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BRIEF COMMUNICATION

The localization of auxin transporters PIN3 and LAX3 during lateral root development in *Arabidopsis thaliana*

F.M. PERRINE-WALKER[1,3]* and E. JUBLANC[2]

Biochimie et Physiologie Moléculaire des Plantes,UMR5004 CNRS/INRA/SupAgroM/UM2, Institut de Biologie Intégrative des Plantes, Place Viala, F-34060 Montpellier Cedex 1, France[1]
UMR866 Dynamique Musculaire et Métabolisme, Place Pierre Viala, F-34000 Montpellier, France[2]
La Trobe Institute of Molecular Sciences, La Trobe University, Melbourne 3086, Australia[3]

Abstract

Fluorophore tagged proteins are used in *Arabidopsis thaliana* to understand their functional role in plant development. This requires the analysis of their spatial localization *in planta*. However, the localization analysis is often perturbed by a significant overlap of the fluorophores used to label proteins of interest and the optical filtering methods available on the confocal microscope. This problem can be addressed by the use of spectral imaging with linear unmixing the image data. We applied this method to help us identify double transgenic *A. thaliana* lines which expressed two fluorescently tagged auxin transporter proteins: the auxin efflux protein PIN-FORMED-3 (PIN3), tagged with green fluorescent protein (GFP), and the auxin influx protein LIKE-AUX1-3 (LAX3), tagged with yellow fluorescent protein (YFP). This method allows the reliable separation of overlapping GFP and YFP fluorescence signals and subsequent localization analysis highlighting the potential benefit of this methodology in studies of lateral root development.

Additional key words: linear unmixing, GFP, YFP, spectral imaging.

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In vivo fluorescent labelling any protein is now possible by tagging a respective protein with a fluorophore (FP) using simple molecular cloning methods and a consequential expression of the gene fusion in living cells. However, the number of proteins that can be imaged simultaneously using different FPs is still limited not only due to the suboptimal spectroscopic and biophysical properties of some FP variants, but also their overlapping emission spectra (Mylle *et al*. 2013). In addition, conventional laser scanning microscopy images are detected using interference filters having a relatively broad bandwidth, which lead to images with poor spectral resolution (for reviews see, Berg 2004, Berg and Beachy 2008). This is advantageous for imaging single FPs and for multi-component imaging in which the component FPs possess adequately separated emissions (Berg 2004). However, this restrains the number of detected components to those that can be readily separated with the different filter bandwidths (Berg 2004). The FPs most successfully used in live imaging, such as cyan fluorescent protein (CFP), green fluorescent protein (GFP), or yellow fluorescent protein (YFP), have strongly overlapping emission spectra which make them problematic to separate in co-localization experiments using optical filtering methods (Zimmermann *et al*. 2003). Spectral imaging offers a means for dealing with these problems inherent to interference filter-based detection**,** and in combination with linear unmixing, it can be an effective tool for separating overlapping signals.

Spectral images are acquired by collecting spectral data covering emission bandwidths of the component fluorophores. Using reference spectra of individual FPs present, derived from pixels in the same image or singly stained samples run in parallel, signals of the component FPs contained in each pixel can be 'unmixed' using simple linear equations incorporated in the microscope

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Abbreviations: GFP - green flurorescent protein; LAX3 - LIKE-AUX1-3; LR - lateral root; MR - main root; PIN3 - PIN-FORMED-3; YFP - yellow fluorescent protein.
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* Corresponding author; e-mail: m.perrine@unimelb.edu.au

software (Berg 2004). FPs, such as fluorescein isothiocyanate (FITC) and GFP, whose emission peaks are separated by 7 nm can be separated computationally (Dickinson *et al.* 2001, Haraguchi *et al.* 2002). The application of such techniques for cell biological research *in vitro* and *in planta* is very useful (Kraus *et al*. 2007, Berg and Beachy 2008, Mylle *et al*. 2013).

In this work, we generated transgenic *Arabidopsis thaliana* seeds expressing both an auxin influx protein LIKE-AUX1-3 (LAX3) tagged with YFP and an auxin efflux protein PIN-FORMED-3 (PIN3) tagged with GFP, generated by crosses. Then we determined the emission spectra for both FPs that were fused to PIN3 and LAX3, respectively. The generated emissions were used as reference spectra and**,** by linear unmixing, determined the presence and location of GFP/YFP tagged auxin transporters in roots of live transgenic *A. thaliana* seedlings.

Arabidopsis thaliana L. wild-type (Col-0) seeds were obtained from the Nottingham Arabidopsis Stock Centre (Loughborough, UK). PIN3::PIN3GFP seeds (Col-0 background) provided by Prof. J. Friml (Institute of Science and Technology, Austria) and LAX3::LAX3YFP seeds (Col-0 background) provided by Prof. M. Bennett (University of Nottingham, UK) were also used. The seeds were surface sterilized in 50 % (v/v) ethanol containing 2 % (m/v) *Bayrochlor* (*Bayrol*, Mundolsheim, France) for 10 min followed by five washes with 100 % ethanol and drying in a laminar air flow. Then they were planted in 12 × 12 cm transparent plates filled with 40 cm^3 of a solid medium (1 %, m/v, agar type A) containing 0.5 mM $CaSO_4$, 0.5 mM $MgCl_2$, 1 mM KH_2PO_4, 2.5 mM 2-(morpholino) ethane sulfonic acid (MES, pH 5.8), 50 μM NaFeEDTA, 50 μM H_3BO_3, 12 μM $MnCl_2$, 1 μM $CuCl_2$, 1 μM $ZnCl_2$, and 0.03 μM NH_4MoO_4 (Remans *et al*. 2006). This basal medium was supplemented with 1 mM KNO_3 as nitrogen source (all chemicals were from *Sigma*, St. Louis, USA). After storage at a temperature of 4 °C in the dark for 2 d, the plates were incubated vertically in a growth chamber at a temperature of 22 °C, a 16-h photoperiod, and an irradiance of 230 μmol m^{-2} s^{-1}.

For growth in a naturally lit greenhouse, seeds were sown in soil and seedlings were transferred into individual pots 10 d after germination. Plants were grown in sterilized compost under a 16-h photoperiod and day/night temperatures of 20-25/10-15 °C.

PIN3GFP/LAX3YFP double transgenic *Arabidopsis* seeds were obtained by crossing respective parent lines. Screening and selecting T1 PIN3::PIN3GFP × LAX3pro::LAX3YFP seedlings were done by verifying fluorescence in main roots (MR) and lateral roots (LR), *i.e.*, PIN3GFP in the LR primordium (Friml *et al*. 2002, Benková *et al*. 2003) and LAX3YFP in the epidermal layer above the emerging primordium or in the root stele (Swarup *et al*. 2008) using an epifluorescence *Olympus BX61* (Tokyo, Japan) microscope. T1 seedlings presumably with both YFP and GFP fluorescence were then transferred to the greenhouse to generate T2 seeds. T2 transgenic seeds were then germinated and tested to verify the presence and location of YFP and GFP constructs using confocal fluorescence microscopy.

GFP and YFP images on LR primordia, LR, and MR were acquired with a *Carl Zeiss* (Jena, Germany) *LSM 510 META Axiovert 200M* inverted microscope with a water immersion objective *C-Apochromat 40/1.2*. GFP and YFP were excited at 488 nm (an Argon laser) and detected at 505 - 530 nm or 505 - 550 nm using band-pass filters (green and yellow colours assigned to respective FPs). Propidium iodide (1 μg cm^{-3}) was used to stain plant cell walls and was excited at 543 nm and detected at 585 nm (a long-pass red filter).

For seedlings expressing both GFP and YFP constructs, a linear spectral unmixing analysis was used as the spectral overlap between GFP and YFP (emission peaks at 510 and 527 nm, respectively) is too large to allow separate detection using the filters mentioned above. We used the software provided with the *Zeiss LSM 510 META* microscope system to perform linear unmixing data by acquiring images in the Lambda mode using a 488 nm argon laser. We collected GFP and YFP emission spectra in plant root cells expressing the individual FPs and used spectral unmixing on transgenic plants expressing both FPs.

For 3-D reconstruction, Z-stack images were acquired using spectral imaging at 1μm intervals and used linear unmixing to determine the location of GFP and YFP fused proteins. Images were further analyzed using *Fiji* (*Rasband W.*, *Image J 1.47h*, the National Institutes of Health, USA, http://imagej.nih.gov/ij/), and *Bitplane Imaris version 7.6.3*, and then processed in *Illustrator CS6* (*Adobe*).

After generating reference spectra for GFP and YFP, Col (0), PIN3::PIN3GFP, and LAX3::LAX3YFP transgenic seedlings were tested using spectral imaging and linear unmixing for both FPs. The Col (0) seedlings (n = 7) showed no signals (data not shown), whereas the seedlings expressing PIN3GFP (n = 10) or LAX3YFP (n = 7) were successfully imaged when using linear unmixing suggesting that GFP and YFP could be detected separately (Fig. 1, Table 1 Suppl. and 2 Suppl.). The localization of LAX3 was similar to that previously described in Swarup *et al*. (2008) and Marhavý *et al*. (2013). The PIN3 localization, using the same method, was similar to that previously described by Guyomarch *et al*. (2012). Spectral imaging the double transgenic *Arabidopsis* seedlings expressing both PIN3GFP and LAX3YFP (n = 14) demonstrated that GFP and YFP signals could be detected (Fig. 2*A*,*B*, Table 3 Suppl.) and separated by linear unmixing (Fig. 2*C*, Fig. 1 Suppl.). To test this further, Z-stack images of the primordium were acquired in the Lambda spectral mode, and 3D reconstruction and linear unmixing applied. Linear unmixing was possible on Z-stack images where PIN3GFP was located in the pericycle and the primordium and LAX3YFP were located in the MR stele, cortical cells, and epidermal cells above the emerging

primordium (Fig. 2*D*). After linear unmixing, we further investigated if there were two distinct signals for GFP and YFP by splitting the two colour channels. GFP and YFP signals which appeared to be in the same cell, *i.e.*, the cortical cell above the emerging primordium could be detected (Fig. 2*E*,*F*).

By generating the transgenic *Arabidopsis* seedlings expressing both PIN3GFP and LAX3YFP, we demon-

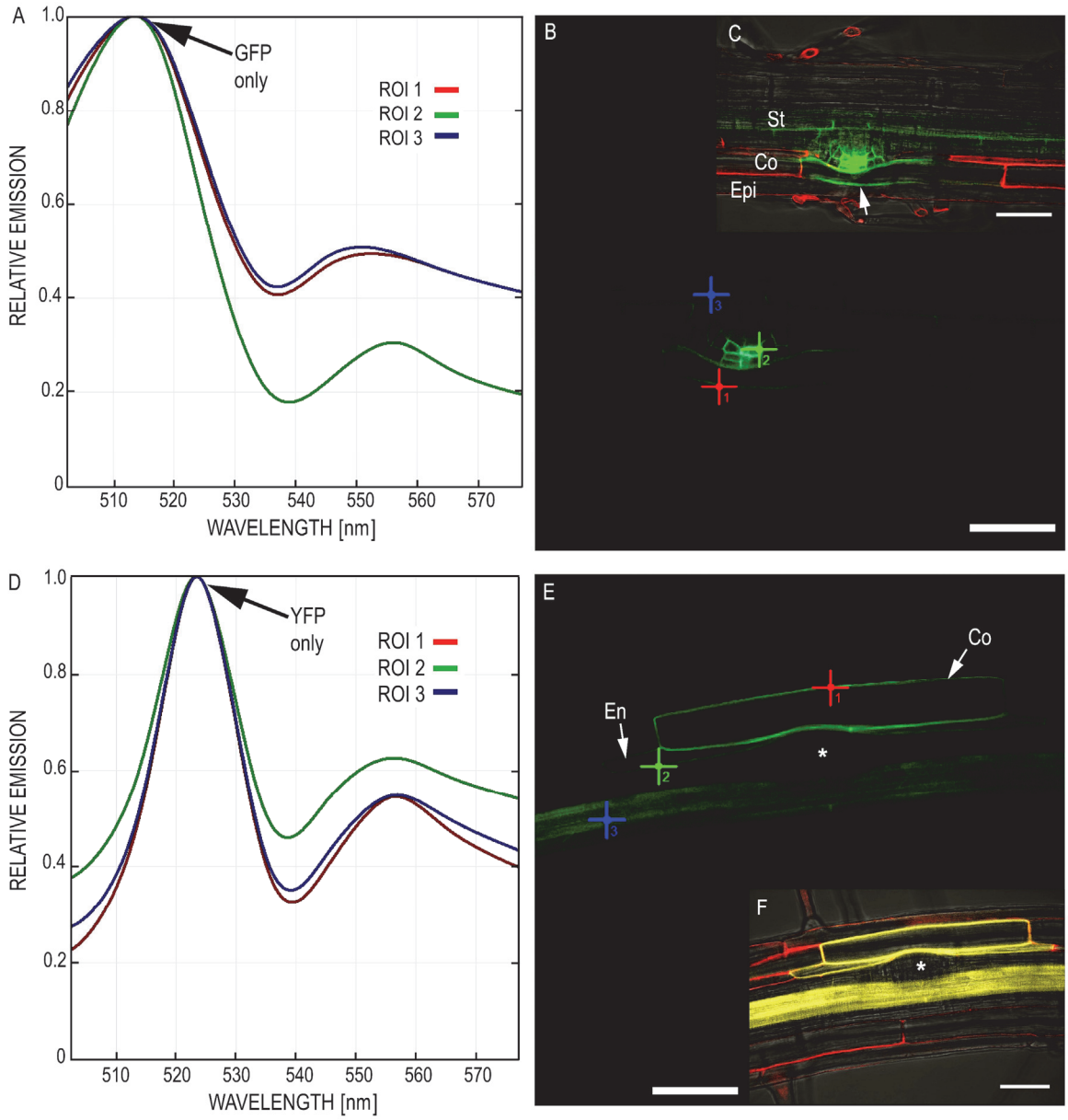

Fig. 1. The spectral profile and linear unmixing PIN3::PIN3GFP and LAX3::LAX3YFP transgenic *Arabidopsis* seedlings in the lateral root primordium region. *A* - The normalised emission spectra of GFP at three different pixel points or regions of interests (ROI 1, 2, 3) demonstrating that they had similar profiles in the lateral root primordium region of a transgenic *Arabidopsis* seedling expressing PIN3::PIN3GFP only. *B* - The spectral confocal image of the lateral root. *C* - The confocal image of *B* acquired using conventional filters with PI staining (*red*) and light transmission highlighting the presence of PIN3GFP in the cortical cell above the emerging primordium. *D* - The normalised emission spectrum of YFP highlighting three different pixel points demonstrating that they had similar profiles in the root primordium region of a transgenic *Arabidopsis* seedling expressing LAX3::LAX3YFP only. *E* - The spectral confocal image of the root primordium. *F* – The confocal image of *E* acquired using conventional filters with PI staining (*red*) and light transmission highlighting the presence of LAX3YFP in the endodermal and cortical cells above the emerging primordium. En - endodermis, Epi - epidermis, Co - cortex, St - stele; the *white asterisk* highlights the LR primordium in *C*; the *bar* = 50 μm. *Crosses* 1, 2, 3 - Crosses 1, 2, 3 correspond to the regions of interests (ROI) 1, 2, 3 selected for spectral imaging and linear unmixing in PIN3::PIN3GFP and LAX3::LAX3YFP transgenic *Arabidopsis* seedlings, respectively, to generate respective normalised emission spectra in *A* and *D*.

strated that YFP and GFP can be detected using spectral imaging and linear unmixing. Using the conventional optical filtering methods available on our confocal microscope, we would not have been able to image and detect the two FPs whose spectral emissions overlapped in the PIN3GFP/LAX3YFP double transgenic lines. Using spectral imaging and linear unmixing, we confirmed that LAX3 was located in the MR stele, the endodermis, the cortex, and the epidermis above LR primordia at different stages of emergence. This is in

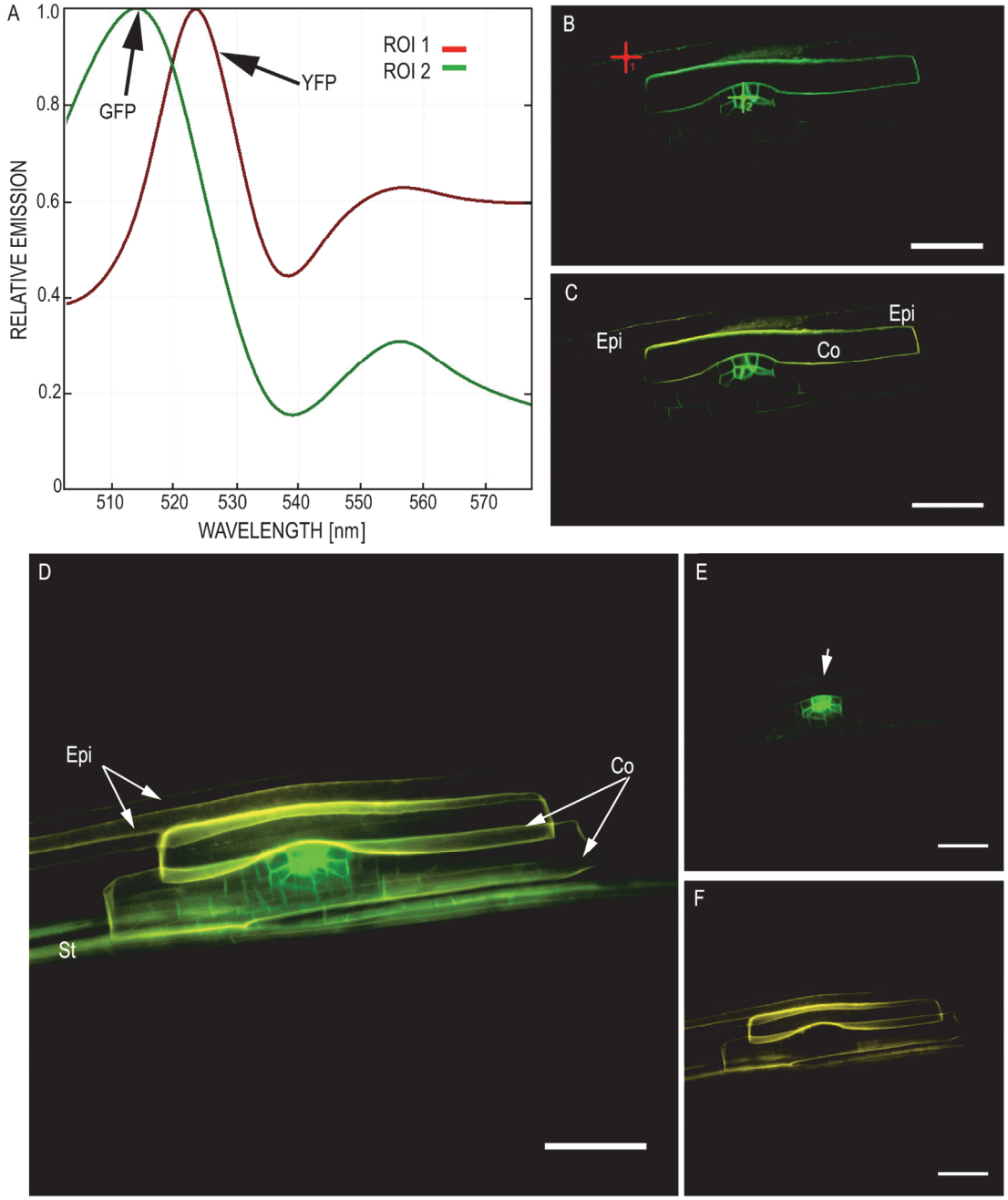

Fig. 2. The spectral profile and linear unmixing a double LAX3YFP/PIN3GFP transgenic *Arabidopsis* seedling in the LR primordium region ROI 1 and 2. *A* - The normalised emission spectra of GFP and YFP acquired from two different pixel points of the root primordium region of a double transgenic PIN3GFP/LAX3YFP *Arabidopsis* seedling. *B* - The spectral confocal image of lateral root primordium highlighting the ROI 1 and 2 (denoted by the crosses 1 and 2, respectively) selected to create the normalised emission spectra in *A*. *C* - The linear unmixing overlay image of *B* showing YFP and GFP. *D* - The Z projection image of the LR primordium consisting of merged Z stacks of 49 images. *E* - The image of *D* showing a GFP (*green*) signal only in the cortical cell above the primordium. *F* - The image of *D* showing YFP only. Epi - epidermis, Co - cortex, St - stele; the *white asterisk* in *E* points to the LR primordium; the *bar* = 50 μm.

agreement with observations made by Swarup *et al*. (2008) and Péret *et al*. (2013). Furthermore, we also found that PIN3GFP was located in the pericycle, the primordium, and cells in the endodermal and cortical layer located above the emerging primordium, as previously shown by Benková *et al*. (2002), Marhavý *et al*. (2013), and Péret *et al*. (2013).

This is advantageous as time spent generating new protein constructs tagged to FPs and the transgenic plants expressing these new constructs by transformation could be saved. Furthermore, spectral imaging with linear unmixing in living tissues could be adapted to live imaging and, in combination with other techniques, to help understand the role of proteins during lateral root development.

A recent work by Péret *et al*. (2013) demonstrated that PIN3 and LAX3 need to be induced consecutively during LR emergence, and that the interplay between PIN3 and LAX3 could create sharp intercellular gradients in LAX3 expression. Our work shows that PIN3 and LAX3 could be expressed simultaneously in the same cell suggesting that certain cells could intake and exude auxin simultaneously, *i.e.*, in the cortical cell above LR primordia. However, we cannot rule out that YFP and GFP fused auxin transport proteins remained in certain cells after the expression of the constructs had been switched off thus highlighting the need of complementary techniques, such as RT-qPCR and live imaging to dynamically monitor auxin membrane proteins in such cells.

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