A protocol for expression of foreign genes in chloroplasts

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Several major costs associated with the production of biopharmaceuticals or vaccines in fermentation-based systems could be minimized by using plant chloroplasts as bioreactors, which facilitates rapid scale-up. Oral delivery of chloroplast-derived therapeutic proteins through plant cells eliminates expensive purification steps, low temperature storage, transportation and sterile injections for their delivery. Chloroplast transformation technology (CTT) has also been successfully used to engineer valuable agronomic traits and for the production of industrial enzymes and biomaterials. Here, we provide a detailed protocol for the construction of chloroplast expression and integration vectors, selection and regeneration of transformants, evaluation of transgene integration and inheritance, confirmation of transgene expression and extraction, and quantitation and purification of foreign proteins. Integration of appropriate transgenes into chloroplast genomes and the resulting high levels of functional protein expression can be achieved in ~6 months in lettuce and tobacco. CTT is eco-friendly because transgenes are maternally inherited in most crop plants.

INTRODUCTION

Plants have emerged as a viable alternative to microbial fermentation and mammalian cell culture for industrial production of biopharmaceuticals; they offer rapid scale-up potential and lower production costs and are free of human pathogens and toxins. For example, it is possible to produce up to 360 million doses of an anthrax vaccine in one acre of tobacco[1]. The combination of maternal inheritance of transgenic chloroplast genomes in most crops[2–4] with cytoplasmic male sterility[5] minimizes the risk of cross-pollination with non-genetically modified crops or their relatives in the wild. Indeed, chloroplast transgenic plants producing human therapeutic proteins have been grown and tested in the field[6].

When stably integrated into the chloroplast genome, transgenes express large amounts of foreign proteins[7]. This is due to the presence of up to 10,000 copies of the chloroplast genome in each plant cell[8,9]. Transgenes integrate at a precise location in the genome by homologous recombination, which is mediated by flanking chloroplast DNA sequences present in the chloroplast vector; this eliminates position effects frequently observed in nuclear transgenic lines[9]. Although techniques have been developed for targeted integration into the nuclear genome[10], these have not yet been used to develop useful traits. Other advantages of transplastomic plants over nuclear transgenic plants include lack of transgene silencing, despite 169-fold higher levels of transgene transcript than in nuclear transgenic plants[11,12], and foreign protein levels up to 46% (wt/wt) of total leaf protein[7]. Multivalent vaccines can be engineered in a single transformation step because polycistrons are translated without processing into monocistrons; several heterologous operons have been expressed in transgenic chloroplasts[13].

It is important that foreign proteins expressed in chloroplasts are post-translationally modified and processed like native proteins. Several human therapeutic proteins have been successfully expressed in transgenic chloroplasts; appropriate functional assays on a range of foreign proteins expressed in chloroplasts have been shown to form disulfide bonds and to be folded correctly[6,14–16]. Similarly, several chloroplast-derived vaccine antigens have been shown to form the correct disulfide bonds and assemble as heterodimers, pentamers (or other suitable configurations)[17–19] or to contain the appropriate lipid modifications[20]. These observations confirm that the chloroplasts have the machinery to fold complex proteins in the chloroplast stroma and form disulfide bonds or other post-translational modifications required for their functionality. Expression of glycoproteins with glycosylation identical to that occurring in humans has been a major challenge in nuclear transformation of plant cells, when targeting such proteins to the endoplasmic reticulum. However, glycosylation does not occur in chloroplasts, which provides a unique opportunity to express therapeutic proteins free of glycosylation.

The tobacco plant is often chosen because of its large biomass, yielding ~170 metric tons of biomass per hectare[21,22]. In addition, it is easy to engineer the tobacco chloroplast genome and regenerate transgenic lines within a few months. Each transgenic plant is capable of producing 1 million seeds and, therefore, it is possible to scale-up from a single transgenic plant to 100 acres within 1 year. Tobacco is a nonfood and nonfeed crop and is self-pollinated, which minimizes transgene escape. Most important, the expression of foreign protein in leaves facilitates their harvest before appearance of any reproductive structures, offering total biological containment of transgenes.

Design of chloroplast vectors

A chloroplast transformation vector contains several critical elements; a schematic diagram of the standard vector design used in our lab is shown in **Figure 1**. Two distinct components are

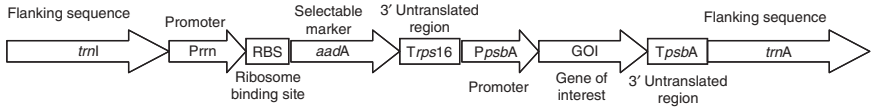

Figure 1 | Schematic representation of the chloroplast integration and expression cassette. This map of chloroplast-expression vector shows the flanking sequence, promoter, regulatory elements, selectable marker gene and the gene of interest.

required to construct the final transformation vector: a vector containing the flanking (targeting) sequence and the sequences required for efficient transgene expression (expression cassette). Guidance on how to construct these components is provided in the Constructing the targeting vector section. While this overall design is effective across most species, the specific sequences should be derived from the chloroplast genome being targeted to ensure that the transgene is integrated and expressed with maximum efficiency. Fully annotated crop plastid genome sequences (**Table 1**) can be found at http://www.bch.umontreal.ca/ogmp/projects/other/cp_list.html, http://www.ncbi.nlm.nih.gov/genomes/static/euk_o.html and http://chloroplast.cbio.psu.edu/cgi-bin/organism.cgi. GenBank (http://www.ncbi.nlm.nih.gov) is also a very useful source of information for designing and constructing plastid transformation vectors. For the purposes of the procedure detailed in this protocol, it is assumed that the required sequence components have been previously assembled; the starting point is the cloning of the expression cassette into the targeting vector to produce the final transformation vector.

Constructing the targeting vector

This vector contains sequence from the chloroplast genome that is homologous to the desired site of integration; it facilitates site-specific recombination and defines the integration site of the transgene. Thus, the sequence must be specific to the plastid genome being targeted and can be PCR-amplified from the relevant genome using suitably designed primers. Attempts have been made to transform potato[23] and tomato[24] plastid genomes using targeting sequences from tobacco but the efficiency of transformation was significantly lower than that observed in tobacco, although some species are inherently more challenging to transform than others. Targeting sequences are generally 1 kb in size and are located on either side of the expression cassette, which is inserted using a suitable restriction enzyme in the spacer region of the targeting

TABLE 1 | Alphabetical list of complete annotated plastid genome sequences of crop plants.

Species	Common name	Accession number	Genome size (bp)
Agrostis stolonifera	Creeping bent grass	NC_008591	136,584
Citrus sinensis	Orange	NC_008334	160,129
Coffea arabica	Coffee	NC_008535	155,189
Cucumis sativus	Cucumber	NC_007144	155,293
		DQ119058	155,527
Daucus carota	Carrot	NC_008325	155,911
Eucalyptus globulus	Eucalyptus	NC_008115	160,286
Glycine max	Soybean	NC_007942	152,218
Gossypium barbadense	Sea Island cotton	NC_008641	160,316
Gossypium hirsutum	Upland cotton	NC_007944	160,301
Helianthus annuus	Sunflower	NC_007977	151,104
Hordeum vulgare	Barley	NC_008590	136,462
Lactuca sativa	Lettuce	NC_007578	152,765
		DQ383816	152,772
Manihot esculenta	Cassava	EU117376	161,453
Nicotiana tabacum	Tobacco	Z00044	155,939
Oryza nivara	Indian wild rice	NC_005973	134,494
Oryza sativa	Rice	X15901	134,525
		AY522329	134,496
		AY522331	134,551
Panax ginseng	Ginseng	NC_006290	156,318
Phaseolus vulgaris	Kidney bean	NC_009259	150,285
Pinus koraiensis	Korean pine	NC_004677	117,190
Pinus thunbergii	Japanese black pine	NC_001631	119,707
Populus alba	White poplar	NC_008235	156,505
Populus trichocarpa	Black cottonwood	NC_009143	157,033
Saccharum hybrid	Sugarcane hybrid	NC_005878	141,182
Saccharum officinarum	Sugarcane	NC_006084	141,182
Solanum bulbocastanum	Ornamental nightshade	NC_007943	155,371
Solanum lycopersicum	Tomato	NC_007898	155,461
Solanum tuberosum	Potato	NC_008096	155,298
		DQ231562	155,312
Sorghum bicolor	Sorghum	NC_008602	140,754
Spinacia oleracea	Spinach	NC_002202	150,725
Triticum aestivum	Bread wheat	NC_002762	134,545
Vitis vinifera	Wine grape	NC_007957	160,928
Zea mays	Maize	NC_001666	140,384

Intergenic spacer regions and regulatory sequences (promoters, 5′ UTRs and 3′ UTRs) provided here are essential for construction of plastid transformation vectors (see http://www.bch.umontreal.ca/ogmp/projects/other/cp_list.html or http://www.ncbi.nlm.nih.gov/genomes/static/euk_o.html or http://chloroplast.cbio.psu.edu/cgi-bin/organism.cgi for access to genomic sequences).

TABLE 2 | Crops in which successful chloroplast transformation has been reported.

Crop	Site of integration	Marker/gene of interest	References
Carrot	*trn*I/*trn*A	*aad*A, *badh*	37
Cauliflower	*acc*D/*rbc*L	*aad*A	57
Cotton	*trn*I/*trn*A	*aph*A6, *npt*II	58
Lettuce	*trn*I/*trn*A	*aad*A, *gfp*	59
Lettuce	*acc*D/*rbc*L	*aad*A, *gfp*	48
Lettuce	*trn*I/*trn*A	*aad*A, *CTB-Pris*	16
Oilseed rape	*rps*7/*ndh*B	*aad*A, *cry*1Aa10	60
Petunia	*acc*D/*rbc*L	*aad*A, *uid*A	61
Poplar	*acc*D/*rbc*L	*aad*A/*gfp*	62
Potato	*acc*D/*rbc*L *rrn*16 *trn*V/3′*rps*7/12	*aad*A, *gfp*	23
Potato	*acc*D/*rbc*L *rrn*16 *trn*V/3′*rps*7/12	*aad*A, *gfp*	63
Rice	*trn*I/*trn*A	*aad*A, *gfp*	64
Soybean	*trn*V/3′*rps*7/12	*aad*A	47
Tobacco	*acc*D/*rbc*L	*aad*A	36
Tobacco	*trn*I/*trn*A	*aad*A, *aro*A	65
Tomato	*trn*fM/*trn*G	*aad*A	24

sequence. Transgenes may be integrated into three types of spacer regions. Transcriptionally silent spacer regions are found at sites where chloroplast genes are located on opposite DNA strands. Read-through spacer regions are found between chloroplast genes located on the same strand and where each gene has its own promoter. Transcriptionally active spacer regions are found in chloroplast operons in which a single promoter drives transcription of several genes. The region most commonly used is the transcriptionally active spacer region between the *trn*I and *trn*A genes. This region is located within the rRNA operon, where the 16S rRNA promoter drives transcription of six genes and each spacer region within this operon is transcriptionally active (**Fig. 1**). The *trn*I gene intron also contains a chloroplast origin of replication, which might facilitate replication of foreign vectors within chloroplasts and enhance the probability of transgene integration[25,26]. Transcriptionally active spacer regions also offer the unique advantage that transgenes lacking promoters or 5′- or 3′-untranslated regions (UTRs) can be inserted and expressed. However, other spacer regions (transcriptionally silent or read-through) may also be used; some examples of integration sites used in a range of crops are listed in **Table 2**. A schematic representation of how new targeting vectors are constructed in our lab is provided in **Figures 2** and **3**.

Constructing the expression cassette

The expression cassette includes a chloroplast operon promoter, ribosome-binding sequences, a selectable marker gene (e.g., antibiotic resistance), a 3′-UTR, gene promoter, a 5′-UTR, the gene of interest (GOI) and its 3′ UTR. The necessary regulatory sequences are PCR-amplified from the genome of interest using primers containing suitable restriction sites, sequenced to identify any errors introduced by PCR and assembled by consecutive rounds of restriction digestion and ligation; the GOI is inserted between the 5′-UTR and the 3′-UTR (**Fig. 3a**). For speed and ease of future cloning, the regulatory cassette (before insertion of the GOI) can be cloned into a suitable vector and maintained. The entire expression cassette (including the GOI) can be inserted into the appropriate restriction site within the targeting sequence to produce the final transformation vector (**Fig. 3b**). In the case of the *trn*I–*trn*A spacer region, a unique *Pvu*II site is used; this *Pvu*II site is present in most of the sequenced chloroplast genomes.

Gene expression in plastids is regulated at both transcriptional and post-transcriptional levels. Protein levels in chloroplasts depend on mRNA abundance, which is determined by promoter strength and mRNA stability. However, high mRNA levels do not necessarily result in high levels of protein accumulation, as post-transcriptional processes ultimately determine levels of foreign proteins within transgenic chloroplasts. Usually, the strong plastid rRNA operon promoter (Prrn) is used to drive transgene expression. In tobacco, the Prrn promoter has binding sites for both the nuclear- and plastid-encoded RNA polymerases[27]. The mRNA is stabilized by a 3′ UTR, usually derived from the *psb*A, *rbc*L or *rps*16 genes; using an alternative 3′ UTR will only marginally improve protein accumulation, as it has been shown that transgenic lines with and without 3′ UTRs accumulate the same level of foreign proteins in transgenic chloroplasts[28]. Therefore, the major focus is on the 5′ UTR to enhance foreign protein accumulation. The most commonly used 5′ regulatory sequences are derived from the *psb*A and *rbc*L genes. The use of 5′ *psb*A UTR is not advised when high-level expression of foreign proteins has deleterious phenotypic effects on transplastomic plants[29]. In this case, a compromise on the level of gene expression must be reached or an inducible expression system should be used. A lac repressor-based inducible expression system has been reported, but transgene repression in the uninduced state is incomplete[30].

The GOI can be expressed with any tag in chloroplasts to facilitate purification. To date, only N-terminal His tags (with protease cleavage sites for removal of tags) have been used to purify chloroplast-derived vaccine antigens or biopharmaceuticals[1,6]. Other approaches used for purification of proteins expressed from chloroplast vectors include fusion of the foreign protein to a protein-based polymer and utilization of the inverse temperature transition property of this polymer for purification[31].

Methods of plastome transformation and plant regeneration

Plastome (plastid genome) transformation was initially thought to be almost impossible due to the physical barrier imposed by the double membrane of the chloroplast envelope. Moreover, there are no viruses or bacteria known to infect chloroplasts that could be used as vectors for gene transfer. Therefore, early attempts to transform plastids involved the introduction of foreign DNA into isolated intact chloroplasts, which could then be re-introduced into protoplasts to obtain transgenic plants[32].

The development of gene gun and biolistic technology has enabled the delivery of foreign DNA directly into living cells. Particle bombardment, in which small gold or tungsten particles are coated with DNA and shot into young plant cells (leaf or callus tissue), is now the most widely used and effective method for transforming plastids. The first successful chloroplast transformation using this method was reported in *Chlamydomonas* by complementation of a native gene fragment in a deletion mutant[33].

An alternative approach is PEG-mediated transformation[34,35]. After protoplast isolation, PEG incubation with foreign DNA facilitates entry of DNA through cell and chloroplast membranes. However, this technique has a lower success rate than biolistic transformation and is not often reproducible.

After transformation, transplastomic plants are regenerated either by direct organogenesis or somatic embryogenesis. Regeneration by direct organogenesis is relatively straightforward and was first achieved in tobacco[36], followed by several other crops (**Table 2**). Regeneration in the first, second or third round of selection via direct organogenesis follows the same time frame in lettuce and tobacco[16]. However, regeneration of homoplasmic transplastomic plants through somatic embryogenesis continues to remain a major challenge because homoplasmy must be achieved before initiation of differentiation in embryogenic cells.

The first stable plastid transformation of nongreen embryogenic cell cultures and regeneration via somatic embryogenesis was established in carrot[37] and then extended to soybean, cotton and rice (**Table 2**). Foreign gene expression has been observed in nongreen tissues such as microtuber[23] (potato), fruit[24] (tomato) and root[37] (carrot). Nongreen plastids (amyloplasts and chromoplasts) are generally known to be less active in gene expression than chloroplasts in photosynthetically active leaves. However, there are currently no well-characterized chloroplast regulatory elements that express in nongreen plastids that can be used to achieve high levels of transgene expression. Recently, the levels of transcription, mRNA accumulation and formation of polysomes were analyzed in amyloplasts of potato tuber. The transcriptional activity was reduced in amyloplasts when compared with chloroplasts, whereas there were small differences in the specific transcript levels. Also, the polysomes associated with the specific transcripts could not be detected[38]. Therefore, further study of gene expression in nongreen plastids is required.

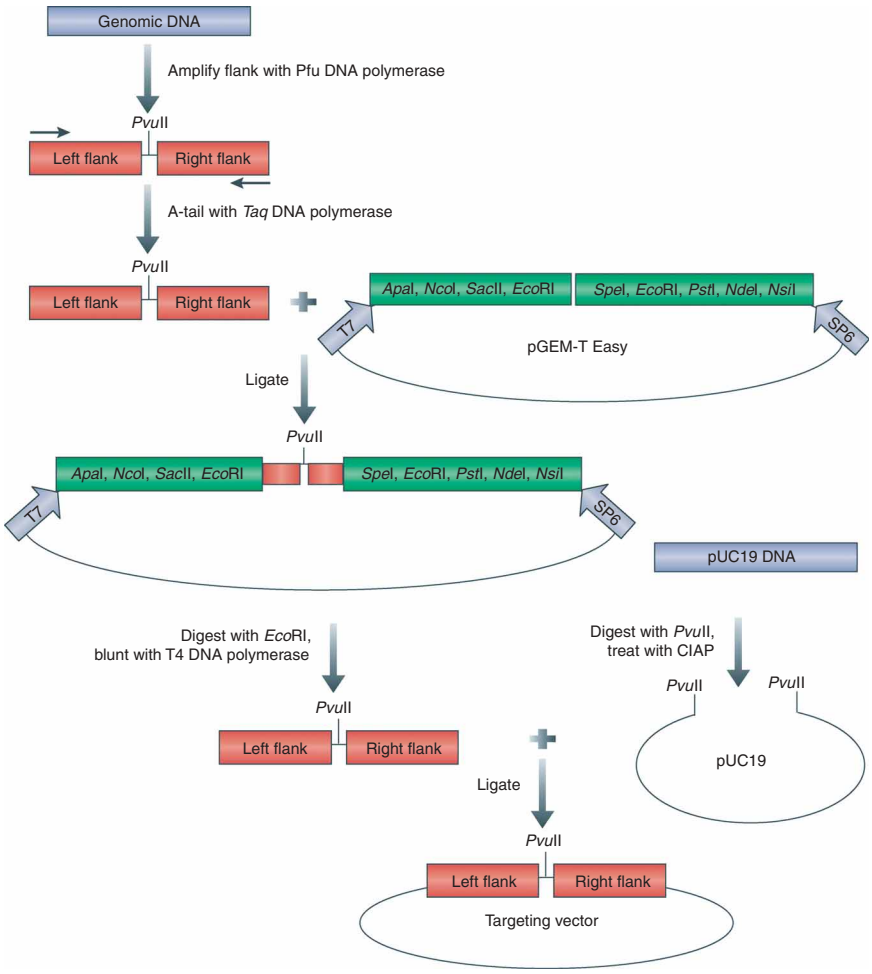

Figure 2 | Schematic representation of cloning process to obtain a plastome-specific targeting vector. Step-by-step procedure to clone chloroplast targeting sequence into the pUC19 vector involves amplification of species-specific chloroplast flanking sequence from genomic DNA. The amplified flanking sequence is A-tailed by *Taq* DNA polymerase and ligated to pGEM-T Easy vector. The cloned flanking sequence is digested with *Eco*RI in this example (restriction site may be different for different plants) and treated with T4 DNA polymerase to make ends blunt. This enables ligation to the blunt-ended *Pvu*II site within the pUC19 vector, resulting in the chloroplast site-specific targeting vector.

Applications of protein expression in chloroplasts

Engineering plants with improved agronomic traits Several foreign genes have been used to engineer agronomic traits via the chloroplast genome, including insect and pathogen resistance, drought and salt tolerance, phytoremediation and cytoplasmic male sterility (**Table 3**). Insecticidal proteins expressed via the chloroplast genome showed the highest levels of expression reported in transgenic plants (up to 46.1% wt/wt of total leaf protein), formed cuboidal crystals[7] and showed 100% mortality when transplastomic leaves were fed to susceptible or resistant insects (up to 40,000-fold resistance was created by breeding insects resistant to the insecticidal protein). Similarly, transplastomic

plants conferred the highest levels of protection against biotic or abiotic stress reported in the literature.

Expression of therapeutic proteins and vaccines Several vaccine antigens have been expressed in tobacco chloroplasts, including the cholera toxin B (CTB) subunit of *Vibrio cholerae*[17], the anthrax protective antigen[1,39], the C terminus of *Clostridium tetani*[40,41], the 2L21 peptide from the canine parvovirus[42,43] and the *Lec*A from *Entamoeba histolytica*[18] (**Table 4**). Tobacco chloroplasts have also been used for the production of valuable therapeutic proteins, such as human somatotropin[14], human serum albumin[44], a broad-spectrum topical agent, systemic antibiotic, wound-healing stimulant, and a potential anticancer agent[45], type I interferons[6,15] and human proinsulin[16] (**Table 5**). Most of these chloroplast-derived proteins have proven to be functional by appropriate tests *in vitro* or in animal studies[4,46].

Soybean, lettuce and carrot plastid transformation have been accomplished using species-specific vectors[37,47,48]. A human therapeutic protein has already been stably expressed in lettuce chloroplasts[16]. Oral administration of transplastomic leaves expressing a CTB–proinsulin fusion protein conferred protection against the development of insulitis in nonobese diabetic mice[16]. These studies open the door for oral therapeutic delivery through plant cells, significantly reducing the cost of purification, processing, cold storage, transportation and sterile delivery. Engineering of the chloroplast genome for expression of vaccine antigens or biopharmaceuticals has ushered in a new era in biotechnology[46,49,50].

Expression of industrially valuable biomaterials In addition to therapeutic proteins and vaccines, plastid genetic engineering offers an ideal opportunity for the cost-effective production of industrially valuable biomaterials (**Table 6**). The successful engineering of several pathways and enzymes has resulted in the effective production of many important biomaterials. For example the *xyn*A gene, which encodes xylanase, was transformed into the tobacco chloroplast genome; the resulting protein was as biologically active as the bacterial-derived enzyme and retained its substrate specificity[51]. Liquid crystal polymer can also be produced to very high levels—up to 25% wt/wt of plant dry weight[52].

Here, we provide a protocol to construct transformation vectors, transform chloroplasts, regenerate transgenic plants using tissue culture, select putative transformants, characterize transgenic plants, extract total proteins, evaluate transgene expression, quantitate foreign proteins and purify foreign proteins using tags. These protocols have been used, and their efficacy and reproducibility have been evaluated, and published in peer-reviewed scientific literature, as mentioned earlier.

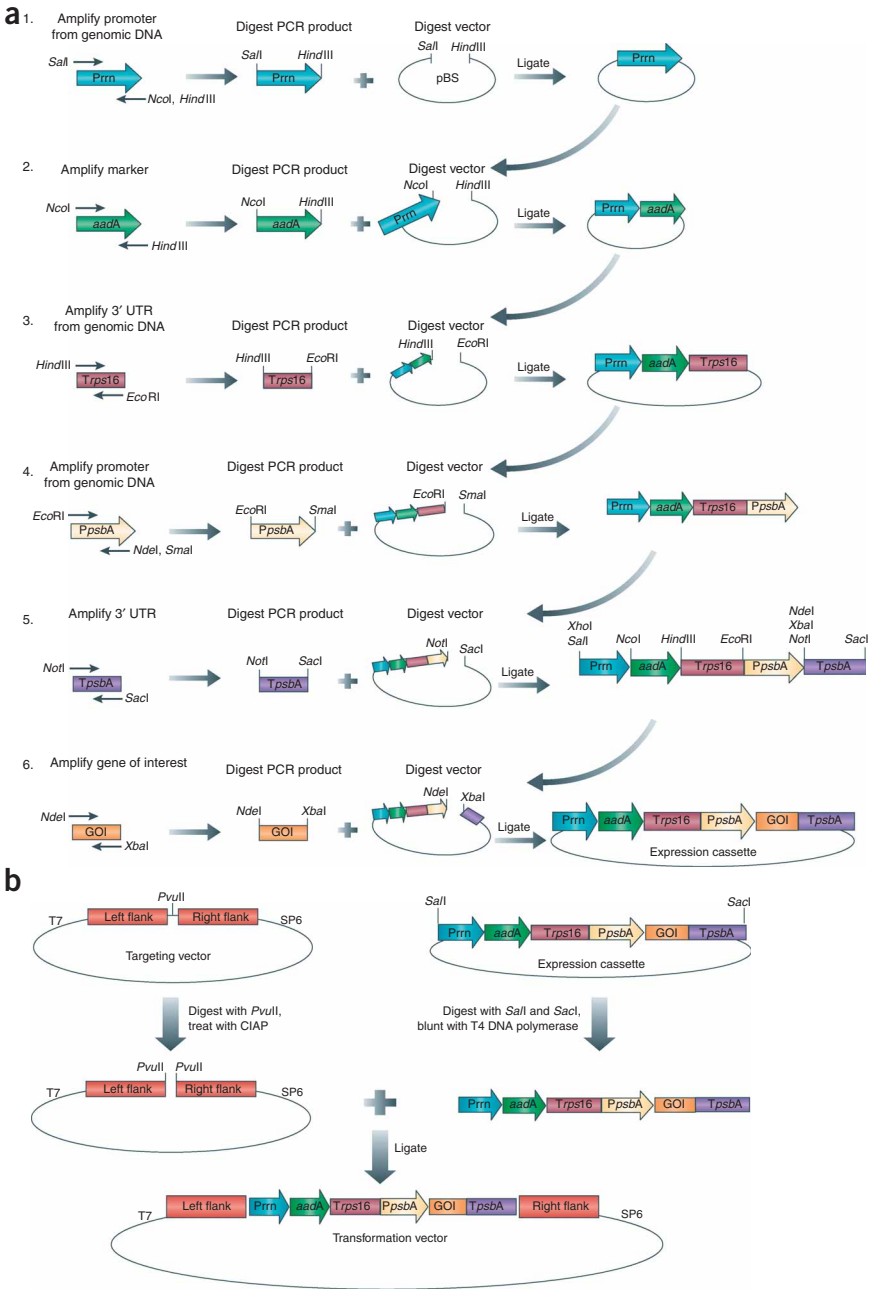

Figure 3 | Schematic representation of the cloning process to obtain a chloroplast transformation vector. (**a**) Steps involved in the creation of gene expression cassette with suitable chloroplast-specific promoters and regulatory elements, amplified from the total cellular DNA using primers designed on the basis of the chloroplast genome sequence information available in the GenBank (provided in **Table 1**). Strategy may vary for different plants, depending on the restriction map of the regulatory elements and the gene of interest. Make sure to include the ribosome-binding site in reverse primer of the Prrn promoter. P*psb*A represents promoter and 5′-UTR. (**b**) Steps involved in the construction of final transformation vector containing species-specific chloroplast flanking sequence and transgene expression cassette.

TABLE 3 | Engineering of agronomic traits via the plastid genome.

Agronomic trait	Gene	Promoter/ 5′/3′ UTRs	Site of integration	Characteristics	References
Insect resistance	*cry*1A(c)	P*rrn*/*rbc*L/*rps*16	*trn*V/ *rps*12/7	Transgenic tobacco leaves accumulated 3–5% of TSP (total soluble protein) as CryIA(c) and were toxic to larvae of *Heliothis virescens*, *Helicoverpa zea* and *Spodoptera exigua*	66
	*cry*2Aa2	P*rrn*/ggagg (native)/*psb*A	*rbc*L/*acc*D	Cry2Aa2 is expressed between 2 and 3% of TSP. When transgenic tobacco leaves were fed to susceptible, Cry1A-resistant (20,000- to 40,000-fold) and Cry2Aa2-resistant larvae of *Heliothis virescens*, *Helicoverpa zea* and *Spodoptera exigua*, 100% mortality was observed against all insect species	67
	*cry*2Aa2 operon	P*rrn*/native 5′ UTR/*psb*A	*trn*I/*trn*A	Cry2Aa2 protein accumulated at 45.3% of TSP and tobacco leaves were toxic (100% mortality) to larvae of *Heliothis virescens*, *Helicoverpa zea* and *Spodoptera exigua*. Electron micrographs showed the cuboidal crystals of insecticidal protein, which enhances stability by protecting it from cellular proteases	7
	*cry*1Aa10	P*rrn*/native 5′ UTR/*psb*A	*rps*7/*ndh*B	Transgenic oilseed rape leaf material was toxic to *Plutella xylostera* larvae, causing 33–47% mortality in 5 d with slight leaf damage	60
	*cry*1Ab	P*rrn*/T7 *gene10*/ *rbc*L	*trn*V/ *rps*12/7	Transgenic soybean plants toxic to caterpillar of *Anticarsia gemmatalis*	68
	*cry*9Aa2	P*rrn*/native 5′ UTR/*rbc*L	*trn*I/*trn*A	Cry9Aa2 accumulated at ~10% of TSP in tobacco and conferred resistance to *Phthorimaea operculella*	69
Herbicide resistance	*aro*A (petunia)	P*rrn*/ggagg/ *psb*A	*rbc*L/*acc*D *trn*I/*trn*A	Transgenic tobacco plants survived concentrations as high as 5 mM glyphosate, whereas untransformed control tobacco plants were extremely sensitive to glyphosate	65
	bar	P*rrn*/*rbc*L/*psb*A	*rbc*L/*acc*D	Transgenic tobacco seeds germinated on media containing 25 μg ml^{-1} glufosinate and the plants survived a herbicide challenge of 2.5% (vol/vol) herbicide solution	70
Disease resistance	*msi*-99	P*rrn*/ggagg/*psb*A	*trn*I/*trn*A	Protein extracts from transgenic tobacco plants inhibited growth of *Pseudomonas syringae* pv *tabaci*, *Aspergillus flavus*, *Fusarium moniliforme* and *Verticillium dahliae*. Planta assays with the bacterial pathogen *Pseudomonas syringae* pv *tabaci* and fungal pathogen *Colletotrichum destructivum* resulted in areas of necrosis and necrotic anthracnose lesions, respectively, around the point of inoculation in control leaves, whereas transformed leaves showed no signs of necrosis or lesions	45
Drought tolerance	*tps1* (yeast)	P*rrn*/ggagg/*psb*A	*trn*I/*trn*A	Transgenic seeds retained chlorophyll when grown in 6% wt/vol PEG, whereas untransformed plants were bleached. After 7-h drying, transgenic seedlings successfully rehydrated while control plants died. There was no difference between control and transgenic plants in water loss during dehydration, but dehydrated leaves from transgenic plants (not watered for 24 d) recovered upon rehydration, turning green while control leaves dried out	12
Phytoremediation	*merA*/*merB*	P*rrn*/ggagg/*psb*A	*trn*I/*trn*A	Transgenic plants showed high levels of tolerance to the organomercurial compound phenyl mercuric acetate (PMA) when grown in soil containing 400 μM PMA	28
Salt tolerance	*badh*	P*rrn*/ggagg/*rps*16	*trn*I/*trn*A	Transgenic carrot plants thrived well in soil pots irrigated with up to 400 mM NaCl, whereas untransformed showed retarded growth in the presence of salt	37
Cytoplasmic male sterility	*phaA*	P*rrn*/*psb*A/*psb*A	*trn*I/*trn*A	Transgenic tobacco plants showed male sterile phenotype lacking pollen. Reversibility of the male-sterile phenotype was observed under continuous illumination, resulting in viable pollen and copious amount of seeds	5

TABLE 4 | Chloroplast-derived vaccine antigens.

Vaccine antigens	Gene	Regulatory elements	Expression % TSP	Functional assays	References
Cholera toxin	*Ctx*B	ggagg/*Tpsb*A	Up to 4.1%	GM1 ganglioside-binding assay	17
Tetanus toxin	*Tet*C bacterial and synthetic	T7 *gene 10*/*Trbc*L *atp*B/*Trbc*L	25.0 10.0	Systemic immune response was observed in mice immunized with extracts from transgenic plants, whereas mice immunized with nontransgenic leaf extract failed to mount a detectable TetC-specific Ab response. All mice immunized with leaf extract harboring the plant-derived TetC survived the tetanus toxin challenge and were free of symptoms	40
Canine parvovirus (CPV)	CTB-2L21 GFP-2L21	P*psb*A/*Tpsb*A	31.1 22.6	GM1 ganglioside-binding assay. Mice and rabbits that received protein-enriched leaf extracts by parenteral route produced high titers of anti2L21 Abs able to recognize the VP2 protein. Rabbit sera were able to neutralize CPV in an *in vitro* infection assay	42,43
Anthrax protective antigen	*pag*	P*psb*A/*Tpsb*A	18.1 14.2	Macrophage lysis assay, systemic immune response, toxin neutralization assay, subcutaneous immunization of mice with partially purified chloroplast-derived or *B. anthracis*-derived protective antigen with adjuvant yielded IgG titers up to 1:320,000, and both groups of mice survived (100%) challenge with lethal doses of toxin	1,39
Amebiasis	*Lec*A	P*psb*A/*Tpsb*A	6.3	Systemic immune response in mice	18
Rotavirus	*vp6*	P*psb*A/*Tpsb*A	Variable	Not reported	19
Lyme disease	*Osp*A *Osp*A-T	P*psb*A/*Tpsb*A	1.0 10.0	Systemic immune response in mice protected mice against challenge with *Borrelia burgdorferi*	20

MATERIALS

REAGENTS

- Chloroplast targeting vector, containing flanking sequences specific to the genome to be targeted (**Fig. 2**)
- Chloroplast expression cassette, containing the necessary genome-specific regulatory sequences and the GOI (**Fig. 3a**), each fragment is verified by DNA sequencing. All published chloroplast vectors from the Daniell Laboratory listed in **Tables 2–6** are available to investigators involved in basic research upon signing of a Material Transfer Agreement
- Young, green, healthy tobacco leaves
- DNeasy Plant Mini Kit (Qiagen, cat. no. 69106)
- dNTPs (Invitrogen, cat. no. 10297018)
- *Pfu* DNA polymerase (Promega, cat. no. M7748) ▲ **CRITICAL** Use high-fidelity DNA polymerase to minimize errors.
- Set of primers for evaluation of site specific transgene integration (**Table 7**) ▲ **CRITICAL** Carefully design using available software such as Primer select module of DNASTAR lasergene program.
- Sterile molecular biology-grade water (Eppendorf, cat. no. 955155033)
- PCR cloning vector (Promega pGEM-T Easy, cat. no. A1360)
- Restriction endonucleases (New England Biolabs) ▲ **CRITICAL** Keep at −20 °C and avoid any cross-contamination.
- T4 DNA polymerase (Invitrogen, cat. no. 18005017)
- T4 DNA ligase (Invitrogen, cat. no. 15224017)
- Calf intestinal alkaline phosphatase (CIAP; Promega, cat. no. M1821)
- QIAquick Gel Extraction Kit (Qiagen, cat. no. 28704)
- QIAquick PCR Purification Kit (Qiagen, cat. no. 28104)
- QIAprep Spin Miniprep Kit (Qiagen, cat. no. 27104)
- Chlorox bleach
- Antibiotics (see REAGENT SETUP) ▲ **CRITICAL** Sterilize all stock solutions with 0.22-μm pore size filter and store at −20 °C.
- Plasmid Midiprep Kit (Bio-Rad, cat. no. 732-6120)
- Ethanol 190 proof for molecular biology (Sigma, cat. no. E7148-1GA) ! **CAUTION** Highly flammable.
- Murashige and Skoog salts 4.33 g for 1 l (MS; Caisson, cat. no. MSP 001)
- 6-Benzylamino purine (BAP; Sigma, cat. no. B3408)
- 1-Naphthaleneacetic acid (NAA; Sigma, cat. no. N0640)
- Sucrose (Sigma, cat. no. S0389-5KG)
- Phytoblend (Caisson, cat. no. PTC 001)
- Soil (Miracle Gro—Pot-mix)
- Water-soluble all purpose plant food (Miracle Gro)
- 1 M phenyl methyl sulfonyl fluoride (PMSF; Sigma; cat. no. P7626; see REAGENT SETUP)
- Plant extraction buffer (PEB; see REAGENT SETUP)
- Coating buffer (CB; see REAGENT SETUP)
- Tween-20 (Sigma, cat. no. P1379)
- Nonfat powdered milk (e.g., Carnation)
- 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate for ELISA (American Qualex, cat. no. K3620)
- Electrochemiluminescent (ECL) substrate for western blot (Pierce, cat. no. 32109)
- Sulfuric acid (dilute to 2M; Sigma, cat. no. 320501)
- BSA (Sigma, cat. no. A7030)
- Bradford reagent (Bio-Rad, cat. no. 500-0006)
- Sample buffer (SDS reducing buffer; see REAGENT SETUP)
- 10× Electrode buffer (EB; see REAGENT SETUP)
- Transfer buffer (see REAGENT SETUP)
- MSO (see REAGENT SETUP)
- RMOP (see REAGENT SETUP)
- Binding buffer (see REAGENT SETUP)
- Wash buffer (see REAGENT SETUP)
- Elution buffer (see REAGENT SETUP)

EQUIPMENT

- Nitrocellulose membrane (Bio-Rad, cat. no. 162-0146)
- PVDF membrane (Bio-Rad, cat. no. 162-0174)
- Whatman filter paper, 70 mm (Whatman; cat. no. 1001-070)
- Sterile 100 × 25 mm^2 Petri dishes (Midwest Scientific, cat. no. TPP 93100)

TABLE 5 | Chloroplast-derived biopharmaceutical proteins.

Biopharmaceutical protein	Gene	Regulatory elements	Expression % TSP	Functional assays	References
Human somatotropin	*hST*	*T7 gene10*/ T*rps*16 P*psb*A/ T*rps*16	0.2–7.0	Growth response of Nb2 cell line in the presence of somatotropin. Rat lymphoma cell line Nb2 proliferated in proportion to the amount of somatotropin in the culture medium, until saturation is reached	14
Insulin-like growth factor	*IGF-1n* *IGF-1s*	P*psb*A/ T*psb*A	Up to 32.0%	Growth response in cultured HU-3 cells	49
Interferon-α2b (IFN-α2b)	IFN-α2b	P*psb*A/T*psb*A	8.0–21.0% in LAMD and 2.0–14.0% in *Petit havana*	Transgenic IFN-α2b protected baby hamster kidney cells against cytopathic viral replication in vesicular stomatitis virus cytopathic effect assay, HeLa cells from HIV-1 entry and mice from a highly metastatic tumor line. Also, it increased the expression of major histocompatibility complex class I on splenocytes and the total number of natural killer cells	6
Human serum albumin	*hsa*	P*psb*A/T*psb*A	Up to 11.1%	Not reported	44
IFN-γ	Gus-IFN-γ	P*psb*A/T*psb*A	Up to 6.0%	Protection of human lung carcinoma cells against infection by encephalomyocarditis virus	15
Monoclonal Ab	*Guy's 13*	ggagg/T*psb*A	Not reported	Not reported	31
Human proinsulin	CTB-Pins (Pins—proinsulin)	P*psb*A/T*psb*A *T7 gene10*/T*rps*16	16.0—Tobacco 2.5—Lettuce	GM1 binding assay, prevention of pancreatic insulitis and preservation of insulin-producing β-cells in cholera toxin B (CTB)-Pins treated NOD mice with lower blood or urine glucose levels. Increased expression of immunosuppressive cytokines such as interleukin-4 (IL-4) and IL-10 in the pancreas of CTB-Pins treated nonobese diabetic mice	16

- 6-Well and 96-well tissue culture plates (Corning, cat. no. 3506 and Corning, cat. no. 3585)
- PTC-100 Peltier thermal cycler (Bio-Rad, cat. no. PTC-1196)
- PDS-1000/He Biolistic particle delivery system (Bio-Rad, cat. no. 165-2257)
- 0.6-μm Gold microcarriers (Bio-Rad, cat. no. 165-2262)
- Macrocarrier holder (Bio-Rad, cat. no. 165-2322)
- Macrocarrier (Bio-Rad, cat. no. 165-2335)
- Stopping screen (Bio-Rad, cat. no. 165-2336)
- 1,100 psi Rupture disks (Bio-Rad, cat. no. 165-2329)
- Helium gas
- Laminar air flow (model no. NU-201-630; Nuaire)
- Vacuum pump (Fisher, cat. no. 01-257-8c)
- Growth chamber fitted with fluorescent lights on a controlled timer (16 h light/8 h dark photoperiod)
- 5-Gallon pots (12 in diameter and 11 in height)
- Commercial mason jars
- Magenta box
- Greenhouse
- Surgical blade #21 (Henry Schein, cat. no. 100-3535)
- Porcelain mortars and pestles (50 ml capacity; e.g., Coors, cat. no. Z247464)
- 1.7-ml Microcentrifuge tubes (Midwest Scientific, cat. no. AVSS1700)
- Thin wall 0.2-ml PCR tubes (Midwest Scientific, cat. no. AVTW2)
- Hand-operated homogenizer (Sigma, cat. no. Z359971) with sterile polypropylene pestle adapters (Sigma, cat. no. Z359947)
- Refrigerated microcentrifuge (Eppendorf 5415R; Fisher, cat. no. 05-401-05)
- Costar 96-well enzymatic immunoassay (EIA) plates for ELISA and protein determination (Corning, cat. no. 3590)
- Syringe-operated filter units, 0.22-μm pore size (Sigma, cat. no. Z359904)
- Mini-PROTEAN 3 (Bio-Rad, cat. no. 165-3323)
- Film developer mini-medical series (AFP Imaging Corp, cat. no. 9992305300)
- Autoradiography cassette (Fisher, cat. no. FBXC810)
- Microtiter plate reader (BioTek Instruments, EL403), equipped with 450-, 570- and 595-nm filters
- 1-ml Nickel chelate-charged columns (Amersham Biosciences, cat. no. 17-1880-01)

REAGENT SETUP

Antibiotics Prepare stock solutions as indicated in the following table.

▲ **CRITICAL** Sterilize all stock solutions with 0.22-μm pore size filter and store at −20 °C.

TABLE 6 | Biomaterials, enzymes and amino acids engineered via the plastid genome.

Desired biomaterial	Gene	Site of integration	Promoter	5′/3′ UTRs	References
Elastin-derived polymer	*eg121*	*trn*I/*trn*A *rbc*L/*acc*D	P*rrn*	ggagg/T*psb*A	71
p-Hydroxybenzoic acid	*ubi*C	*trn*I/*trn*A	P*rrn*	P*psb*A/T*psb*A	52
Polyhydroxybutyrate	*phb* operon	*trn*N/*trn*R	P*psb*A	P*psb*A/T*psb*A	72
Xylanase	*xyn*A	*rbc*L/*acc*D	P*psb*A	P*psb*A/T*psb*A	51
Trp	*asa2*	*ndh*F/*trn*L *acc*D/*orf*184	P*rrn*	*rbc*L/*rp*L32 *rbc*L/*acc*D-*ORF*184	73
Monellin	*monellin*	*trn*I/*trn*A	P*psb*A	P*psb*A/T*psb*A	74

TABLE 7 | Primers used in this protocol (Step 22).

Primer name	Sequence (5′ to 3′)	Comment
3P	AAAACCCGTCCTCAGTTCGGATTGC	To check site-specific integration of selectable marker into the chloroplast genome along with 3M primer; it anneals with the native chloroplast genome
3M	CCGCGTTGTTTCATCAAGCCTTACG	To check site-specific integration of selectable marker into the chloroplast genome along with 3P primer; it anneals with the *aad*A gene
5P	CTGTAGAAGTCACCATTGTTGTGC	To check integration of the transgene expression cassette along with 2M primer; it anneals with the *aad*A gene
2M	TGACTGCCCACCTGAGAGCGGACA	To check integration of the transgene expression cassette along with 5P primer; it anneals with the *trn*A gene

Catalog number	Antibiotics	Stock solution	Working concentration
BP1760-25 (Fisher)	Ampicillin	50 mg ml^{-1} (Water)	50–100 mg l^{-1}
C-1919 (Sigma)	Chloramphenicol	34 mg ml^{-1} (Ethanol)	25 mg l^{-1}
0408-25G (Amresco)	Kanamycin	50 mg ml^{-1} (Water)	50 mg l^{-1}
S4014-25G (Sigma)	Spectinomycin	100 mg ml^{-1} (Water)	100–500 mg l^{-1}
T3258 (Sigma)	Tetracycline	5–12.5 mg ml^{-1} (70% vol/vol ethanol)	25–50 mg l^{-1}

1 M PMSF Dissolve 17.4 mg PMSF in 1 ml methanol. Vortex solution and store at –20 °C for up to 1 month.
PEB 100 mM sodium chloride (NaCl), 10 mM EDTA pH 8 (Sigma, cat. no. 46081), 200 mM Tris–HCl, pH 8 (Fisher, cat. no. BP152-5), 0.05% vol/vol Tween-20 (Fisher, cat. no. BP337-100), 0.1% wt/vol SDS (Fisher, cat. no. BP166-500), 14 mM β-mercaptoethanol (β-ME; Sigma, cat. no. M 3148), 200 mM sucrose and 2 mM PMSF. ! CAUTION SDS and PMSF are harmful to health when inhaled or in contact with the skin and can cause irritation and sensitization. ▲ CRITICAL PEB must be made fresh immediately before use.
Modified PEB 15 mM sodium carbonate (Na_2CO_3; Sigma, cat. no. S7795), 35 mM sodium bicarbonate ($NaHCO_3$; Sigma, cat. no. S6297), 3 mM sodium azide (NaN_3; Sigma, cat. no. S8032), adjusted to pH 9.6 and Tween 0.05% vol/vol and 1 M PMSF.
CB 15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3, adjusted to pH 9.6. ! CAUTION NaN_3 is highly toxic.
Sample buffer (SDS reducing buffer), for 10 ml 3.55 ml H_2O, 1.25 ml Tris–HCl (0.5 M, pH 6.8), 2.5 ml glycerol (Shelton Scientific, cat. no. IB15762), 2 ml 10% wt/vol SDS and 0.2 ml 0.5% wt/vol bromophenol blue (Sigma, cat. no. B8026). Add 50 µl β-ME to 950 µl sample buffer before use.
10× EB, for 1 l Dissolve 30.3 g Tris base, 144 g Gly (Bio-Rad, cat. no. 161-0724) and 10 g SDS in dH_2O.
Transfer buffer, for 1.5 l 300 ml 10× EB, 300 ml methanol (Fisher, cat. no. A411), 900-ml deionized water and 0.15 g SDS.
MSO, for 1 l MS salts, 30 g sucrose, pH 5.8 and 6 g phytoblend.
RMOP[36], for 1 l MS salts, 100 mg myo-inositol, 1 mg thiamine HCl, 1 mg BAP, 0.1 mg NAA, 30 g sucrose, pH 5.8 and 6 g phytoblend (with or without spectinomycin). ▲ CRITICAL This media composition is for tobacco plant regeneration. Different antibiotics may be used depending on the selectable marker in the vector construct; the concentration also varies in different plant species and cultivars. Use 500 µg ml^{-1} of spectinomycin for effective selection of transformants in tobacco (var. Petit Havana). Add antibiotics when medium cools to 45–50 °C.
Binding buffer 20 mM Sodium phosphate, 0.5 M NaCl, 30 mM imidazole, (pH 7.4).
Wash buffer 20 mM Sodium phosphate, 0.5 M NaCl, 5 mM imidazole, (pH 7.4).
Elution buffer 20 mM Sodium phosphate, 0.5 M NaCl, 0.5 M imidazole, (pH 7.4).
Preparation of the gold particle stock for coating DNA ● TIMING **1 h** Take 50 mg of gold particles (0.6 µm) in a siliconized 1.5-ml Eppendorf tube and add 1 ml molecular grade 100% ethanol. Vortex for 2 min and centrifuge at 10,000*g* for 3 min. Carefully discard the supernatant and resuspend the gold particles in 1 ml of 70% vol/vol ethanol by vortexing for 1 min. Incubate at room temperature (24 ± 2 °C) for 15 min and mix intermittently by gentle shaking. Pellet the gold particles by centrifuging at 5,000*g* for 2 min. Discard the supernatant. Wash a total of four times by resuspending the gold particles in 1 ml sterile dH_2O, incubating at room temperature for 1 min followed by centrifugation at 5,000*g* for 2 min. After the final centrifugation step, resuspend the gold particles in 1 ml of sterile 50% vol/vol glycerol and store at −20 °C until ready to use.
Coating gold particles with DNA ● TIMING **40 min (five shots)** Transfer 50 µl gold particles (prepared for coating) from a resuspended stock to a 1.5-ml microcentrifuge tube. While vortexing, add 5 µg of plasmid DNA followed by 50 µl of 2.5 M $CaCl_2$ and 20 µl of 0.1 M spermidine. Continue vortexing for 20 min at 4 °C. Centrifuge the DNA-coated gold particles at 10,000*g* for 1 min. Remove the supernatant. Wash the pellet with 200 µl of 70% vol/vol ethanol followed by 100% ethanol. Resuspend the DNA-coated pellet in 50 µl of 100% ethanol. The DNA-coated gold particles can be stored on ice for 2–3 h and should be used as soon as possible.

PROCEDURE

Preparation of the chloroplast targeting vector for ligation

1| Digest the chloroplast targeting vector with the appropriate restriction enzyme(s). As an example we provide details for the vector shown in **Figure 2**, which should be digested with *Pvu*II (**Fig. 3b**). Assemble the following components in a microfuge tube and incubate at 37 °C for 1 h.

Component	Amount (µl)	Final
10× NEBuffer 2	5	1×
Chloroplast targeting vector (1 µg $µl^{-1}$)	1	1 µg
*Pvu*II (10,000 U ml^{-1})	0.2	2 U
Nuclease-free water	43.8	

▲ CRITICAL STEP To save time, the expression cassette should be prepared (Steps 4–7) at the same time as the targeting vector.

2| Dephosphorylate the vector using CIAP, as instructed by the supplier. This treatment minimizes self-ligation of the vector during later ligation steps (Steps 8–10).

▲ CRITICAL STEP CIAP catalyzes the hydrolysis of 5′ phosphate groups from DNA. This prevents recircularization and self-ligation of linearized vector DNA.

3| Run the entire CIAP-treated sample on a 0.8% wt/vol agarose gel, excise the required band with a clean blade and purify the DNA from the gel using the QIAquick Gel Extraction Kit as instructed by manufacturer.

Preparation of the chloroplast expression cassette for ligation

4| Digest the chloroplast expression cassette with the appropriate restriction enzyme(s). The expression cassette might be a linear DNA fragment assembled by a series of ligated PCR products or part of a previously constructed plasmid (see INTRODUCTION). For illustrative purposes, we will use the example shown in **Figure 3**, in which sequential digestion with *Sal*I and *Sac*I is required. Assemble the following components in a microfuge tube and incubate at 37 °C for 2 h.
▲ CRITICAL STEP To save time, the expression cassette should be prepared at the same time as the targeting vector (Steps 1–3).

Component	Amount (μl)	Final
10× NEBuffer 1 (*Sac*I) or 10× NEBuffer 3 (*Sal*I)	5	1×
BSA (1 mg ml^{-1})	5	100 μg ml^{-1}
Chloroplast expression cassette (1 μg μl^{-1})	2	2 μg
*Sal*I or *Sac*I (20,000 U ml^{-1})	0.4	8 U
Nuclease-free water	37.6	

5| After digestion, run the entire sample on a 0.8% wt/vol agarose gel, excise the required band with a clean blade and purify the DNA from the gel using the QIAquick Gel Extraction Kit as instructed by manufacturer. If a second restriction step is required, repeat Steps 4 and 5 using the purified DNA and the second enzyme.

6| Add the following components to the completely purified restriction digest in a 0.2-ml PCR tube kept on ice; T4 DNA polymerase will 'polish' the ends of each fragment, making them blunt and enabling them to ligate to the blunt-ended *Pvu*II site in the targeting vector (from Step 3). Incubate at 11 °C for 15 min.

Component	Amount	Final
5× T4 DNA polymerase buffer	20 μl	1×
10 mM dNTP mix	1 μl	0.1 mM
Restricted chloroplast-expression cassette	0.5–2.5 μg	0.5–2.5 μg
T4 DNA polymerase (5 U μl^{-1})	2 μl	10 U
Nuclease-free water	Up to 100 μl	

7| Run the entire T4 DNA polymerase-treated sample on a 0.8% wt/vol agarose gel, excise the required band with a clean blade and purify the DNA from the gel using the QIAquick Gel Extraction Kit as instructed by manufacturer.

Ligation of the expression cassette and targeting vector to make the transformation vector

8| Set up 20-μl ligation reactions containing the chloroplast expression cassette (insert, from Step 7) and targeting vector (vector, from Step 3), as detailed below following manufacturer instructions. Set up 1:1 and 3:1 insert:vector ratios, not exceeding a total of 1.0 μg DNA. Also use vector alone as a control to test the efficiency of dephosphorylation. The number of colonies should be minimal in self-ligated vector when compared with the vector and insert ligation. Incubate the reactions at 14 °C for 16–24 h.

Component	Amount	Final
5× Ligase reaction buffer	4 μl	1×
Vector ends	15–60 fmol	15–60 fmol
Insert ends	45–180 fmol	45–180 fmol
T4 DNA ligase (1 U μl^{-1})	1 μl	1 U
Nuclease-free water	Up to 20 μl	

9| Transform competent *Escherichia coli* cells with 2–4 μl of the ligation mix using standard methods ($CaCl_2$ method)[53] and plate on selective plates. Grow overnight at 37 °C.

10| Screen for recombinant clones by colony PCR[54] and confirm the presence of the appropriate insert by restriction analysis. Each clone should be verified by sequencing to rule out any errors introduced by PCR.
▲ CRITICAL STEP Be sure to choose restriction enzymes that determine the direction of the insert; select only clones with the expression cassette in the appropriate orientation; that is, transcription should be from left flank to the right flank.
? TROUBLESHOOTING

BOX 1 | BOMBARDMENT OF LEAF TISSUE ● TIMING 40 MIN (FIVE SHOTS)

(i) Turn on the PDS–1000/He (first red button on the left side).
(ii) Start the vacuum pump and turn the knob on top of the helium gas tank counterclockwise until it feels loose.
(iii) Slowly turn the screw bar clockwise to bring the helium gas to ~250 psi above the desired pressure of the rupture disk.
▲ CRITICAL STEP Use rupture disks of 1,100 psi for tobacco leaf explants. However, rupture disks of variable psi should be tried for optimal transformation of different plant species.
(iv) Place the rupture disk in its holder using sterile forceps. Screw tightly to gas acceleration tube.
(v) Place the stopping screen in the macrocarrier holder assembly with the DNA-coated macrocarrier facing downward, toward the stopping screen. Secure the macrocarrier cover lid and place into the first slot from the top of the PDS chamber.
(vi) Place the uncovered Petri dish containing the leaf sample into the target plate holder and place into the third slot (9 cm) from the top of the PDS chamber. Secure the chamber firmly.
(vii) Press the vacuum button toward the **'VAC'** side to create vacuum inside the chamber. Let the vacuum pressure reach 28 inches Hg as displayed in the vacuum gauge. Bring the same button toward the **HOLD** side. Continue to press the **FIRE** button (red button on the right in the 'Up' position) until the rupture disk bursts.
(viii) Release the **FIRE** button and shift the vacuum button to **VENT**. Open the chamber when the pressure drops to zero and remove the bombarded sample. Replace the Petri plate cover.
(ix) For additional bombardments, repeat Steps (iv)–(viii).
(x) After completing the bombardments, turn off the gas cylinder, vacuum pump and PDS, in that order. To release the gas pressure remaining inside the helium tube, close the helium gas tank by rotating the knob clockwise, and create a vacuum in the chamber by moving the vacuum button toward the **VAC** side. While holding the **VAC** button, repeatedly press the **FIRE** button until the pressure falls to zero. Then turn the screw bar counterclockwise until it feels loose. Release the vacuum and turn off the vacuum pump and PDS.
(xi) Secure the Petri dish containing the bombarded leaves with parafilm and keep in dark for 2 d inside the culture room on racks.

11| Set up an overnight culture to grow the positive clone and isolate the plasmid using a Plasmid Midiprep Kit, as instructed by the manufacturer. The chloroplast transformation vector is now ready for delivery to plants.

DNA delivery into leaf explants and selection of transplastomic shoots

12| Take 100–200 tobacco seeds in a 1.7-ml Eppendorf tube. Wash with 1 ml of 70% (vol/vol) ethanol for 30 s to remove any greasy material.
▲ CRITICAL STEP Leaf explants are used for particle bombardment in tobacco plants. Here, we describe how to harvest leaves from *in vitro* grown plants, but leaves harvested from greenhouse-grown plants[55] can also be used.

13| Add 1 ml diluted commercial bleach, for example, chlorox [1.5% (vol/vol) sodium hypochlorite in water] containing 0.1% (vol/vol) Tween 20. Incubate for 10 min, gently mixing by inverting the microfuge tube.
▲ CRITICAL STEP Perform the remaining steps in a sterile laminar flow hood.

14| Remove the bleach and wash the seeds five times with 1 ml sterile deionized water.
! CAUTION Discard bleach solution according to any regulations stipulated by your institute.

15| Inoculate ~40 seeds per Petri dish in MSO medium for germination. Keep in culture room under white fluorescent lamps (1,900 lux) with 16 h light/8 h dark cycle at 26 °C for 7–10 d.

16| Transfer individual germinated seedlings to magenta boxes containing MSO medium and keep in culture room for 4–7 weeks. Alternatively, transfer nodal segments of aseptically grown plants to magenta boxes containing MSO medium. This latter method decreases the time needed to obtain leaves of an adequate size for particle bombardment.

17| Harvest leaves at five- to seven-leaf stage of plant growth. Place an autoclaved Whatman 70-mm circle filter disk on RMOP medium in a deep Petri dish. Place leaf on filter disk with its adaxial side facing the medium.
▲ CRITICAL STEP Do not use leaves from plants that have been propagated more than five times using the nodal section method, as this will decrease the regeneration potential of the leaf.

18| Load 10 µl DNA-coated gold particles, prepared as outlined in REAGENT SETUP, onto the sterile macrocarrier placed in its holder. Allow it to dry in the laminar flow hood. Proceed with DNA delivery using a standard particle bombardment method[55,56]; our method is outlined in **Box 1**.

19| After 2 d in dark, cut 5-mm^2 pieces of bombarded leaves and place on RMOP selection medium (bombarded side in contact with medium) for the first round of selection. Seal the Petri dish with parafilm.
▲ CRITICAL STEP This procedure for putative shoot regeneration is applicable for other tobacco cultivars (TN90, Xanthi and Burley). However, for LAMD (low nicotine cultivar), use 200 mg l^{-1} of spectinomycin for selection of shoots; shoots will appear 1–2 weeks later than for other cultivars.

20| Keep Petri dishes in culture room under white fluorescent lamps (1,900 lux) with 16 h light/8 h dark cycle at 26 °C. After 4–8 weeks, putative transgenic shoots appear (**Fig. 4**, panel **a**). Screen the putative transplastomic shoots for transgene integration by PCR (**Fig. 5a**,**b**) as described later.

? TROUBLESHOOTING

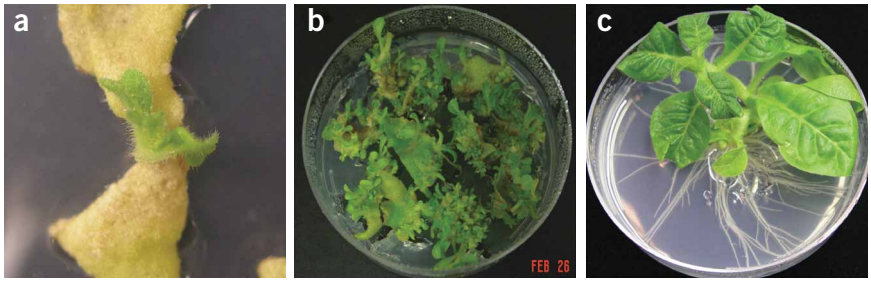

Figure 4 | Selection of transgenic plants. Representative photographs of transgenic tobacco shoots undergoing (**a**) first, (**b**) second and (**c**) third (rooting) rounds of selection.

Confirmation of transgene integration in putative transplastomic plants by PCR

21| Before the second round of selection, harvest 100 mg of leaf material from the putative transplastomic shoots. Isolate DNA using the DNeasy Plant Mini Kit, following the manufacturer's protocol. This procedure yields ~20–30 µg of DNA.

22| Set up two separate 50 µl PCRs in 0.2-ml PCR tubes as detailed in the table below. One reaction (3P and 3M primers) will check integration of selectable marker gene into the chloroplast genome, the second (5P and 2M primers) will check integration of the transgene expression cassette. Primer sequences are given in **Table 7**. Also amplify untransformed leaf DNA in a separate PCR tube for use as control.

Component	Amount per reaction (µl)	Final concentration
10× PCR buffer	5	1×
50 mM $MgCl_2$	2	2 mM
dNTP mix, 10 mM each	1.5	300 µM each
Template DNA (1 µg µl^{-1})	0.5	0.5 µg
3P/5P (10 pmol µl^{-1})	1	0.2 pmol
3M/2M (10 pmol µl^{-1})	1	0.2 pmol
Taq DNA polymerase	1	1 U 50 µl^{-1}
Nuclease-free water	38	

23| Amplify using the following PCR program. Maintain the reaction at 4 °C after cycling.

Cycle number	Denature	Anneal	Extend
1	5 min at 94 °C		
2–31	1 min at 94 °C	1 min at 60 °C	1 min kb^{-1} at 72 °C
32			10 min at 72 °C

■ **PAUSE POINT** PCR products can be stored at 4 °C for several days.

24| Examine 5 µl PCR product by agarose gel electrophoresis. Visualize amplified PCR products by staining with ethidium bromide (**Fig. 5b**). Once the plants have been confirmed for transgene integration by PCR, they are subjected to two further rounds of selection.

? TROUBLESHOOTING

25| For the second round of selection, cut 2-mm^2 pieces of leaves from PCR-positive plants and place them on RMOP selection medium. Grow in culture room under white fluorescent lamps (1,900 lux) with 16 h light/8 h dark cycle at 26 °C. These leaf sections produce transgenic shoots in 3–4 weeks (**Fig. 4b**).

26| Excise the regenerated shoots and transfer to MSO medium containing the appropriate antibiotic. Grow in culture room under white fluorescent lamps (1,900 lux) with 16 h light/8 h dark cycle at 26 °C. This step is termed as the third round of selection, where rooting occurs in 3–4 weeks (**Fig. 4c**). Southern blot analysis (**Fig. 5a**,**c**) can be carried out to confirm integration and determine homoplasmy, as described previously[53,56].

Growth of plants containing integrated transgenes

27| Take homoplasmic plants with roots from Step 26 and wash thoroughly with water to remove the phytoblend or agar.

▲ **CRITICAL STEP** Make sure to remove all the phytoblend or agar, otherwise plants may catch fungal infections and eventually die.

28| Soak jiffy pellet in water for 20 min. Transfer the plant to jiffy pellet in a small container with enough water so that it covers the surface. Cover it with plastic bag to maintain humidity. Keep in growth chamber maintained at 26 °C and 16-h photoperiod of 1,900 lux.

29| After 4 d, make a small hole in plastic bag to facilitate exchange of air. Remove the bag after a total of 7 d.

30| After removing the bag, grow the plant for a week in growth chamber and water the plant every 2 d.

31| Transfer the jiffy pellet with plant to a pot containing autoclaved soil in a greenhouse. Water plants every 2 d and add water-soluble all-purpose plant food every week, according to manufacturer's instructions.

32| After 5 weeks, collect the healthy leaves for characterization of transgenic protein (TP) (Steps 33–59). When the flower heads appear, either check for maternal inheritance, as outlined in **Box 2** (**Fig. 6**), or collect the seeds. To do this, cover the flower heads with a waterproof paper bag (moisture in pods increases fungal infection) until the pods mature. Fasten the mouth of paper bag securely to the stalk below the flower branches using a string or rubber band. When the seed pods have matured, remove the bag, collect the pods and dry them in a desiccator. These seeds can further be used to grow transplastomic plants.

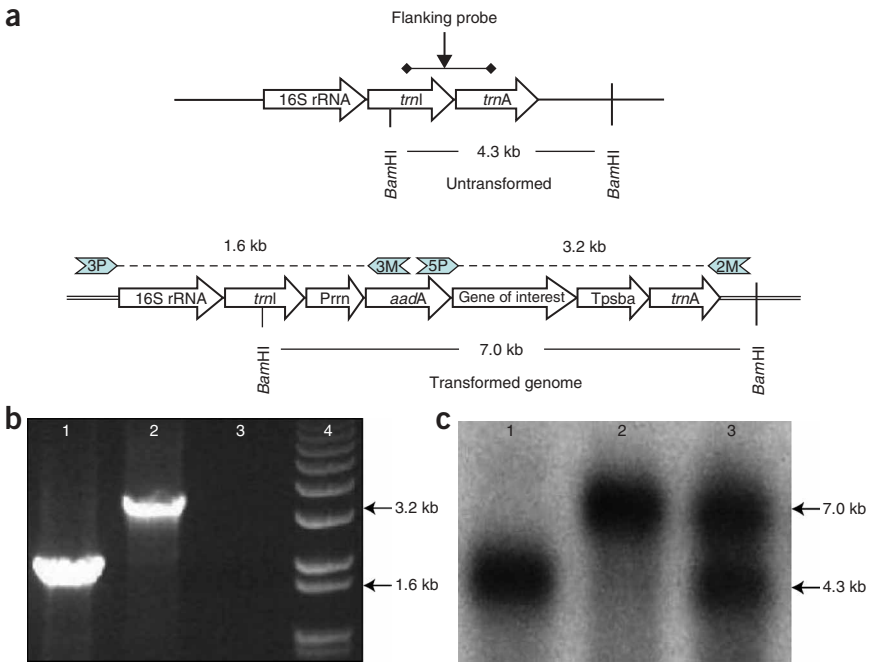

Figure 5 | Evaluation of transgene integration into the chloroplast genome. (**a**) Map depicting the chloroplast transformation vector showing integration site, regulatory elements, selectable marker gene, primer annealing sites and flanking probe fragment for Southern blot analysis. DNA isolated from putative transplastomic shoots was analyzed by PCR and Southern blot analysis. (**b**) 3P/3M and 5P/2M primer pairs were used for PCR analysis; lane 1, PCR product with 3P/3M primers (1.6 kb); lane 2, PCR product with 5P/2M primers (3.2 kb); lane 3, PCR product with untransformed plant; lane 4, DNA marker—1 kb plus DNA ladder. (**c**) The chloroplast genome was probed with a 0.81-kb radiolabeled fragment. Lane 1, untransformed plant; lane 2, transformed plants having reached homoplasmy (contains only transformed chloroplast genome); lane 3, transformed plants having heteroplasmy (contains both transformed and untransformed chloroplast genomes).

■ **PAUSE POINT** Seeds can be stored in airtight Eppendorf tubes for 2–3 years at 24–26 °C. Seeds may be stored for longer periods at 4 °C or at −70 °C.

Extraction of total soluble protein ● **TIMING 1 h**

33| Collect green and healthy leaves from transformed and untransformed plants growing in the greenhouse. Wash soil and debris from leaves and chop off the midrib portion.

■ **PAUSE POINT** Store at −80 °C in a clean Ziploc bag. Remove the leaf samples from storage as and when required.

34| Grind the leaf material in liquid nitrogen to a fine powder. Add 200 µl freshly prepared PEB to each pulverized 100-mg plant sample on ice.

BOX 2 | CONFIRMATION OF MATERNAL INHERITANCE

Emasculate nontransgenic plants and pollinate with pollen derived from chloroplast transgenic plants and vice versa.
(i) Harvest seeds from the F1 hybrid (female nontransgenic X male transgenic and male nontransgenic X female transgenic) and self-pollinated chloroplast transgenic plants.
(ii) Germinate seeds on $^1/_2$ Murashige and Skoog basal medium supplemented with antibiotic to test for maternal inheritance of chloroplast-integrated transgenes. Normally seeds from F1 crosses with male transgenic plants fail to grow on selection medium, whereas all the seeds from F1 crosses with maternal transgenic parent and selfed transgenic progeny seeds germinate and grow well, producing copious roots and leaves. This confirms that the chloroplast transgenic trait is inherited maternally because it is integrated into the chloroplast genome and there is no Mendelian segregation because they are not integrated into the nuclear genome (**Fig. 6**).

35| Homogenize the leaf tissue using a hand-held homogenizer for 5 min, keeping the samples on ice.
▲ CRITICAL STEP Higher temperature leads to proteolysis.

36| Spin the homogenized samples at 15,000*g* for 10 min at 4 °C.

37| Save the supernatant and quantify the total soluble protein (TSP) as outlined in **Box 3**.
■ PAUSE POINT Supernatant can be stored at −80 °C for 6 months.

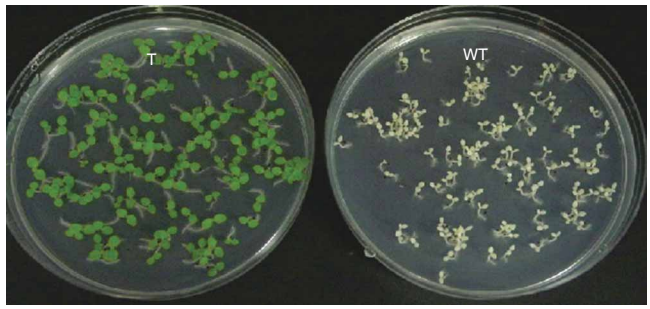

Figure 6 | Maternal inheritance of transgenes. In spectinomycin selection medium (500 mg l^{-1}, MS0), 100% selfed transgenic (T) tobacco seeds germinate and grow normally confirming lack of Mendelian segregation. Selection agent had detrimental effect on the wild-type (WT) seeds (unable to germinate or bleach soon after germination) confirming absence of transgenes.

Confirmation of transgene expression by western blot analysis

38| Boil various quantities (e.g., 100, 10 and 1 μg) of crude extracts (from Step 37) diluted in an equal amount (by volume) of sample buffer for 4–20 min. Some proteins may not be detected after boiling because of destruction of epitopes (e.g., GFP). Note that western analysis may also be performed on purified protein (from Step 72) as described.

39| Load samples (including unboiled control samples) into wells of a 12% wt/vol SDS-polyacrylamide gel[53].
▲ CRITICAL STEP The exact percentage of the resolving gel will depend on the size of the protein. The following table shows the effective range of separation of SDS-polyacrylamide gels.

Acrylamide concentration (% wt/vol)	Linear range of separation (kDa)
15	10–43
12	12–60
10	20–80
7.5	36–94
5.0	57–212

40| Separate the proteins by electrophoresis. Set the initial current at 85 V in 1× electrode buffer until proteins migrate into the resolving gel, then increase the current to 110 V and electrophorese until dye reaches the bottom of the gel.

41| Using electroblotting apparatus, transfer the separated proteins to a nitrocellulose or PVDF membrane at 15 V overnight.
▲ CRITICAL STEP Before use, pre-wet PVDF in methanol for 15 s followed by soaking in water for 2 min. Then carefully equilibrate the membrane in transfer buffer for 5 min.

42| After transfer, soak the membrane in a sufficient volume of PBS-T (PBS supplemented with 0.1% vol/vol Tween-20) to fully cover the membrane for 5 min at room temperature (25 °C). Pour off the PBS-T.
■ PAUSE POINT After transfer, the membranes can be wrapped in plastic cling wrap and stored at −20 °C for a few days.

43| To block nonspecific binding, incubate the membrane at room temperature for 1 h with gentle rocking in PTM (PBS-T supplemented with 3% wt/vol nonfat milk) so that it fully covers the membrane. Pour off the PTM.

44| To detect the TP, fully cover the membrane with primary Ab diluted in PTM (dilution ratio depends on the Ab titer). Incubate the membrane and primary Ab solution at room temperature for 2 h (or overnight at 4 °C) with gentle rocking.

45| Wash the membrane one time with 1× PBS-T for 5 min at room temperature and then add secondary Ab (conjugated to horseradish peroxidase (HRP)) at an appropriate dilution in PTM. Incubate for 1.5 h with gentle shaking.

BOX 3 | QUANTITATION OF TOTAL SOLUBLE PROTEIN ● TIMING 1 H

Perform soluble protein quantitation by following the manufacturer's protocol for the Bio-Rad protein assay (microtiter plate method). Combine 10 μl of various dilutions of sample crude extract (e.g., 1:1, 1:5, 1:10 and 1:20) with 200 μl of diluted protein assay reagent concentrate. Use BSA standards in the following concentrations: 500, 250, 100, 50, 25, 10, 5 and 1 μg ml^{-1}. Read the results of the protein assay on a microplate reader equipped with a 595-nm filter.

46| Wash the membrane three times with PBS-T for 15 min each and one time with 1× PBS for 10 min.

47| Add ECL substrate and incubate at room temperature for 5 min with gentle shaking.

48| Develop the chemiluminescent signal by exposing the membrane to x-ray film (**Fig. 7a**). The initial exposure should be for 1 min; depending on the signal obtained, subsequent exposure times can be extended up to 30 min.
? TROUBLESHOOTING

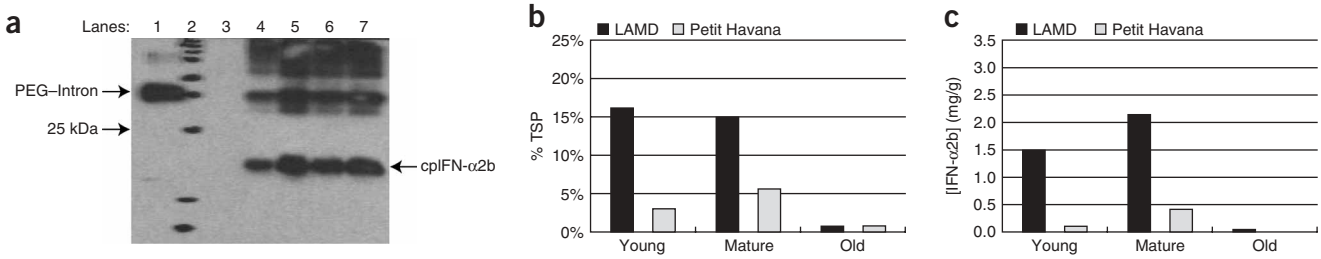

Figure 7 | Confirmation of transgene expression in chloroplasts. LAMD low-nicotine transgenic plants grown in the greenhouse were tested for the expression of IFN-α2b by western blot analysis or ELISA. (**a**) Plant crude extract was first separated by SDS-PAGE, followed by transfer to nitrocellulose and immunoblotting with a mouse monoclonal Ab against human IFN-α. Lane 1, 80 ng PEG–Intron standard; lane 2, protein marker; lane 3, untransformed LAMD; lanes 4–7, transgenic LAMD lines expressing monomers and multimers of IFN-α2b. Quantitation of transgenic IFN-α2b was performed on the T_1 generations of LAMD plants. The standard used was a commercial source of recombinant human IFN-α2b. (**b**) Quantitation of IFN-α2b as a percentage of total soluble protein (TSP). (**c**) Quantitation of IFN-α2b in milligram transgenic protein per gram leaf fresh weight.

Quantitation of TP

49| Prepare various dilutions of crude extract ranging from 1:5 to 1:5,000 in CB. A suitable purified standard for the TP must be selected and an appropriate dilution range must be empirically determined. The standard is also diluted in CB.
▲ **CRITICAL STEP** Mortars and pestles must be autoclaved. Place mortars and pestles in −80 °C freezer for ~30 min before use.

50| Aliquot 100 µl of the diluted plant protein extract and standards into separate wells of a 96-well microtiter EIA plate, in duplicate. Cover the plate with parafilm and incubate either at room temperature for 4 h or at 4 °C overnight. Wash with PSB-T and block with 100 µl of PTM at 37 °C for 1 h.

51| Wash wells three times with PBS-T, followed by three washes with dH_2O. Pat the plate on paper towels to remove excess solution but do not let the wells dry completely.

52| Add 100 µl primary Ab diluted in PTM (appropriate dilution must be empirically determined). Incubate the plate at 37 °C for 2 h.

53| Wash and dry wells as in Step 51.

54| Add 100 µl secondary Ab (conjugated to HRP) diluted in PTM (appropriate dilution must be empirically determined). Incubate the plate at 37 °C for 1 h.

55| Wash and dry wells as in Step 51.

56| Add 100 µl TMB substrate to the wells. When the color begins to change, add 50 µl of 2 M sulfuric acid to stop the reaction.

57| Read the plate immediately on a microtiter plate reader using a 450-nm filter.

58| Use formula 1 to calculate the amount of TP as a percentage of the TSP of transformed leaf material (see **Fig. 7b** for an example). This provides the level of expression and facilitates comparison with expression levels reported in the literature.
Formula 1

$$\%\text{TSP} = \frac{[\text{TP}]}{[\text{TSP}]} \times 100$$

[TP] = Concentration of transgenic protein in ng ml^{-1} (from the ELISA results of Step 57) and [TSP] = concentration of total soluble protein in ng ml^{-1} (from the results of the protein assay of Step 37 and **Box 3**).
▲ **CRITICAL STEP** The concentration units of both the numerator and denominator must be identical.

59| Use formula 2 to calculate the amount of TP relative to the frozen weight of transformed leaf material (in milligrams of TP per 100 mg of freeze-dried leaf material, see **Fig. 7c** for an example). This information is useful for oral delivery of known amount of antigens or other therapeutic proteins.
Formula 2

$$\text{Amount of transgenic protein} = \frac{[\text{TP}] \times V_{\text{PEB}}}{W_{\text{TLM}} \times 10^6}$$

[TP] = Concentration of transgenic protein in nanograms per milliliter (from the ELISA results of Step 57), V_{PEB} = volume of PEB in milliliters used in Step 34 (normally, this value is 0.2 ml) and W_{TLM} = weight of transformed leaf material in grams used in Step 34 (normally, this value is 0.1 g).

Purification of tagged protein (optional)

60| If the GOI was designed to include an affinity tag, the tag can be used to purify the TP, which may be subsequently used for functional assays. Purified proteins are essential for subcutaneous injections or other forms of delivery into the circulatory system or for *in vitro* studies in cultured cells. As an example, we describe affinity purification of a His-tagged TP. Grind 6 g of leaf material in liquid nitrogen to a fine powder. Add 15 ml of modified PEB in a 50-ml Falcon tube to each pulverized plant sample on ice.

61| Homogenize the leaf tissue using a hand-held homogenizer for 5 min, keeping the samples on ice.

62| Centrifuge the homogenized plant samples at 15,000*g* for 10 min at 4 °C. Save the supernatant and proceed directly to protein purification.

63| Dilute the supernatant with binding buffer in a ratio of 1:1. This step ensures the binding of His-tagged protein in the extract to the column.

64| We use an Akta prime machine (Amersham Biosciences) for purification of His-tagged protein as per manufacturer's instructions. In brief, insert inlet tubing A1, B and A3 in the binding buffer, elution buffer and wash buffer, respectively.

65| Connect the column between port 1 on the injection valve and the upper port of the UV flow cell and fill the fraction collector rack with 18-mm tubes.

66| Carefully inject the sample into the sample loop through injection valve.

67| Set up the following purification steps: equilibrate the column with binding buffer, apply the sample, wash the column with wash buffer and elute using elution buffer by gradient elution with a linear gradient of 0–100%.

68| Collect the flow-through from wash step and analyze for any loss of transplastomic protein during washing using ELISA. Use binding buffer and elution buffer as negative control.

69| Identify all the fractions containing purified protein collected in elution step by PrimeView evaluation module showing the corresponding peaks of the protein.

70| Pool together all the fractions containing purified protein and dialyze overnight in the dialysis cassette against cold PBS (pH 7.4) to remove imidazole from collected fractions.
▲ **CRITICAL STEP** It is important to remove most of the imidazole if the purified protein is to be tested in mice, as it causes digestive tract irritation and possible burns.

71| Load the dialyzed protein onto the Amicon Ultra-4 centrifugal filter device and concentrate protein according to manufacturer's guidelines and centrifuge at 2,500*g* at 4 °C until the required concentration is obtained.

72| Run the concentrated protein on SDS-PAGE gel to check the purity of protein and western blot (Steps 38–48) to check for any degradation of purified protein.

73| Determine the protein concentration by ELISA as described in (Steps 49–59). Aliquot concentrated protein and store at −80 °C for further studies or characterization.
? TROUBLESHOOTING

● **TIMING**

Steps 1–3, preparation of the chloroplast targeting vector for ligation: 3–4 h
Steps 4–7, preparation of the chloroplast expression cassette for ligation: 1 d
Steps 8–11, ligation of the expression cassette and targeting vector to make the transformation vector: 5–7 d
Steps 12–20, DNA delivery into leaf explants and selection of transplastomic shoots: 3–5 months
Steps 21–24, confirmation of transgene integration in putative transplastomic plants by PCR: 6–7 h
Steps 25 and 26, second and third round of selection: 6–8 weeks
Steps 27–32, growth of plants containing integrated transgenes: 8–16 weeks
Steps 33–37, extraction of TSP: 1 h
Steps 38–48, confirmation of transgene expression by western blot analysis: 2 d
Steps 49–59, quantitation of TP: 6 h to 2 d
Steps 60–73, purification of His-tagged protein by affinity chromatography: 3–7 d

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 8**.

TABLE 8 | Troubleshooting table.

Step	Problem	Possible reason	Solution
10	No colonies	Cells used for transformation have lost competence	Try different strains of competent cells such as XL10 gold
			Change the method of preparation of competent cells and always use a control to check the efficiency of the competent cells
	Few or no white colonies containing the amplified flanks	The 3′ A overhang is not formed	Try optimizing the A tailing reaction with a different thermostable DNA polymerase such as *Tfl* and *Tth* DNA Polymerase
		Pyrimidine dimers might have formed due to prolonged UV exposure	Reamplify the fragment, A tail and re-ligate
		The inserted fragment might not be disrupting the *lac*Z gene	Check several blue colonies for the presence of the insert
20	Lack of gene integration	Poor coating of DNA on gold particle	Test the efficiency of coating by visualizing on the gel
			For efficient coating of DNA on gold particle, strictly follow the order of addition of DNA, $CaCl_2$ and spermidine as vortexing
			Use freshly prepared $CaCl_2$
			Avoid refreezing and thawing of spermidine
			Use the DNA-coated gold particles within 2 h
		Use of mature leaf	Choose young leaf from *in vitro* grown four- to five-leaf stage plant
		Inadequate DNA delivery	Optimize DNA delivery parameters such as target distance, gap distance and rupture disk pressure
	Poor regeneration or no regeneration of shoots	Bombarded surface (abaxial side) may not be in contact with medium	Ensure the bombarded surface is in contact with selection medium
		Over boiling/sterilization of medium will inhibit shoot regeneration (this will break down phytohormones added to the medium that promotes shoot regeneration)	Autoclave regeneration medium (RMOP) for not >20 min
		Inappropriate concentration of selection agent (antibiotic) used	Use different concentration of selection agent
24	Fragment is of the wrong molecular weight	Nonspecific annealing of primer(s)	Raise the annealing temperature or decrease the annealing time
	Faint amplification of the target DNA	Impure template DNA or inefficient priming	Purify the template DNA by extraction with phenol–chloroform
			Use a different concentration of Mg^{2+} to find the optimal concentration
			Reamplify using faint amplicon as template
48	No signal on the x-ray film	Poor transfer or no transfer Primary, secondary Ab or substrate not working	Stain the gel with Coomassie stain following transfer to determine if transfer is complete or incomplete
			Increase or decrease the transfer time. The duration may vary depending on the protein size. Higher molecular weight proteins require longer transfers and low molecular weight proteins require shorter transfer
			Replace the Ab or substrate if positive control does not work

TABLE 8 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
		Insufficient electrochemiluminescent substrate or less incubation time	Add more substrate and increase the time of incubation
	High background and low signal	Inadequate blocking or insufficient washing	Change the blocking agent to BSA
			Block with 5% milk and wash stringently
60–73	Total protein not binding to the column	Either column could be old or binding buffer may not be added to the plant extract or problem with buffer composition	The binding buffer and plant extract should be in 1:1 ratio. Replace the column if the problem persists. Do not add EDTA to the plant extraction buffer (PEB). EDTA chelates divalent metal ions
	Protein of interest not binding to the column	Could be due to possible change in the confirmation of protein concealing the His-tag or loading too much of total plant protein	Analyze the flow-through to observe if the protein of interest is lost during binding or washing
			Preferably engineer C-terminal His-tag to avoid conformation problems
			Dilute the protein extract if the protein of interest is lost in binding and washing step
	No protein elution peak	Low concentration of imidazole in the elution buffer	Increase concentration of imidazole and use gradient elution
	Too many bands in the eluent	Nonspecific elution of proteins	Increase the washing time and/or decrease the elution buffer concentration
	Degradation products after purification	Possible proteolysis	Maintain low temperature during the entire purification process.
			Add protease inhibitors in the purification buffers
	Column gets clogged	Possibly due to high viscosity of the plant protein extract or the buffer composition	Reduce the viscosity by adding 1:1 binding and modified PEB buffer
			Do not use SDS to extract plant protein intended for further purification

ANTICIPATED RESULTS

Successful construction of a chloroplast-transformation vector should result in a fully functional vector (**Fig. 1**). Determination of complete sequence of the integration cassette should reveal proper alignment of the promoter and UTRs with the coding sequence of the GOI and the selectable marker gene. Before determining DNA sequence, restriction enzyme analysis should provide predicted sizes of restriction fragments. *E. coli* cells harboring the chloroplast vector are expected to grow in Luria–Bertani medium supplemented with 100 mg l^{-1} spectinomycin (or other appropriate selection agent). The protein extract from overnight grown cultures of *E.coli* is expected to show protein of correct molecular weight or their multimers in western blots. Putative transplastomic shoots are likely to appear on RMOP selection medium in 4–8 weeks after bombardment in tobacco (**Fig. 4a**). Efficiency of plastid transformation is highly predictable in tobacco. One set of bombardment with five young leaves should yield at least five independent transformation events. Under optimal conditions, as many as 10–50 transformation events have been obtained. However, the number of transformants may vary depending on the efficiency of DNA delivery, choice of competent leaves and toxicity of the foreign protein to plant cells. Plastid transformation efficiency via direct organogenesis is predictable. For example, under optimized conditions plastid transformation and regeneration via organogenesis in lettuce is as efficient as in tobacco. However, plastid transformation efficiency is not highly predictable in plant species where regeneration requires somatic embryogenesis.

Several shoots are expected within 3–4 weeks of the second round of selection (**Fig. 4b**) from leaves of putative transplastomic shoots, confirmed by PCR for transgene integration (**Fig. 5b**). Rooting of shoots on MSO selection medium occurs within 3–4 weeks (**Fig. 4c**). Southern blot analysis of plants after rooting under selection are likely to show the presence of only transformed chloroplast genomes (homoplasmy; **Fig. 5c**, lane 2), however, a few plants may also show both transformed

and untransformed chloroplast genomes (heteroplasmy; **Fig. 5c**, lane 3). Untransformed plants are anticipated to show a fragment of the native chloroplast genome (**Fig. 5c**, lane 1).

Seeds obtained from self pollination of transplastomic plants and F1 hybrids with pollen from wild type are expected to germinate and grow into uniformly green plants (**Fig. 6**), whereas untransformed plants and F1 hybrid with pollen from transplastomic plants should not grow on spectinomycin containing media. The absence of Mendelian segregation of transgenes should indicate that they are maternally inherited. Western blot analysis of transplastomic plants should show the expected size protein (**Fig. 7a**) and ELISA should quantify the amount of foreign protein. In the case of tobacco, we also determined that the age of the leaf significantly impacts the yield of foreign protein, with mature leaves showing the highest levels of expression. Older leaves show the lowest level of expression, which is correlated with senescence and high proteolytic activity. In the example of interferon expressed in transgenic chloroplasts, both young and mature leaves showed high levels of expression, ranging from 1.5 to 2 mg g^{-1} fresh weight (**Fig. 7b**), or ~15% TSP (**Fig. 7c**), in T_1 transgenic lines. The purification of His-tagged protein from transplastomic plant is essential for functional evaluation of chloroplast-derived proteins by *in vitro* cell culture assays or *in vivo* studies using suitable animal models.

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1. Koya, V., Moayeri, M., Leppla, S.H. & Daniell, H. Plant-based vaccine: mice immunized with chloroplast-derived anthrax protective antigen survive anthrax lethal toxin challenge. *Infect. Immun.* **73**, 8266–8274 (2005).
2. Daniell, H. Molecular strategies for gene containment in transgenic crops. *Nat. Biotechnol.* **20**, 581–586 (2002).
3. Hagemann, R. The sexual inheritance of plant organelles. In *Molecular Biology and Biotechnology of Plant Organelles* (eds. Daniell, H. & Chase, C.) 93–113 (Springer Publishers, Dordrecht, The Netherlands, 2004).
4. Daniell, H. Transgene containment by maternal inheritance: effective or elusive? *Proc. Natl. Acad. Sci. USA* **104**, 6879–6880 (2007).
5. Ruiz, O.N. & Daniell, H. Engineering cytoplasmic male sterility via the chloroplast genome by expression of \{beta\}-ketothiolase. *Plant Physiol.* **138**, 1232–1246 (2005).
6. Arlen, P.A. *et al.* Field production and functional evaluation of chloroplast-derived interferon alpha2b. *Plant Biotechnol. J* **5**, 511–525 (2007).
7. DeCosa, B., Moar, W., Lee, S.B., Miller, M. & Daniell, H. Overexpression of the *Bt cry*2Aa2 operon in chloroplasts leads to formation of insecticidal crystals. *Nat. Biotechnol.* **19**, 71–74 (2001).
8. Grevich, J.J. & Daniell, H. Chloroplast genetic engineering: recent advances and future perspectives. *Crit. Rev. Plant Sci.* **24**, 83–107 (2005).
9. Daniell, H., Kumar, S. & Dufourmantel, N. Breakthrough in chloroplast genetic engineering of agronomically important crops. *Trends Biotechnol.* **23**, 238–245 (2005).
10. Chilton, M.M. & Que, Q. Targeted integration of T-DNA into the tobacco genome at double-stranded breaks: new insights on the mechanism of T-DNA integration. *Plant Physiol.* **133**, 956–965 (2003).
11. Dhingra, A., Portis, A.R. Jr. & Daniell, H. Enhanced translation of a chloroplast-expressed *RbcS* gene restores small subunit levels and photosynthesis in nuclear *RbcS* antisense plants. *Proc. Natl. Acad. Sci. USA* **101**, 6315–6320 (2004).
12. Lee, S.B. *et al.* Accumulation of trehalose within transgenic chloroplasts confers drought tolerance. *Mol. Breed.* **11**, 1–13 (2003).
13. Quesada-Vargas, T., Ruiz, O.N. & Daniell, H. Characterization of heterologous multigene operons in transgenic chloroplasts: transcription, processing, and translation. *Plant Physiol.* **138**, 1746–1762 (2005).
14. Staub, J.M. *et al.* High-yield production of a human therapeutic protein in tobacco chloroplasts. *Nat. Biotechnol.* **18**, 333–338 (2000).
15. Leelavathi, S. & Reddy, V.S. Chloroplast expression of his-tagged GUS-fusions: a general strategy to overproduce and purify foreign proteins using transplastomic plants as bioreactors. *Mol. Breed.* **11**, 49–58 (2003).
16. Ruhlman, T., Ahangari, R., Devine, A., Samsam, M. & Daniell, H. Oral delivery of chloroplast derived cholera toxin B-proinsulin protects against development of insulitis in non-obese diabetic mice. *Plant Biotechnol. J.* **5**, 495–510 (2007).
17. Daniell, H., Lee, S.B., Panchal, T. & Wiebe, P.O. Expression of the native cholera toxin B subunit gene and assembly as functional oligomers in transgenic tobacco chloroplasts. *J. Mol. Biol.* **311**, 1001–1009 (2001).
18. Chebolu, S. & Daniell, H. Stable expression of Gal/GalNAc lectin of *Entamoeba histolytica* in transgenic chloroplasts and immunogenicity in mice towards vaccine development for amoebiasis. *Plant Biotechnol. J.* **5**, 230–239 (2007).
19. Birch-Machin, I., Newell, C.A., Hibberd, J.M. & Gray, J.C. Accumulation of rotavirus VP6 protein in chloroplasts of transplastomic tobacco is limited by protein stability. *Plant Biotechnol. J.* **2**, 261–270 (2004).
20. Glenz, K. *et al.* Production of a recombinant bacterial lipoprotein in higher plant chloroplasts. *Nat. Biotechnol.* **24**, 76–77 (2006).
21. Fischer, R. & Emans, N. Molecular farming of pharmaceutical proteins. *Transgenic Res.* **9**, 279–299 (2000).
22. Cramer, C.L., Boothe, J.G. & Oishi, K.K. Transgenic plants for therapeutic proteins: linking upstream and downstream strategies. *Curr. Top. Microbiol. Immunol.* **240**, 95–118 (1999).
23. Sidorov, V.A. *et al.* Technical advance: stable chloroplast transformation in potato: use of green fluorescent protein as a plastid marker. *Plant J.* **19**, 209–216 (1999).
24. Ruf, S., Hermann, M., Berger, I.J., Carrer, H. & Bock, R. Stable genetic transformation of tomato plastids and expression of a foreign protein in fruit. *Nat. Biotechnol.* **19**, 870–875 (2001).
25. Daniell, H., Vivekananda, J., Nielsen, B.L., Ye, G.N. & Tewari, K.K. Transient foreign gene expression in chloroplasts of cultured tobacco cells after biolistic delivery of chloroplast vectors. *Proc. Natl. Acad. Sci. USA* **87**, 88–92 (1990).
26. Kunnimalaiyaan, M. & Nielsen, B.L. Fine mapping of replication origins (ori A and ori B) in *Nicotiana tabacum* chloroplast DNA. *Nucleic Acids Res.* **25**, 3681–3686 (1997).
27. Allison, L.A., Simon, L.D. & Maliga, P. Deletion of *rpo*B reveals a second distinct transcription system in plastids of higher plants. *EMBO J.* **15**, 2802–2809 (1996).
28. Ruiz, O.N., Hussein, H.S., Terry, N. & Daniell, H. Phytoremediation of organomercurial compounds via chloroplast genetic engineering. *Plant Physiol.* **132**, 1344–1352 (2003).
29. Magee, A. *et al.* T7 RNA polymerase-directed expression of an antibody fragment transgene in plastids causes a semi-lethal pale-green seedling phenotype. *Transgenic Res.* **13**, 325–337 (2004).
30. Muhlbauer, S.K. & Koop, H.U. External control of transgene expression in tobacco plastids using the bacterial lac repressor. *Plant J.* **43**, 941–946 (2005).
31. Daniell, H., Carmona-Sanchez, O. & Burns, B. Chloroplast derived antibodies, biopharmaceuticals and edible vaccines. In *Molecular Farming* (eds. Schillberg, S. & Wiley, V.C.H.) 113–133 (Verlag publishers, Germany, 2004).
32. Daniell, H. & McFadden, B.A Uptake and expression of bacterial and cyanobacterial genes by isolated cucumber etioplasts. *Proc. Natl. Acad. Sci. USA* **84**, 6349–6353 (1987).
33. Boynton, J.E. *et al.* Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. *Science* **240**, 1534–1538 (1988).
34. Golds, T., Maliga, P. & Koop, H.U. Stable plastid transformation in PEG-treated protoplasts of *Nicotiana tabacum*. *Nat. Biotechnol.* **11**, 95–97 (1993).
35. O'Neill, C., Horvath, G.V., Horvath, E., Dix, P.J. & Medgyesy, P. Chloroplast transformation in plants: polyethylene glycol (PEG) treatment of protoplast is an alternative to biolistic delivery systems. *Plant J.* **3**, 729–738 (1993).
36. Svab, Z. & Maliga, P. High-frequency plastid transformation in tobacco by selection for a chimeric *aad*A gene. *Proc. Natl. Acad. Sci. USA* **90**, 913–917 (1993).
37. Kumar, S., Dhingra, A. & Daniell, H. Plastid-expressed betaine aldehyde dehydrogenase gene in carrot cultured cells, roots, and leaves confers enhanced salt tolerance. *Plant Physiol.* **136**, 2843–2854 (2004).

38. Brosch, M., Krause, K., Falk, J. & Krupinska, K. Analysis of gene expression in amyloplasts of potato tubers. *Planta* **227**, 91–99 (2007).
39. Watson, J., Koya, V., Leppla, S.H. & Daniell, H. Expression of *Bacillus anthracis* protective antigen in transgenic chloroplasts of tobacco, a non-food/feed crop. *Vaccine* **22**, 4374–4384 (2004).
40. Tregoning, J.S. *et al.* Expression of tetanus toxin Fragment C in tobacco chloroplasts. *Nucleic Acids Res.* **31**, 1174–1179 (2003).
41. Tregoning, J.S. *et al.* Protection against tetanus toxin using a plant-based vaccine. *Eur. J. Immunol.* **35**, 1320–1326 (2005).
42. Molina, A., Hervás-Stubbs, S., Daniell, H., Mingo-Castel, A.M. & Veramendi, J. High-yield expression of a viral peptide animal vaccine in transgenic tobacco chloroplasts. *Plant Biotechnol. J.* **2**, 141–153 (2004).
43. Molina, A., Veramendi, J. & Hervás-Stubbs, S. Induction of neutralizing antibodies by a tobacco chloroplast-derived vaccine based on a B cell epitope from canine parvovirus. *Virology* **342**, 266–275 (2005).
44. Fernández-San Millán, A., Mingo-Castel, A., Miller, M. & Daniell, H. A chloroplast transgenic approach to hyper-express and purify Human Serum Albumin, a protein highly susceptible to proteolytic degradation. *Plant Biotechnol. J.* **1**, 71–79 (2003).
45. DeGray, G., Rajasekaran, K., Smith, F., Sanford, J. & Daniell, H. Expression of an antimicrobial peptide via the chloroplast genome to control phytopathogenic bacteria and fungi. *Plant Physiol.* **127**, 852–862 (2001).
46. Kamarajugadda, S. & Daniell, H. Chloroplast-derived anthrax and other vaccine antigens: their immunogenic and immunoprotective properties. *Expert Rev. Vaccines* **5**, 839–849 (2006).
47. Dufourmantel, N. *et al.* Generation of fertile transplastomic soybean. *Plant Mol. Biol.* **55**, 479–489 (2004).
48. Kanamoto, H. *et al.* Efficient and stable transformation of *Lactuca sativa* L. cv. Cisco (lettuce) plastids. *Transgenic Res.* **15**, 205–217 (2006).
49. Daniell, H., Chebolu, S., Kumar, S., Singleton, M. & Falconer, R. Chloroplast-derived vaccine antigens and other therapeutic proteins. *Vaccine* **23**, 1779–1783 (2005).
50. Daniell, H. Production of biopharmaceuticals and vaccines in plants via the chloroplast genome. *Biotechnol. J.* **1**, 1071–1079 (2006).
51. Leelavathi, S., Gupta, N., Maiti, S., Ghosh, A. & Reddy, V.S. Overproduction of an alkali- and thermo-stable xylanase in tobacco chloroplasts and efficient recovery of the enzyme. *Mol. Breed.* **11**, 59–67 (2003).
52. Viitanen, P.V. *et al.* Metabolic engineering of the chloroplast genome using the *Escherichia coli* ubiC gene reveals that chorismate is a readily abundant plant precursor for p-hydroxybenzoic acid biosynthesis. *Plant Physiol.* **136**, 4048–4060 (2004).
53. *Molecular Cloning: A Laboratory Manual* (eds. Sambrook, J. & Russell, D.W.) (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York 2001).
54. Costa, G.L. & Weiner, M.P. Bidirectional and directional cloning of PCR products. In *PCR Primer* 2nd edn. (eds. Dieffenbach C.W. and Dveksler G.S.) (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2003).
55. Lutz, A.L., Svab, Z. & Maliga, P. Construction of marker-free transplastomic tobacco using the Cre-*loxP* site-specific recombination system. *Nat. Protoc.* **1**, 1–11 (2006).
56. Daniell, H., Ruiz, O.N. & Dhingra, A. Chloroplast genetic engineering to improve agronomic traits. *Methods Mol. Biol.* **286**, 111–137 (2005).
57. Nugent, G.D., Coyne, S., Nguyen, T.T., Kavanagh, T.T. & Dix, P.J. Nuclear and plastid transformation of *Brassica oleracea* var. *botrytis* (cauliflower) using PEG-mediated uptake of DNA into protoplasts. *Plant Sci.* **170**, 135–142 (2006).
58. Kumar, S., Dhingra, A. & Daniell, H. Stable transformation of the cotton plastid genome and maternal inheritance of transgenes. *Plant Mol. Biol.* **56**, 203–216 (2004).
59. Lelivelt, C. *et al.* Stable plastid transformation in lettuce (*Lactuca sativa* L.). *Plant Mol. Biol.* **58**, 763–774 (2005).
60. Hou, B.K. *et al.* Chloroplast transformation in oilseed rape. *Transgenic Res.* **12**, 111–114 (2003).
61. Zubko, M., Zubko, E., Zuilen, K., Meyer, P. & Day, A. Stable transformation of petunia plastids. *Transgenic Res.* **13**, 523–530 (2004).
62. Okumura, S. *et al.* Transformation of poplar (*Populus alba*) plastids and expression of foreign proteins in tree chloroplasts. *Transgenic Res.* **15**, 637–646 (2006).
63. Nguyen, T.T., Nugent, G., Cardi, T. & Dix, P.J. Generation of homoplasmic plastid transformants of a commercial cultivar of potato (*Solanum tuberosum* L.). *Plant Sci.* **168**, 1495–1500 (2005).
64. Lee, S.M. *et al.* Plastid transformation in the monocotyledonous cereal crop, rice (*Oryza sativa*) and transmission of transgenes to their progeny. *Mol. Cells* **21**, 401–410 (2006).
65. Daniell, H., Datta, R., Varma, S., Gray, S. & Lee, S.B. Containment of herbicide resistance through genetic engineering of the chloroplast genome. *Nat. Biotechnol.* **16**, 345–348 (1998).
66. McBride, K.E. *et al.* Amplification of a chimeric Bacillus gene in chloroplasts leads to an extraordinary level of an insecticidal protein in tobacco. *Biotechnology* **13**, 362–365 (1995).
67. Kota, M. *et al.* Overexpression of the *Bacillus thuringiensis* (Bt) Cry2Aa2 protein in chloroplasts confers resistance to plants against susceptible and Bt-resistant insects. *Proc. Natl. Acad. Sci. USA* **96**, 1840–1845 (1999).
68. Dufourmantel, N. *et al.* Generation and analysis of soybean plastid transformants expressing *Bacillus thuringiensis* Cry1Ab protoxin. *Plant Mol. Biol.* **58**, 659–668 (2005).
69. Chakrabarti, S.K., Lutz, K.A., Lertwiriyawong, B., Svab, Z. & Maliga, P. Expression of the *cry9Aa2 B.t.* gene in tobacco chloroplasts confers resistance to potato tuber moth. *Transgenic Res.* **15**, 481–488 (2006).
70. Iamtham, S. & Day, A. Removal of antibiotic resistance genes from transgenic tobacco plastids. *Nat. Biotechnol.* **18**, 1172–1176 (2000).
71. Guda, C., Lee, S-B. & Daniell, H. Stable expression of a biodegradable protein-based polymer in tobacco chloroplasts. *Plant Cell Rep.* **19**, 257–262 (2000).
72. Lössl, A., Eibl, C., Harloff, H.J., Jung, C. & Koop, H.U. Polyester synthesis in transplastomic tobacco (*Nicotiana tabacum* L.): significant contents of polyhydroxybutyrate are associated with growth reduction. *Plant Cell Rep.* **21**, 891–899 (2003).
73. Zhang, X.H., Brotherton, J.E., Widholm, J.M. & Portis, A.R. Targeting a nuclear anthranilate synthase alpha-subunit gene to the tobacco plastid genome results in enhanced tryptophan biosynthesis: return of a gene to its pre-endosymbiotic origin. *Plant Physiol.* **127**, 131–141 (2001).
74. Roh, K.H. *et al.* Accumulation of sweet protein monellin is regulated by the *psb*A 5′UTR in tobacco chloroplasts. *J. Plant Biol.* **49**, 34–43 (2006).