Preface

The last 10 years have witnessed an explosion in our understanding of plant hormones. The often vague models of hormone action developed over decades have been replaced in short order by detailed molecular models that include receptors and in many cases downstream signal transduction components. Given the rapid progress in understanding the mechanism of action of plant growth regulators, a technical review of hormone methodology is timely. Our book focuses on genetic, biochemical, analytical and chemical biological approaches for understanding and dissecting plant hormone action. The greatest strides in plant hormone biology have come, by and large, from the use of genetic methods to identify receptors and we dedicate a chapter to general genetic methods of analysis using the model system Arabidopsis thaliana. A cluster of chapters focuses on biochemical methods for documenting interactions between hormones and their receptors. The importance of these assays is tremendous; receptor–ligand interactions in animal model systems have been the cornerstones of pharmacological and medicinal chemical assays that have enabled identification of selective and non-selective agonists and antagonists that can be used to further probe and dissect questions of receptor function. This is likely to be a major new frontier in plant hormone research. Given these recent assays for plant hormone receptors, the time is ripe to investigate chemical biological methods for exploiting these assays to develop an understanding the mechanism of action of synthetic plant growth regulators; in this context, we offer methods for conducting chemical genetic screens to identify new growth regulators in Arabidopsis and other plant species. Lastly, the last few years have also seen impressive growth in analytical methods for measuring plant hormones; we offer two chapters on both LC-MS and GC-MS based methods for measuring endogenous plant hormone levels. Collectively, these protocols should empower plant hormone biologists to dissect the ext generation of questions in plant hormone biology and signal transduction.

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Chapter 2

Visualizing Auxin Transport Routes in *Arabidopsis* Leaf Primordia

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Abstract

The phytohormone auxin plays a pivotal role in plant development, regulating a myriad of processes including embryo patterning, root patterning, organ initiation, and vein patterning. Auxin is unique among the plant hormones as it is actively transported from cell to cell in a polar fashion. It has recently been discovered that polar auxin transport generates dynamic, local auxin gradients within plant tissues that appear to provide positional information in patterning processes. Visualization of apparent auxin transport patterns has largely been facilitated by the recent creation of translational fusions of GFP to members of the *Arabidopsis* (At)PIN family of auxin efflux associated proteins. Confocal visualization of these fusion products (PIN:GFPs) enables the tracking of apparent auxin transport patterns in a huge number of samples. This visualization method can be combined with experimental interference, such as local auxin application and inhibition of auxin transport, to deduce possible self-organizing auxin-dependent patterning mechanisms and to make them amenable to mathematical modeling.

Key words *Arabidopsis*, *AtPIN* genes, polar auxin transport, leaf development, vascular patterning, confocal microscopy, auxin application, indole-3-acetic acid (IAA).

1. Introduction

The plant hormone auxin is involved in a wide array of plant processes, including embryo patterning *(1)*, photo- and gravitotropism *(2)*, phyllotaxis *(3)*, vein patterning *(4)*, and root patterning *(5)*. Auxin distribution within the plant depends on auxin efflux from plant cells, which is tightly linked to the expression and subcellular localization of members of the PIN family of transmembrane proteins *(6)*. In plant cells, PIN proteins cycle rapidly and continuously between an endosomal compartment and the basal cell membrane *(7, 8)*, making possible the rapid relocalization of PIN proteins in response to internal and external cues *(9)*.

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Historically, elucidating auxin's precise role in these developmental processes has been difficult due to the difficulty of visualizing auxin directly. However, due to the recent genesis of PIN:GFP translational fusion proteins, one can infer the path and direction of auxin transport from the expression pattern and subcellular localization of PIN:GFPs. Auxin transport routes determined through PIN1:GFP expression patterns are consistent with those determined by other methods, but the use of PIN:GFPs allows one to trace these routes in temporal sequence in huge numbers of samples *(3, 10)*. Recently, this approach has been very successfully combined with live imaging of the shoot meristem to study patterns of auxin transport and gene expression during shoot organ initiation *(11)*. Research involving PIN translational fusions suggests that PIN-dependent auxin distributions are involved in the positioning of lateral organs of the plant root and shoot *(10)*, and the specification of veins of all orders in the plant leaf *(4)*. PIN translational fusions have also been instrumental in revealing the key role of PIN relocation and degradation in root gravitropism *(11)*. All these studies relied on confocal microscopy to examine PIN:GFP expression and subcellular localization in their organ of interest. This basic approach can be used to analyze the role of auxin transport in any developmental process in *Arabidopsis*. Because of high background fluorescence as well as the shape and thickness of the sample, visualization of PIN1:GFP expression in leaf primordia during normal development is particularly challenging. Here, we describe the visualization of auxin-transport patterns during normal leaf development, as well as methods to manipulate those by local auxin application and auxin transport inhibition.

2. Materials

2.1. Seedling Culture

1. Sterilization solution: 15% (v/v) commercial bleach, 0.01% (v/v) Triton X-100 (VWR, West Chester, PA) in sterilized, double-distilled water.
2. Growth medium: Half-strength Murashige and Skoog salts (Sigma, St Louis, MO, USA), 0.5 mg/l morpholino ethane sulfonic acid (MES, Sigma), 0.8% (w/v) agar (BioShop Canada Inc., Burlington, ON), pH 5.7. Note that sucrose is not added to the Growth medium.

2.2. Local Auxin Application

1. IAA paste: up to 10% (w/v) indole-3-acetic acid (IAA; Sigma) mixed in pre-warmed paste consisting of lanolin (Sigma) and a liposoluble red dye (Procter & Gamble; *see* **Note 2**).

2. Insect pins (0.1 mm in diameter; Fine Science Tools, Inc., North Vancouver, BC, CA).
3. Ophthalmic surgical pin holder (Fine Science Tools, Inc.).

2.3. Auxin Transport Inhibition

1. NPA stocks: 1, 10, and 100 mM stock solutions of 1-*N*-naphthylphthalamic acid (NPA; Chem Service).
2. Growth medium (*see* **Section 2.1**).

2.4. Confocal Visualization

1. Double-distilled, sterilized water for mounting (*see* **Note 1**).
2. Microscope slides (76 × 26 mm, VWR).
3. Coverslips (22 × 50 mm, 0.16–0.19 mm thick, VWR).
4. PrecisionGlide needles (30½ gauge, Becton Dickson & Co., Franklin Lakes, NJ, USA).
5. 3 mL Syringe (Becton Dickson & Co.).

3. Methods

The following experiments rely on confocal microscopy [described in detail in ref. *(4)*] to follow PIN:GFP expression and subcellular localization during normal and experimentally challenged leaf primordium development. PIN1:GFP, to which this detailed description specifically refers, is a functional PIN1 protein that is relatively weakly expressed in *Arabidopsis* leaf primordia *(10)*. Experimental manipulations can make PIN1:GFP even more difficult to detect. To obtain high quality images, it is necessary to optimize the following: (a) sample quality – the healthiest seedlings tend to produce the best quality images; (b) mounting – the leaf primordium should be mounted as flat as possible, in the correct orientation, to minimize the number of tissue layers that the light must penetrate; (c) visualization parameters – while others' suggestions help, these must ultimately be determined empirically for each marker and confocal microscope; (d) viability of the sample – samples were routinely visualized immediately after mounting as PIN1:GFP shows signs of decreased expression and relocalization in response to treatment within 5–10 min. Once the sample has been mounted, it is necessary to work quickly and without interruptions. Therefore, optimized parameters and a routine protocol must be established first that can then be used to collect data from large numbers of samples.

3.1. Seedling Culture

1. Leaf synchronization is critical for pattern reproducibility. The following seedling culture protocol incorporates strategies to ensure synchronous leaf initiation and preparation. To

prepare seedlings for confocal visualization, incubate PIN1:GFP seeds *(10)* in 70% ethanol for 1 min and then vortex in sterilization solution for 20 min.

2. Wash 10 times in sterile, double distilled water, incubating for at least 10 min between washes. This incubation period, a modification of the synchronization procedure by Petrov and Vizir *(12)*, allows the seeds to imbibe the water and synchronizes their germination.
3. Sow seeds on growth medium in sterile Petri plates at a density of 1 seed/cm^2. Seal plates and wrap in aluminum foil to exclude light.
4. Stratify seeds at 4°C for 5 days. A 5-day stratification period will provide the best synchronization of germination. Shorter stratification periods will result in progressively less synchronized seedling development.
5. Following stratification, incubate plates in growth chambers with continuous fluorescent light (100 μE/m^2/s^2) at 25°C.

3.2. Local Auxin Application

1. Preparation of IAA paste: Place 1 mL of lanolin in a microcentrifuge tube and place it in a 65°C heating block, to melt lanolin.
2. Add a small quantity (approximately, 25 μl) of liposoluble red dye (*see* **Note 2**), such that mixture turns a deep red color. If necessary, re-adjust the volume to 1 mL by removing a small amount of the lanolin mixture with a pipette. Reserve lanolin-dye paste to treat controls.
3. Remove the microcentrifuge tube from the heating block and allow it to cool slightly, such that it is still liquid but no longer feels warm to the touch (*see* **Note 3**).
4. Add IAA to a final concentration of up to 10% (w/v). Incubate the microcentrifuge tube at 4°C for 15–30 min, to cool and solidify the paste. Due to the lability of IAA, the IAA paste should be used immediately.
5. Application of IAA paste: Using a small spatula, smear a small amount of IAA paste on a piece of weighing paper. Using dissecting microscope to examine the plate of cultured seedlings, select a healthy seedling of the correct stage to treat (*see* **Note 4**).
6. Holding the insect pin in the pin holder, drag the insect pin through the IAA paste. This will cause the paste to form a long thin peak on the tip of the pin.
7. Working under the dissecting microscope, very carefully touch the tip of this peak to one side of the leaf primordium. A small drop of IAA paste will be transferred onto that side of the primordium (*see* **Note 5**).

8. Treat control seedlings in the same way with lanolin-dye paste that lacks IAA.
9. Return the plate of treated seedlings to the growth chamber.
10. After 24 or 48 h post-treatment, proceed with confocal visualization.

3.3. Auxin Transport Inhibition

1. Preparation of NPA growth medium: Prepare plates of growth medium containing 0, 5, 10 or 50 μM NPA by adding an appropriate volume of 1, 10 or 100 mM NPA stock solution to 25 mL molten, solid growth medium (per plate).
2. Sow and stratify PIN1:GFP sterilized seeds as described in ***3.1, Item 4.***
3. Visualize NPA-treated seedlings at the confocal microscope daily from 2 to 5 DAG.

3.4. Confocal Visualization

1. Mounting of samples: Place one seedling on a drop of double distilled, sterile water on a microscope slide.
2. Using the sharp edge of the needle tip as a blade, cut the root above the root-hypocotyl junction and remove. This decreases the thickness of the preparation considerably, enabling better visualization of the leaf primordia. However, without a root, the treatment-induced changes in PIN1:GFP expression will follow 5–10 min post-mounting. Therefore, individual seedlings are imaged immediately after mounting.
3. After removing the root, place one needle on each cotyledon and gently pull them apart, exposing the first leaf primordia that lie between the cotyledon petioles. It may be necessary to slightly damage the cotyledons to force them to lie flat against the slide, as shown in **Fig. 2.1**. If imaging the third leaf or subsequently formed rosette leaves, use the needle to cut away the cotyledons and any leaf primordia covering the

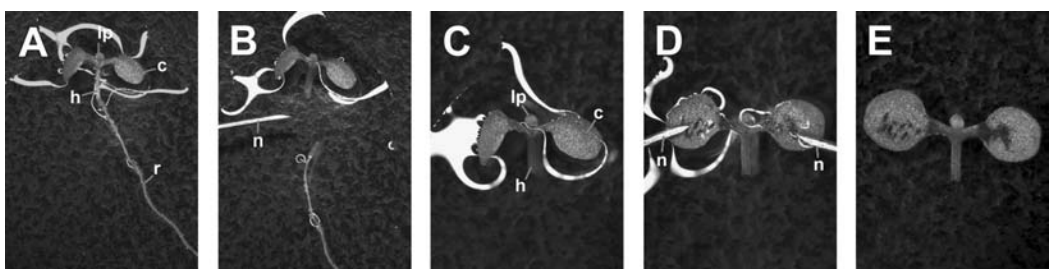

Fig. 2.1. Mounting of *Arabidopsis* seedlings for confocal visualization of the leaf primordia. (c) cotyledon; (h) hypocotyl; (lp) leaf primordium; (n) needle; (r) root. (**A**) Seedling on a drop of water on microscope slide. (**B**) Seedling with hypocotyl cut above the root-hypocotyl junction. (**C**) Seedling prior to flattening. (**D**) Seedling flattened by pulling on the cotyledons with needles. (**E**) Seedling compressed by coverslip.

primordium of interest, and orient the seedling such that this primordium faces up.

4. Place one edge of the coverslip against the slide and, balancing the other edge of the coverslip on the needle, gently and slowly lower the coverslip onto the slide, avoiding the formation of air bubbles (*see* **Note 6**). Sudden dropping of the coverslip onto the preparation will result in damage to the sample.
5. Optimization of visualization settings (*see* **Note 7**): When working with weak fluorescent markers, like the PIN:GFPs, one is typically operating close to the lower detection limit of the microscope. When determining the optimal visualization conditions, begin with all parameters (laser output, laser transmission, pinhole, and gain) at low settings (*see* **Note 8**).
6. Increase gain until an acceptable image is obtained. Only if fluorophore detection is still insufficient, gradually increase the pinhole, as large pinholes will result in a blurred image. If the detection is still unsatisfactory, resort to increasing the transmission and/or output of the excitation laser, keeping in mind that this increases photodamage of the sample.
7. Due to the difference in PIN1:GFP signal intensity in different parts of the leaf primordium, visualization setting that enable the detection of PIN1:GFP in incipient higher order veins will result in total signal saturation in the lateral epidermis, obscuring PIN1 polarity in this area. Take multiple images of the same focal plane, some with higher pinhole/gain settings to capture the weakest areas of PIN1:GFP expression and some with lower pinhole/gain settings to resolve PIN1 localization in areas of very high PIN1:GFP expression (*see* **Fig. 2.2**).

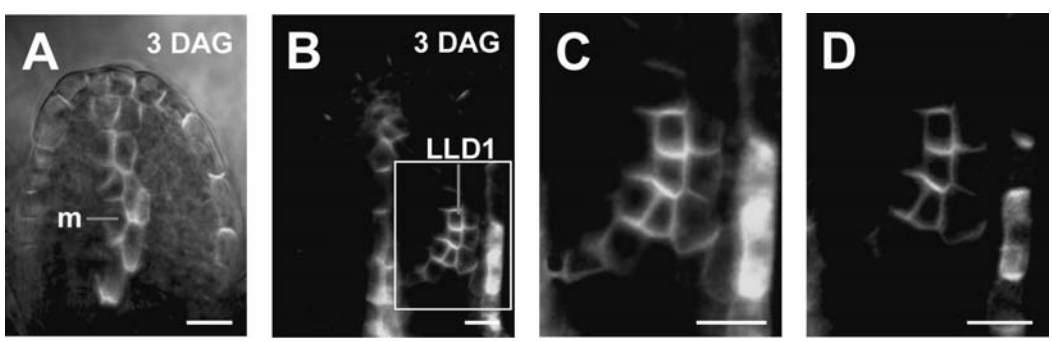

Fig. 2.2. Confocal visualization of PIN1:GFP in *Arabidopsis* leaf primordia. (*Upper right*) Primordium age in days after germination (DAG). (m) midvein; lower loop domain of first (most distal) loop [see ref. *(4)*]. (**A**) Merged DIC and GFP image of a first leaf primordium at 3 DAG, showing PIN1:GFP expression in the midvein and epidermis. (**B**) GFP image of a first leaf primordium at 3 DAG, showing PIN1:GFP expression in the midvein, lateral epidermis, and the lower domain of the first loop. (**C**) Enlargement of the area boxed in (**B**), showing the complete PIN1:GFP expression pattern in the lower loop domain of the first loop. (**D**) Same area shown in (**C**), imaged with a smaller pinhole to visualize PIN1:GFP localization in the areas of highest expression. *Bars*: (**A–D)** 10 μm (Reproduced from ref. *(4)* with permission from CSHL Press).

Those individual images can be used to analyze relative signal intensities. Composite images constructed from single images taken with different settings cannot be used to quantify differences in PIN1:GFP expression, but rather document all polarity features of the PIN1:GFP expression pattern in the developing primordium.

8. Individual images can be used to analyze relative signal intensities using look-up tables (LUTs). The human eye cannot distinguish 256 different levels of brightness, the minimum recorded by all confocal microscopes *(13, 14)*. Therefore, to increase distinction of monochromatic shades, and thus convey the true spectral detection range of the data, critical fluorescence images can be turned into 8-bit gray-scaled images and their intensity expressed as pseudo-colors, using a rainbow spectrum look-up-table (LUT) of ImageJ (National Institutes of Health, http://rsb.info.nih.gov/ij).
9. Since the leaf primordium is not perfectly flat, its complete PIN1:GFP expression pattern cannot be captured in an image of a single focal plane. Cover all features of the expression pattern by taking images at several focal planes, either by focusing manually through the sample and taking images at intervals or by using the z-stack function of the confocal microscope. The resulting images can be compiled and overlaid using microscope software or Adobe Photoshop 7.0 (Adobe Systems). Sample composite images, reconstructed from multiple single images of a IAA-treated and untreated first leaf primordia, are shown in **Figs. 2** & **3**.

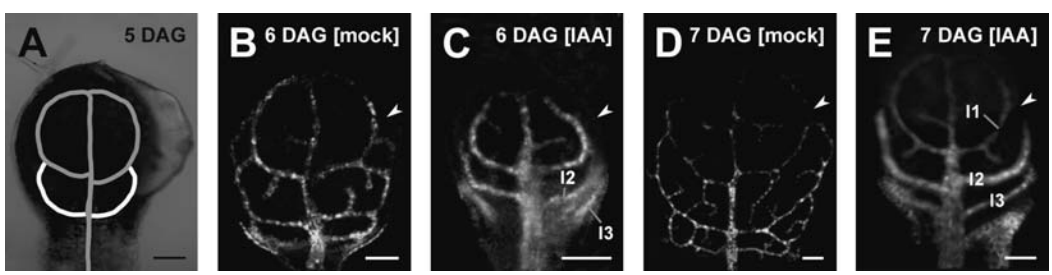

Fig. 2.3. Effects of local auxin application on PIN1:GFP expression in *Arabidopsis* leaf primordia. (*Upper right*) Primordium age in days after germination (DAG) and treatment (IAA/mock, in *square brackets*). (*Yellow arrowheads*) Site of application; (I1, I2, I3) loop 1, loop 2, loop 3 [ordered distal to proximal, see ref. *(4)*]. (**A**) DIC image of a first leaf primordium 5 DAG, showing predicted positions of procambium (*grey*) and marker-expression defined preprocambium (*white*). (**B**) GFP image of a mock-treated first leaf primordium 6 DAG, showing the PIN1:GFP expression in the midvein, in three loops and in higher order veins. (**C**) GFP image of a IAA-treated first leaf primordium 6 DAG, showing increased PIN1:GFP expression in the area of the third loop on the treated side. (**D**) GFP image of a IAA-treated first leaf primordium 7 DAG, showing an extra fourth loop in the PIN1:GFP pattern on the treated side. (**E**) *Bars*: (**A–E**) 50 μm [Reproduced from ref. *(4)* with permission from CSHL Press].

4. Notes

1. Because viscosity is not an issue, distilled water rather than glycerol solutions can be used as a mounting medium.
2. Any brightly colored lipstick could be used as a liposoluble red dye.
3. IAA is heat labile. Therefore, to prevent degradation, the lanolin mixture must be cooled until it no longer feels warm to the touch. Once cooled, IAA is added immediately, because the lanolin paste solidifies quickly at this temperature.
4. To study the effect of local auxin application on loop formation, one would ideally treat those first 4 DAG leaf primordia, in which the second and third loops have not yet formed as preprocambium *(15)*. However, it is very difficult to apply IAA paste unilaterally on such tiny primordia. A preferred stage would therefore be 5 DAG, a stage when the third loop has not yet formed as preprocambium *(15)*. At 4 DAG, the two first leaf primordia lie close together, pointing straight up when viewed from above. By 5 DAG, in contrast, the first leaf primordia have separated and lie perpendicular to the axis of the incoming light.
5. The size of the drop will depend on the width of the IAA paste peak on the pin. Practice drawing the pin through the lanolin paste, to create the thinnest peak possible. This peak can then be used to treat multiple seedlings.
6. If an air bubble forms in the slide preparation, but it does not obscure the leaf primordium, the slide can be used without difficulty. If an air bubble forms over the area of the leaf primordium, it can sometimes be displaced by adding a drop of water to the edge of the coverslip.
7. The visualization protocol described here was developed on a Zeiss Axiovert 100 M microscope equipped with a Zeiss LSM510 laser module confocal unit (Carl Zeiss). The optimal settings have to be determined empirically for other fluorescent markers and confocal microscopes. Once determined, the optimal settings can be re-used throughout a study.
8. On the Zeiss LSM510 confocal microscope, PIN1:GFP can be visualized using GFP was the 488-nm line of an Argon laser at 50–55% of laser output and 4–10% transmission, and with a 505–530-nm band-pass filter. The gain used is 3 (100% of maximum gain) and the pinhole is between 5 and 14 Airy units (36–100% maximum pinhole using 40 × Plan Neofluar oil-immersion objective with a numerical aperture of 1.3.)

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References

1. Weijers, D. and Jurgens, G. (2005) Auxin and embryo axis formation: the ends in sight? *Curr Opin Plant Biol* 8, 32–7.
2. Swarup, R., Kramer, E. M., Perry, P., Knox, K., Leyser, H. M., Haseloff, J., Beemster, G. T., Bhalerao, R. and Bennett, M. J. (2005) Root gravitropism requires lateral root cap and epidermal cells for transport and response to a mobile auxin signal. *Nat Cell Biol* 7(11), 1057–65.
3. Reinhardt, D., Pesce, E. R., Stieger, P., Mandel, T., Baltensperger, K., Bennett, M., Traas, J., Friml, J. and Kuhlemeier, C. (2003) Regulation of phyllotaxis by polar auxin transport. *Nature* 426, 255–60.
4. Scarpella, E., Marcos, D. and Berleth, T. (2006) Control of leaf vascular patterning by polar auxin transport. *Genes Dev* 20(8), 1015–27.
5. Blilou, I., Xu, J., Wildwater, M., Willemsen, V., Paponov, I., Friml, J., Heidstra, R., Aida, M., Palme, K. and Scheres, B. (2005). The PIN auxin efflux facilitator network controls growth and patterning in *Arabidopsis* roots. *Nature* 433, 39–44.
6. Paponov, I. A., Teale, W. D., Trebar, M., Blilou, I. and Palme, K. (2005) The PIN auxin efflux facilitators: evolutionary and functional perspectives. *Trends Plant Sci* 10, 170–7.
7. Friml, J., Benkova, E., Blilou, I., Wisniewska, J., Hamann, T., Ljung, K., Woody, S., Sandberg, G., Scheres, B., Jurgens, G. and Palme, K. (2002a) AtPIN4 mediates sink-driven auxin gradients and root patterning in *Arabidopsis*. *Cell* 108, 661–73.
8. Geldner, N., Friml, J., Stierhof, Y. D., Jurgens, G. and Palme, K. (2001) Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. *Nature* 413, 425–8.
9. Friml, J., Wisniewska, J., Benkova, E., Mendgen, K. and Palme, K. (2002b) Lateral relocation of auxin efflux regulator PIN3 mediates tropism in *Arabidopsis*. *Nature* 415, 806–9.
10. Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D., Jurgens, G. and Friml, J. (2003) Local, efflux-dependent auxin gradients as a common module for plant organ formation. *Cell* 115, 591–602.
11. Heisler, M. G., Ohno, C., Das, P., Sieber, P., Reddy, G. V., Long, J. A., Meyerowitz, E. M. (2005) Patterns of auxin transport and gene expression during primordium development revealed by live imaging of the *Arabidopsis* inflorescence meristem. *Curr Biol* 15(21), 1899–911.
12. Abas, L., Benjamins, R., Malenica, N., Paciorek, T., Wisniewska, J., Moulinier-Anzola, J.C., Sieberer, T., Friml, J. and Luschnig, C. (2006) Intracellular trafficking and proteolysis of the *Arabidopsis* auxin-efflux facilitator PIN2 are involved in root gravitropism. *Nat Cell Biol* 8(3), 249–56.
13. Petrov, A. P. and Vizir, I. Y. (1982) On seed germination synchronization of Arabidopsis thaliana (L.) Heynh. *Arabidopsis Information Service* 19.
14. Russ, J. C. (1995) The image processing handbook. CRC Press Boca Raton, FL.

15. Young, I. T., Gerbrands, J. J. and van Vliet, L. J. (1998) Fundamentals of image processing. In The digital signal processing handbook (eds. V.K. Madisetti and D.B. Williams), pp. 51.01–51.81. CRC Press, Boca Raton, FL.