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Abstract

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Keywords

plastid transformation, edible vaccine, pharmaceutical protein, oligomer assembly, genetically modified crops

Disciplines

Dentistry

Comments

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Expression of the Native Cholera Toxin B Subunit Gene and Assembly as Functional Oligomers in Transgenic Tobacco Chloroplasts

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Abstract

The B subunits of enterotoxigenic *Escherichia coli* (LTB) and cholera toxin of *Vibrio cholerae* (CTB) are candidate vaccine antigens. Integration of an unmodified CTB-coding sequence into chloroplast genomes (up to 10,000 copies per cell), resulted in the accumulation of up to 4.1% of total soluble tobacco leaf protein as functional oligomers (410-fold higher expression levels than that of the unmodified LTB gene expressed *via* the nuclear genome). However, expresssion levels reported are an underestimation of actual accumulation of CTB in transgenic chloroplasts, due to aggregation of the oligomeric forms in unboiled samples similar to the aggregation observed for purified bacterial antigen. PCR and Southern blot analyses confirmed stable integration of the *CTB* gene into the chloroplast genome. Western blot analysis showed that the chloroplast-synthesized CTB assembled into oligomers and were antigenically identical with purified native CTB. Also, binding assays confirmed that chloroplast- synthesized CTB binds to the intestinal membrane GM1-ganglioside receptor, indicating correct folding and disulfide bond formation of CTB pentamers within transgenic chloroplasts. In contrast to stunted nuclear transgenic plants, chloroplast transgenic plants were morphologically indistinguishable from untransformed plants, when CTB was constitutively expressed in chloroplasts. Introduced genes were inherited stably in subsequent generations, as confirmed by PCR and Southern blot analyses. Increased production of an efficient transmucosal carrier molecule and delivery system, like CTB, in transgenic chloroplasts makes plant-based oral vaccines and fusion proteins with CTB needing oral administration commercially feasible. Successful expression of foreign genes in transgenic chromoplasts and availability of marker-free chloroplast transformation techniques augurs well for development of vaccines in edible parts of transgenic plants. Furthermore, since the quaternary structure of many proteins is essential for their function, this investigation demonstrates the potential for other foreign multimeric proteins to be properly expressed and assembled in transgenic chloroplasts.

Keywords

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Introduction

There is currently much enthusiasm for the potential of genetically engineered plants to help control human and animal diseases, through production of edible vaccines.[1,2] Plants expressing bacterial and viral antigens as nuclear transgenes are capable of triggering immune responses when the transgenic tissues are administered orally.[3–6] Indeed, it has been suggested that plant cells containing an oral vaccine may actually potentiate vaccine activity by protecting against premature digestion of the antigen.[7] Despite this promise, transgenes expressed *via* the nucleus often yield insufficient antigen levels, reported as total soluble protein (TSP) or fresh weight (FW): B subunits of enterotoxigenic *Escherichia coli* (0.01% TSP),[3] hepatitis B virus envelope surface protein (0.01% TSP)[8,9] (0.01% FW),[6,10] human cytomegalovirus glycoprotein B (0.02% TSP),[11] and transmissible gastroenteritis coronavirus glycoprotein S (0.06% TSP).[12] Therefore, one ever-present mission is to increase the level of transgene expression within transgenic plants.[7]

Cholera toxin B subunit (CTB) is a candidate oral subunit vaccine for cholera, a disease that causes acute watery diarrhea by colonizing the small intestine and producing the enterotoxin, cholera toxin (CT). Cholera toxin is a hexameric AB_5 protein consisting of one toxic 27 kDa A subunit having ADP ribosyl transferase activity and a non-toxic pentamer of 11.6 kDa B subunits that binds to the A subunit and facilitates its entry into the intestinal epithelial cells. When administered orally, CTB is a potent mucosal immunogen;[13] this is believed to be a result of CTB binding to eukaryotic cell surfaces *via* GM1 ganglioside receptors present on the intestinal epithelial surface, eliciting a mucosal immune response to pathogens and enhancing the immune response when coupled chemically to other antigens.[14,15]

The B subunits of enterotoxigenic *E. coli* (LTB) and cholera toxin of *Vibrio cholerae* genes have been expressed at different levels *via* the plant nucleus. When the native LTB gene was expressed *via* the tobacco nuclear genome, LTB accumulated at levels less than 0.01% of the total soluble leaf protein.[3] To improve LTB expression, a synthetic gene was created that contained plant-preferred codons and eliminated potential mRNA processing signals and destabilizing motifs found in the native gene.[16] Using the native *CTB* gene for comparison, the synthetic gene increased antigen accumulation in leaves and tubers by threefold to 14-fold.[7,16] However, extensive codon modification of genes is laborious and expensive. One of the consequences of these constitutively expressed high LTB levels was the stunted growth of transgenic plants that was eventually overcome by tissue-specific expression in potato tubers.[16] By altering the native *CTB* gene to code for a C-terminal SEKDEL sequence, which targets expression to the endoplasmic reticulum, CTB expression of up to 0.3% TSP was achieved in auxin-induced potato tissues *via* the nuclear genome.[17]

Increased expression levels of several proteins have been attained by expressing foreign proteins in chloroplasts of higher plants.[18 – 20] Recently, human somatotropin (7% TSP)[21] and antimicrobial peptides (21% TSP)[22] have been expressed in transgenic chloroplasts. The accumulation levels of the *Bt Cry2Aa2* operon in tobacco chloroplasts were as high as 46.1% of the total soluble plant protein.[23] Besides the ability to express polycistrons, another advantage of chloroplast transformation is the lack of recombinant protein expression in pollen of chloroplast transgenic plants.[23] Absence of chloroplast DNA in the pollen of most crops reduces pollen-mediated outcross of transgenes.[19–26] Also, stable incorporation of the *CTB* gene into spacer regions between functional genes of the chloroplast genome by homologous recombination eliminates the “position effect” frequently observed in nuclear transgenic plants. Lack of gene silencing in chloroplasts should allow uniform expression levels in different transgenic lines. Integration of the transgene into chloroplast genomes should result in a high level of *CTB* gene expression,

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since each plant cell contains up to 10,000 copies of the plastid genome.[27] Similar to the endoplasmic reticulum, the production of CTB in chloroplasts allows formation of disulfide bridges,[21,28,29] which are necessary for the correct folding and assembly of the CTB pentamer.30

Here, we report that integration of the *CTB* gene into the tobacco chloroplast genome results in high levels of CTB accumulation and assembly of functional oligomers in chloroplasts. This eliminates the need to modify the *CTB* gene for optimal expression in plants. Furthermore, since the quaternary structure of many proteins is essential for their function, this investigation demonstrates the potential for other foreign multimeric proteins to be expressed and assembled properly in the chloroplast.

Results and Discussion

Vector construction and *E. coli* expression

The pLD-LH-CTB (Figure 1(a)) vector integrates the genes of interest into the inverted repeat regions through homologous recombination events between the *trnI* and *trnA* chloroplast border sequences of the transformation vector and the corresponding homologous sequences of the chloroplast genome. Unique advantages of integration of foreign genes at this site include accomplishment of homoplasmy in the first round of selection due to the presence of a complete chloroplast origin of replication within the flanking sequence[31,32] and doubling the copy number of foreign genes per cell. The chimeric aminoglycoside 3′ adenylyltransferase (*aadA*) gene, conferring resistance to spectinomycin and streptomycin, and the *CTB* gene downstream of it are driven by the constitutive promoter of the rRNA operon (Prrn), each with individual ribosome-binding sites (GGAGG), and the foreign transcripts are stabilized by the psbA3′ untranslated region. Since the protein synthetic machinery of chloroplasts is similar to that of *E. coli*,[33] CTB expression of the pLD-LH-CTB vector was initially tested in *E. coli*. Western blot analysis of sonicated *E. coli* cell extracts showed the presence of 11 kDa CTB monomers, similar to commercially available CTB (Figure 2(a)), when blots were probed with rabbit anti-cholera serum. Oligomeric expression of CTB was not observed in *E. coli*, as expected, due to the absence of a leader peptide sequence present in the native *CTB* gene that normally directs the CTB monomer into the periplasmic space allowing for concentration and oligomeric assembly. Inability of *E. coli* to form disulfide bridges in the cytosol is a major limitation in large-scale production of this antigen.

Determination of chloroplast integration and homoplasmy

Five bombardments resulted in 68 independent transformation events. Each transgenic clone was subjected to a second round of spectinomycin selection and putative chloroplast transformants were confirmed by PCR screening. Primers were designed to determine whether the integration of foreign genes had occurred in the chloroplast genome at the directed site by homologous recombination. The strategy employed was to land one primer (3P) on the native chloroplast genome adjacent to the point of integration and the second primer (3M) on the *aadA* gene (Figure 1(a)). The presence of the 1.65 kb PCR product in nine out of the ten putative transgenics screened, confirmed the site-specific integration of the gene cassette into the chloroplast genome (Figure 1(b)). The putative transgenic line 4 (lane 6) is a spontaneous mutant. Such mutations occur in the 16 S rRNA gene and confer high levels of spectinomycin resistance.[34] Nuclear transformants resulting from integration of the *aadA* gene downstream of functional promoters do not confer high levels of spectinomycin resistance and are therefore eliminated in the selection process. To demonstrate the presence of the *CTB* gene in the transgenic lines 1–3 and 5–10, an additional PCR was performed using a primer (5P) that lands upstream of the *CTB* gene, on

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the *aadA* gene, and one (2M) that lands downstream of the *CTB* gene, on the *trnA* gene (Figure 1(c)). The presence of the 1.9 kb PCR product from all the transgenic lines analyzed verifies the expected integration of the *CTB* gene in tandem with the *aadA* gene.

Southern blot analysis of three PCR positive transgenic lines (T_0) was done to verify site-specific integration and to establish copy number. In the chloroplast genome, *Bgl*II sites flank the chloroplast border sequences 5′ of 16 S rRNA and 3′ of the *trnA* region (Figure 3(a)). Either a 6.17 kb or a 4.47 kb fragment from transformed or untransformed plants was obtained when total plant DNA was digested with *Bgl*II. The ^{32}P random primer-labeled 0.81 kb *trnI-trnA* probe fragment, hybridized with the untransformed control giving a 4.47 kb fragment as expected, while a 6.17 kb fragment was observed for the transgenic lines (Figure 3(b) and (c)), indicating that all plastid genomes had the gene cassette inserted between the *trnI* and *trnA* regions. The absence of a 4.47 kb fragment in transgenic lines indicates that homoplasmy has been achieved. Southern blot confirmed that plants transferred to pots were seen to have no adverse pleiotropic effects compared to untransformed plants (Figure 4(a)). Southern blot analysis of T_1 plants (Figure 3(c)) shows that all four transgenic lines analyzed maintained homoplasmy.

Immunoblot analysis of chloroplast-synthesized CTB

Anti-cholera toxin antibodies showed no significant cross-reaction with tobacco plant protein (Figure 2(b), lane 1). In unboiled samples, chloroplast-synthesized CTB appeared as oligomers (Figure 2(c), lane 4) similar to the unboiled, pentameric bacterial CTB, which appeared to have partially dissociated into tetramers, trimers and monomers upon storage at 4 °C over a period of several months (Figure 2(c), lane 7). The absence of monomers in transgenic plant extracts suggests that unassembled monomers are proteolytically degraded. While heat treatment (four minutes, boiling), prior to SDS-PAGE, of pentameric bacterial CTB gave CTB monomeric and multimeric forms (Figure 2(c), lane 6), chloroplast-synthesized CTB dissociated into dimers, trimers and multimers when subjected to similar heat treatment (Figure 2(c), lanes 3 and 5). This is not due to inadequate boiling or low concentrations of dithiothreitol or β-mercaptoethanol. Increasing these agents to reduce disulfide bridges or increasing duration of boiling did not alter polypeptide profiles significantly. However, the same conditions were adequate to dissociate bacterial pentamers (Figure 2(c), lane 6). These results differ from the heat-induced dissociation of potato plant nuclear-synthesized CTB oligomers into monomers.[17] A probable reason for this unusual stability could be a more stable conformation of chloroplast-synthesized CTB, which may be an added advantage in storage and administration of edible vaccines. Proteins larger than 55 kDa are aggregates of oligomers in plant extracts and purified bacterial antigen (Figure 2(c), lanes 4 and 7). Because of variations in the degree of aggregation, boiled samples did not show identical polypeptide profiles. Leaf homogenates from four different transgenic lines showed similar expression levels of CTB protein (Figure 2(b), lanes 3–6) due to lack of gene silencing or position effect that are usually observed in nuclear transgenic plants. This confirms that clonal variation of CTB expression is minimal among independent transgenic lines, as observed in ELISA quantification assays. Consistent expression levels of recombinant proteins in plants (as obtained for CTB in this research) may be essential for production of edible vaccines in plants.

ELISA quantification of CTB expression

Both transgenic T_0 lines tested yielded CTB protein levels ranging between 3.5% and 4.1% of the total soluble protein (40 μg of chloroplast-synthesized CTB protein in 1 mg of total soluble protein, Figure 5(a)). Aggregation of assembled pentamers (Figure 2(c), lane 4) in plant extracts similar to purified bacterial antigen (Figure 2(c), lane 7) should have resulted in underestimation of CTB in ELISA, where protein extracts were used directly without any

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treatment to dissociate aggregated CTB. This inference is supported by observation of very high levels of bacterial genes (up to 46% TSP) with similar AT content in transgenic chloroplasts.[22,23] Also, estimation of CTB expression levels from different stages of leaves (young, mature and old) determined that mature leaves have the highest levels of CTB protein expression. The top three or four leaves were designated as young; large, well-developed leaves collected from the middle of the plant were considered as mature; leaves that were generally bleached or undergoing scenescence, from the bottom of the plant were designated as old. This observation is in accordance with the results obtained when similar experiments were performed with the *Bt Cry2aA2* gene, expressed as a single gene.[23] However, when this gene was expressed with a putative chaperonin gene as the *Cry2aA2* operon, high expression levels in older leaves were observed, probably due to the formation of stable crystalline structure.[23]

GM1-ganglioside ELISA binding assays

Both chloroplast-synthesized and bacterial CTB demonstrated a strong affinity for GM1-ganglioside (Figure 5(b)), indicating that chloroplast-synthesized CTB conserved the antigenic sites necessary for binding of the CTB pentamer to the pentasaccharide GM1. The GM1 binding ability also suggests proper folding of CTB molecules resulting in the functional pentameric structure. Since oxidation of cysteine residues in the B subunits is a prerequisite for *in vivo* formation of CTB pentamers,[30] proper folding is a further confirmation of the ability of chloroplasts to form disulfide bonds. The ratio of CTB expression in transgenic lines 3 (3.5 %) and 7 (4.1 %) in the CTB quantitative assay (Figure 5(a)) is proportional to the GM1 binding measured absorbance ratio of transgenic lines 3 (A = 1.2) and 7 (A = 1.4; Figure 5(b)). This observation indicates that the binding of CTB to GM1 is proportional to the level of synthesis in two different transgenic lines.

High levels of constitutive expression of CTB in transgenic tobacco did not affect growth rates, flowering or seed setting, unlike previous reports for the synthetic LTB gene, constitutively expressed *via* the nuclear genome.[16] Therefore, there is no need to regulate CTB expression in transgenic chloroplasts or express it in specific tissues to overcome pleiotropic effects. Transformed plant seedlings were green, while untransformed seedlings lacking the *aadA* gene were bleached white (Figure 4(b)) when germinated on spectinomycin-containing medium.

The potential use of the chloroplast technology developed in this study is threefold. It can be used for (1) large-scale production of purified CTB, (2) as an edible vaccine if expressed in an edible plant or (3) as a fusion transmucosal carrier peptide, to either enhance mucosal immunity or induce oral tolerance to the products of these peptides.[14] Large-scale production of purified CTB in bacteria involves the use of expensive fermentation techniques and stringent purification protocols,[35] making this a prohibitively expensive technology for developing countries. The cost of producing 1 kg of recombinant protein in transgenic crops has been estimated to be 50 times lower than the cost of producing the same amount by *E. coli* fermentation.[36] If 5% of the alfalfa soluble protein were the target protein, one could harvest about 15 kg of the transgenic-expressed protein/acre per year.[37] While existing expression levels of CTB *via* the chloroplast genome are adequate for commercial exploitation, levels could be increased further (up to 50% total soluble protein) by insertion of a putative chaperonin,[23] which may also aid in the subsequent purification of recombinant CTB due to crystallization. If used as an edible vaccine, a selection scheme eliminating the use of antibiotic-resistance genes should be developed.[38] One such scheme uses the betaine aldehyde dehydogenase (BADH) gene, which converts toxic betaine aldehyde to non-toxic glycine betaine, an osmoprotectant.[39] Other strategies have been developed to eliminate antibiotic-resistance genes from transgenic plants.[40] Development of edible vaccines in transgenic leaves are ideal for animal vaccines. Future development of

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edible vaccines for human consumption would be more appealing if the vaccines were expressed in edible parts of transgenic plants. Expression of a transgene in chromoplasts has been demonstrated,[41,42] and the efficiency of translation of foreign genes in chromoplasts is similar to that of chloroplasts.[43] Therefore, we anticipate that elevated edible vaccine expression in chromoplasts of transgenic fruits is both feasible and forthcoming.

Materials and Methods

Construction of the chloroplast expression vector pLD-LH-CTB

Primers were designed to introduce an RBS site (GGAGG) five bases upstream of the start codon of the coding sequence of the native *CTB* gene, and the leader peptide sequence was deleted. The PCR product was cloned into the multiple cloning site of the pCR2.1 vector (Invitrogen) and subsequently cloned into the chloroplast expression vector pLD-CtV2 after verification of the correct DNA sequence. *E. coli* (transformed by standard $CaCl_2$ transformation procedure) and untransformed *E. coli* cultures (24 and 48 hours) were centrifuged (ten minutes at 10,000 *g*), the pellets were washed twice with 200 mM Tris-HCl, resuspended in 500 μl of extraction buffer (200 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA, 2 mM PMSF) and sonicated. Double-strength SDS sample buffer was added to 100 μl of transformed and untransformed sonicates (containing 50– 100 μg of crude protein extract as determined by Bradford protein assay (Bio Rad)) and purified CTB (100 ng, Sigma). These samples were loaded onto an SDS-15%/PAGE gel and electrophoresed at 200 V for 45 minutes. The separated protein was transferred to a nitrocellulose membrane by electroblotting at 70 V for 90 minutes.

Immunoblot analysis of CTB production in *E. coli*

Blocking by incubation of the membrane in 25 ml of 5% (w/v) non-fat dry milk in TBS buffer (20 mM Tris base (Fisher), 500 mM NaCl (Sigma), pH 7.5) for two hours on a rotary shaker was followed by washing in TBS buffer. Incubation for one hour in a 1:5000 dilution of rabbit anti-cholera antiserum (Sigma) in TBST (TBS buffer with 0.05% (v/v) Tween-20) containing 1% non-fat dry milk and washing thrice in TBST buffer was followed by incubation in a 1:10,000 dilution of alkaline phosphatase conjugated mouse anti-rabbit IgG (Sigma) in TBST. The membrane was washed thrice in TBST, once with TBS followed by incubation in alkaline phosphatase color development reagents, BCIP/NBT in AP color development buffer (Bio Rad) for an hour.

Bombardment and regeneration of chloroplast transgenic plants

Microprojectiles coated with plasmid DNA (pLD-LH-CTB) were bombarded into *Nicotiana tabacum* var. *Petit havana* leaves using the biolistic device PDS1000/He (Bio Rad), as described by Daniell.[44] Following incubation at 24 °C in the dark for two days, the leaves were cut into small (~5 mm × 5 mm) pieces and placed abaxial side up (five pieces/plate) on selection medium (RMOP containing 500 mg/l spectinomycin dihydrochloride). Spectinomycin-resistant shoots obtained after about six weeks were cut into small pieces (~2 mm × 2 mm) and placed onto plates containing the same selection medium.

PCR analysis

Total plant DNA from putative transgenic and untransformed plants was isolated using the DNeasy kit (Qiagen). PCR primers, AAAACCCGTCCTCAGTTCGGATTGC (3P), CCGCGTTGTTTCATCAAGCCTTACG (3M), CTGTAGAAGTCACCATTGTTGTGC (5P), and TGACTGCCCACCTGAGAGCGGACA (2M) were used to perform PCR on both putative transgenic and untransformed plant total DNA. After denaturation for five minutes at 94 °C, samples were carried through 30 cycles using the following temperature sequence:

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94 °C for one minute, 62 °C for 1.5 minutes and 72 °C for two minutes. PCR confirmed shoots, from the second culture cycle, were transferred to rooting medium (MSO medium containing 500 mg/l spectinomycin).

Southern blot analysis

Ten micrograms of plant DNA per sample (isolated using DNeasy kit) digested with *Bgl*II, were separated on a 0.7% (w/v) agarose gel and transferred to a nylon membrane. The chloroplast vector DNA digested with *Bgl*II and *Bam*HI generated a 0.8 kb probe homologous to the flanking sequences. Hybridization was performed using the Ready To Go protocol (Pharmacia). After Southern blot confirmation, plants were transferred to soil. Upon flowering, seeds were germinated on spectinomycin dihydrochloride selection medium. The T_1 seedlings were grown in bottles containing MSO with spectinomycin (500 mg/l) for two weeks, and plants were transferred to pots.

Western blot analysis of plant protein

Transformed and untransformed leaves (100 mg) were ground in liquid nitrogen and resuspended in 500 μl of extraction buffer (200 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA, 2 mM PMSF). Leaf extracts (100–120 μg as determined by Lowry assay), boiled (four minutes) or unboiled, in sample buffer (Bio Rad) were electrophoresed in a 12% (w/v) polyacrylamide gel for five hours at 200 V. The separated proteins were transferred to a nitrocellulose membrane by electroblotting at 85 V for one hour. The immunoblot detection procedure was similar to that done for *E. coli* blots described above. For the chemiluminescent detection, the S. Tag™ P Lumiblot kit (Novagen) was used.

ELISA quantification of CTB

Leaves (transformed and untransformed plants, 100 mg) ground in liquid nitrogen and resuspended in bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) were bound to a 96 well polyvinyl chloride microtiter plate (Costar) overnight at 4 °C. Background was blocked with 1% (w/v) bovine serum albumin (BSA) in 0.01 M phosphate-buffered saline (PBS) for two hours at 37 °C, washed thrice with washing buffer, PBST (PBS containing 0.05% Tween 20) and rabbit anti-cholera serum diluted 1:8000 in 0.01 M PBST containing 0.5% BSA was added and incubated for two hours at 37 °C. The wells were washed and incubated with 1:50,000 mouse anti rabbit IgG-alkaline phosphatase conjugate in 0.01 M PBST containing 0.5% BSA for two hours at 37 °C. Plates were developed with Sigma Fast pNPP substrate (Sigma). The reaction was ended by addition of 3 M NaOH and plates were read at 405 nm. Comparison of the absorbance at 405 nm of a known amount of bacterial CTB-antibody complex (linear standard curve) and that of a known concentration of transformed plant total soluble protein was used to estimate CTB expression levels.

GM1-ganglioside binding assay

The microtiter plate was coated with monosialogan-glioside-GM1 (Sigma) (3.0 μg/ml in bicarbonate buffer) and incubated at 4 °C overnight. As a control, BSA (3.0 μg/ml in bicarbonate buffer) was coated in some wells. The wells were blocked with 1% BSA in PBS, washed thrice with PBST and incubated with soluble protein from transformed and untransformed plants, and bacterial CTB in PBS. Incubation of plates with primary and secondary antibody dilutions and detection was done similar to the CTB ELISA procedure described above.

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Abbreviations used

TSP	total soluble protein
FW	fresh weight
CTB	cholera toxin B subunit
LTB	B subunit of enterotoxigenic *E. coli*
MSO	plant growth medium
RMOP	plant shooting medium

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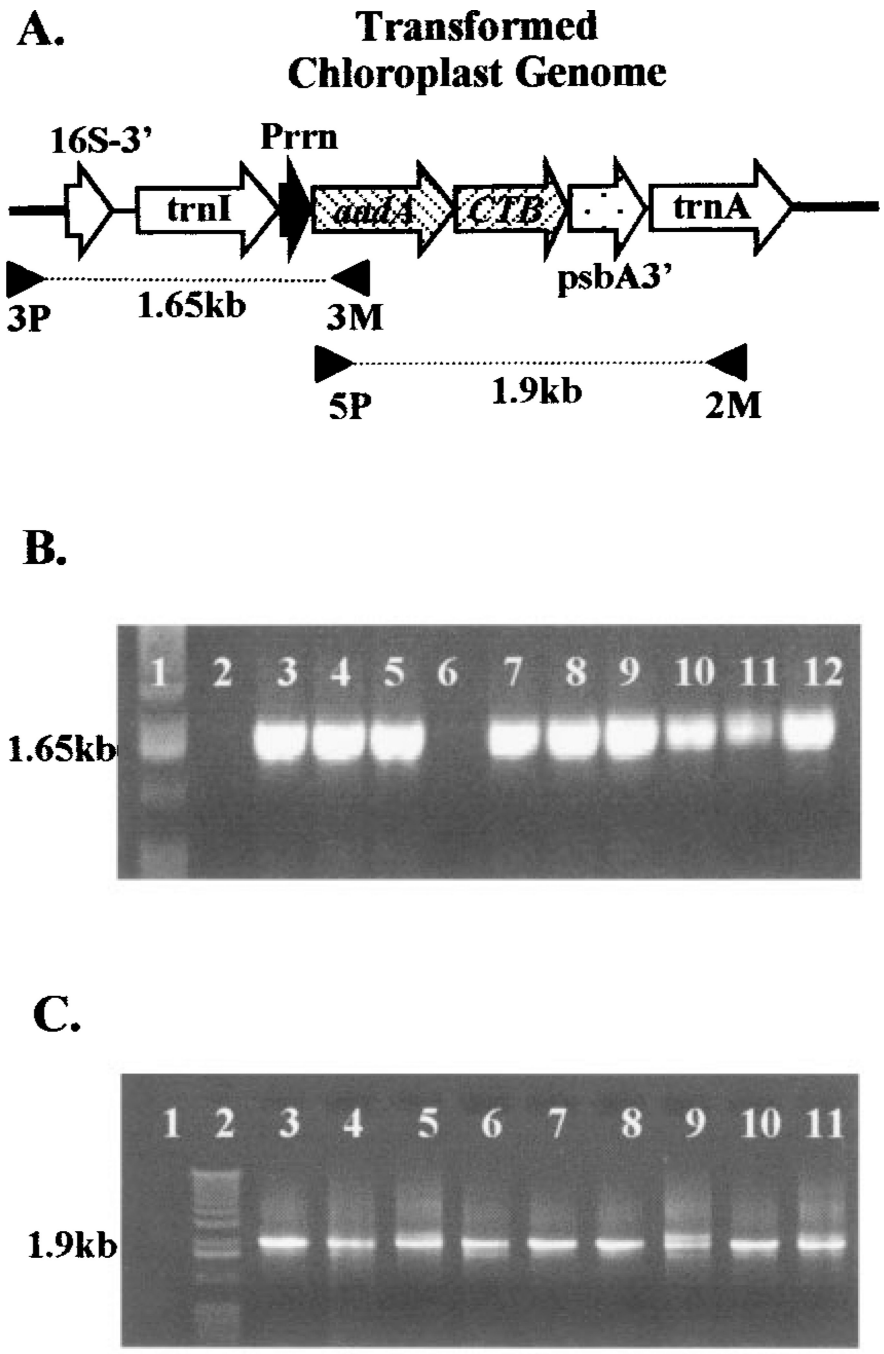

Figure 1.

Transformed chloroplast genome and PCR analysis of control and chloroplast transformants. (a) Chloroplast genome after homologous recombination with the pLD-LH-CTB vector. Flanking sequences involved in recombination are indicated in white. Primer 3P lands on the native chloroplast genome and 3M lands on the *aadA* gene, generating a 1.65 kb fragment. Primer 5P lands on the aadA gene and 2M lands on the trnA flanking sequence, generating a 1.9 kb fragment. (b) PCR analysis: primers 3P 3M generated PCR products using total plant DNA as template. Lane 1, molecular mass marker; lane 2, untransformed plant; lanes 3–12, PCR products with DNA template from independent transgenic lines 1–10. (c) PCR analysis: primers 5P 2M generated PCR products using total plant DNA as template. Lane 1,

untransformed plant; lane 2, molecular mass marker; lanes 3–5, PCR products with DNA template from independent transgenic lines 1–3 and 5–10, lanes 3–11.

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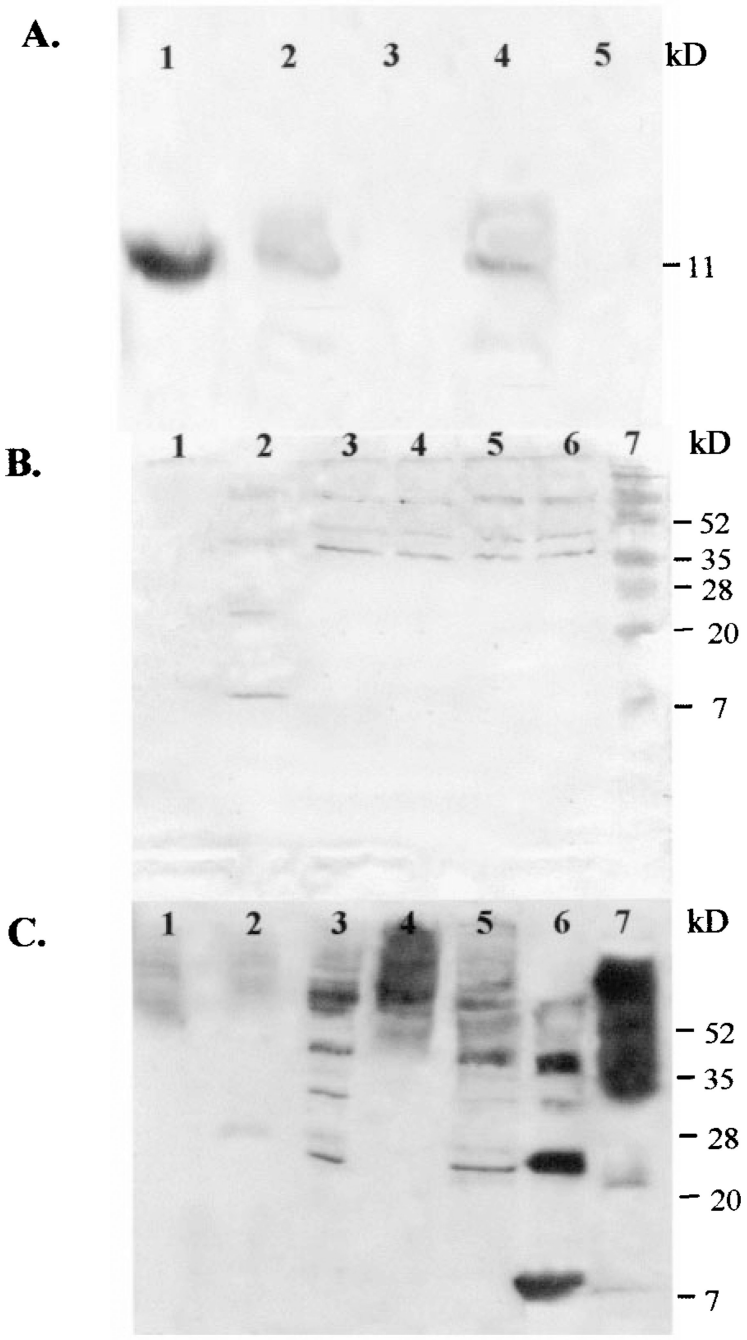

Figure 2.

Western blot analysis of CTB expression in *E. coli* and transgenic chloroplasts. Blots were detected using rabbit anti-cholera serum as the primary antibody and alkaline phosphatase labeled mouse anti-rabbit IgG as the secondary antibody. (a) *E. coli* protein analysis: purified bacterial CTB (100 ng), boiled (lane 1); unboiled 24 hours and 48 hours transformed (lanes 2 and 4) and untransformed (lanes 3 and 5) *E. coli* cell extracts. (b) Plant protein analysis: color development detection. Boiled, untransformed plant proteins (lane 1); boiled, purified CTB antigen (100 ng, lane 2); boiled, proteins from four different transgenic lines (lanes 3–6); molecular mass markers (lane 7). (c) Chemiluminescent detection: Plant proteins: untransformed, unboiled (lane 1); untransformed, boiled (lane 2); transgenic lines 3

and 7, boiled (lanes 3 and 5); transgenic line 3, unboiled (lane 4); purified CTB antigen (100 ng), boiled (lane 6); purified CTB antigen (100 ng), unboiled (lane 7).

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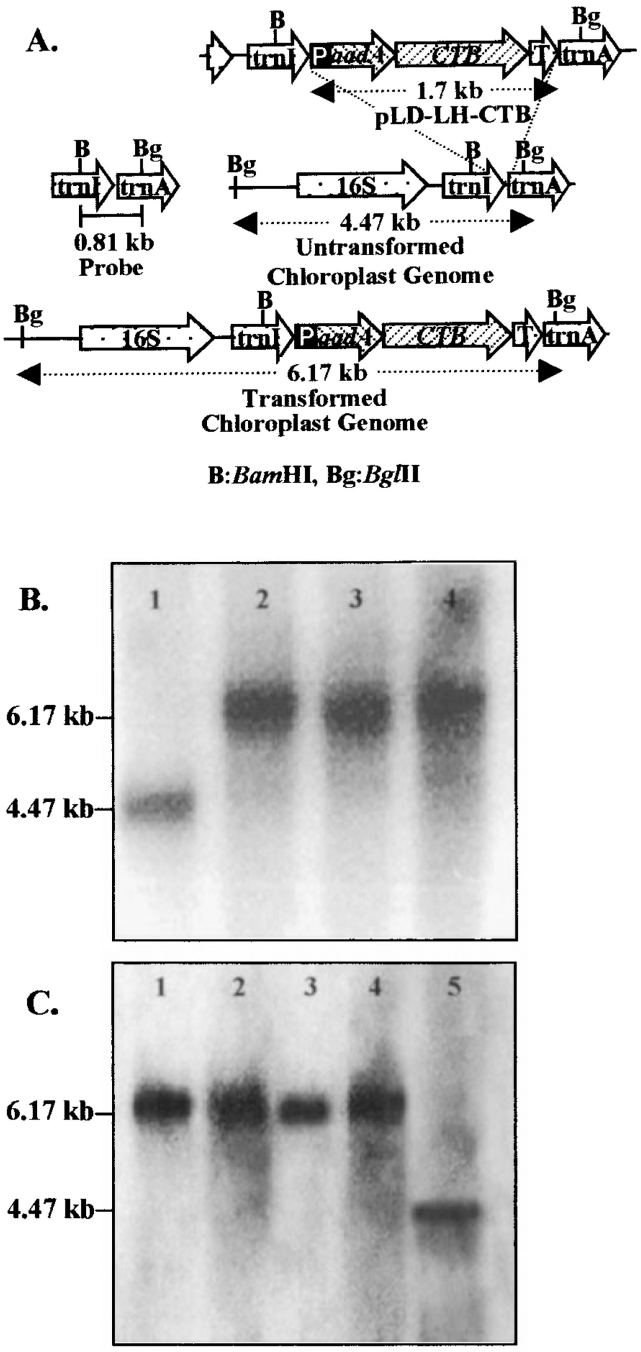

Figure 3.

Southern blot analysis of T_0 and T_1 transgenic plants. (a) A representation of untransformed and transformed chloroplast genomes. Plant DNA was digested with *Bgl*II and hybridized with the 0.81 kb probe, which contained the chloroplast flanking sequences used for homologous recombination. Untransformed control plant chloroplast genomes should generate a 4.47 kb fragment and transformed plant chloroplast genomes should generate a 6.17 kb fragment. Southern blot results of (b) T_0 lines untransformed Petit Havana (lane 1); transgenic plants (lanes 2–4); and (c) T_1 lines transgenic plants (lanes 1–4) and untransformed Petit Havana (lane 5). P is the 16 S rRNA promotor and T is the psbA 3′ UTR.

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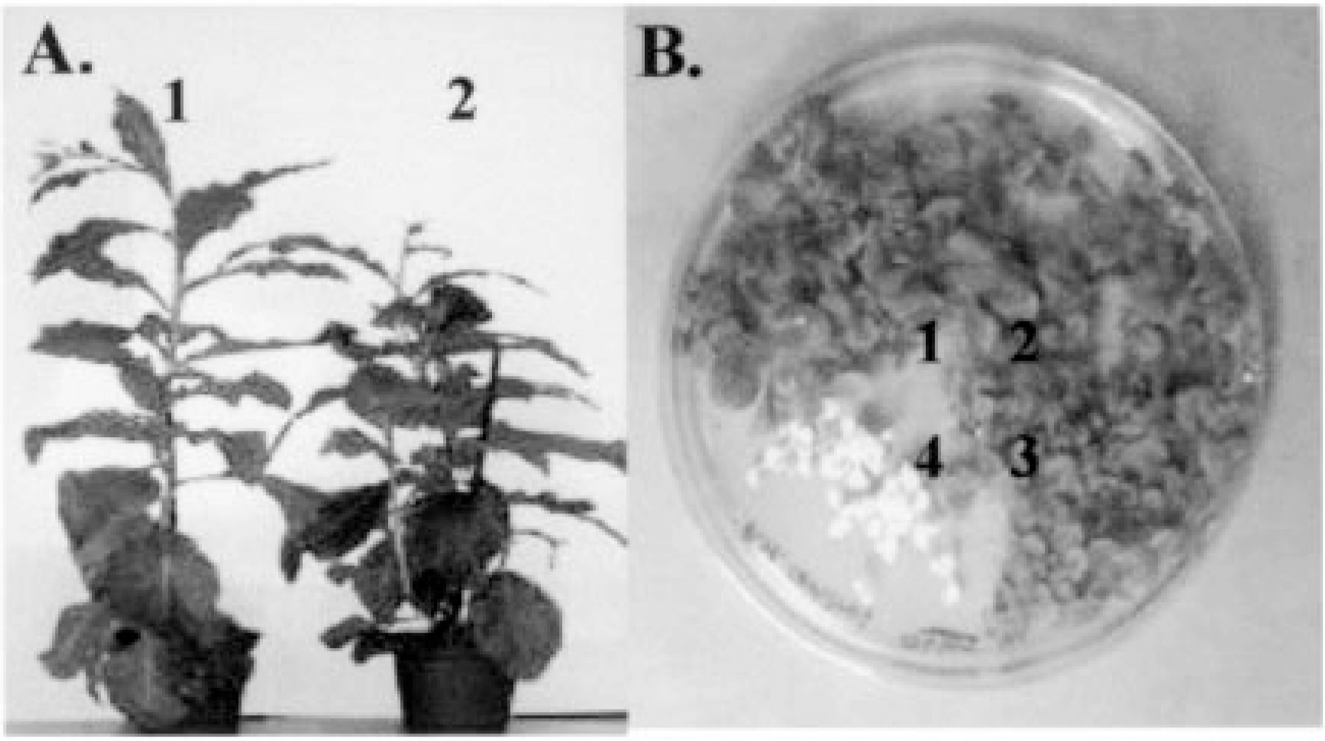

Figure 4.
(a) Plant phenotypes: 1, confirmed transgenic line 7; 2, untransformed Petit Havana. (b) Seedlings (ten day-old) of T_1 transgenic (1, 2 and 3) and untransformed plant (4) plated on 500 mg/l spectinomycin selection medium.

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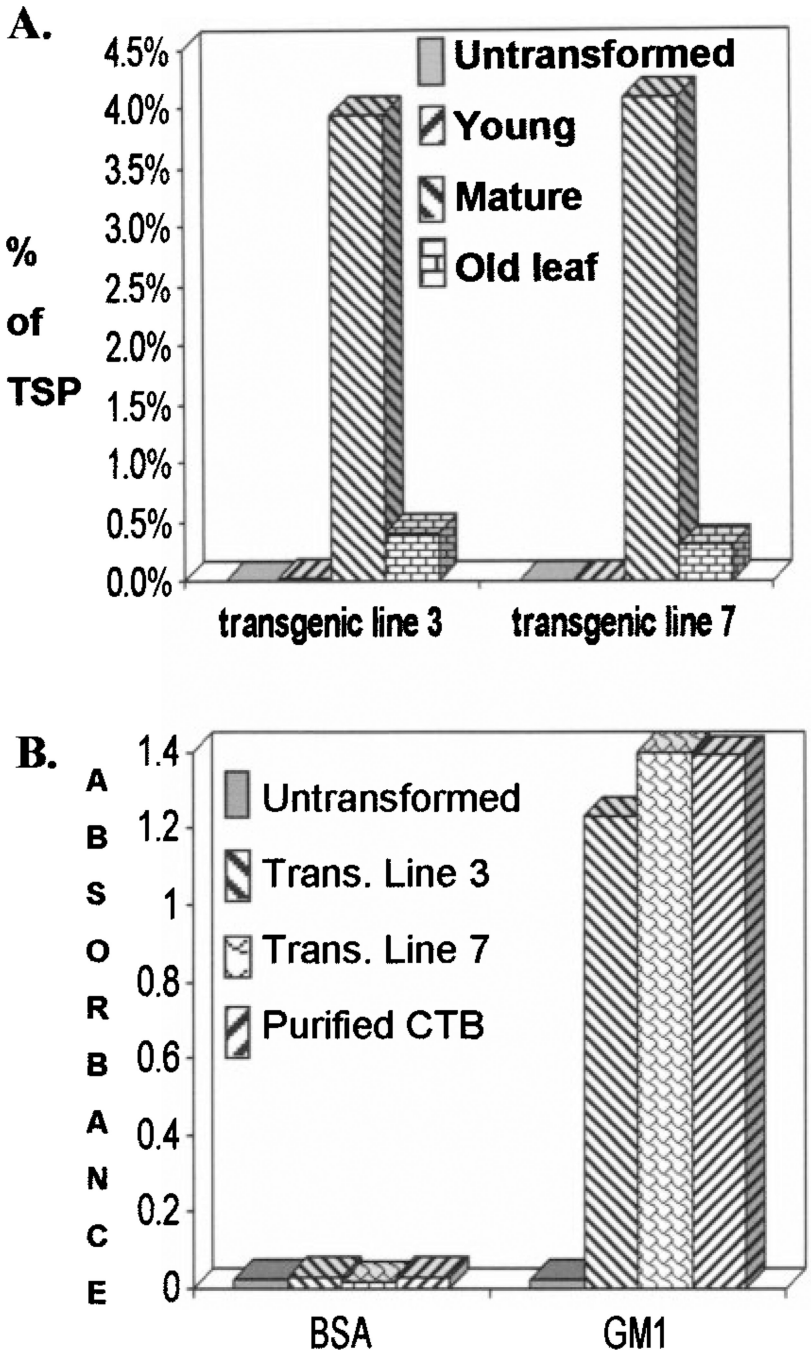

Figure 5.

(a) CTB ELISA quantification: absorbance of CTB-antibody complex in known concentrations of total soluble plant protein was compared to absorbance of known concentration of bacterial CTB-antibody complex and the amount of CTB was expressed as a percentage of the total soluble plant protein. Total soluble plant protein from young, mature and old leaves of transgenic lines 3 and 7 was quantified. (b) CTB-GM1-ganglioside binding ELISA assays. Plates, coated first with GM1-gangliosides and BSA, respectively, were plated with total soluble plant protein from transgenic lines 3 and 7, untransformed plant total soluble protein and 300 ng of purified bacterial CTB. The absorbance of the GM1-ganglioside-CTB-antibody complex in each case was measured.