2024 FINAL REPORT (project ongoing)

PROJECT TITLE: High-throughput Gene Platform for Validating Resistance against Fusarium in Corn
PROJECT NUMBER: 6138-24DD
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ABSTRACT

Fusarium species and other fungal pathogens are a persistent threat to corn production in the United States Corn Belt region, including Minnesota. *Fusarium* infections cause stalk- and ear-rots, and accumulation of poisonous secondary metabolites (mycotoxins) that cause human and livestock health disorders via contaminated corn-based food and feeds. While *Fusarium* species are a problem in the Northern states (which are cooler), another genus of toxigenic fungi, *Aspergillus*, is more of a problem in the warmer Southern states. Unfortunately, climate change is making it possible for *Aspergillus* species to expand their range to include the US Corn Belt states, thereby creating the need for solutions to both pathogens.

The Corn Mycotoxin Mitigation Initiative, led by the non-profit biotech company, 2Blades, is developing corn varieties with resistance to fungi that produce mycotoxins. In this program, 2Blades is using its high-throughput resistance gene discovery platforms, NLRseek™ and PRRseek™, in its laboratory at the University of Minnesota to identify novel sources of genetic resistance to toxigenic fungi such as *Fusarium* and *Aspergillus* species.

For this project, 2Blades utilized the NLRseek™ Platform to source novel resistance to *Fusarium* species. The NLRseek™ Platform is a collection of 985 unique, prioritized receptor proteins from 18 grass families, introduced individually into wheat lines. Initial screening of this Wheat Array using *Fusarium graminearum* in 2023 produced first candidates, which were tested in the field in Year 1 of this project.

In Year 1, the project's principal findings are that two genes potentially confer enhanced resistance to *Fusarium* species. These two genes have been selected for transformation and testing in corn.

INTRODUCTION

Corn is the world's most-produced grain with Iowa, Illinois, Minnesota, and Nebraska responsible for over half of U.S. corn production. Demand for corn is expected to increase significantly by the year 2050 due to rapid population growth[1], but factors including climate change, plant diseases, and pests are a threat to harvests, thus, creating a strong need to develop corn with greater resilience[2]. The total loss due to corn diseases in the US and Ontario, Canada from 2016 to 2019 stood at $20 billion, of which estimated losses due to corn diseases in Minnesota alone were $710 million[3]. Among the most significant disease threats are pathogenic fungi. Fungal diseases affect yields through reduced ear size, poor grain fill, and early eardrop and lodging from stalk rots. In addition, mycotoxins produced by some fungi, including *Fusarium* and *Aspergillus* species, can impact the health of people and animals consuming corn-based food and feeds[3].

Not only are mycotoxins a current and persistent grain issue, but the incidence is increasing with climate change. Severe drought, high summer temperatures and humidity, and extreme precipitation patterns resulting from climate change are extending suitable growth conditions and hence distribution of toxigenic fungi and their associated mycotoxins[4], reaching new agroecological zones or expressing greater pathogenicity. For example, changes in weather patterns are predicted to create increasingly favorable conditions in almost 90% of corn-growing counties in 15 US states, including the Corn Belt. These areas are projected to have increased aflatoxin contamination in 2031–2040 compared to 2011–2020, resulting in tripling of overall indemnities from $10 million to $31 million per year[5]. This will increase mitigation costs associated with fungicides and use of binders, and result in loss of opportunity due to reduced trade[3]. In the US, mycotoxins are already very costly to producers and consumers, with one estimate of mitigation costs and unresolved food and feed losses averaging $2-3 billion each year[6]. Recalls of toxin-tainted food products, drinks, and pet feeds occur regularly, making this a growing consumer issue with potential regulatory implications. For example, in January 2021, around 110 pets died, and more than 210 pets fell sick after consuming aflatoxin-contaminated pet food, leading the FDA to issue a recall of certain products from the market.

Over several decades, scientists and breeders have searched for sources of resistance to toxigenic fungal species through conventional breeding and transgenic strategies, yet thus far, no lines with durable field resistance have reached the market[7]. Although this has been a challenging breeding target, 2Blades brings new insights and tools to test heterologous expression of transgenes from related plant species as an extended gene pool of resistance against *Fusarium* and *Aspergillus* species.

Pathogens inject pathogen virulence factors termed effectors into the plant cells to support disease infection[8]. In response to this, plants activate the resistance (*R*) gene-based immunity, which relies on recognition of effectors by intracellular nucleotide-binding leucine-rich repeat (NLR) receptors[9]. This recognition leads to the activation of effector-triggered-immunity (ETI)[10]. ETI through activation of *R* genes is particularly effective at controlling pathogens[11,12] making the interaction less costly to the plant in terms of yield or growth penalty[13].

2Blades has developed a technology based on 985 nucleotide-binding leucine-rich repeat (NLR) proteins termed NLRseek™. NLRseek™ is a powerful platform that provides a highly efficient way to isolate useful NLRs and has been used to generate a collection of 6,000 transgenic wheat lines expressing these 985 NLRs from 69 accessions of 18 grass species and wild relatives of wheat. This platform has been utilized to discover novel resistance against wheat stem, stripe, and leaf rust.

OBJECTIVE AND GOAL STATEMENTS

The objective of this project was to screen multiple independent transgenic wheat events from 10 NLRs selected based on their reactions to Fusarium head blight infection in a pilot study in 2023. After screening in 2024, two genes were prioritized for transformation in corn germplasm to generate transgenic plants, which will be assessed in pathogen assays in the greenhouse and field by inoculating them with various isolates of *Fusarium* species and assessing gene performance.

MATERIALS AND METHODS

NLRseek™ Platform

The NLRseek™ library of 985 NLRs genes have been transformed individually into wheat lines. In a pilot study in 2023, we tested 100 wheat lines, representing 100 genes. Out of these 100 genes, lines from 10 genes showed a potentially enhanced resistance response when infected with a mixture of *Fusarium* species isolates causing Fusarium head blight in wheat. Outlined below is the process of testing 10 genes represented by 45 independent events, with 105 wheat lines.

Wheat disease field trials

Planting:

Three replicates per transgenic wheat line and their controls were planted and germinated in the field in Rosemount, MN, in a randomized order according to APHIS guidelines. Each plot was composed of four

rows representing four different wheat lines. Each row contained 20 plants, with about a foot of spacing maintained between plots (Fig. 1).

Plot maintenance

Pesticide and cultural practices that are standard for agronomic trials were followed as necessary to prevent crop loss and unusable data. Broadleaf weeds were controlled by applications of Bromate or an equivalent herbicide with application rates, methods, and timing according to the instruction label. Also, we used a hand-sprayer with Round-Up (glyphosate, 6 oz/gallon) to control both grass and broadleaf weeds in the plots.

Fusarium growth

Fusarium isolates were grown from -80°C stocks in 100 ml of 3% mung bean agar in a 250 ml flask covered in foil. Plates were grown for 1 week under fluorescent light with a 12/12 light/dark photoperiod at a temperature of 25°C. After 1 week, plates were flooded with sterile water and gently scraped with a sterile plastic squeegee to collect conidia.

Inoculum preparation

One liter of water was added to one liter of corn kernels and autoclaved. After drying, the autoclaved corn kernels were incubated together with conidial suspensions at room temperature for 2 weeks, and dried. The dried corn spawn was used for field inoculation.

Fusarium inoculations

Transgenic plants and controls were inoculated by evenly spreading dried corn spawn infested with a composite of *Fusarium* isolates on the ground at the base of the plants at about 5g/row. The wild-type wheat cultivars Alsen and MN11394-6 show some resistance against *Fusarium* whereas Roblin is susceptible, and Fielder wildtype is less susceptible compared to Roblin; these lines were used as controls to compare the effect of the *Fusarium* resistance mediated by the transgenes in transgenic lines. Two inoculations were performed starting when 50-75% of the plots were at the heading stage.

Disease assessment

Disease severity was assessed by counting the number of diseased spikelets on 10 randomly selected spikes in a row. The percentage of the spike that is diseased was determined by calculating the average number of diseased spikelets over the entire row (Fig. 1b, c), divided by the total number of spikelets on a spike for that row, and multiplied by 100.

Sampling

Samples from spikes and kernels were obtained for determination of visually scabby score which determines the level of kernel damage.

Data analysis

First, the severity data in the percent value were converted to the proportion (0 to 1). In instances where data was missing, the value zero (0) was assigned. Then, the severity data in proportion were analyzed using beta-distribution generalized linear mixed-effect model (GLMM) with the logit link function, in which the "*Gene*" was the fixed effect and the "*CallusID.Independent.event*" and "*Rep*" were the random effects on the intercept. The R package, glmmADMB, was used for GLMM.

A **B** **C**

Figure 1. The field-based screening pipeline using the NLRseek™ wheat array. **(A)** Wheat lines were grown in the field. (**B**) Scoring. Our scoring assessed percentage Fusarium head blight severity as described in Material and Methods. (**C**) Fusarium head blight infection on a wheat head.

RESULTS AND DISCUSSION

Two genes appear to confer enhanced resistance to Fusarium head blight in wheat

From the pilot study in 2023, preliminary results suggested that 10 genes showed enhanced resistance to Fusarium head blight in wheat. These genes were selected for further testing in more replications and with more independent events in 2024. From these genes, 105 wheat lines representing 45 independent events were tested (Table 1).

Table 1. The list of 10 genes represented by 45 independent transgenic events with 105 wheat lines selected from the 2023 pilot study. Testing these genes was the focus of the project in 2024. The percentage severity score is the average of scores from all lines per gene tested.

Clone ID	# Lines tested	# Independent events tested	Severity score (%)
NLR_05_23	8	3	70
NLR _04_47	4	2	28
NLR _06_27	12	6	79
NLR _05_94	5	2	35
NLR _07_01	13	6	56
NLR _05_92	9	4	32
NLR_05_30	14	6	73
NLR_05_80	16	7	54
NLR_05_81	16	6	54
NLR_06_09	8	3	72
Fielder (Wildtype)	3		52
Roblin (Susceptible check)	3		79
Alsen (Moderate check)	3		33
MN11394-6 (Resistant check)	3		19

The severity score of plants transformed with NLR_05_92 and NLR_05_94 was significantly lower compared to that of Fielder wildtype, suggesting that expression of these two genes conferred enhanced resistance to Fusarium head blight in wheat (Fig. 2).

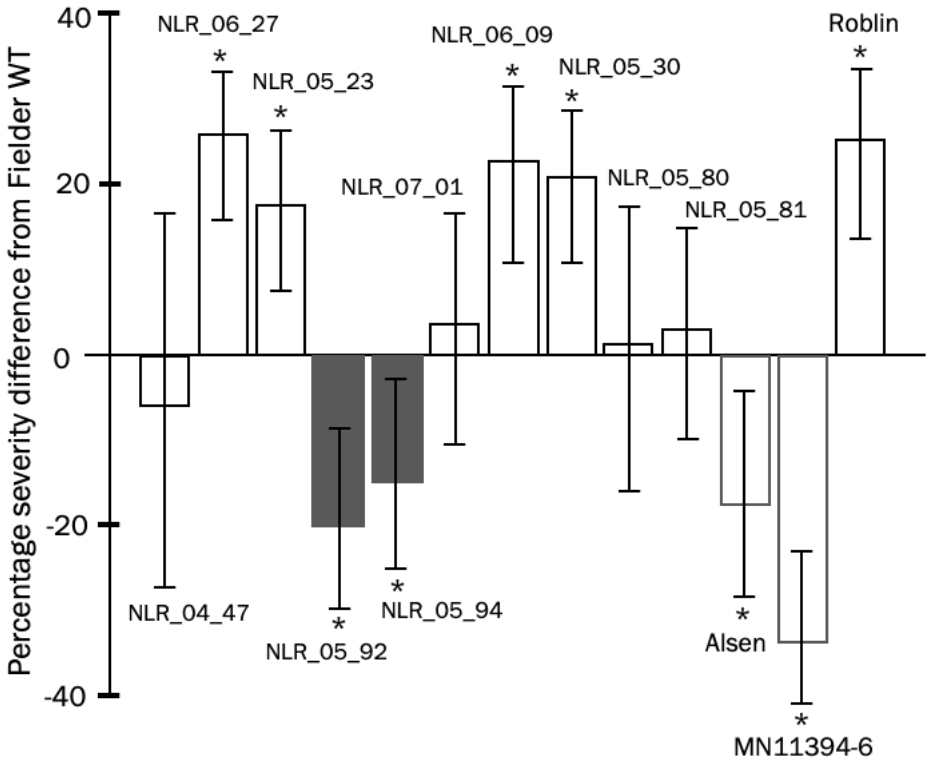

Figure 2. Two genes, NLR_05_92 and NLR_05_94 demonstrate enhanced resistance to Fusarium head blight in wheat. Severity score was determined as the difference in percentage severity between test genes (NLRs) and Fielder wildtype. Data was analyzed as described in Material and Methods using beta-distribution generalized linear mixed-effect model (GLMM) with the logit link function, in which the "*Gene*" was the fixed effect and the "*CallusID.Independent.event*" and "*Rep*" were the random effects on the intercept. The point zero (0) represents the percentage severity values of Fielder wildtype. Any gene with a positive value and an error bar that does not cross the zero line represents significantly higher susceptibility at $P < 0.05$. Any gene with a negative value and an error bar that does not cross the zero line represents significantly higher resistance at $P < 0.05$. Values represent mean ± SD of three biological replicates of all lines tested per gene as shown in Table 1. Asterisks (*) indicate statistically significant differences ($P < 0.05$) between the Fielder wildtype and test genes (NLRs).

Finding effective resistance to *Fusarium* species is complicated by the fact that it is in a group of fungal pathogens called hemibiotrophs. Hemibiotrophic fungi have a dual lifestyle where they infect and initially live on living plant cells (biotrophic phase) before killing and consuming the infected tissue (necrotrophic phase).While NLR-based resistance is effective against a group of fungi that infect and grow in living plant tissue (obligate biotrophic fungi), it has generally been assumed that NLRs may not be effective against hemibiotrophic fungi because they promote cell death as a mechanism to control disease spread, potentially facilitating necrotrophy.

Significantly, in this project, we have successfully identified two NLRs that may provide effective enhanced resistance to *Fusarium*, the pathogen that causes Fusarium head blight in wheat and ear and stalk rot in corn, as well as producing mycotoxins with significant health impacts.

CONCLUSIONS

We have identified two lead genes that appear to confer enhanced resistance to *Fusarium* species in wheat across test periods. These genes emerged from a 2023 pilot study in which 10 candidates showed potentially reduced disease severity compared to Fielder wildtype wheat. The next step is to transform these genes into corn for testing, marking a promising advancement for the project. The goal is to develop corn lines with field-level resistance to *Fusarium* species, which causes stalk- and ear rots, kernel damage and contamination with mycotoxins. Success in wheat and corn is transferable to barley, which is also affected by the same *Fusarium* species.

Genetically encoded resistance can both preserve corn yields and reduce mycotoxin levels in grain, increasing farmers' profits while supporting more sustainable farming practices. These benefits will help ensure that Minnesota corn producers remain competitive in the global marketplace.

EDUCATION, OUTREACH, AND PUBLICATIONS

2Blades was involved in a field exhibition organized by the University of Minnesota where approaches to solve various disease challenges were showcased to Minnesotan growers. 2Blades showcased the work of this project. To prepare for this exhibition, 2Blades produced pamphlets which were distributed to participants.

2Blades is training two students who are pursuing their undergraduate degree at the University of Minnesota. The students are being trained on field plant pathology, molecular biological techniques in plant pathology, sampling and microbiological techniques necessary for maintenance of fungal pathogens. In addition, 2Blades has hired a recent graduate of the University of Minnesota who is currently being trained on testing for disease resistance and susceptibility using various assays. These techniques are directly applicable to the project.

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