Hyper Expression of Active, GFP-fused HFGF21 in Tobacco Chloroplasts

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Abstract

Human fibroblast growth factor 21 (hFGF21) is a promising candidate for metabolic diseases. In this study, a tobacco chloroplast transformation vector, pWYP21406, was constructed. In the vector, the codon optimized encoding gene hFGF21 fused with GFP at its 5' terminal was driven by the promoter of plastid rRNA operon (Prn) and terminated by the terminator of plastid *rps16* gene (Trps16). Spectinomycin resistant gene (*aadA*) as the selectable marker was placed in the same cistron between *hFGF21* and the terminator Trps16. Transplastomic plants were generated by biolistic bombardment method and proven to be homoplastic by Southern Blotting analyses. The expression of GFP was detected under UV light and laser confocal microscope. The expression of GFP-hFGF21 was confirmed by immunoblotting and quantified by ELISA. The accumulation of was GFP-hFGF21 confirmed to be $12.44 \pm 0.45\%$ of total soluble protein (i.e., $1.9232 \pm 0.0673 \text{ g kg}^{-1}$ of fresh weight). The GFP-hFGF21 was confirmed to be capable of promoting the proliferation of hepatoma cell line HepG2, inducing the expression of glucose transporter 1 in hepatoma HepG2 cells and improve the glucose uptakes. The above results suggested that chloroplast expression is a promising approach for bioactive recombinant hFGF21 production.

Introduction

Recombinant proteins are now occupying a great share of therapeutic proteins. There are many platforms that have been developed to produce recombinant therapeutic proteins, including mammal cells (animals), fugal cells, bacterial cells, insect cells, transgenic plant cells (transgenic plants). Since the first transgenic plant was generated in the early 1980s¹, transgenic plants were used to attempt to express exogenous proteins. Up to date, there have been many kinds of recombinant proteins were reported to be successfully expressed in transgenic plants²⁻⁹.

Transgenic plant is considered as an ideal platform for the expression of recombinant proteins due to its advantages compared to other platforms. The merits of plant platform exhibit in varies aspects including integrative-cost-efficiency, powerful production capacity, scale-up easiness, high security, ability of posttranslational modifications, and storage easiness^{2,5,10}. Of transgenic plants, chloroplast (plastid) transformed plants exhibit more merits in recombinant protein expression¹¹⁻¹³, for example, ultra-high expression of heterogenic proteins, site-specific integration of exogenous genes, polycistronic strategy for multiple genes transformation and maternal inheritance of transgenes. Many pharmaceutic proteins have been reported successfully expressed in chloroplasts¹³⁻¹⁶, including therapeutic proteins, antibodies, antigens, etc. Cytokines were also successfully expressed in chloroplasts. For instance, human interleukin-2¹⁷, human TGF β 3¹⁸, tissue plasminogen activator¹⁹ and human epidermal growth factor²⁰.

Fibroblast growth factors 21 (FGF21) is a member of the FGFs family, which regulates cell growth, cell differentiation, embryonic development, metabolism, and triggers many physiological and pathogenic processes²¹⁻²⁴. FGF21 treatment is considered an ideal approach in the therapy of obesity and diabetes as well as other related metabolic diseases, as it does not cause adverse secondary effects, like

hypoglycemia, edema, and liver toxicity²⁵. Although FGF21 is widely found in vertebrates, the production of FGF21 is limited and does not meet the demand in the market due to its low expression level. Therefore, the exploration of efficient methods for FGF21 production is a critical issue waiting to be addressed.

Recombinant human FGF21 (rhFGF21) has been reported to be successfully expressed in microorganisms, e.g., *Escherichia coli*^{26–28}, *Bacillus subtilis*²⁹, and *Pichia pastoris*^{30,31}. The expression of hFGF21 in microorganisms, although relatively high expression levels were obtained, the instability, solubility, and low bioactivity of the product³² are still the issues awaited to be settled; moreover, the downstream purification processing and production cost are also issues needed to be concerned. Human FGF21 was also attempted to expressed in plants, including tobacco (*Nicotiana benthamiana*)^{33,34}, tomato³⁵, Arabidopsis³⁶, carrot³⁷ and rice^{38,39}. However, these expressions in plants, either stable or transient, are of low expression levels.

As mentioned above, transplastomic plants are an ideal platform for recombinant protein production. However, expression of hFGF21 in plant chloroplast is not yet reported, although a member of the FGFs family, bFGF (FGF2) has been demonstrated to be successfully expressed in tobacco chloroplasts⁴⁰. In this paper, the successful expression of hFGF21 fused to GFP at a high level ($12.44 \pm 0.45\%$ of TSP) in tobacco chloroplasts is firstly reported. GFP-FGF21 fusion was proven to be capable of stimulating the expression of glucose transporter 1 (GLUT1) and enhancing the uptake of glucose in hepatoma HepG2 cells, which promoted the proliferation of HepG2 cells.

Results

Vector construction

A tobacco chloroplast transformation vector, pWYP21406 was constructed for the expression of hFGF21 in tobacco chloroplasts. The expression cassette of foreign genes was flanked by *16S rRNA-trnI* sequence at its 5' end and *trnA-23S rRNA* sequence at its 3' end (Fig. 1). In the vector, the gene of interest, *hFGF21* was fused with the reporter gene *smGFP* by a linker coding sequence. The product of the linker, (GSSS)₃-DDDDK, is composed of a triplicate of soft peptide and the enterokinase cleave site. The existence of the linker makes it possible to perform the cleavage of the recombinant hFGF21 from the fusion protein. The fusion of GFP and hFGF21 was designed to enhance the stability of recombinant hFGF21.

Generation of transplastomic tobacco homoplasmy

The biolistic particle bombardment was adopted to carry out tobacco chloroplast transformation. Resistant shoots were regenerated on the selection medium contained spectinomycin, and further screened to generate homoplastomic transgenic plants in subsequently homologous selection (Fig. 2a-b). The evidence from the Southern blotting results further confirmed the homoplasmy of the putative

homoplastic plants in the PCR analysis as the transgenic plants could obtain an approximately 4.8 kb band and the band from the wild-type plant was approximately 2.4 kb (Fig. 1, Fig. 3a).

The *in vivo* green fluorescent testing was performed. The results showed that either *in planta* or in protoplasts (Fig. 2c-j), the green fluorescent emission was observed under VU light, which suggested the expression of the recombinant fusion protein. The seedlings of transgenic lines could survive on the spectinomycin-contained medium while the wild-type seedlings were bleached; likewise, the transgenic seedling on medium could emit green fluorescent but only red fluorescent of chlorophyll was observed from the wild-type seedlings (Fig. 2k-l).

Expression of foreign protein in homotransplastic plants

TSPs were extracted from homologous transplastomic tobacco plants for further investigation of the expression of foreign proteins. The results from SDS-PAGE analysis showed that the fusion protein of interest, GFP-hFGF21, was expressed in tobacco chloroplasts with an expected molecular weight of approximately 48 kDa, which is larger than the free GFP (approximately 27 kDa) (Fig. 3b). The immunoblotting results also suggested the expression of the GFP-hFGF21 fusion (Fig. 3c), which was detected by the antibody against GFP.

The expression level of the fusion protein was determined by the ELISA in the sandwich way. The result suggested that the expression level was around 12.02%-13.04%, of TSP from fresh leaves, and the reached $12.44 \pm 0.45\%$, which means 1.9232 ± 0.0673 mg of GFP-hFGF21 per gram of fresh weight (Fig. 3d).

Bioactivity of GFP-FGF21

The glucose uptake and the expression stimulation of the expression of glucose transporter 1 (GLUT1) in hepatoma cell line HepG2 were tested to assess the activity of the fusion FGF21. The results suggested that the expression of *GLUT1* was also observed to be increased by treatment of GFP-FGF21 fusion (Fig. 4a), and showed a higher expression level than commercial standard FGF21. The upregulation of *GLUT1* resulted in the enhanced the glucose uptake in HepG2 cells after treating with the fusion GFP protein. The glucose uptake increment in the treatment with GFP-FGF21 was larger than that in the treatment with commercial standard (Fig. 4b). These results indicated that the chloroplast-derived fusion FGF21 could stimulate the expression of *GLUT1* in HepG2 cells and enhance the uptake of glucose, which makes the proliferation of HepG2 cells was faster than that in the blank control (Fig. 4c). These results suggested that the fusion protein could display the function of native hFGF21.

Discussion

Given the powerful merits of hFGF21, it is supposed as a promising candidate for metabolic diseases²⁵ and the demand for the growth factor in therapy is increasing. However, the supply of the protein is limited by its low expression level in natural expressing hosts, poor stability, and short half-time. Recombinant hFGF21 has been explored in numerous expression platforms. Yeast (*Pichia pastoris*) was

the first organism for expression of rhFGF21³⁰, and now several platforms have been reported to be capable of expressing rhFGF21, for instance, *E. coli*.²⁷, *Bacillus subtilis*²⁹ and plants^{33,34,38,39}. Plant expression platform is considered to be promising due to the lower cost, higher output, and higher biosecurity concerning^{2,4,11,13,14}.

In this work, the hFGF21 was successfully expressed in common tobacco (*N. tabacum*) chloroplasts in a fusion form at a high level. The expression of the GFP-fused FGF21 reached an average of $1.9232 \pm 0.0673 \text{ g} \cdot \text{kg}^{-1}$ of fresh weight, which is much higher than the previous reported in any of the attempted expression platforms. The previous studies showed that using the microorganism expression platforms, the expression of rhFGF21 in *E. coli* yielded $71 \pm 13.9 \text{ mg l}^{-1}$ ²⁷ or $213 \pm 17 \text{ mg l}^{-1}$ ²⁶ or 46.4% of TPS²⁸, while in the yeast *Pichia pastoris* yielded $6 \mu\text{g l}^{-1}$ ³⁰ or 16 mg l^{-1} ³¹. Although many attempts have been efforted in *Bacillus subtilis*, the data of expression level is not available²⁹. As the expression in plants is concerned, several plant platforms have been tried, including tomato³⁵, carrot³⁷, rice, tobacco (*N. benthamiana*), and *Arabidopsis*. However, the expression levels in tomato, carrot and rice are still unavailable. The expression levels in *N. benthamiana* were varied from $450 \mu\text{g g}^{-1}$ to $500 \mu\text{g g}^{-1}$ of fresh weight by using transient expression strategy with potato virus X (PVX)-based vector^{33,34} and in *Arabidopsis* yielded 3.76% of TSP by using the oil body-based expression system³⁶. The expression level here could yield approximately 0.92 mg g^{-1} fresh weight for free hFGF21, which is much higher than the previous attempts. Although the expression level of human serum albumin (HAS) in rice reached 2.75 g kg^{-1} brown rice⁴¹ (which is a milestone of bioactive recombinant protein expression in plants with nucleus transformation) and $75.60 \pm 4.32\%$ TSP of β -glucosidase accumulation⁴² (a milestone in chloroplast transformation) are higher than that in this work, the data is still promising as a large amount of fresh biomass of tobacco is concerned. There are still many strategies to improve the expression level^{2,11,15}. For example, the application of cis-elements in an expression vector, optimization of 5' UTRs and 3' UTRs, engineering the stability of the recombinant FGF21, using the inducible expression strategy, and so on. The optimized way suggested here is the inducible expression combined with other strategies as the super high expression level in chloroplast may lead to abnormal growth of transgenic plants (e.g., growth retardation), which were reported previously^{42,43}.

FGF21 has been proved to be capable of inducing the expression of GLUT1^{44,45}. The recombinant fusion FGF21 derived from tobacco chloroplasts showed the ability to stimulate the expression of GLUT1 in hepatoma cell line HepG2. As GLUT1 contributes the major glucose influx of HepG2 cells⁴⁶, and increases the uptake of glucose, and finally resulted in a faster proliferation of the cell culture. The stability and the halftime of FGF21 in cells/blood affect its bioactivity²⁸, and efforts to increase the ability of FGF21 have been explored^{28,47,48}. GFP is stable in many occasions⁴⁹, thus it is possible to be utilized as a stabilizer to enhance the stability of the proteins with poor stability. In this work, better accumulation of FGF21 was obtained compared with the previous reports in plants, and better bioactivity was observed compared with the commercial standard FGF21. These may be reasoned to the improvement of the stability of FGF21 by fusion with GFP. However, the intact GFP is too large with strong immunogenicity which may

hinder the application of the recombinant protein. Therefore, the removal of GFP will be necessary in future work. Alternatively, utilization of truncated GFP or other short peptides with less immunogenicity, e.g., peptides of native chloroplast protein⁵⁰, can be explored to get a better expression of rhFGF21 in tobacco chloroplast.

Methods

All the experiments were performed in accordance with relevant guidelines and regulations.

Plant culture

Tobacco (*Nicotiana tabacum*) cv. Petite Havana was used in this work. The seeds were germinated on the plate with agar-solid 1/2 MS medium⁵¹ in a culture room with 16h/8h light/dark photoperiod at 25°C. A week after germination, the seedlings were transferred to transparent containers with agar-solid 1/2 MS medium and cultured in the same condition for 3 weeks.

Vector construction

An expression cassette of PrnT7g10-*smGFP*-linker-*hFGF21*-attP-RBS-*aadA*-attB-Trps16 was designed and artificially synthesized. In the cassette, the promoter of tobacco plastid rRNA operon (Prn) with the leader of gene10 of T7 phage was adopted to drive the transcription of the downstream genes. The green-fluorescent-protein-encoding gene (*smGFP*)⁵² was used as a reporter and fused with the mature human FGF21 (NCBI accession number: NP_061986.1) coding gene (*hFGF21*) by a linker [(GSSSS)₃-DDDDK] coding sequence. The sequence of *hFGF21* and the linker were codon codon-optimized according to the codon usage bias of the tobacco chloroplast genome. The spectinomycin resistant gene, aminoglycoside-3'-adenylyltransferase encoding gene (*aadA*), was adopted as the selectable marker and placed downstream of *hFGF21* with a ribosomal binding site (RBS) from tobacco plastid *rbcL* gene at its 5' terminate. The terminator of the tobacco *rps16* gene was used to terminate the transcription of the cassette. The phage and bacterial ϕ 31 phage integrase recognize sites (attP, attB) were placed at the 5' and 3' terminals of *aadA* respectively for the removal of the selectable gene. The cassette above was inserted into the *Nsi*I site of the plasmid pEASY-Nt⁴⁰ to yield the transformation vector pWYP21406.

Chloroplast transformation and generation of transplastomic plants

The vector pWYP21406 was transformed into the tobacco chloroplasts by using the biolistic particle bombardment as described previously⁴⁰. The leaves of one-month-old tobacco seedlings were used as explants, the plasmid pWYP21406 was coated with 0.66 μ m gold particles, and the bombardment was carried out at 1100 psi with a shooting distance of 6 cm and a vacuum of 28 on MS medium. The bombarded leaves were cultured in the dark at 25°C for 3 days and cut into approximately 5mm $\times$ 5mm slices. The slices were placed on the selection medium (MS medium plus 2 mg l⁻¹ of 6-Benzylaminopurine, 0.2 mg l⁻¹ of α -naphthylacetic acid, and 500 mg l⁻¹ of spectinomycin) and cultured in

a condition of 16h/8h light/dark photoperiod at 25°C. The new medium was replaced every 3 weeks. After the primary resistant shoots were generated, they were screened by observing the existence of green fluorescent. Leaves of green fluorescent emission shoots were then undergone one more round of regeneration for homoplasmy obtainment. The selection would carry out several rounds until homoplastic plants were obtained.

The homogenous process was confirmed by the combination of green fluorescent observation and Southern blotting analysis. The homoplastomic plants were transplanted into soil and cultured in a greenhouse. The plants were cultured until seeds were harvested.

Southern blotting

Total genomic DNA was extracted from transplastomic plants and wild-type plants. The integration of foreign genes was confirmed by Southern blotting analysis. A 988 bp fragment flanking gene *tnI* and *tnA* was used as the probe (Fig. 1). Twenty micrograms of total DNAs were digested by *Bam*HI and *Kpn*I and DNA fragments were separated by 0.8% of agarose gel electrophoresis. The fragments were transferred to a positive-charged Nylon membrane and fixed by the UV-cross-linking method. The probe labeling, hybridization, and detection procedures were followed the user's handbook of DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Switzerland).

Inheritance of transgenes and green fluorescent observation

Transgenic seeds were germinated as described above and moved to 1/2MS medium with or without spectinomycin supplement, and cultured in a 16h/8h light/dark photoperiod at 25°C conditions for two weeks. *In planta* observation of the expression of GFP in either seedlings or plants in soil was carried out by exposing the plant in 365 nm VU light, and a digital camera was used to shoot images. The wild-type plant and seedling were adopted as negative controls.

Protoplasts were isolated from transplastomic and wild-type plants following the previous protocols⁵² and observed on a laser confocal microscope. The green fluorescent was excited by a 488 nm laser and the chlorophyll fluorescent was excited by UV light. The samples were scanned every 0.2 µm in depth and photographed at 1000 × magnification. The series slices of a unique sample were composed into a 3-D image.

Expression of GFP-hFGF21

The leaves of the homo-transplastomic plants, as well as wild-type plants, were collected for protein extraction. Briefly, the leaves were ground into fine powder in liquid nitrogen, and 1 mL of phosphate-buffered saline (PBS) buffer (with proteinase inhibitor mixture) per gram of leaf was added to the powder and mixed thoroughly. The mixtures were incubated on ice for 15 min and mixed 3 more times during the incubation. The supernatants were collected as crude total soluble proteins (TSP) after centrifuging at

15000 rpm at 4°C. The TSPs were used to perform SDS-PAGE analysis, immunoblotting, ELISA, and bioactivity assessment.

The SDS-PAGE was carried out with 15 mg TSP per sample in a 12% separate gel. The gel was stained with Coomassie brilliant blue R250. As for the immunoblotting, the proteins were transferred to 0.22 µm PVDF membrane. The membrane was then blocked by 5% of bovine serum albumin and incubated ordinarily with the monoclonal antibody against GFP (rabbit derived, Sino Biological Inc., Beijing. Cat. #: 13105-R208) and the horse radish peroxidase labeled anti-rabbit IgG antibody (goat derived, Sino Biological Inc., Beijing. Cat. #: SSA005). Metal Enhanced DAB Substrate Kit (Solar Life Science, Beijing, Cat. #: DA1015) was used to visualize the signals on the membrane. ELISA analysis was performed in the sandwich way with a polyclonal antibody against GFP (Sino Biological Inc., Beijing. Cat. #: 13105-RP01) and the same monoclonal antibody against GFP above and detected with an iMARK microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All the above analyses adopted a standard GFP (Sino Biological Inc., Beijing. Cat. #: 13105-S07E) as positive controls and wild-type TSPs as negative controls.

Bioactivity assessment of GFP-hFGF21

The bioactivity of GFP-hFGF21 was tested and determined based on cell proliferation of hepatoma cell line HepG2 (purchased from Cell Bank of Chinese Academy of Sciences, Shanghai, China), the glucose uptake, and the expression stimulation of the expression of glucose transporter 1 (GLUT1) and in HepG2 cells. The HepG2 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in an incubator at 37°C under a humidified atmosphere of 5% CO₂.

For the cell proliferation assessment, The HepG2 cells ($1 \times 10^5 \text{ mL}^{-1}$) were cultured in 96-well plates with 100 µL medium per well. The media contained 50 nmol l⁻¹ of commercialized recombinant hFGF21 (100 nmol l⁻¹, Sino Biological, Beijing, Cat. #: 10911-H07E.), TSP from transplastomic tobacco (containing 50 nmol l⁻¹ of GFP-FGF21), TSP from wild-type tobacco (the same amount to the transgenic) or without supplement. The volumes were adjusted to 150 µL by using PBS. Cells were cultured for 24 h and detected with the thiazolyl blue tetrazolium bromide (MTT) method. Each treatment repeated 12 wells.

The glucose uptake of HepG2 was tested according to Fu et al.³³ with a few modifications as follows: after the adipocytes were starved for 12 h, the tobacco soluble protein was added at the final concentration of hFGF21 100 nmol l⁻¹, and a sample of the culture medium was withdrawn every 12 h to determine the glucose content and the activity. Commercialized recombinant hFGF21 were used as positive controls, and PBS was used as a negative control. Three independent repeats were for each treatment were performed.

As for GLUT1 expression test, the cells treated with 100 nmol l⁻¹ of standard FGF21 or GFP-FGFF21 for 24 h were collected for total RNA isolation. The RNAs were reverse-transcribed into cDNAs and used for real-time quantitative PCR analysis. The PBS treatment was performed as blank control. Three independent repeats of each treatment were carried out. The relative mRNA expression levels were normalized to the β-

actin, the $\Delta\Delta\text{Ct}$ method was used to calculate the expression level. The primers used were *Glut1F* (5'-CTGTCGTGTCGCTGTT-TGTG-3'), *Glut1R* (5'-GGCCACAAAGCCAAAGATGG-3'), *β -actinF* (5'-CCTCGCCTTTGCC-GATCC-3'), and *β -actinR* (5'-CTCGTCGCCCCACATAGGAAT-3).

Declarations

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Author contributions

Y.W., J.F., N.X. and X.W. performed the research, Z.W. and S.X. designed the research and prepared the manuscript. All authors reviewed the manuscript.

Competing interests

The authors declare that they have no conflict of interest.

Data availability. The raw data supporting the results of this article are available in the supplementary files.

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Figures

Figure 1

The expression vector and the integration of foreign genes. The expression cassette of pWYP21406 contains a promoter of *rrn* operon of tobacco plastid genome (*Prrn*) to promote the transcription of the foreign genes and a 5' UTR of the g10 gene of T7 phage (T7-g10) to enhance the expression of the green fluorescent protein (GFP) fusion. The gene of interest (FGF21) was fused to the 3' terminal of smGFP with a linker [(GSSS)₃-DDDDK] coding sequence. The *aadA* gene, which confers the transgene cells the spectinomycin-resistance, is at the downstream of FGF21, with a ribosomal binding site (RBS) of tobacco plastid *rbcL* gene at its 5' terminal. The terminator of tobacco plastid gene *rps16* (*Trps16*) was adopted to terminate the transcription of the cassette. The phage and bacterial ϕ 13 phage integrase recognize sites (*attP*, *attB*) was placed at the 5' and 3' terminal of *aadA* respectively for future removal of the selectable marker gene. The cassette was inserted into the *Nsi* I site between the genes *trnI* and *trnA* and a fragment flanking these two genes (Probe) was used for the probe in southern blotting analysis. The restriction enzyme *Bam* HI and *Kpn* I was used to digest the chloroplast DNA for blotting analysis.

Figure 2

Regeneration, green fluorescent observation and transgene inheritance of transplastomic tobacco plants. **a** Resistant plantlet regeneration on selection medium. **b** Transplastomic plant in rooting medium. **c-d** Transplastomic plant in soil under normal (**c**) and UV (**d**) light. **e-f** Wild-type plant under normal (**e**) and UV (**f**) light. **g-j** Images of a protoplast of transplastomic plant observed with the confocal fluorescent microscope. **k-l** Transplastomic seedlings on media with (**k**) or without (**l**) spectinomycin supplement under UV light. L1, L2 different transplastomic lines; wt, wild-type seedlings.

Figure 3

Molecular analysis and foreign proteins expression of transplastomic plants. **a** Southern blotting of transplastomic plants with the probe indicated in Figure 1. **b, c** PAGE and immunoblotting analysis of the expression of GFP-FGF21. **d** The expression level of GFP-FGF21 in transgenic plants. 1-4, L1-L10, different transplastomic plants; wt, wild-type plant; M1, DNA molecular weight standard (Ziker Biological Technology Co., Ltd, Shenzhen. Cat. #: zk2142-5); M2, "Blue Plus® Protein Marker" protein molecular weight standard (TransGen Biotech, Beijing. Cat. #: DM101-01); M3, Direct-load™ colorful prestained protein molecular weight standard (GenStar Biosolution, Beijing. Cat. #: M221).

Figure 4

Bioactivity assessment of GFP-FGF21. **a** The expression of *GLUT1* in HepG2 cells. **b** The glucose uptake of HepG2 cells. **c** The proliferation of HepG2 cells. PBS, the blank control with PBS buffer treatments; FGF21, commercial human FGF21 standard; GFP-FGF21, the TSP with chloroplast derived GFP-FGF21 fusion from the transgenic plant; wt, the TSP extract from wild-type plant. Significant: **, ##, $p < 0.01$, compared with PBS and wt respectively.

Supplementary Files

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