FEBS 30166 FEBS Letters 579 (2005) 6737–6744

# Transgenic potato expressing Aβ reduce Aβ burden in Alzheimer's disease mouse model

Jung Won Youm[a,1], Hee Kim[b,1], Jee Hye Lo Han[b], Chang Hwan Jang[b], Hee Jin Ha[b], Inhee Mook-Jung[c], Jae Heung Jeon[a], Cheol Yong Choi[d], Young Ho Kim[b,*], Hyun Soon Kim[a,*], Hyouk Joung[a]

[a] *Plant Cell Biotechnology Lab., Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-335, Republic of Korea*
[b] *Digital Biotech Inc., R&D, 1227, Sin Gil Dong, An San City, Kyung Gi Do 425-839, Republic of Korea*
[c] *Department of Biochemistry & Cancer Research Institute, College of Medicine, Seoul National University, Seoul 110-799, Republic of Korea*
[d] *Department of Biological Science, Sungkyunkwan University, Suwon 440-746, Republic of Korea*

Received 7 September 2005; revised 26 September 2005; accepted 2 November 2005

Available online 21 November 2005

Edited by Jesus Avila

**Abstract** **Beta amyloid (Aβ) is believed one of the major pathogens of Alzheimer's disease (AD), and the reduction of Aβ is considered a primary therapeutic target. Immunization with Aβ can reduce Aβ burden and pathological features in transgenic AD model mice. Transgenic potato plants were made using genes encoding 5 tandem repeats of Aβ1–42 peptides with an ER retention signal. Amyloid precursor protein transgenic mice (Tg2576) fed with transgenic potato tubers with adjuvant showed a primary immune response and a partial reduction of Aβ burden in the brain. Thus, Aβ tandem repeats can be expressed in transgenic potato plants to form immunologically functional Aβ, and these potatoes has a potential to be used for the prevention and treatment of AD.**


*Keywords:* Oral immunization; Beta amyloid; Alzheimer's disease; Transgenic plant; Potato

## 1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that is characterized by memory loss and cognitive impairments in the elderly [1]. The pathological hallmarks of AD are neuritic plaques and neurofibrillary tangles. Neuritic plaques are extracellular deposits of fibrils and amorphous aggregates of amyloid β (Aβ) peptides. Neurofibrillary tangles are intracellular fibrillar aggregates of the microtubule-associated protein tau that exhibit hyperphosphorylation and oxidative modifications [2]. Genetic and pathological evidence strongly supports the amyloid cascade hypothesis of AD [3]. The accumulation and aggregation of Aβ appears to damage neurons, which leads to the degeneration of synapses and, eventually, neuronal death. Tau pathology, which contributes substantially to the disease process through hyperphosphorylated tau and tangles, is triggered by Aβ1–42.

The current standard of care for mild to moderate AD includes treatment with acetylcholine esterase inhibitors to improve cognitive function [4], and Memantine, an *N*-methyl-D-aspartate antagonist, has been approved in the US and some other countries for the treatment of advanced AD. All of these drugs are of limited benefit to most patients because they have only modest effects on the symptoms of AD. Currently, no treatment that has strong disease modifying effects is available.

Drugs are currently being developed which target specific sites in the amyloid cascade. Several secretase inhibitors have been tested for their abilities to reduce Aβ generation. γ-Secretase inhibitors, however, often failed this test because of blocking NOTCH signaling pathways [5]. BACE1 (β-secretase) inhibitors may prove beneficial in reducing the production of Aβ, because BACE1 knockout mice showed reduced Aβ production and do not exhibit any abnormal phenotypes [6]. Other approaches to reducing Aβ aggregation in the brain include the use of chelators and small molecules with affinities to Aβ [7]. One promising approach to preventing and treating AD is based upon stimulating the immune system to remove Aβ from the brain [8]. Several strategies, including active and passive immunization, have been examined. The initial reports that immunization with aggregates Aβ1–42 or passive immunization with anti-Aβ antibodies results in the clearance of Aβ plaques from the brains of amyloid precursor protein (APP)-mutant transgenic mice were followed by several reports which found that such immunizations can restore cognitive deficits in transgenic mice [9–12]. Although immunization studies show promising results in APP transgenic mice without any detectable adverse effects, the recent extension of this approach to AD patients resulted in the development of meningoencephalitis, a potentially deadly inflammation of the brain and surrounding membrane, in a small, but unacceptable portion of patients [13]. This called attention to the issue of the refinement of immunization methods to AD researchers. Much progress has been made in designing a vaccine which is appropriate for human use [14–19]. Nonetheless, an effective vaccine

*Corresponding authors. Fax: +82 31 493 1867 (Y.H. Kim), +82 42 860 4599 (H.S. Kim).
*E-mail addresses:* youngho@digitalbiotech.com (Y.H. Kim), hyuns@kribb.re.kr (H.S. Kim).

[1] These authors contributed equally to this work.

*Abbreviations:* AD, Alzheimer's disease; Aβ, beta amyloid; APP, amyloid precursor protein; CTB, cholera toxin B; ELISA, enzyme linked immunosorbent assay; 5Aβ42, tandem repeat 5mer of Aβ1–42; T-DNA, transfer DNA

0014-5793/$30.00 
doi:10.1016/j.febslet.2005.11.003

would be the ultimate solution to AD, and is currently one of the most exciting fields in AD research.

Transgenic plant systems represent an alternative method for the expression of recombinant antibodies, antigens, and therapeutics. During the last 10 years, a host of antigens have been successfully expressed in plants and orally delivered to animals, resulting in enhanced immunogenicity. These antigens include the disease-related virus capsid and surface proteins, and pathogenic bacterial antigens [20–22]. The successful development of a potato-derived vaccine would allow manufactures to meet huge, steady demands.

Here, we report our study of plant-derived human Aβ used in an edible vaccine designed to elicit a specific Aβ immune response. We generated transgenic potatoes which expressed 5 tandem repeats of Aβ1–42 (5Aβ42), under various control schemes. We used the M35I mutated form of Aβ1–42, which causes less oxidative stress in cells than does wild type Aβ1–42. AD model mice (Tg2576) were vaccinated by oral gavage with extracts of transgenic potatoes plus cholera toxin B (CTB). The Aβ-immunized mice were found to have generated antibodies against Aβ, and showed reduced numbers of Aβ plaques in their brains.

## 2. Materials and methods

### 2.1. Generation of the tandem repeats form of Aβ1–42

APP695 cDNA in pCB6 (a gift from Dr. Sisodia at the University of Chicago) was used as a template for the preparation of Aβ1–42 cDNA. In order to ameliorate the toxicity normally associated with wild-type Aβ1–42, we used the mutant β-amyloid, in which the methionine at the 35th sequence has been substituted with isoleucine (M35I). The mutation was generated using the Quickchange mutagenesis kit (Stratagene, La Jolla, CA) according to the instructions of the manufacturer. Specific primers, each of which contained the *Alw*NI enzyme cleavage site (underlined), were designed for the purpose of PCR amplification (forward primer; 5′-TGA CAG ATG CTG GAT GCA GAA TTC CGA-3′, backward primer; 5′-TAA CAG ATG CTG CGC TAT GAC AAC ACC-3′). The amplified Aβ1–42 PCR products were then digested with the *Alw*NI enzyme, and they were then ligated to produce the multimeric form. After gel elution of the multimeric form of DNA (approximately 4–5mer), DNA was introduced into the *Alw*NI site of the pET31b(+) vector (Novagen, Madison, WI). Cloning of the tandem repeats of wild-type Aβ and mutant Aβ was performed at the same time. Each of 5 tandemly repeated Aβ1–42 containing clones (pET31b-5Aβ42) were isolated and confirmed via DNA sequencing and Western blotting, using the Aβ-specific antibody, 4G8 (Signet Lab., Dedham, MA). In order to compare wild-type Aβ with mutant Aβ, the aggregation ability and cytotoxicity of each 5Aβ42 were tested. Aggregation of 5Aβ42 was measured by the thioflavin T (ThT)-binding assay, in which the fluorescence intensity reflects the degree of Aβ fibril formation [23]. Three μM of each of the synthetic Aβ1–42, wild-type 5Aβ42 and mutant form 5Aβ42, was incubated in PBS for 1 h. ThT solution (5 μM) was added to each reaction. The resulting ThT fluorescence was measured using excitation and emission wavelengths of 440 and 485 nm, respectively (Safire, TECAN, Austria). To explore the cytotoxicity, tandem repeat 5Aβ42 was challenged in HT22 murine cell culture. Twenty-five μM of each of the aggregated Aβ was used. Cell viability was determined by MTT (methylthiazol-2-yl-2,5-diphenyl tetrazolium bromide) assay [24]. The survival rate from 5Aβ42-challenged cells was calculated and compared to synthetic Aβ25–35-challenged cells. After the characterization of the Aβ profiles of the wild-type and the mutated form, we used the mutated form of Aβ for further studies.

### 2.2. Construction of a plant expression vector and transformation into potato

The tandem-repeated Aβ gene, intended for ligation into a plant expression vector, was amplified from the pET31b-5Aβ42(M) clone using two primers: 1. Aβ F primer (5′-GGT ACC GGA TCC GCC ATG GAT GCA GAA-3′), to which *Kpn*I, the *Bam*HI restriction site, and the start codon (boxed) were added; 2. AβR primer (5′-GAG CTC GGA TCC AAC TCA CGC TAT GAC-3′), to which *Sac*I, the *Bam*HI restriction site, and a stop codon (boxed) were added. A single amplification product, with an expected size of 5mer of Aβ (denoted as 5Aβ), was purified and subcloned into TopoTA (Invitrogen, Carlsbad, CA) in order to generate Topo5Aβ, which was then confirmed by DNA sequencing. The 694-bp *Bam*HI fragment of Topo5Aβ was then cloned into pMBP1 [25], which yielded pMBP5Aβ. Additionally, the *Kpn*I/*Sac*I fragment of Topo5Aβ was cloned into pAT1 (provided by Dr. G.H. Harn, NongwooBio, Korea), which yielded pAT5Aβ. The 5Aβ PCR product was amplified with another forward primer (5′-TGA GTC GAC GAT GCA GAA TTC CGA CAT GAC TCA-3′), which added the *Sal*I site, and another reverse primer (5′-TAA TCT AGA TCA AAG CTC ATC CTT CTC AGA CGC TAT GAC AAC ACC GCC-3′), which added a sequence coding for SEKDEL (boxed) and an *Xba*I site. We then cut this product with *Sal*I/*Xba*I, and subcloned it into pRTL2 [26] to make pRTL-5AβS. The *Hin*dIII fragment from pRTL-5AβS was then cloned into pBIN19 (Clontech, Mountain View, CA), which yielded the pE5AβS vector.

These vectors were then transformed into *Agrobacterium tumefaciens* LBA4404. The in vitro grown leaf explants (*Solanum tuberosum* cv. Desiree) were then co-cultured in an *Agrobacterium* suspension. The regenerated shoots from the leaf explants were cultured in MS medium (Life Technologies, Gaithersburg, MD), which yielded 1 g of microtubers. At the same time, small plants were transplanted to a greenhouse, in which they were allowed to grow naturally. The microtubers and mature-tubers were harvested after 2 months, and were used in further analyses. The transgenic shoots were initially selected based on the degree of their resistance to kanamycin. To confirm the presence of the 5Aβ gene, PCR analysis was performed.

### 2.3. Nucleic acid extraction and analysis

Total RNA from the tubers of the transformed plants was isolated using the RNAgents® total RNA isolation system (Promega, Madison, WI) according to the instructions of the manufacturer. In order to find Aβ gene expression, Northern blot analysis was conducted with 30 μg of total RNA from each transformed potato. Genomic DNAs from pE5AβS-derived transformants were isolated and digested with *Sal*I and transferred to nitrocellulose filter paper. Southern blot analysis was performed with a Dig-labeled Aβ1–42 gene as a probe.

### 2.4. Protein extraction and analysis

Proteins from the tubers were obtained by homogenization in a mortar and pestle with liquid nitrogen as described in a previous study [27]. Western blot analysis was performed with 4G8 antibody. The recombinant 5Aβ protein levels were determined via direct ELISA assays. The ELISA plates were coated with 100 μg of TSP in 0.05 M carbonate–bicarbonate (pH 9.6) buffer and incubated with monoclonal anti-Aβ1–42 specific antibody (6E10, 1:1000 dilution). The amount of Aβ proteins in TSP was estimated using direct ELISA with Aβ1–42 peptide as a standard.

### 2.5. Vaccination of Tg2576 with transformed plant extract

A group of transgenic mice which expressed human APP (Tg2576), all of which were 15 months old, was used in the immunization experiments. Ten mice from each group were fed tubers from transgenic potato lines pE5AβS #2, and control non-transgenic potatoes plus CTB as an adjuvant. Each mouse was gavaged approximately 25 mg of transgenic plant extracts plus 10 μg CTB (C9903, Sigma, St. Louis, MO) once a week for three consecutive weeks. Blood samples were obtained on day 0, and then 3, 5 and 7 weeks after primary immunization. All blood samples were analyzed by direct ELISA assay. Seven weeks after primary immunization, the transgenic mice were boosted by intraperitoneal injection with 15 μg of synthetic Aβ1–42 peptide emulsified in alum [22]. Sera were collected from the mice 2 weeks after boosting, and were evaluated by direct ELISA assay.

### 2.6. Direct ELISA for anti-Aβ antibody production

In order to measure the anti-Aβ antibody titers in the sera of immunized mice, we coated flat-bottom ELISA plates overnight at 4 °C with Aβ1–42 [0.1 μg/well in 0.05 M carbonate–bicarbonate buffer (pH 9.6)]. The plates were washed three times in PBS-T (phosphate-buffered

saline, 0.05% Tween-20), and blocked for 2 h at room temperature (RT) with 3% BSA in PBS. The plates were then washed three times, and incubated with serum samples diluted to 1:100 in blocking buffer for 2 h at RT, with agitation. The plates were washed three times and incubated with mouse IgG-conjugated horseradish peroxidase (secondary antibody) for 2 h at RT. The plates were washed an additional three times, and were then incubated with 100 μl of TMB substrate (Pierce, Rockford, IL) and $H_2O_2$ for 30 min at RT. After incubation, the reaction was measured at 450 nm with an ELISA reader.

### 2.7. Plaque reduction assay

In order to determine the degree to which the amyloid plaques in the transgenic mice had been reduced, we stained sections from the hippocampal regions of the left hemisphere of the mouse brains with Congo Red. When stained with Congo Red, amyloid plaques appear pink under the microscope. Digital images were taken using a digital camera (Olympus Camedia, C-5050). Images from ten sections of each animal were analyzed using Meta-Morph software. The percentage of the area covered by plaques (Aβ burden) was calculated from the image data. The number of plaques in the defined area and the average plaque size were computed by a computer program in a given parameter [28]. All of the quantitative morphological data were analyzed by one-way ANOVA. A *P*-value less than 0.05 indicated a statistically significant difference.

## 3. Results

### 3.1. Construction of 5 tandem repeats Aβ1–42 and characterization of its product from Escherichia coli

According to recent studies conducted by McGowan et al. [29], mice overexpressing Aβ1–42 develop an AD-like phenotype, but this does not occur in mice expressing Aβ1–40. This finding clearly demonstrates that Aβ1–42 is major causative agent in the pathology of AD. Aβ1–42 aggregates itself and induces oxidative stress in the brain. The 35th methionine of Aβ1–42 seems to lie in the middle of the oxidative stress-signaling pathway associated with AD [30]. Therefore, the Aβ1–42 mutant form is thought to be less toxic than the wild-type peptide. In recent vaccine studies, some of patients have developed meningoencephalitis [13]. We tried to solve this problem by using a mutated form of Aβ1–42 and through the development of an edible vaccine. At the outset, the mutation was introduced to Aβ1–42, replacing Met with Ile at position 35 (Fig. 1A and B). The multimer form of Aβ1–42 was successfully isolated and cloned into pET31b(+) vector (Fig. 1C and D) for 5Aβ42 production in *E. coli*. The multimer form of

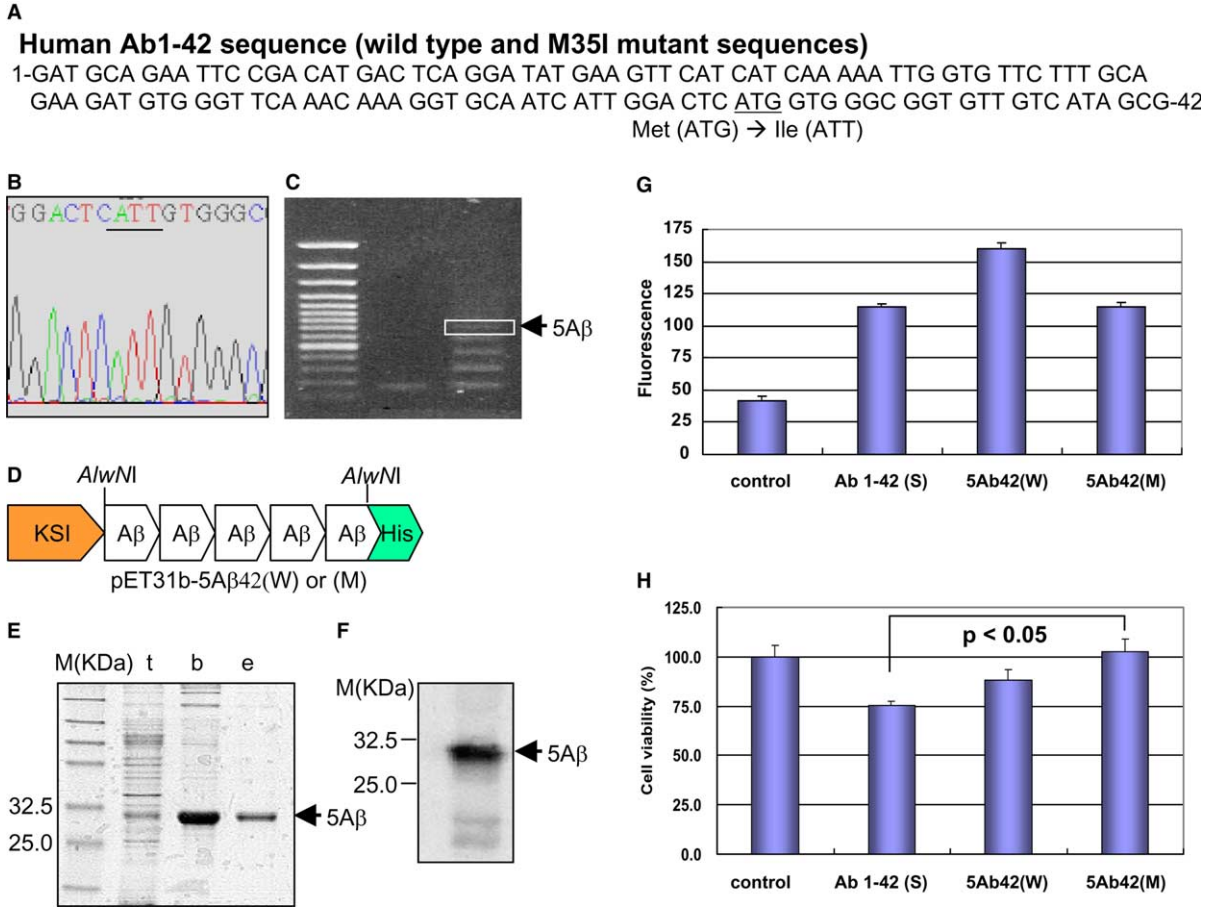


Fig. 1. Construction and characterization of 5Aβ42. (A) Human Aβ1–42 sequence. The 35th Methionine (Met) is underlined. (B) The 35th Met was replaced by Ile. (C) Multimer formation was visualized in agarose gel stained by ethidium bromide. (D) Structure of *E. coli* expression vector for 5Aβ42. KSI, ketosteroid isomerase gene for protein solubility; His, 6×His affinity tag for purification. (E) Production and purification of 5Aβ42. After IPTG induction, total extracts (t) were bound to a nickel affinity column. Column-bound proteins (b) were eluted with imidazole. Eluted protein (e) was analyzed by SDS–PAGE. (F) Western blot result of eluted 5Aβ42. (G) Aβ aggregation activity test. Each of the 3 μM proteins was incubated in PBS for 1hr. The aggregation was monitored by ThT fluorescence. (H) Aβ toxicity test. HT22 cells were challenged with 25 μM of each peptide. The cell survival rates were recorded by MTT assay. The 5Aβ42 peptide has no detectable cytotoxicity (statistically significant result, $P < 0.05$, Student *t* test).

wild-type Aβ1–42 was also cloned into pET31b(+) vector for comparison with mutant Aβ1–42 (data not shown). In order to determine whether the mutated 5Aβ42 retains immunogenicity, Western blot analysis was conducted with purified 5Aβ42 protein with an Aβ-specific antibody, 4G8. The expressed 5Aβ42 exhibited a specific band of the expected size (Fig. 1E and F). Both the mutated form and the wild-type 5Aβ42 aggregated efficiently in PBS. No difference was shown between the aggregation ability of 5Aβ42 and the aggregation ability of synthetic Aβ1–42 at 3 μM for 1 h incubation (Fig. 1G). Additionally, mutant form of the 5 Aβ42 is much less harmful to HT22 neuroblastoma cell cultures than synthetic Aβ1–42 at the same concentration as expected [Fig. 1H, Aβ1–42(S) vs. 5Aβ42(M), $P < 0.05$].

### 3.2. Expression of 5Aβ in transgenic plants

We cloned 5Aβ42 into three different binary vectors: pMBP, pAT and pE35S, all of which possess the nptII gene for selection on kanamycin. We were attempting to develop a vector system with the highest possible 5Aβ42 expression in the transgenic potato tubers. The 35S promoter was applied for strong and constitutive expression (pMBP5Aβ); the patatin promoter was also used for tuber-specific expression (pAT5Aβ). The double 35S promoter, TEV 5′-UTR, the signal peptide linked to the 5′ end of 5Aβ42, and the ER retention peptide linked to the 3′ end of 5Aβ42 were used to enhance accumulation and translation (pE5AβS) (Fig. 2A). Transgenic potatoes were obtained through kanamycin selection and were confirmed by PCR (data not shown). Most of the transformed plants were found to grow successfully with no phenotypic abnormalities, which indicated that the expression of tandem-repeated 5Aβ42 may not interfere with the growth and viability of the plants. These results are consistent with the results of a previous report [27]. The proper transcription and translation of the 5Aβ42 genes from different transgenic plants lines were confirmed by Northern blot and genomic Southern blot analysis. A total of 10 transgenic lines from 3 different constructs were selected: 3 lines of pMBP5Aβ (#1, 3 and 6), 4 lines of pAT5Aβ (#1, 2, 3 and 5) and 3 lines of pE5AβS (#1, 2 and 8). From the results of Northern blot analysis, the transformants containing 5Aβ42 controlled by the double 35S promoter with the TEV 5′-UTR leader sequence evidenced higher 5Aβ42 transcription levels than did the transformants which harbored the 5Aβ42 gene under the control of either the patatin promoter or the single 35S promoter (Fig. 2B, lanes 8–10). We detected no significant differences in the β-amyloid expression levels between the pMBP5Aβ- and pAT5Aβ-expressing transgenic tubers. In order to determine the transfer DNA (T-DNA) copy numbers and the integration pattern of pE5AβS (which shows the highest 5Aβ42 expression among all of the transformants), the genomic DNA was isolated, digested with *Sal*I, and hybridized with a 5Aβ42-specific probe. The pattern of genomic Southern blotting indicated that the insertions had occurred at random sites throughout the genome and copy number was more than 4 (Fig. 2C).

Multiple β-amyloid (5Aβ) proteins expressed in transformed potato tubers were analyzed by Western blotting (Fig. 2D). Total soluble proteins were extracted from the tubers of the individual transformed potato plants expressing pMBP5Aβ, pAT5Aβ, and pE5AβS. 100 μg of TSP was loaded onto 12% SDS–PAGE, and was then subjected to immunoblot analysis with the 4G8 antibody. The amount of 5Aβ42 expression from transgenic plants lines was consistent with the level of transcript determined by Northern blot analysis (Fig. 2B vs. D). Using direct ELISA assays, we estimated the levels of 5Aβ42 protein

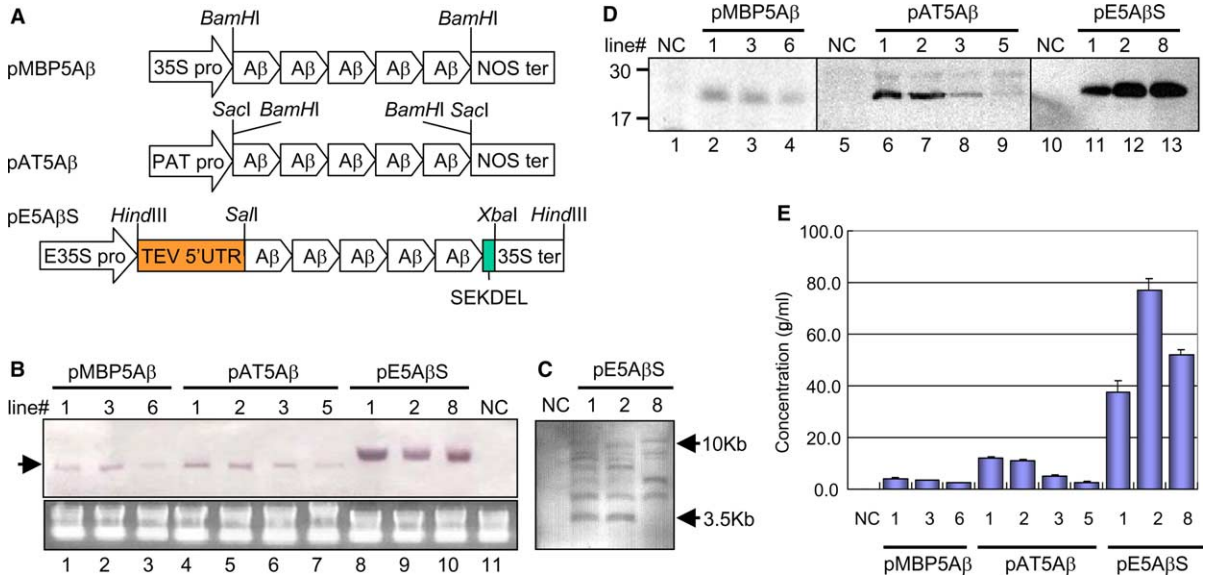


Fig. 2. Construction and production of transgenic potatoes. (A) Schematic diagram of expression cassettes. The 5Aβ42 gene was cloned under the control of 35S CaMV promoter (35S pro), patatin potato tuber-specific promoter (PAT pro), and the dual enhancer 35S (E35S pro) promoter. Transcription was terminated at the 3′ region from the *A. tumefaciens* nopaline synthase gene (NOS ter) and the 35S CaMV gene (35S ter). TEV 5′-UTR, tobacco etch virus 5′-UTR. Hexapeptide ER retention signal (SEKDEL) were used in the pE5Aβ vector. (B) Northern blot analysis of transgenic plant lines. Equal amounts of total RNA were loaded and hybridized by a 5Aβ42-specific probe. All samples contained the 5Aβ42 gene. The intensities of the transcripts indicated the strength of the promoters. NC, non-transgenic potato as negative control. (C) Genomic Southern blot of pE5AβS lines. Multiple and random integration are shown. (D) Western blot analysis of transgenic plant lines. An equal amount of TSP was loaded and 5Aβ42 was detected by Aβ-specific antibody, 4G8. (E) Quantitative analysis of expressed 5Aβ42 in transgenic plants. Direct ELISA was performed with each transgenic plant TSP. The results were consistent with Western blot analysis.

expression in the transgenic tubers expressing each of the three vectors. In the pAT5Aβ transgenic lines, we confirmed slightly higher 5Aβ protein expression levels, as compared to that observed in the pMBP5Aβ transgenic lines. This indicates that the patatin promoter, a tuber-specific promoter which drives Aβ expression in the potato, is stronger than the CaMV35S promoter, a constitutional expression promoter. The most profound β-amyloid expression was observed with pE5AβS line #2, which has the translation enhancer of the tobacco etch virus (TEV) and a cleavage plant signal peptide, which were added at the N-terminus of 5Aβ42. pE5AβS line #2 also possesses an ER retention signal, designated 'SEKDEL', which is added at the C-terminus. The highest 5Aβ42 expression in tuber tissue was approximately 77.0 μg/ml of the applied extract in pE5AβS line #2 (Fig. 2E). These data indicate that the double 35S promoter, along with the TEV 5′-UTR, may trigger an increase in the expression of the 5Aβ protein in pE5AβS transgenic plants. Additionally, a combination of the signal peptide and the SEKDEL extension appears to result in the most pronounced accumulation of the multimeric form of 5Aβ in the plant cells. The antigen, modified with a carboxyl-terminal 'SEKDEL' extension, was found to accumulate in the potato tubers at a higher level than did the unmodified antigen [31,32]. Finally, we selected pE5AβS line #2 as material which would be used for further mice immunization.

### 3.3. Oral immunization of Tg2576 mice with plant-derived 5Aβ

Our first objective was to ascertain whether oral administration of the multimeric form of Aβ1–42 was capable of inducing an immune response, and eventually clearing Aβ deposition in AD model mice. In groups of ten, Tg2576 mice were immunized with extracts from transgenic potato line pE5AβS #2 with non-transformed potato as control. The line #2 tuber extracts was estimated to contain about 0.9 μg of specific Aβ1–42 protein per milligram of TSP. We used about 25 mg of TSP per feeding, and included CTB as an adjuvant. Mice fed on plant-derived 5Aβ42 developed primary serum antibody responses after the first dose. The sera of the immunized mice with the non-transformed plant extracts exhibited no induction of cross-reactive antibodies (Fig. 3A). Antibody serum responses persisted at high levels after the initial feeding of mice with extracts from transgenic line #2 for up to 9 weeks. After that time, the mice were sacrificed to allow for analyses of their brains. After boosting with 15 μg of synthetic Aβ1–42 peptide emulsified in alum at week 7, we noted no further increases in the levels of serum 5Aβ-specific antibodies. Weiner and his colleagues reported that Tg2576 mice showed immune hyporesponsiveness to human Aβ40 peptide because of impaired immune systems [33]. Our results suggest that plant-derived 5Aβ42 plus CTB activated Aβ42-specific T cells to provide assistance in the production of antibodies.

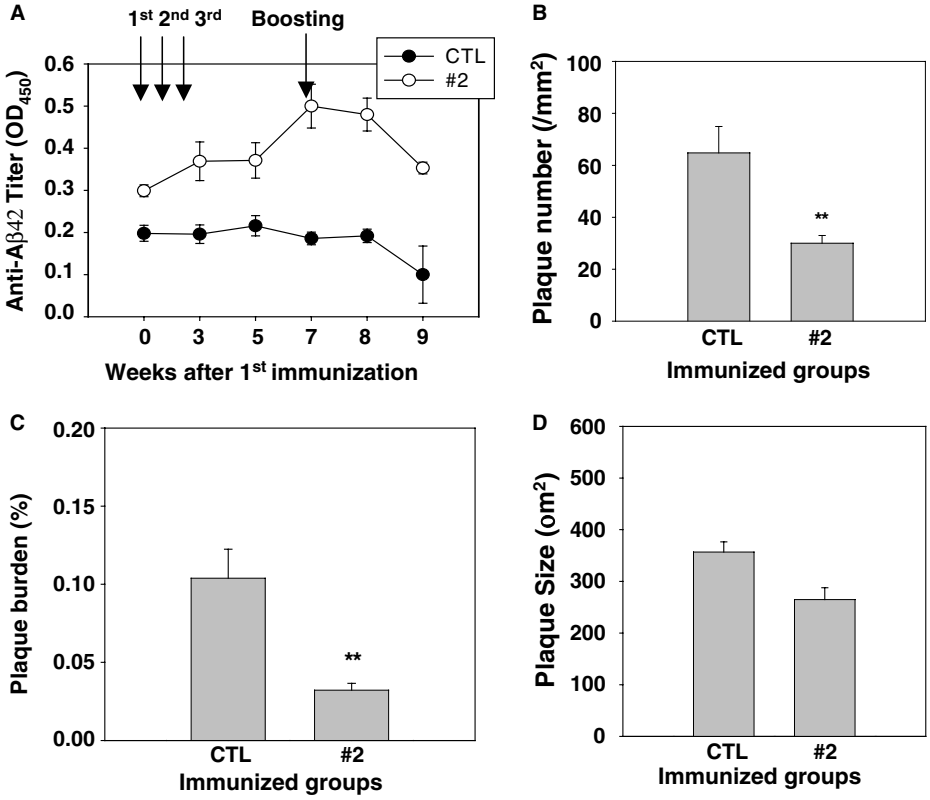


Fig. 3. Immunization and characterization of Tg2576 with plant-derived 5Aβ42. (A) Anti-Aβ antibody responses in transgenic Tg2576 mice, subsequent to the oral uptake of transgenic plant materials. Mice were gavaged by either transgenic tuber lines pE5AβS #2, or non-transformed tubers (CTL) with a CTB adjuvant. Arrows (→) indicate the feeding schedule: day 0, 1, 2 weeks for gavage, and boosting at 7 weeks after initial feeding. (B) Reduction in amyloid plaque in transgenic mice. Fifty sections from each group were stained with Congo Red and the stained sections were then photographed with a digital camera (Olympus Camedia, C-5050) which was linked to the microscope. The images were transmitted to the Meta-Morph program. The area of plaque was calculated. (C) Plaque number per square millimeter was counted. (D) The average plaque size was estimated. Note the statistical significance by one-way ANOVA ($^{**}P < 0.01$).

Several immunization studies for AD mouse models showed a reduction of Aβ levels in animal brains, as well as behavioral changes [11]. In order to determine the effects of plant-derived 5Aβ on the number of amyloid plaques in the brains of challenged mice, we counted the plaques in the brains of Tg2576 mice which had been fed plant-derived 5Aβ42 and CTB (Fig. 3B–D). Our analysis of the brains of the challenged mice revealed an effective reduction of existing plaques in the #2 feeding group, but this reduction was not observed in the control group. The plaque number and the % area of plaque (burden) were significantly reduced in #2 immunized mice (Fig. 3B and C), but the average plaque size was not changed (Fig. 3D). These results indicated that plant-expressed 5Aβ and CTB not only induces a humoral immune response, but also affects the clearance of plaques from the brain.

## 4. Discussions

Few published reports have made special mention of any relationship existing between the expression of foreign proteins and phenotype abnormalities. This would appear to suggest that the expression of foreign proteins exerts no significant adverse effects on the growth of plants [34–36] or interferes with plant growth and viability [37,38]. Beta-amyloid is toxic to cultured cells, but the most of the 5Aβ42-transformed plants were found to grow successfully with no phenotypic abnormalities. These results are consistent with the results of a previous report [27], which indicated that the expression of β-amyloid protein in potato plants exerts no adverse effects on either shoot growth or tuberization, not only under in vitro conditions, but also under the conditions normally observed in a greenhouse.

In our transformed potatoes, the double 35S promoter appears to be more active than any other promoters. Sometimes, however, the presence of 35S promoter resulted in higher levels of expression than did the double 35S promoter [39,40]. This indicates that the expression of a specific antigen, when driven by a different promoter, can also be affected by the nature of the antigen. Additionally, the expression of foreign genes in the different transgenic lines with the same expression vector appeared to vary according to the sites of their integration into the genomic DNA, as well as the copy number (Fig. 2B, C, and D for pE5AβS). The T-DNA insertion patterns reported in this study were different from the simple insertion patterns which are commonly seen in association with *Agrobacterium* transformation. Olhoft and Somers [41] reported that all of the analyzed $T_0$ plants exhibited multiple copies of a number of transgene-hybridizing fragments. They concluded that the observed multiple integration of T-DNA could be attributed to the nature of the binary plasmid.

Our studies indicate that the oral introduction of 5Aβ potato extract with adjuvant into mice stimulated a primary immune response, but we noted no strong or robust response after boosting at week 7. In several reports, an immediate and strong secondary antibody response has been observed in animals receiving a booster of a commercial vaccine, as memory immune cells had apparently been established as a result of oral immunization [22,42]. In the case of Tg2576 transgenic mouse immunization, the impaired adaptive immune response was associated with Aβ1–40 [33].

We used CTB as an adjuvant; CTB is a nontoxic subunit of cholera toxin which has been shown to act as a non-T helper (Th) 1-inducing adjuvant. In an initial clinical trial with Aβ1–42 (AN-1792) conducted by Elan Corp., QS-21, a Th1 adjuvant, was used as adjuvant. Phase IIa trials were halted because some of the patients showed meningoencephalitis due to inflammation in the brain through T-cell hyper-activation. Some researchers predict that Aβ1–42 has both B-cell and T-cell epitopes that would be expected to result in extensive T-cell activation. Therefore, CTB is a more potent adjuvant for Aβ immunization studies [43]. We have not observed any brain inflammatory symptoms in the brains of our mice (unpublished results).

This study is an example of active immunization for AD, and also represents a unique approach, in which transgenic plants are used to produce a vaccine. The clinical peptide vaccine trials were canceled due to adverse effects. As in most other Aβ immunotherapy trials, this trial has taught us that the optimization of a vaccine must circumvent or eliminate deleterious effects, while maintaining the elicitation of a highly specific immune response to Aβ. In this study, we have suggested two possibilities for the elimination or amelioration of adverse effects. One involves the modification of wild-type Aβ1–42. We used the multimeric variant of Aβ, with a modified sequence used as the antigen. Oral vaccines act by stimulating the immune system at effector sites (lymphoid tissue) located in the gut, and the gut immune system suppresses Th1 responses and enhances Th2 responses [21,44]. Therefore, we plan to conduct further studies in order to determine the manner in which transgenic potatoes expressing 5Aβ42 can be used in the development of an edible vaccine against Alzheimer's disease.

*Acknowledgements:* This work was funded by a grant (PF0330302-00) from the Plant Diversity Research Center of the 21st Century Frontier Research Program, which is funded by the Ministry of Science and Technology, a division of the government of the Republic of Korea. This work was also supported by the Korea Health 21 R&D Grant (02-PJ2-PG6-DC04-0001) from Ministry of Health and Welfare.

## References

[1] Price, D.L. and Sisodia, S.S. (1998) Mutant genes in familial Alzheimer's disease and transgenic models. Annu. Rev. Neurosci. 21, 479–505.
[2] Taylor, J.P., Hardy, J. and Fischbeck, K.H. (2002) Toxic proteins in neurodegenerative disease. Science 296, 1991–1995.
[3] Hardy, J. and Selkoe, D.J. (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297, 353–356.
[4] Doody, R.S. (1999) Therapeutic standards in Alzheimer's disease. Alzheimer Dis. Assoc. Disord. 13 (Suppl.), 20–26.
[5] Wong, G.T., Manfra, D., Poulet, F.M., Zhang, Q., Josien, H., Bara, T., Engstrom, L., Pinzon-Ortiz, M., Fine, J.S., Lee, H.J., Zhang, L., Higgins, G.A. and Parker, E.M. (2004) Chronic treatment with the γ-secretase inhibitor LY-411,575 inhibits β-amyloid peptide production and alters lymphopoiesis and intestinal cell differentiation. J. Biol. Chem. 279, 12876–12882.
[6] Luo, Y., Bolon, B., Kahn, S., Bennett, B.D., Babu-Khan, S., Denis, P., Fan, W., Kha, H., Zhang, J., Gong, Y., Martin, L., Louis, J.C., Yan, Q., Richards, W.G., Citron, M. and Vassar, R. (2001) Mice deficient in BACE1, the Alzheimer's β-secretase, have normal phenotype and abolished β-amyloid generation. Nature Neurosci. 4, 231–232.
[7] Citron, M. (2004) Strategies for disease modification in Alzheimer's disease. Nature Rev. Neurosci. 5, 677–685.

[8] Schenk, D., Barbour, R., Dunn, W., Gordon, G., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., Khan, K., Kholodenko, D., Lee, M., Liao, Z., Lieberburg, I., Motter, R., Mutter, L., Soriano, F., Shopp, G., Vasquez, N., Vandevert, C., Walker, S., Wogulis, M., Yedneck, T., Games, D. and Seubert, P. (1999) Immunization with amyloid-β attenuates Alzheimer's disease-like pathology in the PDAPP mouse. Nature 199, 173–177.

[9] Janus, C., Pearson, J., McLaurin, J., Mathews, P.M., Jiang, Y., Schmidt, S.D., Chishti, M.A., Horne, P., Heslin, D., French, J., Mount, H.T.J., Nixon, R.A., Mercken, M., Bergeron, C., Fraser, P.E., St George-Hyslop, P. and Westaway, D. (2000) Aβ peptide immunization reduces behavioral impairment and plaques in a model of Alzheimer's disease. Nature 408, 979–982.

[10] Morgan, D., Diamond, D.M., Gottschall, P.E., Ugen, K.E., Dickey, C., Hardy, J., Duff, K., Jantzen, P., DiCarlo, G., Wilcock, D., Connor, K., Hatcher, J., Hope, C., Gordon, M. and Arendash, G.W. (2000) Aβ peptide vaccination prevents memory loss in an animal model of Alzheimer's disease. Nature 408, 982–985.

[11] Gelinas, D.S., DaSilva, K., Fenili, D., St George-Hyslop, P. and McLaurin, J. (2004) Immunotherapy for Alzheimer's disease. Proc. Natl. Acad. Sci. USA 101, 14657–14662.

[12] DeMattos, R.B., Bales, K.R., Cummins, B.R., Dodart, J.-C., Paul, S.-M. and Holzman, D.M. (2001) Peripheral anti-Aβ antibody alters CNS and plasma Aβ clearance and decreases brain Aβ burden in a mouse model of Alzheimer's disease. Proc. Natl. Acad. Sci. USA 98, 8850–8855.

[13] Orgogozo, J.M., Gilman, S., Dartigues, J.F., Laurent, B., Puel, M., Kirby, L.C., Jouanny, P., Dubois, B., Eisner, L., Flitman, S., Michel, B.F., Boada, M., Frank, A. and Hock, C. (2003) Subacute meningoencephalitis in a subset of patients with AD after Aβ42 immunization. Neurology 61, 46–54.

[14] McLaurin, J., Cecal, R., Kierstead, M.E., Tian, X., Phinney, A.L., Manea, M., French, J.E., Lambermon, M.H.L., Darabie, A.A., Brown, M.E., Janus, C., Chishti, M.A., Horne, P., Westaway, D., Fraser, P.E., Mount, H.T.J., Przybylski, M. and St George-Hyslop, P. (2002) Therapeutically effective antibodies against amyloid-β peptide target amyloid-β residues 4–10 and inhibit cytotoxicity and fibrillogenesis. Nature Med. 8, 1263–1269.

[15] Sigurdsson, E.M., Scholtzova, H., Mehta, P.D., Fragione, B. and Wisniewski, T. (2001) Immunization with a nontoxic/nonfibrillar amyloid-β homologous peptide reduces Alzheimer's disease associated pathology in transgenic mice. Am. J. Pathol. 159, 439–447.

[16] Bard, F., Barbour, R., Cannon, C., Carretto, R., Fox, M., Games, D., Guido, T., Hoenow, K., Hu, K., Johnson-Wood, K., Khan, K., Kholodenko, D., Lee, C., Lee, M., Motter, R., Nguyen, M., Reed, A., Schenk, D., Tang, P., Vasquez, N., Seubert, P. and Yednock, T. (2003) Epitope and isotype specificities of antibodies to β-amyloid peptide for protection against Alzheimer's disease-like neuropathology. Proc. Natl. Acad. Sci. USA 100, 2023–2028.

[17] Lemere, C.A., Spooner, E.T., LaFrancois, J., Malester, B., Mori, C., Leverone, J.F., Matsuoka, Y., Taylor, J.W., DeMattos, R.B., Holzman, D.M., Clements, J.D., Selkoe, D.J. and Duff, K.E. (2003) Evidence for peripheral clearance of cerebral Aβ protein following chronic, active Aβ immunization in PSAPP mice. Neurobiol. Dis. 14, 10–18.

[18] Lombardo, J.A., Stern, E.A., McLellan, M.E., Kajdasz, S.T., Hickey, G.A., Bacskai, B.J. and Hyman, B.T. (2003) Amyloid-β antibody treatment leads to rapid normalization of plaque-induced neuritic alterations. J. Neurosci. 23, 10879–10883.

[19] Wilcock, D.M., Munireddy, S.K., Rosenthal, A., Ugen, K.E., Gordon, M.N. and Morgan, D. (2004) Microglial activation facilitates Aβ plaque removal following intracranial anti-Aβ antibody administration. Neurobiol. Dis. 15, 11–20.

[20] Mason, H.S., Ball, J.M., Shi, J.J., Jiang, X., Estes, M.K. and Arntzen, C.J. (1996) Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. Proc. Natl. Acad. Sci. USA 93, 5335–5340.

[21] Tacket, C.O., Mason, H.S., Losonsky, G., Clements, J.D., Levine, M.M. and Arntzen, C.J. (1998) Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato. Nature Med. 4, 607–609.

[22] Richter, L.J., Thanavala, Y., Arntzen, C.J. and Mason, H.S. (2000) Production of hepatitis B surface antigen in transgenic plants for oral immunization. Nature Biotech. 18, 1167–1171.

[23] Blanchard, B.J., Chen, A., Rozeboom, L.M., Stafford, K.A., Weigele, P. and Ingram, V.M. (2004) Efficient reversal of Alzheimer's disease fibril formation and elimination of neurotoxicity by a small molecule. Proc. Natl. Acad. Sci. USA 101, 14326–14332.

[24] Kim, H., Bang, O.Y., Jung, M.W., Ha, S.D., Hong, H.S., Huh, K., Kim, S.U. and Mook-Jung, I. (2001) Neuroprotective effects of estrogen against Aβ toxicity are mediated by estrogen receptors in cultured neuronal cells. Neurosci. Lett. 302, 58–62.

[25] Suh, M.C., Choi, D. and Liu, J.R. (1998) Cadmium resistance in transgenic tobacco plants expressing the *Nicotiana glutinosa* L. metallothionein-like gene. Mol. Cells 8, 678–684.

[26] Restrepo, M.A., Freed, D.D. and Carrington, J.C. (1990) Nuclear transport of plant potyviral proteins. The Plant Cell 2, 987–998.

[27] Kim, H.S., Euym, J.W., Kim, M.S., Lee, B.C., Mook-Jung, I., Jeon, J.H. and Joung, H. (2003) Expression of human amyloid-β peptide in transgenic potato. Plant Sci. 165, 1445–1451.

[28] Yan, Q., Zhang, J., Liu, H., Babu-Khan, S., Vassar, R., Biere, A.L., Citron, M. and Landreth, G. (2003) Anti-inflammatory drug therapy alter β-amyloid processing and deposition in an animal model of Alzheimer's disease. J. Neurosci. 23, 7504–7509.

[29] McGowan, E., Pickford, F., Kim, J., Onstead, L., Eriksen, J., Yu, C., Skipper, L., Murphy, M.P., Beard, J., Das, P., Jansen, K., DeLucia, M., Lin, W.-L., Dolios, G., Wang, R., Eckman, C.B., Dickson, D.W., Hutton, M., Hardy, J. and Golde, T. (2005) Aβ42 is essential for parenchymal and vascular amyloid deposition in mice. Neuron 47, 191–199.

[30] Butterfield, D.A. (2002) Aβ(1–42)-induced oxidative stress and neurotoxicity: implications for neurodegeneration in Alzheimer's disease brain. Free Radic. Res. 36, 1307–1313.

[31] Haq, T.A., Mason, H.S., Clementes, J.D. and Arntzen, C.J. (1995) Oral immunization with a recombinant bacterial antigen produced in transgenic plants. Science 268, 714–716.

[32] Arakawa, T., Chong, D.K.X., Merritt, J.L. and Langridge, W.H.R. (1997) Expression of cholera toxin B subunit oligomers in transgenic potato plants. Transgenic Res. 6, 403–413.

[33] Monsonego, A., Maron, R., Zota, V., Selkoe, D.J. and Weiner, H.L. (2001) Immune hyporesponsiveness to amyloid β-peptide in amyloid precursor protein transgenic mice: implications for the pathogenesis and treatment of Alzheimer's disease. Proc. Natl. Acad. Sci. USA 98, 10273–10278.

[34] Daniell, H., Lee, S.B., Panchal, T. and Wiebe, P.O. (2001) Expression of the native cholera toxin B subunit gene and assembly as functional oligomers in transgenic tobacco chloroplasts. J. Mol. Biol. 311, 1001–1009.

[35] Aziz, M.A., Singh, S., Kumar, P.A. and Bhatnagar, R. (2002) Expression of protective antigen in transgenic plants: a step towards edible vaccine against anthrax. Biochem. Biophys. Res. Commun. 299, 345–351.

[36] Walmsley, A.M., Alvarez, M.L., Jin, Y., Kirk, D.D., Lee, S.M., Pinkhasov, J., Rigano, M.M., Arntzen, C.J. and Mason, H.S. (2003) Expression of the B subunit of *Escherichia coli* heat-labile enterotoxin as a fusion protein in transgenic tomato. Plant Cell Rep. 21, 1020–1026.

[37] Warzecha, H., Mason, H.S., Lane, C., Tryggvesson, A., Rybicki, E., Williamson, A., Clements, J.D. and Rose, R.C. (2003) Oral immunization of human papillomavirus-like particles expressed in potato. J. Virol. 77, 8702–8711.

[38] Mason, H.S., Haq, T.A., Clements, J.D. and Arntzen, C.J. (1998) Edible vaccine protects mice against *Escherichia coli* heat-labile enterotoxin (LT): potatoes expressing a synthetic LT-B gene. Vaccine 16, 1336–1343.

[39] Joung, Y.H., Youm, J.W., Jeon, J.H., Lee, B.C., Ryu, C.J., Hong, H.J., Kim, H.C., Joung, H. and Kim, H.S. (2004) Expression of the hepatitis B surface S and preS2 antigens in tubers of *Solanum tuberosum*. Plant Cell Rep. 22, 925–930.

[40] Mason, H.S., Lam, D.M.K. and Arntzen, C.J. (1992) Expression of hepatitis B surface antigen in transgenic plants. Proc. Natl. Acad. Sci. USA 89, 11745–11749.

[41] Olhoft, P.M. and Somers, A. (2001) L-Cysteine increases Agrobacterium-mediated T-DNA delivery into soybean cotyledonary-node cells. Plant Cell Rep. 20, 706–711.

[42] Kong, Q., Richter, L., Yang, Y.F., Arntzen, C.J., Mason, H.S. and Tanavala, Y. (2001) Oral immunization with hepatitis B surface antigen expressed in transgenic plants. Proc. Natl. Acad. Sci. USA 98, 11539–11544.
[43] Zhang, J., Wu, X., Qin, C., Qi, J., Ma, S., Zhang, H., Kong, Q., Chen, D., Ba, D. and He, W. (2003) A novel recombinant adeno-associated virus vaccine reduces behavioral impairment and β-amyloid plaques in a mouse model of Alzheimer's disease. Neurobiol. Dis. 14, 365–379.
[44] Faria, A.M.C. and Weiner, H.L. (1999) Oral tolerance: mechanisms and therapeutic applications. Adv. Immunol. 73, 153–264.