



Taming plastids for a green future

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Plant genetic engineering will probably contribute to the required continued increase in agricultural productivity during the coming decades, and moreover, plants can potentially provide inexpensive production platforms for pharmaceuticals and nutraceuticals. With the advent of technologies for altering the genetic information inside chloroplasts, a new attractive target for genetic engineering has become available to biotechnologists. Potential advantages over conventional nuclear transformation include high transgene expression levels and increased biosafety because of maternal organelle inheritance in most crops. This review summarizes the state of the art in chloroplast genetic engineering and describes how reverse genetics approaches enhance our understanding of photosynthesis and other important chloroplast functions. Furthermore, promising strategies by which chloroplast genetic engineering might contribute to the successful modification of plant metabolism are discussed.

The capture of a cyanobacterium by a eukaryotic, mitochondria-possessing cell marked the evolutionary birth of the plant cell. This event dates back more than a billion years and since then the relationship between the eukaryotic host cell and the cyanobacterial endosymbiont has become more and more intimate. The endosymbiont lost its autonomy and most of its genome [1] giving rise to a group of cell organelles, collectively referred to as plastids. Plastids occur in a variety of differentiated forms with the photosynthetic chloroplasts, the starch-storing amyloplasts and the colorful chromoplasts of fruits being the most well known. It was previously thought that plastids are specific to plants but the recent discovery of plastid-like organelles (also called apicoplasts) in certain protozoa highlights the difficulty in pinpointing distinctive features that would set unicellular plants apart from unicellular animals. This is all the more valid because the apicoplasts harbor an unmistakable, although much reduced, plastid genome [2].

What is the information content of a typical plant plastid genome? In the course of evolution, the majority of >3000 genes of the cyanobacterial endosymbiont was either lost or transferred to the nucleus. Extensive sequencing of entire plastid genomes (plastomes) has revealed that, at least in higher plants, plastids possess a remarkably conserved genome. In contrast to plant

mitochondria, genome organization and coding capacity in plastids display relatively little interspecific variation. The 120–130 plastid genes are densely packed in a circular genome and fall into two major categories [3]: (i) photosynthesis-related genes; and (ii) genetic system genes, which comprise for example, genes for rRNAs, tRNAs, ribosomal proteins and RNA polymerase subunits. As might be expected from a former eubacterium, plastid genes and gene expression have retained numerous prokaryotic features. One of these is that most plastid genes are organized in operons and co-expressed to produce polycistronic mRNAs. Regulation of gene expression occurs at all stages, including transcription, RNA processing and stability as well as translation and post-translational processes.

Recently, plastids have become attractive targets for genetic engineering efforts. Compared with conventional transgenic technologies, plastid engineering offers several potential advantages. High protein expression levels [4–6], absence of epigenetic effects (gene silencing, position effects) and easy transgene stacking in operons make the use of plastid genome engineering attractive [7]. From the biosafety perspective, the technology significantly increases transgene containment because plastids are maternally inherited in most crops and are therefore not transmitted by pollen [8].

Genetic engineering of higher plant plastid genomes

Attempts to modify the plastid genome of higher plants encounter two major hurdles: (i) the difficulty in delivering foreign DNA through the double membrane of the plastid; and (ii) the enormous copy number (polyploidy) of the plastid genome. To obtain genetically stable transformed plants (now commonly referred to as ‘transplastomic’), the desired genetic modification must be present in each copy of the plastid genome in each cell. Failure to achieve this so-called homoplasmy results in rapid somatic segregation and genetic instability (Figure 1). Repeated rounds of selection and plant regeneration in tissue culture allow elimination of residual wild-type genomes that are usually still present in primary transformants (Figure 2) [7].

Two DNA delivery methods are currently available for introducing foreign DNA into plastids stably: (i) bombardment of tissues with a particle gun (biolistics) [9,10] (Figure 2) or (ii) treatment of protoplasts with polyethylene glycol (PEG) [11,12]. Biolistics is less time consuming and demanding as far as cell and tissue culture procedures are concerned and it is therefore more widely used at present. Fortunately, integration of foreign DNA into the

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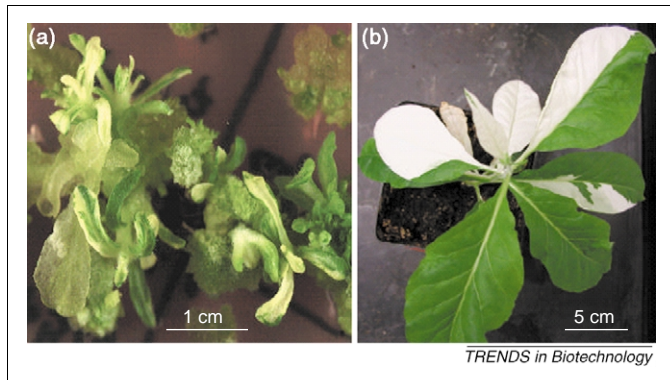


Figure 1. Generation of homoplasmic transplastomic cell lines in tissue culture (a) and whole plants (b). Homoplasmy was made visible by knocking out chloroplast-encoded photosynthesis genes [21] (S. Stegemann and R.B., unpublished). Green sectors still contain wild-type genomes and are therefore photosynthetically active. White sectors are homoplasmic for the transplastome and thus photosynthetically incompetent.

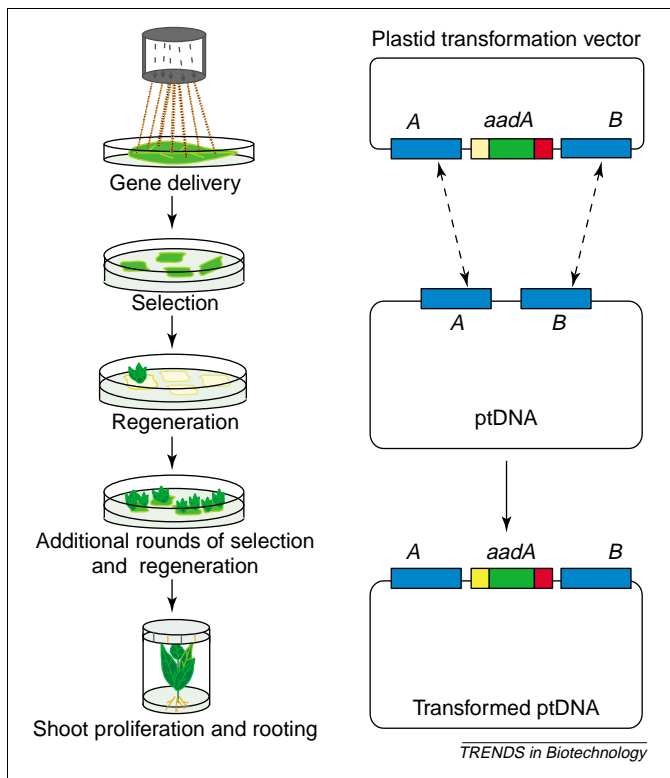


Figure 2. Biolistic chloroplast transformation and transgene integration into the plastid genome via homologous recombination. Left panel: The transformation vector is shot into living leaf cells on the surface of microscopic gold (or tungsten) particles. Small leaf pieces are subsequently exposed to a regeneration medium containing the plastid translational inhibitor spectinomycin. Because the transformation vector contains a spectinomycin resistance gene (*aadA*), transplastomic cells can regenerate despite the presence of spectinomycin. Primary transformants are usually heteroplasmic and, by passing tissue samples through several additional rounds of regeneration under antibiotic selection, residual wild-type genomes are eliminated. Finally, homoplasmic shoots are rooted in phytohormone-free medium. Right panel: Targeting of a transgene to a non-coding intergenic region of the plastid genome (ptDNA). The *aadA* gene is tethered to a plastid promoter and Shine–Dalgarno sequence (yellow) and a plastid 3' untranslated region (UTR; red) conferring transcript stability. Transgene integration into the plastid genome occurs by two homologous recombination events in the flanking regions (dashed lines). Any transgene of interest can be co-incorporated by physically linking it to the *aadA* marker gene. The transgene of interest can either be driven by its own (plastid-derived) expression signals or combined with the *aadA* to form an operon. In the latter case, the transgene cassette consists of the following modules: Promoter → Shine–Dalgarno sequence 1 → Coding region 1 → Shine–Dalgarno sequence 2 → Coding region 2 → 3' UTR.

plastid genome occurs via homologous recombination, which appears to operate in plastids at high efficiency (Figures 2, 3).

The successful generation of tobacco plants with stably transformed plastid genomes (transplastomes) [9,10] was followed by the first biotechnological application of this technology: expression of a *Bacillus thuringiensis* (Bt) toxin gene from the tobacco plastid genome yielded high accumulation levels of the Bt toxin protein of 3–5% of the total soluble protein (TSP) and produced plants that displayed high-level resistance to herbivorous insects [13]. Subsequently, co-expression with two upstream small reading frames further increased Bt protein accumulation, even resulting in crystallization of the protein inside the chloroplast [4]. Another spectacular application of the technology was the production of the human growth hormone, somatotropin, in transgenic tobacco plastids, which gave rise to as much as 7% TSP and demonstrated the remarkable capacity of the chloroplast to correctly form disulfide bonds in a protein of eukaryotic origin [5].

Encouraged by these expression data, the adaptation of plastid transformation protocols for major food crops seemed to be the next logical step. However, extending the crop range was significantly more difficult than initially anticipated [14]. After the first successful transformation in tobacco, it took almost ten years before plastid transformation was achieved for two other Solanaceae species; potato [15] and tomato [16]. An encouraging

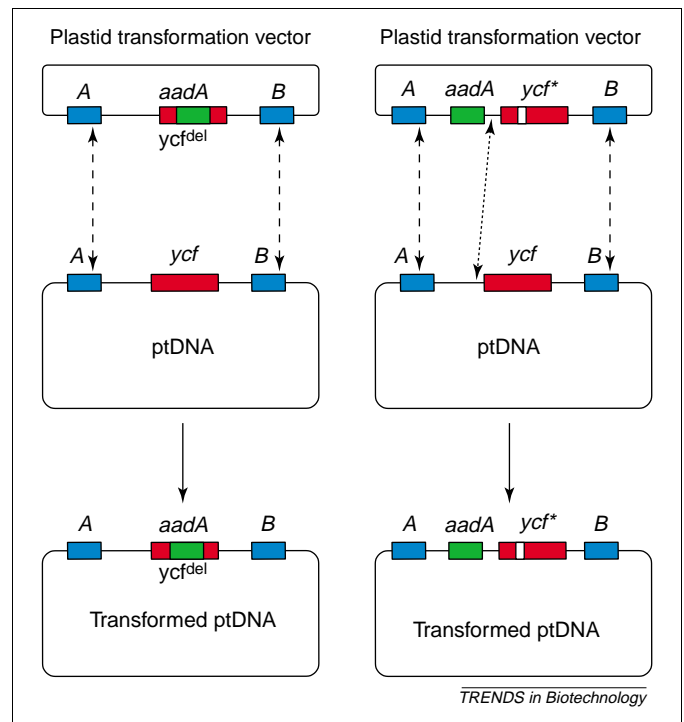


Figure 3. Experimental strategies for reverse genetics in plastids. An open reading frame of unknown function (*ycf*) can be disrupted by insertion of the *aadA* cassette encoding the spectinomycin resistance marker (left panel). Two homologous recombination events in the flanking regions (dashed lines) mediate integration of the *ycf* knockout allele (designated here as *ycf^{del}*) into the chloroplast genome. More-subtle changes (e.g. point mutations) can be introduced by placing the *aadA* marker into an adjacent intergenic region (right panel), which however results in only partial linkage of spectinomycin resistance with the desired change in the *ycf* (designated *ycf**). Consequently, not all chloroplast transformants will carry the mutant *ycf** allele because homologous recombination between the *aadA* and the mutation in the *ycf* (dotted line) can genetically separate the two traits.

feature of the tomato system is the high-level foreign protein accumulation even in ripe red tomatoes, offering great promise for the production of edible vaccines, antibodies and other biopharmaceuticals in plants [16]. Some progress was recently made with plastid transformation in Brassicacea species [17–19] and rice [6] although remaining problems with heteroplasmy [6,19] and plant fertility [17] are yet to be solved.

Reverse genetic analysis of plastid genes and open reading frames

In addition to putting extra genes into plastids, the advent of routine methods for plastid genome engineering has opened up the exciting possibility of introducing almost any desired change into endogenous plastid genes. Although not of immediate practical value, such reverse genetics studies have contributed significantly to our understanding of photosynthesis, its molecular components and their molecular interactions. This knowledge is, in turn, crucial to all attempts to engineer the light reactions of photosynthesis.

The tobacco plastid genome, although fully sequenced more than 15 years ago [20], has kept some secrets, with several plastid gene functions remaining to be determined. In the absence of efficient 'forward genetics' methods (i.e. plastid genome-specific mutagenesis and genetic screens), assigning clear-cut functions to plastid genes and open reading frames (ORFs) remained difficult for many years. The development of facile methods for making transplastomic tobacco plants along with the high homologous recombination activity in plastids has paved the way to functional genomics in higher plant plastids using reverse genetics approaches (Figure 3). Photosynthesis is the most prominent function of chloroplasts and more than half of the protein-coding genes in the plastid genome encode photosynthesis-related proteins, so it was not surprising that the systematic knockout of ORFs in tobacco led to the discovery of several new genes for photosynthetic functions. One of the most remarkable cases was a tiny ORF of just 29 amino acids (*ycf6*, hypothetical chloroplast reading frame no. 6), which was inactivated by targeted replacement of the ORF with the *aadA* selectable marker gene (Figure 3). The resulting *ycf6* knockout plants were photosynthetically incompetent because of the complete absence of the thylakoidal cytochrome *b_{6f}* complex, the redox-coupling multiprotein complex transporting electrons from photosystem II to photosystem I. Subsequent biochemical analyses established that the Ycf6 protein is a genuine subunit of the cytochrome *b_{6f}* complex and the *ycf6* reading frame was therefore renamed *petN* [21]. PetN is one of the smallest functional proteins known to date and its discovery brings to light an important lesson for genome researchers: the size threshold for ORFs currently applied in the bioinformatic evaluation of genome sequences is usually much higher than 29 codons (most often 50–100 codons) and hence an unknown number of small ORFs escapes detection. Consequently, the information content in fully sequenced genomes might be significantly higher than currently believed.

Reverse genetics has also begun to shed light on the enigmatic *ndh* genes, a set of plastid genome-encoded

genes with striking homology to mitochondrial complex I genes. These genes had long been suspected to encode subunits of a putative plastid NAD(P)H dehydrogenase, which was proposed to be involved in chlororespiration, a respiratory electron transport chain that interacts with photosynthetic electron transport and involves the non-photochemical reduction and oxidation of plastoquinone. Interestingly, deletion of *ndh* genes from the tobacco plastid genome had no significant phenotypic consequences under standard growth conditions, but resulted in measurable physiological effects when the knockout plants were exposed to various stress conditions [22–24]. The physiological and biochemical data obtained by the comparison of wild-type and knockout plants indicate that the Ndh protein complex participates in cyclic electron flow around photosystem I [25–27]. This proposed function is compatible with the mild, stress-dependent phenotypic effects of *ndh* gene inactivation.

The simple knockout strategy of disrupting ORFs by targeted insertion of the selectable marker gene (usually a chimeric *aadA* gene conferring resistance to spectinomycin; Figure 3) is most appropriate for probing the functions of freestanding ORFs. In those cases in which the ORF is part of an operon, its disruption with the selectable marker gene cassette (which is usually driven by a strong promoter) bears the risk of interfering with transcript accumulation and/or mRNA folding. A suitable alternative strategy circumventing these potential problems involves integration of the selectable marker outside of the target gene or operon (Figure 3). However, in doing this one must accept incomplete linkage between the marker gene and the mutation to be introduced (e.g. a deletion in the reading frame or creation of a premature stop codon [28]). This strategy is also the method of choice for studying the function of plastid genes by making point mutations, which are often more informative than loss-of-function mutations. For example, knockout of the genes for the α - or β -subunit of cytochrome *b₅₅₉* results in complete loss of photosystem II, indicating that cytochrome *b₅₅₉* is essential for photosystem II assembly, but this does not tell us anything about the role of this enigmatic cytochrome in photosynthetic electron transport. However, it is possible to identify single amino acid changes that impair the electron-transporting function of cytochrome *b₅₅₉* without blocking its assembly function for photosystem II. In this way, cytochrome *b₅₅₉* could be studied in a tobacco mutant in which the conserved phenylalanine at position 26 within the β -subunit (PsbF) was changed to serine by introducing a point mutation into the plastid *psbF* gene [29]. The plastoquinone pool was found to be significantly reduced in dark-adapted leaves of the mutant, demonstrating that cytochrome *b₅₅₉* plays an important role in photosynthesis by keeping the plastoquinone pool (and thereby the acceptor side of photosystem II) oxidized in the dark [30].

Making photosynthesis more efficient, for example by improving its tolerance to abiotic stress conditions has long been the Holy Grail of photosynthesis research. Clearly, we are still a long way from making educated predictions about specific molecular changes in the photosynthetic apparatus that could be beneficial for

environmental adaptation. However, the exploitation of reverse genetics techniques in both the nucleus and the chloroplast, and the exciting progress being made in structural biology, will undoubtedly bring us closer and closer to this ultimate goal.

Reverse genetics approaches have also been used for studying several plastid genes not related to photosynthetic functions. Perhaps the most spectacular results came from knockout experiments with the genes for the four subunits of the plastid-encoded *E. coli*-like RNA polymerase. Analysis of plastid RNA synthesis in RNA polymerase knockout plants revealed the presence of significant residual transcription initiating at a distinct set of promoters [31,32]. This finding provided direct evidence for the existence of a second major RNA polymerizing activity in plastids, which is nuclear-encoded. The discovery of this enzyme activity was soon followed by the cloning of nuclear genes for bacteriophage-type RNA polymerases, which were found to be targeted to plastids [33]. The presence of two distinct transcription systems in plastids and their emerging interplay in the transcriptional regulation of plastid-encoded genes will also have important implications for optimizing transgene expression from plastid genomes.

Can every plastid gene be knocked out? Probably not, as demonstrated by the failure to generate knockout plants for two ORFs in the tobacco plastid genome, *ycf1* and *ycf2* [34]. Similarly, attempts to delete the chloroplast genome-encoded subunit of an ATP-dependent protease (*clpP* gene) were unsuccessful [35]. In both cases, plastid transformants were readily obtained, but could not be purified to homoplasmy. Under antibiotic selection, cells remained heteroplasmic adjusting their plastid genome population to a fairly constant ratio of wild-type and transformed genome copies. This suggests that although the transplastome was essential for expressing antibiotic resistance, the wild-type genome was maintained by the cell for expressing the essential plastid gene deleted in the transplastome. In accordance with this assumption, random genome segregation in the absence of selective pressure (Figure 1) did not produce homoplasmic transplastomic cells but instead, always resulted in loss of the transplastome [34]. Two recent papers have provided direct evidence for plastid genes and gene expression being essential for cell survival and plant development in tobacco. Using the CRE-loxP site-specific recombination system, the gene encoding ClpP could be effectively deleted by crossing a nuclear transgenic line expressing a plastid-targeted CRE recombinase to a transplastomic line harboring loxP sites upstream and downstream of the gene encoding ClpP [36]. Highly efficient *clpP* excision was correlated with arrested seedling development indicating that ClpP-mediated protein degradation in plastids is essential for shoot development. In a second study, homologous recombination was employed to induce loss of plastid translation in a cell line-specific manner. Switching off plastid translation caused an arrest in cell division, which in turn resulted in severe defects in leaf morphogenesis and floral development [37]. This study establishes a crucial role for plastid genes and their expression in plant development.

Thus far, all reverse genetics studies in higher plant plastids were conducted in tobacco, in which plastid transformation is a routine technique. Unfortunately, tobacco is allotetraploid (amphidiploid, i.e. is composed of four chromosome sets derived from the two diploid species *Nicotiana sylvestris* and *N. tomentosiformis*) and not a genetic model species. *Arabidopsis*, the number one model species for nuclear genetics and functional genomics, has the disadvantage that the production of fertile transplastomic plants is not yet feasible. Hence, an efficient integration of nuclear and plastid reverse genetics, although highly desirable, is not feasible at present. However, with the growing investment in genomics and functional genomics tools for tomato and other Solanaceae (<http://sgn.cornell.edu/solanaceae-project/>) a non-*Arabidopsis* model system for the functional analysis of plastid–nuclear interactions might become an attractive alternative.

Can photosynthesis be improved through Rubisco engineering?

Even a slight improvement in the efficiency with which plants use light, water and carbon dioxide to produce organic matter would have tremendous effects on agriculture. The efficiency of photosynthesis is dependent on the performance of the most abundant protein on earth: ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Rubisco makes up 30–50% of the soluble protein in leaves. Because it works very slowly, catalyzing only the reaction of a few molecules per second, large quantities of the enzyme are required. Another property of Rubisco that limits the efficiency of carbon dioxide fixation is the ability of oxygen to bind to the active site of the enzyme. When Rubisco uses oxygen as substrate, ribulose bisphosphate is broken down and carbon dioxide is released, a process known as photorespiration. Photorespiration wastes energy and is of no apparent use to the plant.

Because Rubisco catalyzes the rate-limiting step of carbon dioxide fixation, two seemingly straightforward strategies could theoretically improve the efficiency of photosynthesis: (i) enhancement of the catalytic turnover rate of Rubisco and (ii) increasing the specificity of the enzyme for carbon dioxide relative to oxygen thereby reducing the photorespiratory waste of energy. What are the chances of making a faster Rubisco by increasing its catalytic rate? Evolution had more than a billion years time to experiment with Rubisco sequences, so it seems unlikely that a change of one or a few amino acids would result in a more efficient Rubisco. By contrast, improving the CO₂:O₂ specificity could be a more realistic goal: There is significant natural variation in the specificity factor of different Rubisco enzymes isolated from different species, with the best enzymes being three times as specific as the Rubiscos in crop plants [38]. Thus, transfer of the catalytic properties of the best-performing naturally occurring enzymes onto the Rubisco of important crop plants should be feasible, although this is unlikely to be achievable through a few simple changes.

The availability of high-resolution three-dimensional structures for Rubiscos from a variety of photosynthetic microorganisms and plant species provides a solid basis for all attempts to successfully engineer this enzyme.

Although much mutagenesis work has been done on cyanobacterial Rubisco (reviewed in [38]), until recently, plant Rubiscos were not amenable to *in vivo* analysis for the simple reason that the gene (*rbcL*) for the catalytic large subunit is chloroplast genome-encoded in all plants (whereas genes for the small subunit, *rbcS*, are usually encoded by the nuclear genome). In view of the tremendous importance of Rubisco for both basic and applied research, it is not surprising that once chloroplast transformation technologies had been developed for the green alga *Chlamydomonas reinhardtii* [39] and the higher plant tobacco [9,10], Rubisco genes were among the first targets of genetic engineering [38–40] (Table 1).

One of the higher plant Rubiscos with the highest CO₂:O₂ specificity comes from the sunflower (*Helianthus annuus*). It exceeds the specificity of the tobacco enzyme by ~10% and a seemingly straightforward experiment replaced the *rbcL* gene in the tobacco plastid genome with the corresponding sunflower gene [41]. Although such transplastomic plants could be generated, and even produced hybrid hexadecameric Rubisco consisting of eight sunflower large subunits and eight tobacco small subunits, the hybrid enzyme did not have the catalytic properties that were hoped for. The specificity factor did not reach the sunflower value and, more disappointingly, the turnover rate of the enzyme was severely diminished (by a factor of approximately four; [41]; Table 1). These findings illustrate that although Rubisco is one of the most intensely studied enzymes, it is still difficult, if not impossible, to accurately predict the outcome of protein engineering experiments. What could be the reason for the poor performance of the hybrid sunflower–tobacco Rubisco? Limited compatibility of the sunflower large and tobacco small subunits is certainly one possibility, although alternative explanations, such as inefficient cooperation between the hybrid enzyme and Rubisco activase (an auxiliary enzyme preventing Rubisco from being inactivated upon turnover) must also be considered.

Because our present knowledge about structure–function relationships in the Rubisco holoenzyme seems far too limited to make educated predictions about meaningful molecular changes, engineering strategies that avoid the problem of assembling hybrid enzymes could

hold more promise. Encouraging results in this area come from an experiment in which the tobacco *rbcL* was replaced with the *rbcM* gene from the photosynthetic bacterium *Rhodospirillum rubrum* [42]. Rubisco from *R. rubrum* has a simple homodimeric structure and the gene encoding it (*rbcM*) is the only gene known to be required for assembly and catalytic activity. Transplastomic tobacco plants expressing *rbcM* synthesized fully active homodimeric Rubisco of the *R. rubrum* type (also called ‘form II Rubisco’) but, consistent with the kinetic properties of the *R. rubrum* enzyme (which has a low CO₂:O₂ specificity factor), required CO₂ supplementation for photoautotrophic growth [42,43] (Table 1).

Although all engineering attempts have thus far failed to produce a better Rubisco, these experiments have taught us important lessons about Rubisco function and regulation. Plastid genome engineering will certainly continue to provide a valuable tool for creating crop plants with more-efficient Rubisco enzymes, but similarly precise tools for engineering the plant’s nuclear genome might be required to make this dream come true. There is no obvious reason why transfer of superior Rubiscos, such as the sunflower version, into other crops should not be feasible, even though this could require exchange of all components of the system (including the nuclear genes for the Rubisco small subunit and the Rubisco activase). In this respect, recent progress with gene targeting by homologous recombination in *Arabidopsis* [44] and rice [45] represents an important step forward.

Engineering biosynthetic pathways in plastids

Engineering economically important traits in crop plants has long been a major goal of biotechnological research. Metabolic engineering will probably contribute to the required continued increase in agricultural productivity by securing more-nutritious food, and moreover could provide a cost-effective future production platform for pharmaceuticals and nutraceuticals [46].

Genes for enzymes tapping metabolite pools to synthesize novel compounds as well as quantitative trait loci suitable for increasing the accumulation of health-promoting substances are prime candidates for metabolic engineering strategies. Major challenges are posed by the

Table 1. Attempts to modify Rubisco in tobacco (*Nicotiana tabacum*) with plastid genome engineering^a

Genetic change	Plant phenotype – catalytic properties of Rubisco	Refs
<i>rbcL</i> deletion	Lack of photoautotrophic growth; could be partially complemented by introduction of a plastid-targeted <i>rbcL</i> into the nuclear genome	[56]
<i>rbcS</i> insertion	Low RbcS protein accumulation from the plastid transgene (1% of total RbcS)	[57]
<i>rbcS</i> insertion (<i>rbcS</i> antisense plants)	RbcS protein accumulation not significantly increased; no complementation of the <i>rbcS</i> antisense phenotype	[58]
<i>rbcL</i> replacement with <i>Synechococcus rbcL</i>	Lack of photoautotrophic growth because of absence of RbcL protein accumulation	[41]
<i>rbcL</i> replacement with <i>Helianthus rbcL</i>	Accumulation of hybrid enzyme (tobacco SSU, sunflower LSU); CO ₂ :O ₂ specificity similar to tobacco Rubisco; catalytic rate lowered to 25%	[41]
Insertion of the <i>rbcLS</i> operon from non-green algae	High-level RbcL and RbcS protein accumulation, but no assembly of active Rubisco	[59]
Replacement of <i>rbcL</i> with <i>rbcM</i> from <i>Rhodospirillum rubrum</i>	Plant growth only in CO ₂ -enriched atmosphere; fully active form II Rubisco with catalytic properties identical to the <i>Rhodospirillum</i> enzyme	[42,43]
Codon 335: Leu → Val	Drastically reduced CO ₂ assimilation; plant growth only in CO ₂ -enriched atmosphere; CO ₂ :O ₂ specificity dropped to 25%	[60]

^aAbbreviations: LSU, large subunit; *rbcL*, gene for large subunit of Rubisco; *rbcS*, gene for small subunit of Rubisco; *rbcM*, gene for the homodimeric Rubisco type; SSU, small subunit.

identification of suitable candidate genes and their precise regulation *in planta* to specifically enhance the level of a given metabolite (ideally confined to the plant's edible parts) without negatively impacting plant growth and yield.

Recent progress in plant biotechnology has convincingly demonstrated the great potential of transgenic approaches towards metabolic pathway engineering and micronutrient fortification. Successful synthesis of β -carotene (provitamin A) in 'golden rice' [47] and phytoferritin (an iron-storing protein; [48]) in rice endosperm perhaps provide the two most exciting examples. Vitamin A deficiency is a devastating malnutrition disorder common in countries where rice is the only staple food. Rice endosperm is essentially free of β -carotene, the provitamin A, which can be converted by the human body into active vitamin A. Because the biosynthesis of β -carotene occurs inside the plastid compartment, two approaches seem feasible to engineer β -carotene synthesis into rice endosperm: (i) direct transformation into the plastid genome or (ii) nuclear transformation with gene constructs harboring transit peptide sequences for protein import into plastids. Using the latter strategy, 'golden rice' was produced by incorporating the three genes required to synthesize β -carotene from the carotenoid precursor geranylgeranyl pyrophosphate (which is present in rice endosperm). Two enzymes were taken from daffodil, *Narcissus pseudonarcissus*, and the third one was borrowed from a carotenoid-synthesizing bacterium, *Erwinia uredovora* [47]. Rice seeds with enhanced β -carotene content could in the near future become an inexpensive and effective means of preventing and curing diseases caused by vitamin A deficiency in regions where rice is the only staple food. In addition to β -carotene, other carotenoids, such as lycopene, lutein and zeaxanthin serve as powerful antioxidants and thus are also nutritionally important compounds, highlighting the great attraction of the carotenoid biosynthesis pathway for metabolic engineering. Because all reactions specific to carotenoid biosynthesis are localized in plastids, this pathway is also a suitable target for metabolic engineering directly via chloroplast transformation. This transplastomic approach would offer additional advantages, such as increased transgene containment and simple transgene stacking by constructing multigene operons or directly taking them from prokaryotic source organisms. Several constructs with bacterial, fungal and plant carotenoid biosynthesis genes have recently been inserted into the tobacco and tomato plastid genomes (S. Ruf, D. Wurbs and R.B., unpublished). In addition to testing metabolic engineering strategies, analysis of carotenoid spectra in these transplastomic plants is also hoped to provide new insights into the regulation of this important biochemical pathway, which is currently only poorly understood.

Being the site of photosynthesis as well as sugar and starch syntheses, the chloroplast is also an important compartment for carbohydrate metabolism. Extensive work has been done on engineering carbohydrate metabolism via nuclear transformation (e. g. [49,50]) and similar work using plastid transformation technology is just starting. An encouraging example of successful

carbohydrate engineering using the transplastomic technology is the recent production of trehalose in tobacco chloroplasts [51]. Osmoprotective sugars like trehalose can be used to actively stabilize enzymes, proteins, pharmaceuticals and even human organs for transplantation purposes. A most remarkable example of trehalose usage for the preservation of pharmaceuticals is that drying of an oral polio vaccine in the presence of trehalose at 45 °C resulted in an equally stable vaccine as a liquid vaccine solution kept chilled at 4 °C. In addition, trehalose is of interest to the food industry because it does not engage in chemical reactions with amino acids or proteins, thus preventing browning of food products during processing. Recently, trehalose has been approved as a safe food ingredient by both the US Food and Drug Administration (FDA) and the European Union regulation system (reviewed in [52]). Trehalose can be metabolically derived from plant sugar pools, for example, by expressing the yeast gene encoding trehalose phosphate synthase (*TPS1*). The trehalose phosphate synthase catalyzes the conversion of glucose-6-phosphate and UDP-glucose to trehalose-6-phosphate. Introduction of the yeast *TPS1* into the tobacco chloroplast genome resulted in an ~20-fold increase in trehalose accumulation [51]. It will be interesting to see how precisely the accumulation of trehalose in chloroplasts can be adjusted to the desired levels by testing different expression signals for the *TPS1* transgene. Plants synthesizing large quantities of trehalose could provide a useful production platform for the food and pharmaceutical industries whereas smaller quantities of trehalose could be adequate to confer drought tolerance to the plants [51,53] presumably by protecting cellular membranes, safeguarding enzymes and stabilizing the photosynthetic complexes.

Other worthwhile targets for metabolic engineering are the biosynthetic pathways for fatty acids and essential amino acids. In an effort to increase the amount of fatty acids produced, the plastid-encoded acetyl-CoA carboxylase subunit gene *accD* was overexpressed in tobacco plastids by tethering it to the strong constitutive rRNA operon promoter. Transplastomic plants indeed showed an increase in fatty acid content in leaves and, moreover, displayed delayed leaf senescence and increased seed yield [54]. To overproduce the essential aromatic amino acid tryptophan, the control enzyme of tryptophan biosynthesis, anthranilate synthase, was engineered by plastid transformation in tobacco. Anthranilate synthase is a tetrameric enzyme composed of two α -subunits and two β -subunits, all of which are nuclear encoded. The α -subunits regulate the enzyme activity by being the target of feedback inhibition by the pathway's end product, tryptophan. Feedback-insensitive mutant versions of the anthranilate synthase α -subunits are potentially suitable for deregulating the enzyme and trigger a higher-than-normal accumulation of the essential amino acid tryptophan. Indeed, expression of such a feedback-insensitive α -subunit from the tobacco plastid genome (in addition to the wild-type enzyme still being expressed from the nuclear genome) resulted in increased tryptophan accumulation and made the plants less sensitive to the anthranilate synthase inhibitor 5-methyl tryptophan [55].

Clearly, these first applications of the transplastomic technology do not represent more than a promising beginning. Making metabolic engineering via plastid transformation a routine method will depend largely on progress in simplifying the technology and extending the crop range. This progress remains to be made but with the current intensification of research in this area, substantial strides forward can be expected in the near future.

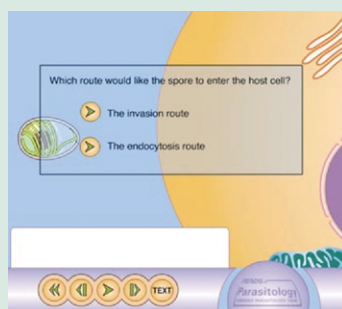
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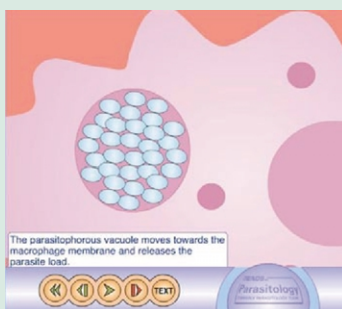
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