Title: Biosensors in plants
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Highlights:

- Of many biosensor types, few have been applied in plant biology
- Sensors suited to adaptation and application in plants are identified
- Genetically-encoded biosensors are proving powerful tools for auxin and a few metabolites, but these remain qualitative tools
- Real-time, quantitative biosensors are the way forward

Summary
Biosensors come in an increasing array of forms and their development is defining the rate of advance for our understanding of many natural processes. Developmental biology is increasingly using mathematical models and yet few of these models are based on quantitative recordings. In particular, we know comparatively little about the endogenous concentrations or fluxes of signalling molecules such as the phytohormones, an area of great potential for new biosensors. There are extremely useful biosensors for some signals, but most remain qualitative. Other qualities sought in biosensors are temporal and spatial resolution and, usually, an ability to use them without significantly perturbing the system. Currently, the biosensors with the best properties are the genetically-encoded optical biosensors based on FRET, but each sensor needs extensive specific effort to develop. Sensor technologies using antibodies as the recognition domain are more generic, but these tend to be more invasive and there are few examples of their use in plant biology. By capturing some of the opportunities appearing with advances in platform technologies it is hoped that more biosensors will become available to plant scientists.

Introduction
The term biosensor has been attached to many technologies. They can be both qualitative or quantitative, genetically-encoded or solid-state physical devices, give real-time or time-fractionated data, *in vivo* or *ex vivo* and of many other designs. Developments in all these areas are advancing apace, led in different fields by the

need for cheap, reliable diagnostic tests, non-invasive recordings and by the ingenuity of researchers.

In essence, a biosensor couples a recognition event by a biological receptor to a transformer which converts this recognition event into a signal that can be captured and interrogated. Two common versions of these configurations are an enzyme coupled to an electrode with the output then electronic, and a ligand-sensitive receptor coupled to fluorescent proteins which are interrogated by confocal microscopy. Both these examples suggest measurements taken in vivo, and both can be real-time, but many other biosensor options are ex-vivo measurements and for these the versatility of antibodies as sensor units is prevalent, although molecular imprinting and many other recognition technologies may also be used. Current advances are in the areas of multiplexing sensors, high-throughput, DNA aptamer sensors, and increasing sensor robustness, resolution and sensitivity with the advent of nanotechnologies.

In order to limit our coverage, we will review progress in the development of biosensors for plant developmental signals and the abundant opportunities awaiting suitable sensors. In particular we will consider phytohormone signalling, although other systems will be covered in order to capture the breadth of biosensor applications. Examples of various biosensor systems are given in Table1.

The ideal biosensor is selective, sensitive with a favourable signal to noise ratio, gives a quantitative (calibrated) dose-response curve over physiologically-relevant concentrations of analyte (usually over several orders of magnitude), gives a localised (spatially resolved) reading in vivo, has a fast-response yielding time-resolved data and yet is not invasive and does not disturb the endogenous responses of the system. Consequently, all biosensors are compromises! Yet the fact that this is true does not necessarily devalue the information they give. The impact of each compromise is determined by the experimental system and, generally speaking, the information they provide is an advance over what has gone before.

Biocatalytic electrochemical biosensors

Biosensor advances have been led by the glucose sensor, now sold globally for blood glucose monitoring by diabetics [1*]. Glucose oxidase has an FAD cofactor and, in early electrodes, the redox transfer of electrons from glucose was channelled via FAD into hydrogen peroxide in the presence of molecular oxygen. Peroxide was then detected at a platinum electrode. Biosensors based on similar oxidases or coupled enzymic reactions record current as peroxide is oxidised back to oxygen at the electrode at an operating potential of around 600 mV. They have been adopted widely, from sensors for pathogen detection, food quality testing (eg using lactate oxidase) [2] through to environmental monitoring, although there are few applications in plant biology. More recent developments incorporate mediators, electron transfer intermediates tethered to the electrode or available to diffuse freely in the electrode matrix, often a silicate sol. The use of mediators bypasses the production of hydrogen peroxide to allow more sensitive, longer-lasting and less damaging sensor systems [1*]).

Opportunities for biocatalytic sensor applications in plants

One of the beauties of enzyme-based electrochemical sensors is that the biosensor is designed on a molecular recognition event that has evolved naturally, often with high

selectivity and of an appropriate affinity for working under endogenous substrate concentrations. A survey shows that several of the plant hormones are catabolised by redox enzymes, raising the potential for selective, enzyme-based biosensors. Auxin, cytokinins and gibberellins are all deactivated by oxidases, for example. IAA oxidase remains somewhat elusive as a specific enzyme, with many peroxidises able to oxidise IAA but with low substrate specificity. Gibberellin is deactivated by GA 2-oxidases [3]. These 2-oxoglutarate–dependent dioxygenases (as is the widely used glutamate oxidase) [4] express in soluble, active form from *E. coli* and so might be suitable for GA sensors, especially with the benefit of recent advances in carbon microfiber electrodes and mediators [1*, 5*]. Cytokinin oxidase is better classed as a dehydrogenase [6], but it is both active and exceedingly stable after expression in heterologous systems. Early work using AtCKX2 has indicated that electrochemical sensors for cytokinin are feasible (Kowalska, Frebort and Napier, unpublished).

Several commercial biosensors use redox enzymes from plants. For example, superoxide dismutase is used for antioxidant assessment in health monitoring [7] and tyrosinase (monophenol monooxygenase) for monitoring pollution by phenolics [8]. Laccase (a copper-containing oxidase) is used in food monitoring to detect plant flavonoids [9]. No record can be found of the use of these sensors to study plant development.

It has become clear that extracellular ATP is an extracellular signal in plants as well as animals, and is involved in elongation growth [10*] and defence responses [11]. One elegant biosensor which could be applied to plant signalling is the ATP biosensor [12] based on a coupled enzyme system. The enzymes are immobilised in a porous layer of polymerised silane on a platinum electrode. The electron flow induced by regeneration of the final, redox enzyme in the pathway is monitored by voltammetry. The electrodes are small, 25-100 μm, fast and stable. Extracellular plant ATP has been studied using a non-electrochemical, genetically-encoded biosensor (see below). By fusing luciferase to a cellulose-binding peptide [10*] the sensor became locked onto the cell wall, allowing monitoring of apoplastic ATP in roots. The system does need the infusion of luciferin, but the signal appeared selective and was sensitive to micromolar ATP concentrations. Although it could not be truly quantitative, this sensor gave high spatial resolution and was temporally dynamic. Other genetically-encoded biosensors are discussed below.

Non-enzymatic electrochemical sensors

Having illustrated the potential and noted the adaptability of enzyme-based (biocatalytic) sensors, it is also true that their application has limitations, not least the fact that access to a specific, stable enzyme is required for each analyte of interest. Consequently, other electrode-based sensor systems have been developed, non-enzymatic electrochemical sensors and affinity-based sensors [1*].

One electrochemical biosensor system is noteworthy, that based on the direct oxidation of the auxin IAA at the electrode surface at high electrode operating potentials [13]. Early carbon (graphite) pastes [13] have given way to carbon nanotube-coated platinum electrodes [14] which essentially increase contact area and electrical connectivity. By incorporating the self-referencing vibrating microelectrode technique, selectivity and sensitivity were improved [15**]. These auxin-sensitive microelectrodes are positioned close to the tissue surface and the tip vibrates in the

boundary solution layer to sample the auxin signal at two positions 10 μm apart. At the position closest to the tissue auxin measurements will represent the activity of auxin as it is lost from the cell wall. This signal is referenced against that from the electrode at its outer extreme of movement, a site representing close to background concentrations of auxin. By calculating the difference (self-referenced) signal, the sensor tends to subtract out noise, cancelling contributions from competing ions etc. giving a measurement of auxin activity at the surface. By calibration and using the dimensions of the electrode with Ficks laws on diffusion, local auxin flux can also be calculated.

Auxin electrodes were sensitive between around 0.1 and 50 micromolar IAA [14], shown to give readings from plant extracts in accord with extraction and analytical techniques, to be stable over hours of recording, and the vibrating electrode version has been used to quantitate fluxes from root apices [15]. These are clearly useful tools, and the flux measurements are in accord with models of auxin movement in this tissue. Unfortunately, the application of these electrodes has been limited, due mainly to operational restrictions. The more advanced self-referencing electrode, for example, may only be suited to records at the surface of 'permeable' tissues like the root apex because of its need for free vibrational movement in the boundary layer, but it is non-invasive.

Affinity-based biosensors

Recognition need not be harnessed to catalysis. Receptor proteins can provide some of the same properties as enzymes, and well-selected antibodies provide highly selective and high affinity recognition for almost any soluble analyte. Receptors are not always suitable, often due to lability or requirement for a membrane. Antibodies on the other hand are robust and versatile and have been adapted into diverse immunosensor systems. Affinity recognition need not be based on biomolecules as the recognition element and some robust sensor systems make use of molecular imprinted materials and volume-sensitive hologram-based biosensor surfaces [16]. The most recent addition to the family of affinity-based sensor technologies is the development of nucleic acid fragments as recognition species, especially the use of selected or designed aptamers (eg short 20-40 nucleotide, single stranded DNA) which bind the analyte [1*,17*]. In all cases there is no enzymatic activity to measure for signal transduction, but a range of quantitative analytical methods are widely available including electrochemical, such as by impedometrics [18*], versions of activity mapping by atomic force microscopy [19**], and optical such as by hologram [20], fluorescent tags or various biophysical platforms, such as microbalance and surface plasmon resonance (Table 2).

In almost all cases the assay formats for affinity-based sensors have been *in vitro* and so to get any record of the response timescale a series of time-fractionated samples must be prepared. Consequently, affinity-based assays generally do not give a dynamic, interactive record and may miss transient changes. There is no doubt that for applications in eg diagnostics or environmental monitoring this is adequate and appropriate. Cost-effective, high-throughput systems are available, some with parallel, multiplexed assays [1, 21, 22].

The plant biology world does have some very good antibodies to eg plant hormones and these are starting to be developed into biosensors. An electrochemical

impedence-based electrode based on an antibody has been described for ABA [23]. This is reported to have a sensitivity range of 1 nM to 1 μM, although there was an unfavourable pH dependence and a slow time resolution because it relied on binding equilibrium being reached. A sensor for auxin based on a competitive immunoassay recorded by QCM (quartz crystal microbalance) has also been described [24] and a voltametric electrode for gibberellins [25], each with similar good dynamic ranges, but poor time resolution. They have yet to yield novel biological information.

Outside the world of diagnostics, time resolution is often a limitation in immunological biosensors because of the need to allow binding to approach equilibrium, usually a matter of tens of minutes. However, redesigning the familiar competition-based assay to analyse data from the dissociation kinetic (as opposed to the normal binding/association curve) does allow quantitative real-time recordings (Badescu and Napier, unpublished). Of course, an assay of this type is *ex vivo*, but the assay platform combined with continuous sampling using microfluidics and microdialysis gives an antibody-based, quantitative real-time biosensor. The versatility, cost and availability of antibodies for immunological sensors carry user advantages. Plant developmental biology could undoubtedly benefit from harnessing the power of these immunosensor systems.

Genetically-encoded biosensors – promoter::reporters

One reason why solid-state sensors have not been widely exploited in plants has been the widespread success of genetically-encoded sensors. Genetic reporters sensitive to the activity of particular hormones have been available for a number of years, and amongst the most widely used has been the auxin-sensitive reporters based on the synthetic promoter DR5 [26]. The first generation used DR5 fused to β–glucuronidase (GUS) and this reporter gene has helped describe some of the many sites of auxin accumulation, in particular after gravitropic stimulation. More recently, optical sensor systems based on green fluorescent protein (GFP) and other FPs have become the fusion partners of choice.

The term auxin biosensor has been used to describe the DR5-GFP reporter [27]. It was used to define the temporal change in auxin activity maximum in the root columella and lateral root cap cells after gravitropic stimulation. Many other groups have also capitalised on the value and versatility of DR5-GFP and versions of it. Coupled with confocal microscopy and co-localisation with reporters for auxin transport proteins or IAA biosynthesis enzymes, for example, these biosensors have provided us with beautiful and detailed accounts of very localised and temporal auxin signals [28**].

The importance of promoter-reporter fusions in developmental biology is not under question, and there is no doubt that DR5 and similar biosensor drivers have contributed greatly to our understanding of plant signalling, but we should ask whether this type of biosensor is as good as we can get.

There are many attractions to the use of genetically-encoded, promoter-driven biosensors. They are free of the need to puncture or otherwise wound the host cells, they can be driven by sensitive, signal-dependent promoters and many can be monitored in living cells in real time. However, they are not perfect. DR5 is considered a fairly faithful reporter for IAA activity [26] but it is also induced by

brassinolide and some tissue selectivity is known [29,30]. Reporter systems for other hormones have been less instructive and even less analyte-specific [31].

There is also the question about time-responsiveness. Clearly, from induction to functional reporter there is a time delay. In gravitropism, auxin activity changes in the root could be detected by DR5::GFP only after 1.5 hours [27]. The need for more rapid sensors might be questioned for some analytes, yet there is no doubt that auxin, for example, redistributes rapidly and polar flow is generally recorded at around 10 mm/h, which means it may cross hundreds of cells each hour. Further, it is unwise to assume that because we have not previously been able to measure rapid changes in analytes that they do not exist or do not matter. GFP is generally a stable protein and so transience in the amplitude of a signal and the frequency of transients may also be missed, yet might be vital for message decoding as they are for intracellular calcium concentrations [32].

Reservations about some of the characteristics of the fluorescent proteins has led to novel, improved versions. Mutagenesis of YFP yielded Venus, a variant with reduced sensitivity to pH and chloride ions, and a much faster maturation time [33]. Triplicated FPs targeted to specific intracellular compartments [34*] have been used to good effect to increase signal amplitude and precision, but the complexities of driving the sensor signal via promoter activation, transcription, translation, maturation and degradation make the promoter-driven sensors qualitative reporters, not quantitative.

Genetically-encoded biosensors – other options

The optical biosensor field has been led and enlightened, in particular, by calcium sensitive reporters such as aequorin and its successors the cameleons [35]. The cameleons utilise the large conformational rearrangement induced by calcium binding to calmodulin to change the proximity of paired FPs fused at either end, frequently CFP and YFP or their variants, and the sensor is interrogated using FRET microscopy. Second generation versions were improved for selectivity by using engineered versions of calmodulin and a calmodulin-binding peptide in the bridge between FPs so that on binding calcium the sensor folds up on itself to promote FRET [35]. Other modifications generated a set of cameleons with differing calcium dose-dependencies to allow measurement over a wide set of concentration ranges suited to differing cellular compartments. FRET can be ratiometric allowing the recordings to be calibrated and quantitative. Furthermore, because expression of the biosensor is constitutive, there is no time delay before a signal can be recorded. Temporal changes can be recorded on the timescale of less than a millisecond [36**], although data acquisition timescales are frequently of the order of seconds due to constraints in the microscope. Likewise, dissociation and reversal of the signal is rapid making these biosensors very dynamic tools.

Examples of the use of cameleons in plants include the spiking signatures of cytoplasmic calcium induced by nodulation in Medicago [37] and intranuclear calcium spikes in root hairs [38**]. It should be noted that other calcium-sensitive chemical dyes such as Oregon Green are also still extremely useful even though they require single cell injections [39]. These allow analyses in plants without the need for transformation.

The depth of accumulated knowledge acquired from successive generations of sensors for intracellular calcium signalling is well reviewed [32] and developments continue [40**]. However, other metal ions are also amenable to genetically-encoded (and other) biosensors, although these have yet to be deployed in plants. Zinc enjoys a set of possible sensors based on both natural zinc-binding protein domains, such as metallothioneins, and on synthetic motifs [41*, 42*] and magnesium sensors can be envisaged based on engineered Troponin C [43]. Staining embryos of Norway spruce with zinc-sensitive chemical probes indicated that intracellular zinc mediates programmed cell death in plants [44]. More versatile analysis with biosensors may reveal additional roles in developmental responses.

Following on the heels of the cameleons has been the elegant engineering of bacterial periplasmic sugar-binding proteins to yield a set of optical metabolite biosensors. As above, the signal is transduced by the fusion of a conformationally-active centre, the metabolite binding domain, with FPs to give FRET reporters [45]. The biggest collection is the FLIPs (FLuorescent Indicatior Proteins) from the Frommer laboratory and these are sensitive to physiologically-relevant concentrations of a set of pentoses, hexoses and disaccharides as well as some amino acids and phosphate [46**]. Such is the power of the FRET sensors that they have greatly improved the science of fluxomics, the record of changes in steady state concentrations over time, with some FRET sensors now allowing more than one analyte to be monitored at one time [45].

One other ratiometric biosensor that has been deployed in plant biology is the redox-sensitive roGFP. In this case excitation efficiency is sensitive to redox state, allowing calibration and a quantitative output. The probe has been used to report on the local redox state after wounding [47*], in various cellular compartments and from inside mitochondria during respiratory challenges [48*].

As discussed above for enzymatic sensor systems, FRET and other fluorescence-based reporters are restricted in use because they have relied on the availability of analyte-specific binding proteins or binding domains. The ability to combine the generic availability of antibodies with FRET-sensitive reporter cassettes would be highly valuable, although unfortunately still a dream. Alternative, widely applicable strategies are starting to emerge however. Many proteins are rapidly degraded once ubiquitinated and this may offer a way to get dynamic data for a wide variety of plant signals for which proteolysis is the natural regulatory process. For example, combining the degron domain of an Aux/IAA protein fused to Venus YFP under a constitutive promoter may yield a much more dynamic reporter for auxin than DR5 (T. Vernoux, ENS Lyon, France; unpublished). Where the size of an FP is troublesome, tetracysteine tags can be used with fluorescent biarsenicals [49]. Although this does require absorption of the fluor from a bathing medium, valuable data have been acquired from both luciferase and aequorin which each need exogenous cofactors.

Opportunities and targets

Modelling development

Plant developmental signalling has been led forward jointly by analytical biochemistry, in particular the advances of mass spectrometry [50**], and qualitative optical sensors. Until the advent of cell sorting to produce quantitative maps of eg IAA, all quantitative techniques lost spatial resolution of the analyte. Even with cell-

specific analysis the opportunities for fluxomics are lost. As noted, the use of qualitative optical sensors has allowed activity mapping with high spatial and increasingly better time resolution. These methods, along with the subcellular distribution maps of PIN and AUX proteins have provided information for several mathematical models of eg auxin signalling and channelling [51, 52**]. However, all such models work around the problem of having no quantitative data on auxin concentration and assume its location. Typically, data for other plant developmental signals trails that for auxin. Biosensors clearly have the potential to illuminate and refine both models and the physiology they mimic.

Biosensors in plant disease detection

Stepping away from hormonal control systems, there are many other areas of plant science for which biosensors could be rewarding. Systemic signalling could be amenable to any of the sensor systems described above [53*] and recent peroxide sensors could help study stress responses [5*], but there are also possibilities in disease detection and diagnosis. Label free biosensors that can detect and quantify specific plant pathogens on-field might enable farmers to target application of pesticides precisely, reducing their use [21*].

Currently antibodies are the preferred detection systems since they offer all the advantages noted above and many pathogen-specific antibodies are available. However, most transducing technologies remain lab-based instruments requiring specialist technicians, such as surface plasmon resonance (SPR), quartz crystal microbalance (QCM) and cantilever-based sensors. Field units need development [21*]. There is better news for volatile sensors and both electronic nose and field asymmetric ion mobility spectrometry (FAIMS) have been shown to be useful early disease and infestation sensors [55*, 56].

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