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From jellyfish to biosensors: the use of fluorescent proteins in plants

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ABSTRACT The milestone discovery of green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*, its optimisation for efficient use *in plantae*, and subsequent improvements in techniques for fluorescent detection and quantification have changed plant molecular biology research dramatically. Using fluorescent protein tags allows the temporal and spatial monitoring of dynamic expression patterns at tissue, cellular and subcellular scales. Genetically-encoded fluorescence has become the basis for applications such as cell-type specific transcriptomics, monitoring cell fate and identity during development of individual organs or embryos, and visualising protein-protein interactions *in vivo*. In this article, we will give an overview of currently available fluorescent proteins, their applications in plant research, the techniques used to analyse them and, using the recent development of an auxin sensor as an example, discuss the design principles and prospects for the next generation of fluorescent plant biosensors.

KEY WORDS: *fluorescent protein, transgenic plant, biosensor, DII-VENUS, confocal microscopy*

Introduction

Genetically encoded fluorescent proteins (FPs) represent powerful tools for biological research. This article will review the development of FPs for use in plants together with current applications and recent advances in microscopy techniques. Fluorescent reporter design and techniques for fluorescence quantification will be discussed using the recently-developed DII-VENUS auxin biosensor as an example, together with perspectives for future developments.

Genetically-encoded fluorescent reporters: from a slow start to a bright future

Three years after the production of the first transgenic plants, the first light-emitting plant was generated (Ow *et al.,* 1986), an event considered newsworthy enough to be reported by both *TIME magazine* and *The Today Show* (Lemonick 1986; Bazell and Palmer 1986). This transgenic tobacco plant contained the gene for the enzyme luciferase from the firefly *Photinus pyralis* and when provided with the appropriate substrate, luciferin, emitted enough light for an image to be captured on photographic film following a lengthy (24h) exposure (Fig. 1A). Reporters based on FPs rather than enzyme-mediated bioluminescence were not available until the cloning of the gene for green fluorescent protein (GFP) six years later (Prasher *et al.,* 1992). GFP had been identified in the jellyfish *Aequorea victoria* thirty years earlier (Shimomura *et al.,* 1962). *A. victoria* emits green light whereas the bioluminescent protein isolated from its photogenic tissues, aequorin, emits blue light in the presence of calcium ions *in vitro*. Co-localised to the same tissues, GFP was shown to be excited by aequorin bioluminescence, emitting the green light which gives the characteristic hue to *A. victoria* luminescence (Morise *et al.,* 1974).

The cloning of the *GFP* gene provided researchers with a marker that could be expressed *in vivo*, detected in real time and which required no substrates or cofactors (Millwood *et al.,* 2008). The first report of successful expression of GFP in the model organisms *Escherichia coli* and *Caenorhabditis elegans* was published two years after its cloning (Chalfie *et al.,* 1994). The next three years saw GFP transiently expressed in plants: in *Arabidopsis thaliana* tissues and in protoplasts of *Citrus sinensis* and *Zea mays (*Sheen *et al.,* 1995; Niedz *et al.,* 1995; Chiu *et al.,* 1996). However, attempts to produce stable transformants in *Arabidopsis* resulted in plants with no detectable fluorescence signal (Sheen *et al.,* 1995). It was

Abbreviations used in this paper: FRET, fluorescence resonance energy transfer; FLIM, fluorescence lifetime imaging; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein.

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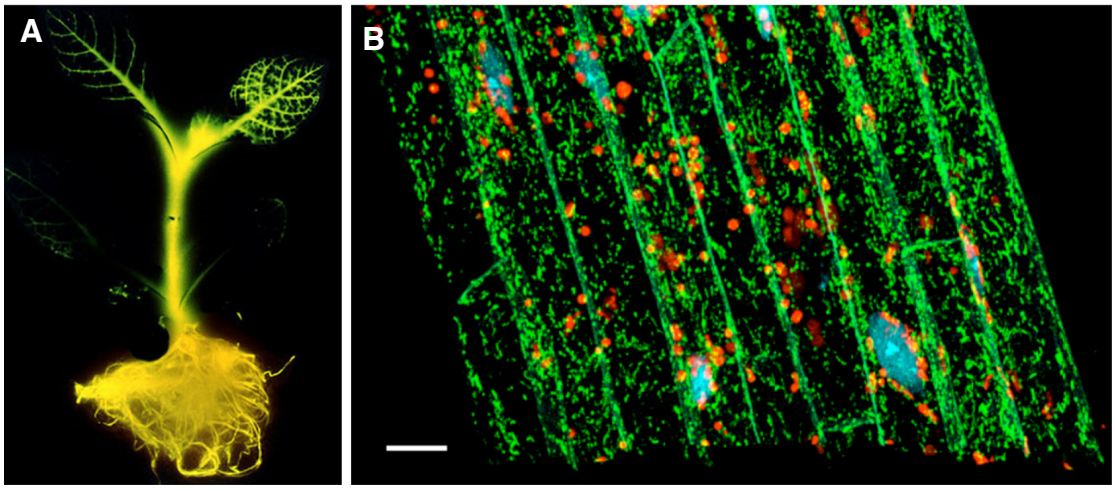

Fig. 1. Transgenic reporter plants. (A) *Autoradiograph of the first transgenic plant to express a light-emitting reporter: a tobacco plant expressing the firefly luciferase gene (image from Ow* et al.*, 1986).* **(B)** *Confocal laser scanning micrograph of a root of the* Arabidopsis *reporter line Kaleidocell (image from Kato* et al.*, 2008) in which cell walls are marked with GFP, mitochondria with YFP, plastids with RFP, and nuclei with CFP. Scale bar: 10 µm. Note that the GFP and YFP signals were collected in the same channel and hence not isolated.*

subsequently found that correct expression of GFP is confounded in *Arabidopsis* by aberrant mRNA processing in which a cryptic intron is excised from the transcript resulting in a non-fluorescent protein (Haseloff and Amos 1995). Once the sequences involved in splice-site recognition of this intron were mutated, full GFP function was restored (Haseloff *et al.,* 1997).

Re-engineering fluorescent proteins

In parallel with the developments in adapting GFP for use *in plantae*, the protein was also the subject of intense research to increase its utility. Wild type GFP has excitation maxima at both 396 and 475 nm (Chalfie *et al.,* 1994; Table 1), preventing its use as a resonance energy transfer acceptor. Replacing the serine at position 65 with a threonine produced an FP with a single excitation peak at 489 nm with an amplitude six times that of wild type and a slight shift of the emission maximum to 511 nm (Heim *et al.,* 1995). Adoption of this re-engineered GFP was aided by the fact that the widely-used fluorescent dye fluorescein isothiocyanate (FITC) has excitation and emission spectral maxima at 495 and 519 nm respectively, making the S65T GFP variant compatible with existing hardware filter sets. Favoured codon optimisation of the S65T sequence produced a modified GFP that fluoresced 100-fold brighter than wild type (Chiu *et al.,* 1996). Researchers at Clontech Laboratories Inc. engineered "enhanced" GFP (EGFP), incorporating several mutations (including S65T) to produce a variant with improved fluorescence intensity. This variant also included 190 silent base mutations to allow more efficient EGFP mRNA translation in mammalian and plant cells and hence higher expression (Yang *et al.,* 1996).

Not just green – creating a fluorescence palette

In addition to the optimisation of GFP, much of the effort in modifying wild type GFP was directed to the production of FPs that emit at different wavelengths (colours). Multiple emission colours allow simultaneous isolation of individual target genes, the creation of donor/acceptor pairs for fluorescence resonance energy transfer (FRET) and multicolour labelling of organelles. Variants of GFP have been produced that fluoresce orange, yellow and cyan (though note that these colour designations do not often follow the recognised spectral wavelength ranges and are commonly used to distinguish groupings of FPs). With a single published exception (Mishin *et al.,* 2008), the emission maxima of GFP variants have been limited to wavelengths up to 529 nm, leading researchers to hunt for GFP homologues in other organisms for suitable candidates with fluorescence emission towards the red end of the spectrum, with most success coming from proteins from corals of the genus *Discosoma* (Bevis and Glick 2002; Shaner *et al.,* 2004). The photoconvertible FP Kaede was isolated from another coral, *Trachyphyllia geoffroyi* (Ando *et al.*, 2002). The utility of FPs emitting at different wavelengths is illustrated by the *Arabidopsis* "Kaleidocell" line (Kato *et al.*, 2008) in which nuclei, plastids, mitochondria, and plasma membranes are tagged with cyan, red, yellow and green fluorescent proteins, respectively (Fig. 1B). The most useful examples of FPs in each colour range are summarised in Table 1.

Applications of fluorescent proteins in plant biology

Following the optimisation of FPs for use in *Arabidopsis*, further challenges had to be overcome before FPs could be widely used in plants. Chlorophyll, lignified secondary cell walls and vacuolar contents all display autofluorescence overlapping with the emission wavelength of GFP which thus has to be taken into account when planning and performing experiments (Gunning and Schwarz, 1999; Berg, 2004; Berg and Beachy, 2008). GFP is a relatively small protein (27 kDa) and can passively diffuse through plasmodesmata (Grebenok *et al.,* 1997, Itaya *et al.,* 2000; Crawford and Zambryski, 2001). Unwanted diffusion (when promoter fusions are used) can be avoided by using repeats, such as three GFPs fused together (3xGFP) or by adding specific subcellular localisation signals as HDEL/KDEL endoplasmic reticulum targeting sequences, nuclear localisation signals (NLS), or others (Gomord *et al.,* 1997; Grebenok *et al.,* 1997; Haseloff *et al.,* 1997; Chytilova *et al.,* 1999). The combination of 3xGFP with an NLS not only increases fluorescence but by concentrating it to the nuclei also facilitates imaging of targets with low expression levels. Cellular retention of 3xGFP can also be exploited to study cell to cell transport of transcription factors (Nakajima *et al.*, 2001).

The use of FP markers in modern plant molecular biology has increased dramatically over the last two decades. With a large selection of FPs and the availability of sophisticated image acquisition hardware and analysis software, not only co-localisation

analyses but also quantitative measurements and determination of polar distribution of FPs are achievable. Numerous FP fusions localizing to specific organelles have been produced (Nelson *et al.,* 2007; Geldner *et al.,* 2009; Kato *et al*., 2008: see Fig. 1B) and the generation of new fusions is simple using freely-available vectors (Karimi *et al.,* 2007; De Rybel *et al.,* 2011; Zhou *et al*., 2011). Once transgenic plant lines expressing one or more FPs have been generated, protein dynamics at the subcellular level (from individual protein to organelle) can be investigated *in vivo*. Using FP fusions, it is possible to visualise the expression pattern and the subcellular localisation of one or more proteins and to compare changes in expression or localization in mutants or samples under different experimental conditions (abiotic stresses, hormone treatments, etc.). Such studies allow detailed examination of protein dynamics, function or interaction in regulatory and developmental processes. In plant developmental research, 3D timelapse imaging with carefully chosen marker proteins allows the monitoring of developmental processes or cell division patterns *in vivo* without disruption to the process under study.

Individual cell types can be labelled by using enhancer- or gene trap lines such as the GAL4-UAS system originally developed in *Drosophila* and subsequently optimised for use in plants (Haseloff, 1999). These lines contain an enhancer trap module consisting of a minimal promoter driving the yeast transcription factor GAL4 with a coding sequence optimized for plants and an endoplasmic reticulum (ER) localised GFP reporter (mGFP5-ER) controlled by the GAL4 response upstream activating sequence (UAS). Upon insertion of the construct in close proximity to a tissue specific enhancer element, GAL4 will be expressed which then activates mGFP5-ER expression (Haseloff, 1999). These lines allow live imaging in genetic and developmental studies and can also be used for tissue specific expression or targeted misexpression of a particular gene (reviewed in Acosta-Garcia *et al.,* 2004). Enhancer- or gene trap lines have also been used for tissue specific transcriptomic and proteomic studies and hormone quantification (reviewed in Moreno-Risueno *et al.,* 2010; Pu and Brady, 2010) using fluorescence activated cell sorting (FACS; Haseloff, 1999; Birnbaum *et al.,* 2003; Petersson *et al*., 2009). This can lead to the identification of tissue specific regulatory sequences (Tsugeki and Fedoroff, 1999). GAL4-UAS enhancer trap transactivation based insertion collections have been generated for *Arabidopsis* and are freely available from donor laboratories and stock centres.

Imaging protein dynamics

Protein dynamics can be visualised in detail *in vivo* by using FPs and the techniques of photobleaching, photoactivation and photoconversion/photoswitching (see review in Sparkes, 2010). In photobleaching experiments, the protein structure of the FP is irreversibly changed by illumination from a high intensity light source (usually a laser). This structural change halts fluorescence (Swift and Trinkle-Mulcahy, 2004). Once targeted FPs have been bleached, fluorescent recovery over time (FRAP) can be monitored. This return of fluorescence is due to the movement of unbleached proteins to the affected area or to the synthesis of *de novo* FPs. To distinguish between those two possibilities, treatment with the protein-synthesis inhibitor cycloheximide can be used to limit recovery only to movement of unbleached proteins. FRAP experiments are often paired with FLIP (fluorescence loss in photobleaching) assays which, instead of measuring the recovery in fluorescence, monitor a decrease in fluorescence in a region adjacent to the bleached area. As an alternative to selective photobleaching, protein mobility can also be assessed by monitoring selective activation of fluorescence in proteins tagged with photoactivatable GFP (PAGFP; Lippincott-Schwartz and Patterson, 2009). Upon irradiation with ultraviolet light, the fluorescence of PAGFP dramatically increases. The use of photobleaching and photoactivation techniques not only allows the dynamics of individual proteins to be studied, it also permits the study of physical membrane properties if integral membrane proteins are tagged (Ward and Brandizzi, 2004; Held *et al.,* 2008). Photoconvertible or photoswitchable fluorophores exhibit a shift in emission wavelength upon excitation, allowing monitoring of both the unconverted and converted pool of proteins in the same sample (Lippincott-Schwartz and Patterson, 2009). For example, fluorescence of the photoconvertible protein Kaede (isolated from the coral *Trachyphyllia geoffroyi*) changes irreversibly from green to red upon activation with ultraviolet light (Brown *et al.,* 2010, Table 1).

Protein-protein interactions

A wide range of techniques are available to study protein-protein interactions in plants. However, many operate only *in vitro* such as yeast two-hybrid screening or co-immunoprecipitation and therefore may not reflect the situation *in planta*. In contrast, FP based methods can be performed *in vivo* and also take protein localisation of putative binding partners into account.

TABLE 1

FLUORESCENT PROTEINS

Fluorescence	Protein	Origin	Excitation nm	Emission nm	Reference
Far-red	mPlum	*Discosoma sp.*	590	649	Wang *et al.,* 2004
Red	mCherry	*Discosoma sp.*	587	610	Shaner *et al.,* 2004
	DsRed	*Discosoma sp.*	556	586	Matz *et al.,* 1999
Orange	mOrange	*Discosoma sp.*	548	562	Shaner *et al.,* 2004
Yellow	EYFP	*Aequorea victoria*	514	527	Tsien 1998
	VENUS	*Aequorea victoria*	515	528	Nagai *et al.,* 2002
	YPet	*Aequorea victoria*	517	530	Nguyen & Daugherty 2005
Green	GFP	*Aequorea victoria*	396, 475	507	Chalfie *et al.,* 1994
	EGFP	*Aequorea victoria*	488	507	Yang *et al.,* 1996
	EmGFP	*Aequorea victoria*	487	509	Tsien 1998
Cyan	mCFP	*Aequorea victoria*	433	475	Zacharias *et al.,* 2002
	Cerulean	*Aequorea victoria*	433	475	Rizzo *et al.,* 2004
	CyPet	*Aequorea victoria*	435	477	Nguyen & Daugherty 2005
Green/red	Kaede	*Trachyphyllia geoffroyi*	508	518	Ando *et al.*, 2002
			572	582	on UV excitation

Commonly-used FPs (adapted from Shaner *et al.,* 2005 and Ckurshumova *et al.,* 2011).

Förster or fluorescence resonance energy transfer (FRET) measures the energy transfer between a donor and an acceptor fluorophore. A prerequisite for FRET is that the donor's emission spectrum overlaps with the excitation spectrum of the acceptor. Potential fluorophore pairs are GFP with monomeric red fluorescent protein (RFP), and cyan fluorescent protein (CFP) with yellow fluorescent protein (YFP). These are each fused to one of the putative interacting proteins. If these proteins are in very close proximity (1 – 10 nm), energy is non-radiatively transferred from the donor to the acceptor fluorophore leading to fluorescence quenching in the donor and increase in the acceptor (Wallrabe and Periasamy, 2005). Over the last decade, FRET has been successfully combined with fluorescence lifetime imaging (FLIM) *in plantae* (Wallrabe and Periasamy, 2005; Swift andTinkle-Mulcahy, 2004; Held *et al.,* 2008). FLIM is based on each fluorophore having an unique lifetime, defined as the average time the molecule remains in an excited state before returning to the ground state (Chen *et al.,* 2003). This lifetime is not affected by the concentration of the fluorophore or excitation intensity (Wallrabe and Periasamy, 2005), but can be influenced by changes in pH, temperature, calcium ion concentration and FRET occurrence (Chen *et al.,* 2003). Importantly, FRET-FLIM can produce additional spatial and temporal information for protein interactions that might not be obtained with other techniques (Osterrieder *et al.,* 2009).

Alternatively, split protein systems or bimolecular fluorescence (BiFC) can be used for quantitative and qualitative studies of protein-protein Interactions *in vivo*. Most existing FPs have been used to create split reporters and have been used to investigate the dynamics of many protein-protein interactions (reviewed in Muller and Johnsson, 2008). Upon protein interaction, the two FP fragments come into close proximity and the FP is reconstituted. The resulting fluorescent signal can then be analysed by confocal laser scanning microscopy. Because of the large range of available fluorophores it is also possible to image multiprotein complex interactions in the same cell (Weinthal and Tzfira, 2009). However, when designing experiments and analysing their results one needs to be aware that the binding of the reassembled GFP domains is irreversible and therefore the temporal dynamics of the investigated protein-protein interactions cannot be monitored. However this can also be an advantage when weak interactions are monitored. (Magliery *et al.,* 2005; Nyfeler *et al.,* 2005). Unlike FP-based approaches, split-luciferase reporters are reversible (Porter *et al.,* 2008) and have been successfully adapted to establish membrane protein topology (Zamyatnin *et al.,* 2006).

Visualising fluorescent proteins in plants

If driven by a strong promoter, GFP fluorescence in transgenic plants can be visualised with the naked eye. This is especially simple if using a variant with the wild type excitation peak at 395 nm as illumination with UV light results in emission that does not require filtering to exclude the exciting light as it is invisible to the human eye. If using one of the engineered variants with a single excitation peak, suitable filters must be used to mask the excitation wavelengths from the detection/viewing system. Benchtop and hand-held fluorometers have been developed to quantify fluorescence and whole plant fluorescence can even be monitored remotely using stand-off laser-induced spectrometry (see review in Millwood *et al.*, 2008). The most widely-used instruments for visualising FPs *in plantae* however are microscopes, ranging from simple epifluorescence devices to highly complex laser scanning confocal instruments.

Advances in fluorescent microscopy

The recent advances in methods based on FP markers have only been made possible by parallel improvements in fluorescent microscopy techniques and image acquisition and analysis software. Researchers now have a choice between a wide range of image-acquisition techniques at different resolutions and throughput, based on their experimental requirements. In this section we will give a brief description of the principles of operation and the advantages and disadvantages of various microscope technologies.

Widefield fluorescence microscopy is the cheapest and simplest fluorescence imaging system available. It generates images by collecting photons from multiple focal planes. As a result, images are often of lower quality compared to the techniques discussed below. Pictures are blurry, as out of focal plane photons are collected as well, leading to lower image contrast and resolution (Swedlow and Platani, 2002).

Confocal laser scanning microscopy (one photon) is based on beam-scanning microscopy in which a single photon provides energy to linearly excite a fluorophore. Regions of the sample above and below the focal plane are also exposed to the excitation illumination, resulting in excitation of fluorescence outside of the focal plane. To overcome the problem of a blurry, unsharp image, a pinhole rejects out of focus fluorescence. Increasing pinhole size to compensate for low excitation and emission results in lower Z-axis resolution. To obtain 3D pictures, the specimen can be imaged in many different layers of the Z-axis (Z-stack), but as a consequence a longer time is required for image acquisition which can result in photobleaching and photodamage of the subject (Pawley, 1995; Periasamy *et al.,* 1999; Rubart, 2004). Spinning disc confocal microscopy overcomes some of the limitations of confocal microscopy by scanning the entire image simultaneously and collecting fluorescence through numerous pinholes. This allows faster image acquisition, and a reduction of photobleaching of up to 15-fold (Ichiara *et al.,* 1996; Wang *et al.,* 2005)

In two-photon and multiphoton microscopy, two or more long wavelength photons are absorbed around the focal point, leading to non-linear excitation of the fluorophore. In contrast to single photon confocal laser scanning microscopy, excitation of fluorescence is restricted to the focal plane and consequently a pinhole is not required. The limited excitation reduces photobleaching and photodamage to the imaged cells. These features make multiphoton microscopy the technique of choice for experiments where repetitive or prolonged laser exposure are required, such as time lapse and live imaging. Another advantage of multiphoton microscopy is the ability of longer wavelength photons to penetrate deeper into the tissue, beneficial when working with thicker samples (Denk *et al.,* 1990; Potter *et al.,* 1996; Centoze and White, 1998; Periasamy *et al.,* 1999; Rubart, 2004).

Another novel microscopy technique that reduces sample bleaching is light-sheet microscopy (also known as selective-plane illumination microscopy or SPIM). A rotating sample is illuminated with a thin light sheet. The resulting fluorescence is collected with an objective positioned perpendicularly to the sheet. This arrangement exposes only the object plane being imaged to the excitation light. Therefore the light-dose is lower than in conventional

microscopes (as only the plane being imaged is illuminated) and as a result specimens suffer far less photodamage, allowing long-term high-resolution imaging of intact plants. The use of the light sheet also detects fluorescence over the entire field of view and therefore makes x-y scanning unnecessary, resulting in a faster image acquisition time (Maizel *et al.,* 2011; Sena *et al.*, 2011).

The development of these highly sophisticated fluorescence microscopes reflects the demand from the scientific community for more complex and detailed imaging. Together with improved fluorescent tags, scientists now have a complete set of tools to study rapid (in the range of seconds/minutes) and dynamic developmental process. Recently, a new fluorescent sensor that quantifies bioactive auxin in plant tissues *in vivo* and over time to a degree not hitherto attained has been developed (Vernoux *et al.*, 2011; Brunoud *et al.*, 2012). The numerous benefits of this new tool for plant scientists raise the fascinating question of whether a similar approach can be applied to develop sensors for other hormones.

Fluorescent biosensors for plant hormones

In order to quantify the dynamic distribution of plant hormones, transgenic plants have been engineered to express FPs in the presence or absence of a specific signal (Jach, 2006). These reporters usually rely on endogenous or, in cases such as auxin and the DR5 sequence where hormone-specific regulatory motifs are known, synthetic promoters (Ulmasov *et al.,* 1997). These promoters drive the expression of an FP, which can have any particular properties of interest (see sections above). As FPs can be followed over time using confocal or fluorescent microscopes and because such reporter lines can easily be crossed into mutant backgrounds of interest, they have allowed the study of detailed dynamic changes in hormone signalling at the cellular level. It is important to note that it is neither the hormone itself nor the input to the signalling pathway which is measured in such systems, but the overall output of the signalling cascade and this can have considerable disadvantages (for a review of these concepts, see Okumoto *et al.,* 2012 and Wells *et al.,* 2013). The transcription, translation and maturation of the FP will have a profound effect on reporter expression as these processes may vary between tissues or environmental conditions. Secondly, if downstream FPs are expressed in cells that are responding to a specific signal, it would be naïve to expect that these are not affected to some degree by additional, non-specific signals. These effects may differ in particular genetic backgrounds due to ecotype specific properties, a common problem in *Arabidopsis* (Fu *et al.,* 2009), and are very hard to control. Finally, and probably of greatest importance, is the time required to express a fully mature FP from mRNA, which under standard plant growth conditions takes about 2 hours at 23°C for the fast maturing FP VENUS (Brunoud *et al.,* 2012; Nagai *et al.,* 2002; Table 1).

This is even more relevant when considering dynamic developmental processes in plants, such as root gravitropism (Swarup *et al.*, 2013). Plant roots usually grow following the gravity vector, regulated by the redistribution of the plant hormone auxin from the root tip into the elongation zone (Swarup *et al.,* 2005). In the "normal" situation, where roots grow following the gravity vector, fluxes of auxin are equal around the root tip. This can be visualised using the synthetic auxin reporter DR5 driving the expression of GFP (Wolverton *et al.,* 2011). If this flux changes, for example following a 90° gravity stimulus, auxin accumulates on the lower part of the root and is depleted in the upper part (Rashotte *et al.,* 2001). This redistribution leads to a tropic response whereby roots re-orientate their growth towards gravity (Thimann, 1935). This is a highly dynamic process: a root starts to respond within 10 minutes after a gravistimulus and grows straight again in a matter of hours (Wolverton *et al.,* 2011). Because of the time delay to produce a mature fluorescent GFP, a DR5::GFP reporter only starts to show a differential distribution when the gravitropic response is well established. To overcome this, Wolverton and colleagues used a rotating platform to maintain roots at a constant angle to the gravity vector and imaged fluorescence at set time points (Wolverton *et al.,* 2011). However, such an experimental set up is only applicable for the study of root gravitropism, which, irrespective of the quality of the data reported, limits its utility. In order to reveal these dynamic processes it is the design of the reporter itself that has to be optimised (Wells *et al.,* 2013).

Recently, Vernoux and colleagues (Vernoux *et al.*, 2011; Brunoud *et al.*, 2012) engineered a new fluorescent auxin sensor that responds within minutes of exogenous auxin application. This new sensor works in a completely different way from the reporters described so far as fluorescence is not induced by the target signal but degraded in its presence. This exploits the fact that genomic auxin responses rely mainly on the degradation of repressor proteins called IAAs (Chapman and Estelle, 2009). IAA proteins have been shown to interact via their domain II (DII or degron) in an auxin-dependent manner with a sub-class of F-BOX Ubiquitin E3 ligase, of which there are 6 members in *Arabidopsis*: AFBs 1-5 and TIR1 (Tan *et al.,* 2007). When the interaction is strong (i.e. abundant bioactive auxin), the half-lives of IAA proteins are dramatically reduced, varying from 11 minutes (for IAA1 and 17) to up to 80 minutes (for IAA28) (Dreher *et al.,* 2006). Therefore, auxin response correlates with the turnover of IAA proteins: If IAA levels are high, auxin responses are low and *vice versa* (Tiwari *et al.,* 2001). Unfortunately, because the maturation time of common FPs is longer than the half-life of IAA proteins it has not been possible to observe IAA-FP fusion proteins on a fluorescent or confocal microscope. However, using VENUS (see Table 1) and the DII domain of the most stable yet auxin sensitive IAA protein (IAA28), Vernoux and colleagues were finally able to "see" a native IAA-FP fusion protein *in vivo* for the first time (Vernoux *et al.,* 2011; Brunoud *et al.,* 2012). Importantly, the DII-VENUS reporter does not measure the output of the auxin response pathway (as do reporters based on DR5) but is directly related to the input to the pathway, i.e. the bioactive signal itself. Fig. 2 shows the distribution of the DII-VENUS input reporter and a DR5::3xVENUS output reporter in *Arabidopsis* root tips (Fig. 2A, left and right panels respectively).

DII-VENUS: a "model" biosensor?

The initial characterisation showed that the DII-VENUS reporter is an ideal auxin sensor (Band *et al.,* 2012, Brunoud *et al.,* 2012, Vernoux *et al.,* 2011). Firstly, it is broadly expressed, at least in root tissues, via the constitutive cauliflower mosaic virus (CaMV) 35S promoter, with fluorescence localised to the nucleus due to an NLS (Fig. 2A left panel). Critically, DII-VENUS is degraded in a dose dependent manner when treated with exogenous auxin (Fig. 2B upper panel) and this is furthermore specific to bioactive auxins, since inactive variants do not have any effect. It also requires active receptors (TIR1 and AFB1-3) and a wild type DII,

as mutations in key amino acids known to stabilise IAA proteins stabilise the sensor as well. The main advantage of this reporter is its speed of response. A decrease in DII-VENUS fluorescence can be detected within minutes of exogenous auxin production (Fig. 2B upper panel); in contrast a DR5-based reporter takes over 2h for an increase in fluorescence to be detected (Fig. 2B lower panel). Using DII-VENUS, the dynamic redistribution of auxin during root gravitropism can thus be visualised even before the root starts to re-orientate its growth towards the gravity vector (Brunould *et al.*, 2012; Band *et al.*, 2012).

As with every new approach there are drawbacks that must be taken into account when designing experiments. It has been shown that DII-VENUS interacts with auxin receptors *in plantae* (Brunoud *et al.,* 2012). Because the transgene is highly overexpressed by the 35S promoter, it is possible that it occupies all the receptors available in the nucleus. However, it has been shown that overexpressing the DII-VENUS sensor does not affect gravitropism itself, as transgenic plants behave identically to their wild type counterparts, suggesting this not to be a problem (Brunoud *et al.,* 2012). Furthermore, although CaMV 35S is a strong promoter, it is not ubiquitously expressed, especially in the embryo (Odell *et al.,* 1985), and very often leads to silencing of the transgene after several generations (Daxinger *et al.,* 2008). These problems may be circumvented by employing a different promoter less prone to silencing. For example, the RPS5A promoter is strongly expressed in the embryo but then reduces in more mature tissues after germination (Weijers *et al.,* 2001). Unfortunately, there are probably no ideal promoters, a limitation shared by both input and output reporter systems.

The degradation of DII-VENUS requires an active receptor which in the case of auxin involves not only the receptor itself but the whole SCF (Skp1, Cullin and F-BOX) complex (Chapman and Estelle, 2009). In the root, it has been shown that the lateral root cap has a lower sensitivity to auxin and that this is most likely due to reduced expression of receptor complexes in this tissue (Brunoud *et al.,* 2012). Therefore, the sensor does not directly reflect the endogenous auxin but also reflects the sensitivity of cells to auxin. To measure actual hormone abundance, one can employ techniques such as gas chromatography followed by mass spectrometry (GC/MS) (Edlund *et al.,* 1995). However, because large amounts of tissues are required, whole plants are often used or, in best cases, specific organs. Consequently, the spatial distribution of the signal being measured cannot be determined. To improve spatial resolution, Petersson and colleagues (Petersson *et al.,* 2009) have successfully combined GC/MS with protoplasting and fluorescent activated cell sorting (FACS) approaches to quantify auxin and its metabolites in specific tissues (Fig. 2E). Even though the quality of the data produced was very high, there are several issues associated with this approach. Firstly, because it relies on the enrichment of individual cell types, the technique does not reveal the tissue specific auxin gradients at the root apical meristem which are key in regulating the transition between the different regions of the root tip. Secondly, the length (2 hours) and harshness of the protoplasting treatment prevents the monitoring of dynamic changes in auxin abundance. Finally, it is a destructive approach, preventing the monitoring of a particular tissue over time. Fig. 2 C-E shows maps of auxin distribution in the root tip produced using the DR5 output reporter (Fig. 2C), DII-VENUS (Fig. 2D) and FACS (Fig. 2E). Note the complementarity between the input and output reporters and the high spatial resolution of the map generated using DII-VENUS.

As DII-VENUS fluorescence reduces with the abundance of its target, it may be difficult to follow the signal over time unless a background nuclear marker, such as histone 2B fused to RFP, is pres-

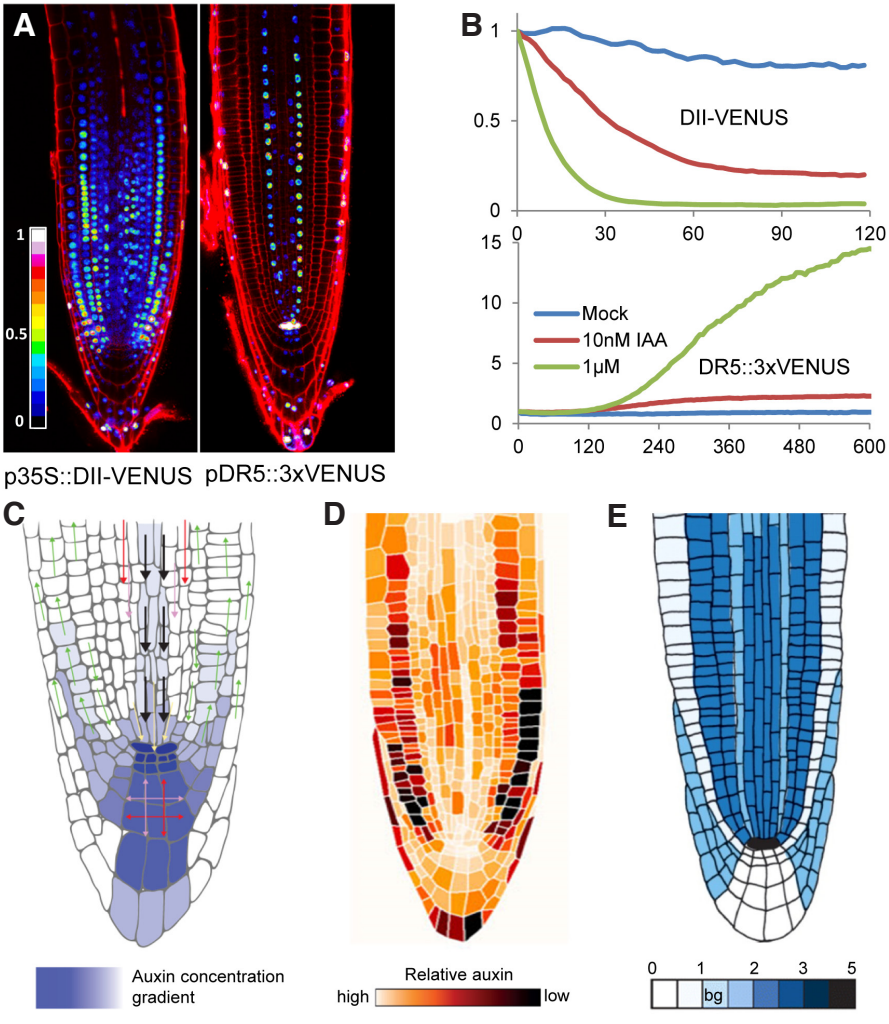

Fig. 2. DII-VENUS responds rapidly to auxin and reveals auxin distribution at high spatial resolution. (A) *Confocal laser scanning micrograph of* Arabidopsis *roots expressing the DII-VENUS (left) and the DR5::3xVENUS reporter (right) (unpublished data).* **(B)** *Changes in VENUS fluorescence in DII-VENUS (top) and DR5::3xVENUS (bottom) in response to various auxin concentrations (unpublished data). X-axis: time (in minutes, note the scale is different between charts); y-axis: normalised relative fluorescence.* **(C-E)** *Maps of auxin distribution obtained using* **(C)** *the DR5 reporter (image from Krecek* et al., *2009),* **(D)** *the DII-VENUS biosensor (Brunoud* et al., *2012), or* **(E)** *by direct quantification of IAA (indole-3-acetic acid) using GC/MS on sorted protoplasts (Petersson* et al., *2009). bg, background level.*

ent (Federici *et al*., 2012). This is not an ideal control, however, since even when using the same promoter for both reporters, there is no guarantee that their expression will be identical and linear. Despite these limitations this new generation of fluorescent biosensors have provided the opportunity to image the dynamics of hormone signalling *in vivo* at a resolution that has not been achieved before. To relate imaged DII-VENUS fluorescence to auxin abundance required the development of parameterised mathematical models as the relationship is non-linear (Band *et al*., 2012). In order to monitor plant roots responding to a gravistimulus in a realistic environment, the authors adapted an inverted confocal microscope to image roots arranged vertically on growth plates. Using experimental time course data together with the parameterised model, they showed that auxin distribution changes rapidly and only transiently between the upper and lower sides of the root and that the system returns to steady state at a set angle from the gravity vector. Such detailed observations have previously not been possible and are the result of combining state of the art confocal microscopy techniques, novel engineered fast-maturing and bright FPs, and mathematical models to simulate complex signalling pathways.

Conclusions and Perspectives

The availability of a variety of multi-coloured FPs has revolutionised many aspects of plant research over the last two decades. The development of both the reporters themselves and the systems used to image and quantify them has progressed at a great pace, and almost certainly will continue to do so.

The data derived from DII-VENUS fluorescence is an excellent demonstration of how FP technology has improved our understanding of fundamental biological processes. Interestingly, the main feature of auxin signalling - the regulated turnover of repressor proteins via an F-BOX E3 ubiquitin ligase complex - is found in the response pathways for other hormones such as jasmonates (JAZ repressors, COI1 F-BOX ligase), and gibberellins (DELLA repressors, SLY F-BOX ligase). The approach used to develop DII-VENUS can potentially be employed to study other hormone responses, offering researchers the prospect of new fluorescent biosensors for the study of plant developmental processes.

New techniques and insights are being brought to bear on both the design of biosensors based on FPs and the techniques used to interpret fluorescence data and it is certain that they will remain an essential part of the plant researcher's toolkit for many years to come.

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