

Seeing is better than believing: visualization of membrane transport in plants

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Recently, the plant transport field has shifted their research focus toward a more integrative investigation of transport networks thought to provide the basis for long-range transport routes. Substantial progress was provided by a series of elegant techniques that allow for a visualization or prediction of substrate movements in plant tissues in contrast to established quantitative methods offering low spatial resolution. These methods are critically evaluated in respect to their spatio-temporal resolution, invasiveness, dynamics and overall quality. Current limitations of transport route predictions-based on transporter locations and transport modeling are addressed. Finally, the potential of new tools that have not yet been fully implemented into plant research is indicated.

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the 80s and 90s, the plant transport community concentrated mainly on biochemical characterization of single transporter isoforms with a focus on a kinetic level (the 'queen of transport experiments' [2]). In the last two decades, deep insights into transporter expression, trafficking, regulation and their role in plant physiology were provided. Recently, plant scientists have shifted their interest toward a more integrative investigation of long-range transport routes or transport networks [3–5] that is of water, nutrients or some hormones (for an overview, see [Figure 1](#)).

Transport was classically quantified on a whole organ or plant level using radiotracers, external microelectrodes or cell-type specific analyses of transport substrates (for reviews on these methods, see Ref. [6]). While these techniques allow for a high level of temporal resolution, they are limited in the spatial aspect. Therefore, direct and indirect techniques have been developed in order to image either local substrate concentrations or intra and intercellular transport *in planta*. Here, we present current methods that allow for a quantitative visualization of transmembrane transport and compare them with classical transport measurements and *in silico* techniques.

Inference of transport routes by transporter imaging

For several substrates, putative transport routes have been deduced from polar transporter localizations [3,5,7–10]. These cell-biological approaches have been partially backed up by analyses of reporter expression and transport modeling (see below).

The best-understood example is probably the polar transport of the auxin, indole-3-acetic acid (IAA), in the Arabidopsis root tip provided by a network of plasma membrane localized im- and exporters of the ABCB, AUX1/LAX and PIN-FORMED (PIN) families [3,11]. Different degrees of tissue-specific and polar locations of members of these families have been established by immunolocalization and fluorescent protein fusions (see [Figure 3](#)) that are in line with current models of a reversed fountain auxin flux pattern (see [Figure 1](#); [8]). Similarly, the boric acid channel, NIP5;1, and the boron exporter, BOR1, have been localized in a polar fashion in the plasma membranes facing toward soil and stele, respectively, and are therefore suspected to function in the uptake and translocation of boron to support growth of various plant species (see [Figure 1](#); [7]). Likewise, the ABCG-type SL transporter, PDR1, exhibits an asymmetrical localization in the plasma membrane of petunia root

Introduction

*Because you have seen me, you have believed;
blessed are those who have not seen and yet have believed.*¹
John 20: 29

The catalyzed movement of substrates across membranes, commonly referred to as transmembrane transport, has always been a hotspot in plant biology [1]. Throughout

¹ Please note that this bible citation is not at all meant as a religious statement but a stylistic element acknowledging the effort of the transport field to promote techniques allowing for a quantitative visualization of transport, which is the content of this review.

Figure 1

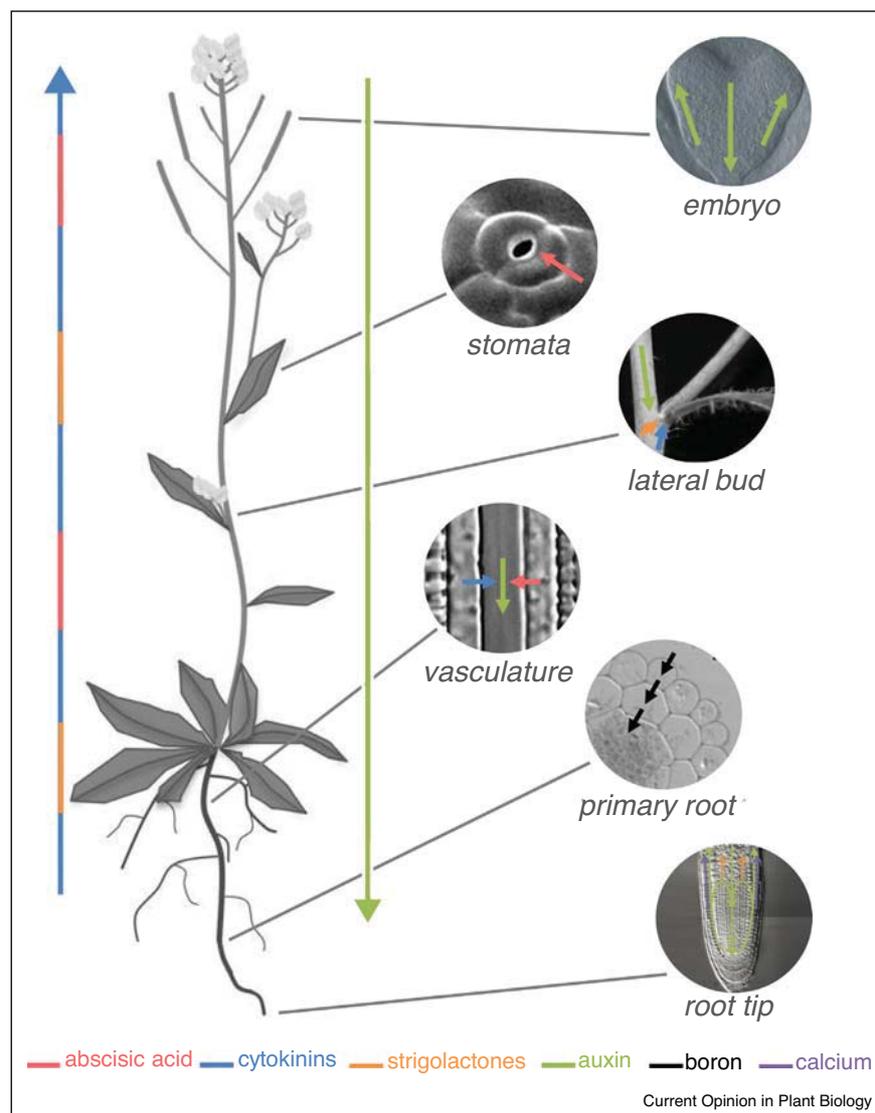


Illustration of trans-membrane, long-range transport routes in plants.

Exemplary long-range transport routes of indicated phytohormones, boron and calcium are indicated by arrows. Note that only long-distance signaling of Ca^{2+} where it acts as a second messenger but not as a nutrient is indicated. In contrast, boron paths are indicating nutrition routes. Overall figure outline is based on [5]; usage of Arabidopsis model was granted by Mary Lou Guerinot.

tips indicating a directional cell-to-cell transport process at least in this region of the root (see Figure 1; [5]). However, presence of a transporter on a cellular subdomain does not automatically reflect local activity levels. Moreover, this approach failed so far in the prediction of transport routes for non-polar transporters known to be involved in long-distance transport [12].

Quantification of transporter activity or regulation

A better proxy might be the recently developed *transport activity sensors*, such as AmTrac and MepTrac [13,14], NiTrac1 and PepTrac [15]. Although these transport

activity sensors do not strictly monitor transport, and have not yet been tested *in planta*, they will most likely become valuable, especially in the context of analytes for which no tracers exist.

An alternative approach might be imaging transporter regulation, such as by protein phosphorylation [16]. AGC kinases were shown to phosphorylate and thus alter transport activities of auxin transporters of the PIN and ABCB families [16–20], but have not yet been imaged *in planta*. A future alternative might be offered by novel GFP-based kinase reporters, called SPARK (Separation of Phases-based Activity Reporter of Kinase). SPARK

offers imaging of fast kinase kinetics and is reversible [21**] but has not yet been adapted to plant systems.

Visualization of transporter substrates: *in vivo* biochemistry

While some transporter substrates (like some secondary metabolites) are by nature fluorescent and can be followed directly, most are not and thus need either be applied as a radiolabeled or fluorescent version (see below) or require an indicating tool [22]. *In vivo* biochemistry [23] has provided us with a series of cell-biological techniques that allow for an imaging of either local substrate concentrations or intra and intercellular transport *in planta* (see Figure 2). However, it is important to keep in mind that most of these techniques indicate local substrate concentrations that are then used as a proxy for transport.

Chemical indicators

Chemical indicators are fluorescent molecules that respond to the binding of substrates by changing their fluorescence properties ([24]; see Figure 2). They exist for different metal cations (such as zinc, potassium, sodium, magnesium, calcium), protons and chloride and their loading and membrane targeting has been optimized by generating hydrophobic versions that are cytoplasmically cleaved and activated. In plants, the most commonly used pH-indicator and calcium-indicator are BCECF

(2',7'-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein) [25] and Fura-2/Calcium Green variants, respectively [24]. The latter can be either non-ratiometric (such as Calcium Green) or ratiometric (Fura-2), allowing one to correct for unequal dye loading, photo-bleaching or focal plane shifts. However, a common problem with chemical indicators in plant cells is that dye loading is hindered by the cell wall or compartmentalization in organelles (mainly vacuoles) avoiding an equal distribution [22,26].

Therefore, genetically encoded (GE) indicators, *reporters* and *sensors* [27–30] have been developed that indirectly or directly indicate local substrate concentrations, respectively (Figure 2). They allow for selective targeting to subcellular compartments (by insertion of signal sequences) or to cell types (by choice of promoters) [31].

Reporters

Expression-based (or signaling) reporters consist of natural or synthetic promoters fused to a reporter gene. Many but not all plant hormones, including auxin, ABA, cytokinin (CK), ethylen, jasmonic acid (JA) and salicylic acid can be visualized in this manner (for reviews, see Refs. [23,27,30–32]). Since publication of the prototype artificial auxin-responsive element, *DR5* [33], several variants have been developed (for a review, see Ref. [22]). *DR5* activation is slow (ca. 2 hour) and reflects an auxin

Figure 2

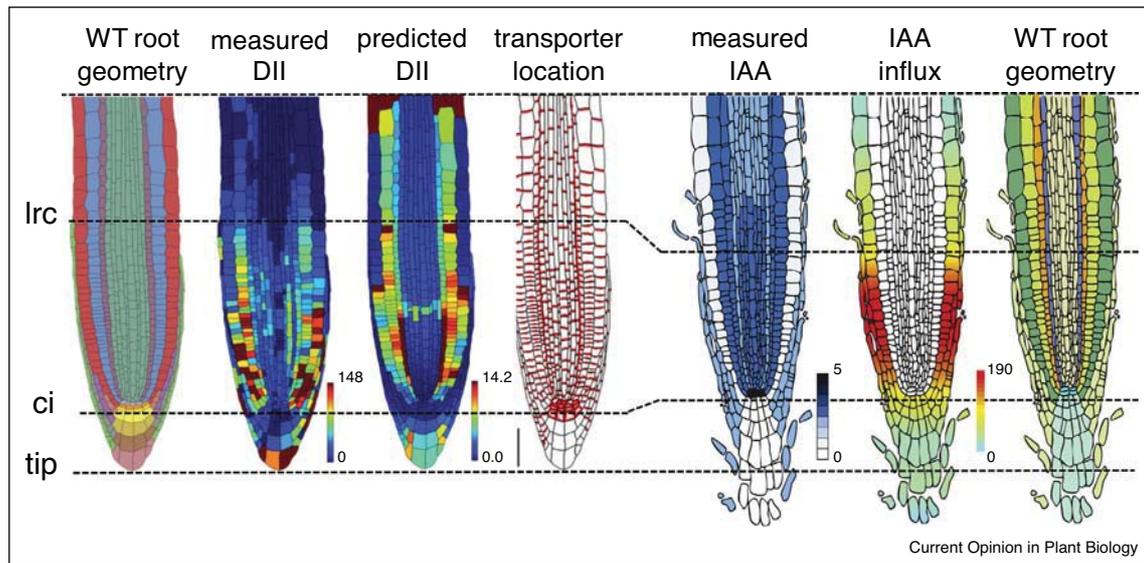
	Chemical Indicator	Reporter	Sensor	Fluorescent substrate	Click-chemistry	Imaging of radioactivity
Principle						
Readout type	Change of fluorescent properties	Promoter activation, reporter degradation	FRET, change of fluorescence intensity	Fluorescence	Fluorescence	(Digital) Radiography, Micro-autoradiography
Dynamic readout	+	+/- ¹	+	+	(+)	-
Subcellular resolution	+/-	-	(+)	+	+	+/-
Calibration	+/-	+/- ¹	+	-	-	-
Noninvasive	+	(+) ²	(+) ²	+	-	-

Current Opinion in Plant Biology

Comparison of methods used to derive transmembrane, long-range transport in plants.

Indicated approaches are evaluated with respect to their readout type and dynamic range, their invasiveness, their offered subcellular resolution and their correlation between the amount of detected target molecule and readout (calibration). Target molecules are in blue; fluorescent proteins are depicted as rounded rectangles. Note that principles are illustrative examples and might be not complete; brackets indicate limitations of methods. Figure outline is inspired by Ref. [27]. ¹ Dynamic range and calibration varies between reporter types; ² Reporter and sensors require genetic access and are thus not suitable for non-model plants (for details, see text).

Figure 3



Comparison of imaged, modeled and measured auxin transport in the root tip.

Measured DII-VENUS signals and transporter locations (here: PINs) were extracted from confocal images and are compared with modeled DII-VENUS distribution and predicted auxin fluxes (all taken from Ref. [89]). As reference, imaged auxin (transporter) distributions are compared with heat map presentation of IAA influx profiles measured using an IAA-specific microelectrode [97] or of high-resolution IAA contents measured by MS after cell-type sorting [97,98]. Note that the latter two quantitative transport techniques are not described in this review. Cell types labeled on the schematics of wild-type root geometry (left and right) are given; for identity refer to Refs. [89] and [97]. Note that the alignment of schematics is based on position of root tips, columella initials (ci) and end of lateral root caps (lrc).

response maximum, and is thus mainly suitable for the root tip [22,34,35].

Reporters-based on the principle of SCF-mediated proteasomal degradation of transcriptional repressors in the signaling pathway of several hormones were developed increasing the temporal (but also spatial) resolution of expression-based reporters. The blueprint consisted of the DII degen sequence of Aux/IAA repressor proteins of auxin signaling, which was fused to a nuclear targeted VENUS [36]. This module provides a decrease of DII-VENUS reporter fluorescence in tens of minutes upon auxin perception and is therefore suitable to image unequal auxin distribution in bending roots [36]. Combining the degen motive fusion with an auxin-insensitive reporter gene resulted in ratiometric reporters. For example, R2D2, now offers semi-quantitative auxin imaging at very reasonable spatio-temporal resolution [37,38,39]. Degradation-based reporters have also been developed for GA (GFP-RGA; [40]), JA (Jas9-VENUS; [41]) and SL (StrigoQuant; [42]).

Despite tremendous improvements in expression and degradation-based reporters with respect to speed and sensitivity, their indirect mode of action allows only for — at best — semi-quantitative *in vivo* imaging of local substrate concentrations. Further, reporters display final outputs that integrate over transport, biosynthesis and

metabolism. An even more limiting aspect is that their sensitivity is primarily determined by the expression of the employed signaling machinery and thus cell and tissue-specific [34].

Sensors

A series of genetically encoded activity sensors that are able to report directly and within seconds changes in calcium, proton, energy metabolite, heavy metal or macronutrient concentrations have been established [27,31]. Like reporters, sensor are used to assess resident substrate concentrations that are then often equalized as transport activities [27,43].

The photoprotein aequorin, from jellyfish has been widely used in plants for more than two decades in order to study calcium dynamics by means of emitted bioluminescence [44]. However, due to its low cellular sensitivity, aequorin was slowly replaced by FRET-based sensors (Figure 2; [45]), such as Cameleon [24]. Versions of Cameleon with improved calcium binding affinity and dynamic range [23,27,28] (YC3-60, [46] and YC-nano), were recently used for demonstrating long-range calcium signaling in the root and between shoot and root, respectively [47–49]. The intensimetric calcium sensor, R-GECO1, exhibits an increased (>10×) sensitivity compared with YC3.6 in response to elicitors of the innate immune response to pathogens, flagelin22 and chitin [50], and R- was used to image auxin-induced calcium

gradients in root-hair tips and long-distance calcium waves between root cells [51**].

For local pH measurements, single fluorescent protein sensors, such as pHluorins [52], using the pH sensitivity of EGFP are mainly employed [53*]. The pH sensor, pHusion, allows for a ratiometric quantification of cytoplasmic and apoplastic pH [28]. Recently, a pHusion variant allowed measuring pH in and outside of the TGN/early endosome network demonstrated the role of the V-ATPase for protein secretion [54**].

The Frommer group was driving forward the development of FRET sensors for a wide range of sugars [23,27,31]. Beside a quantification of intracellular concentrations, these sensors also permitted the kinetic characterization of individual sugar transporters [1] and the identification of novel transporters [55]. Sensors for transport of ATP; [56,57], ROS, heavy metals (eCALWY) (for references, see Refs. [27,31]) and different macronutrients have been developed (for a review, see Ref. [58]). Very recently, ATP sensing in living plant cells using the FRET-based sensor, ATeam1.03-nD/nA [56], revealed concentration gradients in plant tissues and stress-related dynamics of energy physiology [57].

Hormone imaging by sensors has been achieved first for ABA: two independent groups have developed sensors consisting of a FRET pair that is linked by an ABA-responsive sensory module, called ABACUS [59*] and ABAlcon [60*], respectively. Both sensors have proven to be useful in demonstrating kinetics of ABA transport and dynamics upon application of external ABA, [30,32,59*]. The nuclear localized GA sensor, GIBBERELLIN PERCEPTION SENSOR 1 (GPS1), allowed sensing of nanomolar concentrations in Arabidopsis [61].

The intensimetric calcium sensor, R-GECO1, was shown to exhibit an increased (>10×) sensitivity compared with classical YC3.6 in response to elicitors of the innate immune response to pathogens, flagelin22 and chitin [50**]. R-GECO1 was used to image auxin-induced calcium gradients in root-hair tips but also long-distance calcium waves between root cells [51**].

Fluorescently labelled substrates

Fluorescently labeled substrates have only recently been used in plants (Figure 2). The fluorescent BR analog, Alexa Fluor 647-castasterone (AFCS) [62], GA-fluorescein [63] and different fluorescent auxins [34**,35,64] have been developed. Moreover, a quantum dot-based fluorescent probe, CdTe-JA, was employed to label JA binding sites in tissue sections of mung bean and Arabidopsis seedlings [65]. Recently, different fluorescent SL analogs were successfully designed: while EGO10A-BP and CISA-1 [66] are constitutively fluorescent variant making it first choice for transport studies,

Yoshimulactone Green or GC242 were constructed to exhibit only fluorescent after cleavage by the SL receptor [67].

Bioactive auxin derivatives tagged with fluorescein (IAA-FITC) or rhodamine (IAA-RITC) are distributed symmetrically and apoplastically, respectively [35]. Hayashi *et al.* designed fluorescent auxin analogs to be actively transported but to be inactive for auxin signaling [34**], while very recently new fluorescently labeled auxins exhibit an anti-auxin activity [64]. The former is important because auxin signaling is known to influence auxin transporter expression and location [34**]. NBD-IAA (7-nitro-2,1,3-benzoxadiazole-IAA) revealed a similar auxin distribution as found with DII-based reporters [34**] but lacked signals in the quiescent center known to be mainly produced by auxin biosynthesis and not transport. A limitation of NBD-auxins is that they are for unknown reasons only working in root and not in shoots [34**]. Recently, NBD-NAA, whose nuclear import is restricted, was used as a tool to dissect cytoplasmic and ER delivery of nuclear auxin concentrations [39**]. This indicates their usefulness in analyzing intracellular transport, which is usually hard to uncover using classical tools.

Click-chemistry of transport substrates

In animal models, the problem of bulky fluorescent tags interfering with binding to transporter (or receptor) proteins has been partially solved by 'click chemistry' [68]. This technique triggers the selective, covalent connection between a molecule of interest and a detection tag *in situ* (Figure 2). A variety of labeling tags can be used then for microscopy or analytics.

To our knowledge only one study reported on the distribution of a mobile substrate using this technology in plants: the azido derivative of indole-3-propionic acid (IPA, an active auxin), IPA-N₃, was used as an auxin tracer and provided some evidence of the presence of auxin binding sites in the apoplast of elongating cells [69**]. IPA-N₃ was detected mainly in the outer cell layers of the root tip and does thus not fully reflect the auxin accumulation sites. This seems to be less a penetration but a fixation problem [69**]. However, these technical issues will be overcome and as such 'click chemistry' might soon be the method of choice despite the fact that it requires a fixation step preventing *in vivo* imaging.

Imaging of radiolabeled substrates

Radio-autographic analysis of the distribution of transport has a long tradition in plants (e.g. [70]). Recently, polar auxin transport of Arabidopsis stem segments was visualized by detecting ¹⁴C-IAA with a double-sided silicon strip detector [71]. In recent years, the micro-autoradiography method (MAR) has been established to visualize the distribution of ¹⁰⁹Cd and ³³P within sections of rice

tissues [72]. Currently, only fixed material can be used, preventing any dynamic information about substrate distributions. As ^{14}C (and ^3H) emit beta particles at very low energy, most transport substrates could be labelled with suitable isotopes for autoradiography, which would expand the application properties of MAR (Figure 2; [72]).

Immunolocalization of transport substrates

ABA [73,74], IAA [22] and CK [75] have been visualized in different plant species by whole-mount immunolocalization using polyclonal and monoclonal antisera (Figure 2; [76]). Despite requiring of fixation, some of these antisera have become successful tools for the *in situ* localization of local maxima [75,77] or the translocation of hormones in plants [78–82]. Anti-IAA antibody signals were reported to recapitulate signals found with the *DR5* reporter in the mature and lateral root [77,79]. However, a part of these publications have been heavily debated with respect to their interpretation and technical quality [83].

Modeling of transmembrane transport over tissues

Early auxin transport models, called flux-based models, were inspired by Tsvi Sachs in the late 1960s and hypothesized that the flux of auxin through cell membranes reinforces itself [84,85]. The integration of experimentally determined patterns of transporter localization into simulations confirmed this in *Arabidopsis* [86–88,89*]. Gradient-based models were introduced [90,91], provided support for the assumed roles of auxin maxima in shoot development (for an excellent review, see Ref. [92]).

The primary motivation of most of these studies was to predict the movement of a transported substrate (here: auxin; Figure 3) within a developing tissue. In this regard, the development of the OnGuard platform for modeling transport processes in stomata has proven successful in uncovering previously unexpected features of guard cell physiology [93]. However, today's interest lies more in testing if a hypothesized role for a substrate can be supported by *in silico* data [94]. A recent study employing data-driven modeling of intracellular auxin fluxes between the ER or cytoplasm and the nucleus, respectively, indicated that the ER has a dominant role in controlling nuclear auxin uptake [39**]. This and other approaches argue that computer models can provide (or even go beyond) a 'proof of principle'. However, any conclusions-based on human-made, and as such subjective, algorithms will always require an experimental validation.

Conclusions and perspectives

Recently, the plant transport field has made a major conceptual shift away from a *single transporter* analysis toward a more *integrative transporter* approach, aimed at

exploring transporter networks in order to understand their interplay and their incorporation into plant physiology. This progress has been made possible by the development of a series of techniques that allow visualization of local concentrations and movements of substrates in plant tissues. While early quantitative methods (like radiolabeled substrates, micro-electrodes and cell-type specific analyses of transport substrates) provide temporal resolution, visualization of transport by means of chemical indicators, fluorescent analogs and especially reporters and sensors provides good spatial information with reasonable resolution for many substrates. In that respect, doubting Thomas was right: seeing is better than believing.

Most tools have their individual advantages and limitations (see Figure 2). Some allow only for (semi)-quantitative analyses with high spatial but only low temporal resolution (such as reporters and sensors), while some indicators, sensors or fluorescent analogs were found to interfere with plant physiology by high substrate binding affinities or by competing with endogenous signaling [24,32,57]. However, these kinds of problems may to a certain degree be overcome by using non-plant substrate binding domains [95].

As usual, new technology and insight provides us with new challenges. For example, the transfer of sensors from single cell approaches to the plant level has been difficult due to the wide affinity/sensitivity range (nM-mM) that is required for some substrates. A solution might be the co-expression of spectrally non-overlapping, intensimetric sensors with different affinity ranges. Further, in contrast to intercellular transport, transmembrane transport between subcellular compartments has proven difficult to measure directly. In that respect, a directed targeting of sensors to individual subdomains of interest as shown recently [54**] and design of selective permeable fluorescent dyes [39**] will be helpful.

Another issue is the inadequate separation of transport from signaling/biosynthesis by some imaging tools, like reporters or sensors. In that respect it appears as if we have not yet fully used the potential of fluorescent transport substrates or click chemistry. In the future, structure-activity design will allow us to predict fluorophore positions and linker length [63,64] in order to direct uptake, stability and distribution of fluorescent substrates [96].

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as

- of special interest
- of outstanding interest

1. Chen LQ, Cheung LS, Feng L, Tanner W, Frommer WB: **Transport of sugars**. *Annu Rev Biochem* 2015, **84**:865-894.
2. Tanner W, Caspari T: **Membrane transport carriers**. *Annu Rev Plant Physiol Plant Mol Biol* 1996, **47**:595-626.
3. Park J, Lee Y, Martinoia E, Geisler M: **Plant hormone transporters: what we know and what we would like to know**. *BMC Biol* 2017, **15**:93.
4. Kang J, Lee Y, Sakakibara H, Martinoia E: **Cytokinin transporters: GO and STOP in signaling**. *Trends Plant Sci* 2017, **22**:455-461.
5. Borghi L, Kang J, Ko D, Lee Y, Martinoia E: **The role of ABCG-type ABC transporters in phytohormone transport**. *Biochem Soc Trans* 2015, **43**:924-930.
6. Geisler M, Wang B, Zhu J: **Auxin transport during root gravitropism: transporters and techniques**. *Plant Biol (Stuttg)* 2014, **16**(Suppl. 1):50-57.
7. Yoshinari A, Takano J: **Insights into the mechanisms underlying boron homeostasis in plants**. *Front Plant Sci* 2017, **8**:1951.
8. Naramoto S: **Polar transport in plants mediated by membrane transporters: focus on mechanisms of polar auxin transport**. *Curr Opin Plant Biol* 2017, **40**:8-14.
9. Kang J, Park J, Choi H, Burla B, Kretschmar T, Lee Y, Martinoia E: **Plant ABC transporters**. *Arabidopsis Book* 2011, **9**e0153.
10. Adamowski M, Friml J: **PIN-dependent auxin transport: action, regulation, and evolution**. *Plant Cell* 2015, **27**:20-32.
11. Groner P, Friml J: **Auxin transporters and binding proteins at a glance**. *J Cell Sci* 2015, **128**:1-7.
12. Geisler M, Aryal B, di Donato M, Hao P: **A critical view on ABC transporters and their interacting partners in auxin transport**. *Plant Cell Physiol* 2017, **58**:1601-1614.
13. Ast C, De Michele R, Kumke MU, Frommer WB: **Single-fluorophore membrane transport activity sensors with dual-emission read-out**. *eLife* 2015, **4**e07113.
14. De Michele R, Ast C, Loque D, Ho CH, Andrade S, Lanquar V, Grossmann G, Gehne S, Kumke MU, Frommer WB: **Fluorescent sensors reporting the activity of ammonium transporters in live cells**. *eLife* 2013, **2**e00800.
15. Ho CH, Frommer WB: **Fluorescent sensors for activity and regulation of the nitrate transporter CHL1/NRT1.1 and oligopeptide transporters**. *eLife* 2014, **3**e01917.
16. Aryal B, Laurent C, Geisler M: **Learning from each other: ABC transporter regulation by protein phosphorylation in plant and mammalian systems**. *Biochem Soc Trans* 2015, **43**:966-974.
17. Zourelidou M, Absmanner B, Weller B, Barbosa IC, Willige BC, Fastner A, Streit V, Port SA, Colcombet J, de la Fuente van Bentem S *et al.*: **Auxin efflux by PIN-FORMED proteins is activated by two different protein kinases, D6 PROTEIN KINASE and PINOID**. *eLife* 2014e02860.
18. Christie JM, Yang H, Richter GL, Sullivan S, Thomson CE, Lin J, Titapiwatanakun B, Ennis M, Kaiserli E, Lee OR *et al.*: **Phot1 inhibition of ABCB19 primes lateral auxin fluxes in the shoot apex required for phototropism**. *PLoS Biol* 2011, **9**e1001076.
19. Henrichs S, Wang B, Fukao Y, Zhu J, Charrier L, Bailly A, Oehring SC, Linnert M, Weiward M, Endler A *et al.*: **Regulation of ABCB1/PGP1-catalysed auxin transport by linker phosphorylation**. *EMBO J* 2012, **31**:2965-2980.
20. Armengot L, Marques-Bueno MM, Jaillais Y: **Regulation of polar auxin transport by protein and lipid kinases**. *J Exp Bot* 2016, **67**:4015-4037.
21. Zhang Q, Huang H, Zhang L, Wu R, Chung CI, Zhang SQ, Torra J, Schepis A, Coughlin SR, Kornberg TB *et al.*: **Visualizing dynamics of cell signaling in vivo with a phase separation-based kinase reporter**. *Mol Cell* 2018, **69**:334-346 e334.
22. Parizkova B, Pernisova M, Novak O: **what has been seen cannot be unseen-detecting auxin in vivo**. *Int J Mol Sci* 2017, **18**.
23. Jones AM, Grossmann G, Danielson JA, Sosso D, Chen LQ, Ho CH, Frommer WB: **In vivo biochemistry: applications for small molecule biosensors in plant biology**. *Curr Opin Plant Biol* 2013, **16**:389-395.
24. Kanchiswamy CN, Malnoy M, Occhipinti A, Maffei ME: **Calcium imaging perspectives in plants**. *Int J Mol Sci* 2014, **15**:3842-3859.
25. Braun FJ, Hegemann P: **Direct measurement of cytosolic calcium and pH in living Chlamydomonas reinhardtii cells**. *Eur J Cell Biol* 1999, **78**:199-208.
26. Plieth C: **Plant calcium signaling and monitoring: pros and cons and recent experimental approaches**. *Protoplasma* 2001, **218**:1-23.
27. Uslu VV, Grossmann G: **The biosensor toolbox for plant developmental biology**. *Curr Opin Plant Biol* 2016, **29**:138-147.
28. Gjetting SK, Schulz A, Fuglsang AT: **Perspectives for using genetically encoded fluorescent biosensors in plants**. *Front Plant Sci* 2013, **4**:234.
29. Lemke EA, Schultz C: **Principles for designing fluorescent sensors and reporters**. *Nat Chem Biol* 2011, **7**:480-483.
30. Walia A, Waadt R, Jones AM: **Genetically encoded biosensors in plants: pathways to discovery**. *Annu Rev Plant Biol* 2018, **69**:497-524.
31. Grossmann G, Krebs M, Maizel A, Stahl Y, Vermeer JEM, Ott T: **Green light for quantitative live-cell imaging in plants**. *J Cell Sci* 2018, **131**.
32. Waadt R, Hsu PK, Schroeder JI: **Abscisic acid and other plant hormones: methods to visualize distribution and signaling**. *Bioessays* 2015, **37**:1338-1349.
33. Ulmasov T, Murfett J, Hagen G, Guilfoyle TJ: **Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements**. *Plant Cell* 1997, **9**:1963-1971.
34. Hayashi K, Nakamura S, Fukunaga S, Nishimura T, Jenness MK, Murphy AS, Motose H, Nozaki H, Furutani M, Aoyama T: **Auxin transport sites are visualized in planta using fluorescent auxin analogs**. *Proc Natl Acad Sci U S A* 2014, **111**:11557-11562.
35. Innovative design of fluorescent auxin analogs to be actively transported but to be inactive for auxin signaling avoiding unwanted auxin transporter expression and location. Used to dissect cytoplasmic and ER delivery of nuclear auxin concentrations [45].
36. Sokolowska K, Kizinska J, Szewczuk Z, Banasiak A: **Auxin conjugated to fluorescent dyes—a tool for the analysis of auxin transport pathways**. *Plant Biol (Stuttg)* 2014, **16**:866-877.
37. Brunoud G, Wells DM, Oliva M, Larrieu A, Mirabet V, Burrow AH, Beeckman T, Kepinski S, Traas J, Bennett MJ *et al.*: **A novel sensor to map auxin response and distribution at high spatio-temporal resolution**. *Nature* 2012, **482**:103-106.
38. Liao CY, Smet W, Brunoud G, Yoshida S, Vernoux T, Weijers D: **Reporters for sensitive and quantitative measurement of auxin response**. *Nat Methods* 2015, **12**:207-210 202-210.
39. A set of auxin reporters that allow sensitive and semi-quantitative detection of auxin signaling and responses in plants. DR5v2 offers by dual-color imaging an extended range of auxin responses, while, R2D2 allows for a ratiometric analysis of auxin.
40. Wend S, Dal Bosco C, Kampf MM, Ren F, Palme K, Weber W, Dovzhenko A, Zurbriggen MD: **A quantitative ratiometric sensor for time-resolved analysis of auxin dynamics**. *Sci Rep* 2013, **3**:2052.

39. Middleton AM, Dal Bosco C, Chlap P, Bensch R, Harz H, Ren F, Bergmann S, Wend S, Weber W, Hayashi KI *et al.*: **Data-driven modeling of intracellular auxin fluxes indicates a dominant role of the ER in controlling nuclear auxin uptake.** *Cell Rep* 2018, **22**:3044-3057.
- Elegant study that by employing a data-driven modeling of intracellular auxin fluxes between the ER or cytoplasm and the nucleus, respectively, and a fluorescent auxin analog with restricted nuclear passage indicated that the ER has a dominant role in controlling nuclear auxin uptake.
40. Lofke C, Zwiewka M, Heilmann I, Van Montagu MC, Teichmann T, Friml J: **Asymmetric gibberellin signaling regulates vacuolar trafficking of PIN auxin transporters during root gravitropism.** *Proc Natl Acad Sci U S A* 2013, **110**:3627-3632.
41. Larrieu A, Champion A, Legrand J, Lavenus J, Mast D, Brunoud G, Oh J, Guyomarc'h S, Pizot M, Farmer EE *et al.*: **A fluorescent hormone biosensor reveals the dynamics of jasmonate signalling in plants.** *Nat Commun* 2015, **6**:6043.
42. Samodelov SL, Beyer HM, Guo X, Augustin M, Jia KP, Baz L, Ebenhoh O, Beyer P, Weber W, Al-Babili S *et al.*: **StrigoQuant: a genetically encoded biosensor for quantifying strigolactone activity and specificity.** *Sci Adv* 2016, **2**e1601266.
43. Okumoto S, Jones A, Frommer WB: **Quantitative imaging with fluorescent biosensors.** *Annu Rev Plant Biol* 2012, **63**:663-706.
44. Zhu X, Feng Y, Liang G, Liu N, Zhu JK: **Aequorin-based luminescence imaging reveals stimulus- and tissue-specific Ca²⁺ dynamics in Arabidopsis plants.** *Mol Plant* 2013, **6**:444-455.
45. Hillearya R, Choib WG, Kimb SH, Limb SH, Gilroy S: **Sense and sensibility: the use of fluorescent protein-based genetically encoded biosensors in plants.** *Curr Opin Plant Biol* 2018, **46**:32-38 <http://dx.doi.org/10.1016/j.pbi.2018.07.004>.
46. Monshausen GB, Messerli MA, Gilroy S: **Imaging of the Yellow Cameleon 3.6 indicator reveals that elevations in cytosolic Ca²⁺ + follow oscillating increases in growth in root hairs of Arabidopsis.** *Plant Physiol* 2008, **147**:1690-1698.
47. Choi WG, Gilroy S: **Plant biologists FRET over stress.** *eLife* 2014, **3**e02763.
48. Choi WG, Toyota M, Kim SH, Hillearya R, Gilroy S: **Salt stress-induced Ca²⁺ waves are associated with rapid, long-distance root-to-shoot signaling in plants.** *Proc Natl Acad Sci U S A* 2014, **111**:6497-6502.
49. Monshausen GB, Miller ND, Murphy AS, Gilroy S: **Dynamics of auxin-dependent Ca²⁺ and pH signaling in root growth revealed by integrating high-resolution imaging with automated computer vision-based analysis.** *Plant J* 2011, **65**:309-318.
50. Keinath NF, Waadt R, Brugman R, Schroeder JI, Grossmann G, Schumacher K, Krebs M: **Live cell imaging with R-GECO1 sheds light on flg22- and chitin-induced transient [Ca²⁺]_{cyt} patterns in arabidopsis.** *Mol Plant* 2015, **8**:1188-1200.
- The direct comparison between the ratiometric and intensimetric calcium sensors, YC3.6 and R-GECO, revealed a higher dynamic range for R-GECO in response to elicitors of the innate immune response.
51. Dindas J, Scherzer S, Roelfsema MRG, von Meyer K, Muller HM, Al-Rasheid KAS, Palme K, Dietrich P, Becker D, Bennett MJ *et al.*: **AUX1-mediated root hair auxin influx governs SCF(TIR1/AFB)-type Ca²⁺ signaling.** *Nat Commun* 2018, **9**:1174.
- The calcium sensor, R-GECO1, was used to image auxin-induced calcium gradients in root-hair tips but also long-distance calcium waves between root cells.
52. Miesenbock G, De Angelis DA, Rothman JE: **Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins.** *Nature* 1998, **394**:192-195.
53. Gjetting KS, Ytting CK, Schulz A, Fuglsang AT: **Live imaging of intra- and extracellular pH in plants using pHusion, a novel genetically encoded biosensor.** *J Exp Bot* 2012, **63**:3207-3218.
- The pH sensor, pHusion, allowed for a ratiometric quantification of cytoplasmic and especially apoplasmic pH, demonstrated by the immediate alkalinization of the subepidermal apoplast upon external auxin treatment.
54. Luo Y, Scholl S, Doering A, Zhang Y, Irani NG, Rubbo SD, Neumetzler L, Krishnamoorthy P, Van Houtte I, Mylle E *et al.*: **V-ATPase activity in the TGN/EE is required for exocytosis and recycling in Arabidopsis.** *Nat Plants* 2015, **1**:15094.
- Usage of the ratiometric sensor, pHusion, for measuring pH inside and outside of TGN/early endosomes unveiled the importance of acidification for secretion and recycling.
55. Chen LQ, Hou BH, Lalonde S, Takanaga H, Hartung ML, Qu XQ, Guo WJ, Kim JG, Underwood W, Chaudhuri B *et al.*: **Sugar transporters for intercellular exchange and nutrition of pathogens.** *Nature* 2010, **468**:527-532.
56. Imamura H, Nhat KP, Togawa H, Saito K, Iino R, Kato-Yamada Y, Nagai T, Noji H: **Visualization of ATP levels inside single living cells with fluorescence resonance energy transfer-based genetically encoded indicators.** *Proc Natl Acad Sci U S A* 2009, **106**:15651-15656.
57. De Col V, Fuchs P, Nietzel T, Elsasser M, Voon CP, Candeo A, Seeliger I, Fricker MD, Grefen C, Moller IM *et al.*: **ATP sensing in living plant cells reveals tissue gradients and stress dynamics of energy physiology.** *eLife* 2017, **6**.
58. Okumoto S, Versaw W: **Genetically encoded sensors for monitoring the transport and concentration of nitrogen-containing and phosphorus-containing molecules in plants.** *Curr Opin Plant Biol* 2017, **39**:129-135.
59. Jones AM, Danielson JA, Manojkumar SN, Lanquar V, Grossmann G, Frommer WB: **Abscisic acid dynamics in roots detected with genetically encoded FRET sensors.** *eLife* 2014, **3**e01741.
- This study as well as [59*] developed the first phytohormone (ABA) sensor.
60. Waadt R, Hitomi K, Nishimura N, Hitomi C, Adams SR, Getzoff ED, Schroeder JI: **FRET-based reporters for the direct visualization of abscisic acid concentration changes and distribution in Arabidopsis.** *eLife* 2014, **3**e01739.
- This study as well as [59*] developed the first phytohormone (ABA) sensor
61. Rizza A, Walia A, Lanquar V, Frommer WB, Jones AM: **In vivo gibberellin gradients visualized in rapidly elongating tissues.** *Nat Plants* 2017, **3**:803-813.
62. Irani NG, Di Rubbo S, Mylle E, Van den Begin J, Schneider-Pizon J, Hnilikova J, Sisa M, Buyst D, Villarrasa-Blasi J, Szatmari AM *et al.*: **Fluorescent castasterone reveals BRI1 signaling from the plasma membrane.** *Nat Chem Biol* 2012, **8**:583-589.
63. Shani E, Weinstain R, Zhang Y, Castillejo C, Kaiserli E, Chory J, Tsien RY, Estelle M: **Gibberellins accumulate in the elongating endodermal cells of Arabidopsis root.** *Proc Natl Acad Sci U S A* 2013, **110**:4834-4839.
64. Bielezsova K, Parizkova B, Kubes M, Husickova A, Kubala M, Ma Q, Sedlarova M, Robert S, Dolezal K, Strnad M *et al.*: **New fluorescently labeled auxins exhibit promising anti-auxin activity.** *N Biotechnol* 2018, Jun 25. pii: S1871-6784(17)30572-1. doi: 10.1016/j.nbt.2018.06.003. [Epub ahead of print].
65. Gao Y, Yu Y, Hu X, Cao Y, Wu J: **Imaging of jasmonic acid binding sites in tissue.** *Anal Biochem* 2013, **440**:205-211.
66. Rasmussen A, Heugebaert T, Matthys C, Van Deun R, Boyer FD, Goormachtig S, Stevens C, Geelen D: **A fluorescent alternative to the synthetic strigolactone GR24.** *Mol Plant* 2013, **6**:100-112.
67. Kameoka H, Kyojuka J: **Spatial regulation of strigolactone function.** *J Exp Bot* 2018, **69**(9 April):2255-2264 <http://dx.doi.org/10.1093/jxb/erx434>.
68. Horisawa K: **Specific and quantitative labeling of biomolecules using click chemistry.** *Front Physiol* 2014, **5**:457.
69. Mravec J, Kracun SK, Zemlyanskaya E, Rydahl MG, Guo X, Picmanova M, Sorensen KK, Ruzicka K, Willats WGT: **Click chemistry-based tracking reveals putative cell wall-located auxin binding sites in expanding cells.** *Sci Rep* 2017, **7**:15988.
- First study using 'click chemistry' in order image a hormone (auxin) tracer in plants providing evidence for the presence of auxin binding sites in the apoplast of elongating cells.

70. Sabnis DD, Hirshberg G, Jacobs WP: **Radioautographic analysis of the distribution of label from h-indoleacetic acid supplied to isolated coleus internodes.** *Plant Physiol* 1969, **44**:27-36.
71. Boot KJ, Hille SC, Libbenga KR, Peletier LA, van Spronsen PC, van Duijn B, Offringa R: **Modelling the dynamics of polar auxin transport in inflorescence stems of Arabidopsis thaliana.** *J Exp Bot* 2016, **67**:649-666.
72. Hirose A, Kobayashi NI, Tanoi K, Nakanishi TM: **A microautoradiographic method for fresh-frozen sections to reveal the distribution of radionuclides at the cellular level in plants.** *Plant Cell Physiol* 2014, **55**:1194-1202.
73. Pastor ACN, Alegre L: **Immunolocalization of abscisic acid by monoclonal antibodies in Lavandula stoechas L. leaves.** *Plant Growth Regul* 1995, **16**:287-292.
74. Sharipova G, Veselov D, Kudoyarova G, Fricke W, Dodd IC, Katsuhara M, Furuichi T, Ivanov I, Veselov S: **Exogenous application of abscisic acid (ABA) increases root and cell hydraulic conductivity and abundance of some aquaporin isoforms in the ABA-deficient barley mutant Az34.** *Ann Bot* 2016, **118**(4):777-785.
75. Casanova E, Valdes AE, Fernandez B, Moysset L, Trillas MI: **Levels and immunolocalization of endogenous cytokinins in thidiazuron-induced shoot organogenesis in carnation.** *J Plant Physiol* 2004, **161**:95-104.
76. Pasternak T, Tietz O, Rapp K, Begheldo M, Nitschke R, Ruperti B, Palme K: **Protocol: an improved and universal procedure for whole-mount immunolocalization in plants.** *Plant Methods* 2015, **11**:50.
77. Benkova E, Michniewicz M, Sauer M, Teichmann T, Seifertova D, Jurgens G, Friml J: **Local, efflux-dependent auxin gradients as a common module for plant organ formation.** *Cell* 2003, **115**:591-602.
78. Bouchard R, Bailly A, Blakeslee JJ, Oehring SC, Vincenzetti V, Lee OR, Paponov I, Palme K, Mancuso S, Murphy AS *et al.*: **Immunophilin-like TWISTED DWARF1 modulates auxin efflux activities of Arabidopsis P-glycoproteins.** *J Biol Chem* 2006, **281**:30603-30612.
79. Krouk G, Lacombe B, Bielach A, Perrine-Walker F, Malinska K, Mounier E, Hoyerova K, Tillard P, Leon S, Ljung K *et al.*: **Nitrate-regulated auxin transport by NRT1.1 defines a mechanism for nutrient sensing in plants.** *Dev Cell* 2010, **18**:927-937.
80. Nishimura T, Toyooka K, Sato M, Matsumoto S, Lucas MM, Strnad M, Baluska F, Koshida T: **Immunohistochemical observation of indole-3-acetic acid at the IAA synthetic maize coleoptile tips.** *Plant Signal Behav* 2011, **6**:2013-2022.
81. Mettbach U, Strnad M, Mancuso S, Baluska F: **Immunogold-EM analysis reveal brefeldin a-sensitive clusters of auxin in Arabidopsis root apex cells.** *Commun Integr Biol* 2017, **10**e1327105.
82. Schlicht M, Strnad M, Scanlon MJ, Mancuso S, Hochholdinger F, Palme K, Volkmann D, Menzel D, Baluska F: **Auxin immunolocalization implicates vesicular neurotransmitter-like mode of polar auxin transport in root apices.** *Plant Signal Behav* 2006, **1**:122-133.
83. Robinson DG, Hawes C, Hillmer S, Jurgens G, Schwechheimer C, Stierhof YD, Viotti C: **Auxin and vesicle traffic.** *Plant Physiol* 2018, **176**:1884-1888.
84. Sachs T: **Polarity and the induction of organized vascular tissues.** *Ann. Bot. (Lond)* 1969, **33**:263-272.
85. Bennett T, Hines G, Leysner O: **Canalization: what the flux?** *Trends Genet* 2014, **30**:41-48.
86. de Reuille PB, Bohn-Courseau I, Ljung K, Morin H, Carraro N, Godin C, Traas J: **Computer simulations reveal properties of the cell-cell signaling network at the shoot apex in Arabidopsis.** *Proc Natl Acad Sci U S A* 2006, **103**:1627-1632.
87. Grieneisen VA, Xu J, Maree AF, Hogeweg P, Scheres B: **Auxin transport is sufficient to generate a maximum and gradient guiding root growth.** *Nature* 2007, **449**:1008-1013.
88. Peret B, Middleton AM, French AP, Larrieu A, Bishopp A, Njo M, Wells DM, Porco S, Mellor N, Band LR *et al.*: **Sequential induction of auxin efflux and influx carriers regulates lateral root emergence.** *Mol Syst Biol* 2013, **9**:699.
89. Band LR, Wells DM, Fozard JA, Ghetiu T, French AP, Pound MP, Wilson MH, Yu L, Li W, Hijazi HI *et al.*: **Systems analysis of auxin transport in the Arabidopsis root apex.** *Plant Cell* 2014, **26**:862-875.
- An auxin transport model based on actual root cell geometries and subcellular localizations of auxin carriers was tested using DII-VENUS-derived data. This study revealed that AUX1/LAX influx carriers are also required beside efflux carriers to create the pattern of auxin distribution at the root tip.
90. Smith RS, Guyomarc'h S, Mandel T, Reinhardt D, Kuhlemeier C, Prusinkiewicz P: **A plausible model of phyllotaxis.** *Proc Natl Acad Sci U S A* 2006, **103**:1301-1306.
91. Jonsson H, Heisler MG, Shapiro BE, Meyerowitz EM, Mjolsness E: **An auxin-driven polarized transport model for phyllotaxis.** *Proc Natl Acad Sci U S A* 2006, **103**:1633-1638.
92. Voss U, Bishopp A, Farcot E, Bennett MJ: **Modelling hormonal response and development.** *Trends Plant Sci* 2014, **19**:311-319.
93. Hills A, Chen ZH, Amtmann A, Blatt MR, Lew VL: **OnGuard, a computational platform for quantitative kinetic modeling of guard cell physiology.** *Plant Physiol* 2012, **159**:1026-1042.
94. Kramer EM: **Computer models of auxin transport: a review and commentary.** *J Exp Bot* 2008, **59**:45-53.
95. Heim N, Garaschuk O, Friedrich MW, Mank M, Milos RI, Kovalchuk Y, Konnerth A, Griesbeck O: **Improved calcium imaging in transgenic mice expressing a troponin C-based biosensor.** *Nat Methods* 2007, **4**:127-129.
96. Lace B, Prandi C: **Shaping small bioactive molecules to untangle their biological function: a focus on fluorescent plant hormones.** *Mol Plant* 2016, **9**:1099-1118.
97. Wang B, Bailly A, Zwiewka M, Henrichs S, Azzarello E, Mancuso S, Maeshima M, Friml J, Schulz A, Geisler M: **Arabidopsis TWISTED DWARF1 functionally interacts with auxin exporter ABCB1 on the root plasma membrane.** *Plant Cell* 2013, **25**:202-214.
98. Petersson SV, Johansson AI, Kowalczyk M, Makoveychuk A, Wang JY, Moritz T, Grebe M, Benfey PN, Sandberg G, Ljung K: **An auxin gradient and maximum in the arabidopsis root apex shown by high-resolution cell-specific analysis of iaa distribution and synthesis.** *Plant Cell* 2009, **21**(6):1659-1668.