DEVELOPMENT OF RECOMBINANT HUMAN EPIDERMAL GROWTH FACTOR PRODUCTION IN *NICOTIANA BENTHAMIANA*

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การพัฒนาการผลิตรีคอมบิแนนท์อีพิเดอร์มอลโกรทแฟคเตอร์ของมนุษย์ในต้น *Nicotiana benthamiana*

น.ส.อรณิชา หรรษ์อิทธินันต์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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อรณิชา หรรษ์อิทธินันต์ : การพัฒนาการผลิตรีคอมบิแนนท์อีพิเดอร์มอลโกรทแฟคเตอร์ของมนุษย์ในต้น *Nicotiana benthamiana*. (DEVELOPMENT OF RECOMBINANT HUMAN EPIDERMAL GROWTH FACTOR PRODUCTION IN *NICOTIANA BENTHAMIANA*) อ.ที่ปรึกษาหลัก : รศ. ดร.วรัญญู พูลเจริญ

อีพิเดอร์มอลโกรทแฟคเตอร์ของมนุษย์ (hEGF) เป็นโพลิเปปไทด์สายสั้นที่ได้รับความสำคัญในการใช้ประโยชน์ทางคลินิกในช่วงทษวรรษที่ผ่านในด้านการสมานแผล ในปัจจุบันระบบการผลิตโปรตีนด้วยพืชเป็นระบบทางเลือกที่มีต้นทุนต่ำในการผลิตรีคอมบิแนนท์โปรตีน ดังนั้นในการศึกษานี้มีวัตถุประสงค์ที่จะพัฒนาการผลิต hEGF ด้วยต้นยาสูบ *Nicotiana benthamiana* ด้วยวิธีการแสดงออกแบบชั่วคราวด้วยเจมมิไนไวรัลเวคเตอร์ โดยโครงสร้างของยีน hEGF ได้ออกแบบมาทั้งหมด 6 แบบ พบว่าการแสดงออกของโปรตีน hEGF ที่ดีที่สุดนั้นจากโปรตีนที่มีเป้าหมายที่เอนโดพลาสมิก เรติคูลัม และมีตำแหน่งของหมู่ฮิสทิดีนฝั่งปลายซี โดยได้ผลผลิตโปรตีนถึง 15.695 ไมโครกรัมต่อกรัมน้ำหนักใบพืช หรือ 0.499% ของปริมาณโปรตีนทั้งหมด จากนั้นใช้ทำให้ hEGF จากพืชบริสุทธิ์ด้วยเทคนิคโครมาโทกราฟีแบบจำเพาะกับนิกเกิ้ลลิแกนด์และยืนยันเอกลักษณ์โปรตีนด้วยวิธี SDS-PAGE และ Western blot ที่จับด้วยแอนติบอดีที่จำเพาะต่อส่วน hEGF นอกจากนี้ผลจากการศึกษาเบื้องต้นในการเพิ่มประสิทธิภาพของการทำให้โปรตีนบริสุทธิ์ด้วยการสกัดวิธีต่างๆ พบว่าการสกัดโปรตีนด้วยบัฟเฟอร์ปริมาตรมากสามารถช่วยให้จับกับ hEGF จากสารสกัดได้ และสารละลายบัฟเฟอร์ที่ pH 4 สามารถกำจัดโปรตีนอื่นจากเซลล์พืชโดยเฉพาะโปรตีนรูบิสโก ออกจากสารสกัดได้ดีกว่า อย่างไรก็ตามการตกตะกอนโปรตีนด้วยเกลือแอมโมเนียมซัลเฟตนั้นไม่เหมาะสมในการกำจัดโปรตีนอื่นจากเซลล์พืชออกจาก hEGF นอกจากนั้นการศึกษานี้ยังพบว่ารีคอมบิแนนท์โปรตีน hEGF ที่ผลิตจากพืชนั้นไม่ส่งผลความเป็นพิษต่อเซลล์ HaCaT ซึ่งมีผลเทียบเท่ากับผลการศึกษาจากโปรตีน hEGF ที่ผลิตเพื่อเชิงพาณิชย์ ดังนั้นในการศึกษานี้จึงเป็นการสนับสนุนว่าระบบการผลิตโปรตีนจากพืชนั้นเป็นระบบที่สามาถทำได้ง่าย และมีความคุ้มทุนในการผลิต hEGF ในการผลิตระดับอุตสาหกรรม

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Human epidermal growth factor (hEGF) is a short polypeptide that has gained clinical importance in the recent decade for wound healing. Currently, plant-based expression system is considered as an alternative viable platform for low-cost recombinant protein production. Hence, this study aims to produce hEGF in *Nicotiana benthamiana* plants *via.,* transient expression using a geminiviral vector. The hEGF gene constructs were designed into six different constructs. The optimal expression was observed from the construct targeting the endoplasmic reticulum with C-terminal histidine tag at the yield level up to 15.695 μg/g LFW or 0.499% TSP. The plant-produced hEGF was purified by using Nickel affinity chromatography and confirmed its identity by SDS-PAGE and Western blot probed with anti-hEGF antibody. Furthermore, the preliminary study of the protein purification efficiency was found that the high extraction volume allows better hEGF recovery and extraction buffer pH 4 could largely remove host cell proteins (HCP), especially RuBisCO. However, ammonium sulfate precipitation is inapplicable for removing HCP from plant-produced hEGF. Furthermore, the study showed that no cytotoxic effects have been found in HaCaT cells from the plant-produced hEGF which is equivalent to commercial hEGF in HaCaT cells. Hence, this study supports that the potential of plant expression system offers a simple and cost-effective approach for the industrial-scale production of recombinant hEGF.

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Oranicha Hanittinan

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LIST OF ABBREVIATIONS

%	= Percentage
A, T, C, G	= Nucleotide containing the base adenine, thymine, cytosine, and guanine, respectively
bp	= Base pair
BSA	= Bovine serum albumin
cDNA	= Complementary deoxyribonucleic acid
DFU	= Diabetic foot ulcer
DMSO	= Dimethysulfoxide
dNTPs	= Deoxyribonucleotide triphosphates (dATP, dTTP, dGTP, dCTP)
dpi	= Day post-infiltration
ER	= Endoplasmic reticulum
g	= Gram(s)
H, His tag	= Histidine tag
hr	= Hour(s)
H_2O	= Water
hEGF	= Human epidermal growth factor
HRP	= Horseradish peroxidase
kb	= kilobase
kDa	= Kilodalton
L	= Liter(s)
M	= protein marker
min	= Minute(s)
mL	= Milliliter
MTT	= Methylthiazolydiphenyl-tetrazolium bromide
ng	= Nanogram
Ni-NTA	= Nickel-nitrilotriacetic

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OD	= Optical density
PBS	= phosphate buffer saline
PCR	= Polymerase chain reaction
PVDF	= Polyvinylidene difluride
rpm	= Revolution per minute
TAE buffer	= Tris-acetate and EDTA buffer
TSP	= Total soluble protein
SEKDEL	= Ser-Glu-Lys-Asp-Gly-Leu
SD	= Standard deviation
SP	= Signal peptide
μg	= Microgram(s)

Amino acid abbreviations

A / Ala	= Alanine	N / Asn	= Asparagine
C / Cys	= Cysteine	P / Pro	= Proline
D / Asp	= Aspartic acid	Q / Gln	= Glutamine
E / Glu	= Glutamic acid	R / Arg	= Arginine
F / Phe	= Phynylalanine	S / Ser	= Serine
G / Gly	= Glycine	T / Thr	= Threonine
H / His	= Histidine	V / Val	= Valine
I / Ile	= Isoleucine	W / Trp	= Tryptophan
K / Lys	= Lysine	Y / Tyr	= Tyrosine
M / Met	= Methionine		

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CHAPTER 1
INTRODUCTION

1.1 Rationale and significant

Wound healing is a complex process that is collaborated with many cell strains and regulated by numerous signaling molecules including growth factors, cytokines, etc. (Steed, 1997, Barrientos et al., 2008). Generally, many cell strains (such as platelets, keratinocytes, microvascular cells, fibroblasts, etc.) and growth factors are up-regulated in response to tissue injury for the reconstruction of skin integrity. To cure skin wounds in a timely fashion, the healing processes are completed with the help of all parameters (Demidova-Rice et al., 2012).

In some cases, a skin wound might fail to heal in a timely manner leading to chronic wounds and scarring due to pathophysiological factors including inflammation, malnutrition, age, diabetes, overweight, obesity, pressure necrosis, etc (Park et al., 2017). Chronic wounds often remain for years and recur in up to 60-70% of patients (Frykberg and Banks, 2015). Those underlying conditions of chronic wounds can become a significant cause of public health problem and economic burden on patients in terms of productivity losses, medical care costs, and morbidity (Sen, 2019, Olsson et al., 2019) (Frykberg and Banks, 2015). In the United States, the wound repair expenses in the health care system are up to $25 US billion-dollar annually (Olsson et al., 2019). Apart from those factors causing chronic wounds, the presence of growth factors has been reported a deficiency in chronic wound environment compared with acute wounds, which could interrupt the progression of normal wound healing process to further steps (Park et al., 2017, Braund et al., 2007). Hence, wound healing therapy with the administration of exogenous growth factors becomes a promising therapeutic intervention to boost healing processes in a timely fashion which could improve

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the medical costs and also lead to the reduction of the risk of infection (Wong et al., 2019).

To date, on-going developments in biopharmaceutical technology have been considerably explored on recombinant growth factor production. Several growth factors have been exposed a great therapeutic potential in wound management including platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), transforming growth factor-beta (TGF-β) and others (Steed, 1997).

Human EGF (hEGF) is one of the growth factors that have been extensively characterized (Barrientos et al., 2008). hEGF has various biological activities on cell proliferation in an epidermal cell, fibroblast and keratinocyte (Cohen and Elliott, 1963, Barrandon and Green, 1987, Lembach, 1976) leading to clinical applications as a therapeutic agent for wound healing (Brown et al., 1989, Brown et al., 1991) and gastrointestinal diseases (Guglietta et al., 1995). hEGF was firstly discovered from adult mice (Brown et al., 1991, Cohen, 1962) and detected in a variety of body fluids, such as milk, urine, saliva, amniotic fluid, plasma, etc. (George-Nascimento et al., 1988) (Carpenter and Cohen, 1979). However, extracting natural growth factors from living animals or humans remains challenging due to complicated purification procedures with lack purity which could not be applied for industries (Starkey et al., 1975). As a result of numerous hEGF applications, many researchers have been attempted to develop new approaches for the replacement of conventional hEGF production platforms.

In the past century, recombinant DNA technology provides a feasible approach for the development of new biopharmaceutical products as well as achievement in commercial approvals (Khan et al., 2016). Previously, hEGF had been expressed in many platforms such as *Escherichia coli* (*E.coli*) (Oka et al., 1985) (Sivakesava et al., 1999), *Bacillus brevis* (Yamagata et al., 1989),

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Saccharomyces cerevisiae (George-Nascimento et al., 1988, Topczewska and Bolewska, 1993), *Pichia pastoris* (Mohammadian et al., 2013, Eissazadeh et al., 2017) and baculovirus (Yu et al., 2006), etc.

Aside from those platforms, plant molecular farming (PMF) have emerged as a novel branch in the biopharmaceutical industry providing cost-effective technology, inherent absence of human pathogen contamination (Fischer et al., 2004). In particular, plants can introduce post-translational modifications that have an effect on proper folding, assembly and functionality in many valuable agents such as vaccines, hormones, cytokines, enzymes, and antibodies (Obembe et al., 2011).

In the present study, *Nicotiana benthamiana* or tobacco plant was chosen as the hEGF expression system due to non-food crops excluded from the food chain and also providing high biomass in a leafy plant (Fischer et al., 2004). Previously, Higo et al. (1993) reported that expression of hEGF in tobacco plants was shown that the hEGF accumulation level was only 0.001% of total soluble protein (TSP) (Higo et al., 1993). Wirth et al. (2004) tried to improve the expression levels using different genetic constructs to allow accumulation of either the cytoplasm or the apoplast. The hEGF content was expressed up to 0.11% of TSP when the protein was targeted to the apoplastic space. It was exhibited that extracellular localization affects in protein yield increases (Wirth et al., 2004). Besides, Bai JY et al. (2007) attempted to improve the expression level in tobacco plant through usage of optimized codon, the addition of the C-terminal endoplasmic reticulum (ER) retention signal sequence, namely Lys-Asp-Glu-Leu (KDEL) and harboring matrix attachment regions (MARs) at both ends of the hEGF transcriptional unit. The level of hEGF was shown up to 0.3% of the TSP (Bai et al., 2007).

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In this study, we aimed to optimize the hEGF expression *via.*, a geminiviral-based vector system with transient expression in *N. benthamiana* through various strategies including the usage of different gene constructs carrying N- or C-terminal Histidine tag (His tag) location and also different protein target in the plant cell. Additionally, plant-produced hEGF was investigated the cytotoxicity activity compared with commercial hEGF using a cell-based assay.

1.2 Growth factors in Wound healing

Growth factors are polypeptides that control cellular functions including cell growth, differentiation, and metabolism. It can interact specifically with cell surface receptors which evoke intracellular signaling cascades and result in altered gene expression. Growth factors are of particular importance in both normal and abnormal physiological processes such as wound healing, diabetic foot ulcer, respectively (Stone and Varacello, 2020).

Wound healing is a complex biological process which can be divided into the following stages: 1.) Hemostasis and inflammation phase, 2.) Proliferative phase, 3.) Remodeling phase.

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1.) Hemostasis and inflammation phase

The first phase of hemostasis immediately begins after skin damage with vascular constriction and fibrin clot formation that prevents excessive bleeding and protects the wound area (Demidova-Rice et al., 2012). Following skin damage, activated platelets release several growth factors including EGF, hepatin-binding EGF-like growth factor (HB-EGF), PDGF, insulin-like growth factor 1 (IGF-1), transforming growth factor (TGF)-α and β. These growth factors play as a chemotactic agent to recruit neutrophils and monocytes into the wound (Park et al., 2017). Neutrophils clean the wound area by engulfing

bacteria and damaged tissue and also release proteases (such as the matrix metalloproteinases (MMP), elastase and collagenase) to debride the wound by removing damaged extracellular cellular matrix (ECM) components (Bennett and Schultz, 1993). The monocytes become macrophage which phagocytoses bacteria and releases many cytokines and growth factors including interleukin-1 (IL-1), basic fibroblast growth factor (bFGF), PDGF, TGF-α, TGF-β. For uncomplicated wounds, this inflammatory phase lasts 1-2 days (Braund et al., 2007).

2.) Proliferation phase

Growth factors, secreted from macrophage in previous stage, act as the activator of several skin cells such as fibroblast, epithelial cell and vascular endothelial cells to migrate into the wound area (Bennett and Schultz, 1993). The formation of granulation tissue including fibroblast, macrophage, neovasculature in a loose matrix of fibronectin, collagen and hyaluronic acid starts to fill the wound area (Park et al., 2017).

Fibroblast migrates and proliferates into the granulation tissue. In addition, fibroblast produces collagen and glycosaminoglycans, which are major components of the EC, including MMPs to eliminate the fiber which do not contribute to the structural integrity of the wound. Then, fibroblasts are converted into myofibroblasts to enhance collagen deposition and initiate wound contraction.

During re-epithelialization process, keratinocyte becomes activated and migrates through the fibrin and the ECM to encase the wound gap (Cañedo-Dorantes and Cañedo-Ayala, 2019). Growth factors, that are important during epithelialization such as EGF, TGF-α, have been reported to enhance the rate of reepithelialisation (Braund et al., 2007).

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3.) Remodeling phase

In this last phase of wound healing, the stage aim to restore the tensile strength by reorganization, degradation and reconstruction of the ECM. Granulation tissue with a higher type III collagen is replaced with a type I collagen and the tissue is reorganized the structure to increase the mechanical strength of the tissue (Gonzalez et al., 2016) (Park et al., 2017).

1.3 Epidermal growth factor (EGF)

EGF is one amidst the numerous well-characterized growth factors in the wound healing process. It is a single-chain polypeptide containing 53-amino acids which was first identified from the submaxillary glands of adult mice (Cohen, 1962). The protein structure is folded into three loops through the interaction of three intrachain disulfide bonds between six cysteine residues (Cys 6-20, Cys 14-31 and Cys 33-42) which are crucial for the biological activities (Bennett and Schultz, 1993, Carpenter and Cohen, 1979).

Cohen and Carpenter (1975) reported that isolated hEGF from human urine was shown its biological activity at least similar to mouse EGF in the growth stimulation of the cultured human foreskin fibroblasts and corneal epithelial cells from the chick embryo in organ culture as well as the *in vivo* induction of precocious eyelid opening in newborn mice. However, the physical and chemical properties of human EGF (hEGF) are different from mouse EGF (mEGF) (Cohen and Carpenter, 1975). 37 out of 53 amino acid residues are identical between mEGF and hEGF. Comparison of the amino acid sequences between mEGF and hEGF are shown as depicted below (Das, 1982):-

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NH_2-Asn-Ser-Tyr(Asp)-Pro(Ser)-Gly(G1u)-Cys-Pro-Ser(Leu)-Ser-Tyr(His)^{10}-Asp-Gly-Tyr-Cys-Leu-Asn(His)-Gly(Asp)-Gly-Val-Cys^{20}-Met-His(Tyr)-Ile-Glu-Ser(A1a)-Leu-Asp-Ser(Lys)-Tyr-Thr(Ala)^{30}-Cys-Asn-Cys-Val-Ile(Val)-Gly-Tyr-Ser(Ile)-Gly-Asp(Glu)^{40}-Arg-Cys-Gln-Thr(Tyr)-Arg-Asp-Leu-Arg(Lys)-Trp-Trp^{50}-Glu-Leu-Arg

*Remark : The name of amino acid within the patentheses stand for the amino acid derived from hEGF (Das, 1982). The amino acid sequences of hEGF are shown in Figure 1.

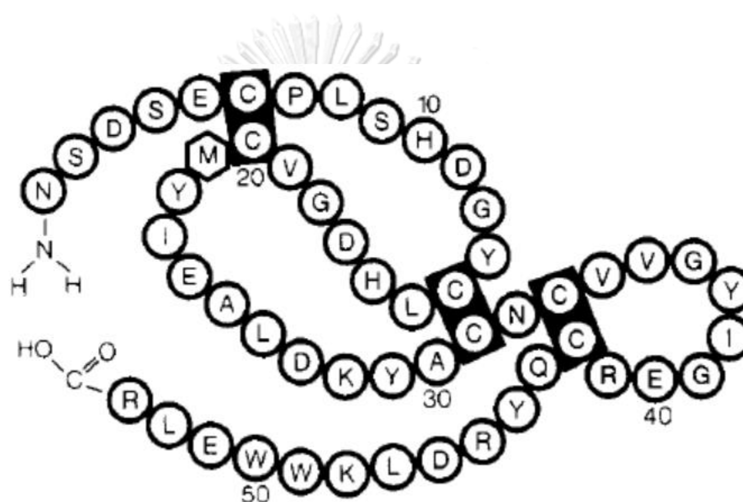

Figure 1. The amino acid sequences of human epidermal growth factor (hEGF) with location of disulfide bond (George-Nascimento et al., 1988)

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1.3.1 Mechanism of action derived from EGF

EGF is a member of the epidermal growth factor receptor (EGFR) ligands, which comprises of seven ligands including EGF, transforming growth factor-alpha (TGF-α), heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC), amphiregulin (AREG), epiregulin (EREG), and epigen (EPGN) (Singh et al., 2016).

The biological activities of EGF rely on the interaction between EGF with its receptor called epidermal growth factor receptor. This receptor is a member of four ErBb family receptors, namely ErbB1 (also classified as EGFR), ErbB2,

ErbB3, and ErbB4 (Pastore et al., 2008). The component of this receptor comprises of three major regions as shown in Figure 2:- (Wee and Wang, 2017)

1.) **An extracellular domain** : composed of 621 amino acids containing two cysteine-rich domain which participate in ligand binding.
2.) **A hydrophobic transmembrane domain** : composed of 23 amino acids which play an important role in receptor dimerization.
3.) **An intracellular domain** : composed of 542 amino acids containing the tyrosine-specific protein kinase domain.

Intracellular signaling pathways are involved in three major steps : a.) the interaction between a receptor-specific ligand with EGFR in the extracellular domain, b.) receptor dimerization of active EGFR-EGFR activates phosphorylation of the intracellular domain or tyrosine kinase, c.) initiation of several intracellular signaling cascades (Bennett and Schultz, 1993). There are two major intracellular pathways activated by EGFR including (Berlanga-Acosta et al., 2009) : (1) RAS-RAF-MEK-MAPK pathway which controls cell-cycle progression from the G1 phase to S phase and cell proliferation, (2) PI3K-Akt pathway which activates a cascade of antiapoptotic and cytoprotective signals.

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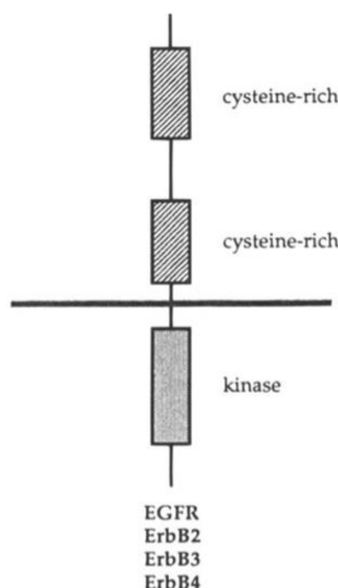

Figure 2. Composition of epidermal growth factor receptor (EGFR) (Wee and Wang, 2017)

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1.3.2 Biological activities of EGF

Biological activities derived from EGF have been extensively studied in a wide range of aspects. According to the study of Cohen S. and Elliott GA., 1962, they firstly reported that the biological activity of mEGF, isolated from submaxillary gland of the male mouse, was able to stimulate the epidermal proliferation and keratinization (Cohen and Elliott, 1963). EGF also promoted the effect on cultured human fibroblasts through stimulation of DNA replication and cell proliferation in cultures (Lembach, 1976). In addition, EGF is active in the induction of a chemotactic response in an endothelial cell (Grotendorst et al., 1989). Since those cells are the major cell strains associated with wound healing process, it is suggested that EGF play vital roles in wound repair (Ziegler et al., 2012).

There are three stages involved in wound healing processes including the phase of hemeostasis/inflammation, proliferation and remodeling. During the initial stage of normal wound repair, blot clotting and platelet degranulation are

the first major process that occurs at injured tissues and initiate wound healing process. Growth factors, especially EGF, are extensively released from platelets and diffused from the injured area to the surrounding system. EGF plays a vital role in epithelial cell migration and increases the number of fibroblasts in the wound. Moreover, it has been reported that EGF directly increase the rate of reepithelialisation and the tensile strength in wounds (Braund et al., 2007) (Zeng and Harris, 2014). In conclusion, EGF is an important growth factor emerging in the injured area at the earliest time point and encouraging initiation of the wound healing cascades in normal wound healing (Ziegler et al., 2012).

There are numerous experimental studies evaluating the biological activities of EGF in wound healing therapy using *in vivo* study model including:-

1.) Epidermal regeneration of porcine epidermal wounds and partial-thickness burns (Brown et al., 1986)
2.) Acceleration of epidermal wound healing in alkali-burned corneas of rabbits (Singh and Foster, 1987)
3.) Reduction of inflammatory effect induced by an inflammation-inducing irritant (12-O-tetradecanoylphorbol-13-acetate, croton oil, and arachidonic acid) on the inner ear surface of female OF1 mice (Casaco et al., 1999).

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However, some patients might face aberrant wound healing due to predisposing factors, such as inflammation, malnutrition, age, diabetes, overweight, obesity, and pressure necrosis, etc. After the occurrence of tissue injury, the inflammatory events are followed with the recruitment of inflammatory cells (such as neutrophils, macrophages) to remove bacterial and release of proteases to clear damaged extracellular matrix components (EMC). In the case of chronic wound, it has a recurrent inflammation which prolongs proinflammatory cytokine cascade and enhances levels of proteases contributing to a deficiency of growth factors which interupts in the healing phase.

The exogenous application of growth factors has been recommended since the growth factors might reciprocate the wound destruction and let the wound healing properly (Park et al., 2017) (Wong et al., 2019).

In clinical studies, hEGF has been developed as a therapeutic agent for wound repair by accelerating the healing rate of partial thickness skin wounds in humans (Brown et al., 1989), and stimulating the healing of chronic wounds in patients who had failed to cure with conventional interventions (Brown et al., 1991).

EGF-containing products have been developed into several dosage forms such as topical gel (Mohan, 2007), topical spray (Park et al., 2018), and gelatin film dressings with EGF (Tanaka et al., 2005), etc. Several companies have also launched products containing EGF indicated for treatment of chronic wounds, especially chronic diabetic foot ulcers (DFU) (Mohan, 2007).

To date, EGF shows not only great potentials in wound healing effect but also promising effects in the treatment of gastrointestinal diseases such as gastrointestinal ulceration, necrotizing enterocolitis, Zollinger-Eliison syndrome, and congenital microvillus atrophy (Guglietta et al., 1995). The biological effects of the epidermal growth factor are summarized in Table 1.

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Table 1. Biological effects of epidermal growth factor (EGF)

Biological effects	References
In vitro	
Stimulation of the epidermal proliferation and keratinization	(Cohen and Elliott, 1963)
Induction of human fibroblast proliferation	(Lembach, 1976)
Promotion of *in vitro* keratinocyte growth	(Barrandon and Green, 1987)
Potent chemoattractant for a rat heart vascular endothelial cell line (RHEC)	(Grotendorst et al., 1989)
In vivo	
Epidermal regeneration of porcine epidermal wounds and partial-thickness burns	(Brown et al., 1986)
Acceleration of epidermal wound healing in alkali-burned corneas of rabbits	(Singh and Foster, 1987)
Reduction of inflammatory effect induced by an inflammation-inducing irritant on the inner ear surface of female OF1 mice	(Casaco et al., 1999)
Clinical effects	
Acceleration of partial thickness skin wound healing in humans	(Brown et al., 1989)
Therapeutic effects in gastrointestinal diseases	(Brown et al., 1991)
Stimulation of chronic wound healing in patients who had failed with the conventional interventions.	(Guglietta et al., 1995)
Effect on healing of diabetic foot ulcers	(Mohan, 2007)
Reduction of severe oral mucositis in patients undergoing radiotherapy for head and neck cancer	(Wu et al., 2009)

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1.4 Recombinant protein expression systems - human epidermal growth factor

EGF has been detected in a variety of body fluids, such as milk, saliva, urine, plasma, and others (Zeng and Harris, 2014). At first, Starkey RH, Cohen S, and Orth DN. (1975) reported that endogenous EGF was derived from human urine containing similar effects with mEGF even the structures are slightly different. However, extracting the growth factors from living organisms remains a great challenge due to a complicated procedure and an insufficient amount (Starkey et al., 1975). Therefore, it is a great opportunity to apply new strategies of large-scale recombinant protein production aiding in the development of further EGF application since it has showed a range of actual and potential uses which has been creating a great demand for applying in research, clinical practice, and industries.

To date, recombinant DNA technology has been extensively growing in the biopharmaceutical industry in terms of production and product approval of several recombinant proteins (Paul and Ma, 2011). Human EGF had been produced in various traditional platforms, including *E.coli* (Oka et al., 1985) (Sivakesava et al., 1999), *B. brevis* (Yamagata et al., 1989), , *S. cerevisiae* (George-Nascimento et al., 1988, Topczewska and Bolewska, 1993), *P. pastoris* (Mohammadian et al., 2013, Eissazadeh et al., 2017), and a mammalian host system *via*., an adenoviral vector (Negahdari et al., 2016), etc.

However, several limitations of these systems should be concerned for recombinant protein production. For instance, *E.coli* is the most employed microorganism for the expression of heterologous proteins offering fast growth, high product yield, cost-effectiveness, and high scalability. However, post-translational modification influencing biological activity does not occur in this system (Baeshen et al., 2015), whereas recombinant proteins produced from

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E.coli sometimes result in aberrant disulfide bonds formation, which have an impact on protein stability (Berkmen, 2012).

In the case of the eukaryotic expression system, yeast is applied a model organism for biopharmaceutical production. Yeast system as *P. pastoris* is able to secrete high content of properly folded proteins. Nevertheless, glycosylation step in yeast system produces a high expression of mannose glycan structures, which may diminish serum half-life and induce allergic reactions in humans (Ahmad et al., 2014).

1.5 Plant Molecular Pharming (PMF)

1.5.1 Plant-based production of biopharmaceuticals

Plants are readily responsible as a recombinant protein expression platform for the large-scale production (Fischer et al., 2004). Many studies have shown the ability of plant systems to produce complex recombinant proteins with a variety of therapeutic functions including hormones, cytokines, enzymes, nutraceuticals, antibodies, and vaccines (Obembe et al., 2011).

Unlike traditional expression system, key advantages of a plant-based expression system for recombinant protein production are inexpensive manufacturing cost, absence of endotoxin or human pathogens (Warzecha, 2008, Fischer et al., 2004) and the ability of plant system to perform post-translational modifications (PTMs), which make the recombinant proteins to assemble with proper folding and retain the structural and functional integrity (Obembe et al., 2011).

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Apart from these advantages, there are several limitations including low protein yields, poor protein stability, difficulties in downstream processing, and the presence of non-authentic glycan structures (Fischer et al., 2004). A regulatory framework for PMF is one of the major burdens for plants to compete the

existing systems in terms of biosafety concerns, product registration and current good manufacturing practice (cGMP), etc. Hence, it is essential that these burdens should be improved to support the potentials of plant-derived pharmaceuticals available in the market (Obembe et al., 2011).

"Elelyso (taliglucerase alfa)" is the first plant-based therapeutic enzymes which is commercialized by Protalix Biotherapeutics for the treatment of Gaucher's disease (Fox, 2012). Also, there is a small number of plant-derived pharmaceuticals approved into the market, such as HN protein of Newcastle disease virus (poultry), therapeutic human proteins gastric lipase (Merispase®), human glucocerebrosidase (prGCD), etc (Obembe et al., 2011).

1.5.2 Plant host species as bioreactors

There are a wide variety of plant species applicable are now available for recombinant protein production such as tobacco, tomato, potato, alfafa, lettuce, rice, spinach, wheat, barley, and maize, etc (Fischer et al., 2004, Warzecha, 2008).

N. benthamiana or tobacco has been widely engineered as a recombinant protein transient expression platform. Tobacco has many desirable attributes, as readily amenable to genetic engineering (Conley et al., 2011), substantial amounts of biomass, high scalability, non-food crop, well-established transformation protocols, all-year-round growth, and harvesting, and available large-scale infrastructure (Obembe et al., 2011). Transformed tobacco leaves are harvested in order to extract recombinant proteins prior to flowering, which avoiding the gene leakage into the environment through the pollen or seed dispersal. These advantages make tobacco consider as an ubiquitous platform that has been adopted by many biotechnology companies, such as Kentucky BioProcessing (KBP), Mapp Biopharmaceutical Inc., Icon Genetics GmbH, and Medicago, etc (Fischer et al., 2004).

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In this study, *N. benthamiana* is selected as an expression platform to produce the human epidermal growth factor (hEGF).

1.5.3 Agroinfiltration as a strategy for recombinant protein production in tobacco

Recombinant protein can be produced by two general approaches which are stable transgenic plants and transient expression (Chen et al., 2013). For stable transgenic plants, this technique is produced by using genetic engineering (GE) techniques (Rani and Usha, 2013) to introduce and stably integrate the transgene into either the plant nuclear or chloroplast genomes, which will address a new trait to the next generations. Genetically modified (GM) plants can be generated through an introduction of transgene *via*., *Agrobacterium tumefaciens*-mediated transformation or bombardment/biolistic method (Chen et al., 2013, Rani and Usha, 2013). Genetically modified organism (GMO) technology is useful in various fields as a result of the improvement of shelf-life, yield, quality, and pest resistance, including tolerance of heat, cold, and drought (Rani and Usha, 2013). However, this technology consumes long development time from several months to a year or more to select and generate several generations until becoming homozygous plants, and the gene is randomly inserted in the plant genome resulting in a low yield of the recombinant proteins (Yao et al., 2015). Moreover, the regulation on genetic engineering has been restricted to cultivation among Thailand since 2003 (Preechajarn, 2018).

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Alternatively, transient expression system is introduced to use routinely as a quick recombinant protein production system within a few weeks resulting in a high yield of biopharmaceutical products (Obembe et al., 2011). A foreign gene can be introduced into plant cells without stable integration in the host genome *via*., the help of *A. tumefaciens* harbouring the gene of interest (Erickson et al.,

2013). *A. tumefaciens* is a gram-negative bacteria or a plant pathogen which has the unique ability to cause crown-gall disease in plants by transferring a portion of its tumor-inducing plasmid (Ti plasmid) (as shown in **Ti plasmid of *Agrobacterium***Figure 3), called a transferred DNA (T-DNA) into plant systems (Krenek et al., 2015) which can integrate or remain as an episome.

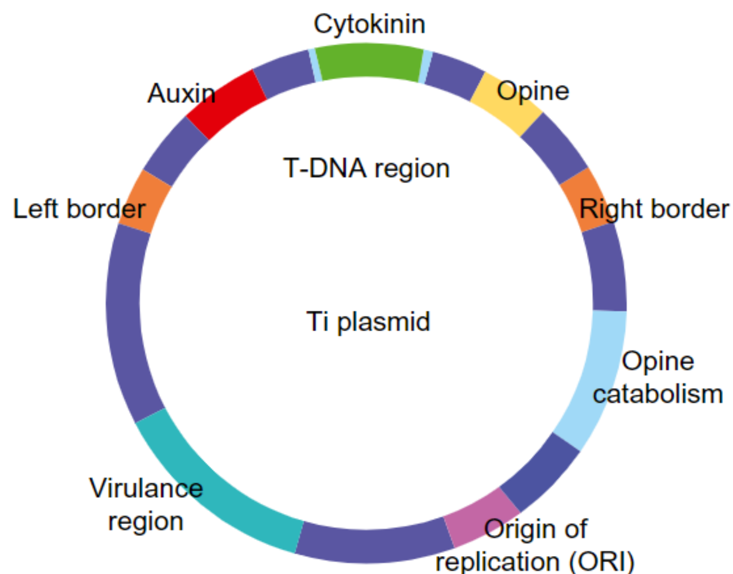

Figure 3. Ti plasmid of *Agrobacterium* (Hull et al., 2009)

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For *Agrobacterium*-mediated plant transformation, the bacterial virulence (*vir*) genes, located in the *Agrobacterium* Ti plasmid, senses plant phenolic substances that are released from wounded sites. Subsequently, *vir* proteins are produced and bind to the 5' end of the processed single-strand DNA. The T-DNA and vir proteins (including VirD2, VirE2, VirE3, VirD5 and VirF) secretes from the bacteria *via.,* a type IV secretion system (Pitzschke, 2013) (Lee and Gelvin, 2008). Since no gene sequence within T-DNA takes part in T-DNA transfer, the wild-type oncogenes and opine synthase genes has been replaced with genes of interests in recombinant *Agrobacterium* (Tzfira and Citovsky, 2006).

In this study, *A. tumefaciens* used for plant transformation is the recombinant *Agrobacterium* strain GV3101 which has been disarmed by splitting the *vir* and T-DNA regions of Ti-plasmid into two separate replicons. The replicon harbouring the *vir* gene is the vir helper plasmid and a T-DNA is located on the binary vector which could function in both *E.coli* and in *Agrobacterium* (as shown in Figure 4) (Lee and Gelvin, 2008).

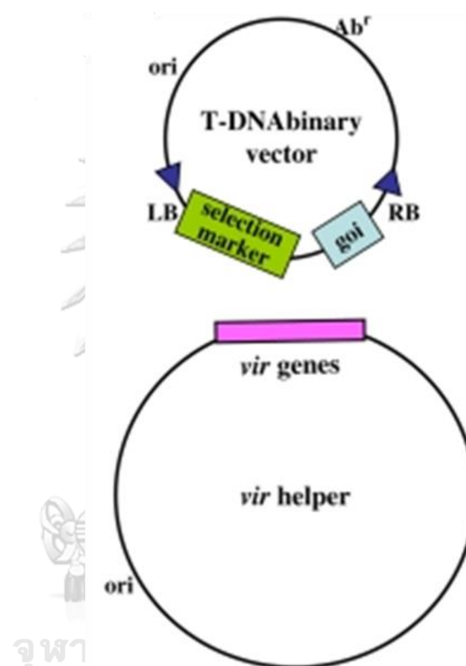

Figure 4. T-DNA binary vector system (Lee and Gelvin, 2008)

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Agroinfiltration method is a common way to introduce recombinant *A. tumefaciens* carrying insert gene into plant cells. As the buffer suspension with *Agrobacterium* enters the intercellular space of the lead, a successful infiltration is indicated by the color change from a light green color to darken (Chen et al., 2013). Syringe agroinfiltration is a simple technique using a needleless syringe to introduce the foreign gene by creating a small nick on the backside of the leaf surface. For large-scale production, a vacuum chamber is a more scalable way which can be applied to introduce the recombinant *Agrobacterium* suspended in

infiltration buffer. The plant leaves are submerged into an infiltration media with the recombinant *A. tumefaciens* harboring the gene of interest. Under negative pressure, the air in the interstitial spaces of the leaves will be pulled out and replaced by the *Agrobacterium*-containing media (Chen et al., 2013).

1.5.4 Expression vector for plant transformation

Plant viral vectors have been widely used as an expression vector for protein expression in plants. First-generation virus expression vectors or complete vectors are developed from a wild-type virus with the entire genome and the engineered sequence for a protein of interest (Gleba et al., 2007). However, the limitation of these vectors consists of the unwanted assembly of virus vectors in plant cells, the rapid loss of the transgene (Gleba et al., 2007), the limit size (less than 1 kilobase (kb)) of the inserted gene (Gleba et al., 2007, Hefferon, 2012), etc.

Second generation virus expression vectors have been designed to overcome the limitation by deconstructing the virus genomes into a replicon which contains only the essential components for robust replication and translation. The sequences coding for coat protein and movement protein were replaced with the sequence that is not derived from a virus (Gleba et al., 2007). Hence, the deconstructed vector can be delivered by *Agrobacterium* harboring a DNA copy into plant cells (Hefferon, 2014). To date, there are many popular deconstructed viral vector systems, such as MagnICON® technology (Chen et al., 2011), geminiviral vector (Chen et al., 2011), and pEAQ vector (Sainsbury et al., 2009).

Geminiviruses are a large family of plant DNA viruses in the genus of *Mastrevirus* which have been widely used as biopharmaceutical production systems. These viruses contain a small single-stranded circular DNA genome varying in size from ~2.5-3.0 kilobases (kb) which can infect many plant species

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and duplicate with very high-copy numbers in the nucleus of infected cells (Hefferon, 2014).

Mastreviruses are monopartite which can infect both monocot plants (such as wheat dwarf virus, maize streak virus) and dicot plants (such as tobacco yellow dwarf virus, bean yellow dwarf virus (BeYDV)) (Chen et al., 2011). The genome of BeYDV consists of two main components including:- 1.) two intergenic regions, namely a long intergenic region (LIR) and a short intergenic region (SIR), 2.) four protein-encoding genes between these regions. The latter can be encoded into only four proteins: a movement protein (MP), a capsid protein (CP) which are translated from the viral-sense (V) strand, and two replication initiator proteins, which are Rep and RepA, produced from the complementary-sense (C) strand (C1/C2 genes).

LIR and SIR are the only cis-elements required for replication of the genome. The LIR possesses a bidirectional promoter element which promotes a rolling-circle replication of the V-strand. In addition, the SIR is indicated as the origin of C-strand synthesis and contains transcription termination and polyadenylation signals (Chen et al., 2011) (Figure 5)

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The Geminiviral DNA genome is initially replicated inside a host cell's nucleus by converting the single-stranded genes into double-stranded DNA intermediate *via.,* host DNA polymerases. This DNA intermediate serves as a template for viral open reading frame (ORF) transcription and the rolling circle replication mechanism. The Rep protein is essential for replication by nicking at a conserved stem-loop sequence (5'-TAATATTAC-3') in the LIR and binding strongly to the 5' terminus. Moreover, the 3'-OH terminus domain serves as the primer for the synthesis of nascent DNA strand. Rep plays role as a terminase to release the displaced plus strand and nick the nascent plus strand to regenerate the origin of replication. (Chen et al., 2011, Mor et al., 2003) (Baltes et al., 2014)

This geminiviral replicon system has been used to produce a wide variety of recombinant proteins such as Ebolavirus glycoprotein (Phoolcharoen et al., 2011), Narita 104 Virus-like particles (Mathew et al., 2014), monoclonal antibodies (Huang et al., 2010), etc.

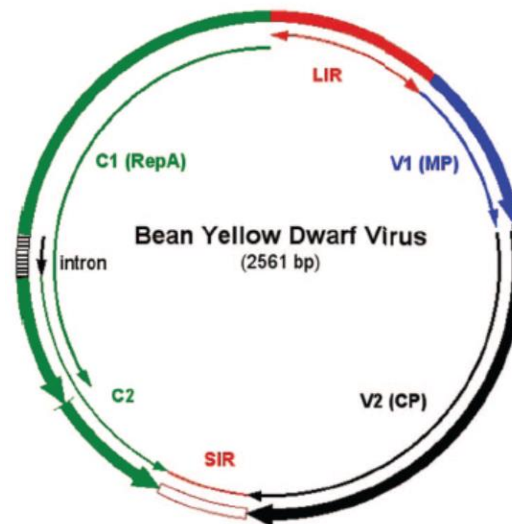

Figure 5. Bean yellow dwarf virus genome structure

The long intergenic region (LIR), Virion sense genes (V1, movement protein; V2, coat protein), The complimentary sense genes (C1, RepA; C1/C2 with spliced intron, Rep protein)

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In this study, the geminiviral replicon system modified from BeYDV is utilized as an expression vector which was exhibited to provide rapid and high mRNA levels for the high expression of protein of interest (Chen et al., 2011, Mason and Diamos, 2018). Modified BeYDV replicon has been developed by deleting the V1 (MP) and V2 (CP) gene and replacing with the Cauliflower Mosaic Virus (CaMV) 35S promoter, a RNA silencing suppressor (p19) from Tomato Bushy Stunt Virus (TBSV) to increase the expression of Rep and RepA relative to wild-type levels (Mason and Diamos, 2018).

1.5.5 Plant-based production for recombinant human epidermal growth factor

The production of recombinant growth factors emerges as an an interest of the biopharmaceutical platform providing a variety of its potential application in regenerative medicine (Mitchell et al., 2016). Numerous growth factors have been reported to produce in plants such as acid fibroblast growth factor (aFGF) (Liu et al., 2007) (Ha et al., 2017), keratinocyte growth factor 1 (KGF1) (Feng et al., 2014), hEGF (Wirth et al., 2004).

As shown in Table 2, hEGF was expressed in various host species, such as tomato (Zhi et al., 2007), soybean (He et al., 2016), and also *Nicotiana* species (Bai et al., 2007) (Wirth et al., 2004) (Higo et al., 1993) (Thomas and Walmsley, 2014). Zhi et al., 2004 showed that the EGF yield expressed in transgenic tomato was shown up to 3.48±1.01 ng per gram fresh weight; whereas the EGF yield in transgenic soybean was shown up to 6.7±3.1 – 129.0±36.7 μg EGF per gram dry seed (He et al., 2016).

In case of tobacco plants, Higo et al. (1993) tried to express hEGF gene in tobacco plants and reported the level of protein accumulation up to 0.001% of TSP (Higo et al., 1993).

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Wirth et al. (2004) tried to construct different vectors targeted either the cytoplasm or the apoplast. Firstly, the study showed that accumulation of hEGF in the cytoplasm resulted in the level of 0.00001% of TSP. To improve yields, AP24 osmotin signal sequence was inserted to the gene construct to target to the endoplasmic reticulum, which allowed the protein levels of up to 0.11% of TSP.

According to the previous findings on protein localization, Bai JY et al. (2007) attempted to gain higher hEGF expression in tobacco plant through the addition of the C-terminal endoplasmic reticulum (ER) retention signal sequence, Lys-Asp-Glu-Leu (KDEL) into the gene. In addition, each organism has a subset of

synonymous code mainly which allows its faster translation rate and higher fidelity (Lienard et al., 2007). Codon optimization was also adopted to modify the coding sequence in accordance with the codon bias of tobacco to optimize translation efficiency (Bai et al., 2007). Matrix attachment regions (MARs) are flanked at both sides of the hEGF transcription unit. MARs can interact specifically to the nuclear matrix and form chromatin loops in eukaryotic cells. Use of MAR is to minimize homologous gene silencing and promote steady protein accumulation derived from exogenous genes (Bai et al., 2007).

However, developing a stable transgenic line requires at least 6 months or a year in order to generate a homogenous transgenic plant line (Yao et al., 2015) (Xu et al., 2018).

Wirth et al. (2004) also used viral vectors as genetic constructs due to less time-consuming than stable plant transformation. Viral vectors were designed by the addition of cytoplasmic gene (PVXEGF) expression and apoplastic gene expression (PVXAPEGF). The protein level reached up to 0.015% of TSP in plants infected with the apoplast version compared to the cytoplasmic version, which the protein level was scarcely detectable. Accordingly, it was clear that the increase of hEGF accumulation can be affected by protein targeting to extracellular localization (Wirth et al., 2004).

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According to the recent study, Thomas ans Walmsley, 2004 expressed the protein into several subcellular compartments including apoplast, ER, and vacuole. This study reported the high yield up to 6.24% TSP on 13 day after post-infiltration (dpi) expressed in 5-week-old *N. benthamiana* plants when targeting the vacuole and co-infiltrating with P19 silencing supressor.

It is highly essential that the hEGF production in *N. benthamiana* by transient expression could rapidly produce in large-scale quantities within less than one week after plant transformation.

Table 2. Plant expression system used for human epidermal growth factor production

Host	Protein yields	Expression conditions	References
Transgenic expression system			
N. tabacum L. cv. F104	~ 0.001% of TSP	N/A	(Higo et al., 1993)
N.benthamiana, *N.tabacum*	0.00001% of TSP	▪ Subcellular targeting : cytoplasm	(Wirth et al., 2004)
	~ 0.11% of TSP	▪ Subcellular targeting : apoplast (AP24 osmotin signal sequence)	
N. tabacum	~ 0.3% of TSP	▪ Codon optimization ▪ C-terminal retention signal peptide (KDEL) ▪ Matrix attachment regions (MAR)	(Bai et al., 2007)
Mini-tomato	3.48±1.01 ng/g fresh weight	▪ Codon optimization	(Zhi et al., 2007)
Soybean	6.7±3.1 to 129.0±36.7 µg EGF/g dry seed	▪ Codon optimization ▪ Subcellular targeting to ER	(He et al., 2016)
Arachis Hypogaes L. (peanut hairy roots)	10.7 µg/g dry weight	N/A	(Yao et al., 2019)

Abbreviation: TSP = total soluble protein

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Table 2. Plant expression system used for human epidermal growth factor production (Continued)

Host	Protein yields	Expression conditions	References
Transient expression system			
N. benthamiana, *N. tabacum*	Not detected	▪ Subcellular targeting : cytoplasm	(Wirth et al., 2004)
	Up to 0.015% of TSP	▪ Subcellular targeting : apoplast	
N. benthamiana	0.269 mg/g of LFW (6.24% of TSP)	▪ Codon optimization ▪ Subcellular targeting : ER, apoplast, vacuole ▪ P19 silencing inhibitor ▪ Day optimization ▪ Plant age optimization	(Thomas and Walmsley, 2014)

Abbreviation: TSP = total soluble protein, LFW = leaf fresh weight

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1.5.6 Optimization strategies for plant-based protein production

There is no standard platform for plant-based expression systems driving to explore a suitable process for increasing productivity and protein yields. Even though numerous key advantages of the plant production system have been established, the limitations make them fail to overcome the existing systems. According to previous studies, there are many reports demonstrate several strategies to obtain an adequate amount of protein in plant systems. However, there remain many strategies to improve the protein yield in this system as shown below:

1.) Optimization of protein expression level

In plant-based expression system, the subcellular tegeting is one of the most important strategies governing the overall protein expression level, stability, and also biological activities due to a multitude of localization choices in plants (Warzecha, 2008).

The protein expression can be targeted to the subcellular localization by the addition of a short N-terminal signal peptide (SP) into the protein. This signal peptide is helpful to guide the proteins to the endoplasmic reticulum (ER) secretory pathway, and then migrate through the Golgi apparatus, vacuole, or apoplast (Benchabane et al., 2008) (Warzecha, 2008). In the ER, there are many chemical reactions occurs by molecular chaperones, such as N-linked glycosylation, disulfide bond formation. Disulfide bonds can stabilize and fold the protein into the correct structure by reducing the entropy of the unfolded conformation.

Besides, the yields can be optimized even further when prolongs the retention time of recombinant proteins in the ER lumen that helps the

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proteins to associate with the various chaperones required for proper folding (Benyair et al., 2011).

Addition of a SEKDEL (Ser-Glu-Lys-Asp-Gly-Leu) retention signal to the C-terminus of the protein can also increase the stability and accumulation of recombinant protein (Obembe et al., 2011). This peptide sequence have been stated that it can reduce the protein exit from the ER without generating absolute retention (Zagouras and Rose, 1989).

2.) Improvement of downstream processing in protein isolation and purification

Downstream processing is an important stage to harvest and purify the target protein. In the case of plant host system, plant tissue disruption is required for releasing and extracting recombinant proteins expressed in plant cell which can contribute to the high down-stream processing cost (Buyel et al., 2015). Moreover, purification step is needed to recover the target protein from insoluble debris and soluble plant host proteins (Schillberg et al., 2019). Hence, the efficient recovery of recombinant proteins from plant expression system should be improved.

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Commonly, affinity tags are used to facilitate the protein recovery in plant systems through specific affinity binding ligands to the tags. Tags that are commonly used include polyhistidine (His tag), StrepII, and Tandem Affinity Purification (TAP) tag, etc (Obembe et al., 2011).

Polyhistadine tag, also known as the His tag, is one of the most common fusion tags used for the purification of recombinant protein. This short peptide can be tagged to the N- or C-terminus to facilitate protein purification and detection. Proteins can specifically bind to immorbilized metal affinity chromatography (IMAC) through the interaction with the metal

ions, such as Ni^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+}, Ca^{2+}, and Fe^{3+}, but Ni^{2+} is the most popular tag (Spriestersbach et al., 2015) (Kimple et al., 2013).

The key advantage of the polyhistidine tag is the relatively small size that barely affects protein function. This suggests that His tag is not required to remove from the target protein and the protein can be eluted out under mild conditions (Kimple et al., 2013).

However, the major limitation of His tag is the nonspecific protein binding to the IMAC column, especially in low expression levels of the tagged protein. Besides, some host proteins carrying two or more adjacent histidine residues may coelute with the protein target, resulting in significant background binding and contamination in the final product (Bornhorst and Falke, 2000).

Other strategies have also reported to improve the purity of recombinant protein from plants such as low pH extraction (Alkanaimsh et al., 2019), ammonium sulfate precipitation (Park et al., 2015). Low pH extraction has been tested in other studies to reduce unwanted native protein impurities, especially RuBisCO from the crude extract (Alkanaimsh et al., 2019).

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Protein precipitation using ammonium sulfate is one of the common methods to aggregate the proteins under high salt condition. Commonly, 35% ammonium sulfate is often applied for precipitation of TSPs from plant extract (Park et al., 2015). However, it should be investigated for an appropriate concentration to precipitate the TSP harboring the protein of interest.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials and Equipment

2.1.1 Plant materials

Tobacco plant (*Nicotiana benthamiana*)

2.1.2 Microorganisms

Escherichia coli strain DH10B

Agrobacterium tumefaciens strain GV3101

2.1.3 Equipment

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Autoclave (Tomy SX-500,)

Balance (ae ADAM®, UK)

Benchtop Centrifuge (Hettich UNIVERSAL 320R, Andreas Hettich GmpH and Co.,)

Blender (OTTO®)

Blotting equipment (Biorad®, USA)

Chemiluminescence imagerQuant LAS4000 (GE Healthcare, USA)

Electrophoresis (Bio-rad®, USA)

Electroporation (MicroPulser™, Bio-rad®, USA)

Heating block (WishTherm®)

Incubator shaker (WiseCube®)

Magnetic stirrer and heater (Stuart, UK)

Microplate reader (SpectraMax M5, USA)

Microplate incubator (Hercuvan Lab systems, UK)

Mini Centrifuge (Bio-Rad®, USA)

Multichannel pipette (Cleaver scientific, UK)

PCR thermocycler (MJ Mini™, Bio-rad, USA)

Pipette (Pipetman®, USA)

pH meter (Starter3100M, OHAUS, USA)

Refrigerator (Meling Biology&Medical, China)

SDS-PAGE electrophoresis (Bio-Rad®, USA)

Spectrophotometer (GENESYS 30, ThermoScientific)

Vortex mixer (Vortex-2 GENIE, USA)

2.1.4 Materials

1.5, 15, 50 mL Centrifuge tube (Axygen®, USA)

96-well microplate (Greiner bio-one, Austria)

Membrane filter 0.45 micron (Millipore, Germany)

Polyvinlidene difluoride (PVDF) membrane (Immun-Blot® PVDF, Bio-rad, USA)

PCR tubes/strips (Axygen®)

Petri dishes plate

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

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2.1.5 Chemical reagents

Agar powder (Titan Biotech Ltd., India)

Agarose (Vivantis®, Malaysia)

Acrylamide/Bisacrylamide 40% (HiGenoMB®, India)

Ammonium Persulfate (APS) (HIMEDIA®, India)

Ammonium sulfate (Carloerbareagets, Italy)

β-merceptoethanol (Applichem, Germany)

Bromophenol blue (Honeywell Fluka™, Finland)

Coomassie Blue blue R-250 (AppliChem®)

Deoxynucleoside triphosphate (dNTP) : dATP, dCTP, dGTP, dTTP

Enhanced chemiluminescence (ECL) (Abcam®)

Ethanol (EMSURE®, Germany)

Ethylenediaminetetraacetic acid (EDTA) (HIMEDIA®, India)

Gel loading dye (Biolab®, USA)

Glacial acetic acid (EMSURE®, Germany)

Glycerol (HIMEDIA®, India)

Glycine (HIMEDIA®, India)

Imidazole (Applichem®)

2-(N-morpholino) ethanesulfonic acid monohydrate (MES) (PanReac Applichem®)

Magnesium sulfate ($MgSO_4$) (KEMAUS®, Australia)

Methanol (Merck, Germany)

Nickel (II) sulphate hexahydrate (EMSURE®, Germany)

Nickel sulphate (KEMAUS®, Australia)

Ni-NTA Affinity Resin (Amintra®)

Peptone (HIMEDIA®, India)

Potassium chloride (Carloerbareagets, Italy)

Potassium dihydrogen phosphate (Carloerbareagets, Italy)

Protein ladder (Bio-red®, USA)

Safegreen Loading Dye (New England Biolabs, USA)

Skim milk (Difco™)

Di-Sodium hydrogen phosphate (Na_2HPO_4) (EMSURE®, Germany)

Sodium chloride (NaCl) (EMSURE®, Germany)

Sodium dihydrogen phosphate (Carloerbareagets, Italy)

Sodium hydroxide (NaOH) (VWR Chemical®)

Sodium dodecyl sulfate (Carloerbareagets, Italy)

Tetramethylethylenediamine (TEMED) (Affymetri®, USA)

Tris-base (Vivantis®, Malaysia)

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Tween-20 (Vivantis®, Malaysia)

VC 1 kb DNA Ladder (Vivantis®, Malaysia)

Yeast extracts (Titan Biotech Ltd., India)

2.1.6 Enzymes and its buffer

Restriction enzymes : *Xba*I, *Xho*I, *Sac*I (New England Biolabs, USA)

T4 DNA ligase (New England Biolabs, USA)

Taq DNA polymerase (Vivantis®, Malaysia)

Q5® High-Fidelity DNA polymerase (New England Biolabs, USA)

2.1.7 Antibiotics

Ampicillin (PanReac AppliChem®)

Gentamicin (Millex®, Germany)

Kanamycin (Millex®, Germany)

Rifampicin (Millex®, Germany)

2.1.8 Antibody

Anti-histidine conjugated with Horseradish peroxidase (HRP) antibody (Abcam, USA)

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Mouse anti-human EGF antibody (R&D system, USA)

Goat anti-mouse IgG antibody conjugated with HRP (R&D system, USA)

Recombinant human EGF protein (Catalog: 236-EGF-01M, R&D system, USA)

2.1.9 Kits and plasmids

AccuPrep® Gel purification kit (Bioneer Corporation, Korea)

AccuPrep® Plasmid Mini Extraction Kit (Bioneer Corporation, Korea)

Human EGF ELISA kit (DuoSet® ELISA Development System, R&D system, USA)

Bradford assay (Bio-Rad®, USA)

pBHA cloning vector (Bioneer corporation, Korea)

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

pBYR2e expression vector

2.1.10 Software and database

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

ExPAsy Bioinformatics Resource Portal (https://web.expasy.org/translate/)

GenBank NCBI (https://www.ncbi.nlm.nih.gov/genbank/)

GeneArt Gene Synthesis Portal

(https://www.thermofisher.com/order/geneartgenes/projectmgmt)

NCBI (https://www.ncbi.nlm.nih.gov/)

NEBcutter V2.0 (http://nc2.neb.com/NEBcutter2/)

2.1.11 Oligonucleotide primers

The oligonucleotide primers were designed and synthesized by Bioneer Cooperation and Bionics, Korea. The lists of primer used in this study are shown in Table 3. Primers no.1-8 were used for amplification of the six different hEGF gene constructs as shown in Figure 5, primers no. 9-10 were used as universal primers for screening the presence of pGEM®-T harbouring the hEGF gene in *E.coli* strain DH10B and primers no. 11-12 were used for screening the presence of pBYR2e harbouring hEGF gene in *E.coli* strain DH10B or *A. tumefaciens* strain GV3101.

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Table 3. The designed primers used for amplification and cloning

No.	Name of primer	Primer sequences (5'⟶ 3')	Direction
1	*Xba*I-SP-F	TCTAGAACAATGGGCTGG	Forward
2	*Xho*I-H-EGF-F	CCTCGAGCATCATCACCACCATCACCATCATAACTC CGACTCCGAGTGC	Forward
3	*Xba*I-ATG-EGF-F	TCTAGAACAATGAACTCCGACTCCGAGTGC	Forward
4	*Xba*I-ATG-H-F	CTCTAGAATGCATCATCACCACCATCACCATCAT	Forward
5	*Sac*I-KD-H-R	CGAGCTCTCAAAGCTCATCCTTTTCAGAATGATGG TGATGGTGGTGATGATG	Reverse
6	*Sac*I-EGF-R	CGAGCTCTCAGCGAAGCTCCCACCACTTG	Reverse
7	*Sac*I-KD-EGF-R	CGAGCTCTCAAAGCTCATCCTTTTCAGAGCGAAGC TCCCACCACTTG	Reverse
8	*Sac*I-H-EGF-R	CGAGCTCTCAATGATGGTGATGGTGGTGATGATGG CGAAGCTCCCACCAC	Reverse
9	T7	TAATACGACTCACTATAGGG	Forward
10	SP6	ATTTAGGTGACACTATAG	Reverse
11	2e-29e F	TGATATCTCCACTGACGTAAGG	Forward

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2.2 Experimental Procedures

2.2.1 Gene design and construction

2.2.1.1 Gene design and codon optimization for *N. benthamiana*

Human epidermal growth factor (hEGF) amino acid sequences were obtained from NCBI database (GenBank Accession No. : AFA26280.1) and also from the reviewed literature (George-Nascimento et al., 1988, Yamagata et al., 1989).

The hEGF amino acid sequences were reversed into nucleotide sequences and optimized codon-usage for *N. benthamiana* expression using Invitrogen GeneArt (ThermoFisher scientific).

To prepare the hEGF DNA for expression, the plant codon-optimized DNA sequences encoding the hEGF was designed using codons that are preferred in tobacco plant. The hEGF gene was fused with a 5' signal peptide (5'-TCTAGAACAATGGGCTGGTCCTGCATCATCCTGTTCCTTGTTGCTACTGCTACCGGCGTTC ACTCTGATGTTCAACTTCTCGAG-3') to drive proteins into the secretory pathway and a 3' ER retention sequence (TCTGAAAAGGATGAGCTT) to keep the protein in the ER. The 3' His tag (5'- CATCATCACCACCATCACCATCA T-3') was added with the hEGF gene to facilitate the protein purification using Nickel affinity column chromatography. Additionally, stop codon and the restriction enzyme sites including *Xba*I (TCTAGA), *Xho*I (CTCGAG), and *Sac*I (GAGCTC) were added to ease the process of cloning and ligation and for the mature polypeptide. The hEGF template was synthesized by Bioneer cooperation, Korea and provided in pBHA plasmid. The hEGF template sequences are shown below:-

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hEGF template : 5’ SP-EGF-H-KD 3’

TCTAGAACA**ATG**GGCTGGTCCTGCATCATCCTGTTCCTTGTTGCTACTGCTACCGGCGTTCACT

CTGATGTTCAACTTCTCGAGAACTCCGACTCCGAGTGCCCCCTCTCCCACGACGGCTACTGCCT

CCACGACGGCGTCTGCATGTACATCGAGGCCCTCGACAAGTACGCCTGCAACTGCGTCGTCGGC

TACATCGGCGAGCGCTGCCAGTACCGCGACCTCAAGTGGTGGGAGCTTCGCCATCATCACCACC

ATCACCATCATTCTGAAAAGGATGAGCTT**TGA**GAGCTC

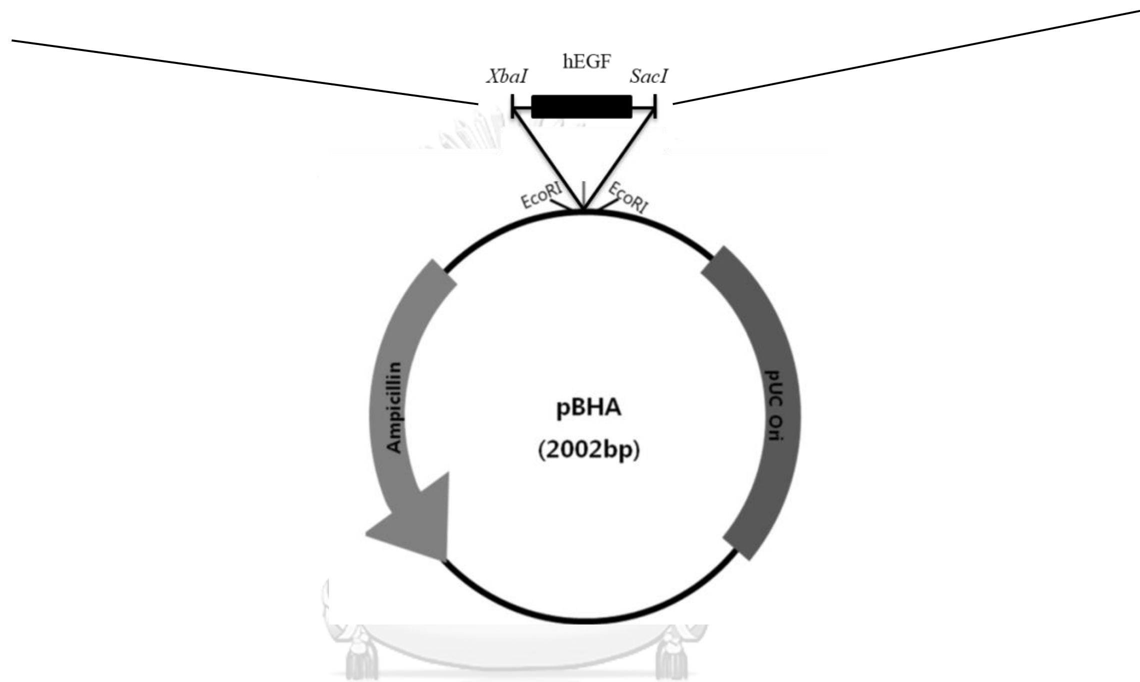

Figure 6. The schematic diagram of hEGF template provided in pBHA cloning vector (pBHA-SP-EGF-H-KD)

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2.2.1.2 Gene construction

1.) Primer design

For the construction of other hEGF gene constructs, these constructs were generated using PCR amplification from the hEGF gene template provided in pBHA cloning vector (pBHA-SP-EGF-H-KD; Figure 6) using specific primers (Table 3). The specific primers were designed in accordance with the hEGF coding sequence in pBHA cloning vector to include or exclude the needed sequences.

For the construct named "**SP-EGF-H**" (Figure 7B), the template was amplified with the primer *Sac*I-H-EGF-R (5'-CGAGCTCTCAATGATGGTGATGGTGG TGATGATGGCGAAGCTCCCACCAC-3') which removed the ER retention sequence (SEKDEL) at the 3' end from the template.

The set of primers including *Xho*I-H-EGF-F (5'-CCTCGAGCATCATCACCAC CATCACCATCATAACTCCGACTCCGAGTGC-3'), which added His tag sequence at 5' end and *Sac*I-EGF-R (5'-CGAGCTCTCAGCGAAGCTCCCACCACTTG-3') were used to amplify the template providing the PCR product named "***Xho*I-H-EGF**".

Additionally, the gene construct of "***Xho*I-H-EGF-KD**" was created using the primer *Xho*I-H-EGF-F for adding His tag sequence at 5' end and *Sac*I-KD-EGF-R (5'-CGAGCTCTCAAAGCTCATCCTTTTCAGAGCGAAGCTCCCACCACTTG-3') for adding the ER retention sequence at 3' end.

Both the PCR products of "***Xho*I-H-EGF** and "***Xho*I-H-EGF-KD**" were joined separately with pGEM®-T cloning vector bearing the signal peptide with same restriction sites (*Xho*I/*Sac*I) becoming as the construct named "**SP-H-EGF**" and "**SP-H-EGF-KD**", respectively (Figure 7B).

The primer *Xba*I-ATG-EGF-F was designed to exclude the signal peptide and added start codons (ATG) at 5' end. The PCR product was carried out as "**EGF-H**". The last gene construct "**H-EGF**" was produced by using the primer *Xba*I-ATG-H-EGF-F to remove the signal peptide and introduce the His tag sequence at 5' end.

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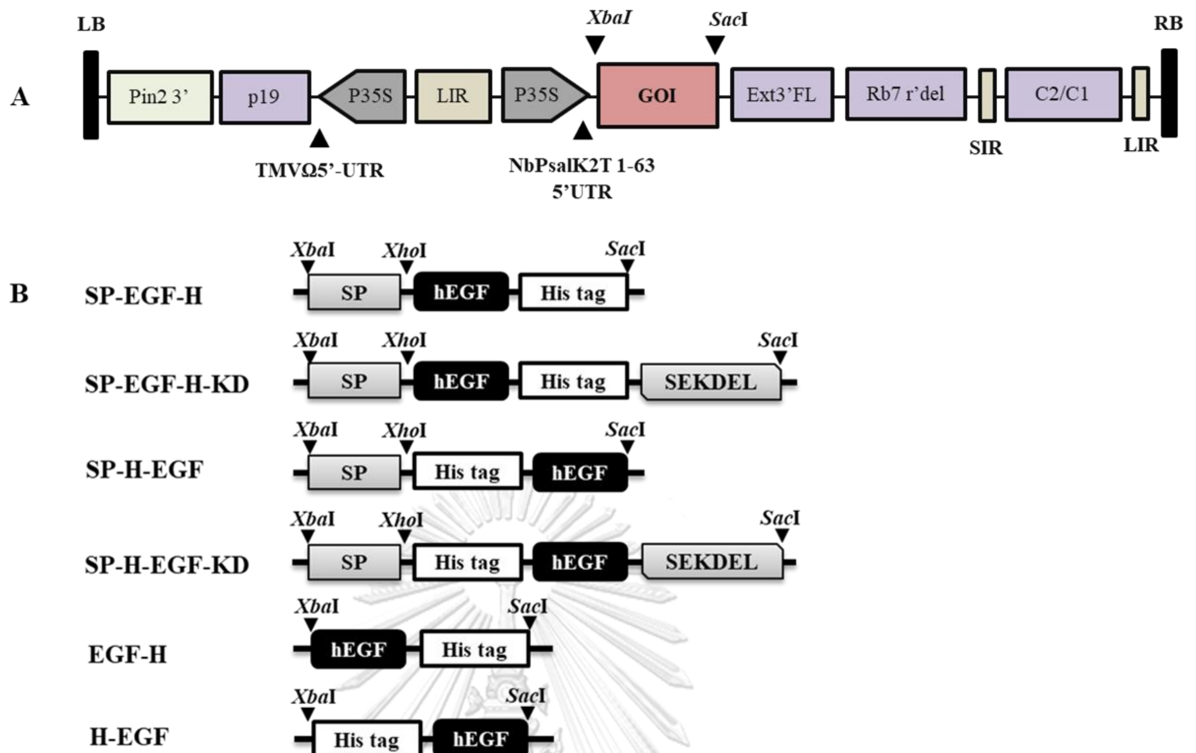

Figure 7. The schematic diagram of the 6 different gene constructs

2.) Gene amplification

In the process of gene amplification, the PCR reaction was performed using Q5^{TM} High-Fidelity DNA polymerase which possess a proofreading activity (3’⟶5’ exonuclease activity) with the highest fidelity amplification. In addition, the Taq DNA polymerase was used to generate deoxyadenosine (dA) overhang at the 3’-terminus to facilitate the ligation of PCR fragment into pGEM®-T cloning vector bearing deoxythymine (dT) (Pezza et al.). The PCR master mix was prepared as shown in Table 4:-

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Table 4. Components of PCR master mix for gene amplification

Components (1x master mix, 25 μL)	Amount (μL)
10 μM Forward primer	1.0
10 μM Reverse primer	1.0
2 mM dNTP	2.5
Q5 Taq polymerase	0.2
Buffer (with $MgCl_2$)	5.0
Ultrapure water	14.8
$MgCl_2$	0.5
Remark : 0.2 μL Taq polymerase (added before the stage of final extension)	

The PCR cycling conditions are described as follows: initial denaturation at 98C for 2 min followed by 30 cycles of 98°C for 30 s, 52°C for 30 s, and 72°C for 30 s, with final elongation step at 72°C for 10 min.

3.) Cloning, Ligation and Transformation

After completion of the PCR reaction, PCR products were separated using 1% Agarose gel electrophoresis alongside with 1 kb DNA ladder (VC 1kb DNA Ladder, Vivantis) to purify the product and determine the size of DNA fragments. The gel was mixed with Safegreen dye staining to visualize the band. The DNA fragments at expected size were purified using AccuPrep® Gel purification kit followed the supplier's protocol.

The eluted PCR products were ligated into pGEM®-T cloning vector using T4 DNA ligase enzyme in 2xligation buffers. The ligated product was incubated overnight and transformed into *E.coli* competent cells strain DH10B.

Competent cell stock solution is stored in the -80°C freezer and gently thawed on ice. 50-100 μL of competent cells were mixed with the ligation

mixture and placed on ice for at least 20 min. The *E.coli* stain DH10B was transformed by heat-shock at ~42°C for short period of time in a heating block (heat shock method). Then, the cells were added with 500-1,000 µL of LB broth and incubated at 37°C for 1 hr. After incubation, the DH10B *E.coli* carrying the plasmid with the gene of interest (pGEM®T-GOI) was spread on a LB agar plate containing Iso-1-thio-β-D-thiogalactopyranoside (IPTG), 5-Bromo-4-chloro-3-indole-beta-D galactopyranoside (X-gal) and ampicillin.

The resulting bacterial colonies on an agar plate were screened through antibiotic-specific selection, blue-white colony selection, and colony polymerase chain reaction (colony PCR).

(1) **Antibiotic-specific selection**: According to pGEM®-T Easy Vector Map shown in Figure 8, this cloning vector contains the ampicillin-resistance gene (Promega, 2018). Hence, the ampicillin-sensitive cells cannot grow in the culture media containing ampicillin antibiotic.

(2) **Blue/White colony selection**: pGEM®-T cloning vector contains the coding sequence of the *lacZ* gene. The activity of *lacZ* gene results in the production of β-galactosidase enzyme which can hydrolyze X-gal and produce blue-coloured colonies. When the *lacZ* is disrupted by insertion of GOI, this enzyme activity cannot be occurred resulting in the bacterial colonies remain white (Promega, 2018).

(3) **Colony polymerase chain reaction (colony PCR):** The selected colonies can be screened using PCR reaction. White colonies from Blue-white colony screening were chosen to amplify with T7/SP6 primers or GOI-specific primers. The PCR master mix was prepared as shown in Table 5:-

Table 5. Components of PCR master mix for colony PCR

Components(1x, 10 µL)	Amount
10 µM Forward primer	1.0
10 µM Reverse primer	1.0
2 µM dNTP	1.5
Taq polymerase	0.2
$MgCl_2$	0.5
Buffer A	1.5
Ultrapure water	4.3

The PCR cycling conditions were as follows: initial denaturation at 98C for 2 min followed by 30 cycles of 98°C for 30 s, 52°C for 30s, and 72°C for 30s, with final elongation step at 72°C for 10 min.

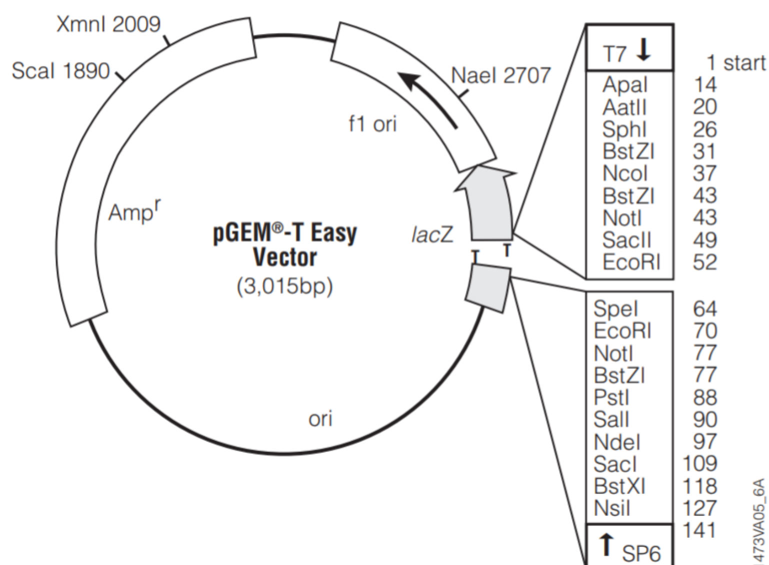

Figure 8. pGEM®-T Easy Vector Map (Promega, 2018)

The PCR products were loaded alongside with DNA ladder into 1% Agarose gel electrophoresis to examine the presence of insert gene with expected size. The selected *E.coli* colonies were inoculated in LB broth supplemented with 50 mg/L of ampicillin at 37°C overnight. After that, recombinant *E.coli* was harvested for plasmid extraction to obtain the plasmid containing GOI using AccuPrep® Plasmid Mini Extraction Kit, following the manufacturer's protocol.

4.) Sequence analysis

Each recombinant plasmid was extracted by Plasmid extraction kit. The purified plasmids were analyzed the nucleotide sequences by Bionics, Korea. The sequencing results were aligned with the template sequence using Clustal Omega (Multiple sequence alignment program). If the sequence results are similar or identical to the template or can be translated into the correct amino acid sequence, the plasmid containing GOI with correct sequence was selected for further experiments.

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2.2.2 Construction of plant expression vector and *A. tumefaciens* transformation

2.2.2.1 Construction of plant expression vector

pGEM®-T cloning vector bearing hEGF gene was digested by *Xba*I and *Sac*I restriction enzymes to obtain the gene of interest. The gene insert was separated on 1% Agarose gel electrophoresis and purified using Accuprep® gel purification kit. In this study, the expression vector is a geminiviral vector named pBYR2e which contains kanamycin-resistance gene, *Xba*I/*Sac*I restriction site. The gene insert was joined together with pBYR2e bearing same restriction sites (*Xba*I/*Sac*I) using T4 DNA ligase and 10xligase buffer. The ligation was allowed to react at 4°C

for overnight. Then, the ligated mixture was transformed into *E.coli* strain DH10B competent cells and grown on an agar plate supplemented with kanamycin.

The colonies harboring recombinant plasmid with GOI are grown on the agar plate supplemented with kanamycin. A single colony was chosen to perform colony PCR reaction with the primer 2e-29e F/2e-29eR or the gene-specific primers. The PCR products were examined for the presence of GOI at the expected size using 1% Agarose gel electrophoresis. After that, the colony was inoculated in LB media supplemented with 50 mg/L of kanamycin at 37°C, overnight. The bacteria culture was harvested for plasmid extraction (pBYR2e-hEGF) using AccuPrep® Plasmid Mini Extraction Kit, following the manufacturer's protocol.

2.2.2.2 *A. tumefaciens* transformation

After plasmid extraction from DH10B *E.coli*, the purified pBYR2e-hEGF plasmid was transformed into *A. tumefaciens* by electroporation. *A. tumefaciens* competent cell stock solution is stored in -80°C freezer and should be gently thawed on ice. The cells were mixed with the pBYR2e-hEGF plasmid before transferring into a dry electroporation cuvette. Then, the *Agrobacterium* cell was transformed using an electroporator. Immediately add 500-1,000 µL of LB broth into the cuvette and incubate the cells at 28°C for 90-120 min.

The transformed *A. tumefaciens* was spreaded on the LB agar supplemented with 50 mg/L of kanamycin, gentamicin and rifampicin and incubated at 28°C for 48 hr. The *Agrobacterium* clones were confirmed by PCR analysis as described above.

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2.2.3 *N. benthamiana* transformation using agroinfiltration

A single recombinant *Agrobacterium*-colony harboring the pBYR2e-hEGF expression vector was inoculated in LB media supplemented with 50 mg/L each of kanamycin, gentamicin and rifampicin and incubated at 28°C for overnight. The overnight grown *Agrobacterium* cells were harvested by centrifugation (6,000 rpm for 10 min) and resuspended with infiltration buffer (10 mM MES, 10 mM $MgSO_4$, pH 5.5) to get an appropriate optical density at 600 nm (OD_{600}) using spectrophotometer (GENESYS™ 30 Visible Spectrophotometer, ThermoFisher Scientific).

The wild-type *N. benthamiana* plants were used for transformation *via*., a simple technique called Syringe infiltration. This technique was performed using a 1 mL syringe without needle in order to infiltrate the recombinant *Agrobacterium* suspension into the abaxial surface of tobacco's leaves. After infiltration, the tobacco plants were kept under controlled conditions with a 16 hr light/8 hr dark cycle at 28°C.

For large-scale production, the plants were upside-down and submerged into the infiltration buffer containing recombinant *A. tumefaciens* using vacuum infiltration with a pump and vacuum desiccator chamber. Under vacuum condition, the air inside the leaves was drawn out and replaced with recombinant *Agrobacterium*-containing media (Chen et al., 2013).

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2.2.4 Plant-produced hEGF expression in *N. benthamiana*

2.2.4.1 Determination of optimal hEGF gene constructs

The expression level of different constructs were compared by infiltration the leaves with *Agrobacteirum* containing either one of the constructs including "SP-EGF-H", "SP-EGF-H-KD", "SP-H-EGF", "SP-H-EGF-KD", "EGF-H", and "H-EGF". *A. tumefaciens* harboring GOI was resuspended with infiltration buffer (the final

OD_{600} at 0.4). Then, the leaves were harvested and blended with extraction buffer (5 mM Imidazole, 20 mM Tris-HCl, 50 mM NaCl, pH 7.4) until the crude extract becomes homogenous. Clear crude extracts were obtained from high speed centrifugation at 13,000 rpm for 30 min to precipitate unwanted fibers. The supernatant was measured the TSP amount by Bradford assay and retained for later analysis by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. The optimal gene construct was chosen for further study.

2.2.4.2 Determination of optimal days post infiltration for high protein expression

For time-course experiment of hEGF expression in plants, infiltration buffer containing recombinant *A. tumefaciens* with the optimal construct in pBYR2e-hEGF were infiltrated into the leaves of *N. benthamiana* plants. At least three plants were tested in order to reduce the batch-to-batch variation of plants. The infiltrated leaves were collected on several days including 2, 4, and 6 dpi in order to examine the optimal incubation day that can highly express the hEGF yield. The clarified crude extracts were analyzed by Western blot.

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2.2.5 Purification of plant-produced hEGF using Nickel affinity column chromatography

Nickel affinity column chromatography is widely used in protein purification for the His-tagged proteins. To prepare a nickel-affinity column, the Nickel-nitrilotriacetic (Ni-NTA) affinity resin (Amintra®) was added into a gravity flow purification column and flushed the beads with deionized water repeatedly to remove 20% ethanol residues. Then, allow the beads settle by gravity and equilibrate with 5 column volumes (CV) of extraction buffer.

Briefly, large-scale production of plant-produced protein was conducted using vacuum chamber by submerging the plant upside down into the infiltration buffer containing *Agrobacterium*-harbouring hEGF gene. Infiltrated leaves were collected for protein extraction by grinding with extraction buffer. Then, the supernatant was obtained after centrifugation at 13,000 rpm for 30 min and clarified by a sterile 0.45-micron membrane filter.

Clarified crude extract was gently loaded into the Ni-NTA column to allow the binding interaction between His-tagged proteins with the Nickel bead. Then, the beads were washed with 10 CV of wash buffer (5 mM and 20 mM Imidazole, 20 mM Tris-HCl, 50 mM NaCl, pH 7.4) in order to remove the non-specific binding proteins prior to elution. To elute the protein, elution buffer (250 mM Imidazole, 20 mM Tris-HCl, 50 mM NaCl, pH 7.4) was added and allowed to equilibrate with the beads. The purified protein samples were examined with SDS-PAGE, Western blot and quantified by enzyme-linked immunosorbent (ELISA) assay. Additionally, the purified plant-produced hEGF solution was desalted to remove high imidazole content and concentrated with Amicon® centrifugal filter -3 kDa cutoff columns and filtered through a 0.2 micron filter membrane for further experiment.

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2.2.6 Preliminary results for optimization of purification strategy

2.2.6.1 Effect of extraction volume on the efficiency of protein purification

The effect of extraction volume on protein purification was assessed in this study. Twenty gram of infiltrated leaves was grinded with liquid nitrogen using a mortar and pestle. The leaf powder was added with 2 mL of extraction buffer per 1 gram of leaf fresh weight and blended until homogenous. After extraction, the extract was divided into two sets. The first set was centrifuged at 13,000 rpm at 4°C for 30 min; whereas the extract in the second set was further

diluted 10 times with extraction buffer, and then collected the supernatant by centrifugation. Both sets of the clarified extracts were further purified by a single-step Ni-NTA affinity chromatography. The purified protein was analyzed by SDS-PAGE and Western blot analysis.

2.2.6.2 Protein purification using Ammonium sulfate precipitation

To improve the protein purification efficiency, the clarified crude extract was added with various concentrations ranging from 30 to 80% ammonium sulfate to determine their effects on removal of host cell proteins (HCP). Briefly, the clarified crude extract was firstly added with 30% ammonium sulfate and incubated for 30 min at 4°C with constant stirring. After centrifugation at 13,000 rpm for 30 min at 4°C, the supernatant was collected and ammonium sulfate was added into the supernatant up to the final ammonium sulfate concentration at 40, 50, 60, 70 or 80%, repeatedly. The pellet was resuspended with extraction volume and analyzed by SDS-PAGE and Western blotting.

2.2.6.3 Protein purification using Acid precipitation

One gram of harvested leaves was grinded with extraction buffer with different pH ranging from 4 to 8. Then, the crude extract was centrifuged at 13,000 rpm for 30 min and the supernatant was collected. The clarified protein was analyzed by SDS-PAGE and visualized by Coomassie Blue staining. For Western blot analysis, the protein was transferred on to PVDF membrane and detected by using rabbit anti-His antibody (Abcam, UK).

2.2.7 Protein characterization and quantification

2.2.7.1 Bradford assay

The clear crude extract was measured the amount of total soluble protein (TSP) using Bradford assay (Bio-rad). This assay can be carried out by preparing the dilution of 1 part Dye Reagent Concentrate with 4 parts deionized

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water. A standard curve was carried out in a range concentration of bovine serum albumin (BSA) from 50-400 µg/mL which the r^2 value should not be less than 0.95. The samples and standard solutions are determined the absorbance at the wavelength of 545 nm. All of protein samples were diluted with extraction buffer to obtain the same amount of total soluble protein before further experiment.

2.2.7.2 SDS-PAGE and Western blot analysis

Plant-produced hEGF protein samples were added with reducing loading buffer (125 mM Tris-HCl pH 6.8, 12% SDS, 10% glycerol, 22% β-mercaptoethanol, and 0.001% bromophenol blue, pH 6.8) and denatured by boiling at 95°C for at least 5 min. The protein samples were loaded into each well in order to separate on 6-18% gradient polyacrylamide gels (Appendix B). Briefly, the SDS-PAGE gel components are shown below:

- **Resolving gel** : 40% acrylamide, 1.5M Tris pH8.8, 10%SDS, 10%APS, TEMED
- **Stacking gel** : 40% acrylamide, 1M Tris pH6.8, 10%SDS, 10%APS, TEMED

Following SDS-PAGE, the protein samples were either visualized by Coomassie Blue staining or electrophoretically transferred and immobilized onto a PVDF membrane at 75 V for 90 min or 30 V for overnight (Appendix C). Then, the membranes were blocked with 5% non-fat skim milk in PBS pH 7.4 as a blocking reagent for 1 hr or overnight to avoid non-specific binding sites on the membrane. After blocking, the membranes were incubated with 3% non-fat skim milk supplemented with 1:5,000 dilutions of rabbit anti-His antibody conjugated to horseradish peroxidase (HRP) for at least 2 hr or overnight in refrigerator. Furthermore, the size and identity of the plant-produced hEGF were confirmed using western blot analysis. The blotted membranes were probed with mouse anti-human EGF antibody as a primary antibody and subsequently added with goat anti-mouse IgG antibody conjugated to HRP as a secondary antibody. The

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membranes were washed thoroughly for 3 rounds of 10 min with PBS-T (PBS and 0.05% tween-20). Then, the blots were developed by chemiluminescence using ECL plus detection reagent.

2.2.7.3 Sandwich ELISA protocol for plant-produced hEGF quantification

The concentration of plant-produced hEGF in plant crude extracts or purified protein samples was quantified using sandwich ELISA technique. This assay was performed in accordance with the supplier's protocol of human epidermal growth factor (hEGF) (DuoSet® ELISA Development System, R&D system) shown as follows:-

1.) Plate preparation

Mouse anti-human EGF Capture antibody was diluted with 1xPBS and coated into a 96-well microplate. The plate was incubated overnight to allow the antibody fully attached on the plate surface.

After that, aspirate the well and wash with wash buffer (0.05% Tween-20 in PBS, pH 7.2-7.4; 1xPBS-T) for 3 times. The plate surface was blocked with Reagent Diluent (1% BSA in PBS pH 7.2-7.4, 0.2 µm filtered). Incubate at room temperature (RT) for at least 1 hr. Then, repeat the washing step 3 times before further steps.

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2.) Standard and sample preparation

Recombinant human EGF standard was prepared using 2-fold serial dilutions (Range concentration = 3.91-250 pg/mL) and plant-produced hEGF protein samples was diluted into several dilutions with 1% BSA in 1xPBS.

3.) Assay procedure

Add plant-produced hEGF protein sample and recombinant Human EGF Standard into each well and incubate for 2 hr at RT. Then, repeat the washing step for 3 times. Biotinylated Goat Anti-Human EGF Detection Antibody was

incubated in each well for 2 hr at RT. Add streptavidin-HRP B to each well and incubates for 20 min at RT in dark place. Repeat the washing step again. Add substrate solution to each well and incubate at RT. Stop the reaction using stop solution (2N H_2SO_4) and carefully tap the plate.

The plate was measured the optical density using a microplate reader (SpectraMax® M5 Microplate Reader, USA) at 450 nm. The standard curve was plotted with the absorbance value as y-axis and concentration as x-axis. The absorbance value of protein sample was used for determining the protein concentration in the sample.

2.2.8 Cytotoxicity assay

Plant-produced hEGF was examined a cytotoxicity activity in an immortalized human keratinocyte (HaCaT) cell lines using MTT assay. MTT (3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide, a yellow tetrazole) assay was used for measuring the activity of mitochondrial enzyme as an indicator in viable cells. MTT is reduced to formazan dyes, giving purple color formazan crystals by NADH. A solution of dimethyl sulfoxide (DMSO) is added to dissolve the insoluble purple formazan crystal into a colored solution. The resulting colored solution can be quantified by measuring at a certain wavelength (Aslantürk, 2018). In this study, HaCaT cells at a density of $1x10^4$ cells per well were cultured in a 96-well plate and maintained in a humidified atmosphere of 5% CO_2 at 37°C for 24 hr. Then, cells were treated with various concentrations (5, 10, 50, and 100 ng/mL) of plant-produced hEGF and commercial hEGF for 24 hr. After that, the cells were added with MTT reagent solution (0.4 mg/mL) and incubated for 3-4 hr at 37°C. After incubation, the MTT reagent was then removed and 100 µl DMSO was added to dissolve the formazan crystals giving the purple

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colored solution which can be read out by a microplate ready (Perkin Elmer Microplate reader #1) at the absorbance of 570 nm.

2.2.9 Statistical analysis

All values are presented as mean ± SD from three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc analysis with a P value less than 0.05 ($p \leq 0.05$) considered as statistical significance.

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CHAPTER 3
RESULTS AND DISCUSSION

3.1 hEGF gene cloning into plant expression vector

The nucleotide sequence encoding for human epidermal growth factor (hEGF) was codon-optimized *in silico* for *N. benthamiana*. The hEGF gene was generated into six different gene constructs as shown in Figure 7. The gene was ligated into pGEM®-T easy vector and transformed in *E. coli* strain DH10B. The pGEM®-T easy vector containing each hEGF constructs was extracted from *E.coli* and confirmed the nucleotide sequences by DNA sequencing (Appendix A). Subsequently, six different hEGF genes were cut from pGEM®-T easy vector with specific restriction enzymes (*Xba*I/*Sac*I) and ligated into a plant Geminiviral expression vector (pBYR2eK2Md) bearing the same restriction enzyme sites, separately. The vector was modified from the bean yellow dwarf virus which was reported to provide rapid and high mRNA levels (Chen et al., 2011) (Mason and Diamos, 2018).

The vector containing hEGF genes were digested with *Xba*I and *Sac*I restriction enzymes prior to *Agrobacterium* transformation. Results showed that the size of each hEGF constructs is at 276 base pair (bp) for SP-EGF-H, 294 bp for SP-EGF-H-KD, 276 bp for SP-H-EGF, 294 bp for SP-H-EGF-KD, 204 bp for EGF-H, and 204 bp for H-EGF as predicted (Figure 9). *Agrobacterium* clones were confirmed by PCR for further experiments.

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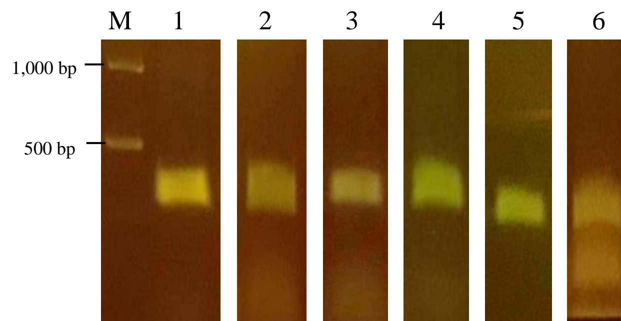

Figure 9. 1% Agarose electrophoresis of digestion of expression vector containing hEGF gene constructs.

Remark: M: DNA marker, Lane 1: SP-EGF-H, Lane 2: SP-EGF-H-KD, Lane 3: SP-H-EGF, Lane 4: SP-H-EGF-KD, Lane 5: EGF-H, Lane 6: H-EGF

3.2 Determination of optimal gene design for effective protein production

In order to determine the optimal expression levels of different hEGF gene constructs, *N. benthamiana* leaves were infiltrated with *A. tumefaciens* bearing one of the six different constructs (Figure 7). The necrotic reactions of infiltrated leaves were monitored for several days including 2, 4, and 6 dpi. Tissue necrosis has been noted as a sign of expressing certain proteins using BeYDV vector which might be due to the hypersensitive response of plant against viral infection causing the burst of reactive oxygen species and the necrotic lesions from programmed cell death (Mason and Diamos, 2018). As shown in Figure 10A, the leaves infiltrated with each constructs cannot be early observed the necrotic reaction on 2 dpi, and gradually exhibited on the infiltrated leaves after 4 dpi, in particular the leaves infiltrated with the construct containing N-terminal signal peptide presented high necrotic reaction on 6 dpi afterward.

In this study, the infiltrated leaves were harvested on 4 dpi and extracted with extraction buffer. The supernatants were measured the TSP amount by Bradford assay and analyzed by SDS-PAGE and Western blot probed with anti-His antibody. According to the Figure 10B, the blot was shown that the protein yield

was higher in the leaves infiltrated with the construct “SP-EGF-H-KD” compared to other six different gene constructs tested. Furthermore, two C-terminal His tagged constructs including “SP-EGF-H-KD” and “EGF-H” were observed by the anti-His antibody and none of the N-terminal his-tagged hEGF constructs were detected which might be due to partial protein digestion or protein folding (Debeljak et al., 2006). Even the his tag is a small size polypeptide which is believed to cause minimal effects on the protein folding, few studies have shown that the localization of histidine residues has differential effects on protein properties and its structure (Panek et al., 2013) (Zhao and Huang, 2016).

Subcellular localization or protein targeting to specific cell compartment plays a critical role in determining the protein stability and yield *in planta* (Benchabane et al., 2008). Many reports have illustrated that utilization of ER retention sequence (K/HDEL) has been shown many advantages in terms of elevated recombinant protein accumulation and protein quality (Heidari et al., 2014, Petruccelli et al., 2006, Benchabane et al., 2008).

The present study also found that the increased yield was obtained from the hEGF gene construct carrying the C-terminal SEKDEL ER retrieval peptide sequence *via.,* transient expression system in *N. benthamiana*. The expression level of hEGF is 15.695 µg/g leaf fresh weight (LFW) or 0.499% TSP obtained from the optimized construct (SP-EGF-H-KD) (Appendix D). In conclusion, this optimized construct (SP-EGF-H-KD) was selected for further experiments.

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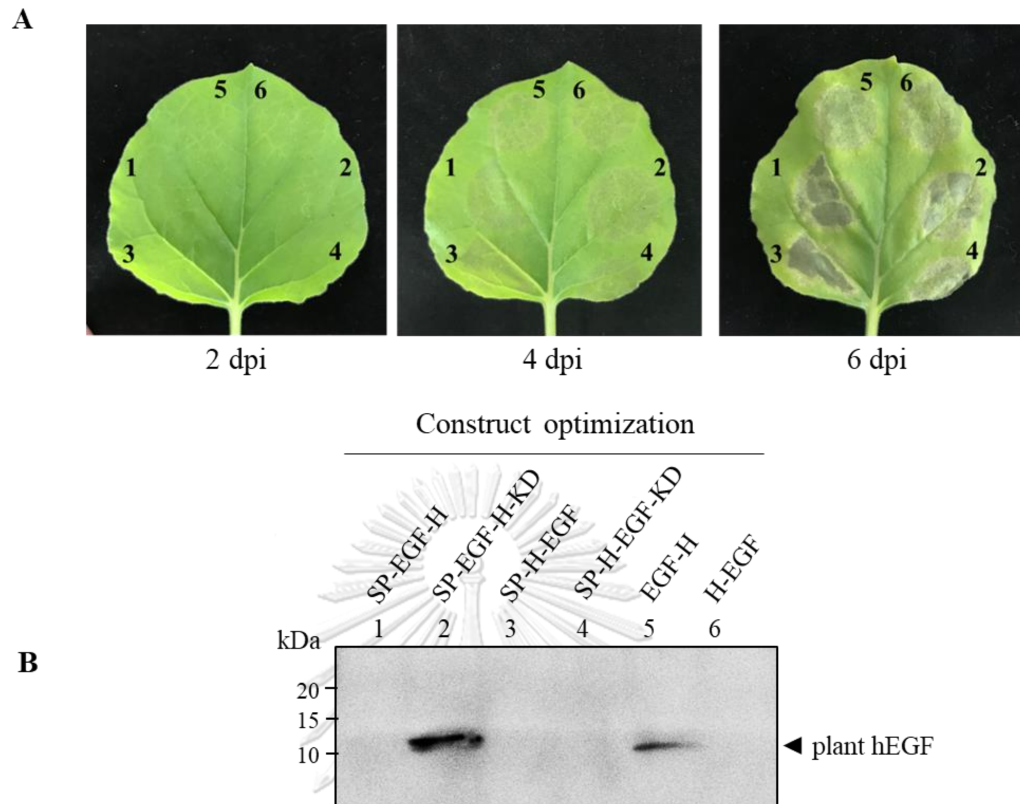

Figure 10. Effect of different gene constructs on recombinant hEGF production in plants

Remark: (A) Typical phenotype of *N. benthamiana* leaves showing necrosis symptoms after infiltration with six different constructs on 2, 4 and 6 dpi. (B) Western blot of plant-produced hEGF crude extract from leaves infiltrated with 6 different constructs under reducing conditions. The protein was transferred to PVDF membrane and the blot was probed with HRP-conjugated anti-His antibody. kDa: kilodalton; Lane 1: SP-EGF-H; Lane 2 : SP-EGF-H-KD; Lane 3 : SP-H-EGF; Lane 4 : SP-H-EGF-KD; Lane 5 : EGF-H; Lane 6 : H-EGF. Arrowhead represents plant-produced hEGF. Equal amounts of total soluble protein were loaded in each lane.

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Among the previous studies of hEGF transient expression in *N. benthamiana*, the average yield of 0.499%TSP in this study was well below the highest hEGF yield record in *N. benthamiana* reported by Thomas and Walmsley, 2014 as shown in Table 2. The difference in %TSP might be due to the amount

of total soluble protein had plunged sharply relied on the harvesting time, increasing the percentage of hEGF as a proportion. Thereby, it is difficult to directly compare the expression level between studies.

Nonetheless, hEGF had been mostly produced in traditional expression systems, especially prokaryotic expression in *E.coli* and also some hosts in eukaryotic expression system as shown in Table 6. In the previous studies, *E.coli,* is the most preferred host for the hEGF production due to several advantages such as fast growth rate with high yield of recombinant proteins compared with other host platforms. *E.coli*-derived proteins are mostly expressed as an insoluble form in inclusion bodies. It is required additional steps for *in vitro* protein refolding which might be leading to improper folding and loss of bioactivity (Zhang et al., 2018). However, some studies have attempted to improve the expression conditions *via.,* the use of fusion tags (Zheng et al., 2016) (Ma et al., 2016) and periplasm system (Oka et al., 1985) (Sivakesava et al., 1999).

In case of fusion tags, it is required addition steps for removing the fusion tag causing low protein yields or failure of protein recovery. In addition, periplasm system might be problematic in some proteins due to the proteolytic degradation of the target protein, an insufficient secretion efficiency and its capacity for protein secretion (Zhang et al., 2018).

In present study, the hEGF yield from *N. benthamiana* is well below than the yield reported in prokaryotic expression system, whereas it shows that the plants can be a host system for hEGF expression which is able to express proteins in form of soluble proteins without the requirement of any other additives for *in vitro* additional refolding step.

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Table 6. Expression hosts used for human epidermal growth factor production

Host	Vector / Gene design	Expression condition	Protein yield	References
Bacteria				
***E.coli* strain C600**	pTA1522 vector / hEGF fused with the signal peptide of *E.coli* alkaline phosphatase	periplasm	2.4 mg/L hEGF secreted from *E.coli*	(Oka et al., 1985)
***E. coli* strain JM101**	pETacEGF vector / hEGF fused with the signal peptide of *E.coli* alkaline phosphatase	periplasm	325 mg/L (continuous fed-batch cultivation)	(Sivakesava et al., 1999)
***E.coli* strain BL21(DE3)RIL**	pET-11b vector / hEGF fused with N-terminal TrpE sequence, C-terminal Arg_6 residues	Inclusion body	223 mg/L	(Sharma et al., 2008)
***E.coli* strain BL21**	pHGB1-TEV / hEGF with different fusion proteins; 1. B1 domain of streptococcal protein G (GB1) 2. Small ubiquitin-related modifier (SUMO) 3. Thioredoxin (Trx) 4. Glutathione S-transferase (GST)	Intracellular	GB1 greatly improved the yield (unspecified yield)	(Zheng et al., 2016)
***E.coli* strain BL21(DE3)**	pET21a vector / hEGF fused with Mxe Gyr intein (Mxe), SUMO, His_{10} tag	Intracellular	29.4 mg/L of native hEGF	(Ma et al., 2016)

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Host	Vector / Gene design	Expression condition	Protein yield	References
E.coli strain BL21	pD881-TorA vector/ hEGF fused with a signal peptide TorA *via.,* the Twin Arginine translocation (TAT) pathway	Inclusion body	0.9625 mg/L	(Maksum et al., 2017)
B. brevis 47	pTB361	Extracellular	240 mg/L of the culture	(Yamagata et al., 1989)
B. subtitis	hEGF fused with signal peptide, T7 promoter	Extracellular	360±9.41 mg/L	(Su et al., 2020)
		Yeast		
S. cerevisiae	-	Extracellular	4.82±0.57 mg/L	(Valdés et al., 2009)
Pichia pastoris GS115	pPIC9K-hEGF	Extracellular	2.27 mg/L of the culture	(Eissazadeh et al., 2017)
		Mammalian		
HEK293 cells	pAdenoVator ΔE1/E3 (adenoviral vector	Extracellular	-	(Negahdari et al., 2016)

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3.3 Optimal day after post-infiltration (dpi) for optimal gene construct

Most of the previous studies utilized the transgenic plants such as tomato, soybean and also *Nicotiana* species for hEGF production. As shown in Table 2, the high hEGF yield at 0.3%TSP derived from transgenic tobacco was reported from Bai et al. 2007 by utilizing a KDEL sequence to translocate the protein into the ER. However, a period of time for developing a stable transgenic line takes for at least 6 months to a year.

It is crucial that the hEGF is transiently produced in *N. benthamiana* for rapid production with large quantities of protein less than one week after plant transformation. In this study, a time-course experiment of hEGF expression in *N. benthamiana* was conducted to determine the optimal time to harvest the infiltrated leaves biomass with high hEGF accumulation after agroinfiltration. The *N. benthamiana* leaves were infiltrated with recombinant *A. tumefaciens* bearing the optimal construct “SP-EGF-H-KD” with the agrobacterium concentration at optical density (OD_{600}) at 0.4. Leaf samples were harvested on 2, 4, and 6 dpi, respectively. The hEGF expression level was analyzed by Western blot analysis and probed with anti-His antibody under reducing conditions.

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As shown in Figure 11, the most band intensity of plant-produced hEGF from infiltrated leaves was apparently shown at 2 dpi before necrosis was evident. Moreover, the band intensity gradually decreased each sequential day. This evidence might implicate that the occurrence of leave necrosis on 4 and 6 dpi caused by the protein accumulation triggering a hypersensitive defense response and ER stress in plants. This reaction is characterized by the burst of reactive oxygen species and the formation of necrotic lesion from programmed cell death leading to the reduction of protein yields (Mason and Diamos, 2018).

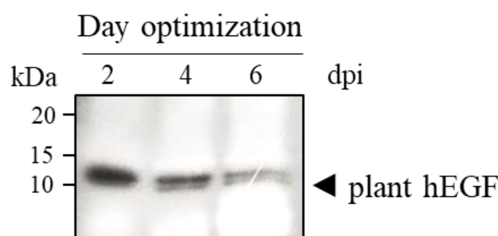

Figure 11. Optimization of transient expression for high level protein expression in plants

Remark: Western blot of crude extract expressing hEGF on 2, 4 and 6 dpi under reducing condition. The membrane was probed with HRP-conjugated anti-His antibody. kDa: kilodalton; dpi: day post-infiltration; the lane number represents the dpi used for harvesting infiltrated leaves. Arrowhead represents plant-produced hEGF. Equal amounts of total soluble protein were loaded in each lane.

3.4 Purification of hEGF from *N. benthamiana* leaves

Recombinant hEGF protein was engineered to contain His-tag in order to facilitate simple protein purification by immobilized metal affinity chromatography. After the extraction process, plant crude extracts were filtered using 0.45 μm and 0.22 μm filters, respectively. Then, crude extract was loaded into a single-step Ni-NTA affinity chromatography which is used to purify hEGF from *N. benthamiana* leaves due to its specific binding to the his tagged protein.

The authenticity and molecular weight size of the purified plant-produced hEGF protein sample was confirmed by SDS-PAGE and Western blot analysis probed with mouse anti-human EGF antibody as a Primary antibody and goat anti-mouse antibodies conjugated with HRP as a Secondary antibody under reducing condition (Figure 12, Lane 2). Furthermore, the non-infiltrated wild-type *N. benthamiana* crude extract was also used as a negative control for the assay (Figure 12, Lane 1).

According to the Figure 12, Lane 2, a hEGF band with a His tag and a SEKDEL ER retention signal was clearly observed at approximately 12 kDa which is

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slightly higher than the expected molecular weight of 8 kDa. Moreover, the Western blot data has confirmed that C-terminal His tag of hEGF can be accessible to the Ni-NTA resin and recovered from plant crude extract.

Overall, rapid expression, a simple purification strategy, and low risk of human pathogen contamination indicate that *N. benthamiana* can be used as a cost-effective system for industrial production scale. However, non-optimized purification may require many subsequent chromatography steps which inflate the production costs. Hence, this present study has extended the preliminary investigation on the optimization of the purification condition in order to improve protein quality and yield.

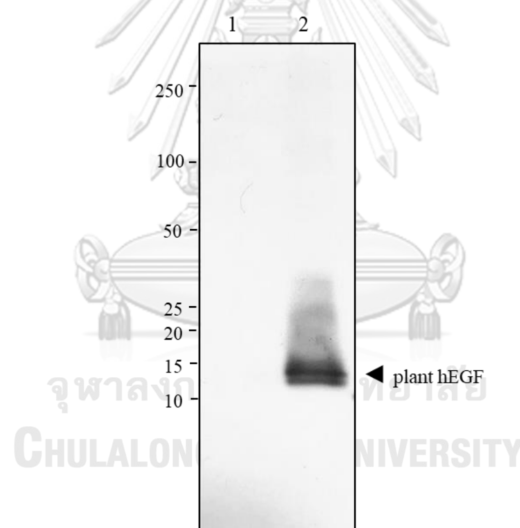

Figure 12. Purification of plant-produced hEGF from *N. benthamiana* leaves

Remark: Western blot analysis of purified plant-produced hEGF under reducing condition: The blot was probed with mouse anti-human EGF antibody. Lane 1, non-infiltrated wild-type *N. benthamiana* crude extract; Lane 2, purified plant-produced hEGF. Arrowhead represents purified plant-produced hEGF.

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3.5 Preliminary investigation on improving the hEGF purification efficiency of Ni-NTA affinity chromatography

The first step of recombinant protein production from plants is the protein extraction which is the important step to disrupt the cell wall and release the protein of interest and other host native proteins into the crude extract and then, followed by purification to recover the target protein. However, this study was also extended the preliminary investigation on improving the purification condition in order to improve protein quality and purity.

3.5.1 Effect of extraction volume

A preliminary attempt to remove host cell contaminants during purification was evaluated. The volume of extraction buffers is an important factor for sufficient cell disruption, subsequent purification step, protein recovery after release into the extract (Grabski, 2009). In this study, we examined the impact of high volume of crude extract in enhancing the purification efficiency during Ni-NTA affinity chromatography. As shown in Figure 13, Coomassie Blue-stained gels showed that the hEGF in eluted protein collected from the higher volume (1:10) was more than the eluted protein from lower volume (1:2) with lower amount of protein contaminants.

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Furthermore, the band intensity of the hEGF recovered from high volume (1:10) (Figure 13, Lane 4) was also found to be slightly higher compared to the other one. This result suggests that the high extraction volume allows the Ni-NTA affinity resin to recover his-tagged protein from plant crude extract effectively.

To our knowledge, this is the first report describing the effect of extraction volume on purification efficiency of plant-produced hEGF using Ni-NTA affinity resin.

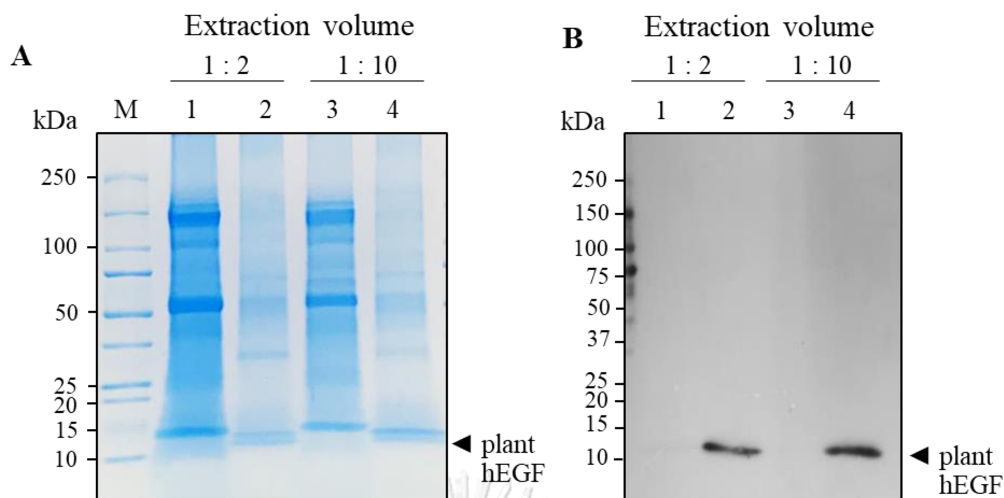

Figure 13. Effect of extraction buffer concentration on protein purification

Remark: (A) SDS-PAGE analysis of plant crude extract extracted from different concentration of extraction buffer. (B) Western blot of plant-produced hEGF crude extract using different extraction buffer concentration (1:2 vs 1:10). kDa: kilodalton; M: protein marker, Lane 1, 3: total soluble protein from plant crude extracts extracted with 1:2 and 1:10 extraction buffer concentration, respectively; Lane 2, 4: purified hEGF protein eluted from Ni-NTA affinity chromatography. Arrowhead represents plant-produced hEGF.

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3.5.2 Effect of ammonium sulfate precipitation in crude extract

Precipitation is a common purification technique to remove the protein from a solution by altering protein solubility in the presence of a high salt concentration in order to purify a target protein from others. Ammonium sulfate is commonly used, due to high water solubility with no adverse effects on the bioactivity of proteins (Evans et al., 2009). This method has been used to remove host cell proteins such as the photosynthetic protein, RuBisCO and others (Wilken and Nikolov, 2012).

According to the previous study, 35% ammonium sulfate was applied to remove native host proteins from plant crude extract (Park et al., 2015). This study aimed to investigate an appropriate saturated ammonium sulfate concentration (30-80%) which has an effect on TSP removal from crude extracts. The protein pellets collected from each precipitation step were dissolved with the buffer and analyzed by SDS-PAGE gel with Coomassie Blue staining and Western blot analysis.

As shown in Figure 14A, the Coomassie Blue-stained SDS gel showed that most of the RuBisCO protein was largely precipitated as pellets at the ammonium sulfate concentration ranging from 30-50%. On the contrary, the plant-produced hEGF protein was initially co-precipitated at 30%, following 40, 50% of ammonium sulfate saturation, whereas low or no proteins were detected at 60-80% ammonium sulfate concentration, respectively (Figure 14B).

This is the first report showing that that ammonium sulfate precipitation is not suitable for removing host cell contaminants from the plant-produced hEGF; but failed to separate the abundant RuBisCO from crude extract. However, further process optimization is highly essential to improve the protein quality.

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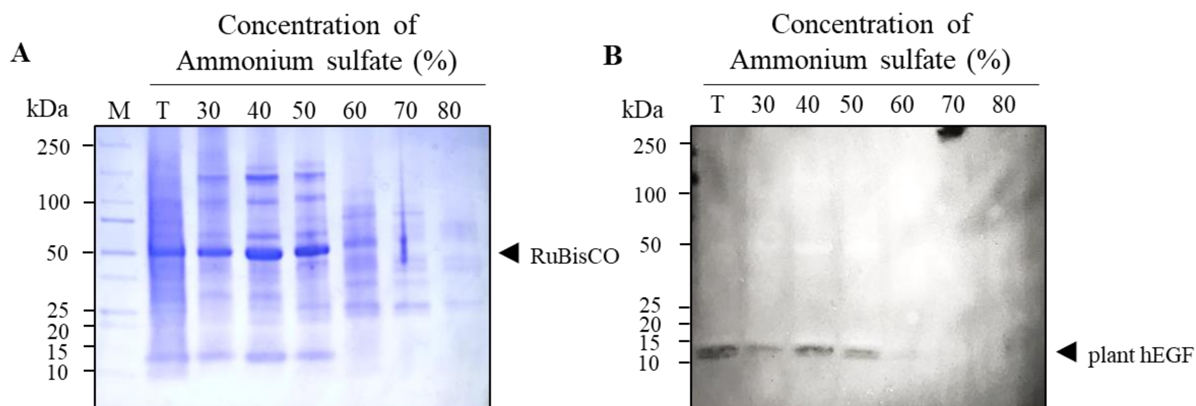

Figure 14. Effect of ammonium sulfate concentration on protein precipitation and purification

Remark: (A) SDS-PAGE analysis of precipitated proteins collected from 30-80% saturated ammonium sulfate concentration in plant crude extract (B) Western blot of plant-produced hEGF crude extract under reducing condition. kDa: kilodalton; M: protein marker; T: total soluble protein from plant crude extract; the lane number represents the ammonium sulfate concentration used for precipitating the proteins. Arrowhead indicates RuBisCO (Figure 14A) and plant-produced hEGF (Figure 14B).

3.5.3 Effect of extraction buffer pH on protein quality

In this study, the harvested plant leaves were extracted with different extraction buffers with pH ranging from pH 4 to 8. As shown in Figure 15A, Coomassie Blue-stained gel showed a prominent ~50 kDa band of RuBisCO protein was slightly reduced when harvested leaves extracted with lower pH buffer, and in particular, it was largely removed from the TSP extracted by using extraction buffer at pH 4. Furthermore, the band intensity of hEGF from pH 4 buffers was slightly reduced compared to other pH buffer conditions (Figure 15B).

Such findings indicate that the plant-produced hEGF can be purified with low pH extraction buffer, especially buffer pH 4, which could largely remove the host cell contaminants and the abundant RuBisCO proteins from the crude extract. Hence, acid precipitation technique can be applied for protein

purification resulting to a much purer final product without loss of significant yield. In addition, this technique might be able to improve the performance of subsequent purification step.

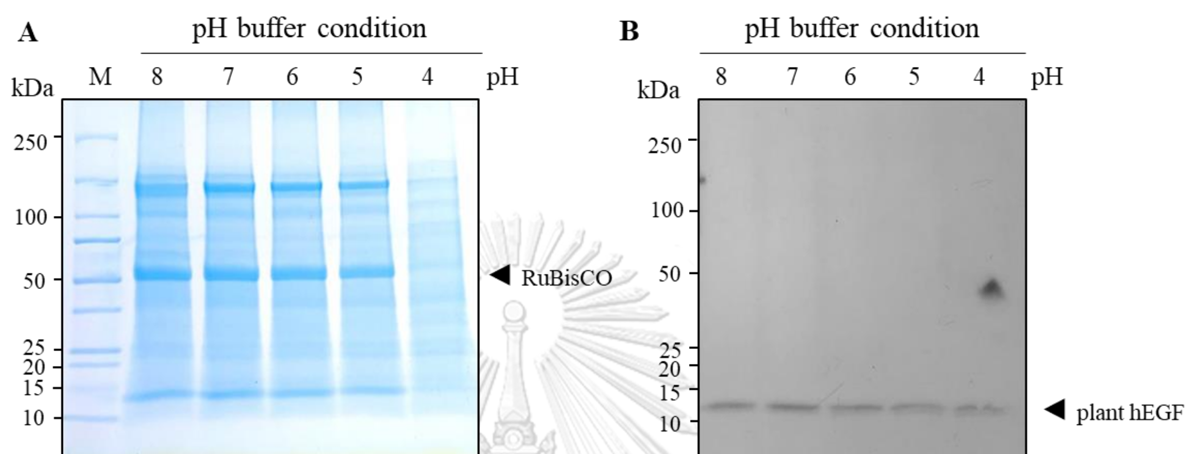

Figure 15. Effect of extraction buffer pH on protein purification

Remark: (A) SDS-PAGE analysis of plant crude extract extracted from different extraction buffer pH (pH 4, 5, 6, 7, and 8) (B) Western blot of plant-produced hEGF crude extract under reducing condition. The blot was probed with HRP-conjugated anti-His antibody. kDa: kilodalton; M: protein marker; the lane number represents the pH of extraction buffer used for removing host protein contaminants from plant crude extract. Arrowhead indicates RuBisCO (Figure 15A) and plant-produced hEGF (Figure 15B).

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However, the ability to investigate the preliminary strategy in optimization of purification condition will enable future detailed studies of the scale-up process to ascertain an industrial manufacturing process that could be helpful to realize the benefits of plant expression system.

3.6 Cytotoxicity assay of hEGF produced from *N. benthamiana*

In order to evaluate the cytotoxic effect of plant-produced hEGF, a HaCaT human keratinocyte cell line was applied as an *in vitro* cell model in the present study. HaCaT cells are considered as an *in vitro* model of skin wound healing (Huang et al., 2018) (Colombo et al., 2017). In the skin epidermis, keratinocytes are a main component which involves in maintaining and restoring skin barrier after injury through re-epithelialization process. Studies have shown that hEGF directly increases the rate of reepithelialization (Braund et al., 2007), which is considered as an essential parameter for a successful wound repair (Pastar et al., 2014).

Here, the cytotoxic effects of the purified plant-produced hEGF were examined in HaCaT cells using the MTT assay. The tetrazolium-based colorimetric (MTT assay) is a tetrazolium compound which will be reduced to a formazan product by mitochondria activity. The concentration-dependent effects of plant-produced hEGF and commercial hEGF on the viability of HaCaT cells were tested by treating the cells with different concentration at 5, 10, 50 and 100 ng/mL and analyzed after 24 hr incubation. The untreated cells were considered as negative control. As shown in Figure 16, the result of cytotoxicity assay indicated that the plant-produced hEGF did not show any cytotoxicity to the HaCaT cells, irrespective of concentration tested which similar to commercial hEGF in all the tested concentrations (Appendix E).

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In conclusion, our recent data might serve as a basis for further experiments to explore the biological activities of purified plant-produced hEGF in wound healing therapy and encourage the translatability of *in vitro* and *in vivo* data for clinical development.

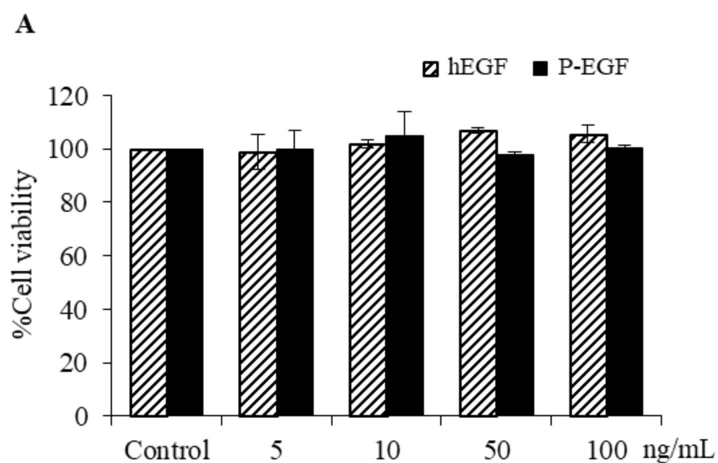

Figure 16. Cytotoxic effect of plant-produced hEGF
in HaCaT cells analyzed by MTT assay

Remark: After 24 h or treatment with plant-produced hEGF (P-EGF) and commercial hEGF (hEGF-control) at the concentration from 5, 10, 50 and 100 ng/mL. Values are shown as mean±SD of the percentage of HaCaT cell viability.

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CHAPTER 4
CONCLUSION

Plant molecular farming (PMF) has been used as a potential platform to produce therapeutic proteins. Besides low production cost and high scalability, the advantages of plants are low risk of human pathogen contamination and the ability of post-translational modifications which are essential for its biological activity. *N. benthamiana* has been shown its benefit in producing a variety of therapeutic proteins including growth factors. In this study, we used *N. benthamiana* as a bioreactor for human epidermal growth factor production.

The present study demonstrates that *Nicotiana*-based expression system is feasible to produce the functional hEGF protein using Agroinfiltration of a Geminiviral expression vector into *N. benthamiana.* Gene construct optimization was performed to improve the hEGF expression by targeting the protein expression in different subcellular organisms and varying the location of an N- or C-terminal His tag. The geminiviral expression vector was utilized for providing rapid hEGF expression. The optimal gene construct is "SP-EGF-H-KD" which provides the highest expression among 6 different constructs. The expression level was shown up to 15.695 μg/g LFW or 0.499% TSP was obtained from the optimized construct. This phenomenon has been demonstrated in Bai et al. 2007 that targeting to the ER system can improve the expression level. In a time course experiment, the hEGF gene also expressed at the highest level on 2 dpi before necrotic reaction was observed on the infiltrated leave.

The plant crude extract containing C-terminal His tagged hEGF protein was further purified using a single-step Ni-NTA affinity chromatography. Besides, the preliminary results in improving of hEGF purification from *N. benthamiana* were also provided including high extraction volume, ammonium sulfate precipitation, and acid

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precipitation. However, rigorous experimentations are required to support these preliminary results including the further study of purification optimization, scale-up experiments. These studies are needed to improve the quality and quantity of recombinant proteins to ascertain an industrial manufacturing process that could be helpful to realize the benefits of plant expression system.

Moreover, *N. benthamiana* is feasible to produce the hEGF protein without any cytotoxic effect in Human keratinocyte (HaCaT) cell treated with the concentration ranging from 5, 10, 50 and 100 ng/mL. Altogether, the present study demonstrates that the potential of using a transient expression system for production of hEGF protein can be exploited in further studies for their application in tissue engineering and cosmeceuticals.

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APPENDICES

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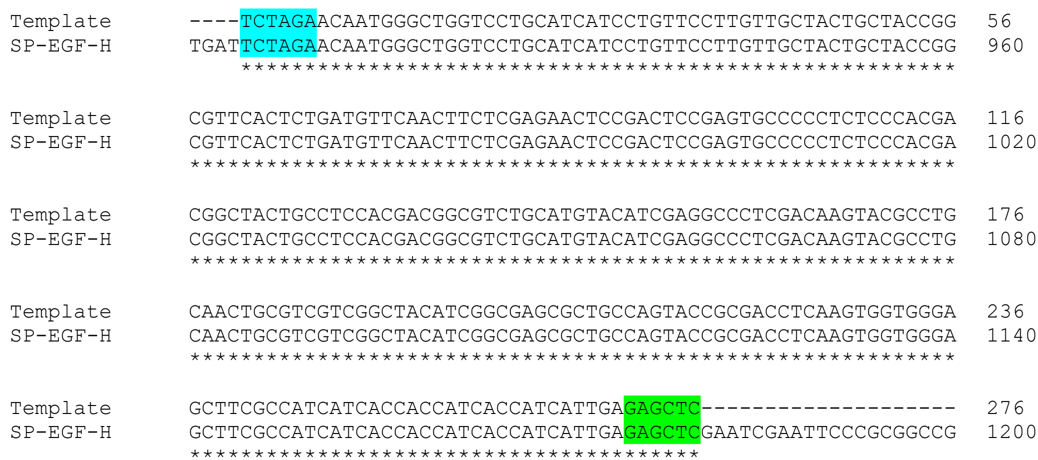
APPENDIX A

The nucleotide sequence alignment of six different hEGF nucleotide sequences

The nucleotide sequence alignment of the six different hEGF sequence for *N. benthamiana* including N-terminal signal peptide (SP), histidine tag (H), and C-terminal SEKDEL ER retention sequence (KD). Dots represent identical nucleotides. Highlight color represents restriction enzyme site of *Xba*I (Blue) and *Sac*I (Green).

1. SP-EGF-H



```
Template        ----TCTAGAACAATGGGCTGGTCCTGCATCATCCTGTTCCTTGTTGCTACTGCTACCGG   56
SP-EGF-H        TGATTCTAGAACAATGGGCTGGTCCTGCATCATCCTGTTCCTTGTTGCTACTGCTACCGG   960
                    ********************************************************

Template        CGTTCACTCTGATGTTCAACTTCTCGAGAACTCCGACTCCGAGTGCCCCCTCTCCCACGA   116
SP-EGF-H        CGTTCACTCTGATGTTCAACTTCTCGAGAACTCCGACTCCGAGTGCCCCCTCTCCCACGA   1020
                ************************************************************

Template        CGGCTACTGCCTCCACGACGGCGTCTGCATGTACATCGAGGCCCTCGACAAGTACGCCTG   176
SP-EGF-H        CGGCTACTGCCTCCACGACGGCGTCTGCATGTACATCGAGGCCCTCGACAAGTACGCCTG   1080
                ************************************************************

Template        CAACTGCGTCGTCGGCTACATCGGCGAGCGCTGCCAGTACCGCGACCTCAAGTGGTGGGA   236
SP-EGF-H        CAACTGCGTCGTCGGCTACATCGGCGAGCGCTGCCAGTACCGCGACCTCAAGTGGTGGGA   1140
                ************************************************************

Template        GCTTCGCCATCATCACCACCATCACCATCATTGAGAGCTC--------------------   276
SP-EGF-H        GCTTCGCCATCATCACCACCATCACCATCATTGAGAGCTCGAATCGAATTCCCGCGGCCG   1200
                ****************************************
```

2. SP-EGF-H-KD

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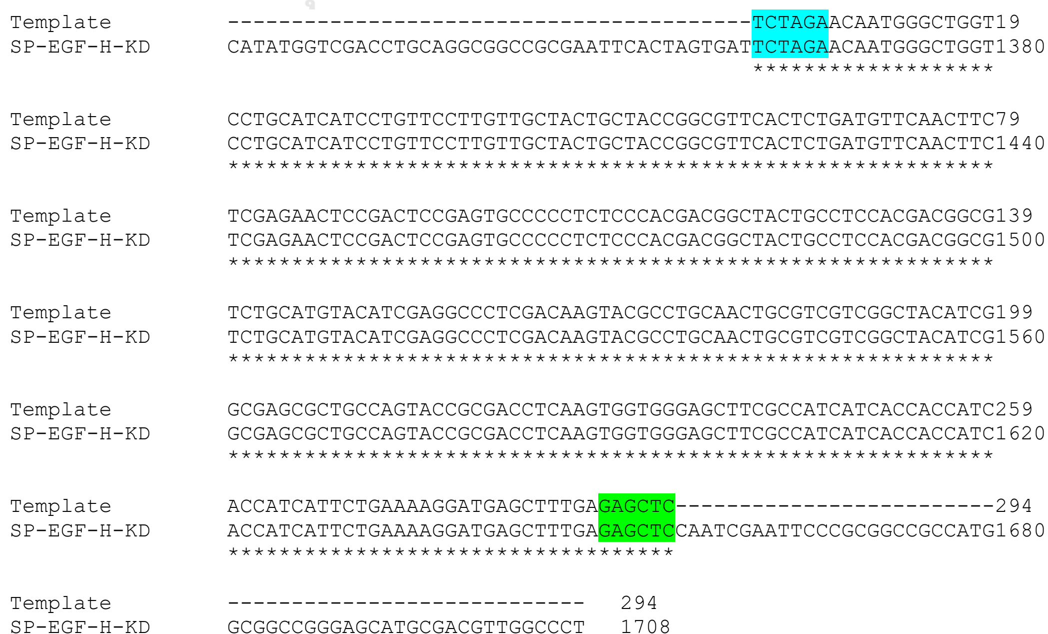

```
Template        -----------------------------------------TCTAGAACAATGGGCTGGT19
SP-EGF-H-KD     CATATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGATTCTAGAACAATGGGCTGGT1380
                                                         *******************

Template        CCTGCATCATCCTGTTCCTTGTTGCTACTGCTACCGGCGTTCACTCTGATGTTCAACTTC79
SP-EGF-H-KD     CCTGCATCATCCTGTTCCTTGTTGCTACTGCTACCGGCGTTCACTCTGATGTTCAACTTC1440
                ************************************************************

Template        TCGAGAACTCCGACTCCGAGTGCCCCCTCTCCCACGACGGCTACTGCCTCCACGACGGCG139
SP-EGF-H-KD     TCGAGAACTCCGACTCCGAGTGCCCCCTCTCCCACGACGGCTACTGCCTCCACGACGGCG1500
                ************************************************************

Template        TCTGCATGTACATCGAGGCCCTCGACAAGTACGCCTGCAACTGCGTCGTCGGCTACATCG199
SP-EGF-H-KD     TCTGCATGTACATCGAGGCCCTCGACAAGTACGCCTGCAACTGCGTCGTCGGCTACATCG1560
                ************************************************************

Template        GCGAGCGCTGCCAGTACCGCGACCTCAAGTGGTGGGAGCTTCGCCATCATCACCACCATC259
SP-EGF-H-KD     GCGAGCGCTGCCAGTACCGCGACCTCAAGTGGTGGGAGCTTCGCCATCATCACCACCATC1620
                ************************************************************

Template        ACCATCATTCTGAAAAGGATGAGCTTTGAGAGCTC-------------------------294
SP-EGF-H-KD     ACCATCATTCTGAAAAGGATGAGCTTTGAGAGCTCCAATCGAATTCCCGCGGCCGCCATG1680
                ***********************************

Template        ----------------------------   294
SP-EGF-H-KD     GCGGCCGGGAGCATGCGACGTTGGCCCT   1708
```

3. SP-H-EGF

```
Template        -------------------------------------------------------TCTAG 5
SP-H-EGF        ATTTGGGCGAACTCGCATGCTCCGGCCGCCATGGCGGCCGCGGGAATTCGATTCCTCTAG 60
                                                                       *****

Template        AACAATGGGCTGGTCCTGCATCATCCTGTTCCTTGTTGCTACTGCTACCGGCGTTCACTC 65
SP-H-EGF        AACAATGGGCTGGTCCTGCATCATCCTGTTCCTTGTTGCTACTGCTACCGGCGTTCACTC 120
                ************************************************************

Template        TGATGTTCAACTTCTCGAGCATCATCACCACCATCACCATCATAACTCCGACTCCGAGTG 125
SP-H-EGF        TGATGTTCAACTTCTCGAGCATCATCACCACCATCACCATCATAACTCCGACTCCGAGTG 180
                ************************************************************

Template        CCCCCTCTCCCACGACGGCTACTGCCTCCACGACGGCGTCTGCATGTACATCGAGGCCCT 185
SP-H-EGF        CCCCCTCTCCCACGACGGCTACTGCCTCCACGACGGCGTCTGCATGTACATCGAGGCCCT 240
                ************************************************************

Template        CGACAAGTACGCCTGCAACTGCGTCGTCGGCTACATCGGCGAGCGCTGCCAGTACCGCGA 245
SP-H-EGF        CGACAAGTACGCCTGCAACTGCGTCGTCGGCTACATCGGCGAGCGCTGCCAGTACCGCGA 300
                ************************************************************

Template        CCTCAAGTGGTGGGAGCTTCGCTGAGAGCTC----------------------------- 276
SP-H-EGF        CCTCAAGTGGTGGGAGCTTCGCTGAGAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCG 360
                **************************
Template        ------------------------------------------------------------ 276
SP-H-EGF        ACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACC 420
```

4. SP-H-EGF-KD

```
Template        --------------------------------------------------TCTAGAACAA10
SP-H-EGF-KD     TTGGCGACTCCATGCTCCGGCCGCCATGGCGGCCGCGGGAATTCGATTCCTCTAGAACAA60
                                                                  **********

Template        TGGGCTGGTCCTGCATCATCCTGTTCCTTGTTGCTACTGCTACCGGCGTTCACTCTGATG70
SP-H-EGF-KD     TGGGCTGGTCCTGCATCATCCTGTTCCTTGTTGCTACTGCTACCGGCGTTCACTCTGATG120
                ************************************************************

Template        TTCAACTTCTCGAGCATCATCACCACCATCACCATCATAACTCCGACTCCGAGTGCCCCC130
SP-H-EGF-KD     TTCAACTTCTCGAGCATCATCACCACCATCACCATCATAACTCCGACTCCGAGTGCCCCC180
                ************************************************************

Template        TCTCCCACGACGGCTACTGCCTCCACGACGGCGTCTGCATGTACATCGAGGCCCTCGACA190
SP-H-EGF-KD     TCTCCCACGACGGCTACTGCCTCCACGACGGCGTCTGCATGTACATCGAGGCCCTCGACA240
                ************************************************************

Template        AGTACGCCTGCAACTGCGTCGTCGGCTACATCGGCGAGCGCTGCCAGTACCGCGACCTCA250
SP-H-EGF-KD     AGTACGCCTGCAACTGCGTCGTCGGCTACATCGGCGAGCGCTGCCAGTACCGCGACCTCA300
                ************************************************************

Template        AGTGGTGGGAGCTTCGCTCTGAAAAGGATGAGCTTTGAGAGCTC----------------294
SP-H-EGF-KD     AGTGGTGGGAGCTTCGCTCTGAAAAGGATGAGCTTTGAGAGCTCCCAACGCGTTGGATGC360
                ********************************************

Template        ------------------------------------------------------------294
SP-H-EGF-KD     ATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGT420
```

5. EGF-H

```
Template      ------------------TCTAGAACAATGAACTCCGACTCCGAGTGCCCCCTCTCCCAC   42
EGF-H         TTTGAAATTTCTGCAACATCTAGAACAATGAACTCCGACTCCGAGTGCCCCCTCTCCCAC   720
                                ******************************************

Template      GACGGCTACTGCCTCCACGACGGCGTCTGCATGTACATCGAGGCCCTCGACAAGTACGCC   102
EGF-H         GACGGCTACTGCCTCCACGACGGCGTCTGCATGTACATCGAGGCCCTCGACAAGTACGCC   780
              ************************************************************

Template      TGCAACTGCGTCGTCGGCTACATCGGCGAGCGCTGCCAGTACCGCGACCTCAAGTGGTGG   162
EGF-H         TGCAACTGCGTCGTCGGCTACATCGGCGAGCGCTGCCAGTACCGCGACCTCAAGTGGTGG   840
              ************************************************************

Template      GAGCTTCGCCATCATCACCACCATCACCATCATTGAGAGCTC------------------   204
EGF-H         GAGCTTCGCCATCATCACCACCATCACCATCATTGAGAGCTCGAAGTGACATCACAAAGT   900
              ******************************************

Template      -----------------------------------        204
EGF-H         TGAAGGTAATAAAGCCCAAATAAATTAAGGTATTC        935
```

6. H-EGF

```
Template      ----------------------------------------------------TCTAGAAT   8
H-EGF         ATGTGGCCAAATCGCATGCTCCGGCGCCATGGCGGCCGCGGGAATTCGATTCTCTAGAAT   60
                                                                  ********

Template      GCATCATCACCACCATCACCATCATAACTCCGACTCCGAGTGCCCCCTCTCCCACGACGG   68
H-EGF         GCATCATCACCACCATCACCATCATAACTCCGACTCCGAGTGCCCCCTCTCCCACGACGG   120
              ************************************************************

Template      CTACTGCCTCCACGACGGCGTCTGCATGTACATCGAGGCCCTCGACAAGTACGCCTGCAA   128
H-EGF         CTACTGCCTCCACGACGGCGTCTGCATGTACATCGAGGCCCTCGACAAGTACGCCTGCAA   180
              ************************************************************

Template      CTGCGTCGTCGGCTACATCGGCGAGCGCTGCCAGTACCGCGACCTCAAGTGGTGGGAGCT   188
H-EGF         CTGCGTCGTCGGCTACATCGGCGAGCGCTGCCAGTACCGCGACCTCAAGTGGTGGGAGCT   240
              ************************************************************

Template      TCGCTGAGAGCTC-----------------------------------------------   201
H-EGF         TCGCTGAGAGCTCGAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAG   300
              *************
```

APPENDIX B

Preparation of polyacrylamide gel electrophoresis

1. **Stock reagent**

1.5 M Tris-HCl pH8.8

Tris-base 181.71 g

Adjust pH to 8.8 with concentrated HCl and adjust volume to 100 mL with distilled water.

10%w/v SDS solution

SDS 10 g

Add distilled water to the final volume of 100 mL

10% Ammonium persulfate ($(NH_4)_2S_2O_8$)

Ammonium persulfate 1 g

Add distilled water to the final volume of 10 mL

2. **6-18% gradient SDS-PAGE gel**

Ingredients	6%	8%	10%	12%	15%	18%
H_2O	2.850	2.600	2.320	2.100	1.720	1.348
40% Acrylamide	0.750	0.980	1.280	1.500	1.880	2.250
1.5 M Tris (pH 8.8)	1.300	1.300	1.300	1.300	1.300	1.300
10% SDS	0.050	0.050	0.050	0.050	0.050	0.050
10% APS	0.050	0.050	0.050	0.050	0.050	0.050
TEMED	0.004	0.003	0.002	0.002	0.002	0.002

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3. 10x reducing loading buffer

125 mM Tris-HCl, pH7.4

12% w/v SDS

10% v/v Glycerol

22% v/v beta-mercaptoethnol

0.001% w/v bromophenol blue

4. 10x running buffer

Tris-base	30	g
Glycine	144	g
SDS	10	g

Adjust volume with distilled water to final volume of 1,000 mL.

5. 1x running buffer

10x running buffer	100	mL

Adjust volume with distilled water to final volume of 1,000 mL.

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APPENDIX C

Preparation of Western blot analysis reagents

1. 20x transfer buffer

Tris-base	30	g
Glycine	144	g

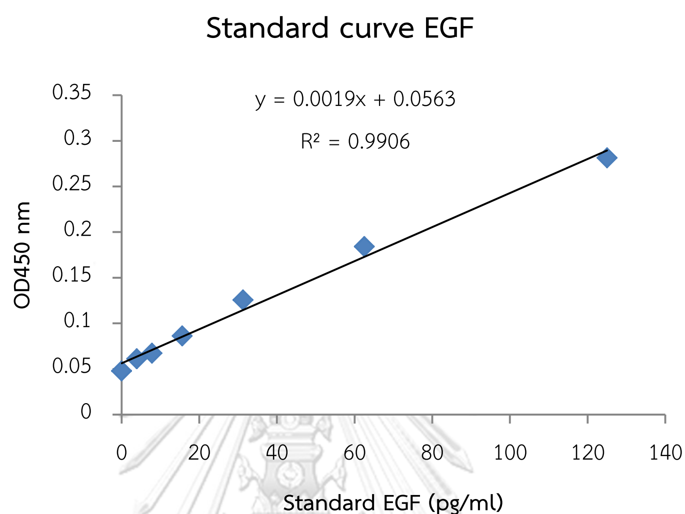
Adjust volume with distilled water to final volume of 1,000 mL

2. 1xtransfer buffer

20x transfer buffer	50	mL
Methanol	150	mL
DI water	850	mL

APPENDIX D

hEGF quantification from the optimal gene construct (SP-EGF-H-KD) using ELISA technique



OD_{450}	µg/ml	Fresh weight(g)	TSP (µg/ml)	%TSP	Volume (ml)	µg/g FW
SP-EGF-H-KD						
0.2011	3.810526	0.04471	855.4	0.445467	0.15	12.78414
0.2729	5.7	0.07052	970.91	0.587078	0.15	12.12422
0.2722	5.681579	0.03843	1222	0.464941	0.15	22.17634
Conclusion %TSP : Average = 0.499162%, SD = 0.076758 µg/g FW : Average = 15.6949%, SD = 5.622783						

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APPENDIX E

Cytotoxicity activity

The percentage of cell viability of HaCaT cells after treatment with plant-produced hEGF (P-EGF) and commercial hEGF (hEGF) at 5, 10, 50 and 100 ng/mL for 24 hr. Values are shown as mean and SD of the percentage of HaCaT cell viability.

Conc. (ng/mL)	P-EGF		hEGF	
	Mean	SD	Mean	SD
Control	100.000	0.000	100.000	0.000
5	99.647	6.976	98.772	6.351
10	104.592	9.321	101.677	1.473
50	97.732	0.780	106.842	0.803
100	100.240	0.873	105.515	3.460

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