

Using a BSMV mediated genome editing system to validate the function of *Fhb1*  
candidate genes in Fusarium head blight resistance

by

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## Abstract

*Fusarium* head blight (FHB) significantly reduces wheat grain yield and quality. It also produces mycotoxins within infected plant tissues that are harmful to human and animal. Using resistance genes in wheat cultivar is a most promising approach to prevent FHB epidemics. To date, more than 500 QTLs have been found to be associated with FHB resistance. Among them, *Fhb1* shows the most stable major effect on FHB resistance in wheat and has been used as a major source of resistance in breeding programs worldwide. Recently *Fhb1* has been cloned as a histidine rich calcium binding protein (*TaHRC*), but its resistance mechanisms remain to be determined. CRISPR/Cas9 genome editing has been widely used for gene function determination. However, delivery of gene editing complex is a challenging step for hexaploid wheat due to low transformation efficiency for most wheat genotypes. In this study, we attempt to use our newly developed genome editing protocol for wheat to create *Fhb1* mutations and evaluate their functions on wheat FHB resistance. We used previously developed Cas9-overexpressed Bobwhite that was generated by transformation as the Cas9 donor. We crossed the Cas9-overexpressed Bobwhite plants to Ning 7840 and selected their F<sub>2</sub> plants with both Cas9 and the target genes for gene editing. Barley stripe mosaic virus (BSMV) was used to deliver guide RNA (gRNA) to selected Cas9-carrying wheat plants. Treated plants were screened for mutations by sequencing target fragments in an ABI 3730 sequencer. Plants with mutations were evaluated for FHB responses in greenhouse conditions. We identified three mutants with two carrying frame shift mutations (mut\_36, mut\_99) and one carrying a nucleotide substitution (mut\_123) in *TaHRC*. Sequence alignment with reference genome indicated that two mutants (mut\_36 and mut\_99) carried *TaHRC* mutations in A subgenome, and one (mut\_123) had a *TaHRC* mutation in D subgenome. Phenotyping the mutants demonstrated no change in FHB susceptibility compared non-edited control plants. Our results indicated that BMSV mediated gene editing system does not require tissue culture step and can be used for delivery gRNA to hexaploid wheat. *TaHRC* homologs in A and D genomes did not affect FHB resistance.

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# Chapter 1 - Literature Review

## Wheat widespread through the world

Wheat is a staple food crop that contributes a main source of dietary carbohydrates and proteins for daily diet of world population (Balfourier et al., 2019). Consumption of wheat reached 66 kg/per capita in 2016 according to FAO (Cheli et al., 2017), accounting for about fifteen percent of calories consumed by human (Hyles et al., 2020). Many food products such as bread, pasta, noodle etc. are made of wheat grains. Additionally, this crop is also used as feed for cattle and swine (Shewry, 2009). Wheat grew in about 38% of land worldwide in 2018 and produced 700 million tons of wheat yearly ( Abhinandan et al., 2018; Ma et al., 2020) with most wheat produced in China, India, Ukraine, European union countries, the US, Canada and Australia (Hyles et al., 2020). About 131 million tons of wheat are produced in China and 99.7 in India, 72.1 million tons in Russia in 2018 ([https://en.wikipedia.org/wiki/International\\_wheat\\_production\\_statistics](https://en.wikipedia.org/wiki/International_wheat_production_statistics)).

Wheat is a monocot species that belongs to *poacea* family. Wheat was domesticated much earlier than other cereal crops (Abhinandan et al., 2018) with the first harvest on southeastern territories of Turkey (Balfourier et al. 2019). Wheat cultivation started spreading across Europe through Balkans peninsula at approximately 8,500 years ago and then reached Mediterranean, Scandinavia, England and North Africa (Balfourier et al., 2019). At the same time wheat was introduced to new areas in Iran, India, China, and other Asian countries. Nevertheless, majority of modern cultivars from Asia contain Italian wheat in their pedigrees (Balfourier et al., 2019). Wheat was first brought to the US territories by the Spanish colonists in 15<sup>th</sup> century ([https://en.wikipedia.org/wiki/Wheat\\_production\\_in\\_the\\_United\\_States](https://en.wikipedia.org/wiki/Wheat_production_in_the_United_States)). New wheat varieties were introduced from North Africa, USSR, Japan and Korea to the U.S. in the middle of 20<sup>th</sup> century in order to improve wheat productivity and resistance to biotic and abiotic stresses in the U.S. (Cox, 1991).

Tetraploid and diploid wheats were first selected for cultivation by farmers ~9,000 years ago (Shewry, 2009). During further domestication process, these crops were occasionally crossed with genetically distant plants, which resulted in new hexaploid wheat with improved productivity (Shewry, 2009). Today, majority of modern wheat varieties in production are hexaploids. Nevertheless, a small portion of tetraploid wheat (*Triticum durum*) is still grown to

maintain diversity of wheat end-use products (Shewry, 2009). Wheat spreads across the world and survives in a wide range of environments demonstrating its high adaptation to these environments. However, climate change incites more wheat diseases and abiotic stresses, which provides big challenges for sustainable wheat production. Germplasm from distant species may serve as valuable sources for improvement of wheat (Shewry, 2009), and modern biotechnologies may provide novel tools to improvement of wheat productivity under these stresses.

### **Wheat *Fusarium* head blight**

*Fusarium* head blight (FHB) is one of the most devastated diseases in wheat that has been recorded throughout the history of human. FHB occurrence was first described in England in 1884 (Z. Ma et al., 2020). The first incidence of FHB infection in the US was recorded in 1890s (Arthur et al., 1891). In the U.S. severe FHB outbreaks occurred in 1919, 1928, 1935 and 1980 and the instances were recorded in Kansas, Nebraska (Menistu et al 2007 abstract), Missouri, Illinois (Nganje et al., 2004), Iowa and New York (McMullen et al., 2012) and many others states. In general, FHB has been recorded in 31 out of 40 states (McMullen et al., 2012). Massive spread of FHB was recorded in Japan and China in 1914 and 1937, respectively (Ma et al., 2020). In China the infection colonized 95 percent of Jinda 2950 wheat which was the most commonly grown wheat variety in 1930's. Thirty new FHB outbreaks has been reported in China since 1950 (Z. Ma et al., 2020). In South America, FHB was first documented in Brazil in 1939 (Z. Ma et al., 2020), followed by 1945, 1972, 1977 epidemics in Argentina, Paraguay and Uruguay, respectively. Dramatic infection of wheat was observed in Poland in 2016 and 2017 (Bilska et al., 2018). Nowadays the FHB outbreaks can be observed annually throughout the world (Z. Ma et al., 2020).

*Fusarium* head blight is a disease that primarily occurs in wheat spikes. The disease is mainly caused by a fungi *Fusarium graminearum*. However other *Fusarium* species may also trigger the disease (Buerstmayr et al., 2009). *F. graminearum* can be characterized as a saprotroph that is pathogenic to many plant species and infects various wheat organs such as roots, stems, grains and seedlings to cause local necrosis in infected tissues. Infection of *F. graminearum* in wheat grain cause damage in starch and protein (Askun, 2018) and make them shrunken and losses their natural color. FHB can reduce crop yield up to 80 percent in some fields (McMullen et al., 2012). Depending on environmental conditions, the first symptoms such

as water soaking spots could be observed on the glume of the inoculated spikelet as soon as two days after infection. Typical FHB symptoms is blight spikelets, but pink mold can also appear in glumes with sporodochia production in field (Osborne & Stein, 2007).

### **Mycotoxins associated with FHB**

Mycotoxins are diverse group of substances that is synthesized by *F. graminearum*. The molecular structure of mycotoxins may vary from molecules with a single heterocyclic ring to molecules with various heterocyclic rings. The range of molecular weight starts with 50 Da and can reach up to 1000 Da (Bezerra et al., 2014). *F. graminearum* is more prone to produce mycotoxins from type B trichothecenes' class. These substances include: 4-acetylnivalenol (4ANIV), nivalenol (NIV), 3-acetyl and 15-acetyl deoxynivalenol (3ADON and 15ADON) and deoxynivalenol (DON) (Bezerra et al., 2014). Trichothecenes are normally produced at later stages of fungi development (Bezerra et al., 2014). *TRI5* is one of the crucial genes for trichothecene production. It has 1186 base pairs in length. Proctor et al. (1995) demonstrated that mutation of *TRI5* reduced virulence of *Fusarium* species. The enzyme encoded by *TRI5* gene is crucial to trichodiene synthase and is an important regulator of mycotoxins production (Liu et al., 2015).

Recent study demonstrates that *F. graminearum* from different regions may vary in their virulence. This virulence variation might be attributed to levels of mycotoxins produced by the *F. graminearum* from these regions because *Fusarium* isolates from different locations showed variation in the ability to synthesize mycotoxins. What is more, species from different geographic locations may produce different mycotoxins (Liu et al., 2017). For example, 3ADON producing *F. graminearum* is mainly in Canada, NIV producing *F. graminearum* mainly in southern United States, and 3ADON producing *Fusarium* mainly in China. It was noticed that *Fusarium* species from Asia are mainly nivalenol producers, while deoxynivalenol producing *Fusarium* is more common in North America and Europe (Zhu et al., 2020). Lee et al., (2015) indicated that geographic distribution of a particular *Fusarium* mating types can be traced by their mycotoxin profiles (chemotypes).

Mycotoxins are also health concern for animals who consumed toxin-contaminated products. Mycotoxins from the trichothecenes group proved to have protein synthesis inhibition properties and consumption of the mycotoxins may cause vomiting, nausea, diarrhea, and weight losses (Bezerra et al., 2014). Consistent exposure to the mycotoxins through food resources may

cause serious chronic effects. In long run, mycotoxins can trigger nephrotoxic, carcinogenic, immunosuppressive, mutagenic effect on animal health (Bezerra et al., 2014). DON consumption may cause IgA nephropathy, and the mycotoxins are also among the most common causes of liver cancer (Osborne et al., 2007).

The accidents of mycotoxin poisoning are documented. For example, consumption of DON contaminated cereal grain has been reported in China and India in 1987 (Bhat et al., 1989), and mycotoxin poisoning incidents were reported for wheat in Kenya in the years 1981, 2001, 2004, and 2005 (Song et al., 2005), and for corn in 1988 in midwest states in the U.S. (Russell et al., 2018). In these cases patients who consumed contaminated products suffered severe disease. Recently, Oliver et al., (2007) has shown that massive consumption of mycotoxins contaminated products resulted in increased number of ergotism and aleukia in 20<sup>th</sup> century in Europe. To prevent health risks linked to consumption of mycotoxin contaminated food products, several countries have established regulations on maximum allowed toxin content in grains and their products. Today the mycotoxins content in food products are much lower in developed countries than in developing countries where governmental regulation on toxin limits is not available. These rules facilitate control over the amount mycotoxins in food and specify the up limit of mycotoxins in food sources used for human consumption (H. Buerstmayr et al., 2009). For example, the maximum concentration of mycotoxins in food sources in European Union is 1.25 ppm, 1 ppm in the USA, 2 ppm in Canada (H. Buerstmayr et al., 2009). These rules help to prevent mycotoxin poisoning of humans. However, they may significantly limit the amount of grains that can be used for food production (Bilska et al., 2018). In addition, diversified diets allows reduced exposure to mycotoxins and decreases concentration of the toxic substances in daily diets, but developing countries tend to have less diverse diets than developed countries (Shephard, 2009)

### **Factors impacting FHB spread**

*F. graminearum* can reproduce sexually as well as asexually. Pattern of spores spread depends on the life cycle of the fungi. During sexual reproduction binucleate cells are generated. Ascospores are usually produced in plant's stomates and silica cells by sexual reproduction. Spores produced in these cells spread with air (Shephard, 2009). Ascospores have a small size of  $21\text{ }\mu\text{m} \times 3.5\text{ }\mu\text{m}$  and can travel long distance from the originally infected plants to destination plants. With the help of wind and water, spores can spread to distant territories to infect more

host plants (Paillard et al., 2004). High rate of lipids synthesis can be observed in the pathogen. These substances are crucial for prolong survival of spores during winter and for further sexual reproduction (Trail, 2009). Asexually generated conidiospores can survive long winter by colonizing on residues of dead plants of maize and cereals (Osborne & Stein, 2007). Conidiospores have high virulence on plants (Dweba et al., 2017) (McMullen et al., 2012). The FHB infection usually occur during flowering stage in wheat. Infected spikes can become completely blighted in a susceptible cultivar (McMullen et al., 2012). Invasion of the pathogen occurs mainly through florets, then spread to nearby spikelet through rachis nodes. The disease development takes place in apoplast where the pathogen gets nutrients for the growth and development (Vaughan et al., 2016). *F. graminearum* initially grows in the intercellular space without damaging cells of wheat and initiates the production of mycotoxins at this stage. Once mycotoxin content reaches to a certain level, a programmed cell death event will be initiated in the plant (Brown et al., 2010). Mycotoxins and lipase are crucial for fungal development as well as FHB symptoms manifestation. These signaling molecules may regulate cell wall degradation in plants (Sella et al., 2014). Fungal colonization in the plant tissues may result in damage in lignin layer of cell walls. *F. graminearum* starts consuming substances from damaged cells by mycotoxins or by the pathogen. Then *F. graminearum* starts to grow laterally (Brown et al., 2010). Many factors have been reported to impact virulence of *F. graminearum* including Gpmk1 and MAP kinases (Dweba et al., 2017).

Several agricultural practices facilitate FHB epidemics including minimum or no tillage, crop rotation and irrigation etc. In minimum- or no-tillage system, the plant residues remain on soil surface, which can serve as a good substrate for ascospore development. Spores can be produced more than 23 months after the first appearance on the residues (Osborne & Stein, 2007). The residues on the soil surface are decomposed much slower than other materials buried in deep layers of soils, thus are ideal media for spore development (Osborne & Stein, 2007). Warm weather and high moisture after anthesis are crucial for FHB development. These conditions are not only critical for development of ascospores (Trail, 2009), also facilitate initial infection and spread of the *F. graminearum* in a spike after initial infection. Therefore, climate changes may dramatically increase global FHB epidemics. High temperature before anthesis in some regions will facilitate dramatic increase of ascospore production. High temperature and high moisture may also increase mycotoxin accumulation (Vaughan et al., 2016), because high

temperature impacts expression of the genes (*TRI4*, *TRI5*, *TRI6*, *TRI10*, *TRI12*, *TRI13*) in the DON biosynthesis pathway (Parra et al., 2011).

Temperature setting also impacts expression of resistance genes. To illustrate this, Sumai 3 is one of the most resistant wheat cultivars identified to date. However, some studies demonstrated its susceptibility in certain environments. This may be attributed to some extreme growth conditions of Sumai 3 (Niwa et al., 2014).

FHB has dramatic economic effect on the US agriculture. FHB outbreak in the US has resulted in \$1.3 billion losses from 1991 to 1997 (Bai & Shaner, 2004), losses caused by FHB reached 21 million tons in the period between 1993 to 2014 (Z. Ma et al., 2020). In particular losses due to the FHB reached 57 million dollars in 2008 in Kansas (Bai et al., 2018). *F. graminearum* has been considered as one of the most threatening fungal pathogens in the world (Dean et al., 2012).

Climate change has resulted in FHB spread to more wheat growing areas where FHB has never occurred before. Wide spread of FHB leads to more yield, economic losses and may pose additional health risk on organisms that consumes wheat contaminated with mycotoxins produced by *F. graminearum*. That is why finding strategies for wheat protection as well as wheat improvement toward the FHB resistance are crucial tasks.

### **Plant resistance mechanisms**

Multiple projects with a goal to screen modern cultivars of wheat and close sub relatives toward resistance to FHB were initiated worldwide. Organization such as Wheat and Barley Scab Initiative helped to facilitate projects linked to resistance gene search. With help of these initiatives 70,000 and 10,500 varieties of wheat and closely related species were evaluated for resistance toward FHB by 2019. Majority of screened wheat germplasm lines were FHB susceptible, only small portion of evaluated lines showed various levels of FHB resistance. Sumai 3 and Wangshuibai were amongst the most resistant germplasms, which have been used as sources of FHB resistance genes in various breeding programs. In general, the most resistant lines were found from the locations where FHB outbreaks have been severe and frequent. Among wheat species, diploids and tetraploids showed lower resistance than hexaploidy wheat. An FHB immune variety of wheat was not found (Z. Ma et al., 2020). Resistance to FHB is controlled by genotype × environment interaction.

Five types of resistance to FHB has been described by Mesterházy,(1995). Type I resistance is described as resistance to initial infection (Liu et al., 2008). In plants with type II resistance *F. graminearum* cannot spread through infected heads after point inoculation of single floret. Crops with type III are resistant to DON accumulation. Type IV resistance can be characterized as resistance to kernel damage, while, tolerant to FHB infection plants can be classified into type V resistance class (Liu et al., 2008). Pyramiding genes that facilitate different types of resistance increase overall tolerance of wheat to FHB (Liu et al., 2008). Medium to high heritability has been reported for different types of FHB resistance. Type I resistance reached 0.7, type II resistance was scored at 0.73 (Z. Ma et al., 2020). However, FHB resistance is controlled by the multiple genes and each gene only contributes partial resistance (Tao Li et al., 2016).

### **Major QTLs for FHB resistance**

To date, more than 500 QTLs across 21 chromosomes were reported for different types of FHB resistance in wheat. *Fhb1*, *Fhb2*, *Fhb4*, *Fhb5*, *Fhb6*, *Fhb7* are QTLs that have large effect on FHB resistance (Z. Ma et al., 2020). These QTLs facilitate different types of resistance in wheat. Wheat with type II resistance are preferred for breeding because such resistance is easy to phenotype and it is more stable (Dweba et al., 2017) Almost 200 QTLs contribute to type II FHB resistance and are distributed on all 21 chromosomes.

*Fhb1* from Sumai 3 and its derivative Ning7840 is a major QTL that is widely used in breeding programs all around the world is crucial for type II resistance (Tao Li et al., 2016). It significantly increased FHB resistance in elite spring wheat (Eckard et al., 2015). A tightly linked marker *UMN10* were discovered and applied for introducing *Fhb1* to elite cultivars in U.S. breeding program (Liu et al., 2008). *Fhb1* resistance appears to be affected by plant height and shorter wheat varieties tend to suffer significantly more FHB damage than taller ones (M. Buerstmayr et al., 2020). *Fhb1* is located on 3BS chromosome (Cuthbert et al., 2006). *Fhb1* from Sumai 3 has been cloned by (Rawat et al., 2016). The size of the cloned fragment reached 350 kb. Thirteen protein genes were found in the cloned region of Sumai3.

Three independent research groups have been working on cloning *Fhb1* and explaining role of *Fhb1* in type II FHB resistance in wheat. Two candidate genes were proposed as results of these works. These two genes were named Histidine-rich calcium-binding protein (HIC or HRC) and Pore-forming-toxin-like domain (PFT) (Su et al., 2019), (Rawat et al., 2016). The

distance between two genes that were claimed to be candidates for FHB resistance reaches 50kb (Moore et al. 2015). Data regarding candidate genes from *Fhb1* presented by research labs of (Su et al., 2019), (Li et al., 2019), (Rawat et al., 2016) is contradicting. Su et al. (2019) has described a large deletion at the start codon of *TaHRC*. This loss of function mutation is a feature of resistant allele of *TaHRC* and it is a cause of resistance (Su et al., 2019). However, the data presented by Li et al. (2019) demonstrates that gain of function mutation in *TaHRC* is a cause of resistance. The frame shift mutation in the gene creates a new start codon and new protein facilitate resistance to FHB, according to Li et al. (2019). Separate from this, Rawat et al. (2016) has demonstrated that *TaPFT* from *Fhb1* is cause of resistance. However, Su et al. (2019) has shown that *TaPFT* was present in accessions with highly susceptible phenotypes to FHB. Due to this, finding candidate genes from *Fhb1* as well as understanding mechanisms that underline FHB resistance are important tasks.

*TaPFT* is a 3472 bp gene that encodes chimeric lectin in wheat. It was first described in Sumai3 variety. Open reading frame of the gene reaches 1437 bp. The protein that corresponds to gene consists of 478 amino acids and have two agglutinin domains. In addition, ETX/MTX2 domains were identified in the protein. Bioinformatic data suggested that the *TaPFT* may be important for pathogen signaling. However, no experimental evidence was presented yet. ABA triggers increase of *TaPFT* expression (Rawat et al., 2016).

*TaHRC* gene was first studied from Wungshibai variety. This gene has three introns, open reading frame reaches 786 b.p. *TaHRC* protein has a calcium binding domain. It is a nuclear protein with no known function in FHB resistance. HRC translated product consists of 261 amino acids (Su et al., 2019).

Genes from *Fhb1* are valuable sources of resistance for wheat breeding due to its large effect in multiple genetic backgrounds and environments (Paudel et al., 2020). Genome editing can be used to determine candidate genes from *Fhb1* and their functions in the disease resistance. In addition to this, it can be helpful for improving resistance in wheat. With help of the technology knock-out of genes can be produced.

## **Gene editing**

Creating mutations in a particular genomic region is an important task for genomic research and breeding. Conventional methods for creating mutations have significant disadvantages. For instance, chemical methods such as ethyl methane sulfonate (EMS) and

physical radiation treatments usually generate multiple random mutations in a plant, which may also disrupt many vital genes for plant growth and development. Biological approaches for generating mutants such as incorporation of transposons are tedious and require special governmental regulations for field testing since they use gene transformation. In addition, they may be not efficient in some species. Gene editing tools overcome these disadvantages, are faster, cheaper, more efficient and can be transgene free to avoid GMO issue (Chaudhary et al., 2017).

All gene editing techniques require two essential steps: creating double stranded breaks in a particular genome locus and repairing these breaks with introduced changes. Double stranded breaks can be introduced to a specific genomic region with help of meganuclease enzymes in a laboratory. Several enzymes can be used for editing (Gupta & Shukla, 2017). Double stranded breaks can cause significant harm to development and normal functioning of cells; however, organisms have gained mechanisms to deal with these issues during evolution process. Two methods of double stranded break repairing were described: non-homologous end joining (NHEJ) and homologous end joining (HEJ). Broken ends of DNA molecules are joined randomly with a single or multiple nucleotides losses in damaged sites by NHEJ, also a new nucleotide sequence can be introduced to damaged DNA after the repairing during HEJ. These mechanisms are necessary for gene editing (Alberts et al., 2005).

### **Gene editing techniques**

Knock-out or knock-in can be achieved by gene editing. Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), cluster regulatory short palindromic repeats (CRISPR) are major gene editing technologies that are widely used these days (Gupta & Shukla, 2017). These technologies differ by the way that double stranded break is produced during their editing process. Fig. 1.1 outlines how the gene editing approaches work.

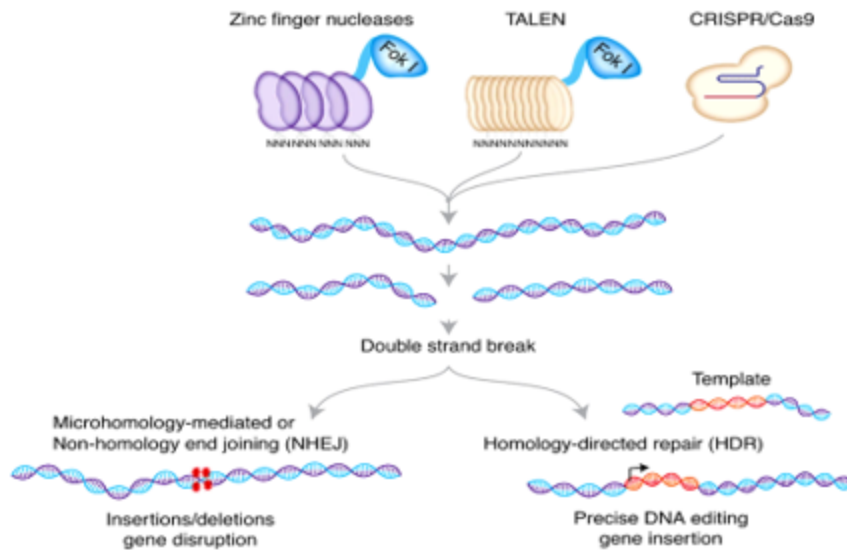


Figure 0.1. Comparison of three major gene editing processes (<https://facellitate.com/genome-editing-methods-explained/>).

Zinc finger nuclease (ZFN) has been effectively used for gene editing in a wide range of organisms such as: human, animals, insects, and plants (Miller et al., 2007). ZFN was first characterized in 1996 and consists of two main protein domains. Zinc finger protein domain is responsible for binding to a targeted DNA sequence, whereas, FokI nuclease has restriction enzyme properties. FokI was derived from *Flavobacterium okeanokoites* and fused to a zinc finger (Y. G. Kim et al., 1996). DNA binding motif can be altered according to various targeted regions of a genome. DNA binding motifs consist of repeated cysteine and histidine repeats. Each repeat connects to 3 nucleotides base pairs in targeted DNA sequence. ZFNs systems can be potentially improved to recognize DNA fragment with size up to 36 bp in length (De Souza, 2012). However, it also has limitations. One is design of nucleases that requires a lot of time and resources. Additionally, some genomic sequence cannot be targeted with ZFN (Gupta & Shukla, 2017). It also has high off-target rates and these unintended gene disruption events significantly disturb well-being of an organism to be edited. Thus, cytotoxic effect may be accompanied with application of ZFNs (Gupta & Shukla, 2017).

Transcription activator-like effector nuclease (TALEN) is another enzyme that can be used for gene editing. This protein was first characterized in *Xanthomonas*. After the bacteria infect plant host, TALEN facilitate activation of expression in a gene cluster (Bogdanove et al.,

2010). Each amino acid in recognition domain of TALEN system binds to a separate nucleotide. That is the main characteristic to distinguish TALEN from ZFN. TALE is a protein domain that takes part in binding targeted DNA region and includes tandem repeats. The length of recognition site varies with the experiment design. However, sequences of 18-24 bp are the most used for recognitions. FokI is fused to TALE domain in order to produce double stranded breaks (Li et al., 2011). Sticky ends are produced by TALEN system. Experimentally used TALEN are mainly from yeasts, plants, animals, and human cells. The TALEN approach has demonstrated different efficiency levels in different gene editing experiments, ranging from 30 - 100% knock-out efficiency and up to 10% knock-in efficiency. One drawback is complexity of TALEN experiment design that requires rearrangement of the protein complex for every nucleotide sequence target (Gupta & Shukla, 2017).

CRISPR Cas9 technology becomes the most popular amongst all gene editing protocols. Unlike other gene editing protocols, CRISPR does not require re-arrangement of protein parts in gene complex for different targets in a genome (Komor et al., 2016). It is an efficient, fast, straightforward technique and used in various genetic engineering projects and can be used edit different eukaryotic and prokaryotic genomes (Zhang et al., 2020). Like most of the genetic engineering techniques, CRISPR system was first discovered in bacteria in *E. coli* in 1987. CRISPR serves as an immune response system in the bacteria and helps them to survive during bacteriophage invasions (Wiedenheft et al., 2012).

### **CRISPR system for gene editing**

CRISPR system plays an important role in defense of bacteria against viruses. CRISPR responds to viral attack through sequence specific RNA binding to the genomes of invading viruses to degrade viral nucleic acids with the help of the specific enzymes that recognize the location where binding occurs. The CRISPR system use diverse mechanisms to defend against viral attacks. The regions of genomes that correspond to CRISPR system can be characterized by significant sequence diversity. Furthermore, some genes from CRISPR loci can serve as a distinctive feature of a particular bacteria (Koonin et al., 2017). There are three major types of CRISPR systems (Makarova et al., 2015)

Type II is the best studied and most used system for genome editing due to the presence of Cas9 protein (Makarova et al., 2015). General outline of mechanism that underline CRISPR. Cas9 is unique bacterial protein that facilitate cut of double stranded DNA after targeted DNA is

found. (Parker et al., 2009). Cas9 have two DNA binding motifs: HNH that cleaves to the complementary to gRNA strand and RuvC that binds non-complementary strand (Jinek et al., 2012). Mutation in the region that corresponds to these domains leads to substantial decrease in enzyme activity (Ribeiro et al., 2018). Protospacer (PAM) with NGG sequence (where N stands for any nucleotide) is important recognition sites in viruses' genomes. They serve as a mark which can be recognized and deactivated by Cas9 protein. PAM sequence is essential for CRISPR system assembly. Arginine residues in 1333 and 1335 positions of Cas9 play a crucial role in recognition of PAM. After binding of these parts occurs protein changes its conformation. After Cas9 recognizes this short sequence gRNA can bind to complementary nucleotide sequence. After these Cas9 can start producing double stranded breaks (Alok et al., 2021).

Another crucial component of CRISPR system is gRNA. Multiple repetitive sequences that are separated by spacers were found in CRISPR loci of bacteria. Repeated sequences on both sides of spacers play a role in distinguishing bacterial DNA from nucleic acids of invading organisms (Wiedenheft et al., 2012). The length of spacer sequences varies from 20 to 50 bp. Spacers are important for recognition of loci where double stranded breaks need to be introduced. Products transcribed by the spacers (crRNA) bind to complementary targeted nucleic acid of viruses and facilitate silencing and degrading the viral nucleic acids. crRNA must bond to tracrRNA before attaching to form a complex or called gRNA and gRNA will attach to a special protein to direct the protein to targeted location.

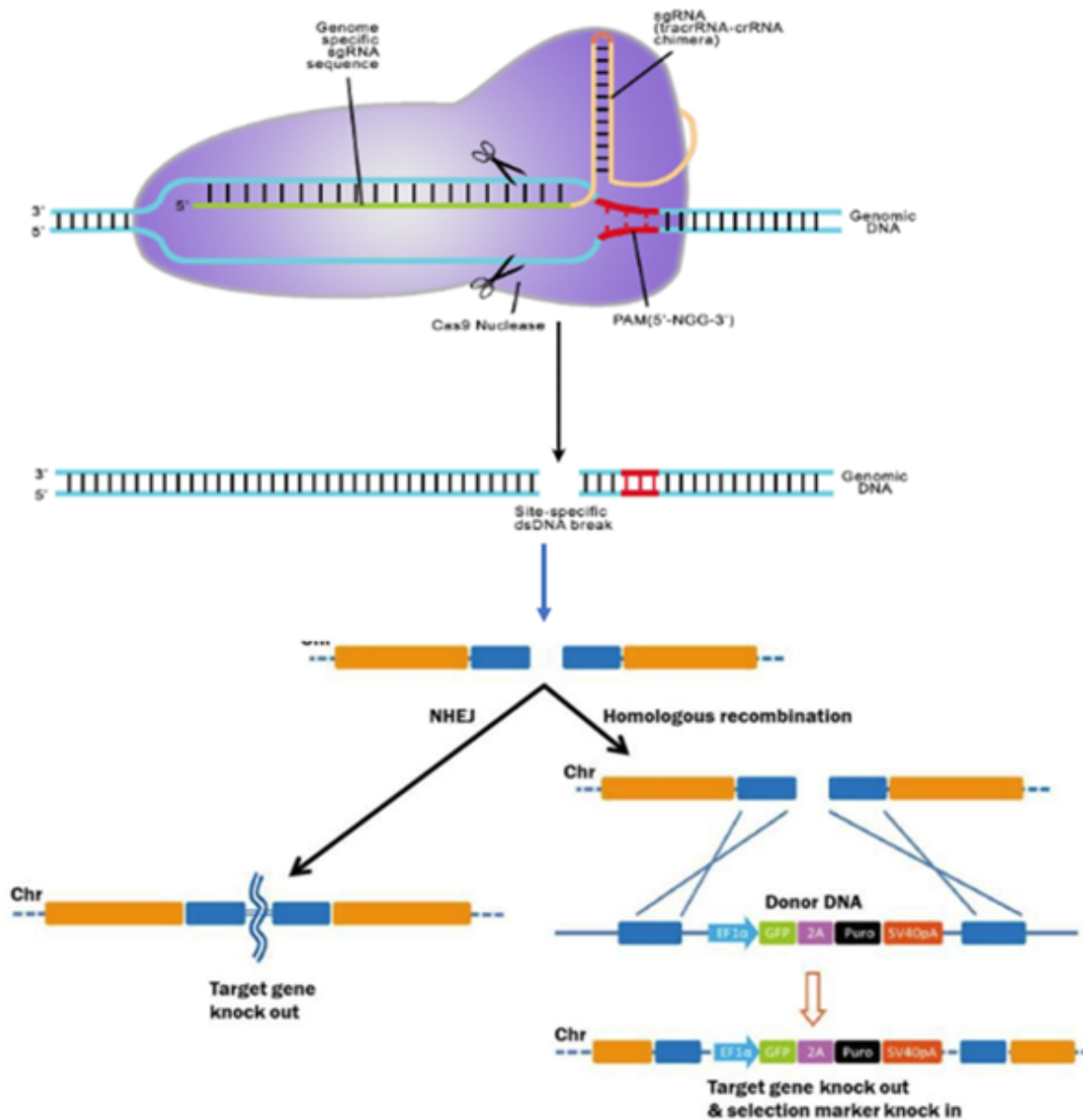


Figure 0.2. Outline of CRISPR Cas9 gene editing (<https://www.genecopoeia.com/resource/genome-editing-talen-or-crispr/>)

Unlike type II CRISPR system, multiple proteins are responsible for gRNA binding in systems I. This system requires assembly of a protein complex called Cascade (Brouns, et al., 2013). Cas 3 is an essential part of this system. It is located in between large and small subunit of Cascade. Cascade involves CasABCDE proteins (Datsenko et al., 2012). CasE is the most important for activation of CRISPR. Earlier it was considered that CasE takes part in transcription modification of crRNA. With help of it 61 nucleotide crRNA is produced

(Datsenko et al., 2012). (Semenova et al., 2011) argued these roles of CasE in such process and states that CasE is important for supporting crRNA and cascade complex. Cascade binds to the crRNA which has matching sites with PAM and gRNA of intruding species. Attachment of cascade system to the targeted genome parts triggers activation of nucleases that produces double stranded cuts (Datsenko et al., 2012). (Wiedenheft et al., 2012). Cas5, Cas7 and Cas6 play role in pre-crRNA transcription processing. Type I system is divided into six subtypes, according to the phylogeny of the genes involved in the system (Makarova et al., 2015).

In type III CRISPR system a unique Cas10 protein is present. This protein has 4 distinct domains. Cyclase-like domain, helical domain that is important for Zn binding, palm domain with catalytic activity and C-terminal alpha helical domain that presumed to have polymerase activity. This protein contributes to large subunit of cascade in type III CRISPR system. In many cases type III system does not have their own Cas1 and Cas2 proteins. That is why it relay on other CRISPR systems for crRNA synthesis which can be produced with system I and II. This type of CRIPR system has two sub-types which has different signature proteins in small subunit of a cascade (Makarova et al., 2015).

In bacteria new genomic sequences can be inserted as repeated region to gain resistance to a new pathogen. Cas7, Cas1 are presumed to be important genes for this purpose (Wiedenheft et al., 2012). Recently potential role of pre-existing spacers in acquiring new sequences was demonstrated by Nussenzweig. In this study a correlation between the number of previously obtained spacers and efficiency of gaining new ones was shown. Presents of functional spacers without mutations is linked to a higher rate of recruitment of new parts to the CRISPR targeting system. At the same, pre-exited spacers does not have to match perfectly to the targeted nucleotide sequence in virus genome in case of type 1 and type 2 CRISPR mediated protection. Degradation of virus sequences occurs slowly for these types of CRISPR systems (Nussenzweig et al., 2019).

CRISPR system has been actively used by researchers for the last decade. By incorporating nucleotide sequence of Cas9 and gRNA targeted mutations can be obtained. gRNA must be constructed in a way that it will match unique sequence of targeted genes. After CRISPR complex produces double stranded breaks in targeted genes mutations will be generated with help of non-homologous end joining NHEJ or homologous end joining HEJ (Fig 1.2) (Nussenzweig et al., 2019).

## **Application of CRISPR system**

Since first discovery of CRISPR system it has been used multiple times for targeted gene editing in various organisms. The advantage of this technology for agricultural industry is that plants modified by it do not fall into transgene containing organism's category. Mutations produced by CRISPR technology are inherited and in most cases are homozygous. Various mutations such as deletion insertion and substitution can be produced with CRISPR. Additionally, different modifications of nucleotide sequences can be introduced with help of CRISPR. These modifications may improve qualitative and quantitative compositional characteristics in plant's harvest. To illustrate this, acrylamide free potato (Haltermann et al., 2016), apples with inhibit synthesis of polyphenol oxidase (Nishitani et al., 2016), pectate lyase inhibited tomato mutants (Wang et al., 2019) etc were produced with help of CRISPR. Additionally, disease tolerance and abiotic resistance can be archived with CRISPR technology. Rice blast tolerance was created with help of this gene editing approach (Upadhyay et al., 2013).

Localization of genomic regions on chromosomes can be determined with help of CRISPR complex. Based on binding sites a localization of genes or QTLs on a chromosome can be estimated. Such system has advantages over traditional method of gene loci determination such as FISH. Fixated cells are not necessary for CRISPR system (Arora & Narula, 2017). CRISPR based method requires catalytically inactive Cas9 protein that is expressed with gRNA within cells. After binding to a targeted genome region affinity precipitation is applied to isolate this region. Next generation sequencing technique can be used next. Additionally, physical interaction between different genes can be estimated. Significant advantage of the approach is that it is highly sensitive. Single copy gene can be found with it. This is especially important for finding genes of resistance in polyploid organisms such as wheat. This technique may alleviate the process of resistance gene screening (Fujita & Fujii, 2013). Previously this approach was shown to be efficient on mice DNA (Bennet-Beker et al., 2017).

One of major application of CRISPR in gene editing is gene knockout. Different from RNAi technology that partially silence a gene expression, CRISPR-based gene knockout allows completely switching off expression of targeted genes. Also, CRISPR-based gene editing is more accurate than RNAi and can target any genome region. Therefore, CRISPR has been used in functional genomics study, and probably, can completely replace reverse genetic approaches in the future (Arora & Narula, 2017).

## **CRISPR delivery system**

CRISPR needs to be delivered to a targeted organism for performing gene editing. This can be achieved with help of vectors. Plasmids can be used as vectors (Arora & Narula, 2017). Transformation step is necessary for delivering plasmids with parts of CRISPR complex. For majority of hexaploid wheat varieties transformation can be performed due to low regeneration efficiency and low callus induction which are essential steps for plant transformation (Arora & Narula, 2017). That is why it is necessary to develop new approaches that do not require genetic transformation for delivery of CRISPR gene editing system to wheat.

Previously Hamada et al. (2018) have presented biolistic CRISPR delivery method that do not require gene transformation. In their protocol shoot apical meristem tissues of embryos were chosen as a delivery target of CRISPR complex. In the experiment a recombinant plasmid was used to carry Cas9 and gRNA. For bombardment authors used gold nanoparticles. Moderate efficiency for this gene editing protocol have been shown. In wheat 5.2% of treated plants demonstrated mutations in targeted genes (Hamada et al., 2018). Separate from this, nanoparticles with Cas9 and sgRNA were previously shown to be efficient for floral DIP method delivery of the gene editing complex (Chen et al., 2021[abstract]). Another approach for delivery of CRISPR gene editing complex may include usage of ribonucleoprotein complex (RNP). RNP is complex that consists of Cas9 protein connected to sgRNA. To transfer RNP to wheat cells nanoparticles were used. The advantages of this method include relatively high efficiency of gene editing, absence of gene transformation step as well as the fact that mutants obtained with help of RNP mediated gene editing is considered transgene free. Nevertheless, the authors mentioned that efficiency of this gene editing protocol may fluctuate depending on the genotype (Liang et al., 2017). That is why there is still needed to find a simple and efficient protocol for gene editing in hexaploidy wheat.

Viruses are other type of vectors that overcome this issue. Barley stripe mosaic virus (BSMV) is an RNA virus with three sub genomes (alpha, beta, and gamma). Alpha part carries RNA dependent RNA polymerase; beta contains sequence for capsid protein and movement proteins; gamma is carrying part of the polymerase. Typical symptoms in BSMV infected plants are yellow mosaic on leaves. Primary hosts for the virus are barley and wheat although it can infect wide variety of plants. BSMV can infect wheat but in most cases, infection would not lead to complete death of the crop. This makes the virus suitable tool for plant genetic engineering.

(Hu et al., 2019). It was previously demonstrated that BSMV can be efficiently as a delivery tool for virus induced gene silencing.

Determining the functions of *Fhb1* candidate genes in FHB resistance is a crucial task. CRISPR gene editing is a valuable tool that can be used for this. In our research we intended to develop a two step CRISPR delivery system that relays on transgenic Bobwhite that was used as a donor of Cas9 and BSMV that carried gRNA to hexaploid wheat. Additionally, generating mutations in *Fhb1* candidate genes and evaluation of FHB response in mutants with non-synonymous mutations were performed.

## **Chapter 2 - Materials and methods**

### **Plant materials and growing conditions**

Hexaploid wheat (*Triticum aestivum*) accessions ‘Bobwhite’, ‘Ning7840’ were used as plant materials for gene editing. Bobwhite is an FHB susceptible cultivar and has high transformation efficiency, therefore it was used as the CRISPR/Cas9 gene donor in this study. Ning 7840 is an FHB resistant variety that carries *TaHRCR* but is not suitable for gene transformation.

Wheat seeds were planted in 50-cell trays (Hummert International, Topeka, KS) with a growing mix (Sungro horticulture, Agawam, Canada), and placed in a cold room at 6 °C for 24 h to break the dormancy. Plants were vernalized at 6 °C for a week after shoot come out from soil. Next, the wheat plants were transplanted to 4×4 individual pots (Hummert International, Topeka, Kansas) in PGR 15 growth chambers (Conviron, Winnipeg, Canada) at 15 °C during the day and 12 °C during night with 12 h of daylight period. The plants were fertilized with Osmocote (24×8×16, NPK) classic slow-release fertilizer (The Scotts Company LLC, Marysville, USA) and then with Miracle-Gro soluble fertilizer (The Scotts Company LLC, Marysville, USA) weekly for four times after transplanting. Three weeks past transplanting, the temperatures in the growth chamber were increased to 20 (night) and 23 °C (day). Bamboo sticks were attached to the wheat plants to provide mechanical support to the plants four weeks after transplanting.

### **Gene editing of *TaHRC* in wheat**

Gene transformation is limited to several wheat varieties. Bobwhite was one of a few cultivars with high transformation efficiency and was used to transform Cas9. Using gene bombardment Cas9 was transferred into Bobwhite to generate Cas9 overexpressed lines following the protocol by Tian et al. (2019). However, Bobwhite variety plants can be described as highly susceptible to FHB. Additionally, this wheat genotype does not have nucleotide sequences from *Fhb1*. That is why Bobwhite was crossed to Ning 7840, wheat varieties to deliver Cas9 nucleotide sequence. Previously, Li et al., (2019) have shown that Ning 7840 wheat variety was carrying unique gain of function mutation which presumed to be cause of resistance to FHB. This allele of the gene was a target for gene editing. Thus, plants with Cas9 and targeted genes were obtained after a cross.

Finally, sgRNAs that were necessary for targeting of Cas9 protein to *TaHRC* genes had to be introduced in wheat with targeted nucleotide sequences and Cas9. BSMV was used to carry this component of gene editing complex. sgRNAs were ligated into plasmids carrying virus genome parts and were transfected into *Nicotiana benthamiana*. This plant was used as a secondary inoculum for Ning 7840 Cas9.

### gRNA design and BSMV vector construction

E-CRISPR (German Cancer Research Center, <http://www.e-crisp.org/E-CRISP/index.html>) online software was used to design gRNA for and *TaHRC* gene editing. The program selected a 20 base-pairs (bp) gene sequence that contains protospacer adjacent motifs (PAM) upstream of the potential gRNAs sequence. The most specific gRNA regions are selected to minimize off target effect. Five regions in *TaHRC* sequences were selected to design gRNA (Fig. 2.1).

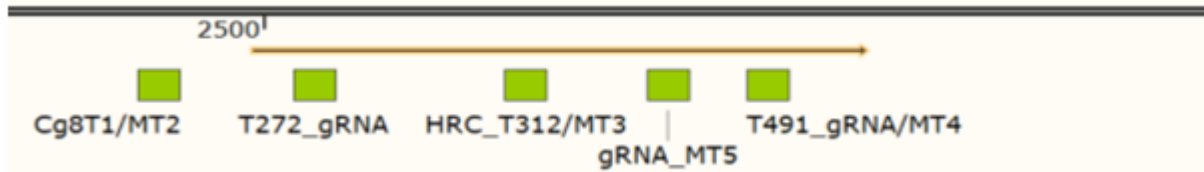


Figure 0.1. Positions of gRNA in *TaHRC*. Guide RNA positions in *TaHRC*, Arrowheads refer to open reading frames (ORF); color bars represented gRNA regions.

Single guided RNAs (sgRNA) were delivered to the targeted wheat plants using BSMV system. The primers sgRNA were synthesized for the target genes. Spacer sequences were incorporated between sgRNAs and  $\gamma$ b stop codon of the virus sequence as previously described (Hu et al., 2019). sgRNA was cloned using *Nco*I and *Spe*I restriction sites near  $\gamma$ b. Virus vector components (RNA $\alpha$ , RNA $\beta$ , RNA $\gamma$ ) from BSMV ND18 strain were reversely transcribed and cloned separately into a pCB301-2X35S-MCS-HDV NOS plasmids with RNA $\gamma$  carrying the sgRNA using Seamless Assembly Cloning Kit (Smarter Technologies Inc., Houston, USA). Assembled vectors were provided by Dr. Dawei Li from China Agricultural University. The final vectors contained pCB301-BSMV $\alpha$ /pCB301-BSMV $\beta$ /pCB301-BSMV $\gamma$ -gRNAs.

### Recombinant plasmid transformation using *Agrobacterium*

Cells of EHA 105 *Agrobacterium* strain was used to prepare *Agrobacterium* competent cells. A single *Agrobacterium* colony was inoculated into 100 ml of a Luria-Bertani (LB) broth medium with rifampicin and kanamycin. After the medium was incubated at 28 °C for 12h with

shaking at 300 revolutions per minute (r.p.m) in a Lab-line Orbit environ-shaker (Lab-line instruments, INC, ILL, USA), the finished cells were spined down at 5,000 r.p.m for 5 min at 4 °C in a Beckman Coulter Allegra X-15R centrifuge (BackmanCoulter, Inc, USA). Precipitated cells were then diluted in 50 ml 10% glycerol that was cooled down on ice before use and then spined down again in the centrifuge. After supernatant was discarded, the cells were resuspended in 1 ml of 10% glycerol and aliquoted into 1.7 ml polypropylene tubes (BioExpress, Lodi, CA, USA) with 40 µl of cells mixture in each tube. Tubes with cells were stored at -80 °C for future use.

For electroporation, 40 µl of electro-competent cells in a tube was placed on ice for 10 min and then added with 80 µl of three plasmid DNA. The competent cell mix with plasmids were transferred to a cold 2 mm gap electroporation cuvette BTX Disposable Cuvettes Plus Model 620 (BTX, division of genomic INC, San Diego, CA, USA) on ice. The cuvette was placed into a 2,510 Eppendorf electroporator (ETL Testing laboratories INC, Cortland, NY, USA) that was set at 2.50 kV, 25 µFD, and 400 Ω. The cells were multiplied in 3 ml of LB broth shaking for 3 hs at a room temperature, and 10 µl of the transformed cells was transferred to a previously prepared Petri dish with LB media, rifampicin and kanamycin antibiotics to eliminate bacteria without inserts. The Petri dish plate was incubated at 28 °C for 12 h. A single colony of *Agrobacterium* grown on the plate was selected and cultivated in LB broth at 28 °C overnight with shaking. Multiplied bacterial cells were pelleted by centrifugation at 2,200 g for 10 min before they were used to infect plants.

LB medium was prepared with agar mixture Lenox (Sigma, St louis, MO, USA) containing amino acids, vitamins, carbohydrates for normal development of bacteria. Fourteen gram of LB powder was dissolved in 400 ml of water and autoclaved in a Tuttnauer 3870E (Tuttnauer Inc, USA). Autoclaved medium was cooled down and stored at 4 °C in a fridge. The LB medium was melted and cooled down to a room temperature and added with appropriate antibiotic at 150 ng per ml before each use. LB media without agar Lenox (Sigma, St louis, MO, USA) was prepared by adding 16 g of mixture to 800 ml of water, and then autoclaved and cooled down at 4 °C in a fridge.

### **Agroinfiltration of *Nicotiana benthamiana* plants**

BSMV inoculum was increased in *N. benthamiana*, a highly susceptible species to BSMV, before it was applied to wheat plants. *N. benthamiana* plants were grown in the growth chamber,

and were infiltrated with the previously prepared *Agrobacterium* at four-leaf stage following Yuan et al. (2011).

Buffer containing 10 mM MgCl<sub>2</sub>, 10 mM 2-N-morpholinoethanesulfonic acid (MES), and 0.1 mM acetosyringone (pH=5.2) was added to the pellet with *Agrobacterium* and mixed. The mixture was incubated at a room temperature for 3 h. The *Agrobacterium* carrying different plasmids with virus components were mixed together in equal amount and used to inoculate two to three young leaves of each *N. benthamina* plant depending on size of the plant. Plants were infiltrated with the bacteria in central vein of each leaf using a syringe without a needle. Infiltrated leaf surfaces were then sprayed with clean water to keep high humidity on the surfaces where the inoculation occurred, and inoculated plants were covered with plastic boxes to maintain humidity for successful virus spread. The inoculated plants were checked for viral symptoms including yellow leaf color and shrunk leaf parts at about 10 days after inoculation.

### **Confirmation of gRNA in infected tobacco plants**

Reverse transcription PCR (RT-PCR) was performed to confirm presence of viral RNA. Viral RNA was extracted from *N. benthamina* plant tissues using a TRIzol kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. All pipettes and working surfaces were sterilized with RNase Away (Molecular BioProducts Inc, San Diego, CA) prior to the RNA extraction. Infected leaf tissues were collected in a 2 ml GeneMate polypropylene tube (BioExpress, Lodi, CA, USA) and were rapidly submerged into liquid nitrogen to prevent RNA degradation. One stainless metal bead was placed into each tube and the tube was shaken at 1,450 cycles per minute (C/M) for one minute in a 2010 Geno/Grinder® (Spex SamplePrep, USA). As soon as plant tissue was ground into a fine powder, and 1 ml TRIzol (Invitrogen, Carlsbad, CA, USA) reagent was added to each tube with 100 mg of tissue powder, and then the tube was incubated at a room temperature for 5 min to dissolve nucleoprotein complex completely.

The sample tube was added with 200 µl of chloroform and shaken firmly for 15 seconds. The samples were left at a room temperature for 3 min and then centrifuged at 12,000 g for 15 min at 4 °C in a Sorval Legend Micro 21 R centrifuge (Thermo Fisher scientific, Osterode am Harz, Germany). After the centrifugation, RNA in the upper aqueous phase was transferred to a new 1.7 ml tube (BioExpress, Lodi, CA, USA) and 500 µl of isopropanol was added to the tube before it was incubated in a room temperature for 10 min. The tube was centrifuged at

12,000 g for 10 min at 4 °C. After the supernatant in the tube was discarded, the RNA pellet in the tube was added with 1 ml 75% ethanol, mixed firmly using Labnet VX100 vortex mixer (Labnet International Inc, Woodbridge, NJ, USA), centrifuged at 7,500 g for 5 min at 4 °C. The pellet was dried at a room temperature for 10 min. and was resuspended in RNase-free water. The tube with RNA was put into a dry bath (Fisher Scientific, Osterode am Harz, Germany) at 55 °C, and then stored at -20 °C until use. RNA quality was checked in a 1% agarose gel following Sambrook and Russell (2001).

To prepare 1% agarose solution, 1.5 g agarose powder (GeneMate, USA) was mixed with 150 ml of 1x TAE buffer (600 ml ddH<sub>2</sub>O, 242g Tris base, 6.71 ml glacial acetic acid, 100ml of 0.5M EDTA pH=8.0) in a 250 ml beaker. The beaker was heated in a microwave until agarose were completely dissolved, then cooled down to about 40 °C before adding GelGreen® Nucleic Acid Gel Stain (Biotium, USA) to the gel solution for visualization of RNA in the agarose gel in a UV transilluminator.

RNA samples were added with a loading buffer to the wells in the agarose gel. RNA samples (4 µl) were mixed with 2 µl of the loading buffer containing 400 ml 10mM Tris-HCl, 37.5 Ficoll-400, 0.03 Bromophenol blue (BFB) and then was loaded into each gel well. Lambda DNA was loaded in a middle well as a size ladder and the gel was run in 1X TAE buffer. Electrophoresis was conducted under 120 volts for 30 min. RNA bands were visualized in a BioRad Gel Doc XR+ system (Bio-Rad Laboratories, USA) using Image Lab (Version 6.0.1) software.

Viral RNA of BSMV was transcribed into DNA for PCR using a Maxima H Minus First Strand cDNA Synthesis Kit (Thermoscientific, Vilnius, Lithuania) following manufacturer's protocol. Extracted RNA was quantified in a Qubit® fluorometer (Life Technologies Corporation, CA, USA). following the manufacture protocol. Next, 5µl of RNA sample was used to quantify the extracted RNA using water as a blank control.

For reverse transcription, 3 ng/µl of RNA was mixed with 1 µg of oligo (dT) primers, 1µl of 10 mM dNTP mix, 4 µl of 5X RT buffer and 1 µl of Maxima H Minus Enzyme Mix. The reaction mix was centrifuged for 10 sec and incubated at 50 °C for 30 min and then at 85 °C for 5 min to terminate the reaction in a PTC 200 Peltier thermal cycler (MJ research, Inc, Massachusetts, USA).

To check the presence of BSMV virus in tobacco tissues, a 13  $\mu$ l PCR master mix included 5 mM dNTPs, 1  $\mu$ l homemade *Taq* polymerase, 25 mM MgCl<sub>2</sub>, 10mM each forward and reverse primer, the primer complementary to viral BSMV $\gamma$  (carries gRNA), 10x ASB PCR buffer (10mM Ammonium sulfate, 670 mM Tris-HCl, 0.1% Tween-20, pH=8.8), and 1  $\mu$ l of the first strand cDNA. The PCR plate was vortexed and centrifuged at 1,450 r.p.m for one min in a Beckman Coulter Allegra X-15R centrifuge (BeckmanCoulter, Inc, USA).

PCR was amplified in a Peltier PT-200 thermal cycler starting at 95 °C for 3 min, then 35 cycles at 95 °C for 30 sec, 61 °C for 45 sec and 72 °C for 1 min varying with the length of targeted DNA sequence (1 kb fragment requires 1 min for amplification) with a final step at 72 °C for 5 min.

For each sample, 4  $\mu$ l of the PCR product was loaded into a 1% agarose gel. The gel was stained with DNA SafeStain (Lambda Biotech, MO, USA) and visualized under UV light in a GelDoc Plus. Samples with a sharp single band of expected size on the agarose gel were considered as the target product.

### **Generation of Cas9-overexpressed transgenic Bobwhite plants**

Cas9 overexpressed transgenic lines were generated using gene bombardment, as described by Tian et al.(2019) in Dr. Trick's lab in the Department of Plant Pathology, Kansas State University. For transformation immature embryos were isolated from 10-14 post anthesis wheat spikes to produce calli. The calli were incubated in dark for 2-3 days, and then 1 $\mu$ g/ $\mu$ l of Cas9 DNA carried by plasmid pAHC2 was mixed with Tungsten particles (99.9% 1 micron M10 equiv., Atlantic Equipment Engineers, Bergenfield, NJ) to bombard calli. The calli were transferred to induction media containing selection agent and then incubated in room temperature in dark for 4 weeks. The calli were transferred to shoot production media with selective agent to grow shoots with 16 h of light (~80  $\mu$ mol) at 20 °C, and 8 h in darkness at 18 °C, and 40–50% relative humidity (RH) for 2 weeks. The calli were transferred to shoot elongation selection media under the same conditions until development of plantlets. Plantlets were transferred to pots containing soil and grown at 16 h in light at 20 °C, and 8 h in darkness at 15 °C in high humidity for 5 days. The quantitative PCR was used to verify presence of Cas9 gene in the transgenic plants.

Cas9-overexpressed Bobwhite was crossed to a wheat variety Ning 7840 to transfer Cas9 nucleotide sequence. The F<sub>2</sub> plants with targeted genes and Cas9 sequence were selected using PCR for the experiment.

### Inoculate BSMV in wheat

*N. benthamiana* served as intermediate host of BSMV for viral inoculum increase. The infected tobacco leaves that were confirmed to carry BSMV with the target sgRNA was used as inoculum to inoculate Bobwhite wheat plants that carry Cas9 gene. BSMV -infected tobacco leaves with BSMV carrying the target sgRNA were cut into small pieces and grinded with a pestle in 20 mM Na-phosphate buffer ( $pH = 7.2$ ) containing 1% celite into uniform sap as inoculum to inoculate Bobwhite wheat plants with Cas9. Inoculation was conducted at two-leaf stage by rubbing the sap on one wheat leaf, then spraying water on the inoculated leaf surface and covering the pot containing the inoculated plants with a plastic sheet for 24 h as outlined in Fig. 2.2.

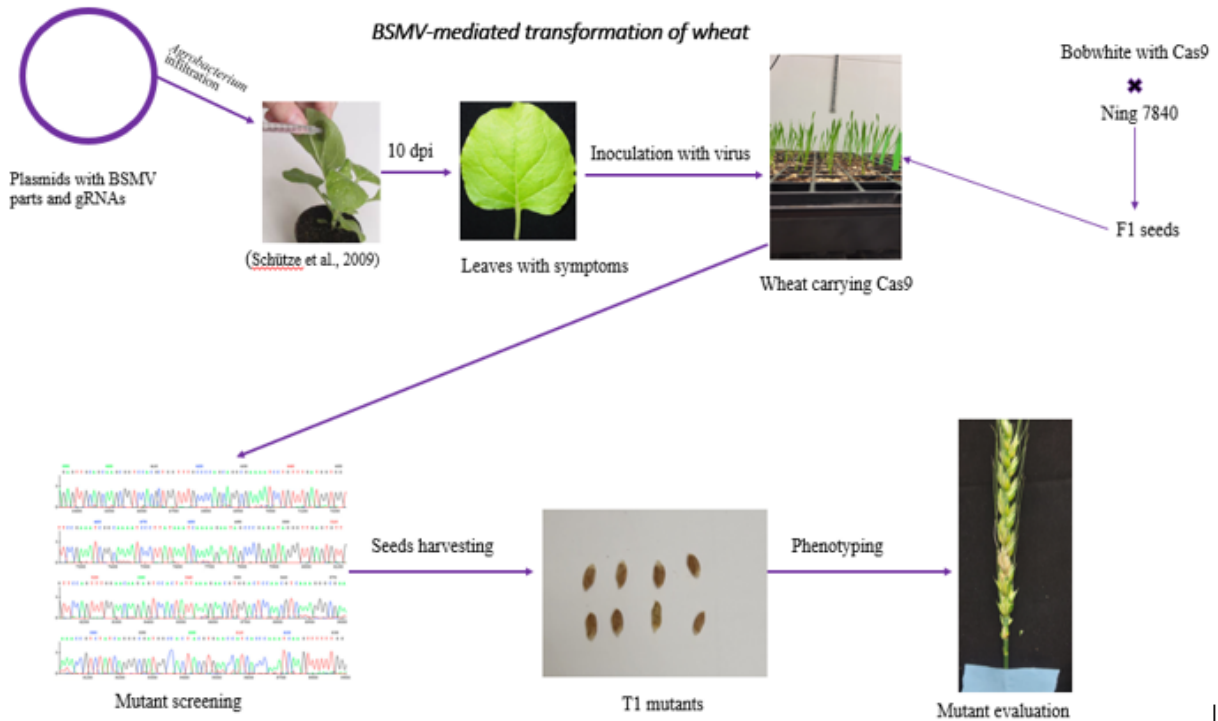


Figure 0.2. Scheme of BSMV-mediated gene editing system. Viral inoculum production and inoculation, gene editing, mutant identification and phenotype evaluation.

## DNA isolation and PCR amplification of targeted genes

Wheat leaf sample from M<sub>0</sub> was collected for mutation screening at 10 days after BSMV infiltration. To extract DNA from wheat plants, 3 pieces of 2 cm of leaf tissue were cut 10 after BSMV infection and placed in GeneMate 96-deep well plate (BioExpress, Lodi, CA, USA). Leaf samples with a metal bead in each well were dried in a freeze drier (ThermoSavant, Holbrook, NY, USA) for 2 days, and ground to a fine powder in a GenoGrinder (SPEX sample prep, USA) at 1,750 cycles per min for 2 min. After grinding, the sample plates were sealed right away to prevent moisture from getting inside of the plate.

A modified Hexadecyltrimethylammonium bromide (CTAB) protocol was used to extract DNA (Murray & Thompson, 1980). The CTAB extraction buffer was made of 300 ml ddH<sub>2</sub>O, 50 ml of 1.0 M Tris-HCl (pH 8.0), 20 ml of 0.5 M EDTA, 10 g CTAB, 40.91 g NaCl, 0.5732 g TCEP and 10 g Polyvinylpolypyrrolidone (PVPP). The newly prepared CTAB (650 µl), together with 2 µl of RNase (Life Technologies, Carlsband, CA, USA) at 20 µg/µl, was added to each well of a leaf tissue plate. The sample plate was covered with mats, mixed at 1,750 C/M for 10 sec in the Geno/Grinder, then put in a Thermo Heratherm OMH-180 oven (Thermo-scientific, Langenselbold, Germany) at 65 °C for 90 min and inverted gently every 30 min. After incubation, the sample plates were centrifuged at 4,000 r.p.m for 1 sec, cooled down to a room temperature, and added 300 µl of chloroform/isopropanol solution (25:1). The mat-sealed sample plate was gently inverted for 45 min under a safety hood. The DNA plate was spined at 5700 r.p.m for 30 min in 4 °C and 450 µl of supernatant was transferred to a 1-ml plate with 450 µl of previously prepared cold isopropanol. The plate was then gently inverted 3 times and then centrifuged at 5,700 r.p.m for 45 min at 4 °C. After supernatant was discarded, the plate containing DNA pellets was dried on paper towers. The plate with dry DNA was added 500 µl of 70 % ethanol to each well and centrifuged at 1,750 C/M for 15 min. The plate was centrifuged at 5,700 r.p.m for 15 min at 4 °C and then ethanol supernatant was discarded. After another ethanol wash step, DNA pellet was dried in an oven for 30 min until there was no visible liquid and then dried in a fume hood for 3 h. Dry DNA in the plate was diluted with 200 µl of autoclaved 10mM Tris + TritonX-100 (10 ml 1M Tris-HCl pH=8.0, 31.25 µl TritonX-100, add ddH<sub>2</sub>O to a liter) to suspend DNA in 65 °C for 60 min., and then centrifuged for 30 sec before stored in 4 °C.

DNA quality was checked using 1% gel as described in previous sections. DNA samples that showed a single sharp band on a gel was used for PCR amplification of *TaHRC*. The

genomic region with gRNA targets was amplified by PCR and 1% agarose gel electrophoresis using the same protocol that was previously described (Table 1) with one pair of primers for *TaHRC* gene.

Table 0.1. Primers and PCR conditions for amplification of gRNA regions of *TaHRC*

| Gene                      | Primer sequence in 5' => 3' orientation              | Primers used for sequencing of PCR products | Length of amplified fragment | Temperature of annealing      | gRNA and its sequence |
|---------------------------|------------------------------------------------------|---------------------------------------------|------------------------------|-------------------------------|-----------------------|
| <i>TaHRC<sub>A</sub></i>  | F:tccttgagaagaaggaagctc;<br>R:gacgggaagcaactcgtgtaa. | F:tccttgagaagaaggaagctc                     | 766 b.p.                     | 55 °C - 45 s<br>72 °C – 1 m   | Mt2-Mt5               |
| <i>TaHRC<sub>3B</sub></i> | F:attcctactagccgcctggt<br>R:tggggcaagcaaacattg       | F: gtctgttttgggctcacgtcg                    | R - 1292 b.p.<br>S - 2044b.p | 59 °C - 45 s<br>72 °C – 1.3 m | Mt2-Mt5               |

### PCR clean up and sequencing

To prepare PCR products for sequencing, 0.5 µl of Exonuclease I (Thermo Scientific, Vilnius, Lithuania) and 1 µl of FastAP Thermosensitive Alkaline Phosphatase (Thermoscientific, Vilnius, Lithuania) were added to 3 µl of the PCR products, and then mixed and centrifuged for 1 min at 1700 r.p.m to settle the liquid. The mixture was incubated at 37 °C for 15 min, and then increased temperature to 85 °C for 15 min to terminate the reaction in a PCR thermocycler. The mixture was stored at 4 °C for sequencing.

For sequencing, the master mix contained 2 µl of 5X sequence buffer, 0.5 µl DMSO, 0.5 µl BigDye v1.1 (ThermoFisher Scientific, Waltham, MA, USA), 3 ng (1 µl) purified DNA, 5 ng (1 µl) forward primers (Table 1), and 5 µl ddH<sub>2</sub>O. The master mix was prepared in dark, mixed, centrifuged, amplified in the 96-wells PCR thermocycler starting at 94 °C for 1 min, then with 25 cycles of 94 °C for 30 sec, 55 °C for 1 min and 60 °C for 4 min. The final products were stored at 4 °C before purification.

Optima DTR Ultra 96-well plates (EdgeBio, USA) were utilized for PCR sample purification. After protective tapes were removed from bottom sides of the Optima plate, purification column was inserted into a 96-well waste plate. The assembly was centrifuged at 2,100 r.p.m for 5 min in a pre-cooled (4 °C) centrifuge. and amplified sequence samples were

pipetted into filter wells and centrifuged at the same conditions as before. After the samples were covered with lids and placed in a sequencing machine.

Sequence data was visualized as chromatogram using a SnapGene software v. 5.3.2. Only the sequence with low error rate, clearly distinguishable peaks and full-length reads were chosen for subsequent alignment to identify mutated sequence using Lasergene SeqMan Pro (v. 7.1.0) from DNASTAR package. The derived *TaHRC* sequences were aligned to the reference sequences of *TaHRC* (GenBank ID: [MK450312.1](#)) from Ning 7840 to identify mutated sequence in the gRNA-targeted regions.

Samples that carried presumable mutation and had double peaks through the chromatogram were selected for cloning. With help of cloning presence of the mutation can be confirmed. Separate alleles of the genes can be differentiated. Consequently, loci where a mutation occurs can be estimated. Separate from this, sequencing of cloned samples allows to reduce background noise which improve chromatogram quality of sequenced samples.

### **Cloning of targeted sequences**

The PCR primers may amplify multiple homologous sequences and direct sequencing of PCR products could not get expected sequence. Therefore, PCR products were cloned before sequencing to separate the individual sequences in the PCR products. For PCR cloning, the PCR products were purified to remove dNTPs and primer residues using Gencatch PCR purification kit (Epoch Life Science, Inc, Sugar Land, USA) before they were ligated into a plasmid. To purify PCR, 25 µl of the PCR products was mixed thoroughly with a 500 µl PX buffer in a 1.7 ml tube and the mixture was transferred to a column containing a silica membrane inside. A collection tube was placed under the silica column before they were centrifugated at 5,000 r.p.m for 60 sec, and liquid waste in the collection tube was discarded. The sample was added 500 µl of WN washing buffer, and centrifugated at 5,000 r.p.m for 60 sec. After The flow-through liquid waste was discarded, 500 µl WS washing buffer was added to the column and centrifuged at 5,000 r.p.m for 60 sec and then at 13,000 r.p.m for 3 min to remove ethanol residues. The column was placed on the top of a new 1.5 ml tube and 15 µl of water was added to the center of the column. After 4 min in room temperature, the column was centrifuged at 13,000 r.p.m for 2 min. Eluted DNA was located at the bottom of the 1.5 ml tube. Agarose gel electrophoresis was used to check the quality of isolated DNA. If a sharp band was observed in the DNA sample, the DNA was used for cloning right away or stored in -20 °C for late use.

The purified DNA was cloned into a Topo PCII vector by ligating the DNA fragments into plasmids (Fig. 2.3) using TOPO TA cloning kit following the manufacturer's protocol (Thermo Fisher Scientific, Carlsland, USA). An adenosine overhang was added to the DNA fragment by polymerase during PCR. The overhang was complementary to the vector to improve ligation efficiency. Cloning kit containing the topoisomerase was used for the vector activation.

For ligation, 2 µl purified PCR product was added to a 1 µl salt solution that contains all necessary components for ligation including 200 mM NaCl and 10 mM MgCl<sub>2</sub> and prevent topoisomerase from binding to the ends of DNA molecules during the ligation process. The ligation mixture was added with 1 µl of vector solution and 2 ul ddH<sub>2</sub>O to bring the final volume to 6 µl, mixed gently and incubated at a room temperature for 30 min to facilitate ligation.

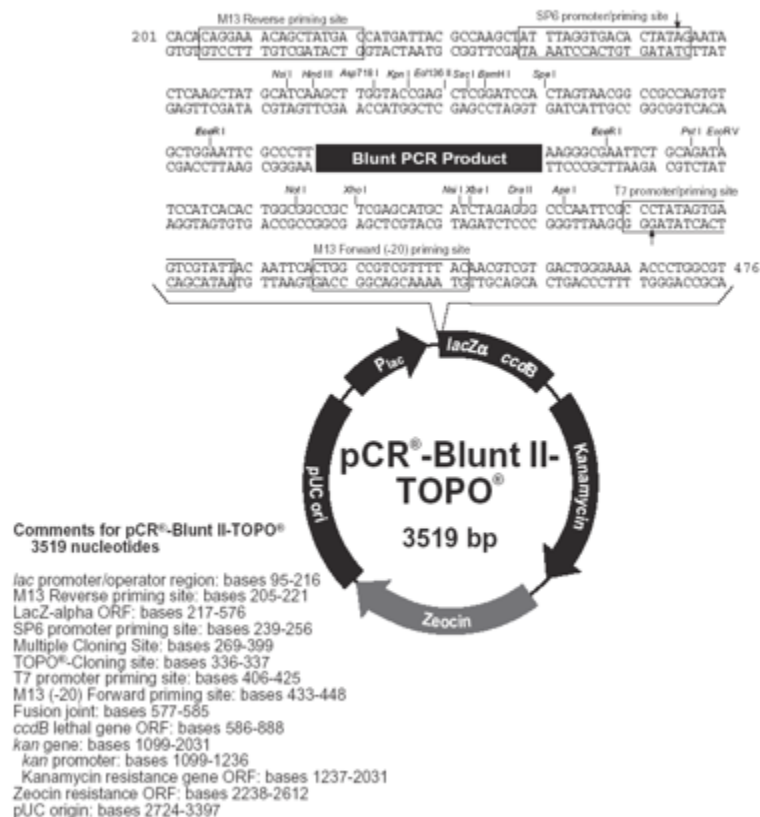


Figure 0.3. Map of Topo PCII vector used for gene sequence cloning (<https://www.thermofisher.com/order/catalog/product/K465001#/K465001>)

## Transformation of competent cell

The ligated products were transformed to previously prepared *E. coli* competent cells (Inoue et al., 1990). For transformation, *E. coli* was grown on a liquid LB medium with 5 ml of ampicillin at 37 °C for 12 h, and then transferred to 50 ml LB medium to grow for another 12 h at 30 °C. The *E. coli* cell concentration in the medium was measured at 600 nm wavelength using a UV-1650 PC spectrophotometer (Shimadzu, Huashan, China). The tube with the *E. coli* cells were placed on ice for 10 min and centrifuged at 4,000 r.p.m for 10 min. After discarded the liquid waste, the remaining pellet was resuspended in a 20 ml cold CC buffer containing 10 mM Hepes (2.38g), 15 mM CaCl<sub>2</sub> (2.21g), MnCl<sub>2</sub>\*4H<sub>2</sub>O (10.89g), and 250mM KCl (18.64g) at pH of 6.7. All the components except MnCl<sub>2</sub> were added and pH was adjusted to 6.7 using KOH. The solution was added with 1M MnCl<sub>2</sub> \*4H<sub>2</sub>O and filtered through 0.22 µm filter Millex GP (Millipore Ltd, Carrigtwohill, Irland).

The pellet was resuspended in a CC buffer, kept on ice for 10 min and then centrifuged at 4,000 r.p.m for 10 min at 4 °C. Pelleted cells were resuspended in 3 ml cool CC buffer and added with 280 µl of DMSO. The mixture was incubated on ice for 10 min and then made 50 µl aliquots using 1.7 ml tubes that were previously frozen in liquid nitrogen. Competent cells were stored at -80 °C for future use.

Tubes containing the competent cells from a -80 °C freezer were defrosted on ice for 10 min, then added with 2 µl of recombinant plasmids and mixed slightly. The first transformation was done by keeping the competent cells on ice for 30 min to increase binding of plasmids to the membranes of competent cells. The transformation mixture was heat shocked at 42 °C for 30 sec in a Isotemp 210 water bath (Fisher scientific, USA). To increase transformation efficiency, the tubes with the cells were incubated on ice for 5 min and then added with Super Optimal broth with Catabolite repression media (S.O.C) (Thermo Fisher Scientific, Carlsbad, CA, USA) that contains all essential components for rapid bacterial growth including 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>, and 20 mM glucose. Bacteria were grown at 37 °C at 300 r.p.m in a Lab-line Orbit environ-shaker (Lab-line instruments, INC, ILL, USA). After incubation, 50 µl of the bacterial solution was spread on the previously prepared 15 ml LB agar medium with kanamycin. Since TOPO PCII plasmid contains a kanamycin resistance gene, only bacteria that were transformed with this plasmid would

survive on the medium. Plates with bacteria were incubated at 37 °C overnight to grow *E. coli* colonies with recombinant plasmids.

Colony PCR containing 10 ng of DNA and 1 µl of 10µM M13 primers at 10 ng/µl was used to verify presence of inserted target gene as described in previous chapters. Primers hybridize with nucleotide sequences of Topo PCII plasmid (Fig. 2.3). Sixteen individual colonies were taken from each Petri plates and added to colony PCR master mix in separate wells.

Amplified fragments were detected by 1% agarose gel electrophoresis. The expected size of the positive clone was calculated by adding 259 bp (length of DNA fragments that flank potential DNA insertion) to the actual size of expected DNA fragment that was ligated into the plasmid. Only colonies that had expected size on the agarose gel were picked and grown in separated culture tubes with 3 ml of liquid LB medium and Kanamycin at 37 °C overnight.

### **Isolation of recombinant plasmids**

To identify edited sequence mutations in the cloned DNA fragments, the recombinant plasmids were sequenced in an ABI 3730 sequencer. The positive colonies of plasmid DNA were isolated using GenCtach plus plasmid DNA miniprep kit (Epoch Life Science, Inc, Sugar Land, USA). RNase A was mixed with MX1 buffer before using the kit to remove RNA. Additionally, 40 and 24 ml of 98% ethanol were added to WS and WN washing buffer, respectively, before plasmid DNA extraction.

The culture tube with bacterial cells was centrifuged at 13,000 r.p.m to get clean pellet with all growth medium residues removed. The tube was added with 200 µl of MX1 buffer to the bottom of the tube and mixed thoroughly till all small particles were fully dissolved. To isolate plasmid DNA, 250 µl of MX2 was added to the tube and mixed gently. The tube was incubated at the room temperature for 5 min and added with 350 µl MX3 to neutralize the lysis of cell components. To separate degraded molecules from plasmid DNA, the tube was centrifuged at 13,000 r.p.m for 10 min before the supernatant was transferred to a GenCatch plus column. The column was subsequently centrifuged at the same speed for 1 min. To further purify DNA, 500 µl of WN washing buffer was added to the column, and the column was centrifuged at 9,000 r.p.m for 1 min. After discarded flow through to a hazardous wastes bin, 700 µl of WS washing buffer was immediately added to the column. After centrifuging the column at 13,000 r.p.m for 3 min to remove ethanol residues, the column with plasmid DNA was placed over a new 1.5 ml tube and added with 50 µl of autoclaved ddH<sub>2</sub>O to the center of the column. After the column

was placed at the room temperature for 3 min, the column was centrifuged at 13,000 r.p.m for 30 sec and liquid in the bottom tube contains plasmid DNA.

DNA quality and quantity were checked in a 1% agarose gel as described previously. Plasmid DNA with expected quality was selected as the template for sequencing in an ABI 3730 sequencer to identify sequence mutations in the target DNA segments. The quality of sequence data was checked using Snap gene (version 5.3.2.) and only the samples with single peaks were selected for subsequent sequence alignment. Samples with sequence mutations at target sites were traced back to original wheat plants. Seeds from these mutant plants were harvested for phenotyping.

### **Phenotyping of mutants**

Mutant seeds were planted and phenotyped for FHB resistance by comparing the phenotypic data between the mutants and the non-edited controls at the same environments. Plants were grown in a growth chamber for FHB evaluation. Leaf tissue was collected at two leaves stage for DNA isolation to confirm target gene mutations in these plants using Sanger sequencing. After the mutation was confirmed in M<sub>1</sub> plants, the mutant M<sub>1</sub> plants were transferred to individual pots, and grown in growth chamber at 10 °C and 15 °C in the first 4 weeks and then changed to 20 °C and 23 °C with a light period of 12 h.

Wheat spikes of the plants were inoculated with a conidia spore suspension at anthesis by injecting 1000 spores of *F. graminearum* isolate GZ3639 from KS (Jin et al., 2013) into a central spikelet of spikes using a Hamilton PB600-1 syringe (Hamilton Company, Reno, NV). For each plant, 3-5 spikes were inoculated depending on number of spikes available in a plant. The inoculated spikes were bagged with Ziploc Sandwich bags for 48 h to maintain high moisture. FHB symptoms were scored 7, 14 and 21 days after inoculation. Blighted spikelets were considered as major FHB symptoms. Number of spikelets with FHB symptoms and total number spikelets in the spike were counted to calculate proportion of scabbed spikelets (PSS) in a spike.

$$PSS = \frac{\text{Number of spikelets with symptoms in a spike}}{\text{Number of spikelets on a spike}} * 100$$

The mean FHB ratings from each line were compared to determine the effects of the mutants.

## Chapter 3 - Results

### Comparison of *TaHRC* homologous sequence among different genomes

The PCR product of *TaHRC* amplified by *TaHRC\_A* primers (Table 2.1) was observed as a single distinctive band of 734 bp on a gel and was cloned into the plasmid vectors. The cloned recombinant DNA was Sanger-sequenced using both forward and reverse primers to fully cover the sequence in gRNA regions of *TaHRC*. Consensus sequence was generated and aligned to *TaHRC* from Ning 7840 (GenBank ID: [MK450312.1](#)) .

After sequence alignment with *TaHRC* from Ning 7840, multiple SNPs were observed in the cloned sequences. These SNPs are mainly in the regions complementary to gRNA as well as in the other parts of coding sequences (Fig. 3.1.A). The distinct peaks without background noise observed on the chromatograms indicated high quality of obtained sequences (Fig. 3.1.B). In general, the *TaHRC* sequence similarity between colonies were from 80 to 99%.

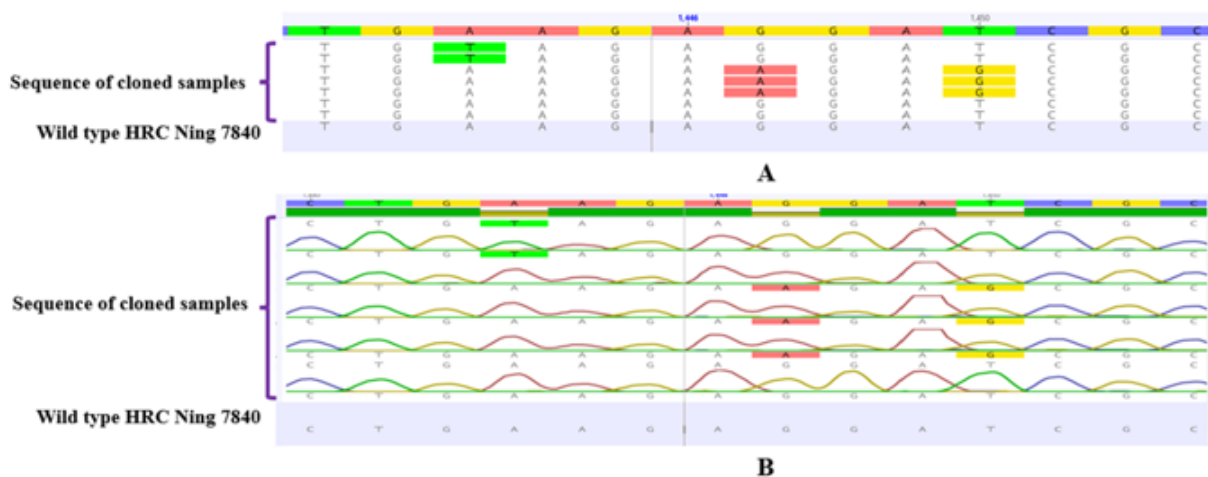


Figure 0.1. Comparison of cloned sequences obtained from one plant sample. A. Alignment of cloned sequences with *TaHRC* sequence from Ning 7840. Colored nucleotides are SNPs. Sequences of cloned samples were compared with *TaHRC* sequence on 3B from resistant variety Ning 7840 (GenBank ID: [MK450312.1](#)); B. Chromatograms of corresponding cloned sequences of *TaHRC* in one plant sample.

In order to determine the genomic regions of mutations, these sequences were blasted in GenBank. The cloned sequences showed high similarity with the sequence in all group 3 chromosomes (3B, 3A 3D). An online platform WheatOmics ( Ma et al., 2021) was used for more detailed comparison in wheat genomes to evaluate sequence diversity of *TaHRC* homologs from 3B 3A and 3D chromosomes. We used *TaHRC* sequence from Ning 7840 (GenBank ID: [MK450312.1](#)) to blast against pan-genome *TaHRC* homologous sequences in Chinese Spring, Fielder, Arina, Jagger, Norin 61, Julius, Lancer, Landmark, Mace, Mattis and Stanley accessions. *TaHRC* sequence ([MK450312.1](#)) from GenBank was found in the 3A, 3B and 3D chromosomes of these pangenome accessions With the sequence identity ranging from 87 to 99.9%.

A phylogenetic tree was constructed for 3B *TaHRC* from Ning 7840 and its homologous sequences from pangenome using the multiple sequence alignment data (Fig. 3.2). The phylogenetic tree formed two groups. Group A contained 3B *TaHRC* sequence from Ning 7840 (MK450312-GenBank), Landmark, Norin 61, Lancer and Arina. This group contained the sequences with the highest identity to previously cloned *TaHRC* gene MK450312. Mean pairwise identity amongst the individuals in the group reached 99.5 %, suggesting this group carries Ning 7840 *TaHRC* resistance allele. The sequence identity between 3B *TaHRC* sequences in Arina and Ning 7840 reached 98%. The 3B *TaHRC* nucleotide sequence in Arina has the 752 bp deletion in the third exon, the same as [MK450312.1](#) in Ning 7840 . However, three SNPs were identified in the non-coding regions of 3B *TaHRC* between Arina and other sequences from group A. Arina is a Swiss winter wheat variety and showed high resistance to FHB (Buerstmayr et al. 2015). Therefore, the group A most likely carry *Fhb1*. Group B contained sequences from 3B chromosomes of Jagger, Mace, Stanley and other pangenome cultivars. Additionally, 3B sequences from Chinese Spring was also in this group B. These varieties carry the susceptible allele of *TaHRC*. Chinese Spring is highly FHB susceptible and does not carry a large deletion in 3B allele of *TaHRC* ( Choulet et al.2010), Schweiger et al.2016). The sequence identity between the 3B sequences in group B and the *TaHRC* sequence from Ning 7840 was 96%. Multiple SNPs and small frame shifts were identified between sequences from group A and group B within coding regions. These changes were spread through the aligned sequences.

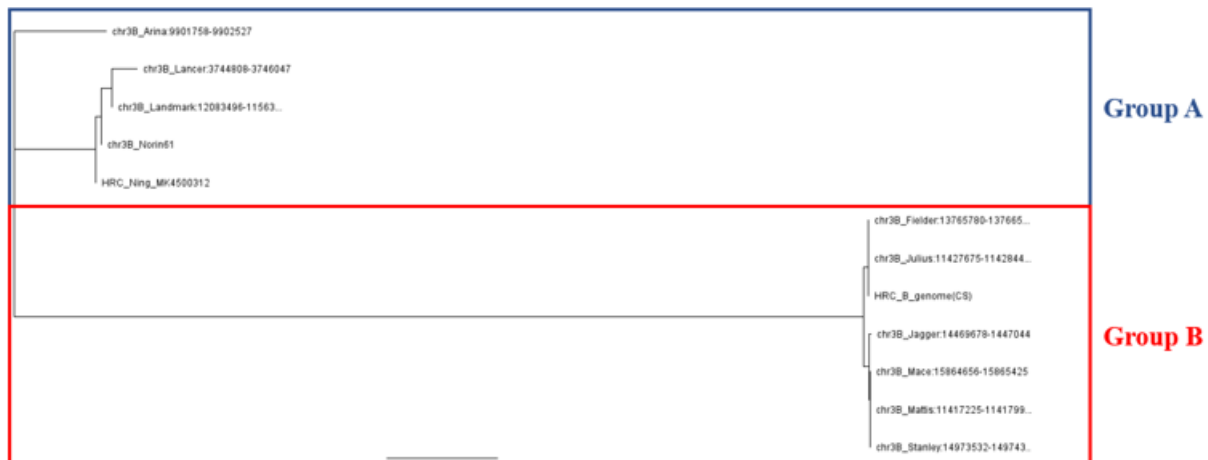


Figure 0.2. Phylogenetic tree of *TaHRC* sequence on 3B of from the wheat varieties of Pangenome.

The 3B *TaHRC<sup>R</sup>* sequence from Ning 7840 was also compared to its homologous sequences in 3A and 3D subgenomes of pan-genome varieties. The sequence identity of *TaHRC* between 3A in the pangenome accessions and 3B *TaHRC<sup>R</sup>* from Ning 7840 were < 94.5%. The *TaHRC* sequences from 3A genomes of Norin 61 and Ariana had the highest similarity to Ning 7840 3B *TaHRC<sup>R</sup>*. The identity of *TaHRC* sequences from 3A of different pangenome cultivars showed 99.8% within coding regions of the gene with only two SNPs (Fig 3.3).

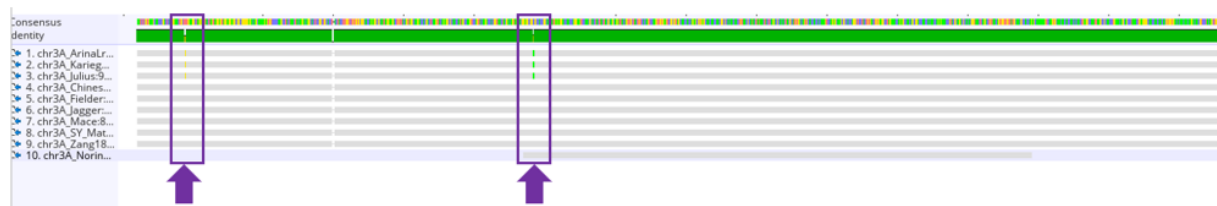


Figure 0.3. Comparison of 3A *TaHRC* sequences among pan-genome wheat accessions. Arrow points to the position of SNPs.

Ning 7840's 3B *TaHRC<sup>R</sup>* sequence was used to blast against the pangenome sequences of 3D *TaHRC* sequence and they showed 97.9% of identity with the highest identity to Mattis's 3D *TaHRC* sequence among the pangenome cultivars. The 3D genome *TaHRC* sequence identity was 98.4% among the pangenome wheat accessions.

Since multiple SNPs were identified in *TaHRC* homologs in each subgenome among the sequences of the pangenome cultivars, we generated *TaHRC* consensus sequences for 3A, 3B and 3D. The consensus sequences were used to determine the locations of mutations.

### Sequencing of edited *TaHRC* gene

A total of 1200 Ning 7840 M<sub>1</sub> plants were screened for mutations after BSMV treatment. The *TaHRC* sequence data from all the treated plants were aligned to previously generated consensus *TaHRC* sequences from chromosome 3A, 3B and 3D. Three plants (Table 3.1) were identified with potential mutations.

Table 0.4. List of mutants induced by BSMV-mediated gene editing.

| <b>Mutant №</b> | <b>gRNA</b> | <b>Mutation</b>           | <b>Amino acid change</b> | <b>Genome and % identity to consensus</b> |
|-----------------|-------------|---------------------------|--------------------------|-------------------------------------------|
| <b>#36</b>      | Mt3         | Deletion and substitution | Frame shift              | A (98)                                    |
| <b>#99</b>      | Mt5         | Deletion                  | Frame shift              | A (98.7)                                  |
| <b>#123</b>     | Mt2         | G->A                      | Asp -> Gly               | D (99)                                    |

Two mutations were confirmed to be in A genome *TaHRC* homolog. They were mutant 36 and mutant 99. The sequence of the mutant 36 had 98% identity to consensus sequence of A genome, 92 % identity to B genome and 94.5% identity to D genome. The sequence had the highest identity to A genome, therefore this mutation most likely occurred in A genome. In mutant 36, a single mutation site had two sequence changes: one nucleotide substitution (A->G) and another single nucleotide deletion in the gRNA site of MT3. Chromatograms of the sequence showed high sequence quality without double peaks in other sites (Fig 3.4 A). Nucleotide sequence of mutant 99 had the highest % of similarity toward consensus sequence of *TaHRC* from A sub-genome (98.7%), while consensus sequences of *TaHRC* from 3D and 3B genome showed 93.8% and 92% similarity respectively. Mutant 99 had 3 nucleotide deletion in MT5 gRNA region (Fig 3.4 A). Mutant 123 demonstrated the highest identity to consensus sequence of *TaHRC* from 3D chromosome (99%) compared to 96% and 94% identity to 3A and 3B *TaHRC* consensus sequences. Mutant 123 carried single nucleotide substitution in MT2 gRNA region (G->A). This mutation altered amino acid residue of the protein from polar asparagine to

non-polar glycine. This change may be critical for folding of a protein and may alter its functions.

To check if these mutations are inheritable, the seeds from the  $M_1$  plants were harvested and planted. The DNA from the seedlings was sequenced again and compared with previous sequence data. The results confirmed the mutations in the target sites of the three lines (Fig. 3.4B).

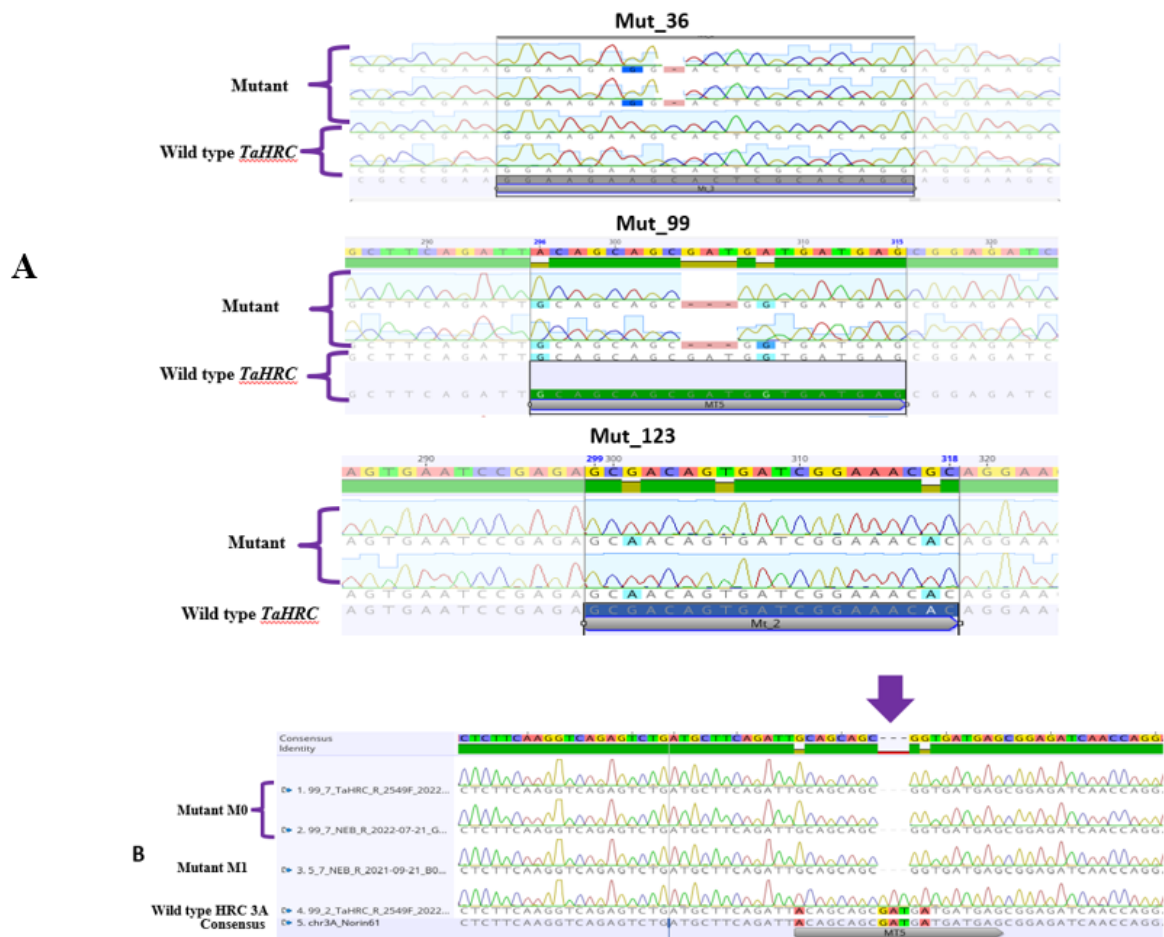


Figure 0.4. Alignment of mutant sequences to wheat consensus sequences of *TaHRC* from 3A,3D chromosomes. A. Alignment of nucleotide sequences of recombinant plasmid DNA from mutant 36, 99 and 123 against consensus *TaHRC* sequence from 3A and 3D genomes. B. Nucleotide sequence alignment of  $M_0$  and  $M_1$  generations of the mutant 99 to consensus *TaHRC* sequence from 3A chromosome. Arrow points to the region with the 3 bp nucleotide deletion.

## Phenotyping of the mutants

Seeds from M<sub>1</sub> mutants were grown, and plants were genotyped and evaluated for the reaction to FHB. Mean PSS scores for the mutant plants reached 8.3 % at 7 days post infection (DPI) while non-edited plants showed 12% score. At 14 DPI both mutant plants and non-edited plants showed the similar PSS score which was about 16%. However, the susceptible control Bobwhite plants showed 65% PSS score at 14DPI. The spikes turned blight with only a few spikelets alive (Fig. 3.5) at this stage. At 21 DPI the average PSS score of mutants reached 40.6%, with the control plants without mutation had 35% infected spikelets in a spike. Bobwhite plants became completely susceptible at 21 DPI.



Figure 0.5. Comparison of FHB phenotype. Comparison among three mutant plants, non-edited F<sub>2</sub> plants with Cas9 and *TaHRCR* alleles and susceptible control Bobwhite at 14 days past infection DPI in the greenhouse.

Eight plants per mutant were phenotyped for FHB resistance with an average of 4-6 inoculated spikes per plant. Mutants 36, 99, 123 had 8%, 10% and 7 % PSS, respectively, at 7 DPI in greenhouse conditions while the non-edited control's PSS was 12%. The difference in means PSS was not significant between each individual mutant and non-edited control in a two-sided *t*-test. The mean PSS of mutant lines and non-edited control plants were not significantly different at 14DPI and 21 DPI. In the mutant line 36, the mean PSS raised from 15% at 14DPI to 32% at 21 DPI (Fig 3.6 A), and Mutant 123 PSS was 17% at 14DPI and 36% at 21 DPI. Mutant 99 PSS were 17% at 14DPI and 54 % at 21 DPI (Fig. 3.6 B). The non-edited plants had 16% PSS

at 14DPI and 35% PSS at 21DPI. The rapid increase in number of infected spikes in mutant 123 for period between 2 and 3 weeks after FHB inoculation might be due to the growing conditions of mutants. The mutants were infected by powdery mildew at the beforementioned time.

The mutants were also evaluated for the plant height and the spike length. The two mutant lines (36 and 123) did not show significant difference in PSS, height and spike length from the no-edited plants (Fig. 3.6 D, E). However, the plants from mutant 99 line was significantly shorter and had smaller spikes than plants from the non-edited control plants.

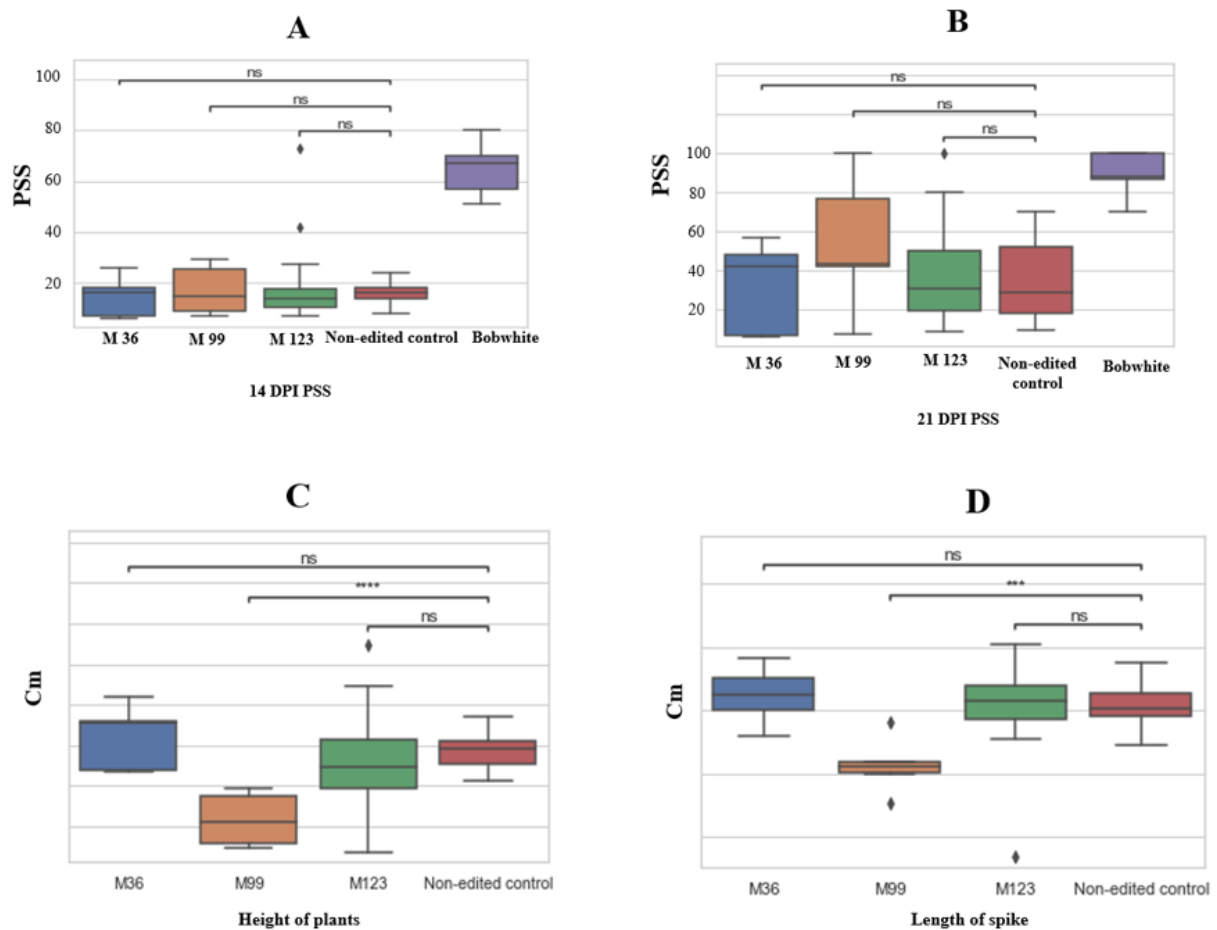


Figure 0.6. Comparison of percentage of scabbed spikelets in a spike (PSS) between mutants and progeny of Bobwhite and Ning 7840 cross plants at 7, 14, 21 days past infection (DPI). Whiskers represent data range; center lines indicate means. Significant difference was claimed based on a two-sided Student *t*-test, A. 7 and 14 DPI after FHB inoculation. B. 21 DPI after FHB inoculation. C. Comparison of plant height between M<sub>2</sub> mutant lines and non-edited progeny of Bobwhite and

Ning 7840 cross plants. For each line 8 plants were compared. D. Comparison of spike length between the mutant plants and non-edited control plants. Five plants per line were measured 21 days after FHB inoculation. whiskers represent data range and center lines indicate means. For each line 8 plants were compared.

## Discussion

CRISPR Cas9 system is a valuable tool for gene editing to change the functions of the target genes. It has been widely used in improvement of crop traits and in functional genomic studies. Today this gene editing system has been tested on multiple species of animals and plants (Barrangou et. al., 2016). However, gene editing process in some crop species such as hexaploid wheat that has large multiple genomes is still a challenging process. Some genes of polyploid wheat have been reported to be successfully knocked out using the CRISPR system (Liu et al., 2021; Li et al., 2021). However, in the conventional gene editing system, Cas9 and gRNA must be delivered to target plants through gene transformation, and most wheat varieties have very low regeneration and callus induction rates during tissue culturing process, which has significantly limited its applications in wheat research and breeding. Additionally, majority of modern wheat gene editing protocols require screening of large numbers of plants with subsequent verification of the mutations in next generations (Wang et al., 2022). To overcome this issue, we developed a BSMV delivery system. This system uses BSMV to deliver gRNA sequences to Cas9- overexpressed wheat to conduct targeted gene editing. If the target genes or alleles are absent in Bobwhite, we crossed Cas9-overexpressed Bobwhite plant to a wheat variety that carries the target gene to be edited and select plants that carry both the targeted gene and CRISPR/Cas9 for gene editing.

The advantages of using BSMV delivery system include absence of tissue culture step, and mutants generated using this approach may not be classified as genetically modified organisms. Relative easiness of BSMV infection step in the virus-mediated gene editing process is another plus of the approach. Previously Hu et al. (2019) has shown that BSMV can infect and spread through wheat tissues. In this study, we generated two plants with frame shift mutations and one plant with a nucleotide substitution in both A and D genomes of hexaploid wheat. The mutations were confirmed to be inheritable by cloning and Sanger sequencing of the target genomic regions. This confirms that BSMV-mediated gene editing system can be used in hexaploid wheat. A minor reduction in height of one line of mutant (mutant 99) versus non-edited control was observed. This can be attributed to growing conditions of the mutants from line 99. Mutants 36 and 123 did not show significant reduction in height. Most of the confirmed mutant plants did

not show symptoms of BSMV infection in the M<sub>2</sub> generation. These results indicated that BSMV-mediated editing may not cause damaging effects on other non-target traits.

BSMV-based CRISPR gene editing has been used as an important tool to generate mutants for functional validation of candidate genes in gene cloning work. The approach proposed in this study is especially valuable for functional genomic study in these wheat varieties that have low gene transformation efficiency due to the low tissue regeneration capabilities. Consequently, using our approach, validation of other important agronomic traits and disease resistance genes function in wheat can be performed. This opens a new perspective for using gene editing in wheat breeding to create new sources of resistance or desirable agronomic traits that cannot be found from conventional breeding lines. These newly created traits can be further incorporated into modern wheat cultivars.

This BSMV-mediated editing system uses overexpressed Cas9 in Bobwhite wheat that was previously transformed. However, based on available genome reference sequence and pangenome sequence, one cultivar may contain only some genes or alleles, not the others. If the target genes/alleles are absent in Bobwhite, then this approach does not work. To solve this issue, we made crosses between Cas9-overexpressed Bobwhite and target cultivars and used markers to identified plants with Cas9 and the target gene for further editing.

FHB is a wheat disease that has caused massive economical losses in wheat (Proctor et al., 1995). Incorporation of resistance genes into modern wheat varieties is the most efficient strategy to minimize FHB spread. Sumai 3 and Ning 7840 are winter wheat variety that carries a major stable FHB resistance QTL *Fhb1* (Tao Li et al., 2016), the most studied FHB resistance QTL (Buerstmayr et al., 2002). Although several groups reported cloning of the QTL (Rawat et al., 2016; Su et al., 2019; Li et al., 2019), the discrepancy in either gene identity or functions remains to be solved. This study was designed to solve the discrepancy. Unfortunately, we did not find mutation in *TaHRC* on chromosome 3B, and only identified mutations in *TaHRC*'s homologs on 3A and 3D subgenomes. The mutations alter amino acid sequence that is transcribed from the gene. The mutants were phenotyped in greenhouse conditions by point inoculation with FHB spores. Mean PSS scores for the mutants carrying frameshift mutation in A genome were 17% and 15% at 14 DPI. The mutant with a sequence substitution in D genome had 17% PSS at 14 DPI. The PSS for non-edited control plants was 16%. Therefore, as expected, protein change of *TaHRC*'s homologs in 3A and 3D did not change FHB resistance in plants,

which supported previous result that *TaHRC*'s homologs in A and D genome did not contribute to FHB resistance due to low expression (Su et al. 2019). Further phenotyping of these mutants will validate the results.

In our study, 0.25% efficiency of gene editing was achieved. A high level of Cas9 expression in wheat is critical for gene editing in wheat and the reduction in Cas9 expression decreased the chances of mutant generation as well as heritability of incorporated mutations (Wang et al., 2021). Moreover, Anand et al. (2003) demonstrated that expression of transgenic constructs in wheat could be reduced or silenced in few generations after gene transformation. In our experiment F<sub>2</sub> plants were derived by crossing Ning 7840 to a Cas9-overexpressed Bobwhite plant and then the F<sub>2</sub> plants with the target *TaHRC* gene and CAS9 were selected for editing. This could affect Cas9 expression level and gene editing efficiency if Cas9 expression was at a low level. Moreover, seeds post BSMV treatment were used for next cycles of mutant screening. In order to increase the gene editing efficiency, Cas9-overexpressed plants need to be prechecked for high level of Cas9 expression before crossing and before editing.

Another possible solution is application of other CRISPR-based gene editing systems. For example, Cas12a gene has been shown to be efficient for plant gene editing. Cas12 nucleotide sequence is 1300 and Cas9 is 1600 b.p (Zetsche et al., 2015). This allows direct incorporation of this Cas12 gene into BSMV genome. This process is not possible for Cas9 due to the limited size of insert that can be ligated into the virus. Additionally, Cas12 requires smaller gRNA regions for targeting gene editing (Bandyopadhyay et al., 2020), therefore, higher specificity to gene homologs for editing.

Complexity of winter wheat genome is another limitation for increase gene editing efficiency in wheat. Wheat is allohexaploid species consisting of A, B and D subgenomes derived from different ancestors. Additionally, the wheat genome is highly repetitive (Walkowiak et al., 2020). *Fhb1* is located on chromosome 3B, however, *TaHRC* has two other homologs in A and D subgenomes. Comparison of *TaHRC* (*Fhb1*) from chromosome 3B to its corresponding A and D homologous sequences showed 94.5% and 97.9% similarity, respectively. The high *TaHRC* sequence identity between 3B and 3A or 3D subgenomes complicates target gene editing. Additionally, current gRNA design tools also limit *TaHRC* gene editing efficiency in wheat. This software was created to identify PAM sequences within coding regions of genes to minimize off-targeted gene editing, therefore the high similarity among three

*TaHRC* homologs makes it difficult for subgenome or chromosome specific editing. Thus, generating specific mutants in hexaploid wheat may be a laborious process and need to screen a large number of treated plants before finding desired mutations. High-throughput DNA isolation and next-generation sequencing system may facilitate quick identification of mutants.

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