

Synthetic Botany

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Plants are attractive platforms for synthetic biology and metabolic engineering. Plants' modular and plastic body plans, capacity for photosynthesis, extensive secondary metabolism, and agronomic systems for large-scale production make them ideal targets for genetic reprogramming. However, efforts in this area have been constrained by slow growth, long life cycles, the requirement for specialized facilities, a paucity of efficient tools for genetic manipulation, and the complexity of multicellularity. There is a need for better experimental and theoretical frameworks to understand the way genetic networks, cellular populations, and tissue-wide physical processes interact at different scales. We highlight new approaches to the DNA-based manipulation of plants and the use of advanced quantitative imaging techniques in simple plant models such as *Marchantia polymorpha*. These offer the prospects of improved understanding of plant dynamics and new approaches to rational engineering of plant traits.

The development of new technologies for the production of larger and improved quantities of goods from less feedstock has defined human innovation for thousands of years, especially in food production. To increase agricultural productivity, plant breeders have been selecting for advantageous traits in crops since 9000–10,000 BC (Zohary et al. 2012). One notable success is the selective breeding of modern maize from teosinte (Beadle 1939; Doebley 2004), in which a handful of genetic differences caused substantial changes in ear morphology, kernel number, and crop yield.

Technologies such as mutagenesis and introgressive hybridization, developed in the early to mid-20th century, are commonly used to in-

crease genetic diversity in breeding populations of food crops, which show lower allelic variation compared with wild populations. Selective breeding still plays a major role in the production of new varieties but the emergence of modern plant biotechnology has led to a more targeted approach to increasing crop yields. Traits of agricultural importance successfully introduced to plants using recombinant DNA technology include herbicide resistance (Comai et al. 1985), drought resistance (Kumar et al. 2014), pest resistance (Bates et al. 2005), pathogen resistance (Brunner et al. 2011; Horvath et al. 2012; Jones et al. 2014), abiotic stress resistance (Jaglo-Ottosen et al. 1998), enhanced photosynthetic capacity (Ku et al. 2001), im-

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proved nitrogen use efficiency (Yanagisawa et al. 2004), and added nutritional value (Ye et al. 2000). However, although plants hold a unique promise for bioproduction at the gigatonne scale, efforts in genetic engineering of plants are lagging compared with microbial systems (Antunes et al. 2009, 2011; Liu et al. 2011, 2013; Koschmann et al. 2012; Wend et al. 2013; Zurcher et al. 2013; Fethe et al. 2014; Müller et al. 2014).

To exploit the unused potential of plants in expressing complex traits, we require (1) efficient tools and methods for genetic engineering, (2) simpler multicellular plant chassis that are amenable to rapid, high-throughput analysis, and (3) control over the biosynthesis, transport, and storage of metabolites in specialized cells within complex plant tissues. In the following, we highlight progress made in these three key areas of concern for the future development of plant synthetic biology.

NUCLEAR TRANSFORMATION IN PLANTS

Implementation of synthetic biology in plants calls for efficient methods for genetic manipulation. The most widely used technique for transformation of most plant species relies on the native capacity of virulent strains of *Agrobacterium tumefaciens* to infect plant tissue and to transfer a segment of its DNA (T-DNA) to the host cell (reviewed in Gelvin 2003). Because this method was adapted to allow delivery of transgenes, it has been used extensively: thousands of transformants can be obtained in a single experiment (Meyerowitz 1989; Ishizaki et al. 2008), providing the throughput required for forward genetic screens. The random nature of this transformation method has been used to mine the underlying biology of plants by mutational T-DNA insertion as well as gene trap methods (reviewed in Springer 2000). Enhancer traps have been used to indicate the presence of nearby endogenous enhancer elements driving an orthogonal transcriptional activator such as GAL4-VP16. T-DNA insertion in the vicinity of an enhancer allows temporal and/or tissue-specific expression of the activator, leading to restricted reporter gene expression according to

the endogenous enhancer (Johnson et al. 2005; Laplaze et al. 2005; Gardner et al. 2009).

In addition to stable nuclear transformation mediated by *Agrobacterium*, transient methods have been developed for quantification of gene expression (Kapila et al. 1997) and bioproduction. For instance, *Nicotiana benthamiana* leaves can be infiltrated with *Agrobacterium* at multiple regions of a single leaf and screened for gene expression a few days after infection. This allows relatively fast characterization of libraries of genetic components in a common genetic background (Sparkes et al. 2006; Engler et al. 2014; Brückner et al. 2015). Transient leaf agroinfiltration in *Nicotiana* has been adopted as an efficient method for the optimization of metabolic pathways, such as artemisinin (van Herpen et al. 2010) and triterpene biosynthesis pathways (reviewed in Thimmappa et al. 2014), and the bioproduction of vaccines (D'Aoust et al. 2008; Mardanova et al. 2015). Large investments have been made in both the private and the public sectors to scale up production for vaccines against viruses like Ebola (ZMapp, Mapp Biopharmaceuticals) and influenza (Medicago). Protoplasts, single cells derived from tissues by digestion of cell walls, can also be transformed transiently. Protoplast transformation has been performed by electroporation (Fromm et al. 1985; Ou-Lee et al. 1986; Hauptmann et al. 1987; Negrutiu et al. 1987; Nishiguchi et al. 1987; Jones et al. 1989) or incubation in a PEG solution (Krens et al. 1982; Potrykus et al. 1985), and is established as one of the preferred methods for studying signaling pathways (reviewed in Sheen 2001). Furthermore, high-throughput protoplast transformation has been used to perform quantitative characterization of large libraries of genetic elements in *Arabidopsis thaliana* (*Arabidopsis*) and *Sorghum bicolor* (sorghum) (Schaumberg et al. 2016).

Precise methods for genomic inspection and reverse genetics screens have also been implemented in plants: for instance, CRISPR-Cas9 genome editing has been applied in a number of plant model systems including *Arabidopsis*, *Nicotiana spp.*, *Solanum lycopersicum* (tomato), *Oryza sativa* (rice), *Triticum aestivum* (wheat), *Zea mays* (maize), *Citrus sinensis* (orange), and



Marchantia polymorpha (*Marchantia*), at varying efficiencies (reviewed in Bortesi and Fischer 2015). Notably, CRISPR-Cas9 genome editing has been successfully used to promote homologous recombination and to simultaneously target multiple loci in *Arabidopsis* and *Nicotiana* (Li et al. 2014), as well as to induce targeted deletions in *Nicotiana* and rice (Lowder et al. 2015). Such methods are enabling unprecedented efficiency of construct delivery, genome refactoring, and chassis engineering in plants.

A COMMON SYNTAX FOR SYNTHETIC PLANT GENES

Increasingly, synthetic biologists rely on the use of modular DNA components to implement genetic circuits, along with a facile chassis for prototyping and troubleshooting, and tools for predicting behavior from mechanistic models. The implementation of assembly standards that allow parts, even those from multiple manufacturers, to be assembled together has underpinned invention in engineering disciplines and the generation of libraries of well-characterized standardized components is at the core of the synthetic biology paradigm.

The BioBrick assembly standard was the first widely adopted biological standard (Knight 2003; Shetty et al. 2008) and large number of parts, primarily for engineering prokaryotes, have been submitted to the Registry of Standard Biological Parts (Endy 2005). Eukaryotic organisms such as plants, however, require a different genetic syntax for the assembly of genes and pathways. Additionally, plasmids used for plant transformation often require specific features, such as the ability to replicate in a shuttle chassis such as *A. tumefaciens*. Recently, several alternative DNA assembly technologies have gained prominence because they offer the advantage of allowing the assembly of multiple parts in a single reaction (Engler et al. 2008; Gibson et al. 2009; Quan and Tian 2009; Li and Elledge 2012; De Kok et al. 2014). Although overlap-dependent methods such as Gibson assembly are powerful, they require custom oligonucleotides and amplification of even well characterized stan-

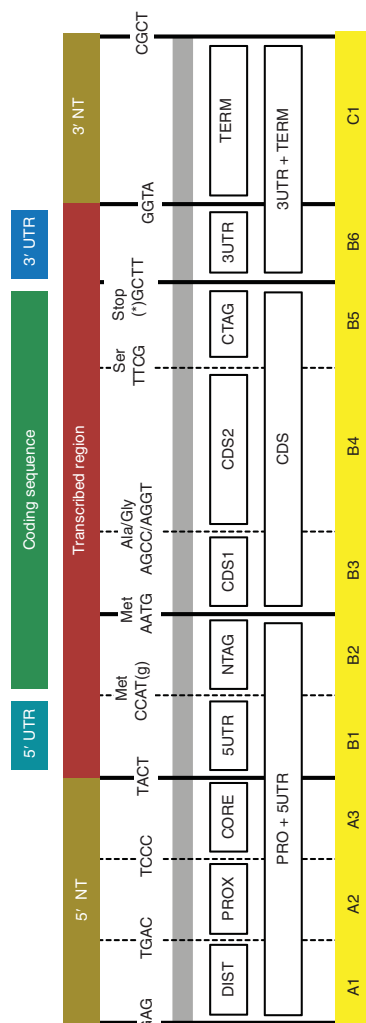
dard parts for each new assembly (Ellis et al. 2009; Liu et al. 2013; Patron 2014) unless standardized overlaps are used (Torella et al. 2013; Casini et al. 2014). The application of Type IIS restriction enzymes for assembling standard parts, known widely as “Golden Gate Cloning,” has become widely used as an alternative approach because parts can be exchanged and assembled cheaply, easily, and in an automatable way without proprietary tools and reagents (Engler et al. 2009; Sarrion-Perdigones et al. 2011; Werner et al. 2012). Many commonly used sequences have been adapted for Type IIS assembly by various plant research laboratories (Sarrion-Perdigones et al. 2011; Weber et al. 2011; Emami et al. 2013; Lampropoulos et al. 2013; Binder et al. 2014; Engler et al. 2014; Vafaei et al. 2014), and a common syntax to enable the exchange of interoperable DNA parts for plants has recently been agreed by a large section of the plant research community (see Fig. 1; Patron et al. 2015).

This common syntax describes 12 fusion sites to enable the facile assembly of eukaryotic transcriptional units and establishes standards that allow standard parts to be exchanged and reused without adaptation. This agreement will aid in establishing registries of genetic components for plants and thus facilitate the design of genetic circuits. The availability of well-characterized parts is the first stage for establishing a higher-order abstraction to implement more complex functions in plants. Combining extensive collections of genetic elements together with high-throughput quantification methods will provide robust measurements to inform mathematical models that describe the statistical normalization methods required for developing parts and devices that will work as expected in complex multicellular organisms.

CONTROL OF TRANSGENE EXPRESSION IN PLANTS

Control of transgene expression can be implemented at multiple levels in plants and other eukaryotes. Regulation can be exerted at the transcriptional, posttranscriptional, and trans-

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Position	Name	Function	5' overhang	3' overhang
A1	DIST	Distal promoter region, <i>cis</i> regulator, or transcriptional enhancer	GGAG	TGAC
A2	PROX	Proximal promoter region or transcriptional enhancer	TGAC	TCCC
A3	CORE	Minimal promoter region, including transcription start site	TCCC	TACT
B1	5UTR	5' untranslated region	TACT	CCAT
B2	NTAG	Amino-terminal coding region	CCAT	AATG
B3	CDS1	Coding region—optional amino-terminal coding region	AATG	AGCC/AGGT
B4	CDS2	Coding region—no start or stop codon	AGCC/AGGT	TTCG
B5	CTAG	Carboxy-terminal coding region	TTCG	GCTT
B6	3UTR	3' untranslated region	GCTT	GGTA
C1	TERM	Transcription terminator, including polyadenylation signal	GGTA	CGCT

Figure 1. Summary of the Phytobrick syntax for standardized plant DNA part composition and assembly. The Phytobrick syntax is a consolidation of Golden Gate, MoClo, and Golden Braid standards (Patron et al. 2015) and defines 12 fusion sites that divide eukaryotic genes into 10 basic functional units (A1–C1). The domains are listed with a brief description of their encoded function. Phytobrick parts can comprise the region between an adjacent pair of fusion sites or span many sites, and consist of portion(s) of a gene cloned into a plasmid flanked by a convergent pair of *BsaI* Type IIS restriction endonuclease recognition sequences. Parts can be assembled into complete transcriptional units in a one-pot, one-step digestion–ligation reaction provided compatible overhangs are produced on digestion and the acceptor plasmid has divergent *BsaI* recognition sites as well as a unique bacterial selection cassette.



lational levels by both endogenous and synthetic orthogonal systems. Among proven orthogonal systems for modulation of gene expression are transactivation systems like the aforementioned GAL4 activator, a tTA tetracycline inducible system based on the *Escherichia coli* tetR repressor (Weinmann et al. 1994), and an IPTG-inducible pOp/LhG4 system based on the *E. coli* lac operon (Moore et al. 1998). The various inducible expression systems implemented in plants to date include the dexamethasone responsive rat glucocorticoid (GR) ligand-binding domain (Aoyama and Chua 1997; Craft et al. 2005; Samalova et al. 2005), ethanol/acetaldehyde-inducible ALCR transcription factor and *alcA* promoter (Caddick et al. 1998; Salter et al. 1998; Roslan et al. 2001), DNA-binding domain of the *lexA* oestrogen receptor (Bruce et al. 2000; Zuo et al. 2000), copper-inducible *ace1* promoter (Mett et al. 1993), and ecdysone receptor (EcR) ligand binding-domain inducible by insecticide methoxyfenozide (Martinez et al. 1999; Padidam et al. 2003; Koo et al. 2004). Other useful circuits comprise the Cre-Lox recombinase system that can be used for induction of transcription (Hoff et al. 2001) or transgene excision (Chakraborti et al. 2008), and a catalytically inactive version of Cas9 fused to regulatory domains. The latter has been shown to perform effective transcriptional activation and repression of an endogenous gene in *Nicotiana* (Piatek et al. 2015) and *Arabidopsis* (Lowder et al. 2015).

Posttranscriptional control over gene expression in plants has been enabled by a variety of RNA-based technologies. Artificial microRNAs (amiRNAs), for example, exploit the endogenous silencing machinery to repress gene expression. The extent of amiRNA-mediated repression can be tuned by altering their expression level (Yu and Pilot 2014). Systems for optimizing the specificity and the efficiency of the amiRNAs (Li et al. 2013) as well as for obtaining inducible multigene silencing (Goh et al. 2012) have also been implemented. Another approach to posttranscriptional control of gene expression in plants is based on riboswitches. Riboswitches are ligand-binding nucleic acid aptamers that are able to modulate

gene expression by sequestering or exposing regulatory regions in transcripts via alternative nucleotide base-pairing (Anthony et al. 2012). The thiamine pyrophosphate riboswitch is an endogenous example in plants: It was identified in the 3' end of the untranslated region of the *Arabidopsis thiC* gene, and exerts control by affecting pre-mRNA splicing of the transcript (Sudarsan et al. 2003; Wachter et al. 2007). Methods have been developed to tune the sensitivities of riboswitches for their cognate ligands (Beisel and Smolke 2009), and to expand their chemical diversity (reviewed in Link and Breaker 2009).

Technologies for nuclear transformation and control over gene expression have been combined to implement synthetic genetic logic systems in plants. Examples include a light-inducible gene expression system activated by red light and switched off by far-red light (Müller et al. 2014) as well as a range of in vivo biosensors to monitor levels of cytokinin (Müller and Sheen 2008; Zurcher et al. 2013), auxin (Wend et al. 2013), plant pathogens (Liu et al. 2011; Fethe et al. 2014), and elicitors (Koschmann et al. 2012). These are examples of synthetic circuits that have been constructed in plants using methods for DNA manipulation, transgene regulation, transformation, and analysis to yield basic systems for chassis engineering and genetic network design in plant synthetic biology.

METABOLIC ENGINEERING IN CELLULAR COMPARTMENTS

Although plant biotechnology has traditionally relied on the cumulative introduction of genes followed by selection (Bates et al. 2005), new technologies promise to streamline this process: methods such as genome-editing (reviewed in Raitskin and Patron 2016; and in Schiml and Puchta 2016), construction of synthetic eukaryotic chromosomes (Annaluru et al. 2014), or the application of operon-like gene clusters (Field et al. 2011; Nützmann and Osbourn 2014) enable unprecedented control over metabolic pathways.

Plants are ideally suited for the implementation of cooperative biosynthetic processes and



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dedicated storage compartments. One example of a highly beneficial metabolic interaction between different plant cell types is the cooperation of bundle sheath and mesophyll, which markedly enhances the efficiency of photosynthesis in C₄ plants (Hibberd and Covshoff 2010). Another prominent instance of multicellular compartmentalization strategy is naturally observed in glandular trichomes of the wormwood *Artemisia annua*, which are specialized for production and storage of the antimalarial compound artemisinin (Graham et al. 2010). The potential of glandular tissues in various plant species for metabolic engineering of alkaloid, terpenoid, and fatty acid metabolism for applications in biotechnology has been reviewed in more detail elsewhere (McCaskill and Croteau 1999; Schilmiller et al. 2008).

Marchantia AS A BASAL MODEL CHASSIS FOR PLANT SYNTHETIC BIOLOGY

One of the major challenges in harnessing the potential of plant systems lies in our limited understanding of complex processes in cellular development. Morphogenetic processes in plants are driven by genetic programs, which shape the collective behavior of cohorts of cells. Vice versa, groups of cells display self-organizing properties, which affect the expression of individual genes. Plant metabolic processes take place in dedicated tissue compartments and are subject to multiscale feedback regulation. Thus, there is a need to develop suitable platforms for elucidating and engineering patterning processes defining the anatomy of plant tissues.

Ideally, such a platform requires a model system that serves as a link between microbes and higher plants. Genetic engineering of crop plants still remains a cumbersome undertaking owing to slow life cycles: Obtaining mature plants from seeds takes ~4 months for tomato (Kimura and Sinha 2008), 5 months for maize (Green and Phillips 1975), and 7 months for wheat (McHughen 1983). Furthermore, polyploidy (Vaughan et al. 2007) and high levels of genetic redundancy in higher plants (Dean et al. 1999) require mutagenesis of several loci to dis-

sect gene function. *Arabidopsis* became popular as a model organism for molecular studies in the 1980s mostly because of its relative simplicity among angiosperm plants (Somerville and Koornneef 2002). Features of *Arabidopsis* include its phylogenetic relevance, a comparatively rapid life cycle of 2–3 months, small stature, abundant seed production, and relative ease of transformation (Flavell 2009). Although easier to work with than many crop species, *Arabidopsis* still shows notable levels of genetic redundancy (Briggs et al. 2006), and crosses to obtain plants homozygous for integrated transgenes (generally integrated hemizygotously) are laborious (Wijnker et al. 2012).

Promising alternative candidate species of lower complexity can be found in the group of bryophytes, descendants of the earliest terrestrial plants (Goffinet and Shaw 2009). The best studied of these are the moss *Physcomitrella patens* and the thalloid liverwort *M. polymorpha*. Although both species show ample promise for bioengineering, *Marchantia* offers several unique advantages including a shorter life cycle, the production of *gemmae* as accessible and robust vegetative propagules (Shimamura 2012), a more streamlined genome (Sasaki et al. 2007; Zobell et al. 2010), and a simple spore-based transformation method for genetic manipulation.

In particular, *Marchantia* represents an excellent model for studying aspects of plant development and morphology. *Marchantia* forms simple, sheet-like tissues that can be characterized in terms of its distinct surfaces (Heberlein 1929): The lower surface shows root-like cells, called rhizoids, which are responsible primarily for the uptake of water and organic nutrients as well as the anchoring of the plant body to the substrate. The body of the thallus features scattered differentiated cells called oil bodies, which have been shown to play a role in isoprenoid metabolism (Suire et al. 2000). Finally, the upper surface is composed of primitive modular complexes for photosynthesis, each of which contains a permanently open pore for gas exchange. The most striking features of the upper surface of the thallus arguably are conical splash cups. Within them, *gemmae*



originate from single cells and are attractive specimens for engineering morphology, given their robustness, plasticity, and accessibility (Vöthcing 1885; Fitting 1935). In contrast to most developing organs in higher plants, *gemmae* are not buried within maternal or support tissues. Furthermore, *gemmae* retain their viability for approximately one year if stored at 4°C in agar, and are capable of vigorous germination on soil as well as artificial and sterile media (Miller 1964).

The morphological simplicity of *Marchantia* is matched by highly streamlined genetics. The sequences of the Y-chromosome, mitochondrial, and plastid genomes are available (Ohyama et al. 1986; Oda et al. 1992; Yamato et al. 2007), whereas an assembled nuclear genome sequence is expected to be published shortly, and can be found in draft form online (marchantia.info). The size of the nuclear genome has been estimated at 280 Mb by flow cytometry, and ~20,000 protein-coding genes have been predicted. In comparison to the 32,670 genes present in *Arabidopsis* and the 35,938 genes in *P. patens* (Yamato and Kohchi 2012), lower levels of gene redundancy appear to be present in *Marchantia*. For instance, a study of receptor-like kinases (RLK) concluded that the *Arabidopsis* genome contains 52 different RLK-families comprised of >600 RLK genes, whereas in *Marchantia* 26 RLK-families comprised of only 29 RLK genes were found (Sasaki et al. 2007). Another example is illustrated by MIKC* MADS box transcription factors: *P. patens* has been shown to include 11 members of this gene family (Rensing et al. 2008) whereas *Marchantia* only encodes one of these.

Marchantia possesses features that qualify it as a plant model chassis for quick and easy genetic engineering. Like moss, the life cycle of *Marchantia* is characterized by a dominant haploid gametophyte phase (O'Hanlon 1926). This allows the immediate expression of mutant genotypes in the phenotype. Through propagation of *gemmae*, haploid isogenic plants are obtained. Because *gemma* development originates from a single cell, transgenic *gemmae* do not display chimeric gene expression patterns. Further, during the gametophytic phase, it is pos-

sible to determine the sex of haploid plants by means of a PCR-based assay (Okada et al. 2000), allowing easy handling and management of female and male lines. To prepare crossing, the sexual phase of the life cycle can be induced by exposure to far-red light (Nakazato et al. 1999), and fertilization is performed by transferring sperm from mature antheridiophores to archegonia. Following fertilization and zygote formation, the life cycle continues in its diploid phase, leading to formation of spores packaged within yellow sporangia. Each cross can produce approximately 7 million spores that can be stored at -80°C for several years. Spore development takes place as soon the spores find a suitable substrate for germination. Unlike seeds of higher plants, spores are unicellular and do not possess a testa, allowing direct visualization of all early processes in development. During spore germination, spores undergo asymmetric cell division, initiate photosynthesis after differentiation of plastids, and begin to develop differentiated tissues within only a few days (see Fig. 2). Following germination, new plants grow vigorously on solid (Takenaka et al. 2000) or liquid media (Ishizaki et al. 2008) in sterile culture, and robustly regenerate from vegetative *gemmae* (Miller 1964), protoplasts (Ono et al. 1979), or tissue cuttings (Kubota et al. 2013). Rapid photoautotrophic growth of *Marchantia* cultures has been reported with a 1.76-day doubling time for culture mass, a faster rate than *Arabidopsis* (Katoh et al. 1979). The high regenerative capacity of liverworts is independent of externally supplied hormones (Vöthcing 1885), and well exceeds the reported regeneration efficiency of *Arabidopsis* (Valvekens et al. 1992) or wheat (McHughen 1983). The life cycle of *Marchantia* takes ~2 months to complete (Shimamura 2012), which is similar to *Arabidopsis* and faster than in *P. patens* (~3 months [Cove 2005]).

Marchantia is one of few plant species for which chloroplast transformation protocols have been confirmed by at least two independent studies (Bock 2015). Stable transplastomic lines of *Marchantia* encoding dual spectinomycin and streptomycin resistance were generated through particle bombardment of sporelings

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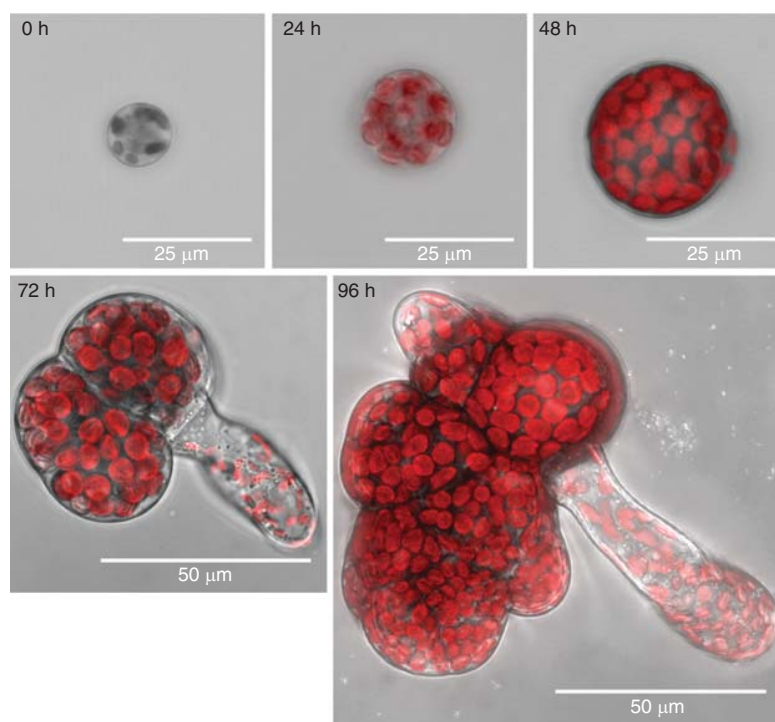


Figure 2. Spore germination. *Marchantia* spores were germinated on a nutrient agar surface. The spores were examined under a 63 \times , NA 1.2 objective at 0, 24, 48, 72, and 96 h after germination using a Leica SP5 confocal laser scanning microscope. A 488-nm laser was used to collect transmission images (grayscale channel), and these were overlaid with images of chlorophyll fluorescence (488 nm excitation, 680–700 nm emission, red channel). Z-series of images (2 μ m apart) were collected and merged to provide views of the developing sporelings at different stages of growth. Scale bars are indicated in each image.

(Chiyoda et al. 2007). This approach was recently extended to the expression of a fluorescent reporter gene from the *Marchantia* plastome (Boehm et al. 2016), which allows characterization of plastid promoters in this model system (see Fig. 3). A notable feature of the *Marchantia* plastome is the apparent absence of mechanisms for RNA editing (Ohya et al. 2009), which may substantially simplify rational engineering of this organellar genome. Although simpler organisms may lack some of the complexity of angiosperms, the basal plant model *Marchantia* can enhance our still fragmented understanding of fundamental aspects of gene regulation, expression, and function in plant metabolism and development as well as provide a more rapid and tractable testbed for synthetic biology.

GENETIC MANIPULATION OF CHLOROPLASTS

In bridging the gap between microbes and plants as platforms for synthetic biology, a particularly attractive chassis is the chloroplast. Sharing its evolutionary heritage with cyanobacteria, this organelle possesses prokaryote-like regulation of gene expression (Mayfield et al. 1995), and is the major biosynthetic compartment in plants. The high biosynthetic capacity of chloroplasts has been used for production of a number of biofuel enzymes (Verma et al. 2010) as well as resistance proteins, antibodies, biopharmaceutical proteins, and vaccine antigens (Wani et al. 2010). Accumulation of large amounts of target protein has been achieved in chloroplasts, exceeding 70% of the

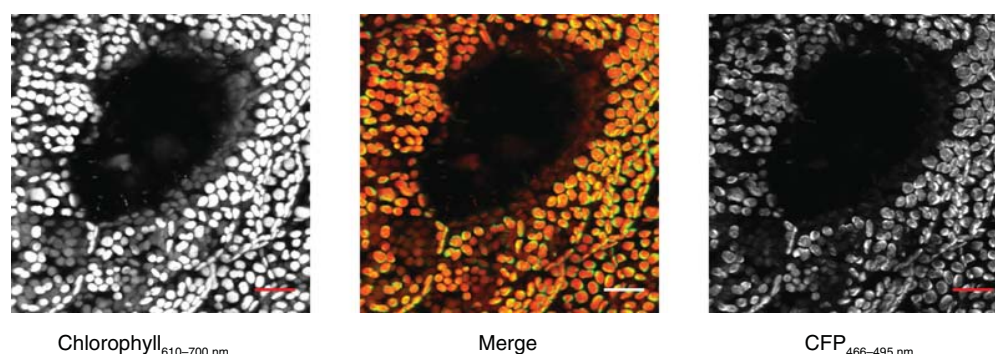


Figure 3. Transgenic chloroplasts of *Marchantia polymorpha* expressing the cyan fluorescent protein mTurquoise2 under control of the tobacco *psbA* promoter. Mature thallus of *Marchantia* was mounted in water under a coverslip, and examined under a $63\times$, NA 1.2 objective using a Leica SP5 confocal laser-scanning microscope. Cells surrounding an air pore were imaged using 458-nm laser excitation. Emission wavelengths were collected for chloroplast autofluorescence (610–700 nm, *left*) and cyan fluorescent protein (CFP) fluorescence (466–495 nm, *right*), with merged images in red and green channels, respectively, shown *center*. Scale bar, 20 μm .

plant's total soluble protein (Oey et al. 2009). No gene silencing has been observed in chloroplasts despite such high accumulation of foreign transcripts (169 times higher than in nuclear transgenic plants, Lee et al. 2003) or foreign protein (46% of total leaf protein [De Cosa et al. 2001]). Metabolic engineering for the production of bioplastic monomers (Bohmert-Tatarev et al. 2011) and compounds of nutritional relevance (Craig et al. 2008; Hasunuma et al. 2008; Apel and Bock 2009) has also been applied to chloroplasts. The high biosynthetic capacity of the chloroplast is closely linked to the polyploid nature of the system: At 10–100 chloroplasts per cell, and 10–1000 genomes per chloroplast (Bendich 1987), stable integration of a transgene into the chloroplast genome enables a substantial amplification in transgene copy number. The high ploidy of the plastome can entail the need for several rounds of selective regeneration following transgene introduction to segregate transplastomic organelles and establish homoplasmy (Maliga 2004). However, homoplasmy has also been achieved immediately after primary selection (Chiyoda et al. 2014).

The process of transgene integration into the plastome following delivery into the chloroplast by means of particle bombardment (Svab et al. 1990) or PEG-mediated transforma-

tion (Golds et al. 1993) is mediated by homologous recombination. This mechanism has enabled the introduction of point mutations in a site-specific manner (Przibilla et al. 1991). As a consequence, the risk of inconsistent gene expression by the positional effect in T-DNA-mediated nuclear transformation is alleviated in the chloroplast environment (Daniell et al. 2002). The high efficiency of homologous recombination in the chloroplast (Blowers et al. 1989) also allows the simultaneous introduction of modifications to several sites of the chloroplast genome by means of cotransformation (Kindle et al. 1991), encouraging the implementation of phenotypic traits based on multiple foreign genes. Multiple genes may be conveniently organized in operon-like polycistronic units (Hasunuma et al. 2008), which can be processed into more efficiently translated monocistronic transcripts by the incorporation of intercistronic expression elements (Lu et al. 2013). Previous studies have also highlighted the activity of chloroplast promoters in bacteria (Brixey et al. 1997) and of bacterial promoters in the chloroplast (Newell et al. 2003). Another bacterial gene expression element, the widely used lac repressor from *E. coli*, has been adapted for IPTG-inducible chloroplast transgene expression (Mühlbauer and Koop 2005). Alternatively, chloroplast-based translational regula-

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tion of transgene expression can be implemented via riboswitches under control of the externally applied ligand theophylline (Verhounig et al. 2010; Emadpour et al. 2015).

Large DNA fragments exceeding 50 kb have been successfully incorporated into the plasmid (Adachi et al. 2007). In fact, on the basis of recent advances in DNA synthesis and assembly technology, synthesis of entire tailor-made plastid genomes has become feasible (Scharff and Bock 2014). Today, metabolic engineering in microbial systems is predominantly driven by generation of large numbers of circuit variants followed by selection of a desired phenotype. Translation of this approach into plants has not been widely adopted primarily because of the requirement for generation and analysis of a sufficient number of transgenic events in the context of long generation times and polyploidy of established model species. Compared with random integration in the nuclear genome by transgenesis or interbreeding (Karunanandaa et al. 2005), integration in the chloroplast genome is achieved by homologous recombination and thus DNA is inserted at a chosen location. This allows a far smaller number of events to be compared as they are free from position effects as well as from epigenetic gene silencing, which is only encountered in the nuclear genome (Lu et al. 2013). Another notable advantage of transplastomic technology over nuclear transformation lies in maternal inheritance of plastid DNA, which greatly reduces the risk of unwanted transgene transmission via pollen and markedly increases the level of containment for field release of transgenic plants.

MODELS FOR MULTICELLULAR GROWTH AND INTEGRATED TECHNIQUES FOR QUANTITATIVE MICROSCOPY AND IMAGE ANALYSIS

Specialized structures in plants carry out a number of highly efficient processes for photosynthesis, secondary metabolite production, and compound storage. These structures are generated during morphogenesis through the concerted behavior of cell populations during development. Our ability to modify plant form

is currently limited by our understanding and control of multicellular processes during morphogenesis. Increasing evidence from mutagenesis studies in angiosperms has suggested that morphogenesis is shaped by a combination of processes embodied in classical organismal (Green 1980) and cellular (Beemster et al. 2003) theories: the “neo”-cell theory proposes that feedback and interplay between cellular and organism-wide processes cooperate in the patterning of tissues (Tsukaya 2003). Evidence indicates that the polarity of cell division and elongation is regulated by the plant cell cytoskeleton and interaction with local cell wall determinants, wall strain, or geometry (Fig. 4). Subsequent genetic interactions mediated by protein or phytohormone traffic between cells can provide positional signals to trigger gene expression, cell proliferation, differentiation, and organ formation (Bohn-Courseau 2010). Consequent cellular growth generates physical strains that are instantaneously transmitted across tissues and constrain growth. The physical constraints on cell size and shape regulate timing and orientation of individual cell divisions and expansion, and therefore guide morphogenesis. This results in hierarchical, highly parallel, and feedback-regulated systems in which the DNA program and phenotype are not directly related, but part of an emergent self-organizing system. DNA-based reprogramming of plants can predictably alter individual cell behaviors, but the grand challenge of plant synthetic biology is to understand and predict how altered cell logic can propagate through large cell populations, modify tissue physics and local patterns of cellular growth, and produce defined morphologies. Effective multi-scale modeling of cellular growth will be a prerequisite for predictable engineering of plant morphogenesis.

The past decade has seen the emergence of increasingly effective models for plant growth in which the software describes the genetic, cellular, and biophysical properties of growing tissues (Jönsson and Krupinski 2010; De Vos et al. 2012; Prusinkiewicz and Runions 2012). Although these models have been developed primarily to provide insight into plant develop-

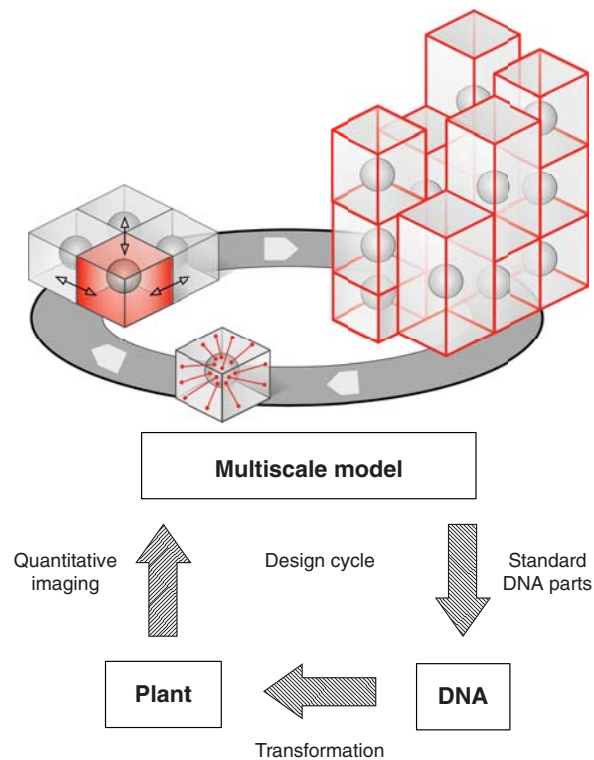


Figure 4. Multiscale model of plant growth for engineering synthetic botanical forms. To model and predict the form of reprogrammed plants, integrated, multiscale models for plant growth are required. These software models need to capture (1) the interaction between cytoskeletal elements and local cell wall determinants, strain or geometry regulating the polarity of cell division and elongation, (2) genetic interactions between neighboring cells that can trigger gene expression and cell proliferation and differentiation, and (3) cellular growth that results in physical strains that are transmitted across tissues and constrain cell growth, because physical constraints on cell size and shape regulate timing and orientation of individual cell divisions and guide morphogenesis. Multiscale models provide an essential tool for engineering multicellular systems. Standardized DNA parts facilitate assembly of DNA circuits that may be introduced into plant systems by transformation, and the performance of DNA-based circuits can be measured using quantitative imaging techniques. Although a genetic circuit may regulate or alter the behavior of an individual cell in an easily predictable fashion, the consequences of altered cell interactions, propagation of changes across large cell populations, changes in tissue-wide physical and chemical interactions, and feedback on the properties of individual cells are difficult to predict. However, this type of system, characterized by cross talk and emergent properties, can be captured accurately by multiscale models. The models form an essential part of any design–build–test cycle for DNA-based engineering of plants.

mental processes, they allow the integration of genetic, cellular, and whole-tissue properties, in a way that facilitates DNA-based reprogramming of large-scale plant growth. To complement these software tools, synthetic biologists require an experimental platform that allows simultaneous measurements of gene expres-

sion, growth, and division of individual cells at the scale of the entire organism. Sporelings and *gemmae*, the reproductive propagules of *Marchantia*, are well suited for such purpose. Their prostrate morphology and exposed mode of development facilitate the application of quantitative imaging techniques (Fig. 5): all

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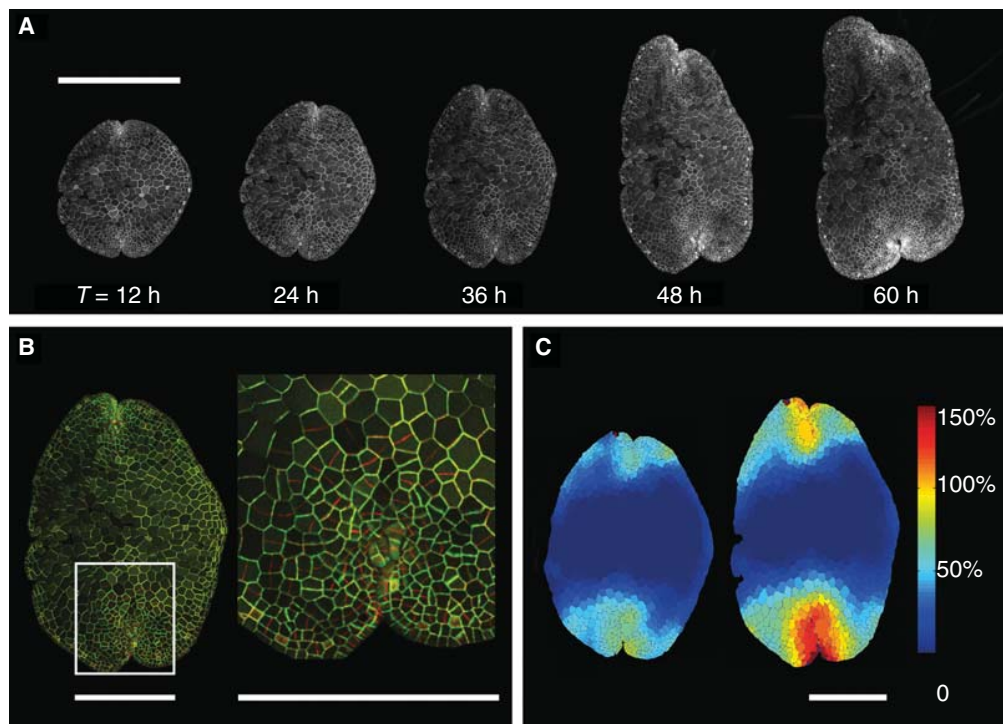


Figure 5. *Marchantia gemmae* as testbeds for quantitative parameterization of plant growth. (A) A transgenic line of *Marchantia* was generated that expressed a green fluorescent protein localized to the plasma membrane. A *gemma* from this transgenic line was transferred to a nutrient agar surface and examined after 12, 24, 36, 48, and 60 h of growth using a Leica SP5 confocal laser scanning microscope. Z-series of optical sections were collected for the same *gemma*, and maximum intensity projections are shown for each point during growth. Scale bar, 500 μm . (B) The 24-h (green channel) and 36-h (red channel) images from the time course were matched using warp-registration image-processing techniques and overlaid. A white box is positioned over one of the apical notches, and this corresponds to the enlarged view shown in the inset. The frequency and orientation of apex-localized cell divisions can be directly visualized in a single *gemma*. Scale bar, 200 μm . (C) The plasma membrane-localized marker allows accurate segmentation of cell geometry during growth of living plants. Quantitative parameters such as cell expansion rate can be mapped across a single *gemma*. Measurements of percent clonal sector expansion per 12 h are shown as a color map. Scale bar, 200 μm .

cells in the surface layer are accessible for imaging to quantify gene expression based on *in planta* cytometry (Federici et al. 2012). Robust protocols to map the dynamics of cell expansion, division, and differentiation on the surface of the *gemma* during the early stages of *gemma* development have been developed (N Purswani, unpubl.).

This combination of advanced imaging techniques and *in silico* models has proven useful for examining and predicting laws for cell division and tissue growth in multicellular algae

(Dupuy et al. 2010; Besson and Dumais 2011). In an engineering context, these methods can form an essential part of a design–build–test cycle for reprogramming plant growth (see Fig. 4). Prototype designs can be assembled from modular DNA parts and transformed into plants, in which the properties of a synthetic gene circuit and impact on plant growth can be measured by quantitative microscopy. Multiscale software models can allow evaluation and even prediction of emergent processes. The simplicity, experimental accessibility, and



morphological plasticity of *Marchantia* sporelings and *gemmae* (Vöthling 1885; Fitting 1935), ease of quantitative measurements, and growing sophistication of software models for cellular growth provide a pathway to the rational design of morphogenetic programs in plants.

CONCLUDING REMARKS

Marchantia provides an experimental model to help bridge the gap between the relative simplicity of microbes and the complexity of higher plants. In particular, *Marchantia* is well-suited for prototyping genetic circuits before their application in a higher plant species of interest because of its amenability to high-throughput transformation and screening of large numbers of circuit variants: As an integral part of its rapid life cycle, *Marchantia* produces millions of spores, which can be transformed within a week using available protocols (Ishizaki et al. 2008; Tsuboyama and Kodama 2014). Owing to the haploid-dominant nature of *Marchantia*, phenotypic effects in transgenic plants can be immediately screened without the need for repeated selective regeneration to obtain homozygous lines. Screening can be performed by means of existing quantitative microscopy methods (Federici et al. 2012), and image processing and parameters can be extrapolated for physico-genetic modeling of multicellular plant tissues (Dupuy et al. 2010) to refine the next iteration of the design cycle. In the future, this process will benefit not only from the improvement of current techniques for DNA assembly, plant transformation, quantitative microscopy, and computational modeling, but especially from the availability of the *Marchantia* nuclear genome. This sequence information will guide the discovery of cell-type- and developmental stage-specific marker genes. The identification of tissue and cell-type-specific promoters in *Marchantia* has particular merit not only for developmental studies but also for metabolic engineering. Notably, compounds derived from plant secondary metabolism still play a dominant role in the discovery of new biopharmaceuticals (Newman and Cragg 2016), despite the advances of modern chemistry. In this light,

the ultimate challenge for biological engineers will not be manipulating individual genes, but exercising control over the collective behavior of metabolic pathways and cohorts of cells and refactoring a multicellular organism's body plan to generate specialized structures and organs for manufacturing and storage of compounds of interest. For example, applications of spatially controlled gene expression in *Marchantia* may include targeted manipulation of carbon fixation in assimilatory filaments (Goffinet and Shaw 2009) or production of bio-fuel components in oil cells (Suire et al. 2000). Control circuits and synthetic pathways proven in this context may serve as valuable tools for some of the largest contemporary challenges in plant metabolic engineering, such as the refactoring of nitrogen fixation pathways in plants (Rogers and Oldroyd 2014) or the introduction of C4 photosynthesis into C3 crops (Leegood 2013).

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