expression level of anti-CMV scFv antibodies.

(A) Positive Control
(B) T_0 transgenic plants
(C) T_1 transgenic plants
(D) T_2 transgenic plants
(E) T_3 transgenic plants
(F) Wild type tobacco plants

Table 4.21 demonstrates the number of detectable dot in the transgenic plants. The results of detected functional genes are presented in Figure 4.28. These results reveal that Line T_{0A} has the least detectable functional gene. Line T_{0D} remained the most promising candidate. A detectable functional gene at T_3 generation was discovered (Table 4.21). Table 4.23 shows the summary of Dot Blot analysis of progenies from parental lines T_{0A} - $T_{0E.}$

Generation	Number of plants analysed	Dot Blot analysis		Percentage
		Detectable	Undetectable	
T_0	5	5	0	100%
T_1	50	32	18	64%
T_2	50	18	32	36%
T_3	50	2	48	4%
Total	155	57	98	38%

Table 4.21: Summary of Dot Blot analysis for $T_{0,}$ T_1, T_2 and T_3 transgenic plants

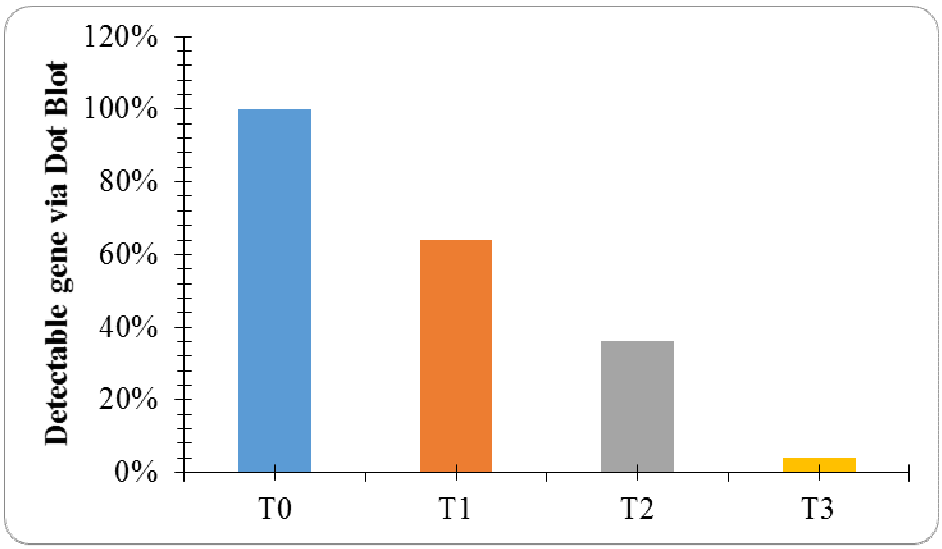


Figure 4.29: Summary of detected functional gene via Dot Blot analysis in 4 generations

	Percentage anti-CMV scFv transgene detected via Dot Blot				
Parental line / Progenies	T_{0A}	T_{0B}	T_{0C}	T_{0D}	T_{0E}
T_1	30%	60%	60%	90%	80%
T_2	10%	50%	20%	60%	40%
T_3	-	-	-	9%	9%

Table 4.22: Summary of Dot Blot analysis for Parental line T_{0A} - T_{0B}

- not detectable

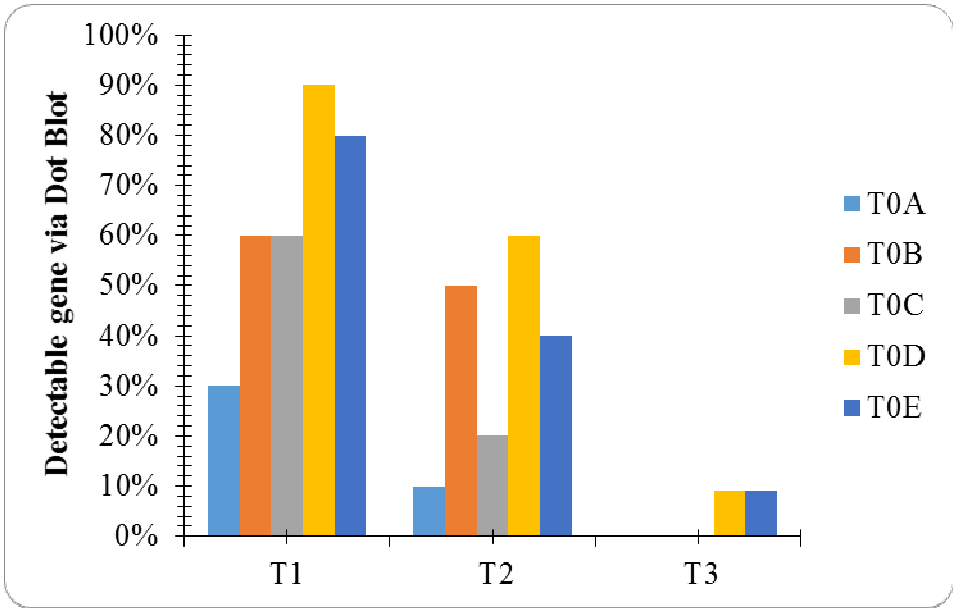


Figure 4.30: Percentage of detectable gene via Dot Blot analysis for 5 parental lines in 3 generations

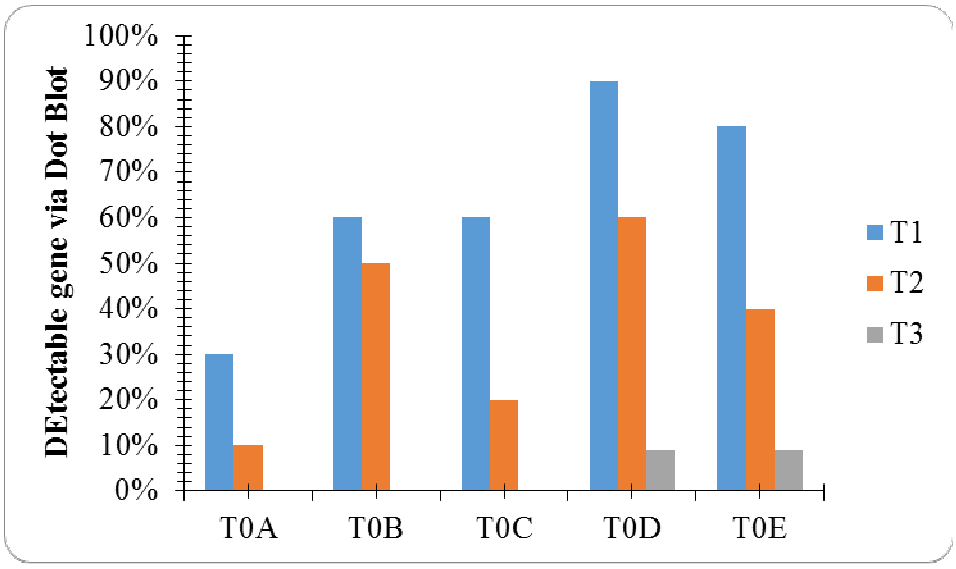


Figure 4.31: Percentage of detectable transgene via Dot Blot analysis for 3 generations in 5 parental lines

Table 4.23: Dot Blot analysis of progenies from parental lines T_{0A} - T_{0E}

Parent Line T_{0A}	Anti-CMV scFv	Parent Line T_{0B}	Anti-CMV scFv	Parent Line T_{0C}	Anti-CMV scFv	Parent Line T_{0D}	Anti-CMV scFv	Parent Line T_{0E}	Anti-CMV scFv
T_{1A2}	+	T_{1B1}	-	T_{1C6}	-	T_{1D1}	+	T_{1E1}	+
T_{1A3}	+	T_{1B2}	+	T_{1C7}	-	T_{1D2}	-	T_{1E4}	+
T_{1A4}	-	T_{1B3}	+	T_{1C8}	-	T_{1D3}	+	T_{1E5}	-
T_{1A5}	-	T_{1B6}	+	T_{1C9}	+	T_{1D4}	+	T_{1E6}	+
T_{1A6}	-	T_{1B8}	+	T_{1C10}	+	T_{1D5}	+	T_{1E7}	+
T_{1A8}	-	T_{1B9}	+	T_{1C11}	+	T_{1D6}	+	T_{1E8}	+
T_{1A9}	-	T_{1B10}	-	T_{1C12}	-	T_{1D7}	+	T_{1E11}	+
T_{1A10}	+	T_{1B12}	+	T_{1C14}	+	T_{1D8}	+	T_{1E12}	+
T_{1A11}	-	T_{1B13}	-	T_{1C15}	+	T_{1D9}	+	T_{1E13}	-
T_{1A12}	-	T_{1B16}	-	T_{1C16}	+	T_{1D10}	+	T_{1E14}	+
T_{2A3}	+	T_{2B1}	+	T_{2C6}	-	T_{2D2}	+	T_{2E1}	+
T_{2A4}	-	T_{2B2}	-	T_{2C7}	-	T_{2D4}	-	T_{2E6}	-
T_{2A10}	-	T_{2B6}	-	T_{2C8}	-	T_{2D5}	+	T_{2E7}	-
T_{2A11}	-	T_{2B8}	-	T_{2C11}	-	T_{2D6}	-	T_{2E8}	-
T_{2A12}	-	T_{2B9}	+	T_{2C12}	-	T_{2D7}	-	T_{2E11}	+
T_{2A13}	-	T_{2B12}	+	T_{2C14}	-	T_{2D8}	+	T_{2E12}	-
T_{2A20}	-	T_{2B16}	+	T_{2C16}	-	T_{2D9}	-	T_{2E15}	-
T_{2A25}	-	T_{2B19}	-	T_{2C19}	+	T_{2D17}	+	T_{2E17}	-
T_{2A27}	-	T_{2B22}	+	T_{2C22}	-	T_{2D19}	+	T_{2E22}	+
T_{2A29}	-	T_{2B23}	-	T_{2C28}	+	T_{2D20}	+	T_{2E23}	+
T_{3A3}	-	T_{3B1}	-	T_{3C16}	-	T_{3D4}	-	T_{3E1}	-
T_{3A4}	-	T_{3B6}	-	T_{3C19}	-	T_{3D5}	-	T_{3E6}	+
T_{3A10}	-	T_{3B9}	-	T_{3C22}	-	T_{3D6}	-	T_{3E7}	-
T_{3A20}	-	T_{3B12}	-	T_{3C28}	-	T_{3D17}	-	T_{3E15}	-
T_{3A25}	-	T_{3B19}	-	T_{3C30}	-	T_{3D19}	-	T_{3E17}	-
T_{3A27}	-	T_{3B26}	-	T_{3C34}	-	T_{3D20}	-	T_{3E22}	-
T_{3A45}	-	T_{3B30}	-	T_{3C46}	-	T_{3D22}	-	T_{3E33}	-
		T_{3B32}	-	T_{3C52}	-	T_{3D28}	-	T_{3E35}	-
		T_{3B37}	-	T_{3C56}	-	T_{3D29}	-	T_{3E39}	-
		T_{3B38}	-	T_{3C63}	-	T_{3D36}	-	T_{3E40}	-
		T_{3B42}	-			T_{3D41}	+	T_{3E42}	-

+ anti-CMV scFv transgene detected
- anti-CMV scFv transgene not detected

4.9 Functionality Test of anti-CMV scFv antibodies with ELISA

Total plant protein was successfully isolated from 155 transgenic tobacco leaves of 4 generations. ELISA method was performed to determine the functionality of anti-CMV scFv antibodies. In this study, expressed CMV coat proteins served as antigen coating the ELISA wells. Anti-CMV scFv antibodies from plant extracts were used as the capture antibody. Detection was carried out using anti-FLAG as the primary antibody and Alkaline Phosphatase (AP) conjugated anti-mouse IgG was used as the second antibody. The positive control used was *E. coli* H2151 carrying anti-CMV scFv construct. Wild type tobacco plants were used as negative control.

Colour development (yellow) and colour intensity were observed after the addition of PNP substrate. Table 4.25 (A) – (G) shows the absorption value on ELISA microplate measured at 405 nm wavelength. The results shown in the Table 4.24 are ratios obtained from absorbance at wavelength 405 over the blank control for different generations of transgenic plants. The overall results are presented in Figure 4.32. These results reveal that detectable functional gene decreased by successive generations. Strong intensity of yellow colour was observed for T_0 and T_1 transgenic plants. A mild yellow colour was obtained for T_2 plant samples. ELISA assays for T_3 transgenic plants did not show any colour development. The raw data tabulated in Table 4.29 (A) – (C) were used to perform Analysis of Variance (ANOVA) which is used to compare the difference between the groups of data.

Plant samples	Absorbance at 405 nm				
	Replicate 1	Replicate 2	Mean	Mean – blank	Standard Deviation
Positive Control	2.326	2.178	2.252	2.079	0.1047
Negative Control	0.310	0.313	0.312	0.139	0.0021
Blank	0.179	0.168	0.173	0.000	
T_0	1.963	1.954	1.959	1.786	0.0064
T_1	1.859	1.886	1.873	1.700	0.0191
T_2	1.364	1.343	1.339	1.166	0.0148
T_3	0.516	0.522	0.519	0.346	0.0042

Table 4.24: Ratio obtained from absorbance of test samples over the mean of blank at 405nm

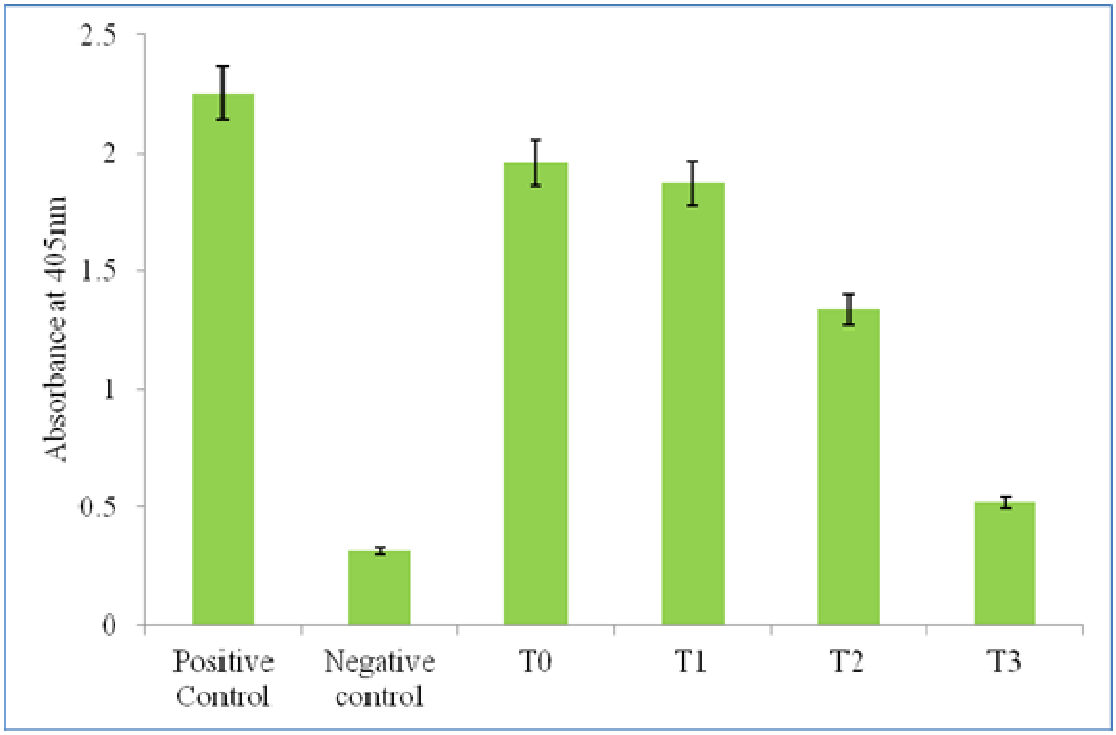


Figure 4.32: Ratio obtained from absorbance of test samples at 405nm is presented in the bar chart above

Table 4.25: Absorbance values at 405nm

(A) Microtiter plate 1 (T_0)

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.365	0.192	0.308	1.851	2.133	1.843	1.974	2.016				
B												
C	2.271	0.216	0.286	1.926	2.111	1.869	1.786	2.078				
D												
E												
F												
G												
H												

(B) Microtiter plate 2 (T_1)

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.302	0.187	0.278	1.987	2.002	1.765	1.945	1.879	1.989	1.766	2.023	1.833
B	1.725	1.590	1.874	1.932	2.003	1.992	1.926	1.768	1.945	1.893	1.835	1.879
C	1.693	1.087	1.900	2.114	1.892	1.691	1.907	1.954	2.017	1.945	1.682	1.754
D	1.989	1.786	1.745	1.809	1.732	1.893	2.035	1.789	1.882	1.673	2.071	1.826
E	1.932	1.880	1.915	1.708	2.102							
F												
G												
H												

(C) Microtiter plate 3 (T_1) - R

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.376	0.157	0.318	2.007	2.134	1.990	1.893	1.995	1.873	1.908	1.997	1.903
B	1.745	1.306	2.082	2.145	1.890	1.986	1.836	1.745	1.962	1.882	1.892	1.994
C	1.859	2.053	2.052	1.854	1.967	2.012	1.795	1.982	2.200	1.565	1.490	2.006
D	1.651	1.793	1.654	1.576	1.732	1.893	2.035	1.915	1.882	1.708	2.071	1.826
E	1.988	1.945	1.837	1.804	2.005							
F												
G												
H												

(D) Microtiter plate 4 (T_2)

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.381	0.128	0.298	1.587	1.143	1.463	1.564	1.113	1.298	1.234	1.219	1.439
B	1.436	1.624	1.119	1.108	1.127	1.378	1.743	1.553	1.236	1.345	1.198	1.256
C	1.213	1.327	1.431	1.116	1.002	1.342	1.521	1.507	1.554	1.578	1.335	1.442
D	1.289	1.209	1.961	1.235	1.589	1.456	1.865	1.673	1.234	1.201	1.115	1.310
E	1.238	1.267	1.281	1.350	1.365							
F												
G												
H												

(E) Microtiter plate 5 (T_2) R

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.997	0.109	0.310	1.501	1.256	1.382	1.286	1.078	1.184	1.371	1.199	1.234
B	1.198	1.871	1.078	1.267	1.212	1.528	1.482	1.397	1.120	1.376	1.318	1.389
C	1.498	1.327	1.431	1.116	1.002	1.342	1.521	1.213	1.554	1.578	1.323	1.598
D	1.304	1.389	1.442	1.176	1.327	1.335	1.478	1.983	1.119	1.210	1.273	1.584
E	1.280	1.209	1.107	1.311	1.382							
F												
G												
H												

(F) Microtiter plate 6 (T_3)

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.254	0.208	0.354	0.504	0.476	0.433	0.564	0.429	0.601	0.447	0.309	0.567
B	0.513	0.528	0.623	0.498	0.402	0.519	0.434	0.593	0.546	0.506	0.599	0.487
C	0.623	0.629	0.507	0.428	0.503	0.607	0.576	0.604	0.602	0.472	0.572	0.430
D	0.527	0.567	0.499	0.525	0.578	0.653	0.406	0.663	0.497	0.390	0.561	0.498
E	0.492	0.510	0.439	0.402	0.440							
F												
G												
H												

(G) Microtiter plate 7 (T_3) - R

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.067	0.189	0.337	0.525	0.493	0.479	0.525	0.477	0.567	0.423	0.606	0.538
B	0.474	0.499	0.596	0.505	0.452	0.549	0.427	0.608	0.598	0.479	0.465	0.521
C	0.601	0.387	0.555	0.390	0.574	0.633	0.528	0.589	0.583	0.494	0.578	0.489
D	0.583	0.504	0.526	0.498	0.599	0.642	0.612	0.633	0.502	0.590	0.441	0.533
E	0.505	0.477	0.473	0.390	0.400							
F												
G												
H												

A1 – Positive Control
A2 – Blank
A3 – Negative Control
} Table 4.11 (A) - (G)

C1 - Positive Control
C2 – Blank
C3 – Negative Control
} Table 4.11 (A)

Analysis of Variance (ANOVA) was performed using Statistica® program to compare the difference between the data obtained. Levene's test was carried out to test the homogeneity of the variances of the groups (Table 4.27) using the data shown in Table 4.26. Unequal HSD (Honest Significant Difference) Test was applied in this case as the sample sizes were unequal. The F statistic is used to reveal whether the means across the groups are significantly different. Significant differences occur between test plants if $P \leq 0.05$, is rejected. Overall, the results indicated that $p = 0.136$ (Table 4.27), therefore there were no statistically significant differences amongst test plants. Table 4.28 shows test samples in 3 homogenous groups according to their significance differences. The visual presentation of the results is shown in Figure 4.33.

	Descriptive Statistics						
Effect	Level of Factor	N	Log10Abs Mean	Log10Abs Std.Dev.	Log10Abs Std.Err	Log10Abs -95.00%	Log10Abs +95.00%
Total		156	0.298914	0.137632	0.011019	0.277147	0.320682
Test Plants	T1A	10	0.438455	0.015181	0.004801	0.427595	0.449315
Test Plants	T1B	10	0.430889	0.028817	0.009113	0.410275	0.451504
Test Plants	T1C	10	0.434465	0.023931	0.007568	0.417346	0.451584
Test Plants	T1D	10	0.417228	0.020817	0.006583	0.402337	0.432120
Test Plants	T1E	10	0.433544	0.019110	0.006043	0.419873	0.447214
Test Plants	T2A	10	0.336881	0.027331	0.008643	0.317329	0.356432
Test Plants	T2B	10	0.347625	0.040832	0.012912	0.318416	0.376834
Test Plants	T2C	10	0.344134	0.033838	0.010700	0.319928	0.368340
Test Plants	T2D	10	0.365762	0.031412	0.009933	0.343291	0.388232
Test Plants	T2E	10	0.342186	0.036454	0.011528	0.316108	0.368264
Test Plants	T3A	7	0.113742	0.018639	0.007045	0.096504	0.130981
Test Plants	T3B	11	0.119774	0.020877	0.006295	0.105749	0.133800
Test Plants	T3C	10	0.123905	0.019802	0.006262	0.109739	0.138070
Test Plants	T3D	11	0.130681	0.018025	0.005435	0.118572	0.142790
Test Plants	T3E	11	0.111753	0.021095	0.006361	0.097581	0.125925
Test Plants	P	3	0.486504	0.014547	0.008399	0.450368	0.522641
Test Plants	N	3	0.079967	0.031780	0.018348	0.001020	0.158914

Table 4.26: Descriptive Statistics of Absorbance Test

	Levene's Test for Homogeneity of Variances Effect: "Test Plants" Degrees of freedom for all F's: 16, 139			
	MS Effect	MS Error	F	p
Log10Abs	0.000357	0.000250	1.430194	0.136044

Table 4.27: Levene's Test indicates P = 0.136

Cell No.	Unequal N HSD; variable Log10Abs Homogenous Groups, alpha = .05000 (Non-Exhaustive Search) Error: Between MS = .00068, df = 139.00 Test Plants	Log10Abs Mean	1	2	3
17	N	0.079967	****		
15	T3E	0.111753	****		
11	T3A	0.113742	****		
12	T3B	0.119774	****		
13	T3C	0.123905	****		
14	T3D	0.130681	****		
6	T2A	0.336881		****	
10	T2E	0.342186		****	
8	T2C	0.344134		****	
7	T2B	0.347625		****	
9	T2D	0.365762		****	
4	T1D	0.417228			****
2	T1B	0.430889			****
5	T1E	0.433544			****
3	T1C	0.434465			****
1	T1A	0.438455			****
16	P	0.486504			****

Table 4.28: Tukey Unequal Honest Significant Test

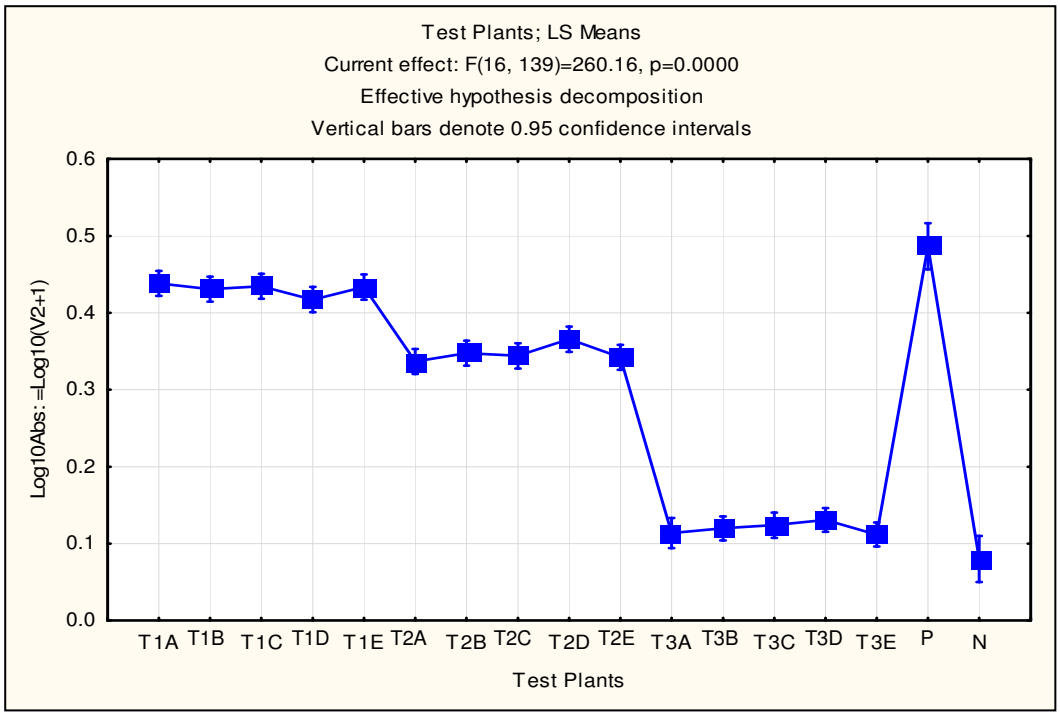


Figure 4.33: Graph Log10 Mean for all test samples

Table 4.29 (A): ELISA assay of progenies from parental lines T_{0A} - T_{0E} (Replica 1)

Parent Line T_{0A}	Anti-CMV scFv	Parent Line T_{0B}	Anti-CMV scFv	Parent Line T_{0C}	Anti-CMV scFv	Parent Line T_{0D}	Anti-CMV scFv	Parent Line T_{0E}	Anti-CMV scFv
T_{1A2}	1.800	T_{1B1}	1.403	T_{1C6}	1.692	T_{1D1}	1.758	T_{1E1}	1.602
T_{1A3}	1.815	T_{1B2}	1.687	T_{1C7}	1.506	T_{1D2}	1.495	T_{1E4}	1.695
T_{1A4}	1.578	T_{1B3}	1.745	T_{1C8}	0.900	T_{1D3}	1.567	T_{1E5}	1.486
T_{1A5}	1.758	T_{1B6}	1.816	T_{1C9}	1.713	T_{1D4}	1.802	T_{1E6}	1.884
T_{1A6}	1.692	T_{1B8}	1.805	T_{1C10}	1.927	T_{1D5}	1.599	T_{1E7}	1.639
T_{1A8}	1.802	T_{1B9}	1.739	T_{1C11}	1.703	T_{1D6}	1.558	T_{1E8}	1.745
T_{1A9}	1.579	T_{1B10}	1.581	T_{1C12}	1.504	T_{1D7}	1.622	T_{1E11}	1.693
T_{1A10}	1.836	T_{1B12}	1.758	T_{1C14}	1.720	T_{1D8}	1.545	T_{1E12}	1.728
T_{1A11}	1.646	T_{1B13}	1.706	T_{1C15}	1.767	T_{1D9}	1.706	T_{1E13}	1.521
T_{1A12}	1.538	T_{1B16}	1.648	T_{1C16}	1.830	T_{1D10}	1.848	T_{1E14}	1.915
T_{2A3}	1.459	T_{2B1}	1.496	T_{2C6}	1.128	T_{2D2}	1.450	T_{2E1}	1.545
T_{2A4}	1.015	T_{2B2}	0.991	T_{2C7}	1.085	T_{2D4}	1.207	T_{2E6}	1.106
T_{2A10}	1.335	T_{2B6}	0.980	T_{2C8}	1.199	T_{2D5}	1.314	T_{2E7}	1.073
T_{2A11}	1.436	T_{2B8}	0.999	T_{2C11}	1.303	T_{2D6}	1.161	T_{2E8}	0.987
T_{2A12}	0.985	T_{2B9}	1.250	T_{2C12}	0.988	T_{2D7}	1.081	T_{2E11}	1.182
T_{2A13}	1.170	T_{2B12}	1.615	T_{2C14}	0.874	T_{2D8}	1.833	T_{2E12}	1.110
T_{2A20}	1.106	T_{2B16}	1.425	T_{2C16}	1.214	T_{2D9}	1.107	T_{2E15}	1.139
T_{2A25}	1.019	T_{2B19}	1.108	T_{2C19}	1.393	T_{2D17}	1.461	T_{2E17}	1.153
T_{2A27}	1.311	T_{2B22}	1.217	T_{2C22}	1.379	T_{2D19}	1.328	T_{2E22}	1.222
T_{2A29}	1.085	T_{2B23}	1.070	T_{2C28}	1.426	T_{2D20}	1.737	T_{2E23}	1.237
T_{3A3}	0.296	T_{3B1}	0.101	T_{3C16}	0.298	T_{3D4}	0.396	T_{3E1}	0.198
T_{3A4}	0.268	T_{3B6}	0.359	T_{3C19}	0.391	T_{3D5}	0.394	T_{3E6}	0.455
T_{3A10}	0.225	T_{3B9}	0.305	T_{3C22}	0.279	T_{3D6}	0.264	T_{3E7}	0.289
T_{3A20}	0.356	T_{3B12}	0.320	T_{3C28}	0.415	T_{3D17}	0.364	T_{3E15}	0.182
T_{3A25}	0.221	T_{3B19}	0.415	T_{3C30}	0.421	T_{3D19}	0.222	T_{3E17}	0.353
T_{3A27}	0.393	T_{3B26}	0.290	T_{3C34}	0.299	T_{3D20}	0.319	T_{3E22}	0.290
T_{3A45}	0.239	T_{3B30}	0.194	T_{3C46}	0.220	T_{3D22}	0.359	T_{3E33}	0.284
		T_{3B32}	0.311	T_{3C52}	0.295	T_{3D28}	0.291	T_{3E35}	0.302
		T_{3B37}	0.226	T_{3C56}	0.399	T_{3D29}	0.317	T_{3E39}	0.231
		T_{3B38}	0.385	T_{3C63}	0.368	T_{3D36}	0.370	T_{3E40}	0.194
		T_{3B42}	0.338			T_{3D41}	0.445	T_{3E42}	0.232

Table 4.29 (B): ELISA assay of progenies from parental lines T_{0A} - T_{0E} (Replica 2)

Parent Line T_{0A}	Anti-CMV scFv	Parent Line T_{0B}	Anti-CMV scFv	Parent Line T_{0C}	Anti-CMV scFv	Parent Line T_{0D}	Anti-CMV scFv	Parent Line T_{0E}	Anti-CMV scFv
T_{1A2}	1.850	T_{1B1}	1.149	T_{1C6}	1.837	T_{1D1}	1.408	T_{1E1}	1.758
T_{1A3}	1.977	T_{1B2}	1.925	T_{1C7}	1.702	T_{1D2}	1.333	T_{1E4}	1.725
T_{1A4}	1.833	T_{1B3}	1.988	T_{1C8}	1.896	T_{1D3}	1.849	T_{1E5}	1.551
T_{1A5}	1.736	T_{1B6}	1.733	T_{1C9}	1.895	T_{1D4}	1.494	T_{1E6}	1.914
T_{1A6}	1.838	T_{1B8}	1.829	T_{1C10}	1.697	T_{1D5}	1.636	T_{1E7}	1.669
T_{1A8}	1.716	T_{1B9}	1.679	T_{1C11}	1.810	T_{1D6}	1.497	T_{1E8}	1.831
T_{1A9}	1.751	T_{1B10}	1.588	T_{1C12}	1.855	T_{1D7}	1.419	T_{1E11}	1.788
T_{1A10}	1.840	T_{1B12}	1.805	T_{1C14}	1.638	T_{1D8}	1.575	T_{1E12}	1.680
T_{1A11}	1.746	T_{1B13}	1.725	T_{1C15}	1.825	T_{1D9}	1.736	T_{1E13}	1.647
T_{1A12}	1.588	T_{1B16}	1.735	T_{1C16}	2.043	T_{1D10}	1.878	T_{1E14}	1.848
T_{2A3}	1.392	T_{2B1}	1.762	T_{2C6}	1.280	T_{2D2}	1.469	T_{2E1}	1.874
T_{2A4}	1.147	T_{2B2}	0.969	T_{2C7}	1.389	T_{2D4}	1.214	T_{2E6}	1.010
T_{2A10}	1.273	T_{2B6}	1.158	T_{2C8}	1.218	T_{2D5}	1.489	T_{2E7}	1.101
T_{2A11}	1.177	T_{2B8}	1.103	T_{2C11}	1.322	T_{2D6}	1.195	T_{2E8}	1.164
T_{2A12}	0.969	T_{2B9}	1.419	T_{2C12}	1.007	T_{2D7}	1.280	T_{2E11}	1.475
T_{2A13}	1.075	T_{2B12}	1.373	T_{2C14}	0.893	T_{2D8}	1.333	T_{2E12}	1.171
T_{2A20}	1.262	T_{2B16}	1.288	T_{2C16}	1.233	T_{2D9}	1.067	T_{2E15}	1.100
T_{2A25}	1.090	T_{2B19}	1.011	T_{2C19}	1.412	T_{2D17}	1.218	T_{2E17}	0.998
T_{2A27}	1.125	T_{2B22}	1.267	T_{2C22}	1.104	T_{2D19}	1.226	T_{2E22}	1.202
T_{2A29}	1.089	T_{2B23}	1.209	T_{2C28}	1.445	T_{2D20}	1.369	T_{2E23}	1.273
T_{3A3}	0.378	T_{3B1}	0.417	T_{3C16}	0.290	T_{3D4}	0.400	T_{3E1}	0.423
T_{3A4}	0.304	T_{3B6}	0.349	T_{3C19}	0.276	T_{3D5}	0.394	T_{3E6}	0.444
T_{3A10}	0.290	T_{3B9}	0.285	T_{3C22}	0.332	T_{3D6}	0.305	T_{3E7}	0.313
T_{3A20}	0.336	T_{3B12}	0.310	T_{3C28}	0.412	T_{3D17}	0.389	T_{3E15}	0.401
T_{3A25}	0.288	T_{3B19}	0.407	T_{3C30}	0.198	T_{3D19}	0.300	T_{3E17}	0.252
T_{3A27}	0.378	T_{3B26}	0.316	T_{3C34}	0.366	T_{3D20}	0.394	T_{3E22}	0.344
T_{3A45}	0.234	T_{3B30}	0.263	T_{3C46}	0.201	T_{3D22}	0.315	T_{3E33}	0.316
		T_{3B32}	0.360	T_{3C52}	0.385	T_{3D28}	0.337	T_{3E35}	0.288
		T_{3B37}	0.238	T_{3C56}	0.444	T_{3D29}	0.309	T_{3E39}	0.284
		T_{3B38}	0.419	T_{3C63}	0.339	T_{3D36}	0.410	T_{3E40}	0.201
		T_{3B42}	0.409			T_{3D41}	0.453	T_{3E42}	0.211

Table 4.29 (C): ELISA assay of progenies from parental lines T_{0A} - T_{0E} (Mean)

Parent Line T_{0A}	Anti-CMV scFv	Parent Line T_{0B}	Anti-CMV scFv	Parent Line T_{0C}	Anti-CMV scFv	Parent Line T_{0D}	Anti-CMV scFv	Parent Line T_{0E}	Anti-CMV scFv
T_{1A2}	1.825	T_{1B1}	1.276	T_{1C6}	1.765	T_{1D1}	1.583	T_{1E1}	1.680
T_{1A3}	1.896	T_{1B2}	1.806	T_{1C7}	1.604	T_{1D2}	1.414	T_{1E4}	1.710
T_{1A4}	1.706	T_{1B3}	1.867	T_{1C8}	1.398	T_{1D3}	1.708	T_{1E5}	1.519
T_{1A5}	1.747	T_{1B6}	1.775	T_{1C9}	1.804	T_{1D4}	1.648	T_{1E6}	1.899
T_{1A6}	1.765	T_{1B8}	1.817	T_{1C10}	1.812	T_{1D5}	1.618	T_{1E7}	1.654
T_{1A8}	1.759	T_{1B9}	1.709	T_{1C11}	1.757	T_{1D6}	1.528	T_{1E8}	1.788
T_{1A9}	1.665	T_{1B10}	1.585	T_{1C12}	1.680	T_{1D7}	1.521	T_{1E11}	1.741
T_{1A10}	1.838	T_{1B12}	1.782	T_{1C14}	1.679	T_{1D8}	1.560	T_{1E12}	1.704
T_{1A11}	1.696	T_{1B13}	1.716	T_{1C15}	1.796	T_{1D9}	1.721	T_{1E13}	1.584
T_{1A12}	1.563	T_{1B16}	1.692	T_{1C16}	1.937	T_{1D10}	1.863	T_{1E14}	1.882
T_{2A3}	1.426	T_{2B1}	1.629	T_{2C6}	1.204	T_{2D2}	1.460	T_{2E1}	1.710
T_{2A4}	1.081	T_{2B2}	0.980	T_{2C7}	1.237	T_{2D4}	1.211	T_{2E6}	1.058
T_{2A10}	1.304	T_{2B6}	1.069	T_{2C8}	1.209	T_{2D5}	1.402	T_{2E7}	1.087
T_{2A11}	1.307	T_{2B8}	1.051	T_{2C11}	1.313	T_{2D6}	1.178	T_{2E8}	1.076
T_{2A12}	0.977	T_{2B9}	1.335	T_{2C12}	0.998	T_{2D7}	1.181	T_{2E11}	1.329
T_{2A13}	1.123	T_{2B12}	1.494	T_{2C14}	0.884	T_{2D8}	1.583	T_{2E12}	1.141
T_{2A20}	1.184	T_{2B16}	1.357	T_{2C16}	1.224	T_{2D9}	1.087	T_{2E15}	1.120
T_{2A25}	1.055	T_{2B19}	1.060	T_{2C19}	1.403	T_{2D17}	1.340	T_{2E17}	1.076
T_{2A27}	1.218	T_{2B22}	1.242	T_{2C22}	1.242	T_{2D19}	1.277	T_{2E22}	1.212
T_{2A29}	1.087	T_{2B23}	1.140	T_{2C28}	1.436	T_{2D20}	1.553	T_{2E23}	1.255
T_{3A3}	0.337	T_{3B1}	0.259	T_{3C16}	0.294	T_{3D4}	0.398	T_{3E1}	0.311
T_{3A4}	0.286	T_{3B6}	0.354	T_{3C19}	0.334	T_{3D5}	0.394	T_{3E6}	0.450
T_{3A10}	0.258	T_{3B9}	0.295	T_{3C22}	0.306	T_{3D6}	0.285	T_{3E7}	0.301
T_{3A20}	0.346	T_{3B12}	0.315	T_{3C28}	0.414	T_{3D17}	0.377	T_{3E15}	0.292
T_{3A25}	0.255	T_{3B19}	0.411	T_{3C30}	0.310	T_{3D19}	0.261	T_{3E17}	0.303
T_{3A27}	0.386	T_{3B26}	0.303	T_{3C34}	0.333	T_{3D20}	0.357	T_{3E22}	0.317
T_{3A45}	0.237	T_{3B30}	0.229	T_{3C46}	0.211	T_{3D22}	0.337	T_{3E33}	0.300
		T_{3B32}	0.336	T_{3C52}	0.340	T_{3D28}	0.314	T_{3E35}	0.295
		T_{3B37}	0.232	T_{3C56}	0.422	T_{3D29}	0.313	T_{3E39}	0.258
		T_{3B38}	0.402	T_{3C63}	0.354	T_{3D36}	0.390	T_{3E40}	0.198
		T_{3B42}	0.374			T_{3D41}	0.449	T_{3E42}	0.222

4.10 Bioassay studies for transgenic plants

The transgenic plants containing anti-CMV scFv antibodies were assayed for resistance to cucumber mosaic virus. The bioassay experiment was designed as described by Boonham and Wood (1998). 3 batches of bioassays were performed in the greenhouse in MARDI, with each batch using T_1, T_2, T_3 transgenic plants (10 each) and 10 seedlings of wild type tobacco plants as control. Two week-old young seedlings (at four leaves stage) of putative transgenic plants were inoculated with cucumber mosaic virus.

All test plants were observed for degree of infection, ranging from healthy (symptomless), tolerant (showing delayed symptoms) and susceptible (severely infected) during a 3-week period. No symptoms were observed for all mock inoculated test plants. On the other hand, virus inoculated plants showed some degree of infection as shown in the Table 4.30. In all 3 batches, almost all wild type test plants developed severe infection symptoms. The first and second generation of progenies showed the highest tolerance percentage and the symptoms were more localised and did not spread to all leaves. The T_3 generation test plants recorded a higher degree of susceptibility to CMV. As presented in Figure 4.34, the wild control plants showed a systemic mosaic after 15 days post-inoculation. On the contrary, no mosaic symptoms were observed in the transgenic plants.

All the plants tested positive for cucumber mosaic virus with ELISA assay (Table 4.31). The amount of CMV present indicates the degree of resistance. As shown in Figure 4.35, the degree of resistance decreases with generations. Transcription of scFv transgene was confirmed with RT-PCR in the test plants (Figure 4.38).

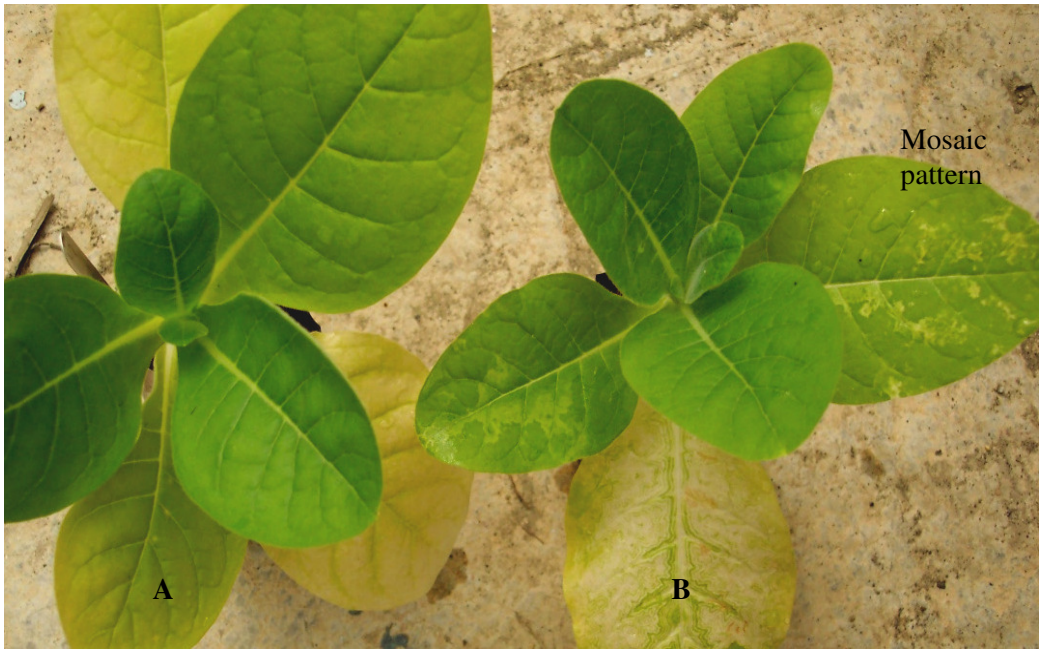


Figure 4.34: Reaction of T_1 transgenic plant (A) and control plant (B) to CMV infection at two-months after virus inoculation

Table 4.30: Degree of infection for transgenic and control plants after infection with cucumber mosaic virus

Batch 1

Generation	Number of test plants	Symptomless	Delayed symptoms	Severe Symptoms
T_1	10	-	8	2
T_2	10	-	7	3
T_3	10	-	4	6
Control	10	-	2	8

Batch 2

Generation	Number of test plants	Symptomless	Delayed symptoms	Severe Symptoms
T_1	10	-	7	3
T_2	10	-	8	2
T_3	10	-	5	5
Control	10	-	1	9

Batch 3

Generation	Number of test plants	Symptomless	Delayed symptoms	Severe Symptoms
T_1	10	1	7	2
T_2	10	-	6	4
T_3	10	-	4	6
Control	10	-	-	10

4.10.1 Spectrophotometric analysis of ELISA assay

Plant samples	Absorbance at 405 nm					
	Batch 1	Batch 2	Batch 3	Mean	Mean – blank	Standard Deviation
Positive Control	2.926	3.154	2.875	2.985	2.800	0.1486
Negative Control	0.265	0.217	0.234	0.239	0.054	0.0243
Blank	0.174	0.194	0.188	0.185		0.0103
Wild type	2.089	2.157	1.944	2.052	1.867	0.1088
T_1	1.423	1.606	1.332	1.457	1.272	0.1396
T_2	1.811	1.733	1.806	1.783	1.598	0.0437
T_3	1.983	1.960	1.895	1.946	1.761	0.0456

Table: 4.31: The results shown in the table were ratios obtained from absorbance at wavelength 405nm

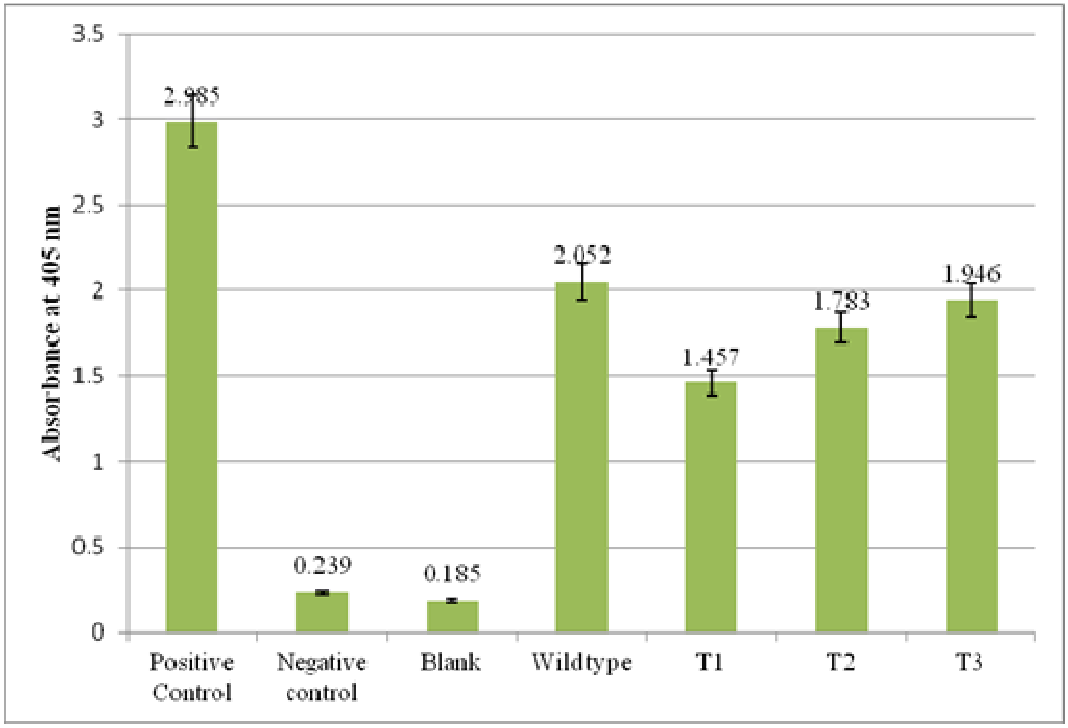


Figure 4.35: Ratio obtained from absorbance of test samples at 405nm is presented in the bar chart above

Table 4.32: Absorbance at 405 nm wavelength

(A) Microtiter plate 1: Batch 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	2,926	0.265	0.174									
B	1.256	1.378	1.502	1.148	1.345	2.032	1.110	2.119	1.211	1.145		
C	1.645	2.212	1.632	1.598	2.187	1.700	1.689	2.296	1.523	1.623		
D	2.304	1.784	1.997	2.095	1.790	1.833	1.769	2.265	1.916	2.080		
E	1.376	1.903	2.235	2.187	2.543	2.338	1.576	2.288	2.302	2.200		
F												
G												
H												

(A) Microtiter plate 2: Batch 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	3.154	0.217	0.194									
B	1.339	1.423	1.342	2.256	2.076	1.549	1.432	1.234	2.131	1.276		
C	1.980	1.675	1.590	1.690	1.597	1.633	1.721	2.233	1.599	1.613		
D	1.798	1.990	2.031	1.823	2.323	1.690	2.102	1.734	1.883	2.221		
E	2.232	2.314	2.512	1.465	2.335	2.178	2.325	2.193	1.980	2.032		
F												
G												
H												

(C) Microtiter plate 3: Batch 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.875	0.234	0.188									
B	1.910	1.234	1.189	1.278	1.356	1.234	1.989	1.325	1.234	0.574		
C	1.832	2.138	1.764	1.973	1.507	1.698	2.008	1.540	1.490	2.115		
D	1.875	2.034	1.862	1.782	1.786	1.976	1.690	1.820	2.020	2.106		
E	1.997	2.076	1.834	2.121	1.890	1.867	1.924	1.932	1.799	2.003		
F												
G												
H												

A1: Positive Control
A2: Negative Control
A3: Blank
B1 - B10: T_1 plants
C1 - C10: T_2 plants
D1 - D10: T_3 plants
E1 - E10: Wild type plants

Analysis of Variance (ANOVA) was performed using Statistica® program to compare differences between the data obtained. All raw data were uploaded to generate the Descriptive Statistic (Table 4.33). Levene's test was then carried out to test the homogeneity of the variances of the groups (Table 4.34). The Tukey HSD (Honest Significant Difference) Test shows the grouping of the test samples (Table 4.35). The F statistic is used to reveal whether the means across the groups are significantly different. Significant differences occur between test plants if $P \leq 0.05$, is rejected. The results indicated that $p = 0.393$. This means there are no statistically significant differences. Hence the values are not rejected. The visual presentation of the results is shown in Figure 4.36.

Level of factor	N	Log10Mean	Log10SD	Log10SE	Log10-95	Log10+95
	18	0.378597	0.178450	0.042061	0.289855	0.467338
positive ctrl	3	0.579544	0.016171	0.009336	0.539372	0.619715
negative ctrl	3	0.022411	0.014195	0.008196	-0.012852	0.057674
WT	3	0.458883	0.016497	0.009525	0.417902	0.499864
T1	3	0.355197	0.025727	0.014853	0.291289	0.419106
T2	3	0.414581	0.008732	0.005041	0.392890	0.436272
T3	3	0.440964	0.008068	0.004658	0.420921	0.461007

Table 4.33: Descriptive Statistics for the test samples

Absorbance at 405nm Degrees of freedom for all F's: 5, 12					
	MS effect	MS error	F	p	
Log10 Abs	0.000061	0.000054	1.136387	0.393232	p> 0.01 ACCEPT
ANOVA					
Effect	SS	Df	MS	F	p
Abs	0.538	5	0.108	419.9	.000*

Table 4.34: Levene's Test for Homogeneity of Variances. P = 0.393

Homogenous Groups, alpha = .05000 Error: Between MS = .00026, df = 12.000							
Cell	Test plants	Log10 Mean	1	2	3	4	5
2	Negative control	0.022411			****		
4	T1 generation	0.355197				****	
5	T2 generation	0.414581	****				
6	T3 generation	0.440964	****	****			
3	Wild type	0.458883		****			
1	Positive Control	0.579544					****

Table 4.35: Tukey Honest Significant Test with variables Log10 Absorbance

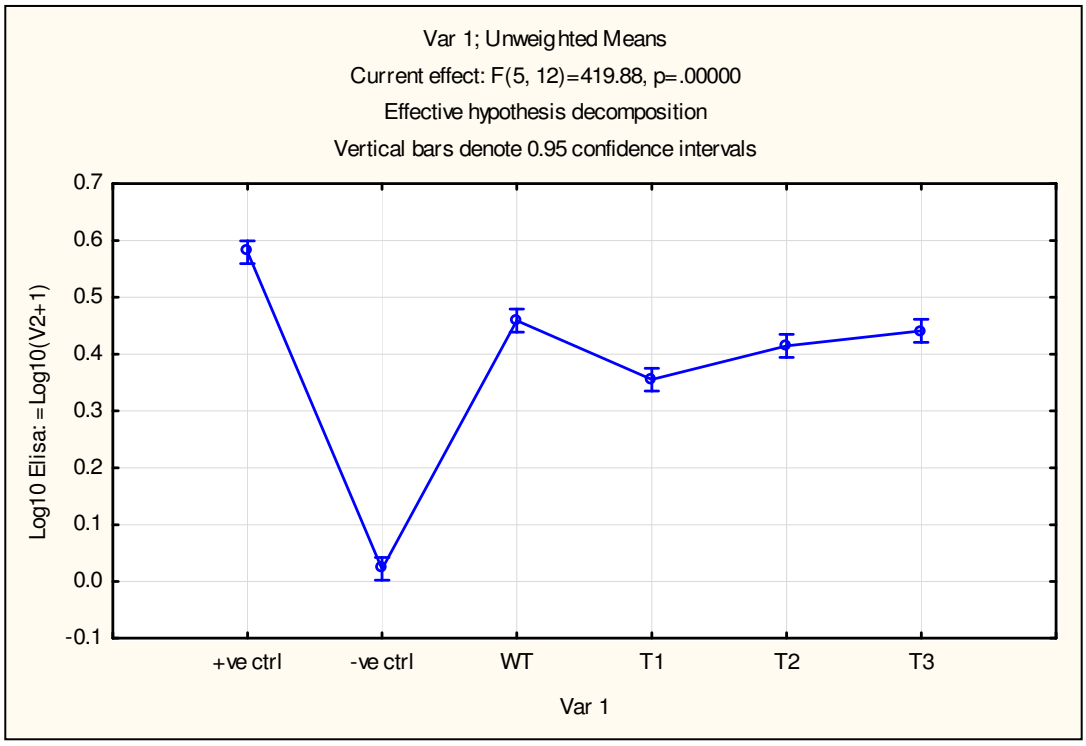
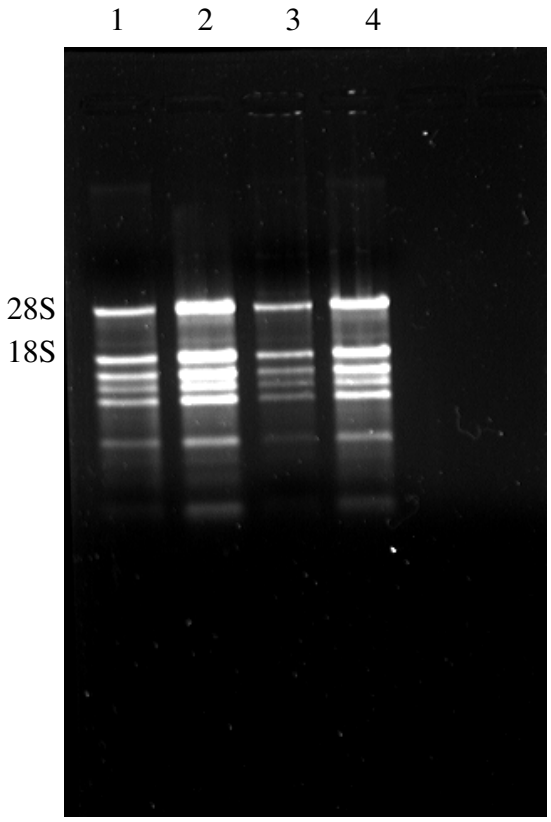


Figure 4.36: Graph showing Log10 Means for all test samples

4.10.2 Detection of anti-CMV scFv gene transcripts in transgenic tobacco plants by RT-PCR

RT-PCR analysis was employed to detect the expression of the anti-CMV scFv mRNA in transgenic tobacco lines. The total RNA was extracted from leaf tissues of tobacco lines transformed with anti-CMV scFv transgene (Figure 4.37). As shown in Figure 4.38, an amplification product of expected size (797 bp) was detected following reverse transcription of total RNA. No RT-PCR products were detected using the total RNA from the wild-type tobacco plants.



Lane 1: wild type tobacco
Lane 2: T_1 plant
Lane 3: T_2 plant
Lane 4: T_3 plant

Figure 4.37: Total RNA was extracted from leaf tissues of individual transgenic lines

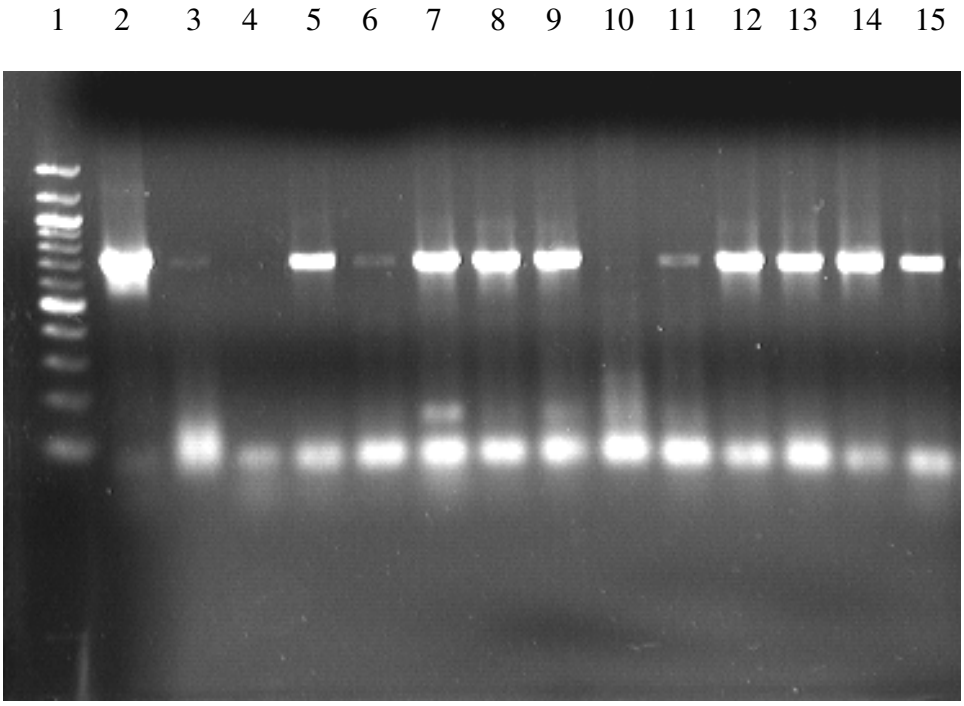


Figure 4.38: Confirmation of anti-CMV scFv transgenes via RT-PCR

Lane 1: 100bp ladder
Lane 2: Positive Control (plasmid pUMSCFV-CMV1)
Lane 3: Positive Control (confirmed transgenic plant)
Lane 4: T_1 wild type tobacco (batch 1)
Lane 5 - 9: T_1 plants (batch 1)
Lane 10: T_1 wild type tobacco (batch 2)
Lane 11-15: T_1 plants (batch 2)

4.11 Protein-protein binding with Autodock

4.11.1 Homology modeling of molecules

In this study, SWISS-MODEL software was applied to predict the structure of light chain and heavy chain of anti-CMV scFv antibody. For heavy chain (V_H), the input sequence is uploaded to compare the heavy chain of a mouse antibody (lae6H.pdb). As for light chain (V_H), mouse antibody (1bafL.pdb) was used as a template to predict the protein structure. BLAST analysis was applied to both sequences (Figure 4.39 and Figure 4.40). The predicted models of both proteins are displayed using DeepView Project (SWISS MODEL) and Accelrys Discovery Studio Visualizer 2.5. The predicted structure of V_H chain and V_L chain are showed in Figure 4.42 and 4.43, respectively. The structure of Cucumber Mosaic Virus used in the docking program was extracted from Gene bank 1f15 pdb (Figure 4.44).

Figure 4.39: BLAST Analysis of V_H chain sequence

The analysed results show that the input sequence is 85% similar to the heavy chain of a mouse antibody (lae6H.pdb).

Model info:	
Modelled residue range:	16 to 121
Based on template	1ae6H (2.90 Å)
Sequence Identity [%]:	85.047
Evalue:	5.93e-38

```
TARGET    16      EIVLTQSP AIMSASPGER VTMTCSASSS IRYIYWYQQK PGSSPRLLIY
1bafL     1       qivltqsp aimsaspgek vtmtcsasss vyymywyqqk pgssprlliy

TARGET              sssss  sssss      ssssssss     sssssss       sssss
1bafL               sssss  sssss      ssssssss     sssssss       sssss


TARGET    64    DTSNVAPGVP FRLSGSGSGT SYSLTINRTE AEDAATYYCQ EWS-GYPYTF
1bafL     49    dtsnlasgvp vrfsgsgsgt sysltisrme aedaatyycq qwssyppitf

TARGET             sss      ssssssss s ssssssss        ssssss ss      ss
1bafL              sss      sssss   s ssssssss        ssssss ss      ss


TARGET    113   GGGTKLELK  ---------- ---------- ---------- ----------
1bafL     99    gvgtklelkr adaaptvsif ppsseqltsg gasvvcflnn fypkdinvkw

TARGET          s  ssssss
1bafL           s  ssssss       sssss    hhhhh s sssssssss        ssss


TARGET          ---------- ---------- ---------- ---------- ----------
1bafL     149   kidgserqng vlnswtdqds kdstysmsst ltltkdeyer hnsytceath

TARGET
1bafL           sss sss  s ssssss          sssss sssshhhh   ssssssss


TARGET          ---------- ------
1bafL     199   ktstspivks fnrnec

TARGET
1bafL                 ssss sss
```

Figure 4.40: BLAST Analysis of V_L chain sequence

The analysed results show that the input sequence is 85% similar to the light chain of a mouse antibody (1bafL.pdb)

Model info:	
Modelled residue range:	1 to 106
Based on template	1bafL (2.90 Å)
Sequence Identity [%]:	85.047
Evalue:	3.02e-36

```
TARGET    1       EIVLTQSP AIMSASPGER VTMTCSASSS IRYIYWYQQK PGSSPRLLIY
1bafL     1       qivltqsp aimsaspgek vtmtcsasss vyymywyqqk pgssprlliy

TARGET              sssss  sssss      sssssssss     sssssss       sssss
1bafL               sssss  sssss      sssssssss     sssssss       sssss


TARGET    49    DTSNVAPGVP FRLSGSGSGT SYSLTINRTE AEDAATYYCQ EWS-GYPYTF
1bafL     49    dtsnlasgvp vrfsgsgsgt sysltisrme aedaatyycq qwssyppitf

TARGET             sss      sssssss s sssssss        ssssss ss      ss
1bafL              sss      sssss   s sssssss        ssssss ss      ss


TARGET    98    GGGTKLELK  ---------- ---------- ---------- ----------
1bafL     99    gvgtklelkr adaaptvsif ppsseqltsg gasvvcflnn fypkdinvkw

TARGET          s  ssssss
1bafL           s  ssssss       sssss    hhhhh s sssssssss        ssss


TARGET          ---------- ---------- ---------- ---------- ----------
1bafL     149   kidgserqng vlnswtdqds kdstysmsst ltltkdeyer hnsytceath

TARGET
1bafL           sss sss  s ssssss          sssss sssshhhh   ssssssss


TARGET          ---------- ------
1bafL     199   ktstspivks fnrnec

TARGET
1bafL                 ssss sss
```

Figure 4.41: The deduced amino acid sequence of V_H chain (A) and V_L chain (B) were obtained through TRANSLATE program

(A)

VQLQESATEL VKPGASVKIS CKASGYSFIV HYINWVKQKP GQGLEWIGCF FPGSGNSKYI ENFRGKATLT VDTSSSTAYM QLSSLTSEDT AVYFCARDDS DGAMDYWGQG TTVTVSS

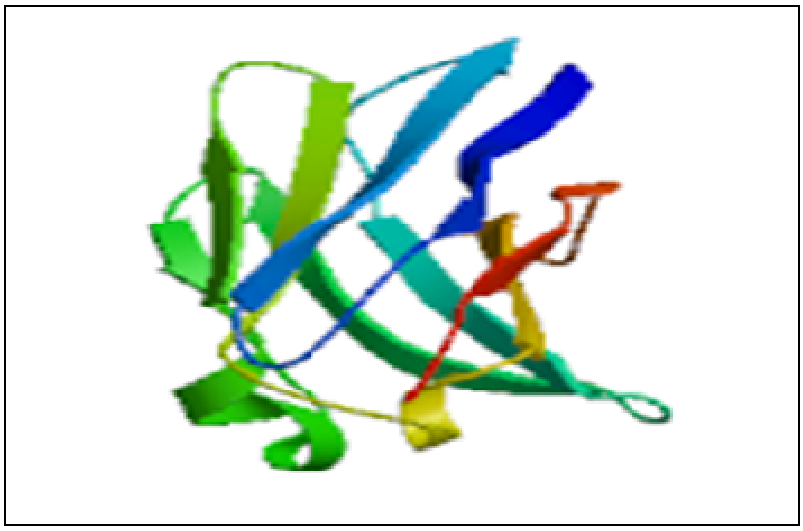
(B)

EIVLTQSPAI MSASPGERVT MTCSASSSIR YIYWYQQKPG SSPRLLIYDT SNVAPGVPFR LSGSGSGTSY SLTINRTEAE DAATYYCQEW SGYPYTFGGG TKLELKR

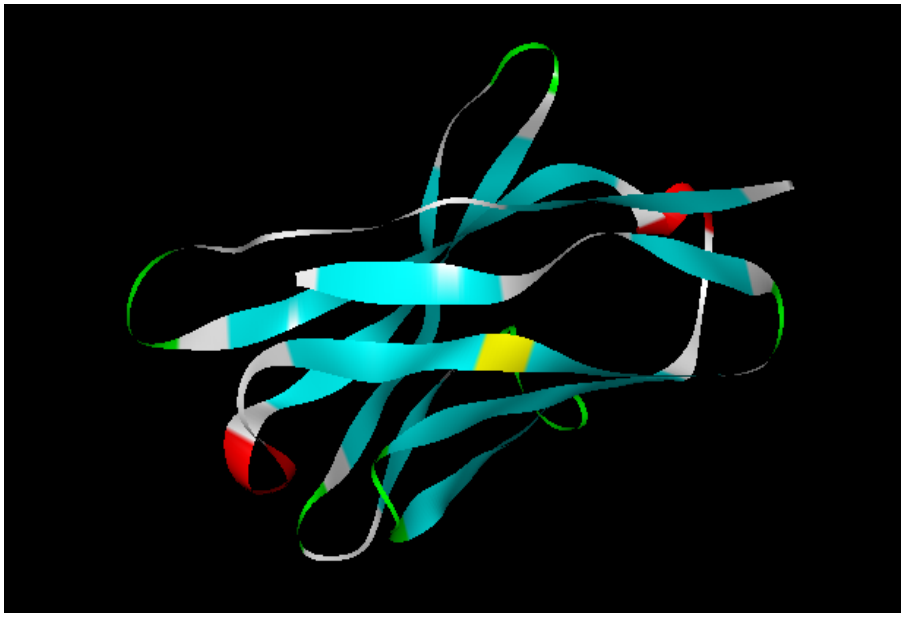
Figure 4.42: Predicted structure of V_H chain (SWISS MODEL)

The structure of V_H chain of anti-CMV antibody was predicted through homology modelling using SWISS-MODEL program. The structure presented in (A) was viewed with DeepView software (formerly called the Swiss PDB Viewer). DeepView colours helical residues red, beta sheet residues (strands) yellow, and all loops grey /white. The green regions represent a turn. V_H chain in (B) ribbon form and (C) ball and stick are also shown using Accelrys Discovery Studio Visualizer 2.5. The α-helices are shown in red, and the β-sheets are shown in cyan.

(A)



(B)



(C)

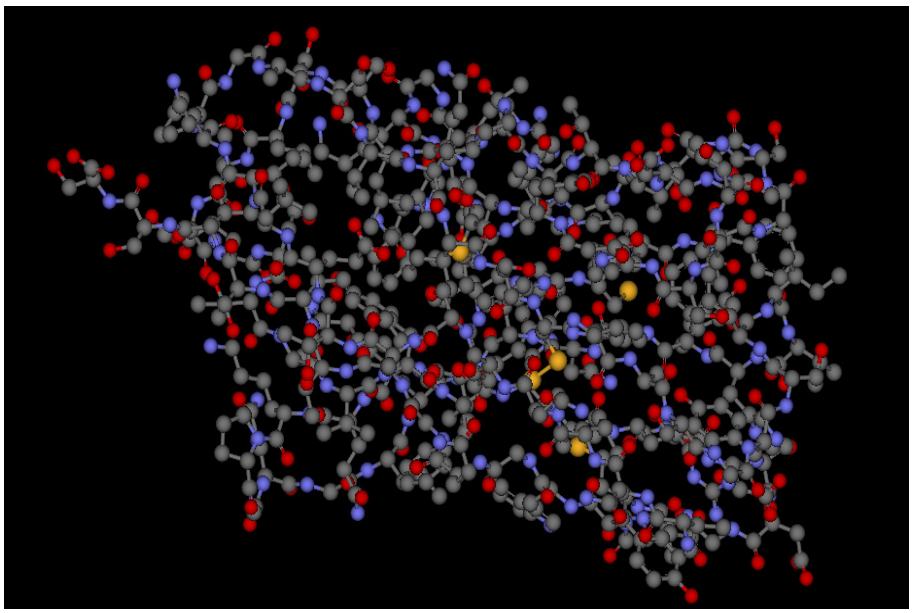
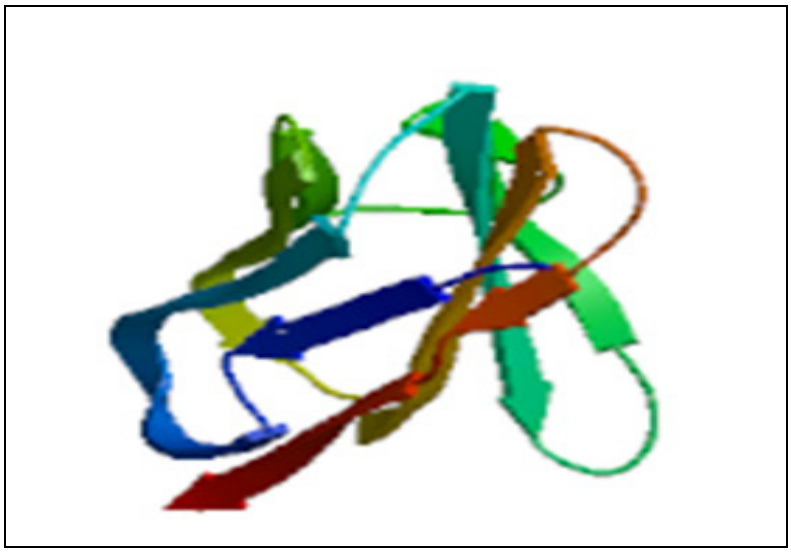


Figure 4.43: Predicted structure of V_L chain (SWISS MODEL)

The structure of V_L chain of anti-CMV antibody was predicted through homology modelling using SWISS-MODEL program. Protein structure presented in (A) was viewed using Deep View software (formerly called the Swiss PDB Viewer). DeepView colours helical residues red, beta sheet residues (strands) yellow, and all loops grey /white. The green regions represent a turn. The models of V_L chain in (B) ribbon form and (C) ball and stick form are also displayed using Accelrys Discovery Studio Visualizer 2.5. The α-helices are shown in red, and the β-sheets are shown in cyan.

(A)



(B)

(C)

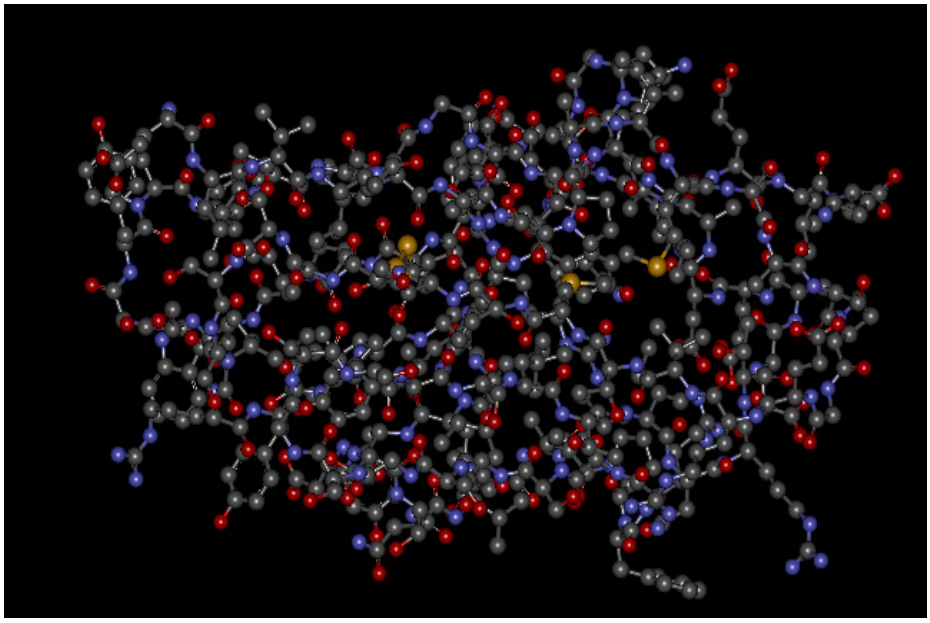
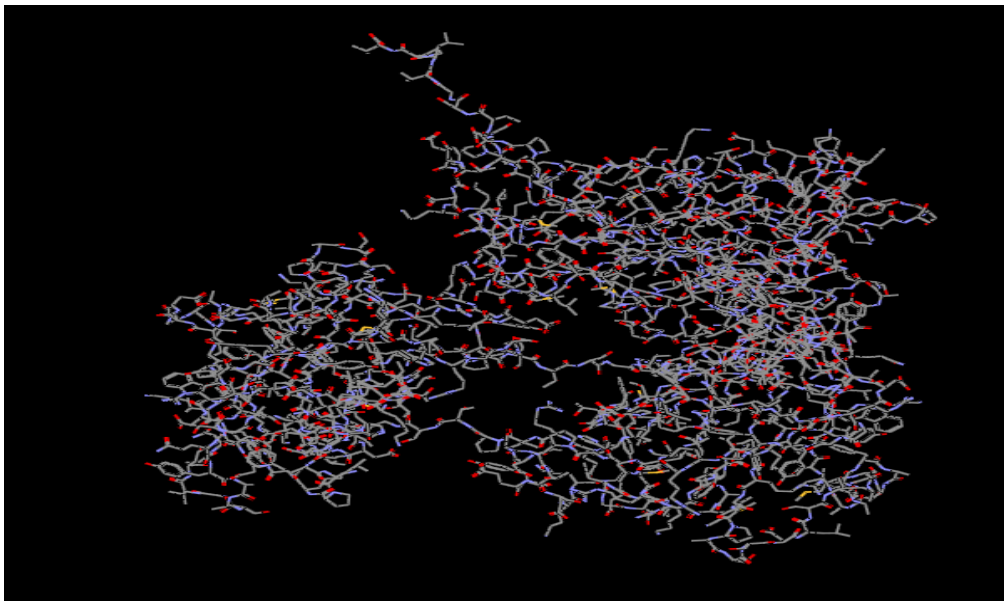


Figure 4.44: Structure of Cucumber Mosaic Virus

The structure of Cucumber Mosaic Virus used in the docking program was taken from Gene bank 1f15 pdb (A). The coat protein used in the docking program to bind to heavy and light chains of anti-CMV scFv antibody is highlighted in yellow (B). The structure was viewed using Accelrys Discovery Studio Visualizer 2.5.

(A)



(B)

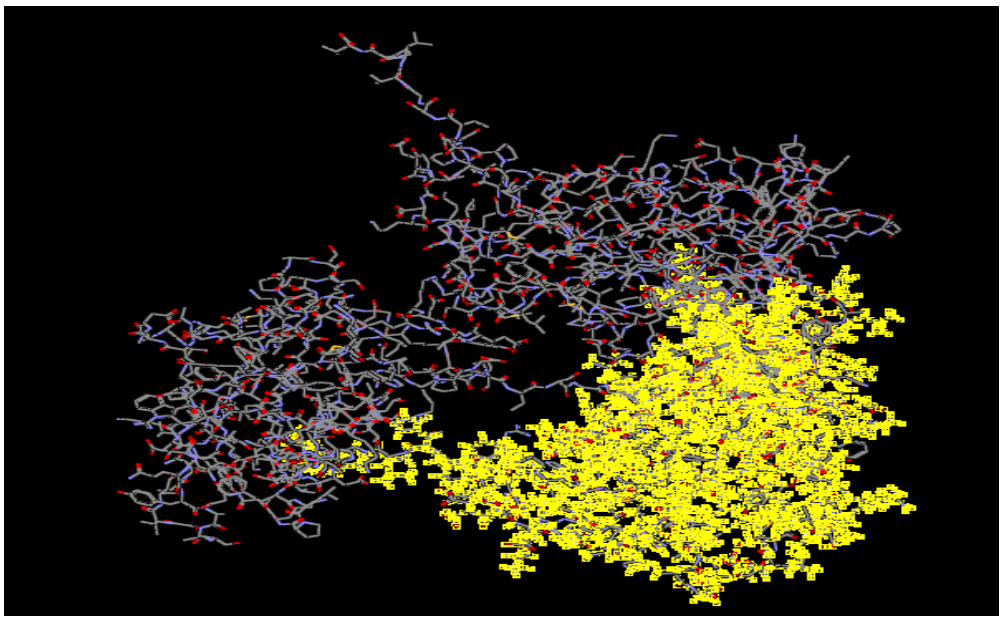


Figure 4.45: The deduced amino acid sequence of CMV (A) and CMV coat protein (B) were obtained through TRANSLATE program

(A)

ERCRPGYTFTSITLKPPKIDRGSYYGKRLLLPDSVTEYDKKLVSRLQIRVNPLPKFDST
VWVTVRKVPASSDLSVAAISAMFADGASPVLVYQYAASGVQANNKLLYDLSAMRADIGD
MRKYAVLVYSKDDALETDELVLHVDIEHQRIPTSGVLPV/DANFRVLSQQLSRLNKTLA
AGRPTINHPTFVGSERCRPGYTFTSITLKPPKIDRGSYYGKRLLLPDSVTEYDKKLVSR
LQIRVNPLPKFDSTVWVTVRKVPASSDLSVAAISAMFADGASPVLVYQYAASGVQANNK
LLYDLSAMRADIGDMRKYAVLVYSKDDALETDELVLHVDIEHQRIPTSGVLPV/ADANF
RVLSQQLSRLNKTLAAGRPTINHPTFVGSERCRPGYTFTSITLKPPKIDRGSYYGKRLL
LPDSVTEYDKKLVSRLQIRVNPLPKFDSTVWVTVRKVPASSDLSVAAISAMFADGASPV
LVYQYAASGVQANNKLLYDLSAMRADIGDMRKYAVLVYSKDDALETDELVLHVDIEHQR
IPTSGVLPV

(B)

DATLRVLSQQLSRLNKTLAAGRPTINHPTFVCSERCKPGYTFTSITLKPPKIDKGSYCG
KRLLLLIQVTEFDKKLVSRIQIRVNPLPKFDSTRVGDGRKVPASSDLSVSAISAMLRTD
LPVWFISTLHLAFKPTQMVDDLSVMRADIGDMRKYAVLVYSKDDALETDELVLHLTLST
NAFPHLGCSQ

4.11.2 Autodock

4.11.2.1 Blind Docking

In this study, blind docking was performed separately for heavy and light chain due to the large molecule size of the scFv antibody. Blind docking was carried out by scanning the entire surface of light chain and heavy chain of Anti-CMV scFv antibody to detect the possible binding sites with Cucumber Mosaic Virus.

The results for the blind docking calculations on the light chain and heavy chain are shown in Table 4.36 and Table 4.37 respectively. In both cases, a population size of 150 and 10 millions energy evaluations were used for 100 times searches, with a 60 x 60 x 60 dimension of a grid box size. For light chain, out of all 100 runs, 11 distinct conformational clusters were found. 24 distinct conformational clusters were discovered in heavy chain (Figure 4.46 and 4.47).

The best 5 conformations in the clustering histogram with the lowest docked energy were chosen to perform specific dockings, with highest numbers of molecules in a particular cluster not more than 2.0 A root-mean square deviation (rmsd). Graphical interpretation and representation of results were performed using Accelrys Discovery Studio Visualizer 2.5.

Cluster Rank	Lowest Binding Energy	Run	Mean Binding Energy	Number in cluster
1	+1.67e+06	82	+1.67e+06	51
2	+1.67e+06	32	+1.69e+06	27
3	+1.75e+06	40	+1.75e+06	3
4	+1.79e+06	87	+1.80e+06	7
5	+1.80e+06	97	+1.82e+06	5
6	+1.83e+06	73	+1.83e+06	1
7	+1.91e+06	58	+1.91e+06	1
8	+1.92e+06	43	+1.92e+06	2
9	+2.02e+06	22	+2.02e+06	1
10	+2.02e+06	53	+2.02e+06	1
11	+2.14e+06	47	+2.14e+06	1

Table 4.36: Clustering Histogram showing conformations of docked energy for ligand light chain scFv

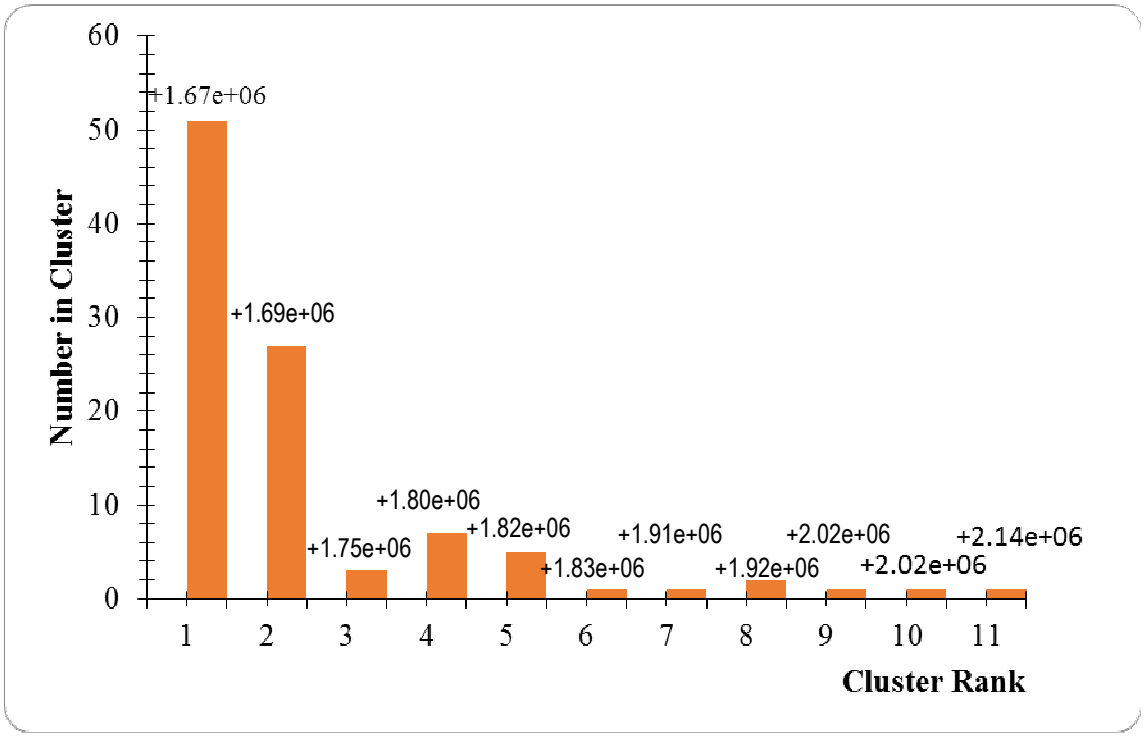


Figure 4.46: Clustering Histogram showing mean binding energy (V_L chain)

Cluster Rank	Lowest Binding Energy	Run	Mean Binding Energy	Number in cluster
1	+1.98e+06	19	+2.23e+06	4
2	+2.30e+06	61	+2.45e+06	6
3	+2.48e+06	41	+1.64e+06	32
4	+2.58e+06	32	+2.66e+06	2
5	+2.68e+06	57	+2.80e+06	2
6	+2.69e+06	49	+2.74e+06	16
7	+2.78e+06	47	+2.78e+06	1
8	+2.81e+06	52	+2.81e+06	1
9	+2.88e+06	11	+2.88e+06	1
10	+2.91e+06	95	+2.91e+06	1
11	+2.91e+06	80	+2.91e+06	1
12	+2.92e+06	25	+3.01e+06	6
13	+2.92e+06	56	+2.94e+06	2
14	+3.02e+06	48	+3.10e+06	7
15	+3.02e+06	82	+3.02e+06	1
16	+3.12e+06	28	+3.20e+06	2
17	+3.21e+06	31	+3.21e+06	1
18	+3.22e+06	64	+3.22e+06	1
19	+3.28e+06	39	+3.28e+06	1
20	+3.29e+06	20	+3.31e+06	5
21	+3.33e+06	60	+3.35e+06	2
22	+3.33e+06	67	+3.34e+06	2
23	+3.51e+06	8	+3.51e+06	2
24	+3.61e+06	72	+3.61e+06	1

Table 4.37: Clustering Histogram showing conformations of docked energy for ligand heavy chain scFv

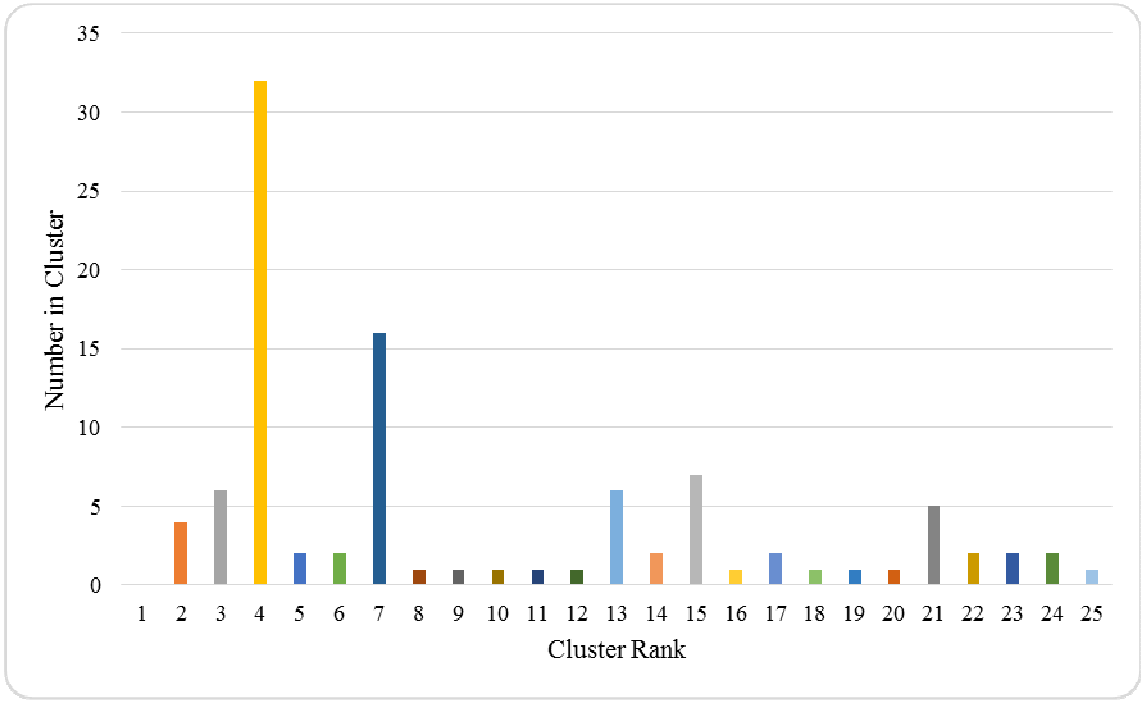


Figure 4.47: Clustering Histogram showing mean binding energy (V_H chain).

4.11.2.2 Specific Docking

The best 5 conformations in the clustering histogram (Figure 4.46 and Figure 4.47), with the lowest docked energy were chosen to perform specific dockings, with highest numbers of molecules in a particular cluster not more than 2.0 A root-mean square deviation (rmsd).

Specific docking for V_H chain was successfully completed in 32 hours. The binding interaction of V_H chain with CMV (highlighted) is shown in Figure 4.48.

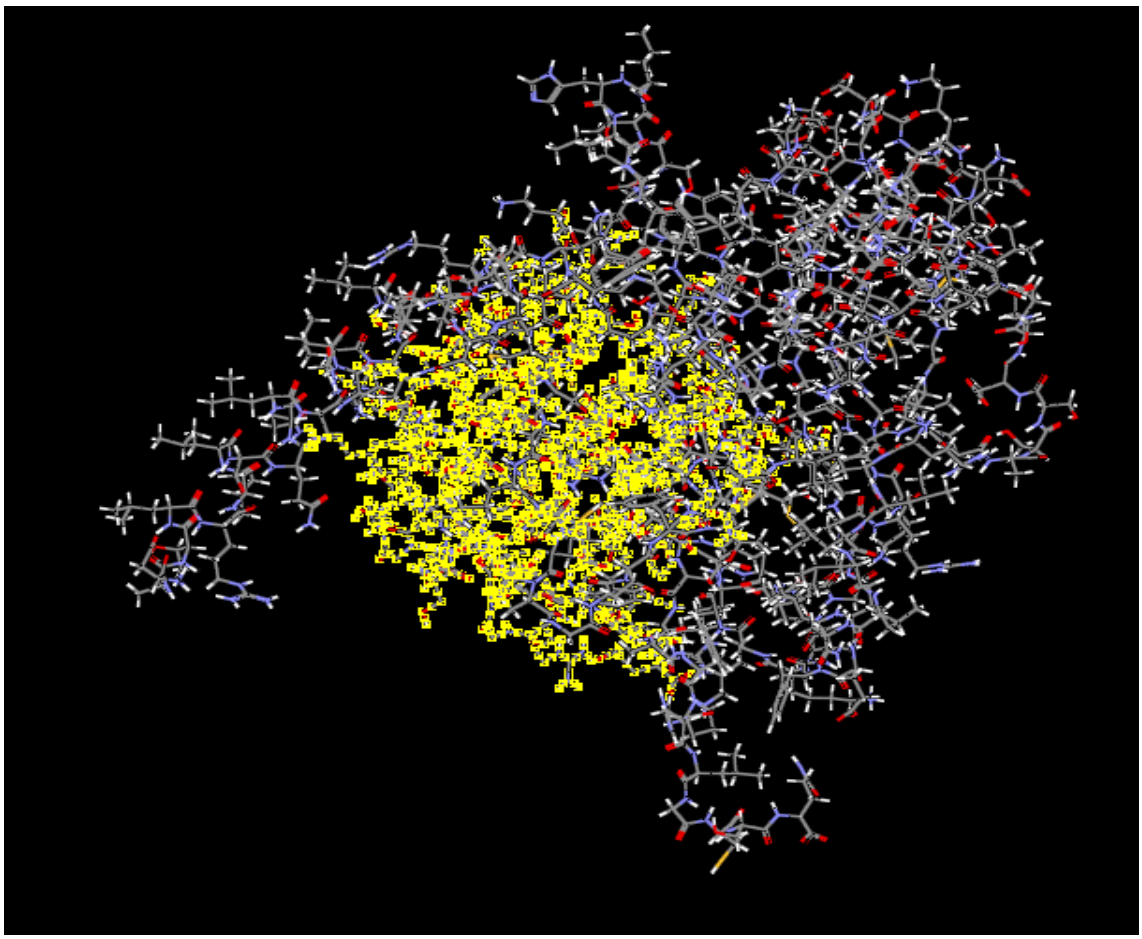


Figure 4.48: Predicted binding site of V_H chain to CMV

Specific docking for V_L chain was successfully completed in 23 hours. The binding interaction of V_L chain with CMV (highlighted in yellow) is shown in Figure 4.49.

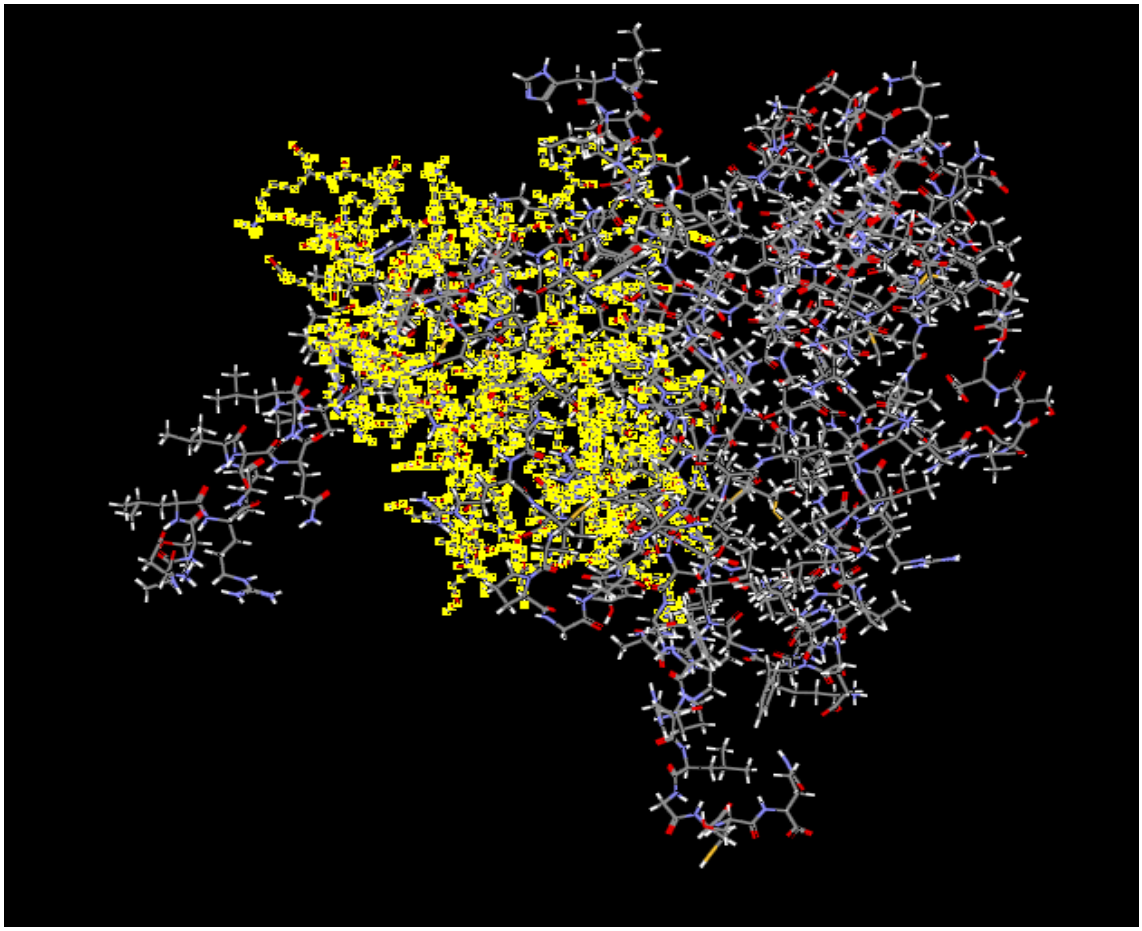


Figure 4.49: Predicted binding site of V_L chain to CMV

5.0 Development of framework for risk assessment and risk management protocol for transgenic plants expressing scFv antibodies

The field application of transgenic tobacco plants expressing scFv antibodies requires compliance to biosafety regulations and licensing conditions. This involves the development of a risk assessment framework which will enable the systematic process of generating the appropriate data that will ensure the safe release of the transgenic plants into the environment.

In Malaysia the approval of transgenic plants is regulated under the Malaysian Biosafety Act which was approved in Parliament in 2007. Core concepts contained in this legislation include the requirement for systemic evaluation of a genetically modified organism (GMO) prior to its approval in Malaysia. The law follows closely the principles stated in Annex III of the Cartagena protocol on Biosafety. This protocol states that the purpose of risk assessment, is to identify and evaluate the potential adverse effects of living modified organisms on the conservation and sustainable use of biological diversity in the likely potential receiving environment, taking also into account risks to human health (Secretariat of the CBD, 2000). A summary of annex III is presented in Figure 5.1.

Risk assessment covers a comprehensive range of science-based methods and tools to identify and mitigate potential risks that the GMO may pose to the environment. A pilot risk assessment and management framework is presented here to evaluate transgenic tobacco plants expressing recombinant scFv antibodies. The risk assessment is based on the following methodology: hazard identification, exposure characterization, exposure assessment and risk characterization (IPCS, 2004). After characterization of potential risks, risk mitigation steps are identified and these methods will form a comprehensive plan to manage the risks.

Figure 5.1: Annex III of the Cartagena Protocol on Biosafety

Annex III

RISK ASSESSMENT

Objective

1. The objective of risk assessment, under this Protocol, is to identify and evaluate the potential adverse effects of living modified organisms on the conservation and sustainable use of biological diversity in the likely potential receiving environment, taking also into account risks to human health.

Use of risk assessment

2. Risk assessment is, *inter alia*, used by competent authorities to make informed decisions regarding living modified organisms.

General principles

3. Risk assessment should be carried out in a scientifically sound and transparent manner, and can take into account expert advice of, and guidelines developed by, relevant international organizations.

4. Lack of scientific knowledge or scientific consensus should not necessarily be interpreted as indicating a particular level of risk, an absence of risk, or an acceptable risk.

5. Risks associated with living modified organisms or products thereof, namely, processed materials that are of living modified organism origin, containing detectable novel combinations of replicable genetic material obtained through the use of modern biotechnology, should be considered in the context of the risks posed by the non-modified recipients or parental organisms in the likely potential receiving environment.

6. Risk assessment should be carried out on a case-by-case basis. The required information may vary in nature and level of detail from case to case, depending on the living modified organism concerned, its intended use and the likely potential receiving environment.

Methodology

7. The process of risk assessment may on the one hand give rise to a need for further information about specific subjects, which may be identified and requested during the assessment process, while on the other hand information on other subjects may not be relevant in some instances.

8. To fulfil its objective, risk assessment entails, as appropriate, the following steps:

(a) An identification of any novel genotypic and phenotypic characteristics associated with the living modified organism that may have adverse effects on biological diversity in the likely potential receiving environment, taking also into account risks to human health;

(b) An evaluation of the likelihood of these adverse effects being realized, taking into account the level and kind of exposure of the likely potential receiving environment to the living modified organism;

(c) An evaluation of the consequences should these adverse effects be realized;

(d) An estimation of the overall risk posed by the living modified organism based on the evaluation of the likelihood and consequences of the identified adverse effects being realized;

(e) A recommendation as to whether or not the risks are acceptable or manageable, including, where necessary, identification of strategies to manage these risks; and

(f) Where there is uncertainty regarding the level of risk, it may be addressed by requesting further information on the specific issues of concern or by implementing appropriate risk management strategies and/or monitoring the living modified organism in the receiving environment.

Points to consider

9. Depending on the case, risk assessment takes into account the relevant technical and scientific details regarding the characteristics of the following subjects:

(a) *Recipient organism or parental organisms.* The biological characteristics of the recipient organism or parental organisms, including information on taxonomic status, common name, origin, centres of origin and centres of genetic diversity, if known, and a description of the habitat where the organisms may persist or proliferate;

(b) *Donor organism or organisms.* Taxonomic status and common name, source, and the relevant biological characteristics of the donor organisms;

(c) *Vector*. Characteristics of the vector, including its identity, if any, and its source or origin, and its host range;

(d) *Insert or inserts and/or characteristics of modification.* Genetic characteristics of the inserted nucleic acid and the function it specifies, and/or characteristics of the modification introduced;

(e) *Living modified organism.* Identity of the living modified organism, and the differences between the biological characteristics of the living modified organism and those of the recipient organism or parental organisms;

(f) *Detection and identification of the living modified organism.* Suggested detection and identification methods and their specificity, sensitivity and reliability;

(g) *Information relating to the intended use.* Information relating to the intended use of the living modified organism, including new or changed use compared to the recipient organism or parental organisms; and

(h) *Receiving environment.* Information on the location, geographical, climatic and ecological characteristics, including relevant information on biological diversity and centres of origin of the likely potential receiving environment.

Source: Secretariat of the Convention on Biological Diversity (2000)

5.1 Malaysian Biosafety Act

In accordance with the Malaysian Biosafety Act, this project needs to undergo a notification process to the National Biosafety Board. Once the application of notification has been received and acknowledged by the Director General (DG) of the Department of Biosafety, the project can commence. The notification form (NBB/CU/10/FORM E) is attached in Appendix. For field trials, an approval for release activity form (NBB/A/ER/10/FORMA) needs to be submitted to the Director General (DG) of the Department of Biosafety. After acknowledgement of receipt, the DG will forward the application to the Genetic Modification Advisory Committee (GMAC). The GMAC is tasked with providing a recommendation on the notification application to the National Biosafety Board (NBB). The NBB will make a decision based on the recommendation of the GMAC. The outcome of the NBB may range from no order, which amounts to an approval to the notification, or issue a cessation order or impose changes in the interests of biosafety.

The information required for the notification process include overall project information such as the objective of the project, list of people involved in the project and the detailed description of the transgenic organisms. The risk assessment and risk management procedures will ultimately result in the formulation of an emergency response plan.

Based on the Biosafety Regulations 2010, three types of risks need to be assessed: risks to the health and safety of humans from the activities associated with genetic modification, risks to the health and safety of humans from an unintentional release of the genetically modified organism and risks to the environment from an unintentional release of the genetically modified organism.

In terms of risk management, information regarding precautions taken in the event of transport outside of contained use premises needs to be provided. Proper

disposal procedures of the transgenic organism and the decontamination of equipment used during the project needs to be elucidated as well.

The emergency response plan will need to detail the procedures to be followed in the event of unintentional release of the transgenic organism beyond the contained use premises. Plans to protect human health and the environment, procedures to remove the transgenic organism in the affected areas, disposal methods for contaminated organisms and isolation of the affected area should be detailed in the emergency response plan. In the event that adverse effects manifest as a result of contained use or unintentional exposure, steps to correct the adverse effects should be demonstrated.

5.2 Risk assessment:

Risk assessment is the process whereby risk is measured. The measurements can be qualitative, quantitative or a mixture of both. The ultimate goal of risk assessment is to decide on how to manage the risk: either by tolerating, mitigating or avoiding the risk altogether. In this context, to assess the risk of the transgenic tobacco, several risk assessment procedures should be performed. Biosafety Guidelines (2012) was referred to develop the framework for Risk Assessment and Risk Management Protocol for Transgenic Plants expressing scFv Antibodies.

Formulation of problem & hazard identification

As with any assessment process, the starting point should be to identify areas for concern and to formulate the datasets required to address the problem. To formulate the problem, complete scientific and regulatory information should be available so that all relevant areas are covered and no significant lapses arise due to incomplete information. The assessment is carried out as a data intensive, scientifically sound and transparent analysis of all available information. The methodology is based on a comparison with

non-modified recipients and in the context of the receiving environment and its intended use (Wolt *et al*., 2010).

It is important to differentiate between hazard and risk. Hazard is the adverse effects on the environment that occurs with the usage of transgenic tobacco and risk is the likelihood that the hazard will occur. Hazard identification is the process to determine whether or not the novel gene products in the genetically modified plant cause adverse effects. Hazard is usually determined experimentally with controlled doses (Poppy, 2000)

Potential adverse effects associated with transgenic plants include toxicity and allergenicity to humans, animals and microorganisms. Apart from this, the cultivation of transgenic crops in the environment poses several risks such as weediness of the transgene plant and gene flow of novel genes to other organisms. It is important to determine whether the genetic modification used to produce the transgenic tobacco produced any unwanted alterations at the molecular level. Furthermore, it has to be ensured that the new genetic material is stably inserted and maintained over several generations. If the inserted genetic modification is unstable or produces unwanted alterations, then it will be very difficult to predict how the transgenic crop will behave when it is released into the wild (Biosafety Guidelines, 2012).

Exposure characterization

Exposure characterization is the determination of the relationship between the magnitude of exposure and the probability of occurrence of the adverse effect. It can be used to identify the severity of the hazard. Many substances lead to adverse effects at high doses and less adverse effects at lower doses.

As the transgenic tobacco will not be used for human consumption, the possibility of the modified plants causing toxicity and allergenicity in humans is deemed to be very

low. There is a chance of ingestion by organisms such as insects in the wild so the hazard level, while still low, are judged to be higher than the possibility of human consumption.

As mentioned previously, gene flow should be investigated as well and information on whether the new transgenes can transfer to other crops should be investigated. Gene flow to other tobacco plants is a real possibility and this should be emphasized in the risk management plan. Gene flow to other plants could occur and gene flow to animals, humans and microorganisms are unlikely.

Exposure assessment

Exposure assessment is the determination of how the extent of exposure to transgenic tobacco leads to environmental and human health risks, if any. Exposure assessment of transgenic tobacco should shed some light on how much of the novel genetic material / proteins that are released into the environment (Biosafety Guidelines, 2012).

To investigate whether the transgenic tobacco is harmful to the environment because of inherent weediness or some other trait conferred to it by its transgenes, information such as decreased ecological diversity, decreased yield of crops, decreased growth rate or increased abundance of organisms should be investigated. The impact of the transgene on soil organisms should also be investigated.

To determine the stability of the genes on a genetic level, tests like ELISA, PCR, Southern and Western blot could elucidate the genetic information of the inserted gene. Southern Blot could be used to determine the location of the insert in the plant cells and can also be used to determine the genetic stability of the insert. ELISA could be used to determine the amounts of the novel protein levels in the cells and hence measure the expression of the insert.

On an observational basis, information on the morphology, seeding, pollen dissemination, disease susceptibility, insect damage, reproduction, survivability can be obtained via data collected in field trials.

Risk characterization

Risk characterization takes into account all the steps mentioned above and is often reported as a quantitative assessment. Hazard identification, exposure characterization and exposure assessment are all essential elements of risk characterization. It ultimately results in the estimation of the risk posed by transgenic tobacco.

5.3 Risk/benefit analysis of transgenic scFv antibody products.

The approach will be to evaluate benefits of the crop in relation to existing production systems against risks identified earlier.

Factors to be considered in applying such analysis:

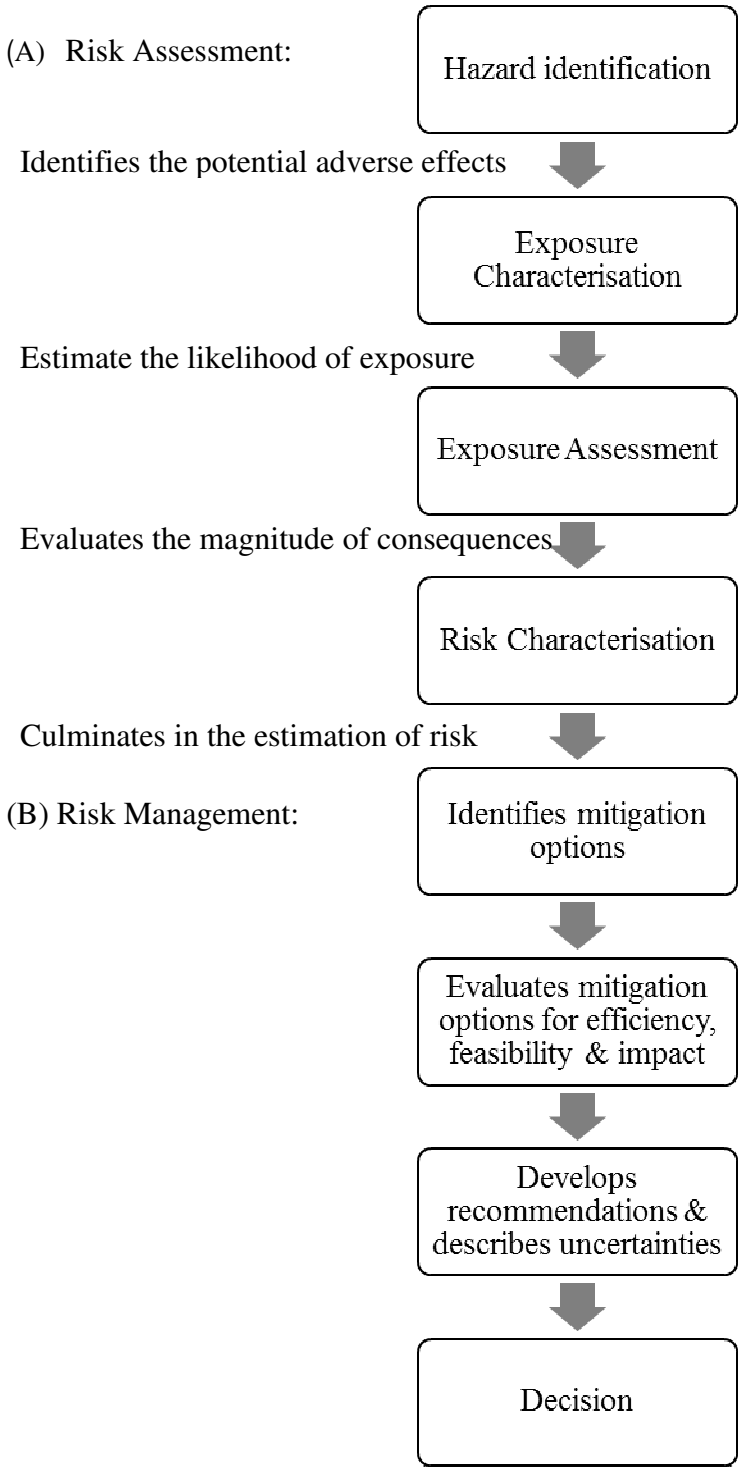
- the magnitude of each potential harm or benefit involved
- the likelihood that it will occur

5.4 Risk management

Risk management is meant to identify mitigation options for any risk identified. The mitigations options are then evaluated for efficiency, feasibility and impact. Once the evaluation is complete, the relevant recommendations are developed. Any uncertainties for formulation of the final risk management report should be mentioned as well. The development of standardized molecular testing kits for traceability and quality control assays would be desirable (Biosafety Guidelines, 2012).

With regards to the characterized risks mentioned earlier, there are several possible risk management strategies. Overall, there is a need to limit the scale of release of the transgenic tobacco plants. There is a need to enclose the plants in a secure trial area to prevent unintended exposure. Proper handling protocols should be in place and in the event that plant materials need to be transferred, proper security measures should be taken. Another point to consider is that the destruction of unusable plant materials should be handled in accordance with established protocols.

Figure 5.2 Flowchart of risk assessment and risk management



Source: EFSA, 2010; Wolt *et al*., 2010.

Toxicity and allerginicity

As the transgenic tobacco in this project is not meant for human or animal consumption, human toxicity through ingestion is likely to be low. For animals there is a slightly higher risk of ingestion. The risk management principle in this instance is to prevent accidental exposure of the plant to humans and animals. One other possibility is the transfer of transgenes to microorganisms, which occurs mostly in the gut of livestock. Again this possibility is remote as the tobacco is not used as feed (Gonsalves *et al*., 2004)

This can be achieved by ensuring that the test area is secure and only authorized personnel have access to the test area. Furthermore, if there is a necessity to transfer plants or plant materials from the test site, utmost care should be taken to ensure that the plant is secure and there is no chance for accidental exposure of the plant to the general environment. Unusable plant material should also be disposed properly.

Gene Flow

Gene flow is defined as the incorporation of genes into the gene pool of one population from one or more other populations (Futuyma, 1998). If transgenic crop genes replace wild genes, the genetic diversity of wild plants will be compromised. If the hybrid offspring are better suited to the environment than their parents then there is a possibility that the hybrid could become an invasive pest. Naturally if the reverse were true the survival of the population in wild could be under threat (Levin *et al.*, 1996). Gene flow occurs via normal plant propagation mechanisms such as seeds, pollen and other propagules. It can occur via humans or animals or via normal environmental vectors like wind, water and air. Methods to control gene flow include physical isolation and plant destruction (NRC 2004b). There have been some suggestions that crops have inherently different rates of gene flow and transgenic plants can be managed by

stratification (Stewart *et al.,* 2003). An alternative view is that ecological factors such as locality and seasonality come into play as well and should be taken into account when devising a management plan (NRC 2004b).

Compared to other plants, tobacco can be classified to have a low probability of gene flow to wild type counterparts. The tobacco crop has difficulty reproducing with the wild type tobacco and even if reproduction is successful the hybrids are sterile, which reduces the likelihood of the transgene escaping into the environment (Ahl Goy and Duesing, 1996). Despite this, steps should be taken to minimize the possibility of gene transfer. Some available methods are to use sterile male plants or to remove the flower. Apart from this, establishing buffer zones around the transgenic plants is also an option. Gene flow to other plants is less likely than gene flow to wild type tobacco and steps should also be taken to minimize this possibility. Gene flow to animals and microorganisms are judged to be negligible so no further precautions are deemed necessary.

In terms of weediness, the modified tobacco plant could potentially be more hardy than normal. An interesting finding from this study was that the transgenic tobacco plant took a longer time to flower so other possibilities relating to the overall weediness should not be dismissed.

The risk management procedures to limit exposure to the environment are to limit the scale of release, enclose and secure the trial area and to destroy unusable plant materials according to proper procedures.

5.5 Framework and suggested protocol for assessment of risks

The following table represents a summary of the full risk assessment and its associated risk management options:

Potential adverse effect	Consequences of potential effects	Estimation of likelihood	Evaluation of identified risk	Consideration of risk management
Toxicity & allergenicity				
1. Human	Very low	Highly unlikely	Very low	• Limit scale of release
2. Other organisms:				
Mammals & wildlifes (fishes and birds)	Low	Unlikely	Low	• Enclose and secure trial area to prevent unintended exposure
Invertebrates (beneficial insects)	Low	Unlikely	Low	• Destroy unusable plant materials in proper manner
Microorganisms	Low	Unlikely	Low	• Ensure secure transfer of plant materials
Environment				
1. Weediness	Low	Unlikely	Low	• Limit scale of release
2. Gene transfer				
Other tobacco plants	Medium	Likely	Medium	• Enclose and secure trial area to prevent unintended exposure
Other plants	Low	Unlikely	Low	• Destroy unusable plant materials in proper manner
Animals (including human)	Negligible	Negligible	Negligible	• Ensure secure transfer of plants materials
Microorganisms	Negligible	Negligible	Negligible	• Not required

6.0 Discussion

Introduction

The Cucumber Mosaic Virus (CMV) is a significant plant pathogen affecting various crops and plants in Malaysia. In 2002, a single chain variable fragment (scFv) antibody, targeted to CMV coat protein, was successfully constructed with mRNA from the spleen of a CMV coat protein-immunized mouse (Chua, 2002). The nucleotide sequence of the variable heavy (VH) and variable light (VL) framework regions of the mouse spleen cDNA were used to design and construct primers for scFv library construction via RT-PCR (Chua *et al.*, 2003). Using the coat protein of a locally isolated chilli strain CMV, a novel soluble Flag-tagged scFv antibody was synthesised after several rounds of panning of the scFv library. The desired anti-CMV scFv fragment was then cloned into a plant expression vector and transformed via *Agrobacterium tumefaciens* into *Nicotiana tabacum* L. cv. White burley tobacco plants (Chua, 2002). In this study, 5 successfully transformed plants were used as individual parental lines to produce progenies for further analysis.

Generations of transgenic plants

Transgenic T_0 tobacco plants were generated by the leaf disc transformation procedure using agrobacteria harbouring recombinant anti-CMV scFv constructs. The plants were grown in a growth room and out of these a total of 5 transgenic plants were allowed to self-pollinate to establish the next generation (T_1). Germination tests were used to study seed viability. Germination rate of 90% was obtained in wild type tobacco plants. Transgenic seeds showed a reduction in germination rate. 72% of T_1 tobacco lines were successfully grown in soil. In subsequent generations, 72% and 71% germination success rates were obtained for T_2 and T_3 progenies respectively. In general, high germination rates were achieved in wild type tobacco plants. These results

suggest that *N. tabacum* has a low level of dormancy consistent with the findings by Finch-Savage and Leubner-Metzger (2006).

Phenotypic characterization of transgenic plants

In comparison to wild type tobacco plants, the T_0, T_1, T_2 and T_3 transgenic plants did not exhibit any unusual physical appearance despite the production of transgenes. However, the flowering time was delayed for transgenic plants as compared to wild type plants. All genetically modified plants took about 5 months to flower while the wild type plants started to flower by 4 months. Soitamo *et al.* (2011) reported that the genes involved in flower induction have been altered in the transgene expressing plants. It is also well documented that the differences observed among transgenics and non-transgenic plants are attributed to the methods employed to generate transgenics and those resulting from breeding (Bhat and Srinivasan, 2002). In transgenic rice plants, R2 generation were reported to be significantly shorter, flowered later and partially sterile as compared with non-transgenic controls (Phillips *et al*., 1994). Liu *et al*. (1996) also observed morphologically abnormal flowers that failed to set seeds in soybeans. Research carried out by Filipecki and Malepszy (2006) indicated that these unexpected phenotypes could be due to pleiotropic effects of DNA integration on the host genome and the side effects of various stresses on the transgenic plants.

Stability and functionality of anti-CMV scFv antibodies

In this study, the putative transgenic plants were analyzed for transgene integration, expression, stability and functional integrity. DNA analysis for transgene integration is one of the basic requirements used to evaluate transgenic plants. The polymerase chain reaction (PCR) is widely used for this purpose. It is one of the most sensitive and simplest methods for detecting integrated genes in transgenic plant

genomes (McGarvey and Kaper, 1991). PCR amplifies specific DNA sequences using designed primers. In this study, the presence of anti-CMV scFv transgene in all 3 generations were observed at a relatively high percentage but in a non-Mendelian segregation manner. It has been reported that transgenes are inherited sexually as a dominant trait (Theuns *et al.*, 2002), with inheritance conforming to a 3:1 Mendelian ratio (Shrawat *et al.*, 2007). Budar *et al*. (1986) revealed that *Agrobacterium*-mediated transformation produces transgenic plants with a low copy number and the transgenes are transmitted to progeny according to Mendelian inheritance. However, in some cases non-Mendelian inheritance has been reported (Deroles and Gardner, 1988). The non-Mendelian inheritance of a transgene has been recorded with a frequency of between 10% and 50% in transgenic plants (Yin *et al*., 2004). The causes of non-Mendelian segregation have been described as due to unstable transmission or poor expression of the transgene results (Limanton-Grevet and Jullien, 2001). Recently, Tizaoui and Kchouk (2012) reported some genetic approaches for studying transgene inheritance and genetic recombination in the successive generations of transformed tobacco can be based on calculation of the theoretical segregation ratios. In the future, in-depth studies are recommended on the mode of transgene inheritance and the recombination frequencies of linked inserts in the generations of transgenic lines.

The integration of anti-CMV scFv gene(s) in the tobacco plants were further confirmed via Southern Hybridization. In this study, at least one copy number of the transgene was detected in 58% of T_1 generation. A slightly elevated percentage of 66% in T_2 generation was observed, but only 52% of the transgene was detected in T_3 generation. Probing the blot with the transgene showed a single band with the size of ~797 bp in all positive test plants. An accurate estimate of the transgene copy number can be made by measuring the signal intensity with an equal amount of DNA from each transgenic plant. Simultaneous probing of the blot with a single copy host gene will

provide an internal standard to determine the single copy transgenic plants (Bhat and Srinivasan, 2002).

Traditionally, Southern Hybridization is one of the conventional methods for transgene copy number determination. However, this technique is time consuming and requires tens of microgram quantities of high-quality DNA (Ingham *et al.*, 2001). Real-time PCR has emerged to be the method of choice for fast and efficient estimation of copy number. External standard curve based method and the ΔCt method involving an internal reference gene were applied to improve the accuracy of real-time PCR (Yuan *et al.*, 2007).

Western blot analysis results revealed that the 32 kDa anti-CMV scFv transgene was expressed at varying levels in 5 transgenic lines across the 3 generations. Of the 5 parental lines analysed, expressed transgene protein was not detected in T_3 generation. In T_1 generation, the T_{1D} line produced the highest amount of detectable transgene proteins, while the T_{1A} line accumulated the lowest amount of protein. In this study, an overall lower expression level was observed in T_2 generation as compared to the previous generation. Apart from this, the T_{2D} line had the highest detectable expressed protein amongst the T_2 generation and the T_{2A} line had the lowest detectable expressed protein. Filipecki and Malepszy (2006) have indicated that independent transgenic lines produce different transgene expressions due to the different position of T-DNA integration or copy number of insertion in the plant genome. Transgene protein was not detected in T_3 generation.

Transgene expression in plants is highly variable. Shrawat *et al.* (2007) reported several factors that influence transgene expression, including the transgene itself, the host genome and the interaction between them. Apart from this, factors such as the tendency of exogenous DNA to undergo rearrangement prior to integration, position effects, the effects of transgene copy number, and the effects of DNA methylation was

reported (Meyer, 1998). The function of genes on a genome-wide scale can be understood via expression profiling by microarrays using formats that include complementary DNA, oligonucleotides or amplicons, as suggested by Xiao *et al.* (2010). Several attempts to improve the expression level of transgenes have been reported. Schouten *et al.* (1996) revealed that the addition of KDEL sequence to an antibody construct improved the accumulation of transgenes significantly. The high accumulation level in cytosol resulting in a high level of protection against CMV has also been reported (Safarnejad *et al.,* 2009).

To further determine the functionality of tobacco expressed scFv, crude leaf extracts of test plants were applied for detection by ELISA and Dot Blot assay. The ELISA results showed that crude extracts containing scFv in T_1 and T_2 generations could specifically bind to CMV coat proteins but failed to do so in T_3 generation as expected. A decrease in functional integrity was observed with subsequent generations based on the results of the Dot Blot Assay. Surprisingly, some positive results were obtained in T_3 generation with Dot Blot Assay. This may be regarded as a false positive as expressed transgene was not detected in T_3 generation. The reason for this could be due to silencing. The phenomenon of gene silencing leading to poor or non-expression of transgene has been shown to be associated with multiple insertions, DNA rearrangements, position effects and over expression (Stam *et al*., 1997; Vaucheret and Fagard, 2001). In addition to this, the relation of transgene to host gene sequences could be an important factor leading to co-suppression (Dorlhac *et al*., 1994). If this is indeed the case, the goal of generating virus-resistant plants will not be successful as RNA viruses will be prevented from accumulating by post-transcriptional process (Stam *et al.,* 1997). Additional successive generations will be needed to study the silencing phenomena.

Bhat and Srinivasan (2002) have suggested picking single copy transgenics to simplify analysis. It was reported that 10 or more independently transformed lines should be included for any kind of meaningful analysis (Birch, 1997). The general practice is to select the progenies (T_1 plants) obtained after self-fertilization of the primary transgenics (T_0 plants) for analysis. However, in most cases, back cross-progenies (BC1F1 plants) would be a better option for analysis, as suggested by Bhat and Srinivasan (2002). The BC1F1 population offers a greater chance of obtaining single copy insertions and allows better comparison among progeny plants to distinguish transgene effect from other effects.

Tissue culture, transformation and breeding are three aspects that need to be considered while analyzing the results. Additionally, it is very important to have appropriate controls in experimentation. Plants regenerated from tissue culture are physiologically very different from their counterparts obtained from traditional propagules (vegetative or seedlings). Flowering and other physiological behaviour have been found to be affected in tissue culture regenerated plants (Bhat and Srinivasan, 2002). To separate the tissue culture effects and transgene effects, untransformed, tissue culture regenerated plants should be used as controls. In addition, non-transgenic controls may be insufficient to identify and exclude effects due to transgene-linked sequences such as the effect of markers (Bhat and Srinivasan, 2002). It has been reported that GUS expressing plants have recorded increased larval growth and up to 50% increase in mean foliage consumption, even though the marker gene introduced along with the gene of interest is generally considered not to affect plant performance or contribute to variation. (Lecardonnel *et al*., 1999). In progeny generations, Bhat and Srinivasan (2002) also recommended that non-transgenic plants derived from primary transgenics to be used as controls.

Challenge of T_1, T_2 and T_3 transgenic plants against CMV

The *N. tabacum* plant is regarded as one of the hosts which are very susceptible to CMV infection due to the high levels of virus accumulation in infected young plants (Safarnejad *et al.,* 2009). Tavladoraki *et al.*, (1993) have reported that scFv antibodies against plant viruses cause a reduction of infection incidence and delay in symptom development by expression in transgenic plants. In this study, the test plants were challenged with CMV to reveal the functionality of the transgene. A bioassay test was performed according to procedure described by Zhang *et al*. (1991). 30 individual transgenic plants at the 4 leaf stage from each generation were inoculated by gently rubbing CMV on the wounded surface. All inoculated plants were kept under similar conditions and a weekly inspection was performed to monitor for symptom development. Early symptoms were discovered 2 weeks after inoculation. The sensitive transgenic lines and wild type plants showed clear symptoms of infection including leaf mosaic and chlorosis. The symptoms induced by CMV on the susceptible test plants were identical to those reported induced by CMV isolates on tobacco plants (Hu *et al*., 2011; Mazidah *et al*., 2012).

To evaluate viral accumulation within the tobacco plants, symptomless plants as well as symptomatic ones were analyzed by ELISA assay. The results showed that only one symptomless plant contained no detectable CMV. In contrast, plants with delayed and severe symptoms showed detectable CMV levels. The ELISA assay demonstrated a direct relationship between symptom severity and the accumulation of CMV. All test plants showed detectable transcripted scFv transgenes. Detectable transcripted scFv transgenes was not found in wild type plants.

Symptomless test plants and those with significant delay of disease symptoms were regarded as resistant. Generally, T_1 and T_2 generations showed high resistance to CMV. This indicates that the resistance phenotype is inherited through T_0 to T_2

generations. A reduction in resistance was observed in T_3 generation. Further studies were conducted on the only symptomless T_1 test plant. It was observed that the T_1 transgenic plant in question remained symptomless. But those initially resistant plants were unable to prevent CMV invasion in the long term. Failure to detect CMV in the plants at early stage was probably due to very low concentration of the virus (Mazidah *et al*., 2012). A few previous studies on the expression of scFv antibodies in transgenic plants have shown that targeting the viral coat protein can lead to improved but incomplete resistance against the challenge virus (Tavladoraki *et al.,* 1993; Zimmermann *et al.,* 1998). Similar results have also been reported for transgenic resistant plants challenged with tomato yellow leaf curl virus (Norris *et al.*, 1998; Zrachya *et al.*, 2007; Safarnejad *et al.*, 2009).

Homology Modeling (HM) and docking

The molecular basis of how proteins function is determined by a number of factors and one important factor is the three-dimensional (3D) structures of the protein. However, it is not easy to determine the 3D structure of a protein from its amino acid sequence. Several methods have been developed to this end. One of the most accurate methods is homology modeling, also known as comparative modelling (Cavasotto *et al.*, 2009). Alternative methods like X-ray crystallography and NMR spectroscopy are inaccurate and time-consuming (Floudas *et al.,* 2006). Homology modeling is based on the fact that proteins which share a similar evolutionary history will have similar structures (Vitkup *et al.*, 2001) and it is the only method which can reliably generate a 3D model of a protein from its amino acid sequence (Tramontano, 2001).

The automated SWISS-MODEL software, available since 1993, was one of the first software in the field of automated modeling and is continuously updated (Schwede, 2003). In this study, SWISS-MODEL program has been applied to predict the V_H and

V_L structures. The analysed results with BLAST show both the input sequences are 85% similar to the template structure in the gene bank. The quality of the homology model therefore is dependent on the quality of the sequence alignment and template structure. A report by Panahi *et al.* (2012) suggested optimization of the predicted model by Modeler 9v8. The predicted protein can be further evaluated using Q-mean score (Benkert *et al.,* 2008) and Ramachandran plot statistics. Structure prediction by HM can help in understanding the 3D structure of a given protein and to elucidate the mechanisms of protein function (Wade *et al.,* 1990). It is important to have a high quality homology model since the function of the protein is determined by its 3D structure. Other programs such as Hidden Markov Model (Karplus, 2009) or Phyre server (Kelley and Sternberg, 2009) could also be used to compare the results.

Molecular docking was carried out in this study to find the potential binding interactions between the anti-CMV scFv antibody and CMV. The Autodock program was selected for use due to its free academic licence and user-friendly software. The newest version of the Autodock package has proved to be efficient even for problematic protein-peptides complex (Hetenyi and Van der Spoel, 2002). The Autodock-based blind docking approach was applied to search the entire surface of V_L chain and V_H chain for binding sites while simultaneously optimizing the conformations of the molecules. The specific binding process was then performed to predict the potential binding sites. We obtained a positive value for lowest energy. This suggests close contact between the anti-CMV antibody and CMV. Due to the limitations of the AutoDock program, other docking programs may need to be used to obtain a better understanding of the interaction between the molecules.

Framework for Safety Assessment of virus resistant transgenic plants

A pilot framework of risk assessment and risk management protocol was presented in compliance with the Malaysia Biosafety Act. As the scFv transgene had not been released for field testing in Malaysia prior to this study, there was no precedent in terms of risk assessment and risk management. With this in mind, the framework developed for this study drew on guidelines from other countries. As the understanding of biosafety improves in Malaysia, the expertise of the scientific community will improve as well. Therefore the safety assessment should be reviewed periodically as new information becomes available.

7.0 Conclusion

The objective of the present study was to evaluate the stability and functionality of the *Nicotiana tabacum* expressing anti-CMV scfv antibodies.

In this study, primary transformants and 3 generations of transgenic tobacco were evaluated for stability and functionality of transgenes. Taken together, all results of T_0, T_1, T_2 and T_3 showed that scFv transgenes have been stably integrated into the plant genome and inherited by successive generations. Even though low expression levels were detected in all test plants, functionality of the transgenes was achieved. The bioassay study provides confirmation of preliminary success in obtaining plants that are resistant to CMV. Overall, areas for improvement for the study include the use of Real-time PCR to determine the expression level and copy number of transgenes. In addition, gene expression studies could also be carried out using microarrays.

In the molecular modeling study, the prediction of binding interaction between CMV and anti-CMV scFv was successfully carried out using the AutoDock program. This prediction should be further evaluated and confirmed with other available Docking programs.

A pilot framework for risk assessment and risk management protocol has been developed within the context of the Malaysian Biosafety Act. The aim of the safety evaluation is to assess if there are any effects on the environment or human health when transgenic tobacco plants are released for field testing.

This proof of concept paves the way for applications to produce diagnostic antibodies to CMV. Future work would include the generation of T_4 for further analysis. In addition, using the guidelines as a reference, biosafety issues on gene flow, toxicity and allergenicity test can be further studied.

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9.0 Appendices

Appendix A:

Buffers and Solutions for small scale isolation of plasmid DNA

LB broth

10g Tryptone

5g Yeast Extraxt

5g NaCl

Dissolved in 1L distilled water

Autoclaved

Solution 1:

50mM glucose

10mM EDTA (pH8.0)

25mM Tris-HCl (pH8.0)

Stored at 4^{o}C

Solution 2:

0.2N NaOH

1% SDS

Prepared freshly

Solution 3:

3M potassium acetate

1.15% (v/v) glacial acetic acid

Stored at 4^{o}C

TE buffer

10mM Tris-HCl, pH 8.0

1.0mM EDTA, pH 8.0

Appendix B

Buffers and stock solutions for Agarose gel electrophoresis

6X Loading Buffer

6ml Glycerol

1.2ml EDTA (0.5M)

2.8ml ddH2O (sterile)

Bromophenol blue

1% Agarose Gel

0.1g Agarose gel powder

Dissolved in 10ml 1X TBE

5X TBE Buffer

54.0g Tris-base

27.5g Boric Acid

20ml 0.5M EDTA (pH 8)

Dissolve in 1L distilled water

1X TBE Buffer

200ml 5X TBE Buffer

800ml distilled water

Stored at room temperature

Appendix C

Buffers for Purification of PCR products

Capture buffer

Buffered solution containing acetate and chaotrope

Wash Buffer

10 mM Tris-HCl, pH 8.0

1mM EDTA

80% (v/v) Absolute Ethanol

Appendix D

Buffers and stock solutions for Southern Blot

Control Labeling Reaction

5µl control DNA2

10µl distilled water

DNA Dilution Buffer

50µg /ml herring sperm DNA

Dissolved in 10mM Tris-HCl and 1 mM EDTA, pH8.0

Depurination Solution

0.25M Hydrochloric acid

Denaturation Solution

1.5M Sodium Chloride

0.5M Sodium Hydroxide

Neutralization Solution

1M Tris (pH7.5)

1.5M Sodium Chloride

20X SSC, pH7.0

175.3g Sodium Chloride

88.2g Sodium Citric acid

Dissolved in 1L distilled water

Stopping Buffer

0.2M Ethylene-Diamino-Tetracetic acid (EDTA), pH 8.0

Dissolved in distilled water

Stored at room temperature

Maleic acid Buffer, pH 7.5

0.1M Maleic acid

0.15M Sodium Chloride

Stored in room temperature

Washing Buffer, pH7.5

0.3% (v/v) Tween 20

Dissolved in Maleic acid buffer

Stored at room temperature

Detection Buffer, pH9.5

0.1M Tris-HCl

0.1M Sodium Chloride

Stored at room temperature

TE-buffer, pH8.0

10mM Tris-HCl

1mM EDTA

Stored at room temperature

10X Blocking Stock solution

10% (w/v) Blocking reagent

Dissolved in Maleic acid buffer

Autoclaved and stored at 4^{o}C

1X Blocking Solution

100ml 10X Blocking Stock Solution

Dissolved in 900ml Maleic acid Buffer

Always freshly prepared

Antibody solution

Anti-Digoxigenin-AP

Diluted 1:5000 in Blocking Solution

Stored at 4^{o}C

Colour-substrate solution

40µl Nitro Blue Tetrazolium (NBT)/ 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)

Added to 2ml of Detection Buffer

Always freshly prepared

Appendix E:

Buffers and stock solutions for SDS-PAGE Electrophoresis and Staining

30% Acrylamide solution (Bio-Rad)

Acrylamide: bis (37.5:1)

Stored in the dark, at 4ºC

10% (w/v) Sodium Dodecyl Sulfate (SDS)

10g SDS

Dissolved in 100ml of distilled water

1.5M Tris-HCl, pH8.8

22.73g Tris base

Dissolved in 150ml distilled water

Stored at 4ºC

0.5M Tris-HCl, pH6.8

6g Tris base

Dissolved in 100ml distilled water

Stored at 4ºC

Sample Buffer

1.25ml 0.5M Tris-HCl (pH 6.8)

2.0ml 10% SDS

2.5ml Glycerol

0.2ml 0.5% (w/v) bromophenol blue

Stored at room temperature

10X SDS Running buffer

30.3g Tris Base

144.0g Glycine

10.0g SDS

Dissolved in 1L distilled water

Stored at room temperature

1X SDS Running buffer

100ml 10XSDS Running buffer

900ml distilled water

10% Ammonium Persulphate (APS)

0.1g APS

Dissolved in 1ml of distilled water

Freshly prepared

4% Stacking Gel

6.1ml Distilled water

2.5ml 0.5M Tris HCl (pH6.8)

1.3ml 30% Polyacrylamide ready-made gel solution

0.1ml 10% SDS

50 µl 10% APS

10µl TEMED

12% Resolving Gel

3.4ml distilled water

2.5ml 1.5M Tris HCl (pH8.8)

4.0ml 30% Polyacrlyamide ready-made gel solution

0.1ml 10% (w/v) SDS

50µl 10% APS

5µl TEMED

Fixing solution

40% Ethanol

7% Glacial acetic acid

Dissolved in distilled water

Coomassie Blue Staining solution

40% Methanol

7% Acetic acid

0.025% Coommasie brilliant blue

Dissolved in distilled water

Destaining solution

20% Ethanol

7% Acetic acid

Stored at room temperature

Appendix F:

Buffers and stock solutions for Western Blot

Transfer Buffer, pH 8.3

3.03g Tris base

14.4g glycine

20ml methanol

Dissolved in 1L distilled water

Stored at 4ºC

TBS-T Buffer, pH7.4

3.0g Tris base

8.0g Sodium Chloride

0.2g Potassium Chloride

0.05% (v/v) Tween 20

Dissolved in 1L distilled water

Blocking solution

5% Skimmed Milk

Dissolved in TBS-T buffer

Alkaline Phosphatase (AP) buffer

1.58g Tris-HCl, pH9.0

0.75g Sodium Chloride

0.10g Magnesium Chloride

Dissolved in 100ml distilled water

Colour Development Solution

33µl Nitro Blue Tetrazolium (NBT)

16.5µl 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)

Added in 5ml AP Buffer

Appendix G:

Buffers and stock solutions for ELISA

General Extraction Buffer, pH 7.4

1.3g Sodium sulfite (anhydrous)

20.0g Polyvinylpyrrolidone (PVP)

0.2g Sodium azide

2.0g Powdered egg albumin

20.0g Tween-20

Dissolved in 1L 1X PBST

Stored at 4ºC

Coating Buffer, pH9.6

1.59g Sodium Carbonate (anhydrous)

2.93g Sodium Bicarbonate

0.2g Sodium Azide

Dissolved in 1L distilled water

Stored at 4ºC

PBS Buffer, pH7.4

8.0g Sodium Chloride

1.15g Sodium Phosphate (anhydrous)

0.2g Potassium Phosphate, monobasic (anhydrous)

0.2g Potassium Chloride

Dissolved in 1L distilled water

PBST Buffer, pH7.4

0.5g Tween-20

Dissolved in 1L PBS Buffer

Blocking Buffer

2% non-fat milk

Dissolved in PBS-T buffer

ECI Buffer, pH7.4

2.0g Bovine serum albumin (BSA)

20.0g Polyvinylpyrrolidone (PVP)

0.2g Sodium azide

Dissolved in 1L PBS-T buffer

Stored at 4ºC

PNP Buffer

0.1g Magnesium Chloride Hexahydrate

0.2g Sodium Azide

97.0ml Diethanolamine

Dissolved in 1L distilled water

Stored at 4ºC

Appendix H:

Buffers and stock solutions for Mechanical Inoculation

0.05M PBS, pH7.0

Stock solution A: 1M Potassium dihydrogen phosphate

Stock solution B: 1M Dipotassium hydrogen phosphate

21.1ml of stock A and 28.9 ml of stock B were mixed and diluted to 1L with distilled water.

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BIOSAFETY ACT 2007

BIOSAFETY REGULATIONS 2010

NBB/A/ER/10/FORM A

APPROVAL FOR RELEASE ACTIVITIES OF LIVING MODIFIED ORGANISM (LMO) (RESEARCH AND DEVELOPMENT PURPOSES IN ALL FIELD EXPERIMENTS) OR IMPORTATION OF LMO THAT IS HIGHER PLANT

NBB/A/ER/10/FORM A shall be submitted to the Director General as an application for certificate of approval of release of LMO [Research and development purposes in all field experiments - Second Schedule of the Act - 1] or importation of living modified organism (LMO) that is a higher plant (not for contained use activities). Any organization undertaking modern biotechnology research and development shall submit the form through its registered Institutional Biosafety Committee (IBC). The IBC should assess the information in the form prior to submission. Application must be accompanied by the prescribed fees as found in Third Schedule of the Biosafety (Approval and Notification) Regulations 2010. Not all parts in this form will apply to every case. Therefore, applicants will only address the specific questions/parameters that are appropriate to individual applications.

In each case where it is not technically possible or it does not appear necessary to give the information, the reasons shall be stated. The risk assessment, risk management plan, emergency response plan and the fulfillment of any other requirements under the Biosafety Act 2007 will be the basis of the issuance of the certificate of approval by the National Biosafety Board (NBB).

The applicant shall submit 1 original and 6 copies of the application to the Director General. A soft copy of the submitted application (including all supporting documents/attachments, if any) shall also be provided in the form of a CD by the applicant. However, all information that has been declared as Confidential Business Information (CBI) should be omitted from the CD.

Accuracy of information

The application should also be carefully checked before submission to ensure that all the information is accurate. If the information provided is incorrect, incomplete or misleading, the NBB may issue a withdrawal of the acknowledgement of receipt of application without prejudice to the submission of a fresh

application. Thus, it is important to provide accurate and timely information that is as comprehensive as existing scientific knowledge would permit, and supported by whatever data available.

Confidentiality

Any information within this application which is to be treated as CBI, as described in the Biosafety Act 2007 in section 59(3) should be clearly marked "CBI" in the relevant parts of the application by providing the justification for the request for CBI. The following information shall not be considered confidential:

a) The name and address of the applicant
b) A general description of the LMO
c) A summary of the risk assessment of the effects on the conservation and sustainable use of biological diversity, taking also into account risks to human health; and
d) Any methods and plans for emergency response

Authorization

Please ensure that if this application is being completed on behalf of the proposed user, that the person completing this application holds proper authority to submit this application for the proposed user. Please provide written proof of authorization.

For further information

Please contact the Director General by:
Telephone: 603-8886 1579
E-mail: biosafety@nre.gov.my

The completed forms to be submitted as follows:

The Director General
Department of Biosafety
Ministry of Natural Resources and Environment Malaysia,
Level 1, Podium 2
Wisma Sumber Asli, No. 25, Persiaran Perdana
Precinct 4, Federal Government Administrative Centre
62574 Putrajaya, Malaysia

Please retain a copy of your completed form.

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APPLICATION CHECK LIST

1. Form NBB/A/ER/10/FORM A is completed with relevant signatures obtained	☐
2. Application assessed and to be sent through the IBC	☐
3. A copy of clearance documents from the Department of Agriculture included (if required)	☐
4. A copy of the clearance document from the state office where the release is to take place	☐
5. Any information to be treated as confidential business information should be clearly marked “CBI” in the application	☐
6. 1 original copy and 6 copies of the completed application submitted. A soft copy of the submitted application (including all supporting documents/attachments, if any) that do not contain any CBI.	☐
7. Fees as prescribed in the regulation: RM_____ Money order/ Bank draft No: _____Made payable to the Secretary General of the Ministry of Natural Resources and Environment	☐

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Preliminary information

1. Organization:	
2. Name of Applicant:	
3. Position in Organization: Telephone (office): Telephone (mobile): Fax number: Email: Postal Address:	
4. Project Title/Unique Identification Code:	
5. IBC Project Identification No:	
6. Is this the first time an approval is being applied for this activity?	Yes ☐ No ☐ if no, please provide information in no 7 below
7. I) Please provide the NBB reference no. for your previous notification/application. II) How is this application different from the previous notification/application submitted for this activity? (please provide an attachment if additional space is required)	

NBB REF.NO :
(For Office Use)

Details of Agent / Importer

8. Organization name:	
9. Contact Person:	
10. Position in Organization: Telephone (office): Telephone (mobile): Fax number: Email: Postal Address:	

NBB REF.NO : (For Office Use)

Institutional Biosafety Committee (IBC) Assessment Report for release of LMO (Research and development purposes in all field experiments) or importation of LMO that is a higher plant (not for contained use activities).

This must be completed by the registered IBC of the Applicant's organization

Section A – IBC Details

1	Name of organization:			
2	Name of IBC Chairperson:			
	Telephone number:		Fax:	
	Email address:			

Section B – IBC Assessment

3	Name of principal investigator:		
4	Project Title:		
5	Date of the IBC Assessment:		
6	Does the IBC consider that the principal investigator and every other person(s) authorized to be involved in the field experiment with the LMO have adequate training and experience for the task?	☐ Yes	☐ No
7	The following information related to this project has been checked and approved		
	a) The objective of the project	☐ Yes	☐ No
	b) The description and genetics of the LMO	☐ Yes	☐ No
	c) The risk assessment and risk management, taking into account the risks to the health and safety of people and the environment from the release of the LMO.	☐ Yes	☐ No

NBB REF.NO :
(For Office Use)

	d) The emergency response plan	☐ Yes ☐ No
8	Has the information been checked by the IBC and found to be complete?	☐ Yes ☐ No
9	Has the IBC assessed the proposed project? ☐ Yes ☐ No If yes, please append a copy of the IBC's assessment report and indicate the attachment in which details are provided.	

NBB REF.NO :
(For Office Use)

Signatures and Statutory Declaration

The proposed release of LMO (Research and development purposes in all field experiments) or importation of LMO that is a higher plant (not for contained use activities) has been assessed as above and endorsed by the IBC. We declare that all information and documents herein is true and correct. We understand that providing misleading information to the NBB, deliberately or otherwise, is an offence under the Biosafety Act 2007.

Applicant:

Signature: _____ Date: _____

Name as in Identity Card/Passport: _____

Official Stamp:

IBC Chairperson:

Signature: _____ Date: _____

Name as in Identity Card/Passport: _____

Official Stamp:

Head of organization/Authorized representative:

Signature: _____ Date: _____

Name as in Identity Card/Passport: _____

Official Stamp:

NBB REF.NO :
(For Office Use)

Part A Risk Assessment

A1 General Information

1. Project Title.

2. Rationale of Project.

3. Project objectives:
   a) Overall Objective
   b) Specific Objective

4. Details of the LMO to be released:
   a) Genus and species
   b) Common name
   c) Modified trait(s)

5. Release site(s) :
   (If more than one location is involved, then the information required in numbers 5, 6, 7, 8 & 9, 10, 11) should be repeated for each location(s) of release)
   a) District(s)
   b) State(s) in which the release(s) will take place

6. Scale of release per release site.
   (Number of LMO involved, size of plot/site etc)

7. Date when the release(s) is expected to commence.

8. Frequency of releases.

9. Date when release(s) is expected to end.

10. For an imported LMO – the date of importation or intended importation, including, if possible, a copy of documentation of clearance or assessment from the relevant authorities like Department of Agriculture (DOA).

11. Description of the proposed activities with the LMO.

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12. Name of person(s) authorized to undertake activities with the LMO.

A2 Risk Assessment Information - Parent Organism

(If more than one parent organism of the same species is involved then the information required in this part should be repeated for each parent organism)

13. Details of the parent organism

If the LMO is the result of a crossing event between more than one species/cultivar/breeding line/variety please include relevant information (for example, LMO crossed with non-LMO or 2 LMOs crossed)

a) Family name
b) Genus
c) Species
d) Subspecies
e) Cultivar/Breeding line/Variety
f) Common name

14. A statement about whether the parent organism has an extended history of safe use in agriculture or in other industries.

15. Information concerning the reproduction of the parent organism:

a) The mode or modes of reproduction
b) Any specific factors affecting reproduction
c) Generation time

16 Information regarding the sexual compatibility of the parent organism with other cultivated or wild plant species.

17. Information concerning the survivability of the parent organism:

a) Ability to form structures for survival or dormancy including seeds, spores and sclerotia
b) Any specific factors affecting survivability, for example seasonability

18. Information concerning the dissemination of the parent organism:

a) The means and extent of dissemination
b) Any specific factors affecting dissemination

19. Details of the natural habitat of the parent organism and its range.

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20. Is the parent organism exotic in Malaysia?

☐ Yes ☐ No

21. Is the parent organism naturalized in Malaysia?

☐ Yes ☐ No

22. Is the parent organism, or a closely related organism, present at, or near, the site of the proposed release(s)?
(If more than one location is involved, then the information required in numbers 22 & 23 should be repeated for each location(s) of release)

☐ Yes ☐ No

23. If yes, please provide details of the population(s) and the estimated distances between them from the proposed release(s).

24. The potentially significant interactions of the parent organism with organisms other than plants in the ecosystem where it is usually grown, including information on toxic effects on humans, animals and other organisms.

25. An assessment of whether the parent organism is capable of causing disease or other ill-health in human, plants or animals and, if so, the details of the possible effects.

26. Details of any known predators, parasites, pests or diseases of the parent organism in Malaysia.

27. Details of pathogenicity, including infectivity, toxigenicity, virulence, allergenicity, carrier (vector) of pathogen, possible vectors, host range including non-target organisms and possible activation of latent viruses (proviruses) and ability to colonize other organisms.

28. Is the parent organism resistant to any known antibiotic and if yes, what is the potential use of these antibiotics in humans and domestic organisms for prophylaxis and therapy?

29. Is the parent organism involved in environmental processes including primary production, nutrient turnover, decomposition of organic matter and respiration?

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A3 Risk Assessment Information - LMO

30. Details of the modified trait(s) and how the genetic modification will change the phenotype of the LMO to be released.

31. What are the gene(s) responsible for the modified trait(s)?

32. Give details of the organism(s) from which the gene(s) of interest is derived:

(If more than one gene is involved then the information required in numbers 32, 33, 34, 35, 36 and 37 should be repeated for each gene)

a) Family name
b) Genus
c) Species
d) Subspecies
e) Cultivar/Breeding line/Variety
f) Common name

33. Indicate whether it is a:

a) viroid
b) RNA virus
c) DNA virus
d) bacterium
e) fungus
f) animal
g) plant
h) other (please specify)

34. Does the gene(s) of interest come from an organism that causes disease or other ill-health in humans, plants or animals? Provide details of the possible effects.

35. Please provide the following information about the gene(s) of interest(s):

a) Size of sequence of the gene(s) of interest inserted
b) Sequence of the gene(s)of interest inserted
c) Intended function of the gene(s) of interest
d) Number of copies of the gene(s) of interest in the construct
e) Details of the steps involved in the construction

f) Provide the map(s) of construct(s) indicating the gene(s) of interests and all other regulatory elements that will finally be inserted in the LMO

36. Please provide the following information about the deleted sequence(s):

a) Size of the deleted sequence(s)

b) Function of the deleted sequence(s)

c) Details of the steps involved in the deletion of sequences from the parental organism

d) Provide the map(s) of construct(s)

37. The following information is on the expression of the gene(s) of interest:

a) Level of expression of the gene(s) of interest and methods used for its characterization

b) The parts of the plant where the gene(s) of interest is expressed, such as roots, stem or pollen

c) Indicate the part(s) of the vector(s) that remains in the LMO

d) The genetic stability of the gene(s) of interest

38. A description of the methods used for the genetic modification:

a) How gene(s) of interest was introduced into the parent organism, or

b) How a sequence of a gene was deleted from the parent organism

39. If no vector was used for the genetic modification please provide details of how the gene(s) of interest is introduced.

40. If vector(s) was used, please provide the following information:

(If more than one vector was used, then the information required in 40 should be repeated for each vector).

a) Type of vector

i. plasmid
ii. bacteriophage
iii. virus
iv. cosmid
v. phasmid
vi. transposable element
vii. other, please specify

b) Identity of the vector(s)

c) Information on the degree of which the vector(s) contains sequences whose product or function is not known

d) Host range of the vector(s)

e) Potential pathogenicity of the vector(s)

f) The sequence of transposons and other non-coding genetic segments used to construct the LMO and to make the introduced vector(s) and insert(s) function in those organisms

41. Details of the markers or sequences that will enable the LMO to be identified in the laboratory and under field conditions. Provide appropriate evidence for the identification and detection techniques including primer sequences of the detection of the inserted gene(s) including marker gene(s).

42. Information (biological features) on how the LMO differs from the parent organism in the following respects:

a) Mode(s) and/or the rate of reproduction

b) Dissemination

43. If there is any possibility that the inserted gene(s) in the LMO could be integrated into other species at the release site(s) and the surrounding environment and if so, please provide the following details:

a) The organism(s) to which the modified trait(s) can be transferred to and the frequency at which it can be transferred

b) The transfer mechanism involved and the techniques that have been used to demonstrate transfer

c) Any possible adverse effects of the transfer including

i. Any advantages the affected organism(s) are likely to have over the number of the species that do not contain the inserted gene(s)

ii. Environmental risks posed by such an advantage

44. The identification and description of the target organism(s), if any.

45. The anticipated mechanism and result of interaction between the released LMO and the target organism(s).

46. The known or predicted interaction on non-target organisms in the release site(s) and the impact on population levels of competitors, prey, hosts, symbionts, predators, parasites and pathogens.

47. A statement on whether the modified trait(s) of the LMO will change the capacity of the plant to add substances to, or subtract substances from, soil (for example, nitrogen or toxic compounds) and, if so, details of all such changes.

48. Details of any other possible adverse consequences.

49. Details whether the LMO compared to the parent organism that will confer a selective advantage that can impact on survival in the release site(s), including a statement on how stable those features are.

50. Details of whether the modified trait(s) will confer a selective advantage on the LMO compared to the parent organism and if so, the nature of the advantages including a statement on how stable those features are and under what conditions.

51. Details of whether the gene(s) of interest or any part of the vector(s) has the ability to reproduce or transfer to other hosts and, if so, details of the host range.

52. In relation to human health:

a) The toxic or allergenic effects of the non-viable organisms and/or their metabolic products

b) The comparison of the organisms to the donor, or (where appropriate) parent organism regarding pathogenicity

c) The capacity of the organisms for colonization

d) If the organisms are pathogenic to immunocompetent persons:

i. diseases caused and mechanisms of pathogenicity including invasiveness and virulence,
ii. communicability,
iii. infective dose,
iv. host range and possibility of alteration,
v. possibility of survival outside of human host,
vi. presence of vectors or means of dissemination,
vii. biological stability,
viii. antibiotic-resistance patterns,
ix. allergenicity, and
x. availability of appropriate therapies.

53. Details of unintended pleiotropic effects (if any), including undesirable effects on agronomic characteristics of the plant which may result from the expression of the gene of interest(s) in the LMO (for example, reduced fertility, increased prevalence, production losses, grain shedding), including an indication of the likelihood of these events.

54. The description of genetic traits or phenotypic characteristics and in particular any new traits and characteristics which may be expressed or no longer expressed.

55. Details of how the genetic modification will change the phenotype of the LMO to be released, including information to demonstrate the effect of the genetic modification.

56. Details of the mechanism of pollen spread (by insect vectors or by other means) in the plant population:
    a) Details of pollen viability for the parent organism and of the LMO
    b) Details of any potential pollinators and their range and distribution in Malaysia
    c) Quantitative data on successful cross-pollination between the parent organism, the LMO and its wild relatives, if available

A4 Information about weeds

57. Details of the members of the family of parent organism that are known to be weeds in any environment.

58. Details of cross-pollination between the species to which the LMO belongs and wild relatives known to be weeds, including a copy of any literature reports that support the information.

A5 Information about the seeds of the LMO

59. A statement on whether the LMO proposed to be released will be allowed to set seed and, if not, whether setting seed is planned for a later release.

60. If the LMO is to be allowed to set seed, will the mature seed normally remain contained within an ear, capsule or pod, so that practically all of the seed can be readily harvested, or is the seed shed soon after it matures?
    If the latter, provide an indication of the proportion of seed likely to remain in the release site(s) following harvest.

61. Details of the length of time that the seeds are capable of being dormant and whether it differs from the parent organism.

A6 Characteristics affecting survival of LMO

62. The predicted habitat of the LMO.

63. The biological features which affect survival, multiplication and dispersal.

64. The known or predicted environmental conditions which may affect survival, multiplication and dispersal, including wind, water, soil, temperature, pH.

65. The sensitivity to specific agents (e.g. disinfectant, pesticides, fertilizers, wind, water).

A7 Information about any secondary ecological effects that might result from the release

66 An assessment of possible effects of the proposed release on:

a) Native species
b) Resistance of insect populations to an insecticide
c) Abundance of parasites

A8 Information about resistance of the LMO to a chemical agent (other than selective agents, such as antibiotics, used in strain construction)

67. Details of any environmental risks related specifically to the resistance of the LMO to a chemical agent (for example, a herbicide, but not a selective agent, such as an antibiotic, used in strain construction), where the resistance is a result of the genetic modification.

A9 Information about resistance of the LMO to a biological agent

68. Details of any environmental risks related specifically to the resistance of the LMO to a biological agent (for example, an insect or a fungal disease), where the resistance is a result of the genetic modification.

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(For Office Use)

A10 Information relating to the release site(s)

(If more than one release site is involved, then the information required in this part should be repeated for each release site)

69. The size of the proposed release site(s).

70. The location of the proposed release site(s). Provide site map(s) with national grid reference(s).

71. Details of the reasons for the choice of the release site(s).

72. Details of the arrangements for conducting any other activities in association with the proposed release(s), such as importation of the LMO and transportation of the LMO, to or from the release site(s).

73. The preparation of the release site(s) before the release(s).

74. The methods to be used for the release(s).

75. The quantity of the LMO to be released.

76. The physical or biological proximity of the release site(s) to humans and other significant biota or protected areas.

77. The size of local human population.

78. The local economic activities which are based on the natural resources of the area.

79. The distance to the nearest drinking water supply zone areas and/or areas protected for environmental purposes.

80. The flora and fauna, including crops, livestock and migratory species in the release site(s).

81. The comparison of the natural habitat of the parent organism(s) with the proposed release site(s).

82. Any known planned developments or changes in land use in the region which could influence the environmental impact of the release.

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(For Office Use)

Part B Risk Management

B1 Information on control, monitoring, post-release plans

83. A description of measures (if any) to minimize the effects of any transfer of the modified genetic trait(s) to other organisms.

84. Details of the proposed release site(s) supervision procedures and if necessary any relevant safety procedures designed to protect staff, including a description of procedures for onsite supervision of the release if the release site(s) is located at some distance from the location of the applicant.

85. Details of proposed measures (if any) for monitoring any risks posed by the LMO(s), including monitoring for:

a) The survival or presence of the LMO, or transferred genetic material, beyond the proposed release site(s), including specificity, sensitivity and reliability of detection methods
b) Impacts on the characteristics, or abundance, of other species
c) Transfer of the gene(s) of interest to other species
d) Any other hazards or deleterious effect

86. Details of proposed procedures for auditing, monitoring and reporting on compliance with any conditions imposed by the NBB.

87. Details of ongoing monitoring to be undertaken after the release(s) are completed.

88. Details of proposed measures to minimize the possible adverse consequences. If no measures have been taken, please give reasons.

89. The methods for elimination or inactivation of the organisms at the end of the experiment and the measures proposed for restricting the persistence of the LMO or its genetic material in the release site(s).

B2 Waste treatment plans

90. Type of waste generated.

91. Expected amount of waste.

92. Possible risks resulting from the waste.

93. Description of waste treatment envisaged and its disposal.

Part C Emergency response plan

94. Methods and procedures for controlling/removing the LMO in case of unintentional release or any adverse effects being realized.

95. Methods for isolation of the area affected.

96. Methods for disposal of other plants, animals and any other thing exposed to the adverse effects

Part D Data or results from any previous release(s) of the LMO

97. Give the following information from the previous applications and releases of the LMO for which the applicant is seeking an approval:
    i. Reference number of each application
    ii. Date of the certificate of approval issued
    iii. Terms and conditions (if any) attached to the approval
    iv. Data and results of post-release monitoring methods and effectiveness of any risk management procedures, terms and conditions and other relevant details
    v. Relevant data if the previous release is on a different scale or into a different ecosystem
    vi. Any other relevant details

98. Details of results of any applications made for approval of the LMO in other countries, including information about conditions (if any) attached to the approval.

99. Details of any previous notifications for contained use activities according to the Biosafety Act 2007 from which the work in this present application has been developed.

100. If the LMO has been previously released overseas, details of any adverse consequences of the release, including identifying references and reports of assessments if any.

NBB REF.NO :
(For Office Use)

BIOSAFETY ACT 2007

BIOSAFETY REGULATIONS 2010

NBB/N/CU/10/FORM E

NOTIFICATION FOR CONTAINED USE AND IMPORT FOR CONTAINED USE ACTIVITIES INVOLVING LIVING MODIFIED ORGANISM (LMO) FOR BIOSAFETY LEVELS 1, 2, 3 AND 4

NBB/N/CU/10/FORM E shall be submitted to the Director General as a notification for contained use and import for contained use (not involving release into the environment of Living Modified Organism (LMO) as specified in Second Schedule of the Act). Any organization undertaking modern biotechnology research and development shall submit the notification through its Institutional Biosafety Committee (IBC) that is registered with the National Biosafety Board (NBB). The IBC should do an assessment prior to submission. Not all parts in this form will apply to every case. Therefore, applicants will only address the specific questions/parameters that are appropriate to individual applications.

In each case where it is not technically possible or it does not appear necessary to give the information, the reasons shall be stated. The risk assessment, risk management plan, emergency response plan and the fulfillment of any other requirements under the Biosafety Act 2007 will be the basis of the decision by the NBB.

The applicant shall submit 1 original and 6 copies of the notification to the Director General. A soft copy of the submitted notification (including all supporting documents/attachments, if any) shall also be provided in the form of a CD by the applicant. However, all information that has been declared as Confidential Business Information (CBI) should be omitted from the CD

NBB REF.NO :
(For Office Use)

Providing information

The information provided in this notification will be used to evaluate the emergency response plan as specified in section 37 of the Biosafety Act 2007 and specific measures to be taken in relation to a contained use activity involving LMO. Thus it is important to provide accurate and timely information that is as comprehensive as existing scientific knowledge would permit, and supported by whatever data available.

The NBB may require additional information, and the applicant will be notified should this be the case. If the applicant fails to provide the additional information requested, the notification shall be deemed to have been withdrawn but it shall not affect the right of the applicant to make a fresh notification.

Accuracy of information

The notification should also be carefully checked before submission to ensure that all the information is accurate. If the information provided is incorrect, incomplete or misleading, the NBB may issue a withdrawal of the acknowledgement of receipt of notification without prejudice to the submission of a fresh notification

Confidentiality

Any information within this notification which is to be treated as CBI, as described in the Biosafety Act 2007 in section 59(3) should be clearly marked “CBI” in the relevant parts of the notification by providing the justification for the request for CBI. The following information shall not be considered confidential:

a) The name and address of the applicant
b) A general description of the LMO
c) A summary of the risk assessment of the effects on the conservation and sustainable use of biological diversity, taking also into account risks to human health; and
d) Any methods and plans for emergency response

Authorization

Please ensure that if this notification is being completed on behalf of the proposed user, that the person completing this notification holds proper authority to submit this notification for the proposed user. Please provide written proof of authorization.

NBB REF.NO :
(For Office Use)

For further information

Please contact the Director General by:

Telephone: 603-8886 1579

E-mail: biosafety@nre.gov.my

The completed forms to be submitted as follows:

The Director General

Department of Biosafety

Ministry of Natural Resources and Environment Malaysia

Level 1, Podium 2

Wisma Sumber Asli, No. 25, Persiaran Perdana

Precinct 4, Federal Government Administrative Centre

62574 Putrajaya, Malaysia.

Acknowledgment of Receipt

Upon receipt of the notification, the Director General shall send to the applicant an acknowledgement of receipt with an assigned reference number. The reference number should be used in all correspondence with respect to the notification.

Exemption

The First Schedule of the Biosafety (Approval and Notification) Regulations 2010 allows exemptions for some types of techniques and contained use activities in relation to LMO posing a very low risk (i.e. contained research activities involving very well understood organisms and processes for creating and studying LMO). Exempted activities should be carried out under conditions of standard laboratory practice. Appropriate biosafety levels as according to Second Schedule of the Biosafety (Approval and Notification) Regulations 2010 should be used for the exempted activities and personnel should have appropriate training. Principal Investigators who believe that the work falls into any of the exemptions should nevertheless notify their IBC of the proposed project. The IBC may review all submitted research projects to determine their exemption or non-exemption status.

Please retain a copy of your completed notification.

NBB REF.NO : (For Office Use)

Notification Check List

1. Form NBB/N/CU/10/FORM E is completed with relevant signatures obtained	☐
2. Notification assessed and to be sent through the IBC (if relevant)	☐
3. A copy of clearance documents from the relevant Government agencies (if required)	
4. Any information to be treated as confidential business information should be clearly marked “CBI” in the notification	☐
5. 1 original and 6 copies of the completed notification submitted. A soft copy of the submitted notification (including all supporting documents/attachments, if any) that do not contain any CBI.	☐

Preliminary information

1. Organization:	
2. Name of Applicant:	
3. Position in Organization: Telephone (office): Telephone (mobile): Fax number: Email: Postal Address:	
4. Project Title:	
5. IBC Project Identification No:	
6. Is this the first time the activity is being notified?	Yes ☐ No ☐ If no, please provide information in number 7

NBB REF.NO :
(For Office Use)

7. I) Please provide the NBB reference number of your previous notification. II) How is this notification different from the previous notification submitted for this activity? (please provide an attachment if additional space is required)	

Details of Agent / Importer

8. Organization:	
9. Contact Person:	
10. Position in Organization: Telephone (office): Telephone (mobile): Fax number: Email: Postal Address:	

NBB REF.NO : (For Office Use)

Institutional Biosafety Committee (IBC) Assessment Report for the contained use and import for contained use of LMO

This must be completed by the registered IBC of the Applicant's organization

Section A – IBC Details

1	Name of organization::			
2	Name of IBC Chairperson:			
	Telephone number:		Fax:	
	Email address:			

Section B – IBC Assessment

3	Name of principal investigator:	
4	Project Title:	
5	Date of the IBC Assessment:	
6	Does the IBC consider that the principal investigator and every other person(s) authorized to be involved in contained use of the LMO have adequate training and experience for the task?	☐ Yes ☐ No
7	The following information related to this project has been checked and approved	
	a) The objective of the project	☐ Yes ☐ No
	b) The description and genetics of the LMO	☐ Yes ☐ No
	c) The emergency response plan and the specific measures to be taken in relation to a contained use activity involving LMO.	☐ Yes ☐ No
8	Has the information been checked by the IBC and found to be complete?	☐ Yes ☐ No

NBB REF.NO :
(For Office Use)

9	Has the IBC assessed the biosafety of the proposed project? ☐ Yes ☐ No If yes, please append a copy of the IBC's assessment report and indicate the attachment in which details are provided.

Signatures and Statutory Declaration

The contained use of LMO within this project has been assessed as above and endorsed by the IBC. We declare that all information and documents herein is true and correct. We understand that providing misleading information to the NBB, deliberately or otherwise, is an offence under the Biosafety Act 2007.

Applicant:

Signature: _____ Date: _____

Name as in Identity Card/Passport: _____

Official Stamp:

IBC Chairperson:

Signature: _____ Date: _____

Name as in Identity Card/Passport: _____

Official Stamp:

Head of organization/Authorized representative:

Signature: _____ Date: _____

Name as in Identity Card/Passport: _____

Official Stamp:

NBB REF.NO :
(For Office Use)

Part A General Information

A1 Information

1. The name and address of the applicant and the name, qualifications and experience of the scientist and of every other person who will be responsible for planning and carrying out the contained use activities and for the supervision, monitoring and safety of the activity.

A2 Project Introduction

In this Part, the applicant is required to describe the proposed activities with the LMO within the context of the project.

2. Project Title:

3. Biosafety Level (BSL) :

   BSL 1 ☐ BSL2 ☐ BSL3 ☐ BSL4 ☐

4. Rationale of activity:

5. Overall Project/Programme Objective:
   Specific Objective(s)**:**

6. Include an estimated time schedule to achieve the objectives:

7. Intended Date of Commencement:

8. Expected Date of Completion:

9. For an imported LMO– the date of importation or intended importation, including, if possible, a copy of documentation of clearance or assessment from the relevant authorities like Department of Agriculture (DOA), Ministry of Health, Malaysia, etc...

10. Categories of people (Research staff, technicians, students etc) authorised to undertake activities with the LMO:

NBB REF.NO :
(For Office Use)

11. Briefly describe the project using non-technical terms:

12. If the experiments are successful are there plans for an application for field experiment?
Yes ☐ No ☐

13. If yes, where would the proposed field experiment take place?

14. Who will undertake the unconfined release?

A3 Description of the LMO

The information requested in the following section is required to help identify any possible hazards associated with the proposed activities with the LMO. Some questions in this section may also relate to risk assessment and risk management, which are addressed in A4.
(If more than one LMO is involved, then the information required in A3 should be repeated for each LMO).

NBB REF.NO :
(For Office Use)

Please fill the specific information in a tabulated form as below

Table 1 Description of the LMO for contained use activities

LMO	**Common and scientific name of donor organism**	**Common and scientific name of parent organism**	**Vector(s) or method of genetic modification**	**Class of modified trait (Refer to Box 1)**	**Modified trait**	**Identity and function of gene(s)of donor organism responsible for the modified trait**	**Target organism(s) of the LMO**	**Target tissues for genetic modification**
1								
2								

NBB REF.NO :
(For Office Use)

Box 1 : Various Classes or Types of Traits

NO	Class (type) of trait
1	Abiotic stress resistance
2	Altered agronomic characteristics
3	Altered nutritional characteristics
4	Altered pharmaceutical characteristics
5	Altered physical product characteristics
6	Antibiotic resistance
7	Foreign antigen expression
8	Attenuation
9	Bacterial resistance
10	Disease resistance
11	Flower colour
12	Fungal resistance
13	Herbicide tolerance
14	Immuno-modulatory protein expression
15	Pest resistance *e.g.* insect
16	Protein expression
17	Reporter/marker gene expression
18	Virus resistance
19	Other (provide details)
20	Unknown

NOTE:

1. If the LMO has more than one modified trait please list all, as according to the list in the Box 1.
2. If the modified trait is not listed in the Box 1, please list it as “other” and provide details of the modified trait.

A4 Risk assessment and management

(If more than one LMO is involved, then the information required in A4.1, A4.2 & A4.3 should be repeated for each LMO)

In order to prepare the Emergency Response Plan, an assessment of any possible risks or potential harm that may be posed by the LMO and the level of risk posed by such hazards based on an assessment of the likelihood and consequence of the hazard occurring must be carried out. The risks that the IBC is required to assess are:

a) risks to the health and safety of humans from the activities associated with genetic modification
b) risks to the health and safety of humans from an unintentional release of the LMO; and
c) risks to the environment from an unintentional release of the LMO

The risk management plan details how any risks posed by the LMO will be managed to ensure that unacceptable risks are not realised.

Summaries of any protocols and/or standard operating procedures can be included to specifically answer the individual questions.

A4.1 Risk Assessment (Basic information)

15. Is there any risk to health and safety of humans occurring from the proposed activity over and above those posed by the donor/parent organism?

    No known hazard ☐ Not relevant ☐ Yes ☐

    If yes, please provide information in question below.

16. What are the possible hazard(s) and the likelihood and consequence of the hazard(s) occurring (i.e. the risk) from the proposed genetic modification(s)?

17. In regard to the health and safety of humans, what are the possible hazard(s) and the likelihood and consequence of the hazard(s) occurring (i.e. the risk) from an unintentional release of the LMO into the environment?

18. In regard to the environment, what are the possible hazard(s) and the likelihood and consequence of the hazard(s) occurring (i.e. the risk) from an unintentional release of the LMO into the environment?

A4.2 Risk Management

19. Do you propose to transport the LMO outside the premises? If yes, describe the precautions taken.

20. How will the LMO be disposed of?

21. What are the procedures for decontaminating equipments used during the proposed activities in order to render any LMO unviable?

A4.3 Emergency Response Plan

22. Plans for protecting human health and the environment in case of the occurrence of an undesirable effect observed during contained use activities.

23. Methods for removal of the LMO in the affected areas in the case of an unintentional release.

24. Methods for disposal of other plants, animals and any other organisms exposed during the unintentional release.

25. Methods for isolation of the area affected by the unintentional release.

26. Details of any other contingency measure that will be in place to rectify any unintended consequences if an adverse effect becomes evident during the contained use activities or when an unintentional release occurs.

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A5 The Premises

Please provide information for all of the facilities being used for the confined activities in the table below.

Information required	**Premise 1**	**Premise 2***	**Premise 3***
1.Name of premises:			
2.Premises type: *(e.g. animal containment premise, laboratory, insect containment premise, etc)*			
3.Biosafety level (BSL):			
4.Who undertook the inspection: *(indicate whether it was* NBB, IBC *or its representative)*			
5.Date of most-recent inspection :			
6.Fill the following if the BSL level is 3 or 4: Date of certification by competent authority (If any) Certificate reference no:			

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7.Premises address:			
8.Premises contact person details/ Biosafety Officer Name:			
9.Business phone number:			
10.Mobile phone number:			
11.Fax number:			
12.Email address:			

Note:

* For notifications with more than one premise; use additional columns if necessary.

A6. Confidential Business Information

Enter in this section any information required in Part A 1 - A 5 for which confidentiality is claimed together with full justification for that claim.

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