Training and Career Development Plan

Results of previous research and scholarships

The final two years of my PhD training were supported by USDA-NIFA predoctoral fellowship project #NYC-153530. Outcomes of this research include the publication of two peer-reviewed manuscripts directly related to the aims of the fellowship. For the first manuscript, after training at a workshop in New York City, I used the Oxford Nanopore Technologies (ONT) MinION to generate long-read sequencing data and create a genome assembly for a disease resistant rice cultivar. The assembly was used to identify a candidate gene responsible for the disease resistance phenotype. In a second manuscript I cloned the candidate gene and demonstrated that it is sufficient to confer disease resistance. In addition to these two manuscripts, I was also able to apply my resistance gene analysis skills to a manuscript describing the genome of the giant sequoia, *Sequoiadendrum giganteum*.

The fellowship supported me for a one-month training at The Sainsbury Laboratory (TSL) in Norwich UK. Here I learned bioinformatic pipelines for RNAseq analysis and disease resistance gene identification in the lab of Dr. Matthew Moscou. The collaborative nature of the work supported by the fellowship greatly expanded my professional network to include peers and mentors at TSL, Johns Hopkins University, and more. Results of this research were presented at domestic and international conferences.

Teaching competency

I was able to further my development as a teacher and mentor with support from my predoctoral fellowship. Building on my previous experience as a teaching assistant, I designed and led a two-day, hands-on, genome editing module for first year plant biology graduate students. I received positive written feedback from students in the class and the material I developed has been used in subsequent iterations of the course. I have also been involved in mentoring several undergraduate summer research students.

As a postdoctoral fellow I will continue to develop my teaching competencies by seeking two teaching experiences each year. These experiences will include guest lectures in Dr. Springer's genetics course and a molecular plant-microbe interactions course. Dr. Springer is supportive of my desire to prioritize the development of teaching skills and has suggested my involvement with the UMN Preparing Future Faculty program. This program is open to postdoctoral researchers and offers courses such as 'Teaching in Higher Education' and 'Multicultural Inclusive Learning and Teaching'. These courses will complement my guest lectures and provide a strong foundation for my development into an effective and inclusive instructor when I secure a faculty position.

Plan for transition to career independence

In order to transition to career independence, it is important that I develop both technical and leadership skills during my postdoctoral training. The development of technical skills related to DNA methylation and gene expression will be accomplished by interaction with Dr. Nathan Springer, his lab members, and other members of the UMN community. In addition, through bi-weekly meetings with Dr. Springer, we will critically evaluate my technical and career development progress. Technical and professional development workshops (virtual or in-person) will be identified by Dr. Springer and myself and attended when appropriate

Dr. Springer's expertise in DNA methylation combined with the resources available through the University of Minnesota will provide a supportive environment for me to acquire the

skills necessary for success in the next step of my career. The generation of fluorescent reporter *Setaria italica* lines, a part of Objective III, will generate a valuable tool to launch my independent research group. These lines represent a novel resource to address plant-biotic interactions that will be critical for several research projects to investigate the basis of plant disease resistance. For example, plants that encode multiple reporters could be used to sort nuclei from cells experiencing different or overlapping stresses, such as heat and disease. Dr. Springer is supportive of me developing these materials and using them as the foundation of my research program when I depart his group.

Career and training goals

My goal is to develop an independent plant science research program at a public university in the US. My research will examine the relationship between disease resistance gene expression and evolution and DNA methylation. I will work to establish collaborations with academic and industry colleagues in order to achieve my overarching goal of developing and deploying effective disease resistance to growers.

I am enthused about teaching undergraduate and graduate level coursework in addition to leading a research group. My current expertise would allow me to teach courses on molecular plant-microbe interactions, general botany, and genomics. In order to increase student engagement, I plan to generate small-scale genomics and evolution projects based on existing, unanalyzed, datasets. Results of these projects will be published on a course website accessible to the plant research community. I believe that practical projects prepare students for their own research, reinforce complex concepts from the course, and provide a resource to other researchers.

Mentoring plan

Dr. Springer has expertise in DNA methylation and gene expression and has worked in several monocot systems. Members of Springer lab have established bioinformatics pipelines for large-scale methylome, transcriptome and genome variation studies. Interactions with Dr. Springer and members of his research group will provide valuable opportunities as I develop my bioinformatics skill-set and perspectives for –omic studies of crop plants. Dr. Springer is involved in a collaborative project with Dr. Feng Zhang to develop genome edited *S. italica* lines. I will work with Dr. Zhang to develop materials needed for Objectives II and III (see attached letter of support). I have also reached out to UMN plant pathology department professor Dr. Brian Steffenson who has agreed to help with sourcing pathogens and development of the assays proposed in Objectives I and II (see attached letter of support). This multi-department team will expand my professional network and facilitate input from a diverse team of experienced researchers.

Dr. Springer has committed to biweekly one-on-one meetings at which professional development and scientific progress will be discussed. At these meetings Dr. Springer will help me to identify development opportunities, provide feedback on experimental design, and help troubleshoot any technical issues I encounter. As mentioned above, I have developed relationships with additional faculty at UMN including Drs. Feng Zhang and Brian Steffenson (see letters of support), both of whom will provide additional access to resources and guidance during the course of the fellowship and beyond. These connections will help me to effectively recruit and mentor undergraduate students. Dr. Springer will actively help identify appropriate

opportunities to participate in scientific meetings and workshops to improve my communication skills and connect me with a broad cross-section of the scientific community.

In addition to the students and post-docs included in Dr. Springer's Biosketch (attached), he has acted as mentor to five graduate students, five post-doctoral researchers, and three visiting scholars, the details of which can be found on Dr. Springer's lab website: maizeumn.github.io/people/

Project Plan

Background

Cereal crops including maize, wheat, barley, and rice make up greater than 40% of the world's caloric intake and are important foundations for global food security (Savary et al., 2019). Despite management strategies, 15-20% of US wheat and maize crops are lost to pests and pathogens annually (Savary et al., 2019). Control of pathogens is often attained through application of pesticides, however this is often not economically feasible for growers. In addition to the economic impact of pesticide use, there can also be negative environmental and health impacts associated with the use of some pesticides (Zhang, 2018). An alternative approach to pathogen control is by growing cultivars that encode genetic resistance. Discovery of resistance often comes from screening domestic and wild germplasm collections (Lenne and Wood, 1991). These screens require large time and energy inputs and have been made more efficient by basic plant immunity research which has provided foundational knowledge on the types of genes and molecular mechanisms often associated with disease resistance (Kourelis and van der Hoorn, 2018). Understanding which genomic regions to focus on speeds up the resistance gene discovery and creates opportunities to engineer synthetic resistance genes.

Model systems are important for the advancement of many fields in biology. In the field of plant immunity much of our knowledge comes from work on the model dicot *Arabidopsis thaliana (Agrawal, 2018)*. The importance of *A. thaliana* as a model cannot be questioned, however many important crop plants are monocots and a more closely related plant may serve as a better model. *Setaria viridis* is a diploid C4 grass that grows in many niches around the globe. *S. viridis* has been developed as a monocot model due to its close phylogenetic relationship to important cereal crops, small genome size, short lifespan, and ease of growth. Importantly, *S. viridis* is transformable and seed can be collected from new transgenics in only eight to ten weeks (Brutnell et al., 2010; Li and Brutnell, 2011). Despite the benefits of *Setaria* as a model, no peer-reviewed studies have examined the *Setaria* response to pathogens or pests. A single preprint demonstrates that the production of serotonin by *Setaria* plays a role in defense against aphid infestation (Dangol et al., 2019). Genomic analysis of the closely related domesticated *S. italica* identified 281 plant immunity genes of the nucleotide binding and leucine-rich repeat (NLR) family (Bailey et al., 2018). It is surprising that the molecular plant-microbe interaction community has not embraced *Setaria* for probing cereal-pathogen interactions.

An emerging area in the study of plant-microbe interactions is the role of dynamic DNA methylation on defense gene expression. In plants DNA methylation occurs at cytosine residues in any context (CG, CH, or CHG where H is any other nucleotide). Studies in *Arabidopsis* have shown that methylation is laid down by DOMAINS REARRANGED METHYLASE 2 (DRM2) with the help of DECREASE IN DNA METHYLATION (DDM1) and maintained by METHYLTRANSFERASE1 (MET1) and CHROMOMETHYLASE2 and 3 (CMT2 and CMT3) (Zhang et al., 2018). Small RNAs guide methylation machinery to selectively methylate genomic regions, often repetitive sequences including transposable elements. Active demethylation occurs

through excision and repair carried out by REPRESSOR OF SILENCING 1 (ROS1), DEMETER (DME), DME-LIKE2 (DML2), and DML3 (Agius et al., 2006; Mok et al., 2010; Zhang et al., 2018).

The methylation status of genic and intergenic DNA sequences changes when plant cells undergo stress (Dowen et al., 2012; Deleris et al., 2016; Kawakatsu and Ecker, 2019). Regions of the genome that are primarily targeted by these methylation changes are transposable elements (TEs). TEs are generally highly methylated which keeps their expression levels low. Demethylation of TEs can lead to increased expression of the TE and nearby genes (Lisch, 2009). Plant immunity genes are often located in TE-rich regions of the genome and differential TE methylation is believed to play an important role in regulating the expression of these genes (van Wersch et al., 2020). Studies linking methylation and plant immunity have shown that decreased methylation is associated with increased resistance to biotrophic bacterial and oomycete pathogens (Dowen et al., 2012; Luna and Ton, 2012; Gohlke et al., 2013). Demethylation deficient *Arabidopsis* mutants are more susceptible to bacterial and fungal pathogens (Le et al., 2014). Little is known about demethylation the mechanism and specificity, but ~80% of regions targeted by demethylases are near annotated genes (Penterman et al., 2007). Demethylation of plant immunity gene promoters may be a mechanism for rapid expression changes. Dynamic DNA methylation/demethylation clearly plays an important role in plant-pathogen interactions.

To date, all studies looking at DNA methylation and gene expression changes in response to stress have used homogenized whole tissues. This approach has been sufficient to detect genome-wide dynamic DNA methylation and differential gene expression, however plant cells that are directly interacting with a pathogen experience and respond differently from cells that are distal to the infection. The ability to parse these pools of cells would allow finer resolution and detection of signal that may be go undetected in whole tissue homogenate. The plant-microbe interaction research community has developed several fluorescent reporter systems that selectively emit signal only in cells that are in direct contact with a pathogen (Henry et al., 2017; Park et al., 2017). These reporters allow the visualization of infected cells and could, potentially, be used for cell sorting prior to sequencing, but sorting these cells would require harsh protoplasting treatment which impacts methylation and gene expression.

As an alternative, the isolation of nuclei from tagged specific cell types (INTACT) system utilizes a transgenic plant with a cell-type or condition specific promoter driving a reporter protein fused to a nuclear membrane targeting motif (Deal and Henikoff, 2010). First developed for use in *Arabidopsis*, the INTACT system has been modified for use in tomato and the monocot rice (Ron et al., 2014; Reynoso et al., 2018). Tagged nuclei are collected with a crude nuclear extraction protocol and sorted via magnetic bead-based separation. The engineering of a reporter driven by a biotic stress inducible gene promoter to drive the INTACT system would allow the sorting of nuclei from cells that are directly experiencing stress for downstream DNA and RNA sequencing. This would improve the sensitivity of detecting differentially methylated regions.

Available Resources

Research in the Springer Lab focuses on monocot genomics, and recent efforts have been made to develop the *Setaria* system as a model for studying monocot DNA methylation. The Zhang lab at UMN has developed robust *Setaria* transformation protocols, enabling efficient generation of transgenic and genome edited *Setaria* lines. The key methylation genes in *Setaria*

have been identified and targeted for disruption using a CRISPR/Cas approach. These include knockouts of the MET1, DRM, CMT, and DDM and functional gene families (Table 1). The DRM1 knockout was prioritized as a proof of concept and, as of July 2020, transgene free DRM1 knockout plants (loss-of-function alleles for both DRM1 paralogs) have been identified. These plants are fertile without major morphological abnormalities. Methylome and transcriptome data for these lines and relevant controls are being generated and will be analyzed prior to the start of the fellowship. Demethylation pathway gene edits are proposed in Objective II (Table 1).

In addition to established transformation protocols, there are excellent genomic resources available for *S. viridis*. A reference genome was published in 2012 (Bennetzen et al., 2012) and a new assembly has been published as a preprint and will be available soon (Mamidi et al., 2020). The success of this fellowship will rely on accurate annotation of plant immunity genes and TEs in the *S. viridis* genome. Both plant immunity genes and TEs are present in high copy numbers in plant genomes and difficult to annotate. Specialized annotation pipelines will be run on the reference genome prior to analyzing differential methylation and expression data. This annotation has been proposed as a resource generation exercise in Objective I and will begin prior to the start of the fellowship.

Rationale and Significance

This project addresses the AFRI area of Plant Health and Production and Plant Products, because a better understanding of DNA methylation and defense gene regulation will empower the discovery and deployment of effective genetic resistance. Several studies have demonstrated the connections between biotic stress, dynamic DNA methylation, and defense gene expression, however each of these studies has been done in the model dicot *Arabidopsis* (Deleris et al., 2016). These *Arabidopsis* studies provide an important foundation, however monocots and dicots diverged 127-175 million years ago (Bell et al., 2010) and there are known differences in monocot and dicot methylation machinery (Springer and Kaeppler, 2005). A study focused on a single resistance gene in rice recently demonstrated that demethylation in reproductive tissues leads to tissue specific expression of the resistance gene expression, allowing disease resistance without a yield penalty (Deng et al., 2017). This demonstrates that dynamic DNA methylation is important in monocot immunity, but global analyses of the interplay between DNA methylation dynamics and disease resistance in monocots are lacking.

In this fellowship I propose to assess the role of DNA methylation in *Setaria* when plants are infected with a bacterial or oomycete pathogen. Undergraduate researchers will be trained to assist me in the automated phenotyping of genome edited *Setaria* plants. Biotic stress reporter lines will be developed for use both in this fellowship and as a foundation to my future research program. In addition to hypothesis driven research, each objective in this fellowship also delivers a resource to the plant science research community including an improved genome annotation, genome edited *Setaria* lines, and transgenic biotic stress reporter lines.

I have built a strong foundation in plant-microbe interaction research through my experience in industry and academia. The laboratory and computational skills I've developed position me well to examine the role of DNA methylation on plant resistance. This is a relatively young field, and a completely new field to me. Dr. Springer has extensive experience and expertise in measuring global DNA methylation and associated gene expression changes in several monocot systems. Dr. Zhang's expertise in *Setaria* transformation will enable the rapid

development of materials needed for this project. Importantly, Dr. Steffenson is well connected to small grain growers and the cereal disease community and will provide guidance in the translation of my basic research to these stakeholders. I am confident that, with their support, I will be able to accomplish the objectives set forth in this proposal, make significant contributions to the field of dynamic DNA methylation, and develop the leadership skills necessary to start an independent research program.

Approach

Hypothesis:
During biotic stress promoter regions of immunity genes will be demethylated and immunity gene expression will increase.

Objectives:

I. Measure changes in DNA methylation and gene expression during biotic stress in *Setaria viridis*.
II. Assess the role of specific DNA methylation genes in biotic stress response of *S. viridis*.
    a. Phenotyping methylation mutants
    b. Gene expression analysis of methylation mutants
III. Sort infected and uninfected cells from homogenized *Setaria* tissue

Methods:

Objective I: Measure changes in DNA methylation and gene expression following biotic stress in *Setaria viridis*.

Resource generation: Annotation of TEs and immunity genes in the *Setaria* genome. Genomic resources exist for *S. viridis*, with additional resources soon to be available (Bennetzen et al., 2012; Mamidi et al., 2020). These high-quality assemblies enable a thorough annotation of TEs and plant immunity genes in the *S. viridis* genome. Pipelines for TE and immunity gene annotation are well established (Stitzer et al., 2019). The annotation will be complete prior to the start of the fellowship and will be a valuable resource for the *Setaria* and plant immunity research communities.

Approach: The bacterial pathogen *Xanthomons vasicola* pv. holcicola (Xvh) and the oomycete pathogen *Sclerospora graminicola* (Sg) have been selected for use in Objective I. Xvh causes bacterial leaf streak of sorghum and is closely related to *X. vasicola* pv. vasculorum the causal agent of bacterial leaf streak of corn, an emerging issue in the US Corn Belt [Lang]. The oomycete pathogen Sg causes downy mildew of foxtail millet and maize and is closely related to several other downy mildews that cause economically important diseases on various other cereals. *S. viridis* is susceptible to both Xvh and Sg and inoculation methods on closely related monocots have been established (Kulkarni et al., 2016; Lang et al., 2017).

S. viridis plants will be grown in controlled growth chambers under standard conditions and inoculated with pathogens at an appropriate growth phase. Xvh will be syringe infiltrated into leaf blade tissue while Sg will be suspended in buffer and spray inoculated onto plants. DNA and RNA will be extracted from diseased tissue samples for bisulfite and RNAseq. RNAseq strategies will be designed to capture both mRNA and small RNAs. Small RNAs are known to play important roles in guiding the methylation machinery. Non-inoculated plants will be grown in the same conditions and sampled at the same time as inoculated plants to serve as

controls for methylome and transcriptome analyses. Data will be analyzed using established best practices currently used by the Springer group. Genome-wide identification of differentially methylated regions (DMRs) will be performed with a focus on DMRs located within 2 kb of annotated immunity genes. Differential expression analyses, with a focus on TEs and immunity genes, will be performed using the RNA and small RNA datasets.

Expected Outcomes: 1) Improved annotation of immunity genes and TEs in the *Setaria* reference genome 2) The first transcriptomic study of *Setaria*-pathogen interactions 3) Association of DMRs and *Setaria* immunity gene expression 4) Expertise in bisulfite sequencing and analysis.

Pitfalls: Previous studies in *Arabidopsis* have demonstrated that methylation changes can be detected in homogenized infected leaf tissue. It is possible that the magnitude of differential methylation in *Setaria* under biotic stress will be too small to be detected in this manner. If preliminary analysis indicates that this may be the case, I will adjust my sampling strategy by using a small punch to collect tissue only from the margins of symptomatic tissue rather than whole tissue homogenate.

Objective II: Assess the role of specific DNA methylation pathways in biotic stress response of *Setaria*.

Resource generation: Genome edited methylation/demethylation pathway *Setaria* lines. CRISPR/Cas mutants of the MET1, DRM, CMT, and DDM methylation gene families are currently in the transformation pipeline at UMN. Transgene free knock-outs of all members of the gene family for MET1 and DRM have been acquired and there is evidence for successful editing at the other targets (Table 1). Transgene free double DRM knockout plants have been sampled to generate sequencing libraries for baseline methylome/transcriptome characterization. These libraries are currently being sequenced at the UMN Genomics Center the results of which will be available prior to the start of the fellowship. It will be important to include mutants in the demethylation pathway as well. I have identified *Setaria* homologs of the *Arabidopsis* demethylation proteins ROS1 and DME and will design CRISPR/Cas constructs to generate these targeted gene disruptions.

		A	B
Methylation	Met1	Sevir.2G06230	Sevir.9G05350
	DDM	Sevir.2G02780	Sevir.9G11100
	DRM	Sevir.9G57480	Sevir.9G49620
	CMT	Sevir.9G30620	Sevir.9G48290
De-Meth	ROS1	Sevir.2g158400	NA
	DME	Sevir.3g228900	NA

Table 1: *Setaria* homologs of methylation and demethylation genes. There are two copies of each of the methylation genes, A and B. Grey rows indicate targets for which gene edited *Setaria* plants have been generated

a – Phenotyping methylation mutants – Wildtype and mutant *Setaria* lines will be grown side-by-side in a growth chamber. Gross phenotype data will be collected with a semi-automated imaging protocol that has been developed by the Springer lab for use on maize (Enders et al., 2019). The imaging protocol will be optimized for *Setaria*. Agronomic traits such as plant height, percent reproductive tillers, and percent fertility will be recorded for wildtype and mutant plants. The quantification of traits and growth rate for plants in control conditions will provide a baseline for assessing these same traits in plants under stress conditions.

Previous studies have shown that when Arabidopsis is unable to maintain methylation, plants are more resistant to pathogens Luna and Ton, 2012; Gohlke et al., 2013). The same pathogens (Xvh and Sg) and inoculation techniques used in Objective I will be used for Objective II. Plants will be photographed over the course of the infection with the imaging pipeline which includes a hyperspectral camera. Undergraduate mentees will be heavily involved in optimizing the imaging pipeline and converting images into quantitative growth and symptom development time courses. These time courses will allow detection of small differences in the wildtype and mutant response to these biotic stresses.

To contextualize differences observed during disease, it will be important to determine if the mutant *Setaria* plants are unable to deal with stress generally. In order to determine this, uninfected plants will be grown under two different abiotic stress conditions, heat and drought. Phenotyping of these plants will be done using the established imaging protocols.

b – Gene expression analysis of methylation mutants – Any *Setaria* methylation mutant that show a detectable difference from wildtype under any condition will be selected for RNAseq analysis. RNA will be extracted from diseased wildtype and mutant lines and used to perform transcriptome profiling. This will allow the association of differential gene expression with the differential disease or abiotic stress phenotypes. We predict that differential methylation of TEs near immunity genes will be associated with increased expression of these genes.

Expected Outcomes: 1) generation of a panel of methylation/demethylation mutants for the *Setaria* research community 2) an advanced phenotyping platform for *Setaria* 3) association of disease symptoms with gene expression data 4) training undergraduate researchers in plant-pathogen interactions and automated phenotyping.

Pitfalls: Preliminary data indicates that the DRM1 disruption plants are fertile and do not display any obvious growth defects, however it is possible that some of the additional proposed knockouts may be lethal. If this is the case, I will use an alternative method to decrease or selectively knock-out expression of these genes. A virus-induced gene silencing (VIGS) strategy would result in reduction, but not elimination, of expression of these genes. Alternatively, it may be possible to engineer inducible suppression of methylation and demethylation genes. There is some risk that VIGS or inducible suppression will not work, will be lethal, or will take too long to generate for use in this fellowship. This seems unlikely, but the existence of the DRM1 mutant indicates that, at the least, I will be able to generate methylation and expression data in this background while troubleshooting.

It is also possible that no mutants will show growth or symptom differences under biotic stress. If this is the case, I will still carry out molecular phenotyping on a select group of plants to determine if this more sensitive technique is able to identify differences in gene expression that do not result in changes that are detectable with the imaging platform.

Objective III: Sorting of infected and uninfected cells from *Setaria* tissue

While the experiments described above will provide valuable insights into the mechanisms of biotic stress response in *Setaria* and the role of DNA methylation in mediating this response these experiments are all focused on crude homogenization of cell populations. Meaningful insights into the responses of plants to pathogens will likely rely upon increased understanding of cell-specific responses. Objective III will focus on developing key resources to enable these types of studies.

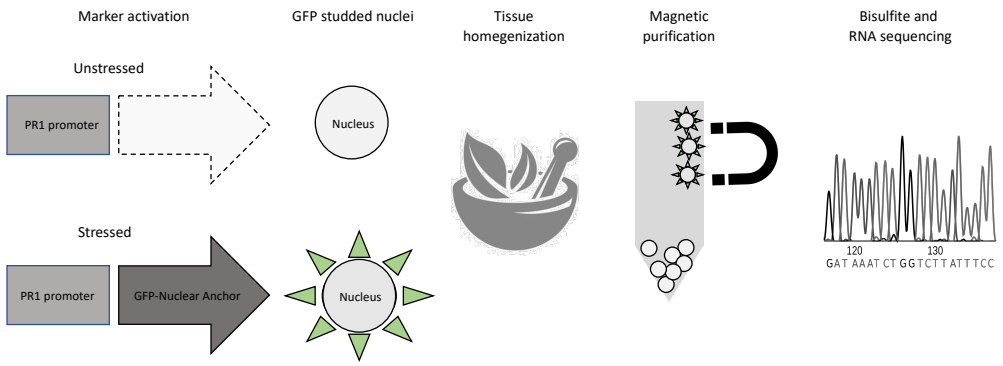

Figure 1: INTACT workflow. Transgenic reporter plants express nuclear anchored GFP when activated. Whole tissues are homogenized. Magnetic antiGFP beads are used to purify only those cells that are expressing the reporter. Stressed and unstressed cells can be sequenced separately.

Resource generation: Two transgenic *Setaria* INTACT biotic stress reporter lines will be generated. Each reporter construct will encode the green fluorescent protein (GFP) coding sequence fused to a nuclear membrane anchoring domain. In this way, when activated, nuclei can be sorted with antiGFP antibody-bound magnetic beads to separate the nuclei from stressed and unstressed cells (Figure 1). A reporter construct will be generated to respond to the plant hormones salicylic acid (SA) and jasmonic acid (JA) associated with biotrophic and necrotrophic infections respectively. The first reporter will use the promoter of the *Setaria* homolog of the SA responsive PATHOGENESIS-RELEATED 1 (PR1) gene. The second reporter will contain the promoter of the ALLENE SYNTHASE OXIDE 2-LIKE (AOS) gene which is induced by JA in the grass *Brachypodium dystachion* (Kouzai et al., 2016). Three positive transformants for each construct will be selected for further characterization. Validation will be carried out by induction of the reporter by exogenous SA or JA application followed by epifluorescence imaging. Transgenic lines that behave as predicted will be advanced to disease assays.

Approach: Following validation, stress reporter lines will be grown in growth chambers and inoculated with the pathogens and assays described above. The PR1 promoter line will be prioritized because both the pathogens selected are biotrophs and should strongly induce the expression of the reporter. Infected plant material will be homogenized, and a crude nuclear purification will be carried out. Purified nuclei will be separated using magnetic anti-GFP beads. These beads will capture nuclei expressing the GFP disease stress reporter. Nuclei from cells not under stress will not bind the beads and can be collected separately. Both populations of cells will be collected and submitted for RNAseq. Gene expression analysis will be conducted to determine differential gene expression. DNA collected from the same nuclei can be submitted for bisulfite sequencing to determine if methylation patterns differ in the two pools of nuclei. The results of both methylation analyses can be compared with that generated in Objective I to determine if there are differences in the cell sorted analysis compared with the whole tissue homogenized samples.

If time allows, the AOS reporter line will be assayed with the fungal wheat head blight pathogen *Fusarium graminearum* (Fg). Fg is a necrotrophy and infection should activate the JA pathway inducing the expression of AOS promoter driven GFP. Analysis of these plants will be carried out in the same manner described above for the PR1 reporter plants.

Expected Outcomes: 1) Generation of biotic stress reporter *Setaria* lines that will be of value to the plant-microbe community and a foundation for my future independent research

program 2) High resolution analysis of DRMs and gene expression in cells sorted from a tissue homogenate 3) Comparison of DNA methylation and expression data from whole tissue and sorted nuclear preperations.

Pitfalls: It will be important to confirm that the selected *Setaria* genes respond as predicted prior to full development of the reporter system. Alternative promoter elements can be selected if PR1 and AOS do not behave as predicted. Initial epifluorescence imaging to validate constructs may be challenging due to leaf blade autofluorescence. Note that this will not impact the nuclear sorting, but it is still important to validate the constructs. As an alternative, I will generate protoplasts from transgenic tissue for reporter induction and validation. It is possible that the RNA content of the nucleus will not be representative of the RNA content of the intact cell, however reports suggest that this is not the case (Palovaara and Weijers, 2019). The DNA and RNA requirements for the sequencing technologies selected are relatively low, however it may be challenging to collect enough stressed cell nuclei for downstream analysis, if this is the case several leaves can be pooled prior to homogenization.

Timeline and Evaluation Plan

Timeline: I will begin research in the Springer Lab in September of 2020, nine months prior to the start of the fellowship. In this time, I will contribute to the analysis of existing maize DNA methylation data and begin resource generation activities of **Objectives I and II**, including annotation of immunity genes and TEs in the *Setaria*. These activities will allow me to begin productive research at the onset of the fellowship.

Efforts on **Objective I through III** will be staggered over the duration of the fellowship. All resources are currently available for the completion of **Objective I**, so this will be prioritized in year 1. **Objectives II and III** require the generation of genome edited or transgenic *Setaria* plants. It will be important that transformation constructs are designed, built, and delivered to Dr. Zhang's facility for transformation in the middle of year 1. Work on **Objective II** will begin before Y2. Ideally the demethylation mutants will be available at this time, but if not, work will begin with the available methylation mutants and demethylation mutants will be assayed as they become available. The biotic reporter lines required for **Objective III** are traditional transgenic plants and should be easier to generate and screen than the methylation/demethylation pathway knockouts. Work on **Objective III** will begin in early Y2 and continue through the end of the fellowship, though lab work will ramp down in the last quarter of Y2 to allow adequate time for data analysis and documentation.

Milestones: Expected outcomes are designated in the description of each Objective above. In addition to these outcomes, I will present my research at a scientific conference each year, either Plant and Animal Genome or the APS Plant Health meetings. Student comments from teaching experiences will be collected and reviewed with Dr. Springer. I will formally mentor one undergraduate and train them in basic lab techniques, plant pathology principles and assays, and scientific communication. I aim to publish the result of each of the three Objectives as three manuscripts submitted to appropriate open access journals.