

# Plant synthetic biology

Wusheng Liu<sup>1</sup> and C. Neal Stewart Jr.<sup>1,2</sup>

<sup>1</sup> Department of Plant Sciences, University of Tennessee, Knoxville, TN 37996-4561, USA

<sup>2</sup> BioEnergy Science Center, Oak Ridge National Laboratory, Oak Ridge, TN 37831-6037, USA

**Plant synthetic biology is an emerging field that combines engineering principles with plant biology toward the design and production of new devices. This emerging field should play an important role in future agriculture for traditional crop improvement, but also in enabling novel bioproduction in plants. In this review we discuss the design cycles of synthetic biology as well as key engineering principles, genetic parts, and computational tools that can be utilized in plant synthetic biology. Some pioneering examples are offered as a demonstration of how synthetic biology can be used to modify plants for specific purposes. These include synthetic sensors, synthetic metabolic pathways, and synthetic genomes. We also speculate about the future of synthetic biology of plants.**

## Simple beginnings: synthetic biology in bacteria and beyond

Synthetic biology found its beginning in bacterial systems and has now advanced to eukaryotes, including plants [1]. With the invention of the first synthetic circuits – the genetic toggle switch [2] and the ‘repressilator’ [3] – the first wave in synthetic biology [4] started with the construction of artificial genetic circuits (see Glossary) and small modules for proof-of-concept demonstration [2,3,5–7]. Advances in microbial synthetic biology have been translated to solving biomedical and industrial problems such as the environmentally controlled cancer cell-invasive bacteria [8] and defense-enhancing bacteriophages as antibiotic adjuvants [9,10]. Synthetic biology projects have also been completed to reconstruct the phage  $\Phi$ X174 [11], refactor the phage T7 [12], and construct an artificial bacterial cell harboring a synthetic 1.08 Mb *Mycoplasma* genome [13]. As synthetic biology moved to yeast (*Saccharomyces cerevisiae*) and mammalian cells, gene switches and designer cells for predictable metabolic and therapeutic functions were generated. Examples include the production of chimeric antigen receptor-modified T cells in non-immune cells [14] and RNA- and cell-based vaccines [15,16].

These examples serve to prove that synthetic biology approaches are tractable and useful, especially when ample research funding is available and systems are adequately understood. However, plant synthetic biology

remains in its infancy. As the second wave of synthetic biology produces and tests systems-level circuitry in established systems [4,17], microbial synthetic biology benefits plant synthetic biology in at least two ways. First, the design principles and concepts developed in microbial synthetic biology are certainly applicable to plants with regard to gene expression and basic cellular function. Second, some microbial parts are being utilized directly in plants to enhance the design and construction of novel plant functions. Following the first plant synthetic circuits for plant metabolite production, such as dhurrin [18], artemisinin [19], and carotenoids [20], and for TNT phytosensing *in planta* [21,22], plant synthetic biology is coming of age. Discussed here are the basic principles of synthetic biology as applied to plants as well as pioneering applications of plant synthetic biology in the generation of artificial plant systems and synthetic genomes.

## Plant synthetic biology: an emerging discipline

Synthetic biology aims to apply engineering principles to the design and alteration of natural systems or to the *de novo* construction of artificial biological devices and systems that exhibit predictable behaviors [23]. Its development processes can be analogous to automobile mechanics.

## Glossary

**Abstraction:** the identification and establishment of hierarchies of functional units for the design process.

**Biological building block:** an individual element/entity occurring in the composition of a biological object and contributing to its function.

**Biomimetics:** the imitation of the structures and functions of systems and elements of nature for the purpose of solving complex problems.

**Chassis:** the host organisms implemented with synthetic devices or gene networks.

**Circuit:** a functional unit rationally designed and assembled with synthetic parts for specific logical functions inside a cell or chassis.

**Conglomerate biological device:** a biological device comprising heterogeneous parts.

**Decomposition:** the breakdown of any object into simpler parts.

**Decoupling:** the breaking down of complicated entities (systems, functions, or problems) into manageable, independent, and simpler constituents.

**Hierarchical function:** functions arranged at different levels.

**Modularity:** functional independence of biological parts and devices.

**Orthogonality:** functionally equivalent and context-free properties of biological parts.

**Patent thicket:** an overlapping set of patent rights used to defend against competitors designing around a licensed patent.

**PhytoBricks:** standard DNA sequences (such as promoters, coding sequences, and terminators) used as Lego-like building blocks for the design and assembly of synthetic biological devices in plants.

**Riboswitch:** a regulatory segment of a mRNA molecule that binds to its effectors, resulting in changes in its own activity.

**Standardization:** the definitive description and characterization of functionally equivalent and interchangeable (i.e., orthogonal) biological parts as well as the standardized conditions for construction and testing.

**Topologies of information:** information coming from different hierarchical levels.

Corresponding author: Stewart, C.N. Jr. (nealstewart@utk.edu).

Keywords: plant synthetic biology; design cycle; enabling tools; engineering principles; pioneering applications.

1360-1385/

© 2015 Elsevier Ltd. All rights reserved. <http://dx.doi.org/10.1016/j.tplants.2015.02.004>

An older model can be tweaked for higher efficiency (top down) or a new model can be built from scratch (bottom up). The top-down approach starts from an existing plant system and aims at making a minimum-size system with the smallest numbers of parts by reducing its complexity. The bottom-up approach starts with individual parts to make artificial biological systems with novel properties [24]. Using either approach, the goal of synthetic biology is to redesign a system for a particular purpose [25] and to better understand biology by reconstruction [26].

Synthetic biologists may view their embryonic field as more engineering than biology in some cases. Biology focuses on scientific discovery and analysis, whereas engineering focuses on design and synthesis; that is, understanding versus building. In this review we discuss the engineering-biology principles that comprise synthetic biology and apply them to plants. Compared with conventional plant biotechnology, which relies mainly on recombinant DNA technology and focuses on recombination of existing heterologous genes and promoters, plant synthetic biology is based on combining the modules of nature in new ways and building new modules. Similar to systems biology, synthetic biology focuses on the interactions and dynamic behaviors of a system's (natural or artificial) parts [27]. Systems biology provides the knowledge base for synthetic biology's design and construction as synthetic biology enhances system-level knowledge through reconstruction [28–30].

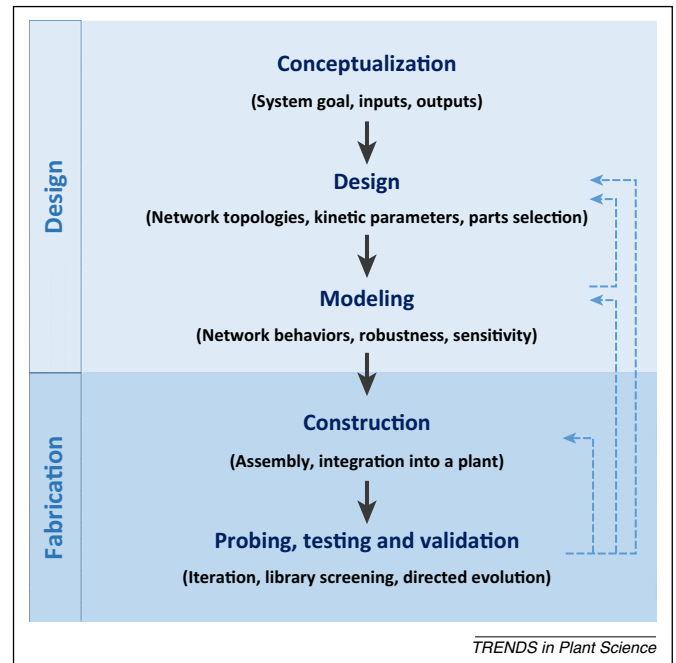
### Design cycle of synthetic biology

The ideal design cycle of synthetic biology encompasses five stages: conceptualization, design, modeling, construction, and, finally, probing, testing, and validation [1,31–33].

Conceptualization specifies the overall goals (i.e., desired features and functions of a device) of synthetic gene networks in response to inputs and expected outputs [1]. The objectives should be rationally formulated so that they can be produced and tested unambiguously. Basic knowledge enables synthetic biologists to build on nature's principles and modules (i.e., using biomimetics). Once an objective is specified, it is necessary to select genetic parts and construction strategies for the fulfillment of the objective. Computer-aided design (CAD) is used to help the determination and optimization of network hierarchies, kinetic parameters, and parts selection [1]. It also helps modeling for the analysis of network behaviors, sensitivity, and robustness to perturbations and the selection of the most promising design for implementation.

Construction entails the fabrication and assembly of synthetic circuits and their integration into a plant host [34]. A trial-and-error approach combined with fine-tuning is required for experimental probing, testing, and validation. Moreover, multiple iterations (Figure 1) might be needed to generate a series of improved approximate solutions to finally obtain the desired functions and properties [1].

Enabling tools to fulfill the design cycle of plant synthetic biology include engineering principles for design, components for parts selection, and plant computational tools for design and modeling. (See [34] for a concise review of some other enabling tools that are used



**Figure 1.** Design cycle for plant synthetic biology driven by engineering principles. The ideal design cycles of plant synthetic biology encompass five stages: conceptualization, design, modeling, construction, and probing and testing [1,32]. Conceptualization specifies the overall goals in response to inputs and expected outputs. Computer-aided design helps determine and optimize network hierarchies, kinetic parameters, and parts selection. Modeling is used to analyze network behaviors, sensitivity, and robustness to perturbations for selection of the most promising designs for experimental testing. Construction is the fabrication and assembly of synthetic circuits involved in standardized cloning and *de novo* DNA synthesis and their integration into a plant chassis by either organelle or nuclear transformation. Optimization is used for probing and testing experimentally. Multiple iterations (broken lines) may be needed to generate a series of improving approximate solutions to finally obtain the desired functions and properties. Decoupling separates design from fabrication.

in plant biotechnology and have great potential in plant synthetic biology, such as synthetic promoters and transcription factors for regulation of gene expression and advanced methods for DNA assembly and synthesis, chassis loading, and precision genome editing.)

### Fundamental engineering principles

To reduce the inherent high degree of plant complexity and redundancy, plant synthetic biology uses the most important foundational engineering principles: decoupling, abstraction, and standardization [1,35,36].

Decoupling allows simplification of complex problems into many smaller problems that can be addressed individually [35]. For example, engineers decouple a design from its fabrication [37] (Figure 1). They can also deconstruct an automobile to, for example, the drivetrain, electronics, and interior. Abstraction separates topologies of information into hierarchical levels and allows limited and principled information exchanges between levels [35]. Each hierarchy level is embedded in a more complex level that provides its context. Abstraction levels include materials such as DNA, parts, devices, and systems. DNA is the primary chemical material that can be assembled rationally to form parts that are combined into devices, which serve a specified function and comprise the synthetic system after they are installed into a plant. Standardization is used to define and characterize orthogonal parts and standardized conditions for

testing [35]. Standardization relies on the modularity and orthogonality of parts and allows parts to be assembled to form a complex system. The simplification and decomposition of a technical process by decoupling, abstraction, and standardization can reduce efforts of design cycles.

### Selecting components for plant synthetic devices

Biological systems can be reduced into parts and modules for reconstruction [38], which requires raw materials be extracted from their natural contexts and refined to be orthogonal and context independent. These parts include *cis*-regulatory elements, promoters, transcription initiation sites, exons, protein domains, protein-coding open reading frames (ORFs), and terminators [20,35,39]. *Cis*-regulatory elements can be standardized for synthetic promoter development, in which the sequence and spacing between various *cis*-elements are important for proper helical orientation to interact with the appropriate transcription factor. Exons and protein domains can be standardized for the generation of synthetic chimeric genes and proteins. Of course, synthetic parts can be assembled modularly resulting in different hierarchical functions, such as synthetic genes, pathways, chromosomes, genomes, and/or conglomerate biological devices and networks [35,39–42]. These synthetic devices can operate at various levels in the Central Dogma and beyond, such as at transcriptional, translational, and post-translational levels. Since plant promoters are more complex than prokaryotic (and some other eukaryotic) promoters, transcriptional regulation and precise control of gene expression in synthetic circuits is a major

challenge and a research priority in plant synthetic biology studies [34,43,44].

Although the number of registered ‘biobricks’ – parts used in synthetic biology – (<http://partsregistry.org/>) is increasing rapidly as a result of the International Genetically Engineered Machine (iGEM) competitions since 2003 [45], their plant subset ‘phytobricks’ (<http://www.plantfab.org>) database only contains a few (~20) parts. One reason for the paucity of phytobricks might be the added complexity of these parts compared with their microbial counterparts. Researchers custom-make parts and devices according to their own standards, which might not be compatible with other laboratories’ catalogs or work as expected in different systems. In addition, intellectual property (IP) issues, including patent thickets [46], are likely to have played a role in the lack of deposition of phytobricks into registries. For example, university technology transfer offices might not approve the deposition of patented genes for free access if a company has licensed them for commercial purposes [46].

### Computational tools for design and modeling

CAD tools are among the most important in synthetic biology [47–49]. Using CAD tools synthetic biologists can improve the functions and properties of synthetic devices *in silico* by optimizing design parameters and testing possible design alternatives before construction. These tools can be grouped into three categories: component design and synthesis [50,51], topology and network design [52–59], and behavior prediction and simulation [60–66] (Table 1). Although many of these tools have been designed

**Table 1. Currently available computational tools for plant synthetic biology**

Software	Description	Adaptability to plants	Refs
<i>Component synthesis and design</i>			
GeneDesign	Web server with algorithms for codon optimization and codon bias graphing so that insertion of restriction sites and design of building blocks are supported.	Ready for plants	[50]
Gene Designer2.0	Software for gene, operon, and vector design, codon optimization, restriction site modification, ORF recoding and primer design.	Ready for plants	[51]
<i>Topology and network design</i>			
GenoCAD	A framework including a formal semantic model that represents the dynamics of multiple part sequences using attribute grammar. It formalizes the context dependency of part functions and translates part sequences to a model to predict their behaviors and to users through interactive ‘grammar checking’ of the design drafts.	Mainly for <i>Escherichia coli</i>	[52]
OptCircuit	An optimization-based framework that automatically identifies components from a list and their connectivity for circuit redesign. It compiles comprehensive kinetic descriptions of promoter–protein interactions using deterministic ordinary differential equations and stochastic simulations.	Can be adapted	[53]
SynBioSS	Software suite for network design and simulation by calculating probability distributions of dynamic biological phenotypes. It contains three components: Designer, WIKI, and Simulator. Designer can transform part sequences into models for simulation in Simulator.	Can be adapted	[54,55]
CellDesigner	Software for graphical drawing of regulatory and biochemical networks.	Can be adapted	[56,57]
e-Cell	A modeling and simulation environment for cellular behavior prediction by building integrative models of the cell based on gene regulation, metabolism, and signaling and running <i>in silico</i> experiments.	Can be adapted	[58,59]
<i>Simulation and behavior prediction</i>			
COPASI	A stand-alone biochemical network simulator that allows easy switches between different simulation approaches.	Can be adapted	[60,61]
CompuCell3D	A multicell, multiscale model for simulation of highly conserved vertebrate somatogenesis by combining specialized hypotheses for specific subcomponent mechanisms into a unified multiscale model.	Can be adapted	[62,63]
CellModeller	A generic tool for the analysis and modeling of multicellular plant morphogenesis by analyzing hierarchical physical and biochemical morphogenetic mechanisms.	Plant specific	[64–66]

and optimized for prokaryotic systems, some are adaptable for plant synthetic biology providing the orthogonal parts for plant synthetic biology are available (Table 1). Moreover, novel tools dedicated for plant synthetic biology are being developed to address plant-specific questions. For example, CellModeller can be used to model cell–cell interactions during plant morphogenesis and is able to perform synthetic plant design [61–63]. Considering the diverse system outputs and limited resources available, computational tool development for plant synthetic biology should be a research priority.

### Pioneering examples of plant synthetic biology

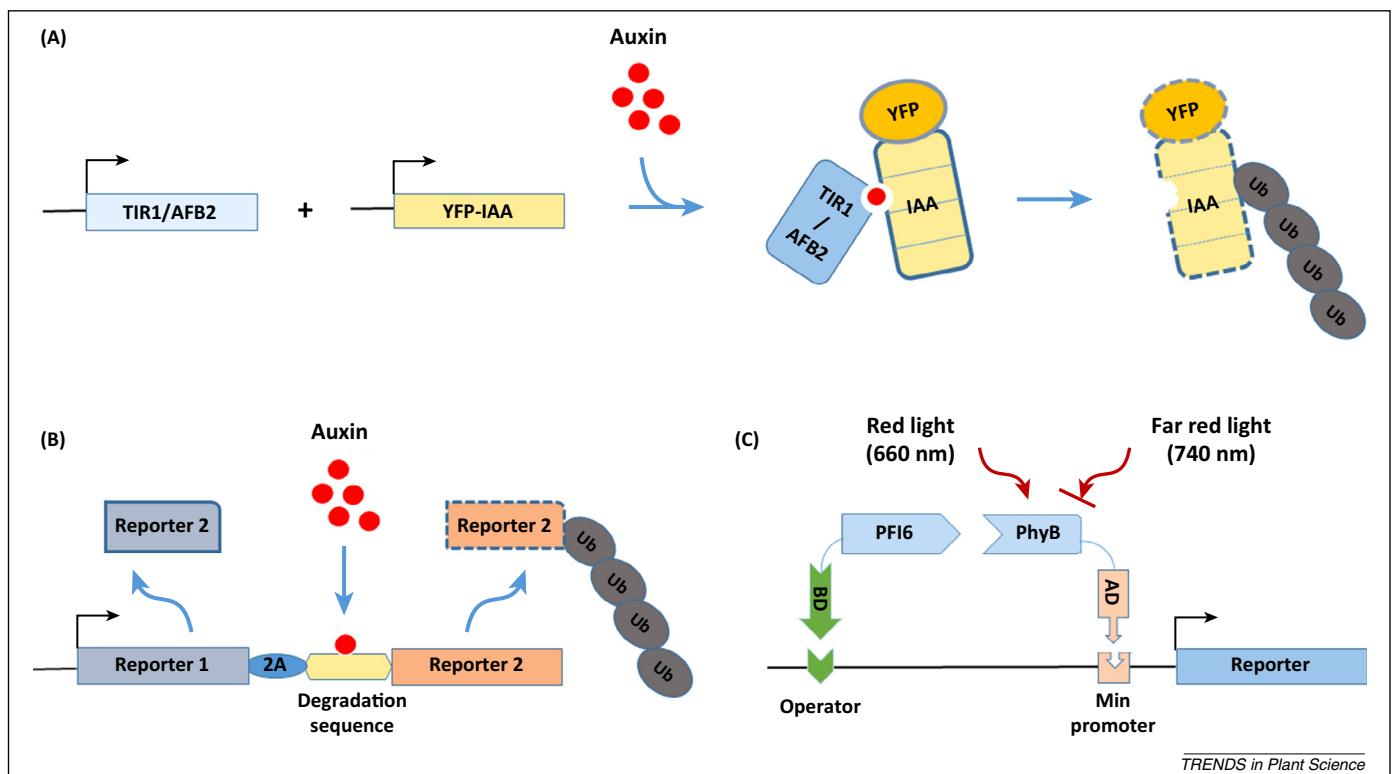
To date, there are only a few published examples of plant synthetic biology such as the production of synthetic sensors and synthetic metabolic pathways. There have been steps toward the production of synthetic genomes in plants, but we remain in the early stages of research.

### Synthetic sensors

Synthetic sensors can be constructed to be transcriptionally or post-translationally controlled when incorporated into engineered organisms and allow cells to identify and report the presence of internal or external stimuli. A good example of post-translationally controlled sensors is the construction of synthetic circuits for monitoring

auxin-induced plant indole-3-acetic acid (IAA) degradation in yeast [67]. Auxin activates gene expression by inducing IAA turnover through interaction with the auxin receptors F-box proteins TIR1/AFBs. The external application of auxin and pairwise mating of yeast expressing either a TIR1/AFB or a YFP-IAA permit auxin to bind to its receptors and IAA subunit II, leading to the ubiquitination and degradation of IAA, which can be monitored by YFP fluorescence (Figure 2A). This device allows precise control of external auxin application (input) for the dynamic measurement of the IAA-coupled YFP fluorescence (output), for the ability to study IAA and TIR1/AFB pairs in isolation, and for the absence of many other intrinsic plant pathways that affect auxin signaling. It also permits the investigation of a complex pathway encoded by large families of genes. Similarly, when linked to two luminescent genes, the 13-amino acid minimal degradation sequences from subunit II of three *Arabidopsis thaliana* IAA genes were used to construct a luminescent sensor for monitoring auxin-induced degradation [68]. Binding of auxin to the degradation sequences resulted in the degradation of the linked luminescent protein in *Arabidopsis* protoplasts (Figure 2B).

Transcriptionally controlled synthetic sensors can be constructed using synthetic promoters and/or transcription factors. A synthetic sensor for monitoring the transcriptional



**Figure 2.** Illustrations of synthetic sensors. (A) Device for monitoring auxin-induced plant indole-3-acetic acid (IAA) degradation in yeast (*Saccharomyces cerevisiae*). The auxin receptors F-box protein TIR1/AFB2 and YFP-IAA hybrid are strongly expressed in various yeast strains. The pairwise mating of yeast strains expressing TIR1/AFB2 or YFP-IAA and the external application of IAA lead to the binding of IAA of plant origin to TIR1/AFB2 via auxin and, thus, ubiquitination and degradation of YFP-IAA. Adapted from [67]. (B) Ratiometric luminescent sensor for monitoring auxin-induced degradation in plants. Two luminescent reporter genes are linked by an 18-amino acid viral 2A peptide that allows cotranslational, intraribosomal cleavage of both reporters and 13-amino acid minimal degradation sequences from subunit II of three *Arabidopsis* IAA genes. Binding of auxin to the degradation sequences resulted in degradation of the linked reporter 2 in *Arabidopsis* protoplasts, which can be monitored as a decrease in reporter 2 gene expression relative to reporter 1 gene expression. Adapted from [68]. (C) A red-light (660 nm)-controlled synthetic switch for light inducible expression in tobacco protoplasts. Red light illumination converts *Arabidopsis* phytochrome B (PhyB) into the active far-red form, which in turn induces heterodimerization with interacting factor 6 (PIF6). When the genes are linked to an activation domain (AD) and a DNA-binding domain (BD), respectively, this heterodimerization results in the activation of reporter gene expression driven by a minimal (Min) promoter and an operator that is bound by BD. This switch can be efficiently turned off by far-red light (740 nm). Adapted from [70]. Abbreviation: Ub, ubiquitin.

output of the plant cytokinin signaling network was constructed in *Arabidopsis* and maize (*Zea mays*) [69]. Cytokinin binds to its cognate receptors and initiates a multistep phospho-relay signaling cascade that ultimately phosphorylates nuclear transcription activators [i.e., the type-B nuclear ARABIDOPSIS RESPONSE REGULATORs (ARRs)]. Using a concatemer of 24 repeats of the consensus sequence recognized by the type-B ARRs to drive a GFP reporter, a synthetic device was constructed to monitor the transcriptional activity of type-B response regulators in stable transgenic *Arabidopsis*, in which strong and dynamic GFP expression patterns correlated with known cytokinin function and potentially revealed novel cytokinin functions [69]. A red-light-controlled synthetic switch was also constructed using the operator site of *Arabidopsis* phytochrome interacting factor 6 (PIF6) upstream of a minimal promoter [70]. Red-light illumination converts phytochrome B (PhyB) into the active far-red form and thus induces heterodimerization with its PIF6. When linked to an activation domain, PhyB binds to the DNA-binding domain-linked PIF6 and activates expression of the reporter gene in tobacco protoplasts (Figure 2C). This switch can be efficiently turned off by far-red light. Other good examples include synthetic circuits for phytosensing of explosives [21,22] or bacterial pathogens [71–73] in transgenic tobacco and *Arabidopsis*, of elicitors in *Arabidopsis* [74], and of soybean cyst nematode (*Heterodera glycines*) in soybean (*Glycine max*) [44] (reviewed in [34]).

### Synthetic metabolic pathways

One goal of plant synthetic biology is to build synthetic metabolic pathways for the production of large amounts of valuable metabolites that are difficult to obtain in natural ways or too costly or complex to produce by chemical synthesis or conventional plant biotechnology [24,75–78]. Expression of individual enzymes constituting biosynthetic pathways can be altered in novel ways. For example, vessel-specific expression of the cinnamoyl-coenzyme A (CoA) 4-ligase (*C4L*) gene and the introduction of an artificial positive feedback loop expressing a master fiber transcription factor (*NST1*) with one of its downstream induced promoters in *Arabidopsis c4l* mutants resulted in reduced lignin content and enhanced polysaccharide deposition for biofuel production [79]. When fused to the transit peptide from *Arabidopsis* ferredoxin (Fd), three sorghum (*Sorghum bicolor*) enzymes comprising the cytochrome P450-dependent pathway (two P450s, CYP79A1 and CYP71E1, and the UDP-glucosyltransferase UGT85B) were relocated to *Nicotiana benthamiana* chloroplasts, resulting in expression of the entire dhurrin pathway in the chloroplasts (Figure 3A) [80]. The reducing power generated by photosystem I was directly used for P450s in chloroplasts.

Individual biosynthetic enzymes can also be combined in novel ways for metabolite production. A good example is the production of sesquiterpenoid artemisinic acid in yeast as an antimalarial drug precursor [19]. A 500-fold higher production of sesquiterpene was achieved using multiple synthetic pathways: (i) overexpression of a truncated 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (*tHMGR*) and downregulation of a squalene synthase (*ERG9*) by a methionine-repressible promoter to increase farnesyl pyrophosphate

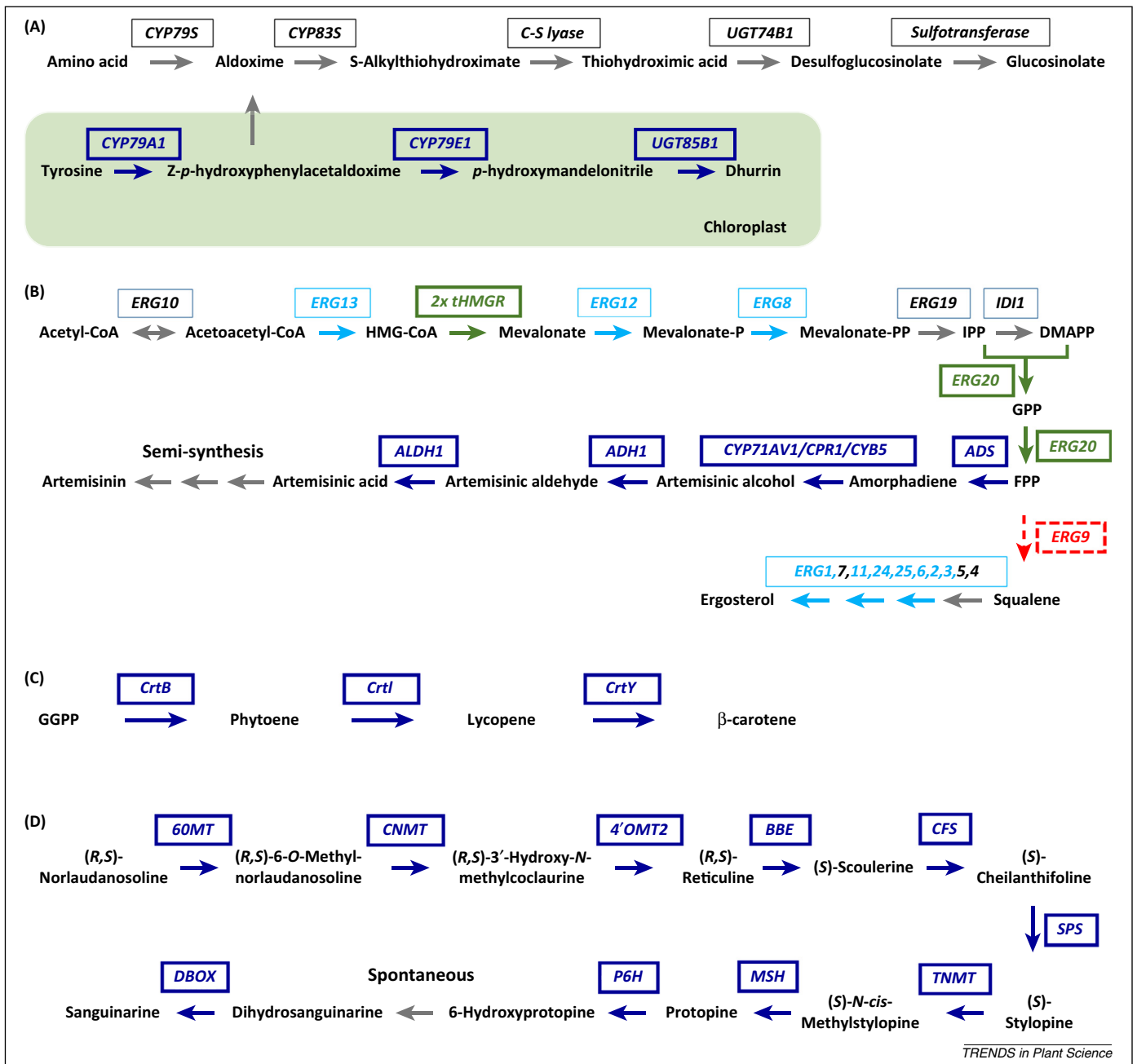
(FPP) production and decrease its use for sterols; (ii) combination of a semidominant mutant allele of a Zn(II)2Cys6 binuclear cluster transcription factor (*upc2-1*) with a downregulating *ERG9* and the addition of the amorphaadiene synthase gene (*ADS*) from the *Artemisia annua* and an additional copy of *tHMGR* for conversion of FPP to amorphaadiene; and (iii) expression of a cytochrome P450 (*CYP71AV1*) and its redox partner *CPR* under the control of galactose-inducible promoters for artemisinic acid production from amorphaadiene (Figure 3B). By replacing and adding selected parts, a more efficient synthetic pathway was developed for potent antimalarial artemisinin production in yeast [81].

Moreover, an entire synthetic biosynthetic pathway can be constructed for novel compound production. For example, tissue-specific expression of a synthetic bacterial mini-pathway containing three *Erwinia* genes encoding phytoene synthase (*CrtB*), phytoene desaturase (*CrtI*), and lycopene beta-cyclase (*CrtY*) under tuber-specific promoter control resulted in golden tubers in potato (*Solanum tuberosum*) in which carotenoids were increased 20-fold and beta-carotene (i.e., provitamin A) 3600-fold (Figure 3C) [20]. Transgenic *Arabidopsis* expressing the entire biosynthetic pathway from sorghum resulted in the accumulation of 4% dry weight dhurrin [18]. Plant alkaloid dihydrosanguinarine was produced in yeast by reconstruction of a ten-gene pathway (Figure 3D) [82].

Increasing the activities of key enzymes and thus the flux of metabolites toward the production of desired metabolites are key points for synthetic metabolic pathway construction. A broad understanding of the biochemical pathways and networks of useful secondary metabolites is needed for future plant metabolic engineering; equally important is to understand plant metabolism. These may later be guided by genome-scale metabolic modeling and metabolic flux analysis.

### Synthetic plant genomes

One far-reaching goal of synthetic biology is the construction of *de novo* genomes. Following the pioneering work in the construction of a bacterial cell harboring a synthesized 1.08 Mb *Mycoplasma* genome [13] and the synthesis of the right arm of chromosome IX and a portion of chromosome VI in yeast [83], three design principles for synthetic genomes have been developed: (i) the resulting synthetic genomes should have (near) wild type phenotypes and fitness; (ii) they should not contain destabilizing elements (tRNA genes and transposons); and (iii) there should exist genetic flexibility for future studies (e.g., genome editing sites) [83]. While still in the distant future, the deployment of a streamlined genome into a plant will be an important accomplishment in plant synthetic biology and is mainly limited by insufficient knowledge about the minimum gene set required to make a minimum genome [24,84]. Compared with the synthesis of synthetic genomes, the successful implementation of synthetic genomes in different hosts will be very challenging [85]. Thus, it makes sense to begin the plant synthetic genome challenge by starting with an already streamlined genome: the plastome toward the production of a synthetic chloroplast.



**Figure 3.** Illustrations of synthetic metabolic pathways. **(A)** A synthetic pathway for production of the cyanogenic glucoside dhurrin in *Nicotiana benthamiana* chloroplasts. Three sorghum (*Sorghum bicolor*) enzymes comprising the cytochrome P450-dependent pathway (two P450s, CYP79A1 and CYP71E1, and the UDP-glucosyltransferase UGT85B) were relocated to *N. benthamiana* chloroplasts resulting in expression of the entire dhurrin pathway (blue arrows) in the chloroplasts. The preexisting glucosinolate biosynthetic pathway (gray arrows) uses CYP79A1-produced *p*-hydroxyphenylacetaldoxime for the production of glucosinolate with the help of the endogenous post-oxime-metabolizing enzymes CYP83B1, C-S lyase (SUR1), and UGT74B1. The presence of CYP79E1 prevents the glucosinolate production from CYP79A1. Adapted from [18,80]. **(B)** A synthetic pathway for a 500-fold higher production of sesquiterpenoid artemisinin acid in yeast (*Saccharomyces cerevisiae*) as an antimalarial drug precursor. Genes from the endogenous mevalonate pathway in yeast that are directly or indirectly upregulated or repressed are shown in green, light blue, and red, respectively. Downregulation of a squalene synthase (*ERG9*) by a methionine-repressible promoter was used to decrease the use of farnesyl pyrophosphate (FPP) for sterols. The biosynthetic pathway from FPP to artemisinic acid in *Artemisia annua* was introduced into yeast for artemisinic acid production and is shown in blue. Adapted from [19]. **(C)** A synthetic bacterial mini-pathway for a 3600-fold increase in beta-carotene (i.e., provitamin A) production. This mini-pathway contained three *Erwinia* genes encoding phytoene synthase (*CrtB*), phytoene desaturase (*CrtI*), and lycopene beta-cyclase (*CrtY*) under tuber-specific promoter control resulted in golden tubers in potato (*Solanum tuberosum*). Adapted from [20]. **(D)** Reconstitution of the sanguinarine biosynthetic pathway from the commercial precursor (R,S)-norlaudanoline to sanguinarine in yeast. The ten heterogeneous genes were from opium poppy (*Papaver somniferum*), with the exception of *P6H* from *Eschscholzia californica*. Metabolites are shown in black, enzymes in italic and boxed, and heterogeneous enzymes in blue. Adapted from [82]. Abbreviations: *ADH1*, alcohol dehydrogenase; *ALDH1*, aldehyde dehydrogenase; *ADS*, amorphaadiene synthase gene; *BBE*, berberine bridge enzyme; *CFS*, cheilanthifoline synthase; *CNMT*, coclaurine *N*-methyltransferase; *CrtI*, phytoene desaturase/carotene isomerase; *DBOX*, dihydrobenzo-phenanthridine oxidase; *DMAPP*, dimethylallyl pyrophosphate; *GGPP*, geranylgeranyl diphosphate; *GPP*, geranyl pyrophosphate; *IPP*, isopentenyl pyrophosphate; *MSH*, (S)-*cis*-*N*-methylstylophine 14-hydroxylase; *P6H*, protopine-6-hydroxylase; *SPS*, stylophine synthase; *tHMGR*, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; *TNMT*, tetrahydroprotoberberine *cis*-*N*-methyltransferase; *4'OMT2*, 4'-*O*-methyltransferase 2; *6OMT*, 6-*O*-methyltransferase.

The prokaryotic nature of the gene structure of the plastome should make it amenable for building synthetic circuits in plants. Pioneering work toward a synthetic chloroplast was conducted in zebrafish embryos when the engineered photosynthetic cyanobacterium *Synechococcus elongatus* PCC 7942 was injected into or taken up by mammalian macrophages [86]. *S. elongatus* expressing a synthetic operon containing invasins from *Yersinia pestis* and listeriolysin O from *Listeria monocytogenes* can invade and replicate inside mammalian macrophages, although at marginal rates, resulting in artificial animal chloroplasts [86]. A plastome engineered for the entire mevalonate pathway was also accomplished in tobacco, in which multiple genes in the pathway were coordinately controlled by a single promoter on an artificial operon [87]. Moreover, synthetic riboswitches have also been successfully implemented in tobacco chloroplast genomes as efficient translational regulators of plastid gene expression in response to exogenously applied ligands [88]. Thus, using the plastome as a vector, high and coordinated expression without the need of multiple promoters should enable synthetic biology in plants for metabolic engineering and subsequent genome editing.

The design of a synthetic plastome as well as synthesizing a 100–150-kb circular genome is no longer a technical challenge given the huge leaps in DNA synthesis and assembly. It remains unclear how researchers might introduce a complete synthetic plastome and replace the endogenous plastome within cells, but this quest is a current target. Advances in synthetic plastomes and plasmids would allow cost-effective and large-scale production of enzymes and biopharmaceuticals of commercial interest among successful synthetic approaches for the precision engineering of lignin synthesis [79], coupling photosynthetic reducing power for dhurrin production in chloroplasts [80], and the biosynthesis of bioactive natural products (i.e., artemisinin [19,81], carotenoids [20], dhurrin [18], alkaloid [82], and vanillin [89]).

### Concluding remarks and future perspectives

The existing, albeit small, plant synthetic biology community has begun to make great strides in applying microbial synthetic biology principles and methodologies for the introduction of synthetic promoters, genes, pathways, and traits into plants for human and environmental benefits. It is expected that plant synthetic biology will play an increasingly important role in conferring stress tolerance and increasing the production of food, biofuels, metabolites, therapeutics, and even completely synthetic life forms. However, its progress is currently slow, costly, and laborious. Plant synthetic biology development is fundamentally limited not only by the availability of well-characterized and interchangeable parts and modules, but also by the modeling, assembly, and fine-tuning of synthetic gene networks [90] (Box 1). Biological parts and modules are sometimes prone to context dependency and might not be completely predictable. In addition, the integration of synthetic devices into a plant host raises compatibility issues including codon optimization, genetic instability, genomic position effects, and regulatory incompatibilities.

### Box 1. Outstanding questions

- How can we scale up the current simple devices to larger and more complex synthetic biological systems while minimizing unexpected networks as the network size and complexity increases?
- How can computational models for design and simulation be significantly improved so that synthetic circuits can better meet inherent biological variability, uncertainty, and evolution?
- What are the advantages and disadvantages of such plant synthetic approaches?
- Will we be able to construct synthetic plants in a similar way as we design cars and computers?
- How far away are we from constructing a living plant from scratch?
- How will the products of plant synthetic biology be regulated?
- Can the plant science community effectively communicate the risks and benefits of new applications and products to consumers?

To overcome these obstacles and limitations, research is needed to improve and accelerate plant synthetic biology design cycles. Developing larger libraries of orthogonal biological parts and modules can be achieved by decomposing networks into the parts and modules that comprise them, mutation library screening, directed evolution [36], rational design, and even the information and insights provided by systems biology. Ongoing development of algorithms, models, and software will assist in better characterization and standardization of the orthogonality of more parts and modules as well as better rational designs. Improvements in the transformation efficiency of crops would enable major advances in synthetic biology in plants. For example, the development of single-cell plant models would enable quicker cycling times for design and implementation. Encouragingly, funding agencies in various countries have started to support plant synthetic biology projects. For example, the US Department of Energy Advanced Research Projects Agency–Energy (ARPA-E) has funded their Plants Engineered to Replace Oil (PETRO) program [91]. The UK Department of Business Innovation and Skills (BIS), Research Councils UK (RUCK), and other UK funds have invested in various plant synthetic biology programs [25].

Given the ‘genetically modified organism (GMO) situation’ in which transgenic crops have been heavily regulated and controversial, it is of interest to understand how synthetic biology might be viewed in applied agriculture. While genome-edited plants that have had a few nucleotides changed or deleted in a gene might not be regulated or viewed as a GMO [92], large DNA inserts and synthetic genomes might be viewed as regulatory nightmares for commercial interests. That would be a pity given the potential benefits of synthetic biology in agriculture and the bioeconomy. Therefore, we recommend frequent communication among scientists interested in applications of synthetic biology and regulators, sociologists, and other interested parties.

### Acknowledgments

The authors thank the University of Tennessee, a US Department of Agriculture Hatch Grant, the US Department of Energy ARPA-E PETRO, and the BioEnergy Science Center (BESC) for funding. The BESC is a Bioenergy Research Center supported by the Office of Biological and Environmental Research in the Department of Energy (DOE) Office of Science.

## References

- 1 Slusarczyk, A.L. *et al.* (2012) Foundations for the design and implementation of synthetic genetic circuits. *Nat. Rev. Genet.* 13, 406–420
- 2 Gardner, T.S. *et al.* (2000) Construction of a genetic toggle switch in *Escherichia coli*. *Nature* 403, 339–342
- 3 Elowitz, M.B. and Leibler, S. (2000) A synthetic oscillatory network of transcriptional regulators. *Nature* 403, 335–338
- 4 Purnick, P.E.M. and Weiss, R. (2009) The second wave of synthetic biology: from modules to systems. *Nat. Rev. Mol. Cell Biol.* 10, 410–422
- 5 Guet, C.C. *et al.* (2002) Combinatorial synthesis of genetic networks. *Science* 296, 1466–1470
- 6 Kobayashi, H. *et al.* (2004) Programmable cells: interfacing natural and engineered gene networks. *Proc. Natl. Acad. Sci. U.S.A.* 101, 8414–8419
- 7 You, L. *et al.* (2004) Programmed population control by cell–cell communication and regulated killing. *Nature* 428, 868–871
- 8 Anderson, J.C. *et al.* (2006) Environmentally controlled invasion of cancer cells by engineering bacteria. *J. Mol. Biol.* 335, 619–627
- 9 Lu, T.K. and Collins, J.J. (2007) Dispersing biofilms with engineered enzymatic bacteriophage. *Proc. Natl. Acad. Sci. U.S.A.* 104, 11197–11202
- 10 Lu, T.K. and Collins, J.J. (2009) Engineered bacteriophage targeting gene networks as adjuvants for antibiotic therapy. *Proc. Natl. Acad. Sci. U.S.A.* 106, 4629–4634
- 11 Smith, H.O. *et al.* (2003) Generating a synthetic genome by whole genome assembly:  $\Phi$ X174 bacteriophage from synthetic oligonucleotides. *Proc. Natl. Acad. Sci. U.S.A.* 100, 15440–15445
- 12 Chan, L.Y. *et al.* (2005) Refactoring bacteriophage T7. *Mol. Syst. Biol.* 1, 2005.0018
- 13 Gibson, D.G. *et al.* (2010) Creation of a bacterial cell controlled by a chemically synthesized genome. *Science* 329, 52–56
- 14 James, J.R. and Vale, R.D. (2012) Biophysical mechanism of T-cell receptor triggering in a reconstituted system. *Nature* 487, 64–69
- 15 Dormitzer, P.R. *et al.* (2013) Synthetic generation of influenza vaccine viruses for rapid response to pandemics. *Sci. Transl. Med.* 5, 185ra68
- 16 Ulmer, J.B. *et al.* (2012) RNA-based vaccines. *Vaccine* 30, 4414–4418
- 17 Weber, W. and Fussenegger, M. (2012) Synthetic gene networks in mammalian cells. *Curr. Opin. Biotechnol.* 21, 690–696
- 18 Kristensen, C. *et al.* (2005) Metabolic engineering of dhurrin in transgenic *Arabidopsis* plants with marginal inadvertent effects on the metabolome and transcriptome. *Proc. Natl. Acad. Sci. U.S.A.* 102, 1779–1784
- 19 Ro, D.K. *et al.* (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* 440, 940–943
- 20 Diretto, G. *et al.* (2007) Metabolic engineering of potato carotenoid content through tuber-specific overexpression of a bacterial mini-pathway. *PLoS ONE* 2, e350
- 21 Antunes, M.S. *et al.* (2009) Engineering key components in a synthetic eukaryotic signal transduction pathway. *Mol. Syst. Biol.* 5, 270
- 22 Antunes, M.S. *et al.* (2011) Programmable ligand detection system in plants through a synthetic signal transduction pathway. *PLoS ONE* 6, e16292
- 23 Schwillie, P. (2011) Bottom-up synthetic biology: engineering in a tinkerer's world. *Science* 333, 1252–1254
- 24 Scharff, L.B. and Bock, R. (2014) Synthetic biology in plastids. *Plant J.* 78, 783–798
- 25 Cook, C. *et al.* (2014) Opportunities in plant synthetic biology. *J. Exp. Bot.* Published online February 6, 2014. <http://dx.doi.org/10.1093/jxb/eru013>
- 26 Chen, Y.Y. *et al.* (2012) Synthetic biology: advancing biological frontiers by building synthetic systems. *Genome Biol.* 13, 240
- 27 Andrianantoandro, E. *et al.* (2006) Synthetic biology: new engineering rules for an emerging discipline. *Mol. Syst. Biol.* 2, 2006.0028
- 28 Barrett, C.L. *et al.* (2006) Systems biology as a foundation for genome-scale synthetic biology. *Curr. Opin. Biotechnol.* 17, 1–5
- 29 Church, G.M. *et al.* (2014) Realizing the potential of synthetic biology. *Nat. Rev. Mol. Cell Biol.* 15, 289–294
- 30 Kitney, R. and Freemont, P. (2012) Synthetic biology – the state of play. *FEBS Lett.* 586, 2029–2036
- 31 Alterovitz, G. *et al.* (2009) The challenges of informatics in synthetic biology: from biomolecular networks to artificial organisms. *Brief. Bioinform.* 11, 80–95
- 32 Rollie, S. *et al.* (2011) Designing biological systems: systems engineering meets synthetic biology. *Chem. Eng. Sci.* 69, 1–29
- 33 Way, J.C. *et al.* (2014) Integrating biological redesign: where synthetic biology came from and where it needs to go. *Cell* 157, 151–161
- 34 Liu, W. *et al.* (2013) Advanced genetics tools for plant biotechnology. *Nat. Rev. Genet.* 14, 781–793
- 35 Endy, D. (2005) Foundations for engineering biology. *Nature* 438, 449–453
- 36 Haseltine, E.L. and Arnold, F.H. (2007) Synthetic gene circuits: design with directed evolution. *Annu. Rev. Biophys. Biomol. Struct.* 36, 1–19
- 37 Heinemann, M. and Panke, S. (2006) Synthetic biology – putting engineering into biology. *Bioinformatics* 22, 2790–2799
- 38 de Lorenzo, V. and Danchin, A. (2008) Synthetic biology: discovering new worlds and new words. *EMBO Rep.* 9, 822–827
- 39 Bashor, C.J. *et al.* (2008) Using engineered scaffold interactions to reshape MAP kinase pathway signaling dynamics. *Science* 319, 1539–1543
- 40 Lim, W.A. (2010) Designing customized cell signaling circuits. *Nat. Rev. Mol. Cell Biol.* 11, 393–403
- 41 Mukherji, S. and van Oudenaarden, A. (2009) Synthetic biology: understanding biological design from synthetic circuits. *Nat. Rev. Genet.* 10, 859–871
- 42 Nandagopal, N. and Elowitz, M.B. (2011) Synthetic biology: integrated gene circuits. *Science* 333, 1244–1248
- 43 Venter, M. (2007) Synthetic promoters: genetic control through *cis* engineering. *Trends Plant Sci.* 12, 118–124
- 44 Liu, W. *et al.* (2014) Computational discovery of soybean promoter *cis*-regulatory elements for the construction of soybean cyst nematode inducible synthetic promoters. *Plant Biotechnol. J.* 12, 1015–1026
- 45 Smolke, C.D. (2009) Building outside of the box: iGEM and the BioBricks Foundation. *Nat. Biotechnol.* 27, 1099–1102
- 46 Yancey, A. and Stewart, C.N., Jr (2007) Are university researchers at risk for patent infringement? *Nat. Biotechnol.* 25, 1225–1228
- 47 Lux, M.W. *et al.* (2011) Genetic design automation: engineering fantasy or scientific renewal? *Trends Biotechnol.* 30, 120–126
- 48 Marchisio, M. and Stelling, J. (2009) Computational design tools for synthetic biology. *Curr. Opin. Biotechnol.* 20, 479–485
- 49 Medema, M.H. *et al.* (2012) Computational tools for the synthetic design of biochemical pathways. *Nat. Rev. Microbiol.* 10, 191–202
- 50 Richardson, S.M. *et al.* (2006) GeneDesign: rapid, automated design of multikilobase synthetic genes. *Genome Res.* 16, 550–556
- 51 Villalobos, A. *et al.* (2006) Gene Designer: a synthetic biology tool for constructing artificial DNA segments. *BMC Bioinform.* 7, 285
- 52 Czar, M.J. *et al.* (2009) Writing DNA with GenoCAD™. *Nucleic Acids Res.* 37, W40–W47
- 53 Dasika, M.S. and Maranas, C.D. (2008) OptCircuit: an optimization based method for computational design of genetic circuits. *BMC Syst. Biol.* 2, 24
- 54 Hill, A.D. *et al.* (2008) Synthetic BiologySS: the synthetic biology modeling suite. *Bioinformatics* 24, 2551–2553
- 55 Weeding, E. *et al.* (2010) SynBioSS designer: a web-based tool for the automated generation of kinetic models for synthetic biological constructs. *Brief. Bioinform.* 11, 394–402
- 56 Funahashi, A. (2008) CellDesigner 3.5: a versatile modeling tool for biochemical networks. *Proc. IEEE* 96, 1254–1265
- 57 Funahashi, A. *et al.* (2003) CellDesigner: a process diagram editor for gene-regulatory and biochemical networks. *Biosilico* 1, 159–162
- 58 Ishii, N. *et al.* (2007) Multiple high-throughput analyses monitor the response of *E. coli* to perturbations. *Science* 316, 593–597
- 59 Tomita, M. *et al.* (1999) E-CELL: software environment for whole-cell simulation. *Bioinformatics* 15, 72–84
- 60 Hoops, S. (2006) COPASI – a COMplex PATHway SIMulator. *Bioinformatics* 22, 3067–3074
- 61 Mendes, P. *et al.* (2009) Computational modeling of biochemical networks using COPASI. In *Systems Biology* (Maly, I.V., ed.), pp. 17–59, Springer
- 62 Chaturvedi, R. *et al.* (2003) Multi-model simulations of chicken limb morphogenesis. *Lect. Notes Comput. Sci.* 2659, 39–49
- 63 Hester, S.D. *et al.* (2011) A multi-cell, multi-scale model of vertebrate segmentation and somite formation. *PLoS Comput. Biol.* 7, e1002155
- 64 Dupuy, L. *et al.* (2008) A system for modelling cell–cell interactions during plant morphogenesis. *Ann. Bot.* 101, 1255–1265



- 65 Federici, F. *et al.* (2012) Integrated genetic and computation methods for *in planta* cytometry. *Nat. Methods* 9, 483–485
- 66 Depuy, L. *et al.* (2010) Coordination of plant cell division and expansion in a simple morphogenetic system. *Proc. Natl. Acad. Sci. U.S.A.* 107, 2711–2716
- 67 Havens, K.A. *et al.* (2012) A synthetic approach reveals extensive tunability of auxin signaling. *Plant Physiol.* 160, 135–142
- 68 Weed, S. *et al.* (2013) A quantitative ratiometric sensor for time-resolved analysis of auxin dynamics. *Sci. Rep.* 3, 1–7
- 69 Zurcher, E. *et al.* (2013) A robust and sensitive synthetic sensor to monitor the transcriptional output of the cytokinin signaling network in *in planta*. *Plant Physiol.* 161, 106–107
- 70 Muller, K. *et al.* (2014) A red light-controlled synthetic gene expression switch for plant system. *Mol. Plant* 10, 1679–1688
- 71 Liu, W. *et al.* (2011) Rapid *in vivo* analysis of synthetic promoters for plant pathogen phytoensing. *BMC Biotechnol.* 11, 108
- 72 Liu, W. *et al.* (2013) Bacterial pathogen phytoensing in transgenic tobacco and *Arabidopsis*. *Plant Biotechnol. J.* 11, 43–52
- 73 Fethe, M.H. *et al.* (2014) The performance of pathogenic bacterial phytoensing transgenic tobacco in the field. *Plant Biotechnol. J.* 12, 755–764
- 74 Koschmann, J. *et al.* (2012) Intergration of bioinformatics and synthetic promoters leads to the discovery of novel elicitor-responsive *cis*-regulatory sequences in *Arabidopsis*. *Plant Physiol.* 160, 178–191
- 75 DePaoli, H.C. *et al.* (2014) Synthetic biology as it relates to CAM photosynthesis: challenges and opportunities. *J. Exp. Bot.* Published online February 24, 2014. <http://dx.doi.org/10.1093/jxb/eru038>
- 76 Fesenko, E. and Edwards, R. (2014) Plant synthetic biology: a new platform for industrial biotechnology. *J. Exp. Bot.* 65, 1927–1937
- 77 Rogers, C. and Oldroyd, G.E.D. (2014) Synthetic biology approaches to engineering the nitrogen symbiosis in cereals. *J. Exp. Bot.* 65, 1939–1946
- 78 Farre, G. *et al.* (2014) Engineering complex metabolic pathways in plants. *Annu. Rev. Plant Biol.* 65, 187–223
- 79 Yang, F. *et al.* (2013) Engineering secondary cell wall deposition in plants. *Plant Biotechnol. J.* 11, 325–335
- 80 Nielsen, A.Z. *et al.* (2014) Redirecting photosynthetic reducing power toward bioactive natural product synthesis. *ACS Synth. Biol.* 2, 308–315
- 81 Paddon, C.J. *et al.* (2013) High-level semi-synthetic production of the potent antimalarial artemisinin. *Nature* 496, 528–532
- 82 Fossati, E. *et al.* (2014) Reconstitution of a 10-gene pathway for synthesis of the plant alkaloid dihydrosanguinarine in *Saccharomyces cerevisiae*. *Nat. Commun.* 5, 3283
- 83 Dymond, J.S. *et al.* (2011) Synthetic chromosome arms function in yeast and generate phenotypic diversity by design. *Nature* 477, 471–476
- 84 Juhas, M. *et al.* (2011) Essence of life: essential genes of minimal genomes. *Trends Cell Biol.* 21, 562–568
- 85 Baker, M. (2011) The next step for the synthetic genome. *Nature* 473, 403–408
- 86 Agapakis, C.M. *et al.* (2011) Towards a synthetic chloroplast. *PLoS ONE* 6, e18877
- 87 Kumar, S. *et al.* (2012) Remodeling the isoprenoid pathway in tobacco by expressing the cytoplasmic mevalonate pathway in chloroplasts. *Metab. Eng.* 14, 19–28
- 88 Verhounig, A. *et al.* (2010) Inducible gene expression from the plastid genome by a synthetic riboswitch. *Proc. Natl. Acad. Sci. U.S.A.* 107, 6204–6209
- 89 Gallage, N.J. and Moller, B.L. (2014) Vanillin – bioconversion and bioengineering of the most popular plant flavor and its *de novo* biosynthesis in the vanilla orchid. *Mol. Plant* 8, 40–57
- 90 Lu, T.K. *et al.* (2009) Next-generation synthetic gene networks. *Nat. Biotechnol.* 27, 1139–1150
- 91 Borak, B. *et al.* (2013) Energy and carbon accounting to compare bioenergy crops. *Curr. Opin. Biotechnol.* 24, 369–375
- 92 Jones, H.D. (2015) Regulatory uncertainty over genome editing. *Nat. Plants* Published online January 8, 2015. <http://dx.doi.org/10.1038/nplants.2014.11>