

Exploring Fusarium head blight (FHB) resistance with nanoparticles and EMS in wheat

by

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Abstract

Fusarium head blight (FHB), a disease caused by *Fusarium* fungi species, affects many small grain crops. It affects crop yield, quality, and safety. Affected wheat results in losses of \$5.6 billion USD per year in North America alone, and outbreaks are only predicted to increase.

Developing and growing FHB resistant wheat cultivars is one of the effective approaches to minimize FHB losses. The *Fhb1* and *Fhb7* genes have shown marked success when it comes to Type II resistance in wheat plants. Using nanoparticles and ethyl methanesulfonate (EMS) mutations, these genes were examined in two projects.

In the first project, lipid nanoparticles were paired with gene-editing plasmids with the goal of knocking out the wildtype *TaHRC-S* region of *Fhb1* gene in Bobwhite wheat seeds. This was done by treating the shoot apical meristems (SAMs) in germinated seeds to mutate the pluripotent cells before plant growth. If successful, this would introduce persistent mutations without integrating any transgenes. While there has been previous success in a pilot study with this method, our plants did not show any mutations once sequenced.

In the second project, Chinese Spring wheat seeds were randomly mutated. Lines with mutations in the *Fhb7* region were isolated and the FHB resistance of these lines was assessed phenotypically. This yielded 32 lines with significant mutations. 18 of these indicated a mutation in an essential area of the *Fhb7* gene that alters the resistance when compared to the wildtype. The remaining 14 lines showed signs of having significant mutations outside of the targeted region. Any synonymous mutations in the susceptible lines were set to the side, for more analysis on missense mutations in the *Fhb7* region.

To further connect these mutations with the phenotypic susceptibility, crown rot trials were correlated with our results. The results of this study will narrow down the essential regions in the *Fhb7* gene for resistance and provided important mutant lines for further FHB research.

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Chapter 1 - Introduction & Literature Review

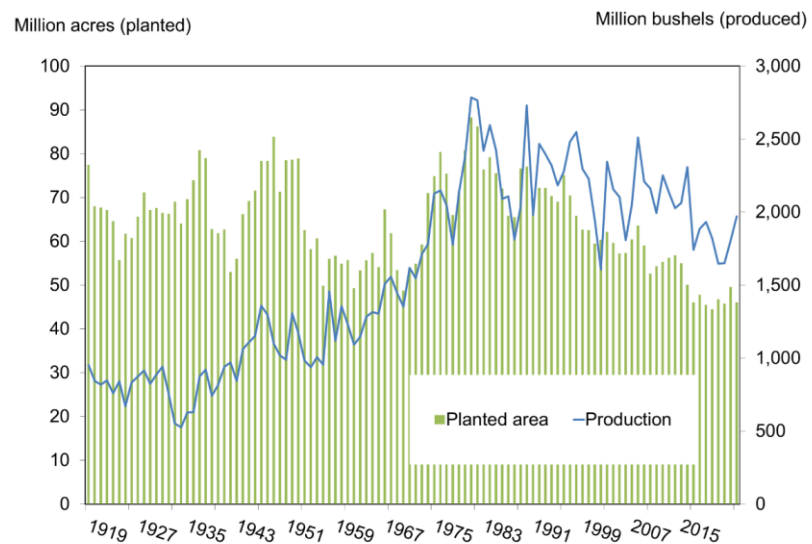
INTRODUCTION

Wheat, rice, corn, sorghum, and millet provide 60% of the total caloric energy consumed by humans. These cereal grains are embedded deeply within human agriculture and nutrition. Bread wheat (*Triticum aestivum*) alone, is the provider of 20% of the calories and protein consumed globally (Shen *et al.* 2025). It has taken its place as a pantry staple since it is easy to store, transport, and can be grown in a variety of climates (Venske 2019).

Wheat ranks third in U.S. field crops in planted acreage and production. In 2024, U.S. farmers were estimated to have produced 1.97 billion bushels of winter, spring, and durum wheat. The overall planted area is down, but this decline is partly made up by increased yields (Figure 1.1.) (USDA 2024).

Wheat farming has a long history, but the wheat that is grown and consumed today is much different than the wheat plant originally grown by our ancestors (Shen *et al.* 2025). As human civilizations developed, so did our cultivars.

U.S. wheat—planted area and production, 1919–2024



Source: USDA, National Agricultural Statistics Service.

Figure 1.1 U.S. Wheat- Planted Area and Production, 1919 – 2024

The land used for wheat production has been decreasing in the last few decades, but the production level has not directly followed this decline (Figure reproduced from: USDA 2024, National Agricultural Statistic Service).

Wild cereal species domestication has been traced as far back as 12,000 years ago, with evidence of wheat domestication as far back as 10,000 years ago (Meyer 2013). The domestication of crop plants began when humans moved from gathering plants to cultivating them (Devore & Lee 2017). Starting with wild plant populations, people have shaped the local flora to fit their needs. From generation to generation of plants, humans selectively bred individuals with specific, desirable traits. Some of the first traits selected were larger grains and non-shattering heads. These allowed for more nutrition from each seed and kept the seeds on the head at harvesting time (Shen *et al.* 2025). These changes made various aspects of agriculture easier, more efficient, and fruitful.

These beneficial traits, and numerous others, arose from random mutations; it took many generations to breed these spontaneous genes into stable crop populations. At first, the domestication process happened unconsciously, but the selective breeding of plants became purposeful once its benefits were discovered (Venske 2019). Humans have domesticated hundreds of plant and animal species for various reasons, such as food, medicine, and materials (Duarte *et al.* 2007).

Mapping quantitative trait locus (QTL), genome-wide association studies (GWAS), and whole-genome resequencing have revealed numerous genes showcasing the history of domestication. These studies show evidence of selection in the genomes of crops like maize, tomatoes, rice, and wheat species (Fuller 2012). ‘Domestication syndrome’ describes the traits that were commonly selected for, changes that improved agricultural production, product quality, or yield (Gepts *et al.* 2018). It can also reduce the overall diversity within a crop and have unintended detrimental effects on other traits.

ARTIFICIAL SELECTION

Domestication is a complex process that results in functional, structural, and genetic changes to the affected plants. When humans first began domesticating wheat, the concept of genes did not exist; they could only work with the observed phenotype (Vittori *et al.* 2017). Selectively breeding on phenotype alone is a time and labor-intensive process, as it depends entirely on the physical manifestation of unknown genes. Even when plants successfully cross, genetic drag can lower the fitness of lines. This process is long, and there is no precise way to insert a single, specific gene into another genome through breeding alone.

Plants with complementary favorable traits are crossed with the goal of identifying progeny with the favorable traits of both parents. When these two parents are mated, chromosomes are mixed, leaving offspring with a combination of both parental genes (Muller 1916). This chromosome crossing-over causes the F1 generation to go from its original state to an all-heterozygous-state. This can cause additivity and dominance effects. In F2, desired genes can get lost, or additional detrimental genes can become fixed. This can cause decreases in the overall health of the crop population. Recurrent backcrossing is traditionally used to fix this problem (Allard 1960).

Recovery of the elite parent's genome takes up to six backcrosses to reach 99.2% similarity (Babu *et al.* 2004). This type of selection maintains the qualities of the host plant while adding only specific genes from the donor. The number of backcross generations for maximum recovery, b_{th} , used to be calculated as: $[1 - (1/2)^{b+1}]$ However, this formula ignores linkage drag (Brinkman & Frey 1977).

Today, we still employ these traditional breeding techniques, but with standard breeding practices alone, it can take 10 plus years to develop and release a new crop variety (Englund 2019). Marker-assisted selection (MAS) and marker-assisted backcrossing selection (MABS) have helped speed this process up by relying on genotype, rather than phenotype (**Figure 1.2**) (Tanksley & Rick 1980).

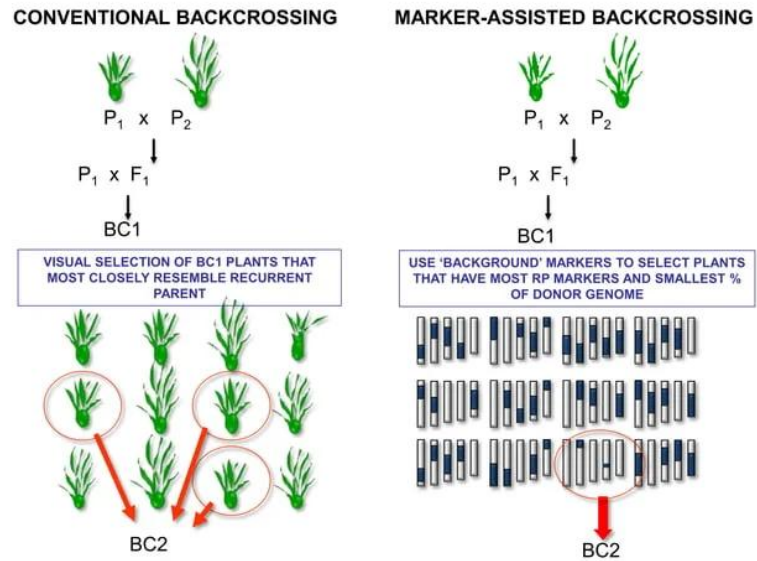


Figure 1.2 Traditional Backcrossing vs. Marker-assisted backcrossing
MAB uses known gene markers near the target gene to intentionally breed individuals with desired genotype. (Figure reproduced from Rani 2014)

MODERN WHEAT

During the “Green Revolution” in the mid-1960s, what we consider our ‘modern’ cultivars, were developed. These lines were bred to be shorter, photoperiod insensitive, and to produce higher yield (Venske 2019). The two main genes responsible for these changes, Reduced height (*Rht*) and Photoperiod (*Ppd*), are widespread in many cultivars today (Borojevic 2005). Since these cultivars were developed, the technology for breeding has only advanced.

Figure 1.3 illustrates a condensed timeline of these breeding innovations.

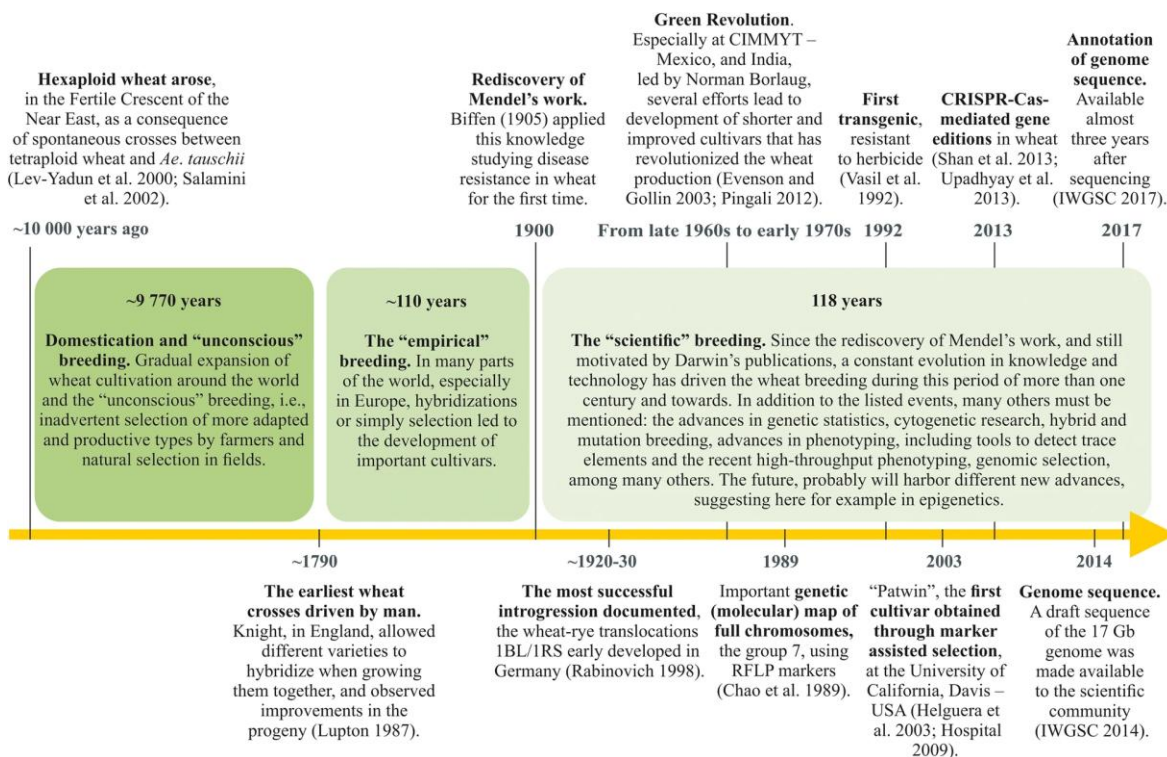


Figure 1.3 Wheat Breeding History

Timeline showing the evolution from unconscious and empirical breeding to modern, scientific breeding phases in wheat (Figure reproduced from Venske *et al.* 2019)

Modern wheat cultivars have deeper roots, higher stress tolerance, and much higher yield than ‘ancient’ classes like emmer, einkorn and spelt (Odone 2025). Bread wheat (*Triticum aestivum*) makes up 95% of current US production, with durum wheat (*Triticum turgidum* ssp. durum) composing the remaining 5% (Gooding *et al.* 2023). There are five main classes of

US Wheat by Region 2020

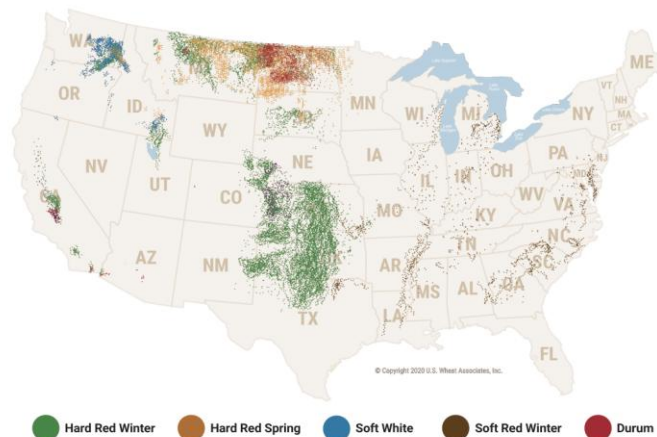


Figure 1.4 U.S. Wheat by Region

Wheat classes grown across the United States in 2020 (Figure reproduced from USW 2020).

wheat grown in the US, each with unique adaptations serving specific purposes (**Figure 1.4**).

Hard red winter (HRW) wheat is grown in the Great Plains and Pacific Northwest. It is the most widely grown type of bread wheat in the U.S. and is popular for its range of uses. Hard red spring (HRS) wheat is grown in the North Central and Southwest regions and is the most popular class after HRW. It is commonly used in bagels, and hard roll recipes (USW 2025).

Soft white (SW) wheat is grown in the Pacific Northwest and is the preferred flour for pastries and biscuits. Approximately 80% of SW grown in the U.S. is exported. Soft red winter (SRW) wheat is grown in the eastern U.S. It is used for cookies and snack food production. Durum, the smallest class of wheat grown in the U.S., is grown in the North Central region. It has a high protein content (12.0-15.0%) and is used to make pasta and couscous (USW 2025).

WHEAT DEMAND

The main objectives of wheat breeding over the last few centuries have been increasing the yield potential, disease resistance, and abiotic stress tolerance (Venske *et al.* 2019). The need for healthy, affordable, and accessible food sources increases alongside our rising population size. The average wheat yield must increase at least 40% by 2050 to provide enough food for the projected growing global population (Reynolds & Braun 2022). Researchers worldwide are dedicating time, energy, and money to find sustainable ways to proactively fulfill this demand. The delicate balance between crop supply and demand requires forethought and planning from scientists, breeders, and farmers cooperating and pooling resources. Many groups and individuals are working to meet this upcoming need, and the approaches used vary widely in scope and execution.

Rapid population growth has defined our ever-evolving approach to agriculture practices and food policies. As a species, we have made many large jumps in population size throughout human history. From 1986 to 1998, the global population increased from 5 billion people to 6 billion; between 2010 and 2020, we saw an increase from 7 billion people to 7.8 billion people. Globally, we are projected to add 1.3 more billion people to the population between 2020 and 2050 (Liu 2020). By 2100, it has been predicted that the world population will reach 10.9 billion people (**Figure 1.5**) (United Nations 2019).

Even as fertility levels decline, the world population will likely continue to grow due to the momentum of population growth (Liu 2020). While education and family planning, the two main factors that contribute to fertility decline, are increasing, a significant population increase is still expected (Hirschman 1994; United Nations 2019).

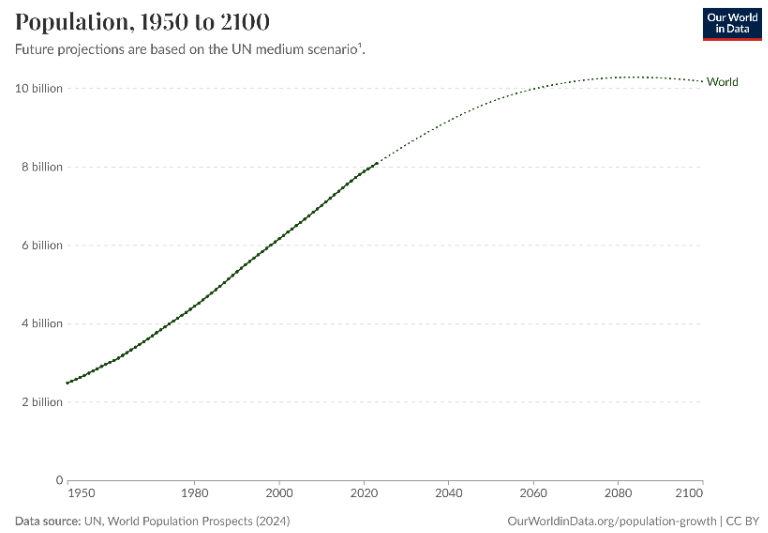


Figure 1.5 Population, 1950 to 2100

Global population future projection based on fertility, mortality, and migration patterns (Figure reproduced from OWD 2024).

Not only is our food demand expected to grow, but our current crop output is also at risk for drastic decreases. A 2 °C temperature increase would reduce wheat yields in the Global South by 10–15% (Reynolds & Braun 2022). Similarly, other crop staples are expected to suffer yield losses as climate patterns change (Lobell *et al.* 2011). As precipitation and temperature patterns change, plants must contend with stresses like salinity, drought, and temperature stress (Mahli *et al.* 2020).

With these projected numbers, there are many facets to be considered, such as human welfare, nutrition, and ecosystem viability (United Nations 2015). While there are suitable locations to increase the expanse of agricultural land, it is also important to consider the preservation of natural resources and ecosystems. It is not realistic to simply increase the amount of land and the scale of our fields. We should seek to use the land we have efficiently, preserving as much as possible. Established ecosystems have an inherent value, and they are also valuable for supporting human health and disposition (Ceballos 2010; Capaldi 2014).

Therefore, we must aim to strike a balance between agricultural needs and the diverse needs within our natural environments. Already, agricultural expansion is tied to diminishing wild lands; research suggests that the 9% increase of cropland in the last two decades is due to expansion attempts to recoup yield losses from drought stress (Zaveri 2020)

As Dr. Gerardo Ceballos, a renowned ecologist, puts it, “Every bit of diversity lost represents a diminution of interesting features of our environment” (Ceballos 2010). It has been well-established that exposure to nature is associated with increased happiness and emotional well-being (Berman *et al.* 2008; White *et al.* 2013). The question that remains is how to ensure that global crop demands are met while also being good stewards to the natural resources afforded to humans.

Land is a limited resource. There is only so much usable land for farming, building, and living. This lack of abundance naturally leads to competition, especially between agricultural and non-agricultural sectors. Current U.S. land totals 2.26 billion acres (Bigelow 2017). The USDA’s Economic Research Service’s (ERS) most recent report found that 53% of those 2.26 billion acres are used for agricultural purposes. Globally, there has been an increase in agricultural

expansion. However, in the U.S. there has been a decreasing trend in farmland use; a 2-million-acre decrease was found between 2012 and 2017, likely due to the difference between cost and profit (USDA 2017). Relatively higher rent from urban land use helps drive the conversion of agricultural lands (Irwin 2007). This agricultural land conversion (ALC) along with limited farmland availability, is a threat to the required food supply (Bigelow 2017).

INCREASING CROP YIELD/MINIMIZING LOSS

Rising agricultural consumption causes water shortages, soil depletion, pollution, deforestation, biodiversity loss, and higher food costs. These devastating impacts will hit the poorest countries first and hardest. Many of these countries have limited agricultural resources, leaving them with few options to fight against these concerns (Alexandratos 2005). To mitigate these effects, breeders and scientists are working to increase yield and minimize crop loss.

Raising overall crop yield has been a goal since the advent of agriculture. Specialized breeding of crops with desirable traits can be traced back as far as 9,980 BC (Devore & Lee 2017). Maximizing yield while minimizing resources and costs is an effective approach to increase crop output and meet demands (Thorntwasite 1953). Increasing a plant's capacity is an appealing idea, but it often takes decades and is limited by potentially unfavorable trade-offs (Sinclair *et al.* 2004).

A different approach to increase yield is the prevention of crop loss. This can come from resistance to common diseases, environmental stressors, and certain pests. For example, 'California Blackeye No. 27' was released in 1999 as a heat-tolerant commercial cultivar of cowpea (Ehlers 2000). This was done by isolating germplasm resistant to high nighttime

temperatures and breeding it into elite cultivars (Hall 1992). This raised yield by removing the negative effects of heat-stress.

While alternative methods for crop development and nutritional increases are necessary, protecting/maximizing our current output will help minimize the need for external resources and increased land usage (Foley *et al.* 2011). By editing the *SlJAZ2* gene, a lab in Madrid, Spain was able to create a tomato line resistant to speck disease (Ortigosa *et al.* 2019). This type of disease resistance is a form of yield preservation and increase.

Weather patterns contribute to disease transmission, emergence/re-emergence, and spread (Semenza & Menne 2009). Higher temperatures can lower incubation times, which increases disease vector populations and increases disease distribution (Lindgren *et al.* 2012). Crop diseases cause massive yield losses, and their prevalence is on the rise. By focusing on disease prevention and resistance, we can mitigate future loss and bolster crop potential.

FUSARIUM HEAD BLIGHT

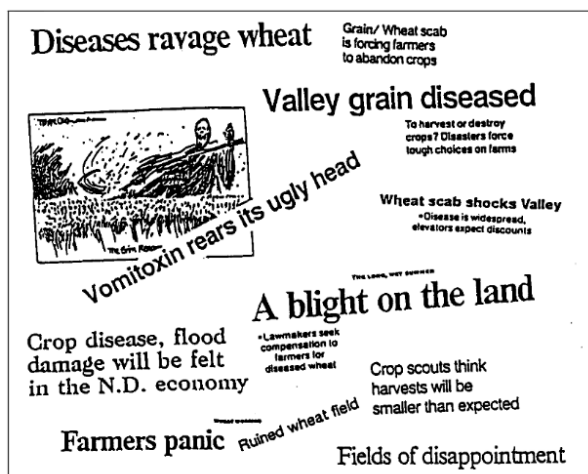


Figure 1.6 FHB Related Stories

Newspaper headlines associated with the 1993 FHB epidemic (Figure reproduced from McMullen *et al.* 1997).

There are many hurdles when it comes to increasing and maintaining wheat yield. Wheat, like all crops, requires water, space, and specific nutrients in adequate amounts to thrive. Even when these needs are met, grain loss can come from uncontrollable or unforeseen forces, such as diseases and pests. There are many diseases and insects that affect wheat fields. Leaf rust, Hessian fly, curl mites, and Fusarium head

blight (FHB) are the most impactful pathogens and insects that affect wheat production globally (Bockus *et al.* 2010).

In 2024, there was 326.7 million bushels of yield losses from wheat diseases in the U.S. and the Canadian provinces of Alberta and Ontario (Crop Production Network 2025). This amount was valued at \$1.8 billion USD. A large proportion of that, 3,641 bushels, were lost to FHB (**Figure 1.6**) (Anderson *et al.* 2025). Every year, wheat affected by FHB results in losses of 28 million metric tons of grain (Savary *et al.* 2019).

FHB, also known as scab disease, is caused by *Fusarium* fungi species. It is a widespread problem for small grain crops (Buerstmayr *et al.* 2020). The disease affects crop yield, quality, and food safety. In 2019, a worldwide survey on pest and pathogen loss across crop species reported that FHB ranked just under leaf rust for bread wheat yield losses (**Figure 1.7**) (Savary *et al.* 2019). FHB was first described by Worthington G. in 1884, and in the 1920s, the US was hit with its first FHB outbreak



Figure 1.7 Global Distribution of FHB

Orange dots represent FHB cases based on documented outbreaks (Figure reproduced from Shude *et al.* 2020).

(Worthington 1884; Powell *et al.* 2021). Following this infestation, FHB outbreaks were reported in many states. The FHB epidemics slowed in the following decades, leading to a lackadaisical attitude towards the disease.

After this brief reprieve, FHB spread rapidly across the US in the 1970s. A 1982 epidemic resulted in a 4% reduction in the total US wheat production; this was equivalent to 2.72

million metric tons, or 5,996,573,531.40 pounds, of loss (McMullen 1997). The outbreaks continued and spread across states into the 1990s. Losses from 1991 to 1996 were estimated to be approximately \$7.67 billion USD. This was the most expensive wheat loss the US had faced at the time (Mielniczuk 2020).

Since the first recorded North American FHB epidemic in the 1920s, various approaches have been used to mitigate the disease and its effects. This has previously included crop resistance, altered agriculture practices, and fungicide applications (Powell & Vujanovic 2021). Despite these efforts, the disease remains a concern for many cereal grains. It is predicted that more outbreaks will occur, driven by climate change and accelerated disease development

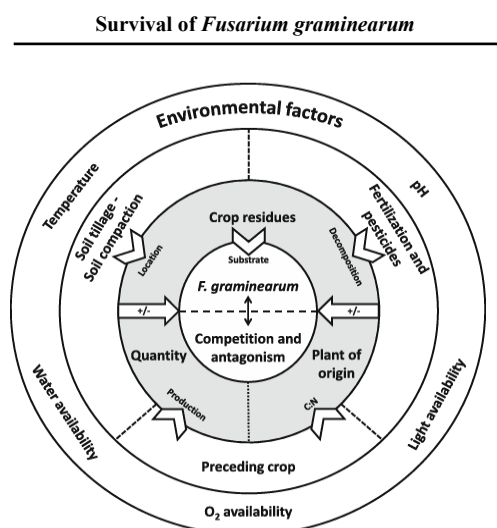


Figure 1.8 Fusarium Spore Survival

Environmental factors affecting the survival of *Fusarium* spores and fungus in the soil (Figure reproduced from Leplat *et al.* 2012).

(Ward, 2008). FHB infections are maximized by warm, humid conditions during flowering and early stages of seed development (Gilbert & Tekauz 2000)

Fusarium species commonly reside in the soil surface (Reyes Gaige *et al.* 2020). This means that certain agricultural practices can expedite and encourage infection within fields (Todorovic *et al.*, 2023). Reduced/no tillage cropping

practices have been shown to increase FHB infection rates (Beyer *et al.* 2006). Certain weather patterns can contribute to the spread of FHB from year to year and crop to crop (Ward 2008).

Increased cases of FHB have been seen when cycling through planting maize followed by wheat, especially during rainy seasons. The fungi can remain on infected corn stalks and then infect the

next year's wheat crop (**Figure 1.8**) (Hoffer *et al.* 1918). This is the primary source of infection, even though *Fusarium* can also be aerially transported (Atanasoff 1920).

This movement allows for spores to infect fields that are miles away from the infected host plants. This adds another layer of complexity to managing FHB infections, since re-introduction is possible due to the fungus spore's ability for atmospheric movement. The spores' range is limited only by how far the wind can take it (Aylor 1986).

Other FHB hosts such as gramineous weeds, sunflowers, and even soybeans are reported to support *F. graminearum* establishment through plant matter residue (Martinelli *et al.* 2001; Pereyra & Dill-Macky 2008). The fungus itself is known to survive for more than two years when infecting plant residue that is above or on the soil- this provides a long-term source for infection (Pereyra *et al.* 2004). *F.*

graminearum in the soil, rather than on plant residue, can survive on its own (McMullen & Stack 1983). However, fungus in the ground does not appear to be a significant source for direct FHB infection, when compared to plant-matter infection (Shaner 2003).

However, *Fusarium* species in the soil can lead to Fusarium root rot (FRR) and

Fusarium crown rot (FCR) infections within many cereal grain crops (Parry *et al.* 1995; Kazan *et al.* 2012).

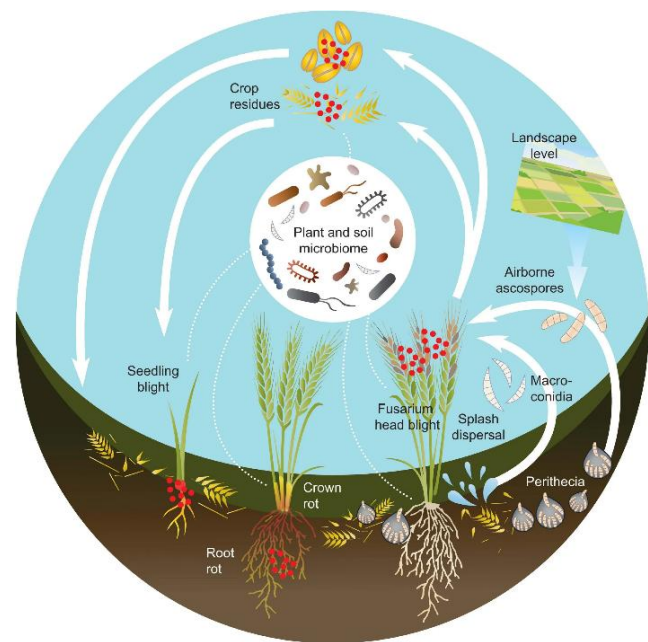


Figure 1.9 FHB Disease Cycle/Interactions

Inoculum originates in crop residues, soil, or from aerial dispersal (Figure reproduced from Karlsson 2021).

F. graminearum produces three types of spores: 1) asexual, non-motile conidias 2) sexually produced ascospores, and 3) chlamydospores (Geiser et al., 2013). Conidiospores rely on wind or rain for dispersion while ascospores are released by the perithecia, a fruiting body, before abiotic transportation (Trail *et al.* 2005). Chlamydospores are larger, with thick cell walls that allow the spores to survive and disperse within the soil (Leslie & Summerell 2006). This diversity of spores leads to *Fusarium* inoculum in the air, in the soil, and in crop residues, as shown in **Figure 1.9**.

MYCOTOXINS

FHB infections in wheat cause bleached heads and ‘scabby,’ low-weight seeds. Infected seeds are toxic for human and animal consumption since *Fusarium* produces mycotoxins (Kazan *et al.* 2012). Mycotoxins are secondary metabolites produced by certain fungal species. They have toxic results when ingested by humans and animals. This toxicity, known as mycotoxicosis, has varying levels of severity based on exposure, age, and other interacting factors (Peraica *et al.* 1999). Every year, hundreds of millions of dollars are lost due to mycotoxigenic *Fusarium* species infecting crops (Windels 2000).

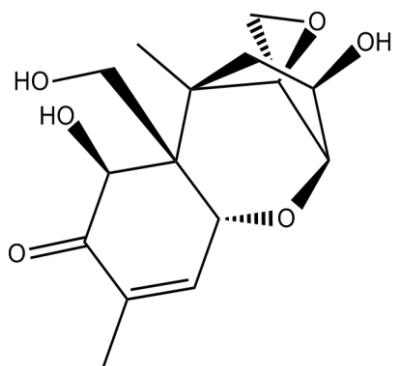


Figure 1.10 DON Chemical Structure
Deoxynivalenol (DON) (Figure reproduced from Pavlina 2010).

Mycotoxins were first described in 1891 by Dr. Sakaki, a Japanese physician. Symptoms from the consumption of fungus-infected rice caused an investigation into the toxic fungal metabolites (Ueno 1988). Deoxynivalenol (DON) is a mycotoxin produced by *Fusarium graminearum*, the main fungus species that causes FHB in wheat (Bottalico & Perrone 2002). DON, also referred to as vomitoxin, is a trichothecene (**Figure 1.10**).

Trichothecenes are epoxide-containing sesquiterpenoid compounds that can slow protein and nucleic acid production in eukaryotes. ‘Sesquiterpenes’ are a category of terpenes, compounds with the formula $(C_5H_8)_n$, that are composed by three isoprene units. By interacting with the 60S ribosomal unit, trichothecenes prevent polypeptide chain initiation and elongation (Cundliffe *et al.* 1974; Carter & Cannon 1977). This effect makes them important compounds for pathogen virulence in crops (Bai *et al.* 2001).

There is a significant relationship between DON levels and visible damage from *Fusarium* species (Tittlemier 2019). Another class of mycotoxins, zearalenones, is produced by *F. graminearum*, but the compounds have not been found to be necessary for FHB development within wheat (Kowalska *et al.* 2016).

DON consumption leads to fever, acute nausea, vomiting, abdominal pain, diarrhea, and dizziness in humans and animals (Sobrova *et al.* 2010). Extremely high concentrations of DON can be fatal or lead to long-term effects (Forsell *et al.* 1987). While such high concentrations are unlikely to be present in food sources, DON is still a cause for health concerns.

DON levels are generally unaffected by common forms of culinary preparation (Sobrova *et al.* 2010). In 2006, a study found that DON has no reduction in concentration after 30 minutes at 170°C (Sugita-Konishi *et al.* 2006). Most food safety guidelines recommend an internal temperature of 165 °C to minimize the risk of potential pathogens (USDA 2025). These mycotoxins make reducing FHB susceptibility a high priority when considering the new cultivars and integrating host resistance.

FHB RESISTANCE

When discussing FHB resistance, there are two categories in which adaptations can fall into: passive or active (Mesterházy *et al.* 1999). Passive mechanisms are based on morphological changes- physical differences that aid in inhibiting disease resistance. For FHB, this includes traits such as longer, loose wheat heads to decrease humidity, and a longer internode between the head and flag leaf to avoid rain splashes, flowering dates, and grain-filling rates (Rudd *et al.* 2001; Ban 2003). Active resistance involves genetic interactions and physiological processes that determine the plant's response to infection (Pritsch *et al.* 2000; Browne & Brindle 2007; Mesterházy 2020).

Five types of active FHB resistance have been reported thus far. In 1963, Type I and Type II FHB resistance were identified. Type I resistance is measured by a plant's resistance to the initial infection (Schroeder & Christensen 1963; Bai & Shaner 1994). This can be measured with inoculum applied evenly over wheat heads, usually through a spraying or natural inoculation. After a successful inoculation, resistance is measured through disease incidence and severity (**Figure 1.11**).

Type II resistance measures disease spread within the spike of the wheat head after successful infection. Type II is the focus of modern testing due to the difficulty measuring Type I resistance. In a greenhouse setting, Type II resistance is easily measured with the phenotypic results of point inoculation. The inoculum is injected into a single floret on the wheat head, and



Figure 1.11 FHB-infested Wheat
Wheat heads infected in the KSU Rocky Ford testing fields (2024).

disease progress is tracked over time (Bai & Shaner 1994). A highly resistant wheat line will show only 1-2 infected spikelets after 21 days, while a more susceptible variety is liable to show near 100% infection rate at just 12 days post infection (Bai & Shaner 1994; Schroeder & Christensen 1963). This allows for discrete categories of the most ‘susceptible’ and ‘resistant’ to be recorded consistently.

A third type of resistance, Type III was later described in 1995. This type deals with resistance to DON toxin accumulation (Mesterházy 1995). This can be measured with gas chromatography (GC) and mass spectrometry (MS). The FDA regulates DON accumulation at grain elevators; grains that have more than 1 ppm are unfit for human consumption, and 5 ppm is the cutoff for animal feed (Food and Drug Administration 2010). Type III resistance is also highly correlated with Type I & II resistance (Paul *et al.* 2006). There have been two other types of resistance described: Types IV & V. Type IV measures resistance to infection within the grain and Type V measures tolerance to yield loss (Mesterházy 1995; Buerstmayr *et al.* 2009; Liu *et al.* 2009).

There have been successful examples of active resistance in recent years. In 2017, a barley UDP-glucosyltransferase was transferred into wheat. This transgenic wheat line had significant Type II resistance and lower DON than the parental line (Li *et al.* 2017). Since 1999, hundreds of QTLs have been identified that are tied to FHB resistance, opening promising avenues for cloning host resistance into elite cultivars (Bai *et al.* 1999; Steiner *et al.* 2017).

FHB R GENES

The aerobiology of *F. graminearum* makes containment and control of FHB difficult. These obstacles led to a focus on finding and using host resistance (Aylor 1986). Host resistance

is a reliable approach for FHB disease resistance (Edwards *et al.* 2001; Mesterházy 2014). However, despite screening thousands of wheat lines, little resistance to *Fusarium* has been found (Wulff 2020). Only two genes with a large effect have been isolated and associated with significant resistance to FHB, with a handful of QTLs with smaller effects. FHB resistance is a polygenic trait; many minor genes work together to increase resistance. This makes it difficult to determine correlation between the many genes and their phenotypic impact (Bai *et al.* 2019).

FHB resistance genes and quantitative trait loci (QTL) have been mapped on all 21 wheat chromosomes, 50 of which have been linked to unique chromosome locations (Liu *et al.* 2009; Steiner *et al.* 2017). Only nine QTLs at this point have qualified as ‘Mendelized’ genes and been named: *Fhb1-Fhb9* (Buerstmayr *et al.* 2019). The *Fhb1*, *Fhb2*, *Fhb4*, and *Fhb5* genes are sourced from common wheat, while *Fhb3*, *Fhb6*, and *Fhb7* are from various ‘alien’ sources (Bai 2018). *Fhb1* and *Fhb7* have shown higher levels of resistance than the other QTLs (Cuthbert 2006; Guo *et al.* 2015). Most other QTLs showed minor effects on resistance, but their effects can be compounded when they are pyramided together (Cai 2016; Liet *et al.* 2016; Hu *et al.* 2023).

Fhb1 was discovered on the chromosome arm 3BS of Chinese germplasm ‘Sumai3’, ‘Ning7840,’ or ‘Wangshuibai’ (Bai *et al.* 1999; Waldron *et al.* 1999; Su *et al.* 2019). *Fhb1* provides large and repeatable resistance to FHB in wheat (Cuthbert 2006; Su *et al.* 2019). Wheat cultivars with *Fhb1* have a much lower percentage of symptomatic spikelets (PSS) when compared to lines without *Fhb1* (Balut *et al.* 2013; Clark *et al.* 2016).

Fhb1 has shown stable resistance in multiple backgrounds, making it a good target gene for breeding (Bai *et al.* 1999; Zhou *et al.* 2002; Cuthbert *et al.* 2006; Schweiger *et al.* 2016).

When bred into different genetic backgrounds, *Fhb1* can reduce the severity of FHB by 20-50%, including in durum wheat. Sometimes though, this introgression came with undesired linkage drag that reduced agronomically beneficial traits (Jin *et al.* 2013; Prat *et al.* 2017). There have been great successes in breeding *Fhb1* into many lines, however, in some backgrounds *Fhb1* is ineffective. The reasons are unknown but speculated to be due to antagonistic interactions with *Fhb1* with other genes in those genomes (Xu *et al.* 2019; Zheng *et al.* 2022).

The area/gene within this QTL responsible for this pathogenic resistance is accepted to be the *TaHRC* gene, although there has been support for a pore-forming toxin (*PFT*) gene being causal (Rawat *et al.* 2016; Su *et al.* 2017, 2019). In a 2018 study, the wildtype (wt) *TaHRC* was deleted from wheat cultivars, and this led to an increase in resistance, providing evidence that wt *TaHRC* acts as a susceptibility gene within *Fhb1* (Chen *et al.* 2018). When this gene is disrupted, a resistant form is created (*TaHRC*-S to *TaHRC*-R). *Fhb1* has been transferred into many cultivars worldwide using marker-assisted selection (MAS) (Bai *et al.* 2018). For example, in the U.S., Pioneer® has released the soft winter wheat (SWW) cultivars ‘25R18,’ ‘25R42,’ and ‘25R51’ that contain *Fhb1* and are available for commercial use (Brown-Guedira *et al.* 2008).

In 2020, the *Fhb7* gene was transferred into *Triticum aestivum* on the 7DL chromosome arm. *Fhb7* confers very effective FHB resistance to many species of wheat, but there is continued debate on the mechanism. This is due to inconsistent resistance between species. The gene originated from *Thinopyrum elongatum*, a distantly related species that is often used in wheat hybridization breeding (Guo *et al.* 2015; Wang *et al.* 2020). *Fhb7* has been recently cloned, and found to encode a glutathione S-transferase (GST), which can provide broad resistance to multiple *Fusarium* species by detoxifying trichothecenes, a mycotoxin class, through de-epoxidation (Wang *et al.* 2020).

There is no homolog of this gene in related wheat species, making this discovery especially important. It is likely that *T. elongatum* acquired this gene through horizontal gene transfer from a fungus in the *Epichloë* family (Wang *et al.* 2020). The fungus most likely thrived with this additional gene as it decreased competition from other fungal species with different modes of infection. (Similar to how antibacterial properties are discovered through bacterial compounds). *Fhb7* does not show a negative association with other agronomic traits, thus it is a good candidate for FHB resistance breeding.

Fhb2-6 and *Fhb8 & 9* do provide resistance to FHB disease, but their effects are much smaller than *Fhb1* and *Fhb7*. *Fhb2* resides on chromosome arm 6BL in Sumai3 (Cuthbert *et al.* 2007). *Fhb3* was found in the alien species *Leymus racemosus* and was transferred to chromosome 7AS in bread wheat (Qi *et al.* 2008). *Fhb4* was found on chromosome 4BL in Wangshuibai, a Chinese landrace (Xue *et al.* 2010). *Fhb5* was discovered on chromosome 5AS, also in Wangshuibai (Xue *et al.* 2011). *Fhb6* was found in *Elymus tsukushiensis* and has been transferred into chromosome 1AS in common wheat (Cainong *et al.* 2015). *Fhb9*, the most recently discovered one, was found on chromosome arm 2DL (Zhang *et al.* 2025).

At this point, only *Fhb1* has been deployed extensively into breeding programs worldwide (Li *et al.* 2019; Su *et al.* 2019; Wang *et al.* 2020). While other named QTLs have not been widely used in breeding. The USDA Central Small Grain Genotyping Lab (USDA-CSGGL) has developed diagnostic markers for *Fhb1*, *Fhb7* and *Fhb9*, so they are ready to be pyramided in new wheat cultivars. Recently, USDA-CSGGL launched marker-assisted backcrossing programs (MAS) and pyramided the three genes into 13 elite hard winter wheat breeding lines or cultivars from six states, and the populations were distributed to these breeding programs for further agronomic traits selection.

EMS MUTAGENESIS

Since the 1930s, radiation, transposons, and chemical mutagens have been employed to induce mutations in plant species (Koornneef *et al.* 1982). Over 3,000 mutant varieties of 242 plant species, including crop species, have been released commercially worldwide. The most common mutant crops according to the Mutant Variety Database (MVD), are rice, barley, wheat, soybean, and maize in that order (Figure 1.12). Out of all the chemically induced mutants registered with the MVD, 80% were created with alkylation agents (MVD 2022).

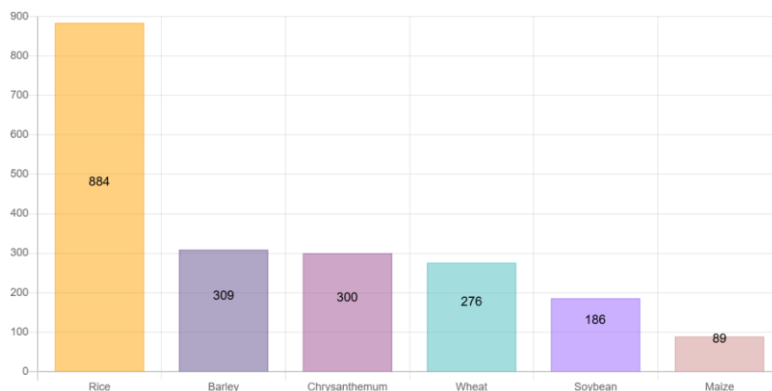


Figure 1.12 Commercially Available Mutant Crops

Number of commercially available mutants in the 6 most popular crop species. The y-axis represents the number of varieties, with the type detailed on the x-axis (Figure reproduced from MVD 2022).

Ethyl methanesulfonate (EMS) ($\text{C}_3\text{H}_8\text{O}_3\text{S}$) is a widely used chemical mutagen that works through alkylation. Its main mechanism is to introduce an alkyl group at the N^7 -guanine and the O^6 -guanine. This forms O^6 -ethylguanine that can pair with thymine (T) but not with cytosine (C). This causes the original G/C pair to be replaced with an A (adenine)/T (thymine) pair during subsequent DNA replication/repair (Figure 1.13) (Hoffmann 1980).

EMS causes mostly G/C to A/T transitions throughout the genome (Greene *et al.* 2003). However, it also generates G/C to C/G or G/C to T/A transversions with 7-ethylguanine hydrolysis and A/T to G/C transition by 3-ethyladenine pairing errors, but at a lower occurrence rate (Krieg 1963).

When EMS mutagenesis was first discovered, the mutation sites were considered completely random (Hoffmann 1980). However, studies suggest that the

locations of mutations from EMS do not follow a completely randomized trend. A 2021 study compared the locations of EMS single nucleotide polymorphisms (SNPs) and natural SNPs. In EMS populations, 26.65% of SNPs were in exons, and in natural populations 18.34% were in exons. Out of these exons, 59.82% of the SNPs found in the EMS population were nonsynonymous, and 58.23% were nonsynonymous in the natural population (Yan *et al.* 2021).

Table 1.1 provides a summary of this data.

Overall, ~9% more EMS-induced SNPs were in coding regions than in the natural population. EMS mutations seem to favor G/C sites, especially when adenine dimers (Two AA nucleotides) are upstream (Yan *et al.* 2021) So, while EMS mutations may not be entirely random, their distributions still have a large enough spread in the genome to be useful for genetic studies.

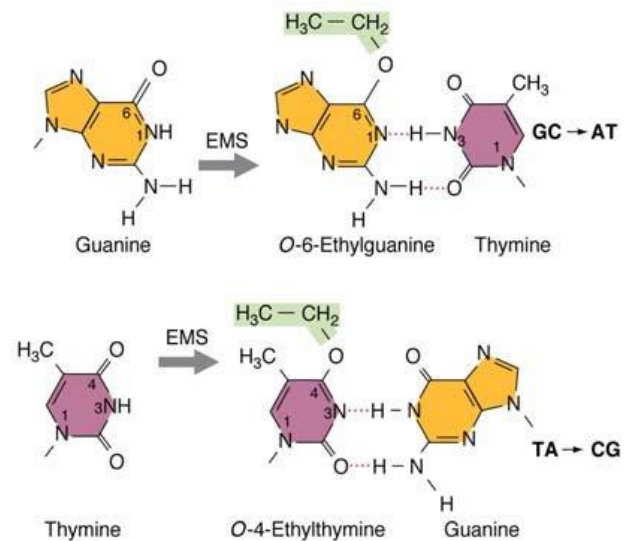


Figure 1.13 EMS Alkylation

The addition of an ethyl group onto the =O of guanine modifies its H-bonds, causing it to pair with thymine rather than cytosine. When this happens to thymine, it bonds to guanine, rather than adenine (Figure reproduced from W. H. Freeman & Company 2002).

Table 1.1 Distribution of EMS SNPs

Genome-wide distribution of EMS-induced SNPs vs. natural SNPs in the Rice3K project. There are differences in spread, which may be explained by preferred sites from EMS (Data from Yan *et al.* 2021)

Distribution Regions	EMS SNPs	Natural SNPs
Intergenic	53.60%	63.19%
Exons	26.65%	18.34%
Introns	15.92%	15.40%
5'UTR	1.25%	0.95%
3'UTR	1.79%	1.52%
Splicing Site	0.78%	0.59%

EMS is still used to generate mutant populations for studies, as it is easy and accessible. It can be used on many types of plant tissue to induce mutations, like seeds, spores, buds, branches, calluses, and pollen. For example, wheat lines resistant to the herbicide imidazolinone (IMI) were selected from EMS mutants. The EMS population produced plants with mutations in the *TaALS* gene. These mutations caused IMI resistance in wheat (Chen *et al.* 2021). This is one of many studies that demonstrate the potential of genetic screening of EMS populations for new agronomic traits. **Table 1.2** details some other examples of successful mutagenesis discoveries.

Table 1.2 Successful Examples of EMS Induced Mutants

EMS mutagenesis study in different crops (Table adapted from Chen *et al.* 2023).

Plant Species	EMS concentration	Additional technology	Traits
Watermelon accession ‘G42’	0%, 0.1%, 0.15% and 0.2%; 0, 40, 60 and 80 min	Incorporating the high-coverage and accurate long-read sequence data.	Elongated fruit shape and male sterility (<i>CIMS1</i>).
Rice (<i>Oryza sativa</i> L.)	3% EMS	Characterized for phenotypic, biochemical and grain qualities	Inducing early flowering mutants
Chickpea (<i>Cicer arietinum</i>)	4% EMS for 10 h	Genetic transformation of tobacco plants	Resistance to IMI herbicides

Dwarf wheat mutants: <i>dm1</i>, <i>dm2</i>, <i>dm3</i> & <i>dm4</i>	1–1.5% EMS for the duration of 4–8 h	Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses	Advancements in yield improvement
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CALLUS CULTURES

A callus culture is an undifferentiated mass of cells that can be regenerated into a full plant when exposed to the correct conditions and hormones (Gautheret 1934). A callus is formed when an explant, a piece of plant tissue, is placed on a nutrient medium to develop into ‘stem’ cells (**Figure 1.14**). Murashige and Skoog (MS) nutrient medium is a common media type for calluses due to its carbon, vitamins, and hormones (Murashige & Skoog 1962). Calluses can be made from the roots, stems, leaves, and even the seeds of numerous plant species.

Calluses can be regenerated through organogenesis or embryogenesis. Organogenesis uses hormones to develop shoots and roots while embryogenesis stimulates the callus to form somatic embryos that can germinate into a whole plant. Callus cells are invaluable to plant preservation and research. They have been widely used for secondary metabolite production, germplasm conservation, and plant propagation. The totipotency of calluses also provides a lot of potential when it comes to genetic engineering.

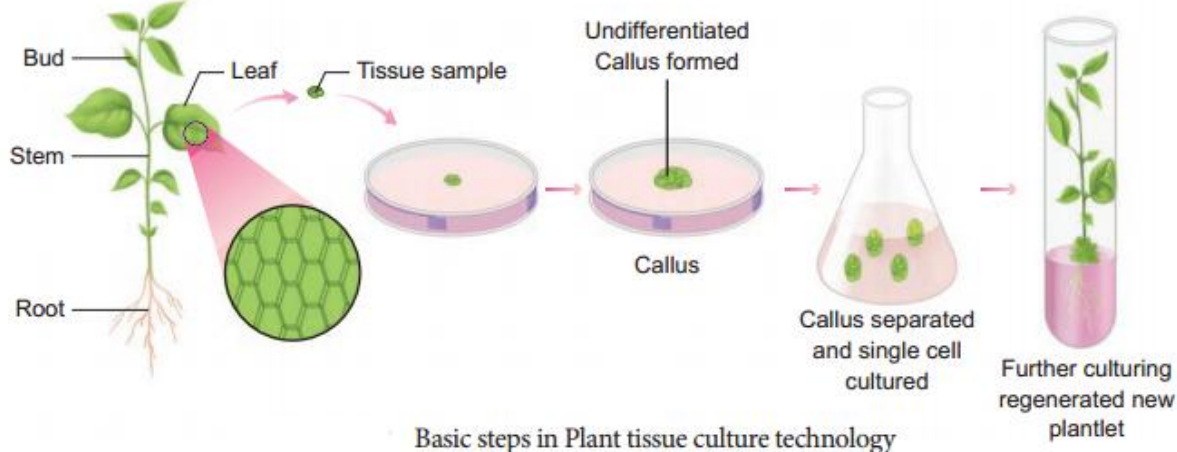


Figure 1.14 Callus Formation

Basic steps in callus formation/regeneration, starting with leaf tissue (Figure reproduced from Kruglova *et al.* 2023).

In 1983, *Agrobacterium tumefaciens*, a plant pathogen that naturally transforms plant cells, was used to make transgenic tobacco. DNA encoding an antibiotic resistance gene was added to the transfer DNA (T-DNA) of the *A. tumefaciens* and then transformed into tobacco cells from a callus (Barton *et al.* 1983; Fraley *et al.* 1983; Zambryski *et al.* 1983). This resulted in chimeric cells, some of which carried the antibiotic resistance gene. This is a popular method of transformation, due to its high success rate and its replication ease. It has even been used to engineer insect-resistant tobacco (Vaeck *et al.* 1987).

In 1990, particle bombardment was successfully utilized to create transgenic maize plants (Klein *et al.* 1987). Genes that conferred chlorsulfuron and phosphinothricin resistance were combined with *E. coli* β -glucuronidase and firefly luciferase genes that acted as reporter genes. These experiments resulted in 15 transgenic plants with persistent mutations (Fromm *et al.* 1990; Gordon-Kamm *et al.* 1990).

Since then, callus-based genetic transformation has been perfected for many crop species. For example, the first commercially available transgenic maize cultivar was approved in 1995 and released in the US in 2009 (United States Department of Agriculture Animal and Plant Health Inspection Service 1995; ISAAA 2009). This cultivar was called ‘Liberty Link’ and it was resistant to phosphinothricin (PPT), an herbicide. A variety of crop cultivars have been released that contain insect resistance genes, stress tolerance genes, and disease resistance genes (Klümper *et al.* 2014; Xiao & Wu 2019).

Calluses can be used in conjunction with many types of genetic transformation tools and are successful in many common crop species. When it comes to wheat species though, this technique is limited. Only a few wheat cultivars can rely on callus cultures due to generally low rates of callus induction and tissue regeneration. To overcome this, different methods must be used to perform wheat transformations.

GENETIC ADVANCEMENTS

Modern genetic understanding and tools have given scientists and breeders the ability to precisely breed, modify, and improve plant lines while saving time and money. It now takes approximately 7-10 years from development to the release of a new or improved cultivar. Instead of painstakingly breeding generations for a quarter of a century, we can rely on modern tools to increase our output. Molecular markers, reference genomes/annotation, genomic selection, speed breeding, and high-throughput phenotyping have all helped decrease the time necessary to develop new cultivars (Venske 2019).

The genetics field has fast improved since Mendelian genetics were discovered. Scientists have delved into genetic studies focusing on all the kingdoms of life, bringing to light crucial mechanisms and knowledge. In 1987, the first insect-resistant transgenic plants were generated with the transformation of a *Bacillus thuringiensis* toxin gene into tobacco, using *Agrobacterium* delivery (Vaecck *et al.* 1987). In the same year, clustered regularly interspaced short palindromic repeats (CRISPR) were discovered. **Figure 1.15** illustrates a condensed timeline of the advancements and intersections of breeding and research methods.

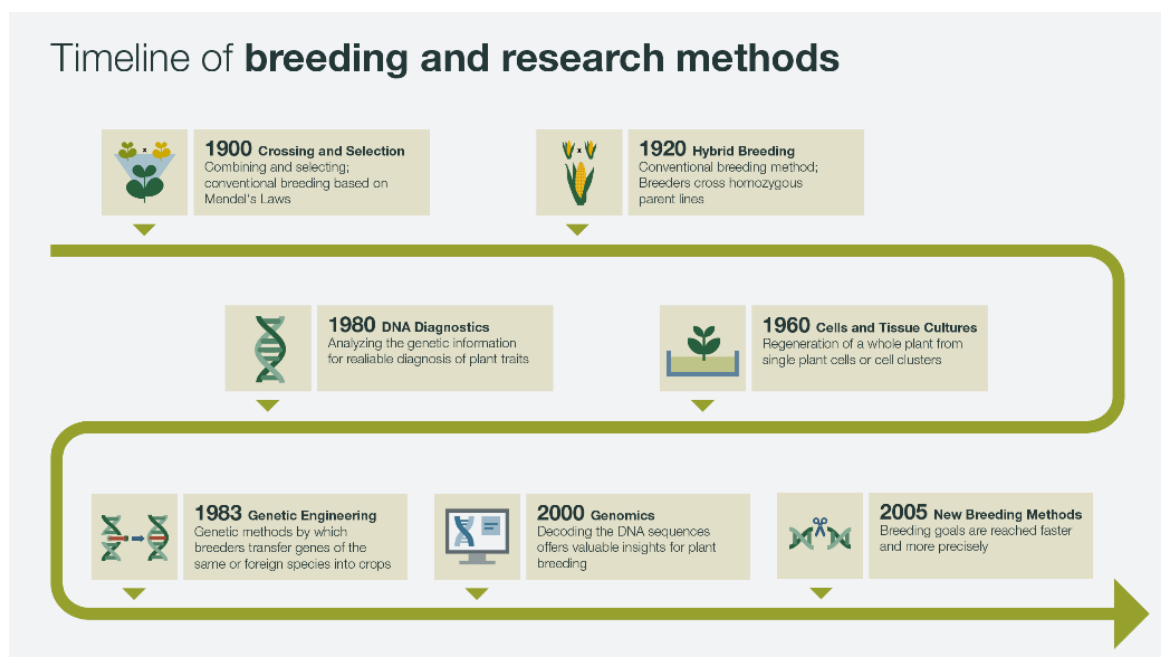


Figure 1.15 Breeding + Technology Timeline

Progress in advancement of breeding and research technologies from 1990 to 2005 (Figure reproduced from Foresight & KWS 2023).

CRISPR-CAS SYSTEM

One of the most influential advancements was the CRISPR-Cas system. CRISPR-clustered regularly interspaced short palindromic repeats- were first found in 1987 in *Escherichia coli* bacteria in a Japanese lab (Ishino 1987). It took many more years before its significance and

scope came to light, with a variety of research groups around the world adding pieces to the puzzle. By 2008, the CRISPR-associated protein 9 (Cas9) and the function of the CRISPR system were accepted, and in 2013, CRISPR-Cas9 was even used to successfully edit human cells (Brouns 2008; Cong 2013).

CRISPR-Cas9 occurs naturally in prokaryotes; this system defends organisms from viruses by cutting up foreign DNA (Hille 2018). The Cas proteins use CRISPR sequences to identify and then cut open specific strands of DNA that complement the CRISPR sequence. Scientists were able to ‘hijack’ this process to create direct mutations within an organism. Using guide RNA to target specific regions or genes, DNA could be inserted, substituted, or even deleted (Zhang 2014).

The original mechanism relies on sequence-specific nucleases (SSNs) and DNA double-strand breaks (DSBs) at locations specified by CRISPR sequences- the ‘protospacer adjacent motif’ (PAM). These DSBs are repaired with the cell’s native mechanisms through non-homologous end joining (NHEJ) and homology-directed repair (HDR) (**Figure 1.16**). NHEJ uses DNA ligase to reconnect the sliced ends, while HDR relies on a template to repair breaks. NHEJ can cause errors, while HDR typically has perfect fixes. Normally, HDR relies on the sister chromatid to act as a template for the repair. However, CRISPR-Cas systems have allowed for outside templates to be used instead with SSNs. Guide RNA can be designed to direct where the DSB occurs to cause insertion or deletion in the gene of choice. The NHEJ pathway can still be useful and is typically used to ‘knockout’ genes by introducing frameshift mutations or large deletions.

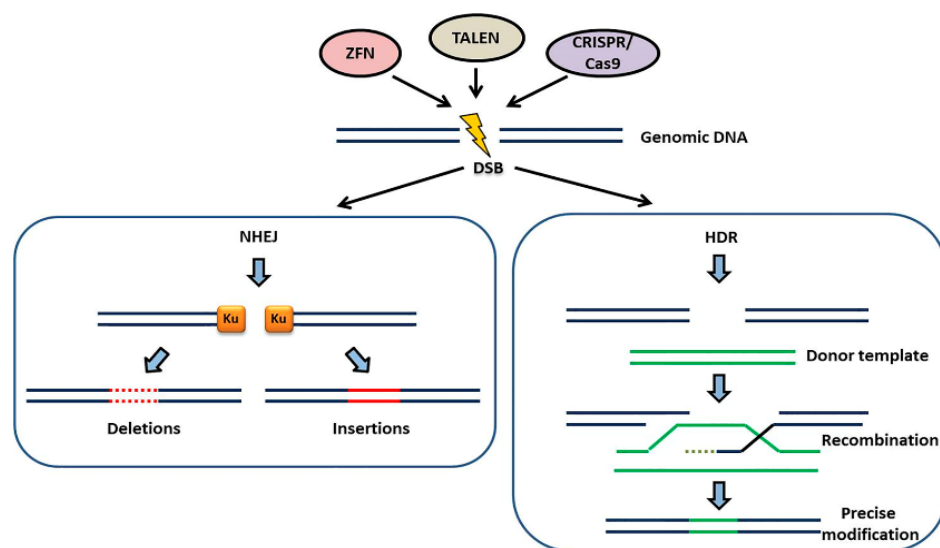


Figure 1.16 NHEJ vs HDR Repair

NHEJ vs HDR repair in the CRISPR system (Figure reproduced from Nekar *et al.* 2022).

Discovery of these molecules and mechanisms allowed for direct gene editing. The ability to modify crops, manipulate genomes in experiments, and even treat genetic diseases was now possible. This technique was so revolutionary that the scientist who discovered it, Jennifer Doudna from UC Berkeley, received a Nobel prize less than 10 years after its discovery (Gostimskaya *et al.* 2022). CRISPR has been used for gene editing in at least 20 crop species for many different traits, such as stress tolerance and yield improvement (Ricroch *et al.* 2017).

Table 1.3 details additional successful mutants created with modern techniques.

Table 1.3 CRISPR-Cas Crops

Examples of successful genome editing using CRISPR-Cas systems to improve disease resistance and abiotic stress tolerance in rice, wheat, maize, & tomato (Table adapted from Nekar *et al.* 2022).

Plant	Gene Modified	Function	Agronomic Trait	Transformation Method
Rice	<i>Bsr-k1</i>	Broad spectrum resistance	Broad spectrum resistance	Agrobacterium-mediated transformation
Wheat	<i>TaMlo-A1</i> , <i>TaMlo-B1</i> , & <i>TaMlo-D1</i>	Mildew resistance	Resistance to powdery mildew	Biolistic transformation

Maize	<i>ALS</i>	Acetolactate synthase	Herbicide tolerance	Biolistic transformation
Tomato	<i>BZRI</i>	Brassinosteroid regulator	Heat stress	Agrobacterium-mediated transformation

NANOPARTICLES

The concept of nanoparticles is credited to Paul Ehrlich, a German physician who believed that targeted delivery could improve drug therapy (Greiling 1954). In the 1960s, the first nanoparticles for drug delivery and vaccines were created by Peter Paul Speiser, professor at the Swiss Federal Institute of Technology (Merkle and Speiser 1973). Speiser was aiming for consistent immune stimulation for diseases like diphtheria and tetanus. These diseases require multiple injections to build up antibody levels,

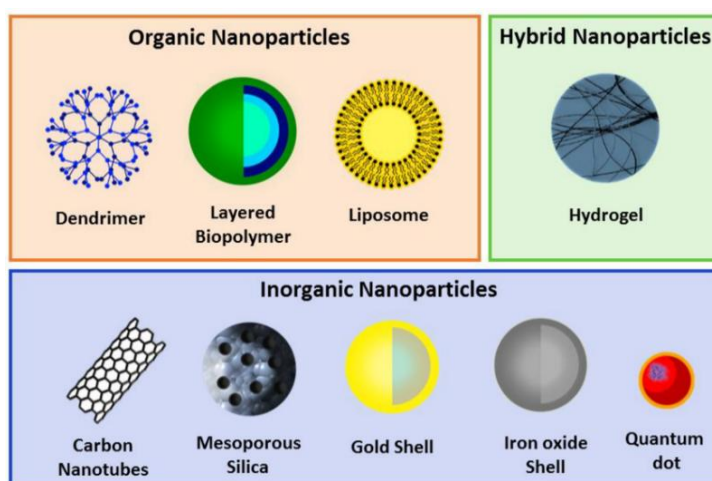


Figure 1.17 Nanoparticle Types

Examples of nanoparticles composed of organic and inorganic materials. (Figure reproduced from Silva *et. al* 2019).

so he theorized that sustained release of the vaccine could prevent this (Birrenbach & Speiser 1976). However, it wasn't until 1978 when these ideas were successfully developed. A visiting scholar in Speiser's lab, Richard Oppenheim, developed gelatin and albumin nanoparticles (Marty *et al.* 1978). Since then, this technology has quickly improved.

Today, the term 'nanoparticle' encapsulates a wide range of structures, from anywhere between 1 nanometer to 1 millimeter in size (Peer *et al.* 2007). They can be composed of organic, inorganic, and/or carbon-based materials (**Figure 1.17**) (Silva *et. al* 2019). Their

composition depends on the intended use. For example, lipid-based nanoparticles are often used for hydrophilic and hydrophobic drugs, and metal particles, *i.e.* gold, are popular for diagnostic imaging and therapeutics (Singh & Lillard 2008; Rosyidah *et al.* 2023). Overall, nanocarriers enable site-specific releases and delivery of various materials to otherwise inaccessible tissues. For drug delivery, they provide stability, solubility, and better bioavailability (Singh & Lillard 2008). A wide range of nanoparticles has been successfully used for delivering vaccines, cancer therapies, and gene editing materials to various cells (Vogel *et al.* 2021; Meng *et al.* 2022; Karlsson *et al.* 2021; Hunter & Tomich 2019).

For example, lipid nanoparticles (LNPs) have been used as carriers to create COVID-19 nucleoside-modified mRNA-LNP vaccines- BNT162b2 (Pfizer) and mRNA-1273 (Moderna) (Vogel *et al.* 2021; Corbett *et al.* 2020). The LNP delivery lets the messenger RNA (mRNA) enter the cells and transiently express the antigen with the host cell's natural mechanisms (Rijkers *et al.* 2021). Using nanoparticles for vaccine deliveries allows for precise targeting and controlled release for inoculation against diseases.

Nanoparticles have even been used to deliver cancer vaccines. The vaccine-particle complexes can activate host immune response against tumor cells using precise targeting to introduce tumor antigens and immune adjuvants (Meng *et al.* 2022). In addition to this approach, nanoparticles carrying nucleic acids and gene-editing tools are also being considered to target tumor pathways, gene expression and metastasis (Xu *et al.* 2021).

BRANCHED AMPHIPATHIC PEPTIDE CAPSULES

Branched Amphipathic Peptide Capsules (BAPCs) are nanoparticles designed to carry and deliver proteins, peptides, and nucleic acids within cells. They were developed by Dr. John Tomich, a professor of biochemistry and molecular biophysics at Kansas State University from 2010 to 2018. In 2018, they were licensed by Phoreus Biotechnology.

BAPCs have positive, hydrophilic headgroups, and hydrophobic, negative tails. This allows them to transport materials with both negative and positive charges. BAPCs are inert with low cytotoxicity, but they can deliver active ingredients. They contain a peptide bilayer that allows the capsules to dissolve while still carrying a variety of compounds (**Figure 1.18**). Surface-bound proteins and nucleic acids are shielded from degradation while attached to the BAPC, and peptides are protected within the capsule.

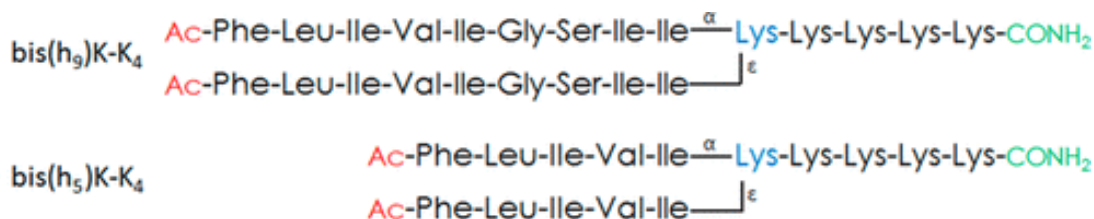


Figure 1.18 BAPC Structure

Structure of BAPCs. The peptiplexes are made of mixture of N-terminal acetylated $\text{bis}(h_9)\text{-K-K}_4$ and $\text{bis}(h_5)\text{-K-K}_4$ (Figure reproduced from Avila *et al.* 2015).

The particles enter cells through the natural endosomal pathway- the cell membrane envelopes the positively charged BAPC, forming an endosome. The endosomal wall is broken down within the cell, releasing the BAPC into the perinuclear cytosol. When the BAPC is exposed to the pH in the cytosol (5.7pH), electrostatic attraction is reduced. This releases the ‘payloads’ from the outside and inside of the BAPC. Proteins, peptides, or nucleic acids can then carry out their intended purpose within the cell (**Figure 1.19**).

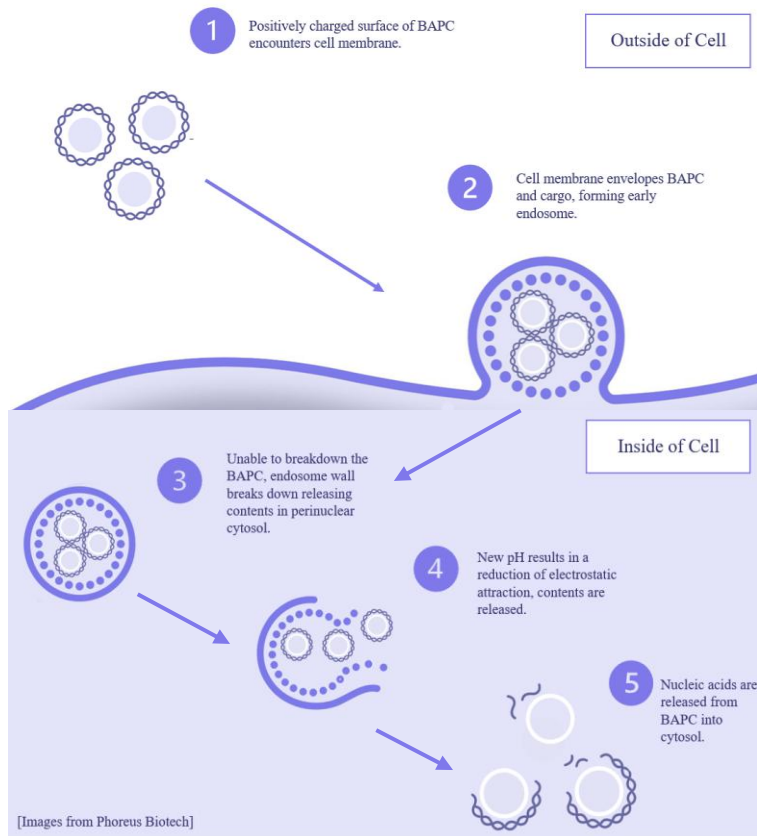


Figure 1.19 BAPC Cell Endocytosis

Illustration of the basic steps in BAPC uptake and delivery system (Figure reproduced from Phoreus Biotech 2018).

Targeting moieties can be attached to the BAPC for accurate delivery. Proteins, peptides, nucleic acids, oligonucleotides, plasmids, and CRISPR-Cas9 components have been successfully bound to BAPC surface, while fluorescent dyes, radionuclides, colloidal gold, and magnetic beads have been encapsulated within the BAPC for delivery.

BAPCs have been employed to improve the delivery of CRISPR components, plasmids, and dsRNA for heritable gene editing. A study from the USDA and KSU

successfully deleted 550 nucleotides in the thioredoxin gene using BAPCs in insect nymphs and adults (Hunter & Tomich 2019). Another paper reports success using BAPCs complexed with double-stranded RNA (dsRNA) for gene silencing in beetles (Carroll *et al.* 2023). BAPCs have a wide range of applications, due to their high temperature stability, low cytotoxicity, and low immunogenicity.

HYPOTHESES/GOALS

As discussed, *Fhb1* and *Fhb7* are two important genes impacting FHB resistance, and host resistance is a reliable way to fight against scab disease. As FHB continues to spread, accelerated by higher temperatures and certain agricultural practices, research on resistance

genes needs to keep pace. With that, finding efficient ways to utilize these genes is also essential for further crop improvement and protection.

Building on the progress of FHB resistance genetics and various mutagenesis processes, we wanted to find new ways to utilize emerging technology to understand and dissect FHB resistance. Methods employed include nanoparticle introduction of vectors to investigate introducing FHB resistance with *Fhb1*/*TaHRC* alteration and testing an EMS population to examine FHB resistance by mutating *Fhb7*.

TaHRC, the active gene in the *Fhb1* QTL, was the first target. The wildtype *TaHRC* is accepted as an FHB susceptibility gene; when portions of the gene are deleted, FHB resistance increases. Another ideal target was the *PDS* gene; when disrupted, this gene causes a yellow, pale color in the plant. An experiment was devised to knock out the wildtype *TaHRC-S* and *PDS* gene in the Bobwhite cultivar. To do this, we tested a novel transformation method for wheat. BAPC nanoparticles were paired with our knockout vectors to neutralize *TaHRC-S* or *PDS*. The nanoparticle complexes were applied to the SAM of germinating wheat seeds, to avoid the need for a callus culture and subsequent plant regeneration. This would open a reliable pathway for using genetic editing tools on wheat, since most wheat is generally recalcitrant to tissue culture formation. In these knockout lines, success of the editing process could be assessed with sequencing and FHB susceptibility.

We are also interested in testing the effects of EMS mutation within the *Fhb7* gene. Studies suggest *Fhb7* carries potential, and to date has not been deployed in any commercial cultivars. It provides a high level of scab disease resistance and was found in a distantly related relative to wheat. We characterized an EMS population derived from the Chinese Spring wheat cultivar containing the *Fhb7* introgression. Utilizing greenhouse genotyping phenotyping trials,

mutations significantly affecting FHB disease resistance were identified. The goal of this research was to identify illuminating mutations in the *Fhb7* gene region and to make a pool of mutants for further study.

Chapter 2 - Editing Wheat Seeds with Nanoparticles

Abstract

FHB is an economically important disease- its yield losses can exceed 50% or between 70-100% in ‘favorable’ conditions. In 2003, \$16 million USD was lost to FHB outbreaks just in Maryland, North Carolina, and Virginia. Since then, outbreak occurrences have only increased throughout the USA.

There have been nine FHB R genes identified, all with varying levels of resistance to FHB. These genes can be a reliable source of protection against this disease, but breeding them into wheat can be a long, expensive process. In many crops, the use of genetic editing tools has sped up this process. However, many of these tools require callus cultures to be successful. Very few wheat cultivars can form calluses, leaving most of these tools inaccessible.

In this project, we tested an alternative method for gene-editing within wheat. By pairing plasmids with lipid nanoparticles, exposed SAM cells are targeted directly on germinated seeds. This would bypass the need for a callus culture while introducing transgene-free, persistent mutations to the germline.

Two different genes were targeted, *TaHRC-S* and *PDS*, with 5 different editing plasmids. These plasmids were complexed with our nanoparticles and applied to the SAM of germinated Bobwhite seeds. Various methods were used to penetrate the SAM cells to allow the genetic tools to knock out the chosen genes, including vacuum infiltration, cellulase degradation, and increased temperature. None of these techniques resulted in successful gene disruption/expression. However, this method is worth exploring further.

INTRODUCTION

Selective breeding has been used for thousands of years to improve crops. Today, we rely on things like genetic markers, reference genomes, and high throughput phenotyping to speed the process up and precisely breed for the desired genotypes. These improved breeding techniques work well, but they cannot insert a single, specific gene into wheat lines. When breeding for a desired gene, especially one from a species whose chromosomes do not freely recombine with wheat, the donor segment can carry deleterious genes, in addition to the target beneficial gene. This creates genetic drag by simultaneously incorporating undesirable traits to the population.

Removing undesirable genes takes many generations of backcrossing. The ability to directly edit an organism's genome was a huge discovery in the genetics field. It pushed the limits at the time, changing what was considered possible. Since the use of CRISPR/Cas9 technology has increased, so has our ability to easily customize our crops.

This is an advantage, particularly for wheat. There is a lack of genetic diversity within the species we rely on today. Bread wheat is a polyploid hybrid crop derived from three highly related diploid species, and it is a relatively young species when compared to other crops (Meyer 2013). These factors contribute to the limited genetic diversity. There has not been enough time to accumulate random mutations in the species. There are few extant sources of genes that breeders can pull from and breed into their lines. Occasionally, distantly related species can act as a gene source, but the genetic drag from these crosses often leads to poor offspring (Allard 1960).

Genetic editing tools save researchers time and money, especially when it comes to plant breeding. Editing a single cell, or a cluster of cells, has been achieved *in vitro* and *in vivo* in many crops. For example, *Agrobacterium* has been used to successfully transform crops such as

asparagus, tobacco, yams, and even maize with transfer DNAs (T-DNAs) (Hernalsteens *et al.* 1984; Vaeck *et al.* 1987; Schäfer *et al.* 1987; Graves & Goldman 1987).

CRISPR/Cas systems have had many successes in gene editing, and introducing persistent, targeted mutations to crop lines. In 2023, the biotechnology company GreenVenus used CRISPR technology to knock-out the polyphenol oxidase gene in various avocado lines. This reduced the browning of the fruit, extending shelf life and cutting down on food waste (Permyakova & Deineko 2023). Many crops such as rice, sorghum, tomato, and sweet oranges have all been successfully edited by CRISPR (Feng *et al.* 2013; Jiang *et al.* 2013; Brooks *et al.* 2014; Jia & Wang 2014). These numerous examples give this editing technology a robust defense for their use in crop modification.

Of course, there are limitations to this technology. In plant breeding, the modifying, deleting, or adding of specific gene sequences is done with the goal of keeping that genotype in future plant lines. Breeders want stable, inheritable phenotypes. This is achieved by ensuring the germline carries the desired gene(s). The germline consists of cells that develop into gametes that are used for plant reproduction. If only somatic, non-germline, cells are edited, the next generation will not carry the altered genome. So, any changes made with modern technology must affect the germline. **Figure 2.1** demonstrates this: An adult with altered somatic cells will not pass that alteration on to their offspring, but germline alterations can persist.

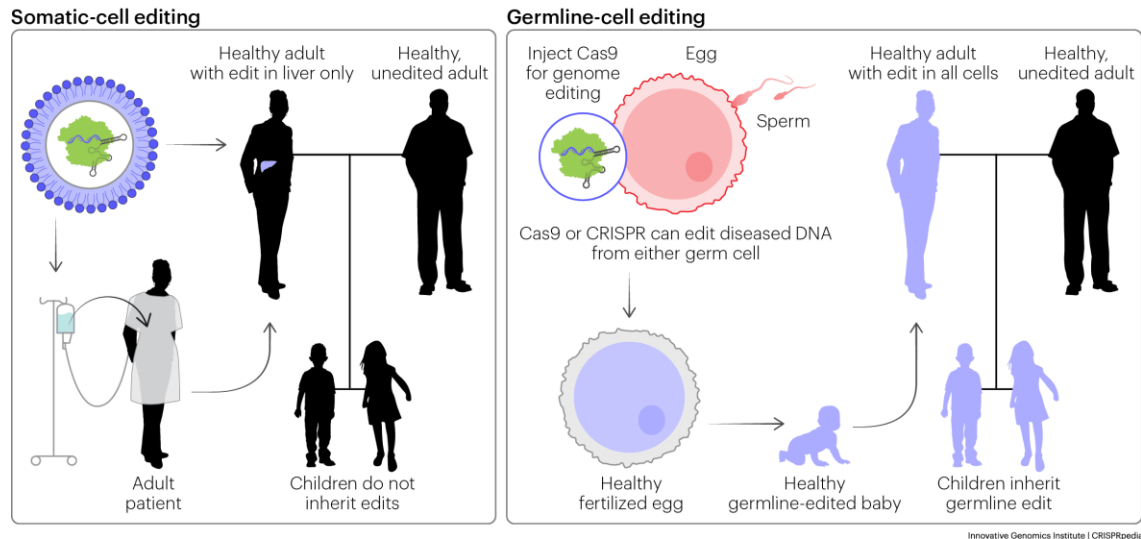


Figure 2.1 Somatic and Germline Mutations

Comparison of somatic and germline cell editing; only germline changes can be inherited (Figure reproduced from Innovative Genomics Institute 2020).

To create heritable mutations, researchers must consider techniques to alter germline cells. An effective approach has been using callused tissues that will eventually develop into germline cells. Callus cultures are a staple of plant gene editing due to their undifferentiated, pluripotent cells. When these cells are modified, the entire organism carries the genetic change introduced. Calluses are a relatively simple way to generate totipotent cells, usually relying on hormones.

Certain wheat lines have previously been edited through calluses, but only a few wheat cultivars can be used for this transformation method. Wheat has low rates of callus induction and callus tissue regeneration, so calluses are not a reliable approach (Reinert & Bajaj 1976). However, like all plants, wheat does have pluripotency in some cells naturally. These cells make up the shoot apical meristem (SAM) and root apical meristem (RAM) within plants.

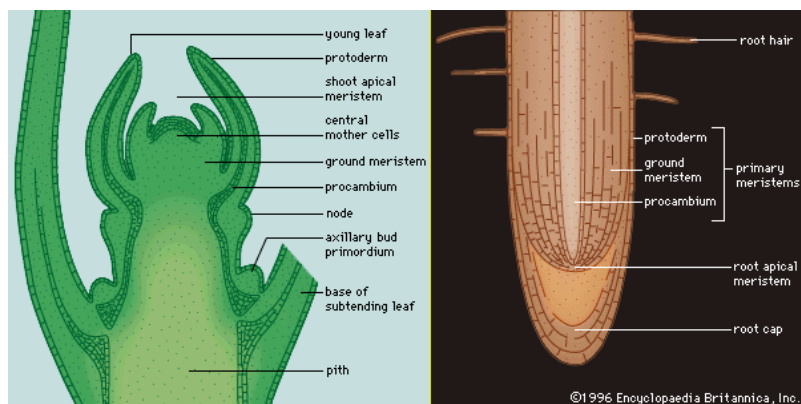


Figure 2.2 Shoot Apical Meristem

Anatomy of the SAM & RAM on a growing plant (Figure reproduced from Encyclopedia Britannica 1996).

The SAM is a cluster of cells at the tip of the shoot that are actively dividing while directing the growth of the plant. They orchestrate formation of leaves, stems, flowers, and other structures, while the RAM directs root

growth and development (**Figure 2.2**) (Kean-Galeno *et al.*). RAM and SAM both have plant stem cells, but the SAM is what leads germline cell production as the plant develops.

Callus cultures still rely on gene transformation to deliver CRISPR/Cas and gRNA into the tissues. This transgene integration causes any resulting lines to be classified as genetically modified organisms (GMOs). This can cause issues when producing new cultivars. Releasing GMO lines can be a lengthy and costly process, due to the numerous regulations in place. In the U.S., GMOs are regulated under the Coordinated Framework for the Regulation of Biotechnology; this framework was created in 1986 and hasn't been updated since 1992. The time to release a GMO crop is influenced by the traits and methods, but on average it takes 8-16 years before lines are released (McDougall 2011). An efficient gene delivery approach that doesn't require tissue culture and regeneration without transgene integration would help solve this problem and put new varieties into farmers' hands much faster.

If these totipotent cells in the SAM were directly edited early enough in development, before germline formation, then the entirety of the organism would carry the new, edited

genotype. This could be a path to bypass callus cultures entirely and ensure that deliberate mutations are passed on from one generation to the next.

Building on these principles, we hypothesized that modification of SAM cells in wheat embryos can create stable, transgene-free mutants, without the need for a callus culture. To test this, we used nanoparticles to deliver Cas9/gRNA components directly to the meristematic cells with hopes of genetic alteration of the *HRC* and *PDS* genes. Experiments were run where the SAM of wheat seeds were exposed through dissection and then treated with these nanoparticle complexes and various editing vectors. The *Triticum aestivum* accession ‘Bobwhite’ was chosen due to its previous gene editing success and high transformation efficiency with the CRISPR/Cas9 systems and Cas9 overexpression (Warburton *et al.* 2002; Chen *et al.* 2019). *HRC* encodes the reticulum histidine-rich calcium-binding protein (GenBank accession CBH32655.1), and it is the susceptible allele that regulates *Fhb1* mediated FHB resistance (Chen *et al.* 2018; Su *et al.* 2019).

With this gene intact, the Bobwhite cultivar is not resistant to FHB infection. Three plasmid vectors were designed to disrupt these genes through deletion at different target sites. The success of the deletions can be tested through sequencing and then confirmed through phenotyping FHB resistance. If *HRC-S* is not functional, the Bobwhite plants will have a higher resistance to scab disease. If successful, this would identify a new method for gene editing plants directly from seed. Two additional editing vectors were designed to knock out the carotenoid synthesizing gene, *PDS*, for a visual indicator of success.

MATERIALS AND METHODS

EXPERIMENT DESIGN

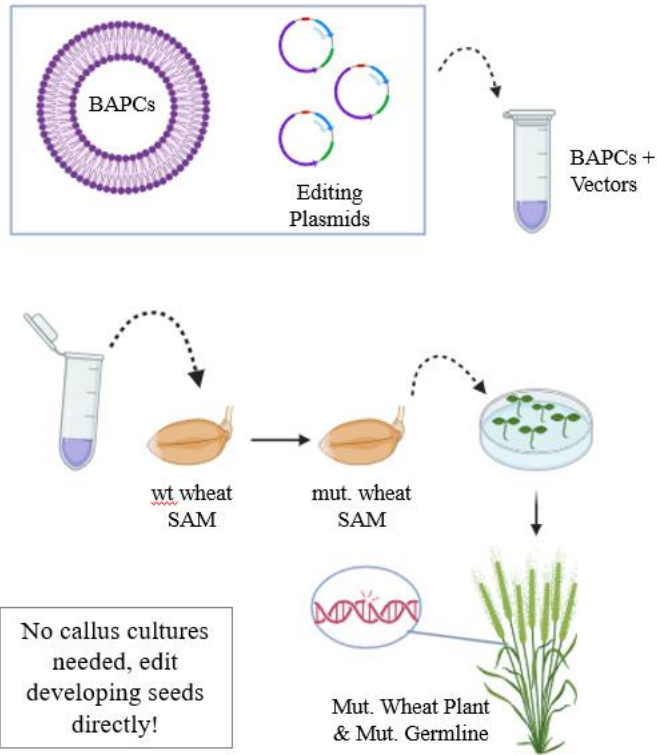


Figure 2.3 Nanoparticle Editing Experiment Overview
(Figure created at biorender.com)

The general steps of the experiment were as follows (**Figure 2.3**):

1) Create RNP complex for editing by combining nanoparticles and the Cas9/gRNA editing vector.

2) Treat the SAM with the RNP complex, delivering the editing vector to the meristematic cells from Bobwhite seeds.

3) Use vacuum infiltration and various methods to assist the complex with penetrating the SAM cells for transformation.

- 4) Grow seedlings from treated embryos, collect tissue for DNA extraction.
- 5) Identify any on-target mutations in treated plants through sequencing.
- 6) Confirm putative editing events by FHB point inoculation in the greenhouse.

RNP COMPLEX ASSEMBLY

THE NANOPARTICLE

Branched Amphipathic Peptide Capsules (BAPC) are nanoparticles (NP) that use lipid-mediated transfection to carry and transfer various materials (Gudlur *et al.* 2012). They were

purchased from the KSU Chemistry Department for this experiment. They can be used to carry proteins, peptides, and nucleic acids to specific targets (Barros *et al.* 2016). The nanoparticles can protect deliverables from lytic degradation, ensuring the vectors make it intact to the target. There have been many successful studies showing the potential of *in vitro* and *in vivo* gene delivery using both 10 nm (BAPC_10nm) and 25 nm (BAPC_25nm) BAPCs (Natarajan *et al.* 2020).

Based on initial test results, 25 nm BAPCs were selected for maximum cell transfection (Phoreus Biotech 2020). These BAPCs combined with the Cas9/gRNA plasmid vectors to form RNP complexes, with the nucleic acids attached to the outside. Different editing vectors were used, with the goal of knocking out two different genes: wt *HRC* and *PDS*.

Cas9/gRNA VECTORS

Five Cas9/gRNA plasmid vectors were designed to knockout the *HRC-S* and *PDS* genes in the wheat material. **Figure 2.4** illustrates one of these vectors, the 1-1 TaHRC-S plasmid. A *GFP* plasmid was also used to visibly track transformation success during the transformation process.

As discussed previously, the

wt *HRC* gene is a susceptible allele that affects FHB resistance, and three different plasmids

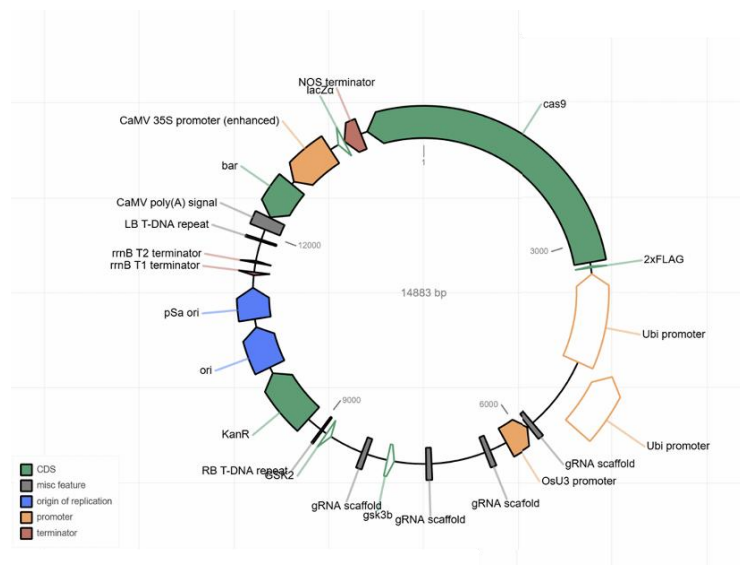


Figure 2.4 1-1 TaHRC-S Plasmid

Snapshot of the 1-1 TaHRC-S plasmid vector and its components (Figure reproduced from Eurofins Plasmid Sequencing).

were made to disrupt this gene in our Bobwhite seeds. *PDS* is a wheat phytoene desaturase gene. Wheat plants with a disrupted *PDS* gene have a bleached, yellow color from degradation of chlorophyll, since the gene is part of carotenoid biosynthesis (Cakir *et al.* 2010). Success with the nanoparticle editing system would be evident early on as the plants were grown from treated seeds. Similarly, *GFP* (green fluorescence protein) has a visible result under UV light, but this vector was used for transient expression (**Table 2.1**).

Table 2.1 Editing Vectors

Plasmids used in the RNP complexes and their respective targets.

Plasmid Vector	Purpose	Target Gene
1-1 TaHRC-S	Knock-out	<i>HRC-S</i>
1-4 TaHRC-S	Knock-out	<i>HRC-S</i>
1-1TaPDS	Knock-out	<i>PDS</i>
2-1 TaPDS	Knock-out	<i>PDS</i>
2-1 TaHRC-S	Knock-out	<i>HRC-S</i>
<u>HBT-SGF TYG-nos</u>	Transient Expression	N/A, GFP

BAPC COMPLEX/RNP

The 5 plasmid vectors were combined individually with BAPC particles to form BAPC complexes (RNP = Cas/gRNA ribonucleoprotein complexes) for seed treatment. The ratios of the BAPC, ddH₂O, and 10 mM CaCl₂ were calculated based on the concentration of the plasmid and the NH₂ (N) /Phosphate (P) charge ratio. BAPCs have a molecular weight of 2000 g/mol and contain 3 Arginine, 2 Lysine, and a free N-terminus. The provided nanoparticle stock was 10 mM or 5.88mg/ml (5882.3ng/ul). Below is an example of the N/P calculations used for the complexes.

Example N/P Calculation for 1 µg DNA mix with 5 µg of BAPC:

Step 1: Find P

- 1 µg of DNA = 3 nmol phosphate
- **P = 3 nmol**

Step 2: Find N

- Charges per peptide (*C_{pep}*) = 3 (Arg) + 2 (Lys) + 1 (N-term.) = 6 positive charges
- Moles of BAPC = (5 µg / 2000 g/mol) = 0.0025 µmol = 2.5 nmol
- Total moles of positive charge (NH₂) = 2.5 nmol x 6 pos. = **15 nmol**

Step 3: Calculate ratio

- N/P = **15/3** = 5 = **5:1 N/P** charge ratio

For vector 1-1 TaHRC-S, the concentration was 248.5 ng/µl. Tables **2.2** and **2.3**, show the calculations and ratios used when creating the RNP complex with the particle and 4000 ng of 1-1 TaHRC-S plasmid DNA. After combining the editing plasmid, BAPC, ddH₂O, and CaCl₂ in a sterile microtube, the contents were mixed via pipetting and briefly spun in a microcentrifuge. Then the solution was incubated at room temperature for 10 minutes to allow complexing of the plasmid and BAPCs.

Table 2.2 BAPC Calculation

N/P ratios and the respective volume of BAPC solution needed for the correct charge

N/P Ratio	ng of DNA	µl BAPC (5882 ng/µl)
7.5	4000	119.7
5	4000	80.2
2.5	4000	40.8
1	4000	16.3

Table 2.3 BAPC Solution Ratios

Ratios used for 100 μ l of the particle complex mixture (RNP) for the first plasmid, 1-1 TaHRC-S

Editing Vector	Vector ng/ μ l	μ l 4000 ng Plasmid	μ l BAPC	μ l ddH ₂ O	μ l 10 mM CaCl ₂	Total
1-1TaHRC-S	248.5	16.1 μ l	16.3 μ l	57.6 μ l	10 μ l	100.00 μ l

After making the BAPC solution, a sample was run on a 1.5% agarose gel with DNA SafeStain to confirm that the plasmids were able to form a complex with the BAPC nanoparticles (Molecular Designs, Birmingham, AL). Samples were loaded with a 3X loading dye, which was made with 200 ml 10mM Tri-HCl, 37.5g Ficoll-400, and 0.03g Bromphenol Blue.

Figure 2.5 shows a picture of one of these gels that was run with different volumes of BAPC. The first well on the right contained a 2 μ l sample of the 1-1 TaHRC-S plasmid alone.

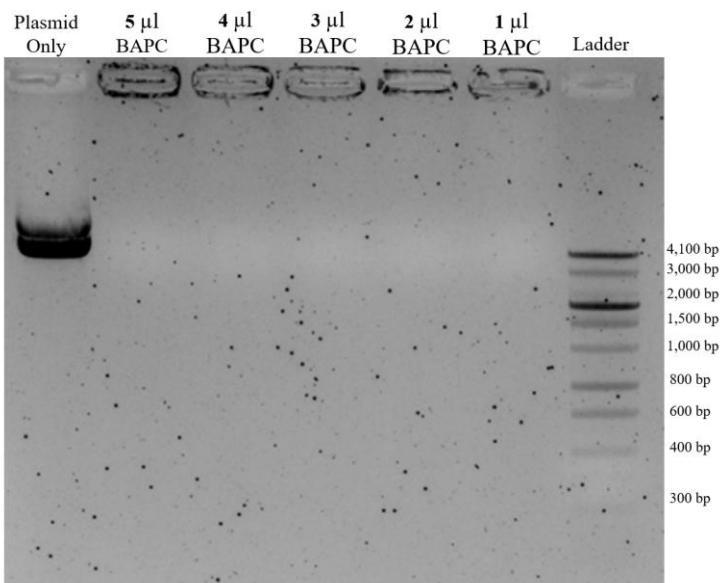


Figure 2.5 BAPC Complexing Test

Gel electrophoresis image of BAPC mixes with varying amounts of BAPC particles combined with the 1-1 TaHRC-S vector.

The subsequent 5 wells contained a series of BAPC mixtures with the 1-1 TaHRC-S vector plasmid and decreasing particle volume, from 5 μ l to 1 μ l. The last well on the right holds a 1 Kb ladder.

Since there is no visible band where the plasmid would be expected in wells 2-6, we were able to conclude that the nanoparticle was properly

complexed with the vector, and that even 1 μ l of 10 mM BAPC was sufficient for binding. The same test was run on the other plasmids as they were used, and each time a new BAPC + plasmid

mixture was made. Once this step was finished, the prepared seeds could be treated with the particle complex solution.

SEED PREPARATION

Seeds were disinfected with a 5% sodium hypochlorite (bleach) solution with a drop of tween. The seed/bleach mixture was gently agitated in a 50 ml conical tube for 30 minutes. After this period, the seeds were rinsed in deionized (DO) water a minimum of five times, or until there were no visible bubbles. The disinfected seeds were then transferred to a sterile, paper-lined petri dish. Tweezers were used to space the seeds out in a single layer. Once this was done, the seeds were placed at 4°C for 3-4 days (**Figure 2.6**).



Figure 2.6 Disinfected Bobwhite Seeds

Seeds after disinfection and germination. Seeds on the right are ready for dissection.



Figure 2.7 SAM Exposure

Bobwhite seed before and after coleoptile dissection to expose SAM

When the coleoptile emerged, the seeds were ready for dissection. Using a standard dissection scope, the coleoptile was carefully removed with a sterile scalpel, leaving the budding SAM intact for treatment, as shown in **Figure 2.7**.

TREATING THE SHOOT APICAL MERISTEM (SAM)

The exposed SAMs were treated with 1.5 µl of the BAPC complex solution under the dissection scope and then were then put under vacuum pressure for 15 minutes at room temperature to force the BAPC complexes into the SAM cells.

As this experiment continued, we evaluated cellulase degradation of the SAM and sonication of the SAM before treatment. After treatment, we also looked at centrifuging rather than vacuum pressure, and an increased temperature (37°C) after treatment to increase cellular uptake of the complexes.

Once the BAPCs enter the SAM cells through cytotaxis, the editing plasmids are transcribed by the cell's natural RNA polymerases before being translated into the Cas9 proteins. These are then complexed with the sgRNA for targeting, forming the RNPs within the cell (**Figure 2.8**). RNPs can then perform the gene editing in the SAM cells. If enough cells are transformed, there is a high chance of germline cells harboring the target mutation as the cells grow and divide.

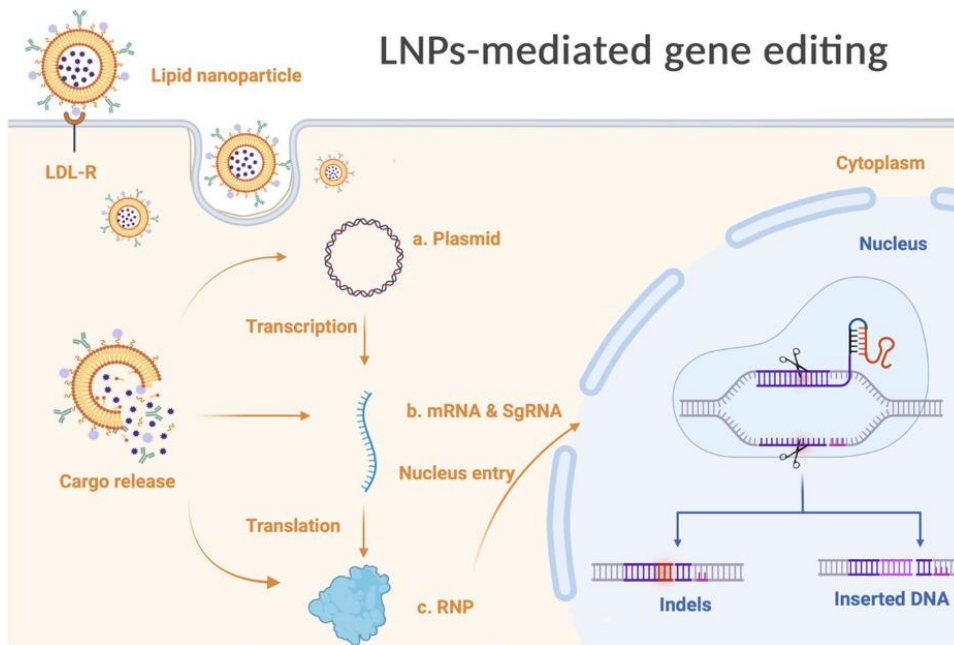


Figure 2.8 LNP-mediated delivery

Lipid nanoparticles (LNPs) enter through endocytosis before releasing encapsulated editing vectors into cytoplasm. This experiment used plasmids, following the 'a' pathway shown (Figure reproduced from Wu *et al.* 2025).

Since the RNP editing process does not integrate the constructs or the transgenes into the wheat genome, any mutants generated will be transgene-free. The Cas-gRNA RNP complexes will degrade over time, leaving only the altered genes behind.

PLANT CONDITIONS

After BAPC treatment, seeds were transferred to another petri dish, one with a thin layer of plain agar on the bottom and 100 μ l of 75% pentachloronitrobenzene for fungus control. The agar and fungicide allowed for stronger root development and a higher seed survival rate (**Figure 2.9**). The dish was left at room temperature for 4-5 days, to allow shoot growth. If the SAM was damaged during dissection, the seed would not grow and was removed from the population.



Figure 2.9 Bobwhite Seeds Growing on Agar

Seeds with normal growth were moved to the greenhouse and continued growing in the agar under a plastic dome in water trays for 2-3 more days (**Figure 2.10**). They were then transplanted into single seedling trays. The greenhouse was set up with 12 hours of light, with average daytime temperature of 26°C and daytime humidity average of 80%. Plant trays were re-filled with water every 2-3 days.



Figure 2.10 Seed Growth

Treated seeds growing on petri dish in water tray, covered by lid to maintain humidity.



Figure 2.11 Plants Growing in KSU Greenhouse.

Leaf tissue for sequencing was collected after 1-2 weeks of plant growth in the greenhouse (**Figure 2.11**). Three pieces of 2 cm-long leaf pieces per plant were cut and placed in GeneMate 96-deep well plates (BioExpress, Lodi, CA, USA). After collection, the plants were transplanted into singular pots. Every week the plants received supplemental nutrients with Miracle-Gro soluble fertilizer (Scotts Company LLC, Marysville, USA), for five weeks.

DNA ISOLATION

The leaf tissue samples were freeze-dried for 2 days at -20 °C under pressure, before being ground into fine powder using the GenoGrinder (SPEX Sample Prep, USA) machine and 1-2 small metal beads per well at 1,750 cycles/minute for 2 minutes (**Figure 2.12**). DNA was extracted from these samples using a modified Hexadecyltrimethylammonium bromide (CTAB) protocol (Murray & Thompson, 1980).

The CTAB extraction buffer consisted of 650 ml ddH₂O, 100 ml of 1.0M Tris-HCL (pH 8.0), 40 mL of 0.5M EDTA, 20.0 g CTAB, 81.82 g NaCl, 1.464g TCEP, and 20g Polyvinylpolypyrrolidone (PVPP).

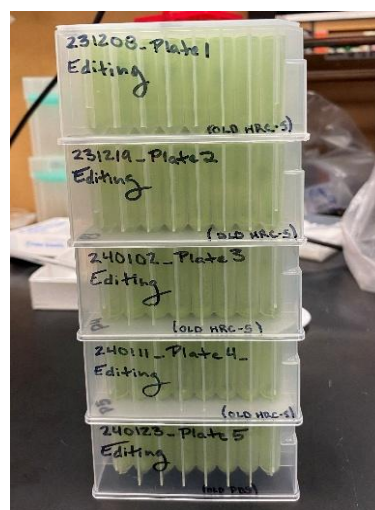


Figure 2.12 Ground Leaf Tissue Samples ready for DNA isolation in GeneMate 96-deep well plates.

Fresh CTAB buffer was added to each well of ground-tissue, along with 2 µl of RNase (Life Technologies, Carlsbad, CA) using the Biomek i5 liquid handler (Beckman Coulter, Brea, CA) and the Mantis Precise Liquid Dispenser (Formulatrix, Bedford, MA), respectively. After covering the wells with silicone mats, the deep-well plates were shaken in the GenoGrinder for

10 seconds at 1,750 cycles/minute (C/M). After removing the mats, these plates were then incubated at 65°C for 90 minutes in a Thermo Heratherm OHM-180 oven. (Thermo-Scientific, Langensfeld, Germany).

After this incubation, sample plates were gently inverted 5 times before being cooled to room temperature. Once at room temperature, 300 µl of a 25:1 solution of chloroform/isoamyl alcohol (ChI/IAA) was added to each well with the Biomek robot. Then, mats were placed back on the plates, and they were gently inverted for 25 minutes at 2 inversions/sec.

Mats were peeled up slowly and placed back on to relieve pressure, before centrifuging (Beckman Coulter Avanti J-E centrifuge) plates at 5,700 rpm at 4°C for 30 minutes. The average distance between the separated, upper aqueous phase and the lower organic phase was measured (**Figure 2.13**). This measurement was used by the Biomek robot to transfer the aqueous phase to new 96-deep well plates with 450 µl of isopropanol in each well to precipitate the DNA.

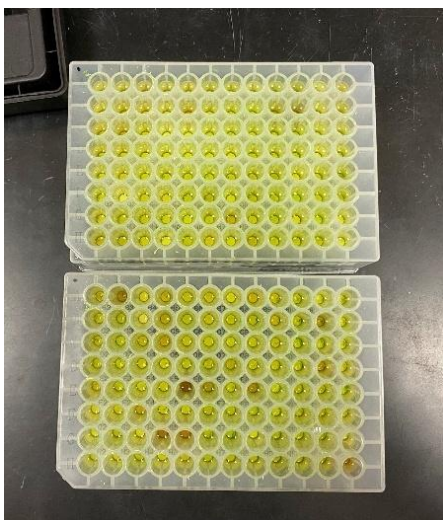


Figure 2.13 Aqueous & Organic Layers
Tissue samples after centrifuging and
before layer separation.

The organic layer was discarded, and the new plates with the aqueous layer were inverted 3 times before resting at 4°C for 30 minutes. After this period, the plates were centrifuged for 60 minutes at 5,700 rpm at 4°C to precipitate the DNA at the bottom. The supernatant was discarded, and the plates drip-dried for one minute.

The extracted DNA was washed with 500 µl of 70% ethanol, and wet-ground at 1,750 C/M for 10 seconds.

After centrifuging again for 60 minutes, the washing step was repeated. The plates were then

centrifuged again and put in the oven at 65°C for 30 minutes for drying. After this, the plates were air-dried for 3 hours to ensure all ethanol is removed before resuspension.

To finish the process, 208 µl of ddH₂O was added to each pellet in the wells and the plate was incubated at 65°C for 60 minutes. The extracted DNA was left overnight at 4°C before use, or kept in long-term storage at -80°C.

DNA QUANTIFICATION/NORMALIZATION

Once DNA was re-suspended in ddH₂O, the samples were quantified with PicoGreen on an Omega fluorometer (BMG Labtech, Germany). The samples were then all normalized to 200 ng/µL with ddH₂O using a Mantis Precise Liquid Dispenser.

The Biomek robot was used to first dilute samples 10X, while a plate was prepared with PicoGreen; 4 µL of 1X PicoGreen was added to each well of a black 384-well Greiner UVStar microplate. For control wells, 4 µl of 10 ng/µl λ-DNA standard was added to 4 wells and 4 µl of water. Four µl of the diluted samples were added to the wells with PicoGreen and mixed through pipetting.

The Omega reader was used to measure the fluorescence of the samples and the standards, and sample concentrations were calculated using the standard curve. The appropriate amount of autoclaved ddH₂O was added to each well to get 200 ng/µl. The plates were capped with mats before gentle inversions to mix the additional water with the samples. After briefly centrifuging, the plates were stored at 4°C for short-term storage, or kept in long-term storage at -80°C. These normalized DNA samples were sequenced using the Illumina NGS sequencer, NextSeq 2000 (GenCore, Orlando, FL).

INITIAL PCR FOR SEQUENCING

Three primer sets were used to amplify the target regions, before a secondary PCR was completed to attach unique sample barcodes. The primer sets were designed based on the targeted loci that each editing vector was designed for, with additional overhangs for the barcode primers. The overhang sequences are as follows, with **Table 2.4** detailing the targeted primer sequences.

F. Overhang (i5): 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[target- seq]

R. Overhang (i7): 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[target- seq]

Table 2.4 Initial Primer Sequences

Primers used for mutation identification within the target site. Primers ordered through Eurofins Scientific, Luxembourg.

Primer Name	Target Sequence	Melting Temp (T _m)
TaHRC-R_gRNA1-F	GTGCCAGGAGGCGCAAGCAC	68°C
TaHRC-R_gRNA1-R	CTCATCATCATCGCTGCTGT	63°C
TaHRC-R_gRNA2-F	TGACAACCATAAGCGTCAC	60°C
TaHRC-R_gRNA2-F	TTACACGAGTTGCTTCCCGTC	63°C
TaPDS gRNA1-F	GACTTCGTGCTCTGCAGGTTA	67°C
TaPDS gRNA1-R	CTAAACGACGAAGAAAGCTGG	65°C

Adding the overhang, the first two primer sets were:

TaHRC-R_gRNA1-F:

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGGAGGCGCAAGCAC

TaHRC-R gRNA1-R:

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTCATCATCATCGCTGCTGT

Table 2.5 PCR Program for Initial Amplification of DNA.

Steps	Temperature	Time
1	95°C	2 min
2	95°C	30 sec
3	65°C	30 sec, -1°C per cycle
4	72°C	30 sec
Go to Step 2		For 8 cycles
5	95°C	30 sec
6	58°C	30 sec
7	72°C	30 sec
Go to Step 2		For 35 cycles
8	72°C	2 min
9	10°C	Forever

Using the adapted primers, PCR reactions were run on standard thermocyclers. For each well, a solution composed of 3.70 μ l ddH₂O, 1.00 μ l 10x ASB Buffer, 0.60 μ l 25 mM MgCl₂Cl₂, 0.40 μ l 5 mM dNTPs, 0.50 μ l 10uM Primers (F&R), and 0.80 μ l of Taq DNA polymerase was used with 3 μ l of 200

ng/ μ l genomic DNA. All PCR solutions were made using the Biomek i7 liquid handler.

Template DNA was taken from the 96-well deep plates and placed in 384-well clear, skirted PCR plates (**Figure 2.14**) (Thermo Fisher, Waltham, MA). Every 384-well plate contained samples from 4 96-well plates, arranged in four quadrants. Then, the rest of the components were added and mixed via machine pipetting. After a brief centrifuge at 1700 rpm to settle the contents, samples were run using the PCR program in **Table 2.5**.

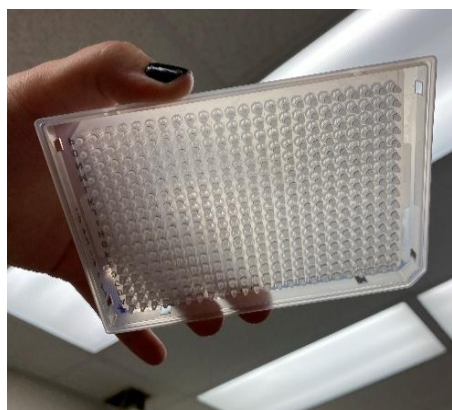


Figure 2.14 Samples in 384-well Plates
Samples with PCR mixture, after centrifuging.

After amplification, PCR products were run on 1.5% agarose gel to check results. To do so, 3 μ l of product was added to 2 μ l of 3X Brophenol Blue loading dye and loaded into the gel wells. The gel was run alongside a 1 Kb ladder for 30 minutes at 80 V. Using the GelDoc XR Molecular Imager (BioRad, Des Plaines, IL), the gel was checked for clear bands at the expected lengths. Any smeared, or missing bands, were re-amplified. **Figure 2.15** shows an

image of a testing gel from various plates. In this example, 5 wells throughout, and the last 15 samples without visible bands were amplified again. When samples were properly amplified, the PCR products were cleaned up before attaching the i5 and i7 tails.

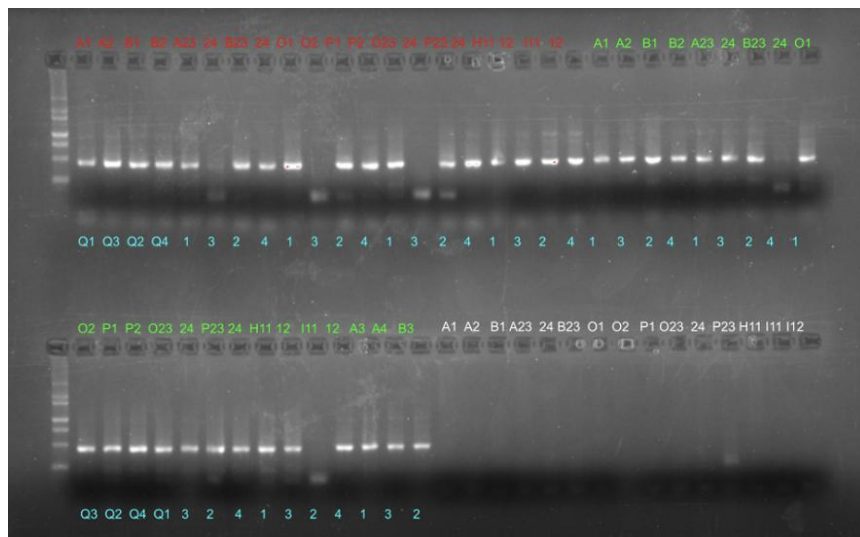


Figure 2.15 PCR Products Check

Picture of 1.5% agarose gel made with DNA SafeStain for illumination of DNA. Used to check success of amplification from each sample.

PCR CLEANUP

To clean up the initial PCR products, Thermoscientific kits were used; 3 μ l of PCR product was mixed with 0.5 μ l Exonuclease1 and 1 μ l of FastAP Thermosensitive Alkaline Phosphatase (Thermoscientific, Vilnius, Lithuania). This solution was mixed and centrifuged at 1700 rpm. Once settled, the products were incubated in a thermocycler at 35°C for 15 minutes and then at 85°C for 15 minutes more to terminate the reaction.

SEQUENCING REACTION

To test if the CRISPR/Cas RNPs induced mutations in the imbibed Bobwhite seeds, the Illumina next-generation sequencer (NextSeq 2000) was used. After the initial PCR reaction, i5 and i7 barcode adapters were added for sample identification. This process allows all samples to be multiplexed once the library was prepared. These i5 and i7 sequences match the terminal P5 and P7 sequences used for clonal amplification on the flow cell, while also ‘indexing’ each sample. **Figure 2.16** illustrates how the Illumina sequencer ‘reads’ samples and i5/i7 barcodes.

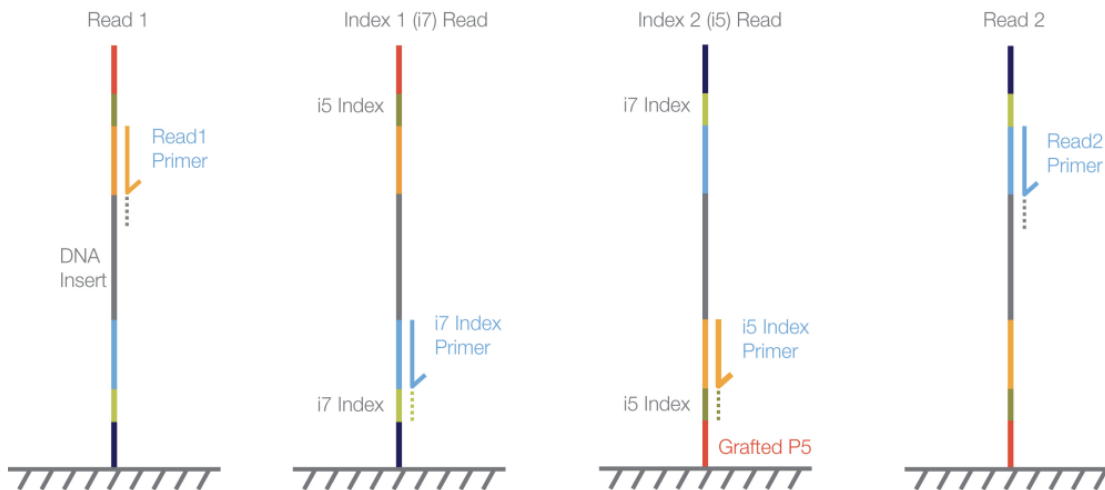


Figure 2.16 NextSeq 2000 Reverse Complement Workflow

This method starts read 1 at the read 1 primer, reads the inserts, and washes the sample off before reading the i7 index primer. The fragment then folds and is read from the grafted p5 oligonucleotide to synthesize the reverse complement before reading the i5 index primer. The sample is then washed, and the process is repeated with read number 2. (Figure reproduced from Illumina Index Adapters Pooling Guide).

There are a couple strategies to attach the necessary adapters. In this project, PCR primers with different combinations of i5 and i7 index sequences were used to amplify our initial PCR products. The primers from the initial PCR had i5/i7-compatible adapter sequences, so only one round of additional amplification was needed. Combinatorial dual indexing (CDI) was used, meaning that each i5 and i7 ‘barcode’ was used multiple times, but each combination of barcodes

was only used once. **Table 2.6** contains the i5 and i7 primers used for the first 5 samples of 2 plates and the first and last 2 samples of the third plate to demonstrate this concept.

Table 2.6 i5 and i7 Sequences

For each plate, a single i5 primer was used in the sequencing reaction, and 96 unique i7 primers were used for each plate. This made individual barcodes for each sample, encompassing all 1440 samples.

Sample Well	Name of i5	Sequence	Name of i7	Sequence
Plate 2				
A01	Next-i5-02	AAGGATCC	Next-i7-01	AACCACTC
B01	Next-i5-02	AAGGATCC	Next-i7-02	AAGGCTCT
C01	Next-i5-02	AAGGATCC	Next-i7-03	AAGTGCAG
D01	Next-i5-02	AAGGATCC	Next-i7-04	ACACTACC
E01	Next-i5-02	AAGGATCC	Next-i7-05	ACAGTGAC
Plate 3				
A01	Next-i5-03	ACGTCAAC	Next-i7-01	AACCACTC
B01	Next-i5-03	ACGTCAAC	Next-i7-02	AAGGCTCT
C01	Next-i5-03	ACGTCAAC	Next-i7-03	AAGTGCAG
D01	Next-i5-03	ACGTCAAC	Next-i7-04	ACACTACC
E01	Next-i5-03	ACGTCAAC	Next-i7-05	ACAGTGAC
Plate 4				
A01	Next-i5-04	ACTTCGGT	Next-i7-01	AACCACTC
B01	Next-i5-04	ACTTCGGT	Next-i7-02	AAGGCTCT
...	...	...	...	...
G12	Next-i5-04	ACTTCGGT	Next-i7-95	TTGCAACG
H12	Next-i5-04	ACTTCGGT	Next-i7-96	TTGGTGCA

To start this process, the cleaned PCR products were diluted with 18 μ l of ddH₂O. The samples were mixed with the Biomek before 3 μ l of the diluted products were transferred to a new 384-well plate. For each 96-well ‘plate’ within the 384-well plate, a single i5 barcode primer was used, and each sample had a unique i7 primer. A total of 15 plates of samples were sequenced, thus 15 different i5 indexes were used. 3 μ l of the specific 2 μ M i5 primer was added to each plate, before 3 μ l of the 2 μ M i7 primers were added to each well within the four

quadrants. This caused the i7 to be paired with a different i5 for each plate and sample, making demultiplexing each sample possible.

Once the correct primers were added to each well, 3.6 μ l of sequencing master mix was added to every sample via the Mantis dispenser. The master mix consisted of 2 μ l of 5X NEB

Table 2.7 'Barcode' PCR
PCR program used to attach unique barcodes to samples

Step	Temperature	Time
1	95°C	1 min.
2	95°C	30 sec.
3	55°C	30 sec.
4	68°C	60 sec.
Go to Step 2		For 14 cycles
5	68°C	3 min.
6	10°C	Forever

Taq Master Mix and 1.6 μ l of ddH₂O. The

Biomek robot mixed the samples, primers, and sequencing mix. Then, the plates were sealed and briefly centrifuged at 1700 rpm to settle contents. The amplification reaction (PCR) was then run on thermocyclers with the program in **Table 2.7**.

POOLING AND LOADING MACHINE



Figure 2.17 Pooled Samples from 15 plates, ready for sequencing.

After the barcoding PCR reaction was complete, samples were pooled for sequencing into a sterile 50 mL conical tube (**Figure 2.17**). This tube was vortexed gently but thoroughly before being loaded into the Illumina NextSeq2000 machine for bridge sequencing

A P1-x 300 flow cell cycle kit was used with PhiX spike (Illumina, San Diego, CA) to increase nucleotide diversity. This kit generated 127.78 million 150bp paired end fastq sequencing reads, of

which 83.5 million were assigned to a sample.

SEQUENCE EXAMINATION

Raw fastq sequences were run through quality control (QC) to retain reads with map quality >20 using bbdduk software (Bushnell 2014). QC reads were aligned to target sequences of *HRC* and *PDS* using Burrow-Wheeler Aligner mem (BWA-mem) for short-read alignment (Heng Li 2009). All base pairs within the amplified region of the target site were checked for deletions or any unusual mutations using a custom Perl script (Wall *et al.* 2000).

RESULTS

Potential mutations were analyzed in the target area for all three genomes (*Fhb1/HRC* and the *PDS* gene are on the 3B chromosome), using BWA-mem (Heng Li 2009). For example, the HRC-targeted plants were aligned to the nucleotide regions below:

B Genome:

TaHRC-S-B sequences

ACTTCCTGTCATCATTTACGGCCCTGCAGGTTTTGAGCTTTTGTCTGTTTCGCTGGGATGGATG CCAA
GAAGTTCCTGCAGATGGT CGAAGACAAGAA GAAGAGACTCCTCGAGAAGAAGC AAGCCCCTCTGAAA
TGGCAGCAGAACTGGAAGCAGCAATCAAGGCCACTGAAGAAAAGGAGAAGAAGCTCAAGTCGAAA
AAGCACAGGAGACGAAGCTATTCTTCCTCAGAATCTGACAGTGAATCCGAGAGCGACAGTGATCGGA
AACACAGGAAGAGGAAGGACCGCAAAAGGCACAAAAGACATGGCCATTCTGACTCTGATGGTGCCAG

The nucleotides in red were the deletion targets for the 1-1 TaHRC-S editing vector. All samples were sorted by their vector plasmids and were aligned to all three genomes. Any polymorphisms within the amplified region were checked with a custom Perl script that analyzed the alignments for insertion or deletion mutations in the target regions. The script reported the number of possible insertions and deletions for each region as the ratio of possible events to total reads for each sample. Overall, very few events were bioinformatically flagged. We visualized the most promising samples using GeniousPrime software (Geneious Biologics 2025.1). This

was done using the alignment feature on the software. Any samples with a higher possibility of deletion were manually aligned to the target region and checked for any deletions in the sequenced region.

Out of the 1440 samples, none showed a true deletion mutation near the target area using GeniousPrime visualization. This forced us to reassess our methods. Despite previous success within the lab with this method on a small scale, our results were unsuccessful (**Table 2.8**).

Table 2.8 Editing Results

Five plasmids used to treat the SAM of Bobwhite seeds, and the number of successfully edited plants grown from vector-treated seeds.

Plasmid Vector	Target Gene	Plants Mutated	Total Plants
1-1 TaHRC-S	<i>TaHRC</i>	0	194
1-4 TaHRC-S	<i>TaHRC</i>	0	190
1-1TaPDS	<i>TaHRC</i>	0	288
2-1 TaPDS	<i>TaPDS</i>	0	384
2-1 TaHRC-S	<i>TaHRC</i>	0	384

We pivoted, working through the methods to see what could have changed from the pilot project that suggested successful editing could be established. The BAPCs were able to complex correctly with the editing vectors, as shown through our gel comparison tests of each mixture. Multiple vectors were designed to target different sites on the genes and theoretically had no issues. Different methods of BAPC penetration into the SAM were experimented with, using a GFP plasmid.

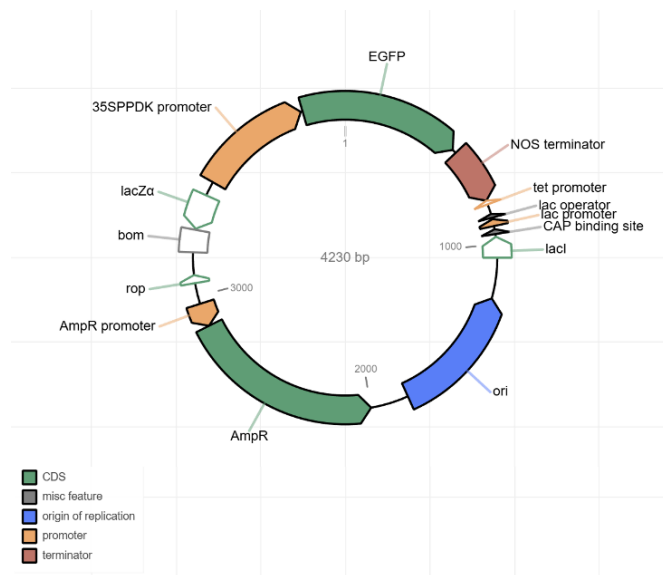


Figure 2.18 HBT-SGF_TYG-nos Plasmid
Different components of HBT-SGF_TYG-nos plasmid
(Figure generated by Eurofins Plasmid Sequencing).

The HBT-SGF_TYG-nos (in pUC18), a synthetic GFP plasmid was provided by Dr. Harold Trick, at KSU (Figure 2.18). This plasmid gives approximately a 20-fold higher protein expression than the jellyfish GFP in plant cells. It is effective with weak promoters and is excited by both blue and UV light. With this plasmid, we should be able to see immediate successes or failures in the SAM

under UV light. The plasmid would be transiently expressed if it was able to penetrate the SAM cells and fluorescence would be visible.

We hypothesized that the step that was not working could be the penetration of the SAM with BAPC, to deliver the plasmid (Figure 2.19). Various techniques were used in conjunction with this GFP plasmid to investigate if proper transport of the BAPC complex into the seeds cells was successful. These included: cellulase degradation of the SAM, sonication of the SAM before treatment, centrifuging rather than vacuum pressure, and an increased temperature (37°C) after treatment.



Figure 2.19 Bobwhite SAM
Exposed SAM on Bobwhite seed,
ready for gene delivery

As a control, to check that the GFP plasmid could fluoresce in the SAM, a gene gun was used to physically insert the plasmid on its own. This was tested on Bobwhite seeds with the SAM exposed. Using a Leica MZFLIII microscope with a GFP filter, (Figure 2.20) fluorescence was successfully detected under UV light 24 hours after delivery as shown in Figure 2.21, showing that this plasmid was able to infiltrate the SAM in Bobwhite and produce proteins.



Figure 2.20 Leica MZFLIII Microscope

Used for GFP detection in Dr. Harold Trick's KSU laboratory.

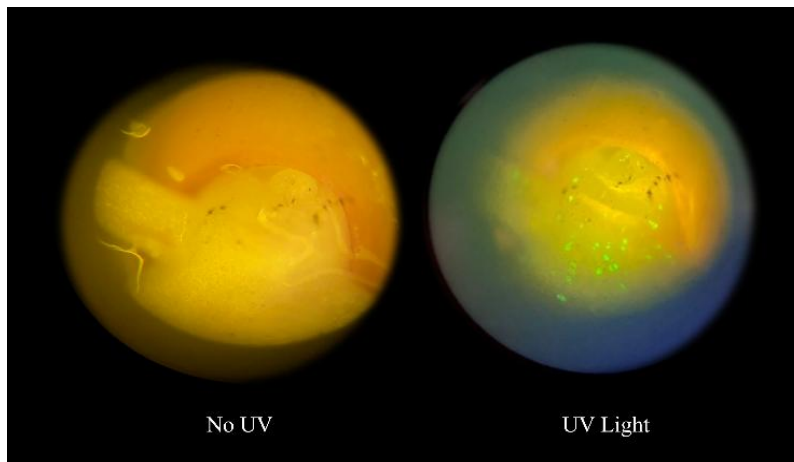


Figure 2.21 GFP Positive Control

Bobwhite seed SAM penetrated with GFP plasmid seen with UV light.

Degradation of the SAM with cellulase was performed in hopes of gently removing the upper layer of SAM cells and the cell wall, that may have been preventing penetration. Cellulase

is used to carefully decompose the natural cellulose in plant cells and expose protoplasts. The manufacturer of the cellulase, bioWorld, suggests a 1.5-2.0% solution. A series of increasing dilutions, 1.5, 2.0, and 2.5% was applied to the SAM for 5, 10, and 15 minutes before being rinsed off with ddH₂O. Once rinsed, the SAMs were treated with the BAPC + GFP plasmid complex. For every treatment, 50 seeds were tested.

After 24 hours, treated seeds were examined for GFP expression. **Figure 2.22** shows an untreated control seed and a treated seed under UV light. No fluorescence was detected for any

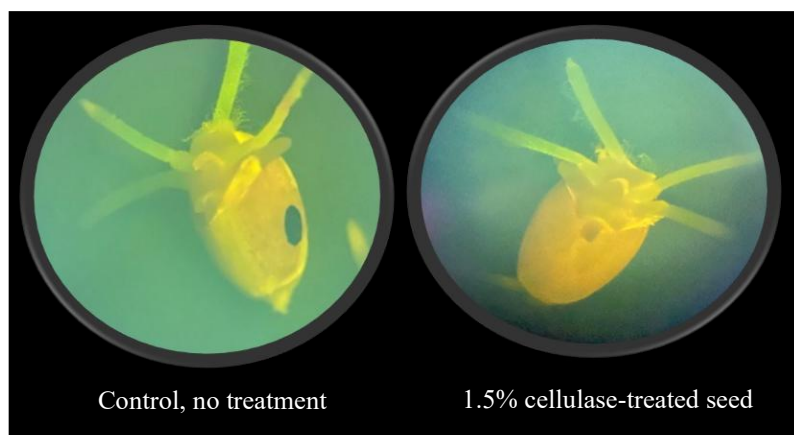


Figure 2.222 Cellulase Results

Control seed (black dot) and seed treated with 1.5% cellulase and GFP/BAPC complex. No visible fluorescence was seen in any of the cellulase treatments after 24 hours.

of the cellulase treatments.

Sonication of the SAM before treatment was completed in hopes of opening larger pores in between the cells, allowing the BAPC complex more room to penetrate the SAM and be absorbed by its cells. Dissected

seeds were placed in sterile 5 ml tubes with ddH₂O, before being floated in a Bransonic

Ultrasonice Bath 3800. Seeds were sonicated for 5,

10, or 15 minutes before the BAPC and GFP

complex were applied to the SAM. For each

sonication duration, 50 Bobwhite seeds were used.

Figure 2.23 shows the seed set-up for floating in

the sonicator. Similarly to the cellulose treatment,

no fluorescence was detected after 24 hours.



Figure 2.23 Sonication Setup

Dissected seeds placed in tubes with ddH₂O, suspended in foam for sonication.

Centrifuging, rather than vacuum pressure, was also tested, in hopes of helping the BAPCs penetrate deeper into the SAM. The SAMs were exposed and treated with the nanoparticle and GFP plasmid, before being centrifuged for 1, 2, or 5 minutes at 1200, 1500, or 1700 rpm. None of these treatments showed successful GFP expression.

Lastly, an increased temperature of 37°C before the initial treatment method was used, in hopes of causing the SAM cells to dry slightly, to encourage the uptake of the liquid BAPC solution. Seeds with exposed SAMs were placed in an enclosed incubator for 10, 15, 30, 45, or 55 minutes before the BAPC/GFP solution was applied. After 24 hours, there was no difference between the treated Bobwhite seeds and the negative control seeds. After all these treatments,



Figure 2.23 Treated Seeds on Agar plates for further growth/examination.

seeds were placed on plain agar petri dishes for ease of examination under the microscope and continued growth, as demonstrated in **Figure 2.24.**

Table 2.9 summarizes these final experiments and their outcomes. These results led us to the conclusion that the method of introducing gene editing vectors using BAPC nanoparticle is unable to produce the desired editing events in wheat currently.

Table 2.9 Alternate BAPC Penetration Methods Results

Treatment	Seeds with Expression	Total Seeds Treated
Vacuum Infiltration	0	1440
Cellulase Degradation	0	450
Sonication	0	150
Centrifuging Infiltration	0	450
Temperature Increase	0	250

DISCUSSION

Although this technology and the initial technique were promising in a pilot study, we were unable to replicate the findings. We tested alternate methods to deliver the BAPC to the SAM, but none proved successful. It is possible that something was different between batches of the BAPCs tested in the pilot project and here. This could also be a case of human error or some other unknown issue. While there were initially issues with molding, it is unlikely that this interfered with the transformation, especially once fungicide was used (**Figure 2.25**). More investigation into this project is needed to effectively troubleshoot and ultimately deliver the editing plasmids to the SAM in the future.

Since there was success with a BAPC editing preliminary study, our first vectors did not have reporter genes or visual cues for success/failure. This means that a

significant amount of time was spent growing, collecting, and sequencing plants before determining that there was an issue with our methods. For future experiments, an early physical indicator would be a better starting point. The TaPDS vector aimed to provide this with a bleaching response, but it was one of the last vectors used. We also batch-tested all samples to minimize the number of runs on the Illumina sequencer.

In this study, only 25 nm BAPCs were used, due to reported high cell transfection rates. In the future, it would be beneficial to test other sizes or types of LPNs (**Figure 2.26**). There are

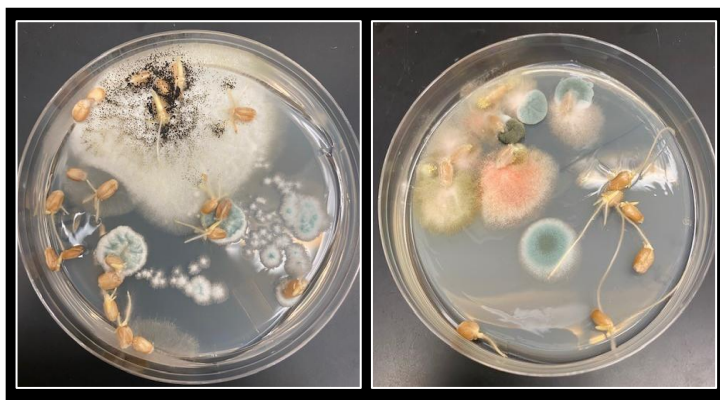


Figure 2.24 “Dirty” Bobwhite Seeds

These are seeds grown on agar without being washed first. When seeds were disinfected with bleach/tween, a small white fungus would still grow, affecting seed survival rate, but still much less than the fungus shown. To combat this, a fungicide was added to the agar plates.

numerous types of nanoparticles, and a comparison study of their use in genetic engineering has not been conducted at this point.



Figure 2.25 Different nanoparticles offered through Phoreus Biotech

While this nanoparticle gene editing study was not successful, this method still holds potential. There are numerous examples of nanoparticles being successfully employed to deliver things such as vaccines, cancer therapies, and gene editing materials to cells (Vogel *et al.* 2021; Meng *et al.* 2022; Karlsson *et al.* 2021).

Our hypothesis that early editing of SAM cells can cause persistent mutations in the germline still follows the logic of tried-and-true editing methods, like callus cultures. Our results are inconsistent with other studies focused on nanoparticle gene editing. There have been other studies focused on callus-free editing techniques.

For example, a study from Germany on *Arabidopsis thaliana* and *Brassica rapa*, successfully transported RNAs from transgenic rootstocks into grafted wild-type shoots and achieved heritable gene editing. This method resulted in transgene-free plants without the need to rely on callus formation (Yang *et al.* 2023). This is an encouraging study, as this method could be integrated with many types of genetic editing tools. It also demonstrates the potential for other transgene-free editing systems.

In 2025, an Australian lab published results supporting that liverwort (*Marchantia polymorpha*) pluripotent cells are shielded from differentiation by the *MpARF2* gene. This single B-class auxin response factor antagonizes auxin responses that would otherwise direct stem cell specialization (Flores-Sandoval *et al.* 2025). This could be a promising avenue for future studies,

especially with crops that need a more reliable stem cell supply. It is a gene that could be integrated into future editing vectors and paired with BAPC transfection.

It is not clear why this project lacked successful editing when the pilot study produced edited embryos. The methods tested with these BAPCs and vectors were ineffective, but a more effective method of gene editing for wheat seedlings would greatly expand wheat breeding opportunities. Further work with nanoparticles and other delivery methods of genetic editing tools directly to growing plant cells should still be explored.

Chapter 3 - Fusarium Head Blight in EMS Mutants

Abstract

Fusarium head blight (FHB), a disease caused by *Fusarium* fungi species, is a widespread problem for small grain crops. The disease affects crop yield, quality, and safety; mycotoxins from the fungi are toxic when consumed. In 2019, wheat affected by *Fusarium* resulted in annual losses of 28 million metric tons of grain. Developing and growing FHB resistant wheat cultivars is one of the most practical and effective approaches to minimize FHB damage.

The *Fhb7* gene has shown marked success in some wheat species when it comes to FHB Type II resistance. However, in some species it has not been as effective. For further study of *Fhb7*, a population of ethyl methanesulfonate (EMS) created mutants was analyzed through phenotypic results. This was done to find significant mutations and add evidence that *Fhb7* is causal for FHB resistance.

Using EMS, 945 Chinese Spring wheat seeds carrying an introgression including the *Fhb7* gene were randomly mutated. Treated lines with mutations in the *Fhb7* region were isolated for this study and assessed phenotypically. Out of these lines, 30 showed a higher susceptibility than wildtype parents, with 14 of those showing a total susceptibility above 50 percent. This could indicate a mutation in an essential area of the *Fhb7* gene that alters the resistance when compared to the wildtype.

These lines were sequenced to isolate the region and type of mutation that affects FHB resistance. We focused on missense mutations in the *Fhb7* region. To further connect these mutations with the phenotypic susceptibility, crown rot trials on significant lines were run. Ultimately, we identified 32 significant mutants affecting FHB resistance. These results will narrow down the essential regions in *Fhb7*, provide mutant lines for further FHB research, and provide support *Fhb7* as an effective FHB R gene.

INTRODUCTION

As previously discussed, FHB, caused by *Fusarium graminearum*, has spread rapidly across the US since the 1980s. These infections cause ‘scabby,’ low-weight seeds that are toxic when consumed. The pathogen can be found on field remnants and in the air, and thrives in warm, humid climates.

Various approaches have been used to mitigate the effects of this disease, including breeding for genetic resistance, altered agriculture practices, and fungicide applications. Despite these efforts, the disease remains a concern for many cereal grains. In 2019, a worldwide survey on pathogen loss across crop species reported that FHB ranked just under leaf rust for *Triticum aestivum* yield losses. These losses are predicted to increase over time, driven by unpredictable weather changes and accelerated disease development.

Host resistance is often a reliable approach for disease control. The *Fhb7* gene provides a high level of Type II resistance to FHB (Guo *et al.* 2015). When plants with *Fhb7* are infected by FHB, the pathogen spreads to nearby spikelets much slower than those without *Fhb7*. This preserves most of the head and reduces yield losses (Bai & Shaner 1994). The *Fhb7* gene was transferred from *Thinopyrum elongatum*, a distantly related species, into *Triticum aestivum*, on the 7DL chromosome arm. (Guo *et al.* 2015). This research examines the resistance associated with the *Fhb7* gene to help further our understanding of the underlying sequence while providing mutant lines for future study.

MATERIALS & METHODS

STARTING POPULATION

We began with a population of EMS treated Chinese Spring wheat lines that carried a 7ES fragment of the *Thinopyrum elongatum* genome harboring *Fhb7*. This population was derived from the *Sr43* cloning project completed by Guotai Yu *et al.* in 2022. In 2015, the USDA and Agricultural Research Service (ARS) completed a material transfer of RWG34, a wheat line with the derived *Sr43* gene, to Dr. Steven Xu, who kindly provided it to our lab. The dominant resistance gene, *Fhb7*, was introgressed into chromosome 7D of *Triticum aestivum*. The RWG34 pedigree is as follows: Chinese Spring (CS)//CS *ph1bph1b**2/KS10-2.

This gene group, located on the 7D chromosome, consists of multiple genes, including:

- 1) *Sr43*- a stem rust resistant gene
- 2) *PSY-E1* – a yellow pigment gene
- 3) *Fhb7*- the FHB resistant gene.

For this project, we were interested in the *Fhb7* gene. The *Thinopyrum elongatum* chromosome and the wheat translocation chromosome is illustrated in **Figure 3.1**. The mutant population in this project was created with germplasm RWG34 (Yu *et al.* 2022).

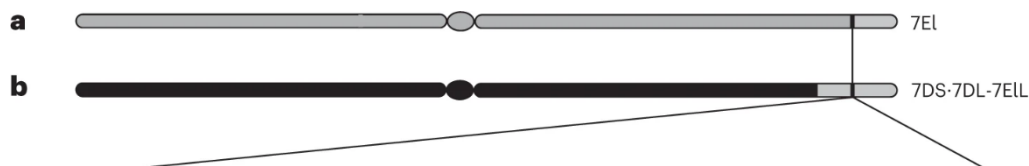


Figure 3.1 Fhb7 Chromosome Translocation

a: *Thinopyrum elongatum* chromosome 7. b: Wheat translocation chromosome, 7D (Figure reproduced from Yu *et al.* 2022).

This Chinese Spring *Fhb7* population was treated with 0.5% ethyl methanesulfonate (EMS) for 6 hours to induce random mutations within the genome (Yu *et al.* 2022). From this

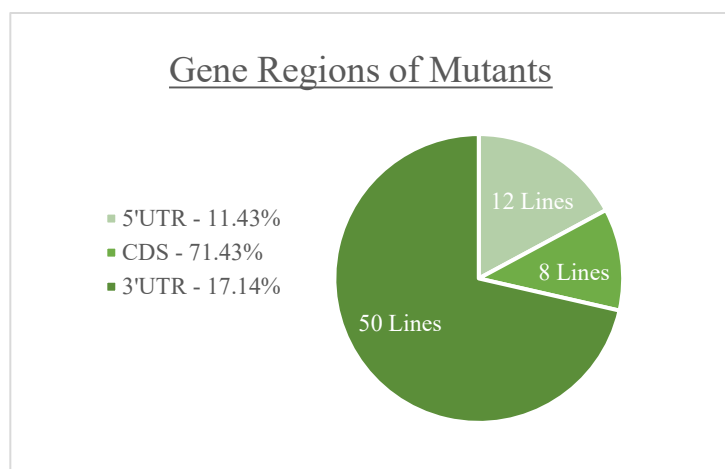


Figure 3.2 SNP Locations in Fhb7 Region from Initial Population

EMS-treated CS-Fhb7 M₀ population, we randomly picked 945 lines. M₁ plants were self-crossed and advanced to M₂ generation. These lines were sequenced in the *Fhb7* and surrounding gene regions with an Illumina sequencer. Plants with related mutations in the *Fhb7* gene region

were selected for further use in this study and self-crossed again, advancing to the M₃ population. This starting M₃ population consisted of 70 different mutant lines, 50 of which were heterozygous. Eight of these lines had mutations in the 5' untranslated region (UTR)/promoter region, 12 contained mutations in the 3'UTR, and the remaining 50 lines had mutations throughout the coding DNA sequence (CDS) region (**Figure 3.2**). The exact positions of the originally identified single nucleotide polymorphisms (SNPs) are detailed in **Table 3.1**.

Table 3.1 *Fhb7* Mutation Locations from EMS Population

Line	Mut. Site (bp)	Location	Homo/Het
Sr43-m2-165	47	5'UTR	Homo
Sr43-m2-1027	62	5'UTR	Het
Sr43-m2-1004	101	5'UTR	Het
Sr43-m2-153	117	5'UTR	Het
Sr43-m2-196	145	5'UTR	Het
Sr43-m2-674	209	5'UTR	Homo
Sr43-m2-4	244	5'UTR	Het
Sr43-m2-1054	261	5'UTR	Homo
Sr43-m2-1149	464	CDS	Homo
Sr43-m2-1068	465	CDS	Homo
Sr43-m2-726	510	CDS	Homo
Sr43-m2-333	558	CDS	Homo
Sr43-m2-127	827	CDS	Het

Sr43-m2-617	875	CDS	Het
Sr43-m2-551	883	CDS	Het
Sr43-m2-954	898	CDS	Het
Sr43-m2-1010	923	CDS	Het
Sr43-m2-403	1071	CDS	Homo
Sr43-m2-1113	1100	CDS	Het
Sr43-m2-243	1111	CDS	Het
Sr43-m2-1075	1145	CDS	Het
Sr43-m2-345	1216	CDS	Het
Sr43-m2-792	1231	CDS	Het
Sr43-m2-662	1259	CDS	Het
Sr43-m2-12	1262	CDS	Homo
Sr43-m2-182	1267	CDS	Het
Sr43-m2-653	1288	CDS	Homo
Sr43-m2-489	1376	CDS	Het
Sr43-m2-507	1392	CDS	Het
Sr43-m2-939	1397	CDS	Het
Sr43-m2-1052	1411	CDS	Het
Sr43-m2-59	1442	CDS	Het
Sr43-m2-600	1445	CDS	Het
Sr43-m2-796	1475	CDS	Het
Sr43-m2-465	1487	CDS	Homo
Sr43-m2-683	1524	CDS	Het
Sr43-m2-1011	1532	CDS	Het
Sr43-m2-358	1534	CDS	Het
Sr43-m2-235	1541	CDS	Het
Sr43-m2-401	1588	CDS	Het
Sr43-m2-527	1616	CDS	Het
Sr43-m2-238	1633	CDS	Het
Sr43-m2-884	1638	CDS	Homo
Sr43-m2-467	1642	CDS	Het
Sr43-m2-993	1643	CDS	Het
Sr43-m2-718	1644	CDS	Het
Sr43-m2-164	1656	CDS	Het
Sr43-m2-598	1668	CDS	Het
Sr43-m2-710	1668	CDS	Het
Sr43-m2-84	1681	CDS	Het
Sr43-m2-74	1684	CDS	Het
Sr43-m2-1138	1684	CDS	Het
Sr43-m2- 983	1751	CDS	Homo
Sr43-m2-248	1782	CDS	Homo
Sr43-m2-387	1820	CDS	Het
Sr43-m2-700	2021	CDS	Het
Sr43-m2-923	2047	CDS	Het

Sr43-m2-1119	2072	CDS	Homo
Sr43-m2-677	2284	3'UTR	Het
Sr43-m2-328	2327	3'UTR	Homo
Sr43-m2-1117	2327	3'UTR	Homo
Sr43-m2-620	2345	3'UTR	Het
Sr43-m2-1066	2362	3'UTR	Het
Sr43-m2-1136	2411	3'UTR	Het
Sr43-m2-495	2486	3'UTR	Het
Sr43-m2-560	2527	3'UTR	Homo
Sr43-m2-624	2557	3'UTR	Het
Sr43-m2-743	2801	3'UTR	Het
Sr43-m2-1032	2816	3'UTR	Homo
Sr43-m2-1129	2898	3'UTR	Homo

We hypothesized that this mutant population might contain mutants with an even higher FHB resistance, as well as mutants that were no longer resistant. These lines could be helpful for identifying new breeding materials, or for narrowing down the crucial regions in the *Fhb7* gene that lead to disease resistance. To test this, a series of phenotyping experiments were conducted.

PLANTING THE POPULATION

These 70 M₃ lines were planted in shared starter trays. Four seeds were planted per homozygous line, and 8 were planted for each heterozygous line. This population of 480 plants



Figure 3.3 Greenhouse Growth

First EMS population shortly after transplanting and tissue collection for DNA extraction, growing in the KSU greenhouse.

transplanted into individual pots (**Figure 3.3**).

was grown at an average day temperature of 18-26°C with an average humidity of 80% in the KSU greenhouse. There were 12 hours of supplemental growth lights each day. When the plants reached ~3-4 inches in height, samples were collected for

DNA sequencing, using the same methods

discussed in chapter 2, before plants were

Miracle-Gro soluble fertilizer (The Scotts Company LLC, Marysville, USA) was added every week for 5 weeks, and watering trays were checked every 2-3 days. Once the plants reached a height of ~8 inches or more, bamboo sticks were added to the pots for extra vertical support.

PHENOTYPING INOCULUM PREPARATION

When the wheat heads began flowering, and the anthers emerged, heads from each plant were infected with *Fusarium graminearum* conidia spores. This inoculum was prepared from a marginal colony on a slant medium. Pure *F. graminearum* isolates were collected with an inoculation loop and transferred onto petri dishes with solid potato dextrose agar (PDA) (**Figure 3.4**). These plates were incubated for 5-7 days at 26°C before refrigerating at 4°C for further use.

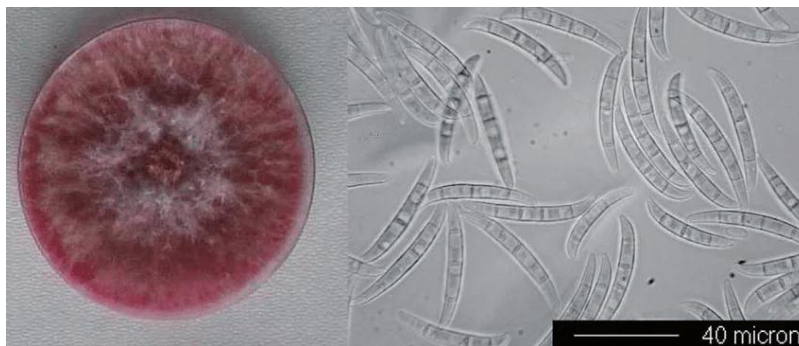


Figure 3.4 Fusarium Plate & Spores

F. graminearum growing on PDA agar next to macroconidia *F. graminearum* spores under microscope (Figure reproduced from Pancaldi *et al.* 2010).

After this, 8-10 fungal plugs were taken from the PDA plates and transferred into a flask with 100 ml of autoclaved liquid mung bean broth. The flask was then put on a shaker and shaken at 250 rpm under

26°C for 72-120 hours. The spore concentration was measured with a hemacytometer and adjusted to a final concentration of 1×10^5 spores/ml. This inoculum was aliquoted into 1.5 µl microtubes and kept at 4°C until use.

GREENHOUSE INOCULATION



Figure 3.5 Bagged Heads Post-Inoculation

Chinese spring wheat heads after point inoculation. Heads enclosed in bags to assist infection.

Once the EMS wheat began flowering, the middle spikelet of each flowering head was injected with 20 μ l of inoculum, which contained ~ 1000 *F. graminearum* spores. After injection, the heads were enclosed with thin plastic bags to support infection with increased humidity (**Figure 3.5**).

After 48 hours, the bags were removed and heads were checked for successful inoculation by observing brown, discolored kernels. Fifteen and twenty-one days after inoculation, the number of infected spikelets and the total spikelets were counted to calculate the percentage symptomatic

spikelets (PSS). Calculation used is as follows:

$$\text{PSS (\%)} = [(\text{Infected Spikelets}/\text{Total Spikelets}) \times 100]$$

Once mature, infected and non-infected heads were collected separately. **Figure 3.6** shows the difference between infected and healthy seeds, while **Figure 3.7** shows examples of different stages of infection that were measured in the greenhouse. A Dunnett's test was performed to identify lines that are significantly different from the parental line PSS at $(\alpha) < 0.05$ level of significance. For significantly different lines, a Cohen's d test was performed to quantify the standardized difference between two means and classify effects as negligible, medium, or large.



Figure 3.6 Scabby vs. Healthy Wheat Grains



Figure 3.7 Severity of FHB infection in Greenhouse after 21 Days
Arrow highlights infected spikelet that indicates successful inoculation.

DNA ISOLATION & NORMALIZATION

After leaf tissue was collected, dried, and ground, DNA was isolated using the modified Hexadecyltrimethylammonium bromide (CTAB) protocol discussed in chapter 2 (Murray & Thompson 1980). The same methods for quantification and normalization were used on the extracted DNA to prepare for Sanger Sequencing.

INITIAL PCR

For ease of sorting, and better sequencing, the *Fhb7* (GST) gene sequence was first amplified by its three parts. Primers for the 5'UTR, CDS, and 3'UTR sections were designed to ensure that the products had some overlapping sequences, so the entire gene could be analyzed. Based on where the initial mutation was located, each sample/line had a different section amplified.

Table 3.2 details the sequences of the chosen primers. **Figure 3.8** shows a primer test done with some of the designed primers. This initial PCR amplification was done using the master mix protocol in **Table 3.3**, using the PCR profile in **Table 3.4**.

Table 3.2 Primers for Initial Amplification

These primers were chosen due to reliable results/strong, single-banded amplicons. Primers ordered from Eurofins.

Primer Name	Sequence	T _m
Fhb7_5UTR_B1_F	AAACCCCCCTCTTTCAGAA	66 °C
Fhb7_5UTR_B2_R	GAGCAGTCAAGTGAATGTTGTATG	64 °C
Fhb7_CDS_C1_F	GGCGATAAGAAATCCACCCAT	65 °C
Fhb7_CDS_C2_R	CGTGACGGATATCTAGCCTAG	64 °C
Fhb7_3UTR_B1_F	AGCTAGCTAGGTCTCGGAG	65 °C
Fhb7_3UTR_B2_R	ATGACGAAAGCCTCCCTC	64 °C

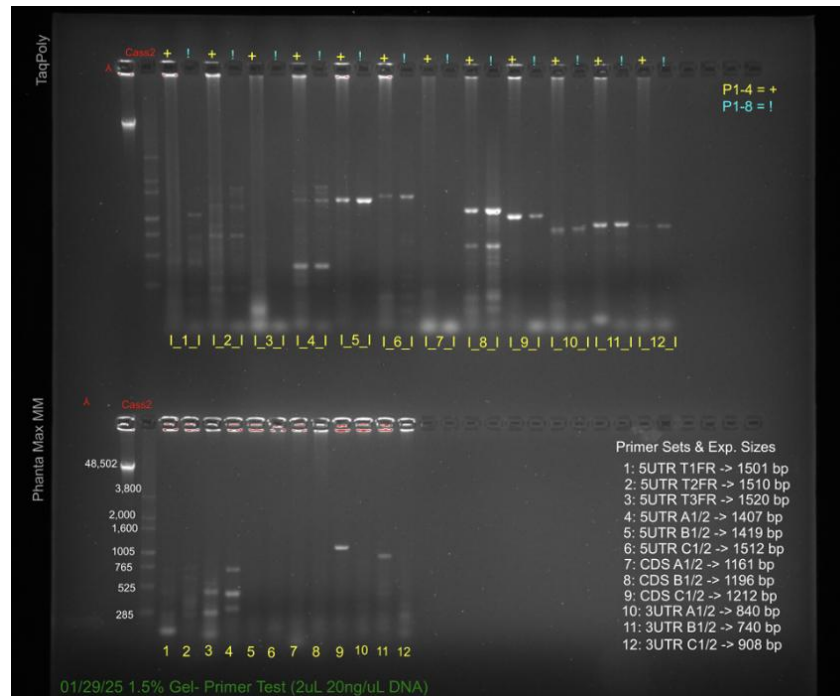


Figure 3.8 Testing Initial Primers

Test gel of various primers designed to amplify the Fhb7 three sections. Sequences that resulted in multiple or weak bands were not used.

Since EMS mutations are typically SNPs, it was important to use a high-fidelity enzyme for amplification. The Phanta Max Super-Fidelity Master Mix from Vazyme was chosen and employed using the product's protocol (Vazyme, Nanjing, China). To start, master mix for each set of primers was created using the ratios in **Table 3.3**. Mixes were vortexed and centrifuged before use.

Table 3.3 PCR Ratios for Initial Amplification of EMS DNA Samples.

PCR Component	Final Conc.	20 µl rxn
Water (ddH ₂ O)		6 µl
Phanta Max Super-Fidelity Master Mix	1x	10 µl
10 µM F primer	0.5 µM	1 µl
10 µM R primer	0.5 µM	1 µl
Genomic DNA	5-100 ng	2 µl

Once the master mixes were ready, template DNA was placed on the bottom of the PCR plate wells. DNA samples were diluted to 30ng/µl, for more accurate pipetting. A total of 60 ng of template DNA was added to each reaction. Then, 18 µl of the prepared master mix was added to each PCR well with 2 µl of DNA. Appropriate master mix + primers were tracked for each DNA sample. Plates were capped, vortexed, and centrifuged before being placed on the thermocycler.

Table 3.4 PCR Program for Phanta Polymerase

Step	Temp. °C	Time
1	98 °C	30 sec
2	98 °C	10 sec
3	60 °C	10 sec
4	72 °C	30 sec/kb
Go to	Step 2	35 Cycles
5	72 °C	5 min
6	15 °C	Forever

The profile in **Table 3.3** was used, based on the Phanta polymerase recommendation and annealing temperatures.

Once the cycles were completed, products were cleaned up and then run in the sequencing reaction.

PCR CLEANUP

Due to poor initial sequencing results, the clean-up of the PCR products was also altered from the process used in chapter 2. Gel extraction of the PCR products was used to better isolate the bands with the GenCatch Gel Purification Kit (Epoch Life Science, Missouri City, TX). Initial PCR products were loaded into 1.5% agarose gel with 6X loading dye. **Figure 3.9** shows the loading process and gel setup. The gel was then run for 30 minutes at 80V to allow the fragments to separate through the gel.

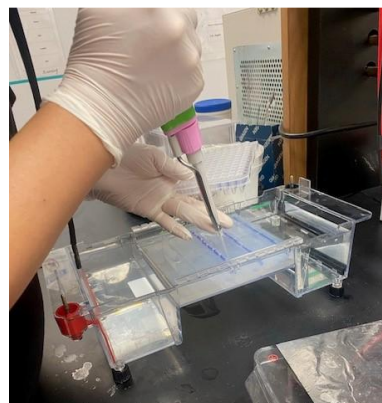


Figure 3.9 Loading PCR Samples for Gel Extraction

In a dark room, the gels were placed on a high performance ultraviolet transilluminator to illuminate the DNA bands. **Figure 3.10** shows a test gel that is ready for extraction. A sterile



Figure 3.10 Samples Ready for Extraction Illuminated with UV

scalpel was used to closely excise the bands before the fragments were placed into individual 1.5 ml microtubes. Once all samples were extracted, 3 volumes of GEX buffer were added to 1 volume of gel by weight.

The samples were incubated at 55°C for 5-10 minutes, until the gel completely dissolved. The dissolved mixtures were then added to gel extraction columns and spun at 5000 rpm for 30 seconds (Legend Micro 21 Centrifuge, Thermo Scientific). After flow-through was discarded, samples were washed with 0.5 ml of WN buffer and spun again. This was repeated with 0.5 ml of WS buffer. After these washes, columns

were spun at 1200 rpm for 3 minutes for ethanol removal. Thirty microliters of ddH₂O were then added to the column's membrane to dissolve the DNA. After another round of centrifuging at 1200 rpm for 1 minute, samples were left at the bottom of the microtubes and stored at 4°C until use.

After this purification, each PCR sample was quantified using the Qubit™ dsDNA Quantification Assay Kit. After quantification, PCR products were diluted to ~16 ng/μl for sequencing.

SEQUENCING REACTION

Sequencing primers were designed and chosen for each sample based on the mutation sites. If the mutations were within 500 bp of a primer, the same primer was used. If they were too far apart, a reverse primer or a separate forward primer was used. When assigning primers to samples, it was ensured that the mutation site was at least 100bp away from primer sequence, since the quality of sequencing near fragment ends tends to be less reliable.

Table 3.5 Primer Names & Targets

Primer Name	Mutation Site Range (bp)	Primer Name	Mutation Site Range (bp)
Fhb7_SectA_1_F	1-363	Fhb7_SectD_3_F	970-1470
Fhb7_SectA_2_F	1-419	Fhb7_SectE_1_F	1209-1709
Fhb7_SectA_3_F	1-444	Fhb7_SectE_2_F	1293-1793
Fhb7_SectB_1_F	186-686	Fhb7_SectE_3_F	1329-1829
Fhb7_SectB_2_F	314-814	Fhb7_SectE_4_F	1372-1872
Fhb7_SectB_3_F	385-885	Fhb7_SectF_1_F	1700-2200
Fhb7_SectB_4_F	456-956	Fhb7_SectF_2_F	1723-2223
Fhb7_SectC_1_F	568-1068	Fhb7_SectF_3_F	1754-2254
Fhb7_SectC_2_F	600-1100	Fhb7_SectG_1_F	2066-2566
Fhb7_SectC_3_F	625-1125	Fhb7_SectG_2_F	2303-2803
Fhb7_SectD_1_F	884-1384	Fhb7_SectG_3_F	2354-2854
Fhb7_SectD_2_F	940-1440	Fhb7_SectG_4_F	2460-2960

Different primers were used outside of the ones used in the initial PCR to account for any shortened sequences. If any ends were not completely amplified, the fragment could still be used for sequencing. **Table 3.5** details the sequencing primer names and their amplified sequence ranges (Supplemental Table 1 details the sequences of each primer).

The sequencing master mix was made by adding components in this order: Water, 5X Sequencing buffer, DMSO, and then Big Dye in the ratios from **Table 3.6**.

Table 3.6 Sequencing Solution Ratios

Reagent	1 rxn	70 rxns	100 rxns
ddH ₂ O	2 µl	350 µl	500 µl
5X Seq. Buffer	2 µl	140 µl	200 µl
DMSO	0.5 µl	35 µl	50 µl
Big Dye	0.5 µl	35 µl	50 µl
Primer	1 µl	70 µl	100 µl
DNA Template	4 µl	-	-

The sequencing PCR was carried out with the appropriate primers for each sample. The purified initial PCR product was added to the bottom of the PCR plate well/tube. Four microliters of PCR products were used, putting the concentration above 50 ng/µl. It is recommended that PCR products longer than 300bp use 5ng/100 bp/µl.

Primers were added to the sides of the PCR plate wells, and 6 uL of master mix was added to each well. Plates

were capped, vortexed, and briefly centrifuged. Samples were placed in the thermocycler and run following the program described in **Table 3.7**.

Table 3.7 Sequencing PCR Profile

Step	Temp. °C	Time
1	94	5 min
2	94	1 min
3	60	2 min
4	60	1 min
Goto	Step 2	25 Cycles
5	4	Forever

PURIFICATION & SEQUENCING

After the sequencing reaction, Optima DTR Ultra 96-well plates (EdgeBio, USA) were used to purify the PCR products. To clear out the excess water hydrating the columns, the plates were centrifuged at 2,100 rpm for 5 minutes at 4°C. The discard plate and caught water was disposed of, and a new PCR plate for the samples was lined up at the bottom.

The samples were carefully loaded in their entirety into individual wells from the top. A protective cover was placed on top of the Optima plate, before the whole set up was centrifuged again at 2,100 rpm for 5 minutes at 4°C. The purified samples were filtered to the bottom of the 96-well plate, where they remained for sequencing. Finally, rubber sequencing mats were placed over the samples, and the plates were loaded into the ABI sequencing tray to start sequencing.

SANGER SEQUENCING ANALYSIS

The putative mutant lines sequencing results were compared to the wildtype parent genome. Sequence mutations were confirmed using the GeniousPrime software. This was done to make sure any initial mutations were persistent and passed onto the next generation.

The *Fhb7* reference sequence was manually loaded in the program. After this, the “Map to Reference” function was selected. This allowed us to select all samples and align them to the original nucleotides. Individual samples were examined by scrolling to the expected site on the reference genome and looking for any SNPs at or around the site. If any samples had a missing SNP, the line was re-sequenced. If the SNP was still ‘missing,’ the line was excluded from any further phenotyping experiments.

CROWN ROT PHENOTYPING

EMS generated mutations are random and spread throughout the genome. The population in this study was selected based on identified mutations within the *Fhb7* region, but there is still a high probability that these lines have additional mutations outside of this area. To assess whether our phenotypic results are due to the *Fhb7* SNPs, a *Fusarium* crown rot experiment was conducted on our significant lines. The *Fhb7* gene also confers a high resistance to crown rot infection. Therefore, we will be able to support our FHB phenotyping results by testing whether the lines are susceptible or resistant to crown rot.

This began with growing *Fusarium graminearum* on Potato Dextrose Agar (PDA) from



Figure 3.11 Sterilized Millet and *Fusarium* on PDA Plate

the previously prepared inoculum. The fungus was grown until healthy and vigorous fungal blocks with hyphae covered the plate. Fungus from the edge of the PDA culture dish was transferred to previously boiled and sterilized millet

(**Figure 3.11**). The millet and fungus were kept at 25 °C for 7 days in an Erlenmeyer flask. The flask was shaken/mixed twice a day to evenly distribute the fungus for material inoculation.

While the inoculum was growing, 150g of sterile soil was placed into small pots measuring 7 cm x 7 cm. Twelve disinfected wheat seeds were placed evenly on the surface and covered with soil. Each line was planted with three repetitions if 36 seeds were available. Pots were watered from the bottom of the trays and grown for 3-4 days in 12 hours of light, at a temperature at 18-26 °C and ~80% humidity.



Figure 3.12 Fungus/Millet on Seedlings

Once the wheat seedlings emerged, a thin layer of contaminated millet was spread in pots, evenly covering the soil and ensuring that there was at least one millet/fungus at the base of each plant (**Figure 3.12**). Plants continued growing for 4 weeks, with the watering trays being refilled every 2-3 days.

After 4 weeks of inoculation, the plants phenotypes were ready to be measured. Wheat seedlings were removed from the pots and washed with water. Plants were placed on black cloth/paper to

Table 3.8 Levels of Disease Severity
Grading scale based on Smiley *et al.* 2005

Grade	Disease Symptoms
Grade 0	The plant does not develop disease.
Grade 1	Brown spots appear at base of the plant stem/mild symptoms appear on first leaf sheath.
Grade 3	The first leaf sheath of the plant has noticeably turned brown.
Grade 5	The second leaf sheath of the plant turns brown.
Grade 7	Brown spots appear on third leaf sheath of plant/ plant is delayed in growth & development due to disease, approaching death.
Grade 9	Plant death.

easily see the infection. From there, the disease index was visually measured using Smiley's disease classification method (**Table 3.8, Figure 3.13**) (Smiley *et al.* 2005).



Figure 3.13 Crown Rot Grading Examples
Grades 0-9 on Chinese Spring Mutants after four weeks of infection.

The crown rot disease index (DI) was calculated with the following equation:

$$DI = \frac{\sum (\text{Number of plants at each disease level} \times \text{Disease grade representative value})}{\text{Total Plants Assessed}}$$

RESULTS

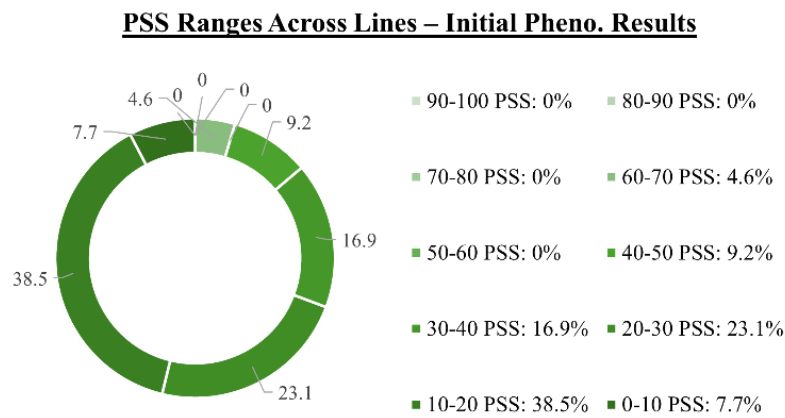


Figure 3.14 PSS Ranges Across Lines
Initial Phenotyping Results at 21 Days Post-inoculation

Figure 3.14 illustrates the spread of PSS ranges in the initial M₃ population. After the data was collected, the grains from the inoculated and healthy seeds were collected separately.

To determine which lines had significantly different FHB

susceptibility, all EMS lines were compared to the parental line, Sr43- m₃-P1. **Table 3.9** shows the parental control's average PSS at 21 days from the first round.

Table 3.9 Average PSS at 21 Days for Parental Control

Control	Avg. PSS 21D	Standard Deviation	Sample Size (n)
Sr43- m ₃ -P1-1	10.278	10.985	21

Figure 3.15 illustrates the spread of lines and their significance when compared to the parental line. The Forest Plot shows the mean difference from the parental line with 95%

confidence intervals. The points crossing the vertical zero line, colored with red, indicate non-significant results. Positive scores indicate an increase in FHB susceptibility, colored in green.

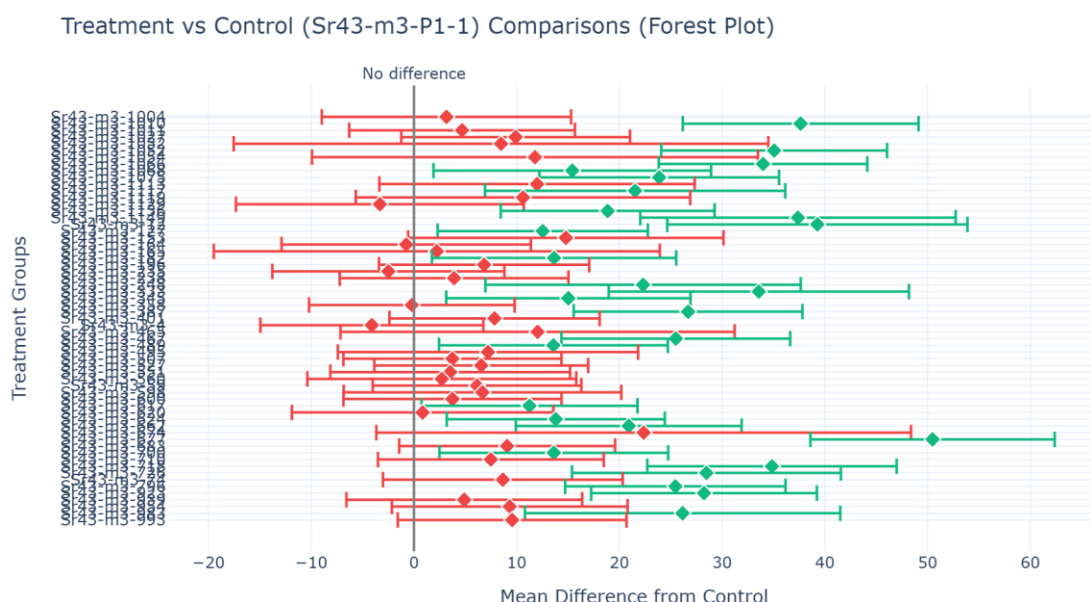


Figure 3.15 Forest Plot of Initial Phenotyping Trial

Green indicates significant differences and red indicates non-significant differences.

The FHB phenotypic data from the 21-day measurement after inoculation for each EMS line was used to identify the significant lines. **Table 3.8** details the results of a Dunnett's test for the lines found to have significant differences in FHB severity compared to the parent (Supplemental Table 2 contains all the tested lines, including ones with negligible results).

Table 3.10 Dunnett's Test Significant Results

Table shows the significant lines when compared to the parental line, Sr43- m3-P1 at significance level (α) 0.05. Lines were chosen based on Dunnett's p-value and Cohen's d results.

EMS Line	<i>Fhb7</i> Region	21D Avg. PSS	Mean Difference	Standard Error	ta-statistic	p-value	Cohen's d	Effect Size
Sr43-m3-1010	CDS	47.936	37.643	5.8544	6.4298	0	2.098	large
Sr43-m3-1052	CDS	46.338	35.0655	5.6065	6.2544	0	1.954	large
Sr43-m3-1066	3'UTR	44.105	33.9808	5.1801	6.5599	0	1.894	large

Sr43-m3-1068	CDS	23.133	15.4097	6.8944	2.2351	0.0256	0.859	large
Sr43-m3-1075	CDS	37.290	23.8709	5.9547	4.0088	0.0001	1.33	large
Sr43-m3-1117	3'UTR	31.808	21.5303	7.4554	2.8879	0.004	1.2	large
Sr43-m3-1136	3'UTR	29.445	18.8566	5.3116	3.5501	0.0004	1.051	large
Sr43-m3-1149	CDS	43.333	37.381	7.8315	4.7731	0	2.083	large
Sr43-m3-12	CDS	67.251	39.2708	7.4554	5.2674	0	2.188	large
Sr43-m3-127	CDS	21.115	12.536	5.221	2.4011	0.0165	0.699	medium
Sr43-m3-182	CDS	26.965	13.6374	6.0663	2.2481	0.0248	0.76	medium
Sr43-m3-248	CDS	36.944	22.3016	7.8315	2.8477	0.0045	1.243	large
Sr43-m3-333	CDS	40.474	33.5732	7.4554	4.5032	0	1.871	large
Sr43-m3-345	CDS	25.385	15.0303	6.0663	2.4777	0.0134	0.838	large
Sr43-m3-387	CDS	38.112	26.6853	5.6816	4.6968	0	1.487	large
Sr43-m3-467	CDS	36.723	25.4892	5.6816	4.4863	0	1.42	large
Sr43-m3-489	CDS	23.846	13.5759	5.6816	2.3894	0.0171	0.757	medium
Sr43-m3-617	CDS	22.229	11.2458	5.3619	2.0974	0.0362	0.627	medium
Sr43-m3-624	3'UTR	23.940	13.8061	5.416	2.5491	0.011	0.769	medium
Sr43-m3-662	CDS	36.541	20.9093	5.6065	3.7294	0.0002	1.165	large
Sr43-m3-677	3'UTR	63.375	50.4823	6.0663	8.3218	0	2.813	large
Sr43-m3-700	CDS	23.037	13.6138	5.6816	2.3961	0.0168	0.759	medium
Sr43-m3-718	CDS	47.036	34.854	6.1914	5.6294	0	1.942	large
Sr43-m3-726	CDS	37.342	28.4713	6.6788	4.263	0	1.587	large
Sr43-m3-796	CDS	34.867	25.4448	5.4744	4.6479	0	1.418	large
Sr43-m3-923	CDS	33.750	28.2381	5.6065	5.0366	0	1.574	large
Sr43-m3-983	CDS	39.444	26.1508	7.8315	3.3392	0.0009	1.457	large

This narrowed down the number of plants for the second run of phenotyping tests. However, none of the significant mutations were in the 5'UTR region, and only 5 mutations within the 3'UTR were flagged. To retain some variety in our gene region mutations, an additional five lines were tested, as shown in **Table 3.11**.

Table 3.11 Extra EMS Lines Added back into Testing Pool

Additional EMS Lines	Fhb7 Region	Mean Difference	Standard Error	ta-statistic	p-value	Cohen's d	Effect Size
Sr43-m3-620	3'UTR	0.8412	6.4936	0.1295	0.897	0.047	negligible
Sr43-m3-4	5'UTR	-4.0996	5.5377	-0.7403	0.4593	-0.228	small
Sr43-m3-153	5'UTR	14.786	7.8315	1.888	0.0593	0.824	large
Sr43-m3-196	5'UTR	6.8254	5.221	1.3073	0.1914	0.38	small
Sr43-m3-1027	5'UTR	9.8957	5.6816	1.7417	0.0819	0.551	medium

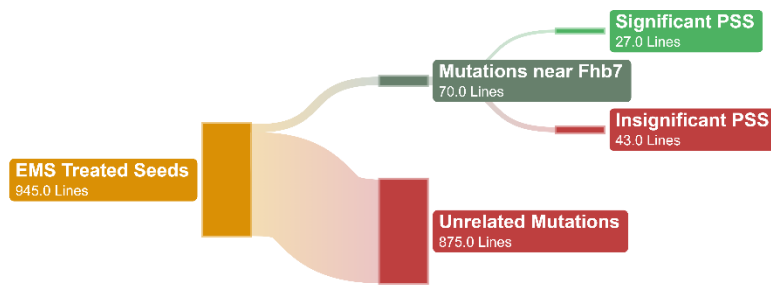


Figure 3.16 Sankey Diagram of Initial Population Narrowing

Figure 3.16 illustrates the paring down that occurred before the second replication of phenotyping took place. There were no lines that showed a significantly higher level of resistance than the parental lines. Individual plants with

higher susceptibility in each significant line were chosen to represent the lines.

A total of 32 lines from the original population were re-assessed for FHB susceptibility/resistance. Seeds from the M₃ self-crossed population were planted, yielding the M₄ population. **Table 3.12** details the average PSS for each line from this second round of greenhouse phenotyping.

Table 3.12 Average PSS on Day 21 for Significant Lines in M₄
Data collected from the second round of phenotyping of EMS mutants based on first round results significance.

Group	Average PSS D21	Standard Deviation	Sample Size (n)
Sr43-m4-P1 (Control)	4.977	3.215	38
Sr43-m4-1010	61.078	11.530	24
Sr43-m4-1027	10.396	10.394	61
Sr43-m4-1052	52.579	7.006	31
Sr43-m4-1066	63.831	12.114	34
Sr43-m4-1075	62.448	25.319	50
Sr43-m4-1113	45.398	8.302	29
Sr43-m4-1117	52.981	9.304	64
Sr43-m4-1149	57.072	16.079	67
Sr43-m4-153	25.250	19.269	64
Sr43-m4-182	41.461	9.335	33
Sr43-m4-248	48.014	7.516	87
Sr43-m4-333	57.810	10.866	38
Sr43-m4-387	41.057	9.095	30

Sr43-m4-677	70.973	23.392	34
Sr43-m4-923	59.485	9.317	68
Sr43-m4-983	44.744	11.253	54
Sr43-m4-1068	22.669	4.685	23
Sr43-m4-1136	31.435	6.142	38
Sr43-m4-12	67.487	5.285	52
Sr43-m4-127	22.339	2.081	47
Sr43-m4-345	29.034	4.330	40
Sr43-m4-467	37.002	2.014	42
Sr43-m4-489	21.112	2.529	28
Sr43-m4-617	20.197	3.397	41
Sr43-m4-624	21.103	3.503	48
Sr43-m4-662	37.094	2.854	34
Sr43-m4-700	21.317	2.338	39
Sr43-m4-718	49.473	2.323	33
Sr43-m4-726	38.427	1.881	52
Sr43-m4-796	35.387	1.877	44
Sr43-m4-620	10.141	3.721	21
Sr43-m4-4	5.551	2.171	33
Sr43-m4-153	20.71	2.619	31
Sr43-m4-196	16.552	3.111	26
Sr43-m4-1027	15.844	2.978	29

All results were found to be significantly different from the control using a Dunnett's test, except for 3 lines. According to Cohen's d, which measures the difference between two groups in standard deviation units, Sr43-m4-620 and Sr43-m5-1027 fell in the 'medium' classification, and Sr43-m4-4 was in the 'negligible' difference category. This is illustrated in **Figure 3.17**.

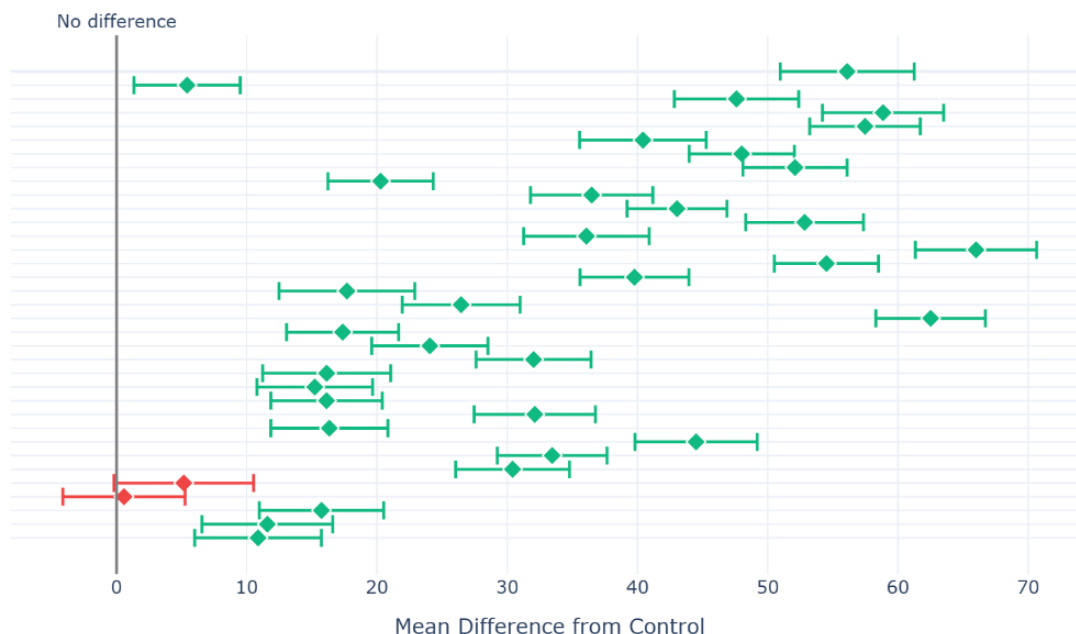


Figure 3.17 Forest Plot of Second Phenotyping Trial (M₄)
Mean differences from parental line with 95% confidence intervals.

A third and final round of greenhouse FHB severity tests was conducted. The coefficient of variance was calculated between the three generations. On average, the severity of susceptibility/resistance increased as the generations progressed. **Table 3.13** details all the averages and the coefficient of variance (CV) between those means.

Table 3.13 Coefficient of Variance between M₃, M₄, & M₅

Degree of variation between generations, based on the ratio of the standard deviation to the mean.
Variation Key: 0-10% **Very Low**; 10-20% **Low**; 20-30% **Moderate**; 30-40% **High**; 40%+ **Very High**

EMS Lines	m3 Avg. 21D PSS	m4 Avg. 21D PSS	m5 Avg. 21D PSS	m3, m4, & m5 Avg. 21D PSS	Sample CV
Sr43-4	5.751	5.552	8.761	6.688	26.88%
Sr43-P1 (Control)	11.418	4.977	5.52	7.305	48.91%
Sr43-620	12.84	10.142	9.845	10.942	15.09%
Sr43-1027	18.594	10.396	14.731	14.574	28.14%
Sr43-196	17.835	16.559	22.646	19.014	16.88%
Sr43-617	22.229	20.197	23.096	21.841	6.81%
Sr43-489	23.846	21.112	22.877	22.612	6.13%
Sr43-700	23.037	21.318	24.788	23.048	7.53%
Sr43-1068	23.133	22.669	24.81	23.538	4.78%

Sr43-153	24.76	20.711	25.25	23.574	10.57%
Sr43-624	23.94	21.103	25.988	23.677	10.36%
Sr43-127	21.115	22.34	31.439	24.965	22.59%
Sr43-345	25.385	29.034	38.838	31.086	22.38%
Sr43-662	36.541	37.095	29.428	34.355	12.45%
Sr43-387	38.112	41.057	24.268	34.479	26%
Sr43-1136	29.445	31.436	46.995	35.959	26.72%
Sr43-796	34.867	35.388	37.677	35.977	4.16%
Sr43-182	26.965	41.462	44.786	37.738	25.11%
Sr43-1113	25.392	45.399	46.722	39.171	30.51%
Sr43-467	36.723	37.002	44.906	39.544	11.75%
Sr43-726	37.342	38.428	44.754	40.175	9.96%
Sr43-248	36.945	48.014	38.558	41.172	14.52%
Sr43-983	39.445	44.745	40.067	41.419	6.99%
Sr43-1149	43.334	57.072	31.482	43.962	29.13%
Sr43-1052	46.338	52.58	36.69	45.202	17.71%
Sr43-1117	31.808	52.982	59.474	48.088	30.09%
Sr43-333	40.475	57.81	52.365	50.217	17.65%
Sr43-1075	37.29	62.449	52.222	50.654	24.98%
Sr43-718	47.0363	49.474	60.742	52.417	13.95%
Sr43-923	33.75	59.485	73.417	55.551	36.23%
Sr43-1066	44.105	63.831	59.715	55.884	18.62%
Sr43-1010	47.936	61.078	61.061	56.691	13.37%
Sr43-12	67.251	67.487	59.97	64.903	6.58%
Sr43-677	63.375	70.973	73.417	69.255	7.56%

This helped separate lines that could have mutations elsewhere in the genome affecting the phenotype. **Table 3.14** shows the Coefficient of Variance between just M₄ and M₅ rounds of phenotyping.

Table 3.14 Coefficient of Variance between m₄ & m₅

Degree of variation between generations, based on the ratio of the standard deviation to the mean.

Variation Key: 0-10% **Very Low**; 10-20% **Low**; 20-30% **Moderate**; 30-40% **High**

EMS Lines	M4 Avg. 21D PSS	m5 Avg. 21D PSS	m4 & m5 Avg. 21D PSS	Sample CV
Sr43-P1 (Control)	6.9775	5.5198	6.24865	16.50%
Sr43-4	5.5518	8.7606	7.1562	31.71%
Sr43-620	10.1419	9.8445	9.9932	2.10%

Sr43-1027	10.3962	14.7311	12.5637	24.40%
Sr43-196	16.5592	22.6457	19.6025	21.96%
Sr43-617	20.1973	23.0965	21.6469	9.47%
Sr43-489	21.1124	22.8769	21.9947	5.67%
Sr43-153	20.711	25.2502	22.9806	13.97%
Sr43-700	21.3175	24.7881	23.0528	10.65%
Sr43-624	21.1034	25.9876	23.5455	14.67%
Sr43-1068	22.6692	24.8099	23.7395	6.38%
Sr43-127	22.3399	31.4394	26.8897	23.93%
Sr43-387	41.0572	24.2677	32.6625	36.35%
Sr43-662	37.0946	29.4276	33.2611	16.30%
Sr43-345	29.034	38.8384	33.9362	20.43%
Sr43-796	35.3878	37.6773	36.5326	4.43%
Sr43-1136	31.4358	46.9949	39.2154	28.06%
Sr43-467	37.0021	44.9056	40.9539	13.65%
Sr43-726	38.4277	44.7544	41.5911	10.76%
Sr43-983	44.7447	40.0667	42.4057	7.80%
Sr43-182	41.4616	44.7861	43.1239	5.45%
Sr43-248	48.014	38.5582	43.2861	15.45%
Sr43-1149	57.0722	31.4815	44.2769	40.87%
Sr43-1052	52.5798	36.69	44.6349	25.17%
Sr43-1113	45.3988	46.7217	46.0603	2.03%
Sr43-333	57.8103	52.3653	55.0878	6.99%
Sr43-718	49.4736	60.742	55.1078	14.46%
Sr43-1117	52.9815	59.4737	56.2276	8.16%
Sr43-1075	62.4485	52.2222	57.3354	12.61%
Sr43-1010	61.0785	61.0606	61.0696	0.02%
Sr43-1066	63.831	59.7153	61.7732	4.71%
Sr43-12	67.4873	59.9698	63.7286	8.34%
Sr43-923	59.4853	73.4167	66.451	14.82%
Sr43-677	70.973	73.4167	72.1949	2.39%

CROWN ROT RESULTS

Table 3.15 details the average grade between both crown rot trials for each tested line, as well as the disease index. Sumai3 is naturally susceptible to crown rot, so it was used as the positive control.

Table 3.15 Average CR Grading and Disease Index
Data from both trials. P1 = Neg. Control, Sumai3 = Pos. Control

EMS Line	Grade Avg Round1	Grade Avg Round2	Overall Average	CR Disease Index
Sr43-m5-1113	0.75	0.441	0.596	6.03%
Sr43-m5-P1	0.659	0.6	0.629	6.94%
Sr43-m5-4	0.967	0.712	0.8395	8.66%
Sr43-m5-718	1.01	0.876	0.943	9.77%
Sr43-m5-467	1.021	1.001	1.011	10.01%
Sr43-m5-983	1.031	1	1.016	10.00%
Sr43-m5-620	1.022	1.054	1.038	10.05%
Sr43-m5-1052	1.419	0.933	1.176	11.01%
Sr43-m5-1027	1.456	1.598	1.527	15.12%
Sr43-m5-196	1.578	1.685	1.6315	15.91%
Sr43-m5-923	2.368	1.276	1.822	17.95%
Sr43-m5-489	2.012	1.989	2.005	20.10%
Sr43-m5-127	2.031	1.989	2.01	20.10%
Sr43-m5-153	2.01	2.104	2.057	20.87%
Sr43-m5-1068	1.986	2.397	2.1915	20.99%
Sr43-m5-617	2.34	2.512	2.426	24.32%
Sr43-m5-700	2.101	2.765	2.433	25.23%
Sr43-m5-624	2.781	2.601	2.691	26.82%
Sr43-m5-345	2.967	3.01	2.9885	30.00%
Sr43-m5-726	3.522	3.021	3.2715	31.98%
Sr43-m5-387	3.844	3.1	3.472	34.99%
Sr43-m5-1136	3.456	3.768	3.612	35.76%
Sr43-m5-662	3.501	3.742	3.6215	35.95%
Sr43-m5-796	3.567	3.699	3.633	36.71%
Sr43-m5-248	4.75	4.882	4.816	47.01%
Sr43-m5-1010	5.313	5.065	5.189	52.35%
Sr43-m5-333	6.25	6.333	6.292	61.56%
Sr43-m5-1117	6.097	6.556	6.326	68.52%
Sr43-m5-1066	6.29	6.655	6.473	74.54%
Sumai3+	6.161	7.154	6.658	66.67%
Sr43-m5-677	6.917	6.586	6.751	75%
Sr43-m5-182	7.387	7.364	7.375	75.07%
Sr43-m5-12	7.142	7.943	7.5425	74.89%
Sr43-m5-1075	8.143	7.903	8.023	79.00%
Sr43-m5-1149	8.81	8.5	8.655	84.97%

Figure 3.18 illustrates a correlation plot between the measured FHB PSS and the crown rot disease index, showing how closely some of the mutant lines are correlated.

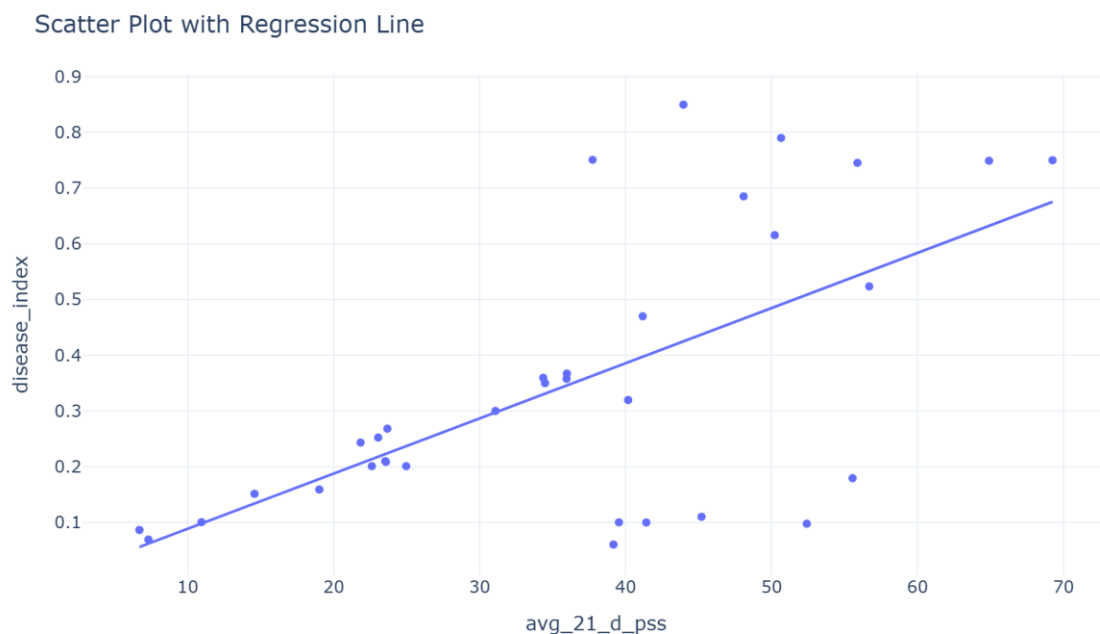


Figure 3.18 Correlation Plot for FHB and Crown Rot Results
Correlation coefficient = 0.6134; p-value = 1.4721e-4; r^2 = 0.3763

Table 3.16 contains the parental line's CR DI and PSS at 21 days, while Table 3.17 details this information for the mutant lines. Six lines differed in severity of response to FHB in the PSS crown rot (highlighted in orange in Table 3.17).

Table 3.16 P1 Phenotypic Data

EMS Line	CR Disease Index	Avg. 21D PSS
Sr43-P1	6.94%	7.305%

Table 3.17 Disease Index vs. PSS of Mutants

Orange boxes contain lines that are different in susceptibility results for the two traits.

Line	CR DI	Avg. 21D PSS %	Line	CR DI	Avg. 21D PSS %	Line	CR DI	Avg. 21D PSS %
Sr43-1010	52.35%	56.691	Sr43-1149	84.97%	43.962	Sr43-387	34.99%	34.479
Sr43-1027	15.12%	14.574	Sr43-12	74.89%	64.903	Sr43-4	8.66%	6.688

Sr43-1052	11.01%	45.202	Sr43-127	20.10%	24.965	Sr43-467	10.01%	39.544
Sr43-1066	74.54%	55.884	Sr43-153	20.87%	23.574	Sr43-489	20.10%	22.612
Sr43-1068	20.99%	23.538	Sr43-182	75.07%	37.738	Sr43-617	24.32%	21.841
Sr43-1075	79.00%	50.654	Sr43-196	15.91%	19.014	Sr43-620	10.05%	10.942
Sr43-1113	6.03%	39.171	Sr43-248	47.01%	41.172	Sr43-624	26.82%	23.677
Sr43-1117	68.52%	48.088	Sr43-333	61.56%	50.217	Sr43-662	35.95%	34.355
Sr43-1136	35.76%	35.959	Sr43-345	30.00%	31.086	Sr43-677	75%	69.255
Sr43-700	25.23%	23.048	Sr43-726	31.98%	40.175	Sr43-923	17.95%	55.551
Sr43-718	9.77%	52.417	Sr43-796	36.71%	35.977	Sr43-983	10.00%	41.419

FINAL MUTANT GROUPS

Persistence of these mutations was confirmed with Sanger sequencing. Using the GeniousPrime software, each confirmed, significant line's mutation was classified as a synonymous, null mutation or a non-synonymous mutation. **Table 3.18** details the significant mutants and their amino acid changes.

Table 3.18 Nonsynonymous Mutations

Green boxes contain lines with mutations that altered the amino acid (AA). Orange boxes have synonymous mutations. White, struck boxes were found to differ in FHB and CR results.

EMS Line	Mut. Site (bp)	Fhb7 Region	Original NT	Mutated NT	OG Amino	Mut. AA
Sr43-m5-1027	62	5'UTR	C/T	G/A	Pro	Leu
Sr43-m5-153	117	5'UTR	C/T	G/A	Ile	Ile
Sr43-m5-196	145	5'UTR	C/T	G/A	Gln	STOP
Sr43-m5-4	244	5'UTR	C/T	G/A	Pro	Ser
~~Sr43-m5-1149~~	464	~~CDS~~	~~C/T~~	~~G/A~~	~~Ser~~	~~Phe~~
Sr43-m5-1068	465	CDS	C/T	G/A	Ser	Ser
Sr43-m5-726	510	CDS	G/A	C/T	Val	Val
Sr43-m5-333	558	CDS	C/T	G/A	Gly	Gly
Sr43-m5-127	827	CDS	G/A	C/T	Gly	Glu
Sr43-m5-617	875	CDS	G/A	C/T	Arg	His

Sr43-m5-1010	923	CDS	G/A	C/T	Gly	Asp
Sr43-m5-1113	1100	CDS	G/A	C/T	Val	Val
Sr43-m5-1075	1145	CDS	C/T	G/A	Ala	Val
Sr43-m5-345	1216	CDS	C/T	G/A	Leu	Phe
Sr43-m5-662	1259	CDS	C/T	G/A	Ser	Leu
Sr43-m5-12	1262	CDS	C/T	G/A	Ser	Leu
Sr43-m5-182	1267	CDS	C/T	G/A	Pro	Leu
Sr43-m5-489	1376	CDS	G/A	C/T	Trp	STOP
Sr43-m5-1052	1411	CDS	C/T	G/A	Pro	Ser
Sr43-m5-796	1475	CDS	G/A	C/T	Ser	Asn
Sr43-m5-467	1642	CDS	G/A	C/T	Gly	Arg
Sr43-m5-718	1644	CDS	G/A	C/T	Gly	Gly
Sr43-m5-983	1751	CDS	G/A	C/T	Trp	STOP
Sr43-m5-248	1782	CDS	C/T	G/A	Ile	Ile
Sr43-m5-387	1820	CDS	G/A	C/T	Gly	Asp
Sr43-m5-700	2021	CDS	G/A	C/T	Cys	Tyr
Sr43-m5-923	2047	CDS	G/A	C/T	Glu	Lys
Sr43-m5-677	2284	3'UTR	G/A	C/T	Phe	Leu
Sr43-m5-1117	2327	3'UTR	G/A	C/T	Arg	Cys
Sr43-m5-620	2345	3'UTR	C/T	G/A	Ala	Thr
Sr43-m5-1066	2362	3'UTR	C/T	G/A	Leu	Leu
Sr43-m5-1136	2411	3'UTR	C/T	G	Glu	Glu
Sr43-m5-624	2557	3'UTR	C/T	C	Gly	Gly

Table 3.19 separates the lines with synonymous mutations, where the amino acid is not altered by the SNP.

Table 3.19 Synonymous Mutant Lines

EMS Line	<i>Fhb7</i> Region	Avg. PSS	CR Disease Index	Original NT	Mutated NT	OG AA	Mut. AA
Sr43-153	5'UTR	23.57%	20.87%	G/C	A/T	Ile	Ile
Sr43-1068	CDS	23.54%	20.99%	A/T	G/C	Ser	Ser
Sr43-726	CDS	40.18%	31.98%	G/C	A/T	Val	Val
Sr43-333	CDS	50.21%	61.56%	A/T	G/C	Gly	Gly
Sr43-1113	CDS	39.17%	6.03%	G/C	A/T	Val	Val
Sr43-248	CDS	41.17%	47.01%	A/T	G/C	Ile	Ile
Sr43-1066	3'UTR	55.88%	74.54%	A/T	G/C	Leu	Leu
Sr43-1136	3'UTR	35.96%	35.76%	G/C	A/T	Glu	Glu
Sr43-624	3'UTR	23.68%	26.82%	G/C	A/T	Gly	Gly

Table 3.20 details the lines carrying nonsynonymous mutations, where the amino acid sequences are altered by the SNP.

Table 3.20 Nonsynonymous Mutant Lines

EMS Line	<i>Fhb7</i> Region	Avg. PSS	CR DI	Original NT	Mutated NT	OG Amino	Mut. AA
Sr43-1027	5'UTR	14.57%	15.12%	A/T	G/C	Pro	Leu
Sr43-196	5'UTR	19.01%	15.91%	A/T	G/C	Gln	STOP
Sr43-4	5'UTR	6.68%	8.66%	A/T	G/C	Pro	Ser
Sr43-127	CDS	24.96%	20.10%	G/C	A/T	Gly	Glu
Sr43-617	CDS	21.84%	24.32%	G/C	A/T	Arg	His
Sr43-1010	CDS	56.69%	52.35%	G/C	A/T	Gly	Asp
Sr43-1075	CDS	50.65%	79.00%	A/T	G/C	Ala	Val
Sr43-345	CDS	31.08%	30.00%	A/T	G/C	Leu	Phe
Sr43-662	CDS	34.35%	35.95%	A/T	G/C	Ser	Leu
Sr43-12	CDS	64.90%	74.89%	A/T	G/C	Ser	Leu
Sr43-489	CDS	22.61%	20.10%	G/C	A/T	Trp	STOP
Sr43-796	CDS	35.97%	36.71%	G/C	A/T	Ser	Asn
Sr43-983	CDS	41.41%	30.00%	G/C	A/T	Trp	STOP
Sr43-387	CDS	34.47%	34.99%	G/C	A/T	Gly	Asp
Sr43-700	CDS	23.04%	25.23%	G/C	A/T	Cys	Tyr
Sr43-677	3'UTR	69.25%	75%	G/C	A/T	Phe	Leu
Sr43-1117	3'UTR	48.08%	68.52%	G/C	A/T	Arg	Cys
Sr43-620	3'UTR	10.94%	10.05%	A/T	G/C	Ala	Thr

Table 3.21 shows the mutants where the FHB and CR susceptibility were different from each other.

Table 3.21 Mutants with Mismatched FHB & CR Susceptibilities

EMS Line	<i>Fhb7</i> Region	Avg. PSS	CR DI	Original NT	Mutated NT	OG AA	Mut. AA
Sr43-1149	CDS	43.96%	84.97%	C/T	G/A	Ser	Phe
Sr43-182	CDS	37.73%	75.07%	C/T	G/A	Pro	Leu
Sr43-1052	CDS	45.20%	11.01%	C/T	G/A	Pro	Ser
Sr43-467	CDS	39.54%	10.01%	G/A	C/T	Gly	Arg
Sr43-718	CDS	52.41%	9.77%	G/A	C/T	Gly	Gly
Sr43-923	CDS	55.55%	17.95%	G/A	C/T	Glu	Lys

DISCUSSION

In this project, a pool of *Fhb7* mutants were tested. While our significant results were all higher susceptibility than the parental line, they still provide information on how the *Fhb7* is expressed and regulated. As previously acknowledged, there is the chance that mutations outside of the gene region examined here are causal SNPs. A mutation outside of *Fhb7* could be crucial to its function. So, while we had a narrow scope with our mutants, there is always the possibility that a mutation somewhere in the initial 945 EMS population greatly affects FHB resistance.

The three cycles of FHB phenotyping provided a large pool of data with more reliable averages than just the M₃ cycle. This allowed for analysis of the consistency between generations. If there was a large discrepancy between a line's M₃ and M₄ generation, then this could be contributed to a high environmental influence or even some selection as the heterozygotes were homogenized.

Five of these lines had a high level of variance (30-40%). The highest variance came from the parental line. This could be attributed to selective seeding; After multiple P1 plants were tested in cycle 1, plants with consistent, high resistance were planted for the next cycle. These cycles also took place in different seasons, and the fluctuations in the environment could explain the variance.

Using phenotypic data, we were able to pare down the EMS population to lines with mutations that exhibited significant effects. The crown rot trials added evidence to whether the *Fhb7*-related SNP was the causal mutation for the phenotypic results. This left us with a pool of 32 mutants for further testing.

These mutants were categorized based on the amino acid changes and the correlation between the FHB and CR phenotyping. Nine mutants had a synonymous mutation in *Fhb7*. These mutants had a significant PSS and CR DI when compared to the control. This could indicate that there is a causal mutation outside of the *Fhb7* region, since synonymous mutations typically have little to no effect. There is the possibility of folding or folding rate differences due to the identified SNP, but most of these changes are too small to explain the significant changes in phenotype. These lines would be great candidates for whole genome sequencing, so other SNPs could be isolated and related to the observed phenotypic differences. Since these had significant FHB resistance deviation, a relevant mutation could be outside of our target region.

Eighteen mutant lines carried nonsynonymous mutations in the *Fhb7* region, where the amino acid was different from the parental line. This could potentially have a larger effect on the protein structure/function, than synonymous mutations. These mutants also had a significant correlation between the FHB and CR disease susceptibility. Together, this indicates that the *Fhb7* related SNPs are causal for the phenotypic differences from the wildtype. These SNPs could be in crucial gene region or influence the gene's expression. This group also provides support for the *Fhb7* gene being responsible for FHB resistance, since the disruption of the gene led to such a drastic difference in susceptibility.

Six lines had a mismatch between the FHB and CR susceptibility, with only one of these lines having a synonymous mutation. The *Fhb7* wildtype gene is also resistant to crown rot infection. If a mutant plant is susceptible to crown rot and FHB, it is likely that the *Fhb7* SNP is altering the phenotypic results in both cases. The lines that did not correlate between the FHB susceptibility and CR susceptibility could still carry a significant mutation outside of the *Fhb7* region, but it is likely that the phenotype results are not caused by the mutation we identified.

However, it is possible that FHB and CR have different regulation mechanisms, and these mechanisms may not agree with each other. For example, if the CR susceptibility was low, but the FHB susceptibility was high, the FHB mechanism alone might be affected by the SNP.

FUTURE DIRECTIONS

Another test was prepared to further link the mutations and the phenotypes. In the m5 generation, heads were collected 24 hours post-inoculation and placed frozen for RNA extraction. This will allow us to look at the expression level of the *Fhb7* gene. For causal susceptible mutants, we would expect to see a decrease in expression when compared to the parental line. If we were to find a more resistant line, we might expect the expression to increase.

The mutants identified are a great place to start for more in-depth examination of *Fhb7* or other FHB-related mutations. Further experiments to find the underlying mechanisms that affect the FHB severity levels in these lines could reveal novel sources of resistance.

CONCLUSION

In these projects, we aimed to examine two QTLs associated with FHB resistance: *Fhb1* and *Fhb7*. Both genes have demonstrated a high level of resistance to FHB disease in their host species. The importance of host resistance will only increase as FHB outbreaks continue and spread. To stay ahead of any major outbreaks, improved sources of resistance are needed.

While the results of our nanoparticle transformation were not successful, the concept behind it still holds merit. With more tests, it could prove to be an effective way to edit wheat cultivars. This would save breeders time and money while building resilience in our crops.

The EMS population yielded 32 mutants in the *Fhb7* region that significantly affect susceptibility; lines that could harbor significant SNPs within and outside of our target area. This opens a pathway for further studies, within these lines and even within the initial 945 EMS lines.

Chapter 4 - Bibliography

- Alexandratos, N. (2005). Countries with Rapid Population Growth and Resource Constraints: Issues of Food, Agriculture, and Development. *Population and Development Review*, 31(2), 201–406. <https://doi.org/10.1111/j.1728-4457.2005.00064.x>
- Alisaac, E., & Mahlein, A.-K. (2023). Fusarium Head Blight on Wheat: Biology, Modern Detection and Diagnosis and Integrated Disease Management. *Toxins*, 15. <https://doi.org/10.3390/toxins15030192>
- Allard, R. W. (1960). Principles of plant breeding. *NY John Wiley & Sons Inc.*
- Anderson, N., Sisson, A., & Turkington, K. (2025). Wheat Disease Loss Estimates from the United States and Canada—2024. *Crop Protection Network*. <https://doi.org/doi.org/10.31274/cpn-20250324-0>
- Avila, L. A., Aps, L. R. M., Sukthankar, P., Ploscariu, N., & Gudlur, S. (2015). Branched Amphiphilic Cationic Oligopeptides Form Peptiplexes with DNA: A Study of Their Biophysical Properties and Transfection Efficiency. *Molecular Pharmaceutics*, 12(3), 706–715. <https://doi.org/10.1021/mp500524s>
- Babu, R., Nair, S. K., & Prasanna, B. M. (2004). Integrating marker assisted selection in crop breeding prospects and challenges. *Current Science*, 87, 607–619.
- Bai, G., Kolb, F., Shaner, G., & Domier, L. (1999). Amplified Fragment Length Polymorphism Markers Linked to a Major Quantitative Trait Locus Controlling Scab Resistance in Wheat. *Phytopathology*, 89(4). <https://doi.org/10.1094/PHYTO.1999.89.4.343>
- Bai, G., Su, Z., & Cai, J. (2018). Wheat resistance to Fusarium head blight. *Can. J. Plant Pathology*, 40(3), 336–346. <https://doi.org/10.1080/07060661.2018.1476411>
- Bajwa, A., Farooq, M., Al-Sadi, A., Nawaz, A., Jabran, K., & Siddique, K. (2020). Impact of climate change on biology and management of wheat pests. *Crop Protection*, 137. <https://doi.org/10.1016/j.cropro.2020.105304>
- Barros, S., Whitaker, S., Sukthankar, P., Avila, L. A., Guldur, S., & Warner, M. (2016). A review of solute encapsulating nanoparticles used as delivery systems with emphasis on branched amphipathic peptide capsules. *Archives of Biochemistry and Biophysics*, 596.
- Birrenbach, G., & Speiser, P. P. (1976). Polymerized micelles and their use as adjuvants in immunology. *Journal of Pharmaceutical Science*, 1763–1766. <https://doi.org/10.1002/jps.2600651217>
- Bockus, W. W., Bowden, R. L., Hunger, R., Morrill, W., & Murray, T. (2010). Compendium of Wheat Diseases and Pests. In *American Phytopathological Society*. APS Press.
- Borojevic, K., & Borojevic, K. (2005). The Transfer and History of “Reduced Height Genes” (Rht) in Wheat from Japan to Europe. *Journal of Heredity*, 96(4). <https://doi.org/10.1093/jhered/esi060>
- Bottalico, A., & Perrone, G. (2002). Toxigenic Fusarium species and mycotoxins associated with head blight in small-grain cereals in Europe: 611-624. *European Plant Pathology*, 108. <https://doi.org/10.1023/A:1020635214971>
- Buerstmayr, M., Steiner, B., & Buerstmayr, H. (2020). Breeding for Fusarium head blight resistance in wheat—Progress and challenges. *Plant Breeding*, 139(3), 429–454.
- Cakir, C., Gillespie, M., & Scofield, S. (2010). Rapid Determination of Gene Function by Virus-induced Gene Silencing in Wheat and Barley. *Crop Science*, 50. <https://doi.org/10.2135/cropsci2009.10.0567>

- Callahan, S. (2026). *Land Use, Land Value & Tenure—Major Land Uses*. USDA Economic Research Service. <https://www.ers.usda.gov/topics/farm-economy/land-use-land-value-tenure/major-land-uses>
- Capaldi, C., Dopko, R., & Zelenski, J. (2014). The relationship between nature connectedness and happiness: A meta-analysis. *Frontier Psychology*, 4. <https://doi.org/10.3389/fpsyg.2014.00976>
- Carroll, E., Kunte, N., McGraw, E., Gautum, S., Range, R., Noveron-Nunez, J., Held, D., & Avila, A. (2023). Gene silencing in adult *Popillia japonica* through feeding of double-stranded RNA (dsRNA) complexed with branched amphiphilic peptide capsules (BAPCs). *Frontiers Insect Science*, 3. <https://doi.org/10.3389/finsc.2023.1151789>
- Chen, Z., Heng, Y., Li, J., Pei, J., & Cao, Y. (2021). Generation of a series of mutant lines resistant to imidazolinone by screening an EMS-based mutant library in common wheat. *Crop Journal*, 9(5), 1030–1038. <https://doi.org/10.1016/j.cj.2020.11.001>
- Corbett, K., Edwards, D., Leist, S., Abiona, O., Boyoglu-Barnum, S., & Gillespie, R. (2020). *SARS-CoV-2 mRNA vaccine design enabled by prototype pathogen preparedness*. 586, 567–571. <https://doi.org/10.1038/s41586-020-2622-0>
- Crist, E., Mora, C., & Engelman, R. (2017). The interaction of human population, food production, and biodiversity protection. *Science*, 356(6335), 260–264. <https://doi.org/10.1126/science.aal2011>
- DeVore, I., & Lee, R. B. (2017). *Man the Hunter*. Routledge. (Original work published 1968, Transaction Publishers)
- Duarte, C. M., Marba, N., & Holmer, M. (2007). Rapid domestication of marine species. *Science*, 316, 382–383.
- Edwards, S. G., Pirgozilev, S. R., Hare, M. C., & Jenkinson, P. (2001). Quantification of trichothecene-producing *Fusarium* species in harvested grain by competitive PCR to determine efficacies of fungicides against *Fusarium* head blight of winter wheat. *Applied and Environmental Microbiology*, 67, 1575–1580.
- Ehlers, J. D. (2000). Registration of ‘California Blackeye 27’ cowpea. *Crop Science*, 40, 854–855.
- Englund, R. (2019). *Wheat Classes, History, and Breeding Timelines*. SDSU Extension. <https://extension.sdstate.edu/sites/default/files/2020-03/S-0005-02-Wheat.pdf> (Original work published South Dakota State University)
- Ezeh, A., Bongaarts, J., & Mberu, B. (2012). Global population trends and policy options. *The Lancet, Family Planning*, 380(9837), 142–148.
- Feng, Z., Wen, Y., & Guo, X. (2014). *CRISPR/Cas9 for genome editing: Progress, implications and challenges*. 23(R1), R40–R46. <https://doi.org/10.1093/hmg/ddu125>
- Flores-Sandoval, E., Suzuki, H., Lazner, J., & Kohchi, T. (2025). The B-class auxin response factor MpARF2 is essential for meristem organization in free-living plant gametophytes. *Current Biology*, 36(2), 261–277. <https://doi.org/10.1016/j.cub.2025.11.015>
- Foresight, & KWS. (2023). *The long journey toward a new variety*. <https://portrait.kws.com/edition-2023/the-long-journey-toward-a-new-variety/>
- Fromme, M. E., Morrish, F., Armstrong, C., Williams, R., Thomas, J., & Klein, T. M. (1990). Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. *Biotechnology*, 8(9), 833–839. <https://doi.org/10.1038/nbt0990-833>
- Fuller, D. Q. (2012). Biodiversity in Agriculture: Domestication, Evolution, and Sustainability. In *Cambridge University Press* (pp. 110–135).

- Gong, X., He, X., Zhang, Y., & Li, L. (2020). Development of an Evaluation System for Fusarium Resistance in Wheat Grains and Its Application in Assessment of the Corresponding Effects of Fhb1. *Plant Disease*, 104(8). <https://doi.org/10.1094/PDIS-12-19-2584-RE>
- Gong, X., He, X., Zhang, Y., Li, L., Sun, Z., & Bai, G. (2020). Development of an Evaluation System for Fusarium Resistance in Wheat Grains and Its Application in Assessment of the Corresponding Effects of Fhb1. *Plant Disease*, 104(8). <https://doi.org/10.1094/PDIS-12-19-2584-RE>
- Gordon-Kamm, W., Spencer, T. M., Mangano, M. L., Adams, T. R., Daines, R. J., Start, W. G., O'Brien, J. V., Chambers, S. A., & Adams, W. R. (1990). Transformation of maize cells and regeneration of fertile transgenic plants. *The Plant Cell*, 2(7), 603–618. <https://doi.org/10.1105/tpc.2.7.603>
- Gostimskaya, I. (2022). CRISPR–Cas9: A History of Its Discovery and Ethical Considerations of Its Use in Genome Editing. *Biochemistry (Mosc.)*, 777–788. <https://doi.org/10.1134/S0006297922080090>
- Greene, E. A., Codomo, C. A., Taylor, N. E., Henikoff, J. G., Till, B. J., & Reynolds, S. H. (n.d.). Spectrum of chemically induced mutations from a large-scale reverse-genetic screen in Arabidopsis. *Genetics*, 731–740. <https://doi.org/10.1093/genetics/164.2.731>
- Greiling, W. (1954). *Under the spell of medicine; Paul Ehrlich*. Dusseldorf: Econ Verlag.
- Gudlur, S., Sukthankar, P., Gao, J., Avila, L. A., Chen, J., Iwamoto, T., & Tomich, J. W. (n.d.). *Peptide nanovesicles formed by the self-assembly of branched amphiphilic peptides*.
- Hall, A. E. (1992). Breeding for heat tolerance. *Plant Breeding Review*, 10, 129–168.
- Hansen, M., Potapov, P., Pickens, A., & Tyukavina, A. (2022). Global land use extent and dispersion within natural land cover using Landsat data. *Environmental Research Letters*, 17. <https://doi.org/10.1088/1748-9326/ac46ec>
- Hao, Y., Rasheed, A., Zhu, Z., & Wulff, Z. (2019). Harnessing wheat Fhb1 for Fusarium resistance. *Trends Plant Science*, (25), 1–3.
- Hasan, M., Rafii, M., & Ismail, M. (2015). Marker-assisted backcrossing: A useful method for rice improvement. In *Biotechnology & Biotechnological Equipment*. <https://doi.org/10.1080/13102818.2014.995920>
- Hille, F., Richter, H., Wong, S. P., & Bratovic, M. (n.d.). The Biology of CRISPR-Cas: Backward and Forward. *Cell*, 172(6), 1239–1259.
- Hoffman, G. R. (1980). Genetic effects of dimethyl sulfate, diethyl sulfate, and related compounds. *Reviews in Genetic Toxicology*, 75(1), 63–129. [https://doi.org/10.1016/0165-1110\(80\)90028-7](https://doi.org/10.1016/0165-1110(80)90028-7)
- Hunter, W., & Tomich, J. (n.d.). BAPC-assisted-CRISPR-Cas9 Delivery into Adult Psyllid Ovaries and Pupae for Heritable Gene Editing (Hemiptera). *Citrus Greening Solutions*. Retrieved <https://citrusgreening.org/disease/researchhighlights/index>.
- Irwin, E., & Bockstael, N. (2007). *The evolution of urban sprawl: Evidence of spatial heterogeneity and increasing land fragmentation*. <https://doi.org/10.1073/pnas.0705527105>
- ISAAA. (2009). *GM Approval Database* (No. T25). Bayer CropScience. <https://www.isaaa.org/gmapprovaldatabase/event/default.asp?EventID=102>
- Karlsson, I., Persson, P., & Friberg, H. (2021). Fusarium Head Blight From a Microbiome Perspective. *Frontier Microbiology*, 12. <https://doi.org/10.3389/fmicb.2021.628373>

- Karlsson, J., Luly, K. M., Tzeng, S. Y., & Green, J. J. (2021). Nanoparticle designs for delivery of nucleic acid therapeutics as brain cancer therapies. *Advanced Drug Delivery Review*, 179. <https://doi.org/10.1016/j.addr.2021.113999>
- Kean-Galeno, T., Lopez-Arredondo, D., & Herrera-Estrella, L. (2024). The Shoot Apical Meristem: An Evolutionary Molding of Higher Plants. *Int J Mol Sci*, 25. <https://doi.org/10.3390/ijms25031519>
- Klümper, W., & Qaim, M. (2014). *A Meta-Analysis of the Impacts of Genetically Modified Crops*. 9(11). <https://doi.org/10.1371/journal.pone.0111629>
- Kreuter, J. (2007). Nanoparticles—A historical perspective. *International Journal of Pharmaceutics*, 331(1), 1–10. <https://doi.org/10.1016/j.ijpharm.2006.10.021>
- Krieg, D. R. (1963). Ethyl methanesulfonate-induced reversion of bacteriophage T4rII mutants. *Genetics*, 48, 561–580.
- Kruglova, N., Zinatullina, A., & Yegorova, N. (2023). Histological Approach to the Study of Morphogenesis in Callus Cultures In Vitro: A Review. *International Journal of Plant Biology*, 14(2), 533–545. <https://doi.org/10.3390/ijpb14020042>
- Leplat, J., Friberg, H., & Abid, M. (2012). Survival of *Fusarium graminearum*, the causal agent of Fusarium head blight. A review. *Agronomy for Sustainable Development*, 33(1), 97–111. <https://doi.org/10.1007/s13593-012-0098-5>
- Li, X., Chen, G., Liu, X., & Liang, X. (2017). A New Global Land-Use and Land-Cover Change Product at a 1-km Resolution for 2010 to 2100 Based on Human–Environment Interactions. *Annals of the American Association of Geographers*, 107. <https://doi.org/10.1080/24694452.2017.1303357>
- Lindgren, E., Andersson, Y., Suk, J., & Sudre, B. (2012). Monitoring EU Emerging Infectious Disease Risk Due to Climate Change. *Science*, 336(6080), 418–419. <https://doi.org/10.1126/science.12157>
- Liu, D., & Raftery, A. (2020). How Do Education and Family Planning Accelerate Fertility Decline? *Population and Development Review*, 46(3), 409–441. <https://doi.org/10.1111/padr.12347>
- Lobell, D., Cassman, K., & Field, C. (2009). Crop Yield Gaps: Their Importance, Magnitudes, and Causes. *Annual Review of Environment and Resources*, 34, 179–204.
- Luo, K., Guo, J., & He, D. (2023). Deoxynivalenol accumulation and detoxification in cereals and its potential role in wheat–*Fusarium graminearum* interactions. *aBIOTECH*, 4, 155–171. <https://doi.org/10.1007/s42994-023-00096-7>
- Malhi, G., Kaur, M., & Kaushik, P. (2021). Impact of Climate Change on Agriculture and Its Mitigation Strategies: A Review. *Sustainability*, 13(3). <https://doi.org/10.3390/su13031318>
- Marty, J. J., Oppenheim, R. C., & Speiser, P. (1978). Nanoparticles—A new colloidal drug delivery system. *Pharmaceutica Acta Helveticae*, 53, 17–23.
- McMullen, M., Jones, R., & Gallenberg, D. (1997). Scab of wheat and barley: A re-emerging disease of devastating impact. *The American Phytopathological Society*, 81(12). <https://doi.org/10.1094/PDIS.1997.81.12.1340>
- Meng, Z., Zhang, Y., Zhou, X., Ji, J., & Liu, Z. (2022). Nanovaccines with cell-derived components for cancer immunotherapy. *Advanced Drug Delivery Review*, 182. <https://doi.org/10.1016/j.addr.2021.114107>
- Merkle, H. P., & Speiser, P. (1973). Preparation and in vitro evaluation of cellulose acetate phthalate coacervate microcapsules. *Journal of Pharmaceutical Science*, 62, 1444–1448. <https://doi.org/10.1002/jps.2600620910>
- Mesterházy, A. (2020). Updating the Breeding Philosophy of Wheat to Fusarium Head Blight (FHB): Resistance Components, QTL Identification, and Phenotyping—A Review. *Plants*, 9(12). <https://doi.org/10.3390/plants9121702>

- Mesterházy, A. (2024). What Is Fusarium Head Blight (FHB) Resistance and What Are Its Food Safety Risks in Wheat? Problems and Solutions—A Review. *Toxins*. <https://doi.org/10.3390/toxins16010031>
- Mesterházy, A., Bartók, T., & Mirocha, C. G. (1999). Nature of wheat resistance to Fusarium head blight and the role of deoxynivalenol for breeding. *Plant Breeding*, 118(2), 97–110. <https://doi.org/10.1046/j.1439-0523.1999.118002097.x>
- Meyer, R., & Purugganan, M. (2013). Evolution of crop species: Genetics of domestication and diversification. *Nature Review Genetics*, 14, 840–852. <https://doi.org/10.1038/nrg3605>
- Meyer, R. S., DuVal, A. E., & Jensen, H. R. (2012). Patterns and processes in crop domestication: An historical review and quantitative analysis of 203 global food crops. *New Phytol*, 196, 29–48.
- Mullan, K., Sills, E., Pattanayak, S., & Caviglia-Harris, J. (2018). Converting Forests to Farms: The Economic Benefits of Clearing Forests in Agricultural Settlements in the Amazon (Pt. 427-455). *Environ Resource Econ*, 71. <https://doi.org/10.1007/s10640-017-0164-1>
- Mutant Variety Database (MVD). (2022). Centre of Nuclear Techniques in Food and Agriculture. <https://nucleus.iaea.org/sites/mvd/SitePages/Home.aspx>
- Nerkar, G., Devarumath, S., Purankar, M., & Kumar, A. (2022). Advances in Crop Breeding Through Precision Genome Editing. *Frontiers in Genetics*. <https://doi.org/10.3389/fgene.2022.880195>
- Neupane, A., Tamburic-Illinicic, L., Brule-Babel, A., & McCartney, C. (2025). Genetic improvement of FHB and DON resistance by combining the Fhb1 gene with additional resistance QTL in winter wheat population. *The Plant Genome*, 18(3). <https://doi.org/10.1002/tpg2.70084>
- Niu, Z., Klindworth, D. L., Yu, G., Friesen, T. L., Chao, S., Jin, Y., Cai, X., & Xu, S. (2013). Development and characterization of wheat lines carrying stem rust resistance gene Sr43 derived from *Thinopyrum ponticum*. *Theoretical and Applied Genetics*, 127, 969–980. <https://doi.org/10.1007/s00122-014-2272-4>
- Odone, A., & Throupe-Kristensen, K. (2025). Modern wheat has deeper roots than ancient wheats: Is this an adaptation to higher productivity? *Annals of Botany*, 136(5), 1193–1201. <https://doi.org/10.1093/aob/mcaf065>
- Ortigosa, A., Gimenez-Ibanez, S., & Solano, R. (2019). Design of a Bacterial Speck Resistant Tomato by CRISPR/Cas9mediated Editing of SIJAZ2. *Plant Biotechnology*, 17, 665–673. <https://doi.org/10.1111/pbi.13006>
- Pancaldi, D., Tonti, S., Prodi, A., & Salomoni, D. (2010). Survey of the main causal agents of Fusarium head blight of durum wheat around Bologna, northern Italy. *Phytopathologia Mediterranea*, 49(2), 258–266. https://doi.org/10.14601/Phytopathol_Mediterr-3442
- Peer, D., Hong, S., Farokhzad, O., & Margalit, L. R. (2007). Nanocarriers as an emerging platform for cancer therapy. *National Nanotechnology*, 2(12), 751–760. <https://doi.org/10.1038/nnano.2007.387>
- Pereyra, S., Dill-Macky, R., & Sims, A. (2004). Survival and inoculum production of *Gibberella zeae* in wheat residue. *Plant Disease*, 88, 724–730. <https://doi.org/10.1094/PDIS.2004.88.7.724>
- Permyakova, N. V., & Deineko, E. V. (2023). Crop Improvement: Comparison of Transgenesis and Gene Editing. *Horticulturae*, 10(57). <https://doi.org/10.3390/horticulturae10010057>
- Petronaitis, T., Simpfendorfer, S., & Hüberli, D. (2021). Importance of Fusarium spp. In Wheat to Food Security: A Global Perspective. In *Plant Diseases and Food Security in the 21st Century* (Vol. 10, pp. 127–159). Springer. https://doi.org/10.1007/978-3-030-57899-2_7

- Powell, A., & Vujanovic, V. (2021). Evolution of Fusarium Head Blight Management in Wheat: Scientific Perspectives on Biological Control Agents and Crop Genotypes Protocooperation. *Applied Sciences*, 11(19), 8960. <https://doi.org/10.3390/app11198960>
- Rani, P. J., Satyanarayana, P., & Chamundeswari, N. (2014). A Review on Marker Assisted Selection in Crop Improvement. *Agricultural and Food Sciences*.
- Reynolds, M., & Braun, H.-J. (2022). *Wheat Improvement- Food Security in a Changing Climate*. Springer Nature. <https://doi.org/10.1007/978-3-030-90673-3>
- Rijkers, G. T., Weterings, N., Obregon-Henao, A., Lepolder, M., Dutt, T., van Overveld, F. J., & Henao-Tamayo, M. (n.d.). Antigen Presentation of mRNA-Based and Virus-Vectored SARS-CoV-2 Vaccines. *Vaccines*, 9. <https://doi.org/10.3390/vaccines9080848>
- Rosyidah, A., Kerdtoob, S., Yudhistrya, W., & Munfadhila, A. (2023). Gold nanoparticle-based drug nanocarriers as a targeted drug delivery system platform for cancer therapeutics: A systematic review. *Springer Nature*, 56, 121–134. <https://doi.org/10.1007/s13404-023-00331-5>
- Savary, S., Willocquet, L., Pethybridge, S. J., Esker, P., McRoberts, N., & Nelson, A. (2019). The global burden of pathogens and pests on major food crops. *Nature Ecology & Evolution*, 3(3), 430–439. <https://doi.org/https%2520://doi.org/10.1038/s41559-018-0793-y>
- Schroeder, H. W., & Christensen, J. J. (1963). Factors affecting resistance of wheat to scab caused by *Gibberella zeae*. *Phytopathology*, 53, 831–838.
- Semenza, J. C., & Menne, B. (2009). Climate change and infectious diseases in Europe. *Lancet Infectious Diseases*, 9(6), 365–375.
- Shan, Q., Wang, Y., Li, J., & Gao, C. (2014). Genome editing in rice and wheat using the CRISPR/Cas system. *Nature Protocols*, 9, 2395–2410. <http://www.nature.com/doi/10.1038/nprot.2014.157>
- Shen, L., Qi, X., & Guo, Z. (2025). Wheat Primer. *Cell Press, Current Biology*, 35, R1029–R1045.
- Silva, S., Almeida, A., & Vale, N. (2019). Combination of Cell-Penetrating Peptides with Nanoparticles for Therapeutic Application: A Review. *Biomolecules*, 9(1). <https://doi.org/10.3390/biom9010022>
- Sinclair, T., Purcell, L., & Sneller, C. (2004). *Crop transformation and the challenge to increase yield potential*. 9(2).
- Singh, R., & Lillard, J. W. (2008). Nanoparticle-based targeted drug delivery. *Experimental and Molecular Pathology*, 86(3), 215–223. <https://doi.org/10.1016/j.yexmp.2008.12.004>
- Sobrova, P., Vojtech, A., Vasatkova, A., & Beklova, M. (2010). Deoxynivalenol and its toxicity. *Interdisciplinary Toxicology*, 3(3), 94–99. <https://doi.org/10.2478/v10102-010-0019-x>
- Sukthankara, P., Avila, A., Whitaker, S., Iwamoto, T., Morganstern, A., & Apostolidis, C. (2014). Branched amphiphilic peptide capsules: Cellular uptake and retention of encapsulated solutes. *BBA Biomembranes*, 1838(9), 2296–2305. <https://doi.org/doi:10.1016/j.bbamem.2014.02.005>
- Tillman, M., Tiedemann, A., & Winter, M. (2017). Crop rotation effects on incidence and diversity of Fusarium species colonizing stem bases and grains of winter wheat. *Plant Disease Protection*, 124, 121–130. <https://doi.org/10.1007/s41348-016-0064-6>
- Todorović, I., Jovičić-Petrović, J., & Moënné-Loccoz, Y. (2023). Microbial diversity in soils suppressive to Fusarium diseases. *Frontier Plant Science*, 14. <https://doi.org/10.3389/fpls.2023.1228749>

- USDA. (2025). *Safe Minimum Internal Temperature Chart*. Food Safety and Inspection Service. <https://www.fsis.usda.gov/food-safety/safe-food-handling-and-preparation/food-safety-basics/safe-temperature-chart>
- Van Esse, P., Reuber, T. L., & van der Does, and Dieuwerthje. (2020). Genetic modification to improve disease resistance in crops. *New Phytologist*, 225, 70–86. <https://doi.org/10.1111/nph.15967>
- Venske, E., Schreinert dos Santos, R., & Busanello, C. (2019). *Bread wheat: A role model for plant domestication and breeding*. <https://doi.org/10.1186/s41065-019-0093-9>
- Vogel, A. B., Kanevsky, I., Che, Y., Swanson, K., Muik, A., Vormehr, M., Kranz, L., & Walzer, K. (2021). BNT162b vaccines protect rhesus macaques from SARS-CoV-2. *Nature*, 592, 283–289. <https://doi.org/10.1038/s41586-021-03275-y>
- Wang, H., Sun, S., Ge, W., & Zhao, L. (2020). Horizontal gene transfer of Fhb7 from fungus underlies Fusarium head blight resistance in wheat. *Plant Science*, 368(6493). <https://doi.org/10.1126/science.aba5435>
- Wang, Y., Cheng, X., & Zhang, Y. (2014). Simultaneous Editing of Three Homoeoalleles in Hexaploid Bread Wheat Confers Heritable Resistance to Powdery Mildew. *Nat. Biotechnol.*, 32(9), 947–951.
- Warburton, M., Skovmand, B., & Mujeeb-Kazi, A. (2002). The molecular genetic characterization of the “Bobwhite” bread wheat family using AFLPs and the effect of the T1BL.1RS translocation. *Theoretical and Applied Genetics*, 104, 868–873.
- Wessel, E., Tomich, J., & Todd, R. (2019). Biodegradable Drug-Delivery Peptide Nanocapsules. *American Chemical Society Omega*, 4(22). <https://doi.org/10.1021/acsomega.9b03245>
- Wu, F., Li, N., Xiao, Y., Palanki, R., Yamagata, H., Mitchell, M., & Han, X. (2025). Lipid Nanoparticles for Delivery of CRISPR Gene Editing Components. *Small Methods*, 10(2). <https://doi.org/10.1002/smt.202401632>
- Xiao, Y., & Wu, K. (2019). Recent progress on the interaction between insects and *Bacillus thuringiensis* crops. *Philosophical Transactions of the Royal Society*, 374. <https://doi.org/10.1098/rstb.2018.0316>
- Xu, X., Liu, C., Wang, Y., Koivisto, J., Zhou, J., Shu, Y., & Zhang, H. (2021). Nanotechnology-based delivery of CRISPR/Cas9 for cancer treatment. *Advanced Drug Delivery Review*, 176. <https://doi.org/10.1016/j.addr.2021.113891>
- Yadava, P., Abhisek, A., & Singh, R. (2017). Advances in Maize Transformation Technologies and Development of Transgenic Maize. *Front. Plant Sci*, 7.
- Yang, L., Machin, F., Wang, S., & Saplaura, E. (2023). Heritable transgene-free genome editing in plants by grafting of wild-type shoots to transgenic donor rootstocks. *Nature Biotechnology*, 41, 958–967. <https://doi.org/10.1038/s41587-022-01585-8>
- Yin, Y., Qin, K., & Song, X. (2018). Transcription Factor Regulates Heat Stress Tolerance through FERONIA Receptor-like Kinase-Mediated Reactive Oxygen Species Signaling in Tomato. *Plant Cell Physiology*, 59(11).
- Yu, G., Matny, O., Gourdoupis, S., & Rayapuram, N. (2022). The wheat stem rust resistance gene *Sr43* encodes an unusual protein kinase. 55, 921–926. <https://doi.org/10.1038/s41588-023-01402-1>
- Zhang, A. M., Yang, W. L., Li, X., & Sun, J. Z. (2018). Current status and perspective on research against Fusarium head blight in wheat. *Hereditas*, 858–873. <https://doi.org/10.16288/j.ycz.18-252>
- Zhang, F., Zhang, H., & Liu, J. (2025). Fhb9, a major QTL for Fusarium head blight resistance improvement in wheat. *Journal of Integrative Agriculture*, 24(11), 4127–4137. <https://doi.org/10.1016/j.jia.2024.03.045>

- Zhang, J., Yu, H., & Li, J. (2023). De novo domestication: Retrace the history of agriculture to design future crops. *ScienceDirect*, 81. <https://doi.org/10.1016/j.copbio.2023.102946>
- Zhenqi, S., Bernardo, A., & Tian, B. (2019). A deletion mutation in TaHRC confers Fhb1 resistance to Fusarium head blight in wheat. *Nature Genetics*, 51, 1099–1105. <https://doi.org/10.1038/s41588-019-0425-8>
- Zhong, Y., Li, C., & Zhang, R. (2018). Precise Base Editing in Rice, Wheat and Maize with a Cas9-Cytidine Deaminase Fusion. *Nat. Biotechnol.*, 35, 438–440.
- Zhu, Z., Xu, D. A., Cheng, S. H., & Gao, C. B. (2018). Characterization of Fusarium head blight resistance gene Fhb1 and its putative ancestor in Chinese wheat germplasm. *Institute of Crop Sciences*, 44, 473–482.

Supplemental Tables

Supplemental Table 1 Primer Sequences for Sanger Sequencing

Primer Name	Nucleotide Sequence
Fhb7 SectA 1 F	CATCTGTCCCGGTTTCGTAAG
Fhb7 SectA 2 F	ATTGTTAGTCCCGGGTCC
Fhb7 SectA 3 F	GGACAAATGGCCCTTTTCTACTAG
Fhb7 SectB 1 F	TCAACTCATAATCACAAGCCCAG
Fhb7 SectB 2 F	GAAGCGACTCTCCTGAATTCC
Fhb7 SectB 3 F	CACACCTCTACTCATGTCACACTA
Fhb7 SectB 4 F	CATCACTCCCAACTAGCCATG
Fhb7 SectC 1 F	GGCCCAAGTGTGTAAAAATCCA
Fhb7 SectC 2 F	CATACACATCACGTCCGCAT
Fhb7 SectC 3 F	ACTCCGTCCCTCATGCTTTT
Fhb7 SectC 4 F	GAAAGGAAAACAGCAGGTAC
Fhb7 SectD 1 F	ACAACCGCATGGAGGAAG
Fhb7 SectD 2 F	GGGCGGTCTTATGTGCAA
Fhb7 SectD 3 F	ACCTGTGTTCTGATCCTT
Fhb7 SectD 4 F	CAAAAATCACGCCTGGGTC
Fhb7 SectD 5 F	CTTACTGTCATGTCTTCCCTC
Fhb7 SectD 6 F	GGACATGGAAGGAAAAATGTTG
Fhb7 SectD 7 F	GTTCCAAGGACACCTGTG
Fhb7 SectD 8 F	GTGGGTGCGTTGTAACATG
Fhb7 SectD 9 F	GAGCTTTTATCCTTCCATTTGTACG
Fhb7 SectE 1 F	CCTTTTCTCTTCATTCATCATCCTGC
Fhb7 SectE 2 F	CCCAATCATCTTCTACGACATAGC
Fhb7 SectE 3 F	CCGTCGCAGAAACATGCT
Fhb7 SectE 4 F	ACTGGCCCTCAACTTCAAG
Fhb7 SectE 5 F	CAGTCAACCCTTTTCTCTTCATTC
Fhb7 SectF 1 F	GACTACGCCCCGCTTCAAC
Fhb7 SectF 2 F	CGTTGACGCAGCCTTTAC
Fhb7 SectF 3 F	CTCATGGTCCACGGAATTC
Fhb7 SectG 1 F	TTGGGCAGCTGCATGATG
Fhb7 SectG 2 F	TTCACTTCGTGGGGTTGG
Fhb7 SectG 3 F	GGTTTTCCGTTATTCAGTTGTAACCC
Fhb7 SectG 4 F	CACTGTATCTTGCGAGAAACTGAG
Fhb7 SectG 5 F	GTTGCCGGTGAGTGAGTG
Fhb7 SectG 6 F	CTGGGCCAGATGAATTTAAC
Fhb7 SectG 7 F	GTGCTCGCTCTTGATCCA
Fhb7 SectG 8 F	CCCAAGGAATAAATAGAGAATCAG
Fhb7 SectG 9 F	CATTGTGTCTCTTTTCTTCACTTCATC
Fhb7 SectG 10 F	CTCGAAGCCCAAGACCCA

Supplemental Table 2 Dunnett's Test Results for all Lines
Compared to parental line's FHB susceptibility.

EMS Line	Mean Difference	Standard Error	t-statistic	p-value	Cohen's d	Effect Size
Sr43-m3-1004	3.1638	6.1914	0.511	0.6095	0.176	negligible
Sr43-m3-1010	37.643	5.8544	6.4298	0	2.098	large
Sr43-m3-1011	4.6815	5.6065	0.835	0.4039	0.261	small
Sr43-m3-1027	9.8957	5.6816	1.7417	0.0819	0.551	medium
Sr43-m3-1032	8.4722	13.279	0.638	0.5236	0.472	small
Sr43-m3-1052	35.0655	5.6065	6.2544	0	1.954	large
Sr43-m3-1054	11.7791	11.0755	1.0635	0.2878	0.656	medium
Sr43-m3-1066	33.9808	5.1801	6.5599	0	1.894	large
Sr43-m3-1068	15.4097	6.8944	2.2351	0.0256	0.859	large
Sr43-m3-1075	23.8709	5.9547	4.0088	0.0001	1.33	large
Sr43-m3-1113	11.9805	7.8315	1.5298	0.1264	0.668	medium
Sr43-m3-1117	21.5303	7.4554	2.8879	0.004	1.2	large
Sr43-m3-1119	10.6134	8.3066	1.2777	0.2017	0.591	medium
Sr43-m3-1129	-3.3179	7.1492	-0.4641	0.6427	-0.185	negligible
Sr43-m3-1136	18.8566	5.3116	3.5501	0.0004	1.051	large
Sr43-m3-1149	37.381	7.8315	4.7731	0	2.083	large
Sr43-m3-12	39.2708	7.4554	5.2674	0	2.188	large
Sr43-m3-127	12.536	5.221	2.4011	0.0165	0.699	medium
Sr43-m3-153	14.786	7.8315	1.888	0.0593	0.824	large
Sr43-m3-164	-0.754	6.1914	-0.1218	0.9031	-0.042	negligible
Sr43-m3-165	2.2222	11.0755	0.2006	0.841	0.124	negligible
Sr43-m3-182	13.6374	6.0663	2.2481	0.0248	0.76	medium
Sr43-m3-196	6.8254	5.221	1.3073	0.1914	0.38	small
Sr43-m3-235	-2.4931	5.7639	-0.4325	0.6655	-0.139	negligible
Sr43-m3-238	3.9066	5.6816	0.6876	0.4919	0.218	small
Sr43-m3-248	22.3016	7.8315	2.8477	0.0045	1.243	large
Sr43-m3-333	33.5732	7.4554	4.5032	0	1.871	large
Sr43-m3-345	15.0303	6.0663	2.4777	0.0134	0.838	large
Sr43-m3-358	-0.2093	5.1055	-0.041	0.9673	-0.012	negligible
Sr43-m3-387	26.6853	5.6816	4.6968	0	1.487	large
Sr43-m3-401	7.8436	5.221	1.5023	0.1334	0.437	small
Sr43-m3-4	-4.0996	5.5377	-0.7403	0.4593	-0.228	small
Sr43-m3-465	12.0328	9.7894	1.2292	0.2193	0.671	medium
Sr43-m3-467	25.4892	5.6816	4.4863	0	1.42	large
Sr43-m3-489	13.5759	5.6816	2.3894	0.0171	0.757	medium
Sr43-m3-495	7.2033	7.4554	0.9662	0.3342	0.401	small
Sr43-m3-507	3.7407	5.416	0.6907	0.4899	0.208	small
Sr43-m3-527	6.5506	5.3116	1.2333	0.2178	0.365	small

Sr43-m3-551	3.5286	5.9547	0.5926	0.5536	0.197	negligible
Sr43-m3-560	2.7101	6.6788	0.4058	0.685	0.151	negligible
Sr43-m3-59	6.1421	5.1801	1.1857	0.236	0.342	small
Sr43-m3-598	6.6692	6.8944	0.9673	0.3336	0.372	small
Sr43-m3-600	3.7478	5.416	0.692	0.4891	0.209	small
Sr43-m3-617	11.2458	5.3619	2.0974	0.0362	0.627	medium
Sr43-m3-620	0.8412	6.4936	0.1295	0.897	0.047	negligible
Sr43-m3-624	13.8061	5.416	2.5491	0.011	0.769	medium
Sr43-m3-662	20.9093	5.6065	3.7294	0.0002	1.165	large
Sr43-m3-674	22.3611	13.279	1.6839	0.0925	1.246	large
Sr43-m3-677	50.4823	6.0663	8.3218	0	2.813	large
Sr43-m3-683	9.0683	5.3619	1.6912	0.0911	0.505	medium
Sr43-m3-700	13.6138	5.6816	2.3961	0.0168	0.759	medium
Sr43-m3-710	7.4842	5.6065	1.3349	0.1822	0.417	small
Sr43-m3-718	34.854	6.1914	5.6294	0	1.942	large
Sr43-m3-726	28.4713	6.6788	4.263	0	1.587	large
Sr43-m3-74	8.6521	5.9547	1.453	0.1466	0.482	small
Sr43-m3-796	25.4448	5.4744	4.6479	0	1.418	large
Sr43-m3-923	28.2381	5.6065	5.0366	0	1.574	large
Sr43-m3-939	4.8982	5.8544	0.8367	0.403	0.273	small
Sr43-m3-954	9.3226	5.8544	1.5924	0.1116	0.52	medium
Sr43-m3-983	26.1508	7.8315	3.3392	0.0009	1.457	large
Sr43-m3-993	9.5594	5.6816	1.6825	0.0928	0.533	medium