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Special issue: Biomass as a path to sustainability

Review

Plant synthetic biology innovations for biofuels and bioproducts

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Plant-based biosynthesis of fuels, chemicals, and materials promotes environmental sustainability, which includes decreases in greenhouse gas emissions, water pollution, and loss of biodiversity. Advances in plant synthetic biology (synbio) should improve precision and efficacy of genetic engineering for sustainability. Applicable synbio innovations include genome editing, gene circuit design, synthetic promoter development, gene stacking technologies, and the design of environmental sensors. Moreover, recent advancements in developing spatially resolved and single-cell omics contribute to the discovery and characterization of cell-type-specific mechanisms and spatiotemporal gene regulations in distinct plant tissues for the expression of cell- and tissue-specific genes, resulting in improved bioproduction. This review highlights recent plant synbio progress and new single-cell molecular profiling towards sustainable biofuel and biomaterial production.

Highlights

Advances in plant synthetic biological techniques allow for the production of various bioproducts and genetic traits.

Synthetic biology can enable sustainability of plant feedstock biomass while decreasing inputs.

Nanobiotechnology for the delivery of synthetic materials allows for effective gene regulation and bioproduct synthesis in plant cells and organelles.

Single-cell techniques will give rise to more versatile control in rationally engineered feedstock plants.

Need for sustainability

Our need for food, fuel, and infrastructure runs counter to the global environmental crisis caused by greenhouse gas emissions, exponential human population growth, and excessive pollution. Therefore, the conversion of biowastes to environmentally friendly materials and the replacement of petroleum-based fuel with **biomass feedstocks** (see Glossary) are considered to be promising routes for environmental sustainability [1]. Synbio is an emerging field at the nexus of computational design of gene circuits, biological parts, and systems. Because of its tremendous potential to synthesize useful products such as medicines and pesticides in transformed biological chassis organisms such as plants, synbio is an attractive biotechnological approach for rapidly addressing the world's sustainability problems.

Synbio as a powerful technique for a sustainable bioeconomy

Recent advances in systems biology include large sets of genome sequences, other omics breakthroughs, and bioinformatics which provide parts lists and putative wiring diagrams of organisms. In contrast to systems biology, which reads life, synbio has the potential to write and rewrite life by installing novel components and circuits into organisms. Thus, synbio is poised to literally transform the bioeconomy by providing new routes of making valuable bioproducts.

Synbio techniques have greatly advanced in the past two decades, with promising success in research, efficient production, and social contributions [2]. Synbio has evolved as an interdisciplinary field integrating biology and engineering to create new biological systems approaches within living cells for more efficient and ecofriendly production methodologies [3,4]. For example, recent research using synbio yielded a sustainable production system for isopropanol and acetone in *Clostridium autoethanogenum* by bioengineering enzymes in the acetone and isopropanol biosynthesis pathways from steel mill off-gas, which also mitigated severe air pollution [4].

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Synbio products already impact our daily lives in many ways. From edible food flavors to medicines, numerous synbio products have been engineered into monocellular and multicellular biological chassis for cost-effective production and purification [5]. Based on the economic success of synbio products, such as vaccines and therapeutics, the demand for more effective techniques and platforms increases every year [6,7]. Here, we review recent successes in plant synbio techniques with an eye towards the manufacturing of environmentally sustainable biofuels and other bioproducts in plant feedstocks.

Synbio techniques for sustainable bioproduction in plants

Crops and trees have high-biomass capacities, making them good candidates for metabolic engineering via synbio [8]. Synbio-enabled rational bioproduction from biomass requires transgene expression to be tuned in space and time. In addition, the reduction or removal of nonessential genetic elements is crucial to minimize metabolic burden [9]. Synbio tools and techniques to accomplish these objectives in plants have made significant progress recently and are characterized herein (Figure 1).

Synthetic promoters

The design, production, and application of synthetic promoters is perhaps the most advanced synbio tool for plants; they can address spatiotemporal expression requirements. Synthetic promoters are typically composed of novel combinations of ***cis*-regulatory elements (CREs)** to tune transgene expression [10].

In addition to synthetic promoters, synthetic gene constructs may also use synthetic genes and terminators as well as distal enhancer or repressor sequence fragments [11,12]. A rational synthetic promoter can be defined as a novel DNA fragment comprising multiple repeats of a single CRE or combinations of several CREs, fused with a minimal promoter that recruits RNA polymerase for transcription; there has been a number of recent successes for constitutive, inducible, and tissue-specific expression [10].

The state of the art encompasses the design of synthetic promoters using CRE databases via computational selectable algorithms. For example, several basic minimal promoters have been synthesized by the combination of biotic or abiotic responsive CREs predicted by motif-selective algorithms with the addition of the 35S CaMV core promoter [13,14]. Moreover, to allow more precise regulatory synthetic promoter construction, other native promoter components surrounding the plant core promoter have been screened [12]. This approach has led to the identification of active enhancers or silencers, resulting in the improvement of basic core promoter activity as screened by transient expression [15]. In addition, orthogonal regulatory systems may be designed for specific control and improvement of synthetic promoter sensitivity. In one such experiment, randomly selected yeast promoter fragments were synthetized to act as activators, repressors, and promoters in *Arabidopsis* sp. and tobacco [16]. Synthetic promoters may be coupled with synthetic transcription factors, such as the *Neurospora* QF system in which bacterial pathogen phytosensors were produced [17].

Genome editing

Advances in plant genome sequencing have enabled more precise DNA manipulation in plants. Unlike transgene integration methods using *Agrobacterium* transformation, genome-editing techniques can minimize possible pleiotropic effects caused by random integration of transgenes. In fact, crops with edited genomes are beginning to find their ways onto the market [18].

Glossary

Biomass feedstock: materials used to make renewable biofuels. Includes plants and lignocellulose compounds from various plant species such as switchgrass, sweet sorghum, hybrid poplar, sugar cane, among others.
Biomass recalcitrance: the impeding factors of biomass saccharification, resulting in reduction of biofuel production. In lignocellulosic feedstock, lignin is the notable compound leading to recalcitrance.
Biorefineries: industrial facilities producing biofuels, power, and biomaterials from biomass feedstocks.
***Cis*-regulatory elements (CREs):** noncoding DNA region located near the transcription initiation sequence containing elements that regulate gene expression such as promoters, enhancers, and silencers.
Epigenetic modification: alterations in gene expression caused not by DNA sequence changes but by other modifications such as DNA methylation. Can lead to downregulation of desired genes in transgenic plants.
Gene expression complex: the set of molecules which work together to initiate gene transcription, including proteins such as transcription factors, RNA polymerase, and chromatin-binding proteins.
Guide RNA: an RNA fragment which guides a DNA cleavage protein, such as Cas nuclease, to the DNA or RNA target region.
Heteroplasmic state: the genetic state in which multiple different copies of plastid DNA or mitochondrial DNA exist within a single cell.
Plastid genome: the collected DNA for plastid genes, typically circular and double-stranded. Encodes many plastid-related processes, most notably photosynthesis.
Polycistronic gene: a characteristic of prokaryotic systems, in which a single mRNA encodes multiple polypeptides simultaneously.
Saccharification efficiency: the ratio of simple sugars produced by enzymatic hydrolysis compared to the original sugar amount in lignocellulose compounds before enzyme treatment. Saccharification denotes the chemical and physical processes which break down complex carbohydrates into ethanol.

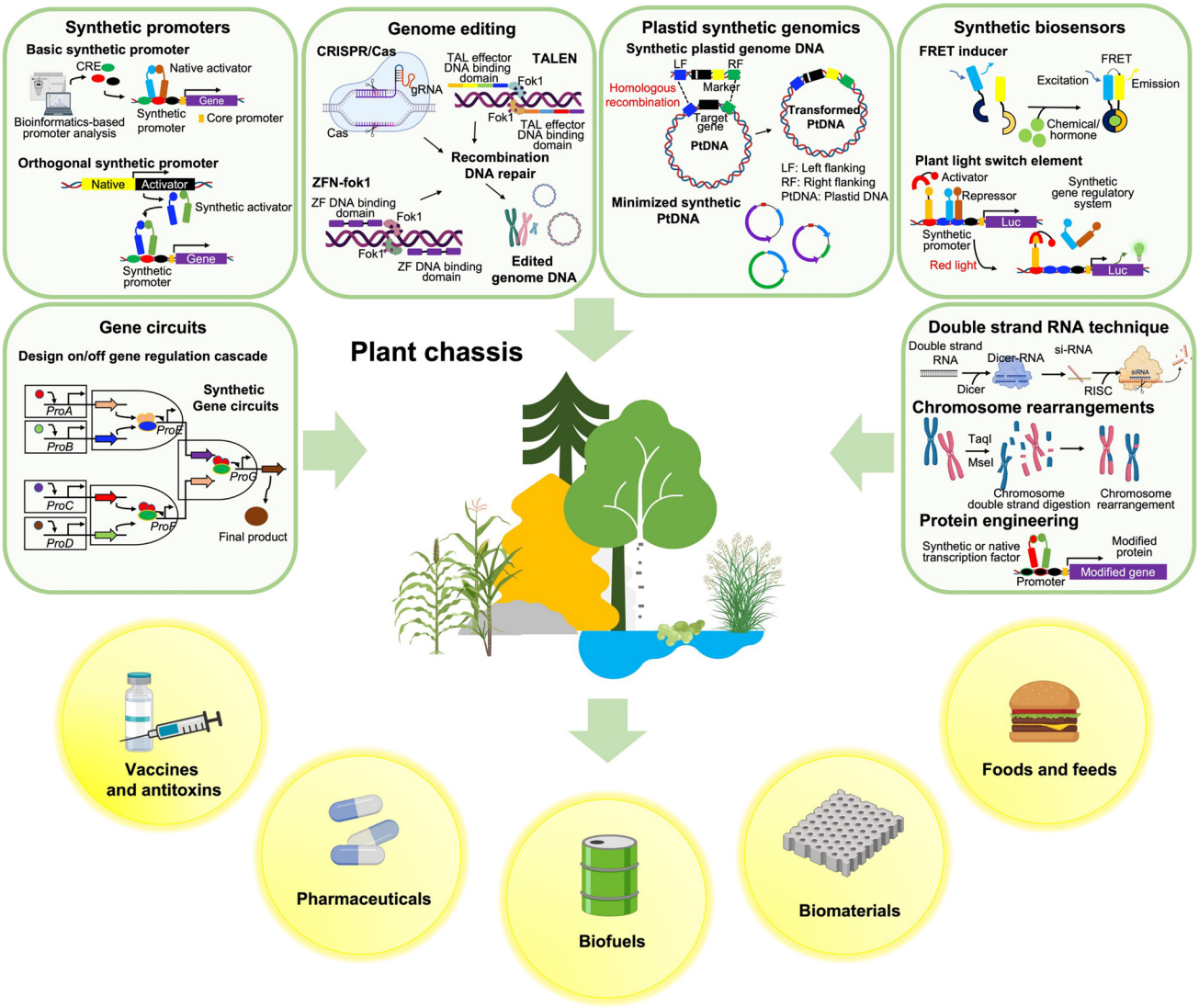

Figure 1. A schematic overview of representative synthetic biology applications using plants. Abbreviations: CRE, *cis*-regulatory element; CRISPR, clustered regularly interspaced short palindromic repeats; FRET, Förster resonance energy transfer; Luc, luciferase; Pro, promoter; RISC, RNA-induced silencing complex; TAL, transcription activator-like; ZF, zinc finger; ZFN, zinc finger nuclease; TaqI and MseI, restriction enzymes. Image was produced with web-based rendering service in www.biorender.com.

Principles of gene editing and its basic applications have been reviewed recently [19,20]. While a number of genome-editing technologies have been used in plants, they have all been largely superseded by clustered regularly interspaced short palindromic repeats (CRISPR) methods.

Owing to its high mutation efficiency, the CRISPR/Cas system is popular in crop genome editing, wherein modifications of the Cas nuclease active protein motif induce single-strand cleavage and longer indel changes at the target of specific **guide RNA** molecules [21,22].

Two types of modified Cas9 protein – Cas9 nickase [nCas9(D10A) or nCas9(H840A)] and deactivated Cas9 nuclease (dCas9) – are also utilized for specific base editing [20]. These Cas

proteins cause precise single- or double-base editing, as well as long indels, and are a powerful mutagenesis tool for creating single- or double-nucleotide polymorphisms at a particular sequence [19,23]. Cytidine to thymine single-base editing was first applied by cytidine deaminase APOBEC1 or APOBEC3A fused with nCas9(D10A) or dCas9 in rice, wheat, maize, and potato [24,25]. Adenine base editors were exploited by a special adenosine deaminase in arabidopsis, rice, and wheat [26,27]. A dual base editing system simultaneously targeting adenine and cytosine with a guide RNA was developed using saturated targeted endogenous mutagenesis editors (STEMEs) [28].

CRISPR/Cas applications in agriculture have been thoroughly reviewed [20,29] for gain-of-function mutations through precise genome editing or deletion of undesired gene traits by sequence-specific indel inductions [27,28]. The tissue-, cell-, and organ-specific targeted CRISPR system-based tissue-specific knockout system (CRISPR-TSKO) spatiotemporally regulates genome editing of the genes involved basic cellular functions and/or reproduction without severe pleiotropic phenotypes [30]. Moreover, cytidine deaminase fused with transcription activator-like effector nuclease (TALEN) DNA binding domains and a single TALEN molecule have been used to successively target and edit plastid and mitochondrial genomes [31,32].

Synthetic genomics in plastids

Synbio techniques utilizing the **plastid genome** for metabolic engineering have several advantages over nuclear transformation. These include precise genetic engineering, **polycistronic gene** stacking, avoiding gene silencing and **epigenetic modifications**, and reduction in gene transmission across generations [33]. Exogenous proteins produced in transplastomic plants may be as high as 75% of total soluble protein (TSP) [34]. For example, higher accumulation of a synthetic nitrogenase Fe protein was shown in tobacco chloroplasts compared with previous attempts using nuclear transformation [35]. However, developing optimal transplastomic DNA vectors, effective biolistic delivery methods, and precise homologous recombination into plastid DNA are still major issues for chloroplast transformation [33,36].

Conventional plastid transformation techniques have been reviewed [33,37]. Many pharmaceuticals, antigen proteins for vaccine production, biofuel precursors, and industrial enzymes can be produced through transplastomic engineering [37–40]. Stable transplastomic approaches in native wormwood led to the exploration of higher efficiency of the artemisinin biosynthetic pathway using the original plant system of *Artemisia annua* rather than model plant alternatives [41]. Similarly, the antioxidant astaxanthin was effectively produced in transplastomic tobacco using synthetic operon constructs stacking three enzyme components of a 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway [40,42]. Several factors – including pentatricopeptide repeat proteins (PRRs), RNA amplification-enhanced riboswitch (RAmpER), and the insertion of intercistronic expression element (IEE) – are known to boost expression of plastid transgenes [36]. It is also possible to introduce exogenous expression in chloroplasts without transgene insertion into the plastid genome, using circular synthetic plastids called minichromosomes or minisynplastomes [43,44]. Such novel episomal vectors may be engineered with synthetic circuits and introduced into plastids without modifying the native plastid genome. Furthermore, they may persist across generations in the absence of selection.

Synthetic biosensors

Synthetic biosensors are used to directly or indirectly recognize environmental cues, including phytohormones, metabolites, and microorganisms [45–47]. Rational design of synthetic biosensors is a pivotal step in leveraging versatile synthetic circuits, allowing for precise recognition of certain input factors and transferring output signals to the next gene circuit [48]. Various molecular

components, including transcription factors, riboswitch regulators, and functional enzymes, can be employed as biosensors coupling with selected fluorescent protein [9,49]. For example, a recent study applied Förster resonance energy transfer (FRET) with the *Escherichia coli* tryptophan repressor (TRPR) to directly detect indole-3-acetic acid (IAA) based on chemically induced dimerization [50]. To date, a wide range of phytohormones have quantified its concentration by synthetic techniques, including FRET, fluorescent protein expression driven by synthetic hormone-sensitive promoters, and fluorescent protein degradation by hormone–receptor complex [51]. Two other biosensors, AtLEA4-5 and SweetTrac1, were engineered to sense osmolarity and sugar substrates [52,53].

Light-sensitive protein sensors are currently the most popular indirect biosensor system employed using synthetic promoters. One such application is the use of plant-usable light-switch elements (PULSE), which can distinguish different light sources and activate gene expression only under specific red light [54]. Similar photoreceptors can be applied via genome-editing technologies to alter expression or even sensitivity thresholds of native photoreceptors, allowing for targeting of aspects of plant growth that can confer superior agronomic value to the engineered crops [55,56].

Synthetic gene circuits

Rationally designed synthetic gene circuits can produce complementary metabolic networks and signal transduction by modular assembly of basic biological parts for gene expression [57,58] which can be used for novel bioproduction [57,59]. However, the advancement of gene circuit design requires further optimizations in synthetic promoters and sensors for precise circuit activation. The recent appearance of a common syntax of plant DNA parts has enabled the assembly of genetic circuits using standardized parts [60,61]. Furthermore, a designed gene circuit was quantitatively characterized by transient screening using protoplasts and stably transformed plants [62,63]. These advances contribute to the development of more powerful gene circuits for the future.

Synthetic toggle switches have been applied to control downstream regulatory circuits for the synthetic design of plant signal transduction pathways [58]. However, further work is needed in the development of advanced genetic circuits to control complex plant regulatory and developmental processes in specific tissues and organs [63].

Other useful techniques

For crop protection, double-stranded (ds) RNA was synthesized to trigger RNAi via bacteriophage φ6 dsRNA generation system [64]. Also, the engineering of functional proteins such as transcription factors modified wild-type gene transcription by reconstruction of **gene expression complexes** [65]. Recently, a genome restructuring method called 'TAQing' has been described for introducing adaptive mutations [66]. Arabidopsis chromosomes were rearranged by TaqI-aided genome rearrangement following DNA double-strand breaks by a TaqI restriction enzyme. Transgenic arabidopsis had higher biomass than controls under stress conditions. When using MseI in place of TaqI, chromosome-rearranged arabidopsis was endowed with new traits such as high salt resistance and hypersensitivity to abscisic acid (ABA) [67]. Protein engineering using computational simulation and/or crystal structure analysis is also a powerful synbio toolbox. Recently, this approach resulted in biocatalysts for the biosynthesis of natural products [68].

Current cooperative synthetic biological techniques

The combination of synthetic biological techniques can enhance the effect of each single application on synthetic events. For example, the growth retardation consequence of the constitutive production of astaxanthin in engineered chloroplasts was alleviated by orthogonal T7 RNA polymerase expression in the nucleus [40]. Additionally, gene knockdown or knockout edits by

CRISPR/Cas9 have been used in tandem with various synthetic processes, including synthetic optical promoter, plastid genome transformation, and synthetic hormone sensor activation [54,69,70]. Herein, approaches must be integrated to produce holistic systems that will be robust in production systems.

Application of nanobiotechnology and novel plant chassis for plant synbio

Current DNA transfer applications of direct DNA bombardment in plant tissue coupled with agrobacterium-mediated transformation, cationic delivery, and viral particles still have limitations [71,72]. Nanoparticle-deliverable gene-editing techniques were shown to be nontoxic to living organisms [73,74] and can mitigate deleterious transgenic effects such as low transformation efficiency in monocots, unintended gene disruption by random insertion, and adverse regulatory system effects [29,71,75–77]. For these reasons, nanoparticles are being considered as an alternative delivery system for DNA and other molecules in plant synbio (Figure 2) [78].

Recently, DNA-grafted nanoparticles were used for nuclear gene delivery in mature leaves of tobacco, arugula, wheat, and cotton, as well as arugula protoplasts, without integration into genomic DNA [78,79]. This study addressed nanoparticle delivery not only as a biomolecule transporter into plant cells, but also as a method for protecting polynucleotides from nuclease degradation *in planta* (Figure 2A). In addition, by using nanoparticle plasmid delivery, the detrimental cell damage caused by biolistic techniques can be avoided. Chitosan-complexed single-walled carbon nanotubes coated with 250 times less plasmid DNA were able to penetrate directly into plant chloroplasts through stomatal pores, replacing the need for damaging physical transport systems (Figure 2B) [80]. Furthermore, nanoparticles engineered with quantum dot (QD) fluorescence and β-cyclodextrin molecules were shown to shuttle diverse chemicals to specific cell organelles. Specifically, the researchers coated nanoparticles with a rationally designed and conserved guiding peptide to target QD transfer into plant chloroplasts [81]. Precise molecule delivery to specific subcellular compartments has been limited in classic transformation systems; however, nanobiotechnology can help [82] (Figure 2C).

In order to extend research and applications alike, synbio needs to be expanded beyond the typical plant biotech models such as species of *Arabidopsis* and *Nicotiana*. Recently, *Wolffia*, a genus of small aquatic monocot plants, has been nominated as a synbio model because of its fast reproduction and relatively simple genome [83]. CRISPR-mediated genome editing of *Wolffia* may be performed to a 95% success rate [84]. If a carbon source and water are continuously provided, *Wolffia* grows similarly to some bacteria species. Therefore, perhaps this small water plant has potential to be used for biofuel and for phytoremediation when growing in chemically contaminated water.

Synbio applications for biomass feedstock production

Many genes associated with the increase in lignocellulosic biomass (LB) and the decrease in **biomass recalcitrance** are regularly engineered in biomass feedstock plants using genetic transformation [85–92]. Diverse synbio approaches have been employed for improving biomass-related traits in plants (Table 1). While chloroplast transformation has been used for introducing single or multiple synthetic operons into plants for biomass synthesis or photosynthesis-related metabolic pathways, it is routinely performed only on a few plant species, none of which are high-biomass crops. Nonetheless, as a stand-alone approach, chloroplast transformation has been shown to enable large quantities of cellulolytic degradation enzymes to be produced and to be easily extracted from chloroplasts [34,93–97].

Cel6A, a bacterial cellulase, was recently produced in high quantities by tobacco chloroplast transformation. Field-grown, two-plastid-engineered tobacco lines had cellulase levels around

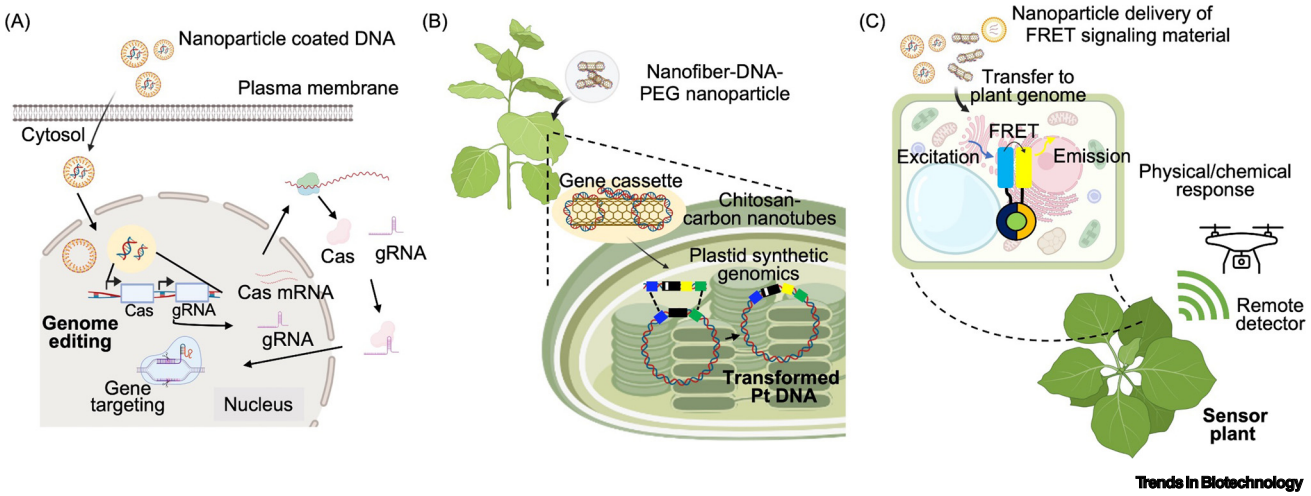

Figure 2. Advances in nanobiotechnology for enhancing plant synthetic biology. (A) Nanoparticle delivery of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas gene construct. (B) DNA delivery by nanofilaments for chloroplast transformation. Chitosan–carbon nanotube complex coated with DNA cassette or plasmid delivered to chloroplasts without physical or chemical damage in the host plant. (C) DNA delivery for biosensing by various nanoparticles. Diverse nanoparticles delivering Förster resonance energy transfer (FRET) or electric signal-expressed genetic materials into plant organelles including genomes located in the nucleus, chloroplasts, and mitochondria. Remote sensing techniques will be applied to monitor biosensing signal against environmental clues and chemicals in the engineered plant. Abbreviations: gRNA, guide RNA; PEG, polyethylene glycol; PtDNA, plastid DNA. Image was produced with web-based rendering service in www.biorender.com.

20% of TSP, without biomass loss, and with little reduction in photosynthetic products [93]. Two other endoglucanases (endo-β-1,4-xylanase and β-glucosidase) were recovered from 2% up to 75% TSP in transplastomic transformed tobacco leaves [34]. Furthermore, the bacterial endoglucanase CelK1 in chloroplast-transformed tobacco accumulated up to 6% of TSP even in a **heteroplasmic state** [95]. In addition to cellulolytic enzymes, hemicellulose degradation enzymes for alcohol conversion, such as xylanase, can also be produced through plastomic transformation [98]. Two recent studies have validated the use of chloroplast-engineered biocatalysts and pectinase enzymes for commercial applications. They displayed long storage durations as freeze-dried powder at ambient temperature without loss of enzymatic activity [97,99]. This demonstrates that chloroplast **biorefineries** are a commercially viable alternative to other purified enzymes. Additionally, they showed that the leaf is a valid tissue of choice for high levels of production.

In addition to their use as enzymatic biofactories, chloroplasts can also be studied for improved photosynthesis, which can lead to increased biomass. The photorespiration process of an important C3 plant can be bypassed or competed with by utilizing synthetic gene circuits and switches to engineer higher carbon fixation rates and obtain more biomass and bioenergy [100]. In a recent study, a photorespiration-bypassing circuit was designed to harness ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) and to establish synthetic routes for minimal CO_2 release [101]. Additionally, engineering for reduced glycolate export from the chloroplast and increased glycolate recycling elucidated another potential pathway for bypassing photorespiration, thereby increasing crop biomass [102,103]. Another possible path is the modification of plant RuBisCO to mimic that of other organisms such as cyanobacteria. One study used tobacco knockouts for native RuBisCO and replaced it with transplastomic expression of a cyanobacterium RuBisCO, leading to a more effective CO_2-capturing mechanisms compared to nontransgenic tobacco [104]. A similar strategy applied RNAi to silence nuclear *RbcS* in tobacco and engineered a combined *rbcL–rbcS* operon within tobacco chloroplasts. Different potato *rbcL–rbcS* operons were

Table 1. The recent applications of synbio techniques for lignocellulosic biomass alteration and bioproduction of feedstock degradable enzymes

Synbio technique	Synthetic target gene or elements	Host plant	Refs
Plastid synthetic genomics	Endoglucanase	Tobacco	[34]
	Bacterial cellulase Cel6A	Tobacco	[93]
	Fungal and bacterial cell-wall degradation enzymes	Tobacco	[94]
	Bacterial glucanase CelK1	Tobacco	[95]
	Bacterial glucanase CelK1	Tobacco	[96]
	Pectinase	Tobacco, lettuce	[97]
	Xylanase	Tobacco	[98]
	Lipase, mannanase, endoglucanase, and exoglucanase	Tobacco, lettuce	[99]
	Glycolate metabolic pathway	Tobacco	[102]
	Glycolate recycling pathway	Rice	[103]
	Exogenous cyanobacterium RuBisCO expression	Tobacco	[104]
	rbcL–rbcS gene	Tobacco	[105]
CRISPR/Cas	AtCCR1; cooperation with SNBE synthetic promoter	Arabidopsis	[70]
	NtAn1	Tobacco	[107]
	NtFAD2	Tobacco	[108]
	PDS homolog	Duckweed	[116]
	Pv 4-Coumarate:coenzyme A ligase (4CL)	Switchgrass	[113]
	Populus CCR2	Poplar	[118]
	SlCLV3 promoter	Tomato	[114]
	SlCLV3 promoter	Maize	[115]
	CLE promoter target	Maize	[116]
TALEN	O-methyltransferase (COMT)	Sugarcane	[110]
	O-methyltransferase (COMT)	Sugarcane	[111]
Synthetic promoter	Ap, Dp, ANDp for drought stress recognition; exogenous gene expression	Arabidopsis	[150]
	SNRE repeat; cooperation with AtCCR1 target CRISPR/Cas	Arabidopsis	[70]
	SNRE repeat and CCR1	Arabidopsis	[117]
Chromosome rearrangement	DNA double-strand break	Arabidopsis	[66]
Combinational transformation	Calcium-dependent protein kinase	Tobacco	[119]
Gene stacking	GALS1, URGT1, UGE2; pectin galactan in stem, and QsuB	Arabidopsis	[120]
Synthetic transcription factors	ViparíNama synthetic protein structure	Arabidopsis; tobacco; tomato	[65]

incorporated in transgenic tobacco, and increased carboxylation rates were demonstrated in some lines and reduced rates in others [105]. It is also possible to create new synthetic pathways to improve CO_2 fixation. One such cycle is the crotonyl-coenzyme A (CoA)/ethylmalonyl-CoA/hydroxybutyryl-CoA (CETCH) pathway, which involves 17 enzymes from nine different organisms [106]. This pathway showed enhanced CO_2 fixation into organic molecules *in vitro*, with rates up to five times higher than the *in vivo* rate of the natural pathways [106].

The genome-editing targets for biomass alteration have focused mainly on lignin and lipid biosynthesis genes in diverse species such as rice, tobacco, arabidopsis, sugarcane, switchgrass, and *Populus* sp. (poplar) [70,84,107–113]. In addition, CRISPR-Cas9 has been used to mutagenize promoter sequences to increase fruit size in tomato and grain yield in maize [114–116].

Previous studies have shown that plants which are genetically transformed for the impairment of lignin synthesis show higher **saccharification efficiency**, but also exhibit dwarf phenotypes as well as a loss of overall biomass. This problem has been mitigated by spatiotemporal regulation of a synthetic secondary wall NAC binding element (SNBE) promoter fused with the lignin biosynthesis gene *CCR1*. Transgenic arabidopsis including this gene construct had a dramatic increase in total stem biomass and demonstrated monolignol transport between vessel cells and neighboring xylary fibers [117]. This inducible synthetic promoter was utilized for xylem-specific restoration of *CCR1* in CRISPR/Cas9-edited *CCR1* knockout arabidopsis. This mutant reduced recalcitrance of lignin in xylem to increase saccharification efficiency while maintaining normal levels of biomass [70]. Poplar, a high-biomass plant for biofuel production, was recently engineered by CRISPR/Cas9 to knock out *CCR2*. Screening of lignin content in gene-edited transgenic poplar had up to 10% lignin reduction without a decrease in biomass, which increased saccharification efficiency by 25–41%, depending on the pretreatment method used [118]. This result is promising as it demonstrates that lignin can be rationally reduced without negative impacts on plant growth and phenotype, thereby allowing for greater biofuel efficiency.

Biodiesel is generally derived from seed oil feedstock. Tobacco *NtAn1*, a homolog of *TT8*, has a negative relationship with lipid synthesis in seeds. When knocked out using CRISPR/Cas9, the resulting mutant had an increase in total lipid productivity [107]. Additionally, it is shown that oleic acid content can be increased in transgenic tobacco seeds that have *FAD2* knockouts introduced by CRISPR/Cas9 [108]. Further studies can apply this knowledge to improve biodiesel crops for increased oil production.

Several new techniques have employed synthetic materials to improve biomass yields. One such method, which differs from those discussed so far, is the introduction of multiple ds breaks within the arabidopsis genome to produce artificial selection. This showed unique phenotypes with increased biomass and higher yields of ethanol produced from xylose [66]. Another route utilizes combinatorial transformation of synthetic gene constructs using multiple calcium-dependent protein kinase genes by bombardment, generating randomly combined genetic networks in tobacco. These transgenic tobacco lines were screened for drought resistance and found to have increased biomass under stress conditions compared to their nontransgenic counterparts [119].

Additionally, protein engineering of hormone-sensitive transcription factors induced the alteration of shoot branching in arabidopsis and plant size in tomato [65]. Finally, gene stacking has been applied to increase C6 sugar content by overexpression of *GALS1*, *URGT1*, and *UGE2*, coupled with *QsuB* gene expression to produce low lignin accumulation in a transgenic arabidopsis [120].

Synbio applications for plant immunity

Pathogens and herbivores are major factors which can negatively impact plant biomass, thereby indirectly reducing sustainability. Therefore, it will prove useful to harness synthetic biological tools that can boost immunity, thereby improving overall biomass. Genome-editing techniques have recently been applied to impair infection processes and reduce viral genome loads. For instance, the CRISPR/Cas9 system was programmed to target beet severe top curly virus and bean yellow dwarf virus, resulting in negation of viral infection symptoms and a decrease in viral genome accumulation in transgenic plants [121,122].

As a further example, researchers have also demonstrated the possibility of synthetically modifying plant immune receptors. In a recent study, the intracellular nucleotide-binding, leucine-rich repeats (NLRs) of a plant immune receptor to pathogen effectors were engineered to improve defense against pathogens by protein engineering. These pathogen-resistant transgenic rice plants were engineered by introducing the integrated decoy domain in the heavy metal-associated domain of RGA5, allowing for nonspecific recognition of three types of *Magnaporthe oryzae* effectors [123].

Future advances of plant synbio through single-cell techniques

To date, systems biology data have been collected from bulk plant cells under environmental stresses or during different developmental stages. This global profiling can reveal new insights for molecular-level regulation of plant biological processes, which are stimulated by environmental cues and developmental signals. Research in bioenergy feedstock plants has gained significant benefits from this information, allowing investigators to increase plant biomass and optimize performance against detrimental biotic and abiotic factors [124]. However, current rational gene construct design relies on bulk profiling methods that sample whole tissues in bulk. This approach limits our ability to understand intricate molecular networks covering whole organisms or complex tissues [125]. Plant tissues are composed of types of cells which serve specialized functions. For any given tissue sample processed using bulk methods, molecular events in one cell type might overlap those in other cell types, limiting the information about specific cellular responses. Therefore, it is important to develop molecular profiling approaches that can distinguish and sample individual cell types in order to accurately describe how plants adapt in different environmental and biological situations [125]. It is also important to find methods to understand why some bioproducts are produced predominantly in a few specific cell types [126]. For example, useful biomass in feedstocks is predominantly composed of secondary cell walls in stems. A deep understanding of biological processes and molecular events in secondary cell-wall tissue would allow for better control of important regulatory networks which are specific to these tissues. Single-cell omics, including transcriptomics, proteomics, and metabolomics, are useful for this purpose (Figure 3).

The most active area of study using plant single-cell techniques is transcriptome profiling for root development. Single-cell RNA sequencing (scRNA-seq) on arabidopsis roots identified genes involved in lateral root initiation and structural maintenance [127–131]. Poplar scRNA-seq data were produced using single protoplast cells of developing xylem [132]. Additionally, a maize scRNA-seq study identified detailed gene expression changes in maize anther germinal cells [133]. Several reviews have summarized cutting-edge processes and analysis protocols for scRNA-seq [126,134,135]. The main issues in this field are separation of single cells without impedance by the plant cell wall, single-cell capture, sequencing techniques using small sample amounts, high-resolution visualization of each cell type, and clustering of scRNA-seq data sets for optimal results.

Current proteomic and metabolic profiling has advanced more slowly than single-cell transcriptomics because of the challenge in analyzing very small amounts of protein within a single cell [136,137]. The reduction of sample preparation volumes and improvement of MS techniques has helped to increase the detectability of proteins in distinct plant single cell types [138]. Furthermore, sample multiplexing techniques using isobaric labeling reagents alleviate the problem of low mass quantities in MS time per cell [139]. Current approaches for tissue preparation, new technologies and analysis methods, and the challenges of plant single-cell techniques in proteomics and metabolomics have been discussed previously [136,137,140,141].

Single-cell transcripts and protein profiling coupled with cell-type-specific MS imaging approaches (such as MALDI imaging) are important components for constructing a complete

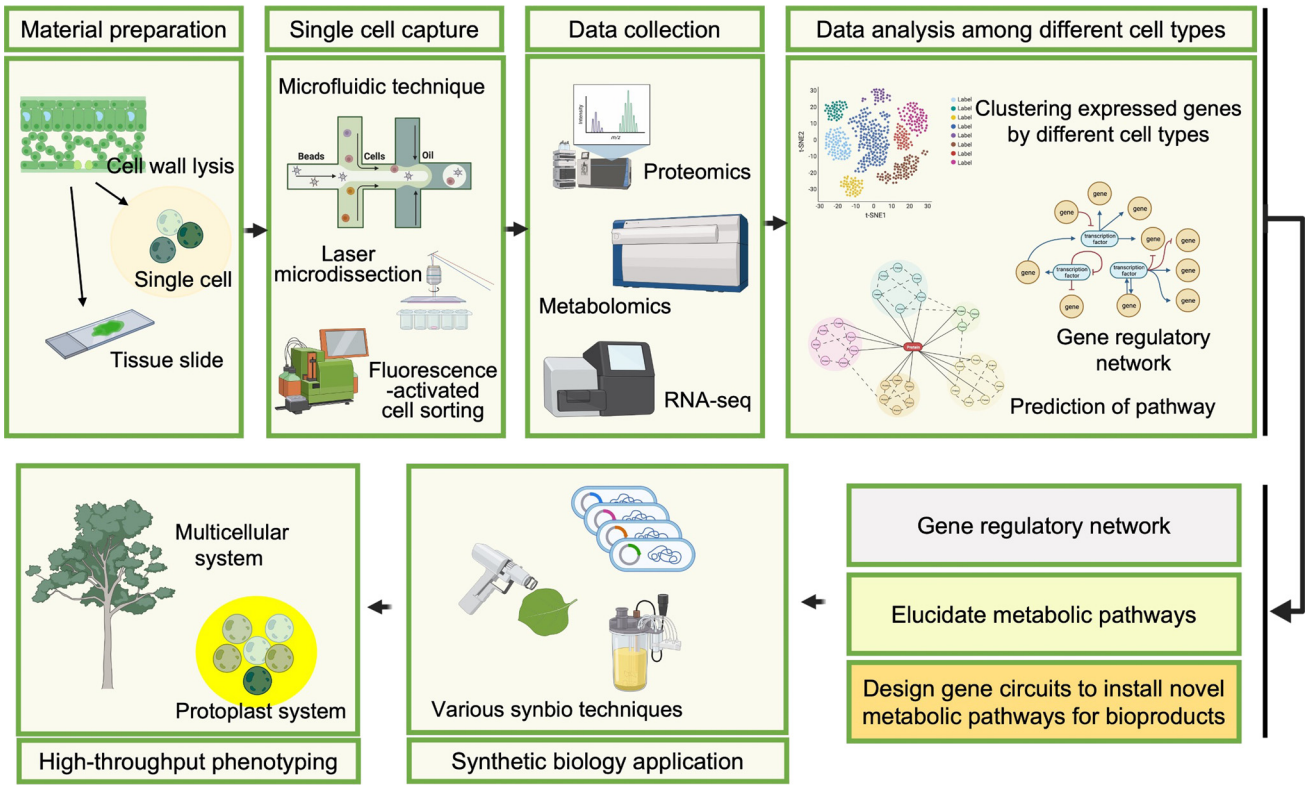

Figure 3. The workflow of application of single-cell techniques into bioengineered plants via synthetic biology (synbio) techniques. Image was produced with web-based rendering service in www.biorender.com.

plant-cell atlas [142,143]. This can allow researchers to map potential gene expression and variation by comparing with single-cell transcriptomes, as well as predicting post-transcriptional and post-translational regulation networks. Computational analysis using powerful algorithms can assist in managing vast amounts of data, leading to better predictions. For example, gene regulatory network (GRN) analysis of scRNA-seq data was recently performed using two types of computational algorithms so far [126]. The first protocol – single-cell regulatory network inference and clustering (SCENIC) – predicted GRNs through three steps, including clustering coexpressed genes with transcription factors, filtering these clusters, and determining regulons for each individual cell [144,145]. A second method, called the Inferelator, was previously tested in yeast scRNA-seq data [146]. These predicted GRNs still require confirmation in practical systems, but this preliminary success is promising. Single-cell omics techniques can provide valuable data for association of individual proteins in the same cellular function at a specific developmental stage. The use of single-cell omics data to engineer typical proteins in hormone regulation was suggested in auxin-related experiments [147]. In addition, the data from forthcoming single-cell techniques can be used in synbio techniques such as gene circuits, useful synthetic promoter prediction, and biosensor design [148].

Although the data of single-cell analysis will give more detailed information to be used in synbio design, it is important to increase data precision in large-scale synbio applications. To improve the efficacy of future synbio projects, we need to understand biological systems (beyond creating

beaucoup of data) in order to rebuild metabolism in plants. Synbio, especially in plants, requires precise modeling with greater predictability than we currently have. These should have detectable device characteristics that allow for error range correction and the precise selection of synbio designs [149].

Concluding remarks and future perspectives

Several synbio techniques – such as synthetic promoters, genome editing, chromosome rearrangement, and synthetic plastid genomics – have been successfully applied for biomass improvement. In addition, synthetic biosensors for monitoring plant growth can synergize with precision agriculture to pinpoint where inputs are needed. In order to reap the fruits of synbio, a more detailed understanding of genetic regulatory mechanisms is needed in order to rationally design systems. We must advance our understanding of the genetic and physiological interactions among different cell types at the spatiotemporal level using single-cell techniques, which will allow for more precise control of synthetic platforms.

Automated systems for synbio design and functional screening in biological systems need to advance, coupled with quantification to select the best performers. Automation for high-throughput screening should speed design–build–test cycles. While the cycle turnaround in plant chassis may never approach those of microbes, synbio will require substantially faster cycling than currently practiced. That said, all technologies have their limitations, and step changes in our understanding in plant biology are needed to affect step changes in plant synbio (see Outstanding questions).

Outstanding questions

How can we reliably develop synthetic gene circuits for spatiotemporal control and reduce host cell damage?

Is it possible to apply current synthetic systems universally in both dicot and monocot plants?

Can we make an automated, high-throughput system flowing efficiently from gene construct generation to quantitative measurement of synthetic systems?

Can we develop artificial single-cell systems containing synthetic circuits for discovering cell-wall composition-to-fuel composition?

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Declaration of interests

No interests are declared.

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