Open Access
Baghdad Science Journal **Vol.16(1)2019**

DOI: http://dx.doi.org/10.21123/bsj.2019.16.1.0018

Expression of Recombinant Human Glucocerebrosidase Protein in Sunflowers

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Received 25/7/2018, Accepted 16/1/2019, Published 11/3/2019

Abstract:

Molecular farming has become one of the most significant implementations of modern biotechnology to generate modified plant crops to produce medicinal proteins. *Agrobacterium* is one plant genetic engineering tool that integrates genes of interest inside a host plant. In recent years, the need to produce recombinant proteins as therapeutics has growing rapidly, and human glucocerebrosidase is one of the proteins that is need to treat disease. In this study, specific primers were designed to amplify *Hu-GBA1 gene* from constructed pGEM-GBA plasmid which was cloned into the plant expression vector pCAMBIA1304. The generated recombinant pCAMBIA1304-GBA plasmid was used to transform *A. tumefaciens LBA4404* and applied for transformation of sunflower cotyledon explants. Colony PCR technique was used to confirm the presence of *Hu-GBA1 gene* in transformed *A. tumefaciens*. *Agrobacterium* containing pCAMBIA1304-GBA was suspended in Infection Medium (IM) supplement with 200 mM acetosyringone. A bacterial suspension was used to transform sunflower cotyledons. After infection, cotyledons were co-cultivated in Co-cultivation medium (CCM), supplied with 200 mM acetosyringone without antibiotics. The cotyledons were then transferred to selection media containing 7.5 mg/L Hygromycin and 250 mg/L Cefotaxime and grown for additional 14 days at 25℃ in photoperiod of 16h L/8h D. The transformed sunflower cotyledons were successfully generated complete plant with used 6-Benzylaminopurine and Naphthalene acetic acid as growth hormones. The presence of the *Hu-GBA1 gene* in the genomic DNA of transgenic sunflower plant was proven by PCR as a band of 1561bp size. The GBA mRNA expression in modified sunflowers was detected by qRT-PCR compared with control GBA mRNA. Enzyme Linked Immunoassay was done on crude recombinant protein that extracted from transformed sunflower using Human Glucosylceramide ELISA Kit, the Elisa test results confirmed the production of recombinant glucocerebrosidase and the concentration of crude recombinant enzyme extracted from transformed sunflower with *GBA1 gene* was 0.45 ng/µl

Key words: Agrobacterium, Gaucher disease, Glucocerebrosidase, Sunflower, pCAMBIA1304.

Introduction:

Recently, genetic engineering has employed plants as new factories to produce biopharmaceuticals. The initial pharmaceutical protein that was expressed in transformed tobacco and sunflower in 1986 is human growth hormone (1). Since then, genetically modified plants were used for expressing vaccines, industrial enzymes, antibodies, nutraceuticals, and other pharmaceutical proteins (2, 3, 4, 5). Both prokaryotic and eukaryotic systems have been used to express recombinant proteins (6) Prokaryotic systems in comparison with mammalian systems are appropriate and inexpensive in terms of equipment requirement.

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However, posttranslational modifications are required in several mammalian proteins for their biological activity such as protein glycosylation, which cannot be achieved by prokaryotic product systems. Moreover, in mammalian cells protein production is very highly in continuous cell cultures. In contrast, protein synthesis and modification pathways in plants have highly similarity to those in animal cells (7), so plants are considered as a promising system to produce human pharmaceutically products on a wide range, and at a lower cost (8, 9). In 2012, first recombinant derivative enzyme, manufactured in carrot cells (Taliglucerase alfa), was confirmed by the FDA for human use (10) is an alternative therapy for treating patients with gaucher disease. Gaucher disease is a group of inherited lysosomal storage disease; (LSD) produced from failure in one of lysosomal enzymes result in accumulation of their substrates which

blocks lysosomal process (11). In patients with gaucher disease, the active form of acid-beta glucocerebrosidase (GBA) is absent, leading to accretion of its substrate glucosylceramide (also called glucocerebroside). In addition, any concerning compounds that are generally decomposed to glucose and lipid materials by glucocerebrosidase inside the lysosomes of cells in patients with GD. Producing effective enzyme replacement therapy (ERT) for patients with gaucher disease (GD), was carried in the end 1980s and early 1990s provide the effectiveness of glucocerebrosidase that preparatory from human placenta as ERT to treat patients with GD1. This experiment elucidated by molecular targeting of the glucocerebrosidase to tissue macrophages through mannose receptors on cell surfaces. Improved targeting of placental glucocerebrosidase by employing the macrophage mannose receptors (MMR) led to the first successful treatment with enzyme replacement therapy in 1991(12). Placental enzyme originated from (Genzyme, Alglucerase, and Ceredase) was later substituted by a recombinant form derived from expressed human glucocerebrosidase gene in Chinas hamster ovary cells (13). The enzyme produced in Chinas hamster ovary must be modified after translation to include that the terminal residuals are recognized by the MMR. In plant cells, such post translational modification is not necessary to expose the mannose residue because 90% of the enzyme is produced with mannose terminated at the natural side chains (14).

A. tumefaciens is an intermediate transforming agent, used as an indirect and efficient method to carry recombinant DNA into genomic plant via the reaction between host plant cells and bacteria (15). This transformation is highly efficient and can be used to modify most cultivating plant species (16). It is a singular pattern system as well as a main tool in biotechnological for genetic manipulate of plant cells (17). The aim of this study is expression of human *GBA1 gene* in the sunflower plant and using *Helianthus annuus L.* as an economical source to produce recombinant glucocerebrosidase.

Materials and Methods:

Construction of pCAMBIA1304-GBA Plasmid

Bacteria Strains and Plasmids

Bacterial Strains

Escherichia coli DH5-alpha and *Agrobacterium tumefaciens* strains. *E. coli DH5α* (Invitrogen) was kindly provided by Prof. Khosraviani Kave (University of Tehran) and used to grow the recombinant binary vector, while *A. tumefaciens LBA4404* Cells (Takara Bio Inc.) was used to transform the plant.

Two vectors were used in this study.

1- **pGEM-T (pGEM-GBA)**: pGEM-GBA recombinant vector Cat. No. HG12038-G (Sino Biological Inc.) is a recombinant plasmid which contains human *GBA1 gene* cDNA size 1611bp. RefSeq: NM_000157.

2- **Plant expression vector pCAMBIA1304**: Product No. M1595 from (Marker Gene Technologies Inc.) Fig.1. This vector carries **LB** and **RB** Left and Right Borders T-DNA fragment from nopaline strain of *Agrobacterium tumefaciens* (18). The plasmid contains a **CaMV35s** promoter, Cauliflower Mosaic Virus promoter to integrate foreign genes into the plant genome **NOS** Nopaline Synthase Terminator, from nopaline synthase gene of *A. tumefaciens*.

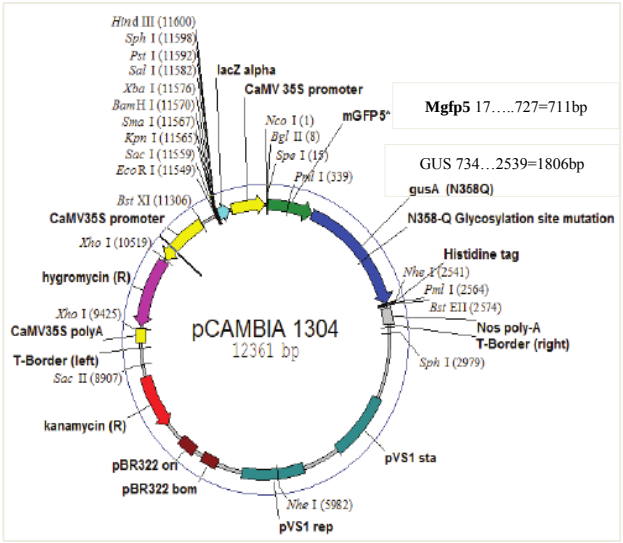


Figure 1. A map of the pCAMBIA 1304 including the right (RB) and left (LB) T-DNA border fragments from a nopaline strain of *A. tumefaciens* with ß-*glucuronidase gene*; gusA: mgfp5 fusion; P35S,CaMV 35S promoter; TEV, tobacco etch virus

Molecular and Biological Methods

Amplification of *Hu-GBA gene* from pGEM-GBA Plasmid

Polymerase chain reaction (PCR) was used to amplify cDNA coding for Hu-GCD (Human glucocerebrosidase) constructed from pGEM-GBA plasmid using the GoTaq®Green Master Mix kit (Promega, USA), with its special signal peptide using the forward primer: F.5-CT**AGATCT**CCATGGCTGGCTGGCATCACA-3 containing *BglII* restriction site and reverse primer: R.5-CC**GGTCACC**TCACTGGCGATGCCACAG-3 contain *BstEII* restriction site in bold. The sequences reference that was used in this study is

NM_000157.3 from Sino Biological Inc. to synthesize the cDNA, this created a fragment of 1561bp. Thermal cycling conditions are given in Table-1 PCR. Conditions were performed according to (19) except annealing temperature by researcher.

Table 1. PCR Conditions for *GBA1 Gene* Amplification

Steps	Temperature	Time	Number of cycle
Pre-Denaturation	94C°	5min	1
Denaturation	94C°	1min	
Annealing	58℃	45sec	30
Extension	68C°	3 min	
Final extension	68C°	7min	1

PCR product was migrated with DNA standard (100bp DNA Ladder) and was verified by electrophoresis on a 1.0 % (w/v) agarose gel (voltage 72V at 60 min) and visualized with UV light.

Sequencing of *Hu-GBA Gene*

The presence of *GBA1 gene* was confirmed in PCR product of purified and unpurified PCR amplicons, determined by sequencing in the forward and reverse direction. Primers and samples were prepared according to Eurofins Genomic GmbH protocol, Germany (support-eu@eurofins.com). The sequences were analyzed using Basic Local Alignment Search Tool "BLAST" to search for homologous sequences in the National Center for Biotechnology Information database (NCBI), http://WWW.blast.ncb.nih.gov (20).

Constructing *Hu-GBA Gene* in pCAMBIA1304 Plasmid

The purified *Hu-GBA gene* and pCAMBIA1304 cloning vector were digested with *BglII* and *BstEII* (Promega®) to insert the *Hu-GBA gene* in reporter gene region of pCAMBIA1304 downstream the CaMV35s promoter and upstream the NOS terminator by ligation process according to the protocol provided along with enzyme system. The purified digested *Hu-GBA gene* and pCAMBIA1304 vector were ligated using AccuPower® Ligation PreMix Kit (Bioneer) and the recombinant plasmid was obtained according to the molar ratio specified by standard ligation protocol: Molar ratio of vector: inserted DNA (gene) was 1:3 in the current study, the ratio was 4µl (pCAMBIA1304): 12µl (*Hu-GBA gene*) total concentration (12ng/µl). The newly constructed plasmid was named as pCAMBIA1304-GBA, Fig2.

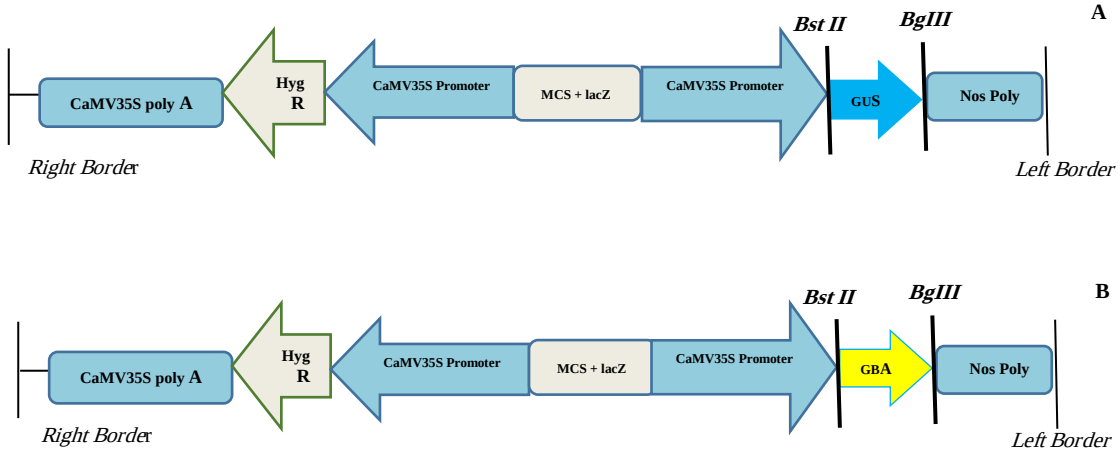


Figure 2. T-DNA region of pCAMBIA1304 LB and RB: Left and Right Borders. A. contains GUS terminator. Gene between, CaMV35s promoter and NOS terminator. B .T-DNA region of pCAMBIA1304 LB and RB: Left and Right Borders, contain *GBA1 gene*, HYG(R): Hygromycin selectable marker, CaMV35s: Cauliflower Mosaic Virus promoter, *BgIII* and *BstEII*: restriction sites, and NOS: Nopaline Synthase.

***Agrobacterium* Transformation**

A. tumefaciens LBA4404 competent cells were prepared using calcium chloride (21). The transformed constructed plasmid was isolated from transformed *E. coli DH5α* into competent *A. tumefaciens LBA4044* by heat shock transformation method with some modification (22). The transformed colonies were selected using kanamycin 50µg/ml and streptomycin 100µg/ ml plates. The colony PCR technique was used to confirm the presence of *Hu-GBA gene* in pCAMBIA1304 after insertion in *A. tumefaciens LBA4404*. The molecular and biological work was

achieved in Genetic Engineering Labs/College of Science /University of Basra/Iraq.

Plant Transformation

Tissue Culture Media

Germinated, infection, pre-culture, co-cultivation, selection and rooting medium were prepared as described by Behnoush *et al*. (2014) (23) used for *Agrobacterium* mediated sunflower cotyledons. All media were maintained in vitro and basic MS salts (24) containing MS micro-macro elements and vitamins, with the addition of sucrose and agar for all plant tissue culture media. The entire medium was dissolved in distilled water and the pH of the medium was adjusted to 5.8 with 0.1N NaOH. Finally, the media was sterilized by autoclaving at 121C ^{0} for 15 minutes. Filter sterilized plant growth regulators and antibiotics (naphtalenaceticacid [NAA], benzylaminopurine [BAP], cefotaxime and Hygromycin) were freshly added to the sterile medium. The composition and the purpose of the media are given in Table-2 below.

Table 2. Composition of Culture Media

	Rooting Medium (RM)	Selection Medium (SM)	Co-cultivation Medium (CCM):	Infection Medium (IM):	Pre-culture Medium (PM):	Germination Medium (GM):
MS	4.4	4.4	4.4	4.4	4.4	4.4
Sucrose (g/L)	30	30	30	50	30	30
Agar (g/L)	8	8	8	-	8	8
Acetosyringone (200mM)	-	-	+	+	-	-
NAA (mg/l)	0.5	0.1	0.1	-	-	-
BAP (mg/l)	1	2	2	-	-	-
cefotaxime (mg/l)	250	250	-	-	-	-
Hygromycin mg/L)	7.5	7.5	-	-	-	-

NAA: 1-naphthalene acetic acid. BAP: 6-benzylaminopurine

Pretreatment of the Explants

Sunflower seeds were obtained from AL-Mussawi nursery in Basra, and classified as *Helianthus annuus L.*by Prof. D. Abd-Ridha Al-Mayah, College of Science /University of Basra/Iraq. The seeds were sterilized according to the steps described by (25). The seeds were soaked for one min in 70% ethanol followed by soaking for 20 min in 14% Clorox, washed with sterilized distilled water three times. The seeds were released out of their coats and soaked again in 70% ethanol for one min, followed by soaking in 14% Clorox for 15 min and wash with sterilized distilled water four times and then left to dry on sterile Whitman filter papers (No.5:11 cm diameter) in sterilized petri plate for 30 min in room temperature.

Seeds Germination

The sterilized seeds were germinated on germination medium GM (approximately 10-15 seeds in each plate). Petri plates were kept in the dark for 3 days at 27℃, in an incubator then the plates were incubated at 25℃ under 16h light/8h dark cycle with florescent light (60µmol m^{-2} $s^{-1)}$ for 3 days according (26).

Pre- incubation of Sunflower Cotyledons

Cotyledons can be used for transformation when there are no or minimal true leaves present on seeding. After 6-7days the cotyledon explants were cut into two halves at the middle region above the cotyledonary node. An additional cotyledon surface was cut near the distal end in parallel lines and then the cotyledons were placed upside down on petri dishes containing pre-culture medium (PM) and were incubated for 2 days before co-cultivation with *Agrobacterium* at 25℃ in photoperiod of 16h light/8h dark (26).

***Agrobacterium* Transformation Procedure**

A. tumefaciens colony carrying the recombinant vector pCAMBIA1304-GBA from YEP plate containing kanamycin (50 mg/l), and streptomycin (100 mg/l) was used to inoculate 2 ml YEP broth contains the same antibiotics in a 50 ml falcon tube, and was incubated at 28°C for 2 days, this *Agrobacterium* suspension was used for inoculation 20 ml YEP medium was allowed to grow overnight at 28°C. After incubation *Agrobacterium* culture was centrifuged at 4000 rpm for 15 min then the bacterial precipitate was re-suspended in infection medium (IM) supplied with 200 mM acetosyringone in 50 ml falcon tube and *Agrobacterium* suspension was further grown for 2 h at 25℃ with shaking in a water bath at 100 rpm. The optical density at 600 nm of culture was measured and adjusted to 0.5OD. The bacterial suspension was used for plant transformation this protocol according to Evans *et al*, (2014) (27) with some modifications. Specifically (the bacteria culture was centrifuged at 4000 rpm and instead 3500 rpm and was used IM media). When the pre-induction step was completed, pre-cultured cotyledons were removed from PM and soaked

directly into *Agrobacterium* suspension followed by gentle shaking at 45 rpm for 30 min at room temperature. Some cotyledons were soaked into infection medium without *Agrobacterium* as a negative control.

Co-Cultivation

After infection, cotyledons were blot dried on sterile filter paper to remove excess *Agrobacterium* bacteria and were transferred to the surface of co-cultivation medium (CCM), supplemented with 200 mM acetosyringone. The cotyledons were put upside down on CCM and the plates were covered and sealed tightly with parfilm and incubated in dark for 3 days at 28°C. The negative cotyledons were cultivation on the same CCM (28). Three days after, the explants were transferred to a sterile cup and washed with sterile water containing cefotaxime (500mg/L) for one minute and washed repeated with shaker for 10 minutes at 75 rpm followed by sterile water for two times to remove excess *Agrobacterium* . Then the explants were dried on a sterile filter paper.

Selection of Transformed Explants

Explants were transferred to the selective shoot medium and incubated at 25 ºC in the dark for 7 days followed by a 16/8h (light/dark) photoperiod at 25 ºC. After 14 days the healthy cotyledons with young green shoots forming were transferred to the same media in order to select transformed explant. The explants were transferred every 2 weeks to a fresh selection medium for shoot regeneration. At all the selection stages, comparative control untransformed explant was prepared in parallel.

Complete Plant Regeneration

The elongated shoots were transferred to new RM medium and after the root were and the explants were regenerated (approximately 40 days), transferred to fresh RM for root development. After 75 days of transformation when the plant had reached 6 cm in length, hygromycin resistant clones, which stand out from the selected cotyledon, were subjected to glucocerebrosidase assay and molecular analysis. Well rooted transformed sunflower plants were hardening under controlled environment conditions for one week at 25˚C under a 16-h photoperiod. The bags were then opened to acclimate the plants for one week before they were transferred to the greenhouse. Plant work was carried out in Plant Tissue Culture Lab/College of Science /University of Basrah/Iraq.

Isolation of Plant Genomic DNA and *Hu-GBA gene* analysis by PCR

Genomic DNA was isolated from 100 mg of fresh sunflower leaves after 75 days of culturing. Extraction was performed using the genomic DNA Mini Plant Kit from Geneaid Company. DNA was quantified using the nanodrop and gel electrophoresis on a 1% agarose gel.

PCR of Genomic DNA

Incorporation of T-DNA, carrying the *GBA gene* in the genomes of transgenic sunflower plant was assessed through polymerase chain reaction (PCR) analysis of total isolated DNA using primers of the selected gene (Hu-GBA). PCR was performed according to the PCR program to amplification of *Hu-GBA gene* from pGEM-GBA Plasmid. Agarose gel electrophoresis was carried out using 1% agar gel and was examined under UV light to reveal the prorated band. Band intensity and location was contrasted with the positive sample constricted plasmid (pGEM-GBAplasmid) and non-transformed plant genomic DNA as negative control.

RNA Extraction from Transformed Plant

Total RNA was isolated from transferred plant using GENEzol™TriRNA Pure Kit from Geneaid Company using the manufacturer's protocol with some modification. RNA concentration and visualization was determined on a 1% agarose gel. Five µl of total purified RNA (50 ng/µl) was reverse transcribed into complementary DNA (cDNA) using AccuPower®RocketScript™RT Premix (Bioneer), and used for amplification *Hu-GBA gene* using primers of the selected *Hu-GBA gene*. PCR was performed according to the PCR program presented in amplification of *Hu-GBA gene* from pGEM-GBA Plasmid.

Quantitative Real-Time PCR (qRT-PCR) Analysis

Real time quantitative polymerase chain reaction (RT-qPCR) was performed to examine the mRNA expression of *GBA1 gene* in transformed sunflowers by using AccuPower® Green Star ™ qPCR PreMix kit (Biooner, Korea). The totals RNA from human blood and from transformed sunflower were used in RT-PCR. The forward primer was 5-CAGCCTCACAGGTTTGCTTCT-3 and reverse primer was R-5GACACACACCGAGCTGTA-3 used for amplification of exon one of the *Hu-GBA gene* (100bp) (29). Gene expression was normalized to that of *Hu-GBA1 gene* from blood healthy human as a positive control gene and the negative control gene was untransformed sunflower sample. Real time PCR instrument was loaded as seen in Table-3 display the condition of program.

Table 3. Real Time PCR Conditions for Detect *GBA Gene* Expression in sunflower

step	Temperature	Time	Number of Cycle
Pre-Denaturation	95℃	10 min	1
Denaturation	95℃	35℃	
Annealing	58℃	45℃	40
Extension	68℃	1℃	
Final Extension	68℃	5℃	1
Melting	-	1 sec	

Extraction Protein from Transformed sunflower

The proteins was extracted according to Shaaltiel *et al*. (2011) procedure (30) from sunflower leaves by grinding the fresh leaf (0.1 g) in liquid nitrogen and the plant powder was mixed with 1ml of extraction buffer (20mM sodium phosphate pH7.2, 20Mm EDTA, 1mM PMSF, 20 mM ascorbic acid, 3.8g polyvinylpolypyrrolidine (PVPP), 1mM DTT AND 1% Triton-x-100). The homogenate was shaken for 30min at room temperature at 25rpm using Comfy Roller (CR-1040) and centrifuged at 17000xg at 4℃ for 20min. The resulting supernatant was adjusted to pH 5.5 using concentrated citric acid and centrifuged at 17000xg at $4C^0$ for 20min. The concentration of recombinant glucocerebrosiase protein was determined by using Recombinant Human Glucosylceramide ELISA Kit (Catalog no. MBS2885128) from (MyBioSource USA). The reagent was accomplished as manufactures protocol and the standard cure was analysis depend on the curve expert program version-4.

Results:

Construction of pCAMBIA1304-GBA Plasmid

The original objective of the work was to produce of recombinant human glucocerebrosidase (Hu-GBA) in transgenic plant. Therefore *Hu-GBA1 gene* was cloned into plant expression vectors. The initial step was amplifying *Hu-GBA1 gene* from pGEM- GBA plasmid. The resultant *GBA1 gene* band is showed in Fig.3. The presence of *GBA1 gene* inserts was screened by direct *A. tumefaciens LBA4044* PCR, specific primers were successful in detecting clones containing *GBA1 gene* Fig.4.

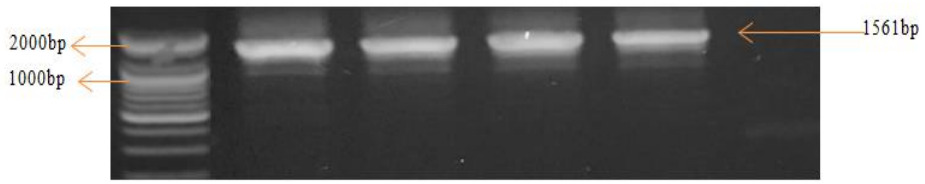


Figure. 3. The analysis of 1% agarose gel electrophoresis for amplification *GBA1 gene* from pGEM-GBA plasmid, M100bp: DNA Ladder, Lane1-4: *GBA1 gene* 1561bp.

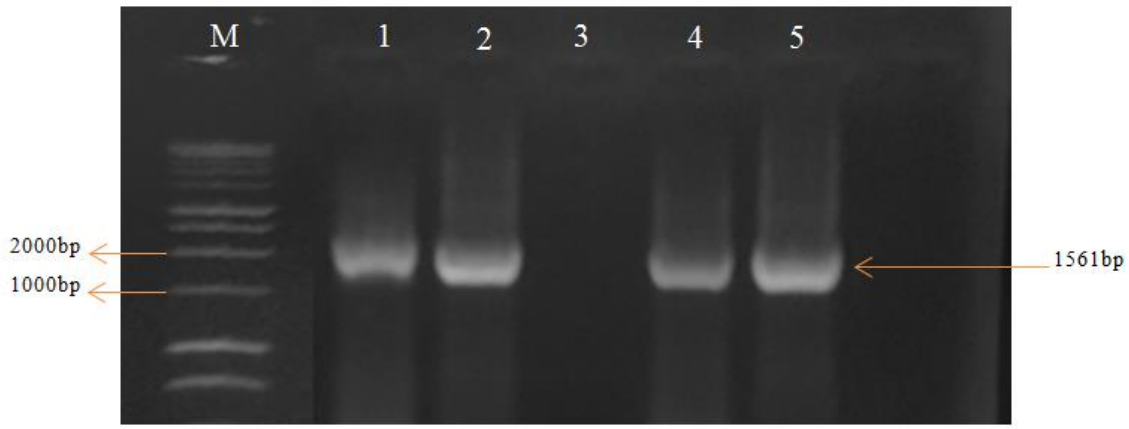


Figure 4. PCR screening for *GBA1 gene* insertions resulting from colony PCR on transformed *A. tumefaciens* LBA4404 M: 1k bp DNA Ladder, Lane. 1, 2, 4: *GBA1 gene* amplify from transformed bacteria. Lane 4: *GBA1 gene* as positive control. Lane 3 is an untransformed colony as negative control (without *Hu-GBA gene*).

Pretreatment of the Explants to Prepare for Transformation

The result of the germination of sunflower seeds on germination media confirmed the success of sterilization method. This sterilization method can be used to produce healthy cotyledons ready for the transformation process. Fig. 5 and 6 show these stages of germinating seeds and preparing seeds cotyledons for transformation.

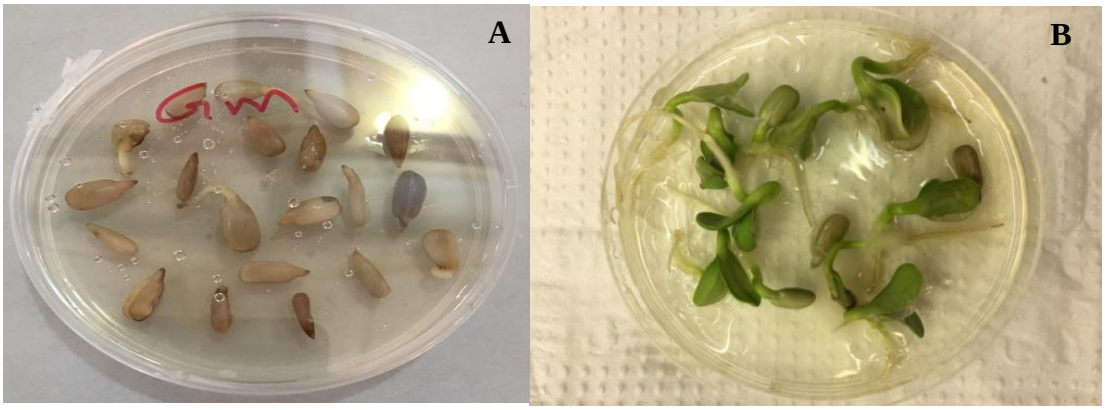


Figure 5. Germination of sunflower seeds A: germination seeds on GM medium in dark condition B: formation young green cotyledons on GM medium after incubated at 25℃ under 16h light/8h dark cycle

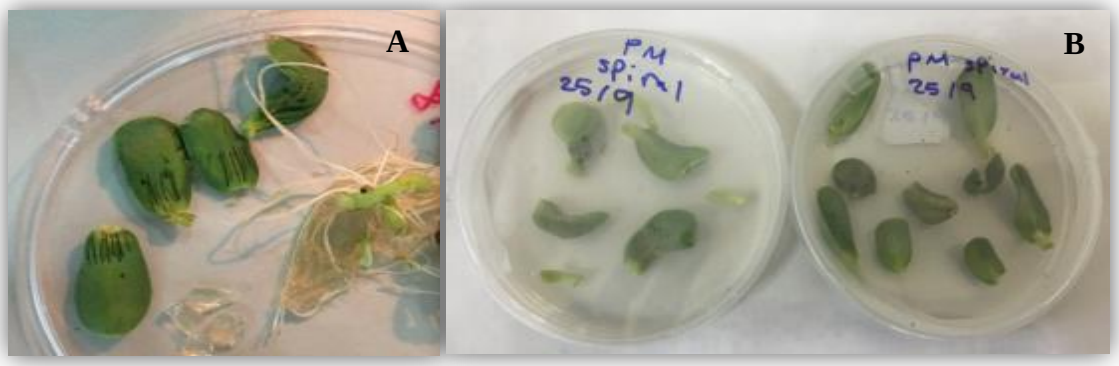


Figure 6. Pretreatment of sunflower seeds A: Cotyledons were cut into two halves at the middle region above the cotyledonary node an additional the cotyledon surfer was cut near the distal end in parallel lines D: cotyledons were placed upside down on petri dishes containing pre-culture medium (PM).

Shuttling *GBA1 Gene* from *A. tumefaciens LBA4404* to Sunflower Cotyledons by Agrobacterium–Mediating Transformation

The cotyledons which were infected with transgenic *Agrobacterium* containspCAMBIA1304-GBA were inoculated on filter paper contains co-cultivation medium supplied with 200 mM acetosyringone for 73h, at 28℃ in full darkness and were selected on selective agar medium for isolation analysis. It was observed that 7.5 mg/L Hygromycin concentration would be sufficient, to kill non-transformed cotyledons Fig.7, so that 7.5 mg/L concentration was used for both selected and negative controls within the study.

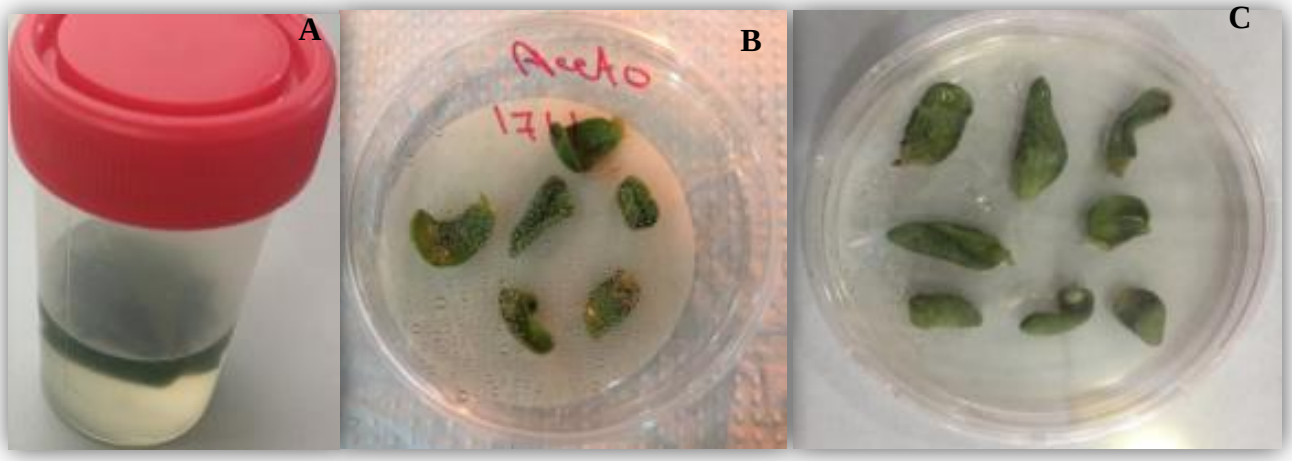


Figure 7. Sunflower cotyledons transformation by *A. tumefaciens* A: Inculpating cotyledons with Infection Medium for 30 minutes; B: cotyledons were incubated on co-cultivation medium for 37h on dark; C: Incubation of cotyledons on selective medium contain cefotaxim and hygromycin antibiotics

Selection of Stable Transformation Cotyledons

The healthy remaining cotyledons were green in color and generated small young shoots while non-transformed cotyledons, became yellow in color and smaller in size. The positive control untransformed cotyledons on selective agar medium containing cefotaxime only without hygromycin were had a young shoot larger than the shoot formed from transformed cotyledons, as seen in Fig. 8.

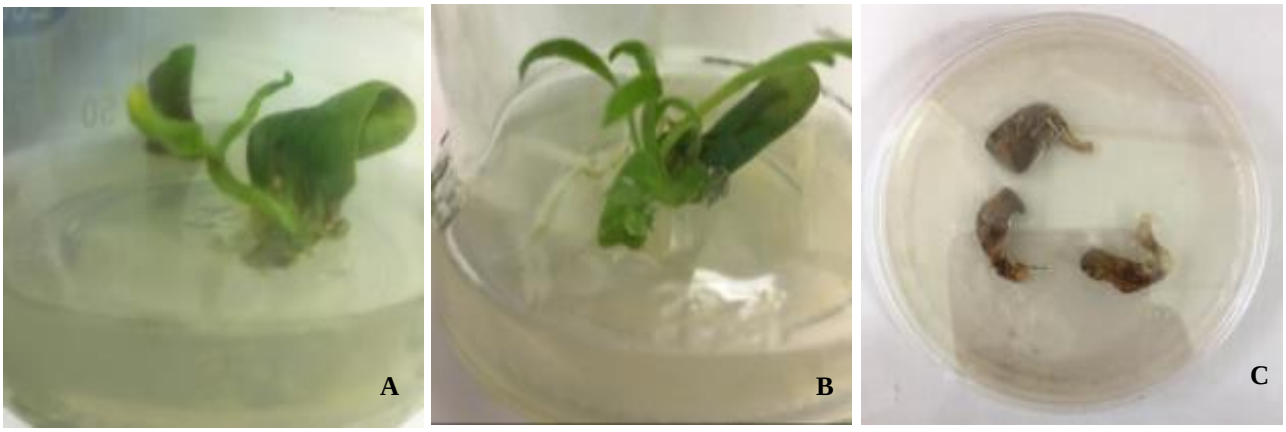


Figure 8. Development of shoot from cotyledon of Sunflower plant A: Two week old regeneration shoot from transformed cotyledons on hygromycin selective medium B: Positive control cotyledons untransformed on the shoot medium without hygromycin C: Untransformed negative control

Roots Development from Transformed Shoots

Use of growth hormones in rooting medium increased both the development of shoots, elongation, and roots produced. The result in Fig.9 show transformed shoots to develop roots on the rooting media supplemented with cefotaxim and hygromycin antibiotics after six weeks, compared to positive control shoots, which forming roots after two weeks on shoot media without hygromycin. The whole negative control were failed to produce any roots. *Agrobacterium* facilitated sunflower transformation successfully to construct transgenic sunflower plants to express human *GBA gene* and regenerated a complete plant after 75 days on selection rooting medium, which was followed by molecular analysis of regenerated whole plant Fig. 10 and 11.

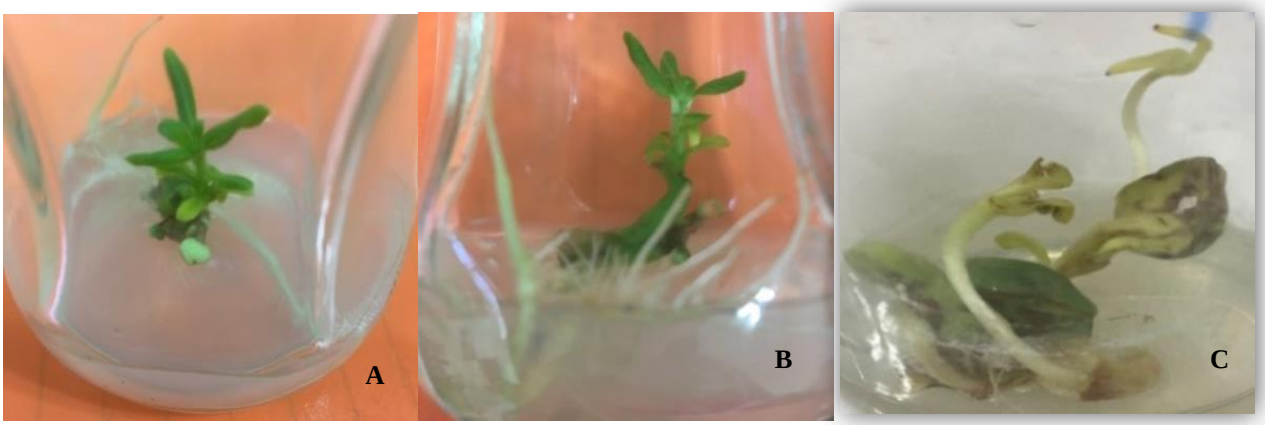
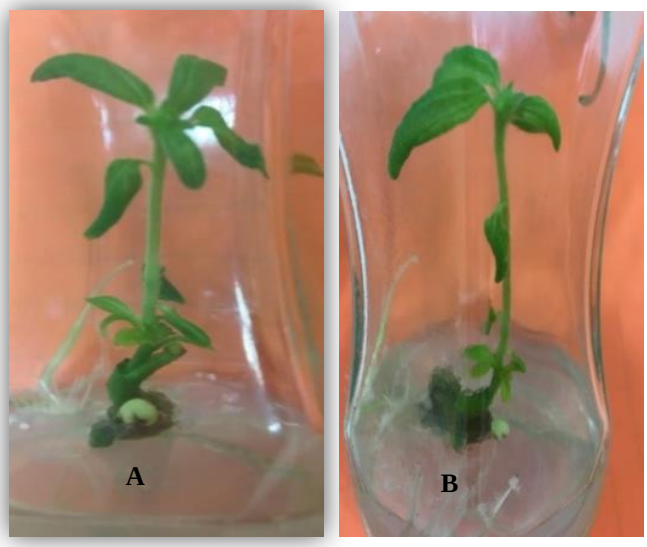


Figure. 9. Developing transgenic sunflower plants increased formation shoot and root from transformed sunflower explants on selective root medium A: Transformed explant B: Positive control untransformed explant C: untransformed sunflower explant was failed to development new plant on selective root medium



Figur10. Complete sunflower plant A: transformed plant regenerated from cotyledons B: untransformed plant regenerated from cotyledons

Figure11. Growth transformed sunflower plant in soil .

Molecular Analysis of Transgenic Sunflower Plant

Positive results of transforming sunflower were revealed by detecting the integrated *GBA1 gene* into the plant genome using PCR. Genomic DNA was isolated from transformed sunflower leaves and *GBA1 gene* was amplified by PCR with *GBA1 gene* specific primer, and the predicted size (1561bp) for *GBA gene* was amplified from transformed plants, as shown in Fig.12.

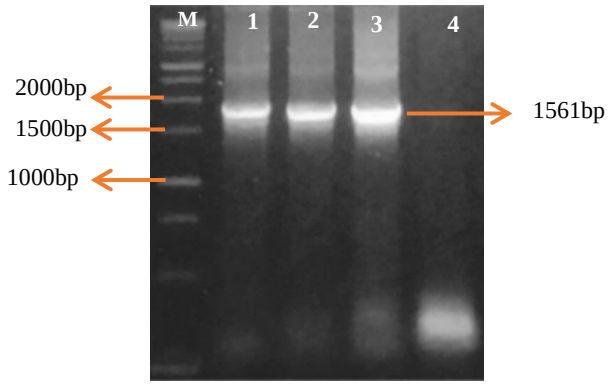


Figure12. PCR product amplification for *GBA1 gene* integration in the plant genome: M, 1K bp ladder; lane 1-2, *GBA1 gene* from transformed sunflower; lane 3, Positive control *Hu-GBA gene*, lane 4, Negative control untransformed sunflower

Quantitative PCR Analysis of Glucocerebrosidase Expression in Transgenic Sunflower

Total RNA was isolated from transgenic and untransformed plants and submitted, to RT-PCR. The resulting cDNA was then amplified by PCR using glucocerebrosidase specific primers. The results of total RNA isolated are shown in Fig.13; a correct size (1561bp) fragment corresponding to the *Hu-GBA1 gene* was amplified in transgenic plant sample. Non amplification was found in the untransformed plant. This result confirmed that the human glucocerebrosidase gene is currently expressed in the transgenic sunflower Fig. 14.

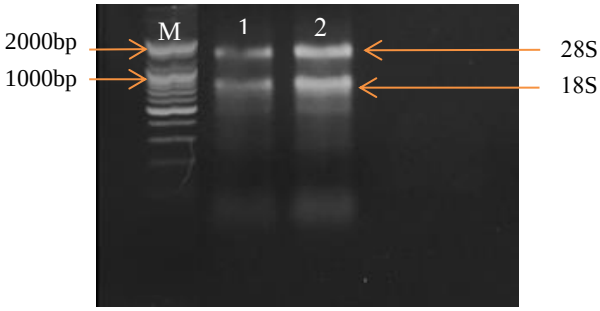


Figure 13. Total Plant RNA; Lane1: RNA isolated from transformed sunflower; Lane2: RNA isolated from untransformed sunflower; M: 100bp DNA ladder

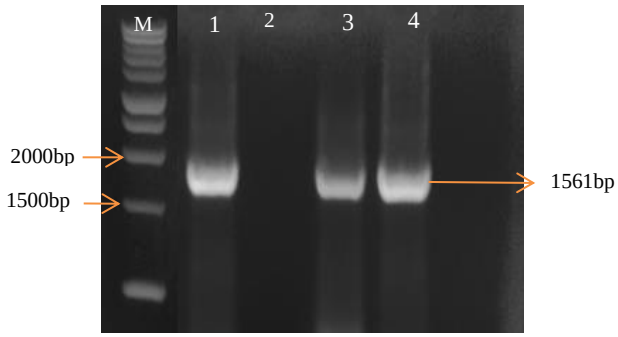


Figure14 . The agarose gel analysis of PCR amplified *GBA1 gene*, lane 1,3: *GBA1 gene* amplified from transformed sunflower lane4: positive control *GBA1 gene* lane 2: negative control from untransformed plant M: 1Kbp DNA ladder

Real Time-PCR Analysis

The real-time PCR transcript data confirmed successful transformation events. These results in Fig.15A,B demonstrated that the target *Hu-GBA gene* had successfully integrated into the sunflower plant genome and was stably expressed in transgenic plants, light cycler melting curve was performed to detect the melting temperature for *GBA gene* in transgenic plant. Real time efficiency was calculated from the given slope in light cycle software. The ratio of gene to the positive control gene was calculated from threshold cycle (CT). GBA mRNA expression from transformed sunflowers was determined according to the CT method using a GBA mRNA expression in healthy human blood as positive control. The result showed that the expression of *GBA gene* transcript in transformed sunflower was increased and it was three folds compared with control Fig.16.

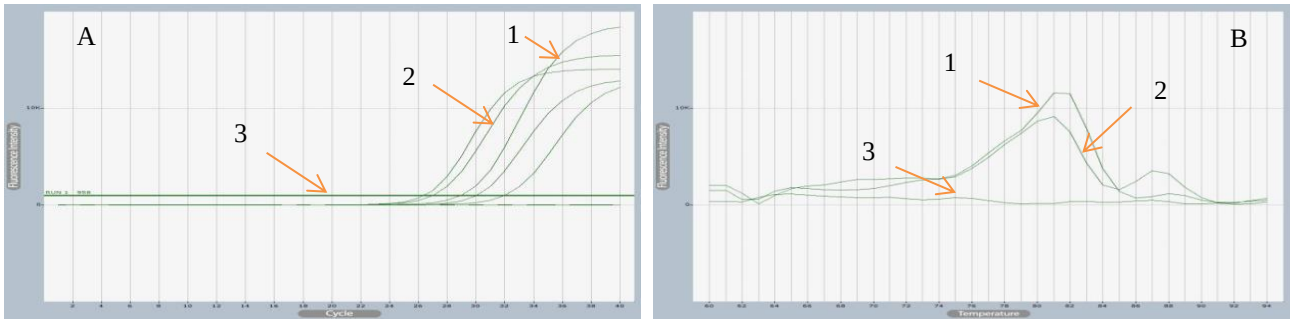


Figure 15. Real time analysis of *GBA1 gene* expression profile in transformed sunflower A: amplification curve for all *GBA genes* B: melting peak for all *GBA1 genes*1-mRNA form human Blood sample 2- mRNA from transformed plant sample3- mRNA from untransformed plant sample

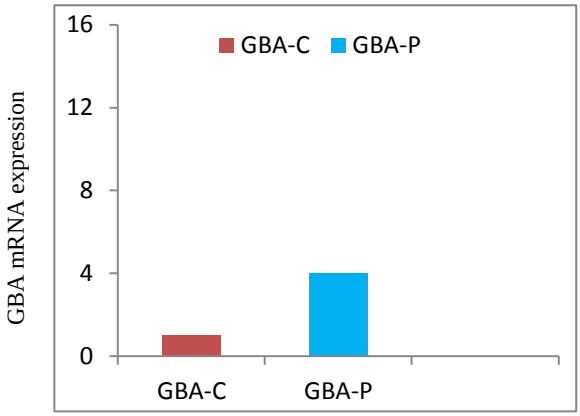


Figure16. Gene expressions of *GBA gene* in transformed sunflower in compare with expression in human blood GBA-C mRNA form human Blood sample as positive control GBA-P mRNA of recombinant GBA gene from transformed sunflower.

ELISA Immunoassay for Recombinant Glucocerebrosidase

The concentration of glucocerebrosidase in protein extraction solutions was assayed by ELISA kit technique, as shown in Fig.17, the standard curve for was made between the concentration of glucocerebrosidase and absorbance at 450 nm. The concentrations of recombinant glucocerebrosidase in this method reach to 0.45 ng/ml.

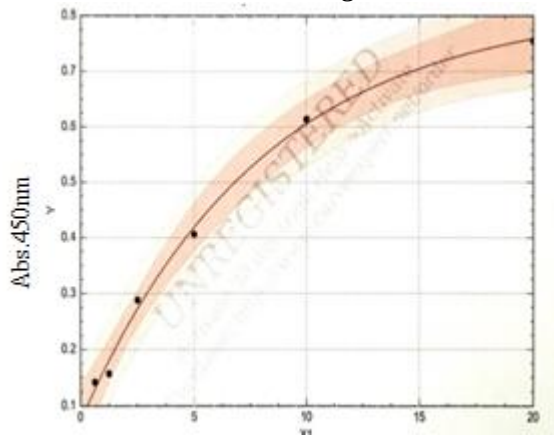


Figure 17. The standard curve for glucocere-Brosidase ng/ml

Discussion:

As an alternative production system, neither bacteria nor mammalian expression systems can be employed here. In contrast, plants offer an efficient eukaryotic system for protein synthesis, and with the coming of modern plant genetic engineering, plants could be used to produce enormous amount of protein with all set established gene expression systems.

Constructed recombinant pCAMBIA1304-GBA Vector

The designed primers resulted in amplified *GBA1 gene* from cDNA which constructed in PGEM-GBA plasmid, and was designed to include *BgIII* site with 5`end and the reverse primer with *BstEII* site at the 5`end. Both enzymes were required to digest the *GBA gene* and integrate it in correct region of pCAMBIA-1304 between the CaMV35s promoter and the NOS terminator. The promoter holds the information to prompt the transcription process and initiate transcription, while the terminator contains information to stop transcription and polyadenylation sign. *A. tumefaciens* has been the most communal plant transformed tool date, and any interest gene can easily integrate inside the T-DNA region using different types of plant specific vectors to generate transformed plants (31, 32).

Transformation Sunflower Cotyledons

Agrobacterium were used for genetic transformation of cotyledonary tissues (33, 34) and because the sunflower cotyledons were quite large and easy to cut near the distal end and could be grown very well in Iraqi environment. Sunflower was selected for genetic modification in our labs and will be considered as the sources for glucocerebrosidase in this study.

Sterilized Sunflower Seeds

In this study, sunflower seeds were sterilized with coat and then the coats were removed and resterilization occurred because the seeds of

sunflower are so large and surrounded by a tough shell consisting of two halves. The embryo has an air chamber that in general attracts fungal spores which makes eliminating them very challenging (35). Therefore, the study the seed coat was removed to avoid fungal contamination when germinated on germination medium and this way was successful in obtaining sunflower cotyledons free of any fungal contamination result in Fig.5.

Incubation of Sunflower Cotyledons with *Agrobacterium*

Transformation via *A. tumefaciens* is the main step facilitated by the physical attachment between *A. tumefaciens* and plant cells. When the *A. tumefaciens* is attached to plant cells, it makes cellulose fibrils at the wounded sites (36), so it was important to make cuts in sunflower cotyledons as in Fig.6A before inoculation with bacteria. The steps in current study the expose sunflower cotyledons to *A. tumefaciens* for 30 min to allow enough physical contact between bacteria and plant cells. *A. tumefaciens* manufactures an exopolysaccharide, unipolar polysaccharide (UPP) that participates in bacterial connection (37, 38). Plant factors are involved in bacterial engagement to the surface of plant cells; vitronectin protein family factor is one of them. Vitronectin is a special receptor for many pathogenic bacteria strains (39). A wide range of chemical compounds secreted from wounded plant act as agents to engage *A. tumefaciens* to the wound sites to infect the affected plant and act as signals for activation virA and virG genes which are required to activate vir gen in *A. tumefaciens* (40), these signals are phenols, aldose monosaccharaides, low phosphate, and low pH (41). The plants excrete sap has acidic distinctive pH (5.0 - 5.8) with elevated content of different phenolic component, which used to prevent microbial attachment, but they actually induce the virulence gene (vir gene) in *A. tumefaciens* to be expressed (42). The most studied and most efficientive vir gene stimulator is 3, 5-dimethoxy acetophenone (acetosyringone, AS) (43). Therefore 200 mM of acetosyrinogen was added to infection and co-cultivation media with pH at 5.5 to provide appropriate conditions for Agrobacterium to express vir gene. In the actual study sucrose was also added to all the media. In infection medium specifically, sugars stimulate of VirA/VirG genes, and also bind to the glucose/galactose binding protein and enhanced activation of vir gene (44, 45) and induce many bacterial genes to be expressed (46).

Expression of Virulence gene (vir gene) leads to produce of a singular strand T-DNA, or so called T-strand, and then it was transported to the plant cell by enhanced *Agrobacterium* to produce proteins VirD1 and VirD2, which act as a post and strand particular endonuclease attached to the coiled Ti plasmid between the third and fourth bases of the T-DNA outlines, and causing it to unwind (47, 48). In this study, sunflower cotyledons were incubated with *Agrobacterium* in an infection medium (IM) supplemented with 200 mM AS for 30 min at 25℃, as see in Fig.7A. This temperature triggers expression of *virB gene* in bacteria (49), where the virB proteins enables T-DNA and Vir proteins to penetrate the bacteria's internal membrane, the peptidoglycan coat, and external membrane, in additional to the host plant membrane and cell wall. Complex VirB is a class of type IV secretion systems (T4SS), that are presented in many range of Gram-negative bacteria and are involved in their pathogenicity (50,51,52,53).

Selecting Transformed Explants

The hygromycin resistance gene is widely used as a selection gene in plant transformation, and the results in Fig.8A shown the concentration of 7.5 mg/ml was sufficient to select transformed cotyledons because that concentration inhibited shoot growing as in seen Fig.8C and this result was in an agreement with (33,54). Who used a hygromycin 7.5 mg /ml to selection transformed sunflower. In this study, two types of plant hormones were used, 6-benzylaminopurine and naphthalene acetic acid (NAA). In tissue culture the plant hormones are important for regenerating explant because they promote growth and elongation plant cells (55).

Frequency of Stable Transformation

The results showed the healthy cotyledons with young green shoots were from transformed sunflower cotyledons and the transformation frequency were 37.5% from 40 transformed cotyledons, but only 15 cotyledons able to produce healthy shoots after two weeks of transformation seen in Fig.9A, B and could produce roots from these shoots as seen in Fig.10A, this result is in agreement with similar work conducted to produce complete transformed plant from cotyledons (56,57) .They produced complete transformed tomato from cotyledons and produced transformed sunflower from transformed cotyledons. Growing whole plant from transformed cotyledon on selection medium confirmed transformed T- DNA from *Agrobacterium* to plant cell due to the expression of T-DNA after a few days (58).

Analysis of *GBA Gene* Expression in Transgenic Sunflower

The transgenic sunflowers were analysed by PCR and real-time RT-PCR. Both techniques were used to prove steady expression of

recombinant gene in the transgenic plant, and in this study used to establish the *GBA gene* expression at the RNA level. *GBA gene* can be amplified from genomic DNA & RNA that was isolated from transformed sunflower leaves using the specific primer, as seen in Fig.12 and 14. They had the same band size for control *GBA1 gene*, and the sequences analysed confirmed that the gene amplified from plant is *GBA1 gene*, the real-time RT-PCR result Fig15 showed That *GBA gene* was expressed in transformed sunflower plants, more or less comparable level of *GBA gene* from the human blood and the modified gene was detected by relative expression. No RNA from the *GBA gene* was found in the non-transformed control plant. Quantitative real-time reverse transcription–polymerase chain reaction (qRT–PCR) is the required method for confirming gene expression and detection the fold change difference in mRNA levels of any target gene, due to its high sensitivity specificity and reproducibility (59) .The quantity of *GBA1 gene* in transformed sunflower was confirmed when we converted its RNA to cDNA and its expression was related to control cDNA compared to reversed total RNA isolated from blood sample of a healthy person. Human Glucosylceramide (GBA) ELISA Kit was used for the quantitative determination of human glucosylceramidase (GBA). This assay has highly sensitivity and specificity for human glucosylceramide (glucocerebrosidase), and no interference was observed between human GBA and analogues. Findings showed the concentration of crude recombinant glucocerebrosidase isolated from transformed sunflower reach to 0.45ng/ml in 0.1g from plant powder.

Conclusion:

In this study, the designed primer successfully amplified the whole the Hu- GBA ORF with its signal peptides and was successful in integrating it within the pCAMBIA-1304 plasmid and forming recombinant pCAMBIA1304-GBA plasmid. Transformed *A. tumefaciens* was able to transfer *GBA gene* to sunflower cotyledons and these transformed cotyledons were efficient in generating whole plant on selection media. The real time analysis showed that transgenic sunflowers were effectively expressing *Hu- GBA gene* with comparable levels of its expression in modified plant and in healthy human blood, and the mature transformed plant was successfully produced glucocerebrosidase protein.

Acknowledgements:

We are thankful to the Director Genetic Engineering Lab/College of Science /University of Basra/Iraq, Prof. Khosraviani Kave (University of Tehran) whose kindly provided *E. coli DH5α* strain, Prof. D. Abd-Ridha Al-Mayah /College of science /University of Basra/Iraq whose classified sunflower seeds and Dr. Zainab Jwad the director plant tissue culture Lab/College of science /University of Basra/Iraq.

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التعبير الجيني للانزيم البشري المركب Glucocerebrosidase في دوار الشمس

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الخلاصة:

تعد الزراعة الجزيئية واحدة من أهم تجارب التقنيه الحيويه لانتاج محاصيل نباتية معدلة وراثيا لإنتاج بروتينات طبية وان بكتريا *Agrobacterium* واحدة من أدوات الهندسة الوراثية التي تستخدم لادخال الجينات المرغوبه داخل النبات. في السنوات الأخيرة زادت الحاجه لإنتاج بروتينات علاجيه بطرق الهندسة الوراثية، ويعد انزيم الـ Glucocerebrosidase المستخدم لعلاج المرض الوراثي Gaucher disease واحد من تلك البروتينات. في الدراسة الحاليه تم تصميم بادئات خاصة لتضخيم الجين البشري *Hu-GBA1* المسؤل عن انتاج انزيم Glucocerebrosidase من البلازميد المركب pGEM-GBA بعدها تم كلونه الجين المضخم في ناقل التعبير النباتي pCAMBIA1304 للحصول على ناقل كلونة يحمل الصفه الوراثيه لتشفير انزيم Glucocerebrosidase. استخدام البلازميد المركب الناتج pCAMBIA1304-GBA لتحويل بكتريا *Agrobacterium tumefaciens* LBA4404 الى بكتريا حامله للبلازميد المركب. اكدت النتائج الحصول على بكتريا محورة وراثيا حامله للجين البشري *GBA1* بواسطة Colony PCR . علقت بكتريا *Agrobacterium* المحوره في وسط Infection Medium (IM) الحاوي على200 ميليمولارمن acetosyringone واستخدم هذا العالق لتحويل فلقات نبات دوار الشمس الى فلقات محورة وراثيا حاوية على الجين البشري *GBA1* ، اذ نميت الفلقات الحاوية على العالق البكتيري في الوسط الزرعي (Co-Cultivation Medium CCM) الخالي من المضادات مع 200 ميليمولارمن acetosyringone ، بعد ذلك تم نقل الفلقات إلى وسط اختيار(Selection Medium SM) يحتوي على 7.5 مليغرام / لتر من Hygromycin و 250 مليغرام / لترمن Cefotaxime والتي نميت لمدة 14 يومًا إضافية بدرجه حرارة25م□ لفترة 16 ساعة ضوء و8 ظلام .اذ تم بنجاح إستزراع نبات دوار الشمس من الفلقات المحور وراثيا وقد ثبت وجود الجين *Hu-GBA1* في الحمض النووي للنبات المحور النامي بواسطه PCR حيث كانت نتيجة التظخيم حزمة بقياس 1561زوج قاعدي. كما تم الكشف عن التعبير الجيني للـ*GBA1* في دوار الشمس المحور بواسطة الريال تايم PCR مقارنه مع *GBA1gene* القياسي. استخدم الاختبار المناعي المرتبط بلانزيم ELISA وباستخدام عدة المحاليل الجاهزة (Human Glucosylceramide ELISA Kit) لتحديد هوية الانزيم المنتج من البروتين الخام المستخلصه من النبات المحور وتحديد تركيزه ايضا، حيث اوضحت النتائج ان تركيز انزيم glucocerebrosidase المستخلص من نبات دوار الشمس المحول بجين *GBA1* هو0.45 نانوغرام/ مليلتر

الكلمات المفتاحية: Agrobacterium ، داء غوشيه ، الغلوكوزيل سيراميديز ، pCAMBIA1304 ، عباد الشمس