Stable and efficient expression of brain-derived neurotrophic factor in tobacco chloroplasts

Yunpeng Wang
Jilin Academy of Agricultural Sciences
Nuo Xu
Wenzhou University
Jieying Fan
Jilin Academy of Agricultural Sciences
Zhengyi Wei
Jilin Academy of Agricultural Sciences
Wen Xin
Beijing TransGen Biotech Co.LtD
shaochen xing (xingsc64@163.com)
Jilin Academy of Agricultural Sciences https://orcid.org/0000-0003-3566-7182

Research Article

Keywords: Tobacco, Chloroplast, Genetic transformation, Brain-derived neurotrophic factor, Green fluorescent protein

Posted Date: June 22nd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1750296/v1

Stable and efficient expression of brain-derived neurotrophic factor in tobacco chloroplasts

Yunpeng Wang[1#], Nuo Xu[2#], Jieying Fan[1], Zhengyi Wei[1], Wen Xin[3], Shaochen Xing[1*]

1. Institute of Agricultural Biotechnology, Jilin Academy of Agricultural Sciences, Changchun, Jilin Province, P.R. China
2. College of Life and Environmental Science, Wenzhou University, Wenzhou, Zhejiang Province, P.R. China
3. Beijing TransGen Biotech Co., Ltd., Beijing, P.R. China

E-mail addresses:

Yunpeng Wang: wangypbio@cjaas.com

Nuo Xu: seer.sino@hotmail.com

Jieying Fan: fanjy08@126.com

Zhengyi Wei: weizy80@163.com

Wen Xin: ceo@transgen.com.cn

# equal contribution

* corresponding authors: Shaochen Xing

E-mail addresses: xingsc64@163.com; ORCID: 0000-0003-3566-7182

Abstract: Brain-derived neurotrophic factor (BDNF) is a factor with many functions such as acceleration of cell proliferation and differentiation and is therefore widely used in clinical applications. In this study, an expression vector named pWYP23402 having a codon-optimized *BDNF* gene was constructed and transferred into chloroplasts of tobacco by gene-gun. After three or four rounds of selection with proper spectinomycin, *BDNF* was integrated into the chloroplast genome of homoplastomic plants confirmed by PCR and Southern hybridization. ELISA assay indicated that *BDNF* fused with GFP represented approximately 15.72%±0.33% of total soluble protein in the leaves of transplastomic plants. Moreover, the chloroplast-derived BDNF displayed similar biological activity to the commercial product. This is the first case report of *BDNF* expression by chloroplast transformation in this model plant, supplying an additional pathway for the production of chloroplast-expressed therapeutic proteins.

Key words Tobacco, Chloroplast, Genetic transformation, Brain-derived neurotrophic factor, Green fluorescent protein

Abbreviations

BDNF	Brain-derived neurotrophic factor
ELISA	Enzyme-linked immunosorbent assay
GFP	Green fluorescent protein
LTP	Long-term potentiation
MCS	Multiple cloning site
NGF	Nerve growth factor
PBS	Phosphate-buffered saline
PMP	Plant-made pharmaceuticals
SCI	Spinal cord injury

SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TrkB	Tropomycin receptor kinase B

Introduction

BDNF (brain-derived neurotrophic factor) is a growth factor studied intensively and belongs to the member of the neurotrophin family of secret proteins that involving in neurotrophin-3 (NT3), NGF, and neutorophin-4 (NT4) [1-5]. It was the first neurotrophic factor explored and purified from pig brain in the year of 1982 since the discovery of NGF. This NGF-like substance was found to be expressed at an extremely low level in the brain [6]. The human *BDNF* gene is composed of 11 exons [7] and its full primary structure showed highly conservative, with 85.9 to100 % identity among the genes of various vertebrates and humans as well [8].

BDNF is first synthesized into pre-pro-BDNF in cell bodies of neurons and glia and then cleaved to become mature BDNF. The mature BDNF will be transported to the terminals where it is released [9]. However, BDNF can also be delivered to the dendrites where the activity-dependent local translation of BDNF takes place [10]. The factor binding with high affinity to the tropomycin receptor kinase B (TrkB) receptor can activate at least three intracellular signaling pathways downstream [11, 12].

BDNF gene was located in No. 11q13 chromosome of the human genome and No. 12 chromosome of rat genome and shared the 50 to 60 percentage of similarity of sequence over other members of the neurotrophic factor family. It is one of the most widely distributed neurotrophic factors in the brain and is mainly expressed in the central nervous system, especially with the highest levels in the regions of the hippocampus and cortex. The mRNA for BDNF was found predominantly in the central nervous system and its sequence of protein indicated that BDNF and NGF are related closely both in structure and function [13].

As a multi-functional chemical molecule, BDNF has been proved to play a critical role in synaptic plasticity, LTP, learning, and memory [14-17, 13].

The more growing evidence demonstrated that BDNF was involved in a wide range of neurophysiological processes [18]. For instance, over the last decade, BDNF has been consistently highlighted as a key linker between the antidepressant drug and the neuroplastic changes, resulting in the improvement of depressive symptoms [19]. It was reported that local hydrogel delivery of BDNF with controlled dosing and duration to the injured cervical spinal cord can repair the respiratory neural circuitry, and this delivery was a safe and robustly-effective strategy to restore diaphragm function after SCI [20].

To be the ideal platform for protein expression, chloroplasts have been regarded to be the favorite organelle for the production of therapeutic proteins including growth factors like bFGF, EGF, and antimicrobial peptides like MSI-99 [21-23].

Tobacco was the firstly engineered flowering plant via chloroplast transformation more than three decades ago [24], and still served as the model plant due to its easy plastom genetic manipulation. Compared with existing prokaryotic and eukaryotic expression systems which have technical and economic limitations, plants have created substantial interest in developing new expression platforms for the effective production of much safer and biologically active complex therapeutic proteins or antigens. More importantly, chloroplast transformation has several advantages like higher expression, generally soluble proteins, and mostly maternal inheritance over nuclear transformation. No sterile laboratories or related equipment is required for protein production as well [25-27].

In this experiment, we engineered tobacco plants to firstly express BDNF via chloroplast transformation. These results will offer solid support for potentially producing BDNF production via growing plants on a large scale to meet the growing clinical demand for therapeutic proteins.

Materials and Methods

Plant materials

Tobacco (*Nicotiana tabacum* cv. Petit Havana) seeds were surface-sterilized and grown on a half-strength MS medium supplemented with 8 g/L agar and 30 g/L sucrose under a 16/8 h light/dark (L/D) cycles. Fully-expanded young leaves were used for subsequent chloroplast DNA extraction and genetic transformation.

Vector construction

According to the nucleotide sequence in the NCBI database, the *BDNF* gene was optimized under the precondition of keeping the amino acid sequence unchanged. The codons were optimized and replaced by Gene Designer based on the codon bias of the tobacco chloroplast genome. The modified gene sequence was analyzed by DNAMAN7.0 and Vector NTI 10.0 to find out enzyme site information. Meanwhile, the optimal codon ATT for serine amino acid was replaced by ATA to eliminate *Xba* I enzyme sites. Two enzyme sites, *Cla* I and *Xba* I, were flanked by the two ends of the optimized gene to fuse with *the gpf* gene.

The expression cassette was chemically synthesized in which a linker amino acid sequence (GSSSGSSSGSSSGSSSDDDDK) combined reporter gene *gfp* with target gene *BDNF* and selectable gene *aadA*, all three cluster genes were driven by promoter *Prrn-T7g10* and terminator *Trps16*.

This artificial cassette was confirmed by sequencing and inserted into the site between *trn I* gene and *trn A* gene to form the expression vector namely pWYP23402.

Tobacco Chloroplast transformation and homoplastomic selection

The tobacco chloroplast transformation was carried out according to the protocol previously described [21]. Young leaves were placed on the MS medium with the adaxial side down, cultured in the dark for 4 h, and then bombarded by gene-gun. The bombarded leaves were cultured at 26°C for 3 days in the dark condition before being cut into 5 mm × 5 mm pieces and transferred to a selective medium that was the MS medium containing 500 mg/L spectinomycin, 2 mg/L 6-BA, 0.1 mg/L NAA,

30 g/L sucrose, and 8 g/L agar at the first round of selection. The medium was renewed every three weeks until the appearance of resistant transformants. During the selection, a UV lamp was used to detect the intensity of GFP expression in the leave cells of transformants, and the plants with high intensity were picked up to do PCR analysis. The PCR-confirmed leaves of resistant transformants were cut into pieces and subjected to additional cycles of selection until the homoplastomic plants were achieved.

Molecular analysis

(1) PCR analysis

For the preliminary identification of homoplastomic plants, total DNA was extracted from young leaves of both transplastomic and wild-type tobacco plants to carry out PCR analysis. The P1 and P2 marked in Fig.1 were designed to check the expression cassette.

(2) Southern blot

Integration of the foreign expression cassette into the tobacco plastid genome was characterized by Southern blot hybridization. The total plastome DNA extracted from young leaves of the transgenic plant was digested with two endonucleases *Bam*H I and *Kpn* I (New England Biolabs, UK) at 37°C overnight and subjected to 0.8% agarose gel electrophoresis, then the digested DNA in gel was transferred onto positive-charged Hybond-N^+ nylon membrane (Roche, Switzerland). DNA was fixed by UV-cross-linking twice for 30 s each time at 2 min intervals. Pre-hybridization and hybridization were carried out by using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Switzerland). The DNA-fixed membrane was hybridized with a ~1.4kb probe in an oven for 16 h at 42°C before washing and visualization. The untransformed wild-type plant was used as the negative control.

Western blot and ELISA assays

To measure the expression level of BDNF, Western blot analysis, and ELISA assay were performed. Briefly, the leaves were grounded by liquid nitrogen, homogenized in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) to extract the crude total soluble protein from leaves of PCR-positive plants, separated by a 15% SDS-PAGE gel, then transferred to a nitrocellulose membrane (0.22 μm, Millipore, USA) via a semi-dry trans-blot electrophoretic transfer cell (200 mA, 30 min, Bio-Rad, USA). The nitrocellulose membrane was then saturated with 5% BSA (w/v) in TBS-Tween 20 (25 mM Tris-HCl pH 7.6, 0.15 M NaCl, 0.05% (w/v) Tween-20) for 1 h. The membrane was incubated with a BDNF-directed antibody (1:100, Santa Cruz Biotechnology Inc. USA), before incubation with the secondary antibody (1:3000, goat anti-mouse IgG alkaline phosphatase conjugate, Novagen, Germany). Labeled proteins were visualized by reaction with BCIP/NBT as a substrate (Roche, Switzerland).

According to the Kit instruction (Abcam, Cambridge, MA, USA), ELISA was performed using commercial anti-BDNF antibodies and commercial BDNF as standard. Wild-type plant was exploited to be the negative control. Three independent duplicates were set to collect data for the statistical analysis.

Elimination of tag from GFP-fusion protein

Purified GFP-fusion protein was digested by enterokinase according to the instruction of the Recombinant Enterokinase Kit (Novagen, Germany) to separate GFP and BDNF. Purification is carried out on AKTA pure150. Equilibrate 3-5 column volumes with balance solution (pH8.0, 20mmol/L Tris-HCl, 0.1mol/L NaCl) for CM Sepharose FF column. Loading raw BNDF on an ion-exchange column. Inflow balance solution 3-5 column volumes to baseline. Elution CM Sepharose FF at 1.5 mol/L NaCl with elution buffer (pH8.0, 20mmol/L Tris-HCl, 2.0mol/L NaCl) and collected effluent. The biological activity was confirmed by the 3T3 cell proliferation assay.

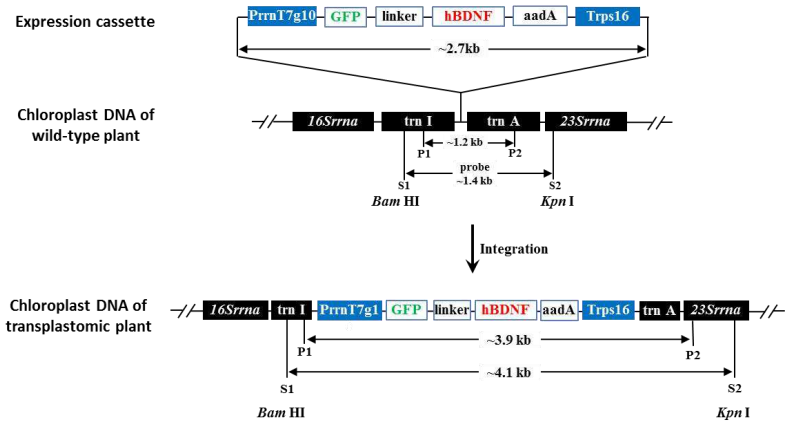
3T3 cell proliferation analysis

NIH3T3 cells were put in 96-well plates with 8×103 cells/well. They were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, China) containing 40ng/mL BDNF for 24 hours. An equal volume of PBS buffer and C-BDNF was added as the blank and positive group, and NIH3T3 cells cultured under the same conditions as the experimental group served as the control and positive group. Cellular proliferative activity was determined via Cell Counting Kit-8 (CCK-8) assays, in which cell survival rate = (A experimental group- A blank group)/(A control group - A blank group). Three independent duplicates were set to perform the statistical analysis. Statistical significance was analyzed with a one-way ANOVA test and the number of asterisks indicates the level of significance (* for $p \leqslant 0.05$ and ** for $p \leqslant 0.01$).

Results

Construction of expression vector

An expression vector named pWYP23402 for chloroplast transformation of tobacco was constructed based on the protocol previously [28] with some modifications (Fig. 1). The homologous fragment from alfalfa was replaced with fragment from tobacco plastid genome, and then the codon-optimized *BDNF* gene was inserted into the MCS of the expression vector. The vector was reconfirmed by sequencing.



195

196 **Fig. 1** Construction of expression vector pWYP23402 for chloroplast transformation. pWYP23402
197 vector flanked with sequences of *16S-trnI* and *trnA-23S* comprised a synthetic expression cassette
198 which contains *gfp* reporter gene, *BDNF* target gene, and *aadA* selectable marker gene. These three
199 genes were jointly driven by the Prrn-T7g10 promoter and the terminator was T*rps16*. P1/P2 indicated
200 the sites of primers used for PCR analysis and S1/S2 indicated the sites of digestion by *BamH* I and
201 *Kpn* I for Southern hybridization. A ~3.9 kb fragment was expected to amplify for transplastomic
202 plants, but only a ~1.2 kb fragment for wild-type plants. Similarly, total DNA was digested by *BamH* I
203 and *Kpn* I for Southern blot analysis. A ~3.9 kb signal was hybridized for transplastomic plants, but
204 only a ~1.4 kb for untransformed plants. The linker was inserted between the *gfp* gene and the *BDNF*
205 gene and digested by enterokinase for BDNF purification subsequently. The amino acid sequence of
206 the linker is GSSSGSSSGSSSGSSSDDDDK.

207

208 Tobacco homoplastomic selection and molecular characterization

209 Young leaves from PCR-positive shoots were cut into small pieces and cultured for two to three
210 more additional cycles to obtain the homoplastomic plants (Fig. 2a).

211 Total DNA was extracted from young leaves of both transgenic and wild-type plants and the
212 integration of the *BDNF* gene into the chloroplast genome was confirmed by Southern blot. As
213 indicated in Fig. 2b, the single ~4.7 kb band in lanes 1, 3 and 4 implied homoplastomic status,

whereas lane 2 displayed an additional ~1.2 kb weak fade band, identically amplified from wild-type plants, indicating its heteroplastomic status.

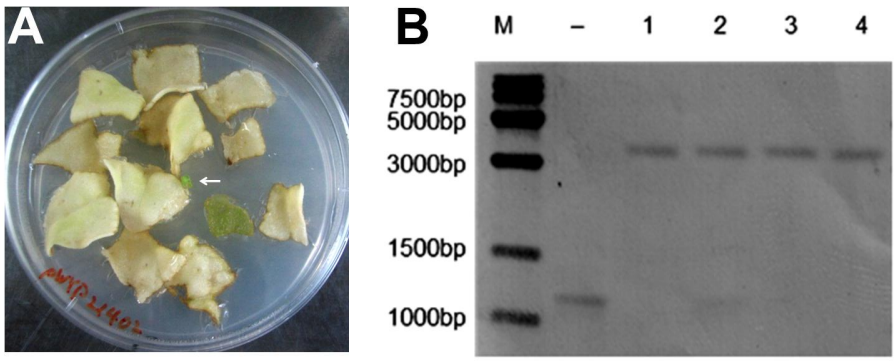


Fig. 2 Homoplastomic resistant callus after selection and molecular testing. **a** Resistant shoots (indicated by white arrow) appear while most leave pieces bleached on antibiotic medium; **b** Southern blot analysis. M, DNA molecular marker, Lane **1-4** The leaves of putative transgenic plants. The single 4.7 kb band in lanes 1, 3 and 4 indicated homoplastomic status, whereas lane 2 with an additional ~1.2 kb band, indicated heteroplastomic status. Line '-' is untransformed tobacco as the negative control.

Assessment of BDNF expression in transgenic plants

The SDS-PAGE gel was used to test the expression of GFP-fused recombinant BDNF. The results showed in Fig. 3a. Approximately 26.9 kD of GFP (lane P) and 40.1 kD of GFP-fused BDNF (lane 1-4) appeared clearly in the gel. In contrast, no such signal was observed in the lane wt which the crude protein was extracted from the untransformed plant.

For the western blot assessment, the results revealed approximately 40.1 kDa signals in lanes 1 to 4, representing BDNF expression in the transgenic lines (Fig. 3b). As expected, no signal was observed for wild-type plants.

The ELISA assay indicated that BDNF fused with GFP represented approximately 15.72% ± 0.33% of total soluble protein, 2.417±0.051g kg^{-1} of fresh leaf.

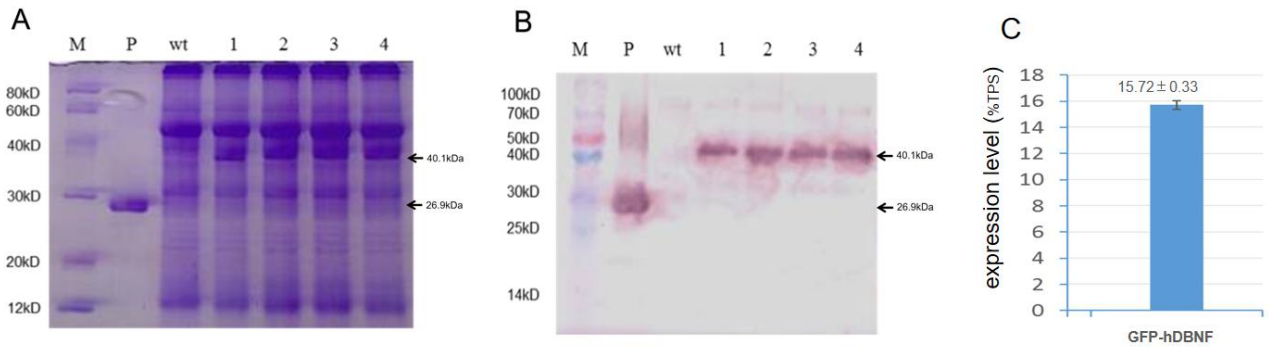


Fig. 3 SDS-PAGE analysis (**a**), Western blot (**b**) and ELISA assay (**c**) for determining the BDNF expression in transplastomic plants. **a** M, protein marker; **p** GFP as the positive control, wt, crude protein from the untransformed plant, **1-4**, crude proteins from four transgenic plants. **b** M, protein marker. Expression of GFP-fused BDNF (40.1 kD) was observed in the leaves of four transgenic plants (lanes 1-4), but absent in the leaves of a wild-type plant (**wt**). Lane **p** indicated the GFP as the positive control. **c** the expression level of GFP-hDBNF in the transgenic plants.

in vivo detection of GFP in transplastomic plants

As the reporter gene, *gfp* gene was inserted in the expression vector, together with the *BDNF* gene, to investigate the integration and the expression of target gene. As the same promoter and terminator was used to drive the *gfp* and *BDNF* genes which connected by a linker, indicating that the *in vivo* detection of GFP in the plant directly reflect the expression of target *BDNF* gene at the same time. The results demonstrated that the transplastomic plant emit strong green light under UV light (Fig. 4d), while the wild-type plant showed red color due to chlorophyll autofluorescence (Fig. 4c).

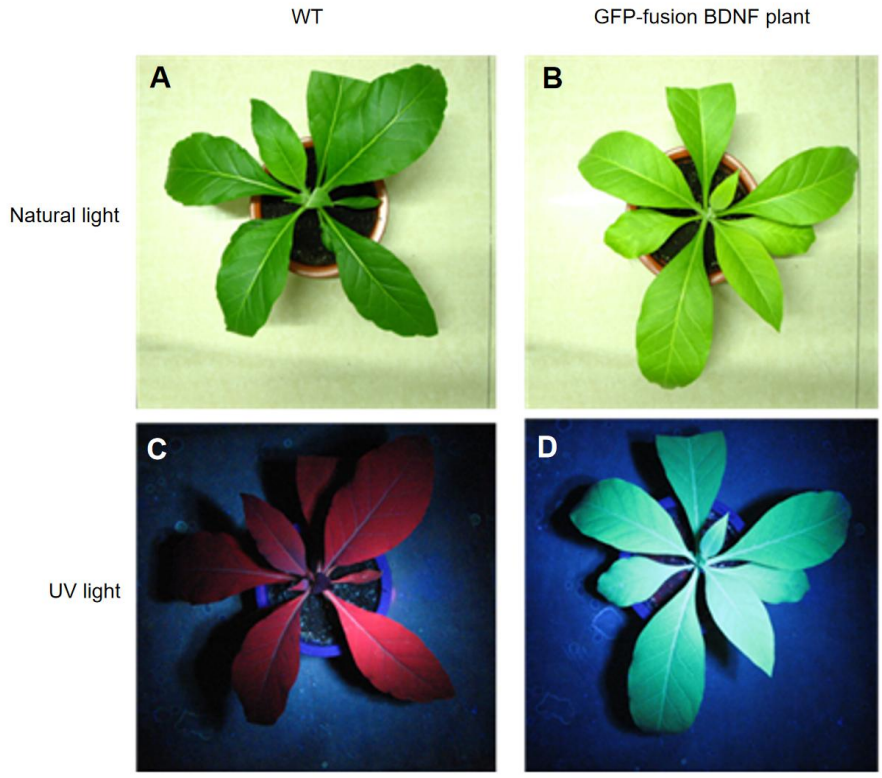


Fig. 4 *in vivo* detection of green fluorescent protein (GFP) in transplastomic plant. **a, b** The growth condition of wild-type and GFP-fusion BDNF plants under natural light, Wild-type plant (**c**) showed red autofluorescence whereas green fluorescence from tranplastomic plant (**d**) under UV light.

Isolation and purification of BDNF and the evaluation of its biological activity

To purify the total soluble protein from the leave, the mature leave was ground in liquid nitrogen, add 5ml PBS buffer per gram. After extraction filtration by 0.22 μm filter membrane, the liquor was put into the 50KD tube, centrifuge for 30 min at 4000 ×g, the outflow was put into fresh 30KD tube, centrifuge for 45 min at 4000 ×g again. The outflow will be the primary product of GFP-fused BDNF in N terminal. The GFP-fused BDNF was digested by enterokinase to separate the GFP from BDNF. The target BDNF was purified by heparin affinity chromatography column. The results showed that the BDNF showed much higher biological activity then the negative control PBS buffer (Fig. 5b),

indicating the potential application in the market of medical proteins of raw materials. Meanwhile, the there is no significant difference between this chloroplast-derived BDNF and the commercial product as standard positive control purchased from Abcam (Cambridge, MA, USA) (See Fig. 5c).

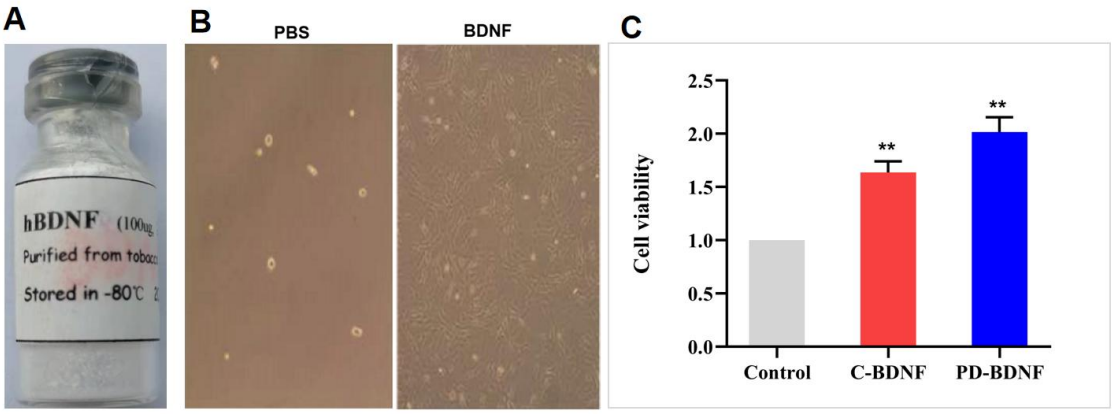


Fig. 5 Purified laboratory product of chloroplast-expressed BDNF and the its cell viability. **a** Cold-dry protein powder of BDNF expressed in chloroplast, **b** 3T3 cell proliferation promoted by BDNF growth factor. The result showed the biological activity of BDNF and the PBS buffer was set as negative control, **c** Comparison of cell viability of three different groups. Control indicated the negative group, C-BDNF indicated the biological activity of commercial product purchased from the company, PD-BDNF indicated the biological activity of BDNF derived from transplastomic tobacco plants. Both C-BDNF (red bar) and PD-BDNF (blue bar) were great significant difference (two asterisks) with the negative control (gray bar).

Discussion

Among existing expression systems of recombinant proteins including microorganisms [29-33], animals [34], and plants [25, 21, 22], plants have shown specific advantages over the other two production systems. The PMPs produced by both nuclear and chloroplast transformation offer some unique advantages over microorganism and mammalian systems like production in large-scale growing plants, cost-efficient, and lack of endotoxins and pyrogens [35, 36].

Current evidence strongly implicates that BDNF is a key and versatile regulator of neural circuit development and function. The secreted BDNF enables to mediate multiple processes in the mammalian brain including sleep deprivation, memory, mode disorder, neuronal differentiation and growth, synapse formation, and plasticity [11, 37-39]. Due to the multiple physiological functions of BDNF in the healthcare industry such as Parkinson's disease [40, 41], the growing supply-demand gap will be a great challenge for the scientists to fill in and the PMPs will be the batter option. For the plant-based expression system, chloroplast transformation has emerged as a new alternative to generate transplastomic plants due to some attractive features over nuclear transformation. The potential for high-level protein expression of this technology makes it feasible to produce therapeutics, vaccines, antigens, and commercial enzymes [42].

Originated from cyanobacterial cells around 1.5 billion years ago, chloroplasts are organelles only existing in the cells of plants. The incredibly high copy number of a cell, reaching up to 10000 copies per mesophyll cell [43], makes chloroplasts feasible to be the bioreactor for the production of therapeutic proteins with high expression levels [35, 44, 36]. Unlike nuclear transformation, gene silencing, variable expression level, and epigenetic regulation have not been observed during the practice of chloroplast transformation.

The expression level is always the concern of the producer. In this research, the expression level of GFP fusion BDNF can reach up to a rather high level of 15.7%. Such a high level of expression maybe benefit from the highly stable characteristics of GFP. Moreover, the target factor is still soluble and shows biological activity after separation with GFP. Considering commercial production in the future, two things should be addressed. One is the expression manner of BDNF. In this research, GFP fusion protein is expressed in tobacco chloroplasts. Subsequently, the enzyme digestion of fusion protein to separate the GFP and BDNF will greatly increase the overall cost and lower the yield of the BDNF. How to promote the yield of BDNF in the tobacco chloroplasts without fusion of GFP will be the challenge in the next step. The second one is the host of expression. The

seed-based system exploits the natural storage properties and therefore has additional merits such as easy transportation and storage, batch processing, highly stability [45]. The large biomass of tobacco mature leaves, however, will be the advantage with an average yield of 2.7 tons per hectare [46]. Moreover, it is practicable to harvest five to seven times annually under field conditions depending on the growing season.

It was reported that the production cost of the tobacco expression system is only one over fifty of the *E. coli* fermentation system [47]. Just recently, there is a report of launching a tobacco leaf-based pectinase production of the commercial-scale platform by Denill's Lab [48], indicating that engineered chloroplasts can offer attractive potential for industrial application just using crude leaf extracts without fermentation, purification, cold-chain transportation that microbial-enzyme products generally have. Moreover, the enzyme can maintain the full function in dried leaves making it much easier during its storage and crude enzyme extract, the competitive low cost, therefore, will be a remarkable feature for this platform. Similarly, plastid-derived growth factors like value-added BDFN will be also expected to develop such a platform to protect jobs and increase income of tobacco-growing farmers.

Compliance with Ethical Standards

Funding

This work was supported by the Agricultural Science and Technology Innovation Program of Jilin Province (CXGC2017TD004), Jilin Province Science and Technology Development Plan Project (20190301074NY), TransGen dream fund for life science (Trans-DFLSci-001) and Scientific and Technological Plan Project of Wenzhou（N20180002).

Author contributions

Yunpeng Wang, Jieying Fan and Nuo Xu were responsible for material preparation and conducting the experiments. Zhengyi Wei and Wen Xin were responsible for data collection and analysis, Shaochen Xing and Yunpeng Wang were responsible for the draft of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that there are no conflicts of interest.

Ethics approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate

Informed consent was obtained from all individual participants included in the study

Consent to publish

All the authors have given the permission for the publication of the manuscript.

References

1. Hohn A, Leibrock J, Bailey K, Barde YA (1990) Identification and characterization of a novel member of the nerve growth factor/brainderived neurotrophic factor family. Nature 344(6264):339–341. http://doi.org/10.1038/344339a0
2. Ip NY, Ibáñez CF, Nye SH, McClain J, Jones PF, Gies DR et al (1992) Mammalian neurotrophin-4: structure, chromosomal localization, tissue distribution, and receptor specificity. Proc Natl Acad Sci USA 89(7):3060–3064. http://doi.org/10.1073/pnas.89.7.3060
3. Maisonpierre PC, Belluscio L, Squinto S, Ip NY, Furth ME, Lindsay RM et al (1990) Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. Science 247(4949 Pt1):1446–1451. http://doi.org/10.1126/science.247.4949.1446
4. Rosenthal A, Goeddel DV, Nguyen T, Lewis M, Shih A, Laramee GR et al (1990) Primary structure and biological activity of a novel human neurotrophic factor. Neuron 4(5):767–773. http://doi.org/10.1016/0896-6273(90)90203-r
5. Rosenthal A, Goeddel DV, Nguyen T, Martin E, Burton LE, Shih A et al (1991) Primary structure and biological activity of human brain-derived neurotrophic factor. Endocrinology 129(3):1289–1294. http://doi.org/10.1210/endo-129-3-1289
6. Barde YA, Edgar D, Thoenen H(1982) Purification of a new neurotrophic factor from mammalian brain. EMBO J 1(5): 549–553. http://doi.org/10.1002/j.1460-2075.1982.tb01207.x
7. Pruunsild P, Kazantseva A, Aid T, Palm K, Timmusk T (2007) Dissecting the human BDNF locus: bidirectional transcription, complex splicing, and multiple promoters. Genomics 90(3):397–406. http://doi.org/10.1016/j.ygeno.2007.05.004
8. Yeh FC, Kao CF, Kuo PH (2015) Explore the features of brain-derived neurotrophic factor in mood disorders. PLoS ONE 10(6): e0128605. http://doi.org/10.1371/journal.pone.0128605
9. Lessmann V, Brigadski T (2009) Mechanisms, locations, and kinetics of synaptic BDNF secretion: an update. Neurosci Res 65(1):11–22. http://doi.org/10.1016/j.neures.2009.06.004
10. Lau AG, Irier HA, Gu J, Tian D, Ku L, Liu G et al (2010) Distinct 3'UTRs differentially regulate activity-dependent translation of brain-derived neurotrophic factor (BDNF). Proc Natl Acad Sci USA 107(36):15945–15950. http://doi.org/10.1073/pnas.1002929107

11. Park H, Poo MM (2013) Neurotrophin regulation of neural circuit development and function. Nat Rev Neurosci 14(1):7–23. http://doi.org/10.1038/nrn3379

12. Soppet D, Escandon E, Maragos J, Middlemas DS, Reid SW, Blair J et al (1991) The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the trkB tyrosine kinase receptor. Cell 65(5):895–903. http://doi.org/10.1016/0092-8674(91)90396-g

13. Leibrock J, Lottspeich F, Hohn A, Hofer M, Hengerer B, Masiakowski P et al (1989) Molecular cloning and expression of brain-derived neurotrophic factor. Nature 341: 149–152. http://doi.org/10.1038/341149a0

14. Lin CC, Huang TL (2020) Brain-derived neurotrophic factor and mental disorders. Biomed J 43(2):134–142. http://doi.org/10.1016/j.bj.2020.01.001

15. Chao MV (2003) Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat Rev Neurosci 4:299–309. http://doi.org/10.1038/nrn1078

16. Cohen S, Greenberg ME (2008) Communication between the synapse and the nucleus in neuronal development, plasticity, and disease. Annu Rev Cell Dev Biol 24:183–209. http://doi.org/10.1146/annurev.cellbio.24.110707.175235

17. Huang EJ, Reichardt LF (2001) Neurotrophins: roles in neuronal development and function. Annu Rev Neurosci 24:677–736. http://doi.org/10.1146/annurev.neuro.24.1.677

18. Kowiański P, Lietzau G, Czuba E, Waśkow M, Steliga A, Moryś J (2018) BDNF: A key factor with multipotent impact on brain signaling and synaptic plasticity. Cell Mol Neurobiol 38: 579–593. http://doi.org/10.1007/s10571-017-0510-4

19. Björkholma C, Monteggiab LM (2016) BDNF-a key transducer of antidepressant effects, Neuropharmacology 102: 72–79. http://doi.org/10.1016/j.neuropharm.2015.10.034

20. Ghosh B, Wang Z, Nong J, Urban MW, Zhang Z, Trovillion VA et al (2018) Local BDNF delivery to the injured cervical spinal cord using an engineered hydrogel enhances diaphragmatic respiratory function. J Neurosci 38 (26): 5982–5995. http://doi.org/10.1523/JNEUROSCI.3084-17.2018

21. Wang YP, Wei ZY, Zhong XF, Lin CJ, Cai YH, Ma J et al (2015a) Stable expression of basic fibroblast growth factor in chloroplasts of tobacco. Int J Mol Sci 17(1):19. http://doi.org/10.3390/ijms17010019

401 22. Wirth S, Calamante G, Mentaberry A, Bussmann L, Lattanzi M, Barañao L et al (2004) Expression of active
402 human epidermal growth factor (hEGF) in tobacco plants by integrative and non-integrative systems. *Molecular*
403 *Breeding,* 13, 23–35.
404 23. Wang YP, Wei ZY, Zhang YY, Lin CJ, Zhong XF, Wang YL et al (2015b) Chloroplast-expressed msi-99 in
405 tobacco improves disease resistance and displays inhibitory effect against rice blast fungus. Int J Mol Sci 16 (3),
406 4628–4641. http://doi.org/10.3390/ijms16034628
407 24. Daniell H, Vivekananda J, Nielsen BL, Ye GN, Tewari KK, Sanford JC (1990) Transient foreign gene
408 expression in chloroplasts of cultured tobacco cells after biolistic delivery of chloroplast vectors. Proc Natl Acad
409 Sci USA 87: 88–92. http://doi.org/10.1073/pnas.87.1.88
410 25. Ahmad N, Mukhtar Z (2013) Green factories: plastids for the production of foreign proteins at high levels. *Gene*
411 *Therapy & Molecular Biology,* 15(1): 14–29.
412 26. Bock R (2021) Engineering Chloroplasts for high-level constitutive or inducible transgene expression. Methods
413 Mol Biol 2021, 2317:77–94. http://doi.org/10.1007/978-1-0716-1472-3_3
414 27. Agrawal S, Karcher D, Ruf S, Erban A, Hertle AP, Kopka J et al (2022) Riboswitch-mediated inducible
415 expression of an astaxanthin biosynthetic operon in plastids. Plant Physiol 188(1):637–652.
416 http://doi.org/10.1093/plphys/kiab428
417 28. Wei Z, Liu Y, Lin C, Wang Y, Cai Q, Dong Y et al (2011) Transformation of alfalfa chloroplasts and
418 expression of green fluorescent protein in a forage crop. Biotechnol Lett 33, 2487–2494.
419 http://doi.org/10.1007/s10529-011-0709-2
420 29. Barr PJ, Cousens LS, Lee-Ng CT, Medina-Selby A, Masiarz FR, Hallewell RA et al(1988) Expression and
421 processing of biologically active fibroblast growth factors in the yeast Saccharomyces cerevisiae. J Biol Chem
422 263(31): 16471–16478.PMID: 2460449
423 30. Gasparian ME, Elistratov PA, Drize NI, Nifontova IN, Dolgikh DA, Kirpichnikov MP (2009) Overexpression
424 in *Escherichia coli* and purification of human fibroblast growth factor (FGF-2). Biochemistry(Moscow) 74, 221–
425 225. http://doi.org/10.1134/s000629790902014x
426 31. Kwong KW, Ng K L, Lam CC, Wang YY, Wong WK (2013) Authentic human basic fibroblast growth factor
427 produced by secretion in Bacillus subtilis. *Appl Microbio Biotechnol* 97, 6803–6811.
428 http://doi.org/10.1007/s00253-012-4592-0

429 32. Mu X, Kong N, Chen W, Zhang T, Shen M, Yan W (2008) High-level expression, purification, and
430 characterization of recombinant human basic fibroblast growth factor in Pichia pastoris. Protein Expr Purif 59,
431 282–288. http://doi.org/10.1016/j.pep.2008.02.009
432 33. Song JA, Koo BK, Chong SH, Kwak J, Ryu HB, Nguyen MT et al (2013) Expression and purification of
433 biologically active human FGF2 containing the b'a' domains of human PDI in *Escherichia coli*. Appl Biochem
434 Biotechnol 170, 67–80. http://doi.org/10.1007/s12010-013-0140-3
435 34. Wu X, Kamei K, Sato H, Sato S I, Takano R, Ichida M et al (2001) High-level expression of human acidic
436 fibroblast growth factor and basic fibroblast growth factor in silkworm (*Bombyx mori* L.) using recombinant
437 baculovirus. Protein Expr Purif 21, 192–200. http://doi.org/10.1006/prep.2000.1358
438 35. He Y, Ning T, Xie T, Qiu Q, Zhang L, Sun Y et al (2011) Large-scale production of functional human serum
439 albumin from transgenic rice seeds. Proc Natl Acad Sci USA 108, 19078–19083.
440 http://doi.org/10.1073/pnas.1109736108
441 36. Sil B, Jha S (2014) Plants: The future pharmaceutical factory. Am J Plant Sci 5(03), 319–327.
442 http://doi.org/10.4236/ajps.2014.53044
443 37. Jin Y, Sun LH, Yang W, Cui RJ, Xu SB (2019) The role of BDNF in the neuroimmune axis regulation of mood
444 disorders. Front Neurol 10:515. http://doi.org/10.3389/fneur.2019.00515
445 38. Patel D, Roy A, Raha S, Kundu M, Gonzalez FJ, Pahan K (2020) Upregulation of BDNF and hippocampal
446 functions by a hippocampal ligand of PPARα. JCI Insight 21,5(10):e136654.
447 http://doi.org/10.1172/jci.insight.136654
448 39. Rahmani M, Rahmani F, Rezaei N (2020) The brain-derived neurotrophic factor: missing link between sleep
449 deprivation, insomnia, and depression. Neurochem Res 45(2):221–231. http://doi.org/10.1007/s11064-019-
450 02914-1
451 40. Motyl J A, Strosznajder JB, Wencel A, Strosznajder RP (2021) Recent insights into the interplay of alpha-
452 synuclein and sphingolipid signaling in Parkinson's disease. Int J Mol Sci 22(12):6277.
453 http://doi.org/10.3390/ijms22126277
454 41. Palasz E, Wysocka A, Gasiorowska A, Chalimoniuk M, Niewiadomski W, Niewiadomska G (2020) BDNF as a
455 promising therapeutic agent in Parkinson's disease.Int J Mol Sci 21(3):1170.
456 http://doi.org/10.3390/ijms21031170

457 42. Bock R (2014) Genetic engineering of the chloroplast: novel tools and new applications. Curr Opin Biotechnol
458 26, 7–13. http://doi.org/10.1016/j.copbio.2013.06.004
459 43. Shaver JM, Oldenburg DJ, Bendich AJ (2006) Changes in chloroplast DNA during development in tobacco,
460 Medicago truncatula, pea, and maize. Planta 224, 72–82. http://doi.org/10.1007/s00425-005-0195-7
461 44. Ding SH, Huang L, Wang YD, Sun HC, Xiang ZH (2006) High-level expression of basic fibroblast growth
462 factor in transgenic soybean seeds and characterization of its biological activity. Biotechnol Lett 28, 869–875.
463 http://doi.org/10.1007/s10529-006-9018-6
464 45. Mikschofsky H, Broer I (2012) Feasibility of *Pisum sativum* as an expression system for pharmaceuticals.
465 Transgenic Res 21, 715–724. http://doi.org/10.1007/s11248-011-9573-z
466 46. Drake MP, Vann MC, Fisher LR (2015) Influence of nitrogen application rate on the yield, quality, and
467 chemical components of flue-cured tobacco, part II: application method. *Tobacco Science,* 52:26–34.
468 47. Kusnadi AR, Nikolov ZL, Howard JA (1997) Production of recombinant proteins in transgenic plants: practical
469 considerations. Biotechnol Bioeng 56(5): 473–484. http://doi.org/10.1002/(SICI)1097-
470 0290(19971205)56:5<473::AID-BIT1>3.0.CO;2-F
471 48. Daniell H, Ribeiro T, Lin S, Saha P, McMichael C, Chowdhary R et al (2019) Validation of leaf and microbial
472 pectinases: commercial launching of a new platform technology. Plant Biotechnol J 17(6):1154–1166.
473 http://doi.org/10.1111/pbi.13119