Laboratory Exercise

An Undergraduate Study of Two Transcription Factors That Promote Lateral Root Formation

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Abstract

We present a lab that enables students to test the role of genes involved in the regulation of lateral roots growth in the model plant *Arabidopsis thaliana*. Here, students design an experiment that follows the effects of the hormone auxin on the stimulation of genes involved in the formation of lateral root initials. These genes, known as lateral organ boundary domain containing protein (*LBD*) genes, are upregulated in the presence of auxin as part of a multistep molecular and biochemically controlled pathway. Depending on which *LBD* gene is tested, and the stage of root development, expression patterns are localized in a discrete and punctate fashion at the site of lateral root initials (*LBD33*), or reveal a broader localization pattern (*LBD16*). Students view expression using the reporter gene *GUS* (beta-glucuronidase). Before GUS staining, students view root growth in a "pseudo-aseptic" agar-based environment that allows complete visualization of whole root development to determine the proper stage to test molecular expression. © 2014 by The International Union of Biochemistry and Molecular Biology, 42(3):237–245, 2014.

Keywords: *gene expression; molecular biology; plant biochemistry; signaling pathways*

Background

Root function is the "other half" of plant studies, but its educational utility is often neglected simply because roots go unnoticed—they are usually underground. This is an unfortunate oversight as molecular root development is a key area of research given our dependence on plant products. Furthermore, root systems are ideal for teaching developmental molecular biology in plants. In seed plants with a "closed type" of root meristem organization (such as *Arabidopsis*), a given cell can be traced back to its original progenitor cell near the root apex [1] allowing investigators to determine key transcriptional events that precede the formation of given tissues. Molecular reporter genes are ideal for these kinds of studies in *Arabidopsis* for undergraduates to study molecular developmental biology.

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Designing experiments that examine molecular root development is a key step in understanding plant function. Lateral root formation provides an excellent phenomenon for students to conduct gene expression studies in the laboratory. In *Arabidopsis* seedlings, young lateral roots have little pigment and therefore serve as an accessible system to view gene expression in whole mount. Green fluorescent protein and GUS are used as reporters that are easily viewed in lateral roots by students. GUS analysis in *Arabidopsis* has been used in the teaching lab to measure the cold-inducible *COR1a* promoter, and students can examine its molecular expression in response to environmental stress of their design [2]. GUS produces a stable, easily viewed signal and can be used to identify expression in a cell- or tissue-specific fashion in the root for marker studies and also gives an opportunity for students to learn about the use of plant transformation techniques using the Ti plasmid/*Agrobacterium* system [3]. With a variety of promoter::GUS lines available from the *Arabidopsis* research community, and in particular the Arabidopsis Biological Resource Center [4] and the Centre for Plant Sciences at The University of Leeds [5], students can explore gene behavior in response to various developmental and environmental signals in different organs and tissues.

The formation of lateral roots shapes the architecture of plant root systems. Appearing soon after germination,

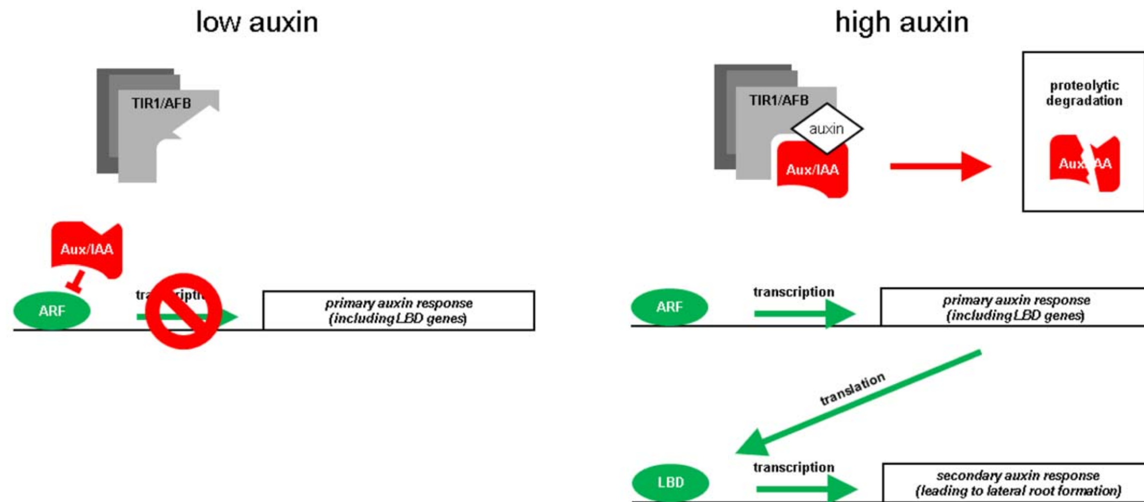

FIG 1 *Molecular and biochemical steps involved in auxin-induced formation of lateral roots. Auxin facilitates the interaction between the TIR1 F-Box protein (TIR1/AFB) of an E3 ubiquitin ligase complex and the Aux/IAA transcriptional repressors leading to proteolytic degradation of Aux/IAA proteins. Degradation of Aux/IAAs frees ARF to activate transcription (primary response) of transcription factors involved in lateral root formation (secondary response), including several LBD genes. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]*

lateral roots are formed from the emerging root via asymmetric, anticlinal divisions in the cell layer immediately outside the vasculature, the root pericycle cells, which lies adjacent to the protoxylem [6]. It is this division of the pericycle founder cells which gives the first morphological event signaling the formation of a lateral root primordium [7]. The molecular mechanisms behind lateral root formation are currently an active area of investigation in the model organism *Arabidopsis,* where data show that initial stages of cell division are preceded by changes in gene expression. Lateral root formation is dependent on signaling downstream of the hormone auxin [6].

Auxin is a reliable tool that has been used previously in biology teaching labs as a trigger for plant responses [8, 9]. As a growth regulator, auxin is the key hormone involved in patterning of molecular events to establish root formation [10]. As shown in Fig. 1, this pathway is initiated through auxin-mediated degradation of Aux/IAA transcriptional repressors [11] via the TIR1 F-Box protein of the E3 ubiquitin ligase complex [12]. High auxin levels lead to ubiquitination of the Aux/IAA proteins and consequent degradation via the proteasome. Because Aux/IAA proteins bind and inhibit auxin response factor (ARF) transcription factors, their degradation frees ARFs to activate transcription of downstream secondary transcription factors involved in lateral root formation, including several *LBD* genes [13].

In this lab, we show that auxin is an effective teaching tool to study gene regulation of lateral root initiation. We develop a lab that uses auxin as a simple tool to induce expression of two genes, *LBD16* and *LBD33*, implicated in the formation of lateral roots [14].

The Concept Behind the Lab

The lab begins by illustrating the usefulness of reporter constructs in transgenic systems to study gene expression. In this lab, several reporter constructs are used to indicate promoter activity in transgenic *Arabidopsis*. In plants a commonly used reporter is the bacterial enzyme GUS. Typically, *GUS* is used to indicate promoter activity by fusing it downstream of the promoter of interest. The construct is stably integrated into the genome of the plant. When the promoter is activated, the *GUS* mRNA is produced, which is then translated to form the GUS enzyme, which converts the exogenously applied substrate X-Gluc (5-bromo-4-choloro-3-indolyl β-D-glucuronic acid) into a blue precipitate. Hence, one can view the local activity of a given gene if its promoter is fused to *GUS*.

For this lab, several promoter::GUS constructs are utilized as viewed graphically in Fig. 2:

1. *pCYCLINB1::GUS*: Here, the *CyclinB1* [15] promoter has been fused upstream of the coding sequence for GUS. The *CyclinB1* promoter is upregulated in actively dividing cells. Students are asked to consider where in plant organs one would expect to see actively dividing cells. The site of lateral root formation would be one such site.
2. *pLBD16::GUS*: *LBD16* is active in tissue involved in the formation of lateral roots. This gene functions in the initiation and emergence of lateral roots and operates genetically downstream of *ARF7* and *ARF19* as a direct

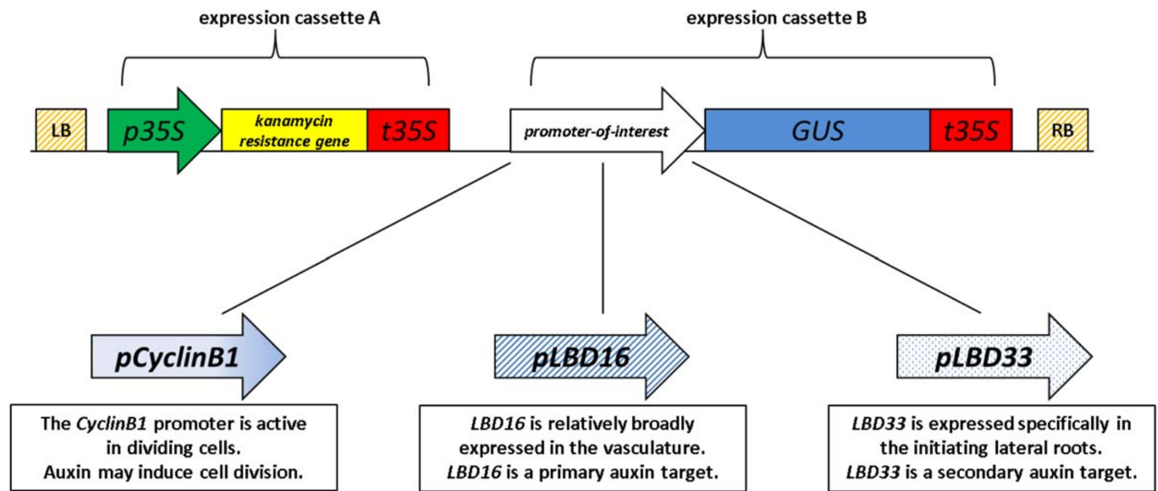

FIG 2 *Reporter-gene constructs. A schematic representation of the transgenic reporter-gene construct used to make the GUS lines. The left border (LB) and right border (RB) sequences are used by Agrobacterium tumefaciens to insert the construct into the plant genome. The construct contains two expression cassettes. Cassette A is for the selection of transgenic plants and consists of a promoter that is active in all tissues (p35S) followed by a gene coding for antibiotic resistance, and sequence that terminates transcription (t35S). Cassette B contains the reporter gene (GUS) in front of which we can insert our promoter of interest (pCyclinB1, pLBD16, or pLBD33); the reporter gene is also followed by a terminator sequence. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]*

target. When the *LBD16* gene is perturbed in *Arabidopsis*, fewer lateral roots form [14]. Lateral root formation is induced in the presence of auxin, hence in *pLBD16::GUS* lines, the blue product of X-Gluc hydrolysis should be apparent in the zone where lateral roots will form or be forming.

3. *pLBD33::GUS*: *LBD33* is also active in tissues involved in the formation of lateral roots and similarly is activated downstream of ARFs, but as an indirect target. It is closely related to *LBD16* based on homology. The most intriguing characteristic regarding *pLBD33::GUS* expression is that it precisely matches the site of where the very first cell division events occur that initiate the formation of lateral roots [14].

During the discussion of plant transgenic reporter genes, students are also given the opportunity to learn about some of the different molecular methods that are used to genetically transform plants. This includes a discussion regarding how to:

1. identify a gene's promoter
2. clone this promoter upstream of a reporter gene to make a reporter construct
3. transform the reporter construct into *Arabidopsis* (via *Agrobacterium*)
4. identify transformed plants using an antibiotic resistance marker
5. analyze the transgenic plant for GUS expression to localize gene activity

This lab begins by asking students to learn and understand the steps involved in the auxin-activated gene expression pathway regulating lateral root formation, and where *LBD* genes might be activated in this pathway. This may be their first experience exploring a gene activation circuit and must therefore be explained clearly. The pathway reveals blocks and activation points so students can conceptualize how a gene network operates by learning the protein-level and transcriptional regulators involved in the formation of lateral roots (Fig. 1).

During class discussion of this regulatory network, students can be asked to consider what happens to *LBD* gene expression when auxin is added (Fig. 1). As they move through the pathway they should expect auxin to induce the degradation of Aux/IAA proteins, which in turn would relieve their repression of ARF function. ARFs, including ARF7 and 19, consequently would activate the primary auxin response, which includes several *LBD* genes. In their own hands, students can then test for upregulation of *pLBD16::GUS* and *pLBD33::GUS* expression in relation to increasing concentrations of exogenously added auxin. Another parameter they can test is the effects of differing periods of auxin exposure on *LBD* regulation.

Axenic Technique Without a Flow Hood: Arabidopsis Plants in Culture

A helpful technique used in this lab is that the plants can be grown on agar in Petri dishes with sterile medium. Typically, root studies in the teaching lab have been hampered by the need for plants to be grown in soil, where one must contend with a myriad of subterranean variables, including the effects of unseen microorganisms, to the tearing of root

material during extraction of the plants from soil. However, plants grown on agar with MS (Murashige and Skoog salts) medium provide the students with a complete view of plant growth and development, with no infection or damage to the roots. The critical element for this method is to omit any supplemental carbon source in the agar medium, such as sucrose, to limit fungal and bacterial growth during the experiment.

In the lab, students induce lateral root initiation by the addition of exogenous auxin to the MS medium on which the seedlings are grown. Students can vary the concentration of hormone or the time of exposure to the hormone. Students design the parameters of this experiment choosing among different treatment variables including time and concentration. The lab runs over several sessions. Students then collect images of their samples to visualize and quantify the effects of auxin on gene expression at the site of lateral root initials (the progenitor cells that will give rise to the lateral root). *An important goal of this lab is for students to have control over as much of the experimental design as possible*.

In Class Testing

This lab module was tested in two different NYU undergraduate classes: Principles of Biology Introductory Laboratory (POB), composed of mainly first-year students, and Genetics and Genomics Lab, composed mostly of juniors and graduating seniors who are majors in the Biology Department. POB is a requirement for all biology majors and by a large number of prehealth students in other departments, most of who aspire to medical- or health-related career. Some students also will later apply to graduate school for a degree in education or to earn a Ph.D. Genetics and Genomics students are all biology majors and take this course to satisfy their "at-the-bench" lab course requirement.

This protocol was run for the POB lab in the fall of 2010, 2011, and 2012, and for the Genetics and Genomics lab in the Spring of 2011 and 2012. Labs were composed of 15–20 students divided into five groups (each group sitting at a research bench) with up to four students. Each group worked as a "scientific" team, pooling data from tests on different plant transgenic genotypes.

Lab Module Origin and Instructor Information and Logistics

This lab-teaching module was designed by Professor Eric D. Brenner, Assistant Clinical Professor in the Biology Department at NYU. The lab was designed also in conjunction with Dr. Kenneth Birnbaum in the Biology Department at NYU, and a post-doc in his lab, Dr. Bastiaan Bargmann (currently in the Biology Department at UC San Diego). *LBD* reporter lines were kindly provided by Hidehiro Fukaki of the Biology Department at Kobe University, Kobe Japan.

This class was taught by Dr. Brenner and has also been taught by POB TAs who are either NYU Ph.D. candidates or Masters students in the biology department. The course has been taught as a module consisting of four consecutive laboratory periods. It has been taught for one lab period per week for four consecutive weeks and it has been taught during two lab periods in a week for two consecutive weeks. In the latter case the lab was held on Tuesdays and Thursdays. All labs are 2 hours and 45 minutes long. It is to be noted that seeds need to be plated at least 5–7 days in advance of the auxin treatment step to ensure that the seedlings are at the right stage of development where the plant has produced a solitary primary root.

Methods

Preliminary Laboratory Preparation

Accumulation of Seed

Seeds of the transgenic constructs are available from the Arabidopsis Biological Resource Center (Ohio State University, Columbus) [4]. Seeds can be requested individually using the following accession number: CS68141 (*pLBD16::GUS*), CS68142 (*pLBD32::GUS*), and CS68143 (*pCyclinB1::GUS*). The seeds can be ordered collectively as an "education kit" with the single following accession number: CS19993.

A successful lab begins with a good batch of seed. That is, seed that is new (collected within the previous year) and grown under environmentally healthy conditions. Otherwise, germination can be aberrant—interfering with the analysis. Although growing *Arabidopsis* is relatively easy, a few comments are important here to ensure seed with high germination efficiency and uniform growth habit. Plants should be grown with high output, cool white fluorescent bulbs, or other suitable light source for cultivating *Arabidopsis*. In our hands, we cultivated *Arabidopsis* for seed production on Metro Mix 360 (soil purchased from Sungro Horticulture). Plants were bottom watered with Miracle Grow fertilizer. This additional fertilizer was critical for producing healthy plants. Seeds must be collected from plants that are allowed to dry after fruit production (in our hands 1 month) so that the siliques (the fruiting structure) turn brown. We habitually test our seeds before use to ensure near uniform germination.

Plant Tissue Culture in a Nonsterile Environment

Plants were cultivated on sterile 1× MS medium (Sigma) in 0.8% Bacto Agar (Difco/Fisher) containing 0.1% MES hydrate (Sigma) at pH 5.7 with 0.1 M KOH. There is no carbon source (sucrose) in the growth medium. Plants manufacture their own carbon molecules via photosynthesis and utilize original food stores in the seed. Omitting carbon from the medium greatly assists in suppressing microbial growth during culture. Thus, seedlings can be germinated on sterile plates *en masse* for large numbers of students—relieving any need of a sterile flow hood. Medium is poured into disposable square tissue culture plates (24 cm × 24 cm).

Seed Sterilization
More than 60 *Arabidopsis* seeds are sterilized by submersion in Clorox (5% sodium hypochlorite) for 4 minutes with periodic mixing in a microfuge tube. In bleach, seeds do not settle quickly and must be pelleted using a 4-minute spin in a microcentrifuge at 10,000 rpm. The bleach is removed and the seeds are washed in sterile water four times. In water, seeds will settle to the bottom of the microfuge more readily than in bleach so that centrifugation after each wash may not be necessary.

LABORATORY 1. *Students initiate the experiment by plating seeds on nutrient agar medium: Time involved: 45 minutes to 1 hour*

Seed Germination
We dispense our seeds on germination medium using a "seed pipetter" made from the bulb of a sterile transfer pipette that has been secured to a p1000 or p200 pipette tip with parafilm (Fig. 3). After seeding the plates were wrapped in parafilm and kept cool at 4°C for 24 h. Typically, 60 seeds of a single genotype are plated onto each plate divided into three rows, each containing 20 seeds. Plates are grown vertically so that roots are mostly aligned. Root development is uniform and visible with brilliant resolution under a dissecting scope, where root hairs, lateral root initials, and lateral root branches can be seen.

GUS expression genotypes (Fig. 2):

- Columbia (wild type)
- pCyclinB1::GUS [15]
- pLBD16::GUS [14]
- pLBD33::GUS [14]

LABORATORY 2. *Prepare hormone induction medium Time involved: Approximately 2 hours*

Students choose experimental parameters: How many seedlings to use per treatment? How long should the auxin treatments be? What auxin concentrations? In our hands we use the synthetic auxin analog, the herbicide 2,4-D (Sigma), because it is simple to work with owing to stability (can be autoclaved). We presume that other auxins, both synthetic and native, can be used.

Step 1: Calculate dilutions

After 5–7 days of growth, seedlings are removed using forceps, or a dissecting needle, and transferred to hormone induction medium that the students have prepared.

Students often struggle with calculating dilutions. We emphasize to the students that calculating dilutions is an important component of laboratory research. Thus, to remediate this issue, as part of this experiment, students have a chance to calculate a simple dilution series. To begin, students are first given the effective concentration range (1 nM–20 μM) where auxin has been shown to affect molecular gene expression. Students then consider the available sample size (number of plants germinated that are healthy from each genotype). If they decided to, for example, create four dilutions (three different auxin concentrations and a negative control) then they must divide the total number of healthy plants of a given genotype by 4. If they have, for example, 40 healthy plants in a genotype, then that can use 10 plants per treatment on this genotype. The following procedure reveals a simple dilution plan that provides an auxin concentration series that covers the effective auxin concentration range. Concentrations are diluted by 1/100 × steps—beginning with 10 μM and ending with 1 nM. In some cases students may choose to use concentrations outside the effective (as in Fig. 5*e* where a concentration as low as 100 pM 2,4-D is utilized).

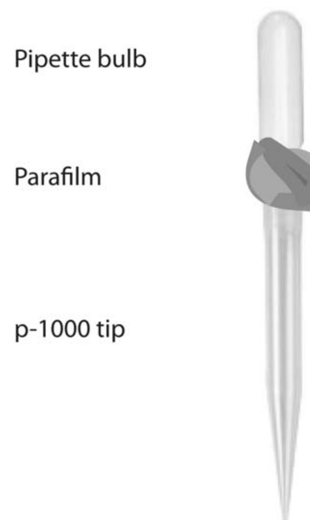

FIG 3 *The* Arabidopsis *seed pipetter.*

Once the students have completed their calculations, their instructor checks over the dilution calculations and approves it when correct. The most significant part of this exercise is that students learn to design their own treatments. We have found that a homework assignment on dilutions is essential to prepare students *prior* to the lab. We emphasize the importance of learning dilutions for their future careers in biology, such as medicine, where proper preparation of dilutions can even be a matter of life and death.

One example of a dilution series we have used follows:

- Beginning with a 2 mM auxin stock (that is prepared before the lab begins), students prepare a series of 100× dilutions creating 20 μM, 200 nM, and 2 nM 2,4-D in 5 mL of sterile water in a square Petri plate (100 × 15 mm); 5 mL of molten 1.2% agar is added to the square Petri plates and mixed (quickly) with each of the 5 mL auxin dilution to create 10 μM, 100 nM, and 1 nM 2,4-D in 0.6% agar.

- For the first dilution, 50 μL of 2 mM auxin stock is mixed into 5 mL of water to make 20 μM auxin. This first dilution can be in a separate tube or even in the square Petri plate that will be used for growth.
- From the first dilution 50 μL is removed and added to 5 mL of water to make 200 nM auxin.
- From this second dilution 50 μL is then added to 5 mL of water to make 2 nM.
- Eighty mL of 1.2% agarose is made by the students in the microwave (note here, it is helpful to use a large flask, say 1 L, and to microwave at low power, or with intermittent pulses, so as not to create a frothy overflow of agar during heating).
- Five mL of the 1.2% agar is transferred to the square Petri plates and mixed (quickly) into each of the 5 mL auxin dilution.
- Five milliliters water is similarly mixed into 5 mL 1.2% agar to create 0.6% water agar to be used as the auxin negative control.
- The agar/auxin solution quickly cools and solidifies so that it is now ready to use in the next step.

Step 2: Transfer plants onto auxin medium

Students mark the bottom of the media plates with a permanent marker dividing it into sectors. Plants of different genotypes are delicately transferred to a given sector with forceps. Because plants are grown on agar the roots are not damaged during transfer. Plates are sealed with parafilm and then incubated for 2–7 days upright. Note, one can also use circular plates. Also note, filter paper can be used in place of agar. Plants are grown upright, but also can be grown flat.

LABORATORY 3. *Staining for promoter activity*
Time involved: approximately 1 hour

Students transfer their auxin-treated plants into prelabeled microfuge tubes. The substrate, 1 mM X-Gluc (Rose Scientific) in 50 mM Na_2HPO_4, pH 7.0, and 0.1% Triton X-100 [2] is then added to the Eppendorf tubes. Just enough X-Gluc buffer is added to fully immerse the plants. The plants are incubated overnight and then stored at 4°C until the next lab. Note, although the substrate, X-Gluc is rather expensive, only small amounts are required. Also note, the X-Gluc buffer used in this lab has omitted ferrocyanide and ferricyanide, as it was deemed unnecessary in our hands. Unused X-Gluc buffer can be frozen and refrozen over months, if not years, and used later.

LABORATORY 4. *Clearing tissues and imaging results*
Time involved: 2–3 hours

Students remove and dispose the GUS staining buffer and replace it with a series of increasing concentrations (15, 30, 50, and 70%) of ethanol using a transfer pipette over a 1-hour period with each wash taking no more than 10 minutes. The seedlings are then fixed in a final solution of 85% ethanol and 15% glycerol. The seedlings are placed on a Petri dish and imaged in this solution. The stained and fixed seedlings can be stored indefinitely, as they may want to reexamine the GUS staining pattern when they write up their results. Stained seedlings are carefully placed onto clear Petri dishes. The organs are carefully spread out and examined under a dissecting microscope. Addition of extra ethanol/glycerol solution to the sample may help improve imaging. If time is limited, then this lab can be stopped at any of the wash stages and imaged later. Note, during imaging, sample preparation requires gentle nudging and general manipulation of the plants. Alignment of the plants side-by-side helps students view the staining patterns better.

Imaging

Two methods are typically used to capture image data. The first method was developed *ad hoc* by the students, where pictures of their stained plants were captured using a smartphone. The camera window of the smartphone is aimed directly through the ocular of the microscope and a picture taken. The second method was one set up for the students at NYU. Here, we used a Canon Rebel XS camera attached to dissecting stereomicroscope (Zeiss Stemi SV6) via a T-mount adapter (model T2–9) (Martin Instruments) linked to SLR camera adapter (model MM-SLR) (Martin Microscope) camera to scope adapter. The camera is attached to an Apple iMac computer and data captured and processed with the EOS Utility software associated with the camera.

Results

Morphology and Expression

Discerning students will notice a pattern of morphological variation on all plants in the presence of changing auxin concentrations. As the auxin concentration increases there is initially an increase in the number of lateral root initials (typically from 2 to 200 nM), and their general morphology becomes unusually swollen. At the highest concentration, in this case 20 μM, lateral root emergence is inhibited.

A technical goal of this lab is for students to observe the effects of auxin on *LBD* gene expression and the corollary events of lateral root formation. Below are images from several students who have performed this lab. Typically, students will generate a composite series of panels displaying their data of GUS staining—attempting to emulate what is typically found in an academic journal. Two images showing GUS expression, produced by two different students, are shown in Figs. 4 and 5 and described below. In Fig. 4 it is noted that the positive control used to test for activation in dividing cells, *pCyclinB1::GUS*, reveals upregulation at the site of dividing cells, including lateral roots, similar to the expression pattern of *pLBD33::GUS*. However, *pLBD33::GUS* is only found in lateral roots, whereas *pCyclinB1::GUS* expression is

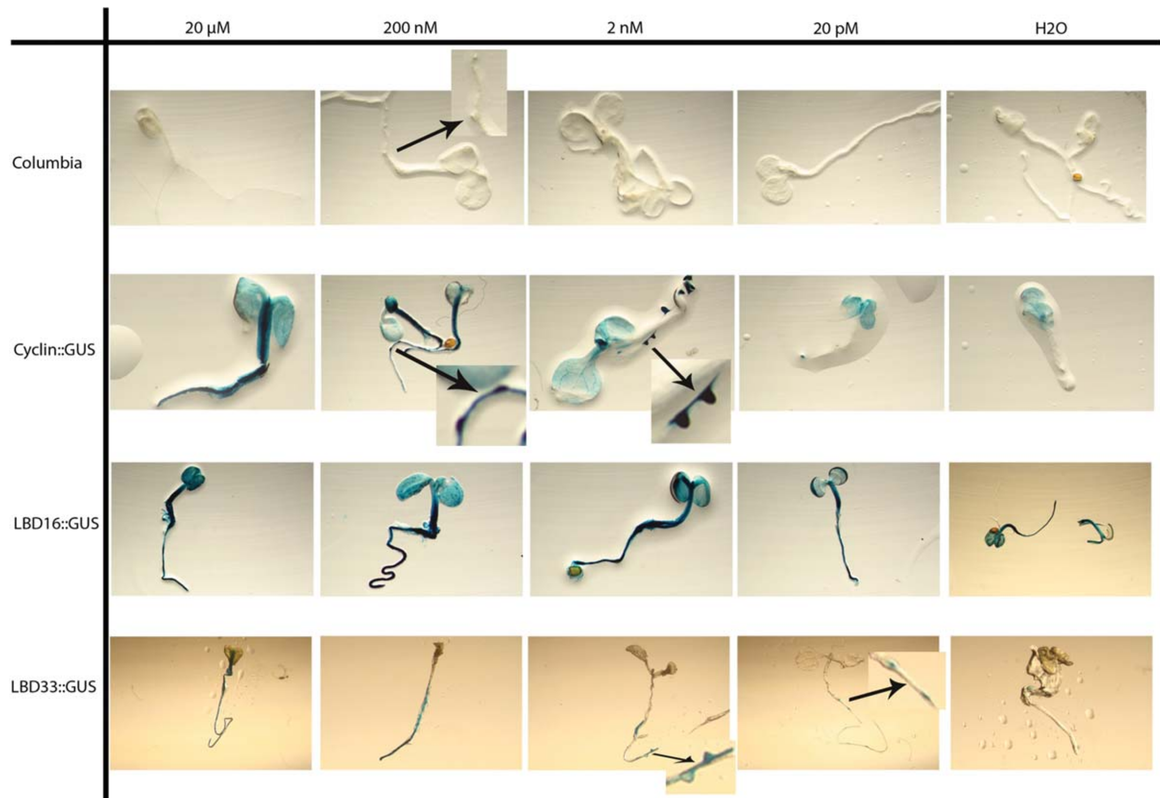

FIG 4 *Data gathered by a student to show the relative effects of increasing auxin concentrations on the promoter activity of genes involved in lateral root formation,* pLBD16::GUS *and* pLBD33::GUS. *The negative control, wild-type Columbia, shows no GUS expression regardless of auxin concentration. The positive control,* pCyclinB1::GUS, *expression is found in tissues with actively dividing cells, including lateral roots. Note, at higher auxin concentrations, lateral roots appear overly developed (2 and 200 nM), where auxin inhibits lateral root elongation.*

observed in all dividing tissues including aerial tissues. Expression of *pLBD16::GUS* is more widespread, indicating that it may play a role in other tissues besides roots. In Fig. 5, expression of *pLBD33::GUS* can be viewed in more detail at the site of lateral initial formation and developing lateral roots.

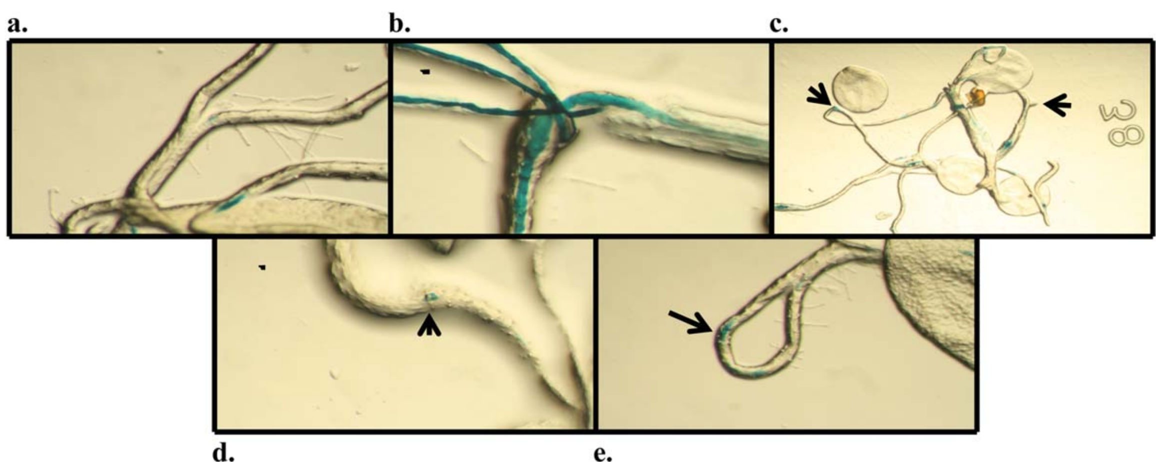

FIG 5 *pLBD33::GUS expression. (a) No 2,4-D control: areas of staining correspond to emerging LR site; (b) 10 µM 2,4-D; (c) 100 nM 2,4-D: lateral root initials indicated by arrows; (d) 1 nM 2,4-D: arrow indicates lateral root initial; (e) 100 pM 2,4-D: arrow indicates area of LBD33 activity.*

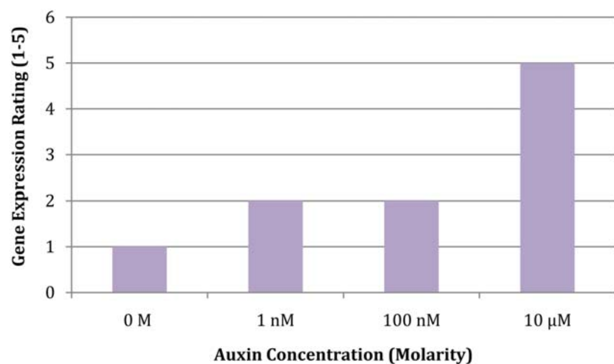

FIG 6 *Qualitative view of gene expression in* pLBD16::GUS *in response to increasing concentrations of auxin.* Arabidopsis *plants were germinated on MS medium for 1 week and then treated for 1 week with different concentrations of 2,4-D. Qualitative estimates regarding the levels of expression were given as relative units on a scale from 1 to 5. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]*

Controls

Expected outcomes reveal that the negative control, wild-type Col-0, as expected, has no GUS staining, regardless of concentration of auxin present. The positive control, *pCyclinB1::GUS*, reveals staining localized in rapidly dividing tissue which can be found in both the root and the shoot. In the primary root and in lateral root primordia, GUS stain is present in untreated plants. As auxin levels increase, GUS levels also increase as cell division is induced. Lateral roots also appear to show increased levels of cell division along with decreased levels of cell elongation leading to rather prominent, blue protuberances, as shown in the magnified images in panels containing 2 and 200 nM auxin-treated *pCyclinB1::GUS*. At the highest auxin concentration 20 μM, nearly all tissues and organs in the *pCyclinB1::GUS* plants stain blue.

pLBD::GUS *Expression Lines*

In the transgenic lines, *pLBD16::GUS* expression is relatively more intense than *pLBD33::GUS*, even in the absence of exogenous auxin. Additional auxin causes even greater GUS expression, as shown for *pLBD16::GUS* (Fig. 6). In the *pLBD33::GUS* line, a more subtle expression pattern is observed. For control plants grown in the absence of exogenous auxin, expression is restricted to the lateral root initials (and the primary root) (Figs. 4 and 5). With increasing levels of auxin, expression becomes more intense and accumulates in the enlarged protuberances where lateral roots are being formed. Higher auxin levels in *pLBD33::GUS* cause GUS expression to appear in other sites in the root besides lateral root primordia, or possibly additional lateral roots are being formed coupled with GUS expression.

Discussion

We present here an undergraduate lab that helps students explore the expression of two genes important for the formation of lateral roots. We use auxin as a simple tool to induce expression of two genes, *LBD16* and *LBD33*, encoding transcription factors implicated in the formation of lateral root initials [16], and then measure their expression with the GUS system. In this process, students learn about the importance of lateral roots to the subterranean plant architecture, as well as the recently uncovered network of genes that are involved in the formation of lateral roots.

Training Pitfalls

From previous experience, certain concepts tied to this lab tend to present a challenge to students. The first is dilutions. It is important that students come to lab with a prepared exercise to help determine planned concentration variations; otherwise, time will be lost in the explanation. Another aspect that students find challenging is the concept of a "promoter reporter-gene expression system." We presume this is difficult because of the multistep/multidimensional aspect of the process. For one thing, students are just beginning to gain a perspective of how the genome is structured and how it functions. It therefore becomes important to explain the process of cloning including how: 1) to make a transgenic construct; 2) to transform this construct into plants; and 3) to test for expression of the reporter gene. To help prompt students to make these connections, we navigate the students carefully through each stage of the transgenic process, beginning with the design of a promoter::GUS construct, to transforming this construct into *Agrobacterium*, followed by transformation of the construct into *Arabidopsis*. We discuss where in the genome the construct might land, and how its genomic position might affect expression, that is, hot spots of expression versus sites of reduced expression. We also mention that more than one insert can reside in the plant genome and ask them to ponder how multiple inserts might affect expression.

Data Interpretation

Once the data have been collected we ask students to consider the following questions:

- In what organs do they see expression?
- In what tissues do they expect to see expression?
- Does (would) treatment duration have an effect on expression?
- Does auxin concentration have an effect on expression?
- Can one quantify changes in gene expression?
- How does staining in the *pCyclinB1::GUS* positive control compare to the *pLBD::GUS* patterns? (A good microscope will show that *CyclinB1* is expressed in dividing cells at the site of lateral root initial formation, whereas

pLBD33::GUS is also expressed in these dividing cells, but in a more diffuse fashion. Furthermore, *pLBD33::GUS*, unlike *pCyclinB1::GUS*, is also found in nondividing cells adjacent to the lateral root initials.)

- Why does *pLBD16::GUS* have a much stronger expression pattern than *pLBD33::GUS*? Given that these are closely related genes that belong to the same gene family, what aspect of the transcriptional cascade might cause expression patterns and intensities to diverge? Also, why is *pLBD16::GUS* expressed in other plant organs besides roots, including aerial organs (when it was originally considered to be a gene involved in lateral root formation)?
- Data in the literature indicate that *LBD16* contains an ARF-binding motif in its promoter and is a direct target of ARFs. *LBD33*, alternatively, appears to be an indirect target of ARFs. One might then ask the students how one could test if other *LBD* genes are direct or indirect targets of ARFs. (For example, one could assess *LBD* transcript levels—by using qPCR, Northern blot analysis, or microarrays in the presence of a translational inhibitor such as cycloheximide, similar to the studies conducted on *LBD16* and *LBD33* by Okushima *et al*. [14]).

These questions are useful to serve as a prompt for a writing assignment that allows students to report their results and interpret their meaning. We ask them to make this report using standard scientific writing style.

Student learning outcomes

A targeted outcome of this lab is for students to develop the conceptual ability to design and execute experiments to study reporter gene expression during lateral root development in a genetic model system in the lab.

In so doing students gain:

- Technical skills:
  - display proficiency cultivating and manipulating *Arabidopsis* in culture.
  - test a reporter-gene system in plant roots.
  - display proficiency working with plant hormones to activate gene expression.
- Conceptual skills.
  - design an experiment to determine the effective concentration of auxin on gene expression using reporter-gene analysis.
  - understand the current scientific knowledge regarding the molecular controls over lateral root initiation in *Arabidopsis*.
  - consider the methods used to understand gene promoter function via plant transgenesis.

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