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Modification of the cholera toxin B subunit coding sequence to enhance expression in plants

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Abstract

The cholera toxin B subunit (CTB) contains five identical polypeptides and targets glycosphingolipid receptors on eukaryotic cell surfaces. Increased expression of CTB in plants is critical for the development of edible vaccines. In this study, the coding sequence of the CTB gene was optimized, based on the modification of codon usage to that of tobacco plant genes and the removal of mRNA-destabilizing sequences. The synthetic CTB gene was cloned into a plant expression vector and expressed in tobacco plants under the control of the CaMV 35S promoter. The recombinant CTB protein constituted approximately 1.5% of the total soluble protein in transgenic tobacco leaves. This level of CTB production was approximately 15-fold higher than that in tobacco plants that were transformed with the bacterial CTB gene. The recombinant CTB produced by tobacco plants demonstrated strong affinity for GM1-ganglioside, which indicates that the sites required for binding and proper folding of the pentameric CTB structure were conserved. This is the first report on the optimization of the CTB-coding sequence to give a dramatic increase in CTB expression in plants.

Abbreviations: CaMV 35S – cauliflower mosaic virus 35S RNA promoter; CTB – cholera toxin B subunit; GM1 – galactosyl-N-acetylgalactosamyl-sialyl-galactosylglucosyl ceramide; TSP – total soluble protein; UTR – untranslated region

Introduction

Cholera is a highly epidemic diarrheal disease that continues to devastate many developing countries with poor socio-economic conditions, in which the sanitation and public hygiene systems are rudimentary, and where safe drinking water is unavailable (Kaper et al. 1995). It is estimated that more than 120,000 people die each year from cholera (WHO 1998). Cholera is caused by *Vibrio cholerae* and disease severity is mediated by the potent action of the cholera toxin (CT), which stimulates the secretion of water and electrolytes into the intestine (Field et al. 1989). The CT has been reported as representative of adjuvants that induce mucosal immunity efficiently (Elson 1989; McGhee JR et al. 1992). The generation of non-toxic CT derivatives that retain adjuvant activity could provide a safe alternative for the evaluation of these toxins as mucosal adjuvants in humans and animals. CT is composed of distinct A and B subunits. The pentameric B subunit (CTB), which contains five identical polypeptides, targets the glycosphingolipid receptors on eukaryotic cell surfaces.

The bacterial CTB has been expressed at total soluble protein levels of 0.3% in potato (Arakawa et al. 1997), 0.095% in tobacco (Wang et al. 2001), and 0.02% and 0.04% in the leaves and fruits of tomato, respectively (Jani et al. 2002). Although the CTB ex-

pressed in tobacco plants has the same antigenic determinants as those of the native CTB (Wang et al. 2001), the low expression levels of foreign antigens in transgenic plants limit the development of effective plant-based vaccines. Therefore, the achievement of high levels of expression is crucial. The expression of vaccine components in plants has been accomplished by modifying a range of leader and polyadenylation signals (Richter et al. 2000), and by optimizing the codon usage for expression in plants (Mason et al. 1998; Tuboly et al. 2000; Streatfield et al. 2001). Higher accumulation levels and enhanced protein stability were achieved *via* the addition of a C-terminal SEKDEL sequence in case of the B subunit of *Escherichia coli* heat-labile enterotoxin (LTB) in tobacco and potato plants (Haq et al. 1995), and measles virus hemagglutinin in tobacco (Huang et al. 2001). The KDEL proteins perform essential functions that are related to protein folding and assembly in the ER (Pelham 1988).

We hypothesized that the native CTB gene, which contains prokaryote-adapted sequence motifs, might not be appropriate for efficient expression in plants. The native CTB gene is more AT-rich than plant genes, and contains sequences that are implicated in the processing and degradation of eukaryotic mRNA. In this study, we report the design and construction of a synthetic CTB gene, the coding sequence of which was optimized for enhanced CTB expression in tobacco plants by incorporating both Kozak and ER-retention sequences. Following the expression of the modified CTB in tobacco plants, GM1 binding analysis was performed to confirm that the recombinant CTB formed a functional pentameric structure.

Materials and methods

Design and assembly of the plant-optimized synthetic CTB gene

The coding sequence of the *Vibrio cholerae ctxB* gene (strain 1854) was analyzed for codon-usage pattern similarities with plant genes and for potential mRNA processing and destabilizing motifs. The codon usage pattern for tobacco was taken from http://www.kazusa.or.jp/codon/.

The strategy for constructing the synthetic CTB gene was based on the overlap extension PCR method, using ten long oligonucleotides (Figure 1). One sense oligonucleotide and antisense oligonucleotides with average overlaps of 18 nt were synthesized with pair-wise combinations. The 25-μl volume PCR mixture contained 10 pmol of oligonucleotide, 0.5 μM dNTP, standard *Pfu* DNA polymerase buffer, and 0.6 U *Pfu* Turbo DNA Polymerase (Stratagene, USA), and the PCR was performed for 25 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. In the continuous steps, the connected products were amplified by PCR using 1 μl of ligation product as the template. The final, full-length products were amplified by PCR using the Ex*Taq* polymerase (Takara Bio Inc., Japan), and cloned into the pGem T-Easy vector (Promega, USA), to yield the pMYO52 plasmid. To ensure accurate gene amplification, both strands of the synthetic CTB gene were sequenced using primers that were specific for the T7 and SP6 promoters.

Construction of the plant expression vector and plant transformation

The synthetic CTB gene was cloned into the plant expression vector pMY27 (Lee et al. 2001). The plasmid pMYO52 was digested with *Bam*HI and *Kpn*I restriction enzymes and the synthetic CTB fragment was ligated into the equivalent sites in the plant expression vector at a position downstream of the CaMV 35S promoter and TMV omega-prime leader (the transcriptional and translational enhancer), and upstream of the nopaline synthase (NOS) terminator, thereby yielding pMYO53 (Figure 2). The ligation reaction mixture was used to transform *E. coli* strain TOP10 (Invitrogen), and kanamycin-resistant colonies were isolated after overnight incubation at 37 °C.

Tobacco (*Nicotiana tabacum* L. cv TI560) plants were cultivated under sterile conditions and a 16-h light/8-h dark cycle at 25 °C on MS medium (Murashige and Skoog 1962) that was supplemented with 3% sucrose and 0.7% Bacto-agar. The binary plant expression vector pMYO53 was transformed into *Agrobacterium tumefaciens* LBA 4404 along with the helper plasmid pRK2013 using the tri-parental mating method (Van Haute et al. 1983). The *Agrobacterium* clone that contained the synthetic CTB gene was propagated under selection pressure and used to infect tobacco plants. The tobacco leaves were cut into 0.5~1-cm^2 sections and placed for 10 min in a culture dish that contained a suspension of $2\sim5\times10^9$ cells ml^{-1} of the *A. tumefaciens* LBA4404 strain that harbored the synthetic CTB gene. The leaf sections were then blotted on sterile filter paper, and co-culti-

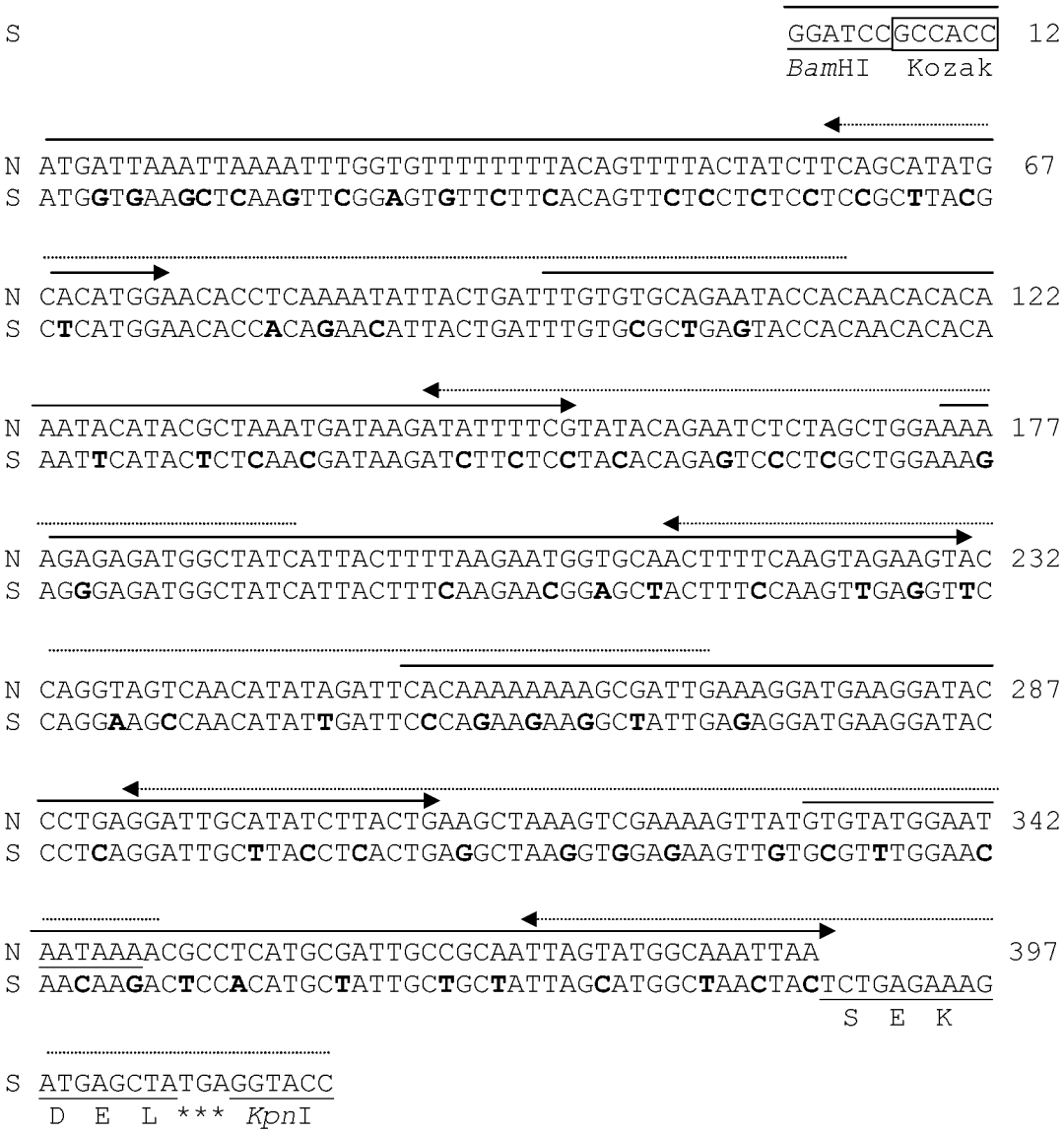

Figure 1. Comparison of the native bacterial CTB gene (N) with the plant codon-optimized synthetic CTB gene (S). The nucleotides shown in bold in the "S" sequence were changed. The sequence of the putative polyadenylation signal (AATAAA) in the native gene is underlined; Normal lines represent the 5' sense oligonucleotides used in the PCR with the 3' antisense oligonucleotides shown as dotted lines.

vated on MS medium that was supplemented with 0.1 mg l^{-1} α-naphthaleneacetic acid (NAA) and 1.0 mg l^{-1} 6-benzylaminopurine (BAP) for 48 h at 28 °C in the dark. After co-cultivation, the explants were transferred to MS medium that was supplemented with 0.1 mg l^{-1} NAA, 1 mg l^{-1} BAP, 300 mg l^{-1} kanamycin, and 500 mg l^{-1} cefotaxime. The explants were transferred to fresh medium every 2 weeks. The developed shoots were transferred into hormone-free MS medium that contained 300 mg l^{-1} kanamycin and 500 mg l^{-1} cefotaxime to induce root formation.

Detection of the CTB gene in the transformed plant genome

Genomic DNA was purified from transformed tobacco leaf tissues according to the method described by Kang and Fawley (1997). The presence of the CTB gene in transgenic plants was determined by

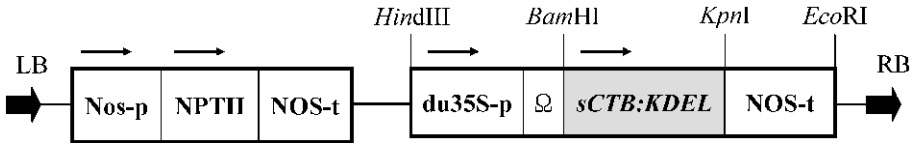

Figure 2. Binary plant expression vector pMYO53 for the expression of sCTB. NOS-p, *nos* gene promoter; NPTII, neomycin phosphotransferase gene; NOS-t, *nos* gene terminator; du35S-p, duplicated cauliflower mosaic virus 35S promoter; Ω, TMV Omega-prime leader; sCTB:KDEL, synthetic CTB gene fused with KDEL; LB, left border; RB, right border.

PCR analysis using the first forward and last reverse primers for the synthetic CTB gene (Figure 1). Genomic DNA samples (100 ng) from the transgenic and wild-type plants, along with 20 ng of plasmid pMYO53 that contained the synthetic CTB gene, were used as templates for the detection of the CTB gene using the PCR conditions described above for amplification of the plant-optimized synthetic CTB gene.

Northern blot analysis

Total RNA was isolated from the leaves of transgenic and wild-type tobacco plants using the Trizol Reagent (Invitrogen) according to the supplier's instructions. The RNA was fractionated on formaldehyde-containing agarose gels, and transferred to a Hybond-N+ membrane (Amersham). Prehybridization was performed at 60 °C for 1~2 h in 6× SSPE, 0.5% SDS, 5× Denhardt's solution, and 100 μg l^{-1} salmon sperm DNA. The blot was hybridized to the probe overnight at 60 °C in a buffer (pH 7.4) that contained 1 mM EDTA, 250 mM $Na_2HPO_4 \cdot 7H_2O$, 1% hydrolyzed casein, and 7% SDS. The membrane was washed twice for 20 min at 60 °C with 2× SSC plus 0.1% SDS, and twice for 20 min at 60 °C with 2× SSC plus 1% SDS. The hybridization signals were detected by autoradiography using X-ray film (Kodak).

Immunoblot detection of CTB protein in transformed tobacco tissues

Transgenic tobacco tissues were evaluated for the presence of the CTB protein by immunoblot analysis using the Bio-Rad Immun-Lite Assay Kit (Bio-Rad 170-6471). Leaf tissues (~0.5 g fresh weight) from transgenic and wild-type plants were homogenized by grinding in liquid nitrogen and resuspended in 1.0 ml of extraction buffer [200 mM Tris-Cl (pH 8.0), 100 mM NaCl, 400 mM sucrose, 10 mM EDTA, 14 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.05% Tween-20]. The tissue homogenates were centrifuged twice at 17,000 × *g* for 15 min at 4 °C to remove the insoluble cell debris. An aliquot of 5~10 μl of supernatant that contained 20 μg of total soluble protein, as determined by the Bradford protein assay (Sigma, USA), was separated on a 15% SDS-PAGE gel at 100 V in Tris-glycine buffer [25 mM Tris (pH 8.5), 200 mM glycine]. Samples of the plant homogenates, along with samples of 30, 150, and 300 ng purified CTB (Sigma C-9903), were either loaded directly to the gel or boiled for 10 min prior to electrophoresis.

The separated protein bands were transferred from the gel to a Hybond C membrane (Promega) using the Mini TransBlot electrophoretic transfer cell (Bio-Rad) for 2 h at 130 mA in transfer buffer [50 mM Tris (pH 8.3), 40 mM glycine, 0.04% SDS, 20% methanol]. Non-specific antibody reactions were blocked by incubation of the membrane in 25 ml of 5% non-fat dry milk in TBST buffer (TBS with 0.05% Tween-20) with gentle agitation at room temperature overnight. The membrane was incubated for 2 h at room temperature with gentle agitation in a 1:5000 dilution of rabbit anti-cholera antiserum (C-3062; Sigma) in TBST buffer that contained 2.5% non-fat dry milk, followed by three washes with TBST buffer. The membrane was then incubated for 2 h at room temperature with gentle agitation in a 1:7000 dilution of alkaline phosphatase-conjugated anti-rabbit IgG (Promega S3731) in TBST buffer, and washed three times with TBST buffer and once with TMN buffer. After washing, the color was developed using BCIP/NBT (USB) in TMN buffer [100 mM Tris (pH 9.5), 5 mM $MgCl_2$, 100 mM NaCl].

Quantification of CTB protein levels in transgenic tobacco tissues by ELISA

The CTB protein levels in transgenic tobacco plants were determined by quantitative ELISA as described in Kang et al. (2003). Total soluble protein samples from transgenic and wild-type plants were coated at 100 μl $well^{-1}$ into a 96-well microtiter plate (Becton)

together with purified bacterial CTB (Sigma C-9903), and the plates were incubated overnight. The plate was washed three times with PBST (PBS plus 0.05% Tween-20). The background was blocked with a 1% (w/v) BSA solution in PBS for 2 h at 37ºC, and the plate was washed three times with PBST. The plates were then incubated with rabbit anti-cholera serum that was diluted 1:8000 in 0.01 M PBS that contained 0.5% BSA for 2 h at 37 °C, followed by three washes with PBST buffer. The wells were incubated with 1:10000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma G-7641) in 0.01 M PBS containing 0.5 M BSA for 2 h at 37 °C, and the wells were washed three times with PBST. The plates were developed by the addition of 100 μl $well^{-1}$ of the TMB substrates (PharMingen 2606 and 2607KC) for 30 min at room temperature in the dark. The plate was read at 405 nm using an ELISA reader (Packard, CT), and the amount of plant-expressed CTB was estimated based on the known amount of purified CTB. To determine the expression level for each transgenic line, four leaf explants were analyzed for CTB expression, and an average expression level was obtained. All measurements were performed in triplicate, and analysis of variance was carried out using the statistical analysis program Excell (Microsoft Corp., USA).

GM1-ganglioside binding assay

The GM1-ELISA assay was performed to determine the affinity of plant-derived CTB for GM1-ganglioside. The microtiter plate was coated with monosialoganglioside-GM1 (Sigma G-7641) by incubating the plate with 100 μl $well^{-1}$ of GM1 (3.0 μg ml^{-1}) in PBS buffer at 4 °C overnight. As a control, the wells were coated with 100 μl $well^{-1}$ of BSA (3.0 μg ml^{-1}). The plates were washed three times with PBST buffer, and blocked with 1% BSA in 0.01 M PBS for 2 h at 37 °C. After washing three times with PBST buffer, the plates were incubated with soluble protein extracts from the transformed and wild-type plants along with purified CTB (Sigma C-9903) for 2 h at 37 °C. The remainder of the procedure was as described above for the ELISA quantification of CTB protein levels.

Results

Design of the synthetic CTB gene

The aim of redesigning the CTB gene was to create a modified gene that would be translated more efficiently than the native gene, and thus give high levels of expression in tobacco plants. Increased levels of protein expression have been reported in plants after modification of gene-coding sequences (Perlak et al. 1991; Adang et al. 1993; Rouwendal et al. 1997; Mason et al. 1998; Horvath et al. 2000; Huang et al. 2001; Huang et al. 2002; Yang et al. 2002). Therefore, we designed a codon-optimized version of the CTB gene, which retained the amino acid coding sequence of the native gene except at position 2, i.e., the ATT triplet for isoleucine (Ile) was replaced with the GTG triplet for valine (Val) (Figure 1). The rationale behind this substitution was that the placement of G immediately following the AUG initiation codon and the Kozak sequence (GCCACC) increased 10-fold the efficiency of translation (Kozak 1989). This change should not alter the sequence of the mature CTB produced in plant cells, since this portion is the signal peptide at the NH_2-terminal. DNA sequences that might contribute to RNA instability in plants, such as the plant polyadenylation signal sequence AATAAA in the native CTB gene, were modified for the synthetic gene (Figure 1). Nucleotides used to modify the DNA sequence increased the overall G+C content, increased plant preferred codons without changing the amino acid sequence, and did not generate consecutive A+T or G+C strings (>5). Codons with CG and TA dinucleotides at codon positions 2 and 3 were replaced by more favourable codons except the last position of CTB coding sequence, CTA (Leu). The C-terminal SEKDEL sequence was used for LTB gene expression in tobacco plants, resulting in approximately three times more recombinant protein accumulation in the transgenic plant than the LTB gene devoid of SEKDEL (Haq et al. 1995). Therefore, the same SEKDEL sequence reported previously was used without modification in the present study. Finally, restriction enzyme sites for *Bam*HI and *Kpn*I were introduced at the 5'- and 3'-ends of the synthetic CTB gene, respectively, to provide convenient restriction sites for cloning into the plant expression vector.

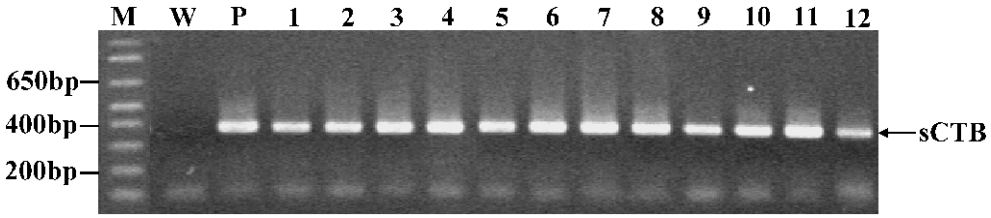

Figure 3. Detection of the synthetic CTB gene in the genomes of transgenic tobacco plants. The amplification products of the synthetic CTB were separated using a 1.2% agarose gel. M, 1-kb DNA ladder; W, PCR generated from the DNA template of the wild-type plant; P, PCR product generated from the DNA template of the pMYO53 plasmid that contains the synthetic CTB; 1-12, PCR products generated from the DNA templates of independent transgenic lines.

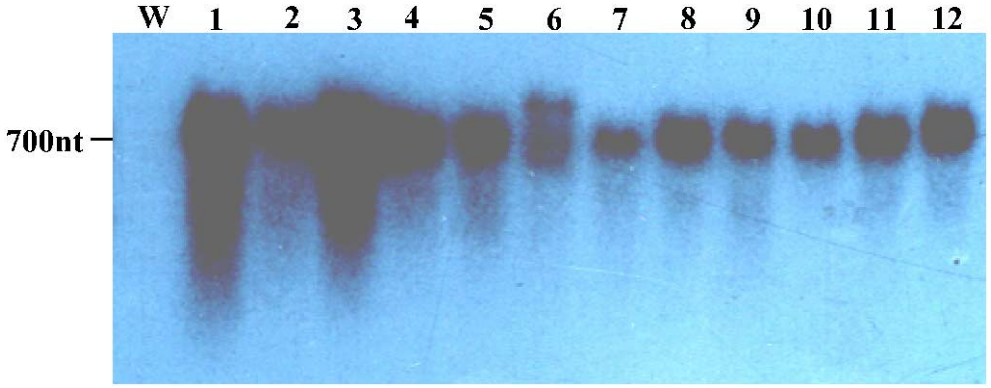

Figure 4. Northern blot of transgenic tobacco RNA using ^{32}P-labeled sCTB as the probe. The samples (20 μg of total leaf RNA) were arranged in the order shown in Figure 3. W, wild-type plant; 1-12, transgenic lines. Samples 1 (O53-1) and 3 (O53-3) showed strong signals.

PCR detection of the CTB gene fusion in transgenic tobacco plants

A total of 12 independent transgenic tobacco plants were regenerated by *Agrobacterium*-mediated transformation. They were analyzed for the presence of the synthetic CTB gene in the genomic DNA of leaf tissues using forward and reverse primers shown in Figure 1. A PCR product of the expected size (414 bp) was obtained from the genomic DNA samples of all 12 transformed tobacco lines, which suggests that the synthetic CTB gene was integrated into the chromosomal DNA of the transgenic plants (Figure 3). As expected, genomic DNA of the wild-type plant was not amplified. Non-specific amplification was not observed, which indicates the high-level specificities of the primers and PCR procedures.

Northern blot analysis

To measure the expression level of CTB mRNA, Northern blot analysis using a synthetic CTB gene probe was performed on total RNA from leaves of transformed plants. The appropriate mRNA species was present in all transgenic plants that contained clearly detectable amounts of CTB. A transcipt of approximately 700 nucleotides hybridized with the CTB probe, which is the size similar to that of LTB (Chikwamba et al. 2002; Mason et al. 1998) (Figure 4). Hybridization of this blot with the CTB probe showed that the probe hybridized strongly with transgenic lines 1 and 3 (O53-1 and O53-3, respectively), thereby indicating high-level expression of the synthetic CTB gene. Thus, these two lines were used for the Western blot, ELISA, and GM1-binding analyses.

Immunoblot analysis of plant-synthesized CTB protein

Plants 1 and 3 (O53-1 and O53-3, respectively), which had the highest levels of CTB transcripts in the Northern blots, were selected for CTB protein characterization. Immunoblot analysis of the transgenic tobacco plants that were transformed with the synthetic CTB gene revealed an oligomeric CTB protein with a molecular weight of 50 kDa (Figure 5).

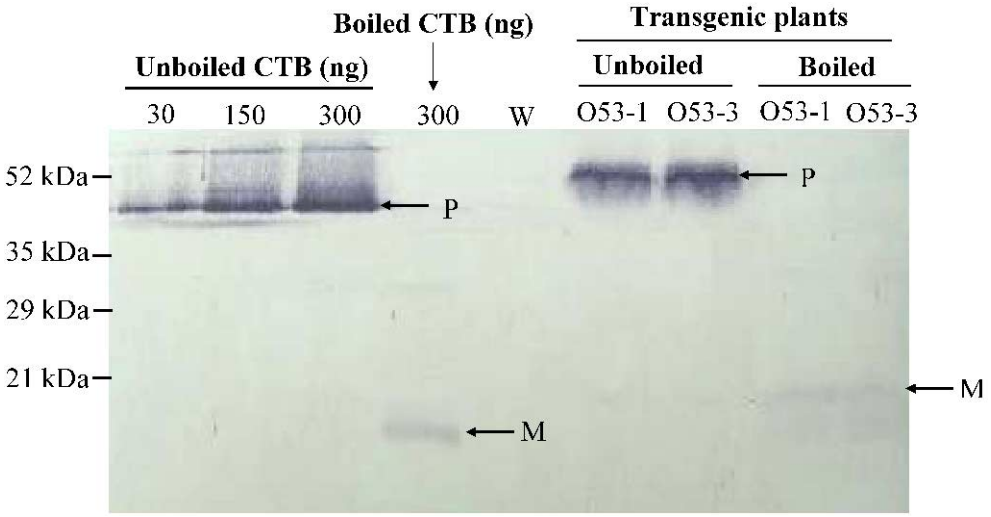

Figure 5. Western blot analysis of CTB protein expression in tobacco leaf tissue. Total soluble protein samples (20 μg) from the wildtype (WT) and two transgenic plants (O53-1 and O53-3), along with 30, 150, and 300 ng of purified CTB protein. The samples from transgenic plants were boiled for 10 min (monomer, M) or not boiled (pentamer, P) prior to SDS-PAGE.

The oligomeric CTB protein dissociated into monomers of 15 kDa when the transgenic plant homogenates were boiled for 10 min. Both the multimeric and monomeric forms of the plant-derived CTB had slightly higher molecular weights than the bacterial CTB (50 kDa versus 45 kDa for the pentamers, and 15 kDa versus 12 kDa for the monomers, respectively). This discrepancy was presumably due to the extra six amino acids that were added at the C-terminus for ER retention, and the failure of the plant cells to remove the leader peptide (Arakawa et al. 1997). The monomers of both the plant-synthesized and bacterial-synthesized CTB proteins were much less immunoreactive than the multimeric forms. The anti-cholera toxin used in the immunoblot analysis was generated in rabbits using the purified toxin from *Vibrio cholerae*. The AB_5 holotoxin is composed of A (27 kDa) and B (12 kDa) subunits. Therefore, the antibody potentially recognizes the native pentamer molecules more efficiently than the dissociated monomers.

Quantification of CTB expression

An ELISA was used to determine the levels of CTB protein in the leaf tissues of the transgenic plant lines O53-1 and O53-3. The amount of plant CTB protein was estimated by comparison of the relative light units (RLU) emitted by a known amount of bacterial CTB protein-antibody complex with that emitted by a known amount of transformed plant total soluble protein. The amount of CTB was expressed as the percentage of total soluble plant protein (% CTB) in the sample (Figure 6A). The optimal concentration of soluble protein loaded in the wells of the microtiter plate corresponded to CTB protein levels that were approximately 1.5% of the total soluble protein in the O53-3 transgenic tobacco leaf tissues. The yield of recombinant CTB protein was additionally estimated in side-by-side comparison of authentic LTB samples of known concentration with experimental samples in Western blot analysis. Direct comparison of band intensities allows simple and reliable visual estimation of protein concentration in the samples. This approach gave us an estimated amount of 300 ng of CTB protein per 20 μg of TSP, which is 1.5% of TSP (Figure 5). Therefore, the estimation of protein concentration comparison by this quantitative Western blot analysis is clearly in agreement with that of quantitative ELISA. Based on the results of the ELISA and immunoblot assays, 1 g of leaf tissue (fresh weight) from the transgenic tobacco plants contained 40~45 μg of recombinant plant-synthesized CTB protein.

GM1 receptor-binding assay for the CTB protein

The ability of the B subunit to bind to gangliosides was examined using 96-well plates that were coated with GM1 gangliosides. In the GM1-ELISA binding

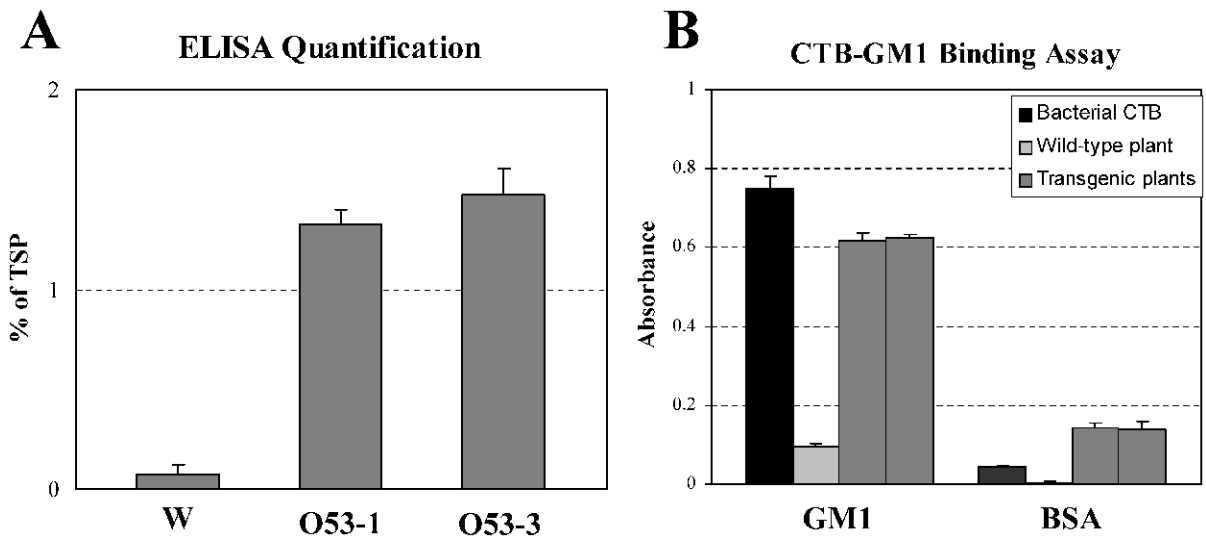

Figure 6. (A) ELISA for the determination of CTB protein levels in the leaves of transgenic plants. W, wild-type plant; O53-1 and O53-3, two transgenic plant lines. (B) GM1-binding ability of bacterial CTB and plant CTB. A plate was coated with GM1 and BSA and incubated with bacterial CTB, and total soluble protein (TSP) of the wild-type and two transgenic plant lines. Absorbance was measured at 405 nm.

assays, both the plant-produced CTB and the bacterial CTB demonstrated strong affinities for GM1-ganglioside, but not for BSA (Figure 6B). The strong binding affinities of the plant and bacterial CTBs to GM1 indicate that binding of the plant-derived CTB subunit to GM1 is co-operative (Schon and Freire 1989; Merritt et al. 1994), and that the sites required for binding of the CTB pentamer to GM1 are conserved. Cooperative binding between plant-produced CTB and GM1 ganglioside reinforces the probability that monomeric B subunits accumulate within the lumen of the ER of plant cells, where self-assembly into the oligomeric, and possibly pentameric, GM1-binding forms of CTB occurs.

Discussion

In an earlier report, CTB:SEKDEL fusion protein expression in potato under the control of *mas* P2 promoter was found to be 0.3% of total soluble protein (Arakawa et al. 1997).

Even though the CTB expression was higher than in other reports (Wang et al. 2001; Jani et al. 2002), perhaps because of the strong *mas* P2 promoter, use of the same promoter to drive an insulin gene resulted in expression levels of 0.05% of total soluble protein (Arakawa et al. 1998b), indicating that stability of RNA or protein could contribute significantly towards achieving high expression levels. [CTB has also been expressed in previous reports using the same promoter CaMV 35S as our experiments.] In tobacco plants, fusion of the PR1b signal peptide-coding sequence to the CTB mature protein gene expressed the protein level of 0.095%, which is much lower than that of our experiment. With the same promoter CaMV 35S, CTB fused with SEKDEL was expressed in tomato. Both tomato leaves and fruits expressed CTB at a level of up to 0.02 and 0.04% of total soluble protein, respectively. All in all, regardless of signal peptide and/or SEKDEL, in our experiments the CTB expression was much higher with synthetic CTB gene compared to wild-type CTB gene. In our other experiments, we tried to express using wild-type and synthetic LTB gene under the control of CaMV 35S promoter. We could not detect any LTB expression by immunoblot analysis by wild-type (data not shown). Synthetic LTB gene devoid of signal peptide coding sequence and SEKDEL did not show any LTB expression either, whereas LTB was highly expressed (2.2% of TSP) in tobacco plants using the synthetic LTB with both signal peptide and SEKDEL (submitted to Biotechnology Letters). It has been reported that LTB and CTB are related protein toxins with similar structure, function and immunochemistry (Sixma et al. 1991). Therefore, we planned to express CTB using only synthetic CTB gene with signal peptide and SEKDEL sequence under the control of

CaMV 35S promoter. CTB has been accumulated at the level of 4.1% of total soluble protein in tobacco leaves (Daniell et al. 2001), and LTB at 2.5% (Kang et al. 2003), also in tobacco plants, by chloroplast transformation, even though the immunogenicity has not been tested yet. The high expression levels of antigen genes using the chloroplast system as well as synthetic genes are of great significance for the development of edible vaccine.

In order to achieve high expression levels of heterologous genes in plants, it is useful to optimize the coding sequence to mimic that of highly expressed plant genes, and to eliminate any mRNA-destabilizing motifs. Many instances of poor expression of foreign genes in plants have been investigated, and several post-transcriptional events that led to low levels of expression, such as improper codon usage (Perlak et al. 1991), abnormal splicing (Haseloff et al. 1997), premature polyadenylation (Jarvis et al. 1997), and mRNA instability (Murray et al. 1991), have been described. The synthetic CTB gene described in this report does not contain any of the rare XCG or XTA codons that are used in dicot plants, since we replaced the rare codons of the bacterial CTB gene with codons that are used frequently in tobacco plants. It has been reported that rare mRNA codons tend to form higher-order secondary structures, which might provide additional time for the ribosome to move through the critical region (Farabaugh 1996; Thanaraj and Argos 1996). In addition, the clusters of rare codons are more likely to lead to translation failure when they are located close to the initiation site of the coding sequence, because the risk of generating an aberrant protein increases as an error occurs in the upstream sequence (Chen and Inouye 1990; Rosenberg et al. 1993; Goldman et al. 1995). It is also known that a strong correlation exists between the frequency of codon usage and the level of its cognate tRNA, since translation is completely blocked when all the rare tRNAs are trapped into translation complexes (Ikemura 1981; Varenne and Lazdunski 1986). It has previously been shown that the expression of foreign proteins may be enhanced by the C-terminal fusion of the ER-retention signal SEKDEL (Wandelt et al. 1992; Haq et al. 1995; Richter et al. 2000; Huang et al. 2001), since the SEKDEL motif is expected to sequester the protein in the ER (Munro and Pelham 1987). The authors proposed that the cellular compartmentation of the SEKDEL protein could have facilitated oligomerization of LTB monomers into pentamers detectable by ganglioside-dependent ELISA. Therefore, we decided to fuse SEKDEL to the C-terminal of the synthetic CTB gene for higher expression in tobacco plants.

Perhaps the best-known mRNA-destabilizing element in higher eukaryotes is the A/U-rich destabilizing element that is found in the 3'-untranslated regions (UTRs) of many labile mammalian mRNAs (Chen and Shyu 1995). It has been shown that certain A/U-rich sequences cause rapid mRNA decay in tobacco (Ohme-Takagi et al. 1993), and rapid degradation of the mRNA that encodes a bacterial insecticidal protein that can be produced in transgenic plants to confer insect resistance (DeRocher et al. 1998). Therefore, consecutive strings of the 5 or more A+T nucleotides were avoided for the DNA sequence of synthetic CTB gene. Premature polyadenylation, which contributes to poor expression of transgenes in foreign hosts, was detected in the AAUAAA motif (Haffani et al. 2000). Accordingly, the use of a synthetic LTB gene with highly modified nucleotide sequences (i.e. increased G/C content and modification of the AAUAAA sequences) resulted in dramatically improved expression in potato plants (Mason et al. 1998). With regard to the synthetic CTB gene described in this report, the G/C content of the CTB-coding sequence increased from 33% to 45%, and the AAUAAA sequence was modified to AACAAG.

The non-toxic CTB pentamer has attracted much attention as an efficient mucosal immunogen, and is a component of a widely licensed oral cholera vaccine (Holmgren and Svennerholm 1990). The antigenicity of purified CTB that is synthesized in tobacco plants is indistinguishable from that of the native CTB protein (Wang et al. 2001), and potato plant-synthesized CTB subunits stimulate significant protective immune responses against the biological effects of CT following oral administration (Arakawa et al. 1998a). Mucosally administered conjugates of CTB and various antigens have been shown to suppress the development or progress of a number of autoimmune diseases in animal models (Sun et al. 1996; Bergerot et al. 1997; Tarkowski et al. 1999; Sadeghi et al. 2002). Fusions between the insulin epitope (Arakawa et al. 1998b) or the neutralizing epitope of hepatitis C virus (Nemchinov et al. 2000) and the C-terminus of CTB were used to deliver plant-synthesized antigens to the gut-associated lymphoid tissues, and shown to elicit immune responses. Oral delivery of plant-based vaccines is an attractive alternative to injection, mainly for reasons of low cost and ease of administration.

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