Developmental Genetics of Root Hair Patterning in *Arabidopsis thaliana*

The purpose of this lab activity is to study gene regulatory networks that affect root hair patterning. Specifically, we will examine how defects to genes that regulate root hair spacing affect the expression of the *GLABRA2* reporter.

Plants extract water and critical nutrients from soil through their roots. Root hairs are tubular root projections that increase surface area and thus provide greater exposure to the soil, enabling more efficient uptake. The regular spacing of root hairs is critical to their activity, and plants control root hair patterning via several mechanisms. In *Arabidopsis*, positional information within the root epidermis dictates root hair outgrowth, and many of genes encoding proteins necessary for transmitting this information have been well characterized [1].

Root hair cell fate determination occurs in the growing tip of the root, also known as the root apical meristem. The *Arabidopsis* root consists of concentric rings of tissue, and root hairs arise from the epidermal layer, which is external to the cortical layer (Figure 1). The epidermal layer produces two cell types: root hairs, which arise from H-cells, and non-root-hair cells, called N-cells. H-cells form mainly from those epidermal cells that touch two different cortex cells simultaneously[2].

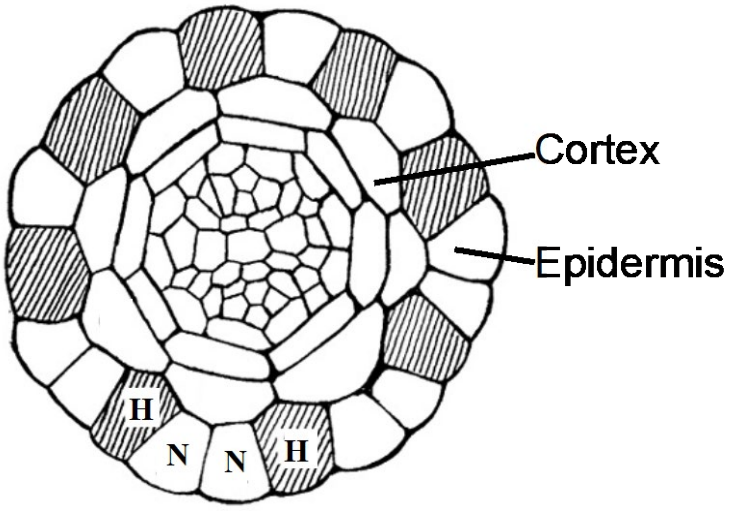

Figure 1 Transverse section through an *Arabidopsis* root. The cortex is internal to the epidermis. The H-cell position within the epidermis is in contact with two cortical cells, and is the normal site of root hair outgrowth. H-cells are shaded. The unshaded cells of the epidermis are in the N-cell position. (Adapted from [2])

Several genes have been identified that affect the positioning of the root hairs in *Arabidopsis*. *GLABRA2* (*GL2*) encodes a homeodomain transcription factor that is expressed specifically in N cells of the epidermis and normally represses the formation of root hairs [3]. Recombinant DNA techniques were used to fuse the 4 KB upstream of the *GL2* gene from *Arabidopsis* to the *GUS* gene from *E. coli*, and this lab-made piece of DNA was transformed into otherwise normal *Arabidopsis* plants. The *GUS* gene encodes β-glucuronidase, an enzyme that cleaves glycosidic bonds in multiple substrates [4]. Since plants do not have detectable levels of native β-glucuronidase activity, the *GUS* gene serves as an excellent gene expression reporter in *Arabidopsis* [5]. β-glucuronidase converts the colorless substrate X-Glc (5-bromo-4-chloro-3-indolyl glucuronide) into diX-indigo (5,5'-dibromo-4,4'-dichloro-indigo), an insoluble blue

pigment [5]. *GL2::GUS* reporter plants produce β-glucuronidase only in those cells that transcribe *GL2*. When these plants are exposed to X-Glc-containing GUS assay buffer, their N-cells turn blue [3].

There are multiple genes involved in the distribution of root hairs in *Arabidopsis*. Mutant plants that are homozygous for defects in the *WEREWOLF* (*WER*) gene produce too many root hairs, and thus aberrantly grow root hairs from the N-cell position [6]. Therefore, the *WEREFOLF* gene is a negative regulator of root hair fate. Mutants that are homozygous for defects in the *CAPRICE* (*CPC*) gene produce fewer root hairs than normal plants. In these mutants, there are epidermal cells in the H-cell position (touching two cortical cells) that fail to produce root hairs [7]. *CAPRICE* is a positive regulator of root hair fate. Mutants that are homozygous for defects in the *SCRAMBLED* (*SCM*) gene produce roots with root hairs and non-root hairs in randomized positions within the epidermis [8,9]. *SCRAMBLED* is thought to act in lateral inhibition of root hair formation between neighboring cells. See Figure 2 for images of the root hair patterns of each of these mutants compared to the wild type.

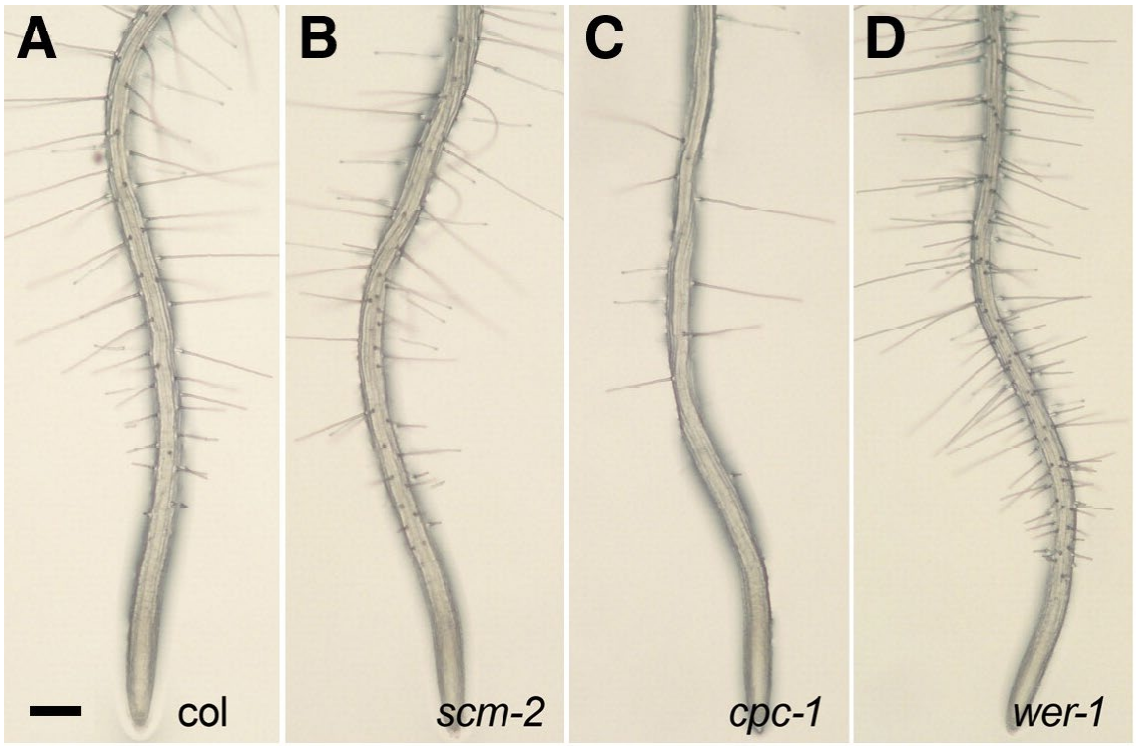

Figure 2 Root hair patterns in the wild type (col) and three mutants. Scale bar = 200 µM (from [8])

You have been given seeds representing four different *Arabidopsis* strains. All of these seeds are homozygous for the *GL2* reporter. The first strain you are given has the wild-type genotype. The other three strains are homozygous for the *wer* mutation, the *cpc* mutation, and the *scm* mutation. You will perform GUS assays on seedlings from each of these four strains and examine the patterns of blue cells in the roots. Based on the pattern you observe, determine which strain is the *cpc* mutant, which is the *wer* mutant, and which is the *scm* mutant.

Week 1:

You have been given four tubes of seeds. Your first objective is to sterilize the seeds, then you will plate them on MS medium.

A. Seed sterilization

1. Add 1 mL of 70% ethanol to each tube, and invert or vortex the tubes to ensure that all interior surfaces of the tubes and all seeds and have been exposed to the 70% ethanol. Do not let the seeds incubate in the 70% ethanol for more than 2 minutes.
2. Allow the seeds to settle to the bottom of the tube, then use a P1000 micropipetter to remove the ethanol to a liquid waste container.
3. Add 1 mL of seed-bleaching solution to each tube, and invert or vortex the tubes to ensure every seed is exposed. Do not allow the seeds to be exposed this solution for more than ten minutes. After five minutes, allow the seeds to settle to the bottom of the tube and use a P1000 pipetter to remove the solution.
4. Add 1 mL sterile water and invert the tubes to wash. Allow the seeds to settle, and remove the water.
5. Repeat the previous step two more times. After the last wash, add 1 mL of fresh sterile water to each of the tubes.

B. Seed Plating

6. You have been given 4 MS plates. Label each with your names, the date, and the strain number.
7. Using a P1000 micropipetter, withdraw the sterilized seeds in water and plate them one by one, keeping about 2-5 mm between each seed. It is easiest to plate a droplet of water with the seed suspended within it. You will find that the seeds settle quickly to the bottom of your pipette tip. It may be easier withdraw a subset of the seeds at a time, or to put the seeds back in their tube to resuspend them in the water in order to get enough separation between seeds to plate them into individual droplets.
8. Once you have plated all the seeds and they have settled onto the agar of the plate, withdraw as much of the extra water droplets from the plates as you can using the micropipetter, and dispense the extra water into the liquid waste container.
9. Allow plates to dry slightly ajar for ~20 minutes.
10. Seal plates with parafilm. They will be placed in a dark box at 4 °C for two days to vernalize before they are placed on their sides under 24-hour light to germinate.

Week 2:

This week you will do GUS assays on the four types of seedlings and examine them to study expression of the *GLABRA2* reporter.

1. Take four 1.5 mL Eppendorf tubes and label each with your name and the strain number.
2. Use the P1000 pipetter to place 250 µl of GUS assay buffer into each labeled tube.
3. Using tweezers, pluck at least five seedlings from the WT *GL2::GUS* plate and place them into the appropriate tube. Repeat with the other three strains of seedlings.
4. Place at 37 °C in a gentle rotator for 30-60 minutes. If no rotator is available, it is sufficient to manually invert the tubes every 10 minutes or so. During the incubation, draw the *GL2* expression pattern you expect to observe in *cpc*, *wer*, and *scm* mutants.
5. Place a droplet of water on a glass slide. Using fine forceps, remove the seedlings from the GUS assay buffer and place them on the slide with their roots in the water. Carefully lower a coverslip on top. Examine the seedlings using the 10X objective on a compound microscope. Draw the pattern you observe for a representative sample of each of the four strains.
6. Which strain represents the *wer* mutant, the *cpc* mutant, and the *scm* mutant? Why do you say so?

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Notes for the preparer:
Each team of students will require about 1 mL of GUS assay buffer, 4 mL of 70% Ethanol, 4 mL of GUS Assay buffer, 4 MS plates, and about 25 ml of distilled water.

GUS Assay buffer (10 ml total)

Mix together the following reagents. Aliquot and store GUS Assay buffer at -20 °C.

1000 µL	1M NaPO4 pH 6.8
200 µL	0.5 M EDTA pH 8.0
1000 µL	5 mM potassium ferricyanide
1000 µL	5 mM potassium ferrocyanide
50 µL	20% triton X-100
200 µL	20 mM X-Glc (RPI # B72200)
6.55 mL	distilled water

***Arabidopsis* seed bleaching solution (50 ml total)**

Mix together the regents below. Store at room temperature.

25 mL	7.5% Sodium hypochlorite solution (household bleach)
250 µL	20% Triton X-100
24.75 mL	distilled water

Murashige and Skoog (MS) plates (1 L= ~35 plates)

Place ~700 mL dI water in a 2L Erlenmeyer flask
4.4 g MS media (Sigma M0404)
pH to 5.7 using 1 M KOH
Add 7 g of agar
Add enough distilled water to bring final volume to 1000 mL.
Autoclave for 20 minutes at 121 psi and cool to ~60 °C.
Pour square plates, if possible. Store at 4 °C

Timing for seedling development is somewhat flexible. Seeds may be bleached at week 1, kept in the dark at 4 °C to vernalize for a week and plated at week 2. The GUS assay can then be done on seedlings at week 3. Using square plates is ideal because the plates can be easily balanced on one side, enabling the roots to grow on the surface of the agar instead of growing into the agar, which makes it easier for students to pull them out for the assay. If a microcentrifuge is available to students during the bleaching and plating, seeds may be pulsed between washes to pellet the seeds and remove the supernatant. This speeds up the sterilization/washing process, but it is not required.