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AMELIORATION OF AMYLOID BURDEN IN ADVANCED HUMAN AND MOUSE ALZHEIMER'S DISEASE BRAINS BY ORAL DELIVERY OF MYELIN BASIC PROTEIN BIOENCAPSULATED IN PLANT CELLS

by

NEHA KOHLI
B.S. State University of New York at Binghamton, NY, 2010

A thesis submitted in partial fulfillment of the requirements
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in the College of Medicine
at the University of Central Florida
Orlando, Florida

Fall Term
2012

Major Professor: Henry Daniell

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ABSTRACT

One of the pathological hallmarks of Alzheimer's disease (AD) is the amyloid plaque deposition in aging brains by aggregation of amyloid-β (Aβ) peptides. In this study, the effect of chloroplast derived myelin basic protein (MBP) fused with cholera toxin subunit B (CTB) was investigated in advanced diseased stage of human and mouse AD brains. The CTB-fusion protein in chloroplasts facilitates transmucosal delivery in the gut by the natural binding ability of CTB pentameric form with GM1 receptors on the intestinal epithelium. Further, bioencapsulation of the MBP within plant cells confers protection from enzymes and acids in the digestive system. Here, 12-14 months old triple transgenic AD mice were fed with CTB-MBP bioencapsulated in the plant cells for 3 months. A reduction of 67.3% and 33.3% amyloid levels in hippocampal and cortical regions, respectively were observed by immunostaining of brain sections with anti- Aβ antibody. Similarly, 70% decrease in plaque number and 40% reduction of plaque intensity was observed through thioflavin S (ThS) staining that specifically stains amyloid in the AD brain. Furthermore, *ex vivo* 3xTg AD mice brain sections showed up to 45% reduction of ThS stained amyloid levels when incubated with enriched CTB-MBP in a concentration dependent manner. Similarly, incubation of enriched CTB-MBP with *ex vivo* postmortem human brain tissue sections with advanced stage of AD resulted up to 47% decrease of ThS stained amyloid plaque intensity. Lastly, lyophilization of plant material facilitates dehydration and long term storage of capsules at room temperature, in addition to increasing CTB-MBP concentration by 17 fold. These observations offer a low cost solution for treatment of even advanced stages of the AD by facilitating delivery of therapeutic proteins to central nervous system to address other neurodegenerative disease.

This thesis is lovingly dedicated to my mother, Reena Kohli, M.S. Her support, encouragement, and constant love have upheld me all through my life.

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LIST OF ACRONYMS/ABBREVIATIONS

*aad*A – Aminoglycoside 3’ Adenyltransferase

AD – Alzheimer’s Disease

BBB- Blood Brain Barrier

BSA –Bovine Serum Albumin

CTB - Cholera Toxin Beta Subunit

CTB-MBP – Cholera Toxin Beta Subunit fused with Myelin Basic Protein

DNA - Deoxyribonucleic Acid

dNTP - Deoxy Nucleotide Triphosphate

DTT - Dithiothreitol

EDTA - Ethylenediaminetetraacetic Acid

ELISA - Enzyme Linked Immunosorbent Assay

GM1 - Monosialotetrahexosylganglioside

HCl -Hydrochloric acid

H_2SO_4 - Sulfuric acid

Kb - Kilobase

kDa – Kilodalton

MBP – Myelin Basic Protein

$MgCl_2$ - Magnesium chloride

MS - Murashige and Skoog

NAA - Naphthalene Acetic Acid

NaCl - Sodium chloride

NaOH - Sodium hydroxide

PBS - Phosphate Buffered Saline

PBST- Phosphate Buffered Saline-Tween 20

PCR - Polymerase Chain Reaction

PMSF -Phenylmethanesulfonylflouride

^{32}P - Radioactive phosphorus

psbA - Photosystem b/A

RMOP - Regeneration Media of Plants

RNA - Ribonucleic Acid

SDS-PAGE - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SSC - Sodium Chloride and Sodium Citrate solution

TEMED - Tetramethylethylenediamine

WT - Untransformed Plant

UTR - Untranslated Region

UV - Ultraviolet

CHAPTER ONE: INTRODUCTION

Alzheimer's Disease

Alzheimer's disease (AD), the most prominent form of dementia, has been estimated to affect 36 million people globally in World's Alzheimer Report (WAR) 2010 (1), with a projected increase of up to 115 million at an annual cost of $604 billion. The 2011 WAR report concludes that up to 28 million people with dementia, lack efficient diagnosis, treatment and care (2), increasing the "treatment gap" from high to low and middle income countries. Many potential therapies for inhibiting cognitive AD symptoms are in development including inhibitors to cholinesterase (3), memantine (4), and other drugs to reduce AD burden. Accumulation of beta-amyloid (Aβ) plaques, attrition of neurons and synapses, rapid clearance of Aβ and neurofibrillary tangles (NFT's) formation (5, 6) are major challenges in treatment of AD. Biomarkers can play an important role in therapeutic treatments by detecting the pathology and biological effects on cerebral amyloid clearance followed by other downstream processes. Early detection of AD by identification and quantification of *in vivo* beta-amyloid through ligands specific to amyloid is a significant advancement. Amyloid imaging has progressed through positron emission tomography (PET) using Pittsburgh compound B as an amyloid ligand (7, 8). Along with magnetic resonance imaging (MRI) that correlates neuro-psychological decline in AD patients (8), other biomarkers facilitate intervention of AD in a large number of patients at preclinical stages and clinical trials. The cleavage of amyloid precursor protein (APP) produces $A\beta_{40}$, $A\beta_{38}$, $A\beta_{42}$ and $A\beta_{46}$ (9), among which $A\beta_{42}$ aggregates to form extracellular plaques. The increase in plaque volume leads to loss in synaptic function and neuronal degeneration (10). Active and passive immunotherapy involves administration of amyloid peptides and anti-

amyloid antibodies respectively to evoke an immune response. Reduction in Aβ levels have been reported in animal models through active and passive immunotherapies (11, 12). However phase II clinical trial of the AN1792 Aβ vaccine was discontinued due to side effects and meningo-encephalitis observed in ~6% of the immunized AD patients (11, 12). Other medications like donepezil or acetylcholinesterase offer only suggestive benefits rather than preventing progression of the disease (13, 14); hence strategies to clear pathologic proteins as the potential targets for AD are needed.

Myelin Basic Protein

Based on insights from AD pathogenesis, several therapeutic approaches are developed to reduce amyloid burden. Liao *et al.* (15) showed the strong interaction of myelin basic protein (MBP) with Aβ peptides, thereby preventing their assembly into mature amyloid fibrils. The "classic" isoform of MBP, 18.5kDa besides seizing the cytoplasmic leaflets of myelin membranes in myelin sheath (16), holds serine proteinase and autocatalytic activity. Moreover, it accounts for 30% of total myelin protein and represents a major structural component of myelin sheaths in the central nervous system. Interaction of MBP with Aβ peptides and their prevention into amyloid fibrils has been shown (17). Furthermore, degrading activity of MBP was demonstrated by incubation with brain sections of Aβ precursor protein transgenic mice *in situ* (15) by binding to Aβ at a site located in the N-terminal domain of MBP. Moreover it has shown to play an important role in amyloid cytotoxic effects in cortical neurons (18). The effect of MBP by delivering it *in vivo* into the brain can be hypothesized to reduce the amyloid plaques as well.

Blood Brain Barrier

To ensure the regulation of transport of substances inside and outside the brain, complex and tight intracellular junctions guard the blood brain barrier (BBB). This regulation further limits the passage of lipophilic molecules, proteins, metabolites across BBB (19). Moreover, the delivery of drugs intravenously does not offer an advantage for neurodegenerative diseases because of the prevention of an optimal uptake across BBB. Recently, studies have shown that polymeric nanoparticles coated with GM1 binding peptide promoted transcytosis as an important process for BBB penetration (20, 21). Further, receptor mediated endocytosis (RMT) and internalization is triggered through involvement of caveolae, which is enriched in ganglioside GM1 (21, 22). Selection of high affinity GM1 binding ligands, like pentavalent CTB address the process of transcytosis across BBB remarkably. Studies have also shown that CTB can bind to GM1 on the surface of neurons and enter through endocytosis (23). The fusion of CTB with therapeutic proteins can offer an optimal delivery system to transport the protein across BBB, *in vivo*. Similarly, MBP fused with CTB was hypothesized to cross through receptor mediated delivery BBB *in* vivo in mice and degrade Aβ aggregates.

Plant Derived Therapeutic Proteins

To address expensive fermentation systems, molecular farming has been developed as an alternative new technology for the production of therapeutic proteins. Recently, FDA approved a carrot cell based system for production of the first human therapeutic protein (24). Expensive chemical synthesis and purification, delivery using injections, and process of cold storage can be eliminated by low cost production of recombinant proteins in plants. The two major processes

for the derivation of proteins from plants include nuclear and plastid derived transformation. Plants provide an excellent bioreactor to produce proteins at a low cost and provide antigen protection by bioencapsulation. Lyophilization process of plant material helps to package capsules in a particular dosage or size, eliminates microbes and allows keeping plant material at room temperature for several months without disrupting its functionality (25).

Chloroplast Transformation

Many advantages are offered by chloroplast transformation and genetic engineering. As each plant cell contains 100 chloroplast organelles and each chloroplast contains up to 100 chloroplast genome; there exists 10,000 copies of chloroplast genome in each plant cells. This phenomenal copy number helps to achieve high expression of transgene levels by chloroplast genetic engineering. Up to 72% total leaf protein (26) has been reported for biopharmaceutical proteins in transgenic chloroplasts. Unlike nuclear transformation, chloroplast genome is not transferred through pollen, offering gene containment through maternal inheritance (27). Through site specific homologous recombination in chloroplast genome, positional effect or gene silencing are prevented. In addition, not only chloroplasts can transcribe polycistronic mRNA of plastids providing efficient expression of multiple genes, but also process posttranslational modifications of eukaryotic protein (28). Many genetically engineered plants for expression of proinsulin (29), insulin and C peptide (30), exendin 4 (25) for treatment of diabetes or blood clotting factor IX for hemophilia B (31), bacterial antigens like anthrax protective antigen (32), viral antigens like human papilloma virus L1 (33), dual vaccine to against cholera and malaria (34) have been expressed ideally in chloroplast system. Moreover, Nicotiana tabacum

chloroplasts have served as a bioreactor for its ability to maintain high biomass, and transformation efficiency. Likewise, edible crop including lettuce are used for transformation not only because it decreases the production cost but also offer ideal system for oral delivery of therapeutic proteins. In a similar fashion, generation of transgenic plants for MBP expression through chloroplast transformation was carried out in this study.

Bioencapsulation and Oral Delivery of Proteins

Oral delivery can be facilitated by bioencapsulation of foreign protein expressed in plant cells. These cells are ensured protection in the stomach from acids and enzymes. As they meet microbes in the gut, plant cell proteins are released in circulatory system. Further, lyophilization of plant cells allows preparation of capsules and long term storage of vaccines at room temperature, eliminating microbes, cold storage, purification and injections. Oral delivery of biopharmaceutical proteins and vaccines efficiently across intestinal mucus membrane is facilitated by GM1 receptors on the intestinal epithelial cells by binding to the CTB half of the fusion protein. Five B subunits monomers in the cholera toxin (CT) are assembled into a pentameric ring structure, which binds specifically to oligosaccharide domain of GM1 receptor (35). Hence, CTB subunit fused to a protein ensures protein delivery to the immune or circulatory system through a receptor mediated mechanism. Moreover, this study focuses on the delivery of CTB fusion protein to the brain. To test the delivery of therapeutic protein, MBP across BBB, MBP was fused with CTB. The GM1 receptors of the brain (36) are anticipated to cross link with CTB portion of the fusion protein and act as a vehicle to deliver MBP across

BBB. The concept of bioencapsulation will allow the protein to remain stable and actively perform its activity of degrading amyloid beta in Alzheimer's disease brain.

CHAPTER TWO: MATERIALS AND METHODS

Southern Blot Analysis

Plant genomic DNA Extraction and Restriction Digestion

Total genomic DNA from untransformed wild type and transplastomic lines was extracted using DNeasy plant mini kit protocol (Qiagen). Extracted genomic DNA was quantitated using Nanodrop (Biorad), then three micrograms of DNA was digested with Afl III enzyme. The reaction mixture contained 4 µl of 10X buffer (New England Biolabs), 3µg of plant genomic DNA, 1µl of enzyme for a final volume adjusted to 40µl with distilled water. The digestion reaction was incubated at 37°C overnight.

Agarose gel Electrophoresis and Transfer of DNA to Membrane

The digestion of DNA samples was confirmed by running them on a 0.8% agarose gel at 60V. The gel was then rinsed twice with distilled water for 5 min each to facilitate efficient transfer. To facilitate the binding of radio-labeled probe to the corresponding genomic DNA fragment, AflIII digested DNA was denatured by soaking the gel in transfer buffer (0.4N NaOH, 1M NaCl) for 20 min. The separated digested genomic DNA in the gel were transferred overnight by placing it facing the presoaked nylon membrane followed by filter paper and gentle pressure by stack of paper towels. Weights were placed on top for balance optimal contact between gel and membrane. Following day, membrane was rinsed with 2X SSC (3M NaCl, 0.3M sodium citrate) 2 times for 5 min each. Membrane was dried on a filter paper and was cross linked using GS gene linker UV chamber at C3 setting.

Hybridization and Autoradiography

After overnight transfer, membrane was prehybridized with 10 ml of QuickHyb prehybridization solution (Stratgene) at 68°C for 1 hr on a rotary chamber. In the meantime 5-10µl of the flanking probe was added to 100 µl of salmon sperm DNA, vortexed and heated at 94°C for 5 min. Next, the probe mixture was added to hybridization bottle containing the prehybridized membrane and incubated for 1 hr at 68°C. Following incubation, the membrane was washed twice with 25ml of wash buffer 1 (2 X SSC & 0.1% SDS) at room temperature for 15 min each and washed twice with 25ml of wash buffer 2 (0.1X SSC and 0.1% SDS) at 60°C for 15 min each. The membrane was wrapped in a saran wrap and radioactivity was checked using a Geiger counter. X-ray film was exposed to the hybridized membrane in dark and kept at -80°C for 12-72 hr depending on the radioactive count. The exposed X-ray film was developed in a film processor.

Confirmation of Inheritance

The plants that were positive from southern blot analysis were multiplied and transferred to Jiffy pots (Harris Seeds). After acclimatization in an incubation chamber for about 2 weeks, plants were transferred to greenhouse for biomass. Seeds from self pollinated transplastomic plants were collected and germinated in ½ MS media containing 2% (w/v) sucrose and spectinomycin (500mg/L) along with wild type seeds in culture room to check for inheritance.

Protein Extraction and Bradford Assay

Expression level of CTB fused MBP protein was examined at different time of harvest and developmental stages. The total leaf proteins of transplastomic plants grown in greenhouse

were extracted according to leaf ages such as young, mature and old at different time points - 10am, 2pm, and 6pm. The leaf material frozen in liquid nitrogen was then grinded to fine powder using motor and pestle. In 300µl of plant extraction buffer (100 mM Nacl, 10 mM EDTA, 200mM tris HCl pH 8.0, 0.05% Tween-20, 0.1% SDS, 14 mM βME, 200 mM sucrose, 2 mM PMSF, 1X protease inhibitor cocktail) 100mg of finely grinded transplastomic leaf was added. The total leaf protein was homogenized by mixing for 10 minutes at 4°C, followed by centrifugation at 14,000 rpm for 5 min to obtain the supernatant and pellet fractions. The pellet was discarded and supernatant was used for quantification. The concentration of the total leaf protein was determined using the Bio-Rad protein assay dye reagent. The standard curve was plotted by serial diluting 0.8µg/µl BSA down to 0.05mg/ml. The supernatants were diluted with water in a ratio of 1:10, 1:20 and 1:40. A 96 well assay plate was used to which 10µl of each sample and BSA standard was added in duplicates. Bradford dye reagent (200µl) was added to each well and absorbance was measured at 595 nm using a plate reader.

Western Blot Analysis

To check for expression of CTB-MBP fusion protein, western blot analysis was performed. The samples were diluted with 2X sample loading buffer (3.55 ml dH_20, 1.25 ml 0.5M Tris-HCl pH 6.8, 2.5 ml glycerol, 2 ml 10% SDS, 0.2 ml 0.5% bromophenol blue), boiled for 10 minutes, centrifuged, loaded on 12% SDS-PAGE gel and ran at 110 V. The proteins were separated according to their molecular weights. Next, the proteins were transferred to a nitrocellulose membrane using Bio-Rad electro blotting transfer apparatus at 85 V for 1 hr. Membrane was then incubated in PTM (PBST with 3.5% dry milk) at room temperature for 1 hr

with gentle shake to block non-specific binding. CTB-MBP protein was detected using anti-CTB rabbit primary polyclonal antibody (Sigma) diluted in PTM (1:12000). The fusion protein was also probed with rabbit anti-human MBP primary monoclonal antibody (1:1000, Abcam). The membrane was incubated with respective primary antibodies overnight at 4°C with gentle shaking. Following day, the membrane was washed twice with PBS-T for 5 min each at room temperature. Next, goat anti-rabbit IgG conjugated to horse radish peroxidase (HRP) secondary antibody diluted in PTM (1:4000) was added to the membrane and incubated at room temperature for 1.5 hr with gentle shaking. Next, membrane was washed thrice with PBS-T for 10 min each followed by a last wash with PBS for 15 min. Supersignal West Pico chemiluminescent substrate (Thermo Scientific) was added to the membrane and a chemiluminescent signal was detected on autoradiography film through an automated X-ray film processor. Similarly native PAGE was carried out with detection of fusion protein in native state as mentioned above, except that DTT and SDS were removed during preparation of gels, sample buffer and plant extraction buffer and the samples were not boiled.

Densitometric Studies for Quantification of Protein

For quantification of the CTB-MBP protein, developed X-ray film against immunoblotted membrane probed with anti-CTB antibody was used for densitometric analysis. To plot the standard curve for CTB protein, known amounts of CTB (50, 100, and 150 ng) were loaded for immunoblot analysis. The concentration of CTB-MBP expressed in transplastomic plants was determined by plotting the detected bands onto the standard curve using the

Alphaimager and Alpha ease FC software. Thetotal soluble protein % was also calculated using formula described before (37).

Lyophilization

CTB-MBP frozen material was lyophilized to obtain higher protein concentration by freeze drying the material using Freezone Benchtop Freeze Dry Systems (Labconco) in vacuum for 48 hrs at -50^{o}C and 0.036mBar. Following lyophilization, freeze dried leaf material was grinded using coffee blender (Hamilton Beach) for 2 min at maximum speed (pulse on for 10 sec and off for 10 sec) then stored under moisture-free condition stored at room temperature. To check the stability and functionality of the lyophilized protein, proteins were extracted described above then immunoblot with anti-CTB antibody was conducted and compare its protein expression to that of fresh weight leaf material.

CTB GM1 Assay

To investigate the proper formation of pentameric structure of CTB-MBP fusion protein, GM1 (monosialotetrahexosylganglioside) assay was done with soluble proteins extracted from the CTB-MBP expressing fresh weight and lyophilized leaf material, and purified CTB. . GM1 ganglioside (Sigma G-7461) was coated by incubating 100µl of GM1 (3µg/ml) in bicarbonate buffer, (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) at 4°C overnight. PTM (100µl) was coated as a control. Plate was washed three times with PBST and distilled water. PTM (200 µl) was added and incubated for 2 hrs at 37°C for blocking non specific binding. The plate was again washed three times with PBST and water. Various concentrations of protein extracted from transplatomic

and untransformed plants diluted in plant extraction buffer were added to the wells and incubated overnight at 4°C, including CTB protein as control. Following day, the plate was washed thrice with PBST and water, and incubated with 1:12,000 dilution of rabbit anti-CTB primary antibody (Sigma) for 1 hr at 37°C. Following set of washings for three times with PBST and water, the plate was incubated with 1:4000 dilution of anti-rabbit IgG secondary antibody conjugated to horse radish peroxidase (Southern biotech, USA) for 1 hr at 37°C. The plate was washed three times with PBST and water. Then 100µl of TMB (Signal Transduction Products) was added and kept in dark for 20-30 min depending on the color reaction. The reaction was stopped by adding 50µl of 2N H_2SO_4. Absorbance was measured at 450 nm by using a microplate reader.

Ex Vivo Studies

Ex vivo brains from 24 months old 3xTg AD mice were received from National Institute of Aging (NIA), for which 7µm cryostat sections were collected on slides. Seven adjacent sections were incubated with 25µg or 50µg of 33% purified CTB-MBP plant protein in PBS. Commercial CTB alone, BSA and untransformed plant protein were also used for concentration-dependent incubation as controls simultaneously. After 2 days of incubation at 37^oC, sections were stained with Thioflavin S (ThS, Sigma), imaged and quantified using NIS Elements for Advanced research software using Nikon Eclipse TE2000-E fluorescence microscope. Threshold intensity was based on the intensity of background staining in control sections. The selected threshold was held constant across all experimental sections. *Ex vivo* post mortem brain tissues from Alzheimer's disease patients, processed and received as described before (38) were deparaffinized and rehydrated to ensure good staining of the tissue. The slides were passed

through xylene followed by graded washes with xylene and ethanol, with a final wash of molecular grade water. Brain sections were incubated with 50µg of CTB-MBP (33% purity) transplastomic plant or untransformed plant material. After 2 days of incubation at 37^{o}C, sections were stained with ThS and DAPI to acquire images as described above.

Animal Studies

Oral delivery of 3xTgAD Mice with Bioencapsulated CTB-MBP

The 3xTg AD mice were received from NIA. These mice were derived by microinjecting two independent transgenes encoding human APP (Sweden) and tau (P301L) into single embryos harvested from the homozygous mutant PS1 (M146V) knockin (PSI-KI) mice, as described previously (72). For these studies, 12-14 months old (at the start of study) 3xTg AD mice were used for oral delivery of CTB-MBP. Animals, housed under pathogen free conditions, were treated under Instituitional Animal Care and Use Committee-approved protocols. The lyophilized leaf material for oral dose (31.2ug/300ul/day) of either transgenic (n=10) or NT (n=4) was delivered three times a week for three months. A set of mice (n=4) were kept unfed. For the oral delivery experiments, the lyophilized material was prepared by adding 4mL of PBS to 500mg of lyophilized and powdered CTB-MBP expressing plant material on ice right before the oral gavage.

Immunohistochemistry and Thioflavin S Staining

On the day of sacrifice, all mice were perfused with saline following which the brains were removed. One hemisphere of the brain tissue from 3xTg AD mice was prepared for histological analysis in 4% paraformaldehyde overnight whereas the three brain regions-

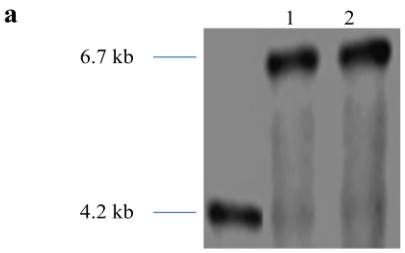
cerebellum, hippocampus, and cortex were dissected from the other hemisphere for Aβ measurements. Brain cryosections (8μm thick) from the coronally mounted other hemisphere of 3xTgAD mice delivered orally with the powdered lyophilized CTB-MBP expressing transplatomic plants was obtained on slides using microtome slicing system (Fischer). Adjacent five sections were exposed for 1 hr to PBS containing 0.1% Triton X-100 (Sigma) and 5% normal goat serum (Invitrogen, Camarillo, CA) to block nonspecific antibody binding, followed by incubation overnight with primary antibody, poyclonal anti-amyloid beta (1:200 Cell Signaling # 2454), overnight at 4^{o}C. To test for nonspecific staining by the secondary antibodies, additional slides were processed in a similar fashion with the primary antibodies excluded. All slides were then rinsed for 1 hr at room temperature in several changes of PBS and incubated in the dark for 1 hr at RT in PBS that contained 5% NGS and the fluorescent secondary antibody, Alexa Fluor 568-conjugated IgG (1:200). Following incubation with secondary antibody, the cryosections were counterstained with Hoescht 33342 (Invitrogen), images were acquired by Nikon Eclipse TE2000-E fluorescence microscope. These images were then processed by NIS Elements for Advanced Research, with the input levels adjusted to span the range of acquired signal intensities exactly. Statistical analysis for the data was analyzed by single factor ANOVA Similarly, following PBS rehydration, adjacent five sections were stained with 0.02% ThS in 70% EtOH for 8 minutes. This was followed by rinsing the slides in 50-80% EtOH and distilled water respectively and cover slipped with DAPI solution for acquiring images as mentioned before.

CHAPTER THREE: RESULTS

Confirmtaion of transgene integration and characterization of CTB-MBP protein expressed in chloroplasts

Transplastomic chloroplast lines were confirmed by Southern blots conferring site specific integration of transgene into the spacer region between trnI and trnA genes. Digestion of untransformed (WT) plant DNA with AflIII showed 4.2kb fragment after hybridizing with 32P-labeled trnI-trnA flanking probe and transplastomic CTB-MBP lines showed only the 6.7kb fragment, confirming homoplasmy through site specific integration of transgene (Fig. 1a). Evaluation of foreign gene expression at different developmental stages - young(Y), mature (M) and old (O) and at time of harvest – 10am, 2pm, 6pm, showed the highest MBP expression in mature leaves at 6pm (Fig. 1b and 1c). Enhanced translation of gene in the light by psbA promoter and 5'UTR regions located upstream of MBP gene cassette should contribute to maximal expression observed later in the evening. Immunoblots probed with CTB (Fig. 1d) and MBP (Fig. 1e) antibody showed ~28.5kDa fusion protein monomer in CTB-MBP transplasomic lines. Fresh weight leaves expressed up to 2% of total leaf protein, where a 17 fold enhanced concentration of the protein was achieved by lyophilization of the plant material (Fig. 1f and j). Equal loading of protein showed a significant difference in accumulation of CTB-MBP between fresh and lyophilized materials (Fig. 1f). Moreover immunoblots probed with CTB antibody showed that lyophilized material maintained stability at room temperature and protection from degradation over 7 months of duration (Fig. 1g). The functionality of CTB-MBP fusion protein was evaluated by GM1 ELISA, where the ability of CTB to bind to the GM1 receptors depends on its pentameric form. GM1 binding assay showed that pentamers of fresh weight and

lyophilized CTB-MBP were formed; this confirms proper folding and formation of disulfide bonds (Fig. 1i). Non-reducing native PAGE immunoblots probed with CTB antibody further confirmed the native pentameric formation (Fig. 1h) within transgenic chloroplasts, attesting functionality of CTB-MBP for further *in vivo* and *in vitro* studies. Lyophilization process preserved the folding and disulfide bonds of CTB-MBP protein even after prolonged storage (Fig. 1j).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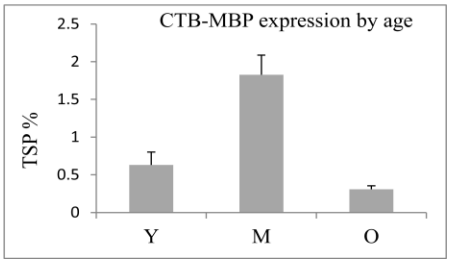
6.7 kb
4.2 kb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CTB-MBP expression by age
TSP %
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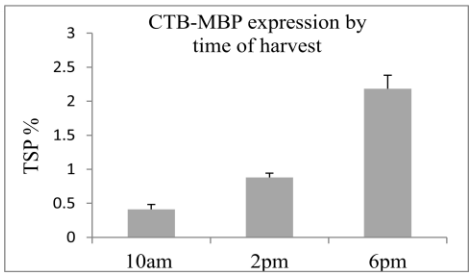
2
1.5
1
0.5
0
Y
M
O


c
CTB-MBP expression by time of harvest
TSP %
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2.5
2
1.5
1
0.5
0
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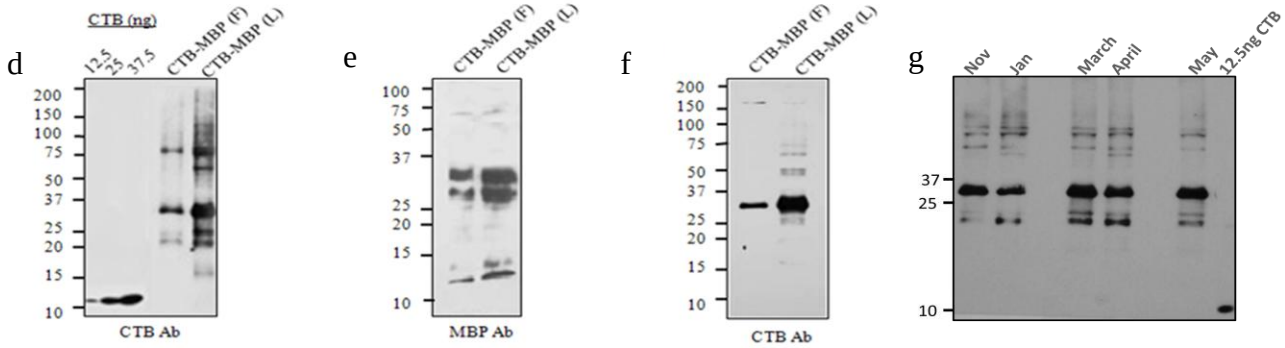
2pm
6pm


d
CTB (ng)
12.5
25
37.5
CTB-MBP (F)
CTB-MBP (L)
200
150
100
75
50
37
25
20
15
10
CTB Ab
e
CTB-MBP (F)
CTB-MBP (L)
100
75
50
37
25
20
15
10
MBP Ab
f
CTB-MBP (F)
CTB-MBP (L)
200
150
100
75
50
37
25
20
15
10
CTB Ab
g
Nov
Jan
March
April
May
12.5ng CTB
37
25
10

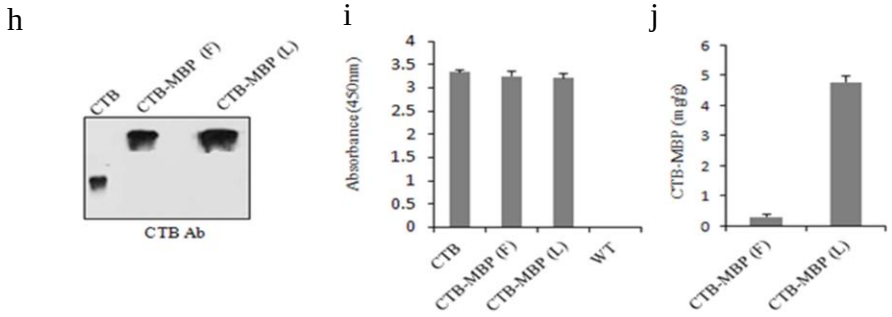

h
CTB
CTB-MBP (F)
CTB-MBP (L)
CTB Ab
i
Absorbance (450nm)
4
3.5
3
2.5
2
1.5
1
0.5
0
CTB
CTB-MBP (F)
CTB-MBP (L)
WT
j
CTB-MBP (mg/g)
6
5
4
3
2
1
0
CTB-MBP (F)
CTB-MBP (L)

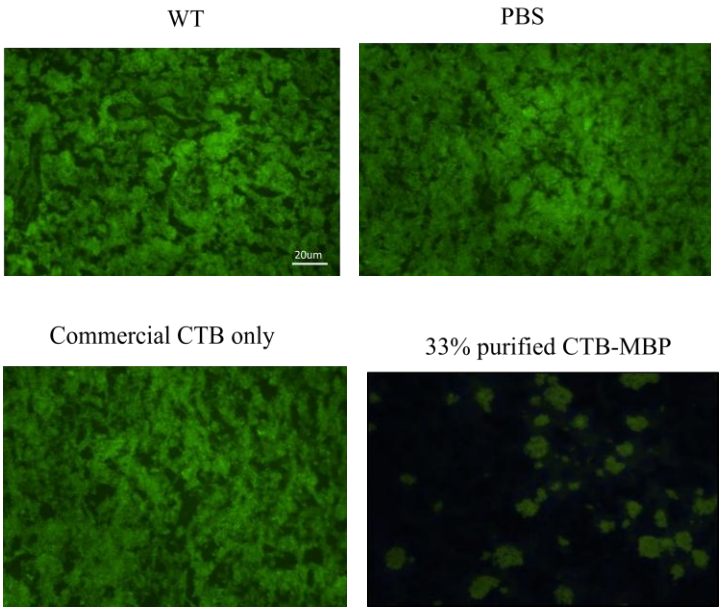
Figure 1: Confirmation of homoplasmy and CTB-MBP protein expression in transplastomic tobacco

(a) Digestion with AflIII yields 4.2kb and 6.7kb fragments in WT and transplastomic lines respectively as shown in southern blot analysis. (b, c) Protein analysis showing CTB-MBP protein expression relative to leaf's age and time of harvest. (d, e)Western analysis of CTB-MBP expression in fresh (F) and lyophilized (L) transgenic plant extracts. SDS-PAGE with anti- CTB antibody (d), Lane 2, 3, 4 - 12.5ng, 25ng, 37.5ng purified CTB standard respectively; lane 5 & 6 - 20ug of F and L respectively. SDS-PAGE with anti-MBP antibody (e), Lane 2 & 3 - 20ug of F and L respectively. (f) SDS PAGE with anti-CTB antibody for normalization of F and L transgenic plant protein. Equal quantity (100mg) of F and L samples that were obtained by adding equal volume of plant extraction buffer (300uL). (g) Western analysis showing long term stability of CTB-MBP (L) after storage at room temperature for seven months. Lane 1, 2, 4, 5, 7 – 20ug of L in stored at different months, lane 8 – 12.5ng CTB standard. (h) Native PAGE with anti-CTB antibody. Lane 1- 37.5ng purified CTB standard; lane 2 & 3 - 2ug of F and L respectively. (i) GM1 ELISA assay for functional pentamers of purified CTB (25ng), transgenic CTB-MBP protein in 20ug of F, L and WT extracts. (j) Quantification of CTB-MBP protein (mg of protein/g of total leaf) in F and L plant extracts.

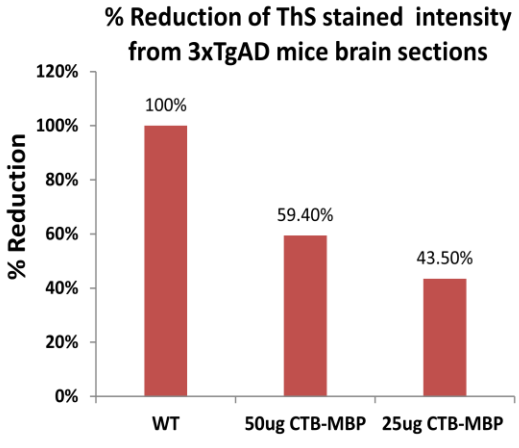
Reduction of amyloid levels in 3xTgAD mice brains by CTB-MBP incubation

Ex vivo studies of thioflavin S (ThS) stained brain sections (Fig. 2a) from 24 months old 3xTg AD mice showed reduction in amyloid plaques in a concentration-dependent manner when incubated with chloroplast-derived CTB-MBP. ThS fluorescence reduction from 44-60% was detected with 2 days incubation with 33% purified 25-50ug of CTB-MBP plant derived protein in adjacent brain sections (Fig. 2b). Control incubations with protein extract from untransformed plants, PBS buffer or purified CTB (commercial source) under identical conditions didn't show any decrease in ThS intensity. The decreased intensity in CTB-MBP incubated sections as compared to WT treated or other controls, shows that CTB-MBP at an optimal concentration of 50μg is able to break and/or remove the amyloid fragments, thereby reducing their thioflavin S fluorescence. Data shown is mean ± S.D of values obtained from seven adjacent sections and evaluated from a total of 63 fluorescence images.

a


WT
PBS
20um
Commercial CTB only
33% purified CTB-MBP

b


% Reduction of ThS stained intensity from 3xTgAD mice brain sections
% Reduction
120%
100%
80%
60%
40%
20%
0%
100%
59.40%
43.50%
WT
50ug CTB-MBP
25ug CTB-MBP

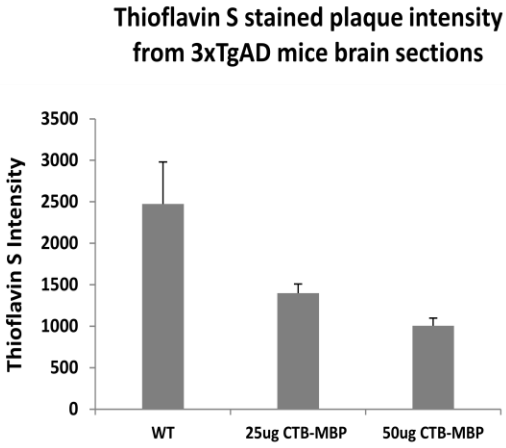
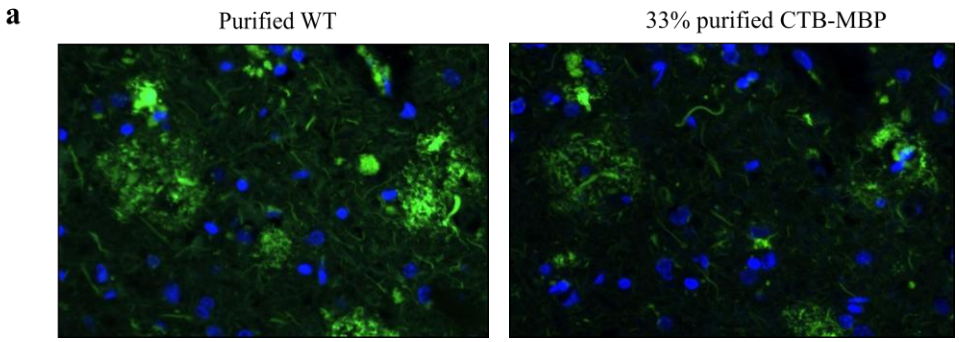

Thioflavin S stained plaque intensity from 3xTgAD mice brain sections
Thioflavin S Intensity
3500
3000
2500
2000
1500
1000
500
0
WT
25ug CTB-MBP
50ug CTB-MBP

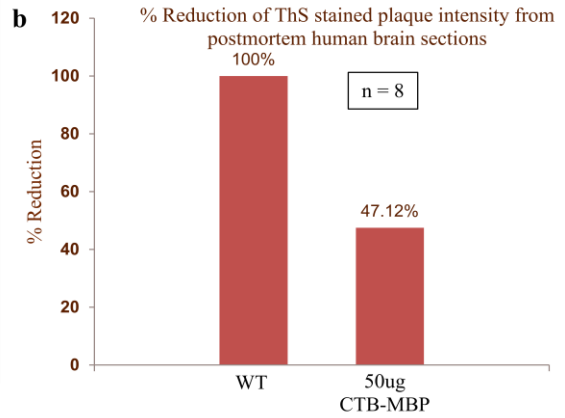
Figure 2 : Reduction of amyloid plaque load in *ex vivo* mouse brain tissue sections incubated with the CTB-MBP chloroplast expressed protein.

(a) Sagittal serial sections of brains from 3xTg AD mice were incubated with 25 and 50μg recombinant CTB-MBP protein partially purified from transgenic leaf extracts or 25 or 50μg commercially available purified CTB for 2 days and then stained with 0.02% ThS solution to visualize the central dense core of compact amyloid (green) and with DAPI to identify cell nuclei (blue) by fluorescence microscopy. As controls, adjacent sections were incubated with 25 or 50μg of untransformed leaf extracts or saline alone and processed for ThS staining in parallel. Shown are sections including the hippocampus and cortex of representative 24 -month-old 3xTg AD mice. (Scale bar: 10um). (b) Quantification of the relative amounts of amyloid plaque load in the ThS stained sections after incubation with the indicated proteins or saline alone as described in (a). The mean plaque counts in the DG, CA1 and CA3 hippocampal and cortical regions per section (in cortex, mantle and pallium regions) were determined with NIS Elements for Advanced Research. Data shown is mean ± S.D of values obtained from seven adjacent sections and two fields per section.

Reduction of amyloid levels in post-mortem human AD brains by CTB-MBP

Ex vivo studies of ThS stained brain sections of post mortem brain tissues from Alzheimer's disease patients (Fig. 3a) were conducted with incubation of CTB-MBP chloroplast expressed protein. An optimal working concentration of 50μg of 33% purified CTB-MBP protein was obtained from *ex vivo* mice studies. Next, adjacent brain sections stained with ThS upon 2 days of incubation with 33% purified 50μg of CTB-MBP, showed 47.5% ThioS fluorescence reduction of stained amyloid plaques (Fig. 3B). As observed in *ex vivo* mice brain sections, the decreased intensity in CTB-MBP incubated human sections when compared to untransformed plant extract shows that MBP through its serine proteinase activity was also able to break down plaques and/or remove the smaller amyloid fragments, thereby reducing their ThS fluorescence in human tissue as well. Data shown is mean ± S.D of values obtained from sections of five AD patients and evaluated from a total of 10 fluorescence images.


a
Purified WT
33% purified CTB-MBP


b
% Reduction of ThS stained plaque intensity from postmortem human brain sections
100%
n = 8
47.12%
% Reduction
120
100
80
60
40
20
0
WT
50ug CTB-MBP

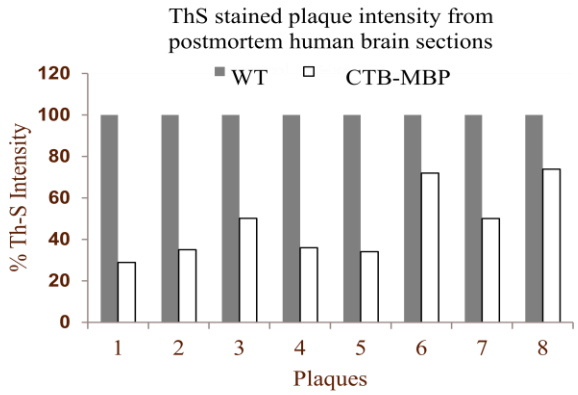
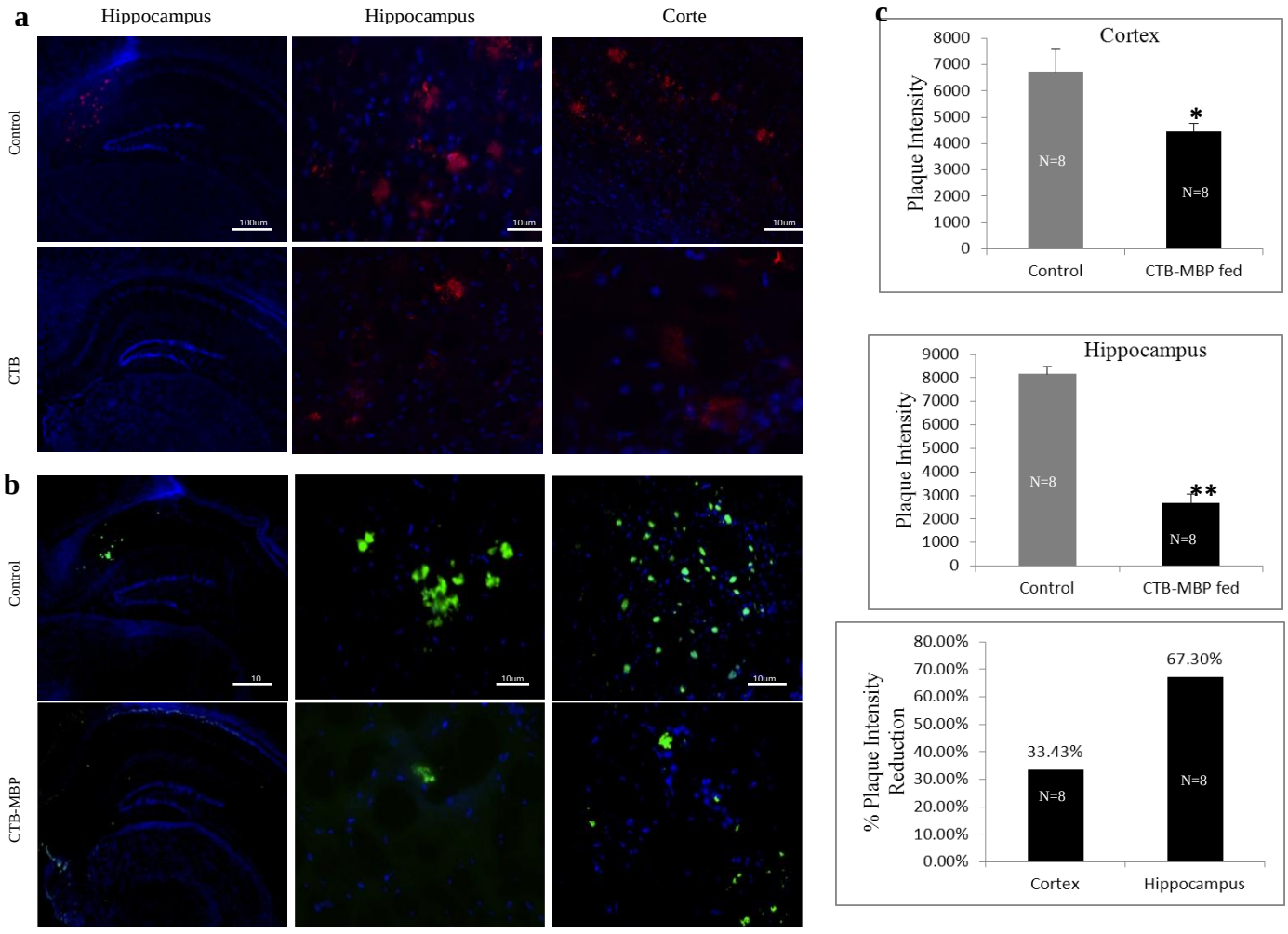

ThS stained plaque intensity from postmortem human brain sections
WT
CTB-MBP
% Th-S Intensity
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
Plaques

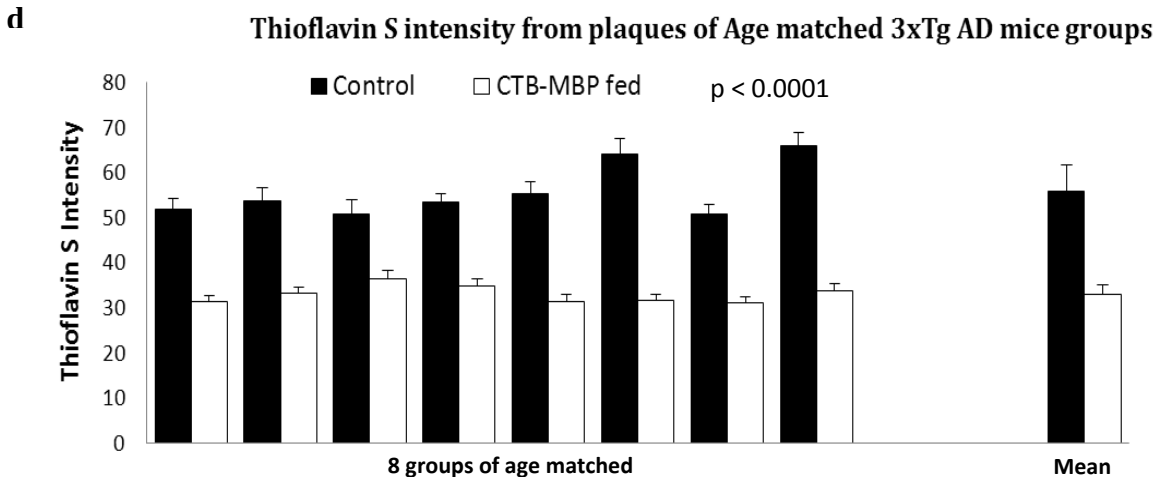
Figure 3: Reduction of amyloid plaque in *ex vivo* post-mortem AD brain tissue sections incubated with the CTB-MBP chloroplast expressed protein

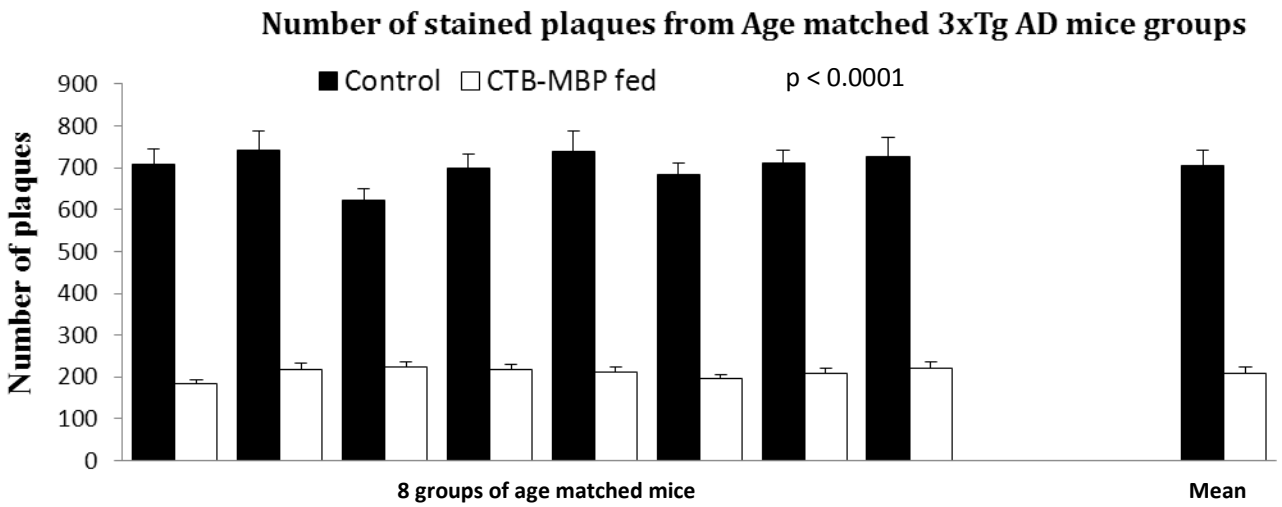
Sections of the parietal cortex from AD patients (a) were incubated with 50μg recombinant CTB-MBP protein partially purified from transgenic leaf extracts or 50μg of partially purified untransformed leaf material for 2 days and then stained with 0.02% Thioflavin S solution to visualize the central dense core of compact amyloid (green) and with DAPI to identify cell nuclei (blue) by fluorescence microscopy. As controls, adjacent sections were incubated with 50μg of untransformed wild-type leaf extracts or saline alone and processed for ThS staining in parallel. (Scale bar: 10um). (b) Quantification of the relative amounts of amyloid plaque load in the Thio-S stained sections after incubation with the indicated proteins or saline alone as described in (a). The mean plaque counts per section were determined with NIS Elements for Advanced Research. Data shown is mean ± S.D of values obtained from sections of five AD patients and two fields per section.

Reduction of Amyloid Levels in 3xTgAD mice upon oral delivery of bioecapsulated CTB-MBP

The effect of oral delivery for a period of three months with either bioencapsulated CTB-MBP (31.2ug/day), untransformed WT plant cells or unfed mice was evaluated in brain sections of 3xTgAD mice, that were 12-14 months old at the start of this study. Stained adjacent cortex and hippocampus sections of control and CTB-MBP treated mice stained with the anti-β-amyloid antibody (Fig. 4a, red) or ThS (Fig. 4b, green) were examined and quantified as described in methods. Most plaques were found in the hippocampus region, particularly DG, CA1 and CA3 sites, from where they started diffusing to the cortex as the age of mice progresses. Aβ level reduction of up to 67.3% and 33.4% in hippocampus and cortex, respectively was observed (Fig. 4c) through immunostaining with anti- Aβ antibody upon oral delivery of CTB-MBP. Data shown is mean ± S.D of values obtained from five adjacent sections from each mouse and evaluated from a total of 120 fluorescence images. Moreover, ThS staining showed up to 70% decrease in plaque number and 40% reduction in plaque intensity (Fig 4d) which was quite consistent in these age matched 3xTgAD mice across the group. These findings address the application of delivery of therapeutic protein, MBP; either through anticipated GM1 mediated receptor binding to the pentameric CTB of the fusion protein in the BBB or through compromised BBB in the AD model of transgenic mice allowing more BBB permeability for CTB-MBP delivery.


a
Hippocampus
Hippocampus
Corte
Control
CTB
b
Control
CTB-MBP
c
Cortex
Plaque Intensity
N=8
N=8
*
Control
CTB-MBP fed
Hippocampus
Plaque Intensity
N=8
N=8
**
Control
CTB-MBP fed
% Plaque Intensity Reduction
33.43%
67.30%
N=8
N=8
Cortex
Hippocampus


d
Thioflavin S intensity from plaques of Age matched 3xTg AD mice groups
Control
CTB-MBP fed
p < 0.0001
Thioflavin S Intensity
8 groups of age matched
Mean


Number of stained plaques from Age matched 3xTg AD mice groups
Control
CTB-MBP fed
p < 0.0001
Number of plaques
900
800
700
600
500
400
300
200
100
0
8 groups of age matched mice
Mean

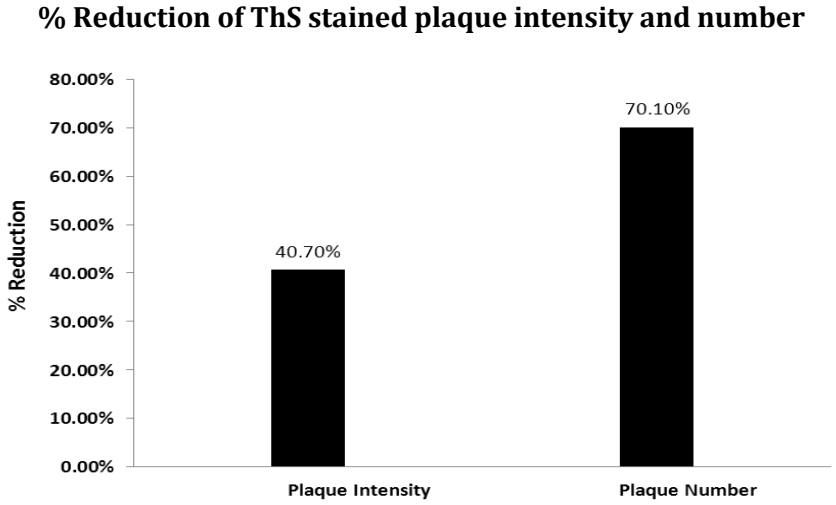

% Reduction of ThS stained plaque intensity and number
% Reduction
80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
40.70%
70.10%
Plaque Intensity
Plaque Number

Figure 4: Oral delivery of CTB-MBP chloroplast expressed protein decreased the amyloid plaque burden in a mouse model of AD.

Representative images (a, b) of the cortical and hippocampal amyloid plaque burden in 3xTg AD mice fed 3 times weekly over 12 weeks starting at 13-15 months of age with either recombinant CTB-MBP protein transgenic leaf extracts (31.2ug/300ul/day; n=10 mice, untransformed (WT) leaf extracts (300ul/day; n=4 mice), or unfed (n=4 mice). Sections were stained for amyloid plaques with either anti- Aβ antibody 2454 (red fluorescence; a) or Thioflavin S (green fluorescence; b) and with DAPI (blue) to label cell nuclei. Scale bar: hippocampus 100um (i) and 10um (ii) at different magnification, cortex 10um (iii)) (c) Quantification of the relative amounts of amyloid plaque load in the anti- Aβ stained sections from the animals treated with CTB-MBP and WT protein extracts as described in (a). (d) Quantification of the relative amounts of amyloid plaque number and intensity in the thioflavinS stained sections from the animals treated with CTB-MBP and WT protein extracts as described in (b). The mean plaque counts in the DG, CA1 and CA3 hippocampal and (mantle and pallium regions) cortical regions per section were determined with NIS Elements for Advanced Research. Data shown is mean ± S.D of values obtained from five adjacent sections per mouse and five fields per section. Single factor ANOVA *$p<0.05$, **$p<0.01$, compared to mice treated with WT leaf extracts and unfed.

CHAPTER FOUR: DISCUSSION

This study shows efficient *in vivo* oral delivery of chloroplast expressed CTB-MBP fusion protein across BBB. The native confirmation of CTB pentamers facilitates binding to the intestinal GM1 receptors and effectively release the fused protein to the circulatory system when orally administered (35). CTB ensures the stability of fused protein through its pentameric confirmation. This work provides evidence for transport and efficient delivery of CTB fusion protein across BBB. Moreover, our findings support therapeutic MBP delivery into the brain *in vivo* potentially to reduce amyloid aggregates. Previous studies show presence of GM1 receptors on the BBB (36). The delivery of plant derived CTB-fusion protein can be anticipated to occur by GM1 receptor mediation through CTB-GM1 interaction on BBB, but additional experiments are required to address this mechanism. Moreover, BBB impairment is associated with more rapid progression in AD over 1 year (39). Clinical studies show that BBB is compromised in AD brains (40). With the damage of anatomical organization of BBB we anticipate increased BBB permeability, facilitating delivery of therapeutic proteins across BBB, further enhancing their delivery. Likewise, ability of bioencapsulated CTB-MBP to reduce amyloid levels *in vivo* indicates another path of efficient delivery of protein across disrupted BBB from site of administration, while maintaining its functional and structural integrity. This provides an inexpensive plant based application of delivering therapeutic protein across BBB.

Both widespread and diffused myelin breakdown (41) in the brains has been reported in patients with AD. Correlation of amyloid deposition with destruction of myelin also contributes the progression of age dependent AD (42). Myelin basic protein covers the myelin membranes and holds the lipid bilayer through electrostatic interactions in the CNS (43), ensuring its

importance in myelination and neurotransmission processes. Along with serine 151 residue serving as an active site for autocatalysis in MBP, its ability to interact with Aβ peptides, or degradation of fibril amyloid deposits have been previously discussed based on *in vitro* studies (15, 17, 18), consistent with our study and showing the capability of MBP to degrade Aβ.

This study also shows an optimal reduction up to 48% of thioflavin S stained amyloid deposits in advanced human AD post mortem brain sections, when incubated with chloroplast derived MBP. The significant reduction in human brains insights the functional degrading activity of plant expressed MBP *ex vivo*. Due to limited availability of human brain sections, anti-Aβ immunostaining could not be performed. Although in each brain section, obtained from different individual suffering with Alzheimer's disease at an advanced stage, consistent ThS staining reduction was noticed upon incubation. Moreover, with a higher purity of plant derived MBP, more effective degradation results could have been observed.

We observed a concentration-dependent degradation and/or reduction of amyloid levels of up to 66% in the brains of old 3xTg AD mice upon MBP incubation. Therefore, in this study, our investigations focus on the role of MBP to reduce the amyloid levels *in vivo* in 3xTg AD mice and *ex vivo* in brains of advanced AD individuals, by CTB mediated delivery of MBP that was expressed in plant cells through chloroplast engineering. We observed that 3xTg AD mice brain showed up to 67% and 33% decrease of amyloid levels when fed with CTB-MBP, particularly in dentate gyrus, CA1 and CA3 regions of hippocampal and in cortical brain regions, that are known to carry the most amyloid burden as a certain pathology in transgenic AD mice as well as in human AD (44). Immunostaining and thioflavin fluorescence, quantified and analyzed by fluorescence microscopy, were used to detect Aβ deposits. Moreover, thioflavin positive

amyloid deposits were observed in the areas of anti-amyloid immunoreactivity by staining hippocampus in adjacent brain sections in *in vivo* studies with anti-amyloid antibody and thioflavin S. This study quantitatively confirms the reduction of immunoreactive amyloid deposits in brain sections from oral delivery of CTB-MBP in old 3xTg AD mice. Histological analyses confirms quantitative as well as qualitative reduction of Aβ accumulation pathology through MBP in 3xTg AD mice and human AD.

Medical imaging techniques like magnetic resonance imaging (MRI) measure the structural integrity of myelin (45). Both widespread and diffused myelin breakdown (41, 46) in the brains has been reported in patients with AD. Correlation of amyloid deposition with destruction of myelin also contributes the progression of age dependent AD (42, 47). Studies report that slow myelin turnover rate could occur (48) because oligodendrocytes in the central nervous system (CNS) may differentiate in the brain development process with condensed myelin repair ability, thereby contributing to myelin degeneration.

Another hallmark of AD observed in 3xTgAD mice is neurofibrillary tangles that comprise of tau proteins (49) which are pathologic in their hyper-phosphorylated state and appears after Aβ deposition. It is possible that contribution of these tangles towards pathology may be reduced by MBP and hence demands further investigations for their reduction in brains of AD patients and transgenic mice model. In addition, the clearance of amyloid load in our investigation also warrants further behavioral studies in 3xTgAD mice over a long term period by oral delivery of bioencapsulated CTB-MBP. However, behavioral changes should be investigated in mice, fed with plant material simultaneously, at an early age of developing AD.

Recent findings suggest that Aβ accumulation is related to stress-induced senescence of astrocytes (50), by losing their ability for homeostasis maintenance in CNS. It remains to be determined from our findings if clearance of Aβ from brain would decrease astrocytes senescence, which contributes to pathogenesis and progression of AD (50). In addition, it has been suggested that zinc sequestration by Aβ deposits is susceptible to AD pathology (51), by inducing Aβ aggregation and disturbing zinc homeostasis. The therapeutic approach suggested by our investigations could also help elucidate mechanistic approaches for zinc distribution associated with pathology in AD.

Active and passive immunotherapies have been reported to target Aβ in clinical studies (11) through immunization, but recent disappointment with bapineuzumab antibody in clinical trial III calls for alternative strategy for Alzheimer's drugs and therapies. The 2011 world Alzheimer's report concludes that up to 28 million people with dementia, lack efficient diagnosis, treatment and care (52), increasing the "treatment gap" from high to low and middle income countries Not only the need for novel strategies are needed but also the urge to halt it at an initial stage will be proven beneficial (53). Our lab used genetically engineered plants for expression and oral delivery of proinsulin (29), insulin and C peptide (30), exendin 4 (25) for treatment of diabetes or blood clotting factor IX for hemophilia B (31). Here we report expression of CTB fused with MBP in tobacco plant chloroplasts and their expression as a fusion protein. Lyophilization of leaf materials increases concentration of bioencapsulated transgenic protein for oral delivery. Lyophilization of plant cells increased CTB-MBP concentration, resulting in CTB-MBP capsules to be stored at room temperature for several months without any degradation of therapeutic protein. Oral delivery of therapeutic protein MBP, through GM1

receptor mediated delivery of CTB to the circulatory system and to the brain across the blood brain barrier in the AD model of transgenic mice in vivo and in advanced human AD brains ex vivo, opens the door for a novel concept and further advances in human clinical studies.

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