MOLECULAR MAPPING OF QTL FOR GENETIC TRANSFORMATION-RELATED TRAIT AND CRISPR/CAS9-MEDIATED GENE EDITING IN WHEAT

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Genetic transformation and CRISPR/Cas9-mediated genome editing have become powerful tools for gene functional characterization and crop improvement. However, their applications in wheat are limited by genotype dependency in genetic transformation and low efficiency in target gene editing. This study aimed to 1) identify the QTL associated with plant regeneration capability from calli derived from immature embryos; 2) mutate the wheat *Tsn1* gene for susceptibility to tan spot disease, using CRISPR/Cas9 vectors delivered by Agrobacterium and particle bombardment mediated transformation; 3) target the wheat *TaHRC* gene (encoding for reticulum histidine-rich calcium binding protein) at the *Fhb1* locus involved in the resistance/susceptibility to Fusarium head blight (FHB) resistance, using haploid induction coupled with the CRISPR/Cas9 genome editing technology. Using phenotypic and genotypic data from a mapping population of 186 recombinant inbred lines derived from the cross between wheat cultivar Bobwhite and wheat line PI 277012, two QTL controlling plant regeneration capability in Bobwhite were detected on chromosome 1A and 6D. To knock out the *Tsn1* gene in wheat, CRISPR/Cas9 vectors with gRNA cassette targeting a coding region of *Tsn1* were used to transform Fielder and Bobwhite through the Agrobacterium-mediated and biolistic transformation methods, respectively. Thirty-two T0 transgenic plants were generated from the transformation experiments. However, no *Tsn1* mutants were identified by screening 1176 T1 transgenic plants through ToxA infiltration. To target *TaHRC* involved in FHB resistance, CRISPR/Cas9 constructs with gRNAs for targeting both two allelic forms (*TaHRC-S* and *TaHRC-R*) of the gene were designed and used for Agrobacterium-mediated transformation of the hybrid corn variety Hi-II. The pollens of transgenic corn plants with high expression of Cas9 and gRNA were used to pollinate emasculated spikes of wheat lines with *TaHRC-S* and *TaHRC-*

R, respectively. Of the 82 haploid plants generated from embryo rescue, 12 plants were identified having mutations at the target sites of *TaHRC-S* and 2 plants at the target site of *TaHRC-R*. Doubled haploid plants are being generated from these gene-edited haploid plants and will be evaluated for FHB resistance. This study may facilitate our understanding of genetics of transformation-related traits and provide a novel approach for improving disease resistance in wheat.

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This dissertation is dedicated to my beloved family:

Parents: Madhu Sudan Karmacharya, Rammati Karmacharya

Sisters: Anju Karmacharya, Sanju Karmacharya, Shreejana Karmacharya, Sajana Karmacharya, Asmita Karmacharya, and Sachita Karmacharya

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LITERATURE REVIEW

Wheat production in the US

Wheat is one of the major staple crops grown worldwide to feed the growing population. In the United States (US), wheat ranks third among field crops in terms of planted acreage and production after corn and soybeans (E. USDA, 2022). In the US, Kansas is the top wheat producing state followed by North Dakota (ND) and Montana (MT) (N. A. S. S. USDA, 2021). In 2021, ND produced 52.8% and 52.7 % of the durum and spring wheat, respectively, in the US. Winter, spring, and durum wheats are the three primary classes that are domestically sown in the US. Winter wheat varieties are sown in fall, stay dormant during winter, and resume the growth until summertime harvesting. Due to harsh winter in the Northern Great Plains, winter wheat plantings are much less as compared to spring or durum wheat. Winter wheat is the largest produced category followed by spring and durum wheat. These three types of wheat can be disaggregated into six major classes: hard red winter, hard red spring, soft red winter, hard white, soft white, and durum. Hard red winter wheat accounts for about 40%, hard red spring wheat accounts for about 25%, soft red winter accounts for about 15%, white wheat accounts for about 15% and durum wheat accounts for 2-5% of total production in the US (E. USDA, 2022).

Wheat evolution and domestication

The Neolithic transition from hunter-gatherer nomadic lifestyle to sedentary agrarian lifestyle, before 10,000 years ago, was a crucial turning point in human history (Childe, 1951). During that time, cereals such as einkorn wheat (*Triticum monococcum* L.) and barley (*Hordeum vulgare* L.) as well as pulses such as pea (*Pisum satiuvum* L.), lentil (*Lens culinaris* Medikus), chickpea (*Cicer arietinum* L.), and bitter vetch (*Vicia ervilia* (L.) Willd.) were domesticated in the Fertile Crescent and considered as Neolithic founder crops (Lev-Yadun et al., 2000). The

Fertile Crescent, also referred as the "cradle of agriculture", is located in the Middle East and encompasses a region extending from Jordan, Israel, Lebanon, and Syria through southeast Turkey and along the Tigris and Euphrates rivers through Iraq and western Iran (Faris, 2014).

The basic *Triticum* genome contains seven chromosomes (1x=7) and the various *Triticum* species consisted of diploids (2n=2x=14), tetraploids (2n=4x=28), and hexaploids (2n=6x=42) (Sax 1922; Kimber and Sears 1987). The genus *Triticum* consists of six species; *Triticum monococcum* L. (AA genome); *Triticum urartu* Tumanian ex Gandilyan (AA genome); *Triticum turgidum* L. (AABB genome); *Triticum timopheevii* (Zhuk.) Zhuk. (AAGG genome); *Triticum aestivum* L. (AABBDD genome); and *Triticum zhukovskyi* Menabde & Ericz. (AAAAGG genome). Among these species, *T. urartu* exists only in its wild form, whereas *T. zhukovskyi* and *T. aestivum* exist only as cultivated forms. Other species, *T. monococcum*, *T. turgidum,* and *T. timopheevii*, have both a wild and a domesticated form (Matsuoka, 2011). The cultivated forms of polyploid wheat were evolved by the amphidiploidization event. During this event, the hybridization of two different species is followed by spontaneous chromosome doubling of F1 hybrid through a mechanism known as "meiotic restitution" where unreduced gametes (2n) are formed in contrast to normal meiosis resulting in reduced gametes (n) (Faris, 2014; Ramanna & Jacobsen, 2003). Wild diploid wheat (*T. urartu*, 2n=2x=14, genome A^uA^u) hybridized with the B-genome ancestor, which is the closest relative of goat grass (*Aegilops speltoides*, 2n=2x=14, genome SS), 300,000-500,000 years before present (BP) to produce wild emmer wheat (*T. turgidum ssp. dicoccoides*, 2n=4x=28, genome A^uA^u BB). However, the origin of the B genome has remained controversial (Dubcovsky & Dvorak, 2007a; Huang et al., 2002). Approximately 10,000 years BP, hunter-gatherers began to cultivate wild emmer. Cultivated emmer (*T. turgidum ssp. dicoccum*, 2n=4x=28, genome A^uA^u BB) spontaneously hybridized with another

goat grass (*Ae. tauschii*, 2n=2x=14, genome DD) 9,000 years BP to produce an early spelt (*T. aestivum ssp. spelta*, 2n=6x=42, genome A^uA^uBBDD) (Dvorak et al., 1998; Matsuoka & Nasuda, 2004). Around 8,500 years BP, natural mutation changed the ears of both emmer and spelt to a more easily threshed type that eventually evolved into the free-threshing ears of durum (*T. durum*) and bread wheat (*T. aestivum*). The gene flow from the ancestors altered the original diversity pattern present in the domesticated wheat. The diversity evolved in the wheat does not reflect the geography of crop origin but parallels the geography of diversity in the progenitor (Dvorak et al., 2011). The process of control and maintenance of the differential diversification rates in polyploid species of wheat is still a big question for the scientific community (Petersen et al., 2006).

Modification of the seed dispersal mode is the major domestication trait in wheat to reduce the spikelet shattering at maturity. Conventionally, wheat domestication practices have focused on a few qualitative traits such as brittle rachis, tough glume, and free threshing controlled by single major genes *Br/br, Tg/tg,* and *Q/q*, respectively (Gill et al., 2007). The domestication process should have been rapid if the ancient farmers only selected the indehiscent, soft glume and free threshing mutants in the wild wheat populations (Peng et al., 2011). Even with rapid domestication, the wild and domesticated forms of the same plant were overlapping for a long time (up to 3,000 years). Indehiscence took over a millennium to become an established event (Tanno & Willcox, 2006). Similarly, selection for the large cereal grain from the wild was slow, as it is controlled by many genes (Peng et al., 2003). This implies that early farmers probably did not focus on important quantitative traits such as indehiscence, spike size, grain size, heading date, growth time, plant height, etc. in the process of harvesting wild wheat (Peng et al., 2011). Domestication of wheat was a series of events occurring at multiple

sites of Fertile Crescent over thousands of years and fitted a gradualist and multi-site model (Feldman & Kislev, 2007; Tanno & Willcox, 2006).

Wheat genome and whole genome sequencing

Common bread wheat (*Triticum aestivum*) has evolved through the series of allopolyploidization via hybridization between the *Triticum* and *Aegilops* species. In nature, polyploid species tend to have more extended geographical distributions than those of their close diploid relatives (Stebbins, 1985). In the same way, polyploidization have contributed to the broad adaptation of wheat to a wide range of climates which is likely because of the genetic diversity captured from the natural populations of its tetraploid ancestors combined with a high rate of evolutionary changes in the wheat genome (Dubcovsky & Dvorak, 2007b; Jordan et al., 2015). Common wheat genome size is approximately 17 gigabases (GB) with each genome (A, B, or D) being approximately 5.5 GB (Marcussen et al., 2014). More than 85% of the wheat genome is made up of repetitive sequences, which make the whole genome sequencing and assembly very challenging (IWGSC et al., 2018).

In 2005, a group of scientists and breeders initiated the International Wheat Genome Sequencing Consortium (IWGSC) for stepwise unraveling the mystery of wheat genome. To simplify the process, the 21 chromosomes from the common wheat landrace Chinese Spring (CS) were sorted by flow cytometry sorting. Physical maps and bacterial artificial chromosome (BAC) libraries were constructed for each chromosome. Because of its large size, chromosome 3B (774 Mb) was the first sorted and a physical map was generated using BAC clones in 2008 (Paux et al., 2008). In 2012, shotgun sequencing strategy with 454 pyrosequencing produced 5.42 Gb of assemblies of CS genome. 94,000 to 96,000 genes were predicted with these assemblies and significant loss of gene family members in common wheat during

polyploidization and domestication was found as compared to the diploid progenitors (Brenchley et al., 2012). The first chromosome-based draft sequence of the Chinese spring genome was released by IWGSC in 2014. This 10.2 Gb genome sequence was obtained by chromosome-based shotgun sequencing using Illumina technology (IWGSC et al., 2014). In 2017, precisely sized mate-pair libraries and optimized algorithms were used to generate a new assembly representing >78% of the genome (Clavijo et al., 2017). By combining Illumina next generation and long-read Pacific Biosciences third-generation sequencing data, 15.4 to 15.8 Gb of final assembly representing >90% of CS genome was generated (Zimin et al., 2017). The first annotated reference sequence of 21 chromosomes of CS (IWGSC RefSeq v1.0) was published on 17 August 2018. This 14.5 Gb reference sequence covered 94% of the genome and contained 107,891 high-confidence gene models. The approximate 1 Gb size reduction in RefSeq v1.0 can be explained by unassembled sequences of highly repeated telomeric and RNA coding sequences (IWGSC et al., 2018). RefSeq v1.0 was further revised, using an optical map based on direct labeling and staining (DSL) chemistry together with the previously constructed nick, label, repair, and stain (NLRS) map, to develop IWGSC RefSeq v2.1. Using optical maps, chimeric scaffolds were detected and resolved, unassigned scaffolds were anchored, ambiguities in positions and orientation of scaffolds were corrected, and gap sizes were more accurately estimated. To accompany RefSeq v2.1, IWGSC annotation v2.1 was completed by using IWGSC Annotation v1.2, which was updated by integrating a set of 117 novel genes and 81 microRNAs into the previous annotation. IWGSC Annotation v2.1 contains 106,913 high confidence and 159,840 low confidence genes (Zhu et al., 2021). This availability of a complete reference genome of wheat provides a great resource for accelerating genomics-assisted wheat research.

Wheat genetic transformation

Genetic transformation is a powerful research tool for identification and functional validation of genes controlling phenotypic traits. It is an indispensable tool for the use of exotic genes for crop improvement that cannot be transferred by sexual means (Haiyan et al., 2007). In contrast to time-consuming conventional breeding techniques, genetic transformation provides a shortcut platform for introducing novel genes directly into the cultivated germplasms for crop improvement. Successful wheat transformation depends on various factors such as the genotype, age, growth condition of the donor plant (Janakiraman et al., 2002). However, wheat is considered recalcitrant to transformation due to the genotype dependency, difficulties pertaining to gene delivery, and recovery of transgenic plants (Yi et al., 2015). Wheat is among the last major crop to be genetically transformed (Vasil et al., 1992). Despite inferior agronomic traits, the spring wheat variety 'Bobwhite' has been used widely in wheat genetic engineering due to its good response in tissue culture with high callus induction and regeneration rates (Fellers et al., 1995). The wheat transformation consists of three major steps: target tissue preparation, gene delivery and selection, and recovery of transgenic tissue or plants. Various explants from wheat have been used for the transformation purposes, including immature embryos (Weeks et al., 1993), mature embryos (Khurana et al., 2002), embryonic calli (Vasil et al., 1992), apical meristems (Zhao et al., 2006), floral organs (Zale et al., 2009), and others. The different gene delivering techniques used for transformation includes biolistic (particle bombardment or gene gun), protoplast-based technologies, *Agrobacterium*-mediated transformation, microinjection, and electroporation (Ingram et al., 2001). Transient and stable transformation by electroporation and polyethylene glycol (PEG) using wheat protoplasts have been discussed by several authors (Marsan et al., 1993; Díaz, 1994; Maheshwari et al., 2011). The stable transformation using

electroporation of protoplasts derived from suspension cultures was first reported by Zhou et al., 1993. In addition, Zhu et al., (1993) used liposomes to deliver DNA into protoplast. However, the protoplast-based transformation had low efficiency, was time-consuming, and was often genotype-dependent. Because of the low reproducibility of other transformation methods, biolistic and *Agrobacterium*-mediated transformation formed the basis for successful wheat transformation.

The first transgenic wheat with herbicide resistance was obtained using the biolistic method with a transformation efficiency of 0.2% (Vasil et al., 1992). Subsequently, this technology was widely used for wheat transformation (Becker et al., 1994; Nehra et al., 1994; Vasil et al., 1992; Weeks et al., 1993). The biolistic method was developed by John Sanford and co-workers at Cornell University in the United States (Sanford et al., 1987). This process involves the delivery of DNA-coated metal particles (0.4 to 1.2 μm) into plant cells. Gold and tungsten particles were used as the microcarrier of DNA. Due to the chemical inertness of gold, gold was preferably used. The equipment has been improved using electrical discharge (Christou, 1995) or helium pressure (Sanford, 1991) instead of gunpowder. The optimization of gene delivery was further facilitated by the commercial availability of Bio-Rad PDS-1000/He device (Kikkert, 1993). The biolistic parameters, such as helium rupture pressure, bombarding distance, vacuum conditions, DNA purity and concentration, calcium chloride ($CaCl_2$), and spermidine concentration to aid adherence of DNA to microcarrier, influence the biolistic-mediated transformation efficiency. In addition, biological parameters such as explant health and type, culture conditions before and after bombardment, screening procedures, and regeneration rates of transformed tissues also play a vital role. Hence, these factors should be assessed and optimized for successful transformation (Ingram et al., 1999; J. Li et al., 2012). Using this

system, transgenes can be introduced to any tissue of any genotype and not host-dependent as in *Agrobacterium-mediated* transformation. Very large DNA fragments of 53 kb linear cassette have been successfully transferred to the wheat genome using this technique (Partier et al., 2017). The major disadvantage of this technology is the integration of multiple copies of an introduced gene into the plant genome at a single locus, which results in silencing of transgenes in subsequent progeny by a tendency to promote homologous rearrangement (Kohli et al., 2003; Sparks & Jones, 2004). One of the solutions for multiple copies integration was using a strategy based on site-specific recombination exploiting the Cre-lox system. The transgene was flanked by recombination sites in an inverted orientation and recombination between the outermost sites resolves the integrated molecules to a single copy (Srivastava et al., 1999). Another solution for multiple integrations was using linear Minimal Expression Cassettes (MECs) instead of the whole plasmid. Transformation with MECs instead of plasmids improved transformation frequency from 0.4% to 1.1% (Yao et al., 2007). The complex transgene integration patterns of biolistic methods have been a driving force in the development of *Agrobacterium tumefaciens*-mediated transformation (ATMT).

Agrobacterium tumefaciens is a gram-negative soil bacterium that can induce crown gall disease in dicotyledonous plants. It can transfer a small segment of its DNA, referred to as T-DNA of tumor-inducing (Ti) plasmid, to the host plant (Ream, 1989). The *virD1* and *virD2* genes of Ti-plasmid encode for endonucleases (VirD1 and VirD2) that nick at the defined ends of 25 bp T-DNA right and left borders. VirD2 covalently links to the 5' end of the T-DNA strand, exits through a type-IV protein secretion system made up of VirB proteins and VirD4, and is transferred and integrated into the recipient plant genome (Fronzes et al., 2009; Ingram et al., 2001). Until the middle of the 1990s, it was generally believed that monocotyledons could

not be transformed by *Agrobacterium*, as they are not the natural host for crown gall disease (de Cleene & de Ley, 1976). Later, it was found that *A. tumefaciens* could attach to monocotyledons in the same manner as dicotyledons, but induction of virulence genes involved in T-DNA transfer was limited (Usami et al., 1988). Potent chemical inducers such as acetosyringone, to induce virulence genes were also identified that aided in transformations in monocots (Stachel et al., 1985). This T-DNA has been exploited extensively and modified into a more easily manipulated disarmed binary vector system for plant transformations (Hoekema et al., 1983). These vector systems consist of two plasmids, one with multiple cloning sites flanked by T-border sequences, a selectable marker gene and an origin of replication for *E. coli*, and another disarmed Ti plasmid without tumor-inducing genes but containing virulence genes. For wheat transformation, *Agrobacterium* strains, LAB4404(Ach_5) and C_{58}, have been used with a wide range of Ti and binary plasmids (H. D. Jones et al., 2005). Some of the important strains such as EHA_{101}, EHA_{105} (Ishida et al., 2015), AGL_0 and AGL_1 (Hensel et al., 2017), GV3101 (Peters et al., 1999), C58C1 (Wang et al., 2009), and LBA4404 (Supartana et al., 2006) have been widely used for wheat transformations. EHA_{101}, EHA_{105}, AGL_0, and AGL_1 contain hypervirulent Ti-plasmid 'pTiBo$_{542}$'. Additional virulence genes form another helper, Ti-plasmids, or other binary vectors in hypervirulent plasmid confer higher transformation efficiencies (Cheng et al., 2004). However, the incorporation of additional virulence genes is not always necessary to produce transgenic lines (Jones, 2005).

The first transgenic wheat generated by ATMT was reported in 1997 (Cheng et al., 1997). However, due to a low transformation efficiency in wheat, particle bombardment remained the method of choice as it generally gave higher transformation frequencies (Harwood, 2012). The comparison study between particle bombardment and ATMT on a large scale showed

a higher transformation efficiency with *Agrobacterium* (Hu et al., 2003). Also, the quality of ATMT was higher than the particle bombardment method. Higher quality usually refers to high single-copy insertion without additional backbone sequences or other rearrangements and with stable expression of transgene over a generation (Harwood, 2012). Generally, most of the literature shows ATMT efficiencies of around 5%. However, the efficiencies have been greatly improved. Risacher et al., (2009) reported efficiencies up to 30%, and Ishida et al., (2015) reported efficiencies of 40-90% for ATMT in wheat. Many approaches have been carried out to increase the DNA delivery by *Agrobacterium*. For instance, sonication and vacuum infiltration improved the transformation in barley (Shrawat et al., 2007). Centrifugation of immature embryos and heat improved transformation efficiency in rice and maize (Hiei & Komari, 2006). The addition of virulence genes on the plasmid backbone improved the rice transformation (Vain et al., 2004). Desiccation treatment of explant after co-culture was found to increase the efficiency of T-DNA delivery (Cheng et al., 2003). Over-expression of histone proteins (H2A-1) in *Arabidopsis* increased the susceptibility to *Agrobacterium* and increased transformation frequency (Gelvin, 2010). Having knowledge and understanding of the role of plant genes in transformation along with all these different factors can contribute greatly to increasing the transformation efficacy (Harwood, 2012).

Tissue culture response and regeneration

Plants show remarkable developmental plasticity to cope with various physical damage caused by biotic or abiotic factors. They respond to the physical wound by proliferating cells adjacent to the injury site to form a soft tissue cushion called callus and cover the cut surface (Sugiyama, 2015). During the wound healing process, these cellular changes were referred to as cell dedifferentiation in early studies. Dedifferentiation is the process by which specialized cells

lose their differentiated character and rejuvenate (Bloch, 1941, 1952). The origin of *in-vitro* plant tissue culture can be traced back to 1902 when Gottlieb Haberlandt hypothesized the idea of culturing vegetative cells of higher plants and plant cells as an autonomous "elementary organism" (Sugiyama, 2015). Subsequently for about 40 years, *in-vitro* culture was only possible with meristematic materials such as root tips and buds, which can be considered as organ culture rather than tissue culture. During the late 1930s, unlimited growth of cells derived from procambial tissue of *Nicotiana glauca x N. langsdorffi* was reported (White, 1936). The long-term maintenance of cell proliferation from normal carrot tissues in culture medium containing indole-3-acetic acid (IAA) was also reported after the 5 years of IAA identified as auxin. Identification of additional phytohormone, cytokinin, drastically changed plant tissue culture techniques in the 1950s (Miller et al., 1955, 1956). High and low ratios of kinetin to IAA favored shoot and root formation respectively. High concentrations of both kinetin and IAA produced an unorganized mass of cells (Skoog & Miller, 1957). The term "callus" originally used for cushion cells around wound healing was extended to include an unorganized mass of dividing cells induced and maintained in presence of phytohormones.

Exogenous phytohormones induce in-vitro organogenesis during tissue culture (Christianson & Warnick, 1985). Regenerants from organogenesis produced only shoot and root but lacked cotyledons and hypocotyls. Regeneration of the entire plant became possible after somatic embryogenesis. Somatic embryogenesis in-vitro was first reported from cultured carrot cells (Steward et al., 1958). This finding established the concept of "cellular totipotency" which means the somatic plant cells have the potential to differentiate into all cells that constitute the whole plant (Steward, 1968). Somatic embryogenesis may be either direct or indirect. Direct somatic embryogenesis is induced on the surface of tissue explants directly without an

intervening callus stage. In indirect somatic embryogenesis, tissue is first cultivated on the high auxin-containing medium to activate cell division and induce embryogenic cells. Then resultant cell mass is transferred to media with reduced or without auxin where somatic embryogenesis occurs (Thorpe & Stasolla, 2001). In both direct and indirect processes, somatic cells dedifferentiate and acquire embryogenic competence (Fehér et al., 2003; Namasivayam, 2007).

Quantitative trait loci (QTL) and genetic markers

Phenotypic traits which are controlled by many genes are known as quantitative traits and the regions within genomes that contain genes associated with a particular quantitative trait are known as quantitative trait loci (QTL) (Collard et al., 2005). A quantitative trait can be measured but depends on the cumulative action of many genes and their interactions with environments, producing a continuous distribution of phenotypes that can vary among the individuals (Sham et al., 2002). The concept of QTL mapping was first described by Sax (1923) using the association of complex bean seed size trait with simple monogenic seed color trait. Thoday (1961) further suggested that mapping of QTL associated with complex traits could be eventually possible by studying the segregation of simply inherited monogenes. The modern QTL mapping technique is based on the same concept that defined sequences of molecular markers act as linked monogenic markers.

Genetic markers are specific DNA sequences that represent genetic differences between individual organisms or species. They do not affect the phenotype of the trait and act as the 'flags' for the target gene (Collard et al., 2005). The three major types of genetic markers are morphological, biochemical and DNA markers (Jones et al., 1997). Morphological markers are usually visual characters such as seed shape, flower color, pigmentation, etc. Biochemical markers are isozymes that can be differentiated by electrophoresis and specific staining. The use

of morphological and biochemical markers is limited because of their limited number and is affected by environmental factors. In contrast, DNA markers are abundant and not influenced by environmental factors (Winter & Kahl, 1995). They are formed by different types of DNA mutations such as point mutations, rearrangements, or errors in replication of tandemly repeated DNA (Paterson et al., 1991). DNA markers that can differentiate species are polymorphic whereas markers that cannot distinguish between genotypes are monomorphic. Polymorphic DNA markers can either be dominant or codominant. Codominant markers can differentiate between homozygous and heterozygous whereas dominant markers cannot (Collard et al., 2005).

Based on the detection method, DNA markers can be further divided into three major categories: (1) restriction fragment length polymorphisms (RFLP) based on restriction digestion and probe hybridization (Williams, 1989); (2) polymerase chain reaction (PCR) based DNA markers consist of a large number of techniques such as random amplified polymorphic DNA (RAPD) (Williams et al., 1990), amplified fragment length polymorphisms (AFLP) (Vuylsteke et al., 2007), inter simple sequence repeats (ISSRs) (Godwin et al., 1997), microsatellites or simple sequence repeats (SSRs) (Akkaya et al., 1992), cleaved amplified polymorphic sequence (CAPS) (Konieczny & Ausubel, 1993); and (3) single nucleotide polymorphisms (SNP) based on DNA sequence (Ganal et al., 2009).

RFLP markers were the most popular markers during the 1980s before PCR-based markers were discovered in the 1990s. RFLP is a highly reliable codominant marker that can distinguish between heterozygotes and homozygotes. Disadvantages of RFLP include labor-intensive and time consuming, high cost, use of radioisotopes, and need for a large amount of DNA (Garrido-Cardenas et al., 2018). PCR-based markers avoid radioisotope hybridization and need a small amount of DNA to generate a high level of polymorphisms. After the availability of

DNA sequencing, sequence-based DNA markers were used for higher throughput and greater genome coverage. SNP arises by the single nucleotide mutations such as transitions, transversions, insertions, and deletions at a specific locus in the DNA sequence (Lander, 1996). Because of their abundance throughout the genome, genomic stability, and amenability to high-throughput automated analysis, they are the most preferred genotyping approach. Recently, high-density SNP genotyping arrays with about 90,000 gene-associated SNPs have been a powerful tool to study genetic polymorphisms in allohexaploid and allotetraploid wheat populations (Wang et al., 2014).

Mapping population and linkage map

Linkage-based QTL mapping depends on the well-defined populations developed by crossing two parents with clear contrasting differences in phenotypic traits of interest. Population sizes of 50 to 250 individuals are generally used in the preliminary genetic mapping (Mohan et al., 1997). For high-resolution mapping, larger populations are required. In self-pollinating species, parents used for making populations are usually highly homozygous. Within a given plant species, several different types of populations have been used for mapping. Each population type has its own advantages and disadvantages (Paterson, 1996). For self-pollinating species, the simplest types of populations are F_2 populations and backcross (BC) populations. Easy to construct and less time to develop are the major advantages of F_2 and BC populations. Individual F_2 plants can be self-pollinated to create recombinant inbred (RI) lines, which are made up of a sequence of homozygous lines with a different combination of chromosomal segments from the original parents. The time necessary to produce RI populations is the most significant disadvantage, as six to eight generations are normally required. Double haploid (DH) populations can be produced by regenerating plants from anther/microspore culture and doubling

their chromosomes. However, this method is limited to plants that are amenable to tissue culture. Both RI and DH populations produce homozygous lines that can be reproduced without genetic change which allows the experiment to be replicated across different locations and years.

The basic principle of 'QTL mapping' is that genes and markers segregate during meiosis (sexual reproduction) via chromosome recombination (called crossing-over), allowing for their study in the progeny (Paterson, 1996). Genes and markers that are tightly linked will be transferred together from parent to progeny more frequently than genes or markers that are positioned further apart. A segregating population has a mixture of both parental and recombinant genotypes. Recombination fractions may be calculated using the frequency of recombinant genotypes, which can be used to infer the genetic distance between markers. The relative order and distances between markers can be estimated by analyzing marker segregation– the lower the frequency of recombination between two markers, the closer they are on a chromosome. Mapping functions are used to convert recombination fractions into map units termed centiMorgans (cM). Linkage maps are produced by analyzing multiple segregating markers. Like signs or landmarks along a motorway, linkage maps show the location and relative genetic distances between markers along chromosomes. Due to varying recombination over the length of the chromosome, there is no exact link between the recombination distance and the physical distance that is represented in base pairs. The major application of linkage maps is to locate chromosomal regions containing genes and QTL linked to traits of interest (Collard et al., 2005; Kumar, 1999; Semagn et al., 2010).

Mapping QTL for tissue culture response and regeneration

Plant regeneration from tissue cultures is influenced by the genotype, physiological status of the donor plant, the plant organ used as an explant, the culture medium, and the interactions

between them (Bolibok & Rakoczy-Trojanowska, 2006). Different studies have suggested that tissue culture response (TCR), which is differentiation and regeneration, is genetically controlled in hexaploid wheat (Lazar et al., 1983; Shimada, 1978). Genetic control of plant regeneration can be qualitative (Reisch & Bingham, 1980), as well as quantitative (Bolibok & Rakoczy-Trojanowska, 2006). Different chromosome arms are associated with varying degrees of TCR which indicated that many genes were responsible (Felsenburg et al., 1987; Galiba et al., 1986; Henry, Vain, et al., 1994). To date, immature embryos are considered the best explants because of their high regeneration ability and are used often for wheat transformation (Jia, Yu, et al., 2009). Friable embryogenic calli (Type II) have better regeneration ability and have been the preferred type of regenerable tissue cultures. Only a minority of the genotypes are capable of developing type-II calli under standard culture conditions (Loyola-Vargas & Ochoa-Alejo, 2016).

Using substitution lines, ditelosomic lines, nullisomic-tetrasomic, and monosomic lines in wheat, chromosomes 4B, 2D, 7B, 7D, 1D, 6BL, 2B, and 2AL have been identified for QTL associated with immature embryo TCR (Felsenburg et al., 1987; Galiba et al., 1986; Kaleikau et al., 1989; Mathias & Fukui, 1986). QTL for plant regeneration from callus derived from immature embryos have been mapped on chromosome 2B and 2D (Amer et al., 1992; 1996; 1997). QTL controlling green-point formations in calli from immature embryos have been detected on chromosome 2AS (Henry, et al., 1994). TCR is also highly influenced by various non-genetic factors such as the growing environment. Even for the same lines of wheat grown in the field and environmental chamber, different quantitative trait loci (QTL) associated with regeneration from immature embryos were mapped (Galiba et al., 1986). QTL analysis of TCR-traits in wheat can be compared to the barley and maize using reference maps or the presence of

common markers due to the syntenic relationships. However, it is still difficult to precisely relate the QTL of interest (Bolibok & Rakoczy-Trojanowska, 2006).

With the advent of DNA-based molecular marker systems and the development of QTL mapping technology, it has become easier to understand the complex traits and characterize their map position and gene function. Finding the molecular markers associated with a QTL of interest has been a valuable tool for the characterization and utilization of any phenotype. Marker-assisted breeding has been used to transfer the high culture response QTL to nonresponsive genotypes of maize, resulting in germplasm with better tissue culture response (Lowe et al., 2016). Such markers can also be used in wheat for marker-assisted recurrent backcross breeding to transfer genes for high plant regeneration from highly responsive genotypes to recalcitrant ones (Bolibok & Rakoczy-Trojanowska, 2006).

CRISPR/Cas9 as a bacterial and archaeal immunity

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) is part of a bacterial and archeal adaptive defense system that has evolved to protect from the invading viruses and plasmids (Wiedenheft et al., 2012). This immune system is mediated by the small RNAs for the sequence-specific recognition and silencing of foreign nucleic acids. CRISPR-Cas loci consist of alternate short partially palindromic DNA repeats occurring at regular intervals and variable spacer sequences. These loci are flanked by accompanying Cas genes. The spacer sequences are derived from the viruses, plasmids, or other invaders from the past (Bolotin et al., 2005; Mojica et al., 2005; Pourcel et al., 2005). The CRISPR-Cas defense process occurs in three steps. The first adaptive step involves the foreign nucleic acid recognition and subsequent integration of short fragments of a foreign sequence, referred to as protospacer, into the proximal end of the CRISPR array. A very short, conserved nucleotide sequence in the

immediate vicinity of the protospacer, known as a protospacer adjacent motif (PAM), is the recognition motif required for the acquisition of foreign DNA fragments (Bhaya et al., 2011; Terns & Terns, 2011; Wiedenheft et al., 2012). In the second expression and interference step, a primary transcript known as pre-CRISPR RNA (pre-crRNA) is transcribed from the CRISPR locus. Next, a specific endonuclease cleaves the pre-crRNAs into small CRISPR RNAs (crRNAs). In the final step, Cas-nucleases are directed at the sites complementary to protospacer of invading viral or plasmid sequence by crRNA, and foreign nucleic acid is silenced (Brouns et al., 2008; Jiang & Doudna, 2015; Makarova et al., 2011; van der Oost et al., 2009; J. Zhang et al., 2012).

Based on the unique set of Cas proteins along with crRNA, the CRISPR system has been classified into six distinct types (I-VI) (Makarova et al., 2015; Shmakov et al., 2015). Cas9 (formerly Csn1) is the hallmark of type II systems. In type II systems, annealing of trans-activating crRNA (tracrRNA) to the repeat sequences of the pre-crRNA is required for primary processing. Then double-stranded RNA (dsRNA) is subsequently cleaved by host RNase III for crRNA maturation. Cas9 is also involved in the crRNA maturation process (Deltcheva et al., 2011). The tracrRNA:crRNA dual RNA directs the Cas9 to the complementary sequence of crRNA and cleaves DNA 3-nucleotide upstream of the PAM sequence. The DNA strand that is complementary to the 20-nucleotide sequence of the crRNA is cleaved by the HNH domain and the strand opposite the complementary strand is cleaved by the RuvC-like domain of Cas9 (Gasiunas et al., 2012; Jinek et al., 2012). Although double-stranded DNA (dsDNA) binding and cleavage by Cas9 is the hallmark of the type II system, Cas9 can also recognize and cleave single stranded RNA (ssRNA) by RNA-guided mechanism which is independent of a PAM sequence in the target RNA (Strutt et al., 2018).

CRISPR/Cas9 as a genome engineering tool

Since the discovery of DNA double helix and the central dogma of molecular biology, many different technologies have been developed for targeted and precise genome editing. For this purpose, the molecular machinery consists of two major parts in general: a DNA-binding domain that mediates sequence-specific DNA recognition and binding, and an effector domain that regulates transcription or enables DNA cleavage near the binding site (Wang et al., 2016). If unrepaired, DNA double strand breaks (DSBs) can cause genomic instability and cell death. There are several pathways to repair double-strand DSBs in eukaryotes. Error-free, template-dependent homologous recombination (HR) (Sung & Klein, 2006) and the error-prone, template-independent non-homologous end joining (NHEJ) (Chang et al., 2017) are the two major pathways to repair DSBs. Other repair pathways such as microhomology mediated end joining (MMEJ) and single strand annealing (SSA) are more error-prone. Introducing double strand break by the recognition of the specific DNA sequences using sequence-specific endonuclease can activate the natural DNA repair mechanism and greatly increase the rate of gene modification at the desired sequence (Plessis et al., 1992; Rouet et al., 1994; Rudin et al., 1989; Wang et al., 2016).

For targeted genome editing, nuclease mediated DSBs approaches have been broadly explored. Meganucleases, also known as homing nucleases, are among the first classes of nucleases used for site-specific gene editing purposes. Meganucleases recognize the long nucleotide sequences occurring only once within a genome and can induce DSBs at the targeted site. However, the probability of finding a meganuclease able to cut a given site is very low, and modifying the recognition sequence through protein engineering is labor-intensive (Grizot et al., 2010; Silva et al., 2011). Other examples for nuclease mediated DSBs include zinc-finger

nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), in which the DNA-binding domain of transcription factors has been fused with the nuclease domain of restriction enzyme FokI. In these two systems, transcription factors can bind to paired adjacent sequences at the target site of DNA and site-specific nucleases form a dimer that activates the nuclease activity, resulting in DSBs near their binding sites. Since these transcription factors are the proteins, targeting and binding to a new site, also requires engineering and cloning a new protein.

In contrast to the above-mentioned nucleases, Cas9 is an RNA-guided nuclease that does not require protein engineering for every DNA target site to be modified. The only change in guide RNA sequence as per the target site is required, which makes this system simpler and more efficient (Doudna & Charpentier, 2014). Cas9 can specifically bind to the target DNA site using Watson-Crick base pairing between the crRNA and DNA, and direct interaction between Cas9 and PAM of DNA. To simplify the system, tracrRNA and crRNA duplex are fused into a chimeric single guide RNA (sgRNA) for gene editing purposes. The 20-nucleotide sequence of sgRNA at 5' end recognizes the DNA target site by Watson-Crick base pairing, and the double stranded structure at 3' end binds to Cas9 (Jinek et al., 2012). Although the term “CRISPR” is not directly related to genome engineering, “CRISPR/Cas9” has been used for the sgRNA-Cas9 system (Doudna & Charpentier, 2014).

Wide hybridization between wheat and maize for production of double haploid plants

Conventional breeding techniques have been used for a long time to bring desired alleles in a single elite variety using genetic crosses of parents with distinct traits. This process involves laborious and time-consuming background screening of large populations. It requires 8-10 generations to fix the target alleles and may be more difficult with a low recombination rate

throughout the crop's genome and for the trait with linkage drag, where the undesirable traits are linked with the desired alleles (Li et al., 2017; Peng et al., 2014). Complete homozygosity at all alleles throughout the genome can be achieved by the process of haploid induction (HI) to generate haploid lines. Sterile haploids (n) should be converted to fertile double haploid (DH) (2n), which can be done by artificial chromosome doubling using an anti-microtubule drug such as colchicine (Kasha, 2005). The DH technology can produce pure homozygous plants within two generations as compared to up to eight generations using conventional selfing and backcrossing. This can save a tremendous amount of time and significantly speeds up the generations of pure homozygous lines (Ren et al., 2017). However, regardless of the approach utilized to generate entire homozygous or isogenic lines, linkage drag remains a challenge for breeding efforts (Bhowmik & Bilichak, 2021).

Haploids in higher plants can occur naturally. The first natural occurrence of sporophytic haploid angiosperm with cytological proof was found in 1921 by Dorothy Bergner in weed species *Datura stramonium* (Blakeslee et al., 1922). *Triticum compactum var. humboldtii* was the first haploid from the cereal species reported by Gaines & Aase, 1926. The occurrence of haploids from at least 71 species, representing 39 genera in 16 families of angiosperms were first recorded by Kimber & Riley (1963) and that number has increased significantly ever since (Dunwell, 2010). Parthenogenesis and uniparental chromosome elimination are the two major mechanisms for *in-vivo* HI (Comai & Tan, 2019). In flowering plants, sexual reproduction is characterized by double fertilization that consists of two parallel fusions of male and female gametes. The diploid embryo is formed by the fusion of male and female gametes. At the same time, the diploid nucleus of the central cell is fertilized by a second haploid male gamete to form a triploid endosperm. During parthenogenesis, the embryo develops from the egg cell within the

embryo sac without the sperm nucleus. This process occurs at low frequency and such rare haploids are difficult to detect among the normal diploid seed offspring (Kendall, 1930). The occurrence of female haploid seeds of angiosperms germinating with two or more seedlings known as 'twin-seedlings' are more common and widely used (Carl & Yawney, 1972; Dunwell, 2010). Parthenogenesis can result in either pseudogamous or autonomous endosperm (with or without central cell fertilization, respectively) and the embryo develops through the process called apomixis that is in absence of meiosis.

Uniparental chromosome elimination, also referred to as selective chromosome elimination, frequently results in haploids of one parent using wide hybridization and is one of the most effective methods successfully used in many species for HI (Wędzony et al., 2009). This phenomenon was first discovered in barley. *Hordeum vulgare* haploids were identified with crosses between *H. vulgare* and *H. bulbosum* (Kasha & Kao, 1970). This technique is routinely used in cereal breeding programs to produce haploids following pollination with maize (Sidhu et al., 2006) or other distantly related cereal species (Komeda et al., 2007; Pratap et al., 2005). Various hypotheses have been presented for the explanation of uniparental chromosome elimination. For instance, asynchronous synthesis of nucleoprotein leads to the loss of the most retarded chromosomes (Bennett et al., 1976; Laurie & Bennett, 1989). Other hypotheses include multipolar spindles formation (Subrahmanyam & Kasha, 1973), host-specific nuclease degradation of alien chromosomes (Davies, 1974), spatial separation of chromosomes during interphase (R. A. , Finch & Bennett, 1982; Linde-Laursen & Bothmer, 1999), and metaphase (Schwarzacher-robinson et al., 1987) of the cell cycle, and parent-specific inactivation of centromeres (R. A. Finch, 1983; Kim et al., 2011; Mochida et al., 2011).

Artificially, haploids in wheat can be produced *in vivo* or *in vitro* by androgenesis, gynogenesis, and wide hybridization (Niu et al., 2014). Androgenesis, also known as anther culture, is an *in vitro* process in which microspore cells (n) develop into an embryo-like structure that further develops into haploid plantlets (Jauhar et al., 2009). Production of haploids through anther culture was first reported in *Datura innoxia* Mill (Guha & Maheshwari, 1964). Gynogenesis, also known as megaspore culture, produces haploids through parthenogenesis (Yang & Zhou, 1982). Large-scale production of haploids is limited due to difficulty in isolating and a small number of megaspores in the plant (Kristóf & Imre, 1996; Wu et al., 2004). As paternal chromosome elimination results only in maternal haploid genome, haploid production through wide hybridization is also considered as gynogenesis (Niu et al., 2014). To produce wheat haploids using wide hybridization, *H. bulbosum* was used in earlier studies (Barclay, 1975; Pickering & Morgan, 1985). However, *H. bulbosum* is limited only to compatible wheat genotypes because of sensitivity to dominant crossability inhibitor genes Kr1 and Kr2, located on the chromosomes 5B and 5A of most wheat cultivars. Kr1 and Kr2 were insensitive to maize and maize pollen could germinate and elongate on wheat stigma and fertilize wheat oocyte and polar nucleus (Laurie & Bennett, 1986, 1988). This led to the establishment of a new wide hybridization technique between wheat and maize and used to produce haploid plants from many commercial wheat cultivars and hybrids (Laurie et al., 1990; Laurie & Bennett, 1988; Laurie & Reymondie, 1991; Suenaga & Nakajima, 1989). Other two crossability inhibitor genes, K3 in Chinese common wheat landrace J-11 (Krolow, 1970) and K4 in Chinese landrace Sichuan White Wheat Complex (Zheng et al., 1992), on chromosomes 5D and 1A were also reported in wheat. Kr1 and Kr2 have no effect on maize crossability in durum wheat, making maize pollination a viable and effective strategy for producing DH. After fertilization of wheat with

maize pollen, zygotes contain one complete chromosome set of each parent. However, maize chromosomes have poorly defined centromeres and less affinity for spindle microtubules in zygotes and young embryos. After the first few cell division cycles, maize chromosomes are lost, and cells contain the haploid complement of wheat chromosomes. The endosperm is either absent or highly abnormal (Laurie & Bennett, 1986, 1988).

Although maize is mostly unaffected by wheat Kr genes, many other factors play an important role in the efficacy of DH production in the wheat × maize system. These factors include genotypes of wheat and maize, temperature, photoperiod, light intensity, the position of spikelet, type and concentration of plant hormones applied, embryo rescue culture media, colchicine concentration, and plant conditions during colchicine treatment (Laurie & Bennett, 1989; Suenaga & Nakajima, 1989; Wędzony et al., 2009).

Haploid induction (HI) and gene editing using wide hybridization

Natural HI systems have been used to develop double haploids in maize (Coe, 2015), barley (Kasha & Kao, 1970), tobacco (Burk et al., 1979), and wheat (Laurie & Bennett, 1988). HI also can be artificially achieved by direct manipulation of centromere-specific histone (*CENH3*). The chromosomes from *cenh3* null mutants were eliminated in haploid progeny produced by crossing *cenh3* null mutants and wild-type *Arabidopsis thaliana* plants. Since *CENH3* is universal in eukaryotes, this system may be used in any plant species (Ravi & Chan, 2010). The commercial feasibility of this system in wheat with an approximately 7% HI rate has been identified by screening genome-edited *TaCENH3α*-heteroallelic combinations. Heterozygous lines triggered higher HI as compared to null homozygous lines (Lv et al., 2020). Two significant QTL, *qhir1* and *qhir8*, associated with HI were identified in maize. The causative allele for *qhir1* was found to be a 4-nucleotide insertion at carboxy (c)-terminal coding

region of *MATRILINEAL (MTL)*, also referred to as *NOT LIKE DAD (NDL)* or *Patatin-like Phospholipase A* (*ZmPLA1*) (Gilles et al., 2017; Kelliher et al., 2017; Liu et al., 2017). For *qhir8*, a single amino acid substitution in the first predicted transmembrane domain of the DOMAIN OF UNKNOWN FUNCTION 679 (DMP) resulted in a 2-3-fold increase in HI rate and 5-6-fold increase in presence of *mtl/zmpla1/nld* (Zhong et al., 2020).

Recently, three different studies reported simultaneous HI and gene editing using CRISPR/Cas9 in plants. This method is named "Haploid Inducer-Mediated Genome Editing" or HI-Edit (Budhagatapalli et al., 2020; Kelliher et al., 2019; Wang et al., 2019). The basic principle relies on the transient expression of Cas9/gRNA cassette in female gametes following pollination and fertilization by maize pollen carrying the transgene. There is no transmission of the transgene into following generations because the spermatid chromosomes are selectively eliminated following fertilization using this method. But the haploid plant may contain the edited gene that is targeted by Cas9/gRNA construct during transient expression. Following chromosome doubling, homozygous DH lines with edited target gene can be generated in a single generation (Budhagatapalli et al., 2020; Kelliher et al., 2019; B. Wang et al., 2019).

In this approach for editing wheat genes using maize pollen expressing CRISPR/Cas9, mutations can be induced at different phases of cell cycle before and after the zygotic mitosis before the elimination of maize chromosomes. Mutations induced during the G1, or early S phase are more likely due to pre-expression of Cas9 and gRNA in the sperm rather than zygotic *de novo* transgene expression. Embryos with such mutations are expectedly non-chimeric. In contrast, after chromatid duplication during G2 phase, mutations can occur in one chromatid or independently in either of the sister chromatids. In this case, the daughter cell with the mutated sister chromatid itself undergoes S phase during first mitosis and the mutated allele becomes

genetically fixed across the two sister chromatids. Whereas daughter cells with non-mutated chromatid or differently mutated chromatid give rise to genetically different alleles. As a result, chimeric embryos produced through mutagenesis during the G2 phase can be expected (Budhagatapalli et al., 2020). This shows that the time of maize chromosome remaining during zygotic stage elimination plays a vital role to induce the targeted mutations.

Fusarium head blight resistance in wheat

Fusarium head blight (FHB) is a devastating fungal disease of wheat and barley worldwide. Among several *Fusarium* spp. that cause FHB, *Fusarium graminearum* Schwabe (syn. Giberella zeae Schw. [Petch]) is the predominant causative agent in North America. It can also infect maize and barley (McMullen et al., 2012). This hemibiotrophic fungi directly infects the spikes, resulting in a complete loss of grain yield during severe epidemics. Also, they produce mycotoxins such as deoxynivalenol (DON) that results in substantially discounted or even rejected grains at sale due to safety concerns (Pestka & Smolinski, 2011). Using host resistance is the most effective way to mitigate the losses caused by FHB (Yang et al., 2005). Resistance to FHB in wheat is a quantitative trait that is usually controlled by genes of small effects and is greatly influenced by the environment. Approximately 500 quantitative trait loci (QTL) for FHB resistance distributed in all 21 chromosomes have been reported (Buerstmayr et al., 2020). Among them only 20% were described as major QTL by the authors and the numbers for minor QTL are likely an overestimation, which still need to be validated (Buerstmayr et al., 2020). However, some major-effect QTL for FHB resistance have been identified in common wheat such as *Fhb1* (Cuthbert et al., 2006), *Fhb2* (Cuthbert et al., 2007), *Fhb4* (Xue et al., 2010), *Fhb5* (Xue et al., 2011), and *Fhb7* (Wang et al. 2020).

Wangshuibai (WSB) and Sumai3 (S3) are the two widely used sources of FHB resistance. WSB is indigenous to the lower reaches of Yangtze valley, China, and S3 is a variety bred at the Suzhou Regional Institute of Agricultural Sciences located in the same region of Wangshuibai with unrelated pedigree. A major-effect QTL was mapped on the chromosome 3BS conferring type-II resistance (resistance to FHB spread within the spike) for both WSB and S3 (Anderson et al., 2001; Lin et al., 2006). The major resistance QTL from S3 was designated as *Fhb1* or *Qfhs.ndsu-3BS* which was originally mapped by RFLP analysis (Waldron et al. 1999) and validated by later studies (Anderson et al., 2001; Bai et al., 1999; Somers et al., 2011; Zhou et al., 2002). *Fhb1* has been regarded as the best source of FHB resistance in the world for half a century (Liu et al. 2006; Cuthbert et al. 2006). Reduction of 20-50% disease severity has been reported in near-isogenic lines (NILs) with *Fhb1* compared to those without *Fhb1* (Bernardo et al., 2012; Jin et al., 2013). The major-effect QTL from WSB was designated as *Qfhs.njau-3B* (Lin et al., 2006), and its introgression into susceptible cultivars greatly reduced diseased spikelet number and diseased rachis length (Xue et al., 2010). From the comparison study using fine mapping, *Fhb1* and *Qfhs.njau-3B* were found to be located in the same genomic region, with only three single nucleotide polymorphisms (SNPs) and were considered as the same FHB resistance allele in the *Fhb1* interval.

Recently, a gene (*TaPFT*) encoding a pore-forming toxin-like (PFT) protein with two agglutinin domains and an ETX/MTX2 toxin domain was identified as the one conferring FHB resistance at *Fhb1* locus (Rawat et al., 2016). However, other later studies contradict this *TaPFT* gene as the resistance gene in *Fhb1* because *TaPFT* is present in many highly susceptible accessions without *Fhb1*-mediated resistance (He et al., 2018). Another fine mapping and sequencing study reported only the *TaGDSL* gene which encodes for GDSL lipase

acylhydrolase, showed a pathogen dependent expression pattern among 28 genes revealed in non-recombining haplotype containing *Fhb1* (Schweiger et al., 2016). Later, two research groups reported that a deletion mutation in *TaHRC* gene which encodes for reticulum histidine-rich calcium binding protein confers *Fhb1* resistance to FHB in wheat (Li et al., 2019; Su et al., 2019).

Li et al., (2019) delimited the *Fhb1* QTL from WSB/S3 to 23.8-kb region with only two open reading frames, encoding a product annotated as histidine rich calcium-binding protein (His) and terpene synthase-like protein (TS). By comparing the sequence of WSB/S3 *Fhb1* region with PH691 (susceptible recurrent parent of R-43 NIL), 752-bp deletion in WSB/S3 sequence was discovered. The marker associated with this 752-bp deletion showed the highest correlation with FHB resistance in association analysis of 151 wheat cultivars. From haplotype analysis of these 151 wheat lines, only WSB/S3 haplotypes (His^R) showed resistance to FHB. Full-length cDNA analysis of His gene showed three and four transcript variants in PH691 and WSB respectively. Induced expression of His^R in WSB spikes during FHB inoculation suggested that His^R is responsible for the FHB resistance in WSB. The deletion in WSB His^R caused loss of the 3' splicing acceptor site and generation of an upstream new acceptor site. This resulted in the change of translational start codon which consequently produced 14 residues longer polypeptide compared to that of His^S from PH691. Transforming highly susceptible cultivar ND183 with a 5,164-bp His^R -containing genomic DNA sequence, resulted in better resistance to FHB than transgene-negative plants. Therefore, it was concluded that the 5' 752-bp deletion in His^R might be a gain-of-function mutation for FHB resistance (Li et al., 2019).

In another study done by Su et al. 2019, *TaHRC* was less expressed in resistant than in the susceptible NILs in RNA sequencing analysis, which agreed with previous studies (Hofstad

et al., 2016; Jia, Cho, et al., 2009; Schweiger et al., 2016). Similar disease severity was observed between the groups of RILs with both *TaPFT* and *TaGDSL* and those RILs without *TaPFT* and *TaGDSL*, which indicated that *TaPFT* and *TaGDSL* did not reduce the disease severity. However, two RIL groups with different *TaHRC* alleles, but the same *TaPFT* and *TaGDSL* alleles had the contrastingly different percentage of symptomatic spikelets per spike. Also, *TaPFT* gene expression was significantly reduced after FHB inoculation suggesting that *TaPFT* and *TaGDSL* can be ruled out from *Fhb1* candidate genes. Further, a 2,650-bp sequence of susceptible (*TaHRC-S*) alleles from susceptible NILs and a 2,041-bp sequence of resistant (*TaHRC-R*) alleles from resistant NILs were cloned. Sequencing of full-length cDNA of both susceptible (*TaHRC-S*) and resistant (*TaHRC-R*) alleles showed that *TaHRC-R* had a large deletion in the corresponding second intron and the beginning of the third exon that removed conserved splicing acceptor site (AG), the translation start codon (ATG) and a 22 bp-additional downstream sequence in the ORF. Knocking down the expression of *TaHRC-S* in susceptible cultivar Bobwhite resulted in significantly lower FHB severity which showed the association of high FHB susceptibility with higher expression of *TaHRC-S* and loss of function of *TaHRC-S* confers *Fhb1* resistance (Su et al., 2019).

The loss-of-function mutation of a *TaHRC-S* conferring FHB resistance and high expression of *TaHRC* induced by *F. graminearum* in susceptible wheat cultivars imply that *TaHRC* is most likely a susceptible gene that regulates the spread of FHB within a wheat spike (Su et al., 2019). Different cases of loss of function of disease susceptibility genes conferring disease resistance have been reported (Faris et al., 2013; Shi et al., 2016; Wang et al., 2014). This *TaHRC* is a new type of gene regulation disease resistance that is unique from the typical R-genes encoding intracellular nucleotide-binding leucine-rich-repeat (NB-LRR) proteins (van

Schie & Takken, 2014). Being conserved in cereal crops, *TaHRC* deserves further study to unravel new mechanisms for disease resistance in other cereal crops. As FHB resistance is controlled by multiple genes, *Fhb1* in combination with other minor resistance QTL from local wheat cultivars is the best approach for durable FHB resistance (Bai et al., 2018).

Tan spot resistance in wheat

Tan spot, also known as the yellow spot, is an economically important foliar disease in wheat. It is caused by the fungus *Pyrenophora tritici-repentis* (Died.) Drechs (anamorph: *Drechslera tritici-repentis*) (Died.) Shoemaker. The diseasse affects both common wheat and durum wheat and causes significant yield loss all over the world where wheat is cultivated (Lamari & Bernier, 1991). The fungus causes large tan-colored lesions often surrounded by chlorotic haloes in susceptible wheat cultivars and the lesions tend to coalesce to large areas of dead leaf tissue in highly susceptible genotypes. Lesions on the leaves directly affect the photosynthesis ability of plants that ultimately lead to yield loss (Faris et al., 2013). Tan spot causes pink smudge in grain and greatly affects the grain quality (Schilder & Bergstrom, 2011). Tan spot epidemics primarily coincided with the adoption of low or no-till farming practices to keep stubble residue in the soil for less soil erosion. The fungus can overwinter in stubble residue, thus this practice of retaining residue has led to an increase in disease incidence (Faris et al., 2013). Another reason for this fungus to cause significant damage in wheat is the acquisition of *ToxA* through horizontal gene transfer from *Parastagonospora nodorum* enabled *P. tritici-repentis* to produce a nectrotrophic effector known as Ptr ToxA (Friesen et al., 2006). *Parastagonospora* (*syn. Septoria, teleomorph Phaeosphaeria*) *nodorum* is a significant wheat pathogen that causes *Parastagonospora nodorum* blotch in many regions of the world (Solomon et al., 2006).

Host interactions with biotrophic pathogens can be explained by gene-for-gene relationships where the incompatible or resistant interaction is due to recognition of pathogen-produced effector (avirulence gene product) by a dominant host resistance (R) gene. The lack of pathogen recognition in these systems leads to a compatible interaction or susceptibility (Flor, 1956). But in the case of the necrotrophic pathogen, when an HST is recognized by a host sensitivity gene, a compatible interaction occurs, leading to susceptibility, whereas when an HST is not recognized by the host, an incompatible interaction occurs, leading to resistance. Resistance response is due to the lack of sensitivity gene in the host or lack of HST in the pathogen. As *P. tritici-repentis* is the necrotrophic pathogen, wheat-*P. tritici-repentis* system can be explained by the inverse of classical gene-for-gene model systems (Wolpert et al., 2003). However, wheat-*P. tritici-repentis* system is more complex than just an inverse gene-for-gene model because of the identification of resistance QTL for broad-spectrum or race non-specific HST produced by the pathogen (Chu et al., 2008; Faris et al., 2012; Faris & Friesen, 2005).

Based on the symptoms produced on a set of wheat differential genotypes, *P. tritici-repentis* can be classified into 8 races (Faris et al., 2013). Race 1 isolates cause both chlorosis and necrosis, race 2 isolates cause only necrosis, races 3 and 5 isolates cause only chlorosis (although on different host lines), while race 4 isolates are avirulent (Lamari et al., 1995). Additional 3 races have been discovered with varying virulence combinations present in races 1–5. Race 1 isolates have virulences from races 2 and3, race 6 isolates have virulences from races 3 and5, race 7 isolates have virulences from races 2 and5, and race 8 isolates have virulences from races 2,3, and 5. (Lamari & Strelkov, 2010). Ptr ToxA is a well-known host-selective toxin produced by races 1 and 2 (Lamari & Bernier, 2010; Tomas & Bockus, 1987; Tuori et al., 1995)

while Ptr ToxC and Ptr ToxB are produced by race 3 and 5 respectively (Effertz et al., 2002; Orolaza et al., 1995).

Ptr ToxA is a small, secreted protein (~13.2 kDa) that was the first HST to be characterized. It is transported to the cells of sensitive hosts and induces necrosis by disrupting the photosynthesis pathway (Ciuffetti et al., 2010). Interactions of ToxA and corresponding host sensitive gene *Tsn1* is the major virulence component for the disease development. Ptr ToxB is also a small, secreted protein (~6.5 kDa) whereas Ptr ToxC is a non-ionic, polar, low molecular mass molecule (Effertz et al., 2002; Strelkov et al., 1999). Unlike Ptr ToxA, the mechanisms of Ptr ToxB and Ptr ToxC are less known. Ptr ToxA and Ptr ToxB have been shown to activate the host resistance to biotrophic pathogens that usually results in hypersensitive response (HR) which include upregulation of WRKY transcription factors, receptor like-kinases and pathogenesis-related (PRR) proteins, activation of phenylpropanoid and jasmonic acid pathways, accumulation of reactive oxygen species, and photosystem disruption. However, both HSTs are important virulence factors associated with the tan spot disease development (W. Zhang et al., 2019).

Various approaches were made to map the host sensitivity gene to Ptr ToxA. Marker-saturated deletion-based physical maps showed that *Tsn1* is located within a gene-dense region of the wheat genome. This region was expected to have 11-fold higher recombination frequency than the genome-wide average which allowed for map-based cloning (Faris et al., 2000). The first high-resolution mapping of this region using AFLP markers delineated the *Tsn1* gene to a 0.2 cM and 0.8 cM interval in a hexaploid and durum wheat respectively (Haen et al., 2004). BAC contigs of 205 and 228 kb flanking *Tsn1* were assembled using an LDN BAC library (Cenci et al., 2003) and PCR-based markers were developed (Lu et al., 2006). BAC contigs were

further expanded and chromosome walking was conducted to assemble a physical map spanning the *Tsn1* locus. *Tsn1* was delineated to an ~350 kb region, and six genes were predicted that co-segregated with *Tsn1*. Further, two predicted genes were eliminated and delineated to 120 kb interval by association mapping using 386 *Triticum* accessions. The remaining four candidate genes were a hypothetical protein, a U2 small nuclear (sn) ribonucleoprotein (RNP) auxiliary factor, and, potentially, a single gene encoding serine/threonine protein kinase (S/TPK) nucleotide binding (NB), and leucine rich repeats (LRR) domains. Subsequent comparison of these four genes in wild-type wheat genotypes and ethyl methanesulfonate (EMS)-induced mutation studies revealed and validated that S/TPK-NB-LRR like gene as *Tsn1*. A total of 13 ToxA-insensitive mutants were identified from screening 2,115 M2 families. Sequence analysis of these EMS mutants indicated that S/TPK-NB-LRR like gene was mutated in each mutant containing missense, nonsense, and splice site mutations. Genotyping of the 386 *Triticum* accessions revealed that only Tox-A sensitive lines harbored the S/TPK-NB-LRR sequence, with six exceptions. These six lines had nonsense and frameshift mutations at different positions within the gene. These EMS-mutation studies verified that all three domains are essential for *Tsn1* function (Faris et al., 2010). The other two HST sensitivity genes before *Tsn1*, *Pc* from *Sorghum bicolor,* and *LOV1* from *Arabidopsis thaliana* were cloned and found to possess NB and LRR domains as classic plant R genes (Lorang et al., 2007; Nagy & Bennetzen, 2008). These sensitivity genes are examples showing that the necrotrophic pathogens are able to hijack and subvert resistance mechanisms acquired by plants to combat biotrophic pathogens (Faris et al., 2013; Shi et al., 2016). Loss of function of these sensitivity genes conferring disease resistance can be explored in many other important crops using gene editing tools like CRISPR/Cas9 to create disease resistant cultivars.

References

Ajmone Marsan, P., Lupotto, E., Locatelli, F., Qiao, Y. M., & Cattaneo, M. (1993). Analysis of stable events of transformation in wheat via PEG-mediated DNA uptake into protoplasts. *Plant Science*, *93*(1–2), 85–94.

Akkaya, M. S., Bhagwat, A. A., & Cregan, P. B. (1992). Length polymorphisms of simple sequence repeat DNA in soybean. *Genetics*, *132*(4), 1131–1139.

Anderson, J. A., Stack, R. W., Liu, S., Waldron, B. L., Fjeld, A. D., Coyne, C., Moreno-Sevilla, B., Fetch, J. M., Song, Q. J., Cregan, P. B., & Frohberg, R. C. (2001). DNA markers for Fusarium head blight resistance QTLs in two wheat populations. *Theoretical and Applied Genetics*, *102*(8), 1164–1168.

Bai, G., Kolb, F. L., Shaner, G., & Domier, L. L. (1999). Amplified length polymorphism markers linked to a major quantitative trait locus controlling scab resistance in wheat. *Phytopathology*, *89*(4), 343–348.

Bai, G., Su, Z., & Cai, J. (2018). Wheat resistance to Fusarium head blight. *Canadian Journal of Plant Pathology*, 40(3), 336-346.

Barclay, I. R. (1975). High frequencies of haploid production in wheat (Triticum aestivum) by chromosome elimination. *Nature 1975 256:5516*, *256*(5516), 410–411.

Becker, D., Brettschneider, R., & Lörz, H. (1994). Fertile transgenic wheat from microprojectile bombardment of scutellar tissue. *The Plant Journal*, *5*(2), 299–307.

ben Amer, I. M., Korzun, V., Worland, A. J., & Börner, A. (1997). Genetic mapping of QTL controlling tissue-culture response on chromosome 2B of wheat (Triticum aestivum L.) in relation to major genes and RFLP markers. *Theoretical and Applied Genetics 1997 94:8*, *94*(8), 1047–1052.

ben Amer, I. M., Worland, A. J., & Börner, A. (1996). The effects of whole chromosome substitutions differing in alleles for hybrid dwarfing and photoperiodic sensitivity on tissue culture response (TCR) in wheat. *Euphytica 1996 89:1*, *89*(1), 81–86.

Bennett, M. D., Finch, R. A., & Barclay, I. R. (1976). The time rate and mechanism of chromosome elimination in Hordeum hybrids. *Chromosoma 1976 54:2*, *54*(2), 175–200.

Bernardo, A. N., Ma, H., Zhang, D., & Bai, G. (2012). Single nucleotide polymorphism in wheat chromosome region harboring Fhb1 for Fusarium head blight resistance. *Molecular Breeding*, *29*(2), 477–488.

Bhaya, D., Davison, M., & Barrangou, R. (2011). CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation. *Annual review of genetics*, 45, 273-297.

Bhowmik, P., & Bilichak, A. (2021). Advances in gene editing of haploid tissues in crops. *Genes 2021, Vol. 12, Page 1410*, *12*(9), 1410.

Blakeslee, A. F., Belling, J., Farnham, M. E., & Bergner, A. D. (1922). A haploid mutant in the Jimson Weed, "Datura stramonium." *Science*, *55*(1433), 646–647.

Bloch, R. (1941). Wound healing in higher plants. *The Botanical Review 1941 7:2*, *7*(2), 110–146.

Bloch, R. (1952). Wound healing in higher plants. II. *The Botanical Review 1952 18:10*, *18*(10), 655–679.

Bolibok, H., & Rakoczy-Trojanowska, M. (2006). Genetic Mapping of QTLs for Tissue-Culture Response in Plants. *Euphytica 2006 149:1*, *149*(1), 73–83.

Bolotin, A., Quinquis, B., Sorokin, A., & Dusko Ehrlich, S. (2005). Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology*, *151*(8), 2551–2561.

Brenchley, R., Spannagl, M., Pfeifer, M., Barker, G. L. A., D'Amore, R., Allen, A. M., McKenzie, N., Kramer, M., Kerhornou, A., Bolser, D., Kay, S., Waite, D., Trick, M., Bancroft, I., Gu, Y., Huo, N., Luo, M. C., Sehgal, S., Gill, B., … Hall, N. (2012). Analysis of the bread wheat genome using whole-genome shotgun sequencing. *Nature 2012 491:7426*, *491*(7426), 705–710.

Brouns, S. J. J., Jore, M. M., Lundgren, M., Westra, E. R., Slijkhuis, R. J. H., Snijders, A. P. L., Dickman, M. J., Makarova, K. S., Koonin, E. v., & van der Oost, J. (2008). Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science*, *321*(5891), 960–964.

Budhagatapalli, N., Halbach, T., Hiekel, S., Büchner, H., Müller, A. E., & Kumlehn, J. (2020). Site□directed mutagenesis in bread and durum wheat via pollination by cas9/guide RNA□transgenic maize used as haploidy inducer. *Plant Biotechnology Journal*, *18*(12), 2376.

Buerstmayr, M., Steiner, B., & Buerstmayr, H. (2020). Breeding for Fusarium head blight resistance in wheat—Progress and challenges. *Plant Breeding*, 139(3), 429-454.

Burk, L. G., Gerstel, D. U., & Wernsman, E. A. (1979). Maternal haploids of nicotiana tabacum L. from seed. *Science*, *206*(4418), 585.

Carl, C. M., & Yawney, H. W. (1972). Multiple seedlings in Acer saccharum. *Bulletin of the Torrey Botanical Club*, *99*(3), 142.

Cenci, A., Chantret, N., Kong, X., Gu, Y., Anderson, O. D., Fahima, T., Distelfeld, A., & Dubcovsky, J. (2003). Construction and characterization of a half million clone BAC library of durum wheat (Triticum turgidum ssp. durum). *Theoretical and Applied Genetics*, *107*(5), 931–939.

Chang, H. H. Y., Pannunzio, N. R., Adachi, N., & Lieber, M. R. (2017). Non-homologous DNA end joining and alternative pathways to double-strand break repair. *Nature Reviews Molecular Cell Biology 2017 18:8*, *18*(8), 495–506.

Cheng, M., Fry, J. E., Pang, S., Zhou, H., Hironaka, C. M., Duncan, D. R., Conner, T. W., & Wan, Y. (1997). Genetic transformation of wheat mediated by Agrobacterium tumefaciens. *Plant Physiology*, *115*(3), 971–980.

Cheng, M., Hu, T., Layton, J., Liu, C. N., & Fry, J. E. (2003). Desiccation of plant tissues post-Agrobacterium infection enhances T-DNA delivery and increases stable transformation efficiency in wheat. *In Vitro Cellular & Developmental Biology - Plant 2003 39:6*, *39*(6), 595–604.

Cheng, M., Lowe, B. A., Spencer, T. M., Ye, X., & Armstrong, C. L. (2004). Factors influencing Agrobacterium-mediated transformation of monocotyledonous species. *In Vitro Cellular & Developmental Biology - Plant 2004 40:1*, *40*(1), 31–45.

Childe, V. G. (1951). The First Waggons and Carts—from the Tigris to the Severn. *Proceedings of the Prehistoric Society*, *17*(2), 177–194.

Christianson, M. L., & Warnick, D. A. (1985). Temporal requirement for phytohormone balance in the control of organogenesis in vitro. *Developmental Biology*, *112*(2), 494–497.

Christou, P. (1995). Strategies for variety-independent genetic transformation of important cereals, legumes and woody species utilizing particle bombardment. *Euphytica 1995 85:1*, *85*(1), 13–27.

Chu, C. G., Friesen, T. L., Xu, S. S., & Faris, J. D. (2008). Identification of novel tan spot resistance loci beyond the known host-selective toxin insensitivity genes in wheat. *Theoretical and Applied Genetics*, *117*(6), 873–881.

Ciuffetti, L. M., Manning, V. A., Pandelova, I., Betts, M. F., & Martinez, J. P. (2010). Host-selective toxins, Ptr ToxA and Ptr ToxB, as necrotrophic effectors in the Pyrenophora tritici-repentis–wheat interaction. *New Phytologist*, *187*(4), 911–919.

Clavijo, B. J., Venturini, L., Schudoma, C., Accinelli, G. G., Kaithakottil, G., Wright, J., Borrill, P., Kettleborough, G., Heavens, D., Chapman, H., Lipscombe, J., Barker, T., Lu, F. H., McKenzie, N., Raats, D., Ramirez-Gonzalez, R. H., Coince, A., Peel, N., Percival-Alwyn, L., … Clark, M. D. (2017). An improved assembly and annotation of the allohexaploid wheat genome identifies complete families of agronomic genes and provides genomic evidence for chromosomal translocations. *Genome Research*, *27*(5), 885–896.

Collard, B. C. Y., Jahufer, M. Z. Z., Brouwer, J. B., & Pang, E. C. K. (2005). An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. *Euphytica 2005 142:1*, *142*(1), 169–196.

Comai, L., & Tan, E. H. (2019). Haploid induction and genome instability. *Trends in Genetics*, *35*(11), 791–803.

Cuthbert, P. A., Somers, D. J., & Brulé-Babel, A. (2007). Mapping of Fhb2 on chromosome 6BS: A gene controlling Fusarium head blight field resistance in bread wheat (Triticum aestivum L.). *Theoretical and Applied Genetics*, *114*(3), 429–437.

Cuthbert, P. A., Somers, D. J., Thomas, J., Cloutier, S., & Brulé-Babel, A. (2006). Fine mapping Fhb1, a major gene controlling fusarium head blight resistance in bread wheat (Triticum aestivum L.). *Theoretical and Applied Genetics*, *112*(8), 1465–1472.

Davies, D. R. (1974). Chromosome elimination in inter-specific hybrids. *Heredity 1974 32:2*, *32*(2), 267–270.

de Cleene, M., & de Ley, J. (1976). The host range of crown gall. *The Botanical Review 1976 42:4*, *42*(4), 389–466.

Deltcheva, E., Chylinski, K., Sharma, C. M., Gonzales, K., Chao, Y., Pirzada, Z. A., Eckert, M. R., Vogel, J., & Charpentier, E. (2011). CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature 2011 471:7340*, *471*(7340), 602–607.

Díaz, I. (1994). Optimization of conditions for DNA uptake and transient GUS expression in protoplasts for different tissues of wheat and barley. *Plant Science*, *96*(1–2), 179–187.

Doudna, J. A., & Charpentier, E. (2014). The new frontier of genome engineering with CRISPR-Cas9. *Science*, *346*(6213).

Dubcovsky, J., & Dvorak, J. (2007). Genome plasticity a key factor in the success of polyploid wheat under domestication. *Science*, *316*(5833), 1862–1866.

Dunwell, J. M. (2010). Haploids in flowering plants: origins and exploitation. *Plant Biotechnology Journal*, *8*(4), 377–424.

Dvorak, J., Luo, M. C., Yang, Z. L., & Zhang, H. B. (1998). The structure of the Aegilops tauschii genepool and the evolution of hexaploid wheat. *Theoretical and Applied Genetics 1998 97:4*, *97*(4), 657–670.

Dvorak, J., Luo, M. C., & Akhunov, E. D. (2011). NI Vavilov's theory of centres of diversity in the light of current understanding of wheat diversity, domestication and evolution. *Czech J Genet Plant Breed*, 47, S20-S27.

Coe Jr, E. H. (1959). A line of maize with high haploid frequency. *The American Naturalist*, 93(873), 381-382.

Effertz, R. J., Meinhardt, S. W., Anderson, J. A., Jordahl, J. G., & Francl, L. J. (2002). Identification of a chlorosis-inducing toxin from pyrenophora tritici-repentis and the chromosomal location of an insensitivity locus in wheat. *Phytopathology*, *92*, 527–533.

Faris, J. D. (2014). Wheat domestication: Key to agricultural revolutions past and future. In Genomics of plant genetic resources (pp. 439-464). Springer, Dordrecht.

Faris, J. D., Abeysekara, N. S., McClean, P. E., Xu, S. S., & Friesen, T. L. (2012). Tan spot susceptibility governed by the Tsn1 locus and race-nonspecific resistance quantitative

trait loci in a population derived from the wheat lines Salamouni and Katepwa. *Molecular Breeding*, *30*(4), 1669–1678.

Faris, J. D., & Friesen, T. L. (2005). Identification of quantitative trait loci for race-nonspecific resistance to tan spot in wheat. *Theoretical and Applied Genetics*, *111*(2), 386–392.

Faris, J. D., Haen, K. M., & Gill, B. S. (2000). Saturation mapping of a gene-rich recombination hot spot region in wheat. *Genetics*, *154*(2), 823–835.

Faris, J. D., Liu, Z., & Xu, S. S. (2013). Genetics of tan spot resistance in wheat. *Theoretical and Applied Genetics*, *126*(9), 2197–2217.

Faris, J. D., Zhang, Z., Lu, H., Lu, S., Reddy, L., Cloutier, S., Fellers, J. P., Meinhardt, S. W., Rasmussen, J. B., Xu, S. S., Oliver, R. P., Simons, K. J., & Friesen, T. L. (2010). A unique wheat disease resistance-like gene governs effector-triggered susceptibility to necrotrophic pathogens. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(30), 13544–13549.

Fehér, A., Pasternak, T. P., & Dudits, D. (2003). Transition of somatic plant cells to an embryogenic state. *Plant Cell, Tissue and Organ Culture 2003 74:3*, *74*(3), 201–228.

Feldman, M., & Kislev, M. E. (2007). Domestication of emmer wheat and evolution of free-threshing tetraploid wheat. *Israel Journal of Plant Sciences*, *55*(3–4), 207–221.

Fellers, J. P., Guenzi, A. C., & Taliaferro, C. M. (1995). Factors affecting the establishment and maintenance of embryogenic callus and suspension cultures of wheat (Triticum aestivum L.). *Plant Cell Reports 1995 15:3*, *15*(3), 232–237.

Felsenburg, T., Feldman, M., & Galun, E. (1987). Aneuploid and alloplasmic lines as tools for the study of nuclear and cytoplasmic control of culture ability and regeneration of scutellar calli from common wheat. *Theoretical and Applied Genetics 1987 74:6*, *74*(6), 802–810.

Finch, R. A. (1983). Tissue-specific elimination of alternative whole parental genomes in one barley hybrid. *Chromosoma 1983 88:5*, *88*(5), 386–393.

Finch, R.A., and Bennett, M.D. (1983). The mechanism of somatic chromosome elimination in Hordeum. *In Kew Chromosome Conference II: Proceedings of the Second Chromosome Conference, P.E. Brandham, ed (London: Allen & Unwin)*, pp. 146–153.

Flor, H. H. (1956). The complementary genic systems in flax and flax rust. *Advances in Genetics*, *8*(C), 29–54.

Friesen, T. L., Stukenbrock, E. H., Liu, Z., Meinhardt, S., Ling, H., Faris, J. D., Rasmussen, J. B., Solomon, P. S., McDonald, B. A., & Oliver, R. P. (2006). Emergence of a new disease as a result of interspecific virulence gene transfer. *Nature Genetics 2006 38:8*, *38*(8), 953–956.

Fronzes, R., Christie, P. J., & Waksman, G. (2009). The structural biology of type IV secretion systems. *Nature Reviews Microbiology 2009 7:10*, *7*(10), 703–714.

Gaines, E. F., & Aase, H. C. (1926). A haploid wheat plant. *American Journal of Botany*, *13*(6), 373.

Galiba, G., Kovacs, G., & Sutka, J. (1986). Substitution analysis of plant regeneration from callus culture in wheat. *Plant Breeding*, *97*(3), 261–263.

Ganal, M. W., Altmann, T., & Röder, M. S. (2009). SNP identification in crop plants. *Current Opinion in Plant Biology*, *12*(2), 211–217.

Garrido-Cardenas, J. A., Mesa-Valle, C., & Manzano-Agugliaro, F. (2018). Trends in plant research using molecular markers. *Planta*, *247*(3), 543–557.

Gasiunas, G., Barrangou, R., Horvath, P., & Siksnys, V. (2012). Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(39).

Gelvin, S. B. (2010). Plant proteins involved in Agrobacterium-mediated genetic transformation. *Annual review of phytopathology*, 48, 45-68.

Gill, B. S., Li, W., Sood, S., Kuraparthy, V., Friebe, B. R., Simons, K. J., Zhang, Z., & Faris, J. D. (2007). Genetics and genomics of wheat domestication-driven evolution. *Israel Journal of Plant Sciences*, *55*(3–4), 223–229.

Gilles, L. M., Khaled, A., Laffaire, J.-B., Chaignon, S., Gendrot, G., Laplaige, J., Bergès, H., Beydon, G., Bayle, V., Barret, P., Comadran, J., Martinant, J.-P., Rogowsky, P. M., & Thomas Widiez, &. (2017). Loss of pollen-specific phospholipase NOT LIKE DAD triggers gynogenesis in maize. *The EMBO Journal*, *36*(6), 707–717.

Godwin, I. D., Aitken, E. A. B., & Smith, L. W. (1997). Application of inter simple sequence repeat (ISSR) markers to plant genetics. *ELECTROPHORESIS*, *18*(9), 1524–1528.

Grizot, S., Epinat, J. C., Thomas, S., Duclert, A., Rolland, S., Pâques, F., & Duchateau, P. (2010). Generation of redesigned homing endonucleases comprising DNA-binding domains derived from two different scaffolds. *Nucleic Acids Research*, *38*(6), 2006–2018.

Guha, S., & Maheshwari, S. C. (1964). In vitro production of embryos from anthers of Datura. *Nature 1964 204:4957*, *204*(4957), 497–497.

Haen, K. M., Lu, H., Friesen, T. L., & Faris, J. D. (2004). Genomic targeting and high-resolution mapping of the Tsn1 gene in wheat. *Crop Science*, *44*(3), 951–962.

Harwood, W. A. (2012). Advances and remaining challenges in the transformation of barley and wheat. *Journal of Experimental Botany*, *63*(5), 1791–1798.

He, Y., Wang, Q., Zeng, J., Sun, T., Yang, G. X., & He, G. Y. (2015). Current status and trends of wheat genetic transformation studies in China. *Journal of Integrative Agriculture*, *14*(3), 438–452.

He, Y., Zhang, X., Zhang, Y., Ahmad, D., Wu, L., Jiang, P., & Ma, H. (2018). Molecular characterization and expression of PFT, an FHB resistance gene at the Fhb1 QTL in wheat. Phytopathology, 108(6), 730-736.

Henry, Y., Marcotte, J. L., & de Buyser, J. (1994). Chromosomal location of genes controlling short-term and long-term somatic embryogenesis in wheat revealed by immature embryo culture of aneuploid lines. *Theoretical and Applied Genetics 1994 89:2*, *89*(2), 344–350.

Henry, Y., Vain, P., & de Buyser, J. (1994). Genetic analysis of in vitro plant tissue culture responses and regeneration capacities. *Euphytica 1994 79:1*, *79*(1), 45–58.

Hensel, G., Marthe, C., & Kumlehn, J. (2017). Agrobacterium-mediated transformation of wheat using immature embryos. In *Wheat Biotechnology* (pp. 129-139). Humana Press, New York, NY.

Hiei, Y., & Komari, T. (2006). Improved protocols for transformation of indica rice mediated by Agrobacterium tumefaciens. *Plant Cell, Tissue and Organ Culture 2006 85:3*, *85*(3), 271–283.

Hoekema, A., Hirsch, P. R., Hooykaas, P. J. J., & Schilperoort, R. A. (1983). A binary plant vector strategy based on separation of vir- and T-region of the Agrobacterium tumefaciens Ti-plasmid. *Nature 1983 303:5913*, *303*(5913), 179–180.

Hofstad, A. N., Nussbaumer, T., Akhunov, E., Shin, S., Kugler, K. G., Kistler, H. C., Mayer, K. F. X., & Muehlbauer, G. J. (2016). Examining the transcriptional response in wheat Fhb1 near-isogenic lines to Fusarium graminearum infection and deoxynivalenol treatment. *Plant Genome*, *9*(1), 1–15.

Hu, T., Metz, S., Chay, C., Zhou, H. P., Biest, N., Chen, G., Cheng, M., Feng, X., Radionenko, M., Lu, F., & Fry, J. (2003). Agrobacterium-mediated large-scale transformation of wheat (Triticum aestivum L.) using glyphosate selection. *Plant Cell Reports*, *21*(10), 1010–1019.

Huang, X. Q., Börner, A., Röder, M. S., & Ganal, M. W. (2002). Assessing genetic diversity of wheat (Triticum aestivum L.) germplasm using microsatellite markers. *Theoretical and Applied Genetics 2002 105:5*, *105*(5), 699–707.

IM Ben Amer, A Börner, & R Schlegel. (1992). The effect of hybrid dwarfing gene D2 on tissue culture. Response of wheat (Triticum aestivum L.). *Cereal Research Communications*.

Ingram, H. M., Livesey, N. L., Power, J. B., & Davey, M. R. (2001). Genetic transformation of wheat: progress during the 1990s into the Millennium. *Acta Physiologiae Plantarum 2001 23:2*, *23*(2), 221–239.

Ingram, H. M., Power, J. B., Lowe, K. C., & Davey, M. R. (1999). Optimisation of procedures for microprojectile bombardment of microspore-derived embryos in wheat. *Plant Cell, Tissue and Organ Culture 1999 57:3*, *57*(3), 207–210.

Ishida, Y., Tsunashima, M., Hiei, Y., & Komari, T. (2015). Wheat (Triticum aestivum L.) transformation using immature embryos. In *Agrobacterium protocols* (pp. 189-198). Springer, New York, NY.

IWGSC, Appels, R., Eversole, K., Feuillet, C., Keller, B., Rogers, J., Stein, N., Pozniak, C. J., Choulet, F., Distelfeld, A., Poland, J., Ronen, G., Barad, O., Baruch, K., Keeble-Gagnère, G., Mascher, M., Ben-Zvi, G., Josselin, A. A., Himmelbach, A., … Wang, L. (2018). Shifting the limits in wheat research and breeding using a fully annotated reference genome. *Science*, *361*(6403).

IWGSC, Lukaszewski, A. J., Alberti, A., Sharpe, A., Kilian, A., Stanca, A. M., Keller, B., Clavijo, B. J., Friebe, B., Gill, B., Wulff, B., Chapman, B., Steuernagel, B., Feuillet, C., Viseux, C., Pozniak, C., Rokhsar, D. S., Klassen, D., Edwards, D., … Dubská, Z. (2014). A chromosome-based draft sequence of the hexaploid bread wheat (Triticum aestivum) genome. *Science*, *345*(6194), 6194.

Janakiraman, V., Steinau, M., McCoy, S. B., & Trick, H. N. (2002). Recent advances in wheat transformation. *In Vitro Cellular & Developmental Biology - Plant 2002 38:5*, *38*(5), 404–414.

Jauhar, P. P., Xu, S. S., & Baenziger, P. S. (2009). Haploidy in Cultivated Wheats: Induction and Utility in Basic and Applied Research. *Crop Science*, *49*(3), 737–755.

Jia, H., Yi, D., Yu, J., Xue, S., Xiang, Y., Zhang, C., ... & Ma, Z. (2007). Mapping QTLs for tissue culture response of mature wheat embryos. *Molecules & Cells (Springer Science & Business Media BV)*, 23(3).

Jia, H., Cho, S., & Muehlbauer, G. J. (2009). Transcriptome analysis of a wheat near-isogenic line pair carrying fusarium head blight–resistant and–susceptible alleles. *Molecular plant-microbe interactions*, 22(11), 1366-1378.

Jia, H., Yu, J., Yi, D., Cheng, Y., Xu, W., Zhang, L., & Ma, Z. (2009). Chromosomal intervals responsible for tissue culture response of wheat immature embryos. *Plant Cell, Tissue and Organ Culture*, *97*(2), 159–165.

Jia, H., Zhou, J., Xue, S., Li, G., Yan, H., Ran, C., Zhang, Y., Shi, J., Jia, L., Wang, X., Luo, J., & Ma, Z. (2018). A journey to understand wheat Fusarium head blight resistance in the Chinese wheat landrace Wangshuibai. *The Crop Journal*, *6*(1), 48–59.

Jiang, F., & Doudna, J. A. (2015). The structural biology of CRISPR-Cas systems. *Current Opinion in Structural Biology*, *30*, 100–111.

Jin, F., Zhang, D., Bockus, W., Baenziger, P. S., Carver, B., & Bai, G. (2013). Fusarium head blight resistance in u.s. winter wheat cultivars and elite breeding lines. *Crop Science*, *53*(5), 2006–2013.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*, *337*(6096), 816–821.

Jones, C. J., Edwards, K. J., Castaglione, S., Winfield, M. O., Sala, F., van de Wiel, C., Bredemeijer, G., Vosman, B., Matthes, M., Daly, A., Brettschneider, R., Bettini, P., Buiatti, M., Maestri, E., Malcevschi, A., Marmiroli, N., Aert, R., Volckaert, G., Rueda, J., … Karp, A. (1997). Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. *Molecular Breeding 1997 3:5*, *3*(5), 381–390.

Jones, H. D. (2005). Wheat transformation: current technology and applications to grain development and composition. *Journal of Cereal Science*, *41*(2), 137–147.

Jones, H. D., Doherty, A., & Wu, H. (2005). Review of methodologies and a protocol for the Agrobacterium-mediated transformation of wheat. *Plant Methods*, *1*(1), 1–9.

Jordan, K. W., Wang, S., Lun, Y., Gardiner, L. J., MacLachlan, R., Hucl, P., Wiebe, K., Wong, D., Forrest, K. L., Sharpe, A. G., Sidebottom, C. H. D., Hall, N., Toomajian, C., Close, T., Dubcovsky, J., Akhunova, A., Talbert, L., Bansal, U. K., Bariana, H. S., … Akhunov, E. (2015). A haplotype map of allohexaploid wheat reveals distinct patterns of selection on homoeologous genomes. *Genome Biology*, *16*(1), 1–18.

Kaleikau, E. K., Sears, R. G., & Gill, B. S. (1989). Control of tissue culture response in wheat (Triticum aestivum L.). *Theoretical and Applied Genetics 1989 78:6*, *78*(6), 783–787.

Kasha, K. J. (2005). Chromosome doubling and recovery of doubled haploid plants. *Biotechnology in Agriculture and Forestry*, *56*, 123–152.

Kasha, K. J., & Kao, K. N. (1970). High frequency haploid production in barley (Hordeum vulgare L.). *Nature 1970 225:5235*, *225*(5235), 874–876.

Kelliher, T., Starr, D., Richbourg, L., Chintamanani, S., Delzer, B., Nuccio, M. L., Green, J., Chen, Z., McCuiston, J., Wang, W., Liebler, T., Bullock, P., & Martin, B. (2017). MATRILINEAL, a sperm-specific phospholipase, triggers maize haploid induction. *Nature 2017 542:7639*, *542*(7639), 105–109.

Kelliher, T., Starr, D., Su, X., Tang, G., Chen, Z., Carter, J., Wittich, P. E., Dong, S., Green, J., Burch, E., McCuiston, J., Gu, W., Sun, Y., Strebe, T., Roberts, J., Bate, N. J., & Que, Q. (2019). One-step genome editing of elite crop germplasm during haploid induction. *Nature Biotechnology 2019 37:3*, *37*(3), 287–292.

Kendall, J. (1930). A parthenogenetic aberrant tobacco plant. *Journal of Heredity*, *21*(8), 363–366.

Khurana, J., Chugh, A., & Khurana, P. (2002). Regeneration from mature and immature embryos and transient gene expression via Agrobacterium-mediated transformation in emmer wheat (Triticum dicoccum Schuble).

Kikkert, J. R. (1993). The Biolistic® PDS-1000/He device. *Plant Cell, Tissue and Organ Culture 1993 33:3*, *33*(3), 221–226.

Kim, N. S., Armstrong, K. C., Fedak, G., Ho, K., & Park, N. I. (2011). A microsatellite sequence from the rice blast fungus (Magnaporthe grisea) distinguishes between the centromeres of Hordeum vulgare and H. bulbosum in hybrid plants. *Https://Doi.Org/10.1139/G01-129*, *45*(1), 165–174.

Kimber, G., & Riley, R. (1963). Haploid Angiosperms. *The Botanical Review 1963 29:4*, *29*(4), 480–531.

Kohli, A., Twyman, R. M., Abranches, R., Wegel, E., Stoger, E., & Christou, P. (2003). Transgene integration, organization and interaction in plants. *Plant Molecular Biology 2003 52:2*, *52*(2), 247–258.

Komeda, N., Chaudhary, H. K., Suzuki, G., & Mukai, Y. (2007). Cytological evidence for chromosome elimination in wheat × Imperata cylindrica hybrids. *Genes & Genetic Systems*, *82*(3), 241–248.

Konieczny, A., & Ausubel, F. M. (1993). A procedure for mapping Arabidopsis mutations using co-dominant ecotype-specific PCR-based markers. *The Plant Journal*, *4*(2), 403–410.

Kristóf, Z., & Imre, K. (1996). Isolation of living megaspores ofTorenia fournieri. *Protoplasma 1996 192:3*, *192*(3), 245–248.

Krolow, K. D. (1970). *Investigations on compatibility between wheat and rye.* Zeitschrift Fur Pflanzenzuchtung.

Kumar, L. S. (1999). DNA markers in plant improvement: An overview. *Biotechnology Advances*, *17*(2–3), 143–182.

Lamari, L. , & Bernier, C. C. (1991). Genetics of tan necrosis and extensive chlorosis in tan spot of wheat caused by Pyrenophora tritici-repentis. *Phytopathology*, *81*(10), 1092–1095.

Lamari, L., & Bernier, C. C. (1989). Evaluation of wheat lines and cultivars to tan spot [Pyrenophora tritici-repentis] based on lesion type. *Canadian Journal of Plant Pathology*, 11(1), 49-56.

Lamari, L., Sayoud, R., Boulif, M., & Bernier, C. C. (1995). Identification of a new race in Pyrenophora tritici-repentis: implications for the current pathotype classification system. *Canadian Journal of Plant Pathology*, 17(4), 312-318.

Lamari, L., & Strelkov, S. E. (2010). Minireview/Minisynthèse The wheat/Pyrenophora tritici-repentis interaction: progress towards an understanding of tan spot disease. *Canadian Journal of Plant Pathology*, 32(1), 4-10.

Lander, E. S. (1996). The new genomics: global views of biology. *Science*, *274*(5287), 536–539.

Laurie, D. A., & Bennett, M. D. (1986). Wheat× maize hybridization. *Canadian Journal of Genetics and Cytology*, 28(2), 313-316..

Laurie, D. A., & Bennett, M. D. (1988). The production of haploid wheat plants from wheat ×maize crosses. *Theoretical and Applied Genetics 1988 76:3*, *76*(3), 393–397.

Laurie, D. A., & Bennett, M. D. (1989). The timing of chromosome elimination in hexaploid wheat × maize crosses. *Https://Doi.Org/10.1139/G89-537*, *32*(6), 953–961.

Laurie, D. A., O'Donoughue, L. S., & Bennett, M. D. (1990). Wheat x maize and other wide sexual hybrids: their potential for genetic manipulation and crop improvement. In *Gene manipulation in plant improvement II* (pp. 95-126). Springer, Boston, MA.

Laurie, D. A., & Reymondie, S. (1991). High frequencies of fertilization and haploid seedling production in crosses between commercial hexaploid wheat varieties and maize. *Plant Breeding*, *106*(3), 182–189.

Lazar, M. D., Collins, G. B., & Vian, W. E. (1983). Genetic and environmental effects on the growth and differentiation of wheat somatic cell cultures. *Journal of Heredity*, *74*(5), 353–357.

Lev-Yadun, S., Gopher, A., & Abbo, S. (2000). The cradle of agriculture. *Science*, *288*(5471), 1602–1603.

Li, C., Liu, C., Qi, X., Wu, Y., Fei, X., Mao, L., Cheng, B., Li, X., & Xie, C. (2017). RNA-guided Cas9 as an in vivo desired-target mutator in maize. *Plant Biotechnology Journal*, *15*(12), 1566–1576.

Li, G., Zhou, J., Jia, H., Gao, Z., Fan, M., Luo, Y., Zhao, P., Xue, S., Li, N., Yuan, Y., Ma, S., Kong, Z., Jia, L., An, X., Jiang, G., Liu, W., Cao, W., Zhang, R., Fan, J., … Ma, Z. (2019). Mutation of a histidine-rich calcium-binding-protein gene in wheat confers resistance to Fusarium head blight. *Nature Genetics 2019 51:7*, *51*(7), 1106–1112.

Li, J., Ye, X., An, B., Du, L., & Xu, H. (2012). Genetic transformation of wheat: Current status and future prospects. *Plant Biotechnology Reports*, *6*(3), 183–193.

Lin, F., Xue, S. L., Zhang, Z. Z., Zhang, C. Q., Kong, Z. X., Yao, G. Q., Tian, D. G., Zhu, H. L., Li, C. J., Cao, Y., Wei, J. B., Luo, Q. Y., & Ma, Z. Q. (2006). Mapping QTL associated with resistance to Fusarium head blight in the Nanda2419 x Wangshuibai population. II: Type I resistance. *Theoretical and Applied Genetics*, *112*(3), 528–535.

Linde-Laursen, I., & Bothmer, R. von. (1999). Orderly arrangement of the chromosomes within barley genomes of chromosome-eliminating Hordeum lechleri × barley hybrids. *Https://Doi.Org/10.1139/G98-158*, *42*(2), 225–236.

Liu, C., Li, X., Meng, D., Zhong, Y., Chen, C., Dong, X., Xu, X., Chen, B., Li, W., Li, L., Tian, X., Zhao, H., Song, W., Luo, H., Zhang, Q., Lai, J., Jin, W., Yan, J., & Chen, S. (2017).

A 4-bp insertion at ZmPLA1 encoding a putative phospholipase a generates haploid induction in maize. *Molecular Plant*, *10*(3), 520–522.

Lorang, J. M., Sweat, T. A., & Wolpert, T. J. (2007). Plant disease susceptibility conferred by a "resistance" gene. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(37), 14861–14866.

Lowe, K., Wu, E., Wang, N., Hoerster, G., Hastings, C., Cho, M. J., Scelonge, C., Lenderts, B., Chamberlin, M., Cushatt, J., Wang, L., Ryan, L., Khan, T., Chow-Yiu, J., Hua, W., Yu, M., Banh, J., Bao, Z., Brink, K., … Gordon-Kamm, W. (2016). Morphogenic regulators Baby boom and Wuschel improve monocot transformation. *The Plant Cell*, *28*(9), 1998–2015.

Loyola-Vargas, V. M., & Ochoa-Alejo, N. (2016). Somatic Embryogenesis. An Overview. *Somatic Embryogenesis: Fundamental Aspects and Applications*, 1–8.

Lu, H. J., Fellers, J. P., Friesen, T. L., Meinhardt, S. W., & Faris, J. D. (2006). Genomic analysis and marker development for the Tsn1 locus in wheat using bin-mapped ESTs and flanking BAC contigs. *Theoretical and Applied Genetics*, *112*(6), 1132–1142.

Lv, J., Yu, K., Wei, J., Gui, H., Liu, C., Liang, D., Wang, Y., Zhou, H., Carlin, R., Rich, R., Lu, T., Que, Q., Wang, W. C., Zhang, X., & Kelliher, T. (2020). Generation of paternal haploids in wheat by genome editing of the centromeric histone CENH3. *Nature Biotechnology 2020 38:12*, *38*(12), 1397–1401.

Maheshwari, N., Rajyalakshmi, K., Baweja, K., Dhir, S. K., Chowdhry, C. N., & Maheshwari, S. C. (1995). In vitro culture of wheat and genetic transformation—retrospect and prospect. *Critical reviews in plant sciences*, 14(2), 149-178.

Makarova, K. S., Haft, D. H., Barrangou, R., Brouns, S. J. J., Charpentier, E., Horvath, P., Moineau, S., Mojica, F. J. M., Wolf, Y. I., Yakunin, A. F., van der Oost, J., & Koonin, E. v. (2011). Evolution and classification of the CRISPR–Cas systems. *Nature Reviews Microbiology 2011 9:6*, *9*(6), 467–477.

Makarova, K. S., Wolf, Y. I., Alkhnbashi, O. S., Costa, F., Shah, S. A., Saunders, S. J., Barrangou, R., Brouns, S. J. J., Charpentier, E., Haft, D. H., Horvath, P., Moineau, S., Mojica, F. J. M., Terns, R. M., Terns, M. P., White, M. F., Yakunin, A. F., Garrett, R. A., van der Oost, J., … Koonin, E. v. (2015). An updated evolutionary classification of CRISPR–Cas systems. *Nature Reviews Microbiology 2015 13:11*, *13*(11), 722–736.

Marcussen, T., Sandve, S. R., Heier, L., Spannagl, M., Pfeifer, M., Jakobsen, K. S., Wulff, B., Steuernagel, B., Mayer, K. F. X., Olsen, O. A., Rogers, J., Doležel, J., Pozniak, C., Eversole, K., Feuillet, C., Gill, B., Friebe, B., Lukaszewski, A. J., Sourdille, P., … Praud, S. (2014). Ancient hybridizations among the ancestral genomes of bread wheat. *Science*, *345*(6194), 6194.

Mathias, R. J., & Fukui, K. (1986). The effect of specific chromosome and cytoplasm substitutions on the tissue culture response of wheat (Triticum aestivum) callus. *Theoretical and Applied Genetics 1986 71:6*, *71*(6), 797–800.

Matsuoka, Y. (2011). Evolution of polyploid triticum wheats under cultivation: the role of domestication, natural hybridization and allopolyploid speciation in their diversification. *Plant and Cell Physiology*, *52*(5), 750–764.

Matsuoka, Y., & Nasuda, S. (2004). Durum wheat as a candidate for the unknown female progenitor of bread wheat: An empirical study with a highly fertile F1 hybrid with Aegilops tauschii Coss. *Theoretical and Applied Genetics*, *109*(8), 1710–1717.

McMullen, M., Bergstrom, G., de Wolf, E., Dill-Macky, R., Hershman, D., Shaner, G., & van Sanford, D. (2012). a unified effort to fight an enemy of wheat and barley: Fusarium head blight. *Plant Disease*, *96*(12), 1712–1728.

Miller, C. O., Skoog, F., Von Saltza, M. H., & Strong, F. M. (1955). Kinetin, a cell division factor from deoxyribonucleic acid1. *Journal of the American Chemical Society*, 77(5), 1392-1392.

Miller, C. O., Skoog, F., Okumura, F. S., von Saltza, M. H., & Strong, F. M. (1956). Isolation, structure and synthesis of kinetin, a substance promoting cell division1, 2. *Journal of the American Chemical Society*, *78*(7), 1375–1380.

Mochida, K., Tsujimoto, H., & Sasakuma, T. (2004). Confocal analysis of chromosome behavior in wheat× maize zygotes. *Genome*, 47(1), 199-205.

Mohan, M., Nair, S., Bhagwat, A., Krishna, T. G., Yano, M., Bhatia, C. R., & Sasaki, T. (1997). Genome mapping, molecular markers and marker-assisted selection in crop plants. *Molecular Breeding 1997 3:2*, *3*(2), 87–103.

Mojica, F. J. M., Díez-Villaseñor, C., García-Martínez, J., & Soria, E. (2005). Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *Journal of Molecular Evolution*, *60*(2), 174–182.

Nagy, E. D., & Bennetzen, J. L. (2008). Pathogen corruption and site-directed recombination at a plant disease resistance gene cluster. *Genome Research*, *18*(12), 1918–1923.

Namasivayam, P. (2007). Acquisition of embryogenic competence during somatic embryogenesis. *Plant Cell, Tissue and Organ Culture 2007 90:1*, *90*(1), 1–8.

Nehra, N. S., Chibbar, R. N., Leung, N., Caswell, K., Mallard, C., Steinhauer, L., Baga, M., & Kartha, K. K. (1994). Self-fertile transgenic wheat plants regenerated from isolated scutellar tissues following microprojectile bombardment with two distinct gene constructs†. *The Plant Journal*, *5*(2), 285–297.

Niu, Z., Jiang, A., Abu Hammad, W., Oladzadabbasabadi, A., Xu, S. S., Mergoum, M., & Elias, E. M. (2014). Review of doubled haploid production in durum and common wheat through wheat × maize hybridization. *Plant Breeding*, *133*(3), 313–320.

Orolaza, N. P., Lamari, L., & Ballance, G. M. (1995). Evidence of a host-specific chlorosis toxin from Pyrenophora tritici-repentis, the causal agent of tan spot of wheat. *Phytopathology*, 85(10), 1282-1287.

Partier, A., Gay, G., Tassy, C., Beckert, M., Feuillet, C., & Barret, P. (2017). Molecular and FISH analyses of a 53-kbp intact DNA fragment inserted by biolistics in wheat (Triticum aestivum L.) genome. *Plant Cell Reports*, *36*(10), 1547–1559.

Paterson, A. H. (1996). Making genetic maps. *Genome Mapping in Plants*, *194*, 23–39.

Paterson, A. H., Tanksley, S. D., & Sorrells, M. E. (1991). DNA markers in plant improvement. *Advances in Agronomy*, *46*(C), 39–90.

Paux, E., Sourdille, P., Salse, J., Saintenac, C., Choulet, F., Leroy, P., Korol, A., Michalak, M., Kianian, S., Spielmeyer, W., Lagudah, E., Somers, D., Kilian, A., Alaux, M., Vautrin, S., Bergès, H., Eversole, K., Appels, R., Safar, J., … Feuillet, C. (2008). A physical map of the 1-gigabase bread wheat chromosome 3B. *Science*, *322*(5898), 101–104.

Peng, J. H., Dongfa, •, Nevo, S. • E., Peng, J. H., Sun, D., & Nevo, E. (2011). Domestication evolution, genetics and genomics in wheat. *Molecular Breeding 2011 28:3*, *28*(3), 281–301.

Peng, J., Ronin, Y., Fahima, T., Röder, M. S., Li, Y., Nevo, E., & Korol, A. (2003). Domestication quantitative trait loci in Triticum dicoccoides, the progenitor of wheat. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(5), 2489–2494.

Peng, T., Sun, X., & Mumm, R. H. (2014). Optimized breeding strategies for multiple trait integration: I. Minimizing linkage drag in single event introgression. *Molecular Breeding*, *33*(1), 89–104.

Pestka, J. J., & Smolinski, A. T. (2005). Deoxynivalenol: toxicology and potential effects on humans. *Journal of Toxicology and Environmental Health, Part B*, 8(1), 39-69.

Peters, N. R., Ackerman, S., & Davis, E. A. (1999). A modular vector for Agrobacterium mediated transformation of wheat. *Plant Molecular Biology Reporter 1999 17:4*, *17*(4), 323–331.

Petersen, G., Seberg, O., Yde, M., & Berthelsen, K. (2006). Phylogenetic relationships of Triticum and Aegilops and evidence for the origin of the A, B, and D genomes of common wheat (Triticum aestivum). *Molecular Phylogenetics and Evolution*, *39*(1), 70–82.

Pickering, R. A., & Morgan, P. W. (1985). The influence of temperature on chromosome elimination during embryo development in crosses involving Hordeum spp., wheat (Triticum aestivum L.) and rye (Secale cereale L.). *Theoretical and Applied Genetics 1985 70:2*, *70*(2), 199–206.

Plessis, A., Perrin, A., Haber, J. E., & Dujon, B. (1992). Site-specific recombination determined by I-SceI, a mitochondrial group I intron-encoded endonuclease expressed in the yeast nucleus. *Genetics*, *130*(3), 451–460.

Pourcel, C., Salvignol, G., & Vergnaud, G. (2005). CRISPR elements in Yersinia pestis acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. *Microbiology*, *151*(3), 653–663.

Pratap, A., Sethi, G. S., & Chaudhary, H. K. (2005). Relative efficiency of different Gramineae genera for haploid induction in triticale and triticale x wheat hybrids through the chromosome elimination technique. *Plant Breeding*, *124*(2), 147–153.

Ramanna, M. S., & Jacobsen, E. (2003). Relevance of sexual polyploidization for crop improvement – A review. *Euphytica 2003 133:1*, *133*(1), 3–8.

Ravi, M., & Chan, S. W. L. (2010). Haploid plants produced by centromere-mediated genome elimination. *Nature 2010 464:7288*, *464*(7288), 615–618.

Rawat, N., Pumphrey, M. O., Liu, S., Zhang, X., Tiwari, V. K., Ando, K., Trick, H. N., Bockus, W. W., Akhunov, E., Anderson, J. A., & Gill, B. S. (2016). Wheat Fhb1 encodes a chimeric lectin with agglutinin domains and a pore-forming toxin-like domain conferring resistance to Fusarium head blight. *Nature Genetics 2016 48:12*, *48*(12), 1576–1580.

Ream, W. (1989). Agrobacterium tumefaciens and interkingdom genetic exchange. *Annual Review of Phytopathology*, 27(1), 583-618.

Reisch, B., & Bingham, E. T. (1980). The genetic control of bud formation from callus cultures of diploid alfalfa. *Plant Science Letters*, *20*(1), 71–77.

Ren, J., Wu, P., Trampe, B., Tian, X., Lübberstedt, T., & Chen, S. (2017). Novel technologies in doubled haploid line development. *Plant Biotechnology Journal*, *15*(11), 1361–1370.

Risacher, T., Craze, M., Bowden, S., Paul, W., & Barsby, T. (2009). Agrobacterium-mediated transformation of wheat via in planta inoculation. *Methods in Molecular Biology, Transgenic Wheat, Barley and Oats.*, *478*, 115–124.

Rouet, P., Smih, F., & Jasin, M. (1994). Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America*, *91*(13), 6064–6068.

Rudin, N., Sugarman, E., & Haber, J. E. (1989). Genetic and physical analysis of double-strand break repair and recombination in Saccharomyces cerevisiae. *Genetics*, *122*(3), 519–534.

Sanford, J. C., Devit, M. J., Russell, J. A., Smith, F. D., Harpending, P. R., Roy, M. K., & Johnston, S. A. (1991). An improved, helium-driven biolistic device. *Technique*, 3(1), 3-16.

Sanford, J. C., Klein, T. M., Wolf, E. D., & Allen, N. (1987). Delivery of substances into cells and tissues using a particle bombardment process. *Particulate Science and Technology*, 5(1), 27-37.

Sax, K. (1923). The association of size differences with seed-coat pattern and pigmentation in Phaseolus vulgaris. *Genetics*, *8*(6), 552.

Schilder, A. M. C., & Bergstrom, G. C. (1994). Infection of wheat seed by Pyrenophora tritici-repentis. *Canadian Journal of Botany*, 72(4), 510-519.

Schwarzacher-robinson, T., Finch, R. A., Smith, J. B., & Bennet, M. D. (1987). Genotypic control of centromere positions of parental genomes in Hordeum × Secale hybrid metaphases. *Journal of Cell Science*, *87*(2), 291–304.

Schweiger, W., Steiner, B., Vautrin, S., Nussbaumer, T., Siegwart, G., Zamini, M., Jungreithmeier, F., Gratl, V., Lemmens, M., Mayer, K. F. X., Bérgès, H., Adam, G., & Buerstmayr, H. (2016). Suppressed recombination and unique candidate genes in the divergent haplotype encoding Fhb1, a major Fusarium head blight resistance locus in wheat. *Theoretical and Applied Genetics*, *129*(8), 1607–1623.

Semagn, K., Bjørnstad, Å., & Xu, Y. (2010). The genetic dissection of quantitative traits in crops. *Electronic Journal of Biotechnology*, *13*(5), 0–0.

Sham, P., Bader, J. S., Craig, I., O'Donovan, M., & Owen, M. (2002). DNA Pooling: a tool for large-scale association studies. *Nature Reviews Genetics 2002 3:11*, *3*(11), 862–871.

Shi, G., Zhang, Z., Friesen, T. L., Raats, D., Fahima, T., Brueggeman, R. S., Lu, S., Trick, H. N., Liu, Z., Chao, W., Frenkel, Z., Xu, S. S., Rasmussen, J. B., & Faris, J. D. (2016). The hijacking of a receptor kinase-driven pathway by a wheat fungal pathogen leads to disease. *Science Advances*, *2*(10).

Shimada, T. (1978). Plant regeneration from the callus induced from wheat embryo. *The Japanese Journal of Genetics*, *53*(5), 371–374.

Shmakov, S., Abudayyeh, O. O., Makarova, K. S., Wolf, Y. I., Gootenberg, J. S., Semenova, E., Minakhin, L., Joung, J., Konermann, S., Severinov, K., Zhang, F., & Koonin, E. v. (2015). Discovery and functional characterization of diverse class 2 CRISPR-Cas systems. *Molecular Cell*, *60*(3), 385–397.

Shrawat, A. K., Becker, D., & Lörz, H. (2007). Agrobacterium tumefaciens-mediated genetic transformation of barley (Hordeum vulgare L.). *Plant Science*, *172*(2), 281–290.

Sidhu, P. K., Howes, N. K., Aung, T., Zwer, P. K., & Davies, P. A. (2006). Factors affecting oat haploid production following oat × maize hybridization. *Plant Breeding*, *125*(3), 243–247.

Silva, G., Poirot, L., Galetto, R., Smith, J., Montoya, G., Duchateau, P., & Paques, F. (2011). Meganucleases and other tools for targeted genome engineering: perspectives and challenges for gene therapy. *Current Gene Therapy*, *11*(1), 11–27.

Skoog, F. , & Miller, C. (1957). Chemical regulation of growth and organ formation in plant tissues cultured. *In Vitro Symp Soc Exp Biol*.

Solomon, P. S., Lowe, R. G. T., Tan, K. C., Waters, O. D. C., & Oliver, R. P. (2006). Stagonospora nodorum: cause of stagonospora nodorum blotch of wheat. *Molecular Plant Pathology*, *7*(3), 147–156.

Somers, D. J., Fedak, G., Savard, M., Somers, D. J., Fedak, G., & Savard, M. (2011). Molecular mapping of novel genes controlling Fusarium head blight resistance and deoxynivalenol accumulation in spring wheat. *Genome*, *46*(4), 555–564.

Sparks, C. A., & Jones, H. D. (2004). Transformation of wheat by biolistics. *Transgenic Crops of the World*, 19–34.

Srivastava, V., Anderson, O. D., & Ow, D. W. (1999). Single-copy transgenic wheat generated through the resolution of complex integration patterns. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(20), 11117–11121.

Stachel, S. E., Messens, E., van Montagu, M., & Zambryski, P. (1985). Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in Agrobacterium tumefaciens. *Nature 1985 318:6047*, *318*(6047), 624–629.

Stebbins, G. L. (1985). Polyploidy, hybridization, and the invasion of new habitats. *Annals - Missouri Botanical Garden*, *72*(4), 824–832.

Steward, F. C., Mapes, M. O., & Mears, K. (1958). Growth and organized development of cultured cells. ii. organization in cultures grown from freely suspended cells. *American Journal of Botany*, *45*(10), 705.

Steward, F. C. (1968). Growth and organization in plants. *Growth and organization in plants*.

Strelkov, S. E., Lamari, L., & Ballance, G. M. (1999). Characterization of a host-specific protein toxin (Ptr ToxB) from Pyrenophora tritici-repentis. *Molecular Plant-Microbe Interactions*, 12(8), 728-732.

Strutt, S. C., Torrez, R. M., Kaya, E., Negrete, O. A., & Doudna, J. A. (2018). RNA-dependent RNA targeting by CRISPR-Cas9. *ELife*, *7*.

Su, Z., Bernardo, A., Tian, B., Chen, H., Wang, S., Ma, H., Cai, S., Liu, D., Zhang, D., Li, T., Trick, H., st. Amand, P., Yu, J., Zhang, Z., & Bai, G. (2019). A deletion mutation in TaHRC confers Fhb1 resistance to Fusarium head blight in wheat. *Nature Genetics 2019 51:7*, *51*(7), 1099–1105.

Subrahmanyam, N. C., & Kasha, K. J. (1973). Selective chromosomal elimination during haploid formation in barley following interspecific hybridization. *Chromosoma 1973 42:2*, *42*(2), 111–125.

Suenaga, K., & Nakajima, K. (1989). Efficient production of haploid wheat (Triticum aestivum) through crosses between Japanese wheat and maize (Zea mays). *Plant Cell Reports 1989 8:5*, *8*(5), 263–266.

Sugiyama, M. (2015). Historical review of research on plant cell dedifferentiation. *Journal of Plant Research*, *128*(3), 349–359.

Sung, P., & Klein, H. (2006). Mechanism of homologous recombination: mediators and helicases take on regulatory functions. *Nature Reviews Molecular Cell Biology 2006 7:10*, *7*(10), 739–750.

Supartana, P., Shimizu, T., Nogawa, M., Shioiri, H., Nakajima, T., Haramoto, N., Nozue, M., & Kojima, M. (2006). Development of simple and efficient in Planta transformation method for wheat (Triticum aestivum L.) using Agrobacterium tumefaciens. *Journal of Bioscience and Bioengineering*, *102*(3), 162–170.

Tanno, K. I., & Willcox, G. (2006). How fast was wild wheat domesticated? *Science*, *311*(5769), 1886.

Terns, M. P., & Terns, R. M. (2011). CRISPR-based adaptive immune systems. *Current Opinion in Microbiology*, *14*(3), 321–327.

Thoday, J. M. (1961). Location of polygenes. *Nature 1961 191:4786*, *191*(4786), 368–370.

Thorpe, T. A., & Stasolla, C. (2001). Somatic Embryogenesis. *Current Trends in the Embryology of Angiosperms*, 279–336.

Tomas, A., & Bockus, W. (1987). Cultivar-specific toxicity of culture filtrates of Pyrenophora tritici-repentis . *Phytopathology*, *77*(9), 1337.

Tuori, R. P., Wolpert, T. J., & Ciuffetti, L. M. (1995). Purification and immunological characterization of toxic components from cultures of Pyrenophora tritici-repentis. *Molecular Plant-Microbe Interactions : MPMI*, *8*(1), 41–48.

Usami, S., Okamoto, S., Takebe, I., & Machida, Y. (1988). Factor inducing Agrobacterium tumefaciens vir gene expression is present in monocotyledonous plants . *Proceedings of the National Academy of Sciences*, *85*(11), 3748–3752.

USDA, E. (2022). *Wheat Sector at a Glance*.

USDA, N. A. S. S. (2021). *Small Grains 2021 Summary (September 2021)*.

Vain, P., Harvey, A., Worland, B., Ross, S., Snape, J. W., & Lonsdale, D. (2004). The effect of additional virulence genes on transformation efficiency, transgene integration and expression in rice plants using the pGreen/pSoup dual binary vector system. *Transgenic Research 2004 13:6*, *13*(6), 593–603.

van der Oost, J., Jore, M. M., Westra, E. R., Lundgren, M., & Brouns, S. J. J. (2009). CRISPR-based adaptive and heritable immunity in prokaryotes. *Trends in Biochemical Sciences*, *34*(8), 401–407.

van Schie, C. C., & Takken, F. L. (2014). Susceptibility genes 101: how to be a good host. *Annual review of phytopathology*, 52, 551-581.

Vasil, V., Castillo, A. M., Fromm, M. E., & Vasil, I. K. (1992). Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. *Bio/Technology 1992 10:6*, *10*(6), 667–674.

Vuylsteke, M., Peleman, J. D., & van Eijk, M. J. T. (2007). AFLP technology for DNA fingerprinting. *Nature Protocols 2007 2:6*, *2*(6), 1387–1398.

Wang, B., Zhu, L., Zhao, B., Zhao, Y., Xie, Y., Zheng, Z., Li, Y., Sun, J., & Wang, H. (2019). Development of a haploid-inducer mediated genome editing system for accelerating maize breeding. *Molecular Plant*, *12*(4), 597–602.

Wang, H., La Russa, M., & Qi, L. S. (2016). CRISPR/Cas9 in genome editing and beyond. Annual review of biochemistry, 85, 227-264.

Wang, H., Sun, S., Ge, W., Zhao, L., Hou, B., Wang, K., ... & Kong, L. (2020). Horizontal gene transfer of Fhb7 from fungus underlies Fusarium head blight resistance in wheat. *Science*, 368(6493), eaba5435.

Wang, S., Wong, D., Forrest, K., Allen, A., Chao, S., Huang, B. E., Maccaferri, M., Salvi, S., Milner, S. G., Cattivelli, L., Mastrangelo, A. M., Whan, A., Stephen, S., Barker, G., Wieseke, R., Plieske, J., Lillemo, M., Mather, D., Appels, R., … Akhunov, E. (2014). Characterization of polyploid wheat genomic diversity using a high-density 90 000 single nucleotide polymorphism array. *Plant Biotechnology Journal*, *12*(6), 787–796.

Wang, Y., Cheng, X., Shan, Q., Zhang, Y., Liu, J., Gao, C., & Qiu, J. L. (2014). Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. *Nature Biotechnology 2014 32:9*, *32*(9), 947–951.

Wang, Y. L., Xu, M. X., Yin, G. X., Tao, L. L., Wang, D. W., & Ye, X. G. (2009). Transgenic wheat plants derived from Agrobacterium-mediated transformation of mature embryo tissues. *Cereal Research Communications 2009 37:1*, *37*(1), 1–12.

Wędzony, M., Forster, B. P., Żur, I., Golemiec, E., Szechyńska-Hebda, M., Dubas, E., & Gotębiowska, G. (2009). Progress in doubled haploid technology in higher plants. *Advances in haploid production in higher plants*, 1-33.

Weeks, J. T., Anderson, O. D., & Blechl, A. E. (1993). Rapid production of multiple independent lines of fertile transgenic wheat (Triticum aestivum). *Plant Physiology*, *102*(4), 1077–1084.

White, P. R. (1936). Plant tissue cultures. *The Botanical Review 1936 2:8*, *2*(8), 419–437.

Wiedenheft, B., Sternberg, S. H., & Doudna, J. A. (2012). RNA-guided genetic silencing systems in bacteria and archaea. *Nature 2012 482:7385*, *482*(7385), 331–338.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., & Tingey, S. v. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, *18*(22), 6531–6535.

Williams, R. C. (1989). Restriction fragment length polymorphism (RFLP). *American Journal of Physical Anthropology*, *32*(S10), 159–184.

Winter, P., & Kahl, G. (1995). Molecular marker technologies for plant improvement. *World Journal of Microbiology and Biotechnology 1995 11:4*, *11*(4), 438–448.

Wolpert, T. J., Dunkle, L. D., & Ciuffetti, L. M. (2002). Host-selective toxins and avirulence determinants: what's in a name?. *Annual review of phytopathology*, 40(1), 251-285.

Wu, C. H., Liang, C. Y., & Ye, X. L. (2004). In vitro megagametophyte development via unfertilized ovule culture in Doritis pulcherrima. *ACTA BOTANICA SINICA-ENGLISH EDITION*-, 46(7), 839-845.

Xue, S., Li, G., Jia, H., Xu, F., Lin, F., Tang, M., Wang, Y., An, X., Xu, H., Zhang, L., Kong, Z., & Ma, Z. (2010). Fine mapping Fhb4, a major QTL conditioning resistance to Fusarium infection in bread wheat (Triticum aestivum L.). *Theoretical and Applied Genetics*, *121*(1), 147–156.

Xue, S., Xu, F., Tang, M., Zhou, Y., Li, G., An, X., Lin, F., Xu, H., Jia, H., Zhang, L., Kong, Z., & Ma, Z. (2011). Precise mapping Fhb5, a major QTL conditioning resistance to Fusarium infection in bread wheat (Triticum aestivum L.). *Theoretical and Applied Genetics*, *123*(6), 1055–1063.

Yang, H. Y., & Zhou, C. (1982). In vitro induction of haploid plants from unpollinated ovaries and ovules. *Theoretical and Applied Genetics 1982 63:2*, *63*(2), 97–104.

Yang, J., Bai, G., & Shaner, G. E. (2005). Novel quantitative trait loci (QTL) for Fusarium head blight resistance in wheat cultivar Chokwang. *Theoretical and Applied Genetics*, *111*(8), 1571–1579.

Yao, Q., Cong, L., He, G., Chang, J., Li, K., & Yang, G. (2007). Optimization of wheat co-transformation procedure with gene cassettes resulted in an improvement in transformation frequency. *Molecular Biology Reports*, *34*(1), 61–67.

Zale, J. M., Agarwal, S., Loar, S., & Steber, C. M. (2009). Evidence for stable transformation of wheat by floral dip in Agrobacterium tumefaciens. *Plant Cell Reports*, *28*(6), 903–913.

Zhang, J., Rouillon, C., Kerou, M., Reeks, J., Brugger, K., Graham, S., Reimann, J., Cannone, G., Liu, H., Albers, S. V., Naismith, J. H., Spagnolo, L., & White, M. F. (2012). Structure and mechanism of the CMR complex for CRISPR-mediated antiviral immunity. *Molecular Cell*, *45*(3), 303–313.

Zhang, W., Zhu, X., Zhang, M., Shi, G., Liu, Z., & Cai, X. (2019). Chromosome engineering-mediated introgression and molecular mapping of novel Aegilops speltoides-derived

resistance genes for tan spot and Septoria nodorum blotch diseases in wheat. *Theoretical and Applied Genetics*, *132*(9), 2605–2614.

Zhao, T. J., Zhao, S. Y., Chen, H. M., Zhao, Q. Z., Hu, Z. M., Hou, B. K., & Xia, G. M. (2006). Transgenic wheat progeny resistant to powdery mildew generated by Agrobacterium inoculum to the basal portion of wheat seedling. *Plant Cell Reports*, *25*(11), 1199–1204.

Zheng, Y. L., Luo, M. C., & Yang, J. L. (1992). Studies on inheritance and gene localization of the crossability of a new material 'J-11'in common wheat with rye. *China Association for Science and Technology First Academic Annual Meeting of Youths Proceedings (Agricultural Sciences), Chinese Press for Science and Technology, Beijing*, 121–127.

Zhong, Y., Chen, B., Li, M., Wang, D., Jiao, Y., Qi, X., Wang, M., Liu, Z., Chen, C., Wang, Y., Chen, M., Li, J., Xiao, Z., Cheng, D., Liu, W., Boutilier, K., Liu, C., & Chen, S. (2020). A DMP-triggered in vivo maternal haploid induction system in the dicotyledonous Arabidopsis. *Nature Plants 2020 6:5*, *6*(5), 466–472.

Zhou, H., Stiff, C. M., & Konzak, C. F. (1993). Stably transformed callus of wheat by electroporation-induced direct gene transfer. *Plant Cell Reports 1993 12:11*, *12*(11), 612–616.

Zhou, W., Kolb, F. L., Bai, G., Shaner, G., & Domier, L. L. (2002). Genetic analysis of scab resistance QTL in wheat with microsatellite and AFLP markers. *Genome*, *45*(4), 719–727.

Zhu, T., Wang, L., Rimbert, H., Rodriguez, J. C., Deal, K. R., de Oliveira, R., Choulet, F., Keeble-Gagnère, G., Tibbits, J., Rogers, J., Eversole, K., Appels, R., Gu, Y. Q., Mascher, M., Dvorak, J., & Luo, M. C. (2021). Optical maps refine the bread wheat Triticum aestivum cv. Chinese Spring genome assembly. *The Plant Journal*, *107*(1), 303–314.

Zhu, Z., Sun, B., Liu, C., Xiao, G., & Li, X. (1993). Transformation of wheat protoplasts mediated by cationic liposome and regeneration of transgenic plantlets. *Chinese Journal of Biotechnology*, *9*(4), 257–261.

Zimin, A. v., Puiu, D., Hall, R., Kingan, S., Clavijo, B. J., & Salzberg, S. L. (2017). The first near-complete assembly of the hexaploid bread wheat genome, Triticum aestivum. *GigaScience*, *6*(11), 1–7.

MOLECULAR MAPPING OF QTL FOR TRANSFORMATION-RELATED TRAIT AND FHB RESISTANCE IN A MAPPING POPULATION DERIVED FROM THE CROSS BETWEEN BOBWHITE AND PI 277012

Abstract

Bobwhite is one of wheat cultivars that have been widely used for wheat transformation, but the genetic mechanism underlying its high transformability is not well understood. To identify quantitative trait loci (QTL) associated with plant regeneration capability, a mapping population of 186 recombinant inbred lines (RILs) derived from the cross between Bobwhite and wheat line PI 277012 were phenotyped for the transformation-related trait. Immature embryos were used as explants for tissue culture to induce calli, and plant regeneration rates from the calli were recorded for the parents and RILs. The average regeneration rate was 0% and 42.5% for PI 277012 and Bobwhite, respectively. The RILs had an average regeneration rate ranging from 0 – 33%. Using genotyping by sequencing approach, a genetic linkage map was constructed with 3,779 SNP markers distributed on all 21 chromosomes, spanning 2,291 cM. QTL analysis detected two QTL (*Qprc.ndwp-1A* and *Qprc.ndwp-6D*) controlling plant regeneration capability in Bobwhite, which were mapped on chromosome 1A and 6D, and accounted for 10.5% and 9.8% of the phenotypic variation, respectively. To confirm the FHB resistance QTL (*Qfhb.rwg-5A.1* and *Qfhb.rwg-5A.2*) previously identified in PI 277012, the Bobwhite × PI 277012 RIL population was phenotyped for FHB resistance in greenhouse and field experiments. However, only one QTL (*Qfhb.rwg-5A.2*) for FHB resistance was identified in this population, which explained 33.0% and 36.2% of the phenotypic variance under the field and greenhouse environments, respectively. The information from this study may facilitate the deployment of the transformability QTL in wheat varieties that are difficult to transform. This study also indicated

that *Qfhb.rwg-5A.2* in PI 277012 is a major QTL for FHB resistance and is stably expressed in different genetic backgrounds.

Introduction

Genetic transformation is a powerful tool for studying gene functions and crop improvement in the modern agriculture system. The potential of genetic manipulation has been widely explored to enhance crop productivity through increasing resistance to various biotic and abiotic factors (Hansen & Wright, 1999). In-vitro tissue culture of explants with an efficient regeneration system is an essential part of genetic transformation in most biotechnological methods for crop improvement. However, wheat has been considered recalcitrant to genetic amenability due to the lack of explants with high regeneration capacity. Along with non-genetic factors such as environmental factors, tissue culture response (TCR) that includes callus formation and plant regeneration in wheat tissue culture greatly depends on genotype (Lazar et al., 1983). Genetic loci for varying degrees of TCR have been mapped to different chromosome arms suggesting TCR is a polygenic or quantitative trait (Felsenburg et al., 1987; Galiba et al., 1986; Henry et al., 1994). Compared to other explants in wheat, immature embryos have been widely used for transformation purposes due to their better TCR. In previous studies, QTL associated with immature embryo TCR were mapped on chromosomes 4B, 2D, 7B, 7D, 1D, 6BL, 2B and 2AL (Felsenburg et al., 1987; Galiba et al., 1986; Kaleikau et al., 1989; Mathias & Fukui, 1986). QTL for plant regeneration from callus derived from immature embryos were detected on chromosome 2B and 2D (Amer et al., 1992, 1996, 1997). QTL controlling green-point formation in calli from immature embryos was reported on chromosome 2AS (Henry et al., 1994). TCR is highly influenced by the environment and culture conditions of immature embryos. Different QTL associated with TCR were identified in an environmental chamber and

the fields even using immature embryos from the same set of lines (Galiba et al., 1986). Therefore, validation of mapped QTL across a larger germplasm basis is essential for gaining a complete picture of the genetic regulation of the immature embryo culture response.

Fusarium head blight (FHB) is an economically important disease in wheat. It causes direct yield loss as well as indirect losses due to the accumulation of mycotoxins such as deoxynivalenol (DON) and zearalenone (ZEA) in the grains (Bai Guihua & Shaner, 1994). Yield reductions due to FHB were estimated as high as 74% (Wegulo et al., 2015). FHB is primarily caused by *Fusarium graminearum* in North America (del Ponte et al., 2017). This disease is favored by prolonged warm and moist conditions during wheat anthesis resulting in shrunken, lightweight, shriveled, and chalky white or pink grains (Wegulo et al., 2013). Identification of novel sources of FHB resistance and introgression of them into the cultivated wheat varieties is a sustainable approach to managing this disease.

FHB resistance is complex and quantitatively controlled by multiple genes and affected by environmental factors (Bai Guihua & Shaner, 1994). Among different types of FHB resistance, type-II resistance that prevents fungal spread within the spike is the most recognized and evaluated type of FHB resistance. Various sources of FHB resistance have been identified, genetically characterized, and successfully utilized in developing FHB resistant cultivars (Buerstmayr et al., 2009; Buerstmayr et al., 2020; Zhao et al., 2018). The hexaploid spring wheat line PI 277012 is one of the FHB resistance sources identified by Dr. Steven Xu's team in the Northern Crop Science Laboratory, USDA-ARS, Fargo, ND (Chu et al., 2011; Oliver et al., 2008; Xu et al., 2007). This wheat line consistently expressed a high level of FHB resistance in both field and greenhouse experiments (Chu et al., 2011; Xu et al., 2007). PI 277012 showed a similar level of type-II FHB resistance as Sumai 3 but does not have the source of resistance

from Sumai 3 (Xu et al., 2007). Further genetic study identified two QTL for FHB resistance in PI277012 using a double haploid (DH) mapping population developed from the cross between Grandin (susceptible to FHB) and PI277012. Those two QTL were designated as *Qfhb.rwg-5A.1* and *Qfhb.rwg-5A.2*, and mapped to 5AS and 5AL chromosomes, respectively (Chu et al., 2011). *Qfhb.rwg-5A.1* peaked at marker Xbarc40 on 5AS in a 40.8 cM interval flanked by markers Xcfa2104 and Xgwm617 and explained up to 20% of the phenotypic variation. *Qfhb.rwg-5A.2* peaked at marker Xcfd39 in a 40.4 cM interval flanked by markers Xwmc470 and Xbarc48 and explained up to 32% of the phenotypic variation (Chu et al., 2011).

Bobwhite is a spring wheat cultivar from CIMMYT (Warburton et al., 2002). It has a high plant regeneration ability during the tissue culture process and is widely used for wheat transformation to generate transgenic wheat plants (Jones, 2005). However, genetic mechanism for high transformability in Bobwhite is not known. Also, it is highly susceptible to FHB (Mackintosh et al., 2007). A preliminary study in our lab showed that PI277012 failed to regenerate plants from calli derived from immature embryo tissue culture. Because of the significant differences in plant regeneration and FHB resistance between Bobwhite and PI 277012, a RIL mapping population derived from the cross were used in the study to identify the QTL associated with plant regeneration from the immature embryo of Bobwhite as well as to verify the FHB resistance QTL previously identified in PI 277012.

Materials and methods

Plant materials

A bi-parental mapping population containing 186 recombinant inbred lines (RILs) (F2:7) was developed from the cross between Bobwhite and PI 277012 using the single seed descent method. This mapping population was used to detect the QTL for transformation-related traits

and verify the FHB resistance QTL previously detected in PI 277012 (Chu et al. 2011). Bobwhite exhibits a high plant regeneration capacity whereas PI 277012 has no regeneration via the callus induction method. Also, Bobwhite is an FHB-susceptible parent whereas PI277012 is an FHB-resistant parent. Other wheat genotypes, Alsen (FHB-resistant), ND2710 (FHB-resistant), and Wheaton (FHB-susceptible), were used as controls in FHB inoculation experiments.

Embryo isolation and callus induction

The RILs and their parents were planted in greenhouse rooms. Plants from each RILs and parents were grown in 15 cm diameter clay pots filled with potting mix (Pro-mix BX; Premier Tech Horticulture, Canada) and supplemented with slow-release fertilizer (Osmocote Plus 15-9-12 N-P-K plus minors; Everris Inc., Dublin, OH) after planting. The greenhouse was supplemented with artificial light provided by 600-watt High Pressure Sodium Lamps (P.L. Light Systems Inc, Beamsville, Canada) for a 14 h photoperiod and the temperature was maintained between 22 and 25 °C. Spikes were collected 14 days after anthesis. Immature kernels were removed from lemma and palea using hands and surface sterilized with 70% (v/v) ethanol for 1 minute. Then, they were treated with 1% sodium hypochlorite for 10 minutes and washed three times with autoclaved distilled water. Immature embryos were isolated under a dissecting microscope using a scalpel and forceps. The embryo axis on immature embryos was removed and placed scutellum down on the callus induction (CI) medium. The protocols of Liang et al. (2018) were adjusted and followed for all tissue culture mediums. Callus induction medium contains 4.4 g/L of Murashige and Skoog (MS) salts including vitamins, 30 g/L sucrose, 2 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D), 0.5 g N-Z-amine A, 600 µl/L of CuSo4 (1mg/ml), 3.2 g/L phytagel and pH adjusted to 5.8. A total of 30 immature embryos were placed

on each of the CI petri plates and two replications were used for each RIL and parent. The petri plates with the immature embryos were incubated in dark at 25 ºC for 2-3 weeks.

Plant regeneration

After callus induction, calli were transferred to the regeneration medium containing 4.4 g/L of MS salt including vitamins, 30 g/L sucrose, 5mg/L zeatin, 3.2 g/L phytagel, and pH adjusted to 5.8. Calli were incubated in a regeneration medium for 3-4 weeks in the culture room with a daily cycle of 16 hours light and 8 hours dark. The number of calli with shoots were counted and the plant regeneration rates in percentage (%) were calculated according to the ratio of the number of calli with shoots to the total number of calli in the petri dish, multiplied by 100:

$$\text{Plant regeneration } \% = \frac{\text{The number of calli with shoots}}{\text{Total number of calli in petri plate}} \times 100$$

Phenotyping of FHB resistance

The 186 RILs and their parents along with controls were evaluated for Type II resistance using the single spikelet inoculation method in both field and greenhouse experiments.

The greenhouse experiment was conducted in the fall of 2020 at NDSU AES greenhouse, Fargo. Plants were grown in 6-inch clay pots filled with potting mix and supplemented with a slow-release fertilizer. The pots were arranged on greenhouse benches in a completely randomized design (CRD) with three replications per wheat line. One pot was used as one replicate and thus each wheat line had three replicates. The greenhouse was supplemented with artificial light provided by 600-W high-pressure sodium lamps for a 14-hours photoperiod. The temperature was maintained between 22 and 25 °C. The inoculum was prepared using four pathogenic strains (Fg 8_13, Fg 10_124_1, Fg 10_135_5, and Fg 13_79) of *F. graminearum* collected from North Dakota and the spore concentration of 100,000 spores mL-1 was used for inoculation (Puri & Zhong, 2010). Inoculation was performed at anthesis (with 50% spikelets

flowering in a spike) by injecting ten microlitres of the spore suspension into the central spikelet using a syringe. 6-10 spikes were inoculated in each pot. The inoculated spikes were misted with water and then covered with a 13 cm transparent polyethylene bag for 48 hours to maintain the high humidity and ensure proper disease development.

The field experiment was conducted in the summer of 2020 at FHB nursery located in Fargo, ND. A randomized complete block design (RCBD) with two replications (hill plots) was used for the field experiment. Each hill plot was planted with 10-15 seeds from each line. The same inoculation method used in the greenhouse experiment was used in the field experiment. At least 8 spikes with similar flowering stage in a hill were inoculated. The flowering date for each hill plot was recorded during the inoculation. An overhead misting system was installed in the field to maintain high humidity for the disease development. The misting system was set up for 12 hours in a pattern of 5 minutes misting in 60 minutes intervals from 6 pm to 6 am. The misting started on the first day of inoculation and ended 14 days after the latest flowering wheat lines were inoculated in the nursery.

FHB disease severity was assessed at 21 days post-inoculation using the visual scale developed by Stack and McMullen (1998) for both field and greenhouse experiments. Disease severity was visually assessed using a modified 1-9 Horsfall-Barrett disease rating scale that expresses the disease severity percentages (0, 7, 14, 21, 33, 50, 67, 80, and 100 %) on wheat spikes (Stack and McMullen 1998). For each replicate, mean FHB severity was calculated by averaging disease severities of all inoculated and rated spikes.

DNA extraction and genotyping by sequencing (GBS)

Fresh young leaves from RILs and their parents were collected into 96-deepwell plates, freeze-dried using liquid nitrogen, and ground using TissueLyser (QIAGEN, USA). The DNA

was extracted using a QIAGEN DNeasy plant mini kit. DNA concentration was measured using nanodrop (Thermo Scientific™, USA). The DNA samples were diluted to 50 ng/ μL and sent to the Center for Grain and Animal Health Research at USDA-ARS, Manhattan, KS, for genotyping using the genotyping by sequencing approach as described by Niu et al (2020). SNPs were called by a universal network-enabled analysis kit (UNEAK) pipeline (Elshire et al. 2011; Glaubitz et al. 2014), and only those SNPs with < 30% missing data were used for genetic linkage map construction and QTL mapping. The physical positions of SNPs on Chinese Spring wheat reference genome (IWGSC et al. 2018) were identified by BLASTn search using the SNP sequences as queries.

Genetic linkage map construction

The computer program MapDisto (v2.1.7) was used to construct a genetic linkage map (Lorieux, 2012). A Chi-square test was conducted for each marker to measure the deviation of allelic or genotypic frequencies from Mendelian expectations, along with their associated probabilities for the loci of the linkage group. The SNP markers were first organized into groups according to the chromosomes to which they belong. The “Find linkage group” command was used with a minimum LOD of 3.0 and maximum recombination frequency (θ) of 0.3 to determine the number of linkage groups for a specific chromosome. Then “order the linkage group” command was used to establish the initial order of markers within a linkage group. The best map order was determined after further analyses using the “check inversions" and “ripple order” commands. For co-segregating markers at each unique locus, only one representative was selected for constructing the genetic linkage map. Several ordering algorithms such as the Kosambi mapping function, classical recombination frequency estimate, SARF ordering criteria, and seriation ordering method were used to calculate map distances in all linkage groups.

QTL analysis

The computer program QGene (v4.4.0) (Joehanes & Nelson, 2008) was used for QTL analysis. The composite interval mapping (CIM) (Jiang et al., 2007) method was chosen in QGene to determine the significantly associated QTL. The LOD threshold for claiming the significance of QTL at $P<0.05$ was 3.0 which was determined by performing 1000 permutation tests (Churchill & Doerge, 1994). The percentage of phenotypic variance explained by a QTL was calculated using the "R^2" function of QGene.

Results

Variations in plant regeneration among RILs and parents

The parents Bobwhite and PI277012 showed significant differences in plant regeneration rate from from calli induced from the tissue culture of immature embryos. Bobwhite had an average regeneration rate of 42.5%, whereas PI 277012 failed to regenerate any plants (Figure 1). The RILs varied in plant regeneration with an average rate ranging from 0 – 33%. The distribution of regeneration percentages was continuous, indicating that this transformation-related trait is quantitatively inherited. No transgressive segregation was observed at both higher and lower levels of regenerations (Figure 2).

Figure 1: Regeneration of shoots from the calli in regeneration medium.

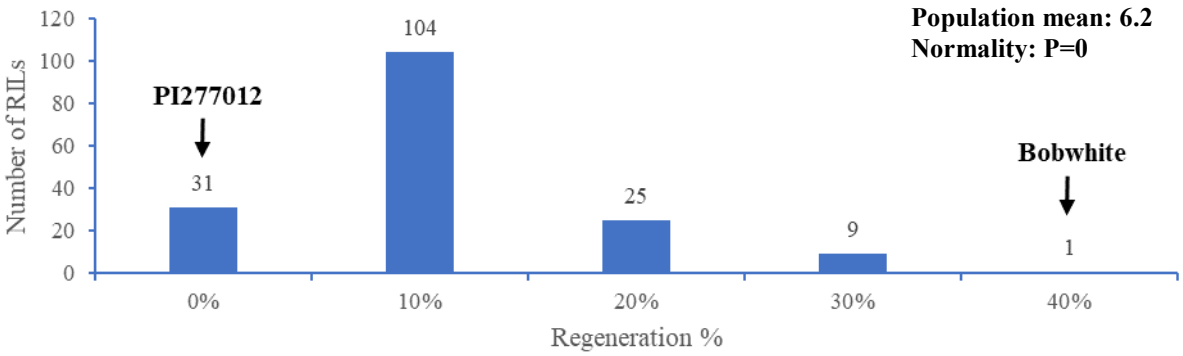

Figure 2: Frequency distribution of plant regeneration in Bobwhite/PI277012 RILs. Arrows indicate the plant regeneration of parents. Normality test was performed using Kolmogorov-Smirnov test (K-S=0.187) in QGene (v4.4.0).

Variations in FHB severity among RILs and parents

The resistant parent PI 277012 showed a high level of resistance whereas the susceptible parent Bobwhite showed moderately susceptible in both GH and field experiments. PI 277012 had an average disease severity of 10% while Bobwhite had average disease severity of 50% in both field and GH. The RIL population showed a continuous distribution of disease severity in both field and greenhouse (GH) experiments (Figure 3 and Figure 4). This indicates that disease severity is a quantitative trait. Disease severities were higher in GH as compared to those under the field conditions. Transgressive segregation was observed at the higher levels of disease severity (Figure 3 and Figure 4).

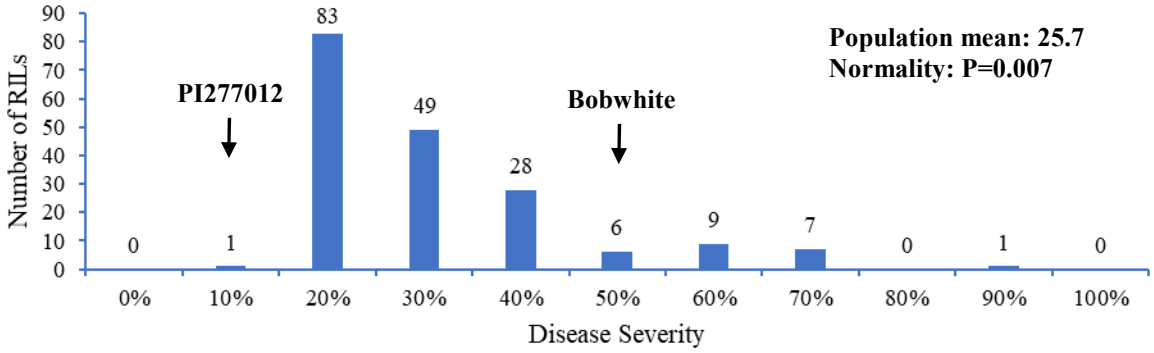

Figure 3: Frequency distribution of FHB severity in Bobwhite/PI277012 RILs in a field experiment. Arrows indicate the FHB severity of parents. Normality test was performed using Kolmogorov-Smirnov test (K-S=0.148) in QGene (v4.4.0).

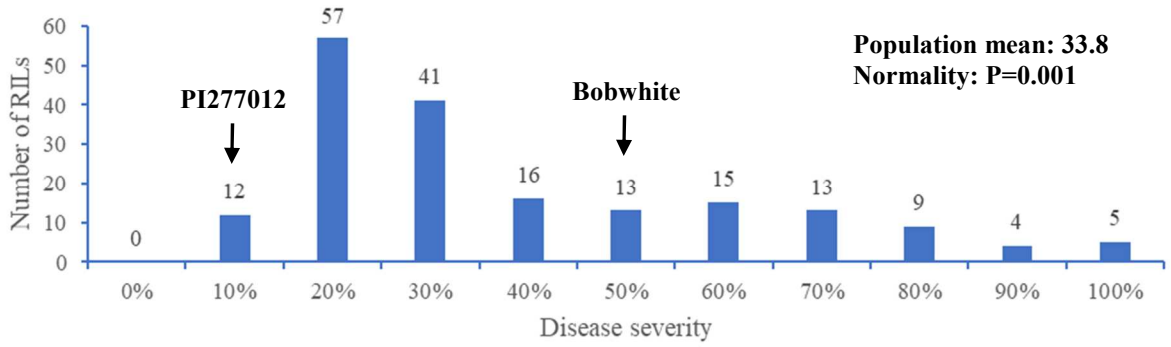

Figure 4: Frequency distribution of FHB severity in Bobwhite/PI277012 RILs in a greenhouse experiment. Arrows indicate the FHB severity of parents. Normality test was performed using Kolmogorov-Smirnov test (K-S=0.167) in QGene (v4.4.0).

Linkage map construction

A total of 3779 polymorphic SNP markers with less than 30% missing data were identified by using the genotyping by sequencing method. After removal of co-segregating markers, 1105 unique SNP markers were used to construct the genetic map, which consisted of 30 linkage groups. 9 of the linkage groups were assigned to genome A, 8 to genome B, and 13 to genome D. The total genetic map length was 2291.48 cM, partitioned into 958.24 cM for genome A, 833.3 cM for genome B, and 499.94 cM for genome D (Table 1). Average distance between markers was 0.61 cM.

Table 1: Total number of markers, linkage group, and linkage distance in all 21 chromosomes.

Chromosome	number of markers mapped	number of linkage group	Linkage distance (cM)
1A	177	2	116.7
1B	53	1	81.59
1D	70	1	82.84
2A	150	2	107.03
2B	468	1	166.06
2D	42	1	77.01
3A	244	1	152.71
3B	264	1	129.35
3D	59	2	77.79
4A	198	1	109.18
4B	69	2	100.54
4D	10	1	30.22
5A	370	1	167.25
5B	268	1	128.78
5D	43	3	103.39
6A	191	1	139.89
6B	305	1	106.43
6D	76	3	44.51
7A	423	1	165.48
7B	272	1	120.55
7D	27	2	84.18
Total	**3779**	**30**	**2291.48**

QTL for plant regeneration and FHB resistance

QTL analysis detected two QTL on chromosome 1A ((*Qprc-ndwp-1A*) and 6D (*Qprc-ndwp-6D*) controlling plant regeneration capability derived from Bobwhite (Table 2). *Qprc-ndwp-1A* accounted for 10.5% of the phenotypic variation with a LOD value of 4.09. 1A_17215908 and 1A_47828499 were the flanking markers for *Qprc-ndwp-1A* with 1A_31257987 as the peak marker. *Qprc-ndwp-6D* explained 9.8% of the phenotypic variation with a LOD value of 3.88. 6D_405818725 and 6D_427415496 were the flanking markers for *Qprc-ndwp-6D* with 6D_427415496 as the peak marker.

One major effect QTL (*Qfhb-ndwp-5A.2*) associated with FHB resistance (type-II) was identified in this population and it explained 33.0% and 36.2% of the phenotypic variance in the field and greenhouse experiments, respectively. *Qfhb-ndwp-5A.2* had a LOD value of 15.84 and 18.05 for the field and greenhouse data, respectively (Table 2, Figure 5). The critical LOD threshold of 3.0 for the 0.05 level of probability was obtained using the 1,000-permutation test.

Table 2: Summary of QTL detected for plant regeneration and FHB severity (type-II resistance) using composite interval mapping (CIM) in Bobwhite/PI 277012 population

QTL	Trait	Chr	Peak marker	Flanking markers	LOD	R2
Qprc-ndwp-1A	PRC	1A	1A_31257987	1A_17215908 - 1A_47828499	4.1	0.1
Qprc-ndwp-6D	PRC	1B	6D_427415496	6D_405818725 - 6D_427415496	3.9	0.09
Qfhb-ndwp-5A.2	FHB (G)	5A	5A_651533373	5A_620541509 - 5A_700162829	18.1	0.36
Qfhb-ndwp-5A.2	FHB (F)	5A	5A_651533373	5A_620541509 - 5A_700162829	15.8	0.33

QTL: quantitative trait loci, Reg: Regeneration, FHB (G): FHB resistance in GH, FHB (F): FHB resistance in the field, LOD: logarithm of odds, R2: proportion of phenotypic variance explained by each QTL.

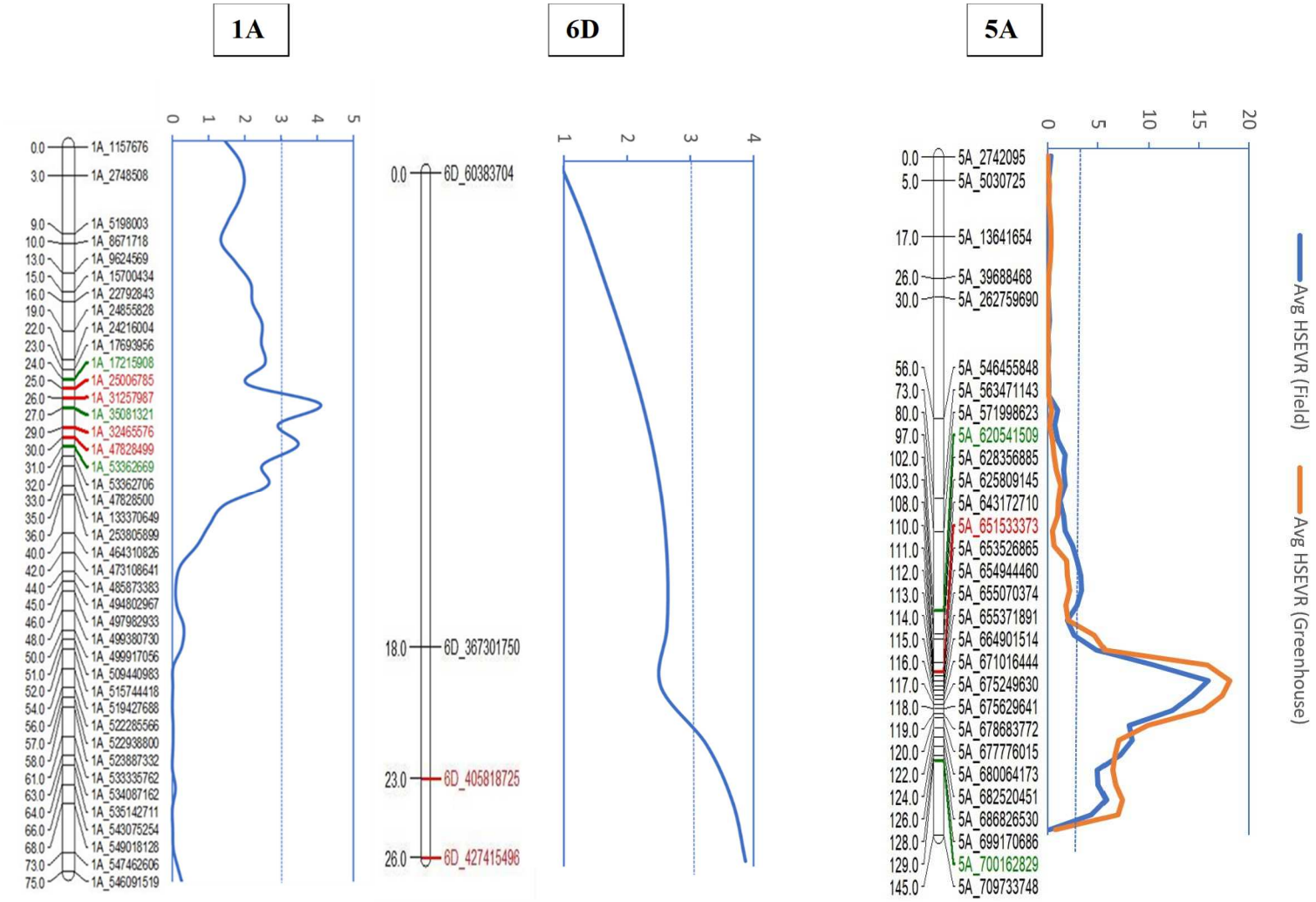

Figure 5: Linkage maps for chromosomes 1A and 6D showing QTL (*Qprc-ndwp-1A* and *Qprc-ndwp-6D*) for plant regeneration capacity, and chromosome 5A for type-II resistance QTL (*Qfhb-ndwp-5A.2*) to FHB detected in the Bobwhite/PI2770122 RILs population.

Discussion

The good TCR of immature embryos refers to the high callus induction, high embryonic callus formation rate, and ultimately high plant regeneration from the embryogenic callus. In a previous study, no significant differences for callus induction from immature embryos among wheat genotypes were reported (Wu et al., 2003). However, other studies showed a wide variation of TCRs between two parents and their RILs (Jia et al., 2009; Ma et al. 2016; Abd El-Fatah et al. 2020), suggesting TCR is a quantitative trait controlled by genetic factors. In the present study, significantly different TCRs were observed between the parents Bobwhite and PI 277012 and in the RILs population derived from them, and two QTL associated with plant regeneration as a TCR from immature wheat embryos were identified on chromosome 1A and 6D, respectively.

In previous studies, various small effect QTL associated with TCR have been identified for both mature and immature embryos. Jia et al. (2007) indicated that wheat group 5 chromosomes (5A, 5B and 5D) play key roles in TCR of Triticeae crops. This has been confirmed by several studies showing QTL for percentage of embryos forming a callus (PEFC) and percentage of calli regenerating plantlets (PCRP) were identified on chromosome 5A and 5B during culturing of both mature and immature wheat embryos (Jia et al. 2007, 2009; Ma et al. 2016). In addition, Nielsen et al. (2015) identified loci on 5A (wPt-6135) and 5B (tPt-4184) associated with embryo formation in culturing of wheat microspores. Mano and Komatsuda (2002) detected a QTL on barley chromosome 5H controlling callus induction from barley immature embryos. However, no QTL for plant regeneration were detected on chromosome 5A, 5B, and 5D in Bobwhite in the present study, instead, the two detected QTL for plant regeneration were mapped on chromosome 1A and 6D. These results suggest that except for

group 5 chromosomes, some other chromosomes may carry QTL for transformation-related traits depending on the wheat genotypes used in the research.

Ma et al. (2016) identified a QTL for differentiation rate (*QDiffr.sau-1A*) on chromosome 1A using mature embryos of wheat cultivar SHW-L1 as explants. This QTL was localized at position 180.71 flanked by the two markers wPt-3698/wPt-730408 and wPt-666607/wPt-665725. According to the positions of the flanking markers, the QTL for plant regeneration identified on 1A in Bobwhite is not in the same region where *QDiffr.sau-1A* is located. Therefore, the 1A QTL detected from Bobwhite represents a novel QTL for TCR. Ma et al. (2016) also detected a QTL for callus induction (*QCallr.sau-6D*) on chromosome 6D. This QTL was localized at position 1.21 near telomere of the chromosome. According to the marker position, this QTL is different from the one identified in Bobwhite. Taken together, the two QTL identified from Bobwhite in the present study are novel.

LOD values for all these QTL ranged from 2.05 to 5.02 with the phenotypic variation ranging from 9.88 to 26.5 %. This indicates that QTL for the TCR are polygenic and quantitatively inherited. This relatively small LOD value for most of the QTL might be due to the small effect of the individual QTL. Because of the heavy workload, the sample size and population size used in these investigations are frequently limited, which may result in a higher rate of experimental errors than other characteristics. Several research have studied genetics of TCR in monocot plants, however, the detailed mechanism for plant regeneration is still not well-studied (Jia et al., 2009).

Chu et al. (2011) identified two QTL for FHB resistance in PI 277012 using a mapping population of 130 DH lines derived from the cross between Grandin and PI 277012. These two QTL were named as *Qfhb.rwg-5A.1* and *Qfhb.rwg-5A.2* and mapped on short and long arms of

chromosome 5A, respectively. However, only one major effect QTL (*Qfhb.ndwp-5A.2*) was detected on chromosome 5AL in the mapping population of 183 RILs derived from the cross between Bobwhite and PI 277012. This QTL mapped to the same region as *Qfhb.rwg-5A.2* on the long arm of chromosome 5A of PI 277012, and thus *Qfhb.ndwp-5A.2* and *Qfhb.rwg-5A.2* are presumably the same QTL. Failure to detect *Qfhb.rwg-5A.1* in the Bobwhite × PI 277012 population is probably due to the fact that this QTL is not consistently expressed across different environments or in different genetic backgrounds. In a large RIL population (n=234) developed from the crosses between PI 277012 and the FHB-susceptible 'Langdon' (LDN) durum, Ren et al. (2020) also failed to detect *Qfhb.rwg-5A.1* although *Qfhb.rwg-5A2* was identified along with several new QTL previously not found. *Qfhb.rwg-5A.2* has been successfully introgressed into durum wheat (Zhao et al., 2018), indicated that this QTL is a major QTL for FHB resistance and is stably expressed in different genetic backgrounds. A major QTL on 5AL was reported by Buerstmayr et al. (2011) in a *T. macha* line and in the cultivated emmer wheat accession PI 41025 reported by Zhang et al., 2014. All these QTL are mapped near the domestication gene Q (Simons et al., 2006), which indicates that they might be localized at the same locus or closely linked together. However, *Qfhb.rwg-5A2* is not the same as the q allele from PI 277012 because resistant RILs with the Q allele were recovered from both mapping populations from the cross between Grandin (susceptible to FHB with the Q allele) and PI 277012 (resistant to FHB with the q allele) (Chu et al., 2011) and the cross between Joppa (moderately susceptible to FHB with the Q allele) and 10Ae564 (moderately resistant to FHB with the q allele) (Zhao et al., 2018).

In summary two QTL associated with plant regeneration from the wheat cultivar 'Bobwhite' were identified. The information from this study may facilitate the deployment of the transformability QTL in wheat varieties that are difficult to transform through gene introgression

combined with marker-assisted selection. The use of transformability QTL along with growth regulator genes such as *GROWTH-REGULATING FACTOR 4* (*GRF4*) (Debernardi et al., 2020), *WUSHEL* (*WUS*) (Zuo et al., 2002), and *BABY BOOM* (*BBM*) (Boutilier et al., 2002) might maximize the transformation efficiency in wheat. This study also indicated that the QTL for FHB resistance *Qfhb.rwg-5A.2* in PI 277012 is a major QTL for FHB resistance and is stably expressed in different genetic backgrounds.

References

Bai Guihua, & Shaner, G. (1994). Scab of wheat: Prospects for control. *Plant Disease*, *78*(8), 760–766.

ben Amer, I. M., A Börner, & R Schlegel. (1992). The effect of hybrid dwarfing gene D2 on tissue culture. Response of wheat (Triticum aestivum L.). *Cereal Research Communications*.

ben Amer, I. M., Korzun, V., Worland, A. J., & Börner, A. (1997). Genetic mapping of QTL controlling tissue-culture response on chromosome 2B of wheat (Triticum aestivum L.) in relation to major genes and RFLP markers. *Theoretical and Applied Genetics 1997 94:8*, *94*(8), 1047–1052.

ben Amer, I. M., Worland, A. J., & Börner, A. (1996). The effects of whole chromosome substitutions differing in alleles for hybrid dwarfing and photoperiodic sensitivity on tissue culture response (TCR) in wheat. *Euphytica 1996 89:1*, *89*(1), 81–86.

Boutilier, K., Offringa, R., Sharma, V. K., Kieft, H., Ouellet, T., Zhang, L., Hattori, J., Liu, C.-M., van Lammeren, A. A. M., Miki, B. L. A., Custers, J. B. M., & van Lookeren Campagne, M. M. (2002). Ectopic expression of BABY BOOM triggers a conversion from vegetative to embryonic growth. *The Plant Cell*, *14*, 1737–1749.

Buerstmayr, H., Ban, T., & Anderson, J. A. (2009). QTL mapping and marker-assisted selection for Fusarium head blight resistance in wheat: a review. *Plant Breeding*, *128*(1), 1–26.

Buerstmayr, M., Lemmens, M., Steiner, B., & Buerstmayr, H. (2011). Advanced backcross QTL mapping of resistance to Fusarium head blight and plant morphological traits in a Triticum macha× T. aestivum population. *Theoretical and Applied Genetics*, *123*(2), 293–306.

Carman, J. G., Jefferson, N. E., & Campbelt, W. F. (1988). Induction of embryogenic Triticum aestivum L. calli. *Plant Cell, Tissue and Organ Cult*, 12(1).

Chu, C., Niu, Z., Zhong, S., Chao, S., Friesen, T. L., Halley, S., Elias, E. M., Dong, Y., Faris, J. D., & Xu, S. S. (2011). Identification and molecular mapping of two QTLs with major

effects for resistance to Fusarium head blight in wheat. *Theoretical and Applied Genetics*, *123*(7), 1107–1119.

Churchill, G. A., & Doerge, R. W. (1994). Empirical threshold values for quantitative trait mapping. *Genetics*, *138*(3), 963–971.

Debernardi, J. M., Tricoli, D. M., Ercoli, M. F., Hayta, S., Ronald, P., Palatnik, J. F., & Dubcovsky, J. (2020). A GRF–GIF chimeric protein improves the regeneration efficiency of transgenic plants. *Nature Biotechnology 2020 38:11*, *38*(11), 1274–1279.

del Ponte, E. M., Valent, B., & Bergstrom, G. C. (2017). A special issue on Fusarium head blight and wheat blast. *Tropical Plant Pathology*, *42*(3), 143–145.

Felsenburg, T., Feldman, M., & Galun, E. (1987). Aneuploid and alloplasmic lines as tools for the study of nuclear and cytoplasmic control of culture ability and regeneration of scutellar calli from common wheat. *Theoretical and Applied Genetics 1987 74:6*, *74*(6), 802–810.

El-Fatah, A., Bahaa, E. S., Sayed, M. A., & El-Sanusy, S. A. (2020). Genetic analysis of anther culture response and identification of QTLs associated with response traits in wheat (Triticum aestivum L.). Molecular Biology Reports, 47(12), 9289-9300.

Elshire, R. J., Glaubitz, J. C., Sun, Q., Poland, J. A., Kawamoto, K., Buckler, E. S., & Mitchell, S. E. (2011). A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. PloS one, 6(5), e19379.

Galiba, G., Kovacs, G., & Sutka, J. (1986). Substitution analysis of plant regeneration from callus culture in wheat. *Plant Breeding*, *97*(3), 261–263.

Glaubitz, J. C., Casstevens, T. M., Lu, F., Harriman, J., Elshire, R. J., Sun, Q., & Buckler, E. S. (2014). TASSEL-GBS: a high capacity genotyping by sequencing analysis pipeline. PloS one, 9(2), e90346.

Hansen, G., & Wright, M. S. (1999). Recent advances in the transformation of plants. *Trends in Plant Science*, *4*(6), 226–231.

Henry, Y., Vain, P., & de Buyser, J. (1994). Genetic analysis of in vitro plant tissue culture responses and regeneration capacities. *Euphytica 1994 79:1*, *79*(1), 45–58.

Jia, H., Yi, D., Yu, J., Xue, S., Xiang, Y., Zhang, C., & Ma, Z. (2007). (2007). Mapping QTLs for tissue culture response of mature wheat embryos. *Molecules & Cells (Springer Science & Business Media BV) ,* 23(3).

Jia, H., Yu, J., Yi, D., Cheng, Y., Xu, W., Zhang, L., & Ma, Z. (2009). Chromosomal intervals responsible for tissue culture response of wheat immature embryos. *Plant Cell, Tissue and Organ Culture*, *97*(2), 159–165.

Jiang, G. L., Dong, Y., Shi, J. R., & Ward, R. W. (2007). QTL analysis of resistance to Fusarium head blight in the novel wheat germplasm CJ 9306. II. Resistance to deoxynivalenol accumulation and grain yield loss. *Theoretical and Applied Genetics*, *115*(8), 1043–1052.

Joehanes, R., & Nelson, J. C. (2008). QGene 4.0, an extensible Java QTL-analysis platform. *Bioinformatics*, *24*(23), 2788–2789.

Jones, H. D. (2005). Wheat transformation: current technology and applications to grain development and composition. *Journal of Cereal Science*, *41*(2), 137–147.

Kaleikau, E. K., Sears, R. G., & Gill, B. S. (1989). Control of tissue culture response in wheat (Triticum aestivum L.). *Theoretical and Applied Genetics 1989 78:6*, *78*(6), 783–787.

Lazar, M. D., Collins, G. B., & Vian, W. E. (1983). Genetic and environmental effects on the growth and differentiation of wheat somatic cell cultures. *Journal of Heredity*, *74*(5), 353–357.

Liang, Z., Chen, K., Zhang, Y., Liu, J., Yin, K., Qiu, J. L., & Gao, C. (2018). Genome editing of bread wheat using biolistic delivery of CRISPR/Cas9 in vitro transcripts or ribonucleoproteins. *Nature Protocols 2018 13:3*, *13*(3), 413–430.

Lorieux, M. (2012). MapDisto: Fast and efficient computation of genetic linkage maps. *Molecular Breeding*, *30*(2), 1231–1235.

Lotan, T., Ohto, M. A., Matsudaira Yee, K., West, M. A. L., Lo, R., Kwong, R. W., Yamagishi, K., Fischer, R. L., Goldberg, R. B., & Harada, J. J. (1998). Arabidopsis LEAFY COTYLEDON1 is sufficient to induce embryo development in vegetative cells. *Cell*, *93*(7), 1195–1205.

Lowe, K., la Rota, M., Hoerster, G., Hastings, C., Wang, N., Chamberlin, M., Wu, E., Jones, T., & Gordon-Kamm, W. (2018). Rapid genotype "independent" Zea mays L. (maize) transformation via direct somatic embryogenesis. *In Vitro Cellular and Developmental Biology - Plant*, *54*(3), 240–252.

Lowe, K., Wu, E., Wang, N., Hoerster, G., Hastings, C., Cho, M. J., Scelonge, C., Lenderts, B., Chamberlin, M., Cushatt, J., Wang, L., Ryan, L., Khan, T., Chow-Yiu, J., Hua, W., Yu, M., Banh, J., Bao, Z., Brink, K., … Gordon-Kamm, W. (2016). Morphogenic regulators baby boom and wuschel improve monocot transformation. *The Plant Cell*, *28*(9), 1998–2015.

Ma, J., Deng, M., Lv, S.-Y., Yang, Q., Jiang, Q.-T., Qi, P.-F., Li, W., Chen, G.-Y., Lan, X.-J., & Wei, Y.-M. (2016). Identification of QTLs associated with tissue culture response of mature wheat embryos. *SpringerPlus*, *5*(1), 1552.

Mackintosh, C. A., Lewis, J., Radmer, L. E., Shin, S., Heinen, S. J., Smith, L. A., Wyckoff, M. N., Dill-Macky, R., Evans, C. K., Kravchenko, S., Baldridge, G. D., Zeyen, R. J., & Muehlbauer, G. J. (2007). Overexpression of defense response genes in transgenic wheat enhances resistance to Fusarium head blight. *Plant Cell Reports*, *26*(4), 479–488.

Mathias, R. J., & Fukui, K. (1986). The effect of specific chromosome and cytoplasm substitutions on the tissue culture response of wheat (Triticum aestivum) callus. *Theoretical and Applied Genetics 1986 71:6*, *71*(6), 797–800.

Oliver, R. E., Cai, X., Friesen, T. L., Halley, S., Stack, R. W., & Xu, S. S. (2008). Evaluation of Fusarium head blight resistance in tetraploid wheat (Triticum turgidum L.). *Crop Science*, *48*(1), 213–222.

Puri, K. D., & Zhong, S. (2010). The 3ADON population of Fusarium graminearum found in North Dakota is more aggressive and produces a higher level of DON than the prevalent 15ADON population in spring wheat. *Phytopathology,* 100(10), 1007-1014.

Qiu, F., Xing, S., Xue, C., Liu, J., Chen, K., Chai, T., & Gao, C. (2021). Transient expression of a TaGRF4-TaGIF1 complex stimulates wheat regeneration and improves genome editing. *Science China Life Sciences 2021*, 1–8.

Simons, K. J., Fellers, J. P., Trick, H. N., Zhang, Z., Tai, Y. S., Gill, B. S., & Faris, J. D. (2006). Molecular characterization of the major wheat domestication gene Q. *Genetics*, *172*(1), 547–555.

Stack, R. W., & McMullen, M. P. (1998). A visual scale to estimate severity of Fusarium head blight in wheat.

Stone, S. L., Kwong, L. W., Yee, K. M., Pelletier, J., Lepiniec, L., Fischer, R. L., Goldberg, R. B., & Harada, J. J. (2001). LEAFY COTYLEDON2 encodes a B3 domain transcription factor that induces embryo development. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(20), 11806–11811.

Tao, L. li, Yin, G. xiang, Du, L. pu, Shi, Z. yuan, She, M. yun, Xu, H. jun, & Ye, X. guo. (2011). Improvement of plant regeneration from immature embryos of wheat infected by Agrobacterium tumefaciens. *Agricultural Sciences in China*, *10*(3), 317–326.

Wegulo, S. N., Baenziger, P. S., Hernandez Nopsa, J., Bockus, W. W., & Hallen-Adams, H. (2015). Management of Fusarium head blight of wheat and barley. *Crop Protection*, *73*, 100–107.

Wegulo, S. N., Bockus, W. W., Nopsa, J. F. H., Peiris, K. H. S., & Dowell, F. E. (2013). Integration of fungicide application and cultivar resistance to manage Fusarium head blight in wheat. *Fungicides - Showcases of Integrated Plant Disease Management from Around the World,* 35-54.

Wu, B. H., Zheng, Y. L., Liu, D. C., & Zhou, Y. H. (2003). Trait correlation of immature embryo culture in bread wheat. *Plant Breeding*, *122*(1), 47–51.

Xu, S., Oliver, R., Cai, X., Friesen, T., Halley, S., & Elias, E. (2007). Searching for new sources of FHB resistance in the relatives of wheat. *In: Canty S, Clark A, Ellis D, Van Sanford D (Eds) Proceedings of the National Fusarium Head Blight Forum, Kansas City, MO, 2–4 December, 2007, University of Kentucky, Lexington*.

Yin, G.-X., Wang, Y.-L., She, M.-Y., Li-Pu, D. U., Xu, H.-J., Ma, J.-X., & Xing-Guo, Y. E. (2011). Establishment of a highly efficient regeneration system for the mature embryo culture of wheat. *Agricultural Sciences in China*, 10(1), 9-17.

Zhang, Q., Axtman, J. E., Faris, J. D., Chao, S., Zhang, Z., Friesen, T. L., Zhong, S., Cai, X., Elias, E. M., & Xu, S. S. (2014). Identification and molecular mapping of quantitative trait loci for Fusarium head blight resistance in emmer and durum wheat using a single nucleotide polymorphism-based linkage map. *Molecular Breeding*, *34*(4), 1677–1687.

Zhao, M., Leng, Y., Chao, S., Xu, S. S., & Zhong, S. (2018). Molecular mapping of QTL for Fusarium head blight resistance introgressed into durum wheat. *Theoretical and Applied Genetics*, *131*(9), 1939–1951.

Zhou, Z., Yang, Y., Ai, G., Zhao, M., Han, B., Zhao, C., Chen, Y., Zhang, Y., Pan, H., Lan, C., Li, Q., Xu, J., & Yan, W. (2022). Boosting transformation in wheat by BBM-WUS. *BioRxiv*, 2022.03.13.483388.

Zuo, J., Niu, Q. W., Frugis, G., & Chua, N. H. (2002). The WUSCHEL gene promotes vegetative-to-embryonic transition in Arabidopsis. *Plant Journal*, *30*(3), 349–359.

TARGETING *Tsn1* GENE IN WHEAT USING GENETIC TRANSFORMATION AND CRISPR/Cas9- MEDIATED GENE EDITING TECHNOLOGY

Abstract

Pyrenophora tritici-repentis causes tan spot, an important foliar disease of wheat. This fungus produces ToxA, a proteinous necrotrophic effector that interacts with a wheat gene (*Tsn1*) to cause the disease. Previous gene cloning study showed that loss of function mutations of the *Tsn1* gene led to resistance to tan spot or insensitivity to ToxA in wheat plants. The aim of this study was to target the *Tsn1* gene for reducing susceptibility to tan spot using genetic transformation coupled with the CRISPR/Cas9 genome editing technology. CRISPR/Cas9-mediated gene editing vectors expressing Cas9 and single guide RNAs (sgRNAs) for targeting the exon-6 of *Tsn1* were constructed and delivered to immature embryos of wheat cv. Bobwhite or Fielder using the *Agrobacterium*- and particle bombardment- mediated transformation methods. A total of 32 transgenic plants (T_0) with the transgene were generated from the embryogenic calli and confirmed by polymerase chain reaction (PCR) using vector-specific primers. These T_0 plants were selfed to produce 1176 T_1 transgenic plants. ToxA infiltration assay showed all T_1 transgenic plants were sensitive to ToxA just like the wild type (Fielder or Bobwhite), suggesting no loss of function mutations occurred at *Tsn1* in the transgenic plants. The failure to produce loss of function *Tsn1* mutants might be due to the low or no expression of Cas9 and gRNA in the transgenic plants. Further research is required to find out the reason and increase gene editing efficiency using the CRISPR/Cas9 system.

Introduction

Growing human population and increasing demand for wheat products worldwide require trait improvement of the crop for increased productivity, better grain quality, and increased

resistance to biotic as well as abiotic factors. Conventional breeding approaches by hybridization have been contributing to the genetic improvement of wheat for many years (Janakiraman et al., 2002). However, classical breeding techniques have some limitations such as the laborious and time comsuming procedure for developing a new variety and decrease in genetic variability of the crop due to long term intravarietal crossing and selection. These limitations can be overcome by the modern genetic engineering tools such as the targeted genome editing technology with the CRISPR/Cas9 system. The CRISPR/Cas9 system has been used to modify many genes in different plants, including crops such as wheat (Shan et al., 2013; Upadhyay et al., 2013; Wang Y.P. et al., 2014), sorghum (Jiang et al., 2013b), rice (Feng et al., 2013; Jiang et al., 2013b; Shan et al., 2013; Xie and Yang, 2013), maize (Liang et al., 2014), tomato (Brooks et al., 2014; Ron et al., 2014), potato (Butler et al., 2015), and barley (Lawrenson et al., 2015). Using this new technology, mutations of small indels and large gene fragment deletions can be introduced at the target genes in various plant species (Jiang et al., 2013b; Zhu et al., 2017; Lawrenson et al. 2015; Li et al., 2013; Mao et al., 2013; Gao et al., 2015; Brooks et al., 2014, Upadhyay et al., 2013; Zhou et al., 2014). The targeted mutagenesis of genes that results in loss of function not only allows for gene function characterization but also facilitates targeted genetic improvements in major crops. For instance, targeting and knocking out the genes for disease susceptibility in plant hosts may lead to improved resistance.

Tan spot of wheat, also known as the yellow leaf spot, is caused by the fungus *Pyrenophora tritici-repentis*. The disease affects all cultivated wheat species and occurs worldwide. Tan spot develops on both the upper and lower surfaces of leaves of wheat plants (Wegulo et al., 2011). The fungus causes large tan-colored lesions often surrounded by chlorotic haloes. These lesions reduce the photosynthetic ability of plants and ultimately result in yield

loss (Faris et al., 2013). The pathogen can infect wheat spikes and eventually the kernels, causing red smudge of seeds. Multiple races of this pathogen have been characterized based on the necrotic and/or chlorotic symptoms produced on the wheat differential genotypes. Race 1 produces both necrosis and chlorosis, race 2 produces necrosis only, race 3 and race 5 produce chlorosis only and race 4 does not produce any symptoms (Lamari et al. 1995). Race 1 is the most prevalent in North Dakota. Race 1 produces the well-characterized proteinous necrotrphic effector Ptr ToxA, which indirectly interacts with a single dominant gene designated *Tsn1*on the long arm of chromosome 5B to cause tan spot disease (Faris and Friesen 2005; Faris et al. 2010). Loss of function mutation of *Tsn1* gene leads to resistance to tan spot in wheat plants (Faris et al., 2010).

The objectives of this study were to 1) Develop plasmid vectors expressing CRISPR/Cas9 machinery specifically targeting the *Tsn1* gene in wheat, 2) Deliver the vectors to wheat regenerable tissue (immature embryo) using *Agrobacterium*- and particle bombardment-mediated transformation methods, 3) Characterize transgenic plants for sensitivity to ToxA and identify loss of function *Tsn1* mutants.

Materials and methods

Plant materials

The spring wheat cultivars ‘Bobwhite’ and ‘Fielder’ were used for genetic transformation experiments. Both Bobwhite and Fielder carry *Tsn1* and are sensitive to ToxA (Faris et al. 2010). The durum wheat cultivar ND Riveland is insensitive to ToxA (Zhaohui Liu, personal communication) and thus was used as ToxA insensitive control in the ToxA infiltration experiments.

Construction of CRISPR/Cas9 gene editing vectors

For *Agrobacterium*-mediated transformation, the vector pLC41-Ubi-Cas9-MCS-Tsn1-HRC (20,758 bp) was constructed for targeting exon-6 of the *Tsn1* gene in wheat (Figure 6, Figure 7). To construct this vector, a DNA fragment containing multiple cloning sites (MCS) (*Pme* I, *Asc* I, *SnaB* I, *Avr* II, *Hpa* I, *Eag* I, *Fse* I, and *Pac* I) was first inserted at the *Acc65* I restriction site of the 163-ubi-cas9 vector (Zhang et al., 2016) to form the construct of 163-ubi-cas9-MCS. The fragment containing Ubi-Cas9 and MCS was released from 163-ubi-cas9-MCS by enzyme digestion with *Pme* I and *Spe* I and cloned into pLC41-Hm (Ishida et al., 2015) to produce pLC41-Ubi-Cas9-MCS. The single guide RNA (sgRNA) scaffold (gRNA-*Tsn1*) with the *Tsn1* exon-6 target sequence TCGATGTGACAAAGAACAAT was cloned into pLC41-Ubi-Cas9-MCS at the *Asc* I and *SnaB* I sites to produce pLC41-Ubi-Cas9-MCS-Tsn1. The wheat gene *TaHRC* was reported to be involved in FHB susceptibility in wheat (Li et al. 2019; Su et al. 2019). To target *Tsn1* and *TaHRC* at the same time, another sgRNA scaffold (gRNA-HRC1) containing the *TaHRC* target sequence CGTCCAACAGCTTGTCTACA was cloned into pLC41-Ubi-Cas9-MCS-Tsn1 at the *Avr* II and *Hpa* I to produce the vector pLC41-Ubi-Cas9-MCS-Tsn1-HRC1 (sent to UC Davis for wheat transformation). Since the wheat gene *TaPFT1* was showed to confer FHB resistance at the *Fhb1* locus (Rawat et al. 2016), the sgRNA scaffold (gRNA-PFT1) containing the *TaPFT1* target sequence CAGCTCATTTGCGGTACCAA was cloned in pLC41-Ubi-Cas9-MCS-Tsn1 at the *Fse* I and *Pac* I sites to produce the vector pLC41-Ubi-Cas9-MCS-Tsn1-PFT1 with the purpose of targeting *Tsn1* and *TaPFT1* simultaneously.

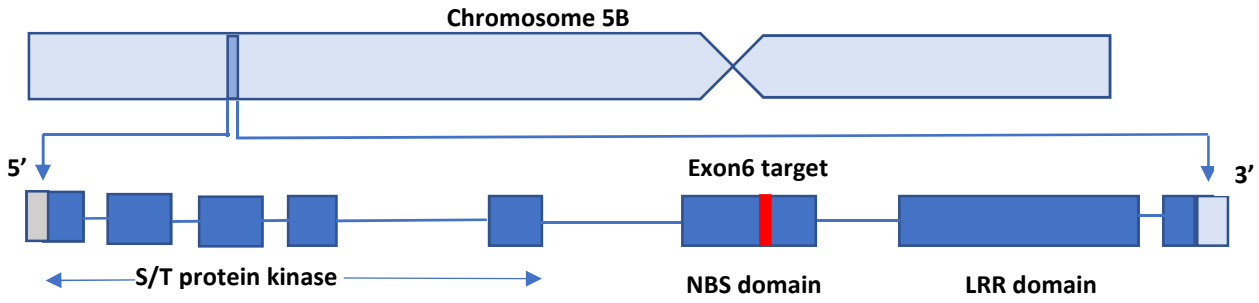

Figure 6: *Tsn1* exon-6 target site in the chromosome 5B by CRISPR/Cas9 vector. Exons and UTRs are shown in blue and gray, respectively (Adapted from Faris et al., 2010)

For particle bombardment transformation, two vectors, pBUN411-Ubi-Cas9-MCS-Tsn1-HRC1 and pBUN411-Ubi-Cas9-MCS-Tsn1-PFT1 were created. These two vectors were constructed by cloning the cassettes containing the two sgRNA scaffolds and ubi-cas9 from pLC41-Ubi-Cas9-MCS-Tsn1-HRC1 and pPL41-Ubi-Cas9-MCS-Tsn1-PFT1 into pBUN411 (Xing et al., 2014), respectively (Figure 8).

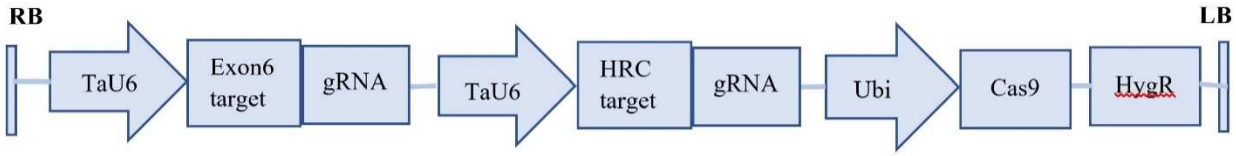

Figure 7: pLC41-Cas9-MCS-Tsn1-HRC vector construct for *Agrobacterium*-mediated transformation. TaU6, wheat U6 promoter; Ubi, maize Ubiquitin promoter ; HygR, Hygromycin resistance; LB, T-DNA left border sequence; RB, T-DNA right border sequence.

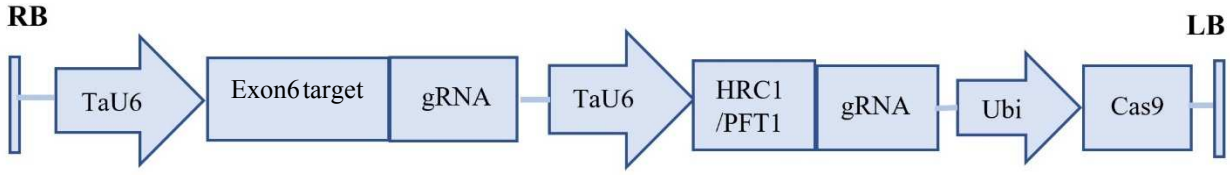

Figure 8: pBUN411-Ubi-Cas9-MCS-Tsn1-HRC1/PFT1 vector construct for particle bombardment transformation. TaU6, U6 wheat promoter; Ubi, Ubiquitin maize promoter; LB, T-DNA left border sequence; RB, T-DNA right border sequence.

Agrobacterium-mediated transformation

The vector pLC41-cas9-MCS-Tsn1-HRC was sent to the Plant Transformation Facility at University of California-Davis, CA, for *Agrobacterium*-mediated transformation using the

protocols of Ishida et al. (2015). Briefly, *Agrobacterium tumefaciens* strain EHA101 was transformed with the vector construct using heat shock method. The inoculum was prepared in LB broth containing 100 µg/ml rifampicin and 100 µg/ml spectinomycin as selection antibiotics with vigorous shaking at 28 ºC for 2 days. Bacteria were collected by centrifugation and resuspended using a WLS-inf medium at the cell density of 0.4 at A_{660} for inoculum. The isolated immature embryos were transferred to a 2 ml WLS-liquid medium and were centrifuged for 10 mins at 14000 rpm. The medium was removed, and 1 ml of inoculum was added to immature embryos in tubes. Tubes were inverted several times to mix inoculum with immature embryos and incubated for 5 mins at room temperature. Then the immature embryos were transferred to WLS-AS medium in a petri plate with scutellum side up. The plate was incubated at 23 ºC in the dark for 2 days. Then immature embryos were transferred to WLS-Res medium and the plates were incubated at 25 ºC in the dark for 5 days. Immature embryos from WLS-Res were transferred to WLS-P5 medium (WLS plus 5mg/l phosphoinothricin) in petri plates and cultured at 25ºC for 2 weeks for callus induction. The callus induced from the immature embryos were cut into two pieces with a scalpel and were transferred to WLS-P10 (WLS plus 10 mg/l phosphoinothricin). The culture plates were incubated at 25 ºC for 3 weeks. The proliferated explants from WLS-P10 were transferred to LSZ-P5 regeneration medium in petri plates and were kept under the light for 2 weeks at 25 ºC in the culture room with a daily cycle of 16 hours light and 8 hours dark. Regenerated plants from the LSZ-P5 medium were transferred to rooting medium LSF-P5 in the glass tubes. The regenerated plants were kept under the light for 2 weeks at 25 ºC in the culture room with a daily cycle of 16 hours light and 8 hours dark. Rooted plants were then transferred to the soil in the clay pots and grown in the greenhouse. The greenhouse temperature was maintained at 22-25 °C and 14 hours of photoperiod.

Particle bombardment mediated transformation

The two gene constructs pBUN411-Ubi-Cas9-MCS-Tsn1-PFT1 and pBUN411-Ubi-Cas9-MCS-Tsn1-HRC1 were sent to the Plant Transformation Facility, Kansas State University for particle bombardment-mediated transformation using the protocol described by Tian et al. (2019). Immature embryos were isolated and only embryo sizes between 0.5 to 1.5 mm in length were placed in CM4 medium in petri plates for callus induction. Embryos were placed with the embryo axis faced down in contact with the medium in petri plates. Petri plates were sealed with parafilm and incubated for 7-12 days in the dark at room temperature to induce callus from the scutellum. 25 vigorously growing calli were separated and placed in the center area of the petri plate with CM4 medium with 36.44 g of mannitol and 36.44 g of sorbitol. The plates were sealed with parafilm and kept in dark at room temperature for 2-3 days to induce more callus. Calli were dried in a laminar flow hood for 20 minutes to reduce the water on the surface before the bombardment. 50 µl (3 mg) of the gold particles (Bio-Rad, USA) suspension in glycerol (50% v/v) solution was transferred to another 1.7 ml microfuge tube and CRISPR/Cas9 plasmid (5 µg), 50 µl CaCl2 (2.5M), and 20 µl spermidine (0.1 M) were added in order while vortexing for 3 minutes. The gold particles were allowed to settle down at room temperature for 10 minutes. The microfuge tubes were centrifuged at 5000 rpm for 2 seconds and the supernatant was removed. The pellet was washed with 150 µl of 70% ethanol first and again washed with 100% ethanol. The final pellet was resuspended in 24 µl of 100% ethanol and vortexed briefly just before use. The biolistic chamber was placed in a laminar flow hood and cleaned using 70% ethanol. 6 µl of final gold suspension aliquots were spread onto the microcarrier disk (Bio-Rad, USA) and allowed to evaporate on a laminar flow hood. The rupture disk of 1100 psi and the microcarrier assembly were loaded. The CM4 plate with the wheat callus in the center of the plate was placed

on the target shelf with a target distance of 6 cm from the stopping plate. The bombardment was carried out using a PDS-1000/He™ device (Bio-Rad, USA). The vacuum in the chamber was maintained at 26 inches of Hg for bombardment and repeated 4 times per plate of calli. After bombardment, CM4 plates were sealed with parafilm and incubated for recovery in the dark at room temperature for 2-4 days. Embryogenic calli were transferred to plates with CM4 medium, plus a selection agent of 5 mg/l glufosinate ammonium and kept in dark at room temperature for 2 weeks. The calli were kept in the CM4 medium with 10 mg/l glufosinate ammonium for another 2 weeks at room temperature. The callus clumps were transferred to shoot production medium (MSP) with 10 mg/l of glufosinate ammonium. The cultured plates with calli were sealed with parafilm and placed under the light (16/8 hours) without stacking for 2-4 weeks at 18-22 ºC for shoot regeneration. When the regenerated shoots were larger than 1 cm in length, shoot generating calli were transferred to root induction medium (MSE) with 5 mg/l of glufosinate ammonium in the tubes. The tubes were sealed and incubated under the light with the same conditions as for regeneration. Well-developed plantlets with more than 7 cm in length and established roots from the tubes were transferred to the soil in clay pots in the greenhouse. Plantlets were covered with clear plastic cups to maintain high relative humidity and promote acclimatization.

PCR amplification of transgenes in transgenic plants

Genomic DNA was isolated from the seedlings of the plants derived from the Agrobacterium- or bombardment-mediated transformation and using polymerase chain reaction (PCR). The plants were first tested for the presence of the cas9 gene using the forward primer, ubi-cas9-F1 (CAAGAGAACCGCAAGGAGAC) and reverse primer, ubi-cas9-R1 (TAACCAGCGTAGCCGTTCTT). Each PCR volume of 50µl contained 30 ng of the template

DNA, 5 pmol of each primer, 200 μM of each dNTP, 1× reaction buffer (20mM Tris-Cl, 2.0mM $MgSO_4$, 50mM KCl, 10 mM $(NH_4)_2SO4$, 0.1% Triton X-100, pH 8.8), and 2.5U of Taq DNA polymerase (New England Biolabs, Ipswich, MA) and run in in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Waltham, MA) using the following conditions: denaturation at 95 °C for 2 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min and a final extension for 5 min at 72 °C. PCR products were subjected by electrophoresis in a 1.2% agarose gel and detected by a gel documentation system after EB straining.

Phenotyping with ToxA infiltration

Since ToxA is produced by both tan spot pathogen *P. tritici-repentis* and Septoria nodorum blotch (SNB) pathogen *Parastagonospora nodorum*, the ToxA cultures were produced from yeast cultures expressing the ToxA (SnToxA) from *P. nodorum* following the protocol previously described (Liu et al., 2012). Using a 1-ml syringe with the needle removed, SnToxA culture filtrates were infiltrated into fully grown secondary leaves of wheat plants. The limits of the infiltrated places were marked using a nontoxic felt pen immediately after infiltration. Three to five days after infiltration, reactions were assessed and classified as insensitive (no necrosis) or responsive (necrosis) (necrosis) (Shi et al., 2016).

Results

Through *Agrobacterium*-mediated transformation using the wheat cultivar 'Fielder' and the vector pLC41-Ubi-Cas9-MCS-Tsn1-HRC, two transgenic Fielder plants (T_0) were generated by the Transformation Facility of University of California-Davis. 196 T_1 plants were generated from these two T_0 transgenic plants and were tested for the ToxA infiltration. None of the transgenic plants showed insensitivity to ToxA.

By particle bombardment mediated transformation using the wheat cultivar ‘Bobwhite’, five transgenic plants were obtained from each of the two vectors ZTC (pBUN411-Ubi-Cas9-MCS-Tsn1-HRC1) and ZTP1 (pBUN411-Ubi-Cas9-MCS-Tsn1-PFT1) (Table 3). The positively transformed plants were screened by PCR using specific primers for vectors (Figure 10). Total 980 T_1 plants were generated from the T_0 plants and tested for ToxA sensitivity by leaf infiltration. None of the plants were insensitive to ToxA (Figure 11).

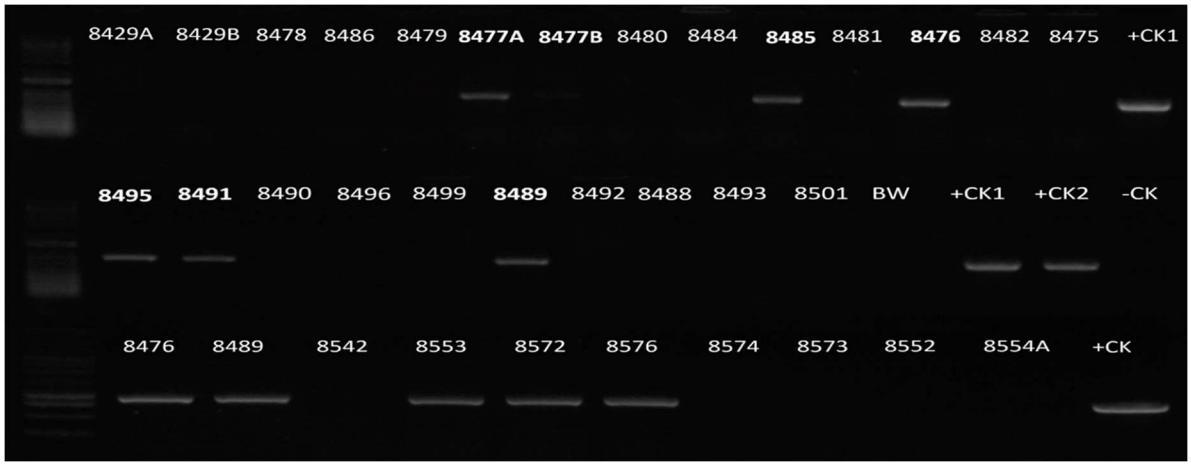

Figure 9: PCR screening of transgenic Bobwhite plants using primers, ubi-cas9-F1 (CAAGAGAACCGCAAGGAGAC) and ubi-cas9-R1 (TAACCAGCGTAGCCGTTCTT). +CK1: plasmid for pBUN411-Ubi-Cas9-MCS-Tsn1-HRC1, +CK2: plasmid for pBUN411-Ubi-Cas9-MCS-Tsn1-PFT1, BW: gDNA for Bobwhite, -CK: water sample for PCR.

Table 3: Transgenic seeds of ZTC (pBUN411-Ubi-Cas9-MCS-Tsn1-HRC1) and ZTP1 (pBUN411-Ubi-Cas9-MCS-Tsn1-PFT1) transformed wheat plants

Construct	Seed samples	PCR_GOI	Seed number for each tiller
ZTC	8485	+	A:53, B:65, C:53, D:58, E:55, F:60, G:52, H:42, I:49, J:41
	8489	+	A:57, B:63, C:50, D:54, E:63, F:23, G:43, H:42, I:7, J:29, K:55, L:106, M:72
	8491	+	A:50, B:40, C:37, D:37, E:27, F:51, G:25, H:42, I:15, J:26, K:16, L:58
	8553	+	A:47, B:55, C:6, D:43, E:20, F:25, G:24, H:28, I:29, J:40, K:60, L:40, M:60, N:41 8576
	8576	+	A:32, B:40, C:45, D:22, E:30, F:26, 6:23, H:19, I:52, J:31
ZTP1	8476	+	A:34, B:31, C:46, D:33, E:11, F:35, G:27, H:16, I:22, J:9, K:35, L:33, M:3
	8477	+	A:72, B:47, C:71, D:67, E:54, F:59, G:70, H:48, I:65, J:64, K:45, L:36, M:49, N:61, 0:54, P:52, Q:38, R:22, S:24, T:70
	8495	+	A:47, B:46, C:61, D:32, E:48, F:46, 6:39, H:29, 1:29, J:41, K:29, L:23, M:52; N:30
	8572	+	A:67, B:60, C:55, D:58, E:56, F:62, 6:36, H:64, 1:52, J:33, K:41, L:79, M:67; N:64
	8674	+	28 tiny seeds

Figure 10: ToxA infiltration assay showing insensitive check- ND Riveland, sensitive check- Bobwhite, T1 sensitive plant 1, and T1 sensitive plant 2 respectively

Discussion

In this study, transgenic plants with CRISPR/Cas9 vectors targeting *Tsn1* gene were generated using both Agrobacterium- and particle bombardment-mediated transformation methods. PCR amplification detected the transgene in the transgenic plants. However, none of the T_0 and T_1 plants showed phenotype change for ToxA sensitivity. This indicates that the

CRISPR/Cas9 system did not target and alter the *Tsn1* gene function in the transgenic plants. If there were any changes in any part of the *Tsn1* gene, causing non-synonymous mutations, plants should have become insensitive to ToxA. No gene mutations detected in the transgenic plants could be due to many factors, including low or no expression of the Cas9 and gRNA in the plants, making the gene editing efficiency very low. Another reason for not getting plants with target gene edited might be due to very low number of transgenic plants generated. Only two and 30 transgenic wheat plants were generated using *Agrobaceterium*- and particle bombardment-mediated transformation, respectively.

The site of integration and the structure of the transgene locus can differ significantly among independent transformants, and each of these characteristics can have a significant impact on transgenic expression levels and stability (Kohli et al., 2003). Rearrangements of transgenes and transgenes with inverted repeats are more likely to be silenced, probably due to the creation of dsRNA through read-through transcription (Muskens et al., 2000). The expression of the transgenes in plants is affected by various factors. One of the important factors is the choice of the promoter. The promoter determines the strength of plant tissue/organ specific gene expression of the transgene (Kopertekh et al., 2009; Sanger et al., 1990). Among five promoters, the double CaMV 35S promoter, figwort mosaic virus (FMV) promoter, the cassava vein mosaic virus (CsVMV) promoter, the sugarcane bacilliform badnavirus (ScBV) promoter, and alfalfa small subunit Rubisco (RbcS) promoter, used to control the expression of a cDNA from *Trichoderma atroviride* encoding an endochitinase (ech42), highest chitinase activity in leaves, roots, and root nodules was obtained in plants containing the CsVMV promoter (Samac et al., 2004). Another comparison study between two viral promoters, CaMV 35T and SCBV with two plant promoters, rice actin1 (OsAct1) and maize ubiquitin 1 (ZmUbi1) to drive aryloxyalkanoate

dioxygenase (*aad-1*) gene in maize inbred line B104, plant-containing constructs showed higher transformation frequencies than the two viral promoter constructs (Beringer et al., 2017). One of the reasons for no targeted mutation might be the promoters used for this study.

The other factors such as local chromatin structure, regulatory sequences at the integration site, transgene copy number as well as epigenetic effects influence the transgene expression levels (Iglesias et al., 1997; Pröls & Meyer, 1992). Transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) are two eminent examples of epigenetic effects (Dietz-Pfeilstetter, 2010). Although epigenetic silencing is a part of the regulation of endogenous genes during plant development, it is also responsible for the transposon silencing, defense of plant viruses and transgene silencing (Baulcombe, 2004) mediated through the distinct micro-RNA (miRNA) genome loci (Mallory & Vaucheret, 2006). The distinction between TGS and PTGS has been discovered to be less clear because both TGS and PTGS contain comparable signaling molecules and an epigenetic flip from post-transcriptional to transcriptional silencing can occur under specific situations (Fojtova et al., 2003). Dicer and Argonaute proteins, as well as RNA-directed RNA polymerase, have been discovered as components of various endogenous PTGS and TGS pathways (Baulcombe, 2004).

The unexpected simultaneous suppression of a CaMV 35S promoter driven chalcone synthase (chs) transgene and the endogenous chs gene in transgenic petunia, also known as co-suppression, was one of the first findings of transgene silencing (Napoli et al., 1990). Transgene silencing has been observed by a phenomenon called trans-inactivation when separate coding sequences regulated by the same promoter are joined via sexual crossing (Matzke et al., 1993). High transgene dose due to strong promoter or high copy number can also be one of the reasons for PTGS which can be regulated by the choice of regulatory areas guiding transcript

accumulation. CaMV 35S promoter having a single copy of upstream activator region (UAR) produced much less co-suppression than those promoters containing two or four copies of UAR (Que et al., 1997).

The use of different selectable marker genes in wheat transformations and their varying efficiencies have been reported. The hygromycin resistance gene (*hpt*) showed better average transformation (5.5 %) as compared to the *bar* gene (2.6 %) (Ortiz et al., 1996). However, no transformed plants were obtained using hygromycin B as a selection as compared to the phosphinothricin acetyltransferase (*bar*) and neomycin phosphotransferase (*aphA*) genes with transformation frequencies of 0.25- 1.2 % of bombarded wheat embryos (Witrzens et al., 1998). Another study of *Agrobacterium*-mediated transformation of wheat reported the highest selection rate (12.6%) with EHA101 on kanamycin selection (nptII) compared to lower average transformation rate with LBA4404 with nptII (2.3%), AGL1 with bar (1 %), and LBA4404 with hpt (0.2-0.4%) (Przetakiewicz et al., 2004).

More than 80% of the wheat genome is composed of transposable elements (TEs) and due to their fast mutation frequency, the short-term suppression is regulated by sRNA directed TGS and PTGS, whereas long-term suppression is regulated by DNA methylation (Cantu et al., 2010). DNA methylation has been associated with the inactive state of the gene, making chromatin structure inaccessible to the transcription factors by the recognition of the methylated residues (Kass et al., 1997; Razin, 1998). HpaII/MspI enzymes digestion of whole genomic DNA from genetically identical lines resulting from the same transgenic event, followed by hybridization with a DNA probe matching to the Ubi1 promoter region, revealed that the silenced line had more extensive methylation of CCGG sites in the promoter (Anand et al., 2003).

In summary, no loss of function *Tsn1* mutants were obtained from Agrobacterium- and bombardment-mediate transformation combined with the CRISPR/Cas9 system. The biolistic transformation may cause multiple copies of integration and chromosomal rearrangements at the integration locations, resulting in transgene inactivation. Although *Agrobacterium*-mediated transformation is more likely to result in single copy integration, the number of transgenic plants is low and more transgenic plants should be generated for mutant identification. Although many efforts have been made for optimizing wheat transformation using both *Agrobacterium*- and biolistic-mediated methods (Fu et al., 2000; Ishida et al., 2015; Kumar et al., 2019; Srivastava et al., 1999), successful transgene expression of the CRISPR/Cas9 system in transgenic plants still remains to be improved for achieving a high level of gene editing efficiency.

References

Anand, A., Trick, H. N., Gill, B. S., & Muthukrishnan, S. (2003). Stable transgene expression and random gene silencing in wheat. *Plant Biotechnology Journal*, *1*(4), 241–251.

Baulcombe, D. (2004). RNA silencing in plants. *Nature 2004 431:7006*, *431*(7006), 356–363.

Beringer, J., Chen, W., Garton, R., Sardesai, N., Wang, P. H., Zhou, N., Gupta, M., & Wu, H. (2017). Comparison of the impact of viral and plant-derived promoters regulating selectable marker gene on maize transformation and transgene expression. *Plant Cell Reports*, *36*(4), 519–528.

Borisjuk, N., Kishchenko, O., Eliby, S., Schramm, C., Anderson, P., Jatayev, S., Kurishbayev, A., & Shavrukov, Y. (2019). Genetic modification for wheat improvement: from transgenesis to genome editing. *BioMed research international*, 2019.

Cantu, D., Vanzetti, L. S., Sumner, A., Dubcovsky, M., Matvienko, M., Distelfeld, A., Michelmore, R. W., & Dubcovsky, J. (2010). Small RNAs, DNA methylation and transposable elements in wheat. *BMC Genomics*, *11*(1), 1–15.

Chang, H. H. Y., Pannunzio, N. R., Adachi, N., & Lieber, M. R. (2017). Non-homologous DNA end joining and alternative pathways to double-strand break repair. *Nature Reviews Molecular Cell Biology 2017 18:8*, *18*(8), 495–506.

Cheng, M., Fry, J. E., Pang, S., Zhou, H., Hironaka, C. M., Duncan, D. R., & Conner, T. W. (1997). Genetic transformation of wheat mediated by Agrobacterium tumefaciens. *Plant Physiol*, *115*, 971–980.

Dietz-Pfeilstetter, A. (2010). Stability of transgene expression as a challenge for genetic engineering. *Plant Science*, *179*(3), 164–167.

Doudna, J. A., & Charpentier, E. (2014). The new frontier of genome engineering with CRISPR-Cas9. *Science*, *346*(6213).

Faris, J. D., Liu, Z., & Xu, S. S. (2013). Genetics of tan spot resistance in wheat. *Theoretical and Applied Genetics*, *126*(9), 2197–2217.

Faris, J. D., Zhang, Z., Lu, H., Lu, S., Reddy, L., Cloutier, S., Fellers, J. P., Meinhardt, S. W., Rasmussen, J. B., Xu, S. S., Oliver, R. P., Simons, K. J., & Friesen, T. L. (2010). A unique wheat disease resistance-like gene governs effector-triggered susceptibility to necrotrophic pathogens. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(30), 13544–13549.

Fojtova, M., van Houdt, H., Depicker, A., & Kovarik, A. (2003). Epigenetic switch from posttranscriptional to transcriptional silencing is correlated with promoter hypermethylation. *Plant Physiology*, *133*(3), 1240–1250.

Fu, X., Duc, L. T., Fontana, S., Bong, B. B., Tinjuangjun, P., Sudhakar, D., Twyman, R. M., Christou, P., & Kohli, A. (2000). Linear transgene constructs lacking vector backbone sequences generate low-copy-number transgenic plants with simple integration patterns. *Transgenic Research 2000 9:1*, *9*(1), 11–19.

H. L. Xing, L. Dong, Z. P. Wang, H. Y. Zhang, C. Y. Han, B. Liu, X. C. Wang, Q. J. Chen. 2014. A CRISPR/Cas9 toolkit for multiplex genome editing in plants. *BMC Plant Biology*, 14: 327.

Iglesias, V. A., Moscone, E. A., Papp, I., Neuhuber, F., Michalowski, S., Phelan, T., Spiker, S., Matzke, M., & Matzke, A. J. M. (1997). Molecular and cytogenetic analyses of stably and unstably expressed transgene loci in tobacco. *The Plant Cell*, *9*(8), 1251–1264.

Ishida, Y., Tsunashima, M., Hiei, Y., & Komari, T. (2015). Wheat (Triticum aestivum L.) transformation using immature embryos. In *Agrobacterium protocols* (pp. 189-198). Springer, New York, NY.

Janakiraman, V., Steinau, M., McCoy, S. B., & Trick, H. N. (2002). Recent advances in wheat transformation. *In Vitro Cellular & Developmental Biology - Plant 2002 38:5*, *38*(5), 404–414.

Kass, S. U., Pruss, D., & Wolffe, A. P. (1997). How does DNA methylation repress transcription? *Trends in Genetics*, *13*(11), 444–449.

Kohli, A., Twyman, R. M., Abranches, R., Wegel, E., Stoger, E., & Christou, P. (2003). Transgene integration, organization and interaction in plants. *Plant Molecular Biology*, *52*, 247–258.

Kopertekh, L., Broer, I., & Schiemann, J. (2009). Developmentally regulated site-specific marker gene excision in transgenic B. napus plants. *Plant Cell Reports*, *28*(7), 1075–1083.

Kumar, R., Mamrutha, H. M., Kaur, A., Venkatesh, K., Sharma, D., & Singh, G. P. (2019). Optimization of Agrobacterium-mediated transformation in spring bread wheat using mature and immature embryos. *Molecular Biology Reports*, *46*(2), 1845–1853.

Liu, Z., Zhang, Z., Faris, J. D., Oliver, R. P., Syme, R., McDonald, M. C., McDonald, B. A., Solomon, P. S., Lu, S., Shelver, W. L., Xu, S., & Friesen, T. L. (2012). The cysteine rich necrotrophic effector SnTox1 produced by Stagonospora nodorum triggers susceptibility of wheat lines harboring Snn1. *PLoS pathogens*, 8(1), e1002467.

Mallory, A. C., & Vaucheret, H. (2006). Functions of microRNAs and related small RNAs in plants. *Nature Genetics 2006 38:6*, *38*(6), S31–S36.

Matzke, M. A., Neuhuber, F., & Matzke, A. J. M. (1993). A variety of epistatic interactions can occur between partially homologous transgene loci brought together by sexual crossing. *Molecular and General Genetics MGG 1993 236:2*, *236*(2), 379–386.

Muskens, M. W. M., Vissers, A. P. A., Mol, J. N. M., & Kooter, J. M. (2000). Role of inverted DNA repeats in transcriptional and post-transcriptional gene silencing. *Plant Gene Silencing*, 123–140.

Napoli, C., Lemieux, C., & Jorgensen, R. (1990). Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. *The Plant Cell*, *2*(4), 279–289.

Ortiz, J. P. A., Reggiardo, M. I., Ravizzini, R. A., Altabe, S. G., Cervigni, G. D. L., Spitteler, M. A., Morata, M. M., Elías, F. E., & Vallejos, R. H. (1996). Hygromycin resistance as an efficient selectable marker for wheat stable transformation. *Plant Cell Reports 1996 15:12*, *15*(12), 877–881.

Partier, A., Gay, G., Tassy, C., Beckert, M., Feuillet, C., & Barret, P. (2017). Molecular and FISH analyses of a 53-kbp intact DNA fragment inserted by biolistics in wheat (Triticum aestivum L.) genome. *Plant Cell Reports*, *36*(10), 1547–1559.

Pröls, F., & Meyer, P. (1992). The methylation patterns of chromosomal integration regions influence gene activity of transferred DNA in Petunia hybrida. *The Plant Journal*, *2*(4), 465–475.

Przetakiewicz, A., Karaś, A., Orczyk, W., & Nadolska-Orczyk, A. (2004). Agrobacterium-mediated transformation of polyploid cereals. The efficiency of selection and transgene expression in wheat. *Cellular & Molecular Biology Letters*, *9*(4B), 903–917.

Que, Q., Wang, H. Y., English, J. J., & Jorgensen, R. A. (1997). The frequency and degree of cosuppression by sense chalcone synthase transgenes are dependent on transgene promoter strength and are reduced by premature nonsense codons in the transgene coding sequence. *The Plant Cell*, *9*(8), 1357–1368.

Razin, A. (1998). CpG methylation, chromatin structure and gene silencing - A three-way connection. *EMBO Journal*, *17*(17), 4905–4908.

Samac, D. A., Tesfaye, M., Dornbusch, M., Saruul, P., & Temple, S. J. (2004). A comparison of constitutive promoters for expression of transgenes in alfalfa (Medicago sativa). *Transgenic Research*, *13*(4), 349–361.

Sanger, M., Daubert, S., & Goodman, R. M. (1990). Characteristics of a strong promoter from figwort mosaic virus: comparison with the analogous 35S promoter from cauliflower mosaic virus and the regulated mannopine synthase promoter. *Plant Molecular Biology 1990 14:3*, *14*(3), 433–443.

Shi, G., Zhang, Z., Friesen, T. L., Raats, D., Fahima, T., Brueggeman, R. S., Lu, S., Trick, H. N., Liu, Z., Chao, W., Frenkel, Z., Xu, S. S., Rasmussen, J. B., & Faris, J. D. (2016). The hijacking of a receptor kinase-driven pathway by a wheat fungal pathogen leads to disease. *Science Advances*, *2*(10).

Srivastava, V., Anderson, O. D., & Ow, D. W. (1999). Single-copy transgenic wheat generated through the resolution of complex integration patterns. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(20), 11117–11121.

Tian, B., Navia-Urrutia, M., Chen, Y., Brungardt, J., & Trick, H. N. (2019). Biolistic transformation of wheat. *Methods in Molecular Biology*, *1864*, 117–130.

Travella, S., Ross, · S M, Harden, · J, Everett, · C, Snape, · J W, & Harwood, W. A. (2005). A comparison of transgenic barley lines produced by particle bombardment and Agrobacterium-mediated techniques. *Plant Cell Rep*, *23*, 780–789.

Vasil, V., Castillo, A. M., Fromm, M. E., & Vasil, I. K. (1992). Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. *Bio/Technology 1992 10:6*, *10*(6), 667–674.

Wegulo, S. N., Zwingman, M. v., Breathnach, J. A., & Baenziger, P. S. (2011). Economic returns from fungicide application to control foliar fungal diseases in winter wheat. *Crop Protection*, *30*(6), 685–692.

Witrzens, B., Brettell, R. I. S., Murray, F. R., McElroy, D., Li, Z., & Dennis, E. S. (1998). Comparison of three selectable marker genes for transformation of wheat by microprojectile bombardment. *Functional Plant Biology*, *25*(1), 39–44.

Zhang Y., Liang Z., Zong Y., Wang Y., Liu J., et al. 2016. Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. Nat. Commun. 7: 12617

TARGETTING WHEAT *TaHRC* GENE FOR FHB RESISTANCE THROUGH HAPLOID INDUCTION COUPLED WITH CRISPR/Cas9-MEDIATED GENOME EDITING TECHNOLOGY

Abstract

Fhb1 is a major quantitative trait locus (QTL) conferring resistance to Fusarium head blight (FHB) in wheat. Previous gene cloning studies show that the *TaHRC* gene (encoding for reticulum histidine-rich calcium binding protein) at the *Fhb1* locus is involved in the resistance/susceptibility to FHB in Sumai3 and its derivatives. Two different *TaHRC* allelic forms, *TaHRC-S* and *TaHRC-R*, were identified, with *TaHRC-S* being present in susceptible genotypes while *TaHRC-R* existing in resistant genotypes. *TaHRC-R* has a 609 bp deletion compared to *TaHRC-S,* and deletion or mutation at *TaHRC-S* leads to improved FHB resistance in wheat. The aim of this study was to target the *TaHRC* gene in wheat using haploid induction coupled with the CRISPR/Cas9-mediated genome editing technology. CRISPR/Cas9-mediated gene editing vectors were designed for targeting *TaHRC* gene and transformed into maize variety Hi-II. The pollens of transgenic maize plants with high expression of Cas9 and gRNA were used to pollinate emasculated spikes of wheat variety Dayn (with *TaHRC-S*) and its near-isogenic line Dayn-Fhb1 carrying *Fhb1* (with *TaHRC-R*), and haploid plants were obtained through the embryo rescue by tissue culture technique. Of 82 haploid plants screened by PCR and sequencing, 12 plants were identified having mutations at the target sites of *TaHRC-S* allele and two plants at the target site of *TaHRC-R* allele. Doubled haploid plants are being generated from these gene-edited haploid plants and will be evaluated for FHB resistance. This study will verify the role of *TaHRC* gene in FHB resistance and may provide a novel approach for improving FHB resistance in wheat.

Introduction

Fusarium head blight (FHB), also called head scab, is a devastating disease in wheat worldwide. The disease is mainly caused by the fungus *Fusarium graminearum* in North America. During anthesis, the fungus can infect spikelets, immature kernels, or full heads of wheat, leading to premature bleaching of heads and peduncle tissues (Bai & Shaner, 1994). FHB can cause huge yield losses in the fields as well as indirect losses due to the accumulation of mycotoxins such as deoxynivalenol (DON) in the grains (Bai & Shaner, 1994). The host resistance is one of the major components in the integrated approach for FHB management (McMullen et al. 2009; Bai et al., 2018)

Resistance to FHB is a quantitatively inherited trait controlled by multiple genes and highly influenced by environmental conditions (Steiner et al., 2017). Five types of resistance to FHB have been described: resistance to initial infection (type I), resistance to spread of infection (type II), resistance to toxin accumulation/ability to degrade toxin (type III), resistance to kernel infection (type III), resistance to kernel infection (type IV) and tolerance to yield loss (type V) (Mesterházy et al., 1999; Miller et al., 1985). Among the many QTL identified for FHB resistance, *Fhb1,* first identified in the Chinese spring wheat cultivar 'Sumai3', is one of the well validated and widely used QTL. *Fhb1* provides the most stable and largest effect on FHB resistance for type II resistance in wheat (Anderson et al., 2001; Bai et al., 1999; Buerstmayr et al., 2009; Waldron et al., 1999). Previous gene cloning studies show that the *TaHRC* gene (encoding for reticulum histidine-rich calcium binding protein) at the *Fhb1* locus is involved in the resistance or susceptibility to FHB in Sumai3 and its derivatives (Li et al., 2019; Su et al., 2019). By comparing the sequence of full-length cDNA of *TaHRC* gene from both resistant and susceptible lines, two different *TaHRC* allelic forms (*TaHRC-S* and *TaHRC-R*) were identified.

The *TaHRC-S* allele was discovered in susceptible genotypes, while the *TaHRC-R* allele was identified in resistant genotypes and had a large deletion of 609 bp compared to *TaHRC-S* (Su et al., 2019). Wheat varieties with deletion or mutation of the *TaHRC* gene showed improved resistance to FHB (Li et al., 2019; Su et al., 2019).

Recently, a new technology combining the CRISPR/Cas9-mediated genome editing technology with haploid induction through the wheat × maize wide hybridization has been used for targeted gene mutagenesis in hexaploid wheat and durum wheat (Budhagatapalli et al., 2020; Kelliher et al., 2019). Using this technology, CRISPR/Cas9 vector targeting the gene of interest in the wheat genome can be designed and used to transform maize plants. Then, maize pollens expressing the CRISPR/Cas9-mediated gene editing machinery are used to pollinate the emasculated wheat spikes. Due to the insensitivity of maize pollen to wheat dominant crossability inhibitor genes *Kr1* and *Kr2*, the maize pollen can germinate and elongate on wheat stigma to fertilize wheat oocyte and polar nucleus (Laurie & Bennett, 1986, 1988). During transient hybrid state of embryo after fertilization, the CRISPR/Cas9 gene editing machinery in the maize chromosome expresses Cas9 and gRNA, which form a complex to edit or mutate the target gene (Budhagatapalli et al., 2020; Kelliher et al., 2019). Since maize chromosome elimination occurs after within first three zygotic divisions due to asynchronous processing in terms of DNA replication, condensation, and centromere formation (Laurie & Bennett, 1989), haploid embryos that contain only the maternal haploid genome are generated without transgenes integrated into wheat chromosomes. This technique can be used to produce transgene free haploid plants with the target gene mutated and double haploid plants with homozygous mutated genes can be produced by colchicine treatment.

The specific objectives of this study were to 1) Develop CRISPR/Cas9 vectors targeting both *TaHRC-S* and *TaHRC-R* alleles of the *TaHRC* gene in wheat, 2) Generate transgenic plants from the maize variety Hi-II using the vector constructs, 3) Verify the expression of Cas9 and gRNA in transgenic maize plants and produce wheat haploid plants by wheat × maize hybridization, 4) Detect mutations at *TaHRC* gene in haploid plants and evaluate doubled haploid plants derived from the *TaHRC*-edited haploid plants for FHB resistance.

Materials and methods

Construction CRISPR/Cas9 vectors

The CRISPR/Cas9 vectors used for maize transformation for targeting the *TaHRC* gene were constructed using pBue411 (Xing et al., 2014) as the backbone vector. Four target sites (T1, T2, T3, and T4) were selected from exon 3 of *TaHRC* for designing the gRNA cassettes (Figure 12). DNA sequence fragment containing one targeted site oligo (T1/T3) with gRNA scaffold, OsU3t (rice U3 terminator), TaU3p (wheat U3 promoter), and another targeted site oligo (T2/T4) was synthesized by Twist Bioscience (South San Francisco, CA). The synthetic DNA fragment contained the tail sequences, ATATATGGTCTCTGGCG at the 5' end and GTTTAGAGACCAATAAT at the 3' end, with a *Bsa* I restriction site in each sequence (the underlined sequence), which was cloned into the *Bsa* I sites of the vector pBue411 using the Golden Gate Cloning method (Xing et al., 2014). Briefly, 15 ul of reaction was prepared, including 200 ng of the synthesized DNA fragment, 200 ng of pBue411 plasmid, 1.5 ul of 10 X T4 ligase buffer, 1.5 ul of 10 X Cutsmart buffer, 1 ul of *Bsa* I, 1 ul of T4 ligase (all buffers and enzymes are from NEB). The reaction was incubated at 37 ºC for 5 hours followed by 5 minutes at 50 ºC and 10 minutes at 80 ºC. 5 ul of the reaction was used for transformation of *E. coli* cells. The plasmid was isolated and confirmed by sequencing. Two vectors, pBue411-HRC1HRC2 and

pBue411-HRC3HRC4 (17,048 bp), were constructed, each targeting two sites in the *TaHRC* gene (Figure 13). pBue411-HRC1HRC2 had gRNA targeting *TaHRC* target 1 (T1) and 2 (T2) whereas pBue411-HRC3HRC4 had gRNA targeting *TaHRC* target 3 (T3) and 4 (T4). All these 4 target sites are conserved cross both the susceptible (*TaHRC-S*) and resistant (*TaHRC-R*) alleles of the *TaHRC* gene (Figure 11).

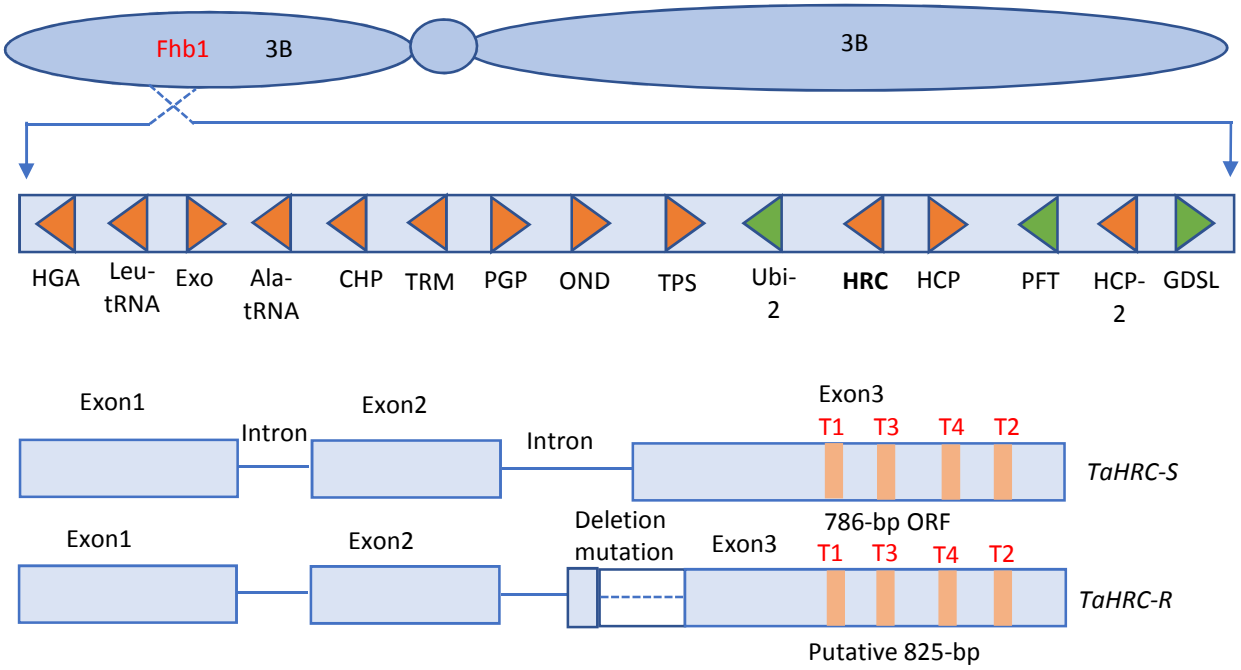

Figure 11: Four different target sites at exon 3 of *TaHRC* gene on the chromosome 3BS (Adapted from Su et al., 2019). *TaHRC* target 1 (T1): AGCTCAAGTCGAAAAAGCAC**AGG;** *TaHRC* target 2 (T2): CTCCTCAGATTCATCGTCTG**AGG;** *TaHRC* target 3 (T3): GCAAGCACAGGTCAAAGAGG**AGG;** *TaHRC* target 4 (T4): GAAGGAAGAAGCACTCGCAC**AGG**.

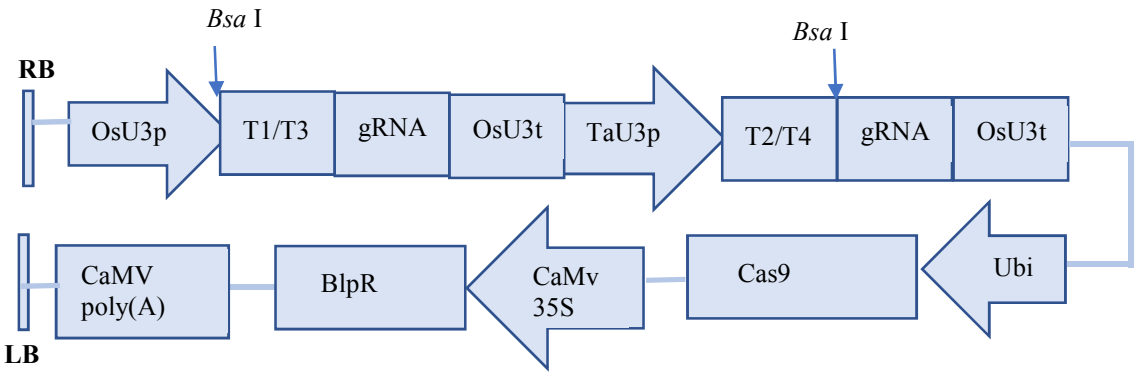

Figure 12: CRISPR/Cas9 vector construct (pBue411-HRC1HRC2 or pBue411-HRC3HRC4). OsU3p, rice U3 promoter; OsU3t, rice U3 terminator; T1/T3, Target 1 & 3; TaU3p, wheat U3 promoter; T2/T4, Target 3 & 4; Ubi, maize Ubiquitin promoter; CaMV 35S, Cauliflower mosaic virus 35S promoter; BlpR, Bialaphos resistance, LB, T-DNA left border sequence; RB, T-DNA right border sequence.

Maize transformation

The two vector constructs pBue411-HRC1HRC2 and pBue411-HRC3HRC4 were sent to the Plant Transformation Facility of Iowa State University for *Agrobacterium*-mediated transformation using the hybrid maize variety Hi-II and the method described by Frame et al. (2002).

Expression analysis of Cas9 and gRNA in transgenic maize plants using quantitative real-time PCR (qRT-PCR)

Expression of Cas9 and gRNA in transgenic maize plants was analyzed using qRT-PCR. Fresh young leaves from the transgenic maize plants were collected in the deep well tubes having beads, and immediately treated in liquid nitrogen. The leaf samples were ground with the Tissue Lyser (Qiagen, USA) and used for mRNA extraction using Monarch® Total RNA Miniprep Kit (New England Biolabs Inc., USA). Reverse transcriptase PCR was performed with the mRNA samples to make complementary DNA (cDNA) using ultrapure SMART MMLV reverse transcriptase for RT-PCR (Takara Bio USA, Inc.). Cas9 gene and gRNA specific primers (Table 4) were used in the qRT-PCR experiments for checking the Cas9 and gRNA

expression, respectively. Specific primers (Table 4) for genes encoding actin and cyclin dependent kinase (CDK) were used for checking actin and CDK expression as an internal reference. Quantitative PCR was performed in a Bio-Rad CFX96 Touch Real-time PCR (Bio-Rad, USA). Each 20 µl of reaction contained 10 µl of SYBR Green master mix (Bio-Rad, USA), 1 µl of each primer, 2 µl of 10x diluted cDNA obtained from reverse transcriptase PCR, and 6 µl of distilled water. The PCR conditions were: 50 ºC for 2 min, 95 ºC for 10 mins, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min. The expression of Cas9 and gRNA was measured with reference to the two housekeeping genes for Actin and Cyclin Dependent Kinase (CDK). Plants with higher expression of Cas9 were prioritized to be used as the pollen source to pollinate emasculated spikes of selected wheat lines.

Table 4: List of primers used for expression analysis of Cas9 and gRNA using qRT-PCR, and PCR amplification of *TaHRC* gene.

Target gene	Primers
Cas9	Cas9-RT-F1: CATGATTAAGTTCAGGGGCC Cas9-RT-R1: AGGTTATCCAGGTCATCGTC
gRNA	gRNA-RT-F1: CGACTCGGTGCCACTTTTTCAAGTTG gRNA-HRC2-R: TCCTCAGATTCATCGTCTGG
Actin	Actin-F2: AGAGGTTACTCCTTCACCACCA Actin-R1: CAGTGATCTCCTTGCTCATACG
CDK	ZmCDK-RT-F2: TCAGTGCTCAGCAGGCTCTA ZmCDK-RT-R2: CATCCCAGAAGGATGTTCGT
TaHRC	HRC-Forward2: ATGGCAGCAGAAACTGGAAG HRC-Reverse1: ATCATCGTGCGAACTCTGCT

Wheat × maize hybridization

Since Hi-II is the maize variety used by the Transformation Facility of Iowa State University for *Agrobacterium*-mediated transformation to produce transgenic maize plants with the two vectors constructed as above, preliminary study was carried out to determine the

production frequencies of haploid embryos in different wheat genotypes after hybridization with this maize variety. Nine wheat lines were used in the wheat × maize hybridization experiments, including Dayn, SD4539, Linkert, MN10201, WA8283, Alsen, Lang_MN, MN10201_BC, and NDHRS_16_13_97. These wheat inbred lines and the Hi-II maize plants were grown in separate greenhouse rooms. The wheat lines were planted about two weeks after the maize plants were planted to synchronize anthesis of wheat and maize plants for crossing. The greenhouse rooms were maintained at 25/18 °C Day/night and 16/8 hours photoperiod. Wheat spikes that fully emerged from the flag leaf and just 2-3 days before anthesis were selected for emasculation. Spikes with slightly yellow or yellow anthers were avoided for emasculation to prevent self-pollination. For emasculation, spikelets located at the tip and base of the spike were first removed using forceps, as they are asynchronous to the rest of the spike and usually sterile. Each spikelet was cut across leaving approximately two-third part of it to facilitate anther removal and easier pollination. All three anthers in each floret were removed using sharp forceps without any damage to the stigma. Small glassine bags labeled with the wheat genotype and emasculation date were used to cover the emasculated wheat spikes to prevent any cross pollination. One day after maize pollen pollination, the pollinated spikes were treated by dipping with a 2,4-D solution (213.05 mg/L, pH at 10.36) with a few drops of Tween-20 in a 50 ml tube. 18-20 days after pollination, the spikes were collected, and green and water filled immature kernels were removed from the spikes using forceps and used for embryo isolation. The dry and dead immature kernels were discarded. The green and water filled immature kernels were surface sterilized with 70% ethanol for two minutes and 20% (v/v) commercial bleach containing 8.5% sodium hypochlorite for 10 to 15 minutes, then rinsed with autoclave water. Haploid embryos were isolated from these immature kernels using forceps and scalpel under stereomicroscope inside a laminar air-

flow hood to maintain aseptic condition. The excised haploid embryos were cultured on MS basal media with 50 g/L sucrose and 3g/L phytagel without any phyto-hormones in 100 mm x 25 mm petri dishes (Fisher scientific). The cultured plates were kept in a dark incubator at 25 ºC temperature. When the coleoptile and small primary roots emerged, the embryos were transferred to new ½ MS basal media with sucrose (30 g/L), and phytagel (3.2 g/L) in small sterile plastic cups with lids. Then the cups with the embryos were incubated in light conditions (16 hours light and 8 hours dark) in room temperature (25 ºC) until green and healthy plantlets developed.

The preliminary study showed that Dayn with the *TaHRC-S* allele had a high haploid embryo formation rate while Alsen with the *TaHRC-R* allele produce few haploid embryos when crossed with Hi-II. To target both *TaHRC-S* and *TaHRC-R* allele, Dayn was crossed with Alsen (with *TaHRC-R*) followed by backcrossing to Dayn and marker-assisted selection with *Fhb1*-specific primers (Su et al. 2019) to produce progenies (BC2 and BC4 generations) carrying *TaHRC-R* in the Dayn genetic background. Dayn and Dayn-*TaHRC-R* (BC2 or BC4) were used to crossed with transgenic maize plants derived from Hi-II using the same procedure described above.

Screening of wheat haploid plants for *TaHRC* mutations using PCR amplification and sequencing

Fresh young leaves were collected from the haploid plantlets in a deep well tube with beads for DNA isolation. The leaf samples were freeze-dried using liquid nitrogen and grinded using the Tissue Lyser (Qiagen, USA). The DNA was extracted using the QIAGEN DNeasy Plant Mini Kit. The target gene (*TaHRC*) was amplified by PCR using primers flanking the whole gene sequence (Table 4). The PCR-products were checked by gel electrophoresis and purified using PureLink™ PCR Purification Kit (Invitrogen, USA) before being sent to Eurofins

genomics (KY, USA) for sequencing. Sequencing results from the samples were aligned with wild type *TaHRC-S* and *TaHRC-R* sequences from Dayn and Alsen using MEGA11: Molecular Evolutionary Genetics Analysis version 11 (Tamura et al., 2021) to identify changes at target sites. The haploid plants with mutation(s) at the target sites were kept and transplanted to 6-inch clay pots filled with potting mix (Pro-mix BX; Premier Tech Horticulture, Canada) in the greenhouse.

Chromosome doubling using colchicine treatment

At 3-4 tiller stage, the haploid plants with mutations at *TaHRC* were treated with colchicine for chromosome doubling. The plant roots were first cleaned with running water to remove soil, dried using tissue paper, and then treated with a colchicine solution that contains colchicine (0.5g/l), DMSO (20ml/l), GA3 (100 mg/l) and one drop of Tween 80 per 100 ml for 8 hours. The colchicine solution was aerated continuously during the 8 hours treatment and covered with aluminium foil to prevent the light exposure. After colchicine treatment, the plants with roots were washed overnight using running water, and then transferred back to soil and kept in milder conditions in the tissue culture room for a week. After that, the plants were returned to the greenhouse for continuous growth and seed production.

Results

Haploid embryo production rates of different wheat genotypes through wide hybridization with maize variety Hi-II

To determine if haploid embryo production rate varies among different wheat genotypes, nine wheat varieties or breeding lines, Dayn, SD4539, Linkert, MN10201, WA8283, Alsen, Lang_MN, MN10201_BC, and NDHRS_16_13_97, were used for crosses with the hybrid maize variety Hi-II maize. The number of spikes used for emasculation and maize pollination along

with the number of haploid embryos isolated were quite different among the nine wheat genotypes: 139 haploid embryos were isolated from 114 spikes of Dayn, 60 haploid embryos from 72 spikes of SD4539, 4 haploid embryos from 20 spikes of Linkert, 28 haploid embryos from 41 spikes of MN10201, 28 haploid embryos from 20 spikes of WA8283, 2 haploid embryos from 8 spikes of Alsen, 30 haploid embryos from 21 spikes of Lang_MN, 18 haploid embryos from 55 spikes of MN10201_BC, and 0 embryos from 7 spikes of NDHRS_16_13_97. As indicated in Figure 14, Dayn, WA8283, and Lang_MN formed more haploid embryos per spike, with a haploid embryo production rate of 1.22, 1.40, and 1.43. Among these three wheat genotypes, Dayn was selected for the further hybridization with the transgenic maize plants derived from the transformation with the two vectors (pBue411-HRC1HRC2 and pBue411-HRC3HRC4) for targeting the *TaHRC* gene.

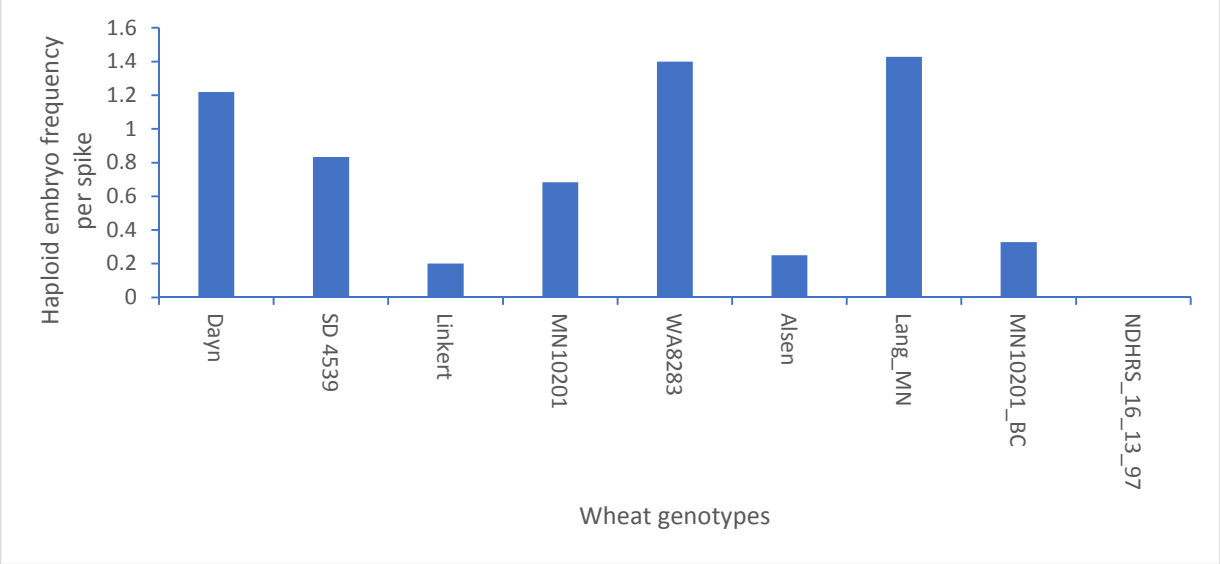

Figure 13: Haploid embryo formation frequency per spike in different wheat genotypes

Expression analysis of Cas9 and gRNA in transgenic maize plants using quantitative real-time PCR (qRT-PCR)

Agrobacterium-mediated transformation of Hi-II with the vector pBue411-HRC1HRC2 generated 26 transformation events (A1011-1 to -26) producing a total of 180 T_0 plants (Table 5).

With the other vector pBue411-HRC3HRC4, 23 transformation events (A1012-1 to -23) were generated, with a total 160 of T_0 transgenic maize plants produced from these events (Table 6).

Table 5: T_0 plants generated from the Agrobacterium-mediated transformation of Hi-II with vector pBue411-HRC1HRC2 by Plant Transformation Facility of Iowa State University

Construct ID	Event ID	Number of Plants
A1011	1	5
A1011	2	10
A1011	3	7
A1011	4	7
A1011	5	9
A1011	6	7
A1011	7	5
A1011	8	5
A1011	9	9
A1011	10	8
A1011	11	6
A1011	12	9
A1011	13	4
A1011	14	9
A1011	15	8
A1011	16	5
A1011	17	8
A1011	18	3
A1011	19	5
A1011	20	9
A1011	21	10
A1011	22	1
A1011	23	8
A1011	24	8
A1011	25	5
A1011	26	10
	Total	180

Table 6: T_0 plants generated from the Agrobacterium-mediated transformation of Hi-II with vector pBue411-HRC3HRC4 by Plant Transformation Facility of Iowa State University

Construct ID	Event ID	Number of Plants
A1012	1	9
A1012	2	5
A1012	3	8
A1012	4	6
A1012	5	5
A1012	6	7
A1012	7	9
A1012	8	6
A1012	9	7
A1012	10	7
A1012	11	10
A1012	12	7
A1012	13	8
A1012	14	10
A1012	15	10
A1012	16	7
A1012	17	6
A1012	18	6
A1012	19	4
A1012	20	5
A1012	21	5
A1012	22	7
A1012	23	6
	Total	**160**

In order to identify the maize plants with high expression of Cas9A and gRNA for the wide hybridization with wheat lines, qRT-PCR was performed with mRNA samples from the transgenic Hi-II maize plants. A total of 60 T_0-plants from A1011 were analyzed for Cas9 expression. 25 plants had higher or similar expression and 35 plants exhibited lower expression for the Cas9 gene with reference to CDK (Table 7). The Cas9 expression levels of representative T_0 plants from A1011 are shown in Figure 14. Among the 25 plants with a high level of Cas9 expression, 21 plants were further screened for gRNA expression, and 9 of the plants showed

higher gRNA expression with reference to CDK. The gRNA expression levels of representative T_0 plants from A1011 are shown in Figure 15. Similarly, 40 T_0-plants from A1012 were screened for Cas9 expression, and 15 plants showed higher or similar expression as compared to CDK. The Cas9 expression levels of representative T_0-plants from A1012 are shown in Figure 16. Among the 15 A1012 T_0 plants with higher Cas9 expression, 12 plants were tested for gRNA expression, and 9 plants exhibited higher gRNA expression (Figure 17).

Table 7: Summary of transgenic maize plants screened for the expression levels of Cas9 and gRNA using qRT-PCR

Generation	Construct	Total screened for Cas9	No of plants with high Cas9	No of plants with low Cas9	Total screened for gRNA	No of plants with high gRNA	No of plants with low gRNA
T_0	A1011	60	25	35	21	9	12
T_0	A1012	40	15	25	12	9	3
T_1	A1011	12	2	10	-	-	-
T_1	A1012	16	8	8	8	4	4

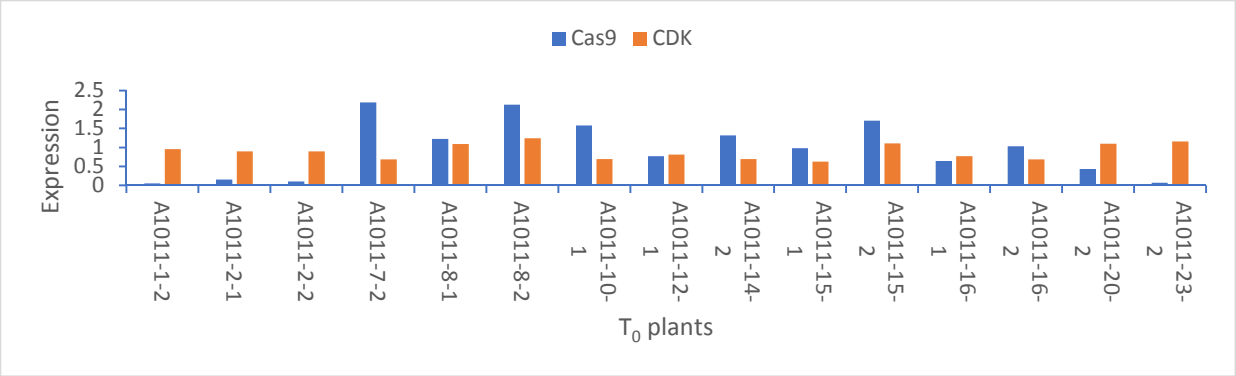

Figure 14: Cas9 expression levels of representative T_0 plants from A1011.

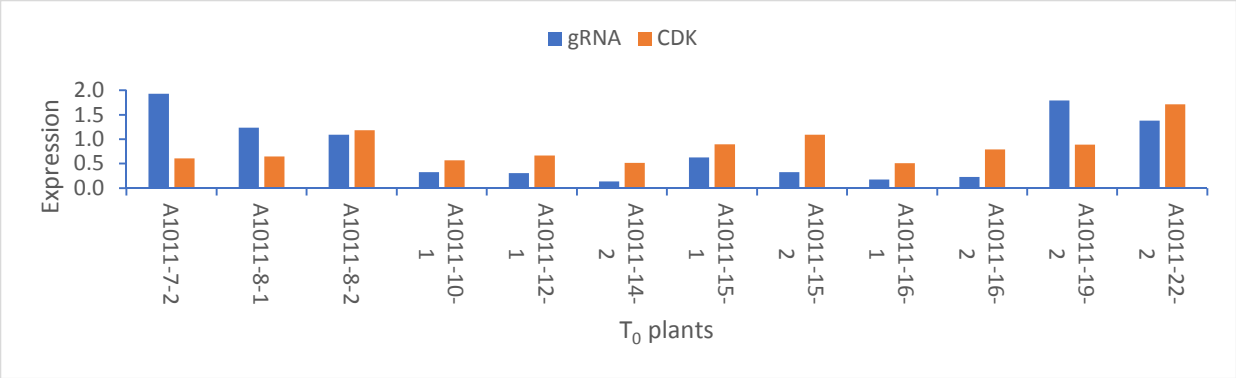

Figure 15: gRNA expression levels of representative T_0 plants from A1011.

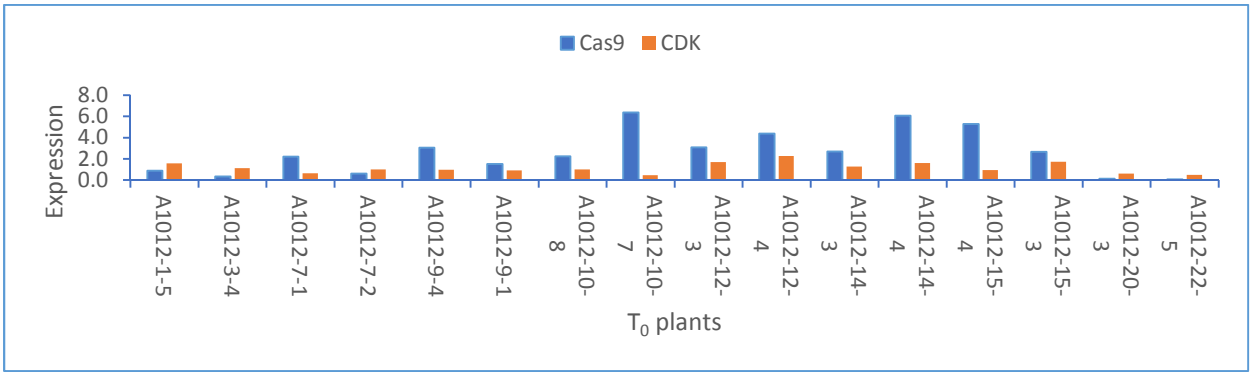

Figure 16: Cas9 expression levels of representative T_0 plants from A1012.

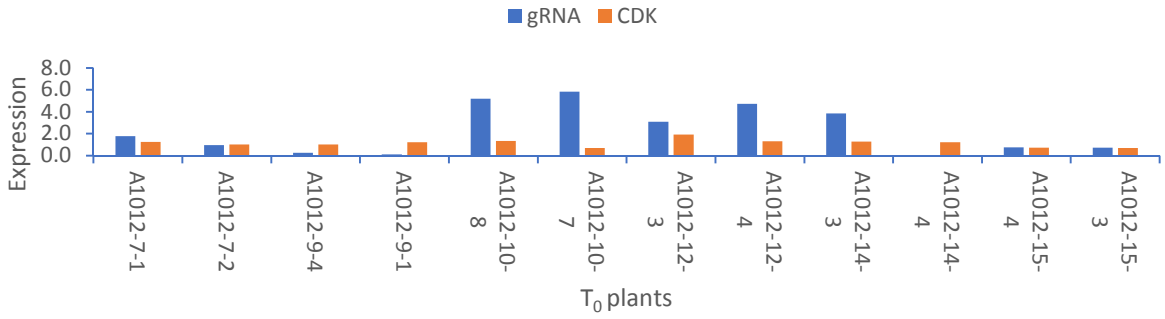

Figure 17: gRNA expression levels of representative T_0 plants from A1012.

12 T_1 plants from seven events (A1011-7, 15, 19, 22, 25, and 26) derived from A1011 and 16 T_1 plants from three events (A1012-7, 14 and 15) derived from A1012 were checked for Cas9 expression. Only 2 T_1 plants (A1011-7-1-7-4-9 and A1011-19-3-7) from A1011 and 8 T_1 plants (A1012-7-1-29, A1012-14-6-30, A1012-14-6-31, A1012-14-6-32, A1012-14-6-33, A1012-14-6-35, A1012-15-4-22, A1012-15-4-23, A1012-15-3-24) from A1012 showed a high

level of Cas9 expression (Table 7, Figure 18, Figure 19). Eight of the 16 T_1 plants from A1012 used for Cas9 expression analysis were checked for the gRNA expression level. The results indicated that four plants (A1012-15-3-19, A1012-15-4-22, A1012-15-4-23, and A1012-15-3-24) had a higher level of gRNA expression in reference to CDK (Figure 20). The three T1 plants (A1012-15-4-22, A1012-15-4-23, and A1012-15-3-24) derived from the same transformation event (A1012-15) had a high level of expression for both Cas9 and gRNA (Figure 19, Figure 20).

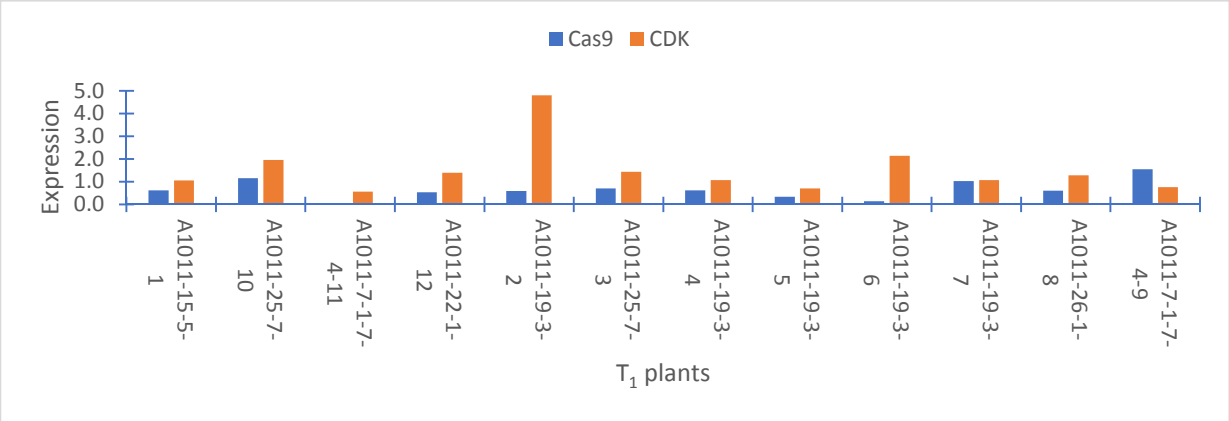

Figure 18: Cas9 expression levels of T_1 plants from A1011.

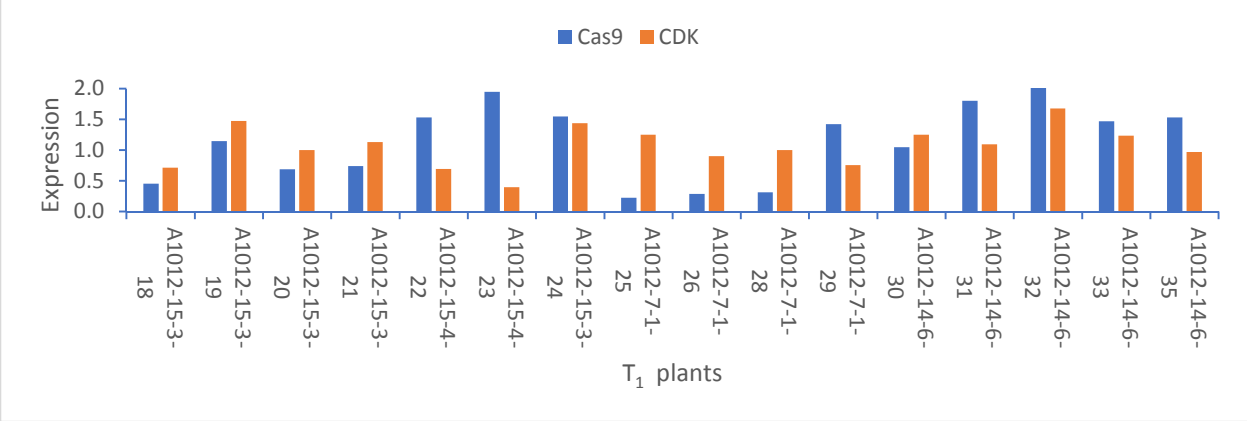

Figure 19: Cas9 expression levels of T_1 plants from A1012.

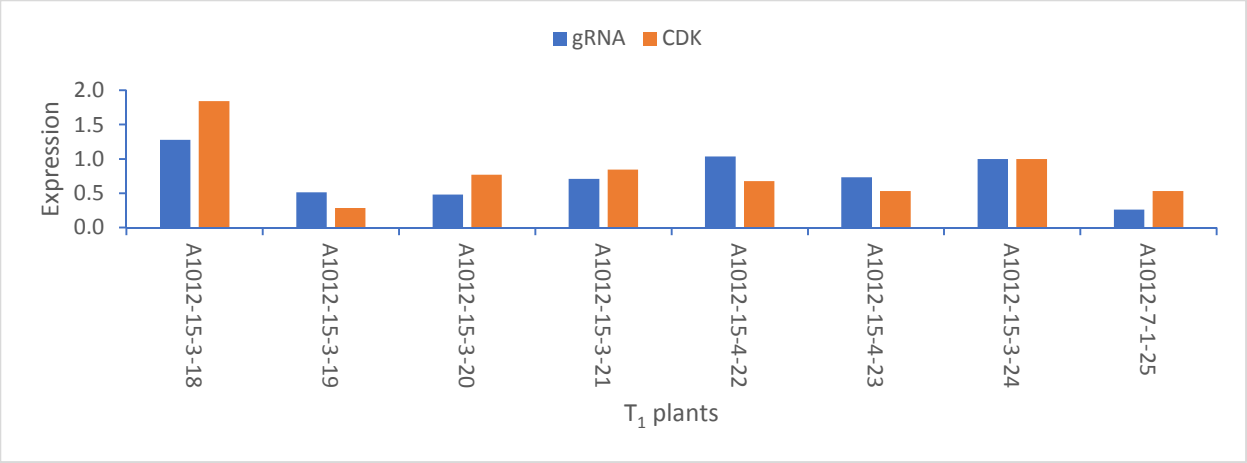

Figure 20: gRNA expression levels of T_1 plants from A1012.

Production of haploid plants from through wide hybridization between Dayn/Dayn-*TaHRC-R* and transgenic maize plants

A total of 238 spikes of Dayn were emasculated and pollinated with pollens of T_0 plants from A1011, from which 62 haploid embryos were isolated with only five haploid plants regenerated (Table 8). A total of 20 haploid plants were generated from 626 haploid embryos isolated from 342 spikes of Dayn pollinated with pollens of the T_0 plants from A1012 (Table 9).

Table 8: Number of wheat spikes emasculated, haploid embryos isolated, and haploid plants generated from the crosses between Dayn and T_0 plants from A1012. Cas9, mRNA expression level of Cas9 gene; gRNA, gRNA expression level; H, Higher expression; L, Lowed expression; S, Similar expression; -, not measured. The relative expression level is compared to Actin or CDK.

Wheat Varieties	Maize pollen source	Cas9	gRNA	Number of spikes	Number of haploid embryos	Number of haploid plants
Dayn x Alsen BC2-9	A1011-22-1	H	L	8	3	0
	A1011-4-2	S	-	3	3	0
	A1011-10-8	-	-	4	3	0
	A1011-19-2	H	L	7	0	0
	A1011-10-6	H	H	9	4	1
	A1011-15-7	-	-	7	1	0
	A1011-7-5	-	-	10	7	0
	A1011-8-4	H	-	2	1	0
	A1011-14-5	-	-	7	5	1
	A1011-26-5	-	-	9	2	0
	A1011-10-5	-	-	9	0	0
	A1011-13-6	-	-	13	6	1
	A1011-7-5	-	-	3	0	0
	A1011-2-6	-	-	2	0	0
	A1011-26-8	H	L	2	0	0
	A1011-13-6	-	-	6	0	0
	A1011-14-5	-	-	2	0	0
	A1011-26-5	-	-	5	0	0
Dayn x Alsen BC2-1	A1011-14-5	-	-	2	0	0
	A1011-10-5	-	-	3	0	0
	A1011-26-5	-	-	1	0	0
	A1011-13-6	-	-	13	1	0
	A1011-2-6	-	-	5	0	0
	A1011-9-7	-	-	9	0	0
	A1011-7-3	-	-	5	0	0
	A1011-7-6	-	-	6	2	0
	A1011-26-5	-	-	1	0	0
Dayn	A1011-13-6	-	-	3	8	0
	A1011-8-3	-	-	3	2	0
	A1011-7-5	-	-	19	0	0
	A1011-15-7	-	-	2	0	0
	A1011-10-5	-	-	4	0	0
	A1011-14-5	-	-	20	2	2
	A1011-10-4	-	-	9	4	0
	A1011-7-6	-	-	6	6	0
	A1011-10-4	-	-	12	2	0
	A1011-14-2	H	L	2	0	0
	A1011-24-3	-	-	5	0	0
Total				**238**	**62**	**5**

Table 9: Number of wheat spikes emasculated, haploid embryos isolated, and haploid plants generated from the crosses between Dayn and T_0 plants from A1012. *, plant with mutation at *TaHRC*; Cas9, mRNA expression level of Cas9 gene; gRNA, gRNA expression level; H, Higher expression; L, Lowed expression; S, Similar expression; -, not measured. The relative expression level is compared to Actin or CDK.

Maize pollen source	Cas9	gRNA	No of spikes	No of haploid embryos	No of haploid plants
A1012-10-5	-	-	14	4	0
A1012-10-6	-	-	11	14	0
A1012-10-7	H	H	3	4	1
A1012-10-8	H	H	9	41	2
A1012-10-9	-	-	5	39	1
A1012-14-3	H	H	25	44	3
A1012-14-4	H	L	18	7	0
A1012-14-5	-	-	43	23	0
A1012-14-6	-	-	12	2	1*
A1012-14-7	-	-	15	6	0
A1012-1-5	L	-	12	20	1
A1012-15-3	H	S	4	2	0
A1012-15-6	-	-	6	14	1*
A1012-21-4	H	-	10	3	0
A1012-21-5	H	-	8	5	0
A1012-2-3	L	-	7	2	0
A1012-23-2	L	-	11	3	0
A1012-23-3	L	-	5	7	0
A1012-4-6	-	-	4	0	0
A1012-4-7	L	-	5	5	0
A1012-7-1	H	H	43	177	4*
A1012-7-2	L	S	13	33	4
A1012-7-3	-	-	24	69	0
A1012-9-1	H	H	13	27	0
A1012-9-2	-	-	5	16	0
A1012-9-3	-	-	9	54	2
A1012-9-5	-	-	8	5	0
Total			**342**	**626**	**20**

Using pollens of T_1 plants from A1011 for pollination, 18 haploid plantlets were generated from 185 haploid embryos isolated from 170 spikes of Dayn (Table 10). Only one haploid plantlet was generated of 6 haploid embryos isolated from 46 spikes of Dayn-*TaHRC-R* (BC4) crosses with the T_1 plants from A1011 (Table 10). Using T_1 plant of A1012 for pollination, 33 haploid plants were regenerated from the 316 haploid embryos, isolated from 126 spikes of Dayn, while 6 haploid plantlets were generated from the 62 haploid embryos isolated

from 47 spikes of Dayn-*TaHRC-R* (BC4) (Table 11). The germination rate of haploid embryos isolated from crosses with T_1-maize pollens of A1011 and A1012 were 9.7% and 12.2 %, respectively (Table 10 and Table 11).

Table 10: Number of wheat spikes emasculated, haploid embryos isolated, and haploid plants generated from the crosses between Dayn or Dayn-*TaHRC-R* and T_1 plants of A1011. Cas9, mRNA expression level of Cas9 gene; gRNA, gRNA expression level; H, Higher expression; L, Lowed expression; -, not measured. The relative expression level is compared to Actin or CDK.

Wheat Varieties	**Pollen source**	**Cas9**	**gRNA**	**No of spikes**	**No of haploid embryos**	**No of haploid plants**
Dayn	A1011-25-7	-	-	6	13	1
Dayn	A1011-26-1	-	-	8	11	0
Dayn	A1011-25-7 (14)	-	-	29	35	4
Dayn	A1011-25-7 (17)	-	-	8	11	1
Dayn	A1011-7-1-7-4 (9)	H	-	25	28	4
Dayn	A1011-25-7 (10)	L	-	14	45	3
Dayn	A1011-22 (13)	-	-	17	5	2
Dayn	A1011-25-7 (15)	-	-	17	31	2
Dayn-*TaHRC-R*	A1011-22 (13)	-	-	7	0	0
Dayn-*TaHRC-R*	A1011-7-1-7-4 (9)	H	-	39	6	1
Total				**170**	**185**	**18**

Table 11: Number of wheat spikes emasculated, haploid embryos isolated, and haploid plants generated from the crosses between Dayn or Dayn-*TaHRC-R* and T_1 plants of A1012. *, plant with mutation at *TaHRC*; Cas9, mRNA expression level of Cas9 gene; gRNA, gRNA expression level; H, Higher expression; L, Lowed expression; S, Similar expression; -, not measured. The relative expression level is compared to Actin or CDK.

Wheat Varieties	Pollen source	Cas9	gRNA	No of spikes	No of haploid embryos	No of haploid plants
Dayn	A1012-15-3 (19)	L	H	16	64	6*
Dayn	A1012-15-4 (22)	H	H	18	57	9
Dayn	A1012-15-4 (23)	H	E	10	13	2
Dayn	A1012-15-3 (20)	L	L	19	56	6*
Dayn	A1012-7-1 (29)	H	L	13	17	2
Dayn	A1012-7-1 (27)	-	-	5	21	2
Dayn	A1012-7-1 (28)	L	S	20	46	2
Dayn	A1012-7-1 (26)	L	H	10	1	
Dayn	A1012-15-3 (24)	H	S	5	10	2
Dayn	A1012-14-6 (31)	H	L	3	1	
Dayn	A1012-7-1 (25)	L	L	7	30	2
Dayn-*TaHRC-R*	A1012-7-1 (25)	L	L	3	2	
Dayn-*TaHRC-R*	A1012-15-3 (24)	S	S	10	28	3
Dayn-*TaHRC-R*	A1012-7-1 (26)	L	S	10	1	
Dayn-*TaHRC-R*	A1012-7-1 (29)	H	L	1	0	
Dayn-*TaHRC-R*	A1012-15-3 (19)	L	H	4	4	
Dayn-*TaHRC-R*	A1012-15-3 (20)	L	L	19	27	3*
Total				**173**	**378**	**39**

Identification of mutations at *TaHRC-S* and *TaHRC-R* in haploid plants

The *TaHRC* gene was amplified from all haploid plants and the parental genotypes (Dayn, Dayn-*TaHRC-R*, and Alsen) by PCR using a pair of flanking primers (Table 4) and subjected to DNA sequencing. No changes were found in the targeted *TaHRC* gene from the five and 18 haploid plantlets, derived from the crosses with the T_0 and T_1 plants of A1011, respectively.

Among 20 haploid plants derived from the crosses between Dayn and T_0 plants of A1012, three had an insertion or deletion mutation at the *TaHRC* gene (Figure 21a, Table 12).

One haploid plant (Dayn-15-6) had one singe base (T) insertion, and the other two (Dayn-7-1 and Dayn-14-6) had a deletion of 19 and 88 bp, respectively, at the target region (Figure 21a, Table 12). The 88 bp deletion in Dayn-14-6 spanned from 3-bp upstream of *TaHRC* target 3 (T3) PAM site to 3-bp upstream of *TaHRC* target 4 (T4) PAM site (Figure 21a), suggesting that this deletion resulted from two cleavages generated by the Cas9 enzyme guided by the two gRNAs containing the oligo (T3 or T4), respectively.

Table 12: Total haploid plants with mutations at *TaHRC* obtained from the wide hybridization between wheat and T_0 plants from A1012.

Sequencing order	Wheat cultivar	Maize pollen	Haploid mutants	Mutation
9	Dayn	A1012-14-6	Dayn_9-14-6	88 bp deletion at TG4
15	Dayn	A1012-15-6	Dayn_15-15-6	"T" insertion at TG4
16	Dayn	A1012-7-1	Dayn_16-7-1	19 bp deletion at TG4

Of the 39 haploid plants generated from the crosses between Dayn/Dayn-*TaHRC-R* and T_1 plants from A1012, 11 haploid plants (~28%) had mutations at the *TaHRC* target sites (Table 13, Figure 21b). Only two haploid plants (Dayn_22-15-3-20 and Dayn_32-7-1-28) contained mutations at both target sites and the rest had mutations at T4 site only. Most of mutations are single base insertions. Five haploid plants had a combination of deletion and insertion (Table 13, Figure 21b).

Table 13: Total haploid plants with mutations at *TaHRC* obtained from the wide hybridization between wheat and T_1 plants from A1012.

Sequencing order	Wheat cultivar	Maize pollen	Haploid mutants	Mutation
19	Dayn	A1012-15-3-19	Dayn_19-15-3-19	'T' insertion at T4
21	Dayn-*TaHRC-R* (BC4)	A1012-15-3-20	BC4_21-15-3-20	19 bp deletion at T4
22	Dayn	A1012-15-3-20	Dayn_22-15-3-20	2 bp deletion at T3 and 'A' insertion at T4
30	Dayn	A1012-15-4-22	Dayn_30-15-4-22	'T' insertion at T4
32	Dayn	A1012-7-1-28	Dayn_32-7-1-28	'G' insertion at T3 & 'T' insertion at T4
35	Dayn	A1012-15-4-22	Dayn_35-15-4-22	7 bp deletion at T4
36	Dayn	A1012-15-4-23	Dayn_36-15-4-23	'G' insertion at T4
43	Dayn-*TaHRC-R* (BC4)	A1012-15-3-24	BC4_43-15-3-24	7 bp deletion at T4
46	Dayn	A1012-15-4-22	Dayn_46-15-4-22	'A' insertion at T4
49	Dayn	A1012-15-4-22	Dayn_49-15-4-22	7 bp deletion at T4
52	Dayn	A1012-15-3-20	Dayn_52-15-3-20	'G' insertion at T4

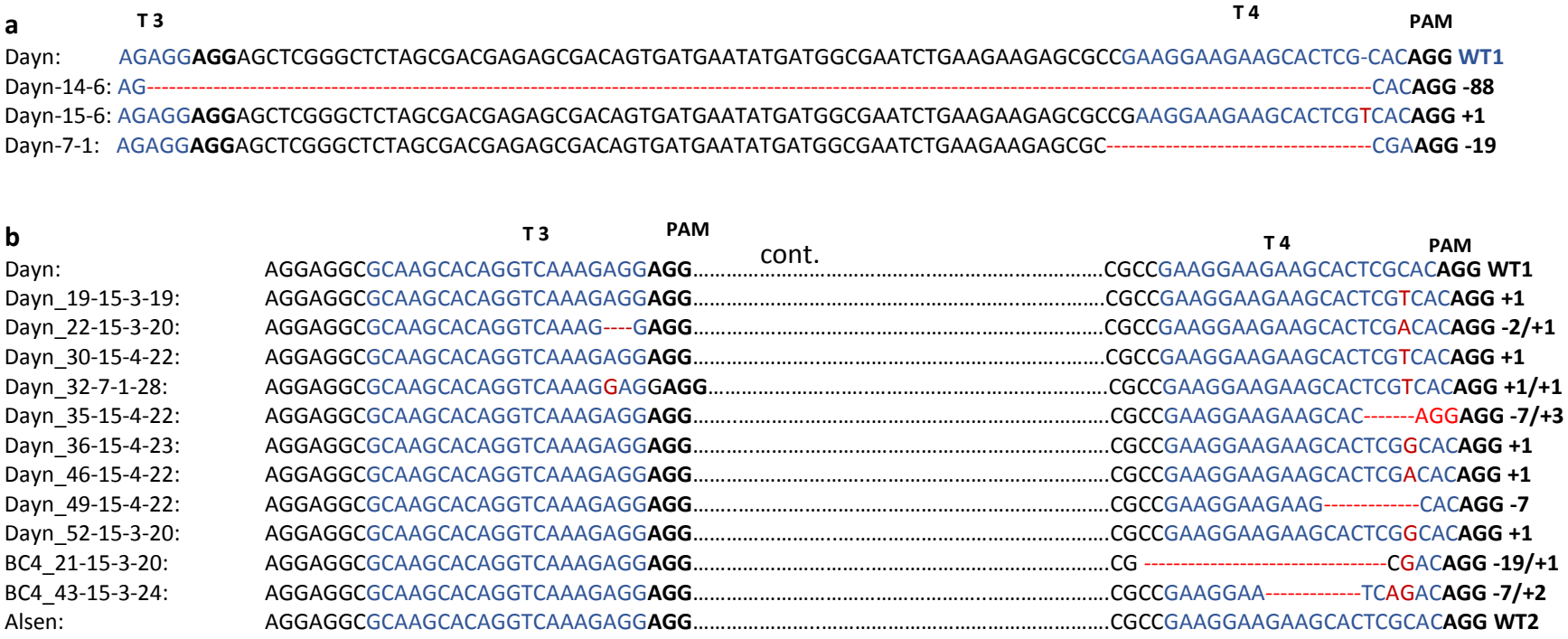

Figure 21: Partial sequences of *TaHRC* gene showing mutations at the two target sites (T3 and T4). a) mutations in haploid plants generated from the crosses between Dayn with *TaHRC-S* allele and T_0 plants from A1012. b) mutations in haploid plants generated from the crosses between Dayn or Dayn x Alsen (BC4) with *TaHRC-R* allele and T_0 plants from A1012. T3, *TaHRC* target 3; T4 *TaHRC* target 4; **AGG**, PAM sequence; red text or -, insertion or deletion mutation.

Discussion

Fhb1 is a QTL that has been widely used in wheat breeding programs for a major source of FHB resistance. It was first mapped on chromosome 3BS back 22 years ago (Anderson et al. 2000). Since then, extensive efforts have been devoted to clone the gene or genes responsible for the FHB resistance at this locus. Rawat et al. (2016) indicated that the wheat gene *PFT* encoding a putative pore-forming toxin-like chimeric lectin protein confers the *Fhb1*-mediated FHB resistance. However, the role of this gene in FHB resistance has now been questioned by several more recent studies based on the fact that many wheat lines with the functional *PFT* gene are susceptible to FHB (Bai et al., 2018; Jia et al., 2018; Li et al., 2019; Su et al., 2019). Li et al. (2019) and Su et al. (2019) demonstrated that different alleles (*TaHRC-R* and *TaHRC-S*) of the *TaHRC* gene (or *His*) near *PFT* at the *Fhb1* locus is responsible for resistance and susceptibility to FHB, respectively. *TaHRC-R* has a 608 bp deletion compared to *TaHRC-S*. However, different mechanisms were proposed by the two studies for the FHB resistance conferred by *TaHRC-R*. Li et al. (2019) considered that *TaHRC-R* was a gain-of-function mutation from *TaHRC-S* and likely acted in a dominant-negative manner. On the other hand, Su et al. (2019) suggested that the wild type *TaHRC* gene (*TaHRC-S*) was a susceptibility gene and a loss-of-function mutation of *TaHRC-S* occurred creating *TaHRC-R* for FHB resistance. In the present study, both *TaHRC-S* and *TaHRC-R* alleles in wheat were targeted using the CRISPR/Cas9-mediated genome editing technology coupled with wide hybridization between wheat and maize to generate double haploid plants containing homozygous mutations for the target allele. Twelve and two haploid plants with mutations at the target sites were generated for *TaHRC-S* and *TaHRC-R*, respectively. Doubled haploid plants are being produced from these haploid mutant plants and further phenotyped for FHB resistance and other traits. Phenotyping of the mutants

obtained from this study will verify the role of both *TaHRC-S* and *TaHRC-R* alleles in FHB resistance. The haploid induction method through wheat × maize hybridization coupled with the CRISPR/Cas9 system may provide a novel approach for improving resistance to FHB and other diseases in wheat.

Previously, the effect of wheat and maize genotypes on the efficacy of haploid embryo formation and haploid plant production has been widely studied. However, the conclusions are not consistent. One study reported that the frequency of haploid embryo formation was only affected by maize genotype but not by the wheat genotypes (Suenaga & Nakajima, 1989). However, several other studies showed that the formation of haploid embryos was significantly affected by wheat genotypes during wheat × maize wide hybridization (Filomena Martins-Lopes et al., 2001; Inagaki & Tahir, 1990; Kumar et al., 2009; Laurie & Reymondie, 1991). Lefebvre & Devaux (1996) used five maize genotypes to cross with 18 wheat F_1 hybrids and showed the parental genotypes interaction was significant for the number of haploid embryos formed per 100 wheat florets after pollinating with pollens from the different maize genotypes. In the present study, the haploid embryo formation rate varied among the different wheat genotypes when crossed with the same maize variety Hi-II. Dayn, WA8283 and Lang_MN wheat genotypes showed higher haploid embryos formation as compared to other wheat genotypes. The same set of wheat genotypes was also used to crosse with another maize variety (B104), which is an inbred line widely used in maize transformation, and the results indicated that different wheat genotypes differed substantially in the rate of haploid embryos formation (data not shown). These results indicate that preliminary screening of wheat genotypes should be conducted using Hi-II to select the right genotype for the haploid induction-gene-editing experiments.

The qRT-PCR analysis of Cas9 and gRNA expression in both Hi-II transgenic plants derived from A1011 and A1012 showed the expression levels of the two genes varied among different transformation events and even among different individuals from the same event. Although a high level of expression for both Cas9 and gRNA was observed in transgenic plants from both vector constructs (A1011 and A1012), only the transgenic plants from a few transformation events derived from A1012 were able to induce the mutation at the target sites of *TaHRC* gene and the mutation types induced by maize plants from different events of A1012 were also different. In general, the chance of producing mutations at the target gene in wheat plants is higher when crossed with transgenic maize plants with high level of Cas9 and gRNA expression. For example, one haploid plant with mutation at the *TaHRC* gene was generated from Dayn pollinated with the T_0 plant A1012-7-1 with a high level of expression of Cas9 and gRNA. However, mutations at target sites of *TaHRC* gene were also observed in the haploid plants derived from the wide hybridization using A1012 T_1 plants with low Cas9 and gRNA expression. This indicate that the expression level of Cas9 and gRNA in maize plant may not be the only factor affecting the CRISPR/Cas9-mediated gene editing efficiency. Also, the expression of gRNA and Cas9 in the leaves of maize plants may not be the same as the expression in wheat embryos where the maize chromosomes remain for a short period of time after fertilization.

The rate of haploid embryo formation from crosses pollinated by T_0 plants from A1011 and A1012 was very much lower compared to that obtained from the crosses pollinated by the T_1 plants. This may be due to the effect of seasonal conditions. The crosses between wheat and the T_0 plants were made in the summer of 2021 starting in July and ending in September while the wide hybridization experiments with the T_1 plants were conducted during winter season starting

from early December of 2021 and ending at late January of 2022. Previous studies reported that the haploid embryo formation efficiency from wheat × maize hybridization is usually higher in the spring (January-April) than in fall (August-December) (Campbell et al., 2010; Niu et al., 2014). The temperature and light intensity play significant roles in the production of haploid embryo numbers by wheat cultivars, and in successful germination of haploid embryos to produce seedlings. The optimal temperature for embryo recovery was 22/17 ºC day/night (Campbell et al., 2010). The temperature affects the fertilization ability of the egg cells, the pollen tube growth in female plants and hybrid seed viability (Niu et al., 2014). The frequency of fertilization was higher at the lower temperature (20 ºC) than at the higher temperature (26 ºC) in the wheat × *H. bulbosum* crosses. The growing environment also influences the viability of maize pollen (Barnabás & Rajki, 1976; Campbell et al., 1998). High temperatures during the summer were the most likely reason for very low numbers of haploid embryos in the present experiment.

In this study, only A1012 maize plants from both T_0 and T_1 generation were able to induce mutations at the *TaHRC* gene. The mutation efficiency using T_0 and T_1-maize from A1012 for pollination was 15% and 28.2%, respectively. Also, most of the mutations occurred at the T4 region of the target site. This indicates that some sites are more prone to the mutations and targeting multiple sites might be an effective strategy for generating mutations using CRISPR/Cas9-mediated genome editing. The Cas9 from *Streptococcus pyogenes* (SpCas9) was found to be more active in creating double stranded DNA breaks at 37 ºC *in vitro* (LeBlanc et al., 2018). Heat stress has been used to increase the efficiency of targeted mutagenesis by CRISPR/Cas9 in plants. In this study, *Arabidopsis* or *Citrus* plants subjected to heat stress at 37 ºC showed much higher frequencies of CRISPR-induced mutations. In maize, the efficiency of

mutations using haploid inducer lines expressing CRISPR/Cas9 may be increased by growing pollinated plants at higher temperature (Kelliher et al., 2019). But in case of wheat × maize wide hybridization, haploid induction is not favorable at higher temperature (Campbell et al., 1998).

The gRNA-guided Cas9 induces the double strand break at target sites which results in the random insertions and deletions (Indels) at target locus through the error-prone NHEJ repair pathway (Feng et al., 2013; Mao et al., 2013; Shan et al., 2013). Most of the mutations in this study showed similar indel mutations. When two gRNA targets are used, it can result in the large fragment deletion between the two target sites. Co-injection of dual sgRNA-guided Cas9 nuclease resulted in the elimination of an interval up to 24 kb between two gRNAs in *Caenorhabditis elegans* (Chen et al., 2014). The large 88 bp deletion mutation in Dayn_15-6 can also be explained by similar phenomena. The deletion occurred between *TaHRC* target 3 and *TaHRC* target 4.

In summary, the haploid induction coupled with CRISPR/Cas9-mediated genome is a novel and efficient approach for targeted mutagenesis of genes that confer disease susceptibility. This study provides a great platform for crop improvement with the opportunity of creating transgene free homozygous mutants in two generations. Phenotypic characterization of the double haploid mutants with the gene editing at *TaHRC-S* and *TaHRC-R* allele will provide the major contribution to clarify the controversy about the *TaHRC* gene as a susceptible or resistant candidate gene responsible for the *Fhb1*-mediated FHB resistance in Sumai3 and its derivatives. As *TaHRC* is a conserved gene in cereal crops, this characterization opens a new avenue to improve FHB resistance in wheat as well as possibly other cereal crops.

References

Anand, A., Trick, H. N., Gill, B. S., & Muthukrishnan, S. (2003). Stable transgene expression and random gene silencing in wheat. *Plant Biotechnology Journal*, *1*(4), 241–251.

Anderson, J. A., Stack, R. W., Liu, S., Waldron, B. L., Fjeld, A. D., Coyne, C., Moreno-Sevilla, B., Fetch, J. M., Song, Q. J., Cregan, P. B., & Frohberg, R. C. (2001). DNA markers for Fusarium head blight resistance QTLs in two wheat populations. *Theoretical and Applied Genetics*, *102*(8), 1164–1168.

Bai, G., Kolb, F. L., Shaner, G., & Domier, L. L. (1999). Amplified fragment length polymorphism markers linked to a major quantitative trait locus controlling scab resistance in wheat. *Phytopathology*, *89*(4), 343–348.

Bai Guihua, & Shaner, G. (1994). Scab of wheat: Prospects for control. *Plant Disease*, *78*(8), 760–766.

Bai, G., Su, Z., & Cai, J. (2018). Wheat resistance to Fusarium head blight. *Canadian Journal of Plant Pathology*, 40(3), 336-346.

Barnabás, B., & Rajki, E. (1976). Storage of maize (Zea mays L.) pollen at −196°C in liquid nitrogen. *Euphytica* 1976 25:1, 25(1), 747–752.

Budhagatapalli, N., Halbach, T., Hiekel, S., Büchner, H., Müller, A. E., & Kumlehn, J. (2020). Site□directed mutagenesis in bread and durum wheat via pollination by cas9/guide RNA□transgenic maize used as haploidy inducer. *Plant Biotechnology Journal*, *18*(12), 2376.

Buerstmayr, H., Ban, T., & Anderson, J. A. (2009). QTL mapping and marker-assisted selection for Fusarium head blight resistance in wheat: a review. *Plant Breeding*, *128*(1), 1–26.

Campbell, A. W., Griffin, W. B., Burritt, D. J., & Conner, A. J. (2010). Production of wheat doubled haploids via wide crosses in New Zealand wheat, *New Zealand Journal of Crop and Horticultural Science*, *28*(3), 185–194.

Campbell, A. W., Griffin, W. B., Conner, A. J., Rowarth, J. S., & Burritt, D. J. (1998). The effects of temperature and light intensity on embryo numbers in wheat doubled haploid production through wheat×maize crosses. *Annals of Botany*, *82*(1), 29–33.

Chang, H. H. Y., Pannunzio, N. R., Adachi, N., & Lieber, M. R. (2017). Non-homologous DNA end joining and alternative pathways to double-strand break repair. *Nature Reviews Molecular Cell Biology 2017 18:8*, *18*(8), 495–506.

Chen, X., Xu, F., Zhu, C., Ji, J., Zhou, X., Feng, X., & Guang, S. (2014). Dual sgRNA-directed gene knockout using CRISPR/Cas9 technology in Caenorhabditis elegans. *Scientific Reports*, *4*.

Ding, M., Zhao, H., Gu, J., Li, H., Liu, K., Yang, M., & Li, S. (2017). Research and breeding application progress of the technique of producing double haploid of wheat by wide hybridization between wheat and maize. *Agricultural Science & Technology*, 18(12), 2202-2208.

Feng, Z., Zhang, B., Ding, W., Liu, X., Yang, D. L., Wei, P., Cao, F., Zhu, S., Zhang, F., Mao, Y., & Zhu, J. K. (2013). Efficient genome editing in plants using a CRISPR/Cas system. *Cell Research 2013 23:10*, *23*(10), 1229–1232.

Filomena Martins-Lopes, P., Guedes-Pinto, H., Pinto-Carnide, O., & Snape, J. (2001). The effect of spikelet position on the success frequencies of wheat haploid production using the maize cross system. *Euphytica 2001 121:3*, *121*(3), 265–271.

Frame, B. R., Shou, H., Chikwamba, R. K., Zhang, Z., Xiang, C., Fonger, T. M., ... & Wang, K. (2002). Agrobacterium tumefaciens-mediated transformation of maize embryos using a standard binary vector system. *Plant physiology*, 129(1), 13-22.

Henry, Y., & de Buyser, J. (1985). Effect of the 1B/1R translocation on anther culture ability in wheat (Triticum aestivum L.). *Plant Cell Reports 1985 4:6*, *4*(6), 307–310.

INAGAKI, M., & TAHIR, M. (1990). Comparison of haploid production frequencies in wheat varieties crossed with Hordeum bulbosum L. and maize. *Japanese Journal of Breeding*, *40*(2), 209–216.

Jiang, W., Zhou, H., Bi, H., Fromm, M., Yang, B., & Weeks, D. P. (2013). Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice. *Nucleic Acids Research*, *41*(20), e188–e188.

Kelliher, T., Starr, D., Su, X., Tang, G., Chen, Z., Carter, J., Wittich, P. E., Dong, S., Green, J., Burch, E., McCuiston, J., Gu, W., Sun, Y., Strebe, T., Roberts, J., Bate, N. J., & Que, Q. (2019). One-step genome editing of elite crop germplasm during haploid induction. *Nature Biotechnology 2019 37:3*, *37*(3), 287–292.

Kohli, A., Twyman, R. M., Abranches, R., Wegel, E., Stoger, E., & Christou, P. (2003). Transgene integration, organization and interaction in plants. *Plant Molecular Biology*, *52*, 247–258.

Kumar, R., Niroula, R. K., & Thapa, D. B. (2009). Response of Wheat Genotypes to Maize Mediated Polyhaploid Production. *Journal of Agronomy*, *2*(3), 156–161.

Laurie, D. A., & Bennett, M. D. (1986). Wheat× maize hybridization. *Canadian Journal of Genetics and Cytology*, 28(2), 313-316.

Laurie, D. A., & Bennett, M. D. (1988). The production of haploid wheat plants from wheat ×maize crosses. *Theoretical and Applied Genetics 1988 76:3*, *76*(3), 393–397.

Laurie, D. A., & Bennett, M. D. (1989). The timing of chromosome elimination in hexaploid wheat× maize crosses. *Genome*, 32(6), 953-961.

Laurie, D. A., & Reymondie, S. (1991). High frequencies of fertilization and haploid seedling production in crosses between commercial hexaploid wheat varieties and maize. *Plant Breeding*, *106*(3), 182–189.

LeBlanc, C., Zhang, F., Mendez, J., Lozano, Y., Chatpar, K., Irish, V. F., & Jacob, Y. (2018). Increased efficiency of targeted mutagenesis by CRISPR/Cas9 in plants using heat stress. *The Plant Journal*, *93*(2), 377–386.

Lefebvre, D., & Devaux, P. (1996). Doubled haploids of wheat from wheat ×maize crosses:genotypic influence, fertility and inheritance of the 1BL-1RS chromosome. *Theoretical and Applied Genetics 1996 93:8*, *93*(8), 1267–1273.

Li, G., Zhou, J., Jia, H., Gao, Z., Fan, M., Luo, Y., Zhao, P., Xue, S., Li, N., Yuan, Y., Ma, S., Kong, Z., Jia, L., An, X., Jiang, G., Liu, W., Cao, W., Zhang, R., Fan, J., … Ma, Z. (2019). Mutation of a histidine-rich calcium-binding-protein gene in wheat confers resistance to Fusarium head blight. *Nature Genetics 2019 51:7*, *51*(7), 1106–1112.

Mao, Y., Zhang, H., Xu, N., Zhang, B., Gou, F., & Zhu, J. K. (2013). Application of the CRISPR–Cas System for Efficient Genome Engineering in Plants. *Molecular Plant*, *6*(6), 2008–2011.

Mesterházy, Á., Bartók, T., Mirocha, C. G., & Komoróczy, R. (1999). Nature of wheat resistance to Fusarium head blight and the role of deoxynivalenol for breeding. *Plant Breeding*, *118*(2), 97–110.

Miller, J. D., Young, J. C., & Sampson, D. R. (1985). Deoxynivalenol and Fusarium head blight resistance in spring cereals. *Journal of Phytopathology*, *113*(4), 359–367.

Niu, Z., Jiang, A., Abu Hammad, W., Oladzadabbasabadi, A., Xu, S. S., Mergoum, M., & Elias, E. M. (2014). Review of doubled haploid production in durum and common wheat through wheat × maize hybridization. *Plant Breeding*, *133*(3), 313–320.

Oury, F. X., Pichon, M., & Rousset, M. (1993). A comparison of 2 haplodiploidization methods in bread wheat: anther culture and interspecific hybridization in maize.

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., & Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nature Protocols 2013 8:11*, *8*(11), 2281–2308.

Sadasivaiah, R. S., Orshinsky, B. R., & Kozub, G. C. (1999). Production of wheat haploids using anther culture and wheat ×maize hybridization techniques. *Cereal Research Communications 1999 27:1*, *27*(1), 33–40.

Shan, Q., Wang, Y., Li, J., Zhang, Y., Chen, K., Liang, Z., Zhang, K., Liu, J., Xi, J. J., Qiu, J. L., & Gao, C. (2013). Targeted genome modification of crop plants using a CRISPR-Cas system. *Nature Biotechnology 2013 31:8*, *31*(8), 686–688.

Sourour, A., Olfa, S. A., da Silva, J. A. T., & Hajer, S. A. (2011). Effect of different factors on haploid production through embryo rescue in durum wheat × maize crosses. *International Journal of Plant Breeding*, *5*(2), 118–121.

Stam, M., Mol, J. N. M., & Kooter, J. M. (1997). The silence of genes in transgenic plants. *Annals of Botany*, *79*(1), 3–12.

Steiner, B., Buerstmayr, M., Michel, S., Schweiger, W., Lemmens, M., & Buerstmayr, H. (2017). Breeding strategies and advances in line selection for Fusarium head blight resistance in wheat. *Tropical Plant Pathology*, *42*(3), 165–174.

Su, Z., Bernardo, A., Tian, B., Chen, H., Wang, S., Ma, H., Cai, S., Liu, D., Zhang, D., Li, T., Trick, H., st. Amand, P., Yu, J., Zhang, Z., & Bai, G. (2019). A deletion mutation in TaHRC confers Fhb1 resistance to Fusarium head blight in wheat. *Nature Genetics 2019 51:7*, *51*(7), 1099–1105.

Suenaga, K., & Nakajima, K. (1989). Efficient production of haploid wheat (Triticum aestivum) through crosses between Japanese wheat and maize (Zea mays). *Plant Cell Reports 1989 8:5*, *8*(5), 263–266.

Sung, P., & Klein, H. (2006). Mechanism of homologous recombination: mediators and helicases take on regulatory functions. *Nature Reviews Molecular Cell Biology 2006 7:10*, *7*(10), 739–750.

Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: molecular evolutionary genetics analysis version 11. *Molecular Biology and Evolution*, *38*(7), 3022–3027.

Waldron, B. L., Moreno-Sevilla, B., Anderson, J. A., Stack, R. W., & Frohberg, R. C. (1999). RFLP mapping of QTL for fusarium head blight resistance in wheat. *Crop Science*, *39*(3), 805–811.

H. L. Xing, L. Dong, Z. P. Wang, H. Y. Zhang, C. Y. Han, B. Liu, X. C. Wang, Q. J. Chen. 2014. A CRISPR/Cas9 toolkit for multiplex genome editing in plants. *BMC plant biology*, 14(1), 1-12.

Zhang, J., Friebe, B., Raupp, W. J., Harrison, S. A., & Gill, B. S. (1996). Wheat embryogenesis and haploid production in wheat × maize hybrids. *Euphytica 1996 90:3*, *90*(3), 315–324.

APPENDIX. SUPPLEMENTARY TABLES

Table A1: Stock solutions and their ingredients used for *Agrobacterium*-mediated transformation (Ishida et al., 2015).

Stock solutions	**Ingredients**
LS Major salts (10X)	Dissolve 19.0 g KNO_3, 16.5 g NH_4NO_3, 4.4 g $CaCl_2 \cdot 2H_2O$, 3.7 g $MgSO_4 \cdot 7H_2O$, and 1.7 g KH_2PO_4, in 900 ml distilled water and filled to 1,000 ml. Store at 4 °C.
Ethylenediamine-tetraacetic acid-iron (FeEDTA, 100X)	Dissolve 2.78 g $FeSO_4 \cdot 7H_2O$ dissolved in 900 ml of hot distilled water and add 3.73 g ethylenediamine- N, N, N′, N′-tetraacetic acid, disodium salt. Cool and fill to 1,000 ml. Store at 4 °C.
LS minor salts (100X)	Dissolve 2.23 g $MnSO_4 \cdot 7H_2O$, 1.06 g $ZnSO_4 \cdot 7H_2O$, 620 mg H_3BO_3, 83 mg KI, 25.0 mg $Na_2MoO_4 \cdot 2H_2O$, 2.5 mg $CuSO_4 \cdot 5H_2O$, and 2.5 mg $CoCl_2 \cdot 6H_2O$ in 900 ml of distilled water and fill to 1,000 ml. Store at 4 °C.
MS vitamins (100X)	Dissolve 10 g myoinositol, 0.2 g glycine, 100 mg thiamine hydrochloride, 50 mg pyridoxine hydrochloride, and 50 mg nicotinic acid and fill to 1,000 ml. Store at 4 °C.
Modified LS vitamins (100X)	Dissolve 10 g myoinositol, 100 mg thiamine hydrochloride, 50 mg pyridoxine hydrochloride, and 50 mg nicotinic acid in 900 ml of distilled water and fill to 1,000 ml. Store at 4 °C.

Table A2: Media and their ingredients used for *Agrobacterium*-mediated transformation (Ishida et al., 2015).

Media	Ingredients
Embryo collection (WLS-liq)	Add 10 ml of the 10× LS major salts, 1 ml of 100× FeEDTA, 1 ml of 100× LS minor salts and 1 ml of 100× MS vitamins, 10 g glucose, and 0.5 g 2-(N-morpholino) ethanesulfonic acid (MES) to 700 ml of distilled water and make up the volume to 1,000 ml. Adjust pH to 5.8. Sterilize using a 0.22 μm cellulose acetate filter and store at 4 °C.
Inoculum (WLS-inf)	WLS-Liq plus 100 μM acetosyringone.
Co-culitvation (WLS-AS)	WLS-inf plus 0.85 mg/l $AgNO_3$, 1.25 mg/l $CuSO_4 \cdot 5H_2O$, and 8 g/l agarose.
Basic composition for selection (WLS)	Add 100 ml of the 10× LS major salts, 10 ml of 100× FeEDTA, 10 ml of 100× LS minor salts, 10 ml of 100× MS vitamins, 5 ml of 100 mg/l 2,4-D, 22 ml of 100 mg/l picloram, 0.5 g glutamine, 0.1 g casein hydrolysate, 0.75 g $MgCl_2 \cdot 6H_2O$, 40 g maltose, and 1.95 g MES to 700 ml of distilled water and make up the volume to 1,000 ml. Adjust pH to 5.8 and add 5 g agarose. Autoclave at 121 °C for 15 min. Cool to 50 °C, and add 100 mg/l ascorbic acid, 250 mg/l carbenicillin, and 0.85 mg/l $AgNO_3$.
Resting (WLS-Res)	WLS plus 100 mg/l cefotaxime (added after autoclaving).
1st selection for phosphinothricin (WLS-P5)	WLS plus 5 mg/l phosphinothricin (added after autoclaving).
2nd selection for phosphinothricin (WLS-P10)	WLS plus 10 mg/l phosphinothricin (added after autoclaving).
Regeneration culture (LSZ)	Add 100 ml of the 10× LS major salts, 10 ml of 100× FeEDTA, 10 ml of 100× LS minor salts, 10 ml of 100× Modifi ed LS vitamins, 50 ml of 100 mg/l zeatin, 20 g sucrose, 0.5 g MES, 2.5 mg $CuSO_4 \cdot 7H_2O$ to 700 ml of distilled water and make up the volume to 1,000 ml. Adjust pH to 5.8 and add 8 g agar. Autoclave at 121 °C for 15 min. Cool to 50 °C and add 250 mg/l carbenicillin and 100 mg/l cefotaxime.
Regeneration after phosphinothricin selection (LSZ-P5)	LSZ plus 5 mg/l phosphinothricin (added after autoclaving).
Rooting culture (LSF)	Add 100 ml of the 10× LS major salts, 10 ml of 100× FeEDTA, 10 ml of 100× LS minor salts, 10 ml of 100× Modified LS vitamins, 2 ml of 100 mg/l IBA, 15 g sucrose, and 0.5 g MES to 700 ml of distilled water and make up the volume to 1,000 ml. Adjust pH to 5.8 and add 3 g Gelrite. Autoclave at 121 °C for 15 min. Cool to 50 °C and add 250 mg/l carbenicillin.
Rooting after phosphinothricin selection (LSF-P5)	LSF plus 5 mg/l phosphinothricin.

Table A3: Stock solutions used for particle bombardment transformation. MS and B5 vitamin stock solutions (100X concentration), [a]Completely dissolve EDTA first, slowly add FeSO4 while stirring, [b]Store in foil-c (Tian et al., 2019).

Stock	**Component**	**Amount (g)/1 l**	**Amount (g)/500 ml**
MS sulfates	$MgSO_4 \cdot 7H_2O$	37	18.5
	$MnSO_4 \cdot H_2O$	1.69	0.845
	$ZnSO_4 \cdot 7H_2O$	0.86	0.43
	$CuSO_4 \cdot 5H_2O$	0.0025	0.00125
MS halides	$CaCl_2 \cdot 2H_2O$	44	22
	KI	0.083	0.415
	$CoCl_2 \cdot 6H_2O$	0.0025	0.00125
MS P, B, MO	KH_2PO_4	17	8.5
	H_3BO_3	0.62	0.31
	$Na_2MoO_4 \cdot 2H_2O$	0.025	0.0125
MS FeEDTA[a,b]	Na_2EDTA	3.725	1.862
	$FeSO_4 \cdot 7H_2O$	2.784	1.392
B5 vitamins[b]	Myoinositol	10	5
	Nicotinic acid	0.10	0.05
	Pyridoxine HCI	0.10	0.05
	Thiamine	1	0.5

Table A4: Media and their ingredients used for particle bombardment transformation (Tian et al., 2019).

Media	Ingredients
Wheat Initiation Media (CM4)	Add 10 mL each of 100X stock solutions of MS FeEDTA (add first), MS halides, MS sulfates, MS P, B, Mo, and B5 vitamins to approximately 800 mL of distilled water. While mixing with stir plate, add 1.65 g of NH_4NO_3, 1.9 g of KNO_3, 0.5 mL of 1 mg/mL 2,4-D, 2.2 mL of 1 mg/mL Picloram, and 40 g of maltose. Adjust pH to 5.7 and add water to 1 L volume. Divide medium into two 1 L media bottles, add 3 g of Phytagel into each bottle, and autoclave for 20–25 min.
CM4 + MS Media	Add 36.44 g of mannitol and 36.44 g of sorbitol to CM_4 medium above. Adjust pH to 5.7 and add water to 1 L volume. Divide medium into two 1 L media bottles, add 3 g of phytagel into each bottle, and autoclave for 20–25 min.
Wheat Shoot Production Medium (MSP)	Add 10 mL 100X stock solutions of MS FeEDTA (add first), MS halides, MS sulfates, MS P, B, Mo, and B5 vitamins to approximately 800 mL of distilled water. While mixing with stir plate, add 1.65 g of NH_4NO_3, 1.9 g of KNO_3, 0.2 mL of 1 mg/mL 2,4-D, 1.9 g of MES, 40 g of maltose, and 100 mg of ascorbic acid. Adjust pH to 5.7 and add water to 1 L volume. Divide medium into two 1 L media bottles, add 3 g of phytagel into each bottle, and autoclave for 20–25 min.
Wheat Root Production Medium (MSE)	Add 10 mL 100X stock solutions of MS FeEDTA (add first), MS halides, MS sulfates, MS P, B, Mo, and B5 vitamins to approximately 800 mL of distilled water. While mixing with stir plate, add 1.65 g of NH_4NO_3, 1.9 g of KNO_3, 1.95 g of MES, 40 g of maltose, and 100 mg of ascorbic acid. Adjust pH to 5.7 and add water to 1 L volume. Divide medium into two 1 L media bottles, add 3 g of phytagel into each bottle, and autoclave for 20 to 25 min.
Selection Media (CM4 + 5G)	Add 250 µL of 10 mg/mL filter-sterilized glufosinate ammonium stock to 500 mL CM4 after autoclaving.
Selection Media (CM4 + 10G)	Add 500 µL of 10 mg/mL filter-sterilized glufosinate ammonium stock to 500 mL of CM4 after autoclaving.
Selection Media (MSP + 10G)	Add 500 µL of 10 mg/mL filter-sterilized glufosinate ammonium stock to 500 mL MSP after autoclaving.
Selection Media (MSE + 5G)	Add 250 µL of 10 mg/mL filter-sterilized glufosinate ammonium stock to 500 mL MSE after autoclaving.