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EMERGING TOOLS FOR SYNTHETIC BIOLOGY IN PLANTS

Synthetic biology in plastids

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SUMMARY

Plastids (chloroplasts) harbor a small gene-dense genome that is amenable to genetic manipulation by transformation. During 1 billion years of evolution from the cyanobacterial endosymbiont to present-day chloroplasts, the plastid genome has undergone a dramatic size reduction, mainly as a result of gene losses and the large-scale transfer of genes to the nuclear genome. Thus the plastid genome can be regarded as a naturally evolved miniature genome, the gradual size reduction and compaction of which has provided a blueprint for the design of minimum genomes. Furthermore, because of the largely prokaryotic genome structure and gene expression machinery, the high transgene expression levels attainable in transgenic chloroplasts and the very low production costs in plant systems, the chloroplast lends itself to synthetic biology applications that are directed towards the efficient synthesis of green chemicals, biopharmaceuticals and other metabolites of commercial interest. This review describes recent progress with the engineering of plastid genomes with large constructs of foreign or synthetic DNA, and highlights the potential of the chloroplast as a model system in bottom-up and top-down synthetic biology approaches.

Keywords: chloroplast, plastid transformation, reverse genetics, synthetic biology, synthetic genomics, metabolic engineering.

INTRODUCTION

All plastids (chloroplasts) go back to a single primary endosymbiosis event that occurred more than a billion years ago through the uptake of a cyanobacterium by a respiring (mitochondria-possessing) protist-like eukaryote (Gray, 1993). Although the exact phylogenetic relationship of the endosymbiont with present-day cyanobacteria is not yet fully resolved, it appears clear that the information content of the genome of the cyanobacterial endosymbiont was not much smaller than that of extant cyanobacteria, in that it harbored at least a few thousand genes (Race *et al.*, 1999; Martin *et al.*, 2002). However, during conversion of the cyanobacterial endosymbiont into a cell organelle, a dramatic genome shrinkage occurred that encompasses approximately two orders of magnitude: a cyanobacterial genome in the low megabase pair range was reduced to a chloroplast genome of 120–250 kilobase pairs. Three processes have contributed to genome shrinkage: (i) the loss of genetic information that became dispensable after the switch from a free-living to an endosymbiotic lifestyle (e.g. genes involved in bacterial cell wall biosynthesis); (ii) the loss of redundant genetic information (e.g. genes for biochemical pathways that were already encoded by the nuclear or mitochondrial genomes of the host cell); and (iii) the massive transfer of genes from the genome of the endosymbiont to the nuclear genome of its host (Timmis *et al.*, 2004; Bock and Timmis, 2008). It has been estimated that up to 4500 nuclear genes of the model plant *Arabidopsis thaliana* are of cyanobacterial origin, which equals approximately 18% of the gene content of the nuclear genome (Martin *et al.*, 2002). What has driven all these genes out of the plastid into the nucleus is not fully understood, but participation in sexual recombination (from which the usually maternally inherited plastids are largely excluded) appears to be one of the forces involved (Blanchard and Lynch, 2000; Khakhlova and Bock, 2006). As the result of genome streamlining, present-day plastid genomes (plastomes) are small and, in seed plants, contain only 120–130 genes (Figure 1; Table 1). This low coding capacity is unlikely to represent the end point of plastid genome evolution. Both phylogenetic (Millen *et al.*, 2001; Rousseau-Gueutin *et al.*, 2013) and experimental evolutionary evidence (Huang *et al.*, 2003; Stegemann *et al.*, 2003; Stegemann and Bock, 2006; Fuentes *et al.*, 2012) indicate that gene transfer to the nucleus is a still an ongoing pro-

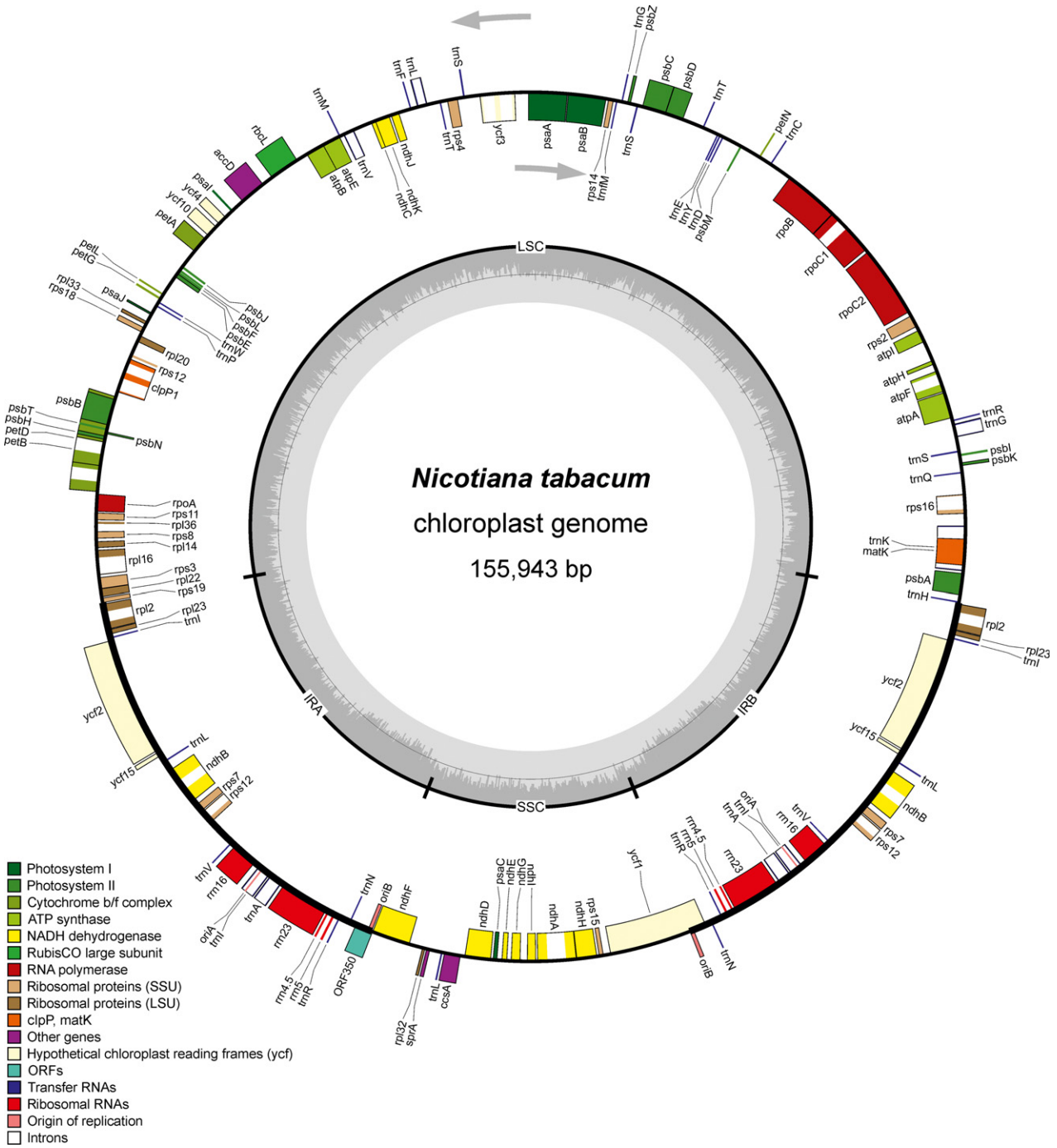

Figure 1. Physical map of the *Nicotiana tabacum* (tobacco) plastid genome. The map was drawn with OrganellarGenomeDRAW (OGDRAW) software (Lohse *et al.*, 2007, 2013) using the complete genome sequence as input (Shinozaki *et al.*, 1986; GenBank accession number Z00044.2). The grey arrows indicate the direction of transcription for the two DNA strands. The inner ring represents the GC content graph, and the circle inside marks the 50% threshold. Abbreviation: LSC, large single-copy region; IRA, inverted repeat A; IRB, inverted repeat B; SSC, small single-copy region.

cess, suggesting that further size reductions are likely to occur in the future, at least on large evolutionary timescales. In addition to genome reduction by gene transfer, there also appears to be selective pressure that keeps intergenic spacers and regulatory elements (promoters, 5′ and 3′ untranslated regions, UTRs) as small as possible. The nature of this pressure is unclear, especially as it does not seem to act on plant mitochondrial genomes and not even on all plastid genomes (Knoop, 2004; Kubo and Mikami, 2007; Bock and Knoop, 2012). Whereas the plastid genomes of seed plants are highly gene dense, at least some algae accumulate significant quantities of presum-

Table 1 Plastid-encoded genes and conserved open reading frames (*ycf*, h*y*pothetical *c*hloroplast reading *f*rame) in seed plants, and their essentiality under heterotrophic and photoautotrophic conditions

Gene	Gene product	Essential for heterotrophic growth	Essential for autotrophic growth	References
psaA	A subunit of PSI	–	+	Redding *et al.* (1999); *C.r.*
psaB	B subunit of PSI	–	+	Bock *et al.* (1999)
psaC	C subunit of PSI	–	+	Redding *et al.* (1999); *C.r.*
psaI	I subunit of PSI	–	–	Schöttler *et al.* (2011)
psaJ	J subunit of PSI	–	–	Schöttler *et al.* (2007)
ycf3	Ycf3 protein, PSI assembly	–	+	Ruf *et al.* (1997)
ycf4	Ycf4 protein, PSI assembly	–	–/+	Krech *et al.* (2012)
psbA	D1 protein of PSII	–	+	Baena-González *et al.* (2003)
psbB	CP47 subunit of PSII	–	+	
psbC	CP43 subunit of PSII	–	+	Rochaix *et al.* (1989); *C.r.*
psbD	D2 protein of PSII	–	+	Erickson *et al.* (1986); *C.r.*
psbE	α-subunit of cytochrome b_{559}	–	+	Swiatek *et al.* (2003a)
psbF	β-subunit of cytochrome b_{559}	–	+	Swiatek *et al.* (2003a)
psbH	H subunit of PSII	–	+	Erickson *et al.* (1986); *C.r.*
psbI	I subunit of PSII	–	–	Schwenkert *et al.* (2006)
psbJ	J subunit of PSII	–	+	Hager *et al.* (2002)
psbK	K subunit of PSII	–	+	Takahashi *et al.* (1994); *C.r.*
psbL	L subunit of PSII	–	+	Swiatek *et al.* (2003a)
psbM	M subunit of PSII	–	–	Umate *et al.* (2007)
psbN/*pbf1*	Photosystem biogenesis	–	–/+	Krech *et al.* (2013)
psbT	T subunit of PSII	–	–	Umate *et al.* (2008)
psbZ	Z subunit of PSII	–	–	Ruf *et al.* (2000), Swiatek *et al.* (2001)
petA	Cytochrome *f*	–	+	Monde *et al.* (2000)
petB	Cytochrome b_6	–	+	Monde *et al.* (2000)
petD	Subunit IV of cyt b_6f	–	+	Monde *et al.* (2000)
petG	G subunit of cyt b_6f	–	+	Schwenkert *et al.* (2007)
petL	L subunit of cyt b_6f	–	–	Fiebig *et al.* (2004)
petN	N subunit of cyt b_6f	–	+	Hager *et al.* (1999)
atpA	ATP synthase α-subunit	–	+	Drapier *et al.* (1998); *C.r.*
atpB	ATP synthase β-subunit	–	+	Karcher and Bock (2002)
atpE	ATP synthase ε-subunit	–	+	Karcher and Bock (2002)
atpF	ATP synthase b-subunit	–	+	
atpH	ATP synthase c-subunit	–	+	Unpublished
atpI	ATP synthase a-subunit	–	+	
ndhA	A subunit of NAD(P)H dehydrogenase	–	–	Kofer *et al.* (1998), Maliga and Nixon (1998)
ndhB	B subunit of NAD(P)H dehydrogenase	–	–	Shikanai *et al.* (1998)
ndhC	C subunit of NAD(P)H dehydrogenase	–	–	Burrows *et al.* (1998)
ndhD	D subunit of NAD(P)H dehydrogenase	–	–	Unpublished
ndhE	E subunit of NAD(P)H dehydrogenase	–	–	
ndhF	F subunit of NAD(P)H dehydrogenase	–	–	Martin *et al.* (2004)
ndhG	G subunit of NAD(P)H dehydrogenase	–	–	
ndhH	H subunit of NAD(P)H dehydrogenase	–	–	Kofer *et al.* (1998), Maliga and Nixon (1998)
ndhI	I subunit of NAD(P)H dehydrogenase	–	–	Kofer *et al.* (1998), Maliga and Nixon (1998)

Table 1. Continued

Gene	Gene product	Essential for heterotrophic growth	Essential for autotrophic growth	References
ndhJ	J subunit of NAD(P)H dehydrogenase	–	–	Burrows *et al.* (1998)
ndhK	K subunit of NAD(P)H dehydrogenase	–	–	Burrows *et al.* (1998)
rbcL	Rubisco large subunit	–	+	Kanevski and Maliga (1994)
rpoA	RNA polymerase α-subunit	–	+	Serino and Maliga (1998)
rpoB	RNA polymerase β-subunit	–	+	Allison *et al.* (1996)
rpoC1	RNA polymerase β′-subunit	–	+	Serino and Maliga (1998)
rpoC2	RNA polymerase β″-subunit	–	+	Serino and Maliga (1998)
matK	Splicing factor (group–II intron maturase)	+	+	Zoschke *et al.* (2010)
rrn16	*16S* ribosomal RNA	+	+	Unpublished
rrn23	*23S* ribosomal RNA	+	+	Unpublished
rrn5	*23S* ribosomal RNA	+	+	Unpublished
rrn4.5	*23S* ribosomal RNA	+	+	Unpublished
trnA-UGC	tRNA-Alanine(UGC)	+	+	Alkatib *et al.* (2012a)
trnC-GCA	tRNA-Cysteine(GCA)	+	+	Legen *et al.* (2007)
trnD-GUC	tRNA-Aspartate(GUC)	+	+	
trnE-UUC	tRNA-Glutamate(UUC)	+	+	
trnF-GAA	tRNA-Phenylalanine(GAA)	+	+	
trnG-GCC	tRNA-Glycine(GCC)	–	–/+	Rogalski *et al.* (2008b)
trnG-UCC	tRNA-Glycine(UCC)	+	+	Rogalski *et al.* (2008b)
trnH-GUG	tRNA-Histidine(GUG)	+	+	
trnI-CAU	tRNA-Isoleucine(CAU)	+	+	Alkatib *et al.* (2012b)
trnI-GAU	tRNA-Isoleucine (GAU)	+	+	Alkatib *et al.* (2012b)
trnK-UUU	tRNA-Lysine (UUU)	+	+	
trnL-CAA	tRNA-Leucine (CAA)	–	–	Alkatib *et al.* (2012a)
trnL-UAA	tRNA-Leucine (UAA)	+	+	Alkatib *et al.* (2012a)
trnL-UAG	tRNA-Leucine (UAG)	+	+	Alkatib *et al.* (2012a)
trnM-CAU	tRNA-Methionine (CAU)	+	+	Alkatib *et al.* (2012b)
trnfM-CAU	tRNA-*N*-formyl-methionine (CAU)	+	+	Alkatib *et al.* (2012b)
trnN-GUU	tRNA-Asparagine (GUU)	+	+	Legen *et al.* (2007)
trnP-UGG	tRNA-Proline (UGG)	+	+	
trnQ-UUG	tRNA-Glutamine (UUG)	+	+	
trnR-ACG	tRNA-Arginine (ACG)	+	+	Alkatib *et al.* (2012a)
trnR-UCU	tRNA-Arginine (UCU)	+	+	Alkatib *et al.* (2012a)
trnS-GCU	tRNA-Serine (GCU)	+	+	Alkatib *et al.* (2012a)
trnS-GGA	tRNA-Serine (GGA)	–	–	Alkatib *et al.* (2012a)
trnS-UGA	tRNA-Serine (UGA)	+	+	Alkatib *et al.* (2012a)
trnT-GGU	tRNA-Threonine (GGU)	–	–/+	Alkatib *et al.* (2012a)
trnT-UGU	tRNA-Threonine (UGU)	+	+	Alkatib *et al.* (2012a)
trnV-GAC	tRNA-Valine (GAC)	–	–	Alkatib *et al.* (2012a)
trnV-UAC	tRNA-Valine (UAC)	+	+	Alkatib *et al.* (2012a)
trnW-CCA	tRNA-Tryptophan (CCA)	+	+	
trnY-GUA	tRNA-Tyrosine (GUA)	+	+	
rps2	Ribosomal protein S2	+	+	Rogalski *et al.* (2008a)
rps3	Ribosomal protein S3	+	+	Fleischmann *et al.* (2011)
rps4	Ribosomal protein S4	+	+	Rogalski *et al.* (2008a)
rps7	Ribosomal protein S7	+	+	
rps8	Ribosomal protein S8	+	+	
rps11	Ribosomal protein S11	+	+	
rps12	Ribosomal protein S12	+	+	
rps14	Ribosomal protein S14	+	+	Ahlert *et al.* (2003)
rps15	Ribosomal protein S15	–	–	Fleischmann *et al.* (2011)
rps16	Ribosomal protein S16	+	+	Fleischmann *et al.* (2011)
rps18	Ribosomal protein S18	+	+	Rogalski *et al.* (2006)

Table 1. Continued

Gene	Gene product	Essential for heterotrophic growth	Essential for autotrophic growth	References
rps19	Ribosomal protein S19	+	+	
rpl2	Ribosomal protein L2	+	+	
rpl14	Ribosomal protein L14	+	+	
rpl16	Ribosomal protein L16	+	+	
rpl20	Ribosomal protein L20	+	+	Rogalski *et al.* (2008a)
rpl22	Ribosomal protein L22	+	+	Fleischmann *et al.* (2011)
rpl23	Ribosomal protein L23	+	+	Fleischmann *et al.* (2011)
rpl32	Ribosomal protein L32	+	+	Fleischmann *et al.* (2011)
rpl33	Ribosomal protein L33	–	–	Rogalski *et al.* (2008a)
rpl36	Ribosomal protein L36	–	–/+	Fleischmann *et al.* (2011)
clpP	Catalytic subunit of the protease Clp	+	+	Shikanai *et al.* (2001)
accD	Acetyl-CoA carboxylase subunit	+	+	Kode *et al.* (2005)
ycf5/*ccsA*	Subunit A of the system–II complex for c-type cytochrome biogenesis	–	+	Xie and Merchant (1996); *C.r.*
ycf10/*cemA*	Inner envelope protein	–	–	Swiatek *et al.* (2003b)
ycf1/*tic214*	214–kDa protein of Tic complex	+	+	Drescher *et al.* (2000), Kikuchi *et al.* (2013)
ycf2	Putative Ycf2 protein	+	+	Drescher *et al.* (2000)
ycf15	Unknown	–	–	Unpublished
sprA	Small non–coding RNA	–	–	Sugita *et al.* (1997)

–/+, borderline case (survival under carefully controlled conditions, but severe mutant phenotype); cyt b_6f, cytochrome b_6f complex; *C.r.*, data available for the green alga *Chlamydomonas reinhardtii*, but not for seed plants; PSI, photosystem I; PSII, photosystem II; unpublished, our unpublished data.
If no reference is given, no experimental data were available, and information on essentiality/non-essentiality is based on reasonable assumptions.

ably non-coding DNA (Maul *et al.*, 2002). With the huge body of knowledge on plastid evolution in nearly all lineages of plants and eukaryotic algae accumulated over the past 30 years, plastids provide a paradigm for the evolution and the design of minimum genomes, in that useful principles for genome streamlining can be derived from studying the reductive evolution of plastid genomes.

Another aspect that makes plastids a particularly attractive target of synthetic biology in plants is the relative ease with which their genomes can be manipulated, at least in the two best-established model systems for plastid transformation: the unicellular green alga *Chlamydomonas reinhardtii* and the seed plant *Nicotiana tabacum* (tobacco). A large toolbox for plastid genetic engineering has been assembled over the years (Day and Goldschmidt-Clermont, 2011; Maliga and Bock, 2011; Bock, 2013, 2014), and the highly efficient homologous recombination machinery present in plastids facilitates both the targeted integration of (large pieces of) foreign DNA and the precise exchange of plastid DNA with modified (mutated) sequences.

Here we review the state of the art in plastid genome engineering, and the salient features of the plastid genome that promise to make the chloroplast an attractive target of synthetic biology in plants. We focus on two areas in which we feel plastids can make particularly valuable contributions: synthetic genomics and the implementation of novel biochemical pathways in plants.

STRUCTURE, FUNCTION AND EXPRESSION OF PLASTID GENOMES

The structure of a typical chloroplast genome of a vascular plant is shown in Figure 1. The plastid DNA maps as a circular genome, although *in vivo* the plastid genome population seems to be structurally quite heterogeneous. In addition to simple circles, multimeric forms (presumably resulting from homologous recombination between genome copies) and linear genome conformations have been identified (Lilly *et al.*, 2001; Scharff and Koop, 2006; Day and Madesis, 2007). The genome occurs at high copy numbers, reaching more than 1000 copies per cell in leaves of the model plant *A. thaliana*, and even several thousand copies in some other species (Zoschke *et al.*, 2007; Liere and Börner, 2013). Thus, although the plastid genome is small compared with the nuclear genome, the plastid DNA can account for up to 10% of the total DNA of the plant cell (Tewari and Wildman, 1966).

In most seed plants, the plastid genome has a tripartite structure, with two inverted-repeat regions separating a

large single-copy region from a small single-copy region (Figure 1). The genome is extraordinarily compact, with intergenic regions accounting for less than one-third of the genome size (Kahlau *et al.*, 2006). Considering that these intergenic regions also contain regulatory elements for gene expression (promoters, 5′- and 3′–UTRs), the proportion of truly non-coding DNA is very small.

The products of most genes encoded in the plastid DNA function in either gene expression or photosynthesis (Shimada and Sugiura, 1991; Figure 1; Table 1). The group of photosynthesis-related genes includes genes for subunits of the thylakoidal protein complexes involved in photosynthetic electron transfer and ATP synthesis [photosystem II, cytochrome b_6f complex, photosystem I, ATP synthase, NAD(P)H dehydrogenase], a limited number of photosystem assembly factors and the large subunit of the CO_2-fixing enzyme Rubisco (Table 1). The so-called genetic system genes (Shimada and Sugiura, 1991) comprise the genes for a complete set of transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs), genes for subunits of the plastid ribosome, the core subunits of the bacterial-type plastid RNA polymerase (PEP), an intron splicing factor and a subunit of the Clp protease (Figure 1; Table 1). A small number of plastid genes do not fall into the two major gene classes and encode other functions, for example, the carboxyltransferase subunit of acetyl-CoA carboxylase (ACCase), the heteromeric enzyme complex that synthesizes malonyl-CoA as a substrate for the *de novo* biosynthesis of fatty acids.

Most of the protein products encoded by plastid genes act in multiprotein complexes and, notably, all of these protein complexes are chimeric in that they are composed of both nucleus-encoded and plastid-encoded subunits. The dual genetic origin of plastid protein complexes necessitates a tight co-ordination of plastid and nuclear gene expression, which is ensured by a multitude of anterograde (nucleus-to-plastid) and retrograde (plastid-to-nucleus) signaling and communication pathways (Bräutigam *et al.*, 2007; Barajas-López *et al.*, 2013).

In line with their cyanobacterial ancestry, plastids possess a gene expression machinery that largely resembles that of bacterial cells; however, at least in some respects, plastid gene expression has become even more complex than bacterial gene expression. For example, whereas the entire Mbp-size genome of bacteria is transcribed by a single RNA polymerase, transcription of the much smaller plastid genome is performed by two RNA polymerase activities: a plastid-encoded bacterial-type RNA polymerase and one or two (depending on the plant species) nucleus-encoded bacteriophage-type RNA polymerases (Liere and Börner, 2007). There is no strict division of labor between the two types of plastid RNA polymerases, in that they transcribe overlapping sets of genes (Zhelyazkova *et al.*, 2012). Most plastid genes are organized in polycistronic transcription units that resemble bacterial operons. Primary polycistronic transcripts undergo a complex series of RNA maturation steps, including the processing of 5′ and 3′ ends, intercistronic cleavage, intron splicing and RNA editing by C → U conversions occurring at highly specific sites (Stern *et al.*, 2010; Barkan, 2011).

Protein biosynthesis in plastids occurs on bacterial-type *70S* ribosomes. As in bacteria, translation initiates at Shine–Dalgarno sequences upstream of the start codon (usually an AUG triplet) or, alternatively, initiates in a Shine–Dalgarno-independent manner at start codons that reside in unstructured regions of the mRNA (Drechsel and Bock, 2010; Scharff *et al.*, 2011). Plastid gene expression is regulated in response to developmental and environmental cues by a complex interplay of transcriptional and post-transcriptional control, the latter being particularly important, and including regulation at the level of RNA stability as well as extensive translational regulation (Eberhard *et al.*, 2002; Kahlau and Bock, 2008; Valkov *et al.*, 2009; Barkan, 2011).

ENGINEERING OF PLASTID GENOMES

The invention of the particle gun provided a universal method for DNA delivery into living cells and subcellular compartments, including organelles. Stable transformation of the chloroplast genome by particle gun-mediated (biolistic) DNA delivery was first accomplished in the unicellular green alga *C. reinhardtii* (Boynton *et al.*, 1988), followed by success in the seed plant species *N. tabacum* (tobacco; Svab *et al.*, 1990; Svab and Maliga, 1993). For more than 20 years, these two species have remained the organisms of choice for plastid transformation, and its application in both basic research and biotechnology. Although a few important crop species can now also be transformed (Sidorov *et al.*, 1999; Ruf *et al.*, 2001; Dufourmantel *et al.*, 2004), progress with developing plastid transformation protocols for additional species has been somewhat slow, and many genetic model species and agriculturally relevant crops are still not transformable, notably including *A. thaliana* and all monocotyledonous species (Maliga, 2004; Maliga and Bock, 2011; Bock, 2014).

The integration of foreign DNA into the plastid genome occurs exclusively via homologous recombination, thus allowing very precise manipulations of the plastid genome, such as the introduction of point mutations at defined positions (Przibilla *et al.*, 1991; Bock *et al.*, 1994). The extraordinarily high activity of the homologous recombination system of plastids also facilitates the simultaneous modification of two distinct regions of the plastid genome by co-transformation experiments, in which two or more plasmid vectors are loaded on the microprojectiles for bombardment (Kindle *et al.*, 1991; Carrer and Maliga, 1995; Krech *et al.*, 2013). Amazingly, this approach can even be used to co-transform the nuclear genome (by non-homologous end

joining) and the plastid genome (by homologous recombination) in a single experiment (Elghabi *et al.*, 2011).

Over the years, plastid transformation in the two model systems, *Chlamydomonas* and tobacco, has become more and more routine, with the efficiency of plastid transformation now approaching that of nuclear transformation. This cannot be ascribed to a single methodological breakthrough, but rather is the result of many incremental improvements in the procedures involved in generating transplastomic cells and plants: the biolistic protocol, the transformation vectors, selectable markers and expression cassettes, and the tissue culture, selection and regeneration protocols. Along the way, a large toolkit for plastid genome engineering has been put together by both the tobacco and the *Chlamydomonas* communities (Maliga, 2004; Day and Goldschmidt-Clermont, 2011; Maliga and Bock, 2011; Bock, 2013, 2014). This toolkit contains, for example, various selectable marker genes, reporter genes, and promoters and untranslated regions (5′- and 3′- UTRs) that confer widely different transgene expression levels. Recently, significant progress has also been made with improving plastid transgene expression in non-green plastid types, such as amyloplasts and chromoplasts (Valkov *et al.*, 2011; Zhang *et al.*, 2012; Caroca *et al.*, 2013), and with developing methods for the inducible expression of plastid transgenes (Mühlbauer and Koop, 2005; Surzycki *et al.*, 2007; Verhounig *et al.*, 2010).

A salient feature of particle gun-mediated transformation is that DNA delivery is entirely based on a physical process. Thus, the biolistic method has no theoretical size limitation and large pieces of foreign DNA can be bombarded into the target compartment (Altpeter *et al.*, 2005). So far, DNA pieces of up to 50 kb have been incorporated into the tobacco plastid genome (Adachi *et al.*, 2007), and there is no reason to believe that much bigger pieces could not be introduced as well. Together with the small genome size (Figure 1) and the ease with which many genetic manipulations can be conducted, the capacity to accommodate large quantities of foreign DNA make the chloroplast an attractive target of synthetic biology. Based on pioneering work in microbial systems (Roodbeen and van Hest, 2009; Delaye and Moya, 2010; Cambray *et al.*, 2011), two main branches of synthetic biology have emerged. Top-down synthetic biology approaches start from an existing biological system, and aim at reducing its complexity, ideally to a minimum-size system that consists of the smallest possible number of parts. Bottom-up synthetic biology approaches start with individual parts (building blocks) and try to construct artificial biological systems from first principles. The overarching goals of both approaches are very similar: (i) to further our understanding of the genetic elements and regulatory principles underlying functional biological systems; and (ii) to design optimized biological systems for engineering applications. The latter goal brings synthetic biology in close proximity to biotechnology, and in fact many applications that nowadays come under the label of synthetic biology also could be viewed as advanced genetic engineering for biotechnological purposes (Peralta-Yahya *et al.*, 2012; Paddon *et al.*, 2013). This semantic issue notwithstanding, the amenability of plastids to large-scale genome manipulations with high precision facilitates both top-down and bottom-up approaches on the road to plant synthetic biology. In the following, the potential of plastids for synthetic biology is illustrated with two examples: (i) the design of minimum-size synthetic plastid genomes, a top-down approach; and (ii) the build-up of new metabolic pathways in plastids via multigene engineering, a bottom-up approach.

TOWARDS A MINIMUM PLASTID GENOME

Designing, synthesizing and booting-up a minimum genome has long been the Holy Grail of synthetic genomics. Recently, exciting progress has been made with the synthesis and assembly of large DNA molecules (Gibson *et al.*, 2008a,b, 2009) and their introduction into microbial cells (Lartigue *et al.*, 2007; Gibson *et al.*, 2010; Karas *et al.*, 2013); however, the rational design of a minimum-size genome and its functional transplantation into a cell have not yet been accomplished. The major hurdle on the road towards a minimum-size synthetic genome is our insufficient knowledge about the minimum gene set required for life. Unfortunately, this set cannot easily be derived from functional genomic studies that determine the essentiality of individual genes in a genome by forward or reverse genetic approaches. Although such approaches have contributed greatly to the systematic identification of essential and non-essential genes in both bacteria (Glass *et al.*, 2006) and plastids (Table 1), classifying a gene as non-essential solely based on the viability of a knock-out mutant can be dangerous, especially if no knowledge is available about the molecular functions of the gene product, its interactions with other gene products and its interconnections with the genetic, metabolic and/or regulatory processes in the cell. The combined removal of functionally connected genes (e.g. genes in which the protein products are functionally redundant or have partially overlapping functions) can result in synthetic lethality, a genetic interaction between two genes (A and B) in which the knock-out of gene A is viable and the knock-out of gene B is viable, but the combined knock-out of both genes is lethal. Identifying (or predicting) synthetically lethal interactions at the genome-wide scale represents the biggest challenge in the endeavor of designing a functional minimum genome and booting it up in a suitable cellular chassis.

Thanks to systematic reverse genetic programs conducted in both *Chlamydomonas* and tobacco, the plastid

genome is now exceptionally well characterized and, with very few exceptions, the functions of nearly all genes in the plastid genome are known (Table 1). Moreover, the small size of the plastid genome and the limited number of biological functions that plastid gene products are involved in (Figure 1; Table 1) are likely to minimize issues with synthetic lethality upon designing a minimum plastid genome. For example, it is well established that, under heterotrophic growth conditions, all photosynthesis-related gene functions are dispensable (Table 1). This conclusion is not only supported by extensive reverse genetic analyses, but also by the analysis of plastid genomes in non-photosynthetic holoparasitic plant species (dePamphilis and Palmer, 1990; Delannoy *et al.*, 2011; Figure 2). Following the acquisition of a parasitic lifestyle, these species have lost most or even all of their photosynthesis-related genes, and therefore possess vestigial plastid genomes that often are less than half the size of the genomes of their photosynthetic relatives (Figure 2). It thus appears relatively safe to eliminate all photosynthesis genes when designing a minimum plastid genome that is functional under heterotrophic growth conditions (i.e. upon growth on sucrose-containing medium in the case of tobacco or acetate-containing medium in the case of *Chlamydomonas*; Figure 3). However, whether the combined deletion of two or more photosynthesis genes, established as non-essential under autotrophic conditions (Table 1), will result in photosynthetically active plants (or leads to synthetic lethality under autotrophic growth conditions), remains to be investigated.

The situation is a bit less clear for the second major class of plastid-encoded genes: the genetic system genes. The reduced plastid genomes of parasitic plants also lost a number of genes encoding components of the gene expression machinery, including some ribosome protein genes and tRNA genes (Morden *et al.*, 1991; Wolfe *et al.*, 1992a,b; Delannoy *et al.*, 2011). However, extensive reverse genetic studies in tobacco have demonstrated that the set of genetic system genes retained in the plastid genomes of parasites does not represent the core set of essential genes. In fact, several tRNA genes and ribosomal protein genes lost from the plastid genomes of parasitic plants turned out to be essential when their deletion from the tobacco plastid genome was attempted (Rogalski *et al.*, 2008a; Fleischmann *et al.*, 2011; Alkatib *et al.*, 2012a). Conversely, a few genes retained in the plastid genomes of at least some parasitic species were found to be non-essential in tobacco. The essentiality of genetic system genes lost from the plastid genome of parasites is best explained with their functional gene transfer to the nuclear genome (Millen *et al.*, 2001; Stegemann and Bock, 2006). It seems conceivable that the accelerated speed of plastid genome evolution in parasites (Wolfe *et al.*, 1991, 1992a,b) also involves an increased rate of gene transfer to the nuclear genome (Fleischmann *et al.*, 2011), although this remains to be confirmed by nuclear genome sequencing. Consequently, the plastid genomes of parasitic plants do not represent a paradigm for minimum plastid genomes, in that gene loss from the plastid genome is not a reliable indicator of non-essentiality.

The putative minimum gene set of a plastid genome that is functional under heterotrophic conditions is shown in Figure 3. The set is largely based on systematic reverse genetic analyses in tobacco plastids (Table 1). Additional coding capacity can be saved by genome-wide changes of the codon usage that make isoaccepting tRNAs dispensable. If the remaining genes are condensed into a compact synthetic genome by removing intergenic spacers and introns, and rearranging genes in new operons, the plastid genome can be shrunken to between one-quarter and one-fifth of its original size (L. B. Scharff and R. Bock, unpublished data); however, a note of caution needs to be added here, because problems with synthetic lethality of non-essential components of the gene expression machinery currently cannot be excluded.

Based on the above considerations, a minimum plastid genome for tobacco plants has been designed by the authors, and its synthesis and assembly is currently underway. It is encouraging that the feasibility of transforming plastids with an entire genome has already been demonstrated in *C. reinhardtii* (O'Neill *et al.*, 2012); however, because of the extensive sequence homology between the transformed genome and the resident plastid genome, no full genome replacement could be achieved. Instead, homologous recombination occurred between the two genome types, resulting in mosaic plastid genomes composed of endogenous and exogenous pieces (O'Neill *et al.*, 2012).

LARGE-SCALE CHLOROPLAST GENOME ENGINEERING WITH SYNTHETIC DNA CONSTRUCTS

The ever-falling costs of DNA synthesis have led to a continuously increasing use of synthetic DNA for chloroplast engineering. Although, initially, synthetic DNA was mainly used to optimize the codon usage of transgenes in order to maximize their expression levels in the chloroplast (Ye *et al.*, 2001; Tregoning *et al.*, 2003; Oey *et al.*, 2009a), it has now become affordable to synthesize entire transformation vectors, including the flanking regions for integration of transgenes into the plastid genome via homologous recombination (Sinagawa-García *et al.*, 2009). Not only does this save much of the time-consuming and labor-intensive cloning work for vector construction, it also offers the advantage that undesired restriction sites can be removed from the flanking regions of plastid DNA, thus simplifying the insertion of multiple transgene cassettes into the vector (Sinagawa-García *et al.*, 2009). More recently, synthetic expression elements have also been developed to address the limited transgene expression capacity in non-green tissues, such as fruits and tubers.

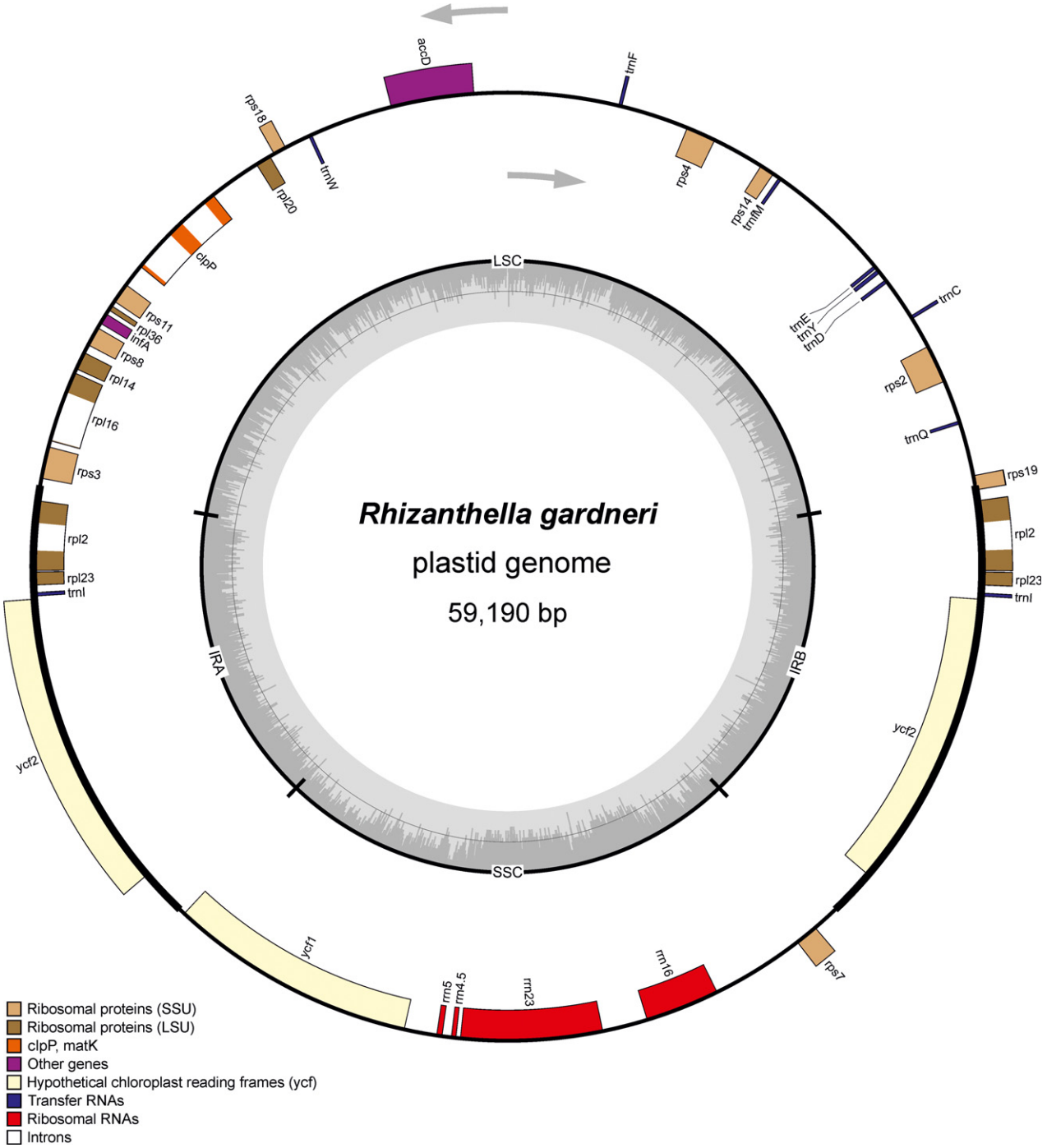

Figure 2. Physical map of the highly reduced plastid genome of the parasitic orchid *Rhizanthella gardneri*. The map was drawn with OGDRAW (Lohse *et al.*, 2007, 2013) using the complete plastid genome sequence as input (Delannoy *et al.*, 2011; GenBank accession number NC_014874.1). The grey arrows indicate the direction of transcription for the two DNA strands. The inner ring represents the GC content graph, and the circle inside indicates the 50% threshold. Abbreviations: LSC, large single-copy region; IRA, inverted repeat A; IRB, inverted repeat B; SSC, small single-copy region.

Based on transcript-profiling and polysome-profiling experiments in amyloplasts and chromoplasts (Kahlau and Bock, 2008; Valkov *et al.*, 2009), new combinations of promoters and 5′–UTRs were designed that boosted transgene expression in non-green plastids to unprecedented levels (Zhang *et al.*, 2012; Caroca *et al.*, 2013).

The introduction of large pieces of foreign DNA into a genome is of particular interest in metabolic engineering,

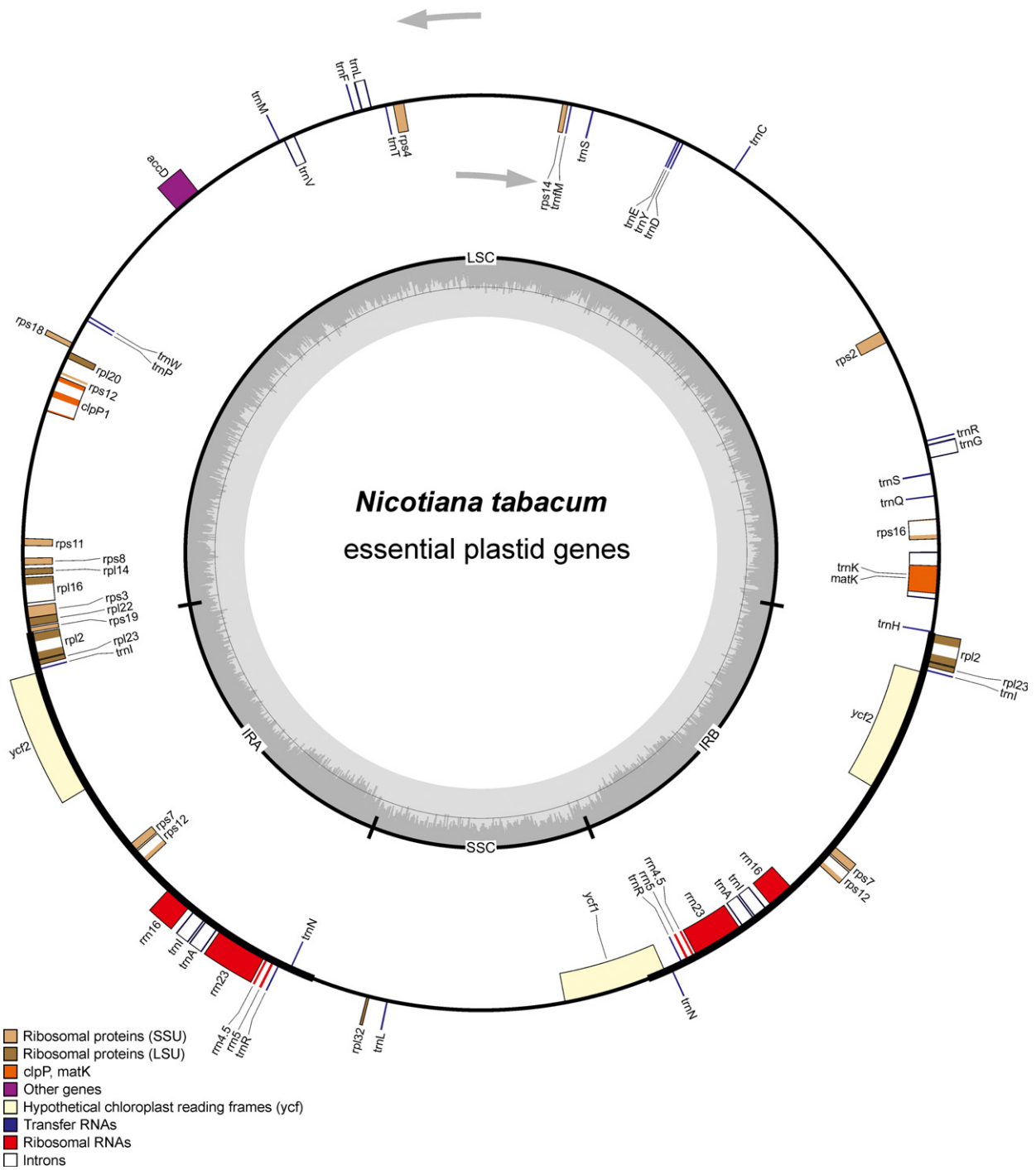

Figure 3. Physical map of the *Nicotiana tabacum* (tobacco) plastid genome, reduced to the minimum gene set required for survival under heterotrophic conditions. The map was drawn with OGDRAW software (Lohse *et al.*, 2007, 2013) using the complete genome plastid sequence as input (Shinozaki *et al.*, 1986; GenBank accession number Z00044.2), and removing all genes identified as non-essential in reverse-genetic studies (cf. Table 1). The grey arrows indicate the direction of transcription for the two DNA strands. Abbreviations: LSC, large single-copy region; IRA, inverted repeat A; IRB, inverted repeat B; SSC, small single-copy region. Note that a redesign of the plastid genome can make additional genes dispensable. These include one copy of the inverted repeat (IR), *matK*, a gene for a splicing factor that would not be needed if all group II introns are removed from the genome, and some tRNA species that would become dispensable if appropriate changes in the codon usage are made throughout the genome.

because the implementation of novel metabolic pathways often requires the introduction of many genes (Bock, 2013; Paddon *et al.*, 2013). As the chloroplast represents the 'metabolic center' of the plant cell, it has become an attractive site of metabolic pathway engineering (Madoka *et al.*, 2002; Craig *et al.*, 2008; Hasunuma *et al.*, 2008; Apel and Bock, 2009). In addition, there are several technical advantages associated with using the chloroplast genome as the target to accommodate transgenes. These include the possibility to attain extraordinarily high expression levels (De Cosa *et al.*, 2001; Oey *et al.*, 2009b), the precise incorporation of foreign DNA into the plastid genome by homologous recombination, the absence of epigenetic gene-silencing mechanisms and the predominantly maternal inheritance of plastid DNA (Azhagiri and Maliga, 2007; Ruf *et al.*, 2007), which greatly reduces the probability of transgene escape via pollen flow. For the engineering of metabolic pathways that require large numbers of transgenes, the arguably biggest attraction of plastid transformation lies in the possibility to stack many genes in synthetic operons (Bock, 2007; Maliga and Bock, 2011). The building of large synthetic operons meets the definition of bottom-up synthetic biology, where different molecular building blocks are engineered together, employing modular design principles (Figure 4).

Given the prokaryotic origin of chloroplasts, the conservation of many parts of the gene expression machinery, and the operon organization of genes in both bacterial and plastid genomes, it would seem possible to transplant entire operons from bacterial genomes into plastid genomes. There has been some success with this strategy (Hasunuma *et al.*, 2008; Krichevsky *et al.*, 2010). For example, the introduction of the entire *lux* operon (comprising six genes) from the bioluminescent bacterium *Photobacterium leiognathi* into the tobacco plastid genome resulted in the expression of the bacterial luciferase pathway, thus creating autonomously luminescent (autoluminescent) transplastomic plants (Krichevsky *et al.*, 2010). However, in a number of cases transgene expression from unmodified bacterial operons in transplastomic plants was rather poor (Nakashita *et al.*, 2001), and this was also the case with some synthetic operons in which construction was based on bacterial-type operons (Magee *et al.*, 2004). The redesign of operons by replacing bacterial expression signals with plastid-specific expression signals (especially plastid 5′–UTR sequences for efficient translation initiation) can significantly improve the expression efficiency of plastid transgenes stacked in operons (Bohmert-Tatarev *et al.*, 2011). Another potential problem with efficient operon expression in plastids comes from a major difference in transcript maturation between bacteria and plastids. Whereas polycistronic transcripts in bacteria usually directly enter translation, many polycistronic transcripts in plastids are only precursor RNAs that need to be cut into monocistronic mRNAs to facilitate efficient translation, especially of downstream cistrons (Hirose and Sugiura, 1997; Drechsel and Bock, 2010). The identification of a minimum sequence element (dubbed intercistronic expression element, IEE) that faithfully mediates the processing of polycistronic primary transcripts into stable monocistronic units (Zhou *et al.*, 2007) has made it possible to incorporate processing signals into synthetic chloroplast operons (Figure 4), thereby minimizing the risk that operon transcripts are not efficiently translatable. Using the vitamin E biosynthetic pathway as a test case, important principles of synthetic operon design were recently elucidated for tobacco and tomato plastids (Lu *et al.*, 2013; Figure 4). Based on these principles, it should now be possible to construct much larger operons (e.g. for more complex biochemical pathways), and express them successfully from engineered plastid genomes.

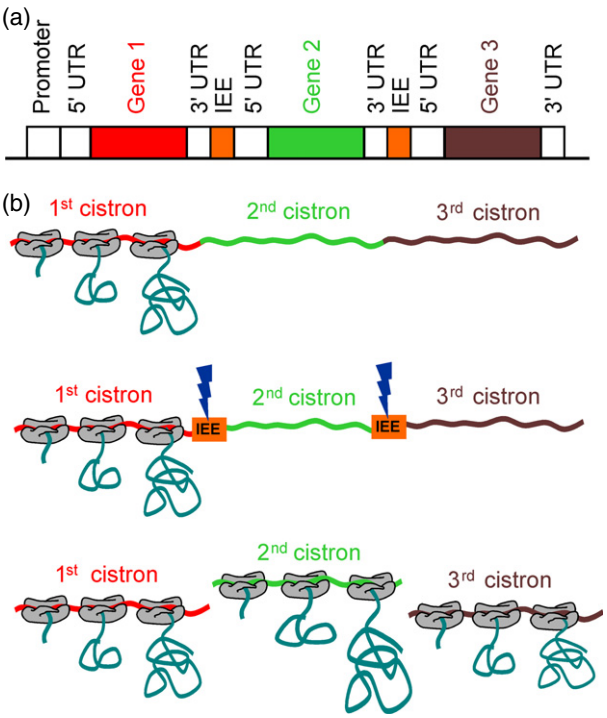

Figure 4. Modular design of synthetic plastid operons for large-scale metabolic engineering in chloroplasts (Bock, 2013; Lu *et al.*, 2013). As an example, an operon comprising three genes is shown.
(a) Schematic operon map showing the coding regions and expression elements involved in efficient operon expression (Zhou *et al.*, 2007; Lu *et al.*, 2013). See text for details.
(b) RNA processing and translation of the operon transcript. The primary transcript is a tricistronic RNA. Translation of the downstream cistrons is often inefficient, a problem that can be solved by the inclusion of a small sequence element (dubbed intercistronic expression element, IEE; Zhou *et al.*, 2007) that triggers the intercistronic processing of the polycistronic primary transcript into stable monocistronic mRNAs that are efficiently translated.

TECHNICAL CHALLENGES ASSOCIATED WITH LARGE-SCALE PLASTID GENOME ENGINEERING

As explained above, the biolistic transformation method predominantly used for plastid engineering relies on an entirely physical DNA delivery process, and therefore is not limited with respect to the size of the transforming DNA. However, the coating of the heavy metal particles used as microprojectiles with the transforming DNA involves relatively harsh mechanical treatments (especially vigorous vortexing) that are necessary to keep the particles in suspension and prevent them from aggregation. Thus, the transformation of the plastid genome with very large DNA molecules will be considerably less efficient, because of the increased risk of mechanical shearing during DNA purification and, especially, during particle coating. In principle, such broken DNA molecules could be repaired *in vivo*, by homologous recombination between multiple copies of the transforming DNA. Successful co-transformation experiments have demonstrated that multiple plasmid copies can enter the plastid upon transformation (Kindle *et al.*, 1991; Carrer and Maliga, 1995). However, how efficiently plastids perform recombination repair of fragmented DNAs remains to be determined. Irrespective of this issue, the development of more gentle protocols for particle coating and, more generally, for DNA delivery into plastids would be highly desirable.

Although generally beneficial for plastid transformation, the high homologous recombination activity in chloroplasts poses a technical problem when whole-genome transplantation is attempted. Recombination between the resident genome and the foreign genome will result in hybrid genomes composed of a largely unpredictable arrangement of pieces from the two genome types (O'Neill *et al.*, 2012). A radical redesign of the synthetic genome can minimize the problem, but is unlikely to fully solve it. Although the protein-coding genes in synthetic genomes can be made very different from the same genes in the resident genome (by playing with the codon usage), there is only limited leeway for sequence changes in rRNA and tRNA genes. As small stretches of sequence homology can be sufficient to trigger recombination (Iamtham and Day, 2000; Rogalski *et al.*, 2006), rRNA and tRNA genes are likely to remain hotspots of unwanted recombination events in whole-genome replacement experiments.

These technical challenges notwithstanding, the insertion into plastid genomes of large pieces of foreign DNA (Adachi *et al.*, 2007) and large multigene constructs (Krichevsky *et al.*, 2010; Bohmert-Tatarev *et al.*, 2011; Lu *et al.*, 2013) for engineering applications at the interface between biotechnology and synthetic biology is already possible now. Thus, plastid transformation is likely to become an important tool for addressing some of the biggest challenges in plant biotechnology, such as the engineering of C4 photosynthesis into C3 crops, or the transfer of nitrogen fixation pathways into plants (Dixon *et al.*, 1997). Fully synthetic and radically redesigned plastid genomes will ultimately offer the exciting possibility to expand the genetic code and, in this way, the amino acid repertoire of life (Guo *et al.*, 2008; Neumann *et al.*, 2010; Young and Schultz, 2010; Neumann, 2012). This would allow the synthesis of proteins with entirely novel properties and catalytic capabilities in plants, which would have enormous potential for molecular farming and, in particular, for the cost-effective and large-scale production of robust industrial enzymes and new therapeutics.

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