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Cloning and transformation human glucocerebrosidase gene in *Agrobacterium tumefaciens LBA4404* strain

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Abstract

Agrobacterium is broad used, after suitable modified, to be the utmost efficient vector for gene transport into plant cells. This study was carried out to produced *Agrobacterium tumefaciens LBA4404* strain contains binary vector has human *GBA gene* to became ready for transforming any desired plant to expression glucocerebrosidase. Recombinant glucocerebrosidase is current treatment for gaucher disease which is lysosomal storage disease caused by mutations in the encode for glucocerebrosidase. In this study, Human whole blood was pivotal source of RNA by using GENEzol™ TriRNA Pure Kit, and the *Hu-GBA gene* was amplified by designed special primers,G*BA gene* was introduced inthe plant expression vector pCAMBIA1304 beneath the domination of the cauliflower mosaic virus 35S promoter and grated recombinant pcambia1304-GBA vector having GBA *gene* by restriction and ligation methods.Calcium chloride heat-shock transformation is used to introduce pcambia1304-GBA into *E. coli DH5α* and plantation on LB agar medium contains kanamycin 50 mg/l. The transformed colonies were proven by colony PCR and amplification *GBA gene* from isolated recombinant pcambia1304-GBA. The recombinant isolation plasmid was transferred to *Agrobacterium tumefaciens LBA4404* using modified freezing-thaw method, transformation Agrobacterium colonies were enhanced by colony PCR. Results demonstrated that The1561bp-*GBA gene* was amplified from human total blood RNA which was confirmed by sequencing the PCR product which gives 100% identified withHomo sapiens glucosylceramidase beta (GBA), transcript variant 1, mRNA NM_000157.3 Gene Bank and colony PCR assured that *Agrobacterium tumefaciens LBA4404* carried *Hu-GBA gene.* .

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Introduction

The gene transmission system, which is the most chosen by the plant biotechnologist is Agrobacterium-mediated transformation because of its available, capability to transport fading scripts of DNA fragments hold the genes of attention with minimum value transport of large fragments of DNA with lower rearranging (Shibata and Liu, 2000; Gelvin, 2003). Thus, plant transformed out of *Agrobacterium* has been a favorite program for several species (Barampuram and Zhang, 2011). So the objective of this study was to introduce human *GBA gene* which encodes the lysosomal enzyme glucocerebrosidase (GCase) into Agrobacterium. *GBA1gene* is located on chromosome 1q21, it has eleven exons, ten introns and is 7.6 kb in total with a close 5.6 kb pseudogene, downstream 16 kb and is 96% homology between them (Horowitz *et al.,* 1989), GCase metabolizes glucocerebroside into glucose and ceramide, changes in *GBA1* lead the autosomal recessive lysosomal storage disorder Gaucher disease (Grabowski, 2008), resulting the commutation of an undegenerated glycolipid in lysosomes of cells of the mononuclear phagocytosis system (Hollak, 2009). Clinical appearance involves enlarged liver and spleen, bone complications, anemia and thrombocytopenia (Pastores *et al.,* 2000; Beutler and Grabowski, 2001). The potency of treatment lysosomal disorder by enzyme replacement therapy. Once the gaucher disease was identified as enzyme deficient disease large scale methods for purification of GBA enzyme from human placenta (Barady *et al.,* 1965; Dale and Beutler, 1976; *Furbish et al.,* 1977). At first; researches began try treating patient with casting, the initial these studies used unchanged enzyme or enzyme enveloped in either liposome (Belchetz *et al.,* 1977), or red blood cells membrane (Beutler *et al.,* 1977), but none were succeeded in improving disease. After this failure in ameliorating disease number of studies has been accomplished rat the mechanism by which hepatocytes are uptake of lysosomal enzyme (Thorpe *et al.,* 1974; Stahl *et al.,* 1978), additional searches of human placental enzyme were appeared it has a high proportion of side chains of galactose-terminated oligosaccharide which bind hardly together with a lectin that expression on membranes of hepatocyte cell (Takasaki *et al.,* 1984). The find that macrophages bearing a receptor (macrophage mannose receptor; MMR) which reacts with terminal mannose side chains supply a mechanization to target the glucocerebrosidase enzyme to action site (Stahl *et al.,* 1978; Furbish *et al.,* 1981), the GC side chain can be adjusted to direct the enzyme to macrophage by eliminated of monosaccharides residue used the enzymes neuraminidase, β-galactosidase and β-N-acetylglucosamines (Barton *et al.,* 1991; Furbish *et al.,* 1981), the first study about introduce mannose-terminated on placental glucocerebrosidease used 60U/kg as a dose administered decrease progressed dangerous events (Barton *et al.,* 1991) the production was confirmed from the food and Drug Administration (Goldman *et al.,* 1992), emporium by Genzyme as aglucerase but its high cost limed supply, and in order to produce protein has high quantity of mannose-terminal side chains wanting the required to process in vitro (Elbein *et al.,* 1990 ; Brumshtein *et al.*, 2010), in 2012 Taliglucerasealfa was generated in carrot cells and be the initial drug produced in plant cells to get FAD confirm (hollak *et al.,* 2012; Traynor, 2012). Produced the enzyme by plant cells decrease the requirement for post translational modification to display the mannose residues as more 90% of the natural side chains are mannose terminated, (Shaaltiel *et al.,* 2007).

The aim of our study was to clone cDNA of human *GBA gene (Hu-GBA gene*) fragment encoding for glucocerebrosidase against Gaucher disease in *Agrobacterium tumefaciens LBA4404 strain*, Molecular cloning was performed in *Escherichia coli DH5-alpha* (Invitrogen) was using for the clone steps and repertory recombinant plasmid and *A. tumefaciens LBA4404* Cells from (Takara Bio Inc.) was used for mediate gene transferred to plant.

Materials and methods

Plasmid construct

The binary plasmid vector pCAMBIA1304 (Fig.1; product No. M1595 from Marker Gene Technologies Inc), was used in this study, Plant expression vector

construct pCAMBIA1304-GBA (Fig.2), was prepared in my laboratory This construct containing *H-GBA genec* DNA sequence cloned under leading of CaMV35S promoter and terminator sequence. This vector holds LB and RB (Left and Right edges) for insertion of foreign genes inside the host genome, a CaMV35s promoter (Cauliflower Mosaic Virus promoter which stimulates height level of transcription), NOS (Nopaline Synthase terminator), a kanamycin resistance gene for election of transformation bacteria colonies, a hygromycinphos photransferase resistance gene as selectable and marker gene for transformed plant, *BgIII* , *Bst EII* restriction sites, GFP , GUS gene (as reporter genes), (Hajdukiewicz *et al.,* 1994)

Sample collection and RNA isolation

Blood sample (2mL) was collected in sterilized EDTA and the tube has been kept on ice and immediately was used for RNA extract. Total RNA was isolated from fresh Peripheral human blood used GENEzol™ TriRNA Pure Kit (100rxns) from Geneaid Company, according to the manufacturer's instruction, the total RNA was quantified using nanodrop/optizen system and the concentration RNA was calculated in ng µL-1. Analyze the RNA banding manner, was performed on 1 % agarose gels and ethidium bromide.

Reversed transcribed cDNA Synthesis and Polymerase chain reactionPCR

Five µl of total purified RNA was reversed transcribed into complementary DNA (cDNA) using AccuPower®RocketScript™ RT Premix (Bioneer), according to manufacturer's instructions, cDNAs were analyzed using 1 % agarose gel electrophoresis and visualized on a UV transilluminator. Polymerase chain reaction (PCR) was using for amplifying cDNA encode to human glucocerebrosidase with its special signal peptide using the forward primer (F.5-CTAGATCTCCATGGCTGGCAGCCTCACA-3) containing *BglII* restriction site in bold and reverse primer (R.5-CCGGTCACCTCACTGGCGATGCCACAG-3) contain *BstEII* restriction enzyme in bold .The sequences reference depending in the our study were NM_000157.3 , this created a fragment of 1561bp. PCR reaction was performing in final volumes of 25 mL containing 50 ng genomic DNA, 1 µl of 1µM each primer (forward and reverse), using GoTaq®Green Master Mix kit (Promega, USA). Thermal cycling conditions were 94C° for 5min followed by 30 cycles of denaturation at 94C°for 1min, annealing 58C° at 45sec. Extension at 68C° for 3 min, and final extension step at 68C° for 7min PCR condition according to Juliana *et al.*, (2012) except annealing TM by (researcher). PCR amplicons (8µl) along with DNA standard (100bp DNA Ladder) were verifying by electrophoresis on a 1.0 % (w/v) agarose gel and visualizing under UV light. The PCR products were purifying by using Gel/PCR DNA Fragment Extraction Kit (Geneaid). According to the manufacture instruction. .

Sequencing of Hu-GBA gene

Confirmed the presence of *GBA gene* in PCR product was by sequenced the purified &unpurified PCR product from blood sample, completely in the forward and reverse direction primers.

Sample Preparation

Samples were preparation according Guide to Eurofins Genomic GmbH protocol, Germany (support-eu@eurofins.com), 10 ng/µl 15 µl for purified PCR Products, 20 ng/µl 15 µl for Unpurified PCR Product and 15µl (10 pmol/µl) for both forward and reverse primers, The tube for each sample and primer tubes was labeled with code number was provided by Eurofins Company as Barcode Label.

The sequences were analyzing used Basic Local Alignment Search Tool "BLAST" in order search for symmetrical sequences in the National Center for Biotechnology acquaintance database (NCBI), http://WWW.blast.ncb.nih.gov (Jenkins *et al.*, 2012).

2-4.Cloning of the Hu-GBA gene in pCAMBIA1304

Cloned the gene of interested in vector requested restriction enzymes and T4 DNA ligase enzyme for digestion and ligation process. The purified *Hu-GBA gene*from blood and pCAMBIA1304 cloning vector

were digested with endonuclease restriction enzymes *BglII* and *BstEII* (Promega®) to insert the *Hu-GBA* gene in reporter genes region of pCAMBIA1304 after the CaMV35s promoter and before the NOS terminator by ligation process according to the protocol provided along with enzyme system and protocol of *Accu Power*® Ligation PreMix Kit (Bioneer, Korea).

2-5. Purification of the digested pCAMBIA1304 and Hu-GBA gene by BglII Restriction enzyme.

The digested pCAMBIA- 1304 vector (12µl) was electrophoresed on an 1% agarose gel and was purified from the gel used Gel/PCR DNA Fragment Extraction Kit (Geneaid) while the digested *Hu-GBA gene*was purified from ligation reaction using the same Kit, the only exception is that 40µl from ligation reaction was used instead of gel slice.

Digestion pCAMBIA1304 plasmid and Hu-GBA gene with BstEII Restriction enzyme

Purified digested plasmid and gene were digested again with *BstEII* Restriction enzyme. The digested pCAMBIA1304 plasmid and *Hu-GBAgene* with *BstEII* Restriction enzyme were purified using the same kit above.

Ligation of GBA gene and pCAMBIA1304 Vector

The purified restricted *Hu-GBA gene* and pCAMBIA1304 vector were ligated using AccuPower® Ligation PreMix Kit (Bioneer) and the construct plasmid was obtained depending to specify molar ratio by standard ligation guide: vector Molar ratio: inserting DNA (gene) was 1:3 in the actual study, the ratio was 4µl (pCAMBIA1304):12µl (*Hu-GBA gene*) total concentration (12ng/µl). The newly constructed plasmid was named as pCAMBIA1304-GBA.

Transformation of E. coli DH5α strain with pCAMBIA1304-GBAby freeze-thaw method

Bacteria was prepared for competent & transformation according to the protocol provided by Novagen Company and Sambrook and Russell (2001) as described below:

Preparation of chemo competent E. coli cells procedure

A single colony of freshly streaked *E. coli DH5α* on Luria-Brentano Agar (LB) was inoculated into 5ml LB culture broth in 50ml falcon tube and incubated at 37C° at overnight; 100µl of culture was using to inoculation 100ml of LB broth in 250ml bottle the next morning and shacked at 37C° for 3h. Then the bottle was putted in ice for 10min (the cells were keeping cold from now on), the cells were collected by centrifuge for 3min at 6000rpm.The supernatant was decanted and gently resuspended on 10ml cold 0.1M CaCl2 and incubated on ice at 20 min. and then was centrifuged for 3min at 6000rpm. The supernatant was discarded and politely resuspended on 5ml cool 0.1 M CaCl2 /15% glycerol, competent cells were distributed in 2ml Eppendorf tubes (200µl/tube) and stored in -80C° for further use.

Transformation Procedure for E. coli DH5α

Tube of competent cells was out of -80°C and was putted on ice for allowing it to thaw to 20min.100 µl of competent cells were transported to pre-chilled tube and remained cells were refrozen in -80°C. 1µl of ligation reaction (cloned plasmid product pCAMBIA1304-GBA.) was adding to the competent cells and the contents were mixing gently by moving the pipette through cells, the competent cells/ plasmid mixture was incubated on ice for 30 min. Each transformed tubes were shock heated by placed the tube into water bath at 42°C for 45 sec. And then the tubes were placed immediately on ice for 2 min. 900µl of LB broth without antibiotic was added to the tube and incubated in a water bath shaker at 37°C with shacked at 150 rpm for 1 h.100 µL of transformed bacteria were cultured on LB plates containing 50 µg/mL kanamycin.

The control tube was making without added plasmid. The plates were incubated at 37°C for 48h. The colonies appeared in the plates were counted and the transformation was confirming by colony PCR and isolated the plasmid from the transformed bacteria. The transformed efficiency was carried according to (Invitrogen).

Confirmation of cloning and transformation Colony PCR

Colony PCR was performed to quickly identify colonies bearing the recombinant DNA according Rasha (2013).

Plasmid isolation and purification from E. coli

The plasmid DNA (pCAMBIA1304-GBA) was isolated and purified from the transformed *E. coli DH5α* cells using AccuPrep® plasmid Mini Extraction Kit (Bioneer, Korea).

Amplification GBA Gene from constructed plasmid pCAMBIA1304-GBA

Using the purified pCAMBIA1304-GBA as template, the *GBA gene* was amplified with appropriate primers and primer conditions as explained. Single recombinant colony of *E. coli DH5α* was transformed to 5ml of LB broth containing 5µl of (50 µg/ml) kanamycin and was grown overnight at 37°C, glycerol stocks were preparing by mixed 700µl of fresh overnight culture with 300µl of 50% glycerol. Bacteria glycerol stocks were stored at -80°C, (Sambrook and Russell, 2001).

Agrobacterium transformation

Preparation of chemo competent Agrobacterium cells

Competent cells of *A. tumefaciens LBA4404* were prepared according to method of Vincze and Bowra, (2006), *A. tumefaciens LBA4404*cells were spread on LB agar plates contain antibiotic (100µg/ml streptomycin) and grow for 48h. At 30°C and were inoculated in 50ml of LB medium containing 50µl of (100µg/ml streptomycin) in 100ml flask and were inoculated at 30°C for48h. The cells were chilled on ice for 15min and then were spun down by centrifuge at 4.500 rpm for 10min at 4°C. The culture medium was discarded and the pellet was dissolved in 2ml of ice-chilled 20mMCaCl2 solution containing 10 %(v/v) glycerol. 200µl aliquots of the suspension were dispensed into prechilled Eppendorf tubes, stored immediately at -80°C.

Agrobacterium Transformation procedure

Transformation *A. tumefaciens LBA4404* was according to Holsters *et al*; (1978) procedure with some modified as fellow:

Tube of competent cells was out of -80°C and was putted on ice to permit it to thaw to 15 min. Number of 2ml Eppendorf tubes were putted in ice to pre-chill, 50 µl of competent cells were transferred into pre-chilled tube and remained cells were refrozen in -80C°, 1 µl /18 ng of (recombinant plasmid isolated from transformation *E. coli DH5α)* was adding to the competent cells and the contents were mixing gently by moving the pipette through cells and the mixture was incubated on ice for 30 min. Each transformation tubes were frozen in liquid nitrogen for 5 min.

This step was modified by research frozen each tube in -80°C (in deep frieze) for 7 min. And then the tubes were heat shocked by placed the tube into 37°C water bath for 5 min. And then placed immediately on ice for 5 min, 900µl of SOC media without antibiotic was added to the tube and incubated at 30°C (temperature degree 30°C was according recommendation of Takara company which provided *A. tumefaciensLBA4404* cells with shaking in a water bath shaker at 100 rpm for 2 h.100 µL of transformed cells were cultured on LB plates containing 50 µg/mL kanamycin and 100 µg/ml to 72 h. At 30°C, the control tube was making without added plasmid.

The colonies appeared in the plates were counted and the transformation was confirmed by colony PCR and isolated the plasmid from the transformed bacteria. 100µg/ml. According to Takara protocol, the transformation efficiency was tested with 1µl/ 1 ng of PRI 900 DNA, and the colonies were selected in plates containing kanamycin 50µg/ml and 100 µg/ml streptomycin.

Colony PCR for A. tumefaciens LBA4404 transformation

Three independent *A. tumefaciens* transformation colonies were randomly chosen and checked for the

insertion by PCR. Colony PCR procedure was depended on (CIMMYT, 2005); glycerol stocks were prepared according to Holsters *et al*; (1978).

Result

RNA isolation and amplification Hu-GBA gene

The highest RNA concentration was achieved from blood with the GENEzol™TriRNA Pure Kit showed in (Fig.3). *Hu-GBA gene* was amplified from human blood sample with its own signal peptide using the forward primer (with its ATC code at 5′ end) and the reverse primer, PCR products were analyzed on 1% agarose gel.

A band of correct size (1561 bp) corresponds to the full-length *Hu- GBA* open reading frame can be seen in (Fig.4).

The sequence data was analyzed by program in the website http://WWW.blast.ncb.nih.gov.

The percentage of identity was found 100% to Homo sapiens glucosylceramidase beta (GBA), transcript variant 1, mRNA NM_000157.3 Gene Bank. (Fig.5)

The nucleotides sequence of the Hu-GBA cDNA was little modification to clone the gene inside pCAMBIA1304 for transformation it in plant cells by engineered *BgIII* restriction site between the 35S CaMV promoter and the GBA cDNA sequence and engineering 7pb including nucleic acids for *BstEII* restriction site between the GBA cDNA sequence and the NOS terminator.

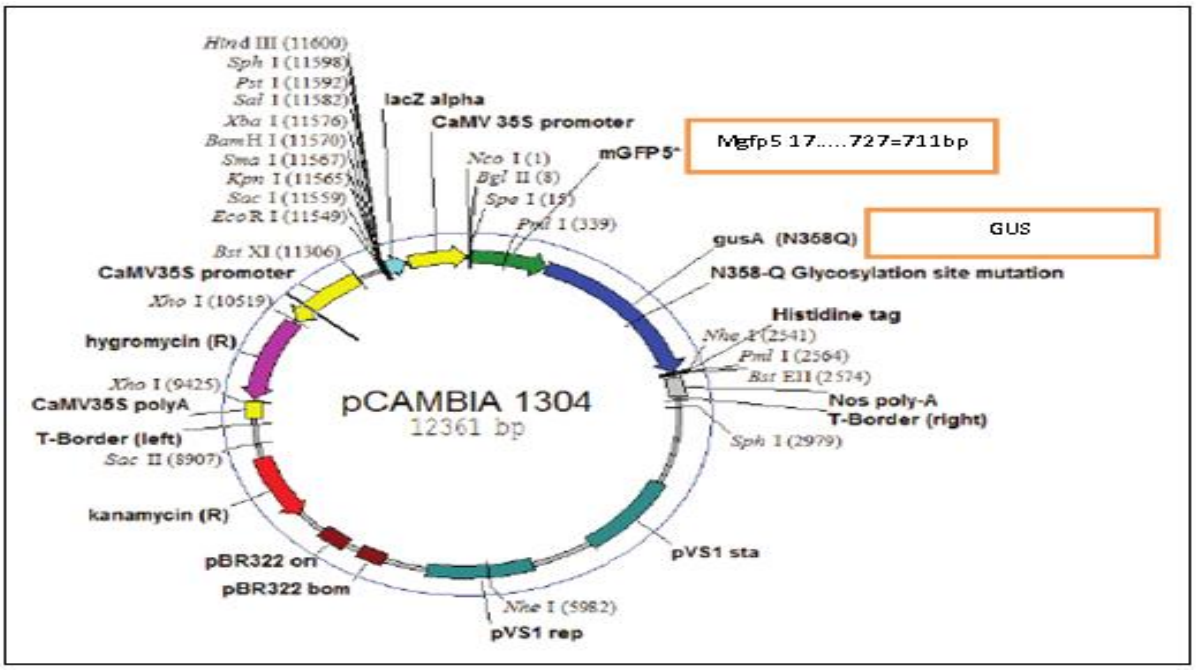

Fig.1.A map of the PCAMBIA 1304 including the right (RB) and left (LB) T-DNA border fragments from a nopaline strain of A. tumefaciens with bar-glucuronidase gene ; gusA:mgfp5 fusion; P35S,CaMV 35S promoter; TEV, tobacco etch virus.

This constructed *GBA gene* was made by using PCR technique and primers were designed to contain these further sequences. Hu-GBA PCR product and the Pcambia1304 plasmid were digested with BgIII/BstEII restriction enzymes. Digested *Hu-GBA gene* generated was cloned into the *BgIII/BstEII*sites of pCAMBIA1304 to produce Pcambia1304-GBA. (Fig.6, a. b) pCAMBIA-1304 was doubled digested with *BgIII* and *BstEII* restriction enzymes, resulted two bands (Fig.7) the upper band (9,791 bp) presented the DNA plasmid and the down (approximately 2,751 bp) represented the *mgfp5 gene* 711bp, *GUS gene* 1806 bp and *6xHis gene* (about

2,751 bp). The final was then realized toplasmid.allow the 1561bp to be introduced into the

Ligation of GBA gene and pCAMBIA1304 vector

The digested *GBA gene* was cloned in the sites *BgIII* and *BstEII* of pCAMBIA1304 to generate recombinant Pcambia 1304-GBA and the ligation product was used to transformation of *E. coli ah5α* cells with pCAMBIA 1304-GBA**.**

The plasmid pCAMBIA 1304-GBA was successful transformed into *E. coli DH5α* using calcium chloride and heat shock transformed method and screening on kanamycin antibiotic containing LB media. The efficiency of transformation was perfect which was enumerating from the colonies number on the plate (Fig.8) shows the transformation of pCAMBIA 1304-GBA into *E. coli DH5α* and transformation efficiency.

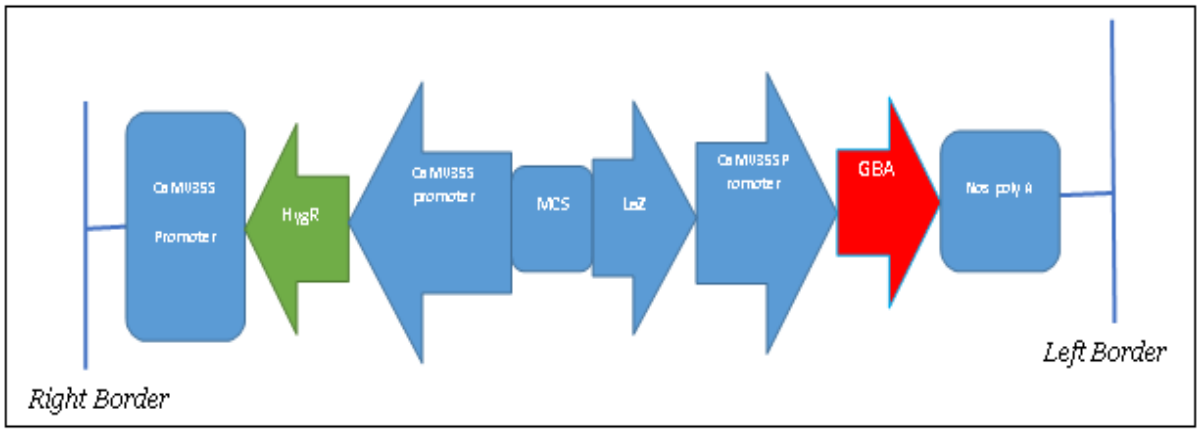

Fig. 2. T-DNA region of construct pCAMBIA1304-GBA.

Screening for the present of GBA Gene inserts by direct transformation E. coli PCR.

The insert- specific primers were successful in identified clone that contained *GBA gene* with its signal peptide by direct bacteria PCR (Fig.9) Shows GBA bands on 1% agarose gel using colony PCR. , 2 colonies were chosen for a screening by colony PCR from transformed bacteria on LB kanamycin plate, all colonies give positive result.

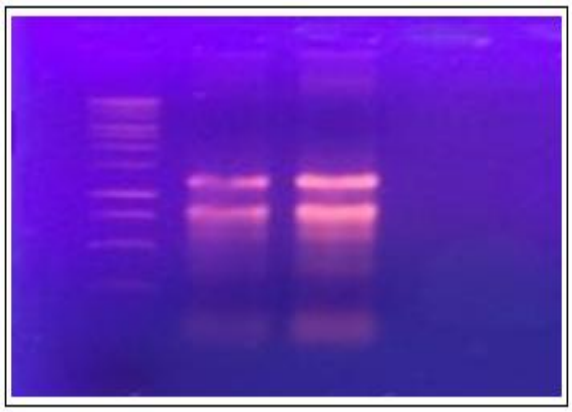

Fig. 3. Total human blood RNA on1% Agarose Gel Electrophoresis.

Isolation of the constricted plasmid Pcambia 1304-GBA from transformed E. coli DH5α and confirmation by Gel electrophoresis

The Pcambia 1304-GBA plasmid was isolated from the transformed bacteria which give positive result with colony PCR and was confirming by gel electrophoresis.

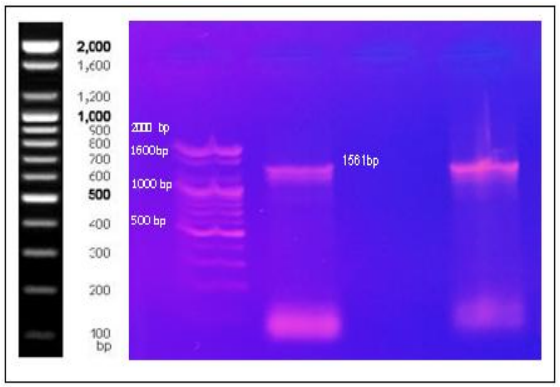

Fig.4. The Analysis of 1% Agarose Gel Electrophoresis for amplification GBA gene from human blood, M100bp: DNA marker, Lane1, 2: GBA gene1556bp.

The (Fig.10) of the DNA plasmid band in agarose gel clearly signals the presences of the pCAMBIA 1304-GBA plasmid, a fragment of correct size 11353 bp, indicated the pCAMBIA1304, and GBA fusion gene compare with entire Pcambia 1304 plasmid without *GBA gene*12361bp.

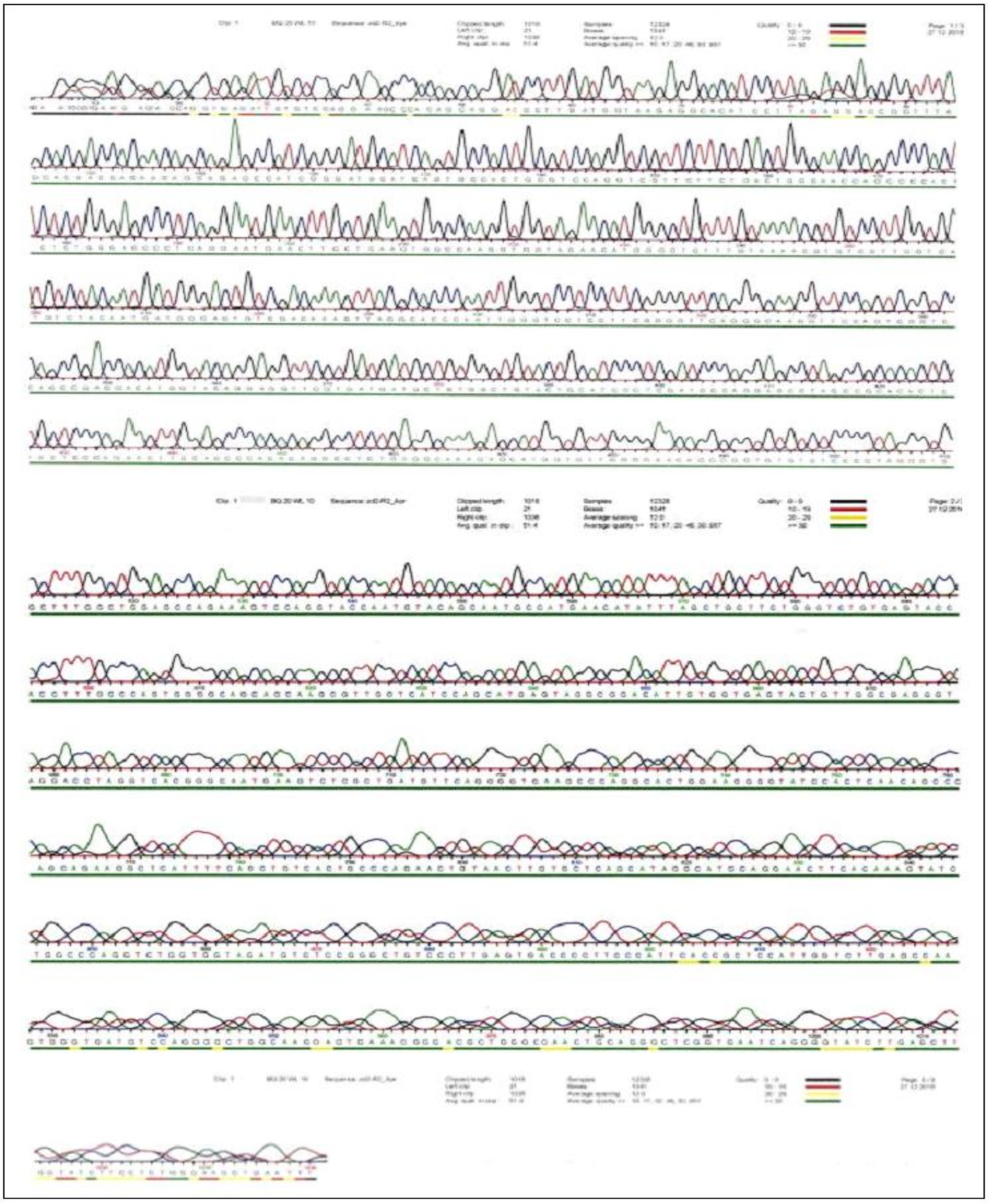

Fig. 5. Analysis of isolated Human GBA gene using gene sequencer.

Confirm integration of GBA gene in Pcambia 1304 plasmid

The fusion *GBA gene* with Pcambia plasmid was PCR amplified from constricted plasmid which was isolated from transformed *E. coli DH5α* using specific primers to amplified GBA (Fig.11) shows the result of the PCR product to amplified GBA fusion gene form constricted plasmid pCAMBIA1304-GBA.

Transformation of Pcanmbia 1304-GBA to Agrobacterium tumefaciens LBA4044

The plasmid including the interested gene (Pcambia-GBA) was successes transformed into *A.tumefaciens LBA4044* by using calcium chloride transformation. *A. tumefaciens LBA4044* harbored the transformed plasmid was selected on YEPmedium contains kanamycin and streptomycin antibiotics (Fig.12a, b,)

shown *A. tumefaciens* untransformation and transformation colonies. And the transformation efficiency was tested by transformation A. *tumefaciens* with pRI 900 DNA using the same protocol for bacteria transformation result of bacteria transformation with pRI 900 DNA showed in (Fig. 12c).

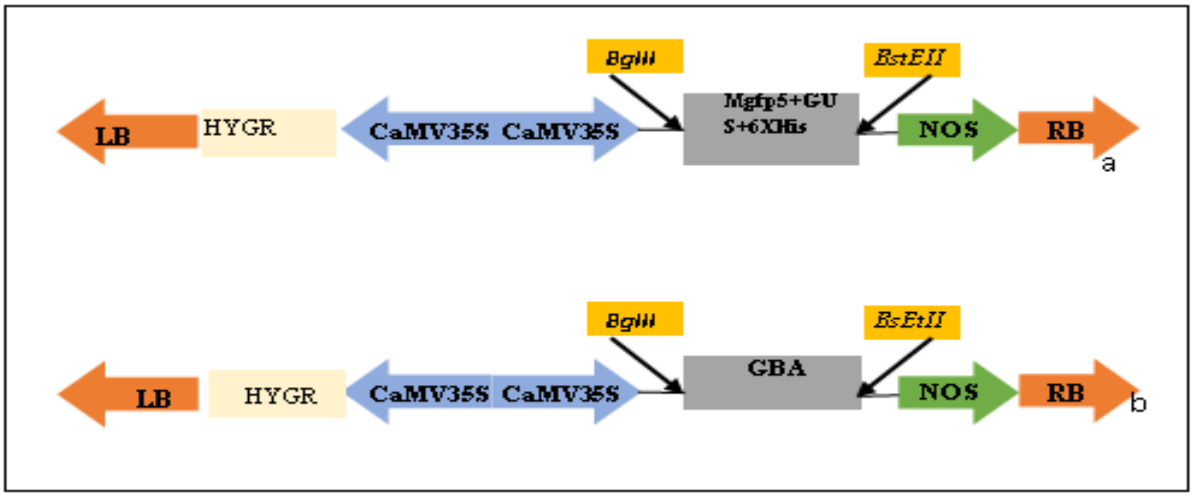

Fig.6.T-DNA region of Pcambia1304 LB and RB: Left and Right Borders, a. contains GUS gene between, CaMV35s promoter and NOS terminator. b .T-DNA region of Pcambia1304 LB and RB: Left and Right Borders, contain GBA gene, HYG(R): Hygromycin selectable marker, CaMV35s: Cauliflower Mosaic Virus promoter, *BgIII* and *BstEII*: restriction sites, and NOS: Nopaline Synthase terminator.

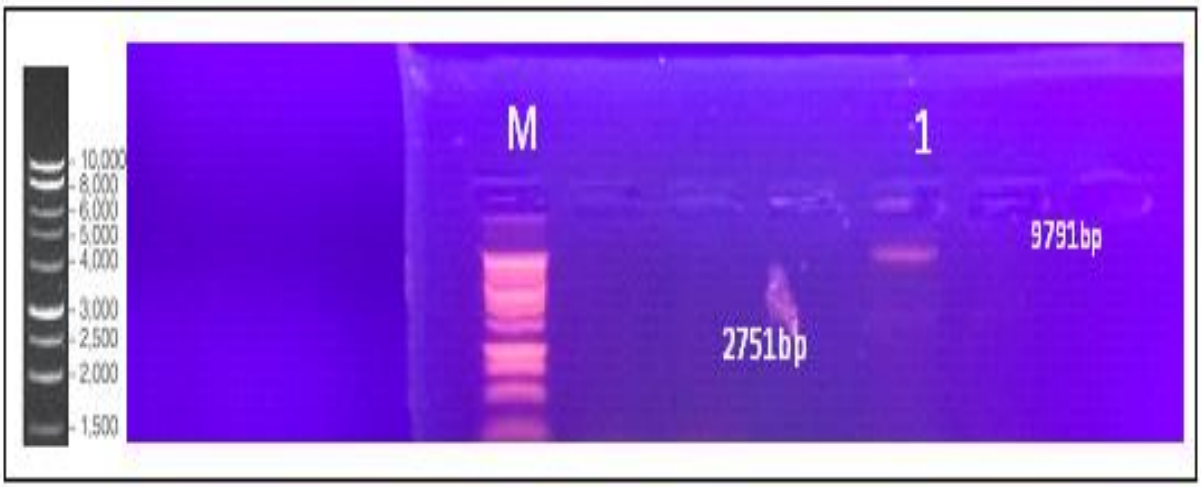

Fig. 7. Digestion of pCAMBIA 1304 with both *BgIII* and *BstEII*. Lanes 1: digested pCAMBIA 1304 resulting in two bands; lane M: Gene Ruler 1 kb DNA ladder.

The presences of *GBA gene* inserts was screened by direct *A. tumefaciens LBA4044* PCR, specific primers were successful in identified clones containing *GBA* (Fig.13), shows amplification using specific primers of the *GBA* portion of integrated pCAMBIA1304-GBA (1561bp) from only two selection colonies. The plasmid pCAMBIA 1304-GBA was isolated from the transformed bacteria which give positive result with colony PCR and was confirming by gel electrophoresis.

The figure of the DNA plasmid band in agarose gel clearly indicates the presences of the pCAMBIA 1304-GBA plasmid, a fragment of correct size 11353 bp, indicated the Pcambia1304, and GBA fusion gen (Fig.14).

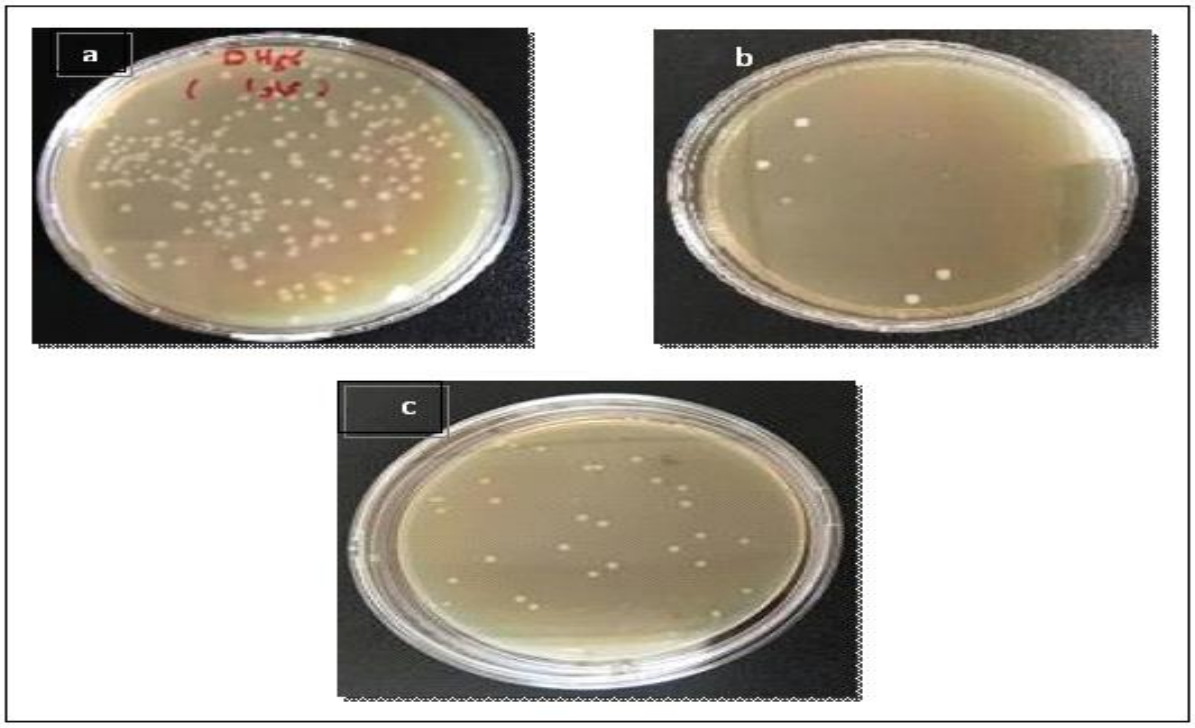

Fig. 8. *E. coli DH5α* colonies, a. Untransformation E. coli DH5α colonies on LB Plate without Kanamycin antibiotic. b. Transformation *E. coli DH5α* on LB Plate with Kanamycin antibiotic**, c.** Transformed efficient of *E. coli DH5α* colonies on LB Plate with Kanamycin antibiotic.

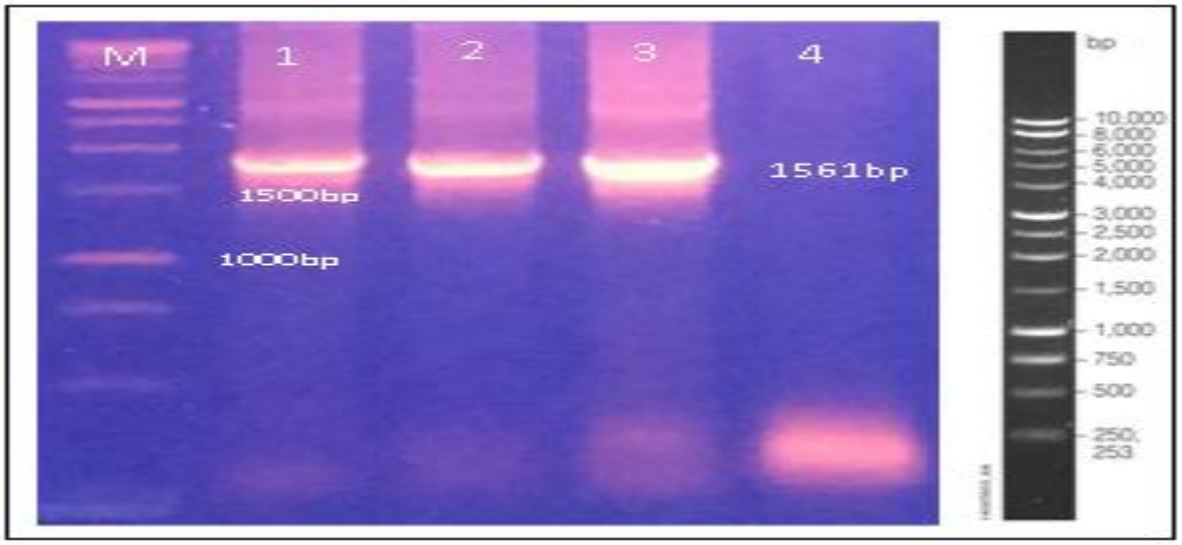

Fig . 9. PCR screening for GBA insertions result from colony PCR on transformed *E. coli DH5α* bacteria M: 1 kb DNA Ladder, Lane 1-2: transformed colony (contain *Hu-GBA gene*), Lane 3: positive control GBA gene Lane 4: untransformed colony as negative control (without *Hu-GBA gene*).

Discussion

RNA Isolation

From figure (3) the safety isolated RNA was determined by 1% gel electrophoresis and the separated bands were distanced on gel and this result was compatible with GENEzol™ TriRNA Pure Kit recommendation, that assure purified and undigested isolated RNA from blood, isolation RNA from cells required for lysis cells and obstruction of cellular nucleases; hence, strong denaturing break down conditions are essential for getting right RNA (Sambrook and Fritsch., 1989). Because the human

blood has a highly percentage of protein that can decrease extraction efficient, protocols included protease digestion must be employed tospring protein contamination, as well as to increasing RNA product with large purity (Birnboim, 1992). Using GENEzol™ TriRNA Pure Kit protocol can increase affect the product and goodness of RNA due to efficient for recovery of minute RNA quantities in peripheral blood and this kit extract the total RNA in high denaturation buffer of guanidine- isothiocyanate-content which instantly Disable RNase activity to insure isolation of right RNA.

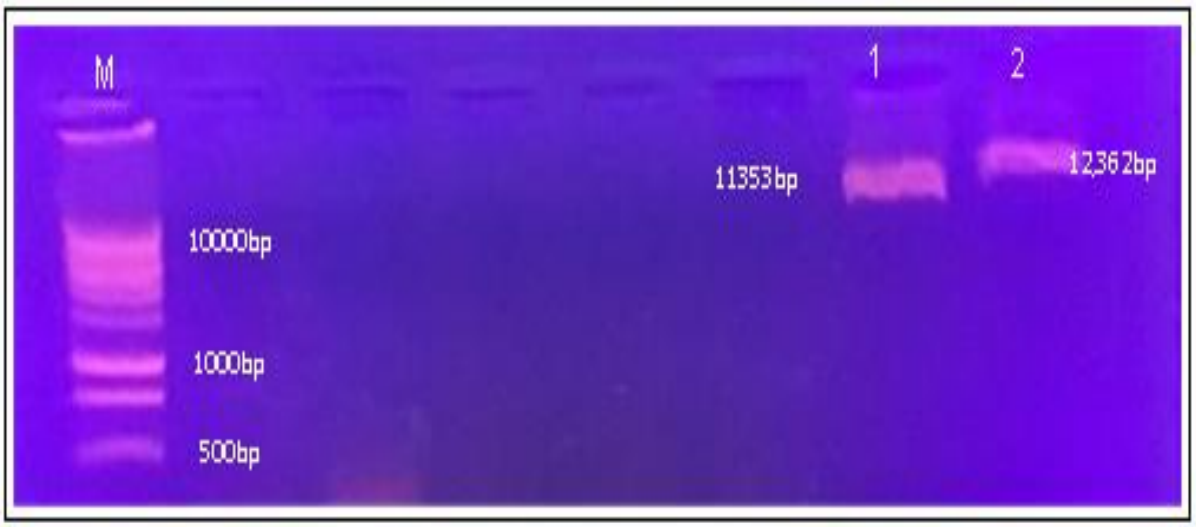

Fig. 10. Recombinant Plasmid Pcambia 1304-GBA isolation from transformation *E.coli* DH5α, Band of correct size (11353 bp, indicated corresponding to the Pcambia 1304, and GBA fused gene was observed in lane 1 ,lane 2, Pcambia1304 Control. (Lane M) ladder was used for size comparison.

Reverse transcription and amplification GBA gene

The result in section (3.1) and figure (4) showed that cDNA resulted from isolated RNA was successful in amplification *Hu-GBA gene* when it used as template in the present of oligodT_{20} and reverse transcriptase enzyme by designed primer pairs specific to amplification this gene and the size of resulted band 1561bp.

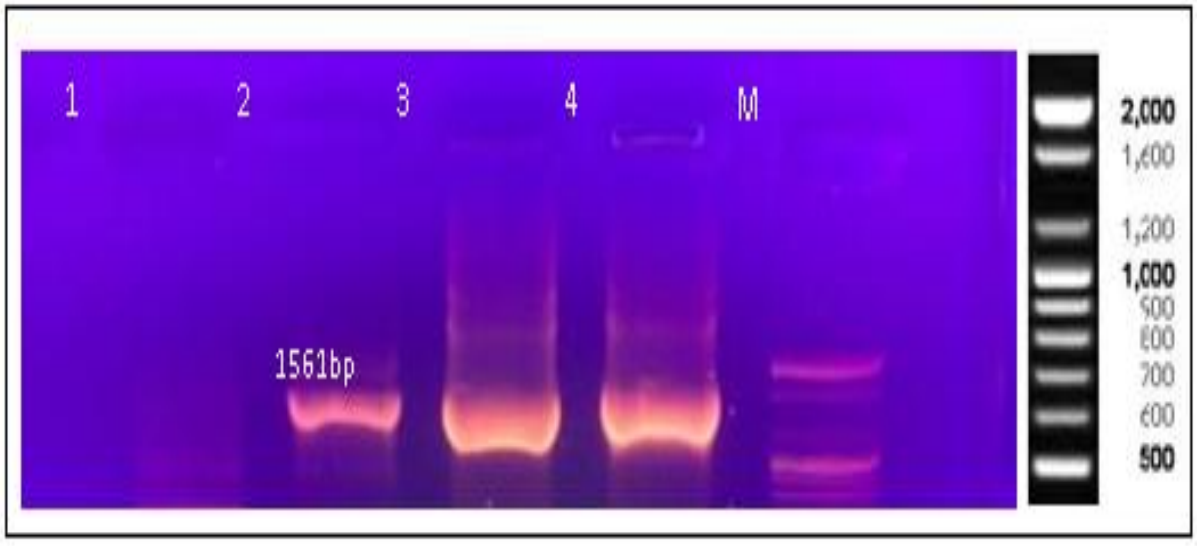

Fig. 11. PCR amplification *Hu-GBA gene* from pCAMBIA1304-GBA isolated from transformed bacteria as template M: 100 kb DNA Ladder. Lane 1: Negative control (pCAMBIA1304 without H*u-GBA gene*). Lanes2: positive control (*Hu-GBA gene*). Lane3-4: amplification was carried using primer with *GBA gene* signal peptide.

This result was agree with the study of Lulu *et al*., (2017),when using blood sample as source for *GBA gene* and the result showed that Amplified was easy accomplished with 50 µg/µl total RNA isolated from fresh human blood with using RT Premix Kit (Bioneer) and PCR Master Mix kit (Promega). In additional the glucocerebrosidase is essential for basal cellular activities and therefore, are constitutively expressed in most cells, it is a housekeeping gene is expressed ubiquitously, though at fading levels so isolated from blood sample(Aerts *et al*., 1987; Doll *et al*., 1993; Ponce *et al*., 2001).

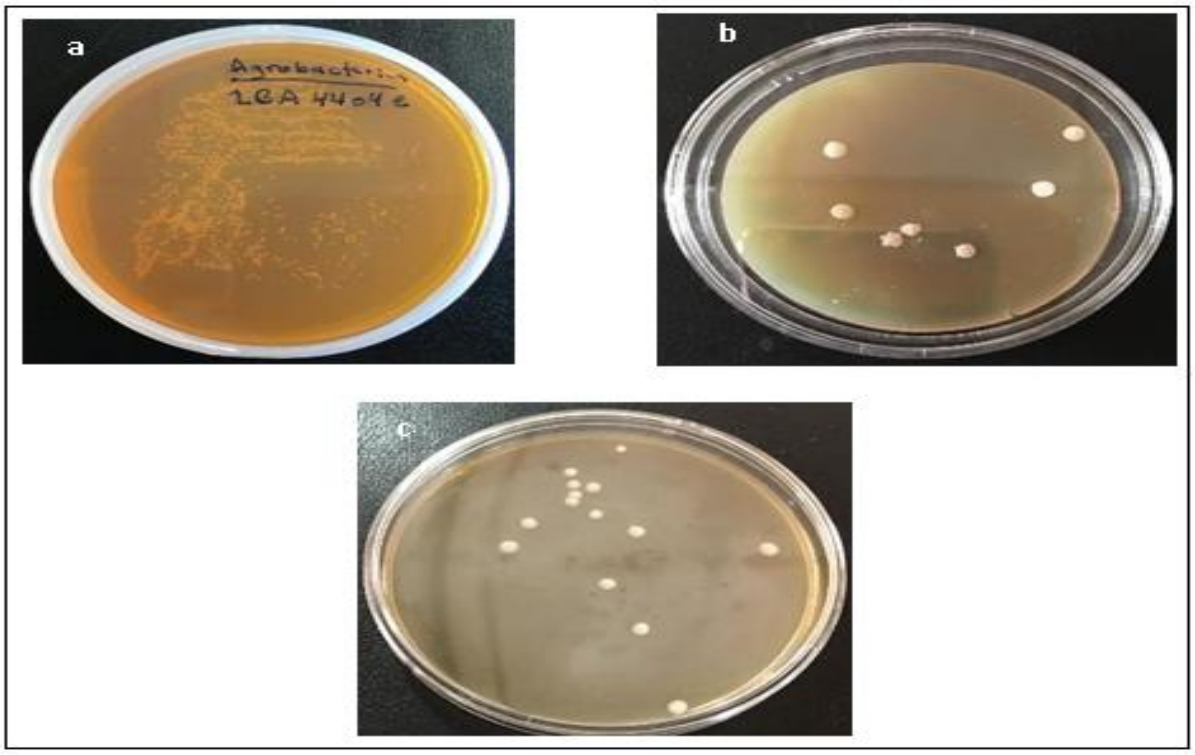

Fig. 12. *Agrobacterium tumefaciens* LBA4404 colonies, a. un transformation colonies on plate contain rifampicin antibiotic, b. transformation colonies with Pcambia1304-GBA on LB plate contain streptomycin and kanamycin antibiotics, c. transformation colonies with pRl 900 DNA on LB plate contain streptomycin and kanamycinantibiotics.

Cloning of Hu-GBA gene into the binary vector pCAMBIA304 In figure (6) *Hu-GBA gene* replace (Mgfp5+GUS+6His) genes because the aim of our search was produced *Agrobacterium* strain contain binary vector has human *GBA gene* to became ready for transform any desired plant to expression this gene, this result work in with result of Behnoush *et al*., (2014) in cloning proinsulin gene in pCAMBIA1304 instead of (Mgfp5+GUS+6His) genes for expressed proinsulin gene in tomato plant, and because the Plant genetic engineeringbuild on promoters example the cauliflower and figwort mosaic virus (CaMV, FMV; Benfey and Chua 1990; Sanger *et al*., 1990) and *Agrobacterium tumefaciens* (Azhakanandam *et al* ., 2015), so to achievement this aim have been used the binary plasmid Pcambia 1304 which contain cauliflower mosaic virus promoter.

The promoter, coding sequence, and terminator are basic ingredientsdesired to successful product mRNA in plant cells.

The promoter contains the requisite information to induct the transcription machinery and begin transcription. The coding sequenceincludes thedesirable stamp which able to take the shape of a protein or RNA.

The terminator supply information to ending transcription and signal polyadenylation (Birch 1997).

Fig. 13.PCR screening for GBA insertions result from colony PCR on transformed *Agrobacterium tumefaciens* LBA4404 M: 1 kb DNA Ladder, Lane 1-3: transformed colony (contain *Hu-GBA gene*), Lane 4: untransformed colony as negative control (without *Hu-GBA gene*).

Digestion GBA gene and pCAMBIA1304 plasmid with restriction enzymes and Ligation of Hu-GBA gene with Pcambia 1304

The result in figure (6) was used *BgIII* and *BstEII* were requisite for *GBA gene* to beintegrated in reporter genes region of pCAMBIA1304 in the correct direction, that is, in a 5`to 3`direction comparative to the 5`to 3`orientation of the GBA gene. As the *BgIII* and *BstEII* sites were Present after the CaMV35s promoter and before the NOS terminator of pCAMBIA1304 vector, the primers were designed to contain *BgIII* site at the 5`end of the forward primer and the *BstEII* site at the 5`end of the reverse primer, in additional using these enzymes do not have recognition sites on full length GBA cDNA depending on the composite restriction map of human glucocerebrosidasec DNA (Sorge *et al.*, 1985), therefore cannot digested the entire *GBA gene*

The insert 1558bp *GBA gene* (1558bp after removing three nucleotides by restriction enzyme) was ligated to the binary vector to generate recombinant Pcambia 1304-GBA, using T4 ligase (Bioneer) which catalyzed binned of an acceptor oligonucleotide that contain adesired 3`OH group, and giver oligonucleotide that contain a desired 5`phosphate group,(Alexander *et al.*, 2003).

Fig. 14. Lane1, constricted plasmid Pcambia 1304-GBA isolated from transformed *A. tumefaciens LBA4044*. M: 1 kb DNA Ladder.

Cloning recombinant vector into E. coli DH5α cells using calcium chloride heat-shock transformation

The result in figure (8.b) shown the successful of introduce recombinant plasmid in *E. coli DH5α* cells and ability these cells to grow on LB plate with kanamycin antibiotic using calcium chloride heat-shock transformation methods this result agree with Nazanin *et al.*,(2017) result in used *E. coli DH5α* cells to multiplication recombinant pCAMBIA-IFN-γ using calcium chloride heat-shock method. This method used to induce bacteria cells artificially to become competence by calcium chloride because some bacteria species are not normally competent for DNA uptake at any stage of life cycle (Dagert, 1979; Hiroaki, 1990). The transformation process by calcium chloride heat-shock promotes bacterial cells to take DNA from the surround environment, the mechanism of this processes how works is yet largely strange, but there are suppositions on the different of the portion procedure the calcium ions role in the cell suspension is form a cation viaduct between the negative charges on phosphate backbone of DNA and phosphorylated lipid A in lipopolysaccharide (LPS), (Delucia *et al.*, 2011; Liu *et al.*,2006). Ice-cold CaCl2 solution eases adherence of DNA to the cell surface, and after short time of heats hock can enters the cell, (Bergman, 1981). *E. coli DH5α* cells were used for the cloning steps and repertory recombinant plasmid (Pcambia1304-GBA gene), and used as base strain for the multiplication the recombinant binary vector (Nazanin *et al.* 2012).

Cloning recombinant vector into A. tumefaciens LBA4044

From the result in figure (12) showed that modified heat-shock method was able to introduce recombinant Pcambia1304-GBA into *Agrobacterium* cells. Using *Agrobacterium tumefaciens LBA4044* as biotechnological tools for genetic transformation with Pcambia1304 was corresponded with many studies like Shahrzad *et al.*,(2012); Behnoush *et al.*, (2014) ; Nazanin *et al.*,(2012) offers many advantages as the chance to transport only one or few copies DNAsegments, contains the interesting gene at higher efficiency with minimum cost and the transfer of very large DNA fragments with lower rearrangement (Shibata and Liu, 2000) and this *Agrobacterium* can be used for transformation of dicotyledonous and monocotyledonous plants (Abne, 2010; Sood *et al.*, 2011).The cloning of Pcambia1304-GBA in *Agrobacterium* was confirmed by amplification *GBA gene* using transformed *Agrobacterium tumefaciens LBA4044* colony as template in colony PCR reaction and plasmid isolated results in Figures (13 and 14) these results showed the bands for GBA amplified from colonies Agrobacterium *tumefaciens LBA4044* and the constricted plasmid which isolated from these colonies were less shine compere with plasmid and GBA bands amplified from transformed *E. coli DH5α* colonies, Colony PCR does not work well with *Agrobacterium* because the great majority of ori in binary vectors that function on *Agrobacterium* willprotect the plasmid at very low copies (1-5 per cell) asdissenting the ori that function in *E.coli* (>300 copies in each cell) pVS1 origins maintain copies plasmid number ranging from three to twelve copies per cell (Oltmanns *et al.* 2010; Sripriya *et al.* 2011; Veluthambi *et al.* 1987) pBR322 origin of replication. Facilitates plasmid replication in *E. coli.* Plasmids carrying this origin exist in low copy numbers (15-20 per cell) in *E. coli* if Rop protein is present, or medium copy numbers (100-300 per cell) if Rop protein is absent(Kazan and Manners,2008).

Conclusion

In this study, total RNA was isolated from fresh Peripheral human blood using GENEzol™TriRNA Pure Kit (100rxns) from Geneaid Company used as source for amplification *Hu-GBA gene* using specific primers, and modified Calcium chloride heat-shock transformation, which provides an efficient inserted of gene into *A. tumefaciens LBA4044*, modified agrobacterium contain recombinant plasmid Pcambia1304-GBA will be act as a source for produced recombinant human glucocerebrosidase in plant.

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