

REVIEW ARTICLE

Transgenic Plastids in Basic Research and Plant Biotechnology

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Facile methods of genetic transformation are of outstanding importance for both basic and applied research. For many years, transgenic technologies for plants were restricted to manipulations of the nuclear genome. More recently, a second genome of the plant cell has become amenable to genetic engineering: the prokaryotically organized circular genome of the chloroplast. The possibility to directly manipulate chloroplast genome-encoded information has paved the way to detailed *in vivo* studies of virtually all aspects of plastid gene expression. Moreover, plastid transformation technologies have been intensely used in functional genomics by performing gene knockouts and site-directed mutageneses of plastid genes. These studies have contributed greatly to our understanding of the physiology and biochemistry of biogenergetic processes inside the plastid compartment. Plastid transformation technologies have also stirred considerable excitement among plant biotechnologists, since transgene expression from the plastid genome offers a number of most attractive advantages, including high-level foreign protein expression and transgene containment due to lack of pollen transmission. This review describes the generation of plants with transgenic plastids, summarizes our current understanding of the transformation process and highlights selected applications of transplastomic technologies in basic and applied research.

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Introduction

The genetic information of plants is distributed among three cellular compartments: the nucleus, the mitochondria and the plastids. The latter two are derived from formerly free-living eubacteria: mitochondria from α -proteobacteria and plastids from cyanobacteria. The prokaryotic progenitors of the present-day cell organelles were engulfed by a pre-eukaryotic cell in an endosymbiosis-like process. During the gradual integration of the acquired endosymbionts into the host cell's metabolism, the organellar genomes underwent a dramatic size

reduction due to both massive gene loss and gene transfer to the nuclear genome.¹ Consequently, present-day organellar genomes are rather small and contain comparably little information. The plastid genome is a circular molecule of double-stranded DNA. In a typical higher plant, it is 120–160 kb in size and contains approximately 130 genes. Identical copies of the plastid genome are present in all plastid differentiation types: proplastids (predominantly present in meristematic tissues), green chloroplasts (present in photosynthetically active tissues), carotenoid-accumulating red or yellow chromoplasts (present in some flowers and fruits) as well as several other plastid types specialized in storage of starch, lipids or proteins.

In spite of the small size of plastid genomes as compared with higher plant nuclear genomes, chloroplast DNA typically makes up as much as 10–20% of the total cellular DNA content.² This is because a diploid plant cell harbors only two

Abbreviations used: ptDNA, plastid DNA; PEG, polyethylene glycol; GUS, β -glucuronidase; GFP, green fluorescent protein; UTR, untranslated region; *ycf*, hypothetical chloroplast reading frame.

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copies of its nuclear genome but thousands of copies of its plastid genome (see Figure 2). A single leaf cell may contain dozens or even hundreds of chloroplasts.² The chloroplast DNA is organized in nucleoids as typical of a prokaryotic system and several such nucleoids are present in each chloroplast. Each nucleoid again harbors several copies of the plastid genome (see Figure 2). This can add up to extraordinarily high ploidy levels of the plastid genome: up to 10,000 (identical) copies of the plastid DNA (ptDNA) can be found in a single pea leaf cell and even up to 50,000 copies in a wheat cell.²

Plastids (as well as mitochondria) have retained numerous eubacterial features, including, for example, gene organization in operons and principally prokaryotic mechanisms of gene expression. Over the past decades, the plastid genome, its structure, expression and evolution was intensely studied using molecular methods. This research has generated a wealth of new information not only about the function of the plastid genetic system but also about the highly sophisticated regulatory mechanisms governing the co-operation of plant cell organelles with their nucleo-cytoplasmic compartment (for reviews see, e.g. Goldschmidt-Clemont,³ Leon *et al.*⁴ and Coleman and Nerozzi⁵). More recently, chloroplast research has benefited enormously from the introduction of transgenic technologies facilitated by the development of reliable methods for plastid genome transformation.⁶⁻⁸ This methodological breakthrough has made feasible the targeted manipulation of the endogenous genetic information of plastids and, in addition, has opened up the exciting possibility to introduce novel information and express it from engineered chloroplast genomes.

Plastid transformation systems

For many years, the genetic transformation of organellar genomes seemed impossible to achieve, since (a) the double membrane of chloroplasts and mitochondria posed a threatening physical barrier to the delivery of transforming DNA into organelle compartments, and (b) no viruses or bacteria were known that would infect chloroplasts or mitochondria and thus could be used as vehicles for gene transfer. This rather pessimistic view changed suddenly when a new "violent" method was introduced into biological research: the shooting with particle-accelerating devices nowadays commonly called "particle guns" or "gene guns".^{9,10} Together with the development of efficient protocols for coating inert metal powder (gold or tungsten) with nucleic acids, this biolistic (biological + ballistic) technique has provided the attractive opportunity to shoot foreign DNA into living cells.¹¹ Encouraged by promising success with nuclear transformation in plants¹² and mitochondrial transformation in yeast,¹³ chloroplasts were the next targets of the cannoners among plant researchers. In 1988, successful chloroplast transformation was reported by

the Boynton and Gillham laboratories for *Chlamydomonas reinhardtii*, a unicellular green alga with a single large chloroplast occupying approximately 60% of the cell volume.⁶ Employing photosynthetically incompetent mutants carrying defective alleles of the chloroplast *atpB* gene (and thus lacking chloroplast ATP synthase activity), the wild-type *atpB* gene was used in this study to complement the mutant phenotype under selection for restored photoautotrophic growth. Stable chloroplast transformants were obtained in which the mutant *atpB* allele had been replaced by the wild-type gene as present in the transformation vector *via* homologous recombination.

A seminal contribution to the further improvement of chloroplast transformation technologies was the development of the first chloroplast-specific antibiotic resistance marker, an originally bacterial aminoglycoside 3'-adenylyltransferase gene (*aadA*) conferring resistance to a number of antibiotics of the aminoglycoside type, including spectinomycin and streptomycin.¹⁴ The antibiotic routinely used for chloroplast transformation is spectinomycin because of its high specificity as a prokaryotic translational inhibitor and its low side-effects on plant cells. The AadA protein catalyzes the covalent transfer of an AMP residue from ATP to spectinomycin, thereby converting the antibiotic into an inactive form (adenylylspectinomycin) that no longer inhibits protein biosynthesis in prokaryotic 70 S ribosomes as present in the chloroplast. In order to convert the *aadA* gene from *Escherichia coli* into a chloroplast-specific selectable marker, its coding region was fused to chloroplast expression signals:¹⁴ a 5' DNA segment providing promoter, 5' untranslated region (UTR) and Shine-Dalgarno sequence as well as a 3' chloroplast DNA segment providing a stable 3' UTR which is required to confer transcript stability *in planta*.

In 1989, Pal Maliga and co-workers were the first gunners to succeed with chloroplast transformation in a higher plant.⁷ Using a chloroplast 16 S ribosomal RNA gene engineered by introducing point mutations that confer resistance to spectinomycin and streptomycin, they demonstrated stable transformation of tobacco (*Nicotiana tabacum*) plastids by biolistic bombardment of sterile leaves followed by selection for spectinomycin-resistant cell lines (Figure 1). In the chloroplast genomes of the transformed plants (also referred to as "transplastomic" plants), the engineered 16 S rRNA allele as present in the transformation vector had replaced the wild-type allele by homologous recombination.⁷ Reciprocal crosses of transplastomic and wild-type plants demonstrated that the introduced antibiotic resistances were uniparentally, maternally inherited as expected for an extranuclear trait.

The initially used antibiotic-resistant 16 S rRNA allele was not an efficient selectable marker and produced on average only one or two tobacco chloroplast transformants per 100 bombarded leaf samples (equaling approximately 400 selection

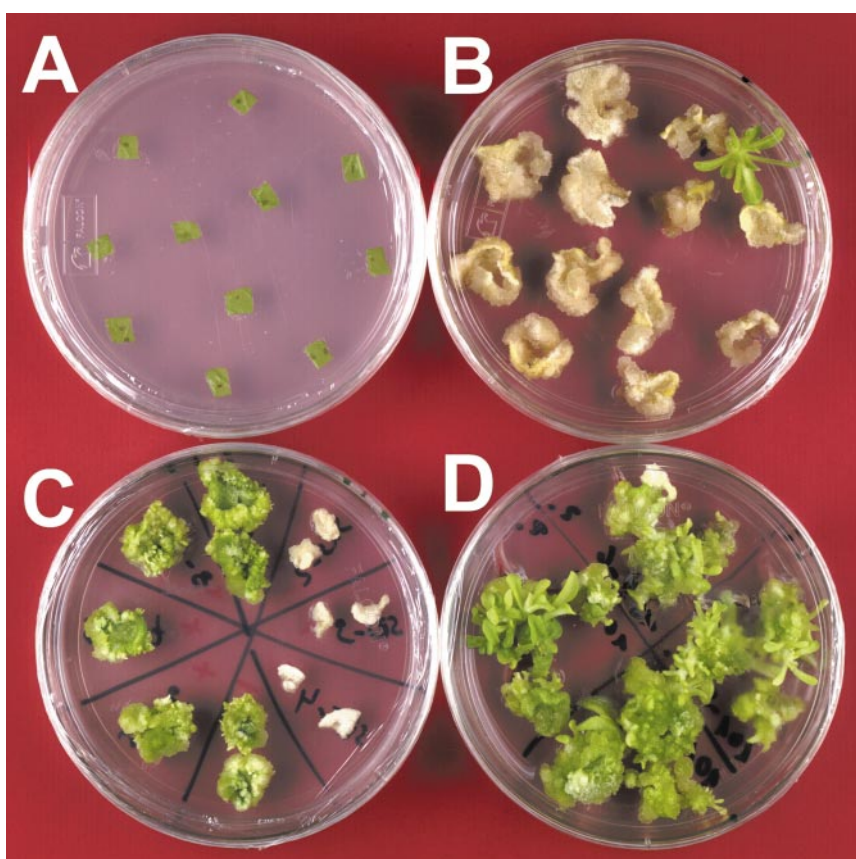


Figure 1. Generation of tobacco plants with transgenic chloroplasts. (a) Selection for chloroplast transformants. Following biolistic bombardment, sterile tobacco leaves are cut into small pieces and exposed to spectinomycin-containing plant regeneration medium. (b) After incubation for four weeks, the leaf pieces are completely bleached out due to effective inhibition of plastid protein biosynthesis by spectinomycin (an aminoglycoside antibiotic specifically blocking translation on prokaryotic-type 70 S ribosomes). A successfully transformed chloroplast expresses the spectinomycin-resistance gene, *aadA*, thus allowing for continued cell and organelle divisions in the presence of the antibiotic. Resistant cell populations initially grow as undifferentiated green callus tissue before the phytohormones present in the synthetic medium eventually induce shoot formation. (c) Elimination of spontaneous spectinomycin-resistant mutants by double selection. Spontaneous spectinomycin resistance occurs through acquisition of specific point mutations in the chloroplast 16 S rRNA gene.¹⁰⁶ Such point mutations act in a strictly antibiotic-specific manner: spontaneous spectinomycin-resistant cells are streptomycin sensitive and *vice versa*. In contrast, the *aadA* transgene confers broad-spectrum resistance to a number of antibiotics of the aminoglycoside type. Consequently, when exposed to double selection on plant regeneration medium containing both spectinomycin and streptomycin, leaf pieces from spontaneous spectinomycin-resistant lines bleach out (right three lines), whereas leaf pieces from true chloroplast transformants remain green, form calli and ultimately regenerate new plantlets. (d) Selection of homoplasmic transplastomic lines. As primary chloroplast transformants are heteroplasmic (see Figure 2), they must be subjected to several additional rounds of regeneration on selective medium in order to eliminate residual wild-type genomes and establish cell lines carrying a uniform population of transformed plastid genomes. To this end, leaf explants are taken in each round and re-exposed to spectinomycin-containing plant regeneration medium.

plates; Figure 1(a)).^{7,15,16} This relatively low transformation frequency is most probably due to the recessive mode of action of the rRNA marker during the selection phase: It confers antibiotic resistance only to those few chloroplast ribosomes that have received their 16 S rRNA molecule from the very few initially present transformed ptDNA copies (Figure 2). By contrast, antibiotic-inactivating marker genes provide dominant drug resistance to the recipient chloroplast and, in theory, a single transformed genome copy is sufficient to detoxify the entire organelle. Two such dominant selectable marker genes for tobacco chloroplast

transformation have been constructed to date (Table 1): (a) a chimeric spectinomycin resistance gene *aadA*⁸ as described above for *Chlamydomonas* chloroplast transformation¹⁴ but containing tobacco chloroplast-specific expression signals; and (b) a similarly designed chimeric *nptII* gene encoding a neomycin phosphotransferase and conferring resistance to kanamycin.^{17,18} While the *aadA* gene is a highly efficient and specific selectable marker, the *nptII* appears to be less efficient and, moreover, produces a significant background of nuclear transformants.¹⁷

Table 1. Foreign genes successfully expressed to date from higher plant plastid genomes

Gene(s)	Gene product and gene source	Function	References
<i>aadA</i>	Aminoglycoside 3'-adenylyltransferase from <i>E. coli</i>	Positive selectable marker (spectinomycin and streptomycin resistance)	8, 107
<i>nptII</i>	Neomycin phosphotransferase from Tn5	Positive selectable marker (kanamycin resistance)	17, 18
<i>uidA</i>	β -Glucuronidase (GUS) from <i>E. coli</i>	Reporter of gene expression	16,108
<i>gfp</i>	Green fluorescent protein (GFP) from <i>Aequorea victoria</i>	(Vital) reporter of gene expression	99, 100
<i>cry1A</i>	Crystal toxin from <i>Bacillus thuringiensis</i>	Insecticidal protein (protoxin)	82
<i>cry2A</i>	Crystal toxin from <i>Bacillus thuringiensis</i>	Insecticidal protein (protoxin)	101
<i>cry2Aa2</i> operon	Crystal toxin, ORF1 and ORF2 (putative chaperonin) proteins from <i>Bacillus thuringiensis</i>	Insecticidal protein (protoxin)	84
<i>codA</i>	Cytosine deaminase from <i>E. coli</i>	Negative selectable marker (5-fluorocytosine sensitivity)	63
<i>EPSPS</i>	5-Enol-pyruvyl shikimate-3-phosphate synthase from <i>Petunia hybrida</i> or from eubacterial species	Herbicide tolerance (glyphosate)	96, 83
<i>bar</i>	Phosphinothricin acetyltransferase from <i>Streptomyces hygroscopicus</i>	Herbicide resistance (glufosinate)	91
<i>hST</i>	Human somatotropin	Therapeutic protein (human growth hormone)	103

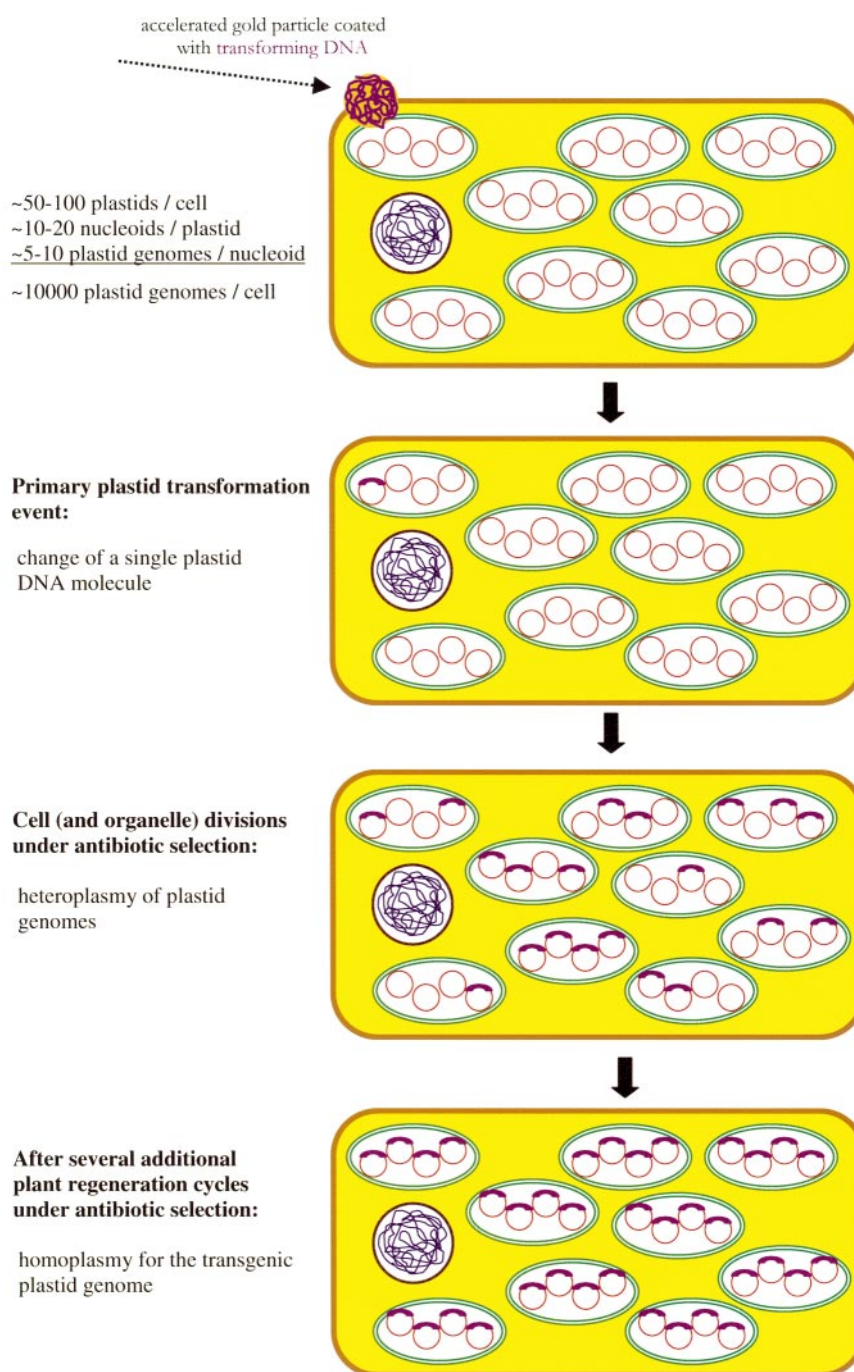


Figure 2. Sorting of plastid genomes and isolation of homoplasmic transplastomic cell lines. The initial chloroplast transformation event involves the change of only a single (or at most a few) out of several thousand plastid genome copies in a leaf cell. During subsequent cell and organelle divisions, the presence of high concentrations of the selecting antibiotic favors multiplication of chloroplasts containing transformed genomes, whereas chloroplasts harboring only wild-type genomes may be eliminated effectively. However, individual chloroplasts may still contain a mixed population of wild-type and transformed plastid genome molecules (intraorganellar heteroplasmy). In additional rounds of plant regeneration on selective medium, gradual sorting out of residual wild-type genomes is achieved, eventually leading to cells with a homogeneously transformed population of plastid genomes commonly referred to as "homoplasmic" or "homoplastidic". See the text for details.

In summary, three types of selectable markers are available for chloroplast transformation experiments: (i) dominant antibiotic-resistance genes (available for *Chlamydomonas* and tobacco^{8,14,17,19}); (ii) recessive antibiotic-resistance markers encoding

antibiotic-insensitive alleles of ribosomal RNA genes (available for *Chlamydomonas* and tobacco^{7,20}); and (iii) recessive markers restoring photoautotrophic growth by complementing non-photosynthetic mutants (currently only available

for *Chlamydomonas*⁶). As outlined above, dominant spectinomycin resistance provided by chimeric *aadA* genes is by far the most effective selection system for chloroplast transformants available to date.

Although direct gene transfer using the biolistic method is undoubtedly the currently most widespread technology for plastid transformation, stable introduction of cloned DNA into chloroplast genomes also has been conclusively demonstrated using two alternative protocols. For *Chlamydomonas*, agitating a suspension of glass beads and cell wall-deficient algal cells in the presence of plasmid DNA produced transplastomic cells, albeit at significantly lower rate than the biolistic protocol.²¹ In tobacco, chloroplast transformation can alternatively be accomplished by chemical treatment of protoplasts with polyethylene glycol (PEG) in the presence of vector DNA.²²⁻²⁴ PEG had long been known to promote uptake of naked DNA by protoplasts and was routinely used to deliver biologically active DNA into the plant nuclear genome.²⁵ Its suitability for the transformation of chloroplasts was somewhat surprising, since it is generally believed that direct exposure of membranes to high PEG concentrations is required to facilitate the passage of DNA molecules. Although in electron microscopy, mesophyll chloroplasts are often seen tightly appressed to the plasma membrane, it is by no means clear how such a membrane permeabilization by PEG could work for the double membrane of the chloroplast when intact protoplasts are treated with PEG. Nonetheless, PEG-mediated plastid transformation in tobacco protoplasts, followed by regeneration of genetically stable transplastomic plants appears to be a reliable and reproducible technique and has been used successfully by several laboratories.²⁶⁻²⁸

In addition to technologies for stable genetic transformation of plastids, several methods for transient gene expression have been described, including *in organello* systems introducing DNA into isolated plastids^{29,30} and *in vivo* methods employing particle bombardment³¹⁻³³ or microinjection techniques.³⁴

The molecular biology of the transformation process

Stable plastid transformation in both *Chlamydomonas* and tobacco appears to be strictly dependent on integration of the transforming DNA into the plastid genome by homologous recombination. Fortunately, plastids have inherited from their cyanobacterial ancestors an efficient RecA-type system of homologous recombination.³⁵ Any plastid genome manipulation therefore requires that the sequence to be introduced into the plastid genome is flanked on both sides by regions of homology with the chloroplast genome.^{15,27} Although the minimum sequence requirements for efficient homologous recombination to occur are currently not very well defined, it is generally assumed that

upwards of approximately 400 bp flanking region on each side, chloroplast transformants are obtained at reasonable frequency. Longer flanks appear to be beneficial, but no careful correlation between size of the homologous regions and transformation frequency has been established to date.

It seems reasonable to assume that the primary plastid transformation event involves the change of only a single or at most a few plastid genome copies (within a single chloroplast) out of the ~10,000 ptDNA copies present in a leaf mesophyll cell (Figure 2). Consequently, primary transplastomic cell lines contain a mixed population of wild-type and transformed plastid genomes (Figure 2). Such cells, tissues or plants are also referred to as heteroplasmic (or, more specifically, "heteroplastomic"). It has been known for almost a century^{36,37} that heteroplasmic situations are genetically unstable and, more or less frequently, resolve spontaneously into either of the two types of genome homogeneity ("homoplasmy"). This sorting-out of extranuclear genetic material is due to random genome segregation upon organelle division as well as random organelle segregation upon cell division.

From this, it appears clear that genetic stability of transplastomic cell lines and plants requires homoplasmy. Homoplasmy can be achieved by allowing for a sufficient number of cell divisions under high selective pressure as exerted by high concentrations of the selecting antibiotic spectinomycin. For *Chlamydomonas*, this is simply done by re-streaking the growing colonies on fresh culture medium containing spectinomycin. For tobacco, plants with a uniform population of transformed genomes are obtained by passing the primary chloroplast transformant through additional cycles of plant regeneration under antibiotic selection: tissue samples are excised from regenerating shoots and re-exposed to regeneration medium with spectinomycin (Figure 1(d)). Typically, homoplasmic (or "homoplastomic") shoots are obtained after two to four such cycles of regeneration under selection. Highly sensitive assays have been developed for confirming homoplasmy and reliably proving the absence of any residual wild-type genome copies, including large-scale seed assays⁸ and PCR-based tests strongly favoring amplification of wild-type genomes.³⁸

In general, two levels of heteroplasmy must be distinguished: (a) interplastidic heteroplasmy, i.e. the presence of chloroplasts with wild-type genomes and those with mutant genomes within one and the same cell; and (b) intrplastidic heteroplasmy, i.e. the simultaneous presence of wild-type and mutant plastid genomes within one and the same chloroplast. Most likely, plastid transformation and gradual sorting out of wild-type genomes involves both types of heteroplasmy (Figure 2). Interplastidic heteroplasmy is likely to disappear rather rapidly, since chloroplasts exclusively harboring wild-type genomes are sensitive to the selecting antibiotic and hence will not multi-

ply as effectively as transformed chloroplasts. In contrast, intraplastidic heteroplasmy is probably more difficult to eliminate since the antibiotic-resistance gene acts as a dominant selectable marker in the sense that one or few copies of the resistance gene are sufficient to confer resistance to the entire organelle. Consequently, there is probably no significant selective advantage of becoming homoplasmic. Why then is it feasible to isolate homoplasmic chloroplast transformants after two to four rounds of plant regeneration on spectinomycin-containing medium? Assuming that, from a certain copy number of transplastomes onwards, antibiotic selection becomes neutral, sorting of genome types will be random upon plastid division. Random distribution of plastid genomes during the organelle division process will give rise occasionally to homoplasmic wild-type chloroplasts or homoplasmic transgenic chloroplasts. Whereas the homoplasmic transgenic chloroplasts will be (at least) as competitive as the heteroplasmic ones, the homoplasmic wild-type chloroplasts will be antibiotic-sensitive and thus may not multiply efficiently during subsequent cell divisions. This model might explain how, over time, wild-type genomes are gradually diluted out and eventually disappear.

There are a few examples of tobacco or *Chlamydomonas* chloroplast transformation experiments resulting in the formation of episomal, plasmid-like elements.³⁹⁻⁴² However, in all these cases, stable integration of the transforming DNA into the plastid genome by homologous recombination also occurred and hence it is not clear whether the episomal elements really replicate autonomously inside the chloroplast or alternatively reflect mini-circle-like recombination products continuously originating from the transformed plastid genome. Interestingly, biolistic transformation experiments in the green alga *Euglena gracilis* have provided evidence for successful chloroplast transformation in the absence of any integration of the transforming DNA into the chloroplast genome.⁴³ Instead, under selective conditions, transforming DNA was maintained as an episomal element at low copy number (approximately one copy per chloroplast). It is currently unclear why *Euglena gracilis* chloroplasts do not integrate transforming DNA into their genome by homologous recombination.

***In vivo* systems for studying plastid gene expression**

The possibility to manipulate plastid DNA sequences *in vitro* and re-introduce the altered sequences into the chloroplast genome has also paved the way to study practically all mechanisms of plastid gene expression in *in vivo* systems. These studies have greatly contributed to our understanding of the rules governing plastid gene expression.^{3,44-48}

The development of chimeric reporter genes for plastid gene expression has provided valuable

tools for systematic studies of the *cis*-acting elements involved in transcription, RNA metabolism and translation.^{49-52,28} The *uidA* gene encoding β -glucuronidase (GUS) has been used in most of these studies, but recent work has shown that the *gfp* gene (encoding the green fluorescent protein, GFP) also functions well in plastids (Table 1). When the coding region of the reporter is fused to plastid gene-specific expression signals, reporter gene expression reliably follows the pattern of the endogenous plastid gene,¹⁶ indicating that the major *cis*-acting sequence elements determining RNA stability and controlling translational efficiency reside within the 5' and 3' UTRs of plastid messenger RNAs.

Transplastomic *in vivo* systems are particularly valuable for the study of all those steps in plastid gene expression for which no faithful or efficient *in vitro* systems are available (or have not been available until very recently), such as group II intron splicing and RNA editing. Tobacco plastid transformation, for example, has been extensively used to study RNA editing, a curious RNA processing step in higher plant cell organelles. RNA editing in plastids of vascular plants is a post-transcriptional process that changes individual cytidine residues into uridine.⁴⁷ Editing events usually result in changes of the coding properties of the affected mRNAs which has the dramatic consequence that amino acid sequences cannot be reliably predicted from DNA sequence analyses. As a faithful *in vitro* system for plastid RNA editing was lacking until very recently,⁵³ chloroplast transformation experiments have been the method of choice to address functional, mechanistic and evolutionary aspects of RNA editing.⁴⁶ Systematic deletional and point mutageneses have allowed to dissect the *cis*-acting elements involved in editing site recognition and to define minimum substrates for plastid editing reactions.^{38,54-56} In addition, *in vivo* studies in transgenic chloroplasts have begun to shed some light on the molecular mechanism of the editing reaction⁵⁷ and on the evolution of editing sites and their *trans*-acting recognition factors.^{58,59}

Transplastomic studies also have been instrumental in advancing our knowledge about mRNA synthesis and transcriptional regulation in plastids. The transcriptional apparatus of plastids comprises a plastid-encoded *E. coli*-like RNA polymerase and a recently identified second, nuclear-encoded transcription system utilizing a bacteriophage-type RNA polymerase.⁶⁰ Comparative studies of *in vitro* capped chloroplast transcripts from wild-type plants and transplastomic plants lacking the plastid-encoded RNA polymerase⁶¹ have assigned transcription initiation sites to the two transcription systems and, in addition, have suggested consensus sequences for promoters recognized by the phage-type enzyme.⁶² These studies have provided novel insights into the regulation of plastid gene expression in response to developmental and environmental cues.

Transplastomic approaches can also contribute to the identification of *trans*-acting factors involved in plastid gene expression. In this respect, negative selectable-marker genes are particularly useful since they allow to devise genetic screens for nuclear genes regulating plastid gene expression. A negative selectable marker for plastids has been developed from the *E. coli* cytosine deaminase gene (*codA*; Table 1) whose expression is lethal to the cell in the presence of exogenously applied 5-fluorocytosine.⁶³ Introduction into the ptDNA of the *codA* coding region fused to chloroplast gene-specific expression signals (promoter, 5' and 3' UTRs) combined with mutagenesis of the nuclear genome would be a highly efficient approach to isolate both general and gene-specific *trans*-acting factors involved in the expression of plastid genes at the transcriptional and post-transcriptional levels.

Chloroplast functional genomics by reverse genetics

Complete plastid genomes have been sequenced from a number of vascular plant and algal species†. The picture that has emerged from these extensive structural genomics studies is that the plastid genomes of green algae and higher plants are remarkably conserved in their coding capacity and genome organization. The majority of plastid-encoded genes can be grouped into two basic classes: genetic system genes (e.g. rRNA, tRNA and ribosomal protein genes) and photosynthesis-related genes.⁶⁴ In addition to the many functionally assigned genes, plastid genomes harbor a number of open reading frames of unknown function.⁶⁵ Those open reading frames that display a significant degree of interspecific conservation are generally considered to be genuine genes and are commonly referred to as *ycfs* (e.g. *ycf3*=hypothetical chloroplast reading frame number 3).

The availability of transgenic technologies for chloroplasts has facilitated the functional characterization of plastid genome-encoded genes and open reading frames using reverse genetics approaches. In contrast to forward genetics, where the (mutated) gene causing an interesting phenotype is aimed to be identified, reverse genetics starts from a known DNA sequence containing an open reading frame of unknown or uncertain function(s) and aims at its mutational inactivation *in vivo*. Lack of the gene product encoded by the reading frame of interest is hoped to produce an analyzable phenotype whose careful characterization is expected to reveal the function of the gene in the wild-type.

Owing to the efficient homologous recombination system in chloroplasts, reverse genetics by targeted knockout analysis or site-directed

mutagenesis has become a powerful tool for plastid functional genomics.⁶⁶ As described above, chloroplast transformation technologies are nowadays routinely available for two model systems, the unicellular green alga *Chlamydomonas reinhardtii* and the higher plant tobacco. Both of these model plants have been used for systematic functional genomics in chloroplasts using reverse genetics strategies.⁶⁷⁻⁷³

A reverse genetics study in chloroplasts starts out with the construction of a mutant allele for the reading frame of interest by using standard *in vitro* techniques for insertional, deletional or site-directed mutagenesis. Linked to a selectable marker, the constructs are then introduced into the plastid genome by chloroplast transformation where the mutant allele replaces the endogenous wild-type allele by homologous recombination. Obviously, homoplasmy of the generated transplastomic lines is an absolute requirement for obtaining stable and clearly interpretable phenotypes.

Construction of a null allele by deletional or insertional mutagenesis is the most appropriate strategy in those cases where the function of an open reading frame (*ycf*) is entirely unknown. Such gene knockouts performed in *Chlamydomonas* and tobacco chloroplasts have led to the discovery of several new gene functions in the plastid genome including a number of small subunits of the large pigment-protein complexes involved in the light reactions of photosynthesis.^{67,68,72,73} Interestingly, chloroplast gene disruptions also have identified novel proteins that are required for the stable accumulation of multiprotein complexes in the thylakoid membrane without being integral components of these complexes. Instead, these proteins may serve as important auxiliary factors in the assembly process of membrane protein complexes. Knockout analysis in chloroplasts have established, for example, that the proteins encoded by the conserved plastid reading frames *ycf3* and *ycf4* are essential factors for the assembly of stable photosystem I complexes in the thylakoid membrane.^{70,74}

Homoplasmic transplastomic cells can be obtained for all knockouts of photosynthesis-related reading frames. This is because photosynthesis is not required under heterotrophic *in vitro* culture conditions: non-photosynthetic *Chlamydomonas* cells can be grown heterotrophically on acetate-containing medium and non-photosynthetic tobacco cells grow on sucrose-containing tissue culture media. However, for several plastid genome-encoded reading frames, the generation of homoplasmic knockout cells has turned out to be impossible indicating that these genes encode essential functions for cellular survival. Under selective conditions, these transplastomic lines remain heteroplasmic with wild-type and transformed genomes co-existing in a relatively constant ratio.^{75,76} This stable heteroplasmy suggests a balanced selection in which the presence of both genome types is required for cell survival: whereas

† Available online (http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/plastids_tax.html).

the transformed genome is essential to express the antibiotic resistance, the wild-type genome is required to provide the gene product of the essential gene (that was knocked-out in the transformed genome). Release of the selective pressure for maintenance of the transformed genome (by cultivation on antibiotic-free medium) allows for random sorting-out of plastid genomes and eventually results in the appearance of homoplasmic wild-type cells.⁷⁶

In addition to knockout experiments with chloroplast open reading frames, reverse genetics analyses are also of great value for the detailed functional characterization of known plastid-encoded proteins by conducting site-directed mutageneses of the respective genes. In this way, crucial protein-protein interaction sites were mapped and important co-factor binding residues in electron-transferring proteins were identified.⁷⁷ These studies have contributed greatly to our understanding of the structure of membrane protein complexes and have significantly advanced our knowledge about the bioenergetic pathways in the chloroplast (reviewed by Hippler *et al.*⁷⁸).

Transgenic chloroplasts in biotechnology

Plastid transformation technologies have attracted biotechnologists, since accommodation of transgenes in the plastid genome instead of the nuclear genome bears a number of inherent advantages for plant genetic engineering.⁷⁹⁻⁸¹

Owing to the polyploidy of the plastid genetic system with thousands of genome copies per cell, extraordinarily high levels of foreign protein accumulation can be achieved in chloroplasts.⁸²⁻⁸⁴ Transgenic plastids are thus ideal expression factories for high-yield protein production. Most remarkably, expression levels of up to more than 40% of the total soluble cellular protein have been obtained⁸⁴ which is ten to 100 times higher than upon nuclear transgene expression in plants. As plastids have in their stroma a limited set of (prokaryotic) protein degradation pathways⁸⁵, it seems conceivable that at least some foreign proteins are not only produced to higher levels but are also more stable inside the chloroplast than in the nucleo-cytoplasmic compartment. However, to what extent protein stability contributes to the enormously high foreign protein accumulation levels in transgenic chloroplasts is currently unknown.

Another advantage of transplastomic technologies is that transgene expression is much more stable and uniform among transgenic lines. Nuclear transformation in plants occurs by more or less random integration of transgenes into unpredictable genomic locations through non-homologous recombination. This results in transgenic lines with widely varying expression levels (position effects) and usually requires screening of large numbers of transgenic plants to identify a line displaying reasonably high transgene expression. Addition-

ally, nuclear transformation experiments in plants frequently suffer from epigenetic gene-inactivation mechanisms commonly referred to as gene silencing.⁸⁶ By occurring somatically, epigenetic gene inactivation may even cause variability in transgene expression levels within one plant.⁸⁷ By contrast, in plastid genomes, transgene integration always occurs by homologous recombination and is neither affected by position effects nor by epigenetic gene-silencing mechanisms. Thus, all transplastomic lines obtained from transformation experiments with a given vector are usually genetically and phenotypically identical and, in theory, the production of a single transplastomic plant per construct is sufficient. This at least partially compensates for the otherwise more laborious and technically demanding use of transplastomic approaches.

Plastid genome engineering also offers unique advantages for the simultaneous expression of multiple transgenes ("transgene stacking"). Transgene stacking is technically difficult in eukaryotic genomes, since multiple transgenes cannot be expressed by co-transcription. This is due to the mechanisms of translation initiation in eukaryotic cells⁸⁸ which normally permit only translation of the first cistron in a polycistronic messenger RNA. By contrast, the principally prokaryotic organization of plastid genomes allows expression of multiple transgenes from operons, since downstream cistrons of a polycistronic messenger RNA are faithfully translated.^{84,89} Because related biosynthetic genes in bacteria are often organized into operons, this opens up the attractive possibility of introducing novel biosynthetic pathways into plastids by expressing entire bacterial operons. As an alternative to transgene stacking by expression as operons, co-transformation can also be used to insert multiple unlinked transgenes into the plastid genome. Moreover, techniques have been developed to recycle the antibiotic-resistance gene after successful plastid transformation. Such strategies for selectable marker removal from transplastomic cells involve either co-transformation or homologous recombination in direct repeats flanking the marker gene.^{90,91}

Transplastomic technologies are also advantageous for ecological reasons. In the vast majority of angiosperm plant species, chloroplasts are passed uniparentally, maternally to the next generation (Figure 3).⁹²⁻⁹⁴ This is due to either exclusion of plastids by unequal cell divisions upon pollen grain mitoses or degradation of plastids and plastid DNA during male gametophyte development.⁹² Consequently, the sperm cell fertilizing the egg is free of plastids and plastid DNA, hence the zygote receives its plastids exclusively from the egg cell without any contribution from the pollen. This lack of pollen transmission of chloroplast genes and transgenes (Figure 3)^{95,96} addresses two major public concerns about transgenic plants: (a) the probability of uncontrolled spreading of transgenes *via* pollen from fields with transgenic crops to fields

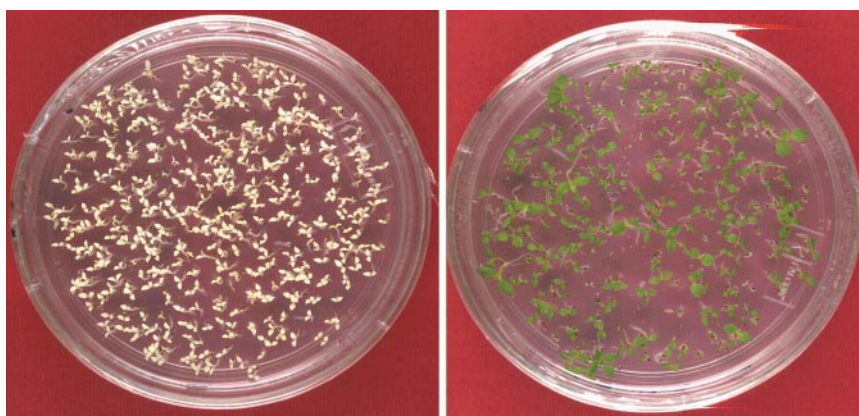


Figure 3. Lack of pollen transmission of chloroplast transgenes due to uniparental, maternal plastid inheritance. Seeds from reciprocal crosses of a transplastomic tobacco plant with a wild-type plant were germinated on spectinomycin-containing synthetic medium. F1 progeny obtained by pollination of wild-type flowers with pollen from a transplastomic plant is free of the chloroplast transgene and hence uniformly sensitive to the antibiotic (left). By contrast, emasculated flowers from transplastomic plants pollinated with wild-type pollen give rise to 100% spectinomycin-resistant progeny (right).

with non-transgenic crops; and (b) the possibility of outcrossing through pollen transmission of transgenes from transgenic crop plants to related wild species (for example, from cultivated oilseed rape, *Brassica napus*, to its weedy relative, *Brassica rapa*).⁹⁵ Thus, by providing transgene containment, transplastomic technologies ensure much higher ecological safety than classical transgenic technologies involving nuclear genome manipulations.

These advantages of transplastomic plants over conventional transgenic plants make chloroplast transformation technologies a promising tool for biotechnologists which has the potential to solve at least some of the technical problems associated with classical transgenic technologies and, in addition, minimizes the ecological risks upon release of transgenic plants into the environment.^{79,97} However, the wide use of transplastomic technologies in plant biotechnology currently encounters one serious drawback: at present, chloroplast transformation is routinely available only for a single higher plant species, tobacco. This is because tobacco is by far the most easy-to-handle species in plant tissue culture, allowing for the development of highly efficient selection and regeneration protocols for the production of transgenic plants. Limitations in the currently available tissue culture systems are considered to be the main obstacle to the extension of transplastomic technologies to other species and, most importantly, to major crop plants. Although recently some progress was made with *Arabidopsis* and potato chloroplast transformation^{98,99} as well as with the generation of (heteroplasmic) transplastomic cell lines in rice,¹⁰⁰ a complete protocol for the production of fertile transplastomic plants has not yet been reported for any other species but tobacco. In fact, of the three chloroplast transformants generated to date for the model species of plant geneticists, *Arabidopsis thaliana*, all were ster-

ile and hence could not be propagated generatively.⁹⁸ However, with the current acceleration of research in this area, rapid progress with plastid transformation systems for agronomically important plant species will undoubtedly be made in the near future.

For the reason discussed above, all biotechnological research conducted to date with transgenic chloroplasts has been carried out in tobacco. In spite of this limitation, the results of these studies have impressively demonstrated the enormous potential of transplastomic technologies for the biotechnology of the future (reviewed by Hager & Bock;⁷⁹ Table 1).

Most crop plants have undergone centuries of breeding. Their efficient cultivation in modern agriculture is largely based on monocultures where plants are exposed to weed competitors as well as viral, bacterial and fungal pathogens. As this results in significant annual harvest losses, the introduction of resistance genes into plant genomes by genetic engineering provides an attractive method of creating highly productive plant varieties not attainable by classical breeding. Taking advantage of the extremely high foreign protein accumulation levels that can be obtained in transgenic chloroplasts, expression of insecticidal proteins^{82,84,101} and herbicide-tolerant enzymes^{96,83} from the chloroplast genome has proven to be a very efficient strategy for successful resistance management and weed control. For example, insecticidal Bt toxin protein expressed from the tobacco plastid genome accumulated to up to more than 40% of the total soluble cellular protein and the transplastomic plants were highly toxic to insect pathogens in bioassays.⁸⁴

Plants also have considerable potential for the production of pharmaceuticals, edible vaccines and antibodies ("plantibodies"), since they provide a cheap source of protein and various secondary

metabolites (for a review see, e.g. Giddings *et al.*¹⁰²). Recently, the human growth hormone (somatotropin) was successfully expressed from the tobacco plastid genome and shown to accumulated to high levels (>7% of total soluble protein). Interestingly, the eukaryotic protein somatotropin was synthesized in chloroplasts in its correct, disulfide-bonded form and proved to be biologically active in bioassays.¹⁰³ This study represents a first promising step towards the use of transplastomic plants as factories for high-yield production of biopharmaceuticals.

For certain applications, it may be desired to restrict plastid transgene expression to a particular tissue or developmental stage. This can be achieved by placing the transgene under the control of a phage T7 RNA polymerase promoter which is normally not recognized by the plastid transcriptional apparatus. Plastid transgene expression can then be switched on by a nuclear-encoded and plastid-targeted T7 RNA polymerase.¹⁰⁴ Expression of the nuclear T7 RNA polymerase gene can, in turn, be controlled by tissue-specific or developmental stage-specific promoters or, alternatively, can be made dependent on chemical inducers of gene expression.⁸⁰

Clearly, transplastomic technologies are still far from being routine tools for plant biotechnologists and some technical limitations have still to be overcome. However, the feasibility studies conducted to date have impressively demonstrated the great potential of plastid genome engineering for a variety of biotechnological applications. High-yield protein and metabolite production as well as effective resistance management are potential areas in biotechnology where transplastomic plants may replace classical transgenic plants in the foreseeable future. Moreover, the increasing need to introduce more than one transgene to express traits determined by multiple genes,¹⁰⁵ will certainly make plastid transformation technologies more and more attractive for plant genetic engineers.

Notes added in proof

A recent report describes the use of a betaine aldehyde dehydrogenase gene as a novel selectable marker for tobacco chloroplast transformation (Daniell, H., Muthukumar, B. & Lee, S. B. (2001). Marker free transgenic plants: engineering the chloroplast genome without the use of antibiotic selection. *Curr. Genet.* **39**, 109-116).

The Cre/lox site-specific recombination system was successfully used by two laboratories for efficient removal of selectable marker genes from transgenic chloroplast genomes (Hajdukiewicz, P. T. J., Gilbertson, L. & Staub, J. M. (2001). Multiple pathways for Cre/lox-mediated recombination in plastids. *Plant J.* **27**, 161-170; Corneille, S., Lutz, K. & Maliga, P. (2001). Efficient elimination of selectable marker genes from the plastid genome by the CRE-lox site-specific recombination system *Plant J.* **27**, 171-178).

The most recent development of a plastid transformation system for tomato (Ruf, S., Hermann, M., Berger, I. J., Carrer, H. & Bock, R. (2001). Stable genetic transformation of tomato plastids: expression of a foreign protein in fruits. *Nature Biotechnol.* **19**, in press) provides a first system for biotechnological applications of plastid genome engineering in a food crop with an edible fruit.

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