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Sensors for the quantification, localization and analysis of the dynamics of plant hormones

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SUMMARY

Plant hormones play important roles in plant growth and development and physiology, and in acclimation to environmental changes. The hormone signaling networks are highly complex and interconnected. It is thus important to not only know where the hormones are produced, how they are transported and how and where they are perceived, but also to monitor their distribution quantitatively, ideally in a non-invasive manner. Here we summarize the diverse set of tools available for quantifying and visualizing hormone distribution and dynamics. We provide an overview over the tools that are currently available, including transcriptional reporters, degradation sensors, and luciferase and fluorescent sensors, and compare the tools and their suitability for different purposes.

Keywords: plant hormone, biosensor, imaging, quantification.

INTRODUCTION

Plant growth regulators, or plant hormones, are chemical messengers that play crucial roles in the control of plant growth and development. A wide range of unique hormones has been characterized in plants, i.e., abscisic acid, auxins, brassinosteroids, cytokinins, ethylene, gibberellins, jasmonic acid, salicylic acid, strigolactone and small peptides (Verma *et al.*, 2016; Stührwohldt and Schaller, 2019; Bowman *et al.*, 2019). The concentration of the hormones can vary between different plant tissues, developmental stages and environmental conditions (Swarup *et al.*, 2007); however, it is not entirely understood how the spatial and temporal distribution of plant hormones is coordinated, nor how these changes trigger diverse responses. In the past, traditional biochemical analyses have been used to gain insight into the distribution and dynamics of plant hormones. For instance, immunohistochemistry using anti-hormone antibodies has been used to determine the distribution of hormones in plant cells and tissues (Forestan and Varotto, 2013; Schlicht *et al.*, 2006; Nishimura *et al.*, 2011).

The need to fix and section the material limits the spatial and temporal resolution, however. Mass spectrometry combined with gas or liquid chromatography has been used to identify and quantify plant hormones at high accuracy and sensitivity (Okamoto *et al.*, 2009; Owen and Abrams, 2009; Gemperline *et al.*, 2016). Although rapid sampling can provide temporal resolution for chromatographic assays, the extracts reflect an average over many cells in an organ, such as a leaf, and thus cannot detect spatial differences, such as gradients. Over the past two decades, advances in the development of fluorescence microscopy technologies and the engineering of biosensors has provided new tools for monitoring plant hormones with minimal invasion and cellular or even subcellular resolution (Walia *et al.*, 2018; Waadt, 2020). Moreover, genetically encoded biosensors are able to detect rapid changes in the concentration and distribution of plant hormones in living cells. In this review, we introduce the current imaging technologies available for plant hormone detection (Tables 1 and 2), and we discuss possible routes to expand and improve biosensors for plant hormones.

Table 1 Biosensors for plant hormones

Analyte	Biosensor	Sensor type	Range of ligand concentrations applied endogenously or exogenously	Reference	
ABA	6xABRE	Transcriptional	approx. 1–25 μM	(Wu <i>et al.</i> , 2018)	
	ABACUS	FRET based	approx. 2–80 μM	(Jones <i>et al.</i> , 2014)	
	ABAleon2.1	FRET based	approx. 100 nM	(Waadt <i>et al.</i> , 2014)	
	ABAleon2.15	FRET based	approx. 100 nM	(Waadt <i>et al.</i> , 2014)	
	ABAleonSD1-3L21	FRET based	approx. 100 nM	(Waadt <i>et al.</i> , 2020)	
	SNACS	FRET based	20 μM ABA (exogenous)	(Zhang <i>et al.</i> , 2020)	
Auxin	DR5:reporter	Transcriptional	10–1000 nM (DR5) 3–1000 nM (DR5v2)	(Ulmasov <i>et al.</i> , 1997; Liao <i>et al.</i> , 2015)	
	pIAAmotif	Transcriptional	10 nM–1 μM 2,4-D (exogenous)	(Lieberman-Lazarovich <i>et al.</i> , 2019)	
	DII-VENUS	Degron based	1–1000 nM	(Brunoud <i>et al.</i> , 2012)	
	R2D2	Degron based	1 μM IAA (exogenous)	(Liao <i>et al.</i> , 2015)	
	L2min17-Luc	Degron based	10–1000 nM	(Wend <i>et al.</i> , 2013)	
	AuxSen	FRET based	5–50 μM	(Herud-Sikimic <i>et al.</i> , 2020)	
Brassinosteroid	BZR1-YFP	Transcriptional	100 μM brassinolide (exogenous)	(Chaiwanon and Wang, 2015)	
	Cytokinin	ARR5	Transcriptional	10–1000 nM (D'Agostino <i>et al.</i> , 2000; Romanov <i>et al.</i> , 2002)	
Ethylene	TCS	Transcriptional	1–1000 nM	(Müller & Sheen, 2007)	
	TCSn	Transcriptional	1–1000 nM	(Zürcher <i>et al.</i> , 2013)	
	EIN3-GFP	Degron based	50 μM ACC (exogenous)	(Guo and Ecker, 2003)	
	EIL1-GFP	Degron based	100 μM ACC (exogenous)	(An <i>et al.</i> , 2010)	
	FP-EBF 3'UTR	Translational	10 μM ACC (exogenous)	(Merchante <i>et al.</i> , 2015)	
	FP-6x EPU	Translational	10 μM ACC (exogenous)	(Li <i>et al.</i> , 2015)	
	EBS:GUS	Transcriptional	10 μM ACC (exogeneous)	(Stepanova <i>et al.</i> , 2007)	
Gibberellin	AEP	Artificial metallozyme	100 μM ACC (exogenous)	(Vong <i>et al.</i> , 2019)	
	GFP-RGA	Degron based	100 μM GA ₃ (exogenous)	(Silverstone <i>et al.</i> , 2001)	
Jasmonic acid	GPS1	FRET based	0.03–1 μM GA ₁ 0.1–2 μM GA ₃ 0.005–0.2 μM GA ₄	(Rizza <i>et al.</i> , 2017)	
	Jas9-Venus	Degron based	50 nM–10 μM coronatine	(Larrieu <i>et al.</i> , 2015)	
	JAI3-FP	Degron based	50 μM jasmonate (exogenous)	(Chini <i>et al.</i> , 2007)	
	Karrikin	DLK2:LUC	Transcriptional	1 μM < KAR1 (exogenous) 10 nM < KAR2 (exogenous)	(Sun <i>et al.</i> , 2016)
pRATIO-SMAX1		Degron based	approx. 200 nM KAR1 (exogenous)	(Khosla <i>et al.</i> , 2020)	
Salicylic acid	NPR1-FP	Transcriptional	0.5 mM INA (exogenous)	(Mou <i>et al.</i> , 2003)	
	Strigolactone	D53-GFP	Degron based	5 μM <i>rac</i> -GR24 (exogeneous)	(Zhou <i>et al.</i> , 2013)
	SMXL6-YFP	Degron based	5 μM <i>rac</i> -GR24 (exogeneous)	(Bennett <i>et al.</i> , 2016)	
	SMXL7-YFP	Degron based	5 μM <i>rac</i> -GR24 (exogeneous)	(Liang <i>et al.</i> , 2016)	
	StrigoQuant	Degron based	10 pM–1 nM	(Samodelov <i>et al.</i> , 2016)	
	rDAD2cpGFP	cpFP based	50–500 nM <i>rec</i> -GR24 (exogenous)	(Chesterfield <i>et al.</i> , 2020)	
	rShHTL7cpGFP	cpFP based	approx. 10–100 nM <i>rec</i> -GR24 (exogenous)	(Chesterfield <i>et al.</i> , 2020)	

Table 2 Comparison of FRET-based direct biosensors

Biosensor	Analyte	K_D	Dynamic range	Ref.
ABACUS1-2 μ	ABA	approx. 2 μM	+60% (500 μM ABA)	(Jones <i>et al.</i> , 2014)
ABACUS1-80 μ	ABA	approx. 80 μM	+160% (500 μM ABA)	(Jones <i>et al.</i> , 2014)
ABAleon2.1	ABA	approx. 79 nM	–8.98%	(Waadt <i>et al.</i> , 2014)
ABAleon2.15	ABA	approx. 600 nM	–10.09%	(Waadt <i>et al.</i> , 2014)
ABAleonSD1-3L21	ABA	approx. 938 nM	n.d.	(Waadt <i>et al.</i> , 2020)
GPS1	GA ₁	approx. 110 nM	+60% (200 nM GA ₁)	(Rizza <i>et al.</i> , 2017)
	GA ₃	approx. 240 nM	+40% (200 nM GA ₃)	(Rizza <i>et al.</i> , 2017)
	GA ₄	approx. 24 nM	+90% (200 nM GA ₄)	(Rizza <i>et al.</i> , 2017)

DETECTION OF PLANT HORMONES IN LIVING PLANT MATERIALS

To understand the physiological effects of plant hormones, it is important to determine the distributions and to quantify the concentrations of plant hormones with high spatiotemporal resolution, which is important for understanding the physiological responses of plant hormones. Fluorescence is an excellent tool for the minimally invasive monitoring of the distribution of bioactive molecules within intact cells, tissues and organs. Fluorescence can be effectively quantified and has femto- and nanosecond excitation and emission rates that allow fluorescence-based probes to faithfully record rapid processes with subcellular resolution. Combined with the development of increasingly sophisticated and sensitive imaging technologies, fluorescence can be used to non-destructively and directly visualize rapid cellular processes. Such fluorescence microscopy provides data with spatial and temporal resolution that is not possible with traditional biochemical methods. The ways that fluorescence can be used to detect plant hormones are discussed below.

FLUORESCENT TAGS AND ANALOGS OF PLANT HORMONES

Organic chemistry has contributed to the progress in plant hormone research (Rigal *et al.*, 2014). Fluorescent dyes can be conjugated to standard forms of plant hormones, and some analogs are naturally fluorescent. Fluorescent conjugates of auxin, 7-nitro-2,1,3-benzoxadiazole (NBD)-naphthalene-1-acetic acid (NAA) and NBD-indole-3-acetic acid (IAA) enabled the visualization of auxin transport dynamics and subcellular auxin distribution in *Nicotiana tabacum* (tobacco) BY-2 cells and *Arabidopsis thaliana* seedlings (Hayashi *et al.*, 2014). Although these fluorescent-labeled auxin analogs are not functional with respect to auxin signaling or metabolism, a fluorescent-labeled brassinosteroid (BR), Alexa Fluor 647-castasterone (AFCS), has been shown to be bioactive. AFCS was used to visualize endocytosis of the BR receptor in living *Arabidopsis* cells (Irani *et al.*, 2012). Bioactive fluorescein-labeled gibberellic acids (GA-fls) accumulated in the endodermal cells of the *Arabidopsis* root elongation zone, and the GA-FI accumulation was inhibited by ethylene signaling (Shani *et al.*, 2013). The strigolactone-agonist probe Yoshimulactone Green (YLG), which has activity similar to strigolactone, becomes fluorescent when hydrolyzed by the hydrolase activity of the strigolactone receptor, thereby enabling the visualization of the perception of applied strigolactone by *Striga* embryos (Tsuchiya *et al.*, 2015).

Peptide hormones have also been successfully labeled with fluorescent dyes to track their localization and uptake. For example, the elicitor-induced peptide pep1 of

Arabidopsis was N-terminally tagged with tetramethylrhodamine (TAMRA) and used to monitor binding to the PEPR receptors followed by clathrin-mediated endocytosis of peptide-receptor complexes (Ortiz-Moreno *et al.*, 2016). In a few cases, peptide hormones were monitored by genetically encoded peptide-FP fusions. Using such tools, the shoot-to-root translocations of C-TERMINALLY ENCODED PEPTIDE DOWNSTREAM 1 (CEPD1) and CEPD-like 2 (CEPDL2) were observed using *GFP-CEPD1* and *GFP-CEPDL2* reporters (Ohkubo *et al.*, 2017; Ota *et al.*, 2020), revealing the signaling pathway that integrates nitrate uptake in *Arabidopsis*. Large fluorescent moieties often interfere with peptide-receptor interactions and reduce bioactivity, however, or are unstable and are processed from the peptide.

The use of synthetic chemistry is an excellent tool that can provide new insights into plant hormone dynamics in living plants. It is noteworthy that these small molecules need to be applied exogenously and are not likely to represent the actual distribution or dynamics of the endogenous hormones, especially because of the differential recognition by transporters, receptors and degradation enzymes in the plant. In addition, often some chemical compounds cannot pass the cuticular layer at the plant surface, and it is difficult to force the entry of compounds into tissues by infiltration in a uniform manner, thereby limiting the reliable quantification of the compounds in the plant.

GENETICALLY ENCODED FLUORESCENT PROTEIN BIOSENSORS

Genetically encoded biosensors have been developed for better quantitative and less invasive imaging of specific plant hormones in genetically transformable plants such as *Arabidopsis*. Genetically encoded biosensors can be categorized as indirect and direct biosensors (Walia *et al.*, 2018). The responses of indirect biosensors is dependent upon changes in gene expression or protein degradation in response to changes in analyte (plant hormone) concentration (Walia *et al.*, 2018; Waadt, 2020). On the other hand, the responses of direct biosensors are dependent on conformational change of the biosensor protein itself (Okamoto *et al.*, 2012). In this section, we introduce different types of biosensors for plant hormone detection and general aspects regarding how they respond to the respective analytes.

Indirect biosensors include two major types: transcriptional reporter and degron-based reporter proteins (Figure 1). Transcriptional reporters consist of a promoter sequence with a conserved motif that is indirectly regulated by analyte concentration, through the activation of a transcription factor that acts within the hormone-dependent signal transduction cascade and controls the expression of a quantifiable reporter gene (Waadt, 2020).

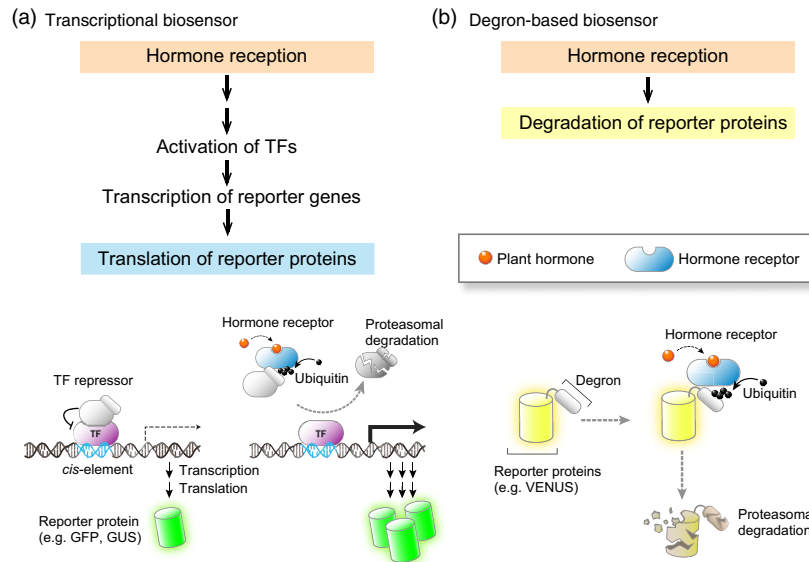


Figure 1. Models of indirect biosensors. Indirect sensors comprise transcriptional or degradation-based biosensors. (a) Typical mechanism commandeered for transcriptional reporters. Plant hormone perception by its receptor induces the proteasomal degradation of repressors for specific transcription factors (TFs). The activated TFs bind to specific sequence motifs (*cis*-elements) and induce the transcription of reporter genes such as GFP, GUS or LUC. The read-out of the transcriptional reporters depends on hormone reception, proteasomal degradation of the TF repressors, transcription, translation, and folding and activity of reporter proteins. Reporter activity can remain even after transcription returns to base levels after hormone levels drop, limiting the temporal resolution of transcriptional reporters; however, transcriptional sensors can be considered memory reporters as they will also report past activity. (b) Typical mechanism commandeered for degron-based biosensors. A reporter protein is fused with a degron. The reporter is active in the absence of hormone, but is rapidly ubiquitinated and degraded by the activities of a specific hormone receptor and the proteasome, respectively. The reporter–degron fusion protein is typically expressed under the control of constitutive and ubiquitous promoters. Therefore, the temporal resolution of the degron-based biosensors is more accurate compared with transcriptional reporters.

Although promoters generally respond to many different cues (Juven-Gershon and Kadonaga, 2010), using specific hormone-responsive *cis*-elements as components of artificial promoters can be effective in achieving high specificity for the hormone of interest. In many cases, a fragment of the cauliflower mosaic virus (CaMV) 35S promoter (minimal CaMV 35S promoter) will be combined with enhancer sequences and specific transcription factor (TF) binding motifs that bind hormone-controlled TFs (Waadt, 2020). Reporter proteins, such as β -glucuronidase (GUS), luciferase (LUC) and green fluorescent protein (GFP), as well as other fluorescent proteins (FPs), can be used to visually represent promoter activity in response to hormone levels (Walia *et al.*, 2018). The read-out of transcriptional reporters is affected by multiple parameters: (i) analyte-dependent activation/deactivation of transcription factors, and (ii) subsequent induction/attenuation of reporter gene expression. The temporal resolution of transcriptional reporters is in the range of minutes to hours (D'Agostino *et al.*, 2000; Brunoud *et al.*, 2012), as multiple steps, including transcription, translation, FP maturation and turnover of reporters affect the actual output. The spatial resolution of transcriptional reporters provides cellular-level resolution but not subcellular-level resolution.

Degron-based biosensors consist of a reporter protein, such as an FP, and a specific domain, the so-called degron.

Degron domains undergo ubiquitination, mediated by specific plant hormone receptors that are associated with SKP1-Cullin-F-Box (SCF) complexes and subsequent degradation by the proteasome (Santner and Estelle, 2010). Constitutive promoters such as CaMV 35S, *UBQ10* or *RPS5A* are typically employed for expressing degron-based biosensors across many cells and stages (Brunoud *et al.*, 2012; Wend *et al.*, 2013; Larrieu *et al.*, 2015; Liao *et al.*, 2015; Samodelov *et al.*, 2016). Therefore, degron-based biosensors typically have a faster temporal resolution (minutes until a degron-sensor response is detectable) compared to transcriptional reporters (estimated to >2 h until FP is formed) (Brunoud *et al.*, 2012). The temporal resolution of degron-based biosensors is determined by not only the transcription rate but also by the FP maturation time and degradation rate of the reporter protein. To maximize the temporal resolution, fast-maturing FPs such as VENUS (with a maturation time of approx. 18 min in *Escherichia coli*; Balleza *et al.*, 2018) are often chosen as reporters for degron-based biosensors.

Direct biosensors consist of a sensory domain that binds directly to a specific plant hormone and two spectral FP variants that function as Förster resonance energy transfer (FRET) donors and acceptors, respectively (Figure 2). The fluorescence read-out generated by direct biosensors is the result of conformational rearrangements in the sensory

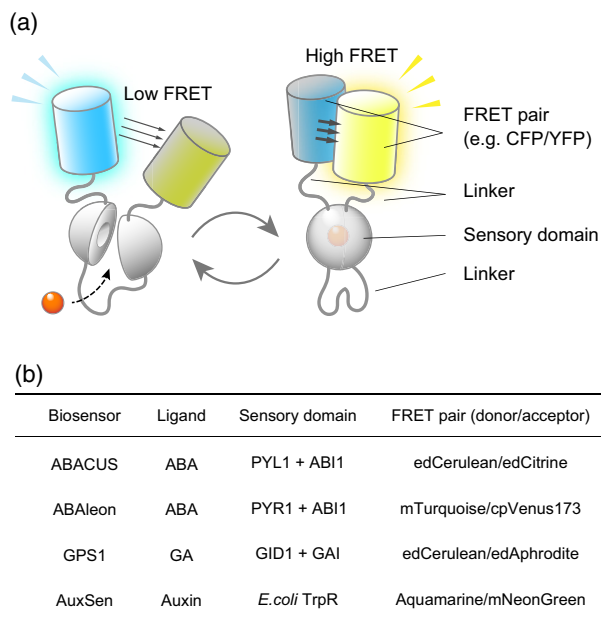


Figure 2. A model of FRET-based direct biosensors. ‘Direct biosensors’ directly bind the plant hormone and report this binding. (a) The typical structure of a FRET-based direct biosensor. The sensor generally consists of a sensory domain sandwiched by two fluorescent proteins (FPs) that differ in emission spectra and that act as a Förster resonance energy transfer (FRET) pair. Ligand binding causes a conformational rearrangement. The conformational change physically alters the distance and orientation between the two FPs and results in a readily detectable change in the emission ratio of the two FPs. Importantly, the FPs and the sensory domains are connected by flexible linkers, which influence the sensor activity. (b) A short list of FRET-based biosensors for plant hormones.

domain that affect the FRET of the sensor and not the abundance of the sensor protein (Walia *et al.*, 2018). Thus, direct biosensors provide rapidly reversible read-outs of plant hormone concentrations with high spatiotemporal resolution. As the read-out of direct biosensors relies on conformational protein dynamics, such sensors are not suitable for analyses of fixed plant specimens. For the engineering of direct biosensors, both endogenous plant hormone receptors and modified synthetic enzymes have been used for the sensory domain (Waadt, 2020). Specific examples are given in the next section.

PLANT HORMONE SENSORS

Abscisic acid

Abscisic acid (ABA) is a monocyclic sesquiterpene that plays key roles in diverse processes, such as seed development and dormancy, stomatal closure, abscission and responses to abiotic stresses in plants (Cutler *et al.*, 2010; Yoshida *et al.*, 2019). In the absence of ABA, protein phosphatase 2C (PP2C) represses the activity of SnRK2-type kinases via dephosphorylation. ABA signaling is initiated by the binding of ABA to a member of the PYL/PYR/RCAR

family and co-receptor PP2C-type protein phosphatases. The binding leads to the activation of a third protein, the SnRK2 protein kinase. SnRK2 then phosphorylates downstream proteins, including ABA-responsive element (ABRE)-binding transcription factors and ion channels. The distribution of ABA has successfully been detected by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) imaging (Shiono *et al.*, 2017) and transcriptional biosensors (Wu *et al.*, 2018). Transcriptional biosensors were developed that consisted of synthetic promoters, based on repeats of the ABRE elements, upstream of GFP fused with an endoplasmic reticulum (ER)-retention signal (erGFP) (Wu *et al.*, 2018). These 6xABRE reporters were used to characterize ABRE-mediated gene expression in the root under stress conditions. Induction of 6xABRE reporters was detected 4 h after treatment with 10 μM ABA in essentially all tissues. 6xABRE reporters have a detection range of at least 1 μM up to 25 μM of exogenously applied ABA. 6xABRE reporters were also induced by osmotic stress (50–150 mM NaCl or 100–300 mM mannitol) (Wu *et al.*, 2018).

To visualize the dynamics of ABA in living tissues, FRET-based direct biosensors, namely ABACUS and ABAlleon, were generated using the ABA perception machinery (Jones *et al.*, 2014; Waadt *et al.*, 2014; Waadt *et al.*, 2020). The sensory domain of ABACUS consists of PYL1 fused to the minimal ABA interaction domain of ABI1 (PP2C) as a recognition element that is sandwiched between the reporter elements edCerulean and edCitrine, as FRET donor and acceptor, respectively (Figure 2). The sensor was developed by testing a large number of variants (Jones *et al.*, 2014). Affinity variants with equilibrium dissociation constant (K_D) values of 2 μM and 80 μM have been engineered (Table 2). ABAlleon2.1 uses PYR1 fused to a truncated ABI1 as the sensory domain linked to mTurquoise and circularly permuted Venus (cpVenus) as FRET donor and acceptor, respectively. The detection range of ABAlleon2.1 was 100–600 nm with a K_D of 79 nM (Table 2). ABACUS showed an increasing emission ratio when ABA levels increased, whereas ABAlleon was characterized by high FRET in the absence of ABA and a decreased ratio when ABA was bound. It is noteworthy that the transgenic Arabidopsis lines expressing the ABA biosensors showed ABA-related phenotypes, however. The primary root growth and germination of ABACUS1 lines were hypersensitive to ABA. As the degree of sensitivity and the binding affinity of the ABACUS1 sensors were correlated, it is likely that the sensor interacts with the endogenous ABA signaling pathway, with effects similar to those found in PYL1 overexpression lines (Jones *et al.*, 2014). Although ABAlleon2.1 is sensitive enough to detect changes in endogenous ABA levels induced by abiotic stress, for example, the Arabidopsis lines that expressed high levels of ABAlleon2.1 and produced bright fluorescence were ABA hyposensitive. The

expression level of ABAlleon correlated with reduced sensitivity, indicating that the sensor scavenged cytosolic ABA (Waadt *et al.*, 2014). ABAlleon has been improved through the screening of deletion variants of ABAlleon2.15 and optimizing FRET pairs, sensory domains and linkers (Waadt *et al.*, 2020). The improved ABAlleon, ABAlleonSD1-3L21, used the amino acids LD and T as linkers between the FPs and sensory domains, instead of the amino acid pairs GP and PG in ABAlleon2.15. When Arabidopsis seedlings expressing ABA indicators were treated with 10 μM ABA, ABAlleon2.15 and ABAlleonSD1-3L21 showed faster responses with a half-life ($t_{1/2}$) of approx. 15 min, whereas ABACUS1-2 μ responded at a slower rate ($t_{1/2} \sim 29$ min).

Recently, another type of FRET sensor, an SnRK2 activity sensor (SNACS) was developed and used to monitor ABA accumulation (Zhang *et al.*, 2020). SNACS is composed of a 48-amino-acid sensory domain surrounding Serine-30 of the Arabidopsis ABA-RESPONSIVE KINASE SUBSTRATE 1 transcription factor (AKS1) and the full-length 14-3-3 protein GF14phi. In SNACS, the binding of the 14-3-3 protein to AKS1, which is phosphorylated by SnRK2, causes a conformational change and changes the FRET efficiency. The emission ratio of SNACS in Arabidopsis guard cells increased in a time-dependent manner after the addition of 20 μM ABA. ABAlleon2.15 and SNACS were both used to test whether CO₂ elevation directly activates ABA signaling; however, neither sensor showed any detectable change in emission ratio in guard cells in response to increasing CO₂ concentrations (Hsu *et al.*, 2018; Zhang *et al.*, 2020).

AUXIN

Auxins, with their key player indole acetic acid (IAA), are structurally related to the amino acid tryptophan and have roles in a wide range of developmental processes in plants, including morphogenesis, cell proliferation and organogenesis. The predominant endogenous auxin IAA is synthesized in proliferating tissues, such as embryos, shoot meristems and young leaves, from where it is transported to roots by polar auxin transport. Auxin gradients play central roles in many phases of plant pattern formation and development (Leyser, 2005; Möller and Weijers, 2009).

Transcriptional reporters, degron-based biosensors and FRET-based direct biosensors have been developed for monitoring auxin (Table 1). The widely used transcriptional reporter *DR5* was the first sensor to be generated, and *DR5*-based transcriptional reporters are still widely used today. The *DR5* promoter contains multiple auxin-response elements (AuxREs), which were first identified by promoter deletion analysis (Ulmasov *et al.*, 1997). The improved version, *DR5v2*, carries AuxREs with a higher affinity for auxin response factors (ARFs), and is thus more sensitive (Liao *et al.*, 2015). *DR5* and *DR5v2* reporters enabled the visualization of the distribution of auxin in embryos, in post-

embryonic root cells such as the quiescent center (QC), in the columella root cap and protoxylem cells, in the shoot apical meristem and in many other tissues. The two artificial *DR5* and *DR5v2* promoters responded to exogenously added auxin concentrations as low as 3 nM, but the reporter amplitude is higher for *DR5v2* compared with *DR5*. This difference in sensitivity may explain why *DR5v2* can detect auxin in a wider range of cell types, including the root metaxylem, pericycle, lateral root cap and root epidermal cells (Liao *et al.*, 2015). In addition to fluorescent proteins such as GFP, the *DR5* promoter has also been combined with GUS and LUC as reporters (Ulmasov *et al.*, 1997; Moreno-Risueno *et al.*, 2010; Van Norman *et al.*, 2013). *DR5*-based transcriptional reporters have been used in a variety of plant species (Pattison and Catalá, 2012; Galli *et al.*, 2015). Based on the conservation of AuxRE sequences (TGTCGG/CC/TC), in particular the pIAA motif, highly sensitive synthetic reporters have been engineered (Lieberman-Lazarovich *et al.*, 2019).

Auxin transport plays an important role in the control of cellular auxin levels. The auxin influx carrier AUX1 and the PIN-family of auxin efflux carriers, which are regulated by protein kinases, determine the level and direction of auxin transport. Therefore, auxin biosensors must have rapid kinetics to be able to follow rapid changes in auxin dynamics, e.g., for the analysis of auxin relocation during blue light phototropic or gravitropic responses. To visualize dynamic changes of auxin, the degron-based auxin reporter DII-VENUS was developed (Brunoud *et al.*, 2012). DII-VENUS uses domain II (DII) of the AUX/IAA repressor IAA28 fused to a variant yellow fluorescent protein (VENUS), which carries nuclear localization sequences (NLSs). DII is ubiquitinated by the auxin receptor TIR1/AFB in the presence of auxin, and this ubiquitination targets AUX/IAA for proteasomal degradation. The CaMV 35S RNA promoter has been used for constitutive expression of the DII-VENUS, such that the expression level of the biosensor should not be affected by auxin concentration changes. An auxin-insensitive variant, mDII-VENUS, with impaired TIR1/AFB interaction, can serve as a negative control.

To enable auxin detection in embryos and meristems, a similar approach was used to engineer the ratiometric auxin reporter R2D2. The R2D2 sensor places both RPS5A: DII-n3xVENUS and RPS5A:mDII-ntdTomato in a single transgene (Liao *et al.*, 2015). As an alternative for efficient quantification, DII of AtIAA17 fused to Firefly was fused to Renilla luciferase via the self-cleaving 2A peptide for normalization, named min17-Luc (Wend *et al.*, 2013). The sensor is ratiometric, as the relative luminescence of Firefly to Renilla luciferase enabled the quantification of the auxin levels in Arabidopsis protoplasts. Three types of sensor modules derived from three different Aux/IAAs provide a wide range of sensitivities and binding affinities for IAA and NAA.

Although degraon-based sensors are likely to be faster compared with transcriptional sensors and to integrate auxin signals over shorter time scales, sensors that transform a binding-induced conformational rearrangement into a change in fluorescence can be orders of magnitude faster and enable subcellular analyses. As the bacterial periplasmic binding protein superfamily covers a wide range of ions and metabolites, this class of proteins is widely used for engineering such sensors (Fukami-Kobayashi *et al.*, 1999). Our group could not identify periplasmic binding proteins that could recognize plant hormones but did make use of repressors that bind tryptophan. As tryptophan and auxin are structurally related, we engineered a sensor for tryptophan as a first step towards building an auxin sensor by subsequent modification of the substrate specificity (Kaper *et al.*, 2007). The Jürgens lab then used the tryptophan sensor FLIPW as a starting point for the screening of mutants that can recognize auxin with high selectivity. The resulting auxin sensor, AuxSen, was then used to measure auxin levels in protoplasts and roots of *Arabidopsis* (Herud-Sikimic *et al.*, 2020). A major advantage of using heterologous proteins as binding domains (here the bacterial Trp repressor) over plant endogenous proteins is that it is unlikely that a fully orthogonal protein domain interferes with plant signaling pathways.

BRASSINOSTEROIDS

Brassinosteroids (BRs) are polyhydroxylated sterol derivatives. BRs are involved in cell elongation, cell division, cell proliferation, seed germination and stress responses, in combination with other plant hormones (Zhu *et al.*, 2013). BR signaling is initiated by the binding of BR to the extracellular domain of the receptor kinase BRI1. Activated BRI1 transiently interacts with BAK1 and then disassociates. In the presence of BR, the GSK3-like kinase BIN1 is inactivated, which leads to the dephosphorylation of BRASSINAZOLE RESISTANT 1 (BZR1)-type transcription factors. Dephosphorylated BZR1 translocates to the nucleus, where it regulates BR-responsive gene expression. Therefore, it is assumed that BZR1 accumulation in the nucleus reports active BR signaling. To monitor the distribution of BR, BZR1-YFP fusions were expressed either from the BZR1 or the CaMV 35S promoter (Chaiwanon and Wang, 2015). BZR1-YFP accumulated to low levels in the nuclei of the stem cell region and at higher levels in the nuclei of epidermal cells in the transition and elongation zone of roots, which indicated a close correlation between rapid cell elongation and the level of BZR1 expression (Table 1). To our knowledge, direct BR biosensors have not been engineered or published yet.

CYTOKININS

Cytokinins are nucleobase or nucleoside analogs that play important roles in cell division and shoot and root

morphogenesis. Cytokinin distribution can be visualized by mass spectrometry-based imaging (Shiono *et al.*, 2017). Cytokinins are perceived by transmembrane two-component receptors that phosphorylate downstream mobile phospho-transmitters (To *et al.*, 2007). In turn, the phospho-transmitters phosphorylate nuclear transcription factors (such as the ARRs) that induce or repress cytokinin-responsive gene expression. To create a cytokinin reporter, the promoter region of ARR5 was fused to GUS or to fluorescent proteins (D'Agostino *et al.*, 2000). Although cytokinin signaling was known at this time to play essential roles in post-embryonic growth and development, its role during early embryogenesis was unclear. To visualize the cytokinin distribution in *Arabidopsis* embryos, a synthetic reporter, two-component-output-sensor (TCS), as well as an improved derivative, TCS-new (TCSn), were engineered. TCSn consists of 24 repeats of DNA-binding sites [(A/G)GAT(C/T)] for cytokinin-responsive transcription factors followed by a fluorescent reporter (Zürcher *et al.*, 2016) that is induced in *Arabidopsis* roots by external addition of *trans*-zeatin or isopentenyl-adenine (Liu and Müller, 2017). TCSn:GFP, TCSn:GUS and TCSn:VENUS:H2B have been used for the analysis of cytokinin responses and cytokinin distribution in *Arabidopsis*, *Oryza sativa* (rice), and *Hordeum vulgare* (barley), respectively (Tao *et al.*, 2017; Kirschner *et al.*, 2018). Although transcriptional cytokinin sensors are highly useful for monitoring cytokinin, a FRET sensor that directly detects the dynamics of cytokinins, and that may be able to differentiate between different cytokinins, might be useful in the future.

ETHYLENE

Ethylene is a gaseous hormone synthesized from methionine that affects plant growth, flower and fruit development, and senescence. Ethylene signaling is initiated by binding to one of the five ETR receptors in *Arabidopsis*, leading to the inactivation of the cytosolic CTR1 kinase, which derepresses EIN2, an ER-bound transmembrane protein. EIN2 is proteolytically cleaved upon ethylene perception, and the C-terminal fragment of EIN2 enters the nucleus to regulate EIN3/EILs transcription factor activities, resulting in the activation or repression of ethylene-responsive gene expression (An *et al.*, 2010; Qiao *et al.*, 2012). EIN3 is a transcriptional activator of ethylene-responsive genes. EIN3-GFP and EIL1-GFP were developed as sensors for ethylene. EIN3-GFP and EIL1-GFP accumulate in *Arabidopsis* seedlings in response to treatment with the ethylene precursor ACC (An *et al.*, 2010). To monitor the distribution of ethylene in plants, a reporter using GUS driven by the synthetic EIN3-responsive reporter EIN3-binding site (EBS) was developed (EBS:GUS) (Stepanova *et al.*, 2007). The distribution of ethylene was reported in the roots of *Arabidopsis* expressing *EBS:GUS* treated with ACC (Stepanova *et al.*, 2007).

In the absence of ethylene, EIN3 is degraded by proteolysis mediated by two F-box proteins, EBF1 and EBF2 (Guo and Ecker, 2003), whereas in the presence of ethylene, EIN3 is stabilized by the degradation of EBF1 and EBF2 induced by ethylene signaling (An *et al.*, 2010). Degradation of EBF1/2 upon ethylene signaling is induced through the recognition of its 3' untranslated region (3'-UTR) being recognized by the C terminus of EIN2 (Merchante *et al.*, 2015; Li *et al.*, 2015). Using the 590-bp 3'-UTR of EBF2 (3'EBF2) and an ethylene-responsive RNA element containing poly-uridylates (EPU) from the 3'-UTR of EBF1, translational regulation probes such as FP-3'EBF2 and FP-6x EPU were developed (Merchante *et al.*, 2015; Li *et al.*, 2015), enabling the monitoring of ethylene dynamics in plant tissues (Fernandez-Moreno and Stepanova, 2020).

As an alternative ethylene detection technology, the enzyme-based chemical biosensor artificial-metalloenzyme ethylene probe (AEP) was developed (Vong *et al.*, 2019). AEP was designed to have an albumin scaffold that would solubilize and protect a metal complex. The metal complex consists of the fluorophore 7-diethylaminocoumarin (DEAC), the second-generation Hoveyda–Grubbs (HG) catalyst and the olefin-containing DABCYL quencher. In the absence of ethylene, the DABCYL quencher suppresses DEAC fluorescence by FRET interaction. When the AEP is exposed to ethylene, cross-metathesis catalyzed by HG releases DABCYL, yielding DEAC fluorescence. This has been applied to the detection of ethylene gas in fruits and Arabidopsis leaves (Vong *et al.*, 2019).

GIBBERELLINS

Gibberellic acids or gibberellins (GAs) belong to the diterpene family. GAs come in many different varieties: some are precursors, whereas others are active or inactive. GAs are involved in seed germination, stem elongation, fruit growth and floral development. GA signaling is initiated through the perception of GA by *GID1* accompanied by *DELLA* proteins in Arabidopsis (Murase *et al.*, 2008). *DELLA* proteins, such as *GAI* and *RGA*, act as repressors of GA-responsive gene expression. When interacting with GA, the *GID1*–*DELLA* complex recruits an E3 ubiquitin ligase to target the complex for degradation, which induces the expression of GA-responsive genes. This ubiquitin-dependent degradation mechanism, which is analogous to the auxin receptor, was used to engineer the degron-based GA biosensor, GFP-RGA (Silverstone *et al.*, 2001). GFP-RGA carrying a nuclear targeting sequence and under the control of the *CaMV 35S* promoter was expressed, and the GFP-RGA sensor showed decreased fluorescence 30 min after the addition of 100 μM GA_3 (Table 1).

The FRET-based GA sensor GPS1 was developed for the high-resolution quantification of spatiotemporal GA distribution (Rizza *et al.*, 2017). GPS1 is composed of domains from the Arabidopsis *GID1* receptor *AtGAI* as sensory

domains. The GA biosensor was optimized by a screening of ligand sensory domains fused to pairs of FRET donor/acceptor variants. Three Arabidopsis *GID1* proteins (*AtGID1A*, *AtGID1B* or *AtGID1C*) and two *DELLA* family proteins (*AtGAI* or *AtRGA*) were tested as sensory domains with five different flexible linkers and CFP and YFP variants (Figure 3). The optimized GPS1 consists of *edAphrodite* (*edAFP*) at the N terminus and *edCelurean* at the C terminus as FRET donor and acceptor, and with the truncated 74-amino-acid *AtGAI* (D28–D101) and the *AtGID1C* linked via a 12-amino-acid linker (L12) as the sensory domains. GPS1 responds to nanomolar concentrations of GA_4 . The K_D values of GPS1 for GA_1 , GA_3 and GA_4 were 110, 240 and 24 nM, respectively (Tables 1 and 2). The binding to GPS1 was partially reversible for GA_1 and GA_3 , but not for GA_4 . To assess the GA distribution *in planta*, a nuclear-targeted variant of GPS1 (*nlsGPS1*) was stably expressed under the control of the ubiquitous constitutive *p16* promoter in Arabidopsis. GPS1 emission was detected in individual cells of both roots and shoots. In seedling roots, a gradient of GA was observed, with a low *nlsGPS1* emission ratio in the apical cell division zone that transitioned to increasing *nlsGPS1* emission ratio in the elongation zone. *nlsGPS1* also showed a higher concentration of GA in the elongation zone of dark-grown hypocotyls. Furthermore, the sensor was successfully used to evaluate hormone levels in Arabidopsis mutants. The FRET-based GPS1 sensor was successfully used to monitor GA levels and investigate the quantitative relationship between the distribution of gibberellin and cell elongation (Rizza *et al.*, 2017).

JASMONIC ACID

Jasmonic acid (JA) is a product of the octadecanoid pathway (Turner *et al.*, 2002). The amino acid conjugate *Ja-Ile* is the bioactive form responsible for signaling (Ruan *et al.*, 2019). JA is involved in plant responses to abiotic/biotic stresses, plant growth and development (Turner *et al.*, 2002). Similar to the case of the auxin and gibberellin signaling pathways, JA is also perceived by a two-component receptor that is part of an SCF complex and is involved in the degradation of downstream effectors like *JAZ*. At low JA levels, *JAZ* represses transcription factors that affect JA-responsive genes. *Ja-Ile* promotes the binding of *JAZ* to the F-box protein *COI1*, leading in turn to the ubiquitination of *JAZ* and the degradation of the complex. As a result, transcription factors of JA-responsive genes are released from repression (Chini *et al.*, 2007; Thines *et al.*, 2007). A JA degron sensor was developed on the basis of the JA-dependent degradation of *JAZ* protein (Larrieu *et al.*, 2015). A specific *Jas* motif in the *JAZ* proteins is responsible for its targeting for degradation. The *Jas* motif of *AtJAZ9* protein was fused to Venus-NLS driven by the *CaMV 35S* promoter (*Jas9-VENUS*). The degradation of

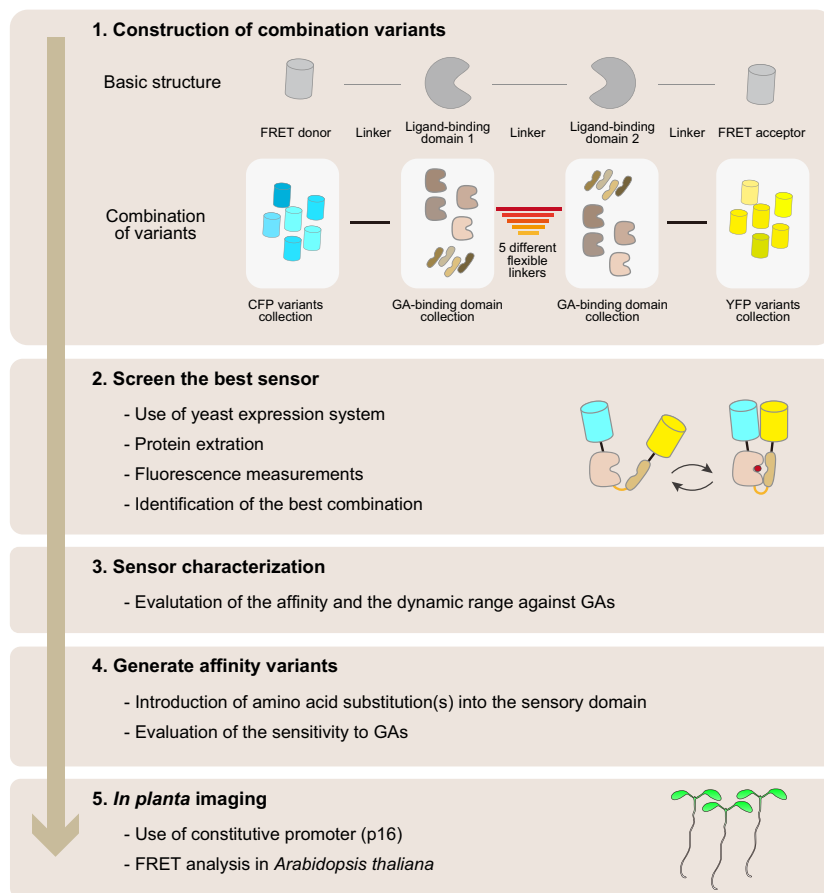


Figure 3. Flow chart for biosensor engineering. We present the engineering procedure for a plant hormone biosensor, in this instance the gibberellin (GA) sensor GPS1 (Jones *et al.*, 2014; Rizza *et al.*, 2017). The biosensor was created by combining domains of different bipartite GA receptors through an artificial linker and flanking the ligand-binding domains with potential Förster resonance energy transfer (FRET) pairs. Linkers and collections of fluorescent protein (FP) variants were tested as FRET pairs and compared in high-throughput yeast expression assays. Candidate GA-binding domains were selected from the receptors AtGID1A, AtGID1B and AtGID1C, and truncated AtGAI or AtRGA domains (DELLA protein domain). The GA-binding domains were linked to each other by different linkers. The combinatorial library was constructed using Gateway® cloning technology, introduced into a protease-deficient yeast strain, expressed and isolated. The best five-way combination was screened by *in vitro* fluorescence measurements of the sensors isolated from yeast. Affinity mutants with reduced or no response to GAs were engineered by rational design. The affinity mutants can be used as negative controls during *in vivo* analysis to exclude artifacts such as those caused by pH changes. GPS1 and the affinity mutant GPS1-NR were expressed in *Arabidopsis* plants under the control of a constitutive promoter (p16).

Jas9-VENUS depends on COI1. Jas9-VENUS was used to measure fluorescence in roots after leaf injury and to investigate the long-distance JA signaling in *Arabidopsis* seedling (Larrieu *et al.*, 2015)(Table 1).

STRIGOLACTONES

Strigolactones (SLs) are a diverse group of plant hormones that share a common core: a tricyclic lactone coupled to a hydroxymethyl butanolide (Waters *et al.*, 2017). SLs are involved in branching inhibition and root development, and also function as signaling molecules that induce symbiosis and parasitic interactions. SL signaling is initiated by binding to the α/β -hydrolase D14/AtD14 (Umehara *et al.*, 2008). SL-binding to D14 leads to the formation of a co-receptor complex comprised of the F-box protein D3/MAX2 and target regulator proteins in the D53/SMXL family (Zhou *et al.*, 2013; Waters *et al.*, 2017). D3/MAX2 engages an SCF complex, leading to the proteolytic degradation of the D53 protein (Waters *et al.*, 2017). Using this SL-triggered degradation mechanism, degron-based sensors were developed. In rice roots, D53-GFP enabled the visualization of D53 degradation in response to the addition of 5 μM of the synthetic SL *rac*-GR24 (Zhou *et al.*, 2013). In *Arabidopsis* roots, the degradation of SMXL6-YFP and

SMXL7-YFP were also induced by *rac*-GR24 treatment (Bennett *et al.*, 2016; Liang *et al.*, 2016). Another degron-based SL sensor is the ratiometric StrigoQuant (Samodelov *et al.*, 2016). StrigoQuant was developed with the same structure as min17-Luc, the ratiometric sensor for auxin, using SMXL6, a homolog of rice D53, as the sensor module instead of the auxin-responsive DII. Other ratiometric SL biosensors, namely rDAD2cpGFP and rShHTL7cpGFP, incorporate circularly permuted GFP (cpGFP) with the SL receptors DAD2 from *Petunia hybrida* (D14 in rice) or HTL7 from *Striga hermonthica* (Chesterfield *et al.*, 2020). The cpGFP can report conformational rearrangements induced by the ligand that cause a change in fluorescence intensity. LSSmOrange was fused to the C terminus of DAD2cpGFP and ShHTL7cpGFP via a rigid linker as reference controls. These ratiometric sensors have been used in tobacco protoplasts (Chesterfield *et al.*, 2020).

PEPTIDE HORMONES

Peptide hormones are small signaling peptides such as phytosulfokine (PSK) and CLAVATA 3 (CLV3) (Murphy *et al.*, 2012). Most known peptide hormones interact with receptors that assemble into heteromeric complexes upon peptide addition. Such induced receptor interactions can

be visualized by FRET between appropriately engineered fusions of receptors and FPs. The use of a peptide receptor as a sensor was conceptually shown using the well-established interaction of the immune receptor FLS2 with its co-receptor BAK1, which is triggered by the presence of the flg22 peptide. Upon peptide addition to *Nicotiana benthamiana* leaf cells expressing FLS2-mCherry and BAK1-GFP, rapid changes in fluorescence lifetime and fluorescence anisotropy indicated the peptide-dependent induction of the assembly of heteromeric and homomeric receptor complexes with high spatial resolution (Somssich *et al.*, 2015). The functionality of this approach has not yet been confirmed in transgenic Arabidopsis plants, however, and improvements in the design of such complex sensor systems may be required to achieve the necessary sensitivity. Furthermore, many peptides do not induce receptor complex assembly but complex rearrangements or redistribution into nanoclusters at the membrane, thus generating a technically more demanding output for further analysis (Bücherl *et al.*, 2017).

OTHER HORMONES

Karrikins (KARs) are butenolides found in wildfire smoke that stimulate seed germination (Nelson *et al.*, 2009). KARs are also involved in photomorphogenesis, root hair development, root density and drought responses. The KAR signaling mechanism is apparently highly similar to that for SLs. The putative KAR receptor, KARRIKIN INSENSITIVE 2 (KAI2), is a homolog of D14 (Waters *et al.*, 2012). In Arabidopsis, KAR signaling is mediated by KAI2, MAX2, SMAX1 and SMXL2, which increase the expression of *D14-LIKE2* (*DLK2*). The first developed transcriptional KAR reporter is *DLK2:LUC* (Sun *et al.*, 2016). Arabidopsis seeds carrying *DLK2:LUC* are incubated with bioactive KARs and the cell lysate can be used for a LUC assay. In this system, the application of 10 nM KAR₂ and 1 μM KAR₁ led to a four-fold increase in luciferase activity after 24 h (Table 1). Recently, a degron-based ratiometric KAR/KL biosensor was developed based on the SMAX1 degradation mechanism (Khosla *et al.*, 2020).

The development of biosensors for other plant hormones, such as salicylic acid (SA) and nitric oxide (NO), is also of major interest. For the detection of SA, a bacterial reporter strain was engineered by inserting a luciferase gene into the chromosome between *salA* and *salR* (*Acinetobacter* sp. ADPWH_1ux). The expression of *salA-luxCDABE-salR* responds to SA. This reporter strain was successfully used to visualize the spatiotemporal distribution of SA in leaves of plants during virus infection (Huang *et al.*, 2006). NPR1-GFP, which is known to migrate from the cytoplasm to the nucleus in response to SA accumulation (Mou *et al.*, 2003), has also been used as an SA reporter. The reporter indicated that defense responses induced by lipopolysaccharides result in SA accumulation in the

leaves (Sun *et al.*, 2012). For NO, several FP-based sensors, including those using FRET, have been developed for mammalian cells, but none has been tested in plants thus far (Eroglu *et al.*, 2018).

HORMONE TRANSPORTER ACTIVITY REPORTERS

The FRET and cpFP-based sensors make use of conformational rearrangements in a binding protein or receptor. We hypothesized that as transport proteins undergo complex rearrangements during the transport cycle, the concepts used for engineering binding protein-based sensors could be used to build reporters for the activity of specific transporters (or enzymes). The approach was successfully used to construct reporters for ammonium and nitrate transport (AmTrac, Amtryoshka and NiTrac) (De Michele *et al.*, 2013; Ho and Frommer, 2014; Ast *et al.*, 2017). Evidence has been provided that members of the CHL1/NRT1/NPF family, which include the nitrate transporter used to build NiTrac, are able to transport a wide range of hormones (Krouk *et al.*, 2010; Chiba *et al.*, 2015; Michniewicz *et al.*, 2019). It is therefore conceivable that NiTrac could also respond to IAA, and that other hormone transporters could be used to engineer transport activity reporters.

FUTURE IMPROVEMENTS THROUGH ENGINEERING THE BIOSENSORS

Sensors that exploit FRET between two spectral variants were the first reporters used to create sensors for calcium and many metabolites (Okumoto *et al.*, 2012). In the simplest form a binding protein was sandwiched between the two fluorophores, as in the prototype maltose and glucose sensors (Fehr *et al.*, 2002; Fehr *et al.*, 2003; Okumoto *et al.*, 2005). Notably, in these sensors, bacterial proteins were used that have a low likelihood of interacting with endogenous plant proteins, thus avoiding artifacts that could occur when using endogenous proteins as recognition elements for sensor construction. Ligand-induced conformational rearrangements change the relative emission of chimeric sensors. Typical FRET sensors have detection ranges of about two orders of magnitude and a rather limited signal-to-noise ratio (SNR). A major advantage of using bacterial periplasmic binding proteins rather than endogenous receptors is that periplasmic binding proteins typically have a high affinity for the ligands, allowing for the rapid development of a series of affinity mutants to cover a broad detection range. Miyawaki and Tsien's original calcium sensor used an endogenous calmodulin combined with an endogenous calcium-calmodulin binding protein as a means to increase the conformational rearrangement (Miyawaki *et al.*, 1997). Later on, they improved the sensor by introducing orthogonal mutations to avoid interaction with the endogenous machinery. A second approach, also developed by Tsien's lab, was the use of a

Matryoshka ratiometric direct biosensor

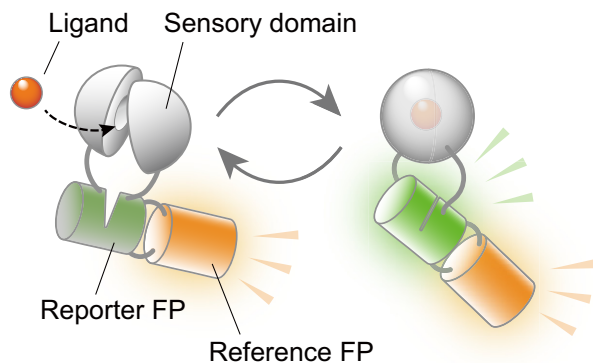


Figure 4. Further optimization of genetically encoded hormone biosensors. A model of a ratiometric direct biosensor. The sensory domain is linked to an environmentally sensitive circularly permuted fluorescent protein (cpFP) that carries an inserted reference FP (like a Matryoshka or nested doll) (Ast *et al.*, 2017). Ligand binding brings the reporter FP components back together and increases the fluorescence. A green–orange (GO) Matryoshka cassette composed of cpsfGFP and LSSmOrange can be used for engineering ratiometric sensors that make use of the large change in intensity caused by analyte binding in the cpFP.

circularly permuted fluorescent protein (cpFP) as a reporter (Baird *et al.*, 1999; Akerboom *et al.*, 2009). These intensimetric sensors often have a much higher sensitivity than other FRET sensors, yet they are sensitive to the changes in sensor levels that arise from promoter activity variability or differential degradation. Recently, Matryoshka technology was developed to convert the intensimetric sensors into ratiometric sensors (Figure 4) (Ast *et al.*, 2017). As an alternative, the fusion of a second FP was used to achieve a comparable result, although specific advantages such as simultaneous excitation and single-step insertion are not possible in this case (Waadt *et al.*, 2017).

As all these sensors make use of fluorescent proteins and operate on the principle of conformational changes in a recognition element, it is worth noting that the conformation of the recognition element and the fluorescent proteins are sensitive to many other parameters, such as ionic strength, pH and redox status (Hochreiter *et al.*, 2015). The maturation time of donor and acceptor fluorescent proteins can also vary the fluorescent read-out (Liu *et al.*, 2018). Affinity mutants are thus important and useful controls for excluding artifacts. Surprisingly, affinity mutants have so far been rarely used in studies and only in the animal/medical field. The selectivity of ligands for a sensory domain should also be considered, because binding proteins have often been tested with only a few analytes. This is especially important for hormones such as GA that exist as many different analogs. We could expect that hormone

sensors could be fine-tuned for each individual analog. Mutations used to create such variants are often in the binding pocket and can generate both high-affinity and low-affinity sensors (Jones *et al.*, 2014), which may impart ligand selectivity to the sensors. An extensive structure–activity relationship (SAR) analysis enables a better interrogation of the ligand–receptor binding machinery (Rigal *et al.*, 2014). If the ligand affinity is too high, the binding protein may interfere with endogenous signaling machinery. This might be avoided if orthologous systems are employed. Although biosensors represent minimally invasive tools, they can have effects on the cell, either by acting as scavengers or by interacting with other cellular components. They thus essentially represent an ‘observer effect’ problem (Buks *et al.*, 1998). From another perspective, such a drawback can be turned into an advantage. The binding protein can be deployed intentionally as a molecular ‘sponge’ that sequesters a ligand (Armbruster *et al.*, 2020), providing an effective tool for the functional analysis of signal transduction. In the absence of even less-invasive technologies, it will be important to keep this in mind and to perform proper controls. For example, when a glucose sensor was used in yeast, the analysis of growth curves and even flux analysis did not reveal any significant impact of the sensors on metabolism (Bermejo *et al.*, 2010), whereas some surface-displayed glutamate sensors clearly impacted processes that led to defects in growth and development (Castro-Rodríguez *et al.*, 2020), and some others did not have any clear impact (Toyota *et al.*, 2018). Affinity mutants are ideal controls, as they often differ only in a single amino acid, and should respond differently if the detected ratio change is caused by a change in the analyte concentration.

Genetically encoded sensors can be targeted to specific compartments, or even attached to specific proteins. For example, a calcium sensor can be attached to a calcium channel to obtain insights into calcium dynamics in the vicinity of the channel. Although a cytosolic biosensor does not require a targeting sequence, it is not always easy to visualize analytes and detect them separately in each cell, as many plant cells carry large vacuoles, squeezing the cytoplasm towards the cell periphery. Nuclear targeting can help with the identification and tracking of individual cells (Rizza *et al.*, 2017), which provides an option for multi-dimensional imaging.

Transcriptional sensors report the result of a treatment and thus report on past activity. As some reporter molecules, like GUS, are extremely stable in living cells, the reporter integrates over comparatively long periods, which can increase sensitivity. By contrast, most genetically encoded binding-dependent sensors report analyte changes with high temporal and even subcellular spatial resolution. For instance, a new type of genetically encoded sensor was developed that provides activity snapshots

within the brain (Fosque *et al.*, 2015). The prototype for such activity snapshot sensors is CaMPARI. CaMPARI carries a photoswitchable FP. When calcium levels increase in a particular cell, the FP in CaMPARI can be red-shifted only when a calcium elevation coincides with user-controlled violet light. CaMPARI has been used successfully in zebrafish, flies and mice. No such sensor for hormones has been reported so far, but the generation of hormone sensors that make use of photoswitchable FPs or Matryoshka variants may provide a promising starting point for engineering sensors that can take hormone activity snapshots.

IMAGING TECHNOLOGIES FOR THE QUANTIFICATION OF SENSOR OUTPUT

Initially, epifluorescence microscopy in conjunction with high-quality electron-multiplying CCD (EMCCD) cameras and simple perfusions systems were used for the quantitative imaging of sensor responses. These systems use either fast switching between multiple excitation and multiple emission wavelengths or devices for image splitting or multiple cameras. Extensive protocols have been published for FRET sensor quantification in yeast and animal cells and contain important information for establishing such imaging systems (Hou *et al.*, 2011; Bermejo *et al.*, 2011). Although confocal microscopy is generally considered to be more advanced, it bears some risks, such as when treatment with an osmotically active substance affects the cell diameter in the z-axis and causes a shift of the focal plane to a different region of the cell. By contrast, epifluorescence integrates over the whole z-axis and can provide information if z-stacks are generated. There are possible artifacts that arise from differences in the thickness of the organs. For example, in roots, one can observe single cells in a single layer in the periphery, but there are multiple tissues overlaying each other in the z-axis in the center. The adoption of nuclear-targeting biosensors may overcome these difficulties.

Plants sense and respond to mechano-stimulation by synthesizing plant hormones and other chemicals (Chehab *et al.*, 2008). As a similar response occurs in plants stimulated by more subtle mechanical cues, such as touch, technological advances for eliminating physical contact are needed. The initial development of a new imaging platform made use of small baths created with Play-Doh and peristaltic pumps (Deuschle *et al.*, 2006; Chaudhuri *et al.*, 2008; Chaudhuri *et al.*, 2011). Such systems can provide substantial throughput, but are typically limited to single seedlings and require substantial set-up time for each seedling. The RootChip, a microfluidic chip platform, allows root growth in a controlled environment on the microscope (Grossmann *et al.*, 2011; Grossmann *et al.*, 2012). Combined imaging using FRET sensors with the RootChip can achieve the non-invasive real-time detection of metabolites such as cytosolic zinc and calcium levels and fluxes in Arabidopsis roots

(Lanquar *et al.*, 2014; Stanley *et al.*, 2018). Although RootChip can provide opportunities to observe the distribution and dynamics of plant hormones in roots, similar imaging platforms must be developed for other tissues.

Another major goal of plant hormone research is to achieve four-dimensional imaging, which can analyze dynamics in an entire cell or tissue over time. Over the last 10 years, light-sheet fluorescence microscopy (LSFM) has contributed substantially to the emerging field of real-time visualization of complex developmental processes. As a result of the low photo-toxicity and high-speed multiview acquisition, LSFM has proven to be a powerful tool for the study of organ morphogenesis and function in zebrafish, *Drosophila* and other model organisms. Although still in its infancy, LSFM is increasingly being used in plant science (Grossmann *et al.*, 2018). Recently, FRET sensors were used with LSFM to monitor the dynamics of MgATP²⁻ in root hairs (De Col *et al.*, 2017) and intracellular calcium release during root or root hair growth (Costa *et al.*, 2013; Candeo *et al.*, 2017). LSFM does still have some limitations in sample conditioning, however, as a result of the spatial arrangement between the lens that produces the single-plane illumination and the objective lens that is used for imaging. Despite these limitations, LSFM imaging technology has the potential to significantly advance the real-time quantitative imaging of hormones in living plants.

OUTLOOK

Plant hormones are highly dynamic and interact in complex ways with each other. The relationships between these dynamics and their physiological phenotypes regulated by plant hormone signaling are not fully understood. Different approaches to follow this dynamism have been undertaken. Imaging mass spectrometry has been developed as a powerful method to detect the distribution of multiple hormones simultaneously (Shiono *et al.*, 2017; Shiono and Taira, 2020), but, for now, the spatial resolution of imaging mass spectrometry is comparatively low. As a complementary technology, biosensors that provide extremely high spatiotemporal information are in great demand. In animal research, the trend is shifting to biosensors that are based on fluorescence lifetime changes (Greenwald *et al.*, 2018). Although not intuitive, this requires the complete redesign of sensors for optimization to the lifetime of fluorescence. Although many 'FRET sensors' appear to carry out non-radiative energy transfer from the donor to the acceptor fluorophore, as evidenced by the emission of the FP with the longer wavelength that is not excited directly, the change seen in the ratio is not necessarily a result of FRET. For example, for the FLIPglu FRET glucose sensors, we have not detected a glucose-induced change in donor FP lifetime. Thus, we do not recommend using terms such as FRET channel or axis labels such as FRET change, as scientists rarely measure absolute

FRET values, but instead measure ratios. NiTrac is a good example of an unexpected although useful effect, i.e., a change in donor quenching or absorption in the sensor caused by the addition of nitrate substrate (Ho and Frommer, 2014). Pioneering work in animal systems conducted by the Zhang lab has enabled the multiplexing and simultaneous recording of many processes, in part through differential subcellular targeting and in part through the development of fluorescence-lifetime imaging (FLIM) sensors (Greenwald *et al.*, 2018). Recently, multiparametric imaging analysis has been achieved by 2-in-1 genetically encoded fluorescence indicators (GEFIs), consisting of two GEFIs fused via a 14-amino-acid linker or the self-cleaving 22-amino-acid P2A linker (Waadt *et al.*, 2020). The type of biosensor, its ligand affinity and the dynamic range should be selected based on the characteristics of the phenomenon to be observed in the plants. The recent intensely active development of plant hormone sensors shows the importance of biosensors, but this field is still at the frontier. Matryoshka technology may help to advance the detection of hormone dynamics in plant cells. Understanding complex hormonal networks with temporal and spatial information is the critical step to a comprehensive understanding of plant hormone science.

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AUTHOR CONTRIBUTIONS

RI, WBF and MN planned the article. All authors wrote the article and prepared the figures.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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