Plant Synthetic Biology: Quantifying the "Known Unknowns" and Discovering the "Unknown Unknowns"[1][OPEN]

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Our knowledge of plant biology has reached the point where we can begin to rationally engineer plant form and function to meet our needs. From a bioengineer's or synthetic biologist's point of view, the goal of studying developmental biology is to generate a predictive model that specifies the molecular circuitry required to move a cell from one state to another. This model could then serve as a guide for harvesting the most useful parts and logic to enable the engineering of novel states and multicell behaviors. Among the most critical parts to understand from this perspective are the signaling molecules that enable intra- and intercellular communication. Several biosensors have been developed in recent years to detect plant-specific signals and secondary messengers. Many other general biosensors have been successfully implemented in plant systems. These biosensors, in combination with single cell "omics" techniques and predictive statistical frameworks, are providing the type of high resolution, quantitative descriptions of cell state that will ultimately make it possible to decode and re-engineer traits associated with higher yields and stress tolerance.

Being a plant developmental biologist today can feel like a lot like being a cryptographer piecing together fragmented messages with only a partial knowledge of the cipher. Biological signaling is rife with redundancy, feedback, and feedforward motifs acting to dampen or amplify each signal, and modulate outputs depending on position and cell identity. To crack the code of these complex genetic signal processors, it is important to be able to measure, as well as manipulate, both signals and responses. Recent advances in synthetic biology have provided a means to access such tools. Sensitive, genetically encoded reporters (biosensors), in combination with emerging single-cell transcriptomics approaches, are providing increasingly detailed molecular descriptions of cells undergoing developmental transitions (Moreno-Risueno et al., 2015; Efroni et al., 2016; Ristova et al., 2016; Cao et al., 2017). However, in many cases we are still unable to measure key signaling molecules directly with fine spatiotemporal resolution.

Several excellent reviews have been published recently that describe the application of biosensors to plant systems (Goold et al., 2018; Hilleary et al., 2018; Walia et al., 2018). Here, we review this state of the art in measuring plant signaling, using principles and tools borrowed from and inspired by engineering, as well as efforts to use this knowledge to enable rapid, rational re-engineering of plant development. We have arranged this review as an engineering cycle in which we will cover "designing" biosensors; "building" biosensors, including technologies to facilitate the use of biosensors in plants; "testing" biosensors; "modeling" signaling and development, including our perspective on integrating biosensors, systems approaches, and optimal experimental design to generate minimal predictive models of plant development; and finally "learning" about plant development, including our outlook on

ADVANCES

- Studies of single-cell and high temporal resolution 'omics datasets paired with biosensors have provided models of key networks in developmental processes.
- Direct biosensors of gibberellins and abscisic acid, along with improvements in indirect biosensors for auxin and abscisic acid signaling, have expanded our understanding of plant hormone biology and developmental signaling.
- Development and application of FRET-FLIM and FCS methods to study protein and protein complex dynamics *in vivo* have advanced our understanding of transcription factor complex formation in meristem maintenance.

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how biosensors paired with synthetic and systems biology approaches will advance knowledge in the future.

DESIGN

The design of any genetically encoded biosensor involves connecting an input modality, which interacts in some way with the species to be measured, to an output modality, which provides some quantifiable product (Fig. 1). These modalities may be DNA, RNA, and/or proteins. The species to be measured (analyte) may be any molecule or complex of molecules. Input modalities may be promoters that respond to the analyte, naturally occurring proteins domains or engineered novel proteins or nucleic acids, which bind (or otherwise respond) to the analyte. Each input modality offers different advantages and drawbacks. As opposed to direct biosensors, which bind to and report the concentration of the desired species, indirect biosensors have input modalities that are natural or engineered responsive promoters or protein domains, such as degrons, which require additional cellular machinery to respond to the analyte (Brunoud et al., 2012; Larrieu et al., 2015). Often referred to as "reporters," indirect biosensors report on the status of the signaling network required to activate the responsive element. Although this complex output can be misinterpreted, indirect biosensors have facilitated numerous discoveries, particularly when paired with systems biology approaches (such as transcriptomic and other genome-scale analyses) to decipher network status (Moreno-Risueno et al., 2010; de Luis Balaguer et al., 2017; Wu et al., 2018). Such advances will be discussed further in the "Test" section.

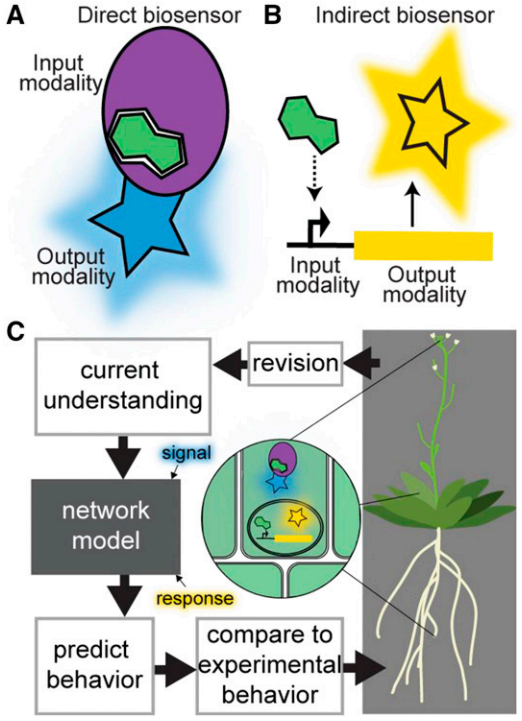

Figure 1. Biosensors link detection of an analyte (such as a signaling molecule) by an input modality to a quantifiable change in an output modality. A, Schematic of a direct biosensor exemplified by a signaling molecule (green) binding protein as the input modality (purple oval) with a fluorescent protein output modality (blue star). This biosensor directly measures the "signal," i.e. concentration of the signaling molecule. B, Schematic of an indirect biosensor exemplified by a signaling molecule responsive promoter of unknown mechanism (dotted arrow) driving expression of a fluorescent protein output modality (yellow star). This biosensor provides a measure of the response of this signaling pathway. C, Using biosensors to measure both the signal and response of a developmental signaling network along with plant phenotype leads to iterative improvement of the developmental network model and our understanding of plant development. Improved understanding of auxin signaling dynamics—realized by multiple biosensors and means of functional quantification—has facilitated rational tuning of plant architecture (Guseman et al., 2015; Je et al., 2016; Wright et al., 2017; Khakhar et al., 2018; Shibata et al., 2018). Newly developed biosensors (Liao et al., 2015; Rizza et al., 2017; Wu et al., 2018), paired with functional and phenotypic quantification of development, will help crack the code underlying developmental signaling and allow rational breeding and engineering of next-generation crops.

Natural binding domains are often part of the signaling pathway one is trying to measure and may interfere with the native pathway components. The laws of thermodynamics dictate that a system cannot be measured without perturbation (Szilard, 1929), but ideally this perturbation will be controlled for and/or minimized. To study normal development, the presence of a biosensor must not alter normal development. Further, protein engineering may be used to render biosensors orthogonal to the native pathway (Rizza et al., 2017). Novel engineered binding proteins or DNA/RNA aptamers require significant investment but are less likely to interfere with the native signaling pathway, especially if potential off-target interactions are controlled for in the design and screening. Numerous methods for directing the evolution of binding modalities have been developed, including phage-display (Smith, 1985; Tan et al., 2016), microbial cell surface display (Charbit et al., 1986; Freudl et al., 1986; Agterberg et al., 1987; Schreuder et al., 1996; Boder and Wittrup, 1997; Daugherty, 2007; Liu, 2015), ribosome display (Mattheakis et al., 1994; Plückthun, 2012), and many in vitro display techniques (Joyce, 1989; Ellington and Szostak, 1990; Tuerk and Gold, 1990; Darmostuk et al., 2015; Tizei et al., 2016). These methods link the genotype and molecular phenotype of large libraries of binding proteins, allowing specific binders to a ligand of choice to be identified and amplified or further characterized. In all cases, expression in the desired host is not guaranteed and further optimization may need to be done, as the expression level of the biosensor combined with the affinity of the input modality for the species of interest determines the dynamic range of the sensor (i.e. the range of input concentrations over which the output of the sensor is quantifiable). Because of these challenges, a transient transformation system for screening expression constructs can expedite biosensor optimization.

Output modalities largely determine the spatiotemporal domain and resolutions of the biosensor measurements. Fluorescent, luminescent, or chromogenic proteins are typical output modalities. Pairs of fluorescent and/or luminescent proteins capable of Förster resonance energy transfer (FRET) or bioluminescence resonance energy transfer are also frequently used. FRET-based biosensors have the advantage of inherent ratiometric output, allowing the expression of the biosensor to be measured by specifically exciting the acceptor fluorophore, and exciting the donor fluorophore to measure the species of interest. Beyond the common issues of photobleaching and phototoxicity, fluorescence measurements in plants can be particularly challenging given autofluorescence and the potential for stimulation of endogenous photoreceptors (Mylle et al., 2013). Luminescence measurements avoid these problems, as they do not require incident light. Bioluminescence resonance energy transfer further allows tuning of the luminescent emission spectra, facilitating ratiometric measurements or measurement of multiple species at once. All light-based measurements are limited by the penetrance of light through tissue, and the numerous light-absorbing structures in some plant cells limit the useful spectrum. Fortunately, dramatic advances are continually being made in microscopy, photo detection, and protein engineering to allow high-resolution imaging across most scales in plants (Rousseau et al., 2015; Clark and Sozzani, 2017; Rios et al., 2017).

Connecting the input and output modalities is generally the most challenging and critical aspect of direct biosensor design, as the connection has a large effect on biosensor resolution and dynamic range. Direct genetically encoded biosensors are typically fusions of the sequences of the input and output modalities (Ostermeier, 2009). The most laborious task in direct biosensor engineering is creating a library of fusions and identifying members that undergo structural changes when exposed to the species of interest, which in turn alter their output. Fortunately, there is a wealth of literature containing numerous case studies (recently reviewed in Bolbat and Schultz, 2017; Sanford and Palmer, 2017), because of early work on engineering of direct biosensors and protein switches (Siegel and Isacoff, 1997; Doi and Yanagawa, 1999; Prehoda et al., 2000; Tucker and Fields, 2001; Dueber et al., 2003; Guntas and Ostermeier, 2004). Ideally the design space of structurally reasonable fusions is thoroughly explored using protein engineering techniques to vary insertional position, linker residues between the modalities, and possibly circular permutation of one or both modalities (Kanwar et al., 2013; Younger et al., 2018). Recently, advances in bioinformatics and decreasing costs of next-generation sequencing have facilitated prediction and experimental determination of sites of potential allosteric regulation (Nadler et al., 2016; Rivoire et al., 2016; Pincus et al., 2017). Folding and stability can be tuned and can also be exploited, either inadvertently or directly, to develop direct fusion biosensors (Tucker and Fields, 2001; Wright et al., 2011, 2014; Choi et al., 2015; Feng et al., 2015; Dagliyan et al., 2016).

Transcription factors are an interesting alternative for connecting input and output modalities of direct or indirect biosensors, by allowing recognition of the species of interest to drive expression of any of the above output domains or another genetic circuit (Feng et al., 2015; Khakhar et al., 2016, 2018; Younger et al., 2016, 2018). The amplification provided by transcription and translation may result in a wider dynamic range. Additionally, this modular connection allows the biosensor to regulate multiple outputs facilitating both measurement and reprogramming of cellular behavior (Faden et al., 2016; Khakhar et al., 2018; Lowder et al., 2018). However, this synthetic gene circuit approach also limits the spatiotemporal resolution of the sensor to the cellular scale and the turnover rate of the output modality.

Biosensors are not limited to detection of monomeric species. Biosensors consisting of short genetic circuits are reminiscent of the enhancer trap (O'Kane and Gehring, 1987) or yeast two-hybrid system (Fields and Song, 1989) and their numerous variants. Advances in microscopy have made possible the in vivo application of well-established methods of quantifying proteins, protein complexes, and protein-protein interactions (Magde et al., 1972; Lakowicz et al., 1992). These methods rely on simple translational fusions, similar to classical FRET-based or protein fragment complementation interaction assays (Pelletier et al., 1999), but utilize highly sensitive confocal microscopes, pulsed lasers, and computational methods to quantify interactions in vivo. It may also be possible to express antibody-like proteins fused to fluorescent proteins, or pairs of antibodies fused to split fluorescent proteins to detect native proteins or complexes (Carlin et al., 2016).

Fluorescence correlation spectroscopy (FCS) measures fluctuations in fluorescence intensity that correlate with the motion of the fluorescently labeled molecule(s) of interest to quantify diffusion (Clark et al., 2016; Clark and Sozzani, 2017). When two different molecules are measured simultaneously in different spectral channels, kinetic parameters of their binding can be inferred from cross-correlation in their diffusion. Another technique, fluorescence lifetime imaging microscopy (FLIM), aims to overcome these issues with overlap in the spectra of the two fluorophores as well as autofluorescence and photobleaching, which can result in poor signal-to-noise ratios in some instances. These issues associated with traditional wave laser microscopy can be abated by using a pulsed laser and by visualizing the time each fluorophore spends in its excited state after the pulse (fluorescent lifetime) instead of intensity. FLIM can be paired with FCS as well as FRET to measure protein-protein interactions (Boer et al., 2014; Long et al., 2017; Rios et al., 2017). These technologies will improve the sensitivity of existing biosensors and facilitate the development of new biosensor approaches.

BUILD

Direct biosensors are generally developed in microbial organisms and then shuttled into organisms less amenable to transformation. This translation between kingdoms and even translation of indirect biosensors between species is not always perfect. This can be due to a combination of issues with expression, folding, stability, and interference with or divergence of endogenous signaling pathways. In most plants, where targeted insertion is not yet possible, there is the additional complexity of integration site variation and frequent silencing (Jupe et al., 2018). Organisms allowing targeted insertion provide an ideal platform for biosensor development, as more direct comparisons of activity can be made between different biosensors. Targeted genetic insertion also allows reporter-tagging of native gene loci, reducing variation. Plants that readily perform homologous recombination, such as *Physcomitrella patens* and *Marchantia polymorpha*, deserve consideration for both the design and application of biosensors, as there is still much to be learned about their development that may inform work in other species (Cove et al., 2009; Ishizaki et al., 2013). To our knowledge, biosensors have yet to be paired with targeted transgene insertion technology (De Paepe et al., 2013) or "landing pads" for plants. This technology is currently low-efficiency and does not allow full specification of the insertion site but does provide more accurate comparison of independent transformants. Homology-directed repair has been demonstrated several times, but usually with low efficiency (Zhao et al., 2016; Čermák et al., 2017; Hahn et al., 2018). Insertional variation in expression can also be mitigated, at least in part, by ratiometric sensors. By expressing a nonfunctional, or constitutively active, version of the biosensor within the same transgene or cistron, expression of the transgene insertion site can be controlled for and higher fidelity achieved (Wend et al., 2013; Liao et al., 2015).

Another challenge across organisms is efficient assembly of unwieldy multigenic constructs. Fortunately, many new toolsets are available for the design and assembly of large and difficult constructs. Several software packages are available for the design and modeling of polycistronic cassettes for biosensors and other applications (Chen et al., 2012a; Hillson, 2014; Harris et al., 2017; Choi et al., 2018; Misirli et al., 2018; Shockley et al., 2018; Watanabe et al., 2018). Several new plant-specific toolkits for assembling the designed constructs have also been developed recently (Engler et al., 2014; Beyer et al., 2015; Shih et al., 2016; Zhu et al., 2017; Pollak et al., 2018).

One of the aspects of these tools that is most critical to the field of biosensor development is the ability to share and reproduce the design, parameterization, and measurement of biosensors between groups and study systems. Common standards for the description of genetic designs and models have been established (Hucka et al., 2015; Martínez-García et al., 2015; Cox et al., 2018), alongside tools for developing and parameterizing (Harris et al., 2017; Zhang et al., 2017; Choi et al., 2018; Shockley et al., 2018; Wandy et al., 2018; Watanabe et al., 2018), as well as visualizing and communicating these designs and models (Merchant et al., 2016; Cox et al., 2017; Der et al., 2017; Medley et al., 2018). Laboratory inventory management and electronic laboratory notebook systems have also been developed to provide a higher degree of organization and reproducibility in the wet lab (List et al., 2014, 2015; Barillari et al., 2016; Craig et al., 2017; Klavins, 2017). The ability of several of these tools to be operated in an integrative notebook environment, containing interleaved narrative with figures and code (possibly of several languages), allows science to be communicated seamlessly and reproducibly (Kluyver et al., 2016; Allaire et al., 2018; Medley et al., 2018). In the future, open sharing of transparent example notebooks documenting complete design-build-test-learn workflows integrating these tools will be the norm. Such examples will provide excellent training and teaching tools, reducing burden, and establishing reproducibility expectations for the field.

TEST

Biosensors have allowed plant biologists to visualize and quantify developmental signals and signaling machinery, as well as provided means to ask better questions as to how development is controlled. To realize our goal of understanding and re-engineering development, we must pair biosensors with systems biology to inform a predictive model of development. Use of systems biology approaches and mathematical modeling paired with transcriptional and translational reporters, cell-type–specific promoters, and enhancers have led to impressive breakthroughs (Vernoux et al., 2011; Bargmann et al., 2013; Efroni et al., 2016; Je et al., 2016; Sparks et al., 2016; de Luis Balaguer et al., 2017; Wendrich et al., 2017; Drapek et al., 2018; Shibata et al., 2018). For example, Shibata et al. used transcriptome and chromatin immunoprecipitation data to develop a gene regulatory network model controlling root hair growth. This model identified both a key positive and negative regulator of root hair growth that formed a feedback loop. This model allowed the authors to identify, and confirm experimentally, genetic manipulations with strong effects on root hair growth. Indirect biosensors paired with systems approaches have also revealed fascinating dynamics of developmental signaling that are still not completely understood, such as oscillations in auxin response within the root meristem, which determine the positions of future lateral roots (Moreno-Risueno et al., 2010; Xuan et al., 2015, 2016; Laskowski and Ten Tusscher, 2017). To track down the unknowns of developmental dynamics will require a better understanding of which signals indirect biosensors are integrating, as well as development of new direct biosensors, simultaneous measurement of multiple

biosensors, and generation of dynamic omics datasets paired with these sensors.

Recently, highly sensitive ratiometric sensors of the auxin signaling network status were developed (Liao et al., 2015) based on improved knowledge of specificity within this network (Boer et al., 2014). These sensors helped revealed new domains of auxin accumulation that were previously predicted by models of auxin transport and production (Scarpella et al., 2006; Grieneisen et al., 2007; Robert et al., 2013). These models were parameterized using translational fusion biosensors, demonstrating the power of the application of multiple biosensors, as the simultaneous measurement of two species facilitates prediction of their dynamic relationship. We highly anticipate proposed future work combining these two high-sensitivity ratiometric sensors (Liao et al., 2015), as well as the development of a direct auxin biosensor (Vernoux and Robert, 2017).

A direct biosensor for gibberellin has recently revealed a strong correlation between gibberellin and cell elongation and helped to decipher the role of the light-responsive PHYTOCHROME INTERACTING FACTORs in regulating gibberellin levels (Rizza et al., 2017). Two indirect abscisic acid signaling biosensors have also recently been developed (Wu et al., 2018). These engineered abscisic acid-responsive promoters complement the detection range of existing direct abscisic acid biosensors (Jones et al., 2014; Waadt et al., 2014). These reporters helped to solidify existing knowledge of abscisic acid's roles in the development of lateral roots and stomata. They also revealed differential regulation depending on the sequence of the core cis-regulatory element and cross-regulation of this promoter by stem cell maintenance transcription factors in the stem cell niche. This important finding highlights the importance and power of characterizing promoter-based reporters thoroughly. In the future, pairing direct and indirect biosensors to measure both signaling inputs and transcriptional outputs may facilitate inference of the intervening network and examination of how these networks interact with cell fate (Fig. 1).

Promoter-based indirect sensors have also been recently used to examine the dynamic relationship between auxin and cytokinin in both barley (*Hordeum vulgare*) and soybean (*Glycine max*; Fisher et al., 2018; Kirschner et al., 2018). These reporters functioned as expected in soybean; however, in barley, the auxin reporters *DR5rev*::*GFP* (Benková et al., 2003) and *DR5v2* (Liao et al., 2015) were poorly expressed and not auxin-responsive (Kirschner et al., 2018). This interesting result compels further examination but may uncover unique paths of evolutionary divergence in auxin signaling components and root development. In soybean, auxin and cytokinin signaling reporters were observed simultaneously in premature root nodules (Fisher et al., 2018). This revealed stark differences in the auxin/cytokinin signaling ratio between premature vascular and parenchyma cells of developing nodules. This pilot study will, we hope, lead to better understanding of the complex roles hormones play in mediating symbioses (Gamas et al., 2017; Betsuyaku et al., 2018; Kunkel and Harper, 2018). Future work integrating multiple biosensors for different developmental signals or different elements within a signaling pathway will greatly improve our understanding of the connectivity and tunability of these signals and the developmental processes they regulate. Integrating nutrient biosensors with developmental signaling will also be crucial to our ability to engineer plants with low resource requirements (Chen et al., 2012b; Upadhyay and Verma, 2015; Okumoto and Versaw, 2017). Novel plant signaling mechanisms are also being revealed by biosensors, such as the recently uncovered Glu-triggered long-distance calcium signaling after wounding (Toyota et al., 2018).

FRET-FLIM and FCS have also helped decipher complex molecular interactions critical to development. FRET-FLIM was recently used to reveal cell-type–specific protein–protein interactions among the SHORTROOT, SCARECROW, and JACKDAW transcription factors, which regulate cell division and patterning in the root (Long et al., 2017). FCS has also been used to track diffusion and interaction of SHORTROOT and SCARECROW (Clark et al., 2016). These studies clearly show cell-type–specific variation in the composition, structure, and activity of complexes of these transcription factors. Future work employing these techniques to examine dynamics of transcription factor complexes, as well as hormone response complexes (Rios et al., 2017), throughout development will provide a mechanistic understanding of cell fate transitions.

MODEL

Measurements of signals alone are of limited use without a predictive framework for linking developmental signals and cell status to transcriptional and phenotypic outcomes. Formulating our current understanding in the framework of a mathematical model allows us to quantify the completeness of our understanding as the deviation between our model and experimental data. An accurate model and understanding also facilitates rational engineering of plant development (Guseman et al., 2015; Khakhar et al., 2018). If the goal of our collective science is to generate the *simplest* model that most completely predicts plant development, then we must accept that our model is, by definition, incomplete. To achieve a maximally informative yet simple model of development, we must carefully design experiments to minimize the uncertainty in both our model selection and parameterization (Smucker et al., 2018). Several groups have developed frameworks for computational design of the optimal set of experiments to identify the mathematical relationship among the signaling inputs, network status, and the developmental outcome, i.e. model selection (Busetto et al., 2013; Apri et al., 2014; Vanlier et al., 2014; Minas et al., 2017; Rougny et al., 2018). Other statistical frameworks aim to design optimal experiments for

OUTSTANDING QUESTIONS

- How can we quantify the levels and dynamics of diverse signals?
- How can signaling data be efficiently integrated from across fields to generate unifying models of development?
- What tools and information are needed to re-engineer or repurpose these signals for novel ends?

determining parameter uncertainty in the chosen model (Dehghannasiri et al., 2015; Fan et al., 2015; Imani et al., 2018; Mohsenizadeh et al., 2018). For example, Dehghannasiri et al. (2015) provides a method for prioritizing future experiments based on existing knowledge of a gene regulatory network and the desired intervention in the network, where intervention in this case is a therapy targeting a pathological network state. Systems biology approaches including similar frameworks have facilitated inference of networks and logic in plant development (Astola et al., 2014; Fisher and Sozzani, 2016; Ristova et al., 2016; de Luis Balaguer et al., 2017; Minas et al., 2017; Shibata et al., 2018; Varala et al., 2018). In addition to optimally improving our knowledge of developmental networks, connecting signaling network models with phenotypic outcome models are of particular importance to the goal of engineering plant development (Prusinkiewicz and Runions, 2012; O'Connor et al., 2014; Landrein et al., 2015; Mellor et al., 2017; Schnepf et al., 2018). One effort critical to the success of systems and synthetic biology in deciphering development will be the continued collaboration among and integration of statistical modeling, optimal experimental design, and dynamic, multivariate molecular genetics techniques.

LEARN

Synthetic biologists' goals for understanding plant developmental biology are within reach. Mathematical models that integrate cell state data from systems approaches with dynamic signal data from biosensors will greatly support efforts to rationally engineer plant form and function. Such models facilitate prioritization and design of experiments to minimize model parameters and improve the certainty of remaining parameters. Implementing statistical tools to design optimal experiments to improve certainty in model selection and parameterization will allow new questions to be addressed efficiently in the context of existing knowledge.

Transdisciplinary approaches combining synthetic, systems, and computational biology are making it increasingly straightforward to quantify the dynamic behavior of signals we already know are important (the "known unknowns") and find new signals and circuits (the "unknown unknowns"). This knowledge will be invaluable in guiding rapid improvements in the quality and quantity of the foods, fuels, fibers, and pharmaceuticals that can be produced by the next generation of crops.

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