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Plastid transformation and its application in metabolic engineering

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Metabolic pathway engineering by transgene expression from the plastid (chloroplast) genome offers significant attractions, including straightforward multigene engineering by pathway expression from operons, high transgene expression levels, and increased transgene containment due to maternal inheritance of plastids in most crops. In addition, it provides direct access to the large and diverse metabolite pools in chloroplasts and non-green plastid types. Here, we review recent progress with extending the toolbox for plastid engineering and highlight selected applications in the area of metabolic engineering, including the combined engineering of nuclear and plastid genomes for the production of artemisinic acid, the direct harness of chloroplast reducing power for the synthesis of dhurrin and the use of an edible host for the production of astaxanthin.

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Introduction

Plastids (chloroplasts), the organelles that define a plant cell, originated more than 1.2 billion years ago when an ancient eukaryotic cell engulfed a photosynthetic bacterium [1]. The endosymbiont then evolved into a semi-contained, semi-autonomous organelle that constitutes a hotspot of cellular metabolic activities. The emerging field of plastid metabolic engineering exploits these features, coupling the natural propensities of the plastid with the introduction of novel metabolic pathways via transformation of the plastid genome (plastome), to facilitate the production of valuable metabolites.

Stable transformation of the plastome was first achieved for the unicellular alga *Chlamydomonas reinhardtii* in 1988, and two years later for the dicotyledonous seed plant tobacco (*Nicotiana tabacum*) [2,3]. Since then, plastid transformation has become increasingly relevant in the field of biotechnology due to the various characteristics that concede advantages to this technology over nuclear transformation. Firstly, while DNA integration in the nucleus is a random process, plastid transformation involves targeted integration by homologous recombination, allowing precise transgene placement and avoiding position effects including interference with endogenous genes. Secondly, due to their bacterial origin, genes in the plastid are transcribed from operons, a feature simplifying the design of the transformation constructs by allowing transgene stacking. Third, transplastomic (plastid-transformed) plants contain transgenes integrated not only into one or two copies of a genome per cell, as with the nucleus, but rather in the approximately 50 copies of the plastid genome in each of the approximately 100 chloroplasts in a cell [4]. Coupled with a fourth and fifth benefit — the innate potential for expression of massive quantities of genes products, and the practical absence of gene silencing or epigenetic modifications [5••] — the accumulation of proteins expressed from the plastome can be up to 10–100 times higher than that of proteins expressed from the nuclear genome [6], and amount up to 70% of the plant's total soluble protein (TSP) [7]. Finally, chloroplasts represent a semi-closed environment with specific biochemical properties. Although it is clear that many metabolites are exchanged with the cytosol, the genetic material and the expressed proteins are for the most part contained, localizing the desired reactions to the plastid compartment and, due to the maternal inheritance of plastids in most crop species, excluding the transmission of transgenes by the pollen.

Recent improvements in the transplastomic technology

As the fundamentals of plastid transformation and the toolbox available for this technology have been extensively reviewed [5••,8,9], we will briefly discuss here only two new tools that were developed most recently.

A new selection system

Plastid transformation requires a selectable marker for the identification of cells with transformed plastids, and the limited availability of reproducible and effective selection systems stands as a major barrier to both super-transformation experiments (i.e. the transformation of an already transplastomic plant) and the expansion of plastid transformation to novel species. Recently, a new selection

system based on the bifunctional enzyme aminoglycoside acetyltransferase(6′)-Ie/aminoglycoside phosphotransferase(2″)-Ia (AAC(6′)-Ie/APH(2″)-Ia) and the antibiotic tobramycin was developed for tobacco [10•], and shown to be equally effective as the currently nearly exclusively used spectinomycin selection system based on the *aadA* gene [11]. It has been speculated by the authors that this new marker gene, which could also be used in combination with other antibiotics, may facilitate development of plastid transformation technology for recalcitrant species, such as monocots, which show natural resistance to spectinomycin [12].

Inducible expression of plastid transgenes

The high innate expression potential of the chloroplast is a double-edged sword: excessive accumulation of foreign proteins can exhaust the gene expression machinery of the plants, drain metabolite precursors, disrupt membrane structures or have toxic effects on the plastid [7,13,14]. To overcome these effects, Verhounig *et al.* [15] had developed a synthetic inducible riboswitch system for the chloroplast, in which application of the metabolite theophylline induces translation by resolving an inhibitory mRNA secondary structure that sequesters the Shine-Dalgarno sequence. The low efficiency of this system was recently improved by the so-called RAmpER (RNA amplification-enhanced riboswitch) method, in which the gene of interest is placed under the control of the T7 promoter, and a T7 RNA polymerase transgene controlled by the synthetic theophylline-responsive riboswitch is additionally incorporated into the plastome [16••]. So far, this system has only been used for the production of recombinant reporter proteins and a pharmaceutical protein, but it holds great promise also for the regulated expression of metabolic pathways in the chloroplast.

Metabolic engineering in transplastomic plants

Plants synthesize a wide range of structurally complex metabolites, including many utilized in human medicine: 25% of the drugs prescribed worldwide are of plant origin [17]. While chloroplasts are primarily recognized as the site of photosynthesis, both photosynthetic and non-photosynthetic plastids act as sites for a wide range of metabolic activities and are involved in massive metabolic flux [18]. For example, the plastid glutamine synthetase/glutamate synthase (GS/GOGAT) pathway is the dominant site of the integration of reduced nitrogen into biomolecules [19], and up to 20% of the plant's fixed carbon enters the plastid shikimate pathway for synthesis of aromatic amino acids, and precursors for pigments, UV protectants, defense compounds and lignin [20].

Interestingly, while certain metabolic pathways are found only or nearly entirely in the plastid, such as the *de novo* synthesis of fatty acids or tetrapyrrole synthesis [21,22], others, like the isoprenoid pathway, occur in multiple compartments. Isopentenyl pyrophosphate (IPP), the isoprenoid building block required for the synthesis of quinones, pigments and several phytohormones (Figure 1), can be synthesised by either the cytosolic mevalonic acid (MVA) pathway or the plastid 2-C-methyl-erythritol 4-phosphate (MEP) pathway [23], and is known to be exchanged between plastids and the cytosol [24]. This partial redundancy suggests that certain metabolic pathways in the plastid may be re-directed to produce novel metabolites with minimal impact on plant development (Figure 1).

Together is better: nuclear and chloroplast transformation for the production of an antimalarial drug

Artemisinin, the mainstay drug for the treatment of malaria [25], is naturally synthesized only in small amounts in the glandular trichomes of the herb *Artemisia annua* [26]. Due to its structural complexity, artemisinin is difficult and costly to synthesize chemically. A recently developed strategy [27••] demonstrated high-level production of its immediate precursor, artemisinic acid, by the transformation of both the plastome and the nuclear genome of the high-biomass crop tobacco. Transplastomic lines expressing the core biosynthetic pathway of artemisinic acid from two synthetic operons accumulated only low levels of the metabolite. Combinatorial transformation, a large-scale co-transformation approach [28], was then used to supertransform these lines, introducing into the nucleus five additional genes whose enzyme products had yet undefined function and dosage requirements in plants (Figure 1). This approach, termed COSTREL (combinatorial supertransformation of transplastomic recipient lines), generated a high number of lines differing in transgene integration patterns, transgene dosages and expression strengths. Their screening for accumulation of the metabolite product revealed an up to 77-fold increase in artemisinic acid, and allowed identification of the ALDH1 enzyme as a key contributor to product yield increase (Figure 1). These results suggest COSTREL as a promising strategy for simultaneously optimizing metabolite production, and clarifying the role of unknown genetic factors and their contribution to flux through a given pathway. The work also demonstrated that an entire pathway in secondary metabolism can be transferred from a medicinal plant to a high-biomass crop, and, moreover, can be relocated from the cytosol of specialized cells to the chloroplasts of all leaf cells.

Harnessing light to power metabolite production: synthesis of the defense compound dhurrin

Dhurrin is a cyanogenic glucoside, naturally produced as a defense metabolite against herbivores in sorghum (*Sorghum bicolor*) by the action of two cytochrome P450 monooxygenases (CYP79A1 and CYP71E1) and a glucosyltransferase (UGT85B1; Figure 1). The two monooxygenases are membrane bound to the ER, and their