**Project FY22-GD-006:** Mitigate FHB in Wheat by Knockdown of Defense Repressors

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1. **What are the major goals and objectives of the research project?**
   The major goal of this project is to enhance resistance against *Fusarium graminearum* by knocking down the activity of *NPR3* and *NPR4*, which encode proteins that repress the defense activator NPR1. NPR1 was previously shown to promote resistance against *Fusarium graminearum* in Arabidopsis and wheat. It is expected that knockdown of *NPR3* and *NPR4* will result in the faster and stronger activation of NPR1-dependent salicylic acid signaling, thus resulting in enhanced resistance to *F. graminearum.* Alternatively, *NPR3* and *NPR4* may function as susceptibility factors to *F. graminearum*, independent of their negative impact on *NPR1* and/or salicylic acid. Three aims were proposed:

   1. Develop RNAi lines to reduce wheat *WhNPR3* and *WhNPR4* expression.
   2. Identify mutations in *WhNPR3* and *WhNPR4* that can be utilized as non-GMO alleles for enhancing FHB resistance in wheat under aim 3.
   3. Characterize response to *F. graminearum* infection in *WhNPR3* and *WhNPR4* knockdown lines identified under aims 1 and 2.

2. **What was accomplished under these goals or objectives?** *(For each major goal/objective, address these three items below.)*

   **What were the major activities?**

   - Two RNAi constructs WhNPR3-RNAi and WhNPR4-RNAi were used to transform hexaploid wheat at the USWBSI-supported central wheat transformation lab at Kansas State University. These constructs were transformed individually and together into a variety of hexaploidy wheat varieties with the expectation of identifying plants that were silenced for (i) WhNPR3, (ii) WhNPR4, and (iii) both WhNPR3 and WhNPR4.
   - Nonsense and missense mutations identified in the homeologs for WhNPR3 and WhNPR4 in the durum variety Kronos were used to identify homozygous mutant plants, which were tested for their reaction to *F. graminearum*, including studying the impact of these mutant alleles on deoxynivalenol (DON) accumulation.
   - To confirm that *NPR3* and *NPR4* function as susceptibility factors, bioassays with *F. graminearum*, were repeated with Arabidopsis *npr3*, *npr4*, and *npr3 npr4* double mutants. These bioassays were conducted with leaf and floral tissues.
   - Crosses were initiated in Arabidopsis to generate *npr1 npr3 npr4* triple mutant. This triple mutant will facilitate future testing to determine if the *npr3 npr4*-conferred enhanced resistance to *F. graminearum* is mediated through the upregulation of *NPR1* function.

   **What were the significant results?**

   - Repeat experiments with the Arabidopsis *npr3*, *npr4* and the *npr3 npr4* double mutant confirmed that knockdown of *NPR3* and *NPR4* results in enhanced resistance to *F.*

*graminearum* in both leaf and floral tissues, thus confirming their contributions as a susceptibility factor in the interaction of this fungus with Arabidopsis. It was also noted that a high percentage of leaves of the *npr3 npr4* double mutant exhibited a hypersensitive-response (HR)-like tissue collapse in response to inoculation with *F. graminearum* that was accompanied by reduced fungal growth as monitored by accumulation of fungal DNA. Additional experiments have suggested that this HR-like tissue collapse is likely due to a fungus-derived factor.

- Similar to the observations with the Arabidopsis *npr3*, *npr4,* and the *npr3 npr4* mutants, missense mutations in wheat *WhNPR3* and *WhNPR4* were associated with enhanced resistance to *F. graminearum* and reduced DON accumulation.

**List key outcomes or other achievements.**

- Multiple missense alleles of WhNPR3 and WhNPR4, which are predicted to result in knockdown of *NPR3* and *NPR4* activity, conferred enhanced resistance to FHB in the durum wheat Kronos background. DON accumulation was also significantly lower in the mutant plants compared to the WT plants. These results are very encouraging, although they need to be repeated in backcrossed lines.
- Experiments with the Arabidopsis *npr3* and *npr4* plants verified that *NPR3* and *NPR4* function as susceptibility factors to *F. graminearum*.
- In the process of studying the impact of *npr3* and *npr4* on Arabidopsis-*F. graminearum* interaction, the mutant plants were observed to respond with a hypersensitive-response that was accompanied by reduced fungal growth. This hypersensitive response was demonstrated to be due to a fungus-derived factor, suggesting that the enhanced resistance of the *npr3 npr4* mutant to *F. graminearum* is likely due to hyper-responsiveness of the mutant to this F. graminearum-derived factor. Whether this factor directly or indirectly targets NPR3/NPR4 remains to be determined.

**3. What opportunities for training and professional development has the project provided?**

**Training:** Two graduate students assisting part-time with this project gained training in plant-pathogen interaction, in particular assessing disease severity and molecular pathology, planning experiments, collecting, recording, and analyzing and interpreting data. A postdoc spearheaded this project, while another senior postdoc helped with training and mentoring of the graduate students. The postdoc spearheading this project gained training in working with *F. graminearum* interaction with Arabidopsis and wheat. In addition, the postdoc gained experience in molecular plant pathology. The senior postdoc received training towards his long-term goal in pursuing a future independent career in academics, including mentoring others, managing lab personnel, ensuring research-related compliance and reporting, and day-to-day function of a research lab.

**Professional Development:** This project contributed to the professional development of the graduate students and postdocs who participated in the weekly group meetings, weekly department seminars, the BioDiscovery Institute research talks, UNT Research day presentations, and the FHB forum. They developed their presentation skills by preparing

posters and/or talks arising out of their work. Co-PI Shah worked individually with the graduate students and postdocs, meeting with them biweekly, to help them prepare towards their long-term professional goals.

**4. How have the results been disseminated to communities of interest?**

Results were disseminated to communities of interest in multiple ways:

- Posters presented at the (i) Annual USWBSI Forum in Cincinnati, December 2023, (ii) at the American Society of Biochemistry and Molecular Biology in March 2023, as well as(iii) local meetings in Denton.
- Flash talk by one of the graduate students at the USWBSI forum in Cincinnati, December 2023.
- Talks presented by the PI at a USDA meeting as well as at another institution, and talks presented by one of the graduate students and the postdoc at the GDER-PBG joint mid-year meeting, which was held virtually in April 2024.
- Outcomes of this work were disseminated to undergraduates in an introductory biology class taught by Co-PI Shah in Spring 2023 at the University of North Texas.

**5. What do you plan to do during the next reporting period to accomplish the goals and objectives?**

- Repeat experiments with the wheat Wh*NPR3* and Wh*NPR4* missense mutants to confirm that resistance to *F. graminearum* and the reduced accumulation of DON observed in these tetraploid wheat plants is reproducibly observed among multiple experiments.
- Backcross the Wh*NPR3* and Wh*NPR4* mutant lines as well as generate lines with mutations in both homeologs of Wh*NPR3* and Wh*NPR4* in the tetraploid wheat Kronos background. Test if the resistance is higher and DON levels lower in lines lacking both Wh*NPR3* and Wh*NPR4* function compared to the single mutants.
- Test if this resistance is also observed in hexaploid wheat that is silenced for Wh*NPR3* and Wh*NPR4*. Multiple RNAi lines have been developed and will be used for this study.
- Determine if the *npr3 npr4*-conferred resistance is dependent on *NPR1* and salicylic acid. To facilitate this, efforts are underway to develop a *npr1 npr3 npr4* triple mutant.