

**UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE BIOCÊNCIAS
CURSO DE GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS**

Trabalho de Conclusão de Curso

**Proposal for the Production of Human β -
Glucocerebrosidase Enzyme in Transgenic Maize Plants**

(Estudo da Produção da Enzima Humana β -Glicocerebrosidase em Plantas
Transgênicas de Milho)

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Porto Alegre, Dezembro de 2011

AGRACEDIMENTOS

Primeiramente, agradeço ao Professor Dr. Giancarlo Pasquali. Ele foi quem pacientemente nos ajudou com a miríade de tarefas demoradas e documentações que tivemos de cumprir ao entrar no Brasil. Seu tempo passado longe de seu laboratório e da sua família foi imensamente apreciado. Além disso, como meu orientador, Giancarlo estava sempre disposto a explicar partes da pesquisa ainda não compreensíveis frente à minha formação prévia, e das quais obtive grande proveito. Ele foi um componente crucial do sucesso de meus estudos no Brasil.

Agradeço aos demais membros do laboratório, Patrícia R.D. Picolotto, Juliana D.K. Borges, Camila B. Scalco, Rochele P. Kirch, Luisa A. de Oliveira e Guilherme Pizzoli, por suas ajudas e orientações na execução correta das técnicas de laboratório.

Finalmente, agradeço à minha Universidade de origem, *The Ohio State University*, junto com a Universidade Federal do Rio Grande do Sul e o Programa FIPES-CAPES, os quais me proporcionaram a oportunidade e a capacidade financeira para estudar no Brasil.

AGRACEDIMENTOS ESPECIAIS

Este trabalho foi desenvolvido no Laboratório de Biologia Molecular de Plantas do Centro de Biotecnologia e Departamento de Biologia Molecular e Biotecnologia do Instituto de Biociências, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brasil, de Julho a Dezembro de 2011.

Eu, Daniel M. Cáceres, sou estudante de graduação em Biologia, Premedicina da *Ohio State University*, Columbus, OH, Estados Unidos da América, selecionado para um período de intercâmbio e treinamento na UFRGS, junto ao Programa de Colaboração e Intercâmbio Brasil-Estados Unidos (FIPSE/CAPES 048/06) promovido pelo *Fund for the Improvement of Postsecondary Education* (FIPSE) do Departamento de Educação Norte-Americano e pela Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) do Ministério da Educação Brasileiro. Agradeço ter tido a oportunidade de realizar esta pesquisa e estudo no Brasil.

SPECIAL ACKNOWLEDGEMENTS

This work was performed at the Laboratory of Plant Molecular Biology of the Center for Biotechnology and the Department of Molecular Biology and Biotechnology of the Biosciences Institute, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil, from July to December 2011.

I, Daniel M. Cáceres, am an undergraduate student of Biology, Pre-Medicine at The Ohio State University, Columbus, OH, United States of America, selected for an exchange training program at UFRGS, within the Collaborating Program and Exchange Brazil-USA (FIPSE/CAPES 048/06) promoted by the Fund for the Improvement of Postsecondary Education (FIPSE) of the U.S. Department of Education and *Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) of the Brazilian Ministry of Education. I am grateful to these programs and those involved for having capacitated the opportunity to perform this research and study in Brazil.

ABBREVIATIONS

- **GBA:** Acid Beta glucosidase
- **GCase:** glucocerebrosidase
- **ERT:** enzyme replacement therapy
- **CHO:** Chinese hamster ovary
- **SRT:** substrate reduction therapy
- **Mt:** megatons, or million metric tons
- **FDA:** Food and Drug Administration
- **PCR:** polymerase chain reaction
- **DNA:** deoxyribonucleic acid
- **cDNA:** complementary DNA
- **mRNA:** messenger ribonucleic acid

RESUMO

A doença de Gaucher é a mais comum das doenças lisossômicas de acumulação. É uma doença rara e hereditária transmitida por herança autossômica recessiva, e é causada por uma deficiência da enzima glicocerebrosidase. A falta desta enzima resulta em acúmulo de glicolipídios no baço, fígado, pulmões e medula óssea. A doença de Gaucher afeta entre 45.000 - 60.000 pessoas em todo o mundo, embora a frequência entre judeus asquenazes sejam de 1 em 850, ou seja, com muito maiores chances de herdar a doença. O tratamento atual para a doença de Gaucher é a terapia de reposição enzimática, pela qual uma forma recombinante da glicocerebrosidase é administrada por via intravenosa a cada duas a três semanas. Esta enzima recombinante é produzida em células de ovário de hamster chinês e vendida sob o nome de *Cerezyme* pela *Genzyme Corporation*. A terapia de reposição enzimática é muito cara, custando aos governos de 200.000 a 300.000 dólares por ano. Plantas transgênicas oferecem uma alternativa mais fácil e menos cara para a produção de enzimas humanas quando comparadas ao emprego de células animais recombinantes. Plantas também são incapazes de servir como hospedeiros a patógenos humanos, o que reduziria um dos riscos de contaminação. Pelo presente Projeto, propõe-se e descreve-se o processo da produção recombinante de glicocerebrosidase humana transgenicamente produzida em plantas de milho. O sucesso nesta produção poderá resultar em uma forma muito mais barata, e uma alternativa para a terapia de reposição enzimática para pacientes da doença de Gaucher.

ABSTRACT

Gaucher's disease is the most common of the lysosomal storage disorders. It is a rare, hereditary disease passed on by autosomal recessive inheritance, and it is caused by a deficiency of the glucocerebrosidase enzyme. This missing enzyme results in glycolipid accumulation in the spleen, liver, lungs and bone marrow. Gaucher's disease affects 45,000 to 60,000 people worldwide, though Ashkenazi Jews have higher chances of inheriting the disease, 1 in 850. The current treatment for Gaucher's disease is enzyme replacement therapy, by which a recombinant form of glucocerebrosidase is intravenously administered every two to three weeks. This recombinant enzyme is produced in Chinese hamster ovary cells and sold under the name of *Cerezyme* by *Genzyme Corporation*. Enzyme replacement therapy is very expensive, costing approximately 200,000 to 300,000 dollars annually. Transgenic plants offer an easier, less expensive alternative for human enzyme production when compared to recombinant animal cells production. Plants are also unable to serve as hosts for human pathogens, which would reduce the risk of contamination. Through the present Project, we propose and describe the process of producing recombinant glucocerebrosidase transgenically in maize plants. Success in the production will result in a much less expensive alternative form of enzyme replacement therapy for Gaucher's disease patients.

BIBLIOGRAPHIC REVIEW

The human GBA gene, encoding acid β -glucosidase, or glucocerebrosidase (GCCase), is 7.5 kb long and consists of eleven exons and ten introns. It is located on the longer arm of chromosome one at position twenty-one (1q21) (1). The human GCCase protein is a lysosomal membrane enzyme. Lysosomal enzymes are synthesized in the endoplasmic reticulum and tagged for lysosomes in the Golgi apparatus. This tag comes in the form of mannose-6-phosphate labels (2). The function of the GCCase is to cleave the β -glucosidic bond of glucosylceramide by hydrolysis (3). GCCase's cleavage of glucosylceramide prevents the unhealthy accumulation of lipid glucocerebrosides. Mutations in the GBA gene can result in a nonfunctional GCCase enzyme. Nonfunctional GCCase results in lipid glucocerebroside accumulation in the body (4). This condition is known as Gaucher's (GOH-SHAY's) disease.

Gaucher's disease is the most common of the lysosomal storage disorders. It is a rare, hereditary disease passed on by autosomal recessive inheritance. Gaucher's disease is caused by deficiency of the GCCase enzyme (5) and is characterized by material accumulation in the spleen, liver, lungs, and bone marrow (4) (**Figure 1**). There are three forms of the disease, types I, II, and III, described as the non-neuropathic, acute neuropathic, and chronic neuropathic forms respectively (6).

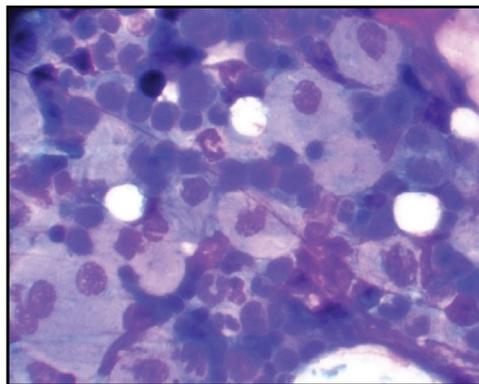


Figure 1: Gaucher's cells. Photomicrograph of bone marrow showing foamy histiocytes characteristic of Gaucher's disease (8).



Figure 2: Osteolytic Lesion
X-ray of osteolytic lesion in humerus bone.

Type I is the most common type and results in the aforementioned symptoms, with the enlargement of the spleen and liver occurring most often. Other symptoms include osteolytic lesions, anemia, and hepatic fibrosis (**Figure 2**). While Type 1 Gaucher's disease only affects 45,000-60,000 people worldwide, 1 in 850 Ashkenazi Jews are afflicted, and an approximated 1 in every 15 Ashkenazi Jews is a carrier of the disease (7).

Type II is the rarest form of the disease and is characterized by rapid neurological deterioration. It usually has a very early onset and the afflicted most often die by the age of two. Type III Gaucher's disease also results in neurological problems, but they tend to progress more slowly and more mildly than type II. Symptoms for type III are onset at varying points in the life of those afflicted (8).

Gaucher's disease was first observed in 1882 by Phillippe Gaucher, a 28 year old French doctor after whom the disease is named. Observing large cells during a splenic aspirate in a spleen, Gaucher thought it was a splenic neoplasm (9). Almost forty years later, in 1924, Epstein recognized the storage of glucocerebrosides (10) and, later, in 1965, Dr. Roscoe Brady and his team described that this storage was due to a lack of the GCCase enzyme (11).

Gaucher's disease has no cure, but treatments of the symptoms for types I and III of the disease have proven successful. The most common treatment for Gaucher's disease is enzyme replacement therapy (ERT), whereby the missing enzyme is

administered intravenously for two hours every two to three weeks (5). This enzyme is a recombinant form of GCCase expressed in Chinese hamster ovary (CHO) cells. It is currently supplied by *Genzyme Corporation* under the name *Cerezyme*[®] (4). ERT with *Cerezyme*[®] has been shown to reduce and relieve symptoms of Type I Gaucher's disease (7). Another form of treatment is substrate reduction therapy (SRT), in which the production of glucocerebrosides is slowed, reducing the glycolipid accumulation (8). *Miglustat*, called *Zavesca*[®], is one such product, sold by *Actelion* for when ERT is not an option. This alternative to ERT can become necessary due to allergic reactions, hypersensitivity, poor venous access, or financial inability to afford ERT (12).

The recombinant form of GCCase supplied by *Genzyme Co.* is expensive. Costs for using ERT range from US\$ 200,000 to US\$ 300,000 annually per person (13). This high cost limits the number of patients able to receive treatment. Recombinant GCCase is also a difficult enzyme to biochemically synthesize, and human cells synthesize small amounts of the protein. Increased levels of production are inhibited by a protein called TCP80. Unfortunately, GCCase and TCP80 analogues are found in all mammals (14). Alternative sources for this recombinant protein have already begun to be explored. One such alternative is protein expression in transgenic plants.

The production of human proteins in transgenic plants offers many economic and qualitative benefits over current forms of production. One such advantage includes reduced health risk, as plants are unable to serve as hosts for human pathogens (15). Another advantage plants offer is that proteins stored in seeds can be stored for longer time and more easily than proteins in animal cells. Transgenic CHO cells, for example, must be processed soon after harvest to prevent significant enzyme loss (5). Transgenic

seeds, on the other hand, have been shown to be able to be stored for weeks (and months or even years at colder temperatures) without experiencing significant enzyme activity loss (16). Besides that, TCP80 analogues have not been found in plants, which could enable higher protein yields due to the lack of GCCase inhibition (5).

Creating recombinant forms of GCCase in plants is an active field of research. A patent as recent as May 2011 was granted by the United States Patent and Trademark Office (USPTO) for the production of recombinant GCCase. This patent (Nr. 7,951,557) by Shaaltiel *et al.* lays claims to the production of a recombinant GCCase in carrot cells. Other similarly based patents lay claims on recombinant forms of other therapeutic lysosomal enzymes, also in plants (17).

Maize, or corn, has been an economically important crop for hundreds of years. Its ancestor, teosinte, was a fraction of maize's size. Selective breeding over many generations created the familiar maize found today (18). Maize sells today for around US\$ 280.00/Mt, or US\$ 7.00/bushel (19). Maize can grow up to 39 feet in height (12 meters), though most produced standardly is bred to grow around 8 feet tall (2.5 meters). Maize is grown in more countries than any other crop, and it is the most widely grown crop in the Americas. Over 800 Mt of maize are produced worldwide annually. The United States of America leads in production, supplying nearly 40% of the global total last year. USA produced 331 Mt (12.1 billion bushels) in 2010, followed by China's maize production of 158 Mt (6.2 billion bushels). The uses for maize are various and include livestock feed, fuel ethanol production and human consumption. Other uses for maize include the production of starches, sweeteners, corn oil, and beverage and industrial alcohol (20).

The use of biotechnology with maize is not a new concept. Maize has been transgenically modified in the past for agricultural reasons. Around 85% of the maize grown in the USA in 2009 was genetically modified. This form of transgenic maize is called *Bt* maize. The *Bt* protein produced by *Bt* maize serves as a pesticide against Lepidoptera larvae. The Bt endotoxin, originally from the entomopathogenic bacterium *Bacillus thuringiensis*, is a very selective protein and does not harm insects of other orders. It is also considered safe for humans, other mammals, birds, and the environment and it was approved by the US Food and Drug Administration (FDA) as nutritionally equivalent to traditional corn (21). Insect resistant *Bt* and herbicide tolerant maize are planted, commercialize and consumed in many other countries as well, including Brazil, Argentina, Canada, Mexico and the European Union (26).

OBJECTIVES

The objective of the proposed work is to transgenically modify *Zea mays* (maize) to contain the human GBA gene and, in turn, produce recombinant GCCase proteins. Human liver cells will first be extracted from healthy human individuals. Messenger RNA from these cells will be used to produce cDNA by reverse transcription, after which a PCR will be run to amplify the cDNA. The cDNA will then be ligated into to the expression plasmid vector pWUbi.tm1, where plant promoter and terminator sequences will be added to regulate gene expression. This expression cassette, made up of promoter, cDNA and terminator, will then be cleaved and newly ligated into the binary vector pWBVec8. Here the cassette will be positioned within the transfer DNA (T-DNA) with

right and left borders, along with a hygromycin resistance gene for transgenic plant cell selection. Recombinant pWBVec8 will be transferred from *Escherichia coli* vector cells to *A. tumefaciens* cells. Following the insertion into *A. tumefaciens*, the recombinant bacteria will be used to transform maize cells. Upon transformation, the transformed maize plants will be regenerated, grown and tested to verify their successful transformation and expression by PCR, Southern blot, Western blot analysis, and a fluorometric enzyme assay.

MATERIALS AND METHODS

A liver biopsy will be performed and human liver cells will be frozen for total RNA extraction. This procedure will be performed by a trained surgeon in a hospital and the liver tissue donor will be a healthy volunteer with no family history of Gaucher's disease. All involved in the procedure will have signed and agreed to an ethics agreement. The agreement and entire procedure will have been submitted to the institutional ethics commission, and no part of any procedures will commence until after their approval.

Messenger RNA will be extracted from frozen liver cells in accordance with Dr. Patrick O. Brown's *Protocol and kit for FastTrack 2.0 mRNA Extraction Human Cells* (22). This mRNA will then be used to perform reverse transcription-polymerase chain reaction (RT-PCR) using the Molecular Bio Products *RT-PCR* protocol and the corresponding kit from *Life Technologies*, resulting in amplified cDNA coding for the GBA gene. Primers flanking the human GBA gene will be designed according to the

sequence available at the GenBank under the accession number "M16328 M11080."

Primers will include start and stop codons of the GBA. The complete GCase sequence is also described in *Molecular cloning and nucleotide sequence of human glucocerebrosidase cDNA* by J Sorge, C West, B Westwood and E Beutler (27).

The expression plasmid vector pWUbi.tm1 (Wang *et al.*, 1998) will be cleaved open with the restriction enzyme *SmaI* (**Figure 3**). The PCR amplified cDNA of the GBA gene will be ligated into pWUbi.tm1 with T4 DNA ligase (Fermentas) according to the instructions of the manufacturer, creating together an expression cassette with the plant promoter *Ubi1-P*, originally regulating a maize ubiquitin gene, and the maize terminator *tm1* ' sequence. The expression cassette will then be cleaved off pWUbi.tm1 with the restriction enzyme *HindIII*. The resulting cleaved plasmid will be resolved by agarose gel electrophoresis and the band specific to the expression cassette will be purified by the *Freeze –Squeeze method* according to Ausubel *et al* (28).

The purified GBA expression cassette will then be ligated into the binary plasmid vector pWBVec8 (**Figure 4**). Plasmid pWBVec8 (Wang *et al.*, 1998) will be cleaved open, also with *HindIII*, and prepared for ligation with the expression cassette. General protocols for the preparation of plasmid vectors for their ligation with DNA fragments, DNA insert ligations into plasmid vectors, and rules for endonuclease digestion will be followed according to Ausubel *et al* (28).

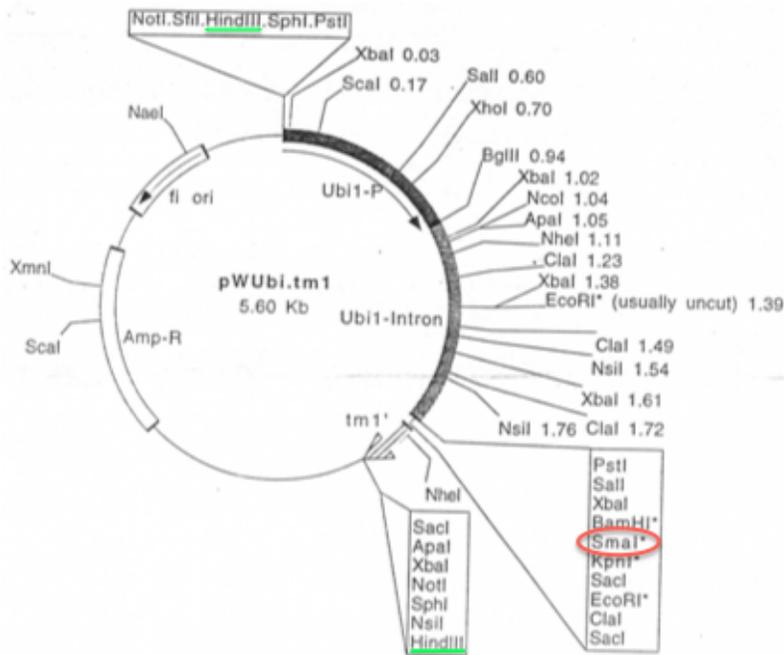


Figure 3: Plasmid *pWUbi.tm1*

Expression plasmid vector containing plant promoter and terminator sequence and restriction enzyme sites. Positions of the restriction site *SmaI* circled in red and *Hind III* underlined in green.

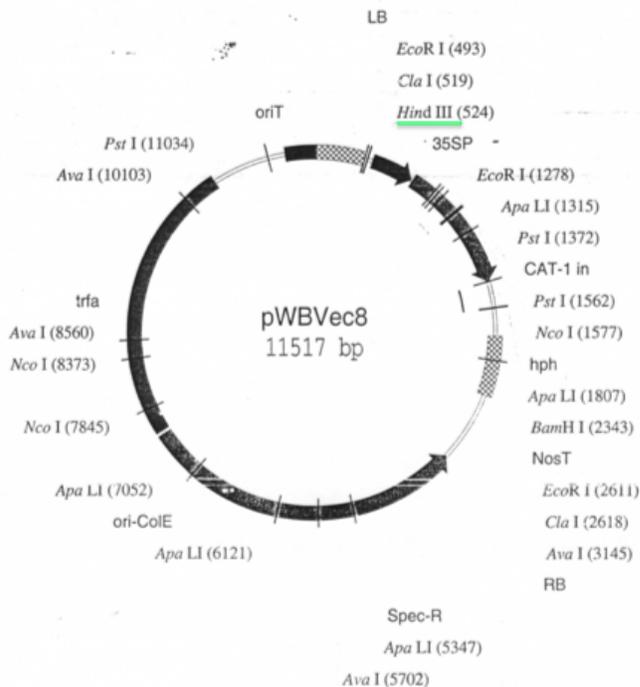


Figure 4: Plasmid *pWBVec8*

Binary plasmid vector containing t-DNA left (LB) and right (RB) borders and the Hygromycin resistance gene for transgenic plant selection. Positions of the *Hind III* restriction sites are underlined in green.

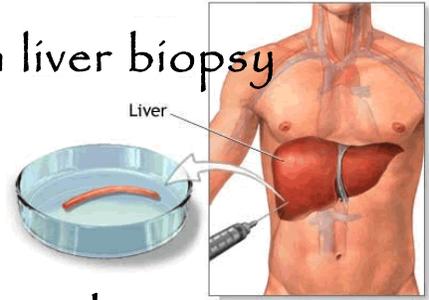
Following the insertion of the expression cassette into pWBVec8, the binary vector will be transformed into *A. tumefaciens*, after which it will be used to transform maize embryos. This transformation will occur by electroporation, by which electricity will be used to increase the permeability of the cells to insert the plasmid (28).

Transformation of maize by *A. tumefaciens* will be conducted based on the protocol followed by Frame *et al.* (23). According to these authors, immature zygotic embryos will be taken from maize harvested ten to thirteen days post pollination. The process of infection, cocultivation, resting, selection of transformed plants, and regeneration will follow. Infection will be accomplished by adding a prepared *A. tumefaciens* suspension to the embryos in tubes and inverting them 20 times, followed by five minutes of resting. This step will allow the *A. Tumefaciens* to infect the maize cells. The ti-plasmid will move into the plant cell and insert DNA into the plant's chromosome.

Cocultivation will include a three-day incubation in the dark at 20 or 23 °C in a cocultivation medium. From here, the embryos will be transferred to 28 °C on resting medium. After four to seven days on the resting medium, the embryos will be transferred to the selection medium. Frame *et al.* protocol will be strayed from here as Frame *et al.* used the herbicide bialaphos for selection and this procedure will be using hygromycin. This is due to the difference in antibiotic resistance genes in the binary vectors used to insert the gene of interest. After selection, maturation and germination will be performed as part of their regeneration. Whole transgenic maize plants will be regenerated from single transformed embryo cells. The transgenic plants will then be grown to maturity in a greenhouse. The general procedures described in Materials and Methods are depicted in **chart 1.**

Chart 1: Flow chart depicting general processes

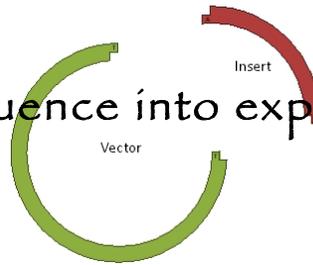
Human liver cell extraction through liver biopsy



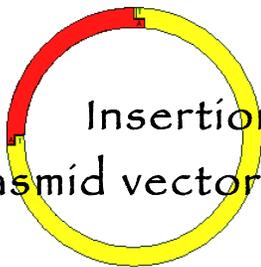
RT-PCR for the production and amplification of cDNA coding for GCase.



Insertion of cDNA sequence into expression plasmid vector pWUbi.tm1



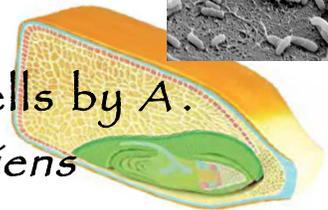
Insertion of cDNA cassette into binary plasmid vector pWBVec8



Insertion of binary plasmid into *A. Tumefaciens*



Transformation of maize embryo cells by *A. Tumefaciens*



Growth of maize plant and further testing



Following the production of transgenic plants will be analyses to measure and confirm the successes of the transformation, GCCase protein production, and GCCase activity. These analyses will be in the form of PCR, Southern blot, Western blot analysis and a fluorometric assay. Protocols for these procedures will be conducted in accordance with Ausubel *et al.* (28).

EXPECTED RESULTS AND DISCUSSION

The liver biopsy is expected to yield healthy liver tissue. After the biopsy, mRNA extraction from the liver tissue and RT-PCR should yield amplified cDNA coding for the GBA gene. This cDNA is expected to ligate successfully first into the expression plasmid vector, receiving the plant promoter and terminator sequences, and then into the binary plasmid vector, receiving T-DNA's left and right borders and a plant marker gene. This plasmid will be inserted into *A. tumefaciens*. This bacterium is in turn expected to transform the immature maize embryos. Recombinant GCCase will be produced by the maize plants and sold as an alternative form of ERT at a lower price than the current one.

The performance of a liver biopsy will be used to ensure that the human cells extracted from the tissue are cells able to produce the GCCase protein. Liver biopsy complications are rare, and recovery time for patients is typically only one to two days (24).

Complementary DNA will be amplified through the RT-PCR. By using cDNA over DNA, the gene of interest will be composed only of its exons, as introns are already spliced out in mature mRNA. This is beneficial in that it prevents errors caused by

incorrect recognition and processing of human introns by plants and smaller DNA is easier to work with.

The primers used for PCR and reverse transcription will be the same, as they will both select for the GBA gene. These primers will be designed using genomic knowledge of GBA so as to only amplify the gene of interest during the processes. The DNA polymerase used to amplify the cDNA will be *Pfu*. This thermostable DNA polymerase, originally from *Pyrococcus furiosus*, possesses exonuclease proofreading activity and resultantly makes fewer mistakes than other polymerases, such as the *Taq* DNA polymerase. *Pfu* also results in blunt-ended PCR products, which are preferable because PCR products with adenine overhangs on the 3' ends (as occurs with ordinary *Taq* DNA polymerase) would require further steps before insertion into the expression vector.

The amplified cDNA will be ligated into the expression plasmid vector pWUbi.tm1. This vector will have been cleaved by the restriction enzyme *Sma*I. This specific restriction enzyme's cleavage creates a blunt ended cut in the plasmid. A blunt cut is necessary so as to correspond to the blunt ends of the amplified cDNA. The same matching of corresponding ends is necessary for insertion into pWBVec8, which is why *Hind*III will be used both in the cleavage of the expression cassette from pWUbi.tm1 and in the cleavage of pWBVec8. This way the overhanging base pairs on one cleaved end will receive the corresponding base pairs from the insert and form a compatible double stranded sequence.

The promoter and terminator sequence added to the GBA gene of interest allow the gene to be expressed in plants. The expression cassette will become a part of The T-DNA with the addition of the left and right borders. Here, a gene coding for

hygromycin resistance will also be included in the T-DNA, which will allow for its selection after transformation. This selection process will occur as part of the transformation protocol of the maize embryos. The transformed maize cells will contain the gene coding for hygromycin resistance. This resistance will enable these cells to grow in a medium containing hygromycin. Non-transformed maize cells will not multiply in this medium, and thus only transformed cells and derived tissues, organs and whole plants will be selected in this step.

For the PCR detection of transgenes maize leaves will be ground and suspended in medium. Both transgenic and non-transgenic leaves will be prepared so as to have a negative control for comparison. The same primers as those used for the PCR conducted with the liver cells may be used, as these primers will select for the GBA gene. The amplified DNA will be run in gel electrophoresis and results will be read from the gel. Bands corresponding to the length of the GCase gene are expected in the transgenic corn, while no band should result there in nontransgenic corn. These bands produced from PCR analysis will prove that the GCase gene has been successfully inserted into the maize plants.

For Southern blot analysis, a fluorescent hybridization probe consisting of the PCR-amplified GBA gene fragment (cDNA) will be used to detect the presence of the GBA gene in the transgenic maize genomes of independently transformed and regenerated plants. This will be performed after digestion with a series of restriction enzymes and separation by gel electrophoresis. The probe will bind to the maize DNA transgene and be detectable due to its fluorescence, proving the independence of the transformation events and showing the transgene copy number in each genome.

The Western blot analysis will also use ground maize leaves as means of extraction, and a gel electrophoresis will isolate the GBA proteins. A primary antibody, produced in rabbits, will be used for the GCCase protein detection. This antibody will recognize and bind to the GCCase protein. A secondary antibody, produced in horses and specific to the conserved domains of rabbit antibodies, will then bind to the primary antibody. This commercially available horse antibody is modified by the fusion to an alkaline phosphatase that, in turn, will convert a colorless substrate into a dark, purple product. These dark bands will be visible at the expected sizes of the recombinant GCCase in total protein extracts of transgenic maize. The density of these bands will be used to determine the amount of protein produced in the maize plants.

The Fluorometric Assay will be used to determine the amount of protein that has been produced in the plants. This method will use the difference in fluorescence of the substrate to the GCCase and measure absorbance to ascertain the activity levels of the protein.

As currently interpreted, no patents' claimed sequences or procedures will be copied in the production of recombinant GCCase in maize. However, patent number 7,951,557 describes similar processes and also involves the use and manipulation of the GCCase gene and protein. If it is determined that claimed content will be copied, money will have to be paid to the inventors of the patent for permission to copy it. This fee, however, would not be enough to prevent the sale of recombinant GCCase for ERT at lower prices than *Genzyme's Cerezyme*[®]. In this way the goal of supplying ERT for people with Gaucher's disease at more affordable prices would still be achieved.

Further testing not previously described in Materials and Methods will also need to be performed and taken into consideration upon the production of transgenic maize plants before their use on humans. Some such tests involve proofs that the plants are safe for humans, animals and the environment. These tests include, but are not limited to, tests to verify the plants are not allergenic, teratogenic, carcinogenic or otherwise harmful in any way. Tests to verify that the protein is active *in vivo* will also have to be made. These tests will commence through animal trials and end in human testing only after consistently successful results. The plants will also need to be approved for sale by the corresponding national agency that reviews biosafety (CTNBio for Brazil), before their commercial sale begins anywhere.

These tests for safety just described briefly, where the protein will ultimately accumulate as well as purification of the protein for testing are all important factors involved in the ultimate production and sale of recombinant GCCase produced in maize plants. This project, however, focused primarily on the production of the protein in maize. Further processes are beyond the current scope of the project.

LABORATORIAL INFRASTRUCTURE

The purchasing of the materials and machines necessary for the completion of the procedures and protocols described, as well as funding for four lab assistants and myself will require a financial support. U\$ 100,000.00 dollars is requested for the funding of this Project, which is estimated to take two years. With his permission, this project will be conducted in the laboratory of Prof. Dr. Giancarlo Pasquali, which is fully equipped with

centrifuges, shakers, glassware, and other equipment necessary to perform the described procedures. **List 1** shows a more detailed description of the destinations of the financial support. A table with the chronology for the completion of objectives within the Project is presented in **Table 1**.

List 1: List of some of the distribution of financial support

- Automated DNA sequencer
- Electronic multi-channel pipettes
- Digital imaging system
- Ultraviolet transilluminator
- Automatic micropipettes
- Refrigerators and freezers
- Gel electrophoresis cells
- Magnetic stirrers
- Analytical balance
- Analytic thermal cycler for PCR microplates
- Microtubes for PCR thermal cycler
- PCR machine
- Autoclave and ovens
- Microcentrifuge
- Ice maker machine
- Digital pH meter

- Vortex mixer
- Greenhouses
- Restriction enzymes
- Hygromycin, buffers, and other solutions
- Primers, dNTPs, reverse transcriptase, and other components for procedures
- Erlenmeyer flasks, beakers, and other glassware

Table 1: Chronology for Project Completion

Objectives	Time (increments of 2 months)											
Obtain human liver tissue	X	X										
Extract mRNA from liver tissue		X	X									
Reverse transcribe to cDNA and amplify with PCR			X	X								
Insert cDNA into expression plasmid vector				X	X							
Insert expression cassette into binary plasmid vector				X	X	X						
Insert binary vector into <i>A. tumefaciens</i> .						X	X					
Transform maize embryo cells with <i>A. tumefaciens</i> .							X	X	X			
Grow transgenic maize plants								X	X	X	X	
Test plants for gene, protein, and protein activity											X	X

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