DEVELOPMENT OF PLANT-PRODUCED ANTI-IL-6R MAB

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น้ำทิพย์ แก้วบรรดิษฐ : การพัฒนาโมโนโคลนอลแอนติบอดีต่อตัวรับอินเตอลิวคิน 6 ในพืช. (DEVELOPMENT OF PLANT-PRODUCED ANTI-IL-6R MAB) อ.ที่ปรึกษาหลัก : รศ. ดร. วรัญญู พูลเจริญ

เมื่อร่างกายเกิดการติดเชื้อ เซลล์ในร่างกายจะตอบสนองโดยการหลั่งกลุ่มโปรตีนชนิดหนึ่งที่เรียกว่า ไซโตไคน์ เช่น อินเตอลิวคิน 6 (IL-6) ที่กระตุ้นการส่งสัญญาณ IL-6/JAKs/STAT3 ผ่านการฟอสโฟริเลชันภายในเซลล์ แต่หากในเซลล์มีการสะสมของ STAT3 ที่ถูกฟอสโฟริเลชันมากเกินไปทำให้เกิดการกระตุ้นแบบย้อนกลับ (positive feedback loop) ส่งผลให้เกิดการหลั่งไซโตไคน์มากเกินและอาจเป็นสาเหตุของการเกิดภาวะพายุไซโตไคน์ (Cytokines storm) ได้ ดังนั้น การยังยั้งการส่งสัญญาณของ IL-6 จึงเป็นอีกหนึ่งเป้าหมายสำคัญสำหรับรักษาภาวะพายุไซโตไคน์ โทซิลิซูแมบเป็นแอนติบอดีต่อตัวรับอินเตอลิวคิน 6 ที่ทำให้มีลักษณะคล้ายคลึงกับแอนติบอดีในมนุษย์ ซึ่งได้รับการรับรองสำหรับการรักษาโรคข้ออักเสบรูมาตอยด์ มีข้อกังวลหลายประการสำหรับการผลิตโปรตีนในเซลล์สัตว์เลี้ยงลูกด้วยนม อาทิ เช่น ต้นทุนการผลิตที่สูง และความเสี่ยงต่อการปนเปื้อเชื้อโรค เมื่อไม่นานมานี้พบว่า ระบบการแสดงออกโปรตีนในพืชได้กลายเป็นอีกทางเลือกสำหรับผลิตรีคอมบิแนนท์โปรตีน จุดมุ่งหมายของงานวิจัยนี้คือ เพื่อพัฒนาโมโนโคลนอลแอนติบอดีต่อตัวรับอินเตอลิวคิน 6 โดยอาศัยพืชอย่าง *Nicotiana benthamiana* สายหนักและสายเบาของแอนติบอดีต่อตัวรับตัวรับอินเตอลิวคิน 6 ถูกสร้างขึ้นโดยใช้เวกเตอร์ pBYK2e สำหรับศึกษาสภาวะที่เหมาะสมต่อการแสดงออกของโปรตีนแบบชั่วคราวใน *Nicotiana benthamiana* ผลการศึกษาพบว่าระดับการแสดงออกของโปรตีนสูงสุดเมื่อใช้สารแขวนลอยอะโกรแบคทีเรียที่มีสายหนักและสายเบาซึ่งมีความเข้มข้นของแบคทีเรีย (OD600) เท่ากับ 0.2 ในอัตราส่วนเท่ากันเพื่อฉีดเข้าสู่ใบพืชและเก็บเกี่ยวในวันที่ 5 หลังการฉีด โมโนโคลนอลแอนติบอดีต่อตัวรับอินเตอลิวคิน 6 ที่ผลิตจากพืชมีการจับอย่างจำเพาะกับโปรตีนรีคอมบิแนนท์ตัวรับอินเตอลิวคิน 6 ของมนุษย์ มีค่าสัมปะสิทธิ์การแตกตัว (dissociation constant, K_D) อยู่ที่ 1.748 µg/mL แม้ว่าการทดลองนี้แสดงให้เห็นถึงความสามารถและความเหมาะสมของแพลตฟอร์มพืชในการสร้างโมโนโคนอลแอนติบอดี แต่ต้องมีการวิจัยในสิ่งมีชีวิตเพิ่มเติม (*in vivo*) เพื่อให้เข้าใจประสิทธิภาพของแอนติบอดีดังกล่าวอย่างสมบูรณ์

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After the body is invaded by pathogens, it responds by secreting cytokines, for example, interleukin 6 (IL-6), which activates an intracellular signaling pathway involving JAKs/STAT3 through phosphorylation. However, overexpression of pSTAT-3 can lead to a positive feedback loop of IL-6, causing the release of more cytokines and potentially leading to cytokine storm. Thus, inhibition of IL-6 signaling is another important target for Cytokine storm treatment. Tocilizumab, a humanized IL-6R antibody has been approved for RA treatment. There are several concerns about mammalian cell protein expression systems, such as higher production costs and pathogen contamination risks. Recently, plants have become increasingly popular as an alternative system for recombinant protein expression. Therefore, the purpose of this research is to develop an antibody that targets IL-6 receptor (IL-6R) using *Nicotiana benthamiana* as an expression system. The HC and LC of the mAb targeting human IL-6 receptor (IL-6R) were constructed using the pBYK2e geminivirus expression vector. The constructs were then transiently co-expressed and investigated the optimal expression conditions in *Nicotiana benthamiana*. The findings indicate that the optimal conditions for expressing the Anti-IL-6R mAb involve infiltrating plant leaves with Agrobacterium containing HC or LC (each bacterial concentration of OD600 0.2) at a 1:1 ratio (HC:LC) at 5 dpi. The plant-produced Anti-IL6R mAb showed specific binding activity to human recombinant IL-6R protein, with KD value of 1.748 µg/mL. In contrast, the negative control did not bind as expected. Although we have demonstrated the capability and appropriateness of the plant platform for creating this

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

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CHAPTER I

INTRODUCTION

Rationale and Significance

Tocilizumab (TCZ), a humanized anti-human IL-6R monoclonal antibody (mAb), is designed to bind to interleukin-6 (IL-6) binding site of the IL-6 receptor (IL-6R) [1]. Since TCZ can bind both membrane-bound IL-6 receptors (mIL-6R) and soluble IL-6 receptors (sIL-6R), it can block classic and trans-signaling. The binding TCZ has been approved for use in treating a range of conditions, including Takayasu's arthritis [2], adult-onset Still's disease [3, 4], Castleman's disease [5], rheumatoid arthritis (RA) [6, 7], giant cell arthritis (GCA) [8], and juvenile idiopathic arthritis (JIA) [9]. It is also used to prevent the cytokine storm from CAR T-cell therapy and COVID-19 [10-12]. However, high production costs of mammalian cell expression system led to higher medical costs. A transient protein expression system in plants is an alternative option to produce proteins efficiently in a cost-effective way. Our goal in this study is to develop the production of anti-IL-6R mAb in plants by using transient protein expression. Each heavy and light chain of anti-IL-6R gene was codon optimized, cloned in geminiviral vector, and transiently expressed in *Nicotiana benthamiana* (*N. benthamiana) via Agrobacterium*-mediated transformation. The plant-produced anti-IL-6R mAb was purified and quantified for its rapid and optimum production in *N. benthamiana*. Moreover, the specific binding function of plant-produced anti-IL-6R mAb was investigated by ELISA.

Research Hypothesis

This study will lead to the production of an effective antibody against interleukin 6 receptor (Anti-IL-6R mAb) in plants.

Objectives of the study

1) To develop Anti-IL-6R mAb production using geminiviral vectors in *N. benthamiana*
2) To determine the specific binding activity of plant-produced Anti-IL-6R mAb

CHAPTER II

LITHERATURE REVIEW

Overview of Interleukin 6 signaling

Structure and Function of IL-6 and its receptor

IL-6 (Interleukin-6)

IL-6 is a multifunction cytokine, also called interferon beta-2 that consists of 184 amino acids [13] to form 21-28 kDa depending on glycosylation [14]. It is made up of 4-helix bundles, with the four long helicases A, B, C, and D arranged in an up-up-down-down structure. There are three binding sites present, the initial one being made up of the C-terminal of the D helix and the C-terminal of the AB loop for the IL-6 receptor. Figure 1 illustrates that site 2 has residues located at the center of helices A and C, and site 3 contains residues in the N-terminal region of the AB-loop and D-helix to interact with gp130 [15].

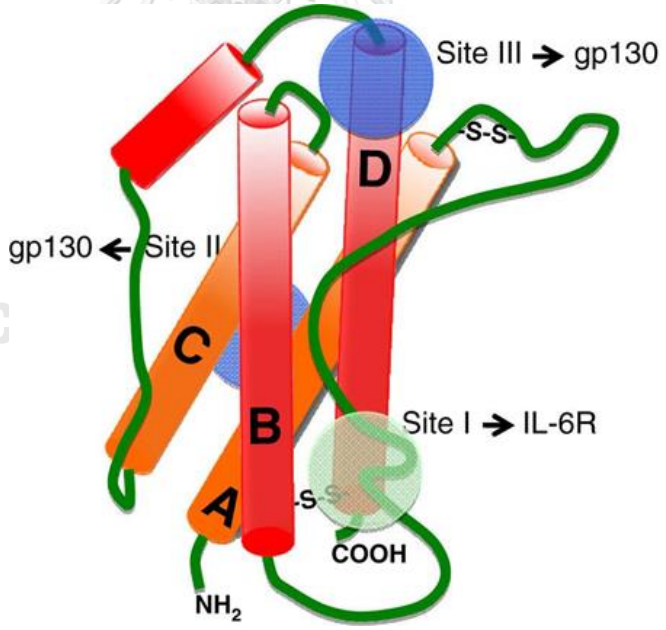


Figure 1 A diagrammatic depiction of the structural domains that make up human IL-6. Source: [16].

During infections, inflammatory conditions, and malignant disorders, Various cell types have been responsible for its release [17], including immune cell such as T cells, B cells, and monocytes and Non-immune cell like dendritic cells, endothelial cells [18, 19], hepatocytes [20, 21], fibroblasts [22, 23], adipocytes [24], keratinocytes [25], mesangial cells [26], and also tumor cells and tumor microenvironment [13, 27-29]. Its gene is controlled by activating nuclear factors including (NF)-**K**B, NF-IL6, hypoxia-inducible factor-1α [30]. In the case of CRS, macrophages that are activated by IFN-γ are the main of IL-6 production [31].

Since IL-6 is a pleiotropic cytokine, it performs several activities depending on its receptor interaction with cells. It is a vital for innate and adaptive immune responses [19]. In humans, it can regulate the acute-phase reaction, haematopoiesis, rate of metabolism, Homeostasis of lipids and neurological development [30]. Functions of IL-6 are shown in figure 2.

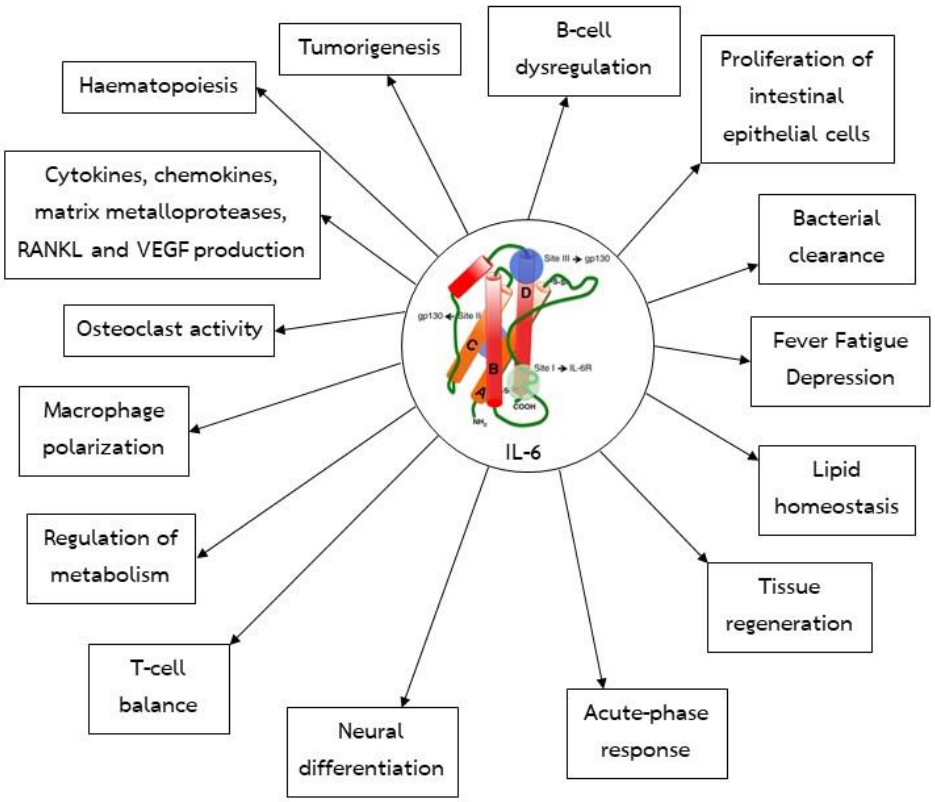


Figure 2 Functions of IL-6. Modified from [16, 30].

Interleukin 6 receptor

IL-6 receptor system composes of 2 subunits: Signal transduction subunit and non-signal transduction subunit [10]. In order to signal with IL-6, an IL-6 ligand have to firstly bind to the IL-6 receptor (non-signal transduction subunit) before the complex can bind to gp130 (transducer subunit) [17]. Affinity of IL-6 is to IL-6R and IL-6 cannot directly bind to gp130 [31]. IL-6Rα alone has low affinity binding [32] whereas IL-6Rα and gp130 which has no ability to bind to IL-6 by itself has high affinity binding [33].

2.1. IL-6R alpha

There are two forms of the non-transducer subunit protein (IL-6Rα), which are the membrane-bound form and the soluble form [13]. membrane-bound IL-6 receptor (mIL-6R, CD126) is a type I transmembrane protein [34] with an 80 kDa molecular weight [35] which express in only some cell types comprising T cells [36, 37], monocytes , macrophage [38] and hepatocytes [19]. The IL-6 ligand is attached to the mIL-6R to begin classical (*cis*-) signaling. IL-6/mIL-6R complex interacts with 130 kDa IL-6 beta receptor (gp130, CD130) [15]. Thus, the classic signaling pathway can be expressed in limited cell types which have mIL-6R. This signaling is crucial for the central homeostasis process, hematopoiesis, and acute-phase immune response [39] and inducing anti-inflammatory process [40]. Binding affinity of soluble IL-6 receptor (sIL-6R) and IL-6 is comparable to that of the IL-6/mIL-6R, and it triggers IL-6 signaling, contrary to other soluble receptors such as IL-1 or TNF receptors that impede the effects of their ligands [41]. There are two mechanisms of sIL-6R generation including

a) **Proteolytic cleavage of mIL-6R** that depends on the metalloprotease activity containing ADAM10/17 (A Disintegrin and Metalloproteinase), which consist of soluble receptors of IL-1, IL-2, TNF-α, CD2l and platelet derived growth factor. PC-sIL-6R (Proteolytic cleavage soluble IL-6 receptor) refers to sIL-6R that is released as the product of this mechanism.

b) In humans, **differential mRNA splicing** can cause the production of soluble factors that do not possess the cytoplasmic and membrane-spanning domains found in the cell-associated protein. This particular mechanism is unique to humans and can lead to the expression of these domains-deficient factors in cells that express receptors for IL-4, epidermal growth factor (EGF), and Leukemia inhibitory factor (LIF) [42].

In the instance of sIL-6R, both mechanisms control the release. Therefore, the whole characteristics of this soluble receptor are influenced by two different isoforms of the sIL-6R (Figure 3). In this context, the isoforms will be called Differential mRNA splicing of soluble IL-6R (DS-sIL-6R) or Proteolytic cleavage of soluble IL-6R (PC-sIL-6R) depending on whether they are created through either differential mRNA splicing (DS) or proteolytic cleavage (PC) that results in shedding of the receptor.

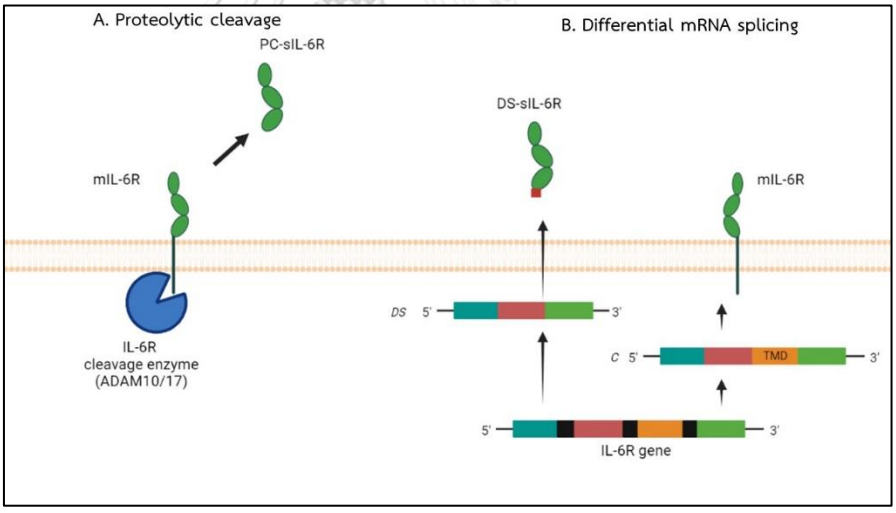


Figure 3 The ways in which sIL-6R is produced are illustrated in diagrams,with one showing how sIL-6R is released through proteolytic shedding (A) and the other showing how it is produced through differential mRNA splicing (B). A) When activated by the appropriate stimuli, a previously cleavage enzyme called IL-6R-CE promotes the release of Proteolytic Cleavage of Soluble IL-6R (PC-sIL-6R) whereas B) Differential mRNA splicing can explain the presence of two different IL-6R mRNA transcripts. One of these transcripts encodes differential mRNA splicing of Soluble IL-6R (DS-sIL-6R)

whereas the other encodes membrane-bound IL-6R (C). TMD: the transmembrane domain. Modified from [43]

Interaction between IL-6 ligand and sIL-6R initiate the trans-signaling (*trans-*) pathway that is important regulator of tumor microenvironment, regulating the inflammatory activation in stromal cells [39, 44]. Proinflammatory activity is activated by trans-signaling. sIL-6R will be reported to increase in the serum of multiple myeloma [45], juvenile chronic arthritis[39] and Graves' disease [46].

2.2. Glycoprotein 130

Signaling transducer subunit protein (gp130, cd130, IL-6Rβ chain) [39, 44] is a transmembrane protein [47, 48], which is ubiquitous in its expression [49]. It is made up of a type I membrane protein [47, 48] with 6 extra-cellular domains that contains 130–150 kDa (Figure 4) [50, 51] including

- D1: Ig-like domain at the N-terminus that can bind to IL-6's site 3.
- D2-3: 2 cytokine binding domains (CBD) that can bind to site 2 of IL-6.
- D4-6: 3 fibronectin-like (FN III) domains that provide signals to the cytoplasmic domain [52].

The N-terminal of gp130 has an Ig-like domain, followed by CBD and three FN-III domains. The intracellular region of the cytoplasmic domain contains two brief non-conserved areas, which separate the conserved region. These domains are referred to as the "Box1" and "Box2" The Box1 domain is composed of a proline-rich amino acid, while the Box2 domain is made up of a group of hydrophobic amino acids and an acidic amino acid residue. These domains serve as docking sites for Janus tyrosine kinases (JAK1, JAK2, TYK2) [53]. Despite their importance in signal transduction, there are only six tyrosine residues present in Box1, Box2, and Box3 of gp130 [54].

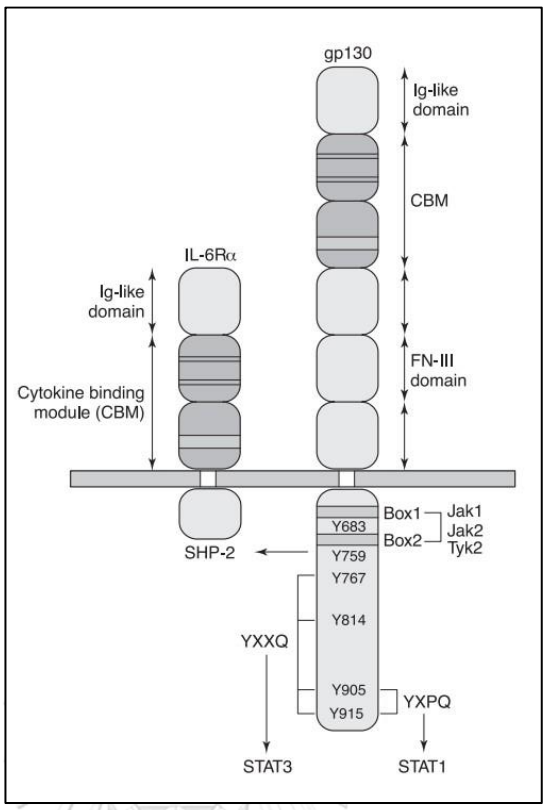


Figure 4 Chimeric structure of IL-6R**α** and gp130. Source: [54].

The IL-6 ligand binding to its receptor initiates the IL-6 signaling pathway [40]. IL-6/IL-6R complex interacts and activates gp130. The membrane proximal domain on gp130 (Box1 and Box2 domain) associated- JAK1, JAK2 or TYK2 disrupts JH2 (JH1 Kinase activity mediated) thereby fully activating JAKs through transphosphorylation exchanging [55]. After that, JAKs will phosphorylated several tyrosine residues at C-terminal of gp130 which is as a docking site for proteins that initiate intracellular signaling (Figure 5) such as the PI3K/AKT [56], RAS/RAF/MEK/MAPK [57] and JAK/STAT3 [53].

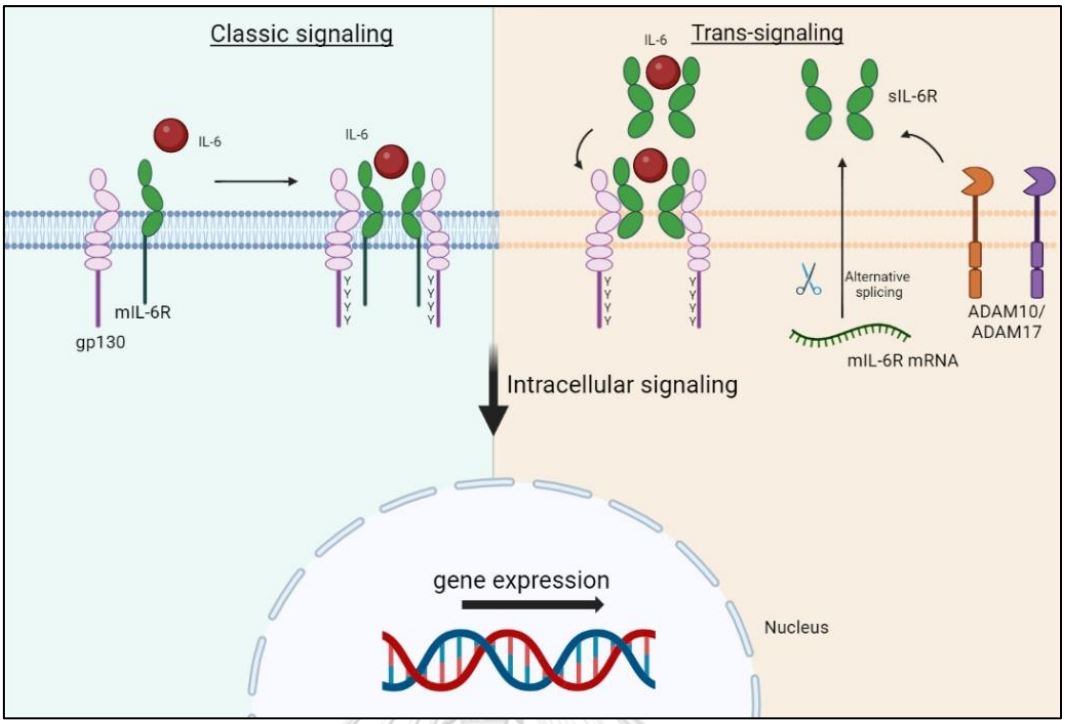


Figure 5 IL-6 signaling pathway composes of 2 pathways including a) the classic (cis-) signaling pathway and b) the trans-signaling pathway.

JAK/STAT3 pathway

JAK/STAT3 pathway, associated with Janus kinase families (JAKs) and Signal transducer and activator of transcription 3 (STAT3) is the main IL-6 signaling involving in immune functions, cell growth, differentiation, and hematopoiesis. Previous studies reported that cell progression of breast carcinoma (BC) [58] and non-small cell lung cancer (NSCLC) [59] are linked to STAT3. This signaling is essential for the genesis, proliferation, and progression of colorectal cancer (CRC) [60].

JAKs (Janus Kinases)

When IL-6 signaling occurs, both classic signaling and trans-signaling that are associated with gp130, activate Janus kinases (JAKs). JAKs (Janus kinases) are enzymes that use ATP for phosphorylation and have specific targets. Four types of JAKs, JAK1-3

and TYK2 [10] whose molecular masses range from 120-140 kDa and are expressed in human cells [49]. JAK1-2 and TYK2 are ubiquitous in expression but, JAK3 is constrained to cells of hematopoietic origin [61]. Interestingly, gp130 interacts with and activates JAK1, JAK2, and TYK2 [62, 63]. JAK1 has an essential role which is evident from the impaired IL-6 signaling in the cells lacking JAK [58, 64]. Inactivated mode of JAK2 and TYK2 can mediate the activation of STATs 1 and 3 in the absence of JAK1 [64]. However, Which JAKs are involved in biological processes and downstream signaling is unclear. For JAKs's general structure, there are 4 domains including FERM domain, SH_2 domain, Pseudo kinase domain and kinase domain and compose of seven homology domains (JH1-7) [10] as shown in figure 6.

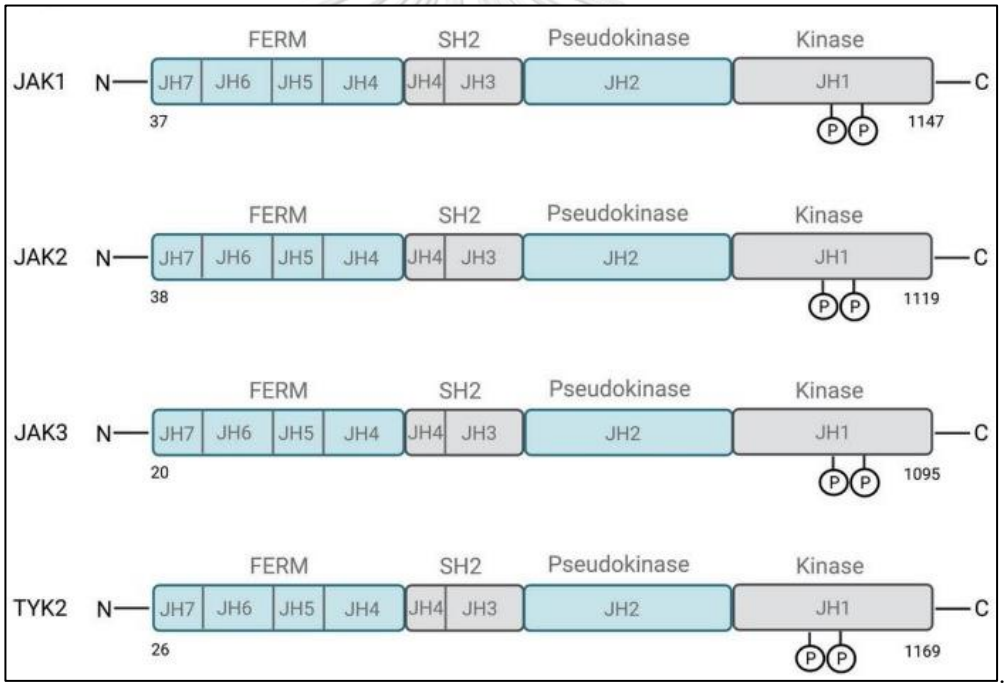


Figure 6 The JAK family is composed of four primary members: JAK1, JAK2, JAK3, and TYK2, all of which are depicted in the figure along with their conserved phosphorylation sites. The JAK family members consist of seven homology domains, with JH1 serving as the kinase domain, JH2 as the pseudokinase domain, and the SH2 domain being formed by a combination of JH3 and JH4. The FERM domain, on the other hand, is composed of JH5, JH6, and part of the JH4 domains. JAK1, JAK2, JAK3, and Tyk2 all

have conserved tyrosine phosphorylation sites, which are Y1038/Y1039, Y1007/Y1008, Y980/Y981, and Y1054/Y1055, respectively. Source: [65].

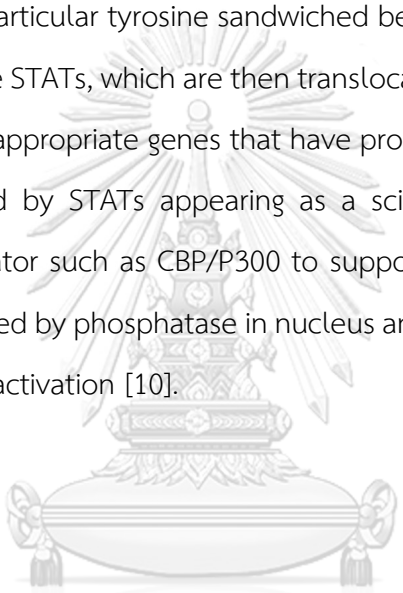
STATs (Signal Transducers and Activators of Transcription)

STATs are transcription regulators containing SH2 domain [66, 67] that can allow STAT binding to phosphorylated cytokine receptor. By using JAKs, a single conserved tyrosine residue is phosphorylated, and it is crucial for STATs activation. There are 7 STATs including STAT1-4, STAT5a-b, and STAT6 [10]. It is evident that STAT signaling pathway can be initiated by several cytokines and growth factors [60]. STATs have a chief role in signal transduction from cytokines and support specific gene transcription. STAT3 is mainly involved in the IL-6 family signaling followed by STAT1 [68]. After STAT3 is phosphorylated via., JAKs, activated structure of unphosphorylated STAT (uSTAT) is different from phosphorylated STAT (pSTAT) (Figure 7).

STAT3 can be activated through several cytokines consisting of IL-6, IL-10, and growth factors (GFs) consisting of EGF and fibroblast growth factor (FGF) [69, 70] (Figure 8). Target genes of STAT3 include vascular endothelial growth factor (VEGF), cyclin D1, Bcl-xL, c-Myc, and MCL1 in combination with conserved DNA sequence [60]. On gp130, there are 4 STATs binding sites that have conserved tyrosine residues. Sequences around this conserved tyrosine residues affect in the affinity of STATs binding whereas PYXXQ is associated with STAT3 [10]. The inactive form of STATs exists as dimer in cytoplasm, and when activated by JAKs, active dimer of STAT3 is translocated to the nucleus. To enhance angiogenesis, proliferation, cancer development, invasiveness, metastasis, and MDR (Multiple Drug Resistance), STAT3 has several target genes that it may activate in tumor cells [49, 62]. For example, MMPs (Matrix metalloproteinase) and Immunosuppression. IL-10 and TGF-β promote IL-6 gene expression resulting in autocrine feedback loop. STAT3 is a positive regulator of Tregs (regulatory T) and MDSCs (myeloid-derived suppressor cell). STAT3 encodes proteins such as Cyclin D1 to drive tumor proliferation and BCL2-like protein 1 for tumor survival. STAT3 is usually

stimulated in immune cells of tumor that negatively regulate effect on neutrophils, natural killer (NK) cells, effector T cells and dendritic cells (DCs), indicating increased STAT3 activation leads to decreased antitumor activity and immunity. Apoptosis in T-cells is prevented by STAT3 dependent cellular signaling that can activate the anti-apoptotic regulators (Bcl-2, Bcl-xL) and by alteration of Fas surface expression [49].

STATs, as inactive dimers present in cytoplasm, are formed by the interaction of the DNA-binding domain and coiled-coil domain of two monomer STAT units. The JAKs-phosphorylate a particular tyrosine sandwiched between SH2 and transactivation domain to stimulate the STATs, which are then translocated into the nucleus to induce transcription activity in appropriate genes that have promoters with STAT binding sites. The DNA is surrounded by STATs appearing as a scissor like structure and recruit transcriptional co-activator such as CBP/P300 to support the transcription. After that, STAT is dephosphorylated by phosphatase in nucleus and shuttle them into cytoplasm for additional series of activation [10].



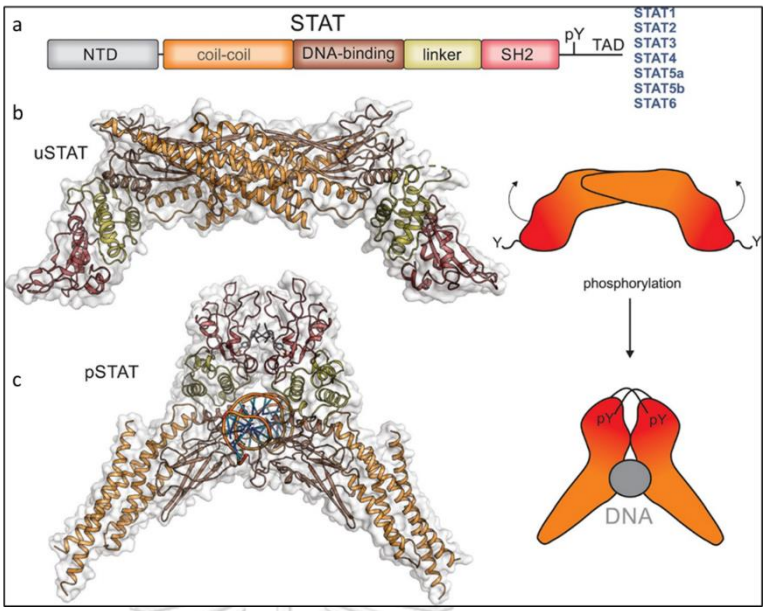


Figure 7 shows a) the schematic representation of Signal Tranducers and Activators of Transcription (STATs). The structures for b) Unphosphorylated of STAT (uSTAT) and c) phosphorylated STAT (pSTAT) of STAT1. For Abbreviation: NTD as N-terminal domain and TAD as transactivation domain. Source: [10].

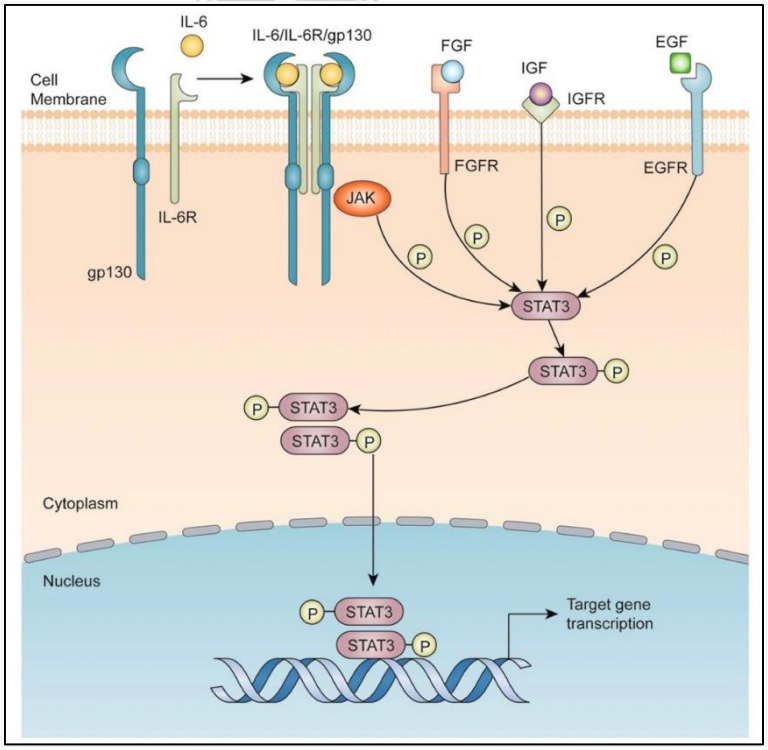


Figure 8 STAT3 activation in cancer cells. Source: [58].

Cytokine Release Syndrome (CRS)

The dysregulation of the immune system known as Cytokine Release Syndrome (CRS)), or cytokine storm, causing by the rapid and excessive secretion of cytokines [19]. Depending on each cytokine level [71], cytokine storms can occur in many bodily parts and cause a wide range of symptoms [72], including common symptoms such as fever, vomiting, diarrhea, headache, dry cough etc.[73], severe calamitous problems including fluid-refractory hypotension, cardiac dysfunction, renal, hepatic, and inflammatory problems, as well as multiorgan failure [74]. that may be life-threatening [75]. Figure 9 demonstrates the signs of a cytokine storm. There are several stimulators that might cause a cytokine storm, such as bacterial or viral infection, certain immunotherapies (such CAR-T cell treatment) [76], autoimmune disorders, and tumor cells [77].

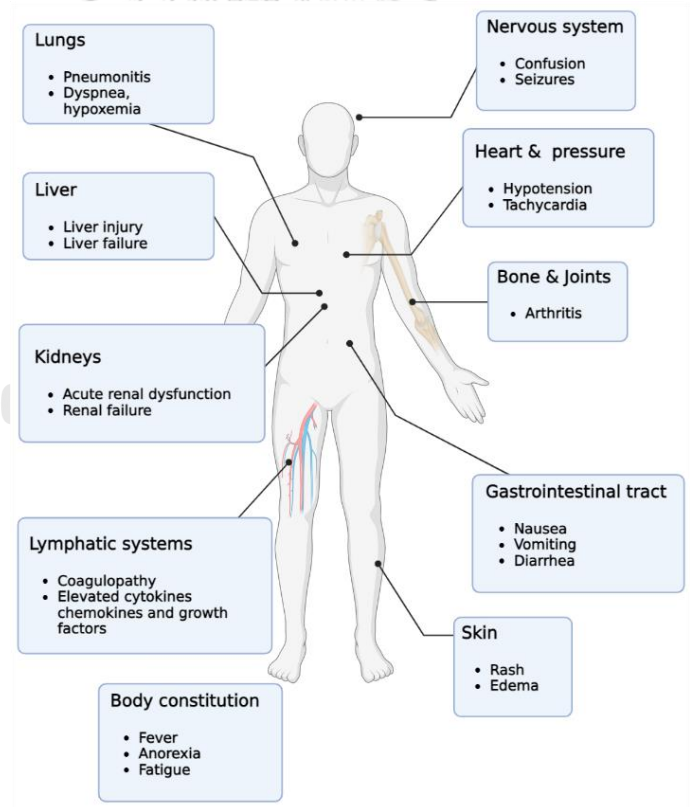


Figure 9 The signs of a cytokine storm.Modified from [72].

The type of cells and cytokines along with their respective signaling pathways play one critical role in the pathogenesis of cytokine storms. Neutrophils, monocytes, and macrophage cells involved in cytokine storm, function by recognizing the pathogens to produce cytokines. Macrophages are stimulated and produce excess amounts of cytokines causing intense tissue damage, leading to organ failure. B cells are not often associated with the pathogenesis of cytokine storms [72]. Cytokine Storm occurs due to excessive cytokine production. The patients who were treated with CAR T-cell therapy or BiTE (bispecific T-cell engager) therapy develop toxicities such as CRS. Monoclonal antibody therapy with alemtuzumab are also associated with CRS [72]. Infection of SARS-CoV-2 virus, also a cause for cytokine storm [78]. This literature focuses on the fundamental mechanism of CRS linked to COVID-19 or CAR T-cell treatment.

CAR T-cell Therapy Mechanism Activating CRS

Immune T-cells engineered to identify antigens on B-cell surfaces and fused to the CAR are employed in CAR T-cell therapy. This therapy is directed to kills lymphoma cells by the cytotoxic functioning of T-cells [19]. When the target tumor cells are bound by CAR T cells or activated T cells, which then kill the cells. IFN- or TNF- is aggressively released from the lysed cells and CAR T cells [79] to activate macrophages, which are essential for the development of CRS [80]. Activated macrophages increase levels of several cytokines (hyperinflammatory state), including IL (interleukin)-1, -2, -2R, -6, -6R (soluble), -10, and -12, TNF- (Tumor necrosis factor alpha), and other cytokines [81] that leading to CRS. Each cytokine interacts with a particular receptor on endothelial cells to create intracellular signaling. Conversely, IL-6 can promote a positive feedback loop by T cell activation [82]. Figure 10 depicts the pathway of inflammation in CRS that is triggered by CAR T cell therapy.

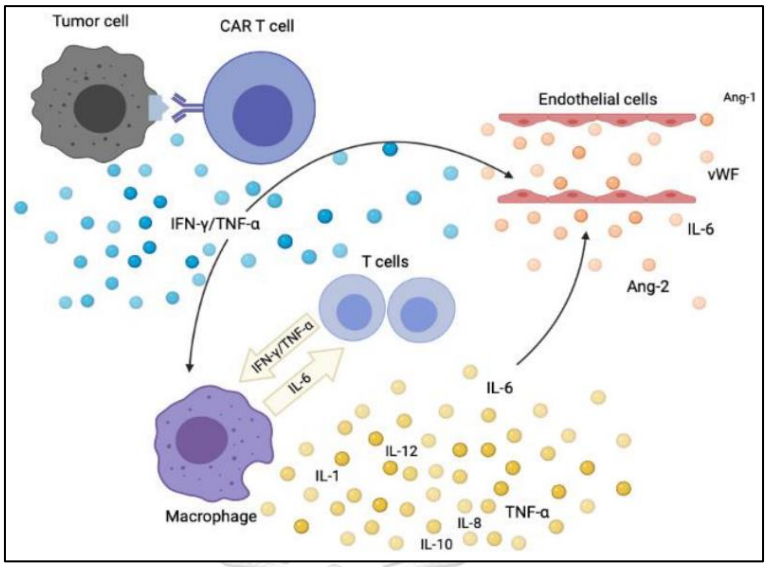


Figure 10 Pathway of inflammation in CRS that is triggered by CAR T cell therapy. Source: [82].

Mechanism of Cytokine storm in case of COVID19

After the SARS-CoV2 virus infects respiratory epithelial cells, TLR (endosomal toll-like receptor) 3, 7, and 8 attach to the virus genome and activate NF-**K**B, which then triggers the transcription of a variety of signaling proteins, such as IFNs (interferon), growth factors, and other proinflammatory cytokines and chemokines [83], which activates macrophages, T cells [84], and dendritic cells [85] to increase cytokines production. Dendritic cells and mononuclear macrophages were infected by the virus, which caused the cells to produce proinflammatory cytokines and chemokines aggressively, resulting in a cytokine storm [86]. These several cytokines comprising IL-1β, IL-6, TNF, IFNα/β, and delayed SECRETION OF IFN-γ and chemokines including ccl (C-C Motif Chemokine Ligand) 2, 7, and 12 [81]. Activated T cell to secrete IFN-γ and TNF-α for activate positive feedback loop [87]. Figure 11 illustrates the pathway of inflammation in CRS that is stimulated by infection of SARS-CoV-2 virus.

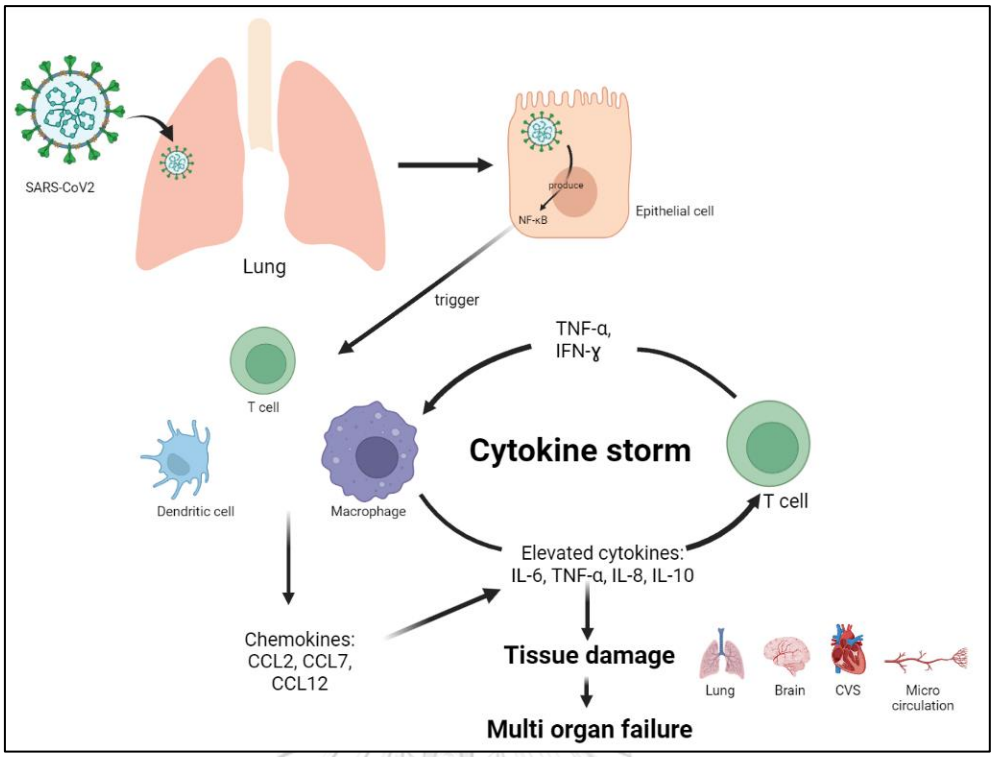


Figure 11 The pathway of inflammation in CRS that is triggered by SARS-CoV-2 virus. Modified from [81, 83, 87].

Tocilizumab

Tocilizumab (TCZ) is a humanized mAb targeting IL-6 binding site of human IL-6R [1], which humanized using complementary determining region (CDR) from a mouse anti-human IL-6 antibody for grafting method [88]. TCZ inhibits both *cis*- and *trans*- IL-6 signaling. Its mechanism of action is by the recognition human IL-6R binding site and inhibit IL-6 signaling through "Competitive IL-6 binding blockage" using IgG1 kappa antibody format that has no cytotoxicity/complement cytotoxicity in cells [56]. By attaching to the IL-6R, TCZ suppresses IL-6 action [27]. It is the first treatment drug approved for Castleman's disease [5] and Crohn's disease [9]. From previous studies, they found that 90% of 21 patients severe COVID19 can be recovered within 2-3 days with TCZ treatment [66] and China's clinical trial has established that TCZ functions in patients who were seriously sick with COVID19 (Chinese Clinical Trail Registry) [89].

Furthermore, several reports shown that TCZ has important anti-carcinogenic roles in several types of cancer via inhibition of JAK/STAT3 pathway [69, 70, 90-93] and discovered that TCZ did not affect to STAT3 expression but can reduced the level of phospho-STAT3 in various cells [94-96]. The combination of Stattic (Stt) and TCZ will be more effective at the suppression of the IL-6/STAT-3 pathway. This could reduce proliferation, migration, and invasive ability of DU145 (metastatic, castoration-resistant prostate cells) and Prostate cancer cells [97].

Systems for Recombinant Proteins Expression

Nowadays, we can express and produce a wide range of recombinant products using a variety of expression systems, which play the essential role in human treatments. Each protein expression system has benefits and disadvantages of its own. The comparison of protein production systems is shown in Table 1. [82].

The plant expression system may be better in comparison to other expression systems in several ways such as ability to perform post-translational protein modification, low operating costs, high protein yield, reduced production time, contamination free from pathogenic microorganisms. There are three different methods of plant protein expression system including stable Expression, transient expression, and cell/tissue culture that are shown as figure 12. Table 2 summarizes the advantages and disadvantages of these three methods.

Table 1 Protein expression systems are compared. Modified from [82].

Parameters	Bacteria	Yeast	Insect cell culture	Mammalian cell culture	Transgenic animals	Plant cell culture	Transgenic plants
Operating cost	low	medium	high	very high	medium - high	high - very high	low
Storage cost	medium	medium	high	high - very high	high	low-medium	very low
Scale-up cost	high	high	very high	high-very high	high	high	very low-medium
Production scale	limited	limited	limited	limited	limited	limited	worldwide
Scale-up capacity	high	high	medium - high	very low	low	medium - high	very high
Speed of production	high	medium - high	medium	low	very low - low	medium high	low
Glycosylation	no	no	no	no	yes	yes	yes
Product quality	low	medium	medium - high	high - very high	high - very high	high	high
Safety	low	unknown	medium	low - high	high	nonspecific	high
Contamination risk	high	low - medium	low - high	high	high	low	low

Table 2 Summarization for the advantages and negatives of plant expression methods. Modified from [98]

	Stable transformation	Transient expression	*In vitro* plant cell/tissue culture
Advantages	• Can apply in Different plant • Can be stored seeds for a long time • Scalability	• Sterile production environment • Compatible with regulatory guidelines • Scalability	• Gene of interest (GOI) does not integrate with plant genome • Easy to manipulation • Rapid protein expression but get high yield (Efficient and saving processing time) • Can be used for quick recombinant protein manufacturing in an emergency.
Disadvantages	• Time-consuming • Requires a large amount of labor • Effect of gene silencing/position • Random gene integration	• Complexity in large-scale production • High cost of cultivation • Genetic instability and reduced productivity over long time period	• Unstable of protein yield • Chances of endotoxin contamination

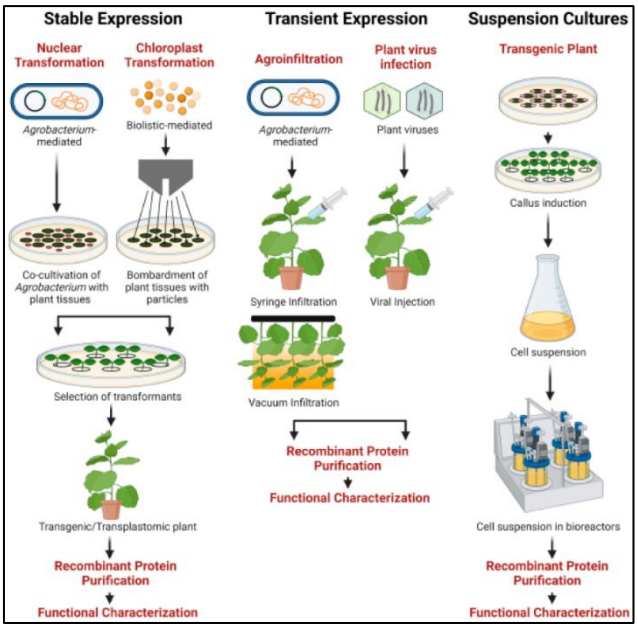


Figure 12 Three different methods of plan protein expression.Source: [69].

Among several plants that are used as hosts for plant expression systems, *N. benthamiana* is the current core production host used by many companies due to its ameable to transformation and innate ability to support high levels and rapid of transgene expression [99, 100]. Moreover, *N. benthamiana* plant is non-food crops for humans or non-feed crops for animal. *N. benthamiana* plant (Figure 13) are usually grown for 4-6 weeks, then infected with *A. tumefaciens* harboring the genes of interest (GOI) by agroinfiltration. 3-7 days after infection, the genes of interest or protein expression typically reach a peak level.

Figure 13 Nicotiana benthamiana plant

***Agrobacterium*-mediated transformation**

To express the heterozygous genes in plants, *Agrobacteria* has been used for transgenes carrier. This strategy is *Agrobacterium*-mediated transformation. *Agrobacterium tumefaciens* (*A. tumefaciens*) is Gram-negative soil bacteria causing of crown gall disease of several plants [101]. Naturally, *Agrobacterium* infects at the plant wound site by wound-plant signaling.

Naturally, *Agrobacterium* enters plants through wounds by induction of plant signaling, which are both spontaneous, such as organic compounds (pH 5.0-5.8), and phenolic compounds including Acetosyringone and hyperacetosyringone. released through plant wounds. When *Agrobacterium* receptors recognized plant signaling, Vir G (Virulence G) protein is activated and induce other vir (virulence) genes expression. Left border (LB) and Right border (RB) refers Transfer DNA (T-DNA); part of Tumor-inducingplasmid (Ti plasmid); were cleaved by endonuclease activity of Vir D2 (Virulence D2) and transfer into plant cell and produce nutrients for *Agrobacterium* (Figure 14) [102].

The use of *Agrobacterium*-mediated transformation provides various benefits, including the ability to transfer a small number of DNA fragments containing genes of interest with greater efficiency and lower cost, as well as the transfer of large DNA fragment with minimal rearrangement (Figure 14) [103].

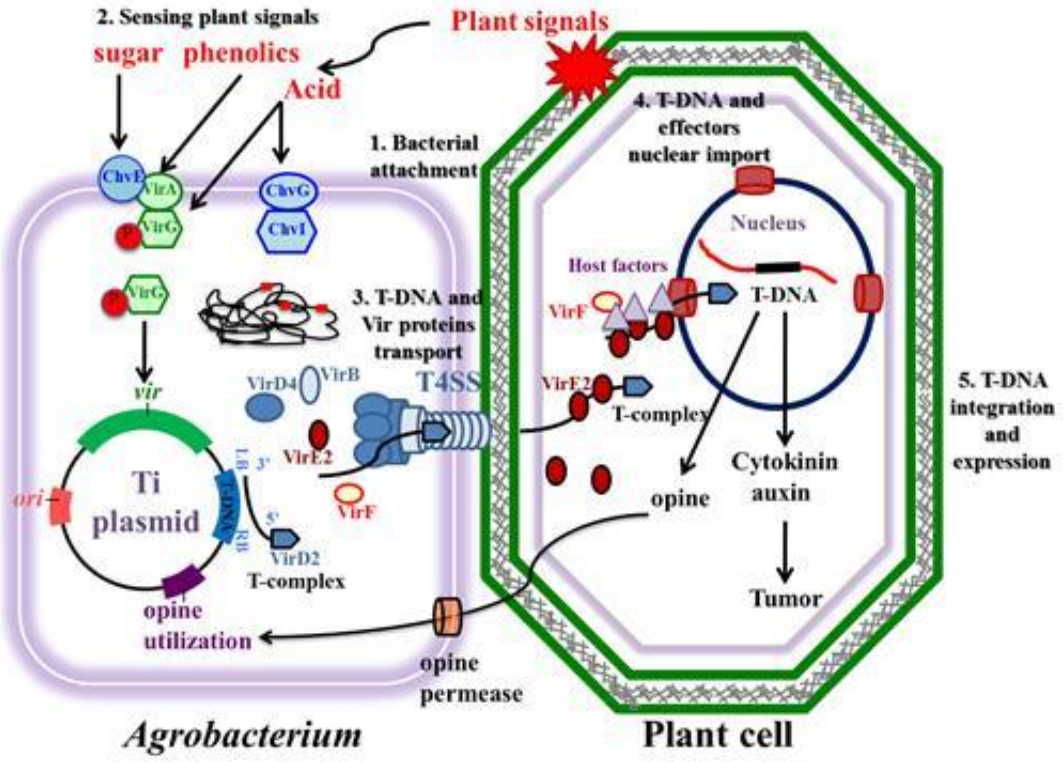


Figure 14 Agrobacterium-mediated transformation.Plant signals including organic compounds and phenolic compounds induce bacterial attachment and activate Agrobacterium Virulence G (Vir G) protein through signaling recognition. Vir G induces expression of other Vir genes in Tumor inducing plasmid (Ti plasmid). Then, plasmid is cleaved at the left and right border and release Transfer DNA (T-DNA) by endonuclease activity of Vir D2. Single strand (ss) T-DNA form ss T-DNA-Vir D2 complex and the complex is coated with VirE2 and transfer into plant cell nucleus. T-DNA complex is uncoated by protein in the nucleus and converts to double strand (ds) T-DNA. ds T-DNA is integrated to plant genome and expresses protein such cytokinin, auxin (for tumor inducing) and opine. Source: [102]

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Recombinant Protein Expression

To enhance protein expression levels in the plant transient expression system, gene expression constructs are an important factor that can impact the strength of protein expression, which includes the addition of promotor sequences, codon usage, protein signaling, and protein retention tags.

Codon optimization

Because of the degenerate nature of the genetic code, the polypeptide chains of most proteins may be encoded by an apparently limitless number of mRNA sequences [104]. Codon use bias is the favored or non-random use of synonymous codons[105], which has been seen in microorganisms, plants, and animals. Genome composition, GC content [106, 107], expression level and size of gene (gene structure), codon location and context in genes, recombination rates, mRNA folding, and tRNA abundance and interactions are some variables affecting codon bias [108]. In addition to the translation elongation rate, codon optimality has been proven to be a major predictor of mRNA stability [109]. Codons that match with abundant tRNA species are present in more stable mRNAs, whereas rare codons are found in unstable mRNAs [110, 111], influence protein translation efficiency and protein folding [112]. Likewise, preferred codon selection is considered to influence mRNA expression via markedly higher amounts of mRNA, indicating controlled translational accuracy and efficiency [113].

Signal peptide

Signal Peptide sequences are sequences of the secretory proteins that act as a postcode to designate the protein secretion pathway as well as the protein target location in all domains of life. They are one of two key compositions that regulate translocation capability and efficiency. The use of different signal peptides from heterologous species might improve translocation efficiency. It indicates that using signal peptides enhanced the production of proteins [114]. Signal peptides generally range from 16 to 30 amino acid residues long, with a hydrophilic, positive-charged at

N-terminal region, a central hydrophobic region, and the signal peptidase cleavage site at the C-terminus. They are the N-terminal extensions of nascent polypeptide chains that enable protein targeting to the endoplasmic reticulum membrane [115]. Previous study has shown that using effective signal peptides might increase recombinant protein yields [116, 117]. In *N. benthamiana* plants, the expression of human mature interferon gamma (mIFN) containing the suitable signal peptide resulted in higher level of expression with appropriate protein folding and assembly [118]. The approach of selecting the most appropriate signal peptide provided a method for increasing protein secretion and enhancing recombinant protein expression levels.

Protein retention tag

The destination or path taken by newly formed recombinant proteins is a critical factor affecting their stability and structure. Generally, proteins undergo post-translational modifications (PTMs) in the endoplasmic reticulum (ER) and Golgi complex [119]. Researchers discovered a C-terminal peptide (SEKDEL/KDEL) in certain ER-sorted proteins, which enables them to be transported back to the ER if they escape. KDEL and SEKDEL are amino acid motifs that are found at the C-terminus of soluble proteins that are resident in the endoplasmic reticulum (ER) of eukaryotic cells. The KDEL motif consists of the sequence Lys-Asp-Glu-Leu, while the SEKDEL motif contains Ser instead of Lys [120]. One study found that adding the hexapeptide motif to lysozyme caused it to bypass the secretory pathway and accumulate in the ER [121]. Subsequent investigations revealed that a protein tagged with SEKDEL could interact with the KDEL receptor, exit the ER, undergo PTMs in the cis-Golgi, and then be retrieved back to the ER through retrograde transport [122].

Geminiviral replicon system

The transient transformation technique has gained popularity due to its ability to quickly increase the amount of recombinant material [123]. This method involves using *A. tumefaciens* to mediate the transient expression through a non-virulent tumor-

inducing plasmid (Ti-plasmid). The plasmid releases transfer DNA (T-DNA) to plant cells. *N. benthamiana* is a common host for this transient expression.

Currently, plant viral expression systems are extensively utilized for gene transformation in plants. These systems provide various benefits, including the ability to rapidly modify the viral system, leading to an improvement in the transient expression level [124, 125].

The use of geminiviral vectors in transient expression systems can enhance the production of recombinant proteins in plants. Geminiviruses are small plant viruses that contain circular single-stranded DNA genomes. The *Geminiviridae* family comprises viruses that infect plants and are transmitted by insects. These viruses have circular, single-stranded DNA (ssDNA) genomes that are enclosed within geminate particles [126]. Through recent advancements, genes responsible for virus replication, spread, DNA transmission in plants, and insect transmission have been identified. Experiments involving gene replacement indicate that these viruses can be utilized to create beneficial expression vectors for plant genes [127]. The *Mastrevirus* genus of the geminiviridae family, specifically, has a circular DNA genome that ranges in size from 2.5 to 3.0 kilo-bases (kb) [128]. *Mastreviruses* possess monopartite genomes and consist of viruses that can infect both monocot plants, such as wheat dwarf virus [129], maize streak virus [130], and digitalia streak virus [131], and dicot plants, such as tobacco yellow dwarf virus and bean yellow dwarf virus (BeYDV) [132].

Several viral systems have been developed for expression vectors, such as Cucumber mosaic virus (CMV) [133], Tobacco mosaic virus (TMV) [96, 97], Plum pox virus (PPV) [134] and Cowpea mosaic virus (CPMV) [135]. The genome of BeYDV (as shown in Figure 15) is single strand circular DNA virus that consists of 2 intergenic regions: long intergenic region (LIR) and short intergenic region (SIR) with 4 coding genes: Capsid protein (CP), Movement protein (MP) and Replication protein (Rep/RepA) [136]. The LIR, which is a long intergenic region, includes a promoter that can activate

transcription in two directions (Bidirectional promoter) and origin of replication on stem loop structure (site for nicking by Rep protein). This promoter drives the expression of both virion sense genes (V1 and V2) that encode for the movement and coat proteins, respectively, as well as complimentary sense genes (C1 and C1/C2 with spliced intron) that encode for the Replication protein (RepA and Rep) which play an important role in rolling replication. On the other hand, the SIR carries the polyadenylation signals required for the protein-coding genes [131].

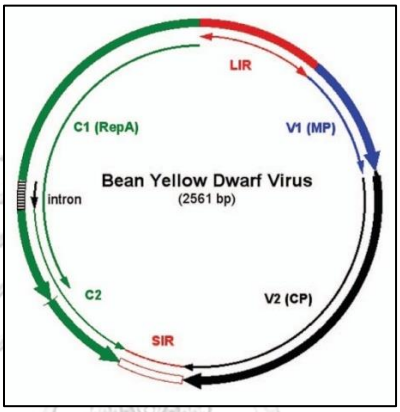


Figure 15 Bean yellow dwarf virus (BeYDV) genome structure. Source: [131].

The pBeYDV (pBY) series of expression vectors were created by modifying the T-DNA from BeYDV DNA and reconstructing it from the Ti-plasmid. One example of this is the pBYR2eK2Md (pBY2eK) vector (Appendix A), which imitates the virus replicon but does not contain any viral structure proteins. T-DNA is the part of Ti-plasmid located from LB to RB which transfers into plant cell (Figure 16). Another part that consists of the genes to support vector replication in bacterium result in binary vector. To achieve strong expression, these vectors use the cauliflower mosaic virus (CaMV) 35S promoter which is strong constitutive promotor [131, 137]. To avoid being silenced by the host's RNA interference (RNAi), the T-DNA cassette was modified by adding the p19 suppressor protein of the tomato bushy stunt virus (TBSV) which increase mRNA accumulation [138]. 5' Untranslated region (5'UTR) from TMV (TMVΩ 5'-UTR) and Tobacco (NbPsaK2T 5'-UTR) were reported that has an importance role in regulation of

translation and increasing mRNA half-life [139]. From all of the above descriptions, it results in the ability to produce recombinant protein from gene of interest (GOI) with high yield.

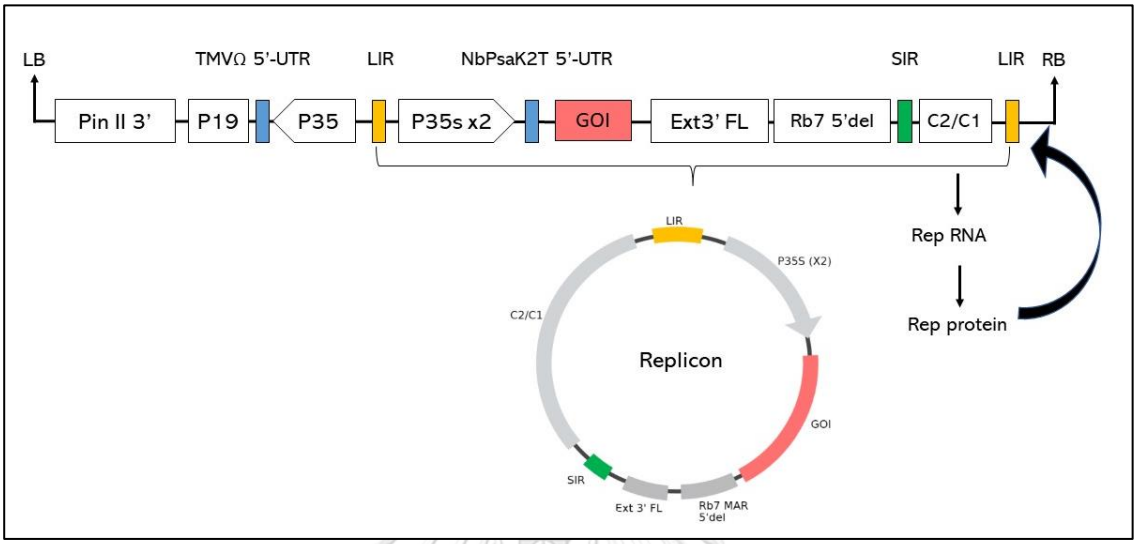
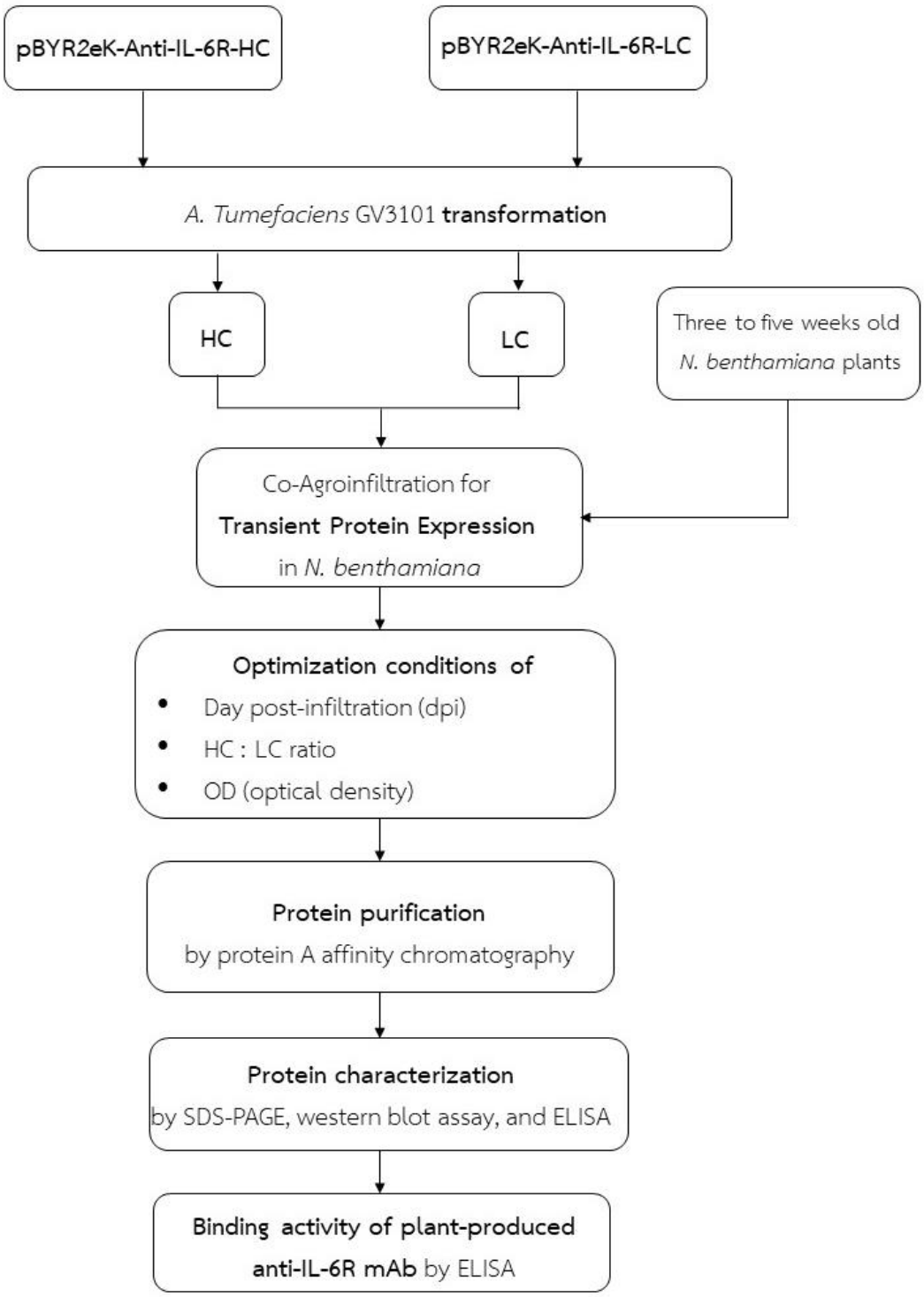


Figure 16 Schematic representation of T-DNA structure and its replicon from pBYR2eK2Md (pBY2eK). LB : Left border; PinII 3′ : Potato proteinase inhibitor II gene terminator; P19 : Tomato bushy stunt virus (TBSV) P19 gene; TMVΩ 5'-UTR : Tobacco mosaic virus Ω 5' untranslated region; P35S : cauliflower mosaic virus (CaMV) 35S promoter; LIR : Bean yellow dwarf virus (BeYDV) long intergenic region; NbPsaK2T 5'UTR : 5′ untranslated region; GOI : Gene of Interest; Ext3′FL : 3′ full length of the tobacco (N. tabacum) extension gene; RB7 : Tobacco RB7 promoter; SIR : Short intergenic region of BeYDV; C2/C1 : BeYDV open reading frames C1 and C2 encoding for replication initiation protein (Rep) and RepA), RB : Right border. Modified from [131, 140].

CHAPTER III

EXPERIMENTAL

Research Framework



Material

1. Antibodies

1) Goat anti-Human IgG-HRP (Southern Biotech, USA)
2) Goat anti-Human kappa-HRP (Southern Biotech, USA)
3) Plant-produced human anti-SARS-CoV-2 H4 mAb [141]

2. Bacteria

1) *Agrobacterium tumefaciens* (GV3101 strain)
2) *Escherichia coli* (DH10B strain)

3. Chemicals

1) 6x Loading Dye-purple (New England Biolabs, USA)
2) 2-(N-morpholino) ethanesulfonic acid monohydrate (MES) (Applichem, Germany)
3) β-merceptoethanol (Applichem, Germany)
4) Acrylamide/Bisacrylamide 40% (Himedia, India)
5) Agar powder (Himedia, India)
6) Agarose powder (Vivantis, Malaysia
7) Ammonium Persulfate (APS) (HIMEDIA®, India)
8) Antibiotics
   - Ampicillin (ITW Reagents, Darmstadt, Germany)
   - Gentamicin (ITW Reagents, Darmstadt, Germany)
   - Kanamycin (Bio Basic, Canada)
   - Rifampicin (Thermo Fischer Scientific, USA)
9) Bovine Serum Albumin Standard (BSA) (Thermo Fisher Scientific, USA)
10) Bradford protein assay (Bio-Rad®, USA)
11) Bromophenol blue (Labochem international®, Germany)
12) Coomassie blue Protein stain (Abcam, UK),
13) Deoxynucleotide triphosphate (dNTP): dATP, dCTP, dGTP, dTTP
14) Di-Sodium hydrogen phosphate (Na2HPO4) (EMSURE®, Germany)

15) Dimethyl Sulfoxide (DMSO; C2H6OS) (Sigma-Aldrich, USA)

16) Ethanol (EMSURE®, Germany)

17) Enhanced Chemiluminescence (ECL) (Abcam, UK)

18) Glycerol (HIMEDIA®, India)

19) Glycine (HIMEDIA®, India)

20) Hydrochloric acid (HCl) (Merck, USA)

21) Human soluble IL-6 receptor recombinant protein (Sino Biological, USA)

22) Magnesium Sulphate (MgSO4) (Merck, USA)

23) Methanol (Merck, Germany)

24) Peptone (HIMEDIA®, India)

25) Potassium chloride (KCL) (Merck, USA),

26) Potassium dihydrogen phosphate (KH2PO4) (EMSURE®, Germany)

27) Protein A resin (Cytiva, USA)

28) Precision Plus Protein™ All Blue Prestained Protein Standards (Bio-Rad®, USA)

29) Skim milk (BD Difco™, USA)

30) Sodium chloride (NaCl) (VWR, USA)

31) Sodium dodecyl sulfate (SDS) (Carloerbareagets, Italy)

32) Sodium hydrogen phosphate (Na2HPO4) (Merck, USA)

33) Sulfuric acid (H2SO4) (RCI labscan, Thailand)

34) Tetramethyl ethylenediamine (TEMED) (Affymetri®, USA)

35) TMB stabilized substrate (Promega, USA)

36) Tris-Acetate-EDTA (TAE) Buffer (Vivantis®, Malaysia)

37) Tris-base (Vivantis®, Malaysia)

38) Tween -20 (Vivantis®, Malaysia)

39) ViSafe Green Gel Stain (Vivantis®, Malaysia)

40) VC 1 kb DNA Ladder (Vivantis®, Malaysia)

41) Yeast Extract (Himedia Laboratories Pvt. Ltd., India)

4. Expression vectors

pBYR2eK2Md (pBYK2e) geminiviral expression vector [131, 142]

5. Enzymes

1) Restriction enzyme (RE) XhoI (New England BioLabs, UK)
2) RE XbaI (New England BioLabs, UK)
3) RE NheI (New England BioLabs, UK)
4) RE AflII (New England BioLabs, UK)
5) T4 DNA ligase (New England BioLabs, UK)
6) Taq DNA polymerase (Vivantis, Malaysia)

6. Equipment

1) Biosafety Cabinet (BSC) (ESCO Lifesciences, Thailand)
2) Blue LED transilluminator (Clare Chemical Research, USA)
3) High-Speed Centrifuge (Beckman Coulter, USA)
4) Microbiological Incubator (Thermo Scientific, USA)
5) Micropipette 2-1000 μg and 5 mL (Thermo Fisher Scientific, USA)
6) Microplate incubator (Hercuvan Lab systems, UK)
7) Microplate reader (BMG LABTECH, Germany)
8) Microplate Reader (Molecular Devices LLC, USA)
9) MicroPulser Electroporator (Bio-Rad®, USA)
10) Mini Centrifuge (Bio-Rad®, USA)
11) Mini Trans-Blot® cell (Bio-Rad®, USA)
12) Multichannel pipette (Costar®, USA)
13) Mini Easy Vertical Electrophoresis Cell (WIX Technology, China)
14) Mixer Mill MM 400 homogenizer (Retsch, Thailand)
15) Nano Scan Microplate Reader (Hercuvan, UK)
16) OTTO® Blender (OTTO, Thailand)
17) pH Bench meter (OHARUS®, China)
18) Refrigerated Centrifuge (Thermo Scientific, USA)

19) Thermo Cycler Machine (Bio-Rad®, USA)

20) TOMY Autoclave sx series (Amuza Inc., Japan)

21) Vertical polyacrylamide gel electrophoresis system (Bio-Rad®, USA)

22) Weighing balance 2 digits (Aczet, India)

23) Weighing balance 4 digits (Aczet, India)

24) WIS-20 Precise Shaking Incubator (WiseCube®, Korea)

7. Material

1) 0.22 μm Syringe filter (MilliporeSigma, USA)

2) 0.45 μm Nitrocellulose Membrane (Bio-Rad®, USA)

3) 15- and 50-mL centrifuge tubes (Corning®, USA)

4) 96-well microtiter plates (U-shape bottom) (Corning, USA)

5) Centrifugal Filter tube (Merck, USA)

6) Centrifuge tube 1.5 mL (QSP®, USA)

7) Cuvette

8) Electroporation cuvette

9) Flask 100, 500 and 1000 mL

10) High binding, half area microplate-96 well (Corning, USA)

11) Medical X-ray Green film (Carestream, China)

12) Membrane filter 0.45 and 0.22 μm (MilliporeSigma, USA)

13) NIPROTM Disposable Syringe 1, 10, 20, and 50 mL and needle (Nipro, Thailand)

14) PCR tubes/strips (Axygen ®, USA)

15) pH - Indicator strips (non-bleeding) (Merck, USA)

16) Pipette Tip sizes: 10, 200, 1000 μL and 5 mL (QSP®, USA)

17) Purification column

18) Sterilized Petri Dish Plastic (MicroQC™, China)

19) Syringe filter 0.22 μm (MilliporeSigma, USA)

8. Gene sequence

Anti-IL-6R monoclonal antibody (Tocilizumab) from previous study [143]

9. Molecular Biology kits

1) Fragment DNA Purification Kit (iNtRONBiotechnology, Inc., Korea)
2) Plasmid DNA Purification Kit (iNtRON Biotechnology, Inc., Korea)

10. Plants

Tobacco plants (*Nicotiana benthamiana*)

Software

1) Expasy DNA sequence to Protein sequence translate tool (Swiss Institute of Bioinformatics, Switzerland)
2) GeneArt Gene Synthesis (Thermo Fisher scientific, USA)
3) GraphPad Prism software (San Diego, CA, USA)
4) NEBcutter (New England Biolabs, UK)
5) Scientific Image and Illustration Software (Biorender)
6) SnapGene Viewer (SnapGene, USA)

Method

Gene Sequence

The sequences for light (VL) and heavy (VH) chain regions of mAb targeting human Interleukin 6 receptor (Anti-IL-6R mAb) were obtained from previous study [143], that corresponds to the originator tocilizumab (Actemra®).

Gene Synthesis and Codon Optimization

The variable region amino acid sequences of light (VL) and heavy (VH) chains encoding for human anti-IL-6R (Anti-IL-6R mAb) were translated to nucleotide sequences. Each nucleotide sequence was added with a codon of restriction enzymes site: *XbaI* at 5' end followed by signal peptide (SP3) and *XhoI*. The restriction enzymes site: *NheI* and *AflI*I were added at 3' end of VH and VL respectively. A linker was added

following to *NheI* of VH and *AflII* of VL at 3' end. These two nucleotide sequences (VH and VL) were codon-optimized to *N. benthamiana* nucleotide sequence via Invitrogen GeneArt® Gene Synthesis. The completely optimized nucleotide sequences were commercially synthesized (Genewiz, Suzhou, China).

Construction of pBYK2e-Anti-IL-6R Expression Vector

Each synthesized region of VH and VL was excised from the commercial cloning vectors by XbaI/NheI and XbaI/AflII (New England Biolabs, Ipswich, MA, USA) for variable-heavy and light chains, respectively. Ligation of the variable regions encoding the human Anti-IL-6R to the human IgG1 constant region with ER retention tag (SEKDEL) and pBYR2eK2Md (pBYK2e) geminiviral expression vector, resulting in the pBYK2e-Anti-IL-6R heavy chain (pBYK2e-Anti-IL-6R-HC, HC) and pBYK2e-Anti-IL-6R light chain (pBYK2e-Anti-IL-6R-LC, LC) constructs. Component of ligation as shown in table 3. The constructs of HC and LC are shown in figure 17.

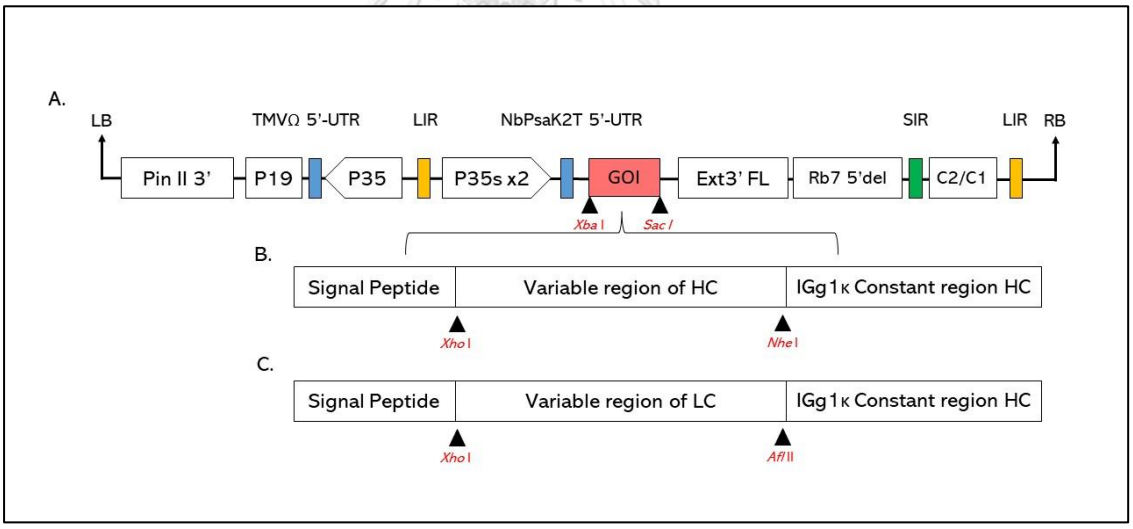


Figure 17 Geminiviral vector: pBYR2eK2Md (pBYK2e) used in the present study. The T-DNA region (A.), which is transferred by Agrobacterium into plant cells, has left and right borders known as LB and RB respectively; PinII 3′ refers to the terminator sequence derived from the potato proteinase inhibitor II gene; P19 refers to the suppressor of RNA silencing that originates from the tomato bushy stunt virus; TMVΩ 5′-UTR refers to 5′ untranslated region of tobacco mosaic virus Ω; 35S refers to Cauliflower Mosaic

Virus (CaMV) 35S promoter; LIR refers to long intergenic region of BeYDV; 35Sx2e refers to CaMV 35S promoter with duplicated enhancer; NbPsalK2T1-63 5′UTR refers to 5′ untranslated region; XbaI refers to XbaI restriction enzyme site; Anti-IL-6R HC refers to HC of Anti-IL-6R antibody (A); Anti-IL-6R LC refers to LC of Anti-IL-6R antibody(B); SacI refers to SacI-HF restriction enzyme site; Ext3′FL refers to 3′ region of tobacco extension gene; Rb75' del refers to tobacco RB7 promoter; SIR refers to short intergenic region of BeYDV; C2/C1 refers to C1 and C2 ORFs of Bean Yellow Dwarf Virus (BeYDV) encoding for replication initiation protein (Rep) and RepA. The heavy and light chain construct were shown in B. and C., respectively.

Table 3 Component of 3 pieces ligation

Component	Volume (µL)
Insertion I (VH/VL)	6.5
Insertion II (CH/CL)	4
Vector	2
10X ligation buffer	1.5
T4 ligase	1.0
Total volume	15

Ligation mixture was incubated at RT overnight.

Gene Transformation

Each ligation mixture harboring the HC and LC constructs was individually transformed into *Escherichia coli* (*E. coli*); DH10B strain by using heat shock technique. Transformed *E. coli* colonies were confirmed for the light and heavy chain Anti-IL-6R genes with colony polymerase chain reaction (PCR) using geminiviral expression vector universal primers to check the integration. Colonies that were confirmed by colony PCR for the Anti-IL-6R constructs were cultured in 3 mL of selective medium (Luria Bertani broth (LB) with 50 µg/mL kanamycin antibiotic), and overnight incubation at 37 °C, 200 rpm in an incubator shaker. *E. coli* colony cultures were further used for plasmid isolation by Plasmid DNA Purification Kit. The isolated plasmids were further confirmed

by restriction digestion with XbaI/BmtI and XbaI/AflII for HC and LC, respectively to release the expected gene fragments from expression vector. The confirmed plasmids were then analyzed by gene sequencing. Then, electroporation was performed for each plasmid, pBYK2e-Anti-IL-6R HC and pBYK2e-Anti-IL-6R LC genes to re-transform into *A. tumefaciens*; GV3101 strain using an electroporator.

The transformed *Agrobacterium* cultures with pBYK2e-Anti-IL-6R HC and LC was plated on Luria Bertani agar (LA) containing rifampicin, gentamicin, kanamycin at a concentration of 50 µg/ml each. The plates were incubated at 28°C for 48 hours.

Transformed *A. tumefaciens* colonies observed on agar plates were confirmed by colony PCR. After that, the positive colonies were cultured in 5 mL of LB containing antibiotics and incubated in temperature-controlled shaker at 250 rpm, 28°C for 20 hours for making the transformed bacterial culture stocks and protein expression.

Colony PCR

Colony PCR was used to confirm colonies of transformed bacteria. 2eR primers were used. The PCR components and cycling conditions are shown in Table 4 and 5, respectively.

Table 4 Components for Colony PCR

Component	Volume (µL)/ reaction
Template	5
Ultrapure water	4.3
10X Buffer A	1.5
dNTP's	1.5
$MgCl_2$	0.5
Forward primer	1

Reverse primer	1
Taq DNA polymerase	0.2
Total	15

Table 5 PCR Conditions for Colony PCR

No.	Condition	Temperature	Time	Cycle
1.	Cell lysis	94 °C	5 min	1
2.	Denaturation	94 °C	0.30 sec	30
3.	Annealing	54°C	0.30 sec	
4.	Extension	72°C	1.30 min	
5.	Hold	25°C	Indefinitely	

Transient expression by co-agroinfiltration into *N. benthamiana* leaves

Three to five weeks old tobacco plants are used for agroinfiltration. The seedlings of *N. benthamiana* were grown in sterile soil pots for 5 weeks at a constant temperature of 28°C with 16 hours of photoperiod cycle.

For protein expression optimization algorithms include day post infiltration (dpi) optimization, HC : LC ratio optimization, and optical density (OD) 600 optimization (for bacterial concentration). To optimize HC and LC ratio, Agrobacterium with each heavy chain or light chain plasmid was cultured with selective media, pelleted, and resuspended with 1X infiltration buffer and were mixed with different ratio immediately prior consist of 1 : 1, 1 : 2 and 2 : 1 (HC : LC) and co-agroinfiltrated by syringe without needle. The infiltrated plants were incubated in the controlled environment. Then,

optimize *Agrobacterium* concentration for infiltration and incubation after infiltration, also called day post infiltration (dpi) was performed, *Agrobacterium* with heavy chain and light chain was cultured with selective media. Each pellet was then resuspended in 1X infiltration buffer to produce final OD600 0.2, 0.4, 0.6, and 0.8. Each different *Agrobacterium* cell concentrations were co-infiltrated into *N. benthamiana* by syringe without needle. The plants were incubated in the controlled environment. The infiltrated leaves were collected on day- 1, 3, 4, 5, 7, and 9 post-infiltration (dpi) in separate 1.5 mL centrifuge tubes. Each sample was extracted with 1X PBS by bead homogenizer and then centrifuged at 13,500 rpm for 10 minutes. Each experiment was performed in quadruplicates. The supernatant (crude extract) of each sample was used for protein analysis by ELISA technique.

Protein Purification

To express anti-IL-6R mAb in *N. benthamiana* for protein purification, *Agrobacterium* with each heavy chain or light chain plasmid was cultured with selective media at 28°C for 20 hr. and dilute to OD600 0.2 with infiltration buffer, then infiltrated into *N. benthamiana via* vacuum infiltration. Infiltrated leaves were harvested on 5 dpi and weighed. To extract protein, infiltrated leaves were blended in 1X PBS extraction buffer at a ratio of 1 g : 2 mL (1g of leaf in 2 mL of extraction buffer). Following a cloth filter clarification step, the blended plant was centrifuged at 15000 x g for 30 minutes at 4°C, and the plant extract (crude extract) was next obtained by filtering the supernatant through 0.45 µm membrane filter.

Protein-A affinity chromatography method was used for protein purification. Crude extract was loaded onto the column containing Protein-A resin. The column was gently washed with 10X column volumes 1X PBS, and then eluted the target protein with glycine buffer (0.1M at pH 2.7) and neutralized with Tris-HCl (1.5M at pH 8.8). The purified elutes were further confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. The total protein was determined by Bradford assay.

SDS-PAGE and Western Blot

The samples were mixed with non-reducing loading dye and reducing loading dye, denature for 5 min at 95℃ and loaded onto SDS-PAGE gel. The protein separation was observed on 4-15% polyacrylamide gel with continuous 1X running buffer at 120 V for 80 minutes. Separated proteins were subjected to either Coomassie staining or Western blot detection. For Coomassie staining, Coomassie® blue stain solution and water were used for staining and destaining the gel, respectively. The protein bands were visualized. For western blotting, separated proteins in the gel were transferred onto nitrocellulose membrane with 1X transfer buffer at 100 V for 2 hours. Subsequently after transfer of proteins, the membrane was blocked with 5% non-fat milk in 1X PBS for 30 minutes at RT and incubated either with 1:10000 Goat Anti-Human IgG-HRP or with 1:2500 Goat Anti-Human Kappa-HRP diluted with 3% non-fat milk in 1X PBS at RT for 2 hours to detect HC and LC protein fragments respectively. The incubated membrane was washed 2 times with 1X PBS-T and followed by single 1X PBS wash for 10 minutes. The protein band was detected on an X-ray film by using enhanced chemiluminescence (ECL).

Bradford Protein Assay

Bradford protein assay was used to measure total soluble protein. As a protein standard, bovine serum albumin (BSA) was employed. 2 mg/mL of BSA stock was diluted with 1X PBS to get the working concentrations ranging from 0.8 - 0.1 mg/mL. Each test sample was diluted with 1X PBS. Bradford protein assay reagent (5X) was diluted to 1X with deionized water. 10 µL of each sample was mixed with 190 µL diluted Bradford protein assay reagent and was added to a 96 well low binding plate, then measure by microplate reader at 595 nm.

Quantification by Sandwich ELISA

To determine the protein quantification, a 96 half well microplate was coated with 25 µL/well of anti-human IgG Fc fragment that was diluted in 1X PBS (1:1000) and incubated at 37℃ for 2 hours. The microplate was washed with 170 µL/well with 1X

PBS-T for 3 times and blocked with 5% of non-fat milk diluted in 1X PBS at 37°C for 1 hours. The microplate was washed with 1X PBS-T. The human IgG1, kappa - Isotype control was subjected to two-fold dilution in 1X PBS whereas plant crude extract and purified were diluted to ratio 1:1000 and 1:2000, respectively. Then, each standard and control was added into the microplate and incubated at 37°C for 2 hours. The microplate was washed with 1X PBS-T. Further, the plate was incubated with 1:2500 Goat Anti-Human Kappa-HRP diluted in 1X PBS. After washing with 1X PBS-T, the microplate was developed by adding 25 µL/well of TMB substrate in dark for 5 minutes for the color development. To stop the reactions, 1M H2SO4 (25 µL/well) was added. The absorbance of each well was then measured at 450 nm using a 96-well plate reader. The experiments were performed in duplicates and data were presented as mean± standard deviation (SD).

Specific Binding Activity by Indirect ELISA

Plant-produced Anti-IL-6R antibody specific binding activity was assessed by ELISA. Each well of a 96 half well microplate was coated with 25 µL of 2 µg/mL human IL-6R recombinant protein and incubated at 4°C overnight. The incubated microplate was washed 3 times with 170 µL/well 1X PBS-T and blocked with 5% skim milk in 1X PBS at 37°C for 2 hours. Known concentrations of purified plant-produced Anti-IL-6R mAb and plant-produced H4 mAb were diluted using two-fold dilution starting with 16 µg/mL until 0.125 µg/mL. Three biological replicates were performed. The PBS-T washed plate was incubated with plant-produced Anti-IL-6R and H4 antibodies at 37°C for 2 hours. After washing, the microplate was incubated with Goat Anti-Human Kappa-HRP (1:2500 in 1X PBS) for 1 hour at 37°C. Finally, the microplate was washed and detected with 25 µL/well of TMB substrate, stopped the reaction with 1 M H_2SO_4 by adding 25 µL/well. And then, the absorbance was read at 450 nm by microplate reader. Plant-produced H4 mAb [73] was used as negative control.

CHAPTER IV
RESULTS AND DISCUSSION

Interleukin 6 (IL-6) is a proinflammatory cytokine released by different cell types, most notably macrophages and T cells. These two cell types are associated with CRS. Symptoms range from fever, vomiting, diarrhea, hypoxia, multiorgan failure to life threatening. Patients may die under various circumstances. Various inhibitors are employed to prevent a worried cytokine storm through IL-6 signaling. Tocilizumab is a humanized mAb that targets the IL-6 receptor and is used to treat RA, giant cell arteritis, polyarticular and systemic JIA, and cytokine release syndrome causing COVID-19. However, the limitations of mammalian cell culture include high production costs, restricted production scale and scale-up capabilities, and a significant danger of contamination. This work aimed to express antibody targeting the IL-6 receptor in a plant platform. In brief, each mab design was ligated to the germinal viral vector, converted into *A. tumefaciens*, and co-infiltrated in *N. benthamiana* for expression host. The plant-produced Anti-IL-6R mAb was purified using protein-A affinity chromatography and then evaluated *in vitro* for specific binding to soluble human IL-6R. The gene sequences encoding the heavy and light chains of Anti-IL-6R mAb were produced in expression construct, as indicated in Appendix A.

Expression and optimization of Anti-IL-6R mAb in *N. benthamiana*

Gene cloning and construction

Each nucleotide sequence from previous research with restriction enzyme sites (*XbaI*/*SacI*), signal peptide, SEKDEL, and constant region was codon optimized and ligated to the pBYR2eK2Md (pBYK2e) geminiviral expression vector. Heavy and light chain plasmids were digested by restriction enzymes to confirm the complete construct. Figure 18 shows agarose gel electrophoresis of heavy chain plasmid, light chain plasmid, and restriction enzyme cleavage products (XbaI and SacI).

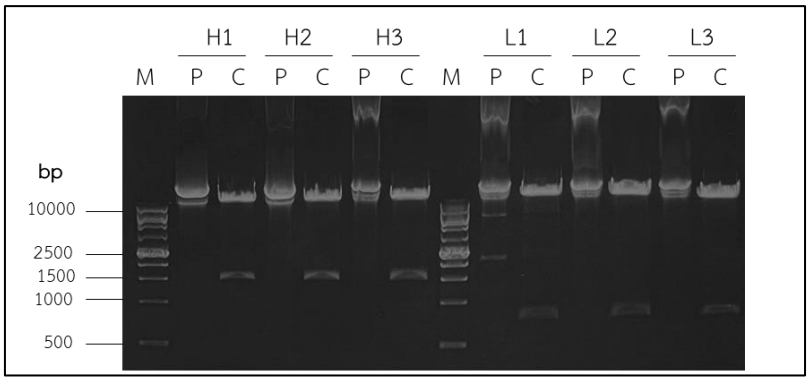


Figure 18 Agarose gel electrophoresis of the Anti-IL-6R HC and LC plasmid and its fragments produced by cleavage of XbaI and SacI restriction enzyme. All of DNA sample was separated by electrophoresis on 0.7% agarose gel and visualized under a blue-light transilluminator. H : Heavy chain DNA construct; L : Light chain DNA construct; P : undigested DNA; C : digested with XbaI and SacI restriction enzyme; M : VC 1 kb DNA ladder.

Then, each construct was transformed into *A. tumefaciens* cells (GV3101 strain) and were screened for successful gene transformation by Colony PCR and determined by horizontal gel electrophoresis. Findings showed positive *Agrobacterium* colonies approximately 1,500 bp for HC and 750 bp for LC (as shown in figure 19)

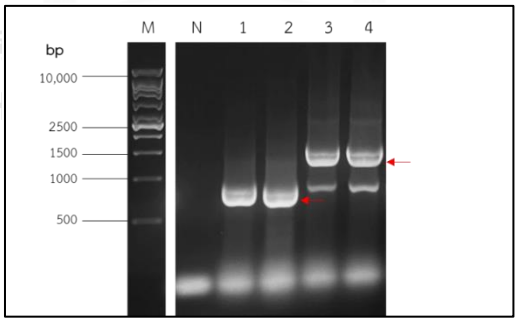


Figure 19 Agarose gel electrophoresis for colony PCR products of transformed A. tumefaciens GV3101 using gene 2eR primers. Autoclaved ultrapure water was used for negative control template. Colony PCR products were separated by electrophoresis on 1% agarose gel and visualized under a blue-light transilluminator. Lane 1-2 : pBYR2eK-Anti-IL-6R-LC vector; Lane 3-4: pBYR2eK-Anti-IL-6R-HC vector; N : Negative control; M : VC 1 kb DNA ladder.

Transient expression of *A. tumefaciens* was the first approach utilized to successfully create recombinant proteins in plants [144]. Therefore, using plants for the transient expression of recombinant proteins provides a viable technological alternative to mammalian cell culture systems. This is because plant-based systems can overcome the limitations of high production and the pathogen contamination risks associated with mammalian systems [145-149]. Recently, there have been successful examples of biopharming in plants, including the production of growth factors like aFGF (Acidic Fibroblast Growth Factor) [150] and VEGF (vascular endothelial growth factor) [151], and antibodies such as mAbs 6D8 [152], and anti-PD-1 mAb (pembrolizumab) [153] which are protective against various diseases. As a result, we produced antibody targeting IL-6R using transient expression in *N. benthamiana*. The use of the geminiviral vector pBY2Ek and the CaMV 35S promoter were essential for optimal gene expression [131]

Optimization of Anti-IL-6R mAb expression in *N. benthamiana*

Different factors were explored, including bacterial culture OD, days post-infiltration (dpi), and heavy chain to light chain ratio. First, ratio of *Agrobacterium* media that contain HC and LC was investigated. *Each cultured* HC and LC *Agrobacterium* with final OD600 = 0.2 were co-agroinfiltrated into *N. benthamiana* in varied ratios of 1 : 1, 1 : 2, and 2 : 1 (HC : LC). The plants that were infiltrated were cultivated in a regulated environment and were harvested at 5 days post-infiltration to assess the extent of protein expression. From *the* preliminary study of HC to LC ratio showed that highest protein expression level in the ratio of 1 : 1, HC to LC at 1001.27 µg/g fresh weight (Figure 20A). Consequently, concentration of bacterial culture and day post infiltration were investigated. Each cultured HC and LC *Agrobacterium* pellet was produced at final OD600 = 0.2, 0.4, 0.6, and 0.8 and co-infiltrated into *N. benthamiana* in the ratio of 1 : 1, HC to LC. Then, the infiltrated plants were incubated in the controlled environment and were harvested within 1, 3, 5, 7, and 9 days of post-infiltration (dpi). Protein expression level was analysed by using ELISA technique. From the results,

expression level of plant-produced Anti-IL-6R mAb from condition of *Agrobacterium* concentration at 5 dpi, OD600 0.2 and 0.4 were not significantly different (P-value > 0.05) (Figure 20B). Due to necrosis depending on *Agrobacterium* concentration and media volume for *Agrobacterium* culture, *Agrobacterium* concentration at OD600 0.2 is the suitable concentration for anti-IL-6R mAb production in plant. Cell necrosis was found in the infiltrated leaves on 3 dpi, but significant necrosis was observed on 5 dpi, as demonstrated in figure 20C.

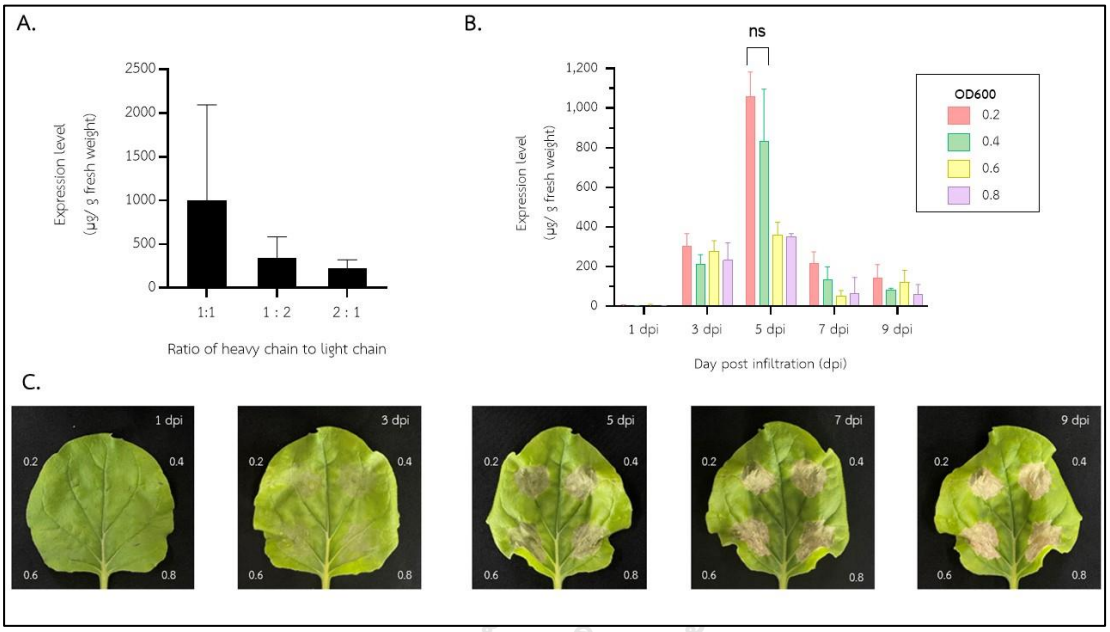


Figure 20 Optimization of HC to LC ratio (A), days post-infiltration (dpi), and A. tumefaciens optical density (OD) (B). The ELISA technique was used to measure the levels of Anti-IL-6R mAb produced by plants, and the results are presented as the mean value ± the standard deviation (SD). A) HC to LC ratio optimization B) Optical density and days post-infiltration optimization and C) The leaf necrosis of plant-expressed Anti-IL-6R mAb at different ODs of A. tumefaciens on day 1, 3, 4, 7, and 9 post-infiltrations.

The levels of Anti-IL-6R mAb that were generated by plants were quantified using ELISA, and the data is reported as the average value with the corresponding standard deviation. Earlier research has indicated that the peak production of several proteins expressed in *N. benthamiana* occurs at different time points. Specifically, the highest yields for anti-Enterovirus 71 (EV71) [154] and anti-PD-L1 (atezolizumab) [155] are reached at 6 dpi, while for anti-human PD-1 mAb (pembrolizumab) [153], anti-IgE (omalizumab)[156], the optimal harvest time is 4 dpi. These short time intervals offer significant benefits in terms of speed for protein expression, distinguishing it from other expression systems [157, 158]. Previous studies reported that the highest yield of various transiently expressed proteins in *N. benthamiana* at OD600 = 0.2 for EGF (Epidermal growth factor) [159], at OD600 = 0.4 for anti-PD-L1 (atezolizumab) [155]. The noticeable decrease in antibody expression observed after 5 dpi could be attributed to the gradual onset of necrosis on the leaves that were infiltrated. Overproduction of recombinant proteins may lead to the misfolding of these proteins, which can initiate necrosis and subsequent degradation of the recombinant proteins [160].

Anti-IL-6R mAb protein purification

From our findings, the highest amount of mAb produced by the plant occurred on the 5-day post-infiltration when it was infiltrated with *Agrobacterium* at a concentration of 0.2 (OD600) using equal amounts of HC and LC. In this research, the vacuum infiltration method was used to achieve the optimal conditions for the lager production in plants. The high-throughput approach described here is a protein production platform that can be quickly scaled up for efficient, cost-effective and safe industrial manufacturing. Previous studies have shown that the vacuum agroinfiltration method can be used to produce many different types of recombinant proteins in plants [153-155, 161, 162].

The plants that underwent vacuum agroinfiltration were collected 5 days post-infiltration and subsequently prepared for the purification stage. The process involved

harvesting leaves that had been infiltrated, extracting them using a solution of 1X PBS, and then centrifuging the mixture to create a plant extract. To obtain purified mAb from this extract, a Protein-A affinity chromatography purification method was utilized due to its strong binding affinity with the Fc region of IgG [163]. Previous studies have demonstrated that monoclonal antibody produced in plants can be effectively purified using a one-step protein A purification process for example capillary morphogenesis gene-2 (CMG2) protein fused Fc (fragment crystallizable) [164], tumour-targeting antibody (mAb H10) [165], PfMSP4-specific mAb [166], and plant-produced SARS-CoV-2 subunit vaccine [162].

To isolate the plant-produced Anti-IL-6R, a plant extract or crude protein was loaded into Protein A resin column, then the column was washed with a washing buffer (1X PBS) to eliminate any non-specific proteins, and then glycine buffer (elution buffer) was used to extract the protein. After that, the plant-produced Anti-IL-6R antibody was identified using SDS-PAGE and western blotting analysis.

To visualize the separated protein band, the SDS-PAGE gel was stained with Coomassie blue staining. IgG antibodies are made up of two types of polypeptide chains and are large molecules with a molecular weight of around 150 kDa. One of these chains is called the heavy chain or H chain and weighs approximately 50 kDa, while the other is called the light chain or L chain and weighs about 25 kDa [167]. Each IgG molecule contains two heavy chains and two light chains. Under non-reducing condition, the plant-produced Anti-IL-6R mAb protein band was showed in stained SDS-PAGE, with the total molecular weight of the antibody approximately 150 kDa, as illustrated in (Figure 21A.) Western blot was performed by detecting of anti-Gamma-HRP and anti-Kappa-HRP with the total molecular weight of the antibody approximately 150 kDa same as SDS-PAGE (as shown in figure 21B and 21C). The reason of glycosylation pattern that results in a shift of the main protein band. The process of glycosylation in the endoplasmic reticulum is similar across almost all species and only involves oligomannose type N-glycans with a specific composition of sugars. On

the other hand, the processing of hybrid and complex type glycans in the Golgi apparatus is highly varied and can differ greatly between different species [168]. Several methods have been developed to modify how N-glycans are processed in plant expression systems in order to boost the production of soluble recombinant proteins and prevent their breakdown in the cytoplasm. These strategies include targeting the biopharmaceuticals to the endoplasmic reticulum to enhance their production or disrupting the activity of the plant's own glycosyltransferases (such as by creating plants that lack fructosyltransferase and xylosyltransferase) and/or introducing foreign glycosyltransferases. These approaches have been described in various studies, including those by Palacpac et al. (1999) [169] , Saint-Jore-Dupas et al. (2007) [170], and Schähs et al. (2007) [171]. One of these methods involves adding an endoplasmic reticulum (ER) retention signal to the C-terminus of a recombinant protein [172]. The KDEL/HDEL motif, which is used for ER retention or retrieval, can be extended (ex. SEKDEL). SEKDEL is a motif that consists of 18 nucleotides and encoding to Ser-Glu-Lys-Asp-Glu-Leu (S-E-K-D-E-L) [173]. If a recombinant protein is linked with SEKDEL signal peptide sequence, it can accumulate within ER [174]. In transgenic plants, retaining proteins in the ER typically leads to increased levels of production. It is uncertain whether a modified glycan structure in mAbP would maintain its effectiveness in providing systemic post-exposure prophylaxis, as glycan processing can impact antibody stability [168].

Under reducing conditions, Anti-IL-6R HC and LC have individual molecular weights of 50 kDa and 25 kDa, respectively as shown in the figure 21D for stained SDS-PAGE. For western blotting, anti-gamma HRP conjugated and anti-kappa HRP conjugated were used to detect heavy chain and light chain, respectively (Figure 21E and 21F). After purification process, plant-produced Anti-IL-6R mAb was used in a specific binding assay.

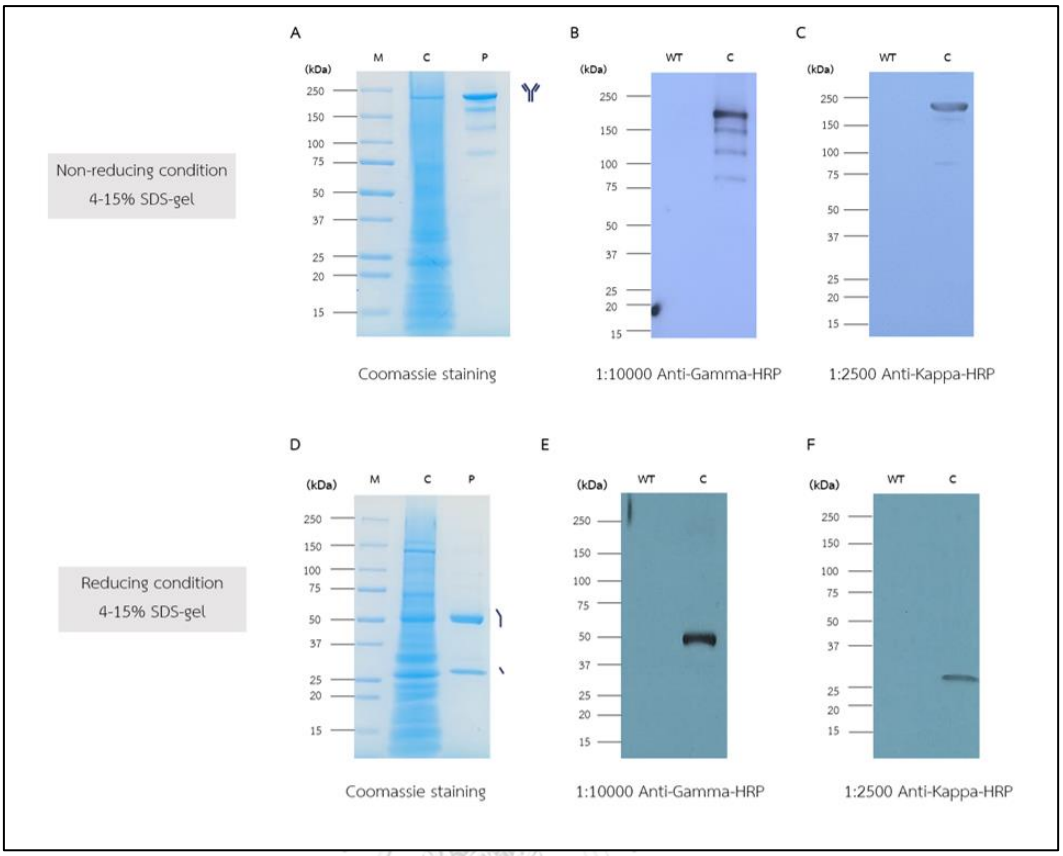


Figure 21 SDS-PAGE and Western blot methods were employed to examine the Anti-IL-6R mAb that was produced in N. bentamiana. Non-infiltrated leaves were used as a negative control (labeled "WT" in the figure). Panels (A&D), (B&E), and (C&F) depict the antibody stained with Coomassie blue and probed with anti-Gamma and anti-Kappa antibodies, respectively, both under non-reducing and reducing conditions. The crude extract was loaded at 20 µg/lane, while purified antibody was loaded at 5 µg/lane.

Assessment of the Binding Activity of Plant-Produced Anti-IL-6R mAb to Human Recombinant IL-6R

To determine the specific binding activity of Anti-IL-6R antibody produced by plants, it was performed in a binding ELISA with its intended target, human recombinant IL-6R. As a comparison, plant produced H4 mAb [141] was included as a negative control in the experiment. To study the binding activity of each antibody,

they were diluted in a series and then incubated with hIL-6R. The binding activity was measured at an Ab450 using an anti-human kappa HRP conjugate that was diluted at a ratio of 1:2500. The results showed that the plant-produced Anti-IL-6R antibody had a dissociation constant (K_D) of 1.748 µg/mL when bound to the human recombinant IL-6R protein, as depicted in figure 22. Conversely, the negative control did not bind as anticipated. The study did not use commercial TCZ as a positive control due to its unavailability in the region [175]. Nonetheless, the active engagement of the plant-produced Anti-IL-6R with its target serves as evidence of its functionality.

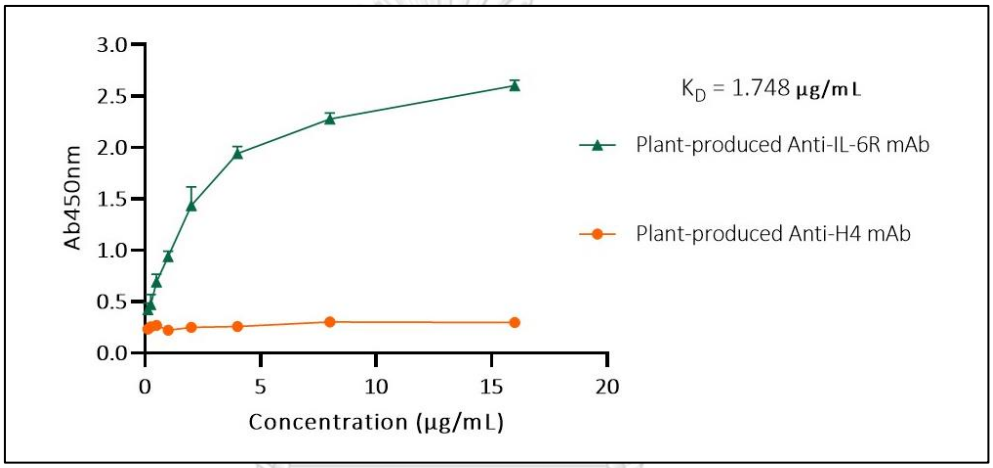


Figure 22 Anti-IL-6R mAb derived from plants binding activityto human recombinant Interleukin 6 receptor (hIL-6R) by indirect ELISA. Plant-produce Anti-H4 mAb was used as negative control. KD: dissociation constant; Ab: Absorbance.

To investigate specific binding activity, plant-produced Anti-IL-6R mAb was used to bind with recombinant human IL-6R by using an immunoassay. The ELISA results demonstrated that the Anti-IL-6R mAb produced by plants can effectively bind to human IL-6R protein. On the other hand, the plant-produced H4 mAb, which lacks specificity, did not display any binding activity towards the target protein. This indicates that the Anti-IL-6R mAb has specific binding activity towards the target protein. Unfortunately, we were unable to compare the mammalian and plant-produced Anti-IL-6R mAb directly in our assay because the commercial TCZ (humanized Anti-IL-6R mAb) was not available.

CHAPTER V
CONCLUSION

Interleukin 6 (IL-6), a multifunction inflammatory cytokine that initiates cellular signaling by binding to its different two receptors, mIL-6R and sIL-6R. Rapid and excessive IL-6 production is caused of cytokine release syndrome (CRS) that can be tissue damage, multiorgan failure and life-threatening. Inhibition of IL-6 intercellular signaling has become a therapeutic target for autoimmune diseases and CRS. However, the production and treatment costs and also contamination risk of mAb expression using mammalian cell culture system are concern. In this study, we produced a recombinant Anti-IL-6R mAb that can recognize human IL-6R *via* *N. benthamiana* as a protein expression host. Each amino acid of Anti-IL-6R HC and LC, which were derived from previous study was translated into nucleotide sequences. Then, each nucleotide sequence was codon optimized, separately constructed in pBYR2eKMd geminiviral expression vector and transiently co-expressed in plants. As the results, plant-produced Anti-IL-6R mAb was optimally expressed at 1056.11 µg/g fresh weight when the plant leaves were co-infiltrated in a ratio of 1 : 1, HC to LC, with a *Agrobacterium* culture OD600 of 0.2. The highest expression level was achieved at 5 dpi. The plant-produced mAb was rapidly expressed at high levels and purified using protein-A affinity chromatography. Plant-purified Anti-IL-6R antibody was confirmed by using anti-Gamma-fused HRP and anti-Kappa-fused HRP antibodies. The molecular weight of plant-based Anti-IL-6R for full form, HC, and LC were approximately 150, 50, and 25 kDa, respectively as predicted. The results of an indirect ELISA demonstrated that the plant-derived Anti-IL-6R mAb exhibited binding activity with KD value of 1.748 µg/mL, while the plant-produced H4 mAb, which served as a negative control, showed no binding activity.

Based on findings, it allows the conclusion that *N. benthamiana* has the potential to be used as a plant transient expression system to produce antibody against human IL-6R in plants. However, in order to determine the efficacy of the plant-

produced Anti-IL-6R mAb, animal studies must be conducted to gain a better understanding of the role played by IL-6 in disease progression and the IL-6 signaling pathway.

REFERENCES

APPENDIX

APPENDIX A

The nucleotide sequences of Anti-IL-6R mAb

Previous research on the characterization of a biosimilar humanized IL-6R mAb, Tocilizumab, provided the amino acid sequences which were then converted to nucleotide sequences and optimized to match the codon-usage of *N. benthamiana*. The nucleotide sequences that were optimized were modified by including a signal peptide at the beginning (N-terminal) and ER retention tag (SEKDEL) at the end (C-terminal) of both HC and LC. *The restriction enzyme sites including XbaI and Sac*I *were also added into the optimized sequences.* To fuse the variable and constant regions, *Nhe*I and *Afl*II were added to the optimized heavy chain and light chain sequences, respectively.

1. **Primer sequences**

   >2e-3R-80bp-Forward

   GGAGAGGACCTCGAGAAAC

   >2e-29e-78bp-Reverse

   GCTTTGCATTCTTGACATC

2. **Restriction enzyme site**

Restriction enzyme	Restriction site
*Afl*II	C^TTAAG GAATT^C
*Nhe*I	G^CTAGC CGATC^G
*Sac*I	GAGCT^C C^TCGAG
*Xba*I	T^CTAGA AGATC^T
*Xho*I	C^TCGAG GAGCT^C

3. Synthesized Sequence of human Anti-IL-6R mAb Heavy chain

```
TCT AGA ATG GGC TGG TCC TGC ATC ATC CTG TTC CTT GTT GCT ACT GCT ACC GGC GTT CAC
 S   R   M   G   W   S   C   I   I   L   F   L   V   A   T   A   T   G   V   H

TCT GAT GTT CAA CTT CTC GAG GAG GTT CAG CTT CAA GAA TCT GGT CCT GGT CTT GTG AGG
 S   D   V   Q   L   L   E   E   V   Q   L   Q   E   S   G   P   G   L   V   R

CCT TCT CAG ACT CTT TCT CTT ACC TGC ACC GTG AGC GGT TAC AGC ATT ACT TCT GAT CAC
 P   S   Q   T   L   S   L   T   C   T   V   S   G   Y   S   I   T   S   D   H

GCT TGG AGC TGG GTT AGA CAA CCT CCT GGT AGA GGT CTT GAG TGG ATC GGC TAC ATC AGC
 A   W   S   W   V   R   Q   P   P   G   R   G   L   E   W   I   G   Y   I   S

TAC TCT GGT ATC ACC ACT TAC AAC CCG AGC CTG AAG TCT AGG GTT ACC ATG CTT AGG GAC
 Y   S   G   I   T   T   Y   N   P   S   L   K   S   R   V   T   M   L   R   D

ACC AGC AAG AAC CAG TTC AGC CTG AGG CTT TCT TCT GTG ACT GCT GCT GAT ACC GCT GTG
 T   S   K   N   Q   F   S   L   R   L   S   S   V   T   A   A   D   T   A   V

TAC TAC TGC GCT AGA TCT CTT GCT AGG ACC ACC GCT ATG GAT TAT TGG GGT CAA GGT AGC
 Y   Y   C   A   R   S   L   A   R   T   T   A   M   D   Y   W   G   Q   G   S

ACC CTC GTC ACC GTC TCC TCC GCT AGC ACC AAA GGT CCA TCG GTC TTT CCA CTG GCA CCT
 T   L   V   T   V   S   S   A   S   T   K   G   P   S   V   F   P   L   A   P

TCT TCC AAG AGT ACT TCT GGA GGC ACA GCT GCA CTG GGT TGT CTT GTC AAG GAC TAC TTT
 S   S   K   S   T   S   G   G   T   A   A   L   G   C   L   V   K   D   Y   F

CCA GAA CCT GTT ACG GTT TCG TGG AAC TCA GGT GCT CTG ACC AGT GGA GTG CAC ACC TTT
 P   E   P   V   T   V   S   W   N   S   G   A   L   T   S   G   V   H   T   F

CCA GCT GTT CTT CAG TCC TCA GGA TTG TAT TCT CTT AGC AGT GTT GTG ACT GTT CCA TCC
 P   A   V   L   Q   S   S   G   L   Y   S   L   S   S   V   V   T   V   P   S

TCA AGC TTG GGC ACT CAG ACC TAC ATC TGC AAT GTG AAT CAC AAA CCC AGC AAC ACC AAG
 S   S   L   G   T   Q   T   Y   I   C   N   V   N   H   K   P   S   N   T   K

GTT GAC AAG AAA GTT GAG CCC AAG TCT TGT GAC AAG ACT CAT ACG TGT CCA CCG TGC CCA
 V   D   K   K   V   E   P   K   S   C   D   K   T   H   T   C   P   P   C   P

GCA CCT GAA CTT CTT GGA GGA CCG TCA GTC TTC TTG TTT CCT CCA AAG CCT AAG GAT ACC
 A   P   E   L   L   G   G   P   S   V   F   L   F   P   P   K   P   K   D   T

TTG ATG ATC TCC AGG ACT CCT GAA GTC ACA TGT GTA GTT GTG GAT GTG AGC CAT GAA GAT
 L   M   I   S   R   T   P   E   V   T   C   V   V   V   D   V   S   H   E   D

CCT GAG GTG AAG TTC AAC TGG TAT GTG GAT GGT GTG GAA GTG CAC AAT GCC AAG ACA AAG
 P   E   V   K   F   N   W   Y   V   D   G   V   E   V   H   N   A   K   T   K

CCG AGA GAG GAA CAG TAC AAC AGC ACG TAC AGG GTT GTC TCA GTT CTC ACT GTT CTC CAT
 P   R   E   E   Q   Y   N   S   T   Y   R   V   V   S   V   L   T   V   L   H

CAA GAT TGG TTG AAT GGC AAA GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC
 Q   D   W   L   N   G   K   E   Y   K   C   K   V   S   N   K   A   L   P   A

CCC ATT GAG AAG ACC ATT TCC AAA GCG AAA GGG CAA CCC CGT GAA CCA CAA GTG TAC ACA
 P   I   E   K   T   I   S   K   A   K   G   Q   P   R   E   P   Q   V   Y   T

CTT CCT CCA TCT CGC GAT GAA CTG ACC AAG AAC CAG GTC AGC TTG ACT TGC CTG GTG AAA
 L   P   P   S   R   D   E   L   T   K   N   Q   V   S   L   T   C   L   V   K

GGC TTC TAT CCC TCT GAC ATA GCT GTA GAG TGG GAG AGC AAT GGG CAA CCG GAG AAC AAC
 G   F   Y   P   S   D   I   A   V   E   W   E   S   N   G   Q   P   E   N   N
```

```
TAC AAG ACT ACA CCT CCC GTT CTC GAT TCT GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC
 Y   K   T   T   P   P   V   L   D   S   D   G   S   F   F   L   Y   S   K   L

ACA GTG GAC AAG AGC AGG TGG CAA CAA GGG AAT GTC TTC TCA TGC TCC GTG ATG CAT GAG
 T   V   D   K   S   R   W   Q   Q   G   N   V   F   S   C   S   V   M   H   E

GCT CTT CAC AAT CAC TAC ACA CAG AAG AGT CTC TCC TTG TCT CCG GGT AAA TCT GAG AAG
 A   L   H   N   H   Y   T   Q   K   S   L   S   L   S   P   G   K   S   E   K

GAT GAG CTT TGA GAG CTC
 D   E   L   -   E   L
```

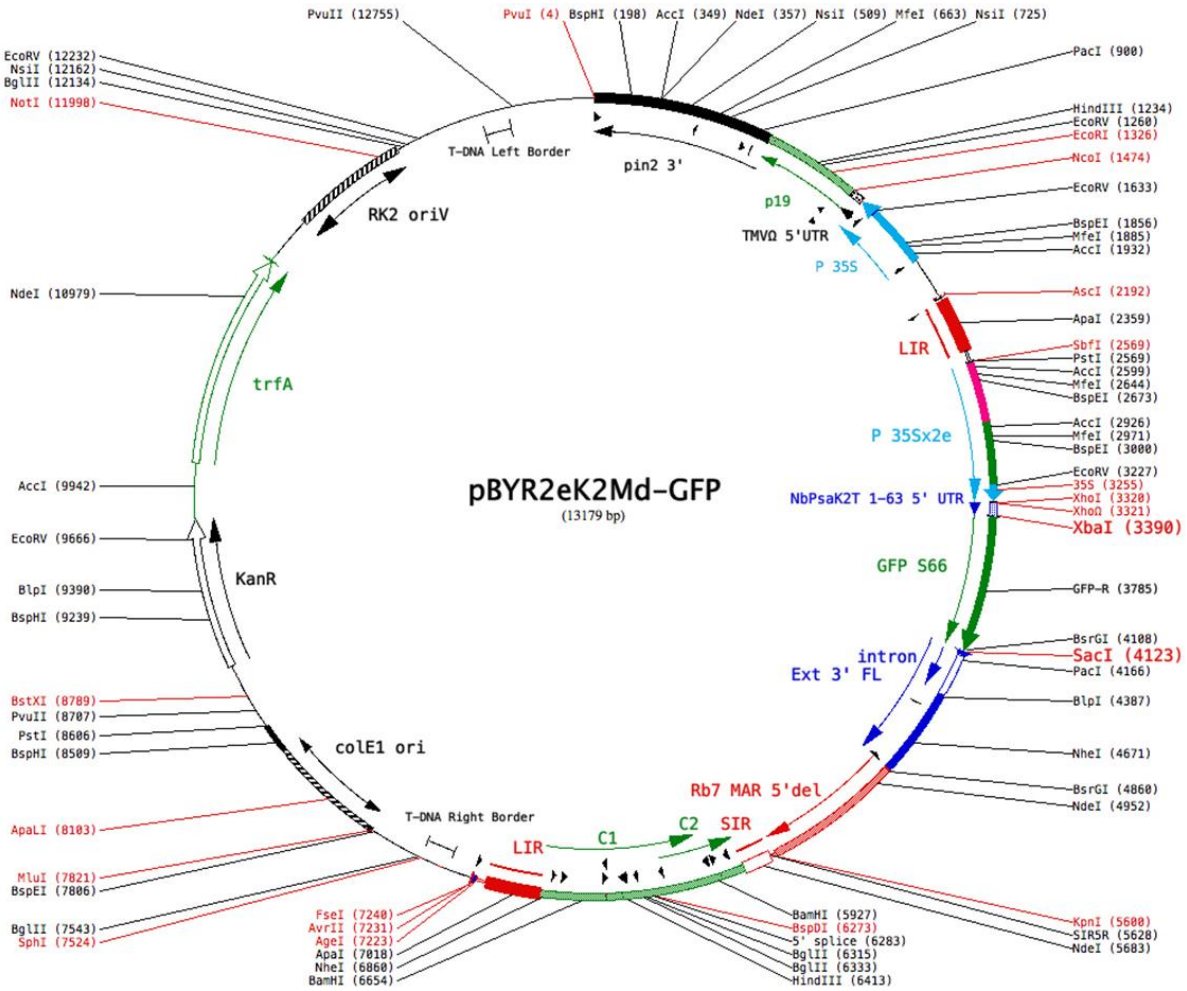
4. Synthesized Sequence of human Anti-IL-6R mAb Light chain

```
TCT AGA ATG GGC TGG TCC TGC ATC ATC CTG TTC CTT GTT GCT ACT GCT ACC GGC GTT CAC
 S   R   M   G   W   S   C   I   I   L   F   L   V   A   T   A   T   G   V   H

TCT GAT GTT CAA CTT CTC GAG GAT ATT CAG ATG ACC CAG TCT CCG TCC TCT CTG TCT GCT
 S   D   V   Q   L   L   E   D   I   Q   M   T   Q   S   P   S   S   L   S   A

TCT GTT GGT GAT AGG GTG ACC ATT ACT TGC AGG GCT TCT CAG GAC ATC AGC AGC TAC CTT
 S   V   G   D   R   V   T   I   T   C   R   A   S   Q   D   I   S   S   Y   L

AAC TGG TAT CAG CAG AAG CCT GGC AAG GCT CCT AAG CTT CTT ACC TAC TAC ACC AGC AGG
 N   W   Y   Q   Q   K   P   G   K   A   P   K   L   L   T   Y   Y   T   S   R

CTT CAC TCT GGT GTT CCG TCT AGG TTT AGC GGT TCT GGT TCT GGA ACC GAC TTC ACC TTC
 L   H   S   G   V   P   S   R   F   S   G   S   G   S   G   T   D   F   T   F

ACC ATC TCT TCA CTT CAG CCT GAG GAC ATT GCC ACC TAT TAT TGC CAG CAG GGT AAC ACC
 T   I   S   S   L   Q   P   E   D   I   A   T   Y   Y   C   Q   Q   G   N   T

CTT CCT TAC ACT TTT GGT CAG GGC ACC AAG GTC GAG ATC AAG AGA ACC GTC GCC GCC CCC
 L   P   Y   T   F   G   Q   G   T   K   V   E   I   K   R   T   V   A   A   P

TCC GTC TTC ATC TTC CCC CCC TCC GAC GAG CAG CTT AAG TCT GGA ACT GCT TCT GTT GTG
 S   V   F   I   F   P   P   S   D   E   Q   L   K   S   G   T   A   S   V   V

TGC CTT CTG AAC AAC TTC TAT CCT AGA GAA GCC AAA GTA CAG TGG AAG GTT GAC AAT GCT
 C   L   L   N   N   F   Y   P   R   E   A   K   V   Q   W   K   V   D   N   A

CTT CAA TCA GGT AAC TCC CAG GAG AGT GTC ACA GAG CAA GAT TCC AAG GAT TCC ACC TAC
 L   Q   S   G   N   S   Q   E   S   V   T   E   Q   D   S   K   D   S   T   Y

AGC CTC TCA AGT ACC TTG ACG TTG AGC AAG GCA GAC TAT GAG AAA CAC AAA GTG TAC GCA
 S   L   S   S   T   L   T   L   S   K   A   D   Y   E   K   H   K   V   Y   A

TGC GAA GTC ACT CAT CAG GGC CTG TCA TCA CCC GTG ACA AAG AGC TTC AAC AGG GGA GAG
 C   E   V   T   H   Q   G   L   S   S   P   V   T   K   S   F   N   R   G   E

TGT TCT GAG AAG GAT GAG CTT TGA GAG CTC
 C   S   E   K   D   E   L   -   E   L
```

5. Signal peptide

```
ATG GGC TGG TCC TGC ATC ATC CTG TTC CTT GTT GCT ACT GCT ACC GGC GTT CAC TCT GAT
 M   G   W   S   C   I   I   L   F   L   V   A   T   A   T   G   V   H   S   D

GTT CAA CTT
 V   Q   L
```

6. pBYR2ek geminiviral vector



APPENDIX B

LAB reagents preparation

1. Buffer

1) 10% w/v Ammonium persulfate (APS)

2) Elution buffer

   0.1 M Glycine pH 2.7

3) Extraction buffer and washing buffer

   1X Phosphate-buffered saline (PBS)

4) 1X Infiltration buffer

   10 mM MES, 10mM MgSO4 pH 5.5

5) 1X Phosphate-buffered saline (PBS)

   137 nM NaCl, 2.7 nM Potassium Chloride (KCl), 1.5 mM Potassium dihydrogen phosphate (KH_2PO_4), 8.1 mM Sodium hydrogen phosphate (Na_2HPO_4) pH 7.4

6) 1X Phosphate-buffered saline-Tween (PBS-T)

   1X PBS, 0.05% Tween 20

7) 1X Running buffer

   192 mM Glycine, 1% SDS, 25 mM Tris

8) 1X Transfer buffer

   192 mM Glycine, 15% Methanol, 25 mM Tris

9) Neutralization buffer

   1.5 M Tris-HCl pH 8.8

10) Z-buffer non-reducing dye

    0.001% Bromophenol blue, 10% Glycerol, 12% Sodium Dodecyl Sulphate, 1.5 Tris HCl pH 6.8

11) Z-buffer reducing dye

    22% Beta-mercaptoethanol, 0.001% Bromophenol blue, 10% Glycerol, 12% Sodium Dodecyl Sulphate, 125 mM Tris HCl pH 6.8

2. Media

1) Luria Bertani Agar

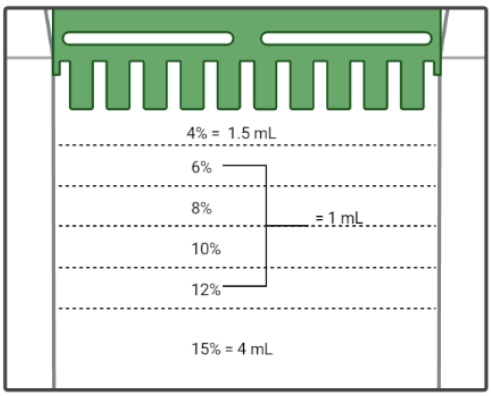
1.5% Agar, 1% NaCl, 1% Peptone, 0.5% Yeast extract

2) Luria Bertani Broth

1% NaCl, 1% Peptone, 0.5% Yeast extract

3. Polyacrylamide gel preparation

Ingredients	Total volume (10 mL)					
	4%	**6%**	**8%**	**10%**	**12%**	**15%**
H_2O	3.22	5.800	5.280	4.820	4.300	3.550
40% Acrylamide mix	0.980	1.500	2.020	2.480	3.000	3.750
1.5 M Tris-HCL (pH 8.8)	2.500	2.500	2.500	2.500	2.500	2.500
10% SDS	0.100	0.100	0.100	0.100	0.100	0.100
10% APS	0.100	0.100	0.100	0.100	0.100	0.100
TEMED	0.008	0.008	0.006	0.004	0.004	0.004



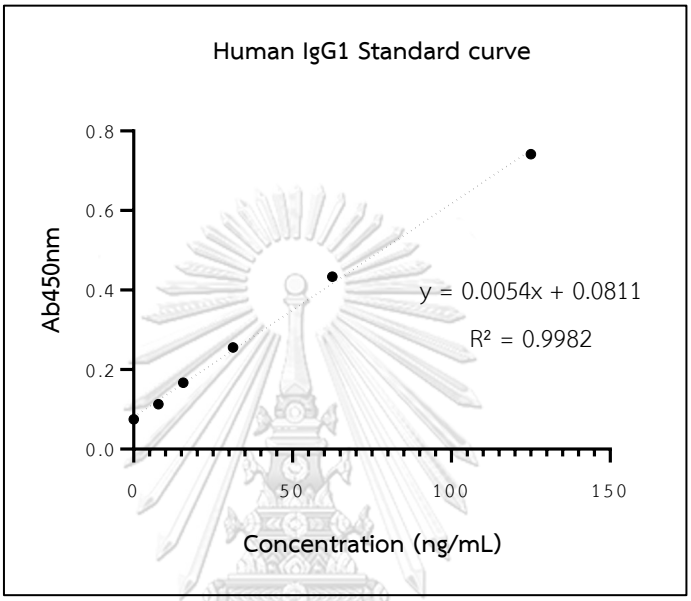
Volume each % polyacrylamide gel for 1 Gradient gel

APPENDIX C

Expression level of anti-IL-6R mAb, quantified by Sandwich ELISA.

1. HC to LC ratio optimization

Human IgG1 Standard curve for HC to LC ratio optimization

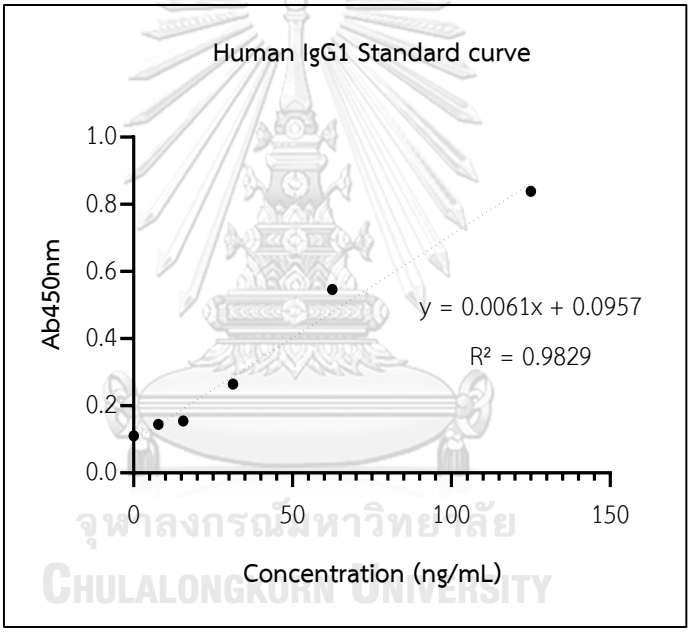


Standard human IgG1 concentration (ng/ml)	Ab450		
	Rep 1	Rep 2	Average
0	0.072	0.078	0.075
7.8125	0.116	0.11	0.113
15.625	0.166	0.168	0.167
31.25	0.261	0.25	0.2555
62.5	0.446	0.421	0.4335
125	0.756	0.728	0.742

Agrobacterium ratio (HC : LC)	Plant-produced Anti-IL-6R mAb concentration in µg/g Fresh weight				
	Rep1	Rep2	Rep3	Rep4	Average
1 : 1	2097.222	1786.881	105.757	15.206	1001.267
1 : 2	644.413	386.106	274.735	57.146	340.599
2 : 1	201.954	173.057	366.126	147.677	222.203

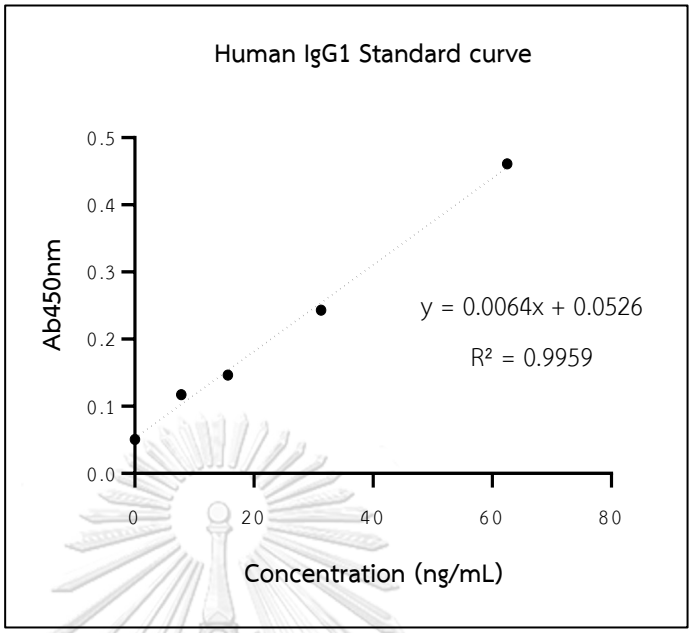
2. ODs and dpi optimization

- Human IgG1 Standard curve (for 1 dpi to 3 dpi)



Human IgG1 Standard concentration (ng/mL)	Ab450		
	Rep 1	Rep 2	Average
0	0.136	0.085	0.1105
7.8125	0.167	0.122	0.1445
15.625	0.16	0.149	0.1545
31.25	0.294	0.235	0.2645
62.5	0.576	0.517	0.5465
125	0.902	0.775	0.8385

- Human IgG1 Standard curve (for 5 dpi to 9 dpi)



Standard human IgG1 concentration (ng/ml)	Ab450		
	Rep 1	Rep 2	Average
0	0.054	0.047	0.0505
7.8125	0.116	0.119	0.1175
15.625	0.154	0.139	0.1465
31.25	0.245	0.241	0.243
62.5	0.462	0.46	0.461

Days-post infiltration (dpi)	*Agrobacterium* concentration (OD600)	Plant-produced Anti-IL-6R mAb concentration in µg/g Fresh weight				
		Rep1	Rep2	Rep3	Rep4	Average
1	0.2	10.189	1.967	-0.682	2.367	3.460
	0.4	-0.909	-2.213	-0.065	0.502	-0.671
	0.6	13.631	-0.587	0.494	-0.855	3.171
	0.8	-2.267	-0.287	-1.220	1.798	-0.494
3	0.2	387.013	254.494	248.916	319.218	302.410
	0.4	199.946	222.295	160.126	272.341	213.677
	0.6	311.149	253.650	211.776	330.357	276.733
	0.8	319.858	131.380	186.588	291.489	232.328
5	0.2	1208.768	1099.049	998.055	918.577	1056.112
	0.4	1017.715	832.810	456.236	1016.393	830.789
	0.6	395.774	342.120	281.781	424.895	361.143
	0.8	326.700	353.043	363.142	358.885	350.443
7	0.2	191.928	297.753	157.573	213.204	215.114
	0.4	143.913	202.132	50.648	146.952	135.911
	0.6	88.920	55.324	20.056	36.089	50.097
	0.8	178.439	64.135	1.796	2.045	61.604
9	0.2	92.193	152.237	97.335	234.478	144.061
	0.4	81.276	95.828	78.565	73.112	82.195
	0.6	57.130	147.058	86.712	192.030	120.732
	0.8	15.267	52.386	130.871	36.919	58.861

APPENDIX D

Determination of binding capacity of plant-produced Anti-IL-6R mAb quantified by indirect ELISA.

Concentration	Ab450 of Plant-produced Anti-IL-6R mAb				
(µg/mL)	Rep1	Rep2	Rep3	Average	STDVA
0.125	0.489	0.412	0.364	0.4217	0.0631
0.25	0.581	0.403	0.43	0.4713	0.0959
0.5	0.778	0.646	0.644	0.6893	0.0768
1	0.997	0.904	0.912	0.9377	0.0515
2	1.608	1.448	1.249	1.4350	0.1799
4	1.996	1.963	1.862	1.9403	0.0698
8	2.339	2.263	2.225	2.2757	0.0580
16	2.649	2.545	2.611	2.6017	0.052

Concentration	Ab450 of Plant-produced H4 mAb				
(µg/mL)	Rep1	Rep2	Rep3	Average	STDVA
0.125	0.212	0.237	0.249	0.233	0.019
0.25	0.215	0.246	0.304	0.255	0.045
0.5	0.266	0.283	0.252	0.267	0.016
1	0.211	0.247	0.201	0.220	0.024
2	0.24	0.256	0.252	0.249	0.008
4	0.254	0.233	0.283	0.257	0.025
8	0.282	0.33	0.29	0.301	0.026
16	0.283	0.29	0.318	0.297	0.019

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