A Novel Horizontally Mounted Confocal Microscope Provides New Insights into Plant Development

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One of the greatest challenges we face in the 21st century is to sustainably feed nine to ten billion people by the year 2050, while simultaneously attempting to reduce the environmental impact of food production. Some options that have been proposed to address these challenges include closing the yield gap (i.e making the difference between attempted and attained yields smaller) and increasing the production potential of crops, largely through the use of new technologies and investing in research in the plant sciences field (Smith and Gregory, 2013). Thus, a crucial overarching aim of the plant sciences is the ability to 'future-proof' crops, i.e. use a combination of molecular breeding and genetic techniques to generate elite crop varieties that are able to withstand environmental stresses such as increased CO_2 levels as well as water and nutrient scarcity, factors which strongly constrain plant growth in soil (Kissoudis, 2016).

While overall plant architecture both above and below ground, is largely determined by the number and length of secondary (or lateral) organs (Reinhardt and Kuhlemier, 2002), recent studies have demonstrated that the *angle* at which these lateral organs grow at is also a crucial determinant of plant architecture, and ultimately overall plant fitness (Uga et al 2015). This is because, nutrients such as water, nitrates and phosphates are most often heterogeneously distributed within the soil strata. For example, nitrates are leached out of soil by precipitation and tend to accumulate within deeper soil layers. Thus, modulation of root growth angle to generate deeper rooting crop species is likely to be a desirable trait, particularly in nitrate deficient soils (Uga et al, 2015). Additionally, since recent research has shown that use of nitrogen fertilisers contributes significantly to global warming, enhancing the ability of plants to take up nitrates from soils more efficiently is also an important aspect of reducing CO_2 emissions and hence, global warming (Tian et al, 2020).

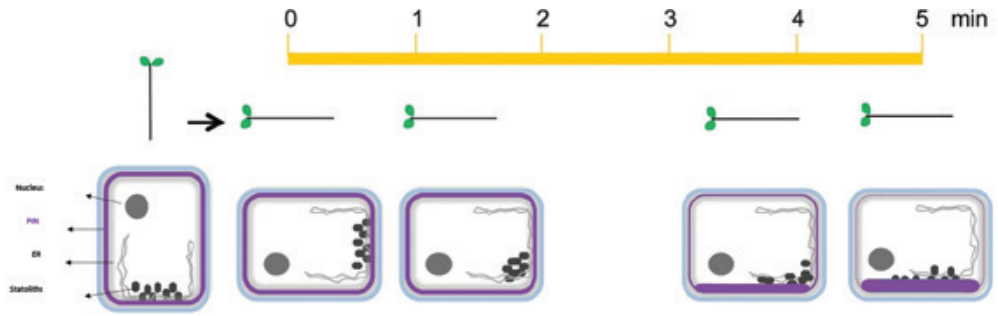

Figure 1: Early molecular events of plant gravitropism (Adapted from Sato et al, 2015). When a vertically growing Arabidopsis seedling (left) is reoriented by 90° and placed perpendicular to the gravity field, this triggers the sedimentation of dense starch filled amyloplasts towards the new physical bottom of the gravity sensing cells in the root tip. The physical signal of amyloplast sedimentation leads to the generation of a biochemical signal that is responsible for repolarisation of the PIN auxin efflux transporter proteins towards the bottom of the gravity sensing cells. The polarisation of PIN proteins leads to the accumulation of auxin towards the bottom of the graviresponding root, which is a key step in the ability of plant organs to respond to gravity.

Considering the importance of root (and shoot) growth angle in determining nutrient acquisition efficiency, and ultimately plant fitness and yield, it is not surprising that a number of researchers have begun to investigate the mechanisms by which oblique growth patterns in flowering'bngn dfl...e4 plant organs are regulated, with particular focus on the molecular genetic pathways, with the aim of identifying specific genes that may be targeted to engineer crops with deeper or shallower root and shoot growth angles. While much about these mechanisms remains unknown, using the model plant *Arabidopsis thaliana*, it has been convincingly demonstrated that the plant hormone auxin, often described as the 'master regulator' of plant development controls nonvertical/ oblique growth patterns in higher plant organs (Roychoudhry et al, 2013; Ruiz-Rosquete et al, 2013).

Recently, a broad overall consensus has emerged relating to the molecular mechanisms of regulation of nonvertical growth in plant organs. In contrast to the main parent body of the plant, both, lateral roots and shoots are growing at an angle to the gravity vector. Thus, it is clear that gravity, which broadly acts to ensure that plant roots grow vertically downward and shoots grow vertically upward, is not acting upon lateral organs in the same manner as the parent axis (reviewed in Roychoudhry and Kepinski, 2015). This has led to several research groups, to further investigate one of the most fundamental processes of plant development – gravitropism. Gravity is the most constant force acting on plants, and the ability of plants to respond to gravity i.e. broadly, for shoots to grow upwards and roots to grow downwards, is known as gravitropism. Because the direction and magnitude of gravity are almost constant on the face of the earth, gravitropism can be regarded as a mechanism of posture control, triggered by sensing the tilt of organs relative to the direction of gravity.

In the model plant *A. thaliana* (and indeed in most multicellular plants), gravity is sensed in specialised cells along the shoot axis and in the root tip known as statocytes. These cells contain dense starch-filled granules called "amyloplasts' which sediment in the direction of gravity, a process that was observed as early as 1900 (Haberlandt, 1900). Thus, when a plant organ, for example, a root is rotated by 90° so as to be placed perpendicular to the earth's surface, gravity ensures that within a few minutes, the amyloplasts sediment towards the new lower side of the root. The sedimentation of these amyloplasts then triggers a signaling cascade that, within a few minutes (reviewed in Morita, 2010; Su et al, 2017), begins with the repolarisation of a set of membrane proteins, known as PIN-FORMED, or PIN proteins towards the lower side of the gravity-

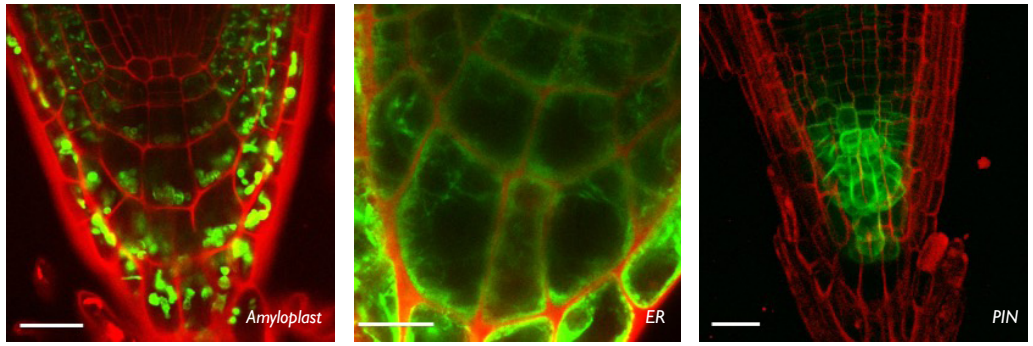

Figure 2. Confocal images of GFP and YFP markers of amyloplasts (left), ER (middle) and PIN proteins (right) in 5-day-old Arabidopsis seedling root tips taken at 40X magnification with the LSM800 horizontal microscope. The seedlings were grown in chambered slides (Labtek) and counterstained with propidium iodide prior to imaging. For imaging the PIN protein GFP marker, a series of Z stacks were taken through the root, and compiled to create a 'sum of stacks' 3D projection in Fiji.

sensing cells, that are now in contact with the amyloplasts (See Figure 1). Because PIN proteins function as transporters of the plant hormone auxin, this process in turn ensures that the auxin is preferentially transported along the lower side of the root. Remarkably, auxin has the ability to inhibit cellular elongation in roots. This means that as auxin accumulates on the lower side of a gravistimulated root, the cells on this side cease to elongate, while those on the upper side continue to do so. This resulting asymmetry in auxin distribution and ultimately cellular elongation results in the root 'correcting' its direction of growth to grow along the gravity vector again.

Although the overall process of gravitropism is so fundamental to regulating overall plant architecture, surprisingly many aspects of the molecular mechanisms underpinning both gravitational sensing and response in plant organs remain poorly understood. In *Arabidopsis* roots, particularly, it is not clear how the first physical signal of amyloplast sedimentation triggers subsequent biochemical pathways leading to PIN repolarisation and auxin redistribution. Furthermore, plants also have the ability to mount a gravitational response that is proportional to the angle of stimulation, according to the so called 'Sine Law', first described by von Sachs in the 1800s. Thus, a root that is reoriented by 90^0 bends downward in the direction of gravity at a faster rate than a root that is reoriented by a smaller angle, such as 45° (Sachs J., 1882).

Amongst the several biochemical and physiological tools that researchers have employed to answer these questions, real-time live cell imaging provides a powerful strategy to elucidate the spatiotemporal processes of gravity sensing and response *in situ*. These approaches have been aided by the generation of several stable transgenic plant lines by multiple research groups over the years, containing fluorescent markers for different organelles involved in the gravitropic response (Friml et al, 2003; Nelson et al, 2007; Geldner et al, 2009, see Figure 2). As some examples, YFP tagged lines for the starch-filled statoliths, as well as the PIN membrane proteins have been utilised to visualise sedimentation kinetics of statoliths and PIN repolarisation in response to gravity and have provided valuable insights into the temporal resolution of these steps. However, a key limitation of all these studies has been the inability to perform dynamic, live cell confocal (or indeed super resolution) imaging on vertically growing plant roots, or those reoriented within the X-Y plane. These challenges have been difficult to overcome, due to the horizontal sample positioning set up of confocal (and other) microscopes, as well as the continuous displacement of the root tip as it undergoes the gravitropic response. Thus, previous work on the kinetics of gravi-dependent organelle repositioning has involved the fixation of *Arabidopsis* root samples prior to imaging, usually using a 4% paraformaldehyde solution. More recently, advanced techniques for sample fixation such as ClearSee (Kurihara et al, 2015) have been developed. While fixation preserves the orientation of the sample with respect to gravity, the overall process is still lengthy, time consuming and often quenches the fluorescent signal. Our innovative solution to overcome all these technical challenges at the University of Leeds was the installation of a novel horizontally mounted confocal microscope with a rotating vertical stage.

Initially funded through a BBSRC equipment alert in 2016, the setup is currently located within the Bioimaging Suite at the Faculty of Biological Sciences at the University of Leeds. Briefly, it consists of an LSM800 confocal microscope (with Airyscan, ZEISS) that has simply been rotated by 90° so that it is mounted horizontally on its back. The microscope is secured in this horizontal position using special metal mounts that were specifically designed for

At ZEISS we recognise that microscopes designed for a production line, even when they are highly modular and configurable, may not always be perfectly adapted for observing all of nature's biological processes. Our Special Customer Solutions (SCS) team focus on designing and developing bespoke microscopy solutions for more challenging applications. In Prof. Kepinski's case when studying plant root growth the challenge is to keep the plant in its natural vertical orientation so that the it exhibits a typical geotrophic response. A typical upright or inverted microscope is therefore not appropriate for observing this growth. The challenge was therefore to rotate the microscope stand with attached laser scanning confocal microscope so it has a horizontal optical axis. The concept to adapt the microscope to the sample's "natural" conditions rather than the other way round was both equally interesting and challenging for our SCS and local application support teams.

The initial on-site installation was completed as normal for an LSM800. Then the microscope was tilted and secured on its back using special mounts designed by a collaborative group in Vienna.

Integrating the control of the motorised rotating stage was performed by our colleagues in software development. This allowed the angle of the observed plants to be fully controllable by the software and macros; and with some trigonometry, the stage position is adapted so the root is still in the centre of the field of view while rotating.

The ZEISS customer Service team on the design and installation of the new horizontally mounted LSM800 at the University of Leeds.

The horizontal microscope configuration was also an issue for the XY stage at first, as gravity was pulling it down under the weight of the stage and insert. A temporary workaround involving pulleys and weights allowed the system to work until ASI provided a stage with a different gear pitch strong enough to work against gravity and solve the issue. Although this was a technically challenging project, we are extremely pleased to have worked closely with the University of Leeds and ultimately delivered on a bespoke microscopy solution that focuses on the sample in its most natural state. ZEISS hope that the horizontal LSM800 will unlock many new opportunities at the University of Leeds to study new areas of plant biology that were previously impossible.

The ZEISS Customer Service team on the challenges of installing the bespoke motorised rotating stage for the horizontally mounted LSM800 at the University of Leeds.

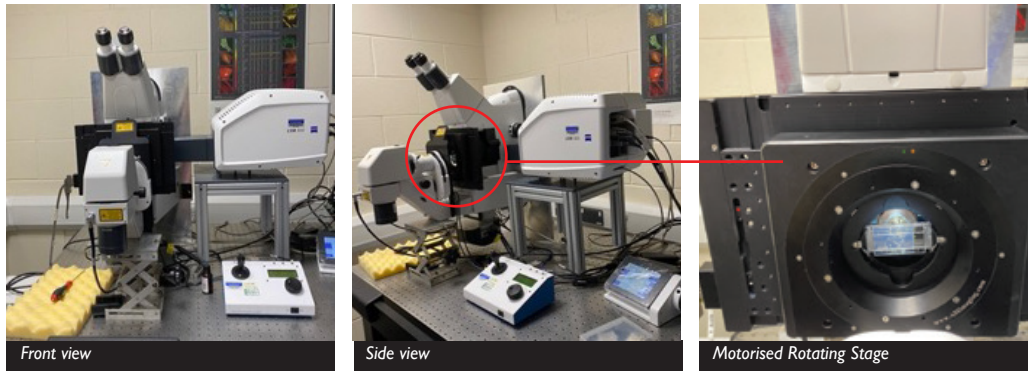

Figure 3. Front (left panel) and side (middle panel) views of the horizontally mounted Zeiss LSM 800 confocal microscope located within the Bioimaging suite at the University of Leeds. The asterisk (*) denotes the specially engineered metal mount to support the microscope in its horizontal position. The red lines (middle panel) show an inset of the motarised rotating stage (right panel) holding a chambered slide containing vertically grown 5-day-old Arabidopsis seedlings.

this purpose by our collaborators, the Friml group at the Institute of Science and Technology (IST), in Vienna, Austria. This configuration preserves access to all the features of the microscope, while now having the additional capacity of being able to hold plants vertically in their native states. Plant samples are grown vertically on commercially available 'Labtek'' chambered slides between the cover slip and the addition of a thin layer of plant growth medium (See Figure 3).

Additionally, the microscope setup also contains a specially engineered bespoke rotating stage with the capacity of holding a range of different types of slides, so that plants (or indeed other samples) can be reorientated at any desired angles ranging from 0-360°, as well as customised LED lighting to carry out experiments related to phototropic (i.e. the movement of plants in response to directional light) responses. Such an approach was first developed by the lab of Prof. Jiri Friml at IST, using an LSM700. However, the newly mounted LSM800 at Leeds is approximately 20 times more sensitive than this system, and the inclusion of Airyscan has enabled up to 1.7x increased resolution in all three axes, making this one of the most advanced microscopes of its kind anywhere in the world. Another advantage of using this system is that the chambered slides can be mounted onto the rotating stage of the microscope directly, without any additional sample preparation. This process has the added benefit of zero perturbation of the samples prior to imaging.

In addition to all this, the ZEISS team have further developed a customised software that enables tracking of the root tip for up to 24 hours. This means that researchers can set up their reorientation experiment with, for example, a number of *Arabidopsis* roots expressing different fluorescent markers and create time lapse image series at high resolution with minimal input.

In order to generate longer time lapse films at magnifications of 40X and 60X however, an added challenge remained initially. Typically, the use of these oil immersion lenses requires the placement of a drop of oil either on the lens surface, or the sample. While this is easily achieved with a typical confocal microscope, placement of oil on the lens of the horizontally mounted LSM800 caused it to drip downwards off the lens. After much trial (and error!) we found that the use of ultrasound gel, of the kind used generally for sonography was a good substitute for oil. The viscosity of the gel ensures that the slide can be coated with a layer of it for imaging at higher magnification with no issues.

This cutting-edge imaging system has already provided invaluable novel insights into the molecular processes of root gravitropism in the model plant *A. thaliana*. For example, we have discovered that contrary to previous data, where amyloplast sedimentation in response to gravity was thought to be a fairly rapid process, the spreading of amyloplasts over the plasma membrane at the new physical bottom of the gravity sensing statocytes can take up to 20 mins. Further, confirming previous studies, we have found that the sedimenting amyloplasts are able to mechanically deform the ER network overlying the lower plasma membrane, and interestingly, that ER structure is restored after 15-20 mins post statolith sedimentation. Finally, we have started to make inroads into elucidating the molecular mechanisms of angle dependent gravitropic response in *Arabidopsis* roots, by demonstrating that the percentage of PIN proteins that polarise to the lower membrane of gravity-sensing cells post reorientation, are proportional to the angle of reorientation.

Taken together, this novel ZEISS LSM800 horizontally mounted microscope has a range of potential benefits and beneficiaries. Besides allowing plants to be imaged in their native vertically growing states with such detail for the first time, this imaging set up has already dramatically enhanced research output at the University of Leeds, where it is located, allowing researchers to develop new skills in high resolution live cell imaging, automation methods and image analysis. Secondly, we envisage that the quality of plant sciences research within the UK will be significantly enhanced. This will subsequently lead to increased economic competitiveness of the UK as well as foster cutting edge interdisciplinary research - not only within the academic field but also within public and private sectors. Finally, in the long term, our findings will be crucial to the understanding of plant gravitropism and eventually the regulation of plant architecture, the modification of which provides an extremely attractive target in the quest for food security in the face of global warming and a rapidly expanding population.

For more information on usage and access to the ZEISS LSM800 horizontal microscope please contact bioimaging-facs@leeds.ac.uk

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Suruchi Roychoudhry (she/her) is a Senior Research Fellow in Plant Developmental Biology currently working within the Centre for Plant Sciences at the University of Leeds. Her PhD work uncovered a novel role of the plant hormone auxin in regulating a critical aspect of plant architecture – oblique growth angles in secondary organs. She then did a short postdoc at the University of Chicago, USA investigating innate immunity in plants through characterisation of protein-protein interactions *in planta* with a heavy focus on bioimaging. Through this work, she discovered a newfound special interest in live cell imaging and image analysis, techniques that are especially useful for her current work on understanding the molecular basis of plant gravitropism. In her spare time she enjoys sharing her research with different audiences and the general public in the form of articles, seminars, outreach events and through her blog 'Plantasia' (https://www.plantasia.online).