Transgenic Plant Journal ©2008 Global Science Books

Expression of a Fusion Protein Consisting of Cholera Toxin B Subunit and an Anti-Diabetic Peptide (p277) from Human Heat Shock Protein in Transgenic Tobacco Plants

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ABSTRACT

A DNA construct encoding cholera toxin B subunit (CTB) and an anti-diabetic peptide from human heat shock protein 60 (hsp60) as a fusion protein was produced and transferred into low-nicotine tobacco through *Agrobacterium*-mediated transformation. Integration of the fusion gene into the plant genome was confirmed by PCR. The transcriptional expression of the fusion gene was demonstrated by RT-PCR. Western blot analysis further verified the synthesis and assembly of the fusion protein into pentamers in transgenic tobacco. G_{M1}-ELISA showed that the plant-derived fusion protein retained G_{M1}-ganglioside receptor binding specificity. The fusion protein accounted approximately for 0.1% of the total leaf protein. The production of transgenic plants expressing CTB-p277 offers an opportunity to test plant tissues for oral peptide antigen therapy against autoimmune diabetes by inducing oral tolerance.

Keywords: anti-diabetic peptide, cholera toxin B subunit (CTB), fusion protein, G_{M1}-ELISA, Low-nicotine transgenic tobacco
Abbreviations: CTB, Cholera toxin non-toxic subunit B; **GAD67**, Glutamic acid decarboxylase 67; **HSP60**, Heat shock protein 60; **IL-4**, Interleukin 4; **NOD**; Non-obese diabetic mouse model; **p277**, Heat shock protein 60 fragment 277; **TSP**, Total soluble protein

INTRODUCTION

Peptide p277 is composed of 24 amino acids from the sequence (437-460) of the human 60 kDa heat-shock protein (Hsp60), with the two cysteine residues of the native sequence replaced by valines (Elias and Cohen 1994). There is accumulating evidence to suggest that like glutamic acid decarboxylase (GAD), insulin or insulin B chain 9-23 peptide, p277 of the Hsp60 protein is an autoantigen serving as a target of T-cells in autoimmune diabetes in humans and NOD (non-obese diabetic) mice, and could therefore represent a potentially effective therapeutic agent for the treatment of autoimmune diabetes. Indeed, a single injection of 100μg of p277 in incomplete Freund's adjuvant can induce a shift in the cytokine profile of the spontaneous T cell response from a damaging Th1-type to anti-inflammatory Th2 type, leading to the permanent arrest of the β cell damage in NOD mice (Elias *et al*. 1997). Furthermore, p277 was shown to be effective even when the treatment was initiated in mice that were already clinically diabetic (Elias and Cohen 1995). The promising results in animal tests have paved the way for clinical trials for the treatment of Type 1 diabetes with peptide p277. A phase 1 human clinical trial showed that subcutaneous administration of p277 is safe, causing no adverse side effects in volunteers with longstanding Type 1 diabetes and with no detectable C-peptide (Cohen 2002), and phase 2 clinical trial in early onset Type 1 diabetes showed that treatment with p277 halted disease progression, prevented further destruction of the beta-cells, and prolonged patients' insulin production (Raz *et al*. 2001). Presently a phase III human clinical trial is in progress. Taken together, these results suggest that the human Hsp60 peptide p277 may hold promise for therapy against Type 1 diabetes.

Oral administration of autoantigens to induce oral immune tolerance represents one of the most desirable methods for the prevention and treatment of autoimmune diseases. In addition to its simplicity and greater patient acceptance, the advantages of oral antigen therapy include increased specificity, as well as reduced toxic side effects associated with systemic therapy (e.g., by injection). Oral administration of autoantigens has been reported to be effective in suppressing disease development in animal models of autoimmunity, including the NOD mouse model of Type 1 diabetes (Strobel and Mowat 1988; Weiner *et al*. 1994). However, the effective use of oral tolerance in the treatment of human autoimmune disease may critically depend on the development of mucosal adjuvants and delivery systems to enhance its efficacy (Ma *et al*. 2004). Another serious limitation in the clinical application of oral tolerance strategies will be the potentially huge cost of producing autoantigens, particularly if repeated regular doses are required to maintain beneficial effects.

Cholera toxin (CT), which is a major pathogenic agent produced by *Vibrio cholerae*, is a potent mucosal immunogen and adjuvant, and most of these activities are retained by the nontoxic B subunit of CT (CTB). This is in part due to its high binding affinity for the GM1-ganglioside receptor present in most cells of the body (Cuatrecassas 1973). CTB has been demonstrated to be an efficient oral adjuvant for mucosal vaccines (Czerkinsky *et al*. 1989; Bergquist *et al*. 1997). Furthermore, CTB has been shown to induce oral tolerance for linked antigens. For example, myelin basic protein (MBP) conjugated to CTB prevented or suppressed experimental allergic encephalomyelitis (EAE), when using lower concentrations of the conjugate than is usually necessary to induce tolerance with the protein alone (Sun *et al*. 2000). Oral administration of insulin or the B chain of insulin conjugated to CTB enhanced oral tolerance, and reduced the dose of antigen and the administration rate necessary for

Received: 23 July, 2008. Accepted: 15 October, 2008.

Original Research Paper

suppression of diabetes in NOD mice (Ploix *et al*. 1999; Sadeghi *et al*. 2002). Promising results against type II collagen-induced arthritis have been obtained by intranasal administration of low doses of type II collagen-CTB conjugate (Tarkowski *et al*. 1999). These results indicate that mucosal administration of autoantigens conjugated to CTB may represent a useful future treatment approach for human autoimmune diseases, such as type I diabetes. At the present time, the mechanism underlying CTB's efficacy as a mucosal adjuvant or carrier molecule has not yet identified with certainty. It has been speculated that the CTB binding to GM1 increases the permeability of the membrane to the antigen (Nashar *et al*. 1996). CTB also induces the major histocompatibility complex (MHC) class II expression on B cells and significantly stimulates antigen presentation in macrophages (George-Chandy *et al*. 2001).

We have developed a new method for inducing oral tolerance, which is based on the use of transgenic plants such as potato and low-nicotine tobacco to express and deliver recombinant autoantigens such as murine GAD67 to the mucosal immune system (Ma *et al*. 1997). Transgenic plants offer several advantages for an oral tolerance strategy, not the least of which is their high production potential for relevant autoantigens with nearly unlimited scale up (Kusnadi *et al*. 1997). As protein purification costs can eliminate the economic advantage of any production system, an additional advantage of transgenic plants for oral tolerance is that plants can also become effective delivery systems without extensive purification. Plant expression also largely eliminates concerns regarding potential pathogens that could be transmitted to humans. Lastly, augmented immune responses to plant produced vaccines may suggest increased stability for plant expressed recombinant proteins to gastrointestinal degradation. Collectively these features make plants an ideal expression and delivery system for oral tolerance. More recently, we have demonstrated that in addition to synthesizing autoantigens, transgenic plants can be used to express immunoregulatory cytokines such as interleukin-4 (IL-4), and that combined oral administration of plant-derived human GAD65 and plant-derived IL-4 had a synergistic effect in inducing robust oral immune tolerance in NOD mice, an animal model for human Type 1 diabetes (Ma *et al*. 2004, 2005).

The aim of the present study was to produce a fusion protein consisting of CTB and the peptide p277 (CTB-p277) in transgenic plants. Our long-term goal is to develop a plant-based oral peptide vaccine to treat Type 1 diabetes via the induction of oral tolerance. Here we demonstrate that the CTB-p277 fusion protein can be efficiently produced in transgenic tobacco plants. Moreover, the plant-derived fusion protein retains important functional characteristics of the native CTB, including pentamerization and GM1 ganglioside receptor binding. The production of transgenic plants expressing the human Hsp60 peptide p277 linked to mucosal adjuvant CTB offers an important first step towards the development of a safe, effective and inexpensive vaccine product for Type 1 diabetes.

MATERIALS AND METHODS

Plasmid construction

An oligonucleotide containing an engineered *Xho*1restriction site followed by the coding sequences for the peptide p277 was custom synthesized by commercial suppliers. The oligonucleotide was rescued by cloning into the *Hinc*II site of the pUC 19 vector to generate pUC19-p277. To create the CTB-p277 fusion protein, the p277 encoding sequence was isolated from pUC19-p277 as a *Xho*I and *Bam*HI fragment and cloned into the same sites of pUC19-CTB. The plasmid pUC19-CTB contains the *ctx*B gene with its signal peptide replaced by a plant signal peptide (i.e., the signal sequence of peanut peroxidase) (Li *et al*. 2006). The in-frame fusion of CTB with p277 was confirmed by sequencing analysis. The fusion gene was isolated as a *Nco*I and *Xba*I fragment and inserted into the same sites of pRTL2-GUS (Carrington and Freed 1990) to replace the GUS gene. The resulting expression cassette comprising the CaMV35S promoter with a double enhancer sequence (2x35S) fused to a 5′ untranslated tobacco etch virus (TEV) leader sequence, *ctx*B-p277, and the nonpaline synthase (NOS) terminator were excised as a *Hind*III fragment and cloned into the pBI101.1 binary vector to create pRT-CTB-p277. Plasmid pBI101.1 is a derivative of pBI101 in which the GUS gene together with the NOS terminator was deleted. The pRT-CTB-p277 binary vector was transformed into *Argobacterium tumefaciens* strain LBA4404 by tri-parental mating (Lige *et al*. 2001).

Plant transformation

Nicotiana tabacum cv. '81V9' leaf discs were transformed by co-cultivation with *A. tumefaciens* LBA4404 containing the pRT-CTB-p277 binary plasmid as described by Horsch *et al*. (1985). Transformed leaf discs were selected on callus-inducing MS104 medium (4.4 g/L Murashige and Skoog (MS, 1962) salts, 3% (w/v) sucrose, 10 mg/L benzylaminopurine, 0.1 mg/L naphthalene acetic acid, pH 5.7, 0.8% (w/v) phytagar) containing 100 mg/L kanamycin. New shoots that developed from the calli were transferred to Magenta boxes containing MS rooting media (4.4 g/L MS salts, 2% (w/v) sucrose, pH 5.7, 0.8% (w/v) phytagar) with 50 mg/L kanamycin and maintained.

PCR analysis of transgenic plants

Transgenic plant DNA was isolated from leaves of tobacco plants grown aseptically in Magenta boxes according to Ma *et al*. (2004). PCR was carried out using forward primer 5′-ATGGCACTTCCA ATTAGCAAG-3′ (corresponding to codons encoding the N′ Terminal engineered signal peptide sequence of CTB) and reverse primer 5′-TTAGTCCTCATTTGCGGGTGTA AGGGA-3′ (corresponding to codons encoding the C-terminal p277 amino acid sequence) to detect the presence of the *ctx*B-p277 fusion gene, or reverse primer 5′-CTCGAGCGGCCCCGGCCCATTTGCCA-3′ (corresponding to the C-terminal end of CTB) to detect the presence of the *ctx*B gene. Cycling conditions comprised an initial step at 94°C for 3 min followed by 30 cycles of 45 s at 94°C, 1 min at 56°C, 1 min at 72°C, and a final extension step at 72°C for 10 min.

RNA purification and RT-PCR analysis

Plant RNA was purified with RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. For RT-PCR, first-strand cDNA was generated in a 20-µl reaction containing 5 µg of total RNA, 0.5 µg of oligo$(dT)_{18}$ and 20 units of SuperScript™ II RNase H Reverse Transcriptase (Invitrogen). A total of 100 ng of RNA-derived first strand cDNA were then used for PCR reaction using the same primer pair utilized above for the detection of *ctx*B-p277 fusion gene. The polymerase used was *Taq* DNA polymerase (Amersham). The PCR cycling conditions were the same as above. RT-PCR products cloned into pUC-19 were sequenced to confirm full length *CTB-p277* expression.

Western blot analysis of transgenic pRT-CTB-p277 plants

Accumulation of the fusion protein in transgenic pRT-CTB-p277 plants was analyzed by Western blotting. Expanded leaves of tobacco plants were ground by mortar and pestle and resuspended in extraction buffer (200 mM Tris pH 8.0, 100 mM NaCl, 400 mM Sucrose, 10 mM EDTA, 14 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.05% Tween-20, 2 µg/ml aprotinin, 2 µg/ml leupeptin). Samples were centrifuged for 10 min at 4°C and the supernatant was collected. The concentration of total soluble protein (TSP) was measured according to the Bradford method using the Protein Assay dye (500-0006, Bio-Rad). Samples were boiled for 5 min or left untreated, separated by SDS-PAGE and blotted onto PVDF membrane (Millipore, Burlington, MA). The membrane was blocked overnight in 5% skim milk-TBST (20mM Tris, 150 mM NaCl, 0.01% Tween-20, pH7.6), then incubated for 2 h in 1:500 dilution (v/v) of rabbit anti-CTB primary antibody (C-3062, Sigma). The blot was then incubated for 1 h with goat anti-

rabbit peroxidase-linked antibody (G-7641, Sigma). Antibody detection was accomplished with SuperSignal® West Pico Chemiluminescent Substrate (34080, Pierce, Rockford, IL). Signals were visualized using a Lumi-imager (Roche Diagnostics).

Quantification of CTB-277 protein accumulation by ELISA

Quantitative ELISA (enzyme-linked immunosorbent assay) determined the level of CTB-p277 fusion protein accumulation in transgenic plants as described previously (Li *et al*. 2006). In brief, triplicate serial dilutions of the plant protein extracts and a CTB standard (C-9903, Sigma) were bound to a 96-well microtiter plate overnight at 4°C. Background was blocked for 2 h in 5% skim milk-PBST (Phosphate buffered saline with 0.05% Tween-20). Plates were washed with PBS, and then incubated with rabbit anti-cholera toxin antibody (C-3062, Sigma) at a dilution of 1:1000 for 2 h at 37°C. Following wash with PBS, plates were incubated with goat anti-rabbit peroxidase-linked antibody (G-7641, Sigma) and developed with the Substrate Reagent Pack (DY999, R&D Systems). Optical density (OD) values were measured at 450 nm, with TSP from untransformed tobacco plants used for background subtraction, and compared to the standards to determine expression level.

GM1-ganglioside binding assay

The GM1-ELISA assay was performed to determine the binding capacity of plant-derived CTB-p277 fusion protein to GM1 ganglioside. Briefly, the microtiter plate coated overnight at 4°C with monosialoganglioside-GM1 (Sigma G-7641) at a concentration of 3 μg/ml (in bicarbonate buffer: 15 mM Na_2CO_3, 35 mM $NaHCO_3$, adjusted pH to pH 9.6) was blocked with 3% fat-free milk in PBS and incubated at room temperature for 2 h, followed by three washes with PBST. The wells were loaded with protein isolated transgenic plants, or bacterial CTB as a positive control. Plates were incubated overnight at 4°C. After washing, the specific binding of plant-derived CTB-p277 to GM1 ganglioside was visualized by the addition of rabbit anti-cholera toxin antibody (Sigma C-3062; 1: 2000 dilution) followed by enzyme-conjugated anti-rabbit IgG (1:5000 dilution) and enzyme substrate 3,3',5,5'-tetramethylbenzidine (TMB; Sigma) as for an ordinary ELISA as described above.

Statistical analysis

Results were analyzed using pair wise t-tests. Values of $p<0.05$ were considered to be statistically significant.

RESULTS

Construction of pRT-CTB-p277 plasmid and plant transformation

The expression vector pRT-CTB-p277, which contains an in-frame fusion of the *ctxB* gene and the p277 fragment of human heat shock protein 60, is shown in **Fig. 1**. Construction of pRT-CTB-p277 has been described in detail in Materials and Methods. The fusion gene was under the transcriptional control of a doubled CaMV 35S promoter.

Following the leaf disc transformation with *Agrobacterium* containing pRT-CTB-p277, more than 25 independent transgenic plants were produced. The presence of the fusion gene was confirmed by PCR analysis of transgenic plants using primers that spanned the entire fusion gene (**Fig. 2**).

Detection of the CTB-p277 fusion gene transcripts in transgenic tobacco plants by RT-PCR

RT-PCR analysis was employed to detect the expression of the CTB-p277 hybrid mRNA in transgenic tobacco lines. When primers spanning the full length of CTB-p277 were used, an amplification product of expected size (480 bp) was detected following reverse transcription of total RNA extracted from leaf tissues of tobacco lines transformed with pRT-CTB-p277. Representative data are shown in **Fig. 3**. No RT-PCR products were detected using the total RNA from the wild-type tobacco plants. To rule out the possibility of amplification of contaminant DNA in the samples, direct PCR amplification without reverse transcription was performed on the RNA preparations. No amplified PCR products were seen under the same conditions (data not shown), confirming the specificity of the RT-PCR reaction. Sequencing of the RT-PCR products confirmed the correct full-length in-frame fusion of the CTB-p*277* fusion (data not shown).

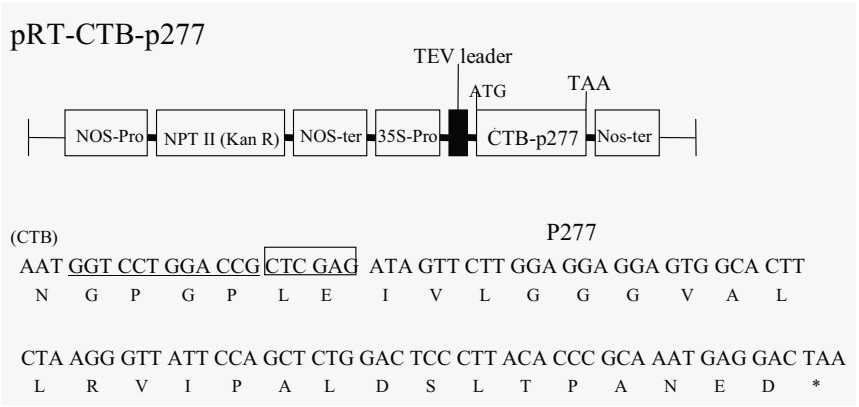

Fig. 1 Schematic diagram of the plant expression vector pRT-CTB-p277. The T-DNA region inserted into the plant genome contains the nopaline synthase expression cassette (KanR), which confers kanamycin resistance on transformed cells, and the CTB-p277 fusion cassette consisting of the CaMV 35S promoter fused to a tobacco etch virus 5′-untranslated region (TEV) and nopalin synthase terminater (Nos-ter). The nucleotide and amino acid sequences of p277 as well as the sequence surrounding the fusion site are shown at the bottom. The underlined sequences correspond to the flexible hinge region. The boxed sequences represent the joining site between the two fusion partners. The stop codon is indicated by an asterisk.

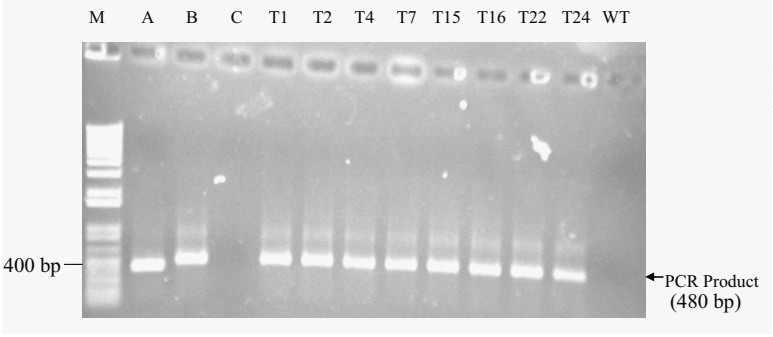

Fig. 2 Detection of CTB-p277 integration into transgenic tobacco genomic DNA by PCR. The CTB-p277 fusion gene was amplified from transgenic plant DNA by PCR, and the PCR products were separated by agarose gel electrophoresis. M, DNA ladder; A, PCR product amplified from pRT-CTB-p277 plasmid DNA with primers specific for full-length CTB (positive control); B, PCR product amplified from pRT-CTB-p277 plasmid DNA with primers specific for full-length CTB-p277 (positive control); C, PCR amplification of pUC19-CTB with primers specific for full-length CTB-p277 (negative control). T1 to T24, independent transgenic lines amplified with primers specific for full-length CTB-p277. WT, wild-type tobacco amplified with primers specific for full-length CTB-p277.

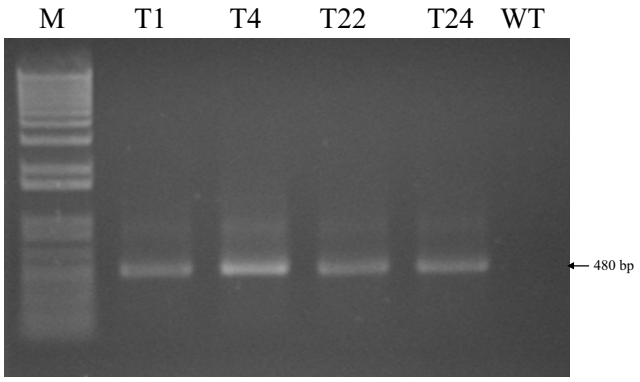

Fig. 3 RT-PCR analysis of CTB-p277 transcripts in RNA from transgenic plants. Total RNA was extracted from leaf tissues of individual transgenic lines. RT-PCR reaction conditions were described in Methods. Primers used were the same as described above for PCR detection of full-length CTB-p277 from plant genomic DNA. M, molecular size marker; T1 to T4, independent transgenic lines; WT, wild-type tobacco.

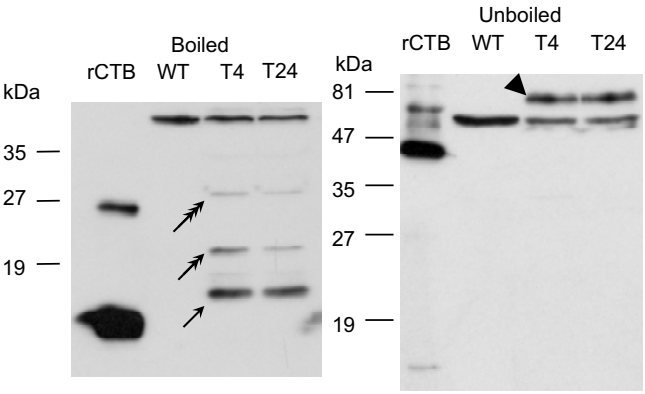

Fig. 4 Western blot analysis of CTB-p277 fusion protein expression in transgenic plants. Total protein extracts (40 μg/lane) from leaf tissues of transgenic plants were fractionated by SDS-PAGE gel, blotted onto PVDF membrane, and probed with anti-CTB antibody. Both boiled and unboiled samples were analysed. T4 and T24 represent two individual transgenic lines; WT, wild-type tobacco; rCTB, recombinant bacterial CTB standard (Sigma). Bands for CTB-p277 monomer (single-headed arrow), dimer (double-headed arrow), trimer (triple-headed arrow) and pentamer (non-tailed arrow) are indicated. Numbers on the left indicate positions of protein size markers in kDa.

Accumulation of CTB-p277 fusion protein in transgenic tobacco plants

To examine the accumulation of CTB-p277 fusion protein in the transgenic plants, Western blot analysis was performed. Proteins extracted from leaves of the pRT-CTB-p277 tobacco plants were probed with anti-CTB polyclonal antibody (**Fig. 4**). The anti-CTB antibody detected a major band of 14 kDa, which is about 2 kDa larger than the control CTB in size, as well as two minor bands between 19 and 35 kDa when the samples were boiled prior to loading. The major band was consistent with the expected size of the monomeric form of CTB-p277, while the two minor bands may represent the dimeric and trimeric forms of CTB-p277, respectively. When the samples were loaded unboiled, a single 81-kDa band was seen in extracts from pRT-CTB-p277 tobacco plants but not from wild-type (**Fig. 4**). The 81-kDa band appears to correspond to pentameric CTB-p277. The presence of a single band suggests that the protein is stable and assembled as a biologically active CTB pentamer. The same bands could not be detected when the extracts were probed with anti-human hsp60 monoclonal antibody (data not shown). This may be due to the low affinity of the anti-human hsp60 antibody towards the peptide p277.

Quantification of CTB-p277 fusion protein in transgenic tobacco leaf tissues

The levels of CTB-p277 fusion protein expression in transgenic leaf tissues were determined by ELISA. Using this method, the amount of plant CTB-p277 fusion protein was calculated by comparison of the optical density (OD) values obtained for transgenic extracts with the OD values for a known amount of bacterial CTB standard. The amount of the fusion protein was then expressed as a percentage of the total soluble plant protein (TSP). Representative data are shown in **Fig. 5A**. Transgenic line T4 showed the highest expression of CTB-p277 fusion protein, accounting for 0.1% of TSP.

GM1 receptor-binding assays of plant-derived CTB-p277 fusion protein

Biological functions of CTB, such as the ability to bind to GM1 ganglioside, depend on the formation of a pentameric structure composed of identical monomers (Hardy *et al*. 1988). To demonstrate that the plant-derived CTB-p277 fusion protein binds to GM1gangliosides, a GM1-ELISA was performed on the leaf extracts from transgenic line T4. The results show that both plant-derived fusion protein and commercial CTB bind gangliosides efficiently (**Fig. 5B**). No binding activity was detected when the plate was coated with irrelevant bovine serum albumin (BSA). These results

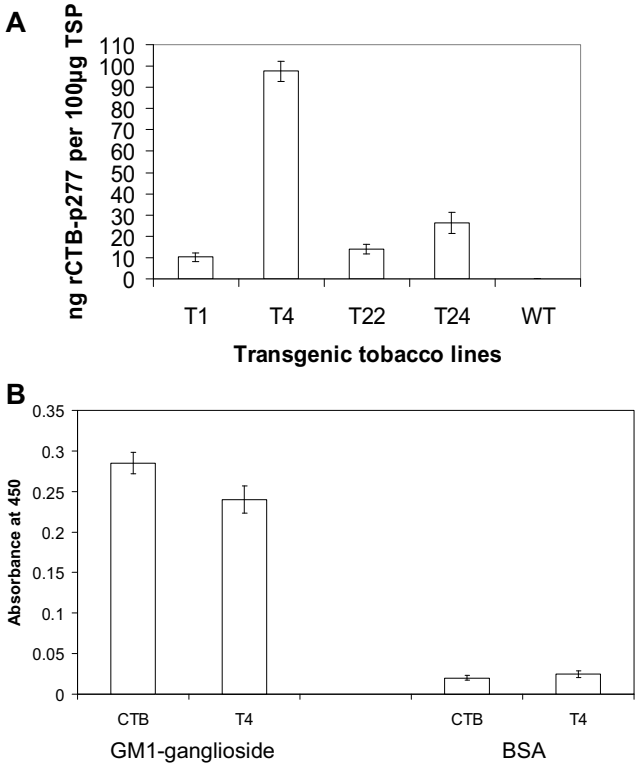

Fig. 5 (**A**) ELISA quantification of the CTB-p277 fusion protein. The amount of the CTB-p277 in total soluble protein (TSP) of transgenic tobacco leaf tissues was estimated by using ELISA as described in the Methods. The CTB-p277 fusion protein concentration was expressed as a percentage of TSP. Data shown here represent averages of three experiments. The numbers on the bottom of the figure represent the different tobacco lines. WT: wild-type tobacco. The error bar represents the standard deviation. (**B**) GM1 binding analysis of plant-derived CTB-p277 fusion protein. The protein samples used in this assay were prepared from transgenic line T4. The amount of total protein added to the microplate well coated with GM1-ganglioside or BSA (control) was adjusted to obtain the concentration of CTB-p277 at approximately 8 ng/ml. The concentration of bacterial CTB used was 10 ng/ml. The absorbance of the GM1-ganglioside-CTB-p277 complex in each well was measured. The values represent the averages of three experiments. The error bar represents the standard deviation.

further suggest that the CTB-p277 pentamer is required for biological activity. Thus, the addition of the p277 peptide to the C-terminus of CTB protein did not affect pentamerization or GM1 receptor binding of the protein.

DISCUSSION

We report here the production of a fusion protein containing the anti-diabetic peptide p277 fused to the C-terminal of the B subunit of cholera toxin (CTB) in transgenic plants, a first step towards the development of a plant-based oral peptide vaccine for the treatment of Type 1 diabetes by inducing oral immune tolerance. The human hsp60 peptide p277 represents a functional important target in Type 1 diabetes, and injection of p277 has been shown to be very effective at treating Type 1 diabetes in both humans and NOD mice (Abulafia-Lapid *et al*. 1999). However, injection is not the preferred route of administration. Injections can cause discomfort and distress, and require trained people to administer it safely. This method also risks introducing harmful microbes into the patient's circulation system. Moreover, as most peptide drugs are chemically synthesized, the cost of their production is high and mass production would be difficult. Administration of p277 by the oral route offers practical advantages over parenteral administration: requiring neither sterile needles nor trained personnel, lower cost, increased quality of life, reduced side-effects, and greater patient acceptability and compliance. However, administration of therapeutic peptides by the oral route represents a major challenge. When administered orally, peptides are subjected to quick degradation by the enzymatic environments of the gastrointestinal tract and especially by the acidic environment of the stomach, thus requiring much higher amounts of peptide than subcutaneous injection. To address these limitations, we have therefore expressed p277 as a fusion with CTB. CTB is a highly stable protein, and has been shown to be an excellent oral delivery carrier and adjuvant for other peptide or protein antigens (Holmgren *et al*. 1994).

As the capacity of CTB as a mucosal adjuvant or carrier molecule for conjugated antigens is critically dependant on its ability to bind GM1 ganglioside in its pentameric form, it is essential to ensure that the CTB-p277 fusion protein retained the ability to form pentamers and bind the GM1 ganglioside receptor. Western blot analysis of unboiled transgenic leaf extracts with anti-CTB antibody showed that the fusion protein was apparently assembled into a pentameric structure (**Fig. 4**). Moreover, GM1-ELISA showed that plant-derived CTB-p277 binds to GM1-receptors as efficiently as the native CTB (**Fig. 5B**). These suggest that the p277 peptide carried by the fusion protein had no demonstrable adverse effect on assembly or GM1 binding of the parental CTB molecule. These results agree with our previous observations that genetic fusion of human insulin B-chain to the C-terminus of CTB had no detectable negative effect on pentamerization and GM1-binding capacity of CTB (Li *et al*. 2006). Previous work by Liljeqvist *et al*. (1997) has suggested that pentamerization and GM1 binding capacity of CTB linked with proteins or peptides can be affected by the length of the partner protein or peptide, or by the conformational changes induced by the fusion partner. To minimize any possible negative perturbation effects of the fusion partner on the pentamer formation and ganglioside-binding capacity of CTB, the p277 was purposely fused to the C-terminus of CTB, as the GM1 binding properties of CTB does not critically depend on its C-terminal amino acid sequences (Zhang *et al*. 1995). Furthermore, a flexible hinge tetrapeptide (GPGP) was introduced between the CTB and p277 to reduce potential sterical hindrance and permit high intramolecular flexibility between the two partners of the fusion protein.

Analysis of boiled transgenic leaf extracts by Western blot using the same anti-CTB antibody revealed one specific band of about 14, and two bands between 19 to 35 kDa (**Fig. 4**). The 14-kDa band with the highest intensity was consistent with the expected size for the CTB-p277 monomer, whereas the two larger bands may correspond to the CTB-p277 dimer and trimer, respectively. The detection of both dimeric and trimeric forms of CTB-p277 in boiled samples suggests that the fusion protein is rather heat stable. The failure to detect the CTB-p277 fusion protein in the same transgenic leaf extracts by using a commercially available anti-human hsp60 monoclonal antibody (Clone 264233, R&D Systems) is not surprising. As this anti-human hsp60 antibody was raised against the whole hsp60 protein, it may not have a high binding affinity towards the peptide p277 that is only 24 amino acids in length. However, two lines of evidence support our conclusion that plant-derived fusion protein contains the amino acid sequence of peptide p277. First, PCR amplification of the genomic DNA isolated from pRT-CTB-p277 transgenic tobacco lines resulted in an anticipated 480-bp DNA fragment, when primers specific for full-length CTB-p277 were used (**Fig. 2**, lanes T1 to T24). The same band was amplified when pRT-CTB-p277 plasmid DNA was a template using the same primer pair (positive control) (**Fig. 2**, lane B), whereas no PCR products were obtained when pCU19-CTB plasmid DNA was amplified with this primer pair (negative control, as this plasmid contains CTB without p277 sequence) (**Fig. 2**, lane C). On the other hand, an anticipated smaller DNA band (400 bp) was obtained when pRT-CTB-p277 plasmid DNA was amplified using primers specific for CTB but not for the fusion gene (**Fig. 2**, lane A), due to the presence of p277 sequence not being amplified. Secondly, RT-PCR amplification of total RNA isolated from these pRT-CTB-p277 transgenic tobacco lines also gave a 480-bp DNA product when the same CTB and p277 specific primer was used (**Fig. 3**), suggesting the expression of full-length CTB-p277 mRNA. As anticipated, RT-PCR amplification of total RNA isolated from wild type tobacco plants gave no product (**Fig. 3**). The in-frame fusion of CTB and p277 was further confirmed via cloning of the RT-PCR product and DNA sequencing (data not shown). Taken together, these results provide solid evidence that the fusion protein contains the amino acid sequence of p277.

The expression level of CTB-p277 fusion protein accounted approximately for 0.1% of total protein in tobacco leaf tissues (**Fig. 4**), which is higher than that reported for CTB or CTB fusion protein expressed in transgenic tobacco by other groups (Wang *et al*. 2001; Jani *et al*. 2004). This may be partly attributed to our use of a plant signal peptide to replace the native signal of CTB. We have previously shown that the use of a plant signal improves the expression of CTB in transgenic tobacco (Li *et al*. 2006). It is anticipated that the current level of CTB-p277 expression can be further improved. One strategy is to express the fusion protein in the chloroplast of tobacco plants. Daniell *et al*. (2001) reported the expression of CTB in transgenic tobacco chloroplasts at levels up to 4.1% of total soluble tobacco leaf protein. Another strategy is to express the CTB-p277 as an ubiquitin fusion. Recently, Mishra *et al*. (2006) demonstrated a significant increase in accumulation of CTB when it was expressed as an ubiquitin fusion in transgenic tobacco plants.

In summary, we have produced transgenic plants expressing CTB-p277 fusion protein. Functional analysis of the plant-derived fusion protein showed that it retains the biological and immunological characteristics of the native CTB including its ability to pentamerize and to bind to GM1 ganglioside receptor. Oral administration of transgenic plants expressing high levels of CTB-p277 may prove to be an effective method for inducing oral tolerance to treat Type 1 diabetes.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. J. Holmgren, Institute of Medical Microbiology, University of Göteborg, Sweden for providing bacterial CTB DNA clone. This research was supported by the Natural Sciences and Engineering Research Council (NSERC).

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