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Breakthrough in chloroplast genetic engineering of agronomically important crops

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Abstract

Chloroplast genetic engineering offers several unique advantages, including high-level transgene expression, multi-gene engineering in a single transformation event and transgene containment by maternal inheritance, as well as a lack of gene silencing, position and pleiotropic effects and undesirable foreign DNA. More than 40 transgenes have been stably integrated and expressed using the tobacco chloroplast genome to confer desired agronomic traits or express high levels of vaccine antigens and biopharmaceuticals. Despite such significant progress, this technology has not been extended to major crops. However, highly efficient soybean, carrot and cotton plastid transformation has recently been accomplished through somatic embryogenesis using species-specific chloroplast vectors. This review focuses on recent exciting developments in this field and offers directions for further research and development.

Introduction

In most angiosperm plant species, plastid genes are inherited uniparentally in a strictly maternal fashion [1,2]. Even though transgenic chloroplasts might be present in pollen, plastid DNA is eliminated from the male germ line at different points during sperm cell development, depending upon the plant species [1]. This minimizes the possibility of outcrossing transgenes to related weeds or crops [3,4] and reduces the potential toxicity of transgenic pollen to non-target insects [5]. Thus, maternal inheritance offers containment of chloroplast transgenes as a result of the lack of gene flow through pollen [3,4].

Another advantage of plastid transformation is the ability to accumulate in the chloroplast any foreign proteins or their biosynthetic products that could be harmful if they were in the cytoplasm [6]. Cholera toxin B subunit (CTB), a candidate oral subunit vaccine for cholera, was non-toxic when accumulated in large quantities within transgenic plastids yet was toxic when expressed in leaves via the nuclear genome, even at very low levels [7,8]. Similarly, trehalose, a pharmaceutical industry preservative, was very toxic when it accumulated in the cytosol but was non-toxic when it was compartmentalized within plastids [9]. Hyper-expression of the industrially important enzyme xylanase in the chloroplast did not cause cell wall degradation and plant growth was not affected, unlike expression of xylanase in nuclear transformants [10].

Additionally, site-specific transgene integration into spacer regions of the chloroplast genome eliminates concerns of position effects that are frequently observed in nuclear transgenic plants. All chloroplast transgenic lines express the same level of foreign protein, within the range of physiological variations [7]. Site-specific integration also eliminates the introduction of vector sequences, which are potential concerns in nuclear transformation [11]. Foreign transcripts do not silence genes in chloroplast transgenic lines despite their accumulation at a level 169 times higher than nuclear transgenic plants [9,12]. Similarly, despite the accumulation of over 46% foreign protein in leaves of chloroplast transgenic lines [5], no post-transcriptional gene silencing has been observed.

Multigene engineering through the chloroplast genome is possible in a single transformation event. For example, introduction of the *cry* operon from *Bacillus thuringiensis* (Bt), coding for the insecticidal protein delta-endotoxin, expressed up to 46% of the total leaf protein [5]. Two bacterial enzymes that confer resistance to different forms of mercury – mercuric ion reductase (*merA*) and organo-mercurial lyase (*merB*) – were expressed as an operon in transgenic chloroplasts and conferred resistance to very high levels of mercury and organomercurial compounds [13]. Three bacterial genes coding for the PHB operon, when expressed via the chloroplast genome, accumulated significant quantities of this polymer [14]. Foreign genes expressed via the plastid genome now bestow useful agronomic traits (Table 1) as well as therapeutic proteins (Table 2). With the exception of carrot, all of these studies reported plastid transformation in tobacco.

Limitations of plastid genetic engineering

Plastid transformation has been highly efficient only in tobacco. Major obstacles to extend this technology to crop plants that regenerate through somatic embryogenesis include inadequate tissue culture and regeneration protocols, lack of selectable markers and inability to express transgenes in non-green plastids [6,11]. The first challenge is to introduce foreign DNA into non-green tissues containing several kinds of plastids, namely proplastids, leucoplasts, amyloplasts, etioplasts, chromoplasts, elaioplasts and gerontoplasts, in which gene expression and gene regulation systems are quite different from mature green chloroplasts [6]. Identification of appropriate regulatory sequences that function in non-green plastids is necessary to achieve foreign gene expression. Yet another major challenge is the ability to regenerate chloroplast transgenic plants through somatic embryogenesis and achieve homoplasmy, which lacks the benefit of subsequent rounds of regeneration offered by organogenesis, because segments of somatic embryos cannot be regenerated into plants.

Transformation of *Arabidopsis*, potato and tomato chloroplast genomes was achieved by organogenesis but the efficiency was much lower than tobacco [15–17]. In *Arabidopsis*, 1 chloroplast transgenic line per 40 or 151 bombarded plates was obtained but they were not fertile. In potato, one chloroplast transgenic line per 25 bombarded plates, and in tomato 1 transgenic line per 10 bombarded plates was obtained (six transformed clones in 540 selection plates). By contrast, up to 14 chloroplast transgenic lines were obtained per bombarded leaf in tobacco [7]. In the case of *Lesquerella*, transgenic clones had to be grafted onto *Brassica napus* rootstock to obtain seeds. Only two chloroplast transgenic clones were obtained from 51 bombarded samples [18]. In soybean, only one heteroplasmic event was recovered out of 984 bombardments performed on embryogenic suspension cultures [19] but this could not be regenerated. In rice, transient integration of the transgene was demonstrated by PCR analysis but stable integration was not achieved [20]; transgenic plants were highly heteroplasmic and the transgenes were not carried over to the next generation. Similarly, in oilseed rape, Southern analysis of the transgenic chloroplast genome was not shown to confirm homoplasmy [21]. Tissue culture limitations and use of

non-green explants have often been cited as primary reasons that have limited chloroplast transformation to solanaceous crops [6].

In spite of the aforementioned failures or limitations, plastid genetic engineering has been achieved recently in several major crops, including cotton and soybean [22,23], and the carrot plastid genome has been transformed [24].

Transformation of the carrot plastid genome

Carrot (*Daucus carota* L.) is one of the most important vegetable crops used worldwide for human and animal consumption, as it is an excellent source of sugars, vitamins A and C, and fiber in the diet [25]. Carrot is classified as a salt-sensitive plant and there is 7% growth reduction for every 10 mM increment in salinity above 20 mM salt [26]. Therefore, carrot is an ideal candidate for genetic manipulation for increased salt tolerance.

Salt stress is a major abiotic stress in plant agriculture. The problem of soil salinity has been compounded by irrigation and excessive use of fertilizers. About 20% of the world's irrigated lands are affected by salinity [27]. Currently, high salinity limits crop production in 30% of the irrigated land in the USA and 20 million hectares globally. One of the metabolic adaptations to salt stress is the accumulation of osmoprotectants. Glycine betaine protects the cell from salt stress by maintaining an osmotic balance with the environment and by stabilizing the quaternary structure of complex proteins. This has been demonstrated in several reports where nuclear transgenic plants accumulating glycine betaine exhibit moderate levels of tolerance to salt stress [28,29].

Kumar *et al.* [24] have recently reported the first successful demonstration of plastid genetic engineering by somatic embryogenesis and the use of non-green cells as recipients of foreign DNA. Transgenic calli derived from cultured cells expressing *betaine aldehyde dehydrogenase* (*badh*) were green in color even in the absence of selection, whereas non-transgenic cells were yellow in color (Figure 1b,c). BADH enzyme activity was enhanced eightfold in transgenic carrot cell cultures in the presence of 100 mM NaCl and they grew sevenfold more (based on fresh weight), when compared with untransformed cells (Figure 1d,e). BADH expression was 74.8% in non-green edible parts (carrots) containing chromoplasts, and 53% in proplastids of cultured cells when compared with chloroplasts (100%) in leaves (Figure 1a). Transformed carrot cells grown in 100 mM NaCl accumulated 50–54-fold more betaine (measured by ¹H-NMR) than untransformed cells. These levels are as high as betaine levels observed in halophytes [24]. Expression of BADH in transgenic plants was adequate to confer very high levels of salinity tolerance (up to 400 mM NaCl; Figure 1f–i). This appears to be the highest level of salt tolerance reported in the literature.

Carrot is ideal for oral delivery of therapeutic proteins

Chloroplast genetic engineering is most suitable for hyper-expression of vaccine antigens and production of valuable therapeutic proteins. Several therapeutic proteins have now been expressed in transgenic tobacco chloroplasts: vaccine antigens for cholera, anthrax, plague and tetanus [7,30–35]; monoclonal antibodies [34,36]; and several human therapeutic proteins, including human somatotropin [37], human serum albumin [38], interferons [34,39], insulin-like growth factor [35] and magainin [40]. However, there is an urgent need for oral delivery of therapeutic proteins and vaccine antigens in order to reduce significantly their cost of production, purification, cold storage and transportation, and to minimize complications associated with intravenous delivery [32].

Carrot is ideal for producing therapeutic proteins owing to the maternal inheritance of transgenes integrated into plastid genomes [41] and the absence of flowering in the first year

when this biennial crop is harvested [42]; both these features help achieve zero-contamination of food crops by Pharm Crops, this being a requirement of various regulatory agencies. Carrot somatic embryos are derived from a single cell and multiply through recurrent embryogenesis, and can be maintained for several years as *in vitro* culture; this provides a uniform source of cell culture, which is one of the essential requirements for producing therapeutic proteins (homogeneous single source of origin). Carrot cells divide rapidly and a large biomass can be produced using bioreactors. When therapeutic proteins are delivered by carrot cells, there is no need to cook, and this should preserve the structural integrity of therapeutic proteins during consumption. With synthetic seed technology, somatic embryos can be cryopreserved for many years. Thus, transgenic carrot with enhanced medicinal or nutritional value can play a crucial role in improving the medicinal value and the health of humans. However, it should be emphasized that fresh vegetables will never be used for oral delivery. Instead, freeze-dried plant cells expressing the therapeutic protein will be used in the form of capsules, to regulate dosage.

High levels of transgene expression were observed in proplastids of cultured carrot cells [24], in sharp contrast to a previous report in which 100-fold less green fluorescent protein (GFP) accumulation in amyloplasts of potato tubers was observed when compared with leaves [16]. Proplastids of carrot cell cultures and carrot chromoplasts showed 53.1% and 74.8% BADH activity respectively when compared with leaf chloroplasts (100%). Regulatory sequences used in this study [16s rRNA promoter, T7 gene 10 5' untranslated region (UTR), rps 16 3' UTR] functioned in all tissue types containing proplastids, chromoplasts and chloroplasts [24]. These observations predict high-level expression of vaccine antigens or other therapeutic proteins in carrot to facilitate oral delivery.

Transformation of the cotton plastid genome

Cotton (*Gossypium hirsutum* L.) is an excellent natural source of textile fiber in the world and is one of the world's most important commercial crops. The USA accounts for over 40% of total world fiber production (\$6.1 billion in annual sales) and is one of the leading exporters in the global trade of raw cotton. Products such as cotton lint and cottonseed are among the top 20 major agricultural products of the USA by value as reported by the Food and Agriculture Organization of the United Nations (FAO) in 2003 (<http://www.nationmaster.com/encyclopedia/USA-agriculture>). The annual business revenue stimulated by cotton in USA economy is about \$120 billion each year, making cotton the highest value crop in the USA according to the International Service for the Acquisition of Agri-biotech Applications (ISAAA; <http://www.isaaa.org>). However, cotton is particularly challenging to manipulate *in vitro* as a result of the difficulties encountered in plant regeneration through somatic embryogenesis.

In 2002/03, insect- and herbicide-resistant transgenic cotton (engineered via the nuclear genome) was planted on 13% of the total area in the world compared with soybean (63%) and corn (19%). In the USA, 77% of the total area of genetically modified (GM) cotton is planted when compared with 81% of GM soybean and 40% GM corn (<http://www.ers.usda.gov>). So far, nuclear transgenic cotton is planted only in restricted areas of the world. For instance, Upland cotton (*Gossypium hirsutum*) has the potential to hybridize with Hawaiian cotton (*Gossypium tomentosum*) and feral populations of *G. hirsutum* in the Florida Keys and *G. hirsutum*/*Gossypium barbadense* in the Virgin Islands and Puerto Rico. For these reasons, restrictions on field plot experimental use permits and commercial planting of Bt-cotton has been instituted in these areas. Similarly, GM cotton is now planted only in regions of the world where there are no wild relatives, to avoid potential outcross with related weeds. Dispersal of pollen from transgenic cotton plants to surrounding non-transgenic plants has been reported [43]. Umbeck *et al.* [44] investigated pollen dispersal

from transgenic cotton embedded in a field of conventional cotton in the USA and observed up to 5.7% outcrossing rates despite buffer rows. Transgene escape could be avoided by chloroplast genetic engineering because of maternal inheritance of transgenes in cotton [1]. Another concern about GM crops expressing Bt toxins is that suboptimal production of toxins might result in an increased risk of pests developing Bt resistance [45]. Furthermore, nuclear-engineered Bt-cotton is not fully protected from attack by insects/pests as a result of low expression of transgenes, and several sprays of pesticides on crop fields are required to minimize the yield loss (<http://www.pmac.net/bt2.htm>).

Kumar *et al.* [22] have recently demonstrated stable transformation of the cotton plastid genome and maternal inheritance of transgenes. *In vitro*-produced transgenic cotton lines were grown in the growth chamber along with non-transgenic plants grown under similar conditions (Figure 2a–d). Growth of chloroplast transgenic lines (Figure 2c), onset of flowering, floral parts, boll formation and seed setting (Figure 2e–g) were similar to the untransformed cotton plants (Figure 2d,h–j). Seedlings (158) from F1 crosses (non-transgenic ♀ × ♂ transgenic) were able to germinate on kanamycin selection medium but failed to grow further (Figure 2k), whereas transgenic seeds were resistant to kanamycin and germinated well, producing copious roots and leaves (Figure 2l). This confirms earlier observations [1] that there is no paternal or biparental inheritance of chloroplast genomes in cotton and that the chloroplast transgenic trait is inherited maternally. All seeds derived from self-pollinated chloroplast transgenic plants germinated on kanamycin and, therefore, no Mendelian segregation was observed among the tested seeds.

Transformation of the soybean plastid genome

Soybean (*Glycine max* L. Merr.), a leguminous crop, is considered as the most important source of proteins. It is widely used as animal feed and for human consumption. The dry matter of soybeans contains ~20% oil and 35–40% proteins of high nutritional quality. It is also the most planted GM crop, representing in 2003 more than half of the soybean-cultivated area worldwide. This corresponds to the adoption by farmers of glyphosate-tolerant cultivars, a trait that has been engineered via the nuclear genome. Presently, much effort is being devoted to the engineering of insect resistance and the improvement of disease resistance and oil quality. The engineering of such traits through the soybean plastid genome, so far not reported, could present the added advantage of transgene containment in the field, since the plastid genome of soybean is inherited maternally [46].

The crucial step of transformation for most plants is the tissue culture. Owing to their ability to regenerate whole plants, cultured soybean embryogenic tissue is the most commonly used material for nuclear transformation by particle bombardment [47]. Nevertheless, soybean transformation still remains difficult, as in other grain legumes [48]. Therefore, the transformation of soybean chloroplasts presents a real challenge because embryogenic undifferentiated cells contain fewer and much smaller plastids [49]. The absence of the complete soybean plastome sequence in the databases up to now does not simplify the task.

Chloroplast genetic engineering of soybean was first attempted by Zhang *et al.* [49], with the objective of increasing its photosynthetic potential. The first successful development of the chloroplast genetic engineering technology by somatic embryogenesis and the generation of fertile chloroplast transgenic plants of soybean was reported by Dufourmantel *et al.* [23]. The transformation efficiency achieved (~2 transformants per bombardment), is 15–100-fold higher than for potato [16], tomato [17], *Lesquerella fendleri* [18] and *Arabidopsis thaliana* [15]. Phenotypically normal transgenic plants were regenerated by somatic embryogenesis (Figure 3a,b). All chloroplast transgenic plants were fully fertile and produced viable seeds. The T1 progenies were uniformly resistant to the antibiotic, confirming the stability of the

transgene and maternal inheritance (Figure 3c,d). The presence of an antibiotic resistance gene under the control of regulatory elements functional in bacteria might constitute a problem for public acceptance. Selection markers such as herbicide tolerance genes or BADH could be developed for soybean [50]. However, selectable markers have their limitations. Herbicide resistance genes cannot be used for the first round of selection; spectinomycin selection is lethal for several crops, including cotton. BADH cannot be used for species that have high levels of endogenous glycine betaine. Alternatively, different systems for marker elimination have been described, relying on homologous recombination between two direct repeats [51], or with site-specific recombination systems such as *Cre-Lox* [52,53], or eliminating integration of marker genes altogether [54].

Reasons for successful plastid transformation in major crops

The aforementioned studies report highly efficient plastid transformation in challenging crops. The vectors employed for chloroplast transformation of potato, tomato and *Lesquerella* contained the flanking sequences from tobacco or *Arabidopsis* [16–18]. This might be one of the reasons for lower transformation efficiency in these crops. Efficiency of tobacco plastid transformation using homologous native flanking sequences has been quite high [38,55]. However, when petunia flanking sequences were used for chloroplast transformation of tobacco, the transformation efficiency decreased drastically [40]. Plastid transformation using less than 100% homologous plastid DNA sequences has been shown in several other cases to result in much lower frequencies [16,17,40,56,57]. Therefore, even though the universal vector concept was proposed several years ago [58] and used to transform several closely related crops (e.g. tobacco, tomato, potato), species-specific vectors have been used for demonstration of plastid transformation, especially in recalcitrant crops. These studies underscore the need to sequence crop chloroplast genomes. Unfortunately, among more than 40 published chloroplast genomes [59] and 200 that are in progress around the world (reported on websites), only six are crop chloroplast genomes.

Understanding and manipulating the somatic embryogenesis system, which lacks the advantage of subsequent rounds of regeneration from heteroplasmic tissues, is a major challenge. During transformation, transformed proplastids in non-green tissues should develop into mature chloroplasts and transformed cells should survive the selection process during all stages of development. Therefore, one major challenge is to provide plastids with the ability to survive selection in the light and the dark, at different developmental stages. This is absolutely crucial when only one or two chloroplasts are transformed in a plant cell after bombardment and these plastids should have the ability to survive the selection pressure, multiply and establish themselves while all other untransformed plastids are eliminated in the selection process.

The ‘double barrel’ vector used for cotton plastid transformation accomplishes this by using genes coding for two different enzymes capable of detoxifying the same selection agent (or spectrum of selection agents) because it is driven by regulatory signals that are functional in proplastids as well as in mature chloroplasts. Both *aphA-6* and *aphA-2* (*nptII*) genes code for enzymes that belong to the aminoglycoside phosphotransferase family but they originate from different prokaryotic organisms. Both enzymes have similar catalytic activity but the *aphA-6* gene product has an extended ability to detoxify kanamycin and provides a wider spectrum of aminoglycoside detoxification, including amikacin [60,61]. Both transgenes (*aphA2* or *nptII* and *aphA6*) are transcribed by the full-length plastid *Prrn* promoter containing the binding site for nuclear-encoded and plastid-encoded RNA polymerase and is expected to function both in proplastids and mature chloroplasts [62]. The *aphA-6* gene is further regulated by the T7 gene 10 5′UTR, which is capable of efficient translation in the dark and in proplastids present in non-green tissues. The rps16 3′UTR was used to stabilize

aphA-6 gene transcripts. The T7 gene 10 5' UTR and *rps16* 3' UTR facilitated 74.8% BADH expression in non-green edible parts (carrots) containing chromoplasts (grown under the ground in the dark) and 48% in proplastids, compared with chloroplasts in leaves (100%) [24]. Therefore, it was anticipated that the *aphA6* gene would be expressed in non-green and green plastids in the light or dark. The *npdI* gene in the cotton plastid transformation vector was driven by the *psbA* 5' and *psbA* 3' UTRs, which have been repeatedly shown to be responsible for light regulated expression of transgenes integrated into the plastid genome [12,38,30]. Thus, it is logical to expect breakdown of kanamycin in both dark and light conditions. Therefore, a combination of both *aphA-6* and *aphA-2* genes, driven by regulatory signals in the light and in the dark, in both proplastids and chloroplasts, provided continuous protection for transformed plastids/chloroplasts from the selection agent. This hypothesis is supported by minimal transformation efficiency (5%, defined as the total number of events $\times$ 100 per total number of plates bombarded) observed in cotton using the single gene, when compared with 42% using the double barrel vector, despite the use of regulatory sequences of the single gene that expressed transgenes efficiently in different types of plastids. By contrast, a single selectable marker gene (*aadA*) driven by light regulatory elements (*rbcL* 5' UTR and the *psbA* 3' UTR) was adequate for soybean plastid transformation because the starting material for bombardment was already green, contained mature chloroplasts and supported photosynthetic activity [23].

Yet another reason for higher transformation efficiency in carrot and cotton might be the presence of the chloroplast DNA replication origin within the long flanking sequence that offered more templates for transgene integration (see Ref. [22] for an in-depth discussion). For example, when chloroplast vectors with or without *oriA* were bombarded into cultured tobacco cells, only the vector with *oriA* located within the *trnI* gene showed prolonged and higher levels of chloramphenicol acetyl transferase (CAT) enzyme activity [63]. When chloroplast vectors with or without *oriA* were bombarded with the same transgenes, the vector with *oriA* present within the *trnI* flanking region achieved homoplasmy even in the first round of selection [64]. The origin of replication has been mapped in the tobacco chloroplast genome and *oriA* is located within the *trnI* gene that forms the left flank in the chloroplast transformation vector used for cotton and carrot transformation [65]. In addition, this transcriptionally active spacer region is a highly preferred site for transgene integration; more transgenes have been integrated at this site than any other site [12,55].

Conclusion

Plastid transformation holds great potential for the introduction of important agronomic traits to plants, as well as the production of biomaterials and therapeutic proteins such as antibodies, biopharmaceuticals and vaccine antigens. High-level gene expression in transgenic chloroplasts should allow the generation of large-quantity but low-cost therapeutic proteins. Purification methods such as chromatography are unnecessary, as oral delivery of therapeutic proteins should completely eliminate this need. Because of this, several major biotechnology companies have initiated research projects to commercialize this technology.

Recent studies highlight the importance of generating plastid transformation systems using somatic embryos or embryogenic calli, which might pave the way to engineer the plastid genome of several major crops in which regeneration is mediated through somatic embryogenesis. Many crops, including cereals (rice, wheat, barley, oats, sorghum, corn), legumes (alfalfa, lentils, peanut, pea, soybean), oil crops (palm, sunflowers, coconut, canola, olive), cash crops (cotton, sugarcane, cassava), vegetable crops (potato, tomato, carrot, sweet potato, sugarbeet, squash, cucumber, lettuce, broccoli, cauliflower, snap bean, cabbage, celery, onion, garlic), fruits/trees and nuts (banana, grape, cantaloupe, muskmelon,

watermelon, strawberry, orange, apple, mango, avocado, peach, grapefruit, pineapple, maple almond), beverages (coffee, tea, coca), timber trees (oak, black walnut, sycamore), and so on, are regenerated *in vitro* by somatic embryogenesis.

In addition, plastid transformation has become a powerful tool for the study of plastid biogenesis and function. This approach has been used to investigate plastid DNA replication origins, RNA editing elements, promoters, RNA stability determinants, processing of polycistrons, intron maturases, translation elements and proteolysis, import of proteins and several other processes [45]. Thus, recent advancements augur well for the production of therapeutic proteins, vaccines and biomolecules in transgenic plastids, and the introduction of agronomic traits into crops using an environmentally friendly approach, and will increase our understanding of plastid biochemistry and molecular biology.

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Figure 1.

Transformation of the carrot plastid genome. **(a)** Complete transgenic carrot plant with orange color of root (edible part) and green shoots. The expression of betaine aldehyde dehydrogenase in carrot cells promoted the green color in transgenic cells, which offers the visual selection of transgenic calli **(b)** versus yellow non-transgenic carrot calli **(c)**. **(d)** Transgenic carrot cells showed proliferic growth in the liquid medium supplemented with 100 mM NaCl, whereas **(e)** untransformed carrot cell culture did not grow in the presence of salt. **(f–i)** Transgenic carrot plants thrived well in soil pots irrigated with 200–500 mM sodium chloride, whereas untransformed carrot plants showed retarded growth in the presence of salt.

Figure 2.

Transformation of the cotton plastid genome using the double barrel vector. Untransformed creamy-color cotton calli **(a)** are transformed to greenish color under selection for 2–3 months, after bombardment **(b)**. Transgenic **(c)** and non-transgenic control cotton plants **(d)** are shown at the stage of flowering and setting seeds. Different floral parts of non-transgenic control cotton **(e–g)** are comparable with floral parts of transgenic cotton **(h–j)**. F1 seedlings produced from crosses between transgenic $\sigma \times \varphi$ non-transgenic cotton showed retarded germination **(k)**, whereas selfed transgenic cotton seedlings **(l)** germinated well on kanamycin selection medium, confirming the maternal inheritance of transgenes.

Figure 3. Transformation of the soybean plastid genome. Untransformed **(a)** and transformed **(b)** soybean plants are shown. Spectinomycin selection medium had a detrimental effect on the untransformed seedlings of soybean **(c)**, whereas transformed seedlings **(d)** grew well, confirming the maternal inheritance of transgenes.

Table 1

First reports of agronomic traits engineered via the chloroplast genome

Trait	Transgene	Promoter	5'/3' UTRs	Homologous recombination site	Laboratory	Refs
Insect resistance	<i>CryIA (c)</i>	Prm	<i>rbcl</i> / <i>trps16</i>	<i>trnN</i> / <i>trps12/7</i>	McBride	[66]
Herbicide resistance	<i>AroA</i>	Prm	ggagg/ <i>trpsbA</i>	<i>rbcl</i> / <i>accD</i>	Daniell	[58]
Insect resistance	<i>Cry2A.a2</i>	Prm	ggagg (native)/ <i>trpsbA</i>	<i>rbcl</i> / <i>accD</i>	Daniell	[67]
Herbicide resistance	<i>bar</i>	Prm	<i>rbcl</i> / <i>psbA</i>	<i>rbcl</i> / <i>accD</i>	Day	[51]
Insect resistance	<i>Cry2A.a2</i> operon	Prm	native 5' UTRs/ <i>trpsbA</i>	<i>trnL</i> / <i>trnA</i>	Daniell	[5]
Disease resistance	<i>MSI-99</i>	Prm	ggagg/ <i>trpsbA</i>	<i>trnL</i> / <i>trnA</i>	Daniell	[40]
Drought tolerance	<i>tps</i>	Prm	ggagg/ <i>trpsbA</i>	<i>trnL</i> / <i>trnA</i>	Daniell	[9]
Phytoremediation	<i>merA^Δ/merB^b</i>	Prm	ggagg/ <i>a.b</i> / <i>trpsbA</i>	<i>trnL</i> / <i>trnA</i>	Daniell	[13]
Salt tolerance	<i>badh</i>	Prm-F	ggagg/ <i>trps16</i>	<i>trnL</i> / <i>trnA</i>	Daniell	[24]
Cytoplasmic male sterility	<i>phaA</i>	Prm	<i>trpsbA</i> / <i>trpsbA</i>	<i>trnL</i> / <i>trnA</i>	Daniell	[68]

^{a, b} Refer to genes and their respective regulatory sequences.

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Expression of vaccine antigens and biopharmaceutical proteins via the chloroplast genome

Table 2

Proteins	Transgene	Promoter	5' / 3' UTRs	Tsp expression (%) ^d	Homologous recombination site	Laboratory	Refs
Elastin-derived polymer	<i>EG121</i>	Prm	T7 gene 10/ <i>TpsbA</i>	ND ^e	<i>trnI/trnA</i>	Daniell	[64]
Human somatotropin	<i>hST</i>	Prm ^a , <i>PpsbA</i> ^b	T7 gene 10 ^a , <i>psbA</i> ^b / <i>Tps16</i>	7.0% ^a , 1.0% ^b	<i>trnV/tps12/7</i>	Staub	[37]
Cholera toxin	<i>CtxB</i>	Prm	Ggagg/ <i>TpsbA</i>	4%	<i>trnI/trnA</i>	Daniell	[7]
Antimicrobial peptide	<i>MSI-99</i>	Prm	Ggagg/ <i>TpsbA</i>	21%	<i>trnI/trnA</i>	Daniell	[40]
Interferon α 2b	<i>INFα2B</i>	Prm	<i>PpsbA/TpsbA</i>	19%	<i>trnI/trnA</i>	Daniell	[31] ^f
Human serum albumin	<i>hsa</i>	Prm ^a , <i>PpsbA</i> ^b	ggagg ^a , <i>psbA</i> ^b / <i>TpsbA</i>	0.02% ^a , 11.1% ^b	<i>trnI/trnA</i>	Daniell	[38]
Interferon γ	<i>IFN-γ</i>	<i>PpsbA</i>	<i>PpsbA/TpsbA</i>	6%	<i>rbcl/accD</i>	Reddy	[39]
Monoclonal antibodies	<i>Guy's 13</i> ; <i>HSBV-Is</i>	Prm; atpA/ <i>rbcl</i>	Ggagg/ <i>TpsbA</i>	ND	<i>trnI/trnA</i> ; <i>psbA/5S/23S</i>	Daniell Mayfield	[31] [36]
Anthrax protective antigen	<i>Paγ</i>	Prm	<i>PpsbA/TpsbA</i>	18.1%	<i>trnI/trnA</i>	Daniell	[30]
Plague vaccine	<i>CaF1w/LcN</i>	Prm	<i>PpsbA/TpsbA</i>	4.6%	<i>trnI/trnA</i>	Daniell	[32] ^g
CPV VP2	<i>CTB-2L2/4</i> , <i>GFP-2L2/b</i>	Prm	<i>psbA/TpsbA</i>	31.1% ^a , 22.6% ^b	<i>TrnI/trnA</i>	Daniell/Veramendi	[8]
Rotavirus VP6	<i>Vp6</i>	Prm ^a , <i>PpsbA</i> ^b , <i>Ptrc</i> ^c	<i>rbcl/TrnB</i> ^a <i>psbA/TrnB</i> ^b <i>lacZ/TrnB</i> ^c	3% ^a , 0.6% ^b , 0% ^c	<i>rbcl/accD</i>	Gray	[69]
Tetanus toxin	<i>Tet C</i>	Prm	T7 gene 10 ^a , atpB ^b / <i>Trbc L</i>	25% ^a , 10% ^b	<i>TrnV/tps12/7</i>	Maliga	[33]

^{a-c} Refer to genes and their respective regulatory sequences and % tsp.

^d Tsp: total soluble protein.

^e ND: Not determined.

^f Falconer, R. (2002) Expression of interferon α 2b in transgenic chloroplasts of a low-nicotine tobacco. M.S. Thesis, University of Central Florida, Orlando, FL, USA.

^g Singleton, M.L. (2003) Expression of CaF1 and LcrV as a fusion protein for a vaccine against *Yersinia pestis* via chloroplast genetic engineering. M.S. Thesis, University of Central Florida, Orlando, FL, USA.