GENERATION OF T. AESTIVUM X AE. SPELTOIDES DOUBLED AMPHIPLOIDS FOR FUTURE USE IN HEAT TOLERANCE RESEARCH, AND ANALYSIS OF THEIR CLONALITY

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Abstract

Wheat is increasing in importance as the global population rises; therefore, abiotic stresses adversely affecting wheat yield, such as heat stress, are of growing concern. Accessions of *Aegilops speltoides*, a relative of the ancestral donor of the B genome of hexaploid wheat (*Triticum aestivum*), have been determined to be highly heat tolerant. Six *Ae. speltoides* accessions were used as male donors in crosses with six hexaploid wheat lines, in all combinations, to obtain F1 amphiploid seed (ABDS = 28 chromosomes). The F1 seedlings were treated with a colchicine solution to achieve chromosome doubling (AABBDDSS = 56 chromosome doubled amphiploids) and grown out to maturity. S1 seed was collected for optimal or heat treatment after anthesis and SPAD readings were taken daily during treatment until maturity. In addition to physiological measurements, leaf tissue samples were collected from S1 plants and their respective parents for marker sequence analysis. Certain doubled amphiploids survived longer in heat treatment than their wheat parents with similar SPAD readings, but had a longer maturation time, similar to their *Ae. speltoides* parents. S2 seed was collected from 20 S1 plants, including one plant from the heat treatment. This heat screen demonstrates variation among the amphiploids, the genetic diversity within pedigree warrants further investigation into the viability and heat stress tolerance of the S2 seeds obtained from this experiment.

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Dedication

This thesis is dedicated to my husband, who stuck with me through the thick and thin of graduate school after I did the same for him. This is also dedicated to my dogs who helped me destress after many long hours of reading and writing, and my Mom who has always been proud of the work I have done in grad school, even though she didn't know what *Aegilops speltoides* was until now. Also to my Dad and Jenna who read my entire thesis in pieces as I wrote. And last but not least, to Jaime who helped me with countless edits.

Chapter 1 - Review of Literature

Wheat Evolution, Population Growth and the Green Revolution

Modern wheat is a very complex species that arose from multiple, independent hybridizations. The first hybridization event is thought to have occurred 10,000 (~8,000 B.C.) years ago between *Triticum uratu* Thumanjan ex Gandilyan (A genome donor) and a relative of *Aegilops speltoides* Tausch (B genome donor), with spontaneous chromosome doubling producing a stable and fertile tetraploid known as *Triticum turgidum* L. (AABB) or Emmer wheat. The second hybridization event happened around 6,500 B.C. between *T. turgidum* and *Aegilops tauschii* Coss. (D genome donor) resulting in hexaploid wheat (*Triticum aestivum* L. (AABBDD)), (Curtis, 2002).

These two events, in combination with human selection for domestication traits, paved the way for early humans to live a more agrarian lifestyle as opposed to the former hunter/gatherer societies. Since the first civilizations in the Fertile Crescent, humans have selected grains that were larger and shattered less, so they could collect the grain and use it as nutritional sustainability for themselves as well as livestock. That was the first step in human wheat breeding efforts. Modern breeding efforts that rely on crossing of parent lines began in the early 1900s.

Wheat is arguably the most important crop in the world. Seventeen percent of all cultivated land is devoted to wheat and it dominates the world trade market, more than all other crops combined (Curtis, 2002). Wheat is a staple grain in the diets of people in many parts of the world, in both developed and developing countries: Wheat provides 20% of caloric intake for more than half the worlds' population (Lucas, 2012). In addition, wheat has many nutritional attributes; it contains carbohydrates, minerals, vitamins, fats, fiber and protein (Johnson, 1978). Due to wheat's highly adaptive nature, it can be grown at all different latitudes and elevations, worldwide.

In the 1950's, 200 million tons of wheat was produced on 200 million hectares of land. Just 40 years later in the 1990's, 550 million tons of wheat was produced on just over 200 million hectares of land (Curtis, 2002). In 2013, world production of wheat reached 715 million

tons (FAO, 2015). This incredible increase in wheat yields was a result of breeding and the Green Revolution.

The world's population has more than doubled since 1961 where it was just over 3 billion. The current population sits at almost 7.5 billion, and it is expected to keep climbing. It has been estimated to reach over 9 billion by 2050 (FAO, 2015). Agricultural advancement has enabled the population growth. The Green Revolution of the mid-20th century had a huge impact on many crops, but especially wheat. With newly available synthetic fertilizers, farmers observed an increase in seed set, but also in plant height (MASWheat). This was detrimental to yield due to the effects of lodging. With the discovery of dwarfing genes in wheat, lodging was no longer a problem and yield increased.

The right combination of *Rht* genes changed the architecture of the plant. Because the dwarfed wheat stood much shorter, the plant could spend more energy on grain filling than on biomass, and lodging was much less of an issue, thus allowing yields to skyrocket in a very short time. This development was aided by the widespread use of synthetic fertilizers and pesticides (Evenson, 2003). Scientists from CIMMYT and around the world were committed to develop wheat varieties tolerant to environment-specific abiotic and biotic stresses. Disease and pest resistance impacting particular parts of the world were bred into these varieties, also increasing yield potential. What took place between the 1940's and 1960's in agriculture was a great leap forward, but we are now on the brink of an even greater population increase, and crop yields need to improve again.

Factors Affecting Yield

Many biotic and abiotic factors cause stress and, thus, yield loss to plants. Biotic stressors include diseases; fungal, bacterial and viral, insect pests, nematodes, and physiologic disorders. Scientists and plant breeders have been discovering and utilizing naturally occurring resistances to some of these factors for decades. Abiotic stress resistances or tolerances are much more difficult to breed into high yielding varieties, as they generally are quantitative in nature. Factors affecting response to abiotic stress usually have low heritability and high genotype-by-environment interactions which, combined with unpredictable field and environmental conditions, makes it difficult to breed resistance, or even tolerance, to these factors. Abiotic stresses include nitrogen, phosphorus and potassium deficiencies, minor element deficiencies,

metal toxicity, salt stress, frost damage and winterkill, herbicide damage, drought stress and heat stress (Duveiller, 2012). In 1982, when analyzing crop insurance usage, it was found that there was an 87% yield decrease in U.S. grown wheat. Only 6% of this decrease was due to biotic factors, the other 94% was due to abiotic stresses, mainly drought and heat (Boyer, 1982). Heat and drought stress are very closely related, and it is difficult to differentiate as both these factors elicit similar defense mechanisms in the plant (Lott, 2011).

Many regions of the world where wheat is grown have periodic high temperature spikes during the wheat growing season, including the Midwest of the U.S., which causes adverse effects on yield. Recently in the U.S., drought and heat stress have caused considerable revenue loss for the wheat crop (Lott, 2011). The United States is the third largest wheat producer (58 million tons in 2013) behind China and India, with 50% of the wheat crop being exported (USDA, 2015). Heat stress can adversely affect the wheat crop at many stages, but terminal stress is generally thought to be the most common form of high temperature stress. Terminal stress is classified as heat stress during the reproductive stage (post anthesis or post flowering). In developing countries, 7 million hectares of wheat are impacted by terminal stress, whereas 36 million hectares of wheat in temperate climate zones experience yield decreases due to heat stress (Reynolds, 2001).

Physiological, biochemical, growth, and yield traits are all affected by high temperature stress. In 2013, Narayanan tested the impact of high temperatures on the following traits in winter wheat. Physiological traits that can be affected include; chlorophyll *a* fluorescence, chlorophyll concentration, leaf level photosynthesis and plasma membrane damage. Reactive oxygen species concentration and total antioxidant capacity in leaves are biochemical traits that may be affected by stress. Growth factors include plant height, tiller number and vegetative dry weight. Yield traits are the most economically relevant and include; spike number, seed set, grain number, grain yield and individual grain weight. It was found that most of these traits are adversely affected by high daytime and nighttime temperatures, causing lower yield and harvest index (Narayanan, 2014).

The Great Plains of the U.S. is the largest adjoined region of low rainfall winter wheat production in the entire world, accounting for 8 million hectares in 2013 (Tack, 2015). Kansas produces 15% of all U.S. wheat production. In 1999, Gibson and Paulsen studied the impact of various high daytime temperatures applied at different stages of wheat growth on grain yield.

Compared to the optimum treatment of 20 °C daytime and 15°C nighttime, the heat treatment (35°C /20°C) applied 10 days post anthesis resulted in a 78% decrease in grain yield. These results showed a much higher yield decrease than any previous controlled-environment studies. Because temperatures regularly surpass 30 degrees Celsius in the Great Plains during grain filling, grain yield in this region is already much lower compared to cooler regions. Due to the existing temperatures in the Midwest of the U.S., wheat is not able to reach its full yield potential: Heat tolerance is a critically important trait, particularly in the face of anticipated climate change (an increase in average day maximum temperature).

Climate Change and its Effects on Agriculture

In 2015, Stratonovitch and Semenov used existing experimental data in the Sirius wheat model to quantify potential wheat yield losses under climate change in Europe. One trait that has been sought after as a heat tolerance trait is extended grain filling period but because of increased periods of high temperature, the increased yield potential of this trait was had not been understood previously (Stratonovitch, 2015). This led the researchers to conclude that heat tolerance around flowering is essential to increasing yield potential under global warming simulations. In a previous study in 2011, Semenov used modelling to project the effects of drought stress and heat stress in the future, accounting for climactic changes in Europe. Using their advanced modelling system, they concluded that yield losses due to drought are likely to decrease with climate change, and that extreme heat events around flowering are more likely to cause significant yield loss due to global warming in Europe (Semenov, 2011).

A study was conducted in 2015 using historical (1985-2013) yield and weather data from Kansas (Tack, 2015). A regression analysis was performed to determine the effect warming global temperatures could have on Kansas wheat yields. The largest temperature effect on wheat yields was attributed to freezing temperatures in the fall causing winterkill, and extreme heat in the spring resulting in sterility and/or poor grain filling. A negative correlation was found between global temperatures and Kansas wheat yields, even accounting for warmer fall temperatures that would reduce winterkill. Looking at historical yield data, Tack also determined that older varieties appeared to be more heat tolerant than recently released varieties, and higher yielding varieties also tend to be less heat tolerant. However, this difference is not statistically significant enough that a farmer could choose to grow certain varieties to negate the effects of

global warming (Tack, 2015). Using climate models, they also found that rainfall will likely increase in the spring, offsetting some heat-caused yield loss. It is known that wheat is the crop most likely to be affected by global warming after potatoes (CIMMYT, 2014). Now that scientists and breeders are aware that the changing climate will require more heat tolerance in wheat varieties, identifying and measuring traits indicative of heat tolerance are critical.

Heat Stress and Tolerance

No single trait is responsible for heat tolerance, just as there is no 'heat resistant' variety of wheat. Heat tolerance is a series of complex interactions that result in higher yields under heat stress. Researchers have been trying to identify what mechanisms result in heat tolerance for decades. Similar yield under optimal and heat stressed conditions is the ultimate goal of heat tolerance.

A recent study in China investigated 14 agronomic and physiological traits under optimal and heat stressed conditions to determine the traits associated with heat tolerance and accurate field measures of these traits for heat tolerance. It was determined that the estimated heat susceptibility index for thousand kernel weight, grain yield per plant, grain weight per spike and flag leaf senescence scale were the criteria to identify heat tolerance (Cao, 2015). Leaf senescence is an age-dependent process that is advanced by heat stress (Fokar, 1998). The flag leaf senescence scale (FLSS) is associated with the stay-green trait and can be used as a measure of heat tolerance (Harris, 2007). Data using this scale can be taken visually in the field, and can assist with choosing varieties that will likely have higher yields under heat stress. In addition, QTL's associated with leaf senescence have been identified (Li, 2015).

Visual ratings can be subjective; therefore, chlorophyll content can also be measured in the field, which shows a significant correlation to thousand kernel weight (Ristic, 2007). Thousand kernel weight is thought to be the best indicator of heat tolerance: If thousand kernel weight shows minimal variation independent of heat condition, the genotype or particular variety demonstrates heat tolerance. The ability of a plant to maintain leaf chlorophyll content under high temperatures has been associated with high yields under stress. A study was done to determine the relative contribution of photosynthesis compared to high carbohydrate reserves in stems under heat stress, which determined that both traits contribute to heat tolerance and suggested that combining these traits could lead to higher heat tolerance (Yang, 2002).

Membrane stability has also been investigated as a potential heat tolerance trait. High temperatures can disturb molecule movement through membranes, interfering with photosynthesis and respiration (Ibrahim, 2001). The genetic variation in modern wheat varieties supports tolerance to high temperatures in certain regions of the world but alternative genetic diversity may provide wheat with more heat tolerance stability.

Diversity in Wild Ancestors of Wheat

Donors of the three wheat genomes (*T. uratu*, *Ae. tauschii*, and *Ae. speltoides*), have been of interest to wheat breeders and geneticists for the inherent reservoir of untapped genetic diversity that can be useful in modern wheat breeding. Because the *Aegilops* genus contains donors of two of the genomes (B and D), it has been an important resource for insect and disease resistance research (Gill, 2006). The D genome within hexaploid wheats has a very narrow genetic base due to the evolutionary bottleneck that began when the D genome entered wheat evolution. For example, the 326bp DNA sequences at the Gss locus showed that *Ae. tauchii* is thirty times more diverse than *T. aestivum* (Caldwell, 2004). The polyploid nature of bread wheat allows a buffered genotype that aids in adaptation to many climates, which is why wheat can be grown all over the world (Slafer, 1994). Because wheat is a hexaploid, there could be three times as many genes controlling stress tolerances in modern bread wheat than in its diploid ancestors. Exploring the vast collections of the diploid ancestors for valuable traits could prove incredibly useful if more robust methods are developed to transfer genes from the wild counterparts into a polyploid wheat background.

An evaluation of 21 species representing 1099 accessions from *Aegilops* were evaluated for resistance to three different leaf rust races and three different stem rust races. It was found that *Ae. speltoides* contained the highest number of accessions (90%) that were resistant to wheat leaf rust and wheat stem rust, as well as powdery mildew (Holubec, 2014). Different physiological responses in difference accessions to each race of rust supported the conclusion that there must be several varied genes for specific resistances. Another study evaluated 44 *Aegilops geniculata* Roth. accessions for powdery mildew and stripe rust resistance. They discovered 37 accessions were resistant to powdery mildew (33 were immune, 1 was highly resistant and 3 were moderately resistant), and 33 were resistant to stripe rust (25 were immune,

4 highly resistant and 4 moderately resistant). Of those, 19 were immune to both powdery mildew and stripe rust (Wang, 2015).

Whereas rusts and mildew are fungal in nature; wheat may also be infected by viruses such as Wheat Dwarf Virus (WDV). A study investigated whether diversity of resistance to WDV was lost in the bottlenecks of wheat evolution. Eighteen wild and domesticated wheats were tested for traits associated with WDV, and it was concluded that the evolutionary bottlenecks did not have a large impact on WDV resistance. It was proposed that WDV resistance may be associated with the D genome and, thus, *Ae. tauschii* could be a valuable genetic resource for WDV resistance improvement in modern wheat varieties (Nygren, 2015).

Pratylenchus thornei Sher & Allen is a root lesion nematode that can reduce yields in wheat crops by more than 50%, and genetic resistance is the most operative way to manage this pest despite no commercial cultivars have resistance. Because wild relatives developed in soils rich with *P. thornei*, they have proven to be a great genetic resource for resistance. Of 251 accessions of wheat and related species that were screened for resistance for this particular nematode, it was found that diploid species as a whole were more resistant than tetraploid species, and tetraploids more resistant than the hexaploids. Twenty-six diploid accessions (11 *Ae. speltoides* (S genome), 10 *Triticum monococcum* L. (A^m genome), and 5 *T. uratu* (A^u genome)) were found to be resistant (Sheedy, 2012). This study confirmed *P. thornei* resistance on the B genome, and discovered the first instance of resistance in the A genome. The authors suggested that resistance is dose-dependent in hexaploid wheat and that resistance genes can be stacked to create a more resilient wheat cultivar (Sheedy, 2012).

Another pest that is prevalent in wheat production is aphids. The three most destructive species are *Rhopalosiphum padi* L., *Schizaphis graminum* Rondani, and *Sitobion avenae* Fabricius. Resistance to these three aphid species was investigated in rye (*Secale cereal* L.) and *Ae. speltoides* by measuring aphid weight, the number of aphids and percent of infected leaf area (chlorosis). Resistance to *R. padi* and *S. avenae* were found in both rye and *Ae. speltoides* (having a decrease in colony weight by 24.2 and 34.3% respectively in the most resistant genotypes), (Crespo-Herrera, 2013). Resistance to *S. graminum* was only seen in *Ae. speltoides* accessions, with the most resistant genotype showing only 3% chlorosis, and aphid colony weight decrease of 67.7%. There is currently no resistance in hexaploid wheat to *R. padi* and *S. avenae*. Similar to the suggestion that *P. thornei* resistance can be achieved through stacking

resistance genes in modern wheat, multiple aphid resistance genes could be transferred into modern wheat from species, such as *Ae. speltoides* that provide a diverse supply of aphid resistances.

In addition to biotic stress resistance, alternative wheat gene pools provide genetic diversity for other traits. Granule-bound starch synthase (*GBSS*), also known as waxy (*Wx*), encodes the enzyme responsible for amylose synthesis in wheat seeds. Null alleles result in reduced or amylose-free starch, which impacts starch quality in the context of functionality and processing of wheat products. Seven diploid *Aegilops* species were investigated for variability in the *Wx* gene. They found nineteen new alleles, which could expand the genetic pool of wheat (Ortega, 2014). In addition to starch quality affecting functionality of wheat, glutenins are critical for gluten formation during wheat dough processing. For bread wheat, particular glutenin subunit alleles are preferable for the pan bread; however, the variability is limited. There were 21 different high molecular weight glutenin subunit (HMW-GS) alleles in the 44 *Ae. geniculata* accessions at the *Glu-1* loci, indicative of germplasm diversity not seen in modern wheat (Wang, 2015).

Diploid relatives of wheat are not the only available resource for genetic improvement. *Triticum dicoccoides* Schrank ex Schübl or wild emmer (AABB), has proven to be a valuable source for genetic diversity. In 2008, Xie et al compiled a comprehensive list of genes that have been identified and mapped in wild emmer. This list includes drought (Peleg, 2006), herbicide (Snape, 1991; Nevo, 1992; Krugman, 1997), powdery mildew (*Pm16*) (Reader, 1991; Chen, 2005), leaf rust (*Lr53*) (Marias, 2005), stripe rust (*Yr15, Yr35*, and *Yr36*) (Sun, 1997; Peng, 2000; Marias, 2005; Uauy, 2005), and fusarium head blight resistances (*Qfhs.ndsu-3AS* and *Qfhs.fcu-7AL*) (Otto, 2002; Kumar, 2007) as well as an alpha-amylase inhibitor (Wang, 2008) and grain protein content (Kahn, 2000). The high grain protein content allele (*GPC-B1*) was identified in *T. diococcoides* and has since been transferred into tetraploid and hexaploid wheat varieties for bread and noodle processing (Avivi, 1978; Joppa, 1990; Khan, 2000).

Heat Tolerance in Wild Ancestors

In the early 90's a study was done over the duration of two hot summers in California to explore survival rates of a collection of wheat and wheat progenitors in the presence of high temperature stresses (Waines, 1994). Diploids (wild and domesticated), tetraploids (wild and

domesticated), and hexaploids (domesticated only) were grown off-season to take advantage of the higher temperatures. *Ae. speltoides* and *Ae. tauschii* had higher survival rates in the field, 98% and 97% respectively, than the other diploid species; *T. monococcum* at 74% and *T. uratu* at 57%. The mean survival rates of the genome-associated species were as follows: D genome species at 97%, B/S genome species at 88% and the A genome species at 66%; suggesting that the *Aegilops* genus may be of particular interest for heat tolerance genes. In this study, the survival rate of wild *T. turgidum* (AABB), 91%, fell between that of its ancestors (*Ae. speltoides* at 98% and *T. uratu* at 57%), but lies closer to its maternal parent, *Ae. speltoides*. This may suggest the dominance of S-genome nuclear genes for cytoplasmic characteristics (Waines, 1994). Heat tolerance in a polyploid may be a combination of the parental tolerances, thus indicating a quantitative nature, with a simpler genetic explanation. Of the 59 *Aegilops* species/accessions screened, ten were determined to be heat resistant during both vegetative and reproductive stages. These 10 accessions originate from the same geographic region (southeast Lebanon, southwest Syria, eastern Israel and western Jordan), suggesting that further collection in that area could assist in discovering heat tolerance in other accessions that could be useful to wheat breeders. The heat-sensitive and tolerant *Aegilops* accessions could be used to research the genetics of reproductive heat tolerance in diploids for further application in hexaploid wheat research.

Yield reduction under stress is an indicative determination of tolerance in wheat, but is not very effective for wild or locally un-adapted species. Gupta et al (2010) tested 129 *Ae. tauschii* accessions for two biochemical traits that have been associated with heat tolerance. They measured cell membrane stability (CMS) at both vegetative and reproductive stages and TTC (2,3,5-Triphenyl tetrazolium chloride) based cell viability. A large variation between accessions for both CMS (ranging from 15.24 - 80.39%) and TTC (ranging from 18.73 – 84.39%) was observed, a wider variation than that was observed between the wheat controls (both susceptible and tolerant). Eight out of 23 *Ae. tauschii* accessions performed better than the most tolerant wheat check for the three categories of tests. A composite cell thermotolerance index (CTI) was calculated based on the three test results for each accession, and ten were identified as heat tolerant. The three tests showed positive correlations over both years with a high coefficient of determination, leading to the conclusion that the genetic component is strong (Gupta, 2010). This variation and high performance led the authors to conclude that *Ae. tauschii* could be used as a

source of genetic diversity for wheat heat tolerance improvement. Due to the variation in heat tolerance performance, a large number of accessions need to be screened in order to find tolerance, no matter the mechanism (biochemical, physiological, etc.).

In the study that inspired this thesis, 52 *Aegilops* accessions (from 5 different species), were screened for yield components and leaf chlorophyll under high temperature terminal stress. Again, much variation was found among and between species for chlorophyll retention. Using the reduction in yield as the final determinant of heat tolerance, it was found that leaf chlorophyll content (measured with a SPAD meter) was a good indicator of tolerance. The detrimental effect of high temperature stress on chlorophyll content was found to be lowest in *Ae. speltoides* having little effect on grain yield. Three *Ae. speltoides* accessions were identified as having high heat tolerance (TA 2348, TA 2342 and TA 2780), (Pradhan, 2010). Another *Aegilops* sp., *Ae. geniculata,* has been shown to be easily hybridized with modern hexaploid wheat, and many genes have been successfully transferred probably by spontaneous pairing (Kuraparthy, 2007). Due to the cross-ability of its relative *Ae. geniculata,* it has been suggested that *Ae. speltoides* should also be able to introgress high temperature tolerance into wheat by direct crossing or backcrossing (Gupta, 2010 and Pradhan, 2012).

Use of Wild Diploids in Wheat Research

Wild wheat relatives possess a plethora of genetic diversity that could be beneficial in wheat improvement; a look at successful gene introgression within cereal crops is forthcoming. The use of amphiploids to transfer genes from one related species to another has been employed. One such example was the transfer of leaf rust resistance from *Ae. taushii* into hexaploid triticale. The goal of the 2015 study was to introduce D-chromosomes from *Ae. tauschii* into triticale by creating *Ae. tauschii* (DD) x *S. cereale* (RR) amphiploids, followed by hybridization of the amphiploids with hexaploid triticale. A series of backcrossing to hexaploid triticale and self-crossing were completed before chromosome analysis was performed to ensure the presence of D-chromosomes and their leaf rust resistance genes (Kwiatek, 2015). This attempt at D-genome introgression into triticale was successful.

Ae. tauschii and wild emmer are not the only success stories in successful gene introgression. The use of *Ae. speltoides* as a genetic resource is not novel. Stem rust resistance (*Sr47*), through *ph1b*-induced homoeologous recombination was transferred from an *Ae.*

speltoides accession into durum wheat line DAS15 by Dr. L.R. Joppa of the USDA-ARS (Yu, 2015). Since then, scientists have been attempting to reduce the *Ae. speltoides* segment in the durum wheat line by backcrossing to reduce linkage drag without losing the resistance gene. Homoeologous recombination was induced between chromosomes 2B and 2S by backcrossing to a durum substitution line, and *Sr47* was confirmed on chromosome 2BL even after the *Ae. speltoides* segments were reduced (Klindworth, 2012). A co-dominant marker for the introgressed gene was developed, which makes screening for rust resistance much simpler (Yu, 2015). Two powdery mildew resistance genes have been derived from *Ae. speltoides*: *Pm12* (Miller, 1988) and *Pm32* (Lapochkina, 1996). Experiments have confirmed that the powdery mildew resistance present in the soft red winter wheat that was derived from *Ae. speltoides* is conferred by a single gene, and markers were identified that can be used in breeding programs (Petersen, 2015).

Aegilops speltoides Research and Potential Transfer

There has been research into the transferring of genes from wild ancestors to hexaploid wheat, even in *Ae. speltoides*. A complete set of both *T. aestivum-Ae. speltoides* chromosome addition lines, and disomic S (B) chromosome substitution lines have been created (Friebe, 2000 and Friebe, 2011). The genetic effects of the *Ae. speltoides* chromosomes on phenotype, gametophytic and sporophytic development were analyzed as well as their cytological characteristics. These studies allow for the examination the genetic effects of alien chromosomes in a hexaploid wheat background. It also creates the possibility of studying the structural and functional variation of the S- genome chromosomes by various genomic and epigenomic methodologies (Friebe, 2011).

Similarly, alien chromosome elimination, which is thought to take place within diploid and hexaploid wheat amphiploids, was analyzed on a sequence level. In 2010 Kumar et al used repetitive DNA sequences derived from *Ae. speltoides* to investigate the fate of this sequence among diploid *Aegilops* species, hexaploid wheat, wheat-*Aegilops* amphiploids and derived chromosome addition lines. Genetic material is often lost during the polyploidization progression (Feldman, 2012), but specific DNA sequence repeats are more likely to be eliminated in amphiploids and chromosome addition lines than others. No *Aegilops* sequence elimination was observed for the particular repetitive DNA sequence analyzed, so additional sequences were

analyzed (Kumar, 2010). This suggests that it will be possible to obtain hexaploid wheat that has genetic material from *Ae. speltoides*; it will require considerable effort to identify genes and their locations on *Ae. speltoides* chromosomes as well as the nature of their homologous pairing within polyploid wheat.

Screening distant relatives for desirable traits can be useful in deciding which species and accessions warrant further research, but that will not indicate whether the trait would be expressed in hexaploid wheat. However, if a trait is expressed in an amphiploid, it is highly likely that in future introgression attempts, the trait would be expressed in wheat. Since F1 hybrids have only one copy of each chromosome, meiosis is abnormal causing very low fertility or even sterility. Thus, the preferable method to be able to screen alien traits within a polyploid background is through chromosome doubling. This results in restored homologous chromosomes reestablishing normal chromosome behavior and consequently, pairing at meiosis. A fertile amphiploid often arises, which can be screened for desirable traits.

In recent work at the University of Nottingham, amphiploids have been generated between wheat and related species from four different genera; *Aegilops, Secale, Thinopyrum* and *Triticum*. Two different methods of chromosome doubling were tested through use of colchicine and of caffeine. Colchicine is a chemical that can be very hazardous to humans and lethal to young plants, so caffeine was also tested to compare the success of doubling. Variability between genera was observed; however, in summary, colchicine produces more successfully doubled seeds but has a higher plant mortality rate whereas caffeine-treated plants have a much higher survival rate but less grains produced (Nemeth, 2015). Genomic in situ hybridization (GISH) was used to determine the genomic constitution of the amphiploids, both multi-color and single color. Chromosome losses and aneuploidy were detected in all the genomes within the amphidiploids (Nemeth, 2015). From their results, they concluded that in amphiploids the B genome of wheat suffers chromosome loss less frequently than the other genomes.

After multiple generations of amphiploids, genetic rearrangements can happen leading to possible breakage of linkage drags associated with certain desirable traits. This in turn can create an entirely new level of increased diversity. The polyploid nature of wheat is one reason that wheat is adaptable to a wide range of climates and environments. The amphiploids will demonstrate similar levels of adaptability, having novel variation and morphologies (Nemeth,

2015). These amphiploids are currently being phenotyped to find traits desired by wheat breeders by the WISP Project (www.wheatisp.org).

Hexaploid and octoploid amphiploids have been screened for high temperature tolerance using chlorophyll content, grain fill time (maturation time) and yield components (in particular thousand kernel weight), to establish tolerance level. The hexaploid amphiploids used were durum wheat x *Ae. tauschii* crosses and the octoploids were crosses between Chinese Spring wheat and several diploid grasses (*Aegilops longissima* Schweinf. & Muschl., *Aegilops searsii* Feldman & Kislev ex Hammer, *S. cereale* and *Thinopyrum ponticum* (Podp.) Liu & Wang). Although the octoploids had higher chlorophyll contents even after heat stress, high kernel weights and low heat susceptibility indices, the yields at optimum conditions were relatively low possibly due to the unbalanced segregation that occurs in these octoploids (Yang, 2002).

Many obstacles remain in the field of interspecific introgression, but advances in high throughput phenotyping, improvement of sequencing technologies and development of whole genome sequences of many plants, including wheat (International Wheat Genome Sequencing Consortium, www.wheatgenome.org), will pave the way for ancestral species to be a trove of genetic diversity for exploitation and implementation by wheat breeding programs around the world.

Chapter 2 - Generation and Testing of Doubled Amphiploids

Introduction

Wheat is a highly adaptive crop that can be grown at different latitudes and elevations; hence, it is a very important staple crop in the global market. Wheat provides 20% of caloric intake for more than half the worlds' population in developing and developed countries (Lucas, 2012). The current population is almost 7.5 billion, and it is expected to keep climbing. It has been estimated to reach over 9 billion by 2050 (FAO, 2015). In 1982, when analyzing crop insurance usage, it was found that there was an 87% yield decrease in U.S. grown wheat. Only 6% of this decrease was due to biotic factors, the other 94% was due to abiotic stresses, mainly drought and heat (Boyer, 1982). Many regions of the world where wheat is grown have periods of high temperature during the wheat growing season, including the Midwest of the U.S., causing adverse effects on yield. Heat stress can adversely affect the wheat crop at many stages of growth and development, but terminal stress is generally thought to be the most common form of high temperature stress. Terminal stress is heat stress at the reproductive stage (post anthesis). In developing countries, 7 million hectares of wheat are impacted by terminal stress, whereas 36 million hectares of wheat crop in temperate climate zones experience yield decreases due to heat stress (Reynolds, 2001).

Climate change is a global threat that will have a huge impact on agriculture, especially as mankind struggles to feed an ever-growing population. Complex climate modelling has shown that yield losses due to drought will likely decrease with climate change, but extreme heat events during flowering events are more likely to cause significant yield loss due to global warming. (Semenov, 2011). Using historical yield data, the net effect of global warming on Kansas wheat yields was found to be negative, despite accounting for warmer fall temperatures that would reduce winterkill. Historical wheat varieties appear to be more heat tolerant than recently released varieties, and higher yielding varieties also tend to be less heat tolerant. However, this difference is not statistically significant enough that a farmer could choose to grow certain varieties to negate the effects of global warming (Tack, 2015).

Heat and drought tolerance in wheat are often highly correlated, since these abiotic stresses frequently occur simultaneously. The estimated heat susceptibility index for thousand kernel weight, grain yield per plant, grain weight per spike and flag leaf senescence scale are the

most effective criteria used to identify heat tolerance (Cao, 2015). The ability of a plant to maintain leaf chlorophyll content under high temperatures has been associated with high yields under stress. Chlorophyll content measured in the field may show a significant correlation to thousand kernel weight depending on the accuracy and precision of measurement (Ristic, 2007). Membrane stability has also been studied as a potential heat tolerance trait. High temperatures can disturb molecule movement through membranes, interfering with photosynthesis and respiration (Ibrahim, 2001). Tolerance to abiotic stresses such as heat and drought are not controlled by a single gene and are a result of genotype by environment; therefore, various measures are used to establish yield correlations. Also complicating the physiological response of wheat in heat and drought conditions is the inherent genome complexity of wheat.

It has been confirmed that the donors of the three wheat genomes (*T. uratu*, *Ae. tauschii*, and *Ae. speltoides*) hold reservoirs of untapped genetic diversity that could be useful in modern wheat breeding. Because wheat is a hexaploid, there could be three times as many genes controlling stress tolerances in modern bread wheat than in its diploid ancestors. Exploring the vast collections of the diploid ancestors for valuable traits could prove incredibly useful if more effective methods are developed to transfer genes from ancestors to a polyploid wheat background. Heat tolerance in a polyploid will be some combination of the parental tolerances, thus making tolerance appear to have a quantitative nature, supporting a simpler genetic explanation of the physiological response. Heat-sensitive and tolerant *Aegilops* accessions could be used to understand the genetics of reproductive heat tolerance in a less complex diploid system; which could in turn be applied to hexaploid wheat research and breeding for abiotic stress tolerance.

Amphiploids created with ancestral grasses have been used to create substitution and addition lines (Friebe 2000, Kumar 2010 and Friebe 2011). Only recently have amphiploids been created with the intention of doubling their chromosomes in order to study their potentially desirable traits. After multiple generations of amphiploids, genetic rearrangements and breakage of linkage drag associated with certain desirable traits are possible if recombination occurs. This represents an entirely new level of increased genetic diversity and physiological response to environmental stresses (Nemeth, 2015). Due to the polyploid nature of amphiploids, they will demonstrate adaptability, having novel variation and morphologies.

Objective

The primary objective of this study is to generate stable octoploids from Ae. speltoides x T. aestivum amphiploids, as well as to establish whether the heat tolerance present in *Ae. speltoides* accessions can be expressed in a polyploid background. Furthermore, marker sequencing validates the non-clonality of the amphiploids and explores the homology between wheat, *Ae. speltoides*, and the amphiploid pedigrees.

Materials and Methods

Plant Material

Five experimental wheat lines from Kansas State University's Wheat Breeding Program and one elite wheat line were used as common wheat parents, along with 6 *Ae. speltoides* accessions obtained from the Wheat Genetics Resource Center (WGRC) in Manhattan, Kansas. The 6 *Ae. speltoides* accessions were chosen based on their Heat Susceptibility Index ratings from Pradhan, et al, 2012. Of these six accessions, one demonstrated low heat tolerance, three demonstrated mid tolerances and two demonstrated high heat tolerance. Overley and Jefimija were used as wheat checks in this research, (Table 2.1).

Crossing Block

Experimental and Treatment Conditions

This research was conducted at the Kansas Wheat Innovation Center in Manhattan, Kansas. Seeds of each parent were germinated in plastic 150mm petri dishes with Terra Coat. The seeds were kept at 4^{o}C for two days, then at 20^{o}C for one day. The germinated seeds were then sown in 1.5" plastic pots containing commercial Sun Grow Metro Mix 300 potting soil (Hummert International, Topeka, KS), at a rate of 5 seeds per pot. The seedlings were raised in a growth chamber (Conviron MTPS Series Multi-Tier Walk-in – MTPS216 with CMP6050 Control System, Winnipeg, MB, Canada) maintained at 25/19^{o}C day/night temperature, 14-hour photoperiod, and 65% humidity. After two weeks, seedlings were vernalized for 56 days at 4^{o}C with an 8-hour photoperiod.

Succeeding vernalization, all seedlings were transplanted into their own 5.5" plastic pot filled with a 1:1 mixture of topsoil and Metro Mix 300, with 2g of Osmocote Plus (Scotts,

Marysville, OH), a slow release fertilizer. The plants were then placed in a greenhouse set to 25/20^{o}C and 85% humidity. They were automatically bottom watered every two days. Five seeds from each of the six wheat parents were germinated every week for five weeks, and ten seeds from each of the six *Ae. speltoides* parents were germinated on the first and third weeks of the wheat parent schedule.

Wide Hybridization

The wheat parent was used as the female parent and, thus, when the spike was about 1" out of the collar, the heads were emasculated: The center florets of each spikelet were removed, and the top of the spikelet was cut straight across at the top of the outer glumes. Using small forceps, the three anthers were then removed from each remaining floret on the head. The head was tagged with an identifier and date, and a pollination bag was placed over the head to prevent cross-pollination. One to two days later, pollen was collected from an *Ae. speltoides* accession that had dehiscing anthers, and using a small paintbrush, pollen was applied to the styles contained in each floret of the wheat head, which was then labeled with the male donor identifier.

As many crosses were made as possible based on pollen availability, ensuring every cross was made (6x6 = 36 possible crosses). After fertilization, the heads were allowed to mature in the greenhouse until physical maturity and grain ripening. The heads with seed were then harvested, dried in an oven for three days, threshed and recorded. Theses F1's should have a chromosome count of 28, with 7 from each genome (ABDS). A list of the successful crosses and seed counts can be found in Table 2.2.

Chromosome Doubling

Experimental and Treatment Conditions

The F1 seeds (excluding a small remnant sample) were germinated in plastic 150mm petri dishes with water. The seeds were kept at 4^{o}C for two days, then at 20^{o}C for one day. The germinated seeds were then sown in 1.5" plastic pots containing commercial Sun Grow Metro Mix 300 potting soil (Hummert International, Topeka, KS), at a rate of 1 seed per pot. The seedlings were raised in a growth chamber (Conviron MTPS Series Multi-Tier Walk-in – MTPS216 with CMP6050 Control System, Winnipeg, MB, Canada) maintained at 25/19^{o}C

day/night temperature, 14-hour photoperiod, and 65% humidity. After two weeks, seedlings were vernalized for 56 days at 4°C with an 8-hour photoperiod. Succeeding vernalization, all seedlings were transplanted into their own 3.5" plastic pot filled with Metro Mix 300. The plants were then placed in a greenhouse set to 25/20°C and 85% humidity where they were automatically bottom watered every two days.

Doubled Amphiploid Creation

At the early tillering stage (approximately twelve days post-transplant), the plants were shaken free of the metro mix, their roots rinsed and cut to 2" from the crown, and individually labelled. The plants then underwent Heartland Plant Innovations' doubled haploid protocol for chromosome doubling. The plants were exposed to the colchicine solution for eight hours, and then rinsed in tap water for fifteen hours. Each plant was then transplanted into their own 5.5" plastic pot filled with a 1:1 mixture of topsoil and Metro Mix 300, with 2g of Osmocote Plus. The pots were then put back in the greenhouse and allowed to grow and fully mature before seed was harvested. Plant counts for those that survived chromosome doubling can be found in Table 2.2. At maturity, each plant was evaluated for seed, which was harvested where present, dried in an oven for three days, then threshed and counted. These S1 doubled amphiploid seeds should have a chromosome count of 56, AABBDDSS. Seed counts can be seen in Table 2.3.

High Temperature Screening

Experimental and Treatment Conditions

The selected S1 seeds (highlighted in Table 2.3), in addition to 10 seeds each of the five represented parents and two checks were germinated in plastic 150mm petri dishes with Terra Coat. The seeds were kept at 4°C for two days, then at 20°C for one day. The germinated seeds were then sown in 1.5" plastic pots containing commercial Sun Grow Metro Mix 300 potting soil (Hummert International, Topeka, KS), at a rate of 5 seeds per pot. The seedlings were raised in a growth chamber (Conviron MTPS Series Multi-Tier Walk-in – MTPS216 with CMP6050 Control System, Winnipeg, MB, Canada) maintained at 25/19°C day/night temperature, 14h photoperiod, and 65% humidity.

After two weeks, seedlings were vernalized for 56 days at 4°C with an 8-hour photoperiod. Succeeding vernalization, all seedlings were transplanted into their own 5.5" plastic

pot filled with a 1:1 mixture of topsoil and Metro Mix 300, with 2g of Osmocote Plus. Each pedigree group of S1 seedlings were randomly divided into two treatment groups (Heat and Normal) and labelled as such. The parents and checks were also randomly divided, five plants in each treatment group from each line or accession (a complete list of plants in the experiment can be found in Table 2.4). All the plants were randomly placed back into the growth chamber (equipped with LED lights at an intensity of 400 micromoles).

Flowering date was recorded for each plant, and ten days later that plant entered its assigned treatment. The two treatments were each held in separate reach-in growth chambers (Conviron PGR15 with CMP6050 Control System, Winnipeg, MB, Canada). The normal treatment chamber was maintained at 25/19^{o}C day/night temperature, 18-hour photoperiod and 50% humidity. The high temperature treatment chamber was maintained at 36/30^{o}C day/night temperature, 18-hour photoperiod and 50% humidity.

Ion Leakage Assay

At a late tillering stage and before flowering, leakage of ions from the leaf were measured according to Djanaguiraman et al (2010). Two leaf punches, each approximately 0.2g, were taken from each plant. One leaf punch was put into 20mL of deionized water in a test tube and kept in a water bath with a constant temperature of 40^{o}C for 30 minutes. Its conductivity (C1) was then measured with a conductivity meter. The second leaf punch was put into an identical tube with 20mL of deionized water and kept in a water bath with a constant temperature of 100^{o}C for 10 minutes, and then its conductivity (C2) was also measured. Membrane damage is expressed as a percentage, calculated: (1 - (C1/C2)) x 100.

Leaf Chlorophyll and Physiological Maturity

When a plant first flowered, the main tiller was tagged. Ten days after flowering was marked as day 0 of treatment. Every day, starting at day 0, leaf chlorophyll content was measured using a Minolta Chlorophyll Meter SPAD-502DL (Agricultural Solutions, LLC; Strong, ME). Every reading was performed on the main tillers flag leaf. A reading consisted of taking three measurements along the length of the main flag leaf. The average was recorded for each plant every day that they were in treatment. Treatment ended when a leaf chlorophyll content could not be obtained from the main flag leaf due to approaching the lowest detection limit of the SPAD meter. Once the plant left treatment, it was placed back in the original growth

chamber and allowed to fully mature. Once the plants main tiller was fully mature, the date was recorded as physiological maturity.

Marker Sequencing

Tissue samples were taken from each plant in the study before being put into treatment. Approximately 4cm of fresh leaf tissue was sampled. The fresh tissue was then lyophilized for 48 hours and then disrupted using a linear mixer mill (Geno/Grinder 2010®). DNA was extracted using the BioSprint™ 96 plant kit (Qiagen; Hilden, Germany). The DNA was then quantified using Picogreen™ dye (Life Technologies; Carlsbad, CA) and fluorescence was measured with a Synergy™ H1 monochromator (BioTek; Winooski, VT). DNA was normalized to 20 ng/µl using the Tecan Freedom EVO™ robotic system (Tecan; Männedorf, Switzerland).

Next-generation sequencing libraries were prepared using the method described by Poland et al (2012) modified for 384-plex reactions. Specifically, 100mg of DNA was digested with 2.5 units each of PstI-HF (a rare cutter) and MspI (a common cutter) restriction enzymes (New England Biolabs; Ipswich, MA). In addition, the 10X NEB CutSmart® buffer was also added. The digestion reaction conditions were 34^{o}C for 2 hours, 65^{o}C for 20 minutes, and a hold step at 4^{o}C. A Mastercycler™ pro 384 (Eppendorf; Hamburg, Germany) thermocycler was used for all thermal reactions. Following digestion, two adapters were ligated to the sticky overhangs of digested fragments. The first adapter was a barcode adapter with a complimentary overhang to the PstI-HF cutsite which allowed for post-sequencing de-multiplexing. The second adapter was a common y-adapter with a complimentary overhand to the MspI cutsite. The ligation reaction volumes were as follows: 10µl of the restriction digest, 2.5µl of the adapter stock (0.02µM of the barcode adapter, 3µM of the common adapter), and 2.5 µl of NEB T4 ligase master mix containing NEB ligase buffer and 100 cohesive end units of T4 ligase. The ligation reaction conditions were 20^{o}C for 2 hours, 65^{o}C for 20 minutes, and a hold step at 4^{o}C. Reactions were then pooled equally. 200µl of the pool were purified using the QIAquick PCR purification kit according to manufacturer directions for a final elution volume of 60µl.

Pooled libraries were then amplified with a forward primer corresponding to the barcode adapter and the reverse primer corresponding to the common adapter. Because the common adapter 5’ priming site was incomplete, only the forward primer was capable of binding in the first cycle of PCR which would then elongate to the end of the 3’ end of the common adapter site

and complete the reverse primer binding site. This was to prevent any adapter dimers or fragments with two MspI cutsites from amplifying ensuring only fragments of interest would amplify. In addition, only 17 cycles were used to improve fragment diversity and prevent any overrepresented digest fragments from dominating the PCR reaction. Reaction volumes were as follows: 10µl of the purified pooled DNA, 2ul of a primer mix containing both primers at 10µM, 8µl of nuclease-free H_2O, and 5µl of 5x NEB MasterMix (1x composition contains 10mM Tris-HCL, 50mM KCL, 1.5mM $MgCl_2$, 5% glycerol, 0.08% IGEPAL® CA-630, 0.05% Tween® 20, 0.2 mM dNTPs, 25 units/ml *Taq* polymerase, pH 8.6 at 25ºC). PCR reaction conditions were an initial step at 95ºC for 30 seconds; 16 cycles of 95ºC for 30 seconds, 62ºC for 20 seconds, 68ºC for 90 seconds; a final elongation at 72ºC for 5 minutes; and a hold step at 4ºC. Amplified DNA was then cleaned up using the Qiaquick PCR purification kit. Because of the low yield due to the reduced cycle number, DNA pools were amplified in duplicate reactions and combined before PCR cleanup. Prior to sequencing, final library integrity was then checked using an Experion™ electrophoresis system (Bio-Rad; Hercules, CA).

Sequencing was performed using the NextSeq™ 500 platform (Illumina; San Diego, CA). A single-read 75 cycle+15 index cycle flowcell was used for sequencing. Because in-line barcodes were used, the 15 indexing cycles were not used. Instead, the flowcell was run for 90 cycles.

Statistical Analyses

Average SPAD readings were calculated for each pedigree in each treatment on each day of treatment. N can be found in Table 2.5. Charts and graphs were made using Stata/SE 12 (College Station, TX). A SPAD value of 0 was entered upon reaching the lower detection limit of the SPAD meter and subsequent days (after 32 days). The data were modified in this way so that when a day was reached that less than N plants were left in treatment, the total average SPAD was still using N values, and thus not creating false peaks.

A modified UNEAK pipeline was used to call GBS SNPs using TASSEL 5 standalone (Bradbury, 2007 and Lu, 2013). A minimum tag sequence count of 5 was required for a tag to be kept after adapter sequences were trimmed. Tags were aligned to the IWGSC wheat genome assembly version 1.0+ popseq (IWGSC, 2014 and Chapman, 2015) using Bowtie2 version 2.2.4 using the --very-sensitive-local command (Langmead, 2012). Marker filtering and the

relationship analysis was performed using JMP Genomics 7.1 (SAS, Cary, NC). Hardy-Weinberg Equilibrium test using the chi-square distribution was used to determine significance of mapped markers. Markers were filtered allowing 10% missing across a marker, 20% missing within an individual, and a minor allele frequency of 5% or greater. Six individuals were removed from further analysis resulting in 1547 markers (1405 with map positions on the hexaploid wheat genome). The relationship of individuals was assessed using a relationship metric based on allele sharing without the assumption that the alleles were inherited from the same ancestor (Gower's Similarity Metric without Range Standardization as part of the SAS Distance Procedure).

Results

Crossing Block and Chromosome Doubling

The results of the successful crossing block can be seen in Table 2.2. The initial crossing block was germinated in February, and thus was pollinated in June. Although the plants were being kept in a state-of-the-art greenhouse, the first crossing block was unsuccessful due to high temperatures in the greenhouse rendering the pollen sterile. The second crossing block was germinated in August so that pollination would fall in November and December.

As many crosses were made as possible, though a few of the *Ae. speltoides* accessions had poor germination so fewer crosses were made with those accessions. TA 2342, TA 2348 and TA 2362 had very low germination, approximately 40%. TA 2348 produced no F1 seeds with any of the wheat parents, which was unfortunate as TA 2348 was one of the accessions that had demonstrated high heat tolerance in a previous study (Pradhan et al, 2012). Of more than 100 crosses made, nineteen successfully produced F1 seed ranging from one to eight seeds per cross. A total of 63 F1 seeds were produced. Remnant F1 seed was saved, ranging from zero to two seeds per cross when available. Crosses that produced only one seed were germinated for the study. Of those with successful germination, 23 F1 plants survived. Of the 23 plants that were treated with colchicine, ten produced doubled amphiploid seed (Table 2.3).

Nine different crosses are represented in the ten doubled amphiploids that produced seed. Of the ten doubled amphiploids, three produced over 100 seeds that appeared morphologically identical to the seeds of their wheat parents, and very little like the other doubled amphiploid seed. The F2 seed counts and tolerance levels of the *Ae. speltoides* parent were taken into

consideration when deciding which F2 seeds to use in the high temperature screening experiment, which are the highlighted crosses in Table 2.3.

Ion Leakage

The results of the ion leakage assay can be seen in Figure 2.1. The membrane damage is expressed as a percent with lower and upper 95% confidence intervals. The two checks had the highest level of membrane damage, around 15%. Generally, the amphiploids had lower levels of membrane damage, similar to the wheat and *Ae. speltoides* parents, though one amphiploid pedigree (TA2780 x KS031009K-4) had the lowest level of damage (around 8%) as well as the tightest confidence interval.

High Temperature Screen

All heat tolerance levels were represented in the doubled amphiploids, TA2097 was rated low tolerant, TA1793 mid tolerant, and TA2780 high tolerant (Pradhan, 2012). The number of days a plant spent in treatment before the SPAD meter was unable to obtain a chlorophyll reading varied between species. The longest a plant was in treatment was 32 days. The amphiploid TA2780 x KS031009K-4 along with both checks (Overley and Jefimija), had plants in the optimum treatment (25/19^{o}C) that survived past the maximum (32 days). The average number of days that wheat (parents and checks) were in optimum treatment was 31.5 days; for *Ae. speltoides,* the average was 17 days. The plant that survived the longest in the heat treatment (36/30^{o}C) was an amphiploid, TA2780 x KS031009K-4, which was 25 days. The average number of days that wheat (parents and checks) was in heat treatment was 21.25 days whereas the average number of days that *Ae. speltoides* was in heat treatment was 9.5 days. These summary statistics can be seen in Table 2.5.

Days in treatment cannot be considered exclusively when determining heat tolerance. Maturation time is also very important when comparing across species, especially when species exhibit different growth habits as is the case with hexaploid wheat and *Ae. speltoides*. Days to Maturity for selected pedigrees can be seen in Figure 2.2. Maturity was measured from planting date to physical maturity date, as noted in Materials and Methods. The wheat in this study ranges from 130-134 days to maturity. The confidence intervals on Figure 2.2 show that the amphiploids had much more variation in maturity time than either the parents or the controls. The doubled amphiploids with pedigree TA2780 x KS031009K-4, had the longest maturation

time of 164 days. This is 2.5% longer than its *Ae. speltoides* parent and 22.4% longer than its hexaploid wheat parent

The maximum SPAD (i.e., starting SPAD) value for each pedigree can be seen in Table 2.5. The three amphiploids in this study with the highest average max SPAD values were TA1793 x KS031009K-4 measuring 70.2, TA2780 x KS061406-LN-39 measuring 67.5, and TA2780 x KS031009K-4 measuring 66.3. The four hexaploid wheat pedigrees followed: Jefimija at 65.7, Overley at 64.9, KS061406-LN-39 at 63.0 and KS031009K-4 at 62.3. And finally, the two *Ae. speltoides* accessions fell on the bottom: TA2780 at 59.25 and TA1793 at 42.6. These SPAD readings were more than likely all taken before any stress was applied to the plants, approximately 10 days after flowering. Flowering date is important to note as well, since the *Ae. speltoides* accessions and the doubled amphiploids all flowered much later than the wheat lines, which can explain the more rapid decrease in chlorophyll content once in treatment; the plants are farther into their life cycle. The amphiploids had high variation in flowering dates, ranging from that similar to the *Ae. speltoides* to much later. Notably, the amphiploids did not have a quick decline of chlorophyll content like the *Ae. speltoides*, but a decline similar to the hexaploid wheat parents and checks, despite their late flowering date. Average SPAD by treatment day sorted by pedigree can be seen in Figure 2.3. It can be noted in this figure that the SPAD reading of amphiploid TA2780 x KS031009K-4 is similar to its wheat parent. However, in this pedigree, the maturation time of the amphiploid is similar to its *Ae.* speltoides parent (Figure 2.2). Figure 2.4 shows selected pedigrees' average SPAD by treatment. The mean SPAD decline on Days 0, 6 and 12 can be seen in Figure 2.5

Once fully mature, seed was harvested from all doubled amphiploids. The seed counts from and pedigree of the S1 amphiploids can be seen in Table 2.6. Twenty doubled amphiploids produced S2 seed, all of the same pedigree: TA2780 x KS031009K-4. Fourteen plants produced more than 40 seeds, of which five produced more than 100. Perhaps most notable is the fact that only one doubled amphiploid in the heat treatment produced seed.

Marker Sequencing

Figure 2.6 is a heat map and clustering dendrogram representing the allele sharing similarity of the filtered marker sequences. The darkest red boxes diagonally through the center of the map demonstrate 100% similarity of markers when a sample is compared to itself. The red

and yellow boxes in the lower right denote the two *Ae. speltoides* accessions, demonstrating very little diversity within each accession as indicated by the color legend. The most notable observation is that the amphiploids of the same pedigree are not clonal. The largest amphiploid pedigree is TA2780 x KS031009K-4 as is labeled. The allele sharing of plants within this pedigree range from 43% to 78% similarity. This information is reiterated in Figure 2.7, a 2D scatterplot matrix of correlation analysis of Principal components. Two principal components account for 92.4% of the variance in allele calls across all sampled individuals. Genotypes are colored by pedigree matching that of the dendogram and relationship matrix. The two tightly clustered pedigrees (light blue and tan) represent the *Ae. speltoides* genotypes. These clustered pedigrees are also represented on the heat map as two distinct groups sharing over 80% allele similarity (red) within genotype group (Figure 2.7). As shown in the PCA and heat map, the amphiploid genotypes within and across pedigrees are not as tightly clustered as the hexaploid wheat (parents and check) or the *Ae. speltoides* parents but nonetheless are more closely related to each other than they are to either of their parental genotypes.

Sequences were also analyzed for enrichment across the wheat ordered contigs with chromosomal assignments. Figure 2.8 shows how the number of significant markers are distributed across the wheat chromosomes. There is enrichment of significant markers across B chromosomes (especially 3B and 5B) which is expected since *Ae. speltoides* S genome is closely related to the B genome donor of wheat. Chromosome 3B has the most significant markers in part due to the fact that it is the most complete and saturated wheat chromosome.

Discussion

Amphiploids have been widely used in wheat research to create addition or substitution lines with translocations to study chromosomal rearrangements of the hybridizations and introgression of disease resistance. Creating amphiploids to use for potential backcrossing into an elite wheat line certainly merits further investigation. The number of rate-limiting steps and success of amphiploid production is a substantial challenge in studying amphiploids. The actuality of the number of S2 seeds produced in this study will allow for much needed further research in heat tolerance of the two amphiploid pedigrees. Of particular interest is the potential of additional study of the six S2 amphiploid seeds that were produced from a heat stressed plant.

These were the only seeds obtained from a heat stressed amphiploid, so there could be a mechanism present in that genotype that aids in survival.

When making wide hybridizations such as those in this study, timing is of critical importance. As demonstrated by the first crossing block that failed to produce any F1 seed, seasonal weather can have a substantial impact on the success of wide hybridizations. If controlled environments (i.e., growth chambers) are large enough and available, then one can make a crossing block potentially any time of year. However, when using greenhouses, seasons and temperature control are very important. During the summer months in Kansas, there can often be outside temperatures of 100^{o}F or higher, which is only magnified in a greenhouse. Cooling systems struggle to consistently keep the greenhouse at optimum cool temperatures to ensure pollen fertility.

In addition to the challenge of temperature control during wide hybridizations, poor germination rates of some *Ae. speltoides* accessions resulted in less genetic material contributed to the number of amphiploid pedigrees. Unfortunately, the poorest germination rate was of the accession rated the highest in heat tolerance, TA 2348. (Pradhan et al, 2012). Due to the limited seed available, and the nature of planting dates, it was difficult to perform additional germinations to compensate for the low germination rates. If more seed had been germinated later in the season, they would not be shedding pollen in time to be used for crosses. In hindsight, when working with non-domesticated grasses, it is preferable to over germinate. If more *Ae. speltoides* seed had been germinated initially, more crosses could have been made and statistical evaluations of heat tolerance between multiple pedigrees would have been possible.

Germination rates of the wild parent and wide hybridization success are only two of the rate-limiting challenges in the creation of amphiploids that arose through this study. Seed and plant counts can be limited by the initial cross success, germination of F1 seed, saving remnant of F1 seed, plant death by colchicine treatment, the percentage of plants that survive colchicine but do not produce seed, the germination of the doubled amphiploids, and finally the fertility of those doubled amphiploids. Due to all these limiting factors, if a larger initial seed set of each *Ae. speltoides* accession was obtained and germinated then we would expect more S1 seed and possibly, more S2 seed for additional heat tolerance studies.

Upon further research after this experiment, a phenomenon called hybrid grass-clump dwarfness was probably present in the doubled amphiploids. This grass-clump dwarfness has

been known to occur in progeny of intervariety wheat crosses, and causes the progeny to fall into one of three categories: Type 1 dwarfs are short and never reach reproductive stages, type 2 dwarfs produced many more tillers than the parents, are short and occasionaly produce seed, and type 3 dwarfs produced many tillers early in the season but essentially returned to normal growth habit later in the life cycle (Canvin, 1976). The doubled amphiploids in this study were probably type 2 dwarfs. In 1976 Canvin and McVetty described an experiment wherein changing the ambient temperature causes these grass-clump dwarves to break their clump and return to normal growth habits and produce viable seed. To treat type 2 dwarfs, plants must be kept at 21^{o}C for both day and night temperatures. In this study, the plants were kept at 25/19^{o}C day/night temperature with a 14-hour photoperiod so the dwarf clump couldn't break due to the fluctuating temperatures. This information will be useful in future research with the S2 seeds if they exhibit similar grass-clump dwarfness. Perhaps, if this temperature treatment had been used on the doubled amphiploids, more of them would have produced seed, or even have had earlier flowering times.

Three doubled amphiploids produced seed that did not have morphological characters that were a combination of wheat and *Ae. speltoides*. Chromosome count of these S2 seed would have confirmed which lines were true doubled amphiploids and if there is any aneuploidy. Additionally, chromosome counting of progeny at each generation would have been interesting. The F1's could have been confirmed as 28-chromsome 'haploids' (ABDS), then chromosomes could be counted again after doubling to confirm the 56-chromsome doubled amphiploids. It could also be useful to germinate and count a further "selfed" generation to see if aneuploidy is present. There are many methods for counting chromosomes. One method currently used by the WGRC in Manhattan, Kansas involves preserving root tips in acetic acid and using an acetocarmine staining technique before squashing the roots on a microscope slide in the hopes of observing chromosomes in late prophase of meiosis 1. If the chromosomes are successfully squashed and separated, they can be manually counted. Another method of karyotyping doubled amphiploids is by multi-color genomic *in situ* hybridization (GISH). Nemeth et al (2015) used this method to determine the genetic constitution of many doubled amphiploids including a *T. aestivum* cv. Chinese Spring x *Ae. speltoides* pedigree. Chromosome counts and recombination can be seen via the mutli-color GISH method. Chromosome counting by this method would have

been an effective and complementary addition to this study to aid in the understanding of doubled amphiploids chromosome preservation with the germplasm created in this study.

The ion leakage assay used in this study (adapted from Djanaguiramen, 2010) was not very successful and is difficult to extrapolate functional application to this study. Additional replication and more advanced equipment may have enabled ion leakage results to support and supplement the current findings. It was required that the hot water bath be kept at a steady 100^{o}C, but neither the manual or electric water bath available at the time of this research was capable of holding such a high temperature. A stove with a pot of boiling water was used instead, which was very difficult when screening the high number of samples. In hindsight, a different method for determining cell membrane thermal stability could have been used, such as that presented in Ullah et al (2014), that only required water baths to be held at 25 and 46^{o}C, then used an autoclave to reach 120^{o}C. Cell membrane thermal stability has been shown to be linked to heat tolerance in wheat and would have been beneficial to this study (Javed, 2014 and Ullah, 2014). Membrane damage may be the best measure for comparing hexaploid wheat to ancestral grasses and their amphiploids due to the diversity in plant development, life cycle and grain fill period.

Without taking into account maturation time and flowering date, the amphiploids screened for high temperature tolerance did not appear to have higher chlorophyll content during treatment than the wheat. The amphiploids had such a wide range of flowering dates even within a single pedigree. The fact that the chlorophyll content of the amphiploids (that were much older than the wheat plants) was so much higher suggests that the amphiploids can maintain high chlorophyll content through their life cycle when not stressed. The plant that survived the heat stress the longest was an amphiploid. This information, in addition to the non-clonality of the amphiploids, suggests there may be more genetic diversity within amphiploids for traits associated with heat tolerance. The amphiploids demonstrated very high levels of variability (genetically, in chlorophyll content, flowering date and maturation time) even within a single pedigree. This alone is reason to continue this research.

Of the 50 amphiploids that were screened, 20 produced seed, which indicates stability and fertility warranting further investigation. It is unlikely that a single amphiploid could capture all of the heat tolerance alleles from its *Ae.* speltoides parent since each is genetically unique. The *Ae. speltoides* accession TA2780 was rated highly heat tolerant (Pradhan, 2012) and

produced the most amphiploids that generally performed well in the heat screen, leading one to conclude that more research is necessary. Even with the limited data in this study, wheat parent KS031009K-4 seemed to perform well in the high temperature screen. This could be due to the fact that its pedigree contains a Missouri soft wheat. Further testing could reveal this line as a source for improved response to heat that is already available as adapted germplasm.

Table 2.1 Plant material used in this study, their species and use in the project.

ID	Species	Use
KS061406-LN-39	*T. Aestivum*	Wheat parent
KS040640K-1	*T. Aestivum*	Wheat parent
KS030887K-6	*T. Aestivum*	Wheat parent
KS031009K-4	*T. Aestivum*	Wheat parent
KS10HW78-1-1	*T. Aestivum*	Wheat parent
Tiger	*T. Aestivum*	Wheat parent
TA1793	*Ae. speltoides*	Speltoides parent
TA2097	*Ae. speltoides*	Speltoides parent
TA2342	*Ae. speltoides*	Speltoides parent
TA2348	*Ae. speltoides*	Speltoides parent
TA2362	*Ae. speltoides*	Speltoides parent
TA2780	*Ae. speltoides*	Speltoides parent
Overley	*T. Aestivum*	Heat susceptible check
Jefimija	*T. Aestivum*	Heat tolerant check

Table 2.2 Crossing block seed counts including F1 seeds produced, F1 seeds selected (the rest saved as remnant), F1 seeds that successfully germinated and the F1 plants that survived colchicine treatment and produced seed.

Cross	# F1 Seeds	# Selected	# Germinated	# Doubled
TA1793x KS040640K-1	4	1	1	
TA1793x KS031009K-4	3	2	2	1
TA1793x KS030887K-6	8	6	4	
TA1793x KS061406-LN-39	2	1	0	
TA1793x KS10HW78-1-1	4	2	2	1
TA1793xTiger	2	1	1	1
TA2097x KS040640K-1	1	1	0	
TA2097x KS031009K-4	3	2	2	1
TA2097x KS030887K-6	3	2	1	1
TA2097x KS061406-LN-39	3	1	0	
TA2097x KS10HW78-1-1	1	1	1	1
TA2097xTiger	5	2	2	1
TA2342x KS040640K-1	1	1	0	
TA2342x KS061406-LN-39	1	1	1	
TA2362x KS040640K-1	2	1	1	
TA2780x KS031009K-4	7	5	4	2
TA2780x KS030887K-6	3	1	0	
TA2780x KS061406-LN-39	8	6	1	1
TA2780x KS10HW78-1-1	2	1	0	
TOTAL	**63**	**38**	**23**	**10**

Table 2.3 S1 doubled amphiploid seed counts (from the 10 doubled plants), their pedigree and appearance notes. The highlighted seeds were those germinated for the high temperature screen based on seed count and heat tolerance level of *Ae. speltoides* parent (low tolerance accession TA2097 progeny was not selected for heat screen).

F1 Pedigree	# Doubled Amphiploid Seeds (S1)	Notes
TA1793x KS031009K-4	2	
TA1793x KS10HW78-1-1	1	
TA1793xTiger	127	Looks like wheat
TA2097x KS031009K-4	6	
TA2097x KS030887K-6	1	Very tiny, probably not viable
TA2097x KS10HW78-1-1	230	Looks like wheat
TA2097xTiger	249	Looks like wheat
TA2780x KS031009K-4	24	
TA2780x KS031009K-4	18	
TA2780x KS061406-LN-39	6	

Table 2.4 Plants in high temperature screen, their treatments, and pedigrees.

				Tissue sampled for	
Plant #	**ID**	**Temperature Treatment**	**Pedigree**	**Ion Leakage**	**Marker Sequencing**
1	H1	Heat	TA1793 x KS031009K-4	yes	yes
2	N1	Optimum	TA1793 x KS031009K-4	yes	yes
3	H2	Heat	TA2780 x KS061406-LN-39	yes	yes
4	H3	Heat	TA2780 x KS061406-LN-39		yes
5	H4	Heat	TA2780 x KS061406-LN-39	yes	yes
6	N2	Optimum	TA2780 x KS061406-LN-39	yes	yes
7	N3	Optimum	TA2780 x KS061406-LN-39	yes	yes
8	N4	Optimum	TA2780 x KS061406-LN-39	yes	yes
9	H5	Heat	TA2780 x KS031009K-4	yes	yes
10	H6	Heat	TA2780 x KS031009K-4	yes	yes
11	H7	Heat	TA2780 x KS031009K-4	yes	yes
12	H8	Heat	TA2780 x KS031009K-4		
13	H9	Heat	TA2780 x KS031009K-4	yes	yes
14	H10	Heat	TA2780 x KS031009K-4	yes	yes
15	H11	Heat	TA2780 x KS031009K-4	yes	yes
16	H12	Heat	TA2780 x KS031009K-4	yes	yes
17	H13	Heat	TA2780 x KS031009K-4	yes	yes
18	H14	Heat	TA2780 x KS031009K-4	yes	yes
19	H15	Heat	TA2780 x KS031009K-4	yes	yes
20	H16	Heat	TA2780 x KS031009K-4	yes	yes
21	N5	Optimum	TA2780 x KS031009K-4	yes	
22	N6	Optimum	TA2780 x KS031009K-4	yes	yes
23	N7	Optimum	TA2780 x KS031009K-4		yes
24	N8	Optimum	TA2780 x KS031009K-4	yes	yes
25	N9	Optimum	TA2780 x KS031009K-4	yes	yes
26	N10	Optimum	TA2780 x KS031009K-4	yes	yes
27	N11	Optimum	TA2780 x KS031009K-4	yes	yes
28	N12	Optimum	TA2780 x KS031009K-4	yes	yes
29	N13	Optimum	TA2780 x KS031009K-4	yes	yes
30	N14	Optimum	TA2780 x KS031009K-4		yes
31	N15	Optimum	TA2780 x KS031009K-4	yes	yes
32	N16	Optimum	TA2780 x KS031009K-4	yes	yes
33	H17	Heat	TA2780 x KS031009K-4	yes	yes
34	H18	Heat	TA2780 x KS031009K-4	yes	yes
35	H19	Heat	TA2780 x KS031009K-4	yes	yes
36	H20	Heat	TA2780 x KS031009K-4	yes	yes
37	H21	Heat	TA2780 x KS031009K-4	yes	yes
38	H22	Heat	TA2780 x KS031009K-4	yes	yes

39	H23	Heat	TA2780 x KS031009K-4	yes	yes
40	H24	Heat	TA2780 x KS031009K-4	yes	yes
41	H25	Heat	TA2780 x KS031009K-4	yes	yes
42	N17	Optimum	TA2780 x KS031009K-4	yes	yes
43	N18	Optimum	TA2780 x KS031009K-4	yes	
44	N19	Optimum	TA2780 x KS031009K-4	yes	yes
45	N20	Optimum	TA2780 x KS031009K-4	yes	yes
46	N21	Optimum	TA2780 x KS031009K-4	yes	yes
47	N22	Optimum	TA2780 x KS031009K-4		yes
48	N23	Optimum	TA2780 x KS031009K-4	yes	yes
49	N24	Optimum	TA2780 x KS031009K-4	yes	yes
50	N25	Optimum	TA2780 x KS031009K-4	yes	yes
51	H26	Heat	TA1793	yes	yes
52	H27	Heat	TA1793	yes	yes
53	H28	Heat	TA1793	yes	yes
54	H29	Heat	TA1793	yes	yes
55	H30	Heat	TA1793	yes	yes
56	N26	Optimum	TA1793	yes	yes
57	N27	Optimum	TA1793	yes	yes
58	N28	Optimum	TA1793	yes	yes
59	N29	Optimum	TA1793	yes	yes
60	N30	Optimum	TA1793	yes	yes
61	H31	Heat	TA2780	yes	yes
62	H32	Heat	TA2780	yes	yes
63	H33	Heat	TA2780	yes	yes
64	H34	Heat	TA2780	yes	yes
65	H35	Heat	TA2780	yes	yes
66	N31	Optimum	TA2780	yes	yes
67	N32	Optimum	TA2780	yes	yes
68	N33	Optimum	TA2780	yes	yes
69	N34	Optimum	TA2780	yes	yes
70	N35	Optimum	TA2780	yes	yes
71	H36	Heat	KS031009K-4	yes	yes
72	H37	Heat	KS031009K-4	yes	yes
73	H38	Heat	KS031009K-4	yes	yes
74	H39	Heat	KS031009K-4	yes	yes
75	H40	Heat	KS031009K-4	yes	yes
76	N36	Optimum	KS031009K-4	yes	yes
77	N37	Optimum	KS031009K-4	yes	yes
78	N38	Optimum	KS031009K-4	yes	yes
79	N39	Optimum	KS031009K-4	yes	yes

80	N40	Optimum	KS031009K-4	yes	yes
81	H41	Heat	KS061406-LN-39	yes	yes
82	H42	Heat	KS061406-LN-39	yes	yes
83	H43	Heat	KS061406-LN-39	yes	yes
84	H44	Heat	KS061406-LN-39	yes	yes
85	H45	Heat	KS061406-LN-39	yes	yes
86	N41	Optimum	KS061406-LN-39	yes	yes
87	N42	Optimum	KS061406-LN-39	yes	yes
88	N43	Optimum	KS061406-LN-39	yes	yes
89	N44	Optimum	KS061406-LN-39	yes	yes
90	N45	Optimum	KS061406-LN-39	yes	
91	H46	Heat	Overley	yes	
92	H47	Heat	Overley	yes	
93	H48	Heat	Overley	yes	
94	H49	Heat	Overley	yes	
95	H50	Heat	Overley	yes	
96	N46	Optimum	Overley	yes	
97	N47	Optimum	Overley	yes	
98	N48	Optimum	Overley	yes	
99	N49	Optimum	Overley	yes	
100	N50	Optimum	Overley	yes	
101	H51	Heat	Jefimija	yes	
102	H52	Heat	Jefimija	yes	
103	H53	Heat	Jefimija	yes	
104	H54	Heat	Jefimija	yes	
105	H55	Heat	Jefimija	yes	
106	N51	Optimum	Jefimija	yes	
107	N52	Optimum	Jefimija	yes	
108	N53	Optimum	Jefimija	yes	
109	N54	Optimum	Jefimija	yes	
110	N55	Optimum	Jefimija	yes	

Figure 2.1 Percent membrane damage measured with ion leakage assay.

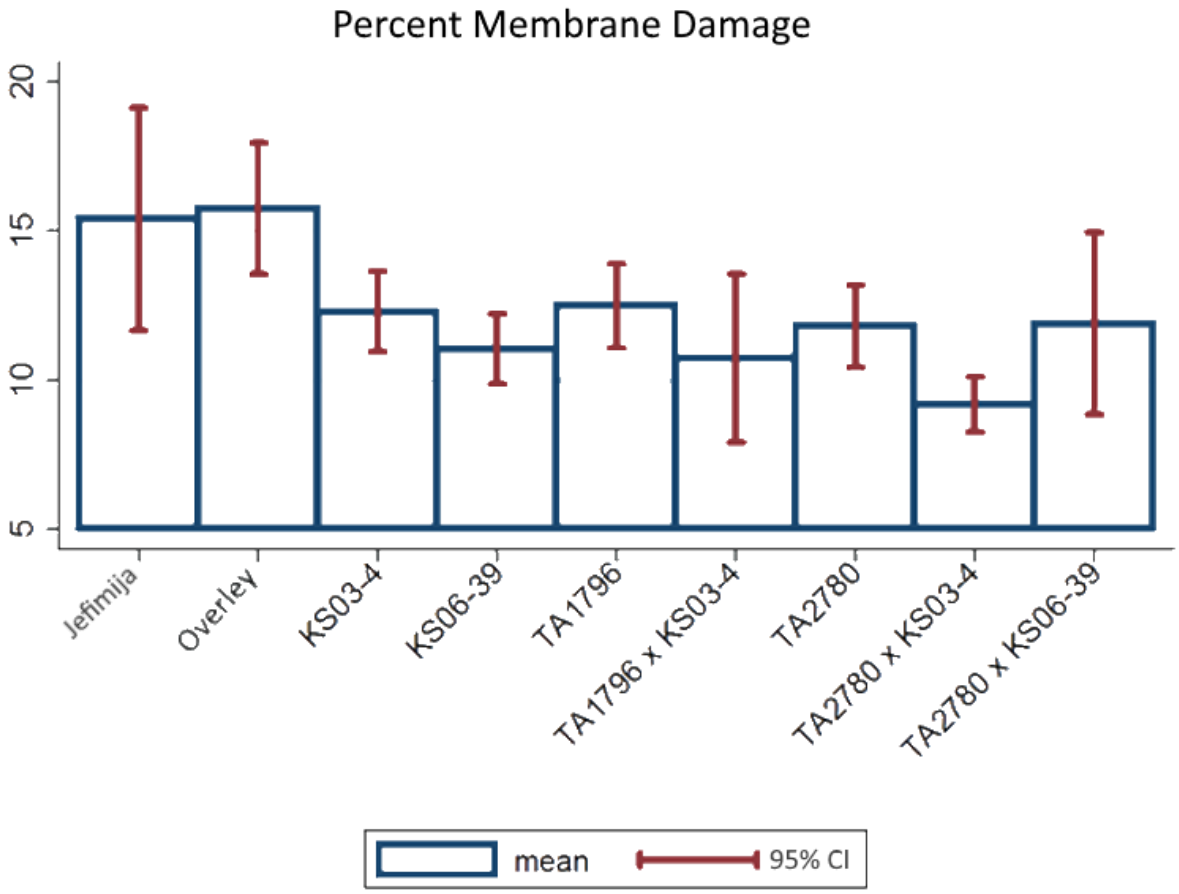


Table 2.5 Summary statistics including minimum SPAD, maximum SPAD, and maximum number of days in treatment for each pedigree in each treatment.

Pedigree	Treatment	N	Max Days in Treatment	Min SPAD	Max SPAD
TA1793 x KS031009K-4	Optimum	1	30	19.6	70.2
	Heat	1	0	N/A	N/A
TA2780 x KS061406-LN-39	Optimum	3	25	3.7	66.3
	Heat	3	7	6.9	68.7
TA2780 x KS031009K-4	Optimum	21	32	0.9	66.2
	Heat	21	25	0.2	66.3
TA1793	Optimum	5	20	2.6	41.1
	Heat	5	9	1.9	44.0
TA2780	Optimum	5	14	0.8	58.8
	Heat	5	10	1.9	59.7
KS031009K-4	Optimum	5	31	1.1	63.2
	Heat	5	23	1.3	61.4
KS061406-LN-39	Optimum	5	31	0.8	62.2
	Heat	5	20	2.2	63.8
Overley	Optimum	5	32	1.4	66.4
	Heat	5	23	3.1	63.5
Jefimija	Optimum	5	32	9.7	69.9
	Heat	5	19	2.1	61.4

Figure 2.2 Days to maturity measured from transplant date to physiological maturity.

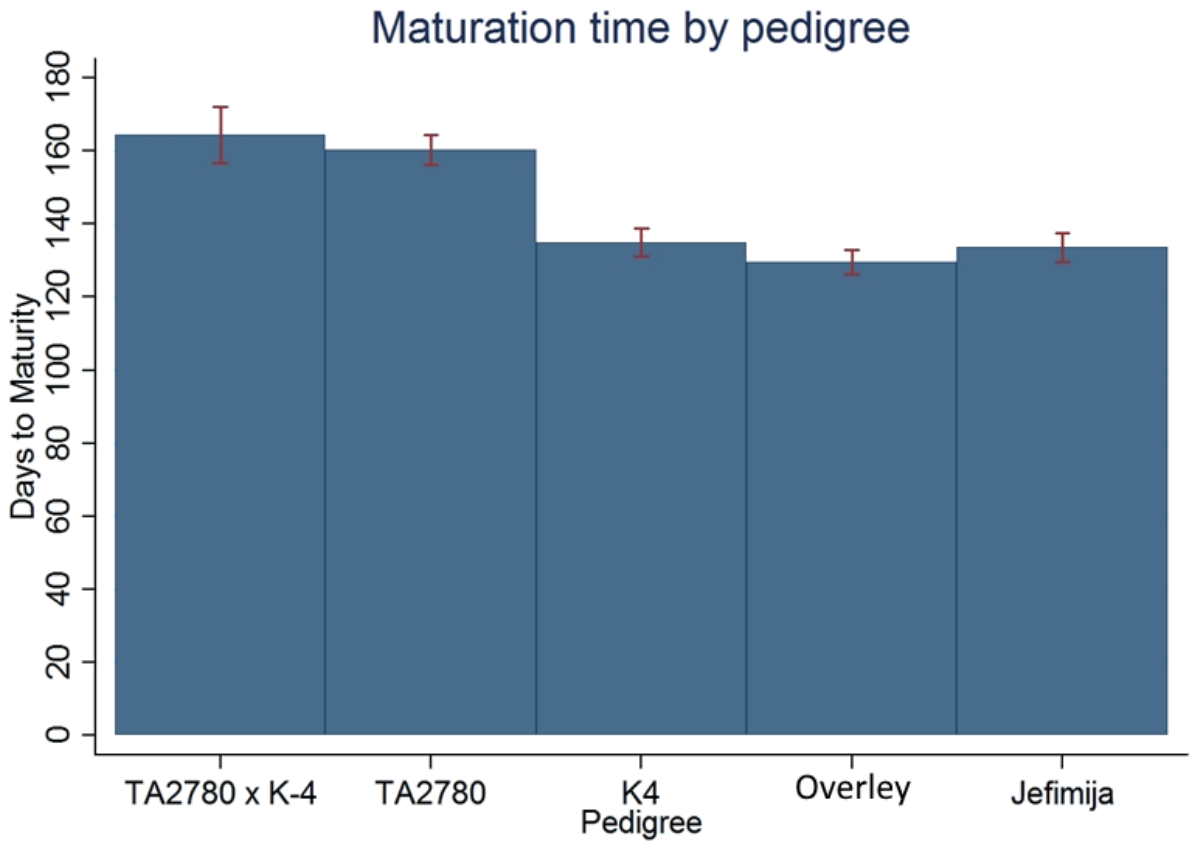


Figure 2.3 Comparison of average chlorophyll content by pedigree and treatment.

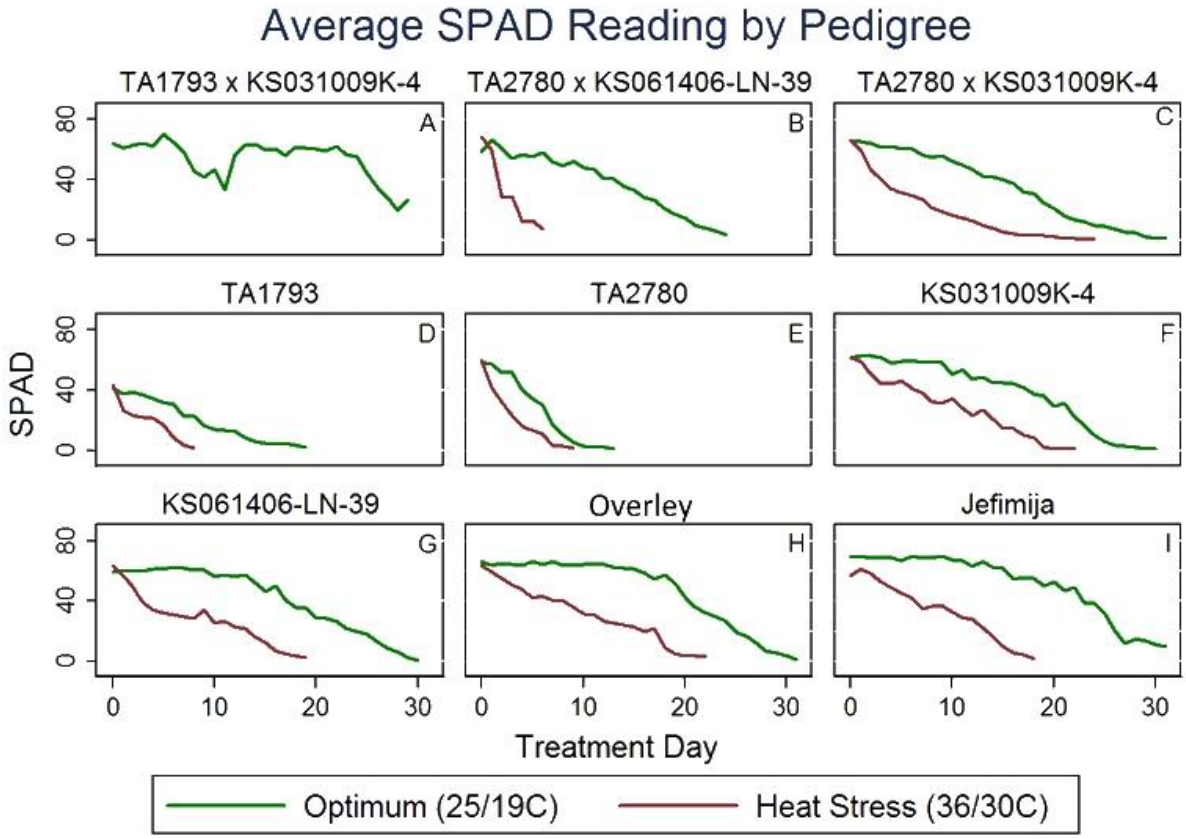


Figure 2.4 Average chlorophyll content by treatment for selected pedigrees.

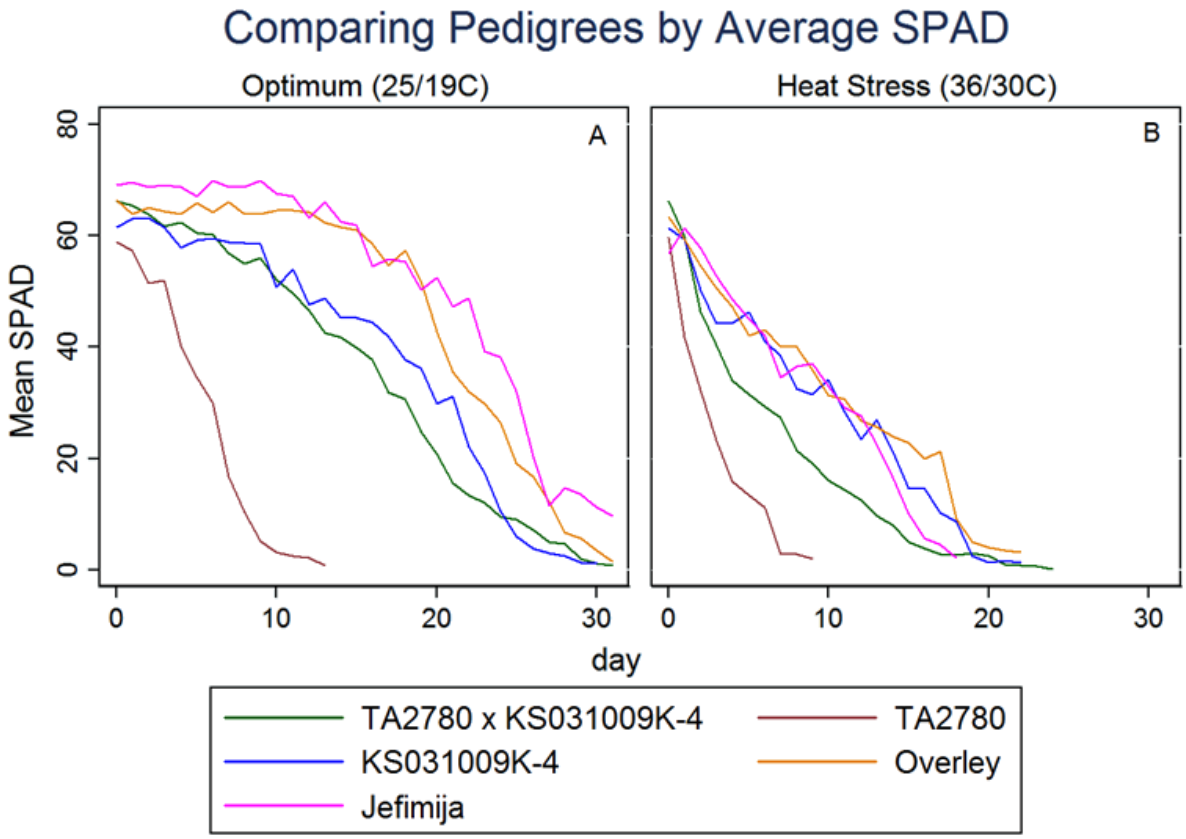


Figure 2.5 Mean chlorophyll content by temperature treatment on days 0, 6 and 12 of treatment.

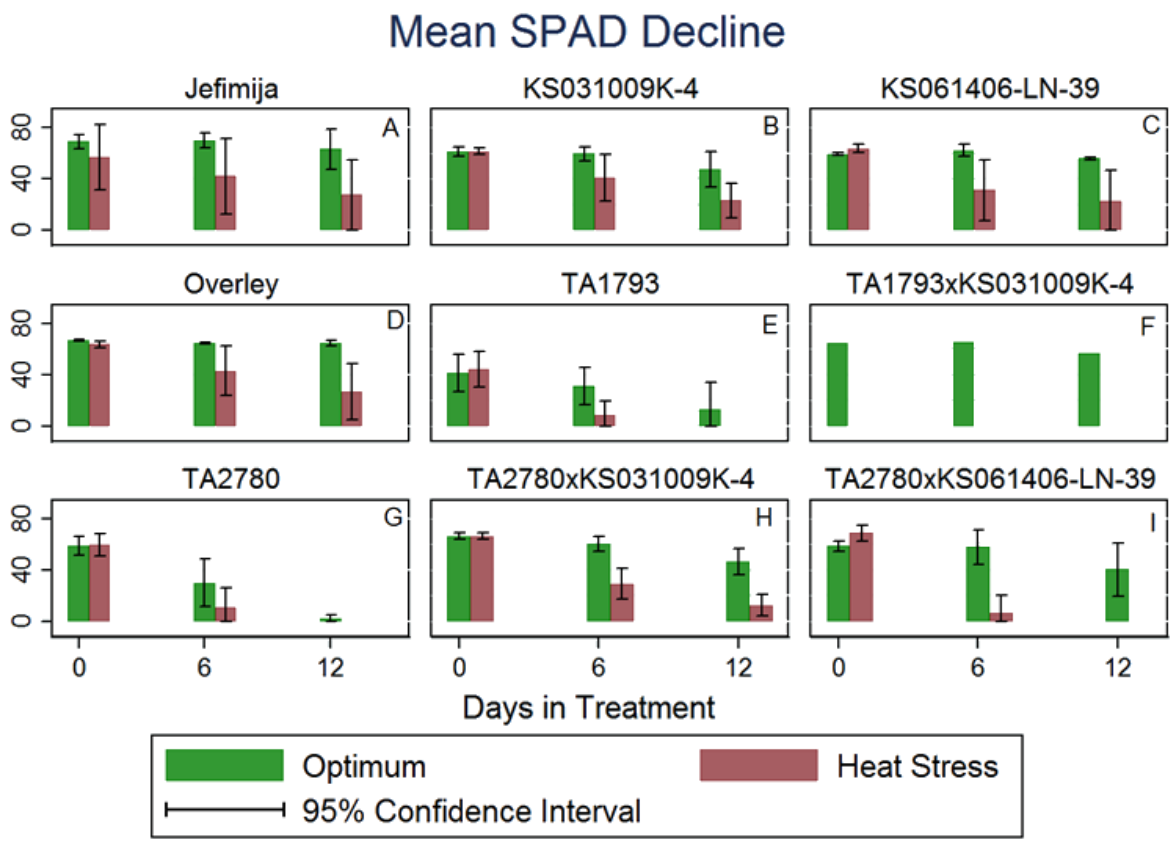


Table 2.6 Doubled amphiploids (S2) seed counts and pedigrees. Highlighted seed was from the only amphiploid that produced seed while in heat treatment.

Plant ID	# S2 Seeds	Pedigree
N6	40+	TA2780 x KS031009K-4
N7	1	TA2780 x KS031009K-4
N8	40+	TA2780 x KS031009K-4
N9	40+	TA2780 x KS031009K-4
N10	100+	TA2780 x KS031009K-4
N11	100+	TA2780 x KS031009K-4
N12	40+	TA2780 x KS031009K-4
N13	40+	TA2780 x KS031009K-4
N14	19	TA2780 x KS031009K-4
N15	100+	TA2780 x KS031009K-4
N16	8	TA2780 x KS031009K-4
N17	40+	TA2780 x KS031009K-4
N18	100+	TA2780 x KS031009K-4
N19	3	TA2780 x KS031009K-4
N20	40+	TA2780 x KS031009K-4
N21	40+	TA2780 x KS031009K-4
N23	40+	TA2780 x KS031009K-4
N24	100+	TA2780 x KS031009K-4
N25	4	TA2780 x KS031009K-4
H22	6	TA2780 x KS031009K-4

Figure 2.6 Sequence comparison heat map and dendrogram.

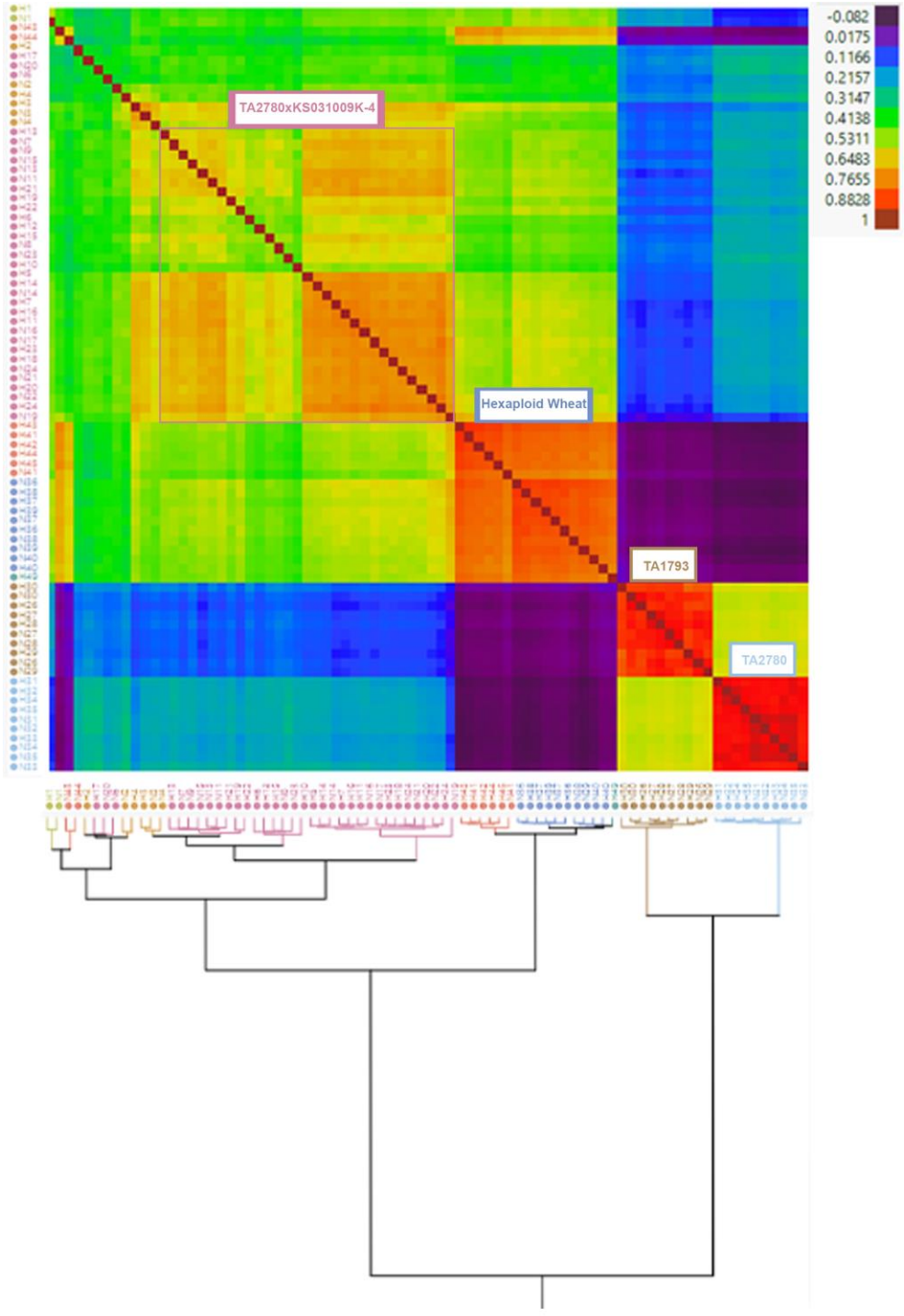


Figure 2.7 2D scatterplot matrix of correlation analysis of principal components.

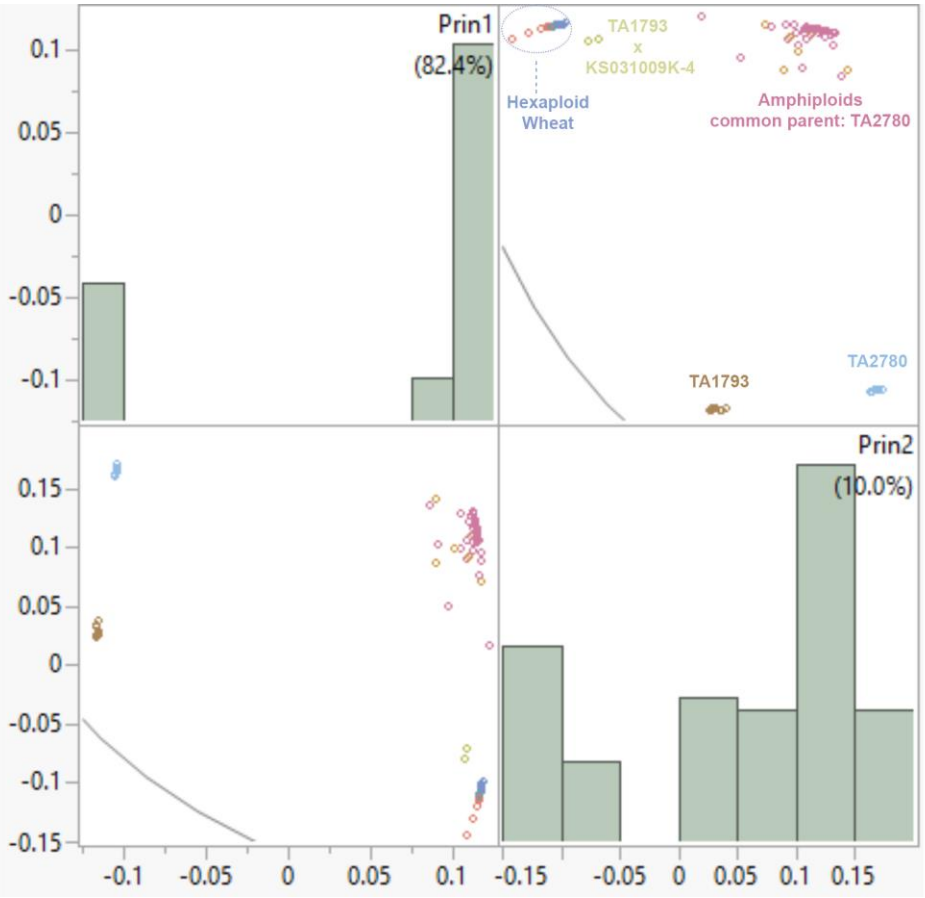
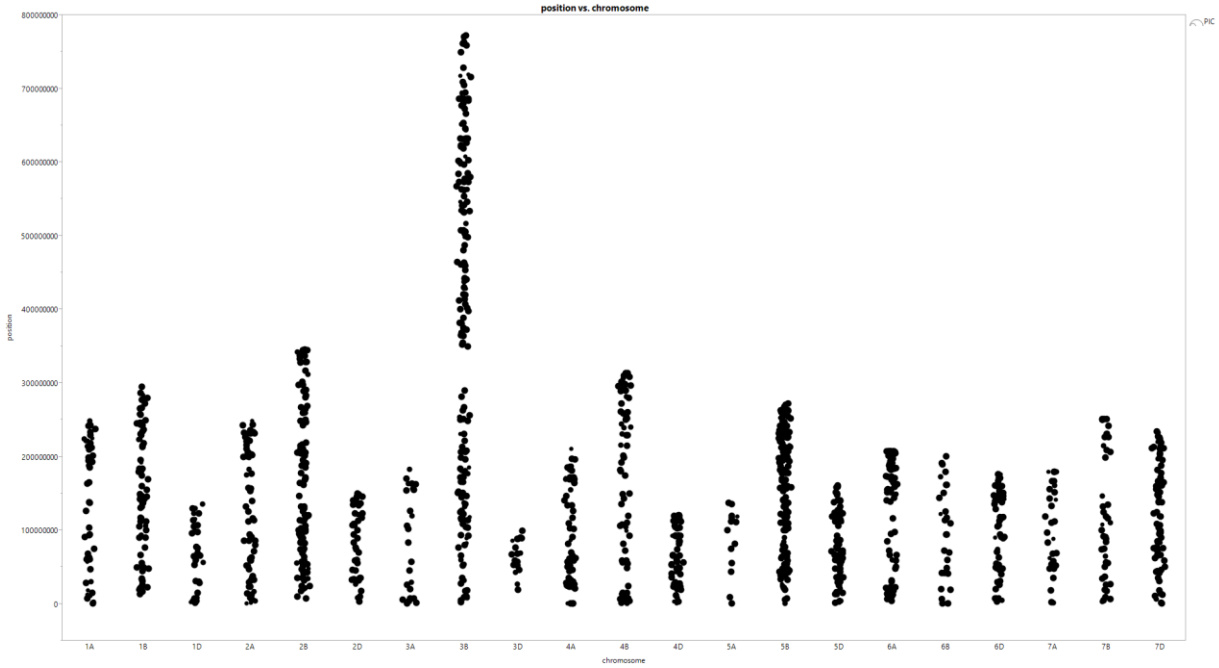


Figure 2.8 Saturation of filtered markers assigned to wheat genome assembly.



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