DEVELOPMENT OF FINE-MAPPING RESOURCES FOR INTERROGATION OF A YIELD IMPACTING QTL ON THE 2D CHROMOSOME IN A BREAD WHEAT (*TRITICUM AESTIVUM*) AND *AEGILOPS TAUSCHII* NESTED ASSOCIATION MAPPING POPULATION

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ABSTRACT

DEVELOPMENT OF FINE-MAPPING RESOURCES FOR INTERROGATION OF A YIELD IMPACTING QTL ON THE 2D CHROMOSOME IN A BREAD WHEAT (*TRITICUM AESTIVUM*) AND *AEGILOPS TAUSCHII* NESTED ASSOCIATION MAPPING POPULATION

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Bread wheat (*Triticum aestivum*) is one of the dominant grain crops across the world. As a mainstay of diets across the world, there is a constant pressure by the breeders and geneticists to identify yield promoting loci. In previous work, significant SNP associations were found on the 2D chromosome (approximately at 23.5Mb and 25.2Mb) for yield variation in a genome-wide association study (GWAS) of the D-genome Nested Association Mapping population (DNAM), an advanced-backcross nested association mapping population of five *Ae. tauschii* lines into an elite bread wheat background. To identify the signal source more precisely, fine-mapping tools were created in this work. Seven Kompetitive Allele Specific PCR (KASP) primer sets were designed for SNP markers between 22Mb and 30Mb, six of which displayed codominant behavior and one dominant. The KASP primer sets were identified the U6718 BC1F1 subpopulation within the DNAM as segregating for bread wheat and *Ae. tauschii* alleles between 22Mb and 30Mb on 2D chromosome. Linkage maps were created for the seven D-genome chromosomes for the U6718 subpopulation. QTL analysis defined the QTL as being between 21.3cM to 28.3cM on the 2D linkage map and 23.3Mb to 30.3Mb on the physical map. Using the defined 2DS QTL and the KASP primer sets, a fine-mapping population was created consisting of five heterozygous inbred families fixed 16 unique recombination events across the 2DS QTL. These developed tools will prove instrumental tools towards refining the understanding of causal loci of this yield QTL.

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CHAPTER 1: INTRODUCTION

The Relevance and Weaknesses of Wheat

Wheat is a name referring to the cultivated grasses belonging to the genus *Triticum*. The widely cultivated members of this genus, bread wheat (*T. aestivum* L) and durum wheat (T. *turgidum* ssp. *durum*), possess many of the valuable agronomic traits seen in other widely grown food crops, including a high yield, the ability to be mechanically harvested, adaptability to a wide array of environments, and being calorically dense. Where wheat differs from most other widely cultivated grain crops is that it produces storage proteins in the grain referred to collectively as gluten. The unusual viscoelastic properties of wheat gluten allow for the production of common food staples, such as leavened and unleavened breads, cakes, biscuits, and noodles, and binders for more processed foods (Shewry, 2009).

These favorable qualities have led to wheat being one of the dominant crops on Earth. In 2017, over 218 million hectares of wheat were harvested worldwide, more than any other crop (FAOSTAT, 2019). These millions of hectares of grain are converted into massive quantities of foodstuffs that provide nearly 20% of the calories consumed by people worldwide (FAOSTAT, 2019). Wheat also has massive commercial value. Wheat is a multi-billion dollar market, with the worth of the United States' yearly production alone fluctuating between $8.1 billion and $17.4 billion over the past decade (USDA NASS, 2018). From both a dietary and monetary perspective, wheat is one the most important crops on the planet.

While wheat possesses a wide range of positive qualities, the crop is not without its weaknesses. As with all plants grown as food crops, new diseases are a persistent threat. For example, Fusarium head blight caused $2.49 billion dollars in losses in just nine states in the United States from 1993 to 2001 (Nganje et al., 2004). Along with disease susceptibility, wheat

protein content is recalcitrant to increasing; agronomic limitations and an inverse relationship with yield make protein difficult to increase in parallel with yield (Shewry, 2009). Wheat is also relatively poor in terms of land-use efficiency. While wheat yielded about 3,500kg/ha worldwide in 2017, the other common grain crops, rice and maize, were much higher at 4,600kg/ha and 5,800kg/ha, respectively (FAOSTAT, 2019). While this may be due in part to being grown on poorer quality land, there is room for improvement for the crop that covers the most land on Earth.

Due to the sheer extent of wheat production in terms of economic and dietary impact on the world, agronomic and genetic improvements directed to this crop will yield rewards at a similarly large scale.

Limits of Genomic Diversity in Bread Wheat

The polyploid wheats are notable in their low levels of genetic diversity. The limited diversity extends even into the wild tetraploid wheats, wild emmer (*T. turgidum* ssp. *diccoides*) and its cultivated counterpart (*T. turgidum* ssp. *dicoccum*) (Haudry et al., 2007) and in hexaploid bread wheat (Akhunov et al., 2010; Chao et al., 1989; Devos and Gale, 1992). Bread wheat diversity is especially restricted; it is estimated bread wheat germplasm as a whole possesses less than 1/3 of the diversity of the diploid progenitor species (Galili et al., 2000) and is even more limited in cultivated varieties (Huang et al., 2002). The genetic variation present in bread wheat is unevenly distributed across the genome, unlike its more diverse tetraploid relatives (Akhunov et al., 2010). These restrictions in bread wheat diversity stem from two factors: its evolutionary origin and inherit restrictions on homoeologous recombination.

Effects of Evolutionary Origin on Bread Wheat Diversity

Bread wheat is an allohexaploid (2n = 6x = 42) possessing three genomes (AABBDD) contributed by three diploid progenitors (Figure 1.1). Between 0.5 and 3 million years ago (Blake et al., 1999; Huang et al., 2002) the wild A-genome contributor, *T. urartu* (AA; 2n = 2x = 14) (Chapman et al., 1976; Dvořák et al., 1993), hybridized with a relative of *Aegilops speltoides* that served as the B-genome progenitor (BB; 2n = 2x = 14) (Salse et al., 2008). While normally such a hybrid would be meiotically unstable due to the differences between the homoeologous chromosomes, a spontaneous whole genome duplication led to the formation of the fertile and stable tetraploid wheat, wild emmer (AABB; 2n = 4x = 28) (Feuillet et al., 2008). Another hybridization occurred between 8,000 and 10,000 years ago, this time between the tetraploid cultivated emmer (AABB; 2n = 4x = 28) (Cox, 1997; Zohary et al., 1969) and the wild grass, *Ae. tauschii* (DD; 2x = 2n = 14) (Kihara, 1944; McFadden and Sears, 1946). Spontaneous whole genome duplications of a few of these sterile, interspecific triploids led to the generation of the primordial hexaploid bread wheat population (AABBDD; 2n = 6x = 42) (McFadden and Sears, 1946).

Severe genetic bottlenecks often follow speciation and polyploidization. This reduced genetic variation can be ameliorated in two major ways: transfer of diversity from the parent species and via natural mutagenesis. Wild emmer benefited from these two processes. Vardi and Zohary (1967) found strong evidence that gene flow from the diploid progenitors into tetraploid wheat was possible. Crossing tetraploid wheat (AABB) and an A-genome progenitor relative (AA) produced a triploid (AAB; 2n = 21) vigorous enough to survive in the field and fertile enough to backcross with the tetraploid. Such triploids served as genetic bridges for movement of genetic diversity from the diploid progenitors to wild emmer population (The and Baker,

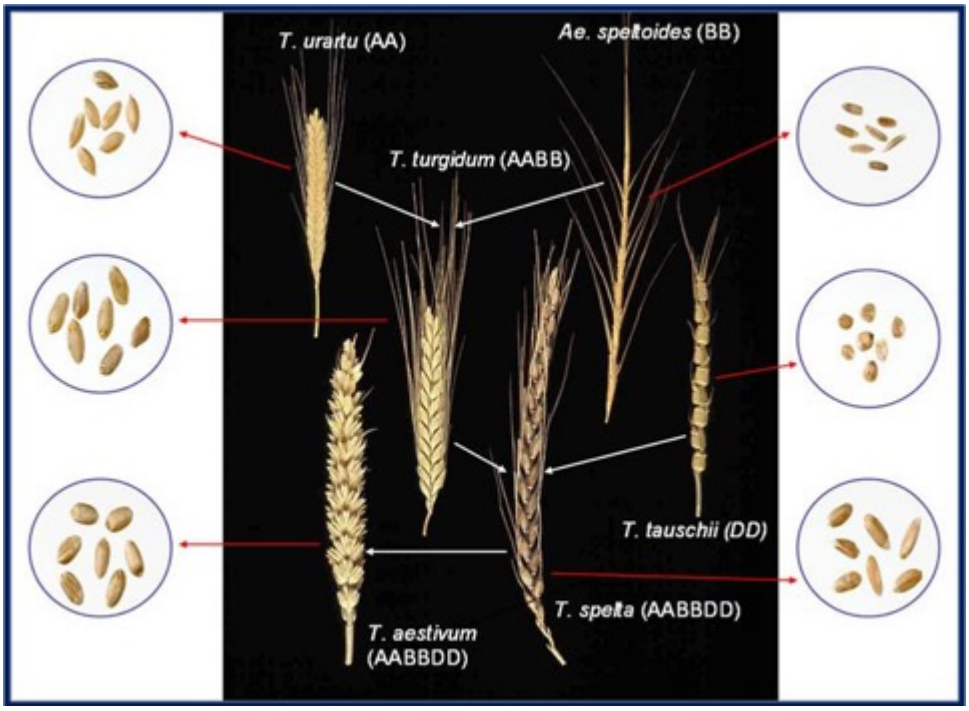

Figure 1.1 – *The evolution of hexaploid bread wheat*.
Bread wheat is an allohexaploid grass whose origin lies in two hybridization events. Initially, the A-genome progenitor, *Triticum uratu* (AA, 2n=2x=14), hybridized with a relative of *Ae. speltoides* (BB, 2n=2x=14), creating a diploid hybrid that, after whole genome duplication, producing the ancestor of *T. turgidum* (AABB, 2n=4x=28). Roughly 8,000 years ago, *T. turgidum* bred with the *Ae. tauschii* (DD, 2n=2x=14) (Kihara, 1944). The resulting triploid (ABD, 2n=21) underwent spontaneous whole genome duplication, producing the hexaploid ancestor of the modern bread wheat (AABBDD, 2n=6x=42) (McFadden and Sears, 1946). Figure from Hussain (2016).

1975; Vardi and Zohary, 1967). While natural mutagenesis is a slow way to increase diversity, wild emmer gradually accumulated mutations across the genome (Cox, 1997). Through both gene flow and natural mutagenesis, the genetic diversity of wild emmer has greatly improved since its inception.

While both gene flow and mutagenesis have contributed towards the diversity of bread wheat, they have not had as great of an effect as in wild emmer. The main reasons for this contrast are due to differences the age and in ploidy. While wild emmer has had the chance to accumulate mutations over its lengthy existence, bread wheat only speciated a short time in the past. As such, bread wheat has not experienced enough natural mutagenesis to significantly impact total genetic diversity (Akhunov et al., 2010). In addition, bread wheat has great difficulty directly accessing the vast reservoirs of genetic diversity found in the diploid progenitors. What allows for the efficient transfer of genetic information between species is a robust, sufficiently fertile hybrid. While the wild emmer could produce such a bridge with its diploid progenitors, hexaploid wheat could not. Crossing bread wheat and *T. urartu* results in a hybrid that is likely to abort, fail to germinate, die prematurely, fail to produce reproductive structures, and have difficulty in setting seed (The and Baker, 1975). Hybrids of bread wheat and the D-genome progenitor, *Ae. tauschii*, are even more difficult to produce; to date, such hybrids (ABDD; 2n=28) have only survived through human intervention (Gill and Raupp, 1987). Only hybrids between hexaploid wheat and tetraploid wheat are vigorous and fertile enough to allow for regular exchange of genomic variation (Martin et al., 2011; Padmanaban et al., 2017). This combination of only existing for a short period of time and only exchanging genetic information with a diversity-restricted species has contributed to the low genetic variation of bread wheat.

Limited Integration of Relative Diversity Due to the Ph1 Locus

Unique to the polyploid wheats, *Ph1* controls which chromosomes can pair with one another. In wheat, almost no recombination occurs between the A, B, and D genomes; only when *Ph1* is knocked out can inter-genomic crossing-over occur (Riley and Chapman, 1958), preventing transfer of variation between the genomes. The activity of *Ph1* is absolutely critical to the wheat species' survival since its loss results in a line becoming genetically unstable after only a few generations (Roberts et al., 1999). The effect of *Ph1* on recombination is so potent it will even reduce the chance of recombination within the genomes if the homologous chromosomes are heterozygous (Dvorák and McGuire, 1981). It also will inhibit crossing over if one of the chromosomes is from a wild ancestor (Dvorak et al., 1998).

A major consequence of the *Ph1* locus has been the prevention of transfer of diversity from the A and B genomes to the D genome. This has been a contributing factor to the D genome suffering from the even lower diversity than the other two genomes (Akhunov et al., 2010; Poland et al., 2012; S. Wang et al., 2014).

The D-genome of Bread Wheat

The Significance of the D-genome

While cultivated tetraploid wheat (durum wheat; *T. durum*) and hexaploid wheat (bread wheat; *T. aestivum*) are closely related and possess many similar qualities, 95% of wheat grown is hexaploid (Dubcovsky and Dvorak, 2007). Bread wheat is favored over its tetraploid counterpart because the D-genome supplied by *Ae. tauschii* imparted favorable qualities to the hexaploid species. One of these qualities is broad adaptability to a wide range of environments and conditions. As reviewed in Feuillet et al. (2008), emmer wheat is adapted to the Middle East and Northern Africa while native range of *Ae. tauschii* is across large portions of central Asia.

These differences resulted in bread wheat being able to grow in a greater number of environments compared to its diploid and tetraploid relatives. The D-genome has also supplied greater resistance to a wide variety of abiotic and biotic stressors. This includes greater salt tolerance, aluminum tolerance, resistance to a number of pests and diseases, frost tolerance, and adaptability to a wider range of vernalization and photoperiod demands (Dubcovsky and Dvorak, 2007). The superior flexibility of bread wheat makes it easier for growers around the world to cultivate it compared to tetraploid wheat.

In addition to making bread wheat easier to cultivate for growers, the incorporation of the D-genome altered wheat grains in ways desirable for consumers. Bread wheat grains can be milled into a flour ideal for the production of leavened bread (bread that is raised using leavening agents, such as yeast), cakes, cookies, crackers, and noodles. Removal of the D-genome has been shown to remove the capacity of bread wheat flour to produce high quality dough (Kerber and Tipples, 1969). This is because the D-genome provides two important qualities that allow for production of these culinary products: superior gluten chemistry and variability in hardness.

Members of the taxonomic tribe, Triticeae (e.g. bread wheat, tetraploid wheats, barley, rye, and oats) produce complex mixture of storage proteins known as gluten. While the plant embryo naturally uses gluten as a source of amino acids and energy, Triticeae glutens possess unique chemical properties that are utilized in bread making. The gluten proteins found in barley and durum, however, are not ideal for bread making (as reviewed in Garg et al, 2009). The highest quality bread making glutens are derived from bread wheat and confer four critical properties to dough: easily deformed, minimally elastic, can be stretched without breaking, and nominally sticky (Cauvain, 2017). These qualities allow for dough to hold onto the carbon dioxide produced by leavening agents and not collapse after baking, producing large, soft loaves.

Bread wheat gluten possesses these characteristics primarily because of one of its components: the high molecular weight glutenin subunit (HMW-GS) proteins. HMW-GS are produced by tightly linked gene pairs at the *Glu-1* loci (*Glu-A1, Glu-B1,* and *Glu-D1*) found on chromosomes 1A, 1B, and 1D (Garg et al., 2009). While the alleles found at any of these loci can have an effect on dough properties (Lawrence et al., 1988), it is the 5 and 10 HMW-GS proteins produced by *Glu-D1* in the D-genome that have the greatest positive impact on gluten quality (Dong et al., 1991; Garg et al., 2009; Kolster et al., 1991). The presence of *Glu-D1* has been shown to improve bread making traits such as mixing time and mixing tolerance (Dong et al., 1991), protein quality (Luo et al., 2001), loaf volume (Kolster et al., 1991), and dough strength (Garg et al., 2009). The influence of *Glu-D1* on gluten quality is so potent, efforts have been made to move favorable alleles into durum wheat and triticale (× *Triticosecale* Wittmack; a bread wheat/rye hybrid) to improve dough qualities (Garg et al., 2009).

Variability in hardness is another valuable trait provide by the D-genome. Pasha et al. (2010) thoroughly reviews the hardness quality. The hardness of a kernel is determined by its quantity of friabilin, a protein that influences how strongly starch granules adhere to the endosperm protein matrix. Softer bread wheats produce kernels with high friabilin levels and thus flour with many starch granules intact, ideal for cakes and crackers. Lower friabilin levels lead to harder wheat grains which are ground into a courser flour due to greater quantities of broken starch granules, suitable for leavened bread making. The level of friabilin produced by a given variety is entirely determined by the genes found in hardness locus (*Ha*) in D-genome. Since tetraploid wheats do not possess the *Ha* locus, kernels lack friabilin and the flour is course and possesses a large number of broken starch granules (i.e. the flour is very hard). This very hard flour is not suitable for leavened bread production due to the excessively damaged starch

granules absorbing too much water (Pauly et al., 2013), limiting the use of durum wheat to unleavened products, such as pasta (Pasha et al., 2010). The effect of the *Ha* locus is so powerful that durum can be made into a soft wheat if *Ha* is translocated into the durum genome (Morris et al., 2011). In summary, the D-genome of bread wheat heightens its ability to tolerate biotic and abiotic stressors and makes it capable of producing the food goods that are in high demand by humans.

The Limitations of the D-genome

While the bread wheat genome has had its genetic diversity restricted, the D-genome has been affected disproportionately more than the A- and B-genomes. This is due to it being especially hard for the D-genome to access genomic variation from other species. First, it is extremely difficult for bread wheat to tap into the vast reservoirs of diversity found in the *Ae. tauschii* species due to bread wheat/*Ae. tauschii* direct hybrids being aborted shortly after pollination (Gill and Raupp, 1987). This differs from the A- and B-genomes' situation where genomic variation can be readily acquired by crossing with tetraploid wheats since such hybrids are vigorous and fertile (Martin et al., 2011; Padmanaban et al., 2017). Second, since the *Ph1* locus prevents crossing over between homoeologous chromosomes (Riley and Chapman, 1958), the D-genome cannot indirectly access tetraploid wheat diversity by recombining with the A- and B-genomes. Because of these two factors, the D-genome is especially isolated from already existing pools of variation.

The isolation of the D-genome has resulted in it possessing especially low genetic diversity. Comparisons between the bread wheat D-genome and its progenitor species, *Ae. tauschii,* have shown a loss in variation. As reviewed by Cox (1997), comparisons in protein profiles of bread wheat and *Ae. tauschii* were the first investigations indicating a relatively low

level of variation in the wheat D-genome. This hypothesis of lost diversity in the wheat D-genome was reinforced by studies using DNA markers, such as RFLP markers (Dvorak et al., 1998; Galili et al., 2000), microsatellite markers (Lelley et al., 2002), single gene sequencing (Caldwell et al., 2004), and large-scale SNP array screenings (J. Wang et al., 2013). These studies found in the regions of the wheat D-genome they investigated possessed anywhere between 3.3% and 61.5% of the variation found in *Ae. tauschii*. Despite the range of estimated change in variation, these comparisons showed the wheat D-genome has limited genetic diversity relative to the wild progenitor *Ae. tauschii.*

D-genome diversity in modern hexaploid wheat is limited compared to the A- and B-genomes. Large-scale SNP screening studies have demonstrated the A- and B-genome are more variable than the D-genome. The screening of a 147-member bread wheat mapping population revealed the D-genome had the least variation, possessing 35-70% less variation than the B-genome and 15-50% less than the A-genome (Poland et al., 2012). A screening of 1,791 bread wheat genes performed by Akhunov et al. (2010) found two major indicators of restricted diversity in the D-genome. 93% of the polymorphic D-genome genes had only two haplotypes and the D-genome genetic variation was more unevenly distributed across its chromosomes compared to the A- and B-genomes. Finally, a massive 2,994 bread wheat panel screening for over 45,000 SNPs found far fewer markers on the D-genome; the D-genome only had 29.5% and 42% of the variation found on the A- and B-genomes, respectively (Y. Wang et al., 2014). Whether compared to the diversity of its progenitor species or to the A- and B-genomes, it is clear the D-genome of bread wheat is especially lacking in genetic diversity.

Improving the Agronomic Qualities of the Bread Wheat D-genome

When the germplasm of a crop lacks the genetic diversity necessary to enhance a desirable trait or restrict an undesirable one, breeders will often turn to wild relatives as alternative breeding material. This is done because wild species tend to have a greater genetic diversity than their domesticated counterparts (Buckler IV et al., 2001). While it is possible to transfer genes from distantly related species, breeders favor species from the primary gene pool for which chromosomes can easily pair and recombine during meiosis (Feuillet et al., 2008). For hexaploid wheat, these relatives are wild einkorn (*T. urartu*), wild and cultivated emmer wheat (*T. turgidum*), and *Ae. tauschii* (Feuillet et al., 2008; Raupp et al., 1993). By crossing with these reservoirs of genetic diversity, wheat breeders increased the genetic variation of the wheat germplasm which had fallen due to the intense selection pressures of the early Green Revolution (Reif et al., 2005; Ren et al., 2013). Since the D-genome had lacked heterogeneity to a greater degree than the other genomes, the influx of *Ae. tauschii* DNA greatly improved many agronomic characteristics of bread wheat.

Despite being a wild weed, *Ae. tauschii* possesses many valuable agronomic characteristics. Certain types of traits can be observed directly in *Ae. tauschii,* allowing for identification of accessions with desirable phenotypes prior to the laborious process of introgression. Screening *Ae. tauschii* before making crosses is often performed when trying to improve disease resistance. Pre-breeders have identified *Ae. tauschii* accessions with resistance to a variety of diseases, including: *Septoria tritici* blotch (McKendry and Henke, 1994), stem rust (Assefa and Fehrmann, 2004; Cox et al., 1992), stripe rust (D. Liu et al., 2010; Rouse et al., 2011), Fusarium head blight (Brisco et al., 2017), tan spot (Cox et al., 1992), powdery mildew, leaf rust (Cox et al., 1994; Gill et al., 1986), Greenbug, and Hessian fly (Gill et al., 1986).

To move such desirable traits from *Ae. tauschii* into hexaploid wheat, a breeder will utilize one of two strategies (Figure 1.2). One method recreates the allopolyploidization event that gave rise to bread wheat (McFadden and Sears, 1946). By crossing a tetraploid wheat (often durum; AABB; 2n=4x=28) with *Ae. tauschii* (DD; 2n=2x=14) and applying colchicine to the resultant triploid hybrid (ABD; 2x=21), a fully fertile amphiploid (AABBDD; 2n=6x=42) is produced (Figure 2A). This synthetic hexaploid wheat (SHW) is then backcrossed into an elite bread wheat cultivar, producing a synthetic backcross line (SBL) that is then used as a parent in a breeding program (Cox et al., 2017). The second method described by Gill and Raupp (1987) is called direct hybridization and involves directly crossing bread wheat and *Ae. tauschii*. While still an embryo, the F1 hybrid (ABDD; 2n=28) is rescued since it will eventually be aborted due to the ploidy differences between the parents. The hybrid is then backcrossed twice and selfed to fixation. The resultant family of RILs have D-genomes that are approximately 12.5% *Ae. tauschii* genetic material but have the same A- and B-genome content as the recurrent wheat parent (Figure 2B).

When moving genetic diversity from *Ae. tauschii* to bread wheat, the breeder must consider the differences between the SHW and direct hybridization methods. As is reviewed in (Cox et al., 2017), both methods produce introgression lines with qualities that may be desirable in certain breeding programs but undesirable in others. The SHW method yields introgression lines with exotic DNA in all three genomes and often possess a spring growth habit. Such plants are especially useful in spring wheat breeding programs aiming to introduce as much non-bread wheat genetics into their germplasm as possible. This has been the case for the International Maize and Wheat Improvement Center (CIMMYT), which primarily focuses on the enhancement of spring wheat germplasm (Thomas Payne, personal communication) and has

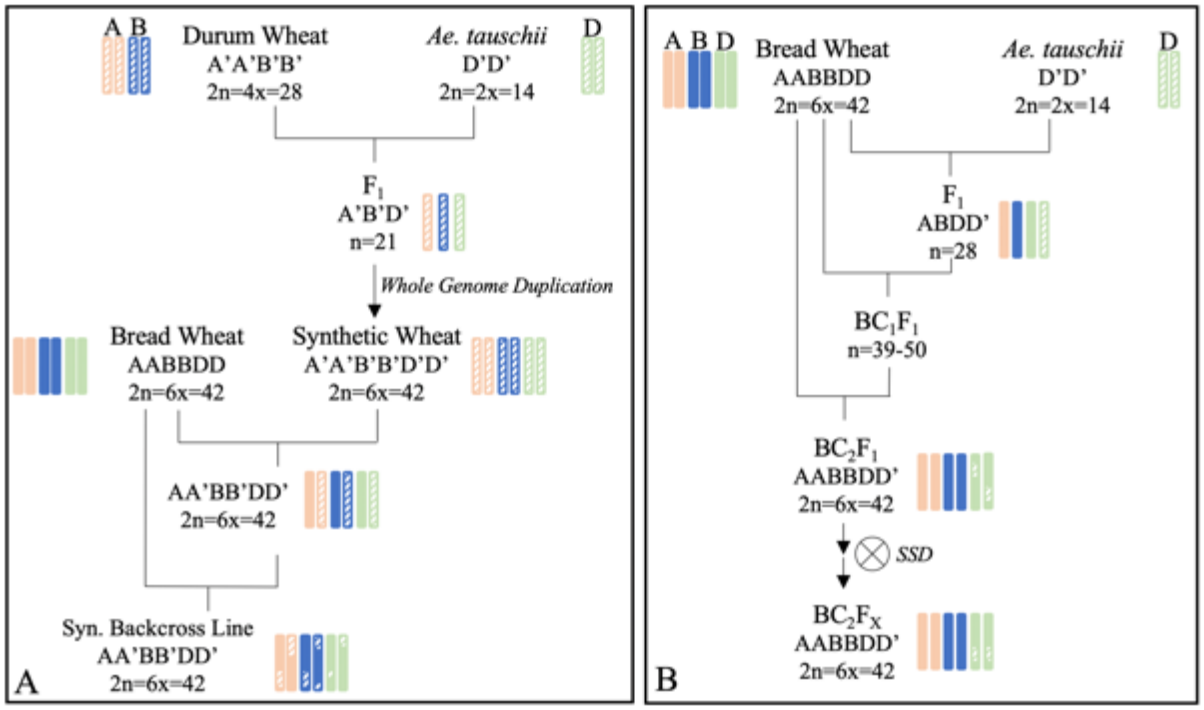

Figure 1.2: *A visual summary of the two most common methods of introgressing Ae. tauschii genetic material into bread wheat*.
Figure 1.2A describes the SHW approach. Here, a triploid hybrid between tetraploid wheat (usually durum) and *Ae. tauschii* has its whole genome duplicated to produce a synthetic hexaploid wheat (SHW). This SHW is then backcrossed twice to a natural hexaploid cultivar to produce a synthetic backcross line (SBL), which is then often used as breeding material. The SBLs possess genetic variation in all three genomes.
Figure 1.2B depicts the direct hybridization method. After embryo rescue, the tetraploid hybrid of bread wheat and *Ae. tauschii* is backcrossed twice to the same bread wheat parent and selfed to fixation. The resultant recombinant inbred lines possess introgressed DNA in D-genome but not in the A- and B-genomes. Information in figures based on Cox et al. (2017) and Gill and Raupp 1987. Figure design inspired by Wiersma 2017.

produced over 1,300 synthetic hexaploids. On the other hand, if a breeder works with winter wheat or seeks to only modify the D-genome, SHW introgression lines may be difficult to work with. Therefore, winter wheat focused groups such as Kansas State University primarily rely upon direct hybridization.

While the two hybridization methods have advantages and disadvantages, both have been used successfully to move beneficial genetic material from *Ae. tauschii* into bread wheat. Cox et al. (2017) and Börner et al. (2015) both provide a list of traits obtained from *Ae. tauschii* introgressions. This includes improved resistance to a wide variety of pests and pathogens, including viruses (e.g. soilborne mosaic virus and wheat spindle streak mosaic virus), fungi (e.g. stem rust and powdery mildew), nematodes (e.g. cereal cyst nematode and root lesion nematode), and insects (e.g. Hessian fly and greenbug). Other relevant traits have been harvested from *Ae. tauschii*, too. *Ae. tauschii* has provided enhanced yield and yield component traits (e.g. kernel size and thousand kernel weight), increased resistance to abiotic stressors (e.g. tolerance to high levels of aluminum, salinity, and boron), and improved dough properties (e.g. new gliadin and glutenin subunits, and resistance to pre-harvest sprouting). Although *Ae. tauschii* is not used directly in agriculture, it has allowed for the improvement of the bread wheat germplasm.

Grain Yield in Wheat

Continuing to Improve Yield in Wheat

A shared goal between nearly all crop breeding programs is to produce higher yielding varieties. While this has been an objective for wheat breeding programs since their inception, modern times and concerns have been raising its importance. One of the most cited reasons for calls to improve yield performance of wheat is the need to meet the demand of a growing world

population. In a 2009 report, the United Nations announced the predicted world population would reach an excess of 9 billion people by 2050 and projected an increase in grain demand by 70% (Food and Agriculture Organization of the United Nations (FAO), 2009). While wheat yields have been improving, the rate of increase has been falling short of the desired projections and thus posing a threat to food security of the future (Reynolds et al., 2012). However, there are other reasons to improve wheat yields beyond trying to meet the needs of a growing human population.

By improving how much grain a single wheat plant can produce less land can be used to produce a given amount of wheat. Wheat easily has the lowest land-use efficiency of the major grain crops; worldwide, wheat yields around 3,500kg/ha while maize and rice yield approximately 5,800kg/ha and 4,600kg/ha, respectively (FAOSTAT, 2019). This disparity is even more extreme in the United States, where yields for wheat, rice, and maize are about 3,200kg/ha, 8,600kg/ha, and 11,000kg/ha (USDA, 2019a). After taking into account that the world devotes more land to wheat than to any other crop (~218 million ha; FAOSTAT, 2019), it shows just how much land area could be devoted to other purposes if growers could get more out of their wheat. In fact, improvements in crop yield are one of the most fundamental ways to ensure as much land as possible remains in its natural state and undisturbed by agriculture (Lambin and Meyfroidt, 2011).

In addition to encouraging the preservation of land in its natural state, certain forms of yield improvement can lead to a reduced carbon footprint per gram of grain product. One way this may be achieved is by increasing the nutrient use efficiency (NUE) of the crop. In combination with a sustainable fertilizer use program, improving the NUE of grain crops may decrease amount of fertilizer required per hectare and, by extension, its carbon footprint (Parry

and Hawkesford, 2010). In addition to protecting the environment, increasing NUE may also directly benefit wheat farmers directly since fertilizers represents one of the largest operational costs for wheat farmers (Tester and Langridge, 2010; USDA, 2019b).

To feed a growing world, to reduce agriculture's effect on the environment, and improve the financial stability of growers, improving the average yield of wheat is an important endeavor. Due to the sheer scale of wheat production, even small improvements in the amount of grain that can be harvested per hectare will have positive effects of a large magnitude.

Past Trends in Yield Improvement in the United States

The history of American growers' efforts to increase and protect the yield potential of their wheat fields shows how yield improvement efforts have evolved. As reviewed by Ball (1930) prior to World War II, wheat yield improvement in the United States was a slow process that gradually built up momentum leading up to and through the advent of the 20th century. Prior to independence from Great Britain, wheat was not as crucial of a crop as it is today. As the grain became increasingly relevant in the 1700's, however, efforts were made to improve yields. It was the 1800's, however, that laid the foundation for wheat to become the most widely grown crop on the planet.

The 19th century and early 20th century saw substantial changes in wheat production (Ball, 1930). Improved machinery in the form of better reapers, planters, tilling equipment, and packing tools allowed for individuals to collect more grain than ever before. Improved milling technology via the invention of roller and purifier allowed for the widespread use of higher yielding varieties. Increased government involvement in crop research through the Morrill Act of 1862 and the Hatch Act of 1887 led to the establishment of land-grant colleges and experimental research stations that would work towards improving agricultural practices.

Time period also saw substantial changes in the varieties utilized by growers (Ball, 1930). This included arrival of a number of foreign varieties such as Mediterranean (arrived in 1819), Pacific Bluestem (arrived in 1850), Red Fife (arrived in 1860), and Turkey (1870's), which would form the basis of the soft red winter, white winter, hard red spring, and hard red winter markets, respectively. The end of the 19th century was marked by breeders actively seeking out foreign germplasm better adapted to the American West. The use of pureline selection and early crossing breeding schemes produced some of the first lines bred for the American climates that would dominate the landscape, including Marquis (hard red spring), Triumph (hard red winter), and Fulcaster (soft red winter).

Despite these efforts, there was very little improvement in yields made between the end of the American Civil War to 1940; yield increased from about 800kg/ha in 1866 and plateauing to roughly just 1,000kg/ha by 1940 (Figure 1.3). This stagnation in progress was also seen in the slow rate of change in the varieties that were utilized and the simplicity of their pedigrees (Dalrymple, 1988; Dobrotvorskiy et al., n.d.). Up to 1929, the top five most widely used varieties were either landraces, pure line selections, or simple hybrids between landraces. Turkey and Marquis (a landrace and simple hybrid variety, respectively), varieties that had been available for more than 50 years, remained as the top two most widely planted varieties through 1939. It would not be until after World War II that Turkey would finally fall out of the top five.

While the first half of the 20th century was marked by stagnation in both the utilized wheat lines and yields, post-World War II was a period of rapid change in the varieties and massive increases in in wheat production (Figure 1.3) that marked the beginning of the Green Revolution. One major source of the increases in yield improvement was the increased availability of chemical fertilizers and pesticides. Prior to 1940, very few farms utilized either of

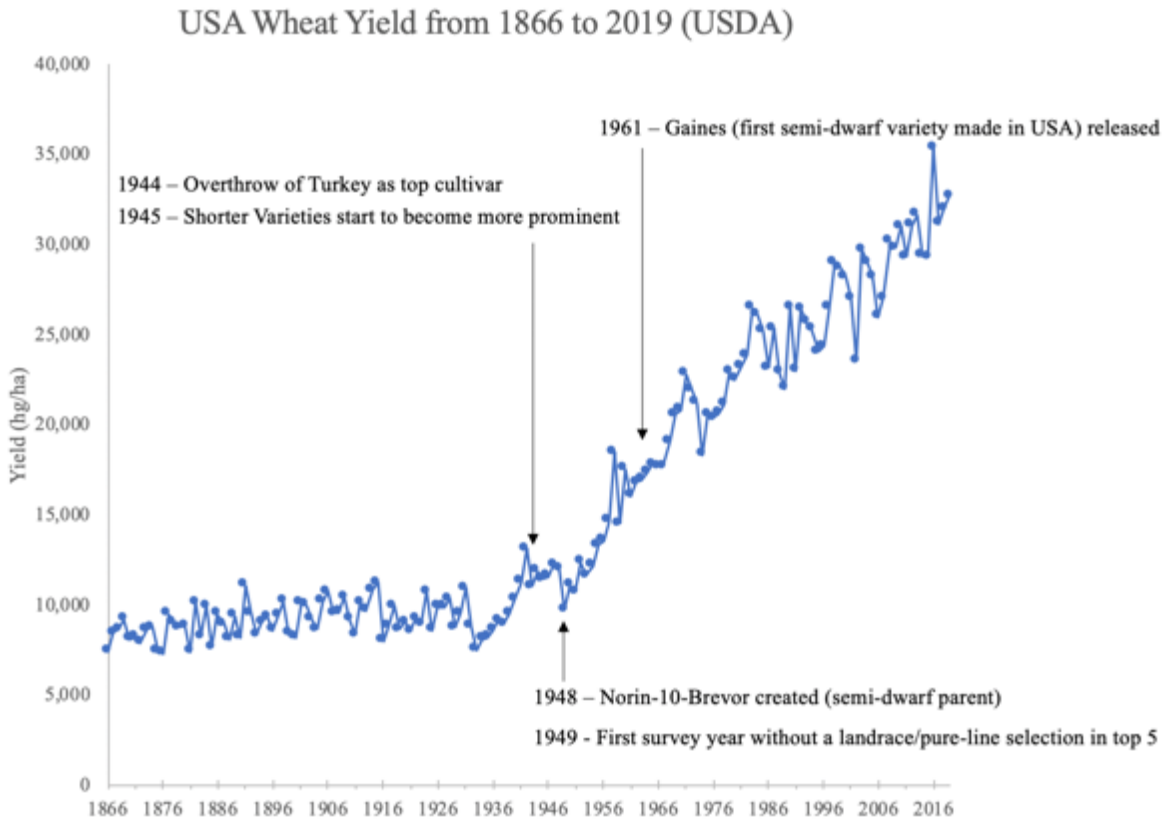

Figure 1.3 – *Bread wheat yield in the United States from the end of the Civil War to 2017 and notable breeding milestones*

these classes of chemicals. From 1940 to 1960, fertilizer use rose from negligible amounts to 6.8 Tg/year. Total fertilizer use rose again to about 21.8 Tg/year by 1980 and then leveled off to around 20 Tg/year (USDA, 2018). The 1940's also saw the utilization of the first mass-produced, synthetic pesticides such as the herbicide, 2,4-D, and the insecticide, DDT. Starting in the 1960's, the side effects of using pesticides were reduced through the development of compounds that were less toxic (e.g. glyphosate) and more target-specific (e.g. insect growth regulators), and were utilized in more efficient ways through integrated pest management programs (Delaplane, 1996). The utilization of agroindustrial fertilizers (Carvalho, 2006) and pesticides (Delaplane, 1996; Warren, 1998) and efficient application methods led to increased yields and played major roles in greatly expanding grain production during the Green Revolution (Carvalho, 2006; Delaplane, 1996; Warren, 1998).

Another major driver of wheat's yield improvement worldwide during the Green Revolution were the changes made to the varieties themselves. Breeding programs became more efficient and improved yields by focusing on three core breeding goals: 1) adapting their varieties to the local environment's photoperiod, 2) incorporating disease resistance alleles from diverse sets of germplasm, and 3) integrating dwarfing alleles into breeding lines (Graybosch and Peterson, 2010). The replacement of the functional *Ppd-D1b* allele on the 2D chromosome with the nonfunctional *Ppd-D1a* allele conferred the trait of photoperiod insensitivity. This trait leads to earlier flowering (Eagles et al., 2010) and greater number of grains per tiller (Worland et al., 1998), conferring a greater yield potential, especially in long-day and drought prone environments (Sun et al., 2014). Similar to pesticides, the incorporation of disease resistance alleles increases yield stability by inhibiting the effects diseases and pests have on yield, reducing farmer economic losses (Borlaug, 1983; Donmez et al., 2001). Introgression of disease

resistance also has the additional financial advantage of reducing the need to purchase disease controlling pesticides (Lamichhane et al., 2016).

While the incorporation of photoperiod insensitivity and disease resistance alleles in elite lines have improved average wheat yields, it was the addition of the semi-dwarfing trait that was most often associated with the large increases in yield over the past 50 years. Originating from the Japanese variety, Norin-10, the *reduced height* (*Rht-1*) alleles confer reduced sensitivity to gibberellic acid, leading to reduced plant height and more grain filled heads (Borlaug, 1983; Hedden, 2003). This improvement in the harvest index translates to a greater proportion of the assimilates produced during photosynthesis leads to an improved harvest index (Nadolska-Orczyk et al., 2017). This was especially effective in environments with high fertilizer inputs since the shorter height made the plants less prone to lodging compared to their taller counterparts. When used in combination with commercial fertilizers, the addition of dwarfing alleles led to large scale increases in yield across the world (Borlaug, 1983; Evenson and Gollin, 2003).

The incorporation these three traits into elite varieties has had a massive impact on wheat production. These genetic improvements have been estimated to be responsible for 50% of the yield improvements seen in developing countries since 1981 (Evenson and Gollin, 2003)) and the main factors in the roughly 1% genetic gain per year seen in the United States since 1959 (Graybosch and Peterson, 2010). Thanks to these past wheat breeding programs and geneticists, the World's wheat production was forever improved.

Methods of Trait Improvement with Potential but also With Uncertain Futures

With fears of a coming yield plateau in wheat (Fehr and Schmidt, 1984; Graybosch and Peterson, 2010), wheat scientists have been seeking less conventional means of increasing grain

yield. Two prominent examples include transgenic measures and hybrid breeding. However, each of these methods possess key weaknesses that will inhibit them from being utilized by the wheat industry.

There exists great potential in improving the yield potential and yield stability of wheat via transformation. In fact, there are numerous transgenic lines of wheat that have been produced with traits that would likely contribute to significant increases in average yields. Glyphosate resistant wheat lines were produced by the Monsanto company at the turn of the century were shown to be full of promise; at a large number of locations, Round-Up Ready wheat displayed the desired resistance to the broad-spectrum, low toxicity herbicide without a loss in yield potential (Zhou et al., 2003). It was estimated this trait would lead to increased returns around $15-$20 per acre (Wilson et al., 2003). Along with increasing yield stability, improvements in yield potential has also been shown to be possible via plant transformation. *Sh2r6hs* transgenic wheat, which possess a modified version of maize's *Shrunken2* gene, has been shown to possess increased seed weight and increased total biomass (Smidansky et al., 2002). In yield trials, *Sh2r6hs* mutants were shown to possess greater yields under favorable conditions (Meyer et al., 2007). Theoretical modifications of the wheat photosynthesis system using transgenesis or gene edit is another potential strategy to improve yields. Methods such as increasing the amount of rubisco in the chloroplast, improving wheat rubisco's efficiency, or replacing wheat rubisco genes with that of a more efficient species are potential paths to increasing total biomass in wheat (Reynolds et al., 2011).

Despite the large potential in using genetic engineering to improve average wheat yields, it is unlikely such technologies will be harnessed in the near future in a commercial capacity in the United States. A major problem is the technology is a non-starter with the many of the major

importers of American wheat. Wilson et al. (2003) explained how Japan, the Philippines, and South Korea (the first, fourth, and fifth top importers of American wheat in the past decade; FAS, 2019) would all likely reject shipments of genetically modified wheat. This would result in a decreased value of the transgenic lines' grain, defeating the commercial value of the transgenic trait. Monsanto recognized this at the turn of century and issued a statement indicating that they would not sell Round Up Ready wheat unless the United States, Canada, and Japan all de-regulated the trait. Combined with a growing domestic anti-GMO movement (Lucht, 2015), there is little chance this technology will be utilized in the United States in the next century.

Another proposed non-traditional method of improving wheat yields is to shift wheat breeding towards hybrid breeding. Like many other crops, wheat displays heterosis. While the flower design of wheat greatly inhibits cross pollination naturally (less than 1% of natural crosses occur due to cross pollination; Singh et al., 2015), large scale methods of hybrid production exist. This includes gametocide, genic-male sterility, and cytoplasmic male sterility systems, each possessing their own positive and negative qualities (Singh et al., 2015). In certain backgrounds, the yield of hybrids can exceed their commercial parent by 10-15% (Gupta et al., 2019).

As with a transgenic approach, there are critical factors preventing hybrid wheat technologies from overthrowing the current system of wheat breeding. (Gupta et al., 2019) reviews some of the current weaknesses with hybrid wheat breeding. First, the cost of hybrid seed development is still too expensive, being 2-3 times more expensive to produce compared to inbred wheat. Next, the hybrid vigor needs to be increased along with the understanding of wheat heterotic groups to make hybrid seed production commercially viable. Perhaps what is the most crippling part of hybrid wheat production, however, is how slow the creation and improvement

of the male-sterile parent lines can be; if wheat breeding efforts continue to return similar yield gains in their inbred programs every year, elite inbred lines will likely outpace hybrid lines in yield improvement (Dawson, 2017). The limited commercial viability of hybrid wheat is evidenced by the actions of leaders in the industry. For example, in 2018 Syngenta pulled its hybrid wheat breeding program from North America, citing uncertain profits in Canada and the United States (Pratt, 2018; Spiegel, 2018)

Reliable Modern Methods of Yield Improvement in Wheat

While transgenic and hybrid breeding strategies are unlikely to make the impacts hoped for, there are a number of modern molecular strategies that have already been successful in improving wheat cultivars. This includes genomic selection (GS) and genome-wide association studies (GWAS) to identify genetic factors behind yield and other valuable traits. The following molecular assisted breeding techniques have a distinct advantage as they are free of the political controversy associated with transgenesis (Tester and Langridge, 2010) and have track records of successfully improving plant traits.

Genomic Selection

Genomic selection is one of the most promising methods being utilized to improve the genetics of crops. As reviewed by Bassi et al. (2015), GS is a statistics-based approach that harnesses both the decreased costs of computers and genetic markers to streamline the breeding process. Part of a breeding population's genomic and phenotypic data (the training set) is provided to the statistical model that is used to predict performance of untested genotypes. The model's prediction accuracy is tested by having it estimate the phenotypes of the other portion of the population (test population). Once a properly calibrated model is generated (i.e. is sufficiently accurate in predicting the phenotypes of the training population), it can be utilized to

estimate the phenotypes of plants using only the genomic marker data and assign a genomic-estimated breeding value (GEBV). With an accurate model, genomic selection can reduce the amount of phenotyping performed and shorten the breeding cycle, both of which have the potential to save time and money. By gradually incorporating more and more germplasm into the training and test populations, the more accurate a model can be at predicting the phenotype of a given line.

Studies and actual applications of genomic selection indicate it will become a major asset to agricultural genetics. Spurred on by its immense success in the dairy industry, applications of GS have been studied in a variety of crops, including in wheat (Voss-Fels et al., 2019). There are a plethora of proof-of-concept studies showing how GS is poised to become a tool for increasing the rate of genetic gain in wheat breeding programs of the future. This includes improving stem rust resistance (Rutkoski et al., 2015), increasing yield (Juliana et al., 2019; Rapp et al., 2018; Rutkoski et al., 2016), enhancing protein content and protein yield (Michel et al., 2016; Rapp et al., 2018), end-use quality traits such as gluten quality (Battenfield et al., 2016), and rapid introgression of valuable alleles from synthetic hexaploids (Dunckel et al., 2017). However, unlike high value crops like maize, not as much as much funds have been directed to GS wheat research and the technology has yet to become a staple of public breeding programs (Bassi et al., 2015; Voss-Fels et al., 2019). Before GS becomes a common breeding tool as it is in the dairy and maize industries, some additional work needs to be done. This includes improving GS models to handle genotype x environment interactions and non-additive genetic effects (Voss-Fels et al., 2019) assessing the long-term impacts of GS on germplasm genetic (Rutkoski et al., 2015; Sweeney et al., 2019), and the execution of studies that quantify the realized gains of longer term use of GS models in selection (Sweeney et al., 2019).

GWAS and QTL Mapping

A common pair of reliable genetic tools utilized by human, animal, and plant scientists alike are GWAS and QTL mapping. As reviewed by Korte and Ashley (2013), QTL mapping was one of the first statistical based approaches for finding associations between specific genetic markers and variations in a given phenotype. Typically involving the use of F_2 or RIL populations generated from biparental crosses with parents of known pedigree, QTL mapping has and continues to provide explanations for fundamental aspects of plant physiology (e.g. the flowering time pathway in *Arabidopsis*) and the discovery of alleles conferring agronomically favorable traits. This technique can detect of minor effects alleles and rare alleles, so long as one of the parent's possesses said allele. QTL mapping, however, has a few weaknesses. A biparental population represents only a tiny portion of the genetic diversity of a species and therefore only a relatively small portion of the loci affecting the trait will be detectable. Also, such populations have only a limited amount of recombination, limiting the resolution of the mapping. GWAS, on the other hand, is an association mapping technique that searches for correlation between genomic markers and phenotypic variation while accounting for population structure and/or kinship. Unlike in standard QTL mapping, GWAS utilizes more complex populations. GWAS panels consist of numerous existing lines from diverse backgrounds or populations created using complex breeding designs. Due to the greater degree of diversity and number of past recombination events, GWAS can provide far finer-mapping resolution than QTL mapping and is more likely to identify major effect alleles due to the greater diversity of the population being analyzed. Due to the size of the population and the nature of the analyses, however, GWAS has difficulty in detecting minor effect QTL and rare alleles. When coupled together, GWAS and QTL mapping can ameliorate their respective flaws. This is often done by initially utilizing

GWAS to perform a broad scan of a diverse set of germplasm to identify potential QTL affecting a given phenotype. QTL mapping is then used as a follow up technique; QTL mapping can be used to fine map a major QTL detected in the GWAS, to determine if a questionable QTL found in the GWAS exists or not, or to clone the causal variant.

While genomic selection is a promising up-and-coming tool for the wheat breeder's toolbox, it has been GWAS and QTL mapping that have identified the bulk of QTL and their associated markers for a wide variety of traits in bread wheat. Due to the eternal arms-race between plants and pathogen, there is a constant demand for new resistance loci, and studies using GWAS and/or QTL mapping have provided. This includes identification of resistance QTL for *Fusarium* head blight (Tessmann and Van Sanford, 2018; Tessmann et al., 2019), *Septoria tritici* blotch (Muqaddasi et al., 2019), stripe rust (Dong et al., 2017), leaf rust (Li et al., 2016), and powdery mildew (Li et al., 2019). Mapping has also found QTL that provide markers for quality characteristics, such as loaf volume for wheats intended for bread production (Battenfield et al., 2018) and starch granule content for optimizing lines intended for noodle production (Li et al., 2017). Finally, GWAS and QTL mapping have been used to identify regions of the genome associated with variation in grain yield and its component traits, such as thousand kernel weight, fertile spikelets-per-spike, kernels per spike, and yield per plant. While many yield QTL are ephemeral, studies have identified QTL associated with variations in grain yield or component traits that are stable across environments and time (Gao et al., 2015; Sukumaran et al., 2014; Turuspekov et al., 2017). By identifying stable QTL and the markers linked to them, breeding programs have been able to use marker-assisted selection to efficiently create improved wheat cultivars.

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CHAPTER 2: DEVELOPMENT OF FINE-MAPPING TOOLS FOR A YIELD QUANTITATIVE TRAIT LOCUS ON THE 2D CHROMOSOME OF BREAD WHEAT (*TRITICUM* AESTIVUM)

Introduction

Bread Wheat Relevance and Weaknesses as a Crop

Bread wheat (*Triticum aestivum* L) is among the most important grain crop cultivated in human history. This importance is borne from a combination of the viscoelastic properties of its gluten storage proteins, a good disposition for mechanical harvesting, broad adaptability, and high caloric density. Due to these and other qualities, bread wheat is a critical part of the food industry, with nearly 20% of human calories being derived from wheat grain (FAOSTAT, 2019). To supply this demand, wheat is grown on more acreage worldwide than any other crop (218 million hectares in 2017; FAOSTAT 2019). As a consequence of its relevance, bread wheat is a multi-billion dollar market, with the worth of the United States' yearly production alone fluctuating between $8.1 billion and $17.4 billion over the past decade (USDA NASS, 2018).

While wheat has been lavished with attention by plant breeders, agronomists, entomologists, pathologists, and (more recently) molecular geneticists, wheat still suffers from a number of weaknesses. Among the most pressing of these flaws is its relatively low yield; while wheat produces 3,500kg/ha on average, the other two major grain crops, rice and maize, produce substantially more per unit area (4,600kg/ha and 5,800kg/ha, respectively) (FAOSTAT 2019). In other words, wheat has poorer land-use-efficiency. As the world continues to grow in population, increase construction of land area intensive green energy (e.g. solar farms and wind power), and give greater value to wilderness conservation, agricultural land may become expendable. In anticipation of increased demand for land and the need to feed an ever-growing population,

efforts need to be made in improving land-use efficiency in all crops. As it is grown on more land area than any other crop, improving the yield of wheat should be a high priority as even the smallest improvement will have far reaching consequences.

History of Improving Bread Wheat Productivity

One route to increasing the yield of wheat is via breeding. As an example, breeding efforts in the United States over the past few centuries have both amplified yield and yield stability. The effort to increase wheat yield via genetic manipulation began with the relatively simple practice of determining what existing variety were best suited for a given environment. These efforts were pioneered by Mark Carleton between the Civil War and the beginning of the 1900's, who brought varieties from Europe and Russia that flourished in field conditions also seen in the American Midwest and West (Ball, 1930). This practice led to the introduction of lines that dominated the pre-World War II United States wheat industry for decades, such as Tukey, Marquis, and Pacific Bluestem. These carefully selected lines possessed attributes such as disease resistance and general adaptability that made them more reliable in terms of yield compared to the various lines brought over by immigrants settling the area.

The next step in genetic manipulation of wheat germplasm was the identification and selection of novel lines. As described by Ball 1930, this began with pure-line selection, leading to the finding of prominent lines such as Blackhull, Kanred, and Lancaster. Foreign introductions and pure-line selection were later replaced with selective crossing, at first slowly before the 1930's and then rapidly during and after WWII. Selective breeding led to creation of landmark American varieties such as Triumph, Wichita, Fulcaster, and Pawnee (Dalrymple 1988).

The combination of selecting lines based on their environmental adaptation as well as the first stabs at breeding programs laid the foundation for modern breeding programs and the

explosive increases in yield that were to come. After WWII, wheat yields in the United States increased at a rate of approximately 28.4kg/ha per year (USDA, 2019a). This was due to simultaneous and continuous improvements in synthetic fertilizers, pesticides, harvesting technology, and crop genetics. Much of the major gains made by breeding programs were due to focusing on three goals: 1) adapting their varieties to the local environment's photoperiod, 2) incorporating disease resistance alleles from diverse sets of germplasm, and 3) integrating dwarfing alleles into breeding lines (Graybosch and Peterson, 2010). By incorporating the *Ppd-D1a* photoperiod insensitivity allele, a number of disease resistance alleles, and the *rht-1* dwarfing allele into elite backgrounds, new varieties possessed earlier flowering periods (Eagles et al., 2010), resistance to a greater number of pests and pathogens, and resistance to lodging under intense applications of synthetic fertilizers (Borlaug, 1983). These three traits lead to varieties with greater yield potential and improved yield stability. These breeding developments in traits such as these contributed greatly to the Green Revolution; these genetic gains were estimated to have been responsible for 21% of the yield increases in developed countries during the Early Green Revolution (1961-1980) and 50% during the Late Green Revolution period (1981-2000) (Evenson and Gollin, 2003).

Origins and Extent of the Low Genetic Diversity of Bread Wheat

To continue to make advances in yield improvements, wheat breeders and geneticists need to continue to identify yield influencing regions of the wheat genome. Once identified and characterized, the knowledge of these yield loci can be leveraged to continue to produce lines with greater yield to keep pace with the needs of the world. However, understanding the effects of loci can be quite difficult if there is little variation at the sites in question. This is especially true for bread wheat, whose diversity is especially restricted, even among the other members of

Triticum (Akhunov et al., 2010; Chao et al., 1989; Devos and Gale, 1992); when compared to its progenitor species, bread wheat has around only 1/3 the genetic diversity of its ancestors (Galili et al., 2000). For those loci with little or no genetic variation within the bread wheat species, their role may be difficult to determine, if not impossible.

A major reason for the relatively low genetic diversity of bread wheat lies in its evolutionary origins. Bread wheat is an allohexaploid with three subgenomes originating from three diploid progenitors (2n = 6x = 42; AABBDD). These ancestors are believed to be *T. urartu* (AA; 2n = 2x = 14) (Chapman et al., 1976; Dvořák et al., 1993), a yet unknown relative of *Aegilops speltoides* (BB; 2n = 2x = 14) (Salse et al., 2008), and *Aegilops tauschii* (DD; 2n=2x=14) (Kihara, 1944; McFadden and Sears, 1946). These diploid ancestors were hybridized in two events. First, was the hybridization of *T. urartu* and the B-genome progenitor around 0.5 and 3 million years ago (Blake et al., 1999; Huang et al., 2002) that led to the creation of the allotetraploid, wild emmer (AABB; 2n = 4x = 28) (Feuillet et al., 2008). Around 8,000 to 10,000 years ago, domesticated emmer hybridized with *Ae. tauschii* to produce the primordial generation of allohexaploid bread wheat (Cox, 1997; Zohary et al., 1969; (Kihara, 1944; McFadden and Sears, 1946).

Due to these series of events, bread wheat has substantially lower genetic diversity than its ancestors. One reason is because the hybridization and duplication event that gave rise to bread wheat only occurred, at most, 10,000 years ago. As such, insufficient time has passed for substantial amounts of natural mutations to accumulate in the species (Akhunov et al., 2010). Another reason is the high ploidy of bread wheat makes it extremely difficult to directly access the vast reservoirs of genetic diversity found in its diploid ancestors. Crosses between bread wheat and diploid progenitors often lead to aborted embryos or the production of weak tetraploid

offspring that are reproductively defective (The and Baker, 1975; Gill and Raupp, 1987). Outside the slow process of natural mutagenesis, the only way bread wheat can naturally acquire genetic variation are via crosses with tetraploid wheats (Martin et al., 2011; Padmanaban et al., 2017). However, due to bread wheat's *Ph1* locus, the infusions of genetic diversity from tetraploid wheats cannot be transferred to D-genome (Riley and Chapman, 1958). Hence, the D-genome suffers from even lower genetic diversity than the A- and B-genomes (Akhunov et al., 2010; Poland et al., 2012; S. Wang et al., 2014). The diversity is so low within the D-genome, in fact, that some diversity panel screenings find the majority of D-genome genes to be monomorphic and those loci that are polymorphic having only two alleles (Akhunov et al., 2010).

To allow for screening of bread wheat genes that have low genetic variation, breeders and geneticists have developed strategies to infuse them with diversity. One method is to recreate the second polyploidization event. This is done by crossing a tetraploid wheat (often x wheat, *Triticum turgidum* ssp. *durum*) with *Ae. tauschii*, duplicating the genome of the resultant triploid (ABD; 2x=21), and producing a fertile amphiploid (AABBDD; 2n=6x=42) known as a synthetic hexaploid (McFadden and Sears, 1946). This synthetic hexaploid can then be crossed to bread wheat and add diversity to all three genomes. Another method is direct hybridization. Under natural conditions, crossing one of the diploid progenitors to bread wheat results in weak and inviable offspring, if any at all. However, using techniques including embryo rescue allow for F1 plants to be recovered; subsequent backcrossing to the wheat parent allows for genetic diversity to be transferred from the diploid to bread wheat (Gill and Raupp, 1987). While the synthetic hexaploid and direct hybridization methods have their strengths and weaknesses, both allow for the transfer of genetic diversity from diploid relatives into bread wheat. Both methods have transferred useful traits into bread wheat; for the D-genome alone, these traits include resistance

to viruses, fungi, nematodes, and insects, improved tolerance for abiotic stresses, better quality dough properties, and enhancement of yield and yield component traits (Börner et al., 2015; Cox et al., 2017). These injections of diversity have also allowed for the mapping and characterization of many novel genes (Dunckel et al., 2015; Joukhadar et al., 2013; Mulki et al., 2013; Olson et al., 2013a; Raupp et al., 1993; Singh et al., 2000; Wiersma et al., 2017).

Recent Example of Improving Yield via Increasing D-Genome Genetic Diversity

One example of an endeavor to leverage the potential of the *Ae. tauschii* gene pool in bread wheat yield improvement has been initiated at Michigan State University. This project has centered around the D-genome Nested Association Mapping population (DNAM), a bread wheat advanced backcross, nested association mapping population infused with *Ae. tauschii* genetics. By identifying regions of the genome associated with yield variation, fine-mapping those regions, and eventually cloning the responsible genes, this project aims to better understand how the D-genome can impact yield in bread wheat.

Prior to the work described in this thesis, the DNAM was created using five accessions of *Ae. tauschii* (Table 2.1). The recurrent parent is KS05HW14, a hard-white winter wheat was developed at Kansas State University, Hays, KS. Five *Ae. tauschii* accessions (DD; 2n=2x=14) including TA1617, TA1642, TA1662, TA1718, and TA10187 were directly hybridized to KS05HW14 (AABBDD; 2n=6x=42), with KS05HW14 serving as the female parent. Figure 2.1 provides a visualization of the breeding scheme. F1 plants were used as the female parent in a backcross to KS05HW14. BC1F1 plants were used as the males in a second backcross to KS05HW14. BC2F1 plants were advanced by single seed descent to the BC2F4 generation. The population is structured into families derived from common *Ae. tauschii* accessions a

Ae. tauschii Accession	Native Country	No. of BC1F1 Individuals Produced	No. of BC2F1 Individuals Produced	No. of BC2F4 derived lines Phenotyped
TA1617	Turkmenistan	3	102	26
TA1642	Iran	1	30	22
TA1662	Azerbaijan	2	150	78
TA1718	Iran	2	167	62
TA10187	Turkmenistan	3	247	64

Table 2.1 – *Ae. tauschii parent accessions used to create the DNAM population*

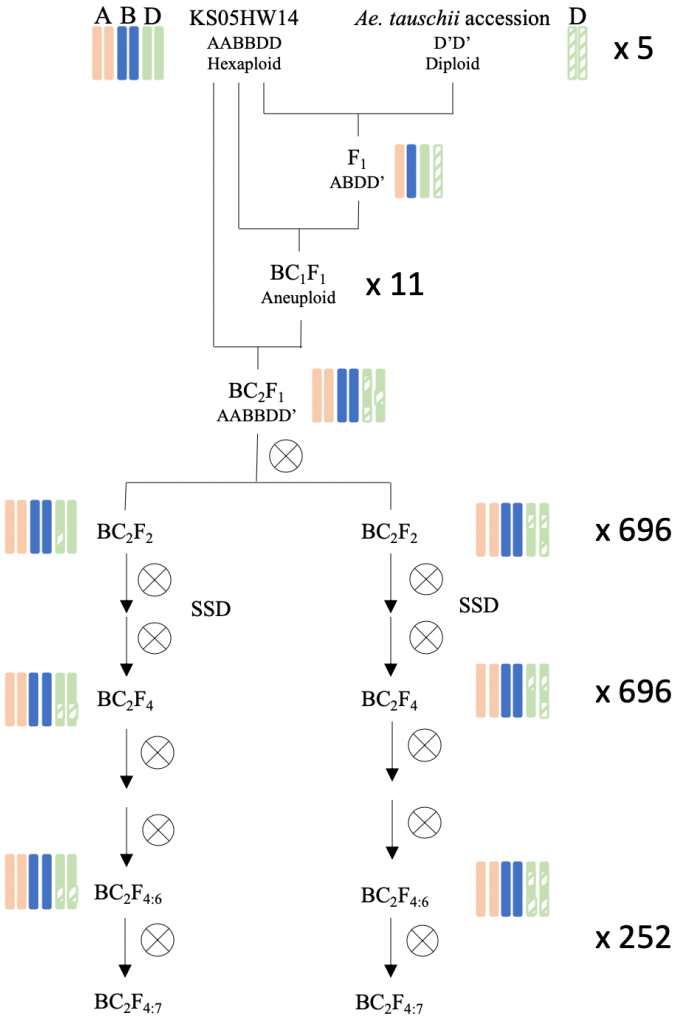

Figure 2.1 – *Graphical representation of the breeding scheme used to create the D-genome Nested associated mapping population*
As described in Olson et al. (2013b), five *Ae. tauschii* accessions (TA1617, TA1642, TA1662, TA1718, and TA10187) were backcrossed twice to the winter wheat line, KS05HW14. BC2F1 lines were selfed to produce 696 BC2F2 lines. BC2F2 lines were advanced by single seed descent for two generations. DNA samples were taken from BC2F4 lines for genotyping-by-sequencing. 252 BC2F4 lines were bulked for two generations. BC2F4:6 lines were phenotyped for yield in 2016 in four locations. BC2F4:7 lines were yield tested in seven locations. BC2F4:8 lines were tested in one location in 2017.

subfamilies derived from common BC1F1 plants. This led to the development of 696 recombinant inbred lines (RILs).

Grain yield trials were performed in six environments in three locations over two years (Table 2.2) using 252 BC2F4-derived DNAM lines. Each environment was plotted in an augmented design across six incomplete blocks. DNAM lines were replicated once across each environment while KS05HW14 and a local check were replicated multiple times across blocks. Block adjustments were made based on the methods used by Hoffstetter et al. (2016). PCA of environments was conducted using eigenvalue decomposition of the adjusted grain yield covariance matrix (RStudio 1.0.136). Heritability of yield across environments was estimated in R Studio (v1.1.453) using the "car" package (Fox and Weisberg, 2014) using the general linear model: $y_{ij} = \mu + g_i + e_j + ge_{ij} + error_{ij}$, where g_i is the fixed effect of the ith genotype, e_j is the fixed effect of the jth environment, and ge_{ij} is the interaction between the ith genotype and the jth environment. Variance components were extracted, and the mean squares were used to estimate heritability using the equation: $h^2 = \frac{MS_g - MS_{ge}}{MS_g}$, where MS_g is the mean square of the genotypic effect and MS_{ge} is the mean square of the genotype-by-environment interaction.

Within each environment there were DNAM lines that outperformed the recurrent parent, KS05HW14; this outperformance of KS05HW14 by DNAM lines ranged from 891.23kg/ha at Richville, MI to as much as 3,374.96kg/ha in Pullman, WA.

A set of reference-based markers were generated after creating genotyping-by-sequencing (GBS) libraries using the two-enzyme approach described by Poland et al. (2012). The TASSEL 5.0 GBSv2 pipeline (Glaubitz et al., 2014) was used to create markers and aligned to the IWGSC reference sequence v1.0 assembly (https://wheat-urgi.versailles.inra.fr/) using BWA version 0.7.12 (Li and Durbin, 2009). Custom Python scripts were made to identify

Location	Minimum Yield	Maximum Yield	KS05HW14 Yield	Broad Sense Heritability
Hays	1,147	4,948	3,489	0.79
Manhattan	1,387	5,322	4,288	0.53
Richville	2,241	5,992	5,168	0.64

Table 2.2 – *Summary of the yield values (kg/ha) of the DNAM population and the recurrent parent (KS05HW14) used in the GWAS*
Testing performed in Hays, KS, Manhattan, KS, and Richville, MI across the 2015 and 2016 harvest seasons

biallelic, differentiating SNP markers that met the following criteria: (1) only D-genome markers were retained, (2) markers were kept only if they were homozygous in the parental lines, (3) the marker must have at least one of the *Ae. tauschii* parents with an allele not identical by state to the bread wheat allele, and (4) no missing data in the parental lines was allowed. Markers segregating in the DNAM lines but had the monomorphic parental calls were set as missing. Markers were kept if they possessed less than 30% missing data, less than 10% heterozygosity, and an MAF were retained. BEAGLE v4.1 (Browning and Browning, 2016) was used to imput missing data within families. After imputation, markers possessing greater than 10% heterozygosity or an MAF of less than 5% were removed. The R package 'VennDiagram' (Chen, 2018) was leveraged to identify common and family-specific sets of markers. Introgression frequency (IF) was calculated as the fraction of reference-based markers not equivalent in state to the recurrent parent's genotype using a sliding window of 2 Mb with a stepsize of 1 Mb. IF was calculated both for the entire DNAM population and within families; while all markers were used in estimating IF for the entire population, family-specific IF was calculated using only markers segregating between the parents of said family. The R package, 'superheat' (Barter and Yu, 2017), was used to visualize IF.

Following filtering and within-family imputation, a total of 10,517 markers within the D-genome were available for association analysis. BC2F4 lines were found to carry anywhere between 0% and 49.86% of alleles originating from their *Ae. tauschii* parent. On average, 17.4% of the D-genome of each line was segregating for alleles from *Ae. tauschii*. The average MAF was 8.7%.

Principal component analysis (PCA) was conducted using eigenvalue decomposition of the covariance matrix in TASSEL 5.0. To measure LD, a subset of evenly distributed markers

was selected. Pairwise marker correlations (r^2) were calculated using TASSEL 5.0. The r^2 data was pooled and averaged based on 1) the physical distance between markers on the same chromosome and 2) across the entire D-genome (L. Wang et al., 2013). A bin size of 50 Mb was used. LD decay was calculated as the value at which the logarithmic regression of the pooled r^2 equals 0.2 (Cabrera et al., 2014). LD decay plots were created in R 3.2.1. Both were conducted using the reference-based marker set. Using the average pairwise correlation (r^2), LD was estimated using 3,789 evenly spaced markers across the D-genome. LD decay was estimated using 50Mb windows and was measured as the distance to which a logarithmic regression decayed to 0.2. Genome-wide LD persisted to 259Mb with significant differences being seen between chromosomes.

The first two principal components explain about 21% of the total variation, indicating a mild population structure. Genome-wide LD persisted to 259Mb with significant differences being seen between chromosomes.

A genome-wide association study (GWAS) was performed by regressing multi-year LS-Means onto the GBS marker set. The optimal models were those with kinship but not PCs. Three specific regions of the genome were deemed significant (Table 2.3). A pair of 2D chromosome markers were significant at both Manhattan and Richville, a 4D marker unique to Hays, and two 6D markers found at both Hays and Richville. Another 6D marker with potential yield association was also found at Manhattan but it was not found to be statistically significant. In all statistically significant markers, the positive effect allele originated from KS05HW14. The effect of the alleles ranged from 349.29 kg/ha to 687.97kg/ha.

By leveraging the vast genetic diversity of *Ae. tauschii,* the Wheat Breeding and Genetics Laboratory at Michigan State University identified multiple regions in the bread wheat D-

genome affecting yield. By doing so, the groundwork was laid for the initiation of fine-mapping of these signals to identify the genes responsible for the observed yield variation.

Chromosome	Physical Position (bp)	MAF	P-value	R2 (%)	Effect(kg/ha)	Environment
2D	23,516,803	0.14	3.03E-10	14.13	559.52	Richville
2D	25,177,561	0.13	1.09E-08	11.18	486.22	Manhattan
4D	498,684,103	0.13	1.58E-10	19.86	340.29	Hays
6D	472,073,634	0.02	1.03E-5	7.03	537.33	Hays
6D	468,008,045	0.04	1.32E-5	4.93	687.97	Richville

Table 2.3 – *Markers identified in the DNAM GWAS significantly associated with grain yield*

Goal of Thesis Project

The purpose of this thesis project was to produce the resources necessary for the fine-mapping of a large effect grain yield QTL identified on the short-arm of the 2D chromosome using the DNAM population. This included characterizing the 2D QTL via bi-parental QTL mapping in a subpopulation segregating for this region, designing of KASP markers spanning the QTL, and development of a fine-mapping population for positional cloning of the 2DS yield gene.

Materials and Methods

Development of Genetic Markers

Source of Genetic Data

Markers were designed based on two datasets of single nucleotide polymorphisms (SNPs). One set was from the entire DNAM population; SNP data for all DNAM RILs had been previously generated by genotyping-by-sequencing (GBS) method using a two-enzyme genotyping by sequencing (GBS) approach (Poland et al., 2011). The second dataset consisted of exome capture data generated by the laboratory of Dr. Eduard Akhunov at Kansas State University. These exome capture data were generated for KS05HW14 and two of the five *Ae. tauschii* accessions (TA1662 and TA1718), along with 57 other lines involved in the Wheat Coordinated Agricultural Project (WheatCAP) that funded this project. These exome capture-based SNPs are publicly available on the Triticeae Toolbox website (https://triticeaetoolbox.org/), a tool generated as part of the WheatCAP grant.

Marker Design

SNP data were extracted from a 10Mb region (20Mb-30Mb) containing the two 2D markers identified in the GWAS to be associated with yield (markers found at 23,516,803bp and 25,177,561bp). Of the SNPs in this region, only those SNPs considered for use as markers were those where there were allelic differences between KS05HW14 and at least one of the *Ae. tauschii* accession parents. Preference was given towards SNPs where all five accessions of *Ae. tauschii* accessions were not identical-by-state (IBS) with KS05HW14. Due to their relative ease of use and amenability to high-throughput screening, SNPs were evaluated for use as Kompetitive Allele Specific PCR (KASP)^{TM} markers (LGC, Teddington, Middlesex, UK).

Multiple strategies were utilized to appraise the suitability of a SNP as a KASP marker. Initially, a combination of desktop applications and web-based tools were used to predict how well a given SNP would work as a primer in PCR. First, formulas generated in Microsoft Excel™ (Microsoft Corporation, Redmond, WA) were used to quantify a number of primer parameters including the distance between SNPs, size of the PCR product, and the GC content. Once those SNPs possessing low quality scores for these values were identified and removed from consideration, the remaining SNPs had possible forward and reverse primers generated and evaluated using the IDT OligoAnalyzer web tool https://www.idtdna.com/pages/tools/oligoanalyzer). This web tool provided estimates of more complex traits of theoretical primers beyond what could be done in the Excel spreadsheets, including prediction of homodimer, heterodimer, and hairpin binding energies. SNPs were then evaluated using a combination of the primer characteristics predicted through these two tools, proximity to the significant GWAS markers, and the number of *Ae. tauschii* accessions that were not IBS with KS05HW14 for that specific marker.

A second approach was utilized for the design of some of the markers. This additional method used the automated primer design tool, SNP Primer Design (https://galaxy.triticeaetoolbox.org/), which was created by Dr. Junli Zhang at the University of California, Davis.

Marker Testing

Once KASP forward and reverse primers were designed, they were synthesized by Millipore-Sigma. Initial testing of primers of new markers was performed using the thermocycling procedure and reagent recipe recommendations of LGC Genomics for KASP genotyping (Table 2.4). An alternative approach was tried later using the procedure and recipe

A

	LGC Procedure[a]		3Cr Procedure[b]	
Step	**Setting**	**No. of Cycles**	**Setting**	**No. of Cycles**
Activation of Enzyme	94°C for 15 minutes	1	94°C for 15 minutes	1
Denaturation Annealing and Extension	94°C for 20 seconds 61-55°C for 60 seconds (dropping 0.6°C per cycle)	10	94°C for 20 seconds 61-57°C for 60 seconds (dropping 0.8°C per cycle)	10
Denaturation Annealing and Extension	94°C for 20 seconds 55°C for 60 seconds	26	94°C for 20 seconds 57°C for 60 seconds	30

B

Component	Volume	Final Concentration
Forward Primer Allele 1 - FAM (100µM)	12µL	12µM
Forward Primer Allele 2 - HEX (100µM)	12µL	12µM
Common Reverse Primer (100µM)	30µL	30µM
Water	46µL	-
Total	100	-

C

Component	Volume
Mastermix Product	2.5µL
Primer Mix	0.07µL
DNA Sample (10ng/µL)	2.5µL
Total	5.07µL

Table 2.4 – *Recommended thermocycling protocols and recipe for KASP genotyping for specific companies' mastermix products*
Table 2.4A describes the recommended thermocycling protocols and how they differ between LGC and 3Cr products. Table 2.4B lists the recipe for the primer mix solution prepared for each KASP marker before genotyping is performed (same recipe for both companies' protocols). Table 2.4C describes the recipe for the genotyping solution for each sample for a given marker (same recipe for both companies' protocols).

for the KASP PCR reagents provided by 3Cr (3Cr Bioscience, Harlow, Essex, United Kingdom). A C1000 Touch Thermocycler (Bio-Rad Laboratories, Inc, Hercules, CA) was used to perform the thermocycling and a BioRad CFX384 camera used to capture the fluorometric results. The behavior of a given KASP primer set was assessed using five DNA samples: 10ng/µL of KS05HW14 DNA, 10ng/µL of an *Ae. tauschii* accession's DNA with the opposite allele, a DNA mixture that was 5ng/µL KS05HW14 and 5ng/µL of the *Ae. tauschii* accession, 10ng/µL DNA of a wheat mutation line lacking the 2D chromosome (Kansas State University Wheat Genetics Resource Center), and a water negative control.

Marker behavior was assessed using the CFX Manager™ software (Bio-Rad Laboratories, Inc, Hercules, CA). Marker behavior was classified as either codominant, dominant, or null (Figure 2.2). The mixture of KS05HW14 and *Ae. tauschii* DNA simulates a heterozygous DNA sample and allows for the detection of codominant markers. The purpose of using the 2D-lacking mutation line is to test if the primers are only binding to the 2D chromosome; if primers are only matching to loci on 2D, the mutation line registers as if it were a water negative control, ensuring primers are not binding to homoeologous chromosomes. If markers did not display codominant behavior, thermocycling parameters and/or the ratio of the two forward primers in the PCR mixture were altered to produce codominant behavior.

QTL Mapping

While statistically significant markers were previously identified in the GWAS of the DNAM population, a QTL analysis was performed to estimate the breadth of the 2D QTL on the chromosome arm using one subfamily of the DNAM derived from direct hybridization with *Ae. tauschii* accession TA1718 called U6718 which included 152 inbred lines. To maximize the

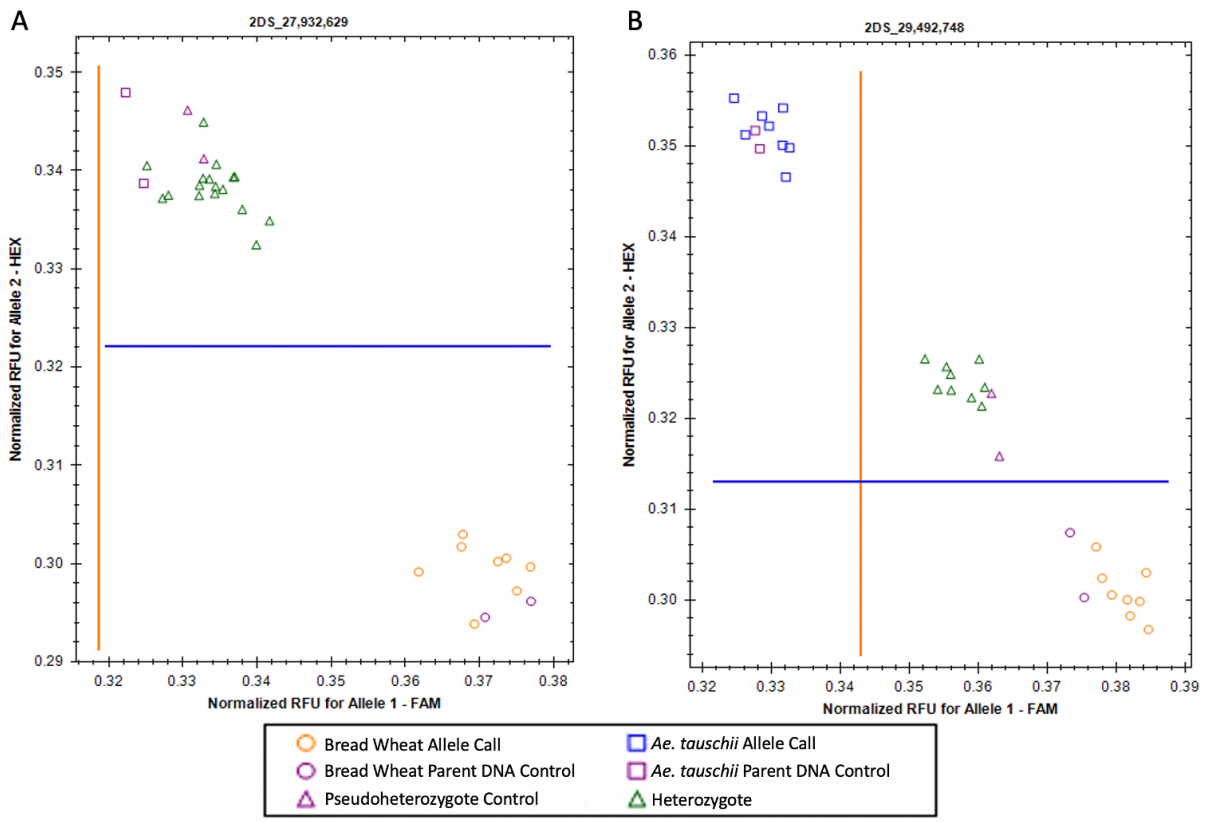

Figure 2.2 – *Examples of visuals of the genotyping results of KASP markers via the CFX Manager™ software.*
Positive controls are designated with the purple color. All other symbols represent experimental samples. Figure 2.2A shows the results of the KASP marker for the SNP at 27,932,629bp (2DS-6). 2DS-6 displays dominant marker behavior because the pseudoheterozygote controls and *Ae. tauschii* controls are too close to one another. Consequently, all the experimental samples labeled as green triangles are either heterozygous or homozygous for the *Ae. tauschii* allele but which exactly cannot be determine. Figure 2.2B shows the results of the KASP marker for the SNP at 29,492,749bp (2DS-7). 2DS-7 displays codominant behavior as all three controls are distinctly separated. Consequently, samples of all three possible genotypes can be clearly distinguished.

number of markers available for analysis, linkage mapping and QTL analyses were performed on individual subpopulations created by individual BC1F1 plants.

Linkage Map Construction

GBS data from the DNAM GWAS were the data used in linkage map construction of D-genome chromosomes. Filtering of the GBS marker data was done using a combination of Bash shell script, R Studio (R Studio Inc., Boston, MA), and TASSEL.

Bash shell scripts were used to perform the initial filtering. GBS data were separated first by the *Ae. tauschii* accession parent and then by BC1F1 line. Any markers matching to the A- or B-genome were removed as the crossing scheme used to create the DNAM only produces variation in the D-genome. Also removed were markers that were identical-by-state between KS05HW14 and the *Ae. tauschii* parent or those where there was no genotyping call for either parent. These pre-filtered data were then uploaded to TASSEL to remove markers possessing a minority allele frequency (MAF) of less than 5%, more than 50% of calls being heterozygous, less than 50% of lines having calls, and those that were indels. After conversion from a nucleotide call format to an ABH format, markers were separated by the chromosome they matched and uploaded to R Studio for linkage mapping.

Linkage mapping was performed in R Studio using the package, “qtl” (Broman et al., 2003). Since DNAM genotyping data were split up by the BC1F1 subpopulations (i.e. KS05HW14 and the BC1F1 treated as the parents of the individual subpopulation), data were treated as “riself” (recombinant inbred lines). Markers with identical calls between all lines, those with apparently switched allele calls, and those that both caused large gaps in their linkage map and had unlikely calls were removed.

Phenotypic Data

The DNAM phenotypic data utilized for QTL analysis included the data used in the GWAS (i.e. 2015 and 2016 data from Ashland, KS; Hays, KS; and Richville, MI) as well as from five other environments. The additional environments were 2016 yield data from Brookings, SD, Champaign, IL, Marianna, AR, and Pullman, WA; and 2017 yield data from Richville, MI. These yield trials were performed in the same manner as described in the methods section of the GWAS summary. As was done in the GWAS, raw phenotypic data were block adjusted relative to KS05HW14 (Figure 2.3).

Block adjusted yield values were processed in QTL analysis in two major ways using the R packages, "car" (Fox and Weisberg, 2014), "lme4" (Bates et al., 2015), and "lsmeans" (Lenth, 2016). Yield data were processed for individual location QTL analyses; least-square means were estimated for each line by location across all years of available data. For performing QTL analyses on the entire dataset, phenotypic data were utilized by extracting best-linear unbiased estimates (BLUEs) from a mixed effects model using genotype as a fixed effect and both location and year as random effects.

QTL Analyses

QTL analyses were performed in R Studio using the "qtl" package. Composite interval mapping (CIM) was performed using a walk speed of 1cM, a window size of 10cM, anywhere between 0 and 3 marker covariates, Haley-Knott regression, and Kosambi mapping. Threshold values were generated for an α of 0.05 at 1,000 permutations. Individual QTL analyses were performed for each location, as well as performing a QTL analysis using all available phenotypic data for all locations.

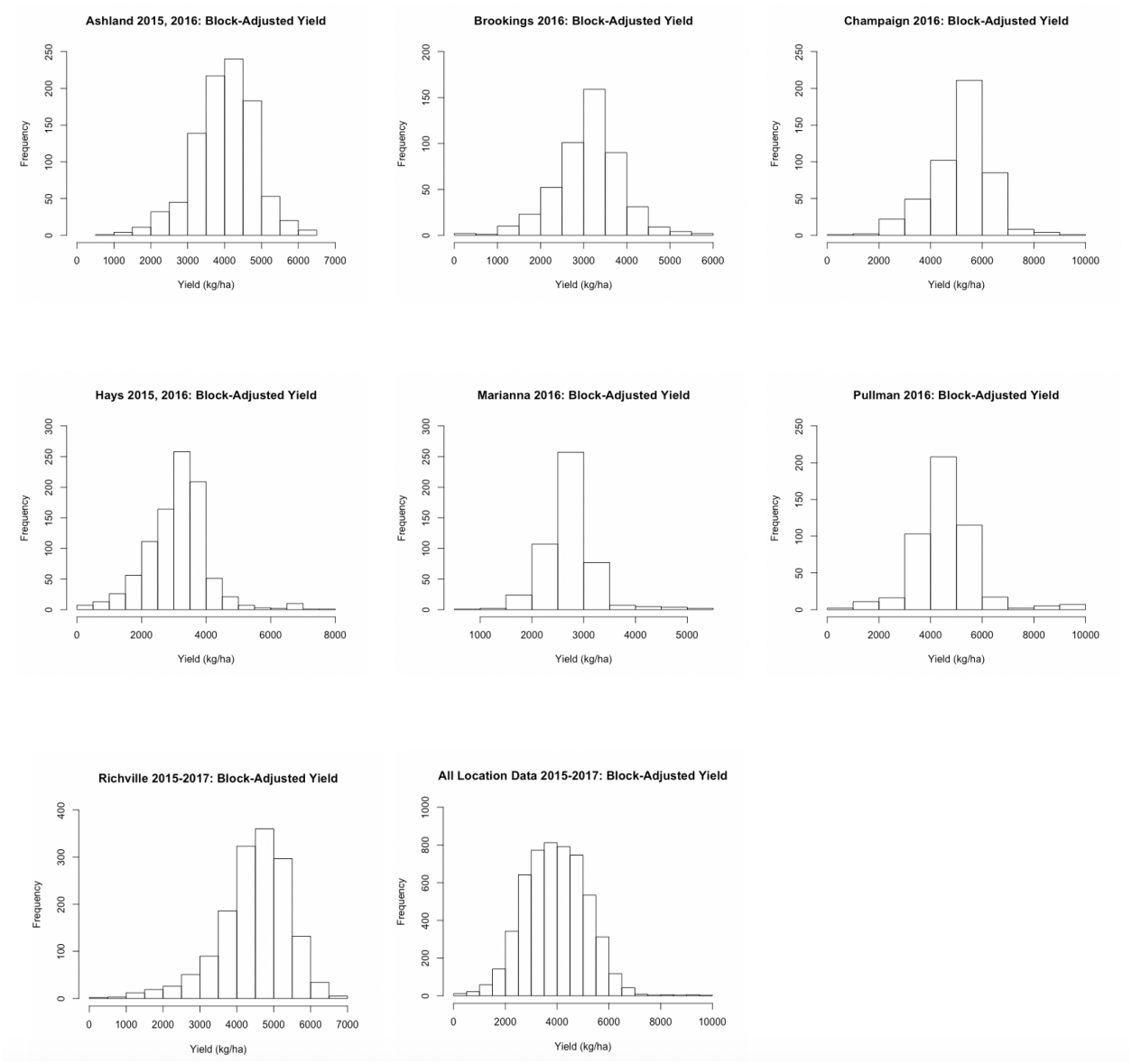

Figure 2.3– *Distributions of the block-adjusted yield values of the DNAM at each of the locations across years and the complete dataset.*

Development of Mapping Population

Identifying Founder Lines to Create Mapping Population

Once KASP markers were developed for the 20Mb to 30Mb region on the 2D chromosome, they were utilized to genotype the DNA of BC2F5 lines for which there was seed available. Lines identified as having heterozygosity in the region of interest were utilized in population development.

Rearing of BC2F6 Lines

All available BC2F6 seed from heterozygous BC2F5 lines were placed into germination boxes and kept at 4°C for 8 weeks under a photoperiod of 8 hours of light and 16 hours of dark. Seedlings were watered as needed with a 2.14mM solution of aqueous tetramethylthiuram disulfide to inhibit fungal growth. After vernalization, seedlings were moved into a greenhouse setting (16-hour photoperiod; 23±2°C) and transplanted into 3.8L pots of Fafard Grow Mix 2 (Michigan Grower Products Inc., Galesburg, MI). Plants were fertilized at regular intervals with a combination of Verdanta® Ecovita® fertilizer (Bioworks Inc, Victor, NY) and Peters Professional 20-20-20 synthetic fertilizer (Isreal Chemicals, Tel Aviv-Yafo, Israel). All plants were allowed to self-pollinate, with their seed being collected after dry-down.

Screening for Recombination in BC2F6 Lines

Approximately 30-50mg of leaf tissue were collected from each BC2F6 line, placed in an 1.1mL extraction tube containing a single, 3.96mm steel ball bearing (Grainger, Lake Forest, IL), and stored at -80°C. Tissue samples were ground to a fine powder by exposing extraction tubes to liquid nitrogen for approximately 1 minute and then transferred to a Retsch MM 400 oscillating mill (Retsch, Newtown, PA) set to 28 oscillations per second. DNA was extracted using the Mag-Bind® Plant dsDNA extraction kit (Omega Biotek, Norcross, GA) and was

performed in a high throughput manner using a Kingfisher Flex automated extraction robot (Thermo Fisher Scientific, Waltham, MA). DNA quantification was performed using the Quant-IT^{TM} Pico-Green^{TM} dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA) and the BioRad CFX384 camera. DNA samples were normalized to 10ng/µL using a PacGen Fit-X1 liquid handler (Integrated Scientific Solutions, Dubai, United Arab Emirates). Finally, genotyping was performed using the KASP marker screening procedures previously stated.

Identification of Lines for Fine-Mapping and Bulking Out

Specific BC2F6 lines were selected for. This included all plants displaying recombination in the 2DS QTL region (henceforth referred to as "heterozygous recombinants"). Also allowed to proceed to the next generation were up to ten sister-lines of each of the heterozygous recombinants fixed for either parental haplotype across the 2DS QTL (five for the KS05HW14 genotype and five for the *Ae. tauschii* genotype). BC2F7 seed were germinated and grown in an identical manner to the previous generation. All BC2F7 lines were genotyped using the 2DS QTL markers to identify lines fixed for the recombination haplotypes (i.e. "homozygous recombinants") and sister-lines fixed for either the KS05HW14 or *Ae. tauschii* parental haplotype. BC2F7:8 seed was planted in the winter of 2018 for bulking out of seed for eventual yield testing in following years. Lines planted in the field as well as lines with novel recombination events were grown in the greenhouse at the same time. An additional year of bulking out lines in the field was performed in Fall of 2019.

Results

Marker Design

Seven KASP markers were created displaying either codominant or dominant behavior (Table 2.5). The markers aligned to regions of the 2D chromosome between 22Mb and 30Mb. Two routes of marker design were used to create these markers: the first being a combination of Microsoft Excel formulas and the IDT OligoAnalyzer tool. The second route used the SNP Primer Design tool designed by Dr. Junli Zhang. One codominant marker was created using the Excel/OligoAnalyzer tool (2DS-7). The SNP Primer Design tool was used to create the remaining six markers. While six markers were codominant, one marker displayed dominant behavior (2DS-6) where a heterozygote is indistinguishable from lines homozygous for the KS05HW14 haplotype. No matter how the thermocycling conditions were altered, 2DS-6 did not display codominant behavior. All markers functioned similarly using either the LGC Genomics procedure and reagents or those of 3Cr. While most markers functioned reasonably well under the recommended thermal cycling protocols, occasionally markers required additional cycles to achieve ideal separation of the clusters.

QTL Mapping

Determining Which DNAM Subpopulations to Use in QTL Analysis

Using the seven KASP markers, available BC2F5 lines from the DNAM were screened for heterozygosity in the 22Mb to 30Mb region on 2D. Of the 95 lines screened, five displayed heterozygosity in the region of interest (Table 2.6; Figure 2.7). All five lines belonged to a single subpopulation within the DNAM, deriving from the BC1F1 line, U6718. U6718 is one of two BC1F1 lines produced through crosses between KS05HW14 and the *Ae. tauschii* accession, TA1718.

Marker Name	Physical Position Approx. Linkage Map Position	SNP Source	Behavior	Forward Primer - KS05HW14 Allele Forward Primer - TA1718 Allele Common Reverse Primer
2DS-1	22,146,307bp 20.2cM	Exome Capture	Co-dominant	FAM Call + TCTCACCATTTGGAACGACG HEX Call + TCTCACCATTTGGAACGACC TCCACGGGCTATTAATTTAGGAAT
2DS-2	22,498,399bp 21.3cM	Exome Capture	Co-dominant	FAM Call + CCTCAAGAAGGTCAAGTGCT HEX Call + CCTCAAGAAGGTCAAGTGCC CGACGCCTTGCATCTTTTGA
2DS-3	23,308,843bp 21.3cM	Exome Capture	Co-dominant	HEX Call + ACCGCTCTCTTAAATCTCACATCA FAM Call + ACCGCTCTCTTAAATCTCACATCG GTTAGCTATGCTGTGAGCCTG
2DS-4	24,925,885bp 23.0cM	Exome Capture	Co-dominant	FAM Call + CAGGCATGTTATCTGCAATTTTTC HEX Call + CAGGCATGTTATCTGCAATTTTTG CATGTAAGTTGTGACAGATGATGAG
2DS-5	25,714,409bp 24.6cM	Exome Capture	Co-dominant	HEX Call + AGACTTTACCAAAGCTTCCGACC FAM Call + AGACTTTACCAAAGCTTCCGACT ACTGCAGAATGTCAAGGGACTAT
2DS-6	27,932,629bp 24.7cM	Exome Capture	Dominant	FAM Call + ACTTGTGGAACTCTGACAGTCA HEX Call + ACTTGTGGAACTCTGACAGTCG AGGAACGATCGGATATATTGCG
2DS-7	29,492,748 27.0cM	GBS	Co-dominant	HEX Call + GCTCCCTGCAGAAATAGAGGTTAACC FAM Call + GCTCCCTGCAGAAATAGAGGTTAACG TCTGCTCCTCTCCAAAGGACTGATG

Table 2.5 – *List of the KASP markers designed for interrogation of the 2DS QTL region, their physical position on the 2D chromosome, the dataset from which they were collected, the marker behavior, and the sequences of the associated primers*

BC2F5 Family	Number of CO Haplotypes	CO Haplotypes Present
U6718-D-096-02	5	5, 7, 13, 15, 16
U6718-D-096-04	5	2, 3, 6, 8, 11
U6718-D-096-06	4	1, 9, 12, 14
U6718-D-096-09	1	10
U6718-D-096-10	1	4

Table 2.6 – *List of the BC2F5 HIF families with the total number of recombination haplotypes identified in each HIF.*
Figure 2.9 provides a visualization of the haplotypes and the location of the crossover events.

Since the fine mapping population is entirely based on the U6718 subpopulation, the only QTL analysis performed was on this subpopulation.

Linkage Mapping of D-Genome Chromosomes

A total of 215,703 markers were identified in the D-genome when the DNAM population was genotype via GBS. Marker filtering for linkage map construction was performed only using data available for the 152 U6718 subpopulation lines. Of these markers, 40,706 were not IBS between KS05HW14 and TA1718 and had calls for both parental lines. Filtering was then performed in TASSEL for markers with the following qualities: had a maximum of 50% of data missing, a minimum minor allele frequency of 5%, a maximum of 50% of calls were heterozygous, and were not indels. This left 6,361 markers. Markers were then separated based on the chromosome they matched to on the reference genome. Final filtering was performed during linkage map construction in R Studio using the "qtl" package; this involved removing markers with likely allele switches, duplicate marker calls, and those whose presence caused unlikely gaps in the linkage map. In the end, 3,625 markers were used to construct the linkage maps that would be used in QTL analysis. Table 2.7 breaks down how many markers were used to create the linkage map for each chromosome, length of the map, physical positions of the first and last markers in the linkage map, and the largest gaps. A visualization of the linkage maps can be observed in Figure 2.4.

Details of the 2D chromosome are provided here as an example. 35,559 GBS markers were available prior to filtering. After removing markers that were IBS between KS05HW14 and TA1718 or lacking a call for either parent, 6,700 markers remained. Filtering in TASSEL left 1,339 markers. Finally, 694 markers remained after filtering out of markers that were duplicate, allele switched, did not meet a maximum recombination frequency of 0.15, did not have a

Chr.	Number of Markers	cM Map Size	Position of First Marker	Position of Last Marker	Actual Chr. bp	Average cM Gap	Average Physcial Gap	Largest cM Gap	Largest bp Gap
1D	283	141.44	5,026,859	479,611,425	495,453,186	0.5Mb	1.68Mb	17.87	56,046,575
2D	694	243.25	1,527,004	650,694,497	651,852,609	0.35Mb	0.94Mb	15.20	14,831,656
3D	735	211.04	328,943	615,212,208	615,552,423	0.29Mb	0.84Mb	5.64	25,802,140
4D	215	99.14	55,346,755	509,437,244	509,857,067	0.46Mb	2.12Mb	23.17	171,474,690
5D	421	181.64	2,786,168	566,067,619	566,080,677	0.43Mb	1.34Mb	27.39	114,144,351
6D	423	214.02	725,011	473,378,572	473,592,718	0.51Mb	1.12Mb	6.77	15,758,685
7D	881	236.72	1,233,019	632,571,867	638,686,055	0.27Mb	0.72Mb	9.08	15,726,030

Table 2.7 – *Characteristics of the linkage maps estimated for the U6718 subpopulation of the DNAM*
A total of 3,652 markers were used to construct the D-subgenome linkage maps, which totaled 1,327.25cM. Average distances between immediately adjacent markers were below 0.6cM/2.5Mb. Except for chromosome 4D, more than 95% of the physical chromosome was captured between the first and last markers. Maps were calculated using the 'qtl' package[a] in R Studio.

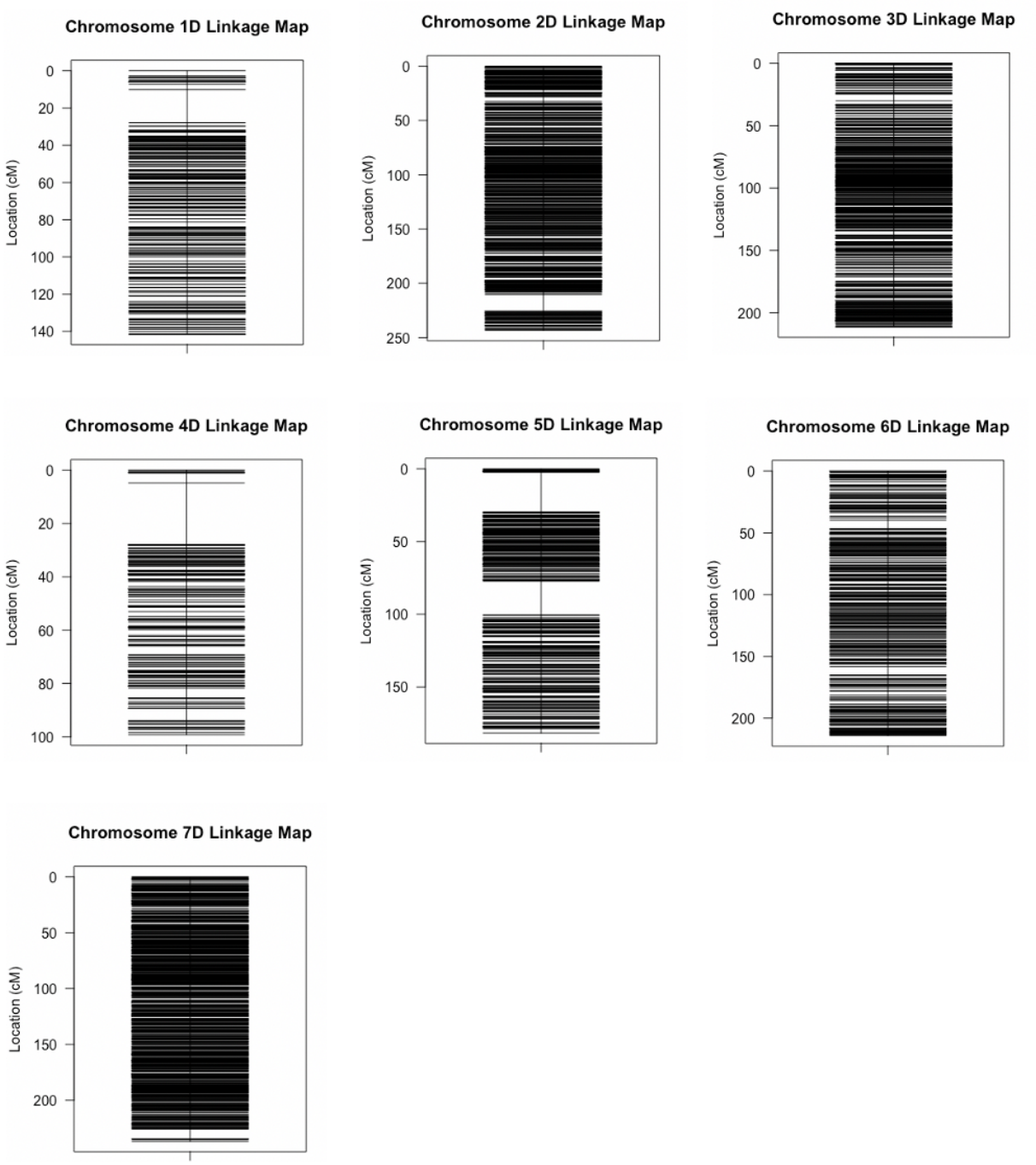

Figure 2.4 – *Linkage maps of the D-genome chromosomes for the U6718 subpopulation*

minimum LOD of 15, or were the cause of inexplicable gaps in the map. The final 2D chromosome linkage map is 243.3cM. The first and last marker mapped to 1,527,004bp and 650,694,497bp, respectively, which captures over 99% of the roughly 652Mb 2D chromosome (Alaux et al., 2018). The average recombination gap size between markers was 0.35cM and an average physical gap of 0.94Mb. The largest gap measured in terms of recombination frequency was 15.2cM wide found between the markers matching to the positions at 628,984,857bp and 635,698,329bp on the chromosome's long arm. The largest physical gap was approximately 14.8Mb between the markers at 332,052,801bp and 346,884,457bp.

QTL Analyses

Of the 152 lines in the U6718 subpopulation, 56 lines were yield tested. In all individual locations, there was at least one U6718-derivied line that exceeded the yield of the KS05HW14 parent; this yield difference ranged from 427.28kg/ha at Ashland, to 1,775.13kg/ha at Pullman. When all the yield data from all locations were assessed collectively as BLUE values, the top ranking U6718-derived line exceeded KS05HW14 by 254.93kg/ha (Table 2.8). Heritability was estimated by location for those with multiple years of data: Ashland, KS has an estimated heritability of 0.557; Hays, KS is 0.654; and the Richville, MI calculated heritability was 0.718. In addition, heritability was estimated for yield data across all locations and was calculated to be 0.885.

Using the linkage maps generated from the marker data of all 152 U6718 BC2F4 lines and the yield data for the 56 yield-tested BC2F4-derived lines, QTL analyses were performed for individual locations and the dataset as a whole. A few significant, location specific QTL were identified: this included a QTL on the long arm of the 5D chromosome in the Hays data, a highly distal QTL on the short arm of the 2D chromosome for Marianna, and a 6D chromosome QTL

	Top Ranking Line	Yield (kg/ha)	KS05HW14 Mean (kg/ha)	Difference (kg/ha)
Ashland	U6718-C-062	4,809.3	4,382.0	427.3
Brookings	U6718-C-071	4,471.0	3,305.9	1,165.0
Champaign	U6718-C-062	6,958.9	5,677.4	1,281.5
Hays	U6718-I-220	4,266.4	3,383.6	882.9
Marianna	U6718-E-143	3,515.9	2,729.0	786.8
Pullman	U6718-C-072	6,223.8	4,448.7	1,775.1
Richville	U6718-I-245	5,570.3	4,850.1	720.2
All Locations	U6718-G-174	4,075.9	3,820.9	254.9

Table 2.8 – *List of top yielding U6718 subpopulation lines and KS05HW14 controls by location in the DNAM yield trials.*
All yield values calculations for individual locations are LS means. When all data were assessed, yield values were estimated as BLUEs. In all locations, KS05HW14 was surpassed by at least one of the U6718 plots.

for Pullman (Figure 2.5). Brookings was the only location displaying no significant QTL. Three locations (Ashland, Champaign, and Richville) displayed a QTL on the 2D short arm that overlapped with the other regions. The significant portions of this 2D QTL (henceforth referred to as the 2DS QTL) varied from location to location (ranged anywhere between 13.0cM/13.8Mb and 28.3cM/30.3Mb) (Figure 2.6). Hays displayed a 2DS QTL in this region as well, but it was not statistically significant.

When the dataset was taken as a whole, QTL analysis revealed only the 2DS QTL as significant (Figure 2.5). The significant region of the 2DS QTL was 7.0cM/7.0Mb wide and ranged from 21.3cM/23.3Mb to 28.3cM/30.3Mb. The positive effect allele was contributed by KS05HW14. The estimated allele substitution effects of the markers in the significant region ranged from 878.2 ± 153.4 kg/ha to 1,227 ± 147.1 kg/ha, with an average effect of 1,026.9 ± 168.9 kg/ha.

Creating the Fine-Mapping Population

Identification of Founder Lines from DNAM

After screening using the developed KASP markers, five of the available DNAM BC2F5 lines were found to display heterozygosity in the 2DS QTL region. These BC2F5 lines were all derived from the same BC2F4 line, U6718-D-096. Four of the five BC2F5 lines (U6718-D-096-02, -04, -06, and -09) displayed heterozygosity for all available markers. U6718-D-096-10 was a heterozygous recombinant with a recombination event appearing to occur between 24.9Mb and 25.7Mb (Figure 2.7).

Development and Characterization of the BC2F6 Generation

All seeds of the five lines were sown for planting, totaling 306 BC2F6 plants. Genotyping using 2DS QTL KASP markers on all plants revealed 12 novel recombination events within the

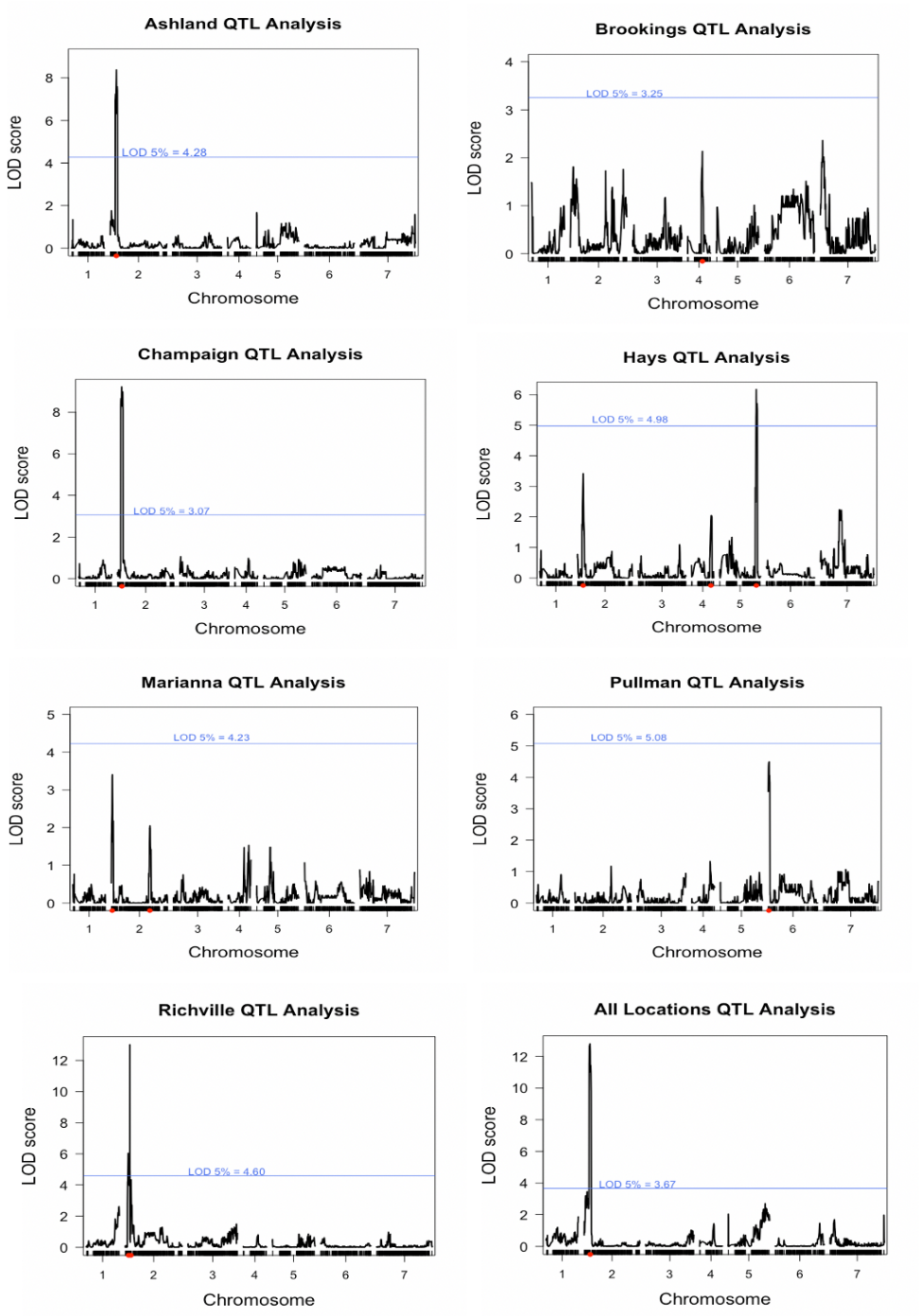

Figure 2.5 – *QTL analyses for yield in the individual seven locations and when the data are taken together*
View is of the entire genome. The blue line indicates the 5% threshold LOD value. The red dots on the x-axis indicate the position of markers used as covariates. The only region of the genome where there were overlapping QTL across regions was a region on the 2D chromosome from approximately 10cM to 30cM. When the data were analyzed as whole, the only significant QTL was on 2D from 21.3cM/23.3Mb and 28.3cM/30.3Mb.

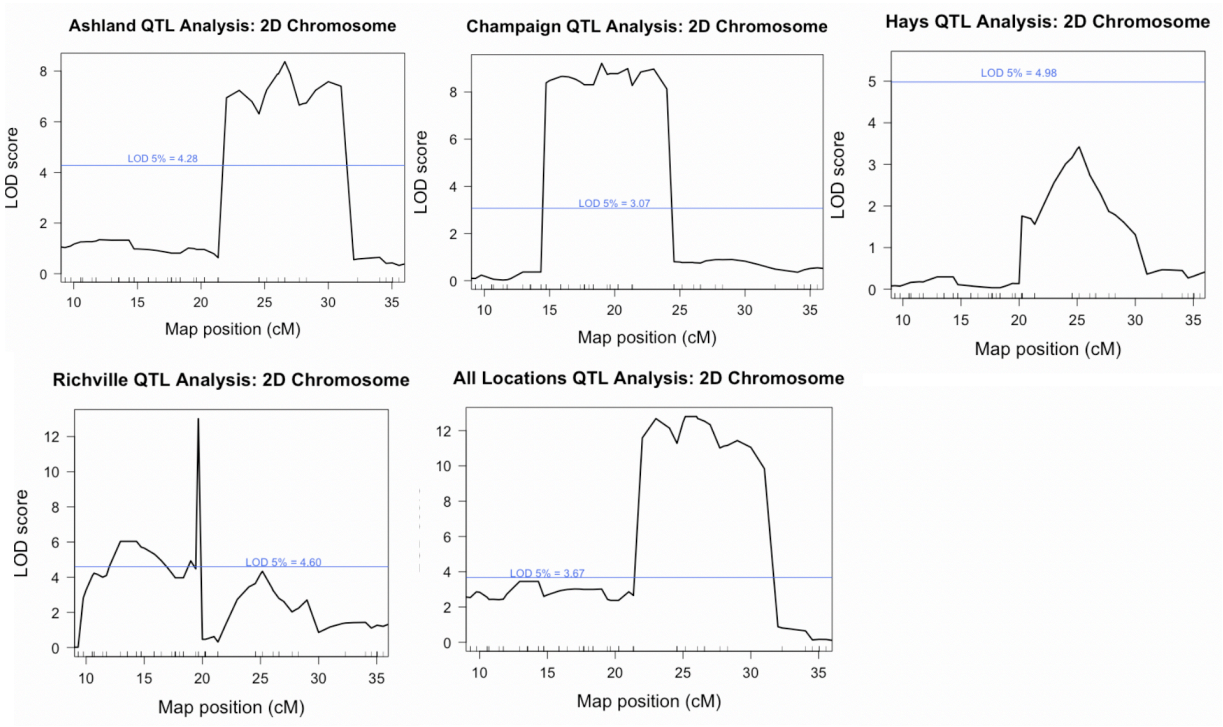

Figure 2.6 – *Close ups of the 2DS QTL in different locations*
The blue line indicates the 5% threshold LOD value. The red dots on the x-axis indicate the position of markers used as covariates. While QTL in multiple regions were detected, the only QTL observed in multiple locations was a QTL found between 10cM and 30cM on the 2D chromosome short arm (2DS QTL). The QTL in this region was significant in datasets from Ashland, Champaign, and Richville. The 2DS QTL was also detected in Hays but it did not exceed the threshold limit. When all yield data were taken together, the 2DS QTL was the only QTL deemed to be significant.

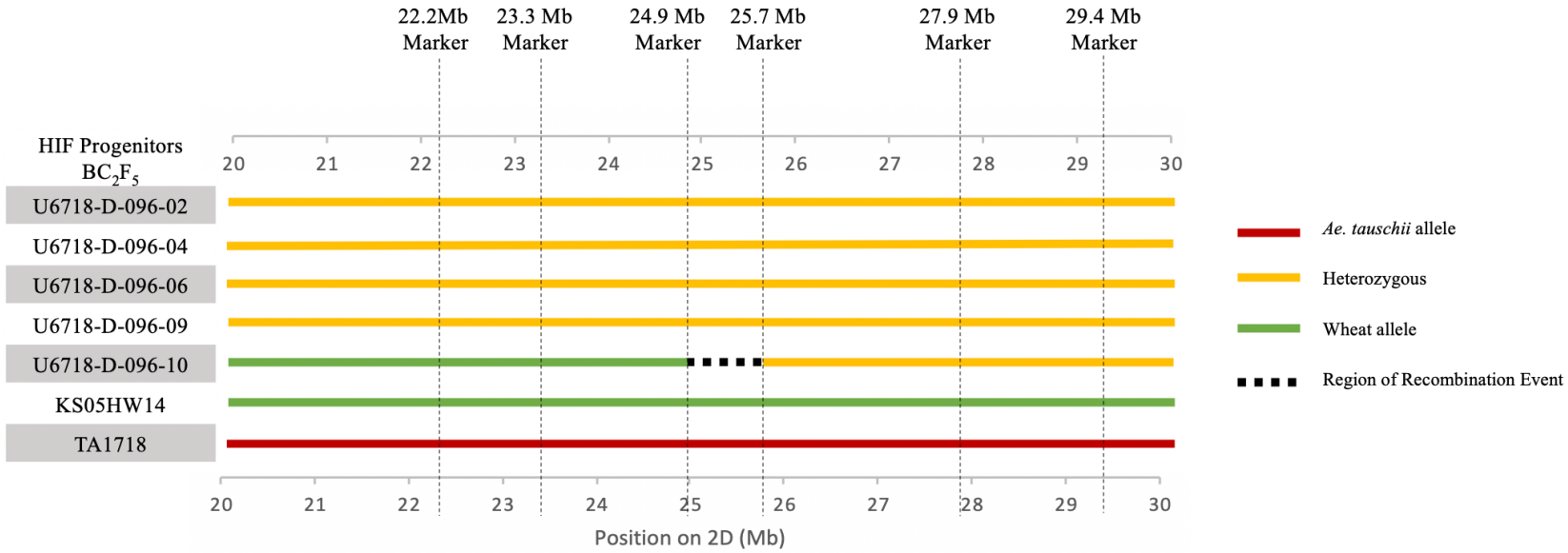

Figure 2.7 – *Haplotypes of the founders of the fine-mapping population.*
Five BC2F5 plants from the DNAM displayed heterozygosity in the region of the genome where the 2DS QTL resides. Each line was used to establish an individual heterozygous inbred family (HIF). The figure depicts the haplotype of the five HIF progenitors based on the genotyping results from markers listed at the top. KS05HW14 (the wheat parent) and TA1718 (the *Ae. tauschii* parent).

region of interest (Figure 2.8). At least one recombination event was identified in between each pair of markers. These recombination events were distributed across 10 heterozygous recombinant lines, with two lines possessing two crossovers (henceforth referred to as "double heterozygous recombinant" lines). Despite the unlikelihood of two gametes with separate novel recombination events coming together to produce a double heterozygous recombinant, their occurrence was confirmed by genotyping their BC2F7 offspring. Homozygote recombinant lines were identified for the crossover event first found in the BC2F5 heterozygote recombinant, U6718-D-096-10.

Development and Characterization of the BC2F7 Generation

The BC2F7 generation consisted of 456 lines generated from the 10 BC2F6 heterozygous recombinant lines, BC2F6 homozygous recombinant lines, and a handful of BC2F6 sister lines homozygous for either the KS05HW14 or TA1718 parental haplotypes. Genotyping of the BC2F7 population led to the identification of at least one homozygous recombinant line for each recombination event. In addition, four novel recombination events were identified (Figure 2.9). While most of the BC2F6 heterozygous recombinants yielded plenty of seed and thus multiple replicates of homozygous recombinant offspring, some did not; the BC2F6 heterozygous recombinants U6718-D-096-02-053, U6718-D-096-04-054, and U6718-D-096-06-001 produced fewer than 10 seeds between them. After being genotyped while being vernalized, it was determined these lines generated only one or two progenies fixed for their respective recombination events.

After genotyping, lines whose seed were intended to be planted in Fall 2018 were transplanted from their 1" x 1" x 4" vernalization containers into one-gallon pots to encourage maximum seed production; these lines included all lines that were homozygous recombinants for

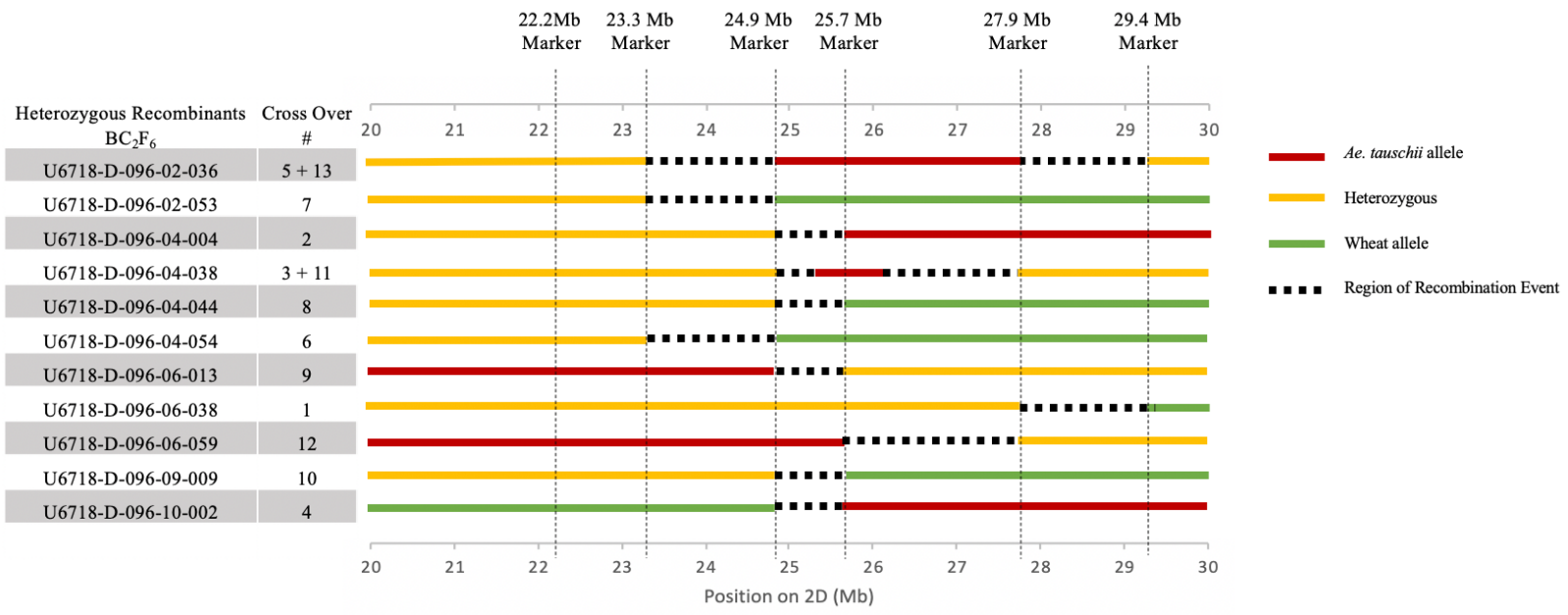

Figure 2.8 – *Haplotypes of recombinant lines identified in the BC2F6 generation*
BC2F6 lines generated from selfing the BC2F5 HIF progenitors were genotyped and heterozygous recombinant lines were identified. A total of 12 novel cross over events were identified in 10 BC2F6 lines (two lines were held two events). The specific cross over events found in each line are indicated.

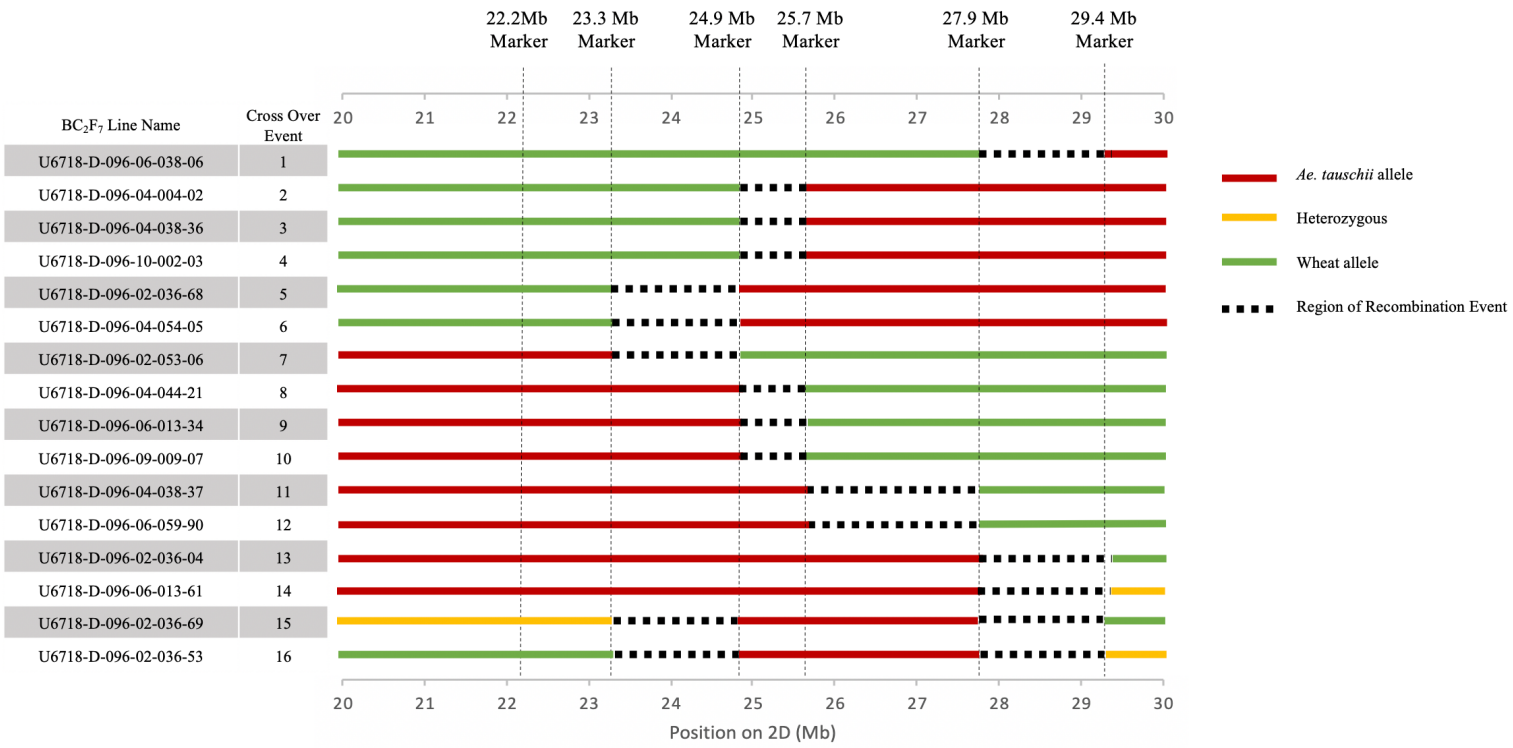

Figure 2.9 – *Haplotypes of homozygous recombinant lines and new heterozygous recombinant liens identified in the BC2F7 generation*

The following BC2F7 haplotypes were produced from selfing heterozygous recombinant BC2F6 parents. Listed are examples of specific lines with the haplotypes indicated as well as the designation given to that haplotype. In addition to identifying plants homozygous for the cross over events identified in the BC2F6 generation, three new cross over events were identified (cross over events 14-16).

their recombination events, select sister lines fixed for either the KS05HW14 or TA1718 haplotype, and the heterozygous recombinant lines displaying new recombination events, totaling 165 plants. Those BC2F7 lines possessing their BC2F6 parents' heterozygous recombinant haplotype were allowed to mature in their small vernalization containers to maintain a backup supply of the crossover events. Remaining lines fixed for either the KS05HW14 or TA1718 haplotypes were culled to conserve greenhouse space.

Fall 2018 Sowing Difficulty and Procedural Modification

The seed produced from the homozygous recombinant BC2F7 lines along with those sister-lines fixed for the parental haplotypes were sown in the field in late 2018. However, the seed sown would largely fail to germinate due to a few factors. First, BC2F7 lines were planted later than would be ideal in preparation for a Fall 2018 planting, meaning the dry-down phase of BC2F7 growth was initiated before all seeds had matured. Second, the entire population was afflicted by an outbreak of *Sclerophthora macrospora*, a fungus responsible for downy mildew disease; the infection led to abnormal growth such as excessive tillering, development of short and limp tillers, inviable flower development, highly shriveled seeds, and delayed flowering. Finally, due to a combination of the previous two events, field planting did not occur until early December. The combination of these three factors were likely the reason for lack of field germination.

A work around was achieved by taking leftover seed from the field planting and again sowing in a greenhouse setting. In addition to allowing for reestablishing a supply of seed of most of the homozygous recombinant and their sister lines, it allowed for the bulking out of a seed supply of the three new recombination haplotypes identified in BC2F7 (events 14-16).

Final BC2F8 Fine-Mapping Population

Fresh seed supplies of the recombination haplotypes 1, 3-5 and 7-16 were reestablished in a Winter 2019-Spring 2020 greenhouse growout. In addition, lines fixed for the KS05HW14 haplotype and lines with the TA1718 haplotypes were reestablished for each BC2F5 family. However, due to the poor seed quality of the lines possessing 2 and 6 crossover haplotypes, sown seed from these lines did not germinate. However, sister lines heterozygous for crossover 2 and 6 are available; as such, the crossover events are not lost and homozygous lines can be reacquired.

Discussion

Marker Development

KASP Marker Design

While both the Microsoft Excel/IDT OligoAnalyzer approach the author designed and the SNP Primer Design tool were able to generate usable KASP markers, the SNP Primer Design tool was more effective and efficient. This was demonstrated by SNP Primer Design tool generated primers being more likely to display codominant or dominant behavior (greater than 70% of generated markers) compared to the author's procedure (lower than 25%) and the SNP Primer Design tool requiring less time and effort. This difference is due to two major reasons. First, the SNP Primer Design tool offers a few additional features; this includes generating suggested primer sequences based on primer physical characteristics (e.g. GC content, melting temperature, hairpins, etc.) and on potential to bind to other regions of the genome. Second, the SNP Primer Design tool is more streamlined and automated, reducing the chance for human-error and the amount of time required by the user; the only human involvement required is formatting the input file listing the SNPs and their location in the wheat genome and selecting the primers to use from the output.

There are a few weaknesses to the SNP Primer Design tool. The most major one is it is that it is specific to bread wheat and cannot be used for other crops. The Microsoft Excel/IDT OligoAnalyzer approach, on the other hand, simply requires potential primers be BLAST against a different reference genome. At the time it was utilized, the SNP Primer Design tool lacked an easy-to-read manual, requiring frequent need to contact the designer. However, for new projects needing either KASP or CAPS markers designed for bread wheat lines, these weaknesses will either be irrelevant or likely be resolved by the time the SNP Primer Design tool is needed.

KASP Marker Procedures

The LGC Genomics and 3Cr based approaches to KASP genotyping were very similar in both ease of use and in the results they produced. The mastermix solutions from both companies had to be handled carefully during aliquoting due to a tendency to form bubbles that were resistant to popping. The reagents and thermocycling parameters of both approaches produced nearly identical genotyping results for the KASP markers. However, the 3Cr-based procedure was the more cost-effective of the two options; while a 25mL bottle of the LGC KASP PCR mastermix reagent (KBS-1016-002-US) cost $1,382.54, the 3Cr equivalent was only $660.00 (PACE 001-0002) (shipping and handling included). Since the mastermix reagent was the highest cost consumable in KASP genotyping and there were no observable differences in result quality, the 3Cr-based procedure was the approach of choice for genotyping.

2DS QTL Markers

The six markers that displayed codominant behavior (2DS-1, -2, -3, -4, -5, and -7) were especially reliable, consistently displaying distinct genotype clusters. 2DS-6, however, would only display dominant behavior, with the heterozygote cluster being indistinguishable from the cluster homozygous for the KS05HW14 allele (Figure 2.2). No matter how thermocycling settings were altered (e.g. increased annealing temperature or additional PCR cycles), the KS0HW14 and heterozygous clusters were not able to be separated.

It is not obvious why 2DS-6 does not display codominant behavior. While the 2DS-6 primers can bind to other regions of the genome other than in the 2DS QTL region, it is with lower affinity; in addition, there is no overlap in non-specific binding sites shared between any two of the three primers, meaning a PCR product should only form if they are binding to the 2DS target site. Also, there does not appear to be a major difference in the binding affinity of the

primers' respective HEX and FAM fluorophore call sequences to the region upstream of the intended binding site.

One reason for the dominant behavior of 2DS-6 may be due to the KS05HW14 allele-specific primer having the potential to form secondary structures. According to the PCR Primer Stats tool (Stothard, 2000), the KS05HW14 allele-specific primer can form self-dimer and a hairpin (Table 2.9). The *Ae. tauschii* allele-specific primer does not form any such structures. It is possible this difference in susceptibility to secondary structures is the cause for this dominant behavior. This may be resolved if a point mutation at the fourth nucleotide from the 3' end in KS05HW14-specific allele primer, changing it from a G to a C. This small change would resolve the formation of secondary structures (Stothard, 2000) without encouraging binding to non-specific regions (Alaux et al., 2018). Additional testing would be required to confirm this would change the marker's dominant behavior, if additional modifications would need to be made to one or both forward primers, or if an entirely new marker should be pursued.

QTL Mapping

Linkage Mapping

The large number of GBS SNPs passing the quality filters (average of 522 markers per chromosome) allowed for high quality linkage maps to be generated, with an average linkage gap size of 0.35cM and physical gap size of 0.94Mb. In general, this allowed for the generation of highly detailed maps that encompassed nearly all of their respective physical chromosomes; when measured from the physical position of the first marker to the last marker used in linkage mapping, nearly all D-chromosome maps represented 95% of the physical pseudomolecules. For most chromosomes, the maximum gap sizes were under 60Mb and 20cM. Especially high-

Primer	Sequence	Is Target Region the Top Hit?	Number of Non-Specific Hits	Negative Primer Characteristics	Visualization
2DS-6-Forward Primer-KS05 Allele	FAM Call + ACTTGTGGAACTCTGACAGTCA	Yes	3	Self-dimer (8 bases) Hairpin (4 bases)	
2DS-6-Forward Primer-TA1718 Allele	HEX Call + ACTTGTGGAACTCTGACAGTCG	Yes	0	None	
2DS-6-Reverse Primer	AGGAACGATCGGATATATTGCG	Yes	1	None	

Table 2.9 – *Characteristics of the 2DS-6 marker's KASP primers. 2DS-6 is a SNP at 27,932,629bp/24.7cM.*
Unlike the other KASP markers generated, 2DS-6 displays dominant behavior; homozygotes for the *Ae. tauschii* allele are indistinguishable from the heterozygotes. A potential cause for this may be the KS05HW14 parent allele primer; other than it's intended target in the 2DS QTL, it possess three non-target matches in the wheat genome (Alaux et al., 2018), more than the other two 2DS-6 primers. Unlike the other two primers, the KS05HW14 allele-specific primer is predicted to produce self-dimer and/or hairpin secondary structures as predicted by the PCR Primer Stats tool (Stothard, 2000).

quality maps included 2D, which possessed the lowest magnitude largest physical gap of all the chromosomes, and 7D which had the smallest average separation between markers.

As is to be expected, those maps with fewer markers that passed filtering were of lower quality. The linkage map of chromosome 4D is the lowest quality of all chromosomes; it was constructed using only 215 markers, encompassed only 89.1% of the physical chromosome (missing the first 55Mb), possessed the widest physical gap of over 171Mb, and the greatest average physical gap of 2.1Mb. The smaller number of markers using in linkage mapping is due to there only being around 26,000 raw markers initially available (average was approximately 30,000 raw markers per chromosome). Also, 4D markers had a lower retention rate through the filtering process; only 0.82% 4D GBS markers were retained for linkage mapping, compared to the other chromosomes which preserved between 1% and 2.3% of the raw markers. Should a higher quality linkage map be desired, it should be constructed from marker information available from another subpopulation.

Improvements Made to QTL Characterization

QTL analyses of the yield trial dataset as a whole and by individual location produced a better understanding of location and size of the 2DS QTL beyond what was found in the GWAS for the DNAM. This allowed for defining the breadth of the QTL as well as its consistency as seen within the U6718 subpopulation.

Several single location QTL were detected in the U6718 subpopulation, including the 5DL QTL at Hays, a highly distal 2DS QTL found at Marianna (not the same as the main 2DS QTL previously discussed), and a 6DS QTL found at Pullman. These QTL were not detected at any other locations and were not significant when all locations' data were assessed simultaneously. The only QTL with any degree of overlap between locations was on the 2D

chromosome between 15cM to 30cM; this QTL was detected in the data at Ashland, Champaign, and Richville. The 2DS QTL also appeared to be present in the Hays data but was not significant. Most notably, the 2DS QTL was the only QTL deemed significant when the yield data were assessed as a whole for the U6718 subpopulation. These qualities of the 2DS QTL indicate it is more substantive than its more location-specific counterparts and less likely to be a fleeting signal.

Potential Gene Candidates in the 2DS QTL

By establishing the boundaries of the 2DS QTL, it allowed for a more targeted investigation into the potential loci that may be responsible for the QTL signal. The coding sequences of the 144 high confidence genes predicted to exist in the 2DS QTL region (from 21.3cM/23.3Mb to 28.3cM/30.3Mb) in the bread wheat reference genome (Alaux et al., 2018) were downloaded. Using the default parameters, the NCBI BLASTx (https://blast.ncbi.nlm.nih.gov/) was then used to identify translated protein alignments matching either to *Arabidiopsis thaliana* (taxid: 3702) or monocots (taxid: 4447). The UniProt/SwissProt (swissprot) database was used in order reduce the number of redundant hits and to focus only on those genes that had been manually annotated and characterized in the literature.

The BLASTx search revealed hits for most of the predicted gene sequences within the database. Three matches are known to be tied to yield or yield related traits (Table 2.10). First is *SENESCENCE-RELATED GENE 1* (*SRG1*) identified in *A. thaliana* (Callard et al., 1996). The *SRG1* protein is part of the Fe(II)/ascorbate oxidase superfamily, which has been shown to be involved in regulating the redox state of ascorbate (Fotopoulos et al., 2006), the

Predicted Gene	Location on 2D (nt)	BLASTx Match	Type of Protein	Predicted Function	Literature
TraesCS2D01G063400	27,276,381 - 27,278,431	SENESCENCE-RELATED GENE 1 (SRG1)	Fe(II)/ascorbate oxidase superfamily	-Regulation of redox of cell -May play role in leaf senescence timing	Fotopoulos et al 2006 Callard et al 1996 Qi-Yan et al 2013
TraesCS2D01G063500	27,318,304 - 27,320,379				
TraesCS2D01G063700	27,330,948 - 27,335,155				
TraesCS2D01G063800	27,451,638 - 27,459,280				
TraesCS2D01G064000	27,493,734 - 27,497,008				
TraesCS2D01G064100	27,521,116 - 27,528,649				
TraesCS2D01G068500	28,762,917 - 28,764,055	SENESCENCE-ASSOCIATED GENE 39 (SAG39)	Cytoseine Protease	-Relocation of nitrogen from roots to seeds -Affects number of seeds	James et al., 2019
TraesCS2D01G071800	30,335,987 - 30,341,720	PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1 (PIE1)	ATP-dependent, chromatin-remodeling protein	-Promotes FLC-mediated and non-FLC-mediated flowering suppression pathways -Embryo development	Noh and Amasino, 2003 Xu et al. (2010)

Table 2.10 – *List of predicted genes within the 2DS QTL that share similarity to genes associated with yield or yield related traits.* Sequences of high confidence genes within the boundaries of the 2DS QTL (Alaux et al., 2018) were used in a BLASTx search (https://blast.ncbi.nlm.nih.gov/) against *Arabidopsis thaliana* and monocot protein sequences in the UniProt/SwissProt (swissprot) database. Three genes' matches may have ties to yield based on studies in the species they were found in: *SENESCENCE-RELATED GENE 1 (SRG1)*, *SENESCENCE-ASSOCIATED GENE 39 (SAG39)*, and *PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1 (PIE1)*. The wheat paralogs of these genes would be worthy of investigation as potential gene candidates.

most prevalent antioxidant in plants. This role in limiting the effect of reactive oxygen species appears to play a role in leaf senescence, to the point where *SRG1* expression is used as a marker for senescence (Jiang et al., 2013). If *SRG1* indeed plays a role controlling senescence, it may be the source of the 2DS QTL yield signal since pre-mature senescence negatively affects yield and harvest quality (Penfold and Buchanan-Wollaston, 2014).

Another potential candidate gene in the 2DS QTL is a predicted gene that matches to the rice gene, *SENESCENCE-ASSOCIATED GENE 39 (SAG39)*. Similar to *SRG1*, the expression of *SAG39* is correlated with senescence, with the highest level of transcripts occurring during the later stages of leaf senescence (L. Liu et al., 2010). However, *SAG39* likely plays a very different role than *SRG1*. *SAG39* is a homolog of the *A. thaliana* gene*, SENESCENCE-ASSOCIATED GENE 12 (SAG12). SAG12* has been shown to be a key component in the reallocation of nitrogen into seeds from other plant organs. When *SAG12* is knocked-out, low-nitrogen conditions lead to both a reduced yield and reduced seed nitrogen content compared to control lines; evidence suggests this reduced seed quantity and quality is due to the *sag12* mutant being unable to effectively remobilize nitrogen in the roots to the seeds (James et al., 2019). Should the wheat *SAG12* homolog be considered a candidate gene for the source of the 2DS QTL signal, experiments such as yield testing and comparisons of root nitrogen content may be useful in the evaluation.

The *A. thaliana PHOTOPERIOD-INDEPENDENT EARLY FLOWERING* gene (*PIE1*) was another prominent match to a predicted 2DS QTL gene matched to by BLASTx that may have ties to yield variation. In *A. thaliana* studies, mutations in *PIE1* have been noted to affect flowering time. Plants with *pie1* knock-outs display earlier flowering due to a suppression of the *FLOWERING LOCUS C* flowering inhibition pathway as well as other, less understood

flowering pathways (Noh and Amasino, 2003). Evidence in rice indicates that *PIE1* is present in monocots and may have a similar effect on flowering; in addition to identifying *OsPIE1* and its role in embryo development, Xu et al. (2010) determined transgenically expressing wildtype *OsPIE1* in *A. thaliana pie1* mutants would recover normal flowering times. Based on this information, it is possible genetic differences in alleles of the bread wheat homolog of *PIE1* may have led to variation in flowering time. Since earlier flowering is often tied with greater grain yields in cereals (Jung and Müller, 2009), the predicted gene matching to *PIE1* may be the source of the signal emanating from the 2DS QTL.

Fine-Mapping Population

While the frequency of recombination was relatively high compared to more centromeric regions, production of the homozygous recombinant lines required hundreds of plants before being identified. The genetic screening of this number of plants was possible because of the efficiency and ease of use of the KASP-based genotyping system and the time-saving nature of automated DNA extraction. Once designed and optimized, the KASP markers produced were consistent in their reliability, allowing for screening of whole subpopulations in hours. With appropriate training in marker selection, primer design and optimization, automation technology, and the selection of less costly reagents, KASP genotyping was an efficient and cost-effective strategy to screening a large number of DNA samples for a small number of markers.

A common problem encountered by researchers attempting to fine map a population is being unable to identify plentiful recombination events in their region of interest. This can be due to a lack of polymorphic markers suitable for genotyping (e.g. primers of the marker are poor quality) or simply a lack of recombination in the region of interest. Fortunately, the 2DS QTL region possessed numerous SNPs to choose from for marker development. Also, the 2DS QTL

region appears to have a relatively high rate of recombination, a phenomenon common in the distal regions of wheat chromosomes (Akhunov et al., 2003). Due to these factors, each of the five subpopulations derived from their respective heterozygous BC2F5 lines produced recombinant genotypes for the 2DS QTL region.

The developed population may allow for a more precise identification of the separate regions of the 2DS QTL. This could be done by analyzing which lines in the fine-mapping population display yield differences. As an example, should the locus responsible for the variation yield be located at approximately 26Mb, fine-mapping lines differing in their alleles for the region between the 25.7Mb and the 27.9Mb markers would likely differ in yield values. Lines with like alleles would not. Due to the recombinant genotypes captured, narrowing of the QTL region is possible. Given a sufficiently strong signal in the environments tested and there only being one locus responsible for the yield variation, homozygous recombinant lines with the *Ae. tauschii* haplotype (crossover events 2-6 and 13-16) would have similar yield while lines with the bread wheat haplotype (crossover events 1 and 7-10) would likely be higher. Lines with the 11 and 12 crossover events may display either higher or lower yields depending precisely where their recombination event occurred. These basic comparisons may prove to be a way to more precisely identify the location of yield variation signal detected in the DNAM as a whole and the U6718 subpopulation.

Thee fine-mapping lines are closely related due to sharing a common BC2F4 ancestor. However, the approximately 3.13% heterozygosity retained within this ancestor may have led to the distribution of different alleles of loci outside the 2DS region that impact yield to the different BC2F5 populations. To control for this possibility, it may be appropriate to find the difference between the yields of the homozygous recombinant lines and their BC2F5 sister lines

fixed for the parental haplotypes. By normalizing the yield measurements using sister lines within the same family, the effects of genotypic variation outside the 2DS region can be reduced. Since the goal is to pinpoint the location of the 2DS QTL signal more precisely, this adjustment would make comparisons between lines from different BC2F5 families more effective.

Summary

A 2D QTL associated with yield variation previously identified in the DNAM population was targeted for deeper interrogation to prepare for eventual identification of the causal loci. The efforts included defining the breadth of the 2DS QTL, development of biallelic markers for genotyping, and the creation of a fine-mapping population. QTL analyses within the U6718 BC1F1 subfamily supported the finding of a previous GWAS that a region in the short arm of the 2D chromosome was associated with yield variation. When yield data from all locations were considered, the analysis defined the 2DS QTL as being located between 21.3cM/23.3Mb and 28.3cM/30.3Mb (7.0cM/7.0Mb wide). Seven KASP markers designed for SNPs identified in this region proved to be reliable. KASP-based genotyping identified five BC2F5 lines with heterozygosity in the 2DS QTL region. These five lines were used to create five subpopulations, each of which produced multiple crossover events in the region of interest (16 in total). Using the KASP markers to guide selection, homozygous recombinant lines were established for each crossover event as well as sister lines fixed for the bread wheat parent and *Ae. tauschii* parent haplotypes. These tools will be useful in fine mapping the 2DS QTL to narrow down the list of potential loci that may be the cause of the signal and in aiding in the selection of DNAM lines for breeding purposes.

Conclusions

A set of fine-mapping tools were generated to investigate a yield QTL previously identified in a GWAS performed in seven locations over two years using an advanced-backcross nested association mapping population known as the DNAM. The DNAM, created by repeatedly backcrossing five accessions of *Ae. tauschii* to KS05HW14, an inbred winter wheat line (Olson et al., 2013b), displayed a pair of markers in the short arm of the 2D chromosome significantly associated with yield variation. These markers were found at the physical positions 23,516,803bp and 25,177,561bp.

To define the borders of the QTL, linkage mapping and QTL analyses were performed using a combination of command line coding, TASSEL, and R Studio. GBS data for the U6718 BC1F1 subpopulation (a line derived from a cross between KS05HW14 and the *Ae. tauschii* accession, TA1718) were used to generate linkage maps all seven chromosomes of the D-genome. These linkage maps possessed exceptionally high marker density with a minimum number of large gaps. U6718 subpopulation QTL analyses detected significant QTL very near the significant marker detected in the DNAM GWAS for the three of the seven locations. While there were other QTL detected, only the 2DS region had a significant QTL when yield data from all locations were assessed simultaneously. This assessment showed the U6718 subpopulation was segregating for a yield associated locus in a roughly 7.0cM/7.0Mb region on the 2D chromosome between 21.3cM/23.3Mb and 28.3cM/30.3Mb. The positive effect alleles in the 2DS QTL are all from KS05HW14 and had an average effect on yield of 1,026.9 ± 168.9 kg/ha.

To allow for the assessment of U6718-derived lines for their potential as founders of the fine-mapping population, SNPs between KS05HW14 and TA1718 were evaluated for use as KASP markers. SNP data was derived from DNAM GBS data and exome capture data generated

at Kansas State University's Dr. Eduard Ahkunov laboratory (https://triticeaetoolbox.org/). Potential primers for SNPs in the 2DS region were evaluated either using a relatively manual approach involving Microsoft Excel and the IDT Oligo Analyzer tool (https://www.idtdna.com/pages/tools/oligoanalyzer) or the automated SNP Primer Design tool (https://galaxy.triticeaetoolbox.org/) designed at the University of California, Davis by Dr. Junli Zhang. The SNP Primer Design tool was determined to be more time efficient and effective for this project. After testing designed markers, seven markers spanning the QTL region were deemed to be robust and reliable enough for use in screening lines. Six of the seven displayed a codominant behavior while the seventh was dominant. It was determined the KASP PCR was best performed using 3Cr's KASP mastermix as it produced equivalent results as LGC's mastermix but at a fraction of the price. The markers themselves will prove valuable for future breeding efforts using DNAM lines in order to select against the negative effect *Ae. tauschii* allele.

Using the 2DS QTL borders defined in the QTL analyses and the designed KASP markers, a high-quality fine mapping population was created. Five BC2F5 lines in the U6718 subpopulation (U6718-D-096-02, U6718-D-096-04, U6718-D-096-06, U6718-D-096-09, and U6718-D-096-10) were selected as the founders of this fine mapping population due to possessing heterozygosity in the QTL region. All BC2F5 lines were offspring of the same BC2F4 parent, U6718-D-096, and therefore were very closely related. The five BC2F5 individuals were used to create five heterozygous inbred families (HIFs), in each of which recombination events were detected. For each HIF, KASP markers were used to guide crossover events to fixation as well as identify lines fixed for the bread wheat haplotype and the *Ae. tauschii* haplotype. In the end, homozygous recombinant lines were developed for the 16

independent crossover events along with their parental haplotype sister lines in their HIF. As the crossover events identified are present across the 2DS QTL region, the population is well designed for the purposes of fine mapping the locus responsible for the QTL signal.

Future Directions

The study successfully produced a fine mapping population suitable for interrogation of the 2DS QTL for purposes of identifying candidate genes. For the fine mapping population to be ready for QTL analysis, however, substantial bulking out of the seed is required. Single replicate hill plots at a single location will likely be possible after single row plots produced from seed of individual fine mapping plants are harvested in Summer 2020. Once these first hill plots are harvested in Summer 2021, there should be sufficient seed for multilocation, multi-replicate experiments. Once data from these experiments are gathered, QTL analysis will be able to be performed to more precisely identify where the signal stems from in the 2DS QTL.

While the fine mapping will be effective with the mapping population as it is, additional efforts can be put forth to increase how much the QTL can be narrowed. One line of pursuit would be to invest in sequence capture of the 2DS QTL region. Sequence capture, a genotyping technology marketed by Arbor Biosciences, allows for genotyping in a manner that is opposite of KASP; while KASP genotyping is optimal for screening a large number of individuals for a small number of markers, sequence capture is ideal for screening a small set of samples for a large number of markers. By using sequence capture on the 16 homozygous recombinant haplotypes, the hundreds of SNPs that lie in the 2DS QTL can be genotyped. This will provide a far more precise determination of where each of the crossover events took place for each homozygous recombination haplotype. This massive increase in genotyping resolution for the recombinant haplotypes will allow for an improved narrowing of the QTL once yield data are collected.

Another way to enhance how much the 2DS QTL region could be narrowed would be by identifying additional recombination haplotypes. There would be two ways to identify novel

crossover events. One way would be by propagating the seed of existing heterozygous lines. This would be especially useful in lines heterozygous for one of the existing crossover events as it may potentially yield a haplotype with two crossover events (as is seen in haplotypes 15 and 16). These “allele island” haplotypes are especially useful since they serve the duel effect of two lines with single crossover events. These “allele island” haplotypes would also be an excellent way to confirm the QTL signal derives from a specific region of the genome. Another way would be to make deliberate crosses between existing recombinant haplotypes; this method would generate an F1 heterozygote for two recombinant alleles and potentially generate an additional recombination point in the F2 generation. Either approach would produce lines that could support the existing fine mapping population.

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