

High-level expression of a human β -site APP cleaving enzyme in transgenic tobacco chloroplasts and its immunogenicity in mice

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Abstract Plastid transformation has to date been applied to the expression of heterologous genes involved in agronomic traits and to the production of useful recombinant proteins. Here, we report a feasibility study for producing the human β -site APP cleaving enzyme (BACE) via transformation of tobacco chloroplasts. Stable integration of human BACE into the plastome was confirmed by PCR. Genomic Southern blot analysis detected the presence of the tobacco *aadA* and human BACE genes between *trnI* and *trnA* in the plastome. Northern blot analysis revealed that the *aadA* and BACE genes were both properly transcribed into a dicistronic transcriptional unit. Human BACE protein expression in transplastomic tobacco was determined by western blot analysis. ELISA analysis revealed that, based on a dilution series of *E. coli*-derived BACE as a standard, transplastomic lines accumulated BACE to levels of 2.0% of total soluble proteins. When mice were gavaged with the transplastomic tobacco extracts,

they showed an immune response against the BACE antigen. The successful production of plastid-based BACE protein has the potential for developing a plant-based vaccine against Alzheimer disease.

Keywords Chloroplast transformation · Plant-derived vaccine · Alzheimer disease · *Nicotiana tabacum* L. · β -secretase

Introduction

Alzheimer disease (AD) is a widespread senile dementia characterized by the progressive formation of insoluble amyloid plaques in the brain. The amyloid plaques comprise a 4 kDa β -amyloid protein ($A\beta$) derived by sequential proteolysis of the amyloid precursor protein (APP) through the activities of β - and γ -secretases (Tanahashi and Tabira 2007; McConlogue et al. 2007). These enzymes are considered to be therapeutic targets important in the treatment of AD (Pastarino et al. 2004). However, γ -secretase is not an ideal therapeutic target because it is also responsible for the cleavage of a number of other important cellular proteins involved in signaling processes. Thus, β -secretase, or β -site APP cleaving enzyme (BACE), is a more attractive therapeutic target (Parsons and Austen 2007). Chang et al. (2007) showed that immunization of transgenic AD mice (Tg2576) with BACE resulted in $A\beta$

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reduction and cognitive improvement. They suggested that immunization may have resulted in anti-BACE antibodies penetrating the blood–brain barrier (BBB) and binding to BACE located on neuronal surfaces, thereby inhibiting enzyme activity. When BACE-antibody complex is endocytosed, BACE cleavage of APP is inhibited by the bound antibody, resulting in a decrease in A β production. Consequently, immunization with BACE to produce antibodies that neutralize β -secretase activity leading to a reduction in A β is being considered as a new therapeutic concept for AD. Previously, it was shown that although BACE knockout mice have reduced A β production, they do not exhibit any abnormal phenotypes (Luo et al. 2001). The BACE gene was cloned and characterized in 1999 (Vassar et al. 1999), and various BACE constructs have been expressed in insect (Bruinzeel et al. 2002), mammalian (Emmons et al. 2008), and *E. coli* cells (Sardana et al. 2004; Tomasselli et al. 2008).

For the production of pharmaceutical proteins, chloroplast transformation has advantages over nuclear transformation approaches, such as high-level transgene expression (Daniell et al. 2002), multi-gene engineering in a single transformation event (De Cosa et al. 2001; Jeong et al. 2004), transgene containment via maternal inheritance (Daniell et al. 2002), and lack of gene silencing and position effects due to site-specific integration (De Cosa et al. 2001; Daniell et al. 2002; Lee et al. 2003; Watson et al. 2004). The recombinant protein yield can be dramatically increased, especially in tobacco, because a leaf cell contains as many as 100 chloroplasts, with up to 100 genomes each; a total of approximately 10,000 plastome copies per cell. Biopharmaceutical compounds, such as human somatotropin (Staub et al. 2000), human papillomavirus L1 protein (Fernandez-San Millan et al. 2008), the B-subunit of the cholera toxin (CTB) (Daniell et al. 2001a), and the tetanus toxin (Tregoning et al. 2005), have been expressed at high levels (4–25% of TSP). Additionally, environmental concerns regarding transgene escape via pollen from nuclear transgenic plants is abrogated by chloroplast transformation due to the maternal inheritance of plastids in most cultivated plants (Molina et al. 2004).

Recently, in our laboratory, human BACE protein was expressed in nuclear transformed transgenic potato and tomato plants in an attempt to develop a plant-derived AD vaccine (Youm et al. unpublished

data). Following analysis of these transgenic plants, the human BACE was shown to accumulate to levels of 0.12–0.19% of total soluble protein (TSP). Low levels of heterologous protein accumulation in plants is regarded as one of the limiting factors in the commercialization of plant-derived vaccines. Here, we introduced a human BACE gene into the plastome of tobacco plants to increase transgenic protein accumulation. Tobacco plants produce a large biomass, yielding approximately 170 metric tons of biomass per hectare (Fischer and Emans 2000). Tobacco, a nonfood and non-feed crop, is self-pollinating, thus minimizing transgene escape. Most importantly, we chose tobacco because it is a model plant for plastid transformation and is closely related to potato and tomato plants. In the current study, we report on the integration and expression of the human BACE gene in the plastome. The accumulation levels and antigenicity of the BACE protein derived from transplastomic tobacco were analyzed by western blot or ELISA. The immunogenic responses of tobacco plastid-derived BACE were observed by oral application in test mice. We discuss the potential use of plastid-derived BACE antigen as a new oral vaccine against AD. This may be the first step toward developing a plant-based vaccine against AD.

Materials and methods

Plant material

Tobacco seeds (*Nicotiana tabacum* L. cv. Xanthi) were surface sterilized with 70% ethanol for 10 min followed by 15 min in 1.0% sodium hypochlorite. Seeds were then washed three times in sterile water before being placed in petri dishes containing solid MS tissue culture medium (Murashige and Skoog 1962) supplemented with 3% sucrose and solidified with 0.8% agar, pH 5.8, at 25°C under long-day conditions (16 h light/8 h dark) with 45 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ white light. Leaves from 8–12-weeks-old plants were used for bombardment.

Construction of the plastid dicistronic expression vector

In accordance with the previously published methods used to construct the plastid transformation vector

pTIG (Jeong et al. 2004), we constructed a new vector for dicistronic expression of the *aadA* and human BACE genes under the control of the plastid *rrn* promoter (only PEP-type) with identical sequence reported by Svab and Maliga (1993). Human BACE was amplified from the pET19b/BACE22460 plasmid (kindly provided by Dr. Inhee Mook-Jung at Seoul National University, Korea) with two primers: forward primer (5'-TTtctagaAGGAGGTATAACA ATG ACC CAG CAC GGC ATC CGG-3'), which included an *Xba*I restriction site and a start codon (underlined); reverse primer (5'-GCCCTctagaTTA ATA GGC TAT GGT CAT GAG-3'), which included an *Xba*I restriction site and a stop codon (underlined). A single amplification product of the expected size of BACE was purified and subcloned into the TOPO TA plasmid (Invitrogen, San Diego, CA, USA) and confirmed by DNA sequencing. The BACE gene was connected by a linker sequence (5'-AGA AGG AGG TAT AAC A-3') including the *rbs* (consensus ribosome binding site GGAGG to enhance translation of BACE) to the 3' end of the antibiotic resistance gene *aadA*.

In summary, the construct included the following sequences: the border sequences with *trnI* and *trnA* genes, which are homologous to the *trnI* and *trnA* genes in the inverted repeat region of the tobacco plastome; *Prrn*, the constitutive promoter of 16S rRNA; the aminoglycoside 3'-adenylyltransferase (*aadA*), conferring resistance to both spectinomycin and streptomycin; the *rbs* fused to the BACE coding sequence; and the 3' UTR of the *psbA* gene. The final vector is referred to as CtVBACE (Fig. 1a).

Chloroplast transformation

The chloroplast transformation method used in this study was published previously by Jeong et al. (2004). Leaf blades were placed abaxial side up on MS medium supplemented with 4.44 μ M 6-benzylaminopurine and 0.54 μ M α -naphthaleneacetic acid (shoot-inducing medium) in plastic petri dishes (87 \times 15 mm). Leaf blades were bombarded by gold particles (0.6 μ m) coated with vector DNA at 1,100 psi and 28 inches Hg using a biolistic particle delivery system (PDS 1000/He; Bio-Rad, Hercules, CA, USA). Following particle bombardment, the tissues were incubated in the dark at 25°C for 48 h on shoot-inducing media. After 2 day, the leaves were

dissected into sections and then cultured for antibiotic selection and regeneration, until shoot development was induced. Homoplasmic transplastomic lines were selected after three rounds of regeneration under selection conditions, and transferred onto MS basal medium containing 500 mg l⁻¹ spectinomycin to induce rooting. Transplastomic plants were transferred to pots and grown in a greenhouse to evaluate the phenotype. PCR was performed on primary regenerated shoots to determine transgene integration into the plastome. PCR primers FI (5'-CCGTAGG TGCGATGATTTACTTC-3') and RA (5'-AGAGT CTTTCAGTGGCACGTTTC-3'), which anneal to regions of the *trnI* gene and the *trnA* gene, respectively, were used to confirm transgene integration into the *N. tabacum* plastome. PCRs were carried out in a DNA thermal cycler (GeneAmp PCR system 9700; Applied Biosystems, Carlsbad, CA, USA) under the following conditions: 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, with a final 10 min extension at 72°C.

Southern blotting

Total genomic DNA was isolated from tobacco leaf tissues (120 mg) using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) and following the manufacturer's instructions. Approximately 5 μ g of genomic DNA was digested with *Bgl*II, separated on 0.8% (w/v) agarose gels, and blotted onto nylon membranes (Zeta-Probe GT genomic tested blotting membranes; Bio-Rad) in 10 \times SSC. For probes, a 0.15 kb *trnA*-specific DNA fragment was amplified by PCR using CtVBACE as a template and *trnA* F1 (5'-TGCGATTACGGGTTGGATGT-3') and *trnA* R1 (5'-GTTCTTGACAGCCCATCTTT-3') primers, and a 0.33 kb BACE-specific DNA fragment was prepared from restriction digestion of the BACE gene with *Xba*I and *Bst*EII. The two probes were then labeled with [³²P] dCTP using the Random Primed DNA Labeling kit (Roche Diagnostics, Mannheim, Germany). Prehybridization and hybridization were carried out overnight in 0.25 M sodium phosphate (pH 7.2) and 7% (w/v) SDS solution at 65°C. The membranes were washed in 20 mM sodium phosphate (pH 7.2) and 5% (w/v) SDS at 65°C for 15 min, then washed in 20 mM sodium phosphate (pH 7.2) and 1% (w/v) SDS at 65°C for 15 min. The

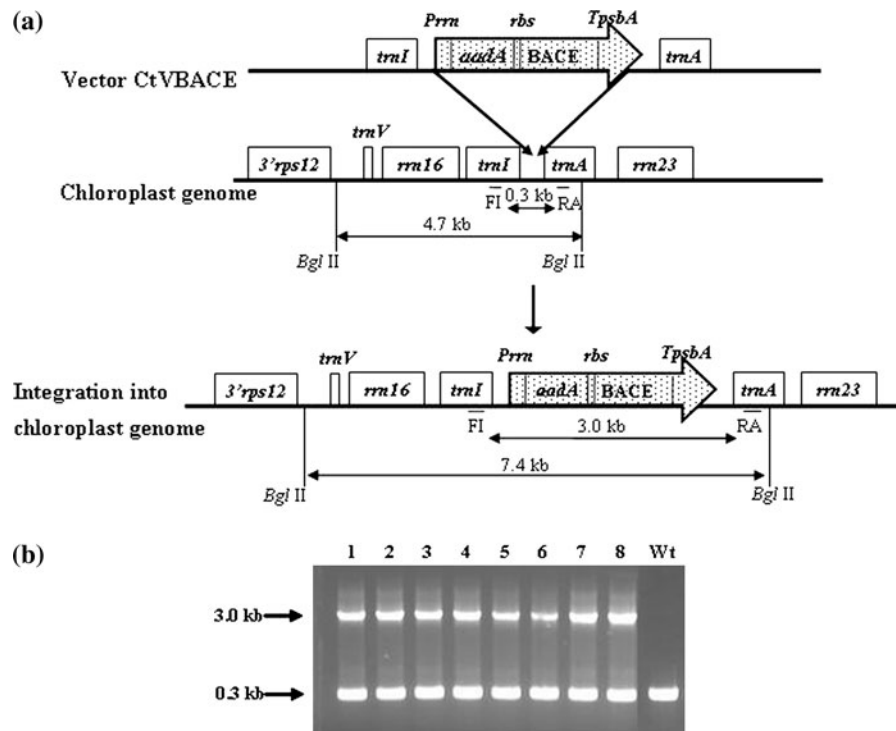


Fig. 1 Introduction of the human BACE gene into the tobacco plastome. **a** Construction of the transformation vector. The *trnI* and *trnA* genes were used as flanking sequences for homologous recombination. The *aadA* gene conferring spectinomycin resistance was used for selection of transgenic shoots. The *aadA* and BACE gene were regulated by the *rrn* promoter (*Prrn*) and the 3'-UTR of *psbA* gene (*TpsbA*) elements. A synthetic ribosome binding site (*rbs*) was used to achieve

translation of BACE. PCR primers are marked as FI and RA with short lines and *Bgl*II restriction sites are indicated with the expected size. **b** Analysis of primary transplastomic plants carrying the human BACE gene integrated into the plastome. Targeted integration of the *aadA*/BACE expression cassette is indicated by a 3.0 kb product generated from all 8 primary transformed lines; Wt-genomic DNA from non-transformed tobacco plants was used as a negative control

membranes were exposed using an Imaging Plate (Fujifilm, Tokyo, Japan) at room temperature (RT).

RNA blotting

Total RNA was extracted from leaves of wild-type (Wt) and transplastomic tobacco plants using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. RNA (5 µg) was denatured with formaldehyde and formamide, fractionated in a 1% agarose gel using 3-(*N*-morpholino) propanesulfonic acid (MOPS) buffer, and then blotted onto nylon membranes (Zeta-Probe GT genomic tested blotting membranes; Bio-Rad) in 20× SSC overnight. A 0.33 kb BACE-specific DNA fragment (probe) was prepared from restriction digestion of the BACE gene with *Xba*I and *Bst*EII. The probe was labeled with [α -³²P] dCTP. Prehybridization,

hybridization at 65°C overnight, and washing of the membrane was carried out according to the manufacturer's instructions.

SDS-PAGE and immunoblotting

Total soluble proteins (TSP) were extracted from leaves of Wt and transplastomic tobacco plants by homogenization in cold protein extraction buffer containing 1× PBS (pH 7.4), 10 mM EDTA, 1 mM proteinase inhibitor cocktail, 0.1% Triton X-100, and 5 mM β -mercaptoethanol (1 g of fresh weight ml⁻¹). The extract was centrifuged for 20 min at 13,000 rpm and the protein concentration was quantified by the Bradford method (Bradford 1976). After separation by 10% SDS-PAGE and transfer to PVDF (Millipore, Bedford, MA, USA), the BACE antigen protein was detected with a specific anti-BACE polyclonal

antibody (Calbiochem, Darmstadt, Germany) followed by a goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (Sigma, St. Louis, MO, USA).

Quantitative ELISA assay of tobacco plastid-derived BACE

The levels of BACE protein in transplastomic plants were determined using a quantitative ELISA analysis. The ELISA plates (Nunc-Immuno Maxisorp, Roskilde, Denmark) were coated with TSP from Wt or transplastomic lines B5, C7, E10, and H3, respectively, and incubated overnight at 4°C. The background was blocked with 5% skim milk in PBST (200 µl per well), and the plate was incubated with a 1:1,000 dilution of goat anti-BACE (Santa Cruz Biotech Inc., Santa Cruz, CA, USA) polyclonal (100 µl per well) for 2 h at RT, and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma). The plates were finally incubated with the chemiluminescent substrate, TMB peroxidase substrate solution (Pierce, Rockford, USA), for 30 min at RT in the dark to maximize the reaction rate. After the confirmation of sufficient color development, reactions were stopped with 2.5 M H₂SO₄. The plates were then assessed for absorbance at 492 nm. The amount of BACE protein in TSPs (µg antigen ml⁻¹ TSP) was estimated by comparing with standard concentrations of *E.coli*-derived BACE.

Protein preparation for mouse administration

Freshly harvested tobacco leaves (500 mg) were quickly powdered in liquid nitrogen, and TSP was extracted with ice-cold buffer containing phosphate-buffered saline (PBS; Sigma), 1 mM EDTA, 0.1% Triton X-100, and 1× proteinase inhibitor (Roche diagnostics). After homogenization, the samples were centrifuged twice at 12,000 rpm for 20 min at 4°C. The supernatant was concentrated by automatic speedvac (AS160; Savant, NY, USA) into 0.5 ml, the appropriate volume for a single dose to mice.

Vaccination of Balb/c mice with tobacco plastid-derived BACE

Balb/c mice of both sexes, weighing 24–25 g, were used in the immunization experiments. The mice

were gaged with the 0.5 ml extracts from transplastomic tobacco line CtVBACE-B5 (five mice) or Wt tobacco plants (five mice) plus 10 µg of Cholera Toxin (CT, Sigma) on days 0, 7, and 14. Immunizations were performed using a 1 ml syringe fitted with a gavage needle. Prior to the oral immunizations, 0.2 ml of sodium bicarbonate was applied by gastric gavage to each of the mice to neutralize stomach acidity. Before the initial immunization with the plastid-derived BACE (day 0) and after the third primary immunization (day 14), blood was drawn from the orbital plexus of each mouse to obtain antiserum samples. Each mouse was given an intraperitoneal booster with 10 µg of yeast-derived recombinant BACE emulsified in alum two weeks after the third administration (day 28). Sera were collected from the mice a week after boosting (day 35), and all blood samples were analyzed by ELISA.

Direct ELISA for anti-BACE antibody

Most of the procedures were performed as previously described by Youm et al. (2005). Briefly, flat-bottom ELISA plates were coated overnight at 4°C with antigen (0.1 µg well⁻¹ or 1.0 µg well⁻¹ BACE with 0.05 M carbonate-bicarbonate buffer [pH 9.6]). The coated plates were incubated for 2 h at RT with serum samples diluted 1:100 in blocking buffer. The plates were then incubated for 2 h at RT with anti-mouse IgG-conjugated horseradish peroxidase (secondary antibody) diluted 1:1,000 in blocking buffer. Tetra methyl benzidine (TMB) substrate 100 µl (Pierce) was added to visualize the color development for 30 min and H₂O₂ was added to stop the reaction at RT. Developed ELISA plates were read on a Microplate reader (Model 680, Bio-Rad).

Results

Generation of BACE-expressing transplastomic tobacco plants

The 1.3 kb human BACE gene (coding for amino acids 22–460 without the transmembrane domain sequence) was placed under the transcriptional control of *Prrn*, the constitutive promoter of 16S rRNA. *Prrn* is the strong plastid rRNA operon promoter (Vera and Sugiura 1995). This gene construct, called

CtVBACE, was introduced into tobacco chloroplasts via a biolistic particle delivery system, and shoots were observed on explants after 5–6 weeks on selection medium. Eight independent green primary-shoots were selected after plastid transformation of tobacco using the vector CtVBACE.

Analysis of transplastomic plants

The eight plant lines regenerated on selection medium were analyzed by PCR using primers designed to detect the *aadA*::BACE gene integrated into the plastome. A 0.3 kb PCR-amplified product was detected in all plants, including Wt ones, and a 3.0 kb PCR-amplified product was detected in the eight transformed primary tobacco lines but not in Wt tobacco (Fig. 1b). This indicated that the gene of interest had integrated into the plastome of the transgenic tobacco and that the lines were heteroplasmic for the transgene insertion. Four homoplasmic lines (B5, C7, E10, and H3) were obtained following two further rounds of regeneration under selection from four primary regenerated shoots (2, 3, 5, and 7, respectively). Correct integration of the BACE gene into the plastome of the four homoplasmic lines was confirmed by Southern blot analysis. The [³²P] dCTP-labeled *trnA* probe hybridized to a 4.7 kb *Bg*III fragment in the untransformed control and to a 7.4 kb fragment in the transplastomic tobacco lines (Fig. 2a). The blot was probed with the BACE coding region probe which only hybridized the 7.4 kb *Bg*III fragment in the transgenic lines (Fig. 2b). These results confirmed that the human BACE gene had integrated into the plastome of the transformants through homologous recombination in the *trnI-trnA* region.

BACE gene transcription in the chloroplast was analyzed by northern blot analysis. The expected length of the *aadA*::BACE dicistronic transcript was approximately 2.0 kb (0.7 kb for *aadA* and 1.3 kb for BACE). The BACE probe detected a major transcript of approximately 2 kb, indicating that the BACE gene was dicistronically expressed in transplastomic plants (Fig. 2c). A second major transcript (4.7 kb) hybridizing to the BACE gene probe is attributed to transgene transcription via read-through from the endogenous 16S rRNA promoter (Jeong et al. 2004; Sugita and Sugiura 1996).

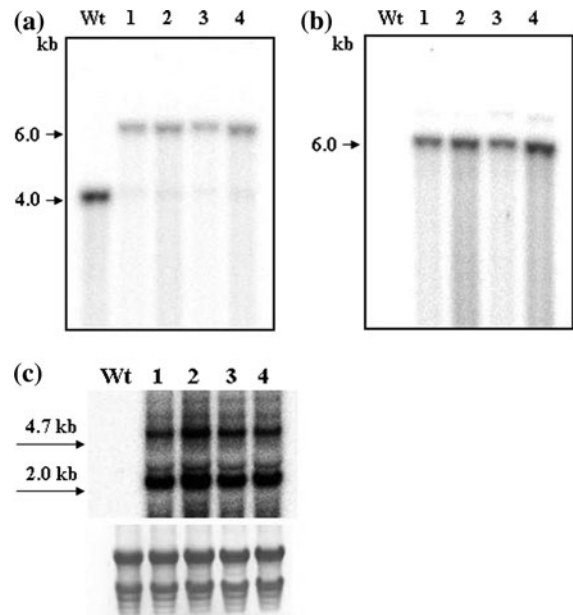


Fig. 2 Analysis of four transplastomic tobacco plants by Southern (a and b) and northern (c) blot. Genomic DNA (5 μ g) isolated from non-transformed wild-type plant (Wt) and transplastomic tobacco lines (1–4 correspond to lines B5, C7, E10, and H3) was digested with *Bg*III. DNA blot hybridized with *trnA* probe (a) and BACE probe (b). c Northern blot analysis of CtVBACE mRNA in transplastomic tobacco plants. Total RNA was extracted from Wt and transplastomic tobacco plants. RNA blot hybridized with BACE probe. Loading was monitored by methylene blue staining of the blot

Accumulation of BACE protein in transplastomic tobacco

The polyclonal anti-BACE antiserum recognized a protein of the expected size for BACE in all four plastid transformed lines (48 kDa, Fig. 3a). No protein was detected in Wt tobacco using this antibody (Fig. 3a).

ELISA analysis was used to quantify the level of BACE protein accumulation in the four transgenic lines. Transplastomic lines B5, C7, E10, and H3 showed significant levels of BACE protein accumulation estimated at 21.05, 22.51, 20.53, and 19.38 μ g mg^{-1} TSP, respectively (Fig. 3b). As expected, the Wt samples showed no change in color development on the ELISA, indicating the absence of BACE protein. The amount of BACE protein produced in each plant line was calculated as the percent of BACE protein detected in triplicate ELISA compared to the TSP. Accordingly, BACE protein accumulation

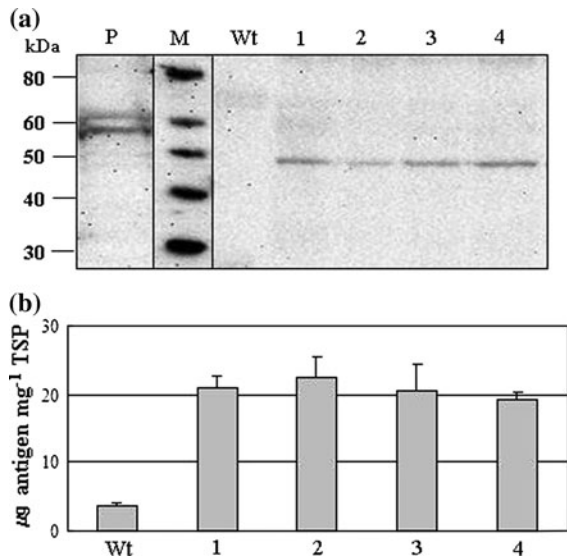


Fig. 3 Analysis of human BACE protein accumulation in transplastomic plants. **a** Western blot analysis of TSP from transplastomic tobacco plants and Wt tobacco using anti-BACE serum. Lanes: M, protein weight marker (kDa); P, AB10 (*E.coli*-produced BACE) cell extracts; Wt, extracts from nontransformed tobacco plant; 1–4, extracts from independent transplastomic tobacco plants B5, C7, E10, and H3, respectively. **b** BACE protein quantification of transplastomic lines by ELISA analysis. The amount of BACE protein in TSPs (μg antigen mg^{-1} TSP) was calculated by comparing of absorbance with standard concentrations of *E.coli*-derived BACE

was estimated at 4.8–5.6 μg 100 mg^{-1} fresh weight in the transplastomic lines.

The transplastomic line B5 was used for further oral administration.

Immune response of mice to tobacco chloroplast-derived BACE

To evaluate the immune response of mice to chloroplast-derived BACE protein, mice were immunized with BACE protein from the transplastomic tobacco line CtVBACE-B5 or from Wt tobacco as a control. Mice were orally gavaged with $\sim 25 \mu\text{g}$ of BACE protein, and then boosted on day 28 with 10 μg of yeast-derived recombinant BACE emulsified in alum. To determine the anti-BACE response, sera was collected before antigen administration as a control, after the third administration, and after the booster. Sera from mice immunized with transplastomic tobacco protein extract had a slight response as measured by ELISA after the third administration,

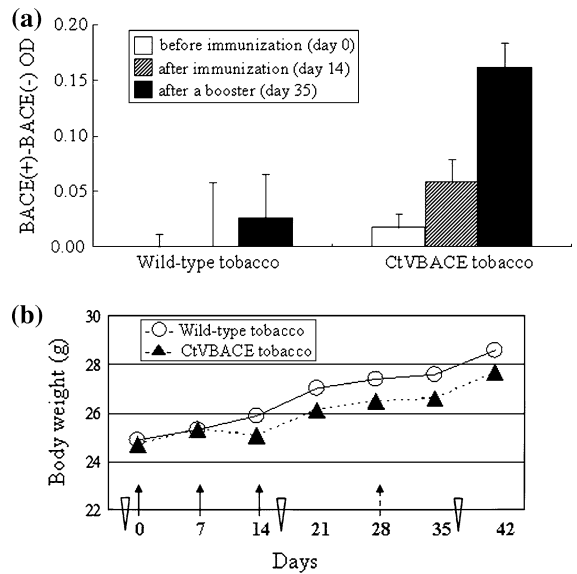


Fig. 4 a Blood serum anti-BACE antibody response to tobacco plastid-derived BACE in Balb/c mice after oral immunization. Mice were gavaged with extracts from Wt tobacco plants or from the transplastomic tobacco line CtVBACE-B5 with Cholera Toxin as an adjuvant on days 0, 7, and 14. Mice sera were collected before inoculation (days 0) (\square); after the third primary immunization (days 14) (hatched square); and after the booster (day 35) (\blacksquare). Sera were analyzed by ELISA for induced antibodies specific to BACE. Each bar represents the average of five immunized mice \pm standard error. **b** Effect of oral immunization of plastid-derived BACE on the body weight in Balb/c mice. The three administrations of plastid-derived BACE are indicated by *solid-line arrows*, and intraperitoneal booster with 10 μg of yeast-derived recombinant BACE is indicated by a *dotted-line arrow*. Blood was drawn from the orbital plexus of each mouse on days 0, 14 and 35, to obtain antiserum samples (∇)

and there was a significant increase in serum reactivity against BACE after boosting (Fig. 4a). Mice inoculated with Wt tobacco extract did not produce any BACE-specific antibodies until boosting. During immunization with plastid-derived BACE antigen, none of the mice died, and weight gain was the same as those inoculated with control extracts (Fig. 4b). We checked the growth pattern of normal, means non-immunized, mice as control group. There was no difference in growth between the non-immunized mice (data not shown) and those immunized with Wt tobacco extracts (Fig. 4b). These findings indicate that oral delivery of plastid-derived BACE was immunogenic in mice.

Discussion

The most likely cause of AD is neuronal cell death induced by the accumulation of toxic $A\beta$ protein in the brain. Thus, a therapeutic agent that could fundamentally inhibit the degeneration of neurons by inhibiting the production and toxicity of $A\beta$ would be a highly effective means of preventing and treating AD. $A\beta$ is produced by the cleavage actions of β -secretase (BACE) and γ -secretase during an amyloidogenic metabolic process. β -secretase inhibition may prevent dementia; this would be more efficient and economical than removing previously formed $A\beta$. Therefore, β -secretase inhibition through vaccination is presently receiving increased attention worldwide (McConlogue et al. 2007).

In the present study, attempts were made to achieve high level expression of human BACE in tobacco plants via chloroplast transformation. Following particle bombardment with the CtVBACE construct and after three rounds of selection, four transplastomic tobacco lines were obtained. Western blot confirmed that plastid-derived BACE protein was recognized by an antibody specific to human BACE, and ELISA analysis estimated BACE protein accumulation in transgenic lines at approximately 2.0% of TSP. This is much higher than the BACE yield from nuclear transformed potato tuber or tomato fruit, estimated at 0.12–0.19% of TSP (Youm et al. unpublished data). However, the level of plastid-based BACE accumulation achieved in this study is lower than what can be expected from chloroplast transformation. The chimeric CTB-2L21 protein was accumulated in tobacco chloroplasts to levels up to 31.1% of TSP (Molina et al. 2004), and the tetanus fragment C toxin to levels of 10–25% of TSP (Tregoning et al. 2005). Furthermore, PA/g for anthrax was accumulated in plastid-transformed plants at levels up to 2.5 mg g⁻¹ fresh weight (Watson et al. 2004). However, other antigens such as FMDV VP1 protein (2.0–3.0% of TSP [Li et al. 2006a, b]), HPV L1 (up to 1.5% of TSP [Lenzi et al. 2008]), and SARS-CoV S1 protein (approximately 0.2% of TSP [Li et al. 2006a, b]) accumulated in transplastomic plants at levels that were similar to, or lower than, those achieved for BACE protein in our study. These markedly different expression levels may be due to the type of antigen being expressed, post-transcriptional events and/or protein stability in

plastids. Herz et al. (2005) reported that protein expression in the plastid depended on several factors including the types of transformation vectors, promoter and translational control elements used, and external factors like plant age or light intensity. Therefore, the levels of BACE expression achieved in this study may be attributable to some of these various factors.

As expected, in stable transplastomic tobacco plants, immunoblot analyses detected a protein that is smaller than the *E. coli*-derived BACE. The *E. coli*-derived BACE has a molecular mass of ~60 kDa, whereas the plastid-derived BACE has a 48 kDa since the transmembrane domain was removed. From this result, we could estimate that the protein size of the chloroplast-derived BACE was not changed due to post-translational process. Chloroplasts have the ability to process eukaryotic proteins, including correct folding of subunits and formation of disulfide bridges (Staub et al. 2000; Daniell et al. 2001b), result in fully functional recombinant protein. In the present study, although we have not analyzed the glycosylation status of the chloroplast-derived BACE protein, the recombinant BACE was immunologically active.

BACE expression in tobacco chloroplasts did not affect plant growth rate, flowering, or seed set, and there was no apparent morphological difference between transplastomic and Wt tobacco plants in the greenhouse (data not shown). This observation concurs with previous reports where transplastomic tobacco plants expressing CTB (Daniell et al. 2001a) or ETEC (Kang et al. 2004) had no pleiotropic effects compared to Wt plants.

Our results show that mice administered with extracts from transplastomic tobacco exhibited a slight induction of primary anti-BACE antibody after three inoculations. However, there was a significant increase in BACE antiserum after boosting compared to the primary antibody response, suggesting that memory immune cells were established as a result of oral immunization. An immediate and strong secondary antibody induction was observed in several studies of small animals that received a boost of commercial vaccines (Richter et al. 2000; Kong et al. 2001). Induction of specific- $A\beta$ antibodies by plant-derived $A\beta$ was reported in a previous study (Youm et al. 2005, 2008). Much work remains to confirm the feasibility of plastid-produced BACE as an AD

vaccine, including characterization of plastid-produced human BACE, purification and processing of BACE, and immunization of a disease model animal.

In conclusion, we successfully expressed human BACE in transplastomic tobacco plants at levels higher than those expressed in transgenic potato or tomato plants. Moreover, this candidate plant-derived antigen was immunogenic via oral route delivery in mice and may be a useful model for developing a broad-spectrum plant-based vaccine against AD.

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