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CODON-OPTIMIZED RECOMBINANT EXPRESSION ASSAY FOR A MONOMERIC 17KDA BASIC FIBROBLAST GROWTH FACTOR (bFGF) IN TRANSGENIC ALFALFA (*MEDICAGO SATIVA* L.): BLUEPRINTS TOWARDS HUMAN THERAPEUTIC PROTEINS

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Abstract

Basic fibroblast growth factor (bFGF) also called as FGF2/FGF-β is an important growth factor and signal protein belongs to heparin binding growth factor family. The naturally occurring bFGF family is capable of stimulating a wide variety of biological activitiesand cellular responses including cell proliferation, angiogenesis, mitogenesis, embryonic development, cellular growth, wound repair process, oncogenic responses and neural development. The present work demonstrated the designing of a preferential codon optimizing strategy for bFGF recombinant protein by recruiting alfalfa as a suitable host bioreactor system. To validate our experimental design the over-expression vector p1390R carrying bFGF gene was efficiently transformed in to alfalfa using *Agrobacterium* mediated transformation. We have confirmed the successful integration of bFGF gene in alfalfa using various molecular techniques. In addition, the transcription regulation of bFGFin transgenic alfalfa was determined through RT-PCRthereby orchestrating gene activity.Subsequently, the recombinant protein of bFGF was separated on SDS-PAGE resulting in a 17kDa product. Moreover, the identification of specific bFGF single protein within a complex mixture of proteins was semi-quantitatively detected on western blot by estimating the size and color intensity of target protein. Altogether, our findings highlight anadvance molecular farming technique resulting in a controlled recombinant expression assay for a monomeric 17kDa bFGF protein in transgenic lines of alfalfa. The present work provides adequate information on the exploitation of plant host systems as bioreactors for essential recombinant therapeutic proteins such as vaccines, hormones, signaling molecules and antibodies with pharmacological potential in future researches.

Key words: Plant engineering, Molecular farming, Plant-microbe interaction, Bioreactor, Plants therapeutics.

Introduction

Basic fibroblast growth factor (bFGF) is considered as an important member of a heparin binding protein family, which control the proliferation, differentiation, and migration of neuroectodermal and mesodermal cell types in several signaling pathways (Ornitz & Itoh, 2015; Zhao *et al*., 2011; Hassan *et al*., 2013; Sun *et al*., 2013). The distinct role of bFGF in tumor angiogenesis (Xiao *et al*., 2015; Kanazawa *et al*., 2010), and its active participation as tissue repaired stimulator was already confirmed in previous studies (Zheng *et al*., 2011). Furthermore the therapeutic potential of bFGF in curing bone damage and Temporomandibular joint (TMJ), healing wound and protecting human heart and brain from injuries of ischemia and reperfusion outlined the multi-function role of bFGF protein (Zheng *et al*., 2000).

The human basic fibroblast growth factor consists of approximately 20 isoforms resulting in 7 subfamilies ranging from 16-24kD monomeric proteins from one mRNA transcript (Itoh & Ornitz, 2008). The expression of these variants differentially occurs in the nucleus, cytosol and cell surface. The induction of optimum expression of recombinant proteins using high frequency codons preference strategies has been determined recently in various host bioreactor systems including plants, mammals and bacteria (Burgess-Brown *et al*., 2010; Fath *et al*., 2011). The conserved sequence homology of human bFGF isoforms and similar binding sites among different species are considered of great interest to address various cellular processes (Penny*et al*., 2018). However it is recommended to preclude certain factors before designing a method for codon-optimized genes including sites of restriction enzymes, cryptic splice sites (GT/AG), and rare codon sequences.

In recent years, the current progress in plant genetic engineering technology is participating pivotal role in finding alternatives to produce low cost therapeutic proteins with the help of transgenic plants (Wang *et al*., 2015). Our previous findings suggested that producing novel transgenic plants can significantly develop a natural bioreactor system for the systematic production of chemicals and industrialproducts with considerably increased product size (Yang *et al*., 2015). In a similar way Alfalfa hold tremendous capabilities that unanimously made them an ideal vehicle for the over-expression of essential recombinant proteins hence overcoming major limitations of traditional molecular farming strategies (Wei *et al*., 2011; Qiang*et al*., 2015).

The aim of the present study was to develop a simple and cost-effective genetic-engineering technique of codon optimization for human bFGF gene to express a recombinant protein in transgenic alfalfa lines by choosing plant preferred codons. In order to testify our hypothesis the plant preferred codons of human bFGF were successfully amplified using RACE-PCR followed

by sub cloning into a plant over-expression vector (p1390R). The transformation of alfalfa lines were efficiently conducted by A. mediated transformation using leaf disc method. Transgenic lines were further detected by Polymerase chain reaction and southern blot analysis confirming the efficient integration of bFGF into alfalfa. RT-PCR analysis revealed that the bFGF gene has a relatively high expression at transcriptional level. A total of 17kD recombinant protein was detected on SDS-PAGE and western blot analysis from transgenic alfalfa thus, indicating the positive regulation and expression of bFGF gene at translational level.

Materials and Methods

Strains, cells and experimental material: *Medicago sativa* L. seeds were purchased from Fuyu Seeds Company, China. *Escherichia coli* DH5α, *A. tumefacien* strain LBA4404, plasmids pUC-bFGF and p1390R were previously constructed and stored stored in our laboratory.Restriction enzymes and DNA ligases were provided by Takara Biotechnology Company Beijing, China. Goat anti-rabbit horseradish peroxidase conjugated immunoglobulins were purchased from Dingguo Biotechnology Company, China.

Gene synthesis and construction of plant over-expression vector: The human bFGF gene (418bp) containing sequences encoding an N-terminal signal peptide sequence (agatcttcc) and C-terminal histidine tag (gaattccttgttccacgtggatct) along with 5′ and 3′ terminal restriction endonuclease sites were optimized for alfalfa codon usage. The synthetic gene fragment of human codon optimized bFGF was successfully obtained by PCR amplification using pUC-bFGF as a template. The appropriate pair of synthetic primers Pb1: GCGGTACCCCAGCTTTGCCAGAGGATG (*KpnI*) and Pb2: GCACTAGTTTAAGACTTAG-CAGACATTGGC (*SpeI*) were used. The amplified (*KpnI-SpeI*) segment of bFGF gene was successfully extracted from 1% agarose gel and inserted into *KpnI*and*SpeI*sites of the linearized p1390R, a plant over-expression vector under the control of a 35S promoter (Fig. 1). The obtained product p1390b-FGF was confirmed with three times restriction digestion analysis and further verified by DNA sequencing. The plant expression vector p1390bFGF was efficiently transformed into *A. tumefaciens* (LBA4404) via Freeze-Thaw method (Zhang & Zeevaart, 1999). Positive *A. tumefaciens* colonies with high carrying bFGF-p1390R were selected on agar plates of yeast extract peptone (YEP) medium containing 50mg/L kanamycin.

Alfalfa transformation and regeneration: The alfalfa (*Medicago sativa* L.) was efficiently transformed with Agrobacterium mediated transformation as described (Tzfira *et al*., 2006; Duan *et al*., 2015). The selected seeds of alfalfa were suspended in ethanol (70%) for a period of 1 min, followed by a four steps washing with sterilized distilled water for 2 min. After this, the seeds were suspended in 10% sodium hypochlorite (NaClO) for 16 minutes followed by another four steps of re-washing for 2 minutes with sterilized distilled water. The inoculations of sterilized seeds were performed on cultured medium with phytohormone-free MS medium of pH 5.8 solidified with 0.7% agar (Murashige and skooge 1962). The cotyledons of the 5 days-old plantlets were cultured in callus induction medium of TM-1+2,4-D (2.0 mg/L)+ 6-BA (1.0 mg/L)+NAA (0.1) mg/L, supplemented respectively with 0/10/20/30 and 40 mg/L hygromycin as a selectable marker to analyze the susceptibility. Hygromycine has been proved a competent marker in alfalfa transformation.

Prior to infection approximately (48 hours), *A. tumefaciens* strain with p1390bFGF was inoculated on LB broth containing 50 mg/L Kanamycin, 100 mg/L Rifampicin and 50 mg/L Streptomycin and incubated overnight at 28°C shaker with 200 rpm speed. Then the bacterial solution was subjected to centrifugation and cultured again with liquid MS medium containing sucrose (3%), AcetoSyringone (l00 μmol/L) with pH adjusted 5.8 for a period of 15 minutes at 4˚C in order to obtain the optimum density between 0.4～0.6 at 600 nm. Cotyledon explants were finally incubated in the previous bacterial solution at room temperature for 5 minutes. After infection, the explants were then placed on co-culture medium including TM-1+ 2.0 mg/L 2, 4-D + 1.0 mg/L 6-BA +0.1 mg/L NAA with 3% sucrose, 0.7% agar for 3 days at 25°C in a complete dark condition. After the successful co-culture incubation, the explants were further re-subjected to the same medium containing 300 mg/L Timentin and controlled concentration of hygromycin. The infected explants were repeatedly sub-cultured for two weeks using the same medium. The resulting mass of calliwere transferred to MS shoot induction medium supplemented with 200 mg/L Timentin, known concentration of hygromycin and 1% sucrose. The green buds were taken together, cut and transferred to the root induction medium until the root length reached to 3~5 cm. After 4-5 weeks, acclimatized rooted plantlets were finally subjected to soil and grown to maturity under control environment in our laboratory green house (25 ± 2°C, 40-200 foot-candle, 16 hour photoperiod).

Extraction of genomic DNA and PCR analysis: Total genomic DNA was extracted from the leaves of both wild-type and transgenic plants using CTAB method (Bressan *et al*., 2014). The wild-type DNA, the negative control, the plasmid (positive control) and DNA from transgenic lines were used as templates in the PCR amplification carried out using GoTaq Flexi DNA polymerase (Promega Corp, Madison, WI). The synthetic primer pair Pb1: GCGGTACCCCAGCTTTGCCAGAGGATG (*Kpn*I) and Pb2: GCACTAGTTTAAGACTTAG-CAGACATTGGC (*SpeI*) were applied to the aforementioned PCR system with the following thermal program: initial denaturation cycle at 94°C for 10min, and the subjected to a total of 30 cycles of 94°C for 55sec, 55°C for 35sec, and 72°C for 35sec and a final extension step at 72°Cfor 3 min. The positive PCR bands were detected in 1% agarose gel under UV light.

Expression analysis of bFGF at transcriptional level: To identify the expression of bFGF gene at transcriptional level in transgenic alfalfa plants, total RNA was isolated from transgenic plants using RNAiso Plus (Total RNA extraction reagent: Cat# 9108/9109) and RT-PCR was performed with gDNA Eraser (Prime Script™ RT reagent Kit, Takara) and reverse transcription enzyme (Clontech) to generate cDNA using mRNA as template. Synthetic primers pair Pb1: GCGGTACCCCAGCTTTGCCAGAGGATG (KpnI) and Pb2: GCACTAGTTTAAGACTTAG- CAGACATTGGC (*SpeI*) was used. The bFGF transcripts were efficiently detected on 1% agarose gel in comparison with positive control (bFGF-Plasmid), negative control and wild-type plants. A specific product of 480bp obtained from 4 out of 5 transgenic lines.

Southern blot characterization of transgenic alfalfa: In order to detect bFGF single copy number at a further molecular level in transgenic alfalfa, we carried out southern blot analysis with (DIG)-labeled probes and fundamental chemiluminescent (Roche) that enabled the detection of nucleic acids more systematically. Total plant DNA was digested with a mono restriction enzyme (*Bam*HI) and run on a 0.8% agarose gel for fractionation at 50 V for 2 hours. The gel was soaked in 0.25 N HCl for 15 minutes and then rinsed 2× with water. The gel was further soaked in transfer buffer (0.4 N NaOH, 1 M NaCl) for 20 minutes and then subjected overnight to a nitrocellulose membrane. The flanking sequence probe was made to generate a 418bp product.

SDS-PAGE and western blot hybridization: Transgenic alfalfa leaves (app. 300 mg) leaves were selected from the previous grown plantlets 15 days prior to protein purification. Leaves of the same masses and size were subjected to further detection in order to avoid mis-comparision. The leaves were ground thoroughly into complete powder by liquid nitrogen, and the resultant extract was resuspended in 0.5 ml PBS buffer (*Sodium phosphate buffer (200ml)* prepared in 0.1M NaH_2PO_4 concentrations and the pH was adjusted to 7. Afterwards we transferred 1.5 ml of the previous solution to micro centrifuge tubes and subjected to a maximum spin at 15000 rpm for 10 min to avoid the presence of large chunks. The supernatant was transferred to 1.5 ml tubes and boiled in hot water for a period of 3 minutes in order to denature the folded proteins. The tubes were cooled at room temperature and then samples were prepared for loading onto a native SDS-PAGE (12% v/v) and run with a constant voltage of 135 V for at least 2h and wait for the tracking dye front to fully reach the bottom of the gel. After a complete run of electrophoresis, the proteins staining were performed with Coomassie brilliant blue G-250. The extracted protein was stored at -80˚C in our laboratory for future use.

A further Western blot analysis was performed to detect the expression level of bFGF in transgenic alfalfa plants. Heterologously expressed bFGF purified from E. coli was used as positive control. The total protein which was separated previously by SDS PAGE was subjected to a nylon membrane (Millipore, Billerica, MA, USA) after the equilibrated treatment. The rabbit polyclonal anti-bFGF antibodies (Abcam) were used as primary antibody followed by a period of 3 hours at 22°C incubation and 3 times washing with PBS containing 0.05% Tween20.

Membranes were further incubated for 40 min at 22ºC with goat anti-rabbit horseradish peroxidase-conjugated immunoglobulins (Promega), as a secondary antibody, followed by a 3 times washing as mentioned above. The positive bands were detected with Western Blotting Luminol Reagent according to the company's instructions. The relative expression of target proteins was obtained by scanning densitometry using Image lab software (version 5.2).

Results

Vector construction: In order to expressbFGF gene fragment for expression in alfalfa host bioreactor , the full length of synthetic codon optimized human bFGF gene was engineered by PCR amplification using PUC-bFGF as template DNA with forward primer Pb1:GCGGTACCCCAGCTTTGCCAGAGGATG (*KpnI*) and reverse primer Pb2: GCACTAGTTTAAGACTTAG-CAGACATTGGC (*SpeI*). This codon optimized bFGFwas designed for its efficient expression in alfalfa by adjusting the plant preferred GC content which inherits the amino acid sequences of bFGF protein. The T-DNA region of the binary vector bFGF-p1390R contained a hygromycin (selectable marker gene) under the control of 35S promoter and terminator, and a synthetic bFGF gene fragment downstream of the CaMV 35S promoter with Nos terminator (Fig. 1). After checking the construct by DNA-sequencing, we proceeded with the genetic transformation of alfalfa via *A. tumefaciens*.

Generation of transgenic bFGF-p1390R alfalfa lines: The construct was introduced into *A. tumefaciens* EHA105 using the heat shock method. Alfalfa seeds were incubated on controlled MS medium supplemented with appropriate concentrations of growth hormones and the A. mediated leaf disc transformation system was established for alfalfa (see material and methods for details). Furthermore, the infected seeds were grown on selection medium with variable concentrations of hygromycin. Our experimental design was set for a period of 15 days and with multiples of 10mg/l, 20mg/l, 30mg/l and 40 mg/l of hygromycin treatment. The results showed that the calli occurred on MS medium without Hygromycin (Fig. 2A), However, when the MS medium was supplemented with 10 mg/L hygromycin, the formation of calliwas retarded (Fig. 2B). No callus formation was observed when the MS medium contains 20 mg/L hygromycin (Fig. 2C). Furthermore, when the concentration of hygromycin was kept more than 30 mg/L, no callus was generated on the explants and lead to a whitish color which deceased gradually (Fig. 2D&E). Therefore, 20 mg/L was used as optimal concentration. The hygromycin susceptible transgenic lines of alfalfa (Fig. 3) were further subjected to elongation and rooting Medium in controlled growth conditions (see Materials and Methods).

Agrobacterium induced transformation *of* bFGF-p1390R in alfalfa resulted in 5 out of 8 independent transformations after the hygromycin *In vitro* assay and was further confirmed with PCR analysis using bFGF-specific primers. A product of 418bp was successfully detected on 1% agarose gel. Plasmid pUC-bFGF was used as a positive control while negative control contains double distilled water as a template. The genomic DNA of wild type alfalfa was also used for comparative analysis as partial evidence for the presence of bFGF gene segment (Fig. 4).

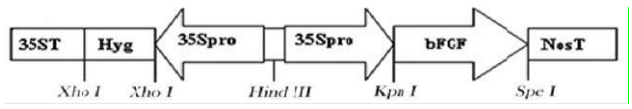

Fig .1. The diagrammatic illustration of plant expression vector p1390bFGF.

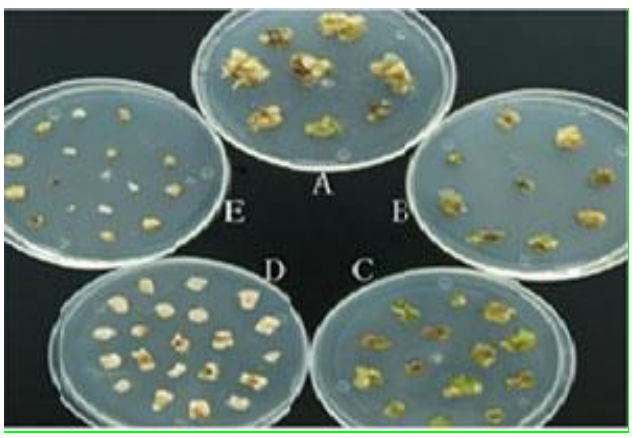

Fig. 2. Susceptibility analysis of alfalfa cotyledons to various concentrations of Hygromycin.A: The calli on the medium without Hygromycin B: The calli on the medium containing 10 mg/L Hygromycin; C: The calli on the medium contained 20 mg/L Hyg; D: The calli on the medium contained 30 mg/L Hyg; E: The calli on the medium contained 40 mg/L Hygromycin.

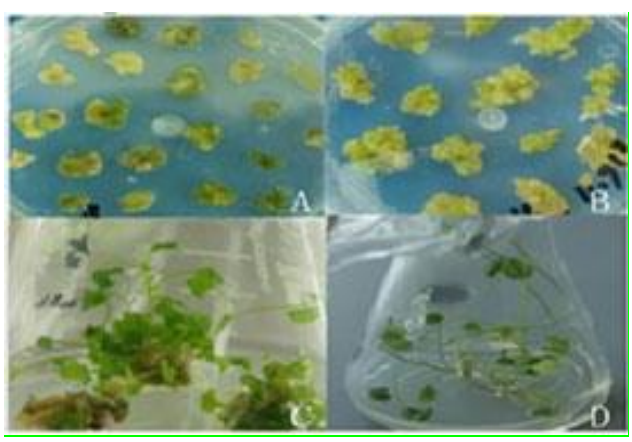

Fig. 3. Shooting and rejuvenation of apical shoots of transgenic alfalfa. A: callus formation supplemented on selection medium. B: Viable callus with active buds incubated on on selection medium. C: Transgenic alfalfa plantlets resistant to hygromycin on shoot induction medium medium. D: Hygromycin resistant plantlets incubated on root induction medium.

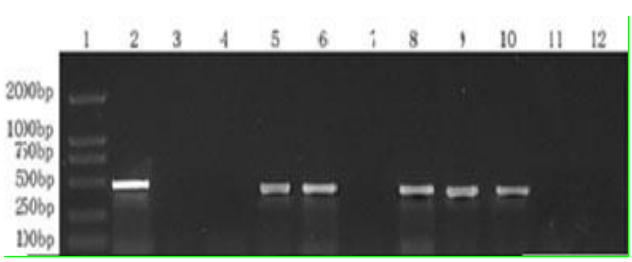

Fig. 4. Identification of bFGF in transgenic alfalfa by PCR amplification. Lane1: DNA Marker DL 2000; Lane 2: Positive control by using plasmid as a template; Lane 3: The wild-type plant; Lane 4: Negative control by using ddH_2O as a template; Lanes 5-12: The transformed plants.

Southern blot hybridization of bFGF in transgenic alfalfa: To verify the integration of specific exogenous DNA fragments in alfalfa mutant plants Southern blot analysis was performed. To find out the bFGF copy number in transgenic plants the total genomic DNA of 5 mutant lines were extracted and completely digested with a single enzyme digestion (BamH1). Three out of five transformation events were detected with (DIG)-labeled probes and chemiluminescent (Roche) separated on 0.8% agarose gel (Fig. 5). After the immobilization of DNA onto a nylon membrane a product of 418bp was obtained of the same size in several copies confirming the presence of bFGF in transgenic lines.

RT-PCR detection, SDS-PAGE and western blot analysis of recombinant bFGF: In order to detect the expression of bFGF gene at RNA level in transgenic alfalfa, total RNA was extracted from the initially detected 5 transformants by PCR and subjected to RT-PCR with gDNA Eraser (Prime Script™ RT reagent Kit, Takara) and reverse transcription enzyme (Clontech). The PCR system was optimized according to previous amplification from genomic DNA using primers Pb1 and Pb2 (See materials and methods). The specific amplification of 418bp fragment was separated on 1% agarose gel from the samples of 4 transgenic plants harboring the expression of recombinant bFGF at mRNA level in the transgenic alfalfa (Fig. 6) thus indicating the efficient expression of bFGF in transgenic alfalfa, except for lane 7. Afterwards, the total protein crude extracts of the transgenic plants detected from PCR positive plants were extracted along with the wild-type plant. The products were separated on 12% SDS-PAGE gel (See material and methods). The results of SDS-PAGE was consisted with that of the expected protein 17 kDa band specifically appeared in 3 of the 4 transgenic lines (Fig. 7, Lane 4, 5 and 6). While it was not seen in one transgenic line and the wild-type plant (Fig. 7, Lane 1 and 3), indicating the phenomenon of transgene silencing might occur at the protein level. To estimate bFGF as a target protein of the total protein extract, Western blot hybridization clearly demonstrates the qualitative expression of bFGF at protein level. Using b-mercaptoethanol free SDS buffer treatment of the incubated protein could convert them into monomeric protein. The western blot analysis was then performed with the conditions of the fixed amount of wild type and transgenic crude extracts. The SDS-PAGE fractions were subjected to a nitrocellulose membrane. The nitrocellulose membrane was fixed and incubated predominantly with the Primary antibody (Rabbit polyclonal anti-bFGF antibodies (Abcam, 1:100,) followed by Goat anti-rabbit horseradish peroxidase conjugated immunoglobulins, (Dingguo Biotechnology Company, China, 1:3000).

A band of the expected size for bFGF was detected in the 3 transgenic lines showing a monomeric 17kDa recombinant protein product by SDS-PAGE Fig. 8 indicatingthe evidence of a considerable level expression at protein level in transgenic alfalfa. No signal was detected in the wild-type plant including one transgenic line. The densitometric analysis of the western blot was investigated which showed deviation from the standard curve that confirms the quantitative accumulation of bFGF. 1g of leaf extracts constituting 17ng while 2g generating curve was recorded at 32.2ng). However, it is important to highlight that signal intensity and percentage was not quantitative in our study.

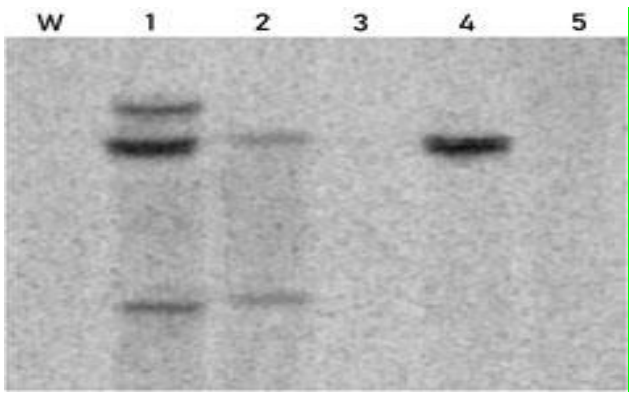

Fig. 5. Southern blot hybridization of bFGF in transgenic alfalfa:Lane 1: The wild-type alfalfa plant; Lane 2-4: The transformed plants.

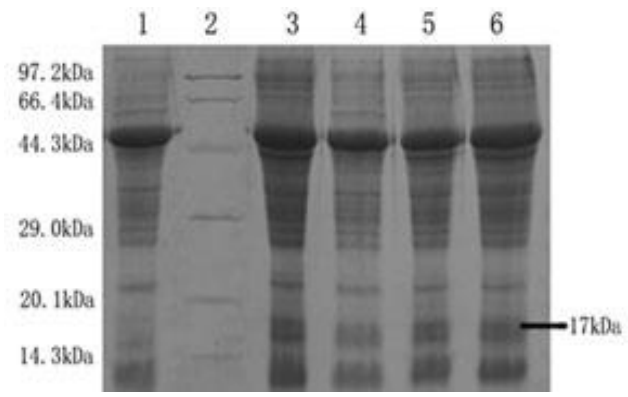

Fig. 7. SDS-PAGE analysis of transgenic plants: Lane 1: The wild-type plant; Lane 2: Protein Molecular Weight Marker (low); Lanes 3-6: The transformed plants.

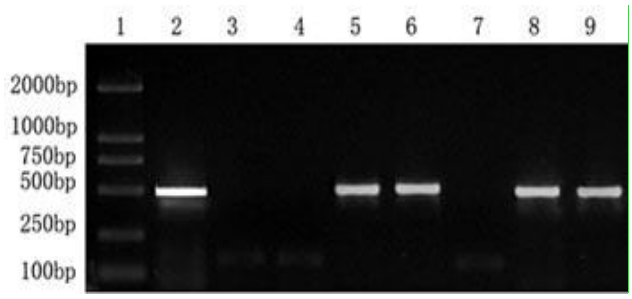

Fig. 6. Expression of bFGF in transgenic alfalfa with RT-PCR: Lane 1: DNA Marker DL 2000; Lane 2: Positive control by using plasmid as a template; Lane 3: The wild-type plant; Lane 4: The negative control by using ddH_2O as a template; Lanes 5-9: The transformed plants.

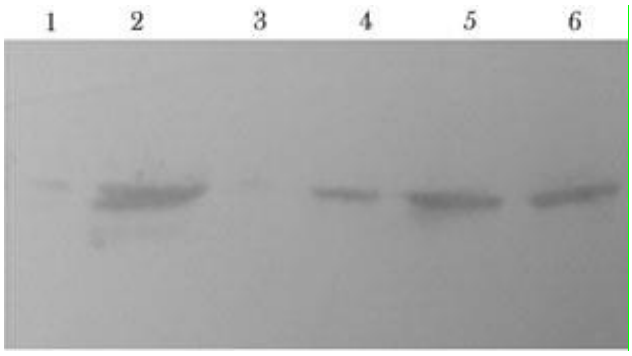

Fig. 8. Protein level analysis of transformed plants with Western blotting: Lane 1: The wild-type plant; Lanes 2: The positive control; Lane 3-6: The transformed plants.

Following are the detail media compositions used in our research work during Agrobacterium mediated transformation.

Media code	Composition
Germination media	MS macronutrients 5ml/L, MS trace 1ml/L, B5 organic 1ml/L, /ferric salt 1ml/L, MES 0.059g/L, sucrose 3g/L, agar 7g/L, (pH5.80-5.86)
Pre-culture media	MS macronutrients 50ml/L, MS trace 10ml/L, B5 organic 10ml/L, ferric salt 10ml/L, MES 0.59g/L, sucrose 30g/L, agar 6.5g/L, (PH5.80). Filter sterilized 6BA 1g/L and NAA 0.5gl/L was added after autoclaving.
infect media	MS macronutrients 50ml/L, MS trace 10ml/L, B5 organic 10ml/L, ferric salt 10ml/L, MES 0.59g/L, sucrose 30g/L, (PH5.80). Filter sterilized 6BA 1g/L, NAA 0.5g/L and AS l00 µmol/L was added after autoclaving.
Co-culture media	MS macronutrients 50ml/L, MS trace 10ml/L, B5 organic 10ml/L, ferric salt 10ml/L, MES 0.59g/L, sucrose 30g/L, agar 6.5g/L, (PH5.80-5.86). placed on shaker for shaking and then Filter sterilized TM-1+ 2.0 mg/L 2, 4-D + 1.0 mg/L 6-BA +0.1 mg/L NAA and AS l00 µmol/L were added after autoclaving.
Selective media 1	MS macronutrients 50ml/L, MS trace 10ml/L, B5 organic 10ml/L, ferric salt 10ml/L, MES 0.59g/L, sucrose 30g/L, agar 6.5g/L, (PH5.80-5.86). Timentin 300 mg/L, Filter sterilized 2, 4-D + 1.0 mg/L 6-BA +0.1 mg/L NAA and Carb 100g/L, and Cef 250mg/L were added after autoclaved.
Selective media 2	Same as selective medium I, in addition with hygromycin with multiples of 10, 15, 20, 30 and 40mg/L, solidified with 6.80 agar at pH 5.8.
Elongation media	MS macronutrients 50ml/L, MS trace 10ml/L, B5 organic 10ml/L, ferric salt 10ml/L, MES 0.59g/L, sucrose 30g/L, agar 6.5g/L, (PH5.80-5.86). Filter sterilized IBA 1g/L, Carb 100g/L, Cef 250mg/L and GA 1g/L were added after autoclaved.
Rooting media	MS macronutrients 50ml/L, MS trace 10ml/L, B5 organic 10ml/L, ferric salt 10ml/L, MES 0.59g/L, sucrose 30g/L, agar 6.5g/L, (PH5.80-5.86). Filter sterilized IBA 1g/L, Carb 100g/L, and Cef 250mg/L were added after autoclaved.
Selective media 1	MS macronutrients 50ml/L, MS trace 10ml/L, B5 organic 10ml/L, ferric salt 10ml/L, MES 0.59g/L, sucrose 30g/L, agar 6.5g/L, (PH5.80-5.86). Timentin 300 mg/L, Filter sterilized 2, 4-D + 1.0 mg/L 6-BA +0.1 mg/L NAA and Carb 100g/L, and Cef 250mg/L were added after autoclaved.
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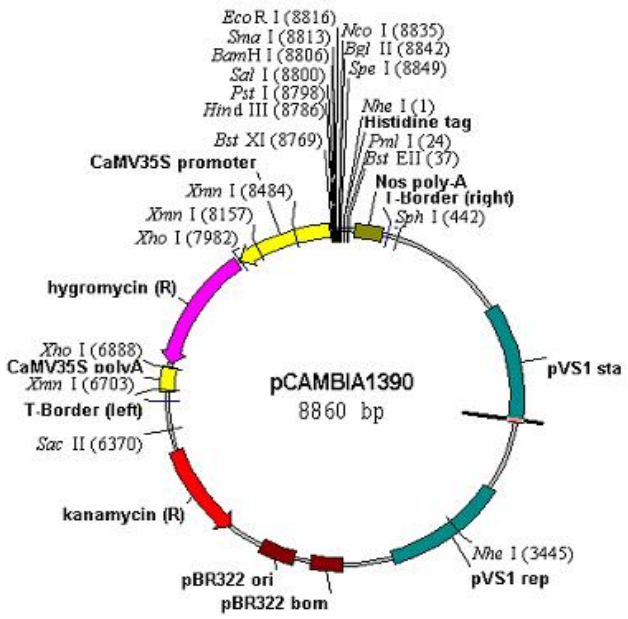

Schematic diagram of plant over-expression vector (bFGF-pCAMBIA1390R) used in our study.

Discussion

Plant cells are ideal bioreactors for the synthesis and oral delivery of vaccines and biopharmaceuticals, minimizing the need for expensive fermentation, purification, cold storage, transportation and sterile delivery. A large family of heparin-binding protein mitogens and inducer factor, with potent pharmacologic utilities due to their broad target cells specificity. Human bFGF has a significant effect on clinical therapy of soft tissue injuries like trauma and empyrosis especially facilitate to the healing of bed sore, ulcer and surgical trauma (Bennell, 1993; Xiong *et al*., 2013). Besides, bFGF is an enhancing factor of the vital movement of cells with the function of slowing down skin ageing and protecting skin coloring and clustering. Studies have proved that bFGF is an angiogenesis factor, with the function of promoting wound healing, tissue repair, and certain cell regeneration (Martin, 1997). Human bFGF is found widely in human tissues, its function as neurotrophic factor and cytokinin in clinical therapy has been proved pivotal, and it also shows a new therapeutic way of many incurable diseases such as Alzheimer's disease, cerebral ischemia, nerve injury, nerve deafness, degeneration of optic nerve etc (Zhao *et al*., 2011).

In the present study, we aimed to establish a plant-based therapeutic platform providing significance over mammalian/microbes systems (Kwong *et al*., 2013) as well as other seasonal crops such as soybean, rice and tobacco overcoming major limitations of a large scale pharmaceuticals production (Ding *et al*., 2006; Yang *et al*., 2003; Kim *et al*., 2008). Human bFGF has been successfully expressed in the aforesaid plant system at a preliminary level by nuclear transformation achieving a moderated amount of yield. Despite previously reported system we employed alfalfa bioreactor system fulfilling the need and rationale for plant platform/bioreactor for production of therapeutic proteins in a more realistic way. This unique system overcomes a number of obstacles over others such as high cost, field harvesting, seasonal variation, and other several plant-derived components require in large bioreactor system (Hellwig *et al*., 2004; (Faraz *et al*., 2019). Alfalfa genome provides great potential of the backlog system enabling high-batch productivity which can sufficiently satisfy the need of a promising and efficient biofactory.

In order to meet the increasing demand of therapeutic protein we expressed a plant expression vector p1390R containing the codon-optimized human bFGF gene in transgenic alfalfa host system by using *A. tumefaciens* and more stable and effective leaf-disc transformation method. The high frequency of a specific protein expression with in a selected host system depends on the usage of individual or a set of identical codons utilization. The most important factor in selection of codon usage is attributed to Darwinian phenomenon of selection between species both from prokaryotes and eukaryotes (Barozai & Din, 2014). The phenomenon of protein expression and accumulation in transgenic plants is also believed to be controlled and regulated at post translational level in order to accumulate a stable protein (Daniell *et al*., 2009). The expression of transgene both at transcriptional and translational level in transgenic alfalfa was efficiently achieved. Western blot analysis and disentometric analysis of the purified protein extract reveals the significance of our research in a broader term. Based on our hypothesis, the transgenic plants obtained in this study might be useful for bFGF production on a large scale, and provide numerous checkpoints to meet the requirement of a potential plant based host bioreactor system. Future attempts may employ significantly to express candidate proteins to enhance the stability, solubility and consumption of plant host cells. Moreover, these plants could be shattered into powder and fluid extract to cure trauma and empyrosis. In addition, it could also be made into cosmetics and facial products to satisfy the requirement of facial market.

Conclusion

A codon optimized human bFGF was successfully isolated and expressed in alfalfa using plant bioreactor system. Surprisingly this system efficiently and accurately supersedes previously existing systems. The unique system of alfalfa provides adequate potential in overcoming major pitfalls related to high batch productivity of human therapeutic proteins. We confirmed the establishment of an alternative method of molecular farming for recombinant proteins directed by a controlled expression system resulting in a 17kDa heterodimericbFGF protein in transgenic alfalfa.

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