# Zooming In on Plant Hormone Analysis: Tissue- and Cell-Specific Approaches

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## Abstract

Plant hormones are a group of naturally occurring, low-abundance organic compounds that influence physiological processes in plants. Our knowledge of the distribution profiles of phytohormones in plant organs, tissues, and cells is still incomplete, but advances in mass spectrometry have enabled significant progress in tissue- and cell-type-specific analyses of phytohormones over the last decade. Mass spectrometry is able to simultaneously identify and quantify hormones and their related substances. Biosensors, on the other hand, offer continuous monitoring; can visualize local distributions and real-time quantification; and, in the case of genetically encoded biosensors, are noninvasive. Thus, biosensors offer additional, complementary technologies for determining temporal and spatial changes in phytohormone concentrations. In this review, we focus on recent advances in mass spectrometry–based quantification, describe monitoring systems based on biosensors, and discuss validations of the various methods before looking ahead at future developments for both approaches.

## Contents





## WHY ARE TISSUE- AND CELL-TYPE-SPECIFIC APPROACHES IMPORTANT?

Plant hormones are highly bioactive signaling molecules that are often present at very low concentrations. They act as chemical messengers, triggering and controlling physiological processes during plant growth and development as well as in response to plant stresses. The occurrence and levels of these compounds depend strongly on the plant organ, plant age, developmental stage, and environmental conditions. Critically, we know that their concentrations are dynamic, changing to trigger and to coordinate responses, but we do not yet fully understand how such changes in spatial and temporal distribution enable diverse responses. New analytical and monitoring tools are adapting rapidly to address the challenges faced by phytohormone researchers, including the ongoing development of instruments and techniques that are capable of recording in smaller numbers of cells, in more precise areas of tissue, and with better time resolution. These advances will help us understand the details and nuances of plant inter- and intracellular communications, allowing informed decisions about, e.g., trait development in crop improvement programs. The pressure to increase analytical performance will undoubtedly continue, and we present this review as a description of the current state of the art.

**Mass spectrometry (MS):** a powerful method of qualitatively and quantitatively analyzing compounds that are converted into gaseous ions and then characterized by their mass-to-charge ratios and relative abundances

## MASS SPECTROMETRY–BASED ANALYSES OF PLANT HORMONES

### Development of Analytical Tools

Improvements in plant hormone identification and quantification are tightly linked to the rapid advances in mass spectrometry (MS) technology that have taken place during the last century

(82, 137). The workhorses of today's plant hormone laboratories are tandem mass spectrometers (MS/MS instruments) combined with the use of either gas chromatography (GC) or liquid chromatography (LC) to separate the analytes. These instruments combine ultrahigh sensitivity and selectivity in the mass analysis with excellent separation of organic molecules from samples that usually contain a very complicated biological matrix (for reviews of methods for plant hormone analysis, see 27, 77, 122). The high selectivity of MS/MS instruments based on specific data acquisition modes, such as selected reaction monitoring, enables the analysis of trace concentrations of analytes in complex matrices. High-resolution MS has recently become popular as an important technique in the field of quantitative bioanalysis (reviewed in 94), as used by Van Meulebroek et al. (128), who were the first to profile the endogenous phytohormones in tomato plants by applying high-resolution MS technology.

Optimized sample purification is equally important for the highest sensitivity possible in the final analysis, and new types of adsorbents, miniaturization, and online purification have the potential to increase the sensitivity even further (93). Regardless of which analytical technology is applied, the proper use of isotopically labeled internal standards is essential to ensure accurate quantitative results, allowing compensations for losses resulting from sample preparation and MS analysis (77). Continued improvements of analytical tools to increase their sensitivity and selectivity are a prerequisite for further developments in tissue- and cell-specific analyses—methods that are essential to gain a better understanding of different biological processes.

On the basis of their physiological functions and structures, the phytohormones are categorized into several major classes: auxins, gibberellins (GAs), cytokinins (CKs), ethylene, abscisic acid (ABA), polyamines, brassinosteroids (BRs), jasmonates (JAs), salicylic acid (SA), and signal peptides (21). Strigolactones were also recently determined to be members of the phytohormone family of compounds (reviewed in 1). All phytohormones are small organic molecules, but they have diverse chemical properties and differ widely in concentration, and different tissues have different matrix properties. Consequently, many new sample preparation techniques have been published over the past decade, such as batch immunoaffinity chromatography (42, 91), in-tip solid-phase extraction (22, 73, 118), magnetic solid-phase extraction (12, 13, 25, 74, 145, 146), monolithic molecularly imprinted solid-phase extraction (26), boronate affinity polymer monolith microextraction (24), and hydrophilic solid-phase extraction (14).

**Gas chromatography (GC):** a convenient method of separating volatile compounds prior to their identification by mass spectrometry

**Liquid chromatography (LC):** an analytical technique used to separate components in a mixture prior to their mass spectrometry–based detection

## Examples of Analyses of Different Classes of Plant Hormones at the Tissue, Cellular, and Subcellular Levels

For auxin [indole-3-acetic acid (IAA)] and its metabolites, methods based on GC- or LC-MS/MS have been the most widely used (reviewed in 93, 102). Tissue-specific profiling of auxin and its metabolites from *Arabidopsis thaliana* seedlings has shown that it is possible to accurately quantify more than 21 different auxin metabolites in very small amounts (<20 mg) of plant material (92). Miniaturized purification methods have been developed to further reduce the amount of biological tissue required (<5 mg). For instance, a powerful one-step, high-throughput, micro-solid-phase extraction approach has been used for CKs from root and shoot tissues (118) and for specific cell populations (2). Better still, a microscale magnetic, immunoaffinity-based method for selective, one-step isolation of CKs from <0.1 mg of plant tissue has recently been developed (L. Plačková, unpublished data).

**Hormone profiling.** Methods for simultaneous quantification of the majority of acidic plant hormones from small amounts of plant material have also been developed. During the last decade, many detailed protocols have been used to identify and quantify different classes of plant metabolites simultaneously (18, 87, 97). Acidic plant hormones are normally extracted from plant tissue

**Hormonomics:** the profiling of a large range of specific but distinct phytohormones and related compounds, such as metabolites, catabolites, and precursors

using a mixture of methanol and water; purification is then done with silica- or polymer-based adsorbents, and analysis is performed using LC-MS/MS (5, 16, 30, 71). These methods make it possible to quantify IAA, ABA, and GAs as well as stress-induced compounds such as SA and JAs in the same extract. In 2002, Müller et al. (85) designed a multiplex GC-MS/MS technique to create organ-specific distribution maps for IAA, ABA, JAs, and SA in *Arabidopsis* at the flowering stage, giving a spatial resolution down to approximately 2 $mm^2$.

Some attempts have also been made to measure an even wider range of plant hormones in the same sample. A sensitive LC-MS/MS method to measure IAA, IAA conjugates, ABA, CKs, and GAs involves derivatization of the acidic hormones with bromocholine to increase the sensitivity for these compounds in the positive ion mode (57). Cai et al. (11) recently applied sequential solvent-induced phase transition extraction to simultaneously quantify 12 basic and acidic phytohormones. The method was validated using different tissues of *Oryza sativa* and *Arabidopsis* plants with very small sample sizes (5 mg fresh weight). In a further example, 20 plant hormones were profiled in rice using a binary solid-phase extraction based on polymer anion and polymer cation exchange resins prior to LC-MS/MS (15).

Seven LC-MS/MS methods have broadened the scope for analysis after sample fractionation by allowing high-throughput quantification of more than 100 primary metabolites, secondary metabolites, and phytohormones from small amounts (10–100 mg) of plant tissue (109). The major obstacles for developing such hormonomics methods (simultaneous quantification of different plant hormones and their metabolites) are the diverse chemical properties, the large concentration ranges of the different compounds, and the diversity of complicated sample matrices. One response has been the development of a new generalized analytical screening method based on selected-reaction-monitoring transitions at specific retention times (J. Šimura, unpublished data). This has also allowed the profiling of more than 100 analytes, including members of all the main classes of plant hormones (auxins, GAs, CKs, ABA, BRs, JAs, and SA), and we anticipate further improvements. Highly efficient extraction and purification methods remain essential if we are to obtain a high enough sensitivity in the MS analyses for all compounds.

**Tissue- and cell-specific analyses.** Comprehensive hormone profiling can help elucidate, at the molecular level, a plant's responses to different conditions, such as stress, mutations, and hormone treatments (102). Although there are an increasing number of applications of phytohormone profiling in whole plants, very few tissue- and cell-specific phytohormone analyses have been published (**Figure 1**). Multiple studies have determined the distribution of auxin and/or CKs in order to investigate the development of shoot and root apical meristems in *Arabidopsis* (8, 19, 75, 76, 90, 142, 147), tobacco (23), pea (120), oilseed rape (121), and maize (141). Moritz & Olsen (84) found large differences in GA content in different parts of *Salix pentandra*, with the highest levels of the bioactive hormone $GA_1$ in the apical and subapical parts of the stem. Moreover, the quantification of GAs in root tips showed that GAs are important for normal root elongation in pea (138). Li et al. (64) recently published a direct derivatization and ultratrace determination of GAs in submilligram amounts of fresh plant organs, matured *Arabidopsis* flowers, and dissected stamens. Reproductive organs (pollen, flowers, and immature seeds) are also well known as main sources of BRs (122). In 1998, Yokota et al. (140) identified endogenous BRs in the anthers of Japanese cedar.

Cell-type-specific comparison of the exact distribution profiles of different hormones within plant meristems is also challenging. The vascular cambium, a wide lateral meristem with an extensive developmental zonation, provides an optimal system for profiling auxins, BRs, CKs, and GAs (46, 47, 56, 81, 125, 126) (summarized in **Figure 1**). These quantifications suggest that CKs and IAA have their maximum in the developing phloem tissue and cambial zone, respectively,

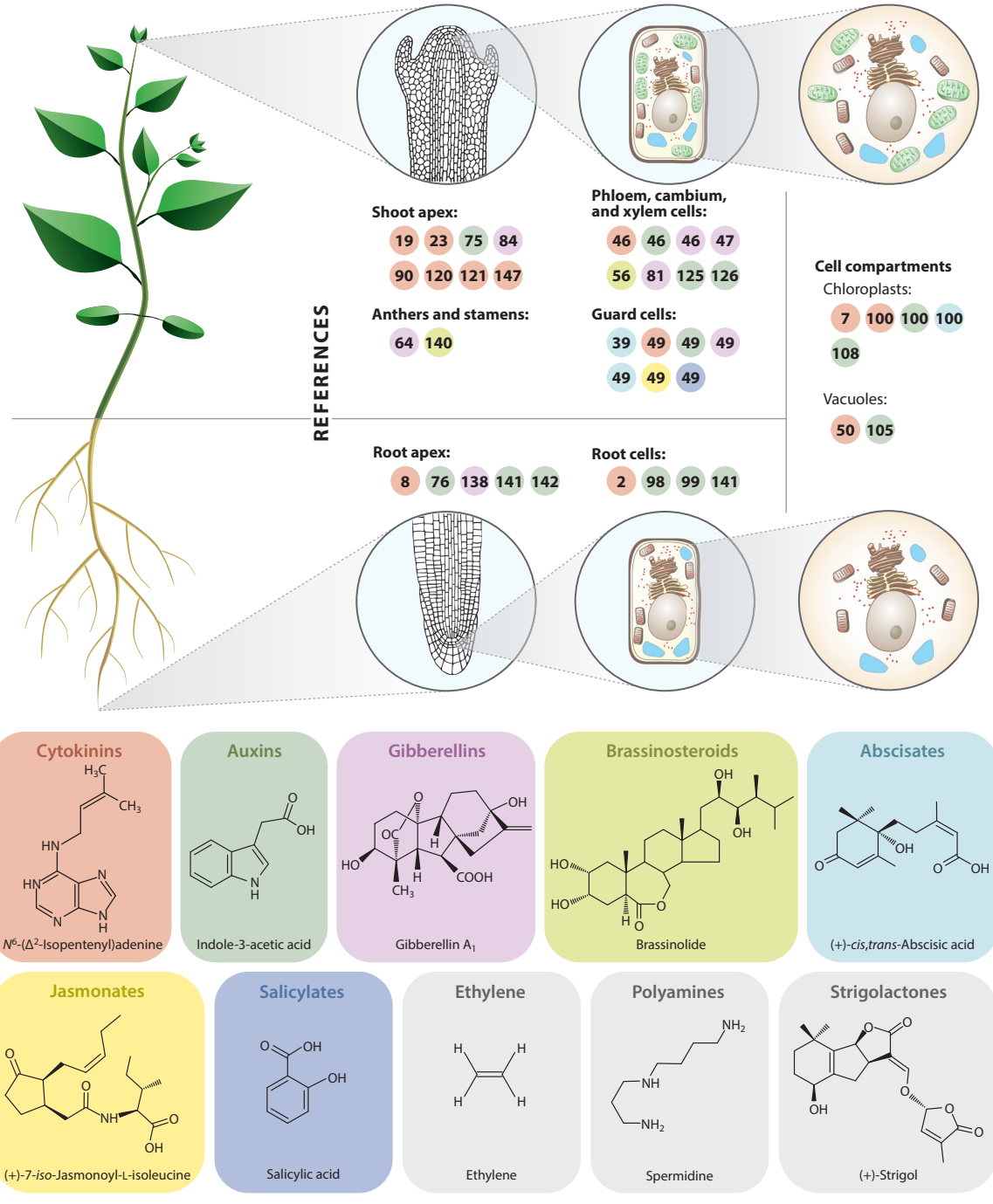



**Figure 1**

Current state of the art of phytohormone profiling based on gas and liquid chromatography–mass spectrometry, with special emphasis on tissue- and cell-specific analyses. The colors of the reference numbers (*top*) correspond with the classes of phytohormones (*bottom*) found in each tissue or cell type.

and that GAs peak in the developing xylem tissue. Advanced MS-based techniques also provided insights into ABA regulation of guard cell signaling and the guard cell metabolome (49). Targeted selected-reaction-monitoring-based phytohormone profiles in *Arabidopsis* stomata provided the first example of single-cell-resolution analysis. Geilfus et al. (39) recently demonstrated stress-induced elevation in the endogenous nanomolar concentration of ABA in *Vicia faba* guard cells. Furthermore, Yu et al. (141) determined that cell-specific auxin profiles address the effects of nitrate on IAA distribution, biosynthesis, and degradation in *Zea mays* root tissues (the root tip, stele, and cortex).

A method developed for cell-type-specific analysis of IAA and its catabolite oxIAA uses a combination of protoplasting, fluorescence-activated cell sorting, and LC-MS/MS quantification (98, 99). A similar method was also recently developed for quantification of CKs (2), which are an especially challenging target because their levels are 10–100 times lower than auxin levels in plant tissues (79). Antoniadi et al. (2) used several *Arabidopsis* lines that expressed green fluorescent protein (GFP) in specific cell types of the root—such as the quiescent center, columella, epidermis, cortex, and endodermis—for protoplasting and cell sorting. The protocol was made possible by improvements in sensitivity in the new generation of triple-quadrupole LC-MS/MS instruments, making it possible to profile IAA and CK metabolites in samples containing only 50,000–200,000 root cells. It is likely that methods for cell-type-specific analysis of other plant hormone classes will be developed in the near future.

**Hormone profiles from live cells.** Methods to quantify metabolites in single cells have been developed for more abundant metabolites, such as amino acids, sugars, and lipids (33, 95). These methods enable investigators to perform in vivo measurements of specified metabolites, although measuring subcellular metabolite concentrations is currently difficult because the whole cell content is normally extracted. So far, quantification of plant hormones at the single-cell level has proved to be even more difficult owing to the very low concentrations of these compounds, but a few attempts have shown that the technology is advancing in this direction. For example, Shimizu et al. (112) used a method combining nano-electrospray ionization of cell contents with direct infusion into an LC-MS/MS instrument to measure ABA and jasmonoyl isoleucine (JA-Ile) in single cells from *Vicia faba* leaves.

**Hormone profiles from subcellular compartments.** Very little is known about the subcellular compartmentation of plant hormones and their distribution between the symplastic space (cytoplasm) and the apoplastic space (cell wall). Several methods can be used to separate different organelles into well-defined fractions, such as density-based fractionation, nonaqueous fractionation, and fluorescence-assisted organelle sorting (3, 34, 62). Some efforts have been made to measure plant hormone concentrations in specific organelles, such as chloroplasts (7, 100, 108) and vacuoles (50, 105).

**Metabolic studies.** Accurate analysis of metabolite concentrations is routine in all MS-based laboratories today. However, measurements of steady-state levels do not provide any information about metabolic fluxes, and methods to measure biosynthesis and degradation rates are therefore needed to obtain a better understanding of dynamic processes in plants (61). Stable labeled compounds can be used to measure metabolic rates by feeding the plants with compounds such as deuterated water, $^{13}CO_2$, or specific labeled precursors. The incorporation of the label into the metabolic pathway is then measured using MS, and the rate of biosynthesis and degradation of the plant hormone and its metabolites can be calculated (60, 76, 114, 123). This method can also measure the turnover of a plant hormone (89). A few attempts have been made to measure

biosynthesis and degradation of plant hormones within different tissues and cell types (76, 98, 99, 101, 116). Interestingly, monitoring a stable isotope fed to *Arabidopsis* seedlings clearly linked the translocation of the $^{13}$C-CKs with the necessity of the CK exporter *Arabidopsis thaliana* ATP-BINDING CASSETTE G14 (AtABCG14) (143). However, short- and long-range transport of plant hormones do make it difficult to calculate metabolic fluxes accurately within a specific tissue or cell type.

### Future Prospects for Mass Spectrometric Techniques

The development of increasingly sensitive and selective mass spectrometers is ongoing, and methods for cell- and organelle-specific analysis of most plant hormones will likely be available in the near future. Nevertheless, higher sensitivity brings its own problems, such as contamination and high background noise. Ultraclean labs and dedicated instruments will be needed to avoid problems with sample contamination, and higher resolution will be needed to reduce the background noise (94). Work in the MS area is already responding to these challenges with a shift from low-resolution to ultra-high-resolution tandem mass analyzers, a shift from conventional LC-MS to ultrafast LC-MS techniques, the adoption of two-dimensional LC-MS for complex samples, and/or use of other dimensions in MS, such as ion mobility spectrometry (43).

Mass spectrometry imaging (MSI) has been used to obtain additional spatial distributions of analytes on the surface of plant tissue (119). The final images show the relative abundances of classes of molecules across the sample area as well as quantitative changes between different tissue sections (111). Matrix-assisted laser desorption ionization, secondary ion mass spectrometry, and desorption electrospray ionization are all commonly used MSI ionization techniques. The spatial resolution of matrix-assisted laser desorption ionization is generally in the range of micrometers, which enables investigations on the cellular or (in some cases) subcellular scales, although secondary ion mass spectrometry can provide spatial resolution below 1 μm and three-dimensional spatial imaging of biological tissues. Clearly, such tissue atlases of phytohormone distributions will become increasingly detailed and will add a new richness to the data on, e.g., hormone gradients over long and short distances.

Desorption electrospray ionization is based on charged droplets impacting on the sample surface; this new ambient ionization technique requires minimal sample preparation or treatment and, hence, less tissue disturbance. In another development, Huang et al. (44) used laser-activated electron tunneling, a photo-generated electron ionization technique, to image endogenous metabolites present in *Cayratia japonica* leaves. The results showed distinct accumulation patterns for different phytohormones, including ABA, GA, and JA. Because of the large number of potentially interfering compounds present in the complex matrices of plant tissue samples and the very low concentrations of phytohormones, MSI of these metabolites remains challenging and will require continuing development of MS instruments with ultrahigh resolution and sensitivity.

## PLANT HORMONE SENSORS

### A History of Different Types of Sensors

Biosensors offer a different approach to hormone measurements. They are distinguished as analytical devices by their use of a biological module for analyte recognition, although the definition does break down in, e.g., some electrochemical devices. Generally, biosensors are used to obtain real-time readings from samples that are not readily compatible with high-end precision instruments, such as those from crude or living tissues. Solid-state biosensors can also be inexpensive

and straightforward to interpret, although industrial use is presently focused only on ethylene biosensors (48). All analytical tools are under such high development pressure that many of these generalizations will soon be outdated, but we use the definition that a biosensor uses a biological module for analyte recognition.

There is a beautiful symmetry in adopting biology's own evolved specificities as tools to identify and quantify other biological molecules, with the prime example being the utilization of antibodies in various immunosensors. Many immunosensors have been devised for plant hormones, some of which we describe below, but unfortunately antibodies do not change or signal when they bind their antigen, which means that the immunosensor readout is indirect and that enzyme-linked immunosorbent assays and radio immunoassays remain more frequently used than immunosensors. By contrast, redox enzymes have proved to be versatile and robust biosensor elements. There are many examples of commercial electrochemical biosensors based on highly selective enzymes, the most widely appreciated being the glucose oxidase–based sensors used by diabetics (131). However, because few plant hormones are metabolized by suitable redox enzymes, there has been limited development in this area. On the other hand, plant hormone biosensors based on genetic reporters have become widely used, with a move from the highly successful expression reporters based on the enzyme β-glucuronidase to more versatile biosensors based on fluorescent proteins, such as GFP. Fluorescence is immensely sensitive, allowing subcellular localization of the sensor signal, but it generally gives a qualitative rather than quantitative output; however, the latest biosensors are based on receptor protein complexes coupled to ratiometric Förster resonance energy transfer (FRET) protein pairs, which can provide quantitative data (53, 129).

Several good reviews have discussed historical plant hormone biosensors (e.g., 32, 107, 122, 133), and we do not wish to duplicate these articles. However, to allow comparisons and illustrate recent developments, we discuss the main types of biosensors below.

**Biosensor:** an analytical device that uses a biological module for analyte recognition

**Immunosensor:** a biosensor that uses an antibody as the selective element

**Förster resonance energy transfer (FRET):** the transfer of fluorescence excitation energy from one fluor to another when the two are in close proximity

**Electrochemistry:** measurement of the progress of an enzymic reaction electrically, generally by using redox reactions to quantify substrate concentrations in sensors

## Electrochemical Biosensors

The readout from electrochemical sensors may be current (amperometric) or changes in resistance/impedance or capacitance. The most widely used format is amperometric detection, which measures the flow of electrons resulting from enzymatic redox reactions, often via intermediate compounds such as hydrogen peroxide ($H_2O_2$). Typically, the analyte is oxidized by an enzyme-bound redox cofactor such as flavin adenine dinucleotide (FAD), which is reduced to $FADH_2$. The FAD is then restored by, e.g., the reaction of $FADH_2$ with molecular oxygen, yielding $H_2O_2$. Electrochemistry has devised many mechanisms to reoxidize $H_2O_2$ at the electrode, giving rise to changes in current (132). Advances in selectivity and sensitivity continue with the adoption of nanoparticles (e.g., 17). Nanoparticles and nanowires do not necessarily lead to nanoscale sensors; they are needed to boost sensitivity by offering very high surface areas. For example, nanoporous gold microelectrodes decorated with platinum nanoparticles have enabled real-time monitoring of $H_2O_2$ release from single cells (135).

Electrochemistry has been applied in several plant hormone biosensors (107). The redox enzyme cytokinin oxidase (CKX, also known as cytokinin dehydrogenase) has been recently applied to platinum wire electrodes coated with Prussian blue (ferric ferricyanide). The purified enzyme, immobilized in a silica gel matrix (78), retains the activity and selectivity of CKX, but the sensitivity is insufficient for use in vivo (59). A second-generation CKX microbiosensor used an electron mediator to couple the FAD redox center to the electrode, increasing sensitivity 1,000-fold, and this sensor was used to measure low nanomolar CK concentrations in tomato root exudate xylem sap, measurements that were then validated by MS (124) (**Table 1**). **Figure 2** shows an example of a biosensor setup for measurements in tomato root exudate.

**Table 1 Recent electrochemical biosensors**

| Plant hormone | Electrode | Sensitivity | Prepurification of the sample needed | Reference |
|---|---|---|---|---|
| Auxin | Antibody and impedance | 30–3,000 μM | Yes | 66 |
| | Electrochemical oxidation and reduced graphene oxide | 0.1–7 μM | Yes | 36 |
| | Antibody and gold nanoparticles | 1 nM–5 μM | Yes | 148 |
| | Antibody and photoelectrochemistry | 0.5 nM–5 μM | Yes | 117 |
| | Self-referencing vibrating electrode | 1–40 μM | No | 83 |
| | Electrochemical oxidation with nanowires | 1–1,000 nM | No | 72 |
| | Molecular imprinting and silver nanoparticles | 0.9–600 nM | Yes | 67 |
| Cytokinin | Cytokinin oxidase | Micromolar | No | 59 |
| | | 4 nM–10 μM | No | 124 |
| | Cytokinin deaminase | 3 nM–2 μM | No | F. Tian, N.E. Dale & R. Napier, unpublished data |
| | Electrochemical oxidation and CuO-$SiO_2$ composite microspheres | 50 nM–100 μM | Yes | 38 |
| | Aptamers | 3–50 μM | Not tested | 104 |
| | | 0.7–6 μM | Not applicable | 115 |
| | Aptamer coupled to fluorescent readout | 0.14–0.6 μM | Not applicable | 70 |
| Abscisic acid | Antibody and impedance | 2 nM–20 μM (claimed) | Yes | 68 |
| | scFv antibody using SPR competitive kinetics | 10 nM–10 μM | No | 4 |
| Gibberellin | Molecular imprinting and electrochemical luminescence | 10 pM–3 nM | Not applicable | 65 |
| | Molecular imprinting and ferrocenecarboxylate dendrimers | 2–100 nM | Not applicable | 31 |
| | Molecular imprinting and Fe-Au composite nanoparticles | 10 pM–10 nM | Not applicable | 144 |
| Ethylene | Cu(I)-doped carbon nanotubes | 1–50 ppm (μL/L) | No | 29 |
| | Multiwalled carbon nanotubes | 10–65 ppm | No | 55 |
| Methyl jasmonate | Electrochemical oxidation and nanoparticle-decorated carbon | 0.7 μM–1 mM | Yes | 35 |
| | Electrochemical oxidation and phosphotungstic acid/graphene oxide | 0.5–80 μM | Yes | 37 |

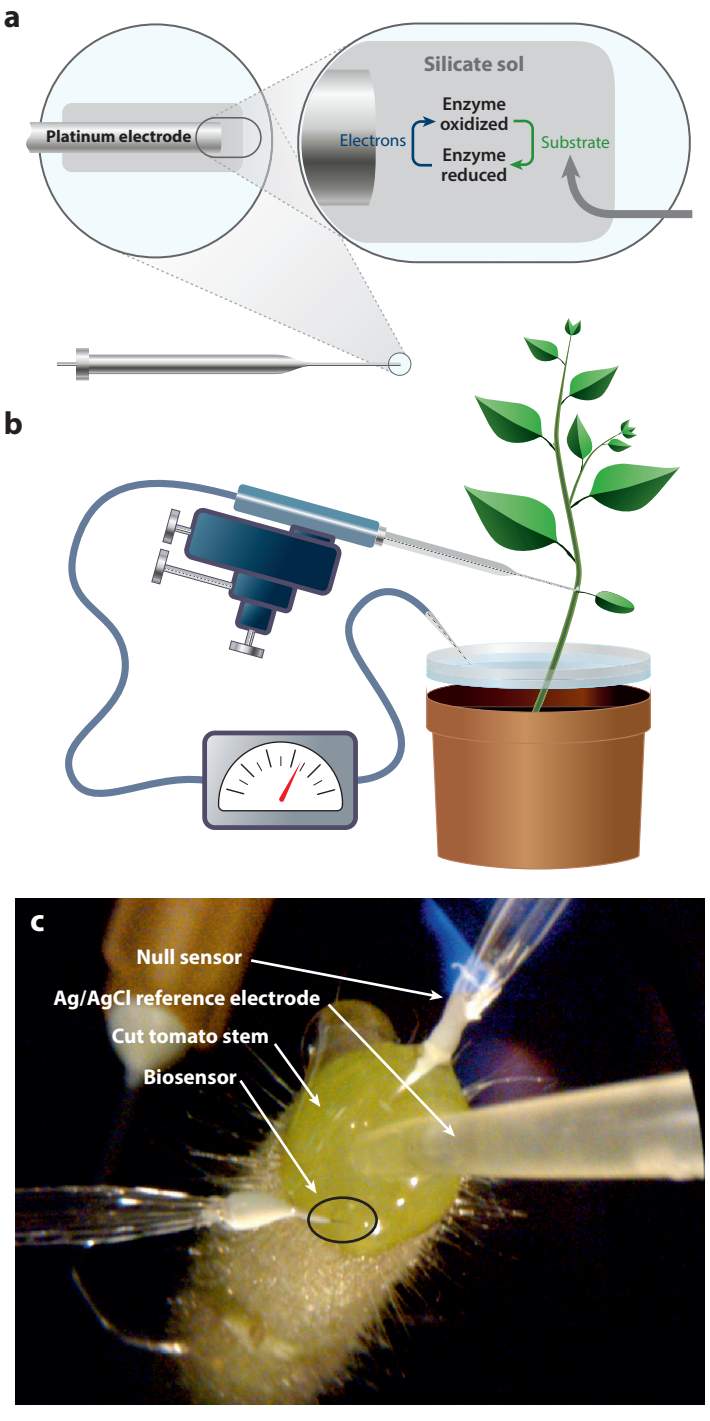
Abbreviations: ScFv, single-chain variable fragment; SPR, surface plasmon resonance. "Not applicable" indicates that the sensors were tested in a biological sample without prepurification but that the sensor probably could not be applied without a sample preparation protocol.

## Antibody-Based Sensors

Antibodies have been incorporated into biosensors in many formats (**Table 1**). An early biosensor for ABA used monoclonal anti-ABA antibodies adsorbed onto glassy carbon electrodes (68). The authors incubated the electrode surface with $[Fe(CN)_6]^{3-/4-}$ and recorded the impedance of the surface. They then added the ABA sample and recorded the impedance again, and the difference in impedance was related to ABA concentration. As with many immunosensors,

**Figure 2**

(*a*) Schematic illustrating a typical electrochemical biosensor. At the top right, the enzyme is encased in a silicate gel, showing electron exchange with the electrode; at the top left, the enzyme sol coats the surface of a platinum wire that in turn is encased in glass to enable precise positioning with micromanipulators. (*b*) Arrangement of an electrode microbiosensor in a plant. (*c*) Photograph of the cut stem of a 14-day-old tomato plant and a drop of root exudate. In the droplet are positioned both null and biosensor electrodes and, in this case, the Ag/AgCl calomel reference electrode. The 25-μm platinum wire sensor (*circled*) may be compared in dimension to the tomato trichomes.



the slow response time caused problems, given that analyte binding needs to be allowed to reach equilibrium, and in most cases (including this ABA biosensor) the sample needs to be extracted and prepurified before measurement. The regeneration of biosensor surfaces between recordings can also cause difficulties or delays. On the positive side, data generally show that biosensor recordings are close to the concentration values measured by a reference technique, such as high-performance LC (e.g., a difference of <10% in Reference 68), and the sensitivity was reported to be 0.5–5,000 ng/mL (approximately 2 nM–20 μM), although the calibration plot suggests that statistically significant values may be obtained over a somewhat smaller and less sensitive calibration range (68).

More work has been done on auxin immunosensors (102), leading to a greater exploration of technical improvements. Li et al. (66) improved immunosensor regeneration times by using a competitive assay between IAA as the analyte and an IAA–horseradish peroxidase (HRP) conjugate. The anti-IAA antibody was immobilized on the surface of a composite-coated electrode, essentially solidified carbon paste. In the absence of free IAA, the antibody bound IAA-HRP, and the HRP reaction rate was recorded by the electrode. In the presence of IAA, the IAA-HRP was displaced, reducing the signal, and regeneration was achieved by bathing the electrode in saline at pH 12. In this case, sensitivity was poor—reported as 5–500 μg/mL (approximately 30–3,000 μM)—and several sample cleanup and concentration steps were required ex planta.

To improve sensitivity, more recent carbon-based electrodes have been coated with nanoparticles to increase surface area (139, 148). In one case, gold nanoparticles were coated with anti-rat immunoglobulins conjugated to HRP, and rat anti-IAA antibodies were loaded onto these antibodies (148). In another case, iron oxide nanoparticles were coated with immunoglobulin G (IgG)–HRP, and the anti-IAA antibodies were coated separately onto gold nanoparticles to obtain a double amplification (139). As with the ABA immunosensor described above, the current was recorded from oxidation of the electron carrier $[Fe(CN)_6]^{3-/4-}$ in the redox reaction of HRP. The presence of IAA, presumably as the anion $IAA^-$, competed with the electron carrier anions to reduce the oxidation signal. Hence, the accumulation of $IAA^-$ by the anti-IAA antibody close to the electrode surface led to quantifiable reductions in electrical signal. Although the large surface area afforded by the nanoparticles increased sensitivity, as with most antibody-based systems, the sensor was slow to reach a steady reading (1 h).

A photoelectrochemical sensor has also been developed for IAA (117). In this technology, light is used to excite, e.g., CdS nanoparticles stabilized in composites on reduced graphene oxide electrodes. Antibodies immobilized on the surface accumulate IAA around the sensor, and the current is recorded after light excites the photoactive surface. In the presence of IAA, the current is reduced, apparently because of increased steric hindrance toward the electron carriers at the electrode surface. The photoelectrochemical sensor in this study was highly sensitive (0.5 nM–5 μM), and the signal stabilized within 10 s during the reading; however, it was still necessary to incubate the analyte with the sensor for 1 h before the reading was taken.

All of the immunosensors for IAA have been tested on plant-extracted analyte solutions. In all cases, the sensors performed favorably against the same samples assayed by alternative analytical techniques, such as high-performance LC. However, the analyte samples required extraction from plant tissues and one or more steps of purification and concentration.

## Alternative Electrode-Based Biosensors

To avoid the time delays associated with equilibration on immunosensors, alternative technologies have been tested (102, 107). Electrode-mediated oxidation of compounds at selected voltages has been used for many analytes and, with the many technologies now available to increase surface

area, can be sufficiently sensitive for plant hormone assays. Because they do not have a biological selectivity element, these technologies are not strictly biosensors, but given the similar terminology, they are often considered together.

Reduced graphene oxide–coated electrodes are popular and have been tested for IAA determinations (36). A wide range of possible contaminating compounds have been tested to evaluate selectivity (although not the important compound ascorbic acid), and the sensitivity is moderate (**Table 1**). Far more impressive has been the use of platinum microelectrodes decorated with nanowires (titanium carbide–carbon quasi-aligned nanofiber arrays) (72). The advantages of amperometric sensors shine through in this application, which combines a rapid response time (milliseconds) with high sensitivity (1–1,000 nM). Liu et al. (72) used such sensors to record IAA efflux from vesicles emanating from protoplasts in real time. Their data appear promising, although they did not provide information on sensor selectivity, in particular the possible contribution to the signals from ascorbic acid—a common problematic molecule for in vivo amperometric measurements and a compound that is certainly prevalent in plants and protoplasts.

Carbon nanotubes doped with a Cu(I) complex have been used with good results for ethylene detection. The ethylene analyte binds to the Cu(I) as it does in its endogenous receptor. In the sensor, ethylene binding decreases the conductivity (increases the resistance) of the nanotubes (55).

Self-referencing microelectrodes for IAA rely on reading and comparing the current as the electrode vibrates between positions close to and distant from the tissue. McLamore et al. (83) improved the early model created by Mancuso et al. (80) by increasing the operating surface area with multiwalled carbon nanotubes. In this case, the travel distance between the two positions was 30 μm, and the inward and outward fluxes of IAA were recorded in vivo from *Z. mays* root tips. The two measurements allow analyte recordings to be taken in dynamic environments because the electrode constantly references itself against the background reading in the distal position. However, these sensors clearly need room to move as well as careful, precise positioning adjacent to the test surface.

Electrochemical oxidation methods have not been widely applied to other plant hormones. A sensor for the aromatic CK benzyladenine, a potential food contaminant, was made using hollow-core and shell-structured CuO-$SiO_2$ composite microspheres (38). These had a reasonable sensitivity (50 nM–100 μM, and a limit of detection of 2.6 nM was claimed), reasonable response time (300 s), and good tolerance of ascorbic acid (other organics not reported) and salts but were somewhat compromised by a steep pH dependence. There is a similar set of sensors for methyl JA (**Table 1**), and some electrochemical oxidation sensors have been reported for ethylene, but photoacoustic techniques for ethylene are more sensitive and are preferred (20).

A further category of nonbiological electrochemical sensors is molecularly imprinted devices. When first developed, this technique had poor sensitivity, but the attractions of robustness and versatility prevailed, and enhanced detection techniques such as electrochemical luminescence have greatly improved performance. The first of these methods to be applied to a plant hormone assay was for $GA_3$ (65), and alternatives for $GA_3$ and IAA followed (**Table 1**). Most rely on doping the molecularly imprinted surfaces with analyte conjugates that are then used in competition with the analyte. Signal amplification is based on the retained conjugate, which is generally interrogated electrochemically. These sensors tend to have good selectivity (and ascorbic acid is not generally a problem) and tolerance to, e.g., salt concentration, although these performance indicator experiments are generally done with near-saturating target concentrations. The sensors are often relatively stable over time in use and in storage, and the probes are cheap. However, given the need to allow binding and equilibration of analyte and competitor conjugate, the signal typically takes tens of minutes to settle, and the signal amplification steps are often nontrivial.

## Aptamers

Immunosensors have had a relatively long history, whereas nucleic acid aptamers and protein affimers are newcomers, especially in the analysis of plant hormones (107). Aptamers are single-stranded RNAs or DNAs that fold to create binding sites for ligand molecules, which might be small or large. There is a rapidly growing industry generating aptamers for diagnostics and therapeutics, but they are also targets for use as switches in vivo. The first report of aptamer activity in plants was the discovery of the natural riboswitch responsive to thiamine pyrophosphate (130). Later, an opinion paper suggested that plant hormones—especially, perhaps, the CKs—bind not only to their known protein receptors, but also to natural, endogenous riboswitches (41). One small step forward from these observations was the application of aptamers as analytical reagents for assaying plant hormones, which has numerous advantages. Once selected, they are cheap, versatile, and highly reproducible, and the chemistry for conjugating reporters at 5′ and 3′ ends is readily available for use in biosensors or, in vivo, in riboswitches to interact naturally with endogenous circuitry.

**Aptamer:** an RNA or DNA molecule that folds into secondary and tertiary structures that provide binding sites for specific analytes; aptamers are the nucleic acid equivalents of antibodies

Artificial aptamers are selected by rounds of affinity-based panning, a process generally called systematic evolution of ligands by exponential enrichment (SELEX) (28). The initial libraries are tremendously diverse (typically starting at $10^{14}$ aptamer sequences) and comprise a variable region (generally 20–60 nucleotides) between constant primer sections. After each round of selection, the sublibrary is amplified by a polymerase chain reaction. To promote selection of high-affinity aptamers, the stringency is increased at each round by additional washes, increased salt concentration, washing with analogs against which activity is undesirable, subtractive capture, and so on.

Diagnostic aptamers to some high-profile small molecules are well characterized [e.g., quinine and cocaine (113)]. For plant hormones, only one group has adapted the ideas and opportunities afforded by aptamers so far, for zeatin (70, 104), although aptamers to, e.g., L-tryptophan and the plant peptide systemin have been known for some time (9, 115, 136). In these reports, aptamers were selected using SELEX with ligand-coated Sepharose beads. For zeatin, histidine-coated beads were used for subtractive selection, and in later rounds, adenine was included in the binding buffer to ensure selectivity. After 24 rounds of SELEX, 40 clones were sequenced and then sorted into five groups based on sequence similarities. Next-generation sequencing and informatics are becoming common, reducing selection times and optimizing recognition of core motifs (110). The next stage is often to use truncation and mutagenesis strategies to minimize the aptamer's size and maximize affinity. It is not uncommon for parts of the primer ends to contribute important bases, both to the recognition motif and to stem, loop, and hairpin structuring elements. Additional structural elements include G-quadruplex folds, and these are important for both systemin and zeatin aptamers (9, 104).

Aptamers have been adopted into classical electrochemical sensors and as fluorescent beacons. Beacons are oligonucleotides linked to fluorophores and have been used in multiple formats for reading aptamer and ribozyme activation. For electrochemical zeatin biosensors, a fluor is attached to a thymine base in the loop region and aptamer binding site. In the absence of a ligand, the aptamers adsorb onto a sensor surface of graphene oxide, which is an efficient quencher. In the presence of zeatin, adsorption is impaired, and the enhanced fluorescence signal is recorded and calibrated. The sensitivity is low compared with that of other biosensor systems (**Table 1**), but the selectivity is reasonable, with high activity against *cis*- and *trans*-zeatin, *trans*-zeatin riboside, and dihydrozeatin and low activity against adenine, adenosine, and ATP (104). The sensitivity was improved (0.7–6 μM; **Table 1**) by further work that also showed simultaneous monitoring of systemin with zeatin using different fluors on the two aptamers and the same technique of beacon quenching by graphene oxide (115). The signal may be read rapidly at the end of the incubation

**Table 2 Genetic reporter sensitivities**

| Plant hormone | Promoter | Sensitivity | Reference |
|---|---|---|---|
| Auxin | DR5 | 10–1,000 nM | 88 |
| | DR5v2 | 3–1,000 nM | 69 |
| | DII-VENUS | 1–1,000 nM | 10 |
| | R2D2 | Not determined | 69 |
| | L2min17-Luc | 10–1,000 nM | 134 |
| Cytokinin | ARR5 | 10–1,000 nM | 106 |
| | TCS | 1–1,000 nM | 86 |
| | TCSn | 1–1,000 nM | 149 |
| Abscisic acid | ABACUS | Various (e.g., 1–100 μM) | 53 |
| | ABAleons | Various (e.g., 20 nM–1 μM) | 129 |
| Jasmonic acid | Jas9-VENUS | 50 nM–10 μM coronatine | 63 |

time, but both equilibration and nuclease digestion require 2 h on the sensor surface. Liu et al. (70) recently reported a direct assay using fluorescence, but both the dynamic range and sensitivity will need improvement for use in vivo.

## Genetically Encoded Biosensors

It is revealing that reviews on analytical methods do not generally consider genetic reporters (e.g., 20, 27, 102), although some recent reviews of selective plant biosensors have appeared (e.g., 127). In the past, this has been justified because transcriptional reporters have been qualitative tools, but more recent work has developed biosensors capable of delivering quantitative data in situ and in real time.

Most plant hormones have had genetic reporters developed for them based on hormone-responsive promoters or promoter motifs (133) (**Table 2**). Minimal promoter elements are preferred because they are less likely to be regulated by additional drivers; the DR5 auxin response element has been the most successful of these, although even this small motif does retain some nonauxin reactivity (133). For the same reason, the CK-driven two-component system (TCS) promoter has succeeded *Arabidopsis* RESPONSE REGULATOR (ARR) reporters. In most cases, β-glucuronidase was the initial reporter element, but the fluorescent protein family has proved far more versatile. Despite the shortcomings of transcriptional reporters, there is no question that the positional and (to a lesser extent) temporal information gained from their use has greatly increased our understanding of hormone physiology.

The challenge of improving temporal resolution has been addressed with, e.g., rapid-folding variants of fluorescent proteins (such as VENUS) and luciferase. Importantly, the sensor strategies have also shifted to monitoring posttranscriptional events, capitalizing on the rapid ubiquitination and degradation of many plant hormone effector elements. This has worked particularly well for auxin with the reporter system DII-VENUS, which couples the degron motif of IAA28 with VENUS (carrying nuclear-localization sequences) behind the 35S promoter to ensure strong and constitutive expression in planta (10). Following auxin addition, the degron binds to the $SCF^{TIR1}$ auxin receptor complex, leading to ubiquitination of DII-VENUS, which then undergoes rapid proteolysis and loss of fluorescence.

The DII-VENUS system has been widely adopted, and in combination with mathematical modeling, it can report changes in auxin gradients within 5 min during root gravitropism (6). Use of the reporter alone could not detect measurable changes in the auxin gradient in less than

30 min, but when multiple records were combined in a model based on cell geometries and auxin carrier distributions, the model allowed the prediction of more rapid initial changes in auxin relocalization.

A similar degron-VENUS reporter has been developed for JA (63). Changes in the Jas9-VENUS signal were recorded within 4 min and showed all of the selectivity characteristics anticipated from CORONATINE INSENSITIVE 1 (COI1)–dependent ubiquitination. The sensor was used to map local changes in JA concentration in *Arabidopsis* roots and to illustrate two phases of JA accumulation distal to wound sites. This exciting work suggested that an initial wound signal traveled at over 1 cm/min from leaf to root, followed by a much more substantial rise in JA in a second, slower wave starting after approximately 30 min. Data from rapid in vivo biosensor monitoring led to a new hypothesis that the first, rapid wave was a conversion from stored conjugates, and the second, slower wave was from newly induced JA biosynthesis.

## Improving Quantitation

Despite the utility of degron-VENUS sensors, they remain essentially qualitative reporters because absolute fluorescence is difficult to quantify in complex tissues. Consequently, DII-VENUS has been redesigned with an alternative promoter, *RIBOSOMAL PROTEIN 5A* (*RPS5A*), in a duplex with a reference fluorescent protein (Tomato) fused to a mutated, auxin-insensitive DII domain to create R2D2 (a ratiometric version of two DIIs) (69). Expression produces equal quantities of DII-VENUS and mDII-Tomato. In the presence of auxin, the VENUS signal decreases. The ratio of the yellow VENUS signal to the red Tomato signal may then be related to the concentration of auxin, and this signal is consistent with known auxin distributions in embryos and roots. As the name implies, R2D2 allows ratiometric analysis and, hence, serves as a quantitative biosensor for auxin that can be read in vivo in real time. Unfortunately, this study did not include a calibration to auxin concentration, but it did show that a significant change in signal can be recorded within 10 min in auxin-treated roots (69).

The R2D2 sensor followed the development of conceptually similar ratiometric biosensors based on DII domains and paired luciferase enzymes (134). In these biosensors, a self-processing linker peptide ensures stoichiometric expression of two luciferase variants. The second luciferase is fused with the DII degron of IAA17, rendering it susceptible to auxin-driven degradation, as for DII-VENUS. The sensor performs strongly in mammalian and plant cells (protoplasts), but this system is not amenable to more widespread use, given the requirement to infiltrate the cells with luciferase substrates and ATP prior to measurement.

## Förster Resonance Energy Transfer and Abscisic Acid

An additional method to retrieve quantitative data from fluorescence is to use FRET, a system that has already been well explored by those studying calcium, redox, and sugar signaling in plants (107, 127). Two similar but distinct FRET-based biosensors for ABA have been developed: abscisic acid concentration and uptake sensor (ABACUS) (53) and ABAleons (129). The ABA sensors use the receptor itself [PYRABACTIN RESISTANCE 1 (PYR1)/PYR1-LIKE 1 (PYL1)/REGULATORY COMPONENT OF ABA RECEPTOR 1 (RCAR1)] as one part of the cassette (**Figure 3**). Following ABA binding, two loops of the receptor fold over the ABA ligand, forming a new surface onto which specific clade A type 2 protein phosphatases (PP2Cs) bind. By creating a single polypeptide with an in-frame cyan fluorescent protein, receptor, flexible linker peptide, truncated phosphatase, and yellow fluorescent protein (CFP-PYR-PP2A-YFP), the two research groups have generated ABA-sensitive FRET biosensors. In the absence of ABA, the two fluorescent proteins are close enough to FRET. ABA binding brings the PP2A complex over

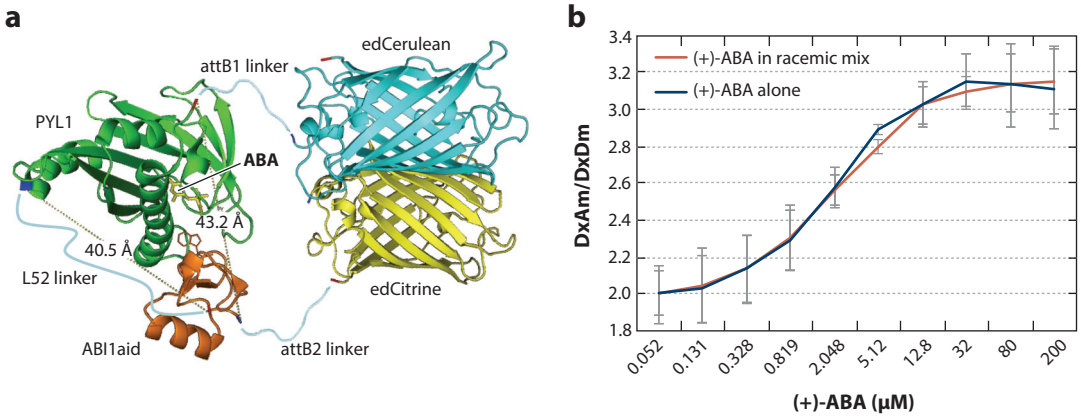



**Figure 3**

The abscisic acid (ABA) concentration and uptake sensor (ABACUS) biosensor (53). (*a*) A diagrammatic representation of the ABACUS protein. (*b*) The dose-response curve for ABACUS, showing low micromolar affinity. The *y* axis represents the magnitude of the ABA-induced change in the ratio of Förster resonance energy transfer (FRET). The value DxAm/DxDm is calculated as the ratio of FRET acceptor emission (Am) to FRET donor emission (Dm) after excitation of the FRET donor (Dx).

the locked ABA-binding site, and steric constraints either force the two FRET partners apart or reorient them, reducing FRET.

The dynamic range of both ABA biosensors appears to be reduced (two decades) (**Figure 3**) compared with those of many of the other sensors discussed above (typically three decades), but the binding data remain consistent with classic Langmuir binding reactions. To widen the dynamic range, both groups developed a set of variants with differing affinities for ABA, thereby broadening the sensitivity range (53, 129).

The ABA biosensors have endogenous receptor-determined specificity and are insensitive to pH over the physiological range (pH 6.6–8.2), and both ABACUS and ABAleons have been expressed in plants, providing novel data on ABA accumulation and transport after challenge with stress or exogenous ABA. There is some concern over effects in unstressed phenotypes, which have been attributed to the biosensor sequestering ABA. Expression levels and targeting may need to be managed carefully, but the temporal and spatial resolution gained by these FRET biosensors makes them impressive and desirable tools.

### Affimers

Affimers are based on small, stable protein scaffolds engineered by mutagenesis to give highly variable binding proteins. As proteins, they can be engineered into single-polypeptide, genetically encoded biosensors or used as alternatives to antibodies in, e.g., impedimetric biosensors (51). The fluorescent indicator protein (FLIP) sugar sensors based on bacterial sugar-binding proteins developed in the Frommer laboratory are highly developed affimer-type biosensors (96). Unfortunately, affimers have not yet been selected for plant hormones.

## COMPARISON BETWEEN AND VALIDATION OF METHODS

It is clear from the discussion above that the two approaches to analytical measurement excel in distinct ways. MS is the benchmark technique, with a long and distinguished history of use with all the plant hormones. In combination with appropriate sample workups, it is exquisitely precise

in assigning and quantitating small molecules and can measure a large number of molecules at once. Increasing sensitivity combined with, e.g., cell sorting will allow measurement of the hormonome in single cell types, as discussed above, although methods to cover both polar and nonpolar molecules in one analysis remain challenging. Similarly, single-cell-resolution analysis is the most difficult task, and MS is still not commonly used for live imaging of phytohormones. It is at this interface—of accuracy with spatial and temporal definition—that biosensors have a place. The solid-state biosensors can be placed accurately and record quantitatively and in real time. However, under almost all measuring circumstances, there will be some tissue trauma. This is likely to be less serious than, e.g., protoplasting, which continues to be a technique of choice for a range of cell-type-specific studies. It is also worth considering that electrochemical biosensors continue to help forge new understanding in, e.g., animal developmental biology. Nevertheless, their use in plant biology remains rare.

One key difference is that of the concentration value derived from the different approaches. MS corrects for losses of analytes during sample workups by adding standards on extraction. It therefore provides absolute quantities of each molecular species per sample, which can be converted to estimates of concentration for each compound in the plant tissue and then contrasted with the concentration values derived from biosensors. Because biosensors are calibrated, their output value is concentration, but this value may not represent the concentration of a single molecular species. For example, Tian et al. (124) showed that the use of CKX confers high selectivity for CKs, excluding other prevalent purines; however, there are many CKs, and a biosensor gives a value that is the integral of all substrates present. The CKX biosensor was calibrated using isopentenyladenosine (iP), and a unit of iP equivalents was then defined to account for the CKX signal gained from CK ribosides as well as free bases (124). Validation by MS showed very good agreement between the biosensor iP equivalent value and the MS data once the latter had been converted to iP equivalents by summing the concentrations of each component compound.

For some experiments, the disadvantage of losing information on each component of a signal is counterbalanced by advantages gained in scale and speed. For example, a CK biosensor fabricated on a 25-μm platinum wire has been placed inside the endosperm of very young embryo sacs in developing *Arabidopsis* seeds (F. Tian, J. Gutierrez-Marcos & R. Napier, unpublished data). To provide sufficient material to validate the biosensor, 50 equivalent seeds were bulked and the sensor records validated by MS. Comparison of the data showed that young embryo sacs from some mutant lines were highly enriched in CKs in both cases, but the biosensor data from inside the seed showed that the concentration there was far higher than that measured by MS from the seed homogenate. The explanation for this result is that the biosensor was recording from a small and very precise compartment, whereas obtaining sufficient material for MS required using the entire early seed, with consequent dilution of the overall concentration.

The latest advance for CK electrochemical biosensors uses a bacterial enzyme, cytokinin deaminase (CKD) (40), which carries a major advantage over the CKX system in that it does not require a mediator (F. Tian, J. Gutierrez-Marcos & R. Napier, unpublished data). The CKD biosensor also has nanomolar sensitivity, is insensitive to ribosides, and yields a signal that represents the concentration of free bases alone. Nevertheless, those applying biosensors need to weigh the disadvantages of, e.g., loss of molecule identity against potential advantages, such as scale and directness of measurement.

Given that some hormones (such as auxin) are essentially only one compound, the example given above for CKs may be an extreme case. In such instances, a biosensor signal may truly reflect the free concentration of that hormone. In this respect, the records of ABA concentration from ABACUS and ABAleons appear to be faithful records of prevailing ABA concentrations throughout these plants (52). The use of genetically encoded biosensors also avoids tissue trauma,

although there remain problems related to local depletion of the analyte and the consequent distortion of the plant's physiology. Nevertheless, genetically encoded biosensors offer detail in time resolution and tissue and cellular distributions for a wide variety of circumstances, and their use will grow (127). They are not yet ready for reporting hormone concentrations from subcellular compartments, and studying the apoplast may remain a challenge, but biosensors suitable for these roles will appear. There is no doubt that biosensor technologies and tools will continue to improve, but it will be wise for biosensor developers to continue to seek validations of their measurements from MS. In turn, MS continues to increase in sensitivity, and protocols for sample preparation are also evolving to address losses during simultaneous isolation of the large range of metabolites.

## CONCLUDING REMARKS AND FUTURE PERSPECTIVES

### Adaptation of Aptamer Technologies for Deployment In Planta

Genetically encoded biosensors have exploited proteins so far, but there are options for engineering genetically encoded RNA-based biosensors. For example, the Spinach aptamers are RNA mimics of the fluorescent proteins and are available in a full spectrum of color variants (103). RNA-based biosensors have the potential advantage of far better time resolution, given that transcription is faster than translation, although this is tempered by more rapid breakdown, which challenges signal intensity. Nevertheless, RNA aptamers coupled to Spinach variants offer exciting possibilities for the future.

Quantum dots and other nanoparticles also offer opportunities for displaying and delivering aptamer biosensors. Quantum dots are generally based on heavy-metal crystals, which provide fluorescence emissions with a high quantum efficiency. Coated with a sulfide layer, such as ZnS, they can carry coatings of biological molecules (such as antibodies) to create efficient diagnostic markers, and some carbon-based nanodots are becoming available (54, 70). An annulus of DNA aptamers is also possible: Conformationally active aptamers coupled to FRET partners for the quantum center could provide nanoprobes, and quantum dots permeate throughout plant tissues (58). The plant sensor world may not yet be prepared for techniques such as DNA point accumulation for imaging in nanoscale topography (DNA-PAINT), which rely on advanced aptamer technologies (45, 54), but there are exciting prospects for those who are working to advance the frontiers of analytical hormonomics.

### Mass Spectrometry–Based Technologies

The rapid development of MS-based analytical techniques is also likely to continue, allowing for organelle- and cell-type-specific analyses of the majority of plant hormones and their metabolites. New technologies, such as MSI and live single-cell analysis, might be available for plant hormone quantification in the future, although at present their sensitivity is too low. The trend toward simultaneous quantification of several plant hormone classes (hormonomics) will continue, making it easier to monitor changes in hormone profiles during development and to identify genes involved in hormone metabolism and signaling.

#### SUMMARY POINTS

1. Mass spectrometry remains the benchmark technology for identifying and analyzing phytohormones, and recent improvements in sensitivity are tightly linked to the rapid advances in mass spectrometry technology.

2. Optimized sample purification is equally important for the highest sensitivity possible in mass spectrometric analysis, and new types of adsorbents, miniaturization, and online purification have the potential to increase the sensitivity even further.
3. Multiple parallel analysis by mass spectrometry is now leading to hormonomics—the profiling of large collections of phytohormones and their metabolites from single samples.
4. Biosensors offer real-time quantitation of specified phytohormones in vivo.
5. Genetically encoded biosensors have huge potential, offering trauma-free, tissue- and plant-wide visualization of prevailing hormone concentrations at cellular resolution.

## DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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