Appl Biochem Biotechnol (2011) 165:465–475
DOI 10.1007/s12010-011-9265-4

High Levels of Expression of Fibroblast Growth Factor 21 in Transgenic Tobacco (*Nicotiana benthamiana)*

Hongqi Fu · Shifeng Pang · Ping Xue · Jing Yang · Xiuming Liu · Yanfang Wang · Tingting Li · Haiyan Li · Xiaokun Li

Received: 21 December 2010 / Accepted: 11 April 2011 /
Published online: 20 April 2011

Abstract Fibroblast growth factor-21 (FGF21) is a hepatic hormone that plays a critical role in metabolism, stimulating fatty acid oxidation in the liver and glucose uptake in adipose tissue. In this study, we produced tobacco plants expressing human recombinant FGF21 (hFGF21) via *Agrobacterium*-mediated transformation using a potato virus X (PVX)-based vector (pgR107). The vector contained the sequence encoding the human FGF21 gene fused with green florescence protein and a histidine tag. The recombinant plasmid was introduced into leaf cells of *Nicotiana benthamiana* (a wild Australian tobacco) *via Agrobacterium*-mediated agroinfiltration. As determined by fluorescence and Western blot of leaf extracts, the hFGF21 gene was correctly translated in tobacco plants. Seven days after agroinfection, the recombinant hFGF21 had accumulated to levels as high as 450 μg g^{-1} fresh weight in leaves of agroinfected plants. The recombinant hFGF21 was purified from plant tissues by Ni–NTA affinity chromatography, and the purified hFGF21 stimulated glucose uptake in 3T3/L1 cells. This indicated that the recombinant hFGF21 expressed *via* the PVX viral vector in *N. benthamiana* was biologically active.

Keywords Agroinfection · Fibroblast growth factor 21 (FGF21) · Fusion protein · Green fluorescent protein · *Nicotiana benthamiana* · Potato virus X

Hongqi Fu and Shifeng Pang contributed equally to this work.

H. Fu · P. Xue · J. Yang · X. Liu · Y. Wang · T. Li · H. Li · X. Li (✉)
Ministry of Education Engineering Research Center of Bioreactor and Pharmaceutical Development, Jilin Agricultural University, Changchun, Jilin 130118, China
e-mail: xiaokunli@163.net

S. Pang
Department of Biology, Guangdong Medical College, Dongguang, Guangdong 523808, China

X. Liu · T. Li · H. Li (✉)
College of Life Sciences, Jilin Agricultural University, Changchun, Jilin 130118, China
e-mail: hyli99@163.com

H. Fu · P. Xue · J. Yang · Y. Wang
College of Traditional Chinese Medicine, Jilin Agricultural University, Changchun, Jilin 130118, China

Abbreviations

CP	Coat protein
dpi	Days post-agroinfection
hFGF	Human fibroblast growth factor
LB	Luria–Bertani
PVX	Potato virus X
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
smGFP	Soluble modified green fluorescent protein
GOD-POD	Glucose oxidase and peroxidase

Introduction

Fibroblast growth factor-21 (FGF21) is a member of the FGF superfamily, which consists of 23 secreted proteins. The highest levels of FGF21 expression are in the liver [1, 2]. FGF21 activates glucose uptake in adipocytes, reduces plasma glucose and triglyceride concentrations in *ob/ob* and *db/db* mice, protects against diet-induced obesity when overexpressed in transgenic mice, and improves lipoprotein profiles in diabetic rhesus monkeys [3, 4]. As a novel member of the FGF family, FGF-21 exhibits the therapeutic characteristics necessary for effective treatment of diabetes. FGF21 is a promising candidate for therapeutic applications because it has an excellent safety profile. It does not show significant mitogenic potential in cell lines and FGF21 transgenic mice. Furthermore, FGF21 administration does not lead to either hypoglycemia or edema, which are two common side effects of current diabetes therapies [5, 6]. Therefore, it could be used as an alternative to insulin in clinical situations.

Plants have been studied extensively for the production of pharmaceutical proteins as an inexpensive and scalable alternative to common expression systems. Plants offer more advantages than microbial or mammalian host systems: the bioprocessing is more cost-effective, they have all of the cellular machinery to complete post-translational modifications of proteins, and they are intrinsically safe. The use of the potato virus X (PVX) vector in such systems is also safe, as there is no evidence that PVX is transmitted by naturally occurring vectors (insects, nematodes, or fungi). Moreover, the virus is not transmitted through seed or pollen, and thus, there is an extremely small risk of its accidental release into the environment. Therefore, environmentally safe conditions are attainable with only a moderate investment in infrastructure. The combination of a plant production system and a PVX-based vector is a promising platform for production of recombinant proteins [7, 8].

Different from stable transgenic plants, plant RNA viruses provide a temporary, transient expression system. These viruses are engineered to carry and replicate foreign genes in susceptible host plants. The sequences delivered by viruses into the infected plant cell remain part of the virus genomic RNA and do not integrate into the plant genome. Moreover, the sequences are amplified by the viral replicase in the cell cytoplasm and are not inherited. Plant viruses are well-suited as transient expression vectors, because the introduced genes are expressed at high levels, the products accumulate rapidly and to high concentrations, and the purification of the products from the plants is simple and inexpensive [9]. There have been many reports on virus-based transient expression of foreign proteins, such as cowpea mosaic virus [10, 11], tobacco mosaic virus [12, 13], tomato bushy stunt virus [14], plum pox potyvirus [15, 16], alfalfa mosaic virus [17, 18], bamboo mosaic virus [19], and PVX [20, 21].

Here, we report an expression system using *Nicotiana benthamiana* to produce human recombinant FGF21. *N. benthamiana* leaves were infiltrated with *Agrobacterium*

tumefaciens strains containing pgR107 binary vectors that were designed to combine the advantages of *A. tumefaciens*-mediated transfection as well as virus infection [22]. We inserted the coding sequence of smGFP and hFGF21 into the cloning sites of the viral binary vector to construct plasmids pgR107-smGFP-hFGF21 and pgR107-hFGF21. Viral replication and expression of the recombinant fusion gene occurred concurrently in agroinfiltrated *N. benthamiana*. The expressed histidine (His)-tagged smGFP-hFGF21 fusion protein was isolated and purified by Ni-NTA affinity chromatography. This technology provided an efficient system for production of the smGFP-hFGF21 fusion protein.

Materials and Methods

Gene and Vector Construction

Plasmids pgR107 and pgR107-smGFP were obtained from Dr. Xingzhi Wang (Institute of Genetics and Cytology, Northeast Normal University, Changchun, China). Plasmid pUC-FGF21, described previously in our laboratory, contains a multiple cloning site (*Cla*I/*Sma*I/*Sal*I) surrounded by two subgenomic viral coat protein (CP) promoters between the triple gene block and the CP coding sequence. The open reading frame of interest was inserted into the cloning site of the PVX within pgR107, and its expression was controlled by one of the CP promoters. The pgR107 plasmid also contained the strong constitutive 35S-promoter-driven synthesis of infectious PVX transcripts in plants [23, 24].

A smGFP-hFGF21 fusion gene was designed using preferred codons, which were optimized for expression in dicotyledonous plants. A polymerase chain reaction product containing the smGFP-hFGF21 sequence flanked by the digestion sites for *Cla*I and *Sal*I was amplified using pgR107-smGFP and pUC-FGF21 as templates, and then cloned into the pgR107 vector resulting in PVX-His-smGFP-hFGF21. The construction of pgR107-HishFGF21 was similar. Correct insertion of sequences was confirmed by DNA sequencing of each plasmid.

Plant Material and Inoculation Conditions

N. benthamiana plants (4–5 weeks old) approximately at the ten-leaf stage were used for *A. tumefaciens* transient assays [25]. The pgR107-His-smGFP-hFGF21, pgR107-smGFP, and pgR107-HishFGF21 plasmids were transferred into *A. tumefaciens* strain GV3101 using a freeze–thaw method, and then the three recombinant *Agrobacteria* containing each of the above plasmids were separately cultured in 50 ml LB (Luria–Bertani) medium [supplemented with kanamycin (50 mg L^{-1}), rifampicin (50 mg L^{-1}), and tetracycline (12.5 mg L^{-1}), 10 mM MES, and 20 μM acetosyringone] overnight at 28 °C. The cells were precipitated by centrifugation for 10 min at 1,500×*g* (Z 36 HK, Hermle Labortechnik GmbH, Germany) and resuspended in liquid minimal medium agar (10 mM $MgCl_2$, 10 mM MES, and 100 μM acetosyringone), and incubated for 2–3 h at room temperature without shaking. The *Agrobacterium* strains were infiltrated onto the underside of tobacco leaves, and then the plants were placed in the dark in a humid atmosphere for 24 h to recover from the treatment. Green fluorescence was monitored under ultraviolet illumination to observe expression of the recombinant protein. The expression of smGFP-hFGF21 and smGFP were also observed under a TCS SP5 confocal laser-scanning microscope (Leica Microsystems CMS GmbH, Germany). Hereafter, the proteins accumulated in plants are referred to as smGFP and smGFP-hFGF21.

Protein Extraction and Purification

N. benthamiana leaf material was harvested at 7 days post-infection. Leaves were frozen in liquid nitrogen and ground in extraction buffer (50 mM PBS, pH 9.5, 1 mM PMSF) at a ratio of 3 ml g^{-1} leaf material. Extracts were clarified by centrifugation at 13,520×*g* for 20 min at 4 °C. The His-tagged smGFP-hFGF21 protein was purified on a Ni-NTA Superflow (Qiagen) column according to the manufacturer's protocol. The proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). To obtain active hFGF21 protein, the fusion protein was cleaved with recombinant bovine enterokinase (rbEK), which is a site-specific endoprotease that preferentially cleaves the end of the C-terminal peptide bond at the recognition sequence, DDDDK. We separated the hFGF21 from His-smGFP using a Ni-NTA superflow column after cleavage.

SDS-PAGE and Western Blot

For Western blot analysis, cellular lysates from *N. benthamiana* leaf material were prepared with extraction buffer (50 mM PBS, pH 9.5, 1 mM PMSF) at a ratio of 3 ml buffer g^{-1} leaf material and separated by SDS-PAGE. Proteins were transferred onto Immobilon-P polyvinylidene difluoride (PVDF) membranes (Perkin Elmer, Boston, MA, USA) using a SEMI-DRY Transfer Cell (Bio-Rad Laboratories, Hercules, CA, USA), and immunoblotted with a monoclonal mouse anti-FGF21 antibody (Abcam Inc., USA) according to the manufacturer's protocol. Immunoreactive bands were visualized with western blotting luminal reagents (Western Blotting Luminol Reagent sc-2048 Trial kit, Santa Cruz Biotechnology, Inc., CA, USA).

ELISA Assay of smGFP-hFGF21 and hFGF21 in *N. benthamiana* Plants

Total soluble protein was extracted from control (not infiltrated with *Agrobacterium*) and agroinfiltrated *N. benthamiana* plants by grinding plant tissue (0.5 g fresh weight) to a fine powder in liquid nitrogen. The powder was homogenized with 1.5 ml of 50 mM PBS (pH 9.5) and centrifuged for 20 min at 13,520×*g*. The concentration of total soluble protein was estimated by the Bradford assay method [26] with bovine serum albumin as the standard.

A direct enzyme-linked immunosorbent assay (ELISA) was used to quantify smGFP-hFGF21 and hFGF21. Successive dilutions of total protein extracts from 1:50 to 1:1,000 in phosphate-buffered saline (PBS; 100 μl per well) were bound to 96-well polyvinylchloride microtiter plates for 2 h at 37 °C. The plates were blocked with 1% fat-free dry milk (DM) in PBS (150 μl per well) for 1 h at 37 °C. The wells were washed three times with PBS containing 0.05% Tween 20 (PBST), and then mouse anti-FGF21 monoclonal antibodies (diluted 1:2,000 in 1% DM/PBST) were added (50 μl per well), and the plate was incubated for 2 h at 37 °C. Subsequently, a rabbit anti-mouse IgG-horseradish peroxidase conjugate (Sigma) (1:2,000 in 1% DM/PBST) was added and the plate was incubated for 1 h at 37 °C. The plate was developed with Slow TMB substrate (Pierce) for 15 min at 23 °C. The reaction was terminated by the addition of an equal volume of 0.5 M H_2SO_4, and absorbance was read at 450 nm. The positive control and the standards consisted of bacteria-derived FGF21, and the negative control consisted of proteins extracted from leaves of control plants (not infiltrated with *Agrobacterium*). To construct the standard curve, bacteria-derived FGF21 was diluted with PBS to concentrations ranging from 0 to 10 μg/ml and processed as above. All of the ELISA assays were carried out in triplicate, and amounts of smGFP-hFGF21 and hFGF21 are expressed as mean values [27].

Culture of 3T3-L1 Cells and Differentiation

The 3T3-L1 cells were obtained from the American Type Culture Collection and were grown and maintained in DMEM high glucose medium (GIBCO Invitrogen Corp, Carlsbad, CA, USA) containing 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. The cells were allowed to grow until post-confluent day 2 and were then induced to differentiate by incubating for 48 h in differentiation medium [growth medium plus 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma), 0.25 μM dexamethasone (Sigma), and 5 μg ml^{-1} insulin (Sigma)]. Thereafter, the medium was changed to maturation medium (growth medium supplemented only with 2.5 μg ml^{-1} insulin) and cells were incubated for a further 48 h. The maturation medium was replaced with fresh maturation medium without inducers every 2 days. Cells were fully differentiated by day 12 with over 90% of the cells containing lipid droplets. The cells were maintained for up to 12 days after the induction of differentiation and sampled every 2–3 days.

Glucose Consumption Determination of 3T3-L1 Cells and Analysis

Purified hFGF21 protein (200 mg L^{-1}) and commercial insulin were diluted to final concentrations of 1, 10, 100, and 1,000 nM with cell culture medium. The mature adipocytes were grown under starvation conditions for 12 h and were then treated with or without these concentrations of FGF-21 and insulin for 24 h. For detection of glucose concentrations, 2 μl culture supernatant was analyzed using the glucose oxidase and peroxidase (GOD-POD) method (GOD-POD kit, E1010, Applygen Technologies Inc., Beijing, China) as described in [28]. In the GOD-POD method, glucose is oxidized to gluconic acid and hydrogen peroxide in the presence of glucose oxidase. Hydrogen peroxide further reacts with phenol and 4-aminoantipyrine by the catalytic action of peroxidase to form a red-colored quinoneimine dye complex, which is quantified spectrophotometrically.

Results and Discussion

Plasmid Construction

We constructed the binary virus vectors pgR107-His-smGFP-hFGF21 (Fig. 1a) and pgR107-HishFGF21 (Fig. 1b). The DNA coding sequences for smGFP-hFGF21 and hFGF21 were controlled by an additional strong subgenomic promoter of the PVX coat protein. Therefore, the genes could be expressed rapidly in *N. benthamiana* concurrently with viral replication. To increase the expression level of FGF21, we inserted the kozak sequence at the 5′ end of FGF21, and changed some of the native hFGF21 sequence codons to plant-preferred codons without altering the amino acid composition of the protein. Furthermore, the DDDDK enterokinase cleavage site was inserted into the *His-smGFP-hFGF21* gene (Fig.1a), which allowed complete removal of the His-tag from hFGF21 *via* a cleavage reaction.

Agroinfection of *N. benthamiana* Plants

The *N. benthamiana* plants agroinfected with *A. tumefaciens* containing pgR107-smGFP, pgR107-His-hFGF21, and pgR107-His-smGFP-hFGF21 developed the characteristic

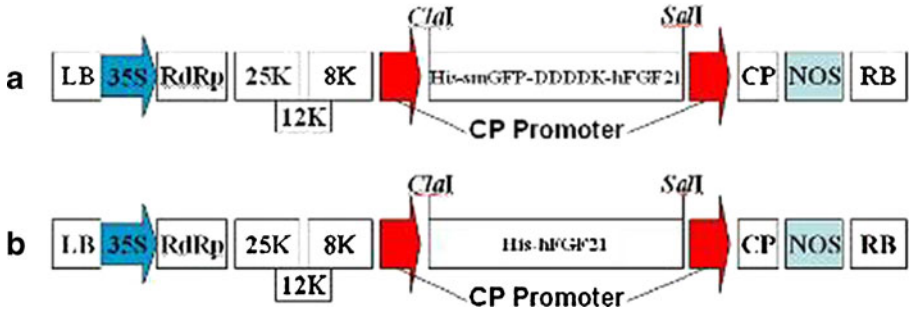

Fig. 1 **a** Schematic map of plasmid pgR107-His-smGFP-hFGF21. **b** Schematic map of plasmid pgR107-HishFGF21. LB and RB, left and right border sequences, respectively, of T-DNA of *A. tumefaciens*; *NOS* transcriptional terminator of nopaline synthase gene of *A. tumefaciens*; *RdRp* viral RNA-dependent RNA polymerase; *K* KDa; *25K*, *12K*, and *8K* viral movement proteins; *CP* viral coat protein; *35S* cauliflower mosaic virus 35S promoter; *His* 6-amino acid histidine tag

mosaic symptoms of PVX infection after agroinfection, in contrast to the healthy plants. The expression of recombinant protein was visible by fluorescence in plants agroinfected

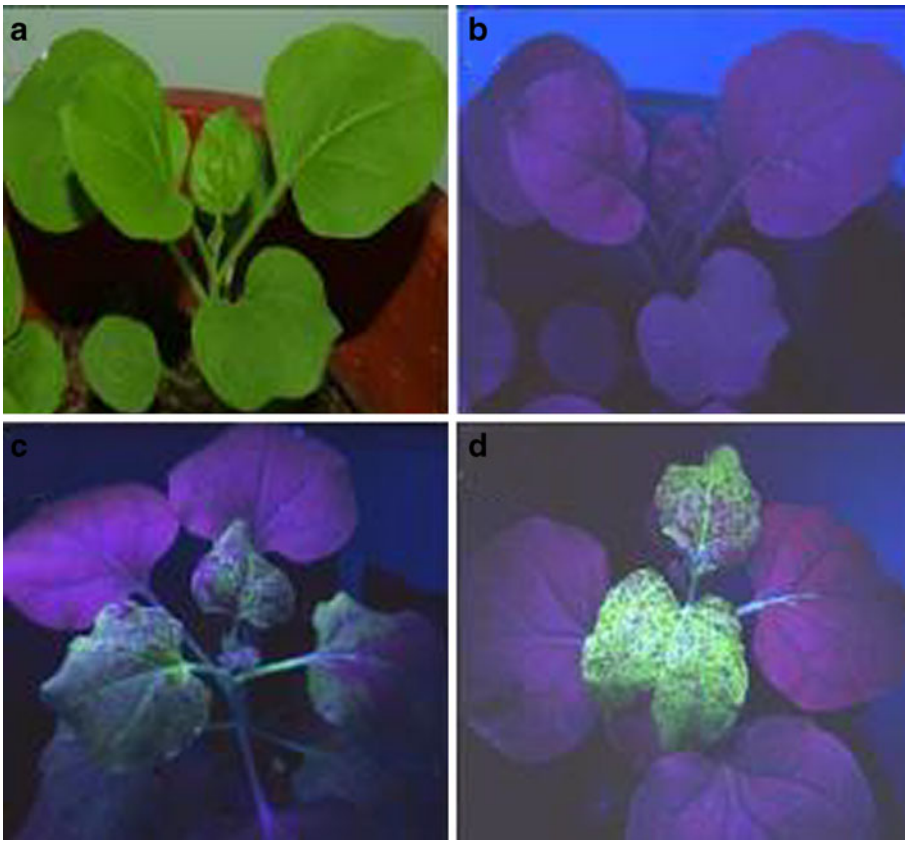

Fig. 2 Green fluorescent images under UV illumination. **a** Control plants (non-infected) under visible light. **b** Non-infected plant under UV illumination. **c** Expressed smGFP-hFGF21 visualized at 7 dpi under UV illumination. **d** Expressed smGFP visualized at 7 dpi under UV illumination

with GV3101/pgR107-His-smGFP-hFGF21. Under ultraviolet illumination, green fluorescent spots were clearly visible on agroinfected leaves of *N. benthamiana* at 3–4 days post-agroinfection (dpi). Diffuse green fluorescence of smGFP and smGFP-hFGF21 was visible in stems and leaves above the infected leaf on the day after agroinfection. The highest accumulation of smGFP (Fig. 2d) and smGFP-hFGF21 (Fig. 2c) was in leaves and stems at approximately 7 dpi, and then the amount of smGFP and smGFP-hFGF21 tended to decrease. Confocal microscopy analysis revealed that the smGFP-hFGF21 and smGFP proteins were localized in the cytoplasm (Fig. 3). At the same time, the luminous intensity ratio of smGFP-hFGF21 was significantly lower than that of smGFP (Fig. 4). These results demonstrated that agroinfection was an efficient way of initiating PVX infection, and that the PVX-based binary vector was an efficient transient expression vector for production of smGFP-hFGF21 in *N. benthamiana*.

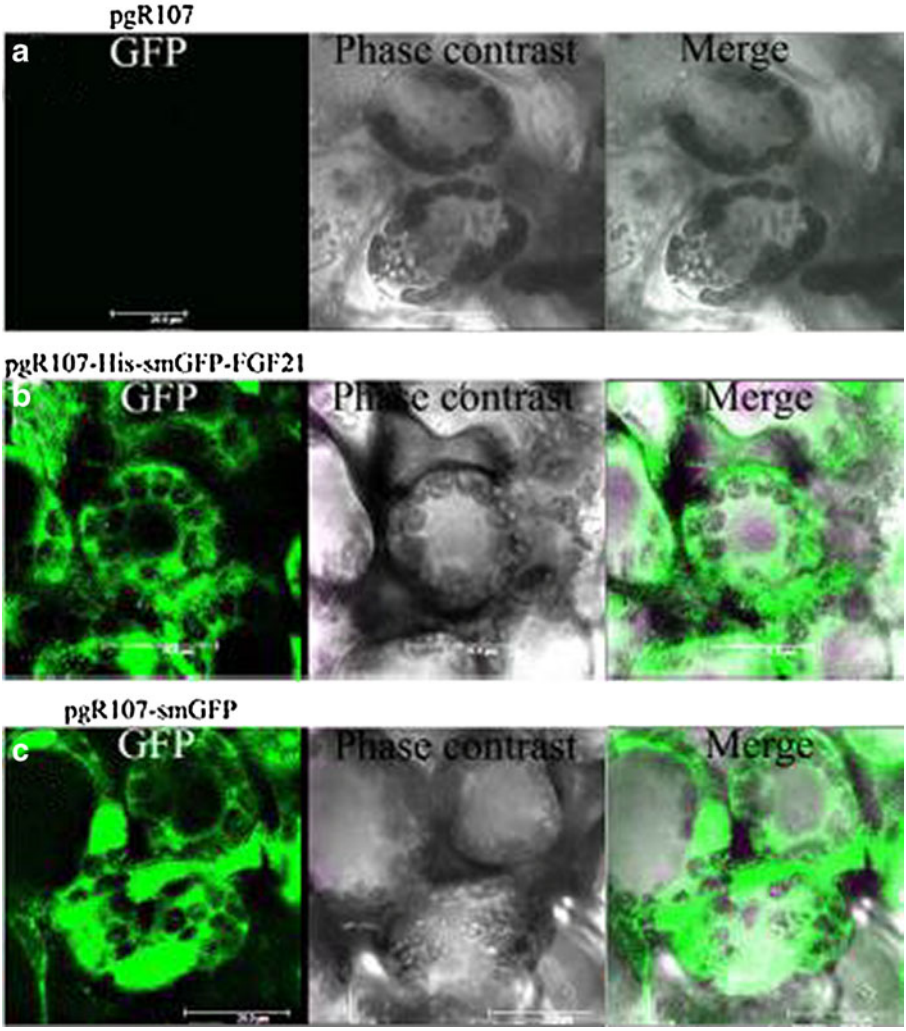

Fig. 3 Confocal microscope images. **a** Non-inoculated control tobacco cells. **b** smGFP-hFGF21 fusion gene expressed in tobacco cells. **c** smGFP alone expressed in tobacco cells. *Bar*=20 μm

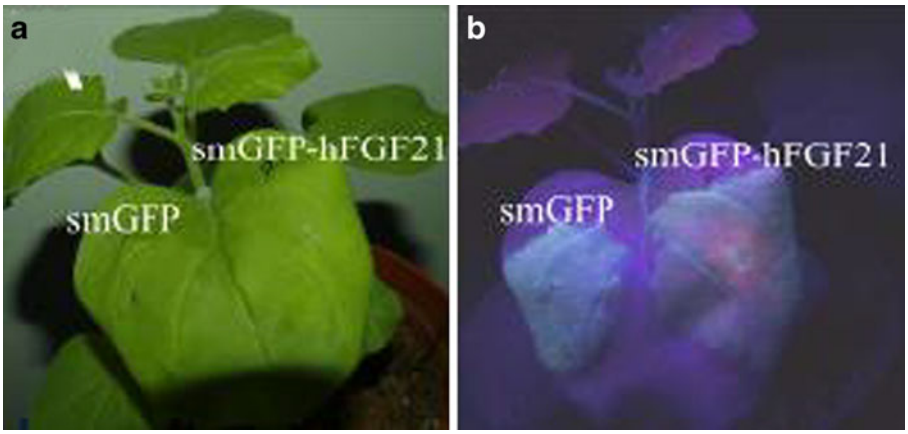

Fig. 4 Luminous intensity ratio of agroinfiltrated *N. benthamiana* leaves with smGFP and smGFP-hFGF21. **a** Agroinfected plant leaves under visible light. **b** Luminous intensity ratio of leaves expressing smGFP (*left*) and smGFP-hFGF21(*right*) at 7 dpi under UV illumination

SDS-PAGE and Western Blot

The analysis of smGFP-hFGF21 (53 kDa) expression by SDS-PAGE (Fig. 5 L3) was difficult because the chimeric protein co-migrated with the large subunit of Rubisco (55 kDa), an abundant leaf protein. However, comparing the intensities of corresponding protein bands in different lanes, we concluded that hFGF21 (Fig. 5 L4) was expressed at relatively low levels. It is likely that cytosolic targeting is not preferred for the native hFGF21. The smGFP-hFGF21 fusion protein was purified on an Ni-NTA Superflow column. Native hFGF21 protein was cleaved from the smGFP-hFGF21 fusion protein via an enterokinase. The results of SDS-PAGE showed that smGFP-hFGF21 (Fig. 5 L1) and hFGF21 (Fig. 5 L5) were purified from the cellular lysates of *N. benthamiana* leaves. Western blots of protein extracts and proteins purified from *N. benthamiana* leaves further confirmed the expression of smGFP-hFGF21 fusion proteins and hFGF21. The result showed that hFGF21 was expressed at low levels (Fig. 6 L3) but smGFP-hFGF21 was expressed at high levels (Fig. 6 L2). To reliably quantify the level of expression, leaf extracts were analyzed by direct ELISA. The ELISA results showed that smGFP-hFGF21 was expressed at high levels in the cytosol, accumulating to approximately 450 μg g^{-1} fresh leaf weight. The expression level of hFGF21 was less than 5 μg g^{-1} fresh leaf weight. The

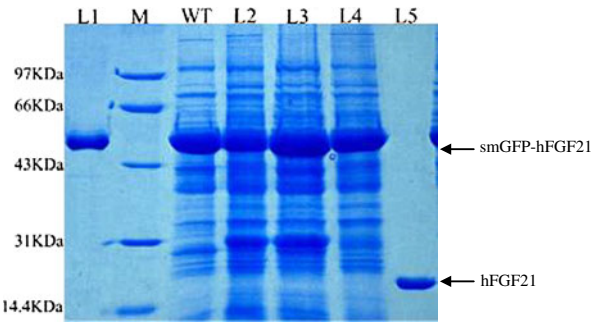

Fig. 5 SDS-PAGE analysis. *M* molecular weight standard. *L1* smGFP-hFGF21 (53 KDa) purified from tobacco leaves at 7 dpi. *WT* non-inoculated control tobacco leaves. *L2* pgR107. *L3* pgR107-His-smGFP-hFGF21. *L4* pgR107-HishFGF21. *L5* hFGF21 (23 KDa) after cleavage and separation from smGFP-hFGF21 fusion protein

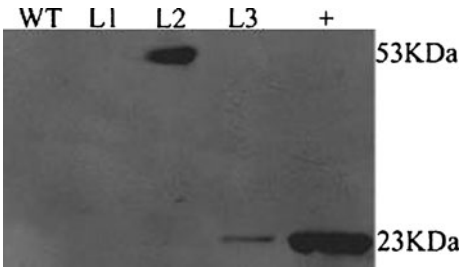

Fig. 6 Western blot of purified smGFP-hFGF21 and hFGF21. smGFP-hFGF21 and hFGF21 from tobacco leaves were resolved by SDS-PAGE, blotted, and probed with mouse anti-FGF21 antibody. *WT* non-inoculated control tobacco leaves. *L1* agroinfected with GV3101/pgR107. *L2* Agroinfected with GV3101/pgR107-His-smGFP-hFGF21. *L3* agroinfected with GV3101/pgR107- HishFGF21. *Plus sign* (+), *E. coli* recombinant hFGF21

stability of the smGFP-hFGF21 fusion protein may well protect its hFGF21 from degradation by hydrolase, whereas the native hFGF21 without the smGFP modification may be susceptible. Similarly, Joensuu et al., 2010 reported transient expression of the hydrophobin HFBI sequence from *Trichoderma reesei* fused to GFP in *N. benthamiana* plants after *A. tumefaciens* infiltration. The HFBI fusion significantly enhanced the accumulation of GFP, with the concentration of the fusion protein reaching 51% of total soluble protein [29]. Other studies have shown that with the fusion strategy, PVX can be used as a presentation system for proteins or peptides such as GFP fusion proteins [23, 30, 31].

The His-tagged smGFP-hFGF21 was expressed in soluble form in the pgR107 expression system. The His-tag simplifies purification of the protein, because the fusion protein can be purified on an Ni-NTA column. Native hFGF21 can be obtained after cleavage of the GFP. We obtained 9-mg final lyophilized product from 20-g fresh leaf material. In other systems using plant virus-mediated transient expression, the reported yields of recombinant pharmaceutical protein purified from tobacco leaves ranged from 0.00003 to 2 mg/g leaf fresh weight [32].

Glucose Metabolism of 3T3-L1 Adipocytes Mediated with Plant hFGF21

The 3T3-L1 preadipocytes and mature adipocytes were treated with different concentrations of hFGF21, the medium supernatants were sampled after 24 h, and the glucose uptake

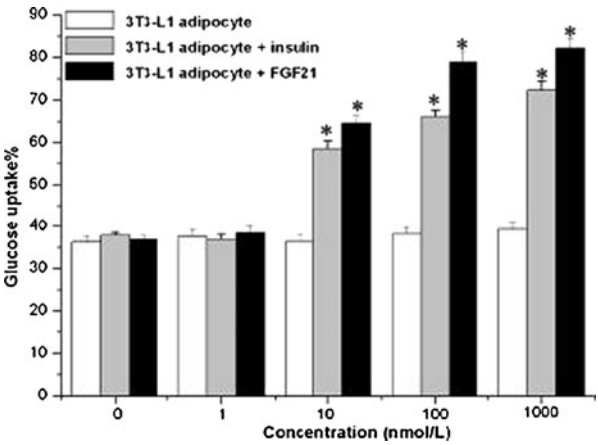

Fig. 7 Glucose uptake of 3T3-L1 adipocytes treated with different concentrations of human FGF-21 and insulin. *$P<0.05$, compared with unstimulated control (3T3-L1 adipocytes group). Values ($\bar{x}\pm s$) represent the average of at least three independent measurements

activity of hFGF21 was examined by the GOD-POD assay. Compared with the unstimulated control, adipocytes treated with hFGF21 showed significantly increased glucose consumption, and there was a significant decrease in the concentration of glucose in the medium ($P<0.05$). In this study, the glucose consumption rate of untreated 3T3-L1 adipocytes (control) was only 37.56%, while that of treated 3T3-L1 adipocytes was significantly higher, reaching 64.5% at a concentration of 10 nM hFGF21. The glucose consumption rate increased significantly with increased hFGF21 concentration in a dose-dependent manner. At a concentration of 1,000 nM hFGF21, the glucose consumption rate was 82.2%, which was 42.9% higher than that of the control. Thus, the plant FGF21 stimulated glucose uptake of 3T3-L1 similarly to insulin (Fig. 7).

The hFGF21 purified from plant leaves stimulated glucose uptake in 3T3-L1 adipocytes, and therefore, the tobacco plant-derived hFGF21 is biologically active. Recently, Wang et al. [33] demonstrated that *E. coli* recombinant FGF21 (rFGF21) can be expressed if directly fused with SUMO (small ubiquitin-related modifier), but the expression level and activity are low. Currently, the expression level of FGF21 is higher in *E. coli*, but most of the protein is contained in inclusion bodies, and so the soluble protein must be obtained via denaturation or renaturation processes. Thus, the purification costs are higher, and the activity of the protein is lower [3].

Whereas prokaryotic expression systems can result in low protein activity and/or complex purification requirements, eukaryotic expression systems have the cellular machinery to complete post-translational modifications, and the purification procedures can be very simple. The use of transgenic plants to produce vaccines, antibodies, and pharmaceutical proteins has gained much attention. In this study, we first expressed and then purified hFGF21 from a plant. Our study demonstrated that the transient fusion strategy allows expression of smGFP-hFGF21 without a significant loss of insert. This procedure yielded a fusion protein that was easily separated into the protein fusion partners. Compared with non-viral transient expression systems, this method is simple, convenient, and easy to scale-up. Moreover, the expression level obtained by fusion expression was higher than that achieved using non-viral transient expression systems. The viral transient system may facilitate rapid and large-scale production of functional recombinant proteins in plants in the future.

Acknowledgments We thank Dr. Xingzhi Wang for the gift of plasmids pgR107 and pgR107-smGFP and for technical advice. This work was supported by the '863' Project of China (Grant No. 2007AA100503), the Important Projects in the Scientific Innovation of Colleges and Universities of China (No.70S018) and the Science and Technology Development Projects of Jilin Province and Changchun City, China (Grant No.20070922).

References

1. Nishimura, T., Nakatake, Y., Konishi, M., & Itoh, N. (2000). *Biochimica et Biophysica Acta, 1492*, 203–206.
2. Mohammadi, M., Olsen, S. K., & Ibrahimi, O. A. (2005). *Cytokine and Growth Factor Reviews, 16*, 107–137.
3. Kharitonenkov, A., Shiyanova, T. L., Koester, A., Ford, A. M., Micanovic, R., & Galbreath, E. J. (2005). *Journal of Clinical Investigation, 115*, 1627–1635.
4. Kharitonenkov, A., Wroblewski, V. J., Koester, A., Chen, Y. F., Clutinger, C. K., & Tigno, X. T. (2007). *Endocrinology, 148*, 774–781.
5. Coskun, T., Bina, H. A., Schneider, M. A., Dunbar, J. D., Hu, C. C., & Chen, Y. (2008). *Endocrinology, 149*, 6018–6027.
6. Xu, J., Lloyd, D. J., Hale, C., Stanislaus, S., Chen, M., & Sivits, G. (2009). *Diabetes, 58*, 250–259.
7. Gomord, V., & Faye, L. (2004). Current Opinion in. *Plant Biology, 7*, 171–181.

8. Huang, T.-K., & McDonald, K. A. (2009). Biochemical. *Engineering Journal., 45*, 168–184.
9. Koprowski, H., & Yusibov, V. (2001). *Vaccine, 19*, 2735–2741.
10. Brennan, F. R., Jones, T. D., Gilleland, L. B., Bellaby, T., Xu, F., & North, P. C. (1999). *Microbiology, 145*, 211–220.
11. Nicholas, B. L., Brennan, F. R., Martinez-Torrecuadrada, J. L., Casal, J. I., Hamilton, W. D., & Wakelin, D. (2002). *Vaccine, 20*, 2727–2734.
12. Turpen, T. H., Reinl, S. J., Charoenvit, Y., Hoffman, S. L., Fallarme, V., & Grill, L. K. (1995). *Biotechnol, 13*, 53–57.
13. Dorokhov, Y. L., Sheveleva, A. A., Frolova, O. Y., Komarova, T. V., Zvereva, A. S., & Ivanov, P. A. (2007). *Tuberculosis, 87*, 218–224.
14. Joelson, T., Akerblom, L., Oxelfelt, P., Strandberg, B., Tomenius, K., & Morris, T. J. (1997). *The Journal of General Virology, 78*, 1213–1217.
15. Fernandez-Fernandez, M. R., Martinez-Torrecuadrada, J. L., Casal, J. I., & Garcia, J. A. (1998). *FEBS Letters, 427*, 229–235.
16. Fernandez-Fernandez, M. R., Mourino, M., Rivera, J., Rodriguez, R., Plana-Duran, J., & Garcia, J. A. (2001). *Virology, 280*, 283–291.
17. Yusibov, V., Hooper, D. C., Spitsin, S. V., Fleysh, N., Kean, R. B., & Mikheeva, T. (2002). *Vaccine, 20*, 3155–3164.
18. Yusibov, V., Mett, V., Davidson, C., Musiychuk, K., Gilliam, S., & Farese, A. (2005). *Vaccine, 23*, 2261–2265.
19. Yang, Z., Liu, Q., Pan, Z., Yu, Z., & Jiao, X. (2007). *Vaccine, 25*, 591–598.
20. Galeffi, P., Lombardia, A., Di Donatoa, M., Latinia, A., Sperandeia, M., & Cantalea, C. (2005). *Vaccine, 23*, 1823–1827.
21. Zeladaa, A. M., Calamanteb, G., de la Paz Santangelo, M., Bigi, F., Verna, F., & Mentaberrya, A. (2006). *Tuberculosis, 86*, 263–267.
22. Jones, L., Hamilton, A. J., Voinnet, O., Thomas, C. L., Maule, A. J., & Baulcombe, D. C. (1999). *The Plant Cell, 11*, 2291–2301.
23. Scholthof, H. B., Scholthof, K. B., & Jackson, A. O. (1996). *Annual Review of Phytopathology, 34*, 299–323.
24. Wagner, B., Fuchs, H., Adhami, F., Ma, Y., Scheiner, O., & Breiteneder, H. (2004). *Methods, 32*, 227–234.
25. Lacorte, C., Ribeiro, S. G., Lohuis, D., Goldbach, R., & Prins, M. (2010). *Journal of Virological Methods, 164*, 7–13.
26. Bradford, M. M. (1976). *Analytical Biochemistry, 72*, 248–254.
27. Elza, F. T., Belo, C. K., Farhat, E. N., & Gaspari, D. (2010). *The Brazilian Journal of Infectious Diseases, 14*, 35–40.
28. Trinder, P. (1969). *J. Clin. Path., 22*, 158–161.
29. Joensuu, J. J., Conley, A. J., Lienemann, M., Brandle, J. E., Linder, M. B., & Menassa, R. (2010). *Plant Physiology, 152*, 622–633.
30. Lacorte, C., Lohuis, H., Goldbach, R., & Prins, M. (2007). *Virus Research, 129*, 80–86.
31. Liu, J., Ma, P., Sun, Y., Yang, M., Yang, L., Li, Y., et al. (2007). *Biotechnology and Applied Biochemistry, 48*, 143–147.
32. Komarova, T. V., Baschieri, S., Donini, M., Marusic, C., Benvenuto, E., & Dorokhov, Y. L. (2010). *Expert Rev. Vaccines, 9*, 859–876.
33. Wang H, Xiao Y, Fu L, Zhao H, Zhang Y, Wan X (2010) BMC biotechnology 10. www.biomedcentral.com/1472-6750/10/14. Accessed 17 February 2010