Membrane stability in times of stress: activation of SENSITIVE TO FREEZING2

A. Training/Career Development Plan

My career in science is the direct result of early on-farm experiences combined with a passion for learning. Through my research experiences, I have observed firsthand the connection between the future of agricultural research and the advancement of biotechnology. Our growing population and changing climate motivates me to continue researching biotechnological techniques we can use to generate crops that will grow and succeed in adverse conditions. My current Ph.D. research focuses on understanding how plants sense and adapt to survive freezing stresses. With an agronomic background and biochemical training, I plan to combine fundamental and applied research to create stress tolerant crops, focusing on efficient enhancement of crop yield by engineering plant metabolism. To make a difference in agricultural production within my lifetime, I plan become a project leader in private industry, such as Monsanto or DuPont Pioneer, or at a governmental agency, such as the USDA. I believe such a position will allow me to accomplish much as a researcher and continue my drive for a marketable product. If permanent job opportunities are lacking when I graduate, I plan to take a post-doctoral research position that allows me to strengthen my skills in stress metabolism and genetic engineering in a more applied setting.

An AFRI Predoctoral Fellowship would give me insight into working at a governmental organization like the USDA by allowing me access to other fellows with similar research interests and career goals and to USDA professionals. It also gives me many opportunities to attend research conferences and travel to specific training opportunities. One meeting I plan to attend with funds from this fellowship is the "Salt and Water Stress in Plants" Gordon Research Conference and Gordon Research Seminar in June 2018. I will also attend "Plant Lipids: Structure Function and Metabolism" in January 2019, where I have agreed to co-chair the Seminar portion. Attending extremely specialized meetings like the two mentioned allows me to interact with some of the best minds in abiotic stress tolerance. With agriculturally focused universities nearby, I can attend regional conferences and training workshops. Previous examples of these at just UNL include: confocal and electron microscopy, metabolomics, proteomics, and a biennial Plant Science Initiative Retreat. I will use additional funding to attend as many of these as are relevant to my intended future career in abiotic stress tolerance.

To gain professional soft skills needed to enter the workforce at a government organization or in the private sector, I will also use funds to attend training sessions in leadership, science communication, and science writing. Through personal conversations with USDA-ARS molecular biologist and biochemist, Gautam Sarath; a mentor, Mark Lagrimini, who was formerly at Syngenta; and multiple plant breeders at Monsanto, including Clint Turnbull; I have identified multiple soft skills important for success in the workplace. I would like to improve my abilities to communicate complex basic research to marketing groups, journalists, upper level management, patent lawyers and the general public. Many workplaces hold internal training, but outside perspectives and methods are also helpful, so I will pursue my own training, as well as leadership and teamwork training. UNL often holds writing and communication workshops and training opportunities through the Department of Graduate Studies.

B. Mentoring Plan

Dr. Rebecca Roston, Assistant Professor at the University of Nebraska-Lincoln (UNL), will provide primary mentorship throughout the course of the work proposed here. Specifically, she will support me in designing and troubleshooting experiments, analyzing results, and interpretation and application. As a fourth year graduate student, I have been transitioning into

independent research and planning projects and experiments with less guidance from Dr. Roston. I will continue on my path of independently designing experiments, attempting new procedures, and arranging meetings weekly to follow-up on their results. Additionally, I will continue to participate and lead bi-weekly journal clubs with topics focused on Roston lab-specific research, and continue attending a weekly journal club made up of plant lipid researchers at UNL.

Successful mentorship in career planning and decision-making has been shown to give a protégé increased performance ratings, salary, and reception of promotions (1,12). In the future I plan to be a group research leader thus I need to acquire leadership and team building skills. Due to my desire to work at the USDA-ARS or in industry, gaining mentoring abilities would improve my ability to coordinate teamwork and foster dynamic group work environments. Informally, I hope to learn from Dr. Roston's mentoring and leadership styles to assist in my own future roles as a mentor. By obtaining these funds, I will be able to attend more training sessions and short courses on how to improve leadership and people management skills. I will also hire an undergraduate worker to mentor in his or her own mini-project contained in the proposed research. The value of a first student mentor can be large, and I well remember my first such relationship and wish to share my positive experiences.

Former mentees of Dr. Roston and current positions include: Undergraduate students contributing to funded research with independent research projects: Jennifer Myers (2015-current), Hope Hersh (2012-2014, graduating MSU this year, George Murphy III (2013-2014, attending U of Michigan for Masters Degree), Peter Hsueh (2012-2013, attending MSU graduate school). Current graduate student mentees: Allison Barnes and Evan LaBrant.

C. Project Plan

1. Introduction: Environmental stresses are intimately connected with plant metabolism and therefore crop yield. Stresses such as freezing and heat stress can damage membranes and cause leakage. One protein responsible for lipid remodeling of membranes is SENSITIVE TO FREEZING 2 (SFR2). By removing the head group off of a monogalactosyldiacylglycerol (MGDG) and adding it processively to other MGDGs, SFR2 creates oligogalactolipids and releases diacylglycerol, which can be converted to oil (2). In Arabidopsis, plants lacking SFR2 (*sfr2* mutants) are unable to sufficiently remodel their membranes and die at temperatures wild type plants can survive (2). SFR2 protein is always present, but only active under membrane stressing conditions. In Arabidopsis, SFR2 activates under freezing stress, mechanical damage, chloroplast isolation, and protoplast isolation (3,4, preliminary data). In tomato, SFR2 also activates under drought and salinity stress (5). Studies of SFR2 in other crop species are lacking.

SFR2 is situated on the outer envelope of the chloroplast, with its active portion in the cytosol (6). During freezing, the cytosol is acidified (3), and we have shown this acidification, along with a high concentration of Mg^{2+}, activates SFR2 (3). However, the specific source of the protons is still unknown. SFR2's location is ideal for sensing this acidification.

Outside of the acidification, not much is known about SFR2's specific mechanism of activation. Previous work from the lab and my own preliminary data show the following: SFR2 protein is present at all temperatures, but only creating oligogalactolipid products after freezing (3). It is tightly regulated at the post-translational level. When Arabidopsis SFR2 is expressed in yeast, oligogalactolipids are constantly produced (7). This suggests that a repression mechanism exists under normal temperatures in Arabidopsis. In a search for protein interactions, no permanent interactors were identified (3). A protein complex of approximately 140 kDa was identified, which is consistent with SFR2 existing as a dimer *in planta* (3).

2. Rationale and Significance: As sessile organisms, plants are exposed to times of environmental stress. Between 1960 and 2008, growing seasons with extreme weather events caused drops in corn yield, including 16% during a year with an early frost, despite the overall increases due to technology (8). Stress tolerance mechanisms must be understood in greater detail to generate stability in crop yields. The short-term goal of this project is to understand the activation and repression mechanism of SFR2 that confers freezing tolerance. The long-term goal of this study is to take advantage of the activation patterns of SFR2 to engineer crops to be more tolerant to freezing and other intense membrane stresses. *Both goals align with the AFRI objective of plant health and production and plant products by understanding a key mechanism for plant membrane stability during stress due to climate variability.*

3. Approach: In this project, my goal is to understand the specific mechanism of activation for SFR2. I will use two complementary approaches to obtain this information. First, I plan to ***elucidate the specific mechanism of SFR2 activation in Arabidopsis***. Second, I will ***examine SFR2's regulation across diverse members of the plant kingdom***. Because so little is known about SFR2's activation patterns, and post-translational activation under membrane destabilizing stresses in general, this project will advance the field of plant stress physiology. Knowledge of this specific mechanism may be exploited to create more stress tolerant crops.

Aim 1: **Elucidate the specific mechanism of SFR2 activation in Arabidopsis**

SFR2 is tightly regulated post-translationally, but we are unsure how or why. Based on preliminary data, we hypothesize that SFR2 is repressed under normal growth conditions. Here, I will test this hypothesis with two approaches: Understanding how SFR2 is regulated as a protein (1.1), and how the cell activates it in times of stress (1.2).

Sub-Aim 1.1: Identify and test SFR2's post-translational modifications

Approach: Previous SFR2 co-immunoprecipitations under SFR2-activating conditions did not identify post-translational modifications or potential protein-protein interactions (3). Here, I propose to use SFR2 fused to yellow fluorescent protein (YFP) to isolate SFR2 under non-active conditions. The SFR2-YFP plants also complement the *sfr2* loss-of-function mutant freezing sensitive phenotype (preliminary data). To capture inactive conditions, Arabidopsis transformed with SFR2-YFP in the *sfr2* background were grown under normal conditions until plants were four weeks old then snap frozen in liquid nitrogen and immediately protease and phosphatase inhibitors were added. Co-immunoprecipitation in collaboration with the Proteomics and Metabolomics Facility (PMF) at UNL identified a phosphorylated residue, threonine 87, in two separate pull down replicates. I plan to repeat these immunoprecipitations and digest with chymotrypsin instead of trypsin to try to improve coverage of the protein, which is currently between 40 and 45%. By changing protein digestion enzymes, SFR2 will be cut at different sites, increasing coverage, and potentially identifying other phosphorylation sites. I will then make single, double and multiple mutants (as needed) by site directed mutagenesis to probe the function of the post-translational modifications in vivo.

For analysis, mutated constructs will be expressed in yeast and in Arabidopsis. Yeast will be the first level of screening for constructs because it is a faster and simple screening method. As stated above, SFR2 is always active in yeast, thus with this assay I am looking for an inactivation of SFR2. The "phosphorylated" mutation to a glutamic acid should inactivate in yeast and the "de-phosphorylated" mutation to a valine should keep SFR2 active. A thin layer chromatograph (TLC) to observe the presence of oligogalactolipids is a simple and fast screening method for SFR2 activity, which I have previously shown (3). "Dephosphorylation" mutants and successfully screened "phosphorylation" mutants will then be stably transformed into

Arabidopsis for further analysis including protein size, complex analysis, ectopic activation, and freezing and acidification tests. Yeast expressing SFR2 is a key tool for identifying the kinase and phosphorylase interacting with SFR2. Kinase libraries are available and more easily screened using yeast than other organisms. In the future, this will begin the screening process to identify the proteins responsible for modifying SFR2.

To determine if other changes such as membrane environment or small molecules interact with SFR2, I am working on relocating SFR2 to the inside of the chloroplast envelope membrane. This allows it to experience any ionic changes occurring due to freezing, but does not allow it to interact with any proteins in the cytosol due to the impermeability of the outer envelope to proteins not targeted to the chloroplast and larger than 10 kDa (13). SFR2 still retains its activity without its transmembrane domain, so by swapping this domain, an active SFR2 can be expressed elsewhere. I have created two constructs with transmembrane domains from proteins known to move fluorescent proteins to the correct locations (14,15).

Expected Outcomes: The experiments in this sub-aim to allow me to unequivocally identify virtually all-possible SFR2 modifications. Understanding SFR2's regulation in Arabidopsis lays the foundation for understanding membrane stabilizing activation mechanisms in other species. As a membrane stabilizing protein, SFR2 has potential applications across a wide variety of stresses. Elucidating regulation mechanisms is the first step in accomplishing crop engineering.

Potential Pitfalls and Alternative Approaches: Although phospho-mimic mutants have long been used (17), there is always the possibility that they may disrupt the structure of SFR2 in addition to preventing or constitutively adding phosphorylation. I can test this using already-defined SFR2 structural assays (7). Yeast or Arabidopsis may struggle to express the specific mutants or fusion constructs proposed here. If that is the case, I will consider using alternative mutations to mimic phosphorylation status (16). PTMs may not play a role in the activation of SFR2, which is why we have designed sub-aim 1.2.

Sub-Aim 1.2: Establish the source of SFR2 activating protons

Approach: Using specific inhibitors of H^+/ATPase pumps in the vacuole and plasma membrane, I will prevent proton flow from one cellular location or the other during freezing and acidification testing. I will use sodium nitrate to inhibit the vacuolar pump (9), and sodium vanadate to inhibit the plasma membrane pump (10). Leaves detached from a soil grown Arabidopsis wild-type plant will be soaked in either vanadate, nitrate, or water (control) then frozen overnight or pH based activation, as previously (3). SFR2 activity will be analyzed as previously (3) and the change in pH in the cytosol will be observed using a pH sensitive fluorophore with which I am also familiar (3,11). The vacuole or apoplast may be individually responsible, or they may both contribute. If the latter is the case, we expect the oligogalactolipid products to still be present in both treatments. As an independent method to verify our results *in vivo*, I plan to obtain Arabidopsis lines that either decrease or increase the proteins responsible for the pumps; both are available from TAIR. Then I will quantify the amount of pump transcript and protein, and compare these to the presence or absence of oligogalactolipids. These plants will also be tested for impairment of ability to survive freezing.

Expected Results: I expect to identify the source of protons activating SFR2. Additionally, the results achieved in this aim will directly tie in to results from Aim 1.1. Knowledge of acidification mechanism can correlate to post translational activation to set up future experiments for analyzing how the acidification occurring changes specific residues or proteins.

Potential Pitfalls and Alternative Approaches: Using an inhibitor does not always ensure an equivalent response from plant to plant. I have anticipated this problem by incorporating both a

chemical and genetic approach. If neither the vacuole nor the plasma membrane contributes to cytosolic acidification, we will have eliminated the two most likely proton sources and can pursue other mechanisms of acidification.

Aim Two: Examine SFR2's regulation across diverse members of the plant kingdom

SFR2 is present in all land plants, but has previously only been analyzed through phylogeny as its classification as a glycosyl hydrolase, and not its function as a galactosyl transferase (8). Also, SFR2 has been studied mostly in Arabidopsis. By comparing SFR2 patterns of activation and protein sequences in other species, we have the potential to learn much about how SFR2 functions in other species. We hypothesize that SFR2's protein in other species will uncover specific motifs important for function as a galactosyl transferase (2.1) and that species of SFR2 activating similarly to Arabidopsis will be able to complement *sfr2* Arabidopsis (2.2).

Sub-Aim 2.1: Compare SFR2 activation patterns across phylogeny

Approach: To compare SFR2's activation across multiple species, I first generated cold kill curves for maize, pea, switchgrass, and wheat and correlated them with oligogalactolipid levels. I observed similar activation patterns to Arabidopsis for maize, pea, and switchgrass, with SFR2 activating before permanent leaf death. Wheat had a different pattern with a constant activation of SFR2. This led to the desire for a better, more complete study of phylogeny. Based on the species already described and tests completed in duckweed, we discovered three patterns to emerge: oligogalactolipid production like Arabidopsis (i.e., during cold/freezing), constant oligogalactolipid production, or a lack of oligogalactolipid production. I expect the activation patterns will correlate to evolution, similarity of SFR2 protein, or cold tolerance. To create a more high-throughput screening, leaf disks are chilled similarly to the method also used in Aim 1, but over a 24-hour period of time. After the chilling, the lipids are extracted and analyzed, comparing each frozen sample to a freshly sampled counterpart. 32 species have already been tested, and 10 more are growing for analysis. After initially sampling across evolutionary diversity, species with sequenced genomes have taken priority for sampling, and necessary plants were ordered from USDA-GRIN or other reputable sources. Evolutionary phylogeny has been filled in where possible with unsequenced species. We still wish to test at least 10 more sequenced and relatively easily available species to supplement protein analysis. Activation patterns will be compared to evolutionary phylogeny, SFR2 protein phylogeny, and cold tolerance. Between sequenced species, we will compare protein sequences to check for any portions of similarity that coordinate to activation patterns. Conserved residues will also assist in informing the evolutionary relevance of post-translational modifications from Aim 1.

Expected Results: We expect that the three patterns of SFR2 activation identified will hold true through other species that have not yet been tested. We also expect to find specific protein motifs or residues conserved that are critical for SFR2's cold/freezing based activation. Further, results from this sub aim will help inform future experiments on SFR2 use in crop species.

Potential Pitfalls and Alternative Approaches: One pitfall that could arise is that -20°C may be too cold for some species and will kill the leaf tissue before SFR2 activation. Based on our maize results, another set of tests going down to just 0°C or may be necessary for more tropical species. Additionally, pH-based activation mimicking freezing can be used to analyze species that are extremely cold sensitive. The possibility of no pattern of protein residues emerging is unlikely, as we have already observed some conservation of threonine 87.

Sub Aim 2.2: Test divergence in SFR2 activation patterns.

Approach: To test divergence in SFR2 activation patterns, we plan to complement *sfr2* loss-of-function Arabidopsis with SFR2-like genes from six other species. SFR2 from two species

that activate similarly to Arabidopsis, two that never activate, and two that are always active will be expressed using the same ubiquitin promoter and YFP tag as the vector used in Aim 1 to complement *sfr2*. Using YFP will allow for a simple localization using confocal microscopy, which is important for confirming SFR2's successful chloroplast targeting. To test complementation, freezing and pH based activation tests will be performed as previously (3).

Expected Results: We expect that SFR2-like genes from species that activate like Arabidopsis will complement *sfr2* Arabidopsis. Additionally, those that are always active in their species will remain always active and those that do not activate will not. We also will be able to explore additional hypotheses about SFR2's tight post-translational regulation in Arabidopsis. It is still unknown if SFR2 allows for freezing survival by the removal of MGDG or the presence of oligogalactolipids. By creating these lines, we can bypass the regulation discovered in Aim 1 and test how SFR2 specifically confers freezing tolerance.

Potential Pitfalls and Alternative Approaches: Because these are genes from other species, we may have a difficult time getting them to express correctly in Arabidopsis. We are already planning to mitigate this is by codon optimization. In the unlikely event that protein expression remains poor, then because we are testing so many different species, we can also complement with alternative species' SFR2-like genes, or we can use transient or inducible expression.

D. Evaluation Plan

To delineate milestones, I have created a timeline for successful progress:

Timeline:	2017	2018			2019
Aim:	F	Sp	Su	F	Sp
1.1 Post-translational modification identification	X	X	X		
1.2 Examine the source of SFR2's activation	X	X	X		
Publishing results of Aims 1.1, 1.2, and 2.2				X	
2.1 Compare SFR2 activation across phylogeny	X	X			
2.2 Test divergence in SFR2 activation patterns		X			
Publishing results of Aim 2.1	X	X			
Dissertation Writing and Defense			X	X	X

F = fall, Sp = spring, Su = summer

To monitor project progress, I will meet with Dr. Roston at regular intervals. We will set up individualized meetings in addition to our weekly lab meetings and bi-weekly journal clubs. I have annual committee meetings, and am welcome to make unofficial meetings with committee members as well.

Each time I give a presentation or make a poster, I will refer back to this timeline to evaluate my progress towards our goals. As part of my PhD program, I am required to take a seminar course each semester. A least one of the semesters in the coming year, I will present my research to other graduate students in my department. I also plan to attend at least one conference per year. Here I will disseminate my research through poster presentations, and apply for giving oral presentations when possible. One such conference, a Gordon Research Seminar, I was elected by a vote of my peers in plant lipid research to co-chair for 2019. The Plant Science Initiative at UNL also has biennial symposia and monthly research update meetings. During months in which I am scheduled to present, I will also share my research there.

I plan to publish two papers from the knowledge gained through this research. Results from aims 1.1, 1.2, and 2.2 will be published together in Fall 2018 and results from aim 2.1 will be published in Fall 2017 or Spring 2018 with other research from the lab. Additionally, all of this research will be part of my dissertation and PhD defense that is planned for Spring 2019.

To meet my mentoring objectives, I will select a talented undergraduate. This will also give me a chance to use the leadership, teamwork, and mentorship skills I plan to gain from attending workshops as a result of being funded through this grant. To date, I have not taken sole responsibility for training someone.