Video Article

Lateral Root Inducible System in *Arabidopsis* and Maize

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Abstract

Lateral root development contributes significantly to the root system, and hence is crucial for plant growth. The study of lateral root initiation is however tedious, because it occurs only in a few cells inside the root and in an unpredictable manner. To circumvent this problem, a Lateral Root Inducible System (LRIS) has been developed. By treating seedlings consecutively with an auxin transport inhibitor and a synthetic auxin, highly controlled lateral root initiation occurs synchronously in the primary root, allowing abundant sampling of a desired developmental stage. The LRIS has first been developed for *Arabidopsis thaliana*, but can be applied to other plants as well. Accordingly, it has been adapted for use in maize (*Zea mays*). A detailed overview of the different steps of the LRIS in both plants is given. The combination of this system with comparative transcriptomics made it possible to identify functional homologs of *Arabidopsis* lateral root initiation genes in other species as illustrated here for the *CYCLIN B1;1* (*CYCB1;1)* cell cycle gene in maize. Finally, the principles that need to be taken into account when an LRIS is developed for other plant species are discussed.

Video Link

The video component of this article can be found at http://www.jove.com/video/53481/

Introduction

The root system is crucial for plant growth, since it ensures anchorage and uptake of water and nutrients from the soil. Because the expansion of a root system mainly relies on the production of lateral roots, their initiation and formation have been widely studied. Lateral roots are initiated in a specific subset of pericycle cells, called founder cells[1]. In most dicots, such as *Arabidopsis thaliana*, these cells are located at the protoxylem poles[2], whereas in monocots, such as maize, they are found at the phloem poles[3]. Founder cells are marked by an increased auxin response[4], followed by expression of specific cell cycle genes (*e.g., CYCLIN B1;1* / *CYCB1;1*), after which they undergo a first round of asymmetric anticlinal divisions[5]. After a series of coordinated anticlinal and periclinal divisions, a lateral root primordium is formed that finally will emerge as an autonomous lateral root. The location and timing of lateral root initiation are however not predictable, since these events are neither abundant nor synchronized. This impedes the use of molecular approaches such as transcriptomics to study this process.

To tackle this, a Lateral Root Inducible System (LRIS) has been developed[6, 7]. In this system, seedlings are first treated with *N*-1-naphthylphthalamic acid (NPA), which inhibits auxin transport and accumulation, consequently blocking lateral root initiation[8]. By subsequently transferring the seedling to medium containing the synthetic auxin 1-naphthalene acetic acid (NAA), the entire pericycle layer responds to the elevated auxin levels thereby massively inducing lateral root initiating cell divisions[6]. As such, this system leads to fast, synchronous and extensive lateral roots initiations, allowing easy collection of root samples enriched for a specific stage of lateral root development. Subsequently, these samples can be used to determine genome-wide expression profiles during lateral root formation. The LRIS has yielded already significant knowledge about lateral root initiation in *Arabidopsis* and maize[9-13], but the need to apply this system to other plant species becomes more apparent as more genomes are sequenced and there is an increasing interest to transfer knowledge to economical important species.

Here, the detailed protocols of the *Arabidopsis* and maize LRISs are given. Next, an example of the use of the system is provided, by illustrating how transcriptomics data gained from the maize LRIS can be used to identify functional homologs that have a conserved function during lateral root initiation across different plant species. Finally, guidelines to optimize the LRIS for other plant species are proposed.

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Protocol

1. *Arabidopsis* LRIS Protocol

Note: The text refers to "small" or "large" scale experiments. Small scale experiments, such as marker line analysis and histological staining[6, 14], require only a few samples. Large scale experiments, such as quantitative real-time qRT-PCR, micro-arrays[9-11] or RNA sequencing, require a larger amount of samples. As such, an amount of ~1000 seedlings per sample was used by Vanneste *et al.*[11] to perform microarray experiment after root segment dissection.

DAY 1

1. **Sterilization of *Arabidopsis* Seeds**
   Note: Select one of the following procedures for seed sterilization. Any of them is suitable for the next steps of the protocol. Gas sterilization (1.1.2) presents two main advantages over liquid sterilization (1.1.1): it takes less time when handling a large number of seeds, since no pipetting has to occur for the individual samples; and the sterilized seeds can be stored for a longer period. However, it requires a desiccator and careful handling.
   1. **Liquid sterilization**
      1. Pour the desired number of seeds in micro-centrifuge tubes. Use up to 20 mg (~800 seeds) in a 1.5 ml tube or 40 mg (~1600 seeds) in a 2 ml micro-centrifuge tubes.
      2. Add 1 ml of 70% ethanol. Invert or gently shake for 2 min.
      3. Replace the 70% ethanol with 1 ml sterilization solution for 15 min. (Prepare fresh sterilization solution: 3.85 ml sodium hypochlorite (NaOCl) stock (12%) (CAUTION: Use fume hood), 5 µl Tween 20, up to 10 ml with water. Invert the tubes several times to make sure that all seeds come in contact with the solution.
      4. In a laminar air flow cabinet, replace the sterilization solution with 1 ml sterile distilled water and rinse the seeds 5 times for 5 min by repeatedly replacing with 1 ml of fresh sterile distilled water.

   2. **Gas Sterilization**
      1. Pour the desired number of seeds in a 2 ml micro-centrifuge tube. Use individual tubes when working with several independent lines, and arrange them opened in a plastic micro-centrifuge tubes box or holder. If the number of seeds in one tube exceeds 500 (12.5 mg), subdivide the seeds in the appropriate number of tubes to ensure successful sterilization. Make sure the caps of neighboring tubes do not cover each other. Fit together the lid of the box on the bottom, as it needs to be in the desiccator as well for sterilization.
      2. Place the box in the bowl of a desiccator, along with a glass beaker (CAUTION: Use fume hood).
      3. Fill the beaker with 100 ml of 12% NaOCl (CAUTION: Use fume hood). Put the lid on the desiccator, but leave a small aperture where the beaker is placed.
      4. Add 3 ml of 37% hydrochloric acid (fuming) (CAUTION: Use fume hood) to the beaker through the small aperture and quickly close the desiccator. The solution will bubble for some time due to Cl_2-gas formation. Leave the system closed O/N (or at least 8 hr).
      5. Remove the lid of the desiccator. Take out the box and cover it with the lid to avoid contamination during transport.
      6. Put the box in a laminar air flow cabinet, remove the lid and leave the seeds for about 1 hr at RT to allow gas release.
         Note: Seeds can be safely stored dry in closed micro-centrifuge tubes for up to 2 weeks at 4 °C.

2. **Lateral Root Induction in *Arabidopsis***
   1. **Lateral Root Inhibition (NPA Treatment)**
      1. Prepare growth medium for *in vitro* growth. The medium contains half strength Murashige and Skoog salt mixture[15], 0.1 g/L myo-inositol, 10 g/L sucrose, and is buffered with 0.5 g/L 2-(N-morpholino)ethanesulfonic acid (MES).
      2. Adjust the pH to 5.7 with 1 M KOH. Add 8 g/L of plant tissue agar and autoclave the medium for 20 min at 121 °C.
      3. Prepare a 25 mM stock solution of NPA in dimethylsulfoxide (DMSO) (CAUTION: Use gloves). In a laminar air flow cabinet, cool the autoclaved medium down to approximately 65 °C (which is the temperature at which the bottle can be held by hand), and add 25 mM NPA stock solution to obtain a final concentration of 10 µM NPA.
      4. Homogenize upon addition by gently shaking the bottle for 10 sec. Pour 50 ml of medium per square petri dish (12 cm x 12 cm).
         Note: In case of large scale experiment, apply a nylon mesh (20 µm) to ensure easy transfer. Prepare a mesh (9 cm x 9 cm) for autoclaving (**Figure 1A**). When autoclaved, apply the mesh to the growth medium using sterile tweezers (**Figure 1B**). Gently push the mesh to the growth medium using a sterilized drigalsky to make sure it is in good contact with the growth medium (**Figure 1B**) (CAUTION: Work in sterile conditions).
      5. Sow the seeds on the NPA-plates.
         Note: In case a large scale experiment is planned, sow 2 dense and well-aligned rows of 50 seeds to facilitate the sampling (see 1.2.2.3).
         1. In case of liquid sterilization, sow the seeds on NPA-plates using a 200 µl pipet. Cut off 3 - 4 mm of the end of the pipetting tips in sterile conditions (or before sterilization of the tips) in order to be able to pipet the seeds. Pipet up and down a couple of times to re-suspend the seeds, then pipet approximately 150 µl sterile water with 10 - 20 seeds and wait till the seeds sediment at the bottom of the tip. (CAUTION: Work in sterile conditions)
            Note: When touching the agar or the mesh gently with the tip, a drop of water with a seed will be released from it (**Figure 1C**).
         2. In case of gas sterilization, sow the seeds using autoclaved toothpicks.Pinch the toothpick into the agar before taking the seeds to ensure a sticky surface. Pick up the seeds one by one using the toothpick and distribute the seeds on the plates

(**Figure 1D**). Alternatively, add sterile water to the seeds, and sow as described in 1.2.1.5.1 (CAUTION: Work in sterile conditions).

6. Seal the plates with breathable adhesive tape and store them plates at 4 °C in the dark for at least 2 days and maximum one week. This is needed for stratification and ensures synchronized and more efficient germination.
   Note: Alternatively, seeds can be stratified before sowing, immersed in distilled water in micro-centrifuge tubes, which requires less refrigerator space. In this case, store the tubes for at least one week at 4 °C, and move the plates to the growth cabinet immediately after sowing (see 1.2.1.7).
   DAY 3
7. Move the plates to the growth cabinet (continuous light (110 µE m^{-2} s^{-1}), 21 °C) for 5 days (2 days of germination and 3 days of growth) in a nearly vertical position (80 to 90 °) to ensure root growth on, and not in the medium.
   DAY 8

2. **Lateral Root Induction (NAA Treatment)**
   1. Prepare the same growth medium as described in 1.2.1.1 and 1.2.1.2. Prepare a 50 mM stock solution of NAA in DMSO (CAUTION: Use gloves). Cool the autoclaved medium down to 65 °C and add NAA solution to the medium to obtain a final concentration of 10 µM NAA (CAUTION: Work in sterile conditions).
      1. Homogenize upon addition by gently shaking the bottle for 10 sec. Pour 50 ml of medium per square petri dish (12 cm x 12 cm).
   2. Transfer the seedlings from the plates containing growth medium supplemented with 10 µM NPA to those supplemented with 10 µM NAA.
      1. Hook the arms of curved tweezers under the cotyledons of the seedling and gently remove it from the plate. Only transfer seedlings of which the roots have grown entirely in contact with the NPA-growth medium and preferably downwards.
      2. Skim the root over the NAA-containing growth medium surface over a small distance. Make sure that the plant roots are in good contact with the growth medium. If needed, press the root gently to the growth medium surface with the tweezers.
         Note: In case of a large-scale experiment, remove all seedlings that are not in contact with the mesh and transfer by lifting the mesh at the two upper corners with tweezers. Make sure the mesh is in good contact with the NAA-containing medium by skimming the mesh over the agar surface over a small distance (**Figure 1E**).
   3. Seal the new plates with breathable tape and place them in the growth cabinet (continuous light (110 µE m^{-2} s^{-1}), 21 °C) in a vertical position for the desired time after induction until sampling.
      Note: In case of a large-scale experiment, use a scalpel to cut the root segments all at once directly on the mesh and sample them by gently scraping the surface of the mesh.

2. LRIS Maize Protocol

1. **Stratification of Maize Kernels**
   1. Incubate the maize kernels at 4 °C in the dark for at least 1 week.
      Note: This protocol has been developed using the B73 maize inbred line.

2. **Sterilization of Maize Kernels**
   DAY 1
   Note: Although maize seedlings do not have to be grown in sterile conditions, it is recommended to sterilize the maize kernels and work with sterile material to prevent fungal growth in the paper roll system.
   1. Put the necessary number of maize kernels in a sterilized glass beaker.
   2. Add 100 ml of sterilization solution (6% NaOCl in water) (CAUTION: Use fume hood).
   3. Add a magnetic stir bar and place the beaker on a magnetic stirrer at low stirring speed (approximately 250 rpm) at RT for 5 min.
   4. Rinse five times for 5 min by replacing the solution with 100 ml of fresh sterile water.

3. **Sowing Maize Kernels**
   1. Put on gloves to reduce the risk of contamination, and take a roll of paper towels.
   2. Tear off two stretches of hand towel paper with a total length of two sheets (approximately 92 cm x 24 cm) and place them on top of each other on a clean surface (**Figure 2A**).
   3. Fold the sheets double over the length (approximately 92 cm x 12 cm) (**Figure 2A**).
   4. Use tweezers to distribute 10 kernels at approximately 2 cm from the top over the entire length of the paper, while keeping an interspace of 8 cm between each kernel and 8 cm free at both ends (**Figure 2B**). Make sure the radicle of the kernel is facing down and toward the paper (**Figure 2B**).
   5. Gently roll the paper over the length, while keeping the seeds in place (**Figure 2B**). To facilitate the rolling, it is optional to first spray the paper with sterile water.
   6. Put the paper rolls in sterilized glass tubes of approximately 6 cm diameter and 14 cm height (*e.g.,* 250 ml centrifuge tubes) and place them in a rack (**Figure 2C**).

4. **Lateral Root Induction in Maize**
   1. Prepare a 50 mM NPA stock solution (dissolved in DMSO) (CAUTION: Use gloves).
   2. For each tube, make a 50 µM NPA solution by adding 125 µl from the 50 mM NPA stock solution to 125 ml sterile water in a sterilized glass beaker.

3. Mix well and pour 125 ml of the 50 µM NPA solution over the paper roll in the tube. The paper roll will absorb the solution and become completely soaked.
   Note: When using paper rolls and tubes with other dimensions, adjust the volume accordingly. The liquid should reach halfway the tube to ensure sufficient uptake.
4. Place the paper roll system in a growth cabinet for three days (27 °C, continuous light, 70% relative humidity). Make sure that the paper rolls remain soaked by adding extra 50 µM NPA solution, since the solution evaporates over time (**Figure 2D**).
   DAY 4
5. Prepare a 50 mM NAA stock solution (dissolved in DMSO) (CAUTION: Use gloves).
6. For each tube, prepare a 50 µM NAA solution by adding 125 µl from the 50 mM NAA stock to 125 ml sterile water in a sterilized glass beaker.
7. Gently squeeze out most of the remaining NPA solution from the paper rolls and place them in sterile water for 5 min to wash it out (CAUTION: Use gloves). Gently squeeze out most of the water from the paper rolls. Repeat this washing step three times.
8. Mix well and pour the 50 µM NAA solution over the paper rolls in the tubes. Make sure they are completely soaked.
9. Place the paper roll system in the growth cabinet (27 °C, continuous light, 70% relative humidity) for the desired duration after induction.

5. **Lateral Root Induction in Adventitious Crown Roots of Maize**
   Note: The LRIS as described above induces lateral roots in the primary root. However, in maize and other monocotyledons, the primary root and the embryonic seminal roots, together with their lateral roots, are mainly important during seedling development. At a later stage in plant growth, the post-embryonic adventitious roots arise on the stem and also develop lateral roots to form a new root system[16]. The LRIS can also be used to induce lateral roots on these post-embryonic adventitious crown roots by following some minor adjustments to the LRIS on the embryonic primary root.
   1. To make the paper rolls, create a sandwich of papers with respectively two layers of hand towel paper on the outer side, and two layers of germination paper within. Place the sterilized kernels (see steps 2.1 to 2.2) in between the two sheets of germination paper. Germination paper, compared to hand towel paper, will be less prone to be penetrated by root hairs and lateral roots, which will make the opening of the paper rolls easier later on. On the other hand, the two external layers of hand towel paper will facilitate absorption of the liquid.
   2. Insert the rolls in 700 ml glass tubes and pour sterile water without NPA over them. Make sure they are completely soaked.
   3. Germinate the sterilized kernels in the growth cabinet (see step 2.4.9).
   4. Grow the seedlings till the adventitious crown roots start to emerge (6 days for B73). Make sure the paper rolls are kept wet at all times by adding sterile water when necessary.
   5. Transfer to a 25 µM NPA solution for 4 days (see steps 2.4.1 to 2.4.4), followed by a similar induction of lateral root initiation by replacing the NPA solution with a 50 µM NAA solution after a washing step (see step 2.4.5 to 2.4.9).

Representative Results

Application of the LRIS to Perform Comparative Transcriptomics of the Lateral Root Initiation Process

One application of the LRIS is the comparison and correlation of gene expression profiles during lateral root formation in different species. Comparative transcriptomics approaches create the possibility to pinpoint orthologous genes involved in the lateral root development process in different species. Lateral root initiation, which consists of the formation of a new organ from a subset of cells contained in an already formed root axis, is the major mechanism shared by angiosperms to control their root architecture. Consequently, it is very likely that it evolved from existing pathways present in a common ancestor and was conserved throughout evolution. Indeed, common potential regulators of lateral root initiation have been found in different species[17, 18].

Sampling Material using the LRIS in *Arabidopsis* and Maize, and Transcriptome Analysis

The LRIS has been established in two different species: *Arabidopsis* and maize[10, 13]. In both species, it was decided to sample the plants just before NAA treatment, and shortly after induction, at the onset of, or during the auxin response, as well as at the onset of, or during the first divisions in the pericycle. Furthermore, Fluorescence Activated Cell Sorting[19] (FACS) was used in *Arabidopsis* or Laser Capture Microscopy[20, 21] (LCM) in maize to select for pericycle cells. In *Arabidopsis*, the time points of 2 and 6 hr after induction were chosen based on the transcriptional characterization of the *pDR5::GUS* auxin response and *pCYCB1;1::GUS* cell cycle marker lines[9] **(Figure 3)**. In maize, the time points 2, 3 and 4 hr after induction, were selected after microscopic characterization of cell division activity and the analysis of several cell cycle marker genes expression using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)[13]. RNA was extracted from the isolated cells and hybridized on microarray platforms as previously described[10, 13].

Finding the Ortholog of *ArabidopsisCYCB1;1* (*AtCYCB1;1)* in Maize

In *Arabidopsis*, the marker line *pCYCB1;1::GUS* has been widely used to track the first divisions of the pericycle during lateral root initiation. Such marker would be very useful in maize. Several homologs of *AtCYCB1;1* were found in maize (www.maizesequence.org, all peptides, BLASTP, default settings). Six of them were present on the microarray performed after LRIS in maize and showed significant enrichment. The best BLAST hit, *GRMZM2G310115*, showed very high transcription levels after LRIS, similar to what was observed in *Arabidopsis* for *AtCYCB1;1* **(Figure 4)**.

Application of the LRIS to Check Individual Gene Expression

To validate *GRMZM2G310115* as a good candidate ortholog of *AtCYCB1;1* in maize lateral root initiation, a real-time qRT-PCR on samples taken at different time points was performed during the course of an LRIS[13]. Plants were treated as described in the above-mentioned protocol and harvested at different time points: before NAA treatment (NPA), and after 2, 3 and 4 hr of NAA treatment. Also, material of plants grown only in water was harvested to compare gene expression between LRIS and neutral conditions. Immediately after harvesting, root segments corresponding to a region comprised between 5 mm and 15 mm above the root tip were dissected under the binocular. Using tweezers, the cortex was separated from the stele (which contains the pericycle), and RNA was extracted from both tissues. This last step was performed instead of LCM, because it is much faster and cheaper for validation. Using the following respective forward and reverse qRT-PCR primers, AGCAGGACGCAGTTGGAGAG and GAGCCGAGAGCACAGAAGAAAG, *GRMZM2G310115* was validated to be up-regulated upon LRIS, and to be specific for the stele tissues **(Figure 5)**. Additionally, this experiment shows that without synchronous induction, the discrete events of lateral root initiation happening in a root growing in water are not detectable, and illustrate the need of the LRIS to reveal differential gene expression related to the process of lateral root initiation.

Figure 1. Lateral Root Inducible System for *Arabidopsis*. (**A**) Preparing the nylon mesh (20 µm): cut the nylon mesh (9 cm by 9 cm), wrap it in aluminum foil and put it in a glass beaker for autoclaving. (**B**) Apply the nylon mesh on an NPA-containing plate (10 µM) using tweezers. Then use a sterile drigalski in order to eliminate air bubbles. (**C**) Sowing seeds on the nylon mesh using a pipet. (**D**) Sowing seeds on the nylon mesh using a toothpick. (**E**) Transfer the nylon mesh to an NAA-containing plate (10 µM) using tweezers. Please click here to view a larger version of this figure.

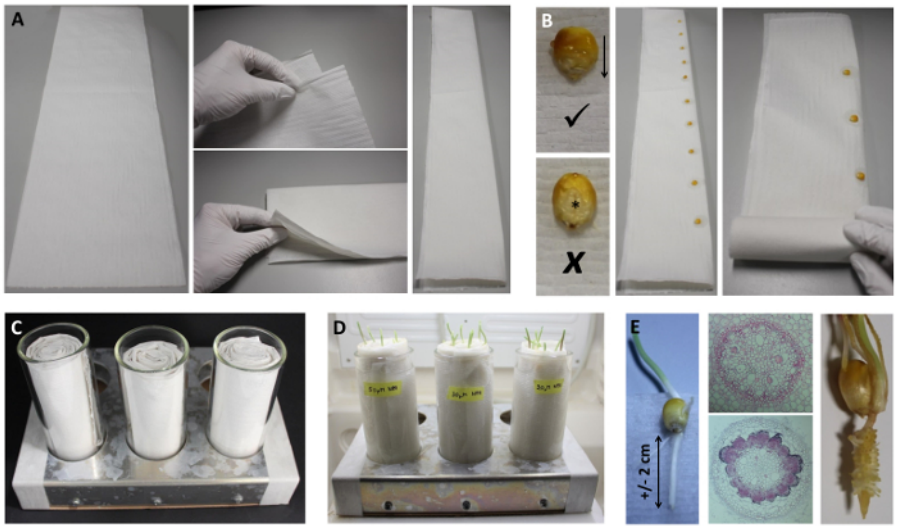

Figure 2. Lateral Root Inducible System for Maize. (**A**) Preparing the paper rolls: place two layers of paper (92 cm x 24 cm, length of two sheets) on top of each other and fold them double over the length (92 cm x 12 cm). (**B**) Put 10 sterilized maize kernels with the radicle facing down on the paper at 2 cm from the top with an interspacing of 8 cm. Then roll up the paper while keeping the maize kernels in place. (**C**) Place the paper rolls in tubes (*e.g.,* 250 ml centrifuge tubes) and put them in a rack. (**D**) Grow the maize seedlings for three days in a 50 µM NPA solution (at 27 °C, continuous light, relative humidity 70%). (**E**) Left: seedling just before transfer to NAA; Middle: microtome transversal sections just before transfer to NAA and 2 days after transfer to NAA; Right: seedling with visible emerged lateral roots 5 days after transfer to NAA. Please click here to view a larger version of this figure.

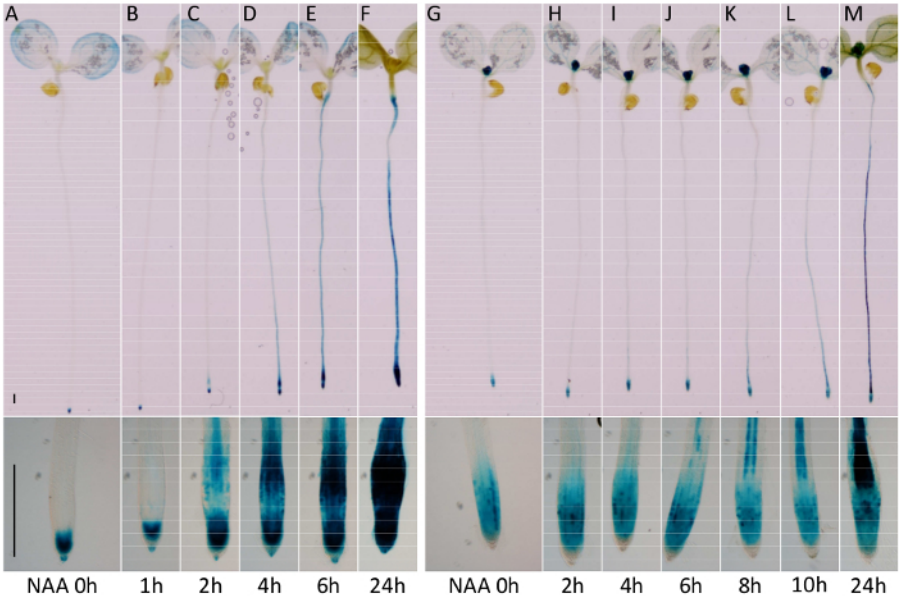

Figure 3. Lateral Root Initiation is Stimulated Upon Prolonged NAA Treatment. (**A-F**) Time course showing the auxin response during an NAA treatment using the *pDR5::GUS* marker line. The auxin response, which is one of the first events triggering lateral root initiation, starts 2 hr after the start of the NAA treatment. In the upper panel, an overview of the whole seedling is given; the lower panel shows the auxin response in the root tip. (**G-M**) Time course of an NAA treatment using the *pCYCB1;1::GUS* marker line. The GUS signal represents the expression of the *CYCB1;1* gene, indicating that the first cell divisions leading to lateral root formation start 6 h after the start of the NAA treatment. In the upper panel, an overview of the whole seedling is given; the lower panel shows *CYCB1;1* expression in the root tip (GUS staining according to Beeckman and Engler, 1994[22]). Please click here to view a larger version of this figure.

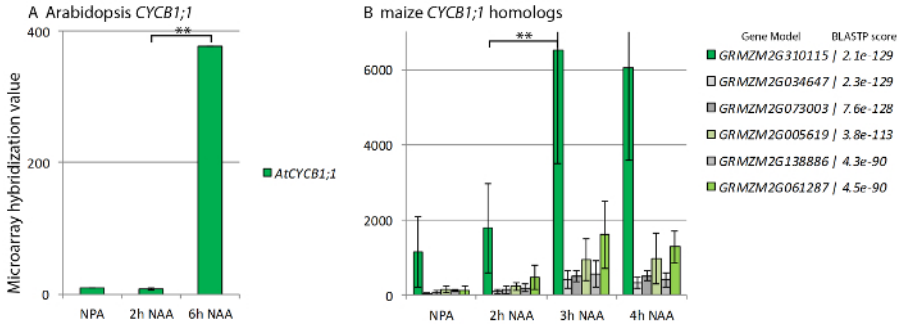

Figure 4. Expression Profile of *CYCB1* Genes in *Arabidopsis* and Maize during LRIS. (**A**) Microarray expression values of *AtCYCB1;1* during LRIS as described by De Smet *et al*. 2008[10] (**B**) Microarray expression values of potential orthologs of *AtCYCB1;1* during LRIS as described by Jansen *et al*. 2013[13] BLASTP score is the score obtained when blasting the protein sequence of *AtCYCB1;1* on the maize genome. Error bars express standard deviation and ** stands for a *p*-value ≤0.01. Please click here to view a larger version of this figure.

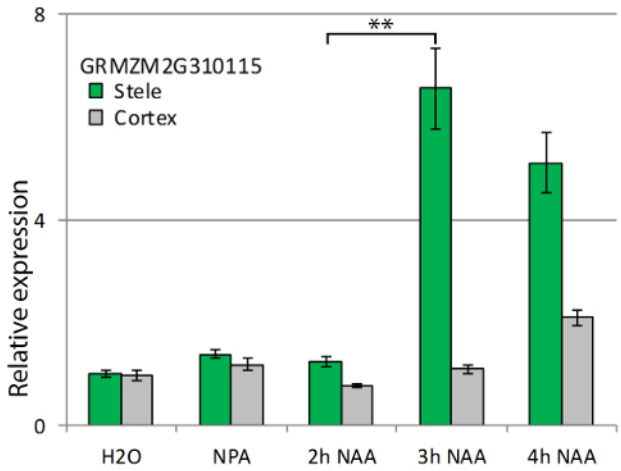

Figure 5. Expression of *GRMZM2G310115* in the Stele and the Cortex of Maize Roots during LRIS. The expression of *GRMZM2G310115* during LRIS in maize was evaluated by quantitative real-time qRT-PCR on dissected stele and cortex samples. A supplementary sampling was performed on plants grown on water. Values were normalized for expression in the stele in water. Error bars express standard deviation and ** stands for a *p*-value ≤0.01 (primers and reference genes according to Jansen *et al*. 2013[13]). Please click here to view a larger version of this figure.

Discussion

In the *Arabidopsis* LRIS protocol, it is important to only transfer the seedlings that have grown entirely in contact with the NPA-containing growth medium. This ensures that lateral root initiation is blocked over the entire root length. In order to prevent wounding the plantlets during transfer, the arms of the curved forceps can be hooked under the cotyledons of the seedling. Upon transfer, make sure that the seedling roots are in sufficient contact with the NAA-containing agar medium. This can be achieved by skimming the root over the agar surface over a small distance. This will ensure an efficient synchronized induction of the lateral root initiation over the total root length. The roots can be grown exposed to light without negatively affecting their growth and lateral root induction.

In the maize LRIS protocol, it is important that the radicle of the kernel faces down and toward the paper to ensure the root will grow downwards and attach to the paper at the correct side[16]. After three days of NPA treatment, the seedlings should have emerged from the kernel with a small shoot, a primary root and seminal roots[16]. The primary root should be approximately 2 cm long before proceeding to the induction of lateral root initiation. This protocol has been developed using the B73 maize inbred line, but in case of a maize line with a different growth rate, adjust the incubation time accordingly. Make sure that the paper rolls remain soaked by adding regularly 50 µM NPA solution when the liquid vaporizes over time, otherwise NPA treatment could be inefficient and unwanted early lateral root development could occur. The system itself prevents exposition of the roots to the light, but light is not a major issue and doesn't affect root growth and lateral root induction.

Alternative ways for controlled induction of lateral roots are the use of mechanical bending[23] or gravistimulation[24]. The main advantage of these systems is that they have a more 'natural' induction compared to the hormone treatments in the LRIS, but the disadvantage is that they are limited in the amount of material that can be harvested at a given time point because they only yield one lateral root initiation event in the bend per seedling compared to a full induction of the pericycle in the LRIS.

The LRIS used in *Arabidopsis* and maize can be used in a variety of plants, though favorable conditions need to be optimized. To install an LRIS, two important successive steps have to be achieved: (1) blocking of auxin transport and (2) accumulation of auxin to induce lateral root initiation. The growing system should allow for efficient and uniform uptake of the compounds and should permit good development of the seedlings. Alternative systems to solid medium (*Arabidopsis*) and paper rolls (maize), such as liquid culture, hydroponics, or aeroponics, could be used for other species. The first stage in the LRIS, *i.e.,* the blocking of the auxin transport, can be achieved by adding an auxin transport inhibitor.

Although NPA leads to an efficient block of lateral root initiation in *Arabidopsis* and maize, alternative compounds such as 2,3,5-triiodobenzoic acid (TIBA) or *p*-chlorophenoxyisobutyric acid (PCIB) might work better in other species. A similar optimization can be done for the second step of the LRIS, *i.e.,* the auxin treatment. The synthetic auxin NAA seems to be the most suited for an LRIS. The auxin precursor indol-3-butyric acid (IBA) might be a good alternative as it also strongly induces lateral roots and has a high bioactivity in different plants[25, 26] . On the other hand, the natural auxin indole-3-acetic acid (IAA) is less stable and more easily metabolized[27], rationalizing its weaker effect on root development in for example maize or rice[25, 26]. The synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4D) accumulates highly in pericycle cells, partially because it is not exported from the cells[28], impairing proper lateral root initiation and inducing artificial fused structures. Also other compounds interacting with auxin pathways, such as naxillin[12], have been shown to induce lateral root initiation in *Arabidopsis* and can be tested in other species.

In some cases, germination is inefficient in the presence of NPA and one might choose to first germinate the seeds in the absence of NPA, to subsequently transfer the seedlings to the NPA-containing system. This induces a possible risk of having early lateral root primordia initiated before lateral root induction with NAA treatment and, hence, it is important to only sample the region of the root that elongated during NPA treatment. Following this general strategy, one should be able to optimize an LRIS for practically any (seed) plant and as such have an easy system to study lateral root development for the plant of interest. Further characterization of the timing of the lateral root initiation can occur via marker lines, as exemplified for *Arabidopsis*. If marker lines are difficult to obtain, a detailed histological study could be done, but this is time-consuming and the first cell divisions are not easily recognizable. Alternatively, expression analysis of cell division markers can be used to indicate the timing of the first cell divisions.

The LRIS can be used for different purposes, such as transcriptome analysis[9-13] as briefly described in the results, as well as histological observations at the macroscopic and microscopic level during lateral root initiation[14]. Different types of sampling, ranging from organ to cell scale[29], might allow unraveling different aspects of the lateral root initiation. In addition, the LRIS can be used to monitor the effect of different compounds on the lateral root initiation[12, 30]. Finally, by using reporter lines, a LRIS can also be used to easily characterize the gene expression and/or protein localization during lateral root development.

Disclosures

The authors have nothing to disclose.

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