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Review

Synthetic biology for plant genetic engineering and molecular farming

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Many efforts have been put into engineering plants to improve crop yields and stress tolerance and boost the bioproduction of valuable molecules. Yet, our capabilities are still limited due to the lack of well-characterized genetic building blocks and resources for precise manipulation and given the inherently challenging properties of plant tissues. Advancements in plant synthetic biology can overcome these bottlenecks and release the full potential of engineered plants. In this review, we first discuss the recently developed plant synthetic elements from single parts to advanced circuits, software, and hardware tools expediting the engineering cycle. Next, we survey the advancements in plant biotechnology enabled by these recent resources. We conclude the review with outstanding challenges and future directions of plant synthetic biology.

Highlights

Plant synthetic biology has gained recognition both in academia and industry for driving significant advances in the fields of agriculture, medicine, and biotechnology.

Unprecedented genetically encoded resources have been developed in plants to control cellular activities as well as engineering novel functions.

Rational design approaches are being implemented to improve conventional plant genetic engineering.

Molecular farming of products with therapeutic and commercial values can be boosted by plant synthetic biology.

Importance of plant genetic engineering and the role of synthetic biology

Plants have extraordinary intrinsic abilities, which, if we can amplify or modify, will greatly improve human health, planet ecosystem, and bioproduction in a sustainable and scalable way. Plants already provide over 25% of our most clinically relevant drugs [1], sequester 30% of emitted CO_2 each year [2], and their genetic engineering can enable food security [3].

Synthetic biology can facilitate precise and complex plant genetic engineering with robust and predictable phenotypes. It represents a comprehensive and rational approach, applying **engineering principles** (see Glossary) to traditional genetic modification. Numerous well-defined **genetic parts, devices, circuits, and systems** with tunable properties have been designed and applied in microbial and mammalian synthetic biology, which enable researchers to program bacterial community behaviors, build mammalian multicellular structures, genetically engineer cells for novel therapeutics, and manufacture valuable bioproducts [4–7]. However, the development of such powerful synthetic biology tools lags behind in plants.

The objective of this review is to showcase the opportunities of synthetic biology to plant researchers and to encourage synthetic biologists to develop resources for plants. Here, we first describe the synthetic biology tools available for reprogramming plants. Next, we discuss the essential role of engineering principles in advancing plant studies. Last, we exemplify impactful applications of plant synthetic biology in molecular farming and for human health and conclude with current challenges and future prospects. Herein we provide a broad survey of the field comprehensively, discussing key advancements, outstanding challenges, and how plant synthetic biology can overcome them. For specific areas of plant synthetic biology with recent detailed reviews, we refer the readers to those reviews throughout the text. This review is a valuable primer to learn about the breadth of research being conducted in plant synthetic biology, and progress and potential in molecular farming.

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Engineering genetically encoded plant synthetic biology tools

Precise control of gene expression is key to perturbing and understanding endogenous gene networks, as well as engineering the genetic blueprint of crop improvement, bioproduction, and environmental sensing [8–10]. The microbial community has designed, constructed, and characterized a large collection of biological parts, devices, and circuits with various functions [11]. Over the past few years, similar efforts have been underway in the plant synthetic biology community to generate functional and robust genetically encoded tools. Here, we describe the established and emerging gene regulation tools at all levels of central dogma in plants. We then highlight the breakthrough of assembling genetic parts into synthetic circuits of varying complexities in plants.

Genetic parts and devices across the central dogma

A standard electronic device functions through routine input signal detection, actuation, and response module that gives rise to a specific output. Similarly, a synthetic biological device is built with genetic circuits comprising input, processing, and output units. Stages across the central dogma exhibit different information processing capabilities, such as input sensing trigger via transcription initiation, relaying and modulating signal via post-transcriptional and translational regulation, and yielding output via the expression product. The aim of synthetic biology is to dependably and consistently design or re-engineer living systems, hence, the initial and necessary step is to establish a catalogue of interchangeable and versatile parts for genetic circuit construction. Information flow in cells begins at transcription, making it desirable to control the transcriptional activity of synthetic circuits. The plant community has engineered a plethora of synthetic chemical- and light-regulated switches for the control of transcription initiation [12]. For a recent comprehensive review of the examples and mechanisms of transcriptional and post-transcriptional controls in plants, readers can refer to Zhong *et al*. [13].

Chemically inducible regulatory systems typically involve a ligand, a receptor, and a **split transcription factor (TF) system**, in which transcription is initiated when ligand binding triggers the transcriptional regulator to recruit RNA polymerase. A variety of endogenous and non-plant originating ligands are engineered into effective inducible regulatory systems in plants (Table 1). Non-plant ligands are typically desirable to eliminate potential **pleiotropic effects** that may result from natural plant responses to plant-originating ligands. Given that the non-plant originating ligands are genetically distant elements, they are less likely to interact with endogenous networks involved in plant physiology, thus minimizing unintended effects.

The copper-inducible strategy was one of the first inducible systems developed for plants in 1993 [14], which was further optimized in 2014 for tighter and more sensitive response [15]. Recently, in 2022, Garcia-Perez *et al*. [16] incorporated yeast transcriptional activation domain (TAD) Gal4 and nuclease-inactivated Cas9 (dCas9) to the copper system to demonstrate efficient and customizable activation of reporter and endogenous genes in *Nicotiana benthamiana* (Figure 1A). Another exciting recent development is the synthetic oxygen sensor in plants generated by mammalian hypoxia-response regulation pairs combined with Gal4/upstream activating sequence [17]. Both transient assays in *Arabidopsis thaliana* mesophyll protoplasts and stable transgenic seedlings validated that this is an effective oxygen-inducible transcriptional regulator, indicating the potential of assisting agricultural practices given oxygen availability (Figure 1B).

Orthogonality is important for preventing crosstalk of circuit components with plant parts and for building intricate gene circuits. CRISPR-based transcriptional regulation techniques were widely leveraged due to their high modularity and orthogonality that results from the specificity in base-pairing between guide RNAs (gRNAs) and target sequences [18]. Kar *et al*. [19]

Glossary

Boolean logic operation: logical operations that operate on Boolean values (i.e., true or false). In biological networks, biomolecule concentrations are represented as binary values and the circuits use logical algorithms to compute outputs based on the presence or absence of inputs.
Chassis: an organism that serves as a platform or host for the engineered system, which is typically chosen based on its growth requirement and ability to support gene expression and bioproduction.
Computer-aided design (CAD): computational software that allows for designing and testing engineered biological systems via *in silico* simulations and helps to optimize experimental setups.
Edible vaccines: vaccines that can be orally administered; they are usually made by expressing the antigen in edible parts of plants and have advantages such as low cost, being needle-free, and facile manufacturing and storage.
Engineering principles: fundamental concepts used for the design and development of engineered systems. (i) Standardization: set standardized requirement for biological parts, to be reused interchangeably across labs; (ii) decoupling: decompose a complicated problem into simpler and more manageable tasks; and (iii) abstraction: simplify the biological system into hierarchies and identify the essential features needed to model within each hierarchy while ignoring less important details.
Genetic parts, devices, circuits, and systems: terms to describe hierarchies of different complexity in genetic engineering with a bottom-up concept. (i) Part: basic biological material with basic functions (i.e., DNA binding, allosteric change, transcription termination); (ii) device: assembly of parts that can perform a desirable function; (iii) circuit: rational integration of devices that can achieve human-defined advanced functions; and (iv) system: combinations of circuits implemented in biological context.
Orthogonality: a desired property that two or more components are unrelated to each other and allow each part of the system to function and to be modified independently.
PhytoBrick standard: a widely accepted standard for interchangeable

developed synthetic promoter elements from scratch to interact via programmed gRNAs with dCas9-VP64 fusions and activated multiple reporter gene expression orthogonally in *A. thaliana* and *N. benthamiana* (Figure 1C).

In addition to the aforementioned synthetic systems, native tissue-specific promoters only activated at certain development stages in certain tissues (e.g., root, vascular, and seed), as well as native plant promoters induced by phytohormones (e.g., salicylic acid, auxin, abscisic acid, and cytokinin) and environmental chemicals (e.g., nutrients, pathogens, microbe-secreted signals) have been extensively studied in recent years [20,21]. By building from these tissue-specific and inducible promoters, synthetic promoters can be reliably designed to achieve spatial and temporal control over transgene expression, allowing for trait and environment-specific gene regulation. In one effort, Hummel *et al*. characterized the TAD domain from over 400 *A. thaliana* TFs for their capacity to modulate transcription together with the GAL4 system, which could lead to future development of regulatory devices for universal and efficient control of transcription in plants [22].

Importantly, further efforts aimed at enhancing inducible systems, with a focus on addressing current limitations such as leakiness, limited dynamic range, and the transferability of these systems to phylogenetically distant species, are highly needed [23].

In addition to chemical regulation, optogenetic (light-regulated) approaches open broad experimental avenues in research and biotechnology as they provide spatiotemporal and reversible control. However, developing optogenetic resources in plants faces several challenges: (i) ambient light required for plant growth inadvertently affects the optogenetic elements, and (ii) spectral compatibility and cofactors are difficult to administer to whole plants. Despite these challenges, several optogenetic systems have been developed in plants, which are reviewed in Shikata *et al*. [24] (Table 1). One outstanding light-regulated gene expression system is the plant usable light-switch elements (PULSE), which uses a blue-light-regulated repressor combined with the red-light-inducible switch to drive a synthetic promoter containing the binding domains of both switches [25] (Figure 1D). PULSE has a low basal activity in blue and white light compared to in darkness, and red-light induction shows a high dynamic range. Having dCas9-TV under the regulation of this synthetic promoter, the switch can be expanded to quantitative and temporally resolved control of any gene of interest in plants.

In parallel, efforts have been put into developing RNA-based switches that act through post-transcriptional mechanisms, such as riboswitches and post-transcriptional gene silencing (PTGS), which could allow faster regulation and diverse structure manipulation.

Riboswitch is an RNA sensor that undergoes conformation change upon ligand binding and modulates gene expression carried by the mRNA (Figure 2A). Given the fast response and versatility in aptamer design, riboswitches regulating transcription termination and translational initiation have been extensively used in bacteria and mammalian cells [26,27]. To date, only one riboswitch has been engineered in plants, where Verhounig *et al*. [28] constructed the theophylline (theo)-inducible riboswitch for GFP accumulation in chloroplasts. Emadpour *et al*. [29] later boosted the efficiency of this riboswitch through the T7 RNA polymerase-based amplification, creating RNA amplification-enhanced riboswitch (RAmpER), which was implemented for high-value but cytotoxic molecule production in *Nicotiana tabacum* [30]. The fact that the theo-inducible riboswitch has now been established for chloroplast- and nuclear-encoded genes [31] in plants indicates similar design strategies could be expanded to generate more varieties of artificial riboswitches.

building blocks that can be assembled into larger genetic circuits in plants.

Pleiotropic effects: a phenomenon where a single gene has multiple effects besides the intended effect on an organism, resulting from the complexity of the biological regulatory network.

Protoplast transformation and leaf agroinfiltration: two standard and practical experimental techniques that can introduce foreign genetic material into plant cells for transient expression of DNA.

Split transcription factor (TF) system: TF has a DNA binding domain (DBD) that binds to specific DNA promoter sequences and a transcriptional activation domain (TAD) that recruits RNA polymerase to activate transcription. Either or both can be fused to conditionally activate receptor protein that allows the expression of the gene of interest to be regulated by the presence of the ligand.

Vertical farms: a type of agricultural system where crops are grown in vertically stacked layers, aiming to reduce land use and expand agricultural practices.

Table 1. Overview of transcriptionally regulated synthetic systems in plants[a]

Chemical-inducible systems						
Ligand	Cis regulator	Transcriptional regulator	Native organism[b]	Plant tested	Application	Refs
Dexamethasone (DEX)	6xGRE-P35Smin	GR	*Rattus norvegicus*	*Arabidopsis thaliana* (arabidopsis)	CAT reporter	[101]
	UAS	Gal4-VP16-GR (GVG)	*Saccharomyces cerevisiae*, *R. norvegicus*	Arabidopsis and *Nicotiana benthamiana* (tobacco)	LUC reporter	[102]
	6xlacO (pOp6)	GR-lacI-Gal4 (LhGR-N)	*Escherichia coli*, *S. cerevisiae*, *R. norvegicus*	Tobacco	GUS reporter	[103,104]
Tetracycline	P35Smin-3xTetO	TetR	Bacteria	Tobacco	GUS reporter	[105]
	7xTetO-P35Smin	TetR-VP16	Bacteria	Tobacco	GUS reporter	[106]
RH5992 (tebufenozide)	6xGRE-P35Smin	GR-HEcR LBD	*Heliothis virescens*	Tobacco	GUS reporter	[107]
Copper (II)	MRE-P35Smin	ACE1	*S. cerevisiae*	Tobacco	GUS reporter, environmental copper sensor	[14]
	4xCBS-PDFRmin	CUP2-Gal4	*S. cerevisiae*	Tobacco	CRISPR/Cas9 transcriptional activation	[16] (Figure 1A)
Ethanol	pAlcA-P35Smin	AlcR	*Aspergillus nidulans*	Tobacco	CAT reporter, manipulation of C metabolism	[108]
Beta-estradiol	8xLexA -P35Smin	LexA-VP16-ER (XVE)	*E. coli*, *Homo sapiens*	Arabidopsis and tobacco	GFP reporter	[109]
Pristinamycin	PIR3-TATA box; P35Smin-PIR3	PIP-VP16	*Streptomyces coelicolor*	Tobacco	GUS reporter	[110]
Methoxyfenozide	5xUAS-P35Smin	GAL4-VP16-EcR (GVE)	*Choristoneura fumiferana*	Arabidopsis and tobacco	LUC reporter	[111]
OOHL	4xtraA box-P35Smin	VP16-TraR	*Agrobacterium tumefaciens*	Carrot and arabidopsis	GUS reporter	[112]
TNT	Synthetic promoter (PlantPho)	PhoB-VP64	Bacteria	Arabidopsis and tobacco	GUS reporter, de-greening circuits	[113]
Macrolide	etr8-PhCMVmin	E-VP16	*E. coli*	Tobacco	LUC reporter	[114]
Digoxin	UAS	Gal4-DIG1-VP16-Meta2	Synthetic	Arabidopsis	LUC reporter, environmental digoxin sensor	[115]
Fentanyl	UAS	Gal4-Fen21-VP16-Meta2	Synthetic	Arabidopsis	LUC reporter, environmental opioid sensor	[116]
Oxygen	4xUAS-P35Smin	HIF1a-Gal4 DBD, pVHL-Gal4 TAD	*H. sapiens*	Arabidopsis	LUC reporter, O_2 availability sensor	[17] (Figure 1B)
Artificial gRNAs	pATF	ATF	Synthetic	Arabidopsis and tobacco	Orthogonal control of fluorescent proteins	[19] (Figure 1C)

Light-inducible systems						
Light	Cis regulator	Transcriptional regulator	Native organism	Plant tested	Application	Refs
Red (660 nm) activate; far-red (760 nm) repress	etr8-PhCMVmin	PhyB-VP16 and PIF6-E	*A. thaliana* and bacteria	Arabidopsis and tobacco	LUC reporter	[25,114]

(continued on next page)

Table 1. (continued)

Light-inducible systems						
Light	Cis regulator	Transcriptional regulator	Native organism	Plant tested	Application	Refs
Green (525 nm) deactivate	8xCarO-PhCMVmin	CarH-VP16	*Thermus thermophilus*	Arabidopsis	LUC reporter	[117]
Blue (461 nm) repress	P35Senh-5x (C120)-PhCMVmin	EL222-SRDX	*Erythrobacter litoralis*	Arabidopsis and tobacco	LUC, dCas9 transcriptional activation	[25] (Figure 1D)

[a]Abbreviations: ACE1, activating copper-metallothionein expression; ATF, artificial TF composed of dCas9 (gRNA)-VP64; CAT, chloramphenicol acetyltransferase; CBS, copper binding site; CUP2, copper responsive factor; DIG1, digoxin binding domain with mutated E83V; E, macrolide repressor protein; EcR, ecdysone receptor; EL222, photoreceptor with light-oxygen-voltage domain and helix-turn-helix domain; ER, estrogen receptor; etr8, 8 copies of MphR(A) operator; Fen21, fentanyl binding protein; Gal4, Gal4-responsive promoter; GAL4, Gal-responsive TF; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HEcR, heliothis ecdysone receptor; LacI, lac repressor; LBD, ligand binding domain; LhGR, lac repressor with His mutation; Mata2, degron from yeast; MphR(A), macrolide repressor protein; MRE, metal responsive element; OOHL, 3-oxooctanyl-L-homoserine lactone; pAlcA, AlcR binding site of the alcohol dehydrogenase I promoter; pATF, ATF promoter followed by P35Smin; PDFRmin, minimal promoter of NADPH-dependent dihydroflavonol reductase; PhCMVmin, minimal human cytomegalovirus promoter; PhyB, phytochrome; PIF6, phytochrome interacting factor 6; PIP, pristinamycin-induced protein; PIR3, 9 copies of PIP binding site; P35Senh, enhancer sequence of pCaMV35S; P35Smin, the minimal CaMV 35S promoter; SRDX, ethylene-responsive element binding factor-associated Amphiphilic Repression (EAR) motif repression domain; TetO, tetracycline operator; TetR, tetracycline repressor; TNT, 2,4,6-trinitrotoluene; UAS, upstream activation sequence; VP16, herpes simplex virus protein; 5x(C120), quintuple-repeat target sequence for EL222.
[b]The original species where the core functional elements are discovered.

In plant PTGS, small-interfering RNAs (siRNAs) and microRNAs (miRNAs) are the two types of small RNAs that promote the cleavage or translational inhibition of targeted and complementary mRNA, with the advantages of versatility and relative ease in design and synthesis (Figure 2B). Since the first artificial small RNAs were demonstrated in *A. thaliana* [32], many more promising strategies with inducible or tissue-specific promoters have been developed and summarized in other reviews [33]. In the meanwhile, there is a need to investigate more naturally existing RNA-based regulation mechanisms and their synthetic counterparts, as well as to address shortcomings, such as programmability, scalability, expression quantity, and off-target effects within the native and surrounding organisms [34].

Translational and post-translational control represents an essential role, as controlling protein depletion or accumulation is a direct approach to tune phenotypes. For instance, 'N-degron' is an N-terminal degradation signal peptide targeting the protein of interest to the ubiquitin/proteasome system [35]. The low-temperature-controlled N-terminal degradation signal (lt-degron) was the first degron established for plants [36] (Figure 2C). When lt-degron was fused to the transcriptional factors regulating the trichome formation and flowering time in *A. thaliana*, the plants exhibited expected temperature-dependent phenotypes [36]. This system is also engineered as a switchable toxin in plants to mediate cell arrest and alter tissue physiology by fusing the lt-degron to a cytotoxic bacterial protein [37]. Degron-mediated protein tuning technique could be a versatile tool for tight modulation of protein activities in multicellular organisms.

Finally, the circuit output is typically a reporter gene to visualize the circuit response for quantification and optimization of circuit performance, or a selectable marker to screen transgenic plants. Commonly used output parts include fluorescent proteins, β-glucuronidase (GUS), luciferase (LUC), and anthocyanin accumulation (reviewed in [38]). One recent reporter, named RUBY, entails coexpression of the pathway that converts tyrosine to vividly red betalain, which is visible to the naked eye and enables easy detection of circuit output or transgenic events [39] (Figure 2D). Additionally, Khakhar *et al*. reconstituted the fungal bioluminescence pathway *in planta* by inserting the enzymes in luciferin synthesis and recycling pathway [40]. This serves as a novel auto-bioluminescence reporter that does not require external substrate addition, thus enabling the rapid study of gene expression at lower cost. Still, there remains a need to develop new

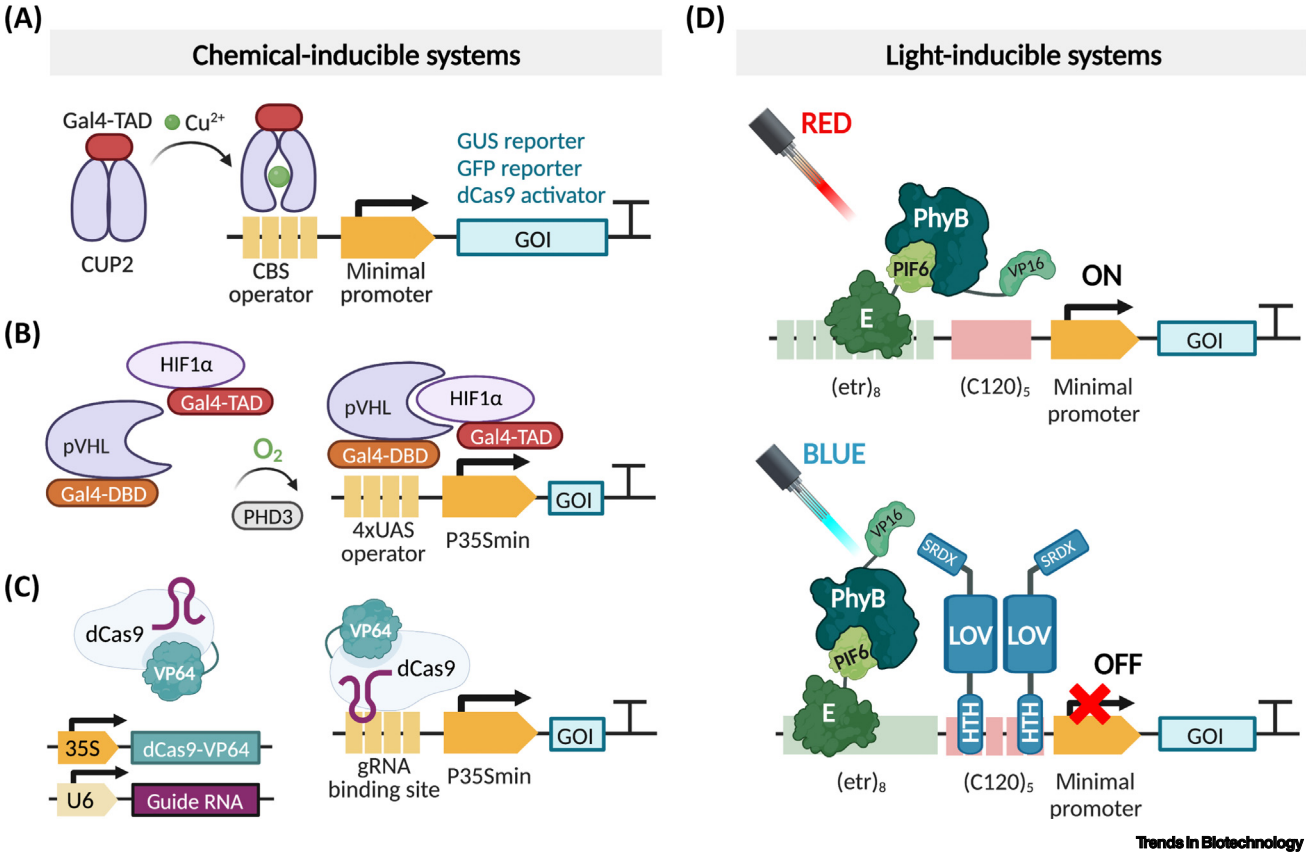

Figure 1. Examples of transcriptional regulation control in plants. Chemically inducible systems. (A) The copper responsive factor (CUP2) is fused with a Gal4 transcription activation domain (TAD), such that when Cu^{2+} is present, CUP2 undergoes conformation change and binds to the copper binding site (CBS) operator, achieving transcription regulation of the downstream gene of interest (GOI). Commonly used GOIs: beta-glucuronidase (GUS), green fluorescent protein (GFP) reporter, and nuclease-deactivated Cas9 (dCas9) activator. (B) The hypoxia-inducible factor (HIF1α) or the von Hippel-Lindau tumor suppressor (pVHL) are fused with Gal4 TAD or Gal4 DNA binding domain (DBD), respectively. In the presence of hydroxylase (PHD3) and oxygen, HIF1a-Gal4 TAD dimerizes with pVHL-DBD to activate GOI expression controlled by the upstream activating sequence (UAS) and minimal CaMV 35S promoter (P35Smin). (C) 35S and U6 promoter drive the expression of dCas9 fused with four tandem repeats of VP16 (VP64) and guide RNA (gRNA), respectively. The artificial promoter, composed of varying number of repeats of gRNA binding sites followed by P35Smin, drives the GOI expression. (D) Light inducible systems. Plant usable light-switch elements (PULSE) integrated a red-light activable switch, based on phytochrome B (PhyB)-VP16 fusion's light-regulated interaction with phytochrome interacting factor 6 (PIF6) and macrolide repressor protein (E) fusion, and a blue-light repressive switch, based on a transcriptional repressor SRDX fused with a photoreceptors EL222 containing light-oxygen-voltage (LOV) domain and helix-turn-helix (HTH) domain. The former binds to eight copies of macrolide repressor protein operator (etr8) under red light, while the latter binds to quintuple-repeat target sequence for EL222 (5xC120) under blue light. Created with BioRender.com.

output parts that can reflect plant cellular activities sensitively, noninvasively, continuously, and reversibly.

Regulatory circuits with advanced functions

While significant progress was made in endowing plants with improved traits, the ability to precisely control the synthetic gene circuits is of undeniable importance. Characteristics of tight control over basal expression, tunable dynamic range upon induction, and responsiveness to multiple stimuli require more sophisticated, versatile, and mathematically validated designs. Analogous to electrical systems, to date bioengineers have achieved toggle switch [41], oscillators [42], Boolean logic gates [43], memory devices for temporal regulation [44], as well as segregation and stripe patterns for spatial regulation [45] in non-plant organisms.

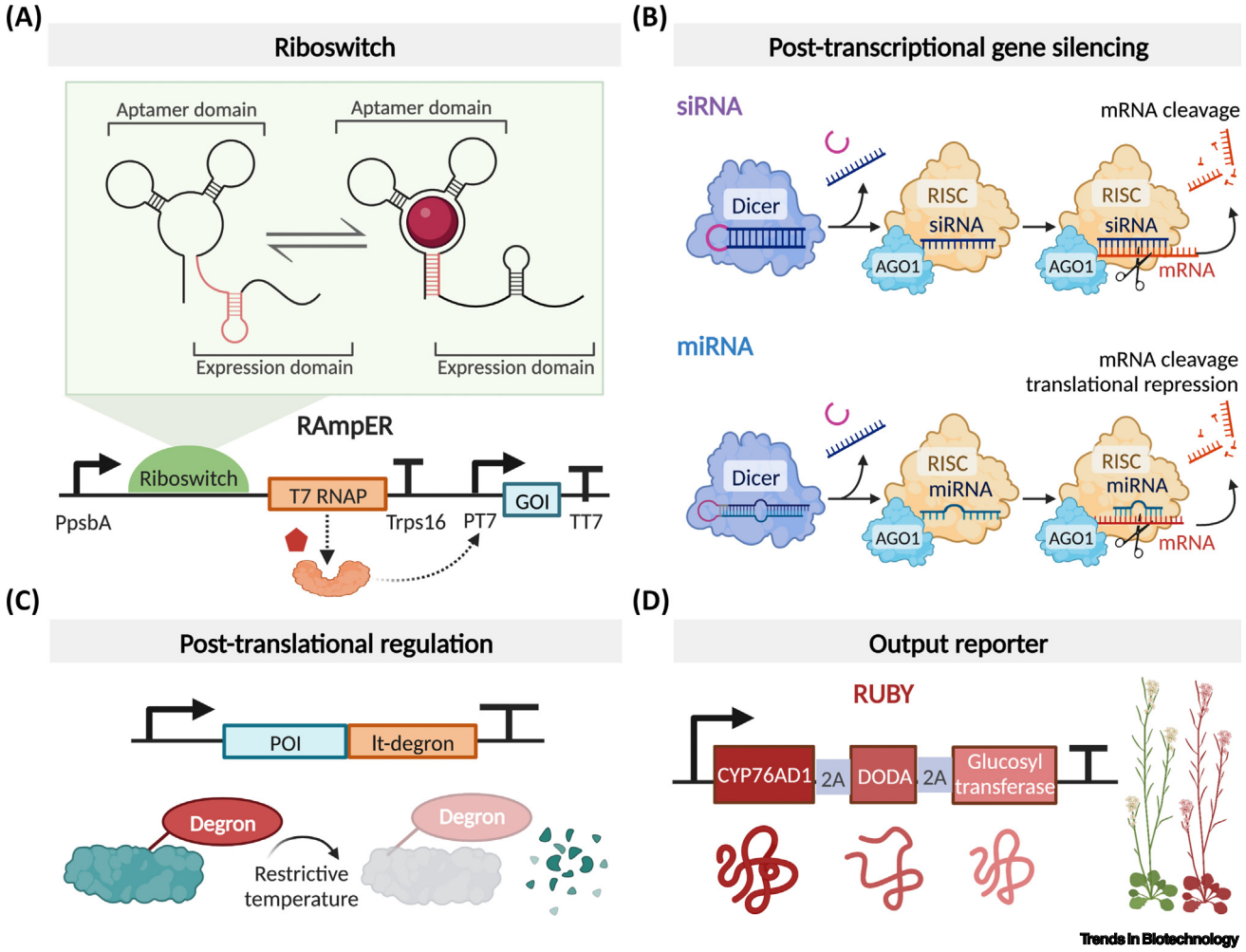

Figure 2. Examples of plant post-transcriptional and post-translational regulation. (A) Allosteric change in aptamer domain and expression platform structure is theophylline-responsive. The RNA amplification-enhanced riboswitch (RAmpER) directly regulates T7 RNA polymerase promoter (T7 RNAP) and further regulates the gene of interest (GOI) downstream of the T7 promoter (PT7). (B) During RNA interfering process, Dicer cleaves long, double-stranded RNA molecules, including small interfering (siRNA) and microRNA (miRNA), into short, single-stranded RNA fragments that are then loaded onto the RNA-induced silencing complex (RISC). Argonaute 1 (AGO1) is responsible for recognizing and cleaving the target mRNA. (C) The low-temperature-controlled degron (lt-degron) is fused to a protein of interest (POI), resulting in the stability of the fusion product dependent on temperature. (D) The betalain biosynthetic pathway expression cassette contains CYP76AD1, L-DOPA 4,5-dioxygenase (DODA), and glucosyltransferase, linked with 2A peptides. Transgenic plants produce vivid red compounds in all tissues, herein named RUBY. Created with BioRender.com. Abbreviations: NtTrps16, 3′UTR of rps16; PpsbA, chloroplast psbA promoter; TT7P, T7 RNA polymerase terminator.

Recently, a reversible memory switch was built with a switchable plant promoter regulating the expression of two genes of interest based on the previously reported bacterial and mammalian memory switch using serine integrases [46]. The switch demonstrated high activity and reversibility by transforming different combinations of constructs into *N. benthamiana* leaves, as well as generating stable transgenic plants. Notably, qPCR analysis revealed that the reversibility was 40% in transient assays and increased to 60% in stable transgenic plants. Such varying performance suggests that an optimization may be required for the switches to operate effectively in different expression and transformation platforms. An estradiol-inducible system was implemented to have better spatiotemporal control of the site-specific recombination elements (Figure 3A).

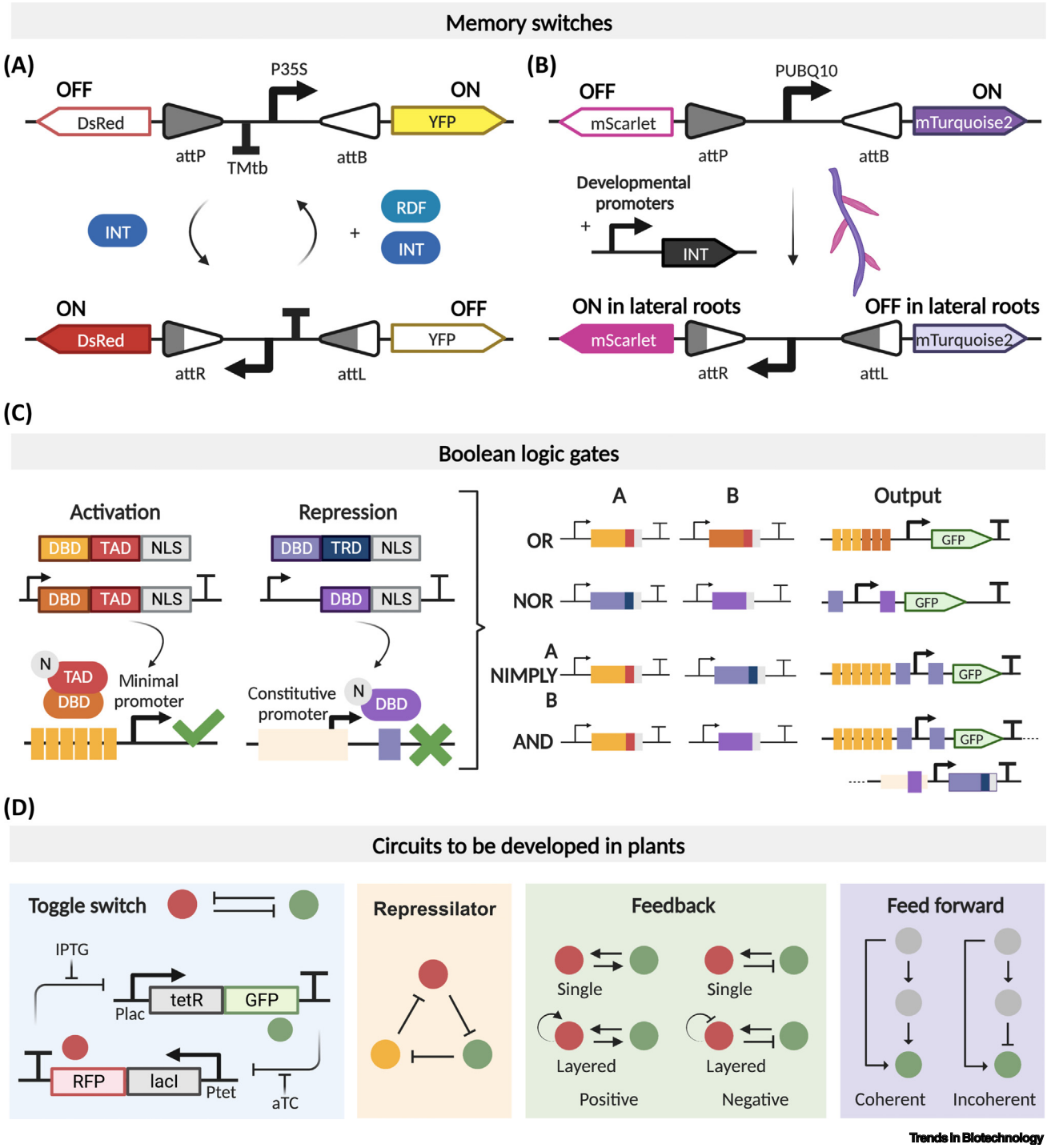

Figure 3. Advanced genetic regulatory circuits in plants. (A) The memory switch has an invertible element: a constitutive CaMV35S promoter (P35S) and a tomato Metallothionein B terminator (TMtb) sequence flanked by recombination sites (triangles). The bacteriophage φC31 integrase (Int) and its cognate recombination directionality factor (RDF) act on the invertible element, switching the expression state of two genes of interest (GOIs): DsRed and yellow fluorescent protein (YFP). (B) The INT is driven by developmental promoters that are activated during early stages of lateral root initiation. The main root expresses mTurqoise2, lateral roots express mScarlet since the INT catalyzes the inversion of PUBQ10 promoter. (C) The activation circuits are composed of synthetic activators [DNA binding protein

(Figure legend continued at the bottom of the next page.)

Apart from external stimuli, memory switches can be activated by internal events. Guiziou *et al*. [47] extended the memory switch technique to record multicellular developmental behaviors in plants. In this study, they expressed integrases with a target that switches between two fluorescent reporters under the control of lateral root developmental promoters in *A. thaliana.* Integrases amplified the fluorescent reporter signal and permanently marked all lateral root descendants (Figure 3B). While the proof-of-concept of analog-to-digital conversion via memory switch has been demonstrated, it is crucial to further evaluate the robustness of the switch under fluctuating conditions and extend to phenotypically relevant marker.

In more realistic scenarios, cells receive input from multiple signals and therefore require combinatorial processing ability to generate complex logical functions. **Boolean logic operation** is an invaluable tool for synthetic biologists, where a collection of regulatory gene circuits can be complied to create logic circuits, and these have recently been built in plants using integrases, CRISPR-interference (CRISPRi), and synthetic transcriptional regulators [48–50]. The first wave of successful implementation of customizable logic circuits in plants indicates their broad potential for programmable manipulation of transcriptional activity. Lloyd *et al*. demonstrated cell type-specific expression of fluorescent markers through integrases that triggered expression using logic combination of stimuli [48]. Khan *et al*. presented a toolkit of reversible CRISPRi-based logic gates and rational design strategies, which have the advantage of simultaneous targeting of multiple loci [49].

Notably, Brophy *et al*. reconstituted the most comprehensive logic gates to-date to confer prescribed spatiotemporal gene expression in plant roots [50]. Starting with a library of activation and repression circuits, which served as a single processing unit, they constructed combinatorial logic of cis and trans elements that perform Boolean logic operations in *N. benthamiana* leaves (Figure 3C). Next, stable *A. thaliana* transformations showed that these gates qualitatively matched the expected patterns when tissue-specific promoters were used to drive transcriptional factor expression together with engineered responsive promoters. This progress constitutes a milestone in plant synthetic biology, where engineering sophisticated expression profiles is not limited to the available tissue-specific promoters, but can be tailored by combinatorial logic functions. However, out of eight logic gates designed, one gate exhibited only a 1.2-fold change between ON and OFF tissue and four gates necessitated additional genetic tuning to operate correctly. The observation of suboptimal performance during the testing phase of these experiments, which was subsequently resolved with alternative strategies, underscores the need for improved models of genetic regulations and tools for high-throughput screening, as well as mechanistic investigation into the biological causes.

Another shortcoming is that current progress has only been achieved in model plants, hence, the translatability of these regulatory circuits with advanced functions to other plant species, such as the agronomically or industrially important crops, is unknown. The translation may also be hindered by several factors, such as varying genetic backgrounds and limited regulatory elements, requiring additional optimization and customization to ensure compatibility. The goal of the

(DBD, light and dark orange), transcriptional activation domains (TAD, red), and nuclear localization signal (NLS, gray)] and activatable promoters (six copies of the operator at the 5′ end of a minimal plant promoter). The repression circuits are composed of synthetic repressors [DBD (light and dark purple), transcriptional repression domains (TRD, blue), and NLS] and repressible promoters (one operator at the 3′ end of a constitutive plant promoter). (D) Examples of future circuits with advanced functions. A toggle switch consists of two mutually repressing transcription factors that have two stable states and can be switched by an input signal (IPTG and aTC). Repressilator is an oscillator circuit that produces oscillations via a series of repressions. Feedback is a regulatory mechanism in which the output of a system affects the input of the system. It can be either single or layered and positive or negative. Feedforward is a regulatory mechanism in which the output of one process serves as the input of the next process. It can be either coherent (output reinforces the input signal), or incoherent (output acts to suppress the input signal). Created with BioRender.com.

aforementioned efforts is to develop a programmable path of achieving beneficial plant development and harness plants as the next generation of bio-foundries.

Although basic parts and devices controlling cellular processes were developed for mammalian systems in the 'first wave' of synthetic biology [51], not many were built for plants. For instance, the toggle switch, which marked the establishment of synthetic biology in the 21st century, is only just being developed in *A. thaliana*, as mentioned in a recent conference abstract [52]. The uncharted space of establishing complex genetic circuits with specific dynamic behaviors in plants lies in front of us, including but not limited to oscillators [53], feedback [54] and feedforward loops [55], pulse generator [56], and fold-change detection [57] (Figure 3D). While acknowledging the difficulties resulting from long time scales required for producing transgenic lines, redundant and polyploid plant genomes, and lack of well-characterized parts across differentiated tissues, we advocate more focus on creating a wealth of regulatory components and systematically analyzing the translation of existing designs to plants.

Essential role of the engineering principle

The core idea of plant synthetic biology is to carry out engineering based on standardized biological components to obtain new biological functions. Rational design of genetic parts using bioinformatics approaches, facile techniques for large and recursive DNA assembly, and automation hardware expedite the design-build-test-learn (DBTL) cycle (Figure 4). Just as standardized toy LEGO bricks can be constructed into complex structures, genetic bricks can be assembled into functional and reliable systems through the DBTL cycle: 'design' specifies the architecture, 'build' is bricks stacking together in a designed order, 'test' requires consistent operation to generate reliable results, and 'learn' is collective information leading to the start of the next cycle. Here, we review the strategies for the fulfillment of each objective and how they integrate synergistically for the goal of rational reconstruction of complex biological systems with desirable input–output functions.

Design

Bioengineers conceptualize a synthetic gene network that can process certain inputs into desired outputs [8]. However, selection of the most promising design for implementation could be cumbersome work. **Computer-aided design (CAD)** alleviates the burden by optimizing network hierarchies, kinetic parameters, and parts selection *in silico* [58]. Automation of design, build, and function prediction of complex genetic circuits is possible in microbes using computational tools, such as Cello 2.0 [58,59]. In microbes, it is also possible to precisely control the growth conditions in high-throughput using frameworks like eVOLVER and design the functions of complex communities [60–62]. Plants, however, pose several challenges due to the composition of differentiated cells and tissues with unique regulatory and developmental contexts and their extreme sensitivity to environmental influences, which can impact the circuit performance [63]. Previous reviews summarized three categories of CAD tools that are ready or can be adapted for plant synthetic biology use, including component design and synthesis, topology and network design, and simulation and behavior prediction [62]

Recently, a general concept has emerged to rationally design and implement transcriptional regulation elements in plants, where cis-regulatory element (CRE) motifs are placed upstream of a core plant promoter to regulate its expression [64]. Regulatory element discovery can be arduous but is greatly beneficial for providing novel and versatile building blocks. Cai *et al*. [65] conducted a *de novo* screening for TF binding sites in common CREs and used this knowledge to develop minimal synthetic plant promoters with varying strength, which indicates that CAD is capable of performing comprehensive analysis to develop a customized suite of genetic elements

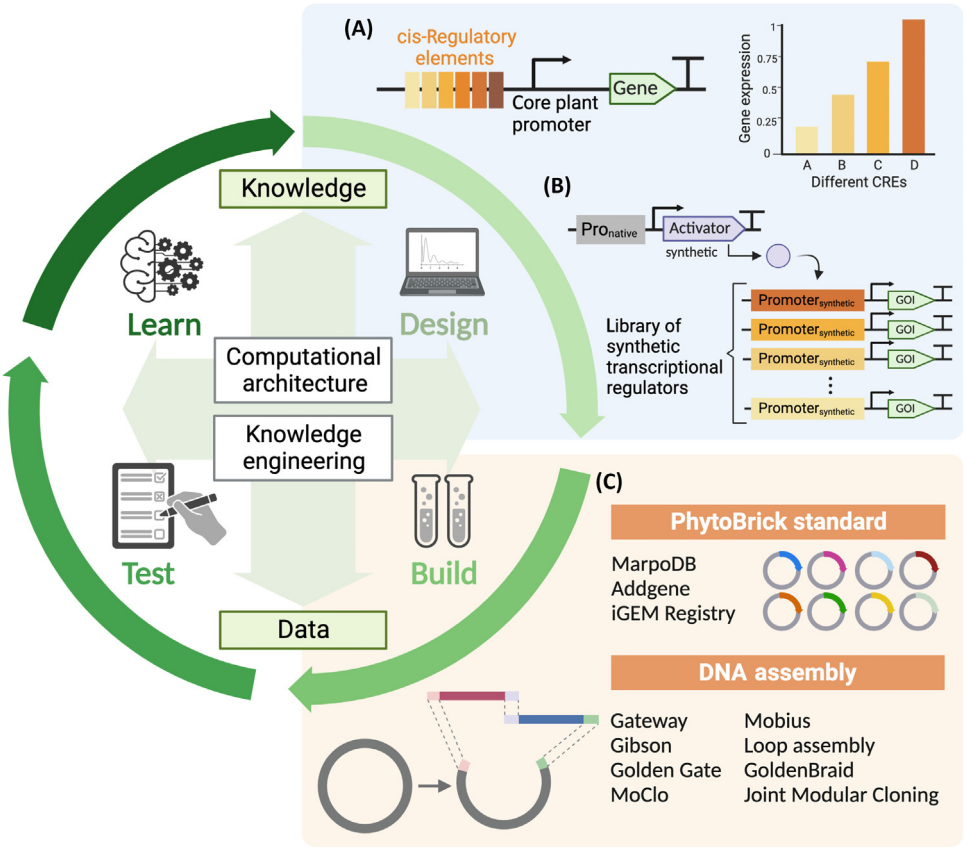

Figure 4. Design-build-test-learn (DBTL) cycle of plant synthetic biology. The design-build-test-learn cycle is a methodology used in synthetic biology to improve and understand the genetic circuits functionality, by iterative cycling through the stages of rational circuit design, construction using molecular biology techniques, testing performance, and learning from the results and collecting information to optimize the design. (A) A common design strategy is to place cis-regulatory elements (CREs) upstream of a core plant promoter to regulate its expression and obtain synthetic plant promoters with varying expression strength. (B) Another design strategy is to create synthetic activators driven by native plant promoters to then act on designed synthetic promoters with varying binding sites. (C) Resources of commonly used PhytoBrick standards and DNA assembly methods in plant synthetic biology. Created with BioRender.com. Abbreviation: GOI, gene of interest.

(Figure 4A). Similarly, by merging components from the yeast TF systems with plant-specific minimal promoter sequences, another team built an expansive and diverse library of synthetic transcriptional regulators and validated them in *N. benthamiana* and *A. thaliana* [66] (Figure 4B). However, these steps represent only an initial stage in the dissection of how individual genetic components affect gene regulation. Further studies and high-throughput testing platforms are necessary to investigate robust design principles and quantitative prediction models.

Build

Techniques for assembling multiple large DNA parts into synthetic pathways or circuits have always been a major requirement in plant synthetic biology. Various cloning approaches, each with their advantages and disadvantages, have been developed, spanning from earlier Gateway and Golden Gate to more recent and capable MoClo and Loop assembly, all of which are extensively discussed in previous reviews [67–69]. One very recently developed framework, Joint

Modular Cloning Toolkit, offers a greater compromise of capacity, flexibility, and extensibility in comparison with previous toolkits for higher-level assemblies in plants [70] (Figure 4C).

Apart from the continuously evolving assembly techniques, standardized DNA elements, such as the **PhytoBrick standard**, have enabled modularized assembly, simplified laboratory protocols, and decreased labor [71]. This was a milestone in the plant synthetic biology field, as it allows a multitude of standard parts to be generated in an off-the-shelf manner and simplifies the design and exchange process. However, the plant PhytoBrick library is currently limited to a few (~20) parts, which is bound to expand soon given the increasing studies in the plant synthetic biology field.

Test

With the capability of rapid, reliable, and inexpensive construction of libraries of parts and devices, the next critical step is to quantitatively define their functional characteristics. A series of functional parameters of switches must be evaluated, including dynamic range (ratio between maximal and basal activation), leakiness (basal activity in the absence of an inducing signal), kinetics, and reversibility, which are critical when assembling building blocks for higher order circuits.

Transient **protoplast transformation and leaf agroinfiltration** are the standard testbeds [72]. A recent study of plant genetic regulatory elements took 37-member combinatorial library comprised of promoters, 3′ untranslated regions (UTRs), promoter-5′UTR fusions, and 5′UTRs and assembled 91 transgene cassettes for relative expression strength evaluation. After *Agrobacterium tumefaciens*-mediated infiltration of *N. benthamiana* leaves with DNA constructs, scanning fluorometric analysis of leaves and single cell flow cytometry analysis of extracted protoplasts allowed the investigation of regulatory elements and their expression activity [73]. Additionally, the lower plant *Marchantia polymorpha* is one of the few species for which chloroplast transformation is well-established and has characteristics of inexpensive culture and fast selection for homoplasmy, making it a simple and facile testbed for assembled DNA circuit [74].

Prototyping at the plant tissue level, such as approaches based on the hairy root and virus-based transgene expression platforms, has the potential to be utilized in the DBTL cycle. Transgenic hairy roots are formed when *Rhizobium rhizogenes* infects plant cells and researchers can use this bacterium to deliver genetic circuits to a variety of plant species. Hairy roots possess unique abilities, including fast growth, high productivity, and ease of maintenance, making them a popular choice for secondary metabolite production, gene function studies, and other applications, as reviewed in [75]. In addition, plant virus-based expression systems have been extensively used to express genes of interest and produce recombinant proteins in a rapid and high-throughput manner [76].

However, the development of reproducible, stable, and low-variability methods for delivering and expressing genetic constructs in plant cell remains a challenge. Automated handling could play a crucial role in limiting the variability from human handling error [77]. For example, the robotic platform, Bright Yellow, is designed to complete protoplast harvesting, transformation, and analysis of fluorescence without any operator in less than 4 hours [78]. Dudley *et al*. [79] developed an automated handling system for a PhytoBrick-compatible DNA assembly and cell-free plant protein expression, which allows for high-throughput testing. Meanwhile, using nanoparticle-based delivery methods and implementing quality control measures for genetic material delivery has the potential to enhance the reliability and consistency of experimental results [80,81].

Learn

Finally, all the information is composed at the learn stage to refine the original hypothesis (represented by a schematic and hypothetic complex metabolic pathway) and enhance the capabilities

in the subsequent cycle. It is unsatisfying when empirically and computationally predicted designs do not align *in planta*, which can be attributed to the lack of quantitative data on parts' functionality, gene silencing effects, and lack of comprehensive mathematic models to account for variable contextual factors [46,50,66,72]. By comparing experimental and theoretical results, we could investigate the mechanistic explanation of variabilities, optimize key parameters, and potentially apply the gained knowledge to create rational design principles for programming plants cells across different tissues and diverse plant species.

Plant synthetic biology advancements translating into applications

Many efforts towards agriculture, with the goal of optimizing plant traits associated with efficient photosynthesis, tolerance to abiotic stress, nutritional improvement, among others, have been discussed thoroughly in recent reviews [9,82,83]. Therefore, here, we focus on the manufacturing of valuable natural products and biopharmaceuticals in plants (Table 2). Reconstruction of biosynthetic pathways for biomolecule manufacturing is a typical route in the biotech industry. Advancements in plant synthetic biology have made abundant functional genetic materials available and conceptualized the engineering strategies, hence now offering tremendous opportunities to create tailored cell factories served for specific purposes.

Molecular farming

Bioproduction in microbial systems, from single gene expression to metabolic engineering, has the benefits of being environmentally friendly, requiring less energy, and featuring diverse genetic background, compared with chemical synthesis [84,85]. Plants have the potential to be the next

Table 2. Overview of landmark and recent plant molecular farming for human health[a]

Product	Host plant	Use/application	Producer/stage	Year	Refs
Taliglucerase alfa	Carrot cell suspension cultures	Gaucher's disease treatment	Protalix and Pfizer/product in market	2012	[118]
ZMApp	Tobacco	Combat Ebola virus outbreak	Mapp Biopharmaceutical/FDA Fast Track	2015	[119]
Immunoadhesin DPP4-Fc	Tobacco	Treat MERS-CoV	Plant biotechnology/clinical	2015	[119]
Hepatitis C virus E1E2 heterodimer	Lettuce	Tested immune response in mice	Academic/R&D	2017	[120]
Pfs25 VLP-FhCMB	*N. benthamiana*	Malaria vaccine	Fraunhofer/clinical Phase 1	2018	[121]
Domain III of West Nile virus (WNV) envelope protein	*N. benthamiana*	Protective immunity against WNV infection	Academic/R&D	2018	[122]
CTB-VP1 fusion protein	Lettuce chloroplasts	Virus-free polio vaccine	Academic/R&D	2019	[123]
bNAb-lectin fusion protein: $VRC01_{Fab}$-Avaren	*N. benthamiana*	Therapeutic agents against HIV-1	Pharma-Planta/clinical Phase 1	2019	[124]
2G12 human monoclonal antibody	*N. tabacum*	HIV-neutralizing therapeutic agent	Fraunhofer IME/clinical Phase 1	2019	[125]
Dengue virus structural proteins	*N. benthamiana*	Tested antibody response in mice	Academic/R&D	2020	[126]
Influenza virus H5 trimer	*N. benthamiana* leaves	Prevent the spread of H5N1 in chickens	Academic/R&D	2020	[127]
Pegunigalsidase alfa	Tobacco cells	Enzyme replacement therapy for Fabry disease	Protalix/clinical Phase 3	2020	[128]
Cathelicidin	Barley grains	Promote closure of chronic wounds	Academic/R&D	2022	[129]
SARS-CoV-2 Spike protein	*N. benthamiana* leaves	COVID-19 vaccine	Medicago/product in market	2022	[130]

[a]Abbreviations: bNAb, broadly neutralizing antibody; COVID-19, coronavirus disease 2019; CTB-VP1, cholera non-toxic B subunit–poliovirus capsid protein; DPP4, dipeptidyl peptidase 4; H5N1, influenza A virus subtype H5N1; MERS-CoV, Middle East respiratory syndrome coronavirus (MERS-CoV); SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; VLP-FhCMB, virus-like particle from Fraunhofer USA Center for Molecular Biotechnology.

generation bio-foundry **chassis** and enable scalable and affordable biosynthesis of valuable products as they have essential advantages compared with microbial platforms. Firstly, the major limitation of microbial platforms is that single-celled microorganisms, including prokaryotic bacteria and eukaryotic yeast, cannot accommodate multiple synthetic pathways requiring multiple compartments. Eukaryotes, such as yeast, often require the introduction of supplemental pathways to obtain the precursors and desired metabolite flux, and prokaryotes lack post-translational modifications that are essential for some bioproducts [84]. Secondly, the requirement of large quantities of nutrient, fermenter infrastructure, fuel tanks, culture medium, and clean rooms to ensure compliance with good manufacturing practice can make microbial production economically infeasible and unscalable.

However, plants naturally offer a myriad of metabolic resources, as they are eukaryotes with cellular compartments, such as chloroplasts that enable high titer production. Moreover, plants can be grown in a scalable manner in **vertical farms**, greenhouses, or in fields without the need of expensive fermenters, media, and sterile conditions. However, some challenges of plant molecular farming that need to be addressed are the higher downstream processing costs and competition with food crops for land and resources. Strategies such as **edible vaccines** can overcome both challenges [86].

In 1989, the first recombinant protein (an immunoglobulin) was produced in tobacco leaves [87]. Since then, many research efforts and accomplishments have been made in molecular farming, which refers to using plants as a factory to produce recombinant proteins, vaccines, small molecules, or other products with potential therapeutic or commercial value. This review will not cover all the achievements of plant molecular farming but focus on several key or recent studies with promising impact on human health (Table 2). For a more comprehensive survey of molecular farming, readers can refer to earlier reviews [10,88–90].

Plant synthetic biology solutions to molecular farming challenges

One challenge in molecular farming is that the production in a heterologous host may decrease the yield of a product. A good example of this is the production of glucoraphanin, which naturally exists in cruciferous vegetables, and extensive efforts have been dedicated to its synthesis in heterologous hosts without success. Suspecting that the reason could be a lack of auxiliary genes, Barnum *et al*. optimized the biosynthetic pathway by screening a combination of previously uncharacterized coexpressed genes and they were able to achieve high glucoraphanin production levels in *N. benthamiana* [91]. Another related problem is the derivatization of some heterologously produced molecules. To address this, Dudley *et al*. identified the host metabolism genes that were upregulated when strictosidine pathway enzymes were transiently expressed and mutated them with CRISPR/Cas9, hence reducing the derivatization and achieving the production of high quantities of strictosidine [92]. These suggest that optimal configuration of the heterologous expression with the host's metabolism could potentially lead to enhanced bioproduction. Another challenge is toxicity and reduced plant growth due to the strong constitutive expression of pathway genes and accumulating product. For instance, when testing *N. benthamiana* plants as constitutive moth pheromone biofactories, it was observed that high pheromone production levels were accompanied with plant growth reduction, causing dwarf plants [93]. To eliminate this problem, Kallam *et al*. innovated synthetic regulatory elements consisting of a copper-inducible expression system and controlled the timing and levels of gene expression to prevent toxicity [94]. Additionally, advanced regulatory circuits mentioned in the first section can be utilized to restrict the spatiotemporal expression of proteins, further mitigating the adverse effect from toxicity accumulation.

Better understanding of plants presents an opportunity to advance bioproduction. Investigation of plant species with desirable characteristics can lead to the identification of optimal chassis

and candidate genes and pathways involved in the natural product synthesis. The rapid development of multi-omics platforms accelerates the process of decoding a plant's prospective relevance to agricultural domestication and biotechnological utilization [95,96]. Similarly, fundamental research to understand the commonly used bioproduction chassis *N. benthamiana* has been extremely helpful. These studies revealed that endogenous proteases that act redundantly and consecutively deplete protein product yield, while the expression of protease inhibitors, changing pH, or gene knockdown, can increase the recombinant protein yield [97].

Concluding remarks and future perspectives

Plant synthetic biology circuits with advanced capabilities have emerged in the last several years, after falling behind microbes and humans for decades. The potential impact of synthetic biology in basic science to understand and manipulate plants, along with its applications in agriculture and biotechnology, is immense [98–100]. Although customers have seen several plant molecular farming products, there remains some technical, economical, and societal issues to be addressed before the full potential can be unlocked (see Outstanding questions). Future work in plant synthetic biology should prioritize developing approaches for optimized and robust circuit function for advanced capabilities in plants, including non-model crops, in addition to improving scale-up and production methods and addressing biosafety and regulatory concerns. We believe we are now on the cusp of the plant synthetic biology blooming stage, whereby in starting to tackle these outstanding questions, we can make a substantial impact on the sustainable food and bioproduct generation for human and planet health.

Outstanding questions

How can we expand the library of plant genetic and regulatory parts that are precise, modular, and tunable?

What knowledge and tools are needed to enable the design of crops with valuable traits to tolerate the environmental stress caused by climate change?

How can we exploit the potential of engineered plants for increasing food quantity and quality while considering biocontamination and regulation?

What efforts are needed to accelerate the procedure of translating bench discoveries into real-world applications?

Will it be possible to scale up these plant synthetic biology approaches to field level?

Can we reduce the high cost of downstream processing on plant molecular farms?

How can scientists communicate globally to standardize plant synthetic biology resources?

What can be done to educate the public and by who?

Declaration of interests

No interests are declared.

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