Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Biotechnology

Genetic engineering of the chloroplast: novel tools and new applications

Ralph Bock

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.

Address
Max-Planck-Institut für Molekulare Pflanzenphysiologie, Am Mühlenberg 1, D-14476 Potsdam-Golm, Germany

Corresponding author: Bock, Ralph (rbock@mpimp-golm.mpg.de)

Current Opinion in Biotechnology 2014, **26**:7–13

This review comes from a themed issue on **Plant biotechnology**

Edited by **Birger Lindberg Møller** and **R George Ratcliffe**

0958-1669/$ – see front matter,

http://dx.doi.org/10.1016/j.copbio.2013.06.004

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