Plant synthetic biology

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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-invading bacteria [8] and defense-enhancing bacteriophage as antibiotic adjuvants [9,10]. Synthetic biology projects have also been completed to reconstruct the phage Φ 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 is still 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 regards 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. 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 in order 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; i.e., understanding vs building. In this review, we discuss the engineering-biology principles that comprise synthetic biology and apply them to plants. Compared to 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 encompass 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 is used to help the determination and optimization of network hierarchies, kinetic parameters and parts selection [1]. It also helps modeling for analysis of network behaviors, sensitivity and robustness to perturbations, and 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 on some other enabling tools that are used in plant biotechnology and have great potentials 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's 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 drivetrain, electronics, the interior, etc. 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 installed into a plant. Standardization is used to define and characterize orthogonal parts and standardized conditions for testing [35]. Standardization relies on 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 in order to interact with the appropriate transcriptional factor(s). 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 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].

Even though 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, ‘phyto bricks’ (www.plantfab.org) database only contains a few (~20) parts. One reason for the paucity of phyto bricks might be the result of 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], have likely played a role in the lack of deposition of phyto bricks into registries. For examples, 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

Computer-aided design (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 prior to construction. These tools could be grouped into three categories, i.e., component design and synthesis [50,51], topology and network design [52-59], and behavior prediction and simulation [60-66] (Table 1). Even though many of these tools have been designed and optimized for prokaryotic systems, some of them 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 on 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 are still 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 for 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 TIR1/AFBs or a yellow fluorescent protein (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 for precise control of external auxin application (input), for the dynamic measurement of the IAA-coupled YFP fluorescence (output), for the ability to study the IAA and TIB1/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 the 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 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, which 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 green fluorescent protein (*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 the *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, a vessel-specific expression of the cinnamoyl-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* mutant resulted in reduced lignin contents and enhanced polysaccharide deposition for biofuel production [79]. When fused to the transit peptide from the *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 the *Nicotiana benthamiana* chloroplast, resulting in expression of the entire dhurrin pathway in chloroplasts (Figure 3A; [80]). The reducing power generated by photosystem I was directly used for P450s in chloroplast.

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 the sesquiterpene was achieved using multiple synthetic pathways: (1) overexpression of a truncated 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (*tHMGR*) and down-regulation of a squalene synthase (*ERG9*) by a methionine-repressible promoter to increase farnesyl pyrophosphate (FPP) production and decrease its use for sterols; (2) combination of a semi-dominant mutant allele of a Zn(II)₂Cys₆ binuclear cluster transcription factor (*upc2-1*) with a downregulating *ERG9* and additions of the amorphadiene synthase gene (*ADS*) from the plant *Artemisia annua* and an additional copy of *tHMGR* for conversion of FPP to amorphadiene; and (3) expression of a cytochrome P450 (*CYP71AV1*) and its redox partner *CPR* under the control of galactose-inducible promoters for artemisinic acid production from amorphadiene (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, the 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 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 accumulation of 4% dry-weight dhurrin [18]. Plant alkaloid dihydrosanguinarine was produced in yeast by reconstruction of a 10-gene pathway (Figure 3D; [82]).

Increasing the activities of key enzymes and thus the flux of metabolites towards 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) wildtype 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 our insufficient knowledge about the minimum gene set required to make a minimum genome [24,84]. Contrary to the synthesis of synthetic genomes, the successful implementation of synthetic genomes into 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.

The prokaryotic nature of 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 engineered photosynthetic cyanobacterium *Synechococcus elongatus* PCC 7942 was injected into or taken up by mammalian macrophages [86]. *S. elongatus* expressing a synthetic operon containing invasin from *Yersinia pestis* and listeriolysin O from *Listeria monocytogenes* can invade and replicate inside mammalian macrophages though 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 into 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 using 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 huge leaps in DNA synthesis and assembly. It is still unclear how researchers might introduce a complete synthetic plastome and replace the endogenous plastome within cells, but this quest is a current target by researchers. Advances in synthetic plastomes and plastids would allow a cost-effective and large-scale production of enzymes and biopharmaceuticals of commercial interest, among those successful synthetic approaches for the precision engineering of lignin synthesis [79], coupling photosynthetic reducing power for dhurrin production in chloroplast [80], and biosynthesis of bio-active 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 would 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 by not only the availability of well-characterized and interchangeable parts and modules, but also 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.

To overcome the above-mentioned 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 information and insights that are provided by systems biology. Ongoing development of algorithms, models and software will assist a 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 advancement in the 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 different countries 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), the Research Councils UK (RUCK) and other UK funds have invested in various plant synthetic biology programs [25].

Given the ‘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 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 well be viewed as regulatory nightmares for commercial interests. That would be a pity given the potential benefits of synthetic biology in agriculture and the bio-economy. Therefore, we recommend frequent communication among scientists interested in applications of synthetic biology and regulators, sociologists, and other interested parties.

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Table 1: Currently available computational tools for plant synthetic biology

Software	Description	Adaptability to plants	Refs
Component synthesis and design			
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]
Topology and network design			
GenoCAD	A framework including a formal semantic model which 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 in order 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]
Cell Designer	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	Can be adapted	[58,59]

	building integrative models of the cell based on gene regulation, metabolism and signaling and running <i>in silico</i> experiments.		
Simulation and behavior prediction			
COPASI	A stand-alone biochemical network simulator that allows easy switches between different simulation approaches.	Can be adapted	[60,61]
CompuCell3D	A multi-cell, multi-scale model for simulation of highly conserved vertebrate somatogenesis by combining specialized hypothesis for specific subcomponent mechanisms into a unified multi-scale 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]

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?

667 **Glossary**

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

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

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

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

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

677 **Conglomerate biological device:** biological device consisting of heterogeneous parts.

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

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

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

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

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

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

686 **Phytobricks:** standard DNA sequences (such as promoters, coding sequences and
687 terminators) used as Lego-like building blocks for the design and assembly of synthetic
688 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.

Figure Legends

Figure 1. Design cycle for plant synthetic biology driven by engineering principles. The ideal design cycles of plant synthetic biology encompasses 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 (dotted 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.

Figure 2. Illustrations of synthetic sensors. (A) Device for monitoring auxin-induced plant IAA degradation in yeast (*Saccharomyces cerevisiae*) (modified from [67]). The auxin receptors F-box protein TIR1/AFB2 and yellow fluorescent protein (YFP)-IAA hybrid are strongly expressed in different 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. (B) Ratiometric luminescent sensor for monitoring auxin-induced degradation in plants (modified from [68]). Two luminescent reporter genes are linked by an 18-amino acid-long viral 2A peptide that allows for a co-translational, intraribosomal cleavage of both reporters, and a 13-amino acid minimal degradation sequences from the subunit II of three *Arabidopsis* IAA genes. Binding of auxin to the degradation sequences resulted in the 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. (C) A red light (660 nm)-controlled synthetic switch for light inducible expression in tobacco protoplast (modified from [70]). Red light illumination converts *Arabidopsis* phytochrome B (PhyB) into the active far-red form, which in turn, induces heterodimerization with its interacting factor 6 (PIF6). When both 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 which is bound by the DNA binding domain. This switch can be efficiently turned off by far-red light (740 nm).

Abbreviations: IAA, Indole-3-acetic acid; Ub, ubiquitin.

Figure 3. Illustrations of synthetic metabolic pathways. (A) A synthetic pathway for the cyanogenic glucoside dhurrin production in the *Nicotiana benthamiana* chloroplast (modified from [18,80]). Three sorghum (*Sorghum bicolor*) enzymes comprising the cytochrome P450-dependent pathway (two P450s CYP79A1 and CYP71E1 and the UDP-glucosyltransferase UGT85B) were relocated to the *Nicotiana benthamiana* chloroplast, resulting in expression of the entire dhurrin pathway (blue arrows) in chloroplasts. The preexisting glucosinolate biosynthetic pathway (gray arrows) uses CYP79A1-produced *p*-hydroxyphenylacetaldoxime for the production of glucosinolate with the help of endogenous post-oxime-metabolizing enzymes CYP83B1, C-S lyase (SUR1), and UGT74B1. The presence of CYP79E1 prevents the interaction of the glucosinolate production from CYP79A1. (B) A synthetic pathway for a 500-fold higher production of sesquiterpenoid artemisinic acid in yeast (*Saccharomyces cerevisiae*) as an antimalarial drug precursor (modified from [19]). Genes from the endogenous mevalonate pathway in yeast that are directly or indirectly upregulated or repressed are shown in green, light blue or red, respectively. Down-regulation of a squalene synthase (*ERG9*) by a methionine-repressible promoter was used to decrease the use of FPP for sterols. The biosynthetic pathway from FPP to artemisinic acid in *Artemisia annua* was introduced into yeast for the artemisinic acid production and shown in blue. (C) A synthetic bacterial mini-pathway for a 3600-fold increase in the beta-carotene (i.e., provitamin A) production (modified from [20]). 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*). (D) Reconstitution of the sanguinarine

758 biosynthetic pathway from the commercial precursor (*R,S*)-norlaudanosoline to
759 sanguinarine in yeast (modified from [82]). The ten heterogeneous genes were from
760 opium poppy (*Papaver somniferum*) except *P6H* was from *Eschscholzia californica*.
761 Metabolites are shown in black while enzymes in italic and boxed, and heterogeneous
762 enzymes in blue. Abbreviations: *ADH1*, alcohol dehydrogenase; *ALDH1*, aldehyde
763 dehydrogenase; *ADS*, amorphadiene synthase gene; *BBE*, berberine bridge enzyme; *CFS*,
764 cheilanthifoline synthase; *CNMT*, coclaurine *N*-methyltransferase; *CrtB*, phytoene
765 synthase; *CrtI*, phytoene desaturase/carotene isomerase; *CrtY*, lycopene beta-cyclase;
766 *DBOX*, dihydrobenzo-phenanthridine oxidase; DMAPP, dimethylallyl pyrophosphate;
767 FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl diphosphate; GPP, geranyl
768 pyrophosphate; IPP, isopentenyl pyrophosphate; *MSH*, (*S*)-*cis*-*N*-methylstylophine 14-
769 hydroxylase; *P6H*, protopine 6-hydroxylase; *SPS*, stylophine synthase; tHMGR, 3-
770 hydroxy-3-methyl-glutaryl-coenzyme A reductase; *TNMT*, tetrahydroprotoberberine *cis*-
771 *N*-methyltransferase; *4'OMT2*, *4'*-*O*-methyltransferase 2; *6OMT*, 6-*O*-methyltransferase.
772

Figure 1
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Figure 2
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Figure 3

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