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Article type : SI Phytohormones 2021

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

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/tpj.15096](https://doi.org/10.1111/tpj.15096)

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Abstract

Plant hormones play important roles in plant growth and development, 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 quantitatively 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, and degradation sensors, luciferase and fluorescent sensors - and compare the tools and their suitability for different purposes.

Keywords: Absciscic acid, auxin, biosensor, cytokinin, ethylene, fluorescence, gibberellin, jasmonic acid, karrikin, nitric oxide, peptides, salicylic acid, strigolactone

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 is not entirely understood. 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). However, the need to fix and section the material limits spatial and temporal resolution. Mass spectrometry combined with gas or liquid chromatography have 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 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.

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 the high spatiotemporal resolution is of importance for understanding the physiological responses of plant hormones. Fluorescence is an excellent tool for 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 recording of 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 analogues 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, 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 visualization of auxin transport dynamics and subcellular auxin distribution in tobacco BY-2 cells and *Arabidopsis thaliana* seedlings (Hayashi *et al.*, 2014). While these fluorescent-labeled auxin analogues are not functional with respect to auxin signaling or metabolism, a fluorescent-labeled brassinosteroids (BR), Alexa Fluor 647-castasterone (AFCS), had been shown to be bioactive. AFCS was utilized to visualize endocytosis of the BR receptor in living *Arabidopsis* cells (Irani *et al.*, 2012). Bioactive fluorescein-labeled gibberellic acids (GA-FIs) 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 visualization of the perception of applied strigolactone by *Striga* embryos (Tsuchiya *et al.*, 2015).

Peptide hormones have also been successfully labelled with fluorescent dyes to track their localization and uptake. For example, the elicitor-induced peptide pep1 of *Arabidopsis* was N-terminally tagged with TAMRA and used to monitor binding to the PEPR receptors followed by clathrin-mediated endocytosis of peptide-receptor complexes (Ortiz-Morea *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 (CEPD) 1 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*. However, large fluorescent moieties often interfere with peptide-receptor interactions and reduce bioactivity, or are unstable and processed from the peptide.

The use of synthetic chemistry is an excellent tool that can provide new insights into the plant hormone dynamics in living plants. It is noteworthy that these small molecules need to be applied exogenously and likely do not represent the actual distribution or dynamics of the endogenous hormones, especially due to differential recognition by transporters, receptors and degradation enzymes in the plant. In addition, some chemical compounds can often not pass the cuticular layer at the plant surface, and it is difficult to force entry of compounds into tissues by infiltration in a uniform manner, limiting 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, responses of direct biosensors are dependent on conformational change of the biosensor protein itself (Okumoto *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 the analyte concentration through activation of a transcription factor that acts within the hormone-dependent signal transduction cascade, and controls expression of a quantifiable reporter gene (Waadt, 2020). Although promoters generally respond to many different cues (Juven-Gershon and Kadonaga, 2010), utilizing 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 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 the promoter activity in response to hormone levels (Walia *et al.*, 2018). The readout of transcriptional reporters is affected by multiple parameters: (i) analyte-dependent activation/deactivation of transcription factors and subsequent (ii) induction/attenuation of reporter gene expression. Temporal resolution of transcriptional reporters is in the range of minutes to hours (D'Agostino *et al.*, 2000; Brunoud *et al.*, 2012), since 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* promoters are typically employed for expressing the 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 compared to transcriptional reporters (for transcriptional reporter, estimated to >2 hours; for transcriptional reporter until FP is formed, at least several minutes are required before a degron-sensor response is detectable) (Brunoud *et al.*, 2012). Temporal resolution of degron-based biosensors is determined by not only transcription rate but also FP maturation time and degradation rate of the reporter protein. To maximize temporal resolution,

fast-maturing FPs such as VENUS [maturation time: ~18 min in *E. 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 of two spectral FP variants that function as Förster Resonance Energy Transfer (FRET) donors and acceptors, respectively (Figure 2). The fluorescence readout generated by direct biosensors is caused by conformational rearrangements in the sensory domain that affect the FRET of the sensor and not on the abundance of the sensor protein (Walia *et al.*, 2018). Thus, direct biosensors provide rapidly reversible readouts of plant hormone concentrations with high spatiotemporal resolution. Since the readout 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

Absciscic Acid

Absciscic 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 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), were developed and named (Wu *et al.*, 2018). These 6xABRE reporters were used to characterize ABRE-mediated gene expression in the root under stress

condition. Induction of 6xABRE reporters was detected 4 h after treatment with 10 μ M ABA in essentially all tissues. 6xABRE reporters have a detection range of at least 1 μ M up to 25 μ M 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 (protein phosphatase type 2C; 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 K_d values of 2 μ M and 80 μ M 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, while ABAlleon was characterized by high FRET in the absence of ABA and a decreased ratio when ABA was bound. However, it is noteworthy that the transgenic *Arabidopsis* lines expressing the ABA biosensors showed ABA-related phenotypes. Primary root growth and germination of ABACUS1 lines were hypersensitive to ABA. Since 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 for example by, for example, abiotic stress, 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 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 μ M ABA, ABAlleon2.15 and ABAlleonSD1-3L21 showed faster responses with $t_{1/2}$ ~15 min, while ABACUS1-2 μ responded at a slower rate ($t_{1/2}$ ~29 min).

Recently, another type of FRET sensor, a 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 SUBSTRATE1 transcription factor (AKS1) and the full-length 14-3-3 protein GF14phi. In SNACS, binding of the 14-3-3 protein to AKS1, which is phosphorylated by SnRK2, causes a conformational change and changes FRET efficiency. The emission ratio of SNACS in Arabidopsis guard cells increased in a time-dependent manner after addition of 20 μ M 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 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 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 to 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 amount 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 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 (NLS). DII is ubiquitinated by the auxin receptor, TIR1/AFBs 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/AFBs 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 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.

While degran-based sensors are likely faster compared to transcriptional sensors and 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. Since 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 repressor that bind tryptophan. Since tryptophan and auxin are structurally related, we engineered a sensor for tryptophan as a first step towards building of 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 (BR) are polyhydroxylated sterol derivatives. BRs are involved in cell elongation, cell division, cell proliferation, seed germination, and stress response in combination with other plant hormones (Zhu *et al.*, 2013). BR signaling is initiated by 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 dephosphorylation of BRASSINAZOLE RESISTANT1 (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 CaMV 35S promoter (Chaiwanon and Wang, 2015). BZR1-YFP accumulated to low levels in 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 postembryonic 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 utilized for analysis of cytokinin responses and cytokinin distribution in Arabidopsis, rice, and barley, respectively (Tao *et al.*, 2017; Kirschner *et al.*, 2018). Although transcriptional cytokinin sensors are highly useful for monitoring cytokinin, a FRET sensor, which directly detects the dynamics of cytokinins and which 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 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 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), while in the presence of ethylene, EIN3 is stabilized by 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'-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 to monitor 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 catalysed 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 that are precursors, while others are active or inactive. GAs are involved in seed germination, stem elongation, fruit growth, and floral development. GA signaling is initiated by perception of GA by GID1 accompanied by DELLA proteins in Arabidopsis (Murae *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 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 a decrease fluorescence 30 min after addition of 100 μ M GA₃ (Table 1).

The FRET-based GA sensor, GPS1, was developed for 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 GA₄. The K_{ds} of GPS1 for GA₁, GA₃, and GA₄ were 110 nM, 240 nM, and 24 nM, respectively (Table 1 and 2). The binding to GPS1 was partially reversible for GA₁ and GA₃, but not for GA₄. To assess the GA distribution *in planta*, a nuclear-targeted variant of GPS1 (nlsGPS1) was stably expressed under 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 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 ubiquitination of JAZ and 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 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 a SCF complex, leading to 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 visualization of D53 degradation in response to the addition of 5 μ M of the synthetic SL *rac*-GR24 (Zhou *et al.*, 2013). In Arabidopsis roots, 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 CLAVATA3 (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 fusion between these receptors and a FP. The use of a peptide receptor as a sensor was conceptionally shown using the well-established interaction of the immune receptor FLS2 with its coreceptor 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 assembly of heteromeric and homomeric receptor complexes with high spatial resolution (Somssich *et al.*, 2015). However, the functionality of this approach has not yet been confirmed in transgenic Arabidopsis plants, 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 strigolactones. 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, the cell lysate can be used for a LUC assay. In this system, 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 detection of SA, a bacterial reporter strain was engineered by inserting a luciferase gene into the chromosome between *salA* and *salR* (*Acinetobacter* sp. ADPWH_{lux}). Expression of *salA-luxCDABE-salR* responds to SA. This reporter strains 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

FRET and cpFP-based sensors make use of conformational rearrangements in a binding protein or receptor. We hypothesized that since 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 used to successfully 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 by 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 prototypes 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 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

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 changes in sensor levels, that arise from promoter activity variability or differential degradation. Recently, the Matryoshka technology was developed to convert the intensimetric sensors into ratiometric sensors (Figure 4) (Ast *et al.*, 2017). As an alternative, 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).

Since 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). Maturation time of donor and acceptor fluorescent proteins can also vary the fluorescent readout (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. Selectivity of ligands for a sensory domain should be also 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 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. While 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 intentionally be deployed as a molecular ‘sponge’ that sequesters a ligand (Armbruster *et al.*, 2020), providing an effective tool for functional analysis of signal transduction. In the absence of even less invasive technologies, it will be important to keep this in mind and perform proper controls. For example when a glucose sensor was used in yeast, analysis of growth

curves and even flux analysis did not reveal significant impact of the sensors on metabolism (Bermejo *et al.*, 2010), while surface-displayed glutamate sensors apparently impacted processes that led to defects in growth and development (Castro-Rodríguez *et al.*, 2020) and some did not have a clear impact (Toyota *et al.*, 2018). Affinity mutants are ideal controls, since 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. While a cytosolic biosensor does not require a targeting sequence, it is not always easy to visualize analytes and detect them separately in each cell since 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. Since 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 was successfully used in zebrafish, flies, and mice. No such sensor for hormones has been reported so far, however the generation of hormone sensors that make use of photoswitchable FP or Matryoshka variants may provide a promising starting point for engineering sensors that can take hormone activity snapshots.

Imaging technologies for quantification of sensor output

Initially, epifluorescence microscopy in conjunction with high quality Electron-multiplying CCD (EMCCD) cameras and simple perfusions systems were used for 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). While confocal microscopy is generally considered more advanced, it bears some risks, such as when treatment with an osmotically active substance affects 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 due to 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). Since a similar response occurs in plants stimulated by more subtle mechanical cues such as touch, technological advances for eliminating physical contact are needed. Initial development of 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 typically are limited to single seedlings and require substantial setup 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 noninvasive 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). While the RootChip can provide opportunities to observe the distribution and dynamics of plant hormones in roots, similar imaging platforms need to 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 ten years, light-sheet fluorescence microscopy (LSFM) has contributed substantially to the emerging field of real-time visualization of complex developmental processes. Due to 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 LSM 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). LSM does still have some limitations in sample conditioning, because of the limitation 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, LSM imaging technology has the potential to significantly advance real-time quantitative hormone 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 simultaneously detect the distribution of multiple hormones (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, it requires complete redesign of sensors for optimization of the fluorescence lifetime change. While many 'FRET sensors' appear to carry out nonradiative 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 due to FRET. For example, for the FLIPglu FRET glucose sensors, we have not detected a glucose induced change in donor FP lifetime. Thus, we recommend not to use terms such as FRET channel or axis labels such as FRET change, since scientists rarely measure actual FRET, but rather just a ratio. NiTrac is a good example for an unexpected, though useful effect, i.e. a change in donor quenching or absorption caused in the sensor by addition of the substrate nitrate. (Ho and Frommer, 2014). Pioneering work in animal systems by the Zhang lab has enabled multiplexing and simultaneous recording of many processes in part through differential subcellular targeting and in part through the development of 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.

Acknowledgements

This research was supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany's Excellence Strategy – EXC-2048/1 – project ID 390686111, the Alexander von Humboldt Professorship, and a Japan Society for the Promotion of Science (JSPS) grant (19H000932) to WBF, and the Human Frontier Science Program to MN. ITbM is supported by World Premier International Research Center Initiative (WPI), Japan. The authors declare no conflict of interest.

Author contributions

RI, WBF and MN designed the manuscript. All authors wrote this manuscript and prepared the figures.

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Tables

Table 1. Biosensors for plant hormones

Analyte	Biosensor	Sensor type	Range of ligand concentrations applied endogenously or exogenously	Reference
ABA	6xABRE	Transcriptional	~1-25 μ M	(Wu <i>et al.</i> , 2018)
	ABACUS	FRET-based	~2-80 μ M	(Jones <i>et al.</i> , 2014)
	ABAlcon2.1	FRET-based	100 nM ~	(Waadt <i>et al.</i> , 2014)
	ABAlcon2.15	FRET-based	100 nM ~	(Waadt <i>et al.</i> , 2014)
	ABAlconSD1-3L21	FRET-based	100 nM ~	(Waadt <i>et al.</i> , 2020)
	SNACS	FRET-based	20 μ M 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 μ M 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 μ M 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 μ M	(Herud-Sikimic <i>et al.</i> , 2020)
Brassinosteroid	BZR1-YFP	Transcriptional	100 μ M	(Chaiwanon &

			brassinolide (exogenous)	Wang, 2015)
Cytokinin	ARR5	Transcriptional	10 - 1000 nM	(D'Agostino et al., 2000; Romanov <i>et al.</i> , 2002)
	TCS	Transcriptional	1 - 1000 nM	(Müller & Sheen, 2007)
	TCSn	Transcriptional	1 - 1000 nM	(Zürcher <i>et al.</i> , 2013)
Ethylene	EIN3-GFP	Degron-based	50 µM ACC (exogenous)	(Guo and Ecker, 2003)
	EIL1-GFP	Degron-based	100 µM ACC (exogenous)	(An et al., 2010)
	FP-EBF 3'UTR	Translational	10 µM ACC (exogenous)	(Merchante et al., 2015)
	FP-6x EPU	Translational	10 µM ACC (exogenous)	(Li et al., 2015)
	EBS:GUS	Transcriptional	10 µM ACC (exogeneous)	(Stepanova et al., 2007)
	AEP	Artificial metallozyme	100 µM ACC (exogenous)	(Vong et al., 2019)
GA	GFP-RGA	Degron-based	100 µM GA ₃ (exogenous)	(Silverstone et al., 2001)
			0.03 - 1 µM GA ₁	
	GPS1	FRET-based	0.1 - 2 µM GA ₃ 0.005 - 0.2 µM GA ₄	(Rizza et al., 2017)
Jasmonic acid	Jas9-Venus	Degron-based	50 nM -10 µM coronatine	(Larrieu et al., 2015)
	JAI3-FP	Degron-based	50 µM jasmonate (exogenous)	(Chini et al., 2007)
Karrikin	DLK2:LUC	Transcriptional	1 µM < KAR1 (exogenous) 10 nM < KAR2 (exogenous)	(Sun et al., 2016)

	pRATIO-SMAX1	Degron-Based	200 nM~ KAR ₁ (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 μ M <i>rac</i> -GR24 (exogeneous)	(Zhou <i>et al.</i> , 2013)
	SMXL6-YFP	Degron-based	5 μ M <i>rac</i> -GR24 (exogeneous)	(Bennett <i>et al.</i> , 2016)
	SMXL7-YFP	Degron-based	5 μ M <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	~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	~ 2 μ M	+ 60% (500 μ M ABA)	(Jones <i>et al.</i> , 2014)
ABACUS1-80 μ	ABA	~ 80 μ M	+ 160% (500 μ M ABA)	(Jones <i>et al.</i> , 2014)
ABAlleon2.1	ABA	~ 79 nM	– 8.98%	(Waadt <i>et al.</i> , 2014)
ABAlleon2.15	ABA	~ 600 nM	– 10.09%	(Waadt <i>et al.</i> , 2014)
ABAlleonSD1-3L21	ABA	~ 938 nM	n.d.	(Waadt <i>et al.</i> , 2020)
GPS1	GA ₁	~ 110 nM	+ 60% (200 nM GA ₁)	(Rizza <i>et al.</i> , 2017)
	GA ₃	~ 240	+ 40% (200 nM GA ₃)	(Rizza <i>et al.</i> , 2017)

	nM		
GA ₄	~ 24 nM	+ 90% (200 nM GA ₄)	(Rizza et al., 2017)

Figure legends

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 proteasomal degradation of repressors for specific transcription factors (TFs). The activated TFs bind to specific sequence motifs (*cis*-elements) and induce transcription of reporter genes such as GFP, GUS or LUC. The readout 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 since 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 control of constitutive and ubiquitous promoters. Therefore, temporal resolution of the degron-based biosensors is more accurate compared to transcriptional reporters.

Figure 2. A model of FRET-based direct biosensors

‘Direct biosensors’ directly bind the plant hormone and report this binding. **(a)** A typical structure of FRET-based direct biosensors. The sensor generally consists of a sensory domain sandwiched by two FPs that differ in emission spectra and which act as a 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.

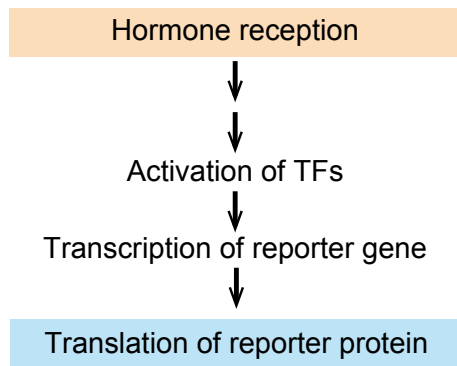
Figure 3. Flowchart for biosensor engineering

Engineering procedure of a plant hormone biosensor, here the GA sensor GPS1 (Jones *et al.*, 2014; Rizza *et al.*, 2017). The biosensor was created by combining domains of different bipartite GA receptor through an artificial linker and flanking the ligand-binding domains with potential FRET pairs. Linkers and collections of FP variants were tested as FRET pairs and compared in high throughput in 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 gibberellins were engineered by rational design. The affinity mutants can be utilized as negative controls during *in vivo* analysis to exclude artifacts like those caused by pH changes. GPS1 and the affinity mutant GPS1-NR were expressed in Arabidopsis plants under control of a constitutive promoter (p16).

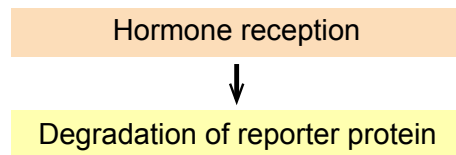
Figure 4. Further optimization of genetically encoded hormone biosensors

A model of a ratiometric direct-biosensor. The sensory domain is linked to an environmentally sensitive circularly permuted 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 increasing 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 intensity change caused by analyte binding in the cpFP.

(a) Transcriptional biosensor

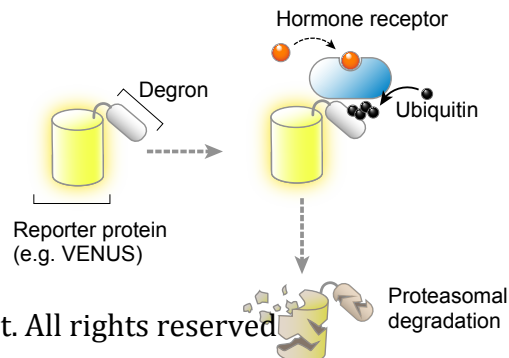
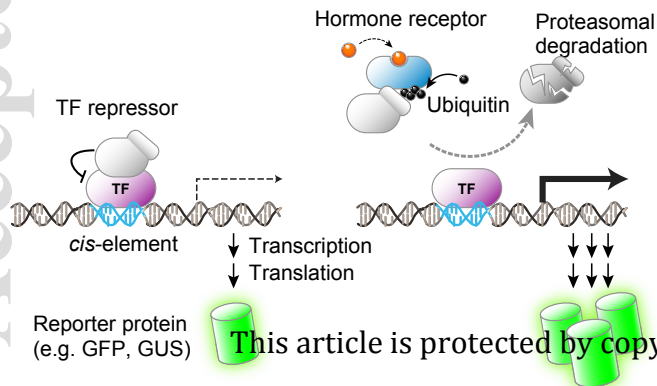


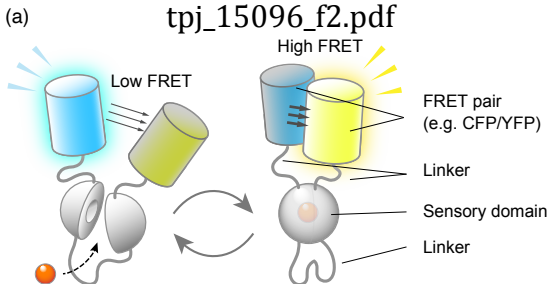
(b) Degron-based biosensor



Plant hormone

Hormone receptor

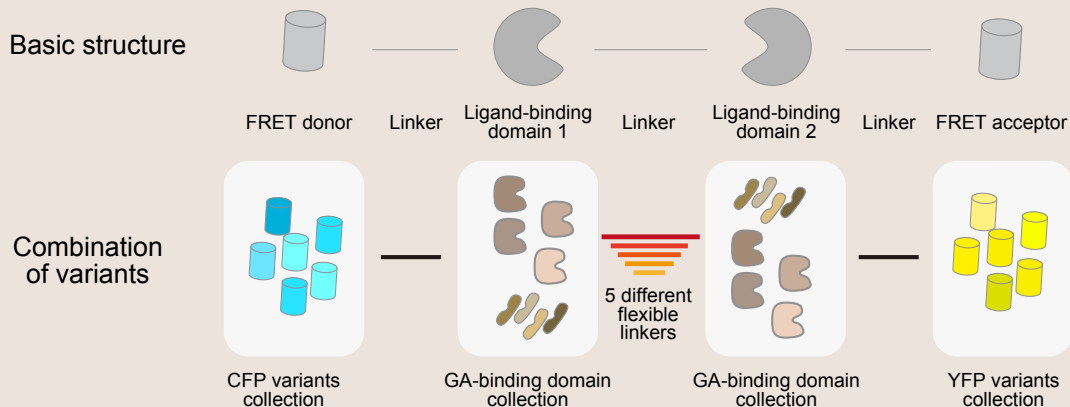




(b)

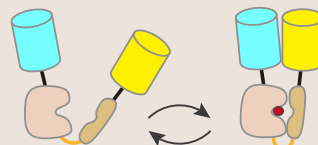
Biosensor	Ligand	Sensory domain	FRET pair (donor/acceptor)
ABACUS	ABA	PYL1 + ABI1	edCerulean/edCitrine
ABALeon	ABA	PYR1 + ABI1	mTurquoise/cpVenus173
GPS1	GA	GLD1 + GAI	edCerulean/edCitrine
AuxSen	Auxin	<i>E.coli</i> TrpR	Aquamarine/mNeonGreen

1. Construction of combination variants



2. Screen the best sensor

- Use of yeast expression system
- Protein extraction
- Fluorescence measurements
- Identification of the best combination



3. Sensor characterization

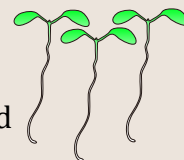
- Evaluation of the affinity and the dynamic range against GAs

4. Generate affinity variants

- Introduction of amino acid substitution(s) into the sensory domain
- Evaluation of the sensitivity to GAs

5. *In planta* imaging

- Use of constitutive promoter (p16)
- FRET analysis in Arabidopsis thaliana



Matryoshka ratiometric direct biosensor

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