PRODUCTION OF RECOMBINANT PROTEIN LTB-CTLA4 BY TRANSIENT PLANT-BASED EXPRESSION USING GEMINIVIRAL VECTOR

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การผลิตรีคอมบิแนนท์โปรตีนแอลทีบี-ซีทีแอลเอ 4 โดยการแสดงออกชั่วครู่ในพืชโดยมีตัวนำยีนจากเจมิไนไวรัส

น.ส.สุธิตา เยี่ยมฉวี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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By	Miss Sutita Yiemchavee
Field of Study	Pharmaceutical Sciences and Technology
Thesis Advisor	Associate Professor WARANYOO PHOOLCHAROEN, Ph.D.

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สุธิตา เยี่ยมฉวี : การผลิตรีคอมบิแนนท์โปรตีนแอลทีบี-ซีทีแอลเอ 4 โดยการแสดงออกชั่วครู่ในพืชโดยมีตัวนำยีนจากเจมิไนไวรัส. (PRODUCTION OF RECOMBINANT PROTEIN LTB-CTLA4 BY TRANSIENT PLANT-BASED EXPRESSION USING GEMINIVIRAL VECTOR) อ.ที่ปรึกษาหลัก : รศ. ดร.วรัญญู พูลเจริญ

โรคมะเร็งเป็นสาเหตุการเสียชีวิตอันดับต้นของประชากรโลก ในปี ค.ศ.2018 มีผู้ป่วยที่เสียชีวิตด้วยโรคมะเร็งถึง 9.6 ล้านคน ในปัจจุบันมีการนำวิธีต่าง ๆ มาใช้ในการรักษามะเร็ง โดยเมื่อไม่นานมานี้ได้มีการนำภูมิคุ้มกันบำบัดมาใช้ในการรักษาโรคมะเร็งได้อย่างมีประสิทธิพล โดยการปิดกั้น immune checkpoint เป็นหนึ่งในภูมิคุ้มกันบำบัดที่สามารถลดการเสียชีวิตของผู้ป่วยมะเร็งได้อย่างมีนัยสำคัญ โดยกลไกล immune checkpoint เป็นระบบที่ควบคุมภูมิคุ้มกันในร่างกายไม่ให้ร่างกายตอบสนองต่อภูมิคุ้มกันตนเองมากจนทำร้ายเซลล์ของร่างกาย ในขณะที่เซลล์ภูมิคุ้มกันบางประเภทสามารถทำลายเซลล์มะเร็งได้การยับยั้งกลไกล immune checkpoint โดยหนึ่งใน immune checkpoint ที่มีการนำมาพัฒนาในการรักษาโรคมะเร็งที่สำคัญ คือ โปรตีน cytotoxic T-lymphocyte antigen-4 หรือ CTLA-4 เป็นโปรตีนที่พบได้บนผิวเซลล์เม็ดเลือดขาวที่มีบทบาทในระบบ immune checkpoint โดยการทำหน้าที่ยับยั้งการเจริญเติบโตและการแบ่งตัวของเม็ดเลือดขาวประเภท cytotoxic T-lymphocyte ซึ่งเป็นเม็ดเลือดขาวที่มีหน้าที่ทำลายเซลล์ติดเชื้อหรือเซลล์มะเร็ง เมื่อยับยั้งหรือลดการทำงานของโปรตีน CTLA-4 จะสามารถเหนี่ยวนำให้เกิดการเพิ่มการสร้างภูมิคุ้มกันเพื่อต่อต้านเซลล์มะเร็งได้ โดยยารักษาประเภทโมโนโคลนอลแอนติบอดีที่จำเพาะต่อโปรตีน CTLA-4 สามารถยับยังการเจริญเติบโตของเซลล์มะเร็งในผู้ป่วยได้อย่างมีประสิทธิผล แต่อย่างไรก็ตามยังมีข้อจำกัดในด้านปริมาณในการให้ยาที่ถี่และปริมาณมาก ในปัจจุบันการผลิตชีวเภสัชภัณฑ์ในพืชได้รับความสนใจมากขึ้น โดยพืชสามารถใช้เป็นฐานการผลิตโปรตีนโครงสร้างซับซ้อนได้โดยไม่ต้องอาศัยสถานที่หรือเครื่องมือซับซ้อนราคาแพง งานวิจัยฉบับนี้ได้มีการออกแบบรีคอมบิแนนท์โปรตีน LTB-CTLA4 โดยมีโปรตีน CTLA-4 เชื่อมต่อกับ heat-labile enterotoxin B subunit (LTB) ผ่านวิธีการแสดงออกแบบชั่วครู่โดยใช้ตัวนำยีนจากเจมิไนไวรัส สามารถผลิตรีคอมบิแนนท์โปรตีนในพืช *Nicotiana benthamiana* ได้ปริมาณสูงสุดที่ 12.9 ไมโครกรัมต่อกรัมของน้ำหนักพืชสด นอกจากนี้โปรตีน LTB-CTLA4 ที่ผลิตในพืชสามารถเหนี่ยวนำหนู BALB/c ให้สร้างแอนติบอดีชนิด IgG แบบจำเพาะต่อ CTLA-4 โปรตีน โดย LTB-CLTA4 สามารถนำไปพัฒนาต่อยอดวิจัยวัคซีนมะเร็งเพื่อใช้เสริมการรักษาหรือป้องกันโรคมะเร็งชนิดต่าง ๆ สำหรับมนุษย์ได้ในอนาคต

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Sutita Yiemchavee : PRODUCTION OF RECOMBINANT PROTEIN LTB-CTLA4 BY TRANSIENT PLANT-BASED EXPRESSION USING GEMINIVIRAL VECTOR. Advisor: Assoc. Prof. WARANYOO PHOOLCHAROEN, Ph.D.

Cancer is one of the leading global causes of death. In 2018, WHO estimated that 9.6 million patients died from cancer, with 18.1 million new cases emerged. In recent years, immunotherapy is considered as a treatment improving cancer-related immune function by manipulating the immune checkpoints in the immune system. Cytotoxic-T-lymphocyte antigen 4 (CTLA-4) is a immune checkpoint transmembrane protein. The expression of CTLA-4 on the surface of activated T-cell could downregulate the production and proliferation of cytotoxic T-lymphocyte cells (CTLs). CTLs are the host-defended immune cells that could retard the growth of cancer cells by recognizing the tumor-associated antigens expressed on the membrane of cancer cells, then eliminate them before spreading and evading throughout the body. The overexpression of CTLA-4 founded in cancer has a suppressive effect on cytotoxic T-lymphocyte cells, encouraging the tumor cell's growth. Recently, the monoclonal antibodies targeting CTLA-4 have successfully proved to be an active agent in immunotherapy. However, the large amount and multiple dosing had been a major hindrance. In this study, we developed heat-labile enterotoxin B subunit (LTB) fused CTLA-4 or LTB-CTLA4 as a cancer vaccine, to reduce the disadvantage in term of quantity, price, and frequency of dosing. LTB-CTLA4 was produced in *Nicotiana benthamiana* plant as a bio-factory, a promising platform for the plant-derived biopharmaceutical production which has abilities to produce high value but cost-effective vaccines. The highest LTB-CTLA4 level reached 1.29 µg per gram plant fresh weight and induced BALB/c mice to produce specific IgG against CTLA-4. LTB-CTLA4 has a potential as a candidate agent for cancer vaccine either to prevent the development of cancer or to treat cancer in the future.

Field of Study:	Pharmaceutical Sciences and Technology	Student's Signature .....
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Sutita Yiemchavee

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ABBREVIATIONS

APC	Antigen-presenting cell
B-cell	B-lymphocyte
BeYDV	Bean yellow dwarf virus
BIP1	Binding protein 1
bp	Base pair
BSA	Bovine serum albumin
CaMV	Cauliflower mosaic virus
CD	Cluster of differentiation
CHO	Chinese Hamster ovary
cm	Centimeter
CMI	Cell-mediated immunity
CR	Common region
CT	*Vibrio cholera* heat-labile enterotoxin
CTA	*Vibrio cholera* heat-labile enterotoxin A subunit
CTB	*Vibrio cholera* heat-labile enterotoxin B subunit
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CTLA4p	Cytotoxic T-lymphocyte antigen 4 peptides
dpi	Days post-infiltration
ED44Her2	Human epidermal growth factor receptor-2 fragment
ELISA	Enzyme-linked immunosorbent assay

ER	Endoplasmic reticulum
ETEC	Enterotoxigenic *Escherichia coli*
FDA	Food and Drug Administration
FW	Leaf fresh weight
g	Gram
GFP	Green fluorescent protein
GM1	Monosialotetrahexosylganglioside
HA	Influenza hemagglutinin
HER2	Human epidermal growth factor receptor-2
HIV	Human immunodeficiency virus
HLT	Heat-labile enterotoxin
His tag	Histidine tag
HSC	Multipotent hematopoietic stem cell
HspL	Small heat-shock protein
IgG	Immunoglobulin G
IFA	Incomplete Freund´s Adjuvant
IL	Interleukin
IMAC	Immobilized metal affinity chromatography
INF	Interferon
IPTG	Isopropyl-β-D-thiogalactopyranoside
IR	Intergenic region

kbp	Kilobase pair
kDa	Kilodalton
kg	Kilogram
l	Liter
LB	Luria Bertani media
LIR	Long intergenic region
LT	*Escherichia coli* heat-labile enterotoxin
LTA	*Escherichia coli* heat-labile enterotoxin A subunit
LTB	*Escherichia coli* heat-labile enterotoxin B subunit
MES	Ethanesulfonic acid
MHC	Major histocompatibility complex
ml	Milliliter
mg	Milligram
µl	Microliter
µg	Microgram
µm	Micrometer
MSV	Maize streak virus
MUC1	Mucin-like glycoprotein 1
Mw	Molecular weight
MWCO	Molecular weight cut-off
Ni-NTA	Nickel-nitrilotriacetic acid

OD	Optical density
p19	RNA silencing suppressor p19
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered Saline
PBST	Phosphate buffered Saline with Tween 20
PCR	Polymerase chain reaction
PD-1	Programed cell death-1
pI	Isoelectric point
PMP	Plant molecular pharming/farming
PRRs	Germline-encoded pathogen recognition receptors
RB	Right border
RCR	Rolling circle replication
Rep	Replication-associated protein
ROS	Reactive oxygen species
rErbB2	Rat epidermal growth factor-related proteins
RubisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SDS	Sodium lauryl sulfate
SIR	Short intergenic region
ssDNA	Single-strand DNA
T4SS	Type IV secretion system
TAAs	Tumor-associated antigens

Tc	Cytotoxic T-lymphocyte
T-cell	T-lymphocyte
TCR	T-lymphocyte receptor
T-DNA	Transfer DNA
Th	T-helper cell
Ti/Ri plasmid	Tumor/root-inducing plasmid
Treg	Regulatory T-lymphocyte
T-strand	Single-strand form of transfer DNA
V1 or MP	Movement protein
V2 or CP	Capsid protein
VIGS	Virus-induced gene silencing
vir	Virulence genes
Vir	Virulence proteins
UK	United Kingdom
USA	United States of America
US FDA	United States Food and Drug Administration
WHO	World Health Organization
WT1	Wilms' tumor antigen

CHAPTER I

INTRODUCTION

Cancer is one of the leading global causes of death. In 2018, WHO estimated that 9.6 million patients died from cancer with 18.1 million new cases emerged. (Bray et al., 2018; “Cancer,” 2018) In recent years, a novel method emerged as an effective strategy to fight against cancer, immunotherapy. Immunotherapy is a treatment aimed to improve immune function by manipulating the immune checkpoints or the pathways involved in the immune system. Cytotoxic T-lymphocyte antigen 4 (CTLA-4 or CD152) is a transmembrane protein. The expression of CTLA-4 on the surface of activated T-cell could downregulate the production and proliferation of cytotoxic T-lymphocyte cells (CTLs) through CD28-CD80/CD86 pathway. (Krummel & Allison, 1995; Walunas et al., 2011) The overexpression of CTLA-4, a phenomenon remarkably founded in cancer, has a suppressive effect on cytotoxic T-lymphocyte cells, reducing the production, proliferation, and survival of CTLs encouraging the tumor cell's growth. (Rowshanravan, Halliday, & Sansom, 2018) In the present, the monoclonal antibodies targeting CTLA-4 have successfully proved to be an effective agent in immunotherapy. Ipilimumab, the first approved immune checkpoint inhibitor, shown a significant improvement of patients in the clinical investigation. The blockage of CTLA-4 demonstrated the enhancement of immune response by blocking CTLA-4 protein, resulted in the increasing the activation of cytotoxic T-lymphocyte cells that could significantly reduce the development of cancer. (Fellner, 2012; Ribas et al., 2013) However, there are disadvantage of antibody-based treatment; the frequency of dosing and the overall expensive cost, preventing the availability to some patients. Molecular pharming, a promising platform for the plant-

derived vaccines, has abilities to produce high value but cost-effective and yielded moderate-to-high for biopharmaceutical products. (Shanmugaraj, I Bulaon, & Phoolcharoen, 2020; Yao, Weng, Dickey, & Wang, 2015) The recombinant protein production using plants gain many useful aspects of low-cost, speed of production and scalability in upstream process allow plant gaining some feasibility in biopharma area such as minimize the risk of animal pathogen contamination when compared to the conventional system of mammalian cell. (Ma et al., 2015) Heat-labile enterotoxin B subunit (LTB) is a subunit that could compose into pentamer of heat-labile enterotoxin (LT) expressed by enterotoxigenic *Escherichia coli* (ETEC) is a pathogenic microorganism that causes children diarrhea, traveler's disease in developing countries and young animal colibacillosis infection. (Nagy & Fekete, 2005) LTB was considered as an immunoadjuvant because of the ability of strong antigenicity when administration with broad-spectrum of foreign antigens in various routes of administration. The many researches demonstrated the fusion of LTB to other antigen, the immunization of those antigens raises significantly. (Martin & Nashar, 2013; Rios-Huerta, Monreal-Escalante, Govea-Alonso, Angulo, & Rosales-Mendoza, 2017; Salazar-Gonzalez et al., 2014)

In this research, the LTB-CTLA-4 was designed, aimed to break self-antigen and enhancing the specific immune response against CTLA-4. LTB-CTLA-4 protein was produced and optimized in plant *Nicotiana benthamiana* by using geminiviral vector and characterized, then purified by Ni-NTA affinity column chromatography. Finally, the plant-produced LTB-CTLA4 was observed immunogenicity of LTB-CLTA4 in female BALB/c mice. In this study, we explored the possibility of plant-produced recombinant LTB-CTLA-4 as a cancer vaccine candidate either to prevent the development of cancer or to treat cancer in the future.

Literature review

Cancer

Cancer is a non-communicable disease and one of the leading global burden of humankind. According to WHO report in 2018, 9.6 million people died because of cancer around the world, and 18.1 million new cases emerged. In the 21st century, cancer was ranked as a hindrance to human's long-life expectancy. (Bray et al., 2018) Cancer is a general word describing a group of diseases, the abnormally grown cells developing and invading the parts of organs or throughout the body. The late stage of cancer spreading frequently is called metastases, which are a significant cause of death. (McGuire, 2016) In low-to-middle-income countries, 70 percent of cancer patients die, and most of them had limited access to the appropriate diagnosis or treatment services Those developing countries also lacked relevant data to run cancer policy compared to the high-income countries. ("Cancer," 2018) This burden worldwide drastically impacts the global economy and this tendency was continuously increasing. (McGuire, 2016)

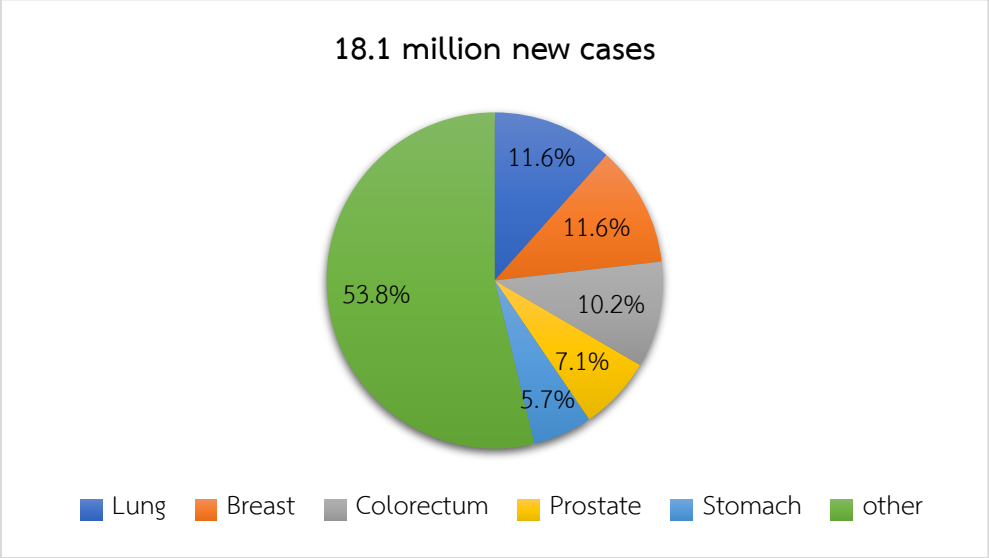

Figure 1. A pine chart indicates total new cases of cancer patient around the world in 2018. (Bray et al., 2018)

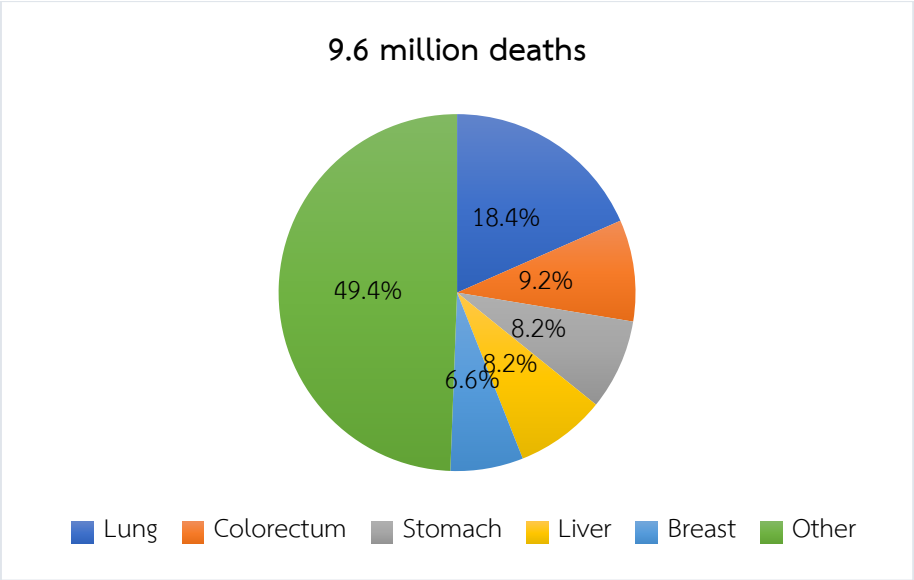

Figure 2. A pine chart indicates total death cases of cancer patient around the world in 2018. (Bray et al., 2018)

Nowadays there are various approach of cancer managements: surgery, chemotherapy, radiotherapy, targeted therapy, hormone therapy, stem cell transplant and immunotherapy. (Sharma, Campbell, Yee, Goswami, & Sharma, 2019)

Immune system

Human immune system could categorize into two main groups: innate immune system and adaptive immune system. The innate immune system is a first-line self-defense mechanism that response to the pathogens and often less specific, comprises of; (i) physical barriers: skin, mucous membrane; (ii) immune cells: neutrophils, macrophages and monocyte; (iii) soluble factors: cytokines chemokines and complements; and (iv) other substances. The pattern recognition receptors or PRRs is germline-encoded pathogen sensors detecting pathological microbe structures called pathogen-associated molecular patterns (PAMPs). The innate immune response also has an important role as an initial of adaptive immune response and inflammatory cytokines secretion. (McDonald & Levy, 2019; Xu, Timares, & Elmets, 2019) Second-line immunity called the adaptive immune system or acquired immune system is a specific foreign antigens recognition system that has

ability to distinguish pathogenic antigens out of host-antigen. It activates immune response to encounter the pathogens or infected cells and developing long-termed immunological memory by memory B-cells. The activation of adaptive immunity initiates the regulation, proliferation, differentiation, and effector functions serially, leading to the collaboration between T-cells and leukocytes by communicating between cell surface receptors and biochemical transduction. After first contact of antigens and initial immune response, the representation of antigens by antigen-presenting cells (APCs) to T-lymphocyte cells transpire. The APCs present the antigen to naïve T-cell via MHC complex then activating naïve T-cell into mature version. If the naive CD8+ T-cells are activated by MHC class I, they would finally mature into cytotoxic-T-cells in which could destroy infected cells with the same specific-presented antigens. However, when the naive CD4+ T-cells are activated by MHC class II on APCs membrane, naïve T-cell would develop into T-helper cells in which can enhance the immune response by activating B-cells, natural killer (NK) cells and macrophages. T-helper cells will facilitate: B-cells proliferation and differentiation into plasma cells, for manufacturing and secreting antigen-specific antibodies; and memory B-cells for long-term remembering those invasive antigens. (Rich & Chaplin, 2019)

Immunotherapy

Immunotherapy is a type of treatment that relies on human immune system for combating with cancer cells. Generally, immune system has been well-known for recognizing foreign antigens then recruiting cellular constituents of innate or adaptive immune response and eradicating those pathogens or invaders. In 19thcentury, the concept of immune response prevents the progression of cancer has been postulated, however, this hypothesis has been proved in the clinical aspects by the recent decades when pharmaceutical agents interfering immune regulation has been developed. (Sharma et al., 2019) The approach of anticancer by using immunotherapeutic schemes were shaped by the knowledge of immunosurveillance

and immune response-cancer relationship, allowing researchers to design mechanism that intervene in the regulation of immune response. The immunotherapeutic strategies against cancer based on the T-cell response regulation, lead to the development of immune checkpoint therapy, oncolytic virus immunotherapy, cancer vaccines, cytokine therapy, monoclonal antibodies, adoptive cell therapy, immune co-stimulatory molecules, and other checkpoint inhibitors. (Sharma et al., 2019)

T-cells response regulation and activation with cancer

The T-cells activation elements compose of complex signaling interactions and co-stimulation of T-cell receptor (TCR) and CD28. When antigen presenting-cells or APCs presented foreign antigens on MHC-peptide complex to TCR, the complex will generate a signal. The signal, however, is inadequate for T-cell activation unless there is co-stimulation signal provided from the complexes of CD28 and its receptors. The CD28 is constitutively presented on T-cell surface that bind to B7 receptor family, B7-1 (CD80) and B7-2 (CD86) which both expressed on APCs. The complexes of CD28/B7-1 or CD28/B7-2 produce a second signal along with co-stimulation of TCR/MHC complex, T-cell activation does occur. To activate the T-cells response against cancer cells, a tumor phagocytosis and cell fragment presentation by APCs are required. When both co-stimulated signals and activation of T-cell transpire, immune cells-related producing cytokines which leads to tumor cells elimination.

Immunosurveillance of cancer depends on immune cells that recognize mutant cell-surface proteins of cancer; however, malignant cells could alter themselves in case of avoiding immune cells. The interaction between cancer and immune cells theoretically divided into three phases: elimination, equilibrium, and escape. The *elimination phase* begin with detected tumors cells are destroyed by innate and adaptive immune system before tumor progressing, follow by the *equilibrium phase* that T cells surveil damaged cancer cells without eradicating

them, which the duration of this phase in some patient could be life-long lasting. The final phase is *escape phase* which tumor cells avoid being detected by immune patrol via adopting various mechanisms, for instance; (i) dropped the expression of MHC or surface antigens, (ii) induction anti-apoptotic mechanisms to increase a resistance toward immune cytotoxic effects, (iii) defection of tumor antigen processing or presentation, (iv) neglection of genes involved immune response against tumor and gains resistance to those immune-related killing mechanism, and (v) tumor microenvironment alteration by recruiting immunosuppressive cells against antitumor responses. (Sharma et al., 2019) These evasion mechanisms of tumor could develop the resistance against host-immunity recognition of T-cells. (Buchbinder & Desai, 2016)

Immune checkpoint blockage therapy

Immune checkpoint is regulation pathways that control the self-tolerance, preventing the immune system from assaulting its own cells. Both stimulatory and inhibitory immune checkpoint molecules had been exploited as a target in immunotherapy in the current research. (Pardoll, 2012) Up until now, the inhibitory immune checkpoint molecules were the most successful in immunotherapy, US FDA approved drug that shown significant clinical results primarily targeted these molecules are: cytotoxic T-lymphocyte antigen 4 (CTLA-4), ipilimumab (Yervoy®); programmed cell death 1 (PD-1) nivolumab (Opdivo®) and pembrolizumab (Keytruda®); and programmed cell death 1 ligand (PD-L1), atezolizumab (Tecentriq®). And the very first pioneer target of the immune checkpoint inhibitor is CTLA-4 molecule. (Alsaab et al., 2017; Pardoll, 2012)

Cytotoxic T-lymphocyte antigen 4

Cytotoxic T-lymphocyte antigen 4 (CTLA-4) or CD152 is a membrane-bound protein constitutively produced in regulatory T-cells (Tregs) but also found on activated T-cells surface. CTLA-4 and CD28 have a homologous structure that share the affinity toward B7 ligand protein, B7-1 (CD80) and B7-2 (CD86) on APC cells. (Krummel & Allison, 1995; Walunas et al., 2011) However, CTLA-4 is considered to be an immune checkpoint and an important key for specific immune responses due to its inhibitory property that downregulate T-cells production, proliferation and survival, opposite to its counterpart CD28. Because CTLA-4 has higher affinity to both B7-1 and B7-2 receptors while CD28 has more specific affinity to B7-1 than B7-2, making CD28/B7-2 complex has least possibility to happen (Buchbinder & Desai, 2016; Rowshanravan et al., 2018), explaining the inhibitory effect of CTLA-4. In normal situation, CTLA-4 likely to upregulated in T-cell after activation to competitively bind to B7 receptors, preventing the overstimulation of immune response from co-stimulated signals of TCR/MHC and CD28/B7 complexes. Before T-cell activation, 90 percent of CTLA-4 generally stays inside T-cells, moving in the contained vesicle. When T-cells co-stimulated activation occurs, CTLA-4 would be transported onto T-cells surface through the exocytosis process. The stronger the signals send from T-cells activation, the more expressive of CTLA-4. When CTLA-4/B7 complexes is excessive, overall negative signal will induce IL-2 repression, resulting in the retardment of T-cell production and proliferation. (Buchbinder & Desai, 2016) In addition, CTLA-4 also constitutively expressed in Tregs, the T-cells subpopulation which has suppressive property that maintain the balance of immune system (Lewis & Blutt, 2019) CTLA-4 also associates with autoimmune disease such as Hashimoto thyroiditis, Graves' disease, and systemic lupus erythematosus (SLE). (Peterson & Maltzman, 2019)

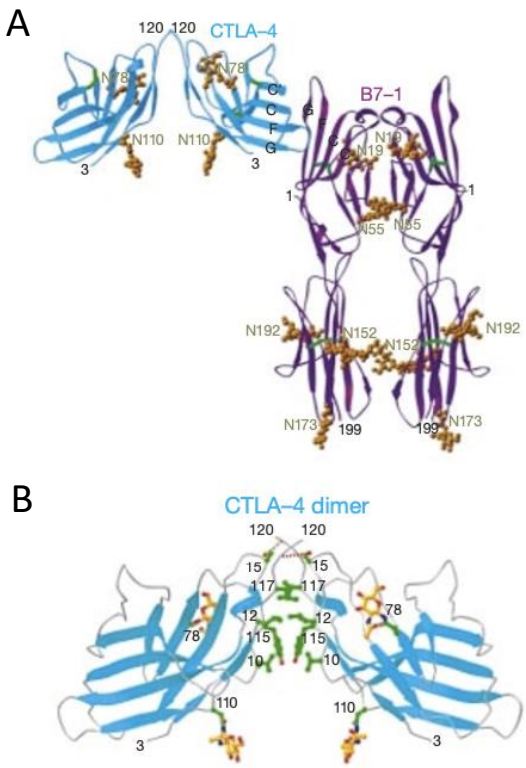

Figure 3. CTLA-4 and B7-1 crystal structure. (Stamper et al., 2001)

A) dimeric human CTLA-4 binds to B7-1 receptor;

B) dimeric human CTLA-4.

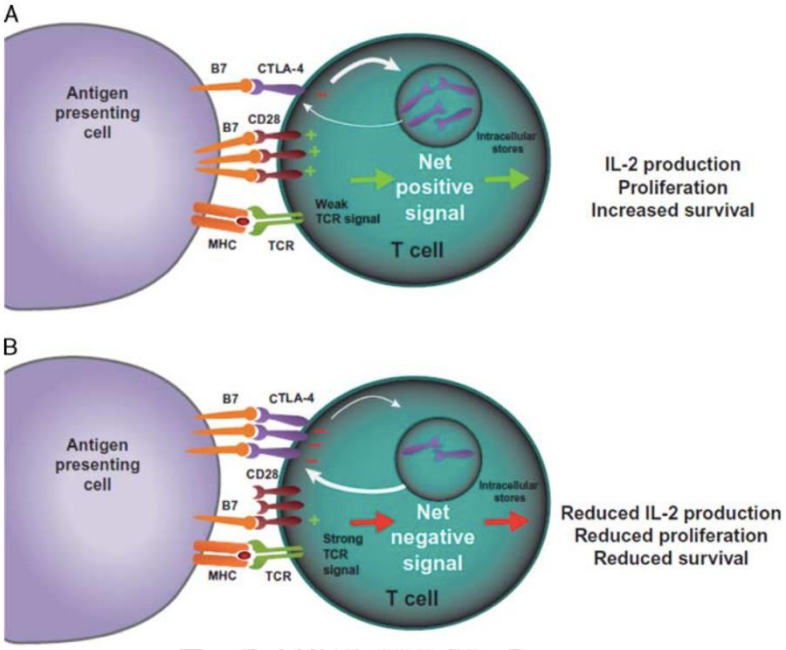

Figure 4. CTLA-4 stimulatory and inhibitory mechanism.The upregulation of CTLA-4 when the co-signals of T-cells activation, the inhibitory of T-cell occurs after B7 receptor preferring to bind with CTLA-4 over CD28, resulting in regression of T-cells production, proliferation and overall survival. Figure 4A shown the larger number of extracellular CD28 than CTLA-4 which are binding to the B7 proteins on APC. The net positive signal in T-cells lead to production of IL-2 and activating T-cells. On the contrary, figure 4B shown the numerous CTLA-4 expression on the cell surface that could compete in B7 binding with CD28, resulting in the net negative signal and the reduction of IL-2. The T-cell activation is impeded. Figure adopted from Buchbinder and Desai, 2016. (Buchbinder & Desai, 2016)

CTLA-4-targeted immunotherapies

The idea of using immune checkpoint inhibitor as a promising anticancer agent has been shown after the preclinical studies of anti-CTLA-4 antibodies that confirmed CTLA-4 blockage influencing the tumor regression and increasing survival in animal models. Anti-CTLA-4 monotherapy effective in various types of tumor-

transplanted mouse model and demonstrated protection when challenged with tumor. (Callahan, Wolchok, & Allison, 2010) The very first anti-CTLA-4 drug "ipilimumab" was approved by US FDA in 2011 for the treatment of melanoma, unresectable or metastatic skin cancer types. Ipilimumab is a full-ranged human monoclonal antibody against human CTLA-4. In clinical trial phase 3, metastatic melanoma patients treated with ipilimumab developed the best overall clinical response compared with peptide vaccine alone or ipilimumab plus peptide vaccine. The one-year survival rates were 45.6% for ipilimumab treated patients and 23.5% for two-year endpoint. (Hodi et al., 2010) Currently, a combination of ipilimumab and nivolumab was also in the clinical trial phase 2 for treating hormone-resistance prostate cancer in metastasis to other organs and tumor cells expressing androgen receptor-variant-7 (AR-V) that demonstrated the resistance to hormone therapy and some chemotherapy. (Silberstein, Taylor, & Antonarakis, 2016) Moreover, ipilimumab has been shown feasible result in combined chemotherapy approach of metastatic urothelial cancer in clinical trial phase 2 (Galsky et al., 2018) Despite good clinical outcomes of ipilimumab, CTLA-4 blockage associated with the raise of effective immune response some patients experienced autoimmune-related side effect which varied on individual of their immune condition. (Buchbinder & Desai, 2016; Callahan et al., 2010) Furthermore, fully human anti-CTLA-4 monoclonal antibody is tremelimumab, but the was discontinued after clinical trial phase 3 in advanced melanoma patients because the interim analysis shown no different than standard treatment. (Ribas et al., 2013)

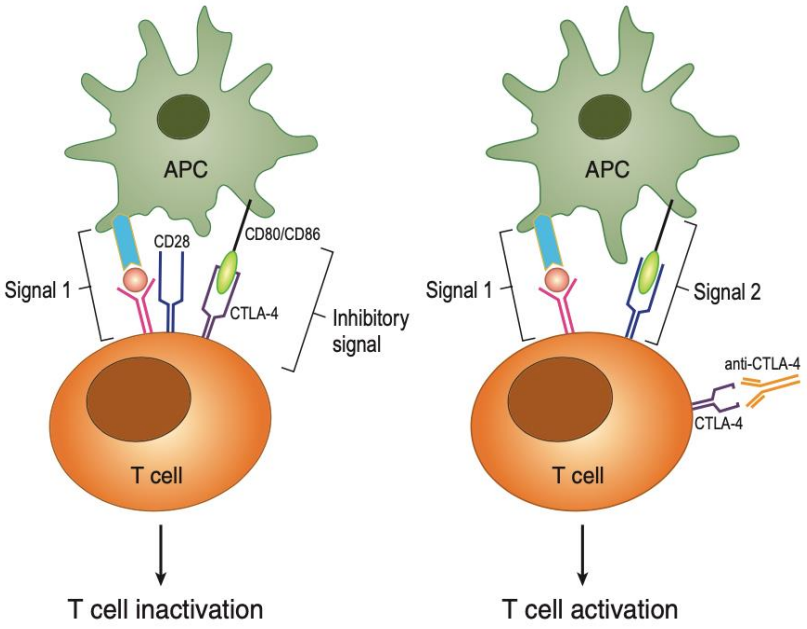

Figure 5. The relationship of ligands involved T-cell activation and inactivation. The costimulatory signals of TCR/MHC complex (signal 1) and CD80/86-CD28 complex (signal 2) promotes T-cell activation. (Left) The binding of CD80/86 and CTLA-4, accompanied with signal 1, leads to overall inhibitory signal and T-cell inactivation. (Right) The blockage of CTLA-4 by anti-CTLA-4 promotes the signal 2 binding, enhancing overall stimulatory signal and T-cell activation. (Brunner-Weinzierl & Rudd, 2018)

Heat-labile enterotoxin

Heat-labile toxin is an enterotoxin expressed by microorganism whose name refers to its heat-sensitive nature. The heat-labile toxin members mainly consist of *E. coli* heat-labile enterotoxin (LT) produced by enterotoxigenic *E. coli* (ETEC), and cholera toxin (CT) produced by *Vibrio cholerae*. Both toxins have similar structure with approximately 80% of amino acid identity (Connell, 2007; Mudrak & Kuehn, 2010), LT and CT have been known as pathogens infected both human and animal. According to World health organization, LT is one of the main cause of diarrhea

disease in young children and traveler's disease in developing countries, and also, a cause of colibacillosis infection in young animal such as piglet and calve. (Nagy & Fekete, 2005) A research by Isidean et al. indicated almost 60% of ETEC isolated from human feces who had diarrheal-associated symptom secreted LT enterotoxin. (Isidean, Riddle, Savarino, & Porter, 2011) This diarrheal symptom relates to the structures of LT's two subunits. The A subunit (LTA) is a single polypeptide which has ADP-ribosylation activity that has ability to increase the intracellular cAMP activity by activating adenyl-cyclase in the intracellular domain of a small intestine. This process leads to an acute outflux of water and electrolytes, a notable symptom of diarrhea disease. (Connell, 2007) Another part of LT, the five B subunits (LTB) form a pentamer and non-covalently bound with LTA, contributes to AB5 structure forming. The B pentamer interacts with ganglioside GM1 receptor on the mucosal membrane, which assist to the entire LT endocytosis. The LT possesses the mucosal adjuvant property that contributes to a strong antigenicity when administrates with broad-spectrum of foreign antigens. (Connell, 2007; Mudrak & Kuehn, 2010)

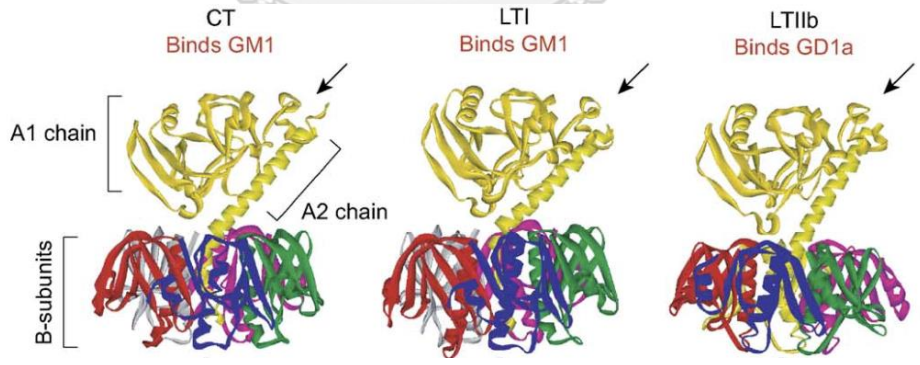

Figure 6. 3D crystal structure of AB5 composition.

Two protein domains assemble into the whole CT or LT toxin; the A domain represented in yellow ribbons, comprises of A1 and A2 chain, A1 responsible for the toxin pathogenicity, and A2 chain anchors the LTA domain to the B domain together; the B domain consists of five B subunits (shown in red, blue, green, pink, and grey) assembling as a holotoxin. The black arrow marks the site of the loop that can be cleavage by proteolytic reaction. (Lencer & Saslowsky, 2005)

E. coli heat-labile enterotoxin B subunit (LTB)

Despites robust mucosal adjuvant property of LT, it still has telltale enterotoxicity due to LTA subunit that impede the clinical test as an adjuvant or immunogen in human, however, the comprehension of LT adjuvanticity could be advantageous for the future of adjuvant innovation. To circumvent LT disadvantage, the adjuvanticity of LTB subunit has been explored due to its adjutancy but non-toxic. The affinity of LTB toward GM1 receptor is critical for inducing antigenic activity, as LTB pentamer or as a whole LT. (de Haan et al., 1998) However, there are mechanisms behind the binding to GM1 receptor. LTB subunit does not only facilitate the entry of LT, but it also indirectly stimulates the immune response. The research by De Haan et al. demonstrated that LTB-fused epitope could delivered into major histocompatibility complex (MHC) class I pathway, which enhanced the ability of the antigen presentation in antigen-presenting cell (APCS) (de Haan et al., 1998) and MHC class II (Bone, Eckholdt, & Williams, 2002; Martin & Nashar, 2013) (i); influencing of dendritic cells (DCs) and other APCs maturation and activation (Pitcovski et al., 2006) ; (ii) eliciting strong humoral and cellular response by activating both B and T lymphocytes, stimulating the secretion of cytokines. (Yamamoto, McGhee, Hagiwara, Otake, & Kiyono, 2001) These properties make LTB one of the promising novel platform of vaccine adjuvant development.

Plant-based molecular pharming

Plant molecular pharming (PMP) is a practice that using plants as a bio-factories for recombinant protein production such as biopharmaceutical products or valuable proteins. Many species of plants, tissue culture systems, and various protein expression strategies had been exploited in these areas. (Spiegel, Stöger, Twyman, & Buyel, 2018) Since biotechnology allows genome editing techniques manipulating or altering the foreign proteins expression in plant, PMP has been viewed as one of

attractive platforms for biopharmaceuticals manufacturing. For these therapeutic macromolecules were considered to be valuable in the pharmaceutical market and rendered a efficacious clinical outcome in the patients, which could be roughly categorized into three classes: antibodies, vaccines, and replacement proteins. (Grilo & Mantalaris, 2019; Spiegel et al., 2018) The early concept of PMP was to produce edible vaccine using fruit or vegetable as a container for oral administration without need of sterile injection or healthcare professionals. (Yao et al., 2015) The first attempt in the production of plant-made biopharmaceutical product was to transform the plants expressing the desirable proteins permanently by transgene insertion of plant genome. Later came the advancement of the expression vectors, plant transient expression technologies continued the development of plant viral vectors which yielded more satisfiable protein amount and more controllable than early concept. (Hefferon, 2014)

Up until now there are three type of platforms refined in the plant molecular pharming. (i) The plant cell suspension culture or aquatic species that could be contained in the controlled system in the same way of conventional platform like mammalian cells or microorganism but required less cost media, for examples: plant cell-free, mosses, algae etc. (Spiegel et al., 2018) (ii) The second one is transient expression platform; the recombinant proteins produce by this method has relatively rapid producing and up-scaling attributes than other plant-produced platforms. The widely recognized plants used is *N. benthamiana* (iii) Lastly, transgenic plant which gains the benefit of large-scale production in greenhouse and yields considerable biomass. (Schillberg, Raven, Spiegel, Rasche, & Buntru, 2019)

The commercial-scale manufacturing facility of plant-made biopharmaceuticals has been rapidly on-progress, some products has reached to the final line of practice in the clinical trials then launch in the commercial market and

companies had been working on the establishment of the milestone for plant expression system. (Buyel, 2018; Schillberg et al., 2019) This make possible for plant-produced platform would be utilized as alternative more solid in the future.

Nowadays, a recombinant glucocerebrosidase enzyme for Gaucher's disease treatment called taliglucerase alfa (Elelyso™), the genetically modified carrot cells-derived biopharmaceutical that receive the approval from US FDA in 2012. This replacement enzyme carrot cell suspension in controlled environment that comply with the regulation (Mor, 2015) The exemplar of the on product that brought *N. benthamiana*-produced chimeric monoclonal antibodies for Ebola virus treatment or ZMapp™ patients. The transient expression in *N. benthamiana* was applied in the production of ZMapp™ (Davey et al., 2016) in to the light. The breakthrough could trait back to the emergency cases of Ebola, the notorious emerging cases of Ebola virus epidemic in West Africa initiated the larger clinical trial in the infected patients. Despite the statistically insignificant results to prove the superior above standard care alone, *N. benthamiana*-derived chimeric monoclonal antibodies had been proved to be safe and well-tolerated in human. (Davey et al., 2016; Dhama et al., 2018) There are also plant-derived bioproducts in current-developed exemplars, tobacco-derived H5N1 influenza hemagglutinin protein (H5) expressing on surface of virus-liked particles (VLP) elicited the humoral and cell mediated response in healthy volunteer in the clinical trial phase 2. The transgenic tobacco Nicotiana tabacum was engineered to express neutralizing monoclonal antibodies (mAb) PG12 against human immunodeficiency virus (HIV). The mAb PG12 formulated as vaginal gel applying before sexual intercourse to prevent HIV infection. Moreover, the data from clinical trial phase 1 provide information as a guideline of plant-made biopharmaceutical production that complied with the regulator. (Ma et al., 2015)

Target diseases	Biopharmaceutical agents	Plants host	Clinical status	References
Gaucher's disease	Taliglucerase alfa (Elelyso™),	Carrot cell culture	FDA approved in 2012	(Aviezer et al., 2009; Mor, 2015; Zimran et al., 2019)
Ebola virus infection	Chimeric monoclonal antibodies against Ebola virus (ZMapp™)	*N. benthamiana* agroinfiltration	Phase 2/3	(Davey et al., 2016)
Influenza	Seasonal influenza plant-based quadrivalent virus-like particle (QVLP) vaccine	*N. benthamiana* agroinfiltration	Phase 3	(Pillet et al., 2016, 2019)
Influenza	H5N1 influenza hemagglutinin protein (H5) expressing on surface of virus-liked particles (VLP) vaccine	*N. benthamiana* agroinfiltration	Phase 2	(Hendin et al., 2017)
Non-Hodgkin's lymphoma	Idiotype IgG-based vaccine	*N. benthamiana* agroinfiltration	Phase 1	(Tusé et al., 2015)
Fabry disease	Alpha-galactosidase-A (Fabrazyme)	Tobacco cell suspension culture	Phase 2	(Kizhner et al., 2015)
Fabry disease	Alpha-galactosidase-A (moss-aGal)	Moss cell culture	Phase 1	(Shen et al., 2016)

Human immunodeficiency viruses (HIV) infection	Anti-HIV IgG	transgenic *N. tabacum*	Phase 1	(Ma et al., 2015)

Table 1. The examples of outstanding plant-derived biopharmaceutical products were in clinical trials. (Donini & Marusic, 2019)

The limitation and opportunity of molecular pharming

In very near future, plants are unlikely to replace the standard platform such as mammalian cells or bacterial platform yet, because of theirs well-established processes and facilities that comply with the regulation, also the yield gained from plants were still not match to those conventional platforms. (Schillberg et al., 2019) Moreover, plant still have its own limitation, for examples, for the PMP technology requires the bio-manufacturing industry to accommodate with new technology, practices, and GMPs for both up-stream and down-stream processing. The PMP track record with the regulator or FDA was still limited, its GMPs is in the initial stage and few agents of plant-produced biopharmaceuticals were introduced to the clinical or commercial scale. (Buyel, 2018) Even now PMP had been considered as an alternative that has been hindered from establishment and development of standard process mainly operated in the academic section. The industrial scale of molecular pharming concept is still very new established hen compare to mammalian cells or microbial system, the conventional platform of biopharma field which were considered as a gold standard. Moreover, there were many researches on PMP in the laboratory scope nowadays, however, not too many publications investigated the prospective of manufacturing in larger scale. (Spiegel et al., 2018) The lack of PMP downstream processing or good manufacturing practice (GMP) exploration make the

investor reluctant to invest in PMP platform because of the regulatory was still not familiar with plant-made platform. (Spiegel et al., 2018) Some plant-made products underwent the clinical trials were discontinued, even less products reached the approval step by FDA regulation and launched in the commercial market. (Schillberg et al., 2019; Shanmugaraj, I Bulaon, et al., 2020)

Nevertheless, plants still have special niche that appealing to pharma market, for examples; (i) the proteins function could be ameliorated in planta. For plant glycosylation pattern slightly diverges from human's and processes the consistency; (ii) plant matrix possibly be a bio-capsule for oral administration of animal therapeutic agents or vaccines; (iii) the rapidness of transient expression technology enables the fast production of recombinant proteins in short period, suitable for emergency emerging cases of epidemic disease. (Schillberg et al., 2019; Shanmugaraj, I Bulaon, et al., 2020)

Nicotiana benthamiana

Nicotiana benthamiana is a plant in the *Nicotiana* "tobacco" genus of Solanaceae family had been well-perceived in the plant biotechnology for a longtime. (Bally et al., 2018) The *Nicotiana* plants are widely explored as a versatile tool for plant-genome editing in the plant genetic modification researches due to theirs herbaceous nature of short life-cycle, and the successful rate in the context of exogenous gene transformation and plant cell regeneration. (Goodin, Zaitlin, Naidu, & Lommel, 2008)

N. benthamiana is one of the species in *Nicotiana* genus which has small-to-medium-flowered plant indigenous to Australia. Because *N. benthamiana* could be infected by the majority of plant viruses, it earned a reputation of becoming a species of choice that played the main role of a plant host-pathogen in the plant virology research. The susceptibility to majority of viruses was considered to be the

main reason of the preferability of *N. benthamiana* over the earlier pioneer in the plant biotechnology such as *Arabidopsis thaliana,* which has smaller size of genome, more understandable and completely elucidated in the genomic aspect. However*, A. thaliana* could not match *N. benthamiana* in the aspect of susceptibility to the wide-range kind of viruses, which there also were reports of *N. benthamiana* was susceptible to the bacteria, fungi, viroid, nematodes, and other stresses. (Christie & Crawford, 1978; Van Dijk, Van Der Meer, & Piron, 1987)

Transient expression of exogenous protein in *N. benthamiana*

The innovation of plant virus-based vector technology brought *N. benthamiana* in to the new light, this technology allows the foreign genes expression, silencing plant endogenous genes and visual reporter genes development *in planta* possible. (Bally et al., 2018) The genomic sequences of viruses and other cassettes, which contribute to the alteration of RNA transcription in *N. benthamiana,* are arranged in the transfer DNA (T-DNA) region on Ti plasmid in *Agrobacterium*-mediated plant transformation system. The Ti plasmid is the former developed tools that agronomically and horticulturally important in plant genome editing by using *Agrobacterium tumefaciens* as a powerful carrier for delivering plasmid contained gene of interest into the plant cells. (Gelvin, 2003) The plant viral vector system possesses ability of plant virus in the context of rapid gene expression in short period that researchers applied this benefit for the rapid production of foreign protein in plant. (Diamos & Mason, 2019; Diamos, Rosenthal, & Mason, 2016; Tusé et al., 2015) These transient expressing abilities differ plant virus-based system from the former time-consuming regeneration procedure of transgenic plant, the method that permanently insert gene of interest into plant genome. (Gelvin, 2003) Moreover, the term ‘agroinfiltration’ was invented to accompany the delivery method of *Agrobacterium* suspension harboring desired viral vector through the intracellular

space between *N. benthamiana* leaf cells, either by using syringe without needle or vacuum pump.

N. benthamiana became more important along with PMP concept. Although the sister in the same genus of *Nicotiana*: *Nicotiana tabacum*, had been widely studied in the plant biotechnology as a transgenic transformation of plant host. The transient expression of recombinant protein in viral susceptible *N. benthamiana* by using plant viral vector becomes increasingly popular and a numerous publication aimed to express therapeutic proteins, vaccines and antibodies. (Bally et al., 2018; Spiegel et al., 2018) In 2014, the emerging cases of Ebola in South African countries leaded to the clinical trial of plant-derived therapeutic antibodies against Ebola virus in human. (Davey et al., 2016) US FDA granted the approval for chimeric murine monoclonal antibodies transiently expressed in *N. benthamiana* plant, ZMapp™, to cure severe cases of American healthcare professional workers who suffering from Ebola virus symptom (Davey et al., 2016) The situation suddenly brought a spotlight on a plant transient expression platform for the potential in the production of therapeutic proteins beyond laboratory scale.

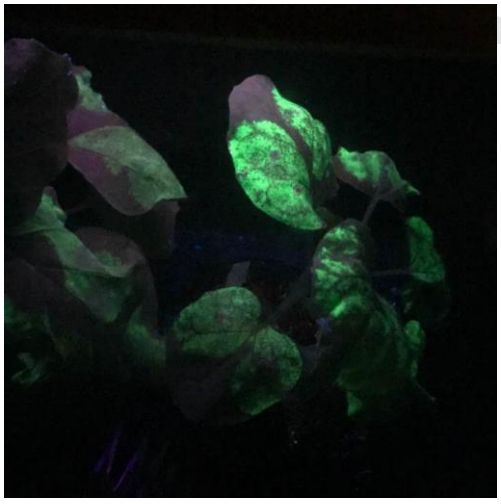

Figure 7. A picture of green fluorescent protein (GFP) expression in *N. benthamiana* leaves. The *N. benthamiana* leaves were agroinfiltrated with plasmid encoded GFP gene. The GFP protein was transiently expressed in *N. benthamiana* leaves, visualized under Ultra-violate light after 4 day after agroinfiltration.

Agrobacterium tumefaciens

Agrobacterium tumefaciens (scientific name: *Rhizobium radiobactor,* synonym: *Agrobacterium radiobactor*) is a common name of rod-shaped, gram-negative, soil-lived bacteria in the genus *Agrobacterium* prevalent all over the world. (Kado, 2014) The members in *Agrobacterium* genus, some were taxonomically categorized in as new genera (*Rhizobium rhizogenes*, *Allorizobium vitis* etc.), are well-recognized for their plant pathogenic virulence causing a crown gall disease, a tumor-like disease mostly found on the base or root of woody plants. (Chen et al., 2013; Gelvin, 2003; Kado, 2014; Nester, 2015) The diversity of *Agrobacteria* in genomic-based taxonomy aspect was not well-defined, it has been currently under revision of the classification. (Gan, Lee, & Savka, 2019; Gan & Savka, 2018) (Although, *Agrobacteria* strains and species naturally infect plants above 600 species, covered many families in which mostly dicotyledons. Later, when viral vector engineering technologies were available, the monocotyledons became more susceptible. Even species were expanded beyond plants overtime, such as fungi, yeasts, ascomycetes, and basidiomycetes. There was also a report of T-DNA was transfer into human cells. (Guo, Ye, Gao, Xu, & Yang, 2019) *A. tumefaciens* became popular because of its ability to horizontally transfer foreign genes into the plant host cells, making it one of the most powerful genetic modification tools in plant molecular biotechnology and especially in PMP field.

The initial research of *Agrobacterium* began in 1987, with the description and isolation of bacterium grapevines' crown gall in the Royal Botanical Gardens of Napoli, Italy, was published. (Kado, 2014) In 1900s, the American researchers isolated causal bacterium and named *Bacterium tumefaciens*, and finally shifted to *Agrobacterium tumefaciens*. At first, the scientists postulated that *Agrobacterium* might produce some typical substances, such as chemical irritants, phytohormones etc., in which disturb plant metabolism, creating abnormal growth in the plant cells.

The scientific evident strongly suggested that *Agrobacterium* harbors encoded-plasmid inside and later named tumor-inducing plasmid or Ti plasmid (in *Rhizobium rhizogenes* called root-inducing (Ri) plasmid, after its potential of causing hairy root disease).

Tumor-inducing plasmid (Ti plasmid)

Ti plasmid has ability to semi-randomly incorporate an oncogenic region of DNA segment into plant host genome, called T-DNA (transfer DNA), in which is responsible for the production of plant-growth factors and *Agrobacterium* energy sources called opines. Opines are small chemical compound found in the gall part of *Agrobacterium*-infected plants. They are unusual nutrients of Agrobacterium Ti plasmid could be divided into two subtypes: octopine- and nopaline-type, categorized by a type of opines syntheses gene encoded in T-DNA region. In figure 8, shown the mapping of Ti plasmid octopine-type. Edged with left (LB) and right border (RB), T-DNA comprises of three subregions: T-DNA left (T_L), T-DNA center (T_C) and T-DNA right (T_R). The T-DNA contains biosynthetic enzyme genes: phytohormones, auxins and cytokinin syntheses; and of *Agrobacterium* nutrient synthesis elements, opines: octopine (ocs), manopine (mas), and agropine (ags) syntheses, respectively. On the left border side is T_L subregion contained auxin, cytokinin and ocs syntheses, separated with T_C, T_R contained mas and ags syntheses at the right end. (Gelvin, 2003; Guo et al., 2019; Nester, 2015) Other significant regions are virulence *vir*-region encoded *vir* genes, which are protein facilitators assisting in T-DNA transportation. The *vir* genetic elements consist approximately 30 genes, about 20 genes are necessary in tumor creation and arranging as operons that composing into regulons. (Nester, 2015)The critical key of Vir proteins are VirA, which is responsible for responding to environmental stimuli, and VirG which initiates the activation of *vir*-genes. Next are a group of Vir proteins require for the processing and T-DNA transportation, for

examples: VirB, VirD, VirC, VirE, and VirE1. The Vir proteins whose roles are minor: VirH, VirF, VirD5, VirE3, VirJ and small heat-shock protein (HspL). Other are miscellaneous proteins such as chromosomal Vir proteins. (Gelvin, 2003; Nester, 2015) Ti plamid was modified and disarmed the oncogene on T-DNA region, then replaces with gene of interest and other cassettes to enhance or facilitate gene expression. (Gelvin, 2003)

Given more than a century of researching, the pathogenesis of *Agrobacterium* was quite well-researched. The complicated mechanism of T-DNA transportation process has been clarified. In summary, the transformation process initiates with: (i) *Agrobacterium*-host recognition and response by virA and VirG sensing phenolic and sugar compounds, causing *vir*-genes activation; (ii) T-DNA region on Ti plasmid is incised by VirD1 and VirD2 on the repeat sequences of left and right border (LB and RB), transporting as single-strand of T-DNA (T-strands); (iii) VirD2 binds to T-strand on 5’ end of RB, generating VirD/T-strand complex; (iv) VirD/T-strand complex along with Vir effectors travel through bacterium membrane into plant cells by type IV secretion system (T4SS), a system facilitating the VIr effectors transportation; (v) VirE2 protein accompanies T-strand, transporting past cytoplasm into plant nucleus then VirE2 detaches from T-strands; (vi) T-strands insert into plant genome, respectively. (Gelvin, 2017)

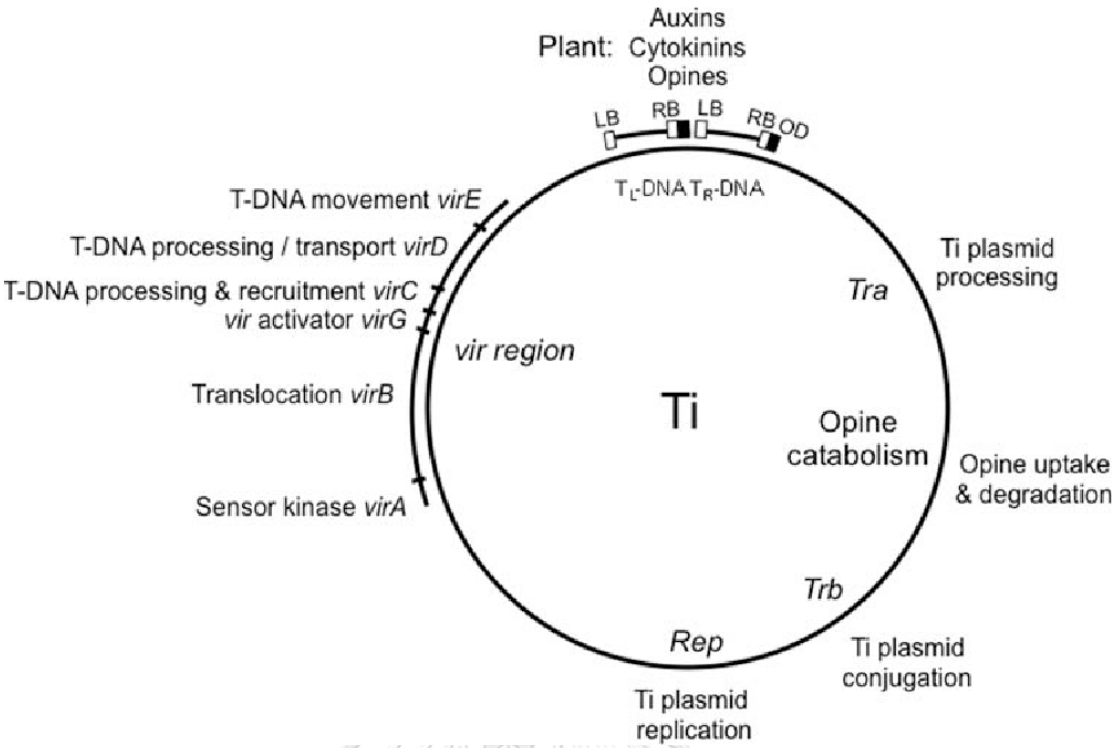

Figure 8: shown the schematic of Ti plasmid inside *A. tumefaciens*. Laid between left (LB) and right border (RB) is T-DNA, containing genes involved opines, auxin and cytokinin syntheses. (Gelvin, 2003; Gordon & Christie, 2014; Nester, 2015)

Geminivirus

Geminivirus is a single-strand DNA (ssDNA) plant virus in family *Geminiviridae*, notable in their morphology of geminate, icosahedral symmetry. The virions of geminivirus composed of single a structural protein called capsid protein (CP) with no other associated protein detected. No lipid or carbohydrate were reported. The double capsid supposed to contain genomic component ranging from 2.5-3.0 kbp in size (Varsani et al., 2017; Zerbini et al., 2017), and could be categorized into two types: monopartite and bipartite. The monopartite type contains one copy of ssDNA, consist of genera*: Mastrevirus*, *Curtovirus*, *Topocuvirus* (and some virus in the genus *Begomovirus*). For bipartite, the genome has two different genetic components which

are required for plant viral infection, the only member of bipartite is *Begomovirus.* (Hefferon, 2014; YANG, DING, & ZHOU, 2017; Zerbini et al., 2017) Both types have the same highly conserved stem looped-nucleotide acid sequences of 3' TAATATT/AC 5' named intergenic region (IR) in monopartite virus, and common region (CR) in bipartite. This region is required for the DNA replication process, called rolling circle replication (RCR). The replication of genomic components of geminiviruses essentially depend on the host factors.

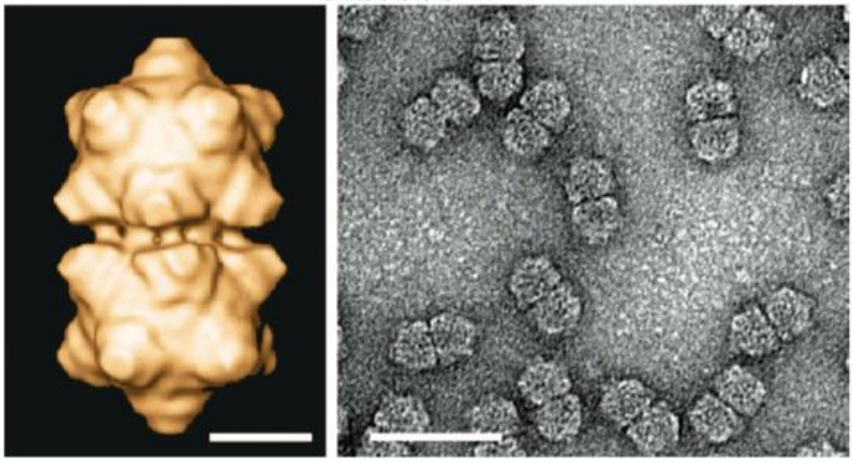

Figure 9. the example of geminivirus structure under electron microscope inspection. (Left) 3D structure of maize streak virus (MSV), a member of *geminiviridae* family shown twin icosahedral symmetric capsules. (Zhang et al., 2001)
(Right) The picture of MSV taken by electron microscopy. (Zerbini et al., 2017)

In the initial stage after plant-viral infection, the crucial factors in DNA synthesis that almost absent in the geminiviral genome (such as DNA polymerase and other DNA synthesis-involved enzymes or protein), are recruited from the plant host. The ssDNA replication initiated with the complementary-sense strand complement on virion-sense to generate double-strand DNA, then the initiator replication-associated protein (Rep) from the virus-encoded sequence cleaves the virion-sense strand on the IR/CR region and performs rolling circle replication (RCR) procedure.

After viral DNA fragment cyclize itself on the IR position by Rep protein and the assistance of RepA protein, a smaller version of Rep that can adjust a proper plant cell environment, readying for replication. (Jeske, 2009) The Rep protein continues producing large amount of virion-sense strand copies by using complementary strand as a model, then the detaching virion-sense strand ssDNAs are nicked, re-circle, and copied multiple times again by Rep/RepA proteins. The ssDNA can either endure repeated RCR process or assemble into novel viral particles in the final infection stage.

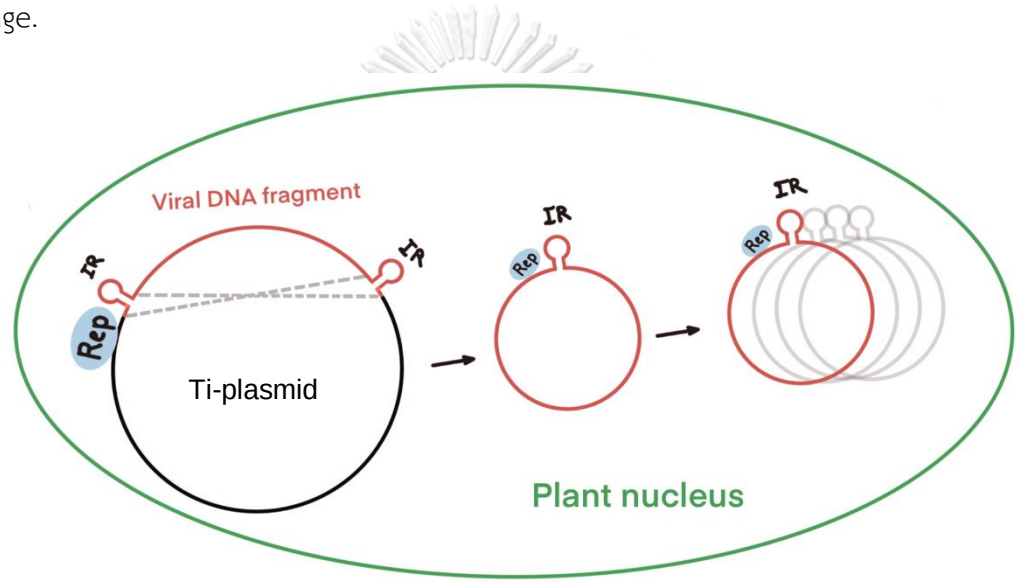

Figure 10. The figure represented a summarize of rolling circle replication (RCR). After geminiviral vectors are incorporated into plant cells, the virion-sense strand ssDNAs are nicked, re-circle, and copied multiple times by the assistance of Rep/RepA proteins, generating a numerous replicon.

An example of geminivirus, bean yellow dwarf virus (BeYDV) in genus *Mastrevirus* that can cause bean yellow dwarf disease. Encapsulated BeYDV genome contains four encoded protein: movement protein (V1 or MP), capsid protein (V2 or CP), Rep (C1), and RepA (C2) proteins. Both MP and CP are structural proteins encoded on viral-sense strand (V). The Rep/RepA are protein involved in RCR process

encoded on complementary-sense (C) strand. Between V and C strands separate with LIR and SIR. In geminiviral-based expression vector, CP gene is deleted for prevention of viral capsid forming and the other cassettes are added to assist recombinant protein production. (Chen, He, Phoolcharoen, & Mason, 2011)

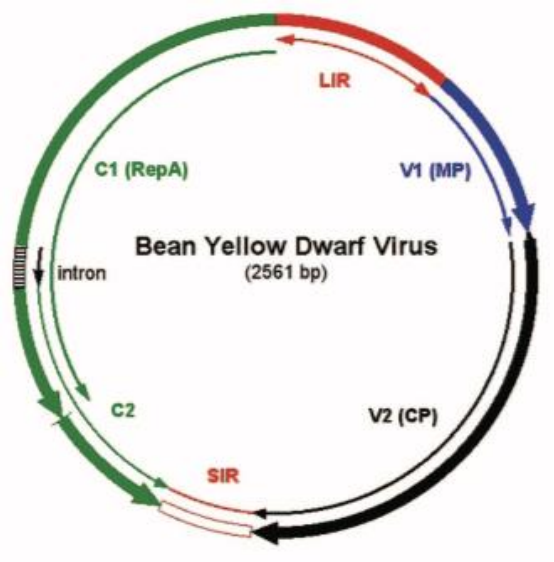

Figure 11. Shown bean yellow dwarf virus (BeYDV) structure. (Chen et al., 2011)

Objective of this study

- To produce recombinant LTB-CTLA4 fused protein by transient plant-based expression using geminiviral vector in *Nicotiana benthamiana.*
- To test the activities of plant-produced recombinant LTB-CTLA4 fusion protein.

CHAPTER II

MATERIALS AND METHODS

Materials

Equipment

Autoclave: Tomy SX-700 (Tomy Digital Biology, Japan)

Blender: High performance commercial blender BE-127A (Otto, Thailand)

DI water generator: Barnstead Pacific TII (Thermo Scientific, USA)

Digital balance: Mettler Toledo AG135 (Mettler Toledo, USA)

Digital balance: Sartorius TE 1502s (Sartorius, Germany)

Centrifuge: Hettich centrifuge UNIVERSAL 320R, (Andreas Hettich, Germany)

Centrifuge: Heraeus Multifuge X3R centrifuge (Thermo Electron LED, Germany)

Chemiluminescent chamber: Chemiluminescent imageQuant LAS4000 (GE Healthcare, USA).

Electrophoresis equipment: Mini-PROTEAN® Tetra System (Bio-Rad, USA)

Electroporation: MicroPulser™ (Bio-Rad, USA)

Hot air oven: Contherm Designer Series Oven (LabChina, Taiwan)

Heat block: WiseThem® HB-R, (Wisd laboratory instruments, Australia)

Hotplate stirrer: LabTech (Daihan Labtech, Indonesia)

Incubator: Memmert (Memmert, Germany)

Lyophilizer: Lyophilizer Labconco® Freezone 6 Plus (Labconco, USA)

Lyophilizer chamber: Labconco Lyophilizer Chamber 600 ml (Labconco, USA)

pH meter: SevenCompactTM pH meter S220 (Mettler Toledo, USA)

Power supply: PowerPac™ Basic power supply (Bio-Rad, USA)

Microplate incubator: Hercuvan (Lab systems, Malaysia)

Microplate reader: SpectraMax M5 microplate reader (Molecular Devices, USA)

Microwave oven (Sharp, Thailand)

Mini centrifuge: Bio-Rad (Bio-Rad, USA)

PCR machine: MJ Mini™ (Biorad, USA)

Refrigerator centrifuge: Hermle Z 383 K (Hermle Labortechnik GmbH, Germany)

Refrigerator shaker: WiseCube® (Wisd laboratory instruments, Australia)

Shaker: Wiggin WS-300R (Wiggens GmbH, Germany)

Ultrapure water generator: Barnstead MicroPure (Thermo Scientific, USA)

Vacuum pump: Rocker 300 Oil-free (Rocker Scientific, China)

Visible spectrophotometer: GENESYS 30 (Thermo Scientific, USA)

Votex: LP Votex Mixture (Thermo Scientific, USA)

Chemicals and reagents

1,2-Bis(dimethylamino)ethane, TEMED (Affymetrix, USA)

2-(N-morpholino) ethanesulfonic acid; MES (PanReac AppliChem, Germany)

5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; X-gal (Himedia, India)

Acetic acid, glacial (Scharlau, Spain)

Agarose (Vivantis, USA)

Ammonium persulfate (Kemaus, Australia)

Ammonium sulfate (Carlo Erba reagents, Spain)

Beta-mercaptoethanol (PanReac AppliChem, Germany)

Bis-acrylamide, 40% (Himedia, India)

Bromophenol blue (Labochem International, Germany)

Coomassie® Brilliant BlueR-250 (AppliChem, Germany)

dATP, dCTP, dGTP, and dTTP (Fermentas, USA)

DNA gel stain SafeGreen™ Loading Dye (Vivantis, USA)

DNA loading dye (New England Biolabs, USA)

EDTA-disodium salt dihydrate (Himedia, Mumbai, India)

Ethanol, 95% (Chemex, Thailand)

Ethanol, purified (Merck, Germany)

Glycerol (Himedia, India)

Glycine (Himedia, India)

HeiterTM (Kao, Thailand)

Hydrochloric acid (Merck, Germany)

Imidazole (PanReac AppliChem, Germany)

Isopropyl-β-D-thiogalactopyranoside; IPTG (Himedia, India)

Methanol, 95% (Chemex, Thailand)

Methanol, purified (Honeywell, Korea)

Magnesium sulphate (Kemaus, Australia)

Miller-Luria Bertani (LB) Agar (Himedia, India)

Ni-NTA affinity resin Amintra® (Abcam, UK)

Nickel sulphate (Kemaus, Australia)

Peptone (Himedia, India)

Potassium chloride (Kemaus, Australia)

Potassium dihydrogen phosphate (Carlo Erba reagents, Spain)

SDS-Lauryl sulphate sodium salt (Himedia, India)

Skim mike (Becton, Dickinson and Company, France)

Sodium chloride (Himedia, India)

Sodium chloride (Himedia, India)

Sodium hydroxide (Himedia, India)

TAE buffer, 50x (Bio-rad, USA)

TMB stabilized substrate for horseradish peroxidase (Abcam, UK)

Tris (Vivantis, USA)

Tween-20 (Vivantis, USA)

ViSafe Green gel stain (Vivantis, USA)

Yeast extract powder (Affymetrix, USA)

Antibiotics

Ampicillin sodium Salt (BioChemica AppliChem, Germany)

Gentamycin Sulfate (BiocChemica AppliChem, Germany)

Kanamycin Sulfate (Biochemica AppliChem, Germany)

Rifampicin (Himedia, India)

Proteins, antibodies, enzymes, and biological substances

All blue ladder, Precision Plus ProteinTM Standards (Bio-rad, USA)

Anti-cholera toxin B-rabbit polyclonal antibodies (Abcam, UK)

Anti-histidine antibody-HRP (Abcam, UK)

Anti-rabbit-goat-HRP antibody (Jackson ImmunoResearch, USA)

Heat-labile enterotoxin B subunits, LTB (Sigma-Aldrich, USA)

Taq polymerase with buffer (New England Biolabs, USA)

T4 DNA ligase (New England Biolabs, USA)

SacI-HF (New England Biolabs, USA)

VC 1kb DNA ladder (Vivantis, USA)

XbaI (New England Biolabs, USA)

XhoI (New England Biolabs, USA)

Plasmids

pBYR2eK2Md (Diamos & Mason, 2019; Diamos et al., 2016)

pGEM®T-Easy vector systems (Promega, USA)

DNA extraction and ELISA kits

DNA-spin™ Plasmid DNA purification kit (iNtRON Biotechnology, Korea)

MEGAquick-spin™ plus Fragment DNA purification Kit (iNtRON Biotechnology, Korea)

Human CTLA-4 Simplestep ELISA kit (Abcam, UK)

Others

0.45 µm nitrocellulose membrane (Bio-Rad, USA)

0.45 µm sterile Filter 47 mm (Merck Millipore, Ireland)

Amicon® Ultra 15 mL Filters 10 kDa (Merck, Ireland)

Disposable cuvettes (BRAND, Germany)

Microplate, 96 wells (Greiner bio-one GmbH, Germany)

MicroPulser electroporation cuvettes, 0.2 cm gap (Bio-Rad, USA)

Millex® - GP 0.22 um filter unit (Merck Millipore, Ireland)

Parafilm® (Bemis, USA)

pH indicator strips (non-bleeding) pH 0-14 universal indicator MColorpHast™ (Kerck KGaA, Germany)

Microorganism and plant expression system

E. coli strain DH10B

A. tumefaciens strain GV3101

N. benthamiana plant

Software and database

BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/)

ExPaSy Bioinformatics Resource Portal, Compute pI/Mw tool (https://web.expasy.org/compute_pi/)

NEBcutter V2.0 (http://www.labtools.us/nebcutter-v2-0/)

Oligo Calculator version 3.27 (http://biotools.nubic.northwestern.edu/OligoCalc.html)

Reverse complement tool (http://www.bioinformatics.org/sms/rev_comp.html)

Translate tool (http://web.expasy.org/translate/)

Methods

LTB-CTLA4 gene construction and molecular cloning

Sequence of LTB-CTLA4

Nucleotide sequence templates of LTB-CTLA4 were a gratitude from Professor Sergio Rosales-Mendoza and was designed by Alejandra Wong-Arce from Universidad Autónoma de San Luis Potosí, San Luis Potosí, México. The Sequence of LTB gained from GenBank GenBank Accession no.: WP024180933.1, fused with GPGP linker, followed by the sequence of CTLA4 extracellular domain, residues 36-161; GenBank Accession no.: BC074893.2. The original LTB-CTLA4 sequence has signal peptide from *Influenza* A virus HA protein or algae *Chlamydomonas reinhardtti* binding protein 1 (BIPI) on N-terminus. The end of C-terminus contains SEKEDL amino acid sequence which allow for recombinant protein to retain in the plant endoplasmic reticulum.

Amino acid sequence LTB-CTLA4 with signal peptide form *Influenza* A virus
MKANLLVLLCALAAADAPQSITELCSEYRNTQIYTINDKILSYTESMAGKREMVIITFKSGATFQVEV PGSQHIDSQKKAIERMKDTLRIAYLTETKIDKLCVWNNKTPNSIAAISMENGPGPKAMHVAQPAVVL ASSRGIASFVCEYASPGKATEVRVTVLRQADSQVTEVCAATYMMGNELTFLDDSICTGTSSGNQVN LTIQGLRAMDTGLYICKVELMYPPPYYLGIGNGTQIYVIDPEPCPDSDSEKDEL

Table 2. Amino acid sequence LTB-CTLA4 with signal peptide form *Influenza* A virus

Amino acid sequence LTB-CTLA4 with signal peptide from *C. reinhardtti*
MAQWKAAVLLLALACASYGFGVWAEEEKLGTVIGPQSITELCSEYRNTQIYTINDKILSYTESMAGKR EMVIITFKSGATFQVEVPGSQHIDSQKKAIERMKDTLRIAYLTETKIDKLCVWNNKTPNSIAAISMENGP GPKAMHVAQPAVVLASSRGIASFVCEYASPGKATEVRVTVLRQADSQVTEVCAATYMMGNELTFLD DSICTGTSSGNQVNLTIQGLRAMDTGLYICKVELMYPPPYYLGIGNGTQIYVIDPEPCPDSDSEKDEL

Table 3. Amino acid sequence LTB-CTLA4 with signal peptide from *C. reinhardtti*

Primers design

The primers were design based on original LTB-CTLA4 sequence using Oligo Calculator version 3.27 (http://biotools.nubic.northwestern.edu/OligoCalc.html) and Reverse Complement tool (http://www.bioinformatics.org/sms/rev_comp.html). The %GC content and melting temperature (Tm) of primers were carefully designed and calculated to minimize the potential formation of primer dimer, hairpin loops or 3' complementary.

primer name	5'to 3' sequence	comments
>XbaI-AgSP1_F	CTCTAGAATGAAGGCCAACCTCCTCG	*Influenza* A virus HA protein
>XbaI-AgSP2_F	CTCTAGAATGGCCCAGTGGAAGGCC	*Chlamydomonas reinhardtti* binding protein 1 (BIP1)
>SacI_KD_H_LTB2_R	GAGCTCTCAAAGCTCATCCTTTTCAGAATGATGGTGATGGTGGTGATGATGGTCCGAGTCGGGGCAGGG	partial CTLA-4 sequence with 8x histidine tag, and SEKDEL sequences
>Plant_SP3_F	ACAATGGGCTGGTCCTGCATCATCCTGTTCCTTGTTGCTACTGCTACCGGCGTTCACTCTGATGTTCAACTT	Murine IgG2 heavy chain leader peptide

Table 4. The table shown the nucleic acid sequences of primers

pBY2e-LTB-CTLA4-8H-KDEL construction

To construct pBY2e-LTB-CTLA4-8H-KDEL murine-SP, LTB-CTLA4 with signal peptide form *Influenza* A virus or *Chlamydomonas reinhardtti* templates were amplified by PCR using forward primer >XhoI_LTB_F which contained *Xho*I cut site. For the construction of pBY2e-LTB-CTLA4-8H-KDEL with *Influenza* A-SP and *C. reinhardtt*-SP, both original templates were amplified by using forward primer >XbaI-AgSP1_F for the *Influenza* A-SP template, and using forward primer >XbaI-AgSP2_F for *C. reinhardtt*-SP template. All three constructs applied similar reverse primer of >SacI_KD_H_LTB2_R which contains the sequence of 8x histidine tag, following by SEKDEL and *Sac*I cut site.

name	amino acid sequence	source
Influenza A-SP	MKANLLVLLCALAAADA	*Influenza* A HA (haemagglutinin HA-1) protein signal peptide (strain A/Puerto Rico/8/1934 H1N1) ,ER-targeted.
C. reinhardtii-SP	MAQWKAAVLLLALACASYGF GVWAEEEKLGTVIG	*C. reinhardtii* binding protein, (BIP 1). ER-localized chaperone of the HSP70 superfamily.
Murine-SP	MGWSCIILFLVATATGVHSDV QLLE	Murine/Human igG2 H heavy chain leader peptide. Leader peptide for protein secretion into extracellular by using hydrophobic core.

Table 5. Shown the amino acid sequences and their sources of each constructs

The 25 µl PCR reaction of one tube consists of: 1 µl DNA templates, 1 µl 10 µM forward primer, 1 µl 10 µM reward primer, 1 µl $MgCl_2$, 0.12 µl *Taq* polymerase, 2.5 µl 2 mM dNTP, and 2.5 µl 10x reaction buffer A. Finally adjusted with nuclease-free water to 25 µl and mixed well. Load the prepared PCR reaction in PCR machine, program of each step was set following *table 6*. After 25 µl of finished reactions were mingled with 3 µl of DNA loading dye, loaded the PCR products into 1% agarose with ViSafe Green gel stain, then underwent the electrophoresis procedure 100 V for 30-45 min. Cut the bands and purified DNA fragments by using MEGAquick-spin™ plus Fragment DNA purification Kit (iNtRON Biotechnology, Korea)

step	cycle	temperature	time
Initial denaturation	1	95°C	3 min
Denaturation	30	95°C	30 sec
Annealing		52°C	45 sec
Extension		68°C	60 sec
Final extension	1	72°C	10 min
Hold reaction at	1	4°C	forever

Table 6. PCR reactions mixture

For all pBY2e-LTB-CTLA4-8H-KDEL constructs, the purified DNA fragments were ligated into pGEM®-T Easy Vector system (Promega, USA), the 10 µl ligation reaction comprised of 4 µl DNA fragment, 0.5 µl pGEM-T vector, 5 µl 2x reaction buffer, 0.5 µl DNA ligase, then adjusted with nuclease-free water to 10 µl. Incubated the reaction overnight at 4°C. Subsequently, mixed the reaction with *E. coli* DH10B competent cells then proceeded with a heat-shock procedure for plasmid

transformation using LB 1.5% agar supplemented with 100 µg/ml ampicillin. Then applied the plate with 40 µl of 20 mg/ml X-gal and 20 µl of 50 mg/ml IPTG on the agar surface for the blue-white colony selection. Only white color colonies were proceeded for the transgene detection with colony PCR. (PCR protocol, *table 6*) After the gene of interest-positive clones was selected, cultured in 5 ml LB broth in incubator shaker overnight at 37°C. Finally, the plasmids of transformed *E. coli* suspension were extracted by using DNA-spin™ Plasmid DNA purification kit (iNtRON Biotechnology, Korea), then purified plasmids were initiated with enzymes digestion.

Each plasmid that contained *Influenza* A-SP or *C. reinhardtii*-SP was digested with restriction enzymes *Xba*I and *Sac*I, for the murine-SP was digested with restriction enzymes *Xho*I and *Sac*I, in the incubator at 37°C for 2 hours. For the use of *Influenza* A-SP or *C. reinhardtii*-SP constructs, the pBYR2eK2Md plasmid (Diamos MS et al 2016) was also cut with enzymes *Xba*I and *Sac*I at the previous condition. After the digestion of plasmids, gel electrophoresis with 1% agarose was conducted, and expected bands were cut and purified with MEGAquick-spin™ plus Fragment DNA purification Kit (iNtRON Biotechnology, Korea). For the murine-SP construct, pre-ligated pGEM®-T Easy Vector that readily contained murine-SP, *Xho*I, and *Sac*I enzyme cut sites was used in the ligation reaction.

Next, the ligation of *Influenza* A-SP or *C. reinhardtii*-SP or murine-SP constructs was conducted. The 10 µl ligation reaction comprised of 1 µl T4 DNA Ligase, 6.5 µl enzymes cut gene fragments elution, 2 µl enzymes cut plasmids elution, 2 µl 10x T4 DNA ligase reaction buffer, and adjusted to 10 µl with nuclease-free water, incubated the reaction overnight at 4°C. Then the ligation reactions were undergone plasmids transformation into *E. coli* DH10B competent cells by using the heat-shock method. Subsequently, spread transformed *E. coli* using LB 1.5% agar (for *Influenza* A-SP and *C. reinhardtii*-SP constructs, supplemented with 50 µg/ml

kanamycin, for a murine-SP construct, supplemented with the previous concentration of ampicillin, PTG, and X-gal). The colonies were selected and proceeded for the transgene detection with colony PCR. (PCR protocol, *table 6*) For increasing ligated plasmid copy number, PCR-confirmed positive colonies were cultured in 5 ml LB broth, supplemented with 50 µg/ml kanamycin or 100 µg/ml ampicillin, in an incubator shaker overnight at 37°C and the plasmid of transformed clones were extracted.

Then purified plasmids of *Influenza* A-SP and *C. reinhardtii*-SP constructs were proceeded with transformation into *A. tumefaciens* cells by using the electroporation method. The transformed *Agrobacterium* was cultured on a 1.5% agar plate supplemented with 50 µg/ml kanamycin, 50 µg/ml gentamicin, and 50 µg/ml rifampicin, at 28°C for two days in darkness. The transformed *Agrobacterium* colonies were selected and confirmed with PCR by using forward primers of >XbaI-AgSP1_F for *Influenza* A-SP construct and >XbaI-AgSP2_F for *C. reinhardtii*-SP construct, for reverse primer, >SacI_KD_H_LTB2_R was used with both constructs (PCR protocol, *table 6*). Positive clones were cultured in 5 ml LB broth with 50 µg/ml kanamycin, 50 µg/ml gentamicin, and 50 µg/ml rifampicin, in an incubator shaker at 28°C for two days. Added glycerol into an aliquot of *Agrobacterium* culture, mixed thoroughly, then stored at -80°C.

With purified plasmids of the murine-SP construct, the transformed plasmid was transformed into *E. coli* by using the heat-shock method and plated on 1.5% agar plate supplemented with 100 µg/ml ampicillin, incubated at 37°C overnight. The positive clones were selected and confirmed with PCR by using >Plant_SP3_F as a forward primer and >SacI_KD_H_LTB2_R as a reverse primer (PCR protocol, *table 6*), then cultured in 5 ml LB broth supplemented with 100 µg/ml ampicillin in an incubator shaker at 37°C overnight. The digested again with restriction enzymes *Xba*I and *Sac*I in the same manner of the previous enzyme digestion reaction. Next, the

electrophoresis was performed, the desired bands were cut, and DNA fragment was purified. Subsequently, proceeded with ligation of murine-SP into pBYR2eK2Md plasmid by using the previous ligation reaction setup. To increase plasmid copy number, the ligated plasmid was transformed into *E. coli* by using the heat-shock method, then plated on 1.5% agar supplemented with 50 µg/ml kanamycin. The selected colonies were selected and confirmed with PCR by using >Plant_SP3_F as a forward primer and >SacI_KD_H_LTB2_R as a reverse primer (PCR protocol, *table 6*), and positive clones were cultured in 5 ml LB broth, supplemented with 50 µg/ml kanamycin, in incubator shaker overnight at 37°C, then the plasmid was extracted. The ligated plasmid was proceeded with transformation into *A. tumefaciens* cells by using the electroporation method following a similar procedure as *Influenza* A-SP and *C. reinhardtii*-SP constructs. Stored glycerol-mixed aliquot of *Agrobacterium* culture at -80°C.

Amino acid sequence of LTB-CTLA4 with signal peptide form *Influenza A* virus
MKANLLVLLCALAAADAPQSITELCSEYRNTQIYTINDKILSYTESMAGKREMVIITFKSGATFQVEVP GSQHIDSQKKAIERMKDTLRIAYLTETKIDKLCVWNNKTPNSIAAISMENGPGPKAMHVAQPAVVLAS SRGIASFVCEYASPGKATEVRVTVLRQADSQVTEVCAATYMMGNELTFLDDSICTGTSSGNQVNLTI QGLRAMDTGLYICKVELMYPPPYYLGIGNGTQIYVIDPEPCPDSDHHHHHHHHSEKDEL

Table 7. Amino acid sequence of LTB-CTLA4 with form *Influenza* A signal peptide, with the addition of 8x histidine tag

Amino acid sequence of LTB-CTLA4 with signal peptide from *C. reinhardtti*
MAQWKAAVLLLALACASYGFGVWAEEEKLGTVIGPQSITELCSEYRNTQIYTINDKILSYTESMAGKR EMVIITFKSGATFQVEVPGSQHIDSQKKAIERMKDTLRIAYLTETKIDKLCVWNNKTPNSIAAISMENGP GPKAMHVAQPAVVLASSRGIASFVCEYASPGKATEVRVTVLRQADSQVTEVCAATYMMGNELTFLD DSICTGTSSGNQVNLTIQGLRAMDTGLYICKVELMYPPPYYLGIGNGTQIYVIDPEPCPDSDHHHHHH HHSEKDEL

Table 8. Amino acid sequence of LTB-CTLA4 with *C. reinhardtti signal peptide*, with the addition of 8x histidine tag

Amino acid sequence of LTB-CTLA4 with murine leader peptide
MGWSCIILFLVATATGVHSDVQLLEPQSITELCSEYRNTQIYTINDKILSYTESMAGKREMVIITFKSGA TFQVEVPGSQHIDSQKKAIERMKDTLRIAYLTETKIDKLCVWNNKTPNSIAAISMENGPGPKAMHVAQ PAVVLASSRGIASFVCEYASPGKATEVRVTVLRQADSQVTEVCAATYMMGNELTFLDDSICTGTSSG NQVNLTIQGLRAMDTGLYICKVELMYPPPYYLGIGNGTQIYVIDPEPCPDSDHHHHHHHHSEKDEL

Table 9. Amino acid sequence of LTB-CTLA4 with murine leader peptide, with the addition of 8x histidine tag.

Transformation *E. coli* by using heat-shock method

Firstly, chemically prepared competent cells of *E. coli* strain DH10B from -80°C glycerol were thawed on ice. After 100 µl of *E. coli* competent cells were pipetted into a 1.5 ml tube, added 10 µl plasmid or plasmid-ligation mixture then gently mixed by tube-tapping and incubated on ice for 20-30 min. Subsequently, place the tube of mixed plasmid and *E. coli* competent cells into heat block at 40°C for 60 sec, then immediately put the tube on ice. Applied 500 µl of LB media into the tube for promoting transformed *E. coli* growth, incubated in the incubator shaker,

setting at 200 rpm, at 37°C for 1 hour. Spread bacterium suspension onto 1.5% LB agar plate supplemented with suitable antibiotics or agents for colony selection. Finally, the plate was incubated overnight in an incubator at 37°C then selected positive colonies for colony PCR.

Transformation of *A. tumefaciens* by using electroporation method

The electrocompetent cells of *A. tumefaciens* strain GV3101 from -80°C glycerol stock was thawed on ice. The 10 µl plasmids were mixed with 100 µl electrocompetent *A. tumefaciens* cells in electroporation cuvettes, size 0.2 cm (Bio-Rad, USA). After applying a cuvette in MicroPulser™ (Bio-Rad, USA), set the electroporation machine program into '*Agr*' then operated the machine with one electro-pulse. After that, instantly applied 500 µl of LB media into the cuvette, mixed gently by pipetting, and transferred into a 1.5 ml centrifuge tube, incubated a tube of transformed *A. tumefaciens* in the incubator shaker, setting at 200 rpm, 28°C for 2.45-3 hours. After reducing the majority of LB media by using a mini-centrifuge, plated the electroporated *A. tumefaciens* cells onto a 1.5% LB agar plate containing appropriated selective media. Incubated the plate in an incubator at 28°C overnight for two days.

LTB-CTLA4 expression in *N. benthamiana*

***N. benthamiana* cultivation**

The seeds of *N. benthamiana* was grown in the water-saturated peat moss with semi-closed system environment of 16-hours photoperiod for 7-9 days in the greenhouse, then transferred to soil with fertilizer. The *N. benthamiana* plants were natured and watered thrice a week until the suitable age of 4-8-week-old.

Transient expression in *Nicotiana benthamiana* by agroinfiltration

A. tumefaciens GV3101 harboring plant expression vector pBRY2e-LTB-CTLA4 was thawed form -80℃ glycerol stock and grown overnight in LB media supplement with antibiotics at 28℃ (50 µg/mL kanamycin, 50 µg/mL gentamicin, and 50 µg/mL rifampicin, or 1 µg of antibiotics per 1 ml LB broth). The overnight-cultured Agrobacterium cells were centrifuged at 6000 rpm for 15 min, and the pellet was resuspended with infiltration buffer (100 mM 2-(N-morpholino) ethanesulfonic acid (MES) and 100 mM $MgSO_4$, pH 5.5). The final concentration of *Agrobacterium* suspension was adjusted to 0.4 at OD_{600}. The 6-8-week-old *N. benthamiana* plants were agroinfiltrated by using a syringe without needle. For vacuum method in large-scale production, after immersing leaves of *N. benthamiana* plants in Agrobacterium suspension in vacuum chamber, close the desiccator then Open the pump until the pressure reaches to 80 psi. Hold the pressure for at least 30 sec, then releases the pump's valve. Re-vacuum the plants if needed, depending on the infiltrated leaves' condition. Consecutively, stored the infiltrated plants in the greenhouse of 16-hours photoperiod. The leaves were harvested on day 1, 2, 3, 4, 5, and 6-day post-infiltration (dpi) to assess the optimal day for high protein production.

Protein extraction and purification

Recombinant Protein extraction and purification

N. benthamiana leaves were harvested and the plant crude protein was extracted with 2 volumes of PBST buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.005% Tween-20, pH 7.4) per leaf fresh weight (FW) into leaf powder. Subsequently, the samples were thoroughly mixed and vigorously vortexed for 5 min. Then proceeded by centrifugation of buffer-mixed samples at 13,000 rpm

for 15 min and, finally, clear supernatant was gathered for the protein analysis or proceeded further in the purification steps.

For large-scale production, *N. benthamiana* plants were agroinfiltrated using a vacuum chamber. The *N. benthamiana* leaves were homogenized with two volume of pre-chilled IMAC 5 buffer (5 mM imidazole, 50 mM NaCl and 20 mM Tris-HCl, pH 7.4), then filtered out the plant biomass using a clean cotton cloth. The filtrate was collected and centrifuged at 13,000 rpm at 4°C for 45 min. After collecting clear supernatant, invisible debris was removed by using a 0.45 µm membrane filter (Merck, Cork, Ireland) connected with a negative-pressure vacuum pump. The filtrate was applied into a column packed with Ni-NTA Affinity Resin-Amintra® (Abcam, Cambridge, UK). The washing step was performed using IMAC 20 buffer (20 mM imidazole, 50 mM NaCl and 20 mM Tris-HCl, pH 7.4), then eluted the bounded protein with IMAC 250 buffer (250 mM imidazole, 50 mM NaCl, and 20 mM Tris-HCl, pH 7.4). The elution was filtered with a 0.22 µm syringe filter (Merck, Cork, Ireland) before loading into Amicon® Ultra 15 mL Filters 10 kDa (Merck, Cork, Ireland). The tubes were centrifuged Amicon® at 4000xg for 15 min and discarded filtrate then added PBS buffer. The step was repeated until the concentration of imidazole salt was the least minimized. Finally, purified protein was stored in at -80°C or lyophilized overnight using Lyophilizer Labconco® Freezone 6 Plus (Kansas City, MO, USA).

Ammonium sulfate protein precipitation

The protocol of ammonium sulfate protein precipitation was adopted from Burgess RR, 2009 (Burgess, 2009), intending to remove RuBisCO, an abundant host protein of *N. benthamiana,* from the protein purification process. In summary, the concentration of protein extract was measured then kept cold on ice. The

ammonium sulfate salt was calculated to make 35% salt concentration of crude extract volume (see Appendix D), then added into the chilled crude extract, continuously stirred by using a magnetic stirrer for 30-45 min. After mixing, centrifuged the saturated solution that contained precipitated salt at 9,500 rpm for 30 min, then separately collected the pellet and supernatant. Subsequently, dried the pellet and dissolved with IMAC 5 buffer equally to former crude extract volume. After the dissolved solution's filtration with a 0.45 µm sterile filter, the filtrate was introduced into the Ni-NTA Affinity Resin-Amintra® column chromatography and proceeded with the purification steps.

For ammonium sulfate-saturated supernatant, it was calculated and adjusted the concentration of ammonium sulfate from 35% to 80% (see Appendix D). Subsequently introduced calculated ammonium sulfate into chilled supernatant then continuously stirred by using a magnetic stirrer for another 30-45 min, then repeated the centrifugation with the previous condition. Subsequently, dried the pellet and dissolved with IMAC 5 buffer equally to former crude extract volume. Then the solution was filtrated and proceeded with the purification steps. The sample of each step was aliquoted for protein detection.

Characterization and quantification of LTB-CTLA4

Bradford assay

The Bradford reagent (Bio-Rad, USA) was prepared by diluting into de-ionized water in the ration 1:4 gaining the brownish color solution. The serial dilution of bovine serum albumin (BSA) protein, at concentrations 25, 50, 100, and 200 µg/ml, was prepared as a standard curve. The 980 µl of diluted Bradford solution was pipette-mixed with 20 µl of protein sample or BSA standard in a disposable cuvette.

The cuvettes were measured absorbance by using a visible spectrophotometer (Thermo Scientific, USA) at 595 nm.

SDS-PAGE and western blot assay

The samples with reducing condition were mixed with 10x reducing SDS loading dye buffer (125 mM Tris HCl, 12% SDS, 10% glycerol, 22% β-mercaptoethanol, 0.001% bromophenol blue, pH 6.8). The proteins were separated in 15% acrylamide gel and the bands were visualized by Coomassie Blue staining. For western blot analysis, the separating proteins were transferred onto 0.2 μm nitrocellulose membrane (Thermo Fisher Scientific, USA). The membrane was blocked with 5% fat-free dry milk in PBS for 30 mins. Then the membrane was washed and probed with anti-cholera toxin B-rabbit polyclonal antibodies (Abcam, UK) with the dilution of 1:2000 in 3% skim milk and incubated for 1.5 hour. Then the membrane was washed thrice and then the peroxidase-conjugated anti-rabbit-goat (Abcam, UK) antibody was as a secondary antibody with a dilution 1:10000 in 3% skim milk and incubated for 1 hour. For histidine-tag probing, used only first antibody which was a peroxidase-conjugated anti-histidine dilution 1:5000 in 3% skim milk and incubated for 1-1.5 hour. After washing membranes like previous washing steps, finally, applied the chemiluminescent ECL substrate(Abcam, Cambridge, UK) onto the membrane, the protein signal was detected using the chemiluminescent detector chamber, ImageQuant™ LAS 4000 biomolecular imager (GE Healthcare, Chicago, IL USA).

CTLA-4 ELISA assay

The human CTLA-4 SimpleStep ELISA® Kit (Abcam, UK) was used for the quantification and binding affinity of plant produced-recombinant protein by following manufacturer instruction, The procedure, in brief, the plant protein extraction were diluted at appropriated dilution with sample diluent. The 50 µl of buffer-diluted preparations were added into the capture antibody pre-coated strips, with three duplicates of each sample. Then introduced 50 µl of CTLA-4 antibodies cocktail and incubated for 1 hour at room temperature. The incubated strips were washed trice with wash buffer and towel blot-dried. Subsequently, the TMB development solution was added and incubated for 10 min. After adding 100 µl of stop solution, measured coloration at wavelength 450 nm with a SpectraMax M5 microplate reader (Molecular Deviced, USA), wild-type *N. benthamiana* crude extract as negative control and human CTLA-4 as a standard. Perform triplicate the data was represented as Mean +/- SD.

GM1-ELISA assay

The expression level of LTB-CTLA4 was determined by a GM1 ganglioside-dependent ELISA assay. Briefly, 96 well polystyrene plates were coated with Type III GM1 ganglioside (1.5 µg per well, Sigma) dissolved in carbonate buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$) and incubated overnight at 4°C. After washing with PBST, plates were blocked with 5% fat-free dry milk dissolved in PBST and incubated for 2 h at room temperature. Then the protein extracts were added, and plates were incubated overnight at 4°C. After incubation, anti-CT-serum (1:500) was added as primary antibody and incubated at 4°C overnight. Horseradish peroxidase-conjugated anti-mouse IgG (1:2000 Sigma, St.Louis, MO) was added and incubated for 2 h at room temperature. Reaction was developed by using ABTS substrate solution [0.6 mM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; Sigma, St. Louis, MO),

0.1 M citric acid, 1 mM of H2O2, pH 4.35] and optical density values were measured at OD 405 nm by using a Thermo Scientific Multiskan® FC microplate photometer (Thermo Scientific, Waltham, MA).A standard curve prepared with CTB standards was used to assess the amount of recombinant protein produced in *N. benthamiana* leaves.

Immunogenicity assessment

Immunization assay

LTB-CTLA4 immunogenicity assay was performed on the Laboratory of Professor Sergio Rosales-Mendoza from Universidad Autónoma de San Luis Potosí, San Luis Potosí, México. Immunogenicity of the pant-made LTB-CTLA4 protein was assessed in 10-week-old female BALB/c mice. Test animals were nurtured under standard conditions with free access to food and water following the procedures suggested by the Federal Regulations for Animal Experimentation and Care (SAGARPA, NOM-062-ZOO-1999, Mexico). The protocol was approved by the Institutional Animal Care and Use Committee (Approval number CEID/201803).

BALB/c mice groups (n=5) were subjected randomly to a subcutaneous immunization scheme on days 1, 15, and 30 with 10 µg of plant-derived LTB-CTLA4 or 10 µg of CTLA4 peptide (CTLA4p). Both immunogens were emulsified in one volume of Incomplete Freund´s Adjuvant (IFA). PBS was administrated for a control group.

Mice sera were collected on days 0, 14, 29, 45, 115, and 130 (T0, T1, T2, T3, T4, and T5, respectively). After mice were bled, the blood was centrifuged at 6000 rpm for 10 min for removing the red blood cells and the supernatant was collected. The sera were analyzed by ELISA to measure the IgG response against LTB or the

CTLA4p. The 96-well polystyrene plates were coated overnight at 4°C with 1 μg per well of the CTLA4 synthetic peptide (sequence: DSQVTEVCAATYMMGNELTFLDD; Synpeptides Co. China) or 1 μg/well of LTB standard. The plates were washed thrice with PBST between each step. After coating the plates with peptide or LTB, the plates were blocked with 5% skim milk at 2°C for 2 hours. The serial dilutions of mice sera were applied and plates were incubated at 4°C overnight. A goat horseradish peroxidase-conjugated anti-mouse IgG was introduced as the secondary antibody (1:2000 dilution, Sigma, St. Louis, MO) and plates were incubated for 2 h at 25°C. Antibody binding detection was determined as described in the previous experiments. Antibody levels were described as the mean OD values measured in a microplate reader. For antibody titer assay, the serial dilutions were determined and the higher dilutions in which the OD value was above the one registered for the PBS group+2×SD was analyzed as titer value. GraphPad Prism 8 software was used for one-way ANOVA ($p < 0.05$) by using immunogenicity test data.

CHAPTER III

RESULTS

The LTB-CTLA4 constructs were successfully cloned

The pBY2e-LTB-CTLA4-8H-KDEL with three different signal peptides, murine-SP, *influenza* A-SP, and *C. reinhardtti*-SP (figure 12), were successfully constructed, ligated into pBYR2eK2Md expression geminiviral vector, and transformed into *A. tumefaciens* strain GV3101. The gene contained the signal peptides on N-terminus end, followed by LTB, GPGP linker and CTLA-4 sequence. The next part was 8x histidine residues on the C-terminus followed by SEKDEL sequence, all three insert genes were flanking with *Xba*I and *Sac*I restriction sites. All constructs were confirmed the size with PCR (figure 12) and the sequences was confirmed with gene sequencing.

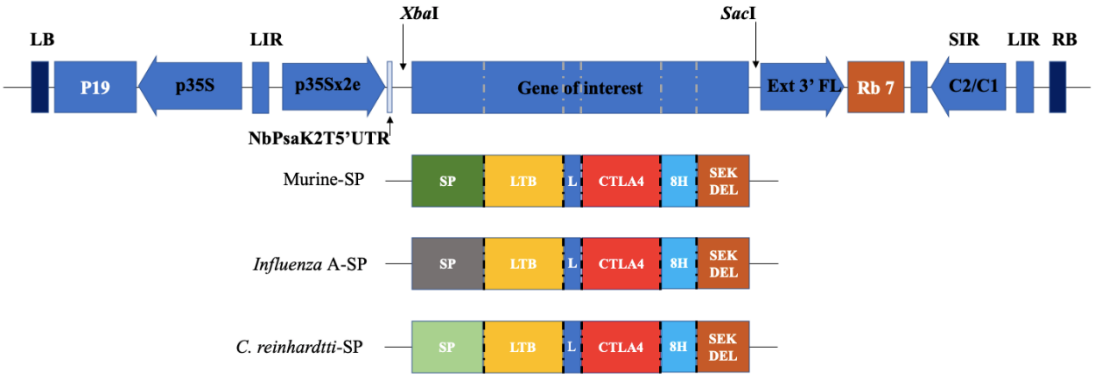

Figure 12. Schematic representation of expression vector.

The cassette consists of 35S: A promoter from cauliflower mosaic virus 35S; three constructs of gene of interest were designed with different signal peptides: murine-SP, *influenza* A-SP, and *C. reinhardtti*-SP; LTB-CTLA4: heat-labile enterotoxin B (LTB) fused human cytotoxic T-lymphocyte-associated

protein 4 (CTLA-4); L: GPGP linker. The gene contained the signal peptide on N-terminus and 8x histidine residues on the C-terminus followed by SEKDEL sequence, flanking with *Xba*I and *Sac*I restriction sites; NbPSaK2T5'UTR: *N. benthamiana* photosystem K subunit 5' untranslated region: Ext 3' FL: extensin 3' flanking region; RB 7: tobacco RB7 promoter; C2/C1: bean yellow dwarf virus (BeYDV) promoter C1 and C2 encoded viral replicon, Rep and RepA proteins; LIR and SIR: long and short intergenic region of the BeYDV geminiviral vector; P19: tomato bushy stunt virus (TBSV) P19 silencing suppressor; LB and RB: the left and the right border, respectively.

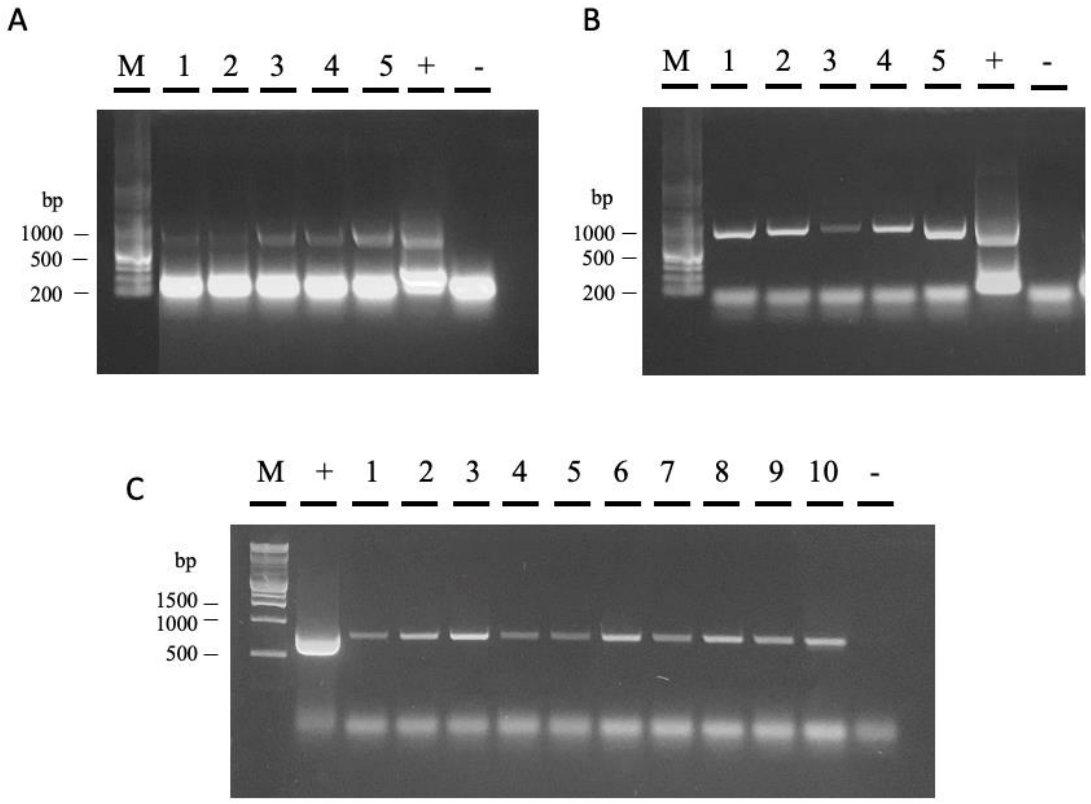

Figure 13. The electrophoresis of colony PCR of *A. tumefaciens* transformed each LTB-CTLA4 construct by using 1% agarose.

A) The colony PCR of *A. tumefaciens* transformed with *influenza* A-SP construct, approximate size was 806 bp. Lane M: DNA ladder; Lane 1-5: selected transformed colonies with *influenza* A-SP construct; Lane +: PCR reaction of LTB-CTLA4 plasmid with *influenza* A-SP as positive control Lane -: PCR reaction without DNA template as negative control.

B) The colony PCR of *A. tumefaciens* transformed with *C. reinhardtti*-SP construct, approximate size was 856 bp. Lane M: DNA ladder; Lane 1-5: selected transformed colonies with *C. reinhardtti*-SP construct; Lane +: PCR reaction of LTB-CTLA4 with *C. reinhardtti*-SP plasmid as positive control; Lane -: PCR reaction without DNA template as negative control.

C) The colony PCR of A. tumefaciens transformed with murine-SP construct approximate size was 825 bp. Lane M: DNA ladder; Lane 1-5: selected transformed colonies with murine-SP; Lane +: PCR reaction of LTB-CTLA4 with murine-SP plasmid as positive control; Lane - : PCR reaction without DNA template as negative control.

Transient Expression of LTB-CTLA4 in *N. benthamiana*

The LTB-CTLA4 fusion gene was *N. benthamiana* codon-optimized and cloned into geminiviral vector pBYR2eK3Md with different signal peptides (figure 12) and expressed in *N. benthamiana*. The plant expression vectors were introduced to *A. tumefaciens* strain GV3101 and infiltrated into *N. benthamiana* leaves. The phenotype of the leaves infiltrated with the expression vector containing different signal peptides did not show many differences. (figure 14A) The enzyme-linked immunosorbent assay (ELISA) showed that the best signal of LTB-CTLA4 expression was with the cassette that containing murine-SP, which shown the completely necrosis leaves on 6 days post-infiltration (dpi). The highest expression level of LTB-CTLA4 was 1.29 µg/g FW on 4 dpi. (figure 14B) The LTB-CTLA4 expressed with ER-targeted signal peptides from *Influenza* A virus and micro-green algae *C. reinhardtti* shown the completely necrosis sign on 4 dpi and both yielded the highest around 200 ng/g FW yield on 2 dpi (figure 14B), relatively six times less LTB-CTLA4 protein than of murine-SP construct. The LTB-CTLA4 protein in plant extracts was detected by western blot analysis using anti-cholera toxin B-rabbit (anti-CTB) with goat-anti-rabbit-HRP. (figure 14C) The LTB-CTLA4 expressed on each dpi with total soluble

protein (TSP) of leaf samples were equally adjusted by using Bradford assay. The figure shown the similar tendency of LTB-CTLA4 expression quantified with ELISA in figure 14B. The construct containing murine-SP was selected to continue with LTB-CTLA4 production.

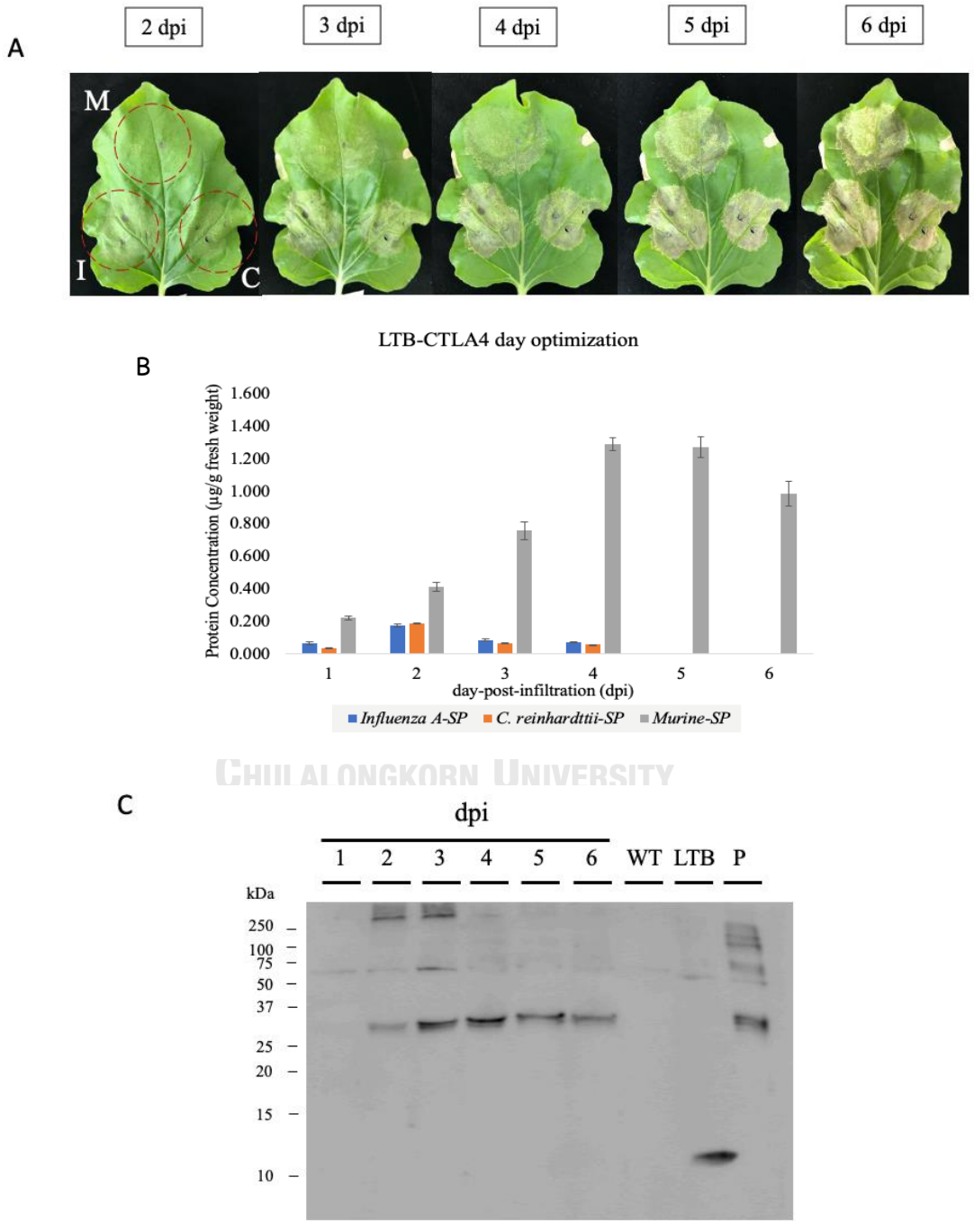

Figure 14. Assessment of the expression of LTB-CTLA4 in plants.

A) Phenotype of *N. benthamiana* leaves expressing LTB-CTLA4 with different signal peptides. (M) murine heavy chain leader sequence (murine-SP); (I) *Influenza* A virus HA signal peptide (*Influenza* A-SP) (C) *C. reinhardtti* ER-targeted signal peptide (*C. reinhardtti*-SP).

B) The accumulation of LTB-CTLA4 in *N. benthamiana* leaves after infiltrating with the vector carrying the *Influenza*-SP and *C. reinhardtti*-SP and murine-SP. The quantitative ELISA was performed by using human CTLA-4 as a standard.

C) Western blot of LTB-CTLA4 construct with murine-SP under reducing condition, using anti-LTB antibody as primary secondary and HRP conjugated anti-rabbit-goat antibody as secondary antibody. Lanes: 1-6, crude extract of day-optimized LTB-CTLA4 from dpi 1 to 6, respectively; WT, *N. benthamiana* wild-type plant crude extract; LTB, commercial heat-labile enterotoxin B subunit (approximate size 11.6 kDa); P, *N. benthamiana*-derived, affinity column-purified LTB-CTLA4 protein. The total soluble protein of each crude extract sample was measured by Bradford assay and equally adjusted.

LTB-CTLA4 assembled as pentamer and bound to GM1 receptor

The expression and integrity of the LTB-CTLA4 fusion protein was tested by western blot probed with anti-CTB and anti-histidine-HRP antibodies. The expected band at ~30 kDa was observed in protein samples extracted from the infiltrated leaves. (figure 15A) In figure 15A and 15B shown LTB-CTLA4 band at ~150 kDa in non-reducing environment indicated that LTB-CTLA4 generally assembled into a pentamer. Moreover, the reducing condition showed that the LTB-CTLA4 could also form various oligomers. In figure 15C shown the result of LTB-CTLA4 that could be purified using the Ni-NTA affinity chromatography. The GM1-ELISA was performed and LTB-GM1 binding was observed, emphasizing the affinity of LTB pentamer toward GM1-receptor.

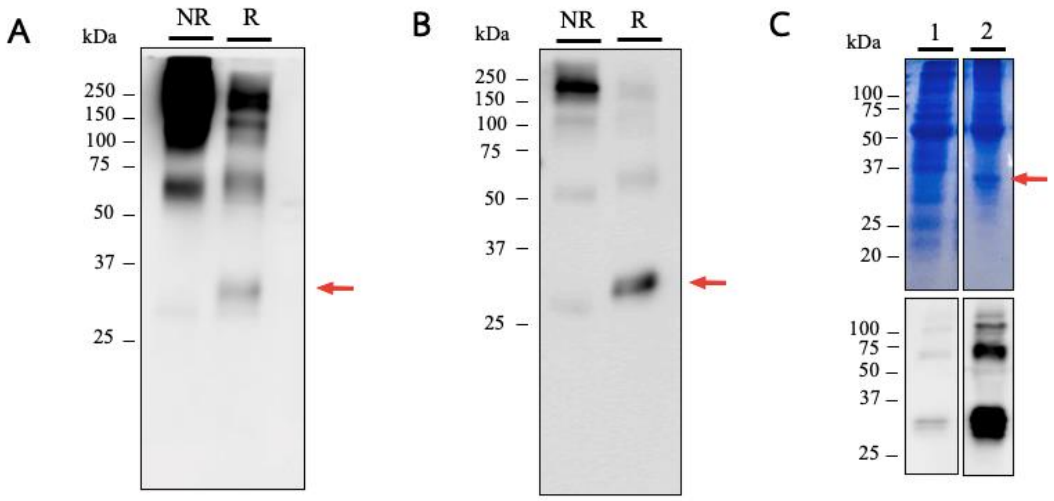

Figure 15. Detection and purification of LTB-CTLA4 from *N. benthamiana* plants. Western blot analyses were performed to detect the recombinant protein in total protein extracts from agroinfiltrated plants using an anti-LTB antibody (A) or an anti-His tag antibody (B). Lanes: NR, LTB-CTLA4 in non-reducing condition; R, LTB-CTLA4 in reducing condition. (C) Coomassie-stained SDS-PAGE gel and western blot were performed under reducing condition, for purified LTB-CTLA4 obtained by IMAC. Lanes: 1, crude extract from *N. benthamiana* biomass expressing LTB-CTLA4; 2, eluate containing LTB-CTLA4 obtained with 250 mM imidazole. The red arrows indicate the ~30 kDa LTB-CTLA4 band.

LTB-CTLA4 was purified using affinity chromatography

After crude extract contained LTB-CTLA4 was separated from *N. benthamiana* biomass, the affinity column chromatography was performed and aimed to optimize concentration of IMAC washing buffer for purification with Ni-NTA affinity column chromatography. In figure 16A, the approximate 25 g leaves FW of *N. benthamiana*-expressed LTB-CTLA4 with murine-SP was extracted with two volume of IMAC 5 buffer and loaded into gravity-flow column chromatography packed with 1 ml of Ni-NTA beads Amintra® (Abcam, UK). The each step was sampled and denatured under

reducing condition, then continued with either western blot or Coomassie-stained SDS-PAGE or both analyses, to confirmed the present of LTB-CTLA4 protein of each purified step., western blot probed with anti-CTB antibodies and goat-anti-rabbit-HRP as a secondary antibodies. The result from western blot analyses confirmed the expression of recombinant LTB-CTLA4 *N. benthamiana* when proceeded with purification steps. The signal was perceived on ~30 kDa, an approximated size of LTB-CTLA4. (figure 16A and 16B) In figure 16A shown the optimization of IMAC washing buffer. The LTB-CTLA4 in crude extract and flow-through were detected on land 1-3. However, when column was washed with IMAC, no detection of LTB-CTLA4 protein found. (figure A, lane 4) When the column was washed with buffer IMAC 50, LTB-CTLA4 protein was detected again, making IMAC 50 unsuitable of washing buffer for LTB-CTLA4 purification (figure 16A, lane 5) and suitable washing buffer of LTB-CTLA4 protein was IMAC 20.

In figure 16B shown the purification of large-scale production of LTB-CTLA4, detecting with western blot and Coomassie-stained SDS-PAGE gel analyses. The approximate 86 g leaves FW of *N. benthamiana*-expressed LTB-CTLA4 with murine-SP was extracted with two volume of IMAC 5 buffer and loaded into gravity-flow column chromatography packed with 1 ml of Ni-NTA beads Amintra®. The leak of LTB-CTLA4 protein was found after the column was washed with IMAC 20 (figure 16B, lane 5) indicating the protein exceeded the column capacity and there also was abundant LTB-CTLA4 protein in the second flow-through (figure 16B, lane 3), therefore, the flow-through was further repeated into new purification step.

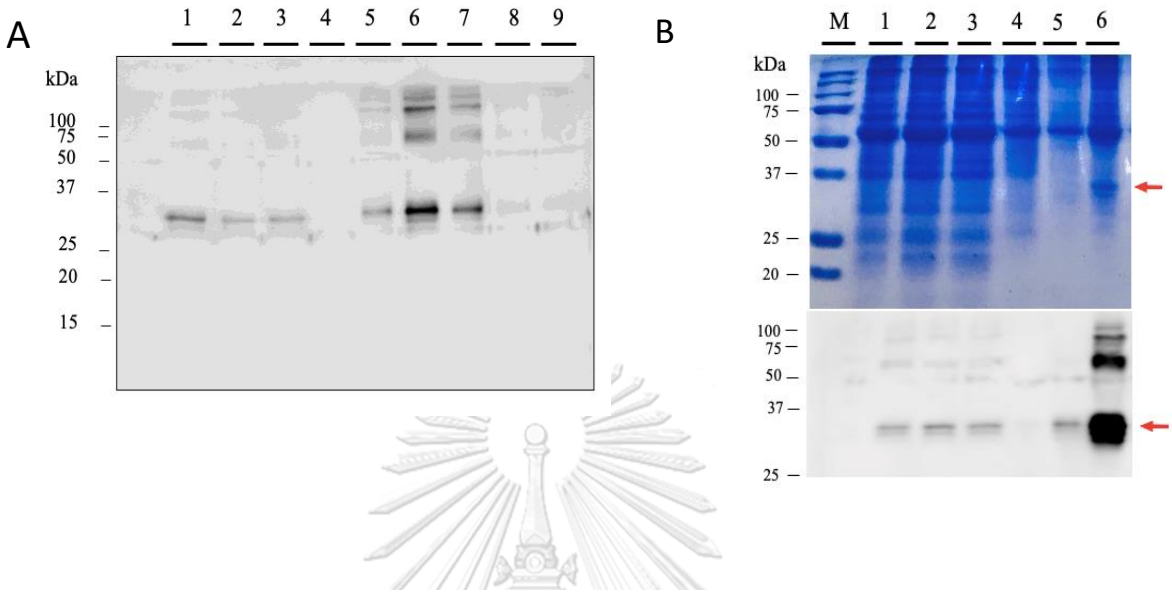

Figure 16. Western blot and Coomassie-stained SDS-PAGE gel of LTB-CTLA4 purification.

A) The western blot of optimization of IMAC concentration as a washing buffer after purified with Ni-NTA affinity column chromatography, using anti-cholera toxin B-rabbit with goat-anti-rabbit-HRP as a secondary antibody. The sample of each step was denatured under reducing condition then continued with western blot and SDS-PAGE analysis. Lane M: protein marker; Lane 1: LTB-CTLA4 crude extract; lane 2: first flow-through; lane 3: second flow-through; lane 4: IMAC 20 wash; lane 5: IMAC 50 wash; lane 6: first 1 ml elution with IMAC 250; lane 7: second elution with 1ml of IMAC 250; lane 8: third elution with 1ml of IMAC 250; lane 9: fourth elution with 1ml of IMAC 250.

B) Western blot and SDS-PAGE of LTB-CTLA4 purification with Ni-NTA affinity column chromatography. Lane M: protein ladder; Lane 1: LTB-CTLA4 crude extract; Lane 2: first flow-through; Lane 3: second flow-through; Lane 4: IMAC 5 wash; Lane 5: IMAC 20 wash; Lane 6: elution with 5 ml of IMAC 250.

Ammonium sulfate protein precipitation of LTB-CTLA4

To reduce non-specific protein, ammonium sulfate protein precipitation of murine-SP LTB-CTLA4 protein was demonstrated and Ni-NTA affinity column chromatography of ammonium sulfate precipitation was performed. The estimate isoelectric point (pI) and molecular weight (Mw) of murine-SP LTB-CTL4 construct was calculated by using bioinformatic tool: ExPaSy Bioinformatics Resource Portal, in which was 5.62 and 29.898 kDa, respectively. The size and pI of Ribulose-1,5-bisphosphate carboxylase/oxygenase or RubisCO, most abundant of plant host protein is ~50 kDa and ~ 4.4–4.7 respectively. The figure 17 shown that Coomassie-stained SDS-PAGE gel and western blot using anti-cholera toxin B-rabbit with goat-anti-rabbit-HRP as a secondary antibodies, the majority of LTB-CTLA4 and RubisCO precipitated at the 35% ammonium salt concentration (low-salt concentration), and minority of both protein was precipitated at the 80% ammonium salt concentration (high-salt concentration). Therefore, the different ammonium salt concentration could not separate LTB-CTLA4 and RubisCO from each other and ammonium sulfate protein precipitation was not suitable method to purify LTB-CTLA4 protein.

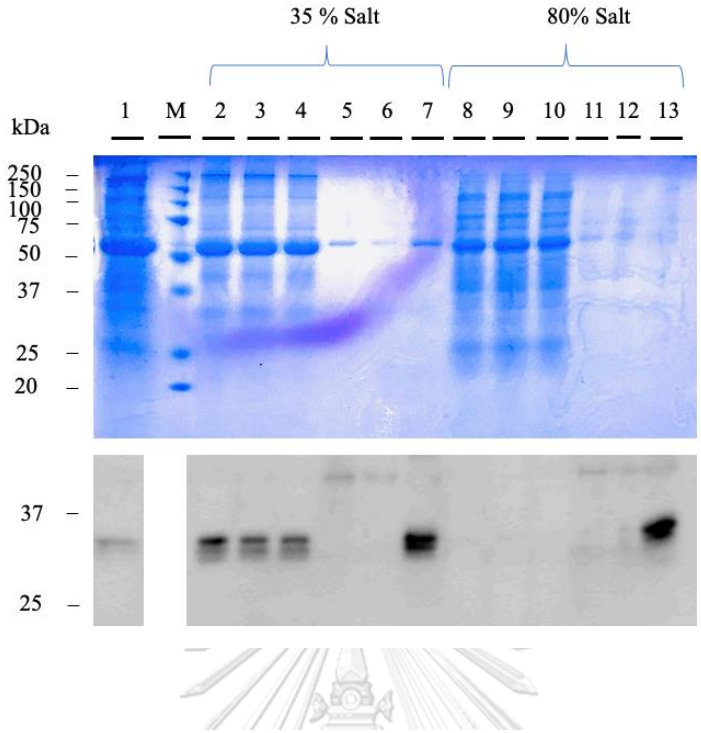

Figure 17. The Ni-NTA affinity column chromatography of ammonium sulfate precipitation of murine-SP LTB-CTLA4.

The LTB-CTLA4 protein was detected with Coomassie-stained SDS-PAGE gel and western blot using anti-cholera toxin B-rabbit with goat-anti-rabbit-HRP as a secondary antibodies. Lane 1-7 is purification of 35% ammonium salt precipitation by using Ni-NTA affinity column chromatography, Lane M: protein marker; Lane 2: crude; Lane 3: first flow-through; Lane 4: second flow-through; Lane 5: IMAC 5 wash; Lane 6: IMAC 20 wash; Lane 7: IMAC 250 elution, respectively. For lanes 8-13 is purification of 80% ammonium salt precipitation by using Ni-NTA affinity column chromatography, Lane 8: crude; Lane 9: first flow-through; Lane 10: second flow-through; Lane 11: IMAC 5 wash; Lane 12: IMAC 20 wash; Lane 13: IMAC 250 elution, respectively.

LTB-CTLA4 induced immunogenic response against CTLA-4 in mice model

To test the immune response of plant-derived LTB-CTLA4 protein, 5 female BALB/c mice were subcutaneously administrated with 10 μg of purified LTB-CTLA4 and synthesized CTLA4p, both IFA-emulsified. The mice were bled, and the sera was collected on T0 to T5 for investigation of humoral responses against CTLA-4 protein. The sera IgG responses antibodies against CTLA4p were measured by ELISA, and the synthetic peptide sequence from human CTLA-4 was used as a target antigen. For the humoral response induced against the LTB carrier, anti-LTB sera IgG responses were determined by ELISA, and the target antigen was pure LTB was used. The OD values of sera dilution at a 1:20 dilution is presented. The titers of anti-CTLA4p antibody titers induced by the plant-made LTB-CTLA4 were determined by assessing serial dilutions in ELISA targeting a CTLA4 synthetic peptide. Titers were defined as the highest dilutions in which the OD value was above the one registered for the PBS group+2×SD.

The result shown that the subcutaneous administration of plant-derived LTB-CTLA4 enhanced a humoral immune response in the murine model. In LTB-CTLA4 immunization group, the sera IgG against CTLA4p significantly increased on T4, and the group received CTLA4p alone shown sparsely signal. Interestingly, the sera IgG response against LTB of LTB-CTLA4 group was early detected on the T1 of the immunization. (figure 18A and 18B) The immunization titer of LTB-CTLA4 significantly raised when compared to the CTLA4p that OD value approached zero. (figure 19)

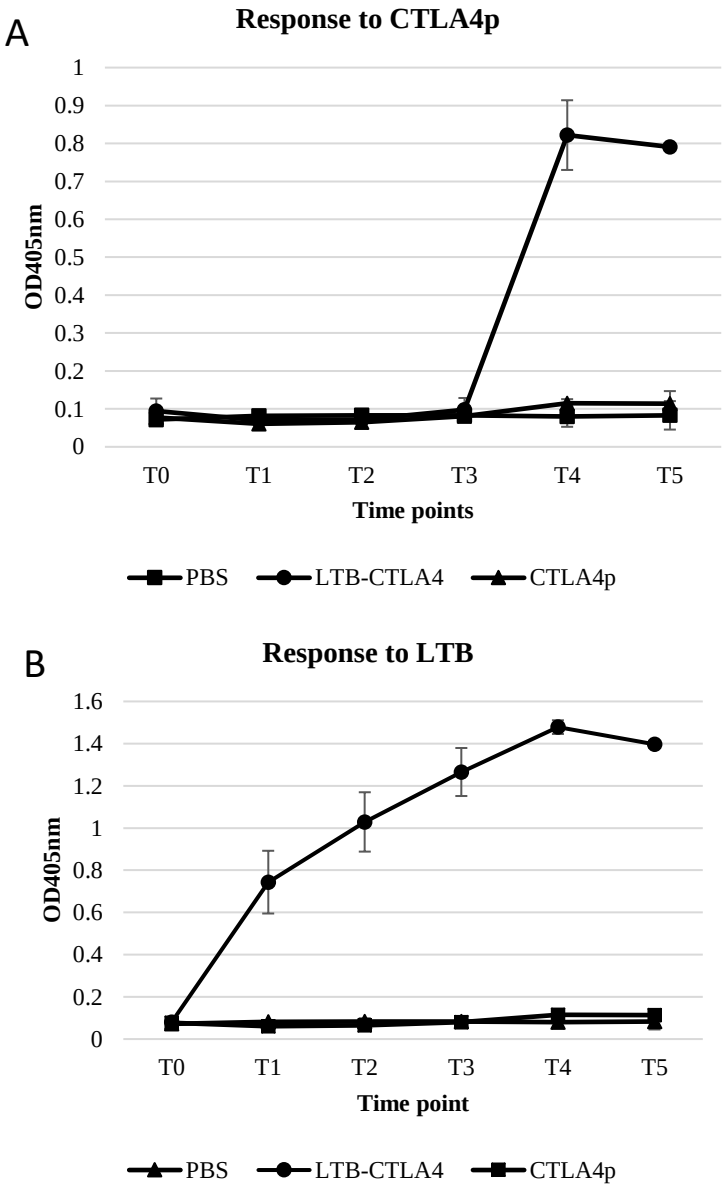

Figure 18. Shown the potential of plant-made LTB-CTLA4 protein to induce humoral responses against both LTB and CTLA4.

ELISA was conducted to determine anti-CTLA4p seric IgG responses. The target antigen was a synthetic peptide from CTLA4 sequence. Mean OD values at a 1:20 dilution is presented. (figure 18A) A humoral response induced against the LTB carrier. (figure 18B) Anti-LTB seric IgG responses were determined by ELISA. The target antigen was pure LTB. Mean OD values at a 1:20 dilution is presented.

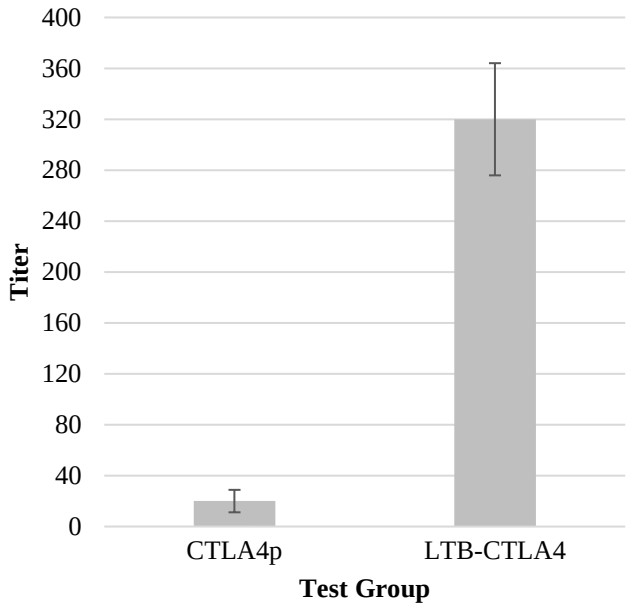

Figure 19. Anti-CTLA4p antibody titers induced by the plant-made LTB-CTLA4. Titers were determined by assessing serial dilutions in ELISA targeting a CTLA4 synthetic peptide. Titers were defined as the highest dilutions in which the OD value was above the one registered for the PBS group+2×SD.

CHAPTER VI

DISCUSSION

In recent years, immunotherapy emerged as an effective strategy to fight against cancer. The immunotherapeutic treatment aims to improve the immune function by manipulating the immune checkpoints or the pathways involved in the immune regulation system. The cytotoxic T-lymphocyte antigen 4 or CTLA-4 is one of the immune checkpoints well-studied in the immunotherapy. The expression of CTLA-4 on the surface of activated T-cell can downregulate the production and proliferation of cytotoxic T-lymphocyte cells (CTLs) through CD28-CD80/CD86 pathway. CTLs are the host-defended immune cells that could retard the growth of cancer cells by recognizing the tumor-associated antigens expressed on the cancer cell membrane and then eliminating them before spreading and evading throughout the body. (Nutt & Huntington, 2019) The overexpression of CTLA-4, an occurrence remarkably founded in cancer, has a suppressive effect on CTLs resulting in the encouragement of tumor cell growth. The blockage of CTLA-4 demonstrated the enhancement of immune response by blocking CTLA-4 protein, increasing the activation of cytotoxic T-lymphocyte cells that remarkably reduce the development of cancer. (Rowshanravan et al., 2018; Vignali, Collison, & Workman, 2008) The very first FDA approved immune checkpoint inhibitor targeted CTLA-4, ipilimumab, shown a significant improvement in metastatic melanoma patients in the clinical studies currently under investigation for more indications. (Buchbinder & Desai, 2016)

Molecular pharming has been investigated for decades and many relevant aspects were observed. (Moustafa, Makhzoum, & Trémouillaux-Guiller, 2016) For

plant-produced platform could minimize the risk of animal pathogen contamination compared to the conventional system such as a mammalian cell. (Yusibov, Streatfield, & Kushnir, 2011) This system also provides inexpensive and relatively fast production of therapeutic proteins that make them affordable for the people in low-to-middle income countries. (Paul et al., 2011) Over past years, this molecular pharming had been on-going in progressive steps and developed favorable results. In 2012, taliglucerase alfa (Elelyso^{TM}), a carrot cells-derived glucocerebrosidase enzyme for Gaucher's disease treatment, was the very first genetically modified plant-produced biopharmaceutical received the approval from US FDA. (Mor, 2015) In 2014, the recent notorious emerging cases of the Ebola virus in West Africa had further initiated the clinical trial of *N. benthamiana*-produced chimeric monoclonal antibodies (ZMapp^{TM}) in the infected patients. (Davey et al., 2016) Another plant-derived vaccine in the current-developed exemplar, tobacco-derived H5N1 influenza hemagglutinin protein (H5) expressing on the surface of virus-liked particles (VLP) elicited the humoral and cell-mediated response in healthy volunteer in the clinical trial phase 2. (Clegg, Rininger, & Baldwin, 2013; Pillet et al., 2019) Furthermore, many were currently exanimated. The present study proposed to produce the plant-derived therapeutic vaccines targeting cancer immune checkpoint. We selected CTLA4 as a protein target, and LTB-CTLA4 was design, the plant transient expression with *N. benthamiana* platform was used as a host bioreactor.

The transient expression based on plant viral vectors benefits from fast production over the time-consuming genetic-modified transgenic plant, allowing a generation of the considerate amount of recombinant protein in a short period. The molecular pharming transient-based is suitable for an urgent call for situations, such as in the pandemic emergency, when urgent demand occurs for rapid development of vaccines or antibodies, both for therapeutic and diagnostic purposes. (Capell et al.,

2020; Shanmugaraj, Malla, & Phoolcharoen, 2020) Moreover plant-made bioproduct also has the potential of producing small quantities for personalized medicines. (Shanmugaraj, Malla, et al., 2020; Tusé et al., 2015). A noticeable number of plant viruses had been meticulously inspected nowadays and extended to the development of transient plant-based tools for vaccines and biopharmaceuticals production platform. (Komarova et al., 2010) The geminivirus replicon vectors had been proved to be efficacious for protein expression in plants, especially with *N. benthamiana*, which serves as a functional plant host. In this study, we manipulated pBYR2e, a geminiviral expression system in plants (Diamos et al., 2016), and obtained the highest expression of LTB-CTLA4 approximately 1.29 µg/g FW on day 4-5 which is an average time in this viral system. (Hefferon, 2014) However, we only observed LTB-CTLA4 amount on micrograms per gram fresh weight level. Nowadays, recombinant protein production in plants still struggles from the yield challenge when compared to the conventional platforms (Schillberg et al., 2019; Spiegel et al., 2018). Nevertheless, the LTB-CTLA4 transient expression in *N. benthamiana* case gains the advantage of the speed of production, from gene construction and molecular cloning process to the protein expression solely consumed approximately 3-4 weeks before up-scaling into the mass production.

The early necrosis sign was observed on *N. benthamiana* leaves of the construct harbored signal peptides from *Influenza* A virus and *C. reinhardtti* resulting in less time for protein accumulation in the plant cells. The LTB-CTLA4 quantification of construct with *Influenza* A-SP and *C. reinhardtti*-SP There were reports that the plant call viability correlated with recombinant protein production; plant cell necrosis is one of the hindrances of protein accumulation in plant cells. (Diamos & Mason, 2019; Diamos et al., 2016; Norkunas, Harding, Dale, & Dugdale, 2018) Plant cells respond to the stress or pathogen invasion by generating reactive oxygen

species (ROS), which often lead to plant cell necrosis. The mitigation of plant cells necrosis by delaying or hindering ROS might enhance the gene transformation efficacy and overall yield of the recombinant protein production in the plant. Moreover, murine-SP is a leader peptide for protein secretion into extracellular by using hydrophobic core, but *Influenza* A virus and *C. reinhardtti* signal peptides are ER-targeted and ER-localized chaperone in which both facilitating protein transportation to ER. When excess protein was coerced into ER-accumulation, this might cause ER-stress resulting in ROS induction and finally plant cells necrosis. (Diamos, Rosenthal, and Mason 2016; Diamos and Mason 2019) One hypothesis that make murine-SP more preferable over *Influenza* A-SP and *C. reinhardtti*-SP is becase of murine-SP might reduced ER-stress by transportating protein to extracellular section. In the process of LTB-CTLA4 protein purification by using Ni-NTA affinity column chromatography, the western blot and Coomassie-stained results indicated the correct size of LTB-CTLA4 around 30 kDa. The 50 kDa band of RuBisCO is a common contaminant found when purified *N. benthamiana*-derived protein with Ni-NTA column chromatography, a notable problem of the plant-derived downstream process. (Phoolcharoen et al., 2011; Schillberg et al., 2019) RuBisCO is found ubiquitously in plant leaves and abundantly produced when the plant cells are stressed with a viral vector. The ammonium sulfate precipitation was conducted in an attempt to remove RuBisCO plant host protein. However, LTB-CLTA4 had a close-range of rubisco precipitation, for pI of *Nicotiana tabacum*'s RuBisCO is approximately 4.4–4.7 (Barbeau & Kinsella, 1988) and calculated pI of LTB-CTLA4 is 5.62, leading to a considerable amount of protein loss after ammonium sulfate precipitation. For the purity is one of the critical concern in the biologic drug in case of undesirable contaminant proteins could interfere or hinder active protein into entirely function. The more sophisticated techniques were recommended for more purity.

CTLA-4 is a membrane-bound protein undergoing post-translational modifications (PTMs) of disulfide bonds and N-linked glycosylation. The human CTLA-4 is conceivable to have natural form as a homodimer. (Stamper et al., 2001) The plant platform gains these advantages in this aspect over the PTM issue in other expression systems, for example, the incorrect folding of complex protein in *E. coli* or over-glycosylation problem in yeast. (Shanmugaraj, I Bulaon, et al., 2020; Shanmugaraj, Malla, et al., 2020) The plant glycoproteins have little difference from those that belong to humans, and some studies also concentrated on the humanization of plant glycans. (Montero-Morales et al., 2019; Montero-Morales & Steinkellner, 2018) The success in clinical response of anti-CTLA-4 or ipilimumab reported its affinity toward CTLA-4, which caused competitive and direct steric effects preventing its binging to B7 receptor. (Ramagopal et al., 2017; Walker & Sansom, 2011) There is a study shown that CTLA-4 glycosylation is not critical for receptor-ligand of CTLA-4/B7 recognition (Peraino et al., 2013); however, the primary purpose of LTB-CTLA4 is to encourage the specific response against CTLA-4 protein, more identical to the native CTLA-4 is required. The western blot data have shown the assemble of LTB-CTLA4 as a pentamer, a behavior similar to the natural conformation of LTB subunits, which implied the LTB holotoxin structure was preferable than homodimer of CTLA-4. The formation of pentamer is important for GM1 binding, which is crucial for eliciting a specific immune response. After the administration of LTB-CTLA4 for three doses in a two-week interval, the IgG response against LTB and CTLA4p were detected, proving the immunogenicity concept of LTB-CTLA4. The highest titer dilution of anti-CTLA4 in mice sera were monitored up to 320, indicated the potential of LTB-CTLA4 as a cancer vaccine. There were promising results from studies of plant-produced cancer vaccines, for example, a tumor-

associated antigen, mucin-like glycoprotein 1 (MUC1), fused with LTB found to be immunogenic and capable of breaking tolerance in MUC1.Tg mice (Pinkhasov et al., 2011), rErbB2 oncoantigen protected mice from mammary cancer development. (Matić et al., 2016) Moreover, research of human epidermal growth factor receptor-2 fragment, ED44Her2, fused with non-toxic part of tetanus toxin also shown trastuzumab-like activity, which demonstrated tumor suppression in the murine model. This study also demonstrated that only Her2-fused with foreign immunogen could induce immunization effect more than administrated with plant-produced ED44Her2 fragment alone. (Chotprakaikiat et al., 2016) Recently, a study by Ramirez and colleagues (Hernández-Ramírez, Wong-Arce, González-Ortega, & Rosales-Mendoza, 2020) constructed LTB -fused multiepitope, called BCB, which expressed in *Schizochytrium* sp. by using algae-based Algevir expression system. The BCB targeted breast cancer contains tumor-associated antigens: human epidermal growth factor receptor-2 (HER2), mucin-like glycoprotein 1 (MUC1), Wilms' tumor antigen (WT1), and mammaglobin. The BCB vaccine shown the induction of immune response in mice that could recognize lysate from T41 breast cancer cell line. This gainly information speaks volumes for the potential of TAAs-targeted cancer vaccines strategy.

In summary, we successfully produced LTB-CTLA4 by using geminiviral transient expression in *N. benthamiana* that could induce an effective humoral immune response in a murine model compared to the group, received CTLA4 peptide alone. We proposed plant-derived LTB-CTLA4 as a vaccine candidate for cancer therapy to prevent and alleviate malignant cell progression, the vaccine that produced with rapid and cost-effective transient plant-based expression for achieving affordable bioproduct. The future studies on LTB-CTL4 will be exanimated further beyond overall humoral immune response, for instance, the investigation of a particular type of T-lymphocyte immune response in mice sera, CTLA-4 challenge,

investigation of the tumor progression and survival in human CTLA-4 genes knock-in murine model to observed whether LTB-CTLA4 could breaching the tolerance or not. Further information is required to evaluate the potential of plant produced LTB-CTLA4 as an effective vaccine candidate for humans in the future.

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APPENDIX A

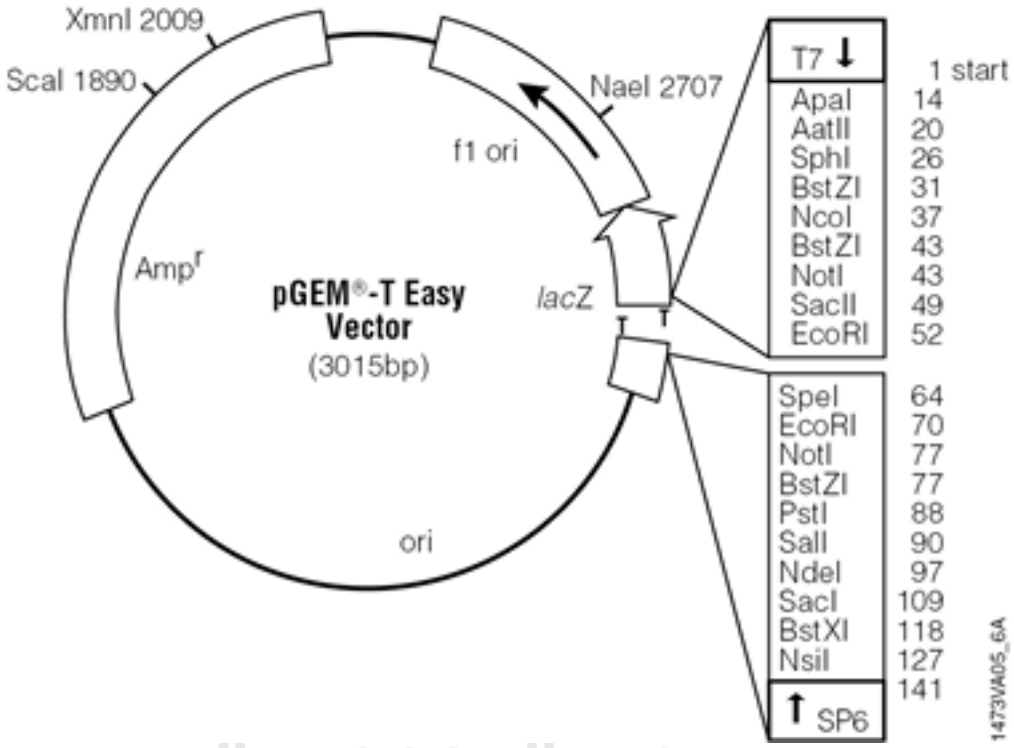
Nucleotide and amino acid sequence of LTB-CTLA4-8H-KEDL

LTB-CTLA4-8H-KDEL
atgggctggtcctgcatcatcctgttccttgttgctactgctaccggcgttcactctgat M G W S C I I L F L V A T A T G V H S D gttcaacttctcgagccgcagtccatcaccgagctttgctcggagtaccgcaacacccag V Q L L E P Q S I T E L C S E Y R N T Q atctacaccatcaacgacaagatcctctcctacaccgagtcgatggccggcaagcgcgag I Y T I N D K I L S Y T E S M A G K R E atggtcatcatcaccttcaagtcgggcgccaccttccaggtcgaggtccccggctcccag M V I I T F K S G A T F Q V E V P G S Q cacatcgactcgcagaagaaggccatcgagcgcatgaaggacaccctccgcatcgcctac H I D S Q K K A I E R M K D T L R I A Y ctcaccgagaccaagatcgacaagctctgcgtctggaacaacaagacccccaactccatc L T E T K I D K L C V W N N K T P N S I gccgccatctcgatggagaacggccctggccccaaggccatgcacgtcgcccagcccgcc A A I S M E N G P G P K A M H V A Q P A gtcgtcctcgcctcctcgcgcggcatcgcctccttcgtctgcgagtacgcctcgcccggc V V L A S S R G I A S F V C E Y A S P G aaggccaccgaggtccgcgtcaccgtcctccgccaggccgactcgcaggtcaccgaggtc K A T E V R V T V L R Q A D S Q V T E V tgcgccgccacctacatgatgggcaacgagcttaccttcctcgacgactcgatctgcacc C A A T Y M M G N E L T F L D D S I C T ggcacctcctcgggcaaccaggtcaacctcaccatccagggcctccgcgccatggacacc G T S S G N Q V N L T I Q G L R A M D T ggcctctacatctgcaaggtcgagcttatgtacccgccgccctactacctcggcatcggc G L Y I C K V E L M Y P P P Y Y L G I G aacggcacccagatctacgtcatcgaccccgagccctgccccgactcggaccatcatcac N G T Q I Y V I D P E P C P D S D H H H caccatcaccatcattctgaaaaggatgagctttgagagctc H H H H H S E K D E L - E L

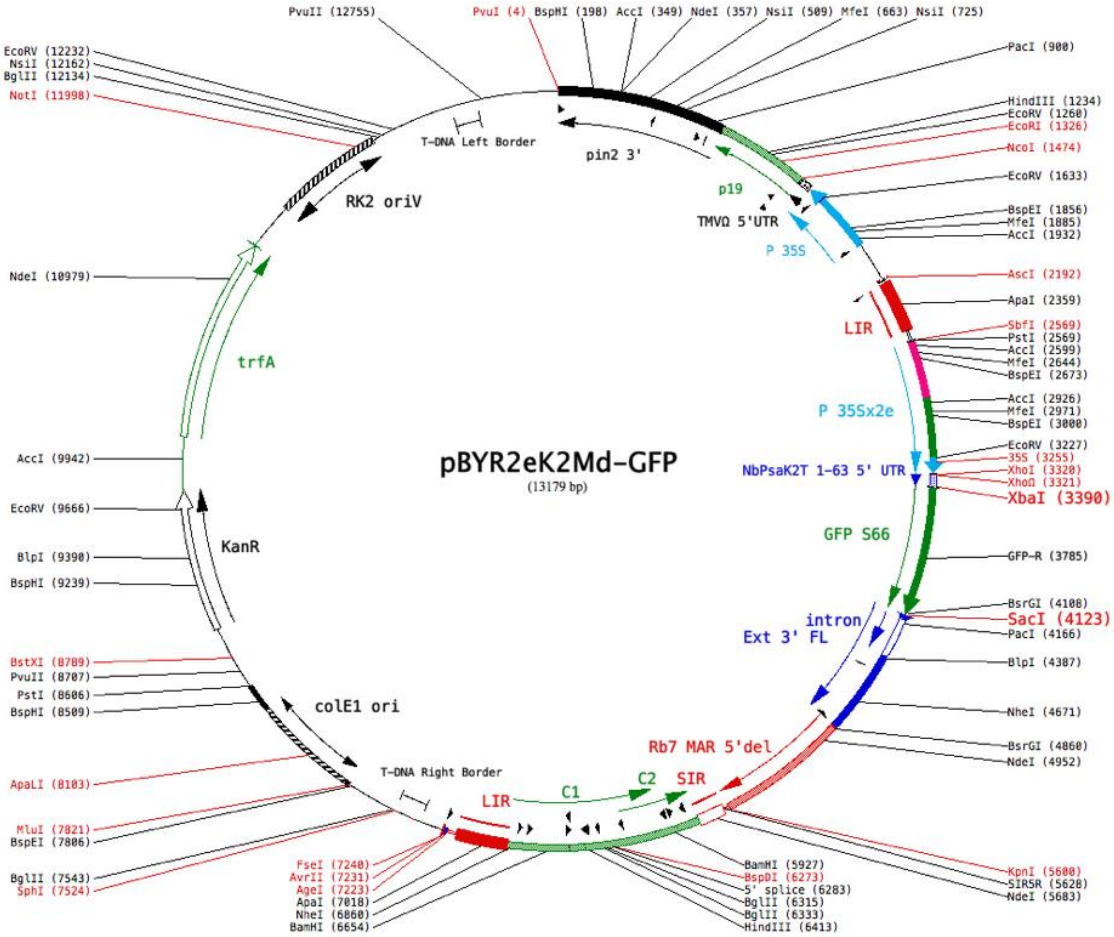
APPENDIX B

Vector Maps

Cloning vector: pGEM®-T Easy Vector



Expression vector in *N. benthamiana*: geminiviral vector pBYR2eK2Md, a gratitude from Diamos MS et. al., 2016. (Diamos & Mason, 2019)



APPENDIX C

Preparation of chemical solutions

Buffer

10x Phosphate buffered saline (PBS) pH 7.4

NaCl	80	g
KCl	2	g
Na_2HPO_4	14.4	g
KH_2PO_4	2.4	
DI water to	800	ml
Adjust pH by 1 M NaOH to	7.4	
Adjust volume to	1000	ml
Then autoclave		

1x PBS pH 7.4

10x PBS pH 7.4	100	ml
DI water to	1000	ml

10x Tris-buffered saline (TBS) pH 7.4

Tris base	60.6	g
NaCl	87.6	g
DI water to	800	ml
Adjust pH by 1 M HCl to	7.4	
Adjust volume to	1000	ml
Then autoclave		

1x TBS pH 7.4

10x TBS pH 7.4	100	ml
DI water to	1000	ml

10x Infiltration buffer

MES	21.925	g
dissolve in DI water	500	ml
$MgSO_4$	24.648	g
dissolve in DI water	500	ml
Mix both solutions well to make	1000	ml
Then autoclave		

1x Infiltration buffer

10x Infiltration buffer	100	ml
DI water to	1000	ml

1 M H_2SO_4

Conc. H_2SO_4 (18.4 M)	2.72	ml
DI water to	50	ml

1 M HCl

Conc. HCl (12 M)	4.17	ml
DI water to	50	ml

50% Glycerol

Glycerol	25	ml
DI water	25	ml
Mix both solutions well to make	50	ml
Then autoclave		

Antibiotics

100 mg/ml Ampicillin

Ampicillin	1	g
DI water	10	ml
Filter by 0.22 um filter		

50 mg/ml Rifampicin

Rifampicin	0.5	g
DI water	10	ml
Filter by 0.22 um filter		

50 mg/ml Kanamycin

Kanamycin	0.5	g
DI water	10	ml
Filter by 0.22 um filter		

50 mg/ml Gentamicin

Gentamicin	0.5	g
DI water	10	ml
Filter by 0.22 um filter		

Bacterial medium

Luria-Bertani (LB) medium

Yeast extract	5	g
Peptone/Tryptone	10	g
NaCl	10	g
DI water to	1000	ml

Luria-Bertani (LB) medium agar

Yeast extract	5	g
Peptone/Tryptone	10	g
NaCl	10	g
Agar	15	g
DI water to	1000	ml

Bradford assay

Bradford reagent

Bradford reagent (Bio-Rad, USA)	50	ml
DI water to	200	ml

SDS-PAGE and western blot

10% APS

Ammonium persulfate	1	g
DI water to	10	ml

1 M Tris-HCl pH 6.8

Tris base (Mw 121.14)	121.14	g
DI water to	800	ml
Adjust pH by 1 M HCl to	6.8	
Adjust volume to	1000	ml

1.5 M Tris-HCl pH 8.8

Tris base (Mw 121.14)	181.71	g
DI water to	800	ml
Adjust pH by 1 M HCl to	6.8	
Adjust volume to	1000	ml

2 M Tris-HCl pH 7.4

Tris base (Mw 121.14)	242.28	g
DI water to	800	ml
Adjust pH by 1 M HCl to	6.8	
Adjust volume to	1000	ml

10x Running buffer

Tris base (Mw 121.14)	30	g
Glycine (Mw 75.07)	144	g
Sodium dodecyl sulphate (Mw 288.08)	10	g
DI water to	1000	ml

1x Running buffer

10x Running buffer	100	ml
DI eater to	1000	ml

20x Transfer buffer

Tris base (Mw 121.14)	30	g
Glycine (Mw 75.07)	144	g
DI water to	1000	ml

1x Transfer buffer

20x Transfer buffer	50	ml
Methanol	150	ml
DI water to	1000	ml

Z-buffer reducing condition

125 mM Tris-HCl pH 6.8	6.25	ml
12% sodium dodecyl sulphate (SDS)	6	g
10% glycerol	5	ml
22% beta-mercaptoethanol	11	ml
DI water to	50	ml
Add Bromophenol blue	qs	drop

Z-buffer non-reducing condition

125 mM Tris-HCl pH 6.8	6.25	ml
12% sodium dodecyl sulphate (SDS)	6	g
10% glycerol	5	ml

DI water to	50	ml
Add Bromophenol blue	qs	drop

Phosphate buffered saline/ Tris-buffered saline with Tween-20 0.05% (PBST/TBST)

1x PBS/TBS buffer	500	ml
Tween-20	250	ul

Coomassie blue stanning

Coomassie R250	1	g
Methanol	1000	ml
Glacial acetic acid	200	ml
DI water to	2000	ml

De-staining solution

Glacial acetic acid	50	ml
Methanol	100	ml
DI water to	500	ml

SDS-PAGE gel preparation

Normal gel 1 glass

Resolving gel	8	ml
Stacking gel	3	ml

Gradient gel 1 glass

15% SDS-PAGE gel	2	ml
12% SDS-PAGE gel	2	ml
10% SDS-PAGE gel	2	ml
8% SDS-PAGE gel	2	ml
6% SDS-PAGE gel adjust to full glass	~2	ml

Immobilized metal affinity chromatography (IMAC)

IMAC 5

2 M Tris-HCl pH 7.4	2.5	ml	(20 mM)
5 M NaCl	2.5	ml	(50 mM)
2 M Imidazole	0.625	ml	(5 mM)
DI water to	250	ml	

IMAC 20

2 M Tris-HCl pH 7.4	2.5	ml	(20 mM)
5 M NaCl	2.5	ml	(50 mM)
2 M Imidazole	2.5	ml	(20 mM)
DI water to	250	ml	

IMAC 50

2 M Tris-HCl pH 7.4	2.5	ml	(20 mM)
5 M NaCl	2.5	ml	(50 mM)
2 M Imidazole	6.25	ml	(50 mM)
DI water to	250	ml	

IMAC 250

2 M Tris-HCl pH 7.4	2.5	ml	(20 mM)
5 M NaCl	2.5	ml	(50 mM)
2 M Imidazole	31.25	ml	(250 mM)
DI water to	250	ml	

APPENDIX D

Ammonium sulfate precipitation

The final concentration of ammonium sulfate, percentage saturation at 0°C, the table was adopted from Burgess RR, 2009. (Burgess, 2009)

Initial concentration of ammonium sulfate (percentage saturation at 0 °C)	Percentage saturation at 0 °C																				
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100				
	Solid ammonium sulfate (g) to be added to 1 l of solution																				
0	106	134	164	194	226	258	291	326	361	398	436	476	516	559	603	650	697				
5	79	108	137	166	197	229	262	296	331	368	405	444	484	526	570	615	662				
10	53	81	109	139	169	200	233	266	301	337	374	412	452	493	536	581	627				
15	26	54	82	111	141	172	204	237	271	306	343	381	420	460	503	547	592				
20	0	27	55	83	113	143	175	207	241	276	312	349	387	427	469	512	557				
25		0	27	56	84	115	146	179	211	245	280	317	355	395	436	478	522				
30			0	28	56	86	117	148	181	214	249	285	323	362	402	445	488				
35				0	28	57	87	118	151	184	218	254	291	329	369	410	453				
40					0	29	58	89	120	153	187	222	258	296	335	376	418				
45						0	29	59	90	123	156	190	226	263	302	342	383				
50							0	30	60	92	125	159	194	230	268	308	348				
55								0	30	61	93	127	161	197	235	273	313				
60									0	31	62	95	129	164	201	239	279				
65										0	31	63	97	132	168	205	244				
70											0	32	65	99	134	171	209				
75												0	32	66	101	137	174				
80													0	33	67	103	139				
85														0	34	68	105				
90															0	34	70				
95																0	35				
100																	0				

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