Shaping Small Bioactive Molecules to Untangle Their Biological Function: a focus on Fluorescent Plant Hormones

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Abstract

Modern biology overlaps with chemistry in explaining the structure and function of all cellular processes at the molecular level. Plant hormone research is perfectly located at the interface between these two disciplines, taking advantage of synthetic and computational chemistry as a tool to decipher the complex biological mechanisms regulating phytohormones mode of action. These small signalling molecules regulate a wide range of developmental processes, adapting the plant growth to ever changing environmental conditions. The synthesis of small bioactive molecules mimicking the activity of endogenous hormones has allowed to unveil many molecular features of their functioning, giving rise to a new field, plant chemical biology. In this frame, fluorescence labelling of plant hormones is emerging as a successful strategy to track the fate of these challanging molecules inside living organisms. Thanks to the increasing availability of new fluorescent probes and advanced and innovative imaging technologies, we are now in a position to assess many of the dynamic mechanisms through which plant hormones exert their action. Such a deep and detailed comprehension is mandatory to develop new green technologies for practical applications. In this review we will summarize the results obtained so far concerning the fluorescent labelling of plant hormones highlighting the basic steps leading to the design and synthesis of these compelling molecular tools and their applications.

Introduction

Many biological problems demand molecular and quantitative approaches that can only be supplied by tools derived from chemistry — such as single-molecule measurements, single-cell imaging, and the use of exogenous small molecules to modulate and to identify the activity of cellular components. Thus, the integration of chemistry and biology has become an essential tool to address most of the still unanswered biological questions. On these grounds not unexpectedly,

chemical biology is now a rapidly growing field that combines the potential of traditional chemistry and biochemistry with the medical and environmental relevance of modern molecular, cellular, organismic, and human biology.

A succesfull application of this new integrated approach that directly probes living systems at the chemical level has recently involved plant hormones research. Plant hormones are low molecular weight mobile compounds with a vital role as signaling molecules as they regulate plant growth and mediate responses to both biotic and abiotic stresses, being effective at low concentrations (Davies, 2010). To date, structurally diverse phytohormones (PHs) have been characterized, deriving from various essential metabolic pathways such as auxin, cytokinin, abscisic acid, ethylene, gibberellin, brassinosteroid, salicylic acid, jasmonate, nitric oxide and the recently discovered strigolactone (Santner & Estelle, 2009). The fine regulation of their response pathways at multiple levels such as metabolism, transport, perception, and signaling allows their proper function, shaping the plant growth. While in the last decade classical genetic approaches in the model plant *Arabidopsis thaliana* has indoubtely increased our comprehension of plant hormones mode of action*,* in recent years, new insights into plant hormone biology have been brought thanks to the inter-connection between chemistry and biology. This implies that essential information such as the hormone's chemical properties and its active moiety can be combined with structural characterization of the target protein, leading to the rational design of novel analog molecules as chemical tools probing PH-regulated responses. Moreover, knowledge of the structural requirements mandatory for bioactivity gained through extensive structure activity studies (SAR) on natural hormones and their synthetic analogs have led to the chemical design of structurally related compounds for commercial application (Rigal *et al.*, 2014). The possibility to affect plant growth through the use of exogenous small compounds optimized for field usage is a new exciting frontier in applied research but demand a deep and detailed comprehension of plant hormone systems.

One of the approaches that has become increasingly attractive for plant scientists actively involved in PHs research is the development of fluorescent or tagged compounds as new tools to trace PHs signaling and distribution. The chemical engineering of labeled analogs coupled to the detection power of fluorescence imaging techniques can enable the isolation of hormone receptors and the monitoring of the hormone's tempo-spatial distribution as well as the ligand-receptor complex localization. Indeed, information provided by the direct visualization of the inner working of these interacting molecules have proved to be extremely useful to dissect their mode

of action (Fridlender *et al.*, 2015; Hayashi *et al.*, 2014; Irani *et al.*, 2012; Shani *et al.*, 2013; Tsuchiya *et al*., 2015).

Our intent here is to provide an overview on the recent advancements in plant hormone fluorescence labeling, defining the chemical tools necessary to design suitable fluorescent labeled bioactive compounds to be used as *in vivo* tracers. Among the phytohormones, we will especially focus on Strigolactones, a unique class of plant signaling compounds recently brought to the limelight for their multifaceted role and potential of applications.

Lighting up the darkness

The rapid development of novel imaging techniques has revolutionized the way scientists look at the structures of life alive, directly visualizing the inner workings of molecules, proteins, protein complexes, organelles, cells, tissues, organs and whole organisms. Fluorescence imaging has been a major player of that revolution, being much more efficient than other imaging technologies based on radioactivity, bioluminescence, electromagnetism, and electrochemistry. This methodology is (i) sensitive enough to visualize biological compounds at physiological concentrations, ranging from the micromolar to the nanomolar scale; (ii) it has sufficient spatial and temporal resolution to analyse dynamic cell processes; (iii) it is not invasive, i.e., detection is possible without altering the samples. These characteristics have made live fluorescence imaging extremely used in cell biology (Terai & Nagano, 2013).

In fluorescence imaging, fluorescent probes are added to the sample either chemically or genetically as labels to report the behaviour of the molecule of interest. These fluorescent labels can be divided into three general groups: (i) small organic fluorophores, (ii) fluorescent proteins and (iii) quantum dots. Of course, the choice of the fluorescent label to be used mostly depends on the given application or experimental system the researcher has to deal with. For instance, the well-known genetically encoded green fluorescent protein (GFP) has become the most commonly used labeling tool for proteins in general, since its discovery in the 1990s (Tsien, 1998). However, to label low molecular weight compounds such as plant hormones, fluorescent proteins are not a suitable option since their molecular size (around 25 KDa) would fully exceed the size of the molecule to be tagged. Instead, chemically shyntesized organic fluorescent molecules represent the most convenient labelling agents to trace small signalling compounds inside a living cell without altering their behaviour. They are basically applicable to any sample, and, most important, their chemical design can be shaped in order to meet specific requirements (solubility,

spectroscopic properties, activation/inactivation of the signal). Recently, fluorescent quantum dots (QDs) are emerging as an important tool for imaging thanks to their outstanding optical properties, such as high quantum yields, broad absorption spectra, narrow and symmetric size-tunable emission, and strong resistance to photobleaching (Li & Zhu, 2013); however, their use as fluorophore to label phytohormones is still very limited (Cao *et al*., 2015). For the above cited reasons, fluorescent proteins (FPs) and quantum dots (QDs) will not be reviewed here (see Chudakov *et al*., 2010 and Wegner & Hildebrandt, 2015, respectively); instead, we will present an overview of the small organic fluorophores used in plant hormone fluorescence labelling and their characteristics.

It is important to stress here that a labelled bioactive compound, as the name suggests, is composed by two different moieties, a fluorophore and a bioactiphore. Thus, when conceiving its synthesis both of them have to be designed to meet specific requirements. For instance, the bioactiphore would be shaped to play the designed role as agonist/antagonist while the fluorophore would have to fit the spectroscopic properties necessary for the target bioimaging study. Finally, and most important, the molecule in a whole would have to work as predicted.

A trip across the visible spectrum: small organic fluorophores

Nowadays, a vast selection of fluorophores provides greater flexibility, variation and fluorophore performance for research applications than ever before. One of the major advantages of chemically synthesized fluorophore is that the fluorescence signal of a molecule can be drastically modulated so, for instance, probes that exhibit activation of the signal as a consequence of enzymatic activity, pH changes or proximity to a target molecule can be exploited (Lavis & Raines, 2008).

Among the multitude of small organic fluorophores that have been discovered and developed, the most commonly used belong to the well-known organic fluorophore families that usually emit beyond 500 nm such as fluoresceins, rhodamines, cyanines, coumarins, NBD and BODIPY dyes (Figure 1). Each fluorophore has distinct characteristics that should be considered when deciding which one to use as label.

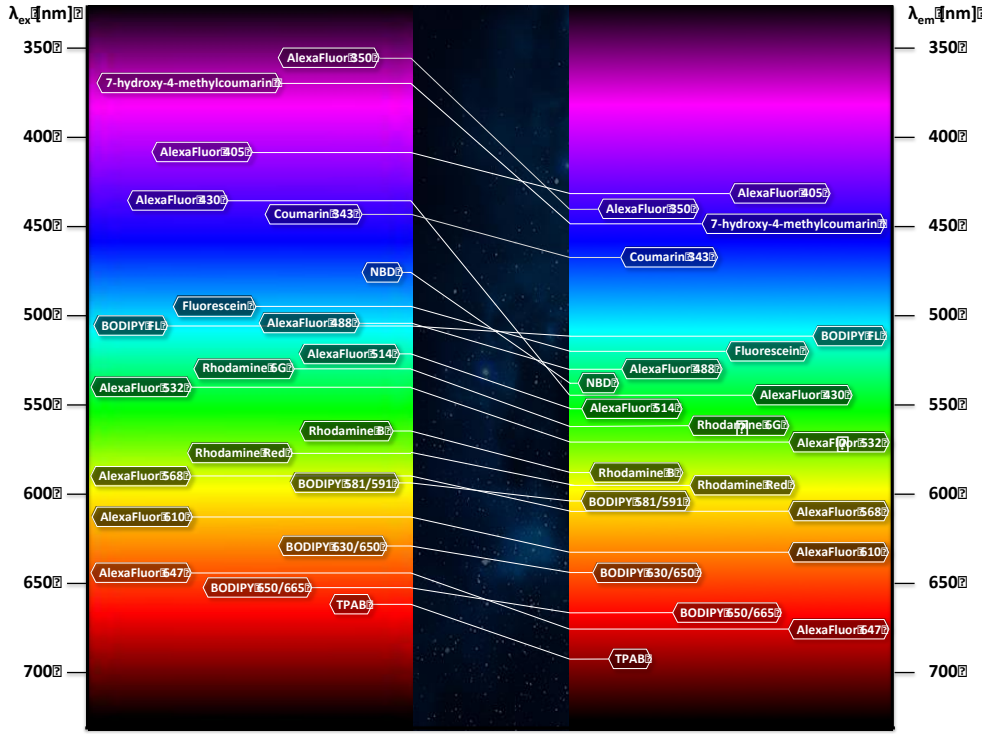

FIGURE 1. Plot of wavelength (nm) of maximum absorption ($\lambda_{ex,}$ left) and emission ($\lambda_{em,}$ right) for selected fluorophores.

Fluorescein and rhodamine (Figure 2) belong to the xanthene class of dyes.

Fluorescein (λ_{abs} 490 nm and λ_{em} 512, in water) is one of the most common used fluorophore in biolabelling because of its relatively high molar extinction coefficient, high fluorescence quantum yield and, above all, good watersolubility. However, fluorescein is also characterized by a high rate of photobleaching, absorption/emission pH-dependency (Figure 3) and tendency of self-quenching after bioconjugation (Songlin, 2011).

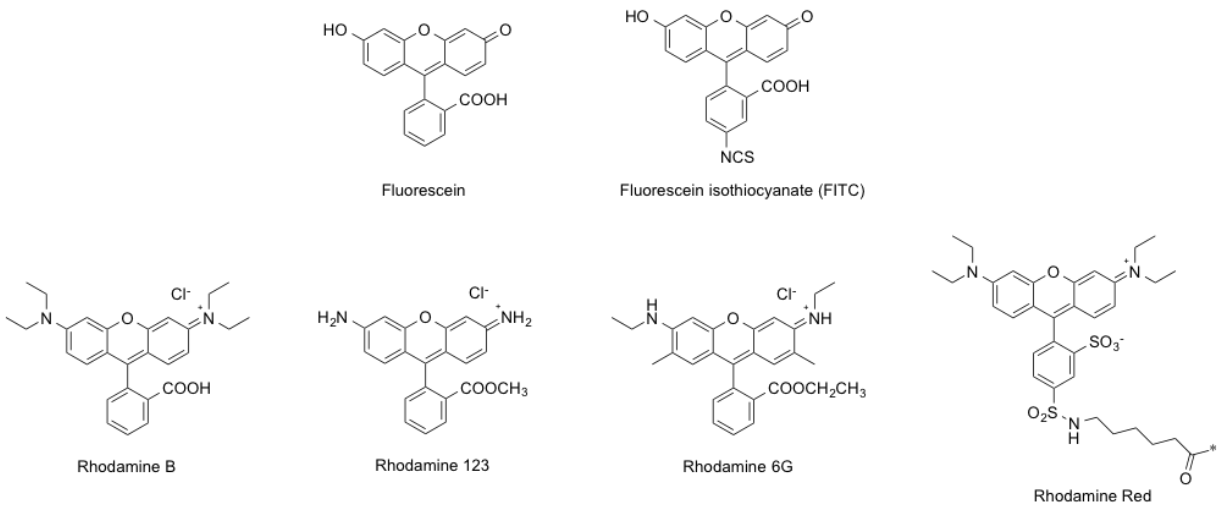

Figure 2. Molecular structure of the most common fluorescein and rhodamine commercial dyes.

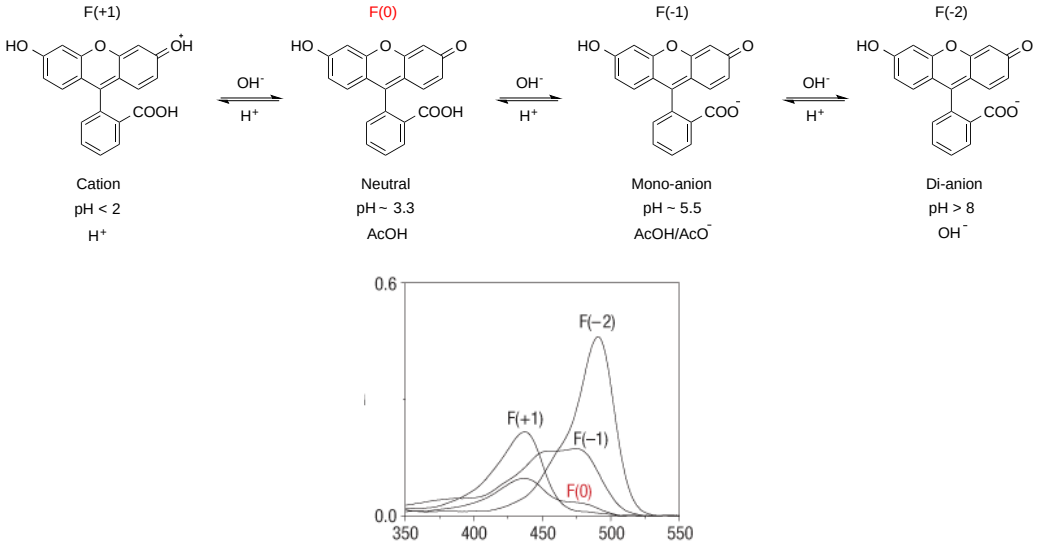

FIGURE 3. Variation of absorbtion/emission properties of Fluorescein depending on pH.

Rhodamine dyes (Figure 2), which comprise rhodamine 6G, rhodamine B, rhodamine red, rhodamine 123 and Texas Red are more photostable and less sensible to pH compared to fluorescein, but their water solubility is poor, which limits the employment in biolabelling applications (Songlin, 2011).

Rhodamine fluorophores tipically represent the alternative to fluorescein when an emission wavelength shifted to red is preferred. For instance, a red emitting fluorophore would offer a better resolution and contrast when working with organisms, especially plants, characterized by a high background signal from cellular autofluorescence in the 300-550 nm range.

The widely employed Alexa Fluor® class of dyes (Figure 4) is obtained through sulfonation of coumarin, rhodamine, xanthene (such as fluorescein), and cyanine dyes (no application regarding the labeling of plant hormones by regular cyanine dyes is reported as far as we know). Sulfonation makes Alexa Fluor® dyes negatively charged and hydrophilic. Moreover, they are less pH-sensitive and more photostable than the original dyes from which they are synthesized, which makes them suitable dyes for biolabelling. However, they are also much more expensive (patented by Invitrogen). The excitation and emission spectra of the Alexa Fluor® series cover the visible spectrum and extend into the infrared (Panchuk-Voloshina *et al*., 1999).

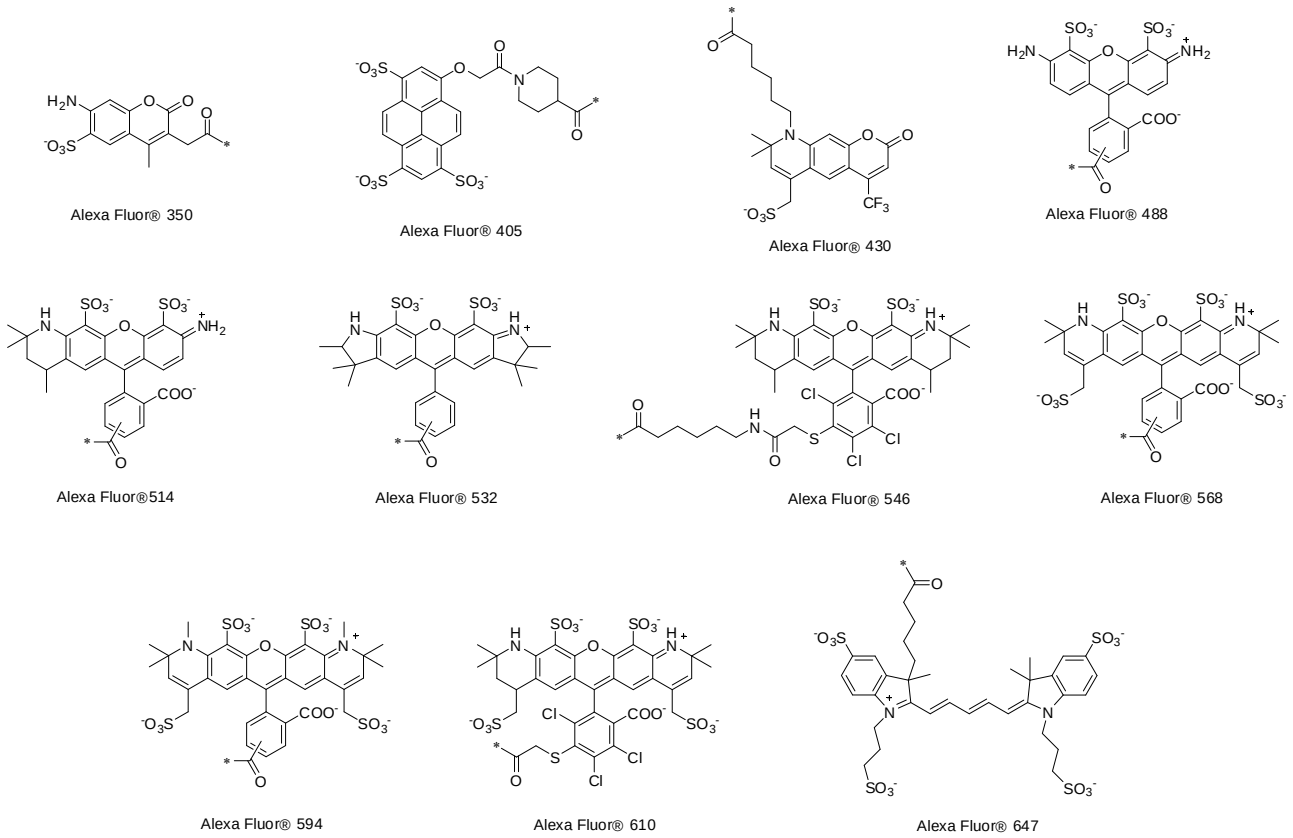

FIGURE 4. Molecular structure of Alexa Fluor® dye series.

The coumarins class comprises natural products, pharmaceuticals, and fluorophores. When the basic scaffold of coumarins is substituted at position 7 with an electron-donating group, the molecule exhibit fluorescence with UV or near-UV excitation wavelengths, being 7-hydroxy-4-methylcoumarin (*i.e.*, 4-methylumbelliferone; 4-MU) the most common example (λ_{abs} = 360 nm, λ_{em} = 450 nm) (Figure 5). The related 7-aminocoumarin molecule is largely adopted as scaffold to obtain derivatives to be used as biomolecular labels. Indeed, different nitrogen substitution patterns yield different spectral characteristics, while other substitutions can fine-tune chemical properties such as solubility or pH sensitivity. Reactive groups are typically inserted at position 3 or 4, allowing the fluorophore to be coupled to multifarious substrates (Figure 5) (Lavis & Raines, 2008). The chemistry of coumarins is highly flexible, making these compounds suitable probe as fluorescent labels, fluorescent sensors, and fluorogenic enzyme substrates (Lavis & Raines, 2014).

NBD (7-nitro-2,1,3- benzoxadiazole) (Figure 5) is a remarkable example of a small heterocyclic fluorophore widely used in biophysical, biochemical, and cell biological studies. Its low molecular weight makes it suitable to label small molecules without affecting their biological activity, serving as an excellent probe for both spectroscopic and microscopic applications.

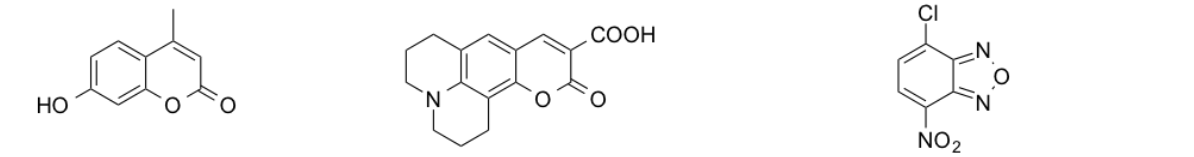

FIGURE 5. Molecular structure of a 7-substituted coumarine derivative and of NBD 4-chloro-7-nitrobenzo[c][1,2,5]oxadiazole.

Derivatives of NBD are particularly used for the preparation of lipid probes and novel kinase substrates. The NBD group fluoresces brightly in the visible range; its fluorescence is quite weak in water but it exhibits a high degree of environmental sensitivity upon transfer to a hydrophobic medium (Lavis & Raines, 2008).

Another class of fluorophores whose excitation/emission wavelenght span the visible spectrum is BODIPY (BOron DIPYrromethens). The molecular structure of BODIPY dyes is based on the 4,4-difluoro-4-borat-3a,4a-diaza-s-indacene, which has been used to build a variety of useful fluorescent labels and other probes (Lavis & Raines, 2008) (Figure 6).

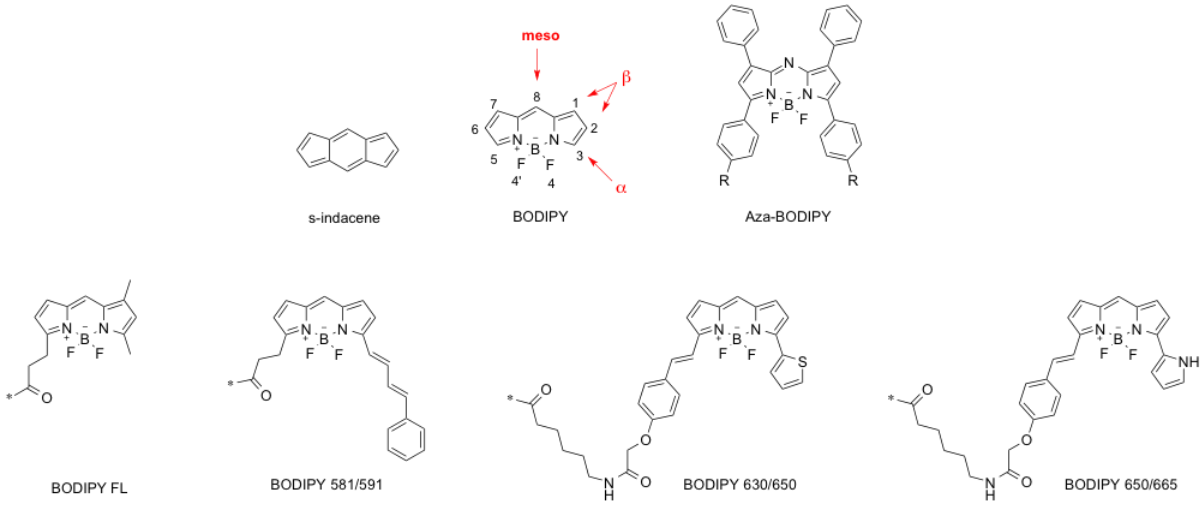

FIGURE 6. Molecular structure of s-indacene, BODIPY core, Aza-BODIPY and a selection of commercially available BODIPYs.

The first member of this class of compound was reported by Treibs and Kreuzer in 1968 (Treibs & Kreuzer, 1968), although relatively little attention was given to the discovery until the end of the 1980s. Then, the potential use of this dye for biological labeling was recognized and several new BODIPY-based dyes were designed and indeed commercialized for biological labeling, the BODIPY

fluorophore becoming a registered trademark of Molecular Probes (Invitrogen) (Ulrich *et al*., 2008). Thanks to the favorable photophysical properties inherent to this family of dyes, BODIPYs have then been considered as substitute for fluoresceins and rhodamines (Songlin, 2011). Depending on precise post-functionalization on the BODIPY core at *meso*, α, β -pyrrolic or even at the 4 position, the emission maximum of BODIPY dyes extend in the 510-800 nm range (Figure 6) (Rohand *et al*., 2006). More recently, interest has been increased in the 4-bora-3a,4a,8-triazaindacene dyes (commonly referred to as aza-BODIPY dyes) owing to their efficient fluorescence in the far-red and near-IR regions of the spectrum. Aza-BODIPYs are basically BODIPY derivatives with the *meso*-carbon atom replaced by an imine type nitrogen atom (Figure 7) (Kamkaew & Burgess, 2015; Ulrich *et al*., 2008; Wu & O'Shea, 2013).

BODIPY dyes are unusual in that they are relatively nonpolar and the chromophore is electrically neutral. These properties tend to minimize dye-induced perturbation of conjugate functional properties. Therefore they are often the preferred choice for labelling nucleotides, amino acids and other low molecular weight ligands (Daly & McGrath, 2003; Middleton *et al.*, 2007; Xie *et al*., 2006). BODIPY dye conjugates of low molecular weight molecules also tend to be more permeant to live cells than are conjugates of charged fluorophores.

Chasing the firefly: probing plant hormones with fluorescence

Binding of a fluorophore to a bioactive molecule allows to track its fate inside a living organism, a step forward to the comprehension of the molecule's functioning. However, it is essential to ensure that the labeled compound effectively mimics the hormone of interest.

As preliminary phase, *in silico* SAR models have become an essential and effective tool for computationally predicting the biological activity of a compound against a certain protein target from its molecular structures. Such models allow to optimize the design of compounds with the desired biological properties and are particulary useful to pinpoint the sites on a molecule suitable for fluorophore binding, that should not compromise the whole molecule activity.

Neverthless, once synthesized, the fluorescent compounds are extensively tested both *in vitro* and *in vivo* to exclude the possibility that the fluorophore itself, even if relatively small, could be responsible for the molecule's bioactivity/distribution pattern, a severe issue to be taken into account when dealing with small bioactive labeled compounds.

If the newly synthesized fluorescent compound satisfies all the requirements in terms of bioactivity, receptor-binding specificity, stability, and spectroscopic properties, it can be successfully used to investigate the functioning of the related hormone, as we will discuss below**.**

Auxin

Auxins are defined as low molecular weight organic acids containing an aromatic ring and a carboxyl group. The most abundant endogenous auxin is the indole-3 acetic acid (IAA). Essential for plant development, auxin has been shown to mediate diverse systemic responses including control of senescence, response to pathogens and abiotic stress while at the cellular level controls cells division and elongation (Sauer *et al.*, 2013).

In 2007 Muscolo and co-authors made the first successful attempt to obtain a fluorescent derivative of auxin. In order to investigate the biological activity of humic substances, they derivatized both primary natural auxin IAA and humic substances by binding with fluorescein isothiocyanate (FITC) and monitored their interaction with carrot cells in culture.

By means of competition tests between labeled and unlabeled compounds, they demonstrate the occurrence of a specific binding between the low molecular weight fraction of humic substances and cell membranes of *Daucus carota* (Muscolo *et al*., 2007).

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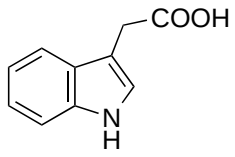
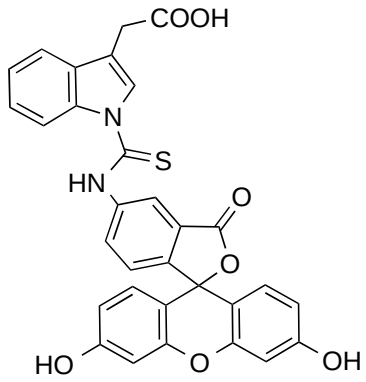
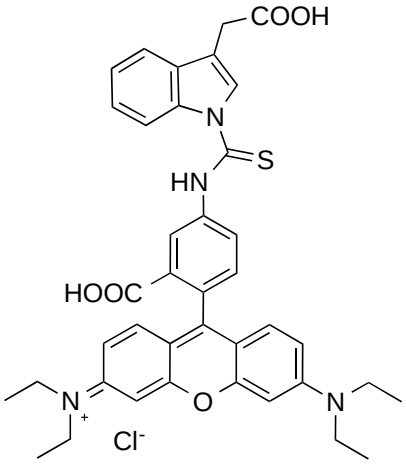


Indole-Acetic Acid (IAA)



IAA-FITC



IAA-RITC

B

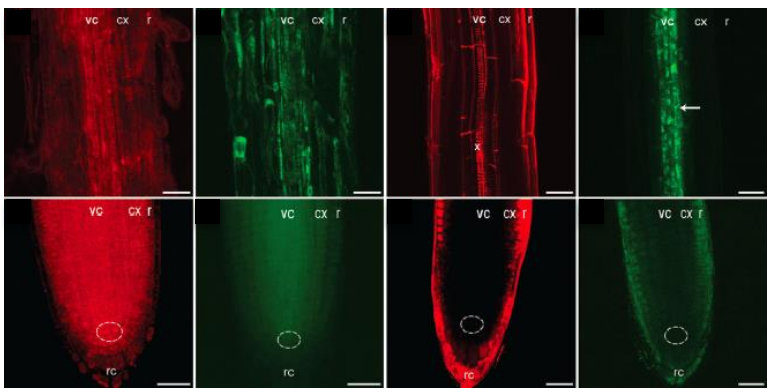


FIGURE 7. Molecular structure of natural auxin, its fluorescent comjugates and their distribution patterns in *A.thaliana* roots imaged in confocal microscopy. A, Molecular structure of Indole Acetic-acid (IAA), Fluorescein (IAA-FITC) and Rhodamine (IAA-RITC) derivatives of IAA. B, Distribution of fluorescent signal in roots after application of fluorescent conjugates IAA-RITC (1 and 5) and IAA-FITC (2 and 6) or fluorescent dyes RITC (3 and 7) and FITC (4 and 8). Scale bar, 50 μm; vc –vascular cylinder; cx – cortex; r – rhizodermis; x – xylem vessels; rc – root cap; circles show the QC region and arrow show pericycle with signal in symplast (From: Sokolowska *et al*., 2014).

More recently, Sokolowska and collegues synthesized both a fluorescein (IAA-FITC) and a rhodamine (IAA-RITC) auxin derivative through a direct conjugation of FITC or RITC to IAA at the indole NH group (Figure 7). Importantly, both compounds proved to have retained auxin-like biological activity in three different biological assay, providing evidence that bioactivity has not been affected by the binding of both fluorophores. In addition, these two fluorescent dyes are reported to differ in their pathway of transport, FITC being considered a symplasmic dye, spreading between cells via plasmodesmata, whereas RITC being regarded as an apoplasmic marker, transported through the system of the cell walls (Figure 7). Direct observation of *Arabidopsis* living tissues treated with the two fluorecent conjugates, however, did not put in evidence any differences in their distribution regardless of whether the apoplasmic or symplasmic dye was used for conjugation; moreover, such distribution pattern was similar to that of free auxin reported in literature.

These evidences suggest that both auxin conjugates could be transported like free auxin. This statement is further supported by the observation that both the fluorescent dyes applied in a free unbound form showed a significantly different pattern of distribution compared to their IAA-conjugated form. Such a negative control is essential to validate the efficiency of a fluorescent conjugate, to verify that the observed distribution pattern could be effectively addressed solely to the molecule bioactiphore. Thus, the authors conclude that both obtained auxin conjugates, IAA-FITC and IAA-RITC possess auxin-like biological activity and auxin-like targeted transport and therefore, represent a new research tools for studying the role of auxin and its transport pathways in developmental processes in plants (Sokolowska *et al*., 2014).

As we mentioned, the determination of the required structure for a molecule's bioactivity is central for the successful design of a labelled compound. Based on SAR analysis they previously carried out on alkoxy-auxin analogs (Tsuda *et al*., 2011), Hayashi and collegues (2014) designed fluorescent auxin analogs to be active in the auxin transport system but inactive for auxin

signaling, in order to monitor the native auxin gradient and transport sites. Indeed, active auxin analogs would affect the localization of auxin transpor proteins as PIN and auxin inactivating enzymes as GH3, and the distribution of the labeled analogs will therefore no longer reflect the native auxin gradient. To reach this aim, they introduced a NBD fluorophore moiety into 5-hydroxy-IAA and 7-hydroxy- naphthalene 1-acetic acid (NAA), the latter being a potent synthetic auxin analog, on the ground that alkoxy substitution on that position did not affect their transport through AUX1, PIN, and ABCBs auxin transporters but rendered the analogs inactive at auxin receptors (Figure 8) (Tsuda *et al.*, 2011). Interestingly, loss of bioactivity which is usually seen as an adverse consequence of fluorophore binding is here a specific requirement of the designed conjugate.

Additionally, fluorescently labeled indole (NBD-indole) and benzoic acid (NBD-benzoic acid) were synthesized as negative controls to confirm the specificity of the fluorescence images of the auxin analogs. The two fluorescent auxins NBD-IAA and NBD-NAA produced similar fluorescence images

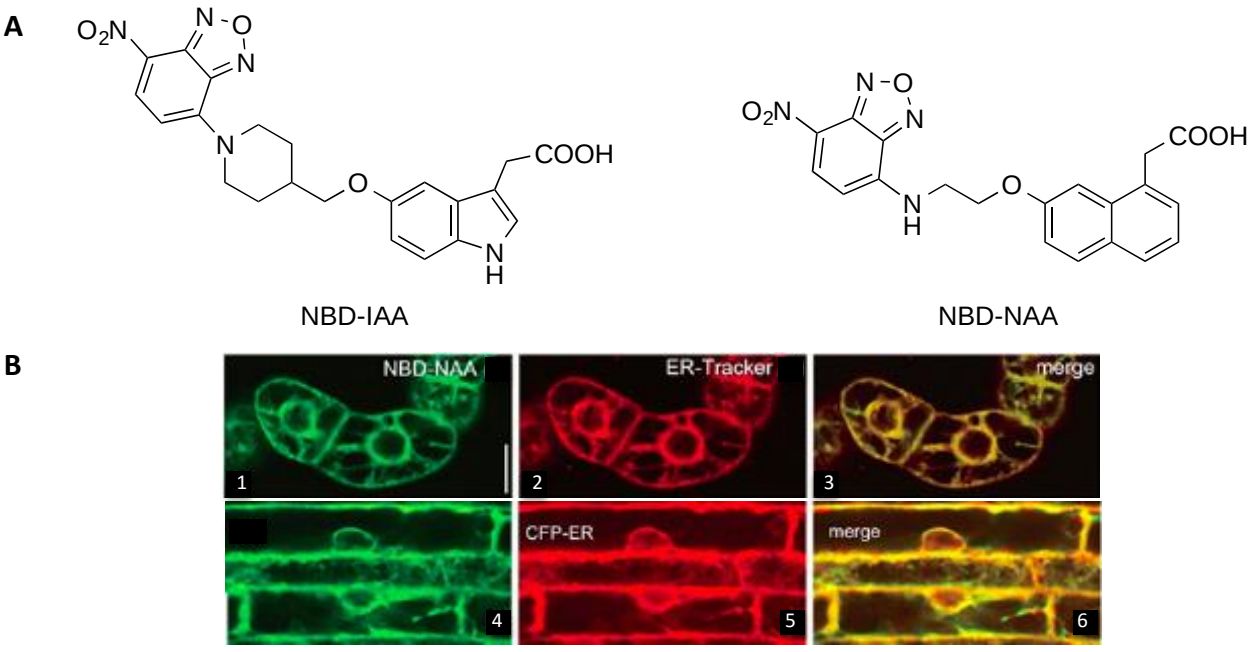


FIGURE 8. Molecular structure of fluorescence-tagged auxins and their distribution pattern in *A. thaliana* root cells imaged in confocal microscopy. A, Molecular structure of NBD-tagged natural (NBD-IAA) and synthetic (NBD-NAA) auxin. B, Subcellular distribution of NBD-NAA in tobacco BY-2 cells: (1-3) incubated with NBD-NAA (1) and ER-Tracker (2) for 20 min or (4-6) expressing the ER marker (5) CFP-HDEL. Scale bars, 20 μm (From: Hayashi *et al*., 2014).

compared with auxin reporter *DR5* expression, while both negative control molecules did not. By means of auxin-regulated reporter gene expression measurments and yeast two hybrid assays the

inactivity of both fluorescent derivatives (NBD-IAA and NBD-NAA) on auxin signaling have been confirmed while auxin transport inhibitors and mutants for auxin transporters have been used to demonstrate that NBD-auxins show the same transport profile as the original IAA and NAA molecules. Together with auxin biosynthesis inhibitors and an auxin biosensor, these analogs indicated a substantial contribution of local auxin biosynthesis to the formation of auxin maxima at the root apex. Moreover, fluorescent auxin analogs mainly localized to the endoplasmic reticulum in cultured cells and roots, implying the presence of a subcellular auxin gradient in the cells (Hayashi *et al.,* 2014).

Gibberellins (GAs)

Gibberellins (GAs) are essential for many developmental processes in plants, including seed germination, stem elongation, leaf expansion, trichome development, pollen maturation and the induction of flowering. Among the plenty of GAs (>130) that have been identified in plants, fungi and bacteria, only a few have biological activity, with GA_1, GA_3, GA_4 and GA_7 being the major bioactive GAs. They are derived from a basic diterpenoid carboxylic acid skeleton, and commonly have a C3-hydroxyl group (Daviere & Achard, 2013). Structure-activity relationships for gibberellins (Gas) are known since the middle of the 1980s, showing that 3β-hydroxyl and 6-carboxyl groups are mandatory for bioactivity, while chemical modification of the 16(17)-double bond usually does not affect it (Figure 9).

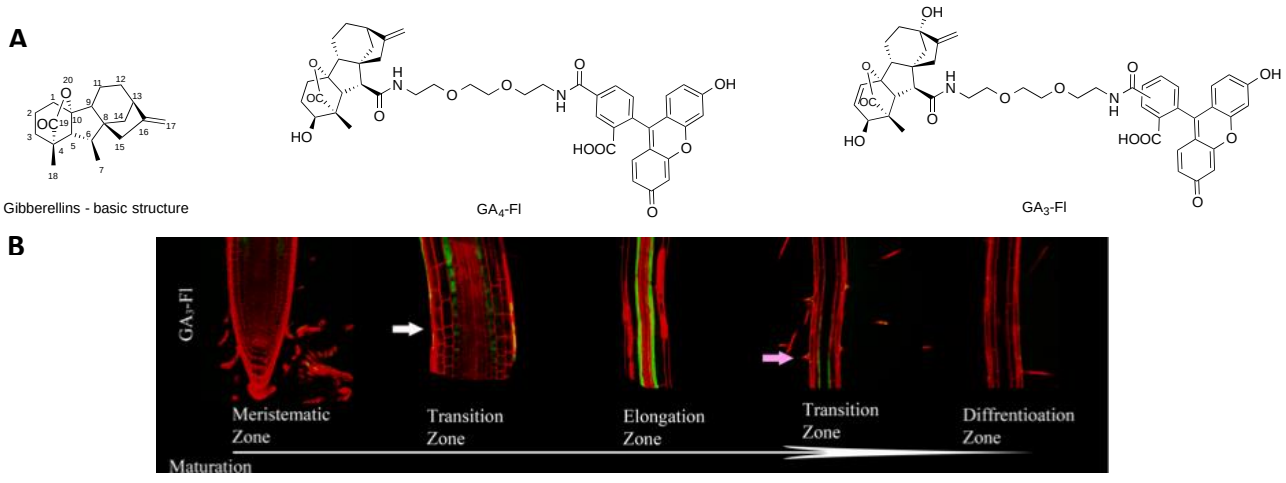


FIGURE 9. Molecular structure of gibberellins, their fluorescence-tagged derivatives and localization during *Arabidopsis* root maturation. A, Basic molecular structure of Gibberellins with carbon atom numbering, molecular structure of fluorescein derivatives GA_4-Fl and GA_3-Fl. B, GA_3-Fl highly accumulates in the

elongating endodermal cells. White arrow mark transition from meristematic to elongation zone; pink arrow marks transition from elongation to differentiation zone (From: Shani *et al*., 2013).

The first fluorescent labelled gibberellins were synthesized by Beale *et al.* (1991), together with biotinylated and photoreactive gibberellin probes, as well as immobilised gibberellins. However, a description of the bioactivity of such derivatives was lacking. To this regard, Pulici and collegues (1996) later prepared fluorescein derivatives of bioactive gibberellins (FLBG) by using the synthetic method reported by Beale, with the aim to provide a description of their bioactivity. They inserted two spacers of different lenght between fluorescein and the gibberellin moiety and measured their ability to induce α-amylase activity in the embryoless half grain, a process known to be normally induced by active GAs synthesised by the embryo. The compound with the longer spacer resulted to be more active presumably because of reduced hinderance by the fluorescent moiety, allowing a better interaction with the receptor. However, to be as active as the free GA_3, the fluorescent derivative had to be about 10 times more concentrated than GA_3. The authors concluded that decrease in activity could be due to reduced ability of the fluorescent derivative to move across the cell wall acting as a physical barrier against it while allowing the unhindered GA_3 to move freely (Pulici *et al.,* 1996).

Very recently, two other bioactive fluorescein conjugates of GAs were generated (GA_3-Fl andGA_4-Fl) (Figure 9) and used to successfully map the spatial distribution and transport mechanisms of GAs in *Arabidopsis* roots (Shani *et al.*, 2013). As for Pulici *et al.,* a positive correlation between linker length and bioactivity has been found.

Beside testing their fluorescent derivative bioactivity *in vivo*, they also performed *in vitro* tests such as pull-down assay and yeast two-hybrid assay and verified that tagged GAs were able to promote the specific interaction between the receptor GID1b and the repressors of GAs response DELLA protein, which is known to occur as part of the GAs perception mechanism. To further establish that bioactivity of GA_3-Fl andGA_4-Fl arised from the intact molecules and not degradation products, they analysed extracts from plants treated with both fluorescent derivative through a HPLC-HRMS (high resolution mass spectrometry) and found that GA_3-Fl was more stable, thus selected it to conduct the distribution pattern mapping. The *in vivo* observation of *Arabidopsis* root treated with the tagged compound led to determine the preferential accumulation of GAs in the vacuole of elongating endodermal cells, and this process was identified as an actively regulated transport (Shani *et al*., 2013).

Brassinosteroids (BRs)

Brassinosteroids include a class of nearly 70 polyhydroxylated sterol derivatives that are essential for the proper regulation of multiple physiological processes required for normal plant growth and development (Figure 10) (Clouse, 2011,).

As for Gibberellins and auxin, the protein responsible for binding of Brassinosteroids and triggering their signalling cascade is known, being a a leucine rich repeat (RLK) plasma membrane receptor named BRI1. However, while the role of BRI1 in initiating the BR response through its kinase activity have been studied in detail, the involvement of endocytosis in the regulation of BRI1 signaling is less understood.

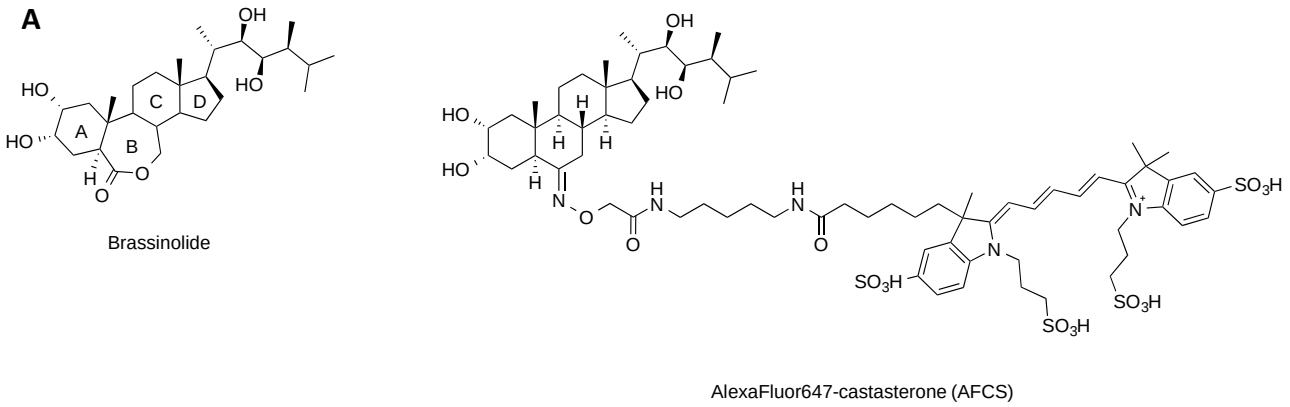


FIGURE 10. Molecular structure of a natural brassinosteroid, the fluorescence-labelled brassinosteroid analogue and its distribution pattern in root meristem epidermal cells of *Arabidopsis* seedlings expressing different fluorescent markers. A, Molecular structure of Brassinolide with rings lettering and of AlexaFluor647-castasterone (AFCS). B, AFCS labels both the GFP-tagged version of BRI1 (BRI1-GFP) and the TGN/EE marker VHAa1-RFP (white arrows). Scale bar 5 μm (From: Irani *et al*., 2012).

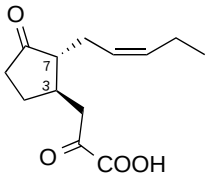
To this regard, Irani and collegues coupled a far-red emitting Alexa Fluor® tracer to the BR analog castasterone at the C6 keto position, known to be left outside of BRI1 ligand-binding pocket, to produce a bioactive fluorescently labeled BR analog named AlexaFluor647-castasterone (AFCS, Figure 10). Taking advantage of a GFP-tagged version of BRI1 and other various fluorescent-tagged endomembrane markers, they followed the endocytic route of receptor-ligand complexes *in vivo*

and analyzed BR signaling processes in living *Arabidopsis thaliana* cells (Irani *et al.*, 2012). Their publication is an example of how different type of fluorophore, i. e. small organic fluorophores and fluorescent proteins, can be used together in an integrated approach to visualize the complex system of small molecules, proteinous receptors and endomembrane machinery which are involved in a plant hormone signalling pathway. It is also interesting to notice here the use of synthetic small molecules to selectively perturb specific cellular processes, such as Tyrphostin A23 (TyrA23), a drug that interfere with the clathrin- mediated endocytosis. These so-called chemical genetics approaches can be used to generate phenotypes by altering protein function directly without the need of stabile mutations as in classical genetic approaches (see Hicks & Raikhel, 2014 for a deeper discussion).

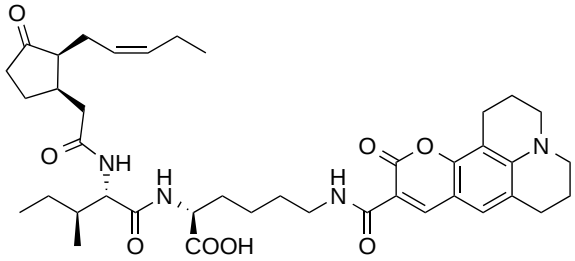
Jasmonic acid (JA)

JA and its derivatives, collectively known as jasmonates, are fatty acid derived cyclopentanones that regulates growth and development, responses to various environmental stresses and gene

A



(-)-Jasmonic Acid



JA-Ile-Coumarin

B

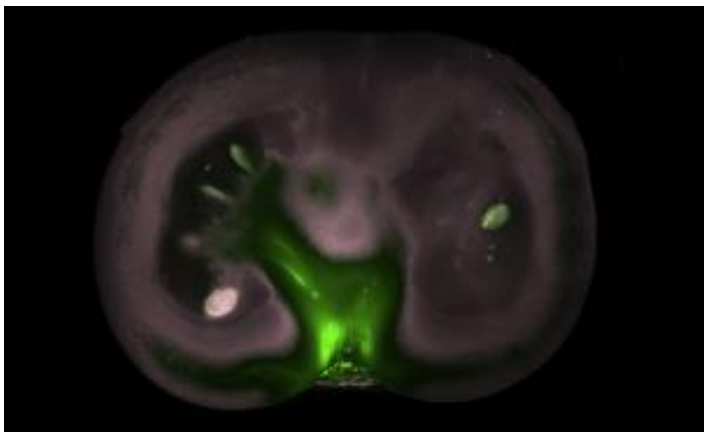


FIGURE 11. Molecular structure of (-)-Jasmonic Acid, its bioactive fluorescent derivative and distribution in tomato fruit. A, (-)-Jasmonic Acid and fluorescent JA-Ile-Coumarin. B, Migration of the fluorescent jasmonate in tomato visualized by fluorescent imaging (CRi Maestro imaging system) 48 h after application (From: Liu *et al*., 2013)

expression, playing a key role in plant defense responses against pathogens and pests (Figure 11) (Dar *et al*., 2015).

Based on examination of the model of interaction between the jasmonate and its receptor COI1, a fluorescent labeled jasmonate has been designed and synthesized in 2012 by Liu and collegues. In particular, the endogenous amino acid conjugate of JA, jasmonoyl-L-isoleucine (JA-Ile), which represents an active jasmonate derivative, has been bound to Coumarin 343 by means of a L-lysine link chain (Figure 11). Being the carboxyl group of isoleucine moiety far away from the active site, it has been selected as suitable position for fluorophore insertion. The efficacy of this design was evaluated by molecular docking, which has become a powerful tool for organic chemists to design modifications on bioactive compounds. The fluorescent jasmonate resulted to fit well within the binding pocket of COI1-JAZ1, with a strong interaction between the tagged molecule and the receptor, supporting that the designed probe would be bioactive. As expected, the effect of the fluorescent probe on the root growth of cabbage seedlings was found to be similar to that of the methyl jasmonate (MeJA), the standard bioactive jasmonate (Liu *et al*., 2012). The fluorescent jasmonate have then been applied to tomato fruits to visualize through fluorescent imaging the distribution and migration progress of jasmonates in vascular tissues (Figure 11) (Liu & Sang, 2013).

Abscisic acid (ABA)

ABA is a sesquiterpenoid plant hormone, which has since been shown to regulate many aspects of plant growth and development, and responses to environmental stresses (Finkelstein, 2013).

Fluorescein isothiocyanate (FITC) was introduced on to the 4' carbonyl group of ABA *via* 4-aminobenzhydrazide as a spacer by Asami *et al.* (1997). The bioactivity of the fluorescent tagged compound has then been evaluated by measuring its antagonistic activity toward the GA-induced α-amylase activity in the aleurone protoplasts. Even if the concentration required to mimic ABA

effect was about 30 times higher for the fluorescent ABA (FABA), the tagged compound was effectively able to inhibit the amylase induction by GA_3 (Asami *et al*., 1997). However, as far as we know it does not appear in literature any application of this compound, or other fluorescence-tagged version of ABA, in further fluorescence imaging studies on plants (Figure 12).

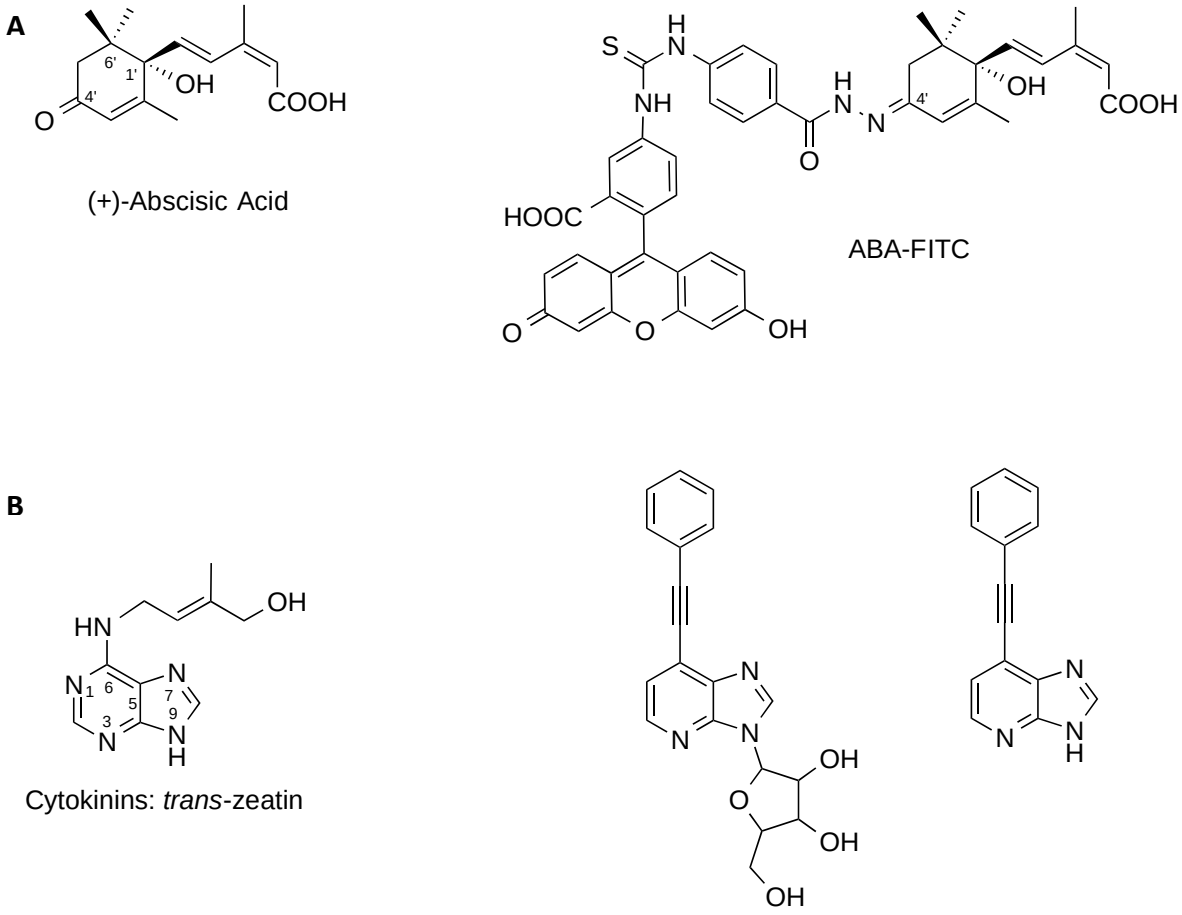


FIGURE 12. Molecular structure of Abscisic Acid, cytokinins and their fluorescent derivatives. A, (+)-Abscisis Acid and its Fluorescein conjugate ABA-FITC. B, *trans*-zeatine, a naturally occurring cytokinin, and carbon-substituted fluorescent cytokinin analogue developed by Nishikawa and collegues.

Cytokinins (CKs)

Naturally occuring CKs are adenine derivatives with a side chain at the N^6 – position; structure and conformation of this chain can markedly influence the biological activity of the cytokinin.

The appearance of fluorescent cytokinins in literature date back to the year 2000, when Nishikawa and collegues reported the synthesis of the fluorescent CK analog 7-Phenylethynylimidazo[4,5-*b*]pyridine and its riboside, where the two N^1- and N^6-atoms of adenine were replaced with carbon atoms. The compounds exhibited fluorescence in the UV part of the spectra as a result of the long

conjugated system involving the two aromatic rings connected with the triple bond. Cytokinin activities of the fluorescent CK analog and its riboside were assessed in three different biological assays by comparison with standard compounds and the 7-Phenylethynylimidazo[4,5-*b*]pyridine showed to have retained a potent bioactivty (Figure 12) (Nishikawa *et al.*, 2000,). However, as for ABA fluorescent analog, no further pubblication regarding the application of this fluorescent CK analogs is reported in literature, as well as any other example of the synthesis of fluorescent labelled CKs.

Strigolactones (SLs) in the spotlight

SLs are relatively newcomers in the PH class. Since the discovery of the first strigolactone in 1966 it took half a century to un-puzzle the story of this intriguing class of molecules. In elucidating the mechanism of action of this carotenoid-derived compounds and their transportation/exudation in plants, it became mandatory to be able to trace these bioactive molecules *in vivo*.

Hereunder, a brief introduction on SLs is provided as functional to the further discussion on the steps that have led to the succesfull design and synthesis of fluorescent SLs analogs by different research group converging on this topic.

SLs in brief: a unique class of phytohormones and communicators

In plants, Strigolactones (SLs) represent a unique example of interkingdom communication overlap. This class of signalling molecules is biosynthetized by plant roots and released in the rizosphere in very small amounts. SLs were originally isolated from plant root exudates as germination stimulants for root parasitic plants of the family *Orobanchaceae* and so were regarded as detrimental to the producing plants (Kim *et al.*, 2010; Yoneyama *et al*., 2010). Subsequently, Akiyama *et al.* (2005) discovered their role as indispensable chemical signals for the establishment of arbuscular mycorrhizas, the widespread symbiosis between land plants and a small group of fungi named Glomeromycota which enhance plant nutrition and stress resistance (Bonfante & Genre, 2010). Thus, SLs became recognized as beneficial plant metabolites. Then, more recently, they have been recognized as real plant hormones, involved in the inhibition of shoot branching, in root development and in a growing list of other developmental processes (Gomez-Roldan *et al*., 2008; Lopez-Obando *et al*., 2015; Umehara *et al*., 2008). Moreover, very recently, an antiproliferative activity of SLs and their analogues on cancer cell lines has been

demonstrated, revealing another significant biological effect brought by the same bioactiphore (Mayzlish-Gati *et al*., 2015; Pollock *et al.*, 2012).

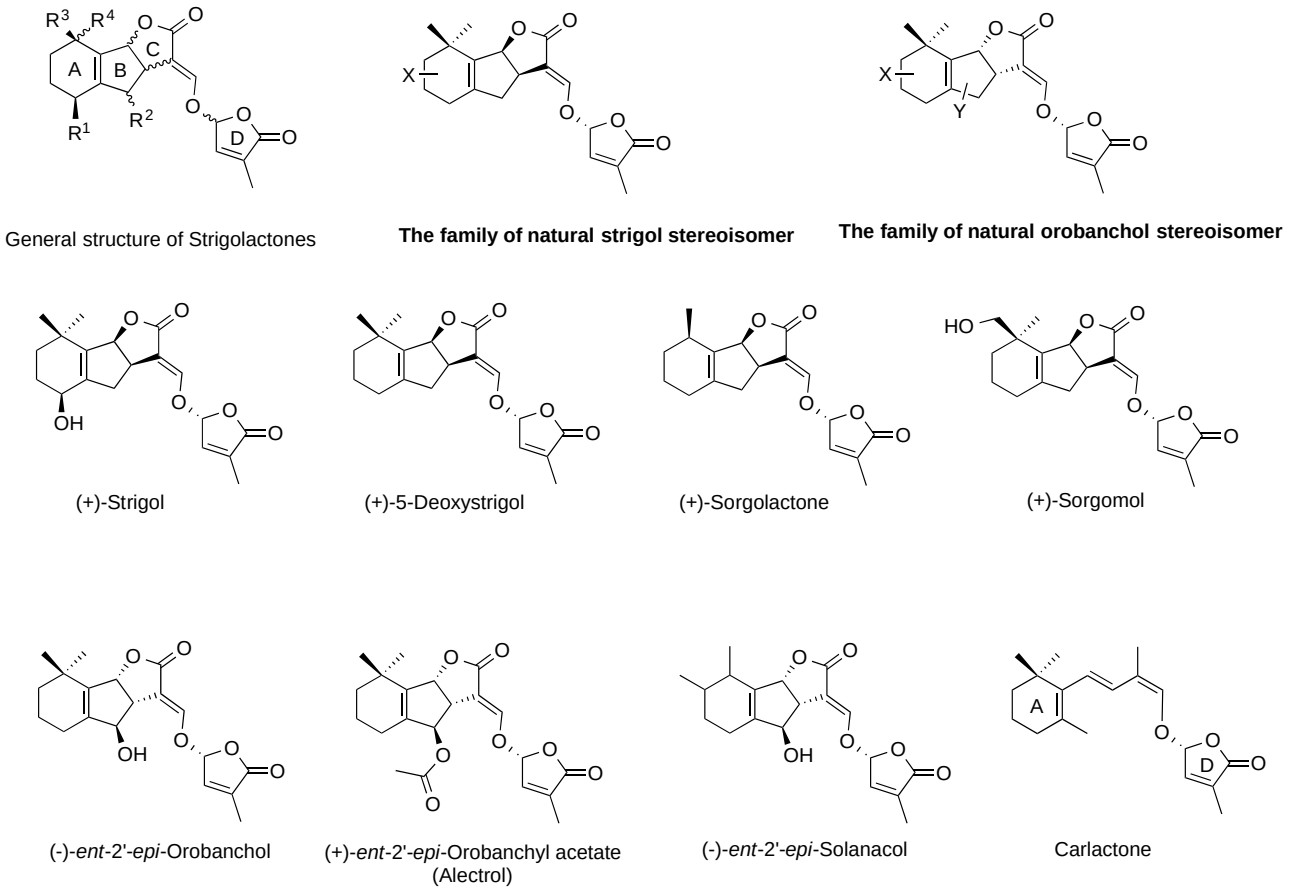


FIGURE 13. General structure of SLs with rings lettering, Strigol-like and Orobanchol-like families, selection of natural SLs and Carlactone.

Naturally occurring strigolactones

To date, at least 19 naturally occurring SLs have been isolated and structurally characterized by different research group (Figure 9), often with difficulties due to the small amounts of those molecules found in tissues and in root exudates (Xie *et al*., 2010; Yoneyama *et al*., 2009; 2010). They all share a common framework composed by a tricyclic lactonic fused system (ABC part) linked through an enol ether bridge to a methyl butenolide (D ring). As discriminating features they bear one or two methyl groups in the A-ring and one or more hydroxyl or acethoxyl group in the A/B ring moiety. Also, the stereochemistry at the B/C junction and at C-2' is a variable. According to a recent structure revision (Xie *et al*., 2013), naturally occurring SLs can be divided in two families that is, one in which the stereochemistry in the BC part is the same as (+)-strigol, and the other in which the stereochemistry in the BC part is the same as (-)-orobanchol. Both families have a conserved *R*-configuration at the C-2' position (Figure 13).

Synthetic SL analogs and structure-activity relationship (SAR) studies

The structural diversity which characterize SLs could be at the base of their versatility with respect to perception, enabling them to be differentially perceived by distinct organism. Indeed, the correlation between the chemical structure of naturally occurring strigolactones and their activity is one of the methods that have been used to gain insights about SLs' bioactivity, especially when collection of mutants were still not available. However, as mentioned, naturally occurring SLs are difficult to obtain in sizeable quantities and their stability is poor therefore, since the beginning of the strigoloactone story, the SL community had actively developed SL analogues that are easily accessible in sizeable quantities and stable enough to be kept in stock for months, greatly facilitating structure-activity relationship studies in different models.

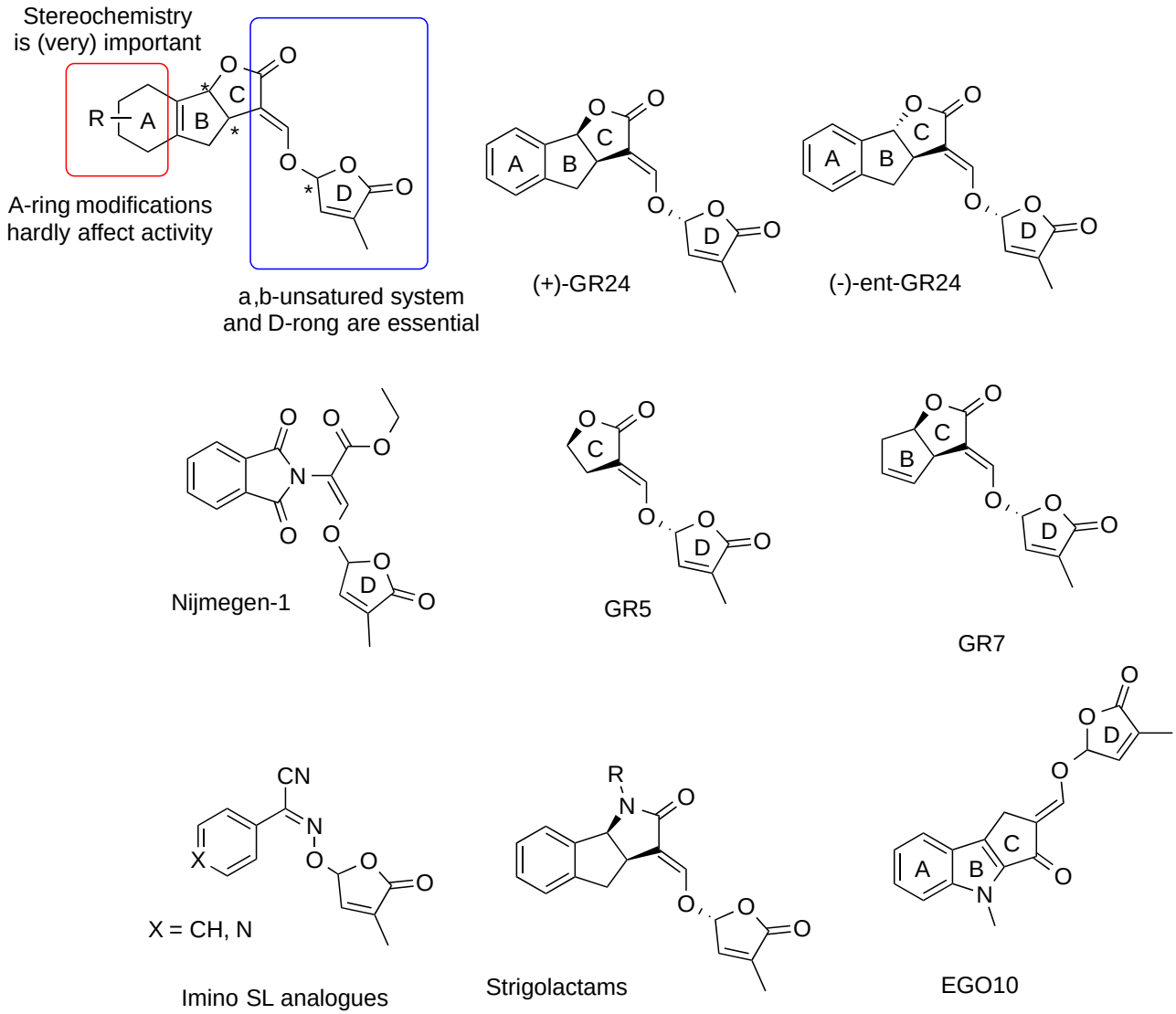


FIGURE 14. Model for designing synthetic SL analogs and molecular structure of synthetic SLs.

Plenty of different SL-like structures have been designed and synthesized, generating modification on the original ABC-D structure (Figure 14) (Boyer *et al*., 2012; Boyer *et al*., 2014; Cohen *et al*., 2013; Kgosi *et al*., 2012; Zwanenburg & Mwakaboko, 2011; Zwanenburg *et al.*, 2013). Besides analogues that usually retains the enol-ether bridge, the synthesis of SL-mimics, lacking both the enol ether moiety and the ABC ring system, have been reported. SL mimics are simpler molecules in which a butenolide (D ring in SLs) is linked to a good leaving group at C'2 (Figure 14) (Fukui *et al.*, 2011; 2013). One of the most potent analogues, the GR24 (Figure 14), is currently widely used as the standard in biological assays (Johnson *et al.*, 1981; Mangnus *et al*., 1992).

Through the comparison of the activity of different SL-like structures in different biological systems, the structural requirements to induce bioactivity have been partially clarified, together with possible molecular mechanisms at the receptor site. Importantly, it has been shown that there are nuances between plants, parasitic weeds and AM fungi, suggesting that each system uses a different perception system, which complicates the scenario.

All currently known natural SLs possess the same CD rings with a methyl at the C4' position, suggesting the importance of this structure for bioactivity. Also, stereochemistry of SLs has been shown to be crucial for bioactivity and the results so far acquired suggest that analogues with stereochemistry similar to those of natural SLs often return the highest activity in all biological system (Akiyama *et al*., 2010; Nakamura *et al*., 2013; Nomura *et al.*, 2013; Scaffidi *et al*., 2012; 2014). Indeed, the (-)-*ent*- GR24 component of *rac*-GR24, showing the stereochemistry of the non-natural SL, have been shown to have activity distinct from that of (+)-GR24 in *A. thaliana* (Figure 14). Thus, racemic mixtures of chemically synthesized SLs and their analogues, should be used with caution because they can activate responses that are not specific to naturally occurring SLs (Scaffidi *et al*., 2014). As support to this hypothesis, it has already been demonstrated that the bioactivity on AM fungi seems to be strongly dependent on the configuration at C2' of the D ring (Prandi *et al*., 2011). Recently in our laboratory we synthesized a series of strigolactone analogues, whose absolute configuration has been elucidated and related with their biological activity, thus confirming the high specificity of the response. Analogues bearing the R-configured butenolide moiety showed enhanced biological activity, which highlights the importance of this stereochemical motif (Artuso *et al*., 2015).

SLs perception

The identification of the receptor protein/s responsible for binding strigolactones and triggering the signaling cascade is a challenging task, especially because of the differences in the way SLs are perceived by different organisms.

In plants, genes encoding SL receptors have been identified from the characterization of SL-insensitive mutants. There is now a substantial body of data regarding the function of DWARF14 (D14) proteins belonging to a clade of the α/β-hydrolase enzyme superfamily as probable SL receptors from rice (D14), Arabidopsis (AtD14) and petunia (DAD2) (Arite *et al.*, 2009; Hamiaux *et al.*, 2012; Waters *et al*., 2012). Interestingly, the gibberellin (GA) receptor GID1 belongs to the same α/β–fold hydrolases family as D14 (Ueguchi-Tanaka *et al*., 2005); while GID1 has lost its catalytic triad and does not exhibit hydrolase activity, D14 possesses the conserved triad Ser-His-Asp (S-H-D) and is predicted to function accordingly. As regards, D14 proteins have been shown to *in vitro* hydrolyze the synthetic SL GR24 into inactive ABC- and D-ring parts (Figure 15).

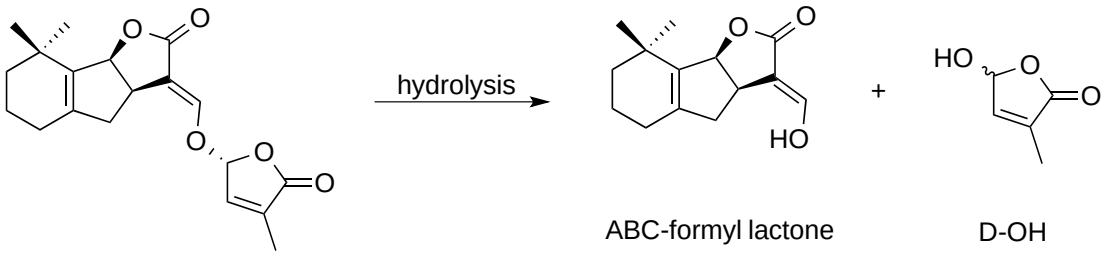


FIGURE 15. Hydrolysis of strigolactones leads to generation of two fragments, the formyl tricyclic ABC core and the hydroxybutenolide.

Mutation of the catalytic triad at the Ser residue hamper the biological response of D14 proteins; however, the role of SL hydrolysis by the receptor is not clear and the hydrolysis products have been shown to be biologically inactive (Hamiaux *et al*., 2012).

The molecular mechanism of SLs' perception occurring at the receptor site has been a fervent discussion topic. Indeed, based on SAR studies different mechanisms have been proposed, among which the most accounted rely on the attack of a nucleophile directly to the butenolide ring (Scaffidi *et al.*, 2012). This last mechanistic hypothesis is fully consistent with the α/β-hydrolasic function of D14: following hydrolysis, the D-OH part of SLs forms part of the recognition surface for interacting proteins. The D14-SL-target protein complex then modulates downstream signaling (Nakamura *et al*., 2013).

From functional SLs analogues to functionalized SLs analogues

Beside deepening the structure–activity relationship in different systems, in recent years the development of new synthetic SL analogues have been directed towards a new research strategy, that is to design fluorescent active SL analogues suitable for bioimaging studies *in vivo* detection as a tool to understand SLs distribution and perception in parasitic weeds, plants and AM fungi.

At the end of the 90s', the preparation of biologically active, labelled strigol analogues was proposed as a strategy to identify the strigol receptor in the seeds of parasitic plants (Thuring *et al*., 1997). Since the CD-part is essential for biological activity, the substituents were introduced on the A-ring, because of its remote position with respect to the bioactiphore. Amino-GR24 was prepared and functionalized with affinity tags or photoaffinity labels, among others, biotin and the fluorescent dansyl and rhodamine groups (Reizelman *et al.*, 2003), but the results remained unpublished (Figure 16) (Zwanenburg & Pospísil, 2013).

Subsequently, the synthesis of SL analogues with a built-in fluorescent moiety were reported (Bhattacharya *et al*., 2009; Prandi *et al.*, 2011). Bhattacharya *et al*. described the synthesis of a new class of SL analogues (PL series) featuring an unprecedented extended conjugated system and whose bioactiphore is an α, β- unsaturated ketone instead of the more common α, β-unsaturated lactone (Figure 16). Beside showing remarkable activity in *Orobanche aegyptiaca* seed germination tests, some of these molecules displayed interesting luminescent properties (Bhattacharya *et al*., 2009). These observations encouraged the researchers to design and develop new fluorescent analogues whose spectroscopic properties could be exploited in bioimaging studies. Even if some molecules displayed a good activity on both parasitic plant seeds and AM fungi, unfortunatly none of them was found to meet the instrumental requirements that fix at 405 nm the minimum wavelength of excitation required for confocal studies (Figure 16) (Prandi *et al*., 2011).

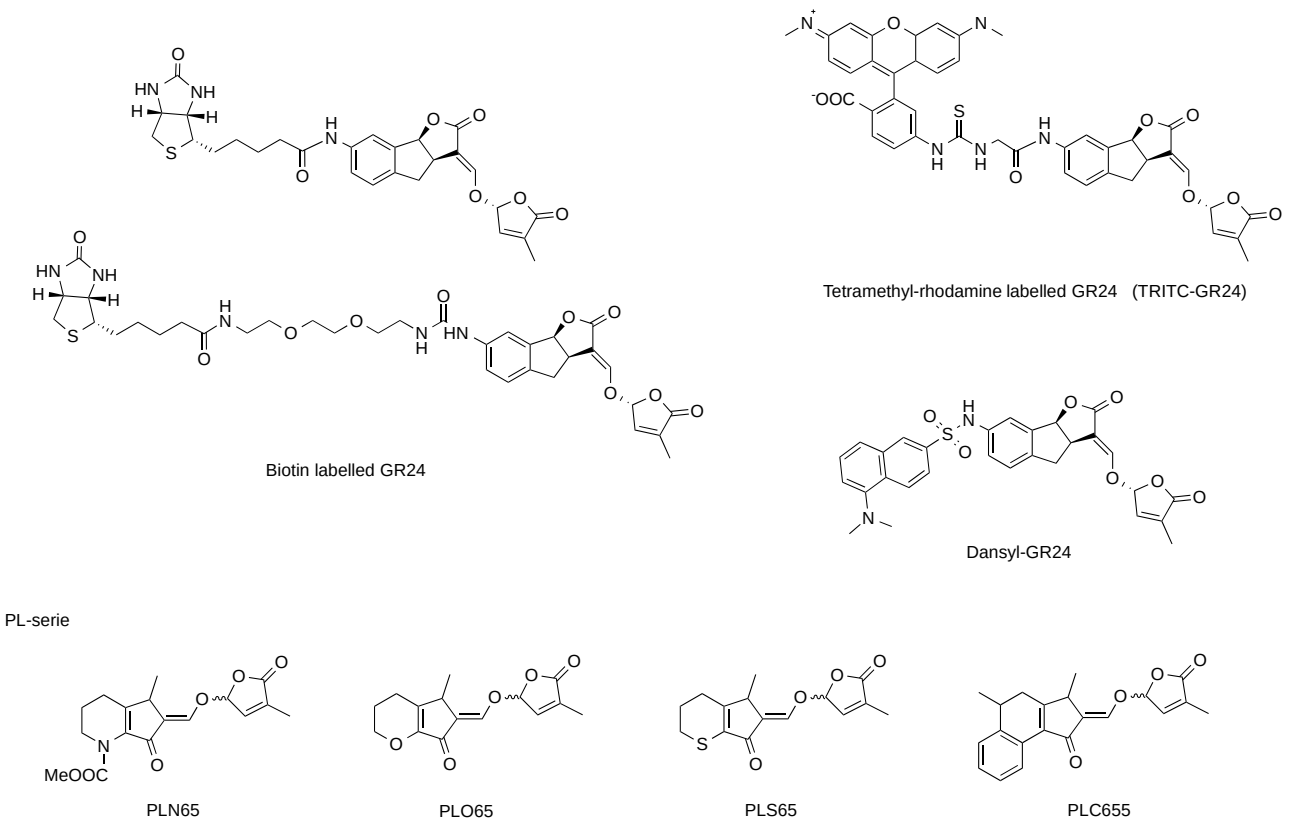


FIGURE 16. Molecular structure of functionalized SL analogues

Based on these results, a multifaceted approach was applied in conceiving a new synthetic design, involving the binding of different fluorophore moieties on the A-ring of the SL analogue core. Indeed, a 3D pharmacophore model based on SAR data for germination inducing activity confirmed that only positions on the A-ring are suitable for bearing a bulky fluorophore, while other positions are not, because they are probably crucial site for the binding of the molecule to the receptor pocket (Lolli *et al*., unpublished results). A computational screening to predict the spectroscopic properties of the newly designed molecules allowed the selection of a reduced set of molecules to be effectively synthesized: a fluorescein derivative (BL), three BODIPY derivatives (EG, HR and EGO), two boranil derivatives (Bor-Ph-EGO and 5-NO_2-Bor-Ph-EGO) and a dansyl derivative (AO) (Figure 17). Eventually, biological activity tests on *Orobanche aegyptiaca* seeds and preliminary observations *in vivo* were performed on both a plant (*Arabidopsis thaliana*) and an AM fungal (*Gigaspora margarita*) system for each different fluorescent-tagged SL-like molecule, leading to identify the most promising candidate for further imaging studies, the molecule BODIPY-EGO10 (EGO10-BP). Notably, this molecule shares the core structure with the SL analog EGO10, which has been shown to retain good biological activity in each biological system, i.e. as

inducer of parasitic seed germination, hyphal branching and root hair elongation (Cohen *et al*., 2013; Prandi *et al*., 2011; 2013).

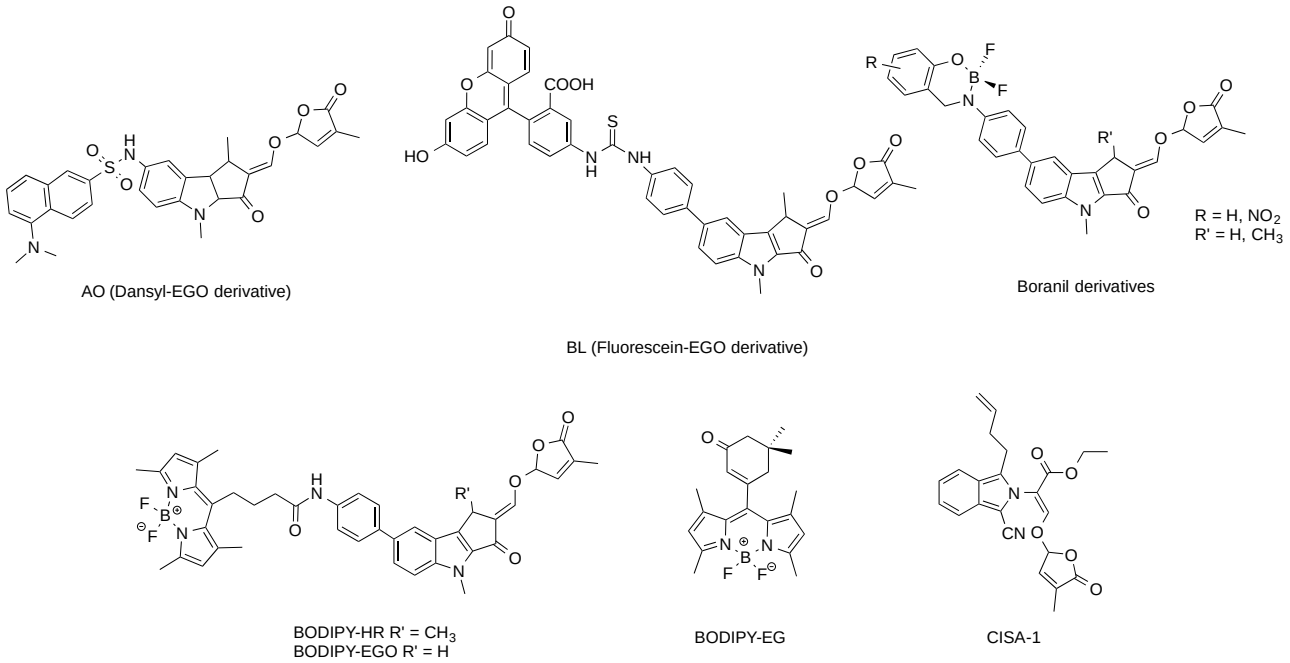


FIGURE 17. Molecular structure of fluorescent SL derivatives.

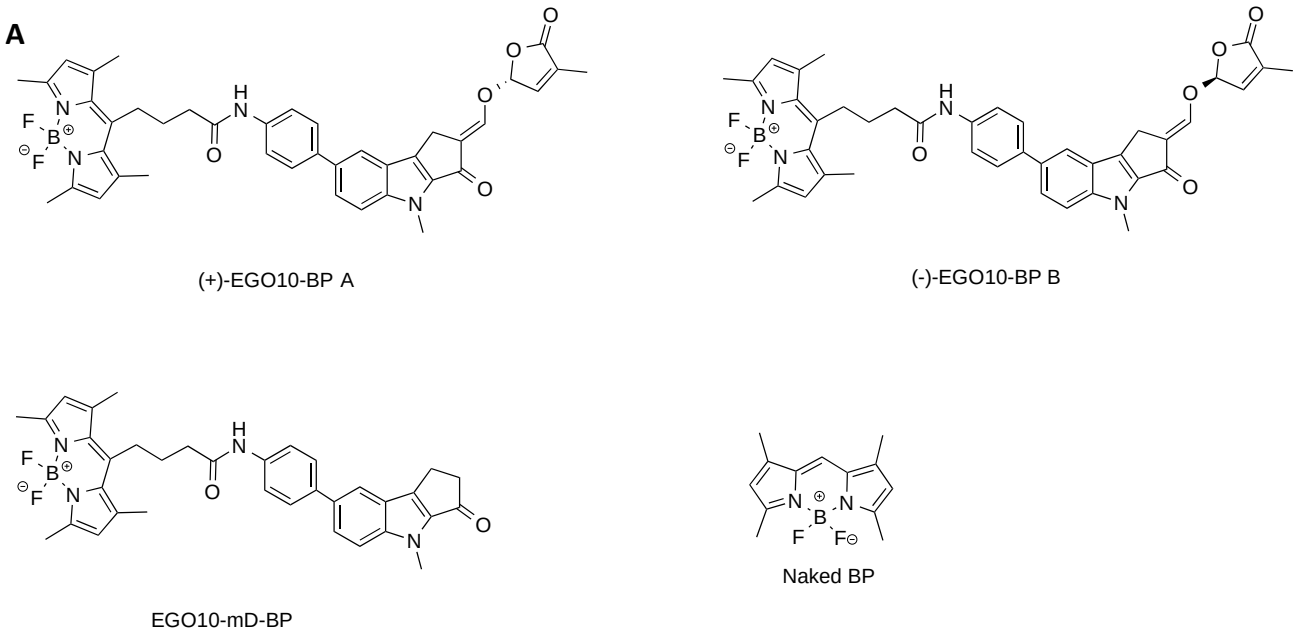
Importantly, following the perception of the above cited fluorescent analogs by D14-type receptor proteins, the ABC part bearing the fluorophore would be cleaved at the enol-ether bond as a consequence of the enzyme hydrolitic activity, possibly leaving the unlabelled D-ring inside the binding pocket. Being the hydrolysis a rapid event, it would therefore be difficult to label the receptor site with these kind of fluorescent analogs. Instead, they constitute a suitable tool to *in vivo* monitor SLs distribution and transport in both plants (see following paragraph) and AM fungi.
During the same time period, a new fluorescent SL named CISA-1 has been synthesized by a simple procedure. A classical genetic approach performed on *Arabidopsis* Columbia wild-type, *max1*/*max4* (SL-deficient mutants) and *max2* (SL-insensitive mutant) confirms its SL-like activity. The fluorescent property of CISA-1 has been observed at 10mM in solution with the excitation and emission spectra between 300-380nm and 400nm, respectively, but unfortunately fluorescence detection *in planta* still needs to be improved (Rasmussen *et al.,* 2013).

Mapping of fluorescent SLs distribution in Arabidopsis thaliana*.*
Central to the role of strigolactones is the plant root. Here they are biosynthesized (Delaux *et al*., 2012; Xie *et al*., 2010) and exert part of their action as endogenous hormones. They increase cell numbers in the primary-root meristem (Koren *et al.*, 2013; Ruyter-Spira *et al.*, 2011), and regulate

lateral-root formation in relation to growth conditions – suppressing it under sufficient phosphate, but inducing it under limiting phosphate (Kapulnik *et al*., 2011a; Ruyter-Spira *et al*., 2011). Strigolactones also increase root-hair elongation in the primary root (Kapulnik *et al*., 2011a; 2011b). From the root, SLs are transported to the aerial part of the plant (Brewer *et al*., 2013), where they act mainly as inhibitors of shoot branching (Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008). The plant root is also primary to SLs exogenous role, being the site of their release into the rizosphere, where they are perceived at nanomolar concentration by AM fungi, a small group of fungi belonging to the Glomeromycota phylum (Schüβler, 2001).

In 2012, the *Petunia hybrida* ABC transporter PDR1 has been isolated and its key role in regulating the development of arbuscular mycorrhizae and axillary branches, by functioning as a cellular strigolactone exporter, has been demonstrated (Kretzschmar *et al*., 2012).

More recently, new evidence for SL distribution have been provided in the plant using a fluorescent BODIPY (BP)-tagged SL analog EGO10-BP. In particular, four different BP derivatives differing in their structure were used: EGO10A-BP (pure enantiomer A), EGO10B-BP (pure enantiomer B), EGO10-mD-BP (SL analog EGO10-BP lacking the D-ring and the enol ether bridge) and naked-BP (fluorophore only) (Figure 18). By comparing the *in vivo* distribution pattern and the bioactivity of the four different fluorescent compounds, the dynamic processes involved in the localization and regulation of SLs in *Arabidopsis thaliana* root system has been investigated.



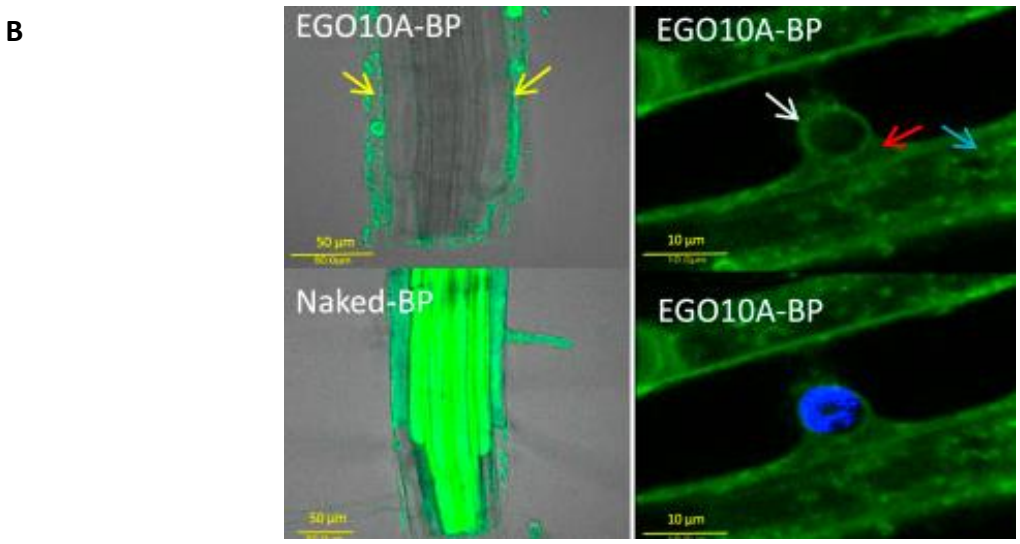


FIGURE 18. Molecular structure of BODIPY-derivatives and mapping of their distribution in *Arabidopsis* root system. A, EGO10-BP enantiomers A and B, EGO10-BP lacking the D-ring and the enol ether bridge (EGO10–mD-BP) and BODIPY fluorophore alone (naked-BP). B, Images of roots treated with EGO10A-BP or naked-BP. Green- EGO10A-BP or Naked-BP signal, Blue staining- DAPI. Yellow arrows denote the epidermis cell layer. Blue, red and white arrows indicate EGO10A-BP staining in endosomes like bodies, cytoplasm and nucleus envelop, respectively (From: Fridlander *et al*., 2015).

According to SAR studies, the enantiomer with natural SL structures (R configuration) EGO10A-BP was found to be the most active analog for root-hair elongation. EGO10A-BP distributed in the roots mainly in the epidermal cell layer, suggesting SLs to be mainly transported in the epidermis cell layer. EGO10A-BP molecules are present mostly in the cell cytoplasm and this transport may be dependent on the structure of the transported molecule, since the un-natural enantiomer and the molecules lacking the D-ring and the enol ether bridge are transported in the root to a lesser

extent. The inhibition of the ATP synthesis brought evidence that both the influx and efflux of EGO10A-BP are ATP dependent, the influx negatively and the efflux positively regulated (Figure 18) (Fridlander *et al*., 2015).

Fluorogenic SLs probe to identify the receptor of parasitic plants

Root parasites of the genera *Striga*, *Orobanche* and *Phelipanche* belonging to the Orobanchaceae family, are considered to cause highest negative impact on food production because of their ability to affect many important food crops (Parker, 2009). Germination of root parastitic plant seeds is triggered by SLs but the mechanism of how minute amounts of structurally diverse strigolactones are sensed by the parasitic seeds is unclear. Very recently, Tsuchyia and collegues evidently boosted the research in that field succesfully probing the SL receptor in *S. hermontica* by means of fluorescence. Their approach has been to develop two structurally simple fluorogenic molecules where fluorescein is coupled to one (Yoshimulactone Green YLG) or two (YLGW Yoshimulactone Green double) bioactive strigolactone moieties (D-ring) through a simple synthetic procedure. The so-obtained probes take advantage of the hydrolitic activity of AtD14-type strigolactone receptors, acting as reporters of the perception. Indeed, following the binding of the fluorogenic probe to its receptor through the D-ring, subsequent hydrolysis occurs, leading to the release of fluorescent products (free fluorescein) whose signal can be visualize and quantified (Figure 19) (Tsuchiya *et al.*, 2015). Bioactivity tests confirmed the ability of YLG molecule to induce germination of *S. hermontica* seeds, which developed fluorecence as a consequence of the fluorogenic molecule treatment (Figure 19). By means of extensive *in vitro* biochemical tests using recombinant AtD14 protein and its homologs in *S. hermontica* ShD14 and ShHTL1 to -11, the latters identified by searching in a public *Striga* RNA-seq database, the group designated *ShHTL7* as the gene coding for the functional strigolactone receptor in *Striga hermontica.*

Tracking signal perception by the strigolactone receptors in intact *Striga* seeds was the final step.

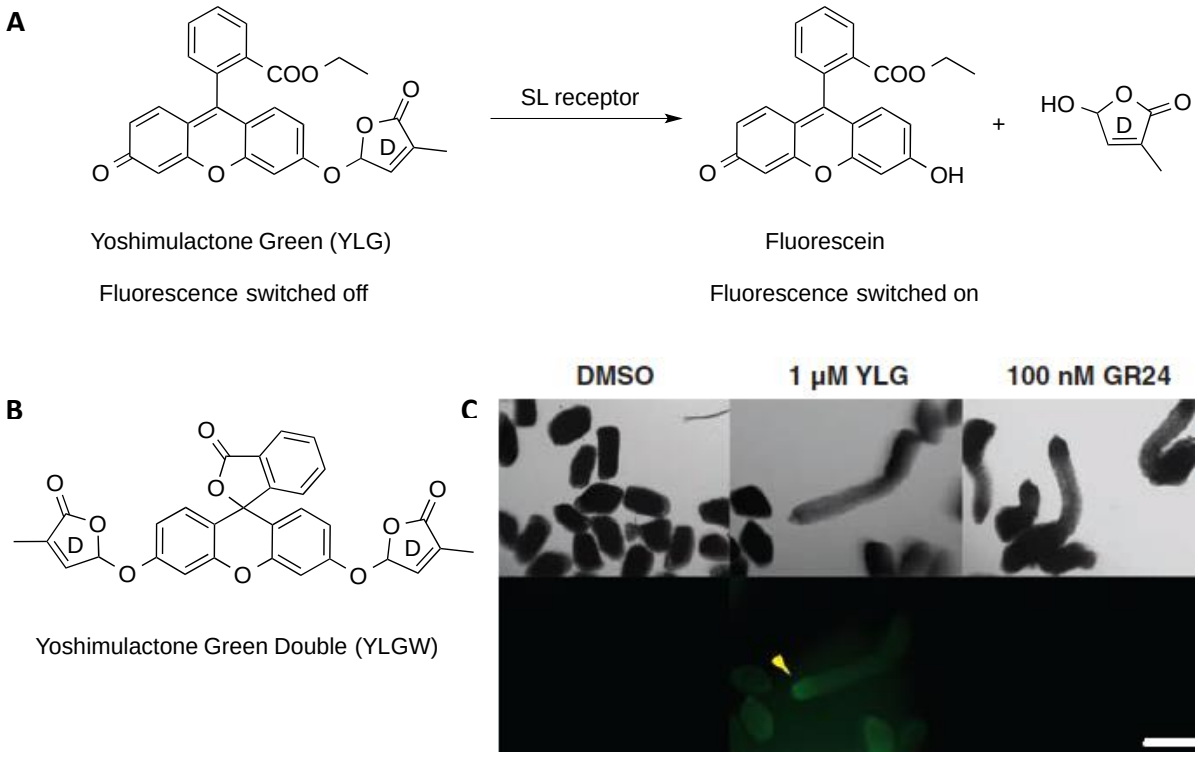


FIGURE 19. Identification of strigolactone receptor in *S. hermontica***.** A, Illustration of the molecular design of fluorescence turn-on molecule YLG for strigolactone receptors. B, Molecular structure of the fluorogenic probe bearing two D-ring (YLGW). C, YLG stimulates *Striga* seed germination with simultaneous release of the fluorophore. Top, bright-field image; bottom, fluorescence image. The arrowhead indicates the root tip. Scale bar, 300 mm (From: Tsuchiyia *et al*., 2015).

To this regard, the molecule bearing two D-ring moieties, YLGW (Figure 19B), was used to perform the imaging because of an increase in the on/off ratio with respect to YLG, as reported by the authors.

Application of YLGW to seeds resulted in fluorecence to appear following a two-phase kinetic: in a first moment, named by the author wake-up wave, fluorescence was visible at the root tip of the *Striga* embryo and diffused through the cotyledon in a six-hours period, finally disappearing. The so-called pre-germination pause was then followed by a second wave of fluorescence, the elongation tide, which accompanied the elongation of the root typical of a germinating seed (Tsuchiya *et al*., 2015).

Fluorescent SLs and AM fungi

Starting from 2005, strigolactones were identified as being the morphogens responsible for triggering hyphal branching, necessary for the AM fungus to enter the so-called pre-symbiotic phase. Indeed, when plants are affected in strigolactones production their capacity to be mycorrhized by AM fungi is strongly reduced, thus indicating that SLs are essential signaling molecules for the establishment of the symbiosis (Gomez-Roldan *et al*., 2007; 2008).

It was demonstrated that strigolactones not only induce hyphal branching (Akiyama *et al*., 2005) but also activate mitochondrial function and spore germination, inducing rapid changes in shape, density, and motility of mitochondria as wells as a rapid enhancement of the energy metabolism (Besserer *et al*., 2006; 2008). In addition, SLs have been proved to stimulate fungal production of chitin oligomers, in particular tetramers and pentamers, which were in turn identified as key fungal signals directed to the host plant (Genre *et al*., 2013).

Therefore, SLs appear to trigger a cascade of molecular and cellular events necessary for hyphae to become infective, but the exact site of action of SLs in AM fungal hyphae is still a big question mark. Synthetic SL analogues have been developed that suppress shoot branching of plants but do not induce hyphal branching of AM fungi (Boyer *et al*., 2014). Thus, the fungal SL receptor is thought to be different from the putative SLs receptors in plant and parasitic weeds, the α/β-hydrolases D14 and HTL, and SL perception in the two different biological systems is most likely a result of convergent evolution (Gutjahr, 2014). To this regard, no apparent D14/HTL homolog has been found in *Rhizophagus irregularis* genome (Koltai, 2014). Uncover the molecular basis of SLs response in AM fungi is made more difficult by the fact that common genetic engineering techniques are unfeasible on these ancient and cryptic class of fungi; in this frame, we designed our fluorescent SL analogues to meet the structural requirements mandatory for bioactivity on AM fungi, i.e. presence of the tricyclic lactone as well as the methylbutenolide (Akiyama *et al*., 2010), in order to used them as a tool to *in vivo* investigate the dynamic mechanism of SL perception.

Conclusions

Our comprehension of plant hormones functioning has been amazingly boosted thanks to small bioactive molecules designed for a specific target or function. The ability to manipulate che hormone's chemistry coupled to the recent advancements in the structural characterization of protein's binding pockets has led to the synthesis of a cospicuos range of effective derivatives, paving the way to a targeted regulation of plant hormone mechanisms. In this frame, plant

hormones fluorescence labelling is succesfully emerging as a powerful technique to directly visualize not simply the presence or location of molecules within cells and tissues, but also many of the complex and dynamic features of plant hormones signalling, transport and distribution in living organisms. Indeed, even in the world of science, eyesight is our most effective and informative channel, as suggested by the sentence “seeing is believing”.

Furthermore, fluorescent tagged bioactive compounds should be considered as a developing tool in the area of PH research. As far as new information about plant hormones mode of action are gathered, the design and synthesis of these molecules can be fine tuned to meet the novel requirements. For instance, the bioactiphore can be specifically designed for a specific function ascribed to the plant hormones, as well as modification of the fluorophore can be generated thanks to the increasing availability of new fluorescent probes and creative ways to use them by means of advanced and innovative technologies.

In this context, to allow the design of perfectly fitting molecules, a close collaboration between chemists and biologists is essential. Going forward, we expect that fluorescence labeled PH will help to dissect different PHs related processes.

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