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Toolboxes for plant systems biology research

Jihyun Park[1], Gozde S Demirer[2,3] and Lily S Cheung[1]

The terms 'systems' and 'synthetic biology' are often used together, with most scientists striding between the two fields rather than adhering to a single side. Often too, scientists want to understand a system to inform the design of gene circuits that could endow it with new functions. However, this does not need to be the progression of research, as synthetic constructs can help improve our understanding of a system. Here, we review synthetic biology tool kits with the potential to overcome pleiotropic effects, compensatory mechanisms, and redundancy in plants. Combined with -omics techniques, these tools could reveal novel insights on plant growth and development, an aim that has gained renewed urgency given the impact of climate change on crop productivity.

Addresses
[1] School of Chemical and Biomolecular Engineering, Georgia Institute of Technology, Atlanta, GA 30332, USA
[2] Department of Plant Biology and Genome Center, University of California Davis, Davis, CA 95616, USA
[3] Department of Chemical Engineering, California Institute of Technology, Pasadena, CA 91125, USA

Corresponding author: Cheung, Lily S (lily.cheung@gatech.edu)

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Introduction

Plant science has experienced a surge of big data, with techniques like laser capture microdissection, Translating Ribosome Affinity Purification Sequencing (TRAP-Seq), Fluorescence-Activated Cell Sorting (FACS), and time-lapse fluorescence microscopy [1–4] providing extraordinary tissue/cell-specific information. Yet, our ability to manipulate genes with similar precision and throughput lacks behind, with most functional studies still relying on knockout mutants and systemic overexpression using the constitutive Cauliflower Mosaic Virus (CaMV) 35S promoter. These perturbations in single genes seldom produce visible phenotypic differences, as comprehensive genetic studies and large-scale genomics projects have shown [5,6]. High similarity in coding sequences among plant gene families often results in complete or conditional functional redundancy, leading to substantial phenotypic plasticity buffering. Moreover, in cases where single gene perturbations do produce a visible phenotype, pleiotropic and compensatory effects oftentimes obscure the molecular mechanisms responsible for changes in transcript or protein levels.

Advancing plant systems biology will require precise perturbations that can overcome gene redundancy, pleiotropism, and compensatory mechanisms in -omics studies. Such an approach could be as effective as experiments that used cell-specific perturbations to validate predictions from single-cell transcriptomic analyses, which have revealed novel roles for known factors in leaves and lateral roots [2,7]. However, such approach requires versatile genetic constructs for precise spatiotemporal control of gene function. Here, we review recent tool kits for vector assembly and transcriptional and post-translational regulation of gene products that could be combined with -omics studies, focusing on those applicable to a multitude of genes.

Vector assembly kits

A crucial challenge in plant biology research is the extensive time necessary for transformation and crosses. Thus, single-step delivery of whole gene circuits or multiple transgenes is ideal, and numerous approaches have been developed to facilitate the task [8,9].

One of the most recently developed tools is loop assembly, a technique for recursive fabrication of large genetic circuits that can theoretically generate plasmids with unlimited transcription units and length [10••]. Loop assembly uses two Type IIS restriction enzymes and corresponding standardized vector sets (Odd and Even receiver plasmids). Assemblies are performed through iterated 'loops.' Two sets of four plasmid vectors are provided, which allow alternating assembly cycles. The iterative process of combining genetic modules, four at a time, can be continued infinitely by alternating between odd and even Loop vectors [11].

Standardized parts

Several libraries of natural or synthetic genetic parts are available for plants. It is important for these parts to be orthogonal and quantitatively characterized [12]. This means the parts should display minimal interaction between each other and endogenous components and have defined input-output relations.

Heterologous and synthetic transcriptional regulators are more likely to be orthogonal than plant derived ones.

Transcriptional activator-like effectors from bacteria like *Xanthomonas* [13] and zinc-finger chimeras [14] were some of the earliest genetic parts used in plants. However, their engineering was laborious. More recently, catalytically inactive Cas9 (dCas9) has been shown to successfully activate and repress transcription of target genes without appreciable off-target effects [15].

The main limitation in the generation of predictable gene circuits is still the time necessary to test and model the outputs of genetic parts in stably transformed plants. Schaumberg *et al.* showed that quantitative characterization in *Arabidopsis* protoplasts can serve as a good proxy for *in planta* performance by testing 128 pairwise combinations of synthetic promoters and repressors [16•]. The system used luciferase as output and Hill functions for modeling. Variability due to random transgene insertion, common in *Agrobacterium*-mediated transformation, was a challenge.

One option to surmount this variability is to use a ratiometric luciferase system [17]. Another option would be to create lines with predetermined genomic landing sites like those in *Drosophila melanogaster* [18]. The bacteriophage ϕC31 DNA site-specific integrase could be used to generate mapped genomic locations for the insertion of transgenes with subtle differences, facilitating comparison. The ϕC31 integration system has already been demonstrated to work as a memory switch in *Nicotiana benthamiana* [19]. Another tool plant biologists could borrow from fly geneticists are balancer chromosomes, which are chromosomes containing multiple inverted regions capable of suppressing crossovers during meiosis that would help the visual tracking of chromosomes carrying transgenes during crosses [20].

Temporal control of gene expression and protein localization with inducible systems

Chemical inducers can be used to initiate the transcription of transgenes at defined developmental stages. Since the creation of the tetracycline-inducible gene expression system [21], numerous chemically inducible systems for plants have followed. The glucocorticoid-inducible system is a primary example, which uses a chimeric transcription factor (GVG) consisting of the GAL4 binding domain from yeast, the VP16 activation domain from herpes, and the glucocorticoid receptor (GR) from rats [22]. This system has been shown to be tightly regulated and rapidly induced in tobacco, *Arabidopsis*, rice, pine, and citrus plants [22–26].

Although the glucocorticoid-inducible system has been demonstrated effective, its orthogonality has been questioned. Activation of the system can cause developmental growth defects and interfere with endogenous gene expression [27,28]. Zuo *et al.* addressed these limitations with the construction of the estrogen-inducible XVE system, which uses a chimeric transcriptional factor consisting of the LexA binding domain from bacteria, VP16, and the human estrogen receptor (ER) [29]. Another alternative is the recently reported dexamethasone-inducible pOp6/LhGR system, which uses the *Escherichia coli* lac repressor *lacI*^{His17}, the GAL4 transcription-activation-domain-II, and the rat GR ligand binding domain [30].

Post-translationally, the rapamycin-inducible KnockSideways in Plants (KSP) system can be used to control protein localization. This system relies on the heterodimerization of the FKBP domain of HsFKBP12 and the FKBP12 rapamycin-binding domain of mTOR. The system was shown efficient at directing bait proteins to the plasma membrane, mitochondria, microtubules, and nucleus in *N. benthamiana* [31].

Spatial control of gene expression with tissue/cell-specific promoters

In the last decade, numerous tissue/cell-specific promoters have been isolated from diverse plant species (e.g. *Arabidopsis*, rice, tomato, soybean) [32–35]. These promoters have typically been employed for TRAP-Seq and developmental studies but are being increasingly used for precise and controlled manipulation of genes with minimum adverse effects. These promoters provide substantial advantage over constitutive ones like CaMV35S, which can cause pleiotropic effects and reduce plant growth. Two examples are worth noting.

Decaestecker *et al.* developed a technique called CRISPR-TSKO, which enables the creation of somatic mutations in desired cell types, tissues, and organs (Figure 1a). To achieve this, researchers used tissue-specific, somatic promoters to drive Cas9 expression and demonstrated root cap-specific, stomata-specific, and lateral root-specific gene knockouts in *Arabidopsis* [36•].

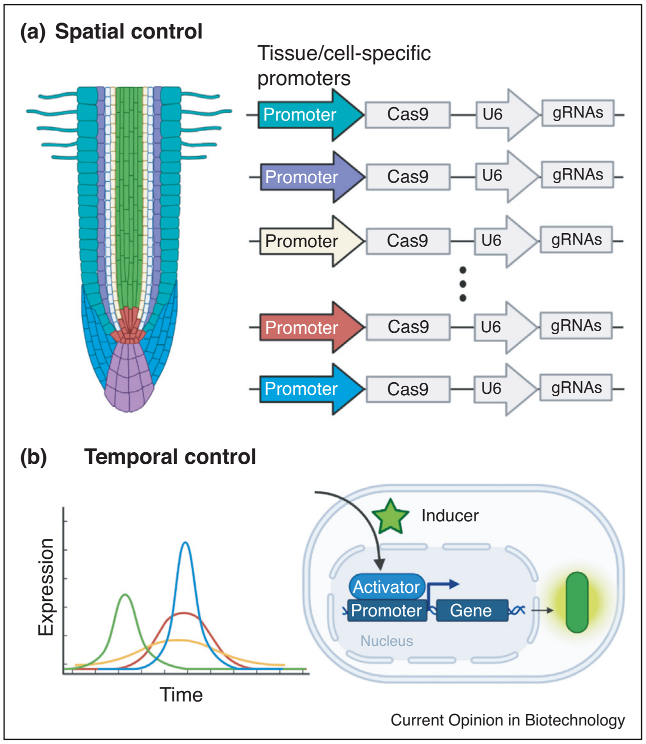
Wang *et al.* improved on this idea by integrating CRISPR/Cas9, the XVE system, and root-cell-type-specific promoters, enabling temporal in addition to spatial control of gene editing (Figure 1b). This allowed the team to trigger somatic gene knockout in *Arabidopsis* root meristem with estradiol [37••].

Engineering of synthetic promoters for precise control of transgene expression in a spatiotemporal manner has been another important advance in plant science. Readers interested in a detailed discussion of their rational design are referred to Cai *et al.* [38], and for a list of all recent plant synthetic promoters to Ali and Kim [39].

CRISPR/dCas9 for multigene regulation

The activation and repression of gene expression using dCas9 in plants has become a common and powerful

Figure 1



Spatiotemporal control of gene expression. **(a)** Tissue/cell-specific promoters can enable spatially controlled expression of Cas9. Different colors represent promoters expressed in different plant cell/tissue types. **(b)** Inducible Cas9 expression in plants confers temporal control and can be merged with cell-specific promoters to provide both spatial and temporally manipulated genes. Different colored peaks represent the ability of expressing genes at the desired time using inducer molecules, and at the desired cells using cell-type-specific promoters.

approach for genetic and epigenetic regulation. In addition to its use for single-gene regulation, CRISPR/dCas9 platforms offer unparalleled multiplex ability by using multiple sgRNAs simultaneously. There exist several tools for multiplexed activation in plants (dCas9-TV, dCas9-SunTag, dCasEV2.1) [40–42]. Recently, Pan *et al.* developed CRISPR-Act3.0 for highly efficient multiplexed gene activation and performed simultaneous activation of many enzyme-encoding genes in rice as well as multigene activation in *Arabidopsis* [43••]. A critical challenge of using dCas9 for gene regulation is the need for correct protospacer adjacent motifs (PAMs) sequence proximal to the promoter. When using SpCas9, it could be challenging to find good target sites with NGG PAMs, given that promoters in plants are often AT-rich. To overcome this limitation, Pan *et al.* successfully adapted the use of dCas12b, a protein that recognizes VTTV PAMs for multigene activation with CRISPR-Act3.0. In addition, they used the near-PAM-less SpCas9 variant, SpRY, and demonstrated that dSpRY-Act3.0 is a highly promising tool for multigene regulation [43••].

CRISPR/dCas9 has also been used for transcriptional repression. However, to our knowledge, repression in plants has not been done commonly in a multigene manner [44], unlike the case in bacteria, yeast, and human cells [44–46]. The one study that performed simultaneous multigene repression in plants by Lowder *et al.* used a synthetic pco-dCas9-3X(SRDX) transcriptional repressor to reduce transcript levels of two microRNAs in *Arabidopsis* [47].

Light control of gene expression

Optogenetics uses light and genetically encoded photoswitches to alter gene expression reversibly, thus offering an alternative in situations where promoters with the desired tissue/cell-specific activities are unavailable or in cells with poor uptake of chemical inducers. The technique traditionally uses photoactivatable channels and light sensory parts from bacteria, algae, and plants. However, because most of these proteins respond to the same wavelengths of light that control plant growth and development, their use can result in undesired side effects. The recently developed Plant Usable Light-Switch Elements (PULSE) system overcomes this limitation and illustrates an elegant strategy for implementing optogenetics in plants [48••].

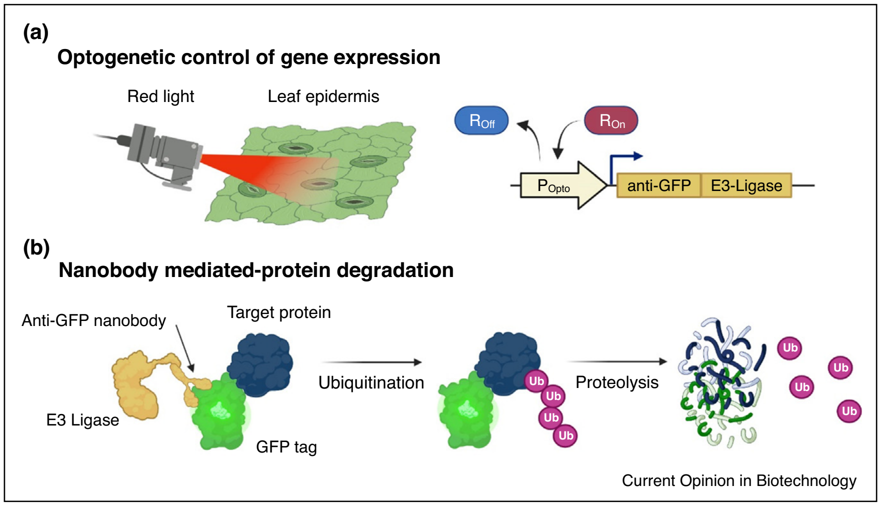
PULSE is comprises two engineered proteins and a synthetic promoter (P_{Opto}) (Figure 2a). In this system, a transcriptional repressor (B_{Off}) prevents gene expression under blue light (~450 nm), while an activator (R_{On}) induces expression under red light (~660 nm). Transgene expression can be tuned by controlling the intensity of monochromatic red light [48••].

PULSE's effectiveness was demonstrated with transient expression in *N. benthamiana* leaves and transgenic *Arabidopsis*. Gene expression was prevented in daylight (250–800 nm) by the B_{Off} repressor and inactive in dark, allowing plants to grow under normal photoperiod. Transfer to monochromatic red light induced expression of luciferase in *Arabidopsis*, reaching maximum levels after ~12 hours; while return to white light decreased luminescence, showing the reversibility of the system. Whether PULSE inadvertently affects endogenous signaling due to several of its components originating from plants remains to be explored.

Nanobodies and inducible systems for post-translational control

Nanobodies are small, single-domain antibodies isolated from camelids that can be transgenically expressed to bind endogenous proteins or small molecules. Researchers demonstrated the use of nanobody for selective protein degradation, which can be completed faster (within

Figure 2



Optogenetic control of protein degradation. **(a)** In the PULSE system, a transcriptional repressor (B_{Off}) derived from the bacterial light-regulated DNA-binding protein EL222 and a plant EAR repression domain prevents gene expression under blue light (~450 nm), while an activator (R_{On}) from a plant phytochrome B (PhyB) and a phytochrome-interacting factor 6 (PIF6) induces expression under red light (~660 nm). The synthetic promoter (P_{Opto}) is composed of repeated binding domains for EL222 and PIF6 upstream of a human cytomegalovirus minimal promoter and can be used to drive expression of anti-GFP nanobodies. **(b)** Anti-GFP nanobody and E3 Ubiquitin Ligase chimeras can be used to selectively target and degrade GFP-tagged proteins.

minutes to few hours) than transcriptional or RNAi-mediated downregulation. The technique fuses a nanobody with an F-box domain, resulting in a chimera that can polyubiquitinate the proteins recognized by the nanobody and thus target it for proteasome degradation (Figure 2b). This prevents compensation effects that could otherwise complicate the interpretation of phenotypic changes. Moreover, when combined with somatic tissue-specific or inducible promoters in multicellular organisms, restricted protein degradation can circumvent the lethality or sterility associated with knockouts of the target gene.

An anti-GFP nanobody allows selective degradation of functional GFP-tagged proteins. Furthermore, the process can be monitored in real-time by the loss of fluorescence. The use of this approach in multicellular organisms was first demonstrated in *D. melanogaster*. The method, named deGradFP, was shown to titrate target proteins in less than three hours and phenocopy loss-of-

Table 1

Summary of plant tools and their availability

Tool kit	Availability	References
Loop assembly	Addgene	[10••,11]
uLoop assembly	Addgene	[10••,11]
Dual luciferase ratiometric reporter system	Addgene	[17]
ϕC31 integration system	Addgene	[19]
XVE inducible transcription factor	Addgene	[29,63]
pOp6/LhGR gene expression system	Nottingham *Arabidopsis* Stock Centre (NASC)	[30]
Rapamycin-inducible KnockSideways in Plants (KSP)	VIB-UGent Center for Plant Systems Biology	[31]
CRISPR-TSKO	Addgene	[36•]
Inducible CRISPR/Cas9 system	Addgene	[37••]
dCas9-TV	Upon request to authors	[42]
dCas9-SunTag	Addgene	[40]
dSpRY-Act3.0	Addgene	[43••]
pco-dCas9-3X(SRDX)	Addgene	[47]
PULSE system	Addgene	[48••]
Anti-GFP nanobody-based degradation system	Upon request to authors	[50,51•]
Anti-GFP nanobody-based delocalization	Upon request to authors	[52]

function mutations in homozygous mutant backgrounds [49].

Similar systems successfully depleted GFP-tagged and YFP-tagged nuclear protein in tobacco due to cross-reactivity of the nanobody [50,51•]. Anti-GFP nanobody has also been used in *Arabidopsis* root epidermal and cortex cells to delocalize plasma membrane-associated protein complexes [52] and combined with PULSE for localization studies [48••]. Newer nanobodies against other fluorescent proteins and peptides offer opportunities for multiplexing, albeit they still need to be tested in plants [53–55].

Discussion

This review highlighted synthetic biology tool kits that in conjunction with -omics could improve the systems-level understanding of plant development and physiology (Table 1). Common features of these kits include their orthogonality, compatibility with genes with diverse functions, and capacity to affect multiple genes simultaneously with spatiotemporal precision. Other tools that are efficient in plants but missed one or more of these features, like those based on ethanol induction, synthetic plant hormone-responsive transcription factors, heat-shock promoters, and light-gated ion channels [56–59], were not discussed.

We cataloged these tool kits for two reasons. First, we wanted to help readers identify tools that could work together, such as using loop assembly and pco-dCas9-3X (SRDX) multiplexed repression to screen for genetic interactions. Another idea would be combining nanobodies, the XVE system, and existing collections of phase-specific reporters to study cell-cycle regulation [60].

The second and more important reason was to encourage the design of easier to interpret -omics studies. Comparing the transcriptional and proteomic profiles of wild type plants with knockout mutants or constitutive overexpression lines often reveals hundreds of differentially expressed candidates. However, it is seldom clear which candidates interact directly with the perturbed gene or whether effects are cell autonomous, no matter how sophisticated the subsequent bioinformatics analysis is. Now, with the proliferation of single-cell analyses, it is our opinion that a better picture of the gene regulatory network underlying a particular phenotype can be obtained from tissue/cell-specific and inducible perturbations.

While -omics studies can be easily conducted in non-model plant species, the traditional transformation methods based on *Agrobacteria* and tissue culture are not. Thus, application of the tools described in this review to crops where current transformation efficiency are poor will require novel methods, such as the one developed by Demirer *et al.* that uses nanoparticles to allow efficient gene expression and silencing in many eudicots and monocots [61•,62].

Conflict of interest statement

Nothing declared.

CRediT authorship contribution statement

Jihyun Park: Writing – review & editing. **Gozde S Demirer:** Writing – review & editing. **Lily S Cheung:** Writing – review & editing.

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