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# Multigene engineering: dawn of an exciting new era in biotechnology

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## Abstract

Development of a rice variety enriched in provitamin A, the accumulation of polyhydroxybutyrate polyester in *Arabidopsis* nuclear transgenic plants (with enzymes targeted to chloroplasts in both), and the expression of bacterial operons via the chloroplast genome are recent landmark achievements in multigene engineering. Hyper-expression of transgenes has resulted in the formation of insecticidal protein crystals or inclusion bodies of pharmaceutical proteins in transgenic chloroplasts, achieving the highest level of transgene expression ever reported in transgenic plants. These achievements illustrate the potential of multigene engineering to realize benefits of the post-genomic revolution.

## Introduction

A vast majority of agronomic traits are quantitative and are controlled polygenetically. Genetic engineering is now moving from the initial phase of introducing single gene traits (e.g. resistance to herbicides, disease or insects) to multigenic traits [1], coding for complete metabolic pathways, bacterial operons or biopharmaceuticals that require an assembly of complex multisubunit proteins.

Multigene engineering via the nuclear genome involves several challenges. First, generation of transgenic lines expressing individual genes is necessary, because the nuclear genome does not process polycistrons. Second, such independent transgenic lines that harbor transgenes need to be brought together within a single host by repetitive breeding. Unfortunately, this step is complicated by gene silencing and position effects observed frequently in nuclear transgenic plants. Gene silencing has been observed because of the use of repetitive regulatory sequences, integration of multiple copies of the transgene or even as a result of the efficient transcription of transgenes; it occurs both at the transcriptional and post-transcriptional levels [2]. Position effects are caused by the random integration of transgenes into the nuclear genome. Screening of multiple transgenic lines might require the use of different selectable markers at each step. It is remarkable that, despite these technical hurdles, multiple genes have been skillfully engineered via the nuclear genome for the expression of vitamins [3••,4]. However, these efforts have been highly time-consuming; for example, it took seven years to engineer three genes for the expression of provitamin A, even though the authors were fortunate to introduce two genes at once [5].

Fortunately, there are a few alternative approaches to overcome the aforementioned challenges. In one such effort, a series of three genes encoding a polyprotein containing three enzymes were introduced via the nuclear genome. The polyprotein consisted of



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tobacco vein mottling virus (TVMV) Nla proteinase and two other reporter genes —namely, acetate kinase and *Tn9* chloramphenicol acetyl transferase — separated by the TVMV Nla proteinase recognition sequence. The Nla proteinase facilitated separation of the two enzymes, which were independently functional [6]. Although this approach has been used previously for multigene engineering, this study attempted to simultaneously express foreign proteins in the cytosol and chloroplasts. Such polyproteins should be modified, however, to ensure efficient and predictable processing of individual enzymes within chloroplasts.

Besides technical challenges in nuclear multigene engineering, there are unfortunate negative perceptions and environmental concerns about genetically modified food crops. Lack of gene containment owing to the pollen-mediated out-cross of transgenes from nuclear transgenic plants to related crops or weeds has been a major concern [5,7]. In addition, the possibility of insects developing resistance to insecticidal proteins, due to low levels of transgene expression and toxicity of transgenic pollen to non-target insects, has raised environmental concerns for transgenic plants engineered for pest resistance [5,7].

To address some of these environmental concerns and to facilitate multigene engineering in a single transformation step, the chloroplast genome has been targeted to express several foreign genes [8,9]. Compartmentalization and expression of the transgenes in the maternally inherited chloroplasts should help to allay public concerns about gene containment [10,11]. The ability of plants with transgenic chloroplasts to kill insects that developed very high levels of resistance (up to 40 000-fold) against *Bacillus thuringiensis* insecticidal proteins should also dispel the fear of insects developing resistance in the field [12]. Further, the lack of toxicity of transgenic pollen to non-target insects is yet another advantage of plants with transgenic chloroplasts [13••]. The capability of breaking expression level barriers without causing harmful effects to the host plant and the ability to engineer multiple genes in a single transformation event, are probably the greatest advantages of chloroplast genetic engineering. Coordinated expression of multiple genes, preferably driven by a single promoter, is especially important for stoichiometric synthesis and assembly of multisubunit proteins like monoclonal antibodies [14]. Observations of nearly 50% foreign protein in the total soluble protein (tsp) [13••] and 17 000% more transcripts in chloroplast transgenic plants than nuclear transgenic plants [15•] assuages the concerns of gene silencing at the transcriptional or post-transcriptional level. Position effects are not observed in chloroplast genetic engineering because of targeted gene integration; several independent chloroplast transgenic lines express foreign proteins to the same level, except for minor physiological variations [16•]. In some cases, manipulation of a pathway or hyper-expression of a transgene is very demanding on nuclear transgenic plants, resulting in deleterious pleiotropic effects including stunted growth and sterility. However, such pleiotropic effects observed in nuclear transgenic plants were alleviated when the same foreign proteins were compartmentalized within transgenic chloroplasts [15•,16•,17]. Other recent developments in chloroplast genetic engineering have been the advent of a plant-derived selectable marker [18••] and transformation of the chloroplast genome of edible plant species, including potato and tomato [19,20•]. This review discusses recent achievements and forecasts the future role of chloroplast and nuclear transformation in multigene engineering of plants.

## Nuclear multigene engineering

A significant recent step in multigene engineering has been the development of a rice variety that accumulates provitamin A [3••]. Vitamin A deficiency results in various diseases like night-blindness or even complete blindness. It is estimated that improved vitamin A nutrition can help to prevent over one to two million deaths each year among children aged one to four years. Employing *Agrobacterium*-mediated transformation, three genes essential

for the synthesis of the enzymes of the β-carotene biosynthetic pathway were targeted to plastids in rice endosperm using three different vectors. The β-carotene precursor, geranyl-geranyl-diphosphate, synthesized in the rice endosperm plastids was efficiently processed into phytoene, by phytoene synthase, and then further converted into lycopene in a reaction catalyzed by phytoene desaturase. Lycopene was eventually converted to β-carotene by lycopene β-cyclase, which humans convert into vitamin A. The transgenic rice plants were fertile with no apparent pleiotropic effects.

Another example of nuclear multigene engineering is the expression of three enzymes of the polyhydroxybutyrate (PHB) pathway [21•]. A quadruple construct [22], comprising a selectable marker and three cassettes (each containing one of the three *phb* genes with a plastid targeting signal) flanked by a 35S promoter and *nos* (nopaline synthase) terminator, was used to introduce three genes involved in this pathway. This approach resulted in a large accumulation of PHB (4% fresh weight) fourfold higher than previous reports [17]; however, this had a severe effect on the phenotype of transgenic plants (proportional to PHB accumulation). Lack of gene silencing, in spite of repetitive use of the same regulatory sequences, goes against current understanding of transgene silencing. Unfortunately, the production of PHB polyesters in transgenic plants has not been commercially feasible so far, because of severe effects on growth/fertility and an inability to achieve high expression in large biomass crops.

## Chloroplast multigene engineering

The concept of chloroplast transformation, conceived in the mid-80s [23,24], has recently blossomed into a safe and environmentally friendly technology [8,9,25]. When the first transgenes were introduced via the chloroplast genome, it was believed that foreign genes could be inserted only into transcriptionally silent spacer regions, amidst divergent chloroplast genes [26]. However, Daniell *et al.* [10] advanced the concept of inserting transgenes into functional operons and transcriptionally active spacer regions. This approach facilitated the insertion of multiple genes under the control of a single promoter, enabling the coordinated expression of transgenes [13••,15•,16•,27•]. Earlier reports, based on *in vitro* studies of chloroplast mutants, established a definite requirement for the processing of dicistrons to monocistrons before translation [28–30]. To test this hypothesis, multiple transgenes were inserted into the rRNA operon of chloroplast genomes to study their transcription, RNA processing and translation. Contrary to previous reports, the following examples unequivocally demonstrate that polycistrons are efficiently translated in transgenic chloroplasts without any requirement for RNA processing. The fact that several foreign proteins are synthesized in large quantities without any detectable monocistrons support this conclusion.

Expression of a protein-based biomedical polymer as a dicistron in transgenic chloroplasts demonstrated, for the first time, the potential of this technology to engineer biopharmaceuticals [31••,32]. Recently, human serum albumin (HSA), expressed under the regulation of the optimal chloroplast ribosome-binding site (GGAGG), could not be easily detected (<0.02% tsp) in transgenic chloroplasts. In the past, the same regulatory sequence has resulted in accumulation of large quantities of several other foreign proteins (up to 21% tsp) [27•]. HSA was, however, successfully hyper-expressed in transgenic chloroplasts as a dicistron or polycistron, by manipulating the 5′ and 3′ regulatory sequences of the transgene (A Fernandez-San Millan, A Mingo-Castel, H Daniell, unpublished results) [33]. HSA accumulated in such large amounts that inclusion bodies formed and increased the size of transgenic chloroplasts (Figure 1a). HSA inclusion bodies were readily purified by simple centrifugation and solubilized to functional monomers. Regulatory sequences used in this study should serve as a model system for enhancing the expression of foreign proteins that

are highly susceptible to proteolytic degradation and in addition should provide major advantages in purification. This study reports the highest level of pharmaceutical protein ever observed in transgenic plants. This is the first report to provide direct evidence for translation of transgene polycistrons, without any requirement for processing to monocistrons. Also, this study identifies a heterologous untranslated region (UTR) that could be used in non-green plastids, free of nuclear control. Searches for such non-green UTRs have been elusive so far.

To combat a disease like cholera that often assumes epidemic proportions and poses a threat as an agent of bioterrorism, there is a need for producing vaccines on an agricultural scale. Therefore, cholera toxin β subunit (CTB) was expressed in transgenic chloroplasts as a dicistron. As the quaternary structure and disulfide bonds of many pharmaceutical proteins are essential for their function, we demonstrated, using CTB, the assembly of functional oligomers in transgenic chloroplasts. Expression of the native β subunit gene (*ctx*B) was 410-fold higher than in nuclear transgenic plants and there were no pleiotropic effects, in contrast to nuclear transgenic plants that showed stunted growth [16•,33]. Western blot analysis and enzyme-linked immunosorbant assay (ELISA) showed that several independent transgenic lines expressed the same amount of CTB, except for physiological variations [16•]. Engineering CTB in transgenic chloroplasts, along with recent success in the chloroplast transformation of edible crops and the availability of plant-derived selectable markers, augur well for producing edible vaccines in transgenic chloroplasts on a cost-effective basis [16•,18••,19,20•].

Chloroplast transformation has also been employed to confer resistance to biotic and abiotic stresses. Expression of an antimicrobial peptide, MSI-99, as a dicistron in transgenic chloroplasts was shown to inhibit the growth of several plant pathogens, including *Pseudomonas syringae, Aspergillus flavus*, *Fusarium moniliformae, Verticillium dahliae* and the multidrug-resistant human pathogen *Pseuodomonas aeruginosa,* when tested using *in planta* and *in vitro* assays [27•,34]. Lysis of transgenic chloroplasts at the site of infection resulted in high-dose release of the antimicrobial peptide (800 μg MSI-99, inhibitory concentration 1 μg MSI-99 for 1000 bacterial cells or fungal spores). In another recent report, the integration of a yeast trehalose-6 phosphate synthase (TPS) gene as a dicistron in transgenic chloroplasts was shown to confer drought tolerance, as evidenced by growth of transgenic plants on 6% polyethylene glycol and ability to rehydrate after dehydration [15•, 35]. Whereas nuclear transgenic plants accumulating trehalose in the cytosol showed stunted growth, sterility and other pleiotropic effects, chloroplast transgenic plants showed normal growth and physiology and no pleiotropic effects (Figure 2). Chloroplast transgenic plants showed 16 699% more *tps*1 transcripts than the best nuclear transgenic plants, alleviating the possibility of gene silencing in transgenic chloroplasts (Figure 3).

Perhaps the most significant accomplishment, which has made chloroplast transformation technology safe, is the use of a plant-derived selectable marker, betaine aldehyde dehydrogenase (BADH), to obtain chloroplast transgenic plants by expression of a dicistron [18••,36]. The selection process involves conversion of toxic betaine aldehyde to glycine betaine by BADH; glycine betaine also serves as an osmoprotectant. The BADH gene derived from spinach not only eliminates the need for the use of antibiotic resistance genes but is also 25-fold more efficient than antibiotic resistance genes, exhibiting rapid regeneration of transgenic shoots within two weeks. These developments should help to allay public concerns and make genetically modified foods more acceptable.

Ever since chloroplast technology was conceived, it was anticipated that the prokaryotic nature of the organelle should allow the expression of bacterial operons. This promise was realized when expression of the *B. thuringiensis cry*2Aa2 operon in transgenic chloroplasts

led to the formation of insecticidal crystals (Figures 1b,c) [13••,37]. The 4.0 kb operon consists of three genes, with *cry*2Aa2 being the distal gene. The open reading frame, *orf*2, immediately upstream of the gene codes for a putative chaperonin that is necessary for folding the protein into cuboidal crystals (that are resistant to proteolytic degradation). Expression of the operon in transgenic chloroplasts resulted in the accumulation of Cry2Aa2 protein at 46.1% of tsp, even in senescing bleached old leaves. Such high levels of insecticidal protein were instrumental in combating insects that are normally difficult to control, including the 10-day old cotton bollworm and beet armyworm. Observed hyper-expression of Cry2Aa2 protein argues against any possibility of gene silencing in transgenic chloroplasts.

The possibility of expressing a pharmaceutical protein, which involves multiple genes, has been explored using the Guy's 13 monoclonal antibody. This antibody against the surface protein of *Streptococcus mutans*, which is the causative agent of dental caries, was successfully expressed and properly assembled in transgenic chloroplasts [14,38]. This is the first demonstration of expression of a multisub-unit foreign protein that is assembled with disulfide bridges. Application of Guy's 13 monoclonal antibody to the dental surface prevented recolonization of the bacterium for up to two years [39]. This multisubunit antibody has been expressed via the nuclear genome by generating independent transgenic lines, followed by subsequent breeding [40]. For commercial application, however, expression levels should be increased further in nuclear transgenic plants.

Phytoremediation is evolving as a safe technology to address the increasing problem of the pollution of soil and water bodies. One of the most toxic pollutants that threatens our health and ecosystem is mercury. In the environment, mercury is rapidly methylated by bacteria producing a 10-fold more toxic organomercurial, owing to its ability to cross lipid membranes [41]. Over 90% of methylmercury is absorbed in blood compared with only 2% of inorganic mercury, causing neurological degeneration in birds, mammals and humans. In photosynthetic organisms, mercury inhibits the oxygen-evolving enzyme (OEE) complex, binds to thylakoid membranes [42] and removes EP33 (one of the proteins of the OEE complex [43]). Mercury reduces the variable fluorescence (which provides a measure of photosynthetic efficiency) owing to additional inhibitory sites on the donor side of photosystem II, causing damage to the light-harvesting complexes and structural changes in the antenna pigments that affect the primary photochemistry; mercury also inhibits plastocyanin [44]. Nuclear codon optimized *mer*A (mercury ion reductase) and *mer*B (organomercurial lyase) genes were used to obtain transgenic plants that are resistant to mercury and organomercurials, respectively (up to 10 μM) [45•]. The low level of tolerance observed might result from the low levels of nuclear expression, compounded by the fact that these enzymes were not targeted to chloroplasts, where mercury is most toxic, requiring continuous detoxification. Therefore, the *mer* operon has been expressed via the chloroplast genome to overcome these problems [46•,37].

## Conclusions

Plant biotechnology is at the threshold of an exciting new era in which the emphasis is on the introduction of traits that require the manipulation of metabolic pathways or coordinated expression of multisubunit proteins. The development of rice varieties enriched in provitamin A is an early success story in this new era. The chloroplast transgenic approach has facilitated expression of bacterial operons and biopharmaceuticals at unprecedented levels, never before reported in the literature. Accumulation of about 50% of foreign proteins in the total soluble protein in chloroplast transgenic plants resulted in the formation of insecticidal protein crystals or inclusion bodies of biopharmaceuticals. Foreign transcripts in transgenic chloroplasts accumulated 17 000% more than the best nuclear transgenic

plants. These exciting achievements not only relieve concerns about gene silencing and position effects, but also eliminate the need for time-consuming breeding to bring multiple transgenes within a single host. In addition, these advances offer several environmentally friendly features including gene containment. The new era will rely heavily on both nuclear and chloroplast multigene engineering technologies to utilize the new knowledge acquired in the post-genomic era for biotechnological applications and to understand complex metabolic pathways.

## Abbreviations

| | |
|---|---|
| **BADH** | betaine aldehyde dehydrogenase |
| **CTB** | cholera toxin β subunit |
| **HSA** | human serum albumin |
| **PHB** | polyhydroxybutyrate |
| **tsp** | total soluble protein |
| **UTR** | untranslated region |

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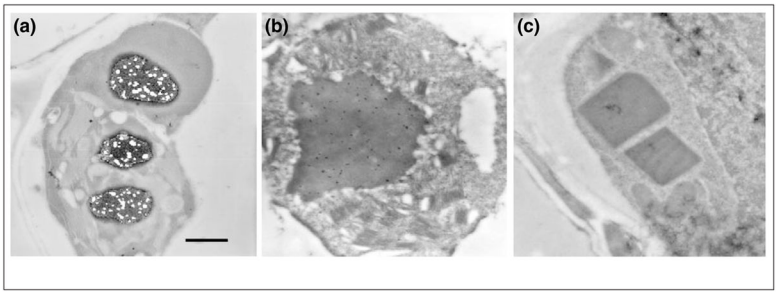


**Figure 1.**
Examples of the highest level of transgene expression. Electron micrographs of the hyper-expression of foreign proteins in transgenic chloroplasts. **(a)** Inclusion bodies of HSA, the most widely used intravenous protein in human therapies. **(b,c)** Immunogold-labeled inclusion bodies and cuboidal crystals of the insecticidal *B. thuringienesis* Cry2Aa2 protein.

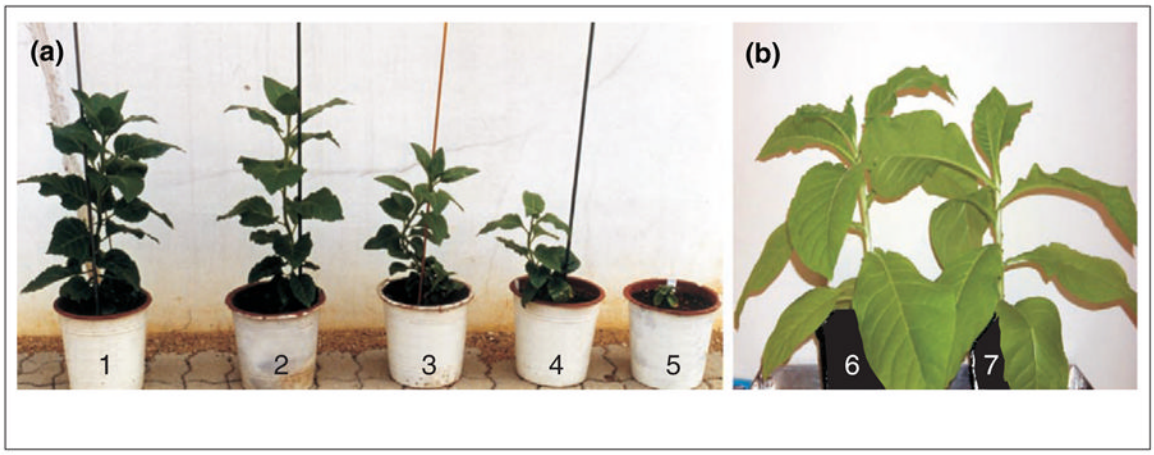


**Figure 2.**
Alleviation of pleiotropic effects. Comparison of the phenotypic effects of trehalose accumulation in the cytosol and chloroplasts of transgenic plants. **(a)** Untransformed wild type (1); nuclear transgenic plants from different, independent transgenic lines (2–5). **(b)** A chloroplast transgenic plant (6); untransformed wild type (7). (Figure reproduced from [15•] *Transgenic Research*, in press.)

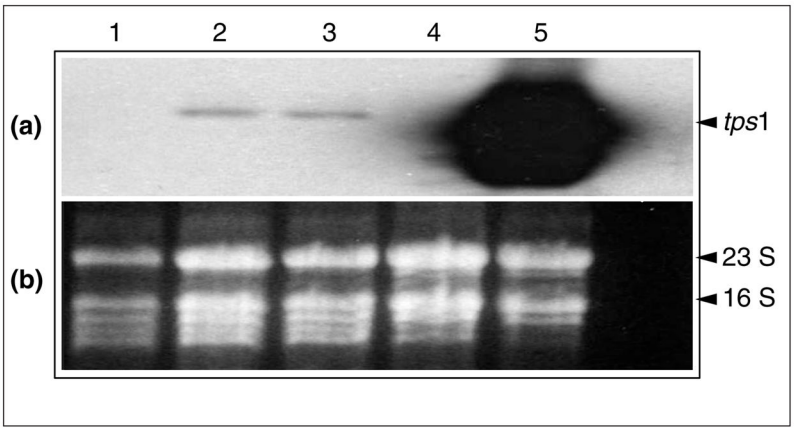


**Figure 3.**
Elimination of gene silencing. Northern blot analysis of nuclear and chloroplast transgenic plants expressing the trehalose phosphate synthase (*tps*1) gene. **(a)** Steady-state transcript levels of *tps1*: (1) untransformed wild type; (2) and (3) highly expressing nuclear transgenic plants; (4) untransformed wild type; (5) chloroplast transgenic plant. **(b)** Ethidium bromide stained total plant RNA to verify equal loading of RNA. (Figure reproduced from [15•] *Transgenic Research*, in press.)