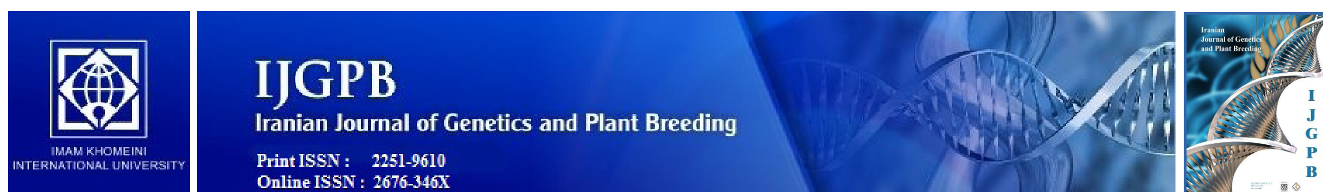

Agrobacterium-mediated transient expression of basic fibroblast growth factor (bFGF) in fenugreek (*Trigonella foenum-graecum* L.)

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ABSTRACT

The basic fibroblast growth factor (bFGF) has the potential applications as a therapeutic agent for tissue repair, skin wound healing, and recovery of the neurodegenerative diseases. In this study, after the optimization of all steps in transient transformation by *Agrobacterium,* the bFGF gene expression was examined in fenugreek leaves infiltrated with different *Agrobacterium* strains under optimal conditions. These include the AS concentration of 200 μM, bacterial density (OD) of 1.0 OD_{600}, and co-cultivation time for 10 days. The transient expression of the insert was verified in the infiltrated leaves by qRT-PCR analysis. The production of the recombinant bFGF protein was also rectified by Western blot analysis. In addition, the production titers of the recombinant bFGF protein were measured by enzyme-linked immunosorbent assay (ELISA) in all infiltrated leaf samples. Although, there were no significant differences in relative levels of bFGF transcript among the different treatments, but the accumulation levels of recombinant bFGF were significantly affected by *Agrobacterium* strains. It is possibly due to the post-transcriptional gene silencing. The highest accumulation level of recombinant protein was obtained in leaf samples infiltrated by the EHA105 strain, estimated to be about 3.85 ng bFGF g^{-1} FW.

Key words: *Agrobacterium*, Fibroblast growth factor, GUS activity, Histochemical assay, Transient expression.

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INTRODUCTION

Fenugreek (*Trigonella foenum-graecum* L.) belonging to the family Fabacecae is an annual dicotyledonous crop with trifoliate leaves and vegetated as a seeded legume. It is widely cultivated in South Africa, Europe and Asian continents. This plant is known as a medicinal plant and used for vegetable and pharmaceutical purposes (Sundaram and Purwar .(2011 Fenugreek is potentially used as antidiabetic (Puri 2002), antipyretic, and hypocholesterolemia (Bhatti 1996), and harbors some therapeutic properties including wound-healing, bust improvement, and aphrodisiac treatment (Tiran 2003). In addition, due to having several essential chemical substances with medicinal value, fenugreek is classified as a health food product or the most universally recognized nutraceutical (Acharya *et al.,* 2006). The fenugreek germplasm indicates the highest level of genetic diversity among other legumes for both qualitative and quantitative traits (Qadir *et al.,* 2017). Its size of genome is almost 685 Mbp with a c-value of about 0.7 (Young *et al.,* 2003), and its transcriptome analysis has been the attractive subject for many researchers (Vaidya *et al.,* 2013).

Fibroblast growth factors (FGF) are a large group of heparin-binding polypeptides comprising 23 members, with potential therapeutic uses (Yamashita *et al.,* 2000). Among the members of the FGFs, basic fibroblast growth factor (bFGF) is the most abundant prototype in the central nervous system (Zechel *et al.,* 2010). Gospodarowicz *et al.* (1978) was the first to purify the bFGF from the bovine brain and it was biochemically characterized in 1984 (Bohlen *et al.,* 1984). This prototype shows pleiotropic effects on different types of cells and organs including proliferation, differentiation, development, migration, phenotypic modulation, and survival (Zou *et al.,* 2008). The bFGF promotes hematopoiesis (Bikfalvi *et al.,* 1997) epithelial cells growth and smooth muscle cells in Mammalia (Zou *et al.,* 2008) and play a critical role in the activity of various organs such as lung, skin, muscle, skeleton, and nervous system (Bikfalvi *et al.,* 1997). In addition, there has been a report on the beneficial effect of bFGF on bone healing in a mouse calvarial defect model and also on its stimulating effect in proliferation and osteogenic differentiation of MC3T3-E1 cells *in vitro* (Poude *et al.,* 2019). Because of its potential effects, the bFGF could be applied as an efficacious therapeutic agent for tissue repair (Imaizumi *et al.,* 2019), skin wound healing (Zhan *et al.,* 2020), and amelioration of the neurodegenerative diseases (Chen *et al.,* 2019).

Various expression systems have been used to produce bFGF including *Bombyx mori* larva (Wu *et al.,* 2001), *Escherichia coli* (Song *et al.,* 2013), and *Bacillus subtilis* (Kwong *et al.,* 2013). However, these systems possess several difficulties including incompatible glycosylation patterns, high production cost, and low yield levels. One of the promising expression systems to produce different recombinant proteins are plants with distinct advantages including cost-effective, scalability, and not being contaminated with animal pathogens (Xu *et al.,* 2012). Plants have been extensively utilized to express a wide range of therapeutic proteins, e.g. human interferon-γ (Heidari-Japelaghi *et al.,* 2019, 2020), human serum albumin (Sedaghati *et al.,* 2020), and human fibroblast growth factor (Yi *et al.,* 2015). Ding *et al.* (2006) generated transgenic soybeans expressing recombinant human bFGF under control of *G1* gene promoter. In the seeds of the transgenic soybean plants they obtained the highest titer of bFGF accumulation as about 2.3% of total soluble protein (TSP). Identical to the native bFGF, the soybean-derived bFGF promoted proliferation of Balb/c 3T3 cells, being biologically active. In rice (*Oryza sativa* L.), recombinant bFGF was produced with an expression level of up to $1.86×10^2$ μg/g in seeds. The recombinant protein was obtained by a simple purification method with a final recovery of 4.49% led to a yield of up to 8.33 μg/g. The bioassay demonstrated that the recombinant bFGF stimulated proliferation of NIH/3T3 cells *in vitro* similar to commercialized bFGF (An *et al.,* 2013). In addition, Wang *et al.* (2016) introduced a codon-optimized bFGF gene into tobacco (*Nicotiana tabacum*) chloroplasts by the biolistic method. The recombinant bFGF protein was successfully expressed in tobacco leaves with yield level about 0.1% of TSP in transplastomic tobacco plants.

This is the first study on the transient expression of a recombinant protein in *T. foenum-graecun* by agroinfiltration. In this research, we first analyzed several affecting factors on transient expression efficiency and optimized the transient transformation conditions in fenugreek by measuring GUS activity (Sandhya *et al*., 2022). Subsequently, the production titer of bFGF was evaluated in the transformed fenugreek treatments by three *Agrobacterium tumefaciens* strains. Finally, we presented that fenugreek is a suitable plant host for the production of recombinant bFGF protein.

MATERIALS AND METHODS

Plant materials and growth conditions

Fenugreek seeds of (*T. foenum-graecun* L.) were purchased from Pakan-Bazr Company, Isfahan, Iran,

and planted in plastic pots containing peat moss and sand (1:1). The pots were transferred into a controlled growth chamber under environmental conditions including 25±2 °C, 70% relative humidity, with 16/8 h light and dark photoperiod, respectively, and under 25 μmol m^{-2} s^{-1} irradiance provided by cool fluorescent lamps. After one week, the seedlings were incubated at 22±2 °C, 60% relative humidity, a photoperiod of 16 h with 75 μmol m^{-2} s^{-1} light intensity (Heidari-Japelaghi *et al.,* 2020). The leaves from six- or eight-week-old plantlets were used for all investigations.

Agrobacterium strains and binary vector

Three *A. tumefaciens* strains used for agroinfiltration included GV3101, LBA4404 and EHA105. In addition, a binary vector, pCAMBIA3301, carrying the human bFGF gene (pCAMBIA-bFGF, kindly donated by Dr. Bastami from Imam Khomeini International University, Qazvin, Iran) under the control of cauliflower mosaic virus (CaMV35S) promoter and nopaline synthase (*nos*) terminator was used for transformation. The recombinant vector was transferred into each *Agrobacterium* strain separately and the integration of the recombinant plasmid was verified by PCR using specific primers and double digestion with *Bam*H I and *Sac* I (Fermentas, Germany).

Bacterial culture preparation for infiltration

The *Agrobacterium* strains were streaked on the YEP medium solidified with agar (5 gL^{-1} NaCl, 5 gL^{-1} Yeast Extract and 10 gL^{-1} Peptone) including 25 mgL^{-1} Rifampicin and 50 mgL^{-1} Kanamycin. A single colony of *A. tumefaciens* strains was separately inoculated into 15 mL LBman medium (2.5 gL^{-1} NaCl, 5 gL^{-1} Yeast Extract, 10 gL^{-1} Peptone, and 10 gL^{-1} Mannitol) composing the above mentioned antibiotics and shaken on an orbital shaker (150 rpm) for 48 h at 28 °C. About 500 μl of each bacterial suspension was individually inoculated into the 50 mL LBman medium containing 20 μM acetosyringone (AS), 10 mM 2-(N-morpholino)-ethanesulfonic acid (MES) the same antibiotics, and kept overnight at 28 °C. Then, the bacterial cells were precipitated by centrifugation at 4000 rpm for 15 min and resuspended in the liquid MMAi medium (½ MS, 10 mM MES, 2% Sucrose, 50–300 μM AS, , pH 5.6) to reach a final optical density (OD) of 0.4–1.2 at 600 nm (Ma *et al.,* 2012). Finally, the bacterial suspensions were shaken on an orbital shaker (150 rpm) at 28 °C for 60 min before use.

Plant agroinfiltration

Fenugreek plants were kept at 20 °C under low light one day prior to infiltration. In the next day, infiltration with *Agrobacterium* strains was carried out on the leaves obtained from of 6- or 8-week-old plants. In order to carry out the agroinfiltration, the underside of the leaf was scratched by a syringe needle. Then, the injection of the bacterial suspension was made into the entire leaf area via a 1 mL needleless syringe (Ma *et al.,* 2012). Finally, the infiltrated plants were transferred in a controlled growth room under conditions including 25±2 °C with 75 μmol m^{-2} s^{-1} light provided by cool fluorescent lamps, a photoperiod of 16/8 h light/dark and 60% relative humidity for 2–10 days.

Optimization of transient transformation efficiency

To optimize the transient transformation efficiency in fenugreek, several factors affecting were evaluated such as AS concentration (50, 100, 150, 200, and 300 μM), the bacterial strain, (in the liquid MMAi infiltration medium, OD_{600}=1.0), bacterial density (0.4, 0.6, 0.8, 1.0, and 1.2) at 600 nm (in the liquid MMAi medium supplemented with 200 μM AS) and co-cultivation time of 2, 4, 6, 8, and 10 days post agroinfiltration (dpa). The leaves with various treatments were collected at 10 dpa, frozen in liquid nitrogen, and kept at −80 °C until use. All parameters were analyzed and optimized according to the relative GUS activity. After all steps of transient transformation by Agrobacterium were optimized, the bFGF gene expression was examined in fenugreek leaves infiltrated with different Agrobacterium strains under optimal conditions including the, AS concentration of 200 μM, bacterial density (OD) of 1.0 OD600, and co-cultivation time for 10 days.

Total RNA isolation, RT-PCR, and qRT-PCR

The infiltrated leaves were ground to a fine powder, in liquid nitrogen, using a pestle and mortar, then, total RNA was isolated by a cetyltrimethylammonium bromide (CTAB) method (Heidari-Japelaghi *et al.,* 2011). For the synthesis of the first strand cDNA, 3 μg of DNase I treated total RNA (Fermentas, Germany) was used as the template, RevertAid™ M-MuLV Reverse Transcriptase (200 U/μl, Fermentas, Germany) and Oligo (dT)18 primer (1 μg/μl, Fermentas, Germany) were incubated for 60 min at 42 °C. Specific primers (forward primer: 5′-ATGGCTGCTGGGTCAATCAC-3′; reverse primer: 5′-TCACGACTTCGCCGACATAG-3′) were employed for the reverse transcription-PCR (RT-PCR) in a PCR apparatus (Techene, UK) set up for 35 cycles. The RT-PCR conditions were set up for each cycle as denaturation at 94 °C for 30 s, annealing at 58 °C for 60 s, extension at 72 °C for 30 s and a final extension (72 °C/5 min). The quantitative real time-PCR (qRT-PCR) was also carried out with the specific primers (forward primer: 5′-CCCACACATCAAGCTCCAGT-3′; reverse primer: 5′-GGTAACGCACTTAGACGCCA-3′)

by a qRT-PCR apparatus (Bio Molecular Systems, Australia). All reactions were performed in 12 µL of the reaction mixture. For the qRT-PCR amplification 40 cycles were run as 20 s at 95 °C, 20 s at 56 °C, and 20 s at 72 °C. Two negative controls included, the cDNA from the leaves transformed by native *A. tumefaciens* with no construct and the cDNA from the leaf samples infiltrated with *A. tumefaciens* containing pCAMBIA3301 binary vector. Relative levels of bFGF transcript in the infiltrated leaf samples were specified using the reference genes of β-actin and α-tubulin as demonstrated by Heidari-Japelaghi *et al.* (2019).

Protein extraction and analysis

Five hundred mg of the infiltrated leaf samples harboring the recombinant bFGF protein were pulverized into a fine powder in liquid nitrogen by a pestle and mortar and added into a micro tube containing 1 mL of extraction buffer including 1 mM EDTA, 2% polyvinyl pyrrolidone (PVP), 50 mM sodium phosphate pH 8.0 and 150 mM NaCl (Reuter *et al.,* 2014). Proteins were separated from the cell debris by centrifugation of the homogenates twice at 14,000 rpm for 30 min at 4 °C. The total soluble proteins (TSP) concentrations were measured using bovine serum albumin (BSA) (Sigma–Aldrich, Germany) as the standard (Bradford 1976). Proteins were assessed by SDS-PAGE analysis on a 12.5% polyacrylamide gel. Then, the gel was stained using Coomassie brilliant blue R-250 (Applichem, Germany).

Western blotting

To perform western blotting, about 20 µg TSP were first separated on a 12.5% polyacrylamide gel. Then, proteins were transferred onto a nitrocellulose membrane by a semi-dry transfer blotting apparatus (Bio-Rad, USA) for 1 h. Subsequently the membrane was blocked by immersing in TBST buffer [l50 mM NaCl, 20 mM Tris-HCl, 0.05% (v/v) Tween 20, pH 7.5] containing 5% non-fat skimmed milk, for 1 h. Afterwards, the membrane was incubated in the blocking buffer containing diluted (1:2000) mouse antibody raised against -polyHistidine-horseradish peroxidase (Sigma, USA) for 2 h. The membrane was then washed three times in TBST buffer and substrate solution [20 mM Tris-HCl, 500 mM NaCl, 5.0% (v/v) H_2O_2, pH 7.5] containing 0.05% 3, 3′-diaminobenzidine (DAB) and used for peroxidase staining. After the color development, the membrane was photographed using a Canon PC1057 camera.

Enzyme-linked immunosorbent assay (ELISA)

A Hu FGF basic ELISA Kit (Life Technologies, USA) was used according to the manufacturer's instructions to estimate the recombinant bFGF protein concentration in the infiltrated leaf samples. Briefly, the absorbance of all 96 wells of a microtiter plate reader (BioTek, USA) was first measured at 450 nm. Then, bFGF concentration in samples was calculated using a standard curve created with a 2-fold serial dilution of the bFGF standard (1000 $pgmL^{-1}$) on the same microtiter plate reader. Relative titers of the recombinant protein were determined by normalization of the fluorescence signal values based on the fluorescence signal emitted from the same amount of TSPs in the control and elimination of the fluorescence signal in the background.

GUS histochemical and GUS activity assay

For GUS histochemical test the shuttle vector pCAMBIA3301 harboring the β-glucuronidase (*uid*A) gene under the control of CaMV35S promoter, *nos* terminator and neomycin phosphotransferase (*npt*II) gene conferring resistance to kanamycin was used (Jefferson, 1987).The infiltrated leaves were incubated in the staining buffer [20 mM EDTA (pH 8.0), 50 mM $NaPO_4$ (pH 7.2), 0.1% Triton X-100, 500 mgL^{-1} of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) and 20% methanol] for 72 h at 37 °C. To remove chlorophyll, the leaf samples were treated in 70% ethanol for 48 h at 37 °C. The relative GUS activity was estimated with 4-methylumbelliferyl-β-D-glucuronide (MUG) as the fluorescent substrate by a microplate reader (BioTek, USA) (excitation at 356 nm, emission at 455 nm) (Wu *et al.,* 2014).

Statistical analysis

The qRT-PCR, ELISA, and GUS activity reactions were accomplished with three biological replicates, using three leaves for each experiment. The analysis of variance (ANOVA), of the data was carried out by the statistical software SPSS ver. 16 (SPSS Inc., Chicago, IL, USA). Mean comparisons were made by the Duncan's test at the $P<0.05$ confidence level. , and the data were shown as mean±standard deviation (SD).

RESULTS AND DISCUSSION

Transient expression of uidA in fenugreek leaves

Reporter genes are widely used to verify primarily the successful *Agrobacterium*-mediated transformation. Especially, intron-containing reporter genes are considered as creditable indicators in transgenic plants analysis, because the gene expression is prevented in *Agrobacterium* cells, whereas it can appropriately be expressed in plant cells (Vancanneyt *et al.,* 1990). Accordingly, different intron-containing reporter

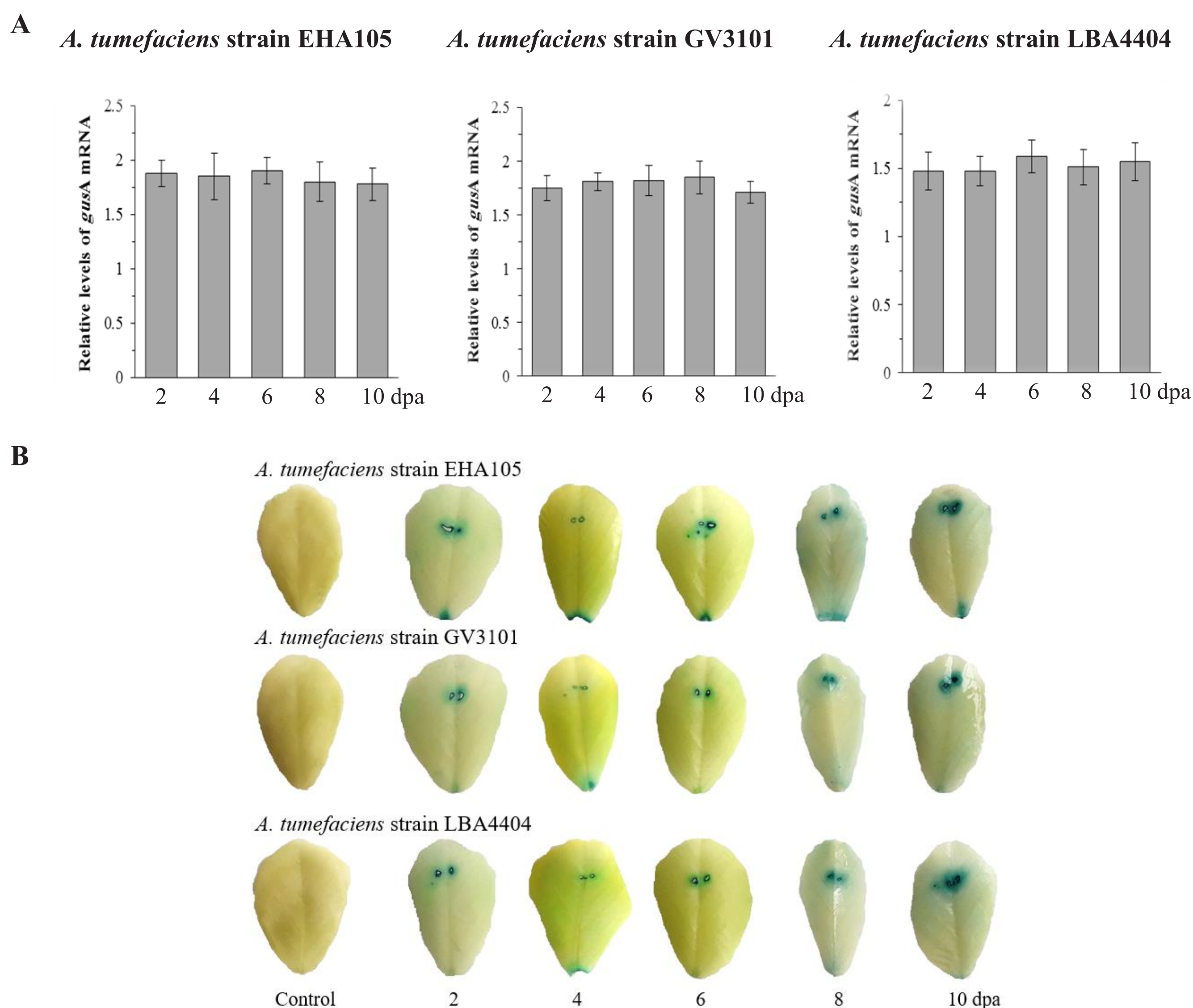

Figure 1. Analysis of transient *uid*A gene expression in fenugreek leaves infiltrated by different *Agrobacterium* strains harboring pCAMBIA3301 binary vector at 2, 4, 6, 8, and 10 dpa. **A:** Confirmation of *uid*A transcript expression in infiltrated leaves by qRT-PCR. The data normalization was carried out by comparing their expression with those of the reference genes. The values present mean±SD of three biological replicates, each replicate coming from three leaf samples. **B:** The transformed leaves stained by GUS assay.

genes have been applied to develop stable or transient transformation protocol of many plants including *gus*A and *uid*A (Heidari-Japelaghi *et al.,* 2020).

The *uid*A reporter gene was utilized to perform GUS histochemical assay in infiltrated leaf samples and its transient expression was analyzed by qRT-PCR and the GUS staining (Figure 1). The presence of *uid*A mRNA was demonstrated in leaf samples by qRT-PCR via the measurement of transcript expression levels (Figure 1A). In the *Agrobacterium* strains, there was not any significant difference between mRNA levels at different times of co-cultivation (2, 4, 6, 8, or 10 dpa). The histochemical GUS assay was also accomplished in fenugreek leaves infiltrated with different *Agrobacterium* strains including LBA4404, EHA105 and GV3101. The shuttle vector pCAMBIA3301 carrying the *uid*A-intron was transferred into the *Agrobacterium* strains to transform fenugreek leaves, the transient expression of *uid*A gene was shown to have occurred at different co-cultivation times (Figure 1B). All leaf samples were successfully infected and no GUS activity was observed in the samples infiltrated with wild type *Agrobacterium* used as a negative control, showing that *uid*A gene expression occurred only inside the transformed plant cells.

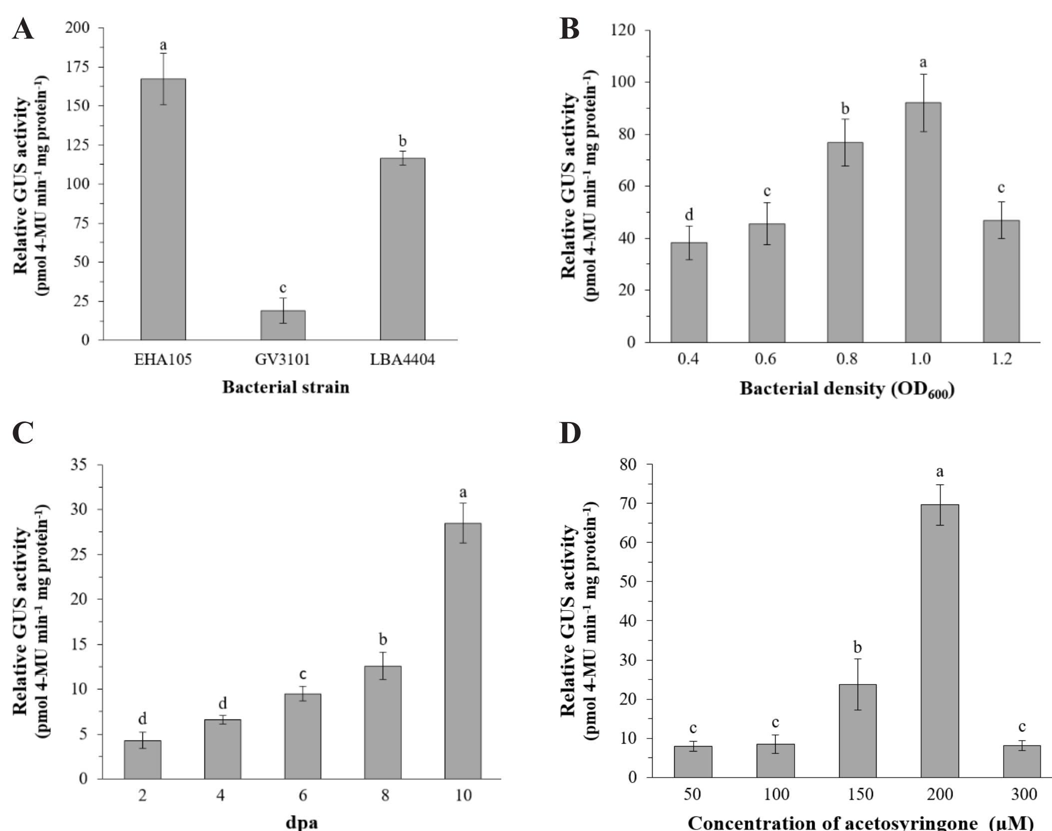

Figure 2. Investigation on the parameters influencing the efficiency of transient transformation by estimation of relative GUS activity; **A:** the bacterial strain, **B:** the bacterial density (OD_{600}), **C:** the co-cultivation time (dpa), and **D:** the concentration of AS. Data show mean±SD of three biological replicates, each replicate coming from three leaf samples. Means represented by different letters differ significantly, revealed by Duncan's test (*P*<0.05).

Transient expression Optimization

The transient expression conditions in fenugreek leaves was optimized through analyzing parameters influencing the transient transformation efficiency including the bacterial density, bacterial strain, , the co-cultivation time , and the AS concentration by estimation of relative GUS activity (Figure 2). The results on the ability of the different *Agrobacterium* strains to infect fenugreek leaves demonstrated that the bacterial strains remarkably varied on the level of *uid*A expression. The EHA105 strain revealed the highest transient expression efficiency with 167.30 pmol 4-MU min^{-1} mg $protein^{-1}$, LBA4404 with 116.60 pmol 4-MU min^{-1} mg $protein^{-1}$ and GV3101 with 19.00 pmol 4-MU min^{-1} mg $protein^{-1}$ strains (Figure 2A). It is shown that different *Agrobacterium* strains possess various bacterial-encoded T-DNA transfer mechanisms and show diverse abilities to attach to plant cell walls. As a result, some strains show more aggressive behavior than others, according to the target plant species (Nam *et al.,* 1997). Thus, genetic makeup of the bacterial strains may significantly affect the transient transformation efficiency. The EHA105 strain was reported to produce good GUS activity levels and to indicate a gentle wound response. In upland cotton (*Gossypium hirsutum*), it was used to optimize a transient transformation system both to gain significant expression levels and to decrease wound-induced genes expression (Li *et al.,* 2018). Use of the highly virulent *Agrobacterium*-EHA105 strain has been also suggested for the transient transformation in tobacco, *Arabidopsis*, and flowering Chinese cabbage (*Brassica campestris*) (Zhong *et al.,* 2016).

To determine the suitable concentration of *Agrobacterium* for *uid*A expression, we used varying concentrations of EHA105 strain (OD_{600} values of 0.4, 0.6, 0.8, 1.0, and 1.2). The highest level of relative GUS activity was observed when the *Agrobacterium* concentration reached an OD_{600} value of 1.0 and was calculated to be about 92.00 pmol 4-MU min^{-1} mg $protein^{-1}$. The level of *uid*A expression was also reduced at lower OD_{600}=0.4 and higher OD_{600}=1.2 *Agrobacterium* cell densities (Figure 2B). It was reported that higher *Agrobacterium* concentrations

lead to cell death and its low concentrations lead to few or no cells for transient gene expression (Li *et al.,* 2018). These results represent that bacterial density is critical in plant transformation and determination of the optimal *Agrobacterium* concentration results in maximum transient transformation efficiency (Dai *et al.,* 2003). Similar to our results, a good transient transformation efficiency was reported to occur with OD_{600} 0.8–1.0 in upland cotton (Li *et al.,* 2018). Furthermore, in the *Tamarix hispida* the transient root-absorption system utilized by Ji *et al.* (2014), the highest transient transformation efficiency was obtained using an OD_{600} value of 0.9, while in tobacco (*N. benthamiana*) root-absorption transient system, an OD_{600}=1.23 was applied in all experiments (Yang *et al.,* 2008). In contrast, Zhong *et al.* (2016) suggested that infection with a bacterial concentration of OD_{600}=0.3 conferred the highest efficiency in transient transformation of Chinese cabbage.

Different co-cultivation times (2, 4, 6, 8 and 10 dpa) were assessed on the transient *uid*A expression levels. The results demonstrated that co-cultivation time significantly affects the relative GUS activity. The *uid*A expression increased continuously after co-cultivation for 10 days and peaked at 10 days with the value of 28.50 pmol 4-MU min^{-1} mg $protein^{-1}$ (Figure 2C). The level of *uid*A expression at 10 days was 6 times higher than that of the second day, demonstrating that co-cultivation time is a crucial parameter in transient transformation efficiency. Depending on the host plant genotype, transient expression usually lasts for 2–4 dpa before the number of expressing cells decrease and the expression level per infected cell is reduced (Lacroix and Citovsky, 2013). However, the application of the selection agent (kanamycin) confirmed the stable expression of GUS gene through the integration of T-DNA into the genome of infected leaves in Petunia, leading to an enhancement in expression levels at 10–14 dpa (Janssen and Gardner, 1990).

AS is known to enhance the transient transformation efficiency by inducing the *vir* operon of *A. tumefaciens* and subsequently cause the T-DNA to be delivered (McCullen and Binns, 2006). This natural phenolic compound is produced by wounded plant cells and can expand the host range of various *Agrobacterium* strains (Huang *et al.,* 2001). We also investigated the effect of various concentrations of AS (50, 100, 150, 200, and 300 μM) on *uid*A expression in fenugreek leaves infiltrated with EHA105 strain. An increase in the concentration of AS from 50 to 200 μM caused a high increase in the transient transformation efficiency. The highest titer of transient *uid*A expression was observed at 200 μM AS with the value of 69.70 pmol 4-MU min^{-1} mg $protein^{-1}$. However, the relative GUS activity declined significantly at concentration of AS more than 200 μM indicating a negative effect on the expression level of *uid*A (Figure 2D). Similarly, the optimum AS concentration in optimization experiments was obtained about 200 μM in persimmon (*Diospyros kaki*) and tobacco (Mo *et al.,* 2015; Heidari-Japelaghi *et al.,* 2020). In upland cotton, Li *et al.* (2018) carried out the optimization of AS concentration for high transient expression of β-glucuronidase gene at three different concentrations of AS ranging from 80 to 300 μM. They reported 165 μM as an optimal concentration for AS. These results proposed that the addition of AS into the inoculation medium improves transient transformation efficiency and that a proper concentration of AS must be opted.

Transient expression of bFGF transcript in fenugreek leaves

After the optimization of *Agrobacterium*-mediated transient transformation, expression of bFGF gene was examined in fenugreek leaves infiltrated with different *Agrobacterium* strains (OD_{600}=1.0) under optimal conditions (AS=200 μM at 10 dpa). The transient expression of bFGF transcript was verified first by RT-PCR (Figure 3). Using RT-PCR, a single clear amplified band with the expected size of 468 bp was observed in all leaf samples transformed with all *Agrobacterium* strains carrying the binary vector, pCAMBIA-bFGF (Figure 3A). In the negative controls, leaf samples transformed with the wild type *A. tumefaciens* or with the *A. tumefaciens* harboring the binary vector pCAMBIA3301, no PCR products were observed.

The quantification of transcript levels of bFGF gene was carried out using qRT-PCR analysis. To specify relative titers of bFGF transcript in different treatments, the β-actin and α-tubulin genes were employed as housekeeping controls (Heidari-Japelaghi *et al.,* 2019). The qRT-PCR analysis corroborated the expression of the bFGF transcript in all leaf samples infiltrated with various *Agrobacterium* strains harboring the binary vector pCAMBIA-bFGF (Figure 3B). However, no bFGF transcripts were detected in the negative controls (data not shown). In addition, no significant differences were detected between different treatments. Our results are in agreement with those of Heidari-Japelaghi *et al.* (2020) who infiltrated tobacco leaves with various *Agrobacterium* strains carrying hIFN-γ gene in different expression constructs and found no significant differences in treatments.

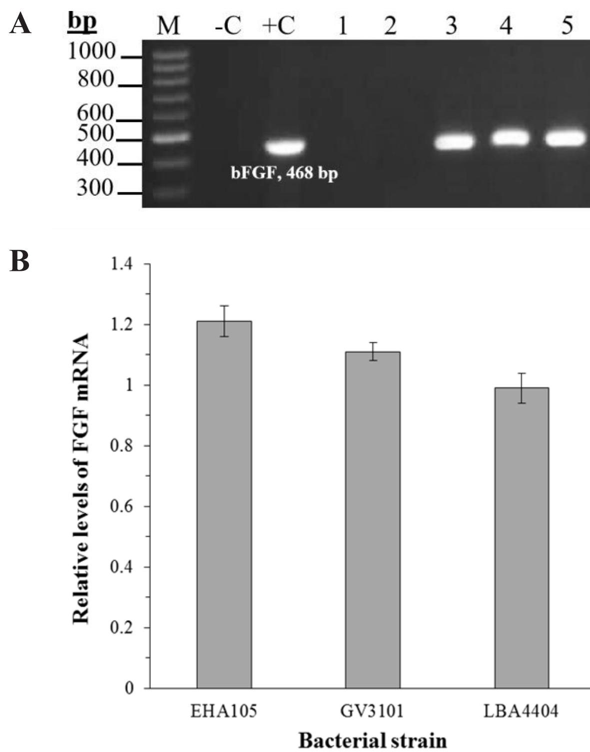

Figure 3. Reverse transcriptase and quantitative real time PCR detection of bFGF transcripts. **A:** The RT-PCR amplification. M, 100 bp DNA ladder; –C, negative control without any template; +C, positive control of PCR reaction containing the recombinant pCAMBIA-bFGF binary vector as template; 1, Result obtained by wild type *A. tumefaciens*; 2, *A. tumefaciens* containing pCAMBIA3301; 3, EHA105 strain harboring pCAMBIA-bFGF; 4, GV3101 strain containing pCAMBIA-bFGF; 5, and result obtained by LBA4404 strain carrying pCAMBIA-bFGF. **B:** The qRT-PCR analysis of bFGF gene at 10 dpa. Data are mean±SD from three biological replicates, each replicate coming from three leaf samples in 3 different bacterial strains.

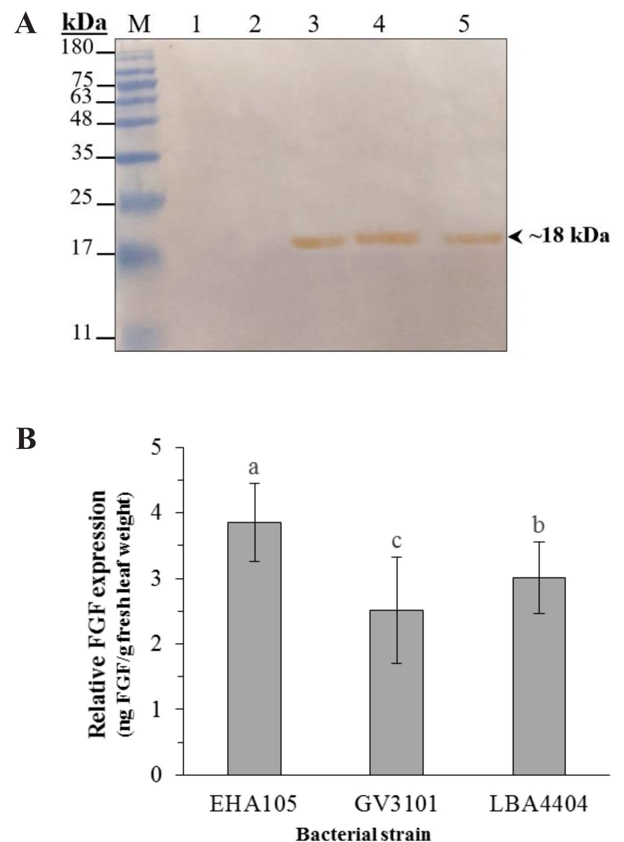

Figure 4. Detection of recombinant bFGF protein in leaf samples infiltrated by *Agrobacterium* strains; **A:** Western blot, and **B:** ELISA assay. M, molecular weight marker; 1, wild type *A. tumefaciens*; 2, *A. tumefaciens* containing pCAMBIA33011; 3, EHA105 strain harboring pCAMBIA-bFGF; 4, GV3101 strain containing pCAMBIA-bFGF; 5, LBA4404 strain carrying pCAMBIA-bFGF. 25 µg total protein/lane were loaded for SDS-PAGE. Data are mean±SD from three biological replicates, each replicate coming from three leaf samples. Means represented by different letters differ significantly, revealed by Duncan's test (P<0.05).

Transient expression of recombinant bFGF

The TSPs isolated from the infiltrated fenugreek leaves were assayed 10 dpa by SDS-PAGE, Western blotting, and ELISA (Figure 4). The leaves sampled from the plants infiltrated with wild type *A. tumefaciens* and with the *A. tumefaciens* carrying the binary vector pCAMBIA3301 were applied as negative controls. Contrary to the negative controls, the expression of recombinant bFGF protein was detected by Western blotting in all treatments. The Western blot analysis demonstrated a polypeptide band with the expected length of ~18 kDa in accordance with the recombinant bFGF protein in all infiltrated leaf samples (Figure 4A). In human, bFGF harbors five isoforms with lengths of 18, 22, 22.5, 24 and 34 kDa. The 18 kDa isoform is known as the prototype of bFGF. It is able to adjust cell migration and proliferation in a paracrine or autocrine mode (Delrieu, 2000). The ELISA assay affirmed the production of the recombinant protein in all infiltrated samples (Figure 4B).The effect of agrobacterium strains on the amount of recombinant protein accumulation maybe due to the post-transcriptional gene silencing mechanism. It has been proposed that this mechanism plays a crucial role on the decrease of the transient expression period (Voinnet *et al.,* 2003). However, the use of a viral-silencing suppressor with transgene may ameliorate the transient expression level (Shamloul *et al.,* 2014). The highest accumulation level of recombinant protein was obtained in leaf samples infiltrated by the EHA105 strain, estimated to be about 3.85 ng bFGF g^{-1} FW.

CONCLUSION

We optimized the *Agrobacterium*-mediated transient

transformation in *T. foenum-graecun* and successfully expressed the recombinant bFGF protein in fenugreek leaves by agroinfiltration. The highest accumulation level of recombinant protein was obtained in leaf samples infiltrated by the EHA105 strain, estimated to be about 3.85 ng bFGF g^{-1} FW. Overall, our results revealed that fenugreek could be a valuable host plant for the production of bFGF and possibly other types of therapeutic proteins especially oral vaccines due to its short growing cycle, lower production costs, direct oral consumption, and stable yield.

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