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Plastid biotechnology: prospects for herbicide and insect resistance, metabolic engineering and molecular farming

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Transgene expression from the chloroplast (plastid) genome offers several attractions to plant biotechnologists, including high-level accumulation of foreign proteins, transgene stacking in operons and a lack of epigenetic interference with the stability of transgene expression. In addition, the technology provides an environmentally benign method of plant genetic engineering, because plastids and their genetic information are maternally inherited in most crops and thus are largely excluded from pollen transmission. During the past few years, researchers in both the public and private sectors have begun to explore possible areas of application of plastid transformation in plant biotechnology as a viable alternative to conventional nuclear transgenic technologies. Recent proof-of-concept studies highlight the potential of plastid genome engineering for the expression of resistance traits, the production of biopharmaceuticals and metabolic pathway engineering in plants.

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Introduction

When the genetic transformation of chloroplasts was accomplished nearly two decades ago [1,2], many researchers were thrilled by the possibility to knockout genes and to study the mechanisms of plastid gene expression *in vivo*, but few thought about biotechnological applications (Figure 1). The wake-up call came when a gene conferring insect resistance that had proven notoriously difficult to express from the plant's nuclear genome was put into the chloroplast genome and its gene product (a *Bacillus thuringiensis* toxin protein) accumulated to extraordinarily high levels [3]. Since then, chloroplast transformation has been the subject of considerable excitement among biotechnologists. Besides the potential for high-level production of foreign proteins, other

attractions of the technology include its effectiveness as a high-precision genetic engineering technique (owing to transgene integration exclusively via homologous recombination; Figure 1), the absence from plastids of epigenetic effects and gene silencing mechanisms, the ease with which multiple transgenes can be stacked by linking them together in operons, and the increase in transgene containment provided by the maternal mode of chloroplast inheritance in most crop plants, which largely excludes chloroplasts (and thus plastid transgenes) from pollen transmission (e.g. see [4,5] for reviews).

Over the past two decades, great progress has been made in understanding the mechanisms that govern transgene expression from the chloroplast genome and in unraveling the potential of the technology for biotechnological applications. The widespread use of chloroplast transformation in biotechnology is still a long way off, but could one day benefit producers, consumers and patients. Here, I review progress in the three areas of applied research that have formed the main focus of chloroplast biotechnologists: the engineering of resistance traits, the modification of metabolic pathways and the production of pharmaceuticals in plants.

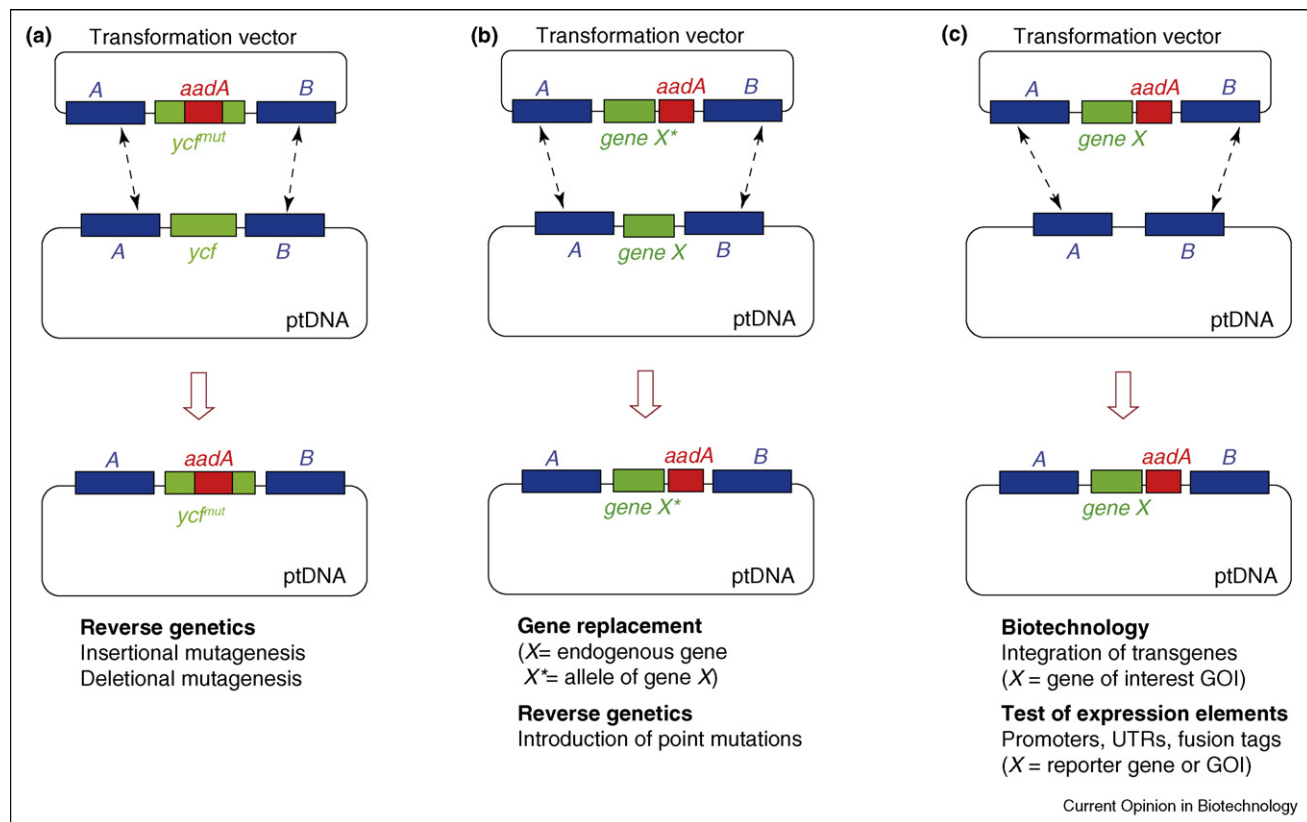
Herbicide and insect resistance

When genetically engineering pathogen or herbicide resistance into plants, the level of resistance often positively correlates with the accumulation levels of the resistance-conferring gene product (protein). In view of the high capacity of chloroplasts to express and accumulate foreign proteins, it was therefore unsurprising that resistance genes were among the first transgenes investigated for high-level expression from the chloroplast genome.

Owing to their huge diversity, the crystal toxin proteins from *B. thuringiensis* strains provide a rich source of insecticidal proteins for pest control. Various *cry* genes encoding toxin proteins active against different insect species have been introduced into plants, and several crops expressing such insecticidal proteins from their nuclear genome have already been commercialized. Initially, the prokaryotic origin of the *cry* genes and their different codon usage caused severe complications for efficient expression from plant nuclear genomes. By contrast, when introduced into the plastid genome, *cry* genes expressed extremely well; there was no requirement to adjust the codon usage nor any need for other sequence manipulation [3,6,7]. Consequently, leaves of these transplastomic plants proved highly toxic to herbivorous insect larvae, as demonstrated by feeding assays.

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Figure 1



Applications of chloroplast transformation technology and the corresponding design of plastid transformation vectors. **(a)** For reverse genetics, the selectable marker gene for plastid transformation *aadA* (a spectinomycin resistance gene) is used to disrupt an endogenous gene of unknown or unclear function. *ycf*, plastid open reading frame; *ycf^{mut}*, mutated plastid open reading frame. **(b)** For gene replacement, an allele of an endogenous gene is linked to the *aadA* marker gene to replace the endogenous allele by homologous recombination. This strategy can also be used to introduce point mutations into plastid genes and open reading frames to study their functions. **(c)** For expression of a transgene of interest (GOI), this passenger gene is linked to the *aadA* gene and targeted to a neutral insertion site, typically an intergenic region of the plastid genome. A, B, flanking plastid DNA regions for integration via homologous recombination; ptDNA, plastid genome; UTR, untranslated region.

The highest expression level, reported to be around 45% of the total soluble protein (TSP), was obtained when the *ery* gene formed part of an operon with two small open reading frames (ORFs) upstream, as is the case in the bacterial genome. This exceptionally high expression rate was attributed to one of the upstream ORFs, which potentially encodes a chaperonin that assists correct folding of the toxin protein and in turn could promote protein crystallization inside chloroplasts [7]. However, a recent report that high-level expression of a *ery* gene (Cry9Aa2) from the plastid genome (to about 10% of TSP) results in severe growth retardation [8^{*}] indicates that, rather than maximizing expression, careful determination of the optimum expression level is required to obtain plants with good insect protection while minimizing yield penalty.

Although all these studies were conducted in the model species tobacco, the recent generation of insect-resistant transplastomic soybean plants offers promise for transfer of the technology to important (food) crops [9^{*}].

The feasibility to use chloroplast genetic engineering for weed control has been explored in several studies that aimed at producing glyphosate-tolerant tobacco plants. Glyphosate (N-(phosphonomethyl) glycine) is a widely used broad-spectrum herbicide that acts as a potent inhibitor of the plant aromatic amino acid biosynthetic pathway by competitively inhibiting the key enzyme 5-enol-pyruvyl shikimate-3-phosphate synthase (EPSPS). In plants, EPSPS is a nuclear-encoded chloroplast-targeted enzyme. Tolerance to glyphosate can be obtained by either overexpression of EPSPS or through the expression of enzyme variants with decreased affinity for the herbicide. So far, only the latter approach has resulted in tolerance levels that were sufficiently high for commercial application in transgenic crops. Bacteria provide a rich natural source of genes for glyphosate-insensitive EPSPS enzymes. Alternatively, point mutations in plant EPSPS genes can drastically lower the affinity of the enzyme for glyphosate and such mutant alleles can also be used to engineer herbicide tolerance into crops. Attempts to

optimize glyphosate tolerance in transplastomic tobacco plants have involved three strategies: testing of glyphosate-tolerant EPSPS genes from different sources, adjusting the codon usage of the EPSPS gene to the codon usage in plastids where usually triplets with A or T in third codon position are strongly preferred, and testing different expression signals (promoters, 5' untranslated regions, N-terminal fusions) to maximize EPSPS expression [10,11]. Of these strategies, the choice of the 5' untranslated region (containing the Shine–Dalgarno sequence and thus determining the rate of translation initiation) had by far the strongest effect on the attainable level of herbicide tolerance [11]. EPSPS accumulation levels varied more than 10 000-fold and, with the best performing constructs, protein accumulation reached values of over 10% of the TSP of the plant. These transplastomic plants were tolerant to very high glyphosate doses, but revealed a so far unresolved paradox: chloroplast localization of the EPSPS gene required 250-fold higher enzyme accumulation to reach similar tolerance levels as nuclear expression of the identical EPSPS gene [11]. Whether or not this phenomenon can be attributed to low EPSPS expression levels in non-green plastid types (e.g. proplastids in the meristems) and their persisting sensitivity to the herbicide, remains to be investigated.

Chloroplast expression has also been demonstrated to be a suitable route for obtaining other herbicide tolerances, including phosphinothricin (glufosinate) tolerance [12,13]. Plastid expression of the *bar* gene encoding the herbicide-inactivating phosphinothricin acetyltransferase (PAT) enzyme led to high-level enzyme accumulation (up to >7% of TSP) and conferred field-level tolerance to glufosinate [13]. Interestingly, despite significant efforts, all attempts to develop herbicide resistance genes as selectable markers for chloroplast transformation have failed [13,14]. It is not clear why the direct selection for herbicide resistance does not produce chloroplast transformants. One possibility is that herbicide application could lead to early lethality, whereas exposure to antibiotics inhibiting plastid translation is generally considered a non-lethal selection scheme [14].

Both the herbicide tolerance studies and the work on insect resistance demonstrate that transplastomic technology might be particularly useful in those cases where successful resistance engineering crucially depends on high expression levels of the resistance gene. They also highlight the importance of testing a variety of expression signals and gene variants to obtain fully optimized transgenic constructs that provide effective plant protection in the field.

Metabolic engineering

In contrast to resistance engineering and molecular farming, massive overexpression of the enzyme of interest is usually not necessary for metabolic pathway engineering (and, in some cases, can even be undesirable).

In chloroplast transformation, the desired expression levels can be adjusted by choosing appropriate combinations of plastid expression signals (e.g. promoters, Shine–Dalgarno sequences, 3' untranslated regions), but this is often a trial-and-error process and requires multiple rounds of construct optimization. This is because, although the strength of the different expression signals has been well defined in reporter gene studies (e.g. [15]), the sequence of the coding region also has a strong influence on foreign protein accumulation. This might be due to differences in translation rates (e.g. owing to aberrant mRNA secondary structure formation) and/or differences in protein stability, which sometimes severely limits protein accumulation in chloroplasts [16] and thus makes transgene expression levels to a certain extent unpredictable. Nonetheless, plastid genome engineering represents an attractive alternative to conventional nuclear transgene expression for metabolic engineering, mainly because of the greatly increased transgene containment and the possibility to stack several transgenes by linking them in operons. The latter feature is of particular relevance when several enzymatic steps are to be engineered or the introduction of entire pathways is attempted.

The plastid harbors a huge number of metabolic pathways and, for this reason, is also commonly referred to as the 'biosynthetic centre of the plant cell'. In view of its outstanding importance and the fact that many of its components are plastid encoded, photosynthesis is an obvious candidate pathway for metabolic engineering. As the efficiency of carbon fixation is the factor that usually limits plant primary production, ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and, in particular, its large chloroplast-encoded catalytic subunit represents a prime target of attempts to engineer photosynthesis. Over the past few years, extensive work has been carried out to engineer Rubisco to alter its enzymatic properties. Although the ultimate goal of improving the performance of Rubisco still seems a long way off, this research has provided novel insights into structure–function relationships and greatly enhanced our understanding of this key enzyme for biomass production on our planet. Much of this work has been covered by recently published comprehensive review articles [17,18] — the interested reader is referred to them.

The most complex metabolic pathway to be introduced into the plastid genome so far is that for the synthesis of the bioplastic polyhydroxybutyrate (PHB). PHB is a biodegradable polyester synthesized by various bacteria, presumably as a storage compound. In the eubacterium *Ralstonia eutropha*, PHB biosynthesis initiates from acetyl-CoA and involves three enzymes: β -ketothiolase (encoded by the *phbA* gene) which condenses two acetyl-CoA molecules to form acetoacetyl-CoA; acetoacetyl-CoA reductase (encoded by the *phbB* gene) which reduces acetoacetyl-CoA to β -hydroxybutyryl-CoA; and PHB

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synthase (encoded by the *phbC* gene) which polymerizes the β -hydroxybutyryl-CoA monomers to form PHB. In *Ralstonia*, the three genes are co-transcribed from an operon. After fusing the operon to a chloroplast promoter and 3' untranslated region, it was integrated into the tobacco plastid genome [19,20]. Although PHB synthesis was obtained (up to 1.7% in dry weight), significant accumulation of PHB in chloroplasts appeared to cause male sterility and severe growth retardation [20]. A recent study has provided evidence that β -ketothiolase expression is responsible for the male sterility of the transplastomic plants [21]. The deleterious effects of *phb* operon expression in plastids could be largely circumvented by placing the operon under the control of a nuclear-encoded ethanol-inducible T7 RNA polymerase that was targeted to plastids. The plastid *phb* operon was driven by a T7 promoter so that spraying of the doubly transgenic plants with ethanol induced expression of the T7 RNA polymerase in the nucleus, which in turn switched on transcription of the *phb* operon in plastids [22^{••}]. This improved inducible PHB production system shows promise, but further optimization will be required to achieve accumulation levels that are sufficiently high to make plants an economically competitive production platform for biopolymers.

Until very recently, the application of plastid transformation to metabolic pathway engineering was restricted to the model species tobacco, a non-food plant the plastids of which can be transformed relatively easily. Success with future applications in food crops will be crucially dependent on efficient transgene expression in edible plant organs, like fruits and tubers, which are often non-green and contain plastid types (amyloplasts and chromoplasts) that are generally believed to be much less active in gene expression than chloroplasts in photosynthetically active tissues. To explore the possibility of engineering a nutritionally important metabolic pathway in non-green plastids, a recent study used plastid transformation in tomato [23] to alter carotenoid biosynthesis towards producing fruits with elevated contents of provitamin A (β -carotene), an important antioxidant and essential vitamin for human nutrition [24^{••}]. Although the expression of fungal carotenoid biosynthesis genes was unsuccessful, plastid expression of a bacterial lycopene β -cyclase gene (from *Erwinia herbicola*) triggered conversion of lycopene, the main storage carotenoid of tomato fruits, to β -carotene and resulted in a fourfold enhanced provitamin A content of the fruits [24^{••}]. This study demonstrates the feasibility of metabolic pathway engineering through plastid transformation in non-green plant organs. However, a systematic analysis of gene expression in such non-green plastid types is urgently needed to identify expression elements (e.g. promoters, 5' and 3' untranslated regions) that are particularly suitable to trigger high-level plastid transgene expression in non-leafy tissues.

Molecular farming

There is significant interest in developing transgenic plants as expression factories for biopharmaceuticals, an area commonly referred to as molecular farming [25,26]. Low production and delivery costs, easy scale-up and high safety standards (owing to low risk of product contamination with human pathogens and/or endotoxins) are considered major attractions of using plants as production platforms for pharmaceuticals.

In view of the high capacity of the chloroplast to express and accumulate foreign proteins, it seems particularly attractive to exploit plastids for the production of proteinaceous pharmaceuticals, such as antigens, antibodies and antimicrobials. Recently, several promising steps have been taken in this direction. So far, most efforts have been focused on the high-level production of antigens for use as vaccines and their tests for immunological efficacy in animal studies.

The first chloroplast-produced antigen that proved to be immunologically active in experimental animals was a candidate subunit vaccine against *Clostridium tetani*, the causal agent of tetanus [27]. In a proof-of-principle study, fragment C of the tetanus toxin (TetC), a non-toxic protein fragment that was already known to be a good antigen, was expressed from the tobacco chloroplast genome [27]. Extraordinarily high levels of antigen protein were obtained: 10% of TSP with a GC-rich version of the *tetC* coding region and even 25% with an AT-rich version of the gene that more closely corresponded to the codon usage in higher plant chloroplasts. Importantly, mucosal immunization of mice with the chloroplast-derived TetC antigen (applied nasally or orally) induced protective levels of anti-TetC antibodies and protected the animals from a subsequent lethal tetanus toxin challenge.

Similarly promising progress has been made with developing a chloroplast-based vaccine for anthrax, an acute infectious disease caused by the spore-forming bacterium *Bacillus anthracis*. The plasmid-borne *pagA* gene of virulent *B. anthracis* strains encodes the so-called protective antigen (PA), a strongly immunogenic protein that forms part of the toxin cocktail produced by the bacterium. PA alone is non-toxic, however, making it an excellent candidate for an anthrax vaccine. Expression of the *pagA* gene from the tobacco chloroplast genome gave rise to stable antigen protein [28,29[•]]. To simplify antigen purification from the plant and, in this way, facilitate downstream processing, a His-tagged version of PA was also expressed [29[•]]. Maximum expression levels were in the same range as for TetC (14% of TSP) and, when combined with another component of the toxin cocktail (the so-called 'lethal factor' LF), the chloroplast-derived PA was equally effective in cytotoxicity assays as the bacterial protein produced in *B. anthracis*. Subcutaneous administration of partially purified PA from tobacco chloroplasts

elicited an immune response that was sufficiently strong to protect mice from a subsequent challenge with a lethal dose of anthrax toxin [29[•]].

Several subunit vaccines require post-translational modifications to be immunogenic. An example is the outer surface lipoprotein A (OspA) from the pathogenic bacterium *Borrelia burgdorferi*, which is used as a Lyme disease vaccine. In the bacterium, the OspA protein undergoes N-terminal lipidation, a modification that is essential for the protein to be antigenic [30^{••}]. Conventional wisdom used to say that chloroplasts are incapable of conducting such modifications, mainly because no endogenous plastid proteins carrying them had been identified. However, when OspA was expressed from the tobacco plastid genome, the protein was found to be lipidated, although the chemical structure of the modification might be slightly different from that in the bacterium [30^{••}]. Moreover, subcutaneous injection of the chloroplast-produced OspA (together with an adjuvant) in mice induced protective antibodies at levels that, according to previous immunological studies, should be sufficient to protect the animals from a challenge with *B. burgdorferi* [30^{••}].

In addition to the three examples discussed here, high-level antigen production in transgenic tobacco chloroplasts has been demonstrated for a variety of other antigens, however, immunological data have not yet been obtained for most of these.

In many cases, the development of efficacious edible vaccines is likely to require adjuvants as immunostimulatory agents. Cholera toxin acts as a strong mucosal adjuvant and its non-toxic B subunit (CTB) has proven to exert an immunostimulatory effect when fused to antigens for mucosal immunization [31]. It is therefore encouraging that CTB can be expressed to high levels in tobacco chloroplasts both alone [32] and as a fusion protein (demonstrated for a fusion to the N terminus of green fluorescent protein [33]). Future efforts to produce vaccines in transgenic chloroplasts will certainly take advantage of the high stability of CTB in chloroplasts, although it remains to be seen whether CTB fusions with proteins that are less stable than GFP (which itself accumulates to enormous levels when expressed from the chloroplast genome [34]) will also give high-level protein accumulation.

Although the high expression levels attainable using the transplastomic technology are vital to the economical production of vaccine proteins in plants, the future commercial success of plant-based vaccines it by no means guaranteed. Many conventional vaccines can be produced already at very low costs and, therefore, the attractions of the plant production system will have to be compelling. This clearly would be the case if antigen-producing plants can be developed into edible vaccines, a concept that has

been validated for some antigens in experimental animals [35], although its applicability to humans still remains to be proven.

Conclusions

Clearly, plastid genetic engineering holds great promise for plant biotechnology; however, formidable challenges remain to be addressed before the technology can reach its full potential. Of utmost importance is the need to extend the crop range of plastid transformation. Although recent progress has been made in developing plastid transformation for several important crops (e.g. [23,36,37]), there are still no workable protocols for cereal species — which include the world's main staple foods. Most probably, it will take significant investments in the optimization of the currently available tissue culture, regeneration and selection protocols before plastid transformation in monocots will become a reality.

Another area where more research needs to be undertaken is in the regulation of gene expression in non-green plastids. To date, the vast majority of studies using transplastomic technology have been conducted in tobacco, largely limiting the analysis of transgene expression to leaf chloroplasts. If the technology is to become widely applicable in food crops, however, we will need a much better understanding of plastid gene expression and its control in non-green plastid types as found in most fruits, tubers and seeds. It is well established that the activity of plastid gene expression is generally lower in non-photosynthetic tissues, but unfortunately we know very little about the bottlenecks that might limit foreign protein accumulation in non-green plastids, such as amyloplasts and chromoplasts. The need to identify plastid expression signals (e.g. promoters, 5' and 3' untranslated regions; Figure 1) suitable to direct efficient transgene expression in non-green tissues and organs therefore represents a vital task.

Finally, there is a clear need to develop tightly controllable systems for transgene expression from the chloroplast genome. For many applications, it is highly desirable to limit transgene expression to certain tissues, organs and/or developmental stages or to be able to induce expression at will by exogenously applying a stimulus. The latter is of pivotal importance to molecular farming for two reasons. First, high-level expression of foreign proteins may have deleterious phenotypic effects [27,38] and/or impose a significant metabolic burden on the plant. Second, if carried out in food crops, inadvertent contamination of the food chain with the plant-produced chemical or pharmaceutical must be prevented; this could be most effectively accomplished by making transgene expression strictly dependent on a chemical inducer. Although such systems are already well established for nuclear transgenes [39], their development for plastid transgenes is still in its infancy. Most existing systems rely on nuclear transgenes,

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usually a T7 RNA polymerase targeted to the chloroplast where it drives expression of a transgene placed under the control of a T7 promoter [22^{**},38]. More recently, a Lac repressor-based IPTG-inducible expression system for plastids has been reported, although transgene repression in the uninduced state was incomplete [40]. Thus, the need to devise chloroplast-only inducible expression systems that do not require nuclear transgenes, but which are tightly controllable, represents one of the major frontiers in plastid biotechnology.

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