OVERVIEW

Most flowers are either radially symmetric (i.e., can be divided into equal halves along multiple axes) or bilaterally symmetric (i.e., with a single plane of symmetry along the dorsal-ventral (D-V) axis) in their final forms [1]. The ancestors of all flowering plants and of all major lineages are predicted to produce radially symmetric flowers, while bilateral symmetry has evolved at least 130 times independently [2], [3]. It is well known that differential organ elaboration along the D-V axis in many taxa is controlled by a developmental program dependent on the *CYCLOIDEA* (*CYC*) gene. **However, what establishes the D-V domains in the floral meristem (FM) at the earliest stage and how *CYC* expression is restricted to the dorsal domain remains unknown.** The goal of this proposal is to address these fundamental questions by employing a new genetic and developmental model system, the monkeyflower species *Mimulus lewisii*. I will focus on two *M. lewisii* mutants with altered *CYC* expression patterns and will characterize the underlying genes to elucidate the molecular pathways upstream of the *CYC* network. **Results from this project will not only be significant from a developmental perspective in understanding the symmetry breaking mechanism in the floral meristem (i.e., how D-V domains are initially established), but also crucial for understanding the genetic bases underlying convergent evolution in floral forms.**

INTRODUCTION

Symmetry is a major element of floral morphological diversity (Fig. 1a-d) [1]. The interest in patterns of floral symmetry has been documented since centuries ago (e.g., [4]). Numerous ontogenetic studies, tracing back as early as the invention of the first microscopes in the early 1800's ([5]–[7]), suggested that **the establishment of symmetry occurred at the earliest phases of development of the floral meristem (FM)**. Meristems are domes of cells that harbor stem cells in the center and organogenic cells on the side [8]. Upon reproduction, the vegetative meristems that produce leaves will transition to inflorescence meristems (IMs), which give rise to FMs [8]. The positional information of the dorsal and ventral domains has already been established at this stage: the side of the FM that is closer to, or further away from, the IM becomes the dorsal or ventral side, respectively [1], [8] (Fig. 1f). Subsequently, the FM produces all floral organs that will form a flower. The patterns of organ initiation, such as the position, number, and timing, might already be distinct along the D-V axis in the FM for a bilaterally symmetric flower (e.g., Fig. 1f). After initiation, organs along the D-V domains often follow different morphogenesis programs, which help to elaborate the divergence along the D-V axis in the final floral forms. Furthermore, **evolution of floral symmetry appears to be highly labile**. The transitions from radial to bilateral symmetry are thought to help attract bilaterally visioned pollinators and considered a key innovation in many species-rich flowering plant lineages, including asterids, legumes, and orchids [1], [9], [10].

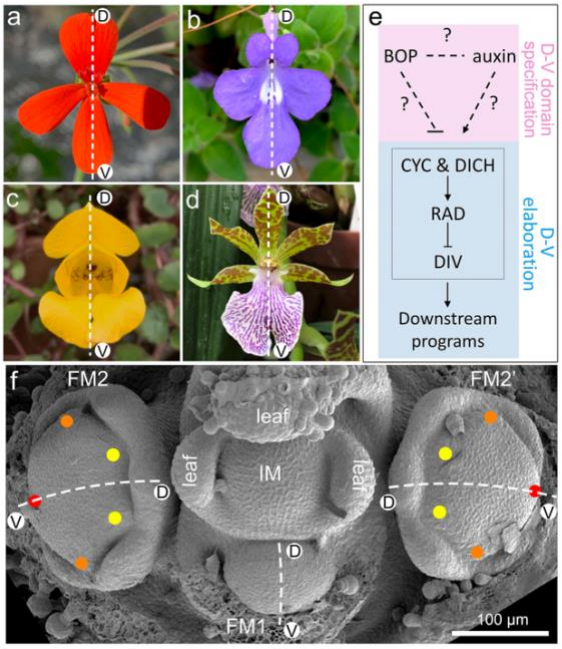

Figure 1. Bilateral symmetry is one of the recurrent patterns in floral forms. (a-d) Examples of bilaterally symmetric flowers in the UConn greenhouse: *Pelargonium tongaense* (a), *Streptocarpus saxorum* (b), *Impatiens repens* (c), and *Zygopetalum maculatum* (d). **(e)** Current knowledge of molecular pathways for D-V patterning. Dashed lines marked with question marks are relationships that will be tested in the current proposal. **(f)** A developing *Mimulus*. FM1 is the youngest FM produced by the IM and no floral organ has been produced yet. FM2 and FM2' are in the process of initiating petal primordia. Yellow, orange, and red dots represent dorsal, lateral, and ventral petal primordia, respectively. Dashed white line represents the D-V axis. Bilateral symmetry is already apparent in FM2 and FM2': the spacing between the two dorsal petals is smaller than the spacing between other adjacent petal primordia.

Through a series of elegant experiments started in the 1990s, the molecular basis of organ differentiation along the D-V axis was first revealed in snapdragon (*Antirrhinum majus*) flowers (Fig. 1e) [11]–[14]. The identity of dorsal organs is controlled by two paralogous genes from the TCP transcription factor family, *CYCLOIDEA* (*CYC*) and *DICHOTOMA*

(*DICH*), while the development of the ventral organs is controlled by a *MYB* transcription factor gene, *DIVARICATA* (*DIV*). *CYC* and *DICH* activate another *MYB* gene, *RADIALIS* (*RAD*), on the dorsal side, which is hypothesized to be mobile and restricts the activity of *DIV* to the ventral side. This discovery of CYC-based D-V developmental network led to intensive research on the *CYC* homologs in various plants. Strikingly, almost in all cases examined to date, floral bilateral symmetry is associated with the differential expression of *CYC*-like genes along the D-V axis [15]–[17], making it an excellent example of how convergent evolution can occur through repeated recruitment of the same developmental network.

The extensive studies in the past 25 years, **however, were not able to resolve the positional information that pre-patterns the expression of *CYC***. The dorsal-specific expression of CYC is established in the FM prior to the initiation of any organ primordium [11], but how is CYC specifically activated in the dorsal domain is still unknown. This also raises the question whether the independent recruitment of *CYC*-based developmental program in a wide range of taxa with bilaterally symmetric flowers is primarily due to *cis*-regulatory changes of the *CYC*-like genes or changes in the upstream regulators of the *CYC-RAD-DIV* network. A major impediment to elucidating the molecular programs upstream of the *CYC-RAD-DIV* network is the lack of appropriate functional tools in most plants with bilaterally symmetric flowers. Even for the classical genetic model system snapdragon, isolation of genes has mainly relied on endogenous active transposons, and functional tools that require stable transgenic transformation remain inaccessible.

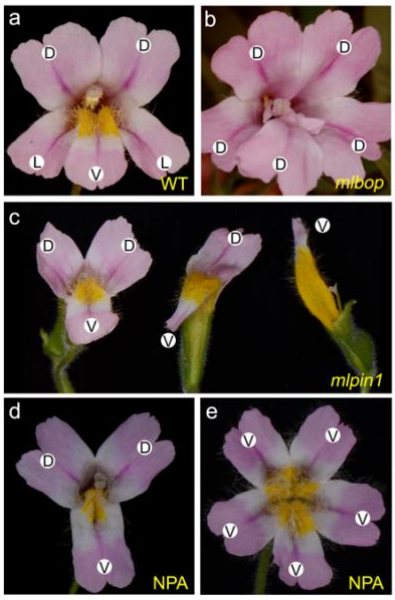

Figure 2. *Mumulus lewsii* as a study system for D-V domain establishment. (a) A WT *M. lewsii* flower. **(b)** A *mlbop* flower. **(e)** *mlpin1* flowers. **(d-e)** WT flowers with NPA application.

In this proposed project, I will take advantage of the myriad of functional tools and genetic resources that are now available in the new model system *M. lewisii*, which has high fecundity (~1,000 seeds per fruit), a short generation time (~ 3 months seed to seed), and copious flower production yearlong in the greenhouse [18]–[20]. *Mimulus lewisii* flowers exhibit typical bilateral symmetry, consisting of two dorsal petals, one ventral petal with contrasting yellow nectar guides, and two lateral petals exhibiting intermediate morphology between dorsal and ventral petals (Fig. 2a). The *M. lewisii* genome contains two genes (*MlCYC1* and *MlCYC2*) that are orthologs of *CYC* and *DICH* in snapdragon [21]. Two intriguing mutants with altered *CYC* expression have been generated in my host lab through EMS mutagenesis.

The first mutant is caused by a loss-of-function mutation in the gene *BLADE-ON-PETIOLE* (*MlBOP*). All petals of the *mlbop* mutant are completely dorsalized and the flower appears radially symmetric (Fig. 2b). Some of the stamens are also petaloid to various degrees in the mutant flower. Preliminary data showed the expression of both *MlCYC* paralogs were upregulated in all *mlbop* petals. **The second mutant** is caused by a loss-of-function mutation in *PIN-FORMED1*, *MlPIN1*, which encodes the auxin efflux carrier responsible for polar auxin transport. The *mlpin1* plants produce flowers with reduced and variable petal numbers; however, the identity and development of the ventral petal is remarkably robust (Fig. 2c). Consistent with the role of MlPIN1 in auxin polar transport, exogenous applications of the auxin polar transport inhibitor, NPA, resulted in floral phenotypes similar to the *mlpin1* mutant (Fig. 2d; [19]). Curiously, some of the NPA-treated flowers become completely ventralized and radially symmetric (Fig. 2e; [18]). The phenotypes of *mlbop* and *mlpin1* raise a series of questions regarding their relationships with the D-V domain determination (Fig. 1e), and **these mutants provide an unprecedented opportunity to investigate the molecular pathways upstream of *CYC*, which formed the foundation to this research proposal**.

RESEARCH OBJECTIVE 1: Determine the genetic relationship among *MlBOP*, *MlPIN1*, and *MlCYC1/2*

Rationale: Although the functions of *BOP* and *PIN1* have been well studied in the model species *Arabidopsis thaliana*, which has radially symmetric flowers, it is unclear how those functions can be

translated into D-V patterning in bilaterally symmetric flowers. The *BOP* genes encode proteins with a BTB/POZ domain and the two *BOP* paralogs in *A. thaliana* function redundantly to control petal numbers, organ abscission, and leaf morphogenesis [22]–[26]. PIN1 is a crucial protein for directing auxin trafficking from cell to cell and is involved in various morphogenesis and organogenesis processes [27]–[30]. The *pin1* mutants in *A. thaliana* are characterized by a naked inflorescence without flowers due to the failure of generating local auxin maxima, which is key to floral primordium initiation [31]. Studies in Asteraceae have shown that exogenous auxin application phenocopies overexpression of the *CYC* homologs [32], suggesting auxin concentration may play an important role in determining D-V patterning, but the mechanisms remain unclear.

Methods: Firstly, I will generate *mlcyc1 mlcyc2* double mutants in *M. lewisii* using CRISPR-Cas9 mediated genome editing, the protocol of which has been employed to generate many mutant lines in my host lab (e.g. [33], [34]). Multiplexed guide RNAs targeting both *MlCYC1* and *MlCYC2* will be cloned into the vector pRGEB32-BAR [35], which carries *Cas9* and the herbicide resistance gene *BAR*. The final plasmid will be transformed into *Agrobacterium*, followed by plant transformation with the well-established "floral spray/vacuum infiltration" method [18]–[20], [33], [34]. Transgenic seedlings will be selected by spraying herbicide. Homozygous mutants will be identified by phenotype (expected to be similar to Fig. 2e) and will be confirmed by DNA sequencing.

To test the genetic relationships between *MlBOP*, *MlPIN1*, and *MlCYC1/2*, I will cross the single mutants to generate *mlbop mlpin1* double mutant, as well as triple mutants of *mlbop mlcyc1 mlcyc2* and *mlpin1 mlcyc1 mlcyc2* (Table 1). For all single, double, and triple mutants, I will carefully document both vegetative and reproductive phenotypes across multiple developmental stages. The phenotypes of *mlbop* and *mlpin1* single mutants, as well as the results from NPA application, indicate that *MlBOP* and *MlPIN1* both function upstream of the *CYC* genes. Therefore, I anticipate both the *mlbop mlcyc1 mlcyc2* and *mlpin1 mlcyc1 mlcyc2* triple mutants phenocopy *mlcyc1 mlcyc2* double mutants to a degree, and flowers of *mlpin1 mlcyc1 mlcyc2* are also most likely to exhibit decreased and variable organ numbers. Meanwhile, although the phenotype of *mlpin1 mlbop* double mutant is difficult to predict, it will be informative to determine whether *MlBOP* and *MlPIN1* function independently or via the same pathway; if the latter, which gene is epistatic to the other.

Table 1. Summary of genetic lines and reporter constructs for each research objective.
Genetic lines marked with asterisks (*) are already available from the Yuan lab. Lines shaded in green or blue in Objective 1 or 2 will be used for Objective 2 or 3, respectively

Objective 1	Objective 2	Objective 3
• WT *	• pMlPIN1::MlPIN1-YFP *	• Co-IP/MS
• *mlbop* *	• DR5rev::RFP *	• ChIP-seq
• *mlpin1* *	• pMlBOP::MlBOP-CFP	
• *mlbop mlpin1*	• pMlCYC1::MlCYC1-mCherry	
• *mlcyc1 mlcyc2*	• pMlCYC2::MlCYC2-mTurquoise2	
• *mlbop mlcyc1 mlcyc2*		
• *mlpin1 mlcyc1 mlcyc2*		

RESEARCH OBJECTIVE 2: Examine the expression dynamics of *MlBOP*, *MlPIN1*, and *MlCYC1/2*

Rationale: To understand the developmental mechanism establishing the D-V domains in the FM and young floral buds, we need a clear knowledge of the spatiotemporal expression dynamics of the key regulators (*MlBOP*, *MlPIN1*, *MlCYC1/2*) in the wild type (WT) and examine how these expression dynamics are perturbed in the various mutant backgrounds (*mlbop*, *mlpin1*, and *mlbop mlpin1*; Table 1).

Methods: The mRNA expression pattern of *MlBOP*, *MlPIN1*, and *MlCYC1/2* will be determined using *in situ* hybridization. Probes of 200-500 bp will be designed in the non-conserved regions of each gene. *MlCYC1* and *MlCYC2* share 49% identity at the amino acid level and are highly divergent from each other at the nucleotide level. Paralog-specific *in situ* hybridization of the two *CYC* genes in *Mimulus guttatus* have been published in [36], which will assist me to determine the best regions to design gene-specific probes. The young inflorescences of WT and mutants will be fixed and embedded in wax. The developmental window that I will focus on starts from the earliest stages of the floral meristem without any organ primordia to the stage that all floral organ primordia are produced (Fig. 1f)

The patterns of protein distribution and auxin response will be analyzed using live confocal microscope imaging. Currently, two reporter lines (in the WT background) are available in my host lab: a PIN1 reporter line carrying a yellow florescent protein tag driven by the native *MlPIN1* promoter

(pMlPIN1::MlPIN1-YFP), and an auxin response reporter line carrying a red fluorescent protein tag driven by the synthetic auxin-responsive promoter DR5rev (DR5rev::RFP; [18], [19]). Distribution of MlPIN1-YFP will inform us the direction of auxin trafficking, while signals of DR5rev::RFP will reveal the position of auxin maxima. Additionally, I will construct florescent reporter lines for *MlBOP*, *MlCYC1* and *MlCYC2* (Table 1). Following the same protocol, I will take the 3-kb promoter region and the coding sequences of the respective genes, and clone the fragments into different pGWB600 vectors, a vector series that carries different florescence protein tags. Different florescent protein tags are chosen for each gene so that signals of multiple genes can be visualized simultaneously when the reporter genes are brought together into the same plant by genetic crosses. The reporter constructs will be transformed into the wild-type and then crossed into different mutant backgrounds.

For live imaging, young inflorescences will be dissected, grown on a tissue culture media with agarose, and stained with FM 4-64 dye, which outlines the shapes of all the cells and its signal can be collected from 640 to 700 nm that will not interfere with signals of other florescent reporters. In this way, I can not only observe the expression dynamics of different proteins, but also compare properties of cells in the primordia and floral meristems in different genetic backgrounds. Changes in cellular properties will be quantified using MorphographX [37], a software that I have good knowledge of and that is specialized for analyzing and quantifying 4D live-imaged confocal data [38].

Results from this objective will help us answer several important questions. For instance, is there any difference in auxin dynamics during dorsal vs. ventral organ initiation in the WT? Is *MlBOP* expressed before or after the formation of auxin maxima? Are the spatial patterns of *MlBOP* and *MlCYC1/2* expression coinciding, overlapping, or complementary? Regarding *mlpin1* flowers that lack dorsal petals, is it due to missed expression of *MlCYC1/2* or due to the lack of auxin maxima?

RESEARCH OBJECTIVE 3: Investigate the molecular mechanism of MlBOP regulating *MlCYC1/2* expression

Rationale: The upregulation of *MlCYC1/2* in the *mlbop* mutant strongly suggests that *MlBOP* functions upstream of *MlCYC1/2*, but the molecular mechanism by which *MlBOP* regulates the expression of *MlCYC1/2* remains elusive. The BOP protein does not contain any DNA binding domain [23], which means that regardless of whether BOP directly or indirectly regulates *CYC*-related or auxin-related genes, it does so by partnering with other transcription factors that can bind DNA. Identifying the protein partners and the direct gene targets of BOP will be crucial for fully deciphering the topology of MlBOP-dependent D-V patterning network.

Methods: Utilizing the pMlBOP::MlBOP-CFP lines produced from Objective 2, I will 1) identify the protein partners of MlBOP using co-immunoprecipitation coupled mass spectroscopy (Co-IP/MS) and 2) identify the DNA targets of the protein complex using chromatin immunoprecipitation followed by sequencing (ChIP–seq). The target tissues for both Co-IP and ChIP-seq will be determined according to the expression analysis from Objective 2. At least three biological replicates will be included and tissues without MlBOP-CFP expression will be used as negative controls. Commercial anti-GFP antibodies, which can recognize CFP, will be used to immunoprecipitate MlBOP-CFP in both experiments.

For Co-IP, total proteins from target tissues will be isolated, incubated with anti-GFP beads, washed for three times (to eliminate non-specific bindings), followed by immunoblotting. For mass spectrometry, protein elutes will be separated and digested with Trypsin, and analyzed using the Triple Quadrupole Mass Spectrometer: 4000 QTrap in the Proteomics & Metabolomics Facility at the University of Connecticut (UConn). I will identify and analyze the peptides using the software Mascot and the protein database from *A. thaliana* as reference.

For ChIP-seq, tissues will be harvested and cross-linked with formaldehyde, and chromatin will be isolated and sheared into 300-500 bp fragments using sonication. The fragmented chromatin will then be immunoprecipitated by anti-GFP antibody. To construct the ChIP-seq libraries, I will follow the manufacturer's protocol of NEBNext Ultra II DNA Library Prep Kit for Illumina. High-throughput sequencing will be carried out using Illumina Novaseq 6000 with a 150-bp read length. One of my Ph.D. thesis chapters focused on transcriptome profiling using RNA-sequencing [39], from which I gained familiarity with processing raw sequencing reads, mapping reads to genome, calling peaks, and various downstream statistical analyses. All these analytical skills will assist me with analyzing the ChIP-seq data to identify the target genes of MlBOP. It will be particularly interesting to examine whether any genes related to auxin biosynthesis or trafficking, or genes related to the *CYC*-network, are the direct targets of MlBOP.

BROADER IMPACTS

The broader impacts of this proposal have three main tenets: 1) raise public awareness of plants and plant sciences, 2) disseminate research results and resources in *Mimulus*, and 3) mentor undergraduate students for both research experience and professional development.

- *Public engagement*

Increasing public awareness and appreciation to plants and plant sciences has been, and will be, a mission for my entire career. After participating in various outreach programs during graduate school, I found one of the most efficient ways to engage with people is through art. For the past few years, I have maintained a consistent presence on social media with routine posts to show the beauty of plants with fun facts, which obtained a wide range of attention. Since I moved to UConn, I have been deeply impressed by the greenhouse collections here and started a hashtag #UConnGreenhouse, featuring one plant every week. Each post attracted 7,000-15,000 engagements (i.e. times that people saw the post on Twitter) within a week. I plan to continue this weekly hashtag, engage with people who interact with the posts, and encourage more people to use the hashtag as well.

In addition, I will partner with the UConn Plant Biodiversity Conservatory and host several outreach events to CT residents, local K-12 schools, and UConn students all year round. This includes greenhouse tours, houseplant care workshops, scavenger hunts, and collaboration with the School of Fine Arts to display plant-inspired art pieces in the greenhouse.

- *Scientific community building*

I will be actively involved in a few professional societies to disseminate results from my research, share resources in *Mimulus*, and promote open science and community building. During my PhD, I spear-headed and participated in a number of outreach initiatives, as well as diversity, inclusion, and belonging initiatives, largely through my capacity as the student representative (2018-2020) and as a member of the Strategic Planning Committee (2021) of the Botanical Society of America (BSA). This experience made me realize that working with professional societies is an excellent way to reach a large number of people in the scientific community, regardless of their locations, career stages, or backgrounds. I will continue and expand this work to not only BSA, but also Society of Developmental Biology (SDB). In addition to presenting the research findings in the annual conferences, I will also host annual virtual workshops in the spring to teach the genomic, genetic and molecular tools in *Mimulus*. Findings from this proposal will also be written as popular science articles and submit to weblogs such as *Plant Science Bulletin* and *Botany One*, both of which I have published editor's notes and opinion pieces before.

- *Student mentoring*

Many experiments in this proposal can serve as a good entry point for undergraduate students to gain experience in molecular biology and plant biology. I will take advantage of the various undergraduate research programs at UConn (e.g., McNair Scholars Program, Summer Undergraduate Research Fund, Work-Study Research Assistant Program) and mentor students, particularly those from underrepresented populations, not only to help them understand more about plant science and the research process in general, but also improve their critical thinking and communication skills. I will help mentees to recognize that the skills they learn from a plant development research project can lead to a variety of career paths, and guide them with resources and connections from the professional societies to prepare for their future careers.

Besides hands-on mentoring in the lab, I have been, and will continue to be, a mentor for the PLANTS program (Preparing Leaders and Nurturing Tomorrow's Scientists: Increasing the diversity of plant scientists) of BSA and will apply to mentor students through the Choose Development! program of SDB. Both programs aim to increase the number of undergraduate students from diverse backgrounds that enter graduate programs in the field of botany or developmental biology.

TRAINING OBJECTIVES AND CAREER DEVELOPMENT

My career goal is to become a tenure-track faculty at a university where I can pursue my scientific curiosity and conduct rigorous research, empower students to be creative, independent, and critical thinkers, and promote a safe and inclusive environment in our scientific community. The NSF PRFB will prepare me for this goal by providing an unparalleled opportunity to facilitate my development and training as a researcher, educator, and advocate.

My ultimate research interest seeks to understand the origin and evolution of floral ground plan at the molecular level. During my PhD, I focused on one major aspect of the floral ground plan, floral

meristem termination, in a non-traditional model system, *Aquilegia*. From my PhD work, I obtained a solid background in plant morphology and evolution and became very familiar with various microscopy techniques. The current proposed research focuses on another major aspect of the floral ground plan, the dorsal-ventral patterning of the floral meristem. The proposed research is very different from my PhD work not only in the specific research questions, but also in the use of various genetic, molecular, and biochemical methods that I did not have experience with. Therefore, a main goal of this fellowship for me will be learning cutting-edge molecular and genetic tools that are crucial for developmental studies. Acquiring these skills will be indispensable to run my own research program in the future.

In addition, my only experience with the US higher education so far was from my PhD institution, which is a private university that receives countless privileges. University of Connecticut, on the other hand, is a public land-grant university with a much larger undergraduate student body. I am looking forward to interacting with the students, faculty, and staff; doing outreach and mentoring students here will help me to gain a more comprehensive understanding of the US higher education system. I will greatly benefit from this experience when forming my teaching philosophy and mentoring plans, and implementing efforts in promoting diversity, equity, and inclusion.

SPONSORING SCIENTIST AND HOST FIT

Dr. Yuan is the ideal host for this project. *Mimulus* has been subjected to intensive ecological and evolutionary studies for many decades, but Dr. Yuan recognized that *Mimulus* is also an excellent system for developmental studies because it is amenable to rigorous genetic analysis, developmental interrogation, and phenotypic perturbation. The Yuan Lab pioneered a wide range of functional tools (e.g., RNAi, CRISPR/Cas9 genome editing, over-expression, fluorescent reporter lines) in several *Mimulus* species over the years [18]–[20], [34], which established *Mimulus* as one of the very few plant model systems that have comparable resources as *A. thaliana*. In addition, his lab has a huge collection of EMS mutants that remain to be characterized. These functional tools and genetic resources will be critical for completing the proposed research objectives. Moreover, the Yuan Lab has a close relationship with the advanced light microscopy facility and the greenhouse facilities at the University of Connecticut, which have an outstanding reputation for staff support and management and will be a tremendous asset not only to achieving my research goals, but also to successfully carrying out the planned Broad Impact activities.

TIMELINE

Gantt chart detailing my proposal timeline. Q1: spring; Q2: summer; Q3: fall; Q4: winter.

Objectives	2022			2023				2024	
	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2
Research Objective 1									
Generate and characterize double mutants									
Generate and characterize triple mutants									
Research Objective 2									
In situ hybridization									
Make reporter constructs and generate transgenic lines									
Characterize expression dynamics in different backgrounds									
Research Objective 3									
Co-IP/MS; ChIP-seq									
Data analysis									
Broader impacts									
Public engagement									
Scientific community building									
Student mentoring									

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