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Genetic engineering of the chloroplast: novel tools and new applications

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The plastid genome represents an attractive target of genetic engineering in crop plants. Plastid transgenes often give high expression levels, can be stacked in operons and are largely excluded from pollen transmission. Recent research has greatly expanded our toolbox for plastid genome engineering and many new proof-of-principle applications have highlighted the enormous potential of the transplastomic technology in both crop improvement and the development of plants as bioreactors for the sustainable and cost-effective production of biopharmaceuticals, enzymes and raw materials for the chemical industry. This review describes recent technological advances with plastid transformation in seed plants. It focuses on novel tools for plastid genome engineering and transgene expression and summarizes progress with harnessing the potential of plastid transformation in biotechnology.

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Introduction

Plant cells have three genomes and, in some seed plants, two of these genomes are transformable: the nuclear genome and the genome of the plastids (chloroplasts). The plastid genome of photosynthetically active seed plants is a small circularly mapping genome of 120–220 kb, encoding 120–130 genes. It can be engineered by genetic transformation in a (still relatively small) number of plant species and this possibility has stirred enormous interest among plant biotechnologists. There are considerable attractions associated with placing transgenes into the plastid genome rather than the nuclear genome. First and foremost, the high number of plastids per cell and the high copy number of the plastid genome per plastid offer the possibility of expressing foreign genes to extraordinarily high levels, often one to two orders of magnitude higher than what is possible by expression from the nuclear genome [1,2]. Secondly, transgene integration into the plastid genome occurs exclusively by homologous recombination, making plastid genome engineering a highly precise genetic engineering technique for plants (which normally integrate foreign DNA into their nuclear genomes by non-homologous recombination). Third, as a prokaryotic system that is derived from a cyanobacterium acquired by endosymbiosis, the plastid genetic system is devoid of gene silencing and other epigenetic mechanisms that interfere with stable transgene expression. Fourth, similar to bacterial genes, many plastid genes are arranged in operons offering the possibility to stack transgenes by arranging them in artificial operons. Finally, plastid transformation has received significant attention as a superb tool for transgene containment due to the maternal mode of plastid inheritance in most angiosperm species, which drastically reduces transgene transmission through pollen [3,4].

Since the development of plastid transformation for the seed plant tobacco (*Nicotiana tabacum*) more than 20 years ago (reviewed, e.g. in [5,6]), the community has assembled a large toolbox for plastid genetic engineering and also made some progress with developing plastid transformation protocols for additional species. Unfortunately, plastid transformation is still restricted to a relatively small number of species and not a single monocotyledonous species (including the cereals representing the world's most important staple foods) can be transformed. Thus, developing protocols for important crops continues to pose a formidable challenge in plastid biotechnology and significant strides forward are likely to require conscientious efforts and long-term investments in both the academic and the industrial sectors.

Here, I review recent progress in plastid genome engineering in seed plants. I focus on new tools that were developed in the past few years and likely will enable new applications of the transplastomic technology. I also briefly highlight new areas in biotechnology that have been explored recently using transplastomic approaches and that show great promise towards a commercial utilization of the technology in the foreseeable future.

New tools for generating transplastomic plants

Over the past 20 years, the basic methodology of plastid transformation has not changed. Particle gun-mediated (biolistic) transformation remains the method of choice and polyethylene glycol (PEG)-mediated protoplast

transformation is occasionally used as an alternative [6,7•]. As all protoplast-based methods, PEG-mediated plastid transformation is technically demanding, laborious and also more time-consuming than biolistics, but has the advantage that the method is not protected by patents. The development of a tissue culture-independent protocol for plastid transformation (similar to vacuum infiltration or floral dipping for *Agrobacterium*-mediated nuclear transformation of *Arabidopsis*) would make the transplastomic technology accessible to a much wider range of users. Recently, there has been some progress with performing manipulations of the tobacco plastid genome in greenhouse-grown plants, especially the post-transformation removal of marker genes by site-specific recombination using phage-derived recombinases targeted to plastids [8]. The recombinase was delivered by *Agrobacterium tumefaciens* injection into axillary buds of soil-grown tobacco plants. Following decapitation, lateral shoot formation from the injected axillary meristem frequently resulted in the appearance of cell lines with marker-free plastid genomes and, in 7% of the cases, led to transmission of the marker-free genome to the seed progeny. Although this result demonstrates that at least some secondary manipulations of the plastid DNA are possible *in planta* through nuclear expression of plastid-targeted enzymes for genome engineering, a truly tissue culture-independent method for primary manipulation of the plastid genome remains a distant goal that will be difficult to achieve.

Similar to the DNA delivery process, the selection procedures for obtaining transplastomic plants have not changed much over the past two decades. The spectinomycin resistance gene *aadA* encoding an aminoglycoside 3″-adenylyl transferase [9] remains the most commonly used selectable marker gene for chloroplast transformation. Although in recent years, several alternative antibiotic resistance markers have been developed [10–12], they appear to be less efficient than the *aadA*, presumably because they require higher expression levels to confer phenotypic resistance. Nonetheless, they may provide attractive alternatives when intellectual property considerations come into play, and they also represent valuable tools for supertransformation (i.e. the transformation of an already transplastomic plant line with additional transgenes).

Unfortunately, plastid transformation technology is still limited to relatively few species [6,7•]. Developing a protocol for a new species often requires significant efforts to optimize tissue culture, regeneration and selection procedures [13–15]. Workable plastid transformation protocols for important model plants (including *Arabidopsis thaliana*) and agriculturally important staple crops (including all cereals) are still lacking and sometimes even switching to a closely related species or a different cultivar of a species amenable to plastid transformation can be challenging [16]. An alternative to establishing a transformation protocol is to transfer transgenic plastids from an easy-to-transform species to a recalcitrant related species or cultivar. This can be done by employing cell biological manipulations, such as protoplast fusion and generation of cybrids. Cybrids (or cytoplasmic hybrids) are produced by elimination of the nuclear genome of one of the fusion partners in a protoplast fusion experiment (e.g. by γ-ray or X-ray irradiation). To combine transgenic chloroplasts with a new nucleus, the nuclear genome of the transplastomic protoplasts needs to be eliminated and, following protoplast fusion, selection and plant regeneration in the presence of the antibiotic that the transplastomic chloroplasts are resistant to will result in replacement of the resident population of (wild-type) plastids with the transgenic plastids from the alien species or cultivar. This method has been demonstrated to work [17–19], but due to the demanding procedures involved in protoplast fusion and plant regeneration from protoplasts, it is laborious, time-consuming and applicable only to a limited number of plant species.

Recently, a simpler method of transferring transgenic plastids between species has been developed. It is based on the surprising discovery that plastid DNA (and presumably entire plastids) can migrate between cells in grafted plants [20]. As even sexually incompatible species can be grafted, this method allows the transfer of transgenic plastid genomes between species by excising the graft site (after establishment of the graft junction) and selecting for transfer of the transgenic plastid into cells of the recalcitrant species [21••,22••]. This method is likely to become useful in expanding the species range of the transplastomic technology, but its applicability will be restricted to closely related species. The combination of a nuclear genome with a new plastid genotype can result in so-called plastome-genome incompatibilities (PGI) which, with increasing phylogenetic distance, become more likely and can result in severe mutant phenotypes [23].

New tools for plastid transgene expression

A main reason for the excitement about chloroplast transformation among biotechnologists lies in the extraordinarily high foreign protein accumulation levels attainable by expressing transgenes from the plastid genome, which in extreme cases reached more than 50% of the total soluble protein in leaves [24,25,2]. However, it is important to realize that, despite many cases where spectacular expression rates could be obtained [26,27], there is also a significant list of proteins whose expression in plastid was problematic in that expression levels were poor or undetectably low. Although the molecular causes of unsuccessful transgene expression are only rarely investigated systematically, the picture emerging from the cases analysed in some detail suggests that protein stability is often the key factor limiting foreign protein accumulation

[28–30]. Unfortunately, while the regulation of transcription and RNA stability in plastids is reasonably well studied, very little is known about the rules governing protein stability. Recent transplastomic studies have uncovered an important role of the N-terminus in determining the stability of chloroplast proteins [31,29,30]. Consequently, manipulating the N-terminus of unstable recombinant proteins or fusing them to the N-terminus of a stable protein can help to alleviate problems with protein stability in transplastomic plants [29,30]. However, it appears unlikely that all cases of low foreign protein accumulation can be explained by an instability-conferring N-terminus. Internal sequence motifs or improper protein folding may also trigger rapid protein degradation. Unfortunately, next to nothing is known about internal determinants of protein (in)stability and also the rules that govern the foldability of proteins expressed in the plastid remain largely elusive. Thus, making the stability of recombinantly expressed proteins in plastids more predictable and providing guidelines for how to stabilize inherently unstable proteins represent major challenges for future research.

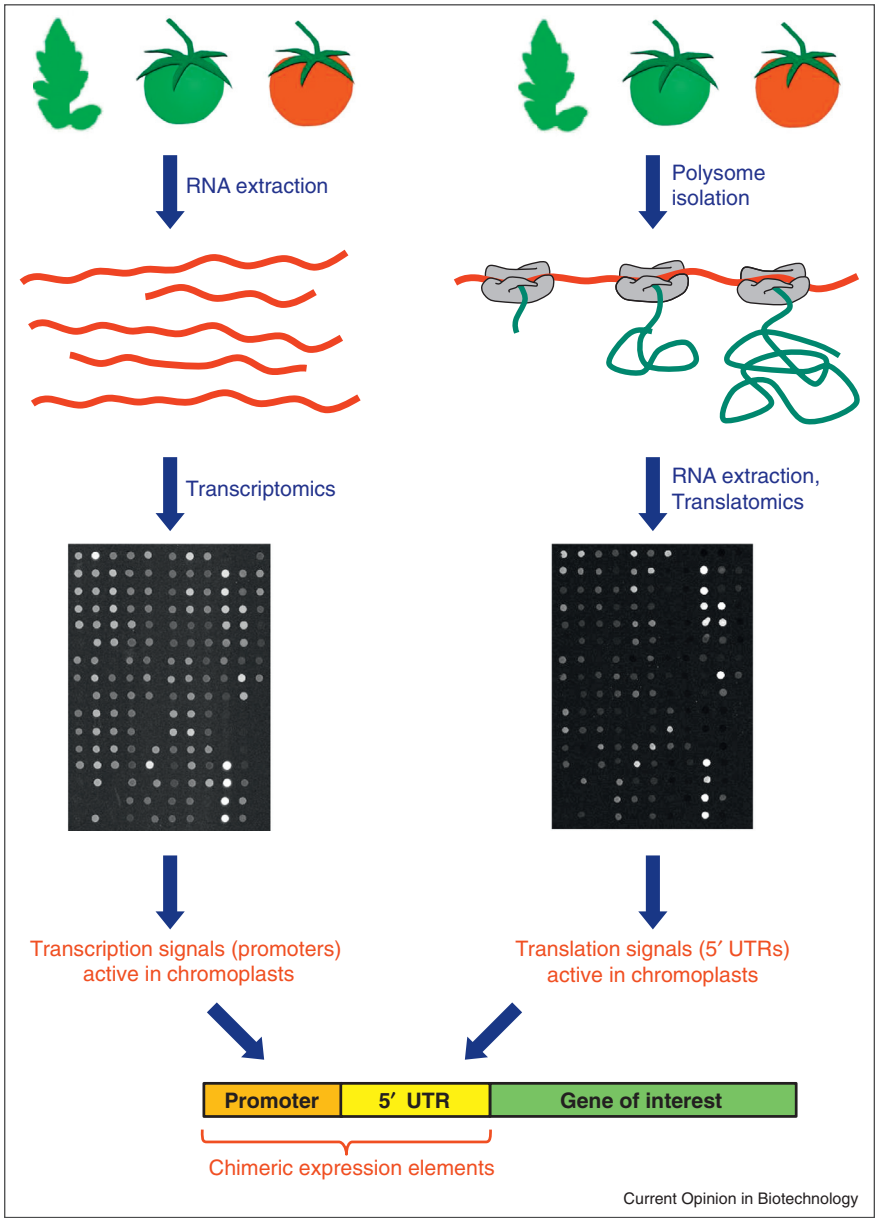
Another attraction of the transplastomic technology relates to the prokaryotic nature of the plastid gene expression machinery. It offers the possibility to stack multiple transgenes in operons and co-express them from a single promoter as a polycistronic mRNA. This is especially useful for the engineering of metabolic pathways (which often requires concerted expression of several enzymes). Although operon expression has been successful in some cases [32,33••], there are also several examples of poor expression of at least some of the transgenes in an operon construct transformed into the plastid genome (reviewed in [34]). Although in bacteria, polycistronic mRNAs made from operons directly enter translation, many polycistronic transcripts in plastids must undergo post-transcriptional cleavage into monocistronic units to facilitate translation especially of the downstream cistrons in the operon [35,34]. To ensure translatability of all cistrons of a transgenic operon in plastids, a small sequence element can be included that mediates intercistronic processing into stable monocistronic mRNAs [36,37••]. This element, derived from a processing site in the plastid *psbB* operon and dubbed IEE (intercistronic expression element), provides a valuable tool for synthetic operon design in that it greatly increases the chances of successful operon expression in transgenic chloroplasts [37••,34].

A serious limitation in plastid biotechnology has been the low expression levels of plastid genes in non-green tissues, such as fruits, tubers and seeds. Genome-wide analyses of transcription and translation in tomato fruits and potato tubers revealed that nearly all plastid genes are strongly down-regulated at both the transcriptional and translational levels in these non-green storage organs [38,39]. Interestingly, while a few exceptional genes maintained relatively high mRNA levels but were poorly translated (e.g. the *psbA* gene in tomato encoding the D1 protein of photosystem II), another small group of exceptional genes had low transcript levels but the mRNAs displayed strong ribosome association suggestive of active translation (e.g. the *accD* gene in tomato encoding a subunit of the acetyl-CoA carboxylase; Figure 1). This observation raised the possibility of combining a promoter from a gene showing high mRNA accumulation in fruits or tubers with the 5′ untranslated region (5′ UTR; containing the *cis*-elements for the regulation of translation initiation) from an mRNA showing strong polysome association. Indeed, the construction of such hybrid expression elements (Figure 1) led to significant improvements in the transgene expression levels attainable in roots, tubers and fruits [40•,41•,42••] and thus opens up new opportunities for metabolic engineering and recombinant protein production in plastids of non-leafy tissues.

Sometimes the expression of transgenes in plastids causes severe mutant phenotypes that result in retarded growth or, in extreme cases, prevent autotrophic growth altogether. This can have different reasons, including enzymatic activities of the recombinantly expressed protein that interfere with metabolic processes in the plastid [43], harmful interactions of the foreign protein with chloroplast membranes [44] or simply the high metabolic burden imposed by extreme accumulation levels of the recombinant protein [2,45]. The problem of transplastomic plants developing drastic phenotypes can be dealt with in two possible ways. An immediate solution is to grow the plants (or cell cultures derived thereof) heterotrophically or mixotrophically in bioreactors. Recently, a temporary immersion bioreactor has been developed that facilitates biomass production from transplastomic plants that display severe mutant phenotypes and, therefore, are difficult to grow in soil [46•]. Cultivation in bioreactors requires aseptic conditions and synthetic media, which makes biomass production significantly more expensive compared to plant production under autotrophic conditions in the field or in greenhouses. However, bioreactors represent a viable option for high-value products (e.g. expensive biopharmaceuticals), where the additional costs of biomass production are negligible. Moreover, bioreactors provide fully contained conditions, which may be desirable for certain products and also may simplify deregulation of transgenic plants.

An alternative strategy to deal with deleterious effects of recombinant protein production in plastids is to make transgene expression inducible. This can be readily achieved by employing nuclear transgenes, such as an ethanol-inducible T7 RNA polymerase gene whose protein product is targeted to plastids and transcribes plastid transgenes that are tethered to the T7 promoter [47]. As the use of nuclear transgenes abrogates the containment advantage of the transplastomic technology,

Figure 1



Workflow for optimizing plastid transgene expression in non-green tissues using tomato fruits as an example. Expression of the plastid genome is strongly down-regulated in all non-green tissues. Comparative transcript profiling (transcriptomics) and comparative polysome profiling (translatomics) of leaves, immature green fruits and mature red fruits identify the patterns of organ-specific (leaves vs. fruits) and developmental (green chloroplast-containing fruits vs. red chromoplast-containing fruits) regulation of plastid genes at the transcriptional and translational levels [38]. From these datasets, information about promoters and translation signals active in chromoplasts can be extracted. Combination of a promoter from a gene that is actively transcribed in chromoplasts with translation signals from an mRNA that is strongly translated in chromoplasts produces hybrid expression signals suitable to drive efficient plastid transgene expression in ripe red tomato fruits [42••].

the development of a plastid-only system for inducible transgene expression was highly desirable. The design of an efficient system that offers both tight control and high induction rates proved to be challenging. So far, two options have become available: (i) a system based on the bacterial lac repressor and lac operator, in which transgene expression is induced transcriptionally by spraying or leaf infiltration with the chemical inducer IPTG [48], and (ii) a synthetic riboswitch that induces translation in response to application of the natural compound theophylline [49].

Last but not least, efficient and cost-effective strategies for the purification of recombinant proteins from

transplastomic plants are important components of the toolbox for chloroplast biotechnology. Recent progress in this area includes the evaluation of a number of purification tags [50] and the successful targeting of foreign proteins to plastoglobules, which facilitates the simple enrichment of the recombinant protein by flotation centrifugation [51].

New applications of plastid transformation in biotechnology

The transplastomic technology has been extensively used to insert resistance genes into the plastid genome (making plants tolerant to herbicides or resistant to insect pests), express recombinant proteins for molecular farming (e.g. vaccines) and engineer metabolic pathways. Much of this work has been comprehensively covered by a number of recent review articles [52–54,27,7•] and, therefore, will not be discussed in detail here. Recent new applications in these areas include the development of a plastid resistance gene against D-amino acids that potentially could be used as herbicides [55•] and the successful expression of enzymes of the antioxidant system to provide increased tolerance to abiotic stresses [56,57]. Although initially, the expression of antigens for subunit vaccines was the main application of the transplastomic technology in molecular farming, over the past five years many more pharmaceutical proteins have been expressed in transgenic plastids. Some of these proteins could be expressed to spectacularly high levels, such as several phage-derived endolysins that potentially can provide next-generation antibiotics [2,58]. Promising progress has also been made with the expression of antibody fragments [59•], blood coagulation factors for the treatment of haemophilia [60] and transforming growth factor β (TGF-β), a cytokine-type protein that promotes wound healing and reduces scarring [61].

The expression of industrial enzymes has become an exciting new area of chloroplast biotechnology. With the growing interest in renewable energy sources, the production of biofuel enzymes has received particularly strong attention. A number of recent studies have demonstrated that many of the enzymes that potentially can be used to convert cellulosic biomass into fermentable sugars can be expressed from the plastid genome to very high levels. These include, for example, various cellulases, xylanases, glucosidases, pectate lyases and cutinases [62–65,43,66–68]. Some of the genes for these enzymes were taken from thermophilic organisms and therefore have thermostable properties, a particularly useful feature for the processing of cellulosic biomass at the industrial scale. Although, for the time being, the requirement for costly thermoacidic pretreatment of lignocellulosic biomass remains the main obstacle to the economically viable production of cellulosic ethanol, having a cheap source of enzymes degrading cellulose and hemicellulose represents an important part of the solution.

There is also growing interest in using transplastomic plants as factories for the production of so-called 'green chemicals': raw materials and building blocks for the chemical industry. The recent production of polyhydroxybutyrate (PHB), a renewable bioplastic, to more than 18% of the dry weight of transplastomic tobacco plants represents a particularly striking example of the successful redirection of plant metabolism towards the massive synthesis of a novel compound [33••]. The high-level accumulation of PHB caused reduced growth of the transplastomic plants, but this problem can potentially be solved by making the expression of the PHB operon inducible (see above).

The rapidly increasing number of proof-of-concept studies employing plastid transformation in biotechnological research and the great progress made in the past few years with both high-level recombinant protein expression and multigene engineering [69,33••,37••,34] hold great promise for the commercialization of the technology. So far, products derived from transplastomic plants have not yet entered the market, but especially in the pharmaceutical sector, this is now expected to happen rather soon.

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