

# Expression of a multi-epitope DPT fusion protein in transplastomic tobacco plants retains both antigenicity and immunogenicity of all three components of the functional oligomer

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**Abstract** Expression of genes in plant chloroplasts provides an opportunity for enhanced production of target proteins. We report the introduction and expression of a fusion DPT protein containing immunoprotective exotoxin epitopes of *Corynebacterium diphtheriae*, *Bordetella pertussis*, and *Clostridium tetani* in tobacco chloroplasts. Using biolistic-mediated transformation, a plant-optimized synthetic *DPT* gene was successfully transferred to tobacco plastomes. Putative transplastomic T0 plants were identified by PCR, and Southern blot analysis confirmed homoplasmity in T1 progeny. ELISA assays demonstrated that the DPT protein retained antigenicity of the three components of the fusion protein. The highest level of expression in

these transplastomic plants reached 0.8% of total soluble protein. To assess whether the functional recombinant protein expressed in tobacco plants would induce specific antibodies in test animals, a mice feeding experiment was conducted. For mice orally immunized with freeze-dried transplastomic leaves, production of IgG and IgA antibodies specific to each toxin were detected in serum and mucosal tissues.

**Keywords** Plastid expression · Transplastomic plants · DPT recombinant protein · Immunogenic polypeptide

## Introduction

Simultaneous vaccination against diphtheria, tetanus, and pertussis using a DPT vaccine during infancy and childhood has markedly reduced the incidence of cases and deaths from each of these serious bacterial diseases (CDC 2006; Kalies et al. 2006; WHO 1999). Concerns over the safety of whole-cell pertussis vaccines have prompted the development of acellular vaccines that are less likely to provoke adverse reactions as they contain purified antigenic components of the causal bacterium *Bordetella pertussis*. However, current acellular pertussis vaccines must be administered in a series of multiple doses, thereby contributing to their high production costs (CDC 1992; Salmaso et al. 2001; Tan et al. 2005). Attempts to produce safer, inexpensive, and more efficient DPT vaccines are underway (Abomoelak et al. 1999; Aminian et al. 2007; Kamachi et al. 2003; Meriste et al. 2006).

In the last few years, plants have been genetically engineered to express various recombinant biopharmaceuticals (Ma et al. 2005). Plants can be used as bioreactors for the production of appropriately safe and inexpensive vaccines,

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and contribute to reduced costs associated with vaccine transportation and storage (Giddings et al. 2000; Koya et al. 2005; La et al. 2007). Plant-based vaccine production has been reported in several plant species, including potato (Mason et al. 1996), tobacco (Liu et al. 2005; Mason et al. 1992; Zhang et al. 2006), tomato (McGarvey et al. 1995; Sandhu et al. 2000), lettuce (Kapusta et al. 1999), carrot (Marquet-Blouin et al. 2003; Rosales-Mendoza et al. 2007), and alfalfa (Dong et al. 2005), among others. Feeding studies conducted in animals (Huang et al. 2001; Rosales-Mendoza et al. 2008; Thanavala et al. 1995) and humans (Tacket et al. 1998, 2000) have demonstrated that candidate vaccines produced in plants are effective in eliciting protective immune responses. This vaccine production approach will also have a positive impact on public health measures in developing countries that lack of proper refrigeration systems for storage of traditional vaccines. As plant-based vaccines are administered orally, these vaccines avoid the use of injections, thus eliminating discomfort and more importantly the risk of disease transmission via re-use of contaminated syringes and needles (La et al. 2007; Richter et al. 2000).

Plastid transformation provides an alternative strategy for expressing foreign genes in plants and offers several advantages over nuclear plant expression systems (Chargelegue et al. 2001; Sala et al. 2003). These advantages include higher levels of foreign protein accumulation, site-specific integration of transgenes, and transgene containment due to maternal transmission of plastids thus alleviating environmental concerns over gene flow (Maliga 2003; Daniell et al. 2005). Moreover, the destination of a protein influences both its stability and modification (Drakakaki et al. 2006). To date, many antigenic proteins have been expressed in chloroplasts, such as the cholera toxin B subunit (Daniell et al. 2001), anthrax protective antigen (Koya et al. 2005), tetanus toxin fragment C (Tregoning et al. 2003), *Escherichia coli* K99 fimbrial subunit antigen (Garg et al. 2007), and the spike (S) protein of the severe acute respiratory syndrome coronavirus (SARS-CoV) (Li et al. 2006). Recently in our laboratory, a fusion protein of the heat labile toxin B subunit (LTB) of the enterotoxigenic *Escherichia coli* along with the heat stable toxin (ST) fusion protein (LTB-ST) has been expressed in transplastomic tobacco plants, and demonstrated its immunogenic characteristic in tested mice (Rosales-Mendoza et al. 2009).

Previously, we have expressed an antigenic polypeptide containing epitopes of diphtheria, pertussis, and tetanus exotoxins in tomato plants via nuclear transformation (Soria-Guerra et al. 2007). Following analysis of transgenic tomato plants, it has been demonstrated that the *DPT* transgene is integrated into the genome, transcribed, properly translated, and accumulating at levels of 0.006% of total soluble protein (TSP) (Soria-Guerra et al. 2007). In addition,

we have demonstrated that three doses of 270 mg each of freeze-dried tomato triggers specific immune responses in mice (Soria-Guerra et al., unpublished). These studies suggest that the DPT could be used as a viable multicomponent DPT subunit vaccine. However, low levels of expression of the DPT protein in tomato plants render this plant-based candidate vaccine less desirable for commercial use as an oral vaccine. In this study, we report on the transfer and expression of the *DPT* fusion gene, re-designed for expression in tobacco chloroplasts. Recovery of transplastomic T1 tobacco plants accumulating high levels of the recombinant DPT fusion protein is reported. The antigenicity of all three components of the DPT fusion protein is also confirmed in these transplastomic tobacco plants by ELISA assays. Following oral feeding of freeze-dried transplastomic tobacco leaves in test mice, immunogenic responses are observed.

## Materials and methods

### Gene and vector construction

As previously described, a multi-epitope DPT fusion protein was selected as the target for plastid expression in tobacco plants (Soria-Guerra et al. 2007). This fusion protein contains six immunoprotective exotoxin epitopes of *Corynebacterium diphtheriae*, *Bordetella pertussis*, and *Clostridium tetani*. A synthetic gene encoding for the DPT fusion protein is designed for optimal expression in tobacco plastome based on codon usage in plants. The 600-bp sequence includes a ribosome binding site and *Xba*I and *Xho*I restriction sites at the 5' and 3' ends, respectively. This gene has been synthesized by GenScript Corp. (Piscataway, NJ), and no changes were made in the amino acid sequence for the toxin subunits, linkers, and adjuvants, except for the deletion of the SEKDEL sequence. The two adjuvant-coding sequences of the heavy chain tetanus toxin were added near the C-terminal.

For plastid transformation, the plasmid pBic was used; it was derived from the chloroplast transformation vector pKCZ (kindly provided by Dr. Hans-Ulrich Koop). This vector is designed for integration of foreign genes into an *Mun*I restriction site between *trnN-GUU* and *trnR-ACG* in the inverted repeat region of the chloroplast genome (Zou et al. 2003). Several modifications have been made to the pKCZ vector in order to express the *DPT* and *aadA* genes as bicistrons under the control of the plastid 16S rRNA promoter (Prrn). First, a synthetic plastid 16S-rRN-promoter (Prrn) fused to the 5'-UTR of gene *10* from the bacteriophage T7 (*T7G10*) was prepared using synthetic oligonucleotides. The 5'-UTR region of this promoter was ligated to the pKCZ vector at restriction sites *Nhe*I and *Bgl*III of the

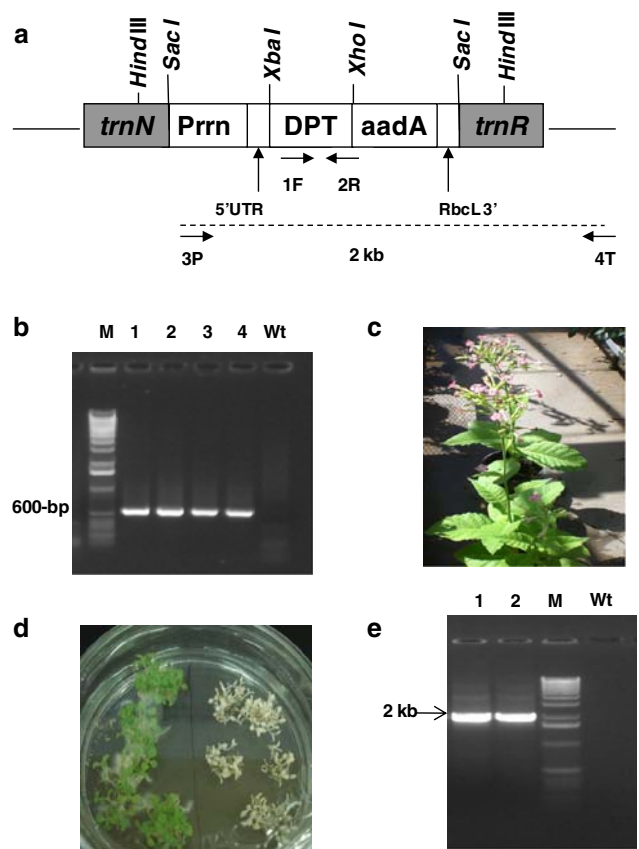
pKCZ-UTR. Then, the vector pKCZ-UTR was digested with *SpeI* and *NheI* in order to delete the *aadA* expression cassette, and self-ligated to generate pKCZ-UTRdel. An *aadA* expression cassette was released from the pKCZ vector by *SmaI*–*EcoRV* digestion, and cloned downstream of the *Prrn*-UTR at *AfeI* and *PmlI* sites of pKCZ-UTRdel to generate the pBic vector. The DPT coding sequence was subcloned into the pBic vector digested with *XbaI* and *XhoI* restriction enzymes. The resulting plasmid was named pBic-DPT (Fig. 1a). A positive clone was selected following restriction analysis and sequenced. All cloning and analysis procedures were performed following standard protocols (Sambrook et al. 1989). DNA for plastid transformation was prepared using the Qiagen Plasmid Midi Kit (Qiagen, Valencia, CA).

Plastid transformation

Tobacco (*Nicotiana tabacum* cv. Petit Havana) seedlings were grown aseptically on Murashige and Skoog (MS) medium for 3–5 weeks. DNA was coated onto 0.6 μm gold particles, and bombarded onto fully expanded leaves using the PDS-1000/He (Bio-Rad, Hercules, CA) biolistic delivery system as previously described (Daniell et al. 2001). Following bombardment, leaves were incubated for 48 h in the dark at 25°C. Then, leaves were cut into small sections (~5 mm × 5 mm), and cultured with the abaxial side in contact with the RMOP medium (Svab and Maliga 1993) and containing 500 mg/L spectinomycin. After 6–8 weeks, leaves from putative transplastomic shoots on selection medium were cut into small sections (0.2 cm<sup>2</sup>), and placed on a selection medium for the next round of selection. Following three selection rounds, spectinomycin-resistant shoots were transferred onto fresh MS medium containing 500 mg/L spectinomycin for rooting. Whole plants were transferred to soil, acclimatized, and grown in the greenhouse to maturity. After blooming, seeds were collected, subject to surface sterilization and germinated on MS media containing spectinomycin.

PCR and Southern blot analysis

Total DNA in T0 plants was extracted from putative transplastomic and non-transformed tobacco leaves using the DNEasy™ Plant Mini Kit (Qiagen Inc., Valencia, CA). For PCR analysis, a 50 μl reaction mixture containing 250 ng DNA, 1.5 mM magnesium chloride, 2.5 U *Taq* DNA polymerase, 1 mM dNTPs, and 1 μM of each of forward and reverse primers was used. The forward primer 1F (5' ggtatgattctagccaccagagg) and reverse primer 2R (5' gagcggctattcaagatgtgaagc) were used to amplify the DPT gene. Primers 3P (5' gctccccgcctgttcaatgaga) and 4T (5' gcattctaagtagtaagccccaccaagatg) were used to detect



**Fig. 1** a Schematic diagram of the plastid transformation vector harboring the synthesized *DPT* gene. This gene is under the control of the *Prrn* promoter; moreover, the 5'-UTR of phage T7 gene 10 contains a GGAGG ribosome binding site. This gene is inserted at the *trnR/trnN* insertion site into the tobacco chloroplast genome. *RbcL* 3', 3' untranslated region of the *rbcL* gene; *aadA*, spectinomycin resistance gene. 1F and 2R correspond to primers used for the detection of the *DPT* gene; while, 3P and 4T, primers used to detect transplastomic lines. b PCR analysis of plants using 1F and 2R primers for the *DPT* gene. A specific 0.6-kb PCR band was detected in putative transplastomic tobacco plants (1–4 correspond to lines B3, C1, D3, and E1), but it was absent in wild-type tobacco (Wt). c T0 mature plants transferred to soil and grown in the greenhouse exhibiting normal phenotypes. d T1 seeds germinating on a medium containing 500 mg/L spectinomycin together along with wild-type (Wt). e PCR analysis using primers 3P and 4T; a 2 kb product is amplified from transplastomic plants (1–2 correspond to lines B3 and E1) which is absent in the wild-type (Wt)

integration of the expression cassette into the tobacco plastome. The PCR protocol included an initial denaturation step at 94°C for 3 min followed by 35 cycles of denaturation, annealing, and extension steps of 30 s at 94°C, 60 s at 55°C, and 2 min at 72°C, respectively. PCR products were analyzed on 1% agarose gels. PCR-positive lines were transferred to the greenhouse for plant growth and seed production. T1 seeds were harvested and germinated on a medium containing 500 mg/L spectinomycin.

Southern blot analysis was performed in T1 progeny as previously described (Sambrook et al. 1989). Fifty micrograms of total genomic DNA from leaves of each transformed and

wild-type plants was digested with *HindIII*, *XbaI*, and *SacI* electrophoresed on a 1% agarose gel, and blotted onto a Hybond N membrane (Amersham-Pharmacia Biotech, Piscataway, NJ). The *DPT* fusion gene and a fragment of the *trnN* region were used as probes, generated using the DIG-DNA labeling mixture (Boehringer Mannheim, Germany). The probe was hybridized with the membrane and resolved using the CSPD substrate for alkaline phosphatase according to the manufacturer's protocol.

#### Western blot analysis

For Western blot analysis, protein samples were separated by SDS-PAGE using 4–20% pre-cast polyacrylamide electrophoresis gels (NuSep Inc., Austell, GA). Proteins were blotted onto BioTrace PVDF membrane using a Mini Trans Blot™ electrophoretic transfer cell (Bio-Rad). Membranes were incubated overnight at 4°C in 10 ml of either 1:1,000 dilution of a rabbit anti-tetanus toxin (Ab34890, Abcam, Cambridge MA) or 1:5,000 dilution of a goat anti-diphtheria toxin (US Biological, Swampscott, MA). After washing, membranes were incubated for 1 h in a 1:10,000 dilution of either an anti-rabbit IgG or anti-goat IgG conjugated with horseradish peroxidase (Sigma A5420). The antibody binding signal was detected with a Lumi-light Western Blotting Substrate (Roche Co., Mannheim, Germany) according to the manufacturer's protocol.

#### ELISA analysis

Total soluble proteins (TSP) were extracted from leaves of transplastomic and non-transformed T1 plants according to Kang et al. (2003). Protein concentration was determined by the Bradford protein assay reagent kit (Bio-Rad).

For ELISA assays, 50 ng TSPs were loaded into a 96-well microtiter plate (Immulux™ HB, Dynex Technologies, Germany) diluted in bicarbonate buffer (15 mM Na<sub>2</sub>CO<sub>3</sub>, 35 mM NaHCO<sub>3</sub>; pH 9.6) in a 100 µl volume, and incubated overnight at 4°C. After washing with PBST and blocking with 5% nonfat dry milk, the plate was incubated with goat anti-diphtheria toxin (US Biological, Swampscott, MA), mouse anti-*Bordetella pertussis* toxin (Abcam, Cambridge, MA), or rabbit anti-tetanus toxin (Ab34890, Abcam, Cambridge MA) antibodies diluted 1:1,000 (100 µl per well). As secondary antibodies, anti-goat IgG alkaline phosphatase conjugate (Sigma A4187), anti-mouse IgG alkaline phosphatase conjugate (Sigma A1902), or anti-rabbit IgG alkaline phosphatase conjugate (Sigma A3687) were used. Following incubation with 100 µl *p*-nitrophenyl phosphate liquid substrate (Sigma N7653) per well for 30 min at room temperature, reactions were stopped with 1 N HCl, and optical density was determined at 450 nm using an ELISA microplate reader (MRX Revelation,

Dynex Technologies). For each assay, a standard curve was included, and a tetanus toxoid (NIBSC 02/126), at levels of 0.5, 1.5, 2.5, and 5 ng, was used for quantification of the tetanus toxoid.

#### Test animals and immunizations

Male BALB/c mice, 12- to 14-week-old, (Harlan Sprague Dawley, Inc., Indianapolis, IN) were used for evaluation of the immune response against DPT protein. All animals were handled according to federal regulations for animal experimentation and care (NOM-062-ZOO-1999, Ministry of Agriculture, Mexico), and approved by the Institutional Animal Care and Use Committee.

Mice were immunized via oral route delivery with 50 mg of freeze-dried tobacco powder from E1 line containing 12.25, 21.5, and 5 µg of tobacco-derived diphtheria, tetanus, and pertussis exotoxin epitopes, respectively. Positive and negative controls included DPT toxoids [diphtheria toxoid (NIBSC 02/176), tetanus toxoid (NIBSC 02/126), or pertussis toxoid (NIBSC 66/303)] and 50 mg of wild-type-tobacco material, respectively. Each test group contained five animals for which three oral doses were administered on days 1, 7, and 14. Tobacco powder was hydrated in 0.5 ml water, and the suspension was administered to test animals via the intragastric route. Mice were fasted overnight prior to immunization. The animals were sacrificed on day 21 to collect serum samples and intestinal fluids.

#### Collection and preparation of samples

Serum samples were obtained from blood extracted by cardiac puncture from ether-anesthetized mice. Fluids from the gut were collected, and contents of the small and large intestines were flushed out with 5 mL of cold RPMI 1640 medium. Then, 500 µl of 10 mM *p*-hydroxymercuribenzoate (dissolved in 150 mM Tris-base) was added. Samples were centrifuged at 4°C for 10 min at 12,000g, their supernatants were immediately frozen, and stored at -70°C until assay.

#### ELISA assays of epitope-specific antibodies

Responses in serum and intestinal fluids were determined by an indirect enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates were coated with either 0.5 limit of flocculation (Lf)/ml diphtheria toxoid (NIBSC 02/176), 0.5 Lf/ml (NIBSC 02/126) tetanus toxoid, or 0.23 international unit (UI)/ml (NIBSC 66/303) pertussis toxoid, and with 100 µl per well of bicarbonate-carbonate buffer (15 mM Na<sub>2</sub>CO<sub>3</sub>, 35 mM NaHCO<sub>3</sub>, pH 9.6) for 2 h at 37°C. Plates were washed, and then blocked with 5% nonfat dry milk in PBS (100 mM NaCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>,

3 mM  $\text{KH}_2\text{PO}_4$ , pH 7.2). The serum was diluted 1:10 in PBST (0.05% v/v Tween-20 in PBS). Intestinal fluid samples were diluted 1:2 in 5% nonfat milk dissolved in PBST, added to wells of sensitized plates, and incubated overnight at 4°C. The following antibodies were used as secondary antibodies: biotinylated goat anti-mouse IgG, IgA, IgG1, or IgG2a (Zymed Laboratories, San Francisco, CA). Plates were incubated for 1 h at 37°C and then washed with PBST. A conjugated horseradish peroxidase–streptavidin was added to each well, and plates were incubated for 1 h at room temperature. After washing, plates were incubated at room temperature with 100  $\mu\text{l}$  volume of a substrate solution (0.5 mg/ml *o*-phenylenediamine, 0.01%  $\text{H}_2\text{O}_2$ , 50 mM citrate buffer, pH 5.2). Following color development (15 min), the reaction was stopped with 25  $\mu\text{l}$  of 2.5 M  $\text{H}_2\text{SO}_4$ . Specific antibody levels were expressed as corresponding optical density values, and measured at 492 nm (A492) using a Multiskan Ascent (Thermo Electron Corporation, Waltham, MA) microplate reader.

ELISA data correspond to the geometric means of  $n = 5$  mice per group and representative of duplicate experiments, and error bars correspond to standard deviations. Statistical significance of differences ( $P < 0.05$ ) was determined using analysis of variance.

## Results

### Construction of the plastid expression vector

The sequence of the plant-optimized synthetic gene encoding the DPT recombinant polypeptide, containing immunoprotective epitopes of tetanus, pertussis, and diphtheria exotoxins along with coding sequences of two adjuvants of the heavy chain tetanus toxin, was optimized as per codon bias for tobacco genes. Following optimization, the GC content was 37%, and although 76% of the codons were changed, the original amino acid sequence of the wild-type bacterial genes was maintained. The gene was synthesized by GenScript, and both mRNA processing and destabilizing motifs were avoided. The DPT gene was inserted into the plastid expression cassette of the pBic vector as described in “Materials and methods”.

The plasmid pBic-DPT utilizes tobacco plastid genome sequences spanning the *trnR*-ACG and *trnN*-GUU regions to target the gene of interest into the chloroplast genome by double homologous recombination. The promoter is derived from the strong  $\sigma$ -70-type plastid rRNA operon promoter (*Prrn*) linked to the 5' untranslated region (UTR) of the T7 phage gene 10 (T7g10). The selectable marker gene *aadA* provides spectinomycin resistance for selecting stable transformants, and the plastid *rbcL* 3' untranslated region is involved in mRNA stability (Fig. 1a).

### Analysis of plastid-transformed tobacco plants

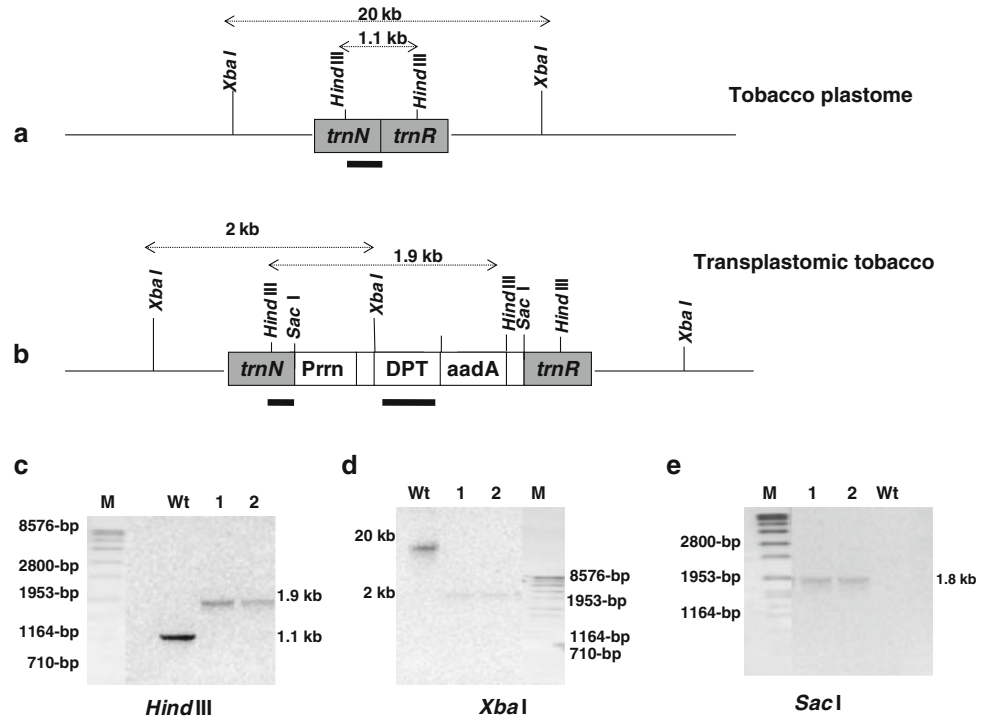
The pBic-DPT construct was introduced into tobacco leaf tissues by microprojectile bombardment, and callus was observed on explants after 7–8 weeks on selection medium. After approximately 6 months, those plantlets regenerated in the third round of spectinomycin selection were allowed to root.

Presence of the transgene in T0 transformed plants was determined by PCR screening using primers specific (1F and 2R) for the DPT transgene. A 0.6-kb PCR-specific band was amplified in all four putative transplastomic plants, but it was absent in wild-type tobacco plants (Fig. 1b). PCR-positive plants were transferred to pots containing soil mix, and grown in the greenhouse until maturity (Fig. 1c). Among these four T0 lines, designated as B3, C1, D3, and E1, three plants, including B3, C1, and E1, were fertile and produced seeds. To check for stability of the DPT transgene, seeds from both wild-type (Wt) and the three fertile transformed lines were surface-sterilized, and allowed to germinate on a 500 mg/L spectinomycin-containing medium. All Wt seeds failed to germinate; while, T1 seeds of transformed lines E1 and B3 readily germinated on the spectinomycin containing medium (Fig. 1d). Seeds of C1 line become bleached under spectinomycin selection and were deemed non-transformed.

PCR reactions were also carried out to assay for presence of the expression cassette using primers flanking the native chloroplast genome, those adjacent to the site of integration (4T), and the *Prrn* promoter (3P) (Fig. 1a). The detection of a 2 kb PCR product in all four putative transplastomic plants confirmed presence of the expression cassette in the chloroplast genome (Fig. 1e).

Transgene integration and homoplasmy were also confirmed in T1 plants, lines B3 and E1 by Southern blot analysis. Total DNA from leaves was isolated from non-transformed and putative transplastomic plants and digested with *HindIII*, *XbaI* and *SacI*. The plastid genome organization of transplastomic tobacco using pBic-DPT and non-transformed tobacco is shown in Fig. 2a and b. When DNA was digested with *HindIII*, a 1.9 kb fragment was detected in spectinomycin-resistant plants, while a 1.1 kb fragment was detected in non-transformed plants (Fig. 2c). When DNA was digested with *XbaI*, a 2 kb fragment was detected in spectinomycin-resistant lines; while, a 20 kb fragment was detected in non-transformed plants (Fig. 2d) using a DIG-labeled fragment of the *trnN* gene. The presence of a 1.8 kb hybridizing band in transformed lines when DNA was digested with *SacI* using a DIG-labeled DPT probe further confirmed the integration of the DPT transgene (Fig. 2e). These results verified that the transgene was inserted within the intergenic region between *trnN* and *trnR* genes and confirmed homoplasmy in both B3 and E1 lines.

**Fig. 2** Southern blot analysis of transplastomic T1 lines. Maps of wild-type (a) and transplastomic (b) genomes showing the positions of *Hind*III and *Xba*I sites; broken lines indicate the expected size fragments. Southern blot analysis confirmed transgene integration in the plastid genome. DNA was digested by either *Hind*III (c) or *Xba*I (d), and blots were hybridized to a probe targeting a fragment of the *trnN* gene. When DNA was digested with *Sac*I (e), the DPT probe hybridized to a 1.8 kb fragment in the transplastomic plants (1–2 correspond to lines B3 and E1); *Wt* wild-type plant



### Western blot and ELISA analysis

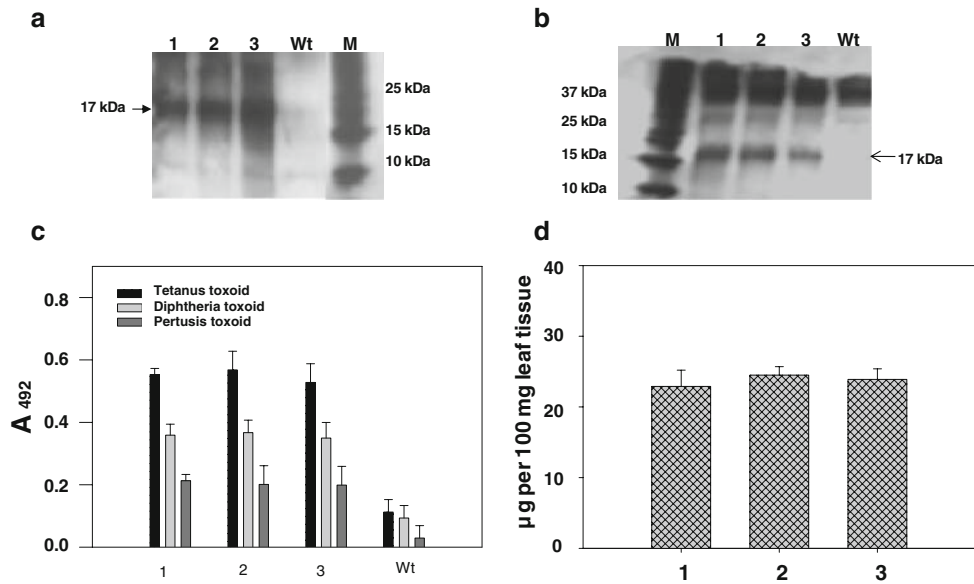
The DPT protein was detected by specific antibodies against DPT toxoids using Western blotting and ELISA assays for TSP extracted from three plants of T1 transplastomic line E1, non-transformed plants, and positive controls (toxoids). A single band of the expected size, 17 KDa, was detected in T1 plants of line E1 using polyclonal antibodies against either tetanus toxoid (Fig. 3a) or anti-diphtheria toxoid (Fig. 3b).

Chloroplast-produced DPT protein showed significant high readings for all three ELISA assays using polyclonal antibodies against the three DPT toxoids. Anti-tetanus toxin assays showed the highest signal, and followed by the anti-diphtheria assay (Fig. 3c). Although signals were weak, significant readings were detected for the anti-*Bordetella pertussis* toxin assay (Fig. 3c). It is likely that the pertussis moiety was either not properly displayed or not as well-displayed as those for the other two toxins. All these findings suggested that native epitopes of the three bacterial components present in the DPT polypeptide were conserved, and were properly displayed. Based on the ELISA assays, protein quantification was carried out by comparing absorbance readings of plant samples with known quantities of the tetanus toxoid. The linear standard curve was used to estimate the amount of the DPT protein present in the transgenic lines. The accumulation of the recombinant DPT protein in three plants derived from transplastomic line E1 was

up to 24.5  $\mu$ g per 100 mg of leaf tissue, and approximately accounting for 0.8% of TSP (Fig. 3d).

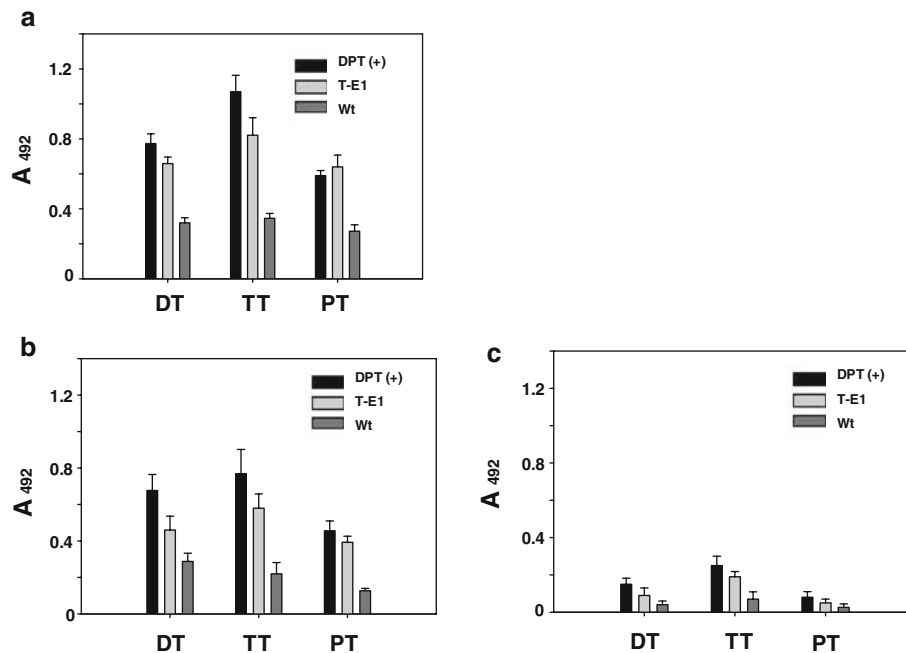
### Serum and intestinal antibody responses in immunized mice

To evaluate immune responses of the recombinant DPT protein produced, we proceeded to feed mice with transplastomic tobacco leaves. Mice were inoculated orally with 50 mg of freeze-dried leaves, collected from the transplastomic E1 line (T-E1), in three doses. Mice fed transplastomic tobacco expressing DPT elicited significant serum IgG antibody responses to tetanus, diphtheria, and pertussis moieties, and similar to those elicited by the positive DPT(+) group (Fig. 4a). While, mice fed wild-type tobacco (Wt) had the lowest IgG responses ( $P < 0.05$ ) (Fig. 4a). Significant specific IgA antibody levels were detected in intestinal tissues (Fig. 4b), but no significant IgG antibody responses were detected (Fig. 4c). Similar high levels of specific IgA responses were induced in mice immunized with E1 tobacco leaves. Specific serum anti-DPT IgG1 (Fig. 5a) and IgG2a (Fig. 5b) antibodies were elicited in mice immunized with the tobacco-derived DPT. Overall, a higher IgG1 than IgG2a antibody response was detected. This suggested that a humoral immune response, rather than a cellular-mediated response, was predominantly elicited.



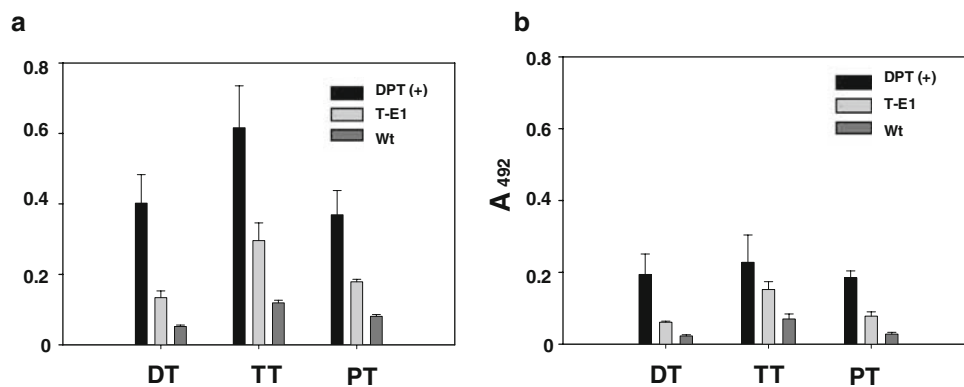
**Fig. 3** Western blot and ELISA analysis. **a** Anti-tetanus toxin or anti-diphtheria toxin **b** Western blot analysis using 20 µg of TSP from three plants of transplastomic tobacco line E1 (lanes 1–3) and non-transformed wild-type plants (Wt). **c** ELISA assay: 50 ng of total soluble protein (TSP) from these plants was analyzed using an anti-tetanus toxin polyclonal antibody, an anti-diphtheria toxin polyclonal antibody, and an anti-*Bordetella pertusis* toxin. Values correspond to

means of three replications along with standard deviations. **d** Levels of DPT protein in leaves of these plants. The amount of DPT was determined using ELISA by comparing the plant sample with a standard curve generated using known amounts (0.5, 1.5, 2.5, and 5 ng) of the tetanus toxoid. Levels of the DPT protein are expressed in µg per 100 mg leaf tissue



**Fig. 4** Antibody responses in serum and intestinal fluids. Three weekly doses of transplastomic tobacco plant tissues (50 mg) as well as untransformed wild-type tobacco were administered to male BALB/c mice via the oral route. Individual samples were run in duplicate. Antibody levels were determined by ELISA in serum samples diluted 1:10 or intestinal fluids diluted 1:2 from groups: DPT (+) (immunized with

DPT toxoids), T-E1 (transplastomic tobacco E1 line), and Wt (wild-type tobacco). DPT-specific serum IgG **a**), mucosal IgA **b**), and mucosal IgG **c**). Mean A492 values ± SD from each experimental group (n = 5 mice) are shown. TT tetanus toxoid, DT diphtheria toxoid, PT pertussis toxoid



**Fig. 5** IgG subclasses of anti-DPT antibodies from serum. IgG1 (a) and IgG2a (b) antibody levels were determined by ELISA in serum diluted 1:10 from the following BALB/c mice: DPT (+) (immunized with DPT toxoids), T-E1 (transplastomic tobacco E1 line), and Wt

(wild-type tobacco). Individual samples were run in duplicate. Mean  $A_{492}$  values  $\pm$  SD from each experimental group ( $n = 5$  mice) are shown. *TT* tetanus toxoid, *DT* diphtheria toxoid, *PT* pertussis toxoid

Altogether, the above findings indicated that oral delivery of tobacco-derived DPT was immunogenic in test animals.

## Discussion

The diphtheria, pertussis, and tetanus (DPT) vaccine is widely used for vaccination of infants and children worldwide. Although its efficacy is well documented, it is expensive due to the production process which involves both scale-up production and purification of recombinant proteins from three different bacteria. In order to reduce side effects, a few attempts have been made to produce a multi-component recombinant DPT vaccine. Boucher et al. (1994) engineered a fusion protein comprising a fragment of the tetanus toxin and the C180 peptide of the S1 subunit of the pertussis toxin (Barbieri et al. 1992). The resulting chimeric protein displayed the protective epitopes of both components. Recently, Aminian et al. (2007) reported on the expression of a fusion protein comprised of the immunoprotective S1 fragment of the pertussis toxin, the full-length nontoxic diphtheria toxin, and the C fragment of the tetanus toxin. The resulting polypeptide appeared to conserve the native epitopes. However, for all these approaches, bacterial fermentation and purification steps were required to purify the recombinant protein, thereby contributing to the high cost of production.

To date, several important human diseases have been targeted for plant-based vaccine development that will contribute to a safe as well as inexpensive vaccine production system (Korban et al. 2002). Recently, a novel polypeptide containing the DPT immunoprotective exotoxin epitopes has been designed and used to demonstrate expression of this fusion protein in transgenic tomato plants (Soria-Guerra et al. 2007). The tomato-derived DPT polypeptide

was recognized by antibodies directed against diphtheria, pertussis, and tetanus toxins; however, expression levels in tomato lines were low,  $\approx 0.01\%$  TSP (Soria-Guerra et al. 2007).

In this study, attempts were made to enhance the expression of the DPT polypeptide in tobacco plants via plastid transformation. A synthetic gene encoding for the DPT polypeptide was designed in order to optimize its expression in tobacco chloroplasts. Following microprojectile bombardment with the *DPT* gene construct and after several cycles of selection, several putative transformants were obtained. PCR analysis of four T0 plants demonstrated presence of the *DPT* transgene. Moreover, presence of the transgene, specific site integration, and homoplasmy were confirmed in the T1 progeny. Western blots and ELISA assays confirmed that the tobacco-derived DPT protein was recognized by specific antibodies against each of diphtheria, pertussis, and tetanus toxins. This also suggested that the three components present in the DPT polypeptide properly displayed the native epitopes. Protein quantitation using ELISA analysis revealed a DPT protein content of 0.8% of TSP. This DPT level was approximately 110-fold higher than that detected previously in our transgenic tomato plants derived via nuclear transformation (Soria-Guerra et al. 2007).

Using transplastomic technologies, Herz et al. (2005) indicated that expression levels of up to 6–10% of TSP could be obtained. In another report, expression of the tetanus fragment C toxin in chloroplast transformed plants reached 10–25% of TSP content (Tregoning et al. 2005). In this study, the *DPT* transgene was expressed under the control of the *Prm* promoter and the 5'-UTR T7G10, reported to mediate high expression in plastids (Kuroda and Maliga 2001a). The relatively low DPT content obtained in transplastomic tobacco plants in this study might be attributed to several factors. Among these, it is likely that the sequence



immediately downstream of the start codon, which has been associated with translation efficiency, might contribute to low levels of expression (Kuroda and Maliga 2001b). Several reports indicated that the amount of recombinant proteins expressed in chloroplasts was lower than 1% of TSP (Maliga 2004). For example, Lee et al. (2006) reported levels of 0.002–0.004% of TSP of the viral capsid protein antigen against Epstein–Barr virus expressed in tobacco plastids. They suggested that this low level of protein accumulation was likely due to post-transcriptional events and protein stability. Herz et al. (2005) reported that expression levels in plastids were very much dependent on the vector, promoter, and translational control element(s), among others. Therefore, these various factors might have contributed to the low levels of expression observed in this study.

Mice feeding studies were conducted using freeze-dried tobacco leaves of transplastomic line E1, expressing DPT. Results suggested that the recombinant DPT protein induced specific systemic and mucosal antibody responses in vaccinated mice. To determine whether effective protective immunity was induced, it will be necessary to assess animal responses and survival to lethal challenge with each of the exotoxins. Moreover, future research would involve targeting the transfer of the *DPT* transgene into edible crops such as lettuce or carrot that have been recently successfully transformed via plastid transformation (Kanamoto et al. 2006; Kumar et al. 2004).

In conclusion, we have successfully expressed the multi-epitope DPT polypeptide in transplastomic tobacco plants at levels higher than those reported in transgenic tomato plants. This further demonstrated successful transformation of plastids with a fusion protein of complex oligomeric structures that remain functional and retain their antigenicities. Moreover, this candidate plant-based vaccine was immunogenic via oral route delivery in mice. This model system would allow for the development of new experimental DPT subunit vaccines having several advantages, including safe and effective production and delivery, reduced side effects, and low cost of production.

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