Dynamics at Different Scales: Hormonal Control in *Oryza Sativa* Root Circumnutation and Gene Regulation in *Arabidopsis thaliana* Cell Differentiation

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Thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science in the
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ABSTRACT

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Abstract

This research spans multiple scales—from the entire organism, down to the genes that created it.

The first project, “Gene Dynamics in Tissue Development”, explores how stem cell differentiation depends on the dynamics of gene networks. In the *Arabidopsis thaliana* root, the SCARECROW (SCR) transcription factor is required for an asymmetric cell division of a stem cell, resulting in two daughter cells that acquire different fates and tissue identities. Although much research has developed the network topology for this division, the dynamics of this process remain unknown. A core feature of the GRN controlling this stem cell asymmetric division is the SCR positive feedback loop. This research develops a synthetic biology approach to systematically and precisely tune various dynamics of SCR protein accumulation. Thus, one can explore the role and function of this positive feedback loop in the developmental process of asymmetric division in the Arabidopsis root.

The following project, “Organ Form for Function” details how organ function depends on cellular form and hormonal signals. As sessile organisms, plants must establish a firm foundation into the terrain wherever the seed lands. Roots, especially the primary root (a seed’s first root), are the only anchor into the terrain. With a multiscale investigation, we identified a molecular pathway required for circumnutation, the circular growth of the root tip. We found the cellular physiology and key hormonal cell signaling events driving this behavior.

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List of Acronyms

Acronym	Full Term
RSA	root system architecture
TF	transcription factor
GRN	gene regulatory network
CEI	cortex-endodermal initial
CEID	cortex-endodermal initial daughter
CDS	coding DNA sequence
TALE	transcription activator-like effector
SCR	SCARECROW, plant transcription factor
SHR	SHORTROOT, plant transcription factor

1. Introduction

1.1 Introduction to Gene Regulatory Networks in Development (Project 1)

A fundamental challenge in biology is understanding how a single cell develops into a multicellular organism. Integral to this is understanding how a stem cell progresses to a specific cell fate from initiating to maintaining its differentiated identity. This process, known as cellular differentiation, is regulated by a network of transcription factors (TFs), which act within gene regulatory networks (GRNs). GRNs are a set of interacting genes that control a specific cell function. Understanding how GRNs coordinate events such as asymmetric cell division, fate specification, and tissue patterning is a leading question in developmental biology. One major challenge is determining which of the variables in the process must act precisely and which may operate loosely within a certain parameter space. Historically, this has been poorly understood in part due to difficulties in tracking developing cells, limitations in imaging, and a lack of methods to precisely control and tune GRN dynamics.

1.2 The Organization of *Arabidopsis* Root for Developmental Research

Arabidopsis is a well-established model organism with a small genome, considerable genetic resources, and translucent roots, which facilitate imaging (Fig. 1A). The *Arabidopsis thaliana* root is an ideal system to study the initiation and maintenance of differentiation, as one can examine every stage of cell development in a single organ (2000 Benfey). During animal development, cells are often mobile, which complicates cell tracking and tracing lineages. As plant cells are sessile, they cannot migrate. The tip of the root is the stem cell niche, containing stem cell populations called “initial cells.” Initials divide toward the direction of the shoot, displacing the root tip further into the

ground. Thus, one can view the root's developmental process in one cell file beginning with the stem cell niche and continuing shootward up to fully differentiated cells (Fig. 1B). Furthermore, tissues are arranged in concentric cylinders, giving symmetry as visible in a transverse section. Typically, development occurs in the three spatial dimensions (x, y, z) as well as the fourth dimension of time. However, we can capture all developmental stages of root cells at one time (Fig. 1B).

The root consists of three outer tissue layers arranged in concentric circles around the inner vasculature bundle (Fig. 1B). These tissues - the epidermis, cortex, and endodermis – are distinct tissue types with unique functions. The SCR transcription factor is necessary for proper development of the cortex and endodermal tissues, together known as the 'ground tissue' layer. The mature endodermis is involved in protecting the root from dehydration and may function in selective uptake and protection from pathogens (2015 Kamiya, 2015 Liberman). The cortex is used to store products from photosynthesis and helps in uptake of water and minerals. The endodermis is particularly appropriate for studying the molecular regulation of cell differentiation because the key players involved in regulating its earliest stages as well as its mature features have been described in detail (2000 Benfey).

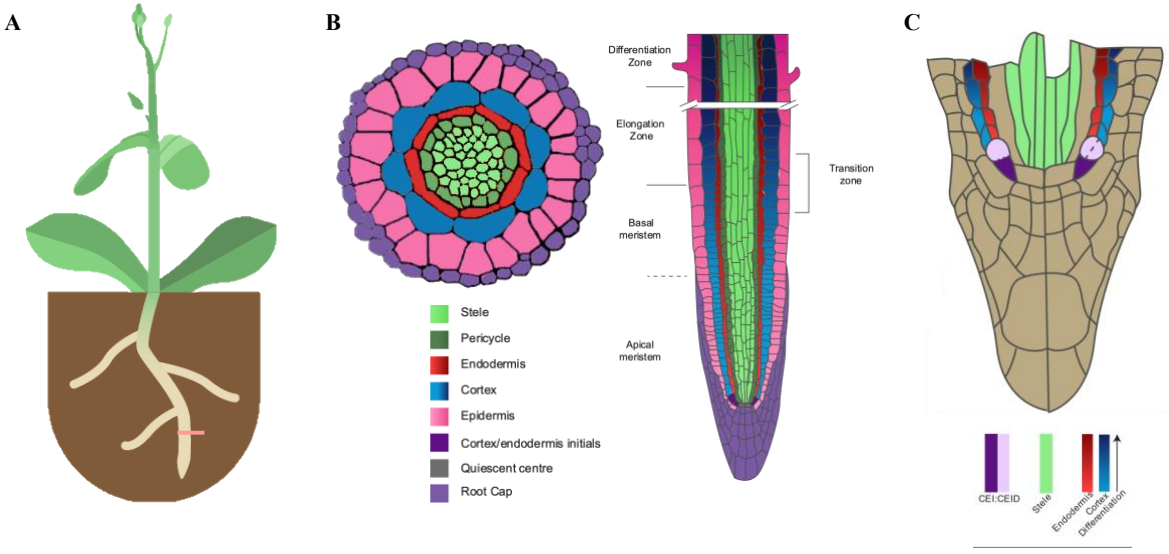


Figure 1. The *Arabidopsis* root as a model for developmental research. A) Arabidopsis plant, marked with a line indicating location of B) transverse section of root. Longitudinal root section (right) with cell types and developmental gradients. C) Depiction of a *Arabidopsis* root with first transverse division on left side of root forming CEI and CEID. The right side of the root illustrates the longitudinal division divisions leading to cortex and endodermis Tissues.

1.3 SCR is Essential for a Developmental Asymmetric Cell Division

In the *Arabidopsis thaliana* root, the SCARECROW (SCR) TF is required for an asymmetric cell division of a stem cell, resulting in two daughter cells that acquire different fates and tissue identities (Helariutta 2000, Laurenzio 1996). Although much research has developed the network topology for this division, the dynamics of this process remain unknown. A core feature of the GRN controlling this stem cell asymmetric division is the SCR positive feedback loop (2007 Cui). In this research, I used a synthetic biology approach so that I may systematically and precisely tune various dynamics of SCR protein accumulation. Thus, I can explore the role and function of this positive feedback loop in the developmental process of asymmetric division in the Arabidopsis root.

Throughout the study of developmental biology, organisms ranging from bacteria to mammals have continually been found to use asymmetric cell division as a strategy for generating cell diversity (1988 Hawkins). In plants, the cortex and endodermis tissues originate from a single stem cell, the "cortex-endodermal initial" (CEI) (Fig. 1C; 2000 Helariutta). This cell first divides transversely to replace itself and produce a CEI daughter (CEID) cell. This CEID cell subsequently undergoes a longitudinal asymmetric cell division (ACD) to generate distinct cortex and endodermis progenitors. Nearly 25 years of research have produced a detailed topology of the GRN regulating these asymmetric divisions, yet much remains to be discovered regarding protein dynamics and how information flows through the GRN to regulate tissue specification.

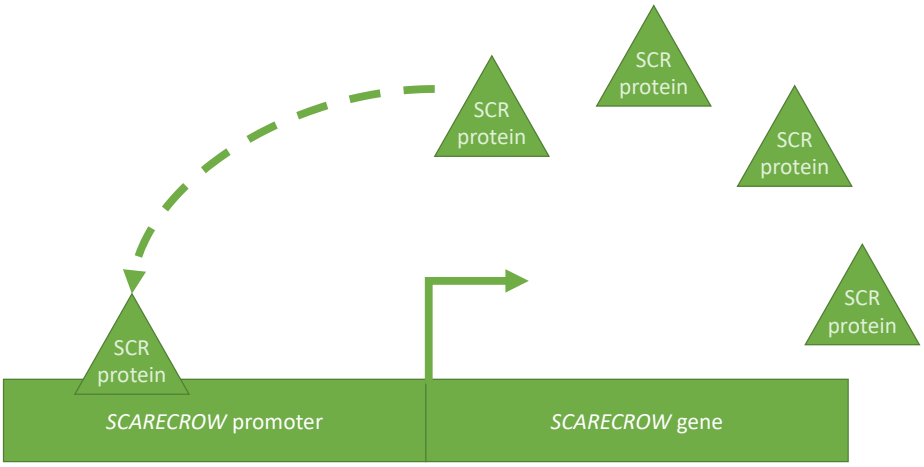


Figure 2. SCR Forms an Autoregulatory Positive Feedback Loop
Schematic of the SCR protein activating SCR transcription to form a positive feedback loop

Two transcription factors required for endodermis formation are SHORTROOT (SHR) and SCARECROW (SCR) (1996 Di Laurenzio, 2000 Helariutta). Our lab and others have identified the molecular mechanisms by which SHR and SCR promote the asymmetric cell division of the ground tissue progenitor cell, the CEID (2004 Heidstra,

2010 Sozzani, 2005 Wildwater). First, SHR is transcribed and translated in the vascular tissue at the center of the root. SHR protein then physically moves to the adjacent tissue, including the CEID. SCR is expressed in the CEID and throughout the endodermal lineage, suggesting that SCR may function in later stages of development. This tissue-specific expression of SCR is regulated at the transcriptional level. SCR protein binds and sequesters SHR in the nucleus of the CEI and endodermal cells. This SHR-SCR complex then activates the SCR promoter. Through a ChIP-PCR assay (2007 Cui), the SCR protein has been shown to bind to the SCR promoter, thus forming an autoregulatory positive feedback loop to drive SCR transcription (Fig. 2). The *scr* mutant is incapable of asymmetrically dividing and the undivided cell file possesses markers from both cortex and endodermal cells (Fig. 3). Thus, SCR is required for the CEID asymmetric cell division.

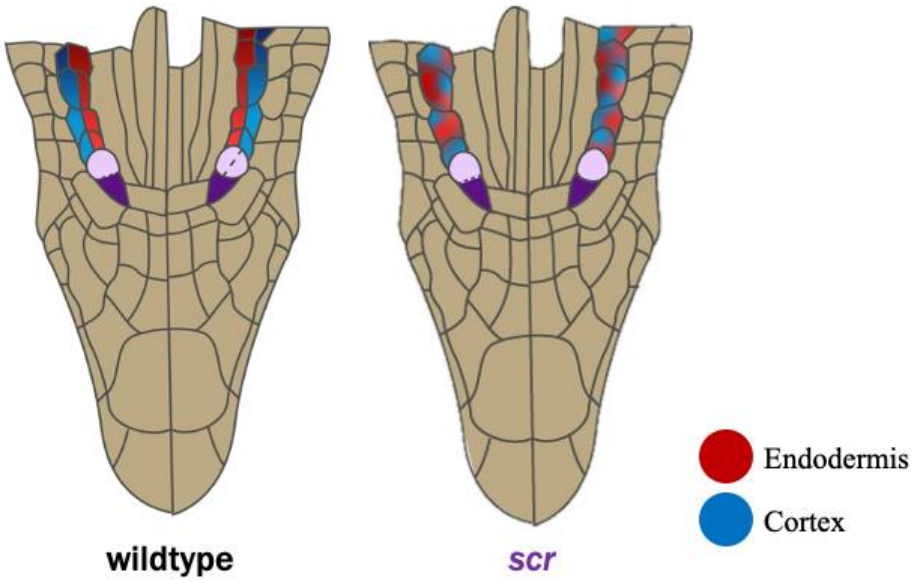


Figure 3. SCR is Essential for Endodermis and Cortex Tissue Formation Comparison of cell files in wildtype and *scr* plants.

1.4 Introduction to Organ Form for Function (Project 2)

As sessile organisms, plants must establish a firm foundation into the terrain wherever the seed lands. Wetland species, such as rice, encounter additional challenges as the intermittent flooding may destroy plants. Roots, especially the primary root (a seed's first root), are the only anchor into the terrain. Circumnutation refers to the circular or elliptical growth of the tip of a plant organ around a central axis. Previously, the function and genetic requirements for circumnutation were unclear. I was part of a team that carried out a multiscale investigation of the regulation and function of this behavior in rice. For complete details of experiments and findings, the paper is available online as a preprint (Taylor 2020). This thesis will detail my contributions to the methods, experiments, and analysis.

2. Project 1: Gene Dynamics in Tissue Development

2.1 Determining the Role of the Autoregulatory SCR Positive Feedback Loop

The SHR-SCR GRN is predicted to involve a bistable switch, existing either ‘on’ or ‘off’ (2012 Cruz). Bistable systems have two stable equilibrium states and can shift between them given enough energy and/or substrate to penetrate the barrier. When this system is ‘off’, mitosis is prevented. Once a certain level of SCR protein has accumulated, the system turns ‘on’ and initiates the division. Mathematical modeling has indicated that, within the known GRN, this SCR autoregulatory PFL plays a central role in regulating the asymmetric division of the CEID.

To test this computationally predicted role of the positive feedback loop in regulating division, an ideal experiment would be to remove the SCR binding sites in the SCR promoter responsible for the positive feedback. However, DNA binding sites for SCR have not been defined despite attempts with bioinformatics inference, ChIP, yeast-one hybrid assays, and protein-binding microarrays. Promoter mutation approaches have difficulty in evaluating the contribution of multiple proteins. Thus, traditional molecular genetic experiments to modify the SHR-SCR GRN would be uninformative.

2.1.1 Utilizing a Yeast System to Characterize Synthetic Circuits

As an alternative approach to determine the function of the SCR positive feedback loop in controlling stem cell asymmetric cell division, I sought to systematically characterize and modulate it. Constructing a range of synthetic circuits to drive SCR expression allows for precise manipulation and thorough exploration of the parameters in which the division can occur. The circuit consists of an inducer which drives a synthetic TF, which drives its own expression in an autoregulatory positive feedback loop akin to

that of SCR (Fig. 4). Finally, this synthetic TF drives expression of the SCR protein. Construction was done using synthetic biology methodology by assembling previously characterized regulatory gene modules, see section 1.1.1 Plasmid Construction for detail.

This synthetic SCR positive feedback loop was constructed in yeast to allow for rapid characterization of many circuits. By optimizing circuits in yeast prior to plant transformations, this design optimizes plant experiments while also providing insight into how network structure impacts function. Future work will determine the essential SCR dynamics for asymmetric cell division. See section 1.4 Future Directions for more detail.

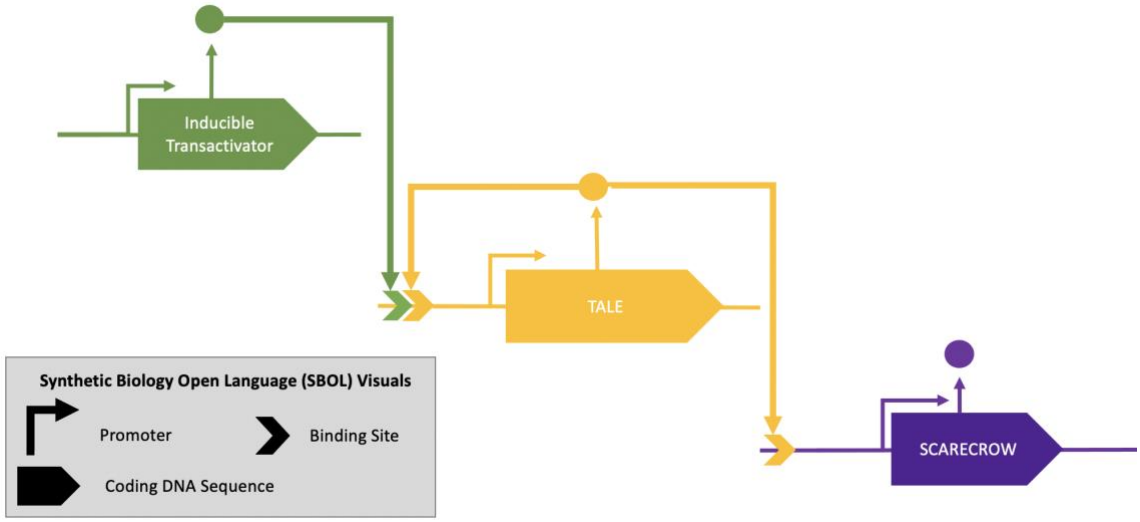


Figure 4. Schematic of Synthetic Circuit for SCR Positive Feedback Loop
Inducible transactivator, XVE, is activated by β -estradiol. The transactivator activates a synthetic TF, the NANOG TALE. The TALE binds to its own promoter, forming a positive feedback loop, as well as the final promoter driving SCR protein expression.

2.2 Materials and Methods

2.2.1 Plasmid Construction

Plasmids containing either promoter or coding DNA sequence (CDS) parts were generated in the UAP1 vector backbone (AddGene #63674) through Gibson assembly (Gibson 2009 Nat Methods). In addition to being compatible with Golden Gate cloning,

these UAP1 parts could then be introduced into either yeast or plant destination vectors. See Figure 5 and Supplementary Table 1 for DNA sequences and plasmids used in this study.

2.2.1.1 Fluorophores

This project used the yeast-optimized GFP and tagRFP (Supplementary Table 1). For the SCR::GFP protein-fluorophore fusion, this project created a C-terminally tagged fusion with the following linker: GGATCCGCTGGATCCGCTGCTGGTTCTGGAGAATTC. For the RFP::TALE protein-fluorophore fusion, this project created an N-terminally tagged fusion with the following linker: GGATCCGCTGGATCCGCTGCTGGTTCTGGAGAATTC.

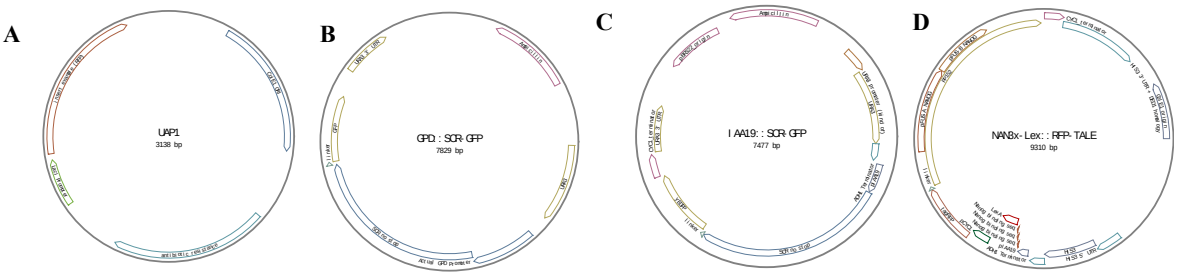


Figure 5. Vectors A) Schematic of UAP1 vector backbone used for 'parts' with RFP insert as example. B) Yeast gateway assembled vector, general yeast promoter driving SCR-GFP fusion protein. C) Yeast gateway assembled vector, minimal promoter driving SCR-GFP fusion protein. D) Yeast gateway assembled vector, 3 TALE binding sites and induction promoter driving RFP-TALE fusion protein.

2.2.1.2 Promoter Variants

To control for stereochemical variables, I maintained the same number of base pairs in promoters with varying TALE binding sites by mutating binding site(s) to render it nonfunctional. In every instance of TALE binding sites, this project uses the minimal promoter IAA19 (Pierre-Jerome 2016). A yeast CYC1 promoter placed

upstream of the induction promoter, LexA, enhanced transcription. See Figure 5 and Supplementary Table 1 for DNA sequences and plasmids used in this study.

2.2.1.3 Transcription Activator-like Effector (TALE) Assembly

The TALE was engineered to bind a NANOG cis-element (2015 Yang). The TALE was constructed by first assembling repeat modules into intermediary arrays then assembled with Golden Gate (Cermak 2011).

2.2.2 Yeast Strain Construction

To grow yeast on solid plates, I used YPD Broth (Sigma Y1375-250G), Adenine (CAS No. 73-24-5; Sigma A2786-5G), and Difco Agar, Granulated (BD 214530). Utilizing the jStack method (Shih 2016), promoter and CDS constructs were assembled into yeast destination vectors (Pierre-Jerome, 2014) using a standard Golden Gate protocol (Engler 2008, Engler 2014). Vectors were transformed into W303-1A ADE2+ *MATa* strains with integration into different amino acid loci (see Supplementary Table 2 for which loci were used per construct). A standard lithium acetate protocol (2002 Gietz) was used for transformations of digested plasmids. For cloning, standard yeast drop-out (Takarabio # 630313, # 630311, # 630315) was used to select for successful transformation with plasmid. See Supplementary Table 2 for detail on what combinations of promoters and CDSs were transformed into yeast.

2.2.3 Cytometry

Cytometry experiments used synthetic complete (SC) media to minimize background fluorescence. SC media was created from mixing Yeast Nitrogen Base (Sigma #Y0626, without amino acids), synthetic drop out media (Sigma #Y2001),

individual amino acid supplements (Ade, His, Leu, Trp, Ura), sterile water and dextrose (Sigma # DX0145-5).

For steady-state expression measurements, a colony was selected from a sample plate and placed into SC. These tubes were grown to stationary phase overnight in a 30 °C shaker rotating at 220 rpm. The next morning, each culture's growth stage was measured using optical density. Cultures were then diluted into fresh SC, and fluorescence measurements taken ~3-6 h later during exponential growth phase. Volumes and length of time varied between experiments but not within experiments.

For induction experiments, yeast grew untreated before supplementing SC media with the inducer, β-estradiol (Siligato 2016). To create an initial concentration of 10mM, I dissolved β-estradiol in EtOH and filter sterilized it using a 0.22uM filter. I diluted this to 1uM, once more using ethanol and filter sterilization. Finally, I diluted this with water and filter sterilized to create my working stock at 500 nM. Samples were dosed with varying amounts of this working stock for varying lengths of time. Volumes and length of time varied between experiments but not within experiments.

Fluorescence measurements were taken at the Duke Flow Cytometry Core using a BD FACS Canto IIs flow cytometer. For experiments using the green fluorescent protein, we used an excitation wavelength of 488 and an emission detection filter at 533 nm. For experiments using the red fluorescent protein, we used an excitation of 633 and an emission detection filter at 550nm. A total of 10,000 events above a 400,000 FSC-H threshold (size filter to exclude debris) were measured for each sample and data exported as FCS files for processing.

2.2.4 Cytometry Analysis

Analysis of cytometry data was conducted using FlowJo software (FlowJo™). First, we created a gate to encompass all haploid fluorescing yeast, excluding items like debris, dead cells, empty droplets, and multi-cell drop clusters (Fig. 6, 7). Though the gates location changes per experiment, the gates are exactly the same within experiments. This analysis delivered data on the relative fluorescence of each yeast strain.

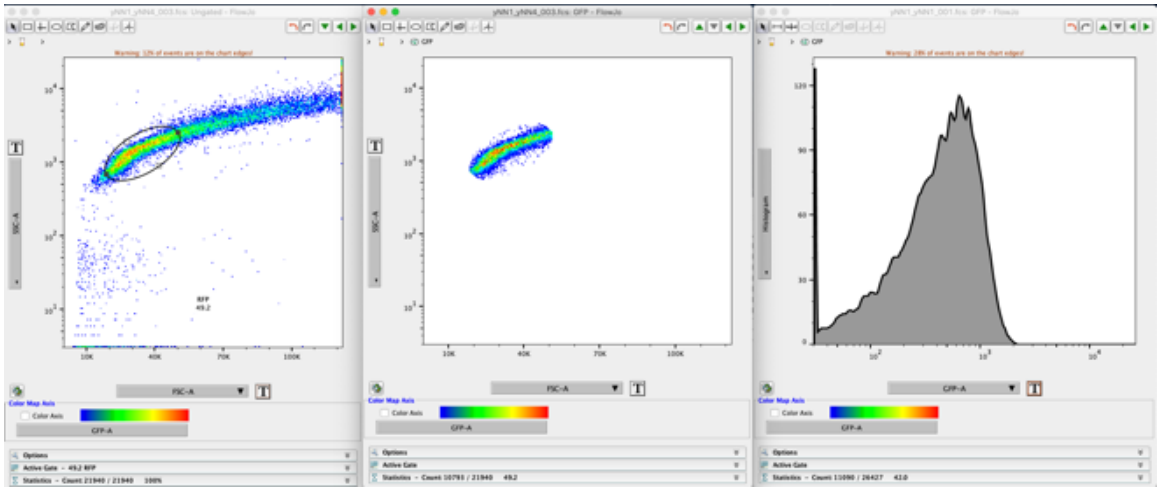


Figure 6. Cytometry Analysis by FlowJo Analysis of cytometry data done in FlowJo. A) entire population of cells, with circle representing the gate B) gated population of live haploid cells C) fluorescence of B.

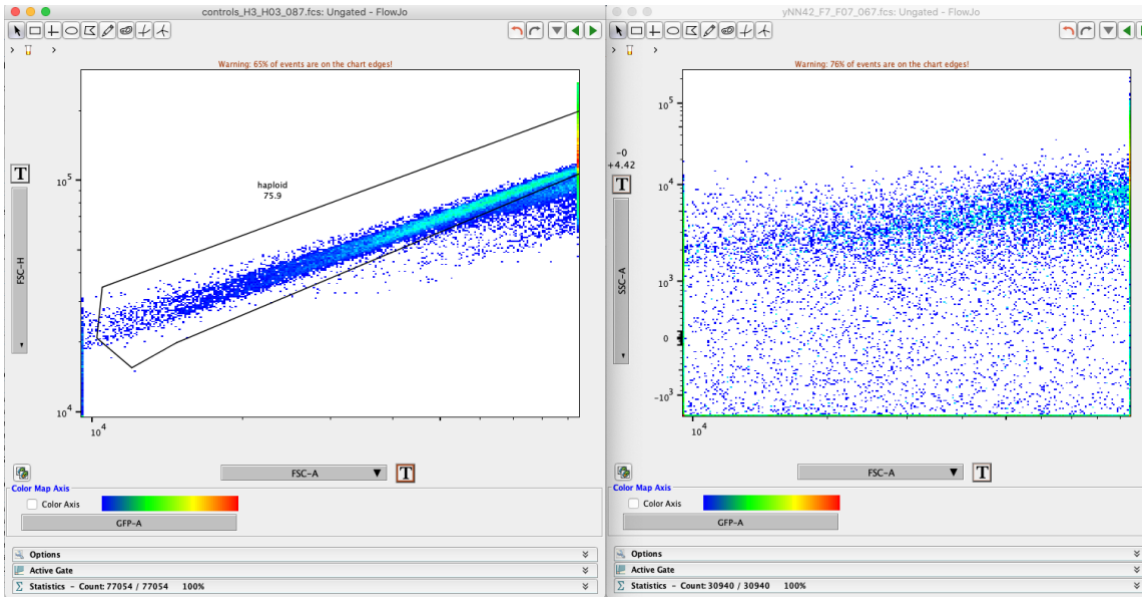


Figure 7. FlowJo Cell Population Comparison (A) Live cells in FlowJo form a clear line whereas (B) dead cells fragment.

2.3 Results

2.3.1 Strategic Cloning Approach for Precise Control and Tuning of GRN

We constructed an inducible synthetic circuit to control the expression of SCR protein (Fig. 4). Here, the inducible system (2000 Zuo) drives expression of a synthetic TF composed of a Transcription Activator-Like Effector (TALE) DNA binding domain and a VP64 activation domain. The TALE-TF was engineered to bind an 18 bp mammalian cis-element (2014 Yang) not found in yeast or plants in order to minimize potential off target effects. The TALE promoter included varying amounts of TALE binding sites, so binding of the TALE to its own promoter generated an autoregulatory PFL, akin to SCR in wild-type plants. By adding the same TALE binding site upstream of SCR, the TALE would also bind and drive expression of SCR.

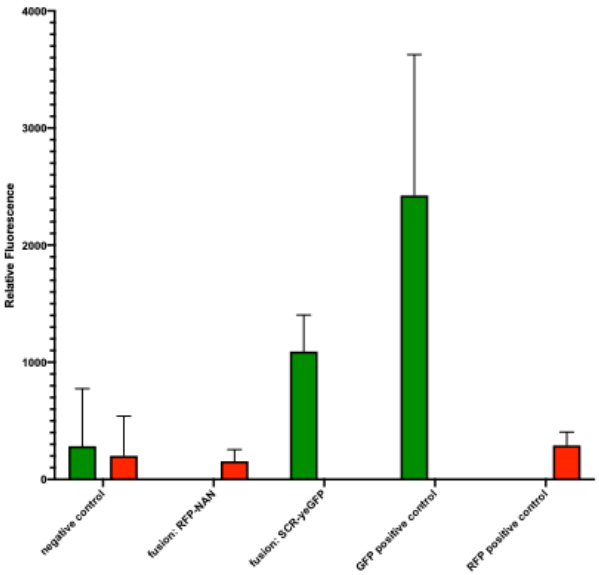
This strategy will aid in determining the role of the positive feedback loop in plants. By varying the dynamics of SCR expression, we can explore the parameter space

in which SCR does and does not result in an asymmetric cell division. Thus, I needed the logic and control given by using synthetic circuits. Experimenting with a range of inducer concentrations allows for testing of different initiation strengths. By controlling induction, we can determine when SCR is expressed, the length of time before cells divide, and whether mutant cells are now capable of division. Altering feedback strength can be accomplished by varying TALE binding sites. Ultimately, this allows for fine-tuning SCR protein expression levels over time.

2.3.2 Testing Circuits in Yeast

To rapidly prototype the synthetic SCR PFL circuit, we characterized the fluorescence of each circuit in yeast (*Saccharomyces cerevisiae*) using flow cytometry. To reduce diploid variance, we did additional transformations into haploid yeast rather than mating haploid yeast. A complete list of yeast strains can be found in Supplementary Table 2.

Firstly, I wanted to verify that fluorescence would remain strong even when linked to a protein. To do so, I compared the fluorescence of GFP and tagRFP when ubiquitously driven by themselves to when fused to proteins. I found a decrease in fluorescence when GFP is fused to SCR (Fig. 8). However, fusing RFP to the TALE did not affect fluorescence.



Yeast Construct	Count	Fluorescence Media
wild type	11090	284
yeast GFP	10793	2424
yeast GFP fused to SCR protein	11633	1092

Yeast Construct	Count	Fluorescence Median
wild type	11090	284
RFP	13231	234
RFP fused to NANOG TALE	12198	247

Figure 8. Protein Fusions to Fluorescent Tags Graph depicting relative fluorescence of wild type yeast, yeast ubiquitously expressing fluorescent protein, and yeast with a protein-fluorophore fusion.

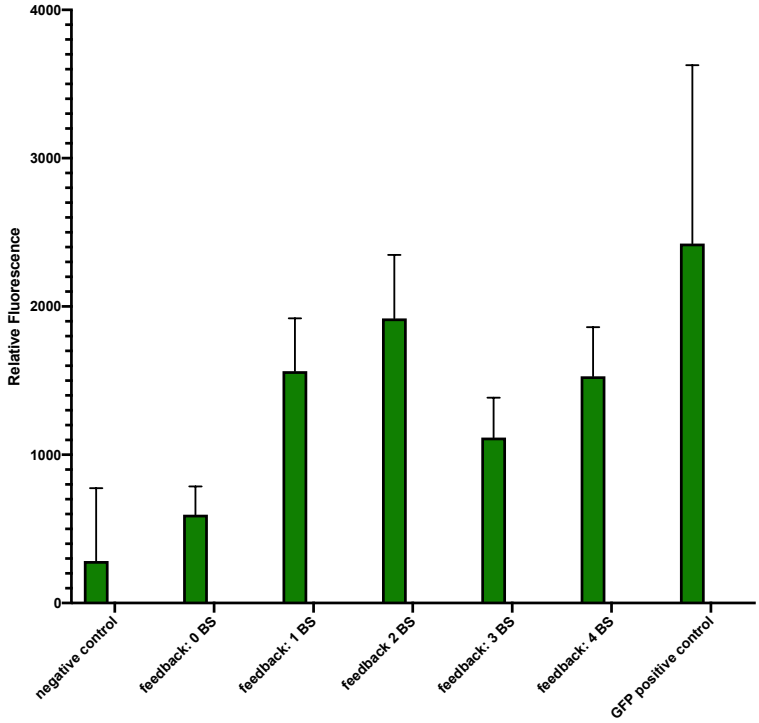


Figure 9. Binding Sites Relative fluorescence of yeast strains with varying binding site numbers. Increasing number of binding sites increases activation with 1 to 2 binding sites, but this is reduced in adding a 3rd or 4th binding site.

Next, I tested TALE-TF binding strength by ubiquitously driving the TALE-TF alongside a promoter driving a fluorophore. This second promoter possessed either 0, 1 ,2 ,3 ,or 4 TALE-TF binding sites. I found that increasing the number of binding sites increases activation with 1 to 2 binding sites, but this is reduced in adding a 3^{rd} or 4^{th} binding side (Fig. 9). This may be due to stereochemistry, so future work could explore increased spacing between sites to increase activation with more than 2 sites.

Next, I tested the induction using varying amounts of inducer molecule (Fig. 10). The induction system was driven under a ubiquitous yeast promoter with a final output of fluorescence. Dosage and length of time varied between experiments but not within experiments. As predicted, increased levels of inducer and longer times of dosage increased fluorescence output. However, I found that RFP fluorescence when induced was much stronger than in the ubiquitously expressed control. Future experiments should alter the fluorescent protein such that the control under the ubiquitous promoter is the strongest fluorescence and include a dose-response experiment (see section 1.4.1).

To generate the full loop, all three promoter – CDS parts of the circuit must be cloned into one yeast strain (Supplementary Table 2). Although experiments were attempted with the full loop, the yeast did not survive. Future cytometry experiments must be optimized for yeast growth and timing of β-estradiol induction.

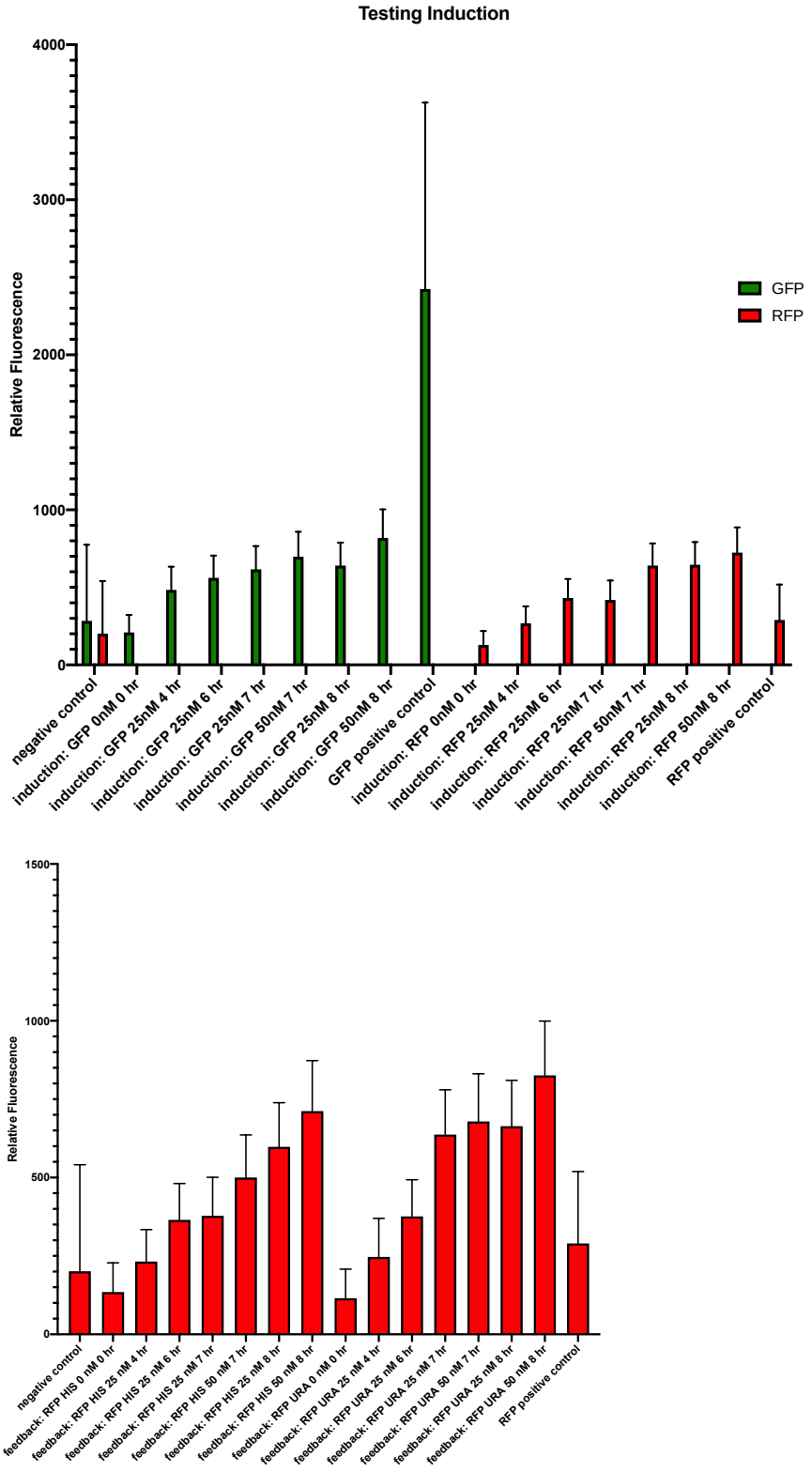


Figure 10. Induction Relative fluorescence activates as predicted, with higher doses and longer times resulting in higher fluorescence with both RFP and GFP tags. Each graph represents an experiment from a different day.

2.4. Future Directions

2.4.1 Optimize Cytometry

To finish characterizing these circuits in yeast, future work should optimize growth and induction conditions. As it is difficult to modulate the amount of yeast used in overnight growth, yeast samples were frequently under- or overgrown. For induction, a dose-response experiment is necessary to determine the minimum and maximum/saturated levels of inducer. All cloning has been completed with the exception of two strains necessary for the full loop (Supplementary Table 2).

The true excitation and emission of tagRFP is at 555 and 584 nm, respectively. However, the available equipment does not match these numbers well, thus the reporter protein tagRFP is too weak to act as control. I suggest the use of Cyan Orange Fluorescent Protein (CyOFP) instead. In addition to withstanding long term imaging, CyOFP excites at 497 nM, the same channel as GFP, but emits at 589nM. Analysis should be repeated using the methods described here.

2.4.2 Characterize the Synthetic Circuit in *Arabidopsis*

The synthetic circuits should be transformed into an *Arabidopsis scr* mutant, which is incapable of asymmetric cell division. In the ideal parameter space, the circuit can rescue the *scr* mutant phenotype, resulting in ACD in a *scr* mutant. The same vectors used to generate yeast strains should be used to generate the circuit in *Arabidopsis*. In lieu of yeast homologous recombination to join multiple promoter and coding DNA sequences together, these circuits must be linked with linkers and plant terminators instead. Previous work demonstrates the ability to induce asymmetric cell division in shr mutant cells by SHR induction (2010 Sozzani). Another dose-response experiment should be conducted,

as plants will likely require a much higher level of inducer. ‘Tuning’ the PFL will be achieved in part by modulating levels of the inducer to alter the initial rate of synthetic TF transcription. The parameter space can be further explored by modifying the number and orientation of TALE binding sites.

I hypothesize that altering SCR expression and kinetics will change the asymmetric cell divisions. If the division fails to occur, a single mutant ground tissue layer will be present. If the division occurs, a cortex cell and endodermal cell will arise. SCR expression, as measured by fluorescence, will dramatically shift from low to high levels with increasing levels of inducer (2012 Cruz). Future work can also test the effect of different promoters on cell division dynamics—cells driven by a weaker promoter may not divide at all, while stronger promoters may be sufficient for division. To determine the minimum SCR kinetics for asymmetric cell division, future experiments must create a range of SCR expression through various circuit designs. I would further test the parameter space of this circuit with alternate circuits by reducing the number of TALE binding sites and removing the positive feedback. If the PFL is necessary for ACD, the latter altered circuit will result in no divisions. Increased synthetic TF binding site numbers may result in faster activation while orientation changes may otherwise perturb the system (2016 Pierre-Jerome). Ultimately, this synthetic PFL network structure should allow scientists to explore a wide range of SCR kinetics to determine the parameters for properly coordinated division.

I foresee the following possible outcomes: a) the SCR PFL works only within a certain range to rescue asymmetric cell division; b) there exists a broad range in which this SCR PFL rescues the mutant; or c) a simple threshold level of SCR is sufficient for

asymmetric cell division. My preliminary work in yeast has demonstrated the functionality of individual components to maximize the likelihood that the parts work together as expected and that the synthetic PFL will be functional in plants. This research will illuminate the dynamics underlying asymmetric cell division in a multicellular organism.

After induction, future work should quantify fluorescently labeled SCR across time and space as well as the rate and number of rescued mutant cells. These kinetic measurements can be made using selective-plane illumination microscopy (SPIM) which provides high spatial resolution, low phototoxicity, and fast image acquisition speeds. Calculating the fluorescence intensity in each individual nucleus allows for quantification of levels of SCR in cells to make correlations to asymmetric cell division. As the division occurs every 24 hours in wild-type plants, roots should be live-imaged for 24-36 hours every 30 minutes to acquire 3D images derived from 1 uM z-stacks. The Benfey lab's custom designed light sheet microscope allows for vertical imaging, eliminating the complications of plant responses to gravity by allowing roots to grow naturally downwards. Using this microscope for these experiments eliminates the phototoxicity typically associated with confocal microscopy.

2.5 Discussion

Determining how gene regulatory networks (GRNs) coordinate tissue development has been a longstanding challenge in developmental biology. Proper development of multicellular organisms requires diverse spatiotemporal patterns of gene expression. In *Arabidopsis thaliana* roots, SCR controls an asymmetric cell division, resulting in two daughter cells that acquire different fates and tissue identities. As the

SCR positive feedback loop is hypothesized to be necessary in this GRN, I developed a synthetic biology approach to systematically and precisely tune various dynamics of SCR protein accumulation. Since the modules used to control this circuit are orthogonal, meaning that they are not endogenous to either organism, this approach enables a parallel analysis of the same circuit, both in yeast and plants. Furthermore, manipulating a specific feature of an endogenous GRN is difficult to interpret as it may have effects elsewhere in the system or other components of the organism may disturb the GRN. These synthetic circuits must still be characterized in yeast and transformed into *Arabidopsis.* By spanning both yeast and plants, this research may also provide novel insights into eukaryotic gene regulation and circuit dynamics in single vs. multicellular organisms.

3. Project 2: Organ Form for Function

3.1 Introduction

Using a gel-based imaging system (Iyer-Pascuzzi 2010) the root system architecture (RSA) phenotypes were measured from *Oryza sativa* lines derived from a sequence-indexed fast-neutron irradiated mutant population in the Kitaake background (Li 2017). X. Kitaake is a rapid cycling, largely photoperiod insensitive Japonica cultivar. These features allow for faster generation times and data acquisition. Although RSA measurements have historically focused on static traits, we sought to capture root growth dynamics to identify novel parameters contributing to root architecture. In our phenotyping system, rice root systems were imaged every 15 min for several days after germination. Images were then analyzed to measure several RSA features including root number, length/depth, and branching patterns (Lynch 1995).

A mutant line was identified that exhibited a much deeper primary root compared to wild type. (Fig. 11). By tracking tip movement, it was found that the wild type regularly circumnutates while the mutant never enters into this pattern. When the tip of a plant organ, such as a root or shoot, begins to grow around a central axis, it has entered into circumnutation. The mutant had a single base substitution that resulted in a premature stop codon of *Oryza sativa histidine kinase 1,* or *OsHK1*. Although this gene encodes a histidine kinase homologous to cytokinin receptors, it does not have a predicted cytokinin binding site. Cytokinin is a multifunctional plant hormone involved in processes including growth, development, and cell proliferation. The plant hormone ethylene also activates cytokinin signaling receptors and HK1 was shown to positively regulate ethylene signaling in rice roots (Zhao 2020). By treating *hk1* mutants with the

naturally occurring cytokinin trans-zeatin, others in lab were able to rescue circumnutation. Thus, HK1 activates downstream cytokinin signaling to regulate circumnutation. However, we wanted to further explore how cell elongation was restricted in this precise pattern.

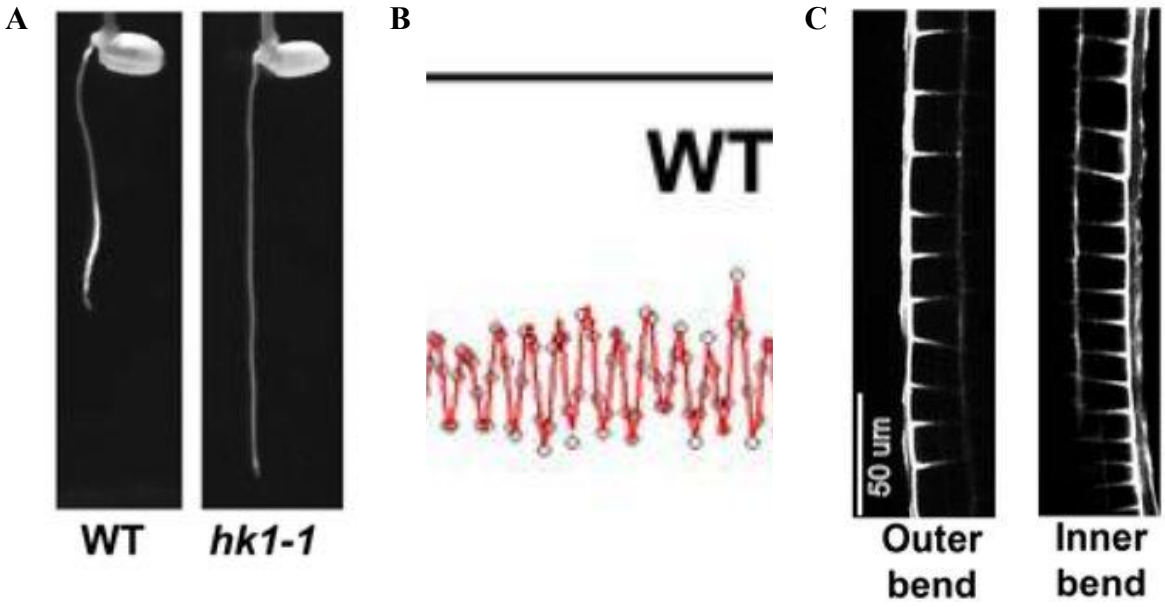


Figure 11. Physiology of Circumnutating Roots A) Maximum projection of a wild type *Oryza sativa* (left) and a mutant (right) incapable of circumnutating. B) Close up of region of cell length measurements. C) Close up of wild type epidermal cells on either side of the root.

3.2 Materials and Methods

3.2.1 Plant Materials and Growth Conditions

Dr. Pamela Ronald from University of California, Davis provided the Kitaake seeds. The wild-type plant is Oryza sativa ssp. japonica variety KitaakeX, a Kitaake plant carrying the rice XA21 immune receptor gene driven by the maize (Zea mays) ubiquitin promoter (Jain 2019, Li 2017). The *Oshk1-1* mutant, referred to as *hk1,* is from the mutant line FN-287 and has a single base substitution at position Chr6:26808461. Genomic coordinates are from Os-Nipponbare-Reference- IRGSP-1.0 (Kawahara 2013).

To plant seeds, we removed the hull, sterilized the seeds using 50% bleach for 7-10 minutes, then rinsed seeds 4-5 times with sterile water. After sterilization, seeds were placed directly in GA-7 Magenta boxes with Yoshida's nutrient solution solidified with 0.15% gellan gum (Gelzan, Caisson Inc.) (Cock 1976). For auxin treatment experiments, seeds were grown hydroponically in liquid Yoshida's medium, floating with sterilized mesh. After planting seeds into boxes, they were first incubated for 2 days in the dark at 30˚C before being moved to constant low light (~650 lux) at 22-23˚C for imaging.

3.2.2 Time Lapse Imaging

Others in lab designed and constructed two robotic automatic imaging systems to acquire images of plants in Magenta boxes every 15 minutes. Time-lapse movies were created using 15 frames/second, or 225 minutes of growth/second.

3.2.3 Root Cell Length

The primary roots of seedlings were imaged using a Zeiss 880 confocal microscope. Plants were 2-3 days old, well past the initiation of circumnutation. In wild type, the primary root begins circumnutating in under 1.5 days. Roots were harvested from media, live-stained with 1mg/mL calcofluor white for 5 minutes, then immediately imaged (Sigma, 18909). Samples were placed on their side to display the angle of maximal bending. To capture the overall root shape and cell organization, I conducted a tile scan and z-stack beginning at the root tip, up to the curve, and into the elongation zone.

To measure individual cell length, I used FIJI image analysis software. First, I created lines parallel and perpendicular to the direction of root growth to determine the angle and region of the root exhibiting maximal bending. Within ±300um from the

center, I measured cell lengths on either side of the root. Because *hk1* mutant roots grow straight, the 'inner' and 'outer' sides were randomly labeled and the region of maximal bending was set to be a comparable distance from the root tip similar to distance wt.

3.2.4 Auxin Treatment Experiments

For auxin treatment experiments, plants grew untreated before supplementing media with auxin, as auxin has been shown to inhibit root elongation. Hydroponically grown rice were dosed approximately 1 day after germination. We diluted 200mM IAA in ETOH with sterile water and brought it to a dosage of 200nM by adding this to the nutrient solution (GoldBio, I-110-25). We diluted 1-NAA and 2-NAA similarly, but created stock solutions in acetone(Sigma, N0640 and N4002). For the auxin efflux inhibitor NPA, 50 mM stock NPA in DMSO was first diluted in sterile water and added to autoclaved media to create a final concentration of 40 nM (Sigma, 33371). An equivalent amount of DMSO was used for the negative control.

3.3 Results

3.3.1 Circumnutation Results from Localized Restriction of Cell Elongation

Roots circumnutate by bending around 1-2mm from the tip in the cellular elongation zone, similar to gravitational stimulus (Su 2017). In response to gravity, auxin influx and efflux carriers directionally transport auxin to the outer cell layers on the lower side of the root. This auxin accumulation restricts cell elongation on one side and orients the root tip downward (Su 2017).

To determine if circumnutation also relies on asymmetric cell elongation, we measured the epidermal cell length on the inner and outer side of 9 wild-type roots from confocal images. For quantification, I created a line bisecting the root, determined the

region of maximal bending, and recorded the epidermal cell length on the inner and outer side ±300um from the center (Supplementary Tables WT and OsHK1). The inner side had a consistent decrease in cell length (Fig. 11C). Because *hk1* mutant roots grow straight, the 'inner' and 'outer' sides were randomly labeled and the region of maximal bending was set to be a comparable distance from the root tip similar to the distance in WT. All 11 mutant controls did not have roots with one side longer than the other. Statistical analysis was done by others in the lab, performing a 2-sided Wilcoxon Signed Rank test resulting in p-values of 0.003906 and 0.5771 for wild type and mutant, respectively (Taylor 2020). Thus, roots circumnutate by initially restricting cell elongation on one side.

3.3.2 Cytokinin and Auxin Can Rescue Circumnutation in the *Hk1* Mutant

We wanted to identify the signaling pathways that regulate cell elongation to create the circumnutation behavior. Regulation of auxin transport and signaling is involved in many Arabidopsis oscillatory root growth patterns (Maher 1930, Simmons 1995, Garbers 1996, Piconese 2003), though it is difficult to determine if these roots circumnutate due to their small size (Mullen 1998, Thompson 2004, Migliaccio 2013). When pea seedlings are treated with auxin transport inhibitors, their roots can be observed to no longer circumnutate (Kim 2016). Ultimately, these results suggest a connection between auxin transport and root bending for circumnutation. Others in the lab examined RNA-seq data and found the *hk1* mutant has a reduction in many auxin response genes which is restored when the mutant is treated with cytokinin.

To test the hypothesis that HK1 controls circumnutation by regulating cell elongation through the auxin pathway, we treated roots with auxin transport inhibitors

and different types of auxin. In the auxin treatment assays, I aided in determining the method of dosage, appropriate hormone concentrations, and conducting the experiments; while the final published measurements are from others in lab. When treated with the auxin transport inhibitor N-1-naphthylphthalamidic acid (NPA), we found that wild-type roots were almost completely unable to circumnutate. A significant reduction in tip displacement amplitude was quantified (Fig. 12A), indicating the importance of auxin transport for circumnutation. If *hk1* is indeed unable to circumnutate due to defects in auxin transport, we hypothesized we might be able to rescue the phenotype by additional auxin. Since auxin can inhibit root elongation, for these experiments we grew plants untreated until about one day post-germination, at whichtime the media was supplemented with auxin. Neither the naturally occurring auxin, indole-3-acetic acid (IAA), nor the synthetic auxin 2-Naphthaleneacetic acid (2-NAA) could rescue circumnutation in the *hk1* mutant. However, treatment with the structurally related 1-NAA resulted in an increase in mean tip displacement and circumnutation in *hk1* mutant roots (Fig. 12B). Notably 1-NAA is lipophilic and thus membrane permeable, so it can easily diffuse across the plasma membrane without an auxin importer and act as a substrate for auxin efflux proteins. Thus, the *hk1* mutant's auxin import, but not export, is compromised. Collectively, these results indicate HK1 controls auxin influx through AUX1, (Fig. 13) which regulates cell elongation for circumnutation.

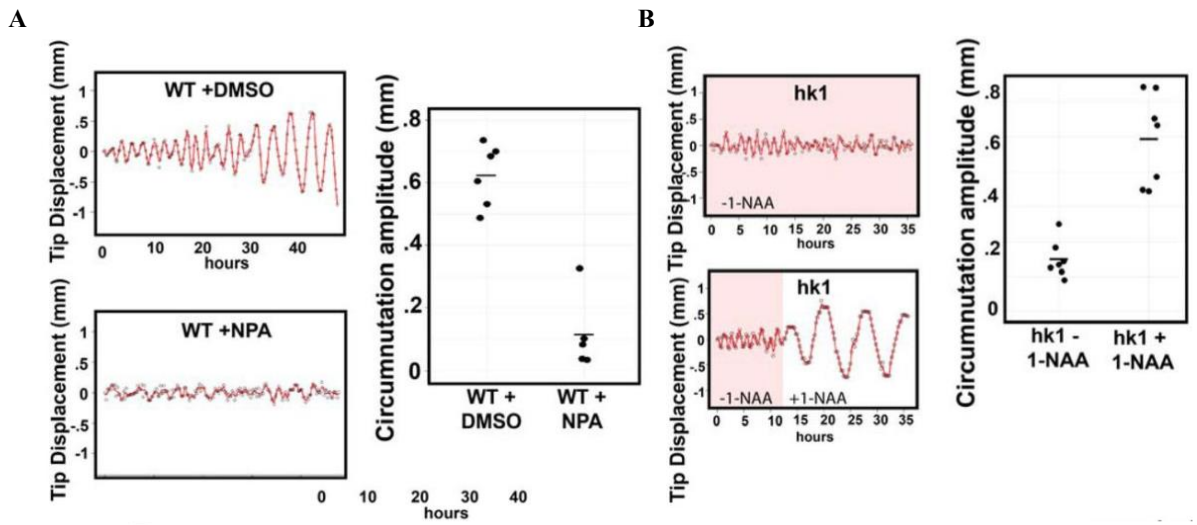


Figure 12. Auxin Hormone Experiments A) Representative root tip traces of the wild type roots treated with and without NPA (left). NPA was solubilized in DMSO, so equivalent amounts of DMSO were used as a control. Quantification of average radius of circumnutation (right). B) Representative root tip traces of the wild type roots treated with 1-NAA (left).

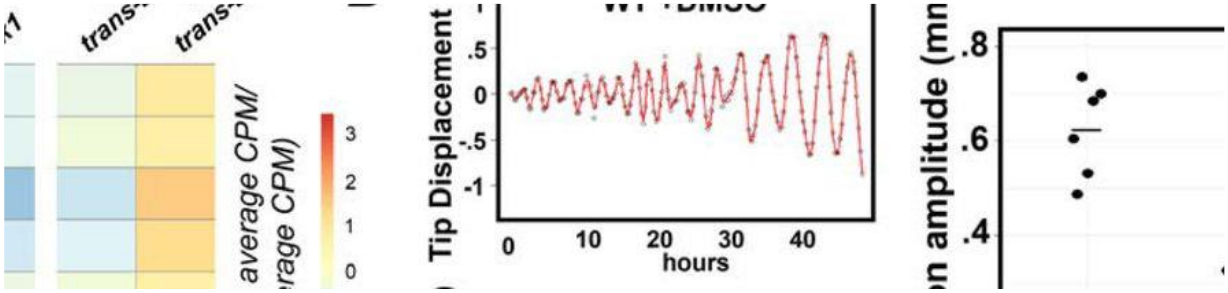


Figure 13. Molecular model of circumnutation

3.4 Discussion

Significant work on this project was performed by other scientists in the Benfey lab and collaborators at Georgia Tech (Taylor 2020). A 30% reduction in transcript levels of the auxin import gene OsAUX1 was observed, which is restored to near wild-type levels in the cytokinin-treated mutant. There was also a lack of circumnutation in an *aux1* mutant. Collaborators found that mutants were not able to effectively explore dense surfaces by growing rice on plastic surfaces with 2.5mm diameter holes equally spaced at different densities in a square lattice and again in soil with dense heterogeneities. These data indicate that wild-type roots are more effective in exploration and less affected by sparse hole density than *hk1* mutants, providing a plausible mechanism to buffer against environmental uncertainty inherent in exploration of soil interfaces. This was also tested by measuring penetration of roots through gravel and found that wild-type roots were significantly more successful at anchoring themselves into soil. Instead of attempting purely downward growth, roots that circumnutate can find a path of least resistance into the soil.

In summary, circumnutation is a dynamic process involving the gene *Oryza sativa Histidine Kinase-1/OsHK1,* the auxin influx carrier gene *OsAUX1*, and hormone signaling by ethylene, cytokinin, and auxin. This behavior appears to require auxin influx and efflux, supported by research indicating auxin inhibits root cell elongation and can be directionally transported. Our results support a model where *HK1* positively regulates an auxin signal transported cell to cell, inhibiting cell elongation, and producing circumnutation (Fig. 13). Robot modeling of roots and experimental testing of root penetration ability has demonstrated the importance of circumnutation for substrate

navigation. This behavior aids in root exploration and may play a critical role in anchoring seeds to their substrate.

3.5 Future Directions

It is yet to be determined if canonical cytokinin receptor signaling regulates circumnutation, or if exogenous cytokinin rescue simply stimulates downstream signaling artificially (Fig. 13).

4. Conclusion

4.1 Conclusions from Gene Dynamics in Tissue Development

By utilizing a synthetic biology method, this work will aid in understanding SCR network dynamics from its initiation of expression in the cortex-endodermal initial cell. Building GRNs quantitatively from the bottom up allows for systematic, thorough testing. By connecting genetic circuit structure to the mechanisms that govern tissue patterning by asymmetric cell division, this project may yield insights which may ultimately aid in building a model. The use of this synthetic circuit allows us to modulate key features to determine the network parameters and dynamics necessary for this asymmetric cell division. This approach will also allow future researchers to directly test how important the PFL is to the bistability of the system as well as systematically test SCR parameters such as feedback strength and rate of protein accumulation. Understanding the nature of cell transitions as they progress from stem cells to differentiated tissue has the potential to impact research areas that require understanding the relationships between large networks of genes, such as agricultural biotechnology, cancer research, and human disease.

4.2 Conclusions from Organ Form for Function

While the molecular mechanisms of circumnutation have begun to be understood, the exact model remains unclear. In this study we analyzed root cell physiology and tested various hormones, identifying key interactions for this behavior. Furthermore, it seems roots must strike a balance between rapid, unidirectional root elongation and multi-dimensional root exploration. Continued study of circumnutation will provide additional insight into how roots regulate their exploratory growth.

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