Establishing *Chlamydomonas reinhardtii* as an industrial biotechnology host

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**SUMMARY**

Microalgae constitute a diverse group of eukaryotic unicellular organisms that are of interest for pure and applied research. Owing to their natural synthesis of value-added natural products microalgae are emerging as a source of sustainable chemical compounds, proteins and metabolites, including but not limited to those that could replace compounds currently made from fossil fuels. For the model microalga, *Chlamydomonas reinhardtii*, this has prompted a period of rapid development so that this organism is poised for exploitation as an industrial biotechnology platform. The question now is how best to achieve this? Highly advanced industrial biotechnology systems using bacteria and yeasts were established in a classical metabolic engineering manner over several decades. However, the advent of advanced molecular tools and the rise of synthetic biology provide an opportunity to expedite the development of *C. reinhardtii* as an industrial biotechnology platform, avoiding the process of incremental improvement. In this review we describe the current status of genetic manipulation of *C. reinhardtii* for metabolic engineering. We then introduce several concepts that underpin synthetic biology, and show how generic parts are identified and used in a standard manner to achieve predictable outputs. Based on this we suggest that the development of *C. reinhardtii* as an industrial biotechnology platform can be achieved more efficiently through adoption of a synthetic biology approach.

Keywords: synthetic biology, industrial biotechnology, *Chlamydomonas reinhardtii*, metabolic engineering, rational design, transgene expression.

**MICROALGAE PRESENT UNIQUE FEATURES FOR APPLIED RESEARCH AND BIOTECHNOLOGY**

Microalgae are a polyphyletic group of unicellular eukaryotic organisms that can occupy both aquatic and terrestrial environments, adopting photosynthetic, heterotrophic or mixotrophic lifestyles. The enormous diversity in the algal lineages is due to their long evolutionary history (Dorrell and Smith, 2011). For instance the green algae, which are ancestral to land plants, diverged 1300 million years ago from the stramenopiles (Yoon et al., 2004), a group that encompasses several microalgal lineages of biotechnological relevance such as diatoms. As a result, microalgae exhibit wide variation in both cellular architecture and biosynthetic capacity, and thus present a number of general and unique features as research models and for commercial exploitation. Specifically, the unicellular physiology combined with rapid cell division and photosynthetic growth mean that microalgae can be more productive per unit land area than any plant system. In addition, using microalgae as feedstocks for low value, high volume products such as biofuels, provides the opportunity to avoid a number of environmental factors that currently affect biofuel production from crop plants (Chisti, 2007). These include issues surrounding land and (fresh)water usage, mono-culture, crop rotation, and input requirements: for example microalgae are able to sequester nitrogen and phosphorus from industrial waste streams, thus reducing the need for fertilisers. Nevertheless, many challenges remain to be overcome before it will be possible to cultivate algae on the very large scales needed for biofuel production (Scott et al., 2010; Klein-Marcuschamer et al., 2013). Instead, microalgae offer considerable potential for the production of low volume, high(er) value compounds, which are characteristically produced by industrial biotechnology (IB). Moreover, the pho-
tosynthetic lifestyle of microalgae means that they offer a more sustainable source of compounds compared to bacterial and yeast hosts used in conventional IB, since there is no need to provide fixed carbon.

Currently microalgae are exploited commercially for compounds that they make naturally, such as the pigments β-carotene and astaxanthin, and polymers alginate, carrageenan and agar for food products. Several research studies have attempted to enhance the production of value-added compounds, including bio-hydrogen from Chlamydomonas reinhardtii (Baltz et al., 2014; Xu et al., 2014), or omega-3 long chain fatty acids such as docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA) from Phaeodactylum tricornutum. Although P. tricornutum naturally makes EPA and some DHA, levels are low and the ratio of the two fatty acids is suboptimal for some commercial applications. In addition, they are present mainly in membrane rather than storage lipids, making extraction inefficient. Hamilton et al. (2014) were able to increase levels of DHA in P. tricornutum eight-fold by heterologous expression of two fatty-acid modifying enzymes, a Δ5-elongase and an acyl-CoA-dependent Δ6-desaturase, from the green alga Ostreococcus tauri using a constitutive promoter. Another desirable feature of microalgae is their cellular complexity, which provides the opportunity to partition or compartmentalise biochemical reactions, something not possible in bacterial hosts. This can facilitate the provision of precursors, or provide intracellular sinks for target products. Indeed, there are already reports of the production of human therapeutic proteins including erythropoietin, interferon β, proinsulin and immunoglobulin A in the chloroplast of C. reinhardtii (Rasala et al., 2010).

Whilst the many benefits afforded by microalgae are yet to be demonstrated at scale, these studies serve to raise the profile of microalgae research and development. The challenge now lies in the application of metabolic engineering strategies to enhance natural features of microalgae, making commercial exploitation economically viable (Klein-Marcuschamer et al., 2013). For example, in dense cultures required for industrial cultivation, light capture is limited. Reduction of the light-harvesting apparatus reduces light absorption per cell, increasing penetration and productivity within the culture (Polle et al., 2003; Cazzaniga et al., 2014). Other targets for optimisation include resource-use efficiency and auto-flocculation to facilitate harvesting. In parallel, to optimise and expand the level and range of novel products made by microalgae, it will be necessary to introduce new biosynthetic capabilities, remove competing pathways, and optimise the provision of precursor substrates and cofactors. Thus for microalgal IB to come of age, we need a suite of molecular biological tools with which to carry out sophisticated metabolic engineering.

For more than 20 years scientists have been able to redirect the metabolism of a host cell in a targeted manner through genetic manipulation, and this has been applied to across the tree of life, in both prokaryotes and eukaryotes, working with well characterised laboratory strains as well as novel environmental isolates. In the commercial sector, several organisms have been developed as robust IB hosts, including bacteria (Escherichia coli, Bacillus subtilis, Corynebacterium glutamicum, Lactobacillus spp.), and yeasts and fungi (Saccharomyces cerevisiae, Aspergillus spp.). These are used as production systems for compounds ranging from organic acids to pharmaceutical proteins (Goel et al., 2012; Buchholz and Collins, 2013). In spite of this success, metabolic engineering seldom demonstrates the design aspect that is so fundamental to engineering per se, since the development of bespoke solutions for each experimental system/product results in parallel development, and limits the transferability of constructs and knowledge. The advent of synthetic biology, whose principles involve a combination of standard parts, predictive modelling, and iterative design and testing, is just beginning to be exploited in the field of metabolic engineering, and holds out real promise for considerable advances in IB for production of compounds to reduce reliance on fossil fuels as feedstocks (Keasling, 2012; Yadav et al., 2012).

In this review, we present an overview of what has been achieved in terms of genetic manipulation of C. reinhardtii, and frame this in the context of bacterial and yeast model systems, drawing comparisons to illustrate current capabilities and limitations. We then discuss the relevance and timely nature of the development of synthetic biology concepts for metabolic engineering and how this represents a step change in capacity and predictive power. Finally we highlight how, through relatively minor changes, synthetic biology approaches might be applied to the development of C. reinhardtii as an IB platform, and also to enhance the microalgal research field more broadly. It is worth mentioning that other microalgae are being explored for similar applications, and we direct the reader to excellent reviews on the subject (Wijffels et al., 2013; Bellou et al., 2014; Chauton et al., 2015). In addition, several cyanobacterial species are being used for metabolic engineering, including for biofuels (Quintana et al., 2011; Berla et al., 2013; Wijffels et al., 2013). As prokaryotes they are not algae, but their simpler genetic system and ease of transformation can offer advantages. Conversely, they are less versatile than algae in terms of metabolic pathways.

**MOLECULAR RESOURCES FOR C. REINHARDTII**

Chlamydomonas reinhardtii (Chlorophyta) is a photosynthetic biflagellate microalga that has been studied for >30 years as a model for basic and applied physiology and biochemistry (see articles in the rest of this issue). The number of resources available for the manipulation of C. reinhardtii has increased markedly over this period (Figure 1). These include well defined protocols for growth,
sexual propagation, and mutagenesis, as well as numerous published biochemical, analytical and reporter assays. Supporting research into various aspects of cell biology is the *Chlamydomonas* stock centre, a collection of >300 plasmids and >2700 strains (www.chlamy.org). Sequences of the nuclear, chloroplast and mitochondrial genomes have been completed (Merchant et al., 2007). The approximately 17 000 genes are well annotated, due to the substantial amount of expression data available, as well as the efforts of the community. The current nuclear genome database, version 5.5, has had most gaps filled and is –93% complete (Blaby et al., 2014). This knowledge base is made accessible by web based tools including JGI Phytozome genome browser (Goodstein et al., 2012), the functional genomic ChlamyCyc portal (May et al., 2009), KEGG metabolic pathway portal (Kanehisa, 2000), and the PlantGDB comparative genomics resource (Duvick et al., 2008). The complexity of cellular metabolism is made accessible in *silico* by the generation of genome-scale metabolic models (GSMMs). GSMMs present a snapshot of metabolism in a network format and allow the topology of system to be investigated. Several GSMMs for *C. reinhardtii* have been generated (Boyle and Morgan, 2009; Christian et al., 2009; Manichaikul et al., 2009; Chang et al., 2011; Cogne et al., 2011; Dal’Molin et al., 2011; Kliphuis et al., 2012), and the efficacy of such models has been reviewed recently (Reinders et al., 2014).

Protocols for transformation of the nucleus, chloroplast and mitochondria are available using biolistics (Debuchy et al., 1989), electroporation (Shimogawara et al., 1998), protoplasts (Boynton et al., 1989), electroporation (Shimogawara et al., 2000), Agrobacterium (Kumar et al., 2004) or even simply vortexing with glass beads (Kindle, 1990). Facilitating nuclear transformation is the availability of several selectable markers, including auxotrophic markers, such as ARG7, which restores arginine prototrophy (Debuchy et al., 1989; Purton and Rochaix, 1995), as well as resistance genes, BLE (resistance against phleomycin; Stevens et al., 1996), APHVI (paromomycin; Sizova et al., 1996), and HYG (hygromycin B; Berthold et al., 2002). Transformation of the chloroplast genome can be by homologous recombination, enabling targeted and specific manipulation, although because of the multiple copies of the chloroplast DNA, several rounds of selection are required to obtain homoplasm (Day and Goldschmidt-Clermont, 2011). Nuclear transformation, on the other hand, is by random integration, and this means that multiple transformants must be screened to obtain stable lines expressing the transgene at the desired level (Debuchy et al., 1989). Important recent developments include the generation of a knock-out library consisting of >1600 single gene knockouts (Zhang et al., 2014) and the description of two strains, generated by random mutagenesis, which demonstrate enhanced protein expression due to an unknown mechanism that reduces the effects of transgene silencing (Neupert et al., 2008).

Complementing these resources are genomics studies of *C. reinhardtii*, of which there an increasing number (Table 1), including several aimed at metabolic engineering. Matthew et al. (2009) analysed the metabolobiology.

**Table 1 Examples of genomic studies completed for the analysis of specific physiological and biochemical characteristics of *C. reinhardtii***

| Study                  | Studies                                | Reference                  |
|-----------------------|----------------------------------------|----------------------------|
| Microarrays           | Tandem repeat discovery                | Zhao et al. (2014a)        |
|                       | Sexual production                      | Ning et al. (2013)         |
|                       | Transcription factors                  | Riaño-Pachón et al. (2008) |
|                       | Alternative splicing                   | Labadorf et al. (2010)     |
|                       | Polyadenylation                        | Zhao et al. (2014b)        |
|                       | Growth adaptation                      | Perrineau et al. (2014)    |
|                       | based on natural selection, serial dilution |                             |
|                       | TAG production improvement              | Goodenough et al. (2014)   |
|                       | Flagella regeneration                  | Stolc et al. (2005)        |
|                       | CO₂ effect                             | Fang et al. (2012)         |
| Proteomics            | Basal body                             | Keller et al. (2006)       |
|                       | Mitochondria                           | van Lis et al. (2003)      |
|                       | Thylakoids                             | Allmer et al. (2006)       |
| Metabolomics          | ChlamyCyc database                     | May et al. (2009)          |
|                       | Partitioning oil and starch            | Johnson and Alric (2013)   |
|                       | S-deprived                             |                             |
|                       | H₂ production                          | Matthew et al. (2009)      |

Figure 1. The rise of *C. reinhardtii* as a model system for molecular biology.

Major breakthroughs include first nuclear and chloroplast transformations (Boynton et al., 1989; Blowers et al., 1989; Kindle et al., 1989), mitochondrial transformation (Randolph-Anderson et al., 1993), systems analysis by proteomics (Hippler et al., 2001), the proposal of *C. reinhardtii* as a model organism (Harris, 2001), the production of the first therapeutic recombinant protein in the chloroplast (Mayfield et al., 2003), the sequencing of the nuclear genome (Merchant et al., 2007) and the publication of genome editing techniques (Sizova et al., 2013; Gao et al., 2014; Jiang et al., 2014) and the creation of a knock-out collection (Zhang et al., 2014).

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me of *C. reinhardtii* following sulfur depletion. This analysis demonstrated that between 0 and 24 h post sulfur deprivation, *C. reinhardtii* rapidly metabolised extra-cellular fixed carbon and synthesised the storage compounds starch and lipids in the form of triacylglycerides (TAGs). Over the next 96 h hydrogen production was achieved, peaking at 48 h post sulfur deprivation, while starch reserves were metabolised. However, amino acid and fatty-acid biosynthetic capacity and cellular stores remained intact, suggesting that the observed decline in hydrogen production was not due to metabolic depletion but instead because of the toxic accumulation of fermentation products, formate and ethanol. More recently, Lv et al. (2013) analysed the transcriptome of *C. reinhardtii* under exponential growth and lipid accumulation, identifying over 2500 genes that were upregulated. Of these 80% were assigned to functional categories which, as well as lipid biosynthesis, included several central metabolic pathways, suggesting an increase in general metabolism in response to the imposed stress. In addition, 41 transcription factors were differentially expressed under tested conditions. Building upon the genomic analyses are several GSMMs (Reijnders et al., 2014), which facilitate predictive attempts to harness the biosynthetic capacity of *C. reinhardtii*. Combined with a legacy of fundamental research and the current impetus placed on microalgae as a potentially sustainable source of energy, these modelling and other systems biology approaches are leading to an enormous increase in our understanding of *C. reinhardtii*, and efforts to manipulate it. At the same time, there has been a rise in the number of other microalgae with sequenced genomes and genomics tools (Figure S1 and Table S1); these data are leading to a much wider knowledge base of these organisms generally.

**Advanced tools for manipulation of the *C. reinhardtii* nucleus**

Efforts to express genes in the *C. reinhardtii* nucleus have been underpinned by the elucidation of mechanisms to regulate transgene expression. Examples include the use of promoters (Table 2) that have been characterised either as constitutive (e.g. *HSP70A/RBCS2*; Schroda et al., 2000) or regulated in response to specific stimuli, such as light (*PSAD*; Fischer and Rochaix, 2001), copper (*CYC6*; Quinn and Merchant, 1995), nitrate (*NIT1*; Ohresher et al., 1997), and most recently vitamin B₂₆ (*METE*; Helliwell et al., 2014). RNA-based elements have also been employed to augment transgene expression (Table 2). Introns of the Rubisco Small Subunit 2 (*RBCS2*) have been used extensively since they were first shown to function as enhancer elements in a manner independent of orientation, and they are effective when positioned either upstream or downstream of the promoter (Lumbreras et al., 1998). Thiamine pyrophosphate riboswitches have been identified in the thiamine biosynthetic pathway of *C. reinhardtii* which cause alternative splicing on binding the ligand, (Croft et al., 2007). The TH4 riboswitch has subsequently been used to regulate expression of a nuclear encoded protein, NAC2, which in turn is required for chloroplast gene expression (Ramundo et al., 2013). Down-regulation of native *C. reinhardtii* genes has taken advantage of RNA interference, where binding of short fragments of RNA (microRNA or miRNA) complementary to specific mRNAs leads to cleavage and thus silencing of the target gene. *C. reinhardtii* encodes all the components of the RNAi pathway (Casas-Mollano et al., 2008), and techniques for artificial-micro-RNA (amiRNA) have been developed (Molnar et al., 2009; Zhao et al., 2009). In one report, combining amiRNA with the NIT1 promoter resulted in an inducible gene knockdown system (Schmollinger et al., 2010).

Several different reporter genes are available, both fluorescent and colorimetric, and in most instances these have been modified for expression in *C. reinhardtii*; the unusually high GC content of ~65% (Merchant et al., 2007) means that most heterologous genes need to be codon optimised before they are expressed efficiently (e.g. Fuhrmann et al., 1999). Expressed proteins can be directed to different subcellular locations, or for secretion, using characterised targeting peptides, and tags for immunodetection or purification of expressed proteins have been developed (Eichler-Stahlberg et al., 2009). Eukaryotic cleavage peptides, such as viral 2A peptides (Rasala et al., 2012), have been demonstrated to work in *C. reinhardtii*, thus in principle allowing expression of two or more proteins from a single transgene construct. This would overcome potential difficulties in the limited number of promoters available, repeated use of which would be likely to increase the problems of gene silencing.

In the last 2 years, reports of advanced techniques for genome editing of *C. reinhardtii* have appeared. The first demonstration of genome editing was via zinc finger nucleases, which work by fusing a DNA binding domain from naturally occurring zinc finger nucleases with a DNA cleavage domain. Sizova et al. (2013) transformed *C. reinhardtii* with a heterologous non-functional APHVIII gene, interrupted with a 24-bp COP3 sequence, the target of the zinc finger nuclease. Subsequent transformation with the gene encoding the zinc finger nuclease, expressed from the *HSP70A* promoter, and a 120-bp sequence of DNA complementary to the non-functional region of APHVIII, followed by selection for paromomycin resistance, generated several colonies, indicating restoration of APHVIII gene function. No spontaneous resistance was apparent in the controls. The methodology was subsequently used to introduce targeted mutations into the native COP3 gene. The second example of genome editing employed Transcription Activator-Like Effectors (TALE) for specific activa-
| Functional element | Name | Phytozyme gene ID | Property | Reference |
|--------------------|------|-------------------|----------|-----------|
| **Promoters**      | HSP70A | Cre08.g372100 (196 bp upstream to ATG) | Typically used as constitutive; expression can be enhanced by high light and temperature | Schroda *et al.* (2000) |
|                    | RBCS2 or HSP70A/RBCS2 | Cre02.g120150 (180 bp upstream to ATG) | Strong constitutive (refer to HSP70A) | Lumbreras *et al.* (1998) |
|                    | PSAD | Cre05.g233332 (822 bp upstream to ATG) | Typically employed as strong constitutive; expression maybe enhanced by high light | Fischer and Rochaix (2001) |
|                    | CYC6 | Cre16.g651050 (127 bp upstream to ATG) | Metal (Cu) responsive | Quinn and Merchant (1995) |
|                    | NIT1 | Cre09.g410950 (282 bp upstream to ATG) | Ammonium responsive | Ohresser *et al.* (1997) |
|                    | ATX1 | Cre09.g392467 (532 bp upstream to ATG) | Iron (Fe) responsive | Fei and Deng (2007) |
|                    | CA1 | Cre05.g248400 (194 bp upstream to ATG) | CO₂ responsive | Villand *et al.* (1997) |
|                    | SQD2 | Cre01.g038550 (75 bp upstream to ATG) | Phosphate (P) responsive | Iwai *et al.* (2014) |
|                    | CAMV 35S | – | Enhancer and minimal promoter only | Ruecker *et al.* (2008) |
|                    | METE | – | Cobalamin (B₁₂) suppression | Hellwell *et al.* (2014) |
| **Regulatory elements** | **RBCS2 intron 1, 2 and 3** | Cre02.g120150 | Enhance gene expression as intron in coding region or 5`UTR | Eichler-Stahlberg *et al.* (2009), Lumbreras *et al.* (1998) |
|                    | THI4 | Cre04.g214150 (1414 bp 5`UTR) | 5`UTR, thiamine (B₁) suppression | Croft *et al.* (2007), Moulin *et al.* (2013) |
|                    | RBCS2 | 32 aa | Rubisco small subunit 2 chloroplast transit peptide | León *et al.* (2007) |
| **Functional peptides** | FD | 32 aa | Ferredoxin chloroplast targeting transit peptide | León *et al.* (2007) |
|                    | ARS2 | Cre16.g671350 (63 bp 5` end from ATG) | N-terminal secretion | Eichler-Stahlberg *et al.* (2009) |
|                    | 2 x simian virus 40 (SV40) | 20 aa | N-terminal nuclear target peptide | Rasala and Mayfield (2014) |
|                    | Foot-and-mouth disease virus [FMDV] 2A | 20 aa | Translational cleavage peptide | Rasala *et al.* (2012) |
| **Reporter genes** | Gaussia luciferase (gLuc) | 555 bp | Luciferase assay | Ruecker *et al.* (2008) |
|                    | GFP, mCHERRY, EYFP, DsRED, tdTOMATO, VENUS | 708-1437 bp | Fluorescent protein | Rasala *et al.* (2013) |
| **Highly expressed host strains** | UVM4 and UVM11 | Cre16.g671400 (3012 bp cDNA) | Chromogenic assay | Davies *et al.* (1992) |
|                    | – | – | High expression host, transgene silencing suppressed | Neupert *et al.* (2009) |
tion of target genes (Gao et al., 2014). In this work, the endogenous arylsulfatase genes, ARS1 and ARS2, were targeted via their promoters. Nuclear expression of the respective TALE, from a HSP70A/RBCS2 promoter and RBCS2 terminator cassette increased the expression of each ARS at the level of both the transcript and protein, a fact confirmed by an assay of arylsulfatase activity. Finally the efficacy of the Clustered Regularly Interspersed Short Palindromic repeat (CRISPR) system was tested (Jiang et al., 2014). CRISPR associated protein 9 (CAS9) is an RNA-guided DNA nuclease that has been employed to introduce targeted mutations into eukaryotic genomes. No stable transformants of C. reinhardtii expressing the CAS9 protein could be recovered, suggesting that the protein was toxic (Jiang et al., 2014). As a result the authors employed transient expression in an attempt to orchestrate targeted mutagenesis of four independent gene targets, HYG, mutant green fluorescent protein (mGFP), Gaussia luciferase (gLUC) and peptidyl-prolyl cis-trans isomerase (FKB12). A PCR assay was employed to enrich for and detect the presence of Cas9 mediated mutations. Although the system demonstrated low efficiency, with only a single FKB12 mutant obtained, it provides a benchmark for future research in this area.

In spite of our ability to manipulate the genome of C. reinhardtii, few examples of actual metabolic engineering are available in the literature. Of these, engineered expression of native genes for diacylglycerol acyltransferases (DGATs) provides a good example of the current status. In this, three type-2 DGAT genes were expressed constitutively with the PSAD promoter and 3′UTR, and their impact on TAG biosynthesis and profile analysed (La Russa et al., 2012). Although expression varied due to random integration, DGAT mRNA levels were increased 1.7- to 29.1-fold compared to controls. In spite of this no significant increase in lipid biosynthesis was observed, possibly due to induction of lipid degradation pathways (Nguyen et al., 2011). A second example is provided by the attempt to engineer C. reinhardtii to make the keto-carotenoids, astaxanthin and canthaxanthin. Constitutive expression of a β-carotene ketolase gene (BKT1), derived from the green microalga Haematococcus pluvialis, in the C. reinhardtii nucleus using the RBCS2 promoter, combined with targeting to the chloroplast via RBCS2 or ferredoxin (FD) transit peptides (Table 2) resulted in the undesired biosynthesis of 4-keto-lutein (León et al., 2007). Possible reasons for this failure include lack of substrate accessibility in the thylakoid membranes where the carotenoids are synthesised, and/or the inability of the heterologous β-ketolase to integrate into the carotenogenic enzyme complexes. For both this, and the example of expression of DGATs, optimisation would require many different genetic constructs to be tested, including down-regulation of endogenous path-ways as well as expression of transgenes in the same cell line. The current tools available for manipulation of C. reinhardtii limit considerably the ability to carry out these combinatorial studies.

**Engineering the C. reinhardtii Chloroplast**

From an engineering perspective, the chloroplast provides a unique cellular compartment that harbours numerous essential processes, including those of biotechnological importance such as carotenoid and fatty-acid biosynthesis. The chloroplast genomes of more than 20 species are now accessible to genetic modification (Day and Goldschmidt-Clermont, 2011; Maliga, 2012). However, chloroplast engineering is routine for only three species, Nicotiana tabacum (Golds et al., 1993), N. benthamiana (Davaranah et al., 2009), C. reinhardtii (Blowers et al., 1999). This further highlights the importance of C. reinhardtii as an IB platform. Working with the chloroplast provides a number of unique advantages, for example, the chloroplast genome is derived from a cyanobacterial genome that has been reduced by two orders of magnitude (Scharff and Bock, 2014). Its prokaryotic origin allows genes to be co-ordinated into operons, and to integrate DNA in a targeted manner via homologous recombination, so that mutant phenotypes can be complemented and resistance markers recycled to generate ‘marker-less’ strains (Fischer et al., 1996; Bateman and Purton, 2000) (Table 3). Moreover, some established bacterial resources also work in the C. reinhardtii chloroplast, including the lac repressor system (Kato et al., 2007). This is useful as it has been reported that native promoters maybe compromised by tight regulation of essential photosynthetic processes. For example, the promoter and 5′UTR of the psbA gene, which encodes the D1 subunit of photosystem II, can only be used in a psbA-deficient background because of D1-dependent auto-repression (Rasala et al., 2011). In spite of this, some combinations of promoter and 5′UTR have been identified that achieve efficient transgene expression in photosynthetic backgrounds, such as the 18S rRNA promoter-aptA 5′UTR hybrid (Rasala et al., 2011), and there are several reports of expression of single proteins being produced within the C. reinhardtii chloroplast (Tissot-Lecuelle et al., 2014). However, examples of metabolic engineering are much fewer in number. The most relevant example is that of enhanced bio-hydrogen production. Wu et al. (2011) expressed codon optimised hemH and lba to enhance cell-ular respiration, reducing the dissolved oxygen concentration in the media, thereby causing an up regulation of hydrogenase genes hydA1 and hydA2. Nonetheless, as with examples in the nucleus, improvements obtained through chloroplast metabolic engineering are limited compared with what has been achieved in other biological platforms.

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Industrial biotechnology employs biological systems, mainly microbes, for the production of commodities. Today these range from fuels, to platform chemicals, and many high-value products, including a vast range of therapeutics and pharmaceuticals. IB has its origins in fermentation practices for beer, wine and breadmaking, but as microbiology emerged as a science, the ability to control these technologies proceeded in parallel, and led to many important compounds being made this way (reviewed in Buchholz and Collins, 2013). A noteworthy example is penicillin from the fungus *Penicillium notatum*, as well as several other naturally occurring antibiotics from soil bacteria. The advent of genetic manipulation enabled the optimisation of biological production systems and enormously expanded the portfolio of compounds that could be produced by IB, including the ability to produce human insulin and growth hormone as recombinant proteins, avoiding the side effects of use of animal forms of the hormones (Vajo et al., 2001). Today, as well as pharmaceuticals, products from IB are an everyday feature of life, from vegetarian cheeses made with recombinant rennet, to plastics made from platform chemicals, to enzymes in washing powders (Goel et al., 2012).

The best known IB platforms are *E. coli* and *S. cerevisiae*, their utility being derived from decades of research that has progressed through various stages, ranging from the introduction of individual genes using standard expression systems, to the regulation of complex biochemical pathways using highly regulated genetic circuits (Figure 2).

Parallel to the development of tools for manipulation, the application of systems biology and GSMMs have enabled rational modification of discrete pathways (Yu et al., 2011; Chen and Zeng, 2013), whilst improvement of platform strains with respect to growth and productivity within the fermenter, as well as downstream processing, has led to optimised hosts for different bioprocesses (Goel et al., 2011).

**Table 3:** Regulatory elements available for the *C. reinhardtii* chloroplast. Chloroplast functional parts are classified as transcriptional leaders (promoter and 5′UTR), chloroplast-targeted peptides, selectable markers and destination sequences for homologous recombination

| Functional element | Name | Properties | Source |
|--------------------|------|------------|--------|
| Transcriptional leaders | AtpA, psbD | Constitutive expression | Barnes et al. (2005) |
| | psaA-exon1 | Strong expression | Michelet et al. (2011) |
| Chloroplast-targeted peptides | psaD | 35 aa N-terminal | Fischer and Rochaix (2001) |
| | rbcS2, FD | 32 aa N-terminal | León et al. (2007) |
| Selectable marker | rnsS and rnl point mutations | Spectromycin and erythromycin resistance | Kindle et al. (1991) |
| | arg9 | Arginine complementation | Remacle et al. (2009) |
| | aadA | Spectinomycin resistance | Goldschmidt-Clermont (1991) |
| | aphA6 | Resistance kanamycin | Bateman and Purton (2000) |
| | GFP | Fluorescent reporter | Franklin et al. (2002) |
| | gusA | β-Glucuronidase activity | Sakamoto et al. (1993) |
| | atpB | 5-Fluorodeoxyuridine resistance | Kindle et al. (1991) |
| | coda | Cytosine deaminase sensitivity to 5-fluorocytosine | Young and Purton (2014) |
| Destination sequences for homologous recombination | p322 | psbA and 16S rRNA | Manuell et al. (2007), Barnes et al. (2005) |
| | patpint-cg11 (atpB-int) | Inverted repeat and atpB 3′UTR | Nickelsen et al. (1994), Michelet et al. (2011) |
| | pLM7 (IR-int) | psbA and 5S/23S | Bateman and Purton (2000) |
| | p72B | psbH and psbN | |
| | p71 | tscA and inverted repeat | Kindle et al. (1991) |

**Figure 2:** Schematic of host optimisation via metabolic engineering and synthetic biology. The chronologic development of a biological host via metabolic engineering (blue), including the recent advent of systems biology and genome-scale metabolic models. Synthetic biology (red) has the potential to expedite this process in new biological systems, including microalgae, providing an opportunity to proceed directly from basic knowledge and capacity to the generation of highly optimised productive hosts.

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This progression is demonstrated by the work on the optimisation of E. coli strains expressing a gene encoding amorphadiene synthase from Artemisia annua, which catalyses the conversion of the isoprenoid intermediate farnesyl pyrophosphate into amorphadiene. Starting with a landmark metabolic engineering paper, Martin et al. (2003) introduced a yeast mevalonate pathway into E. coli to enhance production of the isoprenoid precursor, isopentenyl pyrophosphate. This increased amorphadiene biosynthesis 36-fold, to 24 μg mL⁻¹. This strain was employed in several subsequent studies to achieve iterative improvements in target compound production by elucidating metabolic limitations in precursor biosynthesis and improving carbon flux towards the end products. First, expression of the mevalonate pathway gene 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase was modulated to improve precursor biosynthesis leading to a three-fold (ca. 70 μg mL⁻¹) increase over the original strain (Pitera et al., 2007). Following this, mevalonate kinase and amorphadiene synthase were identified as rate limiting, and optimisation of promoter strength and gene copy number increased productivity seven-fold (ca. 160 μg mL⁻¹) over the original construct (Anthony et al., 2009). This moved the metabolic bottleneck back to HMG-CoA reductase, and the introduction and optimisation of an alternative HMG-CoA reductase, derived from Delftia acidovorans, led to levels of 700 μg mL⁻¹ amorphadiene (Ma et al., 2011). Most recently, Dahl et al. (2013) identified farnesyl pyrophosphate as a toxic intermediate and introduced dynamic control to regulate its synthesis and consumption. The application of systems biology methods identified promoters induced in response to intermediate accumulation, and these were employed to drive the expression of both the farnesyl pyrophosphate biosynthetic pathway and the amorphadiene biosynthetic genes, resulting in a doubling of the amorphadiene titre, to 1.6 mg mL⁻¹. This approach is now routine in IB where iterative improvements enable optimisation of inputs and gene expression to maximise the economic viability of a bioprocess.

Tangential to these developments has been the rise of synthetic biology. Much of the current focus of this discipline is on the development and analysis of genetic circuits, emergent behaviour and minimal genomes (Juhas et al., 2011), as well as the generation of non-natural biological systems with an expanded genetic code (Neumann and Neumann-Staubitz, 2010). Nonetheless, the potential for synthetic biology to contribute to IB is significant (Khalil and Collins, 2010; Yadav et al., 2012).

SYNTHETIC BIOLOGY ALLOWS RATIONAL DESIGN OF BIOLOGICAL SYSTEMS

Synthetic biology draws on principles first employed in the electronics field, and aims to create or (re)design complex biological circuits, networks or whole organisms in a rational way, combining computational models with assembly of standard or modularised components, to create useful outputs. Synthetic biology is characterised by an iterative workflow of continuous improvement, with generated knowledge informing subsequent rounds of design (shown schematically in Figure 3a). The basis is the use of standard parts (Figure 3b) for the design and engineering of novel biological systems, in this example DNA sequences such as promoters, terminators or introns. These parts or pathways may be described as orthogonal, that is they are transferrable between different cellular hosts with little/no impact on function (i.e. platform independent). They may be insulated, so they are unaffected by host cell metabolism, while an approach may be said to lead to abstraction, allowing design at higher levels of complexity because work builds upon well characterised inputs. This makes knowledge transferable, as a combination of elements that lead to a predetermined expression level of a transgene could be employed in subsequent studies to drive the expression of other transgenes. The aim is that, once sufficiently well characterised, the design process no longer needs to consider individual DNA parts. Instead these may be combined into expression cassettes or devices (Figure 3c) with novel but predictable outputs. This will allow the field to progress more efficiently by building directly upon work that preceded it. Table 4 provides a summary of other commonly used synthetic biology terms.

Synthetic biology in action – Escherichia coli

Escherichia coli is the best understood and most advanced biological platform available, demonstrating great utility for the development of ground-breaking molecular, genetic and biochemical techniques. As such it has been widely employed to pioneer synthetic biology (Cameron et al., 2014). For example, there is now a Registry of Standard Biological Parts, or Biobricks (Knight, 2003), the number of which now exceeds 12 000 (Vilanova and Porcar, 2014). Public access allows researchers to explore novel functionalities and applications, in return for contributing to the centralised knowledge database. This foundation provides the ability to engineer strains and create genetic circuits, such as the use of a two-component sensor and response system where genes were regulated in response to different light wavelengths (Olson et al., 2014). The system was composed of a histidine kinase with an N-terminal phytochrome domain sensor and a C-terminal bifunctional kinase-phosphatase signalling domain. Using biological information and modelling, the system was manipulated to regulate protein expression in response to different wavelengths and pulse profiles of light using an automated illumination program. The binary states demonstrated by this system provide a basis for future logic based gene circuits.
Escherichia coli synthetic biology has already advanced to population-scale regulation using synthetic networks. An example of this is the synchronisation of genetic clocks by post-translational events. Prindle et al. (2014) employed intracellular negative-feedback and a quorum-sensing oscillator in combination with proteins tagged for rapid protease catalysed degradation via the CLPXP signal. By coupling these processes the authors engineered a system in which the response delay was reduced by an order of magnitude over systems coupled at the level of transcription. Modification of the linker sequence length between the peptide and the degradation tag made the timing of the response tunable, offering the potential to create optimised expression profiles for synthetic gene circuits. As an illustration, a useful application of this for metabolic engineering would be regulation of key mevalonate biosynthetic genes in the amorphodiene strain discussed above.

Synthetic biology in action – Saccharomyces cerevisiae

The major chassis in eukaryotes is S. cerevisiae. This unicellular yeast benefits from numerous community resources that support fundamental research, including knock-out collections in which each protein-coding region has been systematically disrupted both individually (Dujon, 1998; Winzeler et al., 1999), and in pairs (Tong et al., 2001), allowing investigation of lethality and gene relationships. Aspects of S. cerevisiae research have moved to adopt the principles of synthetic biology. A pioneering example of this is the creation of an entirely artificial yeast chromosome (Annaluru et al., 2014), engineered to streamline and condense the encoded genetic information and allow re-organisation of coding regions on a massive scale. This paves the way for rapid laboratory-based evolution to generate novel phenotypes with biotechnological relevance.

Further examples of how synthetic biology has been applied to S. cerevisiae include the development of novel synthetic inducible promoters, regulated by a ligand that does not influence cell physiology (i.e. is insulated), which are also tunable. McIsaac et al. (2014) constitutively expressed an artificial transcription factor (ATF) in a cell line harbouring a green fluorescent protein (GFP) reporter that was regulated via a synthetic promoter containing one or several binding sites for the ATF. The reporter was expressed in the presence of the inducer, β-estradiol, and modification to the number and position of the ATF binding sites provided tunable expression upon induction, with a dynamic range that spanned three orders of magnitude.

In another study, Yofe et al. (2014) investigated the ability of non-coding sequences to regulate gene expression in S. cerevisiae. Creating a library of 240 reporter lines, each containing a different intron within the reporter gene coding sequence, the authors observed variation in expression spanning 100-fold. These lines demonstrated robustness in expression level and a capacity for tunable gene expression, achieved through the modification of key intron motifs and/or the folding energy of the intron-exon boundary. Finally, to allow rational design, the authors developed a computational model that fitted the experimental performance of tested sequences.

To demonstrate the power of a combinatorial approach, the five genes of the uncharacterised violacein biosynthetic pathways were targeted. In this, five promoters which spanned nearly three orders of magnitude in expression level were employed to drive expression of the violacein biosynthetic genes from BBa_K274002, a plasmid from the Registry of Standard Biological Parts (Knight, 2003). A rapid PCR genotyping method combined with computational modelling to integrate experimental data enabled
DEVELOPMENT OF A C. REINHARDTII INDUSTRIAL BIOTECHNOLOGY PLATFORM

The approaches presented above provide an overview of what is currently possible with well characterised IB platforms, and how a synthetic biology approach would augment both the range and amount of compounds that could be produced, and the rate of progress. For C. reinhardtii, although our ability to manipulate it genetically is advancing rapidly, many limitations remain for effective metabolic engineering. For example, fewer than 100 protein-coding genes from the C. reinhardtii genome have been functionally validated, compared with the approximately 6800 genes that have been characterised experimentally in Arabidopsis thaliana (Koornneef et al., 2004). Additionally, the number of tools to regulate transgene expression (Tables 2 and 3) is small compared with standard IB hosts. There are also the well known complications of integration of transgenes into the nucleus via non-homologous end joining, and extensive gene silencing. A significant additional challenge is the fact that regulatory networks at the genetic, cellular, and metabolic levels in C. reinhardtii are almost completely unexplored, so that attempts at manipulation will be effectively empirical, whereas there are many studies of the interactome of E. coli (Juan et al., 2008) and S. cerevisiae (Ito et al., 2001). Although undoubtedly some of these knowledge gaps for C. reinhardtii will be filled in the near future (as discussed above), it should be noted that no biological chassis evolved to be optimal for scientific research, with extensive engineering required to create today’s E. coli strains for routine protein expression (Goel et al., 2012; Buchholz and Collins, 2013). Thus rather than waiting for this to be true for C. reinhardtii, would it be possible to expedite progress by applying current leading concepts, techniques and technologies from synthetic biology? This might lead to quicker solutions to current research questions and, importantly, provide the opportunity to develop C. reinhardtii production systems that exceed the capacity of current metabolic engineering approaches. Adopting standardisation, modularity, and design/testing does not require drastic changes in approach, nor does it render all prior knowledge obsolete. Instead this progression can be achieved by subtle changes to minimise experimental variables and standardise workflows, creating a cycle of perpetual improvement.

To demonstrate this, let us consider an example of improved TAG biosynthesis for biodiesel production (Figure 4). When cultured under diurnal conditions, i.e. with a light/dark photoperiod, C. reinhardtii will photosynthesise during the light, fixing carbon dioxide and storing any excess as starch. In the dark the accumulated starch is consumed to support cellular activity. When exposed to nutrient stress, such as nitrogen depletion, C. reinhardtii accumulates much higher amounts of storage molecules,
mutants deficient in starch biosynthesis (Wang et al., 2012). Under conditions where the desired product is TAG, starch biosynthesis may be considered an undesired carbon sink. This principle was demonstrated through the analysis of mutants deficient in starch biosynthesis (Wang et al., 2009). However, starchless mutants are not able to be propagated under diurnal conditions because they lack the required starch to survive periods of darkness (Davey et al., 2014). To overcome this and optimise TAG production one may therefore consider a synthetic circuit that links nitrogen depletion and starch biosynthesis in a manner that would enhance carbon flux into TAG production only under nitrogen stress (shown schematically in Figure 4a).

Employing the workflow presented in Figure 3, a regulatory system could be identified and used to regulate the expression of a starch biosynthesis gene in response to light or nitrogen depletion. Once suitable elements are identified data describing expression characteristics could be integrated into an in silico model to generate predicted outputs (Figure 4b), and then tested to find the optimal combination of parts for the device. The next stage would be to engineer the system such that TAG production was induced independently of nitrogen depletion. Complementation of a starchless phenotype using this regulatory system would restore starch biosynthesis to that of a wild-type strain, with expression induced in response to light availability (input; light). Because the same system would be down-regulated in response to nitrogen stress (input; nitrogen depletion), this would over-ride the light-based regulation (OR gate logic). The designed circuit would, when exposed to nitrogen stress, suppress starch biosynthesis as native TAG production pathways are induced, producing more TAG because of increased fixed carbon availability.

**CONCLUSION**

Microalgae are of interest to academic and commercial stakeholders due to their unique biology, which encompasses facets of bacterial and yeast systems, and their potential as a biotechnology platform. Although the microalgal research field is less well developed than for the former organisms, the model green alga, _C. reinhardtii_, is positioned for development as an IB platform. It is evident that the major limitation now lies in our capacity to engineer _C. reinhardtii_ efficiently. By taking a synthetic biology approach, progress within the field should be accelerated.

One final point to make is that the diversity of microalgae means that _C. reinhardtii_ does not provide all of the attributes assigned to ‘microalgae’. That said, its development as an IB platform would undoubtedly benefit the entire microalgal community, and pave the way for subsequent more efficient development of alternative microalgal hosts of biotechnological importance, including _P. tricornutum_, _Thalassiosira pseudonana_, _O. tauri_ and _Nannochloropsis_ spp. (Table S1). These will offer a wider range of products, and possibly other advantages such as homologous recombination, which has been reported for the latter two species. It is important to acknowledge that although _E. coli_ and _S. cerevisiae_ are the best known IB platforms, many other bacteria and yeasts are employed in million dollar biotechnology sectors for the production of value-added products. The time is right to consider extending this to microalgae to address the desire to improve the sustainability of these processes (Langevels et al., 2010).

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