NICOTIANA TABACUM AS A POTENTIAL PLATFORM FOR THE PRODUCTION OF RECOMBINANT ANTI-*TOXOPLASMA* SINGLE-CHAIN VARIABLE FRAGMENT (scFv) ANTIBODY

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ABSTRACT Plant systems have now gained much attention for recombinant therapeutic protein production. In this study, we aimed to evaluate the possibility of producing a single-chain variable fragment antibody (scFv), encoded by the *TP60* gene, against Toxoplasmosis disease in *Nicotiana tabacum* cv. SR1. Toxoplasmosis, caused by infection of a parasitic protozoan known as *Toxoplasma gondii*, is one of the most prevalent parasitic diseases. Leaf explants of *N. tabacum* were infected with *Agrobacterium tumefaciens* strain LBA4404 harbouring binary vector pCAMBIA1304 containing *TP60* gene. Bands observed at the predicted size of 914 bp confirmed the presence of *TP60* transgene, which was stably integrated into both T_0 and T_1 tobacco plant genome. FV12-6 transgenic line produced the highest mRNA expression (7-fold) correlated to the highest accumulation of anti- *Toxoplasma* recombinant scFv antibody (0.52% of the total soluble protein), followed by FV16-10, FV17-7, and FV3-11. Normal growth of the transgenic plants was observed. The segregation analysis of FV3, FV12, and FV16 in T_1 generation confirmed the transgene integration within a single locus according to 3:1 Mendelian's law. These findings indicate the potential of using *N. tabacum* as a host system to produce TP60 recombinant protein without affecting the normal phenotype of the host plant.

ABSTRAK Sistem tumbuhan kini telah mendapat perhatian bagi tujuan penghasilan protein terapeutik rekombinan. Dalam kajian ini, kami bertujuan untuk menilai kemungkinan menghasilkan antibodi serpihan rantaian variasi tunggal (scFv), yang dikodkan oleh gen *TP60*, terhadap penyakit Toxoplasmosis oleh *Nicotiana tabacum* cv. SR1. Toxoplasmosis, akibat jangkitan oleh protozoa parasit yang dikenali sebagai *Toxoplasma gondii*, adalah salah satu penyakit parasit yang paling lazim. Eksplan daun *N. tabacum* telah dijangkiti oleh *Agrobacterium tumefaciens* jenis LBA4404 yang menyimpan vektor dedua pCAMBIA1304 yang mengandungi gen *TP60*. Penjujukan yang kelihatan pada saiz ramalan, 914 bp mengesahkan kehadiran transgen *TP60* yang telah terintegrasi secara stabil ke dalam genom

tanaman tembakau generasi T_0 dan T_1. Individu transgenik FV12-6 menghasilkan ekspresi mRNA tertinggi (7 kali ganda) berhubung kait dengan pengumpulan tertinggi scFv anti-Toxoplasma antibodi rekombinan (0.52% daripada jumlah protein larut), diikuti oleh FV16-10 FV17-7 dan FV3-11. Pertumbuhan normal tumbuh-tumbuhan transgenik diperhatikan. Analisis segregasi FV3, FV12, dan FV16 pada generasi T_1 mengesahkan integrasi transgen di dalam satu lokus menurut undang-undang Mendelian, 3: 1. Penemuan ini menunjukkan potensi *N. tabacum* sebagai sistem perumah untuk menghasilkan protein rekombinan TP60 tanpa menjejaskan fenotip normal tumbuhan perumah.

Keywords: Plant transformation, plant molecular farming, recombinant protein, single-chain variable fragment antibody (scFv), tobacco, toxoplasmosis

1. INTRODUCTION

Toxoplasmosis is a disease caused by the infection of a parasitic protozoan known as *Toxoplasma gondii*, which has economic implications due to neonatal loss and abortion in livestock animals (Elfahal et al., 2013; Stelzer et al., 2019). Efforts have been made to develop vaccines against *T. gondii*; however, only limited vaccines and anti-*Toxoplasma* drugs have been licensed for veterinary use. At present, only one commercial vaccine, Toxovax, which is based on live attenuated S48 strain, has been licensed for use to avoid congenital infection in ewes (Buxton & Innes, 1995; Wen-Bin et al., 2019; Wang et al., 2019). The vaccine was previously developed to prevent the spread of disease in the sheep industry worldwide (Buxton & Rodger, 2008). In addition, Toxovax has previously been used to reduce or eliminate the formation of infective tissue cysts in pigs for human consumption (Innes et al., 2011; Burrells et al., 2015). However, this vaccine is expensive and has a limited shelf-life. Furthermore, it may also revert to a pathogenic strain and may lead to an allergic reaction (Foroutan et al., 2019). *T. gondii* is capable of completing its life cycle without showing any symptoms of infection in feline as the host system. *T. gondii* is zoonotic and could be transmitted through saliva and faeces of infected feline. Although it is usually asymptomatic in adults (Flegr et al., 2014), it can cause complications in immunocompromised persons (Wang et al., 2017) and pregnant women, which leads to congenital effects of newborn babies (Chaudhry et al., 2014). Therefore, this vaccine can have limited application for human use. Further efforts are needed to target this parasitic infection by focusing on the infection mechanisms of this parasite and how the host's immune system could combat this disease. Alternatively, developing recombinant proteins through various host systems would be of great value to human and veterinary medicine (Lim et al., 2018; Puetz & Wurm, 2019). To our knowledge, publications regarding the development of *Toxoplasma* antibody compared to vaccines are very minimal.

Antibodies are an essential component of protective immunity against the non-self antigen (foreign). Recombinant antibody fragments, such as single-chain variable fragment (scFv), an alternative to full-length monoclonal antibodies, have become useful in clinical practices due to the reduced size and simple expression in several systems while retaining their specific paratope (Farajnia et al., 2014; Lim et al., 2018). For instance, scFv with intact paratope has been successfully expressed in both prokaryotes and eukaryotes, such as

bacteria (Sarker et al., 2019), mammalian cells (Omar, 2017), yeasts (Yuan et al., 2019), and insects (Kurasawa et al., 2012; Cérutti, & Golay, 2012). Recently, a recombinant EBOV VP35 scFv monoclonal antibody has been found to significantly hamper the inhibition of the interferon-β response, indicating its capability to control the spread of the virus (Flego et al., 2019).

Each host system has its advantages and disadvantages, depending on the experimental purposes and applications. Although most of the clinically available proteins and drugs are derived from mammalian cells, microbe, and yeast, the high demand for biopharmaceuticals coupled with the high costs and inefficiency of the existing production systems have limited the manufacturing capacity of these conventional cell-based expression systems. Moreover, the use of mammalian cells as a production host also raises concerns about its safety. Therefore, the approach of utilising plant host systems in the production of therapeutic proteins offers a lower production cost and lower risk of contamination compared to mammalian cells (Yao et al., 2015; Lim et al., 2018). In addition, the occurrence of post-translational modifications in the plant system ensures that the expressed recombinant proteins are correctly folded while maintaining their structural and functional integrity. The production of numerous complexes functional mammalian proteins, such as human serum proteins, growth regulators, antibodies, and vaccines, in plants have been reported (Obeme et al., 2011). For example, several therapeutic recombinant proteins against Ebola virus (Maxmen, 2012; Merlin et al., 2014; Sack et al., 2015), high-immunodeficiency virus (HIV; Niemer et al., 2014), West-Nile virus (Lai et al., 2014; Chen, 2015), H1N1 virus and its derivatives (Shoji et al., 2013; Cummings et al., 2014; Takeyama et al., 2015), and dengue virus (Kim et al., 2015; Amaro et al., 2015; Dent et al., 2016) have been published.

We hypothesised that tobacco plants could be used as a host system to produce TP60 protein. To test our hypothesis, we introduced and expressed TP60 protein in *Nicotiana tabacum* cv. SR1 mediated by *Agrobacterium tumefaciens* strain LBA4404. TP60 protein, encoded by *TP60* gene, is a tachyzoite-specific single-chain variable fragment (scFv) antibody that has been reported to show at least 1.8-fold higher binding titres towards the target antigen of *T. gondii* compared to the parental antibody (Lim et al., 2012). The presence of the transgene in the transformed *N. tabacum* plantlets was then determined by transient reporter gene expression (GUS and GFP) and PCR analysis. Analysis of *TP60* transgene in transgenic plants at the RNA and protein levels using semi-quantitative and quantitative real-time PCR (RT-qPCR) as well as Western blot, respectively, were carried out. Upon establishing a stable transformation of anti-Toxoplasma protein in *N. tabacum*, the segregation pattern of T_1 generation has been analysed in addition to the phenotypic assessment between transgenic lines. These findings suggested that *N. tabacum* could be a potential host system to produce TP60 recombinant protein without affecting the normal phenotype of the host plant.

2. MATERIALS AND METHODS

2.1 Vector construction, bacterial strain, and culture preparation

The plant expression vector, pCAMBIA 1304 (http://www.cambia.org; CSIRO, Dickson, Australia) was used in the construction of a cassette containing anti-*Toxoplasma* scFv gene, *TP60*. The *TP60* (accession no: KC508790.1) gene

was obtained from the previous studies by Lim (2012) and Go (2013). The fragment was amplified from a pCANTAB 5E vector and ligated to pCAMBIA 1304 and transformed into *Escherichia coli* before being transformed into *A. tumefaciens* strain LBA4404 using a heat-shock method (Rahimzadeh et al., 2016). The T-DNA sequence in pCAMBIA 1304 vector contained hygromycin-resistant (*hpt*II), m*gfp*5 (GFP), *gus*A (GUS), and target (*TP60*) genes fused with E-epitope tag sequence (GAPVPYPDPLEPR) driven by the CaMV 35S promoter (Figure 1). This construct was named as pTP60. Positively transformed colonies of *Agrobacterium* (verified by colony PCR with gene-specific primers, primer sequence listed in Table 1) were inoculated in 10 mL of liquid Luria-Bertani (LB) medium with a ratio of 1:1000 containing 100 µg/mL kanamycin and 50 µg/mL rifampicin (Duchefa, Haarlem, Netherlands) and incubated at 28 ℃ overnight under continuous shaking of 120 rpm in the dark before transformation. About 10 mL bacterial suspension culture with the optical density (OD_{600}) of 0.8–1.0 was harvested by centrifugation at 5,400 × g, 4 ℃ for 10 min. The pellet was re-suspended with 10 mL co-culture medium consisting of full-strength Murashige and Skoog (MS; Murashige & Skoog, 1962) supplemented with 0.1 mg/L α-naphthaleneacetic (NAA) and 1.0 mg/L 6-benzylaminopurine (BAP).

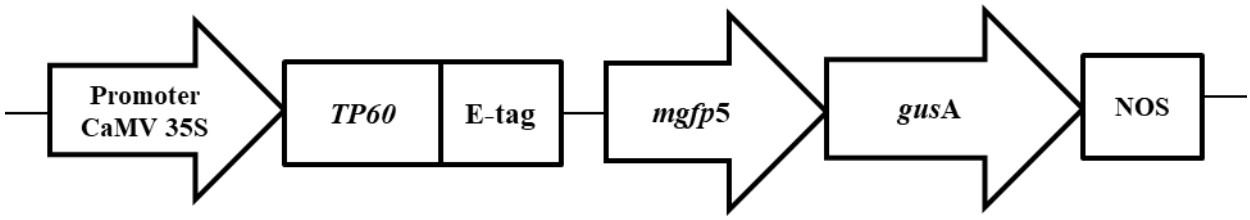


Figure 1. Diagrammatic representation of the pTP60 construct. Binary vector pCAMBIA 1304 as a backbone containing an anti-*Toxoplasma* scFv gene (*TP60*) fused with E-epitope tag (GAPVPYPDPLEPR), green fluorescence protein gene (*mgfp*5), GUS gene (*gus*A), and nopaline synthase terminator (NOS). This construct was driven by Cauliflower mosaic virus 35S (CaMV 35S) promoter.

2.2 Plant materials and seed surface disinfection

The seeds of *N. tabacum* cv. SR1 obtained from Academia Sinica, Taiwan, were soaked in 70% (v/v) ethanol for approximately 30 s followed by rinsing with sterile distilled water twice. Surface disinfection was carried out by submerging and shaking the seeds in 25% (v/v) Clorox^{TM} (containing 0.6% [w/v] of active reagent, sodium hypochlorite) for 20 min on a rotary shaker at 100 rpm and rinsing six times with sterile distilled water (imbibition). The sterilised seeds were transferred to a Petri dish containing 25 mL of semi-solid MS medium (MSO) supplemented with 3% (w/v) sucrose (Sigma Aldrich, Germany) and 0.25% (w/v) Gelrite^{TM} (Duchefa, Haarlem, Netherlands). All cultures were incubated at 25 ± 3 °C under a 16-h photoperiod with a light intensity of 20.3 µmol m^{-2} s^{-1} for about two weeks.

2.3 Transformation and plant regeneration

Prior to transformation, 4-week-old leaves of in vitro *N. tabacum* plantlets were cut into small discs (~1 cm^2 area) and pre-cultured on TSM medium (full-strength MS supplemented with 0.1 mg/L NAA, 1.0 mg/L BAP, 3% [w/v] sucrose, and 0.25% [w/v] Gelrite^{TM}) and incubated at 25 ± 3 °C under 16-h light and 8-h dark cycle

for three days. The leaf discs (nine explants per plate) were infected with *Agrobacterium* containing pTP60 construct for 10 min, blotted dry on a sterile Scott® C-fold paper towel (Kimberly-Clark, USA) and co-cultured on TSM medium in darkness at 25 ± 3 °C for two days. After co-cultivation, the infected explants with *Agrobacterium* harbouring pTP60 or pCAMBIA 1304 empty vector were transferred to TSM-selection medium (MS medium supplemented with 250 mg/L cefotaxime and 20 mg/L hygromycin) until new shoots were formed. Hygromycin-resistant shoots were then transferred to TRM-selection medium (full strength MS medium containing 0.1 mg/L NAA, 3% [w/v] sucrose, 0.25% [w/v] Gelrite^{TM}, 250 mg/L cefotaxime and 20 mg/L hygromycin). The wild-type control plantlets were cultured on the same media without antibiotic supplementation. The wild-type and transformed plantlets with empty vector were used as a control. The transformation event was conducted in triplicate.

2.4 GUS histochemical and GFP qualitative assays

Hygromycin-resistant shoots (10-week-old) were harvested and analysed using histochemical GUS (Jefferson et al., 1987) and GFP visualisation expression assays. GUS-stained tissues were examined under a stereomicroscope (Olympus, UK), whereas the expression of GFP was observed under a confocal laser scanning microscope (LEICA TCS SP5 II) with 20 oil-immersion objective lens. GFP expression was excited at the wavelength of 488 nm and detected through a filter for fluorescence wavelength of 550 nm. Confocal images from the selected section within the upper and lower epidermis (using z-stack) were merged using software (LAS AF version 2.3.1 build 5194 SP5). Wild-type tobacco leaves were used as a negative control to adjust the parameter of auto-fluorescence exclusion.

2.5 DNA extraction and PCR analysis

Total DNA were extracted from both T_0 and T_1 hygromycin-resistant and wild-type control shoots using DNeasy® Plant Mini Kit (Qiagen, USA) according to the manufacturer's instructions. The quantification of DNA were conducted using a NanoDrop 2000 UV-Vis spectrophotometer (Implen, Germany). PCR amplification of T_0 hygromycin-resistant shoots were performed using gene-specific primers, 1304-sk (Table 1) to confirm the presence of transgene with a predicted size of about 914 bp (iNtRON Biotechnology, Gyeonggi-do, South Korea). Amplification were performed using a Bio-Rad T100 Thermal Cycler (Hercules, CA, USA) under the following conditions: 94 °C for 2 min, followed by 29 cycles of 94 °C for 20 s, 58.8 °C for 20 s, and 72 °C for 1 min followed by a final extension at 72 °C for 5 min. The annealing temperatures of each pair of primer are stated in Table 1. The PCR products were visualised on ethidium bromide (EB)-agarose gel electrophoresis under UV Gel Pro Imager (Media Cybernetics, USA). Putatively transformed shoots with pTP60 construct and empty vector were amplified using GFP primers with a predicted size of about 750 bp (Table 1). The germinated T_1 seedlings (~ 4 to 5 weeks of germination) were screened using both 1304-sk and HPT1 primer pairs with the predicted size of 914 bp and 559 bp, respectively. The PCR amplification were carried out similar to the T_0 generation dependent on the annealing temperature, as stated in Table 1.

Table 1. Primer sequences and PCR conditions

Gene	Accession number	Primer, 5'-3'	Annealing temperature and amplicon length
1304-sk[A,B]	Outside target gene region	*5'*-GAGAGAACACGGGGGACTC -*3'* *5'*-GTGCCCATTAACATCACCATC-*3'*	58.8 °C, 914 bp
GFP[A]	AF234300.1	*5'*-GGCTCGAGGACCATGGTAGATCTGACTAGA-*3'* *5'*-CTCGAGTCTAGAGGGGTTTCTACAGGACGTAAACT-*3'*	50.8 °C, 750 bp
HPT1[B]	AF234300.1	*5'*-AGATGTTGGCGACCTCGTATTG -*3'* *5'*-GTTTATCGGCACTTTGCATCGGC -*3'*	55.0 °C, 559 bp
FV-60[C]	KC508790.1	*5'*-TGGAGTCCCTGATCGCTTCACTGGC-*3'* *5'*-GCTCCAGCTTGGTCCCAGCACCG-*3'*	60.0 °C, 149 bp
NtL25[C]	L18908	*5'*-GGTTGCCAAGGCTGTCAAGTCAGG-*3'* *5'*-CCTTCCAGGTGCACTAATACGAGGG-*3'*	60.0 °C, 139 bp

[A] Primers used for amplification of putative transformants (T_0).
[B] Primers used for the amplification of T_1 seedlings.
[C] Primers used for mRNA expression analysis of T_1 seedlings.

2.6 Acclimatisation of transgenic plantlets

Ten PCR-positive individual transformants (represented by ten different lines) of T_0 plantlets (12-week-old) with well-developed roots were washed thoroughly under running tap water and immersed in 1% (w/v) IMAS-THIRAM 80 fungicide (Endona Corp, Malaysia) for at least 10 s before transferred to soil. The acclimatisation was conducted in a greenhouse (Plant Biotechnology Facility, University of Malaya, Malaysia) under 16-h photoperiod and incubated at 25 ± 3 °C. T_1 seeds were collected for gene-stability analysis. Approximately 100–200 T_1 seeds per line were germinated on MSO-selection medium (full strength MS medium containing 3% [w/v] sucrose, 0.25% [w/v] Gelrite™ and 20 mg/L hygromycin). The percentage of T_1 germination of each line was assessed and recorded. In addition, the phenotypes of three individuals of T_1 positive seedlings (four randomly selected lines) were examined and recorded (Table 2).

2.7 RNA extraction

Total RNA from 40-day-old leaves of different T_1 tobacco lines (FV3, FV12, FV16, and FV17) was isolated using the RNeasy® Plant Mini Kit (Qiagen, German). The total RNA was treated with RapidOut DNA Removal Kit (Thermo Fisher Scientific, USA) prior to RT-PCR. RNA integrity and concentration were assessed using EB-stained agarose gel electrophoresis and NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington DE, USA), respectively.

2.8 Semi-quantitative RT-PCR and quantitative real-time PCR analysis

One µg of DNase-treated RNA were used in the cDNA synthesis. The first-strand cDNA was synthesised using RevertAid H minus First-strand cDNA synthesis kit with 10 µM oligo-dT nucleotides (Thermo Fisher Scientific, USA). For reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR), specific primers for FV-60 fragment amplification with a predicted size of 149 bp were used. *N. tabacum* ribosomal protein L25 (NtL25) were used as the reference gene (Schmidt & Delaney, 2010; Table 1). Ten µL of PCR reaction mixtures containing 5 µL of 2 Power SYBR green (Thermo Scientific, USA), 1 µL of 100 ng of cDNA template, and 0.5 µL of each forward and reverse primers at the final concentrations of 500 nM were amplified on an ABI QuantStudio™ 12K Flex real-time PCR system (Applied Biosystems, Carlsbad, CA, USA) under the following parameters: 10 min denaturation step at 95 °C, followed by 40 two-step cycles of 15 s at 95 °C and 1 min at 60 °C. A PCR mixture without template was included as the no template control (NTC). All amplifications were carried out in triplicates, and the data were analysed using the formula, $2^{-\Delta\Delta Ct}$ (Bustin et al., 2009; Livak & Schmittgen, 2001). For semi-quantitative RT-PCR, 100 ng cDNA were used as the template following a 3-step standard PCR amplification (iNtRON Biotechnology, Gyeonggi-do, South Korea) at the annealing temperature of 60 °C for both target (FV-60) and reference (NtL25) gene primers. The amplicon were visualised on EB-agarose gel electrophoresis under UV Gel Pro Imager (Media Cybernetics, USA).

2.9 Protein extraction

The leaves from the third to the seventh axial position of T_0 and T_1 tobacco plants of each line were harvested after 40 days of acclimatisation and ground into fine powder in the presence of liquid nitrogen (Robert et al., 2013; Figure 2). The powder (100 mg) was dissolved in 100 µL of 1 phosphate buffer saline (PBS; Thermo Fisher Scientific, USA) and incubated at room temperature for 5 min before centrifuged at 15,000 × g for 5 min at 4 °C. The supernatant was transferred to a new 1.5 mL microcentrifuge tube and stored at -80 °C until use (Lin, 2011). Quantification of total soluble proteins was assayed, according to Bradford (1976) using Bio-Rad Bradford Dye (Hercules, CA, USA) and bovine serum albumin (BSA) as a protein standard.

Figure 2. Representative of 45-day-old *Nicotiana tabacum* cv. SR1 T_1 plant (aerial and leaves numbered from the apex down). Bar = 1 cm.

2.10 Protein purification and quantification

Total protein of the representatives from each line was purified using Dynabeads® M-280 Sheep anti-Mouse IgG (Invitrogen, USA) by coupling the beads with 2 µg of target-Ig (Abcam E-epitope tag monoclonal antibodies), according to the manufacturer's instructions. The purified protein samples were then subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis.

2.11 SDS-PAGE and immunoblotting

About 25 µg of total soluble protein extract and the purified protein were resolved on 12% (w/v) SDS-PAGE in a reducing condition. The gels were either stained with SimplyBlue™ SafeStain (Invitrogen, USA) or transferred to the Amersham ECL Sensitive Nitrocellulose membrane (GE Healthcare, USA) using Bio-Rad trans-blot apparatus (Hercules, CA, USA) in Advansta Flash blot transfer buffer (Menlo Park, CA, USA), according to the manufacturer's instructions. The

membrane was blocked with TBST buffer (1 TBS [Tris buffer saline; Thermo Fisher, USA] with 0.1% [v/v] Tween 20) containing 5% (w/v) BSA, pH 7.0 ± 0.5 for 1 h followed by incubation in 1:1000 of diluted primary antibody, E-epitope tag monoclonal antibodies from immunised mouse (Abcam, Cambridge, UK) overnight at 4 °C with gentle agitation. The membrane was washed three times with TBST for 5 min each on the following day before incubated with 1:5000 diluted goat anti-mouse polyclonal IgG antibodies conjugated with horseradish peroxidase enzyme (HRP), Fc fragments (Abcam, Cambridge, UK), for 90 min at room temperature with gentle agitation. The membrane was washed three times with TBST for 5 min each and visualised with Advansta ECL Advansta WesternBright Quantum (Menlo Park, CA, USA), according to the manufacturer's instructions and imaged using Bio-Rad ChemiDoc Imager (Hercules, CA, USA) under white light.

2.12 Statistical analysis

The phenotypic assessments were conducted in triplicate and statistically analysed using one-way analysis of variance (ANOVA). The mean values of treatments were subjected to Tukey's multiple comparisons. The statistical analysis was performed using SPSS 16.0 (SPSS Inc., USA) at 5% confidence level.

3. RESULTS AND DISCUSSIONS

3.1 Regeneration and transient analysis of putative transformed shoots

In this study, we aimed to express anti-*Toxoplasma* protein, TP60, in tobacco through *Agrobacterium*-mediated transformation. After co-cultivation, the leaf discs were recovered on the TSM selection media. About 20 ± 0.33 shoots which represent as transgenic lines were successfully regenerated from the transformed leaf discs. Non-transformed leaf discs turned brown and became necrotic on the selection media. Hygromycin-resistant shoots were randomly selected for GUS and GFP qualitative assays (Figure 3a). An intense blue colouration was observed on the putative transformed shoots after GUS staining (Figure 3b, c), whereas the non-transformed region on the same expanded putative transformed leaves remained colourless. The mechanism of mixing transformed and non-transformed cells within expanded putative transformed shoots might be due to the high frequency of chimeras in the leaf disc transformation, which can be eliminated by selecting the positive seedling in T_1 progenies. Shoots transformed with pTP60 construct showed fewer blue spots on the expanded leaf (red arrow) compared to only pCAMBIA 1304 (Figure 3c). This indicated that the expression of *GUS* gene was decreasing due to the addition of *TP60* gene insert after the CaMV 35S promoter. The putative transformed shoots were then examined for green fluorescence to determine the expression of the *mgfp5* gene (Figure 3d).

The non-transformed region of the same expanded putative transformed shoots showed red colouration (Figure 3e) due to auto-fluorescence under UV light in the presence of chlorophyll background signal (Goldman et al., 2003; Zhou et al., 2005). According to previous publications, following the recovery of a new putative transgenic plant, GFP fluorescence was usually visible in new emerging shoots, young tissues or organs, whereas weak signal was observed in older ones (Zhou et al., 2004), such as in *Medicago truncatula* var. A17 (Kamaté et al., 2000) and *Verbena officialis* L. (Tamura et al., 2003).

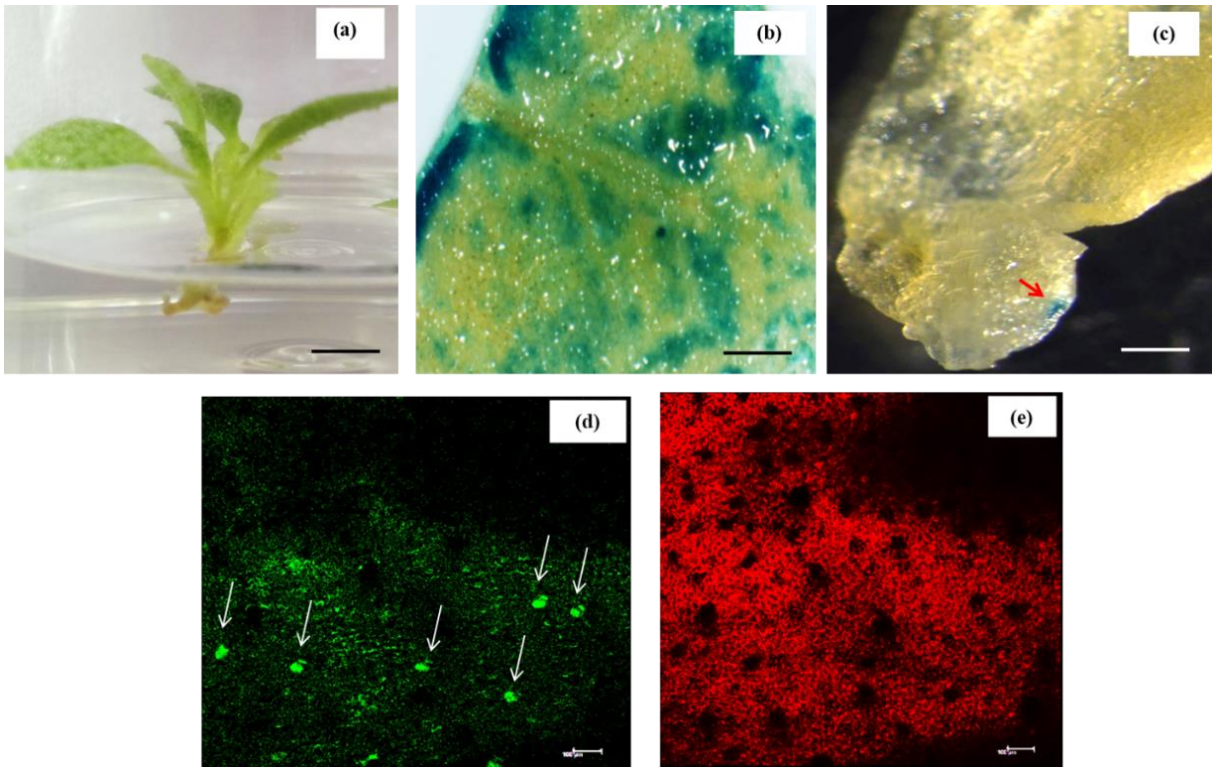


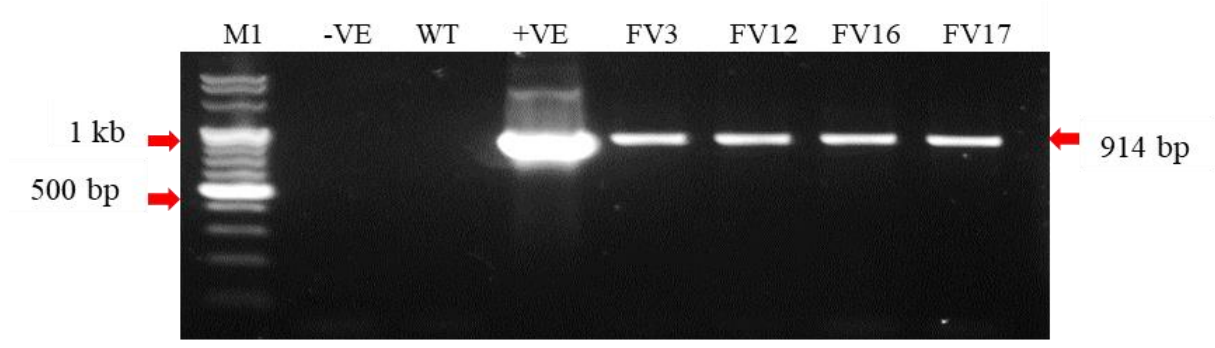
Figure 3. Transient analysis of T_0 putative transformed shoots (10-week-old). (a) Regenerated hygromycin-resistant shoots on the selection medium; (b) GUS histochemical assay of expanded pCAMBIA 1304 putative transformed shoots; (c) GUS histochemical assay of expanded pTP60 putative transformed shoots, red arrow indicates an intense GUS blue colouration; (d) Visualisation of expanded putative transformed shoots with pTP60 construct under a confocal laser scanning microscope (40X-oil immersion objective lens); (e) Detection of red background colour signal on the same putative transformed of the expanded shoots as in (d) within untransformed region under UV. White arrows indicate the GFP signal. Bar = 0.5 cm (a, b, c) and 100 µm (c, d).

3.2 PCR amplification

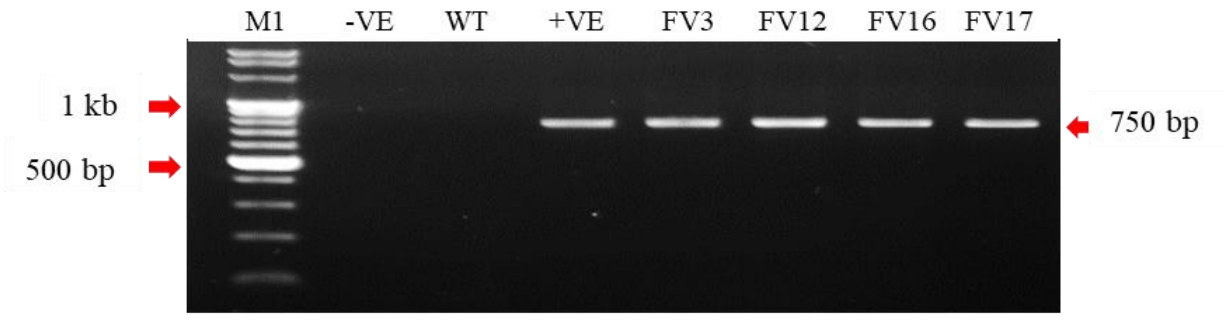
To confirm the presence of the transgene in the T_0 putative transformed shoots, 10-week-old regenerated shoots were analysed by PCR. Detection of about 914 bp and 750 bp of amplified fragments in the putatively transformed shoots confirmed the presence of *TP60* (Figure 4a) and *mgfp5* (Figure 4b) genes, respectively. The expectation of the green fluorescence signal observed on the expanded putative transformed leaves correlated with the presence of *mgfp5*. No amplification was detected in the genomic DNA of wild-type regenerated control plants.

All PCR-positive T_0 plantlets were acclimatised in BSL2 compliant transgenic greenhouse, University of Malaya, Malaysia. The stable integration of heritable *hpt*II gene in T_1 tobacco plant genome was confirmed by PCR using the specific primer, HPT1, whereas, target gene specific primer (1304-sk) was used to confirm the stable integration of *TP60* gene in T_1 progenies. About 559 bp and 914 bp amplified fragments were detected using

both HPT1 and 1304-sk primers, respectively, on hygromycin-resistant T_1 seedlings, including representative seedlings from lines FV3, FV12, FV16, and FV17 (Figure 4c and Figure 4d). This has confirmed the presence of transgene, which was stably inherited in the T_1 generation. Antibiotic-resistance selection marker gene located on the left border of Ti plasmid was used to ensure that truncation will not occur in the T_1 generation. It has been documented that the left border of T-DNA was not inherited in subsequent generation after T_0 (Kemski et al., 2013).



(a)



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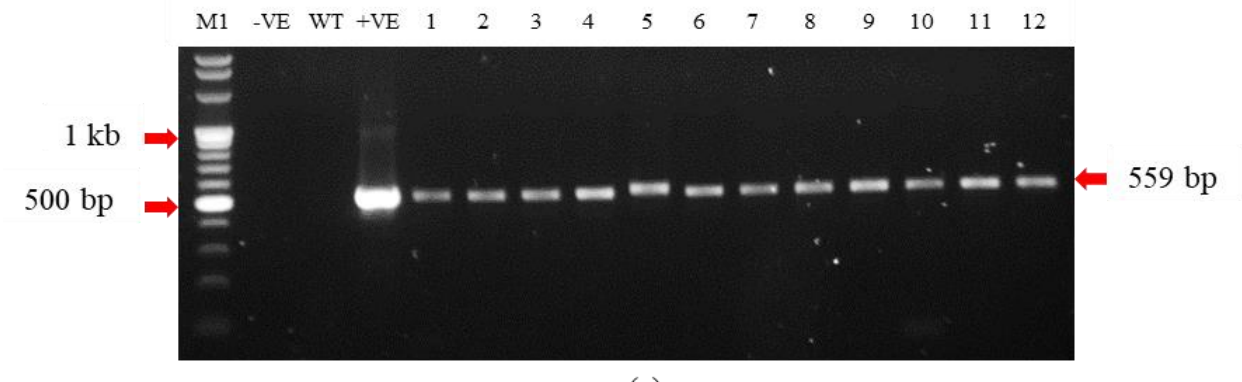


(c)

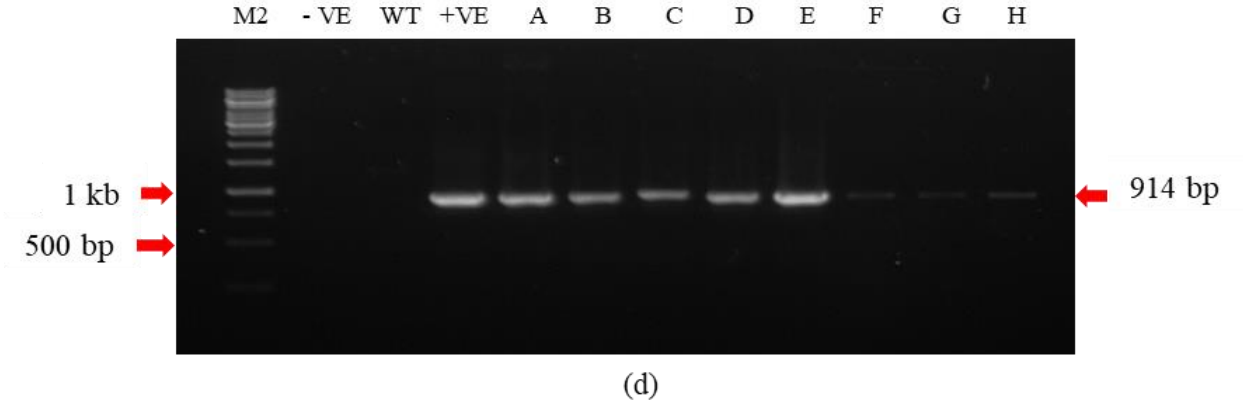


Figure 4. PCR screening of T_0 and T_1 tobacco plantlets and seedlings using specific target gene primers, (a) PCR amplification of T_0 tobacco plantlets using 1304-sk, (b) PCR amplification of T_0 tobacco plantlets using GFP primers (c) PCR screening of 10 week-old of T_1 tobacco seedlings using HPT1 and (d) PCR screening of 10 week-old of T_1 tobacco seedlings using 1304-sk primers. Lanes M1 and M2: Perfect^{TM} 100 bp DNA ladder and Perfect Plus 1 kb DNA ladder (Eur_x, Poland), respectively; –VE: negative control; WT: wild-type tobacco plantlet; +VE: positive plasmid control; FV3, FV12, FV16, and FV17: representatives of T_0 tobacco plantlets from different transgenic lines; 1-12 and A-H: representatives of T_1 tobacco seedlings from different transgenic lines.

3.3 Phenotypic examination and segregation pattern of T_1 seedlings

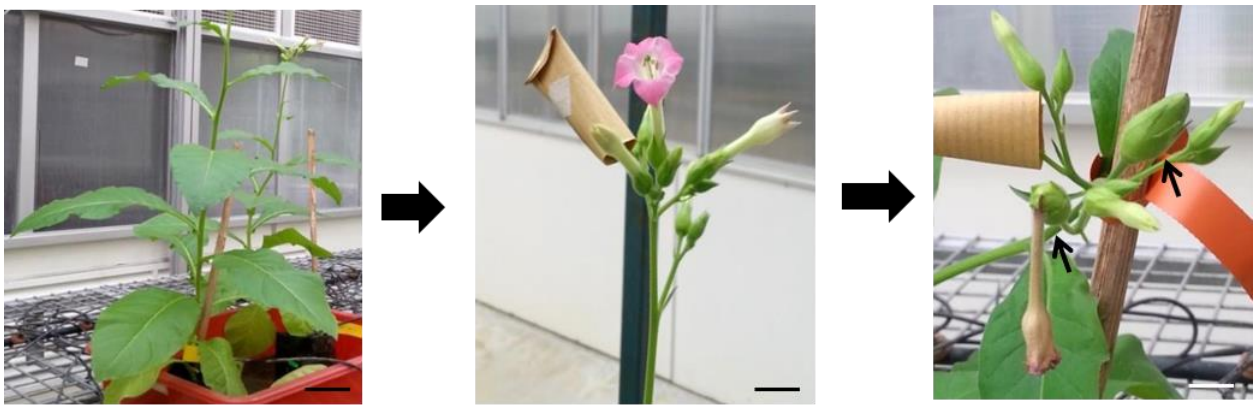
The percentage of germination and phenotypic changes in acclimatised T_1 transgenic tobacco lines was assessed, and the results are tabulated in Table 2. T_1 seeds from each representative line (approximately 100–200 seeds per line) were germinated on the selection media.

The percentage of germination was recorded after seven days. The results showed that wild-type tobacco seeds produced the highest percentage of germination (95 ± 2.9%) compared to other transgenic lines. Among all selected transgenic lines, the highest percentage of germination was recorded in FV17 (90%), followed by FV12 (86%), FV3 (82%), and FV16 (76%). The non- transformant seedlings germinated on the selection media resulted in necrosis and/or showed moderate growth with green-pale colour (Figure 5e). The non-transformed seedlings were then discarded due to their abnormal growth appearance. However, some transgenic seedlings showed delayed germination (more than 14 days), causing the percentage of germination not assessed. The delayed germination might be due to selective pressure on transgenic seeds to adapt to the antibiotic-containing media. As previously reported by Eluk et al. (2016), the introduction of antibiotics, such as enrofloxacin, oxytetracycline, penicillin, and tylosin, in the culture media has delayed germination in the seeds of *Glycine max, Helianthus annus, Sorghum bicolor, Triticum aestivum,* and *Zea mays*, showing a phytotoxic action on crop growth, in contrast to our T_1 transgenic tobacco seeds.

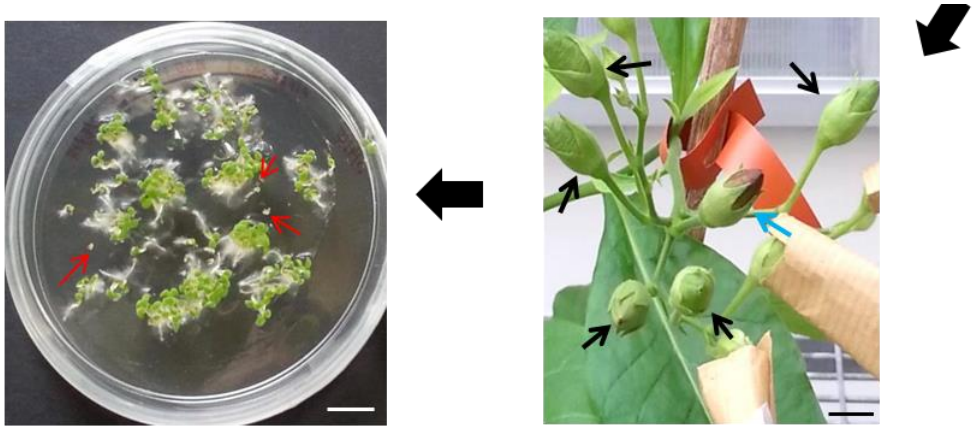
Phenotypic characteristics, such as percentage of germination and stem height, were not significantly different (p <0.05) between the transgenic lines and wild-type tobacco plants, suggesting that this anti-*Toxoplasma* protein did not interfere the growth development and physiology of the tobacco plant itself. These characteristics may be useful for biopharming production (Ahmad et al., 2012b). We then observed the flowering time for the transgenic and wild-type lines by recording the shortest and a mean number of flowering periods. We found that the transgenic line FV16 exhibited the shortest flowering period (26 days), whereas the wild-type tobacco plants displayed longer flowering period (65 days) compared to the transgenic lines. In general, the flowering period for *TP60*-transformants and wild-type was not significantly different (p <0.05), as flowering was observed at 62 ± 2 and 65 ± 6 days, respectively. The introduction of this recombinant protein gene into the tobacco plants has led to early flowering compared to wild-type. Different flowering days, as shown in transgenic lines, might be due to the different positions of the insert into the tobacco host genome. This non-photoperiodic flowering, a facultative response to day length, is probably due to stress (Takeno, 2012). Flowering is a vital life-history trait for plants to ensure seed production required for survival. Plants may alter its flowering time as one of the evolutionary strategies to maximise the chances of reproduction under diverse stress conditions, such as pathogen infection and environmental stress (Kazan & Lyons, 2016).

The number of transgene insertions was identified by the segregation pattern analysis of T_1 seedlings based on *hpt*II gene activity using Chi-square analysis (Table 3). The inheritance pattern of three independent lines, namely FV3, FV12, and FV16, followed Mendelian inheritance by showing a 3:1 ratio (hygromycin-resistance:hygromycin-sensitive). This result indicated that *hpt*II genes were integrated into a single locus in these transgenic lines and stably inherited to T_1 generation (Datta et al., 2001; Chen, 2011).

On the other hand, FV17 did not follow the segregation pattern of 3:1 Mendelian inheritance. This might be due to the integration of *hpt*II gene, which was not within a single locus in this transgenic line or into the unstable transgenic loci, which may reflect complex rearrangement of the integrated genes (Tizaoui & Kchouk, 2012).



(a) **(b)** **(c)**



(e) (d)

Figure 5. The growth stages of transgenic *N. tabacum* cv. SR1 within 3 months of acclimatisation. (a) 6-week-old of acclimatised transgenic tobacco plants; (b) 8-week-old of acclimatised transgenic tobacco plants; (c) Formation of seed pods (black arrows), 4-6 days of post-pollination; (d) Drying seed pods after 1 month of maturity (blue arrow). Ready for the collection of T_1 seeds after the browning of sepals and stem; (e) Approximately 2-week-old of germinated T_1 seeds on selection media. Red arrows indicate the non-transformant of T_1 seedlings germinated on selection media with green-pale colour and abnormal appearance. Bar = 1 cm.

Table 2. The percentage of germination (after 7 days of germination), stem height (20 days of post-acclimatisation) and flowering period of T_1 independent transgenic lines. Same parameters were recorded for wild-type plants as control.

Line	**Percentage of germination (%)**	**Stem height (cm)**	**Flowering period (day)**
Wild-type	95 ± 2.9^{a}	8.97 ± 1.5^{c}	65
FV3	$82 \pm 6.5^{a,b}$	9.2 ± 1.4^{c}	63
FV12	$86 \pm 2.1^{a,b}$	10.2 ± 2.1^{c}	59
FV16	76 ± 5.2^{b}	12.80 ± 1.3^{c}	26
FV17	$90 \pm 5.3^{a,b}$	13.57 ± 7.0^{c}	49

The results represent the mean ± standard error of the mean (SEM) of three replicated experiments. Different letters indicate significant difference between samples at $p < 0.05$.

Table 3. Segregation of *hpt*II gene in the T_1 tobacco plants

Transgenic lines (from T_0)	Total number of T_1 germinated seeds	Segregation Pattern			
		***hpt*II^R**	***hpt*II^S**	**Ratio**	χ^2
FV3	133	106	27	3:1	1.566
FV12	129	90	39	3:1	1.884
FV16	139	105	34	3:1	0.022
FV17	129	71	58	-	-

Each T_0 line represents an independent event of transformation. Total number of T_1 germinated seeds: Number of seeds germinated on the selection media from the corresponding transgenic line of T_0. *hpt*II^R and *hpt*II^S represent the resistant (germinated) and susceptible (non-germinated) seeds on selection media-containing hygromycin respectively. Three lines (FV3, FV12, and FV16) showed a ratio of 3:1 (*hpt*II^R: *hpt*II$^-$). Lines which were not fit the ratio of 3:1 were represented by "-".

3.4 Gene expression and quantification of recombinant anti-Toxoplasma gene

In this study, the expression profiles of the *TP60* gene between three selected individuals of each T_1 transgenic tobacco line were assessed (Figure 6). The third main stem leaves of tobacco plants were used for gene expression analysis. Our quantitative PCR (RT-qPCR) analysis showed that the transcript of *TP60* transgene was detected in all tested transgenic plant samples. A representative of each line, FV3-11 (Figure 6a), FV12-6 (Figure 6b), FV16-10 (Figure 6c), and FV17-7 (Figure 6d) that showed the highest in *TP60* expression in comparison to the other two individual samples were subjected to Western blot analysis. Based on the representative individuals of each line, FV12-6 showed the highest *TP60* expression at 7-fold change, followed by FV16-10 at 2-fold change, and FV17-7 at 1-fold change compared to FV3-11 (Figure 7). The differences in the *TP60* transgene expression level between transgenic lines might be due to the position effect during transformation event.

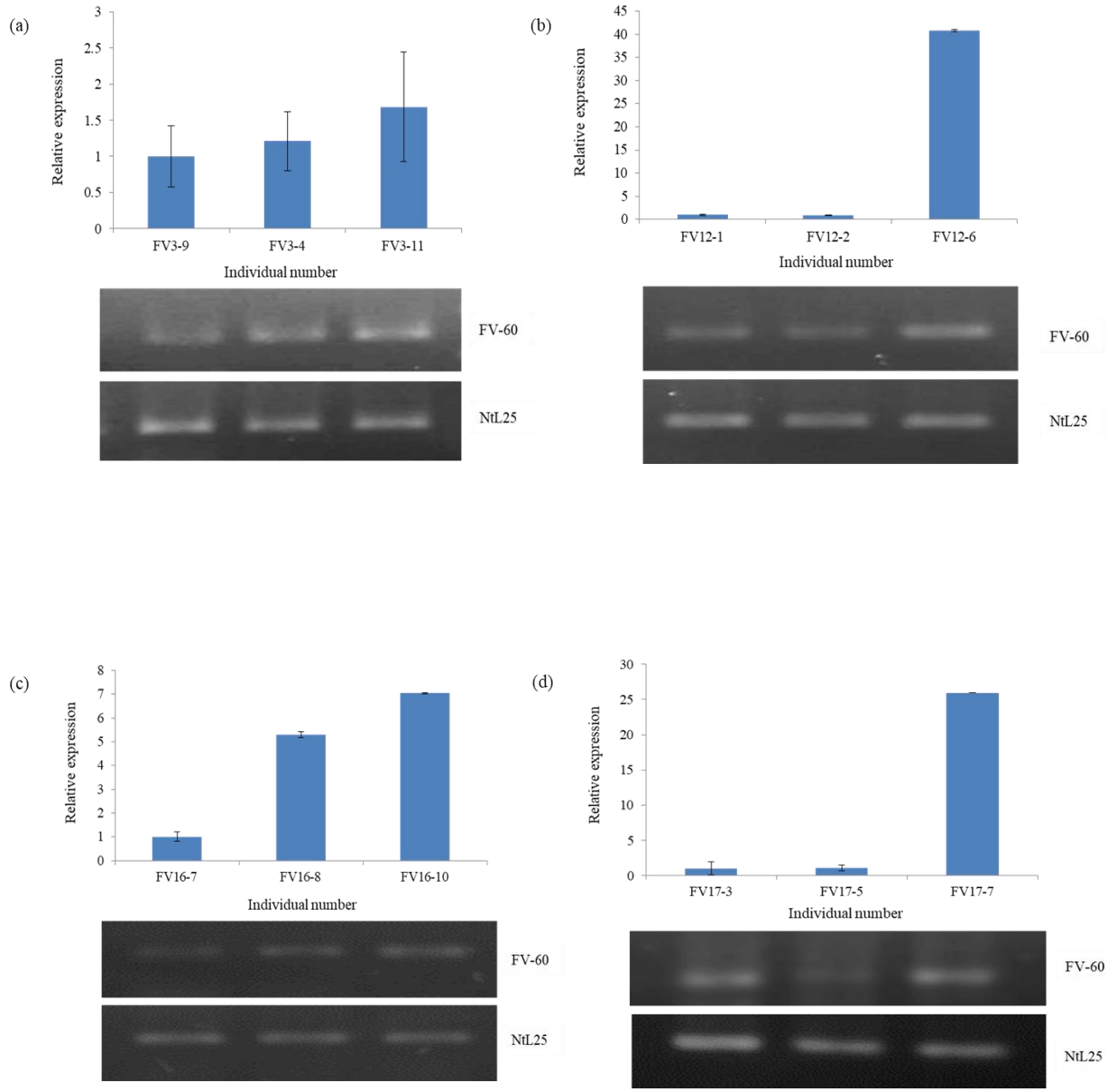


Figure 6. Quantitative PCR (RT-qPCR) and semi-quantitative PCR (RT-PCR) analysis of the transgene expression level for (a) FV3; (b) FV12; (c) FV16; and (d) FV17. Semi-quantitative PCR showed bands at the expected size of 149 bp and 139 bp of the target (FV-60 primers) and internal reference gene (NtL25 primer), respectively. Bars represent the standard errors of three technical replicates. Relative expression on *Y* axis represents *TP60* transcript levels to individual of FV3-9, FV12-2, FV16-7, and FV17-3 for transgenic lines FV3, FV12, FV16, and FV17, respectively.

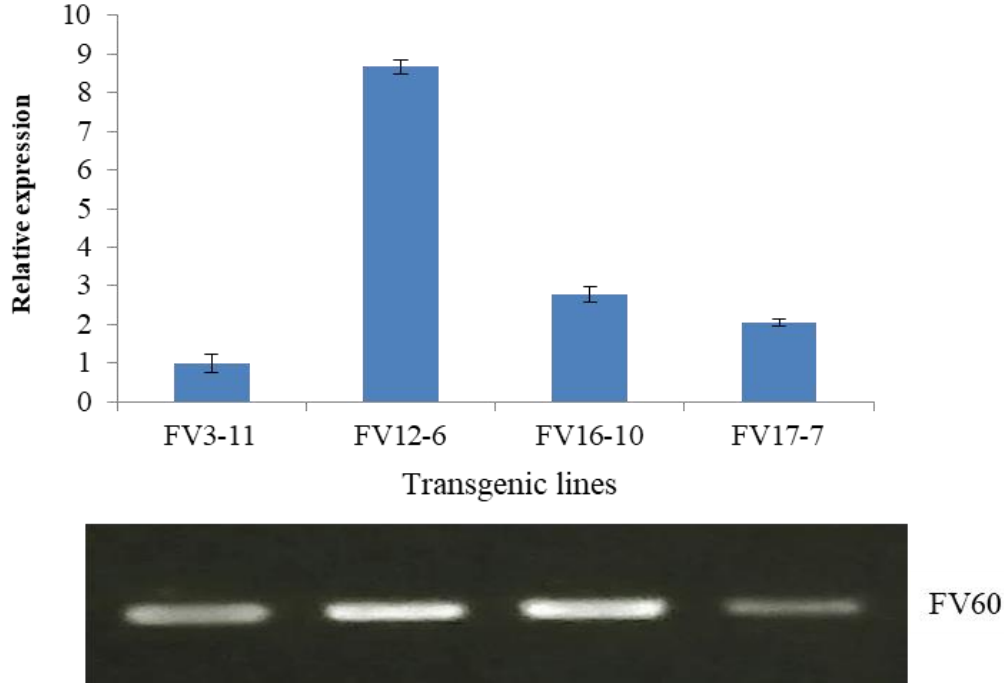


Figure 7. Quantitative PCR (RT-qPCR) and semi-quantitative PCR (RT-PCR) analysis of expression level of *TP60* for FV3, FV12, FV16, and FV17. Semi-quantitative PCR showed a single band at the expected size of 149 bp for the target gene (FV-60 primers). Bars represent the standard errors of three technical replicates. Relative expression on *Y* axis represents *TP60* transcript for FV3, FV12, FV16, and FV17 relative to FV3-11.

3.5 Recombinant anti-Toxoplasma protein expression in tobacco plants

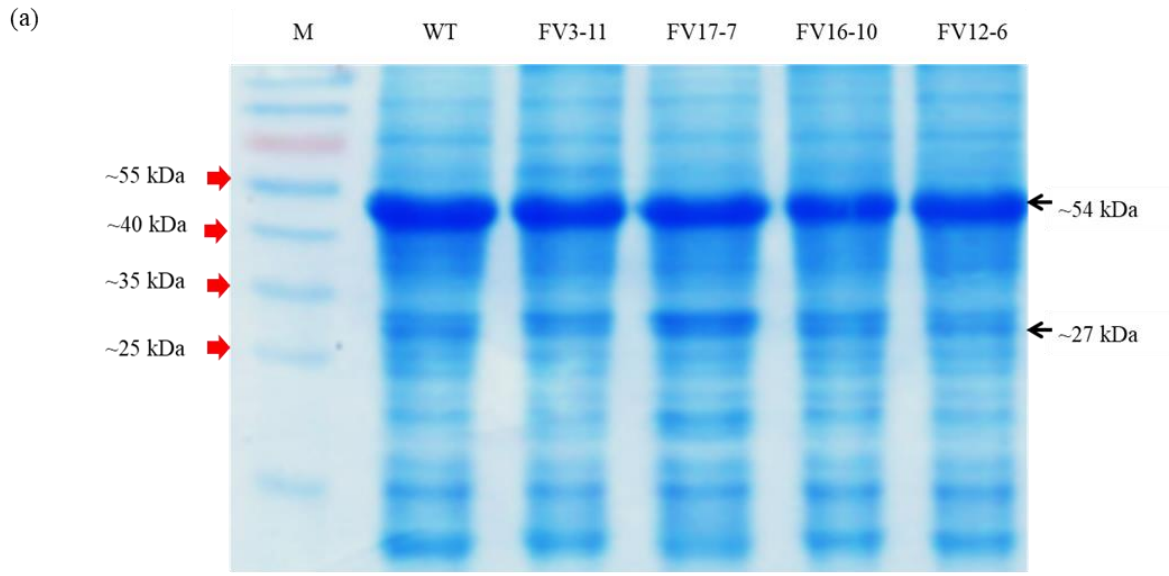
SDS-PAGE and Western blot analyses were performed to confirm the stable production of TP60 protein in the T_1 generation. Total soluble dImer protein showed a clear band at the predicted size of ~ 54 kDa (Figure 8a). An intense band was detected in the wild-type control sample, probably due to the presence of other native protein with a similar size as the recombinant protein. The purified recombinant proteins showed a clear band at the sizes of ~ 60 kDa and ~ 54 kDa (Figure 8b). The presence of unspecific band with the size of ~ 60 kDa might be due to the interference of eluted monoclonal E-epitope tag IgG antibody that covalently bound to the bead together with our target recombinant protein during the elution step.

The detection of anti-*Toxoplasma* TP60 protein using anti-E tag fused protein (Figure 8c) confirmed the expression of the targeted protein at the size of ~ 54 kDa instead of ~ 27 kDa (scFv protein size) without the interference of unspecific band at ~ 60 kDa. The dimeric formation of TP60 protein might be due to serine residue present in the linker peptide

(GGSSRSS), involving dimer formation of the disulphide band under reducing conditions (Schouten et al., 2002). Dobhal et al. (2013) reported that the dimerisation of recombinant protein produced from *N. tabacum* cv. Xanthi was due to cysteine residue present in the linker

peptide of the recombinant protein. The small size of the linker peptide (< 15 amino acids) in scFv tends to form dimers (Yusakul et al., 2015). Although TP60 protein has been previously expressed at ~27 kDa (Lim et al., 2012), we speculate that the dimeric formation of TP60 protein in this study might be due to other factors, such as high ionic strength and pH below than 7.5 during the preparation of samples (Katja et al., 1998). As demonstrated by Giersberg et al. (2010), dimerised anti-^{Nb}TNF-V_HH_{CK} proteins produced from *Nicotiana benthamiana* were able to block the TNFα-activity more effectively than monomeric anti-^{Ec}TNF-V_HH_{CK} protein produced in *E. coli*. Ahmad et al. (2012) demonstrated that dimer proteins had better avidity and stability compared to monovalent scFv. This may increase the yield of protein production. Hence, we speculate that the dimeric formation of TP60 protein in this study could potentially increase the effectiveness and inhibition toward the *T. gondii* tachyzoite antigen. No band was observed in the wild-type tobacco plants (Figure 8c).

Transgenic line FV12-6 showed the highest protein accumulation (1.67 µg/g) compared to the rest of the samples, which correlated with the gene transcript at mRNA level (Table 4). The predicted protein size was confirmed using http://web.expasy.org/translate/onlinesoft ware (Artimo et al., 2012). The TP60 protein yield was calculated by dividing the amount of purified TP60 protein over the total soluble protein (TSP) identified in the plant leaves using Bradford's assay. The purified protein yield ranged from 0.18% to 0.52% (Table 4). The highest amount of scFv recombinant protein (0.52% out of total soluble protein) obtained from FV12-6 was almost the same yield as the study conducted by Wang et al. (2015) on the production of acidic fibroblast growth factor scFv using *N. benthamiana*. However, Makvandi-Nejad et al. (2005) demonstrated that about 41.7 µg/g of dimeric scFv antibody against *Salmonella enterica* Serotype Parathyphi B could be produced in *N. tabacum*. The difference of recombinant protein yield might be due to the different methods of purification and quantification.



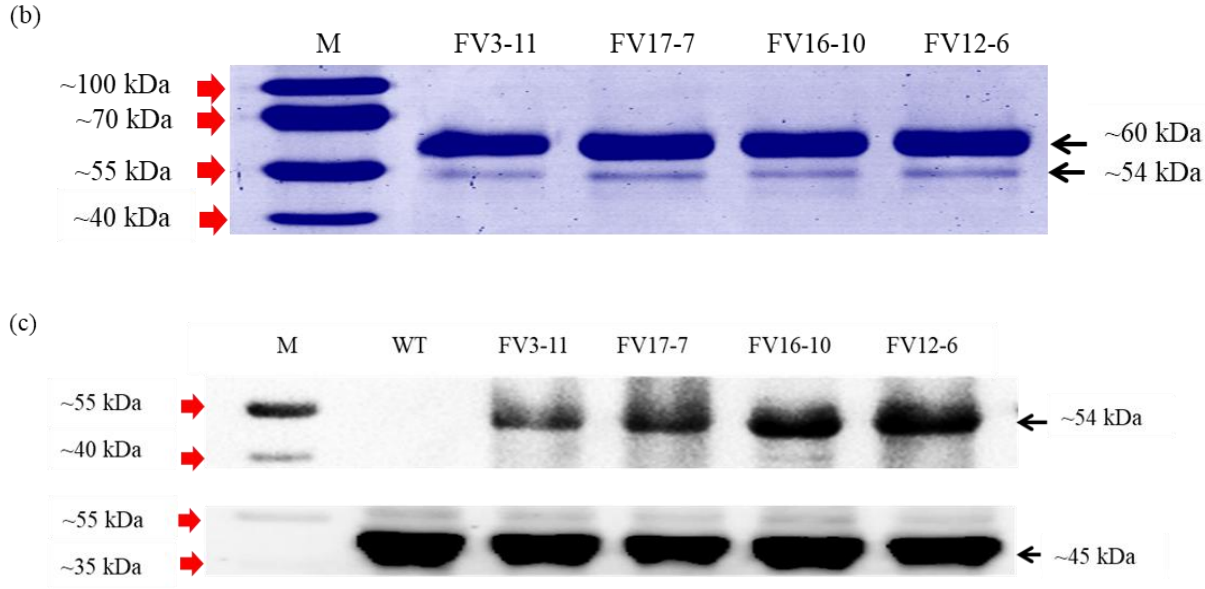


Figure 8. Stable expression of purified TP60 protein in T_1 plant of *Nicotiana tabacum* cv. SR1. (a) Total soluble protein extracted from wild-type and transgenic lines of T_1 tobacco leaves; (b) SDS-PAGE of purified recombinant protein using DyNabeads™; (c) Detection of TP60 recombinant protein fused with E-epitope tag in FV3-11, FV17-7, FV16-10 and FV12-6 lines at 54 kDa except for wild-type control sample following actin-11 as loading control at the predicted size of 45 kDa.; (M) PageRuler™ Pre-stained Protein Ladder, 10-180 kDa (except for loading control). Marker used for loading control was PageRuler™ Plus Prestained Protein Ladder 10-250 kDa (Thermo Scientific, USA).

Table 4. Determination of recombinant anti-*Toxoplasma* scFv protein (TP60) expressed in the T_1 transgenic plants through Bradford's quantification after 40-days of acclimatisation.

Transgenic lines	**Maximum antibody accumulation level**	
	Recombinant protein (µg/g)	**% TSP**
FV3-11	1.16	0.18
FV12-6	1.67	0.52
FV16-10	1.30	0.25
FV17-7	1.14	0.18

Our study demonstrated that the production of recombinant anti-*Toxoplasma* protein in plants is feasible and economical without the need for laborious downstream processes, such as chemical refolding or purification against pathogen contamination of the end protein products. Thereby, the problematic and devastating procedures to obtain the biologically active protein product can be avoided. This technology could also be exploited for the production of pharmaceutically important diagnostic antibodies for the healthcare sector. This will also open up new sectors such as agrochemical and pharmaceuticals industries. Further work will involve a strategy to enhance the production of recombinant protein in terms of yield, protein stability, and functionality.

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5. AUTHOR CONTRIBUTIONS

RYO, NZK, and BCT conceived the idea, designed the experiments and edited the manuscript. FIR conducted the experiments. PSG constructed the plasmid. FIR and BCT wrote the manuscript. NAR edited the manuscript. All authors read and approved the manuscript.

6. CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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