High-level transient production of a protease-resistant mutant form of human basic fibroblast growth factor in Nicotiana benthamiana leaves

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Abstract The human basic fibroblast growth factor (bFGF) is a protein that plays a pivotal role in cellular processes like cell proliferation and development. As a result, it has become an important component in cell culture systems, with applications in biomedical engineering, cosmetics, and research. Alternative production techniques, such as transient production in plants, are becoming a feasible option as the demand continues to grow. High-level bFGF production was achieved in this study employing an optimized Agrobacterium-mediated transient expression system, which yielded about a 3-fold increase in production over a conventional system. This yield was further doubled at about 185 µg g⁻¹ FW using a mutant protease-resistant version that degraded/aggregated at a three-fold slower rate in leaf crude extracts. To achieve a pure product, a two-step purification technique was applied. The capacity of the pure protease-resistant bFGF (PRbFGF) to stimulate cell proliferation was tested and was found to be comparable to that of E. coli-produced bFGF in HepG2 and CHO-K1 cells. Overall, this study demonstrates a high-level transient production system of functional PRbFGF in N. benthamiana leaves as well as an efficient tag-less purification technique of leaf crude extracts.

Key words: bFGF, Nicotiana benthamiana, protease-resistant, recombinant protein, transient production.

Introduction

Human basic fibroblast factor (bFGF) is an essential growth factor that belongs to the vast FGF protein family. It is known to have a strong affinity to heparin and to have bioactivities that include growth regulation, differentiation, migration, and survival of diverse cell types (Barrientos et al. 2008). The specific mechanisms underlying these processes are unknown (Ma et al. 2019), however the Erk1/2 mitogen-activated protein kinase pathway, which plays a central role in cell proliferation control, has been implicated (Zaragosi et al. 2006). Proliferation in human mesenchymal stem cells is known to be triggered by stimulation of the Jun amino-terminal kinase pathway (Ahn et al. 2009). Because of its role in these activities, bFGF is extremely significant in the regenerative medicine and cosmetic industries (Levenstein et al. 2006). Originally, bFGF was purified from the bovine brain and pituitary (Matsuda et al. 1992). However, advancements in bioengineering made possible its production in more accessible platforms like Pichia pastoris (Mu et al. 2008), soybean seeds (Ding et al. 2006), silkworm (Wu et al. 2001) and Bacillus subtilis (Kwong et al. 2013). Now, it is produced commercially in Escherichia coli (Ke et al. 1992) and Corynebacterium glutamicum (Gopinath and Nampoothiri 2014).

Plant molecular pharming 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 (Habibi et al. 2017; Moustafa et al. 2016; Shanmugaraj et al. 2020). The commercialization of taliglucerase alfa (Protalix, Israel), the first FDA approved plant-produced product for enzyme replacement therapy (ERT) using stable plant suspension cells, solidified the potential of plant
molecular pharming. However, as compared to 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-amyrase (Prado et al. 2019), Interleukin-6 (Nausch et al. 2012), antibodies (Vézina 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).

In theory, an ideal production system would have high-levels of recombinant protein accumulation and be created in an expression platform that is easy and inexpensive to cultivate. In soybeans, at 2.3% of total soluble protein (TSP) (Ding et al. 2006) and rice seeds at 9.6% of TSP (An et al. 2013), substantial levels of plant-made bFGF were already attained. However, due to the limited amount of seed biomass produced per unit of land and long growth cycles, this form of production would not be very efficient (Wang et al. 2015). Recombinant production in tobacco leaves, on the other hand, yields more biomass per unit of land. However, the yield of recombinant bFGF previously reported was only about 0.1% of TSP (Wang et al. 2015). Recently, bFGF was transiently produced in N. benthamiana leaves although at a rate of just 2.7 µg g⁻¹ Fresh Weight (FW) (Rattanapisit et al. 2020). This degree of production should be pushed much farther in order for plant-produced bFGF to be more viable for industrial manufacturing.

In this study, effective ways to improve less-explored points in both upstream and downstream processes in Agrobacterium-mediated transient production of bFGF was demonstrated. In comparison to traditional approaches, these enhancements resulted in a three-fold increase in production. Furthermore, the use of a Cys70Ser, Cys88Ser mutant protease-resistant bFGF (PRbFGF) instead of the wild type bFGF further doubled the production. These enhancements can be combined to previously established transient expression systems to boost recombinant bFGF production. The generated PRbFGF was purified using a tagless purification method and demonstrated to be functional in cell proliferation tests.

Materials and methods

Vector construction

The bFGF variants bFGF, GGGbFGF and PRbFGF used in this study (Figure 1A) were derived from the 17.8 kDa isoform of human bFGF (GenBank Accession No: AAQ73204.1) and were synthetically cloned using GeneArt gene synthesis (Thermo Scientific, Waltham, MA). These were then ligated into a plant expression vector pGPTV-bar (Figure 1A) and introduced into A. tumefaciens strain LBA4404 by electroporation (Bio-Rad, Hercules, CA) with voltage, resistance, and capacitance at 2.4 kV, 200 Ω, and 25 μF, respectively. On the other hand, the RNA silencing suppressor p19 (p19) vector-containing LBA4404 used in this study was provided by Prof. Atsushi Takeda from Ritsumeikan University.

Recombinant protein production and optimization

To obtain the optimal conditions for bFGF production and extraction, agroinfiltration buffer supplementation and extraction procedures were optimized using the normal human bFGF variant. First, A. tumefaciens LBA4404 containing a plasmid carrying human bFGF and RNA silencing suppressor p19 were grown in yeast manitol broth containing streptomycin (200 µg ml⁻¹), rifampicin (50 µg ml⁻¹), and kanamycin (100 µg ml⁻¹) for 2 days or until OD₅₆₀ reached 1.0. The Agrobacteria were then washed and resuspended in either the conventional infiltration buffer (10 mM MES, 10 mM MgSO₄, pH 5.8) or supplemented infiltration buffer (10 mM MES, 10 mM MgSO₄, pH 5.8, 0.5 mM acetosyringone, 0.03% Tween-20, and 0.56 mM ascorbic acid) patterned from a previous protocol (Norkunas et al. 2018; Zhao et al. 2017). The Agrobacterium concentration was then adjusted to an OD₅₆₀ of about 0.5 and was incubated at room temperature for 4 h before use. Each setup was co-infiltrated with Agrobacterium containing p19 at a 1:1 ratio. About one-month-old greenhouse-grown N. benthamiana plants were then vacuum infiltrated at 600 mmHg for 3 min using the Agrobacterium suspension combinations and then incubated in a greenhouse. Top and middle leaf samples were collected every day for 7 days and stored at −80°C until use.

For the optimization of the extraction method, the setup using the supplemented buffer and harvested at 6 days were used since this is the setup with the highest yield. Unless specified, the extraction buffer used was 50 mM sodium phosphate pH 6.5, with 100 mM NaCl and 0.025% Tween-20. Three parts of this buffer along with one-part ground liquid nitrogen flash frozen leaves were sonicated in ice for 20 s. NaCl concentration was optimized via comparing the bFGF yield when NaCl concentrations were changed in this procedure. Furthermore, the bFGF yield was compared when sonicated in ice for 20 s or mixed for 10 min. The procedures that yielded the highest bFGF production according to ELISA were combined and used for the comparison of different bFGF variants.

Comparison of recombinant protein production of different bFGF variants

For the comparison of different bFGF variants, the optimized agroinfiltration and extraction procedures were used across the board. In particular, supplemented infiltration buffer was used to agroinfiltrate the plants, harvested after 6 days, and extracted using an extraction buffer containing 50 mM sodium phosphate.
Protein analysis by indirect ELISA

The crude leaf extracts were homogenized and suspended in the extraction buffer with no Tween-20. This was then sonicated briefly (200 W; Kaijo Denki, Tokyo, Japan) on ice. The crude extract concentrations were then adjusted to about 2 µg µl⁻¹ total protein by Bradford assay. Exactly 50 µl of these crude extracts were coated on a 96-well plate, in triplicates. This was then incubated at 37 °C for 30 min and was blocked for another 30 min using 5% bovine serum albumin. Primary antibody incubation was done overnight at 4 °C with shaking using 330 ng ml⁻¹ anti-FGF2 mouse IgG (Santa Cruz Biotechnology, Dallas, TX) in phosphate-buffered saline with 0.05% Tween-20 (PBST). Secondary antibody incubation was done using 1/10,000 anti-mouse HRP IgG from sheep (GE Healthcare, Chicago, IL) at room temperature for 1 h with the secondary antibody anti-mouse-HRP IgG (GE Healthcare, Chicago, IL) at 1:10,000 dilutions. Finally, the bands were visualized by adding Luminata Forte Western HRP substrate (Millipore Sigma, Burlington, MO) to the membrane and the resulting chemiluminescence was detected using iBright 1500 Imaging System (Thermo Fisher, Waltham, MA).

Figure 1. T-DNA of Expression constructs and details of bFGF variants used. (A) Schematic representations of no signal peptide constructs that express 18kDa bFGF in the form of the wild type (bFGF), GGG optimized (GGGbFGF) and protease-resistant (PRbFGF). 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 Agrobacterium-mediated transformation. (B) DNA sequence of GGG optimized bFGF. Underlined sequences are the identified GGGNDD or DGGGND whose GGG sequences were replaced by GGA. (C) Amino acid sequence alignment of wild type (WT) bFGF and protease-resistant bFGF (PR) showing replaced Cys70 and Cys88 to Ser70 and Ser88 respectively.

Protein analysis by SDS-PAGE and western blot

All fractions that were sampled were run through 15% Tris-glycine sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) at 120 V for 90 min. The resulting gels were then analyzed for total protein content by Coomassie brilliant blue (CBB) or silver staining. Meanwhile, bFGF presence was analyzed by western blot. In this case, the proteins from the resulting SDS-PAGE gel were transferred to a PVDF membrane. The membrane was then blocked using 5% skim milk in PBST. Afterward, it was incubated at room temperature for 1 h with the secondary antibody anti-mouse-HRP IgG (GE Healthcare, Chicago, IL) at 1:10,000 dilutions. Finally, the bands were visualized by adding Luminata Forte Western HRP substrate (Millipore Sigma, Burlington, MO) to the membrane and the resulting chemiluminescence was detected using iBright 1500 Imaging System (Thermo Fisher, Waltham, MA).

Stability analysis in crude extracts

To assess the stability of bFGF vs PRbFGF in crude leaf extracts, flash-frozen, ground, 6-day agroinfiltrated leaves expressing bFGF and PRbFGF variants were analyzed for their total soluble protein degradation using SDS-PAGE and ELISA. This method is patterned from an in vitro degradation analysis of antibody with crude tobacco leaf extracts with minor modifications (Stevens et al. 2000). Briefly, the samples of crushed leaves were stored in centrifuge tubes and incubated at room temperature for 6 h and sampled every hour. The
appropriate amount of extraction buffer without Tween-20 was then added to adjust the total protein concentration to 2 µg/µl. This extract was then used for SDS-PAGE and ELISA analysis of bFGF content.

**Purification by SP-ion exchange chromatography followed by heparin affinity chromatography**

Because of the relative instability of bFGF, the resulting crude extract was directly run and semi-purified using sulfopropyl cation exchange column using a procedure previously done in *E. coli* (Seeger and Rinas 1996) with major modifications for *N. benthamiana* leaf extracts. The pH of the leaf crude extracts was readjusted to pH 6.5 and was directly run through 5 ml (1.0 cm × 12.0 cm) SP 650-M cation exchange column (Tokyo Kaken Co., Ltd., Tokyo, Japan) equilibrated with 20 ml extraction buffer. This was done instead of ammonium precipitation followed by dialysis which might incur losses in bFGF yield due to irreversible precipitation. Afterward, an initial washing using the extraction buffer with 0.01% Triton X-100 instead of 0.025% Tween-20 was done at about 3 times column volumes or until all green residues were washed away. Subsequently, additional washes with the extraction buffer without 0.01% Triton X-100 nor 0.025% Tween-20 were done three times. Subsequently, the column was washed with 4 column volumes of wash buffer (50 mM phosphate buffer pH 6.5 with 0.25 M NaCl) and then eluted using elution buffer (50 mM phosphate buffer pH 7.5 with 0.5 M NaCl).

Partially purified SP elution fractions having 0.5 M NaCl concentration in 50 mM phosphate buffer pH 7.5 were diluted with 50 mM phosphate buffer pH 8.0 to reach 0.2 M NaCl concentration. This eluent was then purified through a 1 ml Heparin Sepharose (GE Healthcare, Chicago, IL) column using a protocol patterned from a study for purifying recombinant bFGF in *E. coli* (Seeger and Rinas 1996) with some modifications. After running the partially purified SP eluents, the column was then washed using 4 column volumes of 50 mM phosphate buffer pH 8.0 with 1.0 M NaCl. Lastly, the purified recombinant protein was then eluted using 50 mM phosphate buffer pH 8.0 with 2.0 M NaCl. The final heparin eluent is stored at −80°C for future use.

**Cell proliferation assay**

To test the bioactivity of PRbFGF, cell proliferation assay was performed as in a previous study (Liu and Wu 2009). Briefly, Chinese hamster ovary K1 (CHO-K1) cells and human hepatocellular carcinoma (Hep G2) cells were first seeded in 24-well plates with growth media Hams-F12 and Dulbecco’s Modified Eagle Medium containing 4.5 g/l glucose (DMEM-high glucose) respectively with 10% fetal bovine serum for 24 h. Subsequently, these media were drained, washed three times with PBS and twice with their respective serum free media. CHO-K1 cells were then incubated overnight with serum-free Hams-F12 at 37°C and 5% CO₂. Meanwhile, Hep G2 cells originated from a human liver cancer tissue (Chengye et al. 2017) were incubated with the same conditions but with DMEM-high glucose with 0.1% FBS. Subsequently, recombinant protein was added at 5 ng/ml−1 and 20 ng/ml−1 respectively. The number of viable cells was counted every 2 days for 10 days using Trypan blue exclusion assay and automatic cell counter (Biorad, Hercules, CA).

**Results**

**Optimization of agroinfiltration and extraction procedures**

Agroinfiltration was optimized by comparing the time course of bFGF accumulation in agroinfiltrated leaves when a supplemented buffer containing Tween-20, ascorbic acid and acetosyringone was used as compared to a conventional buffer containing only 10 mM MES and 10 mM MgSO₄. The expression vector pGPTV-bar-bFGF which contained the non-optimized version of bFGF (Figure 1A) was used in these experiments as a constant variable. To quantify the bFGF production, an indirect ELISA quantification method of bFGF was optimized and developed (Supplementary Figure S1). Preliminary ELISA readings of same amounts of bFGF in PBS vs bFGF in crude extracts showed different OD 450 nm values (Supplementary Figure S1A). This suggests a large matrix effect on the readings. Furthermore, dilution of the crude extracts improved the linearity of the standard curve (Supplementary Figure S1B). The total protein concentration of this was measured via Bradford assay and all crude samples in future ELISA experiments were adjusted at this concentration. Lastly, the importance of not having Tween-20 in the crude extracts for ELISA was shown (Supplementary Figure S1C).

The results of the optimization experiments showed that at all time points, the setups where supplemented buffer was used produced more bFGF compared to the setups using the conventional buffer. This increase reached a maximum of up to about 260% at 6 days post-infiltration. Moreover, bFGF production linearly increased day after day until it regressed at some point; with the setups using the conventional buffer regressing 2 days earlier (Figure 2A). This demonstrates how the additives in the supplemented buffer collectively aid in the increase in bFGF production. The higher bFGF yield in the setups using the supplemented buffer in each time points could indicate more efficient T-DNA transfer because of acetosyringone and Tween-20. Ascorbic acid, on the other hand, which has been shown to delay necrosis in agroinfiltrated leaves (Norkunas et al. 2018; Nosaki et al. 2021) could have provided the leaves more time to produce more of the recombinant protein.

The optimization of downstream procedures is vital in maximizing the usable bFGF recovered from the leaf crude extracts, just as the optimization of specific upstream processes is significant in increasing bFGF accumulation. Because of its vulnerability to oxidation...
and the existence of a hydrophobic core, bFGF is known to be prone to aggregation and precipitation (Shahrokh et al. 1994; Zhang et al. 1991). The presence of salt in the buffer has been shown to rearrange exposed residues in proteins and may help its stability and solubility (Dahal and Schmit 2018; Jelesarov et al. 1998). The effect of varying NaCl buffer concentrations on the solubility of bFGF extracted was investigated in this study (Figure 2C). Compared to the extraction buffer with no NaCl, the one with 50 mM already showed about 2-fold increase in yield. However, further increasing the yield to 100 mM still showed an average increase of about 3 µg bFGF/g FW. This could be significant when translated into large scale production. From this concentration, increasing salt to about 150 mM only added about 0.5 µg bFGF/g FW increase only. Meanwhile, sonication has been shown to improve protein recovery from plant biomass (Byanju et al. 2020). The extent to which this procedure boosts the recoverable bFGF when compared to simple mechanical agitation by stirring for 10 min was explored. The results revealed that bFGF recovery from 20-s sonicated extracts was 3-times higher than from 10-min mixed extracts (Figure 2B). Combining these data (Figure 2D), an improved extraction procedure was developed that maximizes bFGF extraction using an extraction buffer containing 100 mM NaCl and 50 mM sodium phosphate pH 6.5 with 0.1% Tween-20, and then subsequent sonication in ice for 20 s.

**Comparison of transient bFGF production across bFGF variants**

After optimization of agroinfiltration and extraction procedures, the difference in recombinant protein yield between wild type bFGF and two additional bFGF variants, GGGbFGF and PRbFGF, was determined. GGGbFGF (Figure 1B) was designed to eliminate the GGGND and DGGGND motifs (Ueno et al. 2020) from bFGF RNA where (N=A/G/C/T and D=G/A/T). These motifs are prone to degradation as previously reported (Ueno et al. 2020). Therefore, optimizing the transgene to not have these motifs may help create more recombinant protein by increasing the amount of bFGF mRNA transcript available. Meanwhile, the bFGF mutant PRbFGF (Figure 1C) was derived from a Cys70Ser, Cys88Ser bFGF mutant previously produced in *E. coli* (Seno et al. 1988) that was thought to have protease resistance, which could boost production by slowing down the rate of degradation of the bFGF created. This mutant was also shown to decrease the likelihood of cysteine interaction-mediated bFGF precipitation (Shahrokh et al. 1994). Both GGGbFGF, and PRbFGF variants were ligated into the plant expression vector pGPTV-bar and used for agroinfiltration.

The yield of the recombinant bFGF produced in these variants was then compared to that of non-optimized bFGF using western blot (Figure 3A), silver staining (Figure 3B), and ELISA (Figure 3C) analyses of the crude extracts. When the yields of the three variations were compared, the GGGbFGF variant did not produce...
High-level protease-resistant bFGF production in *N. benthamiana*

A significant improvement in yield. Possible explanations for this is that because p19 RNA silencing suppressor was already co-expressed, the factors that degrade recombinant mRNA is already neutralized or bFGF recombinant mRNA was already stable. PRbFGF, on the other hand, produced the highest production at around 185 µg g⁻¹ FW and showed the thickest protein bands at around 18 kDa, which correspond to bFGF. With a production of roughly 100 µg g⁻¹ FW, this was 2-fold higher than the non-optimized and GGGbFGF variants.

**Stability analysis of PRbFGF and bFGF in crude extracts**

The mutant PRbFGF was hypothesized to have protease resistance which consequently could increase the recombinant protein yield (Seno et al. 1988). To test this theory and clarify the mechanism by which PRbFGF variant increased recombinant protein production, the degradation of PRbFGF in the homogenized crude extract was investigated. As it is, the homogenized crude extract contains all the proteins in the agroinfiltrated leaves including both the recombinant protein and all the native proteases. Comparing the rate of loss of bFGF with the mutant PRbFGF in these extracts should show the difference in the degree of stability of these variants.

As expected, measurement of recombinant bFGF degradation in the crude extracts at 20°C using ELISA showed a higher percentage of intact soluble PRbFGF as compared to bFGF over time (Figure 4). This strongly suggests the resistance of PRbFGF to degradation by proteases or by precipitation due to oxidation. Calculation of the trend in these setups roughly predicts about a 9.0% decrease in intact soluble bFGF content per hour. This was 3 folds faster than in PRbFGF which is only at around a 3.2% decrease per hour. In addition, for PRbFGF, a smaller truncated bFGF band was seen being generated in the CBB staining of the crude extracts as incubation time progressed (Figure 4B, lower panel and Supplementary Figure S2). A similar formation of this truncated form was reported for purified bFGF (Sauer et al. 2021).

**Recombinant protein purification**

The recombinant PRbFGF synthesized must be pure in order to be employed safely in biological systems. In this study, PRbFGF was successfully purified using SP cation exchange chromatography followed by heparin affinity chromatography. This purification scheme which was previously used to purify the normal recombinant bFGF in *N. benthamiana* leaves (Supplementary Figures S3 and S4) with a recovery of around 34%. It also worked the same when using the protease-resistant variant (Figure 5). This confirmed that the Cys70Ser and Cys88Ser mutations had no effect on bFGF's key bioactivity of heparin binding and that tagless bFGF purification was possible in agroinfiltrated *N. benthamiana* leaf crude extracts.

**Cell-proliferation assay**

In order to test if plant-produced mutant PRbFGF was functional, its ability to stimulate the proliferation of Hep G2 and CHO-K1 cells was tested. The results showed bioactivity and improved stability of recombinant PRbFGF (Figure 6). In the setups using Hep G2 cells (Figure 6A), PRbFGF consistently provided higher viable cell count and percent viability from day 2 to day 10 as compared to the normal bFGF variant produced in *E. coli*. Setups utilizing CHO-K1 cells, on the other hand (Figure 6B), revealed PRbFGF activity similar to that of the conventional bFGF version though higher residual growth was observed in the negative control setup. This phenomenon could be attributed both from the residual bFGF still attached to the receptors in CHO-K1 due to its slow release from it (Moscatelli 1992) and the lower bFGF concentration needed to stimulate CHO-K1 cells than Hep G2 cells. All in all, these results demonstrate that PRbFGF not only works effectively in vitro, but also has an extended effect on cell proliferation. This could be due to the increased stability of PRbFGF in the medium, allowing it to stimulate more cells for longer periods of time.
Discussion

Key upstream and downstream processes must be identified and manipulated in order to boost recombinant protein production. The upstream processes are mainly concerned about the accumulation of the recombinant protein in the expression platform. This accumulation is dependent on the expression platform’s biological reactions and the extrinsic factors that affect them. On the other hand, downstream processes are involved in the level of recovery of the produced protein from the upstream processes. In the context of Agrobacterium-mediated transient production, there could be a vast selection of points in the upstream processes that could be manipulated. In particular, the ‘magnICON’ and ‘Tsukuba system’ involves the use of a designated tobamovirus (TMV) and geminiviral replication system (Klimyuk et al. 2012; Suzaki et al. 2019). Through these systems, large amounts of the transgene could be made available, resulting in high-level expression. However, there are still several steps along the protein production pathway that could still be improved to increase production. In this study, the combination of previously reported yield-increasing agroinfiltration buffer additives acetosyringone, Tween-20 and ascorbic acid was confirmed to be effective.

Continuing through the course of protein expression, the transgenes that were made available through agroinfiltration will be transcribed to mRNA and subsequently be translated into the target protein. RNA silencing is a well-known plant defense mechanism that has the potential to drastically limit the production of recombinant proteins (Davidson and McCray 2011; Mardanova et al. 2017). After translation, the recombinant protein is now exposed to various degradation processes due to the presence of a vast
assortment of proteases in plant cells (Buyel et al. 2021). Protein degradation is often associated with protease and Endoplasmic Reticulum (ER)-associated degradation (ERAD). Production of high-levels of recombinant protein could induce the unfolded protein response (UPR) (Mattanovich et al. 2004) and this could significantly reduce recombinant protein production levels. In addition, SUMO (small ubiquitin-like modifier) proteases (Morrell and Sadanandom 2019) are employed by the plants in response to stress which could also inhibit high-level recombinant protein production. Both high-levels of foreign proteins and agroinfiltration could create the stress needed to induce this phenomenon. These proteases are normally involved in the plant’s homeostasis and defense (Minina et al. 2017; Van Der Hoorn 2008). However, they may obstruct recombinant protein production (Buyel et al. 2021). Silencing of several plant proteases resulted in aberrant phenotypes that inhibit proper growth of the plant (Bae et al. 2013). This suggests that these proteases are essential for plant homeostasis and development, and that removing them would be detrimental to the plant.

Therefore, the problem of the lowering of recombinant protein yield due to protease-degradation should be approached differently. A previous study found that PRbFGF, which contains Cys70Ser and Cys88Ser mutations improved bFGF recovery from inclusion bodies while maintaining bioactivity (Rinas et al. 1992). It was hypothesized that the increased recovery was caused by the stabilization of protease-resistant bFGF intermediates, which resulted in protease resistance. The use of the mutant PRbFGF variant instead of the wild type bFGF resulted in a 2-fold increase in production in this study. The precise mechanism of this increase in production could be protease resistance, precipitation resistance or both. The mechanism of this degradation resistance was hypothesized to be due to the stabilization of folding intermediates (Rinas et al. 1992; Seno et al. 1988) or a lack of protease cleavage sites due to the unnatural content of the protein backbone (Werner et al. 2016). Cysteines on the external domains on the other hand, have been shown to be aggregation-prone sites due to disulfide-linked multimerization (Shahrokh et al. 1994). The removal of these cysteines in mutant PRbFGF may have slowed aggregation. However, it is also possible that both of these mechanisms contribute to the overall availability of intact soluble PRbFGF compared to normal bFGF.
On the other hand, the tagless purification system of bFGF in agroinfiltrated N. benthamiana leaf extracts demonstrated in this study has neutralized potential risks brought by the previous His-tagged purification system (Rattanapisit et al. 2020) demonstrated before. First, His tag is known to promote protein aggregation in some cases (Hamilton et al. 2003; Shahrvan et al. 2008). Since the wild type bFGF is already prone to aggregation (Shahrokhi et al. 1994; Zhang et al. 1991), and that aggregated proteins lead to cytotoxicity (Lee et al. 2020), placing this tag might have contributed to the low yield and early leaf necrosis that was encountered in the previous study in the same expression platform (Rattanapisit et al. 2020).

In conclusion, this study demonstrated a procedure for high-level production of tag-less purified, bioactive PRbFGF, as well as how optimizing agroinfiltration and extraction methods, as well as replacing cysteines on the external domains of bFGF to serines, could be used to significantly increase its production. These strategies of customizing extraction based on specific recombinant protein characteristics as well as protein engineering ‘fragile’ spots of recombinant protein to enhance stability could be promising ways to improve recombinant protein production in a protease-rich expression platform like plants.

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Author contributions
E.M. and K.F. conceived and designed the experiments. E.M. performed the experiments with the technical support from K.F., R.M. and H.K. T.O. did some preliminary experiments in recombinant bFGF production. E.M. wrote the manuscript with the support from K.F., H.K. and R.M.

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Conflict of interest
The authors declare no conflict of interest.

Description of Supplementary files
The supplementary files include the preliminary optimization data for bFGF indirect ELISA and bFGF double purification.

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