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Feature Review

Quantitatively Understanding Plant Signaling: Novel Theoretical–Experimental Approaches

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With the need to respond to and integrate a multitude of external and internal stimuli, plant signaling is highly complex, exhibiting signaling component redundancy and high interconnectedness between individual pathways. We review here novel theoretical–experimental approaches in manipulating plant signaling towards the goal of a comprehensive understanding and targeted quantitative control of plant processes. We highlight approaches taken in the field of synthetic biology used in other systems and discuss their applicability in plants. Finally, we introduce existing tools for the quantitative analysis and monitoring of plant signaling and the integration of experimentally obtained quantitative data into mathematical models. Incorporating principles of synthetic biology into plant sciences more widely will lead this field forward in both fundamental and applied research.

Quantitatively Understanding the Complexity of Plant Signaling: Novel Experimental Approaches

Plants are subject to external stimuli to which they respond and integrate with endogenous growth and developmental programs using highly complex and adapted cellular signaling networks. From signal perception at the receptor level, to signal processing and transduction over a wide array of protein–protein interactions and the resulting control of gene transcriptional activation or repression, regulatory networks are highly intertwined and often multigenic in the individual components [1,2]. In our efforts to understand and moreover modulate these complex processes, quantitative methods of interrogating and monitoring these pathways are essential.

Classical genetic, biochemical, and molecular biology approaches in either model plants or *in vitro* and orthogonal platforms have provided a comprehensive picture of the functionality and connectivity of key components involved in many signaling pathways in mediating plant life [3–7]. However, owing to multiple experimental constraints, detailed insights into signaling kinetics and multifactorial dynamic interactions between multigenic, highly complex networks remain limited [8]. We propose that specific tools for synthetic biology strategies, developed for other eukaryotic systems [9,10], have the potential to move the field of plant research forward. Specifically, towards monitoring but also controlling plant signaling on a much more intricate level at high quantitative and spatiotemporal resolution. The collection of highly quantitative and spatially resolved data, together with the integration into detailed mathematical models on mechanistic and functional descriptions, is increasingly possible with implications in both fundamental and applied plant research (Figure 1, Key Figure).

Trends

Synthetic-biology strategies are yielding molecular switches for the targeted quantitative control, with high spatiotemporal resolution, of regulatory networks, aiming at virtually every level of signaling perception, transduction, and gene expression.

The plant synthetic-biology toolbox comprises molecular devices for the quantitative analysis of hormone and other signaling cascades through the use of genetically encoded biosensors and the concomitantly developed high-end microscopy hardware and analytics. These allow the monitoring of plant signaling with high spatial and temporal resolution.

Mathematical modeling of plant signaling pathways, generated based on quantitative experimental data collected on individual signaling aspects, can contribute greatly to a more complete understanding of network dynamics. This aids not only in optimizing experimental design but also leads to an understanding of the complex regulatory processes taking place upon single or multiple parameter perturbations.

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Key Figure

Synthetic Biology for a Quantitative Understanding of Signaling Networks

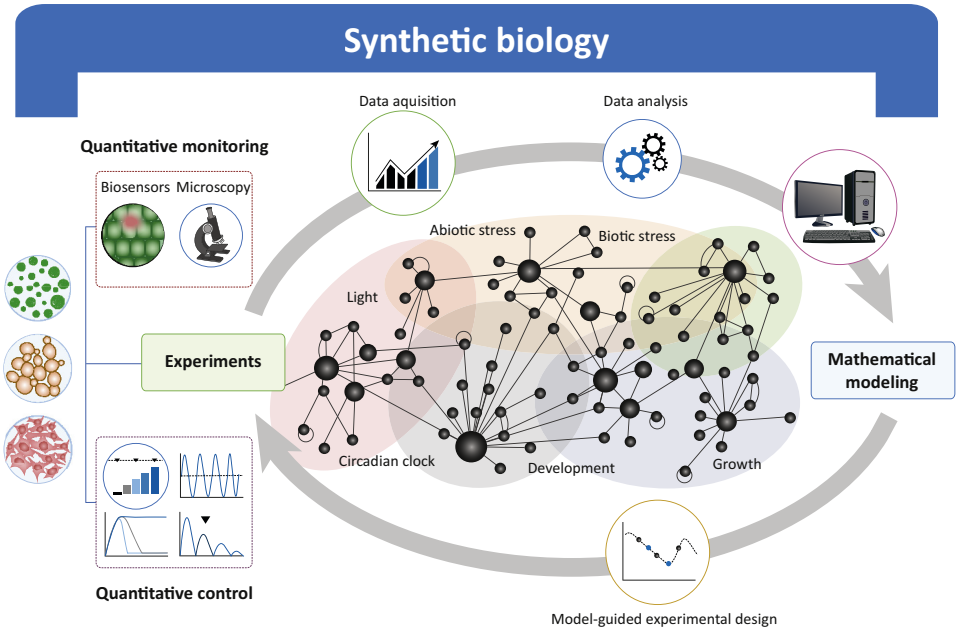

Figure 1. The implementation of synthetic-biology principles and theoretical–experimental approaches aims at a more complete and comprehensive quantitative overview of plant signaling networks. Tightly interconnected regulatory networks sensing, integrating, and responding to environmental and endogenous signals and programs, such as light, circadian clock, abiotic and biotic stress, development, and growth, can be controlled and monitored using experimental methods and strategies in plants or by partial reconstruction in orthogonal cellular platforms (left). These include genetically encoded quantitative biosensors for use in plant cells and tissues, matched with advanced microscopic methods for high spatial and temporal resolution (upper left). The current development of a battery of synthetic molecular switches for the targeted quantitative control of cellular signaling pathways in terms of signal level/amount and kinetics (frequency, duration, and amplitude), based on approaches already widely employed in other organisms (lower left), will lead, with specialized and high-throughput data acquisition and analysis (top), to the collection of quantitative data on single signaling events and parameters to be integrated into mathematical models describing the complexity of the networks (right). Modeling aids experimental design (bottom) and essentially results in an *in silico* plant signaling map (center) that can be used as a predictive tool, guiding future endeavors in crop optimization and fundamental research.

Approaches to Targeted Quantitative Interrogation and Modulation of Plant Signaling: Synthetic-Biology Input

The engineering of a great number of synthetic biology switches and tools in eukaryotes has made it possible to control cellular signaling in a very quantitative and spatially resolved manner at virtually every level of signal perception and transduction. Approaches in yeast and mammalian systems include precise regulation of the transcription of individual components as well as manipulating the abundance, function, and localization of proteins, even allowing dose-dependent control and quantitative outputs [11–14]. However, the implementation of such tools in plants lags behind (Box 1). The introduction of advanced and innovative tools to precisely target signaling networks at multiple levels is needed. To achieve this, plant scientists

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Box 1. What Is (Plant) Synthetic Biology?

As a multidisciplinary, integrative field of research, synthetic biology bridges engineering with natural sciences for the rational and mathematical model-assisted design, construction, and assembly of well-characterized biological modules into higher-order complex synthetic biological systems with new functionalities. These range from metabolic and regulatory networks to whole cells and even tissues and organisms. In a short time, these groundbreaking approaches have already led to major advances in fundamental and applied research including understanding, preventing, and curing diseases, biopharmaceutical discovery, and fine chemical production. However, broad implementation of these engineering strategies by the plant biology community lags behind. Current research employing plant synthetic-biology approaches has mostly concentrated on (i) the generation of synthetic sensors to monitor cellular signaling (discussed in Quantitative and Spatially Resolved Monitoring of Plant Signaling Processes), (ii) the introduction of synthetic metabolic pathways [18,19], oriented towards optimized crop design, for the production of biopharmaceuticals/high-value metabolites [141], or increased stress tolerance, and (iii) the development of synthetic plastomes and minimal plant genomes [5,136].

The successful experience in animal and microbial systems calls for a broad implementation of the synthetic-biology approaches discussed in this review. In particular, the foundations are set for the development of tools and techniques for quantitative and spatiotemporally resolved control and monitoring of signaling processes; the introduction of (semi-) synthetic regulatory networks with *ad hoc* designed capabilities will facilitate, in combination with mathematical models, the understanding of complex regulatory networks and the improvement of relevant agricultural traits.

should take a leaf out of the general book of synthetic biology signaling studies as applied in other organisms (Figure 2) [9,15].

Systems to control and interrogate signaling in plants until now have mostly employed classical strategies at the transcriptional level by the introduction of genes under constitutive expression (over-expression), deletion or disruption of DNA regions to block gene expression (knockout mutants), or through the introduction of tissue-specific/developmentally triggered promoters [16]. These methods, however, offer a static control of gene expression and are unable to be turned off or induced externally once implemented.

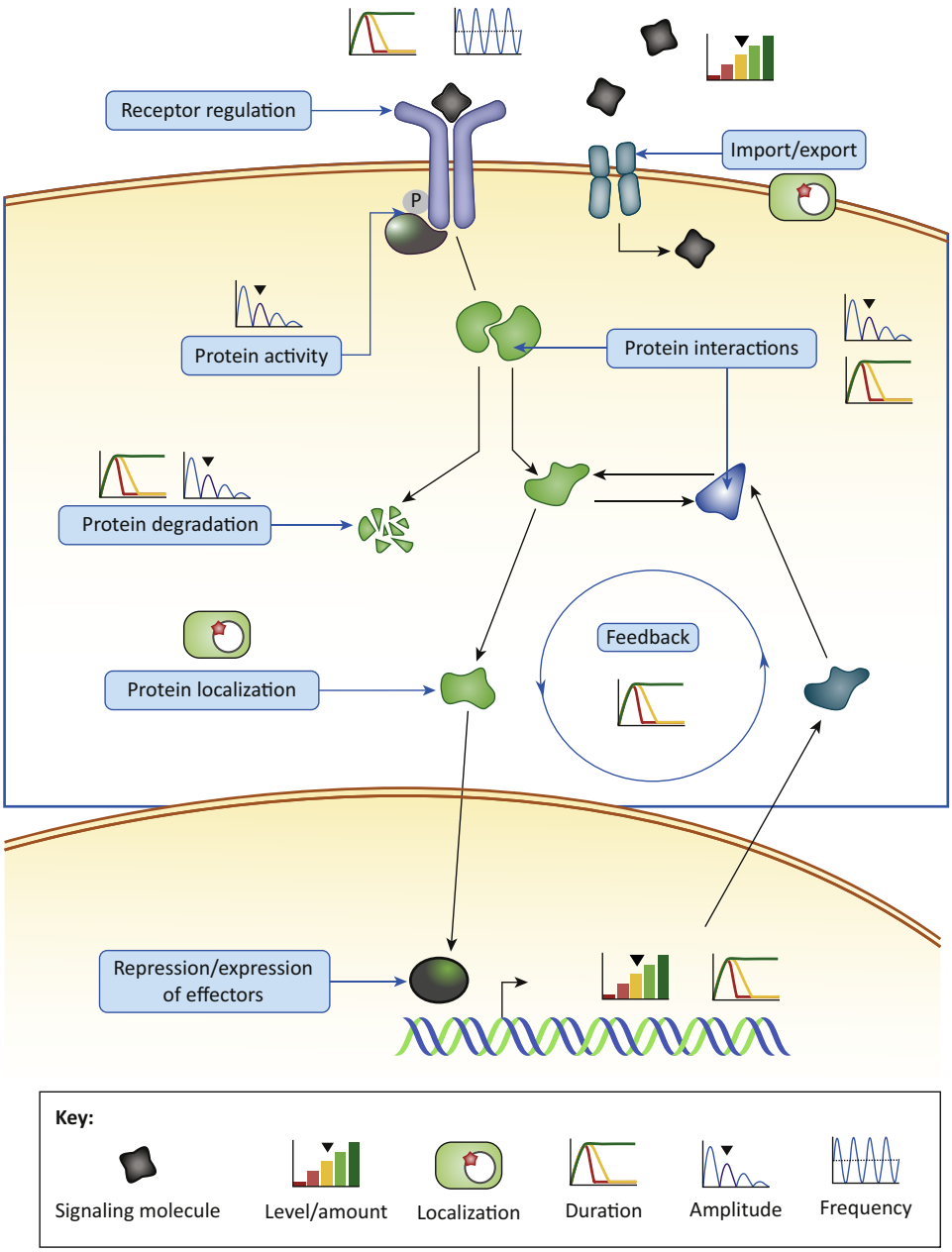
The use of plant endogenous constitutive, tissue-specific, and stress/hormone/light-induced promoters, leading to the more specific expression control of, in particular, metabolic enzymes or stress-related target genes, has been essential for manipulating plant metabolic pathways and in custom crop design [17–20]. Externally inducible systems for gene expression currently used in plants are mostly controlled by temperature shift or with chemical inducers (antibiotics, tetracycline, copper, ethanol, hormones), and have been extensively reviewed [21–27]. Strategies for post-transcriptional control, in other words modulating mRNA abundance or protein synthesis via RNAi/miRNA technology, have also been widely implemented in plants and are induced by similar means (chemical and heat-shock) as gene expression systems [26]. Such systems are intrinsically difficult to turn off once activated dependent on the RNAi/miRNA turnover rates [28], rely on the diffusion/uptake of the inducer if chemical, incur toxic and pleiotropic effects, and/or have limitations for long-term treatment regimens [23,24,29].

Transitioning towards the implementation of novel principles in the design of tools moving beyond traditional means and methods used in basic plant research studies, Faden *et al*. took an elegant approach. They engineered a temperature-switch tool (N-degron) controlling protein abundance in plants by harnessing the cellular machinery of N-end rule-mediated stability of proteins [30]. The switch responds to temperature shifts well within the normal growth conditions of *Arabidopsis* to induce phenotypic changes. It was able to control protein levels of multiple targets (generic, modular design) and illustrated reversible and dose-dependent control (regulatable, tunable), exemplifying synthetic biology principles in the design of a mechanism for targeted signaling control.

Optogenetics is a rapidly growing field of research focusing on engineering photoreceptors and effector proteins to develop synthetic molecular devices for the targeted control of cellular

Glossary

CcaS–CcaR: a cyanobacteriochrome two-component system comprising CcaS and its cognate response regulator CcaR derived from *Synechocystis* sp. PCC6803; responsive to green and red light.
CRISPR/Cas9: clustered regularly-interspaced short palindromic repeats/CRISPR associated protein 9; used in precise genome editing and other DNA-targeting applications.
EL222: an engineered bacterial LOV protein that binds to a cognate DNA sequence upon blue-light illumination.
Fluorescence lifetime imaging (FLIM): an imaging technique based on measuring the fluorescence decay rate of an excited fluorophore.
Fluorescent protein (FP): used in many applications to visualize proteins and substrates.
Förster resonance energy transfer (FRET): energy transferred upon excitation of a fluorophore (donor) to a second fluorophore (acceptor) in close proximity, used in biosensor application.
Light-oxygen-voltage (LOV) domains: light sensors derived from photoreceptor proteins of higher plants, microalgae, fungi, and bacteria that are responsive to blue light. Widely used in optogenetic applications.
Matrix-assisted laser desorption ionization mass spectrometry (MS) imaging (MALDI-MSI): an MS method offering high spatial resolution at the tissue/cellular level.
Phytochrome B (PHYB): a red and far-red light photoreceptor of *Arabidopsis*.
Phytochrome interacting factor 6 (PIF6): binds to phytochromes under red-light illumination.
Synchrotron radiation–Fourier transform infrared (SR-FTIR): used to visualize physical and structural modifications at the molecular level.
Transcription activator-like effector (TALE): used in precise genome editing and other DNA-targeting applications.
UirS–UirR: a cyanobacteriochrome two-component system comprising UirS and UirR, derived from *Synechocystis* sp. PCC6803, responsive to green and red light,



Trends in Plant Science

Figure 2. Targeted Quantitative Modulation of Signaling Dynamics. Molecular tools for the targeted and quantitative modulation of virtually every level of signaling from signal perception, transduction, and integration within the cell to the resulting gene transcriptional effector output. These permit the tight regulation of signaling events in terms of duration, amplitude, and frequency, as well as the level/amount or location of the triggering factors (see bottom for icon description). These approaches are widely employed in other eukaryotic systems but have generally not been widely used in plants. At the level of perception, cellular receptors can be engineered for control of signal duration or frequency, including inducible activation kinetics, and altered substrate affinity/specificity. Signaling molecules can be controlled in level/amount by modulating the transport machinery including substrate import and export proteins. In signal transduction, protein activity can be regulated by targeted and inducible post-translational modifications (e.g., phosphorylation; future targets will include sumoylation, ubiquitination, etc.) resulting in effects on signaling amplitude and duration. Modulation of protein interactions affects signaling amplitude and duration. Tools for facilitated protein degradation or stabilization likewise permit varying signal amplitude and duration. Protein localization can be controlled by sequestration, nuclear import/export, recruitment to membranes, and secretion. Molecular tools targeting the induction or repression of

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and has been engineered to be responsive to UV–violet/green light.
Vascular endothelial growth factor (VEGF): a protein that stimulates angiogenesis and vasculogenesis.
VVD/VIVID: a blue-light flavoprotein (of the LOV domain family) derived from *Neurospora* fungi; responsive to blue light.
X-ray absorption near edge structure (XANES): a type of XAS that can be used to determine the chemical state of elements.
X-ray absorption spectroscopy (XAS): used to determine the local electronic structure of matter with tunable X-ray beams.
X-ray computed tomography (μ-CT): a technique using X-ray optics to generate a 3D model of structures by generating cross-sections of specimens in the micrometer range.
X-ray fluorescence (XRF): an analytical technique used for elemental and chemical analyses.
X-ray microfluorescence (μ-XRF): a technique using X-ray optics to analyze trace elements, where focusing of the X-ray flux with a spatial resolution in the micrometer diameter range is possible.

processes using light [11,13,31,32]. Optogenetic tools already show wide and useful applications in other eukaryotes, overcoming the drawbacks of the currently implemented chemically inducible systems [11]. Light as an inducer is non-toxic and non-invasive, allows high spatiotemporal and quantitative resolution, and an arsenal of optogenetic tools have been developed to choose from [33]. Because plants possess multiple photoreceptors themselves, whose signaling pathways are intricately intertwined with other cascades, and need light as environmental signal and source of energy, one might put off optogenetic control of signaling processes in plants as something that would intrinsically interfere too much with the system to be studied than being a useful tool. Therefore, although possibly not the first inducible system a plant biologist would consider implementing, light-controlled synthetic switches offer highly dynamic and spatially resolved control over multiple cellular signaling processes including gene expression, protein localization, activity, and stability [13]. First optogenetic tools in plants are starting to be reported, as exemplified with the successful implementation of a plant-derived synthetic red/far-red light reversible optical switch for controlled gene expression in *Arabidopsis*, tobacco, and moss protoplast systems (Figure 3) [34,35]. This was a direct transference of a system already established in mammalian cell synthetic biology to plant systems without extensive alteration or optimization required. Utilizing truncated versions of the *Arabidopsis* **phytochrome B** (PHYB, see Glossary) and its interacting factor **phytochrome interacting factor 6** (PIF6) (Figure 3A), the split transcription factor mechanism was used to modulate auxin signaling by placing the auxin receptor, TIR1, as well as a microRNA against TIR1, under the control of the red-light-inducible expression cassette (Figure 3B). Furthermore, the system allows quantitative analysis of the effect of TIR1 overexpression or knock-down on Aux/IAA degradation upon the subsequent addition of auxin. In conjunction with an Aux/IAA-degradation-based biosensor, it constitutes a system that can be dynamically regulated and monitored. This optogenetic tool was also used to drive the ectopic expression of human **vascular endothelial growth factor** (VEGF) in *P. patens* in a dose-dependent manner that was dependent on red or far-red light intensity. The implementation of this light-inducible molecular switch provides the first indication that optogenetic tools can be highly valuable in plant systems for both biotechnological application and targeted signaling studies.

To circumvent potential crosstalk between optogenetic tools and endogenous light signaling in plants, orthogonal photoreceptors or those responsive to light wavelengths less prominently involved in plant signaling might represent a solution. The number of optogenetic switches currently existing in other systems is still expanding, particularly green-light-responsive cobalamin-binding LitR/CarH bacterial systems [36–39], engineered cyanobacterial (e.g., **CcaS–CcaR** and **UirS–UirR** [40,41]) or bacterial phytochromes [42], and bacterial and fungal **light-oxygen-voltage (LOV) domains** (e.g., **EL222** and **VVD**, respectively [43,44]), among other engineered photoreceptors [45,46], could be readily applicable in plant tissues, minimizing the risk of plant light-signaling crosstalk. In addition, microbe-derived opsins, as relatively small and single-component systems, have been implemented to optically control ions fluxes with high specificity (Cl^-, H^+, Ca^{2+} and K^+ ions), primarily in neurons, and are promising for the precise and quantitative modulation of signaling in plants [47,48]. Although some intrinsic questions remain as to their applicability in plant models and characterization would be required, the value that optogenetics would have, with further characterization being necessary, the potential value of optogenetics in studying plant signaling indicates that these approaches will represent an experimental breakthrough for the precise control of cellular signaling at multiple levels, and in a highly dynamic manner, as is already achievable in bacterial, yeast, and animal systems [11,46,49–51]. A wide set of light-inducible tools are implemented in

expression of effector proteins can similarly be used to tightly control the levels/amounts and duration of a signaling response. Feedback in closed-loop synthetic signaling cascades implies control over signal duration and interaction/interconnection between individual signaling cascades.

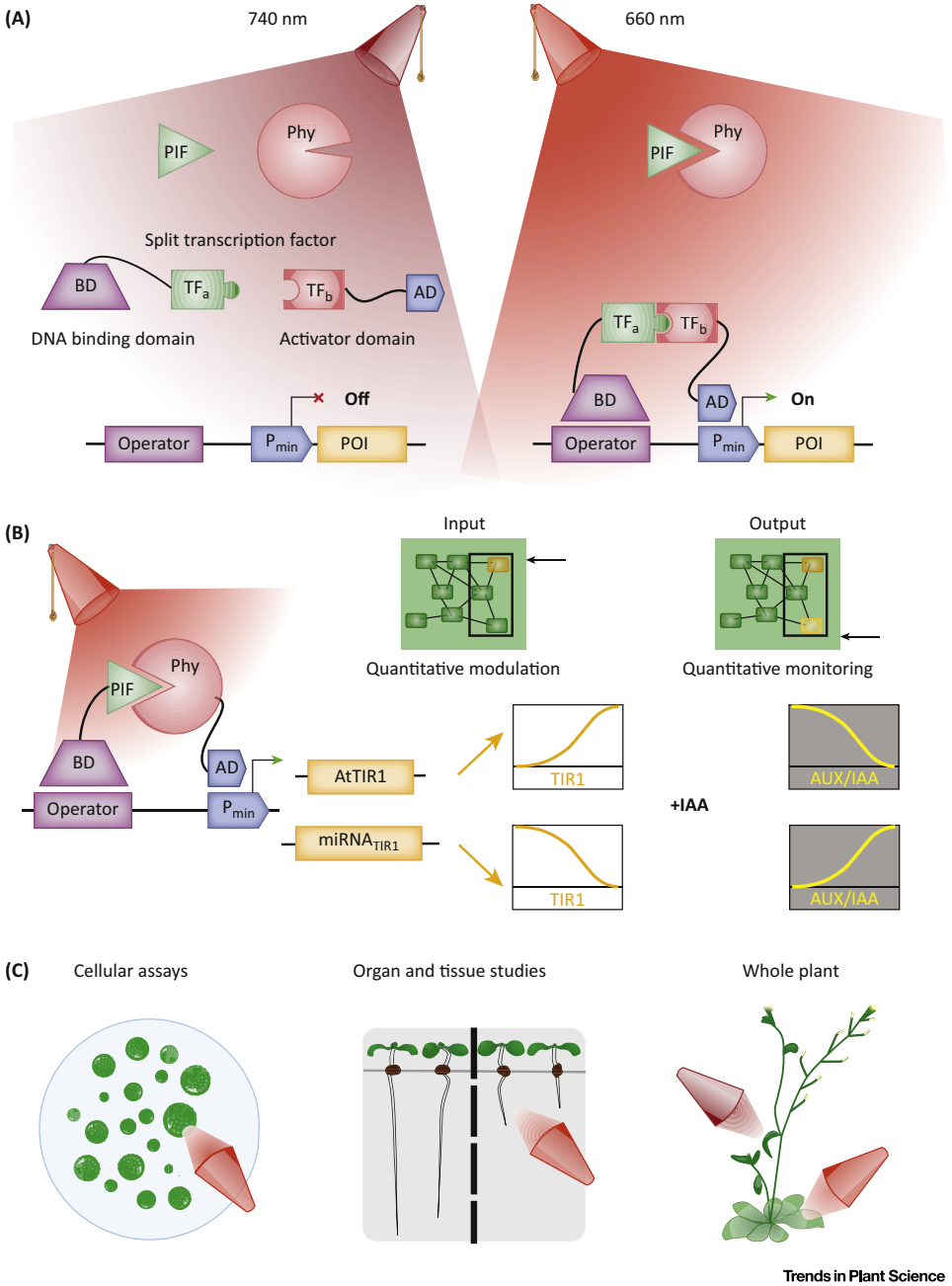

Figure 3. Optogenetics for Quantitative Modulation of Plant Signaling Processes. (A) An example of an optogenetic tool based on the red and far-red light-responsive plant phytochromes, functioning as a split transcription factor system, in which the red-light-responsive phytochrome (PHY) and its interacting factor (phytochrome interacting factor, PIF) are fused to a transcriptional activator (AD) and a DNA-binding domain (BD), respectively. Upon illumination with red light, phytochromes undergo a conformational change after the photoisomerization of the covalently bound chromophore, with an absorption maximum at 660 nm. The conformational change leads to protein interaction between PHY and PIF, which is reversible through illumination with far-red wavelengths, with maximum absorption and reversion at 740 nm. The interaction of PHY and PIF under red light illumination allows binding of the PIF-fused BD to a specific operator sequence located upstream of a minimal promoter and activation of gene transcription via the PHY-fused AD. (B) Müller *et al*. implemented a PHY–PIF red light optogenetic system to drive the expression of TIR1 or miRNA against TIR1 in plant protoplasts [58]. Therefore, fine-tuning the abundance of the key auxin signaling receptor in a highly quantitative and light-dose-dependent manner is possible. The subsequent increase or decrease in the downstream AUX/IAA protein components may then be monitored using a quantitative biosensor, illustrating both targeted quantitative modulation of a

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other organisms to control the activation, phosphorylation, localization, cleavage, secretion, and degradation of signaling proteins, as well as histone modification and chromatin targeting at a DNA level [11,49,52]. Online resources such as www.optobase.org facilitate tool research and selection.

As an illustration of successful signaling studies using optogenetic tools across models, Toettcher and colleagues have developed and implemented both blue- (iLID-SSBP) and red-light (PHY–PIF) systems to control the cellular localization of SOS, an activator of Ras/Raf/MEK/Erk signaling when located at the cell membrane [53,54]. These tools were used to dynamically control Erk signaling in cellular applications in mammalian cells as well as for developmental studies in fruit fly embryos. Signaling pathway activation was controlled with both high spatial and temporal resolution, permitting studies on the dynamics of signal activation in mammalian cells and the effect of local signal induction in *Drosophila* embryonic development. A further example of the modulation of cellular processes is provided by techniques used to control intracellular organelle transport. Optogenetic devices sensitive to both blue light (TULIP, Cry2) and red light (PHY–PIF) have been used to modulate the motility, intracellular distribution, and anchoring of peroxisomes, endosomes, and mitochondria in animal cells, contributing to the study of cell polarization and apoptosis [55–57]. Future extrapolation of these approaches into plant systems could open up novel perspectives for the study of mechanistic processes involved in cellular division, expansion, and differentiation, with potential biotechnological applications.

For plants, one simple strategy would comprise introducing such optogenetic tools under the control of a tissue-specific or otherwise inducible promoter which would allow the controlled expression of the light switch only where (tissue assays) and when (whole-plant assays) needed (Figure 3C). In this manner, transgenic plants with stable optogenetic tools can grow under normal light conditions and be rendered light-responsive on demand.

Synthetic-biology approaches for the development of more orthogonal and controllable expression systems, as well as the generation of promoters with bipartite or tripartite responsiveness, capitalize on *cis*-element promoter engineering, advanced DNA editing, and cloning strategies [5,20,29,58–60]. The categorization and collection of such generated components in open-source registries is an important ongoing initiative in plant synthetic biology, facilitating the transfer of materials and strategies for assembly among researchers worldwide [61,62]. Advanced genome-editing techniques utilizing **CRISPR/Cas9** and **transcription activator-like effector** (TALE) technologies, that are extensively used in mammalian systems [63,64], are also starting to be applied in plant systems (although exceeding the scope of this review; details can be found in [65–68]). Multiple systems for suitable delivery (transient and stable transformation with *Agrobacterium*, transient viral delivery, particle bombardment, polyethylene glycol transformation of protoplasts) and adaptations of mammalian-optimized systems for use in plants have been recently reviewed [7,65]. Beyond genome editing, CRISPR- and TALE-based switches demonstrate the versatility of these DNA-targeting modules to control transcriptional activity and epigenetic status in a target sequence-specific manner [69,70].

The generalized implementation of synthetic tools in plants will open new possibilities for unraveling plant signaling and obtaining a more elaborate description of signaling dynamics.

signaling input as well as quantitative monitoring of the downstream signal output. (C) Using optogenetic approaches not only in cellular setups as already shown but also in targeted plant organ and tissues assays, and up to full transgenic plant applications, is highly feasible given the ability to control the illumination conditions with high spatial and temporal resolution. Abbreviations: At, *Arabidopsis thaliana*; AUX/IAA, auxin responsive proteins; IAA, indole acetic acid; P_{min}, minimal promoter; POI, protein of interest; TF, transcription factor.

Some goals in plant research in the future should be the establishment of generic and adaptable tools, domesticated and characterized for plant models, for the targeted interrogation of individual components within a pathway with both spatiotemporal and quantitative resolution, and to combine them with the means to monitor these processes, as introduced in the following.

Quantitative and Spatially Resolved Monitoring of Plant Signaling Processes

Monitoring and studying plant signaling processes and dynamics in a quantitative manner requires a combination of proper molecular tools, sound analytical methods, and high-throughput data analysis. Many molecular tools have been developed in recent years that facilitate the analysis of individual factors of plant signaling. These include but are not limited to those used in the detection of signaling metabolites (e.g., concentrations and flows of ions, hormones) and small RNAs (abundance and turnover) [71–76]. Likewise, protein conformational changes and interactions can also be monitored [77,78].

The high-throughput technologies of genomics, transcriptomics, proteomics, and metabolomics, with multiple specialized variants thereof, have already contributed a large amount of data on plant signaling over the past decade in the study of physiological, developmental, and stress responses [79–82]. They offer extensive data on regulatory processes at both the signaling (chemical/physical, environmental/endogenous) and protein level. However, the limitation on spatial resolution remains a drawback for such techniques. For the example of metabolomics, traditional mass spectrometry (MS)-based methods for metabolite analysis and quantification are destructive (use of tissue homogenates) with sample preparation being time-consuming and highly specific to the signaling metabolite of interest [79,83,84]. Better spatially resolved methods, based on the utilization of complementary analytics such as in **matrix-assisted laser desorption ionization MS imaging** (MALDI-MSI), present a partial solution to the spatial resolution problem through the targeted local ionization of samples in a 2D and even 3D coordinate system of a plant tissue sample [19,71,85–87]. Among all MS-based methods, data computation and analysis remain a major bottleneck, particularly for those methods incorporating more spatial information [88].

The use of microscopy-based methods to monitor plant signaling at a quantitative level can overcome issues of spatial resolution (Box 2). Fluorescently tagged substrates have been used to follow phytohormone fluxes/kinetics, localization, and distribution *in vivo* for the hormones auxins [89], gibberellins [90,91], brassinosteroids [92], and strigolactones (SLs) [93].

For dynamic analysis of plant signaling on multiple levels, biosensors offer a means for the quantitative analysis at high spatial and even (sub)cellular resolution, and with additional temporal information. Ideally, a quantitative biosensor should not perturb the system in which it is used, have a high signal-to-noise ratio, have a broad range of biologically relevant sensitivity, should be easily detectable, and be able to offer a relative or absolute quantification of the target signaling event or substrate to be studied [76,94]. Biosensors are particularly suited for the detection of small molecules with low abundance, as is the case for most plant hormones, where classical immunochemistry and analytical techniques are limited and signal amplification is not possible. However, they can also be used to monitor protein conformational changes and interactions [77,78]. A large array of biosensors are available for use in plant signaling studies, spanning multiple modes of functionality and readout options from antibody/receptor-based up to genetically encoded [transcriptional reporter-, degradation-, and **Förster resonance energy transfer** (FRET)-based] [94–96]. When implementing a sensor, the underlying molecular mechanism should be evaluated for applicability to the question at hand and the model system to be used (isolated cells vs *in planta*, transient vs stable expression). Likewise, the complexity of design and the type of information obtained in terms of sensitivity

Box 2. High-Resolution Imaging of Plant Cells and Tissues

Plant tissues have both intrinsic benefits and limitations in their compatibility with microscopy methods [108,109]. Autofluorescence over a fairly large span of the light spectrum (chlorophyll- and carotenoid-containing plastids, unwanted excitation of other plant-specific compounds), variable optical properties throughout a single cell (subcellular compartments), extensive light refraction at the cell wall/apoplast, a large sample depth when imaging whole tissues, and developmental responses of whole live-mounts to both gravity and light (limiting long-term mounting and imaging) all pose significant obstacles in obtaining high-resolution, high-contrast, and bright images of plant specimens [109]. Sectioning methods such as laser scanning and spinning disk confocal imaging are commonly used in plant sciences, particularly for live cell imaging. Super-resolution techniques permitting near-molecular resolution of cellular processes have not yet been widely employed in plants because of refraction limitations posed by the cell wall and mounting limitations; however, advances both in sample preparation (oxygenated perfluorocarbon mounting [142,143]) and imaging hardware are making even these techniques more broadly applicable to plant systems (a complete review on super-resolution techniques in plants is given in [144]). In addition, high-resolution imaging of microstructures, speciation, and the localization and concentration of metals and other molecules including structural proteins can be imaged with cutting-edge synchrotron radiation (SR)-based techniques [145]. **Synchrotron radiation–Fourier transform infrared** (SR-FTIR) has been used with intact plant samples to monitor a myriad of physical/structural modifications upon frost and fungal infection [146–148]. These and other SR-methods (**X-ray absorption spectroscopy**, XAS; **X-ray microfluorescence**, μ-XRF; **X-ray computed tomography**, μ-CT; **X-ray fluorescence**, XRF; and **X-ray absorption near edge structure**, XANES) offer an unprecedented subcellular resolution, complementary to spatial MS data, and have the potential to become high-throughput through automation of imaging protocols [145]. The opportunities that these SR-based methods offer, despite requiring specialized facilities, merits a greater interest of the broader plant research community, and investing in these technologies will move the field forward in terms of high-resolution monitoring of plant processes.

towards its target (dynamic range, ratio of signal to noise), the level of quantification possible (absolute or relative), spatial and temporal resolution, and applicability *in vivo* are key factors to consider (Figure 4).

For high spatial resolution, relative ease of use and design, and broad applicability, transcriptional biosensors utilizing synthetic promoters (pDR5, p4D-47, pobs1, and p4xWT-46 for auxin, jasmonate, ethylene, and salicylate, respectively, as reviewed [97]) have proven useful in monitoring the respective hormone signaling pathways. Transcriptional biosensors can be developed for multiple signaling pathways and targets, are modular in design (interchangeable readout), and are generally easy to implement and evaluate. They can offer information regarding signal localization and the general presence or absence of a signal (ON/OFF response), or have a dynamic signal output, depending on the selected readout protein and analysis method. Sensitivity and specificity are crucial for the design and characterization of such sensors because transcriptional activation is a downstream event in signaling processes. Thus, resulting sensor signals may be intrinsically crossregulated through other hormones and pathways [94,98–102]. In addition, transcriptional sensors are limited by a signal time-delay reflecting the induction, expression, and turnover of signal output. Turnover of the readout signal is also one of the factors mediating whether a transcriptional reporter can be used to study the dynamics of a signaling pathway or not. The latest evolution of transcriptional sensors is exemplified in the development of constructs with increased specificity and sensitivity (pDR5v2) and ratiometric design [103].

For a more direct relation of biosensor signal to metabolite concentration, genetically encoded degradation-based sensors have been used extensively. Phytohormone-specific proteasome-mediated degradation of signaling mediator proteins and hormone-specific binding domains have been implemented to generate biosensors for the study of auxin, jasmonate, and most recently SL signaling [98,104–106]. Such sensors are, similarly to transcriptional biosensors, easily implemented, using either **fluorescent protein** (FP) or bioluminescent protein (BP) tags, can be applied in multiple models, and have the potential to become absolutely quantitative when signal degradation is compared with reference samples or integrated into parameterized mathematical models [94]. Ratiometric biosensors that comprise an internal normalization element have been applied in the cases of auxin and SLs [103,104,106], and offer an elegant

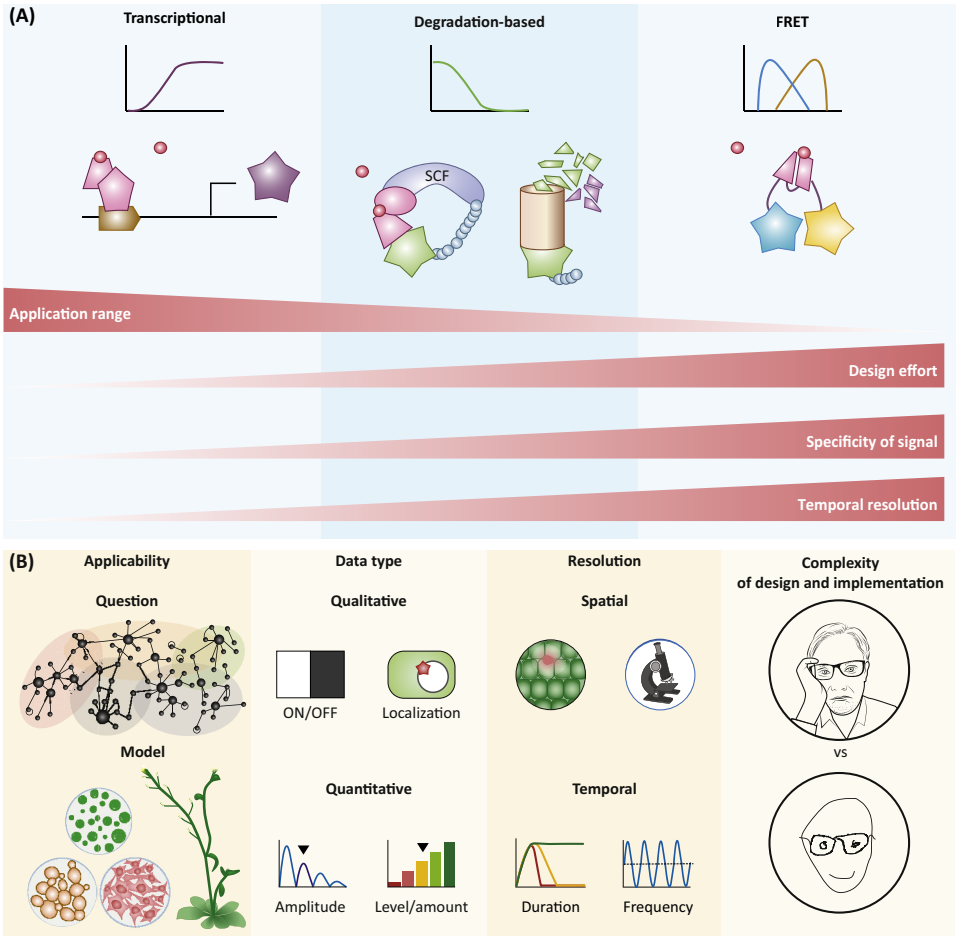

Figure 4. Biosensors for Quantitative Monitoring of Plant Signaling Processes. (A) Three classes of genetically encoded biosensors include transcriptional, degradation-based, and Förster resonance energy transfer (FRET)-based sensors. Transcriptional sensors (left) report the transcriptional activation of a reporter gene upon a signaling event. They are usually fairly simple in design and can be used to study a large range of signaling pathways. They can generate quantitative data and can offer spatial information. Transcriptional sensors can have ON/OFF as well as dynamic signal outputs, with the latter requiring more careful design and characterization. However, a delay in signal perception is intrinsic because gene transcription is a downstream mechanism within the signaling network and time is needed for reporter expression. In addition, transcription-based sensors can be cross-regulated by other signaling pathways. These factors lead to reduced temporal resolution and signal specificity. Degradation-based sensors (middle), for example based on the SCF (Skp1/Cullin/F-box) complex, offer larger possibilities in terms of temporal studies because there is a more direct relation between signal and input, and are also relatively simple in design and use. By the use of ratiometric degradation-based sensors, data can be normalized to generate a quantitative output. The specificity of the signal is affected by potential interactions between sensor components and endogenous signaling, and interference may occur because these sensors are dependent upon cellular protein degradation processes. These sensors can only be developed for signaling pathways that rely on a degradation event (e.g., auxin, strigolactone, gibberellin, and jasmonic acid), decreasing the range of possible applications. FRET sensors (right) can offer high spatial resolution up to the subcellular level, and are a direct measure of a signaling process because the signaling event directly leads to signal output. Thus, both specificity of the signal perceived and temporal resolution are high. However, these sensors are the most difficult to design and optimize, and require knowledge of protein structure/conformation and protein interactions at a molecular level. This reduces the range of applications for signaling studies, although it is noteworthy that these sensors can address additional signaling processes – including protein folding, dimerization, and cluster size determination – that cannot be studied using other genetically encoded sensors. (B) Aspects to consider when choosing a biosensor for a particular question are as follows: (far left) the applicability to the signaling pathway or event of interest, and the applicability to the model system. Plant protoplasts, yeast, or mammalian cells are often more suited for larger-scale quantitative assays in combination with luminescent readouts for sensitive detection. Plant tissues and whole plants are essential for studies on qualitative studies of signaling localization in combination with fluorescent readouts. Most genetically encoded sensors can readily be

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Box 3. Quantitative Imaging of Plant Material

The importance of measuring and analyzing multiple parameters of fluorescence including fluorescence lifetime (via **fluorescence lifetime imaging**, FLIM) and anisotropy when working with FRET and other FP samples has been illustrated in multiple studies to analyze protein interaction parameters and protein cluster size, constitution, and dynamics [149–152]. Much work has been done in recent years describing the difficult empirical design and optimization of FRET sensors and proper signal quantification, calculation, and evaluation of FRET-based data [77,153]. Computation of signal data necessitates algorithms to calculate sensitized FRET, accounting for spectral bleed-through and cross-excitation between fluorophores, which vary between cellular compartments [108,153]. FRET signal interpretation is mostly ratiometric, although photobleaching and FLIM analyses may offer superior quantitative data [77]. Microscopic analysis is, however, intrinsically not high-throughput and is limited by the microscopic setups and equipment that are necessary for plant models, and many biosensors for plant metabolites have not yet been implemented and characterized *in planta* [154]. Application of high-end microscopy allows multidimensional quantitative data to be generated for plant cellular and tissue structures, dynamics, and distributions. To integrate this information into a quantitative description and understanding of biological signaling processes, computer-based bioimage analysis techniques are needed [154–156]. A wide set of computational tools and software packages integrating image analysis workflows and algorithms, for example ImageJ, Macro Language, and Matlab, have been developed to process this high complexity of input image data and produce quantitative and statistically validated information on the correlation between dynamics and spatial parameters.

means of overcoming variations between individual samples, experiments, and models. However, in contrast to the broad range of signaling networks that can be monitored with transcriptional readouts, these sensors can be designed only for signaling pathways that employ targeted degradation of proteins, as is the case with the above-mentioned plant hormones. In addition, they are highly embedded within cellular processes, relying on functional degradation machinery. Depending on the protein or peptide incorporated into the sensor as a sensing element, interaction with other proteins in cellular assays could also lead to increased stability and interference with sensor functionality.

An additional class of genetically encoded sensors are Förster resonance energy transfer (FRET)-based molecular devices. These have been developed for a broad range of plant metabolites and signaling molecules including various ions (Ca^{2+}, Zn^{2+}, Cu^{2+}, Cl^{-}, PO_4^{2-}), metabolites (sugars, cAMP, cGMP, ATP, amino acids), as well as the phytohormone abscisic acid. They have also been used to monitor enzyme activity (kinases, proteases), protein conformational changes, and protein clustering ([96,97,107]; a comprehensive list of FRET-based sensors is given in [77]). They capitalize on protein conformational changes or interactions between proteins upon a signaling event. FRET sensors can be rationally designed for metabolites whose receptor is known and where information on protein structural modifications upon signaling is available [77]. Of all the sensors discussed here, these sensors require the most extensive design, characterization, and expertise for use and analysis (Box 3). However, FRET sensors can be used to study protein interactions and complexes in terms of cluster size, constitution, and dynamics, as well as reporting the presence of a signaling molecule with high spatial and quantitative resolution.

The combination of advanced microscopy techniques with genetically encoded quantitative biosensors, fluorescent-labeled substrates, quality image analysis, and complementation with

transferred between experimental model systems, and the readout can be adjusted depending on the question being addressed. (Middle left) The type of data obtained from a biosensor can be mostly qualitative (localization of signaling, and general induction or lack of a signaling event; ON/OFF) or semi- to fully quantitative (information on the levels/amounts of individual signaling components and the amplitude of a signaling event). (Middle right) The resolution of sensors offering data on localization may offer high spatial resolution at the molecular level (FRET sensors) or simply indicate a tissue or organ of signaling (many transcription- and degradation-based sensors). In addition, with proper design, high temporal resolution of the duration or frequency of a signaling event can potentially be obtained with each sensor class shown here. (Far right) The complexity of design and use are key aspects of biosensor selection to ensure reproducibility and general use throughout the research community.

MS-based omics technologies, will result in the collection of truly quantitative data on individual plant signaling events and processes. This includes information on substrate distribution, ligand–receptor binding, kinase activity, and regulation of gene transcription. Ideally, quantitative and temporally/spatially resolved data of regulatory networks should be captured live and *in vivo.* Although many biosensors and fluorescent substrates have been generated, and appropriate high-end microscopy methods have been developed, perturbations of signaling systems are inherent. This is due to the incorporation of FPs or ectopic expression of the engineered signaling elements in plant cells as well as the microscopy (mounting, illumination) itself [108,109]. Thus, understanding and accordingly engineering the mechanistic principles behind the tools used to either monitor or modulate a signaling event is essential. It is also necessary to differentiate between those tools that remain strictly qualitative and those that offer a means of either relative or absolute quantification, ideally also offering dynamic and/or spatial resolution.

In addition to being able to quantify and monitor hormone substrates and fluxes, quantitative biosensors can also be used as proxies to study signaling pathways in a broader scope. Combination with different genetic backgrounds for signaling elements (e.g., knockout mutants of individual or multiple signaling/hormone biosynthesis components) or coexpression of other factors (e.g., transporters, biosynthetic enzymes, inactivators) can deliver novel quantitative insights into signaling components, effectors, and modulators with high sensitivity [106,110]. For example, the StrigoQuant degradation-based SL sensor was developed by our group to analyze the minimally required molecular components of SL perception and substrate specificity in *Arabidopsis* [106]. By expressing the sensor in protoplasts isolated from various mutant backgrounds for potential SL perception complex members (D14s, MAX2 F-box), it could be determined that, of three members of the D14 protein family, only one was essential for sensor degradation and was thus necessary for perception of a synthetic SL (GR24). Also at the hormone level, the importance of the stereochemistry of a set of natural SLs was analyzed, and stereoselectivity towards a given enantiomeric form was observed. Being ratiometric, the sensor generated normalized data that could differentiate between different test conditions with high quantitative resolution and sensitivity (up to fM levels). The integration of such semi- or fully quantitative and/or kinetic data obtained experimentally, and that can be assigned to the individual factors that influence a signaling system, into mathematical models will provide new insights into complex and highly interconnected plant signaling networks.

Mathematical Modeling of Plant Signaling

To understand how the intricate biological processes integrating environmental and plant endogenous cues such as growth, development, and defense responses are regulated, ideally one would construct a mechanistic representation of the signaling systems underlying these processes. Mathematical models quantitatively describe the connectivity patterns, dynamics, function, and topology of the regulatory networks that directly relate to changes in time and space of signaling molecules and proteins, including their concentrations and fluxes, subcellular and tissue localization, and activation state and function [8,111,112]. Different approaches and modeling principles can be implemented depending on the availability of quantitative experimental data, prior knowledge of the systems parameters involved (or from analogous networks from other organisms), and the nature of the biological question to answer: from bottom-up approaches, starting off with a limited dataset to construct a fairly simple representation of the system components, up to more-complete representations of the temporal and spatial dynamics and topologies of networks in whole tissues ([111,112] for extensive review). The generation and improvement of a model follows an iterative route including cycles of *in silico* tests/parameterization with experimental data/validation/adjustment–improvement until the model performs successfully in explaining the mechanistic basis of a biological phenomenon.

Mathematical modeling has already contributed to our understanding of regulatory mechanisms and the prediction of intricate and 'complex' signaling dynamics, cross-interaction between regulatory networks, and the biological effects at different levels of plant life [113]. Modeling approaches based on quantitative experimental data have integrated environmental, developmental, and growth morphogenetic signals, and suggest that growth patterning mechanisms are mediated by the dynamics and spatiotemporal distribution of auxin signaling across tissues and development (reviewed in [114,115]). In the following we exemplify these strategies with a series of theoretical–experimental approaches. In simultaneous efforts, three groups have employed mathematical modeling, coupled to experimental data obtained by confocal microscopy of the distribution and levels of the auxin efflux pump PIN1, to study the spatial patterns of auxin distribution generated by polarized hormone transport in the apical meristem [111,116,117]. The model predictions were confirmed experimentally, leading to a detailed description of organ initiation in the shoot apex [117], and thereby integrating molecular morphogenetic cues with phyllotaxis mechanisms [111]. More recently, Refahi *et al*. revisited the canonical deterministic model of phyllotaxis at the shoot apex by applying a theoretical approach implementing stochasticity in the patterning mechanism [118]. They could demonstrate that disorders of this self-organizing system arising from noise lead to dynamic phyllotaxis patterns.

Experimental data on auxin directional fluxes generated with the DR5 transcriptional sensor were employed by Wabnik *et al*. to parameterize a model of the spatiotemporal distribution of PIN1 [119]. This approach suggested that directional cell-to-cell transport of auxin is responsible of the establishment of the apical–basal axis in *Arabidopsis* embryos. Finally, by combining quantitative data on the spatiotemporal distribution of auxin gradients generated with the DII-Venus sensor with parameterized mathematical models, the groups of Vernoux and Bennet were able to shed light on the mechanisms involved in the gravitropic response of roots [120]. The model predictions in conjunction with high-resolution kinetic and quantitative information on auxin fluxes showed that a lateral auxin gradient is rapidly and transiently generated upon a gravitropic stimulus, leading to root bending that reverts the asymmetry in auxin distribution, terminating the response (tipping-point mechanism). These examples show how, by employing such multidisciplinary approaches, it is now possible to obtain mechanistic insights into complex developmental and growth processes.

In addition, highly complex regulatory networks controlling the circadian clock [121–123] and flower development [124,125] that integrate temperature, light, and endogenous metabolic and developmental status have been developed and successfully validated. However, most of the models are not spatially resolved owing to the difficulty of gathering high-quality quantitative data from different tissues. A step towards a comprehensive understanding of processes in different cell types is shown in the work of van Esse *et al*. on brassinosteroid signaling. Different models were developed, adjusted, and experimentally validated to predict the differential activity of the BRI1 receptor in root growth and hypocotyl elongation in *Arabidopsis* [126]. Recently, machine-learning approaches have been implemented to obtain a description of intricate plant–pathogen interaction networks [127]. With an ever-increasing computational capacity, most modeling strategies are currently constrained by the availability of quantitative experimental data [128]. In this context, the wide implementation of the technologies and approaches described in this work (sensors, high-end and next generation omics, quantitative microscopy) will contribute to the generation of spatiotemporally resolved experimental data on the dynamics at the metabolite, gene expression/omics, protein activity, and connectivity levels.

Finally, the model-based quantitative understanding of gene regulatory networks and their effects on cellular processes can also be used to guide the targeted experimental interrogation

of the biological systems to obtain deeper mechanistic insights or to assist in engineering strategies for improved stress-tolerance, high-yield, and nutritional quality traits. New-generation chemical switches and, in particular, the development of light-inducible devices, as already widely applied in animal systems [13], will allow model predictions to be precisely translated into targeted modulation of individual components in cells and tissues at high quantitative, spatial, and temporal resolution.

Concluding Remarks and Future Perspectives

The above-mentioned approaches for the study of plant signaling will assist in the collection of quantitative data and promote the understanding of regulatory networks. In addition, the reconstruction of partial or whole-plant signaling pathways in other well-established cellular systems is a valid approach to the quantitative and specific analysis of signaling events. This allows the systematic observation of individual components of pathways that are redundant and/or highly cross-regulated via other pathways in plants. The high level of interconnectivity between light, hormone, and stress signaling networks in plants calls for tools allowing the observation and analysis of crosstalk interactions on an individual basis [7]. An overall reduction of network complexity and elimination of pleiotropic effects is achieved in orthogonal systems by simplifying the (plant) protein environment, thus avoiding interactions with endogenous components that would affect analysis of specific signaling events *in planta* (Figure 5).

The benefits of using orthogonal systems where synthetic circuitry is already well established can be seen in particular applications in yeast cells. One example was the use of synthetic auxin circuits to reconstitute the minimal auxin response machinery in *S. cerevisiae* to permit systematic analysis of the interactions of auxin signaling components without interference with other factors or feedback from other pathways. These assays utilized fluorescence proteins as the readout, allowing high-throughput analysis via flow cytometry and yielding quantitative information as to the activation or repression of DNA binding and signaling over hetero- and homotypic interactions between ARFs and IAAs [129]. Such applications in yeast go beyond the classic two-hybrid systems utilized to study protein–protein interactions, and are instead based on synthetic networks that take advantage of the conservation of basic cellular mechanisms among all eukaryotes, such as protein degradation. These strategies are also being applied in mammalian cells, where plant proteins can be readily expressed with conserved functionality and interaction, and where an extensive toolbox of synthetic-biology molecular devices (inducible switches, reporters, sensors, etc.) and technologies are available. As an example, an optogenetic tool using PHYB and PIF3 in mammalian cells to translocate proteins to the nucleus was able to uncover the molecular mechanism of PHYB cytosol-to-nucleus transport. Previous theories included the unmasking of an intrinsic nuclear localization sequence in the photoreceptor upon red-light illumination. This work showed that PHYB does not locate to the nucleus by itself, and that other factors (e.g., PIF3) are essential for nuclear import [130]. Aside from the production of a useful optogenetic tool that was implemented not only in mammalian cell culture but also in zebrafish embryos, this study shed light on the general PHYB transport mechanism. This was only possible because PHYB could be functionally expressed in a non-plant background, eliminating other interacting components which make this analysis experimentally challenging in plant cells.

The large availability of synthetic tools to very tightly control the levels of expression of components, as well as their cellular localization and function, coupled with established readout systems for quantitative data output, make such platforms ideal for highly quantitative signaling studies. These strategies can be used to dissect the function of single pathway components in a bottom-up approach, allowing the introduction of additional components into the system in a bring-in-and-play manner. In the long run, an understanding of network dynamics, crosstalk between pathways, and of central plant signaling hubs can be based on data obtained for

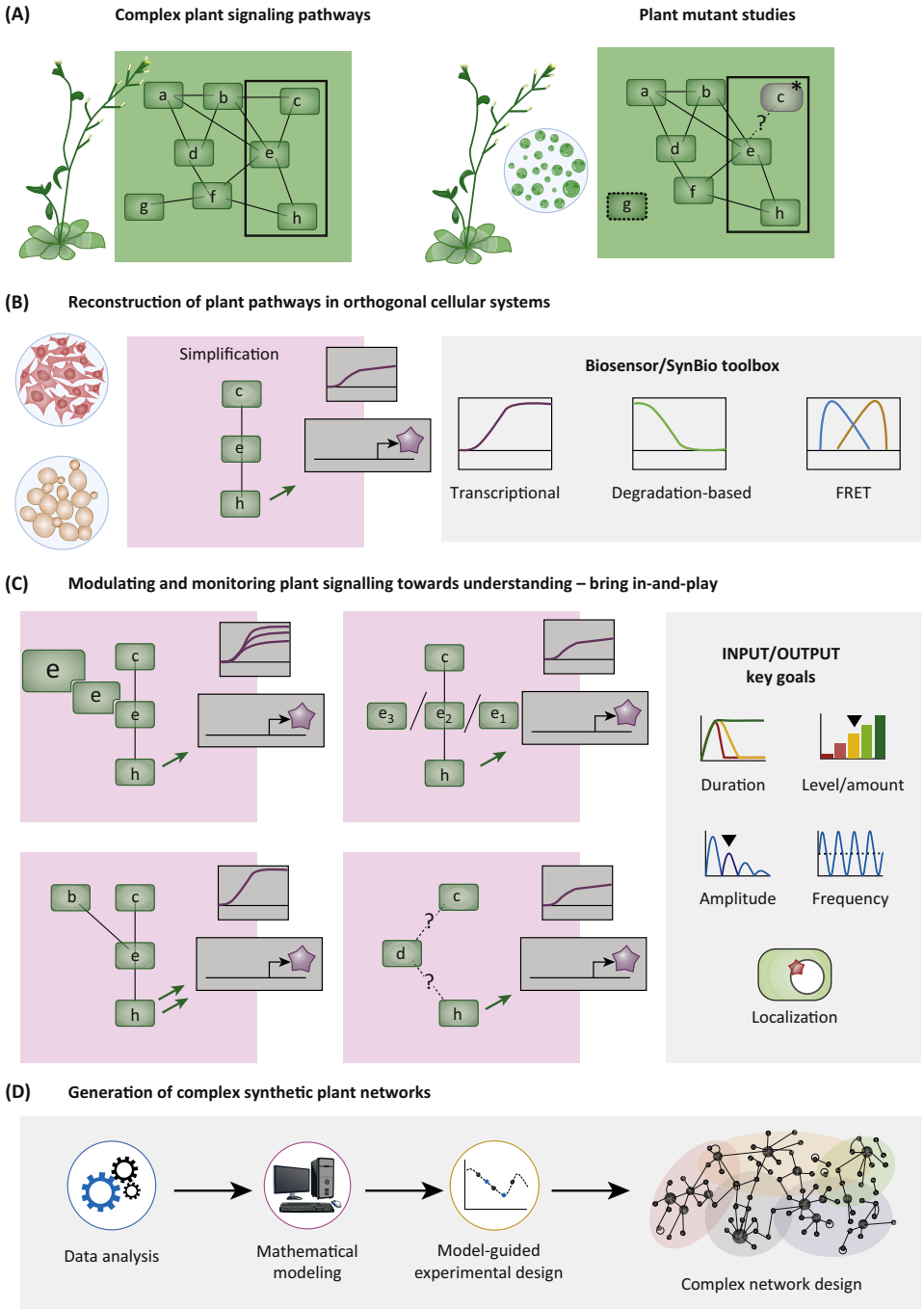
Outstanding Questions

What are the key technical obstacles to the development of better-controlled inducible systems for manipulating plant signaling networks? What are the available strategies to overcome these constraints?

Can unwanted secondary effects on signaling networks be avoided by introducing synthetic switches/biosensors into plant cells? Can fully orthogonal molecular devices be designed that have negligible interference?

What are the socioeconomic implications of developing smart plants? Is enough being done currently towards general acceptance of genetically manipulated organisms (and synthetic-biology concepts), not only in fundamental research but also in applied sciences and crop optimization, and are these sustainable options for the future?

How can the novel theoretical–experimental concepts of synthetic and quantitative plant biology best be incorporated into teaching curricula to prepare future generations of plant researchers for the challenges ahead?



Trends in Plant Science

Figure 5. Orthogonal Systems and Approaches for Reconstructing Plant Signaling To Permit Quantitative Modulation and Monitoring. (A) Complex plant signaling pathways can be simplified for experimental purposes through the use of targeted mutation of signaling components (c, grey*) to alter the functionality of signaling pathway components or via disruption or deletion of DNA regions (g, broken lines). Targeted DNA modifications can be performed using new genome-editing technologies such as CRISPR/Cas9. Studies completed in transgenic plants or protoplasts derived from mutant genotypes can and have been used in the past to simplify pathway analysis. (B) Alternatively to plant-based models, orthogonal systems such as mammalian and yeast cells can be used as a chassis for the reconstruction of simplified plant signaling pathways. This is possible because the fundamental cellular machinery is generally conserved across all eukaryotes, and plant-specific signaling components can be used, in conjunction with the standard toolbox of

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particular signaling events and protein interactions [7]. For example, stemming from the interest in optogenetic tools controllable by red light, the transition rates and binding properties of phytochromes and their interacting factors have been extensively quantified and modeled using *in vitro* data and synthetic signaling circuits in mammalian cells [111,130]. The partial or full reconstruction of plant signaling events would make it feasible, for example, to integrate a reconstructed hormone signaling pathway in mammalian cells with a quantitative readout (biosensor or transcriptional) [104], and then individually bring-in-and-play putative interacting proteins from other pathways to analyze positive or negative effects on hormone signaling or vice versa.

With increased availability of automated facilities for plant transformation/selection and targeted methods of genome editing (CRISPR/Cas and TALE-based technologies [7,64]), the generation of plants with multiple and complex gene traits is increasingly possible. The coming years will witness the development of platforms and tools that are generic, transferable, and plant-domesticated, and that will permit the collection of quantitative and spatially resolved data on individual signaling pathways and the main signaling hubs that interconnect these complex networks. This will lead towards the goal of reconstructing a plant *in silico* [128], and in the future will allow a more comprehensive understanding of the impact of targeted modulation of key signaling pathways on the plant as a whole. This knowledge will be essential in ambitious endeavors such as the introduction of nitrogen fixation into cereals, controlling plant–microbe interactions, receptor hijacking, and general cell–cell, tissue, and organ communication. This will require joint efforts between fundamental plant researchers, synthetic biologists, and theoreticians, to be achieved in the coming decades.

Going beyond interrogating plant signaling at particular points, it should be possible to introduce entire synthetic signaling circuits into plants, using logic design principles, to generate 'smart plants' with improved or new functionalities. These approaches may employ logic gate synthetic circuitry technology following the guidelines and examples developed in bacterial and mammalian models [4,10,14,29,131]. The ultimate objective, inspired by work in animal systems [132–134], is the engineering of prosthetic networks: cellular synthetic closed-loop devices that are able to respond to environmental and endogenous signals, process the information, and effectuate a response accordingly (leveraging a metabolite or generating a developmental, growth, or stress response). First steps to introducing synthetic signal transduction pathways into plants using heterologous components include the engineering of synthetic signal transduction pathways based on cytokinin signaling by replacing endogenous modules with bacterial components with a view to developing sensors for metabolites of interest (e.g., TNT, 2,4,6-trinitrotoluene [135]). In addition, efforts to generate a minimal synthetic plant genome or plastome are ongoing [136–138]. These will facilitate rapid screening

synthetic biology including but not limited to biosensors, to monitor signaling output in a background- and interaction-free cellular environment. (C) Modulation of signaling input can be achieved in a very precise manner by altering the expression levels of individual components, screening multiple protein family members or homologs in different plant species to allow heterologous replacement of single components, and the addition of further signaling modulators, and these systems permit the function of unknown signaling components to be investigated. Once a plant signaling pathway has been reconstituted with a quantifiable synthetic network signal output, the bring-in-and-play approach of adding, removing, mutating, and substituting further plant components is possible. Advanced tools for the precise modulation of signaling molecules and processes are readily available in orthogonal platforms, together with biosensors and other analytic techniques, and will ultimately allow both input modulation and output observation of signaling in a multifaceted way (grey box, right). (D) To design complex plant cellular networks that are responsive to multiple inputs and that deliver a highly dynamic output, quantitative data obtained from signaling pathway analyses should optimally be determined in a high-throughput manner, mathematically modeled, and the model used to aid further experimental design. Calculated and focused analyses of, initially, single signaling events and factors, with progressively increasing complexity, and the incorporation of the data generated into models, will facilitate the design and construction of highly dynamic synthetic plant signaling pathways with applications in both research and industry.

of components and synthetic networks in combination with orthogonal cellular or *in vitro* systems. The use of such simplified platforms has recently been exemplified by Laursen *et al*. for understanding and engineering of the production of the complex secondary defense compound dhurrin by synthetically reconstituting and analyzing the metabolon in liposomes [139]. Finally, a deeper quantitative understanding of plant regulatory networks will allow the implementation of research approaches developed in animal tissue and organ engineering [140]. Synthetic networks can guide the development of synthetic tissues/organs to study developmental processes, architecture, metabolism, and physiological aspects. The final goal would be to engineer a synthetic leaf or even plant with improved traits or even novel functionalities. Obtaining more robust crop plants for the future, and developing photosynthesis-driven production systems for therapeutics and/or food additives, are among the goals of plant science that can be addressed through synthetic signaling network design and the optimization of existing natural processes 141].

Taking the example of the synthetic biology approaches used in other systems, it is time to start developing more complex and dynamic means to regulate plant signaling at virtually every level of signal transduction (optogenetic tools, synthetic signaling pathway construction, closed-loop signaling) and to fully utilize different techniques of monitoring plant signaling (biosensors as proxies to study signaling pathways, high-throughput imaging/image analysis) in a manner that spatially resolved quantitative data can be mathematically modeled, thus advancing signaling studies more rapidly (see Outstanding Questions). The goal of obtaining a comprehensive understanding of the systems that plant biologists have been optimizing for centuries is now achievable, and will allow us to design the 'smart plants' of the future.

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