Expression of protective antigens in transgenic chloroplasts (US)

Henry Daniell

University of Central Florida

Find similar works at: https://stars.library.ucf.edu/patents

University of Central Florida Libraries http://library.ucf.edu

This Patent is brought to you for free and open access by the Technology Transfer at STARS. It has been accepted for inclusion in UCF Patents by an authorized administrator of STARS. For more information, please contact STARS@ucf.edu.

Recommended Citation


https://stars.library.ucf.edu/patents/172
(12) United States Patent
Daniell

(45) Date of Patent: Apr. 8, 2008

(54) EXPRESSION OF PROTECTIVE ANTIGENS IN TRANSGENIC CHLOROPLASTS

(75) Inventor: Henry Daniell, Winter Park, FL (US)

(73) Assignee: University of Central Florida Research Foundation, Inc., Orlando, FL (US)


(11) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: 10/500,351

(22) PCT Filed: Dec. 26, 2002

(36) Provisional application No. 60/344,704, filed on Dec. 26, 2001, provisional application No. 60/393,428, filed on Jul. 3, 2002, provisional application No. 60/393,651, filed on Jul. 3, 2002, provisional application No. 60/400,816, filed on Aug. 2, 2002.

(63) Related U.S. Application Data

(51) Int. Cl. C12N 15/82 (2006.01)

(52) U.S. Cl. 435/320.1

(58) Field of Classification Search None

See application file for complete search history.

(56) References Cited

U.S. PATENT DOCUMENTS
5,484,719 A * 1/1996 Lam et al. ............... 800/292
5,914,123 A 6/1999 Amten et al.

FOREIGN PATENT DOCUMENTS
WO WO 99/10513 * 3/1999
WO WO 00/03022 A3 1/2000
WO WO 01/72959 A2 10/2001

OTHER PUBLICATIONS


(Continued)

Primary Examiner—Anne Kubelik
(44) Attorney, Agent, or Firm—Saliwanchik, Lloyd & Saliwanchik

ABSTRACT

Vectors and methods for plastid transformation of plants to produce protective antigens and vaccines for oral delivery are provided. The invention provides edible vaccines for conferring immunity to a mammal against Bacillus anthracis, as well as Yersinia pestis.

2 Claims, 21 Drawing Sheets
OTHER PUBLICATIONS


* cited by examiner
Native Chloroplast genome

FIG. 1A

FIG. 1B

Native Chloroplast genome

FIG. 1C

FIG. 1D
**FIG. 2A**

```
BgIII
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4 kb</td>
<td>16s</td>
</tr>
<tr>
<td></td>
<td>trnL</td>
</tr>
<tr>
<td></td>
<td>trnA</td>
</tr>
</tbody>
</table>
```

**FIG. 2B**

```
BgIII
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>5.2 kb</td>
<td>16s</td>
</tr>
<tr>
<td></td>
<td>trnL</td>
</tr>
<tr>
<td></td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>aadA</td>
</tr>
<tr>
<td></td>
<td>5' UTR</td>
</tr>
<tr>
<td></td>
<td>pag</td>
</tr>
<tr>
<td></td>
<td>T</td>
</tr>
<tr>
<td></td>
<td>trnA</td>
</tr>
</tbody>
</table>
```

**FIG. 2C**

```
BgIII
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>6.8 kb</td>
<td>16s</td>
</tr>
<tr>
<td></td>
<td>trnL</td>
</tr>
<tr>
<td></td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>aadA</td>
</tr>
<tr>
<td></td>
<td>ORF 1,2</td>
</tr>
<tr>
<td></td>
<td>5' UTR</td>
</tr>
<tr>
<td></td>
<td>pag</td>
</tr>
<tr>
<td></td>
<td>T</td>
</tr>
<tr>
<td></td>
<td>trnA</td>
</tr>
</tbody>
</table>
```

- 0.81 kb Flanking Sequence Probe
- 0.52 kb pag Probe
FIG. 4C

FIG. 5A
**FIG. 5B**

Viability (% of control) vs. Dilution of sample.

- Control Wild Type (extract stored 2 days)
- pLD-JW1 (extract stored 2 days)
- Control Wild Type (extract stored 7 days)
- pLD-JW1 (extract stored 7 days)
- PA 5 µg/ml
- Control Wild Type No LF (extract stored 2 days)
- Control pLD-JW1 No LF (extract stored 2 days)
- Control Wild Type No LF (extract stored 7 days)
- Control Wild Type No LF (extract stored 7 days)
1.65 Kb

FIG. 6A

FIG. 6B

pTOM-BADH2-G10-pag ~8.8 kb

FIG. 7
**FIG. 12**

**FIG. 13A**

**FIG. 13B**
FIG. 17A

pDD34-ZM-gfp-BADH

FIG. 17B

FIG. 18A

pDD33-ZM-aaA-BADH

FIG. 18B
FIG. 20B

16s r-RNA → trnl → Sm-GFP → BADH → trnA → 23S r-RNA

1.5 Kb

FIG. 21
FIG. 22A

16s r-RNA → trnI → aadA → BADH → trnA → 23S r-RNA

Prrm → psebA3' → rpsi63'
gene 10

pDD36-DC-aadA-BADH

FIG. 22B
FIG. 24
EXPRESSION OF PROTECTIVE ANTIGENS IN TRANSGENIC CHLOROPHYTALCI

BACKGROUND

Yersinia pestis is the causative agent of bubonic and pneumonic plague. Bacillus anthracis is the causative agent for the anthrax disease. The Centers for Disease Control and Prevention (hereinafter “CDC”) lists Y. pestis and B. anthracis as two of the six Category A biological agents that pose a risk to national security.

Yersinia pestis

The etiologic agent of plague is the Gram-negative bacterium Yersinia pestis. The natural route of transmission of Y. pestis from one animal host to another is either directly or via a flea vector. Plague is endemic in some regions of the world and outbreaks occasionally occur as a consequence of natural disasters. Y. pestis is also a concern as one of the microorganisms with potential for use against civilian or military populations as a biological warfare/biological terrorism agent. In such a situation, the pneumonic form of plague would be the most likely outcome. This form of plague is particularly devastating because of the rapidity of onset, the high mortality, and the rapid spread of the disease. Immunization against aerosolized plague presents a particular challenge for vaccine developers. There is currently no vaccine for plague.

Both live attenuated and killed plague vaccines have been used in man, although questions remain about their safety and relative efficacy, especially against the pneumonic form of infection. Since plague remains endemic in some regions of the world, and because of the possibility of the illegitimate use of Y. pestis as a biological warfare agent, development of improved vaccines against plague is a high priority. The ideal vaccine should be deliverable in a minimum of doses and quickly produce high titer and long-lasting antibodies. Moreover, such a vaccine should protect against aerosolized transmission of Y. pestis.

The two most recently described approaches to development of improved plague vaccines are 1) attenuated mutants of Y. pestis and 2) subunit vaccines. The potential efficacy of attenuated mutants of Y. pestis as vaccines is supported by experience with the live attenuated vaccine strain EV76. This vaccine has been in use since 1908 and is given as a single dose. Immunization of mice with EV76 induces an immune response and protects mice against subcutaneous and inhalation (aerosolized) infection. However, this vaccine strain is not avirulent and has an unacceptable safety profile. Moreover, multiple variants of the classical EV76 strain exist that differ significantly in passage history and genetic characteristics. Recent studies have focused on creating defined genetically attenuated mutants of Y. pestis, similar to those created in other Gram-negative bacteria (i.e., Salmonella spp.). For unknown reasons, genetic mutations, which markedly attenuate Salmonella spp. do not attenuate Y. pestis. For instance, an araA mutant of Y. pestis was fully virulent in the murine model of disease but avirulent in guinea pigs.

A number of potential subunit vaccines have been evaluated for immunogenicity and protective efficacy against Y. pestis. The two most promising are F1 and V. F1 is a capsular protein located on the surface of the bacterium and the V antigen is a component of the Y. pestis Type III secretion system. These proteins have been produced recombinantly and induce protective immune responses when administered individually. A combination or fusion of F1 and V may have an additive protective effect when used to immunize humans against plague. It is thought that F1-V fusion protein should provide protection against both subcutaneous and aerosol challenge, and will have the potential to provide protective immunity against pneumonic as well as bubonic plague due to either wild type F1+ Y. pestis or to naturally occurring F1-variants. To date no one has been able to express the F1-V fusion protein in transgenic chloroplast. Such an accomplishment would provide a large supply of high-quality antigen for vaccines.

Bacillus anthracis

Bacillus anthracis is the organism that causes the anthrax disease. It is a Gram-positive, nonmotile, aerobic or facultatively anaerobic, spore-forming bacterium. The spores are about 1 μm in size, extremely hardy, resistant to gamma rays, UV light, drying, heat, and many disinfectants. Spores germinate upon entering an environment rich in glucose, amino acids, and nucleosides, such as in animal and human tissues and blood. The vegetative cells enter the spore state when the nutrients are exhausted or when the organisms are exposed to molecular oxygen in the air.

Anthrax is typically a disease of animals, especially herbivores such as cows, sheep, and goats. It affects humans through contact with the spores in one of three ways. Cutaneous anthrax occurs when the spores enter the body through a cut or an abrasion on the skin. Gastrointestinal anthrax occurs when the spores enter the body through consumption of contaminated meat products. Inhalation anthrax occurs when the spores enter the body through inhalation of the spores.

When spores enter the body, macrophages engulf them, migrate to regional lymph nodes and the spores germinate into vegetative bacteria. Macrophages release the vegetative bacteria and they spread through the blood and lymph until there are up to 10^8 bacilli per milliliter of blood. The exotoxins are produced from bacteria and they lead to symptoms and possible death. Spores can survive in the lungs or lymph nodes up to 60 days before germination occurs. In animal experiments, it has been seen that once toxin secretion has reached a critical threshold, death will occur, even if the blood is rendered sterile through the use of antibiotics. From primate studies, the estimated lethal dose of inhaled anthrax spores sufficient to kill 50% of humans exposed to it (the LD50) is 2,500-55,000 spores.

The CDC lists anthrax as a category A disease agent and estimates the cost of an anthrax attack would be $26.2 billion per 100,000 persons exposed. The only vaccine licensed for human use in the U.S., Biothrax (formerly Anthrax vaccine adsorbed, or AVA), is an aluminum hydroxide-adsorbed, formalin-treated culture supernatant of Bacillus anthracis. In addition to the immunogenic protective antigen (PA), the vaccine contains trace amounts of edema factor (EF) and lethal factor (LF) that may contribute to the local reactions seen in 5-7% of vaccine recipients, or reported to be toxic causing side-effects. There is a clear need and urgency for an improved vaccine for anthrax and for improved production methods that allow it to be mass-produced at reasonable cost.

There are two main virulence factors associated with B. anthracis, the polyglutamyl capsule which is believed to prevent the vegetative bacterial cells from being phagocytozed and the exotoxins. Two different exotoxins are produced by three factors. PA binds to the host cell, LF is a zinc metalloprotease which inactivates mitogen-activated protein kinase. The edema toxin is formed when PA binds to EF.
This toxin increases cyclic AMP (cAMP) levels in the cell which upsets the water homeostasis resulting in accumulation of fluid called edema. The lethal toxin is formed from binding of PA and LF. This toxin stimulates macrophages to release interleukin-1b, tumor necrosis factor α, and other cytokines which contribute to shock and sudden death.

Anthrax has become a serious threat due to its potential use in bioterrorism and recent outbreaks among wild-life in the United States. Concerns regarding vaccine purity, the current requirement for six injections followed by yearly boosters, and a limited supply of the key protective antigen (PA), underscore the urgent need for an improved vaccine.

SUMMARY OF THE INVENTION

The present invention pertains to vaccines for conferring immunity in mammals to infective pathogens, as well as vectors and methods for plastid transformation of plants to produce protective antigens and vaccines for oral delivery. The invention further provides transformed plastids having the ability to survive selection in both the light and the dark, at different developmental stages by using genes coding for two different enzymes capable of detoxifying the same selectable marker, driven by regulatory signals that are functional in proplastids as well as in mature chloroplasts. The invention utilizes antibiotic-free selectable markers to provide edible vaccines for conferring immunity to a mammal against Bacillus anthracis, as well as Yersinia pestis. The vaccines are operative by parenteral administration as well. The invention also extends to the transformed plants, plant parts, and seeds and progeny thereof. The invention is applicable to monocot and dicot plants.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 is a schematic view of anthrax chloroplast constructs according to the present invention, and a view of a southern blot. FIG. 1A shows the pLD-JW1 vector used for chloroplast transformation. FIG. 1B shows the pLD-JW2 construct. FIG. 1C shows PCR with the primers 3P and 3M. FIG. 1D shows a PCR analysis of randomly selected clones.

FIG. 2 is a schematic view of anthrax chloroplast constructs according to the present invention, and views of southern blots. FIG. 2A shows expected products from digestion of wild type untransformed plant. FIG. 2B shows expected products from digestion of a plant transformed with pLD-JW1. FIG. 2C shows expected products from digestion of a plant transformed with pLD-JW2. FIG. 2D shows a flanking sequence probe showing heteroplasmy in pLD-JW1 line and homoplasmy in pLD-JW2 lines. FIG. 2E shows a western blot sequence probe showing presence of pag in transgenic lines.

FIG. 3 shows Western blots demonstrating PA expression of transgenic lines containing different constructs. FIG. 3A shows a western blot of pLD-JW1 T1 transgenic lines for PA quantification. Lane 1: wild type; Lane 2: Ladder; Lane 3: 20 ng PA; Lane 4: 10 ng PA; Lane 5: 5 ng PA; Lane 6: 1:10 dilution pLD-JW1 line; Lane 7: 1:20 dilution pLD-JW1 line. FIG. 3B shows a western blot of pLD-JW2 T1 transgenic lines for PA quantification. Lane 1: wild type; Lane 2: Ladder; Lane 3: 20 ng PA; Lane 4: 10 ng PA; Lane 5: 5 ng PA; Lane 6: blank; Lane 7: 1:10 dilution pLD-JW2 line #1; Lane 8: 1:20 dilution pLD-JW2 line #1; Lane 9: 1:10 dilution pLD-JW2 line #2; Lane 10: 1:20 dilution pLD-JW2 line #2. FIG. 3C shows a western blot comparing extraction buffers, containing CHAPS and SDS, and both, and measuring stability of extracts at 4°C, where “Sup” means supernatant fraction. “Hom” means homogenate (soluble and insoluble fractions).

FIG. 4 shows western blots of T1 pLD-JW1 plant in continuous light in FIGS. 4A and 4B, and in FIG. 4C shows histogram of µg PA/g fresh tissue in young, mature, and old leaves after 3 and 5 days of continuous illumination.

FIG. 5 shows graphs of the results of macrophage cytotoxic assays for extracts from transgenic plants. FIG. 5A shows supernatant and homogenate samples from T1 pLD-JW1 tested. FIG. 5B shows Supernatant samples from T1 pLD-JW1 tested.

FIG. 6A is a schematic view of tomato vector construct pLD Tom-BADH, while FIG. 6B shows PCR for transformants using 3P/3M primers of the pLD-TOM-BADH construct.

FIG. 7 is a schematic of the pTOM-BADH2-G10-pag tomato vector construct

FIG. 8 shows PCR analysis of the products of tomato vectors using BADH1 primers, wherein + is pTOM-G10-PA vector control, – is WT Tomato plant, and # 3 is transgenic tomato plant.

FIG. 9A shows tomato seedlings 12 days after seed germination, while 9B shows tomato cotyledons ready for bombardment.

FIG. 10 shows cut and bombarded cotyledons.

FIGS. 11A and B show the response of tomato cotyledons transformed with the pLD-TOM-BADH on RMOP supplemented with 2.5 nm betaine aldehyde, where wild type is illustrated in 11a and putative transformant in 11b.

FIG. 12 shows the serum anti-PA as determined by ELISA and the in vitro toxin neutralization by serum antibodies following intranasal immunization.

FIG. 13A is a schematic view of a pLDS-F1V vector construct, while FIG. 13B shows restriction enzyme analysis of the pLDS-F1V vector.

FIG. 14A shows PCR reactions to determine pLDS-F1V vector integration into the chloroplast genome of Petit Havana, while 14B shows a second PCR reaction, which is also used to determine pLDS-F1V transgene integration.

FIG. 15A shows a Western Blot of pLDS-F1V from XI.1-Blue strain of Ecoli, while 15B shows Western Blots of F1V expression in transgenic chloroplasts.

FIG. 16 is a schematic of constructs to be inserted in edible plants, where “gene X” represents each of LF27-PA63, CTB-LF27, LF27-PA63+CTB-LF27, LF274PA.

FIG. 17A is a schematic view of a pDD34-ZM-gfp-BADH vector construct, while 17B shows the subsequent expression of the construct containing GFP in Ecoli.

FIG. 18A is a schematic of pDD33-ZM-aadA-BADH vector construction, and 18B shows the subsequent expression of the construct in E.coli grown on spectomycin.

FIG. 19(A-C) show GFP expression in embryogenic maize cultures where a is non-transgenic control and b and c are transformed maize embryonic calli.

FIG. 20A shows maize control and transgenic plants on regeneration medium containing spectomycin, while FIG. 20B shows PCR confirmation of chloroplast transgenic plants using appropriate primers.

FIG. 21A is a schematic view of a pDD37-DC-gfp-BADH carrot chloroplast transformation vector, while 21B illustrates GFP expression in E.coli.

FIG. 22A is a schematic of a vector construct pDD36-DC aadA-BADH carrot chloroplast transformation vector, while 22B illustrates the expression of the vector using E.coli cells grown on spectomycin.

FIG. 23 (A-D) shows Expression of GFP in different stages of transgenic cultures of carrot.
FIG. 24 is a schematic view of a Double Barreled Plastid Vector harboring apha-6 and apha-2 genes conferring resistance to aminoglycosides according to the present invention.

DETAILED DESCRIPTION OF THE INVENTION

Chloroplast Engineering

The concept of chloroplast genetic engineering, allows the introduction of isolated intact chloroplasts into protoplasts and regeneration of transgenic plants. Early investigations involving chloroplast transformation focused on the development of in organello systems using intact chloroplasts capable of efficient and prolonged transcription, translation, and expression of foreign genes in isolated chloroplasts. However, the discovery of the gene gun as a transformation device made it possible to transform plant chloroplasts without the use of isolated plastids and protoplasts. Chloroplast genetic engineering has been accomplished in several phases. Studies have been made on the transient expression of foreign genes in plastids of monocots and dicots. Unique to the chloroplast genetic engineering is the development of a foreign gene expression system using autonomously replicating chloroplast expression vectors. Stable integration of a selectable marker gene into the tobacco chloroplast genome was also accomplished using the gene gun. Recently, useful genes conferring valuable traits via chloroplast genetic engineering have been demonstrated. Plants resistant to *Bacillus thuringiensis* (Bt) sensitive and resistant insects were obtained by integrating the cry1Ac and cry2A genes into the tobacco chloroplast genome.

Chloroplast genomes of plants have also been genetically engineered to confer herbicide resistance where the introduced foreign genes were maternally inherited. This was a significant step in the development of commercially viable genetically modified plants because it alleviates any concerns over the problem of out-crossing traits with weeds or other crops.

For large-scale foreign protein production, plants are an ideal choice due to the relative ease of genetic manipulation, rapid scale up (million seeds per plant), large biomass, and the potential to find alternative uses for various crops. A remarkable feature of chloroplast genetic engineering is the observation of exceptionally large accumulation of foreign proteins in transgenic plants (as much as 46% of CRY protein in total soluble protein) even in bleached old leaves. Using chloroplast transformation technology, large quantities of protective antigen can be produced in transgenic plants, due to the presence of thousands of copies of transgenes per cell as opposed to only a few copies in nuclear transgenic plants. By “protective antigen” is meant that the antigen elicits an immunogenic response in mammals when administered by an appropriate route in an appropriate amount. Transgenic chloroplast technology has been used to hyper-express bacterial proteins—up to 46% of total soluble protein from *Bacillus* genes, the highest ever reported in transgenic plants. However, large heterologous proteins are not always processed correctly. Furthermore, such proteins are subject to attack by proteolytic enzymes after formation. The cholera toxin B subunit is about 83 kd in size, presenting a significant challenge to production in a chloroplast. A similar challenge is presented by the F1-V antigen, which is a fusion protein. Such fusion proteins have not heretofore been successfully expressed in chloroplasts. Chloroplasts are prokaryotic in nature and express native bacterial genes (like B subunit of cholera toxin) at very high levels (410-fold higher than nuclear expression). Production in chloroplasts is in sharp contrast to nuclear expression, that often requires extensive codon modifications because of high AT content, unfavorable codons, presence of mRNA destabilizing sequences, and cryptic polyadenylation or splice sites. Chloroplast transformation typically utilizes two flanking sequences that, through homologous recombination, insert transgenes into spacer regions between functional genes of the chloroplast genome, thus targeting transgenes to a known location. This eliminates the “position effect” and gene silencing frequently observed in nuclear transgenic plants. Chloroplast genetic engineering is an environmentally friendly approach, minimizing concerns of out-cross of introduced traits via pollen to weeds or other related crops. The chloroplast genome may be engineered without the use of antibiotic resistant genes as well for the development of edible vaccines. The term “edible vaccine” as used herein refers to a substance which may be given orally which will elicit a protective immunogenic response in a mammal.

The difficulty of engineering the chloroplast genome without antibiotic resistant genes has recently been overcome by modifying chloroplasts without these genes. Engineering genetically modified crops without these genes eliminates their potential transfer to the environment and to microbes in the gut. Antibiotic-free selection can be accomplished by using the betaine aldehyde dehydrogenase (BADH) gene from spinach as a selectable marker. Specifically, the Applicant’s published application, WO01/64023, which is hereby incorporated by reference, demonstrates using an antibiotic free selectable marker. The selection process involves conversion of toxic betaine aldehyde (BA) to non-toxic glycine betaine, which also serves as an osmoprotectant and helps confer drought tolerance.

The Applicant has transformed the chloroplast of edible monocot and dicot species, as is illustrated in the accompanying Figures. Specifically, the figures illustrate the stable transformation of carrot and corn chloroplast genomes, which has not previously been accomplished. The transformation of carrot, tomato, and corn allow the production of edible vaccines. These transformed plants also allow the purification and use of the antigens produced by these plants as high purity parenteral vaccines. However, the production of edible vaccines is preferred for their ease of use and low cost.

FIGS. 17A and 18A illustrate the construction of maize chloroplast transformation vector, where flanking regions were amplified using PCR. The PCR products were cloned and the expression cassette was inserted in the transcriptionally active spacer region between tnl/trnA genes. The expression cassette of FIG. 17A has the Prn promoter driving the expression of GFP and BADH, which are regulated by (5') gene10/psi16 3' and psiA 5'/psi 3' UTRs respectively. The expression cassette of 18A has the Prn promoter driving the expression of adaA and BADH. The latter gene is regulated by (5') gene10/psi16 3' UTRs.

Functions of the genes in the carrot chloroplast transformation vectors were tested in *E. coli*. For observing GFP expression, cells were plated on LB agar (Amp) plates and incubated at 37°C overnight. Cells harboring pDD34-ZM-GFP-BADH were seen to fluoresce when exposed to UV light, as is seen in FIG. 17B. To test the adaA gene expression, cells harboring pDD33-ZM-adaA-BADH plasmid were plated on LB agar plates containing spectinomycin.
transgenic calli. After eight weeks, a number of the healthy growing calli from different bombardment experiments were examined for GFP expression under the fluorescent stereomicroscope and the confocal microscope. Somatic embryos were regenerated on maize regeneration medium containing BA or streptomycin.

Fig. 20A shows maize plants on regeneration medium containing streptomycin or betaine aldehyde. Fig. 20B illustrates maize chloroplast transgenic plants which were capable of growth on the selection agent indicating that construction of transgenic maize, while untransformed maize plants did not grow on the selection medium.

Fig. 20B shows PCR confirmation of chloroplast transgenic plants using appropriate primers. Lanes 1-3, plants transformed with pDD33-ZM-gfp-BADH and Lanes 4-5, plants transformed with pDD33-ZM-aadA-BADH. Lanes - and + represent the negative and positive controls respectively. Genomic DNA was isolated from the leaf tissues and PCR was performed on transformed and non-transformed tissues using appropriate primers.

Figs. 21A and 22A show the schematic construction of carrot chloroplast transformation vectors. The construction of the carrot chloroplast transformation vector illustrated in Figs. 21A and 22A have flanking regions that were amplified using PCR. The PCR products were then cloned and the expression cassette was inserted in the transcriptionally active spacer region between trnI/trnA genes. The expression cassette of Fig. 21A has the Prm promoter driving the expression of GFP and BADH, which are regulated by (5') gene10/rps16 3' and psbA 5'/3' UTRs. The expression cassette of Fig. 22A has the Prm promoter driving the expression ofaadA and BADH. The latter gene is regulated by (5') gene10/rps16 3' UTR.

Functions of the genes in the carrot chloroplast transformation vectors were tested in E. coli. For observing GFP expression, cells were plated on LB agar (Amp) plates and incubated at 37°C overnight. Cells harboring pDD37-DC-GFP-BADH were seen to fluoresce when exposed to UV light (A). To test the aadA gene expression, cells harboring pDD36-DC-aadA-BADH plasmid were plated on LB agar plates containing spectinomycin (100 mg/ml) and incubated at 37°C overnight. Transformed cells grow on spectinomycin (B).

Functions of the genes in the carrot chloroplast transformation vectors were tested in E. coli. For observing GFP expression, cells were plated on LB agar (Amp) plates and incubated at 37°C overnight. Cells harboring pDD33-ZM-GFP-BADH were seen to fluoresce when exposed to UV light as is seen in Fig. 21B. To test the aadA gene expression, cells harboring pDD33-ZM-aadA-BADH plasmid were plated on LB agar plates containing spectinomycin (100 µg/ml) and incubated at 37°C overnight. Transformed cells grow on spectinomycin, as is seen in Fig. 22B.

Fig. 23 (A-D) shows expression of GFP in different stages of transgenic carrot cultures studied under confocal microscope (A) Untransformed control, (B) Embryogenic callus, (C) Embryogenic callus differentiated into globular somatic embryos and (D) Somatic embryo with differentiated cotyledons.
Example 1

Transgene Integration into the Chloroplast Genome by PCR Analysis:

Chloroplast transgenic lines were generated by particle bombardment as described previously. After bombarding *Nicotiana tabacum* cv. Petit Havana tobacco leaves with the chloroplast vectors, the leaves were grown on selective medium containing 500 µg/ml spectinomycin. Three different genetic events can produce spectinomycin-resistant tobacco shoots: (1) transgene integration into chloroplasts, (2) into the nuclear genome or (3) spontaneous mutants. PCR with two specific primers, 3P and 3M, allowed identification of shoots having the desired chloroplast transgene integration. The 3P primer annealed to the native chloroplast genome and the 3M primer annealed to theaadA gene (see FIG. 1C). Nuclear transformants were eliminated because 3P will not anneal and mutants were ruled out because 3M will not anneal. At least 50 shoots were obtained in the initial transformation, a high frequency which suggests that there was no inhibitory effects of PA. Among clones tested, in both constructs, all were chloroplast transformants. No spontaneous mutants or nuclear transformants were observed (see FIG. 1D).

Chloroplast Integration of Transgenes and Homoplasmy:

Southern blots were done to further verify that the transgenes had been integrated into the chloroplast genome and to determine homoplasmy (containing only transformed chloroplast genomes) or heteroplasmy (containing both transformed and untransformed chloroplast genomes). Total plant DNA was digested with the enzyme BglII which generated a 4.4-kb fragment in wild type, 5.2-kb and 3-kb fragments in pLD-JW1 transgenic lines, and 6.8-kb and 3-kb fragments in pLD-JW2 transgenic lines when hybridized with a 0.81-kb probe made from chloroplast flanking sequences (see FIG. 2A-D). The blots were also hybridized with a 0.52-kb probe made from pag coding sequence (see FIG. 2E). All of the pLD-JW2 plants appeared to be homoplasmic. In the pLD-JW1 plants, transgenic line 2 appeared to be heteroplasmic, which is not uncommon to find in *N. tabacum* plants. T₁ refers to first generation transgenic lines and T₂ refers to second generation lines obtained by germination of seeds from T₁.

FIG. 2 shows southern blots to investigate the site of transgene integration and determine homoplasm or heteroplasm. The figure shows a schematic diagram of the expected products from digestion of wild type untransformed plant as follows: FIG. 2A shows plants transformed with pLD-JW1; FIG. 2B shows plants transformed with pLD-JW2. FIG. 2D shows a flanking sequence probe revealing heteroplasm in pLD-JW1 line and homoplasm in pLD-JW2 lines. FIG. 2E shows pag sequence probe showing presence of pag in transgenic lines.

PA Expression in Transgenic Chloroplasts:

Western blots were performed on transgenic lines containing the two different constructs. Full length 83-kDa polypeptide was detected on blots, confirming PA expression in transgenic lines and absence of unique proteases that cleave PA in plant cells (see FIGS. 3A-C). Presence of active furin or trypsin-like proteases would have resulted in a 63-kDa protein due to cleavage at the sequence RKRK (SEQ ID NO:1) (amino acids 164-167). The sequence FFD (SEQ ID NO:2) at residues 312-314 is another site that is highly sensitive to chymotrypsin-like enzymes, and cleavage would have resulted in 47- and 37-kDa fragments. No other cleaved PA products were observed (see full length blots shown), demonstrating stability of chloroplast derived PA. Prior to the Applicant’s discovery, it was widely believed that pro-
teases would cleave long antigenic bacterial peptides, but this invention illustrates that the antigenic bacterial peptides are free from proteolytic degradation.

The T1 transgenic lines showed a greater amount of PA in the pLD-JW1 lines as compared to the pLD-JW2 lines. Additional western blots were done comparing two different detergents in the extraction buffers, CHAPS and SDS, and both were found to extract PA equally well (see FIG. 3C). The supernatant and the homogenate were also found to be comparable suggesting that most of the PA is in the soluble fraction. After storage for two days at 4°C and ~20°C, the PA in plant crude extracts is quite stable (see FIG. 3C). Powdered leaf was stored at ~80°C for several months before performing western blots or functional assays; this did not result in any noticeable decrease in PA quantity or functionality. This facilitates long term storage of harvested leaves before extraction of PA for vaccine production. Native PA has been shown to be highly unstable due to proteolysis-sensitive sites, which have been modified to confer better stability.

Quantification of PA in Transgenic Chloroplasts: In order to quantify the amount of PA in transgenic chloroplasts, western blots were used to observe varying dilutions of the crude extract. PA was quantified using gel documentation software (Bio-Rad). Based on western blot analysis, pLD-JW1 T1 transgenic lines showed 44 µg PA/g of fresh tissue (22 µg/ml). An average tobacco leaf weighed 6.5 g; therefore 286 µg PA could be expressed per leaf. The pLD-JW2 transgenic lines showed lower levels of PA accumulation, probably due to interference of both UTRs, resulting in decrease in translation. We have observed recently that the combination of ORF-psbA5'UTR decreases expression of human serum albumin in transgenic chloroplasts. The psbA regulatory sequences, including the promoters and UTRs, have been shown to enhance translation and accumulation of foreign proteins under continuous light. Therefore, pLD-JW1 T1 transgenic plants were placed in continuous light and young, mature, and old leaves were collected after 3 or 5 days of continuous illumination. Western blots were performed using different dilutions of crude plant extracts (see FIG. 4A-B). The 3 day mature and young leaves contained 80 ng PA or 108 µg PA/g of fresh tissue. The 5 day old, mature and young leaves contained 32 µg PA, 108 µg PA or 156 µg PA/g of fresh tissue, respectively. Thus, young leaves showed the highest accumulation of PA and old leaves showed the lowest, probably due to proteolytic degradation induced during senescence. These assays quantified only full length PA. In spite of loading 500-1000 fold more protein of untransformed plant extracts, no cross-reacting protein was observed with the monoclonal antibody used.

FIG. 4 shows total protein from plant extracts loaded in each lane is shown in parenthesis. FIG. 4A shows pLD-JW1 transgenic line in 3 days of continuous light, 1:20 dilutions. Lane 1: old leaf (187 ng); Lane 2: mature leaf (369 ng); Lane 3: young leaf (594 ng); Lanes 4-5: blank; Lane 6: 10 ng PA; Lane 7: 20 ng PA; Lane 8: ladder; Lane 10: wild type (16,000 ng). FIG. 4B shows pLD-JW1 transgenic line in 5 days of continuous light, 1:20 dilutions. Lane 1: old leaf (214 ng); Lane 2: mature leaf (588 ng); Lane 3: young leaf (745 ng); Lane 6: 10 ng PA; Lane 7: 20 ng PA; Lane 8: blank; Lane 9: ladder; Lane 10: wild type (15,000 ng). FIG. 4C is a histogram of µg PA/g fresh tissue in young, mature, and old leaves after 3 (blue) and 5 (red) days of continuous illumination.

PA accumulation was visualized in crude plant extracts by Coomassie staining. When a capture ELISA was used to quantify PA, it appeared that PA constituted a large percentage of total soluble protein in some extracts. While these values may reflect detection of partially cleaved PA, they do not result from non-specific interaction of the antibodies with any other proteins, because no signal was detected in untransformed plants. However, the data set forth herein is from quantitative scanning of polyacrylamide gels and not from the capture ELISA.

PA Functionality Determined by Macrophage Lysis: Supernatant and homogenate samples from both T0 constructs, pLD-JW1 and pLD-JW2, were tested. Two different buffers were used to extract proteins—one contained CHAPS detergent and one did not have any detergent. The PA produced in chloroplast transgenic lines was able to bind to the anthrax toxin receptor, be cleaved to the 63-kDa fragment, heptamerize, bind LF, be internalized and lyse the macrophage cells. Therefore the transgenic plants were shown to produce fully functional PA (see FIG. 5A). Active PA was found in both the supernatant and homogenate fractions; but was quantitated only in the former. The assay using CHAPS gave a result of the pLD-JW1 supernatant with the best yield. In the absence of any detergent, the supernatants lysed the macrophage cells better than the homogenates. Crude plant extracts contained up to 5 µg per ml functional PA confirming expression of high levels of functional PA. Supernatant samples from T1 pLD-JW1 transgenic lines were tested and they resulted in approximately 12-25 µg functional PA per ml (see FIG. 5B) and the toxicity was entirely dependent on the presence of LF.

The threat of biological warfare and terrorism is real. The most effective way to prevent or deter effective use of anthrax as a weapon would be to produce an efficacious and inexpensive vaccine. Plants are an inexpensive and easy way to produce recombinant proteins, without human or animal pathogen contamination. Because one acre of tobacco yields up to 40 tons of fresh leaves (40,000 kg in three cuttings), the production could be up to 6.24 kg PA per acre based on expression levels reported in this manuscript. There is less than 50% loss during purification from plant extracts (loss of foreign protein is generally between 50 and 90%), and at 5 µg PA per dose (which is roughly equivalent to prior art vaccine which is in a range of 1.75 to 7 µg PA), one can produce 600 million doses of vaccine per acre of tobacco. Thus a few acres of transgenic tobacco can meet the world’s need for the anthrax vaccine.

Experiment Protocol for Example 1

Sterile Nicotiana tabacum cv. Petit Havana tobacco leaves were bombarded using the Bio-Rad PDS-1000/He biolistic device as previously described. The bombarded leaves were placed on RMOP medium containing 500 µg/ml spectinomycin for two rounds of selection on plates and subsequently moved to jars of MSO medium containing 500 µg/ml spectinomycin.

PCR Analysis to Test Stable Integration:

DNA was extracted from tobacco leaves using Qiagen DNeasy Plant Mini Kit available from Qiagen, Valencia, Calif. PCR was performed using the Perkin Elmer GeneAmp PCR System 2400 (available from Perkin Elmer, Chicago, Ill.). PCR reactions contained template DNA, 1 x Taq buffer, 0.5 mM dNTPs, 0.2 mM 3' primer, 0.2 mM...
3M primer, 0.05 units/µl Taq Polymerase, and 0.5 mM MgCl₂. Samples were run for 30 cycles as follows: 95°C for 1 min, 65°C for 1 min, and 72°C for 2 min with 5 min ramp up at 95°C and a 72°C hold for 10 min after cycles complete. PCR products were separated on 1% agarose gels.

Southern Blot Analysis:
Total plant DNA was digested with BglIII and run on a 0.8% agarose gel at 50 V for 2 hours. The gel was soaked in 0.25 N HCl for 15 minutes and then rinsed 2x with water. The gel was soaked in transfer buffer (0.4 N NaOH, 1 M NaCl) for 20 minutes and then transferred overnight to a nitrocellulose membrane. The membrane was rinsed twice in 2xSCC (0.3 M NaCl, 0.03 M Sodium citrate), dried on filter paper, and then crosslinked in the GS GeneElut (Stratagene, La Jolla, Calif.). The flanking sequence probe was made by digesting pUC-CT vector DNA with BamHI and BglII to generate a 0.81 kb probe. Lee, S. B., Byun, M. O., Daniell, H. Accumulation of trehalose within transgenic chloroplasts confers drought tolerance. Molecular Breeding (in press) (2002). The pag probe was made by digesting pag with Neol to generate a 0.52 kb probe. The probes were labeled with 32P using the ProbeQuant G-50 Micro Columns (Amersham, Arlington Heights, Ill.). The probes were hybridized with the membranes using Stratagene QUICK-HYB hybridization solution and protocol (Stratagene, La Jolla, Calif.).

Western Blot Analysis:
Approximately 100 mg of leaf tissue was ground in liquid nitrogen with a mortar and pestle and stored at ~80°C for several months. When it was time to extract the proteins, the powder was removed from ~80°C and 200 µl of plant extraction buffer was added and mixed with mechanical pestle (0.1% SDS, 100 mM NaCl, 200 mM Tris-HCl pH 8.0, 0.05% Tween 20, 400 mM sucrose, 2 mM PMSF). The plant was then centrifuged for 5 minutes at 10,000 g to pellet the plant material. The supernatant containing the extracted protein was transferred to a fresh tube and an aliquot was taken out, combined with sample loading buffer, boiled, and then run on 8% SDS-PAGE gels for one hour at 80 V, then 2 hours at 150 V. Gels were transferred overnight at 10 V to nitrocellulose membrane. The membrane was blocked with PBT (1xPBS, 0.05% Tween 20, and 3% dry milk). PA was detected with anti-PA monoclonal antibody conjugated to horseradish peroxidase (American Qualex Antibodies, A106PN).

The stability assay utilized SDS buffer (0.1% SDS, 100 mM NaCl, 10 mM EDTA, 200 mM Tris-HCl pH 8.0, 0.05% Tween 20, 14 mM β-mercaptoethanol, 400 mM sucrose, 2 mM PMSF) and CHAPS buffer (4% CHAPS, 100 mM NaCl, 10 mM EDTA, 200 mM Tris-HCl pH 8.0, 14 mM β-mercaptoethanol, 400 mM sucrose, 2 mM PMSF). Two hundred µl of each buffer was added to 100 mg powdered leaf tissue. For supernatant fractions, the extraction was centrifuged at 10,000g for 5 minutes and supernatant was removed. For homogenate, the entire extract was used. The samples were stored at 4°C and ~20°C for two days. The rest of the western protocol was the same as described above. Dilutions of 1:10 and 1:20 of the protein extracts were made and run on the gel along with 20, 10, and 5 µg of PA protein standards to generate a standard curve for protein quantification. After the film was developed, the PA was quantified using the Gel-Doc.

Macrophage Lysis Assays:
Approximately 100 mg of powdered leaf tissue was extracted with 200 µl of extraction buffer. For the supernatant fraction, the buffer and tissue were centrifuged for 5 minutes at 10,000xg and the supernatant was placed in a new tube. For the homogenate, it was all of the tissue and the buffer. RAW 264.7 macrophages were plated in 12-well plates in 120 µl DMEM medium and grown to 50% confluence. The medium was aspirated and replaced with 100 µl medium containing 250 ng/ml LF. The control plate received medium with no LF to test toxicity of plant material and buffers. In separate 96-well plates, the plant samples were diluted serially 2-fold and 40 µl of the dilutions were transferred onto the RAW cells so the top row had plant extract at 1:1 dilution. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was added after 5-10 hours to assess cell death. Gu, M. L., Leppla, S. H., Klinman, D. M. Protection against anthrax toxin by vaccination with a DNA plasmid encoding anthrax protective antigen (Vaccine. 17, 340-344 (1999)).

Example 2

Transformation of the Tomato Chloroplast Genome
Prior to the creation of the pTom-BADH2-G10-pag –8.8 kb vector construct (FIG. 7), the Applicant constructed the pLD Tom-BADH vector (FIG. 6A) illustrating the ability to transform the chloroplast genome of edible dicots. The pTom-BADH vector was constructed to with two selectable marker genes (BA and spectinomycin) to test ability of transformed plants to grow on BA as compared to spectinomycin. The pLD-Tom-BADH vector contains the chimeric aadA gene and the BADH gene driven by the constitutive 16 S rRNA promoter and regulated by the 5'UTR region of psbA gene from petunia plastid genome. In this construct both, aadA and BADH possess the chloroplast preferred ribosomal binding site, GAGGG. Another suitable vector used for tomato chloroplast transformation is the pLD-Tom-UTR-BADH vector, which has the constitutive 16 S rRNA promoter driving the expression of the dicistron, in which the BADH is under the regulation of the promoter and the 5'UTR of the psbA gene and the 3'UTR of psbA gene, for enhanced expression. FIG. 6B shows the PCR of tomato chloroplast transformed with the pLD-Tom-BADH using 3P/3M primers. Specifically FIG. 6B shows, Lane 1 and 9-1 Kb plus Ladder; 2-mutant; 3-Clone#8; 4-Clone#9; 5-Clone#11; 6-Wild type; 7-Positive control; 8-pLD-Tom-BADH. From results observed in the gel in FIG. 6B, integration of transgene is quite evident.

FIGS. 11A and B show the response of tomato cotyledons transformed with the pLD-Tom-BADH vector grown on RMOP, which is supplemented with 2.5 nm betaine aldehyde, wherein 11A shows the wild type control and 11B shows the transformatant.

After successful construction and integration into the tomato chloroplast genome using the pLD Tom-BADH vector of FIG. 6A, the Applicant then constructed the pTom-BADH2-G10-pag –8.8 kb vector, which is illustrated in FIG. 7. FIG. 7 shows the schematic construct of the pTom-BADH2-G10-pag –8.8 kb vector, which was constructed containing the selectable marker gene BADH. This construct is also be made using the and gene to confer spectinomycin resistance in place of the BADH gene. After bombarding the tomato cotyledons (seed leaves or embryonic leaves) with the tomato construct of FIG. 7, the cotyledons were put on selection media containing Betaine.
Aldehyde (BA) and calli formed. The calli were transferred to new selection media to obtain shoots.

Fig. 10 shows the first selection after bombardment with the pTom-BADH2-G10-pag -8.8 kb vector, wherein the cotyledons were incubated in the dark for 48 hours and then the bombarded cotyledons were cut. The RMOP medium shown in Fig. 10 is a shoot inducing media. The bombarded cotyledons were grown on 2.5 mM Betaine Aldehyde (BA) for selection.

Fig. 8 shows the PCR test that was performed to determine integration of the pTom-BADH2-G10-pag -8.8 kb vector, where the tomato shoots were tested with PCR to confirm integration of the transgene. In Fig. 8, the + is p1OM-G10-PA vector control, – is WT Tomato plant, and #3 is the transgenic tomato plant. This confirmation utilized appropriate primers.

To apply the plastid transformation technology to edible plants to produce an edible vaccine, a first generation tomato vector is constructed containing pag. Trnl and trmGA are homologous recombination regions in tomato; 5'UTR from pag gene and leave opened pBlue-G vector, where the tomato shoots were tested with PCR to confirm integration of the transgene. In Fig. 8, the + is p1OM-G10-PA vector control, – is WT Tomato plant, and #3 is the transgenic tomato plant. This confirmation utilized appropriate primers.

PCR Confirmation of Transgene Integration:

After bombarding the tomato cotyledons (seed leaves or embryonic leaves) with the tomato construct vector, pTom-BADH2-G10-pag -8.8 kb, the cotyledons are put on selection media containing Betaine Aldehyde (BA) and calli formed (Fig. 8). The calli are transferred to new selection media to obtain shoots. Shoots are tested with PCR to confirm integration of the transgene, which utilizes appropriate primers (Fig. 8).

Mucosal and Transcutaneous Immunization Against Anthrax:

Anthrax vaccine studies focused on mucosal and transcutaneous immunization with rPA as a vaccine antigen when delivered in conjunction with a novel adjuvant, designated LT(R192G). This adjuvant was shown to be effective at augmenting protection against a variety of bacterial, viral, and fungal pathogens when delivered with appropriate antigens intranasally, orally, rectally, or transcutaneously. This adjuvant was developed by the Clements laboratory at Tulane University Health Sciences Center with funding from NIH and the Department of Defense and has been evaluated in a number of Phase I and Phase II clinical trials. In rPA studies, a number of immunologic outcomes were measured, but the studies focused primarily on those associated with protection against inhaled pathogens—serum and bronchial lavage (BAL) fluid antibodies. It was demonstrated that mucosal and transcutaneous immunization of mice with rPA induces high levels of antigen-specific antibodies in serum and in bronchoalveolar lavage fluids. Moreover, circulating anti-rPA antibodies are able to neutralize the cytotoxic effect of anthrax Lethal Toxin when tested in macrophage cytotoxicity assays. Fig. 12 shows the serum anti-PA as determined by ELISA and the in vitro toxin neutralization by serum antibodies following intranasal immunization. Equivalent results were seen following transcutaneous immunization.

Tomato Chloroplast Integration Vector:

Since the expression of the foreign protein is desired in chloroplasts of tomato fruit, the gene of interest needs to be under the control of a regulatory sequence that is free from cellular control. In this context, examples of suitable candidate regulatory sequences are the T7 gene 10 leader sequence and cry2Aa2 UTR. The T7 gene 10 leader sequence is used to express foreign proteins in transgenic chloroplasts. The cry2Aa2 UTR accumulates foreign protein in chloroplasts as efficiently as the psbA UTR. The selectable marker for the future generation vectors can optionally be the BADH gene under the regulation of psbA promoter and 5'UTR as psbA is one of the most efficiently translated chloroplast genes in green tissues. Since green tissue is used for introducing the transgene into the chloroplasts in tomato, it is ideal to use the light regulated psbA UTR for the selectable marker.

Tomato Seed Sterilization and Growth Conditions:

Fig. 9 shows tomato seeds (Moneymaker and Ady varieties) that are surface sterilized with ethanol for 30 s, followed by a 20 min treatment with 1.5% NaOCI and 0.1% Tween 20. Seeds are washed thoroughly with sterile water (at least 3-4 times) and transferred to germination media (Fig. 14). Germination media consists of MS salts with 30% sucrose and 0.8% agar. About 20 seeds are inoculated per bottle and placed under a photoperiod of 16 h light and 8 h dark for 8-10 days to obtain cotyledons for particle bombardment. The cotyledons are then excised either as an explant for bombardment or the resulting seedlings are used for transplantation to obtain leaves.

The cotyledons and leaf material are bombarded using the particle gun. After bombardment the explants are then incubated in the dark for 48 h. The cotyledons and leaves are then cut into small pieces and placed onto RMOP media supplemented with 2.5, 5.0 and 7.5 mM of Betaine Aldehyde for regeneration. In the case of cotyledons, the concentration of 2.5 mM BA is optimal, but not required, as regeneration of putative transformants could be observed after two weeks. Specifically, there is no response on media having higher concentrations of 5.0 and 7.5 mM BA. With leaves, the concentration of 1 and 1.5 mM is optimal for pLD-Tom-BADH and pLD-Tom-UTR-BADH respectively.

Preparation and Analysis of Stable Tomato Plant Transformants:

Selection is optimally performed in the presence of BA, but has also been performed in the presence of antibiotics. After selection, PCR analysis is performed as described above, as is well understood in the art. Finally Southern and northern blot analyses are performed as described above, and well understood in the art, to determine the amount and level of transformation in the chloroplast genome.

Example 3

Transformation of Carrot to Produce Anthrax Vaccine

Carrot (Daucus carota L.) is a biennial plant grown for its edible taproot. It is one of the most important vegetables used worldwide for human consumption. Carrot taproots are rich in vitamin A and fiber and are ideal to genetically manipulate in the chloroplast for the production of edible vaccines. For transformation of carrot, flanking sequences (trnl and trmA) are amplified with the help of PCR. Duration for regeneration of carrot plantlets is shortened to four
months from eight months when replacing the antibiotic selection with BA. The same chloroplast constructs as described above for tomatoes are used for carrot except that homologous recombination regions i.e. trmA and trmE are derived from carrot chloroplast DNA. The advantage of using carrot is that from small clusters of cells or a small piece of carrot one can get thousands of transgenic plants in a limited space. Moreover, single cells are directly in contact with the culture media surface. Therefore, even a small quantity of selecting agent (betaine aldehyde) is more effective in comparison to other larger tissues. Carrot is easy to store for long periods of time.

Plant Material and Tissue Culture:
Seeds of carrot (Daucus carota L. cv. Nantes) are sown in pots and placed under a growth chamber with appropriate growth conditions for as little as four weeks to as long as a year. The hypocotyls are then cut into segments of 1 cm long and placed either on semi-solid callus induction medium or in 50 ml MS medium containing 3% sucrose, 0.1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) having pH 5.7. After 3 weeks of continuous shaking at 24 °C and 120 rpm, liberated cells are collected on a 100 µm mesh, centrifuged (150g for 1 min) and resuspended in fresh medium. Rapidly growing cell cultures can be subcultured weekly. Next, callus formation from hypocotyls segments is established on semi-solid MS medium (Carolina Biological supply company) containing 1 mg/l kinetin and 3 mg/l 2,4-D. Homogenously growing calli is subcultured every 4 weeks on fresh medium. The resulting friable calli is then resuspended in 50 ml MS medium containing 3% sucrose and 0.1 mg/ml kinetin. Finally, suspension-cultured cells are filtered through a 100 µm mesh and subjected to bombardment with chloroplast vectors.

Bombardment and Regeneration of Carrot Chromoplast Transgenic Plants:
Fine cell suspension culture of carrot, evenly spread over MS semi-solid medium is used for bombardment. After bombardment the explants are incubated in the dark for 48 h and later in appropriate light condition (16 h day/night cycle at 24 °C). Somatic embryogenesis is induced in a suspension of single cells and small clusters harvested on sieve and low-speed centrifugation. The harvested cells are washed once with hormone free liquid MS medium and resuspended in 40 ml hormone free MS medium containing different concentrations of betaine aldehyde (1.5, 2.5 and 3.5 mM). Transgenic somatic embryos, visible 2 weeks after induction, are selected manually and transferred onto plates with semi-solid MS medium containing 1% sucrose and variable concentrations of betaine aldehyde (1.5, 2.5 and 3.5 mM). The plates are sealed with parafilm. After two weeks, somatic embryos develop into plantlets which are transferred to soil in pots. Initially, the pots are covered with plastic bags to maintain high humidity and irrigated with progressively reduced concentrations of MS salts for the first week, followed by tap water in the second week. Transgenic plants with stable expression of recombinant protein are then utilized for suitable assays.

Example 4
Construction for the F1V Fusion Protein (the Plague Antigen)
The production of Yersinia pestis vaccine in a low nicotine strain of tobacco (LAMD) is accomplished by expressing in chloroplasts the F1-V antigen fusion protein produced from F1 gene (513 bp/15.5 kDa) and the entire V antigen (980 bp/35 kDa). The entire immunogenic sequence will be (441 +980 +6 for a hinge=1437 bp). With the protein of 478 amino acids having a calculated mass of 53,193 and a pI of 5.1 has shown this fusion protein to be immunoprotective. F1-V was modified to add an EcoR1 site. This fragment is cloned into the universal chloroplast vector, which has been described above, with the psbA 5'UTR upstream of the F1-V fusion. The use of the psbA 5'UTR, is not required, but it has been shown to increase expression of foreign proteins by chloroplast.

Large-scale expression of the fusion protein results in the formation of inclusion bodies as observed with several other foreign proteins expressed in transgenic chloroplasts. These inclusion bodies are easily separated by centrifugation. Another option is use of ammonium sulphate for the precipitation of the protein.

Optionally, a His-tag with an enterokinase cut site was added to the above construct. The His-tag allows for purification on a nickel column with subsequent cleavage of the fusion protein from the His-tag.

The plasmid pHW731 (a pET-24 vector) carrying the gene for the F1V fusion protein was delivered in BLR strain of E. coli. Because of the exonuclease activity in BLR XL1-blue strain of E.coli was transformed with pHW731. Using NdeI and NotI, F1V was cut out of pHW731 and ligated into PCR2.1 with 5' psbA. In order to ligate 5' psbA-F1V into the universal chloroplast vector, pLD-CIV, PCR2.1-5' psbA-F1V was cut with SaeI, and blunt ended, then cut with NotI. This was ligated into pLD-CIV which had been cut with EcoRV (blunt end) and NotI. This produced the chloroplast vector pLD-FIV contained the 5'UTR psbA upstream of F1V, which was then used for bombardment. The use of the psbA 5'UTR has proven to increase expression of foreign proteins by chloroplasts.

FIG. 13B shows PCR restriction enzyme analysis of pLDS-FIV with XhoI, EcoRl, and NdeI, which showed that the psbA-F1V sequence to be in proper orientation in pL.D. (XhoI yielding: 340 bp, 2679 bp, and 4634 bp: EcoR1 yielding 682 bp, and 6953 bp: and XhoI/NdeI yielding 340 bp, 925 bp, 1102 bp, 1620 bp, 3610 bp, and incomplete digestion at 2601 bp, and 4550 bp). Lane 1: 1 KB Ladder, Lane 2: pLDS 37C, Lane 3: pLDS 4C, Lane 4: EcoR1, Lane 5: XhoI, Lane 6: XhoI/NdeI (overnight), Lane 7: XhoI/NdeI. The sequencing of pLDS-(5' psbA)-F1V using the 5'UTR primer, which lands on the 5' psbA, showed no changes during vector construction.

FIGS. 14A and 14B show PCR confirmation of transgene integration into the chloroplast genome. After bombardering tobacco leaves with pLDS-FIV there are three possibilities that might produce shoots: chloroplast transgenic, nuclear transgenic, and mutants resistant to spectinomycin. In order to select chloroplast transgenic plants we utilize two PCR reactions. The first (FIG. 14A), which checks for chloroplast integration, uses 3P and 3M primers which land on the native chloroplast genome and the aarA gene, respectively. Nuclear transformants are screened out because 3P will not anneal. Mutants are screened out
because 3M will not anneal. Positive chloroplast transformants produce a 1.65 Kb PCR product.

The second PCR reaction (FIG. 14B) uses 5P which lands on the aadA gene and 2M which lands on trnA. This produces a PCR product of 1.65 kb+Insert (psbA=203 bp+Fl-V=1437 bp)=3.29 Kb. Plants 2-5, 8, and 10 clearly contain the transgene.

FIG. 15A shows the western blot of pLDS-F1V from XL1-Blue strain of _E. coli_, and 15B shows the western blot of plants 1 and 2.

Turning to FIG. 15A showing the Western blot of F1V expression in _E. coli_, the expression in _E. coli_ was detected by rabbit anti-F1 as the primary antibody and alkaline phosphatase labeled goat anti-rabbit IgG as the Western blot of F1V expression in _E. coli_ was detected by rabbit anti-F1 as the primary antibody and alkaline phosphatase labeled goat anti-rabbit IgG as the secondary antibody. Specifically the western blot in FIG. 15A shows: Lane 1: pLDS-F1V; Lane 2: F1antigen; Lane 3: V antigen; Lane 4: F1V fusion protein.

FIG. 15B illustrates the Western blot of F1V expression in plants as was detected by rabbit anti-F1 and anti-V as primary antibodies and alkaline phosphatase labeled goat anti-rabbit IgG as the secondary antibody. Controls and samples were boiled. Specifically the western blot in FIG. 15B shows: Lane 1: purified F1V fusion protein; Lane 2: Untransformed Petit Havana; Lane 3: Transformed plant line #1; Lane 4: Transformed plant line #2. From this western blot it is clear that transgenic line 2 has surpassed line 1 in growth and is very healthy confirming that the foreign protein is not toxic to plants.

Cloning F1-V Antigen into Tomato:
The F1-V antigen, which is a bacterial antigen, was cloned into the tomato plD vector between gene 10 and rps 16 terminator. This was discussed further above. In this case, the selectable marker, BADH, with psbA 5'UTR and psbA 3' follows the rps16 region. The second vector made contains F1-V attached to the carboxy terminus of CTB. CTB serves as a mucosal carrier for this plague protein.

Example 5

Oral Delivery of Recombinant Proteins via Cholera Toxin B Subunit (CTB)
The increased production of an efficient transmucosal carrier molecule and delivery system in chloroplasts of plants allows the production of plant based oral vaccines and fusion proteins with CTB. CTB has previously been expressed in nuclear transgenic plants at levels of 0.01 (leaves) to 0.3% (tubers) of the total soluble protein. To increase expression levels, the chloroplast genome was engineered to express the CTB gene. We observed expression of oligomeric CTB at levels of 4-5% of total soluble plant protein. PCR and Southern Blot analyses confirmed stable integration of the CTB gene into the chloroplast genome. Western blot analysis showed that transgenic chloroplast expressed CTB was antigenically identical to commercially available purified CTB antigen. Also, GM1-ganglioside binding assays confirm that chloroplast synthetized CTB binds to the intestinal membrane receptor of cholera toxin. Transgenic tobacco plants were morphologically indistinguishable from untransformed plants and the introduced gene was found to be stably inherited in the subsequent generation as confirmed by PCR and Southern blot analyses. Thus chloroplasts form disulfide bridges to assemble foreign proteins. Spontaneously forming CTB pentamers exhibit intact transcytosis to the external basolateral membrane of intestinal epithelium, and have been widely used as oral vaccine vehicles.

Example 6

Double Barrel Plastid Vectors

Chloroplast transformation has been accomplished only in a few Solanaceous crops so far. There are several challenges in extending this technology to other crops. So far, only green chloroplasts have been transformed in which the leaf has been used as the explant. However, for many crops, including monocots, cultured non-green cells or other non-green plant parts are used as explants. These non-green tissues contain proplastids instead of chloroplasts, in which gene expression and gene regulation systems are quite different. During transformation, transformed proplastids should develop into mature chloroplasts and transformed cells should survive the selection process during all stages of development. Therefore, the major challenge is to provide chloroplasts the ability to survive selection in the light and the dark, at different developmental stages. This is absolutely critical because only one or two chloroplasts are transformed in a plant cell and these plastids should have the ability to survive the selection pressure, multiply and establish themselves while all other untransformed plastids are eliminated in the selection process. The Double Barrel Plastid Vectors accomplish this by using genes coding for two different enzymes capable of detoxifying the same selectable marker (or spectrum of selectable markers), driven by regulatory signals that are functional in proplastids as well as in mature chloroplasts.

The plastid vector described here is one among several such examples (non-limiting example). The chloroplast flanking sequence contains appropriate coding sequences and a spacer region into which the transgene cassette is inserted. Any spacer sequence within the plastid genome could be targeted for transgene integration, including transcribed and transcriptionally silent spacer regions. Both aphA-6 and aphA-2 (nptII) genes code for enzymes that belong to the aminoglycoside phosphotransferase family but they originate from different prokaryotic organisms. Because of prokaryotic nature of the chloroplast genome, these genes are ideal for use in transgenic chloroplasts without any codon optimization. Genes of prokaryotic origin have been expressed at very high levels in transgenic chloroplasts (up to 47% of total soluble protein, DeCosta et al., 2001). Both enzymes have similar catalytic activity but the aphA-6 gene product has an extended ability to detoxify kanamycin and provides a wider spectrum of aminoglycoside detoxification, including amikacin. The advantage of choosing kanamycin as a selectable marker is that it has no natural resistance, unlike spectinomycin resistance observed in most monocots or spontaneous point mutation of the 16 S rRNA gene observed during the selection process. In addition, kanamycin is not in human clinical use as an antibiotic and several crops containing kanamycin resistant nuclear transgenes have been already approved by FDA for human consumption (e.g. flavor savors tomatoes) and currently in the market place.

As shown in FIG. 24, in this non-limiting example, all transgenes are regulated by the plastid Prn promoter; this 16S rRNA promoter drives the entire rRNA operon in the native chloroplast genome and contains binding sites for both the nuclear encoded and plastid encoded RNA poly...
merases. Therefore, this promoter is capable of functioning in both plastids and chloroplasts (green and non-green, in the light and dark). The aphA-6 gene is further regulated by the gene 10 5'UTR capable of efficient translation in the dark, in plastids present in non-green tissues (see GFP expression in plastids of non-green cells of corn and carrot in FIGS. 19 and 23 regulated by the 16S rRNA promoter and gene 10 UTR). The rp516 3'UTR has been used to stabilize aphA-6 gene transcripts. The aphA-2 (nptII) gene, on the other hand is regulated by the psbA promoter, 5' and 3' UTRs, which are light regulated and highly efficient in the dark, in plastids (see A. Fernandez-San Millan, A. Mingeo-Castel, M. Miller and H. Daniell, 2003. A chloroplast transgenic approach to hyper-express and purify Human Serum Albumin, a protein highly susceptible to proteolytic degradation. Plant Biotechnology Journal, in press). Therefore, a combination of both aphA-6 and aphA-2 genes, driven by regulatory signals in the light and in the dark in both plastids and chloroplasts, provides continuous protection for transformed plastids/chloroplasts around the clock from the selectable agent. The gene(s) of interest with appropriate regulatory signals (gene X) are inserted downstream or upstream of the double barrel selectable system. Because multiple transgenes are inserted within spacer regions (DeCosa et al 2001, Daniell & Dhingra, 2002), the number of transgenes inserted does not pose problems in transcription, transcript processing or translation of operons (WO 01/64024). Therefore, a combination of both aphA-6 and aphA-2 genes, driven by regulatory signals in the light and in the dark in both plastids and chloroplasts, provides continuous protection for transformed plastids/chloroplasts around the clock from the selectable agent. The gene(s) of interest with appropriate regulatory signals (gene X) are inserted downstream or upstream of the double barrel selectable system. Because multiple transgenes are inserted within spacer regions (DeCosa et al 2001, Daniell & Dhingra, 2002), the number of transgenes inserted does not pose problems in transcription, transcript processing or translation of operons (WO 01/64024). In a variation of this example, aphA-6 and aphA-2 genes, coupled with different transgenes are inserted at different spacer regions within the same chloroplast genome using appropriate flanking sequences and introduced via co- transformation of both vectors.

Example 7

Genetic Engineering of the Corn and Ryegrass Chloroplast Genomes

A. Transformation of Corn Chloroplast Genome

For genetic engineering of the corn chloroplast genome, corn specific sequences, flanking the targeted integration site in the corn chloroplast genome (tm1 and tmnA) were amplified with specific PCR primers and subcloned to flank the betaine aldehyde dehydrogenase (BADH) selectable marker, and green fluorescent protein (GFP) reporter gene expression cassette.

Callus cultures were initiated from aseptically excised immature zygotic embryos (1-2 mm in length), produced on self-pollinated ears of Hill (Fl) maize plants. Ears were maintained in light (16/8 hr photoperiod). Prior to bombardment, nodal sections were placed on the surface of a fresh medium in standard Petri plates (100x15 mm). Gold particles (0.6 gm) were then coated with plasmid DNA as follows: 50 µl of washed gold particles were mixed with 10 µl DNA (1 µg/µl), 50 µl of 2.5M CaCl2, 20 µl of 0.1M spermidine and vortexed. Particles were centrifuged for a few seconds at 3000 rpm and then the ethanol was poured off. Ethanol washing was repeated five times, then the pellet was resuspended in 30 µl of 100% ethanol and placed on ice until it was used for bombardment (the coated particles were used within 2 hours). Bombardment was carried out with the biolistic device PDS1000/He (BioRad) by loading the target sample at level 2 in the sample chamber under a partial vacuum (28 inches Hg). The callus cultures were bombarded with the maize chloroplast transformation vectors using 1100 psi rupture discs. Following bombardment, the explants were transferred to a fresh medium; plates were sealed with micropore tape and incubated in darkness at 25-28°C.

Selection

Selection was initiated two days after bombardment. The bombarded calli were transferred to callus induction medium containing 5-20 mM BA (betaine aldehyde) or 25-100 mg/l streptomycin. Selection was also carried out using 50-150 mM NaCl in combination with the BA to maintain osmotic pressure.

Regeneration

Regeneration was initiated 6 to 8 weeks after bombardment by transferring the calli to a medium R1 containing Ms salts and vitamins supplemented with 1.0 mg/l NAA (α-naphthalene acetic acid), 2% sucrose, 2 g/l myoinositol and 0.3% phytagel at pH 5.8. Regenerated plants were transferred to R2 containing ½ MS salts and vitamins, 3% sucrose and 0.3% phytagel at pH 5.8. Regenerated plants were maintained in light (16/8 hr photoperiod).

Shoot Multiplication

The Surface Sterilization and Germination of Corn Seeds

Corn seeds were surface sterilized in a solution containing 2.6% Sodium hypochlorite (prepared from commercial bleach) containing 0.1% Tween 20 for 20 minutes under continuous shaking, then rinsed four times in sterile distilled water. Seeds were grown on MS medium at pH 5.8 in darkness. Nodal sections were excised aseptically from three day old seedlings. The nodal sections appear as clear demarcations on the germinated seedlings and represent the seventh node. When excised, the nodal cross sections are 1.3 to 1.5 mm in length.

Particle Bombardment of Nodal Sections

Prior to bombardment, 20-30 nodal sections were placed in the center of each petri plate with acropitila end up. Bombardment was carried out with the maize chloroplast vectors, using 1100, 1300 and 1550 psi rupture discs. Multiple Shoot Induction and Selection

Nodal section explants are placed acropitila end up on shoot multiplication medium SM1 composed of Ms salts and vitamins, 1.0 mg/l 6BA (6-Benzyl amino purine), 3% sucrose and 5 g/l phytagel at pH 5.8 under continuous light.
at 25°C. Initiation of the shoot-tip clumps from the original shoot tips occurred 2 to 4 weeks after culture. Two days after bombardment, transformed nodal sections were transferred to shoot multiplication medium containing 5-20 mM BA or 50-100 µg/ml streptomycin selective agents. Subsequent subcultures at two week intervals were carried out by selecting, dividing and subculturing green clumps on selective shoot multiplication medium containing 5-20 mM BA or 25-100 µg/ml streptomycin.

Regeneration

The Multiple shoot clumps were regenerated by transferring them to regeneration medium M1 containing MS salts and Vitamins, 5 mg/l IBA and 3% sucrose at pH 5.8. The developed shoots were regenerated by transferring the shoot tip clumps to M2 medium containing 1/2 MS salts and vitamins, 3% sucrose and 3 g/l phytagel at pH 5.8. It should be further noted that all the regeneration media are supplemented with 5-20 mM BA or 25-100 µg/ml streptomycin as the selective agents.

To engineer the corn chloroplast genome free of antibiotic resistance genes, maize calli were bombarded with a chloroplast expression vector containing the green fluorescent protein (GFP) and the betaine aldehyde dehydrogenase (BADH) genes as selectable or screenable markers. To compare the betaine aldehyde (BA) selection with streptomycin, another chloroplast expression vector was constructed containing the aadA and the BADH genes. The number of putative transgenic events was higher on BA selection than on streptomycin. Transgenic corn tissues screened on BA were examined using a laser-scanning confocal microscope. The GFP fluorescence was observed throughout the somatic embryos of corn. Chloroplast transformation of corn provides a suitable avenue for the production of edible vaccines and oral delivery of biopharmaceuticals.

Corn Chloroplast Transformation Vector

Corn chloroplast transformation vector facilitates the integration of transgene into the inverted repeat (IR) region of the corn chloroplast genome. The vector pLD-Corn-BADH contains the chimeric aadA gene and the BADH gene driven by the constitutive 16 S rRNA promoter and regulated by the 3'UTR region of psbA gene from petunia plastid genome. In this construct both, aadA and BADH possess the chloroplast preferred ribosomal binding site, GGAGG. Another vector used for corn chloroplast transformation pLD-corn-UTR-BADH has the constitutive 16 S rRNA promoter driving the expression of the dicistron, but BADH is under the regulation of the promoter and the 5'UTR of the psbA gene and the 3'UTR of psbA gene, for enhanced expression. When green tissue or non-green embryogenic calli are used for introducing the transgene into the corn chloroplast genome, it is preferred to use the light regulated psbA promoter/UTR or 16 S rRNA promoter/gene 10 UTR, respectively.

Annual ryegrass chloroplast transformation vector facilitates the integration of transgene into the inverted repeat (IR) region of the annual ryegrass chloroplast genome. The vector pLD-Ryegrass-BADH contains the chimeric aadA gene and the BADH gene driven by the constitutive 16 S rRNA promoter and regulated by the 3'UTR region of psbA gene from petunia plastid genome. In this construct both, aadA and BADH possess the chloroplast preferred ribosomal binding site, GGAGG. Another vector used for ryegrass chloroplast transformation pLD-ryegrass-UTR-BADH has the constitutive 16 S rRNA promoter driving the expression of the dicistron, but BADH is under the regulation of the promoter and the 5'UTR of the psbA gene and the 3'UTR of psbA gene, for enhanced expression. When green tissue or non-green embryogenic calli are used for introducing the transgene into the corn chloroplast genome, it is preferred to use the light regulated psbA promoter/UTR or 16 S rRNA promoter/gene 10 UTR, respectively.

Vectors for Production of Edible Anthrax Vaccine in Transgenic Chloroplast of Corn or Ryegrass:

Studies have confirmed the role of PA as the major protective antigen in the humoral response but also indicate a significant contribution of LF and EF to immunoprotection. The LF amino terminal domain, amino acid residues 1-254 (27 kDa) contains all the information necessary for binding PA and mediating translocation, and this domain alone is nontoxic because the catalytic domain of LF, residues 255-776, is responsible for lethality. Titers of antibody to both PA and LF from mice immunized with the combination were 4 to 5 times greater than titers from mice immunized with either alone. Therefore we express the constructs LF27-PA63 (PA63 is the cleaved active form of PA), CTB-LF27 fusion proteins, and LF27 and PA independently within the same edible plant as a standard. The LF27-PA63 and CTB-LF27 constructs are expressed alone and together as an operon in corn and ryegrass. It has been demonstrated that Rotavirus enterotoxin proteins fused with CTB is processed via the MHC II pathway generating a strong T-cell response. Thus CTB-fusion proteins produced in plants are ideal for oral delivery. By expressing the CTB-LF27 and PA-LF27 we maximize immunity to lethal toxin challenge. This is because both Gm1 ganglioside and anthrax toxin receptor (ATR) can be bound by ligands and work synergistically for maximum immune response. A flexible hinge was introduced between fusion proteins to reduce steric hindrance. Specifically a glycine-proline-glycine-proline hinge between CTB-LF27 and proline-glycine-proline-glycine hinge between LF27-PA63 was used. The application of less frequently used codons in plants within the hinge peptide promotes translational arrest during the protein elongation process, facilitating subunit folding prior to translation. The efficiency of folding of some proteins is increased by controlled rates of translation in vivo.

Chloroplast Transformation Protocol for Corn and Ryegrass:

Using either immature embryos (IEs) or embryogenic callus derived from IEs as a target for biolistic gene transfer is a well-established procedure for stable integration into the nuclear genome of corn or ryegrass. For biolistic transfer of integrative chloroplast expression vectors, the gene transfer protocol is adjusted and smaller particle sizes (0.6 µm diameter) are used. Microprojectiles are coated with plasmid DNA (chloroplast vectors) and bombardments are carried
out with the biolistic device PDS1000/He (Bio-Rad) as is well-known in the art relating to the use of the “gene-gun.” Expression levels from chloroplast regulatory sequences and the size of the proplastids are limiting factors for the successful chloroplast transformation using non-green, embryogenic callus tissues as a target for the gene transfer. Therefore, it is most desirable, when using the present invention with plant species not tested here, to compare green shoot meristematic cultures with non-green embryogenic callus as target tissue for chloroplast transformation. Protocols for the establishment of these tissue types are reported for corn and the grasses and are established in the Alpeters laboratory at University of Florida at Gainesville for ryegrass.

The timing of gene transfer after culture initiation and the duration and level of selection affect transgenic events while reducing the number of chimeric plants and achieving homoplasmacy and are best evaluated empirically. BADH and aadA selectable markers are compared with the corresponding selective agents. Selection is to be maintained during the regeneration process of plants. Regenerated plants are then analyzed by PCR and Southern blot for integration in the corn or ryegrass plastome.

PCR is done using DNA isolated from control and transgenic plants in order to distinguish a) true chloroplast transformants from mutants and b) chloroplast transformants from nuclear transformants. In order to test chloroplast integration of the transgenes, the 3’ primer will anneal to the selectable marker gene while the 5’ primer will anneal to the native chloroplast genome. No PCR product is expected with nuclear transgenic plants or mutants using this set of primers. This screening is essential to eliminate mutants and nuclear transformants. Total DNA from wild-type and transgenic plants is isolated and used as a template for PCR reactions. Southern blots allow one skilled in the art to determine the copy number of the introduced foreign gene per cell as well as to test homoplasmacy. There are several thousand copies of the chloroplast genome present in each plant cell. When foreign genes are inserted into the chloroplast genome, not all chloroplasts will integrate foreign DNA resulting in heteroplasmacy. To ensure that only the transformed genome exists in transgenic plants (homoplasmacy), the selection process is continued. In order to confirm homoplasmacy at the end of the selection cycle, total DNA from transgenic plants is probed with the chloroplast restricted DNA from transgenic lines producing epitope tagged PA can be tested for susceptibility to cleavage by trypsin.

Recombinant PA proteins are then asayed for their functional activity in the J774A1 (American Type Culture Collection) macrophage lysis assay. Varying concentrations of PA protein along with LF (1 mg/ml) are added to the cells. The native PA along with LF is kept as the positive control. After 3 h, cell viability is determined using the MTT (3-(4,5-dimethyl thiazol-2-yl)-5-diphenyltetrazolium bromide) dye and the resulting precipitate is dissolved in a buffer containing 0.5% (w/v) sodium dodecyl sulfate, 25 mM HCl and stored frozen at -70° C. in suitable aliquots.

The binding of PA protein to cell surface receptor is analyzed in 24 well plates using constant amount of radioiodinated native PA (0.1 mg/ml), J774A.1 (ATCC) cells are washed twice with cold HBSS for 5 minutes each and then placed on ice. The medium is replaced with cold 25 mM Hepes, 1 mM CaCl2, 0.5 mM EDTA, pH 7.5. The digestion reaction is stopped by adding PMSF to a concentration of 1 mM. Trypsin nicked PA (1.0 mg/ml) is incubated with LF (1.0 mg/ml) and in 25 mM Tris, pH 9.0, containing 2 mg/ml CHAPS (3-(3-cholamidopropyl)dimethyl ammonio-)propanesulfonic acid) for 15 min at room temperature. Samples are applied to nondenaturing 4.5% polyacrylamide gel.

The binding of PA protein to cell surface receptor is analyzed in 24 well plates using constant amount of radioiodinated native PA (0.1 mg/ml), J774A.1 (ATCC) cells are washed twice with cold HBSS for 5 minutes each and then placed on ice. The medium is replaced with cold binding medium (DMEM, Dulbecco’s Modified Eagle Medium, without sodium bicarbonate containing 1% bovine serum albumin and 25 mM, Hepes, pH 7.4). The cells are incubated with 0.1 mg/ml of iodinated PA and varying concentrations of the recombinant PA protein at 4° C. for 3 h and then washed with cold HBSS. The cells are then dissolved in 0.1 N NaOH and radioactivity measured in Gamma counter.

Leaves from transgenic lines producing epitope tagged products are frozen and powdered at 4° C. using a micro-dismembrator and proteins are extracted in PBS with 1% Triton X-100. Fusion proteins are purified by affinity chromatography on a nickel-agarose bed, using standard 6-His methods, as described above.

Assessment of Immunogenic Properties of Transgenic Plant-derived PA:

Corn and ryegrass expressing PA as potential edible vaccines against anthrax are characterized using the protocol
described above. These are then evaluated for the ability of PA-expressing corn seeds or corn or ryegrass leaves or hay to function as edible vaccines for the induction of serum and mucosal (bronchial lavage, nasal, vaginal, and fecal) antibodies by ELISA. Antibodies induced by feeding the transgenic corn or ryegrass to mammals neutralize the biologic activity of anthrax lethal toxin. This activity can be confirmed in an in vitro macrophage cytotoxicity assay. Antibody responses in mice and humans following ingestion of transgenic potatoes and corn expressing recombinant bacterial proteins have been successfully demonstrated.

Oral immunization of mice and other mammals by feeding transgenic plants or plant parts is accomplished as follows. In the case of corn and ryegrass, female BALB/c mice are fed transgenic corn or ryegrass, control corn or ryegrass, or soluble rPA in conjunction with the mucosal adjuvant LT(R192G). The amount of rPA fed to control animals is based upon the amount of PA in the transgenic corn or ryegrass fed to the animals. That amount correlates with the amount of transgenic corn or ryegrass a mouse will consume in a one hour period. Mice tend to eat grass if a small amount of vanilla extract is placed on each leaf. Two additional groups can be included in which the mucosal adjuvant LT(R192G) is administered in conjunction with the transgenic or control corn or ryegrass. Edible vaccines administered to mice often require the presence of a mucosal adjuvant due to the small amount of material that can be consumed by a mouse. However, this is not necessary when using the plants of the present invention to vaccinate humans, or other large mammals due to the volume which can be consumed by the animal. Twenty-five micrograms of the adjuvant should be applied directly to the corn or ryegrass before consumption when testing mice.

Intranasal immunization is accomplished in mice as follows. Mice are first lightly anesthetized with Isoflurane for 35 seconds. The immunizing inoculum (5-10 ml per animal/per dose) is delivered intranasally to the external nares of one nostril with a pipette tip.

Oral immunization: Oral inoculations consisted of 500 ml of the antigen preparation in saline delivered intragastrically with a blunt-tip feeding needle (Popper & Sons, Inc.). Sample collection: Animals are sacrificed following euthanasia by CO₂ inhalation. Blood is collected by cardiac puncture and the serum is separated in Microtainer tubes. Bronchoalveolar lavages (BAL) are obtained by inserting a 45 cm 20 G cannula in the exposed trachea and injecting 1 ml of PBS supplemented with protease inhibitors. The buffer is allowed to bathe the lung for approximately 20 seconds and then it is suctioned out; this procedure is repeated three times in each mouse. The resulting BAL fluid is immediately centrifuged (400 g, 2 min, 4 °C.) and the supernatant is saved. To obtain nasal lavages a flexible 24 gauge cannula is inserted into the posterior opening of the nasopharynx and a total of 150 ml ml PBS+ protease inhibitor is injected into the opening. The outflow is collected as the nasal wash. Vaginal washes are obtained by washing the vaginal mucosa three times with 50 μl of PBS containing 0.01% NaN₃. For determination of fecal IgA, feces are collected and frozen overnight at -70 °C, lyophilized, resuspended in 800 μl PBS containing 0.05% sodium azide per 15 fecal pellets, centrifuged at 1,400xg for 5 minutes, and the supernatant stored at -20 °C until assayed.

Evaluation of humoral and mucosal antibodies: Each serum, BAL, nasal wash, vaginal wash, and fecal extract sample is individually analyzed by ELISA. For all ELISA assays, 96-well plates are coated with 500 ng per well of rPA and incubated overnight at 4 °C. All subsequent steps are carried out at room temperature. After blocking with 1% BSA, twofold serial dilutions of serum, BAL, nasal wash, vaginal wash, or fecal extract from the experimental animals are added. Alkaline phosphatase conjugated rabbit anti-mouse IgG or anti-mouse IgA are used for detection of total IgG or IgA. Biotinylated anti-mouse IgG1, IgG2a, IgG2b or IgG3 followed by alkaline phosphatase conjugated streptavidin are used to quantify antibody isotypes. Optical density at 405 nm is determined using an ELISA reader.

REFERENCES

The following references, along with all other references mentioned herein, and patent applications to which this application may claim priority, are incorporated herein by reference in their entirety.

10. Arntzen, C. www.bio.org/food%26ag/vaccine.htm
11. Arntzen estimates the banana could deliver the vaccine at 2 cents a dose verses $125.00 for an injection.


plague by a recombinant capsular F1-V antigen fusion protein vaccine. Vaccine. 16 (11/12) 1131-1137.


149. Tsafir, T., Breeding volunteers of an oral, inactivated whole cell transformation in tobacco by selection for a chimeric heat-labile enterotoxin adjuvant-LT(R192G); 1997.
What is claimed is:

1. A plastid transformation vector for stably transforming a plastid, said plastid vector comprising, as operably linked components, a first flanking sequence, at least one regulatory sequence operable in a plastid, a DNA sequence coding for a protective antigen, a DNA sequence coding for a chaperonin, and a second flanking sequence.

2. A plastid transformation vector for stably transforming a plastid, said plastid vector comprising, as operably linked components, a first flanking sequence, at least one regulatory sequence operable in a plastid, a DNA sequence coding for a protective antigen, a DNA sequence coding for a chaperonin, and a second flanking sequence, wherein said protective antigen is a bacterial antigen.
It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Specification

Column 1, line 4, under the Title of the Invention section, please insert the following:

--STATEMENT OF FEDERALLY FUNDED RESEARCH

The invention was made with government support under grant no. USDA/ARS 58-3611-2-106 awarded by the United States Department of Agriculture. The government has certain rights in the invention.--

Signed and Sealed this
Seventeenth Day of September, 2013

[Signature]

Teresa Stanek Rea
Deputy Director of the United States Patent and Trademark Office