HEAT TOLERANCE STUDIES FOR WHEAT IMPROVEMENT

by

SHYAMAL KRISHNA TALUKDER

B.Sc., Bangladesh Agricultural University, Bangladesh, 2001

M.S., Bangladesh Agricultural University, Bangladesh, 2002

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Agronomy
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2013

Abstract

Heat stress is one of the major environmental constraints for wheat production worldwide. High temperature during grain filling in wheat leads to a significant reduction in yield. In this research, three different projects were completed. The first project was to study cytoplasmic effects on heat tolerance in wheat, where ten different alloplasmic lines of wheat were backcrossed with four different wheat varieties: 'Karl 92', 'Ventnor', 'U1275' and 'Jagger'. The nuclear genome of the alloplasmic lines was substituted by backcrossing six times using the recurrent parents as males. During the fifth and/or fourth backcross, reciprocal crosses were made to develop NILs (Near Isogenic Lines) for cytoplasm. Sixty-eight NILs and their parents were evaluated in growth chambers for post-anthesis heat tolerance. Plants were grown in the greenhouse and placed under heat stress for 14 days starting at 10 days after anthesis. Growth chambers were maintained at 35°/30°C for heat stress and the greenhouse was maintained at 20°/15°C as the optimum temperature. Effects of high temperature on chlorophyll content and F_v/F_m (a chlorophyll fluorescence measuring parameter) were found to be significant. Cytoplasms 1, 4, 5, 8, 9 and10 provided greater tolerance in one or more nuclear backgrounds. These results indicated that cytoplasmic effects can contribute to heat tolerance of wheat. The second project focused on identification of quantitative trait loci (QTL) for thylakoid membrane damage (TMD), SPAD chlorophyll content (SCC) and plasma membrane damage (PMD), as these traits are found to be associated with resistance to heat stress and contributes to relatively stable yield under high temperature. A RIL (Recombinant Inbred line) population of a cross between winter wheat cultivars 'Ventnor' and 'Karl 92' was evaluated using two different temperature regimes (20°/15°C, 36°/30°C) imposed at ten days after anthesis. The aforementioned traits were evaluated and associated with various molecular markers (SSR, AFLP and SNP). The putative

QTL associated are localized on chromosomes 6A, 7A, 1B, 2B and 1D and have the potential to be used in marker assisted selection for improving heat tolerance in wheat. In the third project, a transgenic approach to increase grain fill during high temperatures was investigated. Grain fill is reduced at temperatures above 25°C in wheat partly due to the inactivity of soluble starch synthase. We isolated a soluble starch synthase gene from rice that has the potential to overcome this deficiency during high temperatures and placed it behind both a constitutive promoter and an endosperm-specific promoter. Transgene expression and the effects of the transgene expression on grain yield-related traits for four generations (T_0, T_1, T_2 and T_3) were monitored. The results demonstrated that even after four generations, the transgene was still expressed at high levels, and transgenic plants produced grains of greater seed weight than Bobwhite control plants under the same environmental conditions. Thousand-seed weight under high temperatures increased 21-34% in T_2 and T_3 transgenic plants when compared to the non-transgenic control plants. In addition, the duration of photosynthesis was longer in transgenic wheat than in non-transgenic controls. Our study demonstrated that expression of rice soluble starch synthase gene in wheat can improve wheat yield under heat stress conditions.

HEAT TOLERANCE STUDIES FOR WHEAT IMPROVEMENT

by

SHYAMAL KRISHNA TALUKDER

B.Sc., Bangladesh Agricultural University, Bangladesh, 2001

M.S., Bangladesh Agricultural University, Bangladesh, 2002

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Agronomy
College of agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2013

Approved by:

Major Professor
Allan K. Fritz

Abstract

Heat stress is one of the major environmental constraints for wheat production worldwide. High temperature during grain filling in wheat leads to a significant reduction in yield. In this research, three different projects were completed. The first project was to study cytoplasmic effects on heat tolerance in wheat, where ten different alloplasmic lines of wheat were backcrossed with four different wheat varieties: 'Karl 92', 'Ventnor', 'U1275' and 'Jagger'. The nuclear genome of the alloplasmic lines was substituted by backcrossing six times using the recurrent parents as males. During the fifth and/or fourth backcross, reciprocal crosses were made to develop NILs (Near Isogenic Lines) for cytoplasm. Sixty-eight NILs and their parents were evaluated in growth chambers for post-anthesis heat tolerance. Plants were grown in the greenhouse and placed under heat stress for 14 days starting at 10 days after anthesis. Growth chambers were maintained at 35°/30°C for heat stress and the greenhouse was maintained at 20°/15°C as the optimum temperature. Effects of high temperature on chlorophyll content and F_v/F_m (a chlorophyll fluorescence measuring parameter) were found to be significant. Cytoplasms 1, 4, 5, 8, 9 and10 provided greater tolerance in one or more nuclear backgrounds. These results indicated that cytoplasmic effects can contribute to heat tolerance of wheat. The second project focused on identification of quantitative trait loci (QTL) for thylakoid membrane damage (TMD), SPAD chlorophyll content (SCC) and plasma membrane damage (PMD), as these traits are found to be associated with resistance to heat stress and contributes to relatively stable yield under high temperature. A RIL (Recombinant Inbred line) population of a cross between winter wheat cultivars 'Ventnor' and 'Karl 92' was evaluated using two different temperature regimes (20°/15°C, 36°/30°C) imposed at ten days after anthesis. The aforementioned traits were evaluated and associated with various molecular markers (SSR, AFLP and SNP). The putative

QTL associated are localized on chromosomes 6A, 7A, 1B, 2B and 1D and have the potential to be used in marker assisted selection for improving heat tolerance in wheat. In the third project, a transgenic approach to increase grain fill during high temperatures was investigated. Grain fill is reduced at temperatures above 25°C in wheat partly due to the inactivity of soluble starch synthase. We isolated a soluble starch synthase gene from rice that has the potential to overcome this deficiency during high temperatures and placed it behind both a constitutive promoter and an endosperm-specific promoter. Transgene expression and the effects of the transgene expression on grain yield-related traits for four generations (T_0, T_1, T_2 and T_3) were monitored. The results demonstrated that even after four generations, the transgene was still expressed at high levels, and transgenic plants produced grains of greater seed weight than Bobwhite control plants under the same environmental conditions. Thousand-seed weight under high temperatures increased 21-34% in T_2 and T_3 transgenic plants when compared to the non-transgenic control plants. In addition, the duration of photosynthesis was longer in transgenic wheat than in non-transgenic controls. Our study demonstrated that expression of rice soluble starch synthase gene in wheat can improve wheat yield under heat stress conditions.

Table of Contents

List of Figures

List of Tables

Acknowledgements

I express my deepest gratitude to my advisor and committee chair Dr. Allan K Fritz, for accepting me into his program and for his continued professional guidance, encouragement, support, kindness, constructive criticism, and enthusiastic help throughout my graduate study at Kansas State University. I must acknowledge his humor, patience and all time positive attitudes, which not only helped me to better continue with my research, but also encourage me to become a good human being. I would like to express my gratitude and appreciation to my advisory committee members, Dr. Robert L. Bowden, Dr. Harold N. Trick, Dr. P.V. Vara Prasad and Dr. Jesse Poland for their time, intellectual support, constructive criticism, encouragement and suggestions during my study and insightful comments to this dissertation.

I must acknowledge the financial support from the Kansas Wheat Commission, the Kansas Crop Improvement Association, Department of Agronomy and Kansas State University. I also thank United States Department of Agriculture for providing me research facilities. A thank to National Small Grain Collection (NSGC) for providing me alloplasmic wheat seeds.

I would like to thank Dr Ali Babar, Timothy C. Todd, Dr. Sunish Sehgal, Dr. Peter Nyori, Adedayo Adeyanju, Chris Gaynor, Dr Jianming Fu, Dr. Shubing Liu, Trevor Rife, Dr. Gautam Pradhan, Dr. JiaRui Li, George Mahama and Sivakumar Sukumaran for their valuable discussion and help to conduct my research.

I must thank Andy Auld, Kimberly Suther, Shuangye Wu, Robert Steele, Angie Matthews, Andrew Green, Sarah Battenfield, Shaun Winnie, Julie Essig, Shiela, Jessica, Hyeonju Lee, Adam and Kelly for their friendly cooperation and help in the lab. I also thank all the previous and present student workers in the KSU Wheat Breeding and Genetics Program, for their direct and/ or indirect contributions to this work.

I express my gratitude to Ranjan Kumar Biswas, Professor Bimal Paul and his wife Anjali Paul and all my friends in Manhattan, who made my stay here as a wonderful experience and helped indirectly to my education.

I am indebted to my parents, brother, sisters, and in-laws for their love, constant support and encouragement. I must acknowledge my wife Smriti, without her support and continuous encouragements my PhD endeavor might have not been possible. Last but not least, Thanks to my surroundings for love, respect, and friendship.

Dedication

I would like to dedicate this dissertation to my late father Harekrishna Talukder and mother Malati Talukder for their never ending love and encouragement.

Chapter 1 - Review of literature

Overview

Wheat (*Triticum aestivum* L. em Thell.) is one of the most important & strategic staple food crops for the majority of world's population. In global consumption, wheat provides almost 55% of the total carbohydrates, 20% of the food calories and 21 % of the protein (Breiman and Graur, 1995; FAO, 2011). On the basis of acreage, wheat production exceeds every other grain crops including maize (*Zea mays* L.), rice (*Oryza sativa* L.) and sorghum (*Sorghum bicolor* L.) (FAO, 2011).

The world food security issue is becoming very critical because of the increase of the world population. In the year 2020, the global wheat demand has been projected to be double the current production level. To cope with the demand, a step–wise annual increase in global wheat production becomes very important (Braun et al., 1998). Over the years, wheat breeding has significantly contributed to increasing the yield potential throughout the world. Along with genetic potential, factors like heat, drought and disease also significantly affect ultimate yield. In addition, an average of 0.5 to 1.0% genetic gain of wheat per year is unable to keep pace with over 2% per year increased demand in developing countries. Though there is genetic potential to increase wheat yield, wheat yield is highly limited under stress conditions. As a result, addressing abiotic factors like heat and drought is very important for wheat improvement (Bahar et al., 2011).

Chronic high temperature is defined as mean temperature during the growth cycle ranging from 18 to 24°C, with maximum day time temperature reaching up to 32°C. Heat shock occurs if the maximum day time temperature exceeds 32°C after the mid-reproductive stage

(Wardlaw and Wrigley, 1994; Cossani and Reynolds, 2012). Only five to six days of 28-32°C average temperature can cause 20% yield reduction in wheat (Stone and Nicolas, 1994). According to Acevedo et al. (1991), every 1°C increase over 17 to 24°C average temperatures during grain filling can causes 4% yield reduction. Almost all the temperate, arid, semi-arid, tropical, and sub-tropical wheat production is affected by heat stress (Paliwal et al., 2012; Fischer, 1986).

High temperature, which is often combined with drought, reduces the yield and quality of wheat (Blumenthal et al., 1995; Maestri et al., 2002; Wardlaw et al., 2002). Heat stress during grain filling leads to a significant reduction in yield, biomass, grain number, harvest index and thousand-kernel weight (Balla et al., 2009). Different traits like early ground cover, leaf rolling, plant height, earliness, grain filling duration, and stay green are associated with tolerance to heat stress (Fokar et al., 1998; Reynolds et al., 2001). Farooq et al. (2011) noted that grain fill rate is accelerated by heat stress, which results in the reduction of grain fill duration (Dias and Lidon, 2009). According to Yin et al. (2009) a 5°C increase over 20°C reduced the grain fill duration by 12 days in wheat even though it increased the grain fill rate. In another study by Streck (2005), it was reported that every for 1°C above a temperature of 15–20°C, grain-filling duration was reduced by 2.8 days.

Grain quantity, grain diameter, protein content and the ratio of protein components are much more sensitive to heat stress in earlier developmental phase of wheat than in late developmental phases (Balla et al., 2009). According to Asseng et al. (2011) high temperature reduces the growth period by accelerating phenological development. In some other studies, wheat yield was found to be reduced under heat stress under well-watered field conditions because of shortening the growing season (Wheeler et al.,1996; Lobell and Ortiz-Monasterio,

2007), less kernels per unit area (Fischer, 1985) and less light interception (Rawson, 1988). An experiment by Ferris et al. (1998) found that 12 days of 40°C heat stress on wheat before and after anthesis, with a temperature gradient tunnel, caused 50% reduction in kernels per square meter resulting in 50% yield reduction. Dias and Lidon (2009) reported a maximum temperature of 31°C, compared with 25°C, caused 15% kernel weight reduction due to shorter grain filling duration, whereas a 35% reduction was reported by Spiertz (1977). After reviewing many experiments, Wardlaw and Wrigley (1994) reported that in Australia and the United States 10 - 15% yield reduction occurred every year because of above optimum temperature during anthesis and grain fill.

High temperature affects wheat production in more than 50 countries that import almost 20 million tons of wheat per year (Reynolds et al., 2001). Moreover, the exponential increase in world population has made us realize the need of expanding production into warmer climates. Considering all these factors, development of heat tolerant cultivar is a major concern for wheat breeding programs around the world (Wardlaw et al., 2002).

Screening for tolerant genotypes is the principal way for the breeders to develop heat tolerant varieties. In most of the cases, yield is evaluated under heat stress (Ozkan et al., 1998). However, this process is not effective in early generations because of cost, time, labor and equipment necessary for such evaluation in a breeding program. Moreover, heat stress is unpredictable in the field and very much associated with drought stress. Additionally, heat and drought stress do not occur evenly in the field.

It has been shown that heat tolerance is a quantitatively inherited trait that follows a continuous distribution (Yang et al., 2002b). Complexity in phenotypic selection for heat tolerance makes marker assisted selection (MAS) an effective approach to improve heat tolerance (Foolad, 2005).

As a result, plant breeders have begun depending on MAS and QTL mapping for traits associated with heat tolerance. This approach requires identification of genetic markers that are associated with genes or QTL affecting plant heat tolerance. Gene manipulation might also be a positive approach to deal with heat stress (Zhang et al., 2001). Recently, significant progress has been made in the identification of genes, enzymes, proteins, mechanisms or compounds with remarkable effect on plant stress tolerance (Apse and Blumwald, 2002; Bohnert et al., 2006). Transgenic approaches have resulted in the development of plants with enhanced stress tolerance in different plant species (Rontein et al., 2002).

The evolution of modern wheat cultivars

Modern cultivars of wheat primarily belong to two types of polyploid species: hexaploid bread wheat-type, and tetraploid durum-type. Common bread wheat (*Triticum aestivum* L.) is a disomic allohexaploid (AABBDD; 2n = 42) having three diploid genomes (A, B, and D) acquired through two separate amphidiploidization events. In the first event (at least 30,000 years ago), wild einkorn wheat**,** *T. urartu* (AA) Tumanian ex Gandilyan hybridized with a goat grass closely related to *Aegilops speltoides* (SS) Tausch to produce the predominantly self-pollinating wild emmer wheat (*T. dicoccoides;* 2n = 4x = 28; genome AABB) after spontaneous chromosome doubling. In the second event (about 9,000 years ago) the domesticated emmer wheat, *Triticum turgidum* spp. *dicoccon* (AABB, 2n = 28) hybridized with *Aegilops tauschii* ssp.

strangulata (syn. *Aegilops squarrosa* L., genome DD, 2n =14) and formed *Triticum aestivum (*2n =6X= 42; genome AABBDD) after chromosome doubling and domestication (Feldman, 2001). (http://www.k-state.edu/wgrc/ and http://www.newhallmill.org.uk/wht-evol.htm).

Cytoplasmic and nuclear genome interaction

Proteins encoded by the nuclear genome are predominantly involved with almost all functional activities and play key roles during growth and development of living cells. Limited, but substantial, numbers of proteins are also encoded from extra nuclear genomes located in the cytoplasm. These extra nuclear proteins interact directly with nuclear proteins for organization and structure and are involved with signaling for the activation of nuclear genes (Pesaresi et al., 2007 and 2006). The redox state of the plastoquinone pool in chloroplasts may influence the regulation of nuclear gene expression. Kinases and phosphatases might be involved in signal transduction from chloroplast to nucleus (Surpin and Chory, 1997; Woodson and Chory, 2008 and 2012).

Reactive oxygen species (ROS) are synthesized as byproducts of various metabolic reactions in plants, and include oxygen ions, free radicals, and inorganic and organic peroxides. ROS production results in plants undergoing oxidative stress and, ultimately, damage to the cell structure. Under stress conditions, ROS accumulation is elevated. Green plants produce singlet oxygen $^1O_2$ in photosystem II (PS II) and the superoxide anion in photosystem I (PSI) under heat stress. Both of these were shown to be involved in signaling and influence the expression of nuclear genes. Based on a review of literature, it was assumed that the singlet oxygen enhanced the expression of 70 nuclear genes and inhibited the expression of nine nuclear genes in plants (Yurina and Odintsovain, 2011).

As the site of two extra-nuclear genomes, the cytoplasm affects various phenotypic traits of crop plants. Cytoplasmic male sterility is a well known phenotype controlled by cytoplasmic genes (Hanson, 1991). The cytoplasm has also been shown to influence yield (Kofoid and Maan, 1982), grain quality (Rao and Fleming, 1978), plant height (Rao and Fleming, 1978), plant vigor (Rao and Fleming, 1978), disease susceptibility (Keane and Jones, 1990), cold tolerance (Sutka et al., 1991), heat tolerance (Shonnard and Gepts, 1994) stay green (Kirk and Bassett, 1978) and other agronomic traits in different crops (Allen, 2005).

Overall effect of high temperature

Among all environmental stresses, heat is the most frequent stress that plants face (Iba, 2002). When a plant is subjected to a temperature beyond its threshold level for a period of time that results in irreversible damage to growth and development, the plant is considered to be heat stressed. Usually 10-15°C temperature elevation over the optimal level can be considered as heat stress (Wahid et al., 2007).

Heat stress is a very common agricultural problem, and most of the plants/crops in the world are exposed to heat during different stages of their life cycle (Stone, 2001). Even though there is variation for heat threshold levels for different developmental stages, heat stress affects plants throughout their ontogeny (Wahid et al., 2007). Cell membrane thermal stability, canopy temperature depression, stomatal conductance, and photosynthetic rate are all physiologically important traits affected by heat stress (Al-Khatib and Paulsen, 1984; Fokar et al., 1998; Reynolds et al., 2001; Cossani and Reynolds, 2012).

Carbon dioxide fixation, photophosphorylation, the electron transport chain, and the *oxygen evolving complex* (OEC) are major sites for temperature-induced damage in plants (Sharkey, 2005). Temperature affects the plants over a broad spectrum of metabolic and cellular

components, and severity of the effect depends on rate of temperature change, intensity and duration (Sung et al., 2003). At very high temperature, cellular organization can be collapsed in a minute due to severe injury to the cell, while it may take a longer for cell death at moderately high temperatures (Schoffl et al., 1999). Very high temperature usually causes direct injury like protein denaturation, aggregation and increased fluidity of membrane lipids. On the other hand, moderately high temperature causes slower/indirect injuries like enzyme inactivation, inhibition of protein synthesis, protein degradation and loss of membrane integrity (Howarth, 2005; Cossani and Reynolds, 2012). Organization, formation and elongation of phragmoplast microtubules are also all affected by heat stress (Smertenko et al., 1997; Barnabás et al., 2008).

High temperature effects on wheat physiology

Chlorophyll content

High temperature affects leaf chlorophyll content in wheat and almost all other crops. An experiment conducted by Efeoglu and Terzioglu (2009) found that a heat shock of wheat at 45°C for 8 hours at the seedling stage reduced chlorophyll accumulation in the leaf. After analysis of wheat populations, Zhao et al. (2007a) found a significant decrease in leaf chlorophyll content under heat stress. Yang et al. (2002a) found 11% to 38% decrease in flag leaf chlorophyll content in hexaploid synthetic wheat using a temperature regime of 30/25°C. The reduction of chlorophyll content influences yield in crop plants (Lopes and Reynolds, 2012). In an experiment on Mexican landraces of wheat, a significant correlation between leaf chlorophyll content and kernel weight was found by Hede et al. (1999).

Photosynthesis and related activities

High temperature has a detrimental effect on plant photosynthesis by changing the structure and function of different apparati involved in photosynthesis (Mathur et al., 2011). In

spring wheat, the leaf photosynthesis rate was decreased by about 15 μmol m^{-2} s^{-1} when night time temperature was increased from 14°C to 23°C (Prasad et al., 2008). In a review paper, Sharkey (2005) explained that the rate of photosynthesis can be decreased even without direct heat injury by increasing photorespiration faster than photosynthesis (Schuster and Monson, 1990). It has been established that, high temperature (35-40°C) reduces photosynthesis rate more than the expected reduction by accelerated photorespiration. In an earlier study, researchers concluded that PSII is the key weak point for high temperature stress (Enami et al., 1994), at very high temperatures (above 45°C) meaning that the photosynthesis rate reduction at 35-40°C cannot be explained by damage to photosystem II (Sharkey, 2005).

Phosphorylation of the light-harvesting chlorophyll complex of PSII (LHCII) and moving the phosphorylated complex from PSII to PSI in the thylakoid is one of the pathways for cyclic electron flow during photosynthesis. There are different proteins in the thylakoid which control phosphorylation and dephosphorylation of LHCII and other core proteins of PSII. Heat stress modifies the capability of some of those proteins and affects phosphorylation (Vener et al., 2001). Long term heat stress thus can cause structural change to thylakoid membranes leading to the inhibition of photosynthetic rate (Sharkey, 2005) by affecting the electron transport (Kouřil et al., 2004).

According to Wise et al. (2004) high temperature primarily damages the photochemical reaction in the thylakoid membrane and carbon metabolism in stroma. All integral membrane proteins, such as the antenna pigment protein complex 18 (carotenoid, chlorophyll a and chlorophyll b) reaction center and electron carrier proteins (cytochrome b cytochrome f and ferredoxin), are harbored in the thylakoid membrane (Taiz and Zeiger, 2006). A study by Al-Khatib and Paulsen (1990) found that high temperature created electrolytic leakage in thylakoid

membranes, resulting in significant reduction of photosynthetic rate. The reduction of chlorophyll content in the thylakoid membrane might be another reason for photosynthetic rate reduction. According to Crafts-Brandner and Salvucci (2000) Rubisco deactivation rate is higher than activase capacity under high temperature. As a result, net photosynthetic rate reduction occurs. Cossani and Reynolds (2012) described the importance and vulnerability of Rubisco during photosynthesis under high temperature. Based on several studies, they also suggested that wheat yield can be increased under high temperature by manipulating Rubisco.

Al-Khatib and Paulsen (1990) reported inhibition of photosynthetic rate might vary among different wheat species. This result was supported by the observation of Dias et al., (2010) that durum wheat showed higher photosynthetic performance than bread wheat under heat stress.

Canopy temperature depression

Canopy temperature depression (CTD) can be defined as the difference between the air temperature (AT) and the canopy temperature (CT). CTD has been used as an important parameter to select for high temperature tolerance in wheat breeding programs (Bahar et al., 2008). According to Munjal and Rana (2003), cooler canopy during the grain filling period was an indicator of heat tolerance. This also supports the idea that CTD is negatively related to heat sensitivity of a plant. Under high temperature, CTD is correlated with leaf area under decline, grain filling duration, grain yield and biomass (Kumari et al., 2007). Because of the ease of calculation and measurement of the trait, along with its application, canopy temperature depression is a commonly used assay (Brennan et al., 2007).

Effect of high temperature on growth and development of wheat

Temperature is an important environmental factor which affects the growth and development of crop plants. In the case of wheat, temperature above 24°C may shorten the life cycle by reducing the duration of growth stages, which results in the reduction of days for assimilate accumulation and total biomass production (Ghazi and Karaki, 2012; Wahid, et al., 2007; Howarth, 2005). In an experiment with 20 durum wheats, Wollenweber et al. (2003) found that the duration of GS1, GS2 and GS3 developmental stages were reduced by a 12°C higher average temperature. In the same study they also found that GS2 was the most sensitive stage to high temperature. Prasad et al. (2008) reported a decrease in time to flowering, grain set, and physiological maturity in spring wheat when grown at high night time temperature. Pradhan et al. (2012) observed that high temperature ultimately decreased the duration of all developmental stages. Shpiler and Blum, (1986) found decreased duration in growth stages GS1 (emergence to double ridge), GS2 (double ridge to anthesis), and GS3 (anthesis to grain maturation) for spring wheat growing during the summer.

Molecular markers and mapping

Molecular markers are useful to assay the variation of DNA sequences and can be helpful tools to utilize new sources of genetic variation by introducing new traits from different gene pools. An ideal marker system should be simple and reliable and cover the whole genome. Molecular markers can facilitate selection of target alleles, minimize linkage drag, accelerate trait introgression in breeding programs, assess genetic diversity and map complex traits. Due to its large genome size, polyploidy and extensive duplication, genome-wide analysis has been very difficult in wheat. The wheat genome contains 16 x 10^9 bp with an average of 810 Mb per chromosome which is 25 fold larger than an average rice chromosome (Argumuganathan and

Earle, 1991). Inspite of these difficulties, RFLP, AFLP, SSR, DArT and SNP markers have been used to construct genetic linkage maps in wheat (Röder et al., 1998; Sourdille et al., 2001; Gupta et al., 2002; and Poland et al., 2012).

Simple Sequence Repeats (SSRs)

Simple-sequence repeats (SSRs), or microsatellites, are tandem repeats of short (1-6 bp) DNA sequences (Tautz and Renz, 1984). Because of hyper-variability, diverse and nonrandom distribution in genomes, abundance, co-dominant inheritance and reproducibility, SSRs are considered useful analytical tools for the construction of genetic maps in wheat (Somers et al., 2004; Sourdille et al., 2004). Using information from various studies Peng et al. (2009) concluded that SSRs can be up to ten-fold more variable in plants than other markers like RAPD, RFLP etc. Furthermore, SSR markers are very useful in polyploid wheat (Peng et al., 2009; Roder et al., 1998). More than 2800 SSRs had been developed in wheat by 2008 (Gupta et al., 2008).

Single Nucleotide Polymorphisms (SNPs)

Because of their ubiquitousness in nature, SNPs have become a marker of choice in recent years. SNPs are very useful for high resolution genetic mapping, marker discovery by association analysis and investigation of evolutionary history of populations (Akhunov et al., 2009; and Zhao et al., 2007b). Development of the Illumina BeadArray platform and GoldenGate SNP assay have made high-throughput SNP discovery feasible for polyploid wheat. As a result, new possibilities have opened up for genome-wide analysis and capture of genetic variation in wheat (Akhunov et al., 2009; Barbazuk et al., 2007).

SNP development using Genotype-by-Sequencing (GBS) is possible for highly diversified, large genome species like wheat because of the advancement of next generation

sequencing technology. This is a very simple, cost effective and rapid approach which is very specific, highly reproducible, and may reach important regions of the genome that are inaccessible to sequence capture approaches (Elshire et al., 2011). The library development for GBS is also very simple, requires minute quantities of DNA, avoids random shearing and can be completed in only two steps (Poland et al., 2012).

QTL mapping for heat tolerance

Heat tolerance is a quantitative trait controlled by a number of genes/quantitative trait loci (QTL) and by interaction among the genes/QTL (Blum, 1988). Because high temperature is an increasing problem, heat tolerance is becoming a very important trait for wheat improvement. Few QTL studies have been reported for heat tolerance in wheat. Using an F_2 population of wheat, Yang et al. (2002b) reported several SSR markers associated with grain fill duration using single marker analysis. Mason et al. (2010) used an F_5 and F_6 generation RIL population and reported five QTL regions on 1A, 2A, 2B, and 3B for heat susceptibility index of yield, flag leaf length, width, and visual wax content. In another study Mason et al. (2011) reported three QTL regions on chromosomes 1B, 5A, and 6D for heat susceptibility index of kernel number, total kernel weight, and single kernel weight. Using senescence related traits, QTL for heat tolerance were reported on 2A, 3A, 3B, 6A, 6B and 7A (Vijayalakshmi et al. 2010). Paliwal et al. (2012) evaluated F_5, F_6, and F_7 generations of a RIL population of wheat for heat tolerance and reported QTL on 2B, 7B and 7D for canopy temperature depression and heat susceptibility index (HSI) of thousand grain weight, grain yield and grain filling duration.

Genetic transformation

Genetic transformation is a tool for crop improvement which also can be used for validation of target genes as a study of reverse genetics (Li et al., 2012). According to Rosegrant et al. (1997) conventional plant breeding alone may not be able to keep up with the increasing demand for food in the future (Guillou and Matheron, 2012). Recombinant DNA technology, along with its allied disciplines, holds a great deal of promise for increasing world wheat production by 1.2 to 1.6% per year. Recombinant DNA technology has been successful for many crops but, among the major cereals, wheat is the last to be genetically modified. Genetic transformation of wheat was initially very difficult because of gene delivering capacity to regenerable explants and totipotency of those explants. Inspite of these difficulties, transgenic technology is a very promising tool to improve stress tolerance, quality and yield of wheat (Bhalla, 2006). Genetic transformation using *Agrobacterium* is generally stable and reproducible. However, the inability of *Agrobacterium* to infect monocots and a prolonged tissue culture process has made the particle gun the preferred approach for genetic transformation in wheat (Abdul et al., 2004). To date, many reports have been published using the biolistic approach for wheat transformation with success rates as high as 7% (Rasco-Gaunt et al., 2001; Abdul et al., 2004).

Hypotheses of the dissertation

According to Budar and Roux (2011) the organellar genomes, and the interaction between organellar and nuclear genomes contribute significantly to the adaptation of plants in different environments. Various studies have shown that thermotolerance is related to photosynthesis and stability of chlorophyll in crop plants. Based on that, we have hypothesized that cytoplasmic variation might be a potential source for providing heat tolerance. Near Isogenic

Lines (NIL) for cytoplasm were developed and tested to assess the role of cytoplasm in thermotolerance.

Vijayalakshmi et al. (2010) reported QTL of significant effects for grain yield, grain weight, grain fill, stay-green and senescence-associated traits under post-anthesis high temperature stress in wheat. Most of the markers reported in the maps were AFLP and SSR. The groups were very small and marker density was very low. In the second project, we hypothesized that we might identify new QTL and better define already identified QTL in this population by adding genome-wide SNP markers along with previously mapped SSRs and AFLPs. The goal is to be able to use associated markers for MAS.

Starch accounts for 75-85% dry weight of wheat grain. Among the starch synthesis enzymes, soluble starch synthase I is the most abundant during grain filling. According to Keeling et al. (1993) temperatures above 25°C reduce soluble starch synthase activity in wheat endosperm. On the other hand, Jiang et al. (2003) found soluble starch synthase activity in rice was very high at around 35°C. Since wheat soluble starch synthase is deactivated at elevated temperature and rice soluble starch synthase withstands high temperature, we hypothesized that expression of a rice soluble starch synthase gene in wheat may increase the sink strength and thereby increase the productivity under heat stress. This was the focus of the third experiment.

Objectives of the dissertation

The primary objective of the dissertation is to identify tools to increase wheat production under heat stress. The specific objectives were:

1. To develop NILs for cytoplasm using ten alloplasmic sources and four adapted varieties.
2. To identify the effect of cytoplasm, and cytoplasmic- nuclear genome interaction on traits associated with heat tolerance.
3. To identify QTL for various traits associated with thermotolerance in wheat.
4. To investigate the effect of expression of a rice soluble starch synthase gene on starch deposition and the yield and kernel weight of wheat under heat stress conditions.

References

Abdul, R., Z. Ma, and H. Wang. 2004. Genetic transformation of wheat (*Triticum aestivum* L): a review. Mol. Plant Breed. 2: 457–464.

Acevedo, E., M. Nachit, and G. Ortiz-Ferrara. 1991. Effects of heat stress on wheat and possible selection tools for use in breeding for tolerance. Pp 401-421, D.A. Saunders (ed.), Wheat for the nontraditional warmer areas. Mexico, D.F: CIMMYT.

Akhunov, E., C. Nicolet and J. Dvorak. 2009. Single nucleotide polymorphism genotyping in polyploid wheat with the Illumina GoldenGate assay. Theor. Appl. Genet. 119: 507–517.

Al-khatib, K. and G.M. Paulsen. 1990. Photosynthesis and productivity during high-temperature stress of wheat genotypes from major world regions. Crop Sci. 30: 112–71132.

Al-Khatib, K., and G.M. Paulsen. 1984. Mode of high temperature injury to wheat during grain development. Physiol. Plant. 61: 363–368.

Allen, J.O. 2005. Effect of teosinte cytoplasmic genomes on maize phenotype. Genetics. 169: 863–880.

Apse, M.P., and E. Blumwald. 2002. Engineering salt tolerance in plants. Curr. Opin. Biotech. 13: 146–150.

Argumuganathan K, and E.D. Earle. 1991. Nuclear DNA content of some important plant species. Plant Mol. Biol. Rep. 9:208–218

Asseng S., Foster, I., and N.C. Turner. 2011. The impact of temperature variability on wheat yields. Global Change Biol. 17: 997–1012.

Bahar, B., M. Yildirim, C. Barutcular and I. Genc. 2008. Effect of canopy temperature depression on grain yield and yield components in bread and durum wheat. Notulae Botanicae Horti Agrobotanici Cluj-napoca. 36: 34-37.

Bahar, B., M. Yildirim and C. Yucel. 2011. Heat and drought resistance criteria in spring bread wheat (*Triticum aestivum L.*): Morpho-physiological parameters for heat tolerance. Scientific Research and Essays. 6: 2212–2220.

Balla, K., I. Karsai, and O. Veisz. 2009. Analysis of the quality of wheat varieties at extremely high temperatures. Cereal Res. Commun. 37: 13–16.

Barbazuk, W.B., S.J. Emrich, H.D. Chen, L. Li, and P. S. Schnable. 2007. SNP discovery via 454 transcriptome sequencing. Plant J. 51: 910–918.

Barnabás, B., K. Jäger, and A. Fehér. 2008. The effect of drought and heat stress on reproductive processes in cereals. Plant Cell Environ. 31: 11–38.

Bhalla, P.L. 2006. Genetic engineering of wheat - current challenges and opportunities. Trends Biotechnol. 24: 305-311.

Blum, A. 1988. Plant Breeding for Stress Environment. CRC Press Inc., Boca Ratan, FL.

Blumenthal, C., F. Bekes, P.W. Gras, E.W.R. Barlow, and C.W. Wrigley. 1995. Identification of wheat genotypes tolerant to the effects of heat stress on grain quality. Cereal Chem. 72: 539–544.

Bohnert, H.J., Q. Gong, P. Li, and S. Ma. 2006. Unraveling abiotic stress tolerance mechanisms-getting genomics going. Curr. Opin. Plant Biol. 9: 180–188.

Braun H.J., T.S. Payne, A.I. Morgounov, M. van Ginkel, S. Rajaram. 1998. The challenge: one billion tons of wheat by 2020. In: A.E. Slinkard, Ed., Proceedings of the 9th International Wheat Genetics Symposium. 1: Saskatoon, Saskatchewan, Canada, Pp. 33–40.

Breiman, A., and D. Graur. 1995. Wheat evolution. Israel J. Plant Sci. 43: 85–89.

Brennan, J.P., A.G. Condon, M. Van Ginkel, and M.P. Reynolds. 2007. An economic assessment of the use of physiological selection for stomatal aperture-related traits in the CIMMYT wheat breeding program. J. Agric. Sci. 145: 187–194.

Budar, F., and F. Roux. 2011. The role of organelle genomes in plant adaptation. Plant Signal. Behav. 6: 635-639.

Cossani, C.M., and M.P. Reynolds. 2012. Physiological traits for improving heat tolerance in wheat. Plant Physiol. 160: 1710–1718.

Crafts-Brandner, S.J., and M.E. Salvucci. 2000. Rubisco activase constrains the photosynthetic potential of leaves at high temperature and CO_2. Proc. Natl. Acad. Sci. USA. 97: 13430–13435.

Dias, A.S., and F.C. Lidon. 2009. Evaluation of grain filling rate and duration in bread and durum wheat, under heat stress after anthesis. J. Agron. Crop Sci. 195: 137–147.

Dias, A.S., J. Semedo, J.C., Ramalho, and F.C. Lidon. 2010. Bread and durum wheat under heat stress: a comparative study on the photosynthetic performance. J. Agron. Crop Sci. 197: 50–56.

Efeoglu, B., and S. Terzioglu. 2009. Photosynthetic responses of two wheat varieties to high temperature. Eur. Asia J. Bio. Sci. 3: 97–106.

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

Enami, I., M. Kitamura, T. Tomo, Y. Isokawa, H. Ohta, and S. Katoh. 1994. Is the primary cause of thermal inactivation of oxygen evolution in spinach PSII membranes release of the extrinsic 33 KDa protein or Mn? Biochimica et Biophysica Acta (BBA) - Bioenergetics. 1186: 52–58.

FAO. 2011. FAOSTAT. Available at http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor. Food and Agriculture Organization of the United Nations, Rome, Italy.

Farooq, M., H. Bramley, A.J. Palta and K.H.M. Siddique. 2011. Heat stress in wheat during reproductive and grain-filling phases. Crit. Rev. Plant Sci. 30: 491–507.

Feldman, M. 2001. The origin of cultivated wheat. In: Bonjean A.P., and W.J. Angus (eds), The World Wheat Book, A history of wheat breeding. Lavoisier Publishing, Paris, France. 3–56.

Ferris, R., R.H. Ellis, T.R. Wheeler, and P. Hadley. 1998. Effect of high temperature stress at anthesis on grain yield and biomass of field-grown crops of wheat. Ann. Bot. 82: 631–639.

Fischer, R.A. 1985. Number of kernels in wheat crops and the influence of solar-radiation and temperature. J. Agric. Sci. 105: 447–461.

Fischer, R.A. 1986. Physiological limitations to producing wheat in semitropical and tropical environments and possible selection criteria. Proc. Internal Symp Wheat for Tropical. Environments. Pp. 209–230. CIMMYT/UNDP, Mexico.

Fokar, M., T. Nguyan, and A. Blum. 1998. Heat tolerance in spring wheat. II. grain filling. Euphytica. 104: 9–15.

Foolad, M.R. 2005. Breeding for abiotic stress tolerances in tomato. In: Ashraf, M., and P.J.C. Harris, (Eds.), Abiotic Stresses: Plant Resistance Through Breeding and Molecular Approaches. The Haworth Press Inc., New York, USA. 613–684.

Ghazi, N. and Al-Karaki. 2012. Phenological development-yield relationships in durum wheat cultivars under late-season high-temperature stress in a semiarid environment. ISRN Agron. 2012: 1–7. doi:10.5402/2012/456856

Guillou, M., and G. Matheron. 2012. The world's challenge- Feeding 9 billion people. (Trans.) F. Bourin. INRA. (Original work published in 2011).

Gupta, P.K., R.R. Mir, A. Mohan, and J. Kumar. 2008. Wheat genomics: present status and future prospects. Int. J. Plant Genomics. 2008. Pp.36. doi:10.1155/2008/896451

Hanson, M.R. 1991. Plant mitochondrial mutations and male sterility. Annu. Rev. Genet. 25: 461–486.

Hede, A.R., B. Skkovm., M.P. Reynolds., J. Crossa., A.L. Vilhelmsen. and O. Stolen. 1999. Evaluating genetic diversity for heat tolerance traits in Mexican wheat landraces. Genet. Res. Crop Evol. 46: 37–45.

Howarth, C.J. 2005. Genetic improvements of tolerance to high temperature. In: Ashraf, M., Harris, P.J.C. (Eds.), Abiotic Stresses: Plant Resistance Through Breeding and Molecular Approaches. Howarth Press Inc., New York.

Iba, K. 2002. Acclimative response to temperature stress in higher plants: approaches of gene engineering for temperature tolerance. Annu. Rev. Plant Biol. 53: 225–45.

Jiang, H.W., W.M. Dian, and P. Wu. 2003. Effect of high temperature on fine structure of amylopectin in rice endosperm by reducing the activity of the starch branching enzyme. Phytochemistry. 63: 53–59.

Keane, E.M. and P.W. Jones. 1990. Effect of alien cytoplasm substitution on the response of wheat cultivars to *septoria nodorum.* Ann. Appl. Biol. 117: 299–312.

Keeling, P.L., P.J. Bacon, and, D.C. Holt.1993. Elevated-temperature reduces starch deposition in wheat endosperm by reducing the activity of soluble starch synthase. Planta. 191: 342–348.

Kirk, J.T.O., and R.A.E. Tilney-Bassett. 1978. The Plastids: Their chemistry, structure, growth and inheritance. Amsterdam, New York: Elsevier/North Holland Biomedical Press. Pp. 980.

Kofoid, K.D., and S.S. Maan. 1982. Agronomic and bread-making performance of fertile alloplasmic wheats. Crop Sci. 22: 725–729.

Kourˇil, R., D. Lazaˊr, P. Skotnica, P. Krchnˇak, and J. Nausˇ. 2004. High temperature induced chlorophyll fluorescence rise in plants at 40–50°C: Experimental and theoretical approach. Photosyn. Res. 81: 49–66.

Kumari, M., V.P. Singh, R. Tripathi, and A.K. Joshi. 2007. Variation for staygreen trait and its association with canopy temperature depression and yield traits under terminal heat stress in wheat. In H.T. Buck, H.T., J.E. Nisi and N. Salomon (Eds.), Wheat production in stressed environments (pp. 357-363). Springer Netherlands.

Li, J., X. Ye, B. An, L. Du, and H. Xu. 2012. Genetic transformation of wheat: current status and future prospects. Plant Biotechnol. Rep. 6: 183-193.

Lobell, D.B., and J.I. Ortiz-Monasterio. 2007. Impacts of day versus night temperatures on spring wheat yields: a comparison of empirical and CERES model predictions in three locations. Agron. J. 99: 469–477.

Lopes, M.S., and M.P. Reynolds. 2012. Stay-green in spring wheat can be determined by spectral reflectance measurements (normalized difference vegetation index) independently from phenology. J. Exp. Bot. 63: 3789–3798.

Maestri, E., K. Natalya, C. Perrotta, M. Gulli, H. Nguyen, and N. Marmiroli. 2002. Molecular genetics of heat tolerance and heat shock proteins in cereals. Plant. Mol. Biol. 48: 667–681.

Mason E.R., S. Mondal, W.F. Beecher, and D.B. Hays. 2011. Genetic loci linking improved heat tolerance in wheat (*Triticum aestivum L.)* to lower leaf and spike temperature under controlled conditions. Euphytica. 180: 181–194.

Mason, R.E., S. Mondal, F.W. Beecher, A. Pacheco, B. Jampala, A.M.H. Ibrahim, and D.B. Hays. 2010. QTL associated with heat susceptibility index in wheat (*Triticum aestivum L.*) under short-term reproductive stage heat stress. Euphytica. 174: 423–436

Mathur, S., A. Jajoo, P. Mehta, and S. Bharti. 2011. Analysis of elevated temperature-induced inhibition of photosystem II using chlorophyll a fluorescence induction kinetics in wheat leaves (*Triticum aestivum*). Plant Biol. 13: 1–6.

Munjal R., R.K. Rana. 2003. Evaluation of physiological traits in wheat (*Triticum aestivum L.)* for terminal high temperature tolerance. In: Proceedings of the Tenth International Wheat Genetics Symposium, Classical and Molecular Breeding, Poestum, Italy, 2: 804–805.

Ozkan, H., T. Yagbasanlar, and T. Genc, 1998. Tolerance and stability studies on durum wheat under drought and heat stress conditions. Cereal Res. Commun. 26: 405–12.

Paliwal, R., M.S. Roeder, U. Kumar, J.P. Srivastava, and A.K. Joshi. 2012. QTL mapping of terminal heat tolerance in hexaploid wheat (*T. aestivum L.).* Theor. Appl. Genet. 125: 561–575.

Peng, J.H., Y. Bai, S.D. Haley, and N.L.V. Lapitan. 2009. Microsatellite-based molecular diversity of bread wheat germplasm and association mapping of wheat resistance to the Russian wheat aphid. Genetica. 135: 95–122.

Pesaresi, P., S. Masiero, H. Eubel, H.P. Braun, S. Bhushan, E. Glaser, F. Salamini, and D. Leister. 2006. Nuclear photosynthetic gene expression is synergistically modulated by rates of protein synthesis in chloroplasts and mitochondria. The Plant Cell. 128: 970–991.

Pesaresi, P., A. Schneider, T. Kleine, and D. Leister. 2007. Interoganellar communication. Curr. Opin. Plant Biol.10: 600–606.

Poland, J.A., P.J. Brown, M.E. Sorrells, and J.L. Jannink. 2012. Development of high-density genetic maps for barley and wheat using a novel two-enzyme genotyping-by-sequencing approach. PLoS One. 7: e32253.

Pradhan, G. P., P.V.V. Prasad, A.K. Fritz, M.B. Kirkham, and B.S. Gill. 2012. High temperature tolerance in Aegilops species and its potential transfer to wheat. Crop Sci. 52: 292–304.

Prasad, P. V.V., S.R. Pisipati, R.N. Mutava, and M.R. Tuinstra. 2008. Sensitivity of grain sorghum to high temperature stress during reproductive development. Crop Sci. 48: 1911–1917.

Rao, A.P., and A.A. Fleming. 1978. Cytoplasmic-genotypic effects in the gt-112 maize inbred with 4 cytoplasms. Crop Sci. 18: 935-937.

Rasco-Gaunt, S., A. Riley, M. Cannell, P. Barcelo, and P.A. Lazzeri. 2001. Procedures allowing the transformation of a range of European elite wheat (*Triticum aestivum L.)* varieties via particle bombardment. J. Exp. Bot. 52: 865–874.

Rawson, H.M. 1988. Effects of high temperatures on the development and yield of wheat and practices to reduce deleterious effects. Wheat production constraints in tropical environments. Proceedings of the International Conference. Chiang Mai, Thailand, 19-23 January 1987, pp. 44–62.

Reynolds, M.P., J.I. Ortiz-Montasterio, and A. McNab. 2001. Application of Physiology in Wheat Breeding. CIMMYT, Mexico. 246 p.

Röder, M.S., V. Korzun, K. Wendehake, J. Plaschke, M.H. Tixier, P. Leroy, and, M.W. Ganal. 1998. A microsatellite map of wheat. Genetics. 149: 2007–2023.

Rontein, D., G. Basset, and A.D. Hanson. 2002. Metabolic engineering of osmoprotectant accumulation in plants. Metab. Eng. 4: 49–56.

Rosegrant, M.W., M.A. Sombilla, R.V. Gerpacio, and C. Ringler. 1997. Global food markets and US exports in 21st century, Paper presented at the Illinois World Food and Sustainable Agriculture Program Conference, Meeting the Demand for Food in 21st century: Challenges and Opportunities, 28th May, Univ. Illionois, Urbana-Champaign, USA.

Schoffl, F., R. Prandl, and A. Reindl. 1999. Molecular responses to heat stress. In: Shinozaki, K., Yamaguchi-Shinozaki, K. (Eds.), Molecular Responses to Cold, Drought, Heat and Salt Stress in Higher Plants. R.G. Landes Co., Austin, Texas, pp. 81–98.

Schuster, W.S. and R.K. Monson. 1990. An examination of the advantages of C3-C4 intermediate photosynthesis in warm environments. Plant Cell Environ.13: 903–912.

Sharkey, T.D. 2005. Effects of moderate heat stress on photosynthesis: importance of thylakoid reactions, rubisco deactivation, reactive oxygen species, and thermotolerance provided by isoprene. Plant Cell Environ. 28: 269–277.

Shonnard, G.C., and P. Gepts. 1994. Genetics of heat tolerance during reproductive development in common bean. Crop Sci. 34: 1168–1175.

Shpiler, L., and A. Blum. 1986. Differential reaction of wheat cultivars to hot environments. Euphytica. 35: 483–492.

Smertenko, A., P. Draber, V. Viklicky, and Z.Opatrny. 1997. Heat stress affects the organization of microtubules and cell division in *Nicotiana tabacum* cells. Plant Cell Environ. 20: 1534–1542.

Somers D.J., P. Isaac, and L. Edwards. 2004. A high-density SSR consensus map for bread wheat (*Triticum aestivum L.).* Theor. Appl. Genet. 109: 1105–1114

Sourdille, P., H. Guyomarch, C. Baron, B. Gandon, V. Chiquet, F. Artiguenave, K. Edwards, N. Foisset, P. Dufour, and M. Bernard. 2001. Improvement of the genetic maps of wheat using new SSR markers. In: Proceedings of the 9th Plant and Animal Genome IX. Final abstracts guide. Applied Biosystems Press, Foster City, California, United States. P. 167

Spiertz, J.H.J. 1977. Influence of temperature and light-intensity on grain-growth in relation to carbohydrate and nitrogen economy of wheat plant. Nether. J. Agric. Sci. 25: 182–197.

Stone, P. 2001. The effects of heat stress on cereal yield and quality. In: A.S. Basra (Ed.) Crop Responses and Adaptations to Temperature Stress, Food Products Press, Binghamton. NY, Pp. 243–291.

Stone, P.J., and M.E. Nicolas. 1994. Wheat cultivars vary widely in their responses of grain-yield and quality to short periods of post anthesis heat-stress. Aust. J. Plant Physiol. 21: 887–900.

Streck, N.A. 2005. Climate change and agro-ecosystems: the effect of elevated atmospheric CO_2 and temperature on crop growth, development and yield. Ciencia Rural. 35: 730–740.

Sung, D.Y., F. Kaplan, K. Lee, and C.L. Guy. 2003. Acquired tolerance to temperature extremes. Trends Plant Sci. 8: 179–187.

Surpin, M., and J. Chory. 1997. The co-ordination of nuclear and organellar genome expression in eukaryotic cells. Essays Biochem. 32: 113–25

Sutka, J., A.J. Worland and O.J. Maystrenko. 1991. Slight effect of cytoplasm on frost resistance in wheat (*Triticum aestivum L.*). Cer. Res. Commun. 19: 311–317.

Taiz, L., and E. Zeiger. 2006. Stress Physiology. 4th ed. Sinauer Asssociaes, Inc., MA, USA. pp. 671–702.

Tautz, D., and M. Renz. 1984. Simple sequences are ubiquitous repetitive components of eukaryotic genomes. Nucleic Acids Res. 12: 4127–38.

Vener, A.V., A. Harmas, M.R. Sussman, and R.D. Vierstra. 2001. Mass spectrometric resolution of reversible protein phosphorylation in photosynthetic membranes of *Arabidopsis thaliana*. J. Biol. Chem. 276: 6959–66.

Vijayalakshmi, K., A.K. Fritz, G.M. Paulsen, G. Bai, S. Pandravada, and B.S. Gill. 2010. Modeling and mapping QTL for senescence-related traits in winter wheat under high temperature. Mol. Breed. 26: 163–175

Wahid, A., S. Gelani, M. Ashraf, and M.R. Foolad. 2007. Heat tolerance in plants: An overview. Environ. Exp. Bot. 61: 199–233.

Wardlaw, I.F., and C.W. Wrigley. 1994. Heat tolerance in temperate cereals: an overview. Aust. J. Plant Physiol. 21: 695–703.

Wardlaw, I.F., C. Blumenthal, O. Larroque, and C. Wrigley. 2002. Contrasting effects of heat stress and heat shock on kernel weight and flour quality in wheat. Funct. Plant. Biol. 29: 25–34.

Wheeler, T.R., G.R. Batts, R.H. Ellis, P. Hadley, and J.I.L. Morison. 1996. Growth and yield of winter wheat *(Triticum aestivum L.)* crops in response to co_2 and temperature. J. Agric. Sci. 127: 37–48.

Wise, R.R., A.J. Olson, S.M. Schrader, and T.D. Sharkey. 2004. Electron transport is the functional limitation of photosynthesis in field-grown pima cotton plants at high temperature. Plant Cell Environ. 27: 717–724.

Wollenweber, B., J.R. Porter, and J. Schellberg. 2003. Lack of interaction between extreme high-temperature events at vegetative and reproductive growth stages in wheat. J. Agron. Crop Sci. 189: 142–150.

Woodson, J.D., and J. Chory. 2008. Coordination of gene expression between organellar and nuclear genomes. Nat. Rev. Genet. 9: 383–95.

Woodson, J.D., and J. Chory. 2012. Organelle Signaling: How stressed chloroplasts communicate with the nucleus. Curr. Biol. 22: 690–692.

Yang, J., R.G. Sears, B.S. Gill, and G.M. Paulsen. 2002a. Growth and senescence characteristics associated with tolerance of wheat-alien amphiploids to high temperature under controlled conditions. Euphytica. 126: 185–193.

Yang, J., R.G. Sears, B.S. Gill, and G.M. Paulsen. 2002b. Quantitative and molecular characterization of heat tolerance in hexaploid wheat. Euphytica. 126: 275–282.

Yin, X., W. Guo, and J.H. Spiertz. 2009. A quantitative approach to characterize sink–source relationships during grain filling in contrasting wheat genotypes. Field Crops Res. 114: 119–126.

Yurina, N., and M. Odintsova. 2011. Plant Organelles-to-Nucleus Retrograde Signaling, Abiotic Stress Response in Plants - Physiological, Biochemical and Genetic Perspectives, (pp 55–74). A. Shanker (Ed.), ISBN: 978-953-307-672-0, InTech, DOI: 10.5772/24241. Available from: http://www.intechopen.com/books/abiotic-stress-response-in-plants-physiological-biochemical-and-genetic-perspectives/plant-organelles-to-nucleus-retrograde-signaling1

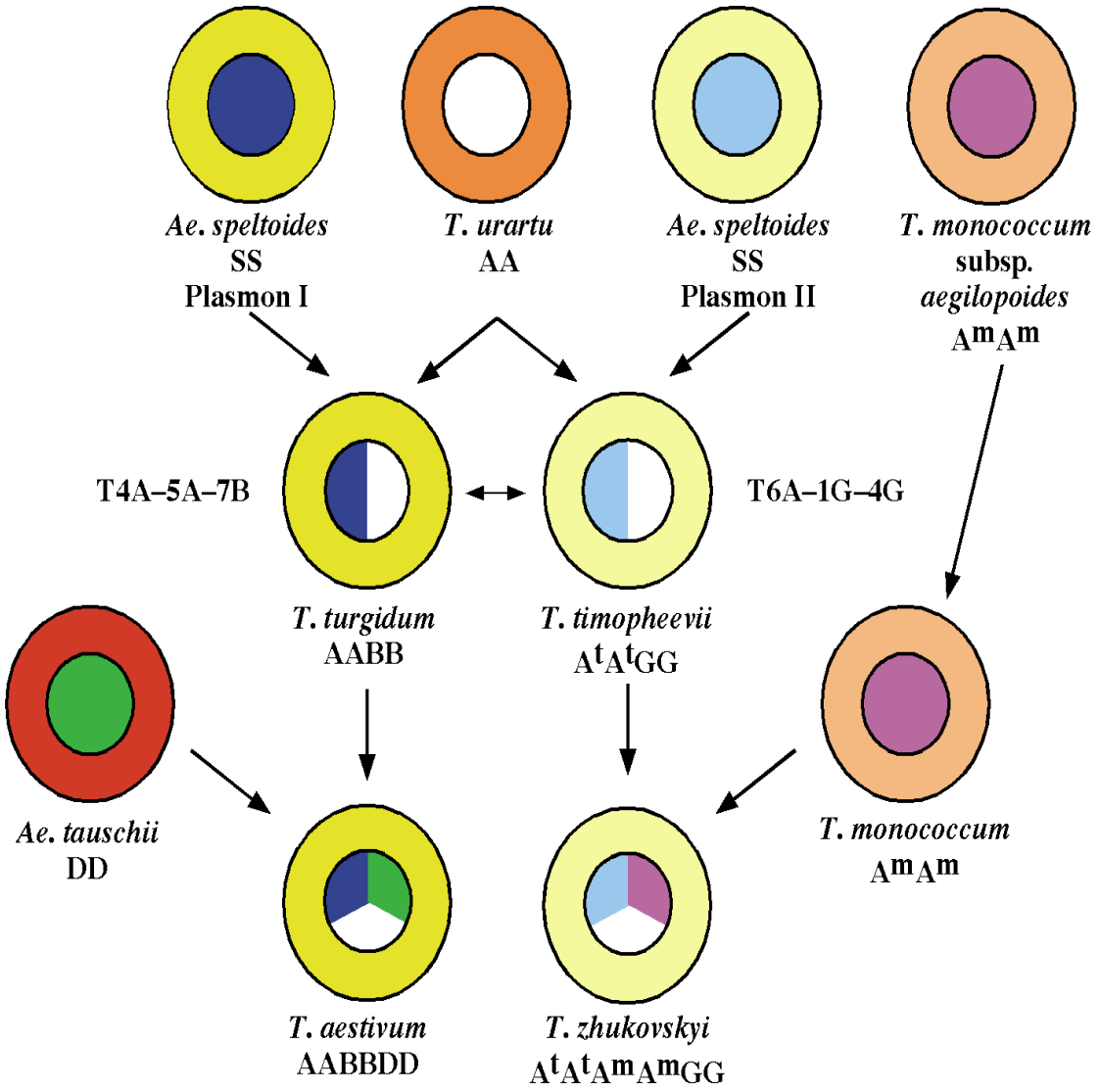
Zhang, H.X., J.N. Hodson, J.P. Williams, and E. Blumwald. 2001. Engineering salt-tolerant *Brassica* plants: characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. Proc. Natl. Acad. Sci. USA. 98: 12832–12836.

Zhao, H., T. Dai, Q. Jing, D. Jiang, and W. Cao. 2007a. Leaf senescence and grain filling affected by post-anthesis high temperatures in two different wheat cultivars. Plant Growth Regul. 51: 149–158.

Zhao, K., M.J. Aranzana, S. Kim, C. Lister, C. Shindo, and C. Tang. 2007b. An Arabidopsis example of association mapping in structured samples. PLoS. Genet. 3: e4. doi:10.1371/journal.pgen.0030004

Figures and Tables

Figure 1.1 Evolution of wheat



Source: Wheat Genetic and Genomic Resource Group (WGGRC), Kansas State University (http://www.k-state.edu/wgrc/)

Chapter 2 - Effect of cytoplasmic source of wheat on heat tolerance

Abstract

The nuclear genomes of ten alloplasmic lines were substituted by backcrossing four or five times using 'Karl 92', 'Ventnor', 'U1275' and 'Jagger' as recurrent parent to study the cytoplasmic effects on heat tolerance. During the final backcross, reciprocal crosses were made to develop NILs (Near Isogenic Lines) for cytoplasm. Sixty-eight lines of BC_5 F_1/ BC_4 F_1 and their parents were evaluated in growth chambers for post anthesis heat tolerance. Plants were grown in the greenhouse and subjected to heat stress at 10 days after anthesis for a period of 14 days. Growth chambers were maintained at 35/30°C for heat stress and the greenhouse was maintained at 20/15°C as the optimum temperature treatment. Effects of high temperature on chlorophyll content and F_v/F_m (a chlorophyll fluorescence measuring parameter) were found to be significant in this experiment. Seven of the cytoplasms provided improved tolerance to heat with at least one recurrent parent. These results indicated that cytoplasmic variation can contribute to stay-green of wheat during high temperature stress and also highlight the importance of interaction between cytoplasmic and nuclear genes. The role of cytoplasm may be considered in wheat breeding programs as they breed for high temperature tolerance, but the nature of interaction between cytoplasm and nuclear gene content needs to be better understood.

Introduction

High temperature is a major concern for crop production throughout the world. Wheat production is affected by high temperature through reduction of yield and quality (Wardlaw et al., 2002). Almost all wheat grown in temperate regions, which accounts for 40% of the total wheat production in the world, is affected by terminal heat stress (Paliwal et al., 2012). Reynolds et al. (1994) demonstrated that above-ground biomass, grains per m^2, days to anthesis, days to maturity, membrane thermostability, flag-leaf photosynthesis, leaf chlorophyll content, stomatal conductance and canopy temperature depression (CTD) were highly affected by heat stress. According to other reports, photosynthesis and photosynthesis-related traits like chlorophyll content, and thylakoid membrane stability were heavily affected by high temperature (Cothren, 1999; Hall, 2004). Bibi et al. (2008) suggested that chlorophyll fluorescence and membrane leakage might represent a practical approach to quantify thermotolerance. To address the heat stress problem, most researchers have concentrated their research on effects of the nuclear genome to identify traits related to thermotolerance, but it is also known that the cytoplasm plays definite roles in physiological and agronomic responses in crops. Roach and Wulff (1987) noted that plastids and mitochondria have small genomes and can directly be transferred from the maternal parent to offspring during ovulation, resulting in a cytoplasmic genetic maternal effect. They also pointed out that the transmission of the cytoplasmic genome is independent of the nuclear genome, and affect heritable quantitative and qualitative traits in plants.

In addition to cytoplasmic male sterility (Hanson, 1991), cytoplasmic influence has been demonstrated for yield (Kofoid and Maan, 1982), grain quality (Rao and Fleming, 1978), plant height (Rao and Fleming, 1978), plant vigor (Rao and Fleming, 1978), disease susceptibility (Keane and Jones, 1990), cold tolerance (Sutka et al., 1991), heat tolerance (Shonnard and Gepts, 1994) stay-green (Kirk and Bassett, 1978) and other agronomic traits in various crops (Allen, 2005).

The sources of extra nuclear genes are the chloroplast and mitochondria. Most proteins are encoded in the nuclear genome but synthesized in the cytoplasm, then shuttled through the membrane into different organelles to be assembled for action. This path leads to a complex interaction between cytoplasmic and the nuclear genomes (Diethelm et al., 1989). As an example, the large catalytic subunit of Rubisco (ribulose 1, 5-bisphosphate carboxylase-oxygenase) is encoded in the chloroplast, while the genes for the small subunits are located in the nucleus (Jensen and Bahr, 1977). Many of the thylakoid proteins involved in photosynthesis are directly encoded in the chloroplast and thus have a significant impact on the photosynthetic process (Wu and Campbell, 2007). In a study on rice Tao et al. (2004) demonstrated that the cytoplasm and cytoplasmic-nuclear interactions played important roles in cold tolerance, yield and other important agronomic traits. The cytoplasm also plays roles in manifesting combining ability and heterosis in different lines and hybrids in some crops (Yadav, 1994; Kumar and Sagar, 2010). A review by Budar and Roux (2011) highlighted that the cytoplasmic genome, and the interaction between cytoplasmic and nuclear genomes contribute significantly to the adaptation of plants in different environments. Vedel et al. (1981) reported that cytoplasmic DNA has significant influence on various traits in wheat. Wheat has a relatively narrow genetic base, therefore cytoplasmic variability may be an important resource to improve various traits

(Ekiz, 1998). Allan (1997) used alloplasmic and euplasmic lines in a Stephens background to demonstrate agronomic effects associated with various cytoplasmic sources. In the current study, the effects of cytoplasmic diversity on tolerance to high temperature stress was explored.

We developed NILs using ten different cytoplasms, and four different backgrounds to study the effect of cytoplasm, and cytoplasmic-nuclear genome interactions on traits associated with heat tolerance.

Materials and methods

Development of NILs

Cytoplasmic NILs (Near Isogenic Lines) were developed by transferring the nuclear genomes from four lines (U1275, Jagger Karl 92 and Ventnor) into the cytoplasms described by Allan (1997). Recurrent parents (U1275, Jagger Karl 92 and Ventnor) were selected based on their diverse reactions to heat stress. Ventnor is considered as heat tolerant (Yang et al., 2002), U1275 and Jagger as moderately tolerant and Karl 92 as heat sensitive (Yang et al., 2002). The pedigrees of U1275, Jagger, and Karl 92 are TAM-107 *3/TA 2460 (TA 2460 is an accession of *Aegilops tauschii*), KS82W418/Stephens and Plainsman V/3/Kaw/Atlas 50//Parker*5/Agent (Sears et al. 1997) respectively. The pedigree of Ventnor is unknown.

A set of ten alloplasmic lines were used as cytoplasmic sources (Allan, 1997). Pedigree with PI numbers of these lines is provided in Table 1. The nuclear genomes of these alloplasmic lines were substituted by backcrossing using the recurrent parents as males. Reciprocal crossing at BC_4F_1 and/or BC_5F_1 using the same plants as parents resulted in the development of 34 pairs of Near Isogenic Lines (Table 2).

Cytoplasmic differences were confirmed between two NILs using a set of 24 chloroplast SSR markers (Table 2.9) described by Ishii et al. (2001). DNA was isolated from leaf tissue using the CTAB method with minor modification. PCR was performed using genomic DNA to screen 34 NIL pairs along with the four recurrent parents. A touchdown PCR program of 95°C for 5 minutes, 4 cycles of 95°C 30s, (68-2/cycle)°C 60s, 72°C 60s followed by, 4 cycles of 95°C 30s, (60-2/cycle)°C 300s, 72°C 60s, 30 cycles of 95°C 30s, 50°C 60s, 72°C 90s and 72°C for 10 minutes was used for all chloroplast SSRs. Amplified PCR products were electrophoresed on 3.0% agarose gel.

Experimental procedure

A total of four separate experiments were performed to phenotype parental and NIL populations. Parental lines were phenotyped to assess the performance of alloplasmic sources along with recurrent parents; meanwhile, NIL populations were phenotyped to compare the cytoplasmic effect on various traits under both heat stress and optimum temperature conditions. Eight to ten seeds from each line were sown in a 7 x 8.5-cm pot filled with metro-mix and placed at room temperature. Once most seeds germinated the pots were transferred to a vernalization chamber at 5°C for 6 weeks. Each of three vernalized seedlings were transplanted to a vinyl pot (10x25-cm.) containing the soil mix of silt loam consisting 1.7 g N, 0.11 g P, 1.4 g K, 4.0 g gypsum, 63.0g perlite, and 400g peat moss per kilogram of soil. Transplanted plants were placed in a greenhouse for growth and development maintaining 16 hours of photoperiod and 20/15°C day/night temperature in the Department of Agronomy, Kansas State University, Manhattan, Kansas, USA. One half teaspoon of systemic insecticide per pot (Marathon II, active ingredient: imidacloprid) and 10 g of controlled release fertilizer (Osmocote Plus, N: P_2O_5:K_2O = 15:9:12; Scotts, Marysville, OH, USA) were applied to the soil at seven days after transplanting.

All the phenotyping under optimum temperature and high temperature was conducted in the greenhouse and growth chambers respectively, maintaining three replications. Parental lines were compared separately at optimum temperature and high temperature following Randomized Complete Block Design (RCBD), where growth chambers were considered as the block for heat stress experiments, and each bench as a block for greenhouse experiments. NILs were phenotyped following a three factor factorial design with three replications, assigning varieties as factor 1, cytoplasm as factor 2 and NIL as factor 3. The greenhouse was set at 20/15°C temperature (day/night), 85% relative humidity, 16-h photoperiod for phenotyping under optimum temperature. Growth chambers were set at 35/30°C temperature (day/night) at 85% relative humidity and 16-h photoperiod for phenotyping under heat stress.

Data measurements

In all experiments, primary tiller/s of plants were tagged during anthesis and relocated for phenotyping at ten days after anthesis based on the most advanced tiller of a pot. Data were collected on grain filling duration (GFD), seeds number per spike, 1000 seed weight, chlorophyll content and chlorophyll fluorescence (F_v/F_m) for both optimum and high temperature conditions. In these experiments, GFD was estimated as the duration between the date of anthesis and date of physiological maturity. The yellowing of the glumes at the bottom of a spike was used as the indication of physiological maturity. After harvest, the spikes from the tagged tillers were dried in an incubator at 45°C for 4 days. Dried spikes were hand-threshed and grains were counted and weighed to determine seed number per spike and thousand seed weight.

Chlorophyll content was measured from the flag leaves of the tagged plants using a self-calibrating SPAD chlorophyll meter (Model 502, Spectrum Technologies, Plainfield, IL). Fluorescence measurements were conducted using a hand-held chlorophyll fluorometer (Model

B/OS-30p, Opti-Sciences, Inc., Hudson, New Hampshire, USA). F_v/F_m was measured from the same flag leaves after 1 hour of dark adaptation, by placing clips one-third up from the leaf base on the abaxial surface. Both Chlorophyll content and chlorophyll fluorescence data were taken every alternate day six times starting from thirteenth days after anthesis.

Data Analysis

Data of the parental lines were analyzed using SAS 9.1.3 PROC MIXED following Randomized Complete Block Design (RCBD). LSMEANS were compared using LSD (Least Significant Difference) (Carmer and Swanson, 1973) for the agronomic traits. Chlorophyll content and F_v/F_m data were treated as time series, with time modeled as a regression variable using the solution option in the PROC MIXED procedure of SAS (Littell et al., 2006). Slopes of the parental lines were compared using a pairwise t-test.

NIL data were analyzed as a three-factor factorial experiment considering genotype, alloplasmic line and NILS as factors and replication as block. Analysis was done using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA) PROC MIXED procedures treating block, genotype, alloplasmic line and NILS as class variables. Block and genotype*alloplasmic line*NILS within blocks were treated as random effects and the remaining variables were treated as fixed effects (Littell et al., 2006). Chlorophyll content and F_v/F_m data were treated and analyzed in the same way as parental data. Slopes of chlorophyll content and F_v/F_m were compared using a pairwise t-test calculated from the common standard error.

Results

Development of NILs

Reciprocal crossing at BC_4F_1 and/or BC_5F_1 resulted in the development of 34 pairs of Near Isogenic Lines (NIL) (Table 2). Alloplasmic line 6 in Jagger, 1, 4 and 8 in Karl 92 and 1 and 2 in Ventnor failed during reciprocal crossing with the recurrent parents.

Marker analysis

Twenty-four chloroplast SSR markers were used to confirm the cytoplasmic differences between the reciprocals of the NIL crosses. All the *Aegilops spp. w*ere found polymorphic by several markers except the reciprocals of *A. ventricosa* (cytoplasm 6) and U1275. We considered this to be a result of a crossing error and did not consider this combination in the results and discussion. None of the twenty-four markers showed polymorphism between *Triticum macha* and euplasmic cytoplasms, while one SSR was polymorphic between *Triticum turgidum* and euplasmic cytoplasms. The cytoplasm of *Haynaldia villosa* was not polymorphic with euplasmic cytoplasms using this set of chloroplast markers (Table 3). This result is most likely due to an error in the process. Therefore results with cytoplasm 7 are not considered in this paper.

Parental lines comparison

Ten alloplasmic lines, along with four euplasmic lines, were phenotyped to assess their performance under both optimum and high temperature conditions. Significant variation was found for seed number per spike and grain filling duration under optimum temperature conditions, while no variability was observed under high temperature (Table 5). Under optimum temperature conditions, alloplasmic line 9 produced the highest number of seed (SN) and Ventnor had the highest grain filling duration (GFD). The differences among the parental lines

for thousand kernel weight (TKW) were not statistically significant for either optimum temperature and heat stress conditions (Table 5).

Among parental lines, alloplasmic line 3 (*Aegilops variabilis* cytoplasm in Stephens background) and euplasmic Ventnor demonstrated lower slope than other parental lines under high temperature meanwhile little variation of slope was found among lines under optimum temperature for either chlorophyll content and Fv/Fm (Table 6).

Cytoplasmic comparison of NILs

Thousand kernel weight (TKW)

The reciprocal NILs were contrasted and tested with the F-test. In no case was an alloplasmic NIL found to be better than its corresponding euplasmic NIL under optimum temperature for TKW. However, cytoplasms 4, 5 and 6 had lower TKW in the Ventnor background. Under heat stress, cytoplasm 10 produced seed with higher thousand kernel weight (TKW) in the Ventnor background (Tables 7 and 11).

Number of seeds per head (SN)

Except cytoplasm 3 in the Karl 92 background, no significant difference was from seed number per head under optimum temperature. Cytoplasm 3 in the Karl 92 background had lower seed number than euplasmic Karl 92. Under heat stress, NILs for cytoplasms 3 and 4 had lower number of seed than euplasmic U1275. On the other hand, NILs for cytoplasm 1 and 10 demonstrated higher number of seeds per head than euplasmic Jagger cytoplasm (Tables 7 and 10).

Grain filling duration

Under optimum temperature, NILs for cytoplasms 4 and 6 in the U1275 background and cytoplasm 6 in the Ventnor background had longer grain filling duration compared to their corresponding euplasmic NILs, but no significant difference was observed between two reciprocal NILs under heat stress (Tables 7 and 12).

Chlorophyll content

Slope was used to characterize changes in chlorophyll content over time. The two slopes of the reciprocal NILs were compared using an LSD value calculated from the common standard error. Non-significant slope variation was observed between cytoplasmic NILs under optimum temperature for all backgrounds (data not shown). Under heat stress, some alloplasmic NILs performed better than the contrasting euplasmic NIL. Specifically, cytoplasms 1 and 2 in U1275, cytoplasms 1, 2 and 4 in Jagger, cytoplasms 5 and 10 in Karl-92 and cytoplasms 8, 9 and 10 in Ventnor demonstrated greater stability of chlorophyll content compared to their contrasting euplasmic NIL (Table 7). Meanwhile, cytoplasm 9 in Jagger and cytoplasms 4 and 6 in Ventnor performed worse than their corresponding euplasmic NILs (Table 7).

F_v/F_m

F_v/F_m data were analyzed and interpreted using the same procedure as chlorophyll content. Under optimum temperature conditions, the performance of reciprocals was not different, however significant differences were found under high temperature. Like chlorophyll content, some of the alloplasmic cytoplasms were showed better performance for Fv/Fm stability compared to their contrasting euplasmic NIL. For example, cytoplasm 1 in U1275, cytoplasms 1 and 8 in Jagger, cytoplasms 5, 9 and 10 in Karl 92 and cytoplasms 5, 8, 9 and 10 in Ventnor had lower slopes than their contrasting euplasmic NILs (Table 7). In contrast, cytoplasm 8 in U1275,

cytoplasm 9 in Jagger, cytoplasm 2 in Karl 92 and cytoplasm 6 in Ventnor performed worse than the corresponding euplasmic NILs (Table 7).

Discussion

The combination of four varieties and ten alloplasmic lines should have produced 40 NILs. Only 34 NIL combinations were developed due to failure during reciprocal crossing where we wanted to use the same plants for both crosses in order to limit any possible heterogeneity of nuclear gene content.

Twenty- four chloroplast SSR markers were used to confirm the cytoplasmic differences in the NIL population. The marker data demonstrated that all the alloplasmic cytoplasms from different *Aegilops spp. w*ere polymorphic to the euplasmic cytoplasms by some of the markers. The exception was *A.ventricosa* (cytoplasm 6) with U1275. Vedel et al. (1981) used restriction fragment length of chloroplast DNA after EcoRI digestion to demonstrate that some bread wheat cytoplasms were maternally inherited from *Aegilops squarrosa*. Another earlier study by Vedel et al. (1978) demonstrated that *Aegilops speltoides* might be the primary source of the cytoplasm of bread wheat, but found distinct variability in mitochondrial DNA between *Triticum aestivum* and *Aegilops speltoides.* Studies by Vedel et al. (1978 and 1981) demonstrated variability among *Aegilops spp.* cytoplasm which clearly supports the variation observed in this study. There was likely a mistake made during development of the NILs for *A. ventricosa* and U1275 resulting in no polymorphism being found between the reciprocals.

Two *Triticum macha* cytoplasms were not very different from euplasmic sources based on this marker set. This is reasonable, as both of the *T. macha* cytoplasms are from the same species. It was surprising to find no polymorphism between *Haynaldia villosa and T. aestivum*

and is likely indicative of a problem with the original alloplasmic stock since the lack of polymorphism was consistent across recurrent parents. One marker showed polymorphism between *Triticum turgidum* and the recurrent parents. This result suggests that *Triticum* cytoplasms are at least somewhat polymorphic.

Based on previous reports we expected Ventnor to be the most tolerant recurrent parent and Karl 92 to be the most sensitive (Yang et al., 2002). Karl 92 actually appeared to be more tolerant than Jagger and U1275 in this study. This result was consistent throughout our experiments and is also consistent with our observations in other heat-related work (data not shown). U1275 would be a better choice as a heat-sensitive parent in constructing populations between parents with contrasting phenotypes.

Number of seed per head, thousand kernel weight and grain filling duration of alloplasmic lines did not show any heterotic effect under any temperature condition. Our results indicate that variation for these traits was due to differences between nuclear gene content of the recurrent parents rather than cytoplasmic influence. This result is in general agreement with Allan (1997) who registered these alloplasmic lines and reported that there was little heterotic potential of these lines for most agronomic traits.

Chlorophyll content and chlorophyll fluorescence are highly associated and might be a practical approach to quantify thermotolerance. Heat stress causes reduction of both chlorophyll content and chlorophyll fluorescence (Djanaguiraman et al., 2010; Bibi et al., 2008; Ristic et al., 2007). The generally greater stability across backgrounds for both chlorophyll content and fluorescence for cytoplasms 1, 5 and 10 indicate these cytoplasms may have greater potential to improve heat tolerance. The variability observed across backgrounds for cytoplasms 2, 4, 8, and 9 indicate there is a significant interaction between the nuclear and cytoplasmic genomes. A

similar effect was noted by Tao et al. (2004) for cold tolerance. The contrasting responses of Jagger and Ventnor using cytoplasm 9 might be a good place to start evaluating the interaction between the cytoplasmic and nuclear genomes

Overall, there is some potential to exploit cytoplasmic variation and cytoplasm-nuclear interactions to improve heat tolerance. The fact that chlorophyll and fluorescence results were not carried through to TKW indicates there are other bottlenecks created by high temperature stress. Any improvement based on nuclear- cytoplasmic interactions would need to be seen as one component of a complex system. Other factors will likely have to be identified to fully exploit this potential.

Conclusions

Using this limited source of alloplasmic cytoplasm, it is clear there is potential to improve thermotolerance associated traits under heat stress by manipulating cytoplasm. This is especially true for chlorophyll content and Fv/Fm that are photosynthesis-related traits and very strong indicators of thermotolerance. It may be of value to explore additional cytoplasmic sources to increase key components of thermotolerance in wheat such as stay-green or photosynthetic capacity. Additional research is needed to fully assess the potential of cytoplasmic variability to contribute to develop of heat-tolerant wheat varieties.

References

Allan, R.E. 1997. Registration of 10 pairs of alloplasmic and euplasmic Stephens wheat germplasms. Crop Sci. 37: 1033–1034.

Allen, J.O. 2005. Effect of teosinte cytoplasmic genomes on maize phenotype. Genetics. 169: 863–880.

Bibi, A.C., D.M. Oosterhuis, and E.D. Gonias. 2008. Photosynthesis, quantum yield of photosystem II and membrane leakage as affected by high temperatures in cotton genotypes. J. Cotton Sci. 12: 150–159.

Budar, F., and F. Roux. 2011. The role of organelle genomes in plant adaptation: time to get to work! Plant Signal. Behav. 6: 635–639.

Carmer, S.G., and M.R. Swanson. 1973. An evaluation of ten pair wise multiple comparison procedures by Monte Carlo methods. J. Amer. Statist. Assoc. 68: 66–74.

Cothren, J.T. 1999. Physiology of cotton plant. p. 207–268. In C.W. Smith and J.T. Cothren (Eds.) Cotton: Origin, History, Technology, and Production. John Wiley & Sons, Inc., Danvers, MA.

Diethelm, R., R. Shibles, D.E. Green, and R.C. Shoemaker. 1989. Cytoplasmic effects on photosynthetic activities of soybean leaves. Crop Sci. 29: 334–337.

Djanaguiraman, M., P.V.V. Prasad, and M. Seppanen, 2010. Selenium protects sorghum leaves from oxidative damage under high temperature stress by enhancing antioxidant defense system. Plant Physiol. Biochem. 48: 999–1007.

Ekiz H., A.S. Kiral, A. Akcin, and L. Simsek. 1998. Cytoplasmic effects on quality traits of bread wheat *(Triticum aestivum L.)*. Euphytica.100: 189–196.

Hall, A.E. 2004. Breeding for adaptation to drought and heat in cowpea. Europ. J. Agron. 21: 447–454.

Hanson, M.R. 1991. Plant mitochondrial mutations and male sterility. Annu. Rev. Genet. 25: 461–486.

Ishii, T.N. Mori, and Y. Ogihara. 2001. Evaluation of allelic diversity at chloroplast microsatellite loci among common wheat and its ancestral species. Theor. Appl. Genet. 103: 896–904

Jensen, G.R., and J.T. Bahr. 1977. Ribulose 1, 5-bisphosphate carboxylase-oxygenase. Ann. Rev. Plant Physiol. 28: 379–400.

Keane, E.M., and P.W. Jones. 1990. Effect of alien cytoplasm substitution on the response of wheat cultivars to septoria nodorum. Anna. Appl. Biol. 117: 299–312.

Kirk, J.T.O., and R.A.E. Tilney-Bassett. 1978. The Plastids: their chemistry, structure, growth and inheritance. Amsterdam, New York: Elsevier/North Holland Biomedical Press.

Kofoid, K.D., and S.S. Maan. 1982. Agronomic and bread-making performance of fertile alloplasmic wheats. Crop Sci. 22: 725–729.

Kumar, R., and P. Sagar. 2010. Effect of cytoplasm on combining ability and yield attributes in pearl millet *(pennisetum glaucum L.*) R.Br**.** Indian. J. Genet. Pl. Br**.** 70: 247–256.

Littell, R.C., G.A. Milliken, W.W. Stroup, R.D. Wolfinger, and O. Schabenberger. 2006. Sas® for mixed models. 2nd ed. SAS Institute Inc., Cary, NC, USA.

Paliwal, R., M.S. Roeder, U. Kumar, J.P. Srivastava, and A.K. Joshi. 2012. QTL mapping of terminal heat tolerance in hexaploid wheat (*T. aestivum L.).* Theor. Appl. Genet. 125: 561–575.

Rao, A.P., and A.A. Fleming. 1978. Cytoplasmic-genotypic effects in the gt-112 maize inbred with 4 cytoplasms. Crop Sci. 18: 935–937.

Reynolds, M.P., M. Balota, M.I.B. Delgado, I. Amani, and R.A. Fischer. 1994. Physiological and morphological traits associated with spring wheat yield under hot, irrigated conditions. Aust. J. Plant. Physiol. 21: 717–730.

Ristic, Z., U. Bukovnik, and P.V.V. Prasad. 2007. Correlation between heat stability of thylakoid membranes and loss of chlorophyll in winter wheat under heat stress. Crop Sci. 47: 2067–2073.

Roach, D., and A.R. Wulff. 1987. Maternal effects in plants. Annual Review of Ecology and Systematics 18: 209–235

Sears, R.G., J.M. Moffatt, T.J. Martin, T.S. Cox, R.K. Bequette, S.P. Curran, O.K. Chung, W.F. Heer, J.H. Long, and M.D. Witt. 1997. Registration of 'Jagger' wheat. Crop Sci. 37: 1010.

Shonnard, G.C., and P. Gepts. 1994. Genetics of heat tolerance during reproductive development in common bean. Crop Sci. 34: 1168–1175.

Sutka, J., A.J. Worland, and O.J. Maystrenko. 1991. Slight effect of cytoplasm on frost resistance in wheat (*Triticum aestivum* L.). Cer. Res. Com. 19: 311–317.

Tao, D.Y., F.Y. Hu, J. Y. Yang, G.F. Yang, Y.Q. Yang, P. Xu, J. Li, C. Ye, and L. Dai. 2004. Cytoplasm and cytoplasm-nucleus interactions affect agronomic traits in *Japonica* rice. Euphytica. 135: 129–134.

Vedel, F., F. Quetier, Y. Cauderon, F. Dosba, and G.Doussinault. 1981. Studies on maternal inheritance in polyploid wheats with cytoplasmic dnas as genetic-markers. Theor. Appl. Genet. 59:239–245.

Vedel, F., F. Quetier, F. Dosba, and G. Doussinault. 1978. Study of wheat phylogeny by ecori analysis of chloroplastic and mitochondrial DNAs. Pl. Sci. Lett. 13: 97–102.

Wardlaw, I.F. 2002. Interaction between drought and chronic high temperature during kernel filling in wheat in a controlled environment. Ann. Bot. 90: 469–476.

Wu, C.A., and D.R. Campbell. 2007. Leaf physiology reflects environmental differences and cytoplasmic background in Ipomopsis (Polemoniaceae) hybrids. Am. J. Bot. 94: 1804–1812.

Yadav, O.P. 1994. Effect of A1 cytoplasm on the combining ability for smut severity in pearl millet. Theor. Appl. Genet. 88: 28–29.

Yang, J., R.G. Sears, B.S. Gill, and G.M. Paulsen. 2002. Quantitative and molecular characterization of heat tolerance in hexaploid wheat. Euphytica. 126: 275–282.

Figure 2.1 Chloroplast primer (Wct2) showing polymorphism between NILs cytoplasm's. NILs are presented pair wise.

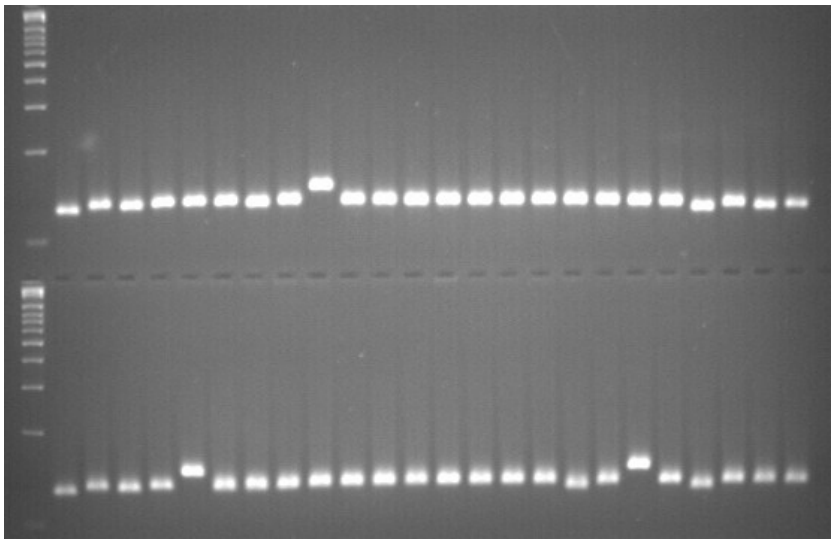


Figure 2.2 Plants at 10 days after heat stress, A) Plants having alloplasmic (PI 590259) cytoplasm and U1275 nuclear genome, B) plants having U1275 nuclear genome and cytoplasm.

A B

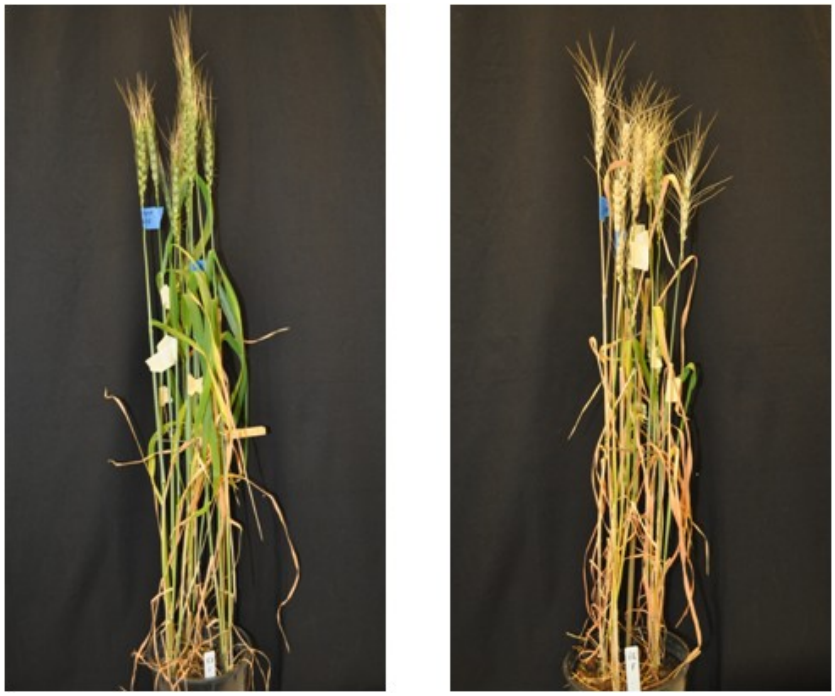


Table 2.1 Pedigree of ten alloplasmic lines in Stephens background (Allen, 1997)

Alloplasmic line	PI Number	Pedigree
1	PI 590259	*Aegilops juvenalis*/6*CHR//9*SK(NDM1)/3/7*SPN
2	PI 590261	*A.cylindrica*/CHR//10*SK(NDM2)/3/7*SPN
3	PI 590263	*A.variabilis*/9*CHR//13*SK(NDM3)/3/7*SPN
4	PI 590265	*A. squarrosa/19*SK(NDM4)//7*SPN*
5	PI 590267	*A.uniaristata*/2**Triticum durum*/10*SK(NDM5)/3/7*SPN
6	PI 590269	*A.ventricosa/T.durum//13*SK(NDM6)/3/7*SPN*
7	PI 590271	*Haynaldia villosa*/*T. durum* //9*SK(NDM7)/3/7*SPN
8	PI 590273	*T.macha/17*SK(NDM8)//7*SPN*
9	PI 590275	*T.macha*/9*SK(NDM9)//7*SPN
10	PI 590277	*T. turgidum*/9*SK(NDM10)//7*SPN

Table 2.2 Pedigree of NIL population in four euplasmic backgrounds

U1275 background	Jagger background	Karl 92 background	Ventnor background
PI590259/TAM-107//6*U1275	PI 590259/KARL 92//5*Jagger	-	-
PI 590261/TAM-107//6*U1275	PI 590261/6*Jagger	PI 590261/6*Karl 92	-
PI 590263/TAM-107//6*U1275	PI 590263/6*Jagger	PI 590263/6*Karl 92	PI 590263/TAM-107//5*Ventnor
PI 590265/TAM-107//6*U1275	PI 590265/6*Jagger	-	PI 590265/TAM-107//5*Ventnor
PI 590267/TAM-107//6*U1275	PI 590267/6*Jagger	PI 590267/6*Karl 92	PI 590267/TAM-107//5*Ventnor
PI 590269/TAM-107//6*U1275	-	PI 590269/6*Karl 92	PI 590269/TAM-107//5*Ventnor
PI 590271/TAM-107//6*U1275	PI 590271/6*Jagger	PI 590271/6*Karl 92	PI 590271/TAM-107//5*Ventnor
PI 590273/TAM-107//6*U1275	PI 590273/6*Jagger	-	PI 590273/TAM-107//5*Ventnor
PI 590275/TAM-107//6*U1275	PI 590275/6*Jagger	PI 590275/6*Karl 92	PI 590273/TAM-107//5*Ventnor
PI 590277/TAM-107//6*U1275	PI 590277/6*Jagger	PI 590277/6*Karl 92	PI 590277/TAM-107//5*Ventnor

Table 2.3 Number of chloroplast SSR markers showing polymorphism between two reciprocals of cytoplasmic NILs using a total of 24 SSR markers.

No	Alloplasmic lines	Number of primers showing polymorphism with euplasmic cytoplasm			
		U1275	Jagger	K-92	Ventnor
1	*Aegilops juvenalis*	15	14	Missing line	Missing line
2	*A. cylindrica*	15	17	10	Missing line
3	*A. variabilis*	7	13	17	15
4	*A. squarrosa*	16	13	Missing line	14
5	*A. uniaristata*	14	14	13	15
6	*A. ventricosa*	0	Missing line	11	11
7	*Haynaldia villosa*	0	0	0	0
8	*T. macha*	0	0	Missing line	0
9	*T. macha*	0	0	0	0
10	*T. turgidum*	1	1	1	1

Table 2.4 F-values from the ANOVA of parental lines comparison for different traits.

	Seed number	1000 kernel weight	Grain filling duration	Chlorophyll content	Fv/Fm
Optimum temperature (20/15°C)	2.49*	1.5	4.96***	1.87*	2.25**
High temperature (35/30°C)	1.38	0.44	1.87	4.99***	3.45***

*, * *, and ***, denote P < 0.05, 0.01 and 0.001 respectively.

Table 2.5 Lsmeans of seed number per head, thousand seed weight and Grain filling duration of alloplasmic and euplasmic parental line under both optimum and high temperature

Treatment	Lines	Seed Number	TKW	GFD
Optimum temperature (20/15°C)	1	56	51.93	44
	2	51	55.29	53
	3	57	47.93	46.67
	4	54.5	57.25	50
	5	56	51.44	53.67
	6	55.67	58.71	30.67
	7	54	64.57	56.67
	8	53.67	72.56	56.67
	9	64.67	62.16	53
	10	56.67	49.69	44
	U1275	36.33	56.16	62.33
	Jagger	40.67	54.22	53.33
	Karl-92	30.33	43.23	54.33
	Ventnor	39.67	58.29	76.67
	LSD	17.82	18.96	13.40
	SE	8.68	9.22	6.54
Heat stress (35/30°C)	1	25.67	15.37	21.67
	2	16.33	12.25	19
	3	30	19.06	26.33
	4	18.33	15.81	17.67
	5	24.33	13.25	19
	6	22.33	9.78	16.87
	7	30.67	19.50	22.67
	8	38.67	17.80	25.33
	9	22	19.07	21.33
	10	31.33	19.01	22
	U1275	36.67	16.168	22.67
	Jagger	35.33	17.98	25.45
	Karl-92	25.67	16.44	25.33
	Ventnor	21	16.85	34.33
	LSD	17.05	13.69	9.67
	SE	8.32	6.65	4.70

TKW- Thousand Kernel Weight, GFD- Grain Filling Duration

Table 2.6 Slope and intercept of parental lines for chlorophyll content and chlorophyll fluorescence under both optimum and high temperature

Treatment	Lines	Chlorophyll content		Fv/Fm	
		Intercept	Slope	Intercept	Slope
Optimum temperature (20/15°C)	1	55.79	0.14	754.1	-6.47
	2	58.75	-0.63	765.89	-6.14
	3	55.14	0.23	750.76	-0.32
	4	55.47	-0.07	746.27	0.21
	5	56.81	-0.29	751.27	-0.93
	6	55.37	0.11	755.8	-2.89
	7	52.76	-0.003	747.73	-7.33
	8	56.16	0.32	752.51	-2.31
	9	55.80	0.22	763.29	-3.97
	10	56.09	0.26	698.89	8.33
	U1275	50.53	0.78	775.37	-0.21
	Jagger	52.76	0.15	777.76	-1.65
	K-92	50.60	0.40	779.72	0.54
	Ventnor	51.56	0.06	784.85	1.13
	LSD	-	0.74	-	7.71
	SE	-	0.38	-	3.93
Heat stress (35/30°C)	1	57.03	-8.03	803.82	-113.3
	2	39.75	-7.21	697.74	-139.8
	3	49.77	0.01	756.19	-4.66
	4	43.73	-7.34	659.71	-122.3
	5	51.25	-8.50	762.11	-139.8
	6	46.68	-8.64	727.62	-145.3
	7	55.08	-4.37	706.84	-44.3
	8	52.77	-6.35	852.84	-116.8
	9	56.76	-8.19	749.11	-106.1
	10	48.27	-3.55	729.84	-81.45
	U1275	67.14	-9.79	920.51	-134.5
	Jagger	54.59	-7.41	724.27	-92.7
	K-92	58.74	-5.80	837.4	-57.3
	Ventnor	52.65	-0.35	778.24	-16.85
	LSD	-	3.66	-	68.06
	SE	-	1.86	-	34.72

TKW- Thousand Kernel Weight, GFD- Grain Filling Duration

Table 2.7 Performance of alloplasmic cytoplasms of NILs under high temperature

Line	Chlorophyll				Fv/Fm				SN				TKW				GFD			
	U	J	K	V	U	J	K	V	U	J	K	V	U	J	K	V	U	J	K	V
1	↑*	↑*	NA	NA	↑ *	↑ *	NA	NA	-	↑	NA	NA	-	-	NA	NA	-	-	NA	NA
2	↑	↑*	-	NA	-	-	↓	NA	-	-	-	NA	-	-	-	NA	-	-	-	NA
3	-	-	-	-	-	↑	-	-	↓*	-	-	-	-	-	-	-	-	-	-	-
4	-	↑*	NA	↓*	-	-	NA	-	↓*	-	NA	-	-	-	NA	-	-	-	NA	-
5	-	-	↑	-	-	↑	↑	↑	-	-	-	-	-	-	-	-	-	-	-	-
6	NA	NA	-	↓*	NA	NA	-	↓*	NA	NA	-	-	NA	NA	-	-	NA	NA	-	-
8	-	-	NA	↑*	↓*	↑ *	a	↑	-	-	NA	-	-	-	NA	-	-	-	NA	-
9	-	↓*	-	↑*	-	↓*	↑*	↑*	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	↑	↑*	-	-	↑*	↑*	-	↑*	-	-	-	-	-	↑*	-	-	-	-

↑ and ↓ indicates positive and negative influence to improve the respective trait. 'NA' indicates missing NIL *, denote P < 0.05. U, J, K, and V indicate U1275, Jagger, Karl 92 and Ventnor respectively.

Table2.8 F-values from the ANOVA of the experiment of NIL population for different parameters under both optimum and high temperature condition

Experiment	Source of variation	Seed number per head	Thousand kernel weight	Grain filling duration	Slope of chlorophyll content	Slope of Fv/Fm
Optimum temperature (20/15°C)	Genotype	16.43***	54.38***	127.26***	1.76	8.31***
	Alloplasmons	1.14	1	3.59***	2.72**	0.73
	G*A	1.26	2.33**	2.81***	1.13	0.65
	NIL	0.07	6.13*	0.06	5.04*	0.7
	G*N	1.92	1.65	0.38	2.88*	0.69
	A*N	1.07	0.58	2.19*	1.52	0.35
	G*A*N	0.86	0.87	1.17	0.99	0.69
High temperature (35/30°C)	Genotype	3.29*	2.37	1.24	19.95***	29.51***
	Alloplasmons	2.16*	1.23	0.28	3.53***	2.31*
	G*A	1.17	0.49	0.98	3.52***	5.21***
	NIL	0.19	0.7	0.55	11.26***	16.33***
	G*N	0.84	0.08	0.22	2.51	4.54**
	A*N	3.73***	1.52	0.86	3.33***	3.41***
	G*A*N	0.69	0.54	0.45	2.79***	3.11***

*, * *, and ***, denote $P < 0.05$, 0.01 and 0.001 respectively.

Table 2.9 Chloroplast specific SSR markers used for discrimination between two NILs (Ishii, 2001)

Primer name	Forward Primer	Reverse Primer	Location	Gene	Size
Wct1	CATCCTTTTCAATCCAAAATCA	GATTAGTGCCGGATACGGG	Intergenic region	*mat*K-5′*trn*K	109
Wct2	CTTATCTAATGACCCAGGACGG	CGAATTGGAAAGAATTCTGACC	Intergenic region	*psb*I-*trn*S	132
Wct3	TCTGGCTCGGTTATTCCATC	TCTAGGAACTATCGAGGGTTCG	Intergenic region	*psb*C-*trn*S	148
Wct4	TCTTCGGAAACGGAAAACC	GGATTTCCCATTATGGGTCC	Intergenic region	5′*trn*G-*trn*T	199
Wct5	TGATATTCTTTCGTGGAGTCCC	TGGTATCCAAAGAAAGGTCCC	Intergenic region	ORF29-*trn*C	83
Wct6	TCACAGGCTGCAAAATTCAG	GGATAATAATGCTGTTCGGACC	Intergenic region	*trn*C*rpo*B	187
Wct7	ATCGTTCCCCACAAGACAAG	AGGGTTAAATGTTAAATGGGGG	Coding region	*rpo*C2	168
Wct8	CTTGGGCAACTGCGTAAATT	ACCAAGAAAGCACATCAGATCA	Intergenic region	*rps*2-*atp*I	150
Wct9	CGCAGCCTATATAGGTGAATCC	TTGCAACCAAGCAGATTATCC	Intergenic region	*atp*I-*atp*H	125
Wct10	TGCCCTTTTTTAACCAATGC	CATGGTCAGCAAAGTTGTTTC	Intron	*atp*F	187
Wct11	TTTTATCTAGGCGGAAGAGTCC	TCATTTGGCTCTCACGCTC	Intron	*atp*F	171
Wct12	CGATCCCTATGTAGAAAGCCC	AACGAAACCCCTTCTTACCG	Intron	*inf*170	149
Wct13	TGAAAATCCTCGTGTCACCA	TGTATCACAATCCATCTCGAGG	Intergenic region	*trn*F-*ndh*J	110
Wct14	TCAACAAGTGACTCGAACTGTG	CGTCATGGAATAGGTGTCTCA	Intergenic region	*rpl*23-*psa*I	199
Wct15	CAATCTGGTTTTGCCTGGTT	ATGGGGTTTTCTATTGATGCC	Intergenic region	*psb*E-ORF31	105
Wct16	TGGTTGGAGTTCGAAAAAGG	AACAAAAGCATTCCACGACC	Intergenic region	*psb*E-ORF31	100
Wct17	GATCGTTCACTCCAAAAAGAGG	ACCCCATTGAATGAAAAAATG	Intergenic region	*psb*E-ORF31	146
Wct18	TGATTCGGAATTAGGGACTCA	GTAAGCATGAAAGAGTTAAATTCCA	Intergenic region	*psb*E-ORF31	199
Wct19	TTTGGAAAAAATAAGTCTCTTCGC	GCGTATCGAAGACTCGAAGG	Intergenic region	*rpl*36-*inf*A	153
Wct20	TTCCATTGGGTAGGGCTTC	GTAATCGCCCCGCCTATAGT	Coding region	*inf*A	178
Wct21	TTCCATTGGGTAGGGCTTC	GTAATCGCCCCGCCTATAGT	Coding region	*inf*A	178
Wct22	GCAATAGTGTCCTTGCCCAT	ACCAAAATAGTTTCATTAGCTCCTG	Intergenic region	*rps*8-*rpl*14	197
Wct23	TCCAGAAAGAAAAACCGGG	TAGCTGCCAGTAAAATGCCC	Intergenic region	*rpl*14-*rpl*16	108
Wct24	AAAAATTAAGAATGGGTTTAGTTGG	AACAAGAGAGCTGTTTCATCTTTG	Intergenic region	*ndh*F-*rpl*32	189

Table 2.10 F- values for comparison of two reciprocals of a NIL in seed number per head under both optimum and high temperature

	Optimum temperature (20/15°C)				Heat stress (35/30°C)			
Cytoplasm	U1275	Jagger	Karl92	Ventnor	U1275	Jagger	Karl92	Ventnor
1	0.27	0	-	-	0.87	7.08*	-	-
2	1.13	1.54	0.22	-	1.16	0.07	0.59	-
3	0.78	1.54	4.52*	0.89	6.8*	1.48	3.54	0.08
4	0.17	0.06	-	2.93	5.59*	0.58	-	0.08
5	1.4	0.68	0.01	2.36	0.5	0.01	1.54	0.17
6	0.09	-	1.4	0.13	0.35	-	2.25	1.24
7	0.13	1.69	0.35	1.4	0.89	0.81	0.59	0.01
8	1.17	1.13	-	1.4	0.55	0.13	-	2.36
9	0.35	0.42	2.54	0.68	0	0.28	0	0.09
10	0.59	1.01	0.03	0	1.4	6.77*	2.55	1.69

*, * *, and ***, denote $P < 0.05$, 0.01 and 0.001 respectively

Table 2.11 F- values for comparison of two reciprocals of a NIL in thousand kernel weight under both optimum and high temperature

Cytoplasm	Optimum temperature (20/15°C)				Heat stress (35/30°C)			
	U1275	Jagger	Karl92	Ventnor	U1275	Jagger	Karl92	Ventnor
1	0.01	0.23	-	-	0.86	0.1	-	-
2	1.45	1.08	0	-	0.02	0.01	0.18	-
3	0.01	0	0	1.64	0.03	0.59	1.16	1.36
4	0.29	0.81	-	5.8*	1.57	0.58	-	1.12
5	0	0.75	0.17	5.14*	0	0.84	0	0.07
6	0.27	-	0.33	6.64*	1.34	-	0.24	1.54
7	0.04	0.47	0.06	0.73	0.02	0.15	0.03	1.41
8	1.49	0	-	0.01	0.06	0.26	-	1.42
9	0.29	0.9	0.01	2.18	0.39	0.27	0.31	3.34
10	3.21	0.54	0.09	1.31	0.77	0.04	0.56	4.55*

*, * *, and ***, denote $P < 0.05$, 0.01 and 0.001 respectively.

Table 2.12 F values for comparison of two reciprocals of a NIL in grain filling duration under both optimum and high temperature

Cytoplasm	Optimum temperature (20/15°C)				Heat stress (35/30°C)			
	U1275	Jagger	Karl92	Ventnor	U1275	Jagger	Karl92	Ventnor
1	1.92	0.56	-	-	3.08	0.09	-	-
2	1.38	1.64	0.73	-	0.02	0.48	0.09	-
3	2.23	0.1	0.28	0.01	0.38	0.01	0.05	0.01
4	4.1*	1.38	-	0.28	1	0.21	-	0.59
5	0.92	0.92	0.28	0.73	0.85	0.15	0.01	0.15
6	11.64***	-	0.01	6.02*	1	-	0.02	0.29
7	0.56	0.05	0.28	0.41	0.72	2.61	0.15	0.85
8	2.32	0.05		0.51	0.02	0.72	-	0.85
9	0.05	0.1	0.04	0.01	0	0.01	0.85	0.85
10	2.91	0.73	0.73	1.64	0.21	0.21	0.02	0.85

*, * *, and ***, denote $P < 0.05$, 0.01 and 0.001 respectively.

Table 2.13 Slope of chlorophyll content and chlorophyll fluorescence (Fv/Fm) of NIL population under both high and optimum temperature

Genotype	Alloplasmic Lines	NIL	Chlorophyll content		Fv/Fm	
			Optimum Temperature (20/15°C)	Heat stress (35/30°C)	Optimum Temperature (20/15°C)	Heat stress (35/30°C)
U1275	1	1	0.833	-4.608	-4.058	-48.220
U1275	1	2	0.577	-10.13	-0.041	-154.76
U1275	2	1	-0.577	-5.881	2.009	-58.371
U1275	2	2	0.390	-8.88	-0.691	-64.105
U1275	3	1	0.537	-9.004	-0.137	-120.2
U1275	3	2	0.510	-8.745	-1.119	-120.36
U1275	4	1	0.159	-10.88	-0.241	-123.79
U1275	4	2	0.035	-9.169	1.908	-103.13
U1275	5	1	0.225	-7.68	-0.461	-78.448
U1275	5	2	0.489	-8.97	0.389	-107.28
U1275	6	1	0.287	-8.826	2.067	-33.01
U1275	6	2	0.307	-5.601	0.299	-40.048
U1275	7	1	0.140	-9.56	0.320	-110.01
U1275	7	2	0.533	-6.406	-0.255	-56.343
U1275	8	1	0.369	-9.219	0.582	-130.11
U1275	8	2	0.560	-8.766	-0.961	-44.695
U1275	9	1	0.100	-4.44	0.325	-58.533
U1275	9	2	0.212	-4.426	-0.670	-40.191
U1275	10	1	0.693	-7.617	-0.640	-74.105
U1275	10	2	0.509	-6.235	-1.428	-57.276
Jagger	1	1	0.293	-4.869	-0.710	-7.876
Jagger	1	2	0.427	-8.436	-1.432	-74.657
Jagger	2	1	-0.004	-2.501	-3.812	-61.695
Jagger	2	2	0.478	-5.935	4.730	-49.486
Jagger	3	1	0.700	-5.079	-2.086	-33.543
Jagger	3	2	0.153	-5.691	-4.486	-83.876
Jagger	4	1	0.578	-0.947	0.041	-11.143
Jagger	4	2	0.127	-5.323	-2.124	-12.329
Jagger	5	1	0.261	-4.057	-5.371	-15.657
Jagger	5	2	0.003	-5.445	-2.240	-42.762
Jagger	7	1	0.071	-2.705	-5.086	-40.695
Jagger	7	2	-0.023	-6.618	-3.524	-89.362
Jagger	8	1	0.198	-3.405	-3.154	-12.752

Jagger	8	2	0.304	-3.691	-7.133	-70.771
Jagger	9	1	0.224	-7.211	-6.003	-112.66
Jagger	9	2	0.207	-3.568	-3.981	-24.505
Jagger	10	1	0.294	-9.757	-2.554	-95.486
Jagger	10	2	0.091	-9.451	-0.486	-111.29
Karl 92	2	1	0.411	-4.951	-2.295	-84.029
Karl 92	2	2	0.302	-3.835	-3.505	-37.524
Karl 92	3	1	0.526	-5.312	-4.952	-4.295
Karl 92	3	2	0.506	-5.977	-2.391	-5.467
Karl 92	5	1	0.070	-3.834	-2.562	-8.457
Karl 92	5	2	0.189	-6.503	-3.476	-62.124
Karl 92	6	1	0.434	-4.283	-0.895	-7.238
Karl 92	6	2	-0.404	-2.964	-2.676	-5.419
Karl 92	7	1	0.900	-3.295	1.512	-9.648
Karl 92	7	2	0.331	-6.754	-3.057	-104.36
Karl 92	9	1	0.647	-5.534	-1.853	-43.876
Karl 92	9	2	0.150	-5.051	-1.205	-110.01
Karl 92	10	1	0.299	-6.911	-2.343	-49.810
Karl 92	10	2	0.420	-8.351	-3.822	-110.49
Ventnor	3	1	0.486	-8.081	2.799	-120.96
Ventnor	3	2	0.405	-10.11	1.403	-116.7
Ventnor	4	1	0.603	-10.45	3.455	-129.07
Ventnor	4	2	0.316	-6.652	0.274	-113.73
Ventnor	5	1	0.431	-6.055	1.022	-88.829
Ventnor	5	2	-0.168	-7.696	-0.700	-120.57
Ventnor	6	1	0.334	-10.84	1.927	-179.5
Ventnor	6	2	-0.095	-5.833	0.152	-110.91
Ventnor	7	1	0.112	-9.207	1.659	-139.61
Ventnor	7	2	0.443	-10.63	-0.151	-188.14
Ventnor	8	1	0.826	-2.829	0.286	-17.295
Ventnor	8	2	0.719	-6.152	-0.057	-73.457
Ventnor	9	1	0.467	-1.041	-0.675	-20.492
Ventnor	9	2	-0.403	-10.08	-0.850	-150
Ventnor	10	1	0.780	-4.711	1.578	-22.238
Ventnor	10	2	0.180	-9.401	-1.884	-123.42
	LSD		0.62	3.40	6.01	57.56

** Under NIL, 1 represents cytoplasm of corresponding alloplasmic line, and 2 represents cytoplasm of corresponding euplasmic genotype. Both “1” and “2” are having common nuclear genome from the corresponding euplasmic genotype.

Table 2.14 Lsmeans of thousand seed weight, seed number per head and grain filling duration of NIL population under both optimum and high temperature condition

Genotype	Allo Lines	NIL	TKW		SN		GFD	
			OT (20/15°C)	HT (35/30°C)	OT (20/15°C)	HT (35/30°C)	OT (20/15°C)	HT (35/30°C)
U1275	1	1	55.03	20.35	32.29	31.54	55.33	29.5
U1275	1	2	55.37	15.73	29	25.67	59.67	21
U1275	2	1	48.24	19.24	29	32.46	55.33	23.67
U1275	2	2	52.96	18.49	23	25.67	59	23
U1275	3	1	53.95	16.67	34.33	10.54	58	19
U1275	3	2	54.39	15.61	29.33	27	62.67	21.67
U1275	4	1	50.10	14.47	33	22.33	63.33	19.67
U1275	4	2	52.21	20.03	35.33	35.67	57	24
U1275	5	1	56.71	16.64	36.33	29.67	59.67	25
U1275	5	2	56.94	16.42	29.67	25.67	62.67	21
U1275	6	1	54.95	16.06	32.33	29.33	63.67	21.67
U1275	6	2	56.99	21.20	30.67	26	53	26
U1275	7	1	58.61	16.73	30	22.33	61	21.33
U1275	7	2	59.37	17.43	32	27.67	63.33	25
U1275	8	1	52.24	19.54	31.33	30.67	65.67	22.33
U1275	8	2	57.60	20.71	24.49	25.99	71	23
U1275	9	1	53.78	14.54	22.67	19.33	65.33	26.67
U1275	9	2	55.90	17.31	26	19.67	66	26.67
U1275	10	1	53.49	18.02	28.67	23.33	66	25.67
U1275	10	2	60.51	14.11	33	16.67	60.67	23.67
Jagger	1	1	52.70	18.44	40	38.67	54.67	24
Jagger	1	2	50.84	17.01	40	23.67	57	25.33
Jagger	2	1	44.64	17.78	40.33	30.67	51.67	26
Jagger	2	2	48.72	17.20	33.33	29.04	55.67	23
Jagger	3	1	48.04	15.42	37.67	16.99	57.33	24

Jagger	3	2	48.30	19.22	30.67	24.67	56.33	23.67
Jagger	4	1	49.16	16.44	36	34.46	61.33	26.67
Jagger	4	2	52.69	20.23	37.33	29.67	57.67	24.67
Jagger	5	1	48.65	16.40	32.33	23	59	24
Jagger	5	2	52.04	20.47	37	23.67	56	22.33
Jagger	7	1	47.39	21.32	45	24.33	53.33	27
Jagger	7	2	50.07	19.40	37.67	29.99	54	34
Jagger	8	1	54.40	22.45	30.67	31.67	54.67	27.33
Jagger	8	2	54.29	20.18	36.67	29.67	55.33	23.67
Jagger	9	1	49.5	19.87	34.33	26	56.33	23
Jagger	9	2	53.31	22.17	38	29	55.33	23.33
Jagger	10	1	52.54	17.41	37	31.67	58.33	23.33
Jagger	10	2	49.66	16.50	42.67	17	55.67	21.33
Karl 92	2	1	47.99	16.42	28.33	27	58.33	22
Karl 92	2	2	47.95	14.52	25.67	22.67	55.67	23.33
Karl 92	3	1	42.13	10.56	22	17.46	58.33	22.67
Karl 92	3	2	42.37	15.90	34	29.33	60	23.67
Karl 92	5	1	42.23	16.09	24	20.67	59.67	21.33
Karl 92	5	2	40.61	16.43	24.67	28.50	61.33	21.67
Karl 92	6	1	40.74	14.55	19.33	17.54	62.33	21.33
Karl 92	6	2	38.50	16.96	26	27	62.67	22
Karl 92	7	1	42.51	16.25	25	18.33	59.33	24.67
Karl 92	7	2	41.51	15.46	28.33	22.67	61	23
Karl 92	9	1	42.84	17.99	23	28.67	61	26
Karl 92	9	2	43.19	15.52	32	28.67	61.67	22
Karl 92	10	1	43.08	16.19	31.67	26.33	60.33	22.33
Karl 92	10	2	41.92	12.84	32.67	17.33	57.67	21.67
Ventnor	3	1	51.61	10.97	32.33	20.67	74.33	23
Ventnor	3	2	56.63	16.79	27	22.46	74	23.33
Ventnor	4	1	47.89	14.62	36.67	22.67	76	24
Ventnor	4	2	57.33	20.40	27	24.50	77.67	20.67

Ventnor	5	1	50.90	16.48	37	24.67	74	23.33
Ventnor	5	2	59.79	15.32	28.33	22.33	76.67	25
Ventnor	6	1	44.50	13.19	25.33	23.96	77.67	21.33
Ventnor	6	2	54.60	19.96	23.33	31	70	23.67
Ventnor	7	1	56.03	4.76	20.33	19.08	68.67	16.67
Ventnor	7	2	52.68	12.69	27	20	66.67	20.67
Ventnor	8	1	45.42	19.16	29.67	21.67	71.82	27.33
Ventnor	8	2	45.83	26.67	36.33	13	68.67	23.33
Ventnor	9	1	54.57	20.57	24.67	23.33	69.33	25
Ventnor	9	2	48.78	11.48	29.33	21.67	69.67	21
Ventnor	10	1	53.81	22.28	28.33	25.67	68.67	26.33
Ventnor	10	2	58.30	11.67	28.67	18.33	72.67	22.33

** Under NIL, 1 represents cytoplasm of corresponding alloplasmic line, and 2 represents cytoplasm of corresponding euplasmic genotype. Both “1” and “2” are having common nuclear genome from the corresponding euplasmic genotype. OT- Optimum Temperature, HT- High Temperature

Chapter 3 - Mapping QTL for the traits associated with heat tolerance in wheat (*Triticum aestivum* L.)

Abstract

High temperature stress during grain filling is a major problem in most of the wheat growing areas. Developing heat tolerant cultivars is becoming a principal breeding goal in the Southern and Central Great Plain areas of USA. Thermotolerance is a well known adaptive phenomenon and an inherent component of heat tolerance mechanism in plants. Traits associated with thermotolerance can be used to develop heat tolerant cultivars in wheat. The present study was done to identify chromosomal regions associated with thylakoid membrane damage (TMD), plasmamembrane damage (PMD) and Chlorophyll content (SCC), which are indicative of thermotolerance. An F_6 derived mapping population, developed from the heat tolerant line, Ventnor and heat sensitive line, Karl 92, was phenotyped for TMD, PMD, and SCC in the F_6 derived F_9 and F_{10} generations. The population was genotyped using SSR, AFLP and GBS-SNP markers. At ten days after anthesis plants were initially exposed to increasing high temperature from 20^{o}C to 36^{o}C over 48 hours time to develop post-anthesis heat tolerance and, subsequently, the heat-adapted plants were exposed to chronic heat treatment at 36^o/30^{o}C day/night temperature for 10 days. Composite interval mapping identified five QTL regions significantly associated with PMD on chromosomes 7A, 2B and 1D, SCC on 6A, 7A, 1B and 1D and TMD on 6A, 7A and 1D. The variability explained by these QTL ranged from 11.90 to 30.62% for TMD, 11.37 to 30.84% for SCC, and 10.53 to 33.51% for PMD. Molecular markers *Xbarc113* and AFLP AGCTCG-347 on chromosome 6A, *Xbarc121* and *Xbarc49* on 7A, *gwm18* and Bin1130 on 1B, Bin178 and Bin81 on 2B and Bin747 and Bin1546 on 1D were associated with these QTL. These QTL can be used for marker assisted selection in breeding wheat for heat tolerance.

Introduction

Wheat is one of the most widely grown cereals globally. Even though there is adaptive plasticity, terminal heat stress has become a common limiting factor for almost all wheat grown in temperate regions, which accounts for 40% (36 million ha) of the total wheat production in the world (Reynolds et al., 1994; Wardlaw and Wrigley, 1994; Reynolds et al., 2001; Hays et al., 2007, Mason et al., 2011; and Paliwal et al., 2012). The southern Great Plains of the USA, is a temperate environment and accounts for 30- 40% of US wheat production and often experiences 32-35^{o}C during grain filling stage (Hays et al., 2007). Exposure to higher than optimum temperature at this stage reduces yield and decreases quality of wheat grain (Fokar et al., 1998; Wardlaw et al., 2002). According to Wardlaw et al. (1989), every 1^{o}C rise above 15 to 20^{o}C can cause 3 to 4% yield reduction. Based on that observation, normal high temperature of 32 to 38^{o}C may decrease harvest by 50% or more every year (Paulson, 1994). The annual occurrence of moderate heat stress, accompanied by periodic extreme heat stress, prevents wheat from reaching its actual yield potential in these temperate regions (Mason et al., 2011).

Thermotolerance is a well known adaptive phenomenon, which is induced by a short acclimation period at moderately high temperatures or by treatment with other non-lethal stress prior to subsequent heat stress (Hong and Vierling, 2000; Larkindale et al., 2005). In the field, thermotolerance occurs under natural conditions and the effect of thermotolerance is an inherent component of heat tolerance (Fokar et al., 1998). Though high temperature is a frequently occurring phenomenon, relatively little is known about the critical genes controlling heat tolerance in plants (Larkindale et al., 2005). To maintain growth and productivity, plants must adapt to stress conditions and exercise specific tolerance mechanisms. The alteration of various photosynthetic attributes under heat stress is a good indicator of heat tolerance as they show correlation with growth (Wahid et al., 2007). Injury to the photosystem can limit plant growth.

Chlorophyll fluorescence, an indicator of photosystem II activity and thylakoid membrane damage, have been shown to correlate with heat tolerance (Moffatt et al., 1990; Yamada et al., 1996). Moffatt et al. (1990) reported that wheat genotypes with higher variable fluorescence (F_v) could also have higher yield potential. Maximum (F_m), base (F_0), variable fluorescence (F_v), and half-time between F_0 and F_m have been reported to have strong genetic correlation with grain yield of durum wheat (Araus et al., 1998). Plasmamembrane stability (also called cell membrane thermostability), which is the reciprocal of plasmamembrane damage, has been reported by various authors to be related to cellular thermotolerance. Increased permeability of membranes is evident by increased loss of electrolytes, an indication of decreased membrane stability and has long been used as an indirect measure of heat-stress tolerance in diverse plant species, including soybean (Martineau et al., 1979), wheat (Blum et al., 2001), cotton (Ashraf et al., 1994), sorghum (Marcum, 1998), and barley (Wahid and Shabbir, 2005). Membrane thermostability has been reported to have strong genetic correlation with grain yield in wheat (Reynolds et al., 1994; Fokar et al., 1998), and a 16-18% expected genetic gain for heat tolerance could be achieved in soybean (Martineau et al., 1979). Marsh et al. (1985) found a large portion of the variability for membrane stability to be controlled by a small number of genes. Heritability of membrane thermostability in maize was estimated to be 73% (Ottaviano et al., 1991). As both thylakoid and membrane thermostability are associated with heat tolerance, it may be wise to combine both characters for the improvement of heat tolerance in wheat.

In spite of being promoted as a promising breeding tool, the use of membrane thermostability and chlorophyll fluorescence for improvement of thermotolerance in wheat is very limited because of time-consuming and labor intensive field evaluation processes. Moreover, membrane stability requires destructive sampling and the potential of high error is

involved in the process of estimating membrane stability. Similarly, measurements of chlorophyll fluorescence require use of expensive instrumentation and, in some cases, necessitates dark adaptation of the leaf tissue, which limits the number of plants that can be screened in a given day. In addition to the complex estimation processes, these traits are influenced by environmental conditions. Thus, improving heat tolerance through traditional breeding methods and use of these tools is difficult. Identification of DNA markers associated with acquired thermotolerance would allow marker assisted selection and an increased efficiency in breeding for these traits. In addition, the identification of QTL will be useful in the identification of genes that are important for tolerance to high temperatures.

Heat tolerance is quantitative trait (Moffatt et al., 1990; Ibrahim and Quick, 2001; Sun and Xu, 1998; and Yang et al., 2002b). Though understanding of molecular mechanisms of wheat plant response to long-term post-synthesis heat stress will be helpful to develop heat-tolerance in wheat, only a few QTL mapping studies have focused on heat tolerance. Yang et al. (2002b) found QTL linked to grain filling duration on the short arms of chromosomes 1B and 5A. In addition, QTL for heat tolerance under hot and dry conditions were detected on chromosomes 2B and 5B in a spring wheat population (Byrne et al., 2002). In another study, conducted under short-term reproductive stage heat stress, Mason et al. (2010; 2011) found several QTL on chromosome 1A, 1B, 2A, 2B, 3B, 5A and 6D for heat susceptibility index of flag leaf length, flag leaf width, visual wax content, kernel number, total kernel weight, and single kernel weight. Paliwal et al. (2012) reported QTL for thousand grain weight, grain fill duration and canopy temperature depression on chromosome 2B, 7B and 7D. Vijayalakshmi et al. (2010) reported QTL with significant effect for grain yield, grain weight, grain filling, stay green and senescence associated traits on 2A, 3A, 4A, 6A, 6B and 7A under post-anthesis high

temperature stress in wheat. In this study we used the same population and marker data of Vijayalakshmi et al. (2010) along with an additional set of Bin markers (SNPs data) developed by using the Genotype-by-Sequencing (GBS) approach.

Most of the reported QTL maps were based on low density SSR and / or AFLP marker. To get a better understanding of the architecture of complex traits, developing a map with high density molecular markers is needed. Genotype-by-sequencing (GBS) is an approach to develop SNP markers which can be used for mapping traits in diverse organisms. This approach is very simple and cost effective and is based on high throughput next generation sequencing. In this method, SNPs are discovered by sequencing a subset of genomic fragments following use of restriction enzymes (Elshire et al., 2011; Poland et al., 2012).

The objectives of the present study were to increase the marker density on the population used by Vijayalakshmi et al. (2010) and identify QTL for membrane stability and chlorophyll content in wheat under heat stress.

Materials and methods

Genetic materials and growth conditions

Ventnor, a hard white Australian wheat, and Karl 92, hard red winter wheat from Kansas were crossed to develop a recombinant inbred line population (RIL). The pedigree of Ventnor is unknown, while Karl 92 is an F_{11} reselection from the cultivar Karl. The pedigree of Karl 92 is PlainsmanV/3/Kaw/Atlas 50//Parker*5/Agent (Sears et al. 1997). Ventnor has been shown to have superior heat tolerance based on its ability to maintain photosynthetic capacity and kernel weight when exposed to post anthesis heat stress (Alkhatib and Paulsen, 1990; Yang et al., 2002a; Yang et al., 2002b). The recombinant inbred line population was developed by advancing from the F_2 through single seed descent (SSD) in the greenhouse to generate a set of $F_{6:7}$ RILs (Vijayalakshmi et al., 2010). The entire population was characterized for thylakoid and plasmamembrane damage, and for chlorophyll content in the $F_{6:9}$ and $F_{6:10}$ generations under optimum (20/15^{o}C) and high temperature stress (36/30^{o}C day/night time temperature) condition at the post anthesis stage.

The plants were grown in a controlled greenhouse at an optimal temperature of 20/15^{o}C, 50/60% relative humidity, 16-h photoperiod, and light intensity of 420 μmol m^{-2} s^{-1} (Yang et al., 2002b). Fertilizer, systematic insecticide, and fungicide were applied as per standard practice. Plants were grown under well watered conditions to avoid water stress. Six pots of each line were planted with three plants per pot. The primary tiller of each plant was tagged at anthesis and was used to estimating traits. Eight days after anthesis, plants were transferred to a controlled growth chamber maintained at optimum growth conditions. The plants were kept 48 hours in the controlled growth chamber to facilitate adaptation to growth chamber conditions.

Heat treatment and physiological characterization

At ten days after anthesis, three pots were left under optimum conditions and three were placed under high temperature stress. The optimum chamber was maintained at 20/15^{o}C, 50/60% relative humidity, 16-h photoperiod, and light intensity of 420 μmol m^{-2} s^{-1} (Yang et al., 2002a). The high temperature chamber was raised from 20/15^{o}C day/night to 36/30^{o}C day/night with 80% relative humidity over a 48 hour period. Those plants were then held at 36/30^{o}C for 10 days. Each pot was treated as a biological replication. Pots were randomly arranged inside the growth chamber for each temperature treatment. The experiments, under both control and temperature treatments, were maintained under well watered conditions to avoid water stress.

Chlorophyll a fluorescence can be used to assess damage to photosystem II (PSII) and thylakoid membranes caused by heat (Moffatt et al., 1990; Maxwell and Johnson, 2000; Sayed, 2003; and Ristic et al., 2007). The ratio of variable (F_v) to maximum fluorescence (F_m), F_v/F_m, was used as an indirect method to assess thylakoid membrane damage (Kadir et al.,2007; and Ristic et al., 2007). F_v/F_m was measured on intact flag leaves one third from the base of abaxial surface after 1 hour of dark adaptation. Fluorescence measurements were conducted using a pulse modular fluorometer (Model OS5- FL, Opti-Sciences, Hudson, NH, USA). The F_v/F_m was measured in plants under control and heat treatment conditions at 4-, 7-, and 10-days after heat treatment. F_v/F_m was estimated from three different plants for each replication and mean F_v/F_m was used for all subsequent analysis. Thylakoid membrane damage (TMD) due to heat stress was assessed by comparing F_v/F_m values between control and heat treated conditions. The relative damage was estimated as follows: % TMD = [((F_v/F_m-heat)-(F_v/F_m-control))/(F_v/F_m-control)]*100. The %TMD values were transformed by Log_2 function, and the transformed data were used for analysis of variance.

Thylakoid harbor chlorophyll, so we also estimated chlorophyll content with the objective determining whether both traits were under similar genetic control. Chlorophyll content was measured in the same flag leaves leaf blade areas where fluorescence measurements were taken. A self-calibrating SPAD chlorophyll meter (Model 502, Spectrum Technologies, Plainfield, IL) was used to measure chlorophyll content. The SPAD chlorophyll content (SCC) was measured on both control and heat treatment plants 4-, 7-, and 10-days after heat treatment. For SCC, the absolute mean value of three primary flag leaves in each replication was used for statistical analysis.

The plasmamembrane damage (PMD) was assessed using the method described by Ristic and Cass (1993). Leaf disks (diameter=5mm) from plants in both treatments were taken from the middle portion of the leaf blade and placed in de-ionized water (4 ml) in sealed vials. PMD was estimated only at 7- and 10-days after heat treatment. Data were collected on two individual flag leaves from two different plants within each replication, and the average value was used to estimate %PMD. The vials were stored overnight on a shaker at 5^{o}C. Electroconductivity of the aqueous solution was measured with a Metter Toledo (SevenMulti S70) conductivity meter. The tissues samples were then autoclaved. The conductivity of the solution was again measured after storing on a shaker at 5^{o}C overnight. The percent electrolyte leakage was calculated based on the conductivity before and after autoclaving. The percent damage was calculated as $100 \times (\%\ \text{leached}_h - \%\ \text{leached}_c) / (X - \%\ \text{leached}_c)$, where h was stressed, c was control, and 'X' was % leached value corresponding to 100% damage which was assumed to be 100% leached. Percent PMD values were transformed by using Log2 function for analysis of variance.

Adjusted mean (Best Linear Unbiased Prediction, BLUP) values were estimated for each sampling date of chlorophyll content, log transformed TMD and PMD data across two generations. Those estimated adjusted mean values of each sampling date were used for QTL analysis.

Statistical analysis

The mean values over three time points (4-, 7-, 10-days) for TMD and SCC, and two time points (7- and 10-days) for PMD were used for analysis of variance (ANOVA) to determine the main effects of genotype (RIL), block and replication factors. During analysis, growth chambers were used as blocks. Analysis of variance and least square means of all traits were estimated using SAS PROC MIXED. Phenotypic correlations and simple regression were calculated for all traits using Microsoft Excel, (2007). Adjusted mean (Best Linear Unbiased Prediction, BLUP) values were estimated by R v2.12.0 statistical programming language (R Development Core 2008).

Molecular markers and map development

A total of 972 molecular markers were used in the mapping effort and included 538 Bin, 258 AFLPs, 175 SSRs, and an EST. The detailed description of the AFLP, SSR and EST markers has been provided (Vijayalakshmi et al., 2010). Bin markers were developed using a genotype by sequencing (GBS) approach (Poland et al., 2012). All the sample DNAs were collected from F_{10} plants leaves and digested by HF-PstI (High- Fidelity) and MspI (New England BioLabs Inc., Ipswich, MA 01938) followed by ligation with a set of 96 adapters (adapter 1) combined with a common adapter (Y adapter) in every reaction. Ligated samples were pooled in a single tube followed by PCR amplification to produce a single library from 96

samples. That library was sequenced on a single lane of Illumina GAII. Barcodes allowed assignment of Illumina raw data to individual samples. Sequences were trimmed to a 64 bp read and SNP-calling was performed using a custom script in Java (www.maizegenetics.net, sourceforge.net/projects/tassel/). To reduce the ratio of missing data, all the SNPs in a bin were called a Bin marker. JoinMap ver. 4.0 (Van Ooijen, 2006) with the Kosambi function (Kosambi, 1944) was used to assemble AFLP, SSR, EST and bin markers into a linkage map at LOD score 5.0. Significantly distorted markers were excluded from the analysis during the group preparation.

Quantitative trait locus (QTL) analysis

The Windows version of QTL Cartographer V2.5 (Wang et al., 2007) was used to conduct composite interval mapping (CIM) analysis based on model 6. The forward and backward regression method was used as a cofactor to control the genetic background while testing a position in the genome. The walking speed chosen for the QTL analysis was 2.0 cM. QTL were verified by LOD scores (2.88-3.28) compared to the threshold calculated from 1000 permutation for $p<0.05$. We also accepted those QTL as significant at a LOD value of 2.5 or more, once it fulfilled the declaration criteria and co localized with other traits described by Paliwal et al. (2012) and Pinto et al. (2010). QTL names were designated following the International Rules of Genetic Nomenclature.

(http://wheat.pw.usda.gov/ggpages/wgc/98/Intro.htm.)

Results

Genetic variations, physiological changes and assessment of heat tolerance

The variance components associated with different effects are presented in Table 1. The genotypes showed strong variation for TMD, SCC and PMD in both generations (F_{10} and F_9) of RIL population. The variance component associated with genotypes contributed more than 92% (deduced from Table 1) of total variation.

The mean values of TMD, SCC and PMD for parents and progenies are presented in Figures 1 and 2. Higher values of TMD and PMD indicate higher damage to thylakoid membrane and plasmamembrane, while higher SCC values indicate higher chlorophyll content in the leaves. The values of TMD and PMD increased with increasing duration of heat stress. On the other hand, values of SCC decreased with the duration of heat stress. The data indicates that the increased exposure of heat stress increases damage to the plasmamembrane, thylakoid membrane and reduces chlorophyll content in the heat stressed plants. The high temperature stress caused damage to thylakoid membrane and plasmamembrane and reduces chlorophyll content in both the tolerant and sensitive parents. However, the damage was lower in the tolerant parent than the sensitive parent. Compared to control, mean TMD values ranged from 12.2% in Ventnor to 32.1% in Karl 92, and mean PMD ranged from 14.3% in the tolerant parent to 42.8% in the sensitive parent (data not presented). The average value of SCC under heat stress (not compared with control) ranged from 43.3 in the tolerant parent to 30.6 in the sensitive parent (data not presented). The mean values for TMD, SCC and PMD in F_9 and F_{10} generations were 21.9 and 24.8%, 38.9 and 36.5%, and 28.6 and 32.9%, respectively (calculated from non

transformed data). The data demonstrated that more damage to thylakoid, chlorophyll and plasmamembrane occurred when the heat treatment duration was increased, which was expected.

The distribution of values for TMD, PMD and SCC are presented in Fig. 2. The F_9 and F_{10} population means were intermediate between the parental means for all three assays. Both positive and negative transgressive segregation was observed for both TMD and SCC (Fig. 2), as well as for PMD (transgressive segregation not shown).

Very strong phenotypic associations were observed among the three traits (Fig. 3). SCC explained 82% and 76% variability in TMD and PMD, respectively, while TMD explained 71% of the variability in PMD. Though all traits were very strongly associated, the association between TMD and SCC was higher than the associations between those two traits and PMD. The associations strongly suggest that the three traits are under similar genetic control and are physiologically associated.

Molecular markers and linkage map

Five hundred sixty of 972 markers were used to produce sizeable linkage groups. Linkage groups without any SSR markers were not considered a viable group in this analysis. Of the 560 group-forming markers, SSRs counts for 91, Bin markers accounted for 391 and AFLPs accounted for 78. The rest of the markers were ungrouped, grouped without an SSR, or distorted. Twenty-two linkage groups were identified and covered a total length of 1044cM, with an average interval of 1.86 cM between markers. All chromosomes except 5D were represented in the linkage groups. Chromosome 2A and 7D each had two groups. Comparing across genomes, the maximum number of markers mapped to the B genome (45.44%) followed by the A genome (42.68%) and the D genome (11.96%). Density of markers was greatest on chromosome 1B, with an average

distance of 0.81 cM between markers, and least on chromosome 2D, with an average interval of 3.25cM between markers.

QTL analysis

Results of QTL analysis for the three traits are shown in Table 2. Five genomic regions (chromosome 6A, 7A, 1B, 2B and 1D) were associated with significant QTL. The QTL were associated with LOD scores ranging from 2.5 to 7.28 and explained of the 10.53% to 33.51% of the phenotypic variability (Table 2 and Fig 4, 5, 6 and 7).

The QTL on chromosome 6A was found associated with SCC and TMD in the first two sampling date. In all the cases, the QTL was flanked by the markers *Xbarc113* and AGCTCG347. Presuming this represents one QTL affecting multiple traits, on an average; it explained 16.48% of phenotypic variation for SCC and 13.39% for TMD in the first two sampling dates (4- and 7- days after heat treatment) (Table 2 and Fig 4).

The QTL on the long arm of 7A showed significant effects for all three traits across all the three sampling date. This QTL was flanked by the *Xbarc121* and *Xbarc49* markers. It explained 19.15 to 30.62% of variability for TMD, 19.53 to 30.84% of variability for SCC, and of 32.03 to 33.51% variability for PMD (Table 2 and Fig 5, 6 and 7). This QTL was the most consistent across all the traits and explained the highest phenotypic variability. It showed significant effects in all sampling dates for TMD and SCC (4-, 7- and 10-days after heat treatment), and for PMD (7- and 10-days after heat treatment).

QTL on 1B chromosome was associated with the first two sampling date (4- and 7- days after heat treatment) of SCC and flanked by *gwm18* and Bin1130 (Table 2 and Fig 4). There was a 0.39cM displacement between the two regions identified for sampling day 4- and 7-.

The QTL identified on chromosome 2B was flanked by Bin 178 and Bin 81 and was significant for both sampling dates for PMD (7- and 10-days after heat treatment). This QTL explained an average of 13.88% of the phenotypic variation for PMD and was remarkably consistent in its effect (Table 2 and Fig 4)

The fifth QTL identified was on chromosome 1D. This QTL was found significant for PMD in the sampling date 10-, SCC in the 7- and TMD in the 4-. The phenotypic variability explained by the QTL was moderate. The highest variability was explained for SCC (16.64%) followed by TMD (14.12%). The QTL was flanked by the marker Bin 747 and Bin 1596. Heat tolerant alleles for PMD, SCC and TMD of all the reported QTL were contributed from the tolerant parent Ventnor (Table 2 and Fig 4).

Discussion

Most of the studies to map QTL associated with heat stress were conducted under short term heat stress. However, under field conditions, temperatures above 32°C are common in the Great Plains of the US. In our study, we first acclimated plants over a 48 hour period so that plants could adapt to the temperature changes and develop thermotolerance. After adaptation, plants were exposed to chronic high temperature for 10 days. Our study was conducted under initially optimal conditions followed by long-term high temperature stress, which is different from most of the other published QTL mapping studies in wheat.

Though there are various complex mechanisms involved in heat tolerance, one of the major effects of high temperature stress on plants is to alter membrane structures, such as plasma membranes (also called plasmalemma or cell membrane) and photosynthetic membranes (thylakoid membrane). Disruption of various photosynthetic and cellular structures causes premature senescence of the plants and, ultimately, reduces yield as assimilate mobilization to the sink is heavily altered. Reynolds et al. (1994) provided physiological evidence that loss of chlorophyll during grain filling is associated with reduced yield in the field. To maintain growth and productivity, plants must adapt to stress conditions and exercise specific tolerance mechanisms. Plant modification for enhanced tolerance is mostly based on the manipulation of genes that protect and maintain the function and structure of cellular components. Identification of genetic variations for these traits can be used to map QTL associated with plasma membrane and thylakoid membrane damage in response to long-term high temperature stress. Heat tolerant lines with more active photosynthetic systems and well maintained cell membranes, can maintain higher green leaf areas and biomass. This will, in turn, lead to a longer grain fill period and increased yield.

Thylakoid membranes and PS II are very thermolabile, and their activity is either greatly reduced or damaged under high temperature stress (Bukhov et al., 1999; and Camejo et al., 2005). The damage to photosystem II may be due to the properties of thylakoid membranes, which harbor chlorophyll, a portion of which is associated with the proteins of PS II (Schreiber and Berry, 1977; Vacha et al., 2007; and Hasegawa, et al., 2010). Heat-induced damage to thylakoid membranes interferes with photochemical reactions, and leads to chlorophyll loss in many crop plants, including wheat (Al-Khatib and Paulsen, 1984; Wise et al., 2004; and Ristic et al., 2007). We used chlorophyll fluorescence (F_v/F_m) and SPAD measurements to estimate thylakoid membrane damage and chlorophyll content of heat stressed plants. Chlorophyll fluorescence is a fast, non destructive and relatively simple way to detect energetic/metabolic imbalance of the photosystem due to water, heat, or combined stresses (Havaux et al., 1988; Moffatt et al., 1990; and Araus and Hogan, 1994). When determined in the dark-adapted state, the ratio of variable (F_v) to maximal fluorescence (F_m) is a measure of the potential quantum yield of PSII. F_v/F_m values are typically in the range of 0.75 to 0.85 in non-stressed plants (Bohlar-Nordenkampf et al., 1989), and are associated with net photosynthesis of intact leaves. Lower F_v/F_m values are an indicator of damage to the PS II and photosynthetic impairment (Araus and Hogan, 1994; Fracheboud et al., 2002). In addition to damage to thylakoid membranes, heat accelerates kinetic energy and can alter lipid properties and increase permeability of membranes (Savchenko et al., 2002). Dias et al. (2010) reported that durum wheat genotypes with higher membrane stability had lower membrane permeability. Cell membranes play a key role in heat- induced damage, as it is the site for many biological activities of plants. Membrane thermostability (MTS) is a fairly heritable trait (Ibrahim and Quick, 2001) and shows good correlation with yield (Reynolds et al., 2001). Shanahan et al. (1990) estimated

membrane thermostability (MTS) in field grown wheat genotypes and found that genotypes with higher membrane stability produced 21% higher yield than the genotypes with lower stability.

Transgressive segregation was observed in our population, meaning that both parents contribute alleles to the phenotype. Saadalla et al. (1990a) estimated genetic effects for MTS by examining 90 F_5 genotypes derived from crosses among heat-tolerant and heat sensitive wheat parents. They observed transgressive segregation in relative injury values determined by MTS, suggesting the parents contributed different genes. In an associated study, Saadalla et al. (1990b) used 144 wheat genotypes to estimate association between membrane thermostability and grain yield. They classified wheat genotypes as heat-tolerant, intermediate and heat sensitive, and found that genotypes in the heat tolerant group produced 9 to 19% more yield than genotypes classified as intermediate and heat sensitive respectively. The results suggest that membrane thermostability or plasmamembrane damage should be a suitable method for selecting heat tolerant wheat genotypes.

Our study demonstrated very strong association among PMD, SCC and TMD. The associations were stronger with increased exposure to heat (data not presented). These strong associations indicate that these traits might be under pleiotropic genetic control. Lin et al. (1985) found that cell membrane disruption under high temperature has the ability to impact photosynthetic or mitochondrial activity and simultaneously decrease the ability of the plasmalemma to retain solutes. Srinivasan et al. (1996) reported eelectrolyte leakage and fluorescence ratio were negatively correlated in legumes, and they concluded that electrolyte leakage and fluorescence tests can be used as screening procedures for breeding heat-tolerant legumes.

This study was done using the same population and marker data of Vijayalakshmi et al. (2010) along with an extra set of GBS SNP markers. The additional markers, and possibly, the use of different mapping software resulted in some variability in group formation compared to Vijayalakshmi et al. (2010). The 2A and 6A groups of Vijayalakshmi et al. (2010) were fused together and were assigned to 6A in this study. They assigned the 2A group based on *Xgwm356* and *Xbarc353*. Those markers have been reported to map to both 2A and 6A (http://wheat.pw.usda.gov/GG2/index.shtml). In our study, the additional markers allowed the identification of a single linkage group. Two markers specific to 6A allowed a more accurate chromosomal assignment.

Vijayalakshmi et al. (2010) and the current study used same mapping population, but varied in their conduct and traits evaluated. We exposed plants to moderate temperature before chronic heat treatment to develop thermotolerance. However, some QTL regions were common in both studies. The similarity of findings increases confidence that these chromosomal regions are truly associated with heat tolerance in this population.

In this study five genomic regions (6A, 7A, 1B, 2B and 1D) were significantly associated with traits related to heat tolerance. Of nineteen QTL identified in this study, twelve QTL explained greater than 15% of the phenotypic variability and should be considered as major QTL.

In the previous study with the same mapping population, Vijayalakshmi et al., (2010) found several QTL on chromosome arms (2A, 3A, 5A, 6A, 7A, 3B, 4B, 6B,7B and 5D) for different senescence related traits under high temperature stress (32/25°C day and night temperature from 10 days after anthesis to physiological maturity). QTL for 75% greenness on

chromosomes 2A and 3B and a region on 2A for 25% greenness were not found in the current work.

In the present study the QTL on 1D was associated with all three traits making it a potentially important QTL for heat. Pinto et al. (2010) reported QTL for anthesis date on 1D under drought and temperate irrigated condition. This QTL was likely not identified in the Vijayalakshmi study because the markers associated with the trait are new SNPs. Addition of new SNPs might be the cause to identify QTL on 1B in this study, which was not identified in the Vijayalakshmi study. Pinto et al. (2010) in their same study reported several QTL on 1B including QTL for canopy temperature, yield and chlorophyll content in the grain fill stage.

The 2B QTL was also not identified in the Vijayalakshmi study due to the lack of markers in the region. The only trait associated with 2B in the present study is PMD. It may be that this locus is only associated with membrane stability and not photosynthetic function. Mason et al. (2010) reported a stable QTL on 2B for Heat susceptibility index (HIS) of grain number.

The QTL identified on chromosome 6A was significant, consistent and co localized for SCC and TMD across the first two sampling dates (4- and 7- days after heat treatment).. Vijayalakshmi et al. (2010) found two QTL on 2A between Xgwm356 and CGT.TGCG-349, and between CGT.TGCG-349 and CTCG.ACC-242 for 75% greenness, 25% greenness, 50% greenness, maximum rate of senescence, and time for maximum senescence. Other markers associated with QTL on 2A and 6A in their study were Xgwm353, GTGACGT-189, GTGCTA-282 and CGACGCT-173. In this study, chromosome 6A encompasses both 2A and 6A of Vijayalakshmi et al. (2010). Most of the trait-associated markers from that study are present in the interval region of our putative QTL. As a result, we strongly believe that the QTL on 6A is

the same QTL previously identified on 2A and 6A and is associated with stay green related traits under high temperature.

The QTL on chromosome 7A was very consistent for all three traits across all the sampling dates with very high LOD values. Phenotypic variability explained by this QTL was also very high and ranged from 18.86% to 33.51%. It was flanked by marker *Xbarc121* and *Xbarc49*. Vijayalakshmi et al. (2010) reported a QTL on 7A for F_v/F_m and time to maximum rate of senescence (TMRS) associated with marker *Xbarc121*. *Xbarc49* marker was physically mapped to 7A by Sourdille et al. (2003) as wheat deletion bin C7AL 1-0.39. EST WHE2105_F08_K15ZS was also located in that bin and was found to be similar to the stress responsive gene (*srg6*) in Hordeum vulgare (NCBI). This mRNA is similar to a DNA binding protein in mouse and Human (Malatrasi et al., 2002). This suggests it may act as a regulatory gene for stress response. EST to a WHE0854_F06_L12ZS is in the same bin and showed homology to a calcium/calmodulin-dependent protein kinase gene in Maize (NCBI). This gene has a role in stress signal transduction in plants. It also acts as a positive regulator for salt and ABA stress tolerance in plants (Yang et al., 2010).

Another EST in that bin, WHE2324_F12_L24ZS, was found to have similarity to a putative DNA topoisomerase I gene in rice. This gene plays a crucial role in stress adaptation of plants by altering gene expression (Jain et al., 2006). This region would be of interest for further investigation.

Even though there was transgressive segregation in the population, we failed to detect beneficial alleles from sensitive parent. This may be attributed to the smaller size of the population and relatively sparse marker coverage throughout the genome. Only 11.96% of our

markers were mapped to the D genome. Most groups in the D genome were small. This might have prevented us from capturing alleles contributed from the sensitive parent.

The overall level of polymorphism in this population is surprisingly low. The pedigree of Ventnor is unknown. The fact that it is an Australian winter wheat. It is possible Ventnor contains US Great Plains wheat through international germplasm exchange. As a result, the genetic diversity between the two parents is very low.

Conclusions

Heat tolerance is a complex trait and influenced by different component traits. Damage to cellular structure and the photosystem are well defined processes, which influence heat tolerance. Our study suggests that function of both systems are very closely correlated when plants are exposed to a long period of stress, and are most likely under similar genetic control. We found four QTL that significantly influence those traits. These QTL are located on chromosomes 6A, 7A, 1B, 2B and 1D. The four SSR markers (*Xbarc121, Xbarc49, gwm18 and Xbarc113*) and five GBS Bin markers (*Bin747, Bin 1596, Bin 178, Bin 1130 and Bin 81*) which were strongly associated with component traits may be useful in marker assisted breeding for heat tolerance in wheat. The AFLP marker, *XCGT.AGCT347* would be a good target for conversion to a more user friendly marker.

References

Al-Khatib, K., and G.M. Paulsen. 1984. Mode of high temperature injury to wheat during grain development. Physiol. Plant. 61: 363–8.

Al-khatib, K., and G.M. Paulsen. 1990. Photosynthesis and productivity during high-temperature stress of wheat genotypes from major world regions. Crop Sci. 30: 1127–1132.

Araus, J.L., and P. Hogan. 1994. Leaf structure and patterns of photoinhibition in two neotropical palms in clearings and forest understory during the dry season. Am. J. Bot. 81: 726–738.

Araus, J.L., T. Amaro, J. Voltas, H. Nakkoul, and M.M. Nachit. 1998. Chlorophyll fluorescence as a selection criterion for grain yield in durum wheat under Mediterranean conditions. Field Crop Res. 55: 209–223.

Ashraf, M., M.M. Saeed, and M.J. Qureshi. 1994. Tolerance to high-temperature in cotton (*gossypium-hirsutum l*) at initial growth-stages. Environ. Exp. Bot. 34: 275–283.

Blum, A., N. Klueva, and H.T. Nguyen. 2001. Wheat cellular thermotolerance is related to yield under heat stress. Euphytica. 117: 117–123.

Bohlar-Nordenkampf, H.R., S.P. Long, N.R. Baker, G.O. quist, U. Schreiber, and E.G. Lechner. 1989. Chlorophyll fluorescence as a probe of the photosynthetic competence of leaves in the field: a review of current instrumentation. Funct. Eco. 3: 497–514.

Bukhov, N.G., and P. Mohanty. 1999. Elevated temperature stress effects on photosystems: Characterization and evaluation of the nature of heat induced impairments. In: Concepts in Photobiology; Photosynthesis and Photomorphogenesis (G.S. Singhal, G. Renger, S.K. Sopory, K.D. Irrgang, Govindjee, Eds.). Narosa Publishers, New Delhi, pp. 617–648.

Byrne, P.F., J.D. Butler, G.R. Anderson, and S.D Haley. 2002. QTL's for agronomic and morphological traits in a spring wheat population derived from a cross of heat tolerant and heat sensitive lines (poster). In: Plant, Animal Genome Conf. San Diego, Calif.

Camejo, D., P. Rodr´ıguez, M.A. Morales, J.M. Dell'amico, A. Torrecillas, and J.J. Alarc´on, 2005. High temperature effects on photosynthetic activity of two tomato cultivars with different heat susceptibility. J. Plant Physiol. 162: 281–289.

Dias, A.S., J. Semedo, J.C. Ramalho, and F.C. Lidon. 2010. Bread and durum wheat under heat stress: a comparative study on the photosynthetic performance. J. Agron. Crop Sci. 197 : 50–56.

Elshire, R.J., J.C.Glaubitz, Q. Sun, J.A. Poland, K. Kawamoto, E.S. Buckler, and S.E. Mitchell. 2011. A Robust, Simple genotyping-by-sequencing (GBS) approach for high diversity species. PLoS One 6: e19379.

Fokar, M., A. Blum, and H.T. Nguyen. 1998. Heat tolerance in spring wheat. II. Grain filling. Euphytica. 104: 9–15.

Fracheboud, Y., J.M. Ribaut, M. Vargas, R. Messmer and P. Stamp. 2002. Identification of quantitative trait loci for cold-tolerance of photosynthesis in maize (*Zea mays L.*). J. Exp. Bot. 53: 1967–1977.

Hasegawa, M., T. Shiina, M. Terazima, and S. Kumazaki. 2010. Selective Excitation of photosystems in chloroplasts inside plant leaves observed by near-infrared laser-based fluorescence spectral microscopy. Plant and Cell Physiol. 51: 225–238.

Havaux, M., Ernez, M., and R. Lannoye. 1988. Tolerance of poplar (*Populus sp*.) to environmental stresses: I. Comparative study of poplar clones using the in vivo chlorophyll fluorescence method. Acta Oecologia/Oecologia Plant. 9: 161–172.

Hays, D.B., J.H. Do, R.E. Mason, G. Morgan, and S.A. Finlayson. 2007. Heat stress induced ethylene production in developing wheat grains induces kernel abortion and increased maturation in a susceptible cultivar. Plant Sci. 172: 1112–1123.

Hong, S.W., and E. Vierling. 2000. Mutants of *Arabidopsis thaliana* defective in the acquisition of tolerance to high temperature stress. Proc. Natl. Acad. Sci. USA. 97**:** 4392–4397.

Ibrahim, A.M.H., and J.S. Quick. 2001. Heritability of heat tolerance in winter and spring wheat. Crop Sci. 41: 1401–1405.

Jain, M., A.K. Tyagi, and J.P. Khurana. 2006. Overexpression of putative topoisomerase 6 genes from rice confers stress tolerance in transgenic *Arabidopsis* plants. FEBS Journal. 273: 5245–5260.

Kosambi, D.D. 1944. The estimation of map distances from recombination values. Ann. Eugenics 12: 172–175.

Larkindale, J., J.D. Hall, M.R. Knight, and E. Vierling. 2005. Heat stress phenotypes of arabidopsis mutants implicate multiple signaling pathways in the acquisition of thermotolerance. Plant Physiol. 138: 882–897.

Lin, C.Y., Y.M. Chen, and J.L. Key. 1985. Solute leakage in soybean seedlings under various heat shock regimes. Plant Cell Physiol. 26: 1493–1498.

Malatrasi, M., T.J. Close, and N. Marmiroli. 2002. Identification and mapping of a putative stress response regulator gene in barley. Plant Mol. Biol. 50: 143–152.

Marcum, K.B. 1998. Cell membrane thermostability and whole-plant heat tolerance of Kentucky bluegrass. Crop Sci. 38: 1214–1218.

Marsh, L.E., D.W. Davis, and P.H. Li. 1985. Selection and inheritance of heat tolerance in common bean by use of conductivity. J. Am. Soc. Hort. Sci. 110: 680–683.

Martineau, J.R., J.E. Specht, J.H. Williams, and C.Y. Sullivan. 1979. Temperature tolerance in soybeans. I. Evaluation of a technique for assessing cellular membrane thermostability. Crop Sci. 19: 75–78.

Mason, R.E., S. Mondal, F.W. Beecher, A. Pacheco, B. Jampala, A.M.H. Ibrahim, and D.B. Hays. 2010. QTL associated with heat susceptibility index in wheat *(Triticum aestivum L.)* under short-term reproductive stage heat stress. Euphytica. 174: 423–436.

Mason, R.E., S. Mondal, F.W. Beecher, and D.B. Hays. 2011. Genetic loci linking improved heat tolerance in wheat (*Triticum aestivum L.*) to lower leaf and spike temperatures under controlled conditions. Euphytica. 180: 181–194.

Maxwell, K., and G.N. Johnson. 2000. Chlorophyll fluorescence - a practical guide. J. Exp. Bot. 51: 659–668.

Moffatt, J.M., R.G. Sears, and G.M. Paulsen. 1990. Wheat high temperature tolerance during reproductive growth. I. Evaluation by chlorophyll fluorescence. Crop Sci. 30: 881–885.

Ottaviano, E., M.S.Gorla, E. Pe, and C. Frova. 1991. Molecular markers (RFLPs and HSPs) for the genetic dissection of thermotolerance in maize. Theor. Appl. Genet. 81: 713–719.

Paliwal, R., M.S. Roeder, U. Kumar, J.P. Srivastava, and A.K. Joshi. 2012. QTL mapping of terminal heat tolerance in hexaploid wheat (*T. aestivum L.*). Theor. Appl. Genet. 125: 561–575.

Paulsen, G.M. 1994. High temperature responses of crop plants. In: K.J. Booth, T. Sinclair and G.M. Paulsen (Eds.), Physiology and Determination of Crop Yield, pp: 365–89. Amer. Soc. Agron., Crop. Sci. Soc. Amer., and Soil Sci. Soc. Amer. WI. Madison, USA

Pinto, R.S., M.P. Reynolds, K.L. Mathews, C.L. McIntyre, J.J. Olivares-Villegas, and S.C. Chapman. 2010. Heat and drought adaptive QTL in a wheat population designed to minimize confounding agronomic effects. Theor. Appl. Genet. 121: 1001–1021.

Poland, J.A., P.J. Brown, M. Sorrells, and J.L. Jannink. 2012. Development of high-density genetic maps for barley and wheat using a novel two-enzyme genotyping-by-sequencing approach. PLoS. One. 7: e32253.

R Development Core Team (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Reynolds, M.P., D.F.Calderini, A.G. Condon, and S. Rajaram. 2001. Physiological basis of yield gains in wheat associated with the LR19 translocation from Agropyron elongatum. Euphytica. 119: 137–141.

Reynolds, M.P., M. Balota, M.I.B. Delgado, I. Amani, and R.A. Fischer. 1994. Physiological and morphological traits associated with spring wheat yield under hot, irrigated conditions. Aust. J. Plant Physiol. 21: 717–730.

Ristic, Z., and D.D. Cass. 1993. Dehydration avoidance and damage to the plasma and thylakoid membranes in lines of maize differing in endogenous levels of abscisic-acid. J. Plant Physiol. 142: 759– 764.

Ristic, Z., U. Bukovnik, and P.V.V. Prasad. 2007. Correlation between heat stability of thylakoid membranes and loss of chlorophyll in winter wheat under heat stress. Crop Sci. 47: 2067–2073.

Saadalla, M.M., J.F. Shanahan, and J.S. Quick, 1990b. Heat tolerance in winter wheat: I. Hardening and genetic effects on membrane thermostability. Crop Sci. 30: 1243–1247.

Saadalla, M.M., J.S. Quick, and J.F. Shanahan. 1990a. Heat tolerance in winter wheat: II. Membrane thermostability and field performance. Crop Sci. 30: 1248–1251.

Savchenko G., C. Wiese, S. Neimanis, R. Hedrich, and U. Heber. 2000. pH regulation in apoplastic and cytoplasmic cell compartments of leaves. Planta. 211: 246–255.

Savchenko, G.E., E.A. Klyuchareva, L.M. Abrabchik, and E.V. Serdyuchenko, 2002. Effect of periodic heat shock on the membrane system of etioplasts. Russ. J. Plant Physiol. 49: 349–359.

Sayed, O.H. 2003. Chlorophyll fluorescence as a tool in cereal crop research. Photosynthetica. 41: 321-330.

Schreiber, U., and J.A. Berry. 1977. Heat-induced changes of chlorophyll fluorescence in intact leaves correlated with damage of the photosynthetic apparatus. Planta. 136: 233–238.

Sears, R.G., J.M. Moff att, T.J. Martin, T.S. Cox, R.K. Bequette, S.P. Curran, O.K. Chung, W.F. Heer, J.H. Long, and M.D. Witt. 1997. Registration of 'Jagger' wheat. Crop Sci. 37: 1010.

Shanahan, J.F., J.B. Edwards, J.S. Quick, and J.R. Fenwick. 1990. Membrane thermostability and heat tolerance of spring wheat. Crop Sci. 30: 247–251.

Sourdille, P., T. Cadalen, H. Guyomarc'h, J.W. Snape, M.R. Perretant, G. Charmet, C. Boeuf, S. Bernard, and M. Bernard. 2003. An update of the Courtot X Chinese spring inter-varietal molecular marker linkage map for the QTL detection of agronomic traits in wheat. Theor. Appl. Genet. 106: 530–538.

Srinivasan, A., H. Takeda, and T. Senboku. 1996. Heat tolerance in food legumes as evaluated by cell membrane thermostability and chlorophyll fluorescence techniques. Euphytica. 88: 35–45.

Sun, Q.X., and R.Q. Xu. 1998. Genetic control of tolerance to high temperature stress in wheat. In: A.E. Slinkard (Ed.), Proc 9th Int Wheat Genet. Symp. 1: 236–244.

Vacha, F., V. Sarafis, Z. Benediktyova, L. Bumba, J. Valenta, and M. Vacha. 2007. Identification of photosystem I and photosystem II enriched regions of thylakoid membrane by optical microimaging of cryo-fluorescence emission spectra and of variable fluorescence. Micron. 38: 170–175.

Van Ooijen, J.W. 2006. Join Map 4.0, software for the calculation of genetic linkage maps in experimental populations. Kyazma, B.V., Plant Research International, Wageningen, Netherlands.

Vijayalakshmi, K., A.K. Fritz, G.M. Paulsen, G.H.Bai, S. Pandravada, and B.S. Gill. 2010. Modeling and mapping QTL for senescence-related traits in winter wheat under high temperature. Mol. Breeding. 26: 163–175.

Wahid, A., and A. Shabbir. 2005. Induction of heat stress tolerance in barley seedlings by pre-sowing seed treatment with glycinebetaine. Plant Growth Regul.46: 133–141.

Wahid, A., S. Gelani, M. Ashraf, and M.R. Foolad. 2007. Heat tolerance in plants: An overview. Environ. Exp. Bot. 61: 199–223.

Wang, S. 2007. Windows QTL cartographer 2.5. Department of Statistics, North Carolina State University, Raleigh, NC. Available at http://statgen.ncsu.edu/qtlcart/WQTLCart.htm. Last accessed date 20th October 2008.

Wardlaw, I.F., and C.W. Wrigley. 1994. Heat tolerance in temperate cereals - an overview. Aust. J. Plant Physiol. 21: 695–703.

Wardlaw, I.F., C. Blumenthal, O. Larroque, and C.W. Wrigley. 2002. Contrasting effects of chronic heat stress and heat shock on kernel weight and flour quality in wheat. Funct. Plant Biol. 29: 25–34.

Wardlaw, I.F., I.A. Dawson, and P. Munibi. 1989. The tolerance of wheat to high-temperatures during reproductive growth 2. grain development. Aust. J. Agric. Res. 40: 15–24.

Wise, R.R., Olson, A.J., Schrader, S.M. and T.D. Sharkey. 2004. Electron transport is the functional limitation of photosynthesis in field-grown Pima cotton plants at high temperature. Plant Cell Environ. 27: 717–724.

Yamada, M., T. Hidaka, and H. Fukamachi. 1996. Heat tolerance in leaves of tropical fruit crops as measured by chlorophyll fluorescence. Scientia Horticulturae.67: 39–48.

Yang L., W. Ji, Y. Zhu, P. Gao, Y. Li, H. Cai, X. Bai, and D. Guo. 2010. GSCBRLK, a calcium/calmodulin-binding receptor-like kinase, is a positive regulator of plant tolerance to salt and ABA stress. J. Exp. Bot. 61: 2519–2533.

Yang, J., R.G. Sears, B.S. Gill, and G.M. Paulsen. 2002a. Growth and senescence characteristics associated with tolerance of wheat-alien amphiploids to high temperature under controlled conditions. Euphytica. 126: 185–193.

Yang, J., R.G. Sears, B.S. Gill, and G.M. Paulsen. 2002b. Quantitative and molecular characterization of heat tolerance in hexaploid wheat. Euphytica. 126: 275–282.

Figures and Tables

Figure 3.1 Mean comparison of tolerant (Ventnor) and sensitive (Karl 92) parents under different time of heat treatment for TMD (thylakoid membrane damage), SCC (SPAD chlorophyll content) and PMD (plasmamembrane damage).

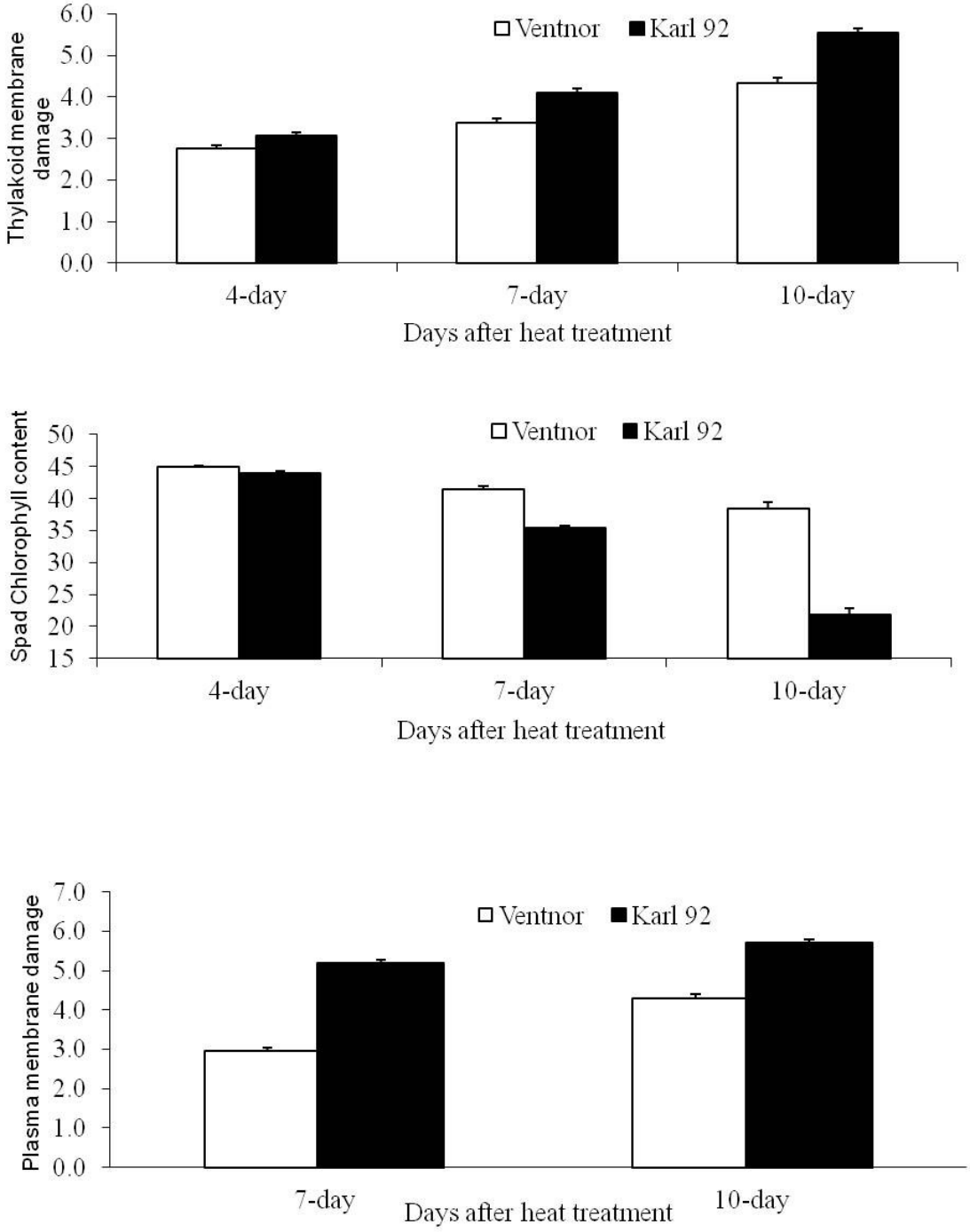


Figure 3.2 Frequency distributions of mean TMD (thylakoid membrane damage), PMD (plasmamembrane damage) and SCC (Spad chlorophyll content) averaged over two generations for 101 RILs.

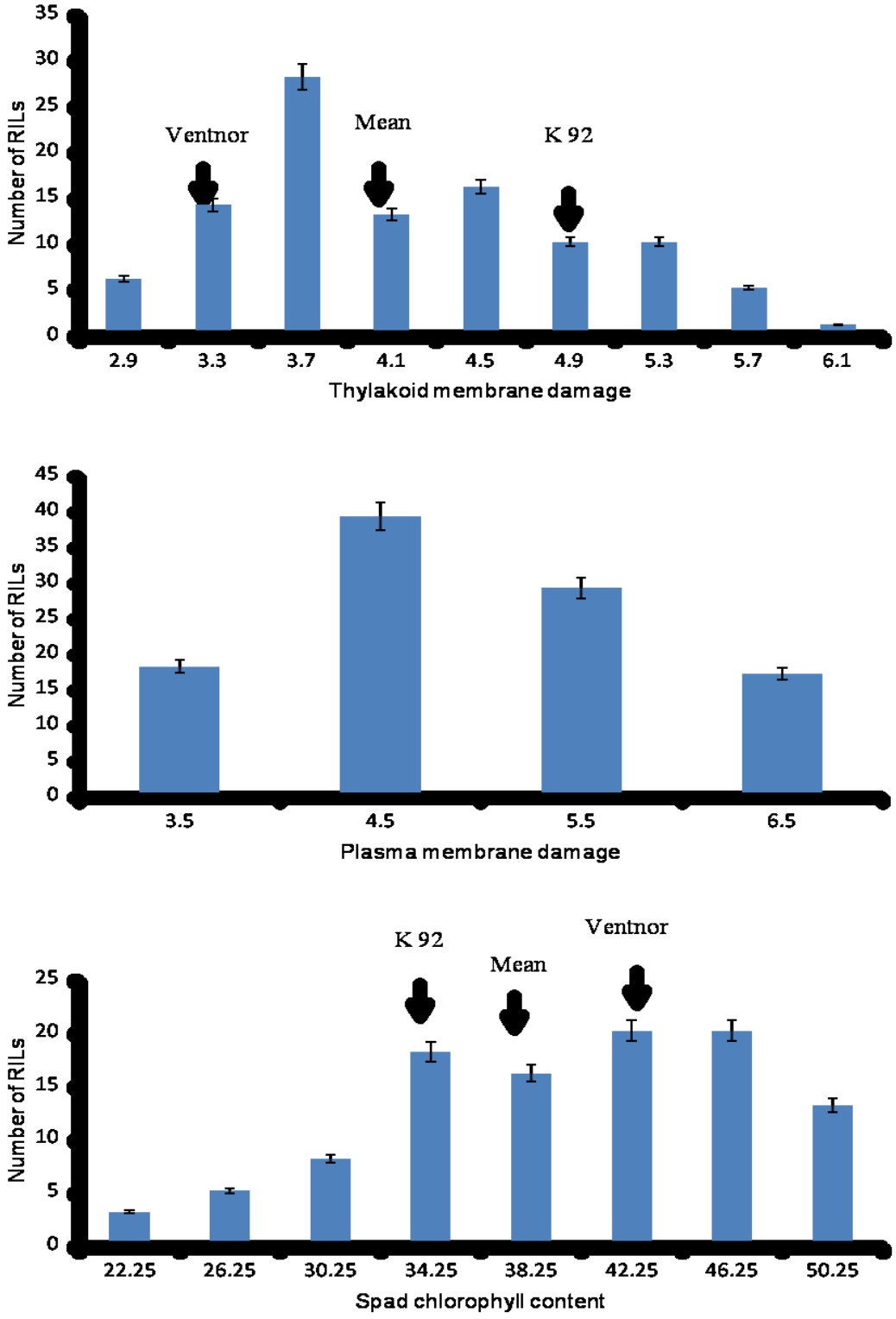


Figure 3.3 The relationships and correlations of thylakoid membrane damage (TMD), SPAD chlorophyll content (SCC) and plasmamembrane damage (PMD) explained by each other in RIL population.

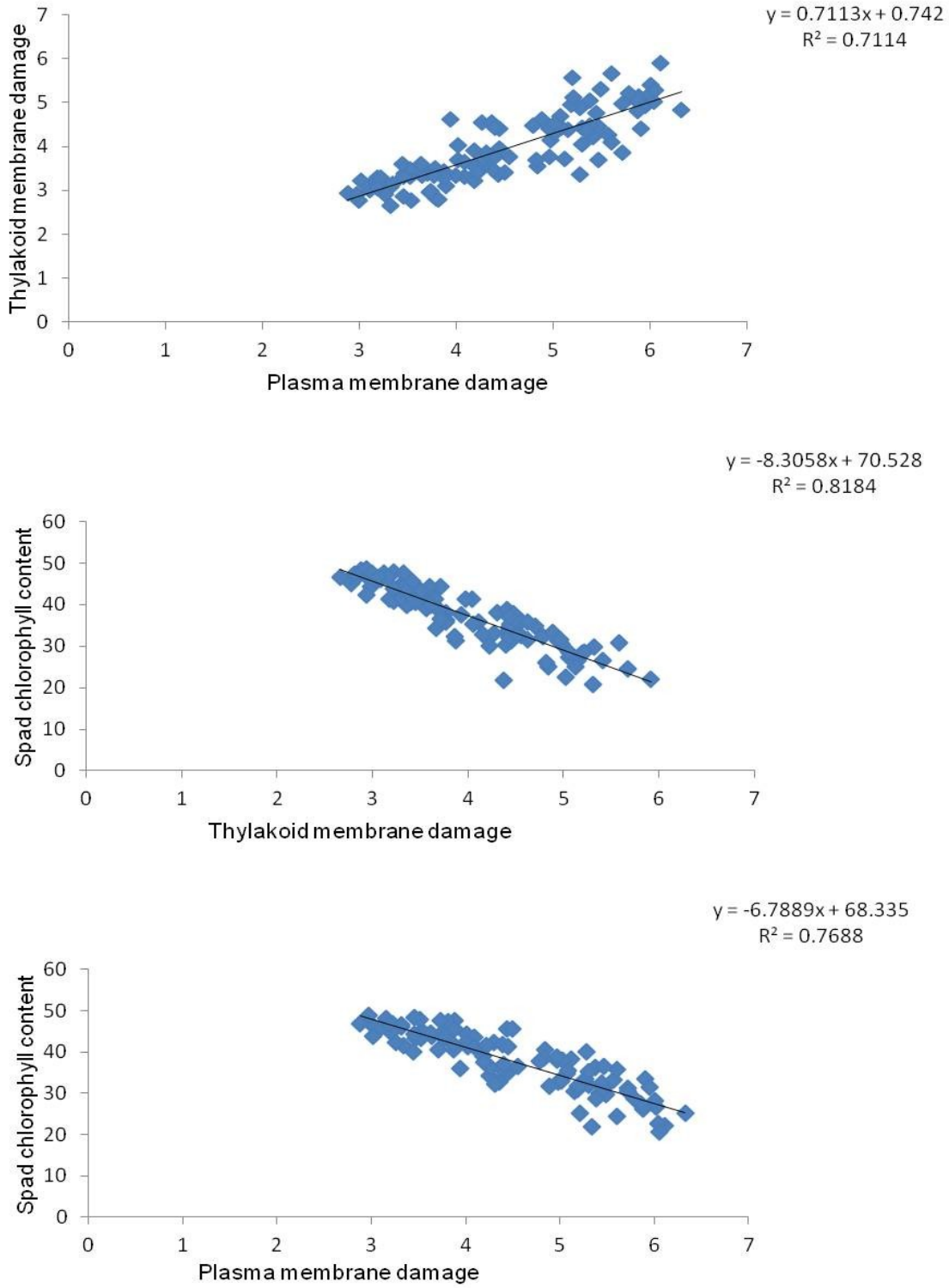


Figure 3.4 Primary genomic regions of heat stress tolerance QTL on 6A, 1B, 2B and 1D identified by composite interval mapping in a Karl92 x Ventnor RIL population. tmd- thylakoid membrane damage, scc- spad chlorophyll content and pmd- plasma membrane damage.

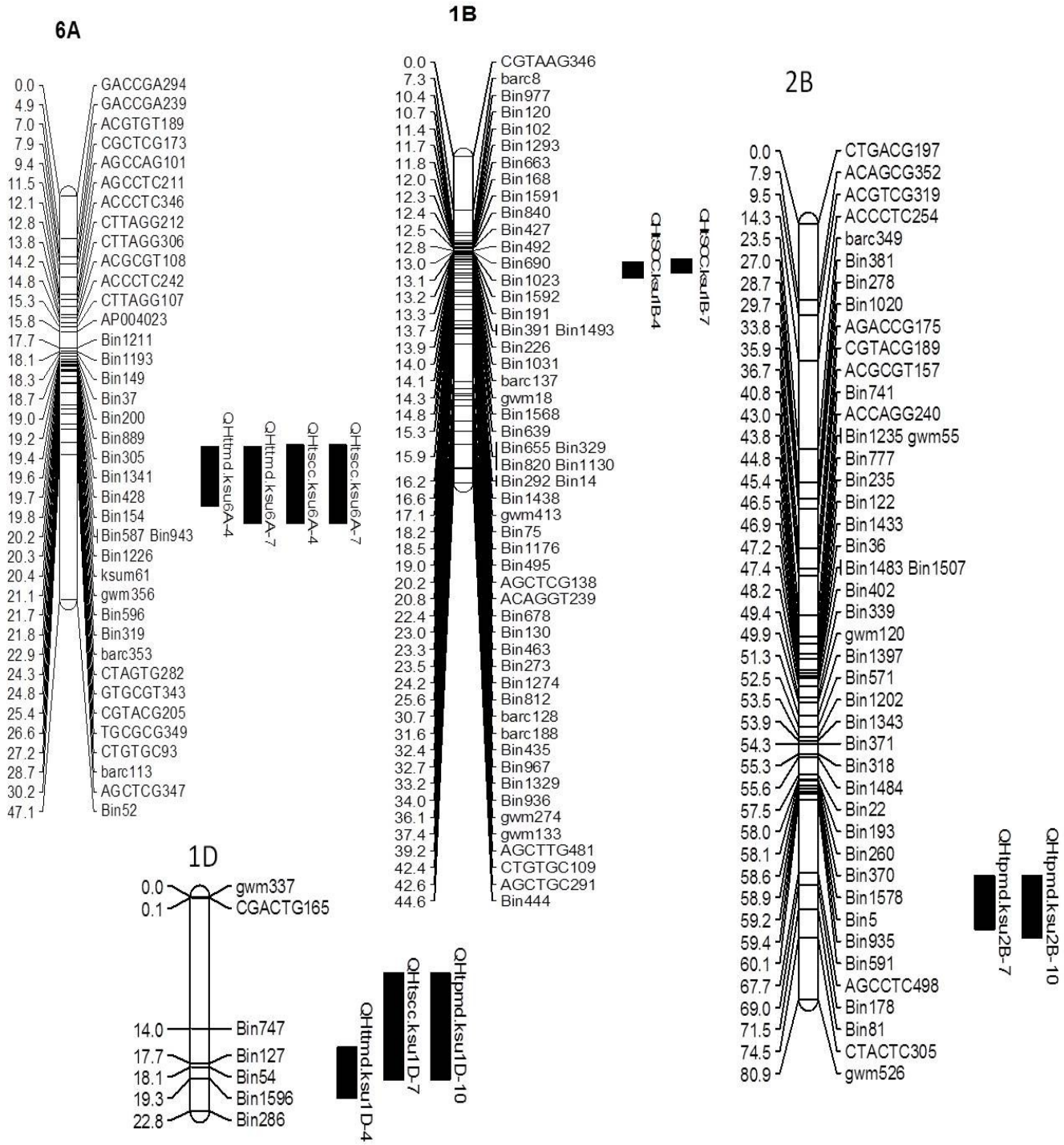


Figure 3.5 Likelihood plots obtained by composite interval mapping for QTL mapped on chromosome 7A for thylakoid membrane damage (TMD). The horizontal line represents a LOD value of 2.5.

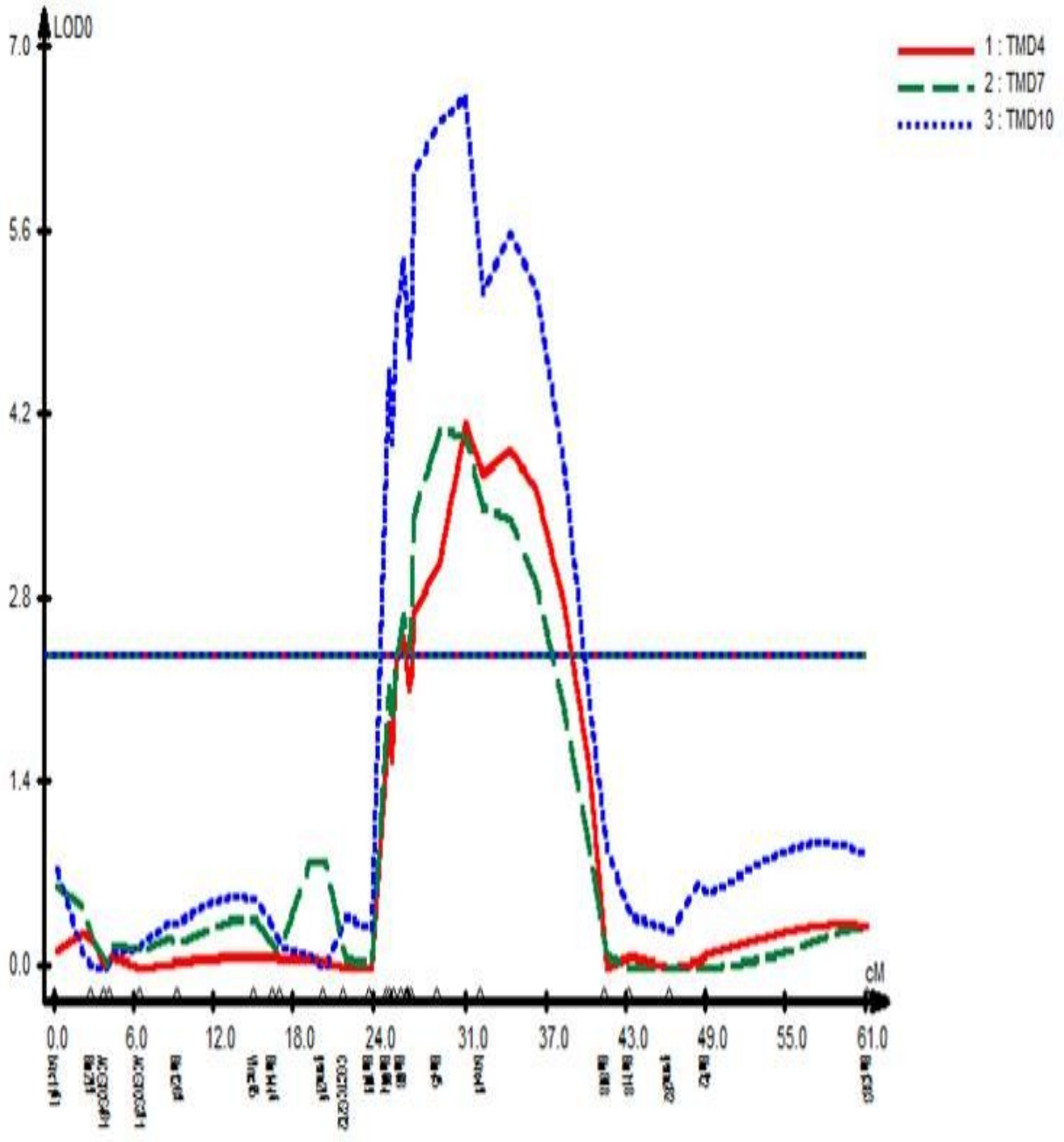


Figure 3.6 Likelihood plots obtained by composite interval mapping for QTL mapped on chromosome 7A for plasmamembrane damage (PMD). The horizontal line represents a LOD value of 2.5.

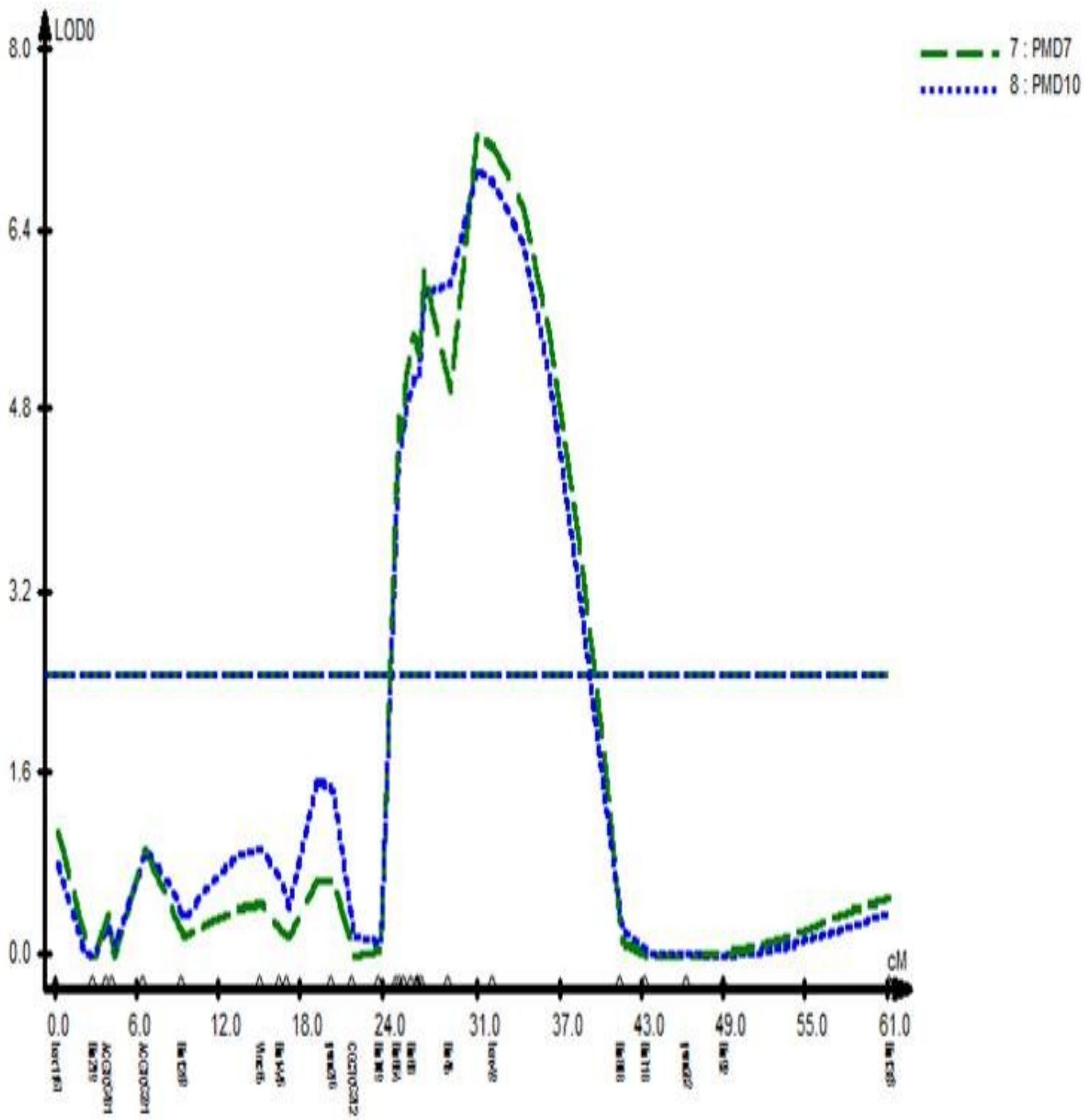


Figure 3.7 Likelihood plots obtained by composite interval mapping for QTL mapped on chromosome 7A for SPAD chlorophyll content (SCC). The horizontal line represents a LOD value of 2.5.

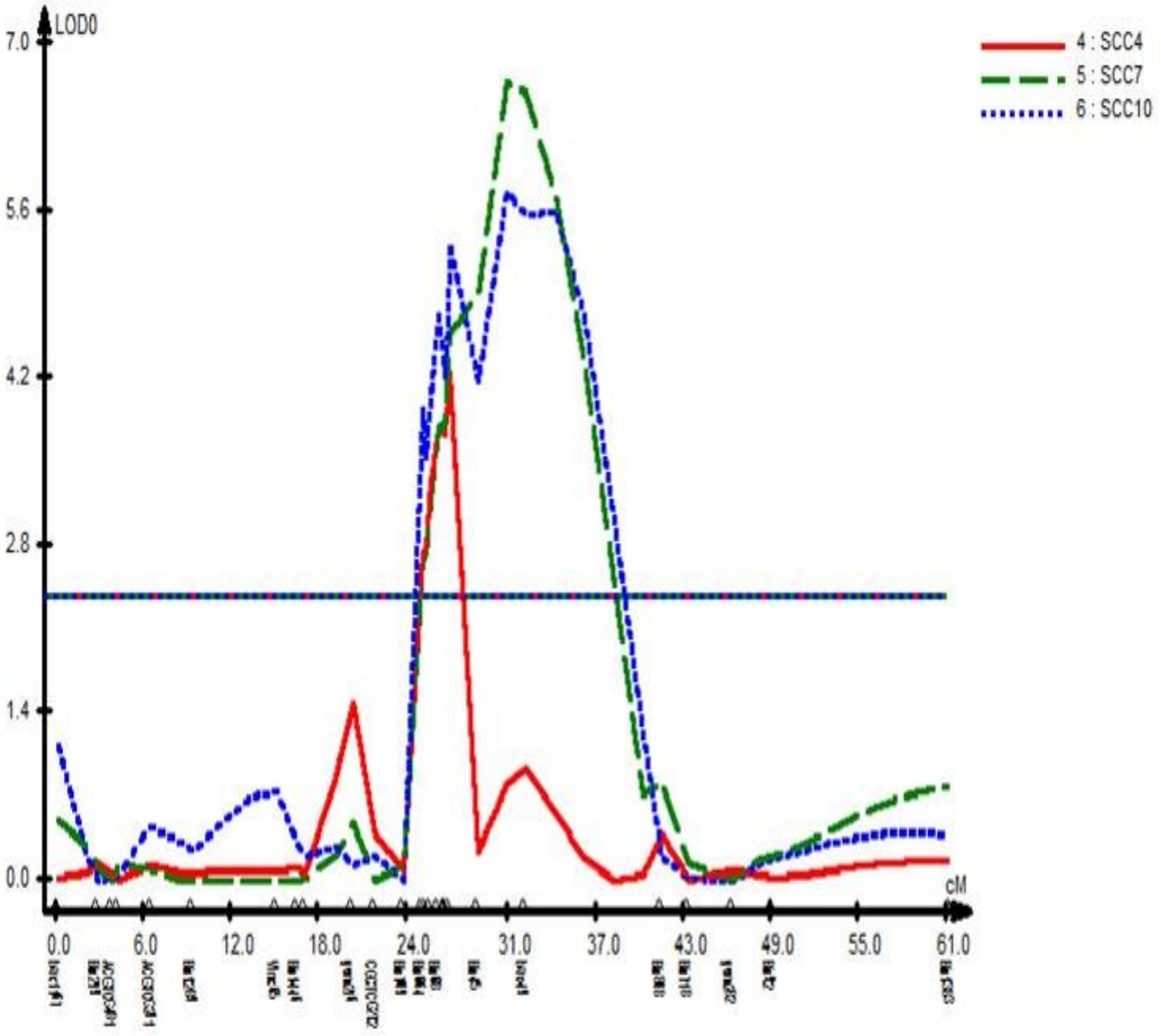


Table 3.1 The variances of Thylakoid membrane damage (TMD), SPAD chlorophyll content (SCC), and Plasmamembrane damage (PMD) under post-anthesis high temperature stress over two generations of RILs.

Sources of variation	DF	F_{10} generation			F_9 generation		
		TMD	SCC	PMD	TMD	SCC	PMD
Block	10	0.004	1.600	0.040	0.015	2.080	0.100
Rep (block)	22	0.000	0.000	0.0004	0.0003	0.000	0.000
Genotypes	102	0.900	52.7	1.22	0.901	53.20	1.04
Residual	174	0.060	0.720	0.010	0.060	0.760	0.070

Table 3.2 Chromosomal locations, marker intervals, determination coefficients (R^2), additive effects and LOD values for significant QTL in Karl/Ventnor 101 recombinant inbred population.

Trait	Chrom	QTL	Flanking Marker	Interval (cM)	LOD	AD	R^2
TMD4	6A	*QHttmd.ksu-6A*	*Xbarc113-AGCTCG347*	6.98	2.58	-0.19	11.90
TMD7	6A	*QHttmd.ksu-6A*	*Xbarc113-AGCTCG347*	8.98	3.21	-0.29	14.87
SCC4	6A	*QHtscc.ksu-6A*	*Xbarc113-AGCTCG347*	9.18	3.8	1.42	17.57
SCC7	6A	*QHtscc.ksu-6A*	*Xbarc113-AGCTCG347*	9.18	3.32	2.34	15.38
TMD4	7A	*QHttmd.ksu-7A*	*Xbarc121-barc49*	11.12	4.15	-0.24	19.15
TMD7	7A	*QHttmd.ksu-7A*	*Xbarc121-barc49*	9.32	4.08	-0.24	18.86
TMD10	7A	*QHttmd.ksu-7A*	*Xbarc121-barc49*	13.05	6.66	-0.48	30.62
SCC4	7A	*QHtscc.ksu-7A*	*Bin754- Bin45*	3.72	4.22	1.53	19.53
SCC7	7A	*QHtscc.ksu-7A*	*Xbarc121-barc49*	11.42	6.7	3.53	30.84
SCC10	7A	*QHtscc.ksu-7A*	*Xbarc121-barc49*	11.42	5.73	4.9	26.59
PMD7	7A	*QHtpmd.ksu-7A*	*Xbarc121-barc49*	13.05	7.28	-0.50	33.51
PMD10	7A	*QHtpmd.ksu-7A*	*Xbarc121-barc49*	13.05	6.95	-0.46	32.03
SCC4	1B	*QHtscc.ksu-1B*	*gwm18- Bin1130*	2.30	2.5	1.07	11.37
SCC7	1B	*QHtscc.ksu-1B*	*gwm18- Bin1130*	2.0	2.75	2.01	12.63
TMD4	1D	*QHttmd.ksu-1D*	*Bin747- Bin1596*	5.31	3.06	-0.18	14.12
SCC7	1D	*QHtscc.ksu-1D*	*Bin747- Bin1596*	11.21	3.58	2.5	16.64
PMD10	1D	*QHtpmd.ksu-1D*	*Bin747- Bin1596*	11.21	2.52	-0.28	11.59
PMD7	2B	*QHtpmd.ksu-2B*	*Bin178-Bin81*	5.55	3.22	-0.30	10.53
PMD10	2B	*QHtpmd.ksu-2B*	*Bin178-Bin81*	6.47	3.75	-0.31	17.22

TMD, thylakoid membrane damage; SCC, SPAD chlorophyll content; PMD plasmamembrane damage. AD, additive effect. For TMD and PMD, negative value of AD, and for SCC, positive value of AD indicates the Ventnor allele having a positive effect on the trait.

Chapter 4 - Expression of a rice soluble starch synthase gene improves the grain yield under heat stress conditions in wheat

Abstract

Heat stress is one of the major environmental constraints for wheat production worldwide. Temperatures above 25°C significantly reduces soluble starch synthase enzyme activity in the wheat endosperm. This enzyme plays a central role in starch deposition. Starch accounts for 75-85% dry weight in wheat grain. Thus, the reduction of starch synthase activity reduces grain yield under high temperature. There are significant differences in sensitivity to high temperatures among soluble starch synthases of cereal species. Rice soluble starch synthase is able to maintain a high enzyme activity at 35°C. In this study, we introduced a modified rice soluble starch synthase gene driven by either a constitutive promoter or an endosperm-specific promoter into wheat and monitored the rice gene expression and the effects of the transgene expression on grain yield-related traits for four generations (T_0, T_1, T_2 and T_3). The results showed that even after four generations, the rice soluble starch synthase gene expressed at a high level and transgenic plants produced grains of greater weight during heat stress. For example, the thousand-seed weight increased 21-34% in T_2 and T_3 transgenic plants when compared to the non-transgenic control plants under high temperatures. In addition we observed the duration of photosynthesis was longer in transgenic wheat than in non-transgenic control. Our study demonstrated that expression of a heat tolerant soluble starch synthase gene can improve wheat yield under heat stress conditions.

Introduction

Wheat (*Triticum aestivum* L.) is the most important staple crops for approximately 36% of the world population (Breiman and Graur, 1995). 40% of the total wheat production in the world is affected by terminal heat stress (Paliwal et al., 2012). Heat stress usually affects wheat production by reducing the yield and quality (Wardlaw et al., 2002). Wheat yield is determined by seed number per unit area and individual seed weight. In a dry wheat seed, starch is the most abundant element accounting for 75-85% of grain dry weight. Thus, the grain yield is largely dependent on starch deposition in the growing endosperm (Jenner et al., 1991). Starch synthesis occurs in plastids of leaves during daylight by using carbon fixed through photosynthesis, and is then mobilized to the storage organs at night (Martin and Smith, 1995). Starch consists of two D-glucose homopolymers, amylose and amylopectin. Amylose is a linear chain of α (1-4) linked D-glucose monomers and generally makes up ~30% of starch. Amylopectin is a highly branched by monomer joining linear chains made by α (1-6) linkages (James et al., 2003; Jong-Seong et al., 2010; Martin and Smith, 1995). In the endosperm of a seed, biosynthesis of amylopectin requires a properly coordinated series of enzymatic reactions which involve the enzymes including ADP glucose pyrophosphorylase (AGPase), four different soluble starch synthases (SSI-IV), starch branching enzyme (BE) and starch debranching enzyme (DBE), whereas amylose biosynthesis requires only AGPase and granule-bound starch synthase (GBSS). There is a possibility that plastidial starch phosphorylase (Pho1) plays an important role in primer formation in the starch biosynthesis reaction (Jong-Seong et al., 2010).

Starch biosynthesis occurs in chloroplasts in green photosynthetic tissues, and in non-green tissue amyloplasts, such as endosperm (Keeling et al., 1988). In higher plants, AGPase produces ADP-Glc and pyrophosphate (PPi) from Glc-1-P and ATP (Jong-Seong et al., 2010; Okita, 1992). Starch synthase (SS) enzymes make linear glucan chains by transferring glucosyl units of

ADP-Glc to the non-reducing end of a glucan chain. In cereal endosperm, a number of isoforms of starch synthase enzymes have been identified, including GBSS, SSI, SSII, SSIII and SSIV (James et al., 2003). GBSS has two isoforms that are mostly confined to storage tissue and involved in amylose synthesis, whereas different SS isoforms, along with some amylose, are predominantly involved in formation of amylopectin (Rahman et al., 2000). In rice, SSIIa and SSIIIa transcripts are most abundant during grain filling phase, meanwhile SSIIb and SSIIIb transcripts are found in the pre-storage phase, indicating that SSIIa and SSIIIa may play vital roles in starch biosynthesis in rice compared to other SS enzymes (Hirose and Terao, 2004). Fujita et al., (2011) found that the presence of any one of the SSI or SSIIIa genes can continue starch biosynthesis in rice. Jong-Seong et al., (2010) demonstrated that rice has only one SSI isoform. Based on anion exchange chromatography results, Fujita et al.,(2011; 2006) concluded that SSI has higher activity than SSIIIa, and accounts for almost 70% of the total SS activity. This supports other, similar findings for the soluble fraction of wheat developing endosperm (Li et al., 2000) and maize endosperm (Cao et al., 1999). These observations suggest that SSI is critical for rice starch biosynthesis. SSI preferentially synthesizes short sequences, while further chain elongation of amylopectin synthesis is performed by other SS enzymes (Jeon et al., 2010). The SSI gene cloned in wheat has 81% amino acid similarity with rice SSI and produces a 75 kDa protein, whereas rice SSI produces a 57 kDa protein (Baba et al., 1993). The wheat and rice SSI are structurally similar, consisting of 15 exons and 14 introns. The exons of both genes are virtually identical in length, but introns 1, 2, 4 and 10 of wheat SSI are longer and introns 6, 11 and 14 are shorter than that of the corresponding rice introns (Li et al., 1999).

Elevated temperature has significant adverse effects on starch deposition during the grain filling stage of different crops (Bhullar and Jenner, 1986a; Bhullar and Jenner, 1986b)). Rijven (1986)

showed that high temperature reduced starch deposition in endosperm possibly by inactivating soluble starch synthase. Prakash et al. (2009) found that soluble starch synthase (SS) is one of the key enzyme components that show sensitivity to high temperature during grain growth of wheat and Keeling et al. (1993) reported that temperatures above 25 °C reduce soluble starch synthase activity in wheat endosperm. On the other hand, Jiang et al. (2003) found that soluble starch synthase activity in rice was highest at around 35 °C, resulting in production of long, linear chain amylopectin in the endosperm. Since wheat soluble starch synthase is deactivated at elevated temperature and rice soluble starch synthase withstands high temperature, expression of a rice soluble starch synthase gene in wheat may increase the sink strength and thereby increase the productivity under heat stress. The objective of this study was to investigate the effects of expression of a rice soluble starch synthase gene on starch deposition and the yield of wheat kernels weight under heat stress conditions.

Materials and methods

Vector construction

A modified rice sequence (Baba et al., 1993; Tanaka et al., 1995) derived from Genbank accession NM_001063416 was used to create the expression vector. The first third of the gene was commercially synthesized by Genscript (Piscataway, NJ) and was digested with XhoI and HindIII to yield a 486bp fragment. The back third of the gene was PCR-amplified from cDNA derived from Katake seeds using a forward primer of (SSR-AF) 5'agcccgatctagaaggtctcacagaa3' and a reverse primer of (SSR-BR) 5'tgaaggaagcagcgaatttctccg3' which amplified a 1675bp fragment and cloned into PCR Blunt vector. This plasmid was then partially digested with XhoI and completely digested with HindIII. The two fragments were then ligated together and transformed into JM109 cells. The fused gene was then sub-cloned between the HMW-GS DY10 promoter (Lamacchia et al., 2001) and terminator for seed expression (Fig.1B) and in pACH17 between the maize ubiquitin promoter (Christensen et al., 1992) and NOS terminator for over expression (Fig.1A). Another construct, pAHC20 contains the *bar* gene controlled by ubiquitin 1 promoter and NOS terminator (Fig. 1C) which confers resistance to the herbicides glufosinate (trade name Liberty; Bayer Crop Sciences, Research Triangle, NC, USA) and bialaphos.

Plant transformation

Bobwhite wheat (*Triticum aestivum* L. cv. `Bobwhite') was used for transformation. Immature embryos 2-3 mm in length were collected from 10-14 day old, surface sterilized caryopses, and placed upside down on CM4 media (Zhou et al., 1995) for two to seven days in a dark room at 25 °C for callus formation. Three to five days after the transfer, the initiated calli were either pretreated four to eighteen hours on 0.4M Mannitol/Sorbitol CM4 media or air-dried in the

laminar hood for 30 minutes prior to transformation. For transformation, plasmid pAHC20 was used in combination with pAHC17 or pJL10P5. Genetic transformation was performed using particle inflow gun (Finer et al., 1992). Recovery and selection of transgenic tissues was similar to Altpeter et al. (1996) and Anand et al. (2003), with slight modification. After bombardment, tissues were kept on the CM4 media for two to five days to allow better recovery. Wheat calli were then transferred onto selection media (CM4 media containing 5 mg/l glufosinate ammonium) and maintained for two weeks in dark. The tissues were transferred to CM4 media containing 10 mg/l glufosinate ammonium in darkness for two cycles of 14 days each. The tissues were then transferred to MSP media containing 10 mg/l glufosinate ammonium in light for shoot production. Tissues developing shoots were transferred to a shoot elongation media, MSE containing 5 mg/l glufosinate ammonium in light for 14 days. When shoots elongated to ~3-6mm, the whole clumps were transferred to large culture tubes with 13 ml of MSE media containing 10 mg/l glufosinate ammonium. Plants with well developed roots and shoots were planted into small peat pots for hardening with high humidity. These hardened plants were selected for herbicide resistance using 0.2% (v/v) liberty solution by painting a marked area of the 3rd leaf, followed by observation at three to five days. The media composition in this experiment was according to Anand et al. (2003).

PCR screening of transgenic lines

In each generation, PCR was performed using genomic DNA to screen transgenic wheat plants for the rice SSI gene. DNA was isolated from leaf tissue using modified CTAB method. A leaf tissue sample of 100-150 mg leaf was taken and placed in a 2ml centrifuge tube and crushed in liquid nitrogen. Eight hundred µl 2X CTAB extraction buffer containing 4µl 2-Mercaptoethanol (Sigma, St. Louis, MO) was added to the sample. Tubes were incubated in a water bath for 60

minutes at 65^{o}C followed by 10 minutes cooling at room temperature. Five hundred µl chloroform: isoamyl alcohol (24:1) was added and the tubes were placed on rotary shaker for 30 to 60 minutes followed by centrifuging for 20 minute at 12000 rpm. Supernatant was transferred to a clean tube and 2 µl RNase was added to remove RNA from DNA. DNA in aqueous phase was precipitated by adding approximately 2/3 volume of isopropanol and placing the tubes at -20 ^{o}C overnight. In each generation, PCR screening was conducted using two primer pairs to screen for transgenic plants containing the SSI gene.

For the first PCR target, the forward primer, 5'- GTGCCTCTGATTGGCTTTATTG, anneals starting 1345 bases downstream from the initiation codon of SSI cDNA, and the reverse primer, 5'- AGGTAAACCCAGCTCCTTCTGCAA hybridizes starting 145 bases upstream of the stop codon of SSI cDNA. This primer pair amplifies a 471 bp PCR product from the 3' end of the gene. The PCR program for this primer was 95 °C for 5 minutes, 30 cycles of 95 °C 30s, 57 °C 30s, and 72 °C 90s followed by 72°C for 10 minutes. For the second PCR target, the forward primer, 5'- TGCCAATTGCTCTTGCTCTTCGTG anneals starting 476 bases downstream of the start codon and the reverse primer 5'- TCAGCAGTTGTGACCTCCCATGA anneals starting 815 bases upstream of the stop codon of the gene, and produces a 670 base pair PCR product. PCR conditions were the same as above except the annealing temperature was 60 °C instead of 57 °C.

Southern blot analysis

PCR was conducted using the second primer pair (F: 5'- GCCAATTGCTCTTGCTCTTCGTG and R: 5'- TTCAGCAGTTGTGACCTCCCATGA) and pAHC17 plasmid DNA as template. The PCR product was purified using QIAGEN gel purification kit, and labeled with dCTP (α-^{32}P) using Megapriming DNA labeling system (Amersham, UK). The labeled PCR product was used

as a probe in Southern blot analysis. Twenty- five µg genomic DNA from T_2 plants were digested with *BamH* 1 and *Eco*R1. Digested DNA were separated on 0.8% agarose gels, and transferred to a hybridization membrane. The membrane was probed with labeled PCR product (Sambrook et al., 1989).

Reverse transcriptase (RT) PCR

RNA was isolated from both leaf and 20 day old developing seeds of T_2 plants. For the leaf sample, QIAGEN RNA isolation kit was used, while isolation from the seed was done by following a previously described protocol (Li and Trick, 2005). RNA was reverse-transcribed to cDNA using a reverse transcription system kit from Promega. PCR was conducted with the first primer pair described above. DNA contamination in RNA was checked by performing PCR using the tubulin primer (F- 5'-ATCTGTGCCTTGACCGTATCAGG-3' and R-5'-GACATCAACATTCAGAGCACCATC-3'). PCR conditions were the same as the first primer pair except the annealing temperature was 58 °C instead of 57 °C.

Western blot analysis

Total soluble proteins of flag leaves and seeds were extracted from each T_2 transgenic wheat event, one non-transgenic wheat variety (BW) and one rice variety (Nipponbare) at 20 days after anthesis. Proteins were extracted by grinding and homogenizing samples in protein extraction buffer (Fu et al., 2008). Protein concentrations were estimated by using Quick Start Bradford Protein Assay kit (Bio Rad). Samples were diluted in the buffer to maintain equal concentration for equal loading. Equal loading was also maintained by checking stained gels. Proteins were separated by loading 40 µg of each sample on a 10% SDS polyacrylamide gels for separation. SDS-PAGE separated proteins were transferred to PDVF membranes for immunoblotting. PDVF membranes with the transferred proteins were probed for rice soluble starch synthase1 (SSI)

protein using rabbit polyclonal anti-SSI antibodies raised by AnaSpec, Inc California, USA. and goat anti rabbit secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc, California, USA).

Heat stress treatment, phenotyping and data analysis

Three experiments were conducted for phenotyping under heat stress and optimum temperature conditions using eight to twenty pots for each event/ line in each experiment. In the first experiment (Exp 1), T_2 plants from an ubiquitin promoter event (Ub-1), a Dy10 promoter event (Dy10) and non-transgenic Bobwhite produced from tissue culture (BWTC) were grown in the greenhouse. Following germination, seedlings were PCR screened for the presence of SSI gene in the laboratory and single seedlings were transplanted to pots (14 cm height, 50 cm top perimeter and 36 cm bottom perimeter) filled with Metro Mix 200 potting soil (Hummert Intl. Topeka, KS). The greenhouse was set a day/night temperature of 22/15°C with 16 hours of light and 8 hours of dark for normal growth. Plants were watered daily. One or two tillers per plant were tagged at anthesis, and half the plants from each event/line were transferred to a high-temperature growth chamber at 10 days after anthesis, as determined using tagged tiller/s. Growth chambers were set at 31/24°C (day/night) with 16/8h (light/ dark) and 70-80% humidity. The remaining plants were transferred to another growth chamber having optimum day/ night temperature (22/15°C) with the same conditions as the high temperature chamber. Thousand kernel weight (TKW) were obtained from the tagged tillers by drying to equivalent moisture content, weighing the seeds and converting to TKW using the seed number of that

tagged head. Effective tillers number was also counted during harvest. Chlorophyll content was measured from flag leaves, started at 12 days after anthesis then every other day for twelve days. Days required for physiological maturity were calculated from date of anthesis to date of physiological maturity. Non-transgenic and transgenic event means were compared using two sample t-tests with unequal sample variance at $\alpha = 0.05$.

In the second experiment (Exp 2), T_2 plants from two ubiquitin promoter events Ub-1, Ub-2, a Dy10 promoter event (Dy10), BWTC and Bobwhite from non-tissue culture (BWNTC) were grown in the greenhouse following the procedure in Exp.1. Heat stress was given in a growth chamber set at 33/26°C maintaining all other conditions similar to Exp.1. Control plants were grown in the greenhouse at optimum temperature (22/15°C) with 16/8h (light/ dark) and 70-80% humidity. Exp.2 was done following the procedure for Exp.1 except that chlorophyll content data were not taken.

In the third experiment (Exp.3), T_3 plants from 2 ubiquitin promoter event Ub-1, Ub-2, a Dy10 promoter event (Dy10), BWTC and BWNTC were grown in the greenhouse. Phenotyping both under heat stress (34/28°C) and optimum temperature (22/15°C) was done in growth chambers following the procedure in Exp 1.

In the final experiment (Exp.4) T_4 plants from the three transgenic events along with non-transgenic Bobwhite (BWNTC) were grown in the green house and transferred into the growth chamber for phenotyping under both high and optimum temperature condition following the procedure in Exp.1. In this experiment, growth chambers were maintained at 32-33/30°C for heat stress and 20/15°C for optimum temperature condition. Data were taken only on thousand seed weight and seed number per head.

Temperature fluctuation of ± 1.5°C was found for highest temperature in growth chamber from the set point, while fluctuation varied (± 2 to 4) in the green house depending on the outside air temperature.

Results

Transgenic plant production, transgene stable insertion, inheritance and copy number estimation

A total of six bar positive transgenic wheat lines were developed using co-transformation with pAHC17/ pAHC20 and pJL10P5/ pAHC20. Five of six lines contained the modified rice SSI gene as determined by PCR analysis from leaf DNA in the T_0 generation (Table 1). Ultimately, two previously SSI-positive lines tested as PCR negative. The remaining three SSI-positive lines were used for further analysis. Two lines contained the ubiquitin promoter, and one contained the Dy10 promoter.

Analysis of SSI gene segregation using X^2–test in T_1 plants showed that the SSI gene segregated in a 3:1 ratio for the Ub1,Ub2 and Dy10 events, indicating insertion of at least one copy of the SSI (Table 1). Southern analysis using genomic DNA from T_2 leaves and the SSI gene sequence as a probe demonstrated that all three transgenic events and the non-transgenic BW had three bands in common (Fig. 2A), indicating that probe hybridized with wheat endogenous SSI gene sequences and, due to it's hexaploid nature, the wheat genome harbors three homeologous SSI genes (copies). Each lane of the transgenic events also showed additional unique bands (Fig. 2A), confirming transgene integration. Based on the uncommon band numbers, Ub1 and Dy10 transgenic events appeared to harbor only a single SSI gene insertion (single copy), while, Ub2 seems to harbor three copies of the rice gene. These results demonstrated the stable insertion of the rice gene into the wheat genome that followed the expected pattern of inheritance.

Transgene expression

Reverse Transcriptase (RT) PCR was conducted using the RNA isolated from both leaf and seed samples of three PCR positive transgenic lines and non-transgenic Bobwhite. The leaf samples from the two ubiquitin promoter events (Ub1 and Ub2) produced a band matching the expected size from the rice SSI gene (Fig. 2B). The Dy10 promoter event and the non-transgenic BW did not show any bands (Fig 2B), indicating that, as expected, the Dy10 promoter is seed specific. The seeds of non-transgenic BW did not show the rice SSI band, while the seeds of all three independent events showed a clear rice SSI band, indicating that both ubiquitin and Dy10 promoters were active in the seeds of the transgenic plants. Single tubulin bands with different sizes between genomic DNA samples and RNA samples demonstrated that RNA samples were not contaminated with DNA (Fig. 2C).

Western blots were used to detect the presence of SSI proteins in wheat. A rice leaf sample was also included. The antibody developed from rice SSI protein sequence recognized SSI proteins in both rice and wheat. In seeds, only one SSI protein band was detected in the three transgenic events, non-transgenic BW and rice. The SSI proteins showed the same molecular weight (75 kD) in both wheat and rice (Fig. 2D). However, the SSI band intensities were much stronger in the transgenic wheat events than in non-transgenic wheat, indicating that the rice SSI protein was produced in the seeds of the three transgenic wheat events.

In leaves, two SSI protein bands (isoforms 60 KD and 75 KD) were detected in three transgenic events and non-transgenic BW. The 75 KD SSI isoform showed stronger band intensity in the Ub_2 event than the Ub_1 event, Dy10 and non-transgenic BW. Only the 75 KD isoform was detected in rice (Fig. 2E).

Yield related traits analysis of transgenic events under heat stress

Based on PCR, RT-PCR, Southern blot analysis and Western blot analysis, three events (Ub1, Ub2 and Dy10) were found positive for transgene (SSI). PCR negative plants from different lines (BWTC) and Bobwhite plants not derived from tissue culture (BWNTC) were compared with different SSI positive lines.

Ub2 was not included in the first experiment and only BWTC plants were considered as control. Significant differences were found for TKW for Ub1 (3.22%) and Dy10 (6.71%) events compared to BWTC (Tables 8 and 2). Effective tiller number per plant (Table 3) and days required for physiological maturity (Table 3) showed no significant difference, but seed number per selected head was significantly lower for the Dy10 event (Table 2). There was no significant difference between transgenic and non-transgenic events for any studied trait (Tables 2 and 3) under optimum temperature conditions.

T_2 plants were used for experiment 2 (Exp. 2) and higher temperatures were used for the heat stress treatment. All three transgenic events were compared with non-transgenic Bobwhite plants. No significant differences between PCR negative Bobwhite (BWTC) and normal Bobwhite (BWNTC) were found for the traits under consideration. Both the tissue culture-derived and non-tissue culture-derived plants were pooled as controls. Transgenic events with ubiquitin (10.70%) and Dy10 (25.67%) promoter showed significant increases in TKW compared to non-transgenic (BW) plants (Table 8 and 4). Individually all events having the transgene had higher TKW than non-transgenic lines (Table 4). Dy10 event produced significantly lower number of seeds than non-transgenic control (BW) in both heat stress (33/26°C) and optimum temperature (25/15°C) conditions (Table 5). Under optimum

temperature conditions, TKW, tiller number and days required for physiological maturity were not significantly different. Under heat stress the Dy10 event took significantly longer to reach physiological maturity (2.66 days) than non-transgenic events (Table 7).

Exp.3 was done using T_3 plants and the stress treatment was imposed using higher temperature (34/28°C) than Exp 2. Transgenic events with both ubiquitin (22.94%) and Dy10 (34.59%) promoters produced seeds having significantly higher TKW than non-transgenic events under heat stress (Table 8 and 4). The Dy10 event produced significantly lower number of seeds per selected head, while events having ubiquitin promoter showed no difference compared to non-transgenic events (Table 5). Tiller number per plant was not significantly different between transgenic and non-transgenic events (Table 6). Days required for physiological maturity differed significantly between transgenic and non-transgenic events, with transgenic events averaging 1.28 additional day of grain filling duration than non-transgenic plants (Table 7).

Some differences in TKW were found under optimum temperatures (22/15°C), in Exp.1 and 3, but not in Exp.2. In Exp.3, Ub2 had significantly higher individual seed weight than non-transgenic BW. No significant variation was found for chlorophyll content in Exp.1 and 3, under either heat stress or optimum temperature conditions (data not shown).

Using T_4 transgenic plants, fourth experiment was done using higher night time temperature and lower daytime temperature (32-33) than Exp.3 for heat stress. Like the previous experiments, Ub1 (13.58%), Ub2 (12.06%) and Dy10 (29.51%) showed considerable increment in thousand kernel weight than non-transgenic control under high temperature (Table 9). There was high variability in kernel weight within the events; as a result, even though there was a remarkable increment in Ub1 and Ub2, they were not significantly different at 5% level. None of the transgenic events showed significant variation for seed number per head in any temperature

condition. Towards the end of the experiment for optimum temperature condition, a malfunction of the growth chamber produced extreme high temperature. As a result, we have disregarded the results of thousand kernel weight under optimum temperature condition.

Discussion

According to Commuri and Keeling (2001), the SSI gene is responsible for the elongation of shorter A and B1 chains during starch biosynthesis in the soluble phase of the amyloplast. As a result, the mutation of the SSI gene or destruction of that enzyme may have a severe impact on crystalline amylopectin matrix formation, as well as grain filling. Here we tested the hypothesis by using the high temperature (35°C) tolerance of the rice SSI gene (Jiang et al., 2003) to increase the grain filling potential of wheat under moderately high temperature. In this experiment we introduced the rice SSI gene into a spring wheat variety (BW) using two different promoters, which was confirmed by PCR and genomic DNA blotting in the T_2 generation (Fig. 2A). Molecular analysis (Southern blot) revealed three copies of the endogenous SSI gene in wheat, because the non-transgenic Bobwhite, along with all the transgenic events, produced three common hybridization bands with the probe for the rice SSI gene (Fig. 2A). This indicates the high level similarity of rice SSI and wheat endogenous SSI. In addition blast analysis of the rice SSI cDNA sequence (Blast 2 sequence program; www.ncbi.nlm.nih.gov) reveals 88% nucleotide identity and 81% amino acid identity (Li et al., 1999) with the wheat SSI gene. The failure of the event with the DY10 promoter to produce a band using cDNA from the leaf sample (Fig. 2B) in Reverse Transcriptase (RT) PCR proved the promoter specific expression of the transgene. In western blot analysis, separation of wheat SSI from Rice SSI was not possible because of the size similarity of the two proteins. According to Baba et al. (1993), the rice SSI protein produced a 57 KD protein band, but in our western gel, it produced a nearly 75KD band which

is similar to the 75 KD wheat SSI band. Using the cDNA sequence of rice SSI (http://web.expasy.org) we found the expected size of the protein as 75.7 KD, which is supportive of our finding. Two protein bands in the leaf protein supports the expression of two isoforms of SSI in wheat leaf tissue (Denyer et al., 1995), but only one isoform in seeds.

Moderately high temperature reduces the SSI activity of wheat at both the transcription (Hurkman et al., 2003) and enzyme activity level (Rijven, 1986), while the reduction of transcription and enzyme activity in rice is lower (Yamakawa et al., 2007). Based on this information, we tested the hypothesis that the rice SSI gene with increased activity will supplement the wheat SSI under high temperature stress and increase the individual grain weight by sustaining starch biosynthesis.

Three transgenic lines expressing the rice SSI gene produced significantly heavier seeds compared to non-transgenic lines under three different experiments at high temperature. Some transgenic events produced slightly heavier seed under optimum temperature in some experiments. This may be due to fluctuation of the highest temperature of the growth chambers. We observed actual temperature were somewhat elevated relative to the set temperature, which might have influenced the SSI activity. There was no variation among transgenic and non-transgenic event in optimum temperature conditions in Exp. 2 which was conducted in the greenhouse during winter. The actual temperature never exceeded the set point in this experiment. It may be possible the wheat SSI activity starts declining at around 25°C or an even lower temperature (Keeling et al., 1993). It was clear that the TKW differential between the transgenic and non-transgenic lines increased (Table 8) as the heat stress was increased from Exp.1 to Exp. 2 to Exp. 3. This result also supports the hypothesis that increasing temperature

decreases wheat SSI activity more than rice SSI and also supports previous research regarding the importance of SSI activity related to grain filling (Prakash et al., 2009).

No significant variation in chlorophyll content (Data not shown) was observed between transgenic and non transgenic events indicating that differences of starch deposition were not due to the differences in photosynthetic capacity. This result is in line with Sofield et al. (1977), who reported that reduced grain filling during high temperature was not due to the supply of the assimilate from photosynthesis.

No variation in tiller number (Tables 3 and 6) and height of the plants (visual measurement) indicates that the transgene did not compromise the morphology of the plants. Significant lower seeds per spike was found with the transgenic event having the Dy10 promoter in experiment 1, 2 and 3 but not in experiment 4. This could be indicative of an interaction between Dy10 promoter and seed setting capability, or simply the product of insertion site of the gene or the effect of somaclonal variation, which might have been fixed with advancing the generation. There is no evidence in the literature supporting the idea that Dy10 promoter can compromise seed setting capability of a plant. Developing more events with the Dy10 promoter need to be done to draw a more definite conclusion.

We did note a slight increase in time to physiological maturity for transgenic plants at the highest temperature tested. Smidansky et al. (2002) reported that pulling sugar from the leaf to seed helps to reduce feedback inhibition of leaf sugar on photosynthesis. Our data suggest that SSI activity was maintained under high temperature and likely allowed continued use of photo-assimilate. The increased grain fill duration we observed may be due to reduction of feedback inhibition of photosynthesis by reducing the accumulation of sugars.

The transgene with the Dy10 promoter produced the heaviest seeds and had longest grain filling duration (GFD). This result may support the feedback inhibition theory but it should be noted that the Dy10 plants also produced fewer seeds per spike and total grain yield was reduced.

Our results suggest that transfer of more heat labile forms of key enzymes may be good strategy for improving heat tolerance in crop plants. This approach should be beneficial for all cool season species that are grown under conditions where heat stress may limit production. It may be useful to explore the heat stability of SSI from other species typically grown under high temperature conditions to identify the most heat labile source of the enzyme.

There is genetic variability within wheat for the ability to maintain greenness (Ristic et al., 2008) under heat stress. The cultivar used in this study is not particularly tolerant to heat stress. Deployment of the rice construct in lines that demonstrate heat tolerance may be an avenue to even greater levels of production under heat stress.

References

Al-tpeter, F., V. Vasil, V. Srivastava, E. Stoger, and I.K. Vasil. 1996. Accelerated production of transgenic wheat (*Triticum aestivum L*) plants. Plant Cell Rep. 16: 12–17.

Anand, A., T. Zhou, H.N. Trick, B.S. Gill, W.W. Bockus, and S. Muthukrishnan. 2003 Greenhouse and field testing of transgenic wheat plants stably expressing genes for thaumatin-like protein, chitinase and glucanase against Fusarium graminearum. J. Exp. Bot. 54: 1101–11.

Baba, T., M. Nishihara, K. Mizuno, T. Kawasaki, H. Shimada, E. Kobayashi, S. Ohnishi, K. Tanaka, and Y. Arai. 1993. Identification, cdna cloning, and gene-expression of soluble starch synthase in rice (*oryza-sativa l*) immature seeds. Plant Physiol. 103: 565–573.

Bhullar, S.S., and C.F. Jenner. 1986a. Effects of a brief episode of elevated-temperature on grain filling in wheat ears cultured on solutions of sucrose. Aust. J. Plant Physiol. 13: 617–626.

Bhullar, S.S., and C.F. Jenner.1986b. Effects of temperature on the conversion of sucrose to starch in the developing wheat endosperm. Aust. J. Plant Physiol. 13: 605–615.

Breiman, A., and D. Graur. 1995. Wheat evolution. Israel J. Plant Sci. 43: 85–98.

Cao, H.P., J. Imparl-Radosevich, H.P. Guan, P.L. Keeling, M.G. James,. and A.M. Myers. 1999. Identification of the soluble starch synthase activities of maize endosperm. Plant Physiol. 120: 205–215.

Cheng, M., J.E. Fry, S.Z. Pang, H.P. Zhou, C.M. Hironaka, D.R. Duncan, T.W. Conner, and Y. C. Wan. 1997. Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. Plant Physiol. 115: 971–980.

Christensen, A.H., R.A. Sharrock, and P.H. Quail. 1992. Maize polyubiquitin genes - structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Mol. Biol. 18: 675–689.

Commuri, P.D., and P.L. Keeling. 2001. Chain-length specificities of maize starch synthase I enzyme: studies of glucan affinity and catalytic properties. Plant J. 25: 475–486.

Denyer, K., C.M. Hylton, C.F. Jenner, and A.M. Smith. 1995. Identification of multiple isoforms of soluble and granule-bound starch synthase in developing wheat endosperm. Planta. 196: 256–265.

Finer, J.J., P. Vain, M.W. Jones, and M.D. McMullen. 1992. Development of the particle inflow gun for dna delivery to plant-cells. Plant Cell Rep. 11: 323–328.

Fu, J.M., I. Momcilovic, T.E. Clemente, N. Nersesian, H.N. Trick, and Z. Ristic. 2008. Heterologous expression of a plastid EF-Tu reduces protein thermal aggregation and enhances CO_2 fixation in wheat (*Triticum aestivum)* following heat stress. Plant Mol. Biol. 68: 277–288.

Fujita, N., M. Yoshida, N. Asakura, T. Ohdan, A. Miyao, H. Hirochika, and Y. Nakamura. 2006. Function and characterization of starch synthase I using mutants in rice. Plant Physiol. 140: 1070–1084.

Fujita, N., R. Satoh, A. Hayashi, M. Kodama, R. Itoh, S. Aihara, and Y. Nakamura. 2011. Starch biosynthesis in rice endosperm requires the presence of either starch synthase I or IIIa. J. Exp. Bot. 62: 4819–4831.

Hirose, T. and T. Terao. 2004. A comprehensive expression analysis of the starch synthase gene family in rice (*Oryza sativa L.*). Planta. 220: 9–16.

Hurkman, W.J., K.F. McCue, S.B. Altenbach, A. Korn, C.K. Tanaka, K.M. Kotharia, E.L. Johnson, D.B. Bechtel, J.D.Wilson, O.D. Anderson, and F.M. DuPont. 2003. Effect of temperature on expression of genes encoding enzymes for starch biosynthesis in developing wheat endosperm. Plant Sci. 164: 873–881.

James, M.G., K. Denyer, and A.M. Myers. 2003. Starch synthesis in the cereal endosperm. Curr. Opin. Plant Biol. 6: 215–222.

Jenner, C.F., T.D. Ugalde, and D. Aspinall. 1991. The physiology of starch and protein deposition in the endosperm of wheat. Aust. J. Plant Physiol. 18: 211–226.

Jeon, J.S., N. Ryoo, T.R. Hahn, H. Walia, and Y. Nakamura. 2010. Starch biosynthesis in cereal endosperm. Plant Physiol. Biochem. 48: 383–392.

Jiang, H.W., W.M. Dian, and P. Wu. 2003. Effect of high temperature on fine structure of amylopectin in rice endosperm by reducing the activity of the starch branching enzyme. Phytochem. 63: 53–59.

Jong-Seong, J., R. Nayeon, H. Tae-Ryong, Harkamal, W. and N. Yasunori. 2010. Starch biosynthesis in cereal endosperm. Plant Physiol. Biochem. 48: 383–392.

Kadir, S. and M.V. Weihe. 2007. Photochemical Efficiency and Recovery of Photosystem II in Grapes After Exposure to Sudden and Gradual Heat Stress. J. Amer. Soc. Hort. Sci. 132:764–769.

Keeling, P.L., J.R. Wood, R.H. Tyson, and I.G. Bridges. 1988. Starch biosynthesis in developing wheat-grain - evidence against the direct involvement of triose phosphates in the metabolic pathway. Plant Physiol. 87: 311–319.

Keeling, P.L., P.J. Bacon, and D.C. Holt. 1993. Elevated-temperature reduces starch deposition in wheat endosperm by reducing the activity of soluble starch synthase. Planta. 191: 342–348.

Lamacchia, C., P.R. Shewry, N. Di Fonzo, J.L. Forsyth, N. Harris, P.A. Lazzeri, J.A. Napier, N.G Halford,. and P. Barcelo. 2001. Endosperm-specific activity of a storage protein gene promoter in transgenic wheat seed. J. Exp. Bot. 52: 243–50.

Li, Z., S. Rahman, B. Kosar-Hashemi, G. Mouille, R. Appels, and M.K. Morell. 1999. Cloning and characterization of a gene encoding wheat starch synthase I. Theor. Appl. Genet. 98: 1208–1216.

Li, Z.W., and H.N. Trick. 2005. Rapid method for high-quality RNA isolation from seed endosperm containing high levels of starch. Biotechniques. 38: 872–-876

Li, Z.Y., G. Mouille, B. Kosar-Hashemi, S. Rahman, B. Clarke, K.R. Gale, R. Appels, and M. K. Morell. 2000. The structure and expression of the wheat starch synthase III gene. Motifs in the expressed gene define the lineage of the starch synthase III gene family. Plant Physiol. 123: 613-624.

Martin, C., and A.M. Smith. 1995. Starch biosynthesis. Plant Cell. 7: 971–985.

Okita, T.W. 1992. Is there an alternative pathway for starch synthesis. Plant Physiol. 100: 560–564.

Paliwal, R., M.S. Roeder, U. Kumar, J.P. Srivastava, and A.K. Joshi. 2012. QTL mapping of terminal heat tolerance in hexaploid wheat (*T. aestivum L.*). Theor. Appl. Genet. 125: 561–75.

Prakash, P., A. Kumari, D.V. Singh, R. Pandey, P. Sharma-Natu, and M.C. Ghildiyal. 2009. Starch synthase activity and grain growth in wheat cultivars under elevated temperature : a comparison of responses. Indian J. Plant Physiol. 14: 364–369

Rahman, S., Z. Li, I. Batey, M.P. Cochrane, R. Appels, and M. Morell. 2000. Genetic alteration of starch functionality in wheat. J. Cereal Sci. 31: 91–110.

Rijven, A. 1986. Heat inactivation of starch synthase in wheat endosperm tissue. Plant Physiol. 81: 448–453.

Ristic, Z., U. Bukovnik, I. Momcilovic, J.M. Fu, and P.V.V. Prasad. 2008. Heat-induced accumulation of chloroplast protein synthesis elongation factor, EF-Tu, in winter wheat. J. Plant Physiol. 165: 192–202.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual.

Smidansky, E.D., M. Clancy, F.D. Meyer, S.P. Lanning, N.K. Blake, L.E. Talbert, and M.J. Giroux. 2002. Enhanced ADP-glucose pyrophosphorylase activity in wheat endosperm increases seed yield. Proc. Natl. Acad. Sci. 99: 1724–1729.

Sofield, I., L.T. Evans, M.G Cook, and I.F. Wardlaw. 1977. Factors influencing rate and duration of grain filling in wheat. Aust. J. Plant Physiol. 4: 785–797.

Tanaka, K.I., S. Ohnishi, N. Kishimoto, T. Kawasaki, and T. Baba. 1995. Structure, organization, and chromosomal location of the gene encoding a form of rice soluble starch synthase. Plant Physiol. 108: 677–683.

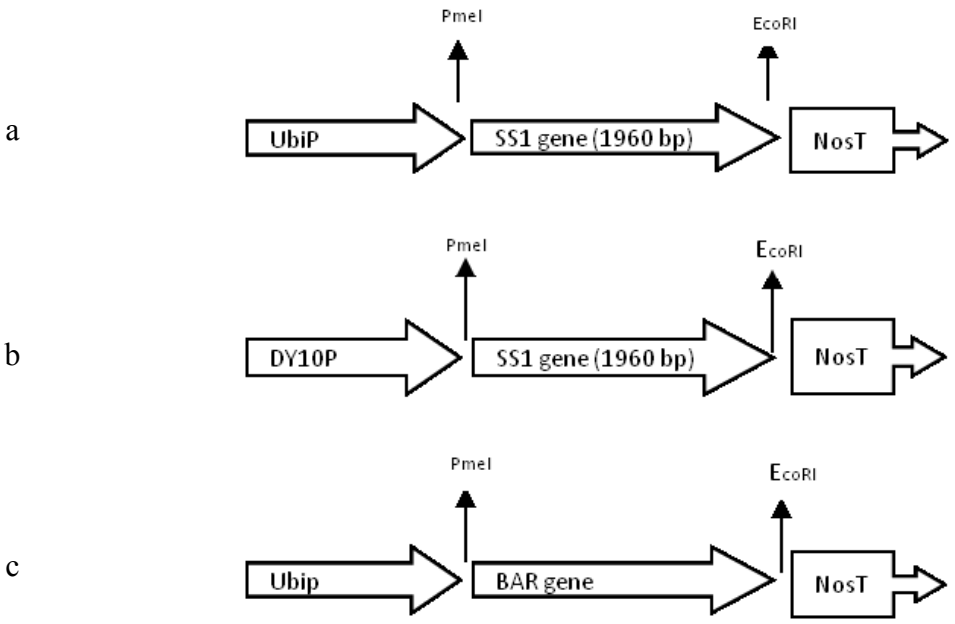
Wardlaw, I.F., C. Blumenthal, O. Larroque, and C.W. Wrigley. 2002. Contrasting effects of chronic heat stress and heat shock on kernel weight and flour quality in wheat. Funct. Plant Biol. 29: 25–34.

Yamakawa, H., T. Hirose, M. Kuroda, and T. Yamaguchi. 2007. Comprehensive expression profiling of rice grain filling-related genes under high temperature using DNA microarray. Plant Physiol. 144: 258–277.

Zhou, H., J.W. Arrowsmith, M.E. Fromm, C.M. Hironaka, M.L. Taylor, D. Rodriguez, M.E. Pajeau, S.M., Brown, C.G. Santino, and J.E. Fry. 1995. Glyphosate-tolerant CP4 and GOX genes as a selectable marker in wheat transformation. Plant Cell Rep. 15: 159–163.

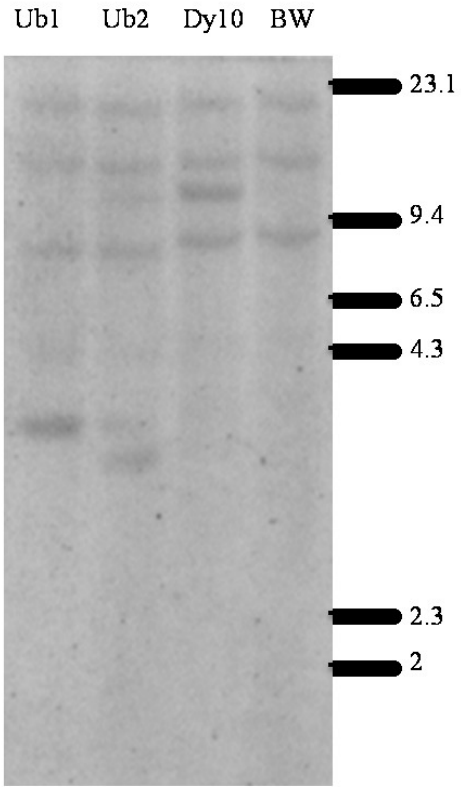
Figures and Tables

Figure 4.1 Schematic representation of the constructs for SSI and BAR gene.



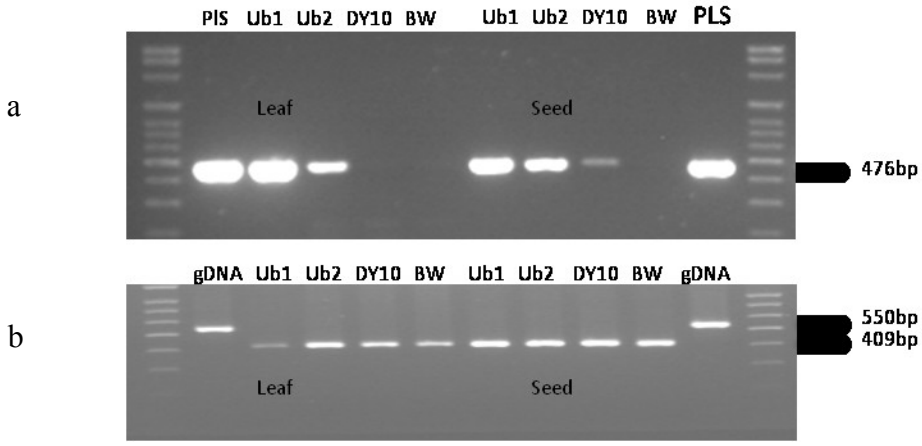
(a) Construct pAHC17. Ubip-Maize ubiquitin promoter, SSI-cDNA from rice soluble starch synthase 1 gene, NosT- Nopaline synthase gene Terminator. (b) Construct pJL10P5 having rice soluble starch synthase1 gene. DY10-High molecular weight glutenin promoter. (c) Construct pAHC20 having BAR gene which confers resistance to the herbicide glufosinate and bialaphos.

Figure 4.2. Southern blot analysis of three T_2 generation transgenic wheat events and a non-transgenic Bobwhite (BW) control.



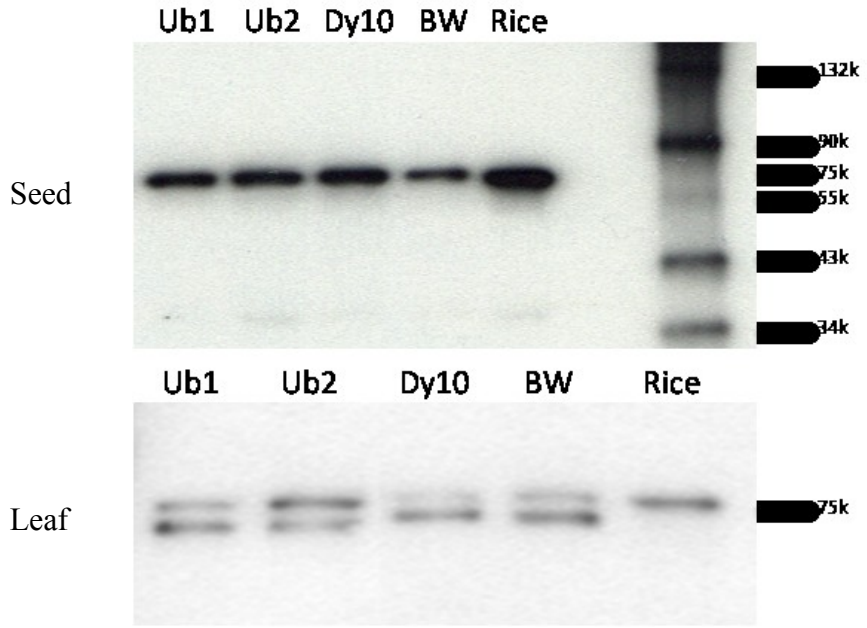
Twenty five micrograms of leaf DNA were digested with BamH 1+EcoR1 enzyme and fractioned on 0.8% agarose gel, transferred to a nylon membrane and hybridized with 32p-labelled PCR product of rice SSI gene. Ub1; Event no 1286 with ubiquitin promoter, Ub2; event no 1700 with ubiquitin promoter, Dy10; event number 2173 with Dy10 promoter, BW; non-transgenic Bobwhite variety.

Figure 4.3 RT- PCR for leaf samples (left) and seed sample (right)



(a) RNA was isolated from both leaf and seed samples. Converted into cDNA. PCR was done with the gene specific primer and found the amplification in 1% agarose gel. Pls; Plasmid DNA having SSI gene, Ub1; Event no 1286 with ubiquitin promoter, Ub2; event no 1700 with ubiquitin promoter, Dy10; event number 2173 with Dy10 promoter, BW; non-transgenic Bobwhite variety.
(b) RT- PCR using Tubulin gene primer for leaf samples (left) and seed sample (right). PCR was done using primer of a house keeping gene called Tubulin. gDNA - genomic DNA of BW. Genomic DNA makes 550 bp band and cDNA makes 409 bp band with that primer. Two bands prove the mixer of DNA and RNA of that sample. For RT PCR, corresponding cDNA were used.

Figure 4.4 Western blot analysis using seed and leaf sample



Three T_2 generation transgenic events, one non-transgenic Bobwhite and one rice samples were used. Total proteins extracted from both leaf and seed samples were fractionated on 10% SDS page gel and transferred to PDVF membrane, probed with polyclonal antibody raised against rice SSI. Equal amount of total protein (40µg) was loaded in each lane. In leaf sample, antibody binds with two SSI isoforms of wheat. Comparing the signal strength of second isoforms of SSI, Ub1, Ub2 and rice leaf sample showed enhanced signal for SSI. In seed samples, all transgenic events along with rice showed stronger signal than non-transgenic wheat. The antibody binds with both wheat endogenous SSI and rice SSI protein.

Table 4.1 Presence of rice soluble starch synthase in six liberty positive wheat event and their X^2 value.

Event No	Promoter	PCR test in T_0	No. of PCR positive T_1plants	No. of PCR negative T_1Plant	X^2 value (based on 3:1 ratio)
1286 (Ub1)	Ubiquitin	+	37	6	2.79
1588	Ubiquitin	+	4	2	1.33
1700 (Ub2)	Ubiquitin	+	7	2	0.037
2036	DY10	+	46	20	0.99
2173 (Dy10)	DY10	+	13	5	0.074
2540	DY10	-	0	10	-

*, * *, and ***, denote P< 0.05, 0.01 and 0.001 respectively.

Table 4.2 TKW and seed number /selected head in transgenic and non-transgenic wheat in T2 generation (Exp 1) using soluble starch synthase gene (SS1) of rice.

Temp.	Event name	TKW (g)			Seed number/selected head		
		Mean"+"	STD	T stat	Mean"+"	STD	T stat
31/24°C	BWTC(6)	29.5	1.21		54.33	11.58	
	UBI (10)	30.45	1.32	1.85*	54.2	14.24	0.02
	Dy10 (4)	31.48	0.83	3.47**	41.25	4.03	2.54*
22/15°C	BWTC (3)	35.62	1.5		59.33	11.5	
	Ub1 (9)	37.71	1.96	1.92	58.55	11.1	0.1
	Dy10(4)	37.53	2.89	1.13	45.75	12.44	1.49

† Number of plants studied in this experiment is accompanied by event name in parenthesis. BWTC –non-transgenic Bobwhite but established through tissue culture, Ub1- event 1286 having ubiquitin promoter, Dy10- Event 2173 having Dy10 promoter.
*, * *, and ***, denote P< 0.05, 0.01 and 0.001 respectively.

Table 4.3 Tiller number/plant and days required for anthesis to physiological maturity (PM) in transgenic and non-transgenic wheat in T2 generation (Exp 1) using soluble starch synthase gene (SS1) of rice

Temp	Event name	Tiller number /plant			Days required for PM		
		Mean	STD	T stat	Mean	STD	T stat
31/24°C	BWTC(6)	5.33	1.03		27.33	1.03	
	UBI (10)	5.7	0.82	0.74	27.4	0.97	0.13
	Dy10 (4)	5.75	0.5	0.85	27.5	1.29	0.22
22/15°C	BWTC (3)	6	1.0		37.33	0.57	
	Ub1 (9)	6.22	0.96	0.34	37.11	0.78	0.53
	Dy10(4)	6.25	2.22	0.2	36.75	0.5	1.4

† Number of plants studied in this experiment is accompanied by event name in parenthesis. BWTC –non-transgenic Bobwhite but established through tissue culture, Ub1- event 1286 having ubiquitin promoter, Dy10- Event 2173 having Dy10 promoter.

*, * *, and ***, denote P< 0.05, 0.01 and 0.001 respectively.

Table 4.4 TKW comparison between transgenic and non-transgenic wheat in T_2 and T_3 generation (Exp. 2 and 3) using rice soluble starch synthase gene (SSI).

Temp	Event name	2nd experiment (high temp 33/26°C)				3rd Experiment (high temp 34/28°C)			
		Mean"+"	Mean"-"	STD	T stat	Mean"+"	Mean"-"	STD	T stat
High temperature	BWTC		22.87 (5)	1.94	0.301		20.23 (5)	2.65	0.134
	BWNTC		22.51 (4)	1.67			19.94 (5)	3.89	
	BW		22.71 (9)	1.73	-		20.09 (10)	3.14	-
	Ub1	24.45 (4)		1.44	1.89*	24.35 (5)		4.31	1.96*
	Ub2	25.17 (4)		2.35	1.88	25.03 (6)		2.46	3.50**
	Ub	24.81 (8)		1.84	2.41*	24.70 (11)		3.24	3.30**
	Dy10	28.54 (6)		1.85	6.14***	27.04 (4)		3.12	3.75**
Optimum temperature 22/15°C	BWTC		40.95 (5)	2.27	0.144		40.91(5)	2.68	0.932
	BWNTC		40.69 (4)	3			39.45(5)	2.25	
	BW		40.84 (9)	2.41			40.18(10)	2.46	-
	Ub1	42.11 (4)		3.37	0.68	43.57(6)		4.28	1.77
	Ub2	40.27 (4)		1.36	1.81	43.17(6)		2.16	2.55*
	Ub	41.20 (8)		2.57	0.292	43.38(12)		3.25	2.63**
	Dy10	40.53 (5)		1.76	0.276	44.68(4)		4.54	1.87

† Number of plants studied in this experiment is accompanied by mean in parenthesis. Mean"+" - positive transgenic plants and Mean"-" are the non-transgenic plants. BWTC –non-transgenic Bobwhite but established through tissue culture, BWNTC, non-transgenic Bobwhite plants not regenerated through tissue culture. Ub1- event 1286 having ubiquitin promoter, Ub2- event 1700 having ubiquitin promoter, The Ub observation combines the Ub1 and Ub2 observation. Dy10- Event 2173 having Dy10 promoter, STD- Standard deviation

*, * *, and ***, denote P< 0.05, 0.01 and 0.001 respectively.

Table 4.5 Comparison of seed numbers per selected head between transgenic and non-transgenic wheat in T_2 and T_3 generation (Exp. 2 and 3 respectively) using rice soluble starch synthase gene (SSI).

Temp	Event name	2nd experiment (high temp 33/26°C)				3rd Experiment (high temp 34/28°C)			
		Mean"+"	Mean"-"	STD	T stat	Mean"+"	Mean"-"	STD	T stat
High temperature	BWTC		47.4	4.56	0.475		46.6	11.74	0.32
	BWNTC		46	4.24			48.8	9.73	
	BW		46.78	4.20	-		47.7	10.23	-
	Ub1	46		4.08	0.314	50.4		12.80	0.41
	Ub2	49.5		13.47	0.395	48.33		4.13	0.173
	Ub	47.75		9.41	0.269	49.27		8.67	0.378
	Dy10	36.83		9.22	2.476*	25.25		5.85	5.14**
Optimum temperature 22/15°C	BWTC		39	8.18	0		50.2	11.26	0.989
	BWNTC		39	10.61			56.4	8.35	
	BW		39	8.70	-		53.3	9.9	-
	Ub1	37.5		9.15	0.276	53.83		10.06	0.103
	Ub2	49.5		11.15	1.67	53.84		9.54	0.106
	Ub	43.5		20.06	0.587	53.83		9.35	0.129
	Dy10	30		7.11	1.96*	36.75		16.52	1.87

Mean"+" -positive transgenic plants and Mean"-" are the non-transgenic plants. BWTC –non-transgenic Bobwhite but established through tissue culture, BWNTC- non-transgenic Bobwhite not regenerated in tissue culture, Ub1- event 1286, having ubiquitin promoter, Ub2- event 1700, having ubiquitin promoter, The Ub observation combines the Ub1 and Ub2 observation. Dy10- Event 2173 having Dy10 promoter, STD- Standard deviation

*, * *, and ***, denote P< 0.05, 0.01 and 0.001 respectively.

Table 4.6 Comparison of Tiller number /plant between transgenic and non-transgenic wheat in T_2 and T_3 generation (Exp. 2 and 3 respectively) using rice soluble starch synthase gene (SSI).

Temp	Event name	2nd experiment (high temp 33/26°C)				3rd Experiment (high temp 34/28°C)			
		Mean"+"	Mean"-"	STD	T stat	Mean"+"	Mean"-"	STD	T stat
High temperature	BWTC		5.6	1.14	0.15		7	0.71	0.69
	BWNTC		5.75	1.71			6.4	1.82	
	BW		5.67	1.32			6.7	1.34	
	Ub1	5.75		0.96	0.128	6.17		1.83	0.62
	Ub2	5.5		1	0.25	6.5		2.16	0.20
	Ub	5.63		0.92	0.076	6.33		1.92	0.53
	Dy10	5.8		1.30	0.182	7		1.58	0.36
Optimum temperature 22/15°C	BWTC		6	1			6.8	1.79	
	BWNTC		6.25	0.95	0.381		7.4	1.14	0.63
	BW		6.11	0.93	-		7.1	1.45	-
	Ub1		6.5	1.29	0.54	6.83		1.17	0.40
	Ub2		6.25	0.96	0.243	7		0.89	0.17
	Ub		6.38	1.06	0.542	6.91		1.0	0.34
	Dy10		6.2	1.30	0.134	7.4		2.07	0.29

Mean"+" -positive transgenic plants and Mean"-" are the non-transgenic plants. BWTC –non-transgenic Bobwhite but established through tissue culture, BWNTC- non-transgenic Bobwhite not regenerated in tissue culture, Ub1- event 1286, having ubiquitin promoter, Ub2- event 1700, having ubiquitin promoter, The Ub observation combines the Ub1 and Ub2 observation.Dy10- Event 2173 having Dy10 promoter, STD- Standard deviation

*, * *, and ***, denote P< 0.05, 0.01 and 0.001 respectively.

Table 4.7 Comparison of days required for physiological maturity between transgenic and non-transgenic wheat in T_2 and T_3 generation (Exp. 2 and 3 respectively) using rice soluble starch synthase gene (SSI).

Temp.	Event name	2nd experiment (high temp 33/26°C)				3rd Experiment (high temp 34/28°C)			
		Mean"+"	Mean"-"	STD	T stat	Mean"+"	Mean"-"	STD	T stat
High temperature	BWTC		26.8	3.27	0.25		22.8	1.30	0.63
	BWNTC		27.25	2.22			22.4	0.54	
	BW		27	2.69			22.6	0.97	
	Ub1	29		3.92	0.93	23.4		0.89	1.59
	Ub2	28		2.45	0.66	23.66		0.82	2.36*
	Ub	28.5		3.07	1.06	23.54		0.82	2.41*
	Dy10	29.66		2.50	1.96*	24.25		0.96	2.90*
Optimum temperature 22/15°C	BWTC		40.8	1.79	1.31		38.8	1.30	0
	BWNTC		43	2.94			38.8	4.82	
	BW		41.78	2.49			38.8	3.33	-
	Ub1	42		3.46	0.115	36.5		4.42	1.10
	Ub2	41		3.37	0.414	37.17		5.19	0.69
	Ub	41.5		3.21	0.20	36.83		4.61	1.16
	Dy10	41.2		1.64	0.52	40.25		4.03	0.64

Mean"+" -positive transgenic plants and Mean"-" are the non-transgenic plants. BWTC –non-transgenic Bobwhite but established through tissue culture, BWNTC- non-transgenic Bobwhite not regenerated in tissue culture, Ub1- event 1286 having ubiquitin promoter, Ub2- event 1700 having ubiquitin promoter, The Ub observation combines the Ub1 and Ub2 observation. Dy10- Event 2173 having Dy10 promoter, STD- Standard deviation

*, * *, and ***, denote $P< 0.05$, 0.01 and 0.001 respectively.

Table 4.8 Percent change in TKW compared to control for the three experiments

Event	1st Exp.		2nd Exp.		3rd Exp.	
	(31/24°C)	(22/15°C)	(33/26°C)	(22/15°C)	(34/28°C)	(22/15°C)
Ub1	3.22 *	5.87	7.66 *	3.12	21.20 *	8.43
Ub2	Absent	Absent	10.83	-1.40	24.59 **	7.44*
Ub	3.22*	5.87	9.25*	0.88	22.94**	7.96*
Dy10	6.71 *	5.36	25.67 ***	-0.75	34.59 **	11.19

*, * *, and ***, denote P< 0.05, 0.01 and 0.001 respectively found in the comparison. The Ub observation combines the Ub1 and Ub2 observation.

Table 4.9 Mean thousand kernel weight (TKW), percent change in TKW compared to BW control and mean seed number in Exp.4

Experiment	Event	Thousand seed weight			% 1000 seed wt increment	Seed number		
		Mean	Std	T-stat		Mean	Std	
Heat stress	BW	23.55	4.88			47.17	8.7	
(32-33/30°C)	UB1	26.75	2.55	1.39*	13.58	52.6	1.95	1.48
	UB2	26.39	3.89	1.11	12.06	51.17	6.55	0.90
	DY10	30.50	3.38	2.94**	29.51	46.57	3.78	0.16
Optimum	BW	34.27	2.1			47	12	
temperature	UB1	34.72	1.0	0.43	1.31	50.4	10.41	0.47
(20/15°C)	UB2	35.21	0.78	0.95	2.74	49.83	9.26	0.43
	DY10	37.59	1.21	2.97	9.67	49	10.1	0.27

* and ***, denote P< 0.1, and 0.01 respectively

Chapter 5 - General conclusion

Three experiments were done to fulfill the objectives of (a) to identify tools to increase wheat production under heat stress (b) to develop NILs for cytoplasm using ten alloplasmic sources and four adapted varieties (c) to identify the effect of cytoplasm, and cytoplasmic- nuclear genome interaction on traits associated with heat tolerance (d) to identify QTL for various traits associated with thermotolerance in wheat (e) to investigate the effect of expression of a rice soluble starch synthase gene on starch deposition and the yield and kernel weight of wheat under heat stress conditions. The conclusion from the each experiments were as follows

Experiment 1 (chapter 2): Using a limited source of alloplasmic cytoplasm, it is clear that there is potential to improve thermotolerance associated traits under heat stress by manipulating cytoplasm. This is especially true for chlorophyll content and Fv/Fm that are photosynthesis-related traits and very strong indicators of heat tolerance. It may be of value to explore additional cytoplasmic sources to increase key components of heat tolerance in wheat such as stay-green or photosynthetic capacity. Additional research is needed to fully assess the potential of cytoplasmic variability to contribute to develop of heat-tolerant wheat varieties. The identified cytoplasmic sources can be useful to develop heat tolerant wheat variety.

Experiment 2 (chapter 3): QTL mapping for heat tolerance supported the idea that damage to cellular structure and the photosystem are well defined processes, which influence heat tolerance. Our study suggests that function of both systems are very closely correlated when plants are exposed to a long period of stress, and are most likely under similar genetic control. We found four QTL that significantly influence those traits. These QTL are located on chromosomes 6A, 7A, 1B, 2B and 1D. The four SSR markers (*Xbarc121, Xbarc49, gwm18 and*

Xbarc113) and five GBS Bin markers (*Bin747, Bin 1596, Bin 178, Bin 1130 and Bin 81*) which were strongly associated with component traits may be useful in marker assisted breeding for heat tolerance in wheat.

Experiment 3 (chapter 4): Soluble starch synthase 1 gene from rice was transferred to wheat to increase grain fill under heat stress in wheat. Our results suggest that transfer of more heat labile forms of key enzymes may be good strategy for improving heat tolerance in crop plants. This approach should be beneficial for all cool season species that are grown under conditions where heat stress may limit production. It may be useful to explore the heat stability of SSI from other species typically grown under high temperature conditions to identify the most heat labile source of the enzyme. The cultivar used in this study is not particularly tolerant to heat stress. Deployment of the rice construct in lines that demonstrate heat tolerance may be an avenue to even greater levels of production under heat stress.

Overall output

1. Cytoplasm from line 1 (*Aegilops juvenalis*/6*CHR//9*SK(NDM1)/3/7*SPN), 5 (*A.uniaristata*/2**Triticum durum*/10*SK(NDM5)/3/7*SPN) and 10 (*T. turgidum*/9*SK(NDM10)//7*SPN) performed better than those corresponding euplasmic cytoplasm. Interaction effect was found with cytoplasm 2 (*A.cylindrica*/CHR//10*SK(NDM2)/3/7*SPN), 4 (*A. squarrosa/19*SK(NDM4)//7*SPN*), 8 (*T.macha/17*SK(NDM8)//7*SPN*) and 9 (*T.macha*/9*SK(NDM9)//7*SPN).
2. QTL were found on chromosomes 6A, 7A, 1B, 2B and 1D associated with membrane damage and chlorophyll content. SSR markers *Xbarc121, Xbarc49, gwm18 and*

Xbarc113 and GBS Bin markers, *Bin747, Bin 1596, Bin 178, Bin 1130 and Bin 81* were strongly associated with component traits.

3. Transfer of greater heat labile forms of key enzymes may be good strategy for improving heat tolerance in crop plants. Soluble starch synthase 1 of rice was found to increase grain fill under heat stress in wheat.

Future direction

1. Explore additional cytoplasmic sources to increase key components of thermotolerance in wheat.
2. Develop and/or identify chloroplast and mitochondrial genome specific markers for membrane stability or stay-green, using genotyping by sequencing (GBS). Extra nuclear genomes of cytoplasmic NILs can be used for sequencing and calling SNPs.
3. Chromosome 7A should be targeted for fine mapping and/or cloning.
4. Developing transgenic wheat to enhance ADP-glucose pyrophosphorylase activity using gene from another crop like maize, along with soluble starch synthase gene from grape to see the grain fill under high temperature in both heat tolerant and heat sensitive varieties.