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?

Project goal: To enhance Fusarium head blight (FHB) resistance in wheat, we proposed to knock down the expression of two known repressors of plant defense, NPR3 and NPR4. These proteins negatively regulate NPR1, a central activator of salicylic acid (SA)-mediated defense responses, which has been previously shown to contribute to resistance against *Fusarium graminearum* in both *Arabidopsis* and wheat. Given *NPR1*'s key role in defense signaling, we hypothesize that reducing *NPR3* and *NPR4* expression will lead to more rapid and robust activation of *NPR1*-dependent SA signaling, thereby enhancing FHB resistance. Alternatively, *NPR3* and *NPR4* may act as susceptibility factors to *F. graminearum* independently of their modulation of NPR1 or SA signaling. Three aims were proposed:

1. Develop RNAi lines to reduce wheat *NPR3* and *NPR4* expression.
2. Identify mutations in *NPR3* and *NPR4* 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 *NPR3* and *NPR4* knockdown wheat 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?

- Several missense mutations were identified in the *NPR3* and *NPR4* homeologs in the tetraploid wheat variety Kronos. Plants homozygous for these alleles were selected and evaluated for their response to *Fusarium graminearum*, including assessments of FHB severity and deoxynivalenol (DON) accumulation.
- Transgenic wheat lines were developed in the hexaploid variety Bobwhite, containing RNAi constructs targeting *NPR3* and *NPR4*, to investigate the impact of gene silencing on FHB resistance.
- Cloning of the coding sequences for wheat *NPR3* and *NPR4* and mutant versions was initiated. This will allow us to determine whether the wheat genes are functionally orthologous to their Arabidopsis counterparts and whether the wheat mutations result in loss-of-function or is the result of dominant alleles.
- Construction of recombinant Arabidopsis *NPR3* and *NPR4* that contain mutations that mimic the FHB-resistance conferring mutant alleles of wheat *NPR3* and *NPR4* was initiated. These mutant Arabidopsis alleles will facilitate testing whether the function of these amino acids in NPR3 and NPR4 function is conserved between wheat and Arabidopsis.
- F_2 progeny from a cross between the Arabidopsis *npr3 npr4* double mutant and *npr1* mutant were screened to identify the *npr1 npr3 npr4* triple mutant. This triple mutant will enable future studies to test whether enhanced resistance conferred by *npr3 npr4* is mediated via upregulation of NPR1 activity.

- Kronos NPR3 and NPR4 mutant lines that consistently exhibit enhanced FHB resistance and reduced DON accumulation have been shared with breeders in the durum Coordinated Project (CP) for independent evaluation of their FHB resistance phenotype and incorporation into elite germplasm.

What were the significant results?

- Repeat experiments with multiple alleles have confirmed that missense mutations, particularly those located within the ankyrin domain of *NPR3* and *NPR4*, confer enhanced resistance to Fusarium head blight (FHB). This resistance is consistently associated with reduced Fusarium-damaged kernels (FDK) and lower deoxynivalenol (DON) accumulation. Notably, mutations substituting glutamic acid (E) with lysine (K) at a conserved position in the ankyrin domain of both *NPR3A* and *NPR3B* homeologs conferred strong resistance phenotypes. The ankyrin domain is involved in interaction with other proteins, suggesting that this mutation likely impacts the interaction of NPR3 and NPR4 with an associated protein.
- In *Arabidopsis*, *npr3*, *npr4*, and the *npr3 npr4* double mutant exhibited increased resistance to *Fusarium graminearum* in floral tissues. This enhanced resistance correlated with elevated expression of the salicylic acid-responsive *Pathogenesis-Related 1 (PR1)* gene, suggesting that the resistance conferred by *NPR3* and *NPR4* knockdown is likely mediated by amplified salicylic acid signaling.
- Although *Arabidopsis npr3*, *npr4*, and *npr3 npr4* mutants show hypersensitive response (HR)-like tissue collapse and ion leakage in leaves upon *F. graminearum* infection, this HR phenotype was absent in infected floral tissues of both *Arabidopsis* and wheat. This suggests tissue-specific differences in the host response to *F. graminearum* infection.

List key outcomes or other achievements.

- Multiple missense alleles of wheat *NPR3* and *NPR4*, predicted to reduce gene activity, conferred enhanced resistance to Fusarium head blight (FHB) in the durum wheat Kronos background. These mutant lines also showed significantly lower levels of Fusarium-damaged kernels (FDK) and deoxynivalenol (DON) accumulation compared to wild-type (WT) plants.
- The hypersensitive response (HR)-like tissue collapse observed in *Arabidopsis npr3* and *npr4* mutant leaves upon *Fusarium graminearum* infection was not observed in floral tissues of *Arabidopsis* or in wheat spikes. As a result, this HR-like response and associated tissue damage is not a concern in wheat, supporting the safe use of *NPR3* and *NPR4* missense mutant alleles as FHB-resistant germplasm in breeding programs aimed at enhancing resistance to FHB.
- Seeds from the most promising Kronos mutant lines, which consistently exhibited strong FHB resistance, were shared with breeders for further independent evaluation and integration into elite durum wheat germplasm.

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

Training: Three graduate students assisting part-time on this project received training in molecular biology and plant–pathogen interactions, including assessing disease severity, conducting molecular pathology analyses, planning experiments, and collecting, recording, analyzing, and interpreting data. The project was led by a postdoctoral researcher, who gained experience working with *Fusarium graminearum* in both Arabidopsis and wheat, as well as in molecular plant pathology. A senior postdoc contributed to the training and mentoring of the graduate students and received professional development aligned with his goal of pursuing an independent academic career. This included mentoring skills, managing lab personnel, overseeing compliance and reporting requirements, and supporting the day-to-day operations of a research laboratory.

Professional Development: This project contributed significantly to the professional development of the participating graduate students and postdoctoral researchers. They regularly engaged in weekly group meetings, departmental seminars, BioDiscovery Institute research talks, UNT Research Day presentations, and the FHB Forum. Through these activities, they honed their presentation skills by preparing and delivering posters and oral presentations based on their research. Co-PI Shah also supported their growth through individualized mentoring, meeting biweekly with each student and postdoc to guide them in preparing for their long-term professional goals.

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

Findings from this project were shared with relevant scientific and agricultural communities through multiple avenues:

- **International Discussion Forum:** Dr. Shah participated as a panelist in an international deep-dive virtual discussion, *Targeting Plant Immune Systems for Sustainable Agriculture* (December 5, 2025), organized by *Frontiers in Science*. The event was attended by over 200 participants from various countries in North America, South America, Asia and Europe. Dr. Shah also contributed an invited article to the associated special issue, published in *Frontiers in Science*.
- **Poster Presentations:** Project results were presented in poster format at the following venues:
  (i) Annual U.S. Wheat and Barley Scab Initiative (USWBSI) Forum, Austin, TX (December 2024)
  (ii) International Symposium on Plant Lipids (ISPL), July 2024
  (iii) University of North Texas (UNT) Research Day, October 2024
- **Invited Talks:**
  (i) Dr. Shah delivered an invited seminar at the Department of Biology, University of Missouri–St. Louis (April 2025)
  (ii) Dr. Shah also presented a seminar to an audience of over 100 undergraduate and graduate students, faculty, and staff as part of the BioFrontiers Seminar Series in the Department of Biological Sciences at UNT (September 2024).

- **Seed Distribution:** Seeds from *Kronos NPR3* and *NPR4* mutant lines that consistently showed the strongest FHB resistance were distributed to durum wheat breeders for further independent testing and incorporation into elite cultivars.

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

- *Functional Orthology Testing*: Genetically confirm whether wheat *NPR3* and *NPR4* genes are true orthologs of the Arabidopsis *NPR3* and *NPR4*.
- *Allelic Function Characterization*: Determine whether the identified wheat *NPR3* and *NPR4* missense alleles are loss-of-function or dominant variants, particularly in the context of their interaction with known protein partners.
- *Functional Conservation of Key Amino Acid Residues in wheat and Arabidopsis NPR3 and NPR4 function*: Arabidopsis *NPR3* and *NPR4* with mutations that mimic the FHB-resistance-conferring wheat *NPR3* and *NPR4* alleles will be tested to determine if the function of these amino acids is conserved between Arabidopsis and wheat.
- *Double Mutant Line Development and Evaluation*: Generate tetraploid wheat (*Kronos*) lines with mutations in both homeologs of wheat *NPR3* and *NPR4* and assess whether combined loss of both genes enhances FHB resistance and reduces deoxynivalenol (DON) accumulation compared to single mutants.
- *Hexaploid Wheat RNAi Validation*: Evaluate whether silencing of *NPR3* and *NPR4* in hexaploid wheat also confers enhanced FHB resistance. Multiple RNAi lines have been propagated and will be used for this analysis.
- *Genetic Dependency of NPR3 and NPR4 mutant allele-conferred FHB resistant phenotypes on NPR1*: Characterize the response of the *npr1 npr3 npr4* triple mutants to *F. graminearum* to determine whether the resistance conferred by loss of *NPR3* and *NPR4* functions is mediated through *NPR1*. If this is the case, then the *npr1* allele is expected to be epistatic to *npr3* and *npr4* and the triple mutant is expected to have relatively higher disease severity like the *npr1* mutant.
- *Publication:* We will disseminate our findings to the broader scientific community by preparing and submitting a manuscript for publication in a high-impact, peer-reviewed journal relevant to plant molecular biology and pathology.