Doctoral Dissertation

High level production methods and unique characteristics of intracellular and secreted acid-stable human basic fibroblast growth factor in *Nicotiana benthamiana*

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DECLARATION

"I hereby declare that this dissertation is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which has been used in it. This dissertation has also not been submitted for any degree in university/institution previously."

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2023

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List of abbreviations

bFGF	human basic fibroblast growth factor
CBB	Coomassie Brilliant Blue
CHO-K1	cell line isolated from the ovary of an adult, female Chinese hamster
ELISA	Enzyme-linked Immunosorbent Assay
ER	Endoplasmic Reticulum
ERAD	Endoplasmic reticulum-associated degradation
FGFR	Fibroblast Growth Factor Receptor
GGGbFGF	human basic fibroblast growth factor with GGGNDD and DGGGND motifs modified
HBS	Heparin binding site
Hep G2	Hepatoblastoma-derived cell line
HS	Heparan Sulfate
MAPK	Mitogen-activated protein kinase
MES	2-(*N*-morpholino)ethanesulfonic acid
PBS	Phosphate-Buffered Saline
PBST	Phosphate-Buffered Saline with 0.05% Tween-20
PCP	Plant Cell Packs
SDS-PAGE	Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis
SS^{HC}	human-derived antibody heavy chain signal peptide
SS^{FGF4}	FGF4 signal peptide
SS^{Ext}	*N. benthamiana* derived extensin signal peptide
SUMO	small ubiquitin-like modifier
T-DNA	Transferred DNA of the tumor-inducing (Ti) plasmid
ASbFGF	Acid-stable basic fibroblast growth factor
UPR	Unfolded-protein response

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Chapter 1 General Introduction

1.1 Recombinant protein expression in plants as an emerging technique for biopharmaceutical production

Plant molecular pharming/farming is defined as the production of recombinant proteins in genetically engineered plants or plant components (Moustafa et al. 2016). It is now an emerging alternative production system. It offers several advantages such as low cost biomass production; proper folding of eukaryotic proteins; and avoidance of animal pathogen contamination during production (Moustafa et al. 2016; Habibi et al. 2017; Shanmugaraj et al. 2020). Shown in Table 1 are the comparison of various parameters across different expression platforms. The overall cost of production in transgenic plants are known to be the lowest across all platforms. In addition, as compared specifically to bacterial production which is the typical production system to date, plant production systems have low risk of endotoxin contamination. This contributes to the lowering of the cost of production as endotoxin contamination could complicate downstream processing which accounts for about 45–92% of the total production costs (Schneier et al., 2020; Straathof, 2011; Saraswat et al., 2013; Wilding et al., 2019). In particular, Table 2 shows the production cost of antibodies across different platforms. It can be seen here that the cost of production of antibodies in plants are about 10-fold lower than in microbial fermentation. These reports all show why plants are indeed a cost-effective way to produce recombinant proteins.

Table 1. Comparison of different expression hosts for therapeutic protein production. (Ma et al. 2003; Shanmugaraj et al. 2014)

System	overall Cost	Production time scale	Scale-up Capacity	Product quality	Glycosylation	Contamination risks	Storage cost
Bacteria	Low	Short	high	Low	None	Endo toxins	Moderate
Yeast	Medium	Medium	High	Medium	Incorrect	Low Risk	Moderate
Mammalian cell culture	High	Long	very low	Very High	correct	Virues,Prions and oncogenic DNA	Expensive
Transgenic animals	high	Very long	low	Very High	correct	Virues,Prions and oncogenic DNA	Expensive
Plant cell cultures	Medium	Medium	Medium	High	Minor Differences	Low Risk	Moderate
Transgenic plants	Very low	Long	very high	High	Minor Differences	Low Risk	Inexpensive

Table 2. Production cost of therapeutic antibodies. (Hood et al. 2002; Shanmugaraj et al. 2014)

Production System	$/g
Chinese Hamster Ovary Cells (CHO)	300
Transgenic Chikens/Eggs	1-2
Transgenic goats/milk	1-2
Microbial fermentation	1.00
Plants	0.10

The commercialization of taliglucerase alfa (Protalix®, Israel; http://protalix.com/), the first FDA approved plant-produced product for enzyme replacement therapy (ERT) using stable plant suspension cells, solidified the potential of plant molecular pharming. On the other hand, stable transgenic production of recombinant proteins in plants, a transient production system offers a faster and higher level of production (Yamamoto et al. 2018). Various industrially important proteins have already been successfully produced via transient expression in plants such as α-amylase (Prado et al. 2019), Interleukin-6 (Nausch et al. 2012), antibodies (Vezina et al. 2009), HIV-1 Nef (Lombardi et al. 2009), eVLPs (Sainsbury and Lomonossoff 2008), and even COVID-19 proteins (Lindsay et al. 2020). Despite its benefits, the plant expression platform has yet to overcome its major challenges in terms of mass production and profitability (Habibi et al. 2017).

1.2 *N. benthamiana* as an efficient platform for protein bio manufacture in plants

N. benthamiana has long been a popular model plant in research. It is a native Australian species that belongs to the Solanaceae family, which also contains pepper, tomato, potato, and tobacco. It is an allotetraploid with a 19-chromosome genome, with *Nicotiana sylvestris* as its supposed maternal progenitor (Kelly et al. 2013). The *Nicotiana* genus' origins can be traced from South America. They subsequently expanded around the world, including Australia, and developed independently.

An intrinsic characteristic of *N. benthamiana* that makes it popular in research is its susceptibility to plant viruses (Bally et al. 2018). The LAB strain of *N. benthamiana* has been discovered to have a dysfunctional gene Rdr1 which codes for an RNA-dependent RNA polymerase. This dysfunction impairs the plant's ability to fight off viral infection (Akhtar et al. 2011) and makes it an ideal host for viruses that are utilized for altered gene expression and gene silencing. Incorporating a functional Rdr1 gene from *M. truncatula* into *N. benthamiana* LAB decreased the plant's hypersusceptibility to Tobamovirus spp. (Yang et al. 2004). This indicates that the plant's hypersusceptibility to more than 500 different plant viruses are indeed caused by the dysfunctional Rdr1 (Clemente 2006).

By taking advantage of the virus' replication system, viral vectors make it possible for foreign messenger RNA molecules to multiply in a plant, which promotes the development of extremely high amounts of proteins or peptides. As a result, *N. benthamiana* serves as a crucial molecular farming production platform due to its extreme viral susceptibility. Furthermore,

because of it is not a plant crop, the likelihood of target products contaminating the food supply is low.

The ability of *N. benthamiana* to quickly and highly express transgenes or gene-silencing signals that are contained within the boundaries of a T-DNA plasmid and delivered into the leaf by the incredibly straightforward method of Agrobacterium infiltration is another crucial characteristic that has given *N. benthamiana* such prominence. After being proven successful for transgenes in *N. benthamiana* in 1997, the method, which had its roots in the agroinfection of plant viruses, was given the new name of agroinfiltration (Kapila et al. 1997). Within 2-3 days, transgene expression starts in the cells of the infiltrated patch and continues for many days (Bally et al. 2018). Researchers have used it for a variety of purposes, such as gene silencing, protein localization, protein expression, protein-protein interactions, and protein purification. The method gives researchers a tool to investigate fundamental biological phenomena and gives biotechnologists a platform for producing proteins at a high level.

1.2 Secretion of recombinant proteins from plant cells

Protein secretion is a fundamental and conserved phenomena common among all life (Wang et al. 2017). This phenomena is used for proper development, homeostasis, cell to cell communication, and response to stress (Chaffin et al. 1998; Agrawal et al. 2010). This function has been utilized in recombinant protein production since this separates the protein of interest from the total protein of the cell (Raskin et al. 2003).

The entry point of the protein secretory pathway is via the endoplasmic reticulum (Brandizzi and Barlowe, 2013). This phenomenon is characterized by the co-translation of nascent proteins with an N-terminal signal peptide into the ER lumen through the Sec61 complex as observed in mammals and yeast (Shao and Hegde, 2011; Park and Rapoport, 2012; Zimmermann et al., 2011). Paralog of this complex has not been determined in plants yet (Wang et al. 2017).

Plant cell suspension cultures have good potential as biofactories for secreted protein. They came from either nontransgenic plants or plants that had been stabilizedly altered. Some of the stable transgenic lines have the genes expressing the desired bioproduct transiently given by agroinfection or agroinfiltration, whereas other stable transgenic lines contain the genes encoding a gene silencing suppressor, such as the extremely efficient P19 from TBSV (Bally et al. 2018).. Despite the fact that suspension cultures have some advantages over whole plants as biofactories in terms of cell type consistency and proliferation, they have so far produced underwhelming yields (Bally et al. 2018)..

Plant cell packs (PCPs) are a relatively new screening platform derived from plant suspension cell cultures devoid of liquid medium which supposedly provide a bridge between the practicality of plant cells and the scalability of full plants (Rademacher et al. 2019). In this method, plant cells are grown as suspension cell culture and the growth medium is drained to make porous, semi-dried plant cell aggregates. Subsequently, an agroinfiltration buffer containing transformed *Agrobacterium tumefaciens* which carries the gene of interest is mixed with the plant cells to initiate transient production of recombinant proteins (Gengenbach et al. 2020; Poborilova et al. 2020; Opdensteinen et al. 2022). This semi dry co-culture is then incubated for several days and the accumulated proteins are collected via a flow through of an extraction through a column

containing the PCPs (Figure 1). These proteins are the actual products or are used to manipulate a particular pathway to produce a certain metabolite. Due to the method's compatibility with microtiter plate formats, several gene variations, expression constructs, and process conditions can be quickly and thoroughly screened either singly or in combination (Gengenbach et al. 2020). PCPs can also be made into multi-milliliter (up to around 150 mL) column or cake shapes, which makes it easier to synthesize goods on a modest scale for preliminary testing (Gengenbach et al. 2019). Furthermore, PCPs are reported to produce secreted recombinant proteins more efficiently than their suspension cell culture counterparts (Gengenbach et al. 2019; Rademacher et al. 2019; Poborilova et al. 2020). This makes this platform a very attractive expression platform for secreted recombinant protein production.

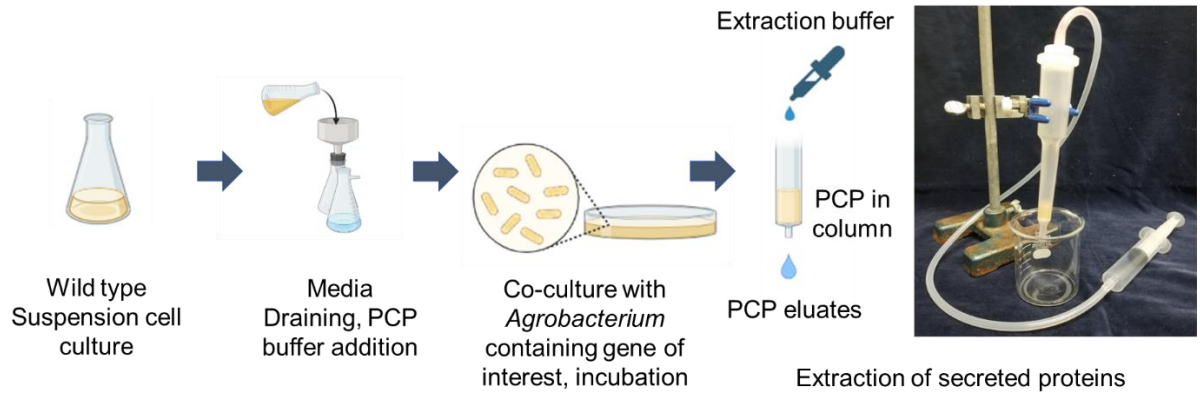


Figure 1. Conventional transient PCP system for producing secreted recombinant proteins.

1.3 Biochemical characteristics of human basic fibroblast factor

In mammalian cells, four different forms of basic fibroblast growth factor (bFGF or FGF-2) are produced as a result of an alternate translational initiation process combining one low molecular weight form (LMW) (155-amino acid form) and three high molecular weight form

(210-, 201- and 196-amino acid forms) (Patry et al. 1997). The intracellular biological activities of these multiple types of bFGF are distinct from one another though all forms activate the canonical FGFR/MAPK pathway and promote mitogenesis (Kole et al. 2017). Nevertheless, it is the cytoplasm-localized LMW form of bFGF that is widely used for commercialization.

The formation of a ternary complex consisting of the FGF, the FGFR, and the heparan sulfate (HS) co-receptor is required for the stimulation of cell proliferation (Yayon et al. 1991; Rapraeger et al. 1991). That the co-receptor is required for the ternary complex has since been demonstrated in vivo (Lin et al. 1999) and in the structures of co-crystalline ternary complexes (Schlessinger et al. 2000). Furthermore, the interactions of FGFs with HS in extracellular matrices regulate their transport from the source to the target cell (Duchesne et al. 2012; Eriksson et al. 1993). Glycosaminoglycans such as HS and its experimental proxy heparin are structurally similar and share the same disaccharide backbone, which is composed of a uronic acid, either L-iduronic or D-glucuronic acid, linked 1,4 to D-glucosamine. Interaction of FGF-2 with heparin is via five putative heparin-binding sites (HBS) which are characterized by the presence of positively charged amino acids like arginine and lysine which bind the sulfate residues of HS. These HBS are conserved in both basic (FGF-2) and acidic (FGF-1) growth factors. (Figure 2) (Xu et al. 2012).

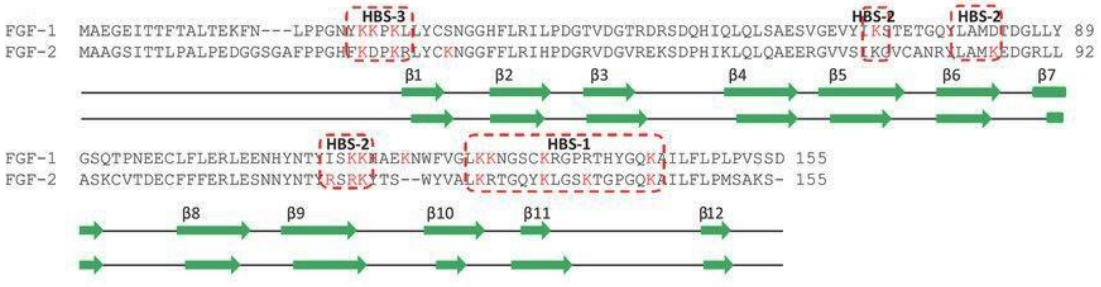


Figure 2. Heparin binding sites of FGF-1 and FGF-2 (Xu et al. 2012)

The bFGF is also a protein notorious for being prone to degradation (Carlini et al. 2016; Masuzawa & Kitazawa 2022) and aggregation. Truncated forms of bFGF has been observed during prolonged storage. A truncated form characterized by the loss of 9 N-terminal amino acids has been observed before by the storage of pure bFGF in pH 7 at 4 ^{o}C for as early as 2 weeks (Sauer et al. 2021). On the other hand, cleavage at Asp15 was observed when pure bFGF is stored at pH 4-5 for 6 weeks (Shahrokh et al. 1994).

1.4 Acid-stable bFGF

Mutations to the cysteine residues of bFGF to serine has been first discovered to improve its solubility and reduce the tendency to localize in exclusion bodies (Seno et al. 1988). One of these mutants, the bFGF-CS23 mutein where the second and third cysteines were replaced by serine was called the acid-stable bFGF. This nomenclature was due to the ability of this mutein to resist HCl degradation and maintain more activity post acid-exposure than the wild type version (Folkman et al 1991). Because of this, this mutant was used for various growth stimulation treatments which involve acidic environments such as the stomach ulcers (Hull et al 1995) and duodenal ulcers (Folkman et al 1991; Szabo et al 1994).

This stability of ASbFGF is not exclusive to acidic environments as the degradation resistance of ASbFGF was also observed at neutral pH (Folkman et al 1991). For context, bFGF contains two cysteines that are buried and two cysteines that are exposed. These second and third external cysteines are involved in disulfide-linked multimerization (Thompson & Fiddes 1991) and may contribute to precipitation (Sluzky et al. 1994). Therefore, replacement of these exposed

cysteines (Cys70 and Cys88) to serine may have decreased the tendency of bFGF to aggregate thus making it more stable.

1.5 Objectives of this study

This study aims to investigate and optimize two general ways to produce human bFGF in *Nicotiana benthamiana*. For the first part, transient production in whole plants was investigated and multi-parameter optimizations in the upstream and downstream processes were examined to maximize production of the cytoplasm localized recombinant protein. Here, the importance of a mutant protease-resistant mutant form of bFGF wherein external cysteines were replaced with serines was also explored to investigate its ability to improve the quantity and quality of the recombinant protein.

For the second part of this study, another mode of production which is secreted production system is also explored. This aims to simplify the extraction and purification process, in hopes of reducing the cost of production of the recombinant protein. Here, apoplast extraction in transiently expressing *N. benthamiana* leaves, suspension culture and PCPs are expression platforms that are investigated.

Chapter 2 High level transient production of acid-stable bFGF in *N. benthamiana* leaves

2.1 Introduction

bFGF is a member of the large FGF protein family. It has a strong affinity for heparin and bioactivities like controlling cell growth, differentiation, migration, and survival (Barrientos et al 2008). The Erk1/2 mitogen-activated protein kinase pathway, which is significant in controlling cell proliferation, has been implicated in these processes, despite the fact that the precise mechanisms underlying them are still unknown (Ma et al. 2019, Zaragosi et al. 2006). Because of its part in these processes, bFGF is very important to the fields of regenerative medicine and cosmetics (Levenstein et al. 2006). Initially, bFGF was isolated from the pituitary and brain of cows (Matsuda et al. 1992). But improvements in bioengineering allowed it to be produced on more widely available platforms, including *Pichia pastoris* (Mu et al. 2008), soybean seeds (Ding et al. 2006), silkworm (Wu et al. 2001), and *Bacillus subtilis* (Kwong et al. 2013). Today, it is produced commercially in *Corynebacterium glutamicum* and *Escherichia coli* (Ke et al. 1992).

Theoretically, the ideal expression platform for a production system would be one that accumulates large amounts of recombinant protein and is simple and affordable to cultivate. Significant levels of plant-produced bFGF were already attained in rice seeds at 9.6% of total soluble protein (TSP) (An et al. 2013) and soybeans at 2.3% of total soluble protein (TSP) (Ding et al. 2006). However, this method of production would not be very effective due to the small amount of seed biomass produced per unit of land and the lengthy growth cycles (Wang et al. 2015). On the other hand, recombinant production in tobacco leaves produces more biomass per

area of land. However, the previously reported yield of recombinant bFGF was only about 0.1% of TSP (Wang et al. 2015). Recently, transient bFGF production was observed in *N. benthamiana* leaves, albeit at a rate of only 2.7 μg/g FW (Rattanapisit et al. 2020). In order to make bFGF produced in plants more commercially viable for industrial manufacturing, this level of production needs to be increased further as much as possible.

Here, various ways to improve less-explored parameters in the production line of the transient production of bFGF in *N. benthamiana* was investigated. This includes the use of a supposedly mRNA-stable variant GGGbFGF, and a Cys70Ser, Cys88Ser mutant acid-stable bFGF (ASbFGF) in place of the wild type bFGF. The use of ASbFGF increased the production two-fold. The ASbFGF produced was purified through a tagless purification system and proven to be bioactive in cell proliferation tests.

2.2 Materials and Methods

2.2.1 Vector construction

The human bFGF variants bFGF, GGGbFGF, and ASbFGF used in this study (Figure 3A) were cloned using GeneArt gene synthesis from the 17.8 kDa isoform of human bFGF (Geenbank Accession No: AAQ73204.1) (Thermo Scientific, Waltham, MA). Following their ligation into a plant expression vector called pGPTV-bar, these were then electroporated (Bio-Rad, Tokyo, Japan) into the *A. tumefaciens* strain LBA4404 using voltage, capacitance, and resistance, settings of 2.4 kV, 25 F, and 200 Ω respectively. The vector containing RNA silencing suppressor 19 (p19)

was kindly provided by Prof. Atsushi Takeda from Ritsumeikan University. All DNA fragments were amplified via KOD polymerase (Toyobo, Osaka, Japan).

2.2.2 Recombinant protein production and optimization

Agroinfiltration buffer supplementation and extraction techniques were examined using the normal human bFGF variant to obtain the best possible conditions for bFGF production and extraction. *A. tumefaciens* LBA4404 was first grown about 2 days or until OD_{600} reached 1.0 in yeast mannitol broth (Hoben and Somasegaran 1982) containing rifampicin (50 μg/mL), streptomycin (200 μg/mL) and kanamycin (100 μg/mL). This strain of *A. tumefaciens* contained a plasmid carrying human bFGF with p19. The *A. tumefaciens* were then rinsed and either resuspended in the standard infiltration buffer (10 mM $MgSO_4$, 10 mM MES, pH 5.8) or in the supplemented infiltration buffer (10 mM $MgSO_4$, 10 mM MES, pH 5.8, 0.03% Tween-20, 0.5 mM acetosyringone, and 0.56 mM ascorbic acid) based on a previous protocol (Zhao et al. 2017; Norkunas et al. 2018). Before use, the *A. tumefaciens* concentration was adjusted to have an OD_{600} of around 0.5. This was followed by a 4-hr incubation period at room temperature. At a 1:1 ratio, *A. tumefaciens* containing p19 was co-infiltrated into each bFGF construct. Then, vacuum infiltrated with the *A. tumefaciens* suspension mixtures for 3 min at 600 mmHg, *N. benthamiana* plants that had been grown in a greenhouse for about a month were incubated. Daily top and middle leaf samples were taken for 7 days and stored at -80 ^{o}C until use. Because it has the highest yield, the leaves agroifiltrated with the supplemented buffer and harvested at 6 days was used for the extraction method's optimization. The extraction buffer was 50 mM sodium phosphate pH 6.5, 100

mM NaCl, and 0.25% Tween-20, unless otherwise stated. Three ml of this buffer was added per gram of ground leaves and were subsequently sonicated (200 W; Kaijo Denki, Tokyo, Japan) in ice for 20 s. The bFGF yield was compared between different NaCl concentrations in this process to determine the best NaCl concentration. The yield of bFGF was also contrasted between the use of 10 min of mixing and 20 s of sonication in ice. The techniques that produced the highest levels of bFGF according to ELISA were combined and used for the succeeding experiments.

2.2.3 Comparison of recombinant protein production of different bFGF variants

The best agroinfiltration and extraction techniques were applied universally for the comparison of various bFGF variants. An extraction buffer containing 50 mM sodium phosphate pH 6.5, 100 mM NaCl, 0.25% Tween-20, and 20 s of sonication on ice was used to extract the total soluble proteins in the leaves after being agroinfiltrated for 6 days with the supplemented infiltration buffer.

2.2.4 Protein analysis by indirect ELISA

The agroinfiltrated leaves were homogenized and suspended in the extraction buffer devoid of Tween-20. This was then placed on ice and given a 20 s sonication (200 W; Kaijo Denki). The concentrations of the crude extract were then fixed to approximately 2 mg/mL of total protein. These crude extracts were coated in triplicates in an exact quantity of 50 μL on a 96-well plate. After being incubated for 30 mins at 37 ^{o}C, this was blocked for an additional 30 mins with 5% bovine serum albumin. Incubation using the primary antibody anti-FGF2 mouse IgG (Santa Cruz

Biotechnology, Dallas, TX) diluted at 1/600 of the total volume was done overnight at 4 ^{o}C with shaking in PBST. Incubation with the secondary antibody was done for an hr at room temperature with 1/10,000 anti-mouse HRP IgG from sheep (GE Healthcare, Chicago, IL) in PBST. The ortho-phenylenediamine substrate (Sigma-Aldrich, St. Louis, MO) was then added to the wells, incubated for 15 min and absorbance is read at 450 nm using a microplate reader (Bio-Rad, Hercules, CA). The absorbance was then read at 450 nm. Creation of the standard curve was made using *E. coli*-derived bFGF (Nacalai Tesque Inc., Kyoto, Japan) mixed with a concentration of 2 μg/L non-agroinfiltrated crude extracts in PBS.

2.2.5 Protein analysis by SDS-PAGE and Western blot

All sampled fractions underwent 15% Tris-glycine, sodium dodecyl sulfate, and polyacrylamide gel electrophoresis (SDS-PAGE) at 120 V for 90 min. Following that, Coomasie Brilliant Blue (CBB) or silver staining was used to visualize the total protein content of the gels. The proteins from the resulting SDS-PAGE gel in this instance were transferred to a PVDF membrane (Merck Millipore, Burlington, MO). The membrane was subsequently blocked using 5% skim milk in PBST. The membrane was then incubated with monoclonal primary antibody anti-bFGF mouse IgG at a dilution of 1:300 overnight at 4 ^{o}C. Following washing steps, it was incubated for 1 hr at room temperature with the secondary antibody anti-mouse-HRP IgG at a dilution of 1: 10,000. Afterwards, Luminata^{TM} Forte Western HRP substrate (Merck Millipore) was added to the membrane to visualize bFGF bands, and the iBright 1500 Imaging System (Thermo Fisher) was used to detect the chemiluminescence.

2.2.6 Stability analysis in crude extracts

SDS-PAGE and ELISA analyses of liquid nitrogen chilled, ground, 6-day agroinfiltrated leaves using bFGF and ASbFGF variants were performed to compare the stability of bFGF and ASbFGF in crude leaf extracts. With a few minor modifications, this technique was based on an *in vitro* analysis of antibody degradation using crude tobacco leaf extracts (Stevens et al. 2000). The samples of crushed leaves were placed in centrifuge tubes and incubated for 6 hrs at room temperature, with samples being taken every hr. The corresponding volume of Tween-20-free extraction buffer was then added to bring the total protein concentration to 2 μg/L. SDS-PAGE and ELISA tests were performed on this extract to determine its bFGF content.

2.2.7 Purification by SP-ion exchange chromatography followed by Heparin affinity chromatography

The resulting crude extract was directly run and semi-purified using an SP cation exchange column using a method previously used in *E. coli* (Seeger & Rinas 1996) with significant modifications for *N. benthamiana* leaf extracts due to the relative instability of bFGF. After being adjusted to pH 6.5, the crude leaf extracts were immediately passed through a 5 mL (1.0 cm x 12.0 cm) SP 650-M cation exchange column (Tosoh Co., Ltd., Tokyo, Japan) that had been pre-equilibrated with 20 mL extraction buffer. This was done as opposed to ammonium precipitation followed by dialysis, which could result in yield losses for the bFGF due to precipitation. Following that, an initial wash using the extraction buffer (50 mM sodium phosphate pH 6.5, 100 mM NaCl, 0.25% Tween-20) containing additional 0.1% Triton X-100 was performed at a volume

that was roughly three times that of the column, or until all green residues were removed. The extraction buffer without 0.1% Triton X-100 was then used three more times for additional washing. The column was then washed with 4 column volumes of wash buffer (50 mM phosphate buffer pH 6.5 with 0.25 M NaCl) and then subsequently eluted using elution buffer (50 mM phosphate buffer pH 7.5 with 0.5 M NaCl).

Dilution using a 50 mM phosphate buffer pH 8.0 was done in the partially purified SP elution fractions to decrease its NaCl concentration from 0.5 M to around 0.2 M. This diluted fraction was then purified using a 1 mL Heparin Sepharose (GE Healthcare) column according to a procedure modified slightly from a previous study on the purification of recombinant bFGF in *E. coli* (Seeger and Rinas 1996). After running through the column, it was then washed with four column volumes of 50 mM phosphate buffer pH 8.0 with 1.0 M NaCl. Elution of the purified recombinant protein was accomplished using 50 mM phosphate buffer pH 8.0 and 2.0 M NaCl. For later use, the heparin eluent was kept in storage at -80 ^{o}C.

2.2.8 Cell proliferation assay

A cell proliferation assay, similar to that used in a previous study, was conducted to evaluate the bioactivity of ASbFGF (Liu and Wu 2009). In a nutshell, around $5x10^3$ human hepatocellular carcinoma (Hep G2) cells and $1x10^5$ Chinese hamster ovary K1 (CHO-K1) cells were first seeded in 24-well plates with growth media Hams-F12 and Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific) containing 4.5 g/L glucose, respectively, for 24 hrs. Both media also contained 10% fetal bovine serum. These media were subsequently drained, washed

twice with their respective serum-free media and three times with phosphate buffered saline. The following day, CHO-K1 cells were incubated with serum-free Hams-F12 at 37 °C and 5% CO_2. The same conditions were used to incubate Hep G2 cells derived from human liver cancer tissue (Chengye et al. 2017), but they were given DMEM-high glucose with 0.1% FBS instead of medium. Purified ASbFGF were quantified via ELISA and were added at 5 ng/mL and 20 ng/mL concentrations for CHO-K1 cells and Hep G2 cells respectively. For comparison, same amounts of commercial *E. coli*-produced bFGF (Nacalai Tesque Inc.) were added as a positive control. Trypan blue exclusion assay was done using an automatic cell counter (Biorad, Hercules, CA) and were used to count the number of viable cells and percent viability every 2 days for 10 days.

2.3 Results

2.3.1 Optimization of agroinfiltration and extraction procedures

Agroinfiltration was improved by comparing the bFGF accumulation time course in agroinfiltrated leaves when a supplemented buffer containing 0.03% Tween-20, 0.56 mM ascorbic acid, and 0.5 mM acetosyringone was used as opposed to a conventional buffer containing only 10 mM MES and 10 mM $MgSO_4$. In these tests, the constant variable was the expression vector pGPTV-bar-bFGF, which contained the non-optimized form of bFGF (Figure 3A).

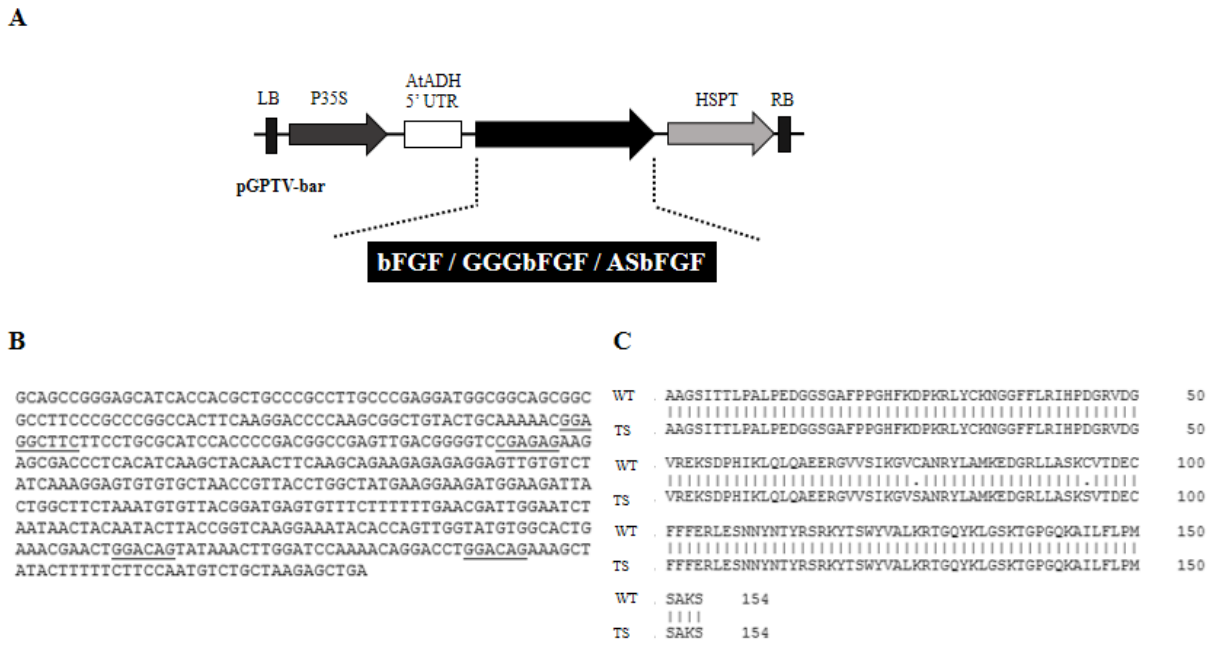


Figure 3. The bFGF variant information and T-DNA of expression constructs. (A) Diagrams of no signal peptide constructs that express 18 kDa bFGF in the wild type (bFGF), GGG-optimized (GGGbFGF), and acid-stable forms are shown (ASbFGF). The recombinant gene is controlled by the *Arabidopsis thaliana* heat shock protein 18.2 terminator (HSPT) and the cauliflower mosaic virus 35S promoter (P35S) (HSPT). The translational enhancer *A. thaliana* dehydrogenase 5' UTR (AtADH 5' UTR) and the T-DNA left and right border (LB and RB), which delineate the boundaries of the transgenes transferred during *A. tumefaciens*-mediated transformation, are additional components. (B) GGG's optimized bFGF DNA sequence. The sequences that have been identified as GGGNDD or DGGGND and whose GGG sequences have been changed to GGA

were underlined. (C) Cys70 and Cys88 have been changed to Ser70 and Ser88, respectively, in the amino acid sequence alignment of wild type (WT) and acid-stable (AS) bFGF. An initiation Met was added at the N-terminal of all bFGF variants.

The findings demonstrated that, at all-time points, bFGF production was higher when using supplemented buffer compared to those using conventional buffer. At 6 days post infiltration, peak yield at about 260% increase was observed. Additionally, bFGF production increased linearly each day until it eventually started to decline, when using the conventional buffer setting the decline 2 days earlier (Figure 4). This demonstrates how the various supplements in the supplemented buffer work together to increase the production of bFGF. The acetosyringone and Tween-20, in the supplemented buffer might have enabled more efficient T-DNA transfer. This resulted in higher yields of bFGF at each time point. Ascorbic acid, on the other hand, might have given the leaves more time to produce more of the recombinant protein because it has been demonstrated to mitigate stress due to agroinfiltration and to postpone necrosis in agroinfiltrated leaves (Norkunas et al. 2018; Nosaki 2021).

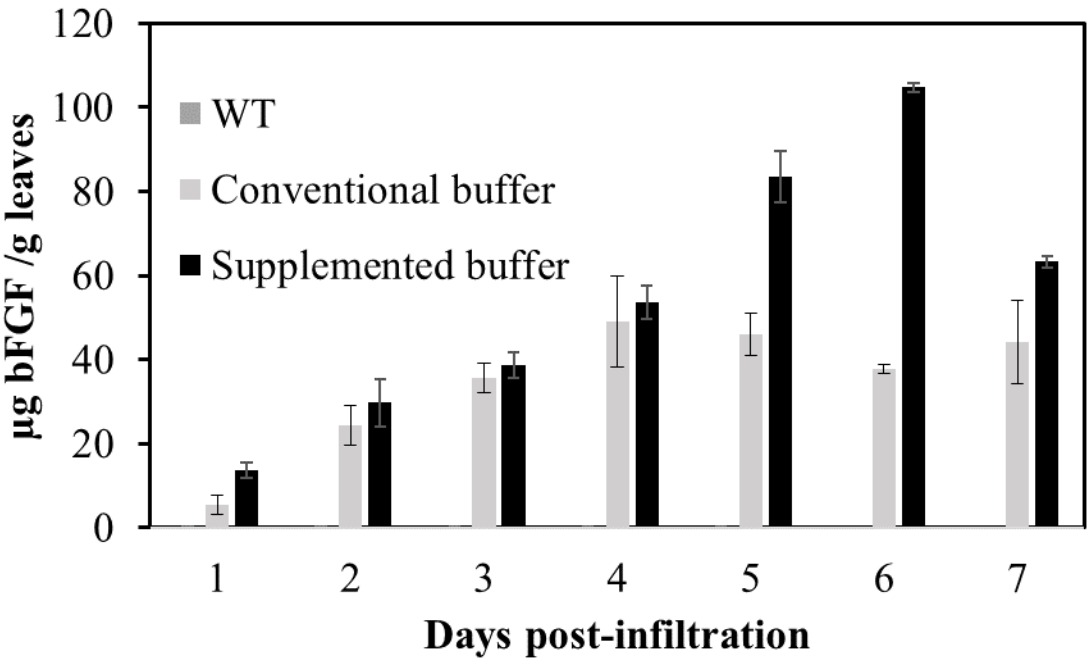


Figure 4. Production of bFGF over time in a supplemented buffer versus a regular buffer after infiltration. Wild type (WT) leaf crude extracts were used as a negative control. Three biological triplicate samples' mean values and standard deviation are displayed.

Similar to how specific upstream processes can be optimized to increase bFGF accumulation, downstream procedures must be optimized to maximize the amount of usable bFGF recovered from leaf crude extracts. The hydrophobic core of bFGF makes it susceptible to oxidation, which makes it more likely to aggregate and precipitate (Zhang et al. 1991; Shahrokh et al. 1994). Proteins' exposed residues have been shown to be rearranged by the presence of salt in the buffer, which may increase their stability and solubility (Jelesarov et al. 1998; Dahal and Schmit 2018). In this study, it was examined how different NaCl buffer concentrations affected the solubility of bFGF extracted. A 100 mM NaCl concentration was found to be the ideal level for the extraction buffer (Figure 5), as salt concentrations above this level no longer affected the

amount of soluble bFGF extracted. Meanwhile, it has been demonstrated that sonication enhances protein recovery from plant biomass (Byanju et al. 2020).

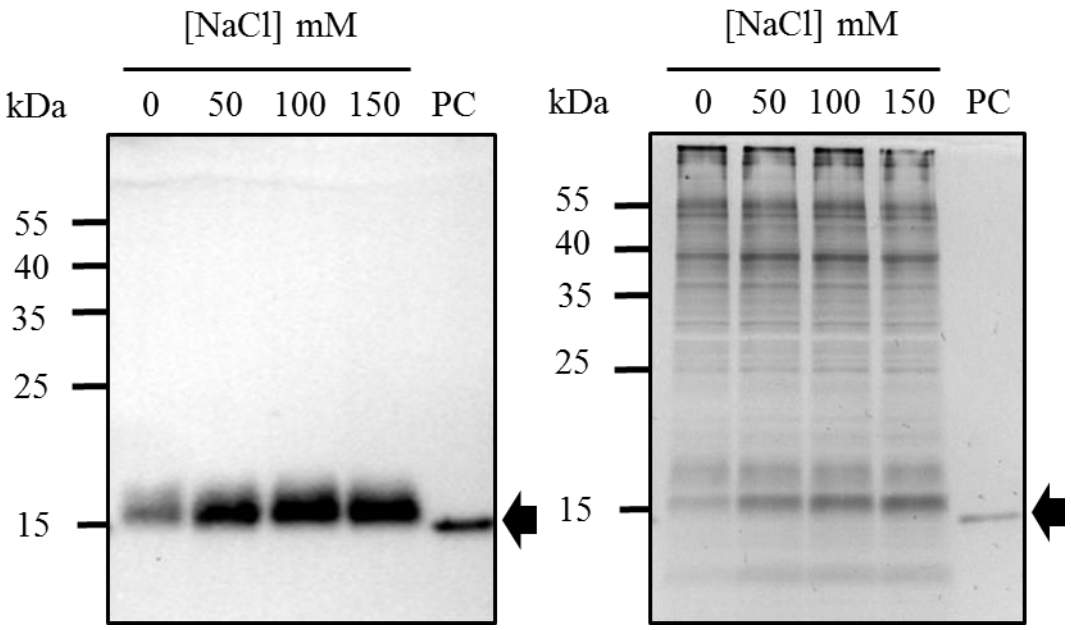


Figure 5. Optimization of NaCl concentration in the extraction buffer. For the positive control (PC), 25 ng of bFGF derived from *E. coli* was loaded.

Comparison of this method to simple mechanical agitation for 10 min, to increases the recoverable bFGF was investigated. The outcome showed that bFGF recovery from sonicated extracts of 20 s was three times greater than that from mixed extracts of 10 mins (Figure 6).

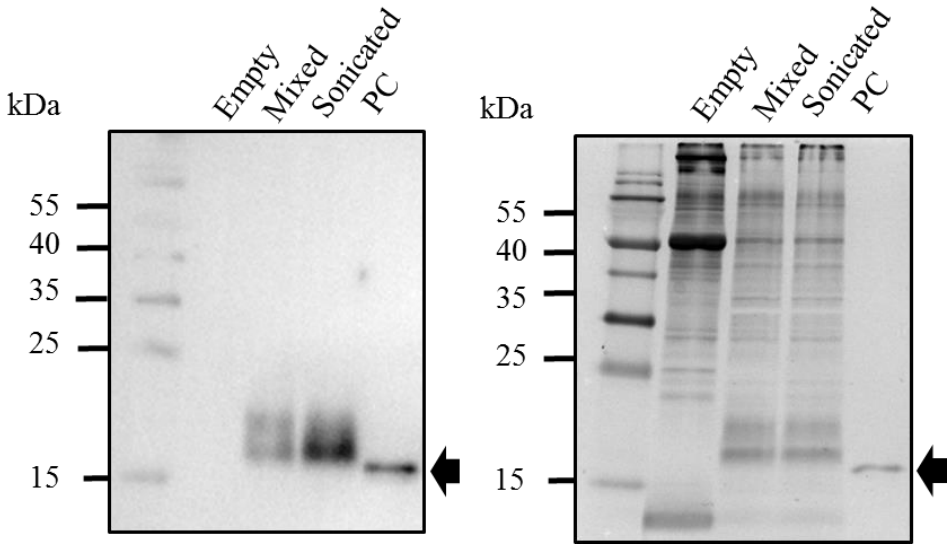


Figure 6. Comparison of the amount of bFGF extracted using 10 mins of mixing vs. 20 s of sonication on ice. For the positive control (PC), 25 ng of bFGF derived from *E. coli* was loaded. For the negative control, crude extracts of leaves agroinfiltrated with only the empty vector (Empty) was used.

Combining these results, a better extraction technique was created that maximizes the extraction of bFGF using an extraction buffer composed of 100 mM NaCl, 50 mM sodium phosphate pH 6.5, and 0.1% Tween-20, followed by 20 s of sonication on ice (Figure 7). These parameters was used in the subsequent experiments.

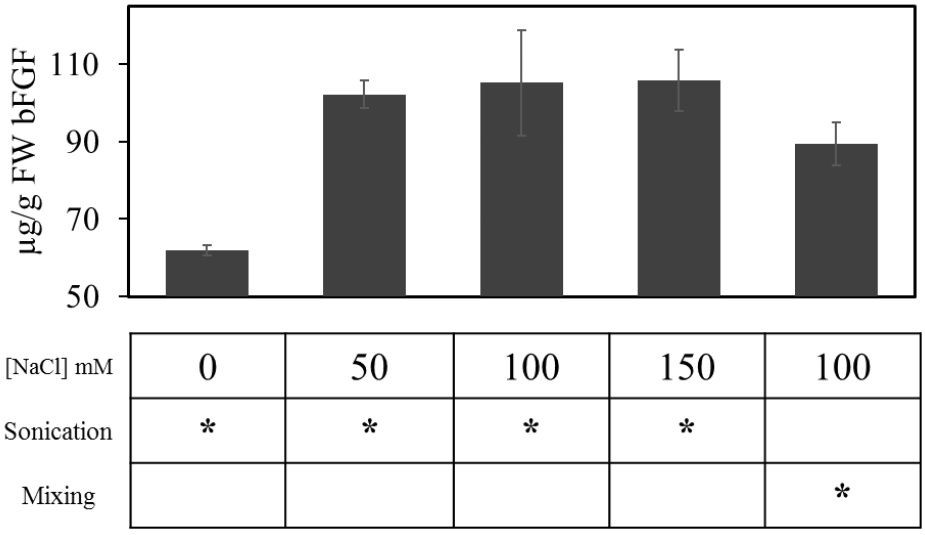


Figure 7. ELISA analysis of the best bFGF extraction buffer in leaf extracts from agroinfiltrated *N. benthamiana*. Three triplicate samples' mean values and standard deviation were displayed.

2.3.2 Comparison of transient bFGF production across bFGF variants

The difference in recombinant protein yield between wild type bFGF and two additional bFGF variants, GGGbFGF and ASbFGF, was identified following optimization of the agroinfiltration and extraction processes. In order to remove the GGGNDD and DGGGND (Ueno et al. 2020) motifs from bFGF RNA, where (N = A/G/C/T and D = G/A/T), GGGbFGF (Figure 3B) was created. As previously reported, these motifs are susceptible to deterioration (Ueno et al. 2020). Therefore, by increasing the amount of bFGF mRNA transcript available, optimizing the transgene to not contain these motifs may aid in producing more recombinant protein. Meanwhile, replacement of external cysteines Cys70 and Cys88 to create the bFGF mutant ASbFGF (Figure 3C) was thought to confer protease resistance properties to the protein, reducing the rate at which it was degraded (Seno et al. 1988). Additionally, it was demonstrated that this mutant reduced the

probability of bFGF precipitation caused by cysteine interactions (Shahrokh et al. 1994). Even though bFGF has four cysteine residues, because Cys23 and Cys93 are located internally and are important for proper folding, replacement of these cysteines to serine led to misfolded bFGF. Therefore, to increase production of bFGF, the Cys70Ser and Cys88Ser mutant ASbFGF was chosen. The plant expression vector pGPTV-bar was modified to incorporate the GGGbFGF and ASbFGF variants for use in agroinfiltration.

Western blot, silver staining, and ELISA analyses of the crude extracts were used to compare the yield of the recombinant bFGF produced in these variants to that of non-optimized bFGF (Figure 8). The GGGbFGF variant produced only a comparable yield to the non-optimized bFGF when the yields of the three variations were compared and did not result to higher yield as expected. The factors that cause recombinant mRNA degradation may have already been neutralized due to the co-expression of the p19 RNA silencing suppressor, or the bFGF recombinant mRNA may already be stable. On the other hand, ASbFGF demonstrated the thickest protein bands at about 18 kDa, which is equivalent to bFGF, and produced the highest production at about 185 μg/g FW. This was approximately twice more productive than the non-optimized and GGGbFGF variants, producing about 100 μg/g FW.

All in all, these data show that production of ASbFGF rather than the normal bFGF would be a more feasible transient production system of bFGF in *N. benthamiana* leaves.

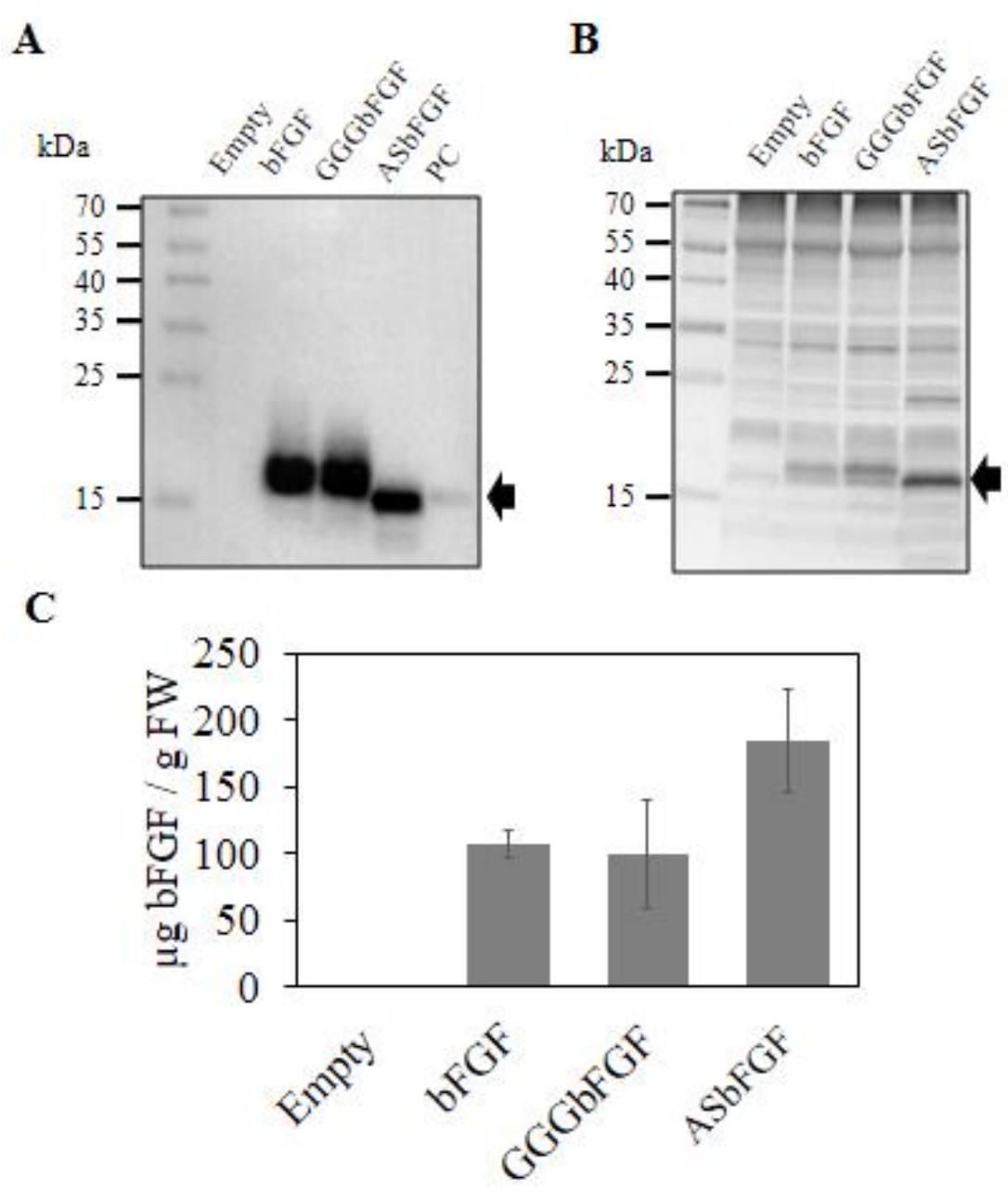


Figure 8. Comparing transient bFGF expression across variants in agroinfiltarted *N. benthamiana* leaves. Shown are here are the Western blot (A), silver staining (B) and ELISA (C). The bFGF variants used are the wild type (bFGF), GGG optimized (GGGbFGF), acid-stable (ASbFGF). For the positive control (PC), 25 ng of bFGF derived from *E. coli* was loaded. For the negative control, crude extracts of leaves agroinfiltrated with only the empty vector (Empty) was used. The data for panel C was presented as the mean and standard deviation of three independent measurements.

2.3.3 Stability analysis of ASbFGF and bFGF in crude extracts

Protease resistance in the mutant ASbFGF was predicted to increase the yield of recombinant proteins (Seno et al. 1988). This hypothesis was put to the test, and the mechanism by which the ASbFGF variant increased recombinant protein production was clarified. To do this, the degradation of ASbFGF in the homogenized crude extract was examined. The agroinfiltrated leaf homogenized crude extract contains all the soluble proteins, including the native proteases and recombinant bFGF. The degree of stability of these variants can be determined by comparing the rate of loss of bFGF and mutant ASbFGF in these extracts.

A higher percentage of intact soluble ASbFGF than bFGF over time was observed when recombinant bFGF degradation in the crude extracts was measured using ELISA at 20 ^{o}C. (Figure 9). This strongly implies that ASbFGF is resistant to protease- or oxidation-induced precipitation of the protein or degradation of the protein. For the non-optimized bFGF, an hrly decrease in intact soluble bFGF content of 9.0% was roughly predicted. ASbFGF only decreases by about 3.2% per hr, which was three times slower than this. Additionally, as the incubation period for ASbFGF progressed, a smaller truncated bFGF band was seen developing in the CBB staining of the crude extracts. Purified bFGF was also reported to produce a similar truncated form (Sauer et al. 2021). These data show how ASbFGF degrades slower in leaf crude extracts compared to the normal bFGF. This provides a mechanism why ASbFGF has 2-fold higher production.

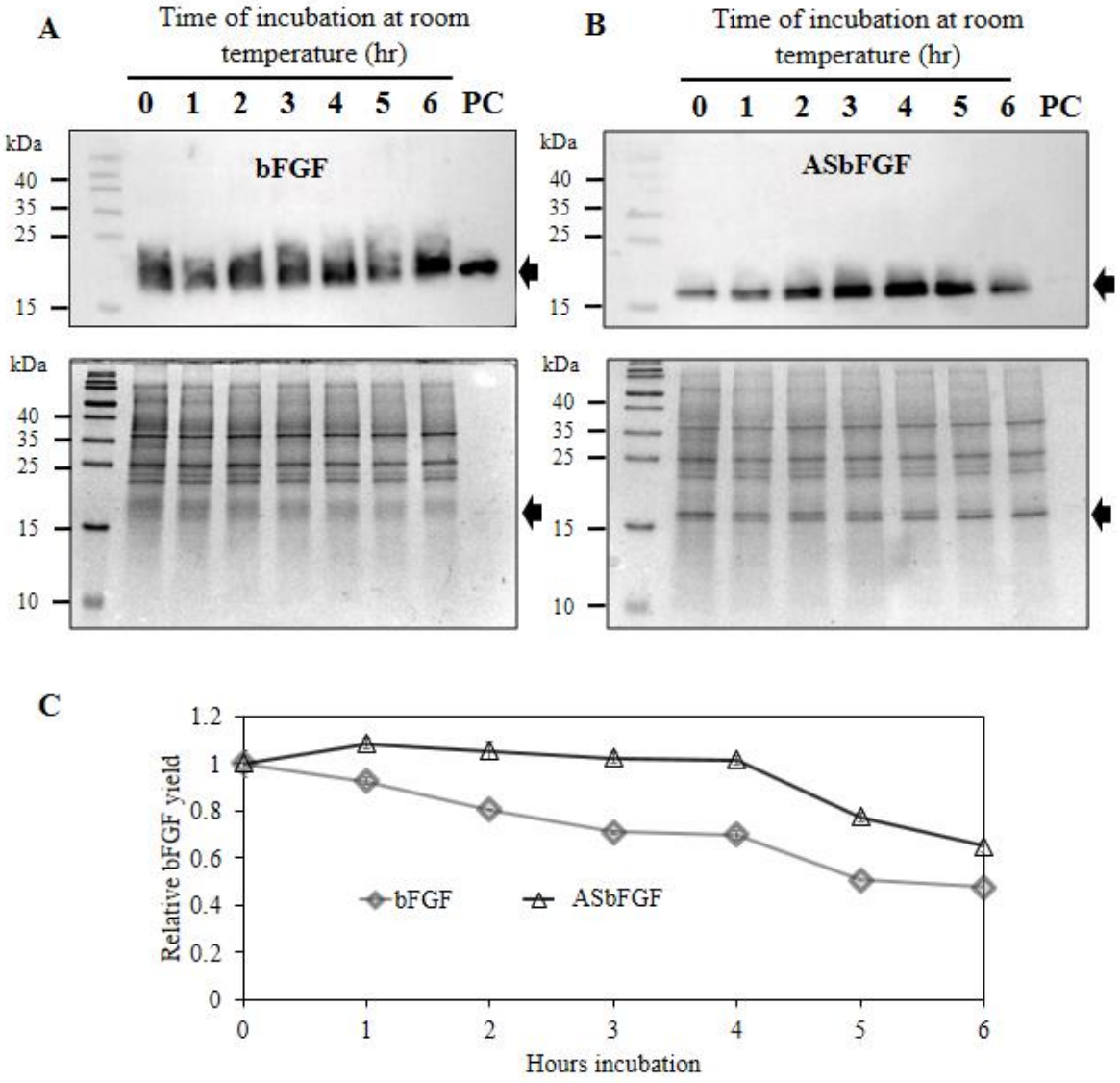


Figure 9. Analysis of the stability of ASbFGF and bFGF in crude extracts. (A)Western blot (top) and CBB staining (bottom) of bFGF in leaf crude extracts, (B)Western blot (top) and CBB staining (bottom) of ASbFGF in leaf crude extracts, (C) ELISA analysis. In panel C, the average data from three replicate experiments were displayed with error bars representing the standard deviation. For (A) and (B), arrows indicate the bFGF and ASbFGF bands in the gel. The positive control (PC) here is 25 ng of bFGF derived from *E. coli*. For (C), To obtain the relative yields, time course yields were divided by the bFGF/ASbFGF yield at 0 hr.

2.3.4 Recombinant protein purification

To be used safely in biological systems, the synthesized recombinant ASbFGF must be pure. In this study, SP cation exchange chromatography was used to successfully purify ASbFGF before being subjected to Heparin affinity chromatography. With a recovery rate of about 34%, this purification method was previously used to purify the normal recombinant bFGF in *N. benthamiana* leaves (Figure 10A). The acid-stable version also performed in the same manner (Figure 10B and 10C). It was established that the Cys70Ser and Cys88Ser mutations had no impact on the crucial bioactivity of bFGF, heparin binding (Seeger & Rinas 1996). This was once again proven here in this study in *N. benthamiana* produced ASbFGF. It was shown here that tagless purification can be obtained via combined SP cation exchange chromatography and Heparin affinity chromatography.

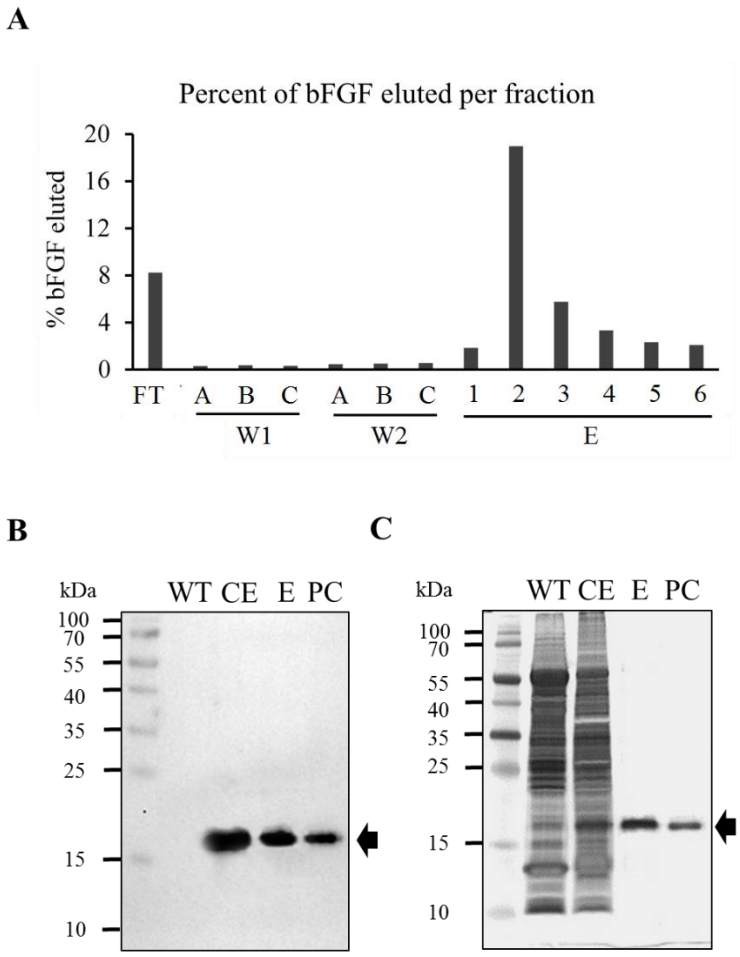


Figure 10. Purification scheme of ASbFGF through SP-Sepharose and Heparin Affinity chromatography. (A) Calculation of recovery of purified bFGF by ELISA (B) Western blot, (C) Silver staining. For (A), FT is the flowthrough, W1A and W1B are the wash fractions using 0.5 M NaCl, W2A-D are wash fractions using 1 M NaCl while E1-E6 are elution fractions using 2 M NaCl. For (B) and (C), the elution fraction following double purification (E), the wild type crude extract (WT), the crude extract of ASbFGF agroinfiltrated leaves (CE), and 25 ng of bFGF derived from *E. coli* (PC) are shown.

2.3.5 Cell proliferation assay

The ability of plant-produced mutant ASbFGF to promote the proliferation of Hep G2 and CHO-K1 cells was examined in order to determine whether it was functional. Recombinant

ASbFGF demonstrated improved bioactivity in the results (Figure 11). From day 2 to day 10, ASbFGF consistently provided a higher viable cell count and percent viability in the Hep G2 cell-based setups (Figure 11A) than the regular bFGF variant made in *E. coli*. However, ASbFGF activity was similar to that of the traditional bFGF version in setups using CHO-K1 cells (Figure 11B). This shows that ASbFGF not only functions well *in vitro* but also has a significant impact on cell proliferation. This might be as a result of ASbFGF's increased medium stability, which enables it to stimulate more cells for longer periods of time.

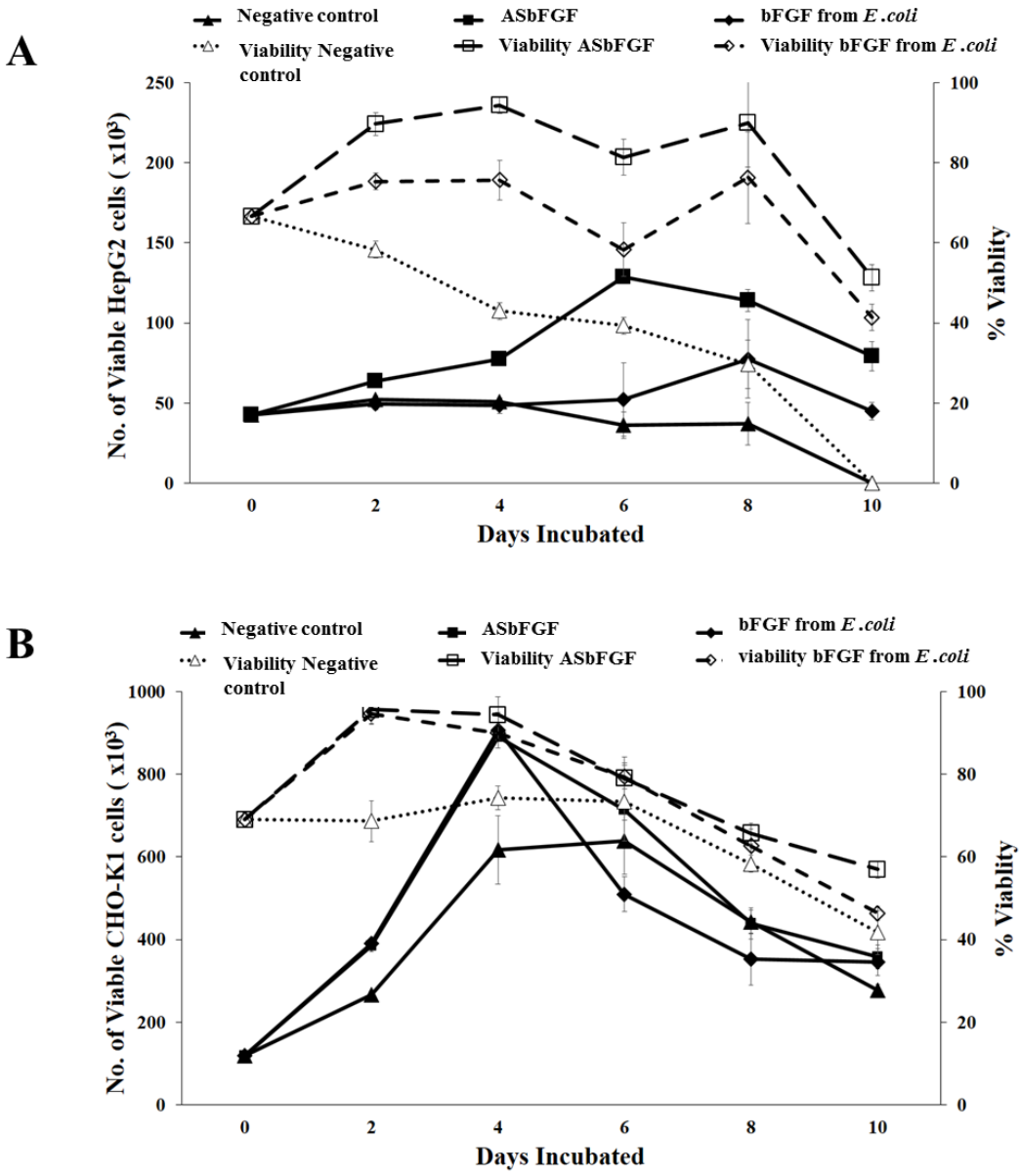


Figure 11. Effect of ASbFGF produced by *N. benthamiana* on the proliferation of Hep G2 cells (A) and CHO-K1 cells (B). For the negative control, PBS is added. The error bars in panels (A) and (B) represent the standard deviation and the mean from experiments done in triplicate.

2.4 Discussion

To increase the production of recombinant proteins, important upstream and downstream processes must be recognized and modified. The accumulation of the recombinant protein in the expression platform is the primary concern of the upstream processes. This accumulation is reliant on the biological responses of the expression platform and the extrinsic variables that influence them. On the other hand, the extent of recovery of the produced protein from the upstream processes is influenced by downstream processes. Numerous points in the upstream processes may be manipulated in the context of *A. tumefaciens*-mediated transient production. The "magnICON" and "Tsukuba system," in particular, use tobamovirus (TMV) and geminiviral replication systems respectively (Klimyuk et al. 2012; Suzaki et al. 2019). The transgene could be hyper expressed through these systems, leading to high levels of expression. Nevertheless, there are other numerous pathways along the recombinant protein production line that could still be improved to boost production like protease reduction and plant incubation parameter optimization.

Many of these steps are predicated on maintaining the health of the plant cells. The agroinfiltration process is intrusive, stressing the plant and possibly resulting in senescence or necrosis (Zhao et al. 2017; Nosaki 2021). To complete the reaction cascade for recombinant protein production, plant cells must first be active and healthy. It has been demonstrated in the past that ascorbic acid can reduce leaf stress and solve agroinfiltration issues when added to the infiltration buffer (Zhao et al. 2017). During the agroinfiltration procedure, the *A. tumefaciens* cells stick to the plant cells and transfer the T-DNA, which contains the transgene and the components needed for its transmission. The effectiveness of this process determines the quantity of recombinant DNA that is readily available, which will be the initial point of production. Tween-20 and acetosyringone

added to the infiltration buffer have been shown to speed up this process (Zhao et al. 2017, Norkunas et al. 2018). This section investigated that these additives might be combined to improve agroinfiltration performance overall.

The transgenes that were made available through agroinfiltration will be translated into the target protein after being translated to mRNA, continuing the process of protein expression. These actions may act as a new point of regulation. A well-known plant defense mechanism called RNA silencing has the potential to significantly reduce the amount of recombinant proteins produced (Davidson and McCray 2011; Mardanova et al. 2017). Due to the presence of a wide variety of proteases in plant cells, the recombinant protein is now exposed to different degradation processes after translation (Buyel et al. 2021). Protease and the endoplasmic reticulum (ER)-associated degradation (ERAD) are frequently linked to protein degradation. High levels of recombinant protein production may trigger the unfolded protein response (UPR) (Mattanovich et al. 2004), which may sharply lower recombinant protein production levels. In addition, plants use small ubiquitin-like modifier (SUMO) proteases in response to stress (Morrell and Sadanandom 2019), which might also prevent the production of high levels of recombinant proteins. The stress required to cause this phenomenon could be produced by both agroinfiltration and high levels of foreign proteins. These proteases typically play a role in the defense and homeostasis of plants (Van Der Hoorn 2008; Minina et al. 2017). But they might prevent the production of recombinant proteins (Buyel et al. 2021). Silencing of several plant proteases led to aberrant phenotypes that prevent the plant from growing normally (Bae et al. 2013). This implies that the removal of these proteases would be harmful to the plant because they are necessary for its development and homeostasis.

Consequently, a different strategy should be used to address the issue of the lower recombinant protein yield caused by protease-degradation. An earlier investigation discovered that ASbFGF, which carries the Cys70Ser and Cys88Ser mutations, enhanced bFGF recovery from inclusion bodies while maintaining bioactivity (Rinas et al. 1992). The stabilization of acid-stable bFGF intermediates, which led to protease resistance, was thought to be the reason for the increased recovery. In this study, production was increased about two-fold when the mutant ASbFGF variant was used in place of the wild type bFGF. Protease resistance, precipitation resistance, or a combination of the two may be the exact mechanism causing this increase in production (Seno et al. 1988; Rinas et al. 1992). The stabilization of folding intermediates or the absence of protease cleavage sites as a result of the protein backbone's unnatural composition were proposed as the mechanisms underlying this degradation resistance (Werner et al. 2016). On the other hand, disulfide-linked multimerization has made external cysteines appear to be aggregation-prone sites (Shahrokh et al. 1994). In mutant ASbFGF, these two cysteines were removed, which might have slowed aggregation. It is also possible that both of these mechanisms work together to increase the overall availability of intact soluble ASbFGF in comparison to regular bFGF.

In conclusion, this study demonstrated a method for high-level production of tag-less purified, bioactive ASbFGF, as well as how optimizing agroinfiltration and extraction methods, as well as replacing external cysteines with serines of bFGF, could be used to significantly increase its production. These methods of tailoring extraction based on particular recombinant protein characteristics as well as protein engineering 'fragile' spots of recombinant protein to enhance stability may be promising ways to increase recombinant protein production in a protease-rich expression platform like plants.

2.5 Summary

The protein bFGF is involved in a number of biological processes, including cell division and growth as well as wound healing. Due to its applications in biomedical engineering, cosmetics, and scientific research, it has become an essential part of mammalian cell culture systems. The demand for alternative production methods is rising, making methods like transient production in plants more attractive as options. In this study, a high-level bFGF production system was developed using an enhanced *A. tumefaciens*-mediated transient expression system, which produced three times as much as a conventional system did. The use of a mutant acid-stable form of the compound, which degraded/aggregated at a threefold slower rate in crude leaf extracts, resulted in a two-fold increase in yield. The product was thoroughly purified using a two-step purification process. It was investigated whether HepG2 and CHO-K1 cells could be stimulated to proliferate by purified ASbFGF. According to the findings, *E. coli*-produced bFGF and purified ASbFGF both have a similar ability to stimulate cell proliferation. A high-level transient production system of functional ASbFGF in *N. benthamiana* leaves as well as a successful tag-less purification method of leaf crude extracts have been demonstrated as a result of this research.

Chapter 3 Production of secreted acid-stable basic fibroblast growth factor in *Nicotiana benthamiana* leaves and PCP

3.1 Introduction

The human bFGF which is known to be heparin-binding; growth-modulating (Himmelfarb & Couper 1997; Liu & Wu 2009); and possessing anti-apoptotic properties (Lynch et al. 2000; Pardo et al. 2002; Barrientos et al. 2008; Liu & Wu 2009) is a growth factor that is crucial in regenerative medicine (Levenstein et al. 2006). These are achieved via the induction of the Jun amino-terminal kinase pathway (Ahn et al. 2009) and regulating calcium homeostasis (Lynch et al. 2000; Peluso 2003).

The native protein bFGF, devoid of a signal peptide is naturally known to be mainly localized in the cytoplasm (Chen et al. 2014) and is known to be unstable. Nevertheless, some portions are believed to be secreted via a non-conventional secretory pathway in mammalian cells (Woodward et al. 1992; Aktas & Kayton 2011). This localization in the cytoplasm is believed to aid in the healing process in cases where epithelial tissue is damaged (Miyoshi et al. 2005) as the damaged cells in that case would liberate bFGF and promote the development of the adjacent cells (Liu et al. 2007; Zhang et al. 2018). Meanwhile, a protein-engineered ASbFGF variant that have its external cysteines changed to serines (Folkman et al. 1991; Szabo et al. 1994) are proven to be effective in increasing recombinant bFGF yield in both *E. coli* (Seno et al. 1988) and *N. benthamiana* (Chapter 2). This increase in yield was thought to be due to mitigation of bFGF's tendency to aggregate, be degraded by proteases, or both.

Secretion of proteins is a basic and vital function common among all life (Wang et al. 2017). Naturally, it is an essential process for cell to cell communication, development, and stress response (Chaffin et al. 1998; Agrawal et al. 2010). Since this process separates the target protein from all of the proteins of inside the cell, signal peptide fusion which makes a recombinant protein be secreted has been employed in recombinant protein production (Raskin, et al. 2003). Various signal peptides have already been fused to bFGF like barley alpha amylase (Rattanapisit et al. 2020) in *N. benthamiana* and rice glutelin (An et al. 2013) in rice. Early necrosis and modest amounts of production were seen, nevertheless, and no evidence of secretion to the apoplast was shown. Currently there is no successful production system for secreted ASbFGF in plants. Nevertheless, with the use of the FGF4 signal peptide fusion, secreted ASbFGF was successfully produced in mammalian cells (Chen et al. 2014) though it is not used for protein manufacture. Meanwhile, *N. benthamiana* extensin signal peptide fusion was reported to be a very efficient method to secrete recombinant proteins in *N. benthamiana* (Jiang et al. 2019, 2020).

So far, the most promising platform to produce secreted recombinant proteins in plants is via plant cell packs (PCPs). PCPs are described as semi-dried plant cells derived from suspension cell culture which are incubated further to produce more recombinant protein (Rademacher et al. 2019; Gengenbach et al. 2020). This platform is believed to produce secreted recombinant proteins at a much higher rate than in previous platforms such as suspension cell culture (Gengenbach et al. 2019; Rademacher et al. 2019; Poborilova et al. 2020) and root culture (Opdensteinen et al. 2022). This method is thought to mimic conditions in the leaves as in leaf apoplast secretion system but with the advantages of convenience and scalability (Rademacher et al. 2019). This is mainly thought to be because of more efficient aeration due to the loose packing of the semi-dried cells

and the PCP buffer which does not contain growth hormones and vitamins and thus would allow cells to divert their metabolic activity less to growth and more on to protein production. In the context of recombinant production of secreted proteins, having them produced in PCPs would also enable accumulation in a more limited volume which is in the plant cell wall and the thin layer of media. Having the recombinant proteins in a more concentrated state helps in a simpler downstream processing. Meanwhile, the loose packing of the cells in PCPs are thought to allow efficient elution of accumulated secreted proteins with negligible leakage of cytosolic proteins (Rademacher et al. 2019).

Currently, all studies about PCPs are transient production systems. This entails that co-culture with *A. tumefaciens* is required every time and this consumes more costs in terms of production time and resources for *A. tumefaciens* culture.

In this study, stable transgenic SS^{Ext}ASbFGF producing *N. benthamiana* suspension cell culture and PCPs were developed which provides for a more continuous secreted SS^{Ext}ASbFGF manufacture (Figure 12). Technologies were combined to develop a production system of secreted acid-stable bFGF in *N. benthamiana* and boosted its production via multi-parameter optimization. Yield and quality was also compared between the PCP and the leaf apoplast secretion system. The bioactivity was demonstrated to be successful in CHO-K1 and HEP G2 cells, and a simpler purification approach was also accomplished.

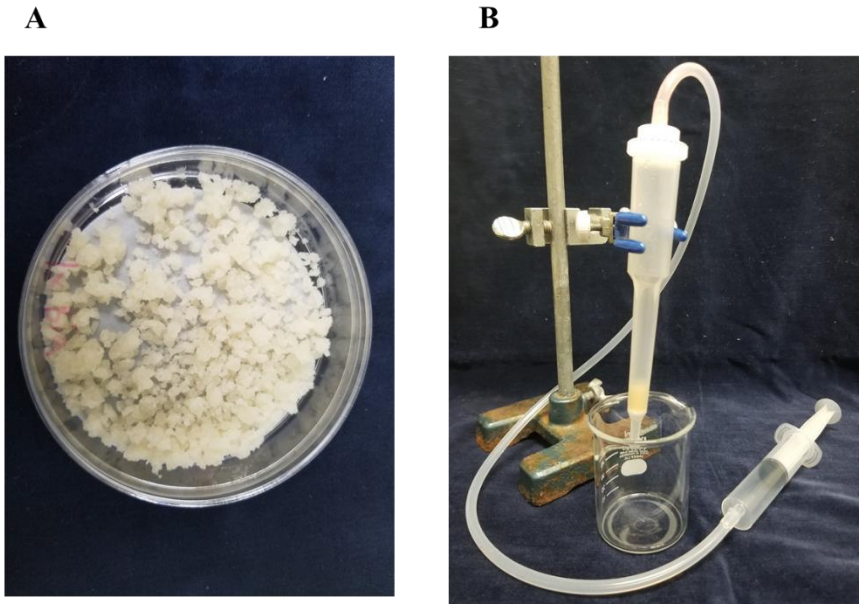


Figure 12. PCP Illustrations. (A) Incubation of PCPs in petri dish. (B) Open column extraction apparatus for secreted ASbFGF in *N. benthamiana* cells.

3.2 Materials and Methods

3.2.1 Vector construction

The bFGF and ASbFGF variants used in this chapter were the same variants used in chapter 2. In order to produce secreted ASbFGF, signal peptide fused variants SS^{HC}ASbFGF (antibody heavy chain signal peptide), SS^{FGF4}ASbFGF (FGF4 signal peptide) and SS^{Ext}ASbFGF (extensin signal peptide), were constructed by fusing ASbFGF into the corresponding signal peptides via NEBuilder® HiFi DNA Assembly (New England Biolabs Inc, MA, USA) and using the primers in Table 1. These were then ligated into a plant expression vector pGPTV-bar (Figure 12A) and introduced into *A. tumefaciens* strain LBA4404 by electroporation with capacitance, voltage, and resistance at 25 μF, 2.4 kV, and 200 Ω respectively. All DNA fragments were amplified via KOD polymerase (Toyobo).

Table 3. Primers used for the Gibson assembly of signal peptide-fused variants of bFGF and ASbFGF

Name	Sequence (5'-3')	Tm (^{o}C)
Mid35SP_F	AAAGCAAGTGGATTGATGT	59
MidHSPT_R	CAAAACATTCACAATGGATTAT	56
F_bFGF_flank_tm62	GCAGCCGGGAGCATCACC	62
R_SSFGF4__flank	GGGGGCGGTGGGGGCGGC	65
R_SSEXT_flank	AGCAGAAGATTCAGAAGCAAGAGAAAGAG	58
R_SSX_flank	GCACTGCACGCCCCTCAG	65
F_SSFGF4_end_15bp	GCCCCCACCGCCCCCTCTCC	60
F_SSEXT_end_15bp	TCTGAATCTTCTGCTTCTCCATCTCC	58
F_SSX_end_15bp	AGGGGCGTGCAGTGCTCT	63
R_bFGF_15bp_tm60	GATGCTCCCGGCTGCTTG	60

3.2.2 Transient production in leaves and apoplastic wall fluid extraction

Transient production in *N. benthamiana* leaves were done as is in 2.2.2. To obtain the apoplast wash fractions containing the secreted ASbFGF, 6-day agroinfiltrated leaves were vacuum infiltrated with ½ X PBST. Apoplastic wash fluid was collected via centrifugation at 1000 *g* and 4 ^{o}C for 15 min. This was done three times to maximize extraction. The volume and soluble protein content of pooled extracts were then analyzed by Bradford assay and ELISA.

3.2.4 Stable production of SS^{Ext}ASbFGF in *N. benthamiana* suspension cells

In order to create a transgenic secreted-ASbFGF-producing *N. benthamiana* cell line, first, *A. tumefaciens* LBA4404 containing the plasmids carrying SS^{Ext}ASbFGF and RNA silencing suppressor p19 grown for 2 days were then washed and resuspended in MS buffer supplemented with 20 mM MES and 10 mM $MgSO_4$. This bacterial suspension was then added to semi-dried 4-

day-old *N. benthamiana* suspension cells. This co-culture was then incubated for 2 days in the dark at 23 ^{o}C. Afterwards, the bacteria were then washed of by repeated washing with MS with 0.1% Plant Preservative Mixture™ (PPM, Plant Cell Technology Inc, Washington, DC) and then centrifuged at 50 x *g* for 1 min until supernatant was clear. The *N. benthamiana* cells obtained were then cultured in 1:1 fresh MS media and supernatant from 4-day-old *N. benthamiana* culture media with 10 μg/mL bialaphos, 200 μg/mL carbenicillin and 0.1% PPM. This was shaken for 2 weeks at 140 rpm at 24 ^{o}C. This resulted in the settling of transformed callus which were then transferred to a solid MS media with 10 μg/mL bialaphos, 200 μg/mL carbenicillin and 0.075% PPM and grown for a month. The largest callus was then suspended in MS media with 10 μg/mL bialaphos until a stable suspension culture was obtained.

3.2.5 Stable production of SS^{Ext}ASbFGF in transgenic *N. benthamiana* PCPs

To make the secreted production system using stable transgenic SS^{Ext}ASbFGF *N. benthamiana* PCPs, stable transgenic SS^{Ext}ASbFGF *N. benthamiana* suspension cells were drained of the MS medium using vacuum filtration. Subsequently, they were suspended in minimal PCP buffer and then semi-dried with sterile tissue. These were then placed in a petri dish with filter paper soaked in PCP buffer (Figure 12A) to provide moisture and nutrition to the cells and incubated in the dark at 23 ^{o}C. For the time-course production data, PCPs were sampled every day.

3.2.6 Extraction of secreted ASbFGF from suspension cells and PCPs

In order to obtain secreted ASbFGF from the plant cells, suspension cells are pelleted by centrifugation at 50 x *g* for 1 min. Excess media was drained using sterile filter paper. For PCPs,

the cells were then weighed to about 0.5 g, put in an open purification column and 1 mL extraction buffer composed of 100 mM NaCl, 50 mM sodium phosphate pH 6.5, and 0.1% Tween-20 was used to wash off the secreted ASbFGF thrice in an open column (Figure 12B). The resulting extract was used for protein analysis.

3.2.7 Growth comparison of SS^{Ext}ASbFGF *N. benthamiana* callus with wild type *N. benthamiana* callus

To investigate the difference of growth characteristics between the transgenic SS^{Ext}ASbFGF *N. benthamiana* callus with wild type *N. benthamiana* callus, about 0.05 g semi dried suspension cells from wild type and SS^{Ext}ASbFGF cells grown in 10/20/40 µg/mL bialaphos were grown in solid MS media and grown in the dark at 24 ^{o}C. Sample callus were then obtained and weighed every 2 days for 20 days.

3.2.8 Protein analysis by Indirect ELISA

To quantify the bFGF produced, tndirect ELISA of secreted ASbFGF and crude extracts from leaves and PCPs were employed as previously done in section 2.2.4. Briefly, leaf/cell lysate crude extracts were homogenized and suspended in an extraction buffer without Tween-20. They were then briefly sonicated (200 W; Kaijo Denki) on ice. After normalizing the total protein concentration and blocking using BSA in PBST. Primary antibody incubation was done using anti-FGF2 mouse IgG (Santa Cruz Biotechnology) in PBST. Subsequently, secondary antibody incubation was done using anti-mouse sheep HRP IgG (GE Healthcare) in PBST. Absorbance at 450 nm was obtained after incubation with Ortho-phenylenediamine (OPD) substrate (Sigma-

Aldrich). For the standard curve, *E. coli* derived bFGF (Nacalai Tesque Inc.) in 2 µg/L non-agroinfiltrated crude extracts in PBS were used.

3.2.9 Protein analysis by SDS PAGE and Western blot

To visualize the bFGF and total soluble protein content of the extracts, SDS-PAGE and Western blot experiments were employed as previously done in section 2.2.5. After running the samples through 16.5% Tris-glycine SDS-PAGE at 120 V for 90 mins, gels were then visualized using CBB or silver staining. For the Western blot experiments, anti-bFGF mouse IgG was used as the primary antibody while anti-mouse-HRP IgG was used as the secondary antibody. The bFGF bands in the membrane were then visualized by adding Luminata^{TM} Forte Western HRP substrate (Millipore Sigma) and the resulting chemiluminescence was observed using iBright 1500 Imaging System (Thermo Fisher).

3.2.10 Purification by Heparin Affinity chromatography

For the purpose of obtaining pure ASbFGF from leaf apoplast and PCPs, further purification was done using Heparin affinity chromatography. Apoplast wash fractions or PCP eluates were run through a 1 mL Heparin Sepharose (GE Healthcare) column using a protocol patterned from a study for purifying recombinant bFGF in *E. coli* (Seeger & Rinas 1996) with some modifications. Washing was done using 50 mM phosphate buffer pH 8.0, 1.0 M NaCl. Finally, pure ASbFGF was eluted using 50 mM phosphate buffer pH 8.0, 2.0 M NaCl. This was then stored at -80 ^{o}C until use.

3.2.11 Investigation of size reduction of secreted ASbFGF

Mobility shifted bands of the secreted ASbFGF in apoplast wash fractions and PCP eluates relative to the non-secreted variant was investigated further via peptide sequencing by Edman degradation (Laursen 1971; Walker 1994; Thakkar et al. 2006). Protein bands in solid phase medium was obtained by running purified fractions by SDS-PAGE and subsequent transferring to PVDF membrane. The corresponding bands where then visualized using CBB staining, dried and were excised. These bands were then analyzed using Procise 491 HT & 494 cLC Protein Sequencing System in the Institute for Protein Research, Osaka University.

3.2.12 In-silico protein structure and cleavage site prediction

In an effort to provide additional background information regarding size-reduction of secreted ASbFGF; in-silico analysis of the protein structure and signal peptide cleavage prediction was done. For the protein structure prediction, amino acid sequences were submitted to the Robetta server (http://robetta.bakerlab.org). To predict protein structure, the submitted sequences were parsed into structural models and putative domains that are made using either homology modeling or de novo structure prediction tools. Homology modelling is done if confident matches were found using BLAST, PSI-BLAST, FFAS03 or 3D-Jury, otherwise, structure predictions were generated using the de novo Rosetta fragment insertion method (Kim et al. 2004). On the other hand, to predict cleavage sites amino acid sequences were submitted to TargetP 2.0 (Emanuelsson

et al. 2007), and SignalP6.0 (Teufel et al. 2022) which predicts signal peptide cleavage via homology.

3.2.13 Cell proliferation assay

The cell proliferation test was conducted as in 2.2.8. Here, 5 ng/mL and 20 ng/mL of PCP-derived, purified secreted SS^{Ext}ASbFGF were subsequently added, respectively. For the positive control, same amounts of commercial *E. coli*-produced were added. Trypan blue assay was done using an automatic cell counter (Bio-Rad) to obtain viable cell counts and percent viability which were performed every two days for ten days.

3.3 Results

3.3.1 Secretion of ASbFGF in *N. benthamiana* leaves by signal peptide fusion

To confirm the best signal peptide among the selected signal peptides for the secreted production system of ASbFGF, plant expression vectors pGPTV-bar-SS^{HC}ASbFGF, pGPTV-bar-SS^{FGF4}ASbFGF, and pGPTV-bar-SS^{Ext}ASbFGF, were constructed and transformed into *A. tumefaciens tumefaciens* LBA4404 (Figure 13A). To do this, the signal peptide sequences were fused to the ASbFGF sequences via Gibson assembly and ligated to pGPTV-bar empty vector. The expression vectors were then used to agroinfiltrate the *N. benthamiana* leaves which were then subsequently harvested after 6-days post infiltration. Secreted bFGF production was then confirmed via Western blotting and silver staining of the apoplastic wash fluid extracts (Figure

13B). Since the acid-stable variants were the ones that produced bFGF (Supplementary Figure 4), they were the variants used for the quantification of signal peptide secretion efficiency. In the Western blot and silver staining analysis of the apoplastic wash fluid extracts (Figure 13B), the secreted bFGF band was only seen in the signal peptide-fused variants. It was also notable that SS^{Ext}ASbFGF had the thickest band pertaining to ASbFGF, which suggests the higher presence of the secreted protein in its apoplast wash fluid compared to the other using mammalian signal peptides. On the other hand, Western blot analysis of the apoplast-washed leaf extracts (Figure 13C) showed that the use of the SS^{HC}ASbFGF variant produced the thickest secreted ASbFGF band, suggesting less efficient secretion. Meanwhile, SS^{FGF4}ASbFGF has comparable yield but more efficient secretion. It could also be observed that generally, bands with lesser mobility was observed in the apoplast-washed leaf extracts (Figure 13C) than the apoplast wash fluid extracts (Figure 13B), as these are the uncleaved unprocessed signal peptide-fused variants yet to be shuttled to the apoplast. Meanwhile, ELISA analysis of both the apoplast wash fluid and the apoplast-washed leaf extracts (Figure 13D and 13E), showed that SS^{Ext}ASbFGF had the highest concentration of secreted ASbFGF in the apoplast wash fluid at around 76 μg/g FW leaves while the setup using SS^{HC}ASbFGF was the lowest at 16 μg/g FW leaves. Furthermore, SS^{HC}ASbFGF also rank lowest with regards to percentage secretion efficiency.

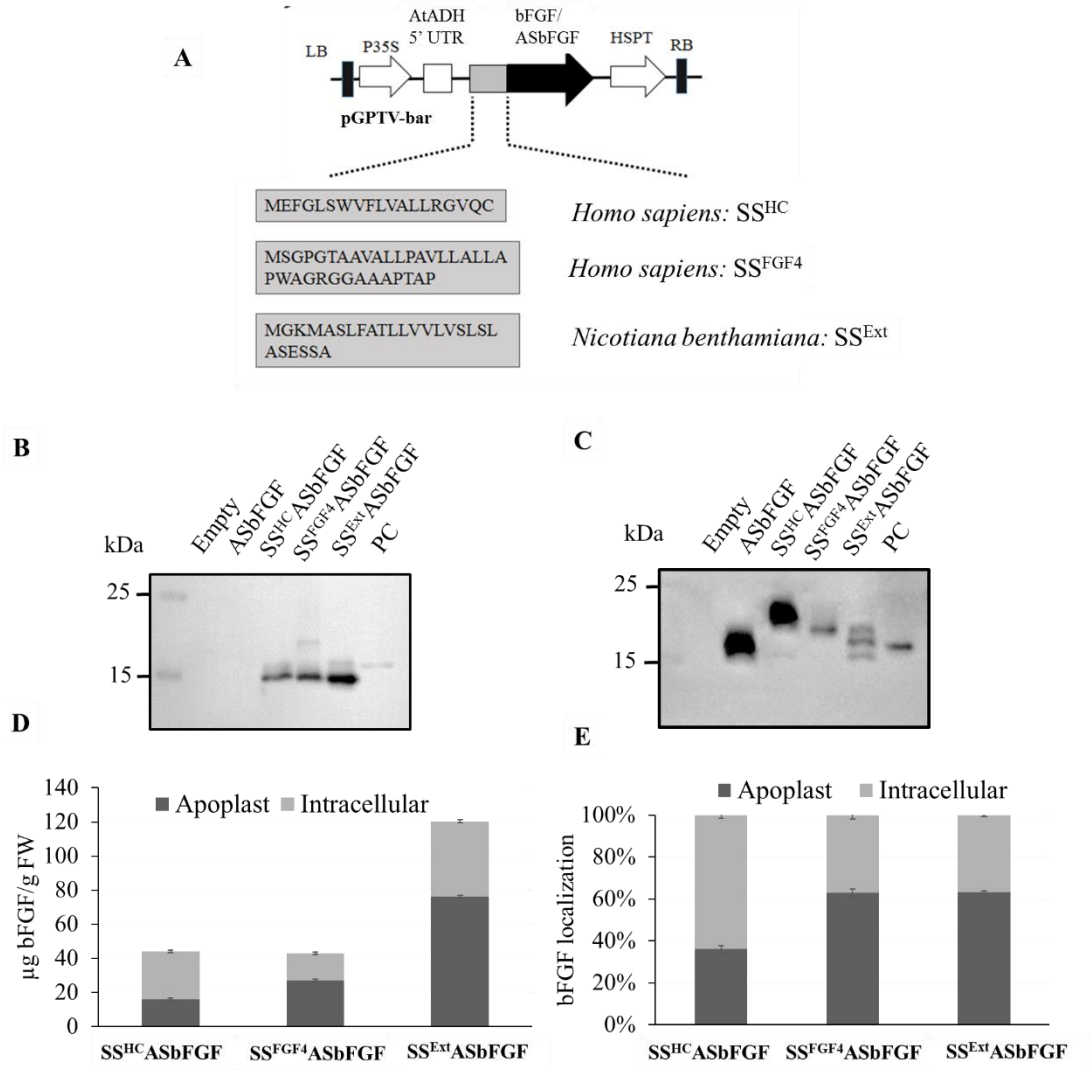


Figure 13. Comparison of production and secretion efficiency of signal peptide fused ASbFGF variants **(A)** T-DNA of expression constructs and details of bFGF variants used. The recombinant gene is under the control of a cauliflower mosaic virus 35S promoter (P35S) and *Arabidopsis thaliana* heat shock protein 18.2 terminator (HSPT). Other elements in the T-DNA are the translational enhancer *Arabidopsis thaliana* dehydrogenase untranslated region (AtADH 5' UTR) and the T-DNA left and right border (LB and RB) which marks the boundaries of the transgenes transferred during *A. tumefaciens*-mediated transformation. The signal peptides used were the human-derived antibody heavy chain signal peptide (SS^{HC}), FGF4 signal peptide (SS^{FGF4}) and *N. benthamiana* derived extensin signal peptide (SS^{Ext}) which were inserted at the N-terminal of the bFGF/ASbFGF gene. (B) Western blot of apoplast wash fractions (C) Western blot of the apoplast-washed leaf crude extracts. (D) Yield of secreted and intracellular ASbFGF among signal peptide fused-variants. (E) Secretion efficiency of the signal peptide-fused variants. For (B) and (C), the

positive control (PC) is 25 ng of bFGF derived from *E. coli*. The negative control is crude extract of leaves agroinfiltrated with only the empty vector (Empty).

3.3.2 Comparison of secreted SS^{Ext}ASbFGF production in *N. benthamiana* suspension culture and PCPs

Secreted production system of ASbFGF was done using the SS^{Ext}ASbFGF which was stably expressed in *N. benthamiana* suspension cells and its PCP derivative. Since no detectable amounts of SS^{Ext}ASbFGF was found in the media of the suspension cell culture, secreted SS^{Ext}ASbFGF was extracted by washing media-drained suspension cells with PBST (Figure 14). This suggests instability/insolubility of the secreted SS^{Ext}ASbFGF in the MS medium and localization around the cell wall of the suspension cells. MS media has no NaCl content in it and it was shown in section 2.3.1 how NaCl concentration in the extraction buffer affected bFGF solubility. However, the yield obtained by washing the suspension cells was only about 0.4 µg/g FW cells (Figure 15). These suggests that further incubating the cells as PCPs that have minimal media might help more in the accumulation and in the recovery of the secreted SS^{Ext}ASbFGF.

Two kinds of stable transgenic PCPs were made in this study: Wet PCPs and Dry PCPs. After draining the MS media of suspension cells, PCP buffer was added at 1.5 mL/g cells. Wet PCPs were made by incubating these cells without media draining. Meanwhile, Dry PCPs were made by draining this PCP soaked cells before incubation up to 7 days.

The results showed that in Wet PCPs, an increased production to about 2 µg/g FW cells was achieved. Meanwhile, in Dry PCPs, a higher production at 13 µg/g FW cells was reached. This increased production might be due to more aeration of the cells and thus higher viability

(Rademacher et al. 2019; Opdensteinen et al. 2022). This allows the plant cells to be more viable for a longer time. Similar findings have been reported previously, citing excess media led to up to about 60-fold decrease of recombinant protein production (Rademacher et al. 2019).

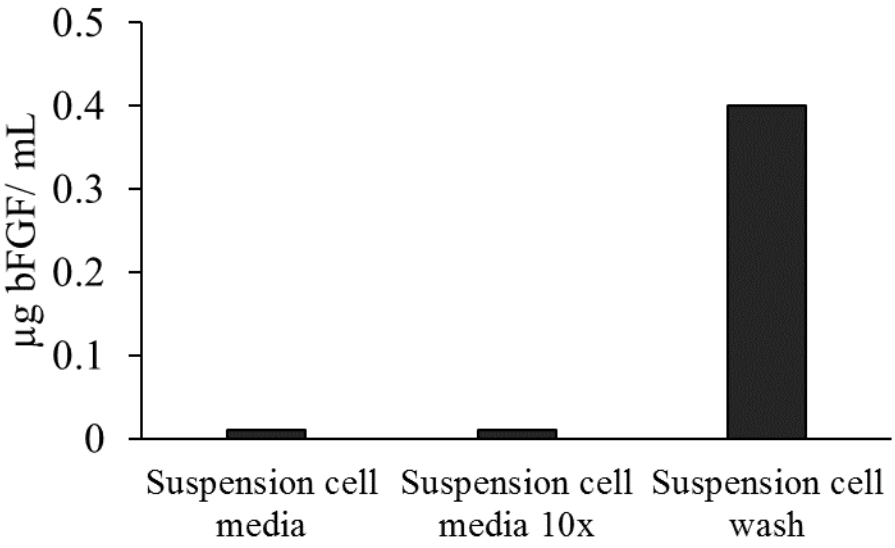
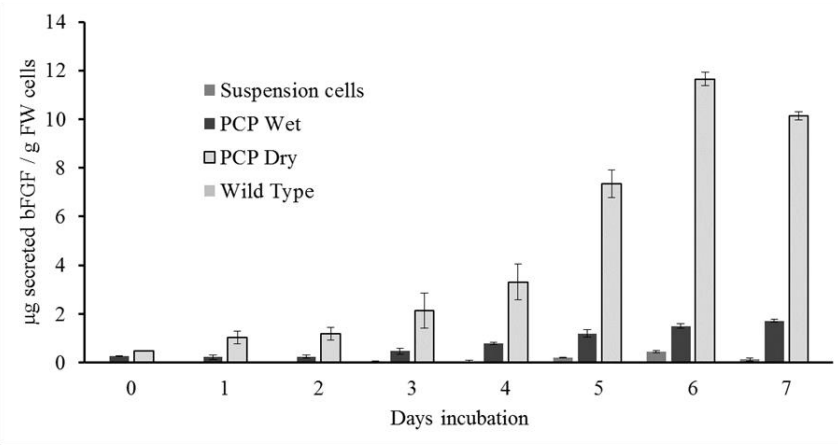
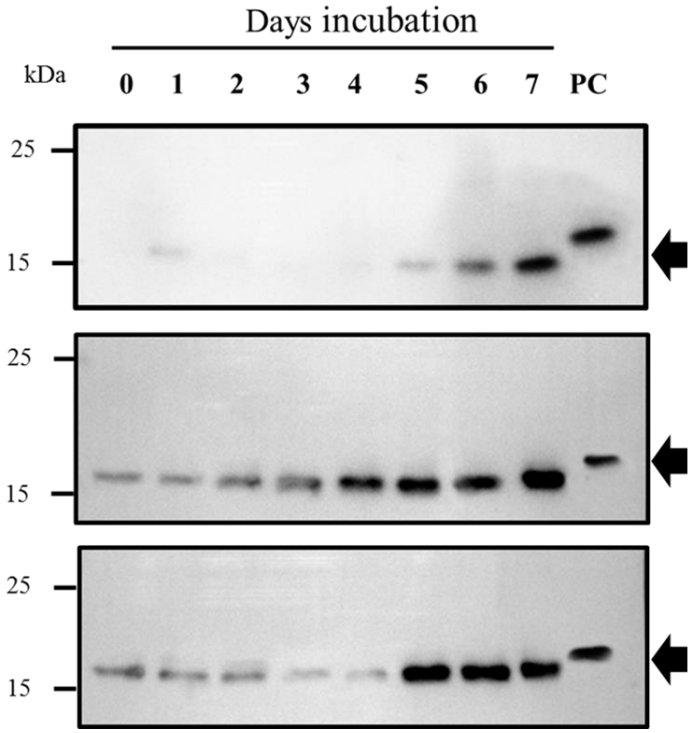


Figure 14. ELISA quantification of bFGF in suspension cell media and suspension cell wash

A


µg secreted bFGF / g FW cells
14
12
10
8
6
4
2
0
Suspension cells
PCP Wet
PCP Dry
Wild Type
0
1
2
3
4
5
6
7
Days incubation

B


Days incubation
kDa
0 1 2 3 4 5 6 7 PC
25
15
25
15
25
15

C

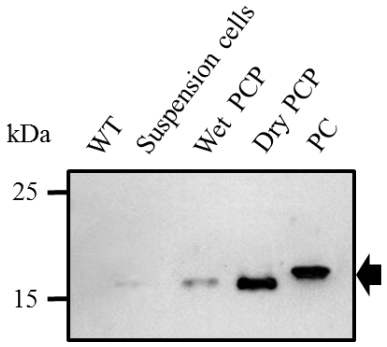

kDa
WT
Suspension cells
Wet PCP
Dry PCP
PC
25
15

Figure 15. Time course production of secreted ASbFGF in suspension cells and PCPs. (A) ELISA quantification. (B) Western blot analysis of secreted ASbFGF in dry PCPs (top), wet PCPs (mid) and suspension cells (bottom). (C) Western blot analysis of 6th day samples of secreted ASbFGF of dry PCPs, wet PCPs and suspension cells. For (A), three triplicate samples' mean values and standard deviation are displayed. For (B) and (C), the arrows indicate the bFGF bands. The positive control (PC) was 25 ng of bFGF derived from *E. coli*.

3.3.3 Heparin affinity purification of secreted ASbFGF

In order to obtain pure secreted ASbFGF, and in hopes of simplifying the purification process, secreted PCP eluates (Figure 16) were subjected to Heparin affinity chromatography alone. Unlike purification from intracellular fractions like in section 2.2.7, purification of secreted ASbFGF skips the initial SP-cation exchange purification step. This simplifies the purification process and provides an alternative way for purification.

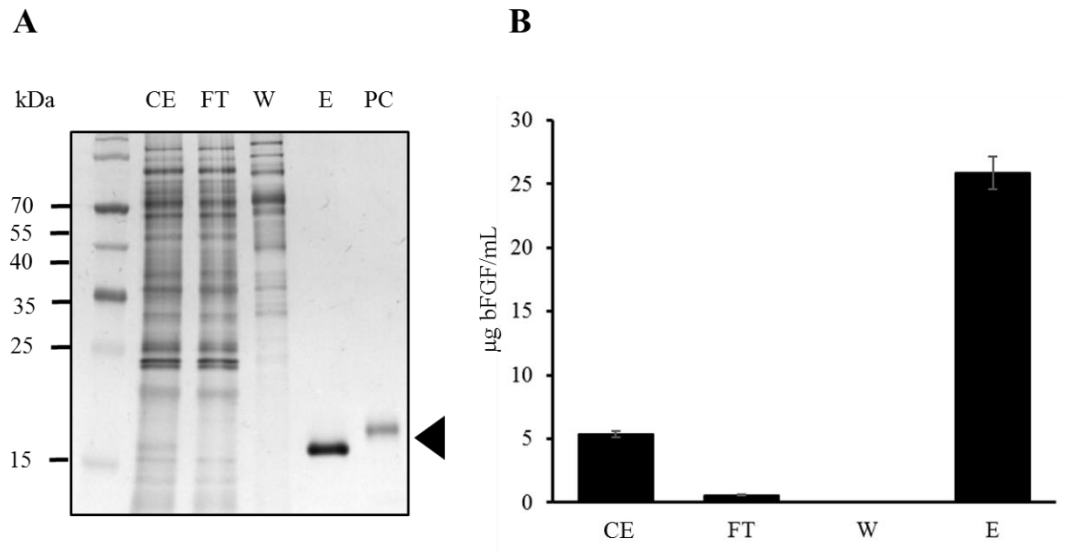


Figure 16. Purification of secreted ASbFGF using Heparin Affinity chromatography. (A) Silver staining, (B) ELISA quantification of bFGF content in the purification fractions. For (A) and (B), the elution fraction following double purification (E), wash fraction (W), flowthrough fraction (FT), crude extract of PCP eluents (CE), and the bFGF derived from *E. coli* (PC) are shown. For (B), three triplicate samples' mean values and standard deviation are displayed.

3.3.4 Investigation of size reduction of secreted ASbFGF

Size reduction of secreted ASbFGF in both leaves and PCPs was observed in the Western blotting of the different purified secreted ASbFGF from leaf and PCPs (Figure 17A). Interestingly, comparing with the original ASbFGF, the overcutting of the signal peptide in leaves (3-4 kDa) was longer than in the PCPs (1-2 kDa). This was then proven by the result of N-terminal peptide sequencing (Figure 17B). Results of this showed that the intracellular ASbFGF is intact, with no truncation whatsoever. Secreted ASbFGF from leaves are truncated at Asp15 and to a lesser extent, Gly16. Interestingly, secreted ASbFGF from PCPs were cleaved less at Pro13. These results were unexpected as signal peptides were supposed to be cleaved at the normal cleavage site. It is also notable that no detectable properly cleaved secreted ASbFGF in the Western blot data were observed. Succinimide cleavage could be a possible explanation for cleavage at Asp15 in the secreted ASbFGF from leaves. Cleavage at asparagine sites specifically at Asp28-Pro and Asp15-Gly were mentioned before in a previous study involving spontaneous cleavage of purified bFGF (Shahrokh et al. 1994). This kind of self-cleavage was also reported before in bacterial flagellar protein FlhB. It involves formation of a succinimide ring occurs through the attack of the -amino group of the adjacent carboxyl amino acid to the carbonyl carbon of the nearby asparagine group (Ferris et al, 2005). However, this reaction took many weeks to make and post agroinfiltration incubation just takes a few days so this could mean that this degradation process could be catalyzed by a chemical reaction in the leaves. Furthermore, this theory cannot explain the cleavage at Gly16 nor the ones at Pro13 in the secreted ASbFGF from PCPs. Exposure of the intracellular ASbFGF in 2.2.6 did not produce truncation of this type. This was only observed in the secreted forms of ASbFGF. Therefore, this suggests that this cleavage is brought about by the process of secretion

in the plant cells. To gain more information, the predicted 3D structure of SS^{Ext}ASbFGF and ASbFGF was made using the Robetta server (Figure 17C). This shows that all the cleavage sites are located around a major beta turn area. This suggests a possible relationship between this structure and the observed cleavage sites.

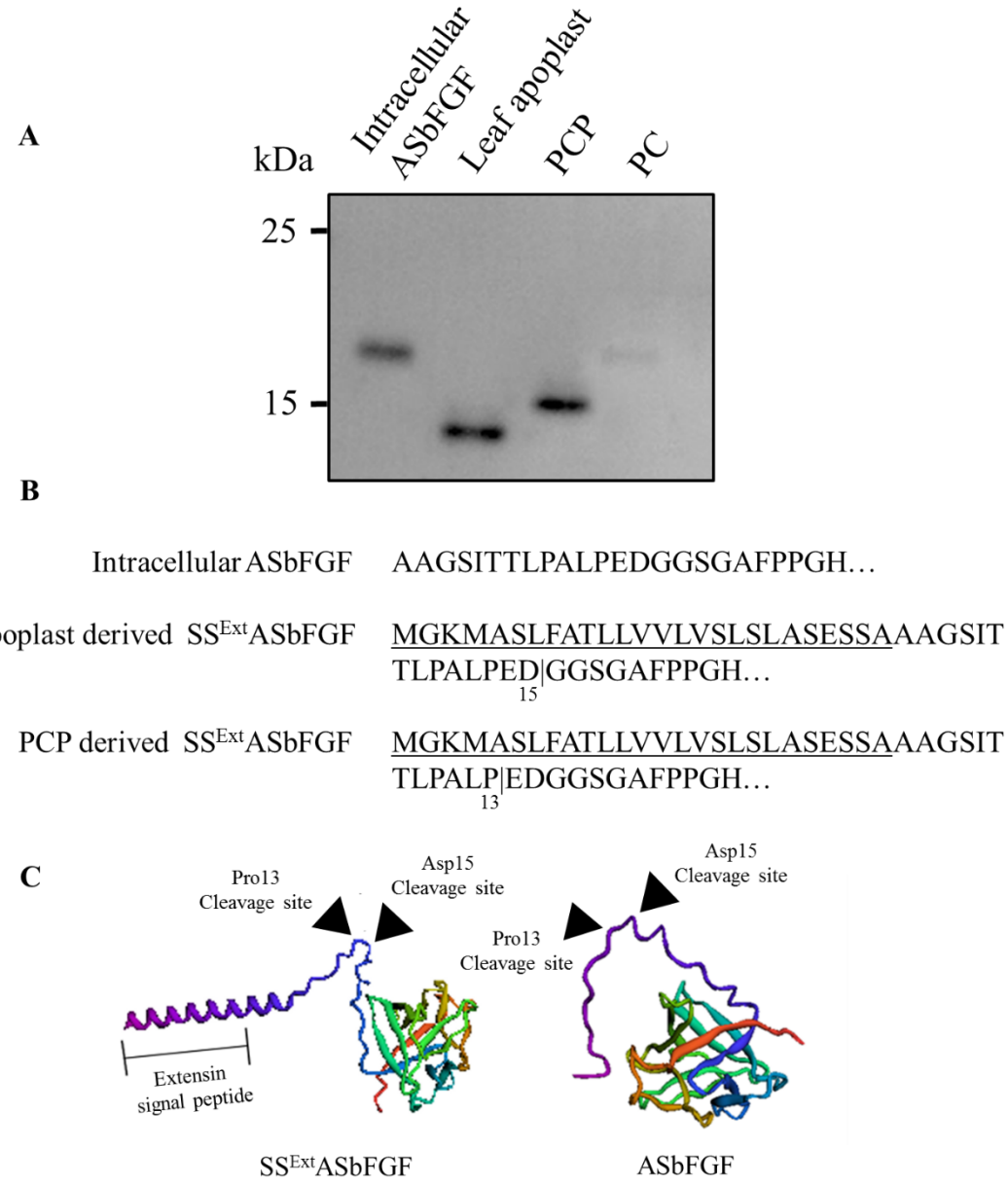


Figure 17. Investigation of signal peptide overcutting. (A) Western blot of purified Intracellular ASbFGF and secreted ASbFGF from leaf apoplast and PCPs. (B) Peptide sequencing results showing the sequence of intracellular ASbFGF and truncation sites in secreted ASbFGF. (C) *In silico* 3D protein modelling of ASbFGF variants using Robetta server. For (A), The positive control (PC) is 25 ng of bFGF derived from *E. coli*. For (C), Underlined amino acids are the ones composing the extension signal peptide.

3.3.5 Prolonged growth in stable transgenic SS^{Ext}ASbFGF producing *N. benthamiana* callus

Extended growth time was observed in the stable transgenic SS^{Ext}ASbFGF producing *N. benthamiana* callus. This phenomenon was first observed in stable suspension cultures which were still healthy after prolonged culture for 2 weeks. Usually, *N. benthamiana* suspension cultures were subcultured every week. Failure to do so led to browning and inability to sustain viability. However, SS^{Ext}ASbFGF producing transgenic counterparts were still yellow which is an indicative of health.

Comparison of the growth curves of the transgenic SS^{Ext}ASbFGF producing cells to the wild type shows that indeed, for some reason, production of SS^{Ext}ASbFGF led to a phenotype that has extended growth phase (Figure 18). In mammalian cells, bFGF is known to have anti-apoptotic properties through both genomic and acute actions (Peluso 2003). These acute actions are reported to be due to bFGF's involvement in calcium homeostasis (Lynch et al. 2000). Currently, there is no known similar mechanism of action of bFGF in plants.

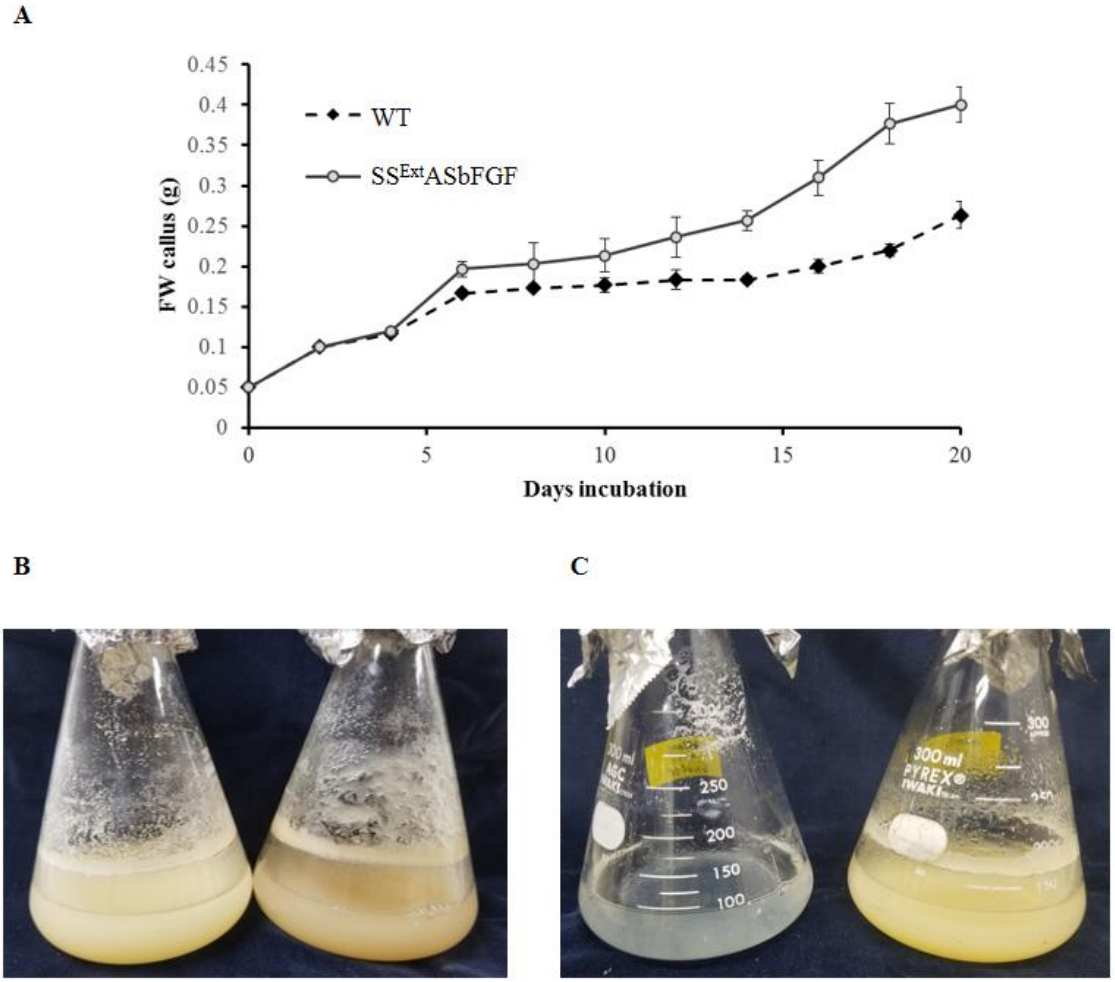


Figure 18. Comparing the phenotype of stable transgenic SS^{Ext}ASbFGF producing *N. benthamiana* callus vs wild type. (A) Comparison of callus growth rate by weight. (B) Color comparison of 20-day-old suspension cultures of transgenic SS^{Ext}ASbFGF producing *N. benthamiana* callus (left) vs wild type (right). (C) Results of subculturing 20-day old suspension cultures of transgenic SS^{Ext}ASbFGF producing *N. benthamiana* callus (right) vs wild type (left). For (A), shown are the means from triplicate measurements from independently grown callus with the error bars representing the standard deviation.

3.3.6 Cell proliferation assay

Bioactivity of the secreted recombinant ASbFGFs was confirmed in the cell proliferation assay using HepG2 and CHO-K1 cells (Figure 19). Heparin binding is reported to have an important role in the biological activity of bFGF. Mutation of the binding sites resulted in compromised bioactivity (Heath et al. 1991). Since heparin binding was not compromised as shown by the purification results, bioactivity was expected. Both using HepG2 cells (Figure 19A), and CHO-K1 cells (Figure 19B), secreted ASbFGFs exhibited a bioactivity comparable to the commercial *E. coli*-produced bFGF.

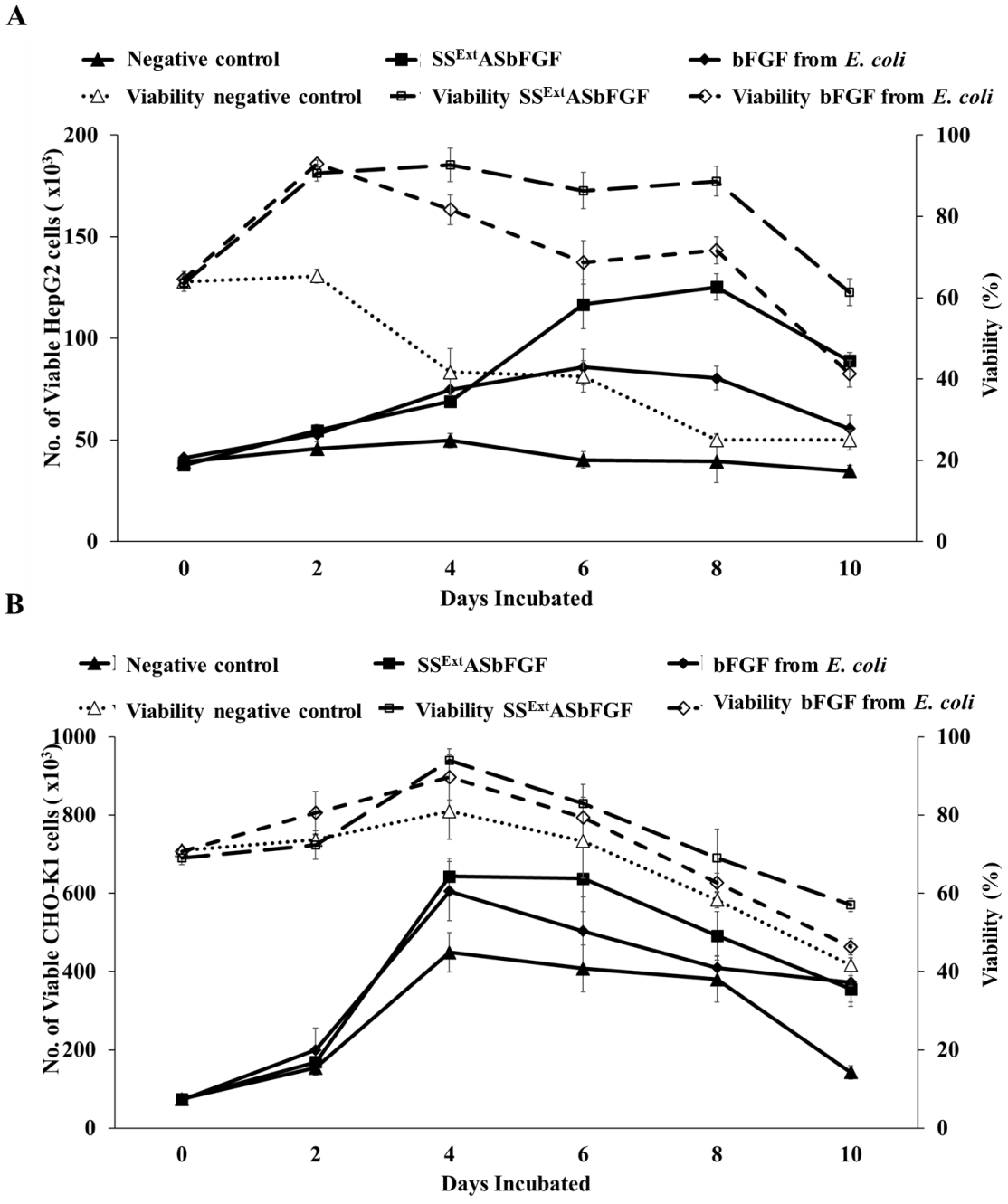


Figure 19. Bioactivity testing of secreted ASbFGF in selected mammalian cell lines. (A) HepG2 cells and (B) CHO-K1 cells. PCP-derived purified secreted SS^{Ext}ASbFGF at 20 ng/mL (Hep G2) and 5 ng/mL (CHO-K1) was used in this experiment. For the positive control, same concentrations of the commercial *E. coli*-derived bFGF was used. For the negative control, PBS is added. The error bars in panels (A) and (B) represent the standard deviation and the mean from experiments done in triplicate.

3.4 Discussion

This study was the first to design and report a viable production system of secreted ASbFGF in plants. bFGF originally is an intracellularly localized protein (Tessler & Neufeld 1990; Caré et al. 1998). that is partially secreted via an unconventional secretion pathway (Aktas & Kayton 2011) in mammalian cells. A previous study produced signal peptide-fused bFGF in *N. benthamiana* leaves but high level production was not achieved (Rattanapisit et al. 2020). This study showed that substantial secreted ASbFGF could be achieved using the acid-stable variant and the use of the signal peptide of native *N. benthamiana* extensin in both PCPs and leaves.

This is also the first study to successfully use stable transgenic cells for PCPs. Secreting proteins with hydrophobic characteristics could be tricky as in bFGF and this PCP based secreted production system could be a model for secreted production of hydrophobic proteins in general. Accumulation rates are not as high as in transient production system in leaves which is 5-times higher, but secreted production in leaf apoplasts pose several logistical disadvantages. First, the need for agroinfiltrating plants everytime, the need to process immediately after harvest, and finally the very tedious process of extracting apoplast wash fractions of individual leaves. This alone hampers the scalability of the production process and renders it unsuitable for industrial scale production. On the other hand, PCPs are easily scalable. They can be grown first as suspension cells in bioreactors. Afterwards, they can be incubated on non-sterile, rooms. Finally the PCP eluates can be collected via large volume glass purification columns (Rademacher et al. 2019; Gengenbach et al. 2020).

Meanwhile, the stable PCP production comparing to transient PCP production gives the obvious advantage of the reduced cost by eliminating the need to agroinfiltrate. This also removes the inconvenience brought by the blockage of *A. tumefaciens* in the columns for eluate collection. In theory, the only advantage of transient PCP production system is the higher accumulation rates. However, this is not observed in the case of bFGF. The stable transgenic secreted ASbFGF producing cells have an unexpected longer-viability rates which might be due to the anti-apoptotic properties of bFGF and this possibly led to the higher accumulation rates.

In this study, it was also observed that the fusion of signal peptides, reduced the production regardless of signal peptide type albeit at varying degrees. This result was similar with a previous publication using amylase signal peptide (Rattanapisit et al. 2020). For other proteins, cytosolic retention led to lower accumulation rates (Conrad & Fiedler 1998; Schillberg et al. 1999). This is not the case for ASbFGF. In theory, the original bFGF variant with no signal peptide should be localized in the cytoplasm while the secreted ASbFGF should be shuttled to the ER-Golgi pathway (Chen et al. 2014) and finally secreted to the apoplast. This region and the secretory pathway as a whole is known to be rich in various proteases (Agrawal et al. 2010; Alexandersson et al. 2013; Minina et al. 2017) and differing types and degrees of proteases where folding bFGF could be exposed to in these two pathways might influence both the reduced secreted ASbFGF yield and the higher production in ASbFGF (Supplementary Figure 4). Another possible reason for this reduced production in secreted variants is the oxidizing environment in the ER which favors disulfide formation (Thorpe et al. 2002; Reznik & Fass 2022). Irreversible aggregation influenced by disulfide formation is a main culprit for bFGF's instability (Shahrokh et al. 1994). Therefore the mutation of 2 external cysteines to serine might have mitigated this phenomenon

and might be the reason why the production of secreted ASbFGF is higher than their non-mutant secreted bFGF counterparts (Supplementary Figure 4). On the other hand, secretion of ASbFGF with a native extensin signal peptide was shown to mitigate the decrease in production. This might be due to the fact that this signal peptide is more recognized by the *N. benthamiana* cells and more likely to be secreted properly instead of directing it for degradation in lysosomes or storage in the vacuole.

N-terminal cleavage of secreted ASbFGF is also an interesting finding in this study. Signal peptide cleavage site prediction softwares TargetP 2.0, and SignalP6.0 (Figure 20 and 21) were not able to predict this overcutting and predicts cleavage at the expected cleavage site which is at the C-terminal of the signal peptide. Interestingly, this specific site is reported to be a degradatory product of purified bFGF at pH 5.0 when aged for 13 weeks (Shahrokh et al. 1994). This degradation could be due to succinimide formation in aspartate regions at acidic conditions. The pH condition of leaves is at about pH 4.3 – 5.2 (Geilfus & Muhling 2011), while MS media is about pH 5.7. This might be a good reason for the cleavage at Asp15 in leaves as succinimide formation is most favorable at pH 4-5. (Shahrokh et al. 1994). However, Ab-initio three-dimensional modeling of the proteins using the Robetta server (Chivian et al. 2003; Kim et al. 2004) shows beta turn where the cleavage site is located. This structure was also observed in the other signal peptide fused ASbFGF variants (Supplementary Figure 6). It is also notable that intracellular ASbFGF did not undergo any observable truncation at the cleavage sites observed in the secreted versions. This is a strong evidence that the secretion mechanism of the plant cells catalyze this process of secreted ASbFGF truncation. However, further studies should be done in order to confirm this

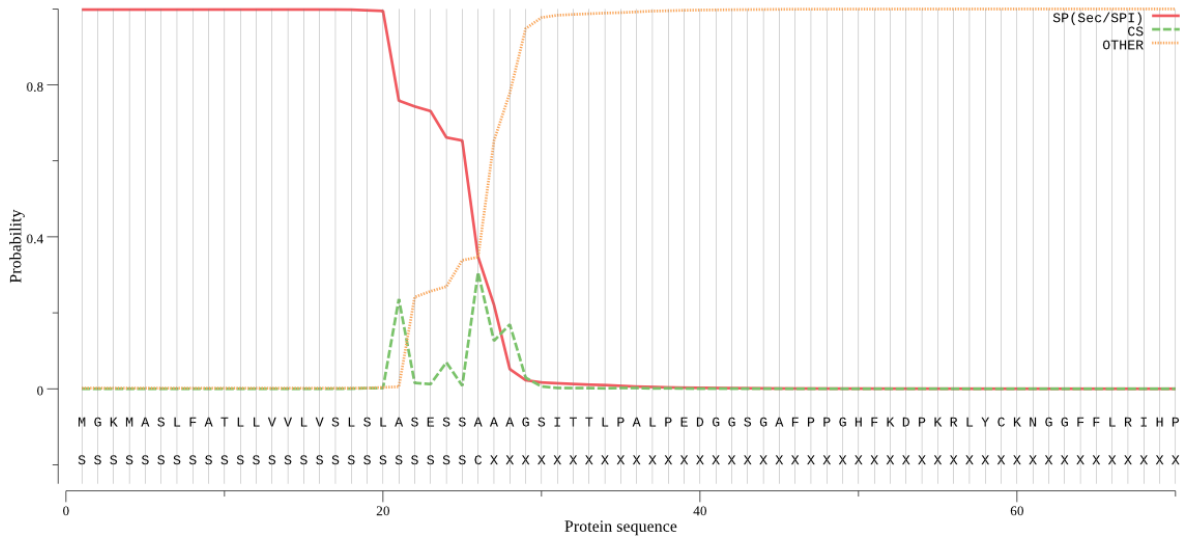


Figure 20. SignalP5.0 prediction of SS^{Ext}ASbFGF cleavage

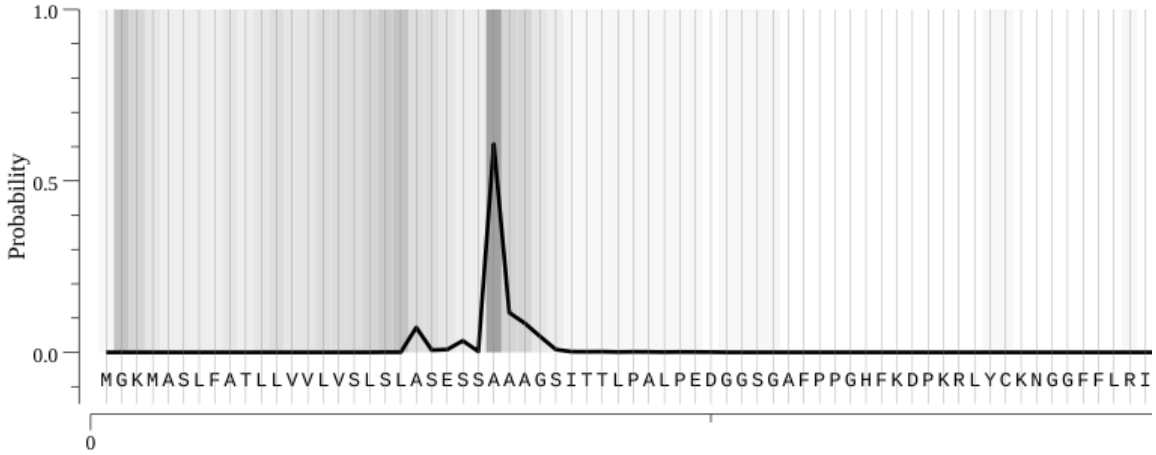


Figure 21. TargetP2.0 prediction of SS^{Ext}ASbFGF cleavage

All in all, this research developed working secreted production systems of bFGF in plants and provided pertinent information regarding the effects of the use of different modes of production in both quantity and characteristics of the recombinant protein produced.

3.5 Summary

The human basic fibroblast growth factor (bFGF) is an important reagent in biomedical engineering, cosmetics, and research. In this study, a *Nicotiana benthamiana* suspension cell-derived, culture media deprived plant cell packs (PCP) were developed for stable transgenic secreted production system of acid-stable bFGF for the first time. This was done by the deriving PCPs from stable transgenic *N. benthamiana* suspension cells producing an *N. benthamiana* extensin signal sequence (SS^{Ext})-fused acid-stable bFGF (ASbFGF) transgene. These PCPs were observed to have about 35-fold increased production compared with their suspension cell counterparts. Furthermore, the purified ASbFGF was obtained via a one-step purification scheme. However, the secreted ASbFGF was seen to be truncated. Nevertheless, they are still found to have bioactivity comparable to that of commercially produced bFGF in HepG2 and CHO-K1 cells. These results show that the *N. benthamiana* PCPs can be a very efficient method for the industrial level production of bFGF and in addition provide some unique observations in the secreted ASbFGF produced and the stable transgenic SS^{Ext}ASbFGF producing cell line produced,

Chapter 4 General Conclusions and Perspectives

As of the writing of this study, bFGF has been produced in a variety of expression platforms such as silkworm (Wu et al. 2016), rice (An et al. 2013), tobacco (Wang et al. 2016), *N. benthamiana* (Rattanapisit et al. 2020*), C. glutamicum* (Ke et al. 1992), and commercially in *E. coli*. This is not surprising as bacterial fermentation is known as the best-characterized and most economical production technology for non-glycosylated proteins in general.

In this study, competitive alternative production systems were established (Table 2). Firstly, a high-level production of intracellularly produced ASbFGF was achieved at 185 µg/g FW leaves. This is competitive if not more competitive than the 40 µg/mL production in *E. coli* (Squirres et al. 1988). However, the downside of this system is the tedious agroinfiltration method and downstream processing A second system developed was a stable transgenic secreted production system of SS^{Ext}ASbFGF in *N. benthamiana* PCP that has a simple purification scheme, low risk of endotoxin contamination while at the same time producing a competitive yield at around 13 µg/g FW cells. Intracellular production in *E. coli* and endotoxin contamination complicates the downstream processing. About 45–92% of the overall manufacturing expenses for recombinant protein products are incurred in downstream processing (Straathof, 2011; Saraswat et al., 2013; Wilding et al., 2019). Therefore, simplifying the downstream processes through the secreted production system in PCP developed in this study could really likely reduce the cost of production of the recombinant protein. In addition, application of this secreted ASbFGF system in *N. benthamiana* PCP though having about 5-fold lower production, still stands as a more attractive option as this method is more scalable and more convenient. However, the exact comparison of costs across the different production systems in the case of bFGF which should also take into

account upscaling is beyond the scope of this study. Further financial studies could be done in order to prove this point.

Table 4. Comparison of recombinant bFGF production across pertinent production systems

Expression platform	**Yield**	**Reference**
E. coli	40 μg/ml	Squires et al. (1988)
N. benthamiana (leaves, normal bFGF)	2.7 μg/g FW leaves	Rattanapisit et al (2020)
N. benthamiana (leaves, ASbFGF)	185 μg/g FW leaves	This study
N. benthamiana (leaves, secreted ASbFGF)	76 μg/g FW leaves	This study
N. benthamiana (suspension cell culture, secreted ASbFGF)	0.4 μg/g FW cells	This study
N. benthamiana (PCP, secreted ASbFGF)	13 μg/g FW cells	This study

In addition to alternative production systems per se, this study also provided new and useful methods and findings. First, this study shows that bFGF can be purified without a tag in *N. benthamiana* crude leaf extracts. This is notable as a proof of concept and also because this might have had indirectly contributed to the higher production as the addition of His-tag to an already aggregation prone bFGF may be the culprit of early necrosis in a previous production method in *N.benthamiana* (Rattanapisit et al. 2020). Secondly, this study developed a new ELISA method for quantifying bFGF in leaf crude extracts. This technology paves the way for a quicker and cheaper quantification method of bFGF produced in plants.

Furthermore, this study is also the first to report novel observations that could be of significance to cell physiology studies. First, the secreted production systems showcase novel truncation pattern of secreted SS^{Ext}ASbFGF that was cleaved at Pro13 which is before the expected Asp15 which challenges the succinimide truncation theory of bFGF truncation at least in the context of secreted ASbFGF production. Combining these findings with the protein structure prediction, these suggest the importance of secondary structures such as a signal peptide alpha helix and a subsequent beta turn in signal peptide cleavage. Another notable observation in this study was a surprising phenotypic side effect of resilience which was observed in stably producing SS^{Ext}ASbFGF *N. benthamiana* cells. This could potentially be harnessed for the development of a more robust *N. benthamiana* suspension cell line.

In general, this study provided important improvements in the technologies regarding recombinant bFGF production especially in plants while discovering unique features of the novel production systems. These methods could potentially lead to more efficient production and studies of bFGF in plants.

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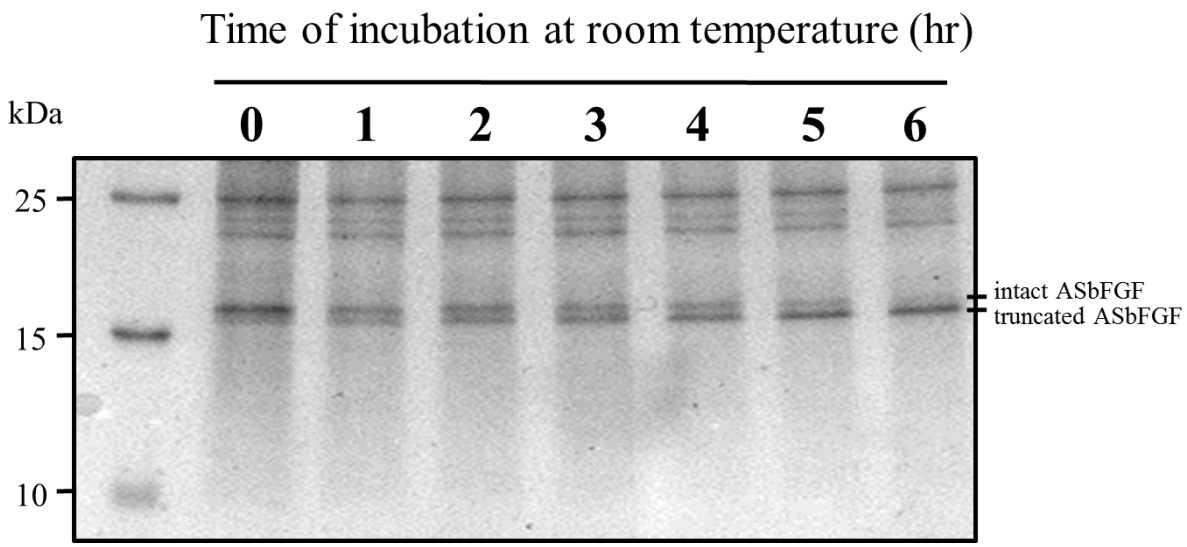
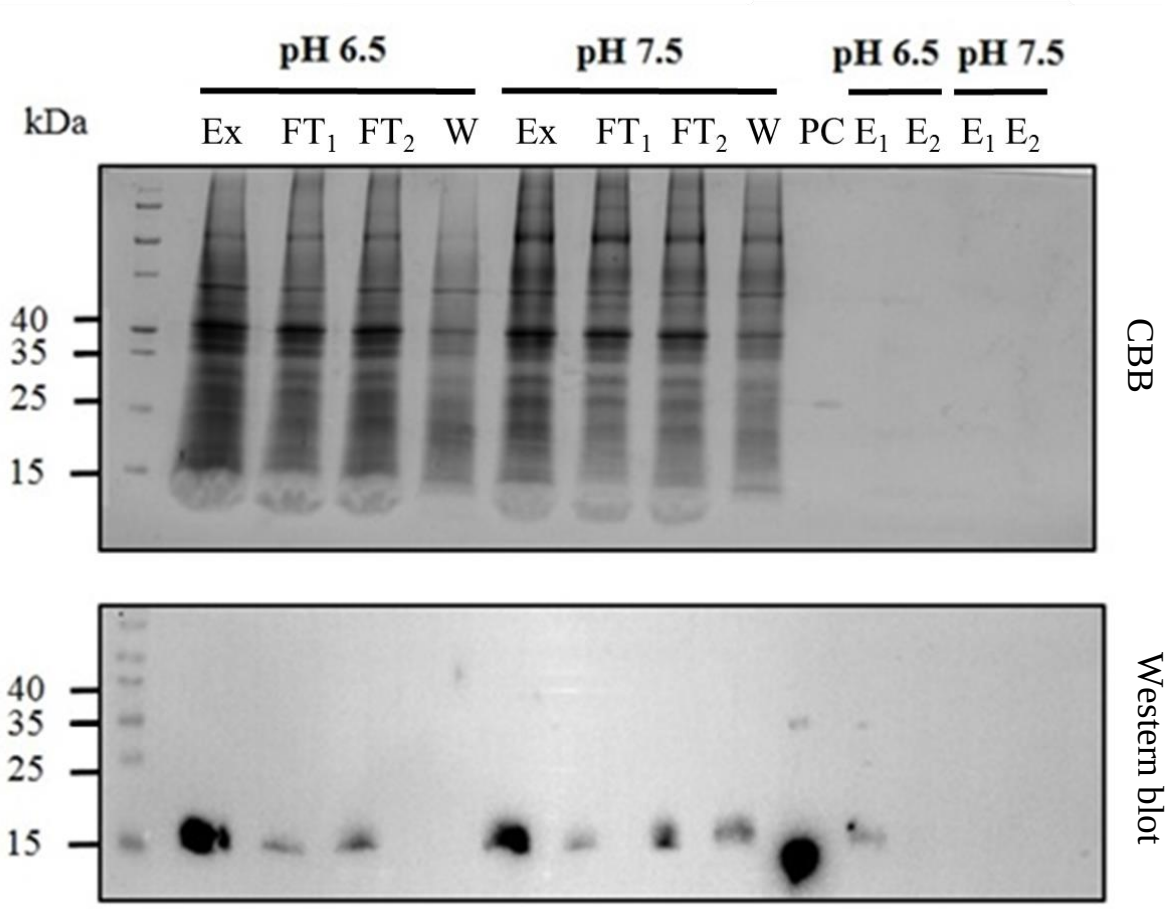
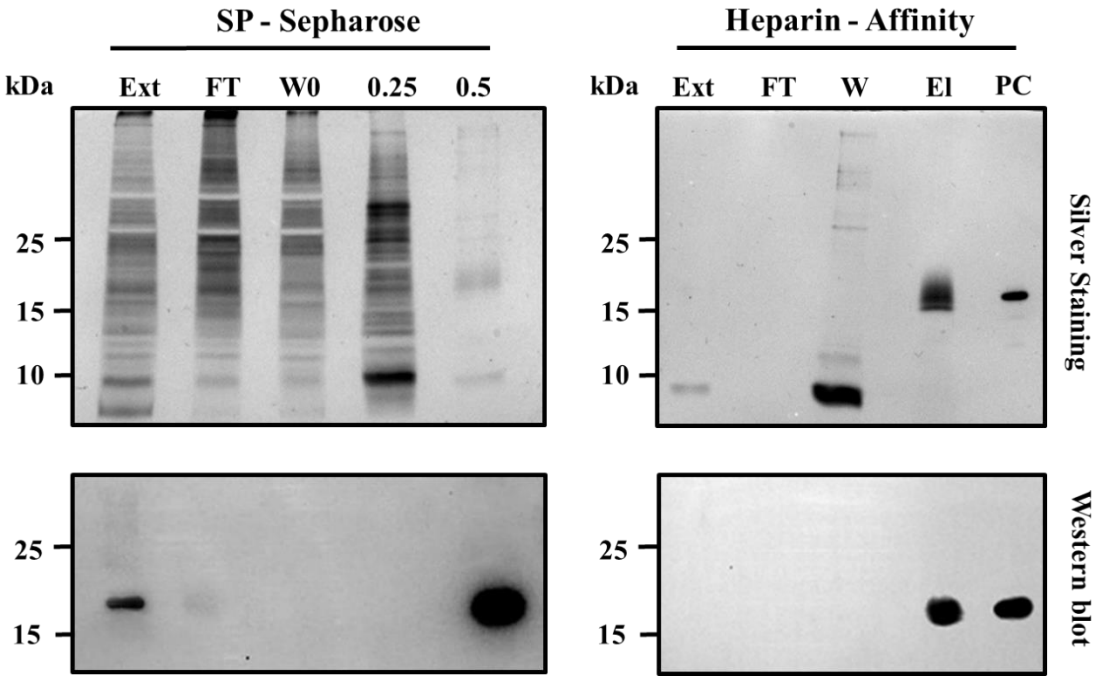
Supplementary Materials



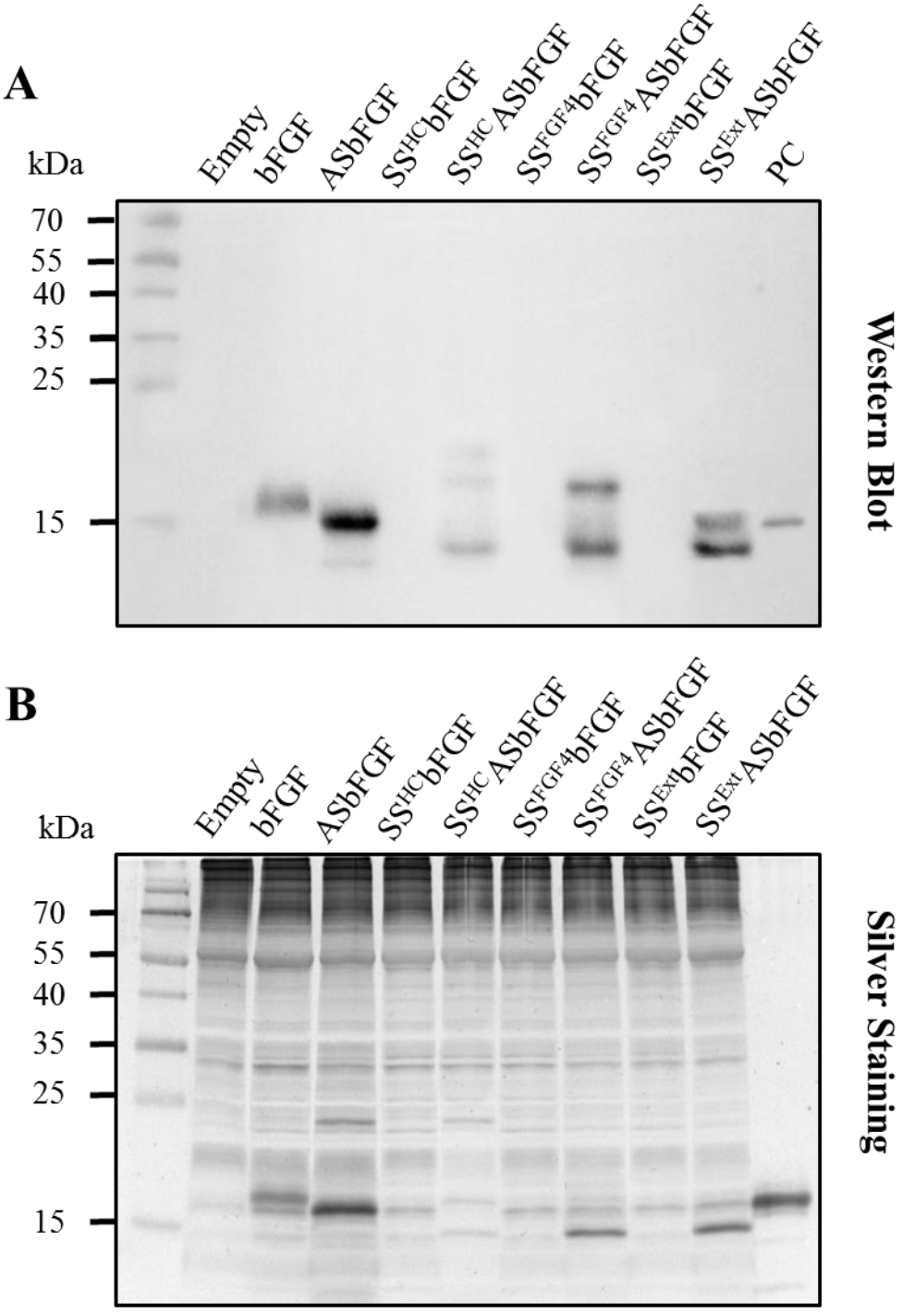
Supplementary Figure 1. Enlarged portion of CBB stained SDS-PAGE gel of the stability analysis of ASbFGF in crude extracts showing production of truncated ASbFGF through time.



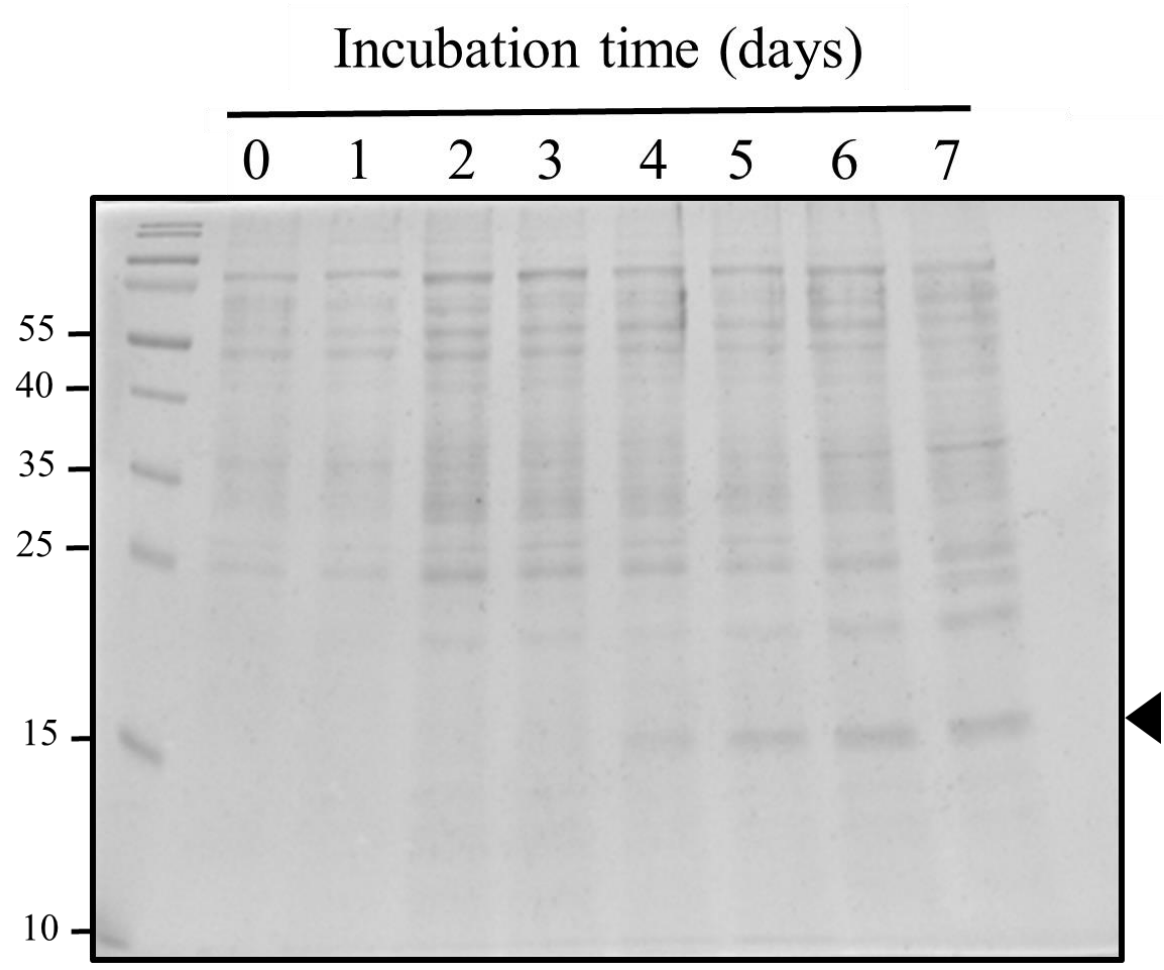
Supplementary Figure 2. Optimal pH testing of wash and elution steps in Sepharose purification. Here, Ext are the crude extracts, FT1 and FT2 are the flowthrough, W0 are wash fractions using extraction buffer and E1 and E2 are elution fractions using 0.5 M NaCl. The positive control (+), was 10 ng E. coli produced bFGF.



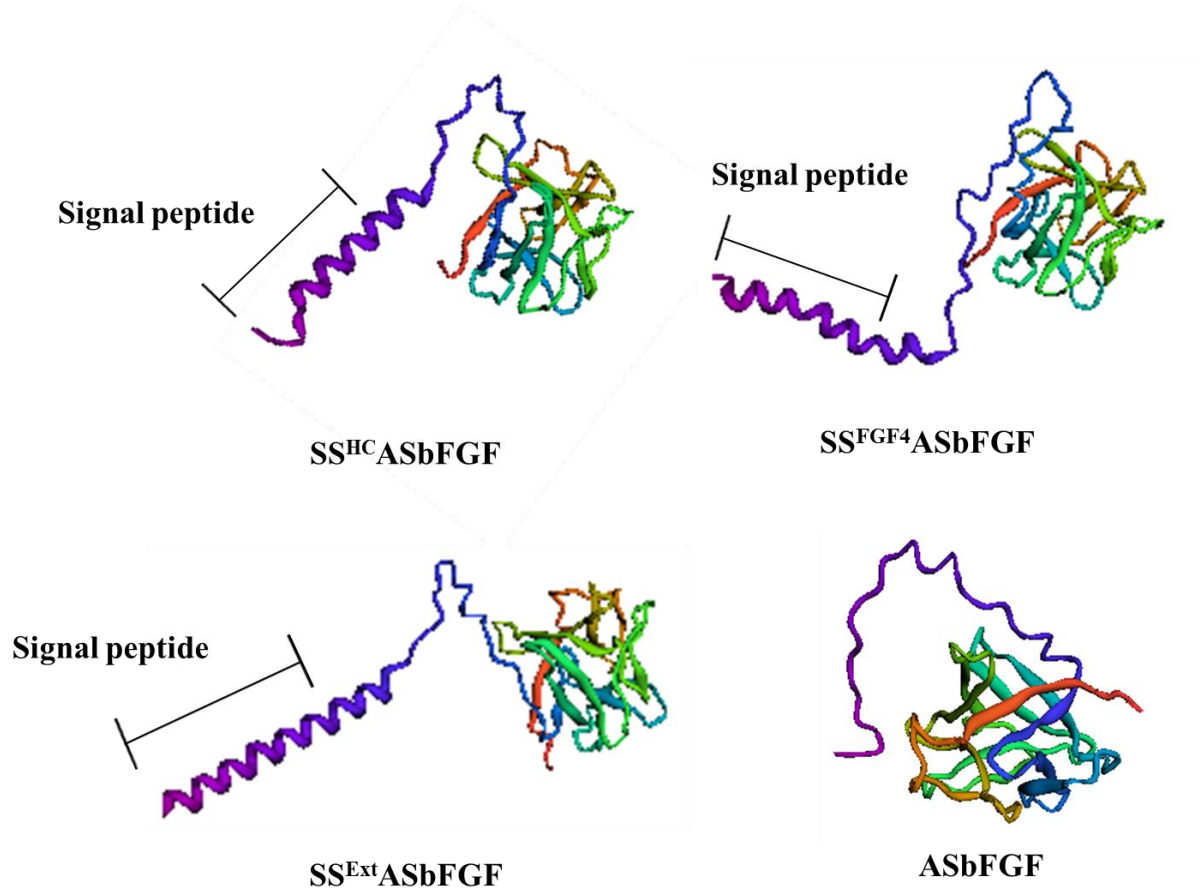
Supplementary Figure 3. SP Sepharose-Heparin affinity purification fractions in bFGF purification. Here, Ext are the initial crude extracts (For heparin affinity Ext is the diluted eluent from SP-Sepharose purification), FT is the flowthrough, W0 is wash fraction using extraction buffer, 0.25 is the wash using 0.25 M NaCl and 0.5 is the elution using 0.5 M NaCl, W is the wash step using 1 M NaCl, E1 is the final elution using 2 M NaCl while PC is 25 ng of *E. coli* made bFGF positive control.



Supplementary Figure 4. Total crude extracts of leaves agroinfiltrated with different bFGF and ASbFGF variants (A) Western blot, (B) Silver staining and (C) ELISA.



Supplementary Figure 5. CBB staining of dry PCP eluates across time.



Supplementary Figure 6. In silico ab-initio 3D modelling of all ASbFGF variants. The 3D structures are the result of de-novo sequencing using the Robetta server. Alpha helix formation of the signal peptide was shown across all signal peptide fused ASbFGF variants.

List of publication

Macauyag EA, Kajiura H, Ohashi T, Misaki R, Fujiyama K. High-level transient production of a protease-resistant mutant form of human basic fibroblast growth factor in *Nicotiana benthamiana* leaves. Plant Biotechnology. 2022. (39):1–11

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