UNIVERSIDADE FEDERAL DO PARANÁ

PEYMAN HABIBI

PRODUCTION OF THE HIV-1 ENTRY INHIBITOR GRIFFITHSIN IN *NICOTIANA BENTHAMIANA AND MOSS SYSTEMS*

CURITIBA

2018

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Coorientadores: Prof. Dr. Carlos Ricardo Soccol

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **PEYMAN HABIBI** intitulada: **Production of novel HIV entry inhibitor griffithsin in Nicotiana benthamiana and moss systems**, após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua Aprovação no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 16 de março de 2018

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DEDICATION

To my parents

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First and foremost I would like to thank my supervisor, Maria Fatima Grossi-de-Sa, for giving me the opportunity to accomplish my Ph.D in her research group. In addition, I would like to thank her for the patient guidance, encouragement and advice she has provided throughout these years. I am grateful to acknowledge my co-supervisor Carlos Ricardo Soccol for giving me the opportunity and the funding to accomplish my Ph.D. in his research group and his kind assistance and useful advices during my PhD years, with many official documents and his constructive suggestions and useful advices during my PhD years.

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RESUMO

O uso de sistemas de plantas como fábricas para a produção de proteínas recombinantes tornou-se uma alternativa proeminente para as indústrias farmacêuticas, devido ao seu alto potencial de acumulação de proteínas. Nas últimas décadas, a aplicação de plantas para produção de proteínas tem ganhado mais atenção, pois as plantas representam uma estratégia econômica que leva a altos níveis de proteínas purificadas e ativas para o setor farmacêutico. Atualmente, a aprovação pela *Food and Drug Administration* (FDA) da primeira geração de proteínas recombinantes produzidas em células de cenoura, a taliglucerase alfa, demonstrou que as células vegetais têm uma capacidade significativa para expressar proteínas complexas para uso terapêutico. A exploração de organismos hospedeiros emergentes para a produção econômica e eficiente de antivirais protéicos contra o vírus da imunodeficiência humana (HIV) é necessária em áreas em situação precária em todo o mundo. Esta objetivou a produção da grifitisina (GRFT), como uma proteína neutralizadora do HIV em *Nicotiana benthamiana,* utilizando três supressores (i.e. silenciadores de genes) e em *Physcomitrella patens* transgênica. Neste estudo, a produção do novo candidato à inibidor da entrada do HIV nas células, proteína GRFT, foi estudada, utilizando *N. benthamiana* como plataforma de expressão, baseada em um vetor não-viral. Para aumentar os níveis de GRFT recombinante, o mecanismo de defesa aplicado ao sistema de RNA de interferência (i.e. silenciamento gênico) de *N. benthamiana* foi abolido usando três supressores virais. Utilizou-se um sistema de expressão transiente transferindo o gene *GRFT*, que codifica 122 aminoácidos, sob o controle do promotor 35S do vírus do mosaico da couve-flor (CaMV) modificado. A presença da GRFT (corretamente enovelada) nas folhas transgênicas foi confirmada, utilizando o ensaio de imunoabsorção enzimática (ELISA). Os dados demonstraram que o uso dos três supressores permitiu o maior acúmulo de GRFT, com um rendimento de 400 μg g^{-1} de peso fresco, e essa quantidade foi reduzida para 287 μg g^{-1} após a purificação, representando uma recuperação de 71,75%. As análises também demonstrouam que a capacidade de GRFT expressa em *N. benthamiana* pode se ligar à glicoproteína 120 do HVI, sendo muito semelhante ao da proteína GRFT, purificada a partir de *Escherichia coli*. Ensaios de

células inteiras usando GRFT purificada mostraram que a proteina GRFT purificada foi ativa contra o HIV. Este estudo demonstra a primeira produção em alto nível do inibidor de entrada do HIV-1 (GRFT) em um sistema de expressão não-viral e ilustra a robustez do sistema de expressão de agroinfiltração melhorada, por meio do uso de três supressores silenciadores de genes. A produção de GRFT foi também estudada no sistema de musgo transgênico, o *P. patens*. A proteina GRFT foi também expressa nesse sistema, mas diferentemente do sistema de expressão transiente, o rendimento da proteína expressa foi baixo.

Palavras-chave: GRFT, HIV-1, *Nicotiana benthamiana*, *Escherichia coli*, agroinfiltração, silenciadores supressores, *Physicomiterella patens*.

ABSTRACT

The use of plant systems as factories for recombinant protein production became a prominent alternative for pharmaceutical industries due to their high potential for protein accumulation. In the last decades, the application of plants for protein production has gained more attention, as plants represent an economic strategy that leads to high levels of purified and active proteins for the pharmaceutical sector. Currently, FDA approval of the first generation of recombinant proteins produced in carrot cells, taliglucerase alfa, demonstrated that plant cells have a significant capacity to express complex proteins for therapeutic use. The exploration of emerging host organisms for the economic and efficient production of protein microbicides against HIV is urgently needed in resource-poor areas worldwide. This thesis focuses on the production of griffithsin (GRFT) as an HIV neutralizing protein in *Nicotiana benthamiana* using three gene silencing suppressors and also in transgenic *Physicomiterella patens*. In this study, the production of the novel HIV entry inhibitor candidate, GRFT, was investigated using *N. benthamiana* as the expression platform based on a non-viral vector. To increase the yield of recombinant GRFT, the RNA silencing defence mechanism of *N. benthamiana* was abolished by using three viral suppressors. A transient expression system was used by transferring the *GRFT* gene, which encodes 122 amino acids, under the control of the *enhanced CaMV 35S* promoter. The presence of correctly assembled GRFT in transgenic leaves was confirmed using immunoglobulin-specific sandwich ELISA. The data demonstrated that the use of three gene silencing suppressors allowed the highest accumulation of GRFT, with a yield of 400 μg g^{-1} fresh weight, and this amount was reduced to 287 μg g^{-1} after purification, representing a recovery of 71.75%. The analysis also showed that the ability of GRFT expressed in *N. benthamiana* to bind to glycoprotein 120 is close to that of the GRFT protein purified from *E. coli.* Whole-cell assays using purified GRFT showed that our purified GRFT was potently active against HIV. This study provides the first high-level production of the HIV-1 entry inhibitor GRFT with a non-viral expression system and illustrates the robustness of the co-agroinfiltration expression system improved through the use of three gene silencing suppressors. The production of GRFT was also

investigated in transgenic moss *P. patens*. The GRFT was successfully expressed in transgenic moss but unlike transient expression system, the yield of expressed protein was low.

Key words: GRFT, HIV-1, *Nicotiana benthamiana*, *Escherichia coli*, agroinfiltration, silencing suppressors, *Physicomiterella patens*

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ABBREVIATIONS

ACE1	- Activating Copper-Metallothionein Expression
Act-1	- Actin1 gene
AIDS	- Acquired Immunodeficiency Syndrome
Alcap	- *Aspergillus nidulans* Alca Promoter
Arc5-I	- Arcelin 5-I,
ART	- Antiretroviral Therapy
APS	- Ammonium Persulfate
AZT	- 3'-Azido, 3'-Deoxythymidine
Bp	- Base pairs
BCECF	- Biscarboxyethyl-5(6)-Carboxyfluorescein Acetoxymethyl Ester
BSA	- Bovine Serum Albumin
Camv 35S	- Cauliflower Mosaic Virus 35S
CCR5	- C-C Chemokine Receptor Type 5
CD4	- Cluster of Differentiation 4
CDS	- Coding DNA Sequence
CHS	- Chalcone Synthase
Cmylcv	- Cestrum yellow leaf curling virus
CS	- Cellulose Sulfate
CV-N	- Cyanovirin-N
DAPI	- 4',6-Diamidino-2-Phenylindole
DMSO	- Dimethyl Sulfoxide
DNA	- Deoxyribonucleic Acid
dpi	- days post infiltration
DTT	-Dithiothreitol
DS	-Dextrin Sulfate
dsRNA	-Double Strand DNA
EC	-The Half Maximal Effective Concentration

EDTA	- Ethylenediaminetetraacetic Acid
ELISA	- Enzyme-Linked Immunosorbent Assay
ER	- Endoplasmic Reticulum
FDA	- Food and Drug Administration
FBS	- Fetal Bovine Serum
GFP	- Green Fluorescent Protein
GM	- Genetically Modified
GHO	- Global Health Observatory
Gp120	- Glycoprotein 120kda
GRFT	- Griffithsin
GUS	- Beta Glucuronidas
HAART	- Highly Active Antiretroviral Therapy
HIV	- Human Immunodeficiency Virus
HPV	- Human Papilloma Virus
HRP	- Horseradish Peroxidase
HSV	- Herpes Simplex Virus
IC	-The Half Maximal Inhibitory Concentration
Ig	- Immunoglobulin
IRES	- Internal Ribosome Entry Sites
Kda	- Kilo Dalton
LB	- Luria-Bertani
MS	- Mass Spectrometry
NCI	- National Cancer Institute
Ngram AVG	- Nanogram Average
NMR	- Nuclear Magnetic Resonance
NNRTIs	- Non- Nucleoside Reverse Transcriptase Inhibitors
NOS	- Nopaline Synthase Gene
NP-40	- Nonidet P-40
NRTIS	- Nucleoside Reverse Transcriptase Inhibitors

OD	- Optical Density
ORF	- Open Reading Frame
OS	- *Oryza sativa*
Osact1	- *Oryza sativa Actin1 Gene*
PBS	- Phosphate Buffered Saline
PCR	- Polymerase Chain Reaction
PEG	- Polyethylene Glycol
PAGE	- Polyacrylamide Gel Electrophoresis
PMA	- Phorbol Myristate Acetate
PMS	- Phenazine Methosulfate
PMSF	- Phenylmethane Sulfonyl Fluoride
PR-1	- PATHOGENESIS-RELATED-1
Prep	- Pre-Exposure Prophylaxis
Pvubi1	- Polyubiquitin Gene 1
Pvubi2	- Polyubiquitin Gene 2
Ramyd3	- Rice A-Amylase Signal Sequence
rpm	- revolutions per minute
RNA	- Ribonucleic Acid
RT	- Room Temperature
RT-PCR	- Real Time- Polymerase Chain Reaction
SE	- Standard Error
SDS	- Sodium Dodecyl Sulfate
SDS-PAGE	- Sodium Dodecyl Sulfate-Poly-Acrylamide Gel Electrophoresis
siRNA	- Small Interfering RNA
SLS	- Sodium Lauryl Sulfate
TBS	- Tris-Buffered Saline
TBST	- Tris-Buffered Saline Tween
TCA	- 2, 4, 6-Trichloroanisole
TEMED	- Tetramethylethylenediamine

TFs	- Transcription Factors
Ti	- Tumor induced
TMB	- 3,3',5,5'-Tetramethylbenzidine Substrate
TMV	- 5' 5'-Leader Sequence of Tobacco Mosaic Virus
TSP	- Total Soluble Protein

1 INTRODUCTION

Plant molecular pharming is the term related to the ability of plant materials to accumulate therapeutic proteins. The emergence of molecular pharming as a reliable and novel production technology in recent decades is the result of years of work, opening new paths to minimize technical problems involved in yeast, bacterial, and mammalian platforms, besides identifying and characterizing a promising regulatory pathway for the large-scale production of biopharmaceuticals (Fischer et al., 1999; Habibi et al., 2017).

Molecular pharming technology presents several advantages, including: (a) production of low-cost biomass; (b) end-product lacking human toxicity; (c) accumulation of complex proteins with correct and proper folding and (d) straightforward ways for protein purification (Moustafa et al., 2015). Furthermore, molecular pharming offers a flexible, scalable, and diverse alternative method for the production of new patent-protected biopharmaceuticals and biosimilars, expanding product opportunities based on rapidly growing 'biobetter' molecules markets (Zimran et al., 2011).

Although the plant-based pharmaceutical industry is still in an early stage of development, many biopharmaceuticals are already in the preclinical and clinical development pipeline. For instance, the commercial development of taliglucerase α (Protalix®, Israel; http: //protalix. com/), used for the treatment of Gaucher's disease, is a significant breakthrough in molecular pharming. Monoclonal antibodies, such as palivizumab and rituximab, moss-GBA (glucerase), moss-aGal (agalsidase), and other biosimilars, are next-generation plant-made recombinant proteins that represent the beneficial attributes of plant cell culture as a promising resource of complex protein production (Grabowski et al., 2014; Niederkrüger et al., 2014). Moreover, the insulin produced in safflower (SemBioSys Genetics, Canada; http: //www.sembiosys. com) and the HIV-neutralizing monoclonal antibody produced in tobacco (Pharma-Planta; Germany; http: //www. pharma-planta.net) were developed based on transgenic plant manufacturing. The Medicago Inc. (Québec, Canada; http://www.medicago. com) is now working on phase II clinical trial with influenza VLP (H5) accumulated in *Nicotiana benthamiana* through agroinfiltration technology. The TABLE 1 summarizes some of the plant-based vaccines for human and animal diseases.

1.1 OPTIMIZATION OF INSIDE AND OUTSIDE FACTORS FOR IMPROVEMENT OF RECOMBINANT PROTEIN PRODUCTION

Although plant platforms have many advantages in the production of biopharmaceuticals, future investigations will be required to invigorate the final product and to overcome the significant challenges and risks associated with large-scale production of biopharmaceuticals. For instance, regulatory unreliability and global concerns regarding the intrinsic yield of recombinant proteins are some of the barriers encountered by molecular pharming users. Hence, the identification and characterization of factors influencing protein accumulation levels are necessary.

The yield may be divided into endogenous and exogenous factors that regulate protein accumulation in plants. Several steps can regulate protein accumulation, including: (a) genetic elements (transcription, translation and post-translation); (b) epigenetic factors inducing gene expression; and (c) environmental factors. Additionally, specific host platforms and the subcellular targeting of proteins to specific compartments are considered critical parameters that contribute to the increase of recombinant protein yield (FIGURE 1).

TABLE 1- SUMMARY OF VACCINES AND RECOMBINANT PROTEINS PRODUCED BY PLANT SYSTEM

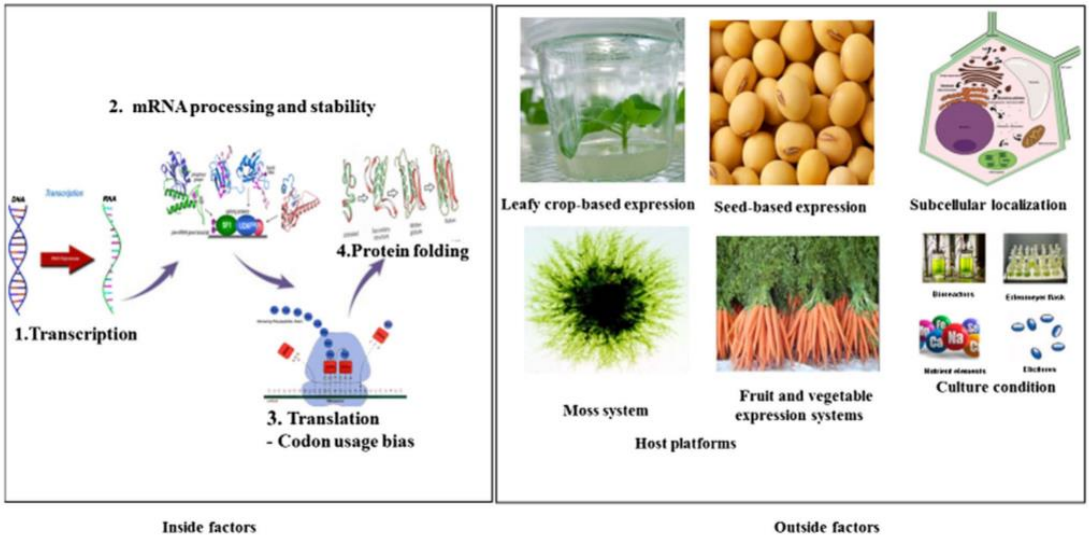
Abbreviation	Full name	Function	*Plant host*	Yield	Reference
NVCP VLP	virus-like particles Norwalk capsid protein	Used as vaccine for Diarrheal disease	*N. benthamiana*	10±0.3 mg/ml	(Lai and Chen, 2012)
VEGF	Vascular endothelial growth factor	Stimulates vasculogenesis and angiogenesis	*P. patens*	480–656 µg/g DW	(Baur et al., 2005)
ESAT-6	Early secretory antigenic target	Immunodiagnosis of Active Tuberculosis	*A. thaliana*	49 µg/g FW	(Rigano et al., 2004)
RHBsAg	Hepatitis B surface antigen	Anti hepatit B	*S. tuberosum*	97.1 ng/g FW	(Sunil Kumar et al., 2003)
Aβ	Human b-amyloid	Alzheimer's disease	*S. lycopersicum*	80 ng/ml	(Youm et al., 2008)
H5 VLP	H5 Virus-Like Particle	H1N1 influenza vaccine candidate	*N.benthamiana*	N/A	(Greer, 2015)
EIII	Domain III of dengue virus E glycoprotein	As a vaccine to prevent infection by the dengue virus.	*N. tabacum cv. MD609*	0.25% of TSP	(Kim et al., 2009)
ETEC	Enterotoxigenic *Escherichia coli*	Used as vaccines for preventing ETEC diarrhoea.	*Zea mays*	1 mg	(Tacket et al., 2004)
Poly HIV	Multi-epitope fusion protein from the human immunodeficienvirus	HIV vaccine candidate	*P. patens*	3.7 µg/ g FW	(Orellana-Escobedo et al., 2015)
VP1-FMDV	Viral protein of foot and mouth disease virus	As feed stuff additives to induce protective systemic antibody response in animals	*Stylosanthes guianensis cv. Reyan II*	0.1–0.5% TSP	(Wang et al., 2008)
IgG1 IGN314	Glyco-optimized version of antibody IGN311	Used to diagnostic tumour-associated glycosylation pattern Lewis Y	*P. patens*	N/A	(Kircheis et al., 2012)
AChE-R	Human acetylcholinester	As organophosphate bioscavengers	*N. benthamiana*	30 mg/kg FW	(Evron et al., 2007)

TABLE 1- SUMMARY OF VACCINES AND RECOMBINANT PROTEINS PRODUCED BY PLANT SYSTEM (CONTINUE)

Abbreviation	Full name	Function	*Plant host*	Yield	Reference
rCV-N	Recombinant cyanovirin-N	Anti-HIV microbicide	*Glycine max*	350 lg/g of dry seed weigh	(O'Keefe et al., 2015)
GRFT	Griffithsin	HIV-1 entry inhibitor	*O. Sativa*	223 µg/g dry seed weight	(Vamvaka et al., 2016b)
FGF	Fibroblast growth factor	Formation of blood vessels	*A. thaliana*	32.9 µg/g dry seed weight,	(Yang et al., 2015)
hPH-20	Human hyaluronidase	Facilitates penetration of the cumulus cell layer by digesting hyaluronic acid	*A. thaliana*	N/A	(Li et al., 2014)
BT-VLP	Bluetongue virus-like particle	As a vaccine against Bluetongue virus	*N. benthamiana*	N/A	(Thuenemann et al., 2013)
EIC	Ebola immune complex	As a anti-Ebola virus	*N.benthamiana*	50 µg⁄g FW	(Phoolcharoen et al., 2011)
HIV-neutralizing antibody 2G12	-	HIV entry inhibitor	*O. Sativa*	42 µg/g dry seed weight,	(Vamvaka et al., 2016a)
hGH	Human growth hormone	Simulates growth, cell reproduction, and cell regeneration	*Glycine max*	2.9% TSP	(Cunha et al., 2011)
Hfix	Human coagulation factor IX	To treat type B christmas disease	*Glycine max*	0.23% TSP	(Cunha et al., 2011)
FH	Complement factor H	Used to treatment of atypical haemolytic uremic syndrome (aHUS), or membranoproliferative glomerulonephritis II (MPGN II) or age-related macular degeneration (AMD)	*P. patens*	25.8 µg⁄g DW)	(Büttner-Mainik et al., 2011)
PRX-105	PEGylated plant-derived recombinant	Parkinson's disease; Poisoning	*N.Tabacum*	N/A	(Atsmon et al., 2015)

SOURCE: Habibi et al. (2017).

FIGURE 1 - SCHEMATIC OVERVIEW OF SEQUENCE FEATURES IMPACTING PROTEIN REGULATION. INSIDE FACTORS INCLUDE TRANSCRIPTION, TRANSLATION, mRNA PROCESSING AND STABILITY, AND PROTEIN FOLDING, AND OUTSIDE FACTORS INCLUDE HOST PLATFORM, CULTURE CONDITION, AND SUBCELLULAR LOCALIZATION.



SOURCE: Habibi et al, (2017).

Therefore, visualization and optimization of these features could be pre-eminent in mediating translational activity and boosting the amount of end-products within plant systems (Silverman et al., 2013).

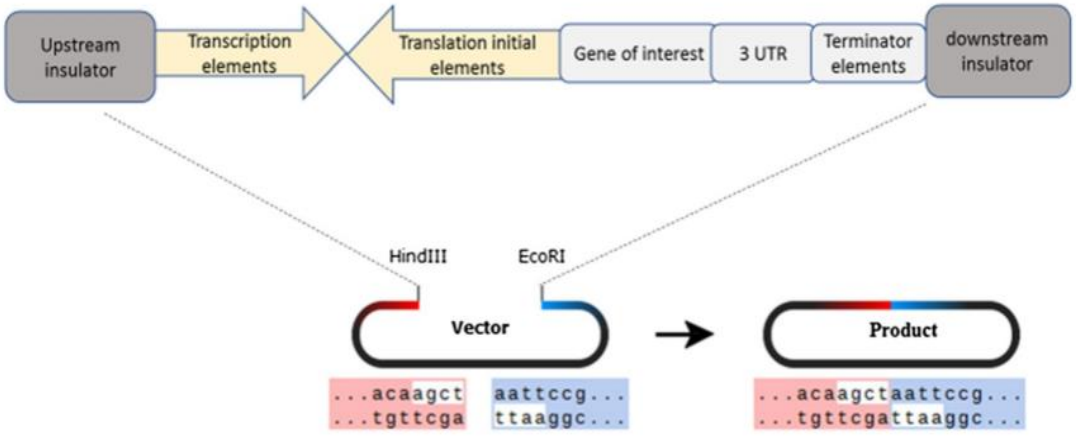
1.2 EXPRESSION CASSETTE COLLECTION

An expression cassette is known as an important construct for high-level production of recombinant proteins in a host plant. Expression cassettes can be classified according to the number of cistrons (recombinant genes) within the corresponding mRNA. Monocistronic cassettes are suitable for expressing single proteins with synthesis driven by their endogenous regulatory sequences (Al-Rubeai, 2011). They can either be provided by separated plasmids or be cloned into a single vector, leading to a higher prospect for the synergistic transfection of all genes of interest (Al-Rubeai, 2011). In the case of single vectors, more than one protein can be expressed in the same cassette,

although the sequence length can be a restriction factor for complete integration into the plant genome.

Bicistronic cassettes commonly use viral-derived internal ribosome entry sites (IRES) (Houdebine and Attal, 1999; Lopez-Lastra et al., 2005) to bypass the 5'-cap-dependent translation process through a ribosomal skip mechanism. However, this approach is not widely used because the ORF expression level downstream IRES is usually decreased (Hennecke et al., 2001). Nevertheless, polycistronic cassettes can be used in an operon-like structure (Osbourn and Field, 2009). It is efficient and very useful in plants for metabolic engineering purposes, such as for secondary metabolite expression via the addition of completely heterologous metabolic pathways (Mozes-Koch et al., 2012). Cassettes carry out the body of required elements in order to increase transcription rate (Clark & Pazdernik, 2015a) (FIGURE 2).

FIGURE 2- SCHEMATIC OF A GENE EXPRESSION CASSETTE. AN EXPRESSION CASSETTE IS A DNA SEQUENCE THAT CARRIES OUT THE BODY OF REQUIRED ELEMENTS TO INCREASE THE TRANSCRIPTION RATE. EACH OF THESE ELEMENTS COULD BE OPTIMIZED TO BOOST THE EXPRESSION RATE



SOURCE: Habibi et al. (2017)

The selection of suitable promoter is an important factor for boosting gene expression via binding and interacting with t*rans-actin* factors (Moustafa et al., 2015). Expression vectors can harbor either endogenous/homologous promoters or exogenous/heterologous promoters regarding the host species. The plant promotor database (http://ppdb.agr.gifu-u.ac.jp/ppdb/cgi-bin/index.cgi) provides variety of core

promotor structure and regulatory element groups for *Physcomitrella patens, Oryza sativa, Arabidopsis thaliana, and Populus trichocarpa (poplar)* (Hieno et al., 2014).

However, some heterologous promoters are commonly used in plants (Twyman et al., 2003a). For example, the CaMV35S (*Cauliflower Mosaic Virus 35S* RNA subunit) is highly compatible to the transcription machinery of dicots and is commonly used for compact expression cassettes, but the *CaMV35S* activity may be decreased in some tissue and cells such as newly developed tissues (Biłas et al., 2016). In this case, the region between -90 and 208 nucleotides act as an enhancer and plays a key role in increasing the promoter activity. *Act-1* and *Ubi-1* are other examples of promoters that show more compatibility with the transcription machinery of monocots(Kang et al., 2008). The efficiency of polyubiquitin promoters *PvUbi1* and *PvUbi2* derived from switchgrass showed several fold higher constitutive expression when it was compared to the efficiency of the *CaMV35S* and *OsAct1* promoters (Mann et al., 2011). Additionally, non-classical promoters were used for both monocots and dicots, such as the *CmYLCV* (*Cestrum Yellow Leaf Curling Virus*) promoter (Stavolone et al., 2003) and the *pPLEX* series promoters (Schünmann et al., 2003), respectively.

The efficiency of several commonly inducible (such as A*lcA, PR-1* and *ACE1*), and tissue-specific promotors (such as 2S albumin promoter, *Arc5*-I promoter, *CHS* and *RB7*) has been reported in plant (Twyman et al., 2003a). Recently, a β–estradiol-inducible promotor has been used to create the TRANSPLANTA collection in *A. thaliana* (Coego et al., 2014) and also to established stable gene expression system in *P. patens* (Kubo et al., 2013) for identification of biological functions of transcription factors (TFs), while TFs can regulate gene expression in all organism. Moreover, the efficacy of synthetic *cis*-acting motifs in transgenic tomato has been investigated and was compared with native *CaMV 35S* and *DECaMV 35S* promoter (Koul et al., 2012). Interestingly, the synthetic *cis*-acting modules led to increased transgene expression in tomato in comparison with native promotors.

In contrast, to the chemically-inducible system developed to control transgene expression, the physical approach is considered more applicable and safer in term of spatiotemporal resolution and toxic effects of gene expression (Kang et al., 1999; Amirsadeghi et al., 2007; Muller et al., 2014). Then, using of chemical inducible transgene

expression in plant cell cultures seems to be undesirable. In comparison with chemical inducer, the light was considered as compatible source for bioproduction of recombinant protein with high temporal resolution. In this context, a red light-switchable promotor has been developed to control transgene expression in the moss *P. patens* (Muller et al., 2014). Additionally, multimeric protein subunits or different independent proteins can be expressed and assembled *in planta* using a single ORF (*sORF*) through linkage to the 2A peptide from foot-and-mouth virus (Luke et al., 2015) or inteins (interspacing polypeptide blocks of functional protein parts) (Evans et al., 2005). Although the 2A peptide is known to trigger the auto-cleavage and release separate subunits, this mechanism occurs before the proteins enter the endoplasmic reticulum (ER). Otherwise, inteins ensure that the entire polypeptide targets to the ER and autocleavage occur in the lumen, to guarantee a balanced protein expression level in an equimolar ratio (Kunes et al., 2009). Once inside the ER, exteins - protein subunit blocks flanking inteins - can be joined via protein splicing through a mechanism that is desirable for protein multimerization (Hauptmann et al., 2013), for instance, or separately released (O'Brien et al., 2010).

Interestingly, this last approach is very suitable for expressing recombinant antibodies. However, it has only been performed for mammalian cells (Gion et al., 2013). Thus, considering all of these possibilities, cassettes can also be shuffled, according to their order in the vector backbone, or rearranged in different orientations for expression (*in tandem* – the 5’ and 3’ ends are kept in the same orientation related to the other cassette – or *sandwich* – the 5’ and 3’ ends of each cassette are inverted to each other in a reversed orientation) (Al-Rubeai, 2011). However, regardless of the vectors setup, it is important to ensure that the coding DNA sequence (*CDS*) of interest has efficient control elements for translation initiation. In this way, it is advisable to provide an appropriate ribosome-binding site (*RBS*) for plant machinery via,the addition of a eukaryote-derived *RBS* (Kozak sequence – consensus: GCCRCCATG) immediately upstream of the initiation codon (Kozak, 1999; Mohammadzadeh et al., 2015).

1.3 CODON USAGE

Codon bias is a crucial step in regulating synthetic gene expression in the plant platform, as it can influence numerous processes in association with protein production

(e.g., RNA processing, protein translation and protein folding). Through evolutionary mechanisms, plant hosts developed similar substitutions of rare codons with favorable ones to reach the desired nucleotide distribution, in which rare codons can inhibit protein translation (Gould et al., 2014). Gene expression-based on codon usage can be predicted using diverse metrics, reviewed by (Lindgreen, 2012). Additionally, the codon usage database (http://www.kazusa.or.jp/codon/) released values about the number of times each codon is used per 1,000 codons, and the total number of times each codon is known to be used.

Codon usage optimization in plants was reviewed (Serres-Giardi et al., 2012) demonstrating that rare codons and AU-rich destabilizing sequences may result in mRNA decline, reducing recombinant protein expression in plants (Laguía-Becher et al., 2010). The correlation between GC content, codon usage, and gene expression has also been reported in plants (Palidwor et al., 2010). These findings show the counterintuitive effect of GC content on determining the codon usage. Similar to these findings, the dominant effect of GC-biased genes on nucleotide distribution was reported in many seed plants (Serres-Giardi et al., 2012). Moreover, the influence of GC bias on codon context has been recognized at the set of codons but not at individual codons. Codon usage optimization boosts gene expression and influences the amount of transgene expression by more than 1,000-fold (Gustafsson et al., 2004). In this context, (Franklin et al., 2002; Gisby et al., 2011) demonstrated significant increases in expression (75- to 80-fold) after codon optimization. In a recent study, the importance of codon usage optimization was shown using increasing of gene expression (4.9- to 7.1-fold or 22.5- to 28.1-fold) in *Lettuce* and Tobacco Chloroplasts, respectively (Kwon et al., 2016). In this way, there are various methods to evaluate the effect of codon usage on gene expression (Box 1) and provide the best tools for codon optimization.

BOX 1. PROPOSED STATISTICAL METHODS FOR THE ANALYSIS OF CODON BIAS

Frequency of optimal codons (Fop) (Ikemura, 1981)

This index represents the rate of favorable codons to synonymous codons. This method has been used for the quantification of codon preferences and for the prediction of gene expression level.

Codon Adaptation Index (CAI) (Sharp and Li, 1987)

This index represents a geometric tool of relative adaptiveness to evaluate each codon. CAI measures the percentage of codons that are the most abundant choice in any organism. This method is also used for the prediction of gene expression level and for the analysis of codon usage in different organisms.

tRNA adaptation index (tAI) (dos Reis et al., 2004)

This index consists on codon adaptation to the intra-cellular tRNA pool. tAI estimates the wobble interactions among tRNAs and codons. This method is good for the prediction of gene expression from any nucleotide sequence.

Effective Number of Codons (ENc) (Suzuki et al., 2004)

This index determines several synonymous codons in the sequence. Compared to CAI, this method does not require a set of references expressing the gene and optimal codons to evaluate codon bias. ENc can evaluate codon usage evenness.

Relative Synonymous Codon Usage (RSCU) (Sharp et al., 1986)

This index evaluates codon bias patterns in whole genomes for a specific codon. It represents the observed number of existing codons divided by the expected number of similar codons. The value of RSCU will be 1 if the frequency of synonymous codons for an amino acid is the same.

Synonymous Codon Usage Order (SCUO) **(Wan et al., 2004)**

This method is based on Shannon's information theory and allows an analysis of the correlation between codon bias and GC composition. SCUO is able to assess the codon bias for different genomes at the same time.

Earlier reports described that the translational elongation step is affected by codon bias optimization (Irwin et al., 1995), and recent works provided evidence of codon

bias optimization on the translation efficiency in prokaryotes and eukaryotes (Duret, 2002; Mueller et al., 2010; Coleman et al., 2011). These findings indicate that codon context effects are significantly correlated with the abundance of tRNA isoacceptor molecules on the ribosome surface. Moreover, post-translation modifications might be affected by codon bias as silent mutations within the transcript, but resulting in unwanted protein instability and misfolding (Brest et al., 2011). Nevertheless, it is important to observe that these complex correlations are still not fully recognized, once that the knowledge about the effects of codon sequence changes during translation and post-translational modifications are somewhat limited.

1.4 SUBCELLULAR TARGETING

The overexpression of a targeted gene via cellular compartments has gained more attention in recent years. However, in addition to the optimization of the exogenous mechanism involved in recombinant protein production, endogenous factors related to housekeeping genes and essential metabolism are features limiting the increased yield of recombinant proteins. This limitation could influence the accumulation of recombinant proteins that require post-translational modification in the ER through the activation of the unfolded protein response (Thomas and Walmsley, 2015). Furthermore, directing recombinant proteins to subcellular organelles creates an environment low in proteases and helps to increase protein production and recovery (Fischer et al., 2012). In addition to the ER and oil bodies, proteins can be targeted to the vacuoles, apoplast, and plastids, and they can even be directed to a hydroponic medium in plant roots (Horvath et al., 2000).

The endoplasmic reticulum (ER) is an ideal organelle to increase protein accumulation and improve protein assembly, in addition to controlling glycosylation (Helenius and Aebi, 2001). The ER is a region with a low quantity of proteases, and the presence of a high concentration of chaperones can assist recombinant proteins in post-translational folding and stability (Nuttall et al., 2002). Previous studies demonstrated that the apoplast targeting of recombinant antibody fragments when retained in the ER, yielded 3.8 µg/g of protein in *O. sativa* cells. The same antibody fragment, when produced in tobacco cells, corresponded to 0.064% of the total soluble protein (Fischer et al.,

1999;Torres et al., 1999). However, ER addressing is not recommended for proteins that require downstream modifications in other organelles, such as oil bodies, vacuoles, and chloroplasts (Doran, 2006)

Furthermore, the accumulation of antibodies directed to the ER using (SE)/(H)/(K)DEL signal peptides increased 2- to 10-fold compared to proteins lacking retention signals (Conrad and Fiedler, 1998). A previous report described that the retention of recombinant Fv antibodies in the ER lumen increased by 10- to 100-fold the antibody concentration when expressed in different plant cells (Conrad and Fiedler, 1998). Also, the use of N-terminal γ-zein proline-rich sequences to target proteins to the ER and protein bodies increased the stable accumulation of proteins in seeds (Torrent et al., 2009).

1.5 HOST SYSTEM

The selection of a suitable host plant and the consideration of its effect on the efficiency of recombinant protein accumulation are important key strategies for boosting intrinsic yield. There are economic issues affecting the selection of plant hosts as suitable benchmarks for the production of recombinant proteins. These economic factors include storage property, scalability, and transportation, cost of downstream processing, potential of a short-time scale and edibility (Obembe et al., 2011). In addition, efficient transformation and regeneration may contribute to the selection of a host plant as an amenable resource for recombinant protein production. However, it is not possible to set a perfect single system with all economic features, as every system has its own advantages and disadvantages that should be considered as signposts to select the best system for protein production.

1.5.1 Leafy Crop-Based Expression

Leafy crops, including tobacco, lettuce, and alfalfa, are well established for the commercialization of biopharmaceuticals due to the many expectations in terms of biomass efficiency and capability at a massive scale. For example, the ability of transgenic tobacco (*N. tabacum*) to produce 1-100 tons of biomass per hectare per year

makes this plant a reliable platform for the production of a high concentration of biopharmaceuticals. Moreover, easy genetic transformation and regeneration are favored by using tobacco as a laboratory model in terms of molecular farming. Therefore, both scalability and successful gene expression history make tobacco a pioneer for the production of various biopharmaceuticals (Twyman et al., 2003b).

Tobacco has long been used as a model system for the production of recombinant proteins and provides promise as a host system for large-scale production of recombinant protein (Fischer and Emans, 2000; Stoger et al., 2002a; Stoger et al., 2002b; Menkhaus et al., 2004; Schillberg et al., 2005a). Transformation protocols for protein expression in tobacco are well established by systems including *A.tumefaciens* mediated DNA-transfer (Kapila et al., 1997) and plant viral vectors (Verch et al., 1998).

Similar to all plants, tobacco is devoid from human pathogens and contamination, eradicating the risk of transmissible diseases. Also tobacco is considered as a non-food and non-feed crop, then minimize the threat of contamination of a food or feed supply (Twyman et al., 2003a). Commonly, the leaf tissue is selected for recombinant protein production, as it provides an abundance of transgenic biomass. Standard farming practices have been already established for large scale production of tobacco biomass and tobacco could be harvested several times a year (Schillberg et al., 2005b). Finally, higher protein accumulation as well as protein stability could be achieved in tobacco chloroplast. Expression of various recombinant therapeutic proteins has been reported in tobacco and numerous of these protein are undergoing clinical trials; however, there is not an approved recombinant therapeutic protein by the U.S. Food and Drug Administration (Menkhaus et al., 2004). The Medicago Inc. (Québec, Canada; http://www.medicago. com) is now working on a phase II clinical trial with influenza VLP (H5) accumulated in *N. benthamiana* through agroinfiltration technology. Planet Biotechnology, Inc. (USA, http://www.planetbiotechnology.com/) and Meristem Therapeutics (Clermont-Ferrand, France) are other two companies, which produce therapeutic recombinant proteins in tobacco.

Next to the well established process for production of recombinant proteins in tobacco , some challenges and barriers should be overcome before tobacco can be considered as a bioreactor system for accumulation of therapeutic proteins in large scale

approach. Seed-based expression systems provide a good platform for the production of recombinant proteins at massive and minute levels. In both cases, the high cost of the process development, such as bioreactor-based production, would be reduced. Moreover, this system could provide a containment lacking proteolytic activity as well as it can resolve problem related with protein instability and decreased activity of recombinant proteins due to long-term storage (Kusnadi et al., 1998; Stöger et al., 2000; Bai and Nikolov, 2001).

However, tobacco seeds could not be efficient and economical for the production of recombinant therapeutics proteins in large scale as tobacco seeds are extremely small. Therefore, protein expression in tobacco is directed to leaves because of the capability for production of large amounts of biomass and ease of scale-up (Menkhaus et al., 2004). Unfortunately, recovery and purification of recombinant proteins by tobacco system encounter some challenges. Firstly, tobacco leaves consist of high level of native phenolic compounds, up to 30 mg/g dry weight, and toxic alkaloids, such as nicotine, (Moloney, 1995; Naidu, 2001). It is extremely could interfere with downstream processing by creation of complexes bonds with target protein in an aqueous fraction (Cheryan and Rackis, 1980; Jervis and Pierpoint, 1989) or by fouling resin during adsorption processes such as chromatography (Menkhaus et al., 2004). Then, these compounds should be removed during downstream process.

Tobacco leaf tissue will also exhibits high content of protease activity creating the environment much less stable during the initial harvest and extraction of the target protein (Moloney, 1995). In this context, fresh leaves should be dried, freeze-dried in nitrogen immediately after harvest, which may increase the stability of the protein (Khoudi et al., 1999; Twyman et al., 2003a). Moreover, the tobacco leaf contains other compounds including native proteins, nucleic acids, and carbohydrates, which should be isolated from the target protein.

Tobacco proteins can be classified into two protein fractions. Fraction 1 contains high amount of the photosynthetic chloroplast enzyme ribulose 1,5-bisphosphate carboxylase-oxygenase (Rubisco), which is measured up to 50% of the total soluble leaf protein (25% total leaf protein) (Garger et al., 2000). This protein consists of eight large subunits and eight small subunits of 55 kD (pI 6.0) and 12.5 kD (pI 5.3), respectively

(Kung, 1976). Like to human or bovine milk proteins, fraction 1 could be utilized as a nutritional protein, and superiority of fraction 1 over soybean protein has been reported. The fraction 2 consists of a combination of soluble proteins and peptides, isolated from both the chloroplast and cytoplasm. The molecular weights of fraction 2 proteins are varied widely, ranging from 3 kD to 100 kD, (Garger et al., 2000). Since tobacco proteins (F1 and F2) show acidic nature, therefore extraction of a basic protein under acidic conditions would result in a lower purification burden (Balasubramaniam et al., 2003).

1.5.2 The Moss *Physcomitrella Patens*

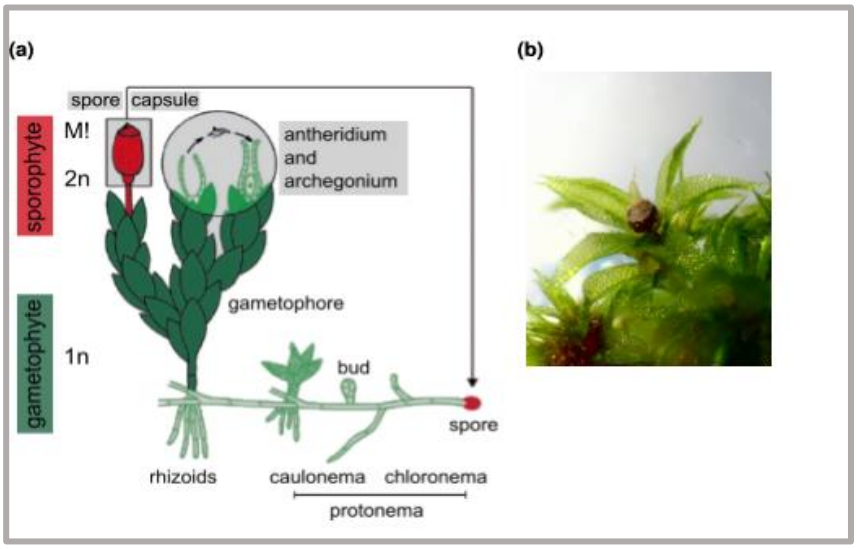
After the transition from aquatic to terrestrial habitat, land plants (embryophytes) began to diverge from a common ancestor more than 450 million years ago (Zimmer et al., 2007). Bryophytes, as remnants of early diverging lineages of embryophytes, comprise the classes hornworts, liverworts, and mosses – including *P.patens*.

Kingdom Plantae
Division Bryophyta
Class Bryopsida
Subclass Funariidae
Order Funariales
Family Funariaceae
Genus Physcomitrella
species *Physcomitrella patens*

Mosses, like ferns and seed plants, show alternation of generations. In its dominant haploid phase (gametophyte generation), *P. patens* produces gametes while in a short diploid phase (sporophyte generation) production of haploid spores occurs (FIGURE 3). In the presence of water and light, germination of the spore is induced, and a filamentous tissue, the protonema, formed. The moss protonema comprises two clearly distinguishable cell types: the chloronema, with relatively short cells and a high chloroplast density (about 48 chloroplasts on average), and the caulonema, made up of longer and thinner cells that contain fewer (about 20) chloroplasts. Both cell types expand by tip growth (Menand et al., 2007) and from subapical cells side branches can be

originated. Some of these side branches are initial cells (three-faced apical cells) and differentiate into buds, which give rise to a more complex structure – the gametophore. These bear leaf-like structures, rhizoids and the sexual organs (Reski, 1998), archegonia (female) and antheridia (male). *P. patens* is monoicous, so that male and female organs arise on the same plant. Fertilization is mediated under water by motile spermatozoids.

FIGURE 3 - SCHEMATIC REPRESENTATION OF *P. PATENS* LIFE CYCLE. A) 1N = HAPLOID, 2N = DIPLOID, M! = MITOSIS. B) MICROSCOPIC PICTURE OF AN ADULT GAMETOPHYTE, WHICH BEARS THE SPOROPHYTE WITH A MATURE SPORE CAPSULE.



SOURCE: Frank et al (2005).

The *P. patens* is a haploid species with a relatively small genome size of 511 Mbp (Schween et al., 2005) distributed among 27 chromosomes (Reski et al., 1994). Transgenic plants can be generated by means of PEG-mediated DNA transfer into protoplasts and homologous recombination occurs in *P. patens* as efficiently as in yeast (Schaefer, 2001), which means it occurs five orders of magnitude more efficiently than in any other plant species tested. Whole genome sequencing has been completed (Rensing et al., 2007) and well-developed molecular tools are available (Lang et al., 2005). Combining these features leads to the outstanding possibility of targeted gene modifications, which can be directly observed without any cross-breeding. Furthermore, culture conditions for *P. patens* are optimized for all differential stage of the moss and

cultivation may range from small (some mL) to large scales (100 L) in photo-bioreactors (Hohe and Reski, 2002; Lucumi et al., 2005). In the context of recombinant protein production, solely chloronema tissue is cultivated under standardized conditions to assure constant product quality. Production strain design is relatively easy in *P. patens* due to the haploid genome and the outstanding rate of homologous recombination. For example, the *P. patens* N-glycosylation machinery, which is organized similarly to that in higher plants (Koprivova et al., 2002), was the first to be humanized in one step by means of directed knock-out of the plant specific enzymes β 1,2-xylosyltransferase, α(1,3)-fucosyl transferase and by a simultaneous site-directed knock-in of human β(1,4) galactosyltransferase (Huether et al., 2005).

3.1.1.1 Recombinant products in moss

Several proteins have been produced in moss. The field started with the expression of the bacterial beta-glucuronidase (GUS) as a quantifiable reporter protein in 2 L moss bioreactors (Reutter and Reski, 1996; Rho et al., 2014). Further reporter proteins that were used to monitor the production process were a bacterial α-amylase and the human placental secreted alkaline phosphatase (Gitzinger et al., 2009) as well as the F-actin marker GFP-talin (Saidi et al., 2005). As a product as well as a stabilizing agent for secreted biopharmaceuticals the human serum albumin (HSA) has been co-expressed in the production process (Baur et al., 2005).

Mosses contain far more genes involved in secondary metabolism than seed plants (Rensing et al., 2007). Some of these metabolites possess well-known human health benefits (Beike et al., 2014). Therefore, one side-aspect of the field is the metabolic engineering of moss to enhance the production of secondary metabolites with commercial value. A breakthrough in this respect was the expression of a taxadiene synthase from *Taxus brevifolia* (Anterola et al., 2009), an enzyme responsible for the synthesis of a precursor of paclitaxel, a widely used anticancer drug (Baird et al., 2010). Another target for engineered mosses is the fragrance industry. In this respect, a patchoulol synthase and an alpha/beta-santalene synthase have been expressed in *P. patens* (Zhan et al., 2014). Patchoulol and alpha/beta-santalol are two sesquiterpenoids used in fragrances (Faraldos et al., 2010).

The first human protein produced in the moss system was the vascular endothelial growth factor (Baur et al., 2005), which has a central function in angiogenesis and in cancer (Goel and Mercurio, 2013). Human complement factor H (FH) is the key regulator of the alternative pathway of complement activation and a protectant against oxidative stress (Goel and Mercurio, 2013). Because it is a large protein of 155 kDa and contains 40 disulphide bonds, it is a difficult-to-express protein. Therefore, attempts are ongoing to produce bioactive but truncated versions (mini FH) in insect cells (Hebecker et al., 2013). Full-length FH has been successfully produced in moss with biological activity in vitro (Büttner-Mainik et al., 2011). After having confirmed full biological activity in different bioassays, this protein will be further evaluated in FH-deficient knockout mice. FH supply is a potential treatment for kidney diseases such as atypical haemolytic uremic syndrome (aHUS) and C3 glomerulopathies (Sethi et al., 2012) and for age-related macular degeneration (Bradley et al., 2011).

A moss-made FH may be a cost-effective and more efficient way to the monoclonal antibody eculizumab, which is limited to the treatment of aHUS, has severe side effects (Schmidtko et al.) and, moreover, is one of the most expensive biopharmaceutical worldwide with treatment costs of about 400,000 Euro per year and patient. Several human growth factors (FGF7/KGF, EGF and HGF) that are used in mammalian cell culture have been produced in the moss system (Orellana-Escobedo et al., 2015).FGF7/KGF (keratinocyte growth factor) is the first commercially available moss-made human protein, intended for *in vitro* use (www.greenovation.com). Based on these experiences, moss has been suggested as a potential production host for vaccines (Rosales-Mendoza et al., 2014). As no adverse effects of moss consumption are known, vaccine-producing moss may be directly administered as an oral vaccine. Thus, expensive protein purification could be avoided. The first moss-made candidate vaccine is a chimeric Env-derived HIV multi-epitope protein that is immunogenic in mice (Orellana-Escobedo et al., 2015).

1.6 TRANSIENT EXPRESSION-BASED SYSTEMS: AGROINFILTRATION AND AGROINFECTION

Transient gene expression is an efficient, cost-effective and time-saving strategy for yielding high amounts of recombinant proteins, as genetic transformation is a slow process, requiring months or years to generate transgenic plants due to regeneration protocols (Hefferon, 2012). In addition, it is a genome integration-independent strategy, and consequently, it is not affected by position effects existing in stable transformation, once that the expression vector remains an episomal DNA molecule. Moreover, transient gene expression can be detected within 3 h after plasmid delivery, reaching an expression threshold after 18 to 48 h, and remaining transcriptionally active for approximately 10 days. Beside these features, transient expression can contribute to overcome concerns with biosafety issues (Komarova et al., 2010).

In plants, transient gene expression is usually accomplished using *A. tumefaciens* in agroinfiltration experiments, by infiltrating bacterial cell suspensions into leaf cells with consequent delivery of T-DNA to the host cells (Circelli et al., 2010). Furthermore, an *A. tumefaciens* approach can be coupled with replicating plant virus genomes in a virus-based replicon system, whose advantages rely on the virus properties: they are small, can be easily manipulated, have a simple infection process and are able to replicate in high levels. These properties make them ideal vectors for heterologous expression and a suitable alternative to stable transgenic systems (Lico et al., 2008).

Next to selection of the method for production of pharmaceutical protein, the target gene should be efficiently integrated into host genome. In this case, direct delivery via biolistics or indirect delivery by using *A. tumefaciens* are considered as the two most widely used methods for plant genetic transformation (Rivera et al., 2012). The direct gene delivery, also known as microparticle -bombardment, involves the coating of transgenes with metal beads such as gold or tungsten particles and direct mechanical firing of them into plant genome (Sanford, 1990). This method can be efficiently used to integrate foreign nucleic acid into both nuclear and chloroplast genomes and, at least in theory, can be applied for transformation of recalcitrant species (Rivera et al., 2012). In addition, biolistics provides a clean gene technology as vector sequences are not required in this method, thus remarkably simplifying the cloning process. Moreover, co-transformation with multiple transgenes that facilitates the introduction of multiple genes

of interest in one simultaneous transformation step requiring only one selectable marker is another advantage of biolistic delivery (François et al., 2002).

In the *Agrobacterium*-mediated transformation system, the exportation of interest gene into host genome carried out by a region of a tumor-inducing (Ti) plasmid resident in *agrobacterium*. Interestingly, the tumor inducing genes in the T-DNA region can be replaced by the gene of interest, and subsequently could be considered as efficient and affordable vectors for introduction of gene of interest into host plant genome by the natural interaction between *A. tumefaciens* and its plant hosts (Rivera et al., 2012). In comparison with biolistics systems, of target gene by *A. tumefaciens* is practical for most dicotyledonous and a restricted number of monocotyledonous plant species (Gelvin, 2003). Based on previous investigations, transformation efficiency, transgene expression, and inheritance of gene delivery methods based on *Agrobacterium*-based transformation is significantly much better than biolistics methods (Rivera et al., 2012). In this context, the low transgene copy numbers and precise gene integration into plant genome could be account as advantage of *Agrobacterium* methods. In the other hand, selective and precise transgene integration into plant genome stem from co-evolutionary ability of *Agrobacterium* and its plant hosts, which result in stable integration and inheritance of target transgenes (Gelvin, 2003). Both, biolistics and *Agrobacterium*-based methods, have been successfully applied for creating stable transgenic lines and transient gene expression of plant species (Rivera et al., 2012). However, gene delivery by *Agrobacterium* is more preferable than biolistics for production of recombinant protein through transient systems, as the biolistics method generally cause significant tissue damage and extremely decrease the biomass productivity available for protein accumulation. Moreover, particle bombardment method requires special equipment for the transfer of genes into plant cells, which complicate the transformation process where the accessibility to this equipment is restricted. In the last years, different protocols have been established for *Agrobacterium*-based gene transformation into plant genome (Gelvin, 2003). Recently, agroinfiltration has gained more attention as the most promising technology transient expression of transgenes, assessment of vector constructs, and production of recombinant proteins in plant cells. (Vaghchhipawala et al., 2011). Agrobacterium-mediated transient expression (agroinfiltration) has been known as

infiltration of *A. tumefaciens* harboring target transgene into plant leaves. Agroinfiltration has been established for studying of various subjects such as protein–protein interaction, protein localization, plant-virus interactions, and gene function. In earlier experiments, whole or partial genomes of plant viruses were cloned into binary vectors and transformed into an *Agrobacterium* host. These exogenous DNA-carrying *Agrobacteria* were then infiltrated into the intercellular space of the plant tissue, allowing the delivery of viral genes into plant genomes (Grimsley et al., 1986). Today, the agroinfiltration has been widely used to deliver DNA of interest from different organisms into plant cells for a broad range of applications (Vaghchhipawala et al., 2011). The agroinfiltration can be processed via syringe and vacuum methods as both of these methods have their advantages and disadvantages. In the syringe method, agrobacterium suspension could be injected into whole or part of leaf through the nick with or without a needleless syringe (Santi et al., 2008). By injection of the *Agrobacterium* suspension into the intercellular space of the leaf, the light green color changes to darken, demonstrating a successful infiltration.

Nowadays syringe infiltration method has been developed for broad range of plant species (Wroblewski et al., 2005) and has shown remarkable advantages. This method is very simple and there is no need for any specialized equipment for infiltration. In addition, by this method, introduction of one target DNA construct or multiple constructs into whole or partial area of leaf could be performed allowing multiple assays on a single leaf (Vaghchhipawala et al., 2011). Therefore, syringe infiltration is considered as efficient and affordable tool in term of transient gene expression for being used in wide range of application such as protein-protein interactions, plant gene functional assay, protein localization, investigation of plant pathogen interactions, and abiotic stresses (Vaghchhipawala et al., 2011).

To investigation of biochemical characterization, purification and preclinical functional of protein of interest in laboratory scale, the agroinfiltration of whole leaf could be considered. This simple method also can be used as a training tool for description of genetic engineering in high school and college education (Chen et al., 2013b).

Vacuum method is an alternative agroinfiltration process, which can able to introduce the *Agrobacterium* deep into entire leaf. This method is based on a vacuum chamber, forcing air out of the intercellular spaces within the leaves (Chen et al., 2013b).

This method was first established for plant species that cannot be efficiently agroinfiltrated by syringe method (Rivera et al., 2012). In this procedure, leaf disc or whole plant leaves are first submerged into backer containing the *Agrobacterium* solution and then the backer is placed in vacuum chamber. By application of vacuum via negative atmospheric pressure in a vacuum chamber, the air out of the intercellular space plant leaf. When the vacuum is release, agroinfiltration could then obtained by the created pressure difference forces. Although, in comparison with syringe infiltration, vacuum infiltration is more sophisticated but this method needs the investment of vacuum pumps, vacuum chambers, and larger volumes of *Agrobacterium* cultures which complicate the agroinfiltration process where the accessibility to equipment is not easy. Moreover, by this method the possibility of multiple assays on a single leaf can be removed. However, its value lies in its enormous scalability potential that cannot be matched by syringe infiltration (Chen et al., 2013b).

Compare with stable transgenic plants, transient expression platform does not result in transgenic lines and only need non-transgenic plant materials for agroinfiltration. Subsequently, transient expression does not cause the potential risk of food contamination or unwanted transgene outflow from genetically modified (GM) plants to non-GM crops or their wild relatives. The most likely factor in causing variability of recombinant protein yield between production batches and between laboratories is the specific condition of plant material (Lai and Chen, 2012). The developmental stage and also physiological state of plants extremely affect their competency in accumulation of the target protein using agroinfiltration. In the agroinfiltration process some critical parameters including the light intensity, supply of fertilizer the temperature, plant inoculation age, and incubation time after leaf infiltration should be optimized for obtaining optimal plant growth and protein production. The amounts of light, water and fertilizer should be considered consistently as a little change in these parameters may drastically change the final size of plants and their ability to produce recombinant proteins. For example, growth under natural light produces much higher leaf biomass, but the yield of recombinant protein from this biomass is lower than that from leaves grown under artificial light (Lai and Chen, 2012). The optimal condition to grow *N. benthamiana* plants is a 16 hr Light/8 hr dark cycle at 25 ± 0.5°C under such artificial lighting (Lai and Chen, 2012). These plants have

already produced sufficient biomass and consistently accumulate high-levels of interest proteins. besides plants older than 6-weeks show more biomass, they have already initiated flower development which interfere the expression of recombinant proteins (Lai and Chen, 2012).

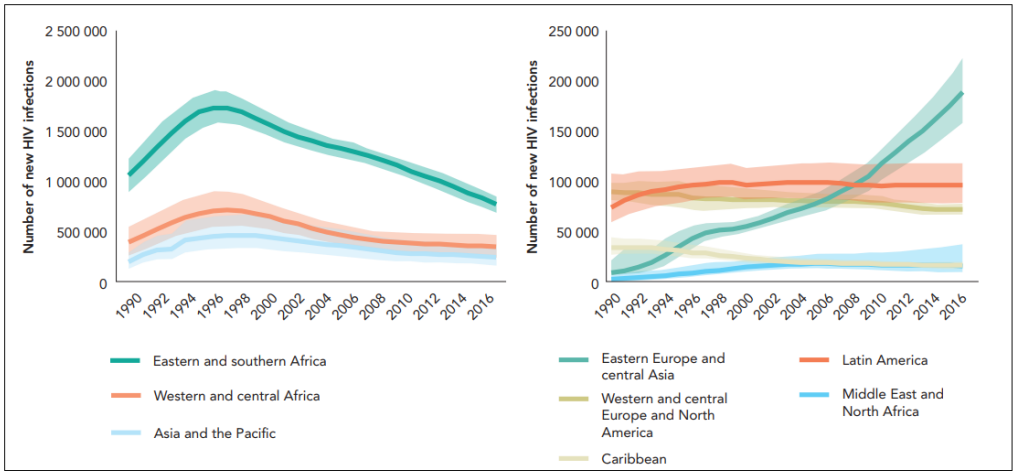
In comparison with viral system in term of agroinfiltration, cost-effectiveness, availability, rapidly and more significantly low induction of immune system and no limitation in size of transgenic DNA have made non-viral system more effective for gene delivery. Beside its speed and high yield, the transient expression platform presents the versatility for expression and accumulation of personalized recombinant proteins, including therapeutics for patient-specific cancers and vaccines for viruses that show rapid antigenic drift and/or multiple strains with unpredictable outbreaks (Nayerossadat et al., 2012; Chen and Lai, 2015). However, limited protein content based on transient expression system remains a significant drawback in term of economic production. In this context the RNA silencing is major limitation to expression of genes in the *Agrobacterium*-mediated transient system as the foreign nucleic acids recognized and subsequently degraded in a sequence-specific manner such as mRNA stability or translational levels (Johansen and Carrington, 2001a). RNA silencing (also known as post-transcriptional gene silencing) is considered as adaptive defense response by eukaryotic whereby invading transgenes and/or viruses are eliminated during highly conserved mechanism. This mechanism starts with formation double stranded (ds)RNA and subsequently the processing of dsRNA to small (s) 20–26-nt dsRNAs with staggered ends and finally terminate with inhibitions of selected sRNA strand within effector complexes acting on partially or fully complementary RNA or DNA (Brodersen and Voinnet, 2006). This mechanism could be inhibited by RNA silencing suppressors. In this case, viruses are developed as remarkable tool to counteract this highly conserved mechanism by providing viral protein (Incarbone and Dunoyer, 2013). RNA silencing suppression activity of viral protein of virus stems from its ability to bind siRNA as well as proteins involved antiviral silencing (Deleris et al., 2006; Lakatos et al., 2006; Mérai et al., 2006; Baumberger et al., 2007; Bortolamiol et al., 2007). The P19 suppressor from the *Tomato bushy stunt virus* (*TBSV*) has been widely used to boost transient or constitutive expression level of recombinant proteins in the plant as it selectively inhibits the 21-nt and

22-nt classes of siRNA as well as reduce the availability of free siRNA for RISC loading (Arzola et al., 2011; Lacombe et al., 2017).

1.7 HIV PREVALENCE

The HIV virus remains to be a major threat to global public health since it was first deciphered in 1981 in the United States (Barre-Sinoussi et al., 1983). Despite comprehensive progress in HIV/AIDS research, about 36.7 million people were living with HIV at the end of 2015 according to the World Health Organization (WHO) (http://www.who.int/mediacentre/factsheets/fs360/en/). (FIGURE 4). AIDS disease have continued the leading cause of death in sub-Saharan Africa and reportedly was responsible for death of 1.1 million people globally in 2105 WHO (World Health Organization). Global Health Observatory (GHO) data - the top 10 causes of death; http://www.who.int/ mediacentre/factsheets/fs310/en/ (accessed March 31, 2017).

FIGURE 4 - A GLOBAL VIEW OF HIV INFECTION. NEW HIV INFECTIONS, ALL AGES, BY REGION, 1990–2016.)



SOURCE :(UNAIDS, 2018).

Current predicts suggest that 5,753 people will become infected with HIV every day—about 240 every hour. Currently there is no cure for HIV. Nevertheless, the disease can be managed with antiretroviral drugs that reduce the rate of HIV replication and hence the mortality and morbidity associated with acquired immunodeficiency syndrome (AIDS). The best current treatment is highly active antiretroviral therapy (HAART), which

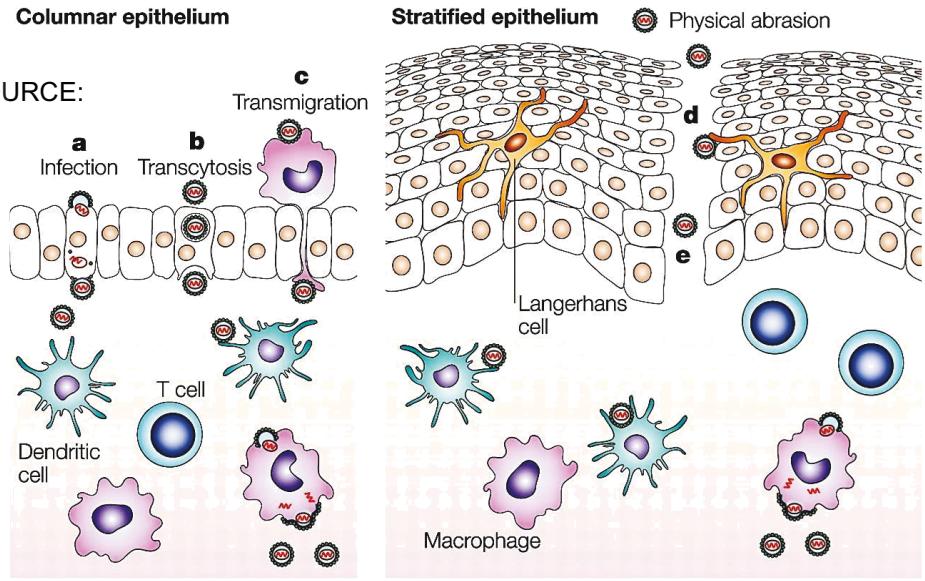
comprises a cocktail of antiretroviral drugs that combat HIV proliferation by inhibiting different components of the virus. However, resistance to HAART is a growing problem and despite measures to increase the distribution of anti-retroviral drugs, the number of new HIV infections is outstripping the number of recipients of HAART by 2:1 (MTN, 2014). For this reason, new strategies are required to prevent the spread of HIV.

1.7.1 Mechanisms of HIV-1 sexual transmission

The infection of HIV virus could be started by passing of virus via the mucosal epithelium of the vagina or rectum, and the occurrence of HIV-1 transmission is reported by males more often than females (Shattock and Moore, 2003; McGowan, 2006). However, understanding of the precise transmission mechanism is complicated and unknown, different strategies have been involved (Shattock and Moore, 2003; McGowan, 2006).(FIGURE 5).

FIGURE 5 - POTENTIAL MECHANISMS FOR HIV-1 TRANSMISSION ACROSS MUCOSAL EPITHELIUM. A) DIRECT INFECTION OF EPITHELIAL CELLS. B) TRANSCYTOSIS THROUGH EPITHELIAL CELLS AND/OR SPECIALIZED MICROFOLD (M) CELLS. C) EPITHELIAL TRANSMIGRATION OF INFECTED DONOR CELLS. D) UPTAKE BY INTRA-EPITHELIAL LANGERHANS CELLS. E) CIRCUMVENTION OF THE EPITHELIAL BARRIER THROUGH PHYSICAL BREACHES. SUCCESSFUL TRANSFER OF VIRUS ACROSS EPITHELIAL BARRIERS WOULD RESULT IN HIV-1 UPTAKE BY MIGRATORY DENDRITIC CELLS (DC-SIGN OR ANOTHER MANNOSE C-TYPE LECTIN RECEPTOR) AND SUBSEQUENT DISSEMINATION TO DRAINING LYMPH NODES, AND/OR

LOCALIZED MUCOSAL HIV-1 INFECTION LEADING TO RECRUITMENT OF ADDITIONAL SUSCEPTIBLE CELLS (SHATTOCK AND MOORE, 2003).



SOURCE: Shattock and Moore (2003).

The virus should invade to some defense barriers, including multiple layers of stratified squamous epithelium in the vagina and ectocervix of females or the inner foreskin, penile glans, and fossa navicularis in males. The success of this hard roadblock was indicated when the epithelial layer of rhesus macaques was thinned by hormonal treatment, subsequently decreasing ‘physical resistance’ to the virus (Marx et al., 1996;Smith et al., 2000).

It is globally considered that HIV infection will be occurred in respond to breakdown of the epithelial stroma during intercourse. Additionally, male circumcision could extremely have a significant impact on HIV as the circumcision may decrease the probability of microtrauma to the large surface area of foreskin (Auvert et al., 2005).

The inhibition of rectal infection has been considered more complicated as the rectum provides just a single layer of columnar epithelium. Also, the incidence of injury during anal intercourse could significantly boost the possibility of HIV-1 infection

(McGowan, 2006). Moreover, the incidence trauma, ulceration or inflammation may result in susceptibility of the epithelium to HIV (Strathdee et al., 1996;Mostad et al., 1997).

1.7.2 Conventional approaches for the treatment of HIV

Today pre-exposure prophylaxis, or PrEP, and antiretroviral therapies allow those patients living with HIV enjoy longer and keep HIV at undetectable levels. The first antiretroviral medication, Zidovudine, also known as azidothymidine was developed and approved in 1987. Nowadays, more than 40 FDA-approved drugs have been released in the market for treatment of individuals carrying HIV virus. Antiretroviral drugs used in the treatment of HIV infection are categorized into protease inhibitors, nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, HIV integrase strand transfer inhibitors, entry inhibitors, and fusion inhibitors. (NAID, 2017). Although these drugs used to prevent the spread of HIV infection and consequently allow patients with healthier lives, they are unable completely remove HIV-infection from patients, cause serious side effects and patients may continue to experience illness associated with HIV infection due to the development of drug-resistance HIV (Cos et al., 2004; Thayer, 2008). Moreover, only 46% of the 36.7 million individuals infected with HIV were received antiretroviral therapy in 2015, according to WHO (http://www.who.int/features/factfiles/hiv/en/), which reminds HIV/AIDS as one of the greatest public health challenges, particularly in low and middle-income countries. On the other hand, infected people in developing countries with the greatest incidence of HIV pandemic, unable to pay medications and/or accessing HIV medications do not simply for most of people.

Sexual transmission is considered as potential way of infection and, in this case, prohibition of sexual transmission may strongly affect the spread of HIV pandemic. In this context, based on (AVERT, 2014) report, heterosexual sex caused approximately two thirds of new HIV infections. Previously, some strategies such as abstinence, a reduction in the number of sexual partners, monogamy, and using male and female condoms have been used to prevent HIV transmission. However, these strategies are not effectively

triumphant against HIV infection. Therefore, invention and development of novel, efficient, accessible, and credible anti-HIV therapeutics would be imperative. In the economic concept of management of HIV prevalence, using competing medications of different types of HIV infection as well as conventional and novel prevention tool, considering a safe and affordable vaccine would be transformative (Smith et al., 2016). In the simple way, our world needs an HIV vaccine more than anything. Additionally, investigation of inhibition strategies, such as pre-exposure prophylaxis, microbicides and voluntary male circumcision (AVAC, 2014) could help to curtail the HIV infection. In this context, the application of microbicides as a self-administered molecules in the vaginal or rectal mucosal surfaces may extremely inhibit HIV infections sexually (Singh et al., 2014).

Truvada (Gilead Sciences, Inc., Foster City, CA) is a prescription medicine for pre-exposure prophylaxis (PrEP) that is used to treat HIV-1 incidence in uninfected people who are at high risk of getting HIV through sex. Because Truvada by itself is not a complete treatment for HIV-1, it must be used together with other HIV-1 medicines (FDA, 2012). Truvada was approved by FDA for being used in combination with other antiretroviral agents for the treatment (not prevention) of HIV-infected adults and children 12 years or older (FDA, 2012). This product consists the RT inhibitor emtricitabine and tenofovir disoproxil fumarate that have been used as antiretroviral drugs. In order to decrease the risk of HIV infection, before start taking Truvada for PrEP , the person should get tested to make sure that he/she does not already have HIV-1 (FDA, 2012).

1.7.3 Griffithsin structure and function

Griffithsin (GRFT), a 121-amino-acid lectin derived from red alga *Griffithsia* spp. (Mori et al., 2005), is among the most promising lectins yet discovered. Griffithsin was discovered as an anti-HIV lead by scientist at the National Cancer Institute (NCI). GRFT was extracted from a marine red alga *Griffithsia* sp. present in the NCI Natural Products Repository. Mass spectroscopic and nuclear magnetic resonance (NMR) data showed the active compound was a protein rather than a small molecule natural product. Its sequence was determined through a combination of N-terminal Edman degradation of the intact protein and N-terminal sequencing of peptide fragments obtained from endopeptidase and cyanogen bromide treatments (Mori et al., 2005). The wild-type

protein from the alga contained an uncommon amino acid of 151.05 Da at position 31 that was replaced by alanine (Ala) in recombinant protein preparations without affecting anti-HIV activity. GRFT has no homology to any other proteins previously reported as well as it has no cysteine in its sequence. It has been shown to have anti-HIV activity against T cell tropic and macrophage-tropic viruses. It is capable of inhibiting cell–cell fusion between chronically infected and uninfected cells and its efficacy as an antiviral agent against other enveloped viruses has also been shown. This protein has a combination of: (i) higher potency than other lectins (Mori et al., 2005;Alexandre et al., 2010), (ii) excellent preclinical properties, including low/no toxicity and lack of significant activation of a variety of cell types (Emau et al., 2007; Kouokam et al., 2011), and (iii) synergy when combined with antibodies and a variety of other lectins (Ferir et al., 2012). Recently, GRFT has also been shown to be active against other viruses, including the hepatitis C virus (O'Keefe et al., 2010; Ishag et al., 2013) and it is resistant to digestion by many commercially available proteases (although it is susceptible to elastase) (Moncla et al., 2011). Recent work specifically addressing lectin cellular toxicity showed that GRFT does not activate T cells, minimally alters gene expression, and minimally induces cytokine secretion. Further, while GRFT can bind some human cells, it still retains antiviral activity (Kouokam et al., 2011). These positive characteristics make GRFT a very promising microbicide candidate and underscore the importance of elucidating the biochemical details of the role of GRFT in HIV inhibition (Balzarini, 2007; Kouokam et al., 2011).

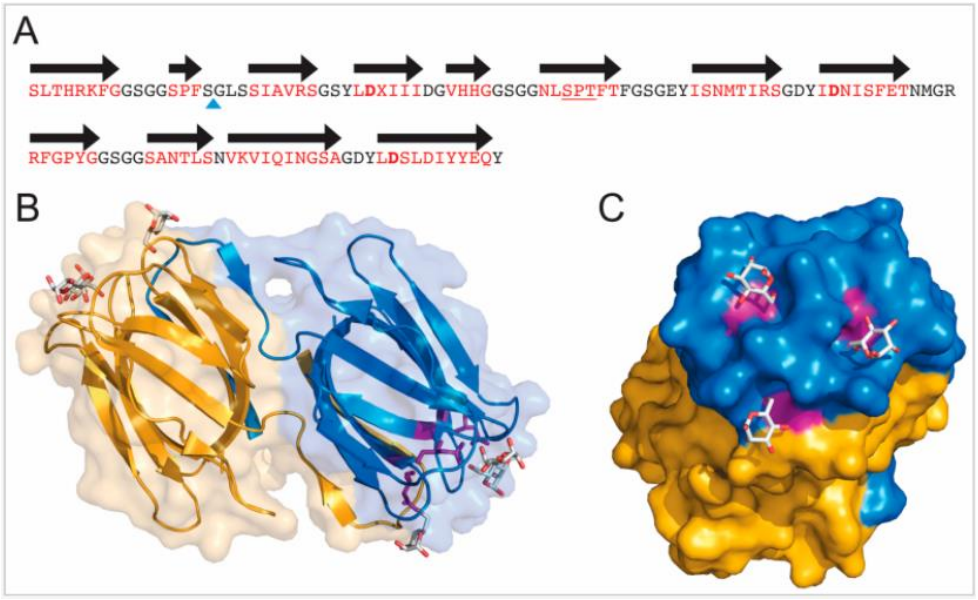
Structural characterization of GRFT showed it to be a domain swapped homodimer with six carbohydrate binding sites that bind to terminal mannose residues on N-linked glycans of the HIV envelope protein, gp120. the FIGURE 8 shows sequence and three-dimensional structure of griffithsin (Lusvarghi and Bewley, 2016a).

Griffithsin exists as a stable homodimer in which each subunit contains 121 amino acids (FIGURE 6A). Structures of GRFT in the absence of any ligand as well as in the presence of different monosaccharides and disaccharides have been solved by X-ray crystallography (Ziolkowska et al., 2006;Ziolkowska et al., 2007a;Ziolkowska et al., 2007b). The GRFT folds into a domain-swapped dimer (FIGURE 6B), where each subunit presents nearly perfect internal three-fold symmetry. The structure is composed by three

repeats of an antiparallel four-stranded β-sheet (Ziolkowska et al., 2006) that superficially resembles a β-prism-I motif found in other lectins of the jacalin family. Two out of 12 β-strands (16 amino acids) swap from one monomer to the other to form a β-prism of three four-stranded sheets. Each subunit of the homodimer griffithsin is capable of binding three monosaccharides. Each binding site is located in an equilateral triangle, with each site separated by approximately 15 Å (FIGURE 6C). Crystal structures with the monosaccharides mannose, glucose and N-acetylglycosamine, and the disaccharides 1-6α-mannobiose and maltose have been reported (Ziolkowska et al., 2006; Ziolkowska et al., 2007a;Ziolkowska et al., 2007b). When interacting with oligosaccharides, it has been shown that GRFT preferentially interacts with the terminal sugar (Ziolkowska et al., 2007a). Crystals with mannose have shown that all binding sites are almost identical and each one contains an aspartic acid (Asp) residue that makes extensive contacts with the sugar. These include Asp30, Asp70 and Asp112 that make hydrogen bonding interactions with O5 and O6 of mannose (Ziolkowska et al., 2006) (FIGURE 6C). Mutations of these residues to Ala do not affect the folding of the protein but weaken the binding to a mannose column (Xue et al., 2012).

FIGURE 6 - SEQUENCE AND THREE-DIMENSIONAL STRUCTURE OF GRIFFITHSIN (GRFT). (A) SEQUENCE OF WILD-TYPE GRFT. AMINO ACIDS LOCATED WITHIN BETA STRANDS ARE COLORED RED AND THE BLACK ARROWS CORRESPOND TO SECONDARY STRUCTURE. X REPRESENTS AN UNKNOWN AMINO ACID OF MASS 151.05 PRESENT IN THE NATURAL ALGA-DERIVED MATERIAL.

THE BLUE TRIANGLE SHOWS THE SITE OF THE GLY-SER INSERTION USED TO PRODUCE THE MONOMERIC VERSION OF GRFT (MGRFT). THE THREE ASP RESIDUES LOCATED IN THE THREE MANNOSE-BINDING SITES ARE SHOWN IN BOLD; (B) RIBBON DRAWING SHOWING THE THREE-DIMENSIONAL STRUCTURE OF GRFT. EACH MONOMER OF THE DOMAIN SWAPPED DIMER IS COLORED YELLOW OR BLUE. MANNOSE RESIDUES BOUND TO THE CARBOHYDRATE BINDING SITES ARE SHOWN IN STICK REPRESENTATION. ASPARTIC ACIDS PRESENT IN THE BINDING SITES ARE COLORED PURPLE; (C) ROTATED VIEW OF GRFT SHOWING THE CARBOHYDRATE BINDING FACE AND MANNOSE BINDING SITES



SOURCE: Iusyarghi and Bewley (2016a).

The HIV can infect cluster of differentiation 4 positive $(CD4)^+$ target cells that include T cells, macrophages, and dendritic cells (DC). The HIV infection can occur cell-free via infection by HIV particles or by cell–cell contact between HIV-1 infected T cells, or dendritic cells and non-infected $CD4^+$ target T cells at a virological synapse (Turville et al., 2008). These synapses are formed when high amounts gp120 present on the surface of HIV-infected cells bind the CD4 receptors on uninfected $CD4^+$ cells. This interaction triggers the same conformational changes needed for further co-receptor binding, followed by gp41 mediated membrane fusion.

It has been shown that GRFT is capable of inhibiting in vitro infection of clade A, B, and C viruses, with clade A being the least sensitive to GRFT (O'Keefe et al., 2009). Additionally, it was shown that the sensitivity to inhibition was highly dependent on the number of glycans on the surface of a gp120 (O'Keefe et al., 2009). It has been shown that single-cycle pseudo types (TZM-bl cell neutralization assay) are generally more

sensitive than neutralization in PMBC assays (Alexandre et al., 2010). The GRFT has been shown to inhibit the fusion between HIV-1 infected T cells and non-infected $CD4^+$ T cells (Mori et al., 2005). GRFT is also capable of preventing infection on cervical explants and cell-to-cell transmission on migratory cells from such explants (O'Keefe et al., 2009). Thus, GRFT has the ability to block HIV infection in numerous assays and platforms.

Interestingly, even though the monomeric GRFT had comparable affinity towards non amannoside, it showed decreased ability to bind to gp120 and even more dramatic decrease in inhibiting HIV infection in CEM-SS cells (Moulaei et al., 2010). This indicates that crosslinking of multiple high-mannose sugars is responsible for the high potency of the lectin GRFT.

The HIV transmission is believed to be mediated predominantly by transmitted/founder (T/F) viruses. These viruses show differential glycosylation when compared with chronic viruses. The effect of GRFT on T/F viruses has been measured and it has been found that there is a great variability on the IC_{50} values for GRFT against T/F viruses (Hu et al., 2015). Interestingly, even though there was no correlation between the number of possible *N*-linked glycosylation sites and the sensitivity to GRFT for T/F viruses, there seemed to be a correlation between the number and location of high-mannose glycan sites and the resistance to GRFT (Hu et al., 2015). Lastly, it has been shown that GRFT can block HIV-2 replication in MT-4 cell cultures with EC_{50}values in the sub-nanomolar range (Ferir et al., 2012).

CHAPTER I GENE-SILENCING SUPPRESSORS FOR HIGH-LEVEL PRODUCTION OF THE HIV-1 ENTRY INHIBITOR GRIFFITHSIN IN *N. BENTHAMIANA*

Abstract

The exploration of emerging host organisms for the economic and efficient production of protein microbicides against HIV is urgently needed in resource-poor areas worldwide. In this study, the production of the novel HIV entry inhibitor candidate, griffithsin (GRFT), was investigated using *N. benthamiana* as the expression platform based on a non-viral vector. To increase the yield of recombinant GRFT, the RNA silencing mechanism of *N. benthamiana* was abolished by using three viral suppressors. A transient expression system was used by transferring the *GRFT* gene, which encodes 122 amino acids, under the control of the enhanced *CaMV 35S* promoter. The presence of correctly assembled GRFT in transgenic leaves was confirmed using immunoglobulin-specific sandwich ELISA. The data demonstrated that the use of three gene silencing suppressors allowed the highest accumulation of GRFT, with a yield of 400 µg g-1 fresh weight, and this amount was reduced to 287 µg g-1 after purification, representing a recovery of 71.75%. The analysis also showed that the ability of GRFT expressed in *N. benthamiana* to bind to glycoprotein 120 is close to that of the GRFT protein purified from *E. coli.* Whole-cell assays using purified GRFT showed that our purified GRFT was potently active against HIV. This study provides the first high-level production of the HIV-1 entry inhibitor griffithsin with a non-viral expression system, and illustrates the robustness of the co-agroinfiltration expression system improved through the use of three gene silencing suppressors.

Key words: GRFT, HIV-1, *N. benthamiana*, *E.coli*, agroinfiltration, silencing suppressors

1 INTRODUCTION

AIDS disease has been considered a major threat to global public health since it was first deciphered in 1981 in the United States (Barre-Sinoussi et al., 1983). According to the World Health Organization, approximately 36.7 million people were living with HIV at the end of 2015 (WHO), (http://www.who.int/mediacentre/ factsheets/fs360/en/). HIV remains the leading cause of death in sub-Saharan Africa and was responsible for the death of 1,1 million people throughout the world in 2105 [World Health Organization; Global Health Observatory (GHO) data, The Top 10 Causes of Death; http://www.who.int/mediacentre/factsheets/fs310/en/ (accessed March 31, 2017)]. The current predictions suggest that 5,753 people will become infected with HIV every day, and this corresponds to approximately 240 people every hour. Macrophages, T cells, and dendritic cells are the preferred destination of HIV.

The viral protein gp120 binds to the CD4 receptor to initiate a series of conformational changes in gp120 that induce fusion of the virus with the host cell membrane. The viral infection progresses by transformation of the viral RNA genome into a proviral DNA, and the integrated provirus is eventually transcribed by cellular RNA polymerases into a set of mRNAs that generate progeny genomic mRNA and spliced mRNA encoding viral proteins (shors, 2011).

The production and manufacturing of HIV entry inhibitors via recombinant DNA technology has been reported (O'Keefe et al., 2009). Despite the discovery of potent HIV prophylactics, their high production costs have prevented the development of corresponding manufacturing processes in the resource-poor areas of the world. Griffithsin is a lectin HIV entry inhibitor that targets the terminal mannose residues on HIV N-linked glycan as long as the HIV is in contact with GRFT. GRFT was structurally characterized by (Moulaei et al., 2010; Fuqua et al., 2015). The anti-activity of GRFT against red algae has been estimated to have an EC_{50} value of 40 pM (Veazey et al., 2003; Veazey et al., 2008). The antiviral activity of GRFT, the fact that it lacks *in vitro* and *in vivo* toxicity, and its environmental stability, such as its stability in media with a broad range of pH values and temperatures, even temperatures close to the boiling point,

indicate that GRFT is a good anti-HIV microbicide candidate (Emau et al., 2007; Kouokam et al., 2011).

The large-scale production of high-quality GRFT is required for the development of this recombinant protein as an anti-HIV microbicide. As a result, plant and bacterial platforms have been used for the expression and accumulation of GRFT (Vafaee et al., 2014; Fuqua et al., 2015). The formation of insoluble inclusion bodies (33%), the high manufacturing cost and the presence of bacterial endotoxin have been considered factors that limit the production of GRFT using an *E. coli* system (Giomarelli et al., 2006).

Moreover, the production of recombinant GRFT has been reported in *N. benthamiana* using a vector based on tobacco mosaic virus (TMV) (O'Keefe et al., 2009) and transgenic seeds of *O. sativa* (Vamvaka et al., 2016a). Regardless of these findings, the high cost of *in vitro* RNA transcription is a marked problem with virus-based system and also the purification of GRFT based on tobacco mosaic virus (TMV) is considered to show some contamination with TMV coat protein and protein degradation (Fuqua et al., 2015).

In addition, seed-based expression systems have been shown to exhibit a high degree of versatility regarding the production of recombinant proteins due to protein stability at ambient temperature (Obembe et al., 2011), but the long-term process of seed production and space requirements might be preclusive (Boothe et al., 2010). In this study, we used a transient expression system based on a non-viral vector to improve the large-scale production of GRFT.

However, the most efficient method for the high-level expression of heterologous protein is a transient expression system, based on vectors with RNA plant virus due to the ability of RNA viruses to replicate to high titres within infected cells. However, this method cannot be used for the insertion of foreign genes without affecting replication and compromising the fidelity of the transcripts. It is due to the lack of RNA-dependent, RNA polymerases for proofreading and the movement of viral replicons throughout the plant, resulting in biocontamination problems and yielding undesirable features (Ahlquist et al., 2005; Castro et al., 2005; Sainsbury and Lomonossoff, 2008).

In comparison with the viral system of agroinfiltration, the non-viral system has better cost-effectiveness, is more widely available, results in significantly lower induction

of the immune system and has no limitation in terms of the size of transgenic DNA. In addition to its speed and high yield, the transient expression platform presents versatility in terms of the expression and accumulation of personalized recombinant proteins, including therapeutics for patient-specific cancers and vaccines for viruses that show rapid antigenic drift and/or multiple strains with unpredictable outbreaks (Nayerossadat et al., 2012; Chen and Lai, 2015).

However, restricted protein production based on transient expression system remains a significant drawback in term of economic production. In this context, the RNA silencing is major limitation for expression of genes by *Agrobacterium*-mediated transient system as the foreign nucleic acids recognized and subsequently degraded through mRNA stability process or translational levels (Johansen and Carrington, 2001b).

RNA silencing (also known as post-transcriptional gene silencing) is considered as adaptive defense mechanism by eukaryotic whereby foreign transgenes and/or viruses eliminate through highly conserved mechanism. This mechanism starts with formation of the double stranded (ds)RNA and subsequently processing of dsRNA to small (s) 20–26-nt dsRNAs with staggered ends, and finally terminate with inhibitions of selected sRNA strand within effector complexes acting on partially or fully complementary RNA or DNA (Brodersen and Voinnet, 2006).

However, this mechanism can be inhibited by RNA silencing suppressors. In this case, viruses are developed as beneficial tool to counteract gene silencing by viral protein (Incarbone and Dunoyer, 2013). RNA silencing suppression activity of viral protein of virus stems from its ability to bind siRNA as well as proteins involved in antiviral silencing (Baumberger, et al. 2007; Bortolamiol, et al. 2007; Deleris, et al. 2006; Lakatos, et al. 2006; Mérai, et al. 2006). The P19 suppressor from the Tomato bushy stunt virus (TBSV) has been widely used to boost transient or constitutive expression level of recombinant proteins in the plant as it selectively inhibits the 21-nt and 22-nt classes of siRNA and also reduce the availability of free siRNA for RISC loading (Arzola, et al. 2011; Lacombe, et al. 2017).

In the present study, a high production level of recombinant GRFT was obtained from transgenic plants seven days after gene delivery. Our transient expression significantly shortened the timeline of GRFT production, i.e., seven days compared with

12 days, as was previously reported. Additionally, in comparison with a stable transgenic seed of *O. sativa*, our transient assays with agroinfiltration showed high levels of transgene expression. Therefore, the short timeline and higher production of GRFT based on our transient expression could be considered advantageous for GRFT development and commercialization.

2 OBJECTIVES

Recombinant production of anti-HIV protein, griffithsin, using transient expression in *N. benthamiana*

2.1 SPECIFIC OBJECTS

(a) Agroinfiltration of *GRFT* transgene by syringe method in intercellular space of *N. benthamiana* leaf.

(b) Co-agroinfiltration of three gene silencing suppressors (P19, P1 and P0) to boost GRFT expression in *N. benthamiana.*

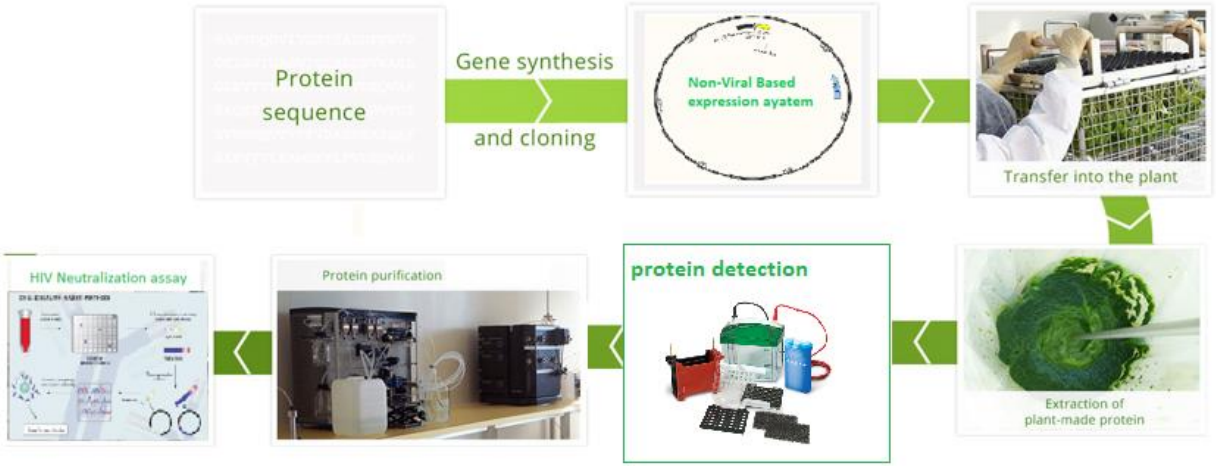
(c) Purification of GRFT produced in *N. benthamiana*.

(d) *In vitro* gp120-binding assay of produced GRFT by *N. benthamiana*.

(e) Whole-cell HIV neutralization assays of GRFT produced by *N. benthamiana*.

3 MATERIAL AND METHODS

3.1 FLOW CHART OF EXPERIMENTS DESIGN



SOURCE: Habibi (2018).

3.2 *IN SILICO* ANALYSIS

3.2.1 Sequence retrieval

DNA sequences of *GRFT* gene was obtained from NCBI website (http://www.ncbi.nlm.nih.gov/gene) (accession number FJ594069). The resulting sequence was submitted to EXPAY Translate tool (http://web.expasy.org/translate/) to achieve protein sequence with all possible open reading frames.

3.2.2 Functional characterization of GRFT

Physico-chemical properties like molecular weight, theoretical IEP (isoelectric point), amino acid composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index, and grand average of hydropathicity (GRAVY) were calculated for GRFT using EXAPASY ProtParam server (http://web.expasy.org/protparam/).

3.2.3 mRNA structure prediction

The mRNA structure was predicted using (http://www.bioinfo.rpi.edu/applications/mfold) (Zuker, 2003).

3.2.4 Codon usage optimization

The codon usage of a DNA sequence of GRFT was optimized using *N. benthamiana* codon usage table (http://www.kazusa.or.jp/codon/) by http://genomes.urv.es/OPTIMIZER/(Puigbò et al., 2007).

3.2.5 Signal peptide prediction

The presence and location of signal peptide cleavage sites in amino acid sequence of GRFT was performed by http://www.cbs.dtu.dk/services/SignalP/(Nielsen, 2017).

3.3 VECTOR CONSTRUCTION

369 bp sequence encoding *GRFT*(accession number FJ594069) was optimized using *N. benthamiana* codon usage table and synthesized by Epoch Life Science Inc (Missouri City, Texas, USA) and then cloned into the pBin61 plant expression vector under the control of double enhanced *Cauliflower mosaic virus* (*CaMV*) *35S* promoter and the nopaline synthase (NOS) terminator sequence. A hexahistidine tag (His) was added to C-terminal of *GRFT* gene to for purification of recombinant GRFT by a single-step affinity chromatography.

3.4 COMPETENT CELL PREPARATION

For preparation of chemically competent cells, a portion from the top of the frozen glycerol stock of *E. coli* strain DH5 alpha was scrape off and subsequently streaked it onto the Luria Bertani medium plate. The plate was incubated at 37ºC overnight. Next day, a single colony was picked into 5 ml of LB medium and was inoculated the culture overnight at 37ºC with shaking at 250 rpm,On day 3, 100 ml LB medium with 1 ml of saturated overnight culture was inoculated and shaked at 37ºC until OD600=0.4. The cell

was placed in an ice bath for 10 minutes and pre-cool solution then was centrifuged at 2700x g for 10 min at 4ºC. The supernatant was discarded and the cell pellet was re-suspended with 1.6 ml ice cold 100 mM CaCl2 by swirling on ice gently. The cell pellet was incubated on ice for 30 min and centrifuged at 2700x g for 10 min at 4ºC. The supernatant was discarded and the cell pellet was re-suspended with 1.6 ml ice-cold 100 mM CaCl3 by swirling on ice gently and incubated on ice for 20 min. In the next step, cells were combined to one tube and added 0.5 ml ice-cold 80% glycerol and swirled to mix. Finally, 100 µl aliquots was froze in liquid nitrogen and stored in -80C.

3.5 TRANSFORMATION OF CHEMICALLY COMPETENT E. COLI

For the transformation of chemically competent cell (strain DH5 alpha), 100 µl of aliquot of cells was thawed on ice, mixed with 5–10 µl of ligation product (cf. 2.2.8) and incubated on ice for 30 min. After 45 sec heat shock at 42℃, the cells were chilled on ice for 3 min. Subsequently, 250 µl of LB medium were added and the tube was incubated at 37 ℃, 300 rpm for 1 h. 50–150 µl of cell suspension were plated onto pre-warmed LB ampicillin (100 µg/ml) plates, which were incubated at 37 ℃ overnight.

3.6 COLONY PCR CONFIRMATION

Bacterial suspensions were prepared from *E. coli DH5* alpha pure cultures and non-transformed bacterial. Suspensions were centrifuged at 12 000 *g* for 5 min and supernatant fluids were removed. Pellets were re-suspended in water and heated at 95 °C for 5 min. Samples were centrifuged again at 12 000 *g* for 2 min and supernatant fluids were collected to be used for PCR amplifications. The PCR reaction was performed based on (TABLE 1.1).

TABLE 1.1 – PCR REAGENTS USED TO AMPLIFY THE GRFT GENE

3 µl	10x PCR reaction buffer
1 µl	forward primer (10 µM)
1 µl	reverse primer (10 µM)
0.3 µl	dNTPs (10 mM each, Fermentas)
0.2 µl	Taq-polymerase
2 µl	template DNA
ad 30 µl	dH_2O

SOURCE: Habibi (2018)

DNA was amplified by specific primers for *GRFT* gene (forward primer: 5'-ATGTCTCTTACTCACAGGAAGTT-3' and Reverse primer: 5′-GTACTGCTCGTAGTAGATATCA-3′) under following condition (TABLE 1.2).

TABLE 1.2 - PCR THERMAL CYCLING CONDITIONS

Step	Temperature	duration	Cycles
Initial denaturation	94°C	3 min	1
Denaturation	94°C	30 s	30
Annealing	59°C	30 s	30
Elongation	72°C	1 min	30
Final elongation	72°C	5 min	1
Storage	4°C	Hold	∞

SOURCE: Habibi (2018).

3.7 PLASMID ISOLATION (PLASMID MINIPREP)

A single colony from a freshly streaked selective plate and was picked and, then inoculated a culture of 5 ml LB medium containing the appropriate selective antibiotic. The plates were incubated for 12–16 h at 37°C with vigorous shaking. On the next day, the bacterial cells were harvested by centrifugation at 6800 x g in a conventional, table - top microcentrifuge for 5 min at room temperature. The pellet was re-suspended in 200 µL solution 1 (TABLE 1.3) and then vortexed to ensure cells are completely re-suspended. There should be no visible clumps. Cells were lysed by adding 200 µL

solution B2 (TABLE 1.3) and the tube inverted immediately and gently 5–6 times until color changes to dark pink and the solution is clear and viscous. The cells were incubated for one minute at the environment. The lysate was neutralized by adding 400 µL of solution 3 (TABLE 1.3) and the tube was inverted until color is uniformly yellow and a precipitate form. The lysate was clarified by spinning for 2–5 min at 16,000 x g. the supernatant was transfer to the spin column and centrifuged for 1 min. Then, flow-through were discarded. Column was re-inserted in the collection tube and added 200 µL of Plasmid Wash Buffer 1 to it. Plasmid Wash Buffer 1 removes RNA, protein and endotoxin. The mixture was centrifuged for 1 min and discarded the flow-through and added 400 µL of Plasmid Wash Solution 2 and centrifuged for 1 min. The column was transferred to a clean 1.5 ml microfuge tube. Finally, was added ≥ 30 µL DNA Elution Buffer to the center of the matrix. Waited for 1 min, then spin for 1 min, to elute DNA.

TABLE 1.3 - LIST OF NEEDED SOLUTIONS FOR PLASMID DNA EXTRACTION.

Solution 1	**Solution 2**	**Solution 3**	**Plasmid wash solution 1**	**Plasmid wash Solution 2**	**DNA Elution Solution**
50 mM glucose	0.2 N NaOH	5 M potassium acetate	Guanidine/isopropanol-based wash buffer	Ethanol-based wash buffer	10 mM Tris
25 mM Tris-Cl (pH 8.0)	1% (w/v) SDS.	Glacial acetic acid	-	-	0.1 mM EDTA
10 mM EDTA (pH 8.0).		H2O	-	-	-

SOURCE: Habibi (2018).

3.8 GEL ELECTROPHORESIS OF DNA

Separation of DNA fragments was performed by agarose gel electrophoresis in 1x TAE buffer. According to the expected size of the fragment, 1–2 % agarose gels were prepared using1xTAE buffer (40 mM Tris base, 1mM EDTA). For subsequent detection of DNA, the fluorescent dye ethidium bromide was added to a final concentration of 0.5 µg/ml. Prior to loading into the gel, samples were mixed with 4x DNA-loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 0.1M EDTA and 30% glycerol). For size determination of DNA-fragments, appropriate DNA ladders (Invitrogen) were used. The

separation of DNA-fragments was visualized under UV light using Gel Doc Universal Hood II with TLUM 100/240V (Bio-Rad).

3.9 *AGROBACTERIUM* COMPETENT CELL PREPARATION AND ANSFORMATION BY ELECTROPORATION METHOD

3.9.1 Preparation of electro-competent *agrobacterium* cells

Two glass culture tubes (16 × 125 mm) each containing 2 mL liquid medium, LB, with single colonies of the *Agrobacterium* strain GV3101 was inoculated with antibiotic 50 µg/ml rifampicin and 50 µg/ml gentamycin in the growth medium. The strain of GV3103 has resistance to Rifamcin and gentamycin antibiotics. The cells were grown overnight at 25–28°C with shaking to aerate. The overnight cultures were used to inoculate two 1-L flasks each containing 200 mL of, LB, with necessary antibiotic. Cells were incubated at 25–28°C with shaking for good aeration (220–250 rpm) until the OD600 of the culture arrived between 0.5 and 1.5. Cells were chilled on ice and transferred to four 250-mL sterilized centrifuge bottles. Cells were centrifuged at 10,000g for 10 min at 4°C and then supernatant was discarded. Pellet cells were washed with 20 mL per bottle of ice-cold sterile water and vortexed well to resuspend cells. Pellet cells were centrifuged at 10,000g for 10 min at 4°C and supernatant was removed. This washing step was repeated one more time. The wash step was repeated using ice-cold 10% glycerol instead of water. Cells were re-suspended in 400 to 800 µL cold 10% glycerol and finally 40-µL aliquots of the competent cells was placed into individual tubes and stored at –80°C.

40 µL of these competent cells were mixed with approximately 1 µg plasmids DNA (pBin61:GRFT) (< 10 µL volume) and electroporated in a 0.1-cm cuvet (ice-chilled) using a Bio-Rad Gene pulser set to parameters 12.5 Kv/cm, 25 µF, 400 ohms. Prior to plating onto agar medium, supplemented with antibiotics, cells were diluted with 1mL liquid medium and grown for 1 h at 28 ºC in an orbital incubator. Rifampicin and gentamicin are *A. tumefaciens* (GV3101) resistance markers, which insure all transformed *agrobacterium* contained pBin61 vector. Transformed *A. tumefaciens* was screened in selective LB medium supplemented with 50 mg l^{-1} kanamycin. *A. tumefaciens*

harboring pBin61:p19; pBin61:p0 and pCAMBIA 1300:p1 were kindly provided by Dr. Severine Lacombe (Institut de Recherche pour le Développement, Montpellier, France)

3.9.2 Plasmid isolation (plasmid miniprep)

Plasmid miniprep from *agrobacterium* was performed based on 1.3.7 section.

3.9.3 PCR amplification from extracted plasmid

To confirm plasmid orientation with target GRFT gene, the extracted plasmid from *A. tumefaciens* (GV3101) was subjected to PCR analysis based on (TABLE 1.1).

DNA was amplified by specific primers for GRFT gene (forward primer: 5'-ATGTCTCTTACTCACAGGAAGTT-3' and Reverse primer: 5′-GTACTGCTCGTAGTAGATATCA-3′) based on (for detail see TABALE 1.2)

3.10 PLANT GROWTH

Almost 200 *N. benthamiana* seeds were planted into a germination tray for 2 weeks. After seed germination, 60 pots were placed into a propagation tray and added 2 *N. benthamiana* seedling into each pot and allowed them to growth in a 25 °C and 84% humidity environment with a 16/8 h day/night cycle. After two weeks, germinated plants were transferred into big pots and to a new growth tray that host plants to provide adequate space for further growth and plants growing were continue in a 25 °C, 50% humidity environment with a 16/8 h day/night cycle, until they are ready to be infiltrated at 6 weeks of age.

3.11 *AGROBACTERIUM* PREPARATION

The *A.tumefaciens* GV3101 strains containing the vectors pBin61: GRFT, pBin61:P19, pBin61:P0 and pCAmbia1300:P1 on LB agar plates containing kanamycin were streaked (100 µg/ml). One strain per plate was streaked and grown at 28 °C for 48 hr. From a single colony on the LB agar plate, inoculate GV3101 strains harboring gene transgene GRFT and silencing vectors was inoculated into 3 ml of LB media with

kanamycin (50 µg/ml), rifamycin (50 µg/ml) and gentamicin (50 µg/ml) in a 15 ml round-bottom culture tube, grown the liquid culture at 28 °C in a shaker overnight with a 250 rpm rotation rate. 30 µL of the overnight culture was transferred to 30 ml of LB media with appropriate antibiotics for each strain. The new culture was grown overnight at 30 °C in a shaker with a 250 rpm rotation rate until the OD_{600} value is in the range of 1-2.0. OD_{600} values for each liquid culture were measured and used the formula below to calculate the necessary volume (V_{inf}) to be diluted in 50 ml of infiltration buffer to give a final OD_{600} of 0.5 for each strain. V_{inf} (ml) = (40 ml) x (0.5) / OD_{600}. Transferred V_{inf} (ml) of each culture to a microcentrifuge tube and spin down the cells by centrifugation at 12,000 x *g* for 2 min, removed supernatant and then resuspended the cells in 10 ml infiltration buffer (10 mM $MgCl_2$, pH 5.5 and 100µM acetosyringone) by vortex briefly. Resuspended cells of pBin61: GRFT+ pBin61:P19+ pBin61:P0+ pCAmbia1300:P1 (TABLE 1.4) were mixed and centrifuged the cells at 12,000 x g for 2 min, and resuspended in total of 40 ml of infiltration buffer.

TABLE 1.4 - PROCEDURE OF MIXING *AGROBACTERIUM* HARBORING GENE OF INTEREST AND GENE SILENCING SUPPRESSORS.

Bacterial culture	Control	GRFT construct
Supressor1 # P0	3.3 ml	6.6 ml
Supressor2 # P1	3.3 ml	6.6 ml
Supressor3 # P19	3.3 ml	6.6 ml
pBin61	10 ml	-
pBin61+GRFT	-	20
Tween	0.015% 0.03%	0.015% 0.03%

SOURCE: Habibi (2018).

3.12 SYRINGE INFILTRATION

6-week old *N. benthamiana* plants with five leaves each were selected. The first three leaves counting from the top of each plant were infiltrated entirely with combinations of *A. tumefaciens* strains (pBin61: GRFT +P19+P0+P1) in infiltration buffer. 10 plant were infiltrated with combinations of negative controls (pBin61+P19+P0+P1). We also assayed the effect of Tween-20 at a concentration of 0.015 % and 0.03 % without any suppressor application in syringe co-agroinfiltration method for studying of expression efficiency.

A firm hold of the front side of the leaf was taken and while applying gentle counter pressure to the nick with the thumb of one hand (FIGURE 1.1A), injected the *Agrobacterium* mixtures in infiltration buffer into the intercellular space with a syringe without a needle (FIGURE 1.1B). Note: As the *Agrobacterium* mixture enters the intercellular space of the leaf, the infiltrated area will turn visibly darker green (FIGURE

1.1C). The injection of the *Agrobacterium* mixtures into the spot was continued until the darker green circle stops to expand.

FIGURE 1.1 - SYRINGE INFILTRATION OF *N. BENTHAMIANA* LEAVES WITH *A. TUMEFACIENS*. THE STRAIN GV3101 OF *A. TUMEFACIENS* HARBORING PBIN61: GRFT +P19+P0+P1 AND PBIN61 +P19+P0+P1 VECTORS WERE RESUSPENDED IN INFILTRATION BUFFER AND LOADED INTO A SYRINGE WITHOUT A NEEDLE. A FIRM HOLD OF THE FRONT SIDE OF THE LEAF WAS TAKEN 6-WEEK OLD PLANT LEAF (A). THE OPENING OF THE SYRINGE WAS POSITIONED AGAINST THE A LEAF SPOT (B) AND AGROBACTERIA IN INFILTRATION BUFFER WERE INJECTED INTO THE INTERCELLULAR SPACE OF THE LEAF (C).

SOURCE: Habibi (2018).

3.13 EXTRACTION OF TOTAL SOLUBLE PROTEIN

The *N. benthamina* leaf samples were harvested at seven days post agroinfiltration and snap frozen in liquid nitrogen. Transgenic leaves were homogenized by Tissue Lyser Adapter Set 2 x 24 (QIAGEN, Germany), according to the recommended kit protocol. We used modified protocol for preparation of protein extraction buffer which described by (Sarnighausen et al., 2004), previously. Briefly, 400 mg of grounded material were extracted with 2 ml ice cold 20 % TCA/aceton. Samples were standing at -20 ºc for 30 min and were subsequently centrifuged at 10,000 g for 20 min at 0 ºC. The flow-through was discarded and the pellet was gently resuspending on 2ml of ice cold acetone. The washing process with acetone was performed two times at -20˚C for 30 min and centrifuged at 14,000g for 20 min at 0˚C. Finally, the collected pellet was air-dried and total protein were extracted by 700 µl of protein extraction buffer (0.2M sodium acetate, 0.5 M sodium chloride (PH 6.8), 50 mM ascorbic acid, 20 mM sodium metabisulfite, 1mM DTT, and 1mM PMSF).

3.14 TOTAL SOLUBLE PROTEIN QUANTIFICATION

Protein concentration was assayed based on (Bradford, 1976) method using SpectraMax® 190 Absorbance Plate Reader (Molecular Devices, USA).

3.15 SDS-POLYACRYLAMIDE (SDS-PAGE) GELS

Protein extracts were mixed with 5 x loading buffer including 200mM Tris (PH 6.8), 10% glycerol, 10% SDS, 10 mM Dithiothreitol, 0.05% Bromophenol Blue and samples were heated in 95°C for 10 min and then separated by 15% glycine-SDS page at 0.02 mA and 80V by Protean II Minigel Apparatus (BioRad). A BenchMark Protein Ladder (Thermo Fisher Scientific) was used to estimate the size of the resulting bands. SDS- polyacrylamide gels (TABLE 1.5) were visualized using Coomassie blue solution or used for Western blot analysis.

TABLE 1.5 - RECIPE SUFFICIENT FOR POLYACRYLAMIDE GELS.

Chemical	**Stacking gel (6 %)**	**Resolving gel (15%)**
dH_2O	2.6 ml	1.8 mL
Acrylamide 30%	1 ml	4 mL
Tris buffer	2 ml 1.5M Tris pH 8.8	1.25 ml 0.5M Tris pH 6.8
10% SDS	50 µl	80 µl
10% APS	50 µl	80 µl
TEMED	5 µl	8 µl

SOURCE: Habibi (2018).

3.16 WESTERN BLOT ANALYSIS

The nitrocellulose membrane (Amersham Protran 0.45 NC 300mm×4m, GE Healthcare Life Sciences) and paper filters were incubated in 5 ml transfer buffer (Tris-based 125 Mm (pH 8.3), Tricin 960 Mm), 35 ml H20 and 10 ml methanol for 15. Finally, proteins were transferred electrophoretically using Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad, USA) at constant condition 2 mA/cm^2 for 25 min. Next, the transferred proteins

to nitrocellulose membrane were blocked overnight at room temperature in 3% non-fat dry milk, Tris-buffered saline (TBS) (50 mM Tris, 150 mM NaCl, pH 7.4) containing 0.05% Tween (TBST). In the next day, after three washing with TBST, the membrane was probed with primary anti-GRFT rabbit polyclonal antibody (National Cancer Institute-Frederick Cancer Research and Development Center, USA) with dilution at 1:1000 in blocking solution on an orbital shaker at room temperature for 4 h at room temperature. The membrane was washed three times for 15 min with TBST buffer, then incubated with the secondary antibody (horseradish peroxidase (HRP)- conjugated) diluted 1:5,000 in blocking solution on an orbital shaker at room temperature for 60 min.

Finally, the membrane was developed with developing buffer (CAS No. Nr77861, Bio-Rad, USA), buffer A (10mM HEPES, 1.5mM MgCl2, 10mM Kcl, 0.5mM DTT, and 0.05% NP-40, pH 7.9) and buffer B (5mM HEPES, 1.5mM MgCl2, 0.2 mM EDTA, 0.5mM DTT, 26% glycerol) prior to signal detection based on the manufacturer's instructions.

3.17 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

To determine the accumulation of GRFT in transgenic plants, ELISA protocol was performed as described previously (Mori et al., 2005). In summary, a 96 well plate was coated by 100 ng of extracted protein from transgenic plants and incubated in carbonate/bicarbonate buffer (15 mM Na2CO3 and 35 mM NaHCO3, pH 9.6) overnight at 4°C. Wells were rinsed three times with PBS containing 0.1% Tween-20 (PBST) and then blocked with 1% bovine serum albumin (BSA) for 2 h at 37°C. Plates were added with primary anti-GRFT rabbit polyclonal antibody with dilution of 1:1,000 for 2:30 h at 37°C and then was washed 3 times with PBST. The wells were incubated with the secondary horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody with dilution of 1:5,000 for 1 h in 1% PBST. Finally, after washing 3 times with PBST, the substrate 3,30,5,50-tetramethylbenzidine (TMB) in buffer acid citric /sodium phosphate dibasic (pH 5) and H_2O_2 (30 %) was added to the reaction and incubated at least for 15 min in the dark and the reaction was stopped with 5 M H_2SO_4 and the absorbance was read on OD 450 nm.

3.18 PURIFICATION BY AFFINITY CHROMATOGRAPHY

Transgenic leaves of *N. benthamiana* were grounded in liquid nitrogen and extracted at 4 °C into lysis buffer (50 mM sodium phosphate, 50 mM ascorbic acid, 10 mM di-sodium EDTA, 1 mM PMSF, pH 7.4). The insoluble material was removed by centrifuging at 14000 rpm for 30 min at 4 °C, and the extract was passed through a 0.45 µm filter and loaded on a Profinity IMAC column at the rate of 1 ml/minute. The column was washed with washing buffer (50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole, pH 7.4). The GRFT protein was eluted from the column for two times by elution buffer (50 mM NaH2PO4, 300 mM NaCl, 500 mM imidazole, pH 7.4). Fractions containing GRFT were mixed and concentrated by ultrafiltration by spin columns with a 10 kDa molecular weight cut-off (Amicon Ultra, EMD Millipore, Darmstadt, Germany). The purity of concentrated GRFT was confirmed by SDS-PAGE and western blot analysis (for detail see item 1.3.15 and 1.3.16).

3.19 *IN VITRO* GP120-BINDING ASSAY

To detection of specific antigen-binding activity of GRFT, 100 ng of HIV-1 gp120 Protein (group M, subtype CRF07_BC) (Sino Biological Inc. China) was coated on the 96 wells of ELISA plates and was incubated overnight at 4° C. The plates were washed with PBST-0.05% for 3 times and were blocked with 1% bovine serum albumin (BSA). After washing with PBST-0.05%, serial dilutions of plant- produced GRFT were added to plates and incubated with GRFT anti-rabbit primary antibody (1:1,000 in PBST), and HRP anti-rabbit secondary antibody (1:2,000 in PBST). Plates were washed with PBST, and the TMB substrate was added to each well. The reaction was stopped with 5M H2SO4 and the absorbance was read on OD450 nm.

3.20 WHOLE-CELL HIV NEUTRALIZATION ASSAYS

A whole-cell cytopathicity assay was conducted to evaluate the protective effect of the GRFT preparations as described previously but modified for a 384-well assay plate format (Gulakowski et al., 1991). Briefly, a quantity of 2,000 exponentially growing CEM-

SS cells, maintained in RPM1 1640 medium (Lonza) without phenol red and supplemented with 5% fetal bovine serum (FBS) (Hyclone), 2 mM t-glutamine and 50 µg/ml gentamicin (Gibco), were combined with serial dilutions of GRFT in assay medium and incubated with or without HIV-1_{RF} in a final volume of 50 µl. After six-days incubation, cellular viability was assessed spectrophotometrically by measuring the reduction of XTT to the chromogenic formazan product at 450 nm. Experiments were carried out in quadruplicate. The tetrazolium reagent XTT was kindly supplied by the Developmental Therapeutics Program at the NCI-Frederick. The CEM-SS cell line was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: CEM-SS Cells from Dr. Peter L. Nara (Foley et al., 1965; Nara et al., 1987; Nara and Fischinger, 1988). To evaluate functional activity of NT-GRFT- 450 mM NaCl at ambient temperature, purified NT-GRFT- 450 mM NaCl in PBS solution was subject to lyophilization process by Alpha 1-4 LD plus freeze-dryer (Martin Christ, Germany) based on manufacturing instruction and whole – cell cytophathicity assay was conducted for lyophilized GRFT (Lyo-GRFT-450 mM NaCl).

4 RESULTS

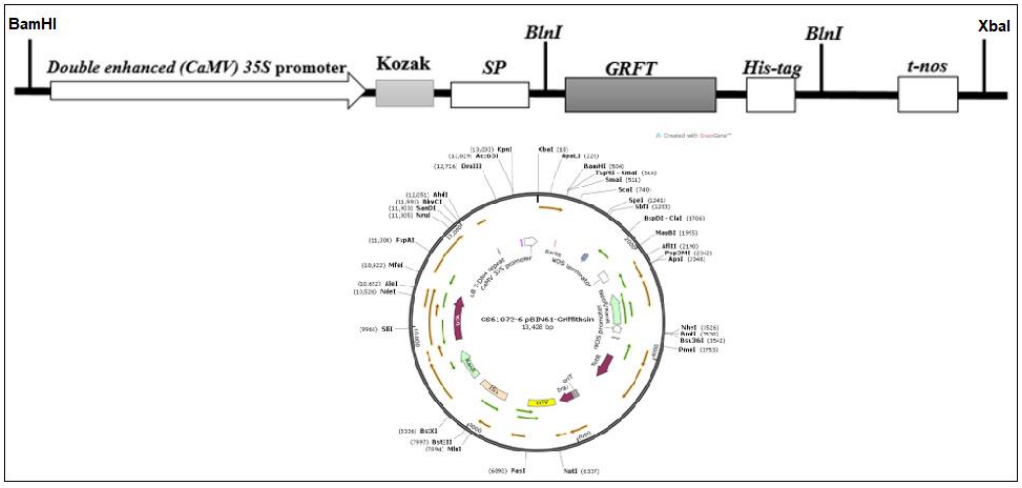
The 369 bp sequence encoding *GRFT* (accession number FJ594069) was optimized using *N. benthamiana* codon usage table (FIGURE 1.2) and then synthesized by Epoch Life Science Inc (Missouri City, Texas, USA). The sequence encoding GRFT was cloned into the pBin61 plant expression vector under the control of double enhanced *Cauliflower mosaic virus* (CaMV) 35S promoter and the nopaline synthase (NOS) terminator sequence (FIGURE 1.3). Subcellular localization prediction for GRFT gene was performed by signal peptide prediction of thaumatin-like protein To32. In the case of GRFT, the *in silico* targeting prediction was confirmed in the secretory pathway (FIGURE 1.4).

FIGURE 1.2 - OPTIMIZATION OF THE CODON USAGE OF A *GRFT* DNA SEQUENCE TO INCREASE ITS EXPRESSION LEVEL CAI: CODON ADAPTATION INDEX IS EFFECTIVE MEASURE OF SYNONYMOUS CODON USAGE BIAS. THE INDEX USES A REFERENCE SET OF HIGHLY EXPRESSED GENES FROM A SPECIES TO ASSESS THE RELATIVE MERITS OF EACH CODON, AND A SCORE FOR A GENE IS CALCULATED FROM THE FREQUENCY OF USE OF ALL CODONS IN THAT GENE. THE INDEX ASSESSES THE EXTENT TO WHICH SELECTION HAS BEEN EFFECTIVE IN MOULDING THE PATTERN OF CODON USAGE. ENC: EFFECTIVE NUMBER OF CODONS. % GC: G+C PERCENTAGE. % AT: A+T PERCENTAGE.

Type	Sequences	CAI	ENc	%GC	%AT
Query	ATGTCTCTTACTCACAGGAAGTTCGGAGGATCTGGAGGTTCTCCATTCTCTGGACTTTCT TCTATTGCTGTGAGGAGTGGATCTTACCTTGATGCTATTATTATTGATGGAGTGCACCAC GGAGGAAGTGGTGGAAATCTTTCTCCAACTTTCACTTTCGGATCTGGAGAGTACATTTCT AACATGACTATTAGGAGTGGAGATTACATTGATAACATTTCTTTCGAGACTAACATGGGA AGGAGATTCGGACCATACGGAGGTTCTGGAGGATCTGCTAACACTCTTTCTAACGTGAAA GTGATTCAGATTAACGGATCAGCTGGAGACTACCTTGATTCTCTTGATATCTACTACGAG CAGTACTGA	0.871	22	42.5	57.5
Optimized	ATGTCTCTTACTCATAGAAAGTTTGGTGGTTCTGGTGGTTCTCCTTTTTCTGGTCTTTCT TCTATTGCTGTTAGATCTGGTTCTTATCTTGATGCTATTATTATTGATGGTGTTCATCAT GGTGGTTCTGGTGGTAATCTTTCTCCTACTTTTACTTTTGGTTCTGGTGAATATATTTCT AATATGACTATTAGATCTGGTGATTATATTGATAATATTTCTTTTGAAACTAATATGGGT AGAAGATTTGGTCCTTATGGTGGTTCTGGTGGTTCTGCTAATACTCTTTCTAATGTTAAG GTTATTCAAATTAATGGTTCTGCTGGTGATTATCTTGATTCTCTTGATATTTATTATGAA CAATATTGA	1.000	20	32.5	67.5

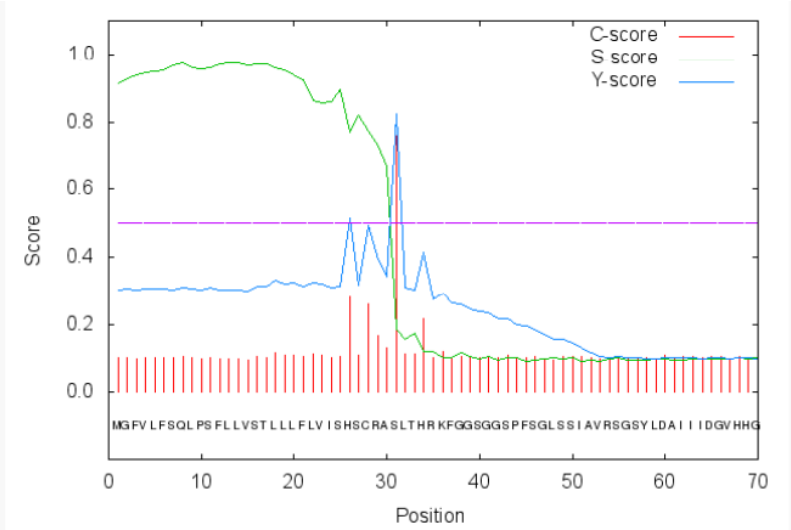
SOURCE: Puigbo et al (2003).

FIGURE 1.3 - SCHEMATIC REPRESENTATION OF THE EXPRESSION CASSETTE AND VIRAL SILENCING SUPPRESSORS USED FOR THE AGROINFILTRATION OF *N. BENTHAMIANA* LEAVES. (A) THE EXPRESSION CASSETTE CONTAINED THE **double-enhanced *cauliflower mosaic virus* (*Camv*) *35s* promoter**, THE **kozak** CONSENSUS SEQUENCE TO INFLUENCE THE INITIATION OF THE TRANSLATION PROCESS, (**SP)** THE SIGNAL PEPTIDE OF THE TOBACCO PR1A PROTEIN, THE **GRFT** CODING SEQUENCE, **his-tag$_6$**, AND THE NOS TERMINATOR (**t-nos**).



SOURCE : Habibi (2018).

FIGURE 1.4 - SIGNAL PEPTIDE PREDICTION OF THAUMATIN-LIKE PROTEIN TO32. THE FIGURE CONFIRMS THE PRESENCE OF THE SIGNAL PEPTIDE, REPRESENTED BY THE CLEAVAGE SITE IN THE POSITION BETWEEN RESIDUES 30 AND 31, C-SCORE DISTINGUISH SIGNAL PEPTIDE CLEAVAGE SITES FROM EVERYTHING ELSE. THE C-SCORE IS TRAINED TO BE HIGH AT THE POSITION IMMEDIATELY AFTER THE CLEAVAGE SITE (THE FIRST RESIDUE IN THE MATURE PROTEIN). S-SCORE (SIGNAL PEPTIDE SCORE) THE OUTPUT FROM THE SP NETWORKS, WHICH ARE TRAINED TO DISTINGUISH POSITIONS WITHIN SIGNAL PEPTIDES FROM POSITIONS IN THE MATURE PART OF THE PROTEINS AND FROM PROTEINS WITHOUT SIGNAL PEPTIDES. Y-SCORE (COMBINED CLEAVAGE SITE SCORE).A COMBINATION (GEOMETRIC AVERAGE) OF THE C-SCORE AND THE SLOPE OF THE S-SCORE, RESULTING IN A BETTER CLEAVAGE SITE PREDICTION THAN THE RAW C-SCORE ALONE. THIS IS DUE TO THE FACT THAT MULTIPLE HIGH-PEAKING C-SCORES CAN BE FOUND IN ONE SEQUENCE, WHERE ONLY ONE IS THE TRUE CLEAVAGE SITE. THE Y-SCORE DISTINGUISHES BETWEEN C-SCORE PEAKS BY CHOOSING THE ONE WHERE THE SLOPE OF THE S-SCORE IS STEEP. THE AVERAGE S-SCORE OF THE POSSIBLE SIGNAL PEPTIDE (FROM POSITION 1 TO THE POSITION IMMEDIATELY BEFORE THE MAXIMAL Y-SCORE). D-SCORE (DISCRIMINATION SCORE). A WEIGHTED AVERAGE OF THE MEAN S AND THE MAX. Y SCORES. THIS IS THE SCORE THAT IS USED TO DISCRIMINATE SIGNAL PEPTIDES FROM NON-SIGNAL PEPTIDES.FOR NON-SECRETORY PROTEINS ALL THE SCORES REPRESENTED IN THE SIGNALP OUTPUT SHOULD IDEALLY BE VERY LOW (CLOSE TO THE NEGATIVE TARGET VALUE OF 0.1).




```
# Measure  Position  Value    Cutoff   signal peptide?
  max. C    31       0.760
  max. Y    31       0.825
  max. S    14       0.976
  mean S     1-30    0.909
       D     1-30    0.871   0.450   YES
Name=Sequence   SP='YES' Cleavage site between pos. 30 and 31: CRA-SL D=0.871 D-cutoff=0.450 Networks=SignalP-noTM
# data
```

SOURCE: Nielsen (2017).

TABLE 1.6 - STRUCTURAL PHYSICOCHEMICAL OF GRFT PROTEIN.

Features	GRFT protein
Number of Amino Acids	122
Molecular weight	12822.05
Theoretical Pi	5.39
Total number of negatively charged residues (ASP+GLU)	10
Total number of positively charged residues (ARG+LYS)	7
Total number of atoms	1756
Instability Index	43.22
Aliphatic Index	70.33
Grand Average Hydropathicity (GRAVY)	-0.223
Ext. Coefficient	11920

SOURCE: Habibi (2018).

The functional characterization of GRFT protein is shown in TABLE 1.6. The hydrophilic nature of protein is associated with a low GRAVY value. The GRAVY value for a peptide or protein can be calculated as the sum of hydropathy values of all the amino acids, divided by the number of residues in the sequence.

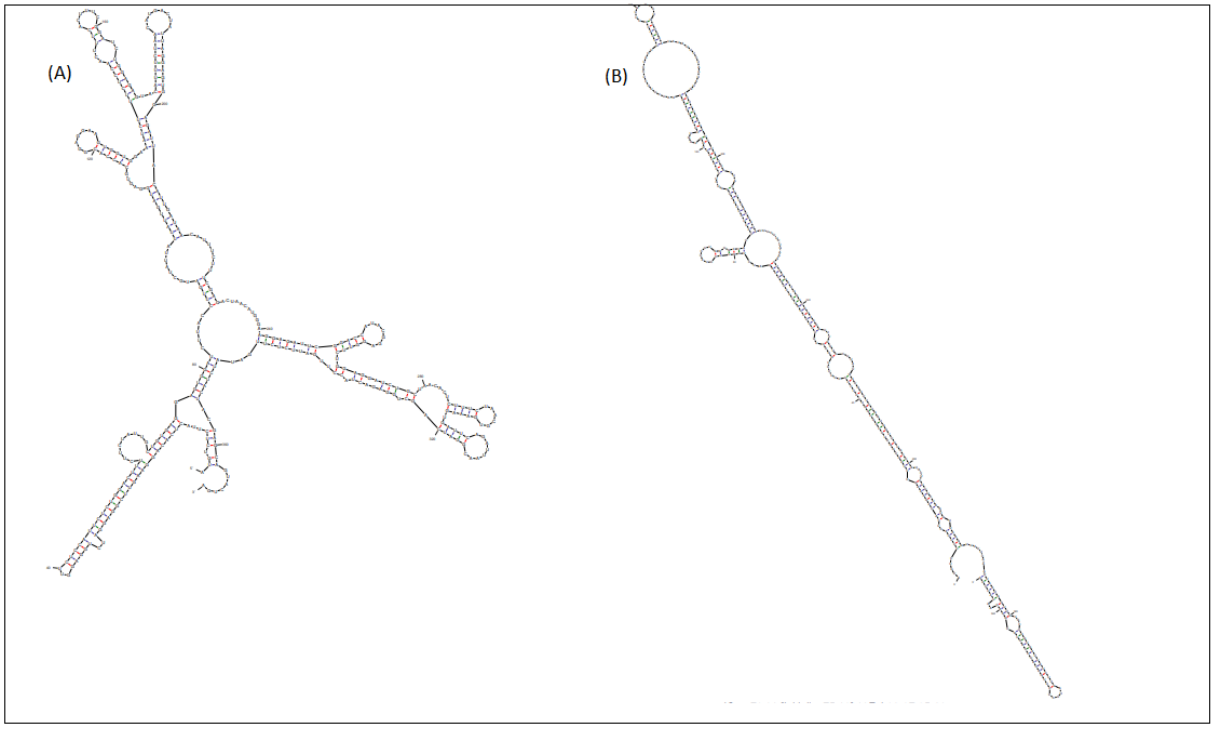
The proteins which have large negative values have relatively more hydropathicity compared to proteins those possess less negative values (Roy et al., 2011). The instability index of GRFT protein was calculated as more than 40 which classified it as an unstable molecule (Roy et al., 2011). The pI value of GRFT proteins was determined to be less than 7 indicating an acidic nature. The aliphatic index for GRFT suggested that this protein has a high thermal stability (Gasteiger et al., 2005).

Generally, mRNA secondary structures like hairpin, loop, and stem will cause interference with the translation of proteins. The mRNAs are the most complex group of cellular RNAs that originated not only from transcription itself but also from numerous modification reactions, such as precursor mRNA (pre-mRNA) splicing, capping, polyadenylation, and 3´ end processing.

Furthermore, the association of mRNAs with protein complexes and factors results in the regulation of mRNA translation and metabolism (Wachter, 2014).Therefore, screening the functional capabilities of RNA folding might identify sequence features that contribute to gene regulation in plant molecular farming. Given a DNA or RNA sequence,

the secondary structure can be predicted, and thus the relative translation efficiency (eg, translation initiation rate) can be predicted. The mRNA folding prediction with the mfold software was performed to compare the structure of GRFT sequence in optimized and non-optimized status (FIGURE 1.5).

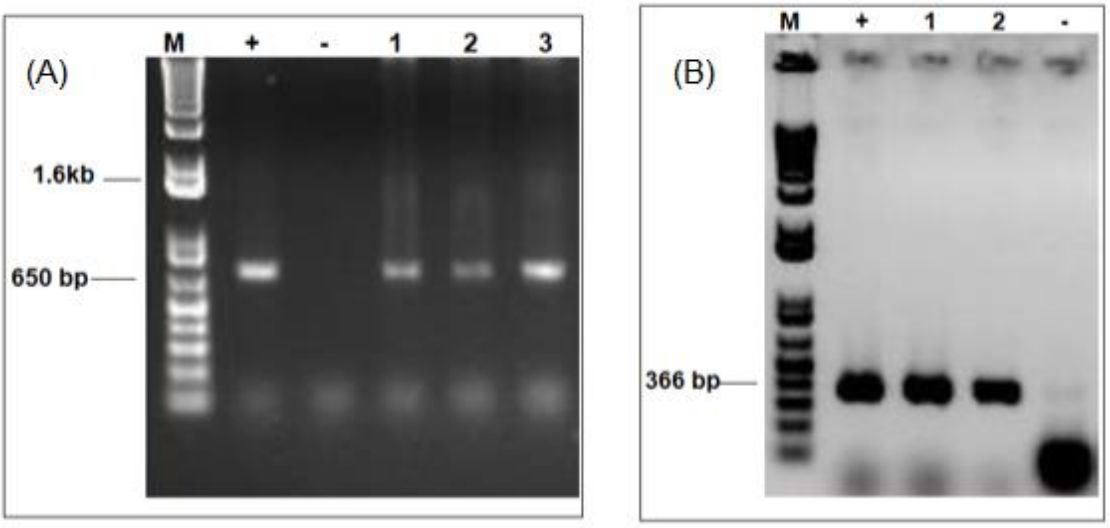
FIGURE 1.5 – THE mRNA SECONDARY STRUCTURE (A) mRNA SECONDARY STRUCTURE OF QUERY (GRFT) WITH INITIAL ΔG = -106 KCAL/MOL AND (B) mRNA SECONDARY STRUCTURE OF OPTIMIZED *GRFT* GENE WITH INITIAL ΔG = -120.20 KCAL/MOL. ΔG IS DEFINED MINIMUM FREE ENERGIES FOR FOLDING THAT MUST CONTAIN ANY PARTICULAR BASE PAIR.



SOURCE: Zuker (2003).

The presence of *GRFT* gene in bacterial system including *E.coli* and *agrobacterium* was confirmed by PCR amplification (FIGUER 1.6).

FIGURE 1.6- COLONY PCR CONFIRMATION FROM *E. COLI* DH5 ALPHA M= LADDER 1 KB PLUS, + = PBIN61: GRFT AS POSITIVE CONTROL; - = H_20 AS NEGATIVE CONTROL; 1, 2 AND 3 ARE COLONIES HARBORING PBIN61: GRFT. (B) PCR AMPLIFICATION OF EXTRACTED PLASMID FROM *A. TUMEFACIENS* (GV3101): M = LADDER 1 KB PLUS, + = PBIN61: GRFT AS POSITIVE CONTROL; 1 AND 2 COLONIES HARBORING PBIN61: GRFT; - = H20 AS NEGATIVE CONTROL



SOURCE: Habibi (2018).

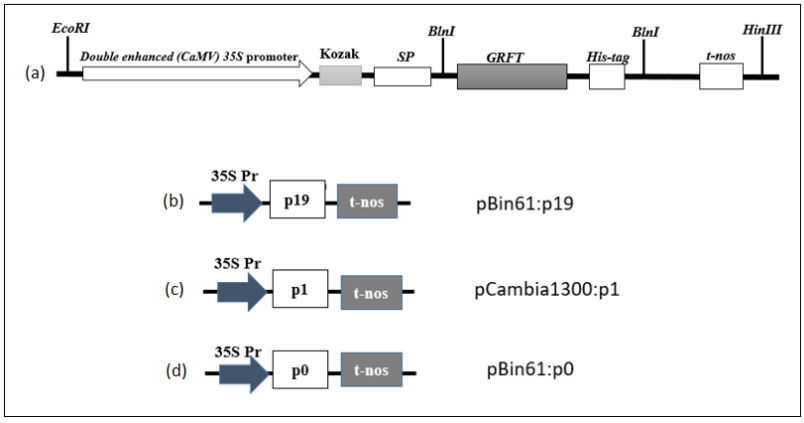
4.1 A COMBINATION OF P19, P0, AND P1 SUPPRESSERS HIGHLY IMPROVES THE EXPRESSION EFFICIENCY

In this study, we investigated the effect of use three gene-silencing suppressors (P19, P0 and P1) with the syringe co-agroinfiltration method could increase the expression level of the GRFT protein. To demonstrate that, the widely and classically used P19 protein from *TBSV* was selected to boost gene expression level (Lombardi et al., 2009b; Garabagi et al., 2012). We further selected the P1, suppressor from the *Rice yellow mottle virus* (*RYMV*), (Siré et al., 2008) and the P0 suppressor from Beet western yellows virus (*BWYMV*) to inhibit the accumulation of 24 nt siRNA (Hamilton et al., 2002; Siré et al., 2008; Lacombe et al., 2010). RISK activity on RNA silencing pathways (Baumberger et al., 2007; Bortolamiol et al., 2007), respectively, in combination of P19. To figure out if our transient expression system based on three gene-silencing suppressors could be successfully used to boost the level expression of GRFT, six weeks old *N. benthamiana* plants were infiltrated with *A. tumefaciens* strain GV3101 containing

the pBIN61 vector harboring the sequence encoding the GRFT under control of double enhanced *Cauliflower Mosaic Virus* (*CaMV*) 35S promoter (FIGURE 1.7).

Plants were also co-infiltrated with either three *A. tumefaciens* clones carrying pBIN61: P19, pBIN61: P0, and pCambia1300: P1 gene silencing suppressors**.** We also assayed the effect of Tween-20 at concentration of 0.015 % and 0.03 % without any suppressor application in syringe co-agroinfiltration method for studying of expression efficiency. Leaves from the infiltrated points were harvested at 7 days' post infiltration (d.p.i) and were analyzed for protein expression level.

FIGURE 1.7 - SCHEMATIC REPRESENTATION OF THE EXPRESSION CASSETTE AND VIRAL SILENCING SUPPRESSORS USED FOR AGROINFILTRATION OF *N. BENTHAMIANA* LEAVES. (A)THE CASSETTE EXPRESSION CONTAINED OF DOUBLE ENHANCED *CAULIFLOWER MOSAIC VIRUS* (*CaMV*) 35S PROMOTER, KOZAK CONSENSUS SEQUENCE TO INFLUENCE THE INITIATION OF THE TRANSLATION PROCESS, SIGNAL PEPTIDE OF THE TOBACCO PR1A PROTEIN, THE *GRFT* CODING SEQUENCE, HIS-TAG6, AND NOS-TERMINATOR (T-NOS). (B)(C)(D) *TOMATO BUSHY STUNT VIRUS P19* GENE, *SWEET POTATO FEATHERY MOTTLE VIRUS P1* GENE AND BEET-INFECTING POLEROVIRUSES BEET CHLOROSIS VIRUS (BCHV) AND *BEET MILD YELLOWING VIRUS* (*BMYV*) *P0* GENE, RESPECTIVELY, UNDER 35S PROMOTER AND NOS TERMINATOR. ALL PLASMIDS WERE BASED ON THE VECTOR PBIN61 BACKBONE EXCEPT P0, WHICH WAS BASED ON PCAMBIA 1300.

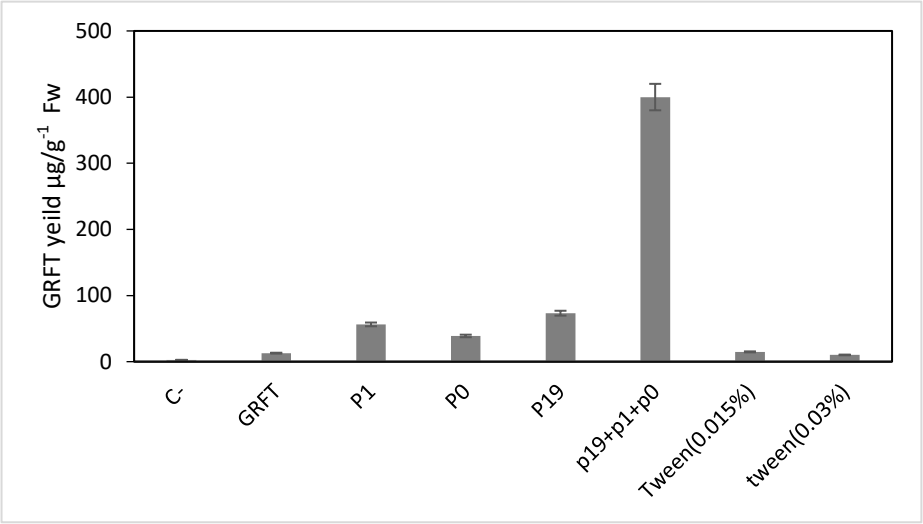


SOURCE: Habibi (2018).

To detect correctly folded, and expression level of GRFT, quantitative ELISA was applied by the grounding of agroinfiltrated leaf (200 mg) in liquid nitrogen. The well-grounded leaf was homogenized in PBS buffer supplied with 1 mM PMSF as a proteases inhibitor. The leaf extracted protein was centrifuged at 14000 rpm and then quantified using the Bradford colorimetric assay (Bradford, 1976). The ELISA experiments were

performed on three biological replicas. Primary anti-GRFT rabbit polyclonal antibody with dilution of 1:1,000 and the secondary horseradish peroxidase (HRP)-conjugated antirabbit IgG antibody with dilution of 1:1,000 were used as capture antibodies. The result of ELISA showed that the maximum expression level was obtained with combination of three gene-silencing suppressors (FIGURE 1.8). The results demonstrated that the application of tween-20 at different concentration without any suppressors did not increased the expression efficiency suggesting the importance of suppressors in agroinfiltration. We then ignored the plant extracts expressed GRFT at a low level for further experiments.

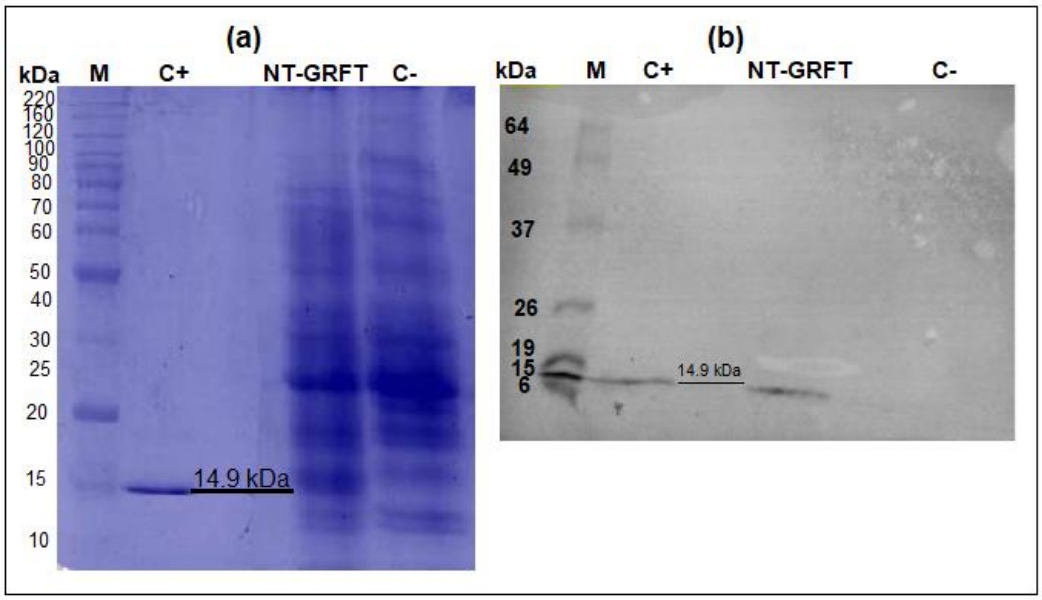
FIGURE 1.8 - ANALYSIS OF THE EFFECTS OF 0.015% AND 0.03% TWEEN-20 SEPARATELY AND THREE GENE SILENCING SUPPRESSORS (P19+P1+P0) TOGETHER AND SEPARATELY ON SYRINGE AGROINFILTRATION EFFICIENCY BY GRFT EXPRESSION. C- = *N.BENTHAMIANA* LEAVES WITHOUT EXPRESSION CONSTRUCT AS NEGATIVE CONTROL. GRFT= NT-GRFT WITHOUT CO-AGROINFILTRATION WITH SUPPRESSORS AND TWEEN-20 WAS USED AS CONTROL. VALUES ARE THE AVERAGE OF THREE EXPERIMENTS ± SD.



SOURCE: Habibi (2018).

To confirm the presence of a band of expected size (14.9 kDa) of GRFT expressed, plant extracts were analyzed by SDS-PAGE and western blotting analysis. The presence of expressed GRFT with the correct size for His_6-tagged GRFT, (14.9 kDa) was detected by western blot analysis representing the correctly folded and soluble form of protein by our transient expression system (FIGURE 1.9).

FIGURE 1.9 - PRODUCTION AND DETECTION OF ANTI- HIV INHIBITOR GRFT IN *N. BENTHAMIANA*. THE PRESENCE OF EXPECTED RECOMBINANT GRFT WAS DETECTED BY WESTERN BLOT ANALYSIS. A) SDS PAGE ANALYSIS OF EXPRESSED GRFT IN LEAF OF *N. BENTHAMIANA* HARVESTED AT 7 DPI. LANE M, 4 µL OF THE BENCHMARK PROTEIN LADDER (THERMO SCIENTIFIC); LANE C+ =GRFT EXPRESSED FROM *E. COLI* AS THE POSITIVE CONTROL; LANE NT-GRFT= 30 µG OF TOTAL SOLUBLE PROTEIN EXTRACTED FROM *N. BENTHAMIANA* PLANTS AGROINFILTRATED WITH THE PBIN61:GRFT CONSTRUCT AND THREE GENE-SILENCING SUPPRESSOR PROTEINS (P19, P0, AND P1); AND LANE C-= 30 µG OF TOTAL SOLUBLE PROTEIN EXTRACTED FROM *N. BENTHAMIANA* PLANTS AGROINFILTRATED WITH PBIN61 CONSTRUCTION AND THREE GENE-SILENCING VIRAL SUPPRESSOR PROTEINS (P19, P0 AND P1) AS A CONTROL. B) WESTERN BLOT ANALYSIS OF EXPRESSED GRFT IN *N. BENTHAMIANA* PLANTS CO-AGROINFILTRATED WITH THREE GENE-SILENCING VIRAL SUPPRESSOR PROTEINS. LANE M, BENCHMARK PRE-STAINED PROTEIN LADDER (THERMO SCIENTIFIC); LANE C+ =GRFT EXPRESSED FROM E. COLI AS THE POSITIVE CONTROL; LANE NT-GRFT= 30 µG OF TOTAL SOLUBLE PROTEIN EXTRACTED FROM *N. BENTHAMIANA* PLANTS AGROINFILTRATED WITH THE PBIN61:GRFT CONSTRUCT AND THREE GENE-SILENCING VIRAL SUPPRESSOR PROTEINS (P19, P0 AND P1); AND LANE C-, 30 µG OF TOTAL SOLUBLE PROTEIN EXTRACTED FROM *N. BENTHAMIANA* PLANTS AGROINFILTRATED WITH PBIN61 CONSTRUCTION AND THREE GENE-SILENCING VIRAL SUPPRESSOR PROTEINS (P19, P0 ,AND P1) AS A CONTROL.



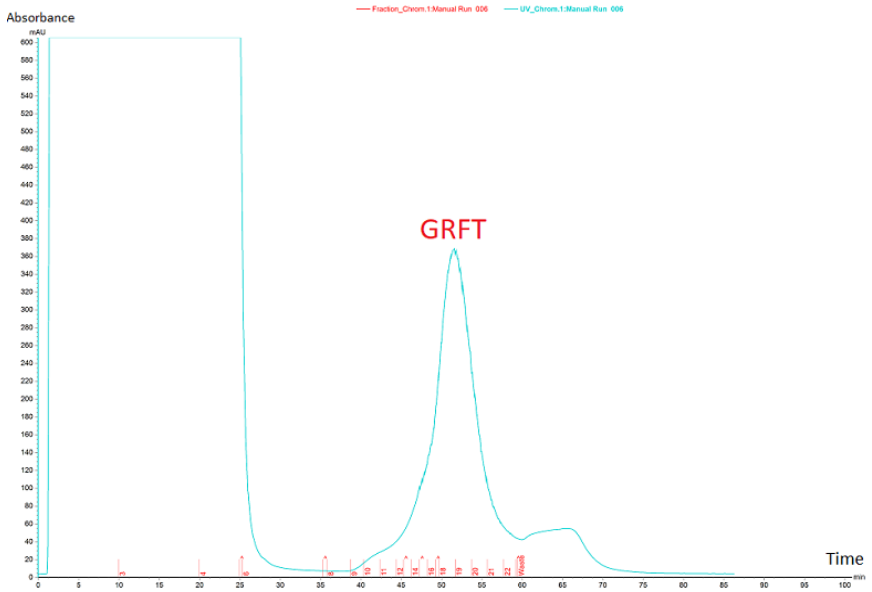
SOURCE: Habibi (2018).

4.2 A ONE STEP-PROTOCOL USED TO PURIFY GRFT EXPRESSED FORM EXTRACT CRUDE

The recombinant protein GRFT was purified from total soluble protein (TSP) of *N. bentaminana by* an immobilized metal affinity chromatography (IMAC) column using an AKTA^{TM} Prime Plus system (GE Healthcare Bio-Sciences, Uppsala, Sweden). Protein

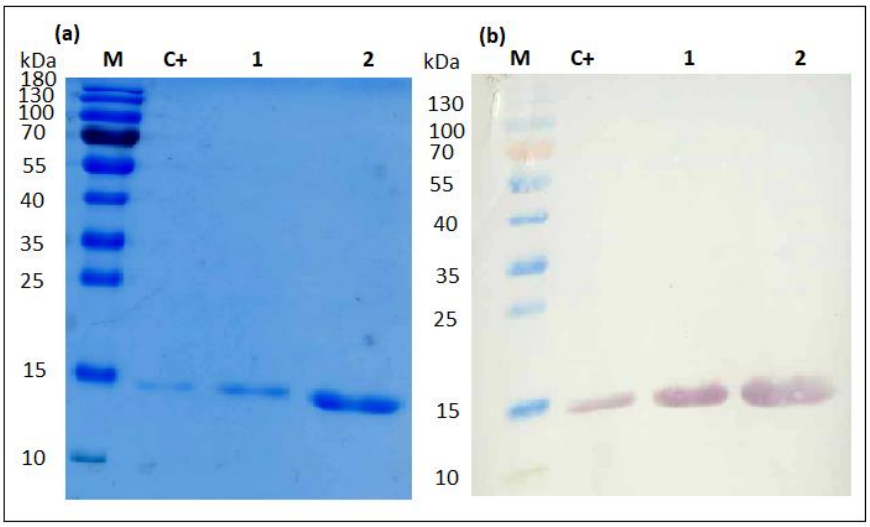
capturing was carried out by 1 ml HisTrap FF column (GE Healthcare Bio-Sciences, Uppsala, Sweden) and eluting it with buffer containing 500 mM imidazole(FIGURE 1.10). The final yield of purified GRFT was 238 µg/g FW which represent a recovery of 59.5%. The presence of purified GRFT was confirmed with SDS-page and immunoblotting analysis (FIGURE 1.11). The immunoblotting analysis revealed the corrected size and folded His6-tagged GRFT. No degradation was seen representing the protein remained stable during upstream and downstream process.

FIGURE 1.10 IMAC CHROMATOGRAM OF THE GRFT PROTEIN SHOWING CLEAR SEPARATION OF THE GRFT-HIS TAG FROM EXTRACTED FRACTION FROM *N. BENTHAMIANA* LEAVES.



SOURCE: Habibi (2018)

FIGURE 1.11 - (A) SEPARATION OF PURIFIED NT-GRFT UNDER DENATURING CONDITION BY SDS-PAGE SHOWING A 14.9-KDA BAND REPRESENTING GRFT. LANE M=5 µL OF THE PAGE RULER PRESTAINED PROTEIN LADDER (THERMO SCIENTIFIC); LANE C+ =500 NG OF GRFT PURIFIED FROM *E. COLI*, LANE 1= 1.5 MG OF NT-GRFT; LANE 2= 3.5 MG OF NT-GRFT; (B) WESTERN BLOT ANALYSIS OF EXPRESSED GRFT IN *N. BENTHAMIANA* PLANTS CO-AGROINFILTRATED WITH THREE GENE-SILENCING VIRAL SUPPRESSOR PROTEINS. LANE C+ =500 NG OF GRFT PURIFIED FROM *E. COLII*; LANE 1= 1.5 MG OF NT-GRFT; LANE 2= 3.5 MG OF NT-GRFT.

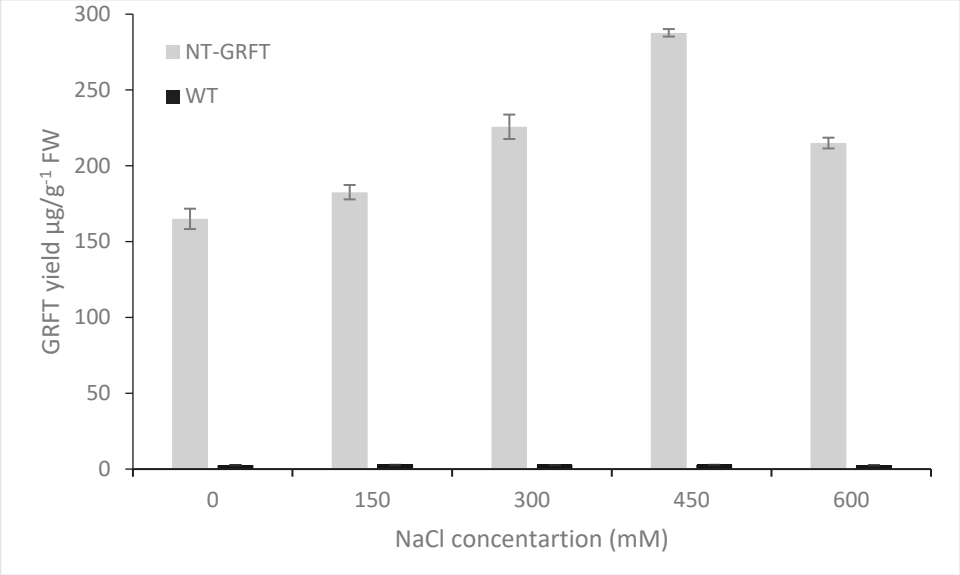


SOURCE: Habibi (2018).

4.3 EFFECT OF SALT CONCENTRATION ON GRFT EXTRACTION

To study the effect of salt concentrations on recovery of GRFT, the addition of different concentrations of NaCl on extraction buffer (50mM sodium phosphate, 50mM ascorbic acid, 10mM EDTA, 1mM PMSF) was performed. The figure 5 shows that by increasing concentration of NaCl to 450 mM, the GRFT content raised from 238 µg/g to 287 µg/g representing a recovery of 69.5%. However, the GRFT yield was decreased above 450 mM salt suggesting the optimal concentration of salt for GRFT recovery could obtain in 450 mM (FIGURE 1.12).

FIGURE 1.12 - EFFECT OF SALT CONCENTRATION IN EXTRACTION BUFFER ON GRFT RECOVERY FROM TSP OF *N. BENTHAMIANA* EXTRACTS. WILD TYPE (WT) WAS USED AS NEGATIVE CONTROL.THE EXTRACTION BUFFER (PH 7.4) CONTAINED 50 MM SODIUM PHOSPHATE, 50 MM ASCORBIC ACID, 10 MM DI-SODIUM EDTA, 1 MM PMSF AND DIFFERENT CONCENTRATIONS OF NaCl VALUES ARE THE AVERAGE OF THREE EXPERIMENTS ± SD.

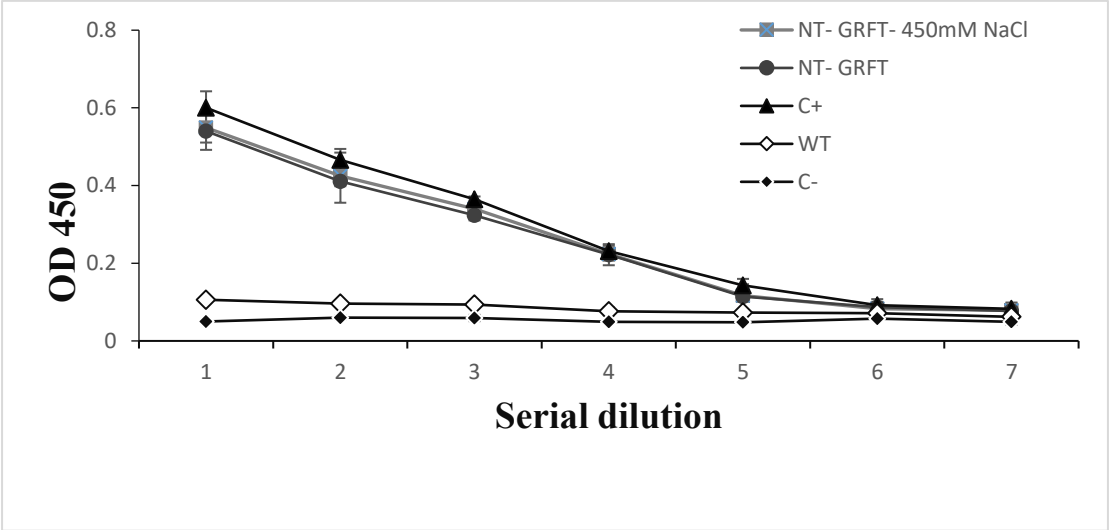


SOURCE: Habibi (2018).

4.4 THE PURIFIED NT-GRFT SHOWED GP-120 BINDING ACTIVITY COMPARABLE WITH GRFT PRODUCED IN *E. COLI*

In vitro binding activity of purified GRFT from leaf of *N. benthamiana* harvested at 7 d.p.i was analyzed using ELISA against HIV gp120. In this experiment, purified GRFT from *E. coli* (EC- *E. coli*) as positive control and PBS as negative control were used. The result of ELISA showed higher gp120-binding activity of purified NT-GRFT than negative control. The gp120-binding activity of NT-GRFT was consistent with the concentration of purified GRFT from *E. coli* suggesting near identical activity to GRFT produced in *E. coli* (FIGURE 1.13). Our result demonstrate that assembled GRFT accumulated in leaf of *N. benthamiana* was functional.

FIGURE 1.13 - ANTIGEN-BINDING ACTIVITY OF CRUDE EXTRACT FROM LEAVES OF *N. BENTHAMIANA* HARVESTED AT 7 D.P.I. AND PURIFIED GRFT FROM E. COLI. TO DETECT THE SPECIFIC ANTIGEN-BINDING ACTIVITY OF PLANT GRFT, PLATES WERE COATED WITH 100 NG OF HIV-1 GP120 PROTEIN, AND BOUND OF PURIFIED GRFT WAS DETECTED WITH GRFT ANTI-RABBIT PRIMARY ANTIBODY (1:1000 IN PBST) AND HRP ANTI-RABBIT SECONDARY ANTIBODY (1:2000 IN PBST). NT-GRFT- 450MM NACL =GRFT EXPRESSED BY EXTRACTION BUFFER CONTAINED 450MM NACL; NT-GRFT =GRFT EXPRESSED BY THE PBIN6: GRFT CASSETTE IN *N. BENTHAMIANA*; C+=GRFT EXPRESSED FROM *E. COLI* AS THE POSITIVE CONTROL; WT = WILD-TYPE PLANTS; C−=NEGATIVE CONTROL (PBS). OD, OPTICAL DENSITY AT 450 NM. BOTH PURIFIED NT-GRFT AND NT GRFT 450MM NACL SHOWED HIGHER BINDING ACTIVITY COMPARED WITH PBS AS THE NEGATIVE CONTROL AND WT.VALUES ARE THE AVERAGE OF THREE EXPERIMENTS ± SD.

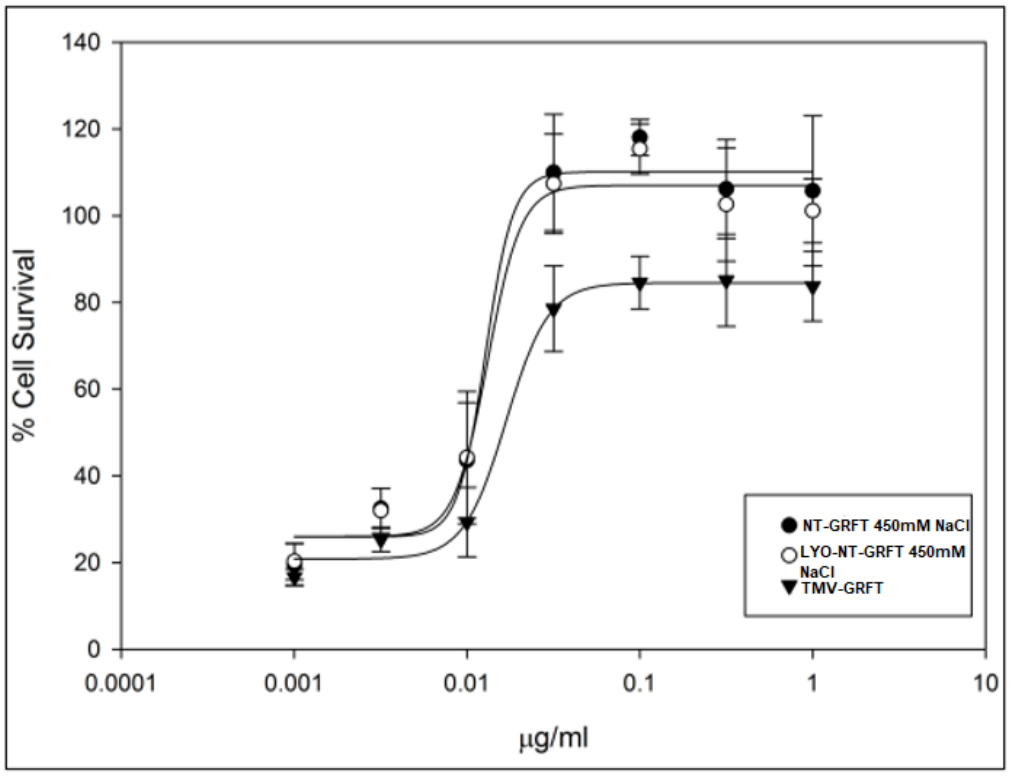


SOURCE: Habibi (2018).

4.5 PURIFIED GRFT WAS POTENTLY ACTIVE AGAINST HIV IN WHOLE-CELL HIV NEUTRALIZATION ASSAYS

GRFT produced by our system and GRFT produced by (O'Keefe, et al. 2010) were tested for their ability to inhibit the cytopathic effects of HIV-1RF against T-lymphoblastic CEM-SS cells. Previously, native GRFT and GRFT produced in *N. benthamiana* showed remarkably potent activity against HIV-1, with EC50 values of 0.054 and 0.156 nM, respectively (O'Keefe et al., 2009). In our experiments, GRFT at a concentration of 100 µg/ml showed potent activity against HIV-1, with an EC50 value of 1.15 ± 1.2 nM comparable to the 1.33 ± 0.17 nM (FIGURE 1.14) of the positive control. No IC50 value was measured, confirming that no toxic constituents had co-purified with the protein.

FIGUER 1.14 - ANTI-HIV ACTIVITY OF NT-GRFT-450MM NACL, LYO-GRFTAND TMV BASED VECTOR GRFT. THE ACTIVITY OF PURIFIEDGRFT WAS ANALYSED BASED ON CEM-SS CELLULAR VIABILITY INFECTED WITH HIV-1RF. THE ACTIVITY OF NT-GRFT WAS COMPARABLE TO THAT OF GRFT PROTEIN PRODUCED USING A TMV-BASED VECTOR (O'KEEFE ET AL., 2010). IN ADDITION, THE ACTIVITY OF LYOPHILIZED NT-GRFT-450MM NACL AGAINST HIV-1 WAS COMPARABLE TO BOTH NON-LYOPHILIZED NT-GRFT-450MM NACL AND TO TMV-BASED VECTOR GRFT. THE CELL VIABILITY WAS ASSESSED USING THE XTT ASSAY AS DESCRIBED IN THE METHOD SECTION. ALL DATA POINTS ARE AVERAGES (±SE) OF QUADRUPLICATE (N=4) MEASUREMENTS.



SOURCE: Habibi (2018)

5 DISCUSSION

The lectin griffithsin is one of the potent HIV inhibitory candidate isolated from red algae, which effectively binds to the HIV envelope protein gp120 and prevents virus infection (Xue et al., 2013; Moulaei et al., 2015). Based on cell analysis, inhibitory effects of GRFT against HIV infection has been indicated at picomolar concentrations (EC50 of ~50 pM) (Mori et al., 2005).

Next to inhibitory activity of GRFT against HIV viral entry, antiviral activity of GRFT against several glycosylated viruses such as the murine herpes simplex virus type 2 (Nixon et al., 2014), the Japanese encephalitis virus (Ishag et al., 2013), the hepatitis C virus (Meuleman et al., 2011), the coronavirus responsible for SARS (O'Keefe et al., 2010) has been demonstrated. Besides lacking toxicity and immunogenicity of GRFT, its thermostability characteristic in a wide range of conditions, make GRFT an interesting candidate in development of antiviral therapeutic concept (O'Keefe et al., 2009; Kouokam et al., 2011; Barton et al., 2014). The production and purification of native GRFT from red algae have been reported, but only at low concentration (Mori et al., 2005). Therefore, the large-scale production of GRFT using plant platform might open up a new avenue for the commercialization of this protein, as a potent HIV inhibitory candidate.

GRFT production was previously shown with a yield of 819 mg/L, but the formation of insoluble inclusion body (33%), just recovery of 66 % protein in soluble protein, high manufacturing cost and bacterial endotoxin have been considered as limitation factors for GRFT production by *E. coli* system (Giomarelli et al., 2006). Moreover, the reduction of recombinant GRFT has been reported in *N. benthamiana* (O'Keefe et al., 2009) and *O. sativa* (Vamvaka et al., 2016a). Even so, recovery of 30 % protein after the purification of GRFT in *N. benthamiana*, contamination with TMV coat protein (Fuqua et al., 2015), and degradation are considered as some drawbacks. In addition, the high cost of *in vitro* RNA transcription is also a marked problem with this system. (Vamvaka et al., 2016a) achieved a yield of 301 µg/g dry seed weight, which was reduced to 223 µg/g dry seed weight after purification, representing a recovery of 74%. In this case, as described above long-term process of seed production and space requirements may be preclusive (Boothe et al., 2010).

We investigated the production of GRFT in transient expression system based on agroinfiltration by non-viral vector in *N. benthamiana* as convenient platform for accumulation of biopharmaceuticals. This is the first report of expression and production of GRFT in transient expression system based on agroinfiltration in *N. benthamiana* using three gene silencing suppressors. High level of recombinant GRFT was recovered from transgenic plants 7 days after gene delivery by syringe infiltration. Thus, our transient expression significantly shortened the timeline of GRFT production as it was previously reported 12 days via viral vector system. Also, in comparison with produced GRFT based on stable transgenic seed of *O. sativa* our transient expression with agroinfiltration showed the high levels of the transgene expression. Therefore, the short timeline and higher production of GRFT based on our transient expression could be considered as advantageous towards GRFT development and commercialization. Transient gene expression based on agroinfiltration is an efficient, cost-effective, and time-saving strategy for yielding high amounts of recombinant proteins, as stable genetic transformation is a slow process, requiring months or years to generate transgenic plants due to regeneration protocols.

In addition, it is a genome integration-independent strategy, and consequently, it is not affected by position effects existing in stable transformation, once that the expression vector remains an episomal DNA molecule (Habibi et al., 2017). Our result is in accordance with those previous works, which indicated that agroinfiltration could efficiently result in transferring of *Agrobacterium* into the plant leaves and robust expression recombinant proteins (Chen et al., 2013b; Abdelghani et al., 2015; Chen and Lai, 2015). The infiltration of *Agrobacterium* into the intercellular space of the leaf was performed with syringe method (FIGURE 2 b). Syringe infiltration has demonstrated as favorable tools that provides remarkable advantages such as simple procedure without the requiring for any specialized equipment, possibility of either infiltrating the whole leaf with one transgene construct or transferring multiple constructs into different areas of one leaf, allowing multiple assessment in one leaf (Vaghchhipawala et al., 2011; Chen et al., 2013a).

In this work, we studied the addition of three gene-silencing viral suppressor proteins (P19, P0, and P1) combined with the syringe co-agroinfiltration method for high GRFT protein expression. We also assayed the effect of syringe co-agroinfiltration method with Tween-20 at concentrations of 0.015% and 0.03% and without any suppressor application on expression efficiency. The results illustrated herein demonstrated that leaves co-agroinfiltrated with the three gene-silencing suppressors exhibited the highest level of GRFT at 7 d.p.i. compared with the application of P19, P1 and P0 individually as well as in the absence of suppressors. Our results suggest that suppressing the post-transcriptional gene silencing mechanism with three gene silencing suppressors in *N. benthamiana* increased the number of *GRFT* gene transcripts and subsequently elevated the transient expression of recombinant GRFT. RNA silencing is critical process involved in decreasing of foreign genes expression in plant system. In this context using of plant virus encoding suppressors of RNA silencing are considered as an applicable strategy to counteract RNA silencing mechanism and subsequently boost protein expression content in plants (Lombardi et al., 2009a;Vézina et al., 2009;Garabagi et al., 2012).

Use of RNA silencing suppressors has been widely reported for increasing of recombinant proteins content in plant. In this context, P19 from *TBSV* involved in siRNA sequestration has been gained more attention. The P19 protein is a strong silencing suppressor protein and can selectively inhibits the 21-nt and 22-nt classes of siRNA (Arzola et al., 2011). The P1 protein has own mechanism to inhibit the accumulation of the 22-nt and 24-nt siRNA (Li and Ding, 2006). The P0 protein is a silencing suppressor, which inhibits local and systemic RNA silencing through AGO1 degradation (Fusaro et al., 2012).

Our result is comparable to Lacombe et al., 2017, who recently showed the effect of combination of P19, P1, and P0 to enhance expression levels of anti-leishmaniasis vaccine candidate by transient expression system in *N. benthamiana*. The synergetic effect of P0 and P1 on RNA silencing suppression was shown in combination with P19. It is hypothesized that the P1 suppressor could affect local RNA silencing suppression via inhibition of 24 nts siRNA production. As 24 nts siRNA might induce the formation of systemic RNA silencing signal and combination of this suppressor with P19 and P0 would

strongly affect the RNA gene silencing, and consequently increase recombinant protein production in plant not only locally but also on systemic level through viral based tool (Lacombe et al., 2017). Based on our result we can speculate that combination of P19, P0, and P1 suppressors could be efficiently applied to boost expression level of other recombinant protein by transient expression system in plants. However, the use of three P19, P0, and P1 suppressors together might not be recommended for stable transgenic plants since combination of RNA silencing suppressors could trigger harmful developmental damage on plant cell (Saxena et al., 2011; Lacombe et al., 2017).

The effect of Tween-20 on GRFT production through syringe agroinfiltration was investigated. In the present study, the application of Tween-20 did not improve the expression efficiency. Our data contrasts those reported by (Zhao et al., 2017), who found that Tween-20 increased GUS expression by influencing the agroinfiltration efficiency. Co- agroinfiltration with Tween-20 caused fast tissue necrosis and cell death, potentially due to the induction of damage by Tween 20 to plant cells in the agroinfiltration system.

To confirm the expression of GRFT by using the transient expression system, SDS-PAGE and immunoblotting analyses were performed. The results demonstrated a specific band of His-tagged GRFT, representing the expression of GRFT under the control of the strong *CaMV 35S* promoter, and in the presence of gene silencing suppressors. Our results are in accordance to those reported previously by O'Keefe et al., 2009 and Vamvaka et al., 2016a.

Recently, Vamvaka and collaborators showed data of an immunoblotting analysis for GRFT detection, which showed a pair of intense bands at 14.6 kDa and 16–17 kDa, and concluded that incomplete removal of the rice α-amylase (RAmy3D) signal peptide might have resulted in the presence of additional band at 16-17 kDa (Vamvaka et al., 2016a). In our study, the results obtained by immunoblotting using primary anti-GRFT rabbit polyclonal antibody does not showed any additional band under denaturing conditions, suggesting complete removal of the signal peptide used in the transient expression system. The purification of the His-tagged GRFT was performed by one-step purification through affinity chromatography, which was performed under native conditions and without ammonium sulphate precipitation. We first obtained a calculated yield of 238 µg/g FW that represent a recovery of 59.5%. The number and nature of a

purification step are considered significant factors affecting the viability of recombinant proteins produced in plants (Vamvaka et al., 2016a). The GRFT produced, based on a viral vector in *N. benthamiana* decreased from 1 mg/g to 300 µg/g after purification, representing a 30% recovery. The presence of the TMV vector coat protein required further purification to remove the coat protein, resulting in a low recovery of GRFT as was demonstrated by O'Keefe et al., 2009.

It was demonstrated that protein extraction using a phosphate buffer combined with IMAC achieved 74% recovery of GRFT expressed in the rice endosperm, based on a stable expression system, and this value was slightly higher than the recovery obtained using our system (Vamvaka et al., 2016a). To study the effect of the salt concentration on GRFT recovery, we added different concentrations of NaCl to the extraction buffer, and the results clearly illustrated that the yield of GRFT was significantly increased by the addition of salt at a concentration of 450 mM. After salt treatment, the yield of GRFT increased from 238 µg/g to 287 µg/g, representing a recovery of 71.75%. This data is in accordance to the Mayani et al., 2011, who showed that the addition of salt at a concentration of 450 mM to the extraction buffer can improve the recovery of recombinant protein in *N. benthamiana*. The protein extraction process is as essential step of the recovery process because it tailors the total extract volume, concentrations and purity of the recombinant protein as well as the isolation of the desired protein from impurities or/and contaminations that should be removed during the purification process (Hassan et al., 2008;Nikolov et al., 2008). Our results demonstrate that protein recovery increases after salt treatment due to the combination of a reduction in electrostatic interactions between the recombinant protein and plant protein components, such as cellulase, which carry a negative charge, and/or increases in osmotic pressure in the extraction buffer, which would result in the dehydration of tissue components (Mayani et al., 2011).

However, increasing the salt concentration in the extraction buffer to a concentration above 450 mM decreased the GRFT yield. It is possible that the solubility of GRFT was reduced at a high salt concentration, which would subsequently increase the hydrophobic interactions with plant tissue components, and this mechanism can feasibly occur in the presence of a higher concentration of salt. Our results demonstrate that optimization of the early steps in the recovery and purification processes are crucial

for obtaining viable plant-based recombinant protein production. We used a non-viral-vector-based transient expression system to eliminate the step for purifying the expressed GRFT. The expression of GRFT in the non-viral vector was prepared under non-denaturing conditions to maintain the native form of GRFT and thus avoid an additional refolding step. The GRFT protein expressed by our system was obtained at a yield of 287 μg/g, which is higher than that obtained in transgenic seed rice (223 μg/g dry seed weight). Our transient expression system significantly shortened the timeline of GRFT production compared with the previously reported timeline of 12 days. Therefore, the short timeline and the higher production of GRFT obtained with our transient expression could be considered advantageous toward GRFT development and commercialization.

The GRFT is a potent HIV inhibitor candidate that tightly binds to high mannose-saccharides on the surface of the glycoproteins gp120, gp41, and gp160 and efficiently inhibits virus infection (Meuleman et al., 2011;Xue et al., 2013). The GRFT has been shown to bind the HIV glycoprotein by blocking CD4 binding as well as by binding other anti-HIV antibodies (Lusvarghi and Bewley, 2016b). GRFT has a smaller recognition epitope and lower binding stoichiometry compared with another HIV lectin. The suitable content of GRFT for binding to a single gp120 glycoprotein has been reported approximately to equal 10 GRFT units (O'Keefe et al., 2010).

In this study, we therefore analyzed the biological activity of NT-GRFT- 450mM NaCl protein which exhibited a comparable EC50 value of 0.9 nM to tobacco-produced GRFT transduced with a viral vector (TMV-GRFT EC50 1.3 nM). Our analysis showed that the ability of GRFT expressed in *N. benthamiana* to bind to gp120 was close to that of the protein purified from *E. coli*. Similarly, O'Keefe et al., 2009 and Vamvaka et al., 2016a showed that the binding characteristics of plant-based GRFT exhibited similar or even better gp120-binding activity than GRFT expressed in *E. coli*.

We also estimated the anti-HIV activity of GRFT through a whole-cell HIV cytopathicity assay. We found that GRFT produced in *E. coli* presented whole-cell HIV cytopathicity with an EC50 value of 0.089 nM as well as EC50 values of 0.054 nM for native GRFT. Additionally, O'Keefe et al., reported an EC50 value of 0.156 nM for GRFT. But in our experiments, we report the anti-HIV activity of TMV-GRFT at an EC50 value of 1.3 nM (O'Keefe et al., 2009). In this case, the discrepancy in EC50 values is the result

of reformatting the whole-cell neutralization assay from a 96-well to a 384-well higher throughput format, as described in the material and methods section. Recently, an EC50 value of 0.27 was obtained for GRFT expressed in the rice endosperm. The differences among the EC50 values obtained in these studies reflect the variability of the syncytium inhibition assay, which is used as a comparison tool in an experiment. *In vivo* safety and efficacy are two critical issues related to potential anti-HIV microbicide activity. The HIV neutralization activity observed in this study confirmed that GRFT is correctly folded in plants and maintains its biological activity.

Physico-chemical modifications such as modification of amino acid side chain in aqueous solution can extremely affect stability and /or functional activity of protein and subsequently influence the risk of adverse side effect in term of protein drug. Therefore, the inherent instability protein-based pharmaceuticals as well as product storage and shipping condition preclude the preparation of protein as shipping and storing product at controlled condition are not technical and economically practicable. Lyophilisation process was used to prepare dehydrated GRFT. In this context lyophilized GRFT was showed functional activity in the room temperature. This characteristic allow the product to be handled and stored conveniently to wider market.

In conclusion, here series of experiments were carried out to boost GRFT transient production in *N. benthamiana* leaves and to demonstrate that this protein maintained its immunogenic properties. In this study, RNA silencing suppression with combination of three gene slinging suppressors as well as the effect of the salt concentration on GRFT recovery were performed to establish the most effective situation to effectively accumulate the recombinant immunogenic GRFT protein in a rapid, efficient and low-cost way for further its development and commercialization.

6 PERSPECTIVE

Several plant-produced molecules are in different stages of clinical development. Furthermore, the future of plant-made pharmaceuticals was recently strengthened by the FDA approval of carrot produced glucocerebrosidase. The carrot produced pharmaceutical proved to be as effective as the CHO produced (Shaaltiel et al., 2007).

Given that several plant-produced pharmaceutical proteins has entered clinical trials and that their regulatory approval processes are smoothed out it probably will not be long before we see an established plant-made pharmaceutical industry.

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CHAPTER II – MOSS-BASED EXPRESSION SYSTEM IS A COST-EFFECTIVE PLATFORM FOR PRODUCTION OF NOVEL HIV ENTRY INHIBITOR, GRIFFITHSIN.

Abstract

The 369 bp sequence encoding *GRFT* was optimized using *P. patens* codon usage table under control of the physicomiterella actin-5 promoter (act5P), the signal

peptide encoding thaumatin-like protein To32 and finally the plasmid was transformed in *agrobacterium*. 150 gametophors of 11-day-old *P. patens* were transformed via *A. tumefaciens* infection. Transgenic gametes were selected in culture medium supplemented with kanamycin for at least eight weeks, and finally 80 selected gametophytes were regenerated and transferred in non-selective medium. The primary identification of the transformants was confirmed by the presence of the 366 bp *GRFT* gene in the genomic DNA of *P. patens* by PCR cDNA library was synthesized and transcription level was analysis by RT-qPCR. To detect correctly folded and expression level of GRFT, quantitative ELISA was applied by grounding of transgenic gametophores (6, 7 and 8 lines). The result of ELISA showed low amount of GRFT expression in three lines. The highest level of expression (0.00018 µg protein per gr fresh weight of protonema was obtained in the moss line 8.

Key words: *physicomiterella,* protonema *,* GRFT, ELISA

1 INTRODUCTION

Infections with the HIV virus are a major public health issue. The World Health Organization estimates that globally 35 million people lived with HIV infections in 2013, including 3.2 million children less than 15 years, and 1.5 million people died of AIDS in 2013. Even though 2.5 million new infections are 22 % less than what was reported for the year 2001, an effective prophylactic HIV vaccine remains the best long-term strategy for preventing HIV/AIDS (WHO 2014).

Today pre-exposure prophylaxis, or PrEP, and antiretroviral therapies allow those patients living with HIV enjoy longer and keep HIV at undetectable levels. The first antiretroviral medication, Zidovudine, also known as azidothymidine was developed and approved in 1987. Nowadays, more than 40 FDA-approved drugs have been released in the market for treatment of individuals carrying HIV virus. Antiretroviral drugs used in the treatment of HIV infection are categorized into protease inhibitors, nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, HIV integrase

strand transfer inhibitors, entry inhibitors and fusion inhibitors. (FDA (U.S. Food and Drug Administration). Drugs used in the treatment of HIV infection can be found at http://www.fda.gov/forpatients/illness/hivaids/ucm118915.htm (accessed March 31, 2017).

Although these drugs used to prevent the spread of HIV infection and consequently, allow patients with healthier lives, they are unable completely remove HIV-infection from patients. This drugs cause serious side effects and patients may continue to experience illness associated with HIV infection due to the development of drug-resistance HIV (Cos et al., 2004;Thayer, 2008). Moreover, only 46% of the 36.7 million individuals infected with HIV were received antiretroviral therapy in 2015, according to WHO (http://www.who.int/features/factfiles/hiv/en/). Infected people in developing countries with the greatest incidence of HIV pandemic, unable to pay medications and/or accessing HIV medications do not simply. Moreover, despite 30 years of efforts to development of a vaccine, for eliciting antibodies that neutralize HIV-1 and, consequently, provides a high level of protection immunity, the truth is, unfortunately, we have not achieved yet (Klein et al., 2013;McCoy and Weiss, 2013). Therefore, invention and development of novel, efficient, and accessible technologies to prevent sexually transmitted HIV infection would be imperative.

Girls and women make up more than half of 36.7 million people living with HIV. So adolescent girls and women aged 14-24 are more susceptible to HIV infection than other group of people. They are accounted for 20% of new HIV infections among adults globally in 2015 (UNAIDS). In this context, critical factors such as lack of education and sexual and reproductive health services, high incidence of rape, poverty, economic gender inequalities, food insecurity and domesticate violence are contributed increased HIV risk of young women and adolescent girls (UNAIDS). To decline the vulnerability of women as well as to make them more stronger against HIV/AIDS, the development of new technologies has been promoted (Piret and Bergeron, 2010). In this context, application of microbicides that would efficiently prohibit the initiation transmission of HIV virus is ideal strategy. The molecular farming field has gained industry interest as an attractive alternative for the production of vaccines using different expression hosts and strategies.

The non-seed plant *P. patens*, a moss, is a well-established model system for evolutionary and functional genomics approaches (Khraiwesh et al.; Mosquna et al., 2009; Sakakibara et al., 2013). It can be grown throughout its complete life cycle under contained conditions *in vitro* in a simple mineral medium (Strotbek et al., 2013). The germination of the haploid spores leads to the growth of protonema a branched filamentous tissue which comprises two distinct cell types, chloronema and caulonema. Every cell is in direct contact with the culture medium, allowing efficient nutrient uptake and product secretion (Schillberg et al., 2013). This young tissue can be maintained in suspension cultures without any addition of phytohormones, only by mechanical disruption of the filaments. In contrast to immortalized or de-differentiated mammalian or higher-plant cell cultures, which are prone to instability or somaclonal variation fully differentiated protonema tissue is genetically stable (Decker et al., 2014). In the next developmental step, buds differentiating from protonema cells give rise to the adult plant, the leafy gametophore, consisting of shoot-like, leaf-like, and root-like tissues .After fertilization of the gametes, the sporophyte, the only diploid tissue in the life cycle of mosses, grows on and is sustained by the gametophore (Decker et al., 2014). *In vitro* cultivation of all stages can be performed either on agar plates or as suspension cultures in liquid media. The availability of efficient protocols for protoplast isolation and transfection (Strotbek et al., 2013), and an excellent regeneration capacity of single transfected cells to whole plants make genetic engineering of moss a straight-forward and frequently used approach (Khraiwesh et al.; Mosquna et al., 2009; Sakakibara et al., 2013). The created moss strains can be preserved by cryo-conservation (Schuster et al., 2007), and thus can serve as Master Cell Banks. The International Moss Stock Center IMSC, a reference center for moss ecotypes and transgenic lines, provides a service for long-term storage (http://www.moss-stock-center.org).

In the area of molecular pharming, the moss *P. patens* has been employed for the production of antibodies with superior lytic potential (Schuster et al., 2007). Other biopharmaceuticals such as *Erythropoietin* (Parsons et al., 2012), human Complement Factor H (Büttner-Mainik et al., 2011), human Vascular Endothelial Growth Factor VEGF (Baur et al., 2005) and human a-galactosidase, which is actually in clinical trials

(www.greenovation.com). The distinctive features of this host are: it can be grown in contained bioreactor cultures (Hohe et al., 2002) up to 500 L batches under full GMP conditions for pharmaceutical production, and it can be genetically modified by gene targeting due to its yeast-like efficient homologous recombination. Precise genome alterations by gene targeting have been employed for the glycoengineering of production lines based on deletion and/or heterologous expression of specific glycosyltransferase genes (Parsons et al., 2013). Moreover, key components of the mammalian transcription, translation and secretion machineries are functional in moss (Gitzinger et al., 2009) as are tunable synthetic promoters for transgene expression (Mueller et al., 2014). Here, we report for the first time production of GRFT inhibitor in transgenic moss lines.

2 OBJECTS

Recombinant production of anti-HIV protein, griffithsin using stable expression in moss *P. patens*

2.1 SPECIFIC OBJECTS

(a) Genetic transformation of *P. patens* mediated by *A. tumefaciens* for *GRFT* gene transfer.
(b) Expression of GRFT gene using stable expression into *P. patens.*

3 MATERIALS AND METHODS

3.1 *IN SILICO* ANALYSIS

3.1.1 Codon usage optimization

The codon usage of a DNA sequence of *GRFT* was optimized using *P. patens* codon usage table based on chapter I section of 3.2.4.

3.1.2 mRNA structure prediction

The mRNA structure was predicted based on chapter I section of 3.2.3 .

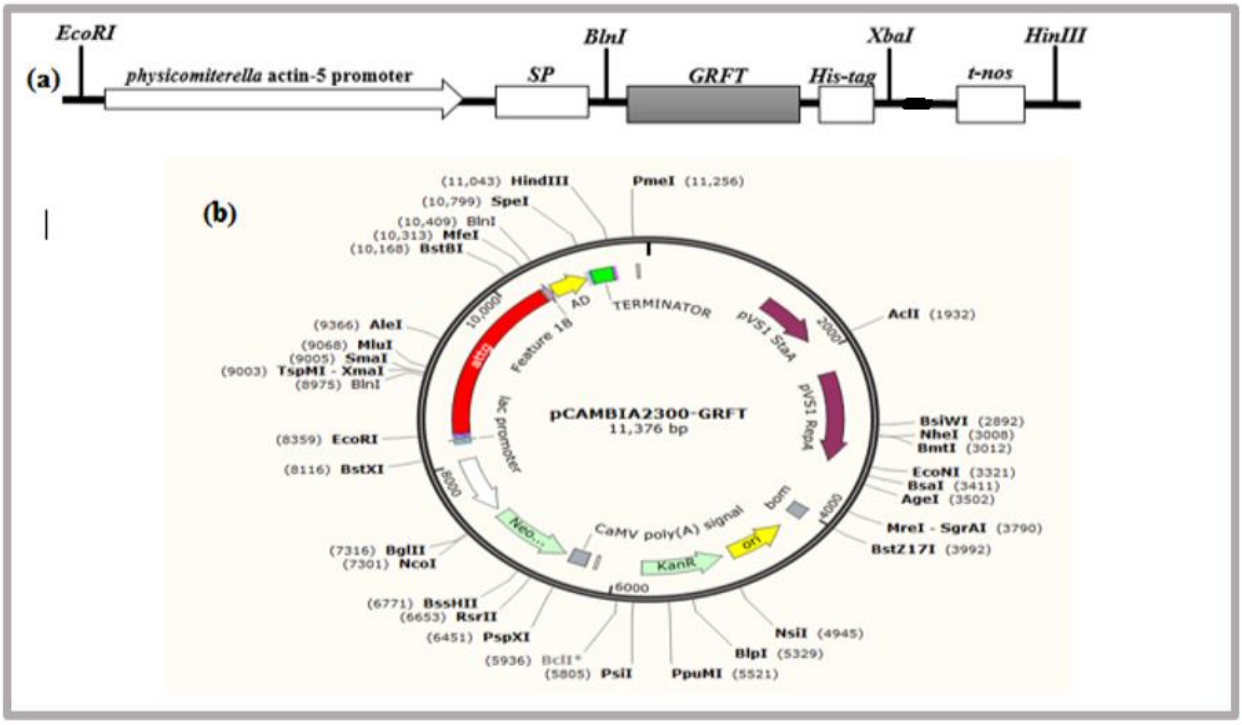
3.1.3 Signal peptide prediction

The presence and location of signal peptide cleavage sites in amino acid sequence of GRFT was performed based on chapter I section of 3.2.5.

3.2 PLASMID CONSTRUCTION

The 369 bp sequence encoding *GRFT* (accession number FJ594069) was optimized using *P. patense* codon usage table under control of the *physicomiterella* actin-5 promoter (act5P), the signal peptide encoding thaumatin-like protein To32 (GenBank accession no. AY95850) which direct protein in secretory pathway and 35S-terminator sequence were also synthesizes by Epoch Life Science Inc (Missouri City, Texas, USA). Then, the construction was cloned into expression vector pCAMBIA2300 between *EcoR*I and *Hind*III. The Histidine- tag was fused in C –terminal of *griffithsin* gene and finally the plasmid was introduced into *A. tumefaciens* (FIGURE 2.1).

FIGURE 2.1 - SCHEMATIC REPRESENTATION OF THE EXPRESSION CASSETTE USED FOR AGROBACTERIUM-MEDIATED MOSS TRANSFORMATION. (A). THE CASSETTE EXPRESSION CONTAINED THE PHYSCOMITRELLA ACTIN-5 GENE PROMOTER SIGNAL PEPTIDE OF THAUMATIN-LIKE PROTEIN TO32, THE GRFT CODING SEQUENCE, HIS-TAG_6, AND NOS-TERMINATOR (T-NOS). (B) SYNTHETIC GENE WAS CLONED INTO *ECO*RI/*HIND*III DIGESTED PCAMBIA2300.



SOURCE: Habibi (2018).

3.3 COMPETENT CELL PREPARATION

Preparation of competent cell was performed based on chapter I section of 3.9.1

3.4 PRE-TRANSFORMATION OF CHEMICALLY COMPETENT *E. COLI*

Transformation of competent cell with pCambia 2300: GRFT was performed based on chapter I section of 3.5.

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3.5 COLONY PCR CONFIRMATION

Colony PCR confirmation was performed based on chapter I section of 3.6

3.6 PLASMID ISOLATION (PLASMID MINIPREP)

Plasmid miniprep was performed based on chapter I section of 3.7.

3.7 GEL ELECTROPHORESIS OF DNA

Separation of DNA fragments was performed by agarose gel electrophoresis in 1x TAE buffer. According to the expected size of the fragment, 1–2 % agarose gels were prepared using1xTAE buffer (40 mM Tris base, 1mM EDTA). For subsequent detection of DNA, the fluorescent dye ethidium bromide was added to a final concentration of 0.5 µg/ml. Prior to loading into the gel, samples were mixed with 4x DNA-loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 0.1M EDTA and 30% glycerol) . For size determination of DNA-fragments, appropriate DNA ladders (Invitrogen) were used. The separation of DNA-fragments was visualized under UV light using Gel Doc Universal Hood II with TLUM 100/240V (Bio-Rad).

3.8 *AGROBACTERIUM* COMPETENT CELL PREPARATION AND TRANSFORMATION BY ELECTROPORATION METHOD

Agrobacterium competent cell preparation and transformation with pCAMBIA 2300:GRFT were performed based on chapter I section of 3.9.1

3.9 PCR AMPLIFICATION FROM *AGROBACTERIUM* CULTURES

The presence of *GRFT* gene into agrobacterium was confirmed based on chapter I section of 3.9.3.

3.10 PLANT CELL CULTURE AND TRANSFORMATION

In this study, a wild type (WT) strain of the moss *P. patens* (Hedw.) Bruch & Schim. was used. *P. patens* plants were axenically cultivated either on solidified Knop medium or in liquid culture (Reski and Abel, 1985). Standard cultivation conditions were at 25 ℃ undera16/8h light/dark photoperiod with a light intensity of 55 µmol/m2s (Philips

TLD 25). Liquid cultures were started by inoculation of liquid Knop medium (TABLE 2.1) with 50mg gametophore tissue. For small volumes (30–200 ml) these cultures were maintained in Erlenmeyer flasks and transferred weekly to fresh medium after mechanical disruption. Plants on solid medium were maintained by monthly subculturing onto fresh plates. For 1 L Knop medium 10 ml of the solutions 1–4 and 50 ml of solution 5 were mixed, filled ad 1 L with H2O and pH was adjusted to 5.8 with 1 M KOH.

TABLE 2.1- KNOP MEDIUM

Component	**Concentration g/L**
KH2PO4	25
KCl	25
MgSO4	25
Ca(NO3)2	100
FeSO4 x 7H2O,	250

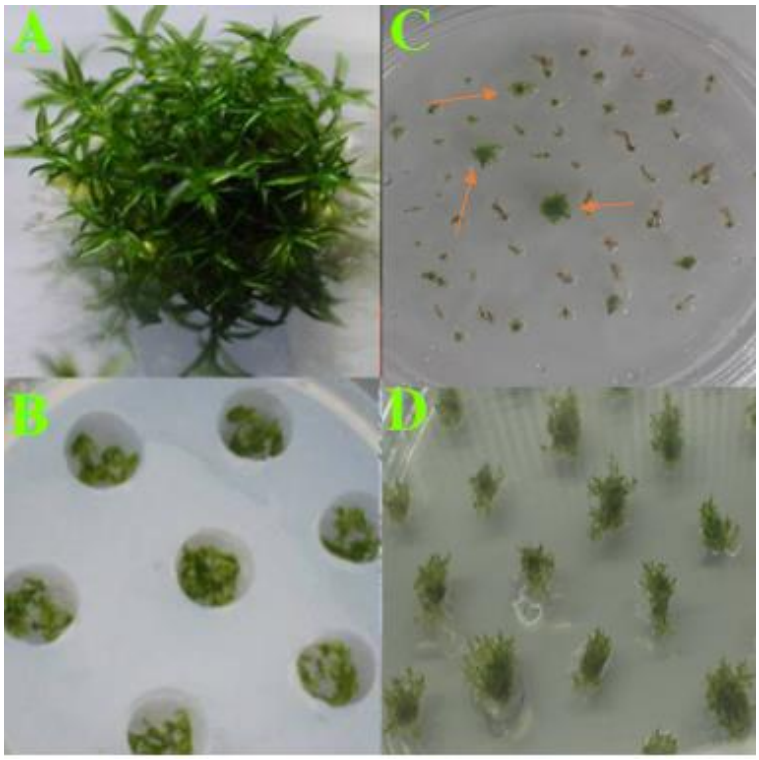
SOURCE: Reski and Abel (1985).

For the preparation of solid Knop medium 1.2 % (w/v) purified agar (sigma, USA) was added.

3.11 TRANSFORMATION OF *P. PATENS*

First, sterile Knop agar medium was poured into 12 cm Petri dishes, 10 wells in every Petri dish were drilled with 12 mm depth and 10 mm diameter at intervals of about 20 mm after the medium cooled down, then the gametophores of gametophytes about 12 leaves were inoculated into the wells with four gametophores in each well. After the gametophores were incubated for 11 days, and developed into protonema at 25°C under continuous light energy of 30 µmol m -2 s -1 from cool-white fluorescent lamps, *A. tumefaciens* at OD600 0.3 was first added into the wells three times at an interval of 12 h for infection and co-cultivation with plants. When the juvenile gametophytes grew up to about 12 leaves in the co-culture wells, their gametophores were transferred into medium supplemented with cefotaxime antibiotic to kill agrobacterium and then they were transferred into selective medium to select transgenic plants (FIGURE 2.2).

FIGURE 2.2 - GROWTH PROGRESS OF THE NORMAL AND TRANSFORMED MOSS P. PATENS. (A) NORMAL GAMETOPHYTES; (B) GAMETOPHORES INOCULATED AND CULTIVATED IN WELLS; (C) SURVIVAL PUTATIVE TRANSGENIC GAMETOPHYTES AND ALBINISTIC GAMETOPHYTES ON SELECTIVE MEDIUM CONTAINING 50 MG L -1 KANAMYCIN; (D) TRANSGENIC GAMETOPHYTES AFTER 3 TIMES OF SUBCULTURE.



SOURCE: Habibi (2018).

3.12 SCREENING OF TRANSGENIC PLANTS

To select the transgenic plants, the survival gametophores of gametophytes from co-culture wells were picked out and immediately inoculated into wells made up of selective Knop medium (FIGURE 6C). To ensure the real transgenic plants were picked out, all the survival gametophores of gametophytes from the first selection were transferred to Knop medium containing 50 mg L^{-1} kanamycin for three generations of selection, then all the transgenic plants were random picked out for molecular analysis.

3.13 RNA EXTRACTION AND cDNA SYNTHESIS

For the screening of transgene expression using reverse transcriptase- PCR, 100-200 mg fresh weight of moss extracts were frozen and grinded on liquid nitrogen and total RNA was extracted by RNeasy® plant mini kit (Qiagen- Germany) according to the manufacturer's protocol. Extracted RNA was assessed for concentration and purity with absorbance ratio of A260/A280 using NanoVue Plus Spectrophotometer and 1% agarose gel electrophoresis for checking of integrity (Data was not showed).

For the synthesis of cDNA library, 50ng of RNA were first treated with 0.2µl DNase I (Boehringer Mannheim, Germany) and incubated at 37°C for 20 min and then incubated with 1µl of primer $Nvdt_{(30)}$ (GAAGACGATTGCTCAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN) at 72°C for 3 min and immediately transferred to ice. In the next step, 4 µl of reaction buffer 5x M-MLVRT(Invitrogen-USA), 2µl of DTT 0.1M and 1µl dNTP were added to reaction and incubated in 42°C for 2min. Finally, the reaction was incubated with 1 µl of M-MLV Reverse Transcriptase (Invitrogen-USA) and incubated at 42°C for 90min and stopped at 70°C for 15min. 2 µl of synthesized cDNA was used for PCR reaction.

3.14 POLYMERASE CHAIN REACTION PCR END POINT

Genomic DNA was extracted by DNA extraction kit (Qiagen, Germany) based on manufacturer's instructions and the extracted DNA was amplified by specific primers for GRFT gene (forward primer: 5'-ATGTCTCTTACTCACAGGAAGTT-3' and Reverse primer: 5′- GTACTGCTCGTAGTAGATATCA-3′) under condition based on section

3.15 QUANTIFICATION OF NUCLEIC ACID BY SPECTROPHOTOMETRIC

Determination of nucleic acid concentration was performed with a spectral photometer using NanoVueTM UV/Visible Spectrophotometer (GE Healthcare). DNA was diluted 1:100 in H2O (or 1:50 for RNA) and absorption (A) was measured at 260 and 280 nm. Calculation of nucleic acid concentrations was based on the assumption that A260 = 1 corresponds to a DNA concentration of 50 µg/ml or a RNA concentration of 40 µg/ml, respectively.

3.16 ETHANOL PRECIPITATION OF NUCLEIC ACIDS

In order to concentrate or sterilize DNA or RNA, samples were mixed with 1/ 10 volume of 3 M sodium acetate, pH 5.2, and 2.5 volumes of ice cold 100 % ethanol and incubated on ice or at -80 ℃ for 30 min. Afterwards, the sample was centrifuged at 14000 rpm at 4 ℃ for 30 min. The supernatant was discarded and the pellet was washed with 1 ml of ice-cold 70 % ethanol and centrifuged (14000 rpm, 4 ℃, 15 min). The supernatant was removed and the pellets were dried under the clean bench (ca. 5–10 min). The pellet was resuspended in an appropriate volume of H2O or TE buffer.

3.17 REAL-TIME QUANTITATIVE PCR

For the determination of transgene expression level, RT qPCR was carried out in a 7500 fast cycling 5-color, 96 well PCR system (Life Biotechnology) using SYBER® Green plus Master Mix 2X (LGC Biotechnologia). The synthesized cDNA (1µl) was diluted in 20µl of H_2O and each sample of cDNA was run thrice on thermocycler well for amplification. The system was ran at 94°C for 15s, 59°C for 30s and 30 cycles and 72°C for 30s. Quantitation-comparative analysis of each cDNA sample was performed using GRFT-specific primer (Forward: 5’-ATGTCTCTTACTCACAGGAAGTT-3’ and Reverse: 5'- GTACTGCTCGTAGTAGATATCA-3') and specific primers of two candidate reference genes serine threonine protein phosphatase 2a (ST-P 2a) and v-Type h+-translocating pyrophosphatase (vH+PP) based on previous work by (Le Bail et al., 2013).

TABLE 2.2 - PRIMERS FOR REFERENCES GENES OF *P.PATENS*

vH+PP forward	GCAAGCCAATCAGTACACTC
vH+PP reverse	5´ATCTTAGCCAACAACCAATAACC3´
Ade PRT forward	AGTATAGTCTAGAGTATGGTACCG
Ade PRT reverse	5´TAGCAATTTGATGGCAGCTC 3´

SOURCE: Habibi (2018).

3.18 PROTEIN EXTRACTION

Fresh frozen moss tissue was ground with mortar and pistil in liquid nitrogen and aliquots of 1g were transferred to a 15 ml falcon tube. Aliquots were either stored at -80 ℃ or directly used. 2 ml of different protein extraction buffer (TABLE 2.3) were pipetted to 1g aliquot and buffer and tissue powder were homogenized by vigorous vortexing and afterwards centrifuged at 8000g in a precooled (4 ℃) centrifuge in order to pellet the cell debris. The supernatant was transferred into a new 15 ml falcon tube and directly used in SDS-PAGE (cf. 2.3.4) or stored at -20 ℃ for later use.

TABLE 2.3 – LIST OF SOLUTION USED FOR PROTEIN EXTRACTION

Buffer 1	**Buffer 2**	**Buffer 3**	**Buffer 4**	**Buffer 5**	**Buffer 6**	**Buffer 7**
Urea 9M	HEPS 1M	Mgcl2 5mM	Tris 100 Mm	k2HPO4	NA2HPO4	NaH2PO4 5mM
SDS1%	PVPP 2%	DTT 4mM	Potassium fluoride 100Mm	KH2PO4	NaHPO4	NaCl 300mM
DTT 1%	TRITON 0.5M	$Na_2S_2O_5$	EDTA 5Mm	ASCORBIC ACID	Urea 9M	Imidazole 20mM
Triton 2%	E.64 1mM	Sucrose 10%	Sodium ascorbate 40 Mm	EDTA	EDTA	
Pepstatin A 5µM	DTT2mm	PVP 3%	BSA 0.2%	PMSF 2Mm	SDS 10%	
EDTA	-	-	PVPP 1%			
PMSF	-	-	-			

SOURCE : Habibi (2018).

3.19 TOTAL SOLUBLE PROTEIN QUANTIFICATION

Protein concentration was assayed based on (Bradford, 1976) method using SpectraMax® 190 Absorbance Plate Reader (Molecular Devices, USA).

3.20 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

To determine accumulation of GRFT in transgenic plants, ELISA protocol was performed as described previously (Mori et al., 2005). A 96 well plate was coated by 100 ng of extracted protein from transgenic plants and incubated in carbonate/bicarbonate

buffer (15 mM Na2CO3 and 35 mM NaHCO3, pH 9.6) overnight at 4°C. Wells were rinsed 3 times with PBS containing 0.1% Tween-20 (PBST) and then blocked with 1% bovine serum albumin (BSA) for 2 hours at 37°C. Plates were added with primary anti-GRFT rabbit polyclonal antibody with dilution of 1:1000 for 2:30 hr at 37°C and then washed 3 times with PBST. Wells were incubated with the secondary horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody with dilution of 1:5000 for 1 hr in 1% PBST. Finally, after washing 3 times with PBST, the substrate 3,30,5,50-tetramethylbenzidine (TMB) in buffer acid citric /sodium phosphate dibasic (pH 5) and H2O2 (30 %) was added to reaction and incubated at least for 15 min in the dark and the reaction was stopped with 5M H2SO4 and the absorbance was read on OD450 nm

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Carbonate/bicarbonate buffer: 50mM C03/HCO3 pH 9.6

1-Na2Co3 1.32 gr

2-NaHco3 1.05 gr

Adjust to 250 mL H20

4 RESULTS

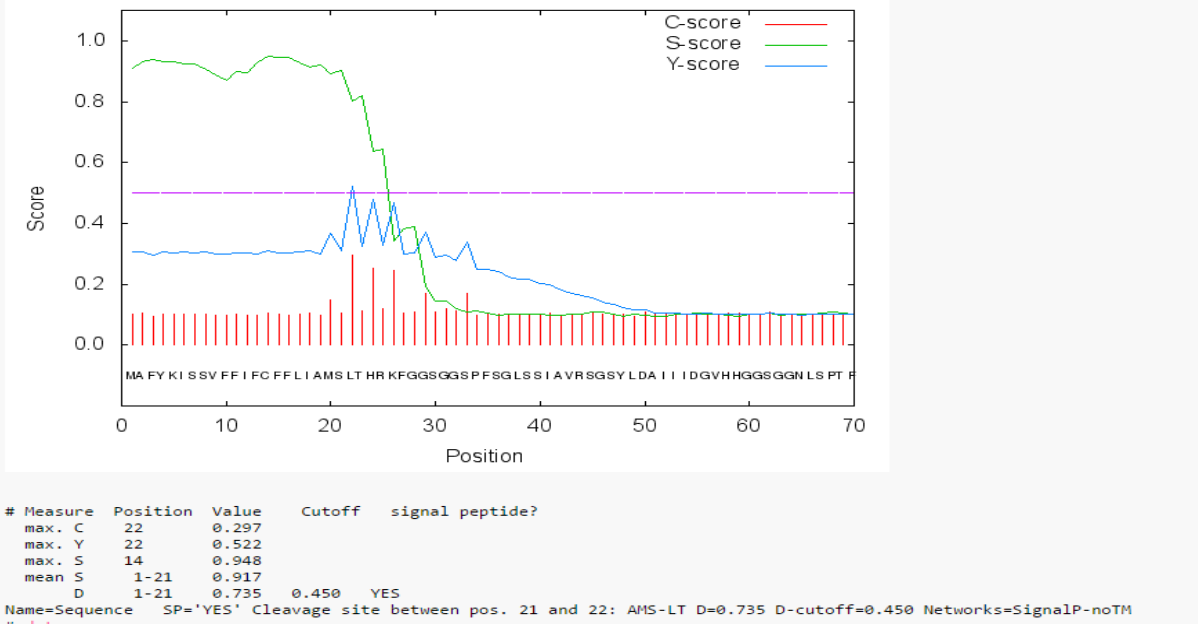
The 369 bp sequence encoding *GRFT* (accession number FJ594069) was optimized using *P. patens* codon usage table (FIGURE 2.3) and then the protein obtained after translation with maximum amino acid sequences was selected by open reading frame finder (FIGURE 2.4). Subcellular localization prediction for *GRFT* gene was performed by signal peptide prediction of thaumatin-like protein To32. In the case of GRFT, the in *silico* targeting prediction was confirmed in secretory pathway (FIGURE 2.5).

FIGURE 2.3 - OPTIMIZATION OF THE CODON USAGE OF A GRFT DNA SEQUENCE TO INCREASE ITS EXPRESSION LEVEL CAI: CODON ADAPTATION INDEX IS EFFECTIVE MEASURE OF SYNONYMOUS CODON USAGE BIAS. THE INDEX USES A REFERENCE SET OF HIGHLY EXPRESSED GENES FROM A SPECIES TO ASSESS THE RELATIVE MERITS OF EACH CODON, AND A SCORE FOR A GENE IS CALCULATED FROM THE FREQUENCY OF USE OF ALL CODONS IN THAT GENE. THE INDEX ASSESSES THE EXTENT TO WHICH SELECTION HAS BEEN EFFECTIVE IN MOULDING THE PATTERN OF CODON USAGE. ENC: EFECTIVE NUMBER OF CODONS. %GC: G+C PERCENTAGE. %AT: A+T PERCENTAGE.

Type	Sequences	CAI	ENc	%GC	%AT
Query	ATGTCTCTTACTCACAGGAAGTTCGGAGGATCTGGAGGTTCTCCATTCTCTGGACTTTCT TCTATTGCTGTGAGGAGTGGATCTTACCTTGATGCTATTATTATTGATGGAGTGCACCAC GGAGGAAGTGGTGGAAATCTTTCTCCAACTTTCACTTTCGGATCTGGAGAGTACATTTCT AACATGACTATTAGGAGTGGAGATTACATTGATAACATTTCTTTCGAGACTAACATGGGA AGGAGATTCGGACCATACGGAGGTTCTGGAGGATCTGCTAACACTCTTTCTAACGTGAAA GTGATTCAGATTAACGGATCAGCTGGAGACTACCTTGATTCTCTTGATATCTACTACGAG CAGTACTGA	0.952	22	42.5	57.5
Optimized	ATGTCTTTGACCCACAGGAAGTTCGGAGGATCTGGAGGATCTCCTTTCTCTGGATTGTCT TCTATTGCTGTGAGGTCTGGATCTTACTTGGATGCTATTATTATTGATGGAGTGCACCAC GGAGGATCTGGAGGAAACTTGTCTCCTACCTTCACCTTCGGATCTGGAGAGTACATTTCT AACATGACCATTAGGTCTGGAGATTACATTGATAACATTTCTTTCGAGACCAACATGGGA AGGAGGTTCGGACCTTACGGAGGATCTGGAGGATCTGCTAACACCTTGTCTAACGTGAAG GTGATTCAGATTAACGGATCTGCTGGAGATTACTTGGATTCTTTGGATATTTACTACGAG CAGTACTGA	1.000	20	44.4	55.6

SOURCE: Puigbo et al. (2005).

FIGURE 2.4 - SIGNAL PEPTIDE PREDICTION OF THAUMATIN-LIKE PROTEIN TO32. THE FIGURE CONFIRMS THE PRESENCE OF THE SIGNAL PEPTIDE, REPRESENTED BY THE CLEAVAGE SITE IN THE POSITION BETWEEN RESIDUES 21 AND 22. C-SCORE DISTINGUISH SIGNAL PEPTIDE CLEAVAGE SITES FROM EVERYTHING ELSE. THE C-SCORE IS TRAINED TO BE HIGH AT THE POSITION IMMEDIATELY AFTER THE CLEAVAGE SITE (THE FIRST RESIDUE IN THE MATURE PROTEIN). S-SCORE (SIGNAL PEPTIDE SCORE) THE OUTPUT FROM THE SP NETWORKS, WHICH ARE TRAINED TO DISTINGUISH POSITIONS WITHIN SIGNAL PEPTIDES FROM POSITIONS IN THE MATURE PART OF THE PROTEINS AND FROM PROTEINS WITHOUT SIGNAL PEPTIDES. Y-SCORE (COMBINED CLEAVAGE SITE SCORE).A COMBINATION (GEOMETRIC AVERAGE) OF THE C-SCORE AND THE SLOPE OF THE S-SCORE, RESULTING IN A BETTER CLEAVAGE SITE PREDICTION THAN THE RAW C-SCORE ALONE. THIS IS DUE TO THE FACT THAT MULTIPLE HIGH-PEAKING C-SCORES CAN BE FOUND IN ONE SEQUENCE, WHERE ONLY ONE IS THE TRUE CLEAVAGE SITE. THE Y-SCORE DISTINGUISHES BETWEEN C-SCORE PEAKS BY CHOOSING THE ONE WHERE THE SLOPE OF THE S-SCORE IS STEEP.THE AVERAGE S-SCORE OF THE POSSIBLE SIGNAL PEPTIDE (FROM POSITION 1 TO THE POSITION IMMEDIATELY BEFORE THE MAXIMAL Y-SCORE).D-SCORE (DISCRIMINATION SCORE).A WEIGHTED AVERAGE OF THE MEAN S AND THE MAX. Y SCORES. THIS IS THE SCORE THAT IS USED TO DISCRIMINATE SIGNAL PEPTIDES FROM NON-SIGNAL PEPTIDES.FOR NON-SECRETORY PROTEINS ALL THE SCORES REPRESENTED IN THE SIGNALP OUTPUT SHOULD IDEALLY BE VERY LOW (CLOSE TO THE NEGATIVE TARGET VALUE OF 0.1).

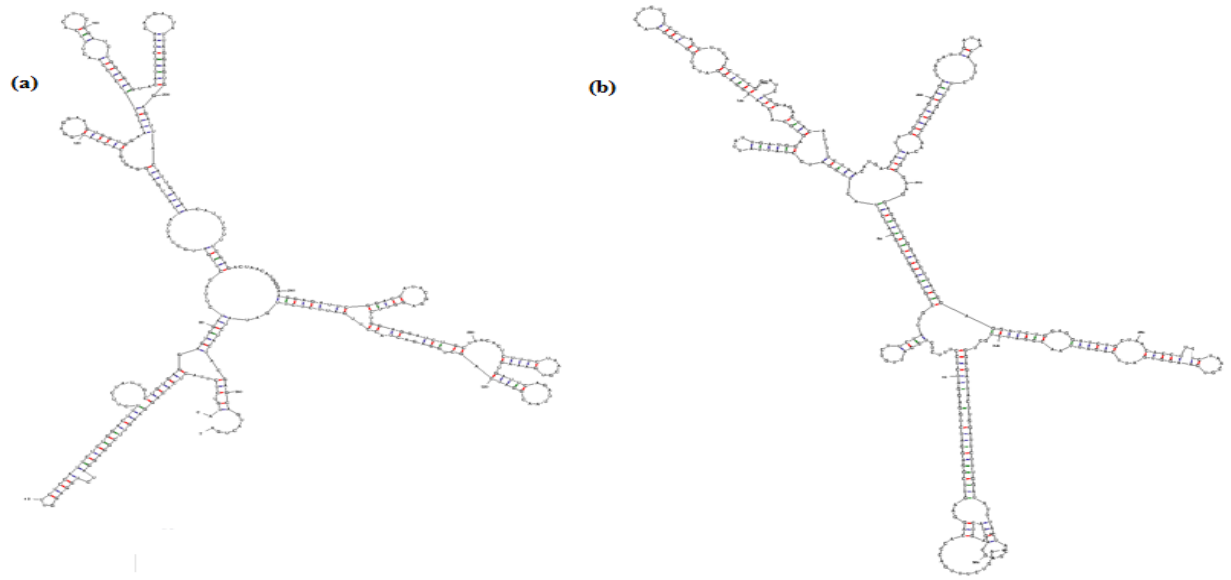


SOURCE: Nielsen (2017).

Generally, mRNA secondary structures like hairpin, loop, stem will cause interference with the translation of protein. The mRNAs are the most complex group of cellular RNAs that originated not only from transcription itself but also from numerous

modification reactions, such as precursor mRNA (pre-mRNA) splicing, capping, polyadenylation, and 3´ end processing. Furthermore, the association of mRNAs with protein complexes and factors results in the regulation of mRNA translation and metabolism (Wachter, 2014).Therefore, screening the functional capabilities of RNA folding might identify sequence features that contribute to gene regulation in plant molecular farming. Given a DNA or RNA sequence, the secondary structure can be predicted and thus the relative translation efficiency (eg, translation initiation rate) can be predicted. mRNA folding prediction with the mfold software was performed to compare the structure of GRFT sequence in optimized and non-optimized status(FIGURE 2.5).

FIGURE 2.5 - MRNA SECONDARY STRUCTURE (A) MRNA SECONDARY STRUCTURE OF QUERY (GRFT) WITH INITIAL ΔG = -106 KCAL/MOL AND (B) MRNA SECONDARY STRUCTURE OF OPTIMIZED GRFT GENE WITH INITIAL ΔG = -120.20 KCAL/MOL. ΔG IS DEFINED MINIMUM FREE ENERGIES FOR FOLDING THAT MUST CONTAIN ANY PARTICULAR BASE PAIR.



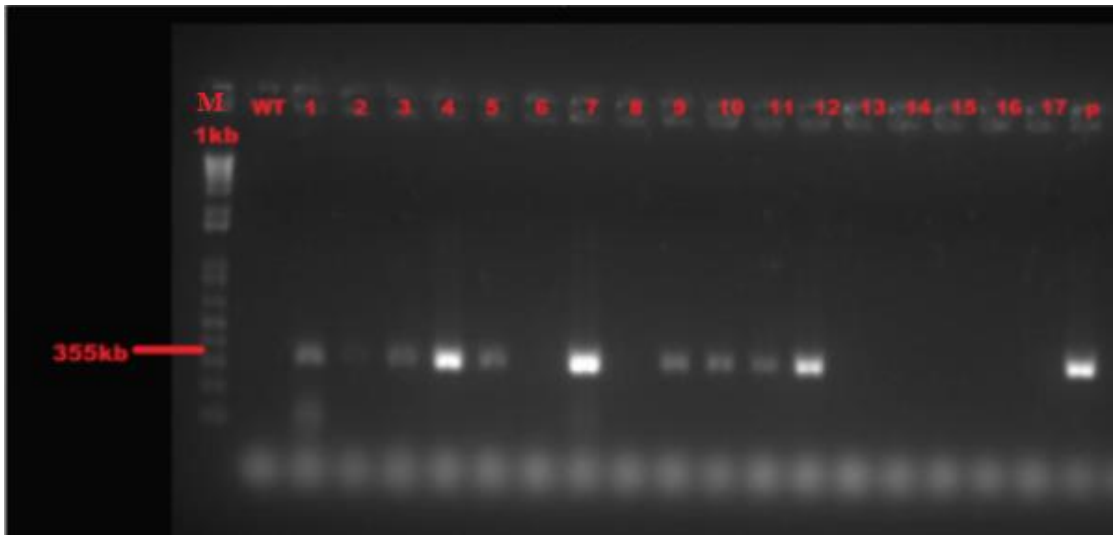
SOURCE: Zuker (2003).

4.1 STABLE PRODUCTION OF GRFT IN MOSS BIOREACTOR

An expression cassette with specific regulatory elements was constructed for moss transformation. The GRFT gene encoding 122 amino acid synthesized under control of *physicomiterella* actin-5 gene promoter (Ppact5-P) and signal peptide of

thaumatin-like protein To32. For protein retention in the endoplasmic reticulum, a synthetic tetrapeptide sequence of KDEL was fused to the 3′ end of GRFT gene. The resulting expression cassette was transferred into the pCambia 2300 vector (pCAMBIA) and finally transferred into *Agrobacterium* by electroporation (Main et al., 1995). Eleven day old gametophores of *P.patens (*150 independent gametophytes*)* were transformed with *agrobacterium* strategy to generate transgenic plants. Transgenic gametophytes were selected on kanamycin-supplemented medium for at least 8 weeks and finally 80 survival putative gametophytes were regenerated and subcultured into medium lacking antibiotic (FIGURE 2.2). Primary identification of transformants was carried out to confirm the presence of 366bp of *GRFT* gene into DNA genomic of *P. patens* using direct PCR (FIGURE 2.6) and the stable integration of GRFT cDNA was approved for 17 transgenic plants.

FIGURE 2.6- AGAROSE GEL ELECTROPHORESIS OF PCR PRODUCTS FOR *GRFT* GENE. M AS 1KB DNA LADDER, LANES 1,2,3,4,5,7,9,10,11,12 : AMPLIFICATION PRODUCT OF 355BP GRFT, LANE WT: NEGATIVE CONTROL. LANES 6,8,13,14,15,16,17: TRANSGENIC PLANT LACKING DESIRED 355BP, LANE P POSITIVE CONTROL.



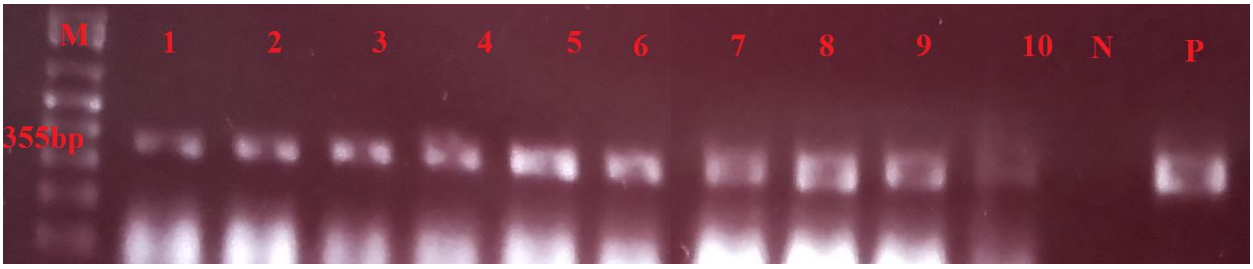
SOURCE: Habibi (2018).

4.2 RT-PCR

Total RNA samples were prepared from tissues of *P.patens* and used for RT-PCR analysis. For reverse transcription (RT) first strand cDNA synthesis was performed.

Subsequently, the primers GRFT for and GRFTrev corresponding to the constitutively expressed gene for *griffithsin* gene were used in a standard PCR with the synthesized cDNA as a control (FIGURE 2.7). cDNA intended for the construction of expression constructs was amplified according to standard PCR.

FIGURE 2.7 AGAROSE GEL ELECTROPHORESIS OF RT-PCR-AMPLIFIED 355-BP GRFT CDNA. LANE M AS 1KB MARKER, LANES 1-10 TRANSGENIC PLANT WITH DESIRED 355BP BAND, LANE N NON-TRANSGENIC PLANT AS NEGATIVE CONTROL AND LANE P PLASMID AS POSITIVE CONTROL.

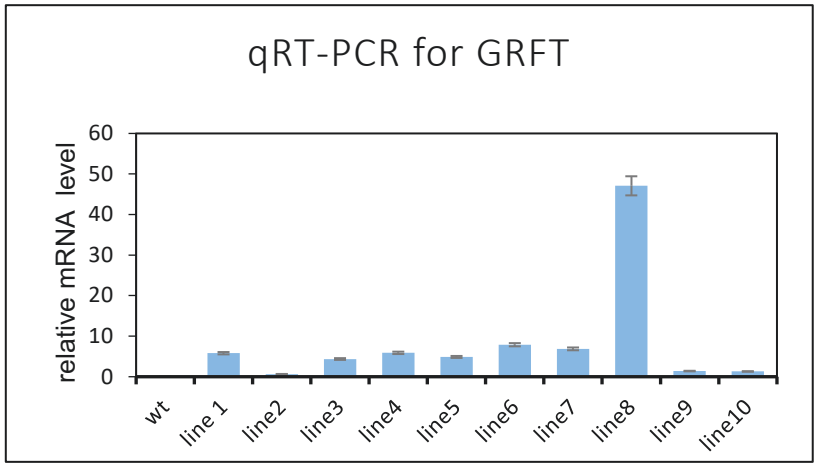


SOURCE: Habibi (2018).

4.3 REAL-TIME QUANTITATIVE PCR

The steady state of GRFT transcript levels was determined for ten transgenic *P.patens* cell lines by quantitative real-time RT-PCR. RNA was extracted and used as a template for qRT-PCR with primers specific for the *GRFT* coding sequence. Delta cycle threshold values were calculated and graphed in FIGURE 15. No *GRFT* transcript was detected in wild-type tissue. Low levels of *GRFT* transcript were detected in line 2 and *GRFT* transcript level was highest in transgenic line 8 ($P < 0.05$) (FIGURE 2.8).

FIGURE 2.8 - RELATIVE MRNA EXPRESSION LEVELS OF GRFT TRANSCRIPT IN 10 TRANSGENIC *P.PATENS*. LINE WT AS NON-TRANSGENIC PLANT. VALUES ARE THE MEAN ± SD (STANDARD DEVIATION) OF 3 INDEPENDENT QPCR EXPERIMENTS.

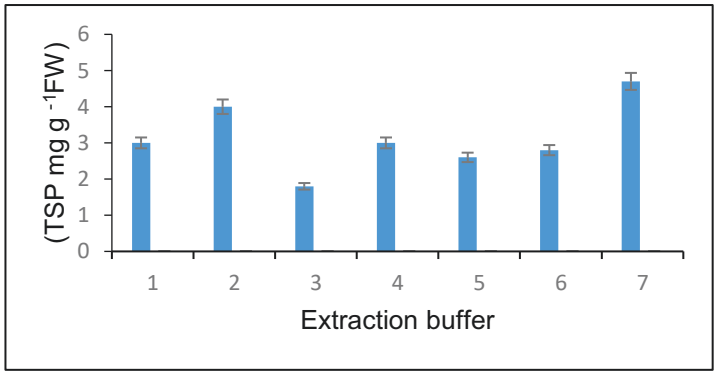


SOURCE: Habibi (2018).

4.4 OPTIMIZATION OF BUFFER EXTRACTION FOR TOTAL SOLUBLE PROTEIN

For extracting high total soluble protein from moss plant, transgenic gametophytes (1g) were grounded in liquid nitrogen and suspended into 500 µl of extraction buffers. The high level of total soluble protein was achieved by buffer 7 (NaH2PO4 5 Mm, NaCl 300 mM, Imidazole 20 Mm, pH 7) (FIGURE 2.9). Then this buffer was used for extraction of total soluble protein.

FIGURE 2.9 - THE EFFECT OF DIFFERENT EXTRACTION BUFFERS ON TOTAL SOLUBLE PROTEIN IN TRANSGENIC MOSS.

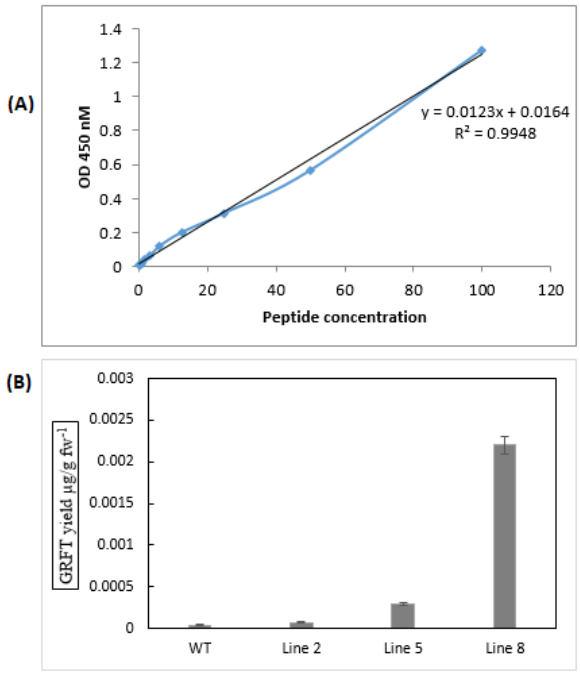


SOURCE: Habibi (2018).

4.5 ELISA ANALYSIS

To detect correctly folded and expression level of GRFT, quantitative ELISA was applied by grounding of transgenic gametophores (line 6, 7 and 8) (200 mg) in liquid nitrogen. The well-grounded gametophores were homogenized in PBS buffer supplied with 1 mM PMSF as a proteases inhibitor. The extracted protein was centrifuged at 8000g and then quantified using the Bradford colorimetric assay (Bradford, 1976). The ELISA experiments were performed on three biological replicas. Primary anti-GRFT rabbit polyclonal antibody with dilution of 1:1,000 and the secondary horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody with dilution of 1:1,000 were used as capture antibodies. The result of ELISA showed low amount of GRFT expression in three lines. The highest level of expression (0.00018 µg protein per gr fresh weight of protonema were obtained in the moss line 8 (FIGURE 2.10).

FIGURE 2.10- (A) STANDARD CURVE USING GRFT SYNTHETIC PEPTIDE TO ESTIMATE GRFT CONTENT. (B) GRFT YIELDS ESTIMATED IN THE TRANSFORMED MOSS CLONES.



SOURCE: Habibi (2018).

5 DISCUSSION

The moss *P. patens* meet the requirements for a safe and flexible biopharmaceutical production platform. Outstanding genetic resources and well developed molecular tools are available and the predominantly haploid life cycle combined with a high rate of homologous recombination in mitotic cells makes custom designed, targeted modifications possible (Decker and Reski 2007, 2008; Mueller et al. 2014). The present study focused on moss as a convenient platform with the following advantages: strains can be propagated in vitro under full containment according to GMP conditions and moss biomass has no apparent toxic effects, allowing for immunization with raw moss material. In addition, *P. patens* performs post-translational protein N-glycosylation preferentially of the complex type (Koprivova et al. 2003), identical to that of higher plants, which may have effects on immunogenicity (reviewed in Rosales-Mendoza et al. 2014a).

The lectin GRFT is one of the potent HIV inhibitory candidate isolated from red algae which effectively binds to the HIV envelope protein gp120 and prevent virus infection (Xue et al., 2013; Moulaei et al., 2015).Based on cell analysis, inhibitory effects of GRFT against HIV infection has been indicated at picomolar concentrations (EC50 of ~50 pM) (Mori et al., 2005; Giomarelli et al., 2006). Beside lacking of toxicity and immunogenicity of GRFT, its thermostability characteristic in a wide range of conditions as well as low cost and large scale production of GRFT by plant platform, make GRFT an interesting candidate in development of antiviral therapeutic concept (O'Keefe et al., 2009;Kouokam et al., 2011;Barton et al., 2014).

Although the production and purification of native GRFT from red algae has been reported but only at low concentration (Mori et al., 2005;Giomarelli et al., 2006). Therefore, the large scale production of GRFT using plant platform might open up new avenue for commercialization of this protein as potent HIV inhibitory candidate. Previously, the production and purification of GRFT has been reported in cytoplasm of *E. coli* system (Giomarelli et al., 2006) .The production of GRFT into inclusion bodies of *E. coli* is a main challenge in development of *E. coli* system for production of GRFT in large scale as using various detergents and highly polymerized cyclo-amylose as a refolding

agent or co-expression of His-GRFT with chaperone components such as DnaK-DnaJ-GrpE and/or GroEL-GroES did not result in a sufficient recovery of biologically active GRFT (Giomarelli et al., 2006). To improvement of GRFT production, using transient expression system in *N. benthamiana* and *O. sativa* has been addressed by (O'Keefe et al., 2009 and Vamvaka et al., 2016), respectively. Even so, the purification of GRFT by *N.benthamiana* involved with some significant barriers including contamination with large and small subunits of RuBisCO, contamination with TMV coat protein (Fuqua et al., 2015), protein degradation which was reduced to 0.3 mg/g after purification and the presence of toxic and endotoxin metabolites (Vamvaka et al., 2016). Although, seed based expression system was showed a high degree of versatility for production of recombinant proteins because of protein stability at ambient temperature (Obembe et al., 2011), but the long-term process of seed production and space requirements may be preclusive (Boothe et al., 2010). In concept of technical advance, *P.patens* is one of the promising expression platform which provided many advantageous in terms of molecular farming including protein accumulation in cell suspension, gene targeting by homologous recombination for glycoengineering and quantitative optimization of moss as a bioreactor for protein production (Lienard and Nogue, 2009). Our expression approach was based on the secretion signal peptide that directs the protein to the complex processing machinery, allowing for glycosylation and complex folding in the ER and Golgi apparatus.

Transgenic gametophores of *P. patens* were induced to express GRFT under control of *physicomiterella* actin-5 gene promoter (Ppact5-P) and signal peptide of thaumatin-like protein To32. In this study, we considered the transformation of *P. patens*, via Agrobacterium. The transformation of *P. patens* via *A. tumefaciens* was reported previously (Li et al., 2010). Similarly, the integration of exogenous GRFT to genomic DNA *P. patens* was confirmed by PCR.

Around 80 survival putative transgenic gamatophores were regenerated and line 8 was selected for further studies as it showed highest amount of GRFT production (µg/g dry weight). Previously, the best performance of GRFT production after purification in *N. benthamiana* (300 µg/g) and *O. sativa* (223 µg/g dry seed weight) was reported. In this study, GRFT was produced in significantly lower amounts in *P. patens* plants.

Direct comparison of recombinant protein production rates in plants is limited as the plant species used not only fundamentally differ (e.g. tobacco, rice, carrot, duckweed, moss). But, moreover, approaches greatly vary in terms of expression strategy (e.g. transient/stable, type of promoter/enhancer), subcellular (e.g. extracellular, ER, apoplast, chloroplasts) or tissue specific (e.g. Leaves, seed, roots) targeting of the recombinant protein, the protein of interest itself (e.g. size, complexity) or culture conditions (e.g. green-house, bioreactor). Furthermore, the declaration of recombinant protein yields is not standardized and may e.g. be given as grams per dry or fresh weight, as units of activity or perceptually per total soluble protein.

In this context, the amounts of recombinant product need to be increased by several techniques. This include using of components of the transcription, translation and secretion machineries, originally developed and optimized for recombinant production in moss. Additionally, using of bioreactor for large-scale processes have to be implemented. Since light intensity and illumination wavelength are key cultivation factors due to their effect on growth and chlorophyll formation kinetics of the moss *P. patens*, then further optimization could be considered as the most effective way to reach high growth and subsequently high level of recombine production.

6 PERSPECTIVES

Using of other strong promoters as well as other expression vector for moss transformation and also optimization of *P. patens* cultivation using of bioreactor system may help to improve the amount of GRFT yield. In this context, in our laboratory we are studying the effect of other expression vector and bioreactor system on production of recombinant GRFT. Moreover, the characterization of the produced GRFT by moss transgenic is in progress.

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