

Sense and Sensibility: The Use of Fluorescent Protein-Based Genetically Encoded Biosensors in Plants

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Abstract

Fluorescent protein-based biosensors are providing us with an unprecedented, quantitative view of the dynamic nature of the cellular networks that lie at the heart of plant biology. Such bioreporters can visualize the spatial and temporal kinetics of cellular regulators such as Ca^{2+} and H^+ , plant hormones and even allow membrane transport activities to be monitored in real time in living plant cells. The fast pace of their development is making these tools increasingly sensitive and easy to use and the rapidly expanding biosensor toolkit offers great potential for new insights into a wide range of plant regulatory processes. We suggest a checklist of controls that should help avoid some of the more cryptic issues with using these bioreporter technologies.

Introduction

Fluorescent biosensors, i.e., fluorescent molecules introduced into an organism to monitor some parameter of its biological activity, have been used for decades to quantify the real-time spatiotemporal dynamics of myriad signaling processes in biology. Although reporter dyes (small organic molecule-based sensors) have been foundational in developing this area [1], the advent of fluorescent protein (FP) technologies, with the associated possibility of engineering genetically encoded indicators, has led to an explosion of research using this approach. Most of these probes have been developed for biomedical applications and then subsequently applied to plant systems. However, we are now beginning to see biosensors designed to report on plant-specific features such as phytohormones [2-6] or transceptor activity [7-10], offering a new and unique insight into the remarkably dynamic cellular life of the plant.

The field of FP-based reporters of biological processes is now immense and so we will have to limit our discussion to a subset of these technologies. Thus, there are approaches to look at plant hormone signaling responses using *promoter::Green Fluorescent Protein (GFP)* expressing plants (e.g. auxin using *DR5rev::GFP* [11] or cytokinin with *TCSn::GFP* [12]) or through the use of protein:GFPs that are degraded upon hormone perception (e.g., *DII-VENUS* for auxin [13], *Jas9-VENUS* for jasmonate [14]; Figure 1). These are important tools to explore plant cell function and for some plant regulators such as auxin and jasmonic acid remain essentially the only available approaches to follow their cellular dynamics. However, these methodologies are monitoring the output of the cellular response system triggered by e.g., auxin to infer auxin dynamics (Figure 1). In this article we will be focusing on a different family of FP-based sensors where the fluorescence signal is reversibly altered by either direct interaction with the molecule being investigated or in response to a physical parameter of the cell such as

membrane voltage (Figure 1C). These sensors allow real-time imaging of the dynamics (both rise and fall) of cellular components down to the subcellular level.

Genetically-encoded biosensors in plants

The use of genetically-encoded fluorescent biosensors in plants has experienced a renaissance in recent years, owing to the prevalence of sensors developed for animal studies that have been successfully co-opted for plant biology research (Table 1). Generally, sensor design takes an endogenous protein or protein domain sensitive to the parameter of interest and then fuses it to one or more fluorescent proteins. The fusion is made in such a way that the fluorescence of the FP is altered by the conformational change in the sensor domain upon binding of that domain to its cellular ligand or its structural rearrangement in response to change in a cellular parameter such as membrane potential (Figure 1).

There are several approaches that have been developed to translate this conformational change in the sensor domain to altered fluorescence of the attached FP. For sensors based of Förster resonance energy transfer (FRET) the sensory domain changes the relative distance between and/or the relative orientations of two attached FPs [15]. The FPs are chosen such that the energy normally emitted by the ‘donor’ FP of the pair is at the excitation energy needed to excite the “acceptor” partner FP. Thus, upon excitation of the donor, the energy it would normally emit as fluorescence emission is non-radiatively (i.e. no photon is emitted) transferred by resonance transfer as excitation energy to the acceptor which now begins to emit fluorescence. The efficiency of this FRET is proportional to the 6th power of the distance between the fluorophores and is also sensitive to their relative orientations, making FRET emission highly sensitive to changes in their relative positions of the FPs governed by the sensor domain’s conformation. In practice the donor is excited and the emission of donor and acceptor recorded. As FRET

increases, the emission of the donor is reduced and the acceptor increased. The ratio between these two gives a measure of FRET efficiency and so of the activity of the sensor domain. Such ratio imaging comes with the added benefit of being highly quantitatively reliable, correcting for many optical artifacts and differences in expression level and reporter localization in parts of the plant or cell being monitored [15]. Table 1 lists some examples of FRET-based sensors such as the yellow cameleon family of Ca^{2+} sensors (e.g., YC3.6, YCNano and D4) and the hormone sensors (ABACUS, ABAleon and GPS1) that have been successfully used in plants.

One other major class of biosensors being used by plant biologists are built around a single FP. Circularly permuted (cp) FPs have their N- and C-terminal halves transposed and this modified structure allows their insertion into sensor domains such that as the sensor region changes conformation, interaction with the attached cpFP's chromophore (responsible for its ability to fluoresce) is changed. The movement of the cpFP either opens up the structure such that solvent can gain access to the chromophore group quenching its fluorescence, or excludes the solvent, leading to increased fluorescent emission [16]. Thus, these sensors are generally intensimetric in nature, becoming brighter or dimmer depending on the conformation of the sensory domain. Single FP sensors are quantitatively more difficult to use than the FRET/ratiometric versions outlined above as increase in signal brightness from activation of the sensor is complex to distinguish from artifacts such as accumulation of biosensor protein in a particular region of the cell, or changes in the optics of the specimen under study. However, some of the single FP sensors are ratiometric (e.g., Hyper 1-3 [17-19], although the closely related Hyper-red is not). These sensors are also often very sensitive to pH changes [20]. However, despite these potential drawbacks, the single FP sensors show large signal changes in response to the cellular parameter they monitor (e.g., GCaMP, CASE and GECO sensors for Ca^{2+} and the Hyper family of H_2O_2

sensors, Table 1) and are becoming increasingly valuable tools for monitoring plant cellular dynamics.

Although biology has provided the sensor domains, i.e., the protein scaffolds necessary to make the initial version or ‘first-generation’ biosensors, these subsequently undergo significant modifications. For example, alterations in the linkers between FP and sensor domain, random mutation, targeted changes to specific amino acids and incorporation of independent reference FPs have all been used to increase signal-to-noise, fine-tune ligand affinities, improve the quantitative accuracy of measurements and limit, if possible, crosstalk with endogenous biology (see below).

Genetically-encoded calcium indicators

Genetically-encoded calcium indicators (GECIs) are probably the most broadly used FP-biosensors in plants and have been demonstrated to work in a wide range of organisms from mosses to angiosperms (Table 1). Since their first introduction in the late 1990s (e.g., [21,22]), GECIs have accumulated a series of modifications that have led to increased fluorescent intensity and dynamic range and variation in their Ca^{2+} affinity to fine-tune the sensors to specific subcellular compartments. Most GECIs in plants have been used under control of a constitutive promoter (e.g. *CaMV35S::* or *AtUBQ10::*) to observe changes in plant cytosolic Ca^{2+} . However, several groups have been moving from using GECIs as indicators for whole seedling or tissue level analyses, to targeting sensors to specific subcellular locations (Table 1). Sello et al. [23] have shown the potential of this kind of subcellular analysis by targeting a GECI (in this case the luminescent Ca^{2+} reporter aequorin) to chloroplast sub-compartments (stroma, thylakoid membrane and thylakoid lumen). Using this approach, they were able to ask how Ca^{2+} response

to various environmental stimuli is sub-structured within a single organelle. A recent example of the potential power of combining this targeting approach with the quantitative capabilities of the bio-reporter measurements was reported by A Kelner, et al. [24], where two GECIs of the GECO family [25] with different excitation/emission spectra encoded within a multi-cistronic vector. These sensors were used in *Medicago truncatula* to simultaneously monitor Myc factor induced Ca^{2+} changes in both the cytoplasm and nuclear lumen [24]. R-GECO (red) was tagged with a nuclear-localization signal (NLS) while G-GECO1.0 (green) was expressed in the cytosol. allowing the fine scale quantification of differences in the timing of response, down to a sub-second resolution, between these compartments to be defined.

The GECIs represent a prime example of sensor development largely driven by biomedical researchers that has yielded probes that are highly useful for plant biology research. However, plant biologists are now beginning to contribute to the evolution of this sensor family, e.g., adapting them for the quantitative rigor that is needed in the complex world of cellular dynamics and rapidly changing autofluorescence that characterizes plant cells. This next generation of GECIs [6,9] offers the high dynamic range of their parent biosensors but with an internal reference that allows for techniques such as ratio imaging [15] to make quantitatively accurate mapping of plant cell Ca^{2+} dynamics possible.

The development of a generation of plant-related biosensors

Although biomedical research has yielded many probes suitable for use in plants there remain significant gaps in the available biosensor toolkit for many plant-related factors such as phytohormones and key plant nutrients and ions. However, several research labs have now begun to take on the important challenge of developing these plant-focused biosensors. For example,

Ho and Frommer [7] have developed the NiTrac and PepTrac biosensors for plant nitrate and peptide transport and De Michele, et al. [10] developed a sensor named AmTrac capable of imaging ammonium transport activity. This first generation of ammonium sensors has already begun its journey down the road of adaptation and optimization with e.g., the development of deAmTracs showing a dual emission readout [8] and a recent version based on insertion of a nested reference fluorescent protein yielding a high dynamic range, ratiometric (i.e., quantitatively more rigorous) sensor protein (AmTryoshka [9]).

First generation phytohormone sensors for ABA and GA are now also available: ABACUS [2] and ABAleon [3,6] monitor ABA and Gibberellin Perception Sensor 1 (GPS1, [5]), GA. Plants perceive ABA via a signaling cascade where an ABA receptor of the PYR/PYL/RCAR family binds to a protein phosphatase (ABI1) to trigger downstream events. Both of the ABA biosensors capitalize on this physical association between receptor and phosphatase. They are built around a fluorescent donor protein and a fluorescent acceptor protein joined in a single peptide by an ABA responsive ‘hinge’ based on the receptor-phosphatase interaction. ABA binding alters the conformation of this hinge region and so changes the interactions between donor and acceptor fluorescent proteins, altering the FRET between them. Changes in FRET therefore reflect changes in ABA levels (Figure 1). The hinge region consists of a full length endogenous ABA receptor (either PYR1 in ABAleon [3] or PYL1 in ABACUS [2]) along with either the full-length ABI1 (ABALEON) or its 49 aa core receptor binding domain (ABACUS). Although both ABA sensors are FRET-based, the differences in their design lead to important differences in response and sensitivity to ABA. For example, ABAleons show a high FRET signal without ABA that decreases upon ABA binding, whereas ABACUS shows increasing FRET as ABA levels rise.

Gibberellin Perception Sensor 1 represents a similar FRET-based biosensor but in this case for GA [5]. When plants naturally perceive GA, the hormone binds to a receptor of the GID1 family, triggering GID1 association with a DELLA protein that then elicits downstream signaling events. Thus, similar to the ABACUS sensor design, the hinge region of GPS1 is made up of the receptor (GID1C) and a 76 aa core receptor binding domain, in this case from the DELLA protein GA INSENSITIVE 1. Increasing GA levels cause a positive FRET ratio change and the sensor has the capacity to bind to several bioactive forms of GA such as GA₁, GA₃, and GA₄ with K_ds in the 20-200 nM range. However, *in vitro*, GA appears to remain associated with GPS1 even after GA is removed from the surrounding medium, indicating GPS1 may have limitations when monitoring rapid decreases in GA levels *in vivo*.

The dark side of biosensor fluorescence

The biosensors outlined above are important tools for exploring cellular regulatory networks and their increasing ease of use is ensuring the widest group of researchers can apply this technology to understanding the cellular dynamics of plant regulatory networks. However, as with all techniques, there are important caveats that need to be kept in mind when beginning to design biosensor-based experiments. Thus, most of these biosensors are being used in stably transformed plants. This is an important advantage of the genetically encoded reporter approach as it allows for ease of use once the transgenic is established and for reproducibility in subsequent experiments. Most lines are developed using *Agrobacterium*-mediated transformation and so have the potential for positional effects of the insertion both at the levels of affecting expression patterns of the sensor and also the potential to generate an insertional mutant

phenotype due to the integration of the reporter cassette into the genome. Using multiple independent reporter lines to confirm results is one straight forward way to overcome this issue.

By definition, biosensors have to interact with cellular features or molecules in order to sense them. Therefore, these reporters also have the potential to alter the very biology they are designed to monitor. For example, one popular GECI named GCaMP [26,27] was recently shown to lead to phenotypes in animals at both the cellular and whole organism levels through disruption of mammalian L-type Ca^{2+} channels [28]. The effect was thought to be through the biological activity of the calmodulin domain that forms part of the Ca^{2+} sensory hinge region within the sensor. A GCaMP variant, GCaMP-X, has been engineered with an extra linker region to specifically limit the ability of the sensor to interact with these endogenous channels [28]. Palmer et al., [29] addressed similar potential issues of interactions with endogenous cellular signaling networks by mutating both the calmodulin domain and its binding partner within the Cameleon Ca^{2+} sensor's hinge to exclusively tailor the binding of one to the other.

In plant cells, Waadt et al., [6] reported that all the GFP-based Ca^{2+} reporter lines they generated (Yellow Cameleon, Twitch, GECO and GCaMP) exhibited detectable growth inhibition that varied with plant age and reporter expression levels and we too have seen many biosensor-expressing lines with clear growth and developmental phenotypes that make them unusable for subsequent experimentation. Similarly, ABAlleon and ABACUS lines show disruption of ABA-related events, pointing to an interaction of the sensor with the endogenous ABA signaling network [2-4]. All of these observations highlight the requirement to carefully screen reporter lines for phenotypes with the goal of finding lines that show negligible effects on the plants to be studied.

Conclusions

GFP-based biosensors offer an unparalleled quantitative window into the inner dynamics of the plant's regulatory networks, from whole plant responses down to sub-cellular resolutions. These tools are under constant development and improvement and the advent of an array of high dynamic range sensors operating at the wavelengths available on standard fluorescence microscopes offers access to this technology to a wide research community. However, as noted above, there are some key features of each biosensor that can impact on interpretation of the changes seen. Therefore, we propose some minimal controls that could help avoid some of the, often cryptic, artifacts that these sensors can generate.

1. Assess at least two independent reporter lines to avoid potential insertional effects of the biosensor transgene.
2. Assess the expression patterns of the sensor to define if differential expression will bias subsequent measurements towards a specific cell type.
3. Measure growth and development and discard lines that show biosensor-induced phenotypes.
4. Assess the imaging and experimental protocols on untransformed controls to assess if changes in autofluorescence are superimposed on any potential biosensor responses.

Despite these possibilities for artefacts, the FP-based biosensors represent a remarkably powerful toolkit for plant biologists wishing to explore the cellular dynamics that drive control systems within the plant body. Although most of the probes in use at present were designed for animal models and then transferred to plant biology, we now have several research groups producing bioprobes for plant-focused regulators such as plant hormones. We can predict an increasingly bright future for this field of research with families of plant-related bioprobes shedding light on some of the darkest regions in our current models of plant regulatory networks.

Acknowledgements

The work in the authors' laboratories is supported by grants from the National Science Foundation (MCB1329723 and IOS1557899), The National Aeronautics and Space Administration (NNX17AD52G and NNX14AT25G) and the Graduate School of the University of Wisconsin-Madison. RH was supported by NSF GRF and the Graduate School of the University of Wisconsin-Madison. We apologize to our many colleagues whose work could not be included due to length restrictions of this manuscript.

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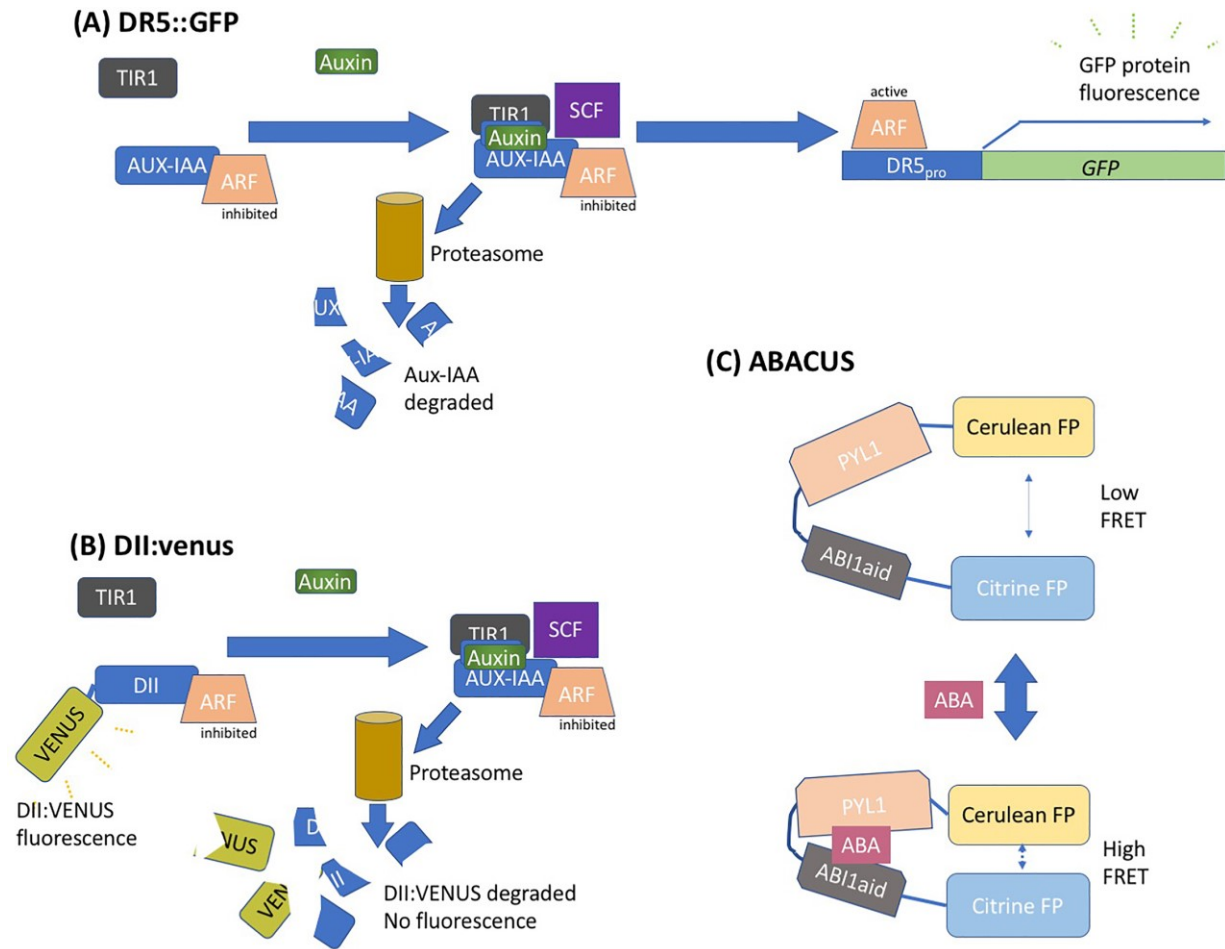


Figure 1. FP-based sensors for plant hormone dynamics. (A) With *DR5::GFP*, auxin activates its endogenous signaling network. Normally the auxin response factor/transcriptional activator (ARF) is repressed by its association with an AUX-IAA protein. When auxin binds to its receptor (a TIR/AFB family member protein) a complex forms between the receptor, AUX-IAA and the SCF complex that targets AUX-IAA for degradation in the proteasome [30]. This loss of the AUX-IAA protein relieves ARF repression and allows induction of auxin responsive promoters such as the synthetic *DR5* promoter element. The *DR5* element can then drive GFP production and so GFP fluorescence represents a proxy for auxin activation of the system. (B) *DII:VENUS* uses the same signaling cascade but substitutes a synthetic AUX-IAA fused with the VENUS FP variant. The DII:VENUS protein is constitutively expressed but is degraded as auxin

levels rise. Loss of fluorescence is used as a measure of auxin activity. DII:VENUS response times are in the minutes time-frame making it more temporally responsive than a transcriptional activator-based reporters such as *DR5::GFP*. (C) In the ABACUS bioreporter, The ABA receptor PYL1 forms a protein hinge with a fragment of the ABI1 phosphatase (ABI1aid). Upon ABA binding, the receptor domain binds to the ABI1 fragment causing a conformational change in the biosensor. This change alters the interaction of the two attached fluorescent proteins such that the FRET energy transfer between them is enhanced. Monitoring FRET signal therefore provides a direct, real-time measure of ABA levels. FP, fluorescent protein.

Table 1. Examples of biosensors used in plants

Target	Biosensor	Species	Subcellular Localization	reference
H ⁺	Ratiometric pHluorin	<i>Arabidopsis. thaliana</i>	thylakoid lumen	[24]
	H148D	<i>A. thaliana</i>		[25]
	PEpHluorin/PRpHluorin	<i>A. thaliana</i>	nucleus, mitochondrion, peroxisome, plastid, Er, Golgi, multivesicular body	[26]
Ca ²⁺	YFP-Aequorin	<i>A. thaliana</i>	chloroplast stroma, thylakoid lumen	[18]
	GFP-Aequorein (G5A)	<i>A. thaliana</i>		[27]
	R-GECO1	<i>A. thaliana</i>		[28]
	R-GECO1-mTurquoise ¹	<i>A. thaliana</i>		[6]
	MatryoshCaMP6s	<i>A. thaliana</i>		[9]
	CASE12	<i>A. thaliana</i>		[29]
	GCaMP3	<i>Nicotiana benthamiana</i>		[30]
		<i>N. tabacum</i>		[30]
		<i>A. thaliana</i>		[31]
	YC3.6	<i>A. thaliana</i>		[32]
	YCNano	<i>A. thaliana</i>		[33]
	D4	<i>A. thaliana</i>	ER	[34]
	YC3.6/YC4.6	<i>A. thaliana</i>	plastid	[35]
	YC3.6	<i>A. thaliana</i>	mitochondrion	[36]
	YC3.6/D3cpv	<i>A. thaliana</i>	mitochondrion	[37]
	D3cpv	<i>A. thaliana</i>	peroxisome	[38]
	NRCG-GECO1	<i>Medicago truncatula</i>	nucleus	[16]
	GCaMP3/YC3.6	<i>Physcomitrella patens</i>		[39]
H ₂ O ₂	HyPer1	<i>A. thaliana</i>		[40]
	HyPer2	<i>N. benthamiana</i>	chloroplast, nucleus	[41]
	HyPer1	<i>A. thaliana</i>	peroxisome	[38]

ATP	ATeam1.03-nD/nA ¹	<i>A. thaliana</i>	plastid, mitochondrion	[42]
ABA	ABAlon2.1 ²	<i>A. thaliana</i>		[3]
	ABACUS-1 ²	<i>A. thaliana</i>		[2]
GA	nlsGPS1 ³	<i>A. thaliana</i>	nucleus	[5]
NH ₄ ⁺	AmTRAC	<i>A. thaliana</i>		[10]
	deAmTrac	<i>A. thaliana</i>		[8]
	AmTryoshka 1;3	<i>A. thaliana</i>		[9]
Zn ²⁺	eCALWY	<i>A. thaliana</i>		[43]
NO ₃ ⁻	NiTrac	<i>A. thaliana</i>		[7]
Peptides	PepTrac	<i>A. thaliana</i>		[7]
glucose	FLIPglu	<i>A. thaliana</i>		[44]
sucrose	FLIPsuc	<i>A. thaliana</i>		[45]
redox	roGFP2	<i>A. thaliana</i>	chloroplast, mitochondria, peroxisome	[46]

This table provides examples of the broad range of biosensor usage and their potential for subcellular targeting in plants. It is important to note that this is not an exhaustive survey of all the plant biosensor literature but is intended to give an overview of the possibilities of this technology. There are many other studies that have very successfully used these approaches that due to space limitations are not covered in the table. Localization is to the cytosol unless otherwise noted. Phenotypes reported associated with biosensor expression: ¹growth inhibition, ²ABA hypersensitivity; ³possible GA hypersensitivity.